A new plant-parasitic nematode species associated with coffee, *Rotylenchus bunae* n. sp. (Nematoda: Hoplolaimidae) from Jimma, Ethiopia

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Summary – *Rotylenchus bunae* n. sp. was discovered in the coffee rhizosphere in Gera district of Jimma, Ethiopia, and has been morphologically and molecularly characterised. The new species is identified by a female body length of 725-876 μm, hemispherical lip region with 4-5 lip annuli, stylet length of 28-30 μm, lateral field areolation only in the pharyngeal region, no cuticular striations, pharyngeal gland overlapping intestine dorsally by 15-26 μm, double epiptygma, no clear spermathecae, vulva position at 54-58% of body length from anterior end, prominent fasciculi in mid and posterior body, rounded or sometimes slightly truncated, coarsely crenated tail with 11-13 annuli, and phasmids located at 7-16 annuli anterior to anus. No males were detected. This species was found closely related to other African *Rotylenchus* species, such as *R. unisexus* and *R. wimbii*; however, it could be well separated from them by both morphology and molecular data (D2-D3 of 28S and partial 18S of rDNA).

Keywords – Gera, morphology, morphometrics, nematodes, new species, spiral nematodes, systematics, taxonomy.

*Rotylenchus* Filipjev, 1936 is a relatively well-known plant-parasitic nematode (PPN) genus with 105 nominal species described to date (Singh et al., 2021; Guo et al., 2022). These obligate ectoparasites are found worldwide and infect various plants (Castillo & Vovlas, 2005; Manzanilla López & Marbán Mendoza, 2012; Sikora et al., 2018). They can be readily separated morphologically from other closely related and similar looking PPN such as *Helicotylenchus* Steiner, 1945, *Hoplolaimus* von Daday, 1905 and *Scutellonema* (Steiner, 1937) Andrássy, 1958, based on a combination of body length, stylet shape and size, position of pharyngeal gland overlapping in intestine, position of female vulva, tail shape, and phasmid position (Manzanilla López & Marbán Mendoza, 2012). However, morphological identification of *Rotylenchus* to species level requires additional detailed observations, especially the morphology of the lip region, the presence or absence of striations on the cuticle at pharynx level, lateral field areolation, the dorsal gland orifice (DGO) position, the tail annuli number and general morphometrical data (Castillo & Vovlas, 2005; Nguyen et al., 2019). In addition to these, molecular data of ribosomal and mitochondrial gene fragments, host and locality information are also generally employed in their identification (Subbotin et al., 2007; Vovlas et al., 2008; Cantalapiedra-Navarrete et al., 2013; Noruzi et al., 2015; Tzortzakakis et al., 2016; Guo et al., 2022; Singh et al., 2022).

To date, 16 *Rotylenchus* species have been described from Africa, and another eight species have been reported from the continent (Castillo & Vovlas, 2005; Singh et al., 2022).
Also, a table of comparative morphology data of 23 Rotylenchus species reported from Africa can be found in Singh et al. (2021) to aid in their identification. Recently, an unknown population of Rotylenchus was detected in a survey of PPN from coffee plantations in Gera and Gomma districts of Jimma in Ethiopia (Singh et al., 2023). Based on our morphological and molecular phylogenetic analyses, this population appears to represent a new species to science and is herein described as Rotylenchus bunae n. sp. using light microscopy (LM), molecular phylogenetic data of ribosomal genes (18S and D2-D3 of 28S), host information and geographic distribution.

Materials and methods

SOIL SAMPLING AND NEMATODE EXTRACTION

The bulk soil sample containing the new species was collected from rhizospheres of four randomly selected coffee shrubs (Coffea arabica L.) (Sample code: Ge29D), using a 3 cm diam. auger, from 20-30 cm soil depth within a 10 m² area in Gera district, Jimma, Ethiopia, in July 2019. The assigned GPS coordinates of the type locality were 7°45′11.1″N, 36°19′39.4″E. Nematodes were extracted from 100 ml of soil by the modified Baermann’s tray method (Whitehead & Hemming, 1965) at the Nematology Research Unit, Ghent University, Belgium. The extracted nematode suspension was stored at 4°C during analysis.

MORPHOLOGICAL CHARACTERISATION

Nematodes were fixed by adding a few drops of Trump’s fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M Sorensen buffer (sodium phosphate buffer, pH 7.5)) in a glass cavity block followed by immediately heating it in a microwave (700 W) for 3-4 s. The glass block was left to rest for 1 h at room temperature and then kept for 48 h at 4°C. Following Seinhorst’s (1959) method, the fixed nematodes were gradually transferred to anhydrous glycerin and mounted on glass slides for light microscopy study. The nematodes were examined, measured and photographed at a magnification of 10-100× using an Olympus BX50 DIC microscope (Olympus Optical), equipped with an UCMOS 5MP camera and a drawing tube for morphological and morphometric studies (Singh et al., 2021).

MOLECULAR CHARACTERISATION

Individual nematode specimens were used for genomic DNA extraction. A single specimen was cut into two pieces using a metallic pin and transferred into a polymerase chain reaction (PCR) tube containing 20 μl of worm lysis buffer (50 mM KCl, 10 mM Tris at pH 8.3, 2.5 mM MgCl₂, 0.45% NP-40 (Tergitol Sigma), 0.45% Tween-20). The tubes were frozen for 10 min at −20°C followed by adding 1 μl proteinase K (1.2 mg ml⁻¹). Then, the tubes were subjected to a thermocycler thermal profile of 65°C for 1 h and 95°C for 10 h. Finally, the lysate was centrifuged at 14 000 g for 1 min (Singh et al., 2021). PCR amplifications of the D2-D3 expansion segment of 28S ribosomal DNA (rDNA) were done using the primer pair D2A: 5′-ACA AGT ACC GTG AGG GAA AGT TG-3′/D3B: 5′-TCC TCG GAA GGA ACC AGC TAC TA-3′ (Nunn, 1992) following the thermal profile described in Etongwe et al. (2020). The successful PCR amplicons were stained using GelRed (Biotium) and visualised in 1% agarose gel under UV light illumination. The amplicons were finally cleaned using alkaline phosphatase (1 U ml⁻¹) and exonuclease I (20 U ml⁻¹) as described in Singh et al. (2020) and sequenced at the Macrogen sequencing facility (Amsterdam, The Netherlands). Contigs were produced from the forward and reverse sequences using Geneious Prime 2020.0.5 (www.geneious.com).

PHYLOGENETIC ANALYSIS

Phylogenetic relationships of the new species with related species were analysed based on the partial sequences of 28S and 18S using the Geneious Prime phylogenetic programs. For this purpose, all the available Rotylenchus sequences of the two gene fragments were retrieved from the NCBI and loaded on Geneious Prime. The newly obtained and published sequences for each gene were aligned using MUSCLE of Geneious Prime, followed by manual trimming of the poorly aligned ends. Bayesian phylogenetic analysis (MrBayes 3.2.6; Huelsenbeck & Ronquist, 2001) was done using the GTR + I + G nucleotide substitution model for each alignment file; analyses were run under 1 × 10⁶ generations (4 runs), and Markov chains were sampled every 100 generations and 20% of the converged runs was regarded as burn-in.
Results

Rotylenchus bunae n. sp.  
(Figs 1, 2)

Measurements

See Table 1.

Description

Females

Body spiral or curved into 6-shape when heat-relaxed. Lateral field differentiation starting as three incisures forming two bands and gradually developing to four incisures forming three bands; mid-band slightly wider than outer bands. Fasciculi present mostly in matured females and prominent around mid- and posterior body. Lateral field areolation only at pharynx region. Longitudinal cuticular striations in anterior body absent. Labial region hemispherical with 4-5 lip annuli, continuous with rest of the body. Well-sclerotised cephalic framework. Stylet robust, cone about half of stylet length, knobs strong and rounded. Pharynx with well-developed median bulb, valves, slender isthmus and gland overlapping intestine dorsally. Secretory-excretory (SE) pore at level of anterior part of pharyngeal gland. Hemizonid often two cuticular annuli long and just above SE pore. Reproductive tract didelphic-amphidelphic, both branches equally developed with outstretched ovaries containing developing oocytes. Vulva just posterior to middle of body length. Epitygma prominent and double. Spermaticca empty and weakly visible to indistinct. Tail generally rounded to occasionally slightly truncated. Cuticle at tail tip thickened and coarsely annulated. Phasmids 7-16 annuli anterior to anus.

Male

Not found.

Juveniles

Body smaller than adult females, spiral or curved into 6-shape when heat-relaxed. Lateral field differentiation, lip region and pharynx similar to that of females. Stylet robust, slightly shorter than that of females. Reproductive tract not developed. Tail length, shape and tip similar to that of female. Phasmid four annuli anterior to anus.

Morphological diagnosis and relationships

Rotylenchus bunae n. sp. can be morphologically diagnosed by the female body length of 725-876 μm, hemispherical lip region with 4-5 lip annuli, stylet length of 28-30 μm, lateral field areolation only in the pharyngeal region and without cuticular striations, pharyngeal gland overlapping intestine dorsally by 15-26 μm, prominent double epitygma, no clear spermathecae, vulva at 54-58% of body length from anterior end, prominent fasciculi in mid and posterior regions of the body that is more prominent in matured females, rounded to sometimes slightly truncated tail with 11-13 annuli and coarsely annulated terminus, and phasmids at 7-16 annuli anterior to anus. The identification code of this species based on the tabular key/matrix for identification of Rotylenchus spp. of Castillo & Vovlas (2005) is A3, B1, C1, D4, E1, G (2,3), H2, I2, J2, K1.

Rotylenchus bunae n. sp. is morphologically close to R. brevicaudatus Colbran, 1962, R. cypriensis Antoniou, 1980, R. minutus (Sher, 1964) Germani, Baldwin, Bell & Wu, 1985, R. unisexus Sher, 1965 and R. wimbii Singh, Karssen, Gitau, Wanjau, Couvreur, Pili, Gheysen & Bert, 2021. They all have female body length within 0.5-0.9 mm, a rounded to hemispherical lip region with four lip annuli, lateral field areolation only at pharyngeal region, absence of body striations, stylet length shorter than or equal to 30 μm and ratio V of 50-70%. However, the new species can be separated from R. brevicaudatus based on the female lip region (hemispherical vs rounded), stylet length (28-30 μm vs 18-27 μm), spermatheca (indistinct vs distinct) and absence vs presence of males. It can be separated from R. cypriensis based on the female lip region (not set off vs well set off), stylet length (28-30 μm vs 21-24 μm), tail length (14-22 μm vs 11-14 μm) and absence vs presence of a ventral mucron at tail tip. Rotylenchus bunae n. sp. can be separated from R. minutus based on the female stylet length (28-30 μm vs 20-27 μm), spermatheca (indistinct vs distinct and rounded), epitygma (prominent vs indistinct), phasmids positions (always anterior to anus vs anterior or posterior to anus), and absence vs presence of males. It can also be separated from R. unisexus based on the female stylet length (28-30 μm vs 20-29 μm), SE-pore around the beginning of the gland vs SE-pore around the mid to end of the gland level and the position of phasmids (7-16 annuli anterior to anus vs 6-7 annuli anterior to anus). Finally, R. bunae n. sp. can be separated from R. wimbii based on the female stylet length (28-30 μm vs 22-27 μm), presence vs absence of...
Fig. 1. Photomicrographs of *Rotylenchus bunae* n. sp. in lateral views. A-E: Anterior bodies showing lip regions, stylet and knobs, secretory-excretory pores (se), hemizonid (h) and cuticle at pharynx level; F-I: Mid bodies around vulva region showing epiptygma (ep), fasciculi (f), lateral lines, vulva, and vagina; J: posterior body showing fasciculi (f); K-P: Tails showing anus, tail annuli, and phasmids (p).
Fig. 2. Line illustrations of *Rotylenchus bunae* n. sp. in lateral views. A, B: Anterior bodies showing lip regions, stylets and knobs, gland overlapping, hemizonid and secretory-excretory pore; D: Mid-body showing epitygma, vulva, vagina and lateral lines; C, E, F: Posterior bodies showing fasciculi, tail shapes, tail annuli and phasmid.
Table 1. Morphometric data of *Rotylenchus bunae* n. sp. from fixed specimens and mounted in glycerin medium. All measurements are in μm and presented in the form mean ± s.d. (range).

<table>
<thead>
<tr>
<th>Character</th>
<th>Female holotype</th>
<th>Females</th>
<th>Juveniles</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>1</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>Body length</td>
<td>778</td>
<td>801 ± 54 (725-876)</td>
<td>539 ± 33.2 (495-587)</td>
</tr>
<tr>
<td>a</td>
<td>25.5</td>
<td>27.6 ± 1.8 (24.9-29.9)</td>
<td>25.4 ± 1.8 (23.9-29.2)</td>
</tr>
<tr>
<td>b</td>
<td>6.8</td>
<td>6.7 ± 0.3 (6.2-7.4)</td>
<td>5.1 ± 0.2 (4.8-5.3)</td>
</tr>
<tr>
<td>b'</td>
<td>5.5</td>
<td>5.7 ± 0.3 (5.4-6.3)</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>35.7</td>
<td>44.5 ± 8.5 (35.7-61.6)</td>
<td>29.4 ± 1.5 (27.7-31.7)</td>
</tr>
<tr>
<td>c'</td>
<td>37.7</td>
<td>38.1 ± 4.9 (31.5-46.3)</td>
<td></td>
</tr>
<tr>
<td>Stylet length</td>
<td>28.8</td>
<td>28.5 ± 0.6 (27.8-29.8)</td>
<td>24.4 ± 0.2 (24.2-24.8)</td>
</tr>
<tr>
<td>Cone length</td>
<td>14.9</td>
<td>14.2 ± 0.6 (13.4-14.9)</td>
<td>12.1 ± 0.5 (11.3-12.5)</td>
</tr>
<tr>
<td>Cone% of stylet</td>
<td>51.7</td>
<td>49.7 ± 1.3 (48.0-51.7)</td>
<td>49.5 ± 2.0 (46.1-50.8)</td>
</tr>
<tr>
<td>Knob height</td>
<td>2.7</td>
<td>3.0 ± 0.3 (2.7-3.6)</td>
<td></td>
</tr>
<tr>
<td>Knob width</td>
<td>6.1</td>
<td>5.6 ± 0.7 (4.8-6.8)</td>
<td></td>
</tr>
<tr>
<td>Lip height</td>
<td>4.2</td>
<td>5.2 ± 0.6 (4.2-6.5)</td>
<td></td>
</tr>
<tr>
<td>Lip width</td>
<td>8.7</td>
<td>9.0 ± 0.4 (8.6-9.8)</td>
<td></td>
</tr>
<tr>
<td>Anterior end to valves</td>
<td>86.3</td>
<td>83.5 ± 3.6 (78.3-89.2)</td>
<td></td>
</tr>
<tr>
<td>Anterior end to pharyngo-intestinal junction</td>
<td>115</td>
<td>119 ± 4.7 (112-127)</td>
<td>107 ± 2.8 (104-110)</td>
</tr>
<tr>
<td>Anterior end to gland end</td>
<td>142</td>
<td>141 ± 7.2 (131-153)</td>
<td></td>
</tr>
<tr>
<td>Anterior end to SE-pore</td>
<td>121</td>
<td>121 ± 6.2 (114-131)</td>
<td></td>
</tr>
<tr>
<td>Gland overlapping length</td>
<td>26.3</td>
<td>19.4 ± 3.6 (15.0-26.3)</td>
<td></td>
</tr>
<tr>
<td>Anterior end to vulva</td>
<td>438</td>
<td>447 ± 28.3 (402-487)</td>
<td></td>
</tr>
<tr>
<td>V%</td>
<td>56.3</td>
<td>55.9 ± 1.2 (54.4-58.3)</td>
<td></td>
</tr>
<tr>
<td>Maximum body diam.</td>
<td>30.5</td>
<td>28.8 ± 2.6 (25.0-32.7)</td>
<td>21.3 ± 1.3 (19.2-22.4)</td>
</tr>
<tr>
<td>Anal body diam.</td>
<td>23.2</td>
<td>21.1 ± 1.8 (18.5-23.2)</td>
<td></td>
</tr>
<tr>
<td>Tail length</td>
<td>21.8</td>
<td>18.5 ± 2.9 (14.2-22.2)</td>
<td>18.3 ± 0.9 (17.3-19.5)</td>
</tr>
<tr>
<td>Tail annuli number</td>
<td>13</td>
<td>11 ± 1 (11-13)</td>
<td>12 ± 1 (11-13)</td>
</tr>
<tr>
<td>Annuli number between phasmid and anus</td>
<td>16</td>
<td>12 ± 3 (7-16)</td>
<td>4</td>
</tr>
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</table>

epitygma, tail length (14-22 μm vs 11-14 μm) and tail tip (more rounded vs more truncated).

**Type specimen**

Holotype female and six paratype females have been deposited at the National Plant Protection Organization, Wageningen Nematode Collection (WaNeCo), Wageningen, The Netherlands, and two paratype females and one juvenile have been deposited at the Ghent University Museum, Zoology Collections, Ghent, Belgium.

**Etymology**

The species epithet is derived from the Oromo word for coffee, *i.e.*, ‘Buna’, the host plant of this new species having been detected in the Gera district of Oromia.

**Type location and host**

The new species was associated with coffee (*Coffea arabica* L.) in Gera district of the Jimma zone in southwest Ethiopia with the GPS coordinates 7°45′11.1″N, 36°19′39.4″E.

**Molecular characterisation and phylogenetic analysis**

Two identical sequences of D2-D3 of 28S rDNA (OQ540741-OQ540742) were generated for this new species. The D2-D3 sequences were found closest to *R. wimbii* (MW074365; 94.4% similarity and 43 out of 765 bp difference) and the 18S were also found closest to *R. wimbii* (MW074383; 98.6% similarity and 12 out of 865 bp difference). Three partial sequences of 18S rDNA (MZ681496-MZ681498) have already been published in Singh *et al.* (2023) as an unidentified *Rotylenchus* sp.
Fig. 3. Phylogenetic relationship of *Rotylenchus hunaee* n. sp. with other related species as inferred from Bayesian analysis of the D2-D3 of 28S rDNA sequences using GTR + I + G nucleotide substitution model. Posterior probabilities of above 0.50 are given next to the clades.
Fig. 4. Phylogenetic relationship of *Rotylenchus buneae* n. sp. with other related species as inferred from Bayesian analysis of the partial 18S rDNA sequences using GTR + I + G nucleotide substitution model. Posterior probabilities of above 0.50 are given next to the clade.
According to the D2-D3 tree, *R. bunae* n. sp. has a maximally supported sister relationship with *R. wimbii* and *R. brevicaudatus* (Fig. 3). In the 18S tree, the new species has a maximally supported sister relationship to *R. wimbii* and *R. unisexus* (Fig. 4).

**Discussion**

A nematological investigation in coffee rhizospheres from southwest Ethiopia (Singh et al., 2023) uncovered an unidentified *Rotylenchus* population in one of the samples. This population is herein morphologically and molecularly characterised and found to be closely related to other *Rotylenchus* species found in Africa including *R. brevicaudatus* (reported from South Africa; Van den Berg & Heyns, 1974), *R. cypriensis* (reported from Algeria and South Africa; Van den Berg, 1998; Germani & Scotto La Massese, 2002), *R. minutus* (reported from South Africa; Van den Berg, 1981), *R. unisexus* (described from Kenya and reported in Ethiopia, South Africa and Zaire; Sher, 1965; Ali et al., 1973; Van den Berg, 1986; Fourie et al., 2001) and *R. wimbii* (described from Kenya; Singh et al., 2021). The morphometrical data of the new species appears to be closest to that of *R. unisexus*; however, they are clearly different in their molecular data. Furthermore, the new species can be easily separated morphologically and molecularly from the remaining species, except for molecular differences with *R. minutus*, as its sequences are not yet available. Of the various morphological differences to distinguish these species (see relationships above), stylet length seems to be the most useful feature, as the new species has relatively the longest stylet. Hitherto, with the characterisation of *R. bunae* n. sp., the total number of nominal species under *Rotylenchus* now stands at 106. Additionally, the presence of fasciculi has yet to be reported in all the species compared above, while distinct fasciculi were regularly observed in the new species.

There are no reports available on the real economic impact of *Rotylenchus* on coffee; however, this nematode has been found associated with the crop in several major coffee-growing countries – for example, *R. robustus* in India (Kumar, 1988) and Indonesia (Souza, 2008), *R. unisexus* and some unidentified *Rotylenchus* spp. in Ethiopia (Mekete et al., 2008a, b), and also more unidentified *Rotylenchus* spp. in Brazil (Kubo et al., 2001; Castro et al., 2008) and Vietnam (Souza, 2008). Similarly, *R. bunae* n. sp. was found abundantly (ca 50 individuals per 100 ml soil) in the coffee rhizospheres in Gera, Ethiopia. This species was also uncovered together with other PPN such as *Cryphodera* sp., *Helicotylenchus* sp., *Paratylenchus leptos*, *Tylenchorhynchus* sp. and *Xiphinema* sp. in the same sample. Some wilting and drying of the coffee plants was visually observed during sampling; however, no link could be established between the observed plants’ symptoms and the nematode parasitism. Nevertheless, it would be fundamental to investigate whether the new species can cause damage to coffee plants.

**References**


