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Morphological and molecular characterisation of one new and several known species of the reniform nematode, *Rotylenchulus* Linford & Oliveira, 1940 (Hoplolaimidae: Rotylenchulinae), and a phylogeny of the genus

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Summary – The reniform nematodes of the genus *Rotylenchulus* are semi-endoparasites of numerous herbaceous and woody plant roots and are mainly distributed in tropical and subtropical regions. In this study, we provide morphological and molecular characterisation of six out of ten presently known valid species of *Rotylenchulus*: *R. clavicaudatus*, *R. leptus*, *R. macrodoratus*, *R. macrosoma*, *R. reniformis* and *R. sacchari* from South Africa, USA, Italy and Spain. *Rotylenchulus parvus* was only studied morphologically. A new species, *R. macrosomoides* sp. n., isolated from soil and roots of sugarcane in South Africa, is described. The phylogeny of *Rotylenchulus*, as inferred from the analyses of D2-D3 of 28S rRNA, ITS rRNA, *coxI* mtDNA and *hsp90* gene sequences, is presented. The study revealed that *R. reniformis* and *R. macrosoma* have a sister relationship, but that relationships between other *Rotylenchulus* species remain unresolved. The phylogenetic analysis also confirmed the hypothesis that this genus originated from the Afrotropical zoogeographical region. Our study revealed that *R. reniformis* and *R. macrosomoides* sp. n. have two distinct rRNA gene types and *R. macrosoma* have three rRNA gene types in their genomes. PCR with species-specific primers was developed for rapid diagnostics of *R. reniformis*.

Keywords – *coxI* gene, *hsp90* gene, morphometrics, new species, PCR with specific primer, phylogeny, rRNA gene, *Rotylenchulus clavicaudatus*, *Rotylenchulus leptus*, *Rotylenchulus macrodoratus*, *Rotylenchulus macrosoma*, *Rotylenchulus macrosomoides* sp. n., *Rotylenchulus parvus*, *Rotylenchulus reniformis*, *Rotylenchulus sacchari*, SEM, taxonomy.

The reniform nematodes of the genus *Rotylenchulus* Linford & Oliveira, 1940 comprise ten valid species. These nematodes are semi-endoparasites of numerous herbaceous and woody plant roots and are mainly distributed in tropical and subtropical regions. *Rotylenchulus reniformis* Linford & Oliveira, 1940 is the most important species in the genus and is considered as a major pest of cotton and other crops in the USA and several other countries (Robinson *et al.*, 1997).

Several morphological characters of immature females are currently used for diagnostics of *Rotylenchulus* species (Loof & Oostenbrink, 1962; Dasgupta *et al.*, 1968; Ger-

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mani, 1978; Van den Berg, 1978; Lehman & Inserra, 1990; Robinson *et al.*, 1997; Siddiqi, 2000). However, high intraspecific variability of some diagnostic features makes identification of this group based on morphology a difficult task. In the last few decades, several studies have been done to characterise nuclear ribosomal RNA (rRNA) genes of *R. reniformis* (Agudelo *et al.*, 2005; Subbotin *et al.*, 2006; Tilahun *et al.*, 2008; Zhang *et al.*, 2011; Nyaku *et al.*, 2013a, b; Deng *et al.*, 2015). One of the interesting results of these works was the presence of high levels of intraspecific and intra-individual variations of rRNA genes with two distinct types of 18S rRNA gene and two types of ITS rRNA gene. The latter of these genes differed also in length: ITS1S (short) and ITS1L (long) (Nyaku *et al.*, 2013a, b). Conventional and real-time PCR with species-specific primers were developed for diagnostics of *R. reniformis* using sequences differences between species in rRNA and β-tubulin genes (Showmaker *et al.*, 2011; Sayler *et al.*, 2012). Several other studies revealed other peculiarities of the genome organisation in this nematode. Genetic analysis of isolates of *R. reniformis* from several states of the USA and Japan using microsatellites revealed a high level of genetic diversity between populations (Arias *et al.*, 2009; Leach *et al.*, 2012). The size of the *R. reniformis* genome based on flow cytometry was estimated to be 190 Mb, which was almost twice as large as the genome of *Caenorhabditis elegans* and 3-4 times larger than the root-knot nematode (*Meloidogyne incognita*) genome (Ganji *et al.*, 2013). Recently, the genome of this species was characterised by shotgun sequencing (Nyaku *et al.*, 2014; Sanders *et al.*, unpubl.) and the complete mtDNA sequence was also deposited in GenBank (Sanders *et al.*, unpubl.).

The 18S, ITS and the D2 and D3 expansion segments of 28S rRNA genes have been shown to be markers in the characterisation of phylogenetic relationships within Hoplolaimidae (Subbotin *et al.*, 2007) and, especially, in cases where morphological characters may lead to cryptic diversity (Vovlas *et al.*, 2008; Cantalapiedra-Navarrete *et al.*, 2013; Palomares-Rius *et al.*, 2014). Similarly, mitochondrial DNA (mtDNA), particularly the protein-coding mitochondrial gene, cytochrome *c* oxidase subunit 1 (*coxI*), has proved to be a powerful tool for providing accurate species identification and assessing phylogenetic patterns across the family Hoplolaimidae (Cantalapiedra-Navarrete *et al.*, 2013; Van den Berg *et al.*, 2013). However, although *R. reniformis* has been intensively sequenced because of its economic importance, little or no information on molecular markers is available for other *Rotylenchulus* species. The partial 18S rRNA gene sequences were given for *R. borealis* Loof & Oostenbrink, 1962 (van Megen *et al.*, 2009) and *R.* cf. *anamictus* Dasgupta, Raski & Sher, 1968 (Powers *et al.*, unpubl.), and the D2 and D3 expansion segments of 28S rRNA gene sequence were provided for *R. macrodoratus* Dasgupta, Raski & Sher, 1968 (Subbotin *et al.*, 2006). Thus, the phylogenetic relationships of *Rotylenchulus* species have not been properly analysed and remain unknown.

Nematode surveys of plant-parasitic nematodes undertaken during the last few years on several crops, including sugarcane, sorghum, maize, grapevine, olive and wild olive in Italy, South Africa and Spain, have revealed severe feeder-root infections and heavy soil infestations by several reniform nematodes, including *R. clavicaudatus* Dasgupta, Raski & Sher, 1968, *R. leptus* Dasgupta, Raski & Sher, 1968, *R. macrodoratus*, *R. macrosoma* Dasgupta, Raski & Sher, 1968, *R. parvus* (Williams, 1960) Sher, 1961, *R. reniformis* and *R. sacchari* Van den Berg & Spaull, 1981, and an unknown species. Preliminary morphological observations indicated that this unknown species appeared to be morphologically similar to *R. macrosoma*, leading us to conduct a detailed morphological and molecular comparative study with previously reported data. Therefore, the objectives of the present study were: *i*) morphologically to characterise seven known *Rotylenchulus* collected in several countries; *ii*) to describe a new species of *Rotylenchulus* isolated from soil and roots from sugarcane in South Africa; *iii*) to characterise *Rotylenchulus* species using ITS rRNA, the D2-D3 of 28S rRNA, *coxI* and *hsp90* gene sequences; *iv*) to study phylogenetic relationships within the genus using these gene sequences; and *v*) to develop PCR with species-specific primers for quick and reliable diagnostics of *R. reniformis*.

Materials and methods

Nematode populations

Nematode populations used in this study were collected from geographically diverse locations in South Africa, Spain, Italy and USA (Table 1). Nematode specimens from the soil samples were extracted using the centrifugal-flotation method (Coolen, 1979) or the rapid centrifugal-flotation method (Jenkins, 1964). Specimens were fixed in hot TAF (no more than 70°C) or 4% formaldehyde + 1% propionic acid. Adult specimens of each sample were processed to glycerin (Seinhorst, 1962, 1966) and mounted on glass slides for species identification.

Table 1. *Rotylenchulus* species used in the present study.

Species	Locality	Plant host	Sample code	GenBank accession numbers				Collector/ Identifier
				D2-D3 of 28S rRNA	ITS rRNA	*coxI*	*hsp90*	
R. clavicaudatus	KwaZulu-Natal Province, South Africa	Sugarcane	CD1357, KZN 1	KT003739	KT003785, KT003786	–	–	E. Van den Berg
R. leptus	Brits, North West Province, South Africa	Sorghum	Tvl 2055	–	–	–	–	E. Van den Berg
R. macrodoratus	Palombaio-Terlizzi, Bari Province, Italy	Grapevine	TOPIT	KT003758	KT003797	KT003722	–	N. Vovlas
R. macrodoratus	Bari, Bari Province, Italy, topotypes	Grapevine	GRAIT	KT003760, KT003761	KT003794	KT003720, KT003721	KT003768	N. Vovlas
R. macrodoratus	Bari, Bari Province, Italy	Olive	OLVIT	KT003762	KT003795, KT003796	KT003719	KT003769	N. Vovlas
R. macrodoratus	Maruggio, Taranto Province, Italy	Olive	TARIT	KT003759	–	–	–	N. Vovlas
R. macrosoma	Vejer, Cadiz Province, Spain	Wild olive	BAET	KT003748, KT003749	KT003800, KT003801, KT003804, KT003806, KT003807, KT003809-KT003811	KT003724	KT003765	P. Castillo
R. macrosoma	Jerez de la Frontera, Cádiz Province, Spain	Olive	J96	KT003747	KT003805, KT003808	KT003725	KT003766	P. Castillo
R. macrosoma	Huévar del Aljarafe, Sevilla Province, Spain	Olive	ST79V	KT003750, KT003751	KT003802, KT003803	KT003726	KT003767	P. Castillo
R. macrosomoides sp. n.	Komatipoort, Mpumalanga Province, South Africa	Sugarcane	TVL2063; CD1482	KT003752-KT003757	KT003787-KT003792, KT003798	KT003723	KT003764	E. Van den Berg
R. parvus	Winterton, KwaZulu-Natal Province, Natal, South Africa	Maize	CD317, N803	KT003734-KT003738	KT003771-KT003779	KT003732	KT003770	E. Van den Berg
R. reniformis	Homestead, Florida, USA (intercepted in CDFA)	Unknown	CD746, CD747	–	KT003793, KT003799	KT003727	KT003763	S.A. Subbotin
R. reniformis	Lake Worth, Florida, USA (intercepted in CDFA)	*Yucca elephantipes*	CD997	KT003744	–	KT003731	–	K. Dong
R. reniformis	Homestead, Florida, USA (intercepted in CDFA)	*Euphorbia* sp.	CD1153	KT003743	–	KT003730	–	S.A. Subbotin
R. reniformis	Mount Dora, Florida, USA (intercepted in CDFA)	*Sansevieria* sp.	CD1395	KT003745	–	KT003729	–	K. Dong
R. reniformis	Jefferson county, Arkansas, USA	Cotton	CD1748	KT003746	–	KT003728	–	R.T. Robbins
R. sacchari	Northern Cape Province, South Africa	Maize	CD1493, NC 2	KT003740-KT003742	KT003780-KT003784	KT003733	–	E. Van den Berg

Light and scanning microscopic study

Nematode specimens were examined, measured and photographed in two laboratories (ARC-Plant Protection Research Institute, South Africa and IAS-CSIC, Spain) using Nikon Labophot-2 or Zeiss III compound microscopes, respectively, equipped with a Nomarski differential interference contrast and a drawing tube.

For scanning electron microscopy (SEM), samples were fixed in 70% ethanol for at least 12 h, and then dehydrated in an ethanol series of 80, 90 and 100% for 15 min each. The samples were critical point-dried using liquid carbon dioxide in a critical point dryer. The dried samples were mounted on SEM stubs with double-sided carbon tape and sputter coated with 15 nm gold/palladium (66/33%). The coated samples were viewed under a FEI Quanta FEG 250 SEM under high vacuum mode at 5-10 kV.

DNA extraction, PCR and sequencing

For molecular analyses, nematode DNA from *Rotylenchulus* samples was extracted from single or several individuals using proteinase K as described by Castillo *et al.* (2003a, b). PCR and sequencing was completed in two laboratories: IAS-CSIC, Spain and CDFA, USA. Detailed protocols were described by Castillo *et al.* (2003a) and Tanha Maafi *et al.* (2003). The primer sets for amplification of the nuclear ribosomal RNA (D2-D3 of 28S rRNA, ITS rRNA), protein coding (*hsp90*) and mitochondrial (*coxI*) genes are given in Table 2.

PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products) or QIAquick (Qiagen) gel extraction kits and used for direct sequencing in both directions with the primers referred above or for cloning. The PCR products were cloned into the pGEM-T vector and transformed into JM109 High Efficiency Competent Cells (Promega). Several clones of each sample were isolated using blue/white selection. PCR products from each clone were sequenced in both directions at the Stab Vida sequencing facilities (Caparica) and Davis Sequencing facilities (Davis, CA, USA) using the same primers. The newly obtained sequences were submitted to the GenBank database under accession numbers KT003719-KT003811 (Table 1).

Table 2. Primer sets used in the present study.

Primer code*	Sequence (5′ → 3′)	Amplified gene	Reference
TW81 (f)	GTT TCC GTA GGT GAA CCT GC	ITS rRNA	Tanha Maafi *et al.* (2003)
AB28 (r)	ATA TGC TTA AGT TCA GCG GGT		
F194 (f)	CGT AAC AAG GTA GCT GTA G	ITS rRNA	Ferris *et al.* (1993); Vrain (1993)
5368(r)	TTT CAC TCG CCG TTA CTA AGG		
D2A (f)	ACA AGT ACC GTG AGG GAA AGT TG	D2-D3 of 28S rRNA	Nunn (1992)
D3B (r)	TCG GAA GGA ACC AGC TAC TA		
U831 (f)	AAY AAR ACM AAG CCN TYT GGA C	*hsp90*	Skantar & Carta (2005)
L1110 (r)	TCR CAR TTV TCC ATG ATR AAV AC		
D2A (f)	ACA AGT ACC GTG AGG GAA AGT TG	D2-D3 of rRNA	Nunn (1992) This study
R_renif_R1A (r)	GAA AAG GCC TAC CCA ATG TG		
D2A (f)	ACA AGT ACC GTG AGG GAA AGT TG	D2-D3 of rRNA	Nunn (1992) This study
R_renif_R2A (r)	CCC GAT ACC ATT TCC ATA CAA G		
D2A (f)	ACA AGT ACC GTG AGG GAA AGT TG	D2-D3 of rRNA	Nunn (1992)
R_renif_R1B (r)	CAC AGA CRC CCR AGC AGC CA		This study
JB3 (f)	TTT TTT GGG CAT CCT GAG GTT TAT	*coxI* mtDNA	Bowles *et al.* (1992)
JB4.5 (r)	TAA AGA AAG AAC ATA ATGA AAA TG		
JB3 (f)	TTT TTT GGG CAT CCT GAG GTT TAT	*coxI* mtDNA	Bowles *et al.* (1992) Derycke *et al.* (2005)
JB5 (r)	AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG		

* f: forward primer; r: reverse primer.

PHYLOGENETIC ANALYSES

The D2-D3 region of 28S rRNA, ITS1 rRNA, 5.8S-ITS2 rRNA, *hsp90* and *coxI* mtDNA gene sequences of several *Rotylenchulus* from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen according to previous published data (Subbotin *et al.*, 2006). The newly obtained and published sequences for each gene (Agudelo *et al.*, 2005; Subbotin *et al.*, 2006; Tilahun *et al.*, 2008; Zhang *et al.*, 2011; Nyaku *et al.*, 2013a; Deng *et al.*, 2015; Kushida & Kondo, unpubl.; Zhou & Zhang, unpubl.) were aligned using ClustalX (Thompson *et al.*, 1997) using default parameters (gap opening = 15.00; gap extension = 6.66) for 28S rRNA, *hsp90* and *coxI* and modified parameters (gap opening = 5.00; gap extension = 3.00) for ITS1 rRNA, 5.8S + ITS2 rRNA gene sequence alignments. Identical sequences were removed using DNAcollapser (http://users-birc.au.dk/biopv/php/fabox/dnacollapser.php) from the Fabox online toolbox (Villesen, 2007). The alignments were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The general time reversible substitution model with estimation of invariant sites and assuming a gamma distribution with four categories gene (GTR + I + G) was selected as the optimal nucleotide substitution model for the analyses. BI analysis for each was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations for *hsp90*, *coxI* and 28S rRNA gene sequence datasets and 4.0×10^6 for ITS rRNA gene sequence datasets. Two runs were performed for each analysis. The Markov chains were sampled at intervals of 100 generations. After discarding burn-in samples other trees were used to generate a 50% majority rule consensus tree. Sequence analyses of alignments were also performed with PAUP* 4.0b 10 (Swofford, 2003). Pairwise divergences between taxa were computed as absolute distance values and as percentage mean distance values based on whole alignment, with adjustment for missing data.

SECONDARY STRUCTURE RECONSTRUCTION FOR THE D2 OF 28S RRNA

Mfold software Version 3 (Zuker, 2003) (http://mfold.rna.albany.edu/?q=mfold/rna-folding-form) was applied to predict the secondary structures for the D2 expansion region of 28S rRNA using the energy minimisation approach. Structures were visualised using VARNA (Darty *et al.*, 2009) and drawn with Adobe Illustrator CS6 and Adobe Photoshop CS6. Consensus sequence was obtained using Consensus Maker (http://www.hiv.lanl.gov/cgi-bin/CONSENSUS_TOOL/consensus.cgi). Helix name codes of the secondary structure rRNA model were given according to Subbotin *et al.* (2007).

PCR WITH SPECIES-SPECIFIC PRIMERS FOR *ROTYLENCHULUS RENIFORMIS*

Species-specific primers were designed using sequence alignment of the D2-D3 region of the 28S rRNA gene. Several species of *Rotylenchulus* were used to test the specificity of PCR. The PCR mixture was prepared as described by Tanha Maafi *et al.* (2003). The PCR amplification profile consisted of 4 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 55°C and 1.5 min at 72°C, followed by a final step of 10 min at 72°C. Two μl of the PCR products were run on a 1.4% TAE buffered agarose gel, stained and photographed.

Results

Within the samples studied we distinguished and characterised seven known valid species: *R. clavicaudatus*, *R. leptus*, *R. macrodoratus*, *R. macrosoma*, *R. parvus*, *R. reniformis*, *R. sacchari* and a new species, *R. macrosomoides* sp. n. As *R. reniformis* was morphologically previously well characterised in several publications, this species was studied molecularly only. *Rotylenchulus leptus* was morphologically re-described because its original description did not include illustrations of the swollen female, which was found in this study. Unfortunately, the lack of additional specimens prevented the validation of this re-description with molecular data. Morphological and morphometric characterisations of *Rotylenchulus* species are given below (Figs 1-13; Tables 3-9).

Rotylenchulus macrosomoides[*] sp. n.
(Figs 1-3)

These specimens were collected on 2 July 2012 from sugarcane in the Komatipoort area, Mpumalanga Province, South Africa.

MEASUREMENTS

See Tables 3, 4.

[*] The species epithet refers to *Rotylenchulus macrosoma* and the Greek suffix εἶδος which means 'similar to'.

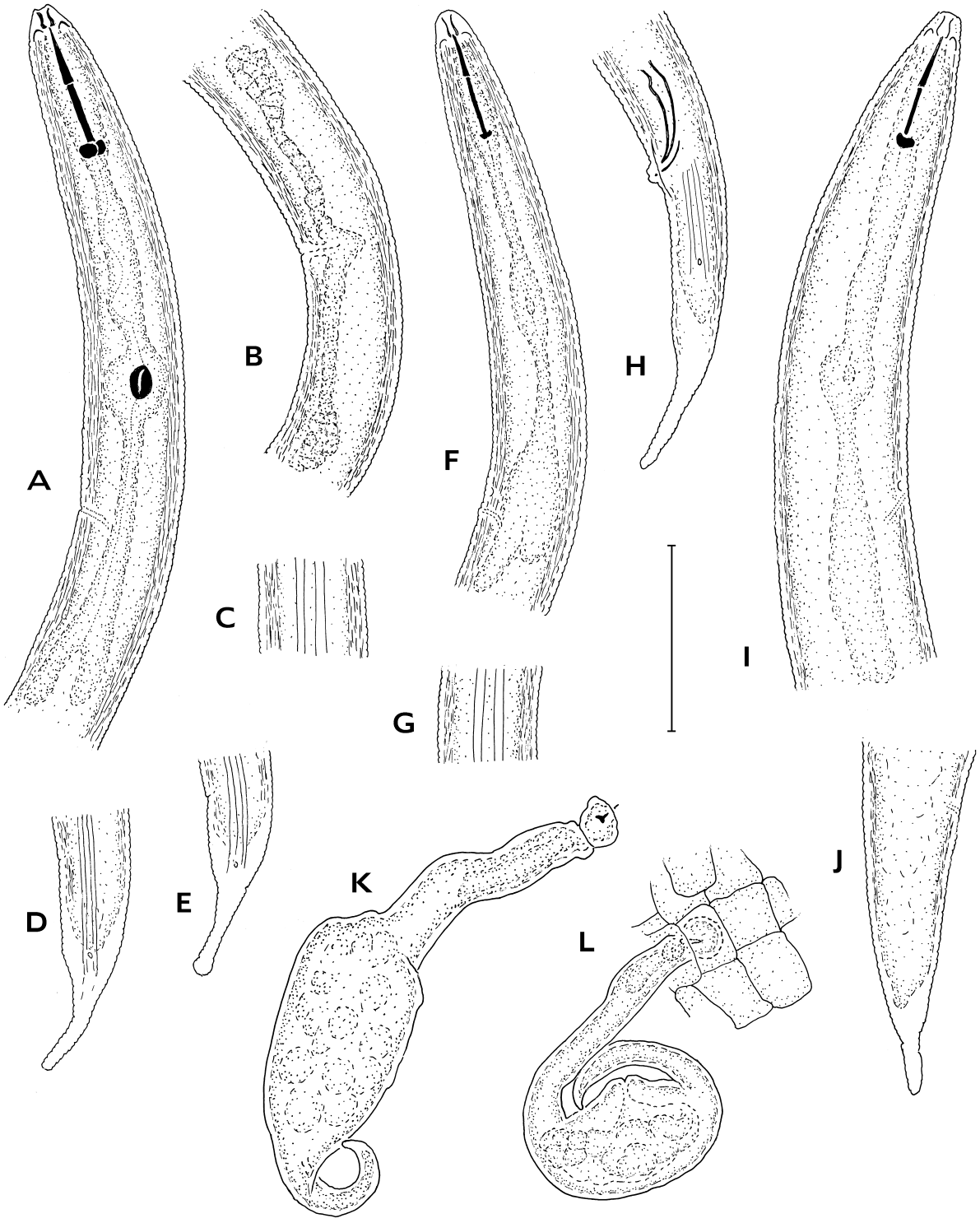


Fig. 1. *Rotylenchulus macrosomoides* sp. n. A-E, immature female. A: Anterior part of body; B: Vulval region with reflexed ovaries; C: Lateral field at mid-body; D, E: Tails. F-H, male. F: Anterior region; G: Lateral field at mid-body; H: Tail. I, J, juvenile. I: Anterior part of body; J: Tail. K, L, mature female. K, L: Habitus. (Scale bar: A-J = 30 μm; K, L = 100 μm.)

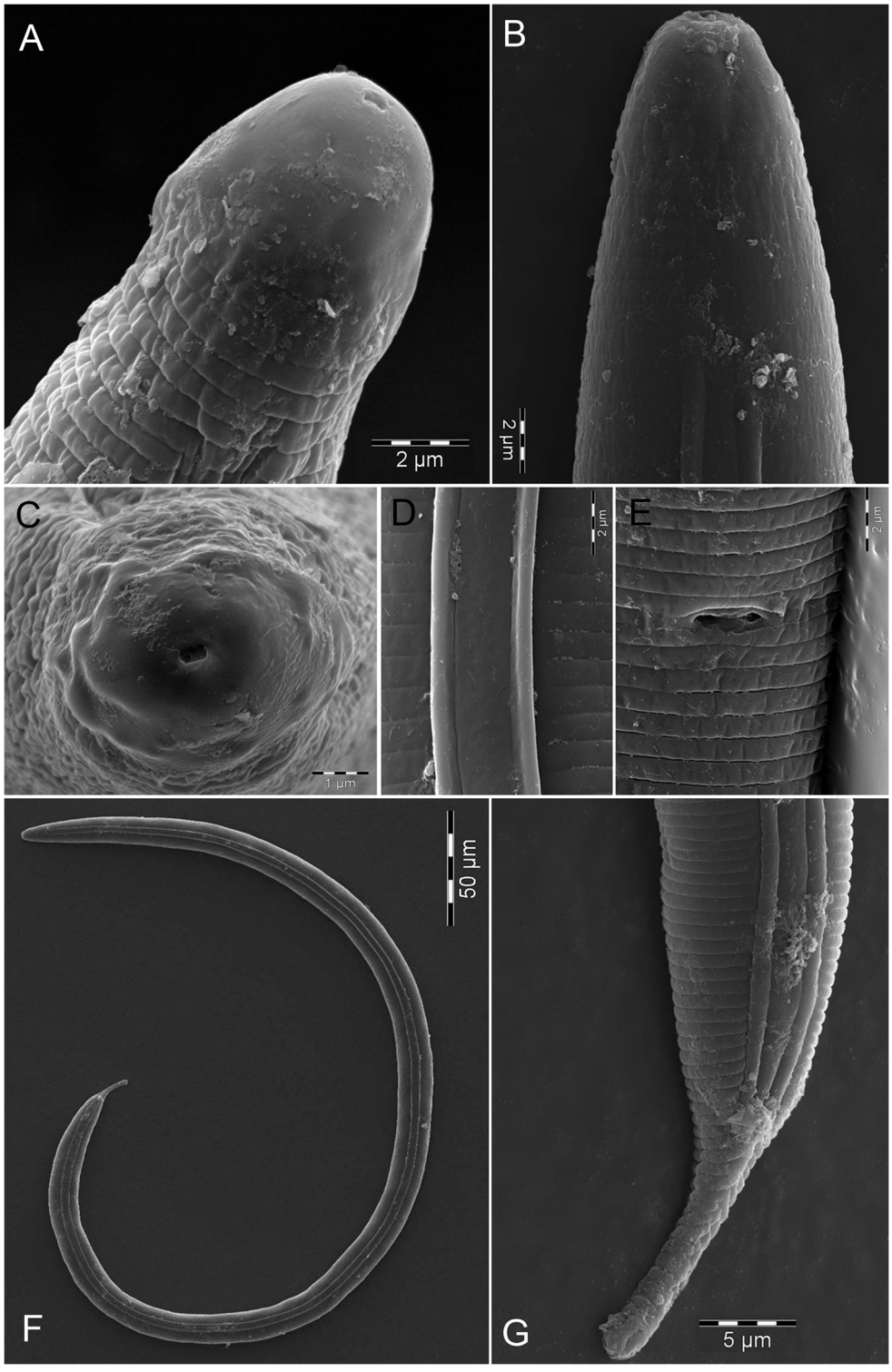


Fig. 2. *Rotylenchulus macrosomoides* sp. n. A-D, immature female. A, B: Lateral view of lip region; C: *En face* view; D: Lateral field at mid-body. E-G, female. E: Vulval area; F: Entire; G: Tail region.

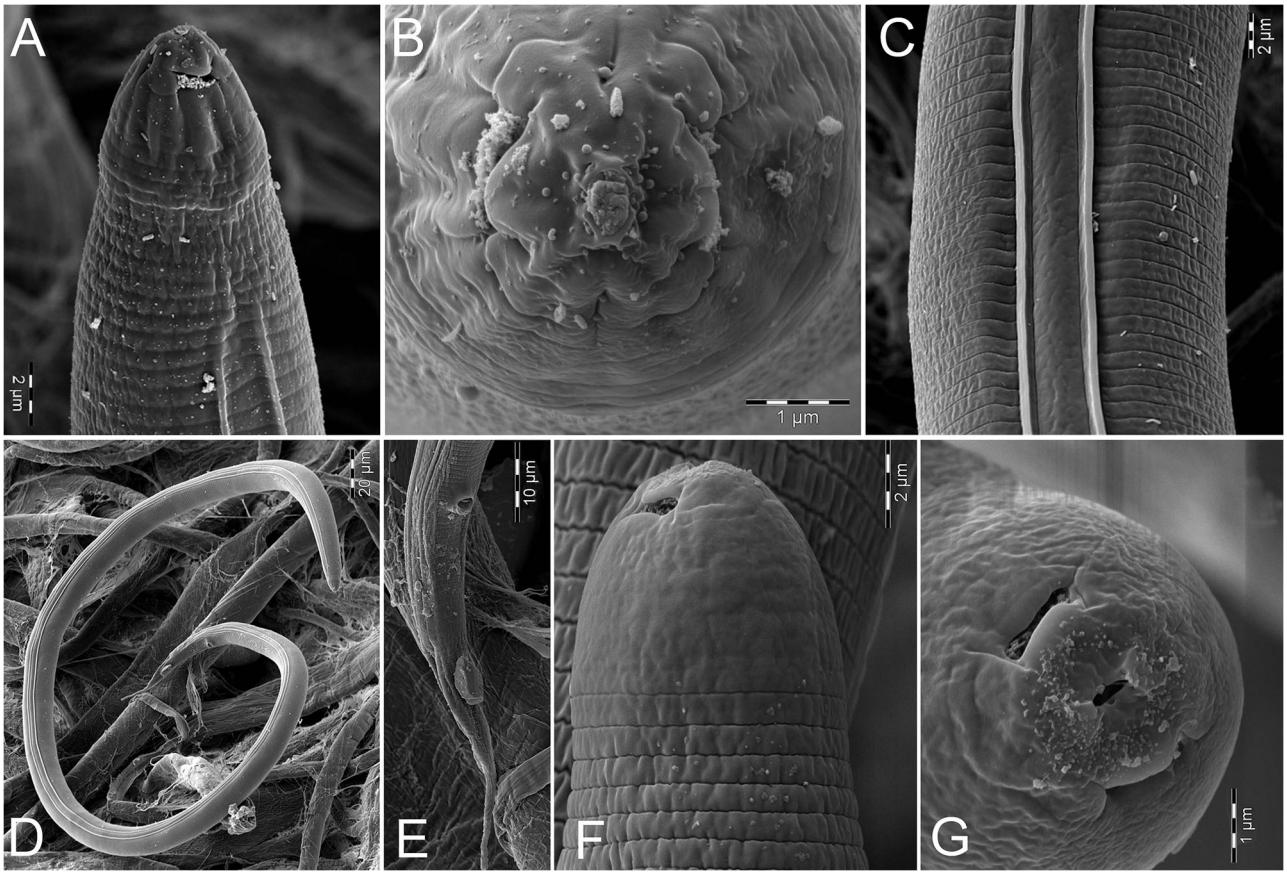


Fig. 3. *Rotylenchulus macrosomoides* sp. n. A-E, male. A: Lateral view of lip region; B: *En face* view of lip region; C: Lateral field at mid-body; D: Entire body; E: Tail region. F, G, immature female. F: Lateral view of lip region; G: *En face* view.

Description

Immature female

Body shape varying from a letter C to curved into 1.5 circles. Lip region high, sloping slightly to a slightly flattened or rounded tip, with no visible annuli. Fusion of lip region sectors and obliteration of amphidial apertures not seen due to fixation problems. Labial framework well developed, stretching *ca* 2-3 annuli posterior from basal plate. Anterior and posterior cephalids seen in a few specimens, situated six and 13-16 annuli posterior to base of lip region. Stylet very slender with knobs rounded posteriorly and mostly slightly sloping anteriorly with outer tips turned upwards. Dorsal pharyngeal gland opening very difficult to see but apparently mostly situated *ca* 1.25 stylet lengths posterior to base. Median bulb distinct, slightly longer than wide with a prominent valve. Excretory pore situated from opposite anterior part of isthmus to opposite anterior part of pharyngeal lobe. Hemizonid 2-3 annuli long situated 4-6 annuli anterior to excretory pore. Hemizonion seen in two specimens only, situated 12-15 annuli posterior to hemizonid. Annulation distinct over entire body up to tail tip. Ovaries outstretched with reflexed tips. Lateral fields distinct with four incisures and three equal bands clearly seen on mounted material, but on SEM photographs outer two bands appearing pushed up into form of a ridge. Lateral fields ending more or less opposite start of hyaline region of tail just posterior to phasmid. Tail with 38-45 annuli, tapering very slightly to about middle of tail, then narrowing suddenly into a long projection with a rounded or very slightly clavate tip; annulation on tail continuing to and around tail tip. Hyaline portion on tail very long ranging from half to almost two-thirds of tail length. Phasmid situated 10-17 annuli posterior to anus.

Table 3. Morphometrics of female *Rotylenchulus macrosomoides* sp. n. from Komatipoort, Mpumalange Province, and *R. macrosoma*. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*R. macrosomoides* sp. n., Komatipoort, South Africa			*R. macrosoma*, Cohn & Mordechai (1988)	*R. macrosoma*, wild olive, Spain, Castillo *et al.* (2003a)	
	Immature female		Mature female	Immature female	Immature female	Mature female
	Holotype	Paratypes	Paratypes			
n	–	10	4	52	12	8
L	508	525 ± 45.7 (463-590)	384-410	470-640	408-510	476-730
a	36.4	34.4 ± 2.3 (31.4-37.3)	5	24.5-38	26.3-34.2	4.5-7.1
b	4.7	4.3 ± 0.4 (3.8-4.7)	5.6-5.9	3.3-5.7	3.5-4.4	–
c	14.1	16.8 ± 2.1 (14.1-21.4)	21.5	11.8-16	11.7-16.8	16.1-24.5
c′	3.3	2.8 ± 0.5 (2.1-3.4)	2.6	3.7-5	2.8-4.4	1.3-2.5
o	141	137 ± 6.1 (130-141)	–	122-188	126-183	138-148
DGO	30	32 ± 1.7 (30-33)	–	–	22-27	21-22
V	78.0	79.0 ± 2.4 (75.5-82.5)	57.5-70.0	58.9-68.0	59.0-64.0	57.0-69.0
G_1	9.7	8.0 ± 1.4 (5.0-10.0)	–	–	–	–
G_2	10.3	7.5 ± 1.7 (4.0-10.5)	–	–	–	–
OV1 length	49	41 ± 8.4 (24-52)	–	–	–	–
OV2 length	51	38 ± 9.7 (21-51)	–	–	–	–
Stylet length	21.5	23.0 ± 1.5 (21.5-25.5)	13.0-13.5	18-22	15-18	14-16
Metenchium length	9.0	10.0 ± 0.8 (9.0-11.0)	6.5	–	–	–
Telenchium length	12.5	13.0 ± 0.9 (12.0-14.5)	6.5-7.0	–	–	–
m	41.3	43 ± 1.8 (40.7-45.5)	48.5-50	–	–	–
Stylet knob width	4.0	3.5 ± 0.5 (3.0-4.0)	2.0	–	–	–
Stylet knob height	2.0	2.0 ± 0.6 (1.0-3.0)	0.75-1.5	–	–	–
Pharynx length	108	123 ± 9.0 (108-139)	64.5-72.0	–	138-181	105-108
Excretory pore from anterior	86	88 ± 5.1 (80-95)	–	–	85-98	–
Diam. at mid-body	14	15.5 ± 1.4 (13.0-18.5)	77-82.5	–	14-17	95-134
Diam. at anus	11	11.0 ± 1.3 (9.0-13.0)	7.5	–	8-11	14-18
Median bulb length	12	12.0 ± 0.6 (11.0-13.0)	14.0-14.5	–	–	–
Median bulb diam.	8.5	9.5 ± 0.9 (8.0-10.5)	12.0-12.5	–	–	–
Valve length	3.5	4.5 ± 0.4 (3.5-5.0)	4.0	–	–	–
Valve width	3.0	3.0 ± 0.9 (3.0-3.5)	3.0	–	–	–
Lip region diam.	7.5	7.0 ± 0.4 (6.5-7.5)	–	–	–	–
Lip region height	3.5	4.5 ± 0.5 (3.5-5.0)	–	–	–	–
Annulus width	0.75	1.0	1.5	–	–	–
Lateral field width	5	4.5 ± 0.6 (3.5-5)	–	–	–	–
Tail length	36	31.5 ± 3.4 (26.5-36.0)	19.0	–	26-40	23-36
h	25.5	21.5 ± 2.6 (17.0-25.5)	–	11.5-18.2	9-12	10-12

Male

Similar to immature female. Body form ranging from a complete circle to 1.5 circles. Lip region high, tapering slightly to a rounded tip, annulation indistinct. Lip pattern, as seen with SEM, consisting of a rounded oral disc containing a slit-like oral opening. Oral disc fused dorsally and ventrally with submedian, dorsal and ventral sectors in a dumb-bell configuration laterally delimiting amphidial openings. Anterior and posterior cephalids very indistinct but where seen they are situated 5-7 and 14-15 annuli posterior to base of lip region. Stylet very slender with small knobs, sloping anteriorly and rounded posteriorly. Opening of dorsal pharyngeal gland very difficult to see. Where seen, ranging from *ca* 1.25-1.75 stylet lengths from base of stylet. Median bulb and valve much smaller than and not as prominent as in female. Excretory

Table 4. Morphometrics of male and juvenile *Rotylenchulus macrosomoides* sp. n. from Komatipoort, Mpumalange Province, South Africa and *R. macrosoma*. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*R. macrosomoides* sp. n., Komatipoort, South Africa		*R. macrosoma*, Cohn & Mordechai (1988)	*R. macrosoma*, wild olive, Spain, Castillo *et al.* (2003a)
	Male	Juvenile (J4)	Male	Male
	Paratypes	Paratype		
n	17	1	21	11
L	661 ± 37.8 (581-719)	511	500-680	449-495
a	39.8 ± 2.6 (35.8-43.7)	25.7	30-41	27.5-34
b	6.4 ± 0.5 (5.1-7.2)	4.1	–	3.5-5.2
c	11.9 ± 0.8 (10.6-14)	10.7	12-16	13.1-15
c′	4.5 ± 0.3 (3.8-5.1)	3.6	–	2.6-3.9
o	150 ± 15.3 (128-174)	136	–	142-188
DGO	27 ± 2.2 (23-30)	26	–	19-26
T	22.5 ± 6.5 (11.2-31.4)	–	20-33	25-42
Stylet length	18.5 ± 1.2 (17.0-21.5)	19.5	13-16	12-15
Metenchium length	9.0 ± 1.1 (7.5-11.5)	8.0	–	–
Telenchium length	9.5 ± 0.8 (8.0-11.5)	11.5	–	–
m	47.9 ± 3.6 (43.5-56.0)	41.5	–	–
Stylet knob width	2.5 ± 0.5 (1.5-3.0)	3.5	–	–
Stylet knob height	1.5 ± 0.4 (0.75-2)	2	–	–
Pharynx length	106 ± 7.6 (93-125)	142	–	110-157
Excretory pore from anterior	87 ± 4.9 (73-94)	81	–	81-96
Diam. at mid-body	16.5 ± 1.3 (14.0-19.0)	20.0	–	14-18
Diam. at anus	12.5 ± 0.8 (10.5-14.0)	13.0	–	9-12
Median bulb length	8.5 ± 1 (7.5-10.5)	11.0	–	–
Median bulb diam.	6.0 ± 0.6 (5.0-7.0)	7.5	–	–
Valve length	2.5 ± 0.5 (1.5-3.0)	3.0	–	–
Valve width	1.5 ± 0.3 (1.0-2.0)	2.5	–	–
Lip region diam.	6.5 ± 0.5 (6-7.5)	7.5	–	–
Lip region height	4.5 ± 0.3 (4.5-5.0)	4.0	–	–
Annulus width	1.0 ± 0.2 (0.5-1.5)	0.75	–	–
Lateral field width	4.5 ± 0.4 (4.5-5.0)	4	–	–
Tail length	55 ± 4.9 (45-64)	48	–	30-36
h	28.0 ± 4.3 (18.5-34.5)	17.5	15-23	8-14
Testis length	148 ± 41.5 (72-209)	–	–	–
Spicule length	23 ± 1.2 (21-25)	–	21-24	19-25
Gubernaculum length	8.5 ± 1.1 (5.0-10.5)	–	8-10	8-10

pore situated from opposite anterior to opposite posterior part of pharyngeal lobe. Hemizonid and hemizonion as in female. Lateral field consisting of four lines with three equal bands. With SEM, outer two bands appearing as two ridges probably due to preparation process. Tail tapering very slightly to *ca* two-thirds of tail length, then narrowing suddenly into a long, narrow projection with annuli continuing around rounded tail tip. In a few cases, tip very slightly clavate. Phasmids situated in middle of tail, more or less opposite where hyaline portion starts. Bursa consisting of two small flat folds in cuticle, very indistinct. Cloacal opening with prominent protruding lips. Spicules and gubernaculum slightly curved.

Juvenile (J4)

Very similar to immature female but with a more prominent flattened lip region with small indistinct annuli. Entire pharyngeal region not as prominent as that of immature female. Tail tapering gradually to last quarter

then continuing as a straight projection with a rounded tip, not distinctly annulated.

Mature female

Body irregularly curved ventrad. Stylet short with small anteriorly sloping stylet knobs, rounded posteriorly. Most internal characters not clearly seen due to being attached to root. Vulva with two raised lips. Tail tapering to a finger-like tip with a longish hyaline portion.

TYPE HOST AND LOCALITY

Rhizosphere and roots of sugarcane collected by Mr M. Masilela, Biosecurity supervisor on a sugarcane farm, near Komatipoort, Mpumalanga Province, South Africa, on 2 July 2012, 148 m a.s.l., 25.45°S, 31.96°E.

TYPE MATERIAL

Holotype female (Slide no. 50065), paratypes – 28 males, two juveniles and four mature females (Slide nos 50066-50077) deposited in the National Collection of Nematodes of the Nematology Unit, ARC-PPRI, Pretoria, South Africa.

DIAGNOSIS AND RELATIONSHIPS

Rotylenchulus macrosomoides sp. n. immature females are characterised by a long, slender body usually curved into 1.5 circles, tail tapering very slightly to about middle of tail, then narrowing suddenly into a long projection with a rounded or very slightly clavate tip with a very long hyaline portion. Males very similar with a longer tail, narrowing less than in immature female at *ca* two-thirds the tail length to a narrow tip and also with a long hyaline region. Juvenile very similar to immature female with a slightly thicker body and narrowing on tail not quite so prominent. Mature females with variously swollen and irregularly curved bodies.

When following the various keys of the genus (Loof & Oostenbrink, 1962; Dasgupta *et al.*, 1968; Germani, 1978; Van den Berg, 1978; Robinson *et al.*, 1997) this species seems to be close to *R. macrosoma* (Dasgupta *et al.*, 1968; Cohn & Mordechai, 1988; Castillo *et al.*, 2003b). There are several differences in some of the characters of the new species specimens and *R. macrosoma* (Tables 3, 4, 8). It differs in the immature female by: DGO = 30-33 *vs* 22-27 μm; V = 75.5-82.5 *vs* 58.9-68; stylet length = 21.5-25.5 *vs* 15-22 μm; pharynx length = 108-139 *vs* 114-181 μm; h = 17-25.5 *vs* 9-18.2 μm; mature female: L = 384-410 *vs* 476-730 μm; pharynx length = 64.5-72 *vs* 105-108 μm; diam. at mid-body = 77-82 *vs* 95-134 μm; tail length = 19 *vs* 23-36 μm; male: L = 581-719 *vs* 446-680 μm; stylet length = 17-21.5 *vs* 12-17 μm; tail length 45-64.5 *vs* 29-36 μm; h = 18.5-34.5 *vs* 8-23 μm. *Rotylenchulus macrosomoides* sp. n. is also close to *R. macrodoratus* because the long stylet for immature females ranges from 21-26 μm, but it can be separated by several characters. It differs in immature female: DGO = 30-33 *vs* 13-18 μm; V = 75.5-82.5 *vs* 57-72; tail length = 26-36 *vs* 20-26 μm and h = 17-25.5 *vs* 6-12; male: L = 581-719 *vs* 450-540 μm; c′ = 3.8-5.1 *vs* 1.8-2.2; DGO = 23.5-30 *vs* 16-18 μm; tail length = 45-64 *vs* 20-24 μm; h = 18.5-34.5 *vs* 10-11. From *R. sacchari* the new species differs in immature female: stylet length = 21-26 *vs* 26-34 μm; L = 463-590 *vs* 574-906 μm; c′ = 2.1-3.4 *vs* 1.1-1.9; DGO = 30-33 *vs* 8-15 μm; V = 75.5-82.5 *vs* 61.5-73; tail length = 26-36 *vs* 15-30 μm; h = 17-25 *vs* 4.5-12.0 μm. Apart from the difference in tail length, the new species has a tail tapering slightly to *ca* two-thirds of tail length, then narrowing suddenly into a long slender projection with a rounded or very slight clavate tip *vs* a broadly rounded tail.

Rotylenchulus clavicaudatus Dasgupta, Raski & Sher, 1968

(Figs 4, 5)

This species was originally described by Dasgupta *et al.* (1968) from immature females and one male from the roots of a *Strelitzia* sp. from virgin soil on the beach at Port St Johns, Eastern Cape Province, South Africa. Van den Berg & Spaull (1981) described several immature females and males and one mature female from sugarcane in KwaZulu-Natal Province, South Africa. So far the species have been found only on the eastern side of South Africa in four provinces mainly on sugarcane. The present specimens were collected from a sugarcane farm at Empangeni, KwaZulu-Natal Province, on 12 March 2013 by H. Dagutat. The present specimens were studied morphologically and, for the first time, molecularly.

MEASUREMENTS

See Table 5.

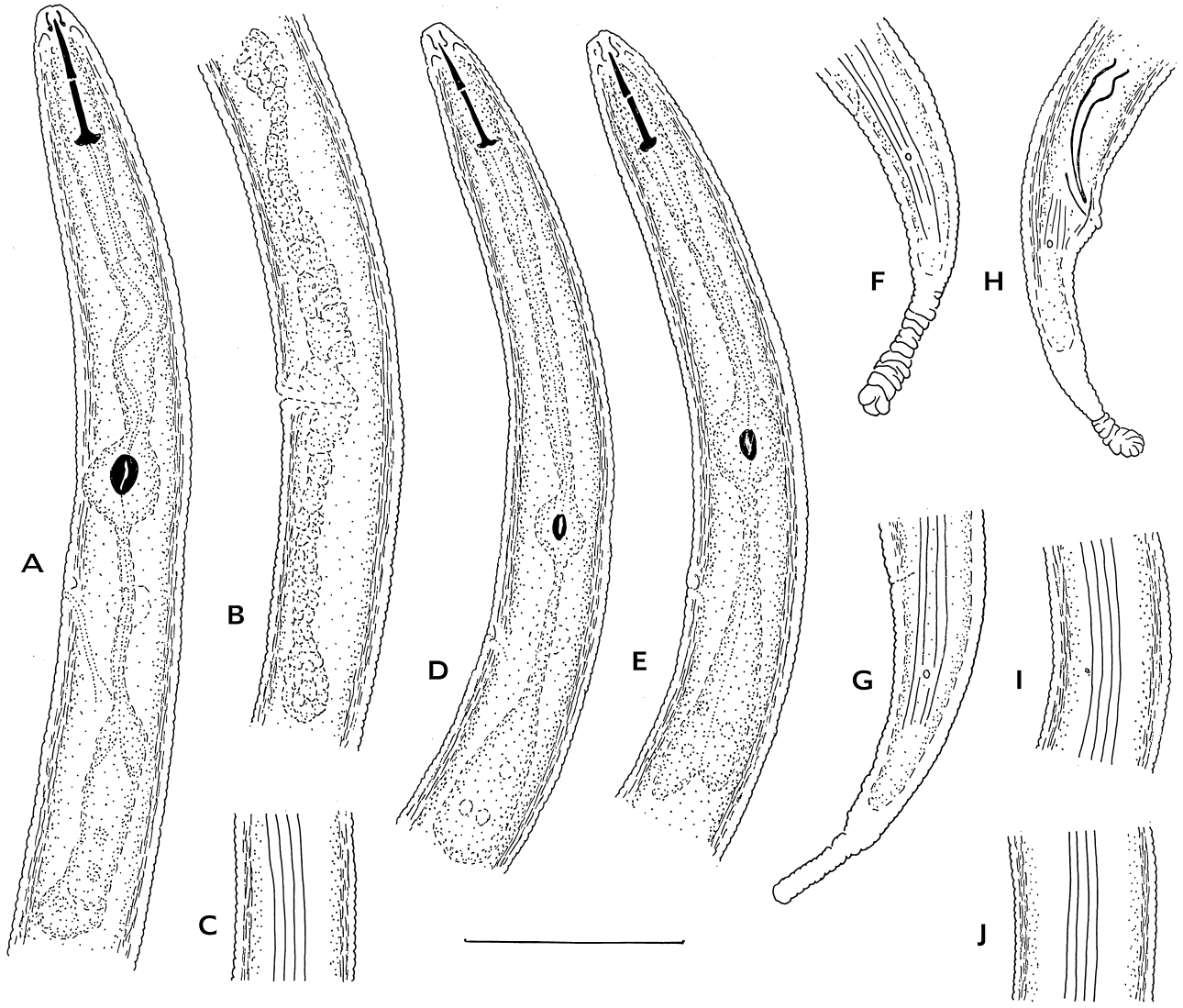


Fig. 4. *Rotylenchulus clavicaudatus*. A-C, F, immature female. A: Anterior part of body; B: Vulval area with reflexed ovaries; C: Lateral field at mid-body; F: Tail. D, H, J, male. D: Anterior part of body; H: Tail; J: Lateral field at mid-body. E, G, I, juvenile. E: Anterior part of body; G: Tail; I: Lateral field at mid-body. (Scale bar = 30 μm.)

DESCRIPTION

Immature female

Body form ranging from an open letter C to a complete circle. Lip region slightly sloping anteriorly to a slightly rounded tip. Not set off with indistinct annuli. SEM photographs showing annuli not to be present. Lip pattern consisting of an oral disc fused with submedian sectors dorsally and ventrally and delimiting amphidial apertures. Labial framework well developed, stretching posteriad two annuli from basal plate. Anterior and posterior cephalids not seen. Stylet with metenchium slightly longer than telenchium, stylet knobs sloping slightly with outer tips slightly curved upward, rounded posteriorly. Dorsal pharyngeal gland opening situated *ca* three-quarters of stylet length posterior to base of stylet. Median bulb distinct, slightly longer than wide with prominent valve. Excretory pore situated from opposite middle of isthmus to opposite anterior part of pharyngeal lobe. Hemizonid two annuli long, situated from five annuli anterior to one annulus posterior to excretory pore. Hemi-

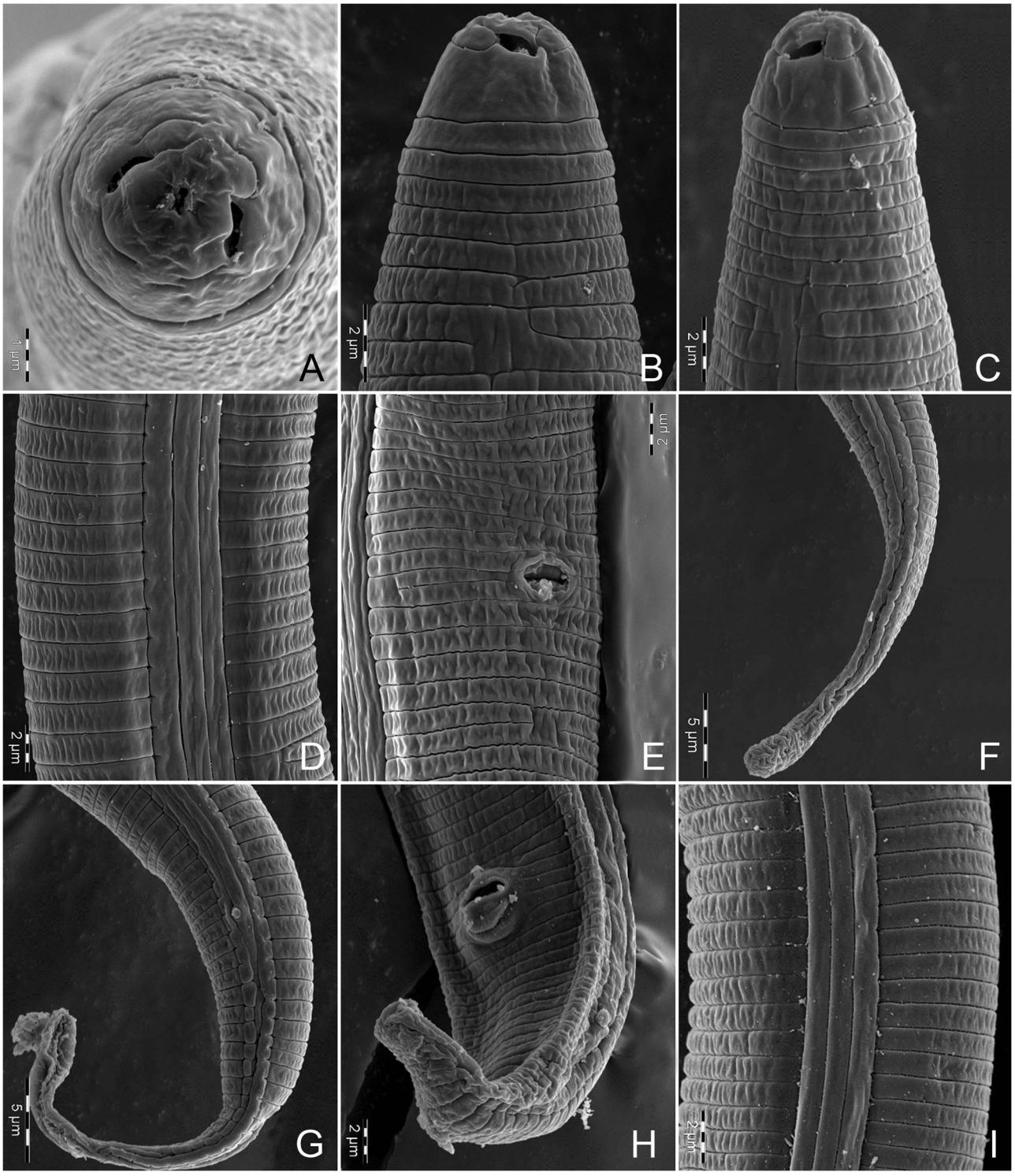


Fig. 5. *Rotylenchulus clavicaudatus*. A-G, immature female. A: *En face* view; B, C: Lip region, lateral view; D: Lateral field at mid-body; E: Vulval region; F, G: Tails. H, I, male. H: Tail region; I: Lateral field at mid-body.

Table 5. Morphometrics of *Rotylenchulus clavicaudatus*. All measurements are in μm and in the form: mean ± s.d. (range).

Character	Empangeni, KwaZulu-Natal Province, South Africa			South Africa, Dasgupta *et al.* (1968)		South Africa, Van den Berg & Spaull (1981)	
	Immature female	Male	Juvenile (J4)	Immature female	Male	Immature female	Male
n	11	4	6	4	1	46	51
L	563 ± 43.2 (483-624)	530 ± 32.2 (488-560)	500 ± 38.1 (446-539)	460-590	540	495 (460-523)	531 (427-569)
a	32.1 ± 2.4 (27.6-36.9)	33.1 ± 3.5 (30.1-37.4)	28.5 ± 2.7 (25.3-31.2)	28-30	29	28.3 (26.3-31.3)	30.6 (23.7-34.7)
b	4.2 ± 0.3 (3.8-4.7)	4.5 ± 0.3 (4.2-4.7)	4.3 ± 0.4 (3.7-4.7)	4-5	–	3.4 (2.7-3.7)	4.4 (3.5-4.7)
c	10.6 ± 0.9 (9.1-11.8)	13.6 ± 0.9 (12.7-14.7)	11.4 ± 2.2 (9.3-12.9)	12-16	15	11.2 (10.1-12.6)	13.0 (10.9-14.1)
c′	4.8 ± 0.7 (3.7-6)	3.1 ± 0.3 (2.8-3.4)	4.2 ± 0.3 (3.9-4.6)	3.9-5.3	–	3.9 (2.5-4.4)	3.4 (2.9-4.1)
o	77.8 ± 11.9 (64-100)	65.2 ± 6.1 (61-72.7)	125 ± 11.1 (114.3-136.4)	75-85	–	73.9 (58.2-121.7)	66.4 (56.8-71.1)
DGO	14 ± 2.5 (11.0-18.0)	11 ± 0.8 (10.5-12.0)	–	–	–	13.2 (10.3-20.2)	11.3 (9.6-12.5)
V or T	57.0 ± 2 (54.5-60.5)	37.5 ± 6.1 (32.5-46.0)	–	57-59	30	59 (55-63)	–
G_1	11 ± 1.7 (8.0-13.5)	–	19.5 ± 2.4 (17.5-22)	–	–	–	–
G_2	10.4 ± 1.6 (8.3-14)	–	16.5 ± 2.3 (13-20)	–	–	–	–
OV1 length	62 ± 8.9 (48-72)	–	–	–	–	–	–
OV2 length	58 ± 7.9 (48-70)	–	–	–	–	–	–
Stylet length	18.0 ± 1.3 (16.0-20.0)	16.5 ± 1.3 (14.5-17.5)	15.5 ± 0.8 (14.5-16.0)	17-20	–	18.0 (16.6-18.8)	16.8 (15.1-18.4)
Metenchium length	11.5 ± 1.2 (10.0-13.0)	10 ± 0.4 (9.5-10.5)	9.5 ± 0.8 (9.0-10.5)	–	–	–	–
Telenchium length	7.0 ± 1.5 (5.5-9.5)	7 ± 0.5 (6.5-7.5)	6.0	–	–	–	–
m	60.8 ± 6.3 (51.8-67.9)	58.1 ± 1.5 (56.5-59.5)	61.8 ± 1.9 (59.9-63.6)	–	–	44-52	–
Stylet knob width	3.0 ± 0.5 (2.5-4.0)	1.5 ± 0.6 (1.0-2.0)	2.5 ± 0.3 (2.0-2.5)	–	–	3.0 (2.2-3.7)	–
Stylet knob height	1.5 ± 0.3 (1.5-2.0)	2.5 ± 0.4 (2.0-3.0)	1.5	–	–	1.5 (0.7-1.8)	–
Pharynx length	131 ± 12.2 (117-156)	115 ± 3.4 (111-118)	116 ± 5.1 (109-122)	–	–	–	–
Excretory pore from anterior	92 ± 4.6 (85-100)	86 ± 2.8 (82-89)	88 ± 3.8 (84-93)	–	–	86 (78-93)	86 (80-94)
Diam. at mid-body	17.5 ± 1.4 (16.0-20.0)	16.0 ± 1.1 (14.5-17.5)	18.5 ± 1.7 (16.5-21.5)	–	–	–	–
Diam. at anus	11.0 ± 0.8 (9.5-12.0)	13.0 ± 0.9 (12.0-14.0)	10.5 ± 1.7 (9.0-12.5)	–	–	–	–
Median bulb length	13.0 ± 1 (12.0-14.5)	10.0	13.0 ± 2.4 (11.0-15.5)	–	–	12.7 (11.0-14.3)	–
Median bulb diam.	9.5 ± 0.8 (8.0-11.0)	7.5	9.2 ± 1 (8.0-10.5)	–	–	8.8 (7.4-9.9)	–
Valve length	4.5 ± 0.7 (3.5-5.5)	3.0	4.0 ± 0.5 (3.5-4.5)	–	–	–	–
Valve width	3.0 ± 0.5 (2.0-3.5)	2.0	2.5 ± 0.3 (2.0-3.0)	–	–	–	–
Lip region diam.	7.0 ± 0.5 (6.5-8.0)	7.5	7.0 ± 0.4 (6.5-7.5)	–	–	5.5 (4.8-5.9)	–
Lip region height	4.5 ± 0.4 (3.5-5)	4.0 ± 0.4 (3.5-4.5)	4.5 ± 0.6 (3.5-5.0)	–	–	3.7 (2.6-4.4)	–
Annulus width	1.5	1.5	1 ± 0.3 (0.75-1.5)	–	–	–	–
Lateral field width	4.5 ± 0.5 (3.5-5.0)	4.0 ± 0.4 (3.5-4.5)	4 ± 0.6 (3.0-4.5)	–	–	4.3 (3.7-5.2)	4.0 (3.3-5.9)
Tail length	54 ± 6.8 (43-66)	39 ± 5 (33-44)	46 ± 10.7 (34-60)	43 (35-50)	–	44 (38-48)	41 (36-49)
h	27.5 ± 5.4 (19.0-35.5)	19.5 ± 1.8 (17.5-21.5)	17.5 ± 1.1 (15.5-18.5)	16-23	1-8	–	–
Testis length	–	199 ± 38.5 (164-253)	–	–	–	–	–
Spicule length	–	24.5 ± 1.1 (23.5-25.5)	–	–	20	–	23.4 (22.1-25.0)
Gubernaculum length	–	9.0 ± 1 (8.0-10.5)	–	–	9	–	11.1 (9.9-13.6)

zonion not distinct, one annulus long, situated 7-15 annuli posterior to excretory pore. Annulation distinct over whole body, right up to tail tip. Ovaries outstretched with reflexed tips. Lateral field distinct with four incisures and three equal bands. Phasmid distinct, situated from 4-12 annuli posterior to anus. Tail with *ca* 25-32 annuli, narrowing gradually posterior to anus and ending in a more broadly rounded, clavate, annulated tip. Hyaline portion of tail long, *ca* 45-55% of tail length.

Male

Similar to immature female. Body form ranging from an open figure 6 to 1.5 circles. Pharyngeal region slightly less developed. Excretory pore slightly more anterior. Tail and hyaline portion slightly shorter. Phasmids situated 8-11 annuli posterior to anus. Tail tip slightly clavate. Bursa with a flat cuticular fold.

Juvenile (J4)

Similar to immature female. Pharyngeal region slightly less developed. Excretory pore slightly more anterior. Hemizonid one or two annuli long, situated directly anterior or one annulus anterior to excretory pore. Hemizonion one annulus long, situated nine or ten annuli posterior to hemizonid. Tail with more annuli than that of immature female but not easy to count, tip not quite as clavate as in immature female. Phasmids situated eight or nine annuli posterior to anus. Hyaline portion of tail *ca* 26-51% of tail length.

Mature female

Not found.

REMARKS

The present specimens correspond well with the descriptions given by Dasgupta *et al.* (1968) and Van den Berg & Spaull (1981). However, since no topotype material of this species was included in this study, molecular differences between this new population and that from the type locality cannot be excluded. *Rotylenchulus clavicaudatus* immature females have a stylet longer than 16 μm, like *R. macrosoma*, *R. macrodoratus*, *R. reniformis* and *R. sacchari*. It differs from *R. macrosoma* in immature females by having shorter DGO = 11-20.2 *vs* 22-27 μm, and a clavate *vs* bluntly rounded tail terminus. This species differs also from *R. reniformis*, *R. macrodoratus* and *R. sacchari* in having immature females with a clavate *vs* rounded tail terminus.

***Rotylenchulus leptus* Dasgupta, Raski & Sher, 1968**

(Figs 6, 12)

This species was originally described from around bamboo, grass and weed soil from Gwelo and Chipinga, Zimbabwe, and was later reported from an unidentified host from Mooi River, KwaZulu-Natal Province, South Africa (Van den Berg, 1978). The present specimens were collected by J. Swart on 28 January 2010 from a sorghum field and an unidentified grass from a farm near Brits, North West Province, South Africa. The small number of specimens collected allowed their morphological analysis, but was not sufficient for molecular analysis.

MEASUREMENTS

See Table 6.

DESCRIPTION

Immature female

Body posture ranging from an open figure 6 to 1.5 circles. Lip region not set off, sloping anteriorly to a flattened tip, with four or five annuli. SEM photographs showing a roundish labial disc with first annulus divided into sectors, number not distinguishable. Labial framework well developed, stretching two annuli posterior from basal plate. Anterior and posterior cephalids not seen. Stylet with metenchium slightly shorter than telenchium. Stylet knobs flattened to slightly sloping anteriorly and rounded posteriorly. Median bulb distinct, slightly longer than wide with prominent valve. Excretory pore situated from opposite middle of isthmus to opposite anterior part of pharyngeal lobe. Hemizonid two annuli long situated opposite or directly anterior to excretory pore. Hemizonion not seen. Annulation distinct over whole body up to tail tip. Ovaries with reflexed tips. Lateral field distinct with four incisures and three equal bands. Phasmids distinct, situated 8-15 annuli posterior to anus opposite about middle of tail. Tail with *ca* 22-34 tail annuli, tapering gradually to a finely rounded annulated tip. Hyaline portion very variable, *ca* 7.6-16.7% of tail length.

Juvenile (J4)

Similar to immature female except for having a slightly broader rounded tail with no obvious hyaline region.

Male

Not found.

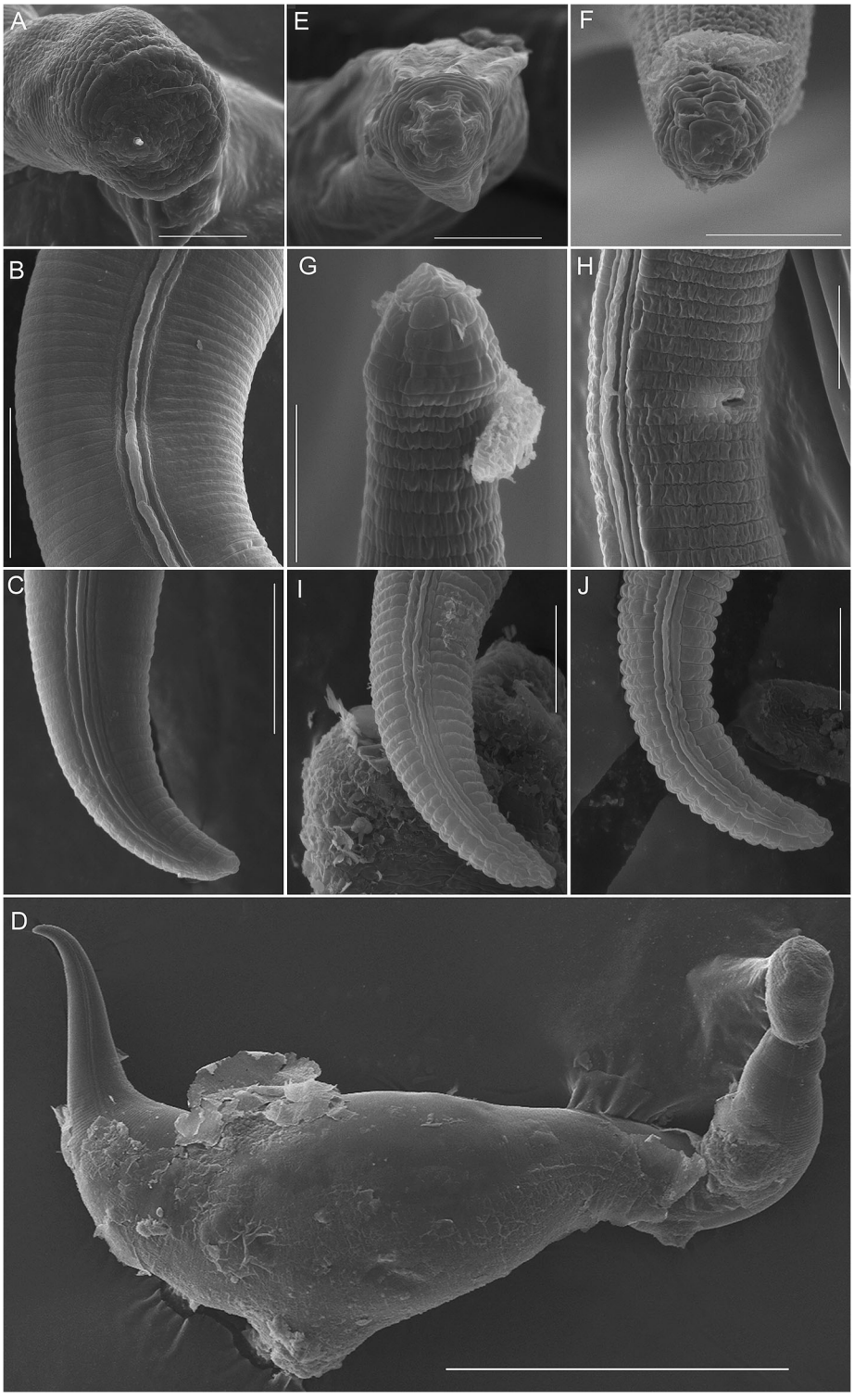


Fig. 6. *Rotylenchulus leptus*. A-D, mature Female. A: *En face* view; B: Lateral field near tail; C: Tail; D: Entire. E-J, immature female. E, F: *En face* view; G: Lateral view of lip region; H: Vulval area; I, J: Tail. (Scale bar: A-C = 10 μm; D = 100 μm; E-J = 5 μm.)

Table 6. Morphometrics of *Rotylenchulus leptus*. All measurements are in μm and in the form: mean ± s.d. (range).

Character	Brits, North West Province, South Africa		Zimbabwe, Dasgupta *et al.* (1968)	South Africa, Van den Berg (1978)
	Immature female	Juvenile (J4)	Immature female	Immature female
n	14	8	43	2
L	378 ± 27.4 (321-434)	397 ± 15.5 (383-428)	290-370	411, 422
a	28.2 ± 1.4 (25.7-29.9)	28.6 ± 3.4 (26.2-36.4)	27-33	21.9, 28.0
b	3.6 ± 0.3 (2.9-4.3)	4.0 ± 0.3 (3.8-4.6)	3.2-4	3.5, 4.0
c	15.9 ± 0.8 (14.5-17.3)	15.4 ± 0.7 (14.7-16.7)	11-14	14.0, 15.0
c′	2.9 ± 0.3 (2.6-3.4)	2.5 ± 0.09 (2.4-2.7)	3.4-4.7	3.2, 3.4
o	163 ± (150-178)	152 ± 24.5 (120-179)	123-160	80
DGO	22.0 ± 1.7 (20.0-24.5)	20.5 ± 3.2 (16.0-25.5)	–	11.0, 11.5
V	61.5 ± 1.8 (59.0-65.0)	–	57-64	57, 58
G_1	8 ± 1.3 (4.5-10.5)	–	–	10, 12
G_2	8.5 ± 1.3 (7.5-11.5)	–	–	10, 11
OV1 length	31 ± 4.9 (14-34)	–	–	–
OV2 length	32 ± 5.5 (17-42)	–	–	–
Stylet length	13.5 ± 0.6 (12.5-14.5)	13.5 ± 0.8 (12.5-15)	11-14	15.1
Metenchium length	6.0 ± 0.5 (5.0-6.5)	6.5 ± 0.8 (5.0-7.5)	–	–
Telenchium length	7.5 ± 0.5 (6.5-8.0)	7.0 ± 0.4 (6.5-7.5)	–	–
m	45.5 ± 2.5 (40.8-48.5)	48.0 ± 3.6 (41.0-50.0)	–	48, 51
Stylet knob width	3.1 ± 0.4 (3.0-3.5)	2.9 ± 0.1 (2.5-3.0)	–	3.7, 2.9
Stylet knob height	1.5 ± 0.2 (1.0-1.5)	1.5 ± 0.3 (1.0-2.0)	–	1.5, 2.2
Pharynx length	107 ± 6.7 (96-120)	100 ± 6.6 (91-108)	–	111, 121
Excretory pore from anterior	75 ± 3.2 (70-82)	80 ± 2.3 (76-83)	65-76	81
Diam. at mid-body	13.5 ± 1 (12-14.5)	14.5 ± 1.1 (13-16)	–	–
Diam. at anus	8.0 ± 0.8 (7.5-9.5)	10.0 ± 0.7 (9.5-11)	–	–
Median bulb length	11.5 ± 0.9 (10.0-13.0)	10.5 ± 0.6 (9.5-11.0)	–	10.0, 10.5
Median bulb diam.	8.5 ± 0.7 (7.5-10.5)	7.5 ± 0.6 (6.5-8)	–	8.0, 9.0
Valve length	3.5 ± 0.3 (3.0-4.0)	2.5 ± 0.6 (1.5-3.0)	–	4.0, 4.5
Valve width	2.5 ± 0.4 (2.0-3.5)	2.0 ± 0.5 (1.5-3.0)	–	2.5
Lip region diam.	6.5 ± 0.5 (5.0-6.5)	6.0 ± 0.6 (5.5-7.5)	–	5.2
Lip region height	3.5 ± 0.5 (3.0-4.5)	3.5 ± 0.3 (3.0-3.5)	–	2.9
Annulus width	1.5	1.1 ± 0.2 (1.0-1.5)	–	1.1
Lateral field width	3.5 ± 0.4 (3.0-3.5)	4.0 ± 0.5 (3.0-4.5)	–	4.4
Tail length	24 ± 2.4 (20-28)	26 ± 1.4 (23-28)	22-28	30, 29
h	3.0 ± 0.6 (1.5-4.0)	–	3-7	3.5, 5.0

Mature female

One found, used for SEM observation and not measured. Vulval opening bulging and clearly seen at mid-body. Posterior body portion ending in a hook-like tip.

Remarks

The two specimens of *R. leptus* described by Van den Berg (1978) from South Africa were very similar to those of Dasgupta *et al.* (1968) from Zimbabwe except for having 4-5 lip annuli *vs* not seen and being slightly longer and having a smaller o value (80 *vs* 123-160) (Table 6). Our studied specimens of immature females of *R. leptus* also fit well with those described by Dasgupta *et al.* (1968) and Van den Berg (1978), except for minor differences. Dasgupta *et al.* (1968) reported larger c′ values (3.4-4.7) for the paratypes and additional materials than those of the present *R. leptus* population (c′ = 2.6-3.4) and two specimens (c′ = 3.2, 3.4) described by Van den Berg (1978) (Table 6). The observed variation in c′ value disproves the differentiation of *R. leptus* with morphologically similar *R. parvus* on this index as previously proposed in the key by Lehman & Inserra (1990).

Although the present specimens of *R. leptus* can fit well with several descriptions of *R. parvus* (Williams, 1960; Dasgupta *et al.*, 1968; Heyns, 1976; Germani, 1978; Van den Berg, 1978; Lehman & Inserra, 1990; Jatala, 1991) there are a few slight differences in immature females between these species, *viz.*, having a higher, conical *vs* low, rounded lip region, and an o value of 150-178 *vs* 60-135. The lack of molecular data for *R. leptus* makes the separation of these two species difficult. The *R. leptus* immature female has a stylet length less than 16 μm like *R. anamictus* and *R. borealis*. It differs from these two species in the lack of males, which are present in the other two species.

Rotylenchulus macrodoratus Dasgupta, Raski & Sher, 1968
(Fig. 7)

This species was described parasitising grapevine (*Vitis vinifera* L.) in Bari, Italy (Dasgupta *et al.*, 1968). Subsequently, the species was also reported from almond (*Prunus amygdalus* L.), Torre Tresca, Italy (Dasgupta *et al.*, 1968), common ivy (*Hedera helix* L.), Saint-Jean-Cap-Ferrat, France (Scotto La Massèse, 1973), olive and other hosts in several localities in Italy (Talamé *et al.*, 1970; Vovlas & Lamberti, 1974), in plum (*Prunus domestica* L.) and apricot (*Prunus armeniaca* L.) in Malta (Vovlas & Lamberti, 1974), Italian oak (*Quercus frainetto* Ten.) in Greece (Vovlas & Lamberti, 1974) and soybean (*Glycine max* (L.) Merr.) in Israel (Cohn & Mordechai, 1977). Populations of this species from olive and a topotype population from grapevine in Bari, Italy, were included in this study.

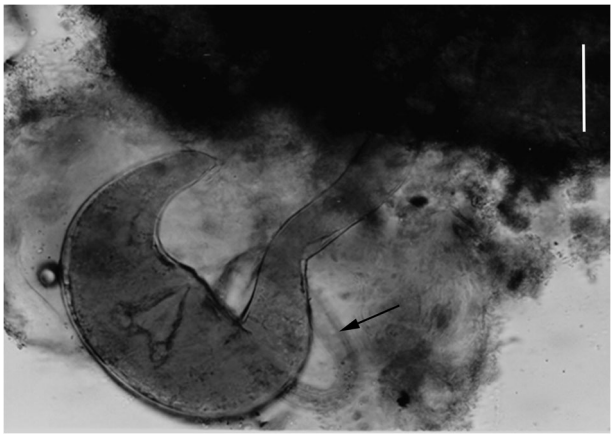


Fig. 7. Olive root parasitised by a mature female of *Rotylenchulus macrodoratus*. Note the gelatinous matrix covering the female body and a male (marked by an arrow). (Scale bar = 100 μm.)

Measurements

See Table 7.

Description

Immature female

Body shape usually in closed C-shape when heat-relaxed. Lip region conoid-rounded not set off, finely annulated. Labial framework well developed with a heavy sclerotisation and extending three or four annuli posterior from basal lip annulus. Stylet long and well developed with anchor-shaped stylet knobs. Dorsal pharyngeal gland opening situated *ca* 1.5 stylet lengths posterior to base of stylet. Median bulb rounded-oval, large, with prominent valves (3-5 μm long). Excretory pore situated from opposite middle of isthmus to opposite anterior part of pharyngeal lobe. Pharyngeal glands overlapping intestine laterally and mostly ventrally. Annulation distinct over entire body, *ca* 1 μm wide. Ovaries outstretched with reflexed tips. Vulva located at more than 63% of body length. Lateral field distinct with four lines and three equal bands. Tail with bluntly rounded terminus, annulation around terminus not prominent. Hyaline portion comprising about half of tail length.

Male

Similar to immature female except for genital system and a more curved posterior part of body. Stylet and pharynx reduced. Median pharyngeal bulb and valves less developed. Tail broadly rounded with rounded tip. Gubernaculum and spicules well developed, ventrally arcuate.

Remarks

The morphology of the new Italian populations of *R. macrodoratus* from cultivated olive and grapevine was almost identical to that described for this species in the original description, confirming that there is only slight morphological and morphometric diversity within populations of this species in that area. Consequently, no morphological drawings of these new populations are provided. The detection of the populations from cultivated olive and grapevine in the same area of the type locality

Table 7. Morphometrics of *Rotylenchulus macrodoratus* from cultivated olive and grapevine in Italy. All measurements are in μm and in the form: mean ± s.d. (range).

Character	Olive, Bari, Bari Province, Italy		Grapevine, Bari, Bari Province, Italy	
	Immature female	Male	Immature female	Male
n	9	5	7	3
L	455 ± 30.0 (407-489)	503 ± 36.7 (452-540)	456 ± 24.9 (414-487)	507 ± 26.2 (478-529)
a	26.1 ± 1.5 (22.6-27.4)	31.4 ± 1.2 (30.1-33.1)	26.4 ± 0.7 (25.5-27.4)	31.7 ± 1.4 (30.2-33.1)
b	3.5 ± 0.4 (2.9-4.0)	3.8 ± 0.5 (3.1-4.6)	3.5 ± 0.3 (2.9-4.0)	3.7 ± 0.1 (3.6-3.8)
c	20.5 ± 1.3 (18.5-22.2)	23.6 ± 2.6 (20.5-27.0)	20.5 ± 1.1 (18.8-21.6)	23.4 ± 1.7 (21.7-25.2)
c′	2.2 ± 0.1 (2.0-2.4)	2.1 ± 0.2 (1.8-2.2)	2.2 ± 0.1 (2.0-2.4)	2.1 ± 0.1 (2.0-2.2)
o	68.0 ± 6.8 (61.9-81.8)	91.6 ± 2.5 (89.5-94.4)	69.7 ± 6.8 (62.5-81.8)	91.5 ± 2.6 (90.0-94.4)
DGO	15.1 ± 1.6 (13.0-18.0)	17.2 ± 0.8 (16.0-18.0)	15.4 ± 1.6 (14.0-18.0)	17.7 ± 0.6 (17.0-18.0)
V or T	64.9 ± 1.6 (62.0-67.0)	25.0 ± 2.7 (21.7-28.0)	65.4 ± 1.3 (63.0-67.0)	25.2 ± 2.1 (23.8-27.6)
G_1	10.5 ± 2.1 (7.8-12.6)	–	10.1 ± 2.2 (7.8-12.6)	–
G_2	10.0 ± 1.8 (8.0-12.0)	–	9.5 ± 1.6 (8.0-11.6)	–
Stylet length	22.2 ± 1.2 (21.0-24.0)	18.8 ± 1.3 (17.0-20.0)	22.1 ± 1.1 (21.0-24.0)	19.3 ± 1.2 (18.0-20.0)
Stylet knob width	3.7 ± 0.4 (3.0-4.0)	3.5 ± 0.5 (3.0-4.0)	3.6 ± 0.4 (3.0-4.0)	3.8 ± 0.3 (3.5-4.0)
Pharynx length	131 ± 9.3 (116-142)	134 ± 10.9 (118-144)	130 ± 10.0 (116-142)	136 ± 7.2 (128-141)
Excretory pore from anterior	91 ± 6.6 (81-102)	92 ± 7.1 (80-98)	90 ± 6.6 (81-100)	94 ± 2.5 (91-96)
Diam. at mid-body	17.4 ± 0.9 (16.0-18.0)	16.0 ± 1.0 (15.0-17.0)	17.3 ± 1.0 (16.0-18.0)	16.0 ± 1.0 (15.0-17.0)
Diam. at anus	10.1 ± 0.8 (9.0-11.0)	10.4 ± 0.5 (10.0-11.0)	10.0 ± 0.8 (9.0-11.0)	10.3 ± 0.6 (10.0-11.0)
Lip region diam.	6.7 ± 0.7 (6.0-7.5)	6.7 ± 0.7 (6.0-7.5)	6.6 ± 0.7 (6.0-7.5)	6.7 ± 0.6 (6.0-7.0)
Annulus width	1.0	1.0	1.0	1.0
Lateral field width	3.6 ± 0.6 (3.0-4.5)	3.5 ± 0.6 (3.0-4.0)	3.6 ± 0.6 (3.0-4.5)	4.2 ± 0.3 (4.0-4.5)
Tail length	22 ± 1.1 (20-24)	21 ± 0.9 (20-22)	22 ± 1.3 (20-24)	22 ± 0.6 (21-22)
h	10.3 ± 1.5 (8.0-12.0)	10.6 ± 0.5 (10.0-11.0)	9.8 ± 1.5 (8.0-12.0)	10.7 ± 0.6 (10.0-11.0)
Spicule length	–	20.0 ± 1.0 (19.0-21.0)	–	20.7 ± 0.6 (20.0-21.0)
Gubernaculum length	–	9.4 ± 0.9 (8.0-10.0)	–	9.7 ± 0.6 (9.0-10.0)

confirms the common occurrence of this species on fruit trees in southern Italy.

The stylet of *R. macrodoratus* immature females is longer than 16 μm like that of *R. clavicaudatus*, *R. macrosoma* and *R. reniformis*. It differs from *R. clavicaudatus* and *R. macrosoma* in the more posterior position of the vulva at 62-72 *vs* 55-66%. It differs from *R. reniformis* in the longer stylet of 22-26 *vs* 16-21 μm.

Rotylenchulus macrosoma Dasgupta, Raski & Sher, 1968
(Fig. 8)

This species was originally described from specimens collected from olive trees in Israel in 1968 and studied in that country by Cohn & Mordechai (1988). Subsequently, it was found in Syria from an unidentified host (Sikora & Greco, 1990) and in Spain on cultivated and wild olive trees. The response of these hosts to *R. macrosoma* has been studied by Castillo *et al.* (2003b). Two new Spanish populations of this species from olive trees were used in this study.

Measurements

See Tables 3, 4, 8.

Description

Immature female

Body shape usually in closed C-shape when heat-relaxed. Lip region conoid-rounded not set off, finely annulated. Labial framework well developed, extending two or three annuli posterior from basal annulus. Stylet long and well developed with metenchium usually slightly shorter than telenchium. Stylet knobs rounded, sloping posteriorly. Dorsal pharyngeal gland opening situated *ca* 1.5 stylet lengths posterior to base of stylet. Median bulb rounded-oval, large, with prominent valves. Excretory

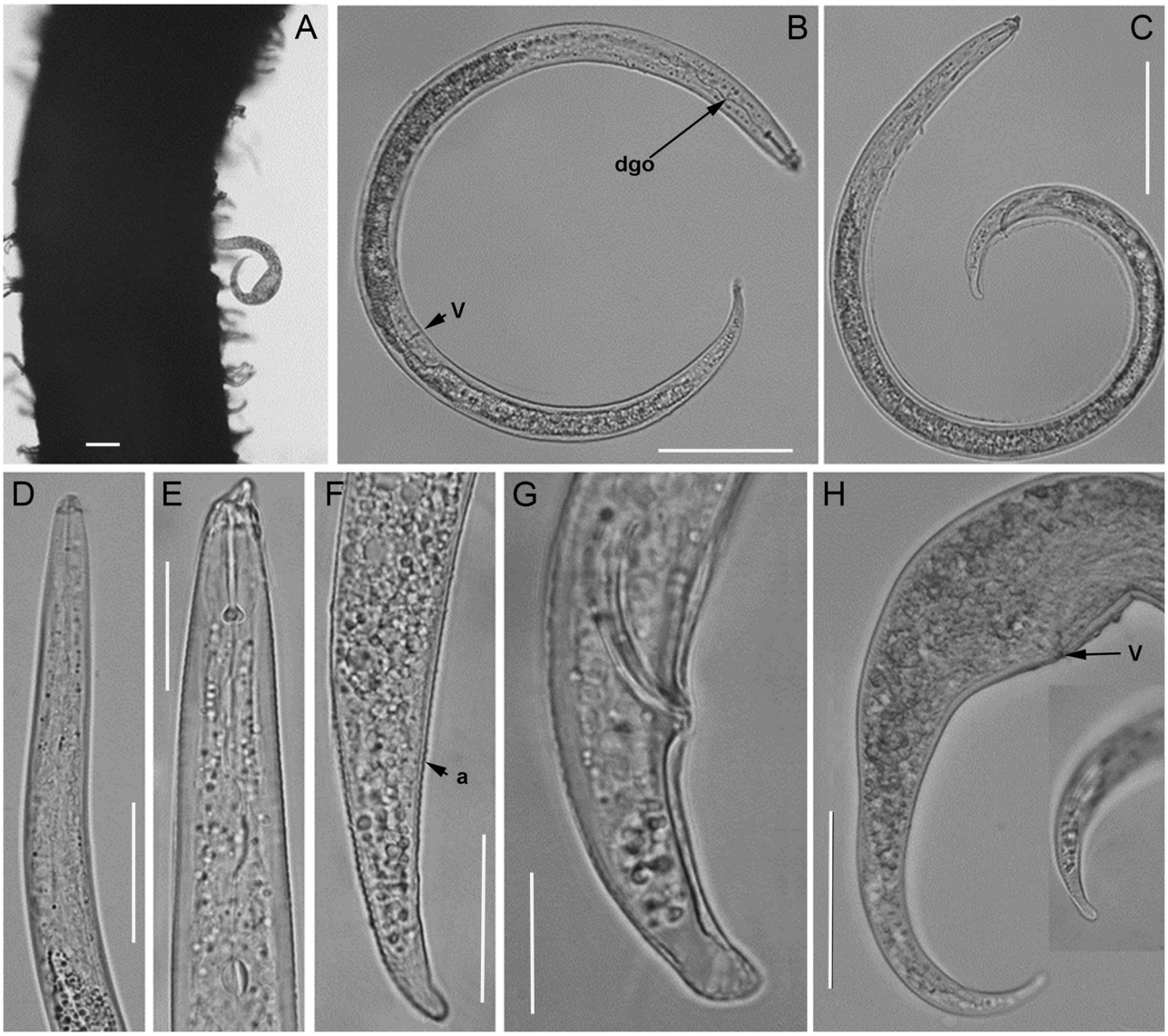


Fig. 8. *Rotylenchulus macrosoma*. A: Olive root parasitised by a swollen female; B, C: Entire immature female and male, respectively; D, E: Immature female anterior region; F: Immature female tail region; G: Male tail region; H: Mature female tail region, inset = tail tip. Abbreviations: a = anus; dgo = dorsal gland orifice; V = vulva. (Scale bars: A-D = 50 μm; E, F = 20 μm; G = 10 μm; H = 100 μm.)

pore situated from opposite middle of isthmus to opposite anterior part of pharyngeal lobe. Hemizonid very indistinct, two or three annuli long, situated from opposite to two annuli anterior to excretory pore. Hemizonion not seen. Pharyngeal glands overlapping intestine laterally and mostly ventrally. Annulation distinct over entire body, *ca* 1 μm wide. Ovaries outstretched with reflexed tips. Lateral field distinct with four lines and three equal bands. Tail with bluntly rounded terminus, annulation around terminus prominent. Hyaline portion *ca* one-third of tail length.

Male

Similar to immature female except for genital system and a more curved posterior part of body. Stylet and pharynx reduced. Median pharyngeal bulb and valves less

Table 8. Morphometrics of *Rotylenchulus macrosoma* from cultivated olive in Spain and *P. parvus* from South Africa. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*R. macrosoma*				*R. parvus*
	Olive, Jerez de la Frontera, Cádiz Province, Spain		Olive, Huévar del Aljarafe, Sevilla Province, Spain		Winterton, KwaZulu-Natal Province, South Africa
	Immature female	Male	Immature female	Male	Mature female
n	6	3	9	5	11
L	476 ± 25.8 (438-502)	475 ± 27.6 (446-501)	484 ± 30.4 (432-520)	480 ± 31.3 (432-514)	275 ± 95.5 (246-361)
a	30.1 ± 1.2 (28.7-31.4)	31.7 ± 0.3 (31.3-31.9)	29.9 ± 1.5 (27.6-32.1)	30.1 ± 1.6 (28.1-31.9)	4.7 ± 0.6 (3.9-5.7)
b	3.7 ± 0.3 (3.3-4.3)	3.4 ± 0.3 (3.1-3.6)	3.7 ± 0.3 (3.3-4.3)	3.6 ± 0.4 (3.0-4.1)	3.4 (n = 1)
c	15.6 ± 1.3 (13.8-17.6)	15.2 ± 0.3 (14.9-15.4)	15.5 ± 1.3 (13.8-17.6)	14.8 ± 0.7 (13.6-15.4)	19.3 ± 2.8 (15.7-23.4)
c′	3.3 ± 0.4 (2.8-4.0)	3.1 ± 0.2 (3.0-3.3)	3.2 ± 0.4 (2.6-4.0)	3.0 ± 0.1 (2.8-3.1)	1.3 ± 0.9 (0.9-1.7)
o	136 ± 17.4 (116-156)	147 ± 8.5 (137-154)	139 ± 10.1 (126-156)	135 ± 4.0 (129-140)	–
DGO	24.0 ± 1.4 (22.0-26.0)	21.0 ± 1.0 (20.0-22.0)	23.8 ± 1.4 (22.0-26.0)	21.6 ± 1.1 (20.0-23.0)	–
V or T	61.5 ± 1.9 (59.0-64.0)	25.0 ± 4.1 (20.9-29.1)	62.4 ± 2.3 (59.0-66.0)	27.0 ± 3.9 (21.5-31.7)	62.0 ± 4.3 (56.0-68.0)
G_1	8.9 ± 1.8 (7.7-11.0)	–	9.3 ± 1.4 (7.7-11.1)	–	–
G_2	8.5 ± 1.0 (7.9-9.6)	–	8.9 ± 0.9 (7.9-9.7)	–	–
Stylet length	17.8 ± 1.7 (16.0-20.0)	15.0 ± 1.0 (14.0-16.0)	17.1 ± 1.1 (16.0-19.0)	16.0 ± 1.0 (15.0-17.0)	13.5 (n = 1)
Metenchium length	–	–	–	–	6 (n = 1)
Telenchium length	–	–	–	–	7.5 (n = 1)
m	–	–	–	–	44.4
Stylet knob height	–	–	–	–	3 (n = 1)
Stylet knob width	3.6 ± 0.4 (3.0-4.0)	3.5 ± 0.5 (3-4)	3.6 ± 0.4 (3.0-4.0)	3.7 ± 0.4 (3.0-4.0)	1.5 (n = 1)
Pharynx length	128 ± 9.9 (114-138)	139 ± 6.1 (132-144)	131 ± 9.3 (116-142)	135 ± 12.7 (117-148)	90 (n = 1)
Excretory pore from anterior	90 ± 3.9 (84-96)	89 ± 5.7 (83-94)	90 ± 5.6 (81-98)	90 ± 7.1 (80-96)	60 ± 6 (55-72)
Median bulb length	–	–	–	–	13 ± 1.3 (12-15.5)
Median bulb diam.	–	–	–	–	10.5 ± 1.3 (9-12.5)
Diam. at mid-body	15.8 ± 0.8 (15.0-17.0)	15.0 ± 1.0 (14.0-16.0)	16.2 ± 1.0 (15.0-18.0)	16.0 ± 1.0 (15.0-17.0)	65 ± 5.7 (59-74)
Valve length	–	–	–	–	3.5 ± 0.4 (3-4)
Valve width	–	–	–	–	2.5 ± 0.3 (2-3)
Diam. at anus	9.5 ± 1.4 (8.0-13.0)	10.0 ± 1.0 (9.0-11.0)	10.1 ± 1.8 (8.0-13.0)	11.0 ± 1.0 (10.0-12.0)	14.0 ± 2.6 (12.0-18.5)
Lip region diam.	6.6 ± 0.7 (6.0-7.0)	6.3 ± 0.6 (6.0-7.0)	6.6 ± 0.6 (6.0-7.5)	6.5 ± 0.6 (6.0-7.0)	–
Cuticle width	–	–	–	–	3.5 ± 1.2 (2.5-6.5)
Annulus width	1.1 ± 0.2 (1.0-1.5)	1.1 ± 0.2 (1.0-1.5)	1.1 ± 0.2 (1.0-1.5)	1.1 ± 0.2 (1.0-1.5)	–
Lateral field width	3.5 ± 0.8 (3.0-3.5)	3.5 ± 0.7 (3.0-4.5)	3.5 ± 0.8 (3.0-5)	3.7 ± 0.6 (3.0-4.0)	–
Tail length	31 ± 3.5 (27-36)	31 ± 1.5 (30-33)	31 ± 3.5 (27-37)	32 ± 2.4 (29-35)	16 (n = 1)
h	10.5 ± 1.4 (9.0-12.0)	10.0 ± 1.0 (9.0-11.0)	10.6 ± 1.6 (9.0-13.0)	10.6 ± 0.5 (10.0-11.0)	–
Spicule length	–	21.3 ± 1.5 (20.0-23.0)	–	22.6 ± 1.5 (21.0-24.0)	–
Gubernaculum length	–	9.0 ± 1.0 (8.0-10.0)	–	10.0 ± 1.0 (9.0-11.0)	–

developed. Tail broadly rounded with rounded tip, in few specimens wider than that of female. Gubernaculum and spicules well developed, ventrally arcuate.

REMARKS

Since the morphology of the two new Spanish populations of *R. macrosoma* from cultivated olive was almost identical to that described for this species in the original description, as well as for the Spanish populations, no morphological drawings of these new populations are provided. The detection of the two populations from cultivated olive in Cadiz and Seville provinces constitutes new records of this species for cultivated olive in Spain. Minor morphometric differences of the two populations from those of the previous description reported in wild olive in Spain include a smaller c′ ratio (2.8-4.0 and 2.6-4.0 *vs* 2.8-4.4), slightly longer stylet (16-20 and 16-19 *vs* 15-18 μm), shorter tail (27-36 and 27-37 *vs* 26-40 μm), and shorter spicules (20-23 and 21-24 *vs* 19-25 μm). These differences may be a result of geographical intraspecific variability. However, since no topotype material of this species was included in this study, molecular differences between these two new populations and that from the type locality in Israel cannot be excluded.

Rotylenchulus parvus **(Williams, 1960) Sher, 1961**
(Figs 9, 10)

As part of the project "*The role and importance of soil-borne diseases and microbial diversity on maize production as well as interactive effects of crop rotation and biocides*", numerous specimens of mature females, immature females and juveniles were collected from maize on a farm west of Winterton, KwaZulu-Natal Province, South Africa, on 11 December 2006 by M. Marais and A. Swart. Much information has been given by various authors on the immature females and juveniles (Dasgupta *et al.*, 1968; Heyns, 1976; Germani, 1978; Van den Berg, 1978; Lehman & Inserra, 1990). We therefore give morphological information on the mature females which were analysed molecularly for the first time.

MEASUREMENTS

See Table 8.

DESCRIPTION

Mature female

Body obese and twisted into various shapes. Lip region form and stylet very seldom seen due to lip region being embedded into plant tissue and thus fixed for mounting. Body annuli seen in some specimens on anterior part of body ranging from 1 to 5 μm in length. Lateral field not seen. Cuticle distinct and fairly broad over entire body. Pharyngeal region mostly distorted or not seen. Where seen, median bulb well developed with a distinct valve. Excretory pore in vicinity of isthmus or basal lobe. Body widest at vulva with vulva situated on a prominent rounded bulge. Vagina conspicuous and large, often heart-shaped. Two ovaries profusely reflexed and convoluted with many females containing one or two large eggs. Tail tapering gradually to a rounded tip without any projections. Anus visible in most specimens.

Immature female

Not found.

Male

Not found.

REMARKS

Rotylenchulus parvus mature females have a stylet shorter than 16 μm like the immature females. Other *Rotylenchulus* species having the stylet shorter than 16 μm are *R. anamictus*, *R. borealis* and *R. leptus*. This species differs from *R. anamictus* and *R. borealis* in the usual absence of males, which have been found only rarely. Since no molecular data are available for the topotype specimens of this species, we cannot exclude molecular differences between this *R. parvus* population from South Africa and that from the type locality in Mauritius.

Rotylenchulus sacchari **Van den Berg & Spaull, 1981**
(Figs 11-13)

This species was described from nine immature females and three males collected from sugarcane near Heatonville, KwaZulu-Natal Province, South Africa, by Van den Berg & Spaull (1981). Recently numerous immature females, males and a few juveniles were collected from a maize field next to the Vaal River on a farm in the

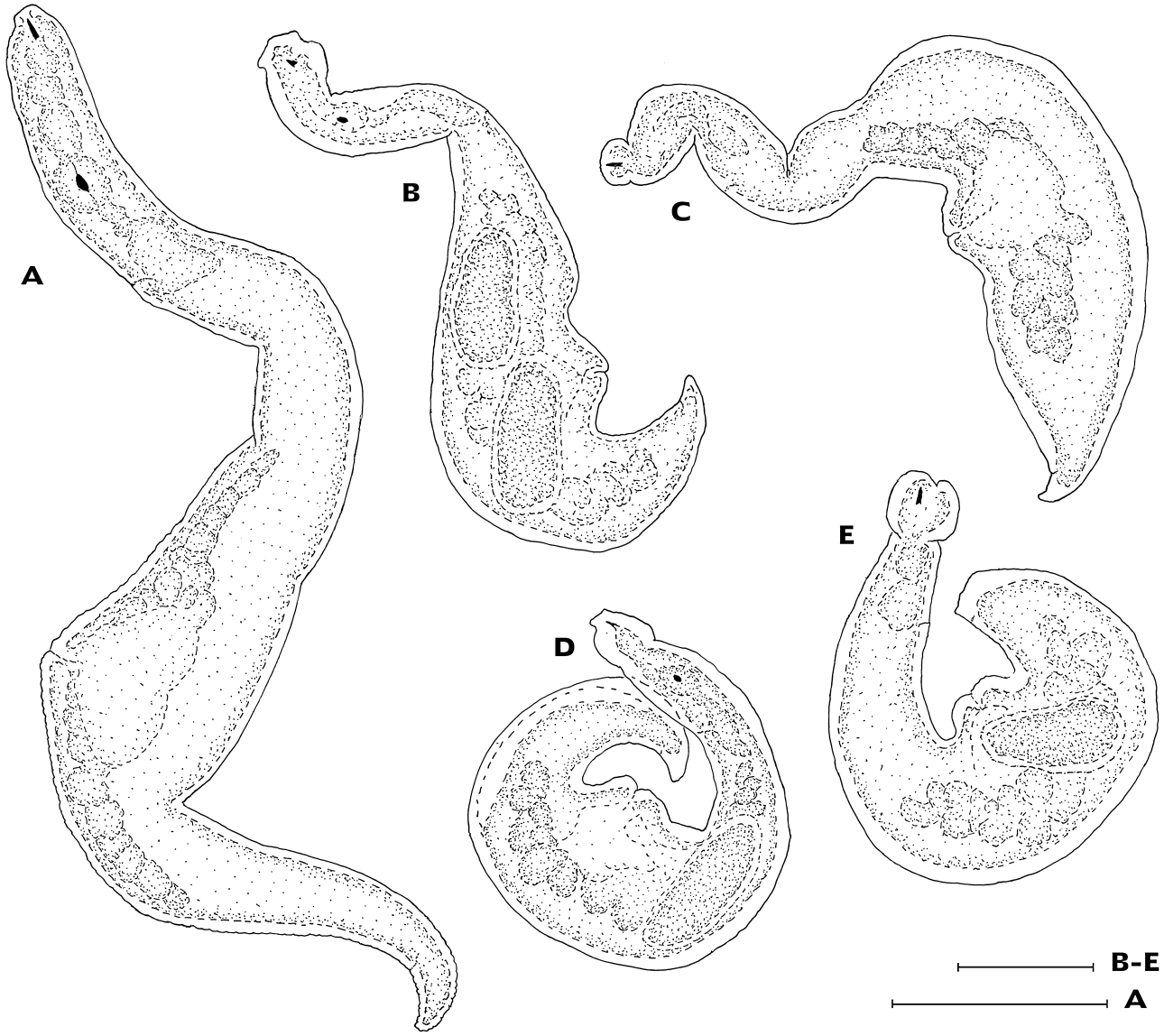


Fig. 9. *Rotylenchulus parvus*. Mature female. A-E: Various body postures. (Scale bar: A = 5 μm; B-E = 30 μm.)

Northern Cape Province on 31 January 2014, very distant from the original locality and from a very different biome. Previously, three collections of the same nematode have been done in the Northern Cape area but not enough material was obtained for the study. The present specimens were studied morphologically and, for the first time, molecularly.

Measurements

See Table 9.

Description

Immature female

Body posture ranging from almost straight to an open letter C to, rarely, 1.5 circles, but mostly in the form of an open letter C. Lip region not set off, sloping slightly to a flattened tip with five or six faint annuli. SEM photographs showing five to be present. Labial framework well developed, stretching two or three annuli posterior from basal plate. Anterior and posterior cephalids rarely seen, situated 2-5 annuli and 11-13 annuli posterior from

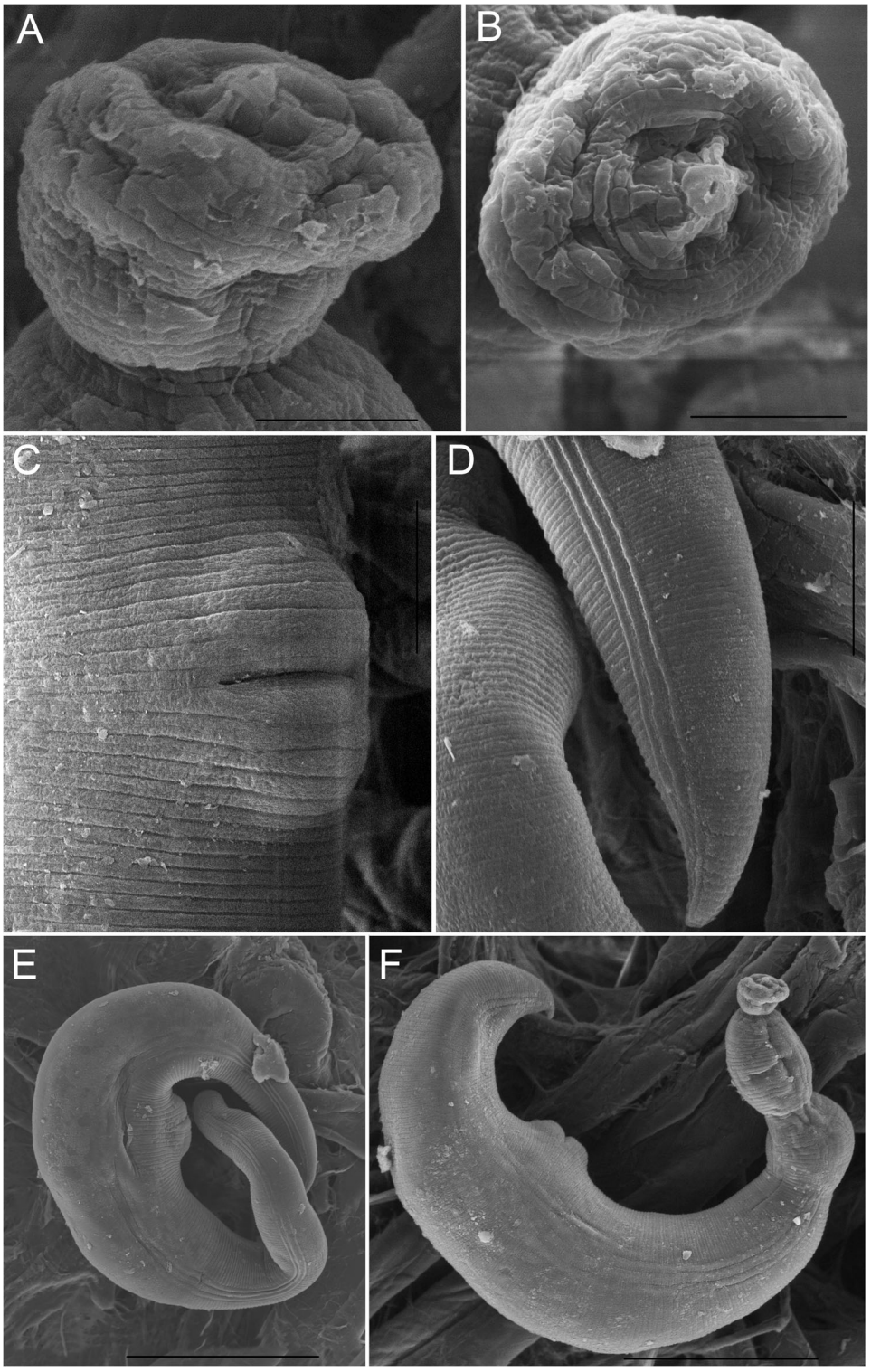


Fig. 10. *Rotylenchulus parvus*. Mature female. A, B: Lip region lateral and *en face*; C: Vulval bulge; D: Tail region; E, F: Different body postures. (Scale bar: A, B = 5 μm; C, D = 10 μm; E, F = 50 μm.)

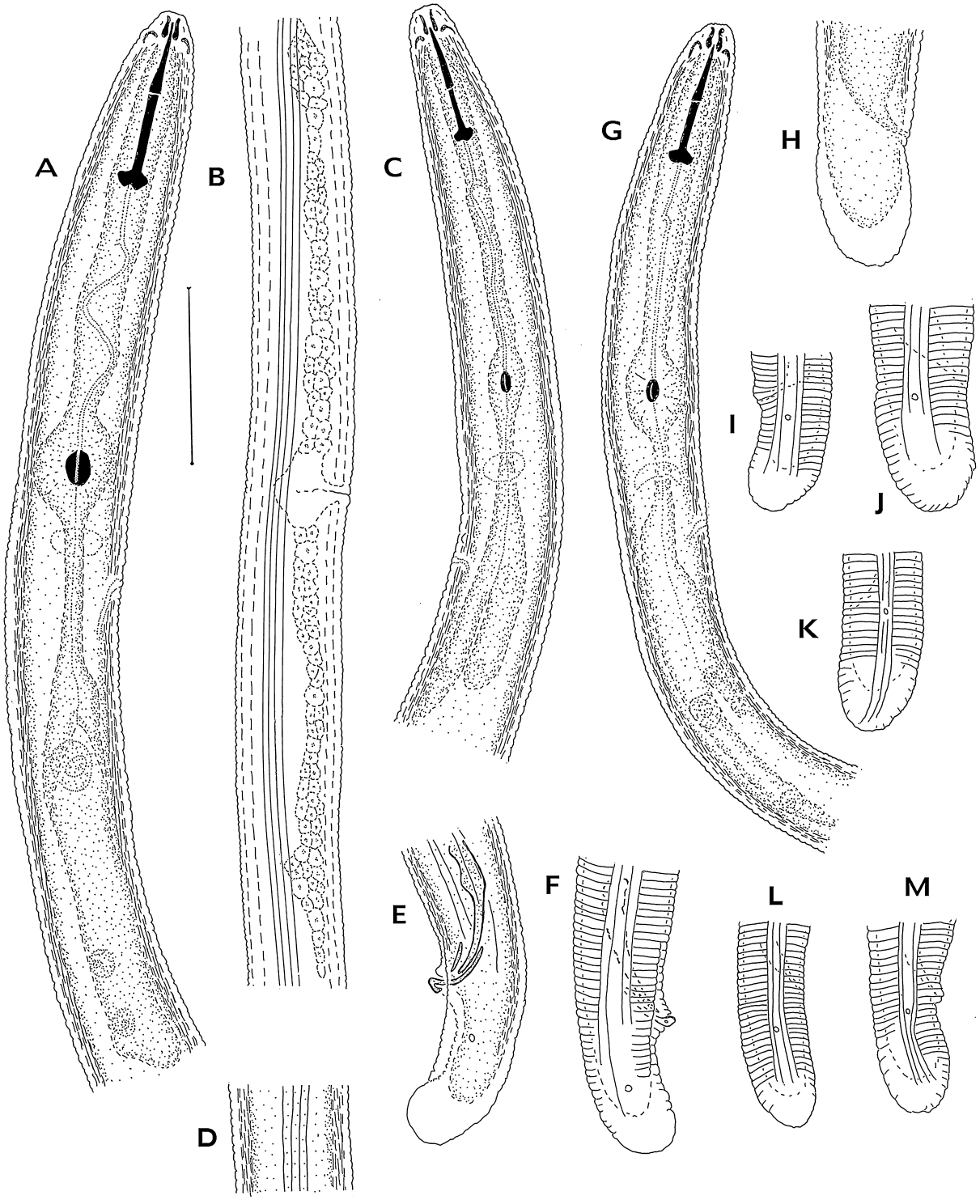


Fig. 11. *Rotylenchulus sacchari*. A, B, H-K, immature female. A: Anterior part of body; B: Vulval area with reflexed ovaries; H-K: Tail regions. C-F, male. C: Anterior part of body; D: Lateral field at mid-body; E, F: Tail region. G, L, M, juvenile J4. G: Anterior region; L, M: Tail region. (Scale bar = 30 μm.)

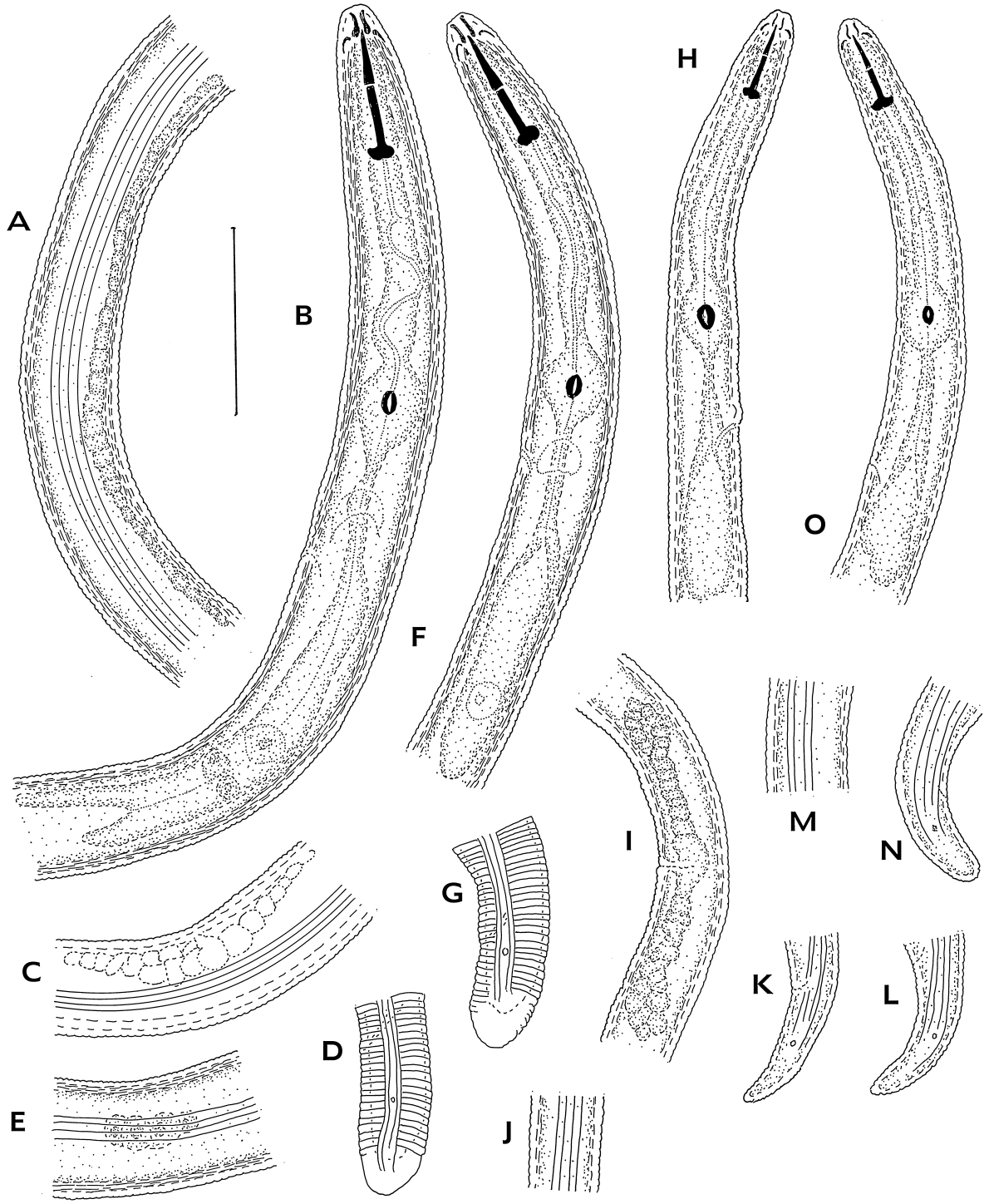


Fig. 12. *Rotylenchulus sacchari*. A-D, juvenile (?J2). A, C: Ovarial primordial area; B: Anterior part of body; D: Tail. E-G, juvenile J2. E: Ovarial primordial area; F: Anterior part of body; G: Tail. *Rotylenchulus leptus*. H-L, immature female. H: Anterior part of body; I: Vulval region; J: Lateral field at mid-body; K, L: Tails. M-O, juvenile. M: Lateral field at mid-body; N: Tail; O: Anterior region. (Scale bar = 30 μm.)

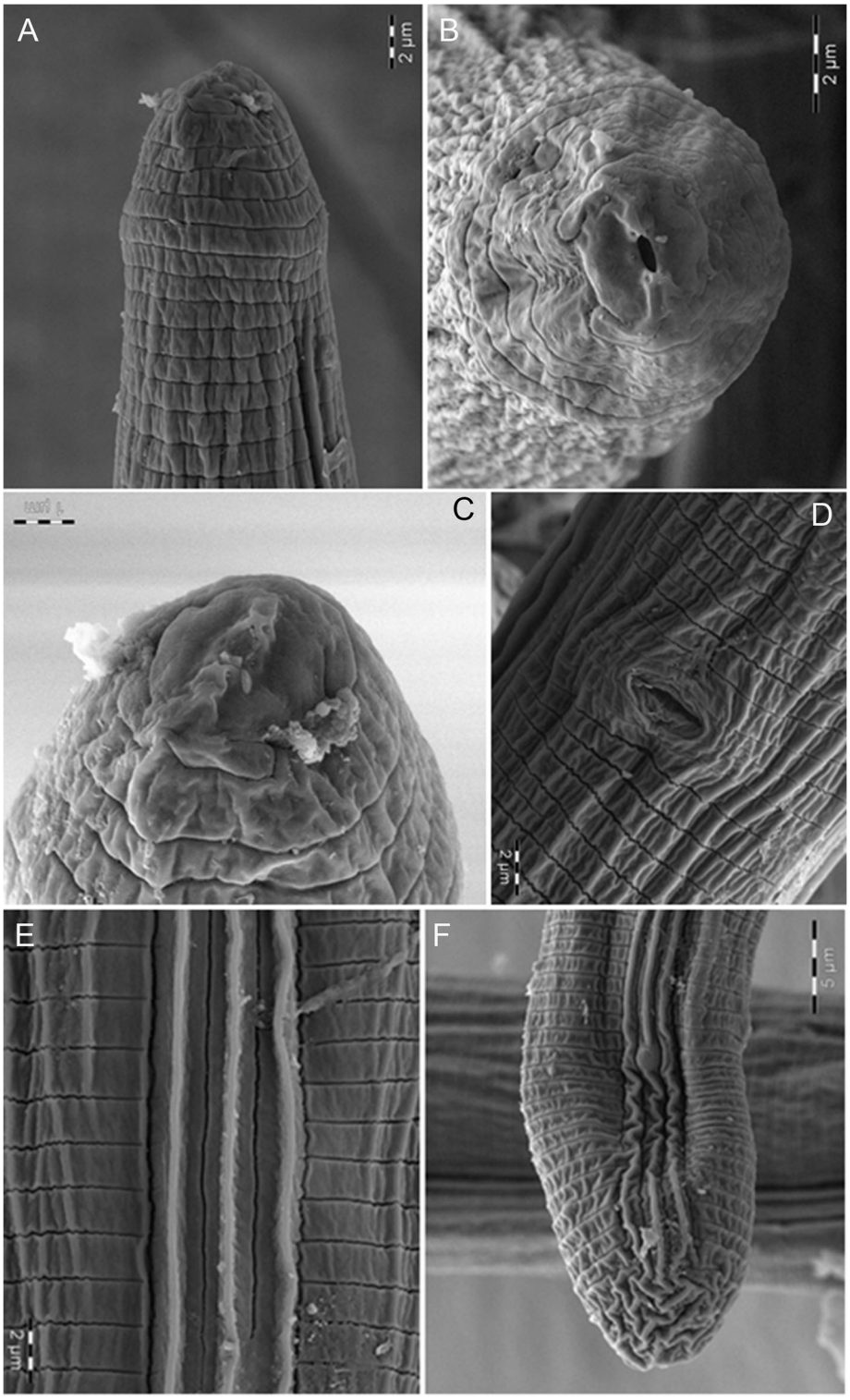


Fig. 13. *Rotylenchulus sacchari*. Immature female. A: lateral view of lip region; B, C: Two *en face* views; D: Vulval area; E: Lateral field at mid-body; F: Tail.

Table 9. Morphometrics of *Rotylenchulus sacchari* from South Africa. All measurements are in μm and in the form: mean ± s.d. (range).

Character	Northern Cape					Natal, Van den Berg & Spaull (1981)	
	Immature female	Male	Juvenile			Immature female	Male
			J2	J3	J4		
n	25	24	1	1	2	9	3
L	704 ± 50 (574-796)	816 ± 47.3 (712-875)	546	566	622, 765	733 (644-906)	908 (805-1015)
a	34.1 ± 2.4 (28.8-36.9)	37.7 ± 2.7 (33.3-42.8)	29.7	33.5	32.5, 39.3	34.9 (32.2-38.5)	38.9 (37.8-39.6)
b	3.9 ± 0.4 (3.1-5.0)	5.7 ± 0.5 (4.9-6.6)	3.3	4.6	5.3	3.5 (3.1-4.5)	7.5 (7.0-8.1)
c	32.2 ± 4.1 (26.1-43.0)	30 ± 3.6 (23.8-37.0)	32.3	22	36.4 (n = 1)	31.9 (28.2-35.5)	31.2 (28.6-34.5)
c′	1.4 ± 0.2 (1.1-1.7)	1.9 ± 0.3 (1.5-2.4)	1.4	2.1	1.9 (n = 1)	1.5 (1.3-1.9)	1.7 (1.6-1.9)
o	37.2. ± 6.0 (28.5-47.5)	45.7 ± 5.2 (34.5-55.6)	37.2	45.2	36.4, 37.1	42.2 (38.2-46.5)	53.4 (51.4-56.2)
DGO	11.0 ± 2 (8.0-15.5)	9.5 ± 0.9 (7.0-11.0)	8.1	10.5	9, 10.5	13.1 (12.0-15.0)	12.4 (11.5-13.0)
V or T	65.0 ± 1.9 (61.5-70.5)	43.8 ± 9.6 (27.1-57.6)	–	17	45, 72	70 (66-73)	–
G_1	12.7 ± 1.6 (10.5-15)	–	–	–	–	6 (3-12)	–
G_2	13.8 ± 1.6 (11.5-17.5)	–	–	–	–	6 (4-9)	–
OV1 length	88 ± 9.7 (75-111)	–	–	–	–	–	–
OV2 length	93 ± 7.9 (78-107)	–	–	–	–	–	–
Stylet length	30.5 ± 1.9 (26.5-34.5)	21.5 ± 1.1 (20-24)	–	23	24.5, 25.5	31.1 (29.1-32.7)	23.2 (21.7-24.4)
Metenchium length	14.5 ± 1.2 (11.5-16.0)	10.5 ± 0.8 (9.5-13.0)	–	10.5	11.5, 12.5	–	–
Telenchium length	16.5 ± 1 (14.5-18.5)	11 ± 0.8 (9.5-12.5)	–	12.5	13.0	–	–
m	46.3 ± 2.3 (42.0-51.0)	50.0 ± 2.4 (44.0-54.5)	42.4	45.2	47.0, 49.0	44-48	49-53
Stylet knob width	5.0 ± 0.3 (4.5-5.5)	3.0 ± 0.4 (2.0-3.5)	1.5	3.5	3.5, 4.5	5 (4.4-5.5)	3.2 (2.6-3.7)
Stylet knob height	3.0 ± 0.5 (2.0-3.5)	1.5 ± 0.3 (1.0-2.0)	3.3	1.5	2	2.7 (2.5-3.0)	1.5 (1.1-1.8)
Pharynx length	182 ± 18 (132-206)	144 ± 13.2 (121-167)	164.7	123.5	144 (n = 1)	209 (181-236)	157 (143-167)
Excretory pore from anterior	108 ± 8 (95-140)	104 ± 3.8 (96-110)	92	94	93.5, 100	113 (107-123)	110 (106-116)
Diam. at mid-body	21.0 ± 1.4 (18.5-24.5)	21.5 ± 1.3 (20.0-25.0)	18.5	17	15.5, 23.5	20.9 (18.5-23.5)	–
Diam. at anus	15.5 ± 1 (14-17.5)	14.5 ± 0.9 (13-16)	12	12.5	10.5	–	–
Median bulb length	16.5 ± 0.9 (14.5-17.5)	12.5 ± 0.5 (11.0-14.0)	14	14	10, 14	16 (13.6-17.7)	–
Median bulb diam.	13.0 ± 0.8 (11.0-14.5)	7.5 ± 0.5 (6.5-8.5)	10.5	10.5	7, 12	12.8 (12.0-14.0)	–
Valve length	5.5 ± 0.5 (4.5-6)	3.5 ± 0.5 (3-4.5)	3.5	3	3.0, 3.5	–	–
Valve width	4.0 ± 0.6 (3.0-5.5)	2.0 ± 0.4 (1.5-3.5)	3.0	2.0	2.5	–	–
Lip region diam.	9.5 ± 0.6 (8.0-10.5)	8.5 ± 0.4 (7.5-9.0)	8.0	8.0	8.0	8.1 (7.5-9.0)	–
Lip region height	6.0 ± 0.4 (5.0-6.5)	5.5 ± 0.4 (5.0-6.0)	5.0	4.5	6.5	5.4 (5.0-6.0)	–
Annulus width	1.5	1.5 ± 0.2 (1.5-2.0)	–	0.75	1	1.3 (1.0-1.5)	–
Lateral field width	5.5 ± 0.7 (4.5-6.5)	5.5 ± 0.7 (4.5-6.0)	–	3.5	4.5	5.6 (4.5-6.0)	7.1 (6.0-8.0)
Tail length	22.0 ± 2.8 (15.5-28.0)	28.0 ± 3.2 (23.0-34.0)	17.0	25.5	19.0	23.2 (18.5-30.0)	29.2 (26.5-31.5)
h	7.5 ± 1.6 (4.5-10.5)	9.0 ± 2 (6-15.5)	5.0	10.5	6.5	8.6 (7.0-12.0)	9.8 (9.0-11.0)
Testis length	–	365 ± 85.9 (214-527)	–	–	–	–	–
Spicule length	–	28.5 ± 1.3 (25.5-31)	–	–	–	–	27.3 (25.0-30.5)
Gubernaculum length	–	13.0 ± 1.0 (11.0-15.5)	–	–	–	–	12.0 (11.0-12.5)

basal plate. Stylet long and well developed with metenchium mostly slightly shorter than telenchium. Stylet knobs varying from slightly sloping to slightly hollow anteriorly and rounded posteriorly. Dorsal pharyngeal gland opening situated *ca* 0.33-0.50 stylet lengths posterior to base of stylet. Median bulb large, prominent, slightly longer than wide with a prominent valve. Excretory pore situated from opposite middle of isthmus to opposite anterior part of pharyngeal lobe. Hemizonid very indistinct, two or three annuli long, situated from opposite, to three annuli anterior, to excretory pore. Hemizonion not seen. Annulation distinct over entire body. Ovaries outstretched with reflexed tips. Lateral field distinct with four lines and three equal bands ending various distances from tail tip in posterior half of tail. Phasmids distinct situated from opposite to seven annuli posterior to anus. Tail broadly rounded with 12-23 annuli, mostly not distinctly annulated on tip but in some specimens annuli appearing very faintly around tip. Hyaline portion comprising *ca* 0.25-0.50 of tail length.

Male

Similar to immature female. As with original description they have a longer body than immature females and considerably shorter stylet. Excretory pore situated mostly opposite anterior part of pharyngeal lobe. Hemizonid two or three annuli long, situated opposite, to three annuli anterior, to excretory pore. Phasmids distinct, situated 4-9 annuli posterior to anus. Tail broadly rounded with 13-20 ventral annuli and with a smoother tip than that of female.

Juvenile (J2, J3, J4)

All stages similar to immature female with same broadly rounded, smooth or slightly annulated tail terminus.

Mature female

Not found.

Remarks

The present specimens fit very well with those of the original description (Van den Berg & Spaull, 1981). Among the reniform nematodes, *R. sacchari* has the longest stylet (>26.5 μm). This study has confirmed this peculiarity in this species. Since no molecular data are available for topotype specimens of this species, we cannot exclude molecular differences between this *R. sacchari* population and that from the type locality.

Sequence and phylogenetic analysis

D2-D3 of 28S rRNA

Twenty-nine new *Rotylenchulus* sequences of the D2-D3 of 28S rRNA gene were obtained in the present study. In total, 121 sequences of the D2-D3 of 28S rRNA gene from *Rotylenchulus* species were initially included in the dataset and then were collapsed into 97 unique sequences. Thus, the D2-D3 of 28S rRNA gene sequence alignment contained 95 sequences of *Rotylenchulus* species and two outgroup taxa and was 759 bp in length. Phylogenetic relationships within *Rotylenchulus*, as inferred from Bayesian analysis, are presented in Figure 14. Sequences of *R. reniformis* were distributed into two distinct clades, which are named as type A (samples from China, USA and Japan) and type B (samples from China) in the tree. Only type A sequences were obtained from all USA *R. reniformis* samples subjected to direct sequencing of the PCR products in the present study. The type A of *R. reniformis* had a sister relationship with *R. macrosoma*, and the type B had a sister relationship with *R. macrosomoides* sp. n. *Rotylenchulus parvus* had a sister relationship with *R. clavicaudatus*. Relationships between other species were not well resolved. Sequence differences between *R. reniformis* type A and *R. reniformis* type B of the D2-D3 of 28S rRNA gene were 13.6-15.6% (98-116 bp), within type A 0.1-4.8% (1-40 bp) and within type B 0.1-4.6% (1-34 bp). GC (guanine-cytosine) content for type A was 54.7% and for type B was 58.8%. Putative secondary structures for the D2 expansion segment of 28S rRNA of type A (Fig. 15A) and type B (Fig. 15B) for *R. reniformis*, reconstructed using Mfold 3.0 software and comparative analysis, showed similar folding. Mapping of mutations occurred in both types revealing that they were mainly compensatory.

ITS1 of rRNA

Forty-one new *Rotylenchulus* sequences of the ITS1-5.8S-ITS2 rRNA gene were obtained in the present study. In total, 322 sequences of the ITS1 rRNA gene from *Rotylenchulus* species were initially included in the dataset and then collapsed into 229 unique ITS1 rRNA gene sequences. Two sequences of *Rotylenchus* species were used as outgroups. The ITS1 rRNA alignment was 884 bp in length. Phylogenetic tree as inferred from BI analysis of the ITS1 rRNA gene sequence alignment is given in Figure 16. *Rotylenchulus reniformis* and *R. macrosoma* sequences formed the major clade I. The

Fig. 14. Phylogenetic relationships between *Rotylenchulus* species. Bayesian 50% majority rule consensus tree as inferred from the analysis of the D2-D3 of 28S rRNA gene dataset under the GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are in bold font.

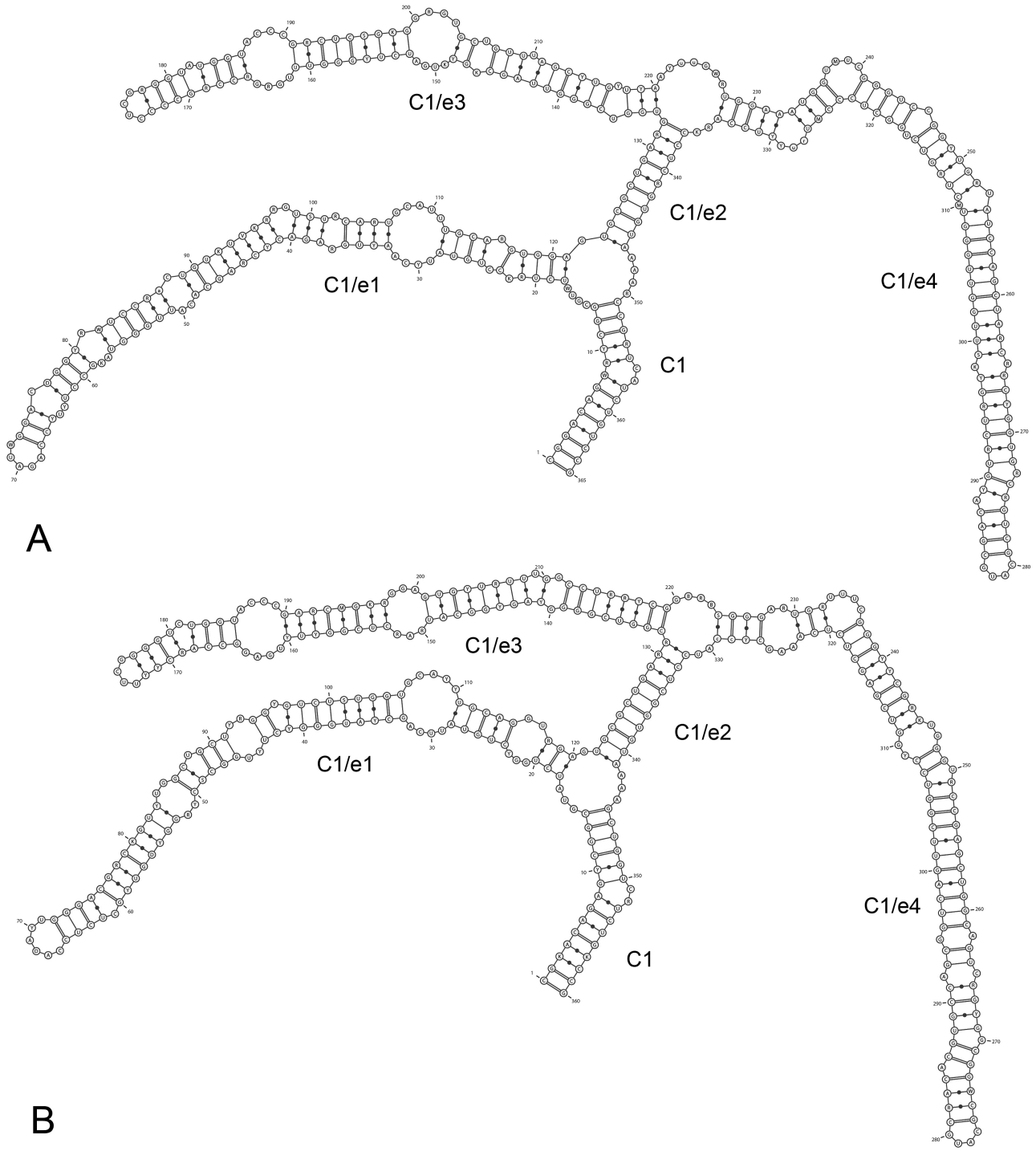
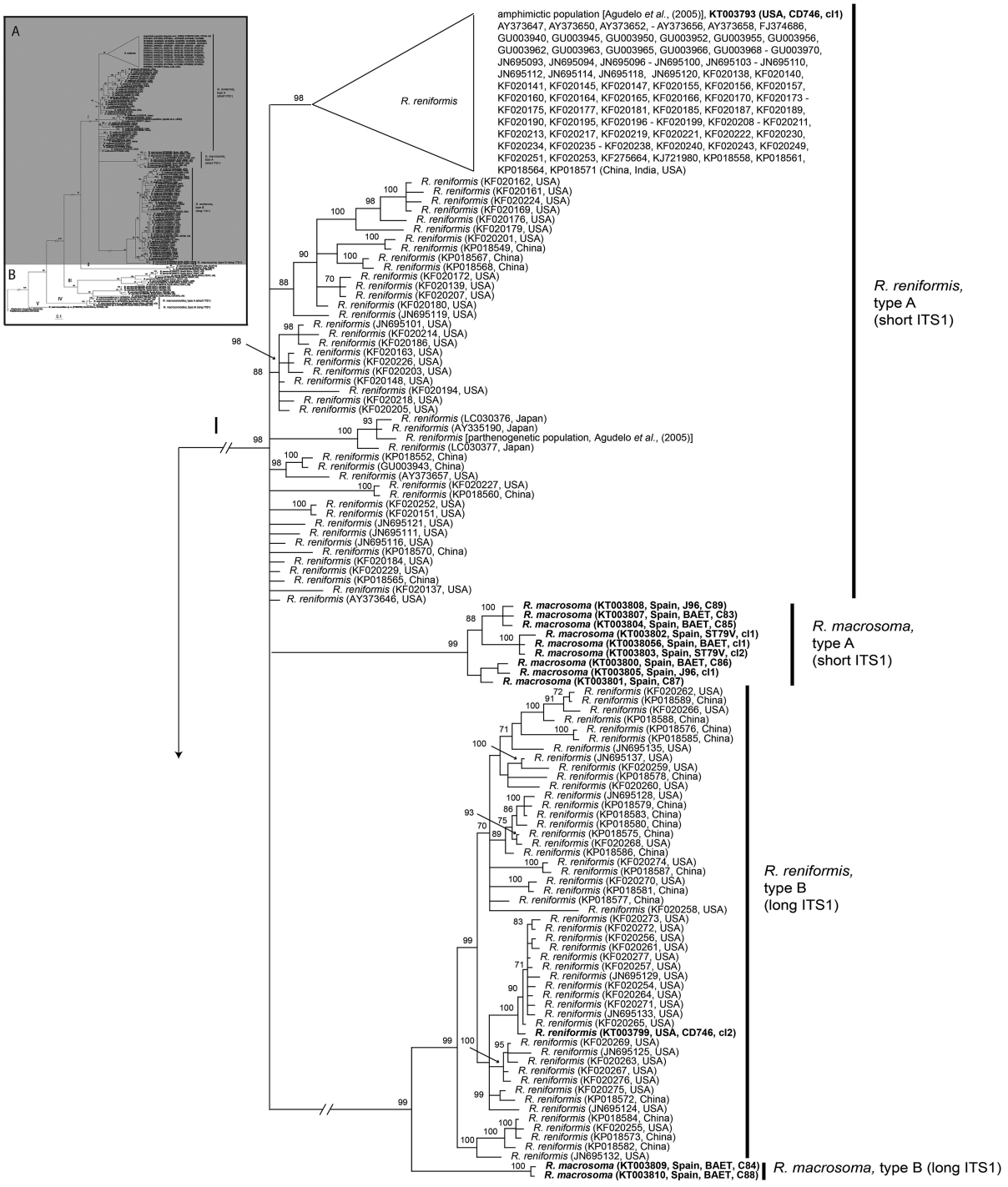
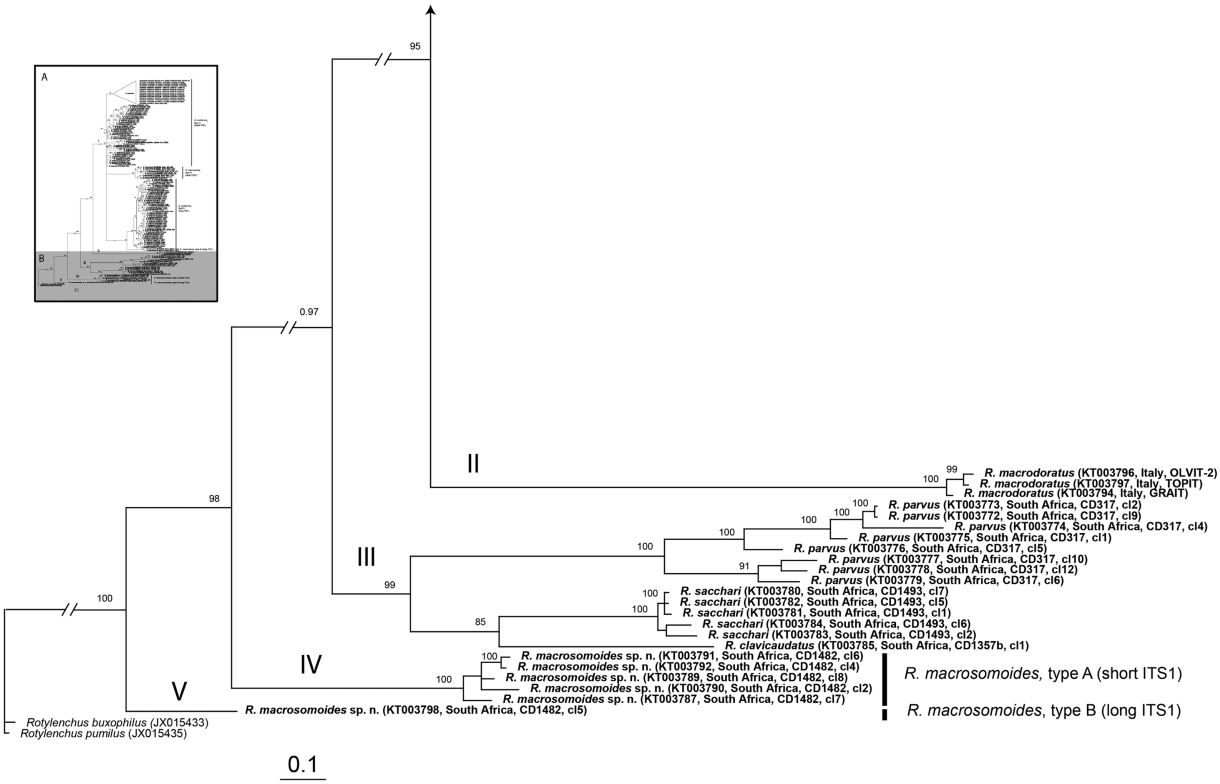


Fig. 15. Putative consensus secondary structures of the D2 expansion segment of 28S rRNA for *R. reniformis*. A: Type A; B: Type B.



(A)

Fig. 16. Phylogenetic relationships between *Rotylenchulus* species. Bayesian 50% majority rule consensus tree as inferred from the analysis of the ITS1 region dataset under the GTR + I + G model. The large clade of *R. reniformis* with unresolved relationships between sequences is indicated as a triangle. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are in bold font.



(B)

Fig. 16. (Continued.)

R. reniformis type A containing a short ITS1 (ITS1S) (323-336 bp), which included both amphimictic and parthenogenetic populations (Agudelo *et al.*, 2005), were distributed into several subclades. The *R. reniformis* type B containing a long ITS1 (ITS1L) (488-502 bp) clustered with the long ITS1 of *R. macrosoma* (type B). Sequence variation within *R. reniformis* type A reached 17.6% (62 bp), and within *R. reniformis* type B was up to 11.4% (60 bp), these types differing up to 24.5% (75 bp) from each other. *Rotylenchulus macrosoma* sequences were distributed among two groups within the major clade I: the type A (ITS1S), which varied from 301-307 bp, and the type B (ITS1L) which was 497 bp in a sequence length. Sequence differences between two groups reached 20.4% (71 bp).

Clade II included only sequences of *R. macrodoratus*, whereas clade III contained sequences of *R. parvus*, *R. sacchari* and *R. clavicaudatus*. The ITS1 sequence length for *R. macrodoratus* was 335-337 bp (intraspecific diversity 1.0%), for *R. parvus* 427-511 bp (up to 20%), for *R. sacchari* 517-518 bp (up to 4.1%) and for *R. clavicaudatus* 542 bp (0%). *Rotylenchulus macrosomoides* sp. n. was paraphyletic and occupied a basal position (clades III and IV). Sequences of clones obtained from one population of *R. macrosomoides* sp. n. were also distributed into two groups: the type A (ITS1S) length varied from 501-507 bp and the type B (ITS1L) was *ca* 660 bp. Sequence variation within the type A reached 5.4% (28 bp). Sequence differences between these two groups were 20.5-21.8% (104-110 bp).

5.8S AND ITS2 RRNA

In total, 130 sequences of the 5.8S and ITS2 rRNA gene from *Rotylenchulus* species were initially included in the dataset and then collapsed into 94 unique sequences. Two sequences of *Rotylenchus* species were used as outgroups. The alignment was 448 bp in a length. Phylogenetic relationships within *Rotylenchulus* is given in Figure 17. Sequences of *R. reniformis* were distributed among two clades I and II (type A and type B) and differed up to 22.9% (73 bp) from each other and within type A

0.2-7.7% (1-29 bp) and type B 0.2-8.4% (1-28 bp). Sequences of *R. macrosoma* were distributed among three groups (A, B, C) within two clades I and II. The *R. macrosoma* type A differed from 27.1-29.4% (89-92 bp) and from 27.0-27.9% (82-89 bp) from type B and type C, respectively, and type B differed up to 26.0% (84 bp) from type C. The *R. reniformis* type A and type B clustered with the corresponding types of *R. macrosoma*. The *R. macrosoma* type C formed a highly supported clade with *R. macrosomoides* type A.

The type A and type B ITS sequences of *R. macrosomoides* sp. n. were distributed into two distant clades (II and IV) and they differed up to 24.1% (74 bp) from each other.

Hsp90

Eight new *Rotylenchulus* sequences of the *hsp90* gene were obtained in the present study. The *hsp90* alignment contained eight sequences of *Rotylenchulus* and two sequences of *Rotylenchus* used as outgroups and was 217 bp (introns excluded) in length. Phylogenetic relationships within the genus as inferred from BI are presented in Figure 18. *Rotylenchulus reniformis* clustered with *R. macrosoma* with a high PP value, whereas relationships between other species were unresolved.

CoxI

The *coxI* mtDNA alignment contained 15 newly obtained sequences of *Rotylenchulus* and two sequences of *Rotylenchus* used as outgroups and was 393 bp in length. Intraspecific sequence variation for *R. reniformis* reached 0.8% (3 bp). Phylogenetic tree reconstructed using BI is presented in Figure 19. Phylogenetic relationships between species were not well resolved.

PCR with *Rotylenchulus reniformis* specific primers

Species-specific primers were developed for *R. reniformis* based on differences in the D2-D3 of 28S rRNA gene sequences (Table 2). Results of PCR with the species-specific primers are given in Figures 20 and 21. The combination of the universal primer D2A with the species-specific primer R_renif_R1A or R_renif_R2A yielded a single PCR product of *ca* 142 bp (Fig. 20A) or 320 bp (Fig. 20B), respectively, for all studied *R. reniformis* samples. These primers amplified fragments of the type A for D2-D3 of 28S rRNA gene.

The primer R_renif_R1B was designed to amplify the type B of D2-D3 of 28S rRNA gene. The multiplex PCR with the universal primer D2A and the species-specific primer R_renif_R1B and R_renif_R2A yielded two PCR products of 179 bp and 320 bp (Fig. 21) and, thus, the results showed that both rRNA gene types existed in extracted DNA of all studied *R. reniformis* populations.

Discussion

In this study we provide morphological and molecular characterisation of eight *Rotylenchulus* species. Molecular characterisation of five species, including *R. macrosomoides* sp. n., was made for the first time. The analysis of ribosomal and protein coding gene nucleotide sequences gave distinct support for the separation from all other studied *Rotylenchulus* spp. of a new species, *R. macrosomoides* sp. n. Phylogenetic analyses placed the ITS sequence of this species at basal positions in both ITS trees and this position is in agreement with the *hsp90* tree. The basal position of *R. macrosomoides* sp. n. within phylogenetic trees supports Siddiqi's opinion about the origin of *Rotylenchulus* in the Afrotropical (Ethiopian) zoogeographical region, comprising Africa south of the Sahara, the southern part of the Arabian Peninsula, and various islands, including Madagascar, where most *Rotylenchulus* speciation seems to have occurred (Siddiqi, 2000). Our study showed that phylogenetic relationships between other *Rotylenchulus* species is still not well resolved, except for a sister relationship of *R. reniformis* and *R. macrosoma*, which clustered together in trees inferred from analyses of *hsp90*, D2-D3 of 28S rRNA and ITS rRNA gene sequences. Dasgupta *et al.* (1968) placed these species in group III and the group IV, respectively, two of five morphological groups, which were erected for reniform nematodes based on the structure of the lip region in combination with tail characters of immature females. Both of these groups are characterised by high and conoid lip region with or without annulation. *Rotylenchulus reniformis* and *R. macrosoma* also share the same type of feeding cell induction in host plant roots – a syncytium (Castillo *et al.*, 2003b) – in contrast with *R. macrodoratus* which induces a uninucleate giant cell (Vovlas & Inserra, 1976; Cohn & Mordechai, 1977).

The presence of two distinct classes of small subunit rRNA gene in a single genome has been reported in organisms belonging to all three domains of life. For example, the genomes of the thermophilic actinomycete *Ther-*

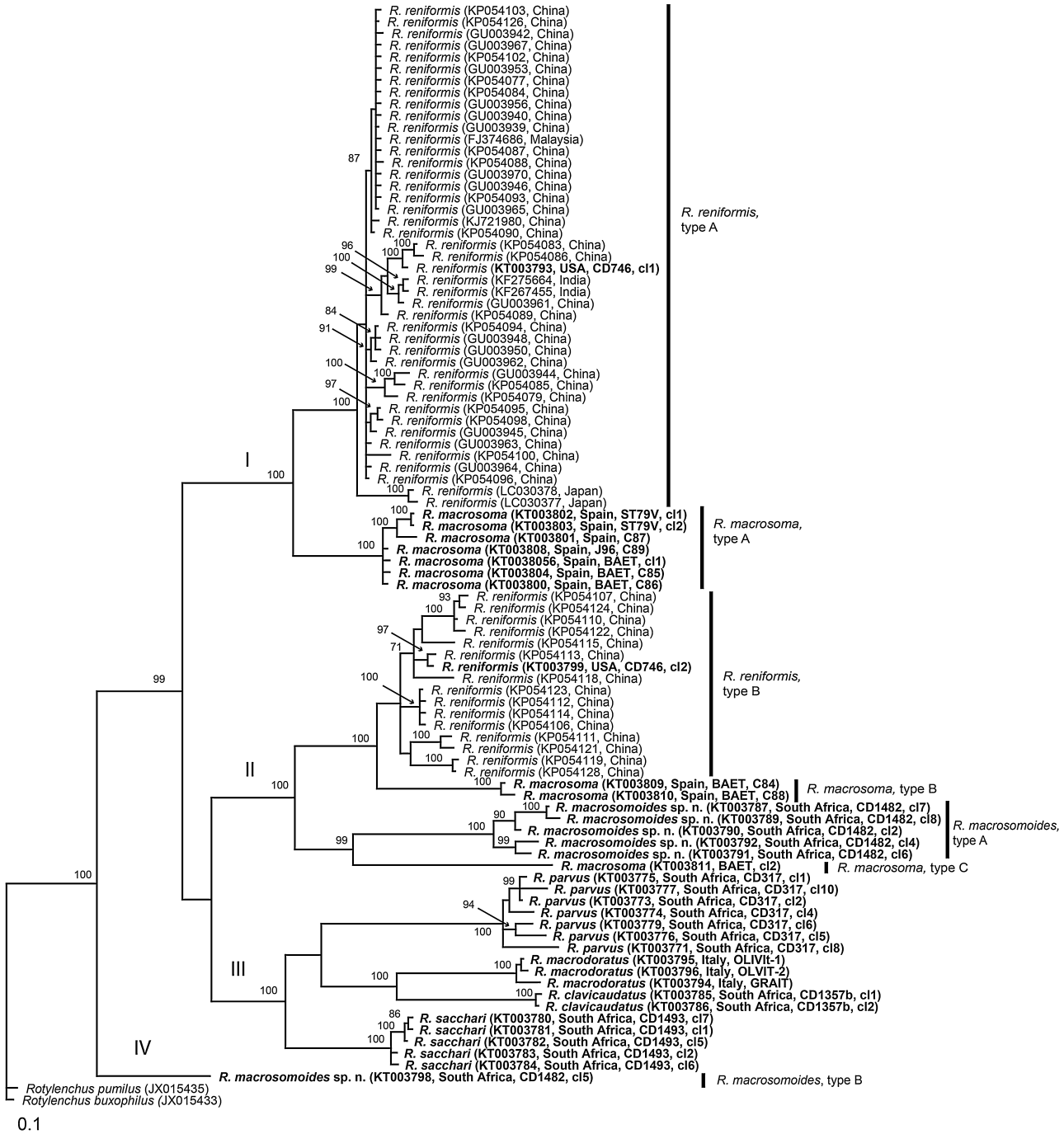


Fig. 17. Phylogenetic relationships between *Rotylenchulus* species. Bayesian 50% majority rule consensus tree as inferred from the analysis of the 5.8S + ITS2 region dataset under the GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are in bold font.

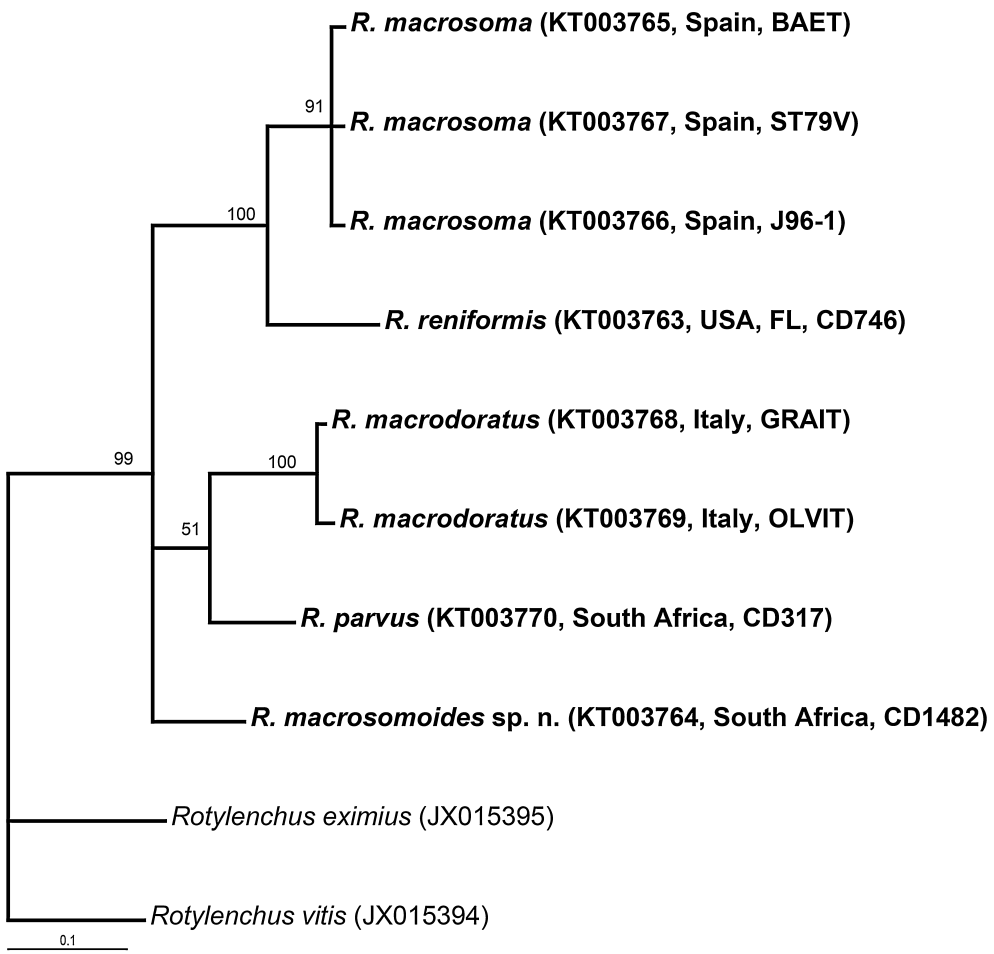


Fig. 18. Phylogenetic relationships between *Rotylenchulus* species. Bayesian 50% majority rule consensus tree as inferred from the analysis of the partial *hsp90* gene dataset under the GTR + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are in bold font.

momonospora chromogena, the eukaryotic parasite *Plasmodium berghei* and the metazoan *Dugesia mediterranea* contain two types of 16S or 18S rRNA genes differing at 6.0, 3.5 and 8.0% of the nucleotide positions, respectively (Gunderson *et al.*, 1987; Carranza *et al.*, 1996; Yap *et al.*, 1999; Torres-Machorro *et al.*, 2010). In most reports, the comparison of sequences was largely limited to the small subunit rRNA genes and it is therefore not clear whether and how far the same level of sequence heterogeneity extends into other regions of the operon (Yap *et al.*, 1999). Our present study confirmed the results of the previous studies by Nyaku *et al.* (2013a, b) and Deng *et al.* (2015), who found two distinct types of rRNA operons (18S, ITS and 28S) in the *R. reniformis* genome. To the best of our knowledge, *R. reniformis* is the only organism among the phylum Nematoda for which this phenomenon has been described so far. These rRNA types showed substantial differences in sequences from each other: the 18S rRNA – 5.5% (Nyaku *et al.*, 2013b), the D2-D3 of 28S rRNA up to 15.6%, the ITS rRNA up to 24.5% (this study). In our study we also found two distinct ITS rRNA types for *R. macrosomoides* sp. n. and three ITS types for *R. macrosoma*. Reconstruction of secondary structure models for two types of the D2 of 28S rRNA for *R. reniformis* and mutation mapping showed that both models have similar conservative folding and most point mutations were compensatory, confirming that both gene rRNA types are functional. Nyaku *et al.* (2013b) also revealed that both 18S rRNA types were expressed in nematode tissues as their sequences were present in ESTs.

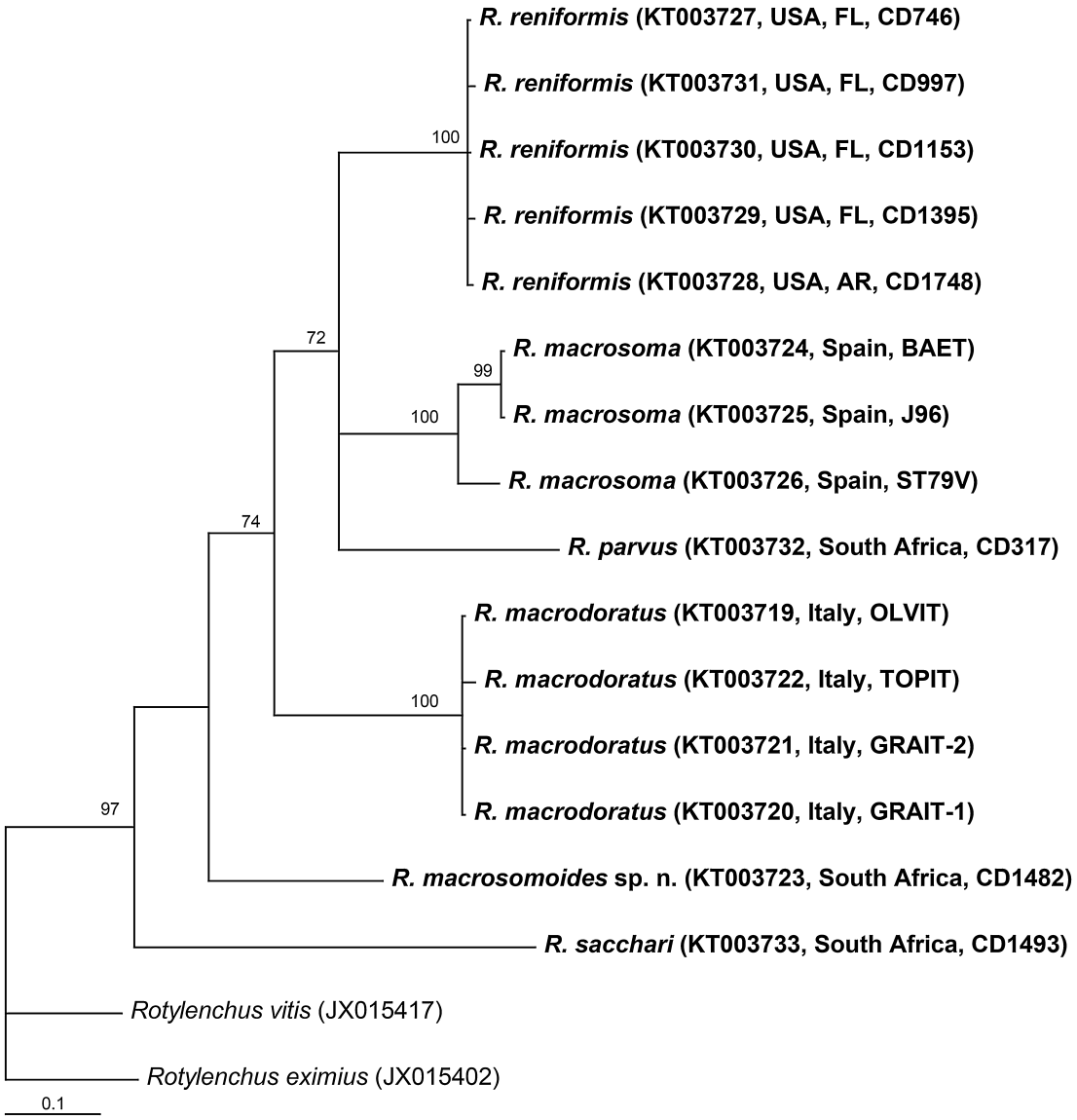


Fig. 19. Phylogenetic relationships between *Rotylenchulus* species. Bayesian 50% majority rule consensus tree as inferred from the analysis of the partial *coxI* gene dataset under the GTR + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are in bold font.

Wang *et al.* (1997) concluded that the presence of distinct types of an rRNA gene in a single organism could be a common rather than unusual phenomenon in nature. In our study, two rRNA types were found in at least three *Rotylenchulus* species. Most probably this phenomenon may occur in other species of the genus, although we did not detect it. It is remarkable that using the universal D2A and D3B primer set we amplified only type A of the D2-D3 of 28S rRNA gene in *Rotylenchulus* but not type B. However, the results of PCR with *R. reniformis* specific primers targeting A and B types clearly showed the presence of both types in all studied *R. reniformis* samples. The negative result of PCR with universal primers with amplification of the type B could be explained by the presence of some mismatches in sequences of the universal D2A primer and the type B of the rRNA gene. Thus, we suggest that further PCR work using various universal rRNA gene primers and a

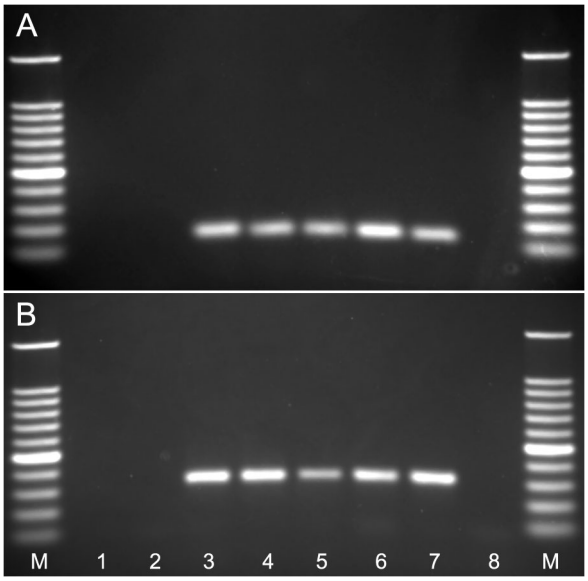


Fig. 20. PCR with the *Rotylenchulus reniformis* specific primers. A: D2A and R_renif_R1A primer combination; B: D2A and R_renif_R2B primer combination. Lanes: M = 100 bp DNA marker (Promega); 1 = *R. sacchari*; 2 = *R. macrosomoides* sp. n.; 3 = *R. reniformis* (CD997); 4 = *R. reniformis* (CD1153); 5, 6 = *R. reniformis* (CD1395); 7 = *R. reniformis* (CD747); 8 = control without DNA.

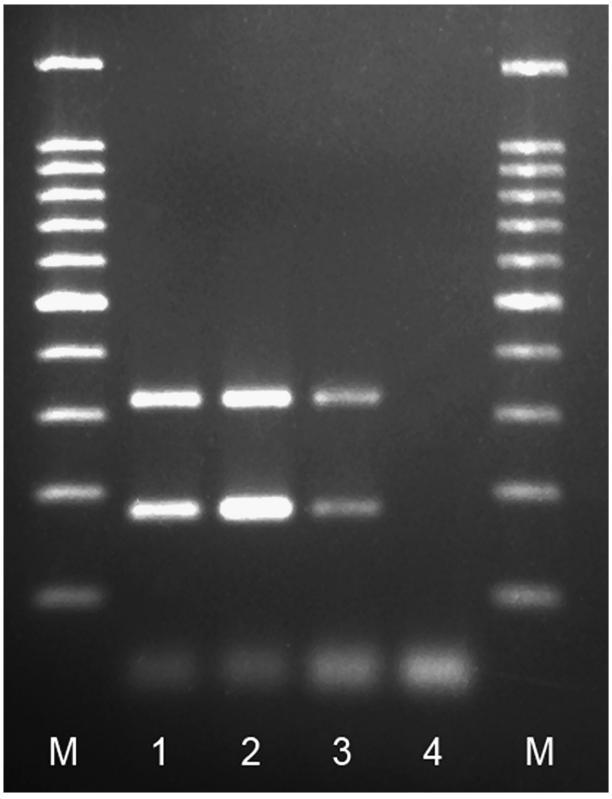


Fig. 21. PCR with the *Rotylenchulus reniformis* specific primers. Multiplex PCR with D2A and R_renif_R1B and R_renif_R2A primer combination. Lanes: M = 100 bp DNA marker (Promega); 1 = *R. reniformis* (CD747); 2 = *R. reniformis* (CD997); 3 = *R. reniformis* (CD1153); 4 = control without DNA.

whole genome sequencing approach should be used to explore the presence of rRNA gene types in genomes of *Rotylenchulus* species.

In several articles, the origin of distinct types of rRNA operons in a single genome has been explained by either divergent evolution following gene duplication or by lateral gene transfer between different species (Gunderson *et al.*, 1987; Mylvaganam & Dennis, 1992; Carranza *et al.*, 1996; Wang *et al.*, 1997). In our opinion, this phenomena may have occurred in an early stage of the evolution of *Rotylenchulus*, either due to ancestral genome duplications or, as Deng *et al.* (2015) suggested, by an ancestral interspecific hybridisation with following rapid divergence of rRNA operons escaping concerted evolution of a multi-gene family.

Our present study also provided more information on intraspecific variation of rRNA genes for *Rotylenchulus* spp., data that can help in species delimitation for this nematode group. Agudelo *et al.* (2005) observed 11.8% divergence in ITS1 sequences between a parthenogenetic form from Japan and 20 amphimictic populations of *R. reniformis* from the USA, Brazil, Colombia, and Honduras. As a result, they believed that this ITS1 sequence difference gave support for the validity of *R. nicotiana* (Yokoo & Tanaka *in* Tanaka & Tsumagori) Baker, 1962, a taxon presently considered by many authors as a junior synonym of *R. reniformis* (Siddiqi, 2000). However, our analysis of the ITS rRNA gene showed that the sequences of parthenogenetic and amphimictic forms studied by Agudelo *et al.* (2005) clustered within sequences of type A and sequence differences between these forms did not exceed the intraspecific variation for *R. reniformis*. Thus, our molecular analysis of rRNA genes does not provide evidence to support the validity of *R. nicotiana* as a separate species.

The results of the present study have significant implications for the reliability of rRNA sequence-based phylogenetic analyses and suggests that analysis of several genes should be applied for reliable reconstruction of phylogenetic relationships of organisms.

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