

Description of *Tylenchulus musicola* sp. n. (Nematoda: Tylenchulidae) from banana in Iran with molecular phylogeny and characterisation of species of *Tylenchulus* Cobb, 1913

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Summary – During a survey conducted on banana plantations in Sistan and Blouchestan province, south-east Iran, a new species of *Tylenchulus* was extracted from the soil and roots of banana plants. This species, named *Tylenchulus musicola* sp. n., is characterised by mature females having a swollen, hook-shaped body with a conical and elongate post-vulval portion ending in a round terminus, males having a weak stylet and a cylindrical and thick tail ending in a bluntly rounded and smooth terminus, and by second-stage juveniles having a slender body and a posterior body portion ending in a finely pointed or mucronate terminus. The results of glasshouse host tests indicated that the new species does not parasitise sugarcane ratoons or sour orange seedlings. *Tylenchulus musicola* sp. n. is distinguished from other known *Tylenchulus* species by the sequences of D2-D3 expansion segments of 28S rRNA and ITS rRNA genes. Phylogenetic relationships within *Tylenchulus* were reconstructed based on rRNA gene sequences using Bayesian inference. Diagnostic PCR-ITS-RFLP profiles are presented for *T. musicola* sp. n., *T. furcus*, *T. graminis*, *T. palustris*, *T. semipenetrans* and *Trophotylenchulus floridensis*. PCR with species-specific primers and genus-specific primer are tested and developed for rapid identification of five *Tylenchulus* species. An identification key to *Tylenchulus* species is provided.

Keywords – key, morphology, morphometrics, *Musa*, new species, taxonomy, *Trophotylenchulus floridensis*, *Tylenchulus furcus*, *Tylenchulus graminis*, *Tylenchulus palustris*, *Tylenchulus semipenetrans*.

Tylenchulus Cobb, 1913 presently contains four nominal species: *T. semipenetrans* Cobb, 1913, *T. furcus* Van den Berg & Spaull, 1982, *T. graminis* Inserra, Vovlas, O'Bannon & Esser, 1988a and *T. palustris* Inserra, Vovlas, O'Bannon & Esser, 1988a. The citrus root nematode, *T. semipenetrans*, has a worldwide distribution and is one of the most common and important plant parasites of citrus in citrus groves. *Tylenchulus semipenetrans* parasitises most *Citrus* species, *Poncirus trifoliata* and other citrus

relatives from the Rutaceae. Several non-rutaceous plants, such as grape, olive and persimmon, are also reported as good hosts. Several biotypes of the citrus nematode are presently recognised based on host specificity testing (Inserra *et al.*, 1980). For many years, *T. semipenetrans* was considered as the only species of this genus and was believed to have the ability to parasitise a wide range of host plants (Chawla *et al.*, 1980; Inserra *et al.*, 1988a). However, nematological surveys and a more detailed morpho-

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logical analysis of *Tylenchulus* populations from different hosts revealed the presence of other species. *Tylenchulus furcus* was described in South Africa from sugarcane, *Saccharum* hybrid plants and poorly growing grass on a golf course (Van den Berg & Spaull, 1982). Previously known as a grass race and a bush race of the citrus root nematode, *T. graminis* and *T. palustris*, respectively, were described from native plants in Florida, USA (Inserra *et al.*, 1988a, b). Later, *T. palustris* was also found infecting peach in Alabama, Arkansas, Georgia and Virginia and some non-cultivated plants including sea oxeye (*Borrichia arborescens* and *B. frutescens*) along the shorelines of Florida and Bermuda (Dow *et al.*, 1990; Inserra *et al.*, 1990; Eisenback & Reaver, 2007).

During a survey of plant-parasitic nematodes in banana plantations in Zarabad, Sistan and Blouchestan province, south-east Iran, a *Tylenchulus* population was isolated from soil and roots of banana plants. Preliminary morphological and molecular analyses showed that the population differs from all four of the described species of *Tylenchulus* and represents a new species. The presence of tylenchulid second-stage juveniles (J2) in soil samples collected in banana plantations has been reported in Venezuela (Haddad *et al.*, 1975) and Burkina Faso (Sawadogo *et al.*, 2001), although the ability of these nematodes to infect banana roots was not ascertained.

The present diagnostics of *Tylenchulus* species is based on the analysis of a few morphological characters of the male, mature female and J2 (Inserra *et al.*, 1988a, c, 1994) and requires taxonomic expertise. *Tylenchulus* J2 could be easily misidentified with J2 of the closely related genera *Sphaeronema*, *Trophonema* and *Trophotylenchulus*, or even with those of root-knot nematodes, *Meloidogyne* spp. Identification procedures might also be complicated by the presence of several *Tylenchulus* species in a sample. For example, *T. semipenetrans* was found to occur in soil in association with other *Tylenchulus* species in Florida (Inserra *et al.*, 1994) and with the new *Tylenchulus* species parasitising banana roots in Iran. *Tylenchulus graminis* and *T. palustris* are often found together in some native areas in Florida.

Wang *et al.* (2004) and Park *et al.* (2009) developed a diagnostic PCR-ITS-RFLP profile for *T. semipenetrans* and Liu *et al.* (2011) were the first to propose species-specific primer sets for detection of this nematode. However, these primers were tested with a few citrus root nematode populations only. Thus, the development of rapid, sensitive and reliable diagnostic tools for regulatory and

diagnostic purposes to separate *Tylenchulus* species becomes a very important task.

The main objectives of the present study are therefore to: *i*) describe a new species of *Tylenchulus* isolated from soil and roots of banana plants in Iran; *ii*) characterise *Tylenchulus* species using ITS-rRNA and the D2-D3 expansion segment of 28S rRNA gene sequences; *iii*) study phylogenetic relationships within the genus using rRNA gene sequences; *iv*) design PCR with genus-specific primers for identification of *Tylenchulus*; and *v*) test and develop PCR-ITS-RFLP and PCR with species-specific primers for quick and reliable diagnostics of all known *Tylenchulus* species.

Materials and methods

NEMATODE POPULATIONS

Soil and root samples containing the new *Tylenchulus* species were collected from a banana plantation in Zarabad about 200 km west of Chabahar, one of the largest cities in the Sistan and Blouchestan province. Seven populations of *T. semipenetrans*, three populations of *T. palustris*, two populations of *T. graminis* and one population each of *T. furcus* and *Trophotylenchulus floridensis* were included in this comparative molecular study. Locations and hosts for samples are indicated in Table 1. Females of the new species were recovered from roots. Males and J2 from soil samples were extracted using a centrifugal flotation technique (Jenkins, 1964).

LIGHT MICROSCOPE OBSERVATIONS

Nematodes were fixed in heated TAF (2 ml triethanolamine, 7 ml formaldehyde and 91 ml distilled water) and transferred to dehydrated glycerin (De Grisse, 1969). The males and J2 were mounted in a small drop of dehydrated glycerin. Banana roots were stained with acid fuchsin-lactophenol, cleared in a lactophenol solution and the stained mature females were then detached from the roots and mounted in glycerin. Morphological and morphometric characters were studied on fixed specimens by using a *camera lucida* installed on a light microscope Olympus BH-2. No observations to ascertain the position of the rectum were made on live J2 mounted in water agar. Measurements of characters for species description were made as proposed by Inserra *et al.* (1988a). Light micrographs of females and J2 were taken with an automatic Olympus camera attached to compound microscopes Olympus

Table 1. Tylenchulus and Trophotylenchulus species sequenced in the present study.

Species	Location	Host	Sample code	rRNA gene sequences		Collector/ Identifier
				D2-D3 of 28S	ITS	
Tylenchulus musicola sp. n.	Zarabad, Sistan and Blouchestan province, Iran	Banana (<i>Musa acuminata</i>)	CD348, CD707, CD721	JN112247, JN112248	JN112278- JN112283	Z. Tanha Maafi
<i>T. semipenetrans</i>	Gerber, Tehama, CA, USA	Olive (<i>Olea europaea</i>)	CD710	JN112252	–	S.A. Subbotin
<i>T. semipenetrans</i>	Riverside, CA, USA	Grapevine (<i>Vitis</i> sp.)	CA27	JN112249	–	S.A. Subbotin
<i>T. semipenetrans</i>	Lake Alfred, FL, USA	Citrus (<i>Citrus limon</i>)	CD294	JN112250	JN112272, JN112276, JN112277	R. Inserra
<i>T. semipenetrans</i>	Nelspruit, South Africa	Citrus (<i>Citrus</i> sp.)	CD614	JN112251	–	E. van den Berg
<i>T. semipenetrans</i>	Ventura, CA, USA	Orange tree (<i>Citrus</i> sp.)	CD1	–	JN112270, JN112273	S.A. Subbotin
<i>T. semipenetrans</i>	TX, USA	Grape fruit (<i>Citrus</i> sp.)	CD435	–	JN112271, JN112274	S.A. Subbotin
<i>T. semipenetrans</i>	Glenn, CA, USA	Citrus (<i>Citrus</i> sp.)	CD153	–	JN112269, JN112275	S.A. Subbotin
<i>T. graminis</i>	Paines Praire, FL, USA	Broomsedge (<i>Andropogon virginicus</i>)	CD227	JN112259, JN112260	JN112286, JN112287	R. Inserra
<i>T. graminis</i>	Cedar Island, FL, USA	Unknown grass	CD750	–	JN112288	R. Inserra
<i>T. furcus</i>	South Africa	Unknown grass	CD292	JN112257, JN112258	JN112284, JN112285	E. van den Berg
<i>T. palustris</i>	Perry, FL, USA	Pop ash (<i>Fraxinus caroliniana</i>)	CD254	JN112255, JN112256	JN112266- JN112268	R. Inserra
<i>T. palustris</i>	Cedar Island, FL, USA	Sea oxeye (<i>Borrachia frutescens</i>)	CD750	–	JN112265	R. Inserra
<i>T. palustris</i>	Horseshoe Beach, FL, USA	Sea oxeye (<i>Borrachia</i> sp.)	CD751	–	JN112263, JN112264	R. Inserra
Trophotylenchulus floridensis	Crystal River, FL, USA	Pine (<i>Pinus elliottii</i>)	CD233	JN112253, JN112254	JN112261, JN112262	J. Stanley

BX50 and BX51 equipped with a Nomarski differential interference contrast.

HOST TEST

Nematode-free sugarcane (*Saccharum officinarum* L.) ratoons, a susceptible host of *T. furcus*, and sour orange (*Citrus aurantium* L.) seedlings, a susceptible host to *T. semipenetrans*, were kept in sterilised sand and then transplanted into 15 cm diam. polyethylene pots containing soil infested with the new *Tylenchulus* species at a density of two J2 (cm soil)⁻³. The pots were maintained in glasshouse conditions at 28 ± 5°C for 6 months. Afterwards, the sprouted ratoons and seedlings were removed from the pots, the shoots were cut and the soil was gently washed from the roots. The root systems were stained

with boiling acid fuchsin-lactophenol, cleared in lactophenol, and examined under a stereomicroscope to detect the swollen female nematodes and egg masses on the root surface.

DNA EXTRACTION, PCR AND SEQUENCING

DNA was extracted from 5-8 J2 using proteinase K protocol. DNA extraction and PCR protocols were as described by Tanha Maafi *et al.* (2003). The forward TW81 and the reverse AB28 primers were used for amplification of ITS-rRNA region (Tanha Maafi *et al.*, 2003) and the forward D2A and the reverse D3B primers were used for amplification of the D2-D3 expansion segments of 28S rRNA gene (Table 2) (Subbotin *et al.*, 2006). The PCR products were purified using the QIAquick Gel Extraction

Table 2. Primer sets used in the present study.

Primer code	Sequence 5' → 3'	Amplified gene	Amplicon length (bp)	References
TW81	GTTTCCGTAGGTGAACCTGC	ITS-rRNA	809-841	Tanha Maafi <i>et al.</i> (2003)
AB28	ATATGCTTAAGTCAGCGGGT			
D2A	ACAAGTACCGTGAGGGAAAGTTG	D2-D3 of 28S rRNA	774-777	Subbotin <i>et al.</i> (2006)
D3B	TCGGAAGGAACCAGCTACTA			
D2A	ACAAGTACCGTGAGGGAAAGTTG	D2-D3 of 28S rRNA	361-364	This study
<i>Tylenchulus</i> specific	TAGGYAAAGCKTGCTTRCCA			
TW81	GTTTCCGTAGGTGAACCTGC	ITS-rRNA	113	This study
<i>Semipenetrans</i> specific	GGACTCTGCTAACCTGGTAGA			
TW81	GTTTCCGTAGGTGAACCTGC	ITS-rRNA	190	This study
<i>Musicola</i> specific	CGCAGACTGCCAGTCGTAG			
TW81	GTTTCCGTAGGTGAACCTGC	ITS-rRNA	690	This study
<i>Furcus</i> specific	ACCAAGACGCACAAAGTACAGAC			
TW81	GTTTCCGTAGGTGAACCTGC	ITS-rRNA	767	This study
<i>Graminis</i> specific	GCTATTGCGAGTCTCCTAGGG			
TW81	GTTTCCGTAGGTGAACCTGC	ITS-rRNA	344	This study
<i>Palustris</i> specific	AGGCTGTCGTAACGCTTCGCA			
Ts-SF	TACCAGGTTGAGCAGAGTTCTT	ITS-rRNA	293	Liu <i>et al.</i> (2011)
Ts-SR	TCCTACCCTTCCACAGCG			

Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction and cloned as described by Tanha Maafi *et al.* (2003). PCR products of one or more clones from each sample were sequenced in the Genomic Center, University of California, Riverside, CA, USA. The newly obtained sequences have been submitted to the GenBank database under accession numbers JN112247-JN112288 as indicated in Table 1.

RFLP-ITS-rRNA

Three to five μl of purified PCR product was digested by one of the following restriction enzymes: *AvaI*, *BseNI*, *BsuRI* or *Hin6I* in the buffer stipulated by the manufacturer. The digested DNA was run on a 1.4% TAE buffered agarose gel, stained with ethidium bromide, visualised on UV transilluminator and photographed. The length of each restriction fragment from the PCR products was obtained by a virtual digestion of the sequences using Web-Cutter 2.0 (www.firstmarket.com/cutter/cut2.html) or estimated from a gel.

PCR WITH GENUS AND SPECIES-SPECIFIC PRIMERS

Genus-specific and species specific primers were designed using sequence alignment of D2-D3 of 28S rRNA

and ITS-rRNA gene, respectively (Table 2). All *Tylenchulus* and *Trophotylenchulus floridensis* samples were used to test the specificity of PCR with genus-specific and newly designed species-specific primers. The PCR mixture was prepared as described by Tanha Maafi *et al.* (2003). PCR for detection of all species of the genus was run with a *Tylenchulus*-specific primer and the D2A primer. PCR for species-specific detections were run in a multiplex condition with all five specific primers and the TW81 primer in a tube and also separately with each species-specific primer set in a tube. The newly designed species-specific primer for *T. semipenetrans* were compared with primer set proposed by Liu *et al.* (2011) (Table 2) using different numbers of J2 in samples. The PCR amplification profile consisted of 4 min at 94°C; 30 cycles of 1 min at 94°C, 45 s at 57°C and 45 s 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.

SEQUENCE AND PHYLOGENETIC ANALYSIS

The newly obtained sequences for each gene were aligned using ClustalX 1.83 (Thompson *et al.*, 1997) with default parameters with their corresponding published gene sequences, ITS-rRNA or D2-3 of 28S rRNA, re-

spectively (Subbotin *et al.*, 2006; Park *et al.*, 2009; Liu *et al.*, 2011; Chen *et al.*, unpubl.). Outgroup taxa for each dataset were chosen according to the results of previous published data (Subbotin *et al.*, 2005, 2006; Bert *et al.*, 2008; Holterman *et al.*, 2009). Sequence datasets were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) under the GTR + I + G model. BI analysis for each gene 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 10^3 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. Sequence analyses of alignments were performed with PAUP* 4b10 (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.

Results

*Tylenchulus musicola** sp. n. (Figs 1-4)

MEASUREMENTS

See Tables 3 and 4.

DESCRIPTION

Mature female

Body swollen for 45.6-54.0% of total body length. Entire body hook shaped, curved ventrally, widest portion at excretory pore, narrowing abruptly posterior to vulva. Lip region hemispherical, not set off from body, lip annuli indistinct, cephalic framework weakly developed. Stylet well developed with large rounded knobs. Transverse striae very faint, not visible in swollen portion. Median bulb large, oval, isthmus slender, nerve ring posterior to median bulb. Basal bulb pyriform, dorsal gland nucleus prominent, subventral nuclei smaller. Hemizonid

* Specific epithet derived from *Musa*, the genus of the host and *colo* = dweller.

not observed. Excretory pore situated at 61-93% from anterior end of body. Excretory-secretory cell not discernable in specimens examined, cuticular lobes visible around pore. Ovary single, convoluted, extending to basal bulb. Spermatheca spherical, filled with rounded sperm. Uterus swollen, ovate often containing eggs 44.9 ± 5.0 (40-52) μm long $\times 21.3 \pm 1.1$ (20-21) μm diam. Vagina almost straight, large. Vulval lips conspicuously protruding. Post-vulval uterine sac absent. Anus and rectum not visible in fixed specimens. Body posterior to vulva elongate, conoid, with rounded terminus.

Male

Body vermiform, straight or slightly arcuate after fixation, slender, and with faint transverse striae. Lip region hemispherical, annuli not visible. Labial framework not developed. Stylet delicate, with small rounded knobs. Pharynx not well developed, but with visible median and terminal bulbs. Nerve ring posterior to median bulb. Excretory pore situated at 52.1-58.7% from head. Testis single, occupying 30-42% of body length, spicules slender, arcuate, gubernaculum slightly curved, bursa absent. Tail cylindrical and thick, ending in a bluntly rounded terminus.

J2 female

Body slender, curved slightly ventrally. Body transverse striae very faint, almost not recognisable. Lip region rounded, not set off from body. Cephalic framework weak. Stylet well developed with large rounded knobs. Median bulb oval, basal bulb pyriform. Nerve ring posterior to median bulb. Excretory pore situated at 49.8-58.4% from anterior end. Genital primordium with 2-4 cells, 61-70% from anterior end. Anus and rectum not visible in fixed specimens. Hyaline posterior body portion ending in a finely pointed or mucronate terminus.

J2 male

Not examined.

Remarks

Observations to detect the rectum on live J2 mounted in water agar were not conducted. So far we do not know whether the rectum is visible in live specimens of this new species. Also, no attempt was made to detect plasmids to confirm the report by Sturhan and Geraert (2005) of the presence of these sensory organs in *T. semipenetrans*.

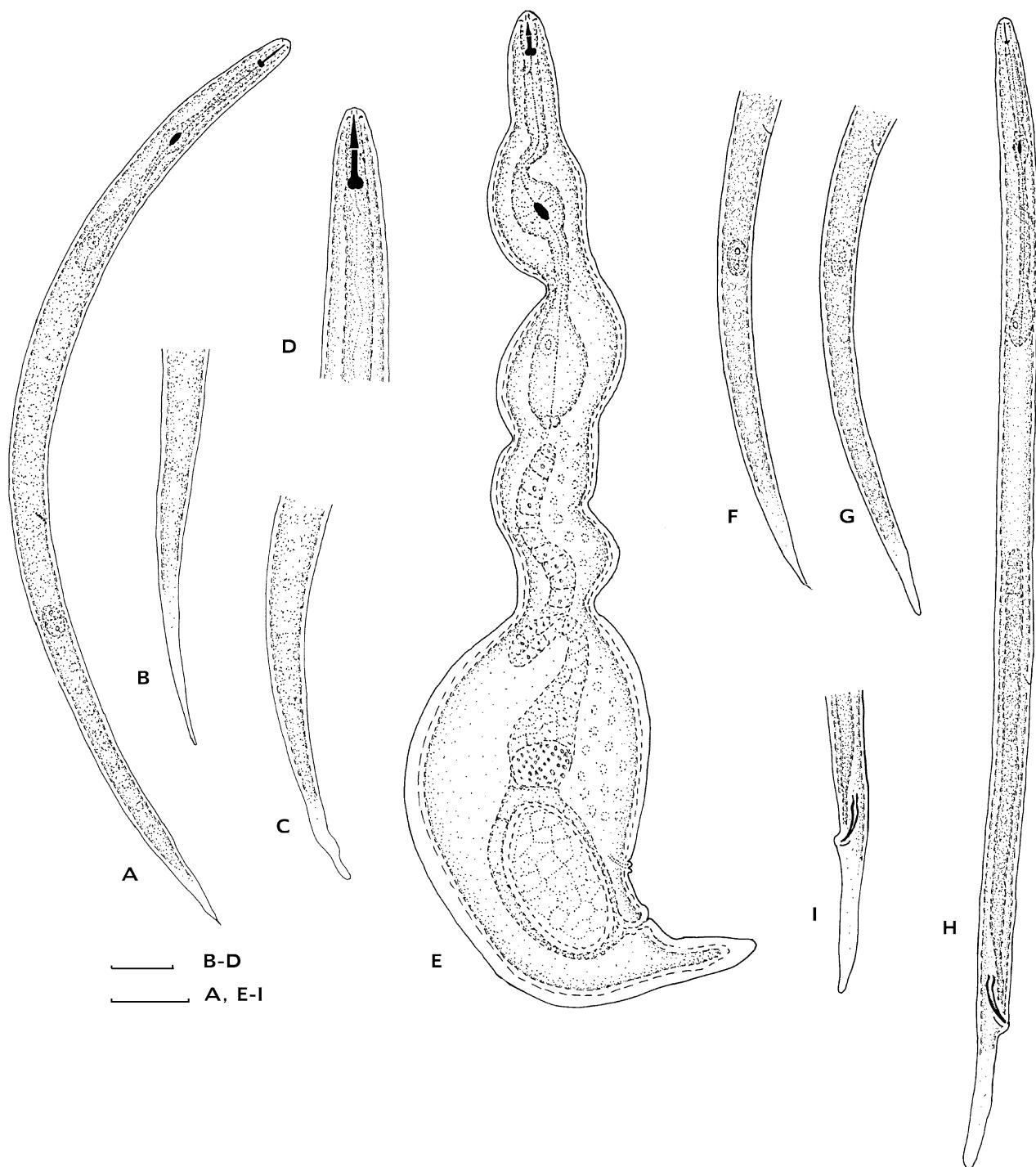


Fig. 1. *Tylenchulus musicola* sp. n. A: Entire body of second-stage juvenile; B, C, F, G: Posterior body portion of second-stage juveniles; D: Anterior body portion of second-stage juvenile; E: Female; H: Entire body of male; I: Tail of male. (Scale bars: A, E-I = 20 μm ; B-D = 10 μm .)

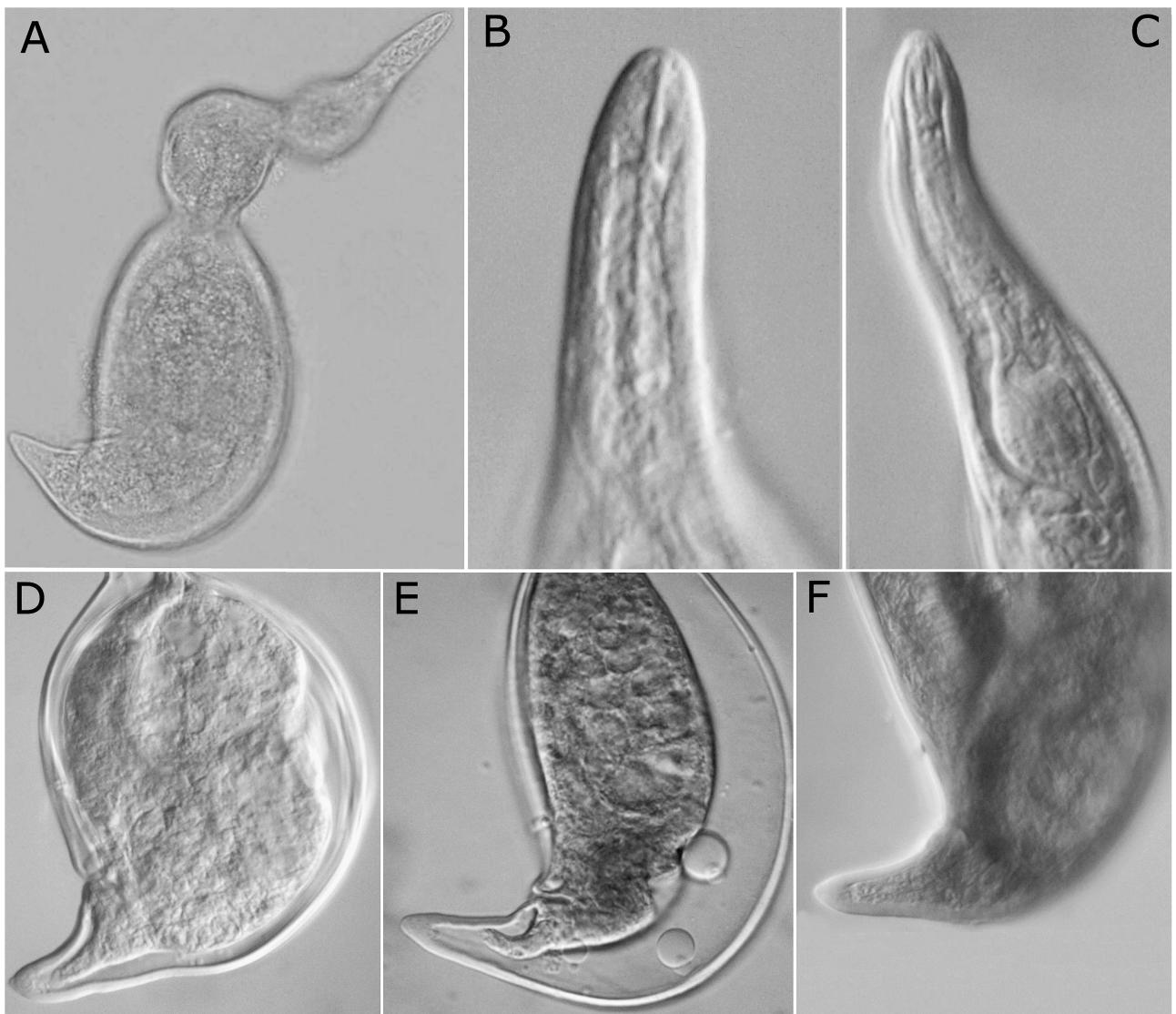


Fig. 2. Light micrographs of females of *Tylenchulus musicola* sp. n. A: Entire body; B, C: Head region; D-F: Vulva and tail region.

TYPE HOST AND LOCALITY

Collected from rhizosphere and roots of banana cv. Harichal (*Musa acuminata* Colla), Zarabad ca 200 km west of Chabahar, one of the largest cities in Sistan and Blouchestan province, Iran.

TYPE MATERIAL

Holotype female, 12 paratype females, 31 paratype J2, five paratype males (Slides 83153/1-19) deposited in the National Nematode Collection of the Nematology De-

partment, Iranian Research Institute of Plant Protection (IRIPP, Tehran, Iran). Additional paratypes are distributed as follows: nine J2 (two slides) and three mature females (three slides) at the Nematology Collection of University California (Riverside, CA, USA); 12 J2 (three slides) and three mature females (three slides) at the United States Department of Agriculture Nematode Collection (Beltsville, MD, USA); ten J2 (two slides) and three mature females (three slides) at the Wageningen Nematode Collection, Plant Protection Organisation (Wageningen, The Netherlands).

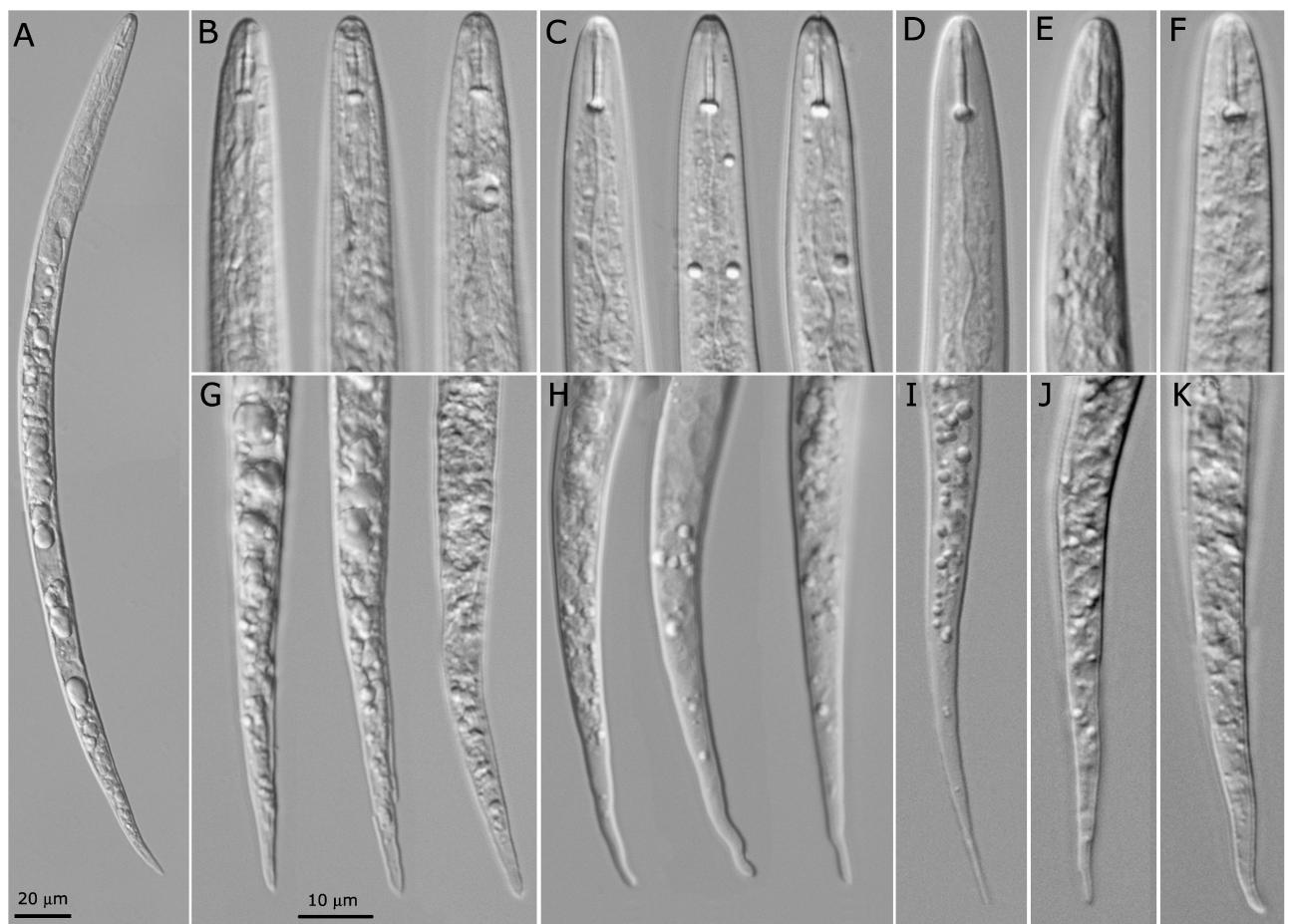


Fig. 3. Light micrographs of second-stage juveniles of *Tylenchulus* species. A: Entire body; B-F: Head; G-K: Posterior body portion and tail; A, B, G: *T. musicola* sp. n.; C, H: *T. semipenetrans*; D, I: *T. furcus*; E, J: *T. graminis*; F, K: *T. palustris*.

DIAGNOSIS AND RELATIONSHIPS

Tylenchulus musicola sp. n. is characterised by the mature female having a swollen hook-shaped body with a conical and often elongate post-vulval portion ending in a round terminus, male having a weak stylet and a cylindrical and thick tail ending in a bluntly rounded and smooth terminus, and by the J2 female having a slender body and a posterior body portion ending in a finely pointed or mucronate terminus.

Tylenchulus musicola sp. n. differs from known *Tylenchulus* species by a combination of morphological and morphometric characters of the female, male and J2. It differs from *T. furcus* by the mature female having a conical post-vulval body portion with a round terminus vs sharply pointed and mucronate, by the male having a bluntly rounded tail terminus vs peg-like, by the J2 female

having a shorter body length (278-334 vs 328-477 μm) and posterior body with pointed and mucronate terminus vs forked (Fig. 4). It differs from *T. graminis* by the mature female having a shorter section of the body that is swollen (45.6-54.0 vs 59-85%), obscure anus vs visible, conoid post-vulval body portion with rounded terminus vs pointed or peg-like or mucronate, by the male having a shorter body length (286-326 vs 422-519 μm), shorter stylet length (8-9 vs 9.9-10.7 μm), shorter tail length (35-40 vs 48.9-65.2 μm) and a cylindrical tail with bluntly rounded terminus vs elongate conoid with mucronate or rounded, and by the J2 female having a shorter body length (278-334 vs 337-441 μm), shorter stylet length (10-12 vs 12.7-13.4 μm), an obscure anus vs visible and posterior body terminus finely pointed or mucronate vs rounded (Fig. 4); from *T. palustris* by the lower female post-vulval section diam. (PVSd) mean value (11 vs 14)

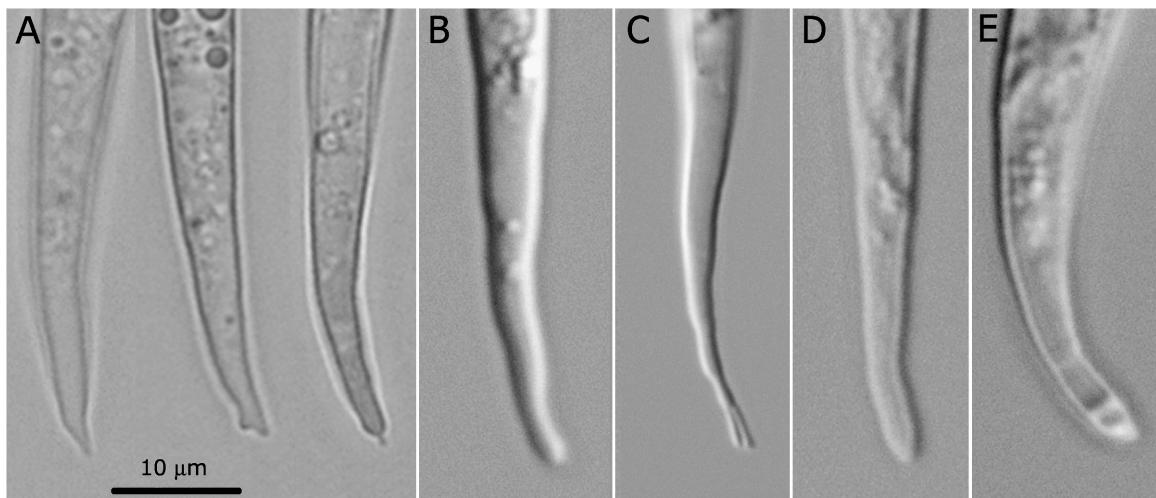


Fig. 4. Light micrographs of posterior body portion and tail terminus of second-stage juveniles of *Tylenchulus* species. A: *T. musicola* sp. n.; B: *T. semipenetrans*; C: *T. furcus*; D: *T. graminis*; E: *T. palustris*.

Table 3. Morphometrics of mature female of *Tylenchulus musicola* sp. n. All measurements are in μm and in the form: mean \pm s.d. (range).

Character	Holotype	Paratypes
n	—	12
L	298	287 \pm 32.6 (240-370)
a	4.6	4.8 \pm 1.4 (3.7-8.2)
b	27	3.1 \pm 0.5 (2.5-4.0)
Stylet	11.0	10 \pm 1.2 (8-12)
DGO	5.0	4.0 \pm 0.8 (3-5)
Anterior end to median bulb	55.0	46 \pm 7.7 (35-55)
Median bulb length	15.0	15.3 \pm 2.4 (12-19)
Median bulb diam.	14.0	14.2 \pm 2.0 (10.0-18.0)
Pharynx length	110	94 \pm 15.2 (66-112)
Basal bulb length	26	21.5 \pm 3.4 (15-27)
Basal bulb diam.	14	12.6 \pm 2.0 (10-16)
Anterior end to excretory pore	240	222 \pm 37.7 (150-280)
Excretory pore from anterior end as % of body length	80.5	77.4 \pm 8.8 (61.2-93.3)
Vulva-excretory pore distance	13	14.1 \pm 3.3 (10-20)
Post-vulval section diam.	10	11.0 \pm 1.4 (9-14)
Post-vulval section length	30	27 \pm 6.0 (17-36)
Post-vulval section cavity	6.0	6.6 \pm 0.7 (6-8)
Swollen posterior body as % of total body length	46.3	45.6-54.0
Body diam. at vulva	20	23 \pm 3.8 (15-29)
Body diam. at mid-body	65	64 \pm 16.8 (30-80)
Cuticle thickness	5.0	4.2 \pm 0.8 (3-5)

and by the J2 female posterior body terminus being finely pointed or mucronate vs bluntly rounded (Fig. 4); and from *T. semipenetrans* by the shorter (17.0-36.0 vs 26.5-52.0 μm) and conical mature female post-vulval body portion vs digitate, by a cylindrical male tail with bluntly rounded terminus vs tapering, and by the finely pointed or mucronate posterior body terminus of J2 female vs rounded (Fig. 4).

The results of the morphological analysis indicate that *T. musicola* sp. n. is a well defined morphological species that can be separated from other *Tylenchulus* species by examining all the life stages of the nematode. However, regulatory and survey samples usually contain mainly soil without roots. If the samples contain only J2, the morphological identification of *T. musicola* sp. n. J2 is very difficult and unreliable without the additional examination of the mature females and males. For regulatory and survey purposes molecular tools should be used to obtain an accurate identification of *T. musicola* sp. n. J2.

MOLECULAR CHARACTERISATION AND PHYLOGENIC POSITION OF *T. MUSICOLA* SP. N.

The ITS-rRNA gene sequence alignment was 877 bp in length and contained 61 sequences of *Tylenchulus*, 21 of them being newly obtained for this study and six for *T. musicola* sp. n. Intraspecific ITS sequence variation for *T. musicola* sp. n. was 1-10 bp (0.1-1.0%) and ITS sequences of this species differed from those of other *Tylenchulus* species by 106-181 bp (13-23%). In

Table 4. Morphometrics of paratype second-stage juvenile and male of *Tylenchulus musicola* sp. n. All measurements are in μm and in the form: mean \pm s.d. (range).

Character	Second-stage juvenile	Male
n	31	5
L	306 \pm 13.8 (278-334)	310 \pm 19.2 (286-326)
a	30.1 \pm 1.6 (27.7-33.4)	27.2 \pm 3.8 (23.8-32.5)
b	3.5 \pm 0.2 (3.2-4.0)	3.5 \pm 0.2 (3.3-3.7)
c	—	8.1 \pm 0.1 (8.0-8.2)
Stylet	11.1 \pm 0.6 (10-12)	8.5 \pm 0.6 (8-9)
Anterior end to median bulb	43.6 \pm 2.5 (38-48)	36.0 \pm 1.4 (35-37)
Pharynx length	87 \pm 4.3 (78-100)	89 \pm 1.5 (87-90)
Anterior end to hemizonid	65 \pm 4.3 (57-71)	57 \pm 6.7 (50-65)
Anterior end to excretory pore	169 \pm 8.4 (148-184)	174 \pm 4.0 (170-178)
Excretory pore to genital primordium	28.5 \pm 6.8 (15-40)	—
Anterior end to genital primordium	198 \pm 9.9 (179-217)	—
Genital primordium to posterior end	109.2 \pm 7.6 (90-124)	—
Max. body diam.	10.1 \pm 0.3 (10-11)	11.5 \pm 1.0 (10-12)
Excretory pore from anterior end as % of body length	55.3 \pm 2.0 (49.5-58.4)	54.9 \pm 3.4 (52.1-58.7)
Genital primordium (%)	64.7 \pm 2.0 (61-70)	—
Spicules	—	15.7 \pm 2.1 (14-18)
Gubernaculum	—	4.5 \pm 0.7 (4-5)
Tail	—	38.3 \pm 2.4 (35-40)

BI phylogenetic tree, these six sequences of *T. musicola* sp. n. clustered together with high PP value (Fig. 5). Intraspecific ITS sequence variation for *T. semipenetrans* reached 22 bp (2.8%). There are no groupings amongst *T. semipenetrans* isolates regarding geographical location or host plant. Relationships between *T. semipenetrans* and other species were not well resolved from the ITS-rRNA gene dataset. Intraspecific ITS sequence variation for *T. palustris* reached 24 bp (3.1%) and for *T. graminis* was 1-14 bp (0.1-1.9%). *Tylenchulus palustris*, *T. graminis* and *T. furcatus* formed a highly supported clade (PP = 100).

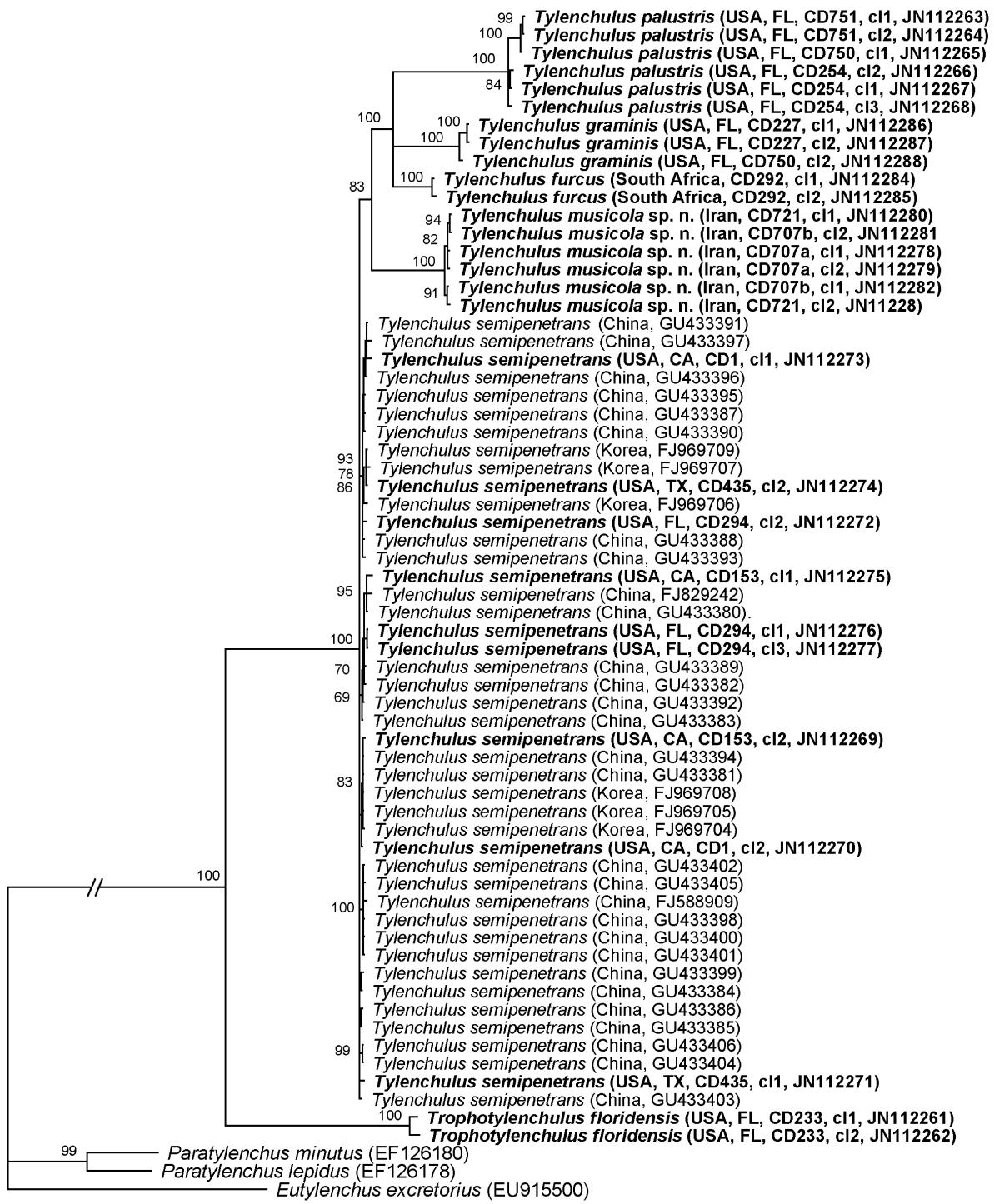
The D2-D3 of 28S rRNA gene sequence alignment was 669 bp in length and contained 20 sequences of *Tylenchulus*, 12 of them being newly obtained for this study and two, which were identical and differed from other *Tylenchulus* species by 51-105 bp (8-16%), being of *T. musicola* sp. n. *Tylenchulus musicola* sp. n. formed a highly supported clade (PP = 100) with *T. semipenetrans* (Fig. 6). Intraspecific D2-D3 sequence divergence for most *T. semipenetrans* samples varied from 0 to 6 bp (0 to 1.5%) and reached a maximum of 14 bp (2.3%) between the California isolate from olive trees and others. The phylogenetic analysis did not reveal any grouping amongst *T. semipenetrans* regarding location or host-plant.

MOLECULAR DIAGNOSTICS OF *TYLENCHULUS* SPECIES

PCR-ITS-RFLP profiles for five species of *Tylenchulus* and *Trophotylenchulus floridensis* are given in Figure 7 and Table 5. Heterogeneity of the ITS-rRNA region for *T. floridensis* was revealed by enzymes *BsuRI*, *BseNI* and *Hin6I*.

Wang *et al.* (2004) and Park *et al.* (2009) were the first to present diagnostic PCR-ITS-RFLP profiles for the citrus root nematode, *T. semipenetrans*, from China and Korea, respectively. Park *et al.* (2009) revealed the heterogeneity of the ITS region after digestion by *MseI* and *MspI* and the presence of two ITS haplotypes. Some differences in PCR-RFLP profiles were also observed between some populations of *T. semipenetrans*. In the present study, we selected four restriction enzymes which do not generate complex profiles and which do not complicate the identification procedure for *T. semipenetrans* and other species.

Comparative sequence analysis of the D2-D3 expansion regions of 28S rRNA gene sequences for *Tylenchulus* and other nematode genera revealed regions with a short unique sequence for representatives of this genus and allowed a genus-specific primer to be designed. Results of PCR with this genus-specific primer are given in Figure 8.



0.1

Fig. 5. The 50% majority rule consensus tree from Bayesian analysis generated from the ITS rRNA gene sequence dataset for *Tylenchulus* species using the GTR + I + G model. Posterior probability more than 70% is given for appropriate clades. Newly obtained sequences indicated in bold.

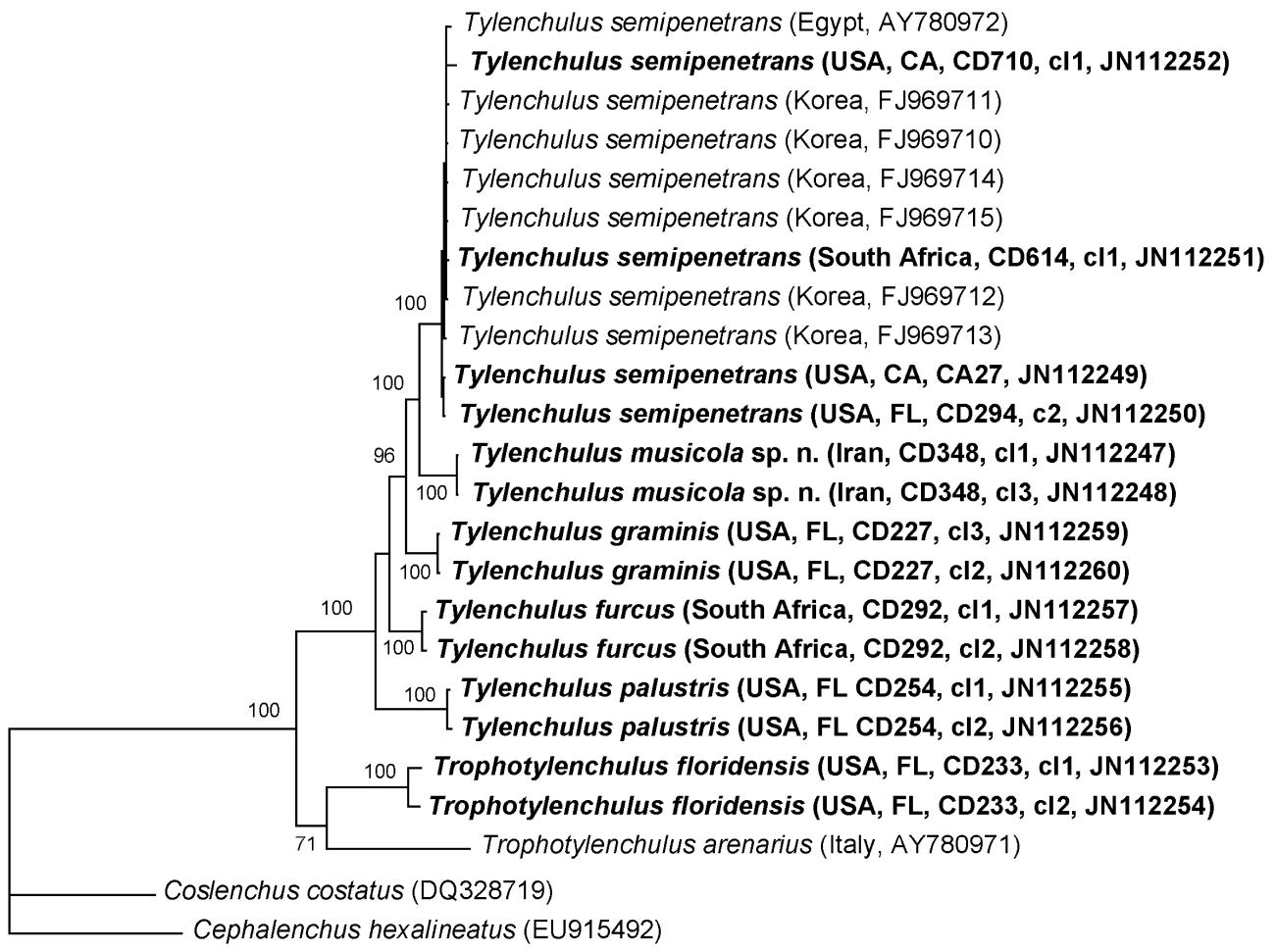


Fig. 6. The 50% majority rule consensus tree from Bayesian analysis generated from the D2-D3 of 28S rRNA gene sequence dataset for *Tylenchulus* species using the GTR + I + G model. Posterior probability more than 70% is given for appropriate clades. Newly obtained sequences indicated in bold.

The genus-specific and universal D2A primers yielded an amplicon of ca 360 bp in length for all tested *Tylenchulus* samples. No PCR products were detected in the samples of *Trophotylenchulus floridensis* and other parasitic nematodes (data not shown).

Species-specific primers were developed for each *Tylenchulus* species based on differences in the ITS-rRNA gene sequences (Table 2; Fig. 9). Results of PCR with species-specific primers are given in Figure 10. The combination of universal primer TW81 with corresponding species-specific primers yielded a single PCR product in 113 bp for *T. semipenetrans*, 190 bp for *T. musicola* sp.

n., 344 bp for *T. palustris*, 690 bp for *T. furcus* and 767 bp for *T. graminis*. All these primer combinations were successfully tested in a multiplex PCR.

Comparative sensitive testing of our specific primer for *T. semipenetrans* and the primer set designed by Liu *et al.* (2011) showed that both PCR methods are able successfully to detect a single J2 in a sample (Fig. 11). Analysis of the sequence alignment (Fig. 9) revealed that primer Ts-SF by Liu *et al.* (2011) targets only *T. semipenetrans* and thus, *in silico* analysis including all known *Tylenchulus* species confirms the specificity of the method of detection for *T. semipenetrans* developed by Liu *et al.* (2011).

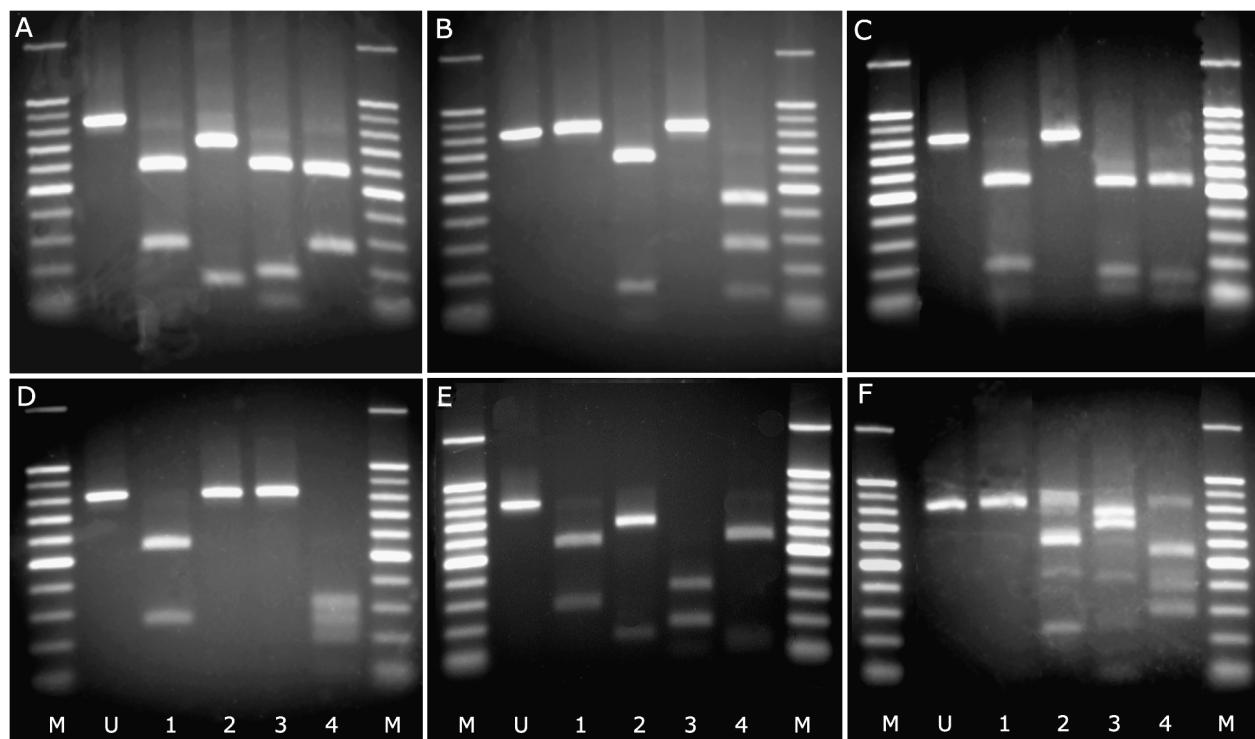


Fig. 7. PCR-ITS-RFLP. *A*: *Tylenchulus semipenetrans*; *B*: *T. musicola* sp. n.; *C*: *T. graminis*; *D*: *T. furcatus*; *E*: *T. palustris*; *F*: *Trophtylenchulus floridensis*. Lanes: *M* = 100 bp DNA ladder (Promega); *U* = unrestricted PCR product; *1* = *AvaI*; *2* = *BseNI*; *3* = *BsuRI*; *4* = *Hin6I*.

Table 5. Approximate lengths (bp) for fragments of PCR-ITS-RFLP for *Tylenchulus* species and *Trophtylenchulus floridensis*.

Species	Unrestricted PCR product	Restriction enzymes			
		<i>AvaI</i>	<i>BseNI</i>	<i>BsuRI</i>	<i>Hin6I</i>
<i>Tylenchulus semipenetrans</i>	841	575, 266	690, 151	583, 181, 77	571, 270
<i>T. musicola</i> sp. n.	828	828	647, 147, 34	828	440, 269, 119
<i>T. graminis</i>	809	543, 196, 70	809	531, 164, 98, 16	539, 146, 65, 59
<i>T. furcatus</i>	812	549, 263	812	812	330, 298, 247, 192, 75
<i>T. palustris</i>	831	557, 274	661, 153, 17	325, 194, 188, 78, 46	553, 127, 76, 75
<i>Trophtylenchulus floridensis</i>	814	814	814, 591, 433, 223, 158	747, 682, 67, 65	531, 380, 283, 151

HOST TEST

Results of the host test experiment did not reveal any juveniles or females parasitising roots of sugarcane or sour orange.

Discussion

Cobb (1913) erected *Tylenchulus* with *T. semipenetrans* as type species. Raski (1957) proposed *Trophtylenchulus*

Raski, 1957 which was separated from *Tylenchulus* by the more anterior position of the excretory pore and the presence of a prominent circumoral elevation, lacking in *Tylenchulus*. The species of both genera have a large excretory and secretory cell that produces a gelatinous matrix through the excretory pore. Maggenti (1962) considered *Trophtylenchulus* a junior synonym of *Tylenchulus* because they share the same excretory system. However, this synonymisation was rejected by Hashim (1983) and supported by Raski and Luc (1987). The results of the molec-

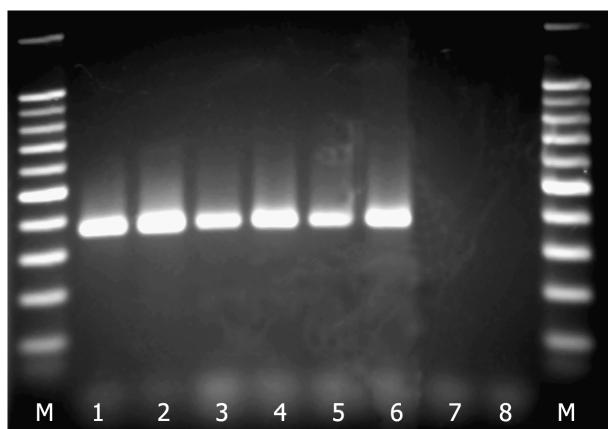


Fig. 8. PCR with the *Tylenchulus* genus-specific primer. Lanes: M = 100 bp DNA marker (Promega); 1 = *Tylenchulus musicola* sp. n.; 2 = *T. semipenetrans* (Ventura, CA, USA); 3 = *T. graminis*; 4 = *T. palustris* (Cedar Island, FL, USA); 5 = *T. palustris* (Perry, FL, USA); 6 = *T. furcatus*; 7 = *Trophotylenchulus floridensis*; 8 = control without DNA.

ular analysis of our study support the validity of *Trophotylenchulus* as a separate taxon from *Tylenchulus*.

Separation of *Tylenchulus* species has posed a very challenging task to many nematologists involved with the identification of these nematodes, especially in Florida. After the description of *T. semipenetrans*, Florida nematologists encountered *Tylenchulus* J2 that were indistinguishable from those of *T. semipenetrans*. These J2 were considered ‘wild races’ of *T. semipenetrans*. Chitwood was also deceived by the morphological similarity of these wild races with those of *T. semipenetrans* and considered these ‘wild races’ as native *T. semipenetrans* populations (Chitwood & Birchfield, 1957). Only in 1988, and after the description in South Africa of a new *Tylenchulus* species *T. furcatus*, were these wild races of *T. semipenetrans* separated from *T. semipenetrans* as two different species, *T. graminis* and *T. palustris* (Inserra *et al.*, 1988a). The description of these two new species facilitated the separation of the three Florida *Tylenchulus* whose J2 can be identified by the position of the rectum, which is visible in live specimens placed in water agar. The results of the molecular analysis of our study confirm the validity of the two species and also provide molecular tools for the identification of these *Tylenchulus*.

Phylogenetic analysis did not reveal any groupings amongst *T. semipenetrans* isolates regarding geographical location or host plant. However, this analysis showed two groups within *T. palustris* samples collected from different hosts. Moreover, the results of our preliminary

and unpublished analysis revealed the presence of another undescribed species in Florida, which is morphologically very similar to *T. palustris*, but significantly different in the ITS sequences. This finding is still under study.

Molecular analysis using rRNA genes does not provide any clear pattern for the evolution and dispersal of *Tylenchulus* species. We can only speculate that the presence of *T. musicola* sp. n. on banana and *T. semipenetrans* on citrus might indicate that these *Tylenchulus* species probably evolved with these plants in their native habitats in south-east Asia. It was not until later that they were distributed with infected propagative materials to other countries and became adapted to parasitise other crops. The preliminary results of the host test suggest that *T. musicola* sp. n. is not able to infect sugarcane and citrus, although these results cannot be considered definitive until they are confirmed by further tests and extensive field observations.

A modified version of the key to *Tylenchulus* species published by Inserra *et al.* (1988a) is given here.

Key to species of *Tylenchulus* Cobb, 1913

1. J2 and mature female with distinct rectum and anus. Mature female with body swollen posteriorly for 60% or more of total body length 2
- J2 and mature female without distinct rectum and anus, except for *T. palustris* and *T. semipenetrans* J2, which have discernible rectum in live specimens. Mature female with body swollen posteriorly for 58% or less of total body length 3
2. J2 with furcate or bifid tail tip *T. furcatus* Van den Berg & Spaull, 1982
- J2 with tail tapering and ending in a bluntly pointed terminus *T. graminis* Inserra, Vovlas, O'Bannon & Esser, 1988
3. Mature female with short and conoid post-vulval body section ending in a rounded and smooth terminus. PVSD = 14.3 (11.2-17.3) μm . Male with stylet knobs = 1.9 (1.7-2.1) μm wide, basal bulb = 9.1 (8.1-11.2) μm wide and tail cylindrical, almost truncate with rounded terminus. Live J2 with discernable rectum; tail = 50 (44-54) μm long, conoid ending in a rounded, occasionally indented, terminus *T. palustris* Inserra, Vovlas, O'Bannon & Esser, 1988
- Mature female with elongate and conoid post-vulval body section ending in a rounded terminus. PVSD = 11 (9-14) μm . Male tail cylindrical, thick, ending in

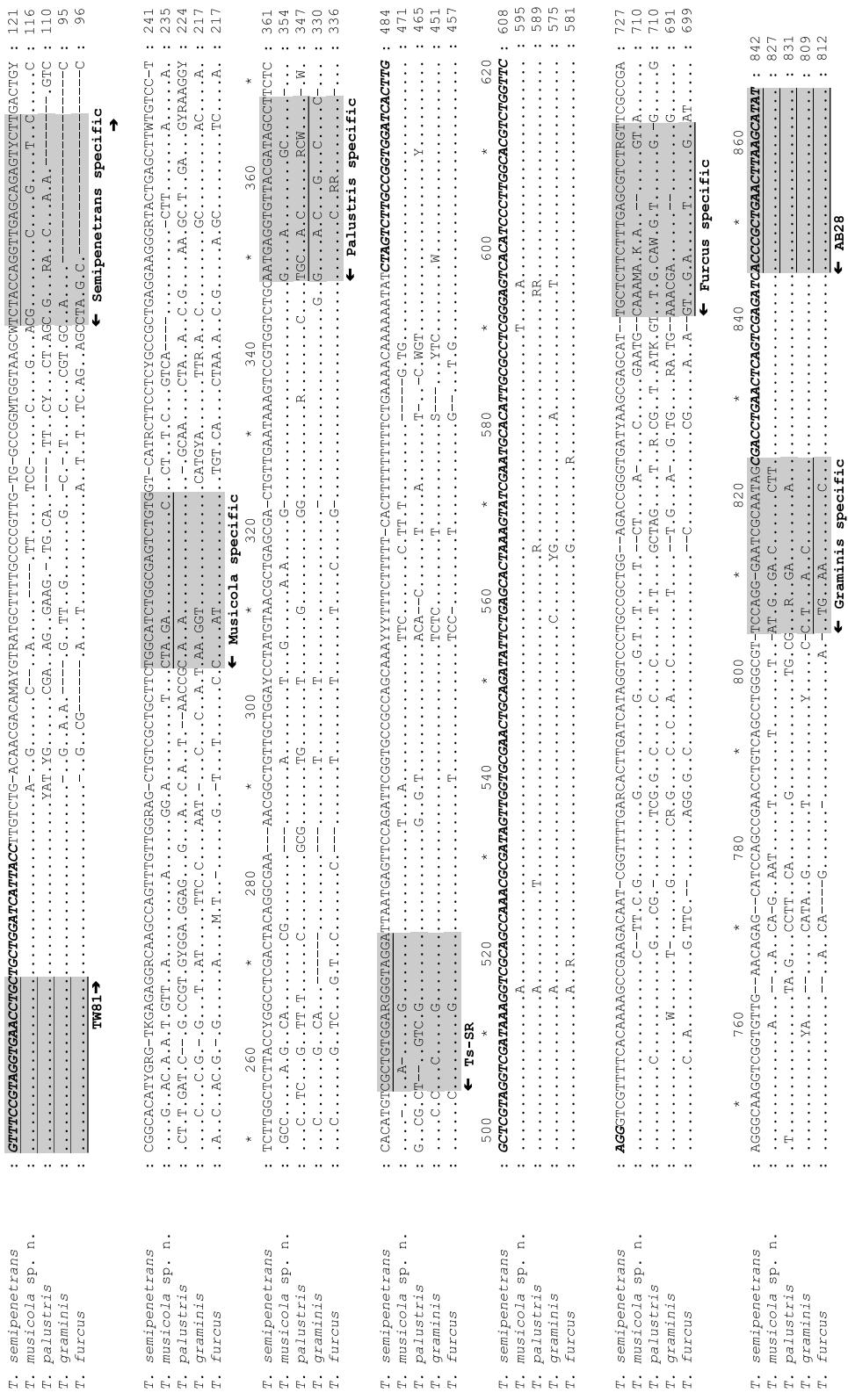


Fig. 9. ITS rRNA gene sequence alignment for *Tylenchulus* species with indication of primer positions. Primer positions for certain species are underlined and marked in grey for alignment. Ribosomal genes of 18S, 5.8S and 28S are marked by bold italic letters.

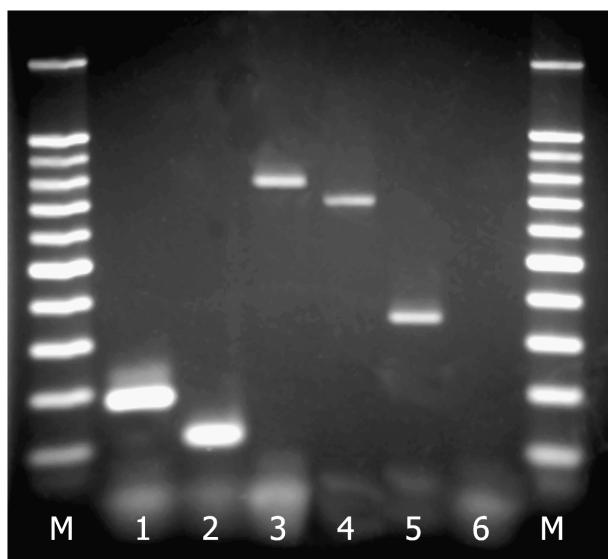


Fig. 10. PCR with the *Tylenchulus* species-specific primers. Lanes: M = 100 bp DNA marker (Promega); 1 = *Tylenchulus musicola* sp. n.; 2 = *T. semipenetrans*; 3 = *T. graminis*; 4 = *T. furcatus*; 5 = *T. palustris*; 6 = control without DNA.

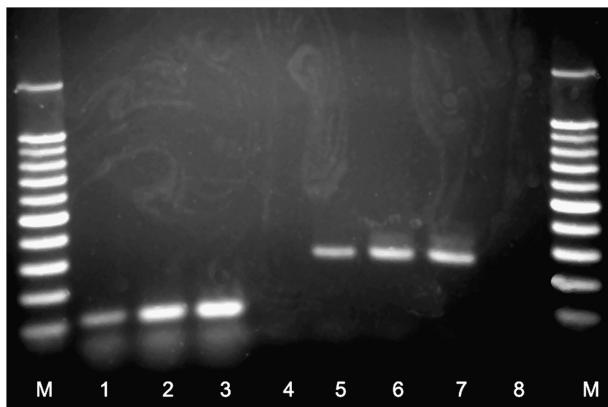


Fig. 11. PCR with the *Tylenchulus* semipenetrans species-specific primers. Lanes: M = 100 bp DNA marker (Promega); 1-4 = primer combination: semipenetrans specific + TW28; 5-8 = primer combination: Ts-SF + Ts-SR; 1 = 8: PCR amplicon obtained from different numbers of J2 per reaction tube; 1, 5 = 0.25 J2; 2, 6 = 0.5 J2; 3, 7 = 1 J2; 4, 8 = negative control without DNA.

a bluntly rounded terminus. J2 with posterior body portion conoid, ending in a finely pointed or mucronate terminus.....*T. musicola* sp. n.
– Mature female with digitate post-vulval body section ending in a round smooth terminus. Male stylet knobs = 1 (0.9-1.2) μm wide, basal bulb = 6.4 (5.1-8) μm wide and tail tapering and ending in a rounded

terminus. Live J2 with discernable rectum; tail = 60 (55-66) μm long, tapering to a rounded terminus
T. semipenetrans Cobb, 1913

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