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Description and molecular characterisation of *Paralongidorus litoralis* sp. n. and *P. paramaximus* Heyns, 1965 (Nematoda: Longidoridae) from Spain

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Summary – *Paralongidorus litoralis* sp. n., a new bisexual species of the genus, is described and illustrated by light microscopy, scanning electron microscopy and molecular studies from specimens collected in a coastal sand dune soil around roots of lentisc (*Pistacia lentiscus* L.) from Zahara de los Atunes (Cadiz), southern Spain. *Paralongidorus litoralis* sp. n. is characterised by the large body size (7.5-10.0 mm), a rounded lip region, clearly offset from the body by a collar-like constriction, and bearing a very large stirrup-shaped, amphidial fovea, with conspicuous slit-like aperture, a very long and flexible odontostyle *ca* 190 μm long, guiding ring located at 35 μm from anterior end, and males with spicules *ca* 70 μm long. In addition, identification data of a Spanish population of *P. paramaximus* Heyns, 1965 recovered from sandy soil of a commercial citrus orchard at Alcala de Guadaira (Seville), southern Spain, agree very well with the original description of the species from South Africa. The 18S rRNA and D2 and D3 expansion regions of 28S rRNA gene sequences were obtained for *P. litoralis* sp. n. and *P. paramaximus*. Phylogenetic analyses of *P. litoralis* sp. n. and *P. paramaximus* rRNA gene sequences and of Longidoridae sequences published in GenBank were done using maximum likelihood and Bayesian inference. In trees generated from the 18S data set *Paralongidorus* clustered as an external clade from *Longidorus*, and in trees generated from D2-D3 of 28S dataset *Paralongidorus* was monophyletic and nested within *Longidorus*. Maximum likelihood test supported the hypothesis of validity of the *Paralongidorus* genus.

Keywords – 18S rRNA, 28S rRNA, Bayesian inference, D2-D3 expansion segments, description, morphology, morphometrics, needle nematode, phylogeny, taxonomy.

During nematode surveys in cultivated and indigenous vegetation in southern Spain, two nematode populations of the genus *Paralongidorus* Siddiqi, Hooper & Khan, 1963 were found for the first time in this area. The nematodes were associated with sandy soils in the rhizosphere of the lentisc or mastic tree (*Pistacia lentiscus* L.) in Zahara de los Atunes (Cádiz province), and citrus (*Citrus aurantium* L.) in Alcalá de Guadaira (Seville province), respectively. These two populations of needle nematodes morphologically resemble *Paralongidorus maximus* (Bütschli, 1874) Siddiqi, 1964, which prompted us to do a detailed morphological and molecular comparative study between both populations with *P. maximus* and closely related species of the genus. These studies identified the citrus population as *Paralongidorus paramaximus* Heyns, 1965 but the population from lentisc differed from all known *Paralongidorus* species and is herein described as a new species. This report extends the geographical

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distribution of *P. paramaximus* as it was previously only known from South Africa.

The genus *Paralongidorus* is well established and widely accepted by nematologists, although its definition is not always consistent. Taxonomy of the genus has been controversial as the genera *Siddiqia* Khan, Chawla & Saha, 1978, *Longidoroides* Khan, Chawla & Saha, 1978 and *Inagreius* Khan, 1982 have been synonymised with it and/or recognised as distinct in several review papers (Luc & Doucet, 1984; Coomans, 1985, 1996; Hunt, 1993; Siddiqi *et al*., 1993; Arias & Bravo, 1997; Escuer & Arias, 1997). Siddiqi *et al.* (1993) synonymised *Longidoroides* with *Paralongidorus* based on a new interpretation of the amphid structure of *P. sali* Siddiqi, Hooper & Khan, 1983, the type species of the genus. However, the interpretation of the amphidial fovea by Siddiqi *et al.* (1993) was questioned by Coomans (1996) after detailed study of the type material. Whatever the status of *Longidoroides*, Coomans (1996) established that it represents an intermediate condition between *Paralongidorus* species with typical amphids and *Longidorus* species with pouch-like amphids and pore-like openings. Coomans (1996) also concluded that *Paralongidorus*, *Longidoroides* and *Longidorus* form a complex, with the primitive forms, *viz*., *Paralongidorus*, including *P. maximus*, having offset lip regions and stirrup-shaped amphids with wide slit-like openings. Escuer and Arias (1997) did not recognise the subgenera *Paralongidorus* and *Siddiqia* within *Paralongidorus* and considered *Longidoroides* as a synonym of *Paralongidorus* since the amphidial fovea of some species of *Paralongidorus* do not correspond to the typical funnel or bilobed shape. Consequently, until additional detailed molecular and phylogenetic analyses are done on species of these genera we consider *Longidoroides* as a valid genus, *Paralongidorus sensu stricto* comprising 53 nominal species at present.

Of the nominal species of *Paralongidorus s. str*., five have been described from Europe with two species from Spain (*P. iberis* Escuer & Arias, 1997 and *P. monegrensis* Escuer & Arias, 1997), one from France (*P. remyi* (Altherr, 1963) Siddiqi & Husain, 1965), and another from Hungary (*P. rex* Andrássy, 1986). *Paralongidorus maximus* is the most common and widespread species of the genus and is a vector of plant viruses, such as arabis mosaic virus (ArMV) (Jones *et al*., 1994). This species has been reported in several countries, including Austria, France, Germany, Hungary, Poland and the UK (Heyns, 1975 with overview till 1975), Portugal (Macara, 1988), Bulgaria (Lamberti *et al*., 1983), Italy (Roca *et al*., 1988), Slovak Republic (Lišková *et al.*, 1992; Lišková, 1995; Lišková & Brown, 1998, 1999, 2003) and South Africa (Swart *et al*., 1996).

Sequences of nuclear ribosomal RNA genes have been recently used for molecular characterisation and reconstruction of phylogenetic relationships of nematodes from *Longidorus*, *Paralongidorus* and related genera (Rubtsova *et al*., 2001; Neilson *et al*., 2004; Ye *et al*., 2004; Handoo *et al*., 2005; He *et al*., 2005). Using the D2 and D3 expansion segments, Rubtsova *et al*. (2001) revealed close relationships of *P. maximus* with a group of *Longidorus* species, including *L. profundorum* Hooper, 1966, *L. sturhani* Rubtsova, Subbotin, Brown & Moens, 2001, *L. carpathicus* Lišková, Robbins & Brown, 1997, *L. intermedius* Kozlowska & Seinhorst, 1979, and *L. elongatus* (de Man, 1876) Micoletzky, 1922. Moreover, this study showed that *P. maximus* formed an internal clade within the genus *Longidorus.* These results were also confirmed by more detailed ML analyses including 23 *Longidorus* and two *Paralongidorus* species (He *et al*., 2005). Nevertheless, the need for further molecular analysis, including additional species of *Paralongidorus*, was suggested in order to confirm these results and clarify the validity of the genus.

The objectives of this work were: *i*) to characterise morphologically and molecularly a Spanish population of *P. paramaximus* from citrus and a new species, *P. litoralis* sp. n., from lentisc; and *ii*) to study the phylogenetic relationships of *Paralongidorus* spp. with *Longidorus* spp. (with *Xiphinema* Cobb, 1913 and *Xiphidorus* Monteiro, 1976 as outgroups) using sequences from the D2-D3 expansion regions of 28S rRNA and the 18S rRNA as inferred from maximum likelihood and Bayesian inference approach.

Materials and methods

Nematode populations used in this study were obtained from sandy soils at a depth of 35-50 cm in the rhizosphere of a commercial citrus grove in Alcalá de Guadaira (Seville province, Spain), 37°17′35.72″N latitude, 5°48′16.73″O longitude at 42 m a.s.l.; and a coastal sand dune with lentisc in Zahara de los Atunes (Cádiz province), 36°07′06.56″N latitude, 5°49′55.45″O longitude at 4 m a.s.l.

Nematodes were extracted from soil samples by magnesium sulphate centrifugal flotation (Coolen, 1979) and by the sieving method described by Flegg (1967). Spec-

imens for light microscopy (LM) were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid, and processed to pure glycerin using Seinhorst's (1966) method.

Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at powers up to ×1000 magnification. Measurements were done using a camera lucida attached to a light microscope. In addition, a detailed morphological study on type specimens of *P. paramaximus* from the nematode collection of the Istituto per la Protezione delle Piante, Sezione di Bari, Consiglio Nazionale delle Ricerche, (C.N.R.), Bari, Italy, was done. Morphometric data were processed using Statistix 8.0 (NH Analytical Software, Roseville, MN, USA).

For scanning electron microscopy (SEM) studies, fixed specimens were dehydrated in a graded ethanol series, critical point dried, sputter-coated with gold and observed with a JEOL JSM-5800 microscope (Abolafia *et al*., 2002).

DNA EXTRACTION, PCR AND SEQUENCING

Nematode DNA was extracted from single individuals as described by Castillo *et al*. (2003), using two adult females of samples from citrus (Alcala de Guadaira) and one male and one female from coastal sand dunes (Zahara de los Atunes), in order to confirm that the latter specimens are conspecific. Protocols for PCR are 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), and the 18S rDNA using the SSU_F_04 (5′-GCTTGTCTCAAAGATTAAGCC-3′) and SSU_R_81 (5′-TGATCCWKCYGCAGGTTCAC-3′) primers (Griffiths *et al*., 2006). The PCR products were purified after amplification with a gel extraction kit (Geneclean turbo; Q-BIOgene, Illkirch, France), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct DNA sequencing. For the 18S gene the internal primer SSU_R_23 (5′-TCTCGCTCGTTATCGGAAT-3′) (sequence available in http://www.nematodes.org/barcoding/sourhope/nemoprimers.html) was also used. DNA fragments from two independent PCR amplifications from two different samples were sequenced in both directions using the same primers with a terminator cycle sequencing ready reaction kit (BigDye; Perkin-Elmer Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3100 genetic analyser; Applied Biosystems, Foster City, CA, USA) at the University of Córdoba sequencing facilities. Sequences were deposited in GenBank under accession numbers EU026155 and EU026158-EU026159 for *P. litoralis* sp. n. and EU026156 and EU026157 for *P. paramaximus*, for the D2-D3 of 28S rRNA and 18S rRNA, respectively.

PHYLOGENETIC ANALYSIS

The D2-D3 of 28S rRNA and 18S rRNA gene sequences of *P. litoralis* sp. n. and *P. paramaximus* were aligned using ClustalX 1.83 (Thompson *et al.*, 1997) with default parameters with corresponding rRNA genes for *Longidorus* and other *Paralongidorus* published in GenBank (Rubtsova *et al.*, 2001; Neilson *et al*., 2004; Handoo *et al.*, 2005; He *et al.*, 2005; Griffiths *et al*., 2006; Holterman *et al*., 2006). The species *Tylencholaimus mirabilis*, *Xiphidorus minor* and *Xiphinema rivesi* were chosen as outgroup taxa (He *et al*., 2005; Holterman *et al*., 2006). Sequence alignments for D2-D3 of 28S and 18S were analysed with maximum likelihood (ML) method using PAUP* 4b 10 (Swofford, 2003) and Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Best fit model of DNA evolution for ML and BI analyses was obtained using the program MrModeltest 2.2 (Nylander, 2004) with the Akaike Information Criterion in conjunction with PAUP*. This analysis indicated that the SYM + I + G and the GTR + I + G model were selected as the most appropriate for the 28S and 18S rRNA gene, respectively. BI analysis was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilised after approximately 1000 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. In ML analysis the estimation of the support for each node was made using a bootstrap analysis with 100 faststep replicates. For testing of alternative topologies in ML, we used the Shimodaira-Hasegawa test as implemented in PAUP*.

Paralongidorus litoralis[*] **sp. n.**
(Figs 1-4)

MEASUREMENTS

See Tables 1, 2.

DESCRIPTION

Female

Body very long and rather robust. Habitus ventrally arcuate usually in an open C when relaxed by gentle heating. Cuticle appearing smooth, 3.5 (3.0-4.0) μm thick, 10.5 (8.0-12) μm thick at tip tail, and marked by very fine superficial transverse striae mainly in tail region. Lip region rounded in lateral view, and clearly set off by a collar-like constriction at level or slightly posterior to amphidial aperture, and 12.5 $\pm$ 1.0 (11-14) μm high. SEM observations showed protruding inner labial papillae and outer labial papillae surrounded by a cuticular ring. Amphidial fovea very large, stirrup-shaped, with conspicuous aperture *ca* three-fourths as wide as lip region. Cephalic papillae appearing as small apertures, each located just anterior to a distinct cephalic lobe (2.5-3.0 μm long). Stylet guiding ring single, 7-8 μm wide, located 1.24 $\pm$ 0.06 (1.14-1.40) times lip region diam. from anterior end. Lateral chord 16.5 (14-20) μm wide at mid-body or 28% of corresponding body diam. Odontostyle long and slender, straight or slightly arcuate, *ca* 3-3.5 μm wide towards its base; odontophore with weak basal swellings. Nerve ring encircling pharynx, located slightly behind middle of pharynx. Pharynx typical of genus. Anterior slender part of pharynx usually with loop overlapping basal bulb. Basal bulb cylindrical, 122$\pm$10.9 (112-143) μm long, 23-27 μm diam. Dorsal pharyngeal gland nucleus in anterior part of bulb, 14-17 μm posterior to gland outlet, one subventral pair of nuclei near middle of bulb. Cardia hemispherical clearly visible, 8-12 μm long. Reproductive system with both genital branches equally developed. Vulva located slightly anterior to mid-body, round in ventral view with 19-20 μm diam. Vagina 34-41 μm long, surrounded by well developed muscles. Each oviduct separated from uterus by elongate spermatheca. Prerectum long and variable, 483-539 μm long. Rectum 33 (28-40) μm long. Tail bluntly rounded, barely dorsally convex-conoid with broadly rounded terminus.

Male

Very common and as abundant as female. Habitus mostly similar to female but with posterior region slightly curved ventrally. Lip region as in female, 12.5 $\pm$ 1.4 (11-15) μm high. Male genital tract diorchic with testes opposed, occupying *ca* 40% of body length. Tail rounded, dorsally convex conoid, ventrally slightly concave with broad blunt terminus and thickened outer cuticular layer. Spicules robust, *ca* two times longer than tail length; lateral guiding pieces 17-20 μm long. One pair of adanal supplements and 10-13 midventral supplements.

Juveniles

All four juvenile stages were found, being distinguishable by relative lengths of body and functional and replacement odontostyles (Table 2). First-stage juveniles were characterised by an elongate-conoid tail with a central peg (Fig. 3), an odontostyle length *ca* 100 μm, and shorter distance from anterior end to stylet guiding ring than that in adult stages. However, morphology in all three juvenile stages (except for genital tract) was similar to that of female, including tail shape of second- to fourth-stage juveniles which was bluntly rounded, yet differed in shorter distance from anterior end to guiding ring.

TYPE HOST AND LOCALITY

Rhizosphere of lentisc or mask tree (*Pistacia lentiscus* L.) from coastal sand dunes, Zahara de los Atunes (Cádiz province), southern Spain.

TYPE MATERIAL

Holotype female and four female and five male paratypes deposited in the Nematode Collection of the Istituto per la Protezione delle Piante, Sezione di Bari, Consiglio Nazionale delle Ricerche, (C.N.R.), Bari, Italy. Twelve female and 12 male paratypes deposited at the Nematode Collection of the Institute of Sustainable Agriculture, CSIC, Córdoba, Spain; and one female and one male paratype deposited at each of the United States Department of Agriculture Nematode Collection (USDANC), Beltsville, MD, USA, slide number T-5632p; University of California Riverside Nematode Collection, USA, slide number UCRNC #30470; and the Nematode Collection of the Landbouwhogeschool, Wageningen, The Netherlands.

[*] The species epithet is derived from the Latin *litoralis* = pertaining to the seashore, and refers to the coastal environment of the nematode.

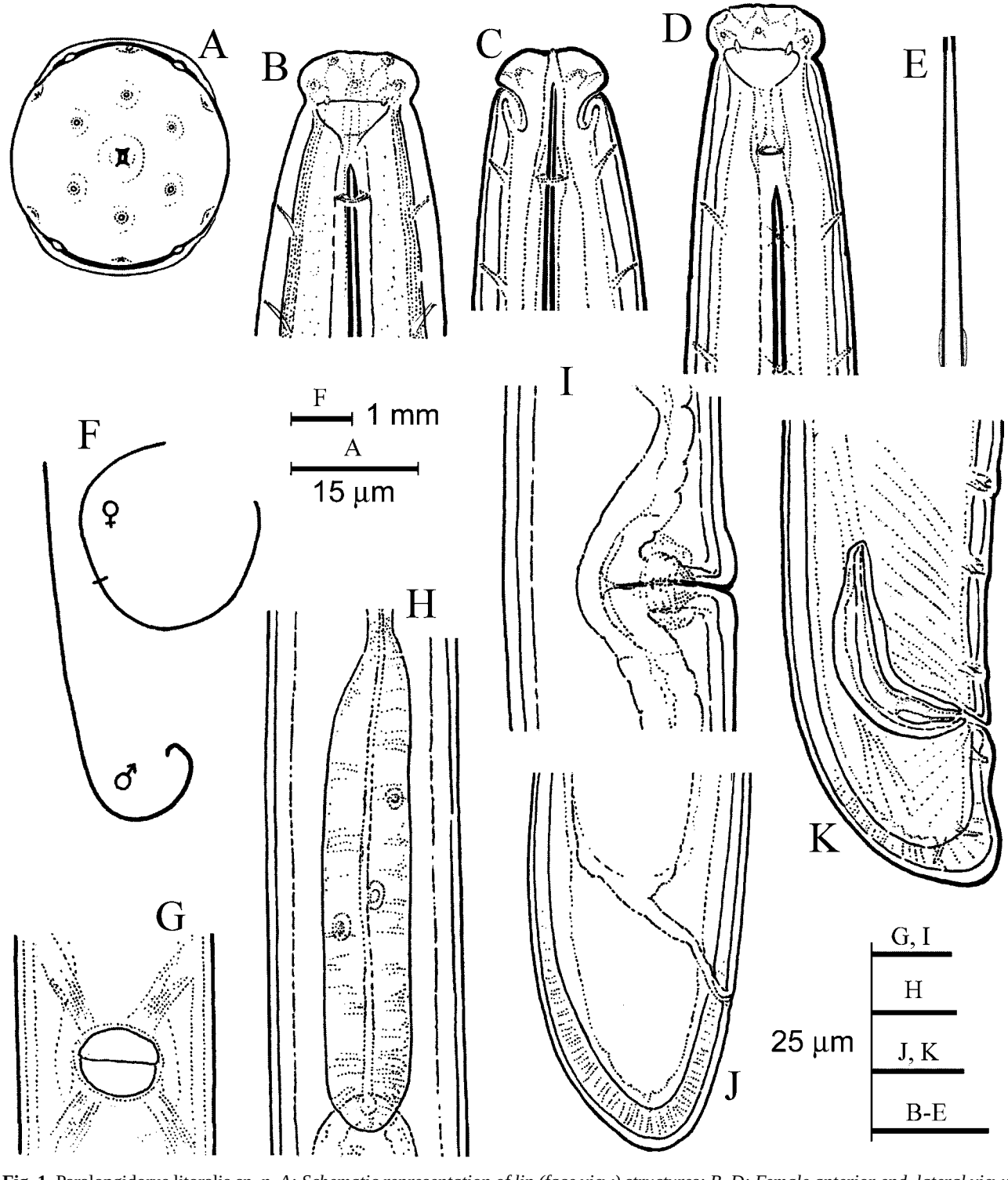

Fig. 1. Paralongidorus litoralis *sp. n. A: Schematic representation of lip (face view) structures; B, D: Female anterior end, lateral view; C: Female anterior end, dorsal view; E: Detail of odontophore; F: Habitus; G, I: Vulval area, ventral (section through vagina) and lateral view; H: Pharyngeal bulb; J: Female posterior region; K: Male posterior region.*

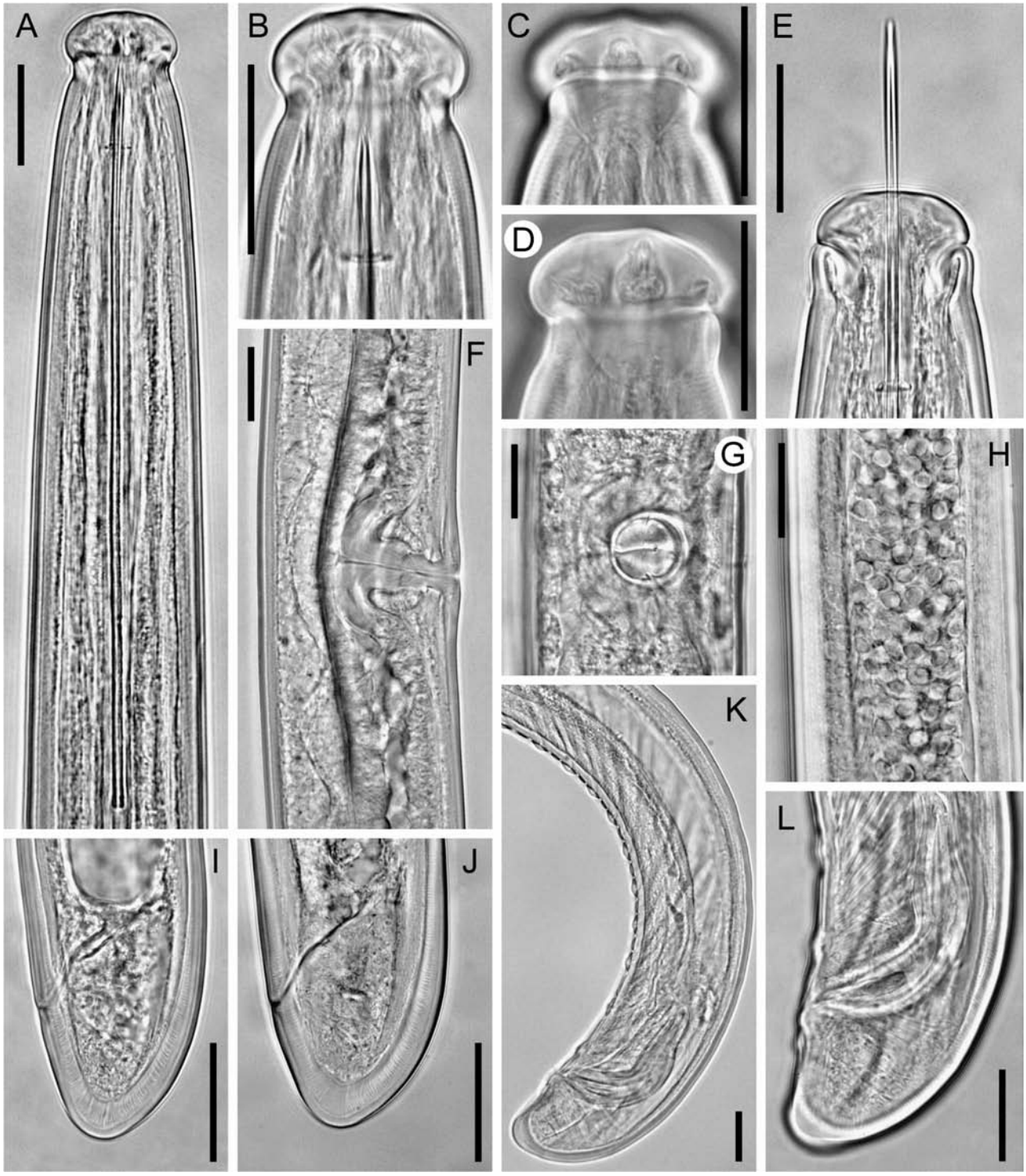

Fig. 2. *Light micrographs of* Paralongidorus litoralis *sp. n. A: Female anterior region; B: Head end; C, D: Amphid at different focus; E: Female anterior end, ventral view showing amphidial fovea; F, G: Vulval region in lateral and ventral view; H: Sperm; I, J: Female tails, lateral view; K, L: Male posterior body portion and tail region. (Scale bars = 25 μm.)*

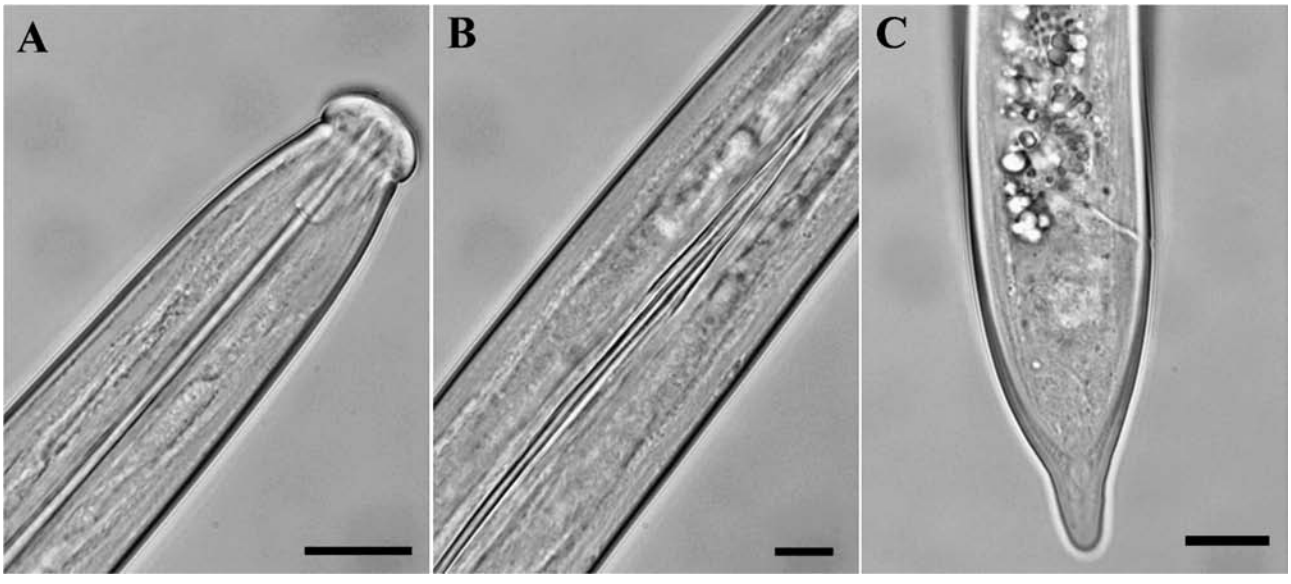

Fig. 3. *Light micrographs of* Paralongidorus litoralis *sp. n. First-stage juvenile. A: Anterior region; B: Detail of odontophore; C: Tail showing central peg. (Scale bars: A = 20 μm; B, C = 10 μm.)*

DIAGNOSIS AND RELATIONSHIPS

Paralongidorus litoralis sp. n. is characterised by a long body (7.5-10.0 mm), a rounded lip region clearly offset from the body by a marked constriction and bearing a very large, stirrup-shaped amphidial fovea, with conspicuous slit-like aperture, a very long and flexible odontostyle *ca* 190 μm long, stylet guiding ring located at 35 μm from anterior end, and males with spicules *ca* 70 μm long. The matrix code according to the polytomous key by Escuer and Arias (1997) is A1, B1, C4, D2, E2, F6, G7, H1, I2, J1, K5, L4, M2, N2, O2.

On the basis of body length, habitus, lip region and amphid, *P. litoralis* sp. n. is close to *P. maximus* and *P. paramaximus* (Table 3). *Paralongidorus litoralis* sp. n. differs from *P. maximus* in the habitus (open C-shape *vs* open spiral), lip region diam. (25-30 *vs* 34-39 μm), lip region shape (collar-like constriction behind the lip region present *vs* absent), distance from oral aperture to guiding ring (32-37 *vs* 37-47 μm), size of amphidial aperture (three-fourths *vs* two-thirds as wide as lip region), odontostyle length (169-206 *vs* 152-187 μm), female tail shape (bluntly rounded, barely dorsally convex-conoid *vs* bluntly rounded), a ratio (114-164 *vs* 72-133), c′ ratio (0.64-0.83 *vs* 0.38-0.60), odontostyle length of the first-stage juvenile (97-104 *vs* 73-89 μm), tail shape of the first-stage juvenile (elongate-conoid tail with a central peg *ca* 12 μm long *vs* relatively long and somewhat digitate), c′ of first-stage juvenile = 1.6 (1.37-1.84) *vs* 1.15 (1.00-1.27), and presence of males (very common *vs* extremely rare). Finally, under SEM observations *P. litoralis* sp. n. showed distinct cephalic lobes (Fig. 4) whereas *P. maximus* consistently showed small cephalic lobes in a population from South Africa (Swart *et al*., 1996) and in a European population (Barsi *et al*., 2007). *Paralongidorus litoralis* sp. n. most closely resembles *P. paramaximus* in morphology and morphometrics (Table 3). However, detailed study of paratypes, as well as specimens of the Spanish population from a citrus grove, revealed the following differences from *P. litoralis* sp. n.: odontostyle length (169-206 *vs* 122-173 μm), c ratio (235-335 *vs* 170-293), and c′ ratio (0.64-0.83 *vs* 0.60-1.00).

Paralongidorus litoralis sp. n. also differs markedly from the other known European *Paralongidorus* species (*P. iberis*, *P. monegrensis*, *P. remyi* and *P. rex*) in lip region morphology (wider in *P. litoralis* sp. n.), amphid size (larger in *P. litoralis* sp. n.), odontostyle length (longer in *P. litoralis* sp. n.), position of the vulva, female and male tail shape, and length of spicules. *Paralongidorus rex* has a clear, narrower, collar-like part posterior to the lip region, thus setting off the narrower lip region from the broader body and the male is unknown; *P. remyi* has a lip region set off by a non-collar-like constriction, the male is unknown and body length is less than 5.6 mm.

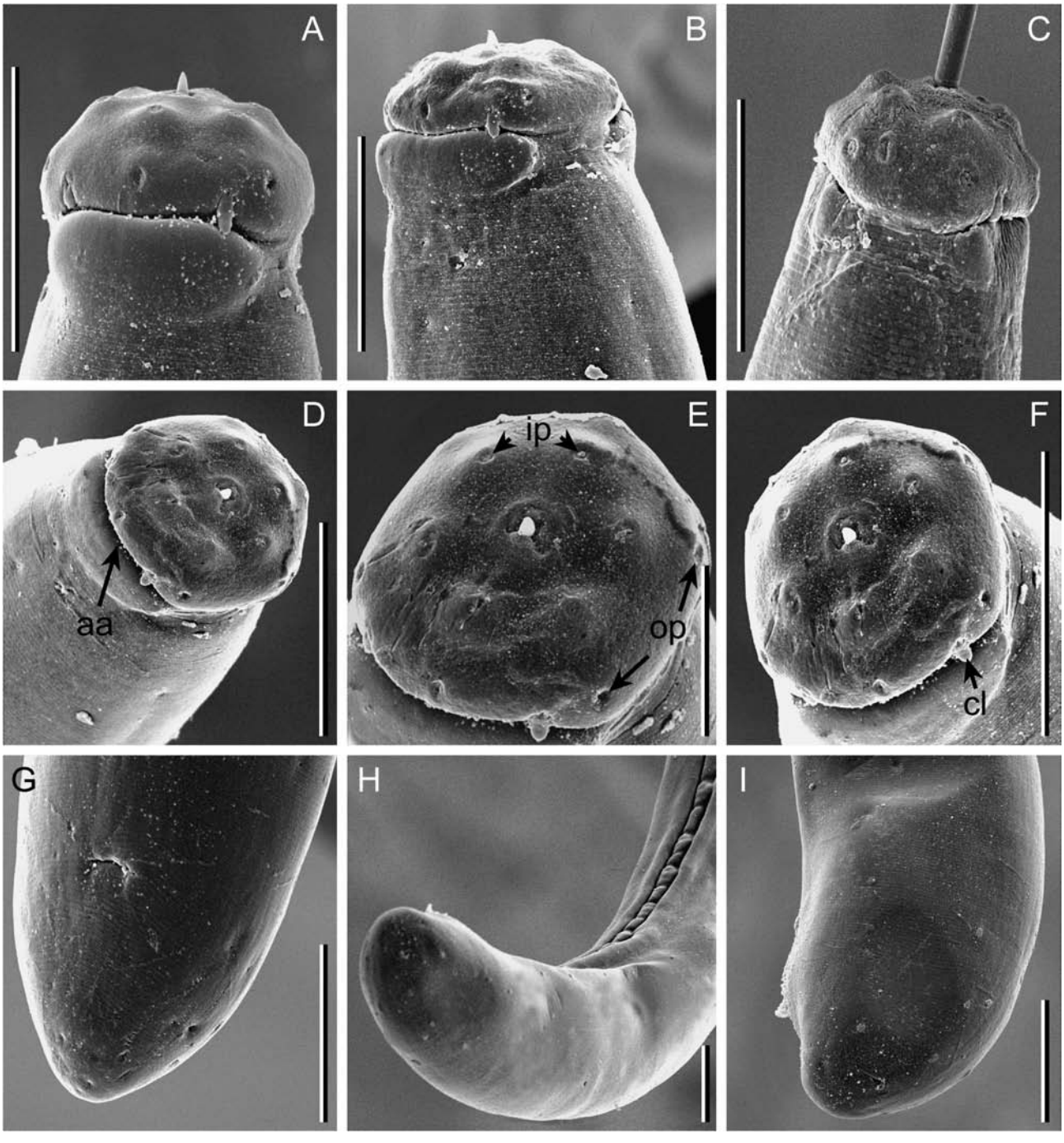

Fig. 4. SEM *micrographs of* Paralongidorus litoralis *sp. n. A, B: Female anterior ends in lateral and ventro-lateral view; C: Male anterior end; D, E, F:* En face *view showing internal (ip) and outer labial papillae (op), cephalic lobe (cl), and amphidial aperture (aa); G: Female tail ventral view; H, I: Male posterior body portion and tail, oblique ventral and lateral view respectively. (Scale bars: E = 10 μm; all others = 20 μm.)*

Table 1. *Morphometrics of females and males of* Paralongidorus litoralis *sp. n. and* P. paramaximus *from Spain. Measurements are in* μm *(except for L) and in the form: mean* $\pm$ *standard deviation (range)*.*

Character	*Paralongidorus litoralis* sp. n.			*Paralongidorus paramaximus*	
	Zahara de los Atunes (Cádiz province)			Alcalá de Guadaira (Seville province)	
	Females		Males	Females	Males
	Holotype	Paratypes	Paratypes		
n	–	19	20	6	3
L (mm)	9.37	9.07 ± 0.69 (7.51-10.07)	8.89 ± 0.81 (7.50-10.01)	7.95 ± 0.46 (7.22-8.58)	7.78 ± 0.63 (7.30-8.49)
a	144.1	146.1 ± 11.9 (113.7-164.4)	159.1 ± 13.9 (138.9-182.1)	150.4 ± 5.00 (144.5-158.7)	183.2 ± 34.8 (162.2-223.4)
b	18.8	18.0 ± 2.9 (14.6-25.0)	18.7 ± 2.3 (15.2-24.3)	19.2 ± 2.4 (15.2-22.1)	18.7 ± 3.4 (16.2-22.5)
c	334.5	284.3 ± 26.3 (234.6-334.5)	289.0 ± 25.6 (244.3-343.5)	256.6 ± 17.0 (233.0-284.9)	238.5 ± 52.8 (187.2-292.7)
c′	0.64	0.74 ± 0.05 (0.64-0.83)	0.70 ± 0.05 (0.58-0.79)	0.81 ± 0.04 (0.76-0.86)	0.88 ±0.12 (0.76-1.00)
V or T	44	41 ± 1.7 (37-44)	42 ± 3.4 (37-50)	44 ± 1.8 (41-46)	46 ± 1.0 (45-47)
G_1	6.9	6.7 ± 1.7 (5.0-8.8)	–	7.4 ± 0.3 (7.0-7.7)	–
G_2	7.1	7.4 ± 1.2 (5.8-8.7)	–	7.4 ± 0.4 (7.0-7.8)	–
Odontostyle	192	193 ± 8.5 (169-206)	190 ± 8.5 (176-206)	168 ± 2.9 (164-173)	162 ± 9.5 (156-173)
Odontophore	89	80 ± 9.4 (70-92)	81 ± 9.9 (65-104)	50 ± 5.0 (44-59)	54 ± 8.6 (46-63)
Lip region diam.	27	28.1 ± 1.1 (25.0-30.0)	27.9 ± 1.2 (27.0-30.0)	26.3 ± 1.6 (24.0-28.5)	28.5 ± 0.5 (28.0-29.0)
Oral aperture-guiding ring	35	35 ± 1.5 (32.0-37.0)	35 ± 1.5 (32.0-36.0)	30 ± 2.2 (27-33)	32 ± 2.6 (30-35)
DO	15.1	13 ± 1.7 (10.2-15.1)	14 ± 2.0 (10.8-16.6)	11 ± 0.6 (10.4-11.2)	11 ± 0.6 (10.2-11.1)
DN	32.6	34 ± 1.9 (30.9-36.4)	33 ± 2.3 (28.1-34.6)	30 ± 0.5 (29.5-30.2)	31 ± 0.6 (30.2-31.0)
SN_1 and SN_2	55.6	55 ± 4.8 (45.2-58.3)	57 ± 3.4 (53.8-62.7)	53 ± 2.5 (51.2-54.8)	53 ± 0.6 (52.1-53.0)
SO_1 and SO_2	84.9	86 ± 1.8 (83.3-87.7)	83 ± 2.9 (80.2-88.8)	87 ± 0.6 (86.2-87.1)	82 ± 1.4 (81.5-83.5)
Nerve ring to anterior end	297	269 ± 62.4 (214-327)	272 ± 27.0 (219-274)	–	–
Pharynx length	498	349 ± 29.5 (311-384)	418 ± 77.7 (331-498)	419 ± 52.0 (373-522)	421 ± 38.7 (377-450)
Tail length	28	32 ± 1.9 (27-34)	31 ± 1.5 (28-34)	31 ± 1.4 (29-33)	33 ± 5.1 (29-39)
Spicule length	–	–	69 ± 2.2 (63-72)	–	60 ± 3.0 (57-63)
Supplements	–	–	1** 11 ± 1 (10-13)	–	1** 11 ± 1 (10-12)

* Abbreviations as defined in Jairajpuri and Ahmad (1992).
** Number of adanal supplements and number of midventral supplements.

Table 2. *Morphometrics of first-stage (J1), second-stage (J2), third-stage (J3), and fourth-stage (J4) paratype juveniles of* Paralongidorus litoralis *sp. n. from Spain. Measurements are in μm (except for L) and in the form: mean ± standard deviation (range)*.*

Character	J1	J2	J3	J4
n	6	6	3	3
L (mm)	1.59 ± 0.46 (1.54-1.66)	3.25 ± 0.23 (2.95-3.47)	3.95 ± 0.39 (3.64-4.39)	5.84 ± 0.37 (5.42-6.11)
a	44.4 ± 5.4 (33.8-49.8)	69.7 ± 5.6 (59.0-74.0)	80.0 ± 5.3 (76.3-86.1)	116.0 ± 6.8 (108.5-121.9)
b	4.9 ± 0.6 (4.0-5.7)	9.4 ± 1.0 (8.4-11.0)	9.6 ± 1.3 (8.6-11.0)	12.5 ± 2.1 (10.4-14.6)
c	36.3 ± 2.1 (33.6-38.8)	108.1 ± 9.5 (89.7-115.7)	126.2 ± 3.6 (123.1-130.1)	189.5 ± 20.3 (170.6-210.9)
c′	1.6 ± 0.18 (1.37-1.84)	0.78 ± 0.05 (0.68-0.83)	0.76 ± 0.09 (0.68-0.85)	0.73 ± 0.04 (0.69-0.78)
Odontostyle	99 ± 2.5 (97-104)	143 ± 4.5 (139-151)	149 ± 6.5 (142-155)	159 ± 3.8 (155-162)
Replacement odontostyle	119 ± 4.1 (113-124)	158 ± 10.9 (138-168)	171 ± 7.8 (162-177)	196 ± 5.5 (191-202)
Lip region diam.	17.6 ± 1.1 (16.5-19.0)	23.5 ± 0.7 (23.0-24.0)	26.7 ± 2.5 (24.0-29.0)	26.3 ± 1.1 (25.0-27.0)
Oral aperture-guiding ring	21 ± 1.3 (20-23)	28 ± 2.1 (26.5-31.0)	27 ± 0.8 (26.5-28.0)	32 ± 0.8 (31.0-32.5)
Tail	44 ± 2.4 (41-46)	30 ± 2.3 (26-33)	31 ± 3.5 (28-35)	31 ± 3.5 (29-35)
Tail peg	13.2 ± 1.5 (11-15)	–	–	–

* Abbreviations as defined in Jairajpuri and Ahmad (1992).

Paralongidorus paramaximus **Heyns, 1965**
(Fig. 5; Table 1)

MEASUREMENTS

See Table 1.

ADDITIONAL INFORMATION

Female

Body very long and rather robust, usually assuming an open C-shape in habitus. Cuticle of midbody 2-2.5 μm thick, 12-14 μm thick at tail tip, marked by very fine superficial transverse striae. Lip region rounded in lateral view, clearly set off by a deep constriction at level of or slightly posterior to amphidial aperture, 9.5 ± 1.5 (7.5-12) μm high. Amphidial fovea very large, stirrup-shaped, broad, slit-like aperture, 19-21 μm long. Stylet guiding ring single, 8-9 μm wide. Basal bulb cylindrical, 118-135 μm long. Dorsal pharyngeal gland nucleus in anterior part of bulb, one subventral pair of nuclei near middle of bulb. Cardia hemispherical to conoid-rounded, 8-10 μm long. Vagina 20-26 μm long. Prerectum long and variable, 483-539 μm long. Rectum 28-31 μm long. Tail dorsally convex-conoid with broadly rounded terminus.

Male

Common. Body with posterior region slightly hooked ventrally; lip region similar to female. Male genital tract diorchic with testes opposed. Tail conoid, dorsally convex conoid, ventrally slightly concave with a broad, blunt, terminus and thickened outer cuticular layer. Spicules robust, *ca* twice as long as tail length. One pair of adanal supplements and 10-12 midventral supplements.

REMARKS

Paralongidorus paramaximus is morphologically very close to *P. maximus* (which is widely distributed and found in Europe and South Africa). The Spanish population of *P. paramaximus* recovered from sandy soil of a commercial citrus grove at Alcala de Guadaira (Seville) agrees very well with the redescription of the species

Table 3. *Morphological and morphometrics differences between* Paralongidorus litoralis *sp. n.,* P. maximus, *and* P. paramaximus*.

Character	*P. litoralis* sp. n.	*P. maximus*	*P. paramaximus*
Habitus	open C-shape	open spiral	open C-shape
Lip region shape	rounded, collar-like constriction present	rounded, collar-like constriction absent	rounded, collar-like constriction present
Lip region diam. (μm)	25-30	34-39	23-29
Oral aperture-guiding ring (μm)	35 (32-37)	41 (37-47)	*ca* 30 (28-39)
Amphid size and aperture	very large (aperture three-fourths as wide as lip region)	large (aperture two-thirds as wide as lip region)	very large (aperture four-fifths as wide as lip region)
Female body length (mm)	9.07 (7.51-10.07)	9.84 (7.60-12.35)	*ca* 7.50 (5.64-10.13)
a	146 (114-164)	72-133	133-182
Odontostyle length (μm)	194 (169-206)	172 (152-187)	*ca* 140 (122-169)
Female tail shape	bluntly rounded, barely dorsally convex-conoid	bluntly rounded	convex-conoid
Female tail length (μm)	32 (27-34)	*ca* 38 (36-41)	*ca* 30 (22-36)
c′	0.74 (0.64-0.83)	*ca* 0.50 (0.38-0.60)	*ca* 0.80 (0.60-1.00)
First-stage juvenile tail shape	elongate-conoid tail with a central peg (*ca* 12 μm); c′ = 1.60 (1.37-1.84)	relative long and somewhat digitate; c′ = 1.15 (1.00-1.27)	unknown
Presence of males	common, as abundant as females	extremely rare (only 2 described)	common, *ca* 50% as numerous as females
Spicule length (μm)	63-72	100-106	54-86

* Data from Heyns (1975), Liebenberg *et al*. (1993), Swart *et al*. (1996) and the present study.

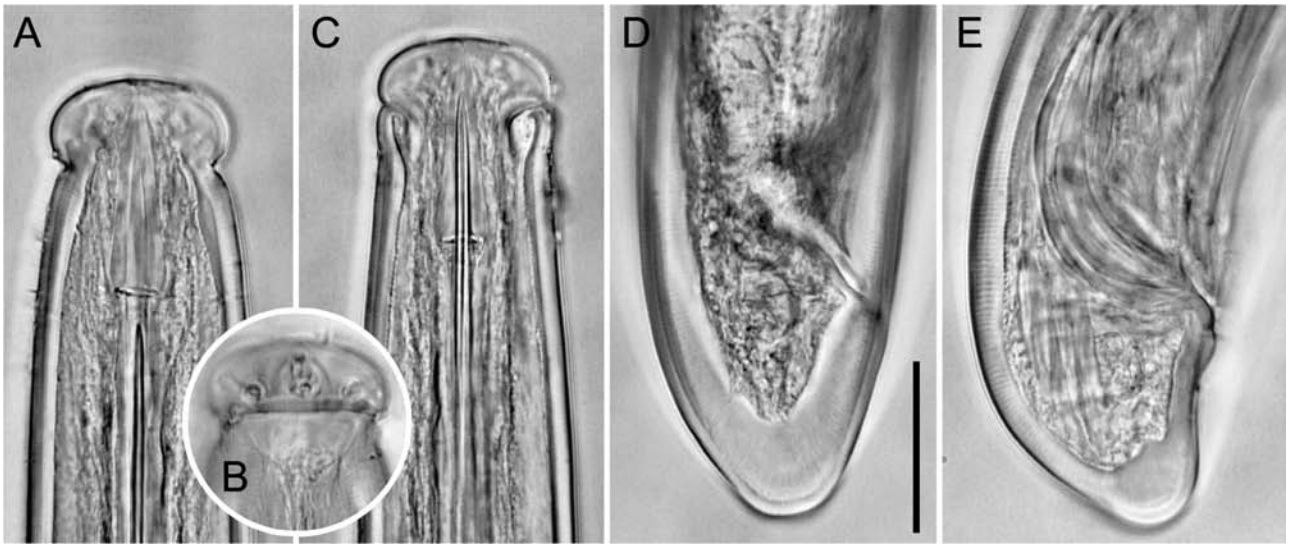

Fig. 5. *Light micrographs of* Paralongidorus paramaximus *Heyns, 1965. A: Female anterior region, lateral view; B: Female anterior region, lateral view showing amphid; C: Female anterior region, ventral view showing amphidial fovea; D: Female tail region; E: Male tail. (Scale bar = 25 μm.)*

by Liebenberg *et al*. (1993). Nevertheless, small differences in de Man ratios (a, b, c, c′) were detected, which confirm specific variability as indicated by Liebenberg *et al*. (1993) who compared several populations from South Africa. The Spanish population of *P. paramaximus* also showed a slightly longer odontostyle than populations from South Africa (164-173 *vs* 122-169 μm), but showed a quite similar lip region diam. (24-28.5 *vs* 24-29 μm),

Fig. 6. *Maximum likelihood trees for Longidoridae. A: As inferred from D2 and D3 expansion fragments of 28S rRNA (Ln L = –7601.67934); B: As inferred from 18S rRNA gene sequences (Ln L = – 4363.46382). Bootstrap values greater than 50% are given for appropriate clades. Newly obtained sequences are indicated by bold text.*

and a similar relative position of the guiding ring (27-33 *vs* 28-39 μm from the anterior end). Finally, the length of the spicules of the Spanish population (57-63 μm) is relatively close to all known South African populations (53-69 μm), except for a male from Kruger National Park with longer spicules (86 μm long).

Molecular characterisation of *Paralongidorus* species and phylogenetic position of genus within Longidoridae

Amplification of the 18S and D2-D3 expansion segments of 28S rDNA from *P. paramaximus* and *P. litoralis* sp. n. yielded a single fragment of approximately 800 and 1700 bp, respectively. The variation in sequences between samples was obtained only for *P. litoralis* sp. n. for the 18S gene; the two samples differing from each other by three nucleotides. Intraspecific sequence diversity for the 18S rRNA gene between three *Paralongidorus* species varied from five to 14 nucleotides (0.3-0.8%). The sequences of D2-D3 fragments for two independent samples of *P. litoralis* sp. n., as well as for two samples of *P. paramaximus*, were identical within each species (only one sample sequence for each species was submitted to GenBank). Sequence variation in D2-D3 fragments between four *Paralongidorus* species ranged from 19-44 nucleotides (2.5-5.7%).

Phylogenetic trees reconstructed by the ML method for the two rRNA genes (18S rRNA and D2 and D3 expansion regions of 28S rRNA gene) are presented in Figure 6. The phylogenetic trees obtained were generally congruent with those given by He *et al*. (2005) and Neilson *et al.* (2004) for D2-D3 of 28S and 18S genes, respectively, with the exception of the position of some poorly supported clades. No significant difference in topology was obtained using the ML or BI approach. In ML and BI trees generated from the 18S rRNA sequences, *Paralongidorus* spp. clustered as an external clade of the genus *Longidorus*. In ML and BI trees generated from the D2-D3 of 28S sequences dataset, *Paralongidorus* nested within *Longidorus*. However, the Shimodaira-Hasegawa test supports the hypothesis ($P = 0.227$) that *Paralongidorus* species are constrained to be a group clustering outside *Longidorus*. Thus, our result based on a wider sampling of longidorids and selection of appropriate outgroup taxa showed that *Paralongidorus* is a valid taxon, in contradiction to the results reported by He *et al*. (2005).

Notes on the geographical distribution of *Paralongidorus* species

Paralongidorus appears to be distributed worldwide, occurring on every continent with the exception of Antarctica. The highest biodiversity of the genus occurs in Asia, where 26 species have been reported from several countries (Table 4), followed by Africa with 22 reported species. However, in other continents the genus shows a low diversity, including Europe, with eight reported species (Table 4), Oceania with three species, and North and South America with only one species each (Table 4), where the genus was recorded from California (Robbins, 1978) and Chile (Roca & Rios, 2006), respectively. These data support the hypothesis that the probable origin of *Paralongidorus* is in the region of South-East Africa to India, the genus probably evolving before these areas became

Table 4. *Occurrence of* Paralongidorus s. str. *species in each continent arranged in alphabetical order.*

Continent (no. of species)	*Paralongidorus* species
Africa (22)*	*buchae, bullatus, capensis, cebensis, christiani, deborae, duncani, epimikis, erriae, fischeri, georgiensis, hanliae, hooperi, lutosus, maximus, namibiensis, paramaximus, sandellus, spasskii, spaulli, wiesae, xiphinemoides*
Asia (26)	*agni, beryllus, buckeri, ciaressi, citri, dasturi, distinctus, esci, fici, flexus, halepensis, inagreinus, indicus, lemoni, lutensis, major, mediensis, microlaimus, nudus, oryzae, rotundatus, sacchari, sali, seclipsi, similis, zenobiae*
Europe (8)	*georgiensis, iberis, litoralis* sp. n., *maximus, monegrensis, paramaximus, remyi, rex*
North America (1)	*microlaimus*
South America (1)	*sacchari*
Oceania (3)	*australis, eucalypti, sacchari*

* Number of species recorded in each continent.

separated by plate tectonics (Coomans, 1985). The most widely distributed and commonest species is *P. maximus*, which has been reported from Africa, Asia and Europe (Table 4). The occasional occurrence of *Paralongidorus* in certain regions of the world, including North and South America, probably represent introductions rather than being a consequence of inadequate sampling and extraction methodology (Robbins & Brown, 1991). The present record of *P. paramaximus* in a citrus orchard in southern Spain may indicate its introduction in Europe through human activities, although a natural dispersal of this species from Gondwanaland cannot be excluded.

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