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First record of *Helicotylenchus varicaudatus* Yuen, 1964 (Nematoda: Hoplolaimidae) parasitizing *Ammophila arenaria* (L.) Link in Portuguese coastal sand dunes

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Summary. A spiral nematode, *Helicotylenchus varicaudatus* Yuen, 1964, parasitizing *Ammophila arenaria* (L.) Link, the dominant grass in the Portuguese coastal sand dunes, is reported from Portugal for the first time and raises to seven the number of *Helicotylenchus* species detected in Portugal. A redescription of the species, with illustrations, and light and scanning electron microscope images of both female and male specimens, is presented. The rDNA containing the internal transcribed spacer regions (ITS) of *H. varicaudatus* was analysed with ITS-RFLP using the restriction endonuclease *Hinf* I. Molecular data from the ribosomal small subunit (SSU) (18S) confirmed the identification.

Key words: marram grass, ITS-RFLP, morphobiometry, SSU (18S), taxonomy.

Introduction

The genus *Helicotylenchus* was established by Steiner (1945) and more than 200 species are now attributed to this genus (Marais, 2001). *Helicotylenchus* is cosmopolitan, occurring in both cultivated and uncultivated soils. Firoza and Maqbool (1994) reported that more than one species often occur in the same sample and that the nematodes sometimes occur in large numbers. Due to the large number of species and to the intraspecific variation of some characters, it is difficult to identify species of *Helicotylenchus* (Fortuner *et al.,* 1981; Fortuner, 1984a, 1984b; Marais, 2001). *Helicotylenchus* species have been classified as ectoparasites, semi-endoparasites and even migratory endoparasites (Decraemer and Hunt, 2006). Plant damage directly attributed to *Helicotylenchus* has been reported for *H. digonicus, H. dihystera, H. indicus, H. labiatus, H. multicinctus*, *H. oleae* and *H. pseudorobustus* (Inserra *et al.*, 1979; Mancini *et al.*, 1983; Saeed *et al.*, 1989; Sarah, 1989, Vovlas and Larizza, 1994; Wouts and Yeates, 1994). In most plants with which *Helicotylenchus* is associated, the damage is insidious rather than severe, although nematode attack may lead to secondary infections from other pathogens (Yeates and Wouts, 1992).

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During an investigation of nematodes associated with marram grass, *Ammophila arenaria* (L.) Link, in Portuguese coastal sand dunes, a population of *Helicotylenchus* was found. *Helicotylenchus* is the most abundant genus of plant parasitic nematodes associated with *A. arenaria* in Portuguese sand dunes along the northern and south-western Atlantic coast (Schreck Reis *et al*., 2005). It is most abundant in the northern part of the country during the summer months, when population numbers of more than 100 nematodes g^{-1} of dry root are found, and interestingly the nematodes are more abundant in the roots than in the soil (Schreck Reis *et al*., 2005). According to Schreck Reis *et al*. (2008) this *Helicotylenchus* species does not cause any negative effects on *A. arenaria* above or below-ground.

The Portuguese *Helicotylenchus* population was studied and some considerable differences in some morphometric parameters of the females were found, e.g. they had a greater body length, raising the possibility that more than one species was involved. However, molecular data from ITS-RFLP analysis and the rDNA SSU (18S) partial sequence indicated that only one species occurred. The *Helicotylenchus* species infecting marram grass was identified as *Helicotylenchus varicaudatus* Yuen, 1964 and is here redescribed and illustrated. Seven *Helicotylenchus* species are now reported from Portugal (Table 1).

Table 1. *Helicotylenchus* species identified in Portugal.

Species	Associated plant	Reference
H. digonicus	Sugar cane	Lima (1962)
H. dihystera (syn. of *H. dihysteroides*)	*Begonia* sp., *Colocasia esculenta, Cactus* sp., *Mentha* sp., *Pelargonium* sp., Poligonaceae	Macara (1962)
	Musa sp.	Sher (1966)
	Maize and beans	Siddiqi (1972b)
	Tomato	Siddiqi (1972a)
	_	Krall (1985)
H. erythrinae	*Amaryllis* sp.	Macara (1962)
H. paraplatyurus	_	Siddiqi (1972a)
	_	Krall (1985)
H. pseudorobustus	Sugar cane and soybean	Lima (1962)
	Carrot and tomato	Abrantes *et al*. (1978)
	Leek and millet	Fortuner *et al*. (1984)
H. varicaudatus	*Ammophila arenaria*	-
H. vulgaris	*Olea europaea*	Abrantes *et al*. (1987)

Materials and methods

Origin and nematode propagation

Nematodes were obtained from the roots of *A. arenaria* collected by the first author (C. Schreck Reis) in the coastal sand dunes of the São Jacinto Natural Reserve, Aveiro (40º 41´ N, 8º 44´ W), a well preserved sand dune system in the northern Portuguese coast. Roots were cut into c. 1 cm pieces and nematode specimens were recovered using the modified Baermann funnel method (Abrantes *et al*., 1976). Nematodes were also propagated on *A. arenaria* plants. Three-week-old seedlings were transferred to 1.5 L pots containing sterilized sand (four seedlings per pot), and each seedling was inoculated with 30 nematodes recovered from *A. arenaria* roots. The pots were placed in a controlled-climate chamber, watered twice a week with tap water and once a month with Hoagland´s nutrition solution. Every six months the cultures were transferred to new seedlings.

Morphological and morphometrical examination

The nematodes were extracted from the *A. arenaria* roots using the modified Baermann funnel method, killed by gentle heat and fixed in triethanolamine formalin water solution (TAF), processed with anhydrous glycerol and mounted in desiccated glycerine (Seinhorst, 1959; Hooper, 1986; Santos and Abrantes, 1988). Two male paratypes (slides RIT765 and RIT766) and two female paratypes (slides RIT767 and RIT768) were deposited with accession No. IG 31510, in the nematode collection of the National Museum of Natural History, Royal Belgian Institute of Natural Sciences, Brussels, Belgium. All remaining nematodes, 8 males (slides 53/1 to 53/8) and 38 females (slides 53/9 to 53/46) were deposited in the nematode collection of the Nematology Laboratory, Department of Life Sciences, University of Coimbra, Portugal. For scanning electron microscopy (SEM), nematodes fixed in gluteraldehyde were transferred to a 2% osmium tetroxide solution and dehydrated in increasing concentrations of ethanol. After critical point drying with carbon dioxide and gold sputter coating, the specimens were viewed and photographed in a FEI Quanta 400 FEG/EDAX Genesis X4M scanning electron microscope at 10 kV (Eisenback and Hirshmann, 1979).

Molecular studies

Molecular studies were carried out using specimens established and multiplied on *A. arenaria* plants.

DNA of the Portuguese isolate was extracted according to Williams *et al*. (1992) with some modifications: 10 and 15 females of larger size, 15 and 20 females of smaller size, and 15 and 20 males were individually hand-picked and transferred to 0.2 mL PCR tubes containing 2.5 μL of lysis buffer (50 mM KCl, 10 mM Tris pH 9, 1.5 mM $MgCl_2$, 60 mg mL^{-1} proteinase K). The tubes were incubated at -70ºC for one hour and then transferred to 60ºC for 60 min followed by 15 min at 95ºC in a thermal cycler. The ITS region was amplified by adapting the methods of Cherry *et al*. (1997), Powers and Harris (1993) and Berry *et al*. (2007) using the forward primer rDNA2 (5´-TTGATTACGTCCCTGCCCTTT-3´) and the reverse primer rDNA1.58S (5´-ACGAGCCGAGTGATCCACCG-3´) (Vrain *et al*., 1992; Cherry *et al*., 1997). A PCR mixture (22.5 μL) containing 0.3 μM of each primer, 1 U Taq polymerase (Q-Biogene), 50 mM KCl, 10 mM Tris pH 9, 1.5 mM $MgCl_2$ and 200 μM dNTPs was added to the tubes. Amplification was done in a GeneAmp PCR System 2700 (Applied BioSystems, Darmstadt, Germany) thermal cycler using an initial denaturation at 94ºC for 5 min, 30 reaction cycles of 94ºC for 1 min, 57ºC for 1 min, 72ºC for 2 min and a final extension at 72ºC for 5 min. Ten μL of the reaction mixture was resolved by electrophoresis in a 1% agarose gel stained with ethidium bromide. The amplified ITS region (10 μL) was digested for four hours at 37ºC using 2.5 U of *Hinf* I (Amersham BioSciences, Little Chalfont, UK). Evaluation was performed in a 2% agarose gel stained with ethidium bromide.

For SSU (18S) amplification and sequencing, DNA was extracted from three samples containing 100 males, 100 females and a mixture of 100 males and females. Nematodes were lysed by thermal shock by immersing them in liquid nitrogen for 5 minutes after which they were incubated in a water bath at 100°C for 5 minutes. The lysate was then purified with the DNeasy Blood and tissue kit (Qiagen, Cologne, Germany) according to the manufacturer's instructions. The rDNA SSU (18S) was amplified using the universal primer 988F (5'-CTCAAAGATTAAGCCATGC-3') and a nematode-specific primer 1912R (5'-TTTACGGTCAGAACTAGGG-3') (Holterman *et*

al., 2006). PCR was performed in a final volume of 25 µL and contained 2 µL of DNA, 0.2 µM of each PCR primer, 0.2 mM dNTPs 1.8 mM $MgCl_2$, 1× buffer and 2.5 U FideliTaq DNA polymerase (USB). The following PCR program was used: 94ºC for 5 min.; 35× (94ºC, 40 sec.; 50ºC, 45 sec.; 68ºC, 90 sec.) followed by 68ºC, 10 min. The purified amplification products (High Pure PCR Product purification kit, Roche Diagnostics, Indianapolis, IN, USA) were sequenced using standard procedures at Eurofins MWG Operon (Ebersberg, Germany). Sequences were then manually reviewed to confirm and correct automatic base calling. Sequences from males, females and mixed samples were aligned by Clustal W (default parameters) to check for sequence similarity. Sequences of closely related species were identified by searching the GenBank nucleotide database (National Center of Biotechnology Information, NCBI, www.ncbi.nlm.nih.gov) and used to generate a Clustal W alignment (Thompson *et al*., 1994), with the mixed sample sequence from the Portuguese isolate, and *Globodera pallida* (U855119) and *G. rostochiensis* (AY593877) as outgroup. The sequences used are listed in Table 2. Maximum likelihood was performed using PAUP* 4b10 (Swofford, 2002). The robustness of the inferred tree was tested using nonparametric bootstrapping (with 1000 pseudo-replicates).

The sequence data from the 18S ribosomal RNA gene partial sequence reported in this study were deposited in the GenBank database (NCBI) under accession number HM237044.

Results and discussion

***Helicotylenchus varicaudatus* Yuen, 1964**

Female

Habitus C-shape (12.5%) or spiral (87.5%) (Figures 1A, E and 2A). Body length with large amplitude, 692.5±82.5 (510–890) µm long (Table 3). Lip region hemispherical, not offset, with two to five lip annules, occasional anastomosis (Figures 1A, B, 2E and 3B). Prestoma rectangular, centrally located on the labial disc, and surrounded by six pore-like openings of the inner labial sensilla (Figure 3A). Labial disc rounded in frontal view (Figure 3A). First lip annule with six longitudinal incisions, corresponding to two lateral lips and four medial lips (two sub-dorsal and two sub-ventral) (Figure 3A). Amphidial openings oval shaped, located between the labial disc and the lateral lips (Figure 3A). Cephalic framework well developed. Cephalids not seen. Stylet slender with anterior faces of stylet knobs rounded (57.5%) or flattened (42.5%) (Figures 1A, B and 2E). Medium bulb elongated, oval shaped, 9.1±0.7 (8–11) µm wide and 12.3±1 (9–12.5) µm long (Table 3). Hemizonid two annules long, located two to three annules anterior to the excretory pore (n=9). Hemizonion not seen. Excretory pore located anterior to level of esophago-intestinal valve at 16±1.6 (12.5–21.4) % of body length (Table 3). Oesophageal glands forming a wrap-around over anterior end of intestine, longest overlap being ventral, 52.1±9.8 (31–74) µm long (Table 3, Figures 1A and 2B). Lateral field with four lines, areolated opposite the oesophageal region. The inner two lines end in a v- or u-shaped tail (Figures 1C–D and 3C). Fasciculi not seen. Reproductive system with two functional genital branches, without regression of the posterior branch (Figures 1A and 2A). Anterior genital branch 20±3.5 (15.4–26.7) and posterior genital branch 19.1±3.7 (13.2–26) as a percentage of body length (n=15) (Table 3). Spermatheca offset, rounded, filled with sperm. Epiptygma folded into vagina. Phasmids pore-like, smaller than the corresponding ventral tail annule, located posterior to anus (Figures 1C, D and 2D). Tail 13.4±1.9

Table 2. GenBank accession numbers of the closely related species sequences used for alignment.

Species	Accession number (GenBank)
Helicotylenchus pseudorobustus isolate HeliPse	AY284606
H. multicinctus strain HeliMul1	FJ969124
H. canadensis isolate HeliCan	AY284605
H. vulgaris isolate HeliVul	AY284607
H. varicaudatus isolate wb14	EU306354
H. dihystera	AJ966486

(9.5–17.5) μm long (Table 3), with four to eight ventral tail annules; shape variable, asymmetrically rounded (35%) having a slight ventral indentation (Figures 1C, D, 2D and 3D,E).

Male

Habitus straight (10%) or arcuate (90%) (Figures 1H and 2F). Heads of males slightly higher than heads of females. Lip region (Figures 1F, 2G and 3F,G), prestoma (Figure 3G), labial disc (Figure 3G), first lip annule (Figure 3G), amphidial openings and cephalic framework (Figure 3G) similar to those of the females. Stylet slender with anterior face of stylet knobs posteriorly rounded (40%)

Table 3. Morphometric data of *Helicotylenchus varicaudatus* Yuen, 1964. All measurements are in µm and in the form: mean ± SD (range).

Character	Portugal		Belgium, New Zealand, Poland, Russia, The Netherlands, Turkey, UK [a]
	Females (n = 40)	Males (n= 10)	Females
L	692.5±82.5 (510–890)	631±52.6 (530–700)	520–910
a	29.9±2.4 (23.5–35.8)	33.9±2.4 (30.3–37.4)	17.4–29
b	7.6±0.8 (5.8–8.7)	7.5±0.6 (6.6–8.5)	4.3–7.7
b’	4.8±0.6 (3.6–6.3)	4.4±0.3 (3.9–4.7)	3.4–6
c	52.2±7.5 (39.4–70.7)	34±2.6 (34–37.3)	32.5–77
c’	1±0.1 (0.7–1.3)	1.9±0.2 (1.6–2.2)	0.5–1.2
m (5)	46.3±2.6 (40.9–52.1)	47.2±2.5 (42.9–50)	44–57
o (%)	23±5 (15–31)	24.5±6.1 (18.2–34.1)	10–39.5
Excretory pore as a percentage of total length	16±1.6 (12.5–21.4)	15.7±0.9 (14–16.6)	–
V (%)	64±1.5 (61–67)	–	57.2–67
OV_1 (%)	20±3.5 (15.4–26.7)	–	18–32
OV_2 (%)	19.1±3.7 (13.2–26)	–	17.5–30
Head region height	3.6±0.6 (3–5.5)	3.6±0.5 (2.5–4.5)	–
Head region width	6.5±0.8 (5–8)	6.4±0.6 (5.5–7.5)	–
Stylet length	23.7±1.1 (22–26)	21.2±1.2 (20–23)	23.8–33.6
Stylet cone length	10.9±1 (9–12.5)	10±0.9 (8.5–11)	–
Stylet knobs height	2.9±0.4 (2.5–4)	2.2±0.3 (2–2.5)	–
Stylet knobs width	5±0.8 (3.5–7)	3.6±0.7 (2.5–4.5)	5–6
Stylet base to dorsal gland opening (DGO)	5.4±1.1 (3.5–7)	5.1±1.1 (4–6.5)	3–13

continued on the next page

Table 3 continued

Character	Portugal		Belgium, New Zealand, Poland, Russia, The Netherlands, Turkey, UK [a]
	Females (n = 40)	Males (n= 10)	Females
Medium bulb length	12.3±1 (9–12.5)	11.2±1.5 (9–13)	–
Medium bulb width	9.1±0.7 (8–11)	7.8±0.9 (7–10)	–
Medium bulb valve length	3.4±0.4 (2.5–4)	2.8±0.3 (2.5–3)	–
Medium bulb valve width	2.5±0.3 (2–3)	2.1±0.2 (2–2.5)	–
Head to esophagus-intestine junction	91.6±6.1 (80–107)	84.7±5.7 (76.5–93)	104–133
Head to excretory pore	109.7±7 (94–121)	98.9±6.3 (86–108)	112–137
Head to posterior end of esophageal gland	144±10.9 (120–167)	145.1±16.6 (126–172)	140–183
Head to vulva	440.1±48.7 (335–550)	–	365–500
Body width at stylet knobs	14.4±1.5 (12.5–18.5)	11.4±0.8 (10–12)	–
Body width at excretory pore	18.1±1.3 (16–22)	15.6±1.1 (13.5–17.5)	–
Greatest body width at mid-body	23.3±2.9 (15–28.5)	18.6±1 (17–20)	22–34
Body width at vulva	21.1±2.9 (14–26)	–	–
Body width at anus	13.6±1.5 (10–17)	9.9±0.6 (9–11)	–
Width of one body annule at mid body	1.9±0.3 (1–2.5)	1.9±0.2 (1.5–2)	1.3–1.9
Spicule length	–	22.4±1.8 (20–25)	–
Gubernaculum length	–	5.9±0.9 (4.5–7)	–
Number of annules from phasmids to anus/cloaca	One to four posterior	Two to three posterior	Seven anterior to four posterior
Distance of phasmids to tail terminus	10.8±1.9 (7–14)	14.2±1.5 (12–16)	–
Tail length	13.4±1.9 (9.5–17.5)	18.6±1 (17–20)	8–19
Number of ventral tail annules	6.1±1.2 (4–8)	9.3±0.9 (8–11)	5–11

[a] Yuen (1964); Sher (1966); Volkova (1987); Bongers (1988); Eroshenko and Volkova (1988); Yeates and Wouts (1992), Brzeski (1998), Kepenekci and Ökten (1996); Bert and Geraert (2000).

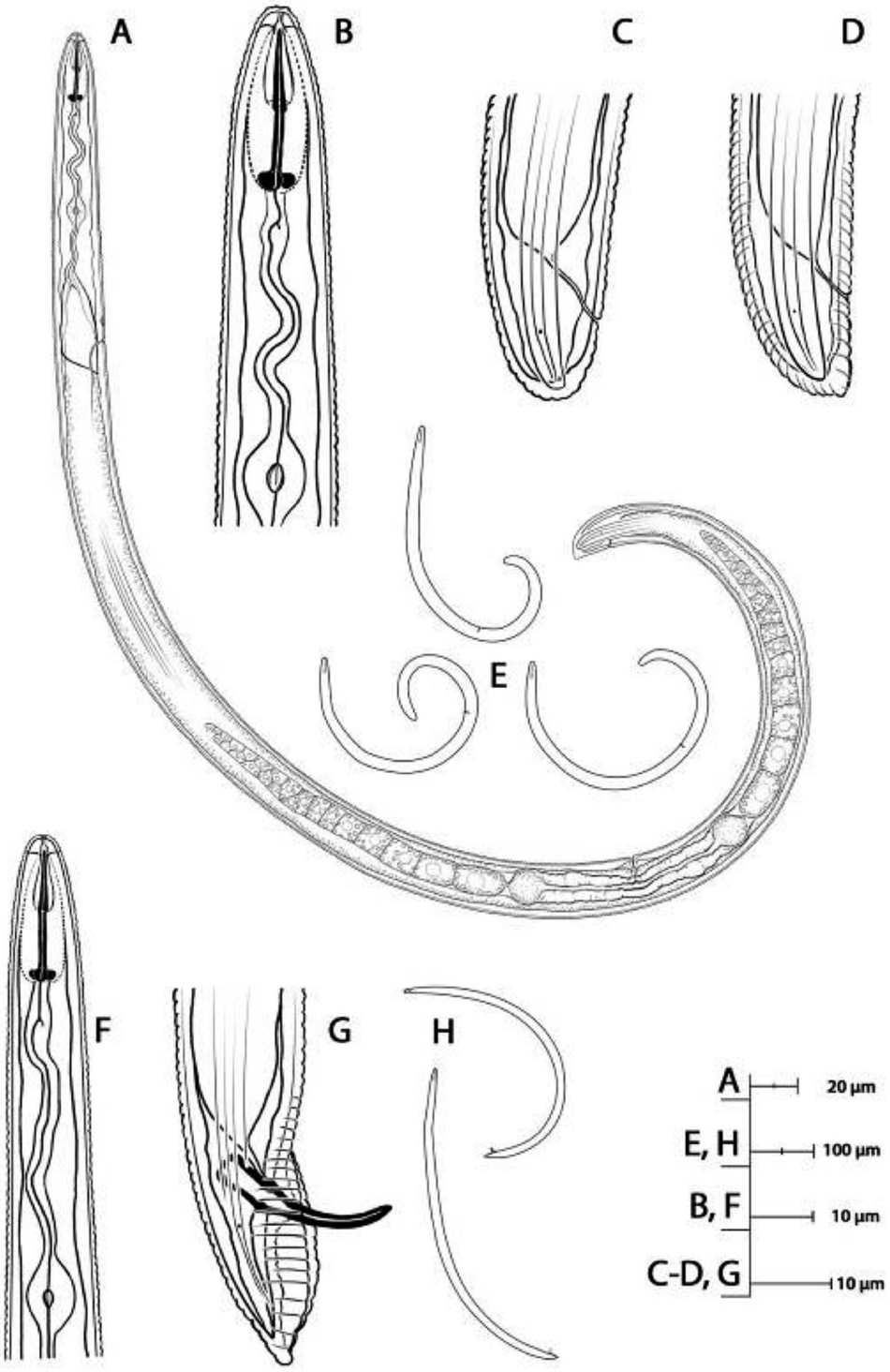


Figure 1. *Helicotylenchus varicaudatus*. Females (A–E). Whole female (A), head, lateral view (B), tails (C,D) and habitus (E). Males (F–H). Head, lateral view (F), tail, lateral view (G) and habitus (H). (Drawings by Fernando Correia).

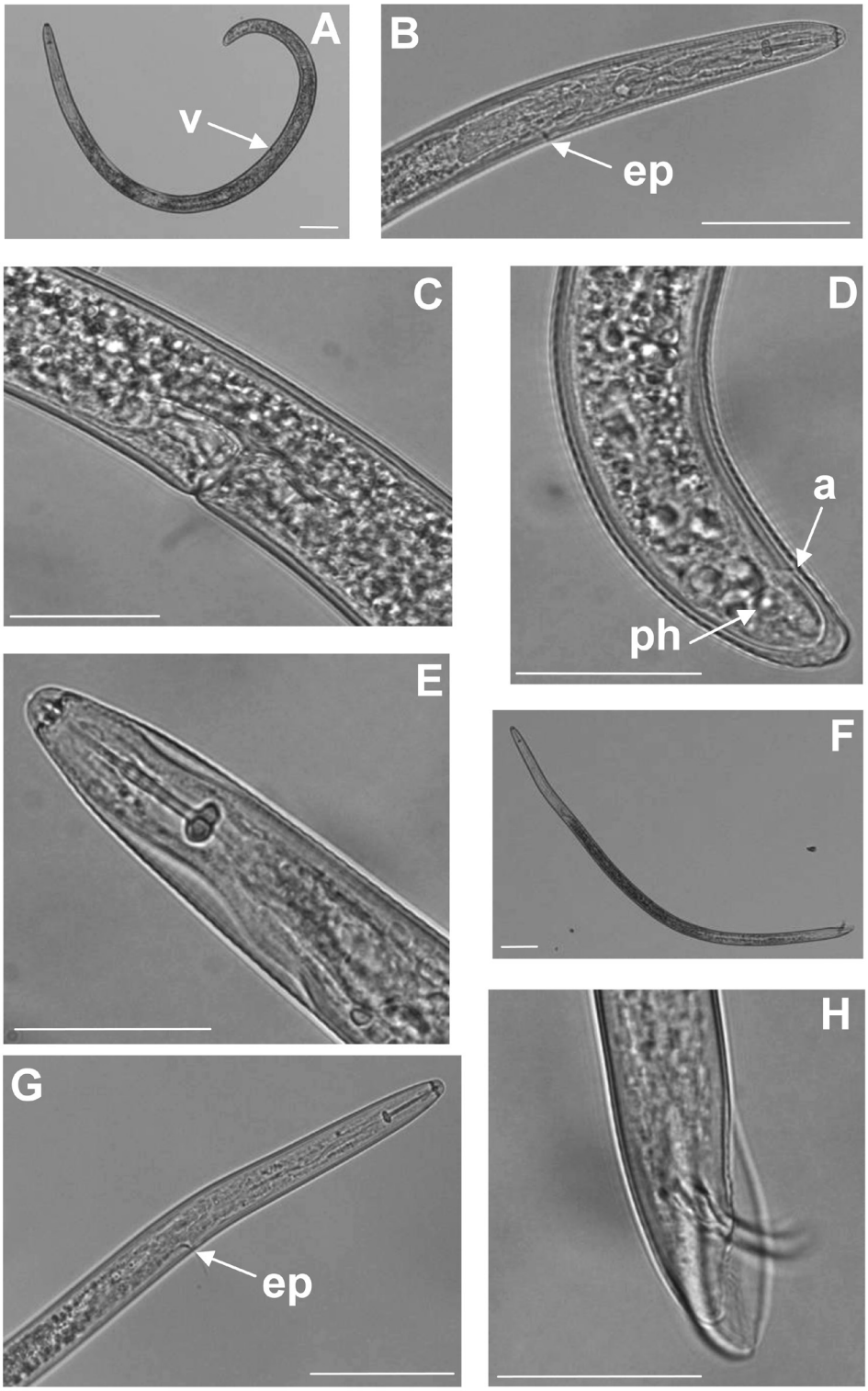


Figure 2. *Helicotylenchus varicaudatus*. Females (A–E). Habitus (A), anterior region, lateral (B), vulva region (C), tail (D) and head (E). Males (F–H). Habitus (F), anterior region (G) and tail (H). v, vulva; ep, excretory pore; a, anus; ph, phasmid. Scale bars: A, B, F, G=50 μm; C, D, E, H=25 μm.

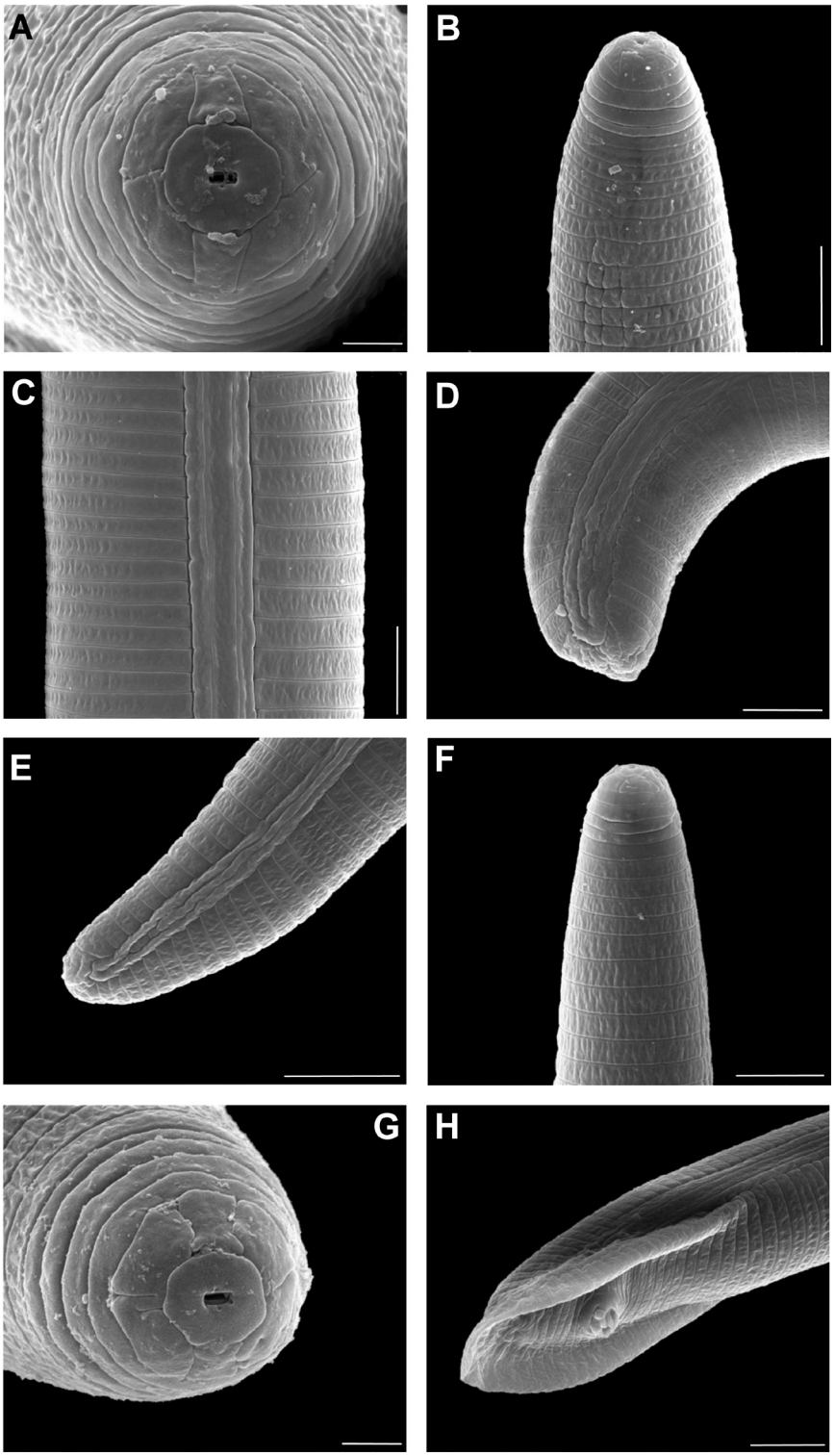


Figure 3. *Helicotylenchus varicaudatus*. Females (A–E). Lip region, face view (A), lip region, lateral view (B), lateral fields (C) and tails (D,E). Males (F–H). Lip region, lateral view (F), lip region, face view (G) and tail (H). Scale bars: A, G=1 μm; B, C, D, E, F, H=5 μm.

or flattened (60%) (Figures 1F and 2G). Hemizonid two annules long, located two to three annules anterior to excretory pore (n=2). Oesophageal glands forming a wrap-around over the anterior end of the intestine, with a 61.7±16.1 (38.5–90) μm long ventral overlap (Table 3 and Figure 2G). Lateral field areolated opposite the oesophageal region and the bursa. Phasmids one to two annules posterior to the cloaca level (Figure 1G). Spicule length exceeds tail length (Figures 1G and 2H). Bursa extends to end of tail (Figures 1G, 2H and 3H). Tail with rounded projection curved ventrally (Figures 1G and 2H).

Remarks

The description of the Portuguese *H. varicaudatus* population agrees with previous descriptions of *H. varicaudatus* from various locations. The specimens of this study ranged widely in body length, 510–890 μm, but they conformed to the body length reported in the literature, 520–910 μm (Kepenekci and Ökten, 1996; Brzeski, 1998). The V value showed the least variation with a coefficient of variation (CV) of 2% and a range of 61–67%, which agreed with V values previously reported for *H. varicaudatus*, 56.9–68 % (Volkova, 1987; Bert and Geraert, 2000). The stylet length of the current specimens, 22–26 μm, was equal to the lower end of the range reported in the literature, 23.8–33.6 μm (Eroshenko and Volkova, 1998; Bert and Geraert, 2000). The coefficient of variation for stylet length was 5%. Stylet length usually has the smallest CV among the quantitative characters, 1.7% in the progeny of a single *H. dihystera* female, and 1.6 to 4% in field populations of a single species (Fortuner, 1979; Fortuner *et al*., 1981). The character of a subdivided first lip annule is reported only for a few species, *H. varicaudatus*, *H. silvicola* Van den Berg and Marais, 1995, *H. curatus* Marais, Van den Berg, Swart and Tiedt, 2004 and to an Italian population of *H. multicinctus* (Cobb, 1893) Golden, 1956 (Vovlas, 1983). No other *Helicotylenchus* species has been reported as having a division of the first lip annule (Sher and Bell, 1975; Loof, 1984; Abrantes *et al*., 1987; Marais and Buckley, 1992; Geraert, 1997; Marais, 1998; Marais and Quénéhervé, 1999; Orion *et al.,* 1999; Bert and Geraert, 2000; Marais *et al*., 2000; Van den Berg and Marais, 1995; Van den Berg *et al.,* 2003; Marais *et al*., 2004) and the only *H. multicinctus* population reported as having a subdivided first lip annule is the Italian population. The Italian *H. multicinctus* population was not available for study, but according to the published data it may be an Italian population of *H. varicaudatus*. Yuen (1964) stressed the variability of the tail shape in his description of *H. varicaudatus*. The specimens examined in this study showed less variation than the original description, and mostly showed only two tail shapes. The Portuguese population differed from most other populations in that it contained male nematodes. Males were found at all locations, although usually only in small numbers. Males were not usually found in the other populations; they have only been reported in populations from The Netherlands (Loof, 1984; Bongers, 1988) and Poland (Brzeski, 1998).

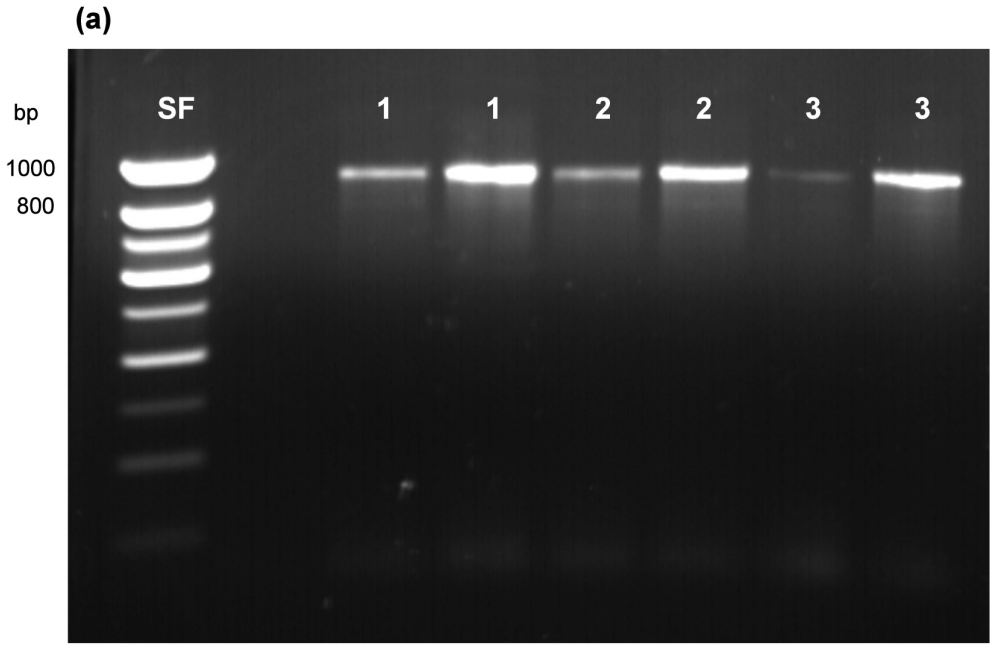
Helicotylenchus varicaudatus has been reported from various countries: Austria, Belgium, Bulgaria, the Czech Republic, England, France, Germany, India, Italy, New Zealand, Poland, Russia, Slovakia, Spain, The Netherlands, Turkey, and the United States (Yuen, 1964; Sher, 1966; Krall, 1985; Yeates and Wouts, 1992; Brzeski, 1998; Bert and Geraert, 2000; Stollarova, 2001; Lehman, 2002; Talavera and Navas, 2002; Háněl, 2007). Only Brzeski (1998) reported *H. varicaudatus* from sand dunes on the Baltic coast, without however specifying the host plant.

Molecular studies

Amplification of the ITS region of rDNA produced a 980-bp product. Restriction digestion with *Hinf* I produced a pattern having a strong band with 400 bp, and a fainter band with ca. 160 bp for all samples regardless of the specimen used (bigger females, smaller females or males) (Figure 4).

Amplification of DNA 18S produced a 1100-bp product for the three samples: males, females and a mixture of males and females. The sequences used are listed in Table 3. All the sequences were identical when aligned by Clustal W, showing that the same species occurred in all three samples (misalignments were found only with the homopolymers, and these were sequencing artefacts). Only the sequence from the mixture of males and females was therefore used for further analysis.

The smallest differences were in the 18s rDNA sequence of *H. varicaudatus* accession number EU306354. The Portuguese *H. varicaudatus* isolate differed from the Belgian isolate in 10 SSU rDNA nucleotides (1%) with a 912 bp sequence overlap.



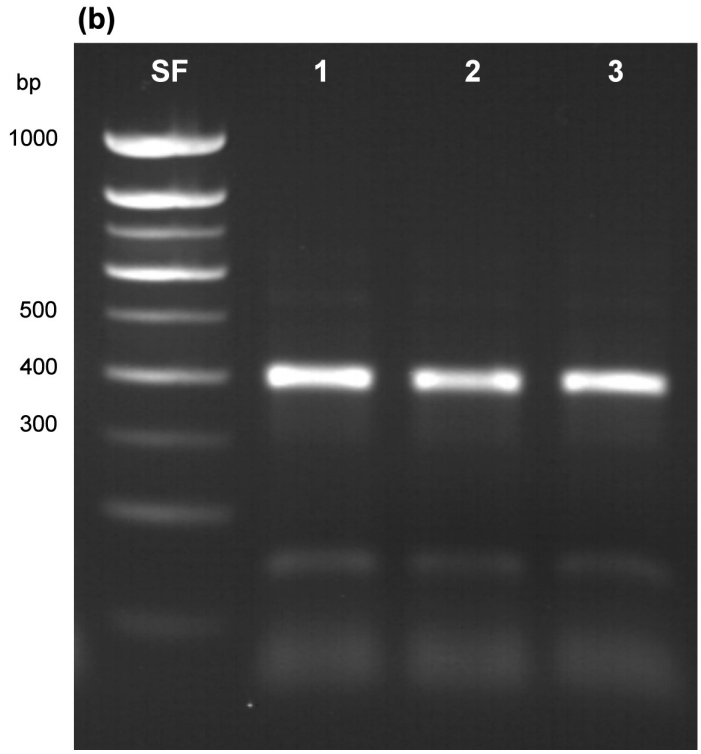


Figure 4. (A) ITS-PCR amplified products on 1.5% agarose gels. 1. *Helicotylenchus varicaudatus* bigger females (10 and 15 nematodes), 2. *H. varicaudatus* smaller females (15 and 20 nematodes) and 3. *H. varicaudatus* males (15 and 20 nematodes). (B) *Hinf*I-digested PCR-RFLP patterns on 2% agarose gels. 1. *Helicotylenchus varicaudatus* bigger females (15 nematodes), 2. *H. varicaudatus* smaller females (20 nematodes) and 3. *H. varicaudatus* males (20 nematodes). SF=DNA marker, Smart Ladder SF (100 bp ladder, Eurogentec, Belgium).

However, phylogenetic analysis placed these isolates together with high support (bootstrap=98%) and placed it as sisters to a clade of *H. canadensis* and *H. vulgaris* with maximal support (Figure 5). Phylogenetic analysis with *H. varicaudatus* accession number EU306354 (Bert and Geraert, 2000; Bert *et al*., 2008) therefore supported the identification of the Portuguese isolate as *H. varicaudatus*.

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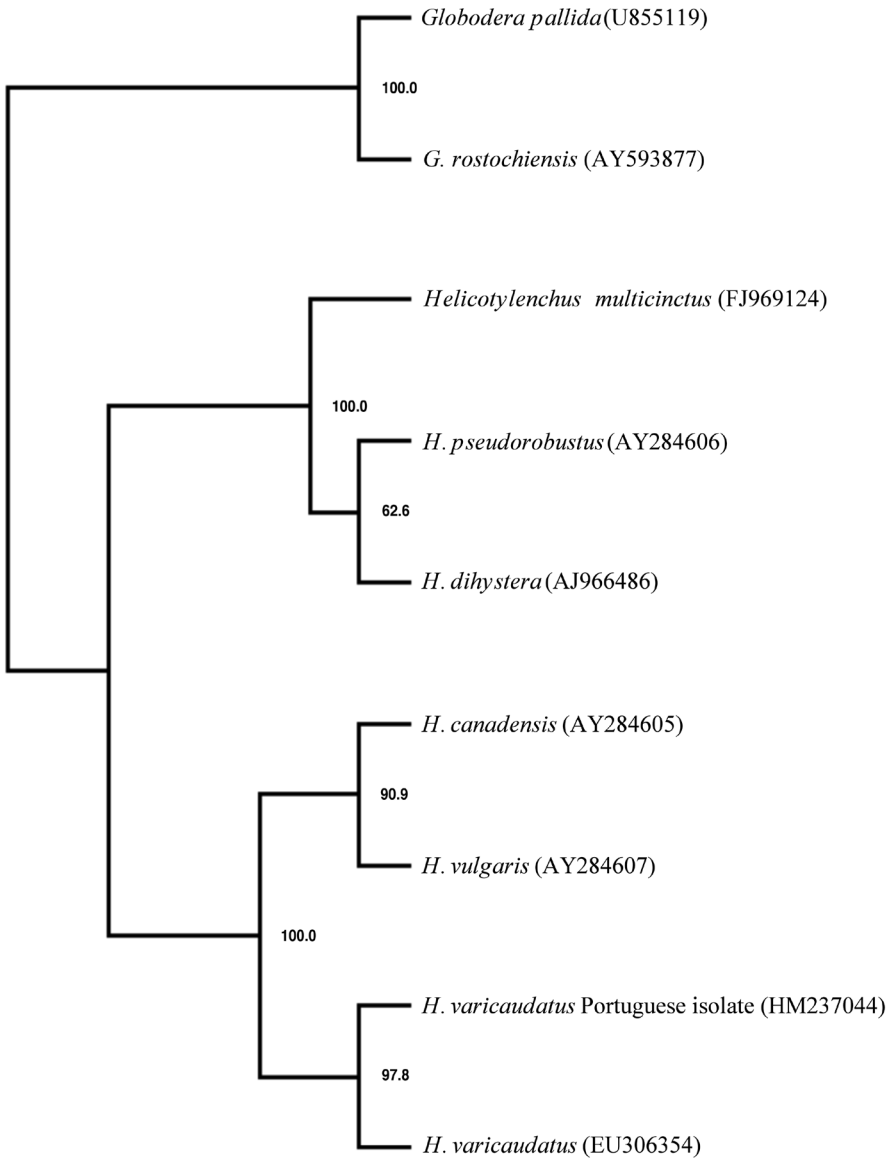

Figure 5. Maximum likelihood (ML) tree of *Helicotylenchus* species retrieved from the GenBank database and from the Portuguese isolate aligned with Clustal W (Thompson *et al*., 1994) with *Globodera pallida* and *G. rostochiensis* as outgroup. ML was performed using PAUP* 4.0b10 (Swofford, 2002). Robustness of the inferred tree was tested using nonparametric bootstrapping (with 1000 pseudoreplicates). GenBank accession codes are shown in parentheses. Bootstrap support values are indicated at nodes.

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Literature cited

Abrantes I.M.O., M.M.N. Morais, I.M.P.F.R. Paiva and M.S.N.A. Santos, 1976. Análise nematológica de solos e plantas. *Ciência Biológica, Ecology and Systematics* 1, 139–155.

Abrantes I.M.O., M.M.N. Morais and M.S.N.A. Santos, 1978. Nemátodos e plantas hospedeiras identificados em Coimbra, Portugal, durante 1972–1977. *Ciência Biológica, Ecology and Systematics* 4, 23–44.

Abrantes I.M.O., N. Vovlas and M.S.N.A. Santos, 1987. Morphological studies on six tylenchid nematode species associated with olive in Portugal. *Ciência Biológica, Ecology and Systematics* 7, 1–9.

Berry S., M. Fargette, S. Morand, and P. Cadet, 2007. Reliability of PCR-based techniques for detection and discrimination of plant-parasitic nematodes of sugarcane. *Nematology* 9, 499–514.

Bert W. and E. Geraert, 2000. Nematode species of the order Tylenchida, new to the Belgian Nematofauna with additional morphological data. *Belgian Journal of Zoology* 130, 47–57.

Bert W., F. Leliaert, A.R. Vierstraete, J.R. Vanfleteren and G. Borgonie, 2008. Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Nematoda: Rhabditida). Molecular Phylogenetics and Evolution 48, 728–744.

Bongers A.M.T., 1988. *De Nematoden van Nederland*. Pirota Schoorl, Bibliotheek uitgave KNNV, nr. 46, 408 pp.

Brzeski M.W., 1998. *Nematodes of Tylenchina in Poland and Temperate Europe*. Muzeum i Instytut Zoologii Polska Akademia Nauk, Poland, 397 pp.

Cherry T., A.L. Szalanski, T.C. Todd and T.O. Powers, 1997. The internal transcribed spacer region of *Belonolaimus* (Nemata: Belonolaimidae). *Journal of Nematology* 29, 23–29.

Decraemer W. and D.J. Hunt, 2006. Structure and Classification. *In: Plant Nematology* (R.N. Perry and M. Moens, eds.) CAB International Publishing, Oxfordshire, UK, 3–32.

Eisenback J.D. and H. Hirschman, 1979. Morphological comparison of second-stage juveniles of several *Meloidogyne* species (root-knot nematodes) by scanning electron microscopy. *Scanning Electron Microscopy* 3, 223–230.

Eroshenko A.S. and T.V. Volkova, 1988. Parasiticheskie nematody rastenii iuga Dal'nego Vostoka. Vladivostok: DVO AN SSSR.

Firoza K. and M.A Maqbool, 1994. A diagnostic compendium of the genus *Helicotylenchus* Steiner, 1945 (Nematoda: Hoplolaimidae). *Pakistan Journal of Nematology* 12, 11–50.

Fortuner R. 1979. Morphometrical variability in *Helicotylenchus* Steiner, 1945. 1. The progeny of a single female. *Revue de Nématologie* 2, 179–202.

Fortuner R. 1984a. Morphometrical variability in *Helicotylenchus* Steiner, 1945. 5. On the validity of ratios. *Revue de Nématologie* 7, 137–146.

Fortuner R. 1984b. Morphometrical variability in *Helicotylenchus* Steiner, 1945. 6. Value of the characters used for specific identification. *Revue de Nématologie* 7, 245–264.

Fortuner R., A.R. Maggenti and L.M. Whittaker, 1984. Morphometrical variability in *Helicotylenchus* Steiner, 1945-4: Study of field populations of *H. pseudorobustus* and related species. *Revue de Nématologie* 7, 121–135.

Fortuner R., G. Merny and C. Roux, 1981. Morphometrical variability in *Helicotylenchus* Steiner, 1945. 3. Observations on African populations of *Helicotylenchus dihystera* and considerations on related species. *Revue de Nématologie* 4, 235–260.

Geraert E., 1997 Comparison of the head patterns in the Tylenchoidea (Nematoda). *Nematologica* 43, 283–294.

Háněl L., 2007. Species and genera of soil nematodes inhabiting tree plantations on colliery spoils near Sokolov. In: *9th Central European Workshop on Soil Zoology,* April 17–20, 2007, České Budějovice, Czech Republic, 53–58.

Holterman M., A. van der Wurff, S. van den Elsen, H. van Megen, T. Bongers, O. Holovachov, J. Bakker, and J. Helder, 2006. Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward Crown Clades. *Molecular Biology and Evolution* 23, 1792–1800.

Hooper D.J., 1986. Handling, fixing, staining and mounting nematodes. In: *Laboratory methods for Work with Plant and Soil Nematodes* (J.F. Southey, ed.), Ministry of Agriculture, Fisheries and Food, London, UK, 59–80.

Inserra R.N., N. Vovlas and A.M. Golden, 1979. *Helicotylenchus oleae* n. sp. and *H. neopaxilli* n. sp. (Hoplolaimidae), two new spiral nematodes parasitic on olive trees in Italy. *Journal of Nematology* 11, 56–62.

Krall E.L., 1985. *Root Parasitic Nematodes – Family Hoplolaimidae* [Translated from Russian]. Nauka Publishers, Leningrade Branch, Leningrad, Russia, 1978, 508 pp.

Kepenekci I. and M.E. Ökten, 1996. Beypazari (Ankara) İlçesi'nde havuf ile münabeye giren domates ekiliú alanlarinda saptanan *Helicotylenchus* (Tylenchida, Hoplolaimidae) cinsine baģli türler. *Türk Entomoloji Dergisi* 20, 137–148.

Lehman P., 2002. *Phytoparasitic Nematodes Reported in*

Florida. Nematology Circular, Division of Plant Industry, Florida Department of Agriculture and Consumer Service, CA, USA, 18 pp.

Lima M.B., 1962. *Introdução ao Estudo dos Nemátodes de Portugal Continental* – Relatório Final do Curso de Engenheiro Agrónomo. Universidade Técnica de Lisboa, Instituto Superior de Agronomia, Lisboa, Portugal, 141 pp.

Loof P.A.A., 1984. Observations on *Helicotylenchus varicaudatus* Yuen, 1964 (Nematoda: Hoploaimidae*). Mededelingen van de Faculteit. Landbouwwetenschappen. Rijksuniversiteit Gent* 49, 621–627.

Macara A.M., 1962. *Contribuição para o estudo de algumas espécies do género Heterodera Schmidt 1871 encontradas em Portugal* - Relatório Final do Curso de Engenheiro Agrónomo. Universidade Técnica de Lisboa, Instituto Superior de Agronomia, Lisboa.

Mancini, G., A. Cotroneo and F. Moretti, 1983. Response of three pines to parasitism by *Helicotylenchus digonicus* (Nematoda: Hoplolaimidae). *European Journal of Forest Pathology* 13, 245–250.

Marais M., 1998. Some species of *Helicotylenchus* Steiner, 1945 from South Africa (Nematoda: Hoplolaimidae). *Fundamental and Applied Nematology* 21, 327–352.

Marais M., 2001. A monograph on the genus *Helicotylenchus* Steiner, 1945. Ph. D. Agriculture Dissertation, University of Stellenbosch, Stellenbosch, South Africa.

Marais M. and N.H. Buckley, 1992. External morphology of eight South African *Helicotylenchus* species (Hoplolaimidae: Nemata). *Phytophylactica* 24, 297–306.

Marais M. and P. Quénéhervé, 1999. A new species of *Helicotylenchus* from French Guiana, with notes on two known species (Nemata: Hoplolaimidae). *Journal for Nematode Morphology and Systematics* 2, 81–88.

Marais M., E. Van den Berg, P. Quénéhervé and L.R Tiedt, 2000. Description of *Helicotylenchus kermarreci* n. sp., with notes on some *Helicotylenchus* Steiner, 1945 and a *Rotylenchus* Filip'ev, 1936 species (Nemata: Hoplolaimidae) from the Guadeloupe Islands, French West Indies. *Journal for Nematode Morphology and Systematics* 2, 159–172.

Marais M., E. Van den Berg, A. Swart and L.R. Tiedt, 2004. Plant nematodes in South Africa. 7. A check list of plant nematodes from the Fynbos Biome, with a description of *Helicotylenchus curatus* sp. n. *Koedoe* 47, 67–78.

Orion D., Y. Levy, Y. Israeli and E. Fischer, 1999. Scanning electron microscope observations on spiral nematode (*Helicotylenchus multicinctus*) – infested banana roots. *Nematropica* 29, 179–183.

Powers T.O. and T.S. Harris, 1993. A Polymerase Chain Reaction method for identification of five *Meloidogyne* species. *Journal of Nematology* 25, 1–6.

Saeed M., A. Khan, S.A. Khan and M. Aslam, 1989. Pathogenicity of the spiral nematodes *Helicotylenchus indicus* Siddiqi, 1963 on sugar cane (*Saccharum officinarum*). *Sarhad Journal of Agriculture* 5, 77–78.

Santos M.S.N.A. and I.M.O. Abrantes, 1988. Morphological characters and methods for preparing nematodes. In: *Nematode Identification and Expert System Technology*. (R. Fortuner, ed.). Plenum Press, New York, NY, USA, 201–215.

Sarah J.L., 1989. Banana nematodes and their control in Africa. *Nematropica* 19, 199–216.

Schreck Reis C., H. Freitas and W.H. van der Putten, 2005. Plant-parasitic nematodes associated with *Ammophila arenaria* (L.) Link in Portuguese coastal sand dunes. *Nematologia Mediterranea* 33, 11–18.

Schreck Reis C., H. Freitas and W.H. van der Putten, 2008. Responses of root-feeding nematodes (*Helicotylenchus* spp.) to local and non-local populations of the host plant *Ammophila arenaria*. *Applied Soil Ecology* 39, 245–253.

Seinhorst J.W., 1959. A rapid method for the transfer of nematodes from fixative to anyhydrous glycerin. *Nematologica* 4, 67–69.

Sher S.A., 1966. Revision of the Hoplolaiminae (Nematoda) VI. *Helicotylenchus* Steiner, 1945. *Nematologica* 12, 1–56.

Sher S.A. and L. Bell, 1975. Scanning electron micrographs of the anterior region of some species of Tylenchoidea (Tylenchida: Nematoda). *Journal of Nematology* 7, 69–83.

Siddiqi M.R., 1972a. On the genus *Helicotylenchus* Steiner, 1945 (Nematoda: Tylenchida), with descriptions of nine new species. *Nematologica* 18, 74–91.

Siddiqi M.R., 1972b. *Helicotylenchus dihystera*. *Commonwealth Institute of Helminthology, Descriptions of Plant-Parasitic Nematodes*, Set 1, No. 9, 3 pp.

Steiner G., 1945. *Helicotylenchus*, a new genus of the plant parasitic nematodes and its relationship to *Rotylenchus* Filipjev. *Proceedings of the Helminthological Society of Washington* 12, 34–38.

Stollarova I., 2001. Free-living and plant parasitic nematode communities of two forest nurseries in Slovakia. *Biologia* 56, 131–139.

Swofford D.L., 2002. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4. Sinauer Associates, Sunderland, Massachusetts.

Talavera M. and A. Navas, 2002. Incidence of plant-parasitic nematodes in natural and semi-natural mountain grassland and the host status of some common grass species. *Nematology* 4, 541–552.

Thompson J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673–4680.

Van den Berg E. and M. Marais, 1995. New species of *Tylenchida* Chitwood (Nemata) from the Amazonas Province, Brazil. *African Plant Protection* 1, 25–39.

Van den Berg, E., M. Marais, S. Gaidashova and L.R. Tiedt. 2003. Hoplolaimidae Filip'ev, 1934 (Nemata) from Rwandan banana fields. *African Plant Protection* 9, 31–42.

Volkova T.V., 1987. [Nematodes of the genus Helicotylenchus Steiner, 1945 from the rhizosphere of conifers in the southern Far-East]. In: *Gel'minty I Vyzyvaemye imi Zabolevaniya,* (Y.L. Mamaev, ed.). Dal'nevostochnyi Nauchnyi Tsentr AN CCCP, Vladivostok., Russia, 115–

122 (in Russian).
Vovlas N., 1983. Morphology of a local population of *Helicotylenchus multicinctus* from Southern Italy. *Revue de Nématologie* 6, 327–329.
Vovlas N. and A. Larizza, 1994. Embryogenic patterns of parasitic habits of *Helicotylenchus oleae* and *H. pseudorobustus*. *Afro-Asian Journal of Nematology* 4, 17–21.
Vrain T.C., D.A. Wakarchuk, A.C. Lévesque and R.I. Hamilton, 1992. Intraspecific rDNA restriction fragment length polymorphism in *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563–573.
Williams B.D., B. Schrank, C. Huynh, R. Shownkeen and R.H. Waterston, 1992. A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. *Genetics* 13, 609–624.
Wouts W.M. and G.W. Yeates, 1994. *Helicotylenchus* species (Nematoda: Hoplolaimidae) from native vegetation and undisturbed soils in New Zealand. *New Zealand Journal of Zoology* 21, 213–224.
Yeates G.W. and W.M. Wouts, 1992. *Helicotylenchus* spp. (Nematoda: Tylenchida) from managed soils in New Zealand. *New Zealand Journal of Zoology* 19, 13–23.
Yuen P.H., 1964. Four new species of *Helicotylenchus* Steiner (Hoplolaiminae: Tylenchida) and a redescription of *H. canadensis* Waseem, 1961. *Nematologica* 10, 373–387.

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