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ORIGINAL ARTICLE

Morphological and molecular characters of two *Helicotylenchus* species from South Africa and relationship of selected soil parameters with *H. pseudorobustus*

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Abstract
During a survey of plant-parasitic nematodes in South Africa's Limpopo Province, two species of *Helicotylenchus* were identified, namely *H. dihystera* and *H. pseudorobustus*. The morphological and molecular characteristics of these species were found to be consistent with those of the known species. A phylogenetic analysis of *Helicotylenchus* populations based on 28S rDNA was conducted, and it was found that the *H. dihystera* identified in this study belonged to the same group as other *H. dihystera* specimens with a 1.00 posterior probability support. Moreover, phylogenetic analysis of *H. pseudorobustus* based on 18S rDNA placed the test population close to other *H. pseudorobustus* specimens with 0.97 posterior probability. Scanning electron microscopy (SEM) for *Helicotylenchus* species also revealed noticeable dissimilarities in the labial disc and lateral field of the tail region between the two species from the present study, including *H. pseudorobustus*, and *H. dihystera*. The redundancy analysis (RDA) showed that *H. pseudorobustus* had a correlation with pH and clay of the soil. In conclusion, despite the challenges associated with identifying *Helicotylenchus* species, SEM and rDNA markers can be considered as highly effective tools to distinguish the species correctly and accurately.

Keywords 28S rDNA · *Helicotylenchus* · Morphology · Phylogeny · South Africa

Introduction

The genus *HeIicotylenchus* Steiner, 1945, with 200 nominal species, comprises a group of semi-endoparasitic nematodes that are known to cause significant damage to various crop plants worldwide (Marais 2001; Subbotin et al. 2011). These nematodes have the ability to manipulate the physiology of their host plant to meet their requirements. As a result, nutrient uptake is inhibited, which leads to stunted growth and reduced crop yield (Riascos-Ortiz et al. 2020). Moreover, the feeding process of these nematodes creates an opening for other pathogens to enter the plant, further exacerbating the damage caused. Studies conducted by Barham et al. (1974) and Hamiduzzaman et al. (1997) confirmed the severity of the damage caused by these nematodes, highlighting the need for effective management strategies to protect crops from their negative impact. Some species, such as *H. vulgaris* have been recognized as severely damaging to sugar beets in England (Spaull 1982). *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961, as the type species of *Helicotylenchus* was described by Cobb in 1893 from sugar cane field in Australia. *Helicotylenchus dihystera* is the most commonly distributed species in the Afro and Neotropical region (Marais 2001). This species is considered a sedentary parasite (Yeates 1971), whereas Machado et al. (2019) and da Silva et al. (2023) indicated that *H. dihystera* could behave both as ectoparasite and endoparasite. According to Chapuis-Lardy et al. (2015), *H. dihystera* is one of the main plant-parasitic nematodes present in western Africa.

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In South Africa, Marais (2001) studied *Helicotylenchus* species using morphological traits, showing that *H. dihystera* and *H. pesudorobustus* (Steiner, 1914) Golden, 1956 was widely distributed. However, the identification of species using morphological characters alone has some limitations due to the overlap of morphometric and morphological characters, especially in genera with a large number of species. For instance, *H. dihystera* and *H. pesudorobustus* showed similar tail morphology, as was shown by Marais (2001). Therefore, molecular studies using rDNA (Subbotin et al. 2015; Shokoohi et al. 2018) may assist in establishing trustworthy relationships of species in such genera. Therefore, the present study aimed 1) to characterise *H. dihystera* and *H. pseudorobustus* using morphological, morphometric, and molecular techniques and 2) to discuss morphometric variation among *H. dihystera* and *H. pseudorobustus* from South Africa, and 3) to study the relationship between *H. pseudorobustus* and selected soil parameters.

Material and methods

Morphological study

Soil samples were collected in January 2023 from the rhizosphere of a Kikuyu grass field located in Magoebaskloof and Haenertsburg, Limpopo Province, South Africa. Nematodes were extracted from the soil samples using the modified tray method (Shokoohi 2023). To preserve the nematodes, they were fixed in a hot 4% formaldehyde solution, except for the specimens used for molecular analyses. After fixing, the nematodes were transferred to glycerin (De Grisse 1969) and mounted on permanent glass slides.

Light microscopy (LM)

The mounted specimens underwent detailed examination and measurement (De Man 1881) procedures using a high-quality VWR microscope (model BL384: Italy). The microscopic images were captured using a state-of-the-art Nikon Eclipse 80i light microscope that was equipped with differential interference contrast optics (DIC) and a powerful Nikon Digital Sight DS-U1 camera (Tokyo, Japan). To ensure the final drawings were as detailed and accurate as possible, the captured micrographs were then meticulously edited using Adobe® Photoshop® CS.

Scanning electron microscopy (SEM)

For scanning electron microscopy an adapted method of Green (1967) was used, and the process of critical point drying was excluded. Mounted nematode specimens were removed from slides, hydrated in distilled water, cleaned in an ultrasonic bath, and dehydrated in a graded ethanol series (Shokoohi et al. 2007). Thereafter, specimens were chemically dried using hexamethyldisilazane (HMDS, Sigma) (Shively and Miller 2009). This method replaces the complicated drying techniques of critical point drying (CPD) preventing shrinkage as well as chances of losing specimens in the CPD process. After this, the specimens were mounted on copper conductive tape, coated, and observed with a Field Emission Scanning Electron Microscope (FE-SEM; model: JEOL JSM-7800F; Japan) at 5 kV.

Phylogenetic analysis

DNA was extracted from three *Helicotylenchus* specimens using the Chelex method (Walsh et al. 1991). The process involved placing the nematodes in a 1.5 ml Eppendorf tube with 5 µl of double distilled water, followed by crushing them with a fine needle and vortexing. Afterward, 30 µl of 5% Chelex® 50 and 2 µl of proteinase K were added to the tube, mixed, and incubated at 56 °C for two hours. The tube was then deactivated at 95 °C for 10 min to inactivate the proteinase K. Finally, the supernatant was extracted and stored at -20 °C.

For partial amplification of the 18S rDNA regions, SSU F04 (5'-GCTTGTCTCAAAGATTAAGCC–3') and SSU R26 (5'-CATTCTTGGCAAATGCTTTCG–3') (Blaxter et al. 1998) were used in the PCR reactions. For 28S rDNA amplification, D2A (5'-ACAAGTACCGTGAGGGAAAGTTG–3') and D3B (5'-TCGGAAGGAACCAGCTACTA–3') (De Ley et al. 1999) were used. The amplification was carried out in a Bio-Rad master cycler using 8 µl of the DNA template, 12.5 µl of 2X PCR Master Mix Red (Promega, USA), 1 µl of each primer (10 pmol µl-1), and ddH_2O to make a final volume of 30 µl. The program involved an initial denaturation step for 3 min at 94 °C, followed by 37 cycles of denaturation for 45 s at 94 °C, 54 °C, and 56 °C annealing temperatures for 18S and 28S rDNA, respectively. This was followed by extension for 45 s to 1 min at 72 °C, and a final extension step of 6 min at 72 °C, followed by temperature hold at 4 °C.

After amplification, 4 µl of the product from each tube was loaded onto a 1% agarose gel in TBE buffer (40 mM Tris, 40 mM boric acid, and 1 mM EDTA) to evaluate the DNA bands. The bands were stained with safeview classic (Applied Biological Materials Inc; Richmond, Canada), visualized, and photographed on a UV transilluminator. The PCR products were purified for sequencing by Inqaba Biotech (South Africa).

For 18S and 28S rDNA trees, *Hoplolaimus galeatus* (Cobb, 1913) Thorne, 1935 (MK809261; EU626788) were selected as an outgroup. The ribosomal DNA sequences were analyzed and edited with BioEdit (Hall 1999) and aligned using CLUSTAL W (Thompson et al. 1994). The GTR + G model was selected using jModeltest 2.1.10

(Guindon and Gascuel 2003; Darriba et al. 2012), and then initiated with a random starting tree and ran with the Markov chain Monte Carlo (MCMC) for 10^6 generations (Ronquist and Huelsenbeck 2003).

Soil parameters relationship with *H. pseudorobustus*

Soil properties analysis was conducted at the laboratory of the Aquaculture Research Unit at the University of Limpopo. The analysis involved measuring the pH and EC of the soil. The pH was measured using the Thermo Scientific Orion 3 Star pH Benchtop (USA), while an EC meter was used to measure EC. Soil texture was also measured using the method developed by van Capelle et al. (2012). To evaluate the relationship between soil factors like pH, EC, soil texture, and *H. pseudorobustus*, a Redundancy Analysis (RDA) was conducted using Past 4.03 (Hammer et al. 2001).

Results

Helicotylenchus dihystera (Cobb, 1893) Sher, 1961

(Fig. 1).

Measurements

See Table 1.

Description

Female ($n = 10$). Habitus C-shaped (3%) to spiral (97%) (Fig. 1c). Lip region 3.2 ± 0.5 (3.0–4.0) µm high and 6.8 ± 1.0 (6–9) µm wide, anteriorly hemispherical and separated from body by a slight constriction, with four to five annules. Labial framework well sclerotised (Fig. 1a), outer margins of labial framework extend 2 µm backward from basal plate. Cephalids not observed. Stylet 22.6 ± 1.7 (21–25) µm long (Fig. 1b), length of conus 8.1 ± 0.7 (7–9) µm; stylet knobs slightly projected towards the anterior region (55%) or indented (45%). Position of dorsal pharyngeal gland opening (DGO) 10.6 ± 1.5 (9–12) µm posterior to stylet knobs. Median pharyngeal bulb oval, 10.5 ± 1.0 (9.5–12.0) µm long and 8.3 ± 0.4 (8–9) µm wide; median bulb valve 2–3 µm long and 2.0–2.5 µm wide. Neck 119 ± 6.2 (112–126) µm long. Pharynx with 24–34 µm long ventral overlap. Deirids not seen. Hemizonid situated one to two annules anterior to excretory pore. Hemizonion not seen. Fasciculi not seen. Nerve ring at isthmus, at 64–71% of neck length. Excretory pore opening opposite isthmus at bulb level, at 75–85% of neck length. Two branches of the reproductive system are both functional, length of posterior branch 168 ± 4.2 (165–171), 95–97% of the corresponding anterior branch. Spermatheca oval, set-off, thick wall and empty (Fig. 1e). Oviduct seemingly short, less than a half of the corresponding body diameter, not well differentiated from the ovary. Uterus tubular without quadricolumella, about twice the corresponding body diameter. Vulva without protruding lips (Fig. 1f), located at 63.9 ± 2.3 (60.1–68.2) of body length. Inner two lines of lateral end on tail in u-shaped pattern. Caudalid not seen. Rectum 9.5 ± 0.6 (9–10) µm long, less than the anal body diameter. Intestine does not overlap rectum. Phasmids located 8–10 annules anterior to anus. Tail 13.0 ± 1.0 (12–14) µm long (Fig. 1g), with nine to twelve ventral tail annules, slightly curved dorsally, dorsal side joins the ventral side at an angle, mostly without ventral projection.

Male. not found.

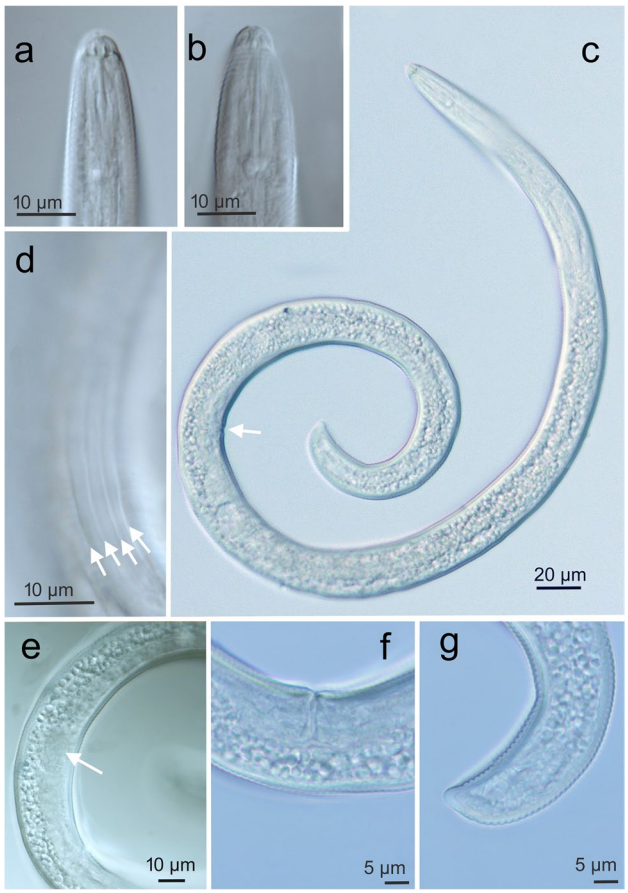

Fig. 1 *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961. **a**, **b** Anterior end (stylet). **c** Entire female (arrow pointing to vulva). **d** Lateral field incisures (arrow). **e** Spermatheca (arrow). **f** Vulva. **g** Female posterior end

Locality and habitat Specimens were collected from the rhizosphere of Kikuyu grass (GPS coordinates: 23°56′29.5"S 29°56′09.8"E) in Haenertsburg, Limpopo Province, South Africa.

Table 1 Measurements of *Helicotylenchus dihystera*, and *H. pseudorobustus* from Limpopo Province, South Africa. Measurements in µm and in the form: mean ± standard deviation (range)

Species	*H. dihystera*		*H. pseudorobustus*	
n	10 females	8 females	10 females	7 females
Location	Haenertsburg	Magoebaskloof	Haenertsburg	Nooyenskopje
L (body length)	629.8 ± 96.7 (535–770)	627.8 ± 99.1 (529–780)	490.2 ± 60.8 (414–576)	515.7 ± 66.2 (475–592)
a (L/midbody diameter)	27.9 ± 4.9 (23.3–35.0)	27.6 ± 5.0 (23.0–35.5)	21.5 ± 2.2 (20.0–24.0)	27.7 ± 0.7 (26.9–28.2)
b (L/neck length)	5.3 ± 0.7 (4.6–6.2)	5.2 ± 0.7 (4.5–6.2)	4.5 ± 0.3 (4.3–4.8)	4.1 ± 0.4 (3.9–4.6)
MB (median bulb to anterior end/neck length%)	54.4 ± 2.3 (51.0–66.0)	54.5 ± 2.4 (51.0–57.0)	50.0 ± 1.6 (50.0–52.0)	55.0 ± 2.4 (54.0–58.0)
c (L/tail length)	48.5 ± 6.8 (38.7–55.0)	46.8 ± 6.1 (39.3–55.7)	34.0 ± 1.6 (31.8–36.0)	35.2 ± 8.8 (33.9–39.3)
c' (tail length/anal body diameter)	1.0 ± 0.1 (0.9–1.2)	1.0 ± 0.1 (0.9–1.3)	1.1 ± 0.1 (0.9–1.2)	1.1 ± 0.1 (1.1–1.2)
O (dgo/stylet length%)	47.2 ± 8.2 (36.0–57.0)	47.2 ± 8.2 (34.0–58.0)	50.5 ± 0.1 (40.0–60.0)	47.2 ± 9.2 (40.0–48.0)
V (vulva anterior end/L%)	62.6 ± 3.8 (56–65)	62.9 ± 4.4 (54–66)	67.0 ± 0.1 (64–78)	63.2 ± 0.8 (62–64)
G1 (anterior genital length/L%)	30.4 ± 5.0 (27–34)	29.7 ± 4.6 (26–33)	30.0 ± 0.1 (20–50)	33.1 ± 20.1 (19–47)
G2 (posterior genital length/L%)	28.6 ± 4.1 (26–32)	27.5 ± 3.7 (25–30)	20.0 ± 0.1 (20–30)	25.4 ± 7.5 (20–31)
Lip region width	6.8 ± 1.0 (6–9)	6.5 ± 1.2 (7–9)	6.0 ±0.9 (5-7)	7.2 ± 0.3 (7-8)
Lip region height	3.2 ± 0.5 (3–4)	3.4 ± 0.6 (3–4)	5–3.8 ± 0.8 (3-5)	4.6 ± 1.1 (4-6)
Stylet length	22.6 ± 1.7 (21–25)	23 ± 2.0 (22–26)	19.7 ± 1.5 (18–21)	22.3 ± 0.6 (22–23)
Stylet conus length	8.1 ± 0.7 (7–9)	8.3 ± 0.6 (8–9)	9.3 ± 0.6 (9–10)	7.3 ± 0.6 (7–8)
Stylet shaft length	10.1 ± 1.1 (9–11)	10.0 ± 1.0 (10–11)	8.0 ± 1.0 (7–9)	9.0 ± 0.8 (8–9)
Stylet knobs height	2.9 ± 0.3 (2–3)	2.8 ± 1.3 (3–4)	1.7 ± 0.6 (1–2)	2.6 ± 0.2 (2–3)
Stylet knobs width	4.8 ± 0.3 (4–5)	4.9 ± 0.2 (3–5)	3.3 ± 0.6 (3–4)	3.7 ± 1.2 (3–5)
DGO	10.6 ± 1.5 (9–12)	10.8 ± 1.8 (9–13)	9.3 ± 2.5 (7–12)	8.3 ± 2.3 (7–11)
Median bulb length	10.5 ± 1.0 (9–12)	10.7 ± 1.4 (10–13)	10.7 ± 0.6 (10–11)	11.3 ± 2.9 (8–13)
Median bulb width	8.3 ± 0.4 (8–9)	8.5 ± 0.5 (8–9)	7.7 ± 0.6 (7–8)	7.0 ± 0.6 (7–8)
Mid of median bulb to anterior end	64.8 ± 5.3 (59–70)	65.2 ± 5.8 (59–72)	58.0 ± 5.6 (52–63)	68.7 ± 1.6 (66–69)
Pharyngeal gland tip to anterior end	96.8 ± 8.2 (88–104)	97.0 ± 8.4 (88–107)	79.3 ± 16.7 (66–98)	96.0 ± 1.7 (95–98)
Pharynx length	98.0 ± 4.9 (91–103)	98.2 ± 5.2 (91–104)	82.7 ± 18.4 (67–103)	92.3 ± 4.0 (90–97)
Pharynx overlapping length	29.2 ± 4.5 (24–34)	29.6 ± 5.1 (24–36)	32.7 ± 4.9 (27–36)	29.3 ± 4.0 (27–34)
Neck length (stylet + pharynx)	119 ± 6.2 (112–126)	119.4 ± 6.8 (112–128)	114.0 ± 12.3 (105–128)	124.7 ± 2.9 (123–128)
Nerve ring from anterior end	80 ± 2.9 (75–82)	80.2 ± 3.1 (75–83)	75.3 ± 10.7 (66–87)	74.3 ± 0.6 (74–75)
Secretory-excretory pore from anterior end	94.5 ± 0.7 (94–95)	95 ± 2.7 (95–98)	88.0 ± 8.7 (83–98)	90.0 ± 5.2 (87–96)
Hemizonid from anterior end	92 ± 0.1 (92–93)	92.5 ± 0.7 (92–95)	86.0 ± 8.7 (81–96)	87.3 ± 4.0 (85–92)
Body diameter at neck base	20.0 ± 1.2 (19–22)	21.4 ± 1.7 (18–24)	22.7 ± 4.0 (19–27)	18.0 ± 1.7 (17–20)
Body diameter at mid body	22.6 ± 0.5 (22–23)	22.8 ± 0.8 (22–24)	24.0 ± 2.0 (22–26)	18.7 ± 2.9 (17–22)
Body diameter at anus	13 ± 1.0 (12–14)	13.2 ± 1.3 (12–15)	13.8 ± 1.9 (11–17)	11.7 ± 0.6 (11–12)
Lateral field width	4.3 ± 0.2 (4–5)	4.4 ± 0.4 (4–5)	4.7 ± 1.3 (3–6)	5.0 ± 0.2 (5–6)
Vagina length	10 ± 0.7 (9–11)	10.2 ± 0.8 (10–11)	11.3 ± 1.5 (10–13)	8.7 ± 1.2 (8–10)
Annuli width	1.0 ± 0.1 (1.0–1.2)	1.1 ± 0.3 (1.0–1.5)	1.6 ± 0.3 (1–2)	1.1 ± 0.1 (1.0–1.2)
Cuticle thickness	1.4 ± 0.2 (1–2)	1.6 ± 0.3 (1–2)	1.7 ± 0.4 (1–2)	1.1 ± 0.1 (1.0–1.2)
Anterior branch of reproductive system	178.5 ± 7.8 (173–184)	175 ± 8.5 (169–181)	144.7 ± 51.6 (110–204)	168.5 ± 79.9 (112–225)
Posterior branch of reproductive system	168 ± 4.2 (165–171)	162.5 ± 4.9 (159–166)	124.0 ± 12.2 (116–138)	132.5 ± 19.1 (119–146)
Rectum length	9.5 ± 0.6 (9–10)	10 ± 0.8 (9–11)	11.0 ± 1.7 (10–13)	9.0 ± 1.7 (7–10)
Tail length	13 ± 1.0 (12–14)	13.4 ± 0.9 (13–14)	14.5 ± 1.4 (13–16)	13.3 ± 1.2 (12–14)

Other material examined The other population (GPS coordinates: 23°51′24.0"S 29°57′25.6"E) was recovered from kikuyu grass in Magoebaskloof, Limpopo Province, South Africa, which no significant differences were observed.

Remarks This particular species was studied in South Africa by Marais in 2001. The identification of this species was made based on its spiral habitus. However, Marais (2001) found that 1% of the populations previously studied in South

Africa had a straight habitus, which was not observed in the present study. The present population of *H. dihystera* was identified based on certain features that matched with the records of *H. dihystera*, such as its body length (535–770 µm), position of vulva (average 63%), location of phasmids, inner incisures of lateral fields fused on the tail, Y-shaped, dorsally curved tail tip with or without projection in the angle of the ventral side, empty spermatheca, and no males. The morphometrics of *H. dihystera* from South Africa confirm the identification of this particular population. There were no significant differences found between the present population and the previously studied populations of *H. dihystera* by Marais (2001). Recently, a population of *H. dihystera* has been reported from Pakistan (Ali et al. 2023) with the same morphometric as the South African population, except for tail length (12–14 vs 19.9–21.1 µm).

DNA characterization The new partial 18S rDNA (PP825418) and 28S rDNA (PP528700) were deposited in GenBank for *H. dihystera*. The 28S rRNA gene sequences of the South African *H. dihystera* exhibited a 99% similarity with the Florida population of *H. dihystera* (HM014261). Moreover, when compared with other populations from South Africa of the same species collected from Nelspruit (HM014260; HM014256), they also showed a 99% similarity. In addition, 28S rDNA marker for *H. pseudorobustus* showed 99% similarity between South African and a population of *H. pseudorobustus* (MG925220) from USA.

Helicotylenchus pseudorobustus (Steiner, 1914) Golden, 1956

(Figs. 2 and 3).

Measurements

See Table 1.

Description

Females ($n = 10$). Habitus spiral (Fig. 2e). Lip region 3.8 ± 0.8 (3–5) µm high and 6.0 ± 0.9 (5–7) µm wide, anteriorly hemispherical and continuous with body contour (Fig. 3a,c), with six to seven annules (Fig. 3a). Labial framework sclerotised, outer margins extend 1–2 µm backward from basal plate. Cephalids not seen. Stylet 19.7 ± 1.5 (18–21) µm long, conus 9.3 ± 0.6 (9–10) µm. Stylet knobs 1.7 ± 0.6 (1–2) µm high and 3.3 ± 0.6 (3–4) µm wide, anterior faces flattened (86%) to indented (14%). Dorsal pharyngeal gland located 9.3 ± 2.5 (7–12) µm posterior to stylet knobs. Median pharyngeal bulb oval to rounded, 10–11 µm long and 7–8 µm wide; valve 2–3 µm long and 2–3 µm wide. Hemizonid one to two annlues long, located one to two annules anterior to secretory-excretory pore. Hemizonion not seen. Nerve ring at isthmus level, located at 63–68% of neck length. Secretory-excretory pore opening opposite isthmus level, located at 76–79% of neck length. Deirids not observed. Two branches of reproductive tract, both functional, length of posterior branch 20–31% of body length, anterior branch length 21–46% body length. Epiptygma rounded, spermatheca axial; vulva situated at 64–78% of body length. Lateral field 4.7 ± 1.3 (3–6) µm wide; inner two lines end on tail in a U-shaped pattern (Fig. 3g, h). Lateral field begins 12–13 annulus from anterior end; aerolated in anterior (Fig. 3c) and mid body (Fig. 3d). Rectum 11.0 ± 1.7 (10–13) µm long. Intestine not extended over rectum. Caudalid not seen. Phasmid pore like, located from five to seven annules anterior to anus, between second and third line (Fig. 3g, h). Tail 14.5 ± 1.4 (13–16) µm long, with six to eight ventral annules, tail more curved dorsally with rounded projection (2–4 µm long) (Fig. 3e, g, h).

Male: not found.

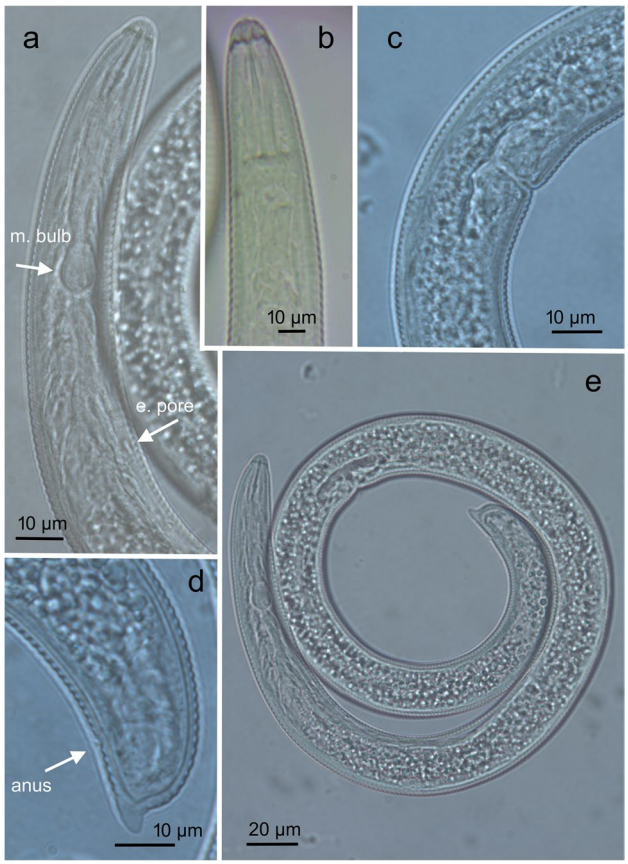

Fig. 2 *Helicotylenchus pseudorobustus* (Steiner, 1914) Golden, 1956. **a** Anterior end (arrow pointing to median bulb, and excretory pore). **b** Stylet, **c** Vulva. **d** Female posterior end (arrow pointing to anus). **e** Entire female

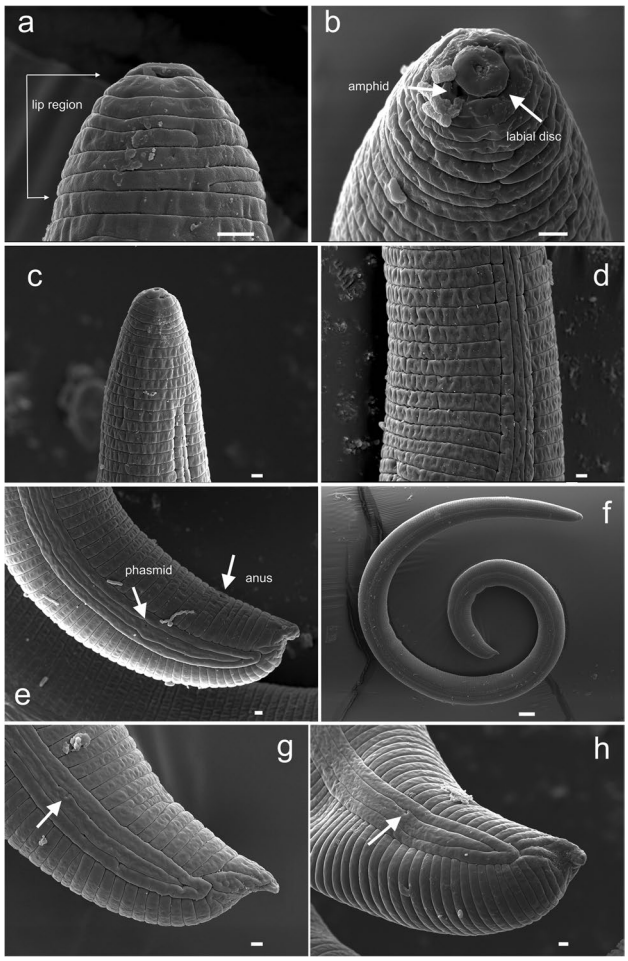

Fig. 3 *Helicotylenchus pseudorobustus* (Steiner, 1914) Golden, 1956. **a** Lip region (lateral view; arrow). **b** Lip region (frontal view; arrow pointing to amphid and labial disc). **c** Anterior end (sublateral view). **d** Lateral field. **e**, **g**, **h** Female posterior end (arrow pointing to phasmid and anus). **f** Entire female. Scales 1 µm, except F 10 µm

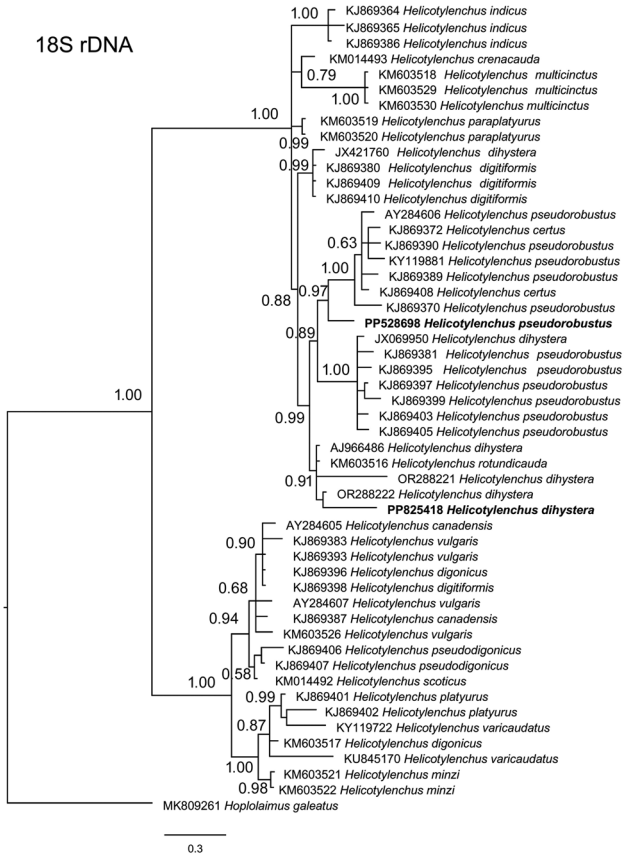

Fig. 4 Phylogenetic tree based on the sequences of 18S rDNA, including the South African *H. dihystera* and *H. pseudorobustus*

Locality and habitat Specimens collected from the rhizosphere of Kikuyu grass (GPS coordinates: 23°56′43.9"S 29°56′35.5"E) in Haenertsburg, Limpopo Province, South Africa.

Other materials examined The other population (GPS coordinates: 23°52′48.2"S 29°56′13.3"E) was recovered from kiwi farm soil in Nooyenskopje, Magoebaskloof, Limpopo Province, South Africa, which no significant differences were observed.

Remarks The specimens observed in the current study were identified as *H. pseudorobustus* based on their spiral shape, rounded lip region, body length (ranging from 414–576 µm), positioning of the vulva (average of 67%), phasmids located 5–7 annules anterior to the anus, fused inner incisures of lateral fields on the tail, Y-shaped, dorsally curved tail tip with a projection in the angle of the ventral side, axial and empty spermatheca, and absence of males. These specimens of *H. pseudorobustus* are in agreement with the updated descriptions provided by Fortuner et al. (1984), Marais (2001), and Subbotin et al. (2015).

DNA characterization The new partial 18S rDNA (PP528698) and 28S rDNA (PP826554) were deposited in GenBank for *H. pseudorobustus*. The 18S rRNA gene sequences of *H. pseudorobustus* exhibit 98% similarity with a population that was collected from Ireland (KY119881). Furthermore, the population that was studied showed 98% similarity with the *H. pseudorobustus,* which has already been deposited in the NCBI. This is the first 18S rDNA sequence of *H. pseudorobustus* from South Africa, specifically Limpopo Province. Besides, 18S rDNA of *H. dihystera* showed 99% similarity with a population of *H. dihystera* (OR288222) from Vietnam.

Phylogenetic analysis Phylogenetic analysis using 18S rDNA (Fig. 4) grouped species of *Helicotylenchus* in two clades, viz. I) *H. multicinctus* (Cobb, 1893) Golden, 1956; *H. crenacauda* Sher, 1966; *H. paraplatyurus* Siddiqi, 1972;

H. pseudorobustus, H. indicus Siddiq, 1963; *H. dihystera, H. rotundicauda* Sher, 1966 and *H. certus* Eroshenko and Tkhan, 1981 with 1.00 posterior probability, and II) *H. scoticus* Boag and Jairajpuri, 1985; *H. vulgaris* Yuen, 1964; *H. canadensis* Waseem, 1961; *H. digitiformis* Ivanova, 1967; *H. pseudodigonicus* Szczygiel, 1970; *H. digonicus* Perry In Perry Darling and Thorne, 1959; *H. minzi* Sher, 1966; *H. platyurus* Perry in Perry et al., 1959 and *H. varicaudatus* Yuen, 1964 with 1.00 posterior probability.

Fig. 5 Phylogenetic tree based on the sequences of 28S rDNA, including the South African *H. dihystera* and *H. pseudorobustus*

Phylogenetic analysis using 28S rDNA (Fig. 5) grouped species of *Helicotylenchus* in nine main clades, viz. I) *H. dihystera* with 1.00 posterior probability; II) *H. leiocephalus* Sher, 1966; *H. platyurus, H. pseudorobustus, H. digonicus, H. paxilli* Yuen, 1964, with 0.99 posterior probability; III) *H. paraplatyurus* Siddiqi, 1972 with 0.99 posterior probability; IV) *H. vulgaris,* and *H. digonicus* with 1.00 posterior probability; V) *H. labiodiscinus* Sher, 1966; *H. multicinctus* and *H. depressus* Yeates, 1967 with 1.00 posterior probability; VI) *H. oleae* Inserra et al., 1979 with 1.00 posterior probability*;* VII) *H. brevis* (Whitehead, 1958) Fortuner, 1984 and *H. cuspicaudatus* Saha et al., 2000 with 0.75 posterior probability; VIII) *H. martini* Sher, 1966 with 1.00 posterior probability; and IX) *H. vulgaris* and *H. minzi* with 1.00 posterior probability (Fig. 5). The results showed *H. dihystera* as a monophyletic group with 1.00 posterior probability. In addition, *H. pseudorobustus* was divided into two groups.

Soil parameters relationship with *H. pseudorobustus*

The RDA result of the soil parameters relationship with *H. pseudorobustus* (Fig. 6) explained 100% variation, in which 98.4% by RDA1, and 1.6% by RDA2. The result indicated that this species had a correlation with clay percentage of the soil. In contrast, there is no correlation between *H. pseudorobustus* and EC of the soil.

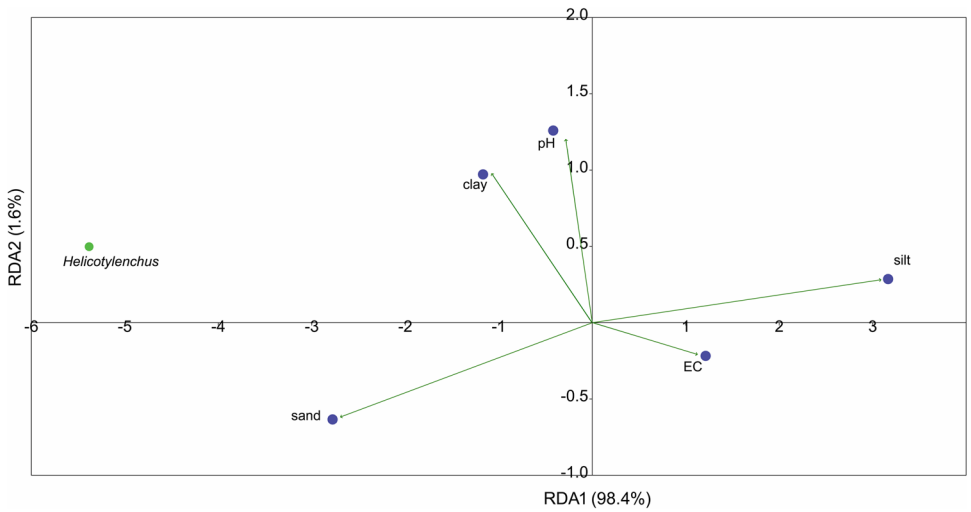

Fig. 6 Redundancy analysis of the relationship between *H. pseudorobustus* and soil parameters

Discussion

Helicotylenchus is a spiral nematode containing more than 200 nominal species (Marais 2001). *Helicotylenchus pseudorobustus* is a spiral species of nematode that was first discovered in soil collected from under moss in the Altmatt region of Switzerland. It has since been found in many different parts of the world. Among the many species belonging to the genus *Helicotylenchus*, *H. pseudorobustus* is one of the most commonly reported worldwide, followed by *H. dihystera* and *H. multicinctus* (Shokoohi et al. 2018).

Various sequences of 28S rDNA have been used to determine the phylogenetic position of *Helicotylenchus* species. This has been studied by several authors, including Schreck Reis et al. (2010), Subbotin et al. (2011, 2015), and Divsalar et al. (2020). As for the family Hoplolaimidae (insert taxon authority), the consensus tree inferred from 28S rDNA in the present study has demonstrated that the genus *Helicotylenchus* is a monophyletic group. This finding agrees with the reports of Subbotin et al. (2011, 2015) and Chen et al. (2024a).

With respect to the clades, our study indicated that some species of *Helicotylenchus* form a paraphyletic group, agreeing with reports by Bae et al. (2009), Subbotin et al. (2011, 2015), and Divsalar et al. (2020). Based on the present result of 28S rDNA phylogeny, the placement of *H. dihystera* in one clade indicates its monophyletic nature. This agrees with the results obtained by Chen et al. (2024a).

However, the 18S rDNA phylogeny of the present study placed populations of *H. dihystera* and *H. pseudorobustus* together in one clade as was reported by Subbotin et al. (2011). In a research study conducted by Subbotin et al. (2015), the morphological differences between various populations of *H. pseudorobustus* were explored. Utilizing advanced molecular techniques such as 28S rDNA (Divsalar et al. 2020), the study revealed a clear separation between *H. pseudorobustus* and *H. dihystera*. In addition, Ali et al. (2023) have indicated that *H. pseudorobustus* and *H. dihystera* are grouped together in the same clade. The same result was obtained in the present study. The findings of this research have provided new insights into the taxonomy of these two closely related species and could potentially be used to aid in their classification and identification in the future.

On the identity of South African *H. dihystera* and *H. pseudorobustus*

Marais (2001) reported the presence of two distinct species of nematodes, *H. dihystera* and *H. pseudorobustus*, in South Africa. These two species share several common characteristics, such as similar morphometrics and general morphology, including empty and offset spermatheca. Interestingly, no males were reported for either species. However, the species mentioned above reported with a tail projection, which causes confusion regarding their identification. Tail projections were mostly less than two annuli in *H. dihystera vs* mostly more than two annuli in *H. pseudorobustus*. Moreover, the lip region differs in both species. In *H. dihystera,* lip regions were reported to be rounded (98%) to slightly flattened (2%), whereas they were mostly round in *H. pseudorobustus* (Marais 2001). Furthermore, in a study conducted by Marais (2001), the labial discs of *H. dihystera* were provided using scanning electron microscopy (SEM). The SEM findings in the presented study revealed that there is a distinct difference in the shape of the labial discs between the two species. *Helicotylenchus pseudorobustus* was observed to have a rounded labial disc (Fig. 7a), while in *H. dihystera* specimens, the labial disc was oval-shaped (Fig. 7c). These results provide important insights into the morphological differences between the two species, which could be helpful in distinguishing them from one another in the future.

In addition, the present study found a significant difference between *H. pseudorobustus* and *H. dihystera* tail regions based on SEM analysis. The lateral field of *H. pseudorobustus* (Fig. 7b) was u-shaped with a small straight line, while that of *H. dihystera* (Fig. 7d) was y-shaped with a long straight line. Despite the similarity in morphometrics and tail projections of the South African specimens, this feature clearly distinguishes the two species.

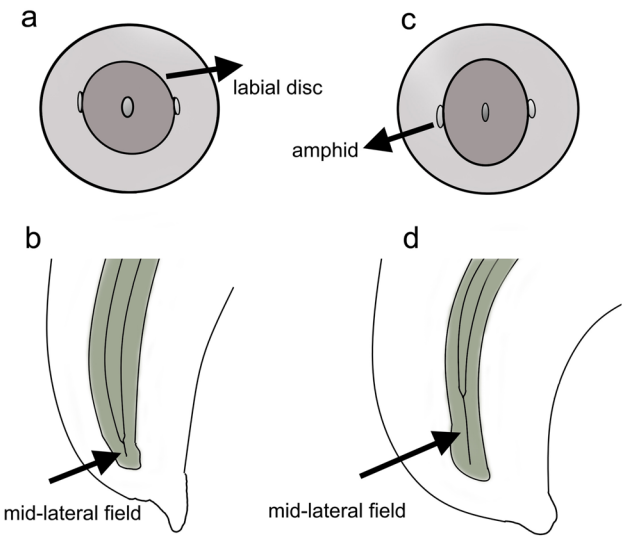

Fig. 7 Schematic view of lip region and tail with lateral field incisures. **a**, **b** *H. pseudorobustus*. **c**, **d** *H. dihystera*

Soil relationship with *H. pseudorobustus*

Soil texture affects the movement of nematodes in the soil (Norton 1989). The results of the present study showed a positive correlation between *H. pseudorobustus* and clay in the soil. Norton et al. (1971) showed a positive correlation between *H. pseudorobustus* and clay of the soil in Iowa soybean fields. They have indicated that pH also had a positive correlation with *H. pseudorobustus*. The same results were obtained in the present study. A survey of plant-parasitic nematodes in Limpopo Province, South Africa (Shokoohi and Masoko 2024) showed that *Helicotylenchus* had a positive correlation with pH. In addition, soil pH was indicated to slightly affect the number of *Helicotylenchus* in soybeans and cotton, respectively (Leiva et al. 2020; Chen et al. 2024b). The same results were obtained in the present study. However, depending on the host plant and nematode feeding type, soil texture may have a different impact on nematode movement and development. The sandy soil was reported to affect the movement and number of *Helicotylenchus* in Portugal and The Netherlands (Schreck Reis et al. 2008). However, they have indicated no close relationship with the host plant. In addition, Leiva et al. (2020) reported no correlation between *Helicotylenchus* and sand particles in the soybean soil. In the present study, sand was not correlated with the number of *H. pseudorobustus*. Soil electrical conductivity (EC) is primarily influenced by soil salinity, clay content, and water content. Additionally, adding nutrients to the soil has been observed to increase soil EC (Kim and Park 2024). Soil EC was reported to have no significant correlation with *Helicotylenchus* in rice fields (Mokuah et al. 2023). Furthermore, the surveys did not show a significant correlation between *Helicotylenchus* and soil EC (Escalante Ortiz et al. 2023). The same result was obtained for the present study.

Conclusion

The present study highlights the significance of using advanced microscopy techniques such as scanning electron microscopy to improve our understanding of the morphological characteristics of different species of *Helicotylenchus*. The results of the phylogenetic analysis showed that 28S rDNA is a reliable marker for identifying *H. dihystera*. This means that the genetic information in the 28S rDNA sequence can be used to determine the species accurately. However, the analysis also revealed that *H. pseudorobustus*, despite being grouped in one clade based on 28S rDNA, showed some degree of variation. This suggests that further research is needed to fully understand this species' genetic variability and identify other reliable markers for accurate species identification.

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Author's contribution ES conducted the study, identified the species, analyzed the data, and wrote the manuscript. CR provided the SEM and some LM photographs. ZH, PM, and CR revised the manuscript. All authors attended the final draft of the manuscript.

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Data availability All relevant data are within the manuscript. The data for molecular study is deposited and available in the NCBI GenBank. The new partial 18S rDNA (PP528698; PP825418) and 28S rDNA (PP826554; PP528700) were deposited in GenBank for *H. pseudorobustus* and *H. dihystera*, respectively.

Declarations

Ethical statement The paper reflects the authors' own research and analysis in a truthful and complete manner.

Conflict of interest The authors declare that there are no conflicts of interest.

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