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Morphological and molecular characterisation of *Hemicycliophora lutosa* Loof & Heyns, 1969 and *H. typica* de Man, 1921 from South Africa (Nematoda: Hemicycliophoridae)

Esther VAN DEN BERG [1,*], Sergei A. SUBBOTIN [2,3] and Louwrens R. TIEDT [4]

[1] *National Collection of Nematodes, Biosystematics Division, ARC-Plant Protection Research Institute, Private Bag X134, Queenswood, 0121 South Africa*

[2] *Plant Pest Diagnostics Center, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, CA 95832-1448, USA*

[3] *Centre of Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninskii Prospect 33, Moscow, 117071, Russia*

[4] *Laboratory for Electron Microscopy, North West University, Potchefstroom Campus, Potchefstroom, 2520 South Africa*

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Summary – Two *Hemicycliophora* species, *H. lutosa* and *H. typica*, found in samples from fallow soil and sugarcane soil in South Africa, were studied morphologically and, for the first time, molecularly. Diagnostic PCR-IT-rRNA-RFLP profiles generated by five restriction enzymes are provided. Study of phylogenetic relationships using D2-D3 expansion segment of 28S rRNA gene sequences revealed that *H. lutosa* was related to *H. poranga*. *Hemicycliophora lutosa* and *H. poranga* are compared morphologically. SEM photographs are given for *H. typica* and for *H. lutosa* for the first time. The male of *H. typica* represents a first report for South Africa.

Keywords – description, molecular, morphology, morphometrics, SEM, taxonomy.

Recently, two *Hemicycliophora* species, *viz*., *H. lutosa* Loof & Heyns, 1969 and *H. typica* de Man, 1921, were found in soil samples from fallow soil near Tarlton in the Gauteng Province, and from sugarcane near Nelspruit in the Mpumalanga Province. Enough material was found to study the species morphologically and also molecularly for the first time. Molecularly, *H. lutosa* and *H. poranga* Monteiro & Lordello, 1978 appear to be related. In this study they are also compared morphologically. SEM photographs are given for *H. typica* and for *H. lutosa* for the first time. The male of *H. typica* is described from South Africa for the first time.

Materials and methods

NEMATODE POPULATIONS

Soil samples were collected using a bucket auger and the specimens were extracted from the soil using the elutriation technique of Seinhorst (1962).

LIGHT MICROSCOPE AND SEM STUDY

Nematodes were killed in FPG (Netscher & Seinhorst, 1969) and mounted in pure glycerin using the slow method described by Hooper and Evans (1993). For scanning electron microscopy, part of the material was transferred to TAF, before being dehydrated in increasing concentrations of amyl acetate in pure alcohol and finally in pure amyl acetate. Following conventional critical point drying and gold/palladium coating (15 nm), specimens were viewed with a Philips XL30 DX41 stereoscan microscope at 10 kV.

DNA EXTRACTION, PCR, CLONING, SEQUENCING AND PCR-RFLP

Several specimens from each sample were put into a drop of 20 μl water on a glass slide and cut under a binocular microscope. Nematode fragments were transferred with 16 μl water into Eppendorf tubes containing 2 μl 10 $\times$ PCR buffer and 2 μl Proteinase K (600 μg ml^{-1}) (Promega, Madison, WI, USA). The tubes were incubated

* Corresponding author, e-mail: VDBergE@arc.agric.za

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at 65°C (1 h) and then at 95°C (15 min). Detailed protocols for PCR, cloning and automated sequencing are as described by Tanha Maafi *et al*. (2003). The following primers sets were used for amplification and sequencing of ribosomal RNA gene fragments: *i*) forward D2A (5′-CAAGTACCGTGAGGGAAAGTTG-3′) and reverse D3B (5′-TCGGAAGGAACCAGCTACTA-3′) for the D2-D3 of 28S rRNA gene (Nunn, 1992); and *ii*) forward TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and reverse AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) for the ITS1-5.8S-ITS2 (Curran *et al*., 1994). The newly obtained sequences were submitted to the GenBank database under the following numbers: GQ406240, GQ406241 for the D2-D3 of the 28S gene for *H. lutosa*, and GQ406237-GQ406239 for the ITS rRNA for *H. lutosa* and *H. typica.*

The PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Purified product of the ITS of *Hemicycliophora* species (4-6 μl) was digested by one of following restriction enzymes: *Ava*I, *Bsh*1236I, *Dra*I; *Hin*fI or *Hin*6I in the buffer stipulated by the manufacturer. The digested DNA was run on a 1.5% agarose TAE buffered gel, stained with ethidium bromide, visualised with a UV transilluminator and photographed. The exact lengths of each restriction fragment from the PCR products were obtained by a virtual digestion of the sequences using WebCutter 2.0 (www.firstmarket.com/cutter/cut2.html).

Molecular phylogenetic analysis

The newly obtained sequence for the D2-D3 expansion segments of 28S rRNA gene for *H. lutosa* was aligned with default parameters with other sequences of the same gene for five *Hemicycliophora* sequences deposited in GenBank and sequences of two *Paratylenchus* species and *Tylenchulus semipenetrans* considered as outgroup taxa (Subbotin *et al.*, 2005). Sequence alignment was analysed using Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). BI analysis under the GTR + I + G model 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. Posterior probabilities (PP) are given on appropriate clades.

Hemicycliophora lutosa Loof & Heyns, 1969
(Fig. 1A–D)

Numerous females and juveniles of *H. lutosa* collected by V. Sequeira from fallow soil in a grassland biome on Vlaktplaats farm near Tarlton (26°04′05″S, 27°38′01″E), 1500 m a.s.l., Gauteng Province.

Hemicycliophora lutosa was described by Loof and Heyns (1969) from three localities in South Africa and was subsequently found in Zambia by Brzeski (1974). Loof (1984) described *H. lutosoides* from Iran, a species which Costa Manso (1998) synonymised with *H. lutosa.* The present material afforded the opportunity to take SEM photographs of the South African specimens and to do molecular studies.

Females are similar to those previously described from South Africa (Loof & Heyns, 1969; Van den Berg, 1981) and from those described by Brzeski (1974), Loof (1984) and Costa Manso (1998).

Males appear to be very rare in this species and only one male was found in the original isolation. No males were found by Brzeski (1974) or Loof (1984). At present there are specimens of 13 populations in the National Collection of Nematodes, Pretoria, and amongst them are only two males, one still within a juvenile cuticle. The measurements of the male are as follows: L = 921 μm; a = 36.3; c = 5.2; c′ = 8.5; excretory pore from anterior end = 145 μm; mid-body diam. = 25.5 μm; lip region diam. = 12.5 μm; lip region height = 7 μm; annulus width at mid-body = 1.5 μm and on terminal part of tail 3 μm; tail length = 175.5 μm; spiculum length (arc) = 50.5 μm; gubernaculum length = 9.5 μm; penial tube length = 13 μm. Loof and Heyns (1969) gave the spiculum length as 26 μm but in the description the distance is quoted as the chord. In the present specimen the chord length of the spicules is 25 μm. The present measurements fit well those of the original male.

Because molecular studies showed *H. lutosa* and *H. poranga* (see descriptions by Monteiro & Lordello, 1978; Chaves, 1983; Chitambar, 1994; Costa Manso, 1996; Crozzoli & Lamberti, 2006) to be closely related, the two species were also compared morphologically and morphometrically. The following differences were noticed: the tail of *H. lutosa* is longer (108-178 *vs* 94-110 μm) giving a lower c value of 5.7-11.2 *vs* 8.9-13.2 and a lower VA%T value of 25.6-56.3 *vs* 36-81. Chitambar (1994) and Chaves (1983) gave the dorsal pharyngeal gland outlet for *H. poranga* as being located 10-19 μm from the stylet base compared with 5-7.5 μm for *H. lutosa*. Morphologically, the main differences are in the *en face* view of *H.*

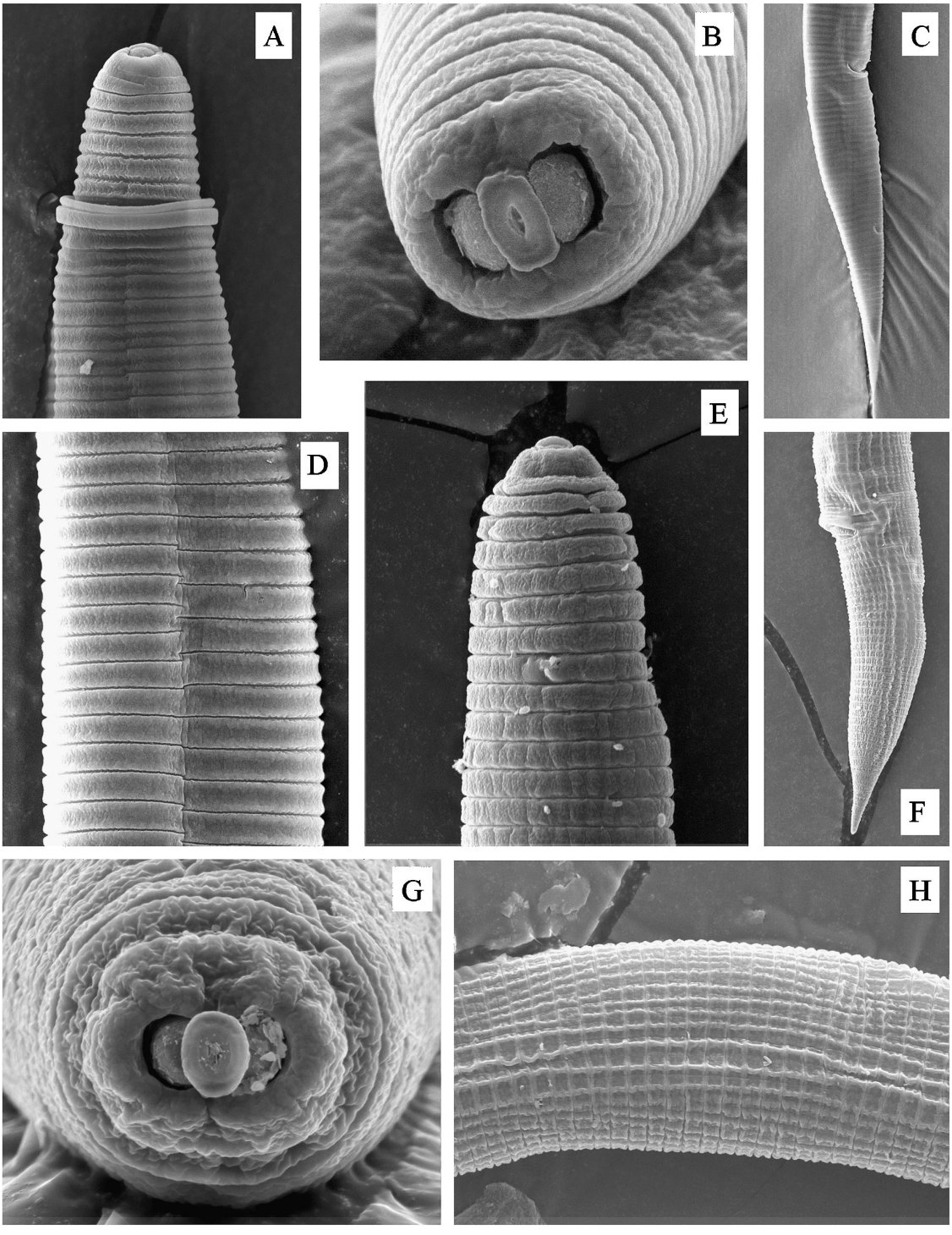

Fig. 1. Hemicycliophora lutosa *Loof & Heyns, 1969. Female, SEM. A: Anterior region; B: Lip region,* en face *view; C: Posterior region; D: Cuticula and lateral field at mid-body.* Hemicycliophora typica *de Man, 1921. Female, SEM. E: Anterior region; F: Posterior region; G: Lip region,* en face *view; H: Cuticular sculpture at mid-body. (Scale bars: A, D, E, H = 20 μm; B = 5 μm; C, F = 50 μm; G = 10 μm.)*

lutosa where the first lip annulus is rectangular with a narrow rectangular labial disc which is not markedly raised above the lip annulus *vs* first lip annulus in the form of a figure eight in *H. poranga* with a rounded to oval, distinctly raised, labial disc. The lateral field of *H. lutosa* consists only of breaks in irregularities in the striae *vs* irregularities and breaks in the striae with ovate markings suggesting the presence of two indistinct to distinct longitudinal lines.

Hemicycliophora typica de Man, 1921
(Fig. 1E–H)

Numerous females of *H. typica* collected by S. Berry from sugarcane in a sandy loam soil on the Laeveld Agricultural College farm, 5 km from Nelspruit, Mpumalanga Province (25°27′23″S, 30°58′31″E), 700 m a.s.l.

Females are identical to those described previously from South Africa (Heyns, 1962; Loof & Heyns, 1969; Van den Berg, 1981).

Hemicycliophora typica was originally described from one male specimen from The Netherlands (de Man, 1921). Based on this scant information the validity of this species was uncertain until a redescription was given from topotypes and their progeny by Loof (1968). Although various authors have described males in this species (de Man, 1921; Tarjan, 1952; Thorne, 1955; Loof, 1968; Costa Manso, 1996) they seem to be very scarce in South Africa. Brzeski (1998) mentioned that males were less common than females. Amongst hundreds of female specimens from more than 100 localities housed in the National Collection of Nematodes, only five males exist. The present Nelspruit population also contained no males. The five males correspond well with the various descriptions of the above authors. As a first report for South Africa the male morphometrics are as follows: L = 551 (514-620) μm; a = 27 (24.5-28.8); b = 5.8 (5.4-6.4); c = 6.7 (6.4-7.6); length of degenerate pharynx = 95.5 (81-109.5) μm; mid-body diam. = 20.5 (18.5-21.5) μm; lip region diam. = 9 (9-9.5) μm; lip region height = 5 (5-5.5) μm; annulus width = 1.5 (1.5-2) μm; lateral field width = 4.5 (4.5-5) μm; tail length = 82.5 (68.5-96.5) μm; spiculum length (arc) = 41 (37-42.5) μm; gubernaculum length = 8 (7.5-9.5) μm; penial tube length = 5.5-6.5 μm. This corresponds very well with the data given by the above authors for males from other countries, *viz*., L = 470-1000 μm; a = 26-41.5; b = 5-7.1; c = 5.1-8; annulus width = 1.7-2 μm; lateral field width = 3 μm; tail length = 71-153 μm; spiculum length (arc) = 36.5-44 μm; gubernaculum length = 6.5-10 μm; penial tube length = 5-13 μm.

Molecular characterisation and phylogenetic relationships within *Hemicycliophora* species

Amplification of DNA from *Hemicycliophora* samples yielded a single fragment of *ca* 790 bp in length for the ITS-rRNA gene. ITS rRNA sequences of these two species differ in 136 nucleotides. PCR-ITS-RFLP diagnostic profiles for *H. lutosa* and *H. typica* generated by five restriction enzymes are given in Figure 2 with approximate sizes of these fragments cited in Table 1.

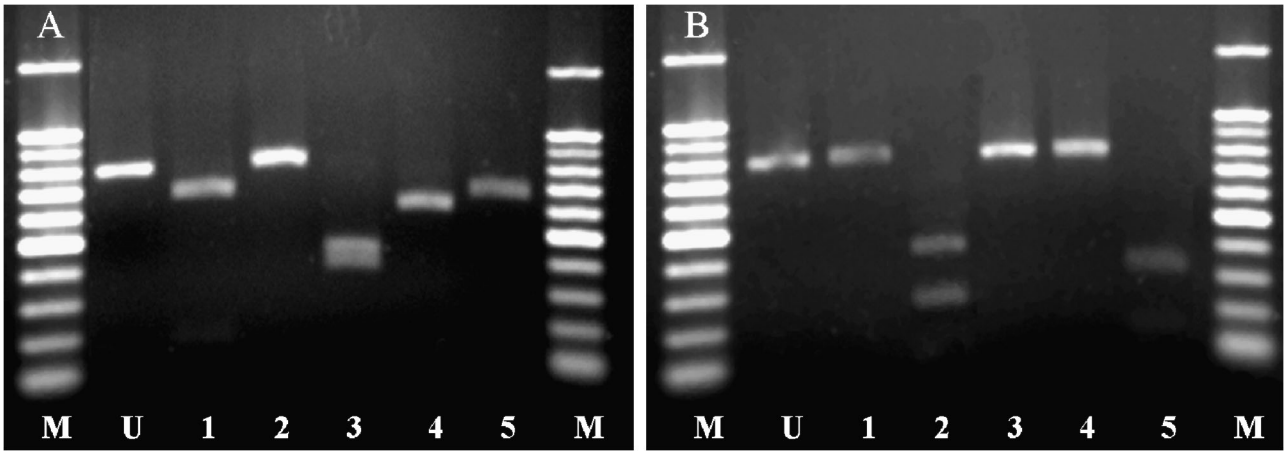

Fig. 2. *Diagnostic PCR-ITS rRNA-RFLP profiles for* Hemicycliophora lutosa *(A) and* H. typica *(B). Code: M = 100 bp DNA marker (Promega), U = unrestricted PCR product; 1 =* Ava*I; 2 =* Bsh*1236I; 3 =* Dra*I; 4 =* Hin*fI; 5 =* Hin*6I.*

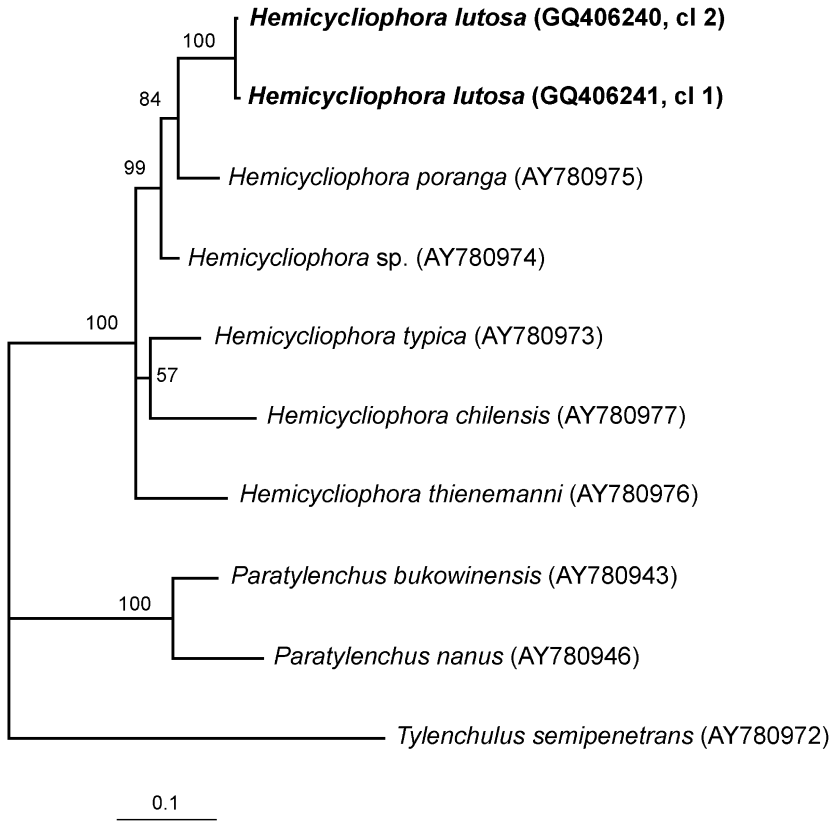

Fig. 3. *Phylogenetic relationships within the genus* Hemicycliophora *as inferred from Bayesian analyses of sequences of the D2-D3 of 28S rRNA using GTR + I + G model of DNA evolution. Posterior probability values are given on appropriate clade. Newly obtained sequences are indicated in bold.*

Table 1. *Approximate sizes (in bp) of restriction fragments generated by five restriction enzymes after digestion of PCR-ITS products amplified by TW81 and AB28 primers for two* Hemicycliophora *species.*

Enzyme	*H. lutosa*	*H. typica*
Unrestricted PCR product	788	791
*Ava*I	655, 133	791
*Bsh*1236I	788	419, 260, 112
*Dra*I	418, 370	791
*Hin*fI	593, 82, 68, 33, 12	756, 35
*Hin*6I	670, 118	354, 175, 87, 67, 45, 39, 24

A phylogenetic tree reconstructed by the BI method for the D2-D3 of 28S rRNA genes is presented in Figure 3. The phylogenetic relationships within *Hemicycliophora* were generally congruent with those for this genus as reconstructed by Subbotin *et al.* (2005) in the tree for the suborder Criconematina, with the exception of the position of some poorly supported clades. *Hemicycliophora lutosa* formed a moderately supported clade (PP = 84) with *H. poranga*.

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