Molecular phylogeny of the nematode genus *Longidorus* (Nematoda: Longidoridae) with description of three new species

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The genus *Longidorus* includes a remarkable group of animals invertebrates of the phylum Nematoda being polyphagous root-ectoparasites of many plants including various agricultural crops and trees that damage either by direct feeding on root cells as well as by transmitting nepoviruses. Species discrimination in *Longidorus* is complicated by the phenotypic plasticity (intraspecific variability and minor interspecific differences) leading to potential mis-identification. We conducted nematode surveys in cultivated and natural environments in southern Spain detected 11 species of *Longidorus*. We developed a comparative study among these related species by considering morphological and morphometrical features together with molecular data from nuclear ribosomal RNA genes (D2-D3 expansion segments of 28S, ITS1, and partial 18S). The results of molecular and phylogenetic analysis confirmed the morphological hypotheses and allowed the delimitation and discrimination of three new species of the genus described herein as *Longidorus baeticus* sp. nov., *Longidorus oleae* sp. nov., and *Longidorus andalusicus* sp. nov.) and eight known species including *L. alveus*, *L. crataegi*, *L. fasciatus*, *L. intermedius*, *L. iuglandis*, *L. magnus*, *L. rubi* and *L. vineicola*. Phylogenetic analyses of *Longidorus* spp. based on the three molecular markers resulted in a general consensus of these species grouping, since lineages were maintained for the majority of species (i.e. species with a conoid-rounded lip region, amphidial fovea asymmetrically bilobed, female tail bluntly rounded), but not in some others (i. e. position of *L. crataegi*, *L. intermedius*, *L. rubi* were quite variable). This is the most complete and with higher number of species included in a phylogenetic analysis for *Longidorus* and *Paralongidorus* species. No correspondence between phylogenetic trees and morphological characters were found for ribosomal markers at exception of amphidial shape. Thus, polyphasic identification, based on integration of molecular analysis with morphology is a tool beyond doubt in *Longidorus* identification.

ADDITIONAL KEY WORDS: cryptic species, D2-D3, integrative taxonomy, ITS1, morphometry, needle nematodes, 18S.

INTRODUCTION

The phylum Nematoda Rudolphi 1808 tends to be greatly conserved in gross morphology which makes species identification a very difficult task. Nematodes are abundant, diverse and ubiquitous members of the meiofauna and play a significant role in nitrogen mineralization and organic matter decomposition in soil food webs, and impact negatively on human agricultural endeavour as well as human health (Blaxter *et al.*, 1998). The genus *Longidorus* Micoletzky, 1922 includes a number of long (2–12 mm) ectoparasitic nematode species polyphagous of many plants including various agricultural crops and that damage either by direct feeding on root cells as well as by transmitting nepoviruses (Taylor & Brown 1997). Some *Longidorus* spp. are cosmopolitan whilst others may have a limited distribution (Coomans 1996). Of the about 150 nominal species of *Longidorus*, only nine species (6.0%) have been reported as virus vectors, transmitting seven out of 38 known nepoviruses (Taylor & Brown, 1997; Decraemer & Robbins 2007). Therefore, correct identification of *Longidorus* species is essential to establish appropriate control measures. Currently, species discrimination in *Longidorus* is mainly based on morphology and morphometrics. However, the species characterization is complicated by the phenotypic plasticity due to a high degree of intraspecific variability within morphometrics and minor interspecific differences leading to substantial overlap among *Longidorus* species increases the potential for mis-identification. As a result, taxonomic difficulties often arise from under- or over-estimation of intraspecific variability of certain morphological characters that are currently being used for species diagnosis. Recently, 52 *Longidorus* species (about 35%) have been molecularly characterized which constitute a useful tool for molecular-based species identification. However, the application of molecular methods to study nematode population structure and systematics has revealed that some long-assumed single species are in fact cryptic; *i.e.*, species that are morphologically indistinguishable and may be phylogenetically distant to one another (Gutiérrez-Gutiérrez *et al.* 2010; Ye *et al.*, 2004).

The polytomous keys for *Longidorus* spp. have been an effective morphological and morphometric mean for identifying species, since they permits a range of characters to be used simultaneously, which makes it more effective for identifying closely related species with overlapping features (Chen *et al.* 1997; Loof & Chen 1999). Nevertheless, the genus *Longidorus* tends to be greatly conserved in gross morphology which makes species identification a very difficult task. To help with this problem, Ye and Robbins (2004b) developed a hierarchical cluster analysis based on female morphometric character mean values and provided a computerized statistical approach to assist by helping to identify and distinguish among the

different *Longidorus* species. However, currently, molecular approaches using ribosomal DNA (rDNA) sequences including 18S, ITS regions and the D2 and D3 expansion segments of the 28S have been shown to be more useful diagnostic markers in the characterisation and phylogenetic relationships within Longidoridae, especially in cases where morphological characters may lead to ambiguous interpretation (De Luca *et al.* 2004; 2009; Neilson *et al.* 2004; Ye *et al.* 2004; He *et al.* 2005; Palomares-Rius *et al.* 2008; Gutiérrez-Gutiérrez *et al.* 2011). Thus, recent molecular phylogeny of dagger and needle nematodes based on the D2-D3 expansion segments of 28S rRNA and partial 18S genes resolved three major clades: Clade I comprise *Longidorus* spp. and *Paralongidorus* spp.; Clade II comprise *Xiphinema americanum*-group including species of *Xiphidorus*; and Clade III comprising the remaining *Xiphinema* species (Gutiérrez-Gutiérrez *et al.* 2011). Furthermore, maximum likelihood (ML) analysis using Shimodaira-Hasegawa-test for the validity of *Paralongidorus* showed the validity of the genus using the D2-D3 expansion segments of 28S and partial 18S (Palomares-Rius *et al.* 2012). D2-D3 expansion segments of 28S rRNA and ITS1 rRNA have been showed to be more useful for species identification compared to partial 18S, since both markers showed more species variability than partial 18S. In fact, in general, partial 18S sequences does not have enough resolution to distinguish species, since different species showed only one or few nucleotide differences, e.g., *Longidorus elongatus* (de Man 1876) Thorne and Swanger 1936, *Longidorus paravineicola* Ye and Robbins 2003, *Longidorus piceicola* Lisková, Robbins and Brown 1997, *Longidorus uroshis* Krnjaic, Lamberti, Krnjaic, Agostinelli and Radicci 2000, and *Longidorus vineicola* Sturhan and Weischer 1964), but it is useful for discriminating other species, e.g. *Longidorus henanus* Xu & Cheng, 1992, *Longidorus paralongicaudatus* Ye and Robbins, 2003 (Neilson *et al.* 2004; Pedram *et al.* 2008).

Up to date, 22 species of the genus *Longidorus* have been recorded from Spain (Peña Santiago *et al.* 2003; Palomares-Rius *et al.* 2010; Gutiérrez-Gutiérrez *et al.* 2011). During nematode surveys conducted in cultivated and natural environments in southern Spain, 17 populations of *Longidorus* species characterized by large to medium body and stylet length appeared to be morphologically related to other known *Longidorus* spp. This prompted us to carry out a morphological and molecular comparative study among those related species, since a combination of both methods is considered a prospective approach in diagnostics of this nematode group.

The objectives of this study were: i) to identify and compare morphologically and morphometrically the 17 Spanish populations of *Longidorus* spp.; ii) to make a molecular characterisation of these *Longidorus* populations based on sequences of the D2-D3 expansion

segments of the 28S nuclear ribosomal RNA gene, the ITS1 of rRNA, and partial 18S rRNA sequences; and *iii*) to study the phylogenetic relationships of *Longidorus* spp.

MATERIAL AND METHODS

NEMATODE POPULATIONS AND MORPHOLOGICAL STUDIES

Nematode surveys were conducted from 2010 to 2011 during the spring season in cultivated and natural environments in southern Spain, including several host plants (Table 1). Samples were collected with a shovel from the upper 50 cm of soil of four to five plants arbitrarily chosen in fifteen localities of Andalusia. Nematodes were extracted from 500 cm³ 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 afterwards from the same locality for completing the necessary specimens for morphological and/or molecular identification.

Specimens for light microscopy were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid and processed to pure glycerine using Seinhorst's method (1966). Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at powers up to 1,000x magnification. . Morphometric study of each nematode population included classical diagnostic features in longidoridae (i.e. de Man body ratios, lip region and amphid shape, oral aperture-guiding ring, odontostyle and odontophore length) (Jairajpuri & Ahmad, 1992). All measurements were expressed in micrometers (µm), unless otherwise indicated in text. For line drawing of the new species, light micrographs were imported to CorelDraw software version X5 and redrawn. All other abbreviations used are as defined in Jairajpuri and Ahmad (1992). In addition, a comparative morphological and morphometrical study of type specimens of some species were conducted with specimens kindly provided by Dr. A. Troccoli, from the nematode collection at the Istituto per la Protezione delle Piante, Sede di Bari, Consiglio Nazionale delle Ricerche, (C.N.R.), Bari, Italy (viz. *Longidorus crataegi*, *Longidorus fasciatus*, and *Longidorus iuglandis*) and Dr Z.A. Handoo, from the USDA Nematode Collection, Beltsville, MD, USA (viz. *Longidorus intermedius*).

DNA EXTRACTION, PCR AND SEQUENCING

For molecular analyses two live nematodes from each sample were temporarily mounted in a drop of 1M NaCl containing glass beads and after taking measurements and photomicrographs the slides were dismantled and DNA extracted. Nematode DNA was extracted from single individuals and protocols for PCR were conducted as described by Castillo *et al.* (2003). The D2-D3 expansion segments of 28S rDNA was amplified using the D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (Castillo *et al.* 2003; He *et al.* 2005; Palomares-Rius *et al.* 2008). The ITS1 region was amplified using forward primer 18S (5'-TTGATTACGTCCCTGCCCTTT-3') and reverse primer rDNA1 (5'-ACGAGCCGAGTGATCCACCG-3') as described in Wang *et al.* (2003). Finally, the 18S rDNA gene was amplified using the SSU_F_07 (5'-AAAGATTAAGCCATGCATG-3'), SSU_R_81 (5'-TGATCCWKCYGCAGGTTTCAC-3') and 13R (5'-GGGCATCACAGACCTGTTA-3') primers (<http://www.nematodes.org/barcoding/sourhope/nemoprimer.html>).

All PCR used the following conditions: one cycle of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, annealing temperature of 57°C for 45 s, 72°C for 3 min and finally one cycle of 72°C for 10 min. Sequencing of some of the ITS1 and partial 18S rRNA genes of some known *Longidorus* spp. identified herein were not successful despite several attempts (Table 1). PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing in both directions using the primers referred 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 SCAI, University of Córdoba sequencing facilities (Córdoba, Spain). The newly obtained sequences were submitted to the GenBank database under accession numbers indicated on the phylogenetic trees and Table 1. For some samples, sequencing of the ITS1 or the partial 18S rRNA sequences was not successful despite several attempts (Table 1).

PHYLOGENETIC ANALYSIS

D2-D3 expansion segments of 28S and partial 18S rDNA sequences of different *Longidorus* spp. from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen according to previous published data (He *et al.* 2005; Holterman *et al.* 2006; Palomares-Rius *et al.* 2008; Coomans *et al.* 2012). The newly obtained and published sequences for each gene were aligned using ClustalW (Thompson *et al.* 1997) with default parameters.

Sequence alignments were manually edited using BioEdit (Hall 1999). Phylogenetic analyses of the sequence data sets were performed with ML using PAUP * 4b10 (Swofford 2003) and Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The best fit model of DNA evolution was obtained using jModelTest v. 0.1.1 (Posada 2008) 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. BI analysis under GTR + I + G model for D2-D3 expansion segment of 28S rRNA, ITS1 rRNA, and partial 18S rRNA, were run with four chains for 3×10^6 generations, 2×10^6 generations, and 2×10^6 generations, respectively. The Markov chains were sampled at intervals of 100 generations. Two runs were performed 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 were visualised using TreeView (Page 1996). In ML analysis the estimation of the support for each node was obtained by bootstrap analysis with 200 fast-step replicates.

RESULTS

SYSTEMATICS

GENUS *LONGIDORUS* MICOLETZKY 1922

LONGIDORUS BAETICUS SP. NOV.

(FIGS. 1-3, TABLE 2)

Holotype. Female extracted from soil samples collected from vineyards in Montemayor, Córdoba province, Spain, (37°39'42.11'' N latitude, 4°39'24.36'' W longitude) by J. Martín Barbarroja and G. León Roperro, 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 M121-04).

Paratypes. Female, male and juvenile paratypes extracted from soil samples collected from vineyards in Montemayor, Córdoba province, Spain, and additional female paratypes collected in Niebla, Huelva province, Spain, associated with citrus were deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers M121-01-M121-15); two females at Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (C.N.R.),

Sezione di Bari, Bari, Italy (M121-10); three females at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (M121-16); and two females at USDA Nematode Collection, Beltsville, MD, USA (T-6168p)

Etymology. The species name is derived from the Latin word *Baetica*, the Roman province of the Iberian Peninsula where the species was collected.

Description of female. Body long and rather robust, slightly tapering towards anterior end, usually assuming an open C-shape when heat relaxed. Cuticle appearing smooth, 4.8 (4.5-5.0) μm thick, 19.2 (16.0-22.0) μm thick at tail tip, and marked by very fine superficial transverse striae mainly in tail region. Lip region conoid-rounded, continuous with body contour. Amphidial fovea pocket-shaped slightly asymmetrically bilobed. Labial papillae prominent. Stylet guiding ring single, 7.5 (7-8) μm wide, located 2.7 ± 0.1 (2.4-2.8) times lip region diam. from anterior end. Lateral chord 16 (14-18) μm wide at mid-body or 20 (15-25)% of corresponding body diam. Odontostyle long and narrow, 1.7 (1.3-1.9) times as long as odontophore, straight or slightly arcuate, *ca* 3.0-3.5 μm wide towards its base; odontophore weakly developed, with rather weak basal swellings. Nerve ring encircling cylindrical part of pharynx, 4.9 (3.8-6.1) times body width at neck base far from anterior end. Anterior slender part of pharynx usually coiled in its posterior region. Basal bulb cylindrical, 160 ± 15.6 (138-199) μm long or *ca* one-third to one-fifth of neck length, 33.1 (27-39) μm diam. Dorsal pharyngeal gland nucleus (DN) and ventro-sublateral pair of nuclei (SN) situated at 22.6 ± 4.1 (17.2-27.6)%, 47.7 ± 2.9 (43.8-50.9)% of distance from anterior end of pharyngeal bulb, respectively. Glandularium 139.6 ± 12.9 (125-163) μm long. Cardia conoid-rounded, 11.9 ± 1.0 (10.5-13.5) μm long. Reproductive system with both genital branches equally developed, each 853 (818-888) μm long, with reflexed ovaries very variable in length. Vulva in form of a transverse slit, located about mid-body, vagina perpendicular to body axis, 20 (17-22) μm long, or 16-34% of corresponding body width, surrounded by well developed muscles. Genital branches equally developed, 11.2 ± 5.4 (6.1-17.0), 10.7 ± 4.6 (6.2-15.2)% of body length, respectively. Uteri 226 (204-242) μm long, differentiated, with sperm cells in most of the females examined; well developed sphincter between uterus and oviduct. Anterior and posterior oviduct of similar size. Ovaries equally developed 115.0 ± 7.1 (110-120) μm long, both of them with a single row of oocytes. Prerectum very variable in length, 9.4 (4.4-10.7) times anal body diam., and rectum 0.6 (0.4-0.7) times as long as anal body diam., anus a small rounded slit. Tail short, bluntly conoid, with rounded terminus, bearing three pairs of caudal pores.

Male. Common, but less frequent (40%) than female. Morphologically similar to female except for genital system, but with posterior region slightly curved ventrally. Male genital tract diorchic with testes opposed, containing multiple rows of different stages of spermatogonia. Tail rounded, dorsally convex conoid, ventrally slightly concave with broad blunt terminus and thickened outer cuticular layer. Spicules arcuate, robust, *ca* 2 times longer than tail length, lateral guiding pieces more or less straight or with curved proximal end. One pair of adanal supplements and 18 (16-19) midventral supplements.

Juveniles. Morphologically similar to adults, but smaller. All four juvenile stages were found, being distinguishable by relative lengths of body and functional and replacement odontostyle (Table 2, Robbins *et al.* 1995; 1996). First stage-juveniles (J1) were characterised by a bluntly rounded to cylindrical tail with a *c'* ratio ≥ 1.7 (Fig. 3), an odontostyle length *ca* 80 μm , and shorter distance from anterior end to stylet guiding ring than that in adult stages.

Diagnosis. *Longidorus baeticus* sp. nov. is a gonochoristic species characterized by a long body (6.5-9.4 mm), assuming an open C-shape when heat relaxed; lip region conoid-rounded continuous with body contour, 12.0-14.5 μm wide; guiding ring located 34-39 μm from anterior end; long odontostyle (115-133 μm); amphidial fovea pocket-shaped, slightly asymmetrically bilobed; vulva almost equatorial; female tail short, bluntly conoid, and bearing three pairs of caudal pores; *c'* ratio (0.5-0.7); males frequent with long spicules (80-95 μm) and 16-19 ventromedian supplements; and specific D2-D3, ITS1 rRNA and partial 18S rRNA sequences (GenBank accession numbers JX445106 and JX445107, JX445093, and JX445119, respectively). According to the polytomous key Chen *et al.* (1997) and the supplement by Loof and Chen (1999), the new species has the following code (codes in parentheses are exceptions): A45-B2-C3-D1-E3-F4(35)-G12-H1-I2.

***LONGIDORUS OLEAE* SP. NOV.**

(FIGS. 3-5, TABLE 3)

Holotype. Female extracted from soil samples collected from olive in Marchena, Sevilla province, Spain, (37°18'22.61'' N latitude, 5°23'31.97'' W longitude) by M. Montes-Borrego and G. León Ropero, 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 Ol57-04).

Paratypes. Female, male and juvenile paratypes extracted from soil samples collected from olive orchards in Marchena, Sevilla province, Spain (collection numbers Ol57-01-Ol57-17); one female, one male, and one first-stage juvenile at Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (C.N.R.), Sezione di Bari, Bari, Italy (Ol57-02); two females and one male at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (Ol57-14); and one female, one male, and one first-stage juvenile at USDA Nematode Collection, Beltsville, MD, USA (T-6169p).

Etymology. The species name is derived from the Latin word *oleae* gen. fem. = olive (*Olea europaea* subsp. *europaea*), the plant from which the new species was isolated.

Description of female. Body spiral to close C-shaped when heat-relaxed, tapered anteriorly. Cuticle appearing smooth, 4.3 (4.0-5.0) μm thick, 15.4 (12.0-20.0) μm thick at tail tip, and marked by very fine superficial transverse striae mainly in tail region. Lip region wide, truncate and slightly concave, continuous with body contour, anteriorly almost flat. Amphidial fovea pocket-shaped, slightly asymmetrically bilobed. Labial papillae prominent. Stylet guiding ring single, 9.5-10.0 μm wide, located 2.2 ± 0.3 (1.8-2.8) times lip region diam. from anterior end. Lateral chord 14 (12-17) μm wide at mid-body or 16.6 (12.0-22.7)% of corresponding body diam. Odontostyle long and narrow, 1.8 (1.5-2.2) times as long as odontophore, straight or slightly arcuate, ca 3.5-4.0 μm wide towards its base; odontophore weakly developed, with rather weak basal swellings. Nerve ring encircling narrower part of pharynx. Anterior slender part of pharynx usually coiled in its posterior region. Basal bulb cylindrical, 136.6 ± 9.9 (125-151) μm long, 27.8 (23.0-34.5) μm diam. Dorsal pharyngeal gland nucleus (DN) and ventro-sublateral pair of nuclei (SN) situated at 27.0 ± 6.8 (14.9-37.9)%, 48.8 ± 2.8 (43.8-53.3)% of distance from anterior end of pharyngeal bulb, respectively. Glandularium 119.2 ± 9.0 (104-132) μm long. Cardia conoid-rounded, 11.2 ± 3.1 (7.0-15.0) μm long. Reproductive system with both genital branches equally developed, each 628 (478-806) μm long, with reflexed ovaries very variable in length. Vulva in form of a transverse slit, located at mid-body, vagina perpendicular to body axis, 20-26 μm long, or 24-29% of corresponding body width, surrounded by well developed muscles. Genital branches equally developed, 8.8 ± 2.1 (6.3-11.1), 8.2 ± 2.1 (6.2-10.8)% of body length, respectively. Uteri 284 (256-313) μm long, differentiated, with

sperm cells in most of the females examined; well developed sphincter between uterus and oviduct. Anterior and posterior oviduct of similar size. Ovaries equally developed, both of them with a single row of oocytes. Prerectum very variable in length, 6.4 (3.1-8.4) times anal body diam., and rectum 0.67 (0.6-0.7) times as long as anal body diam., anus a small rounded slit. Tail short, broadly conoid to hemispherical, with rounded terminus, bearing three pairs of caudal pores.

Male. Common, but less frequent (50%) than female. Morphologically similar to female except for genital system, but with posterior region strongly curved ventrally when heat relaxed. Male genital tract diorchic with testes opposed, containing multiple rows of different stages of spermatogonia. Tail conoid-rounded, with broad blunt terminus and thickened outer cuticular layer. Spicules arcuate, robust, 2.3 (2.0-2.5) times longer than tail length, lateral guiding pieces more or less straight or with curved proximal end. One pair of adanal supplements and 16 (13-19) midventral supplements.

Juveniles. Morphologically similar to adults, body assuming C- or J-shape. All four juvenile stages were found, being distinguishable by relative lengths of body and functional and replacement odontostyle (Table 3, Robbins *et al.* 1995; 1996). Lip region of all juvenile stages similar to that of adults. First stage-juveniles were characterised by a conoid-rounded tail with a c' ratio ≥ 1.5 (Fig. 5I), odontostyle length *ca* 70 μm , and shorter distance from anterior end to stylet guiding ring than that in adult stages (Table 3).

Diagnosis. *Longidorus oleae* sp. nov. is a gonochoristic species characterized by a long body (6.9-9.4 mm), assuming a spiral to closed C-shape when heat relaxed, tapered anteriorly; lip region wide, truncate and slightly concave, continuous with body contour, anteriorly almost flat, 16.0-21.0 μm wide; guiding ring located 36.5-46.0 μm from anterior end; long odontostyle (107-125 μm); amphidial fovea pocket-shaped, slightly asymmetrically bilobed; vulva equatorial; female tail short, broadly conoid to hemispherical, and bearing three pairs of caudal pores, c' ratio (0.6-0.8); males less frequent than females with long spicules (79-100 μm) and 14-20 ventromedian supplements; and specific D2-D3 rRNA, ITS1 rRNA and partial 18S rRNA sequences (GenBank accession numbers JX445103, JX445100, and JX445119, respectively). According to the polytomous key Chen *et al.* (1997) and the supplement by Loof and Chen (1999), the new species has the following code (codes in parentheses are exceptions): A45-B3(4)-C3(4)-D3-E3-F4(35)-G2(1)-H1-I2.

***LONGIDORUS ANDALUSICUS* SP. NOV.**

(FIGS. 3, 6-7, TABLE 4)

Holotype. Female extracted from fallow soil samples collected in Sanlúcar de Barrameda, Cádiz province, Spain, (36°49'01.88'' N latitude, 6°15'28.61'' W longitude) by G. León Ropero and J. Martín Barbarroja, 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 J172-01).

Paratypes. Female, male and juvenile paratypes extracted from fallow soil samples collected from Sanlúcar de Barrameda, Cádiz province, Spain (collection numbers J172-01- J172-14); and two females, two males and one first-stage juvenile at Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (C.N.R.), Sezione di Bari, Bari, Italy (J172-15); and three females, two males, and one first-stage juvenile at USDA Nematode Collection, Beltsville, MD, USA (T-6167p).

Etymology. The species name refers to Al-Andalus, the Arabic name of Andalusia (Spain), the region where the new species was found.

Description of female. Body assuming an open spiral to close C-shaped when heat-relaxed, narrowing towards anterior end. Cuticle appearing smooth, 3.5-4.0 µm thick at midbody, 10.5 (8.5-12.0) µm thick at tail tip, and marked by very fine superficial transverse striae mainly in tail region. Lip region narrow, conoid-rounded, continuous with body contour. Amphidial fovea pocket-shaped, symmetrically bilobed. Labial papillae prominent. Stylet guiding ring single, 8.5-9.0 µm wide, located 2.6 ± 0.2 (2.3-3.2) times lip region diam. from anterior end. Lateral chord 17.3 (17-18) µm wide at mid-body or 30.5 (28.3-31.8)% of corresponding body diam. Odontostyle narrow, 1.4 (1.2-1.5) times as long as odontophore, straight or slightly arcuate; odontophore weakly developed, with rather weak basal swellings. Nerve ring encircling narrower part of pharynx. Anterior slender part of pharynx usually coiled in its posterior region. Basal bulb cylindrical, 100.3 ± 8.3 (85-115) µm long, 21.6 (18.0-26.0) µm diam. Dorsal pharyngeal gland nucleus (DN) and ventro-sublateral pair of nuclei (SN) situated at 27.7 ± 2.7 (24.5-30.5)%, 55.9 ± 5.7 (49.0-62.5)% of distance from anterior end of pharyngeal bulb, respectively. Glandularium 82.8 ± 3.9 (78-88) µm long. Cardia conoid-rounded, 8.6 ± 1.4 (7.0-

10.0) μm long. Reproductive system with both genital branches equally developed, each 290-505 μm long, with reflexed ovaries very variable in length. Vulva in form of a transverse slit, located at mid-body, vagina perpendicular to body axis, 18 (17-21) μm long, or 26-36% of corresponding body width, surrounded by well developed muscles. Genital branches equally developed, 8.8 ± 2.4 (6.3-11.1), 8.2 ± 2.3 (6.2-10.8)% of body length, respectively. Uteri 103-124 μm long, differentiated, with sperm cells in most of the females examined; sphincter between uterus and oviduct weakly developed. Anterior and posterior oviduct of similar size. Ovaries equally developed, both of them with a single row of oocytes. Prerectum very variable in length, 11.4 (6.3-13.6) times anal body diam., and rectum 0.8 (0.7-0.9) times as long as anal body diam., anus a small rounded slit. Tail short, bluntly convex-conoid to hemispherical, with rounded terminus, bearing three pairs of caudal pores.

Male. Common, but less frequent (63%) than female. Morphologically similar to female except for genital system, but more coiled in the posterior region. Tail conoid-rounded, with broad blunt terminus and thickened outer cuticular layer. Spicules arcuate, robust, 2.1 (1.7-2.5) times longer than tail length, lateral guiding pieces more or less straight or with curved proximal end. One pair of adanal supplements preceded by a row of 13-14 ventromedian supplements.

Intersex: Similar to female in general morphology and morphometric data, except having weakly developed spicules, 30 μm long, one weakly developed precloacal supplement located at 11 μm from cloacal opening, and a row of 7 ventromedian supplements.

Juveniles. Morphologically similar to adults, except for smaller size and a relatively longer tail. All four juvenile stages were found, being distinguishable by relative lengths of body and functional and replacement odontostyle (Table 4, Robbins *et al.* 1995; 1996). Lip region of all juvenile stages similar to that of adults. First stage-juveniles were characterised by a cylindrical-rounded tail with a c' ratio ≥ 1.5 (Fig. 7F), an odontostyle length *ca* 40 μm , and shorter distance from anterior end to stylet guiding ring than that in adult stages (Table 4).

Diagnosis. *Longidorus andalusicus* sp. nov. is a gonochoristic species characterized by a medium body (3.4-5.0 mm), narrowing towards anterior end and assuming an open spiral to closed C-shape when heat relaxed; lip region narrow, conoid-rounded, continuous with body contour, 10.0-12.0 μm wide; guiding ring located 26.0-31.5 μm from anterior end; odontostyle 67.0-80.0 μm long; amphidial fovea pocket-shaped, symmetrically bilobed; vulva equatorial;

female tail short, bluntly convex-conoid to hemispherical and bearing three pairs of caudal pores, c' ratio (0.7-1.0), males as frequent as females with spicules 55.0-68.0 µm long and 14-16 ventromedian supplements; and specific D2-D3 and partial 18S sequences (GenBank accession numbers JX445101 and JX445102, and JX445118, respectively). According to the polytomous key Chen *et al.* (1997) and the supplement by Loof and Chen (1999), the new species has the following code (codes in parentheses are exceptions): A2-B1(2)-C2(3)-D1-E2-F2-G1(2)-H1(2)-I2.

MORPHOLOGY AND MORPHOMETRICS OF SPANISH POPULATIONS OF *LONGIDORUS* SPECIES

(FIGS. S1-S6, TABLES S1-S2)

Morphological and morphometrical data as well as molecular delineation (rDNA) of *Longidorus alveus* Roca, Pereira and Lamberti 1989 and *Longidorus magnus* Lamberti, Bleve-Zacheo and Arias 1982, were previously studied and compared with original descriptions and paratype specimens within a study of dagger and needle nematodes infesting vineyards in southern Spain (Gutiérrez-Gutiérrez *et al.* 2011). Thus, only D2-D3 sequences were reported for these samples since other morphological data were identical to previous data (Gutiérrez-Gutiérrez *et al.* 2011). For other known species studied, a brief description and a morphometric comparison with previous records is provided below.

LONGIDORUS CRATAEGI ROCA AND BRAVO 1996

(FIG. S1, TABLE S1)

The *Longidorus* population from grapevine at Montemayor (Córdoba province) agrees fairly well with studied paratypes and original description of *L. crataegi*. This population was characterised by a lip region continuous with the rest of the body, rounded laterally and almost flattened frontally; amphidial fovea pouch-shaped, distinctly asymmetrically bilobed; tail conoid-rounded, dorsally convex with rounded terminus (Table S1, Fig. S1). Morphometrics are similar with those provided in the original description, except for some differences in c ratio, odontophore length, diameter of lip region and spicules length, which may be due to few specimens originally studied or geographical intraspecific variability (Roca and Bravo 1996). This is the first report for Spain and confirms a wider distribution in the Iberian Peninsula, apart from original description in Portugal. According to the polytomous key Chen *et al.* (1997) and the supplement by Loof and Chen (1999), this species has the following code: A4 B34 C3 D1 E3 F345 G12 H1 I2.

LONGIDORUS FASCIATUS ROCA AND LAMBERTI 1981

(FIG. S2, TABLE S1)

The gonochoristic population of *Longidorus* from grapevine at Monturque (Córdoba province) agrees fairly well with the original description and examined paratypes of *L. fasciatus*. Females were characterized by a narrow conoid-rounded lip region; amphidial fovea pouch-shaped and asymmetrically bilobed; and tail bluntly-rounded. Males were almost as common as females and were reported for the first time in this study. They showed a habitus mostly similar to that of female in open C but with posterior region curved ventrally, and were characterized by a genital tract diorchic with testes opposed; tail short, bluntly rounded, dorsally convex and ventrally slightly concave; spicules ventrally arcuate; adanal pair of supplements preceded by a row of 16-17 ventromedian supplements (Table S1, Fig. S2). Only one first-stage juvenile specimen was found, and it is reported here for the first time and characterized by a lip region similar to adults and tail cylindrical with rounded terminus (Table S1, Fig. S2). Morphometrics of grapevine population was coincident with original description, except for minor differences in odontophore length, viz. 60 (54-71) μm vs 58 (46-70) μm (Roca and Lamberti 1981). The species has been reported in Italy and Greece and it is the first report in Spain, suggesting a Mediterranean distribution (Roca and Lamberti 1981). According to the polytomous key Chen *et al.* (1997) and the supplement by Loof and Chen (1999), this species has the following code (codes in parentheses are exceptions): A4 B1(2) C3(4) D1 E3 F4(3) G2 H1 I2.

LONGIDORUS INTERMEDIUS KOZLOWSKA AND SEINHORST 1979

(FIG. S3, TABLE S1)

The *Longidorus* population from Cortes de la Frontera (Cádiz province) agrees fairly well with studied paratypes and original description of *L. intermedius*. This population was characterised by a lip region very slightly set off by shallow depression, rounded anteriorly; amphidial fovea pouch-shaped, not or slightly bilobed; tail with dorsal curvature, almost straight ventrally, bluntly conical with rounded terminus (Table S1, Fig. S3). Morphometrics are coincident with those provided in the original description and also similar to data reported subsequently for populations from several other European countries (Kozłowska and Seinhorst 1979; Andrés and Arias 1987; Roca *et al.* 1991; Peneva *et al.* 2001; Kumari *et al.* 2006). This record confirms that this species is closely associated with the genus *Quercus* in general, although the first record in Spain (also in Cádiz province) was associated with olive (Andrés and Arias 1987). The alphanumeric codes for this population of *L. intermedius* to be applied to the polytomic identification

key for *Longidorus* species by Chen *et al.* (1997) and the supplement by Loof and Chen (1999) are (codes in parentheses are exceptions): A4(3)-B12-C2(3)-D3-E2-F2-G12-H2-I1.

LONGIDORUS IUGLANDIS LAMBERTI AND AGOSTINELLI 1984

(FIG. S4, TABLE S1)

The *Longidorus* population from grapevine at Bonares (Huelva province) agrees fairly well with studied paratypes and original description of *L. iuglandis*. This population was characterised by a narrow lip region, continuous with body contour, conoid-rounded; amphidial fovea pouch-shaped, more or less asymmetrically bilobed; long odontostyle (118-131 μm); tail conoid-rounded to hemispherical with rounded terminus (Table S1, Fig. S4). Morphometrics are coincident with those provided in the original description and also similar to data reported subsequently for populations from Italy and Slovak Republic (Roca *et al.* 1990, 1991; Lisková and Brown 1999). Therefore, this is the first report for Spain, suggesting a Mediterranean distribution. The alpha-numeric codes for this population of *L. iuglandis* to be applied to the polytomic identification key for *Longidorus* species by Chen *et al.* (1997) and the supplement by Loof and Chen (1999) are (codes in parentheses are exceptions): A45-B2(1)-C3-D1-E2-F3(4)-G1(2)-H1-I2.

LONGIDORUS RUBI TOMILIN & ROMANENKO 1993

(FIG. S5, TABLE S1)

The *Longidorus* population from Stone pine at Hinojos (Huelva province) agrees fairly well with the redescription of *L. rubi* (Romanenko 1998). This population was characterised by a rounded and somewhat expanded lip region, offset by a depression (Fig. S5); amphidial fovea pouch-shaped, symmetrically bilobed; moderately long odontostyle (82-90 μm); tail conoid-rounded, slightly curved ventrally with rounded terminus (Table S1, Fig. S5). The male of *L. rubi* is described here for the first time. The male was less common than females but morphologically similar to female except for reproductive system. Amphidial fovea pouch-shaped, symmetrically bilobed; body ventrally arcuate, more strongly curved in posterior region due to well developed copulatory muscles (Fig. S5). Ten to eleven ventromedian precloacal papillae at equal distance from each other, and anterior to the adanal pair (Fig. S5). Spicules ventrally curved, weakly sclerotized. Copulatory muscles and spicule protractor and retractor muscles well developed (Fig. S5). Tail narrowly conoid to a rounded terminus, with two or rarely three caudal pores (Fig. S5). Morphometrics are coincident with those provided in the redescription except for slightly higher a, and c ratio, and shorter distance between oral aperture

and guiding ring (Romanenko 1998). The alpha-numeric codes for this population of *L. rubi* to be applied to the polytomic identification key for *Longidorus* species by Chen *et al.* (1997) and the supplement by Loof and Chen (1999) are (codes in parentheses are exceptions): A3 B1(2) C2 D2 E2 F32 G3 H56 I1.

LONGIDORUS VINEACOLA STURHAN AND WEISCHER 1964

(FIG. S6, TABLE S2)

The three gonochoristic populations of *L. vineacola* from vineyards and Stone pine in Montilla (Córdoba province), Almonte (Huelva province), and El Cuervo de Sevilla (Sevilla province) in southern Spain (Table S2) agrees fairly well with *L. vineacola*. The three studied populations were characterised by a long body (8.0-11.0 mm); a slightly offset lip region; amphidial fovea pouch-shaped and asymmetrically bilobed; odontostyle 90-100 µm long; and broadly rounded tail (Fig. S6). Morphometrics are nearly coincident with those provided in the original description and rather similar to data reported subsequently for other European populations, except for minor intraspecific variations in a, and c ratio, and spicules length (Boag and Brown 1987; Andrés *et al.* 1991; Roca and Bravo 1996). This species was originally described from grapevine in Germany (Sturhan and Weischer 1964), but later on it has been reported sporadically from several European countries and host [*i.e.*, Belgium, England, Greece, Israel, Netherlands; almond tree, celery, grapevine, grasses, hop, maize, onion, pine, potato (Brown and Taylor 1987)]. The alpha-numeric codes for these populations of *L. vineacola* to be applied to the polytomic identification key for *Longidorus* species by Chen *et al.* (1997) and the supplement by Loof and Chen (1999) are: A3-B34-C23-D2-E3-F45-G3-H1-I2.

MOLECULAR CHARACTERISATION OF *LONGIDORUS BAETICUS* SP. NOV., *LONGIDORUS OLEAE* SP. NOV., AND *LONGIDORUS ANDALUSICUS* SP. NOV.

Amplification of the D2-D3 expansion segments of 28S rDNA, 18S rDNA and ITS1 rRNA from the three new and the known *Longidorus* spp. yielded single fragments of approximately 800 bp, 1500 bp and 1100 bp, respectively, based on direct fragment sequencing. D2-D3 sequences of *Longidorus baeticus* sp. nov. (JX445106, JX445107) matched well with the *Longidorus* spp. deposited in GenBank. These sequences were 90% (74 nucleotide differences) to 88% (98 nucleotide differences) similar to *L. goodeyi* (AY601581) and *L. dunensis* Brinkman, Loof and Barbez 1987 (AY593057), respectively.. Intra-specific variation of D2-D3 segments detected between the two studied populations (grapevine and citrus) was 13

nucleotides (98% similarity) and 2 indels (0.26%). The partial 18S of *L. baeticus* sp. nov. (JX445121) matched closely (99% similarity, 19 nucleotides differences) with those for *Longidorus magnus* Lamberti, Bleve-Zacheo and Arias, 1982 (HM921345) and *L. vineacola* (AY283169); and 98% similarity with several species of *Longidorus* such as *Longidorus fragilis* Thorne 1974 (AY283172), *Longidorus grandis* Ye and Robbins, 2003 (AY283165) or *L. elongatus* (GU199044), and varying from 28 to 31 nucleotides. However, the ITS1 of *L. baeticus* sp. nov. (JX445093) did not find homologies with sequences deposited in GenBank.

D2-D3 expansion segments of 28S rDNA from *L. oleae* sp. nov. (JX445103) matched well with the D2-D3 sequences of *Longidorus* spp. deposited in GenBank, being 92, 91, 90% similar to *L. orientalis* (GQ988722), *L. goodeyi* (AY601581) and *L. magnus* (HM921361), respectively; and varied from 65, 70, to 76 nucleotides, respectively. The partial 18S of *L. oleae* sp. nov. (JX445119) showed a high homology with *L. vineacola* (AY283169), *L. magnus* (HM921345) or *L. fragilis* (AY283172), and differing in 2, 8, to 12 nucleotides, respectively. However, the ITS1 of *L. oleae* sp. nov. (JX445100) did not find homology with sequences deposited in GenBank.

D2-D3 expansion segments of 28S rDNA from *L. andalusicus* sp. nov. (JX445101, JX445102) matched well with the D2-D3 sequences of *Longidorus* spp. deposited in GenBank, being 94% to 88% similar to *L. orientalis* (GQ988722), *L. goodeyi* (AY601581) and *L. magnus* (HM921361), respectively; and varied from 40 to 83 nucleotides, respectively. Intra-specific variation of D2-D3 segments detected among the two studied populations (fallow and glasswort) was 2 nucleotides (99% similarity) and 1 indel. The partial 18S of *L. andalusicus* sp. nov. (JX445118) matched closely (99% similarity) with those for *L. vineacola* (AY283169), *L. magnus* (HM921345) and *L. elongatus* (EU503141), and varying from 4 to 12 nucleotides. Using the D2-D3 expansion segments of 28S rDNA and ITS1 rRNA the new species could be clearly separated from all other *Longidorus* spp.

MOLECULAR CHARACTERISATION OF OTHER KNOWN *LONGIDORUS* SPECIES

D2-D3 of *L. crataegi* from vineyards (JX445114) was 96% and 95% similar to *L. goodeyi* (AY601581) and *L. magnus* (HM921361), respectively, and varied from 30 to 36 nucleotides. The partial 18S of *L. crataegi* (JX445124) matched closely (99% similarity) with several species of *Longidorus* such as *L. piceicola* (AY687993), *Longidorus elongatus* (de Man 1876) Thorne and Swanger, 1936 (AY687992, GU199044, AF036594), *L. uroshis* (EF538760), or *Longidorus attenuatus* Hooper 1961 (AY687994), and varying from 3 to 9 nucleotides. D2-D3 of *L. fasciatus* from vineyards (JX445108) was 91% and 90% similar to *L. magnus* (HM921361), and

L. orientalis GQ988722) respectively, and varied from 71 to 83 nucleotides. The partial 18S of *L. fasciatus* (JX445122) matched closely (99% similarity) with several species of *Longidorus* such as *L. vineicola* (AY283169), and *L. magnus* HM921345) or 98% similarity with *L. fragilis* (AY283172) or *Longidorus tabrizicus* Niknam *et al.*, 2010 (FJ009678). The ITS1 of *L. fasciatus* (JX445097) showed scarce homology with other ITS1 of *Longidorus* spp. sequenced and available in GenBank (i. e. 76-83% similarity with *L. orientalis* but coverage of 53-80%). D2-D3 sequence of *L. intermedius* from cork oak (JX445117) was 99% similar to *L. intermedius* (AF480074) from Planegg, Germany, and varied in 9 nucleotides and 2 indels. The partial 18S of *L. intermedius* (JX445126) matched closely (99% similarity) with several species of *Longidorus* such as *L. piceicola* (AY687993), *L. elongatus* (AY687992, GU199044, AF036594), *L. uroshis* (EF538760), or *L. paravineicola* (AY283157), and varying from 5 to 15 nucleotides. D2-D3 sequences of *L. iuglandis* from grapevine and sunflower (JX445104 and JX445105) were 91% similar to *L. magnus* (HM921361), 90% to *L. goodeyi* (AY601581), 89% to *Longidorus athesinus* Lamberti, Coiro and Agostinelli 1991 (AY601574) or 88% to *Longidorus moesicus* Lamberti, Choleva and Agostinelli 1983. No intra-specific variation of D2-D3 segments was detected between the two studied populations. ITS1 of *L. iuglandis* (JX445099) showed scarce homology with other ITS1 of *Longidorus* spp. sequences available in GenBank (i. e. 73-84% similarity with *L. orientalis*). Partial 18S of *L. iuglandis* (JX445120) matched closely (99% similarity) with several species of *Longidorus* such as *L. magnus* (HM921345), *L. vineicola* (AY283169), *L. fragilis* (AY283172), or *L. elongatus* (EU503141), and varying from 8 to 14 nucleotides. D2-D3 of *L. rubi* (JX445116) was 94% similar to *L. goodeyi* (AY601581), 93% to two isolates of *L. magnus* (HM921361, HM921362), 91% to two isolates of *L. orientalis* (GQ988722, GU001823) and *L. euonymus* Mali & Hooper 1974 (AY601573), and varying from 35 to 52 nucleotides. The partial 18S of *L. rubi* (JX445125) matched closely (99% similarity) with several species of *Longidorus* such as *L. fragilis* (AY283172), *L. elongatus* (EU503141), *L. uroshis* (EF538760) or *L. dunensis* (AY284819), and varying from 8 to 9 nucleotides. The ITS1 of *L. rubi* showed also a scarce homology with other ITS1 of *Longidorus* spp. sequenced and available in GenBank (i. e. 85% similarity with *L. arthensis* but coverage of only 46%). D2-D3 of *L. vineicola* (JX445009-JX445111) matched well with the D2-D3 sequences of *Longidorus* spp. deposited in GenBank, being 92% to 89% similar to some of them, including *L. orientalis* (GQ988722) and *L. apulus* Lamberti & Blevé-Zacheo 1977 (AY601571), respectively; and varied from 67 to 82 nucleotides, respectively. Intra-specific variation of D2-D3 segments detected among the three studied populations of *L. vineicola* (grapevine, stone pine and wheat) was from 3 to 7 nucleotides (99% similarity) with

no indels. The partial 18S of *L. vineacola* (JX445123) matched closely (99% similarity) with several species of *Longidorus* such as *L. vineacola* (AY283169), *L. magnus* (HM921345), *L. elongatus* (AY687992, GU199044, AF036594), *L. fragilis* (AY283172) or *L. paravineacola* (AY283157), and varying from 2 to 23 nucleotides. The ITS1 of *L. vineacola* (JX445094, JX445096) showed also scarce homology with other ITS1 of *Longidorus* spp. available in GenBank (70–75% similarity) and varying from 97 nucleotides with *L. orientalis* (GU001821) to 141 nucleotides with *L. magnus* (HM921340).

PHYLOGENETIC RELATIONSHIPS OF THE GENUS *LONGIDORUS*

Phylogenetic relationships among *Longidorus* spp. inferred from analyses of D2-D3 expansion segments of 28S rRNA, ITS1 rRNA and the partial 18S rRNA gene sequences using BI and ML are given in Figures 8, 9, and 10, respectively. No significant differences in topology were obtained using the BI or ML approach and only a few species in some minor clades with low bootstrap support were not congruent with the general topology tree. The 50% majority rule consensus BI and ML trees of a multiple alignment including 75 D2-D3 sequences and 753 bp consisted of 5 moderate to highly supported major clades in the genus *Longidorus* (Fig. 8): (i) nine *Longidorus* spp. from Southern Spain, including the three new species (*baeticus* sp. nov., *fasciatus*, *iuglandis*, *andalusicus* sp. nov., *orientalis*, *oleae* sp. nov., *magnus*, *crataegi*, and *vineacola*) and *L. goodeyi* (AY601581) from Peebles, Scotland (UK) (PP = 100%; BS = 89%); (ii) nine *Longidorus* spp. mostly from central Europe (*piceicola*, *carpathicus*, *uroshis*, *elongatus*, *juvenilis*, *distinctus*, and *leptocephalus*) and *L. intermedius* from Germany and southern Spain (PP = 100%; BS = 89%); (iii) twelve *Longidorus* spp. from several geographic origins, including Switzerland, Italy, Germany, Netherlands, Hungary, Bulgaria and Cuba (*arthensis*, *sturhani*, *raskii*, *apulus*, *athesinus*, *edmundsi*, *euonymus*, *kuiperi*, *attenuatus*, *dunensis*, *profundorum*, and *moesicus*); (iv) a moderately supported clade (PP = 77%; BS = 99%) including the *Paralongidorus* spp. with the exception of *P. bikanerensis* (JN032584), and *L. mindanaoensis* (HQ735098) from Philippines as sister species; and (v) a well supported clade (PP = 100%; BS = 99%) with six species (*poessneckensis*, *carniolensis*, *Longidorus* sp. FDL-2011, *helveticus*, *macrosoma*, and *caespiticola*) mostly from Slavic countries. Phylogenetic inferences based on D2-D3 also suggest that *L. alveus* and *L. rubi* are sister species (PP = 100%; BS = 93%), *L. breviannulatus* (AY601576) from USA is a sister species of clade (ii), *L. africanus* from USA (AY601583) is a sister species of clade (iii), and *P. bikanerensis* is a sister species of clade (v), and *L. camelliae* (AY601585), *L. henanus* (HQ822267), and *L. diadecturus* (AY601584) were not associated to any of the above referred clades (Fig. 8).

Phylogenetic analysis (BI and ML) of *Longidorus* spp. based in ITS1 of a multiple edited alignment including 45 sequences and 1223 total characters showed five clades clearly separated (Fig. 9): (i) a moderately supported clade (PP= 87%; BS=84%) formed by 8 species, including *pius*, *Longidorus* sp. FDL-2011, *raskii*, *alveus*, *profundorum*, *arthensis*, *elongatus*, and *diadecturus*; (ii) a moderately supported clade (PP= 66%; BS=71%) including two new species from Spain (*baeticus* n. sp. and *oleae* n. sp.) and others known species from southern Spain (*vineacola*, *magnus*, *orientalis*, *iuglandis* and *fasciatus*); (iii) a moderately supported clade (PP= 78%; BS=100) formed by 4 species, including *macrosoma*, *helveticus*, *africanus* and *rubi*; (iv) a moderately supported clade (PP=86%; BS=60%) formed by *paralongicaudatus*, *biformis*, *fragilis*, *breviannulatus*, *sturhani* and *kheirii*; and the last low supported clade (PP= 72%; BS=56%) (v), which includes 3 species, *grandis*, *tabrizicus* and *kuiperi*. ITS1 from *Paralongidorus* spp. were avoided for phylogenetic analysis because they showed a very low homology with ITS1 sequences from *Longidorus* spp.

Similarly, the 50% majority rule consensus BI and ML tree of a multiple alignment including 53 18S sequences and 1721 bp consisted of six low to highly supported major clades in *Longidorus* (Fig. 9): (i) a major clade well supported (PP = 95%; BS = 75%) formed by 13 species, including *biformis*, *americanus*, *paralongicaudatus*, *breviannulatus*, *crassus*, *tabrizicus*, *sturhani*, *kheirii*, *profundorum*, *euonymus*, *grandis*, *paravineacola* and *fragilis*; (ii) a low supported clade (PP = 59%; BS = 75%) formed by 8 species, including cf. *intermedius*, *elongatus*, *attenuatus*, *crataegi*, *uroshis*, *piceicola*, *intermedius* and *leptocephalus*; (iii) a well supported clade (PP = 100%; BS = 87%) formed by 8 species, including the three new species and others known species from southern Spain (*fasciatus*, *vineacola*, *iuglandis*, *magnus* and *orientalis*); (iv) a moderately supported clade (PP = 68%; BS = 85%) formed by 6 species, including *helveticus*, *macrosoma*, *poessneckensis*, *africanus*, *ferrisi* and *mindanaoensis*; (v) a moderately supported clade (PP = 66%; BS = 85%) including all *Paralongidorus* species; and (vi) a well supported clade (PP = 98%; BS = 67%) including three species (*diadecturus*, *litchi*, and *henanus*).

DISCUSSION

We described here three new species of the genus *Longidorus* from Southern Spain, based on morphological and molecular data. The main purpose of this research was to understand the phylogenetic relationships among the new and known species of the genus *Longidorus* spp. based on nuclear rDNA.

MORPHOLOGICAL COMPARISON OF *LONGIDORUS BAETICUS* SP. NOV., *LONGIDORUS OLEAE* SP. NOV.,
AND *LONGIDORUS ANDALUSICUS* SP. NOV. WITH RELATED TAXA

According to the polytomous key Chen *et al.* (1997) and the supplement by Loof and Chen (1999), and on the basis of body and odontostyle length, a and c' ratio, lip region, amphidial fovea, female and J1 tail, *L. baeticus* sp. nov. is close to *L. crataegi*, *L. fasciatus*, *L. iuglandis*, *L. moesicus*, *Longidorus olegi* Kankina & Metlitskaya 1983, *Longidorus raskii* Lamberti & Agostinelli 1993, and *Longidorus silvae* Roca 1993 from which it can be differentiated by a combination of these characters, but particularly in the tail shape of J1 (bluntly rounded to cylindrical vs conoid) and odontostyle length (115-133 µm vs 102-111, 96-106, 106-108, 99-104, 131-146 µm) (Table 5). Since there is a general agreement about the intraspecific morphometric variability of *Longidorus* species (Ye and Robbins, 2004b), differences among *L. baeticus* sp. nov. with *L. fasciatus* and *L. iuglandis* result in great overlap of morphometrics and upon rDNA analyses showing clear differences in D2D3 and ITS1 need to be considered a complex of cryptic species. Similarly, *L. oleae* sp. nov. is close to *Longidorus balticus* Brzeski, Peneva and Brown 2000, *Longidorus belloii* Andrés and Arias 1988, *Longidorus kheirii* Pedram, Niknam, Robbins, Ye & Karegar 2008, *L. paravineacola*, *Longidorus pauli* Lamberti, Molinari, De Luca, Agostinelli and Di Vito 1999, *Longidorus profundorum* Hooper 1965, and *L. uroshis*, from which it can be differentiated by a combination of diagnostic characters included in the polytomous key, but particularly in odontostyle length (107-125 µm vs 91-105, 85-107, 113-130, 101-114, 102-118 µm), the shape of amphidial fovea, and the tail shape of J1 (Table 5). Finally, *Longidorus andalusicus* sp. nov. is close to *Longidorus congoensis* Aboul-Eid 1970, *L. crataegi*, *Longidorus igoris* Krnjaic, Lamberti, Krnjaic, Agostinelli & Radicci 2000, *Longidorus jiangsuensis* Xu & Hooper 1990, *L. orientalis*, and *Longidorus psidii* Khan & Khan 1972, from which it can be differentiated by a combination of diagnostic characters included in the polytomous key, but particularly in odontostyle length (67-80 µm vs 66-81, 96-106, 77-106, 82-91, 99-104, 88-105 µm) and the shape of amphidial fovea (Table 5).

The occurrence of intersex in *L. andalusicus* sp. nov. confirms that this phenomenon is quite common in Longidoridae, since it has been reported in several *Longidorus* species, viz. *L. biformis* (Ye and Robbins 2004a) and *L. elongatus* (Robbins 1986), and recently in *P. bikanerensis* (Pedram *et al.* 2012).

The comparative morphological and morphometrical studies of the 17 Spanish *Longidorus* populations confirmed that diagnostic and identification of these species based solely on diagnostic morphometric features is quite complex since there is almost a continuous range of characters measurements within populations as well as among species. The present

results (including new and known species) enlarge the biodiversity of *Longidorus* in the Iberian Peninsula and agree with the results obtained for the phylogeny and biogeography of the genus *Longidorus* in the Euro-Mediterranean region (Navas *et al.* 1993), in which a dispersalist model was one of the primary explanations for the large groups of *Longidorus* species found in that region.

MOLECULAR AND PHYLOGENETIC RELATIONSHIPS IN *LONGIDORUS*

Sequences of nuclear ribosomal RNA genes, particularly D2D3 and ITS1, have proven to be a powerful tool for providing accurate species identification and assessing phylogenetic patterns useful for molecular characterisation and reconstruction of phylogenetic relationships of Longidoridae (He *et al.*, 2005; Gutiérrez-Gutiérrez *et al.* 2012; Palomares-Rius *et al.*, 2012). Our results confirm the usefulness of these markers, since nucleotide differences among species ranged from 40 to 98 nucleotides for D2D3 and 305 to 370 nucleotides for ITS1. However, our findings also confirm that partial 18S sequence does not have enough resolution to distinguish species, since different species showed as low as 2-4 nucleotide differences among species. The phylogenetic relationships inferred in this study based on the D2-D3 and the partial 18S sequences mostly agree with the lineages obtained by Gutiérrez *et al.* (2011) and Palomares-Rius *et al.* (2012) with the phylogeny of dagger and needle nematodes. Only one reference was found for phylogenetic relationships of *Longidorus* spp. based on the ITS1 region (Ye *et al.* 2004), which showed quite similarity with the tree obtained in this study. Small differences may be due to the different phylogenetic methods and additional sequences added in our study. ITS1 diversity among *Longidorus* spp. was higher than that for 18S and D2-D3 fragments. Consequently, this marker should be considered as a very useful tool for the species identification within this genus, and only is useful for resolving relationships among close related species because of nucleotide variation and indels.

Phylogenetic analyses based on D2-D3, ITS1 and partial 18S using BI and ML (Figs. 8, 9, 10) showed a congruent position of the new sequenced species of *Longidorus* spp. from Spain, grouping in a separate clade, except for *L. intermedius* (JX445117, JX445119) and *L. rubi* (JX445125, JX445100) in D2-D3, partial 18S and ITS1 trees, and *L. crataegi* (JX445124) in partial 18S trees which grouped separately (Figs. 8, 9, 10). However, the majority of the species with common sequences in all the ribosomal markers used showed congruence in the clade obtained. Only some species showed not congruence between the different ribosomal markers (i. e. *L. profundorum*, *L. diadecturus*, *L. kuiperi*). This could be due to the sequence origins of these species, different rates of nucleotide differences in the markers or more

difficulties in sequence alignment in ITS1 sequences. In our case, the use of different markers using the same individual showed the congruence of the markers used. This may suggest that southern Spain may be a centre of speciation for this group of *Longidorus* species. Similar observations for this geographical region have also been detected in other genera such as *Xiphinema americanum*-group complex (Gutiérrez-Gutiérrez *et al.* 2012) or *Rotylenchus* spp. (Cantalapiedra-Navarrete *et al.* 2012). He *et al.* (2005) detected a correspondence between shape of amphids and the molecular phylogeny of *Longidorus* based on D2-D3 fragments. Our results mostly agree with He *et al.* (2005) since clade (i) included species with an amphidial fovea pouch-shaped with asymmetrical lobes except for *L. andalusicus* sp. nov. which showed pocket-shaped symmetrically bilobed amphids (Figs. 6-8); clades (ii and iii) included species with amphidial fovea pouch-shaped with symmetrical lobes, except for *L. uroshis*, *L. distinctus*, and *L. moesicus* which are asymmetrically bilobed, and *L. kuiperi* with pocket-shaped not bilobed amphid (Fig. 8); clade (iv) included *Paralongidorus* species with stirrup- or funnel-shape, except for *L. mindanaoensis* with long oval amphid (Fig. 8); and clade (v) with large pouch-shaped not bilobed amphids. Similar congruence results were also observed for ITS1 and the partial 18S (Figs. 9, 10) with more exceptions in the partial 18S tree. Coomans *et al.* (2012) reported that the stirrup and funnel shaped amphidial fovea could be the plesiomorphic shape in longidorids, while amphidial fovea bilobed (symmetric or asymmetric) could be a derived feature. Future phylogenetic studies should include other additional genetic markers such as mitochondrial DNA genes and nuclear protein coding genes in order to resolve the relationships within *Longidorus* and *Paralongidorus*. Others characters (i. e. lip region shape, body and odontostyle length, female tail shape, etc.) did not show a clear relationship with the phylogeny showed in this study. Also, a more comprehensive molecular analysis on such genetic markers based on a worldwide sample of *Longidorus* isolates may clarify the correspondence of morphological and molecular analyses which have been pointed out in this and previous studies.

CONCLUSIONS

Consequently, these data strengthened that *Longidorus* species delimitation should be the result of integrated studies based on morphology, morphometry and molecular taxonomic identification and phylogeny of D2-D3 region ITS1 of rRNA, and in a lesser extend of partial 18S-rRNA sequences. Future phylogenetic studies should include other additional genetic markers as mitochondrial DNA genes and nuclear protein coding genes such as cytochrome c oxidase subunit 1 (*COI*) or heat shock protein (*hsp90*) genes, in order to resolve the relationships within *Longidorus*. And also, the discovery of several new records of *Longidorus*

spp. for Spain suggests that the biodiversity of these nematodes is still not fully clarified, and need some additional studies. Phylogenetic analyses based on the three molecular markers resulted in a general consensus of species grouping, since lineages were maintained for the majority of species (i.e. species with a conoid-rounded lip region, amphidial fovea asymmetrically bilobed, female tail bluntly rounded), but not in some others (i. e. position of *L. crataegi*, *L. intermedius*, *L. rubi* were quite variable).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Light micrographs of *Longidorus crataegi* Roca & Bravo, 1996. A, anterior region. B-D, lip regions. E, detail of vulva. F-I, K, female tails. J, first-stage juvenile tail. L, detail of male reproductive system. M, male tail. Abbreviations: a = anus; af = amphidial fovea; t = testis. (Scale bars: A-M = 20 µm).

Fig. S2. Light micrographs of *Longidorus fasciatus* Roca & Lamberti, 1981. A-E, lip regions. F-H, female tails. I, male tail. J, first-stage juvenile tail. (Scale bars: A -J = 20 µm).

Fig. S3. Light micrographs of *Longidorus intermedius* Kozłowska and Seinhorst, 1979. A, whole female body. B, C, anterior region. D-E, lip regions. F, detail of pharyngeal bulb. G, H, female tails. I, first-stage juvenile tail. (Scale bars: A = 100 µm; B-I = 20 µm).

Fig. S4. Light micrographs of *Longidorus iuglandis* Roca, Lamberti and Agostinelli, 1984. A, anterior region. B-C, lip regions. D, detail of pharyngeal bulb. E, vulval region. F, detail of lateral cord at mid-body. G-H, female tails. Abbreviations: a = anus; af = amphidial fovea; lhc = lateral cord; V = vulva. (Scale bars: A- H = 20 μ m).

Fig. S5. Light micrographs of *Longidorus rubi* Tomilin & Romanenko, 1993. A-F, lip regions. E, detail of vulva. G-J, female tails. K, L, male tail. (Scale bars: A-K = 20 μ m).

Fig. S6. Light micrographs of *Longidorus vineacola* Sturhan & Weischer, 1954. A-E, lip regions. E, detail of vulva. F-H, female tails. I, first-stage juvenile tail. J, male tail. Abbreviations: a = anus; af = amphidial fovea. (Scale bars: A-J = 20 μ m).

Table S1. Morphometrics of *Longidorus crataegi*, *L. fasciatus*, *L. intermedius*, *L. iuglandis*, and *L. rubi* from southern Spain.

Table S2. Morphometrics of three populations of *Longidorus vineacola* from southern Spain.

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Figure legends

Fig. 1. Line drawings of *Longidorus baeticus* sp. nov. A, Pharyngeal region; B, C, Detail of lip region; D, Vulval region; E, F, Female tail; G, Male tail.

Fig. 2. Light micrographs of *Longidorus baeticus* sp. nov. A-E, anterior regions. F, detail of pharyngeal bulb. G, vulval region. H-I, female tails. J-K, male tail. L-O, first-, second-, third-, and fourth-stage juvenile tails, respectively. Abbreviations: a = anus; af = amphidial fovea; n = subventral nuclei; v = vulva; sp = spicules; spl = ventromedian supplements. (Scale bars: A-O = 20 µm).

Fig. 3. Relation of body length with length of functional and replacement odontostyle (ost and rost, respectively) length in all developmental stages from J1 to mature females of: (A) *Longidorus baeticus* sp. nov.; and (B) *Longidorus oleae* sp. nov.; and (C) *Longidorus andalusicus* sp. nov.

Fig. 4. Line drawings of *Longidorus oleae* sp. nov. A, Pharyngeal region; B, C, Detail of lip region; D, Vulval region; E, F, G, Female tail; H, Male tail.

Fig. 5. Light micrographs of *Longidorus oleae* sp. nov. A, anterior region. B-C, lip region. D, vulval region. E-F, female tails. G, male tail. H, anterior region of first-stage juvenile. I-L, first-, second-, third-, and fourth-stage juvenile tails, respectively. Abbreviations: a = anus; af = amphidial fovea; rost = replacement odontostyle; sp = spicules; v = vulva. (Scale bars: A-L = 20 µm).

Fig. 6. Line drawings of *Longidorus andalusicus* sp. nov. A, Pharyngeal region; B, C, Detail of lip region; D, Vulval region; E, F, Female tail; G, Male tail.

Fig. 7. Light micrographs of *Longidorus andalusicus* sp. nov. A-C, lip region. D, vulval region. E, anterior region of first-stage juvenile. F-I, first-, second-, third-, and fourth-stage juvenile tails, respectively. J-L, female tails. M-N, male tail. O, intersex tail showing reduced spicules. Abbreviations: a = anus; sp = spicules; v = vulva. (Scale bars: A-N = 20 µm).

Fig. 8. Phylogenetic relationships of the *Longidorus* species studied within *Longidorus* and *Paralongidorus* for D2 and D3 expansion segments of 28S rRNA. Bayesian 50% majority rule consensus tree as inferred from D2 and D3 expansion segments of 28S rRNA sequences alignments under the GTR + G + I model. Posterior probabilities more than 65% are given for appropriate clades; bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequences in this study are in bold letters.

Fig. 9. Phylogenetic relationships of the *Longidorus* species studied within *Longidorus* for ITS1 rRNA. Bayesian 50% majority rule consensus tree as inferred from ITS1 rRNA sequences alignments under the GTR + G + I model. Posterior probabilities more than 65% are given for appropriate clades; bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequences in this study are in bold letters.

Fig. 10. Phylogenetic relationships of the *Longidorus* species studied within *Longidorus* and *Paralongidorus* for the partial 18S rRNA. Bayesian 50% majority rule consensus tree as inferred from the partial 18S rRNA sequences alignments under the GTR + G + I model. Posterior probabilities more than 65% are given for appropriate clades; bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequences in this study are in bold letters.

1 **Table 1.** *Longidorus* species studied and sequences used.

2

Nematode species	Locality of sample	Host	GenBank accession		
			D2-D3	ITS1	partial 18S
<i>L. alveus</i> Roca <i>et al.</i> , 1989	El Cuervo (Sevilla, Spain)	<i>Vitis vinifera</i> L.	JX445115	-	-
<i>L. andalusicus</i> sp. nov.	Sanlúcar de Barrameda (Cádiz, Spain)	Fallow	JX445101	-	JX445118
<i>L. andalusicus</i> sp. nov.	Jerez de la Frontera (Cádiz, Spain)	<i>Salicornia ramossissima</i> L.	JX445102	-	-
<i>L. baeticus</i> sp. nov.	Montemayor (Córdoba, Spain)	<i>Vitis vinifera</i> L.	JX445106	JX445093	JX445121
<i>L. baeticus</i> sp. nov.	Niebla (Huelva, Spain)	<i>Citrus aurantium</i> L.	JX445107	-	-
<i>L. crataegi</i> Roca & Bravo, 1996	Montemayor (Córdoba, Spain)	<i>Vitis vinifera</i> L.	JX445114	-	JX445124
<i>L. fasciatus</i> Roca & Lamberti, 1981	Monturque (Córdoba, Spain)	<i>Vitis vinifera</i> L.	JX445108	JX445097	JX445122
<i>L. intermedius</i> Kozłowska and Seinhorst, 1979	Cortes de la Frontera (Cádiz, Spain)	<i>Quercus suber</i> L.	JX445117	-	JX445126
<i>L. iuglandis</i> Roca, Lamberti and Agostinelli, 1984	Bonares (Huelva, Spain)	<i>Vitis vinifera</i> L.	JX445104	JX445099	JX445120
<i>L. iuglandis</i> Roca, Lamberti and Agostinelli, 1984	Jerez de la Frontera (Cádiz, Spain)	<i>Helianthus annuus</i> L.	JX445105	-	-
<i>L. magnus</i> Lamberti <i>et al.</i> , 1982	Lucena (Córdoba, Spain)	<i>Vitis vinifera</i> L.	JX445112	-	-
<i>L. magnus</i> Lamberti <i>et al.</i> , 1982	La Rambla (Córdoba, Spain)	<i>Vitis vinifera</i> L.	JX445113	-	-
<i>L. oleae</i> sp. nov.	Marchena (Seville, Spain)	<i>Olea europaea</i> subsp. <i>europaea</i> L.	JX445103	JX445100	JX445119
<i>L. rubi</i> Tomilin & Romanenko, 1993	Almonte (Huelva, Spain)	<i>Pinus pinea</i> L.	JX445116	JX445098	JX445125
<i>L. vineacola</i> Sturhan & Weischer, 1954	Almonte (Huelva, Spain)	<i>Pinus pinea</i> L.	JX445110	-	-
<i>L. vineacola</i> Sturhan & Weischer, 1954	El Cuervo (Sevilla, Spain)	<i>Vitis vinifera</i> L.	JX445111	JX445096	-
<i>L. vineacola</i> Sturhan & Weischer, 1954	Montilla (Córdoba, Spain)	<i>Vitis vinifera</i> L.	JX445109	JX445094	JX445123

3

4 (-) Not obtained or not performed.

5

6

7 **Table 2.** Morphometrics of females, males and juvenile stages of *Longidorus baeticus* **sp. nov.** from southern Spain^a.

8

Locality/host-plant		Paratypes Montilla (Córdoba, Spain) Grapevine						Niebla (Huelva province) Citrus
Characters/ratios ^b	Holotype	Females	Males	J1	J2	J3	J4	Females
N		19	8	3	3	3	3	3
L (mm)	9.4	7.7 ± 0.69 (6.5-9.4)	7.3 ± 0.68 (6.5-8.3)	2.23 ± 0.76 (2.15-2.30)	3.41 ± 0.42 (3.06-3.87)	3.79 ± 0.60 (3.06-4.44)	5.90 ± 0.61 (5.50-6.60)	7.5 ± 0.78 (6.8-8.3)
a	100.5	89.0 ± 10.2 (73.4-106.3)	102.8 ± 9.3 (86.7-116.8)	69.1 ± 3.0 (67.2-72.6)	76.3 ± 8.9 (66.0-82.4)	72.7 ± 6.4 (64.9-80.4)	75.9 ± 3.6 (73.3-80.0)	105.2 ± 6.7 (97.8-110.9)
b	19.5	14.1 ± 2.2 (10.0-19.5)	14.3 ± 1.1 (12.4-16.0)	8.2 ± 1.6 (7.0-10.0)	9.1 ± 2.2 (6.7-11.1)	8.6 ± 1.6 (6.7-10.6)	14.9 ± 4.7 (9.6-18.7)	16.5 ± 1.3 (15.3-17.8)
c	286.2	207.5 ± 23.8 (180.0-286.2)	177.9 ± 19.3 (156.0-212.7)	52.2 ± 2.3 (50.6-54.8)	77.9 ± 10.8 (69.4-90.0)	82.3 ± 9.6 (69.4-92.5)	139.1 ± 13.2 (124.4-150.0)	206.7 ± 15.7 (196.8-224.2)
c'	0.5	0.6 ± 0.1 (0.5-0.7)	0.8 ± 0.03 (0.7-0.8)	1.8 ± 0.2 (1.7-2.0)	1.2 ± 0.1 (1.2-1.3)	1.1 ± 0.1 (1.1-1.3)	0.8 ± 0.0 (0.8-0.8)	0.7 ± 0.1 (0.6-0.8)
V	49	50.7 ± 1.6 (48-54)	-	-	-	-	-	51.0 ± 2.0 (49-53)
Odontostyle	129.0	123.0 ± 5.1 (115.0-133.0)	122.2 ± 1.9 (120.0-125.0)	78.3 ± 1.5 (77.0-80.0)	85.5 ± 5.2 (82.0-91.5)	94.4 ± 2.0 (91.5-96.0)	112.7 ± 0.6 (112.0-113.0)	115.7 ± 6.4 (111.0-123.0)
Odontophore	67.0	74.1 ± 8.3 (67.0-93.0)	62.0 ± 4.4 (57.0-67.0)	44.8 ± 5.0 (40.0-50.0)	52.7 ± 2.1 (51.0-55.0)	56.9 ± 3.7 (53.5-62.0)	69.7 ± 5.7 (65.0-76.0)	67.0 ± 4.6 (63.0-72.0)
Replacement odontostyle	-	-	-	86.0 ± 1.7 (84.0-87.0)	98.5 ± 2.2 (97.0-101.0)	107.3 ± 4.5 (101.0-111.0)	120.0 ± 1.0 (119.0-121.0)	-
Lip region diam.	13.5	13.1 ± 0.6 (12.0-14.5)	12.3 ± 1.2 (11.0-13.5)	5.3 ± 0.3 (5.0-5.5)	6.5 ± 0.0 (6.5-6.5)	7.0 ± 0.4 (6.5-7.5)	9.7 ± 0.3 (9.5-10.0)	13.3 ± 0.8 (12.5-14.0)
Oral aperture-guiding ring	38.0	36.3 ± 1.7 (34.0-39.0)	36.3 ± 1.3 (34.0-38.0)	24.7 ± 0.6 (24.0-25.0)	27.8 ± 0.3 (27.5-28.0)	27.3 ± 1.0 (26.5-28.5)	32.5 ± 1.5 (31.0-34.0)	38.2 ± 2.5 (36.5-41.0)
Tail length	33.0	37.1 ± 3.3 (30.0-43.0)	41.1 ± 2.2 (38.0-45.0)	42.8 ± 1.0 (42.0-44.0)	43.8 ± 0.8 (43.0-44.5)	45.9 ± 2.2 (44.0-48.0)	42.5 ± 3.5 (38.5-45.0)	36.3 ± 2.1 (34.0-38.0)
Spicules	-	-	87.1 ± 4.5 (80-95)	-	-	-	-	-
Supplements	-	-	18.9 ± 1.0 (17-20)	-	-	-	-	-

9 ^a Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).

10 ^b Abbreviations as defined in Jairajpuri & Ahmad (1992)

11

12 **Table 3.** Morphometrics of females, males and juvenile stages of *Longidorus oleae* **sp. nov.** from southern Spain^a.

13

Locality/host-plant	Marchena (Sevilla, Spain) Olive						
	Holotype	Paratypes					
		Females	Males	J1	J2	J3	J4
Characters/ratios ^b							
N		21	11	6	2	3	4
L (mm)	8.0	7.8 ± 0.67 (6.9-9.4)	7.47 ± 0.76 (6.5-9.2)	1.89 ± 0.15 (1.77-2.08)	2.77 ± 0.13 (2.68-2.86)	4.22 ± 0.29 (3.98-4.55)	5.71 ± 0.61 (5.07-6.75)
a	90.4	89.5 ± 8.7 (77.1-106.9)	97.5 ± 10.9 (83.0-116.5)	57.1 ± 3.8 (50.5-59.1)	67.6 ± 0.9 (66.9-68.2)	78.6 ± 11.8 (64.9-86.2)	75.0 ± 4.7 (66.7-81.4)
b	15.0	15.7 ± 1.7 (12.2-19.0)	14.8 ± 2.0 (12.0-19.7)	7.4 ± 1.8 (5.0-8.9)	7.9 ± 0.4 (7.6-8.2)	11.6 ± 1.4 (10.0-12.5)	12.0 ± 1.3 (11.0-14.3)
c	201.1	202.7 ± 21.7 (173.5-262.2)	187.1 ± 21.6 (143.9-214.1)	51.6 ± 10.4 (40.3-62.8)	66.9 ± 2.5 (65.1-68.6)	89.5 ± 8.6 (79.7-96.2)	130.0 ± 17.6 (103.4-150.0)
c'	0.7	0.7 ± 0.04 (0.6-0.8)	0.8 ± 0.04 (0.7-0.8)	1.8 ± 0.3 (1.5-2.1)	1.3 ± 0.0 (1.3-1.3)	1.2 ± 0.1 (1.1-1.2)	0.8 ± 0.1 (0.7-0.9)
V	49	49.8 ± 2.0 (46-53)	-	-	-	-	-
Odontostyle	122.0	118.6 ± 5.3 (107.0-125.0)	117.2 ± 4.9 (108.0-124.0)	69.3 ± 0.5 (69.0-70.0)	76.5 ± 3.5 (74.0-79.0)	90.7 ± 3.2 (87.0-93.0)	100.3 ± 3.1 (96.0-105.0)
Odontophore	72.0	66.7 ± 7.5 (54.0-77.0)	62.5 ± 9.2 (51.0-78.0)	47.5 ± 3.7 (44.0-53.0)	48.5 ± 0.7 (48.0-49.0)	64.7 ± 9.0 (59.0-75.0)	67.3 ± 5.7 (59.0-74.0)
Replacement odontostyle	-	-	-	81.0 ± 4.4 (75.0-85.0)	92.0 ± 0.0 (92.0-92.0)	108.7 ± 7.6 (102.0-117.0)	116.2 ± 5.1 (109.0-122.0)
Lip region diam.	17.5	18.0 ± 1.5 (16.0-21.0)	17.6 ± 1.5 (16.0-21.0)	9.5 ± 0.6 (9.0-10.5)	11.0 ± 0.0 (11.0-11.0)	14.3 ± 0.4 (14.0-14.5)	15.3 ± 1.0 (14.0-16.5)
Oral aperture-guiding ring	40.0	39.6 ± 2.3 (36.5-46.0)	40.4 ± 3.2 (35.0-45.0)	22.5 ± 0.8 (22.0-24.0)	27.3 ± 1.1 (26.5-28.0)	27.0 ± 6.1 (23.0-34.0)	34.3 ± 2.7 (30.5-37.5)
Tail length	40.0	38.6 ± 2.3 (34.5-43.0)	40.1 ± 2.7 (35.0-45.0)	38.0 ± 5.4 (32.0-44.0)	41.5 ± 3.5 (39.0-44.0)	47.7 ± 8.1 (43.0-57.0)	44.2 ± 3.1 (41.0-49.0)
Spicules	-	-	92.0 ± 6.4 (79.0-100.0)	-	-	-	-
Supplements	-	-	16.6 ± 1.9 (14-20)	-	-	-	-

14 ^a Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).

15 ^b Abbreviations as defined in Jairajpuri & Ahmad (1992)

16

17 **Table 4.** Morphometrics of females, males, and stage juveniles of *Longidorus andalusicus* **sp. nov.** studied from southern Spain^a.

18

Locality/host-plant	San Fernando (Cádiz, Spain)		Sanlúcar de Barrameda (Cádiz, Spain)							
	Purple Glasswort		Holotype	Fallow						
				Paratypes						
Characters/ratios ^b	Females	Males		Females	Males	J1	J2	J3	J4	Intersex
N	8	5		15	10	5	1	3	3	2
L (mm)	4.38 ± 0.46 (3.76-5.10)	3.77 ± 0.23 (3.42-4.05)	4.6	4.39 ± 0.41 (3.38-4.99)	4.24 ± 0.40 (3.40-4.77)	0.99 ± 0.10 (0.90-1.14)	1.75	2.1 ± 0.14 (1.96-2.24)	3.03 ± 0.123 (2.94-3.17)	4.93 ± 0.102 (4.86-5.00)
a	78.1 ± 9.1 (69.9-95.0)	76.3 ± 7.9 (68.5-87.9)	71.7	75.3 ± 7.6 (60.7-86.6)	76.0 ± 6.9 (64.2-83.8)	44.4 ± 3.2 (41.9-49.9)	50,0	59.2 ± 3.0 (56.3-62.2)	67.4 ± 3.5 (63.5-70.0)	82.9 ± 3.2 (80.6-85.2)
b	13.5 ± 1.6 (11.2-16.2)	11.0 ± 1.2 (9.6-12.6)	11.7	12.4 ± 2.5 (9.4-20.0)	12.3 ± 3.5 (9.0-21.1)	5.0 ± 1.1 (4.1-6.7)	6,3	8.5 ± 0.4 (8.0-8.9)	8.2 ± 0.8 (7.6-9.1)	14.5 ± 0.5 (14.1-14.9)
c	141.5 ± 15.7 (121.0-164.8)	119.6 ± 10.9 (110.4-134.8)	151.7	141.2 ± 16.0 (112.6-177.6)	143.9 ± 22.2 (113.3-194.1)	38.2 ± 2.7 (35.8-42.3)	47,3	64.6 ± 3.1 (61.1-66.8)	96.0 ± 8.3 (86.9-103.2)	157.1 ± 23.0 (140.8-173.4)
c'	0.8 ± 0.1 (0.7-0.9)	0.9 ± 0.1 (0.82-0.97)	0.7	0.8 ± 0.1 (0.7-1.0)	0.8 ± 0.1 (0.7-0.9)	1.6 ± 0.1 (1.5-1.6)	1,5	1.2 ± 0.1 (1.2-1.3)	1.0 ± 0.1 (0.9-1.1)	0.8 ± 0.1 (0.7-0.9)
V or T	47.9 ± 2.4 (44-50)	46.0 ± 2.8 (44-48)	49	49.5 ± 1.7 (46.5-51.5)	36.6 ± 2.5 (30.5-39.5)	-	-	-	-	-
Odontostyle	82.5 ± 11.0 (78.0-86.0)	77.3 ± 4.7 (72.0-84.0)	80.0	74.0 ± 3.7 (67.0-80.0)	72.5 ± 5.2 (64.0-80.0)	40.7 ± 2.1 (38.5-44.0)	44	51.7 ± 2.4 (49.0-53.5)	62.0 ± 1.0 (60.0-65.0)	75.3 ± 1.8 (74.0-76.5)
Replacement odontostyle	-	-	-	-	-	47.1 ± 2.1 (45.0-49.0)	57	61.2 ± 2.5 (59.5-64.0)	73.0 ± 1.0 (72.0-74.0)	-
Odontophore	50.6 ± 9.2 (48.0-54.0)	51.0 ± 2.6 (48.0-54.0)	60.5	54.8 ± 3.7 (50.0-61.0)	54.8 ± 3.8 (50.0-62.0)	22.5 ± 2.0 (21.0-26.0)	28	35.7 ± 3.2 (32.0-38.0)	46.5 ± 0.9 (46.0-47.5)	52.3 ± 0.4 (52.0-52.5)
Lip region diam.	11.7 ± 0.6 (10.5-12.5)	11.8 ± 0.3 (11.5-12.0)	10.0	10.9 ± 0.8 (10.0-12.0)	11.0 ± 0.7 (10.5-12.0)	5.6 ± 0.4 (5.0-6.0)	8.5	9.2 ± 0.3 (9.0-9.5)	10.0 ± 0.0 (10.0-10.0)	11.8 ± 0.4 (11.5-12.0)
Oral aperture-guiding ring	25.8 ± 0.8 (25.0-27.0)	26.1 ± 1.9 (23.0-28.0)	31.5	28.5 ± 1.6 (26.0-31.5)	29.1 ± 2.0 (26.0-32.0)	16.2 ± 1.2 (15.0-18.0)	20.5	21.8 ± 0.6 (21.5-22.5)	23.8 ± 0.3 (23.5-24.0)	26.5 ± 0.7 (26.0-27.0)
Tail length	31.3 ± 4.9 (25.0-38.0)	31.6 ± 1.8 (30.0-34.0)	30.0	31.2 ± 2.8 (25.5-36.0)	29.9 ± 4.3 (22.0-36.0)	25.9 ± 1.9 (24.0-28.0)	37	32.7 ± 1.2 (32.0-34.0)	31.8 ± 4.2 (28.5-36.5)	31.8 ± 5.3 (28.0-35.5)
Spicules	-	59.6 ± 3.6 (56.0-65.0)	-	-	61.6 ± 3.3 (55.0-68.0)	-	-	-	-	30.0 ± 2.8 (28.0-32.0)
Supplements	-	10.4 ± 0.5 (10-11)	-	-	15.0 ± 0.9 (14-16)	-	-	-	-	8.0 ± 0.0 (8.0-8.0)

19

20 ^a Measurements are in μm (except for L) and in the form: mean ± standard deviation (range).

21 ^b Abbreviations as defined in Jairajpuri & Ahmad (1992)

22

23 **Table 5.** Differential morphometrics of *Longidorus baeticus* sp. nov., *Longidorus oleae* sp. nov., and *Longidorus andalusicus* sp. nov. from closely
24 related *Longidorus* species. (All measurements in μm , except L in mm).

Charactors/Ratio <i>Longidorus</i> spp.	L	a	c'	Odontostyle length	Lip region shape	Lip region width	Oral aperture- guiding ring	Amphidial fovea	Female tail shape	J1 tail shape	J1 c'
<i>baeticus</i> sp. nov.	6.5-9.4	73.4-106.3	0.5-0.7	115-133	conoid-rounded	12.0-14.5	34.0-39.0	asymmetrically bilobed	bluntly conoid- rounded	bluntly rounded to cylindrical	1.7-2.0
<i>crataegi</i>	6.5-8.0	72.5-88.5	0.7-0.9	96-106	conoid-rounded	14.5-16.5	32.0-36.0	asymmetrically bilobed	bluntly conoid- rounded	elongate conoid with peg	1.6-1.8
<i>fasciatus</i>	6.6-8.5	121.0-143.0	0.6-0.9	102-119	conoid-rounded	12.0-14.0	33.0-39.0	asymmetrically bilobed	bluntly rounded	-	-
<i>iuglandis</i>	5.9-8.3	73.0-96.0	0.6-0.7	112-128	conoid-rounded	14.0-16.0	31.0-41.0	asymmetrically bilobed	rounded to hemispherical	bluntly rounded to cylindrical	1.0-1.1
<i>moesicus</i>	6.4-8.0	96.0-147.0	0.8-1.2	115-124	subacute	11.0-13.0	32.0-38.0	asymmetrically bilobed	conoid narrowly rounded terminus	conoid-rounded	2.0-2.7
<i>raskii</i>	6.5-8.1	71.9-96.0	0.7-0.8	90-103	conoid-rounded	15.0-19.0	32.9-38.2	asymmetrically bilobed	rounded to hemispherical	conoid-rounded	1.3-1.8
<i>silvae</i>	5.9-8.0	87.5-123.5	0.7-0.8	113-133	subacute	14.0-17.0	37.0-44.0	asymmetrically bilobed	rounded to hemispherical	elongated-conoid with a long peg	2.5-3.3
<i>oleae</i> sp. nov.	6.9-9.4	77.1-106.9	0.6-0.8	107-125	wide, truncate	16.0-21.0	36.5-46.0	asymmetrically bilobed	broadly conoid to hemispherical	bluntly rounded to hemispherical	1.5-2.1
<i>balticus</i>	6.7-9.0	119.0-163.0	0.7-1.0	91-105	rounded set off by depression	20.0-23.0	23.0-29.0	asymmetrically bilobed	bluntly conoid to hemispherical	-	1.3-1.9
<i>belloi</i>	5.4-8.8	84.0-130.0	0.5-1.1	85-107	truncate	9.5	24.0-36.0	asymmetrically bilobed	bluntly rounded to hemispherical	elongate conoid digitate tip	2.0
<i>kheirii</i>	6.7-9.0	60.3-82.0	0.6-0.9	113-130	wide, truncate	19.5-23.0	36.5-45.0	slightly bilobed at the base	bluntly rounded	conoid digitate tip	2.1-2.8
<i>paravineacola</i>	7.0-9.9	105.2-161.3	0.7-1.0	101-114	slightly expanded, hemispherical	22.5-24.5	28.5-34.5	symmetrically bilobed	bluntly rounded	-	-
<i>pauli</i>	6.5-8.6	120.3-143.5	0.8-1.0	102-118	rounded, set off by depression	14.0-17.0	27.0-36.0	asymmetrically bilobed	bluntly conoid- rounded	subdigitate tip	2.6-3.0
<i>profundorum</i>	6.0-8.3	81.0-119.0	0.7-1.0	91-104	narrow, truncate	13.0-17.0	35.0-41.0	symmetrically bilobed	roundly convex- conoid	pointed, subdigitate tip	2.6-3.1
<i>uroshis</i>	6.1-9.5	79.0-134.0	0.6-1.1	120-151	rounded, flattened	14.0-19.0	34.0-46.0	asymmetrically bilobed	conoid-rounded	elongate-conoid with digitate tip	1.9-3.4
<i>andalusicus</i> sp. nov.	3.4-5.0	60.7-86.6	0.7-1.0	67-80	narrow, conoid	10.0-12.0	26.0-32.0	symmetrically bilobed	bluntly conoid to hemispherical	cylindrical-rounded	1.5-1.6
<i>congoensis</i>	2.8-3.5	50.0-61.0	0.7-0.9	66-81	smoothly rounded	6.0-7.0	25	symmetrically bilobed	dorsally convex, rounded	convex-conoid	1.2-1.9
<i>crataegi</i>	6.5-8.0	72.5-88.5	0.7-0.9	96-106	conoid-rounded	14.5-16.5	32.0-36.0	asymmetrically bilobed	bluntly conoid- rounded	elongate conoid with peg	1.6-1.8
<i>igoris</i>	4.2-6.5	103-132	0.8-1.1	77-106	conoid-rounded	9.5-12.0	29.5-37.0	asymmetrically bilobed	bluntly rounded	bluntly rounded	1.7-2.3
<i>jiangsuensis</i>	3.0-3.7	94.0-107.0	0.7-0.9	82-91	narrow, rounded	6.5-8.0	31.0-33.0	slightly bilobed at the base	bluntly rounded	bluntly rounded to hemispherical	1.2-1.9
<i>orientalis</i>	4.0-5.0	82.0-105.0	0.7-0.9	99-104	conoid-rounded	10.0-11.0	27.0-33.0	symmetrically bilobed	rounded to hemispherical	bluntly rounded to cylindrical	1.9-2.4
<i>psidii</i>	3.0-3.2	62.0-78.0	0.9-1.0	88-105	rounded	12.0	33.0-35.0	symmetrically bilobed	dorsally convex- conoid	-	-

25 (-) unknown