

Paralongidorus spp. from Iran

Nem-11-00057

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3 **Molecular and morphological characterisation of *Paralongidorus***  
4 ***iranicus* n. sp. and *P. bikanerensis* (Lal & Mathur, 1987) Siddiqi,**  
5 **Baujard & Mounport, 1993 (Nematoda: Longidoridae) from Iran**  
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23 Received: 2011; revised: September 2011

24 Accepted for publication: September 2011  
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1 **Summary** - *Paralongidorus iranicus* n. sp., a new bisexual species of the genus, is  
2 described and illustrated by light microscopy, scanning electron microscopy and  
3 molecular studies from specimens collected in the rhizosphere of Scotch pine (*Pinus*  
4 *sylvestris*) from the Kaspian (Khazar) seashore, Nour, northern Iran. *Paralongidorus*  
5 *iranicus* n. sp. is characterised by the large body size (7.8-11.4 mm), a rounded lip  
6 region, clearly set off by a collar-like constriction at level of, or slightly posterior to, the  
7 amphidial aperture, and bearing a very large stirrup-shaped, amphidial fovea, with  
8 conspicuous slit-like aperture, a very long and flexible odontostyle *ca* 170  $\mu$ m long,  
9 guiding ring located at 34  $\mu$ m from anterior end and males with spicules *ca* 80  $\mu$ m long.  
10 In addition, data from an Iranian population of *P. bikanerensis* recovered from the  
11 rhizosphere of palm (*Phoenix dactylifera*) in Bam, Kerman province, south-eastern Iran,  
12 agrees very well and is very close to the original description of the species from India.  
13 The D2 and D3 expansion regions of 28S rRNA gene, ITS1, and 18S rRNA sequences  
14 were obtained for *P. iranicus* n. sp. and *P. bikanerensis*. Phylogenetic analyses of *P.*  
15 *iranicus* n. sp. and *P. bikanerensis* rRNA gene sequences and of *Longidorus* spp.  
16 sequences published in the GenBank were done using Maximum Likelihood and  
17 Bayesian inference. *Paralongidorus* species (including *P. iranicus* n. sp.) clustered  
18 together; however, *P. bikanerensis* clustered within *Longidorus* spp. and was clearly  
19 separated from all other *Paralongidorus* spp. in trees generated from the D2-D3  
20 expansion segments of 28S and partial 18S data set, respectively.

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22 **Keywords** – description, molecular, morphometrics, morphology, needle nematode,  
23 new record, new species, *Pinus sylvestris*, *Phoenix dactylifera*, phylogeny, taxonomy.

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1           *Paralongidorus* comprises migratory ectoparasites that spend their entire life  
2 cycle outside the host plant roots, feeding on an extensive range of herbaceous and  
3 woody-crops, as well as weeds and natural vegetation. *Paralongidorus* spp. are  
4 commonly known as needle nematodes because of their very long stylets and are of  
5 special scientific and economic interest because they directly damage the roots of the  
6 host plant and at least one species is vector of an economically important plant virus  
7 (Decraemer & Robbins, 2007). The genus is well established and widely accepted by  
8 nematologists, although its definition is not always consistent. Taxonomy of the genus  
9 has been controversial as the genera *Siddiqia* Khan, Chawla & Saha, 1978,  
10 *Longidoroides* Khan, Chawla & Saha, 1978, and *Inagrei* Khan, 1982 have been  
11 synonymised with it and/or recognised as distinct in several review papers (Luc &  
12 Doucet, 1984; Coomans, 1985; Hunt, 1993; Siddiqi *et al.*, 1993; Coomans, 1996; Arias  
13 & Bravo, 1997; Escuer & Arias, 1997). Siddiqi *et al.* (1993) synonymised  
14 *Longidoroides* with *Paralongidorus* based on a new interpretation of the amphid  
15 structure of *P. sali* Siddiqi, Hooper & Khan, 1963, the type species of the genus.  
16 However, the interpretation of the amphidial fovea by Siddiqi *et al.* (1993) was  
17 questioned by Coomans (1996) after detailed study of the type material. Whatever the  
18 status of *Longidoroides*, Coomans (1996) established that it represents an intermediate  
19 condition between *Paralongidorus* species with typical amphids and *Longidorus* species  
20 with pouch-like amphids and pore-like openings. Coomans (1996) also concluded that  
21 *Paralongidorus*, *Longidoroides* and *Longidorus* formed a complex, with the primitive  
22 forms, *viz.*, *Paralongidorus*, including *P. maximus* (Bütschli, 1874) Siddiqi, 1964,  
23 having offset lip regions and stirrup shaped amphidial fovea with wide slit-like  
24 openings. In this sense, Decraemer and Coomans (2007) revised several longidorid  
25 species and revealed misinterpretations of the amphid structure with respect to shape of  
26 the amphidial fovea and amphidial aperture, including species such as *Longidorus boshi*  
27 (Khan, Chawla & Saha, 1972) Decraemer & Coomans, 2007, *Longidorus cedari* (Khan,  
28 Saha & Seshadri, 1972) Decraemer & Coomans, 2007, *Longidorus monegrensis* (Escuer  
29 & Arias, 1997) Decraemer & Coomans, 2007 and *Longidorus spiralis* (Khan, Saha &  
30 Seshadri, 1972) Decraemer & Coomans, 2007. In addition, Decraemer and Coomans  
31 (2007) studied female paratype specimens of *Paralongidorus bikanerensis* (Lal &  
32 Mathur, 1987) Siddiqi, Baujard & Mounport, 1993 with different orientations of the  
33 body, which revealed an elongate funnel-shaped amphidial fovea, showing a refractive  
34 outer lining in dorsoventral view, and maintained the species as *Longidoroides*

1 *bikanerensis* Lal & Mathur, 1987. However, all these studies were conducted without  
2 scanning electron microscopy (SEM).

3       Recent studies on molecular phylogeny of dagger and needle nematodes based  
4 on D2-D3 region of 28S and partial 18S genes resolved three major clades: clade I)  
5 *Longidorus* spp. and *Paralongidorus* spp.; clade II) *Xiphinema americanum*-group  
6 including *Xiphidorus minor* Rashid, Coomans & Sharma, 1986; and clade III) the other  
7 *Xiphinema* species (Gutiérrez-Gutiérrez *et al.*, 2011). In this study, the tree topology  
8 analysis by Shimodaira-Hasegawa test of D2-D3 and partial 18S of a broad number of  
9 sequences did not refute the monophyly of the genus *Xiphinema*, which agreed with the  
10 results obtained by He *et al.* (2005). However, in the paper of Gutiérrez-Gutiérrez *et al.*  
11 (2011), the genus *Paralongidorus* was not accepted as a valid taxon, which also agreed  
12 with He *et al.* (2005) but disagrees with a more restricted study with fewer sequences  
13 conducted by Palomares-Rius *et al.* (2008). Nonetheless, no molecular data exist on any  
14 species of the genus *Longidoroides*.

15       During 2008-2010 several extensive studies on systematic of Longidoridae were  
16 performed in Iran (Pedram *et al.*, 2008a,b; 2009; 2011; Niknam *et al.*, 2010). All  
17 identified species belonged to *Longidorus* and *Xiphinema* and hitherto no population of  
18 *Paralongidorus* was found. The only report of *Paralongidorus* in Iran was by Kheiri  
19 and Barooti (1985) which reported *P. georgiensis* Tulaganov, 1937. Following our  
20 studies on longidorids, an extensive study on the presence of species of *Paralongidorus*  
21 in Iran yielded a species having very long body and stirrup-shaped amphidial fovea and  
22 morphologically resembling *P. litoralis* Palomares-Ruis, Subbotin, Landa, Vovlas &  
23 Castillo 2008, and another population resembling *L. bikanerensis*, a fact which  
24 prompted us to undertake a detailed morphological and molecular comparative study  
25 with previous reported data.

26       The objectives of this work were: *i*) to characterise morphologically and  
27 molecularly the two Iranian populations of *Paralongidorus*; and *ii*) to study the  
28 phylogenetic relationships of these populations with *Paralongidorus* spp. and  
29 *Longidorus* spp. (with *Xiphinema* Cobb, 1913 and *Xiphidorus* Monteiro, 1976 as  
30 outgroups) using sequences from the D2-D3 expansion regions of 28S rRNA and the  
31 18S rRNA as inferred from Maximum Likelihood and Bayesian inference approach.

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### 33 **Materials and methods**

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## 1 NEMATODE POPULATIONS

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3 Nematode populations used in this study were obtained from sandy soils at a  
 4 depth of 10-50 cm in the rhizosphere of pine (*Pinus sylvestris* L.) from Nour,  
 5 Mazandaran province, northern Iran, and palm (*Phoenix dactylifera* L.) from Bam,  
 6 Kerman province, south-eastern Iran. Nematodes were extracted by the sieving method  
 7 described by Flegg (1967). Nematodes were observed and hand-picked directly under a  
 8 stereomicroscope Nikon SMZ1000. The specimens were killed by adding hot 4%  
 9 formaldehyde solution, transferred to anhydrous glycerin according to De Grisse (1969)  
 10 and mounted on permanent slides. Specimens were examined using a Zeiss III  
 11 compound microscope with Nomarski differential interference contrast at up to  $\times 1000$   
 12 magnification. Measurements were done using a drawing tube attached to a Nikon  
 13 Eclipse E600 light microscope. For line drawing, handmade drawings were scanned and  
 14 imported to CorelDraw software version 12 and redrawn. Morphometric data were  
 15 processed using Statistix 9.0 (NH Analytical Software, Roseville, MN, USA). The  
 16 location of pharyngeal gland nuclei is given following Loof and Coomans (1972).

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

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## 21 DNA EXTRACTION, PCR AND SEQUENCING

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23 Nematode DNA was extracted from single individuals and protocols for PCR  
 24 were conducted as described by Castillo *et al.* (2003). The D2-D3 expansion segments  
 25 of 28S rDNA was amplified using the D2A (5'-  
 26 ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-  
 27 TCGGAAGGAACCAGCTACTA-3') primers (Castillo *et al.*, 2003; He *et al.*, 2005;  
 28 Palomares-Rius *et al.*, 2008). The ITS 1 region was amplified using forward primer 18S  
 29 (5'TTGATTACGTCCCTGCCCTTT-3') and reverse primer 26S (5'-  
 30 TTTCACCTCGCCGTTACTAAGG-3') as described in Wang *et al.*, (2002). Finally, the  
 31 18S rDNA gene was amplified using the SSU\_F\_07 (5'-  
 32 AAAGATTAAGCCATGCATG-3') and SSU\_R\_81 (5'-  
 33 TGATCCWKCYGCAGGTTAC-3') primers  
 34 (<http://www.nematodes.org/barcoding/sourhope/nemoprimer.html>).

1 PCR products were purified after amplification with GeneClean turbo (Q-  
2 BIOgene SA, Illkirch Cedex, France) or QIAquick (Qiagen, USA) gel extraction kits,  
3 quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington,  
4 DE, USA) and used for direct sequencing in both directions with the primers referred  
5 above. The resulting products were purified and run on a DNA multicapillary sequencer  
6 (Model 3100 genetic analyser; Applied Biosystems, Foster City, CA, USA) at the  
7 STABVIDA sequencing facilities (Monte da Caparica, Portugal). The newly obtained  
8 sequences were submitted to the GenBank database under accession numbers  
9 JN032584-JN032589 as indicated on the phylogenetic trees.

#### 11 PHYLOGENETIC ANALYSIS

13 D2-D3 expansion segments of 28S and 18S-rRNA newly obtained sequences  
14 and sequences obtained from GenBank were used for phylogenetic reconstruction.  
15 Outgroup taxa for each dataset were chosen according to previous published data  
16 (Palomares-Rius *et al.*, 2008). The newly obtained and published sequences for each  
17 gene were aligned using ClustalW (Thompson *et al.*, 1997) with default parameters.  
18 Sequence alignments were manually edited using BioEdit (Hall, 1999). Phylogenetic  
19 analysis of the sequence data sets were performed with maximum likelihood (ML)  
20 using PAUP \* 4b10 (Swofford, 2003) and Bayesian inference (BI) using MrBayes 3.1.2  
21 (Huelsenbeck & Ronquist, 2001). The best fit model of DNA evolution was obtained  
22 using the program jModelTest ver. 0.1.1 (Posada, 2008) with the Akaike Information  
23 Criterion (AIC). The Akaike-supported model, the base frequency, the proportion of  
24 invariable sites and the gamma distribution shape parameters and substitution rates in  
25 the Akaike information criterion (AIC) were used in phylogenetic analyses. BI analysis  
26 under GTR + G + I model for both genes was initiated with a random starting tree and  
27 was run with four chains for  $1.0 \times 10^6$  generations. The Markov chains were sampled at  
28 intervals of 100 generations. Two runs were performed for each analysis. After  
29 discarding burn-in samples and evaluating convergence, the remaining samples were  
30 retained for further analysis. The topologies were used to generate a 50% majority rule  
31 consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees were  
32 visualised using the TreeView program (Page, 1996). In ML analysis, the estimation of  
33 the support for each node was made using a bootstrap analysis with 100 fast-step  
34 replicates.

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## Results

### *Paralongidorus iranicus*\* n. sp.

(Figs 1-4)

#### MEASUREMENTS

See Tables 1, 2.

#### DESCRIPTION

##### *Female*

Body very long, rather robust. Habitus ventrally arcuate usually in an open C when relaxed by gentle heating. Cuticle appearing smooth,  $4.3 \pm 0.5$  (4.0-5.0)  $\mu\text{m}$  thick,  $16.3 \pm 2.0$  (13.0-19.0)  $\mu\text{m}$  thick at tip tail, marked by very fine superficial transverse striae mainly in tail region under SEM. Lip region rounded in lateral view, clearly set off by a collar-like constriction at level with, or slightly posterior to, amphidial aperture,  $11.4 \pm 1.1$  (10-13)  $\mu\text{m}$  high. SEM observations showing a rounded to oval oral aperture surrounded by six inner labial papillae and six outer labial papillae in *en face* view. Cephalic papillae hardly visible on SEM pictures, appearing as small apertures, each located just anterior to a distinct cephalic lobe (2.0-2.5  $\mu\text{m}$  long). Amphidial fovea very large, stirrup shaped, with conspicuous aperture *ca* three-fourths as wide as lip region. Stylet guiding ring single, 6-8  $\mu\text{m}$  wide, located  $1.2 \pm 0.07$  (1.1-1.4) lip region diam. from anterior end. Body diam. at guiding ring level  $37.8 \pm 2.0$  (33-40)  $\mu\text{m}$ . Lateral chord 16.5 (14-22)  $\mu\text{m}$  wide at mid-body or  $21.5 \pm 3.3$  (15.0-24.5)% of corresponding body diam. Odontostyle long and slender, straight or slightly arcuate in posterior half, *ca* 3-3.5  $\mu\text{m}$  wide towards its base, odontophore slightly swollen at base. Nerve ring encircling pharynx, located slightly posterior to middle of pharynx. Pharynx dorylaimoid, typical of genus. Anterior slender part of pharynx usually with loop overlapping basal bulb. Basal bulb cylindrical,  $140 \pm 7.5$  (120-150)  $\mu\text{m}$  long, 28-31  $\mu\text{m}$  diam. Dorsal pharyngeal gland nucleus in anterior part of bulb, 11.5-17.5  $\mu\text{m}$  posterior

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\* The species epithet refers to the country where the species was found.

1 to gland outlet, one ventrosublateral pair of nuclei near middle of bulb. Cardia  
 2 elongated, clearly visible,  $21.5 \pm 6.1$  (15-34)  $\mu\text{m}$  long,  $14 \pm 2$  (10.0-17.5)  $\mu\text{m}$  wide,  
 3 prerectum long and variable, 562 (365-780)  $\mu\text{m}$  long and rectum 42 (35-60)  $\mu\text{m}$  long.  
 4 Reproductive system with both genital branches equally developed, vulva in form of a  
 5 transverse slit, located slightly anterior to mid-body, vagina  $61.5 \pm 5.0$  (53-70)  $\mu\text{m}$  long,  
 6 surrounded by well developed muscles, each oviduct separated from uterus by a well  
 7 developed *pars dilatata oviductus*. Tail short, barely dorsally convex-conoid with  
 8 broadly rounded terminus.

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10 *Male*

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12 Almost as common and as abundant as female. Habitus mostly similar to that of  
 13 female but with posterior region curved ventrally. Lip region as in female,  $11.5 \pm 1.5$   
 14 (10.0-12.5)  $\mu\text{m}$  high. Male genital tract diorchic with testes opposed, occupying *ca* 40%  
 15 of body length. Tail short, dorsally convex conoid, ventrally slightly concave with broad  
 16 blunt terminus and thickened outer cuticular layer. Spicules robust, *ca* twice as long as  
 17 tail length, lateral guiding pieces  $22.5 \pm 2.0$  (20-25)  $\mu\text{m}$  long. One pair of cloacal  
 18 supplements located at  $17.5 \pm 2.0$  (15-20)  $\mu\text{m}$  from cloacal opening and a series of 12-14  
 19 ventromedian supplements ending  $20.5 \pm 3.5$  (15-26)  $\mu\text{m}$  from cloacal pair.

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21 *Juveniles*

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23 Three juvenile stages were found, being distinguishable by relative lengths of  
 24 body and functional and replacement odontostyles (Table 2). First-stage juvenile not  
 25 detected. Morphology in all three juvenile stages (J2, J3, J4) similar to that of female  
 26 (except for undeveloped genital structures). Tail shape of J2 conoid-rounded. J3 and J4  
 27 tail shape barely dorsally convex-conoid, but more elongate than that of female, shorter  
 28 body length, and shorter distance from anterior end to guiding ring (Table 2).

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## 30 TYPE HOST AND LOCALITY

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32 Rhizosphere of Scots pine (*Pinus sylvestris* L.) from the Caspian (Khazar)  
 33 seashore, Nour, northern Iran.

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## 1 TYPE MATERIAL

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3 Holotype female and five female and male paratypes deposited in the Nematode

4 Collection of the Faculty of Agriculture, Tarbiat Modares University, Tehran-Iran.

5 Three female and two male paratypes deposited at each of the following collections:

6 CABI Europe-UK, Egham, Surrey, UK; Istituto per la Protezione delle Piante (IPP) of

7 Consiglio Nazionale delle Ricerche (C.N.R.), Sezione di Bari, Bari, Italy; USDA

8 Nematode Collection, Beltsville, MD, USA. Specific D2-D3, partial 18S, and ITS1-

9 rRNA sequences deposited in GenBank with accession numbers JN032587, JN032588,

10 and JN032589, respectively.

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## 12 DIAGNOSIS AND RELATIONSHIPS

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14 *Paralongidorus iranicus* n. sp. is characterised by a long body (7.8-11.4 mm), a

15 rounded lip region clearly offset from the body by a marked constriction and bearing a

16 very large, stirrup-shaped, amphidial fovea, with conspicuous slit-like aperture, a very

17 long and flexible odontostyle *ca* 170  $\mu$ m long, stylet guiding ring located at *ca* 34  $\mu$ m18 from anterior end, and males with spicules *ca* 80  $\mu$ m long, and a specific D2-D3, ITS1,

19 and partial 18S-rRNA sequence (GenBank accession numbers JN032587, JN032588,

20 and JN032589, respectively). The matrix code according to the polytomous key by

21 Escuer and Arias (1997) is: A1, B1, C4, D2, E2, F6, G7, H12, I22, J1, K45, L23, M3,

22 N23, O2.

23 On the basis of body and odontostyle length, lip region, and amphidial fovea, *P.*24 *iranicus* n. sp. is close to *P. australis* Stirling & McCulloch, 1984, *P. litoralis*, *P.*25 *maximus*, *P. paramaximus* Heyns, 1965 and *P. rex* Andr assy, 1986. *Paralongidorus*26 *iranicus* n. sp. is morphological and morphometrically almost undistinguishable from *P.*27 *litoralis* (both taxa should be considered cryptic species, *i.e.* morphologically28 undistinguishable but genetically distinct) (Palomares-Rius *et al.*, 2008). Even so, both29 species differ slightly in their juvenile characters:  $a = 101.9-137.7$  vs  $113.7-164.4$ ,30 odontostyle length =  $155-184$  vs  $169-206$   $\mu$ m,  $c^{\circ} = 0.5-0.7$  vs  $0.64-0.83$ , odontophore =31  $82-100$  vs  $70-92$   $\mu$ m, all of which could be consider as intraspecific variability in the32 absence of molecular data (Palomares-Rius *et al.*, 2008). From *P. australis* it differs in

33 lip region shape (collar-like constriction posterior to lip region present vs absent),

34 distance from the oral aperture to the guiding ring ( $31.5-39.0$  vs  $58-70$   $\mu$ m), a ratio

1 (101.9-137.7 vs 85.5-116.0), odontostyle length (155-184 vs 146-170  $\mu\text{m}$ ), odontophore  
 2 length (82-100 vs 101-124  $\mu\text{m}$ ), and spicule length (74-85 vs 112-134  $\mu\text{m}$ ) (Stirling &  
 3 McCulloch, 1984). From *P. maximus* it differs in lip region diam. (25-30 vs 34-39  $\mu\text{m}$ ),  
 4 lip region shape (collar-like constriction posterior to lip region present vs absent),  
 5 distance from oral aperture to guiding ring (31.5-39.0 vs 37-47  $\mu\text{m}$ ), female tail shape  
 6 (bluntly rounded, barely dorsally convex-conoid vs bluntly rounded), odontophore  
 7 length (82-100 vs 42-70  $\mu\text{m}$ ), presence of males (common vs extremely rare), and  
 8 spicule length (74-85 vs 100-106  $\mu\text{m}$ ) (Heyns, 1975). From *P. paramaximus* it differs in  
 9 range of odontostyle length (155-184 vs 122-173  $\mu\text{m}$ ), c ratio (221.3-314.8 vs 170-285),  
 10 c' ratio (0.5-0.7 vs 0.60-1.00) and spicule length (74-85 vs 57-69  $\mu\text{m}$ ) (Heyns 1965;  
 11 Palomares-Rius *et al.*, 2008). From *P. rex* it differs in V ratio (37-44 vs 46.2-47.0), c  
 12 ratio (221.3-314.8 vs 230-250), body diam. at guiding ring level (33-40 vs 55  $\mu\text{m}$ ) and  
 13 presence of males (common vs absent) (Andrássy, 1986; Barsi *et al.*, 2007).

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15 ***Paralongidorus bikanerensis* (Lal & Mathur, 1987) Siddiqi, Baujard & Mounport,**

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1993

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(Figs 4-7)

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19 MEASUREMENTS

20

21 See Tables 1, 2.

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23 *Female*

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25 Body long, tapering very gradually towards anterior end, usually assuming an  
 26 open C-shape in habitus. Cuticle appearing smooth, 3.5  $\mu\text{m}$  thick between anterior end  
 27 and guiding ring, varying to 2-3  $\mu\text{m}$  at mid-body and 5.0-6.5  $\mu\text{m}$  at tail tip, with fine  
 28 transverse striae as observed under SEM. Lip region wide, 2.3-2.5 times as long as high,  
 29 anteriorly flat, set off by a sharp constriction at level of amphid aperture and with a  
 30 shallow depression slightly further posterior. SEM photographs revealing a rounded oral  
 31 aperture surrounded by six inner labial papillae and six outer labial papillae. Cephalic  
 32 papillae appearing as small apertures, each located just anterior to a minute cephalic  
 33 lobe (0.5-1.0  $\mu\text{m}$  long). Amphidial fovea elongate-funnel shaped, with conspicuous and  
 34 very fine aperture *ca* three-fourths as wide as lip region, with slit like aperture, 70-74%

1 of lip region diam. Odontostyle simple at base, 1.7-2.0 times as long as odontophore,  
 2 odontophore with slightly swollen base, guiding ring simple, 2.1-2.3 times lip region  
 3 diam. posterior to anterior end. Body diam. at guiding ring level  $25 \pm 1$  (23.5-26.0)  $\mu\text{m}$ .  
 4 Lateral chord 20.5-25.5% of corresponding body diam. wide. Nerve ring encircling the  
 5 narrow anterior part of pharynx, 5.0-5.3 times body diam. at neck base from anterior  
 6 end. Anterior slender part of pharynx usually with loop overlapping basal bulb, basal  
 7 bulb cylindrical, 5.5-6.5 times as long as wide. Basal bulb cylindrical,  $117 \pm 7.2$  (110-  
 8 123)  $\mu\text{m}$  long, 24-26  $\mu\text{m}$  diam. Dorsal pharyngeal gland nucleus in anterior part of bulb,  
 9 7.0-15.5  $\mu\text{m}$  posterior to gland outlet, one ventrosublateral pair of nuclei near middle of  
 10 bulb. Cardia hemispherical,  $12.3 \pm 1.6$  (10-14)  $\mu\text{m}$  wide, prerectum long and variable,  
 11 11.5-12.5 anal body diam. long and rectum 0.8-1.0 anal body diam. long. Reproductive  
 12 system with both genital branches equally developed, each 307-433  $\mu\text{m}$  long, composed  
 13 of a 72.5-120.0  $\mu\text{m}$  long ovary, 150-270  $\mu\text{m}$  long reflexed oviduct, a sphincter and a  
 14 145-167  $\mu\text{m}$  long uterus with thin wall, vagina perpendicular to body axis, 27-35  $\mu\text{m}$   
 15 long, or 55-62% of corresponding body diam., composed of *pars distalis vaginae*, 15-20  
 16  $\mu\text{m}$  long, and *pars proximalis vaginae* as long as high and  $17-20 \times 14-20$   $\mu\text{m}$  in size.  
 17 Vulva a transverse slit and 13  $\mu\text{m}$  or 23% corresponding body diam. long. Tail dorsally  
 18 convex, with rounded terminus, hyaline region 10.5-14.0  $\mu\text{m}$  thick.

19

20 *Male*

21

22 Not found.

23

24 *Intersex*

25

26 Similar to female in general morphology and morphometric data, except having  
 27 weakly developed spicules, 19.0  $\mu\text{m}$  long and 4.5  $\mu\text{m}$  wide, one weakly developed  
 28 precloacal supplement located at 13  $\mu\text{m}$  from cloacal opening, and one ventromedian  
 29 supplement 46  $\mu\text{m}$  far from cloacal pair.

30

31 *Juveniles*

32

33 All four juvenile stages were found and described for the first time, since  
 34 original description include morphometrics of ten juveniles without specifying life-

1 stage. Juvenile life-stages were distinguished by relative lengths of body and functional  
 2 and replacement odontostyle (Table 2, Robbins *et al.*, 1995; 1996). First-stage juveniles  
 3 characterised by an elongate-conoid tail, odontostyle length *ca* 60  $\mu\text{m}$  and shorter  
 4 distance from anterior end to stylet guiding ring than that in adult stages. However,  
 5 morphology in all three juvenile stages (except for undeveloped genital structures)  
 6 similar to that of female, including tail shape of third- to fourth-stage juveniles which  
 7 was bluntly rounded, yet differed by a shorter distance from anterior end to guiding  
 8 ring.

9

## 10 REMARKS

11

12 To the best of our knowledge, *P. bikanerensis* is known only from the type  
 13 population from India in association with palm trees. The Iranian population of this  
 14 species was also found in association with palm. When comparing the morphology and  
 15 all morphometric characters from the Iranian population of *P. bikanerensis* they agree  
 16 very well with the original description (Lal & Mathur, 1987). Nevertheless, small  
 17 differences in de Man ratios (a, b), max. body diam. and odontophore length were  
 18 detected, which confirm intraspecific variability: *i.e.* a = 98.5-140.8 *vs* 125-140; b =  
 19 11.3-14.2 *vs* 13.5-18.5; max. body diam. = 58-90 *vs* 92-95  $\mu\text{m}$ ; odontophore length = 66-  
 20 76 *vs* 54-65  $\mu\text{m}$  and tail length = 25-37 *vs* 40-45  $\mu\text{m}$ . Decraemer and Coomans (2007)  
 21 studied paratype specimens of *P. bikanerensis*, concluding that the shape of the  
 22 amphidial fovea instead of being a bilobed pouch (as illustrated in the original  
 23 description), was elongate funnel-shaped and showed a refractive outer lining in  
 24 dorsoventral view. Our detailed observations on specimens mounted in glycerin, as well  
 25 as in fresh specimens, agree with Decraemer and Coomans (2007) on the morphology  
 26 of the amphidial fovea. However, our SEM observations clearly demonstrated a  
 27 conspicuous and very narrow amphidial aperture which may justify the difficulty for  
 28 distinguishing this structure under light microscopy. Consequently, we maintain this  
 29 species under *Paralongidorus* instead of *Longidoroides*, which agrees with Siddiqi *et al.*  
 30 (1993) on the synonymy of *Longidoroides* with *Paralongidorus*. However, additional  
 31 SEM and molecular studies, are needed to clarify the validity of *Longidoroides*. Our  
 32 SEM data on the amphidial apertures of these Iranian populations of *Paralongidorus*  
 33 clearly showed different morphology to those previously reported for *Longidorus* and  
 34 *Longidoroides* (Swart & Heyns, 1987; Roca, 2006). All these features demonstrate the

1 need for integrating morphological and molecular data for the diagnosis of this complex  
2 species group.

3

4 MOLECULAR CHARACTERISATION OF *PARALONGIDORUS IRANICUS* N. SP. AND *P.*

5 *BIKANERENSIS* FROM IRAN AND PHYLOGENETIC POSITION WITHIN *LONGIDORUS* AND

6 *PARALONGIDORUS*

7

8         Amplification of the partial 18S, D2-D3 expansion segment of 28S rDNA and  
9 ITS 1 rRNA from *P. bikanerensis* and *P. iranicus* n. sp. yielded a single fragment of ca  
10 1700, 800, and 1500 bp, respectively. Sequence variability for the D2-D3 region among  
11 the four *Paralongidorus* sequences retrieved from GenBank and *P. iranicus* n. sp. or *P.*  
12 *bikanerensis*, varied from 45 to 62 nucleotides (6-8%) and 147 nucleotides (18%),  
13 respectively. The 18S rRNA gene showed a lower diversity than D2-D3 segments of  
14 28S rRNA, varying from 14 to 26 nucleotides (1-2%) for both species. No homologies  
15 in GenBank were found for these species using the ITS1 region from the rDNA among  
16 *Longidorus* spp. There are no sequences available for this region in GenBank for  
17 *Paralongidorus*. Both species are clearly separated by differences of 865 nucleotides  
18 (50% similarity from the aligned sequences), 234 nucleotides of these differences being  
19 related to insertions-deletions between both species and mainly related to *P. iranicus* n.  
20 sp (188 vs 46 nucleotides insertions). Using these three molecular markers both species  
21 were clearly separated from all other *Paralongidorus* spp.

22 Phylogenetic trees reconstructed by the ML method for the two rRNA genes (D2-D3  
23 expansion regions of 28S rRNA gene and partial 18S rRNA) are presented in Figures 8  
24 and 9, respectively. The phylogenetic trees obtained were generally congruent with  
25 those given by Gutierrez-Gutierrez *et al.* (2011), He *et al.* (2005) and Neilson *et al.*  
26 (2004) for D2-D3 of 28S and 18S genes, respectively, with the exception of the position  
27 of some poorly supported clades (Figs 8, 9). No significant difference in topology was  
28 obtained using the ML or BI approach for both markers and only a few species in some  
29 minor clades with low bootstrap values were not congruent with the general topology  
30 tree. In ML and BI trees generated from the D2-D3 of 28S sequences dataset (Fig. 8), *P.*  
31 *iranicus* n. sp. formed a well supported clade with the other *Paralongidorus* sequences,  
32 clustering as an additional clade of the genus *Longidorus*. The closest related species to  
33 *P. iranicus* n. sp. is a unidentified species (AY601582) followed by *P. maximus*  
34 (AF480083). However, the position of *P. bikanerensis* is more closely related to some

1 *Longidorus* spp. than to *Paralongidorus* spp. These more closely related species are *L.*  
 2 *Helveticus* Lamberti, Kunz, Grunder, Molinari, De Luca, Agostinelli & Radicci 2001  
 3 (EF538753), *L. macrosoma* Hooper, 1961 (AY601565), *L. poessneckensis* Altherr, 1974  
 4 (EF538750), *L. caespiticola* Hooper, 1961 (AY601567) and *L. latocephalus* Lamberti,  
 5 Choleva & Agostinelli 1983 (AY601569) with a high support value clade clade in BI  
 6 and ML trees. Trees generated using partial 18S using BI and ML (Fig. 9) showed a  
 7 congruent position of *P. iranicus* n. sp. with the other *Paralongidorus* spp. with a closer  
 8 relationship to *P. maximus* (AJ875152) than to *P. litoralis* (EU026159) and *P.*  
 9 *paramaximus* (EU026157). *Paralongidorus bikanerensis* occupied a separate position to  
 10 the other *Paralongidorus* spp., which were grouped in the same cluster with high  
 11 posterior probabilities and bootstrap values. Nevertheless, the cluster formed with  
 12 *Longidorus* spp. was not well supported in BI analyses, but well supported by ML  
 13 analysis. Morphologically related species to *P. iranicus* n. sp., such as *P. litoralis*, are  
 14 clustered together yet clearly separated phylogenetically.

15 Consequently, on the basis of the present morphological and molecular results,  
 16 as well as considering the previous molecular data by Gutiérrez-Gutiérrez *et al.* (2011)  
 17 and Palomares-Rius *et al.* (2008), additional integrative studies are needed for clarifying  
 18 the validity of *Longidoroides* and *Paralongidorus*.

19

## 20 **Acknowledgements**

21

22 The authors thank J. Martín Barbarroja (IAS-CSIC) for excellent technical  
 23 assistance, Zohreh Lori and Azam Houshmand for helping with the sampling in Iran,  
 24 and two anonymous reviewers for critical reviews of the manuscript.

25

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- 29

1 **Table 1.** Morphometrics of *Paralongidorus iranicus* n. sp. and *P. bikanerensis* (Lal &  
 2 Mathur, 1987) Siddiqi, Baujard & Mounport, 1993 from Iran. All measurements are in  
 3  $\mu\text{m}$  (except for L) and in the form: mean  $\pm$  s.d. (range)\*.

Character	<i>Paralongidorus iranicus</i> n. sp.			<i>Paralongidorus bikanerensis</i>	
	Nour (Mazandaran province)			Bam (Kerman province)	
	Holotype	Females	Males	Females	Intersex
n	–	20	12	9	1
L (mm)	9.60	8.79 $\pm$ 0.829 (7.75-11.41)	8.20 $\pm$ 0.5 (7.41-8.85)	6.23 $\pm$ 0.54 (5.37-6.90)	6.4
a	106.7	116.2 $\pm$ 11.3 (101.9-137.7)	123.5 $\pm$ 7.5 (113.5-138.5)	117.2 $\pm$ 12.4 (98.5-140.8)	115.7
b	22.3	15.6 $\pm$ 2.1 (13.3-22.3)	13.0 $\pm$ 1.0 (11.0-14.5)	12.5 $\pm$ 1.1 (11.3-14.2)	12.5
c	307.2	277.1 $\pm$ 26.0 (221.3-314.8)	232.0 $\pm$ 22.0 (204.0-283.0)	190.4 $\pm$ 17.9 (153.4-218.0)	192.8
c'	0.5	0.6 $\pm$ 0.05 (0.5-0.7)	0.7 $\pm$ 0.1 (0.5-0.7)	0.9 $\pm$ 0.1 (0.8-1.1)	0.9
V or T	43	40.5 $\pm$ 2.1 (37-44)	42.2 $\pm$ 5.1 (37-50)	45 $\pm$ 1.3 (43-47)	45
G <sub>1</sub>	6.9	6.7 $\pm$ 1.7 (5.0-8.8)	–	7.4 $\pm$ 0.3 (7.0-7.7)	–
G <sub>2</sub>	7.1	7.4 $\pm$ 1.2 (5.8-8.7)	–	7.4 $\pm$ 0.4 (7.0-7.8)	–
Odontostyle	178	169.5 $\pm$ 7.4 (155-184)	165.0 $\pm$ 5.5 (157-176)	127.4 $\pm$ 3.4 (121-132)	132
Odontophore	100	90.0 $\pm$ 5.2 (82-100)	92.0 $\pm$ 5.5 (83.0-101.5)	70.8 $\pm$ 3.9 (66-76)	76
Lip region diam.	30	27.8 $\pm$ 1.6 (25.0-30.0)	27.0 $\pm$ 1.2 (25.0-29.0)	16.1 $\pm$ 0.4 (15.5-17.0)	16.5
Oral aperture- guiding ring	39	34.1 $\pm$ 2.2 (31.5-39.0)	35.0 $\pm$ 2.0 (32.0-39.0)	35.7 $\pm$ 1.4 (32.5-37.0)	37
DO	12.5	10.8 $\pm$ 1.6 (9.3-12.5)	10.7 $\pm$ 0.6 (9.8-11.2)	14.6 $\pm$ 3.6 (9.6-16.7)	–
DN	23.5	28.4 $\pm$ 4.2 (23.5-31.0)	21.4 $\pm$ 0.7 (20.5-22.3)	22.1 $\pm$ 0.4 (22.0-22.8)	–
SN <sub>1</sub> & SN <sub>2</sub>	59.2	56.9 $\pm$ 1.9 (54.5-59.2)	55.2 $\pm$ 0.7 (54.3-55.9)	54.8 $\pm$ 2.3 (52.5-57.0)	–
SO <sub>1</sub> & SO <sub>2</sub>	85.5	86.7 $\pm$ 1.9 (85.5-88.9)	85.0 $\pm$ 1.2 (83.5-86.3)	86.7 $\pm$ 1.4 (85.3-87.8)	–
Nerve ring to anterior end	314	329 $\pm$ 12.4 (308-350)	228.5 $\pm$ 122.3 (142-315)	223 $\pm$ 7.4 (218-238)	–
Pharynx length	430	566.7 $\pm$ 55.6 (430-655)	624.0 $\pm$ 46.5 (509-677)	495 $\pm$ 27.2 (455-541)	509
Tail length	31	31.9 $\pm$ 2.8 (25-37)	36.0 $\pm$ 3.0 (31.5-40.0)	32.8 $\pm$ 1.8 (31.0-36.5)	33
Spicule length	–	–	79.0 $\pm$ 3.0 (74-85)	–	19

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

1 **Table 2.** Morphometrics of first-stage (J1), second-stage (J2), third-stage (J3), and fourth-stage (J4) paratype juveniles of *Paralongidorus iranicus*  
 2 *sp. n.* and *P. bikanerensis* (Lal & Mathur, 1987) Siddiqi, Baujard & Mounport, 1993 from Iran. All measurements are in  $\mu\text{m}$  (except for L) and in  
 3 the form: mean  $\pm$  s.d. (range).  
 4

Character- Ratio	<i>Paralongidorus iranicus</i> n. sp.			<i>Paralongidorus bikanerensis</i>			
	J2	J3	J4	J1	J2	J3	J4
n	3	6	7	2	6	5	4
L (mm)	2.38 $\pm$ 0.10 (2.29-2.49)	4.03 $\pm$ 0.20 (3.76-4.34)	5.84 $\pm$ 0.65 (4.90-6.66)	1.24 (1.23-1.25)	2.87 $\pm$ 0.26 (2.42-3.14)	3.86 $\pm$ 0.67 (3.76-3.92)	4.65 $\pm$ 0.38 (4.23-5.16)
a	63.0 $\pm$ 3.3 (59.0-65.5)	83.1 $\pm$ 24.2 (66.3-131.0)	88.4 $\pm$ 6.4 (80.4-96.5)	58.4 (56.9-59.9)	83.8 $\pm$ 1.8 (80.8-85.4)	101.8 $\pm$ 4.6 (96.4-106.2)	108.8 $\pm$ 6.2 (99.6-111.7)
b	6.5 $\pm$ 0.5 (6.0-6.8)	8.2 $\pm$ 1.1 (6.4-9.6)	10.4 $\pm$ 1.3 (8.4-12.1)	5.6 (5.1-6.1)	7.7 $\pm$ 0.8 (6.6-8.9)	8.9 $\pm$ 0.7 (7.9-9.7)	10.1 $\pm$ 1.5 (9.0-12.2)
c	75.0 $\pm$ 6.5 (71.5-83.0)	132.7 $\pm$ 14.0 (109.5-151.0)	191.8 $\pm$ 18.5 (168.9-214.7)	31.8 (31.3-32.3)	74.5 $\pm$ 9.3 (63.8-90.9)	108.8 $\pm$ 5.0 (103.4-115.7)	146.4 $\pm$ 21.0 (127.2-176.4)
c'	1.0 $\pm$ 0.1 (0.9-1.1)	0.7 $\pm$ 0.06 (0.6-0.8)	0.6 $\pm$ 0.05 (0.47-0.63)	2.5 (2.5-2.6)	1.4 $\pm$ 0.2 (1.2-1.7)	1.1 $\pm$ 0.07 (1.1-1.2)	0.9 $\pm$ 0.16 (0.7-1.0)
Odontostyle	104.4 $\pm$ 0.9 (103.5-105.0)	124.4 $\pm$ 5.3 (116-130)	147.3 $\pm$ 7.3 (136-155)	59.8 (59.5-60.0)	92.4 $\pm$ 3.9 (87-98)	102.6 $\pm$ 4.3 (95.5-106.0)	110.4 $\pm$ 2.5 (107.0-113.0)
Replacement odontostyle	122.0 $\pm$ 1.6 (120.5-124.0)	142.8 $\pm$ 4.1 (139-148)	167.7 $\pm$ 14.3 (151-194)	75.8 (74.5-77.0)	107.2 $\pm$ 4.3 (102-113)	119.0 $\pm$ 4.6 (112-123)	126.0 $\pm$ 2.9 (123-130)
Lip region diam.	16.0 $\pm$ 0.5 (15.5-16.0)	21.7 $\pm$ 1.1 (20.0-23.0)	24.5 $\pm$ 0.7 (24.0-25.5)	9.8 (9.5-10.0)	12.8 $\pm$ 0.6 (12.0-13.5)	14.6 $\pm$ 0.2 (14.5-15.0)	15.3 $\pm$ 0.5 (15.0-16.0)
Oral aperture-guiding ring	22.5 $\pm$ 2.1 (20-24)	27.7 $\pm$ 1.8 (26.0-31.5)	32.8 $\pm$ 4.0 (24.0-36.0)	18.5 (18.0-19.0)	27.8 $\pm$ 1.0 (26.0-29.0)	31.6 $\pm$ 1.1 (30.0-33.0)	33.0 $\pm$ 0.9 (32.0-34.0)
Tail	31.5 $\pm$ 1.5 (30-33)	30.6 $\pm$ 3.1 (29-37)	30.5 $\pm$ 3.4 (25.5-36.0)	39.0 (38-40)	38.8 $\pm$ 4.3 (31.0-42.5)	35.5 $\pm$ 1.5 (34-37)	32.4 $\pm$ 5.8 (24.0-36.5)

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6

1

2 **Figure legends**

3

4 **Fig. 1.** *Paralongidorus iranicus n. sp.* A, B: Habitus of female and male, respectively; C: Detail  
5 of the anterior genital branch; D: Female pharyngeal region; E-H: Female lip region showing  
6 amphidial fovea (F in dorso-ventral view); I: Vulval region; J: Detail of odontostyle-  
7 odontophore junction; K: Male tail showing spicules and midventral supplements; L, M, N: Tail  
8 of J2, J3, and J4, respectively; O: Female tail; P, Q, R: Details of basal pharyngeal bulb.

9

10 **Fig. 2.** Light micrographs of *Paralongidorus iranicus n. sp.* A: Female pharyngeal region; B:  
11 Female anterior region; C-H: Lip region showing amphidial fovea at different focus; I: Detail  
12 of anterior genital branch; J, K, L: Tail of J2, J3, and J4, respectively; M-O: Female tail; P,  
13 Male posterior body region. Abbreviations: a = anus; af = amphidial fovea; V = vulva; vs =  
14 ventromedian supplements. (Scale bars: A, I, O, P = 50  $\mu\text{m}$ ; B, C, F, G, H, J-N = 25  $\mu\text{m}$ ; D, E =  
15 10  $\mu\text{m}$ .)

16

17 **Fig. 3.** SEM micrographs of *Paralongidorus iranicus n. sp.* A, C: Female anterior ends in lateral  
18 and ventrolateral view showing internal (ip) and outer labial papillae (op), cephalic lobe (cl),  
19 cephalic papillae (cp), and amphidial aperture (aa); D: Vulval region; E, F: Female tail, lateral  
20 and ventral view; G: Male posterior body portion. (Scale bars: A-F = 20  $\mu\text{m}$ ; G = 50  $\mu\text{m}$ .)

21

22 **Fig. 4.** Relation of body length with length of functional and replacement odontostyle (ost and  
23 rost, respectively) length in all detected developmental stages to mature females. A:

24 *Paralongidorus iranicus n. sp.* B: *P. bikanerensis* (Lal & Mathur, 1987) Siddiqi, Baujard &  
25 Mounport, 1993 from Bam, Iran.

26

27 **Fig. 5.** *Paralongidorus bikanerensis.* A: Female lip region, en face view; B-D: Female anterior  
28 end in lateral and ventrolateral view showing amphidial fovea; E: Female habitus; F: Female  
29 anterior region; G: Detail of basal pharyngeal bulb; H: First-stage juvenile anterior region; I,  
30 J: Female tail; Tail of J4; K-N: Tail of J1-J4 respectively; O: Intersex tail showing reduced  
31 spicules.

32

33 **Fig. 6.** Light micrographs of *Paralongidorus bikanerensis* (Lal & Mathur, 1987) Siddiqi,  
34 Baujard & Mounport, 1993. A, B: Female anterior region; C: Female anterior region, showing

1 double constriction; D, E: Female lip region, showing amphidial fovea at different focus; F-H:  
 2 Female tails; I-L: Tail of J1, J2, J3, and J4, respectively; M: Tail of intersex, showing weakly  
 3 developed spicules. Abbreviations: a = anus; af = amphidial fovea; sp = spicules. (Scale bars  
 4 A, B = 50  $\mu\text{m}$ ; C-H, I-M= 25  $\mu\text{m}$ .)

5  
 6 **Fig. 7.** SEM micrographs of *Paralongidorus bikanerensis* (Lal & Mathur, 1987) Siddiqi,  
 7 Baujard & Mounport, 1993. A-C, E: Female anterior end in lateral view, showing amphidial  
 8 aperture (aa), internal papillae (ip), outer labial papillae (op), and cephalic lobe (cl); D: En  
 9 face view showing oral aperture (oa) and papillae; F, G: Female tail, lateral and ventral views,  
 10 showing anus (a). (Scale bars: A, F, G = 20  $\mu\text{m}$ ; B-E, = 10  $\mu\text{m}$ .)

11  
 12 **Fig. 8.** Phylogenetic relationships within Longidorus and Paralongidorus. Bayesian 50%  
 13 majority rule consensus trees as inferred from D2 and D3 expansion segments of 28S rRNA  
 14 sequences alignments under the GTR + G + I model. Posterior probabilities more than 65% are  
 15 given for appropriate clades; bootstrap values greater than 50% are given on appropriate  
 16 clades in ML analysis. Newly obtained sequences in this study are in bold letters.

17  
 18 **Fig. 9.** Phylogenetic relationships within Longidorus and Paralongidorus. Bayesian 50%  
 19 majority rule consensus trees as inferred from partial 18S rRNA gene sequences alignments  
 20 under the GTR + G + I model. Posterior probabilities more than 65% are given for appropriate  
 21 clades; bootstrap values greater than 50% are given on appropriate clades in ML analysis.  
 22 Newly obtained sequences in this study are in bold letters.

23