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Characterization of the Cystoid Nematode *Meloidoderita kirjanovae* (Nemata: Sphaeronematidae) from Southern Italy

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Abstract: A population of the cystoid nematode *Meloidoderita kirjanovae* was detected parasitizing water mint (*Mentha aquatica*) in southern Italy. The morphological identification of this species was confirmed by molecular analysis using the internal transcribed spacer 1 (ITS1) and 5.8S gene sequences of nuclear ribosomal DNA (rDNA), which clearly separated it from the closely related species *Meloidoderita polygoni*. A phylogenetic analysis of *M. kirjanovae* with species of related genera was conducted using sequences of the D2-D3 expansion segments of the 28S nuclear ribosomal RNA gene. The resulting phylogenetic tree was congruent with trees from an extended dataset for Criconematina and Tylenchida. The basal position of the genus *Meloidoderita* together with *Sphaeronema* within the Criconematina clade in this tree may indicate their close relationships. The anatomical changes induced by *M. kirjanovae* population from Italy in water mint were similar to those reported for a nematode population infecting roots of *M. longifolia* in Israel. Nematode feeding caused the formation of a stellar syncytium that disorganized the pericycle and vascular root tissues.

Key words: histopathology, host-parasite relationships, *Mentha aquatica*, molecular analysis, morphology, SEM, taxonomy, phylogeny.

A population of the cystoid nematode *Meloidoderita kirjanovae* Poghossian, 1966 was detected parasitizing a new host, water mint (*Mentha aquatica*), in southern Italy. This is the first record of this plant-parasitic nematode in Italy.

The genus *Meloidoderita* Poghossian (1966) comprises three valid species, including *Meloidoderita kirjanovae* Poghossian, 1966, *M. polygoni* Golden & Handoo, 1984, and *M. safrica* Van den Berg & Spaull, 1982, which can be differentiated according to a combination of morphological and morphometrical characters (Table 1). *Meloidoderita* females retain the eggs inside a hypertrophied uterus that becomes a protective and persistent cystoid sac after the nematode's death.

Whereas *M. kirjanovae* has been commonly reported parasitizing mint and grass pastures in several countries, including Armenia, Germany, Iran, Israel, Portugal (Azores), and Uzbekistan, *M. polygoni* and *M. safrica* parasitize Polygonaceae species in Beltsville, MD, and sugar cane in the Mposa area of Natal, South Africa, respectively (Van den Berg and Spaull, 1982; Sturhan, 1983; Golden and Handoo, 1984; Siddiqi, 1985). Although a complete morphological description of *M. kirjanovae*, based on light microscopy (LM), has been previously reported (Poghossian, 1966; Kirjanova and Poghossian, 1973; Golden and Handoo, 1984), no scanning electron microscopy (SEM) studies have been carried out on this nematode.

No molecular characterization of *Meloidoderita* species is available to establish a more accurate identification approach and phylogenetic relationships with related genera. According to the classification of the Criconematina proposed by Siddiqi (2000), the genus *Meloidoderita* is phylogenetically related to genera of the superfamily Tylenchuloidea (Skarbilovich, 1947) Raski and Siddiqui, 1975, such as *Paratylenchus* Micoletzky, 1922, *Sphaeronema* Raski and Sher, 1952, *Tylenchulus* Cobb, 1913, and *Trophotylenchulus* Raski, 1957 (Siddiqi, 2000). As a consequence of this relationship, the genus *Meloidoderita* is included in the family Sphaeronematidae along with the genus *Sphaeronema*. The other related genera mentioned above are included in the families Paratylenchidae and Tylenchulidae. Recently, Sturhan and Geraert (2005) proposed reconsidering the Criconematina and Tylenchuloidea classification because these authors observed minute phasmid-like structures in tylenchulids, *Sphaeronema* and *Meloidoderita* spp., which were absent in the species of the family Paratylenchidae. These morphological observations of important taxonomical significance were supported by molecular studies conducted by Subbotin et al. (2005) and cast doubt on the monophyly of Tylenchuloidea. The objectives of this study were to corroborate these findings and to provide information on the morphological and molecular characterization of *M. kirjanovae* population from Italy. Additional objectives included the phylogenetic analysis of this population with species of related genera based on sequences of the D2-D3 expansion segments of the 28S nuclear ribosomal RNA gene and a study of the anatomical alterations induced by the nematode in water mint roots.

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TABLE 1. Morphometric characters distinguishing *Meloidoderita* species.

Character[a]	*M. kirjanovae* (Siddiqi, 1985)	*M. polygoni* (Golden and Handoo, 1984)	*M. safrica* (Van den Berg and Spaull, 1982)	*M. kirjanovae* (southern Italy)
Cystoid body length	268 (180–355)	388 (301–477)	256.1 (146–333)	295 (244–411)
Female stylet length	(15–19)	15.3 (15–17)	19.2 (17–22)	15 (14–16)
Distance from lip region to excretory pore	63 (44–88)	112 (81–214)	75 (35–104)	61 (53–80)
Length of spines in cystoid body	8.5 (6.5–13)	22 (13–30)	(not measured)	10.5 (8.0–13.5)
Distance from vulval slit to anus	90 (44–180)	52 (32–86)	(22–24)	68 (59–80)
Lateral fields incisures in juveniles	4	3	0	3

[a] All measurements are in μm unless otherwise stated.

Materials and Methods

Nematode identification and SEM studies: Specimens for morphological characterization used in this study comprised juveniles, males, cystoid bodies, and adult females collected from Laceno Lake at Avellino province (southern Italy) parasitizing water mint. The morphological and morphometric parameters of this population fitted those reported for *M. kirjanovae*.

For SEM studies, mature females and cystoid forms were dissected from naturally infected roots of water mint, and migratory stages (including males and second-stage juveniles) were extracted from infested soils by the centrifugal flotation method (Coolen, 1979) and by incubation of cystoid bodies and egg masses. Specimens were killed by gentle heat, fixed in a solution of 4% formaldehyde, 1% propionic acid, and processed to pure glycerin using Seinhorst's method (Seinhorst, 1966). Fixed specimens were dehydrated in a graded ethanol series, critical point dried, sputter-coated with gold, and observed with a JEOL JSM-5800 microscope (Abolafia et al., 2002).

A population of *M. polygoni* naturally infecting roots of smartweed (*Polygonum hydropiperoides* Michx.) near the steam plant at Beltsville Agriculture Research Center-West, Beltsville, MD, was selected for comparative morphological and molecular analyses.

DNA extraction, PCR amplification, RFLP, sequencing and phylogenetic analysis: Juveniles, cystoid bodies and females of *M. kirjanovae* and *M. polygoni* from host and localities indicated above were used for molecular analyses. Total DNA was extracted from single adult females or juveniles of *M. kirjanovae* and also from *M. polygoni* according to Subbotin et al. (2000). The detailed protocol for PCR was described by Tanha Maafi et al. (2003). The following primer pairs were used for amplification and sequencing of the D2-D3 fragment of the 28S rRNA gene and the ITS region of rRNA gene (ITS1 for *M. polygoni* and ITS1–5.8S-ITS2 for *M. kirjanovae*), respectively: forward D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3' and reverse D3B (5'-TCGGAAGGAACCAGCTACTA-3') (Subbotin et al., 2005); forward TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and reverse 5.8SM5 (5'-GGCGCAATGTGCATTCGA-3') (Zheng et al., 2000) or forward 18S (5'-TTGATTACGTCCCTGCCCTTT-3') and reverse 26S (5'-TTTCACTCGCCGTTACTAAGG-3') (Vrain et al., 1992). The ITS1–5.8S-ITS2 PCR products of *M. kirjanovae* and *M. polygoni* were purified with a gel extraction kit (Geneclean turbo; Q-BIOgene, Illkirch, France) and quantified using the Quant-iT DNA Assay Kit Broad Range fluorometric assay (Molecular Probes, Inc., Leiden, The Netherlands) with a Tecan Safire fluorospectrometer (Tecan Spain, Barcelona, Spain) according to manufacturer's instructions. The D2-D3 amplification products of *M. kirjanovae* were cloned into the pGEM-T vector and transformed into JM109 High Efficiency Cells (Promega Corporation, Madison, WI). PCR products of the ITS region and several D2-D3 clones were submitted for DNA sequencing. Amplicons were sequenced in both directions with a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Madrid, Spain) using the amplification primers listed above according to the manufacturer's instructions. The resulting products were purified and run on a Model 3100 DNA multicapillary sequencer (Applied Biosystems) at the University of Córdoba sequencing facilities. The sequences reported here for *M. kirjanovae* and *M. polygoni* have been deposited in the GenBank database under the accession numbers DQ768428 (D2-D3 fragment for *M. kirjanovae*), DQ768427 (ITS1–5.8S-ITS2 region for *M. kirjanovae*), DQ768425 and DQ768426 (ITS1 region for two clones of *M. polygoni*).

The D2-D3 sequence of *M. kirjanovae* was aligned using ClustalX 1.83 (Thompson et al., 1997) with published D2-D3 sequences of 29 species of tylenchid nematodes and three species of Aphelenchida chosen as outgroup taxa (Subbotin et al., 2006). After removing ambiguously aligned regions from an alignment, we applied a Bayesian interference analysis (BI) for the data set using MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). We used a general-time-reversible (GTR) model of nucleotide substitution and a gamma distribution (G) of among-site rate heterogeneity with six rate categories estimated as the best-fit model by ModelTest to the present data set. Bayesian analyses were initiated with random starting trees and were run with four chains for 1.0×10^6 generations. Markov chains were sampled at intervals of 100 generations. The log-likelihood values of the sample points stabilized 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, with posterior probabilities (PP) given as support for appropriate clades.

The ITS1 region sequences of *M. kirjanovae* and *M. polygoni* were aligned with ClustalX 1.83 (Thompson et al., 1997) with default options.

Histopathology: Naturally infected water mint roots segments were gently washed free of adhering soil and debris, and individual infected root portions were selected together with healthy roots. Root tissues were fixed in formaldehyde chromo-acetic solution for 48 hr, dehydrated in a tertiary butyl alcohol series (40–70–85–90–100%), and embedded in 58°C (melting point) paraffin wax for histopathological observations. Embedded tissues were sectioned with a rotary microtome. Sections of 10 to 12 µm thickness were placed on glass slides, stained with safranin and fast-green, mounted permanently in 40% xylene solution of a polymethacrylic ester (Synocril 9122X, Cray Valley Products, NJ), examined microscopically, and photographed (Johansen, 1940).

Results

Morphological and SEM studies: Meloidoderita kirjanovae was identified by means of LM and SEM examinations. Measurements of females, cystoid bodies, males, and juveniles from glycerin mounts of the Italian population of *M. kirjanovae* are presented in Tables 1 and 2. Females are rather pear-shaped, have protruding terminal vulva, and have a smooth, thick cuticle (3–3.5 µm). Only the neck and anterior region of the female extend into the root, and the exposed body is surrounded by a gelatinous matrix up to two times the size of the female. The gelatinous matrix becomes filled with eggs, and, as the second-stage juveniles hatch from these eggs, a brown cystoid uterine sac filled with eggs develops within the female. The uterine sac has a thick surface with prominent spines and might occupy almost the entire female body (Figs. 1F-L;2C-F). Second-stage juveniles of the Italian population of *M. kirjanovae* showed a lip region lacking annuli and a lateral field marked by three lines (Figs. 1F-L;2C-F). Males were common and lacked stylet and bursa (Fig. 1M,N). These observations indicated that the ornamentation (spines) of the uterine cystoid sac and the biology of the Italian population of *M. kirjanovae* were similar to those reported for a population of this species from Israel (Cohn and Mordechai, 1982; Golden and Han-

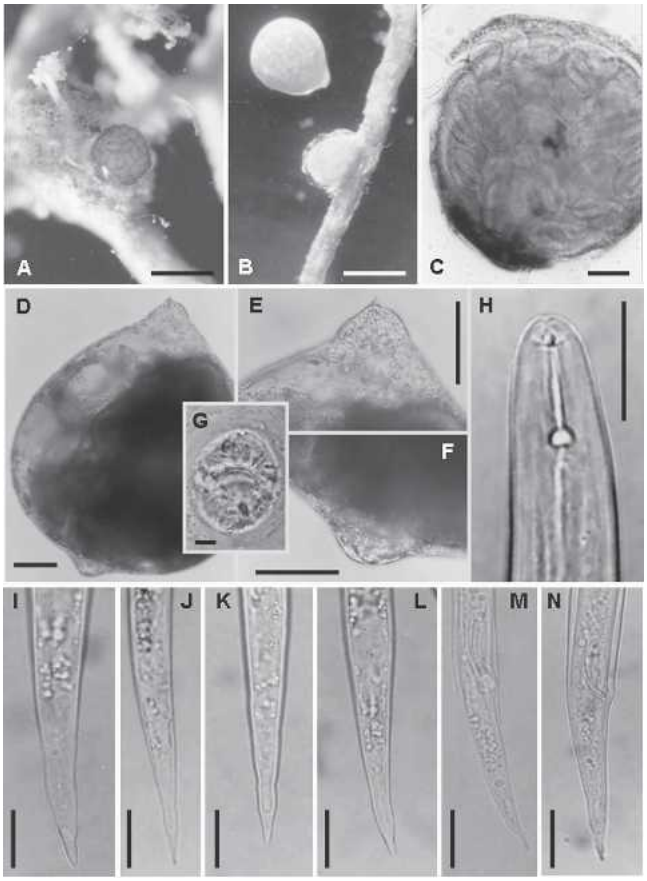

Fig. 1. Photomicrographs of specimens of *Meloidoderita kirjanovae*. A-C) Cystoid bodies on mint root. D) Adult female, whole specimen. E,F) Details of anterior and posterior female body portions. G) Detail of vulval region. H) Second-stage juvenile lip region. I-K) Second-stage juvenile tail regions. L,M) Male tail region. Scale bars: A,B = 250 µm; C-F = 50 µm; G-M = 15 µm.

Table 2. Morphometrics of *Meloidoderita kirjanovae* parasitizing water mint (*Mentha aquatica*) at Laceno at Avellino province (southern Italy).

Measurements and ratios	Females			Males			Juveniles		
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
n	8			10			10		
L	261	13.5	240–278	322	29.8	267–365	337	18.5	298–366
a	—	—	—	22.4	1.5	20.5–24.3	23.5	2.0	20.8–26.8
b	—	—	—	—	—	—	3.2	0.4	2.7–3.9
c	—	—	—	6.7	0.5	5.7–7.3	7.2	0.8	6.1–8.9
Stylet length	15	0.8	14–16	—	—	—	13.5	0.8	12–15
Excretory pore from anterior end	61	9.2	53–80	—	—	—	76.1	9.7	60–91
Distance from vulval slit to anus	68	7.3	59–80	—	—	—	—	—	—
Tail length	—	—	—	48	1.8	44–50	47.1	3.3	41–50
Spicules	—	—	—	14.5	0.7	13–15	—	—	—

[a] All measurements are in µm unless otherwise stated.

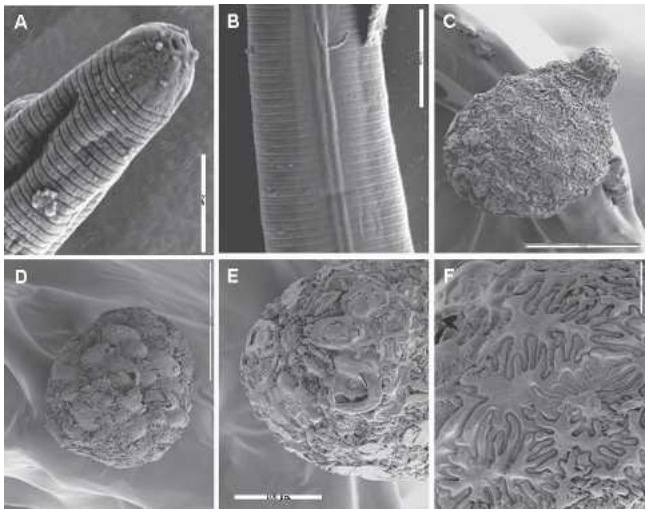

FIG. 2. SEM micrographs of juveniles and cystoid bodies of *Meloidoderita kirjanovae.* A,B) Juveniles. C) Whole cystoid body. D) Gelatinous matrix containing eggs. E) Surface of cystoid body showing the posterior portion of a second-stage juvenile. F) Spines and surface markings of cystoid body. Scale bars: A = 5 µm; B = 10 µm; C = 200 µm; D,F = 20 µm; E = 100 µm.

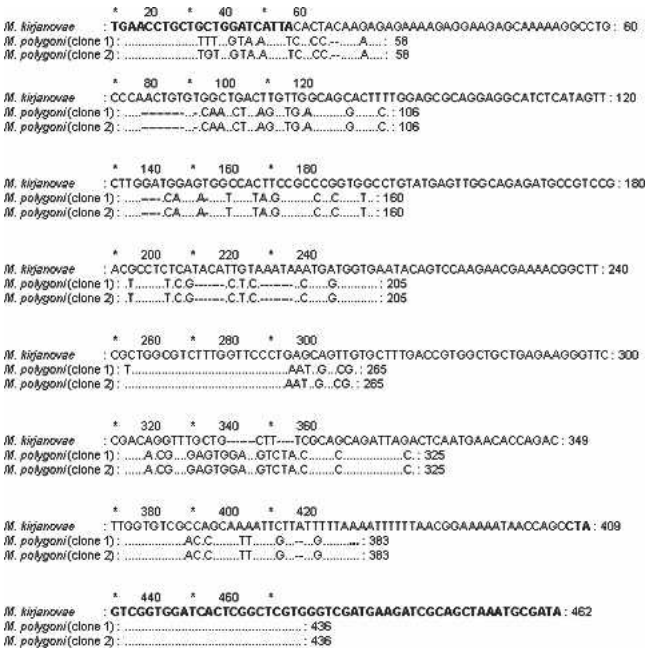

FIG. 3. Partial (473 bp) ITS1-rRNA sequence alignment for two species of *Meloidoderita* with 18S and 5.8S rRNA gene sequences shown in bold. Identities with *M. kirjanovae* are indicated by a period; gaps are denoted by dashed lines.

doo, 1984). In general, morphometry of all life stages of the Italian population of *M. kirjanovae* was shown to be slightly shorter than that of populations from Israel and Russia in body length and distance from excretory pore to anterior end, but was quite similar in other characters such as stylet or spicules length. The body length of juveniles from *M. kirjanovae* from Italy and Israel is clearly shorter than that from *M. polygoni* (298–417 µm vs. 408–504 µm), which could be used as an additional diagnostic character to differentiate these species. However, additional measurements of a higher number of specimens and populations should be considered before a clear decision on that matter can be made.

Molecular characterization and phylogenetic relationships with other nematodes: The nematode was identified by molecular diagnostics using the internal transcribed spacer 1 (ITS1), 5.8S gene sequences of nuclear ribosomal DNA (rDNA), and sequences of the D2-D3 expansion segments of the 28S rDNA gene. Amplification of the ITS region rRNA gene of *M. kirjanovae* using primer combination of 18S and 28S generated a PCR product with a gel-estimated length of approximately 1,000 bp. A portion of the ITS alignment, including the 18S and 5.8S rRNA genes from *M. kirjanovae* and *M. polygoni*, is presented in Figure 3. The length of the ITS1 region of *M. polygoni* was shorter that those of *M. kirjanovae* and differs in 63 to 64 nucleotides (15%) from this species.

To analyze the position of the genus *Meloidoderita* within tylenchids and avoid influence of the possible high level of data saturation on phylogenetic reconstruction, we used a conservative approach to create a sequence data set known as a culled alignment. The culled alignment was created from an automatic alignment containing 713 bp by manually removing 147 ambiguously aligned nucleotide positions, comprising 21% of the alignment length. A Bayesian interference analysis majority consensus tree indicated the division of Tylenchida into several main clades (Fig. 4). *Meloidoderita kirjanovae* clustered with high PP (99%) with representatives of the suborder Criconematina and occupied a basal position in this clade together with *Sphaeronema alni.*

Histopathology: Severe infections of *M. kirjanovae* were detected on young roots of *M. aquatica.* Adult females of *M. kirjanovae* protruded from the surface of all infected root segments (Fig. 1A,B) occurring individually or in clusters, but did not cause distortion of the entire root diameter (Fig. 1B). Usually, there was a single female in each of the numerous, randomly selected infected sites that were microscopically observed (Fig. 1B), but two or three mature females occasionally were found together. Eggs were laid in a gelatinous matrix regularly protruding from the root surface (Fig. 5A), but the cystoid body was often located within the root cortex (Fig. 5A). Histological observations of *Meloidoderita*-infected mint roots (Fig. 5A-E) showed formation of a syncytium at the nematode feeding site. Syncytium expansion induced alterations of the endodermis, pericycle and vascular cylinder as well as a disorganization of the root cortex (Fig. 5A-E). Commonly, the nematode feeding sites comprised six to eight syncytial cells surrounding the nematode's lip region, but in some cases up to 12 syncytial cells which gradually decreased in size with increased distance from the nematode lip region were induced by a single female, (Fig. 5C). Syncytial cells showed the characteristic cytological features

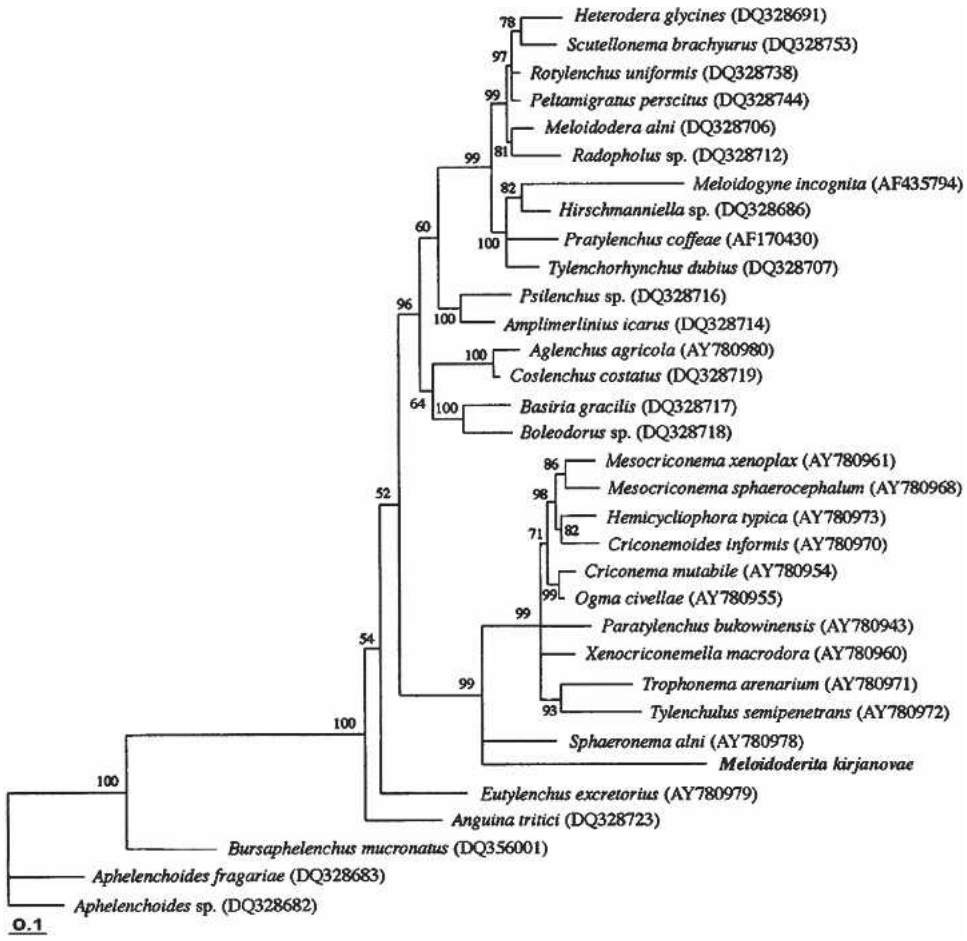

FIG. 4. The 50% majority rule consensus tree from Bayesian analysis generated with the GTR+I+G model. Trees were obtained for a culled (566 bp) alignment of the 28S-rRNA D2-D3 expansion region from 33 taxa of the Tylenchida and Aphelenchida. Posterior probability is given as a percentage for each appropriate clade.

of granulated cytoplasm, thickened cell walls, and a hypertrophied nucleus and nucleolus (Fig. 5D,E). In some cases, some parenchymatic cells in the stele close to the pericycle contained dense protoplasm, but generally no change in their size was observed (Fig. 5C). These anatomical changes did not differ from those described by Cohn and Mordechai (1982) in *M. longifolia* roots infected by a *M. kirjanovae* population from Israel.

DISCUSSION

Accurate identification of *Meloidoderita* spp. is a hard task because some of the differential diagnostic characters are difficult to observe in second-stage juvenile or adult stages (e.g., lateral fields). In fact, Sturhan (1983) identified second-stage juveniles and males of *Meloidoderita* from Iran as *M. kirjanovae*; but did not speciate with certainty the populations from Germany and Azores.

Based on morphometrical and morphological characteristics, the cystoid nematode population from southern Italy infecting water mint was identified as *M. kirjanovae*. Although we have no exact data on the possible origin of this population of *M. kirjanovae* in Italy, because of its finding in a naturally isolated environment, such as Laceno Lake at Avellino province (southern Italy), we hypothesized that this population is native to Italy. The morphological and morphometric parameters of *M. kirjanovae* populations from different geographical origins listed in the literature do not match exactly with our population. Kirjanova and Poghossian (1973) reported in their redescription that *M. kirjanovae* had four incisures in the lateral field of the male and juveniles, but Golden and Handoo (1984) observed only three incisures in the lateral field of specimens from Armenia and Israel. Our SEM data supported these observations confirming the presence of three incisures. Similarly, the lip region of second-stage juveniles of the Italian population of *M. kirjanovae* was smooth under SEM observations, while in the type population three to four annuli were reported (Kirjanova and Poghossian, 1973). The results of our examination also agree with those reported for a population of *M. kirjanovae* from Israel (Cohn and Mordechai, 1982). The secretion of the gelatinous matrix from the

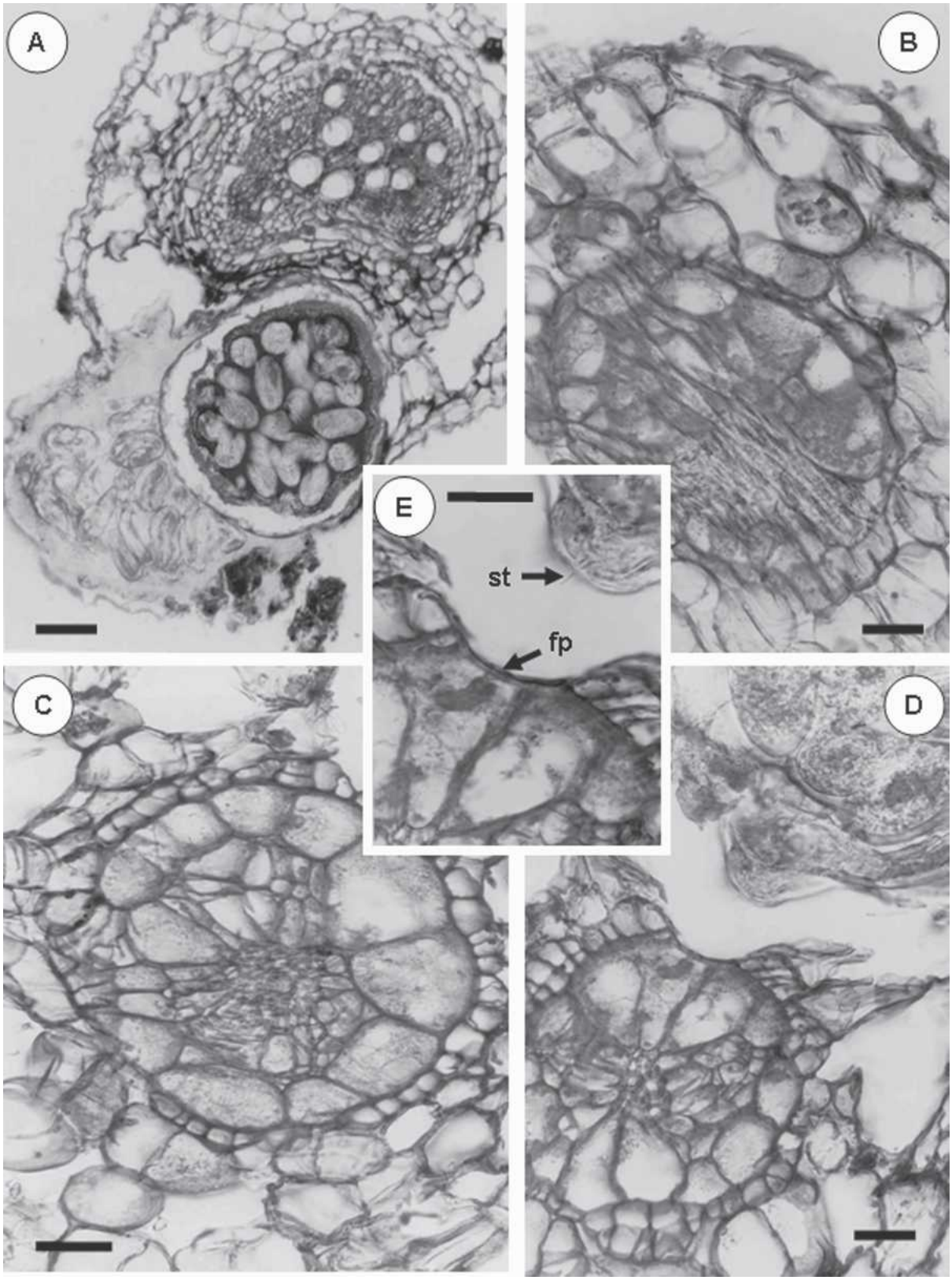

Fig. 5. Histopathology of *Meloidoderita kirjanovae* in roots of *Mentha aquatica*. A) Transverse section of root of mint infected by *M. kirjanovae* showing the nematode female (N) inside the cortical parenchyma. B-E) Reactions of cortical and pericycle cells to nematode infection showing syncitial formation. E) Details of the feeding cell on which distinct feeding peg (fp) is formed, st = Stylet. Scale bars: A = 100 µm; B-E = 25 µm)

vulva in *M. kirjanovae* differs from that of other tylenchulids such as *Tylenchulus* and *Trophonema* which secrete the gelatinous matrix from the excretory pore. This physiological characteristic may corroborate the results of the phylogenetic analysis.

The molecular analysis based on the internal transcribed spacer 1 (ITS1) and 5.8S gene sequences of nuclear ribosomal DNA (rDNA) clearly separated *M. kirjanovae* from *M. polygoni*. This molecular approach is a very useful and reliable tool for the identification of cystoid nematodes, especially when a small number of specimens is available. The phylogenetic (BI) tree from the present analysis was congruent with trees obtained from extended dataset for Criconematina (Subbotin et al., 2005) and Tylenchida (Subbotin et al., 2006). The basal position of the genus *Meloidoderita* together with *Sphaeronema* within Criconematina clade in our tree may indicate their close relationships. The hypotheses considering *Meloidoderita* as member of the family Sphaeronematidae (Siddiqi, 2000) or as member of the family Meloidoderitidae as earlier proposed by Kirjanova and Poghossian (1973) require further testing and analyses using other genetic markers and more representatives of sedentary nematodes of the suborder Criconematina.

Although the general pattern of parasitism found in this study is in general agreement with that described for *M. kirjanovae* by Kirjanova and Poghossian (1973) and Cohn and Mordechai (1982) in *Urtica dioica* L. and *Mentha longifolia* L., respectively, parasitism of *M. kirjanovae* on *M. aquatica* did not show any swelling of the root tissue around the nematode infection point, which was reported by Cohn and Mordechai in *M. longifolia* roots. Andrews et al. (1981) reported a similar parasitism pattern from a population of *Meloidoderita* sp. from Maryland (later described as *M. polygoni*) on *Polygonum hydropiperoides* Mild. Comparison of our results on histopathology with those reported by Subbotin and Chizhov (1986) indicated that *M. kirjanovae* infecting mint induced formation of syncytia which comprise about 400 cells. These cells had enlarged nuclei, vacuolated cytoplasm, and a high number of organelles. Syncytia clearly differ from those induced by *Heterodera* spp. (absence of protuberances on cell wall) or *Rotylenchulus reniformis* (larger size and more intensive lysis of a cell wall).

In conclusion, the biological and morphological observations and the molecular analyses conducted in this study suggest that *M. kirjanovae* populations from Italy and Israel may have originated from the same source.

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