Multi-locus phylogenetic analyses uncover species boundaries and reveal the occurrence of two new entomopathogenic nematode species, *Heterorhabditis ruandica* n. sp. and *Heterorhabditis zacatecana* n. sp.

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Abstract

Species of the nematode genus *Heterorhabditis* are important biological control agents against agricultural pests. The taxonomy of this group is still unclear as it currently relies on phylogenetic reconstructions based on a few genetic markers with little resolutive power, specially of closely related species. To fill this knowledge gap, we sequenced several phylogenetically relevant genetic loci and used them to reconstruct phylogenetic trees, to calculate sequence similarity scores, and to determine signatures of species- and population-specific genetic polymorphism. In addition, we revisited the current literature related to the description, synonymisation, and declaration as *species inquirendae* of *Heterorhabditis* species to compile taxonomically relevant morphological and morphometric characters, characterized new nematode isolates at the morphological and morphometrical level, and conducted self-crossing and cross-hybridization experiments. The results of this study show that the sequences of the mitochondrial cytochrome C oxidase subunit I (*COI*) gene provide better phylogenetic resolutive power than the sequences of nuclear rRNA genes and that this gene marker can phylogenetically resolve closely related species and even populations of the same species with high precision. Using this gene marker, we found two new species, *Heterorhabditis ruandica* n. sp. and *Heterorhabditis zacatecana* n. sp. A detailed characterization of these species at the morphological and morphometric levels and nematode reproduction assays revealed that the threshold for species delimitation in this genus, using *COI* sequences, is 97% to 98%. Our study illustrates the importance of rigorous morphological and morphometric characterization and multi-locus sequencing for the description of new species within the genus *Heterorhabditis*, serves to clarify the phylogenetic relationships of this important group of biological control agents, and can inform future species descriptions to advance our efforts towards developing more tools for sustainable and environmentally friendly agriculture.

Keywords

Biocontrol agents, Dichotomous key, Entomopathogenic nematodes, Nematode morphology, *Photorhabdus*, Phylogenetics, Species description, Taxonomy.
Nematodes of the genus *Heterorhabditis* Poinar, 1976 are soil-dwelling organisms that parasitize and kill certain small arthropods, mainly insects (Kaya and Gaugler, 1993). Their lifestyle is particularly interesting as they establish an obligate, mutualistic symbiosis with entomopathogenic bacteria of the genus *Photorhabdus* (Clarke, 2020; Machado et al., 2018). Nematodes colonize their prey, and upon sensing unknown chemical cues, they release their symbiotic bacterial partners inside the bodies of the infected organisms (Ciche et al., 2008; Dillman et al., 2012). The bacteria establish, multiply and produce an arsenal of immunosuppressors, lytic enzymes, and toxins that kill the infected organism and pre-digest its tissues, which serve as food for the bacteria and the nematodes (Shankhu et al., 2020; Tobias et al., 2016; Vlisidou et al., 2019). The nematodes grow, reproduce, and, upon resource depletion, reestablish symbiosis with *Photorhabdus* bacteria, and abandon the consumed cadavers in search for new prey (Somvanshi et al., 2012). Given this peculiar lifestyle, this deadly symbiotic pair is commonly used as a biocontrol agent in agricultural settings (Kajuga et al., 2018; Paddock et al., 2021; Toepfer and Zellner, 2017; Zhang et al., 2019). In addition, given the enormous biosynthetic capacity of *Photorhabdus* bacteria, they are of great medical, agricultural, and biotechnological importance (Blackburn et al., 1998; Bode, 2009; Hill et al., 2020; Joyce and Clarke, 2003; Lacey and Georgis, 2012; Machado et al., 2018; Machado et al., 2020; Tobias et al., 2018).

The number of described species of the genus *Heterorhabditis* is steadily growing, mainly boosted by recent advances in genomics. Up to now, the genus includes between 16 and 21 valid species, several synonymized species and some species inquirendae (Boemare et al., 1993; Hunt and Nguyen, 2016; Maneesakorn et al., 2011; Sudhaus, 2011; Tóth and Lakatos, 2008). Given the discrepancy in the number of recognized valid species and the increasing number of synonymized species, a thorough revision of the current literature related to the description, synonymisation, and declaration as species inquirendae of *Heterorhabditis* species may help to determine the actual number of valid species in this genus. As some species were described prior to the discovery of modern molecular techniques, and therefore the sequences of phylogenetically relevant gene markers are not available, morphological characters play an important role in this context (Andaló et al., 2006; Edgington et al., 2011; Hunt and Nguyen, 2016; Liu and Berry, 1996; Li et al., 2012; Malan et al., 2008; Malan et al., 2014; Nguyen et al., 2004, 2006, 2008; Pereira, 1937; Phan et al., 2003; Poinar and Veremchuk, 1970; Poinar, 1971, 1976; Poinar et al., 1987, 1992; Poinar, 1990; Stock et al., 2002).

Ribosomal RNA (rRNA) gene sequences such as ITS sequences and the sequences of the D2–D3 expansion segments of the 28S rRNA are traditionally used for identification purposes and for novel taxonomic status descriptions of the species of the genus *Heterorhabditis* (Adams et al., 1998; Campos-Herrera et al., 2011; Li et al., 2012; Malan et al., 2008; Nguyen et al., 2008; Rana et al., 2020; Spiridonov and Subbotin, 2016). As a recently evolved group, marginal variations in the rRNA gene sequences are expected in this genus, which limits the use of these genetic markers for taxonomic purposes, especially of closely related species (Blaxter et al., 1998; Blouin, 2002; Haag et al., 2018). In addition, the use of sequences containing several ambiguous nucleotides, potentially arisen from sequencing errors and/or poor quality-control, leads to erroneous taxonomic affiliations, as it is exemplified by the relatively high number of synonym species in the genus *Heterorhabditis* (Dhakal et al., 2020; Hunt and Nguyen, 2016). The use of mitochondrial DNA such as COI sequences, the gold standard taxonomic marker for species delimitation in the Kingdom Animalia, may help to overcome the taxonomic limitations of rRNA gene sequences. However, this taxonomic marker has been used only sporadically for identification purposes, barely used for taxonomy, and never used to describe new *Heterorhabditis* species (Chaubey et al., 2016; Hebert et al., 2003; Joyce et al., 1994a; Kuwata et al., 2007). As a consequence, the availability of COI sequences for this genus remained very limited for several years, limiting our understanding of the phylogenetic relationships of this genus (Chaubey et al., 2016; Dhakal et al., 2020; Kuwata et al., 2007).

To improve our understanding on the phylogenetic relationships of *Heterorhabditis* nematodes, to determine the most suitable genetic markers for the rapid and reliable identification of the species of this genus, specially of closely related species, and to determine species boundaries in this genus, we generated nucleotide sequences of several phylogenetically relevant gene markers and used them to reconstruct phylogenetic trees, to calculate sequence similarity scores, and to determine signatures of species- and population-specific genetic polymorphism. To improve our understanding on the taxonomic relationships of *Heterorhabditis* nematodes, we revisited the current literature related to the description, synonymisation, and declaration as species inquirendae of *Heterorhabditis* species to compile taxonomically relevant morphological and morphometric characters, characterized new nematode isolates at the morphological
and morphometrical level, and conducted self-crossing and cross-hybridization experiments. Our study illustrates the importance of multi-locus sequencing for the characterization of new species within the genus *Heterorhabditis*, serves to clarify the phylogenetic relationships of these important biological control agents, and can inform future species descriptions to advance our efforts towards developing more tools for sustainable and environmentally friendly agriculture.

**Materials and methods**

**Nematode origin**

*Heterorhabditis* nematodes used in this study were collected by us during different nematode collection campaigns carried out in Rwanda, Mexico, and India, or were collected by different collaborators at different locations around the world (Table S1) (Bai et al., 2013; Bhat et al., 2021b; Bruno et al., 2020; Carrera, 2015; Fallet et al., 2020; Mukuka et al., 2010; Rana et al., 2020; Yan et al., 2016).

**Nematode morphological and morphometrical characterization, light, and scanning electron microscopy**

One representative nematode isolate of each new species, MEX-39 and Rw14_N-C4a, was selected for detailed morphological and morphometrical characterization. First- and second-generation adult nematodes were obtained by dissecting infected *G. mellonella* larvae in Ringer’s solution. Infective juveniles (IJ) were collected after their emergence from *G. mellonella* larvae in White traps (White, 1927). Nematodes were killed with water at 60°C, then fixed in triethanolamine formalin (7 ml formalin, 2 ml triethanolamine, 91 ml ddH₂O), then dehydrated by the Seinhorst’s method, and finally transferred to glycerine (Bhat et al., 2019b; Courtney et al., 1955; Seinhorst, 1959, 1962). Nematodes were mounted in small drops of glycerine on permanent glass slides with extra layers of paraffin wax to prevent the flattening of the nematodes (Bhat et al., 2021a). Morphological measurements were taken using the Nikon DS-L1 software built in a phase contrast microscope (Nikon Eclipse 50i). Between 20 and 25 specimens at each developmental stage were measured. Light microscopy photographs were taken using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) equipped with differential interference contrast optics (DIC) and a Nikon Digital Sight DS–U1 camera. For the scanning electron microscopy (SEM), nematode specimens preserved in glycerine were processed as described by Abolafia (2015). For this, the nematodes were re-hydrated in distilled water, dehydrated in ethanol-acetone, critical-point dried with liquid carbon dioxide, mounted on SEM stubs with copper tape and coated with gold in a sputter coater. Specimens were observed and microphotographs were captured using a Zeiss Merlin microscope (5 kV) (Zeiss, Oberkochen, Germany). All micrographs were processed using Adobe® Photoshop® CS. The obtained morphometrical characters were compared with those published in previous studies describing all the species of the genus, independently of their current status (valid, species inquirendae, synonym, etc) (Abd-Elgawad and Ameen, 2005; Agüera de Doucet and Doucet, 1986; Andaló et al., 2006; Bhat et al., 2019a; Bhat et al., 2021b; Edgington et al., 2011; Gardner et al., 1994; Hunt and Nguyen, 2016; Kajol et al., 2020; Kakulia and Mikaia, 1997; Khan et al., 1976; Liu, 1994; Liu and Berry, 1996; Li et al., 2012; Malan et al., 2008; Malan et al., 2014; Maneesakorn et al., 2015; Nguyen et al., 2004; Nguyen et al., 2006; Nguyen et al., 2008; Pereira, 1937; Phan et al., 2003; Plchta et al., 2009; Poiliar and Veremchuk, 1970; Poiliar, 1976; Poinar et al., 1987; Poinar et al., 1992; Rana et al., 2020; Sagun et al., 2015; Shahina et al., 2017; Shamseldan et al., 1996; Stock, 1993; Stock et al., 1996; Stock, 1997; Stock et al., 2002; Stock et al., 2009; Turco, 1970; Vanlalhlimpuia et al., 2018; Wouts, 1979).

**Self-crossing and cross-hybridization experiments**

Self-crossing and cross-hybridization experiments were carried out on lipid agar plates as described by Dix et al. (1992). *Heterorhabditis ruandica* n. sp. Rw14_N-C4a and *H. zacatecana* n. sp. MEX-39 were self-crossed, hybridized with each other and with *H. bacteriophora* CH21 (Rana et al., 2020). For this, one second–generation male and one second–generation virgin female were placed on lipid agar plates (35 mm diam.) and incubated at 27°C. Ten independent plates per crossing type were set. Progeny production was observed daily for a period of five consecutive days. Experiments were repeated three times under the same conditions.

**Nematode molecular characterization and phylogenetic relationships**

Genomic DNA from about 10 to 20 thousand nematodes was extracted using the genomic DNA
isolation kit following manufacturer’s instructions (Norgen Biotek Corp., Thorold, Ontario, Canada). The following genes/genomic regions were amplified by polymerase chain reaction (PCR): the D2–D3 expansion segments of the 28S rRNA, the internal transcribed spacer (ITS) region of the rRNA, the cytochrome c oxidase I (COI), the thin filament (F-actin)-associated protein (unc-87), and the calmodulin 1 (cmd-1). Primers used were selected based on previous publications (Dhakal et al., 2020; Joyce et al., 1994b; Regeai et al., 2009; Subbotin et al., 2006) (Table S2). PCR reactions consisted of 1 µL of genomic DNA, 12.5 µL of EmeraldAmp GT PCR Master Mix (Takara Bio, Shiga, Japan), 0.5 µL of both forward and reverse primers at 10 mM and 10.5 µL of dH₂O. The PCR reaction was performed using a thermocycler (Mastercycler nexus gradient, Eppendorf, Germany) with the following settings: (i) for ITS and D2–D3, 1 cycle of 1 min at 98°C followed by 35 cycles of 10 sec at 98°C, 30 sec at 50°C, 1 min 30 sec at 72°C, and by a single final elongation step at 72°C for 10 min; (ii) for cmd-1 and unc-87, 1 cycle of 1 min at 98°C followed by 40 cycles of 10 sec at 98°C, 30 sec at 50°C, 30 sec at 72°C, and by a single final elongation step at 72°C for 10 min; (iii) for COI, 1 cycle of 1 min at 98°C followed by 40 cycles of 10 sec at 98°C, 30 sec at 40°C, 30 sec at 72°C, and by a single final elongation step at 72°C for 10 min. PCR was followed by electrophoresis (45 min, 100 V) of 5 µL of PCR products in a 1% TBA (Tris–boric acid–EDTA) buffered agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, California, USA). PCR products were purified using the FastGene Gel/PCR extraction kit (Nippon Genetics Co., Japan) and sequenced using reverse and forward primers by Sanger sequencing (Microsynth AG, Balgach, Switzerland). Obtained sequences were manually curated and trimmed and deposited in the NCBI under the accession numbers given in Table S3. Sequences of the following nematode strains were obtained in this study: *Heterorhabditis ruandica* n. sp. (isolates Rw14_N-C4a and *H. zacatecana* n. sp. MEX-39 nematodes were isolated as described by Machado et al. (2019), (2021b). Briefly, *Galleria mellonella* larvae (Lepidoptera: Pyralidae) were exposed to 100 nematode infective juveniles. Three to four days later, insect cadavers were surface-sterilized and cut open with a blade. Bacteria-digested internal organs were spread onto LB agar plates and incubated at 28°C for 24 to 48 h. *Photorhabdus*-like colonies were sub-cultured until monocolonies were obtained. A single primary form colony was then selected and used for further experiments. Bacteria primary forms were determined by examining colony characteristics and by examining pigments uptake on NBTA plates (LB agar plates supplemented with 25 mg l⁻¹ bromothymol blue and 4 mg l⁻¹ triphenyl-2,3,5-tetrazolium chloride). The strains were further sub-cultured and maintained on LB agar plates at 28°C. To establish their taxonomic identities, we reconstructed phylogenetic relationships based on whole genome sequences of the isolated bacteria and all the different species/
subspecies of the genus *Photorhabdus* (Machado et al., 2021a, b). To obtain genomic sequences, genomic DNA was extracted and purified using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Switzerland) following manufacturer’s instructions. The resulting DNA was used for library preparation using the TruSeq DNA PCR-Free LT Library Prep (FC-121-3003) kit. Indexed libraries were then pooled at equimolar concentrations and sequenced (2 × 150 bp) on an Illumina HiSeq 3000 instrument. Genomes were assembled using the Bactopia pipeline (Petit and Read, 2020). Briefly, the raw Illumina reads were quality trimmed using Trimmomatic 0.39 (Bolger et al., 2014). The resulting reads were assembled with SPAdes 3.14.1 (*k*-mer sizes of 31, 51, 71, 91, and 111 bp) (Bankevich et al., 2012). Scaffolds with a mean read-depth smaller than 20% of the median read-depth of the longer scaffolds (≥5,000 bp) as well as scaffolds that were shorter than 200 bp were removed. The final assemblies were polished using Pilon 1.22 (Walker et al., 2014). Genome sequences were deposited in the National Centre for Biotechnology Information. Accession numbers are listed in Table S4. Phylogenetic relationships were reconstructed based on the assembled genomes and the genome sequences of all validly published species of the genus (Machado et al., 2021a, b). For this, core genome alignments were created using Roary 3.6.2 (Page et al., 2015). Using this alignment, a maximum likelihood tree was constructed using Fasttree 2.1.10 based on the Jukes-Cantor + CAT nucleotide evolution model (Price et al., 2009, 2010). These analyses were carried out in Galaxy (Afgan et al., 2018). Whole genome sequence similarities were calculated by the digital DNA-DNA hybridization (dDDH) method using the recommended formula 2 of the genome-to-genome distance calculator (GGDC) web service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Auch et al., 2010a, 2010b; Meier-Kolthoff et al., 2013, 2014).

**Results and discussion**

### *Heterorhabditis ruandica* n. sp.

Figures 1–4, Tables 1 and 3–6.

#### Males

Body 0.65 to 0.86 mm long, C-shaped after fixation. Cuticle almost smooth, with transversal striae poorly developed. Lateral field not visible. Lip region with six lips developed but not fused bearing six acute labial papillae at oral margin and four rounded cephalic papillae at the base of lips. Oral opening almost rounded with thick margins. Amphidial apertures pore-like, ovoid and located posterior to lateral labial papillae. Stoma rhabditoid type, 1.2 to 2.3 times the lip region width, with short cheilostom with poorly refringent rounded cheilorhabdia, short gymnostom with refringent bar-like rhabdia, and long stegostom surrounded by the pharyngeal collar and bearing bar-like pro-mesorhabdia and small poorly refringent meta-telorhabdia. Pharynx poorly developed with robust corpus without differentiated metacorpus, short and slightly narrow isthmus and pyriform bulb with poorly visible valvular apparatus. Nerve ring encircling the isthmus at 58 to 75% of neck length, just anterior to basal bulb. Excretory pore located at basal bulb level, located at 61 to 97% of neck length. Cardia poorly developed, surrounded by intestinal tissue. Intestine without differentiations. Cardiac anterior end with thin walls. Genital system monorchic, laterally reflexed. Spicules well-developed, separate, with small angular manubrium, calamus poorly developed, and robust lamina with acute tip, scarcely prominent dorsal hump and poorly developed ventral velum. Gubernaculum with manubrium straight and slightly ventrally curved corpus, 40 to 50% of spicule length. Tail conoid with acute tip, ventrally curved posteriorly, flanked by the bursa. Bursa peloderan, with nine pairs of genital papillae (1 + 2/3 + 3), one of them probably the phasmid: three pairs pre-cloacal (GP1–GP3) and six pairs post-cloacal being three pairs at mid tail length (GP4–GP6) and three pairs (GP7–GP9) terminal; GP1 and GP2 more spaced, GP2 and GP3 closely spaced (Figs. 1–4).

### Hermaphroditic females

Body 2.91 to 4.12 mm long, arcuate with general morphology similar to male, having labial papillae very acute and prominent. Nerve ring encircling the isthmus at 56 to 78% of neck length. Excretory pore located at or posterior to basal bulb, located at 67 to 103% of neck length. Genital reproductive system didelphic-amphidelphic with ovaries well developed, reflexed, oviducts and uteri not well visible, vagina very short and vulva small having transverse slit opening. Rectum slender, 0.8 to 1.3 times longer than the anal body diameter. Anus with prominent lips. Tail conoid with acute tip lacking mucro, having cellular part simple at its junction with the hyaline part. Phasmids inconspicuous (Figs. 1–4).

### Amphimictic females

Body similar to, but usually smaller than hermaphroditic females, 1.13–1.61 mm long. Rectum very
Multi-locus phylogenetic analyses uncover species boundaries: Machado et al.

Figure 1: Line drawings of *Heterorhabditis ruandica* n. sp. (A) A hermaphroditic female. (B) Cephalic region of a hermaphroditic female. (C) Pharyngeal region of a hermaphroditic female. (D) Anterior part of the reproductive system of a hermaphroditic female. (E) Posterior end of a hermaphroditic female. (F) An amphimictic female. (G) Pharyngeal region of an amphimictic female. (H) Posterior end of an amphimictic female. (I) A male adult. (J) Pharyngeal region of a male adult. (K) Posterior region of a male adult. (L) Pharyngeal region of an infective juvenile. (M) Posterior end of an infective juvenile. (N) An infective juvenile.
Figure 2: Light microscope micrographs of *Heterorhabditis ruandica* n. sp. (A) An amphimictic female (black arrow pointing at the position of the vulva, white arrow pointing at the anus). (B) Pharyngeal region of an amphimictic female. (C) Posterior end of an amphimictic female. (D) Vulva of an amphimictic female. (E) A male adult. (F) Pharyngeal region of a male adult. (G) Posterior end of a male adult (arrows pointing at the genital papillae).
Figure 3: Light microscope micrographs of *Heterorhabditis ruandica* n. sp. (A) A hermaphroditic female. (B) Anterior end of a hermaphroditic female. (C) Pharyngeal region of a hermaphroditic female. (D) Posterior end of a hermaphroditic female. (E) A sheathed third stage juvenile (J2). (F) Pharyngeal region of a sheathed third stage juvenile (J2). (G) Posterior end of a sheathed third stage juvenile (J2) (arrow pointing the anus). (H) A non-sheathed third stage juvenile (J3). (I) Pharyngeal region of a non-sheathed third stage juvenile (J3). (J) Posterior end of a non-sheathed third stage juvenile (J3).
Figure 4: Scanning electron microscope (SEM) micrographs of *Heterorhabditis ruandica* n. sp. (A, B) Lip region in lateral and frontal views, respectively, of a hermaphroditic female. (C) Excretory pore of a hermaphroditic female (pointed by an arrow). (D) Vulva of a hermaphroditic female (pointed by an arrow). (E) Tail of a hermaphroditic female in lateral view. (F, G) Lip region of a female adult in lateral and frontal views, respectively. (H) Excretory pore (pointed by an arrow). (I) Vulva of a female adult (pointed by an arrow). (J) Tail of a female adult in ventral view. (K, L) Lip region of a male adult in sublateral and frontal views, respectively. (M, N) Posterior end of a male adult in ventral and lateral views, respectively (arrows pointing the genital papillae). (O) Lip region of a second-stage juvenile (J2) in lateral view. (P) Cuticle of a second-stage juvenile (J2). (Q) Tail of a second-stage juvenile (J2) in lateral and ventral views, respectively. (R) Lip region of a third-stage juvenile (J3) in ventral view (arrow pointing the frontal tooth). (S) Cuticle (arrow pointing the cuticle of a third-stage juvenile).
Table 1. Morphometrics of infective juveniles and adult generations of *Heterorhabditis ruandica* n. sp.

| Characters                  | Holotype | Paratypes | Hermaphrodite (1<sup>st</sup> Gen) paratypes | Female (2<sup>nd</sup> Gen) paratypes | Infective juvenile paratypes |
|-----------------------------|----------|-----------|---------------------------------------------|--------------------------------------|-------------------------------|
| n                           | 1        | 20        | 20                                          | 20                                   | 25                            |
| Body length (L)             | 760      | 769 ± 60 (652-863) | 3295 ± 286 (2907-4123) | 1366 ± 123 (1131-1608) | 544 ± 29 (496-591) |
| a (L/BD)                    | 20.3     | 17 ± 1.5 (15-21) | 14.1 ± 1.1 (11.7-16.1) | 18 ± 1.4 (15-20) | 24 ± 3.0 (20-29) |
| b (L/NL)                    | 7.8      | 8.1 ± 1.0 (5.8-9.7) | 23 ± 1.8 (21-27) | 11.4 ± 1.2 (9.0-13.6) | 4.7 ± 0.4 (4.1-5.4) |
| c (L/T)                     | 26.2     | 31 ± 3.6 (23-36) | 42 ± 5.7 (34-51) | 20 ± 2.2 (16-24) | 8.2 ± 1.0 (7.6-8.6) |
| c’ (T/ABW)                  | 1.1      | 1.4 ± 0.2 (0.6-1.7) | 2.2 ± 0.3 (1.7-2.6) | 2.8 ± 0.5 (1.9-3.6) | 4.6 ± 0.8 (3.4-5.8) |
| V (VA/L × 100)              | –        | –         | 48 ± 2.5 (45-55) | 48 ± 2.3 (41-51) | –                            |
| Max. Body Width (MBD)       | 37.5     | 44 ± 3.0 (40-51) | 233 ± 17 (209-274) | 77 ± 4.0 (68-83) | 23 ± 2.7 (18-27) |
| Lip region width            | 6.5      | 7.2 ± 0.8 (5.7-8.4) | 12.4 ± 0.8 (11.0-14.0) | 10.3 ± 0.9 (8.8-12.2) | –                            |
| Stoma length                | 9.5      | 11.1 ± 1.6 (8.7-13.9) | 14.9 ± 1.4 (13-18) | 13.6 ± 1.8 (10.4-16.0) | 13.8 ± 1.2 (12.1-16.0) |
| Bulb length (BL)            | 18.5     | 20 ± 1.8 (18-25) | 35 ± 3.6 (29-42) | 27 ± 2 (23-30) | 13.8 ± 1.8 (11.0-19.0) |
| Pharynx length (PL)         | 95.2     | 84 ± 7.1 (74-107) | 128 ± 6.3 (118-142) | 107 ± 6.9 (91-120) | 102 ± 7.0 (91-115) |
| Nerve ring – ant. end (NR)  | 68       | 63 ± 5.2 (56-74) | 93 ± 7.5 (78-108) | 81 ± 6.4 (69-97) | 55 ± 3.6 (52-64) |
| Excretory pore– ant. end (EP)| 84.3  | 81 ± 10.1 (61-109) | 121 ± 11 (106-153) | 111 ± 10.8 (92-129) | 78 ± 3.4 (70-89) |
| Neck length (Stoma+Pharynx, NL)| 98  | 96 ± 7.3 (84-117) | 143 ± 6.3 (134-159) | 120 ± 6.0 (107-132) | 115 ± 7.3 (103-131) |
| Body width at neck base     | 36       | 34 ± 1.9 (30-37) | 119 ± 8.9 (101-138) | 58 ± 4.3 (50-66) | 18 ± 3.0 (15-24) |
| Vagina length               | –        | –         | 28 ± 4.0 (20-38) | 19.2 ± 2.9 (15-26) | –                            |
| Body width at vulva         | –        | –         | 240 ± 21 (199-278) | 78 ± 3.8 (72-85) | –                            |
| Vulva – ant. end (VA)       | –        | –         | 1581 ± 151 (1369-1882) | 655 ± 47 (572-706) | –                            |
| Vulva – post. End (PV)      | –        | –         | 1713 ± 178 (1453-2241) | 710 ± 89 (559-949) | –                            |
| Rectum length               | –        | –         | 36 ± 4.6 (29-49) | 30 ± 3.8 (24-35) | 8.5 ± 1.9 (6.1-13.7) |
| Anal body diam. (ABD)       | 26.1     | 18 ± 2.4 (15-25) | 37 ± 5.5 (29-51) | 25 ± 4.5 (18-34) | 12.4 ± 1.8 (9.2-16.0) |
| Tail with sheath length (T) | –        | –         | –                        | –                        | 56 ± 4.9 (49-64) |
| Tail without sheath length  | 29       | 25 ± 3.2 (21-29) | 80 ± 7.9 (63-98) | 68 ± 6.5 (62-88) | 30.4 ± 4.5 (22-39) |
long, almost twice longer than the anal body diameter. Anus with posterior lip very prominent. Tail conoid with acute tip lacking mucro, having cellular part bifurcated at its junction with the hyaline part (Figs. 1–4).

**Infective sheathed juveniles (J3 stage envolved by the J2 stage cuticle)**

Body 0.5 to 0.6 mm long, with habitus slightly ventral curved after fixation. Cuticle with transversal striae at anterior end, with both transversal and longitudinal striae at neck region and only with longitudinal striae at rest of body. Lip region lacking differentiate lips, bearing six labial papillae and cephalic papillae not visible. Amphidial apertures very reduced. Oral opening closed, having triradial symmetry. Stoma tubular, about twice the lip region wide. Pharynx slender, with long and narrow corpus, very narrow isthmus and pyriform basal bulb. Nerve ring surrounding the isthmus. Excretory pore at or just posterior to basal bulb. Cardia reduced, surrounded by intestinal tissue. Reproductive system absent. Rectum poorly visible. Anus closed. Tail conoid elongate with acute tip without mucro. Terminal hyaline part 37 to 54% of tail length (Figs. 1–4).

**Infective non-sheathed juveniles (J3 stage)**

Body 0.47 to 0.56 mm long, with habitus almost straight after fixation. Cuticle with only transversal striae. Lip region lacking differentiate lips, and labial and cephalic papillae not visible. Oral opening rounded, closed, bearing a large, very refringent dorsal tooth. Amphidial apertures very prominent. Stoma tubular, slightly longer than the lip region wide. Pharynx, nerve ring and excretory pore location similar to the sheathed stage. Cardia reduced, surrounded by intestinal tissue. Rectum poorly visible. Anus closed. Tail conoid with very acute tip without mucro. Terminal hyaline part absent (Figs. 1–4).

**Diagnosis of *Heterorhabditis ruandica* n. sp. and morphological relationships with other species**

*Heterorhabditis ruandica* n. sp. is characterized by having hermaphrodite females 2.91 to 4.12 mm long, amphimictic females 1.13 to 1.61 mm long, males 0.65 to 0.86 mm long, and IJs 0.50 to 0.59 mm long. Cuticle with poorly visible annuli in adults, with longitudinal crests in IJ2 and with well-developed...
The males of *Heterorhabditis ruandica* n. sp. can be distinguished from *H. beicherriana* males by the anterior end to the excretory pore (61-109 vs. 101-145 µm) and the anterior end to the nerve ring (56-74 vs. 72-93 µm), and by the tail (21-29 vs. 29-41 µm) and gubernaculum (15-21 vs. 20-28 µm) length, and by the D% value (61-97 vs. 102-120). Several other morphometric differences were also observed in hermaphroditic and amphimitic females (Tables 3–6).

**Heterorhabditis ruandica** n. sp. can be distinguished from *H. georgiana* by the anterior end to the excretory pore (67-90 vs. 97-113 µm) and the anterior end to the nerve ring (52-64 vs. 74-94 µm) distances, and by the tail length (49-65 vs. 86-108 µm) of IJs. The males can be distinguished by the anterior end to the excretory pore (61-109 vs. 101-145 µm) and the anterior end to the nerve ring (56-74 vs. 72-93 µm) distances, and by tail (21-29 vs. 29-41 µm) and gubernaculum (15-21 vs. 20-28 µm) length, and by D% values (61-97 vs. 100-122). Several other morphometric characters of hermaphroditic and amphimitic females differ between these two species (Tables 3–6).

**Type host and locality**

The type hosts are unknown as the nematodes of this genus can be hosted by different insect species and were isolated from soil samples by the Galleria baiting technique (Bedding and Akhurst, 1975; White, 1927). Nematode strains *H. ruandica* n. sp. RW18_M-Hr1a and RW18_M-Hr1b were collected in the district of Karongi, Western province of the Republic of Rwanda (Decimal degrees coordinates: -2.131500, 29.325467) in a moist habitat along a river bench covered with sweet potato plants. *Heterorhabditis ruandica* n. sp. RW14_N-C4a nematodes were collected in a ploughed cropland on terraces in a hilly area near Kanyirandori village, Tare sector, Nyamagabe district, Southern province of the Republic of Rwanda (Decimal degrees coordinates: -2.500000, 29.483333).

**Type material**

RW14_N-C4a nematodes are the type material for *Heterorhabditis ruandica* n. sp. Holotype male, and 15 paratype hermaphrodites, males and amphimitic females and 15 third stage juveniles were deposited in the National Nematode Collection of India, IARI, New Delhi, India. Additional specimens were deposited at...
the nematode collection of the Department of Animal Biology, Plant Biology and Ecology of the University of Jaén, Spain, under the following slide numbers: Rwa001-01 to -12 (25 hermaphrodite females and 6 juveniles), Rwa002-01 to -05 (8 amphimictic females and 9 males), and Rwa003-01 to -02 (8 juveniles). Nematode cultures are maintained in the Institute of Biology, University of Neuchatel, Switzerland and in the Rwanda Agriculture and Animal Resource Development Board, Rubona, Rwanda.

**Etymology**

The specific name refers to the country, the Republic of Rwanda (Africa), where the type material, *Heterorhabditis ruandica* n. sp. Rw14_N-C4a nematodes, used to phenotypically characterize the species, were collected.

*Heterorhabditis zacatecana* n. sp.

Figures 5–8, Tables 2 and 3–6

**Males**

Body 0.81 to 0.91 mm long, J-shaped after heat killing and body arcuate posteriorly. Cuticle almost smooth, with transversal striae poorly developed. Lateral field not visible. Lip region with six lips poorly developed bearing six acute labial papillae at oral margin and four rounded cephalic papillae at base of lips. Oral opening almost rounded with thick margin. Amphidial apertures pore-like, ovoid and located posterior to lateral labial papillae. Stoma rhabditoid type, 0.9 to 1.6 times the lip region width, with short cheilostom with poorly refringent rounded cheilorhabdia, short gymnostom with refringent bar-like rhabdia, and long stegostom surrounded by the pharyngeal collar and bearing bar-like pro-mesorhabdia and small poorly refringent meta-telorhabdia. Pharynx poorly developed with robust corpus without differentiated metacorpus, short and slightly narrow isthmus and robust pyriform bulb with poorly visible valvular apparatus. Nerve ring encircling the isthmus at 61% to 96% of neck length, just anterior to basal bulb. Excretory pore located at or posterior to the basal bulb, located at 78% to 134% of neck length. Cardia poorly developed, surrounding by intestinal tissue. Intestine without differentiations. Genital system monorchic, laterally reflexed. Spicules well developed, separate, with more or less rounded manubrium, calamus poorly developed, and thinner and slender lamina with acute tip, scarcely prominent dorsal hump and poorly developed ventral velum. Gubernaculum with manubrium slightly ventral curved and straight corpus, 40% to 60% of spicule length. Tail conoid with acute tip, ventrally curved posteriorly, flanked by the bursa. Bursa peloderan, with nine pairs of genital papillae (1 + 2/3 + 3), one of them probably the phasmid: three pairs pre-cloacal (GP1–GP3) and six pairs post-cloacal being three pairs at mid tail length (GP4–GP6) and three pairs (GP7–GP9) terminal; GP1 and GP2 more spaced, GP2 and GP3 closely spaced (Figs. 5–8).

**Hermaphroditic females**

Body 4.41 to 6.18 mm long, arcuate with general morphology similar to male, having labial papillae more acute and prominent. Genital reproductive system didelphic–amphidelphic with ovaries well developed, reflexed, oviducts and uteri not well visible, vagina very short and vulva small having transverse slit opening. Rectum slender, about 1.5 times longer than the anal body diameter. Anus with prominent lips. Tail conoid with acute tip lacking mucro, having cellular part simple at its junction with the hyaline part. Phasmids inconspicuous (Figs. 5–8).

**Amphimictic females**

Body similar to, but usually smaller than hermaphrodites, 1.95 to 2.80 mm long. Rectum very long, about twice longer than the anal body diameter. Anus with posterior lip more prominent. Tail conoid with acute tip lacking mucro, having cellular part simple at its junction with the hyaline part (Figs. 5–8).

** Infective sheathed juveniles (J3 stage envolved by the J2 stage cuticle)**

Body 0.49–0.58 mm long, with habitus slightly ventral curved after fixation. Cuticle with transversal striae at anterior end, with both transversal and longitudinal striae at neck region and only with longitudinal striae at rest of body. Lip region lacking differentiate lips, bearing six labial papillae and cephalic papillae not visible. Amphidial apertures very reduced. Oral opening closed, having triradiial symmetry. Stoma tubular, about twice the lip region wide. Pharynx slender, with long and narrow corpus, very narrow isthmsus and pyriform basal bulb. Nerve ring surrounding the isthmus. Excretory pore at or just posterior to basal bulb. Cardia reduced, surrounded by intestinal tissue. Reproductive system absent. Rectum poorly visible. Anus closed. Tail conoid elongate with acute tip without mucro. Terminal hyaline part 31% to 56% of tail length (Figs. 5–8).
Multi-locus phylogenetic analyses uncover species boundaries: Machado et al.

Figure 5: Line drawings of *Heterorhabditis zacatecana* n. sp. (A) A hermaphroditic female. (B) Pharyngeal region of a hermaphroditic female. (C) Anterior part of the reproductive system of a hermaphroditic female. (D) Posterior end of a hermaphroditic female. (E) An amphimictic female. (F) Pharyngeal region of an amphimictic female. (G) Posterior end of an amphimictic female. (H) A male adult. (I) Pharyngeal region of a male adult. (J) Posterior end of a male adult. (K) Pharyngeal region of an infective juvenile. (L) Posterior end of an infective juvenile. (M) An infective juvenile.
Figure 6: Light microscope micrographs of *Heterorhabditis zacatecana* n. sp. (A) An amphimictic female (black arrow pointing the vulva, white arrow pointing the anus). (B) Pharyngeal region of an amphimictic female. (C) Posterior end of an amphimictic female. (D) A male adult. (E) Pharyngeal region of a male adult. (F) Posterior end of a male adult (arrows pointing at the genital papillae).
Multi-locus phylogenetic analyses uncover species boundaries: Machado et al.

Figure 7: Light microscope micrographs of *Heterorhabditis zacatecana* n. sp. (A) A hermaphroditic female. (B) Pharyngeal region of a hermaphroditic female. (C) Posterior end of a hermaphroditic female. (D) A sheathed third stage juvenile (J2). (E) Pharyngeal region of a sheathed third stage juvenile (J2). (F) Posterior end of a sheathed third stage juvenile (J3). (G) A non-sheathed third stage juvenile (J3). (H) Pharyngeal region of a non-sheathed third stage juvenile (J3). (I) Posterior end of a non-sheathed third stage juvenile.
Figure 8: Scanning electron microscope (SEM) micrographs of *Heterorhabditis zacatecana* n. sp. (A, B) Lip region in lateral and frontal views, respectively, of a hermaphroditic female. (C) Broken cuticle of a hermaphroditic female with a juvenile emerging. (D) Vulva of a hermaphroditic female (pointed by a white arrow). (E) Tail of a hermaphroditic female in lateral view. (F, G) Lip region of a female adult in lateral and frontal views, respectively. (H) Excretory pore of a female adult (pointed by a white arrow). (I) Vulva of a female adult. (J) Tail of a female adult in ventral view. (K, L) Lip region of a male adult in lateral and frontal views, respectively. (M, N) Posterior end of a male adult in lateral and ventral views, respectively (arrows pointing at the genital papillae). (O) Lip region of a second-stage juvenile (J2) in lateral view. (P) Cuticle of a second-stage juvenile (J2) (arrow pointing the excretory pore). (Q) Tail of a second-stage juvenile (J2) in lateral and ventral views, respectively. (R) Lip region of a third-stage juvenile (J3) in dorsal view (arrow pointing the frontal tooth). (S) Cuticle of a third-stage juvenile (J3) (arrow pointing the excretory pore).
Table 2. Morphometrics of infective juveniles and adult generations of *Heterorhabditis zacatecana* n. sp.

| Characters                        | Holotype | Paratypes | Hermaphrodite (1st Gen) paratypes | Female (2nd Gen) paratypes | Infective juvenile paratypes |
|-----------------------------------|----------|-----------|-----------------------------------|---------------------------|-----------------------------|
|                                   |          | 20        | 22                                | 22                        | 25                          |
| n                                 |          |           |                                   |                           |                             |
| Body length (L)                   | 808.1    | 861 ± 29 (811-914) | 5127 ± 494 (4408-6179)             | 2244 ± 203 (1954-2798)     | 539 ± 21 (493-578)          |
| a (L/BD)                          | 19.0     | 18 ± 1.6 (15-22) | 16 ± 2.0 (13-20)                  | 12.3 ± 1.2 (10.5-15.0)     | 22 ± 1.2 (19-24)            |
| b (L/NL)                          | 8.1      | 9.1 ± 1.1 (7.6-12) | 26 ± 4.3 (20-34)                  | 18 ± 1.8 (16-21)          | 5.0 ± 0.4 (4.4-5.9)         |
| c (L/T)                           | 28.9     | 34 ± 4.2 (26-43) | 70 ± 10.4 (52-90)                 | 39 ± 7.4 (31-63)          | 9.4 ± 0.6 (8.2-10.5)        |
| c' (T/ABW)                        | 1.4      | 1.6 ± 0.3 (1.2-2.5) | 1.6 ± 0.3 (1.2-2.4)               | 1.7 ± 0.2 (1.3-2.0)       | 5.3 ± 0.6 (4.3-6.7)         |
| V (VA/L × 100)                    | –        | –         | 48 ± 4.3 (36-57)                  | 53 ± 4.2 (43-61)          | –                           |
| Max. Body Width (MBD)             | 42.5     | 48 ± 3.6 (41-56) | 319 ± 41 (235-358)               | 183 ± 23 (160-228)        | 24 ± 0.9 (23-27)            |
| Lip region width                  | 6.2      | 7.4 ± 0.7 (6.2-8.8) | 11.7 ± 2.4 (9.2-19.2)           | 10.1 ± 1.0 (7.7-11.4)     | 4.0 ± 0.5 (3.2-5.2)         |
| Stoma length                      | 10       | 9.3 ± 1.0 (6.3-11) | 19 ± 2.0 (14-23)                 | 11.5 ± 1.7 (8.0-15.2)     | 13.5 ± 1.0 (12.0-15.3)      |
| Bulb length (BL)                  | 20.2     | 22 ± 2.4 (19-28) | 40 ± 4.6 (28-49)                 | 30 ± 2.6 (28-38)          | 20 ± 1.4 (17.1-23.0)        |
| Pharynx length (PL)               | 95.2     | 86 ± 9.8 (57-100) | 182 ± 23 (155-211)              | 113 ± 9.5 (101-133)      | 95 ± 7.2 (82-111)           |
| Nerve ring – ant. end (NR)        | 65.4     | 66 ± 5.3 (60-78) | 131 ± 22 (96-169)                | 83 ± 7.3 (71-96)          | 81 ± 6.3 (69-72)            |
| Excretory pore– ant. end (EP)     | 96.2     | 93 ± 9.6 (77-109) | 150 ± 24 (108-190)               | 113 ± 11 (100-133)       | 89 ± 6.8 (72-99)            |
| Neck length (Stoma+Pharynx, NL)   | 99.3     | 96 ± 9.6 (71-108) | 201 ± 21 (174-231)               | 124 ± 10 (112-148)       | 109 ± 6.9 (96-124)          |
| Body width at neck base           | 34.5     | 36 ± 2.3 (31-40) | 167 ± 13 (133-188)               | 95 ± 13.9 (74-121)       | 23 ± 1.3 (19-26)            |
| Vagina length                     | –        | –         | 31 ± 4.0 (24-36)                 | 25 ± 6.4 (17-42)         | –                           |
| Body width at vulva               | –        | –         | 331 ± 33 (257-379)               | 185 ± 27 (153-230)       | –                           |
| Vulva – ant. end (VA)             | –        | –         | 2470 ± 279 (1959-3038)           | 1182 ± 129 (910-1397)     | –                           |
| Vulva – post. end (PV)            | –        | –         | 2657 ± 279 (1990-3938)           | 1062 ± 147 (860-1455)    | –                           |
| Rectum length                     | –        | –         | 36 ± 4.6 (30-41)                 | 27 ± 4.1 (19-39)         | –                           |
| Anal body diam. (ABD)             | 19.6     | 17 ± 2.3 (13-22) | 47 ± 8.1 (34-58)                 | 35 ± 3.2 (31-41)         | 11.1 ± 1.3 (8.6-14.1)      |
| Tail with sheath length (T)       | –        | –         | –                                 | –                        | 58 ± 3.1 (52-63)           |
**Infective non-sheathed juveniles (J3 stage)**

Body 0.47 to 0.55 mm long, with habitus slightly ventral curved after fixation. Cuticle with only transversal striae. Lip region lacking differentiate lips, and labial and cephalic papillae not visible. Oral opening rounded, closed, bearing a small dorsal tooth. Amphidial apertures very prominent. Stoma tubular, slightly longer than the lip region wide. Pharynx, nerve ring and excretory pore location similar to the sheathed stage. Cardia reduced, surrounded by intestinal tissue. Rectum poorly visible. Anus closed. Tail conoid with acute tip without mucro. Terminal hyaline part absent (Figs. 5–8).

**Diagnosis of Heterorhabditis zacatecana n. sp. and relationships with other species**

*Heterorhabditis zacatecana* n. sp. is characterized by having hermaphrodite females 4.41 to 6.18 mm long, amphimictic females 1.9 to 2.7 mm long, males 0.81 to 0.91 mm long, and IJs 0.49 to 0.57 mm long. Cuticle with poorly visible annuli in adults, with longitudinal crests in IJ2 and with well-developed annuli in IJ3. Lip region with six low lips having thick and acute lipplets in adults. Lips are poorly developed in IJ2 and bearing a small refringent dorsal tooth in IJ3. Stoma reduced in adults and tubular in IJs. Pharynx robust and short in adults, and narrow and slender in IJs. Female reproductive system didelphic–amphidelphic. Anal body diameter in hermaphrodites 34 to 58 µm long, in amphimictic females 31 to 41 µm long, and in males 13 to 22 µm long. Tail short and conoid with acute terminus at cellular part in hermaphrodite females (63-87 µm long, c = 52-90, c′ = 1.2-2.4), and in amphimictic females (45-75 µm long, c = 31-63, c′ = 1.3-2.0). Tail conoid-elongate in IJ2 (52-63 µm long, c = 7.9–9.8, c′ = 4.0-6.5) and in IJ3 (25-34 µm long, c = 8.2-10.5, c′ = 4.3-6.7). Male reproductive system monorchic, with spicules 38 to 55 µm long having conoid manubrium 15 to 25 µm long, bursa peloderan bearing nine pairs of genital papillae (1 + 2/3 + 3).

*Heterorhabditis zacatecana* n. sp. is morphologically similar to *H. ruandica* n. sp., *H. amazonensis*, *H. bacteriophora*, *H. georgiana* and *H. beicheriana*, and can be distinguished from these species mainly by adults and infective juvenile characters (Tables 3–6). *Heterorhabditis zacatecana* n. sp. can be distinguished from *H. ruandica* n. sp., one of the morphologically most similar species, by the shape of the male spicule (slender vs. robust) and the manubrium size (large vs. small).
small), the size of hermaphrodites (4.41-6.18 vs. 2.91-4.12 mm), the hermaphrodite neck length (174-231 vs. 134-159 µm), and the hermaphrodite c ratio (52-90 vs. 34-51). The size of amphimictic females (1.95-2.80 vs. 1.13-1.61 µm), the shape of the tail tip (acute and longer vs. with micro), the type of cellular–hyaline junction part (simple vs. bifurcated), the body diameter (160-228 vs. 68-83 µm), the a (11-15 vs. 15-20), b (16-21 vs. 9-14), and c ratios (31-63 vs. 16-24) and the anal body diameter (31-41 vs. 18-34 µm) differ also between H. zacatecana n. sp. and H. ruandica n. sp. IJs anterior ends also differ between these two species (small vs. large), and the presence of a cephalic tooth (small or absent vs. refringent and large).

Morphologically, the IJs of H. zacatecana n. sp. can be distinguished from the IJs of H. amazonensis by their size (493-578 vs. 567-612 µm), the distance from the anterior end to the nerve ring (59-72 vs. 76-93 µm), the neck length (96-124 vs. 107-132 µm), the tail length (52-63 vs. 98-115 µm), the a (19-24 vs. 24-29), c (8.2-10.5 vs. 5.1-6.1), and c’ (4.3-6.7 vs. ca. 7.3 µm) ratios and the E% (128-184 vs. 89-109). Moreover, hermaphroditic females differ in body size (4.41-6.12 vs. 3.52-5.59 mm), tail length (62-87 vs. 104-154 µm) and anal body diameter (34-58 vs. 59-85 µm). Amphimictic females of these two species differ also in body size (1.95-2.80 vs. 1.28-2.07 mm), tail length (45-75 vs. 25-38 µm), and body diameter (160-228 vs. 70-122). Male sizes differ between these two species (Tables 2–6).

Heterorhabditis zacatecana n. sp. IJ can be distinguished from H. bacteriophora by the distance from the anterior end to the nerve ring (59-72 vs. 72-93 µm), and the tail length (52-63 vs. 83-112 µm). In the case of males, they differ in the distance from the excretory pore to the anterior end (77-109 vs. 114-130 µm) and in body diameter (41-56 vs. 38-46 µm). Several morphometric differences were also observed in hermaphrodites and amphimictic females (Tables 2–6).

Heterorhabditis zacatecana n. sp. IJs can be distinguished from H. beicherriana IJs by the distance from anterior end to the excretory pore (72-99 vs. 100-122 µm) and the distance from the anterior end to the nerve ring (59-72 vs. 85-106 µm), the tail length (52-63 vs. 86-111), values of a (19-24 vs. 24-29), c’ (4.3-6.7 vs. 6.0-7.4), and c (8.2-10.5 vs. 5.9-6.8) ratios, and the E% value (128-184 vs. 103-121). Males can be differentiated by differences in neck (71-108 vs. 116-143 µm) and tail (21-33 vs. 32-35 µm) lengths, the distance from the anterior end to the excretory pore (77-109 vs. 130-157 µm) and from the anterior end to the nerve ring (60-78 vs. 81-100 µm). Several morphometric differences were also observed in hermaphrodites and amphimictic females of these two species (Tables 2–6).

Heterorhabditis zacatecana n. sp. IJs can be distinguished from H. georgiana IJs by differences in body diameter (23-27 vs. 17-26 µm), tail length (52-63 vs. 86-108 µm), and anterior end to excretory pore (72-99 vs. 97-113 µm) and anterior end to nerve ring distances (59-72 vs. 74-94 µm). The a, b and c ratios, E% and D% of IJs differ also in these two species. The males of these two species differ in anterior end to excretory pore (77-109 vs. 101-145 µm) and anterior end to nerve ring distances (60-78 vs. 72-93 µm), and neck (71-108 vs. 100-122 µm) and tail (21-33 vs. 29-41 µm) lengths. Several morphometric characters of hermaphroditic and amphimictic females differ between these two species (Tables 2–6).

Type host and locality

The type host are unknown as the nematodes of this genus can be hosted by different insect species and were isolated from soil samples by the Galleria bai ting technique (Bedding and Akhurst, 1975; White, 1927). Heterorhabditis zacatecana n. sp. MEX-39 and MEX-40 nematodes were collected in maize fields in Villanueva (Zacatecas, Mexico; decimal degrees coordinates: 22.161371, -102.887940), and Heterorhabditis zacatecana n. sp. MEX-41 nematodes were collected in maize fields in Apaseo el Alto (Guauajaujo, Mexico; decimal degrees coordinates: 20.470774, -100.59571).

Type material

MEX-39 nematodes are the type material for Heterorhabditis zacatecana n. sp. Holotype male, 15 paratype and 15 third stage juveniles were deposited in the National Nematode Collection of India, IARI, New Delhi. Additional specimens were deposited in the nematode collection of the Department of Animal Biology, Plant Biology and Ecology of the University of Jaén, Spain, under the following slide numbers: Mex001-01 to -03 (6 hermaphrodite females), Mex002-01 to -04 (8 amphimictic females and 3 males), and Mex003-01 to -04 (14 juveniles). Nematode cultures are maintained in the Institute of Biology, University of Neuchatel, Switzerland.

Etymology

The specific name refers to the Mexican state, Zacatecas, where the type material, Heterorhabditis zacatecana n. sp. MEX-39 nematodes, used to pheno-typically characterize the species were collected.
Cross-hybridization experiments

No progeny was observed when males and females of *H. ruandica* n. sp. Rw14_N-C4a and of *H. zacatecana* n. sp. MEX-39 were left to interact. No progeny was observed when males and females of *H. ruandica* n. sp. Rw14_N-C4a and of *H. bacteriophora* CH21 were left to interact. When males and females of *H. ruandica* n. sp. Rw14_N-C4a were crossed, fertile progeny was observed. When males and females of *H. zacatecana* n. sp. MEX-39 and of *H. bacteriophora* CH21 were left to interact. When males and females of *H. ruandica* n. sp. Rw14_N-C4a were crossed, fertile progeny was observed. When males and females of *H. bacteriophora* CH21 were crossed, fertile progeny was observed. Similarly, *H. zacatecana* n. sp. MEX-39 and *H. zacatecana* n. sp. MEX-40 nematodes produced fertile progeny, and *H. ruandica* n. sp. Rw18_M-Hr1a and *H. ruandica* n. sp. Rw14_N-C4a nematodes produced fertile progeny. These results provide further support for the heterospecific status of the Rwandan and the Mexican nematode populations.

Nematode molecular characterization and phylogenetic relationships

Phylogenetic reconstructions based on nuclear and mitochondrial genes (ITS, D2–D3, COI, umc-87, and cmd–1), either individually or concatenated, confirm that the nematodes of the genus *Heterorhabditis* are grouped into three major clades: the "Megidis-group", the "Indica-group" and the "Bacteriophora-group", which is consistent with previous studies (Dhakal et al., 2020) (Fig. 9, Fig. S1). The clade of the "Bacteriophora-group" is, in turn, separated into five subclades. Three of them are composed of already described species: *H. beicherriana*, *H. georgiana*, and *H. bacteriophora*, and two of them are composed of two new, undescribed species, which we named here *H. zacatecana* n. sp., and *H. ruandica* n. sp. (Fig. 9, Fig. S1). Clearer phylogenetic separations within the species of the clade of the "Bacteriophora-group" were observed when phylogeny were reconstructed based on COI, ITS, or on concatenated sequences of COI, ITS, and D2–D3 (Fig. 9, Fig. S1). Closer inspection at the ITS, D2–D3 and COI sequences reveals unambiguous genetic differences between the nematodes of the "Bacteriophora-group" (Fig. 10). Sequence similarity scores and nucleotide difference counts show a closer relationship between *H. bacteriophora*, *H. ruandica* n. sp., and *H. zacatecana* n. sp. nematodes (Fig. 11 and Figs. S2-S6). *Heterorhabditis ruandica* n. sp. and *H. bacteriophora* share 99.1% and differ in 6 nucleotide positions in the ITS sequences flanked by primers TW81 and AB28, share 99.8% and differ in 1 nucleotide position in the D2–D3 sequences flanked by primers D2A and D3B, and share 94.1 to 94.7% and differ in 18 to 19 nucleotide positions in the COI sequences flanked by primers HCF and HCR (Fig. 11 and Figs. S2-S6). *Heterorhabditis zacatecana* n. sp. and *H. bacteriophora* share 99.4% and differ in 4 nucleotide positions in the ITS sequences flanked by primers TW81 and AB28, share 98.9% and differ in 1 nucleotide position in the D2–D3 sequences flanked by primers D2A and D3B, and share 94.1 to 94.4% and differ in 19 to 20 nucleotide positions in the COI sequences flanked by primers HCF and HCR (Fig. 11 and Figs. S2-S6). *Heterorhabditis ruandica* n. sp. and *H. zacatecana* share 99.7% and differ in 2 nucleotide positions in the ITS sequences flanked by primers TW81 and AB28, share 100% and differ in no nucleotide position in the D2–D3 sequences flanked by primers D2A and D3B, and share 97.6% to 98.2% and differ in 6–8 nucleotide positions in the COI sequences flanked by primers HCF and HCR (Fig. 11 and Figs. S2-S6). Noteworthy, we observed almost no intraspecific variations within the nematodes of the "Bacteriophora-group" at different genetic loci (Figs. 10, 11, and Figs. S2–S6). However, the sequences of the COI gene show very interesting signatures of population–specific polymorphism (Figs. 10D–F, 11). Specifically, *Heterorhabditis ruandica* n. sp. Rw18_M-Hr1a and Rw18_M-Hr1b nematodes that were collected in the same western Rwandan region differ from the *Heterorhabditis ruandica* n. sp. Rw14_N-C4a nematodes collected in a southern Rwandan region in a transitional nucleotide change (g.1212A > G) (Fig. 10D). Moreover, *H. zacatecana* n. sp. MEX-39 and MEX-40 nematodes collected in north-central Mexico and *H. zacatecana* n. sp. MEX-41 nematodes collected in central Mexico differ in three transitional nucleotide changes (g.1257T > C, g.1324T > C, and g.1464A > G) (Fig. 10D–F). Hence, due to its highly conserved species–specific polymorphism, and the consistent population-specific polymorphic patterns, the COI gene emerges as an important phylogenetic marker also for the genus *Heterorhabditis*, in a similar manner as it is for many other taxonomic groups (Hebert et al., 2003; Pentinsaari et al., 2016).

Interspecific genetic variability within the *H. bacteriophora* clade

In a recent study, Dhakal et al. (2020) studied several hundreds of ITS sequences of *Heterorhabditis* nematodes and recognized that nematodes
Figure 9: Maximum-likelihood phylogenetic tree reconstructed from: (A) the sequences of the cytochrome c oxidase I (COI) of different *Heterorhabditis* species. A total of 343 nucleotide positions, flanked by primers HCF and HCR, were analyzed; and (B) the concatenated sequences of the following genes/genetic regions of different *Heterorhabditis* species: the D2–D3 expansion segments of the 28S rRNA (D2–D3), the internal transcribed spacer (ITS) of the rRNA (ITS), and the cytochrome c oxidase I (COI). A total of 1673 concatenated nucleotide positions were included in the reconstruction. Accession numbers of the nucleotide sequences used for the analyses are shown in Table S3. *For* *H. marelatus*, *H. indica*, and *H. mexicana*, the sequences that were concatenated are derived from different nematode isolates. *Heterorhabditis safricana*, and *H. tayserae* were not included as their COI or their D2–D3 sequences, respectively, are not publicly available. Numbers at nodes represent bootstrap values based on 100 replications. Bars represent average nucleotide substitutions per sequence position.
Figure 10: Polymorphism in the sequences of the ITS region (A, B), the D2–D3 region (C), and the COI gene (D-F) showing taxonomically relevant nucleotide positions for *Heterorhabditis* nematodes of the “Bacteriophora-group”. Nucleotide position numbers of rRNA genes are according to the sequences of *C. elegans* N2 (NCBI accession number: MN519140) and of mitochondrial genes are according to the sequences of *C. elegans* N2 (NCBI accession number: AY171203).
Multi-locus phylogenetic analyses uncover species boundaries: Machado et al.

**Figure 11:** Pairwise nucleotide similarities (%) in the sequences of the cytochrome c oxidase I (COI) gene of different *Heterorhabditis* species. A total of 344 nucleotide positions, flanked by primers HCF and HCR, were analyzed. Accession numbers of gene sequences used are shown in Table S3.
(Figs. S7 and S8). Phylogenetic reconstructions show a clear phylogenetic separation between all these haplotypes (Fig. S8). Hence, some of the haplotypes described by Dhakal et al. (2020) represent new species, closely related to *H. bacteriophora*, and some others likely represent new species, which highlights the power of statistical parsimony network analyses to uncover undescribed species of the genus *Heterorhabditis*, and supporting previous hypothesis regarding the taxonomic status of these nematode isolates (Bruno et al., 2020; Dhakal et al., 2020; Fallet et al., 2020).

**Symbiotic relationships**

Up to now, the bacterial genus *Photobacterium* Boemare, Akhurst and Mourtant 1993 contains 27 taxa, including species and subspecies (Machado et al., 2021b). Phylogenetic relationship reconstructions based on whole genome sequences show that the bacterial symbionts isolated from *H. zacatecana* n. sp. MEX-39 and *H. ruandica* n. sp. Rw14_N-C4a nematodes, named here as MEX-39 and RW14-46, respectively, show high similarity with two of the already described *Photobacterium* species: Photo-

![Figure 12: Phylogenetic reconstruction based on core genome sequences of *Photobacterium* bacterial strains. Numbers at the nodes represent SH-like branch supports. Bar represents average nucleotide substitutions per sequence position. Accession numbers of the genome sequences used for the reconstruction are shown in Table S4.](image-url)
Machado et al. multi-locus phylogenetic analyses uncover species boundaries: Machado et al.

Photorhabdus kleinii and P. laumondii subsp. laumondii, respectively (Fig. 12). Photorhabdus kleinii MEX-39 shares 87–88% digital DNA–DNA hybridization (dDDH) with other members of the same species, while P. laumondii subsp. laumondii RW14-46 shares 89% digital DNA–DNA hybridization (dDDH) with other members of the same species, (Fig. S9).

On the synonymization and declaration of species inquirendae of some species

We revised the original publications of all synonymized species and based on their morphology and molecular data (when available), we reinforce the synonymized status of most of them (Khan et al., 1976; Wouts, 1979; Stock, 1993; Gardner et al., 1994; Liu, 1994; Stock et al., 1996; Plichta et al., 2009; Stock et al., 2009; Maneesakorn et al., 2015; Hunt and Nguyen, 2016; Shahina et al., 2017; Dhakal et al., 2020). However, the original description of H. bacteriophora provided by Poinar (1976) shows males with very anterior GP1 while in its synonymized species H. heliothidis (Khan, Brooks & Hirschmann, 1976) Poinar, Thomas & Hess, 1977 (=Chromonomema heliothidis Khan et al., 1976) the GP1 appears more posterior (Khan et al., 1976; Poinar, 1976). Hence, it is likely that both species are not conspecific. Therefore, we declare H. heliothidis (Khan, Brooks & Hirschmann, 1976) Poinar, Thomas & Hess, 1976 as species inquirenda.

Heterorhabditis hoptha and H. poinari were poorly described (Turco, 1970; Kakulia and Mikaia, 1997). Original descriptions lack differentiated description of all diagnostic characters of adult and larval stages. According to this, both species should remain in the list of species inquirendae. Heterorhabditis egyptii and H. hambletoni were described showing all diagnostic characters of adults and larvae stages. According to this, both species are considered valid herein (Pereira, 1937; Abd-Elgawad and Ameen, 2005). The lack of molecular data, however, impairs their inclusion in future phylogenetic studies. Nevertheless, new species description should contrast morphological characters with these species. An updated dichotomous key to identify the species of the genus Heterorhabditis is provided (Fig. 13, Tables 3-6).

On the species of the genus Heterorhabditis

Considering the results of this study and the analyses of all the literature that describes new species of the genus Heterorhabditis, the updated list of the species of the genus, including their status, is as follows.

Key to species identification

1a – Hermaphroditic female with tail wider than longer ........................................ downesi
1b – Hermaphroditic female with tail longer than wider ........................................ 2
2a – Hermaphroditic female with tail usually swollen near to tip, ending in a thinner acute terminus .................................................... 3
2b – Hermaphroditic female with tail conoid, not swollen .................................... 5
3a – Male bursa with GP1 more posterior, at spicule lamina level ......................... moreletius
3b – Male bursa with GP1 more anterior, at spicule manubrium level ................... 4
4a – Male excretory pore more anterior, at 75–102 μm from the anterior end ... nomieptensis
4b – Male excretory pore more posterior, at 108–145 μm from the anterior end ...... mexicana
5a – Male bursa with GP1 more anterior than the spicules level .......................... 6
5b – Male bursa with GP2 at spicules level ............................................................... 9
6a – Amphimictic female with tail thicker, about 1.5 times longer than wide .......... 8
6b – Amphimictic female with tail thinner, about 2-3 times longer than wide ....... 7
7a – Amphimictic female with rectum slightly longer than the anal body diameter beichoniana
7b – Amphimictic female with rectum about 2.0-2.5 times the anal body diameter ....... 8
8a – Male with GP2 and GP3 very close ................................................................................ bacteriophora
8b – Male with GP2 and GP3 separated ........................................................................ hambletoni
9a – Hermaphroditic female with tail scarcely longer than wide ....................... 10
9b – Hermaphroditic female with tail twice or longer .......................... 13
10a – Hermaphroditic female with tail slightly curved ventrally, spicules longer, 48–55 μm ........ zeaolindica
10b – Hermaphroditic female with tail very curved ventrally; spicules shorter, 33–49 μm ....... 11
11a – Male with excretory pore more anterior, at 71–93 μm from the anterior end ... bajardi
11b – Male with excretory pore more posterior, at 101–145 μm from the anterior end .... 12
12a – Amphimictic female with tail with finely rounded tip; sheath juvenile with long hyaline part tail, about one half of the tail length ................................................................. georgonella
12b – Amphimictic female with tail having acute tip; sheath juvenile with short hyaline part at tail, about one third of the tail length ......................................................... indica
13a – Male tail with pseudopelodaner tail ............................................................. 14
13b – Male tail with pelodaner tail ................................................................. 14
14a – Spicules with lamina ventrally curved in lateral view .................................. floridensis
14b – Spicules with lamina almost straight in lateral view .................................. 15
15a – Amphimictic female with tail longer (c’ about 4); spicules with lamina anteriorly wider having dorsal hum ................................. 16
15b – Amphimictic female with tail shorter (c’ about 2–3); spicules with lamina having similar width in all length ................................................. 16
16a – Amphimictic female with tail longer, c’ about 3 .......................................... 17
16b – Amphimictic female with tail shorter, c’ about 2 .................................... 18
17a – Juvenile L3 with larger cephalic tooth, hermaphrodites with more anterior excretory pore, at 106–153 μm from the anterior end ......... ruandica n. sp.
17b – Juvenile L3, apparently, with smaller cephalic tooth; hermaphrodites with more posterior excretory pore, at 154–205 μm from the anterior end ....................... egyptii
18a – Spicules thinner and slender ................................................................. 19
18b – Spicules wider and robust ................................................................. 19
19a – Hermaphroditic females with tail shorter, less than 90 μm; amphimictic females with longer tail, more than 40 μm; males generally longer (811–914 μm) ........ zoeoconae n. sp.
19b – Hermaphroditic females with tail longer, more than 100 μm; amphimictic females with shorter tail, less than 40 μm; males generally smaller (692–826 μm) ........ zoeoconae n. sp.

Figure 13: Dichotomous key to identify the species of the genus Heterorhabditis based on morphological and morphometrical characters of L3 juveniles, of male and female adults, and of hermaphroditic females.
Table 3. Comparative morphometrics of adult males of *Heterorhabditis ruandica* n. sp., *H. zacatecana* n. sp., and of different closely related *Heterorhabditis* species. All measurements are in µm (except ratios and percentages).

| Species               | L      | BD     | EP     | NR     | NL     | T      | SL     | GL     | a      | b      | c      | c’     | SW%    | GS%    | D%     | Country       | Reference       |
|-----------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|----------------|-----------------|
| *H. amazonensis*      | 692–826| 36–43  | 96–116 | 71–88  | 97–114 | 29–41  | 35–45  | 19–23  | 18.7*  | 7.7**  | 27.5** | 1.3**  | 120–187 | 44–56  | 95–109 | Brazil        | Andaló et al. (2006) |
| *H. atacamensis*      | 842–1025 | 42–55  | 116–149 | 69–93  | 99–119 | 24–36  | 40–49  | 17–22  | 19.7*  | 9.6**  | 23.9** | 1.5**  | 179–249 | 38–51  | 108–126 | Chile         | Edgington et al. (2011) |
| *H. bacteriophora*    | 780–960 | 38–46  | 114–130 | 65–81  | 99–105 | 22–36  | 36–44  | 18–25  | 20.8*  | 9.1*   | 34.3*  | 1.8*   | 174     | 50     | 117    | Australia    | Poinar (1976) |
|                       | 700–940 | 37–50  | 113–140 | 70–85  | 95–110 | 20–27  | 39–47  | 18–24  | –      | –      | –      | –      | –      | –      | –     | Argentina    | Agiáera de Doucet and Doucet (1986) |
|                       | 689–880 | 38–46  | 78–123  | 55–90  | 92–124 | 21–32  | 34–48  | 17–26  | –      | –      | –      | –      | 1.2    | 147–256 | 41–49  | 68–106 | Australia    | Sagun et al. (2015) |
|                       | 782–927 | 92–120 | 103–139 | 58–76  | 84–105 | 28–37  | 51–53  | 17–26  | 6.6–8.5 | 8.5–10 | 23–32  | 1.4–2.2 | 194–282 | 37–57  | 108–157 | India        | Bhat et al. (2019a) |
|                       | 805–1075 | 42–57  | 84–111  | 80–119 | 84–75  | 24–39  | 39–51  | 17–27  | 16–22  | 7.1–12 | 22–41  | 1.0–1.7 | 170–225 | 40–62  | 77–136 | India        | Rana et al. (2020) |
| as *H. argentinensis* | 1000–2000 | 42–70  | 145–170 | 64–82  | 103–120 | 28–49  | 42–49  | 20–26  | 16.7*  | 8.3*   | 14.3*  | 1.4*   | 198*   | 62*    | 92*   | Argentina    | Stock (1993) |
| as *H. heliothidis*   | 1000–1200 | 32–60 | 125*    | 125*   | 113–131 | 29–36  | 42–52 | 22–27 | 19–35 | 8–11   | 28–38  | 1.3*   | 185*   | 51*    | 95*   | USA         | Khan et al. (1976) |
| *H. baujardi*         | 818–970 | 45–53  | 71–93   | 54–77  | 105–132 | 28–38  | 33–45 | 18–22 | 16–22  | 6.4–8.8 | 24–33  | 1.5**  | 138–208 | 44–61  | 79**  | Vietnam     | Phan et al. (2003) |
|                       | 710–903 | 40–50  | 83–93   | 53–68  | 98–110 | 33–40  | 43–48 | 20–28 | 16–20  | 6.7–9.3 | 18–28  | –      | 154–200 | 47–61  | 80–90 | India       | Vartiachlimpua et al. (2018) |
| as *H. somssockae*    | 737–870 | 37–44  | 68–93   | 72–83  | 90–120 | 20–30  | 32–45 | 17–23 | 20.7*  | 8.3**  | 32.3** | 1.2**  | 133–198 | 42–59  | 74–99 | Thailand    | Maneesakom et al. (2015) |
| *H. beicherriana*     | 889–1192 | 51–73  | 130–157 | 81–108 | 116–143 | 32–45  | 40–49 | 22–27 | 15–23  | 7.2–10 | 22–34  | 1.3–2.3 | 153–208 | 48–59  | 102–120 | China       | Li et al. (2012) |
| *H. downesi*          | 699–876 | 33–40  | 86–91   | 62–78  | 97–106 | 29–34  | 41–47 | 17–19 | 26.6*  | 8.8**  | 27.4** | 1.4**  | 170–220 | 36–47  | 90    | Ireland     | Stock et al., 2002 |
| *H. egyptii*          | 594–848 | 31–56  | 80–97   | 56–84  | 96–109 | 23–34  | 25–50 | 16–22 | 17.1*  | 6.6**  | 19.5** | 1.5*   | 120–220 | 40–65  | 84–91 | Egypt       | Abd–Elgawad and Ameen (2005) |
| *H. floridensis*      | 785–294 | 43–50  | 104–128 | 73–90  | 97–111 | 29–40  | 36–46 | 17–30 | 19.9*  | 7.9**  | 24.1** | 1.4**  | 133–209 | 47–65  | 112    | USA         | Nguyen et al. (2006) |
| *H. georgiana*        | 721–913 | 43–55  | 101–145 | 72–93  | 100–122 | 29–41 | 41–49 | 20–28 | 16.5*  | 7.7**  | 26.1** | 1.4**  | 150–200 | 51–64  | 100–122 | USA         | Nguyen et al. (2008) |
| *H. hambletoni*       | 510–800 | 38–60  | 80–100  | 80–90  | –      | –      | –      | –      | –      | –      | –      | –      | –      | –      | –     | Brazil       | Pereira (1937) |
| *H. hoptha*           | 554–837 | –      | –      | –      | –      | 30.9*  | 43–60 | 26–30 | 18–22  | 5.9–8.2 | 18–37  | 1.1*   | 167**  | 55**  | –   | USA         | Turco (1970) |
| *H. indica*           | 573–788 | 35–46  | 109–138 | 72–85  | 93–109 | 24–32  | 35–48 | 18–23 | 17.6*  | 6.7**  | 23.0** | 1.1**  | 187     | 49     | 121    | India        | Poinar et al. (1992) |
| Species             | Localities | Males   | Females | Male:Female | Malan et al. (1996) | Stock et al. (1977) | Kajol et al. (2020) | Liu et al. (1994) | Liu (2008) | Plichta et al. (2009) | Gardiner et al. (1994) | Shahina et al. (2017) |
|---------------------|------------|---------|---------|-------------|----------------------|---------------------|---------------------|-------------------|--------------|------------------------|------------------------|------------------------|
| H. brevicaudis*     | 40-48      | 92-100  | 80-88   | 104-112     | 28-36                | 44-48               | 20-24               | 2.9*              | 170*        | 47*                    | 84*                    | 5.8-9.7                |
| H. baujardi*        | 40-48      | 92-100  | 80-88   | 104-112     | 28-36                | 44-48               | 20-24               | 2.9*              | 170*        | 47*                    | 84*                    | 5.8-9.7                |
| H. hawaiiensis*     | 46-54      | 71-146  | 67-112  | 100-149     | 26-40                | 40-51               | 18-26               | 1.4**             | 144-191    | 48-65                  | 110-126                | 7.6-9.8                |
| H. pakistanense*    | 38-43      | 112-133 | 80-110  | 100-105     | 30-42                | 35-42               | 20-22               | 19-25             | 144-191    | 48-65                  | 110-126                | 7.6-9.8                |
| H. marelatus        | 48-56      | 110-168 | 61-95   | 99-123      | 24-38                | 41-49               | 18-22               | 15.5*             | 7.8**       | 30.0**                 | 113                     | 6.8**                  |
| H. hepialus*        | 65-98      | 102-131 | 84-114  | 113-139     | 37-49                | 42-52               | 17-24               |                   | 26.7**      | 1.1**                  |                        | 130-196                |
| H. meigs            | 139-176    | 96-112  | 122-134 | 35-43       | 46-54                | 17-24               | 18-22               | 1.6*              | 188         | 43                     | 122                    | USA                    |
| H. mexicana         | 34-46      | 75-102  | 64-75   | 88-106      | 21-32                | 37-49               | 17-24               | 14-18             | 5.6-7.9     | 21-33                  | 1.1-1.7                | 130-196                |
| H. noenepusinensis  | 34-46      | 75-102  | 64-75   | 88-106      | 21-32                | 37-49               | 17-24               | 14-18             | 5.6-7.9     | 21-33                  | 1.1-1.7                | 130-196                |
| H. poinarini*       | 43-70      | 36-65   | 43-55   | 24-32       | 95-100               | 51-95               | 11-97               | 202-301           | 38-56       | 81-108                 | S. Africa              | USA                    |
| H. ruandica Rw14_NC4a | 40-51     | 61-109  | 56-74   | 84-117      | 21-29                | 34-50               | 16-23               | 15-21             | 5.8-9.7     | 43.0**                 | 150-306                | Rwanda                 |
| H. safricana        | 77-1009    | 50-58   | 104-147 | 52-61       | 105-126              | 27-49               | 35-54               | 19-27             | 20.1*       | 150-306                | 43-62                  | S. Africa              |
| H. tayaseae         | 38-48      | 78-120  | 54-88   | 85-123      | 20-29                | 30-42               | 12-21               | 15.1*             | 6.5**       | 14.0**                 | 156                    | Egypt                  |
| H. sonorensis*      | 32-42      | 60-84   | 60-80   | 80-100      | 25-45                | 31-45               | 20-31               | 110-180           | 40-75       | 72-91                  | Mexico                 |
| H. zealandica       | 36-45      | 130-150 | 30-41   | 48-55       | 19-25                |                    |                    | 1.7*              | 246         | 44                     | 118                    | N. Zealand             |
| H. heliothidis*     | 36-45      | 130-150 | 30-41   | 48-55       | 19-25                |                    |                    | 1.7*              | 246         | 44                     | 118                    | N. Zealand             |
| H. zacatecana MEX-39| 41-56      | 78-100  | 71-108  | 35-55       | 15-25                | 7.6-12              | 26-43               | 1.2-2.5           | 7.6-12      | 1.2-2.5                 | 170-320                | Mexico                 |

Note: *Synonymized species. H. bacteriophora (Syn.: H. argentinensis); H. baujardi (Syn.: H. somsokoeae); H. indica (Syn.: H. brevicaudis, H. gerrardi, H. hawaiiensis, and H. pakistanense); H. marelatus (Syn.: H. hepialus); H. tayaseae (Syn.: H. sonorensis); and H. zealandica (Syn.: H. heliothidis apud Wout, 1979 nec Khan, Brooks & Hirschmann, 1976). +Re-instated as valid species herein based on morphological evidence. H. egypti and H. hambletoni (=Rhabditis hambletoni) are declared herein valid species as their original descriptions provide all the diagnostic characters to differentiate them morphologically from all species of the genus. 1Species inquirendae. H. hoptha (= Neoleptodora hoptha) and H. poinari are maintained as species inquirendae because their original descriptions do not include important diagnostic characters to fully differentiate them from the other species of the genus. 2H. heliothidis apud Khan et al., 1976 (=Chromonema heliothidis) is declared herein species inquirendae as the male morphology of H. bacteriophora and H. heliothidis differ. *Data calculated from the drawings provided in the original publication. **Data calculated from other measurements provided in the original publication. --Data not provided in the original publication.
Table 4. Comparative morphometrics of hermaphrodite females of *Heterorhabditis ruandica* n. sp., *H. zacatecana* n. sp., and of different closely related *Heterorhabditis* species.

| Species                | L     | BD    | EP    | NR    | NL    | T     | a    | b    | c    | c'    | V     | ABD   | D%   | Country     | Reference                  |
|------------------------|-------|-------|-------|-------|-------|-------|------|------|------|-------|-------|-------|------|-------------|-----------------------------|
| *H. amazonensis*       | 2686-4893 | 131-241 | 150-379 | 80-196 | 162-302 | 70-120 | –    | –    | –    | –    | 1.8* | 36-52 | 43-76 | 76-126 | Australia | Sagun et al. (2015)          |
|                        | 3086-5492 | 221-352 | 127-260 | 79-162 | 101-200 | 71-123 | 9.2-28 | 23-37 | 25-75 | 1.3-3.7 | 37-52 | 34-75 | 112-155 | Brazil | Andaló et al. (2006)         |
| as *H. argentinensis*  | 5000-7500 | 250-340 | 132-196 | 235-300 | 100-140 | –    | –    | –    | 1.8* | 40-50 | 70-120 | 102* | Argentina | Stock (1993)                 |
| as *H. heliothidis*    | 3000-5100 | 200-344 | 250*   | 163-286 | 76-100 | 11-18 | 11-25 | 30-63 | 2.2* | 45-52 | 62.5* | 80* | USA | Khan et al. (1976)            |
| *H. baujardi*          | 3135-4170 | 180-240 | 156-192 | 186-206 | 66-114 | 15-19 | 16-21 | 36-50 | 2.0* | 43-48 | 47-63 | 88* | Vietnam | Phan et al. (2003)           |
|                        | 3250-3970 | 190-250 | 98-115  | 120-135 | 180-205 | 80-105 | 13-19 | 16-20 | 31-45 | –    | 41-49 | 50-65 | 73-92 | India | Vanlalhlimpuia et al. (2018) |
| as *H. somsookae*      | 2275-3952 | 108-183 | 156-214 | 118-144 | 158-193 | 56-87 | –    | –    | –    | 2.3* | 41-56 | 30-53 | 86-113 | Thailand | Maneesakorn et al. (2015)    |
| *H. beicherriana*      | 3671-5543 | 198-374 | 165-297 | 135-243 | 192-343 | 68-130 | 13-20 | 13-25 | 34-62 | 1.0-2.3 | 41-49 | 51-92 | 76-94 | China | Li et al. (2012)              |
| *H. downesi*           | 3030-5051 | 183-291 | 200-254 | 175-230 | 230-244 | 60-70 | –    | –    | –    | 1.1* | 50-55 | 57-65 | 117* | Ireland | Stock et al. (2002)          |
| *H. egyptii*           | 2100-3100 | 107-164 | 154-205 | 101-147 | 144-192 | 83-115 | –    | –    | –    | 2.7* | 46-59 | 33-51 | 104* | Egypt | Abd-Elgawad and Armeen (2005)|
| *H. floridensis*       | 3731-5865 | 217-331 | 211-301 | 169-271 | 271-301 | 84-126 | –    | –    | –    | 2.5* | 44-49 | 42-78 | 104* | USA | Nguyen et al. (2006)          |
| *H. georgiana*         | 3232-4928 | 157-267 | 200-277 | 143-217 | 132-271 | 65-96 | –    | –    | –    | 1.2* | 44-55 | 42.6* | –    | USA | Nguyen et al. (2008)          |
| *H. hambletoni*        | –    | –    | –    | –    | –    | 65-96 | –    | –    | –    | 1.2* | 44-55 | 42.6* | –    | Brazil | Pereira (1937)               |
| *H. hopthe*            | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | USA | Turco (1970)                 |
| *H. indica*            | 2300-3100 | 107-145 | 163-187 | 104-123 | 163-179 | 72-110 | –    | –    | –    | 45-50 | 38-51 | –    | India | Poinar et al. (1992)         |
| Species | Common Name | Type (Genus) | Description | Location | Year | Authors |
|---------|-------------|--------------|-------------|----------|------|---------|
| *H. bacteriophora* | | | | USA | 1976 | Kajol et al. (2020) |
| *H. argentinensis* | | | | USA | 1976 | Kajol et al. (2020) |
| *H. brevicaudis* | | | | China | 1994 | Liu (1994) |
| *H. gerrardi* | | | | Australia | 2009 | Picht et al. (2009) |
| *H. hawaiiensis* | | | | USA | 1994 | Gardner et al. (1994) |
| *H. magellanicus* | | | | USA | 1996 | Liu and Berry (1996) |
| *H. malanii* | | | | USA | 1996 | Liu and Berry (1996) |
| *H. megalopiper* | | | | Pakistan | 2017 | Shahina et al. (2017) |
| *H. marelatus* | | | | USA | 2014 | Malan et al. (2014) |
| *H. mexicana* | | | | Mexico | 1997 | Kailula and Mikael (1997) |
| *H. noenieputensis* | | | | South Africa | 2014 | Malan et al. (2014) |
| *H. poinari* | | | | USA | 1996 | Liu and Berry (1996) |
| *H. ruandica* | | | | Rwanda | 2016 | This study |
| *H. safricana* | | | | South Africa | 2008 | Malan et al. (2008) |
| *H. tayseareae* | | | | Egypt | 1996 | Shamseldin et al. (1996) |
| *H. sonorensis* | | | | Mexico | 1996 | Stock et al. (2009) |
| *H. zealandica* | | | | New Zealand | 1990 | Poinar (1990) |
| *H. zacatecanae* | | | | Mexico | 1999 | Wouts (1997) |
| *H. barberophila* | | | | USA | 1976 | Kajol et al. (2020) |
| *H. heliothidis* | | | | USA | 1976 | Kajol et al. (2020) |

Note: All measurements are in mm (except ratios and percentages). Synonymized species. *Species inquirendae.**Data calculated from drawings provided in the original publication. +Data calculated from other measurements provided in the original publication. –Data not provided in the original publication.
### Table 5. Comparative morphometrics of adult females of *Heterorhabditis ruandica* n. sp., *H. zacatecana* n. sp., and of different closely related *Heterorhabditis* species.

| Species                  | L       | BD      | EP       | NR       | NL       | T       | a       | b       | c       | V       | ABD     | D%      | Country   | Reference      |
|--------------------------|---------|---------|----------|----------|----------|---------|---------|---------|---------|---------|---------|---------|----------|----------------|
| *H. amazonensis*         | 1279-2070 | 70-122  | 103-126  | 68-100   | 119-142  | 25-38   | –       | –       | –       | 2.4*    | 46-50   | 25-38   | Brazil    | Andaló et al. (2006) |
| *H. atacamensis*         | 1754-2628 | 86-129  | 154-182  | 79-119   | 129-167  | 80-108  | –       | –       | –       | 3.8*    | 43-49   | 24-33   | Chile     | Edington et al. (2011) |
| *H. bacteriophora*       | 3180-3850 | 160-220 | 174-214  | 93-118   | 155-183  | 71-93   | 21.4*   | 18.8    | 41.5*   | 3.1*    | 42-53   | 22-31   | Australia | Poinar (1976) |
| *H. beicherriana*        | 1800-2400 | 100-162 | 122-162  | 83-102   | 108-145  | 40-65   | –       | –       | –       | –       | 41-50   | 23-40   | Argentina | Agúeira do Doucet and Doucet (1986) |
| *H. baujardi*            | 1690-3214 | 100-224 | 101-212  | 67-103   | 120-163  | 54-101  | –       | –       | –       | 2.4*    | 44-50   | 21-24   | Australia | Sagun et al. (2015) |
| *H. bacteriophora*       | 1513-2290 | 84-150  | 128-181  | 71-99    | 113-135  | 41-79   | 11-22   | 11-19   | 26-42   | 1.6-2.5 | 38-51   | 24-39   | 108-150  | India     | Bhat et al. (2019a) |
| *H. bacteriophora*       | 1226-1819 | 58-115  | 108-157  | 68-91    | 101-127  | 29-94   | 16-25   | 7.8-16  | 16-48   | 1.0-3.4 | 44-58   | 24-31   | 83-116  | India     | Rana et al. (2020) |
| as *H. argentinensis*    | 2000-3500 | 90-180  | 105-240  | 88-140   | 162-200  | 75-108  | 12.5*   | 7.8*    | 31.2*   | 2.0*    | 42-48   | 33-35   | Argentina | Stock (1993) |
| as *H. heliothidis*      | 2000-3300 | 184-240 | 146*     | 126*     | 148-177  | 71-93   | 11-15   | 14-21   | 26-46   | 2.8*    | 48-53   | 33*     | 95*     | USA       | Khan et al. (1976) |
| *H. baujardi*            | 1335-2130 | 90-150  | 104-149  | 75-122   | 131-185  | 68-89   | 12-16   | 10-12   | 19-32   | –       | 46-51   | 27-41   | Vietnam   | Phan et al. (2003) |
| *H. baujardi*            | 2060-2290 | 120-150 | 98-115   | 80-95    | 123-148  | 78-108  | 15-17   | 16-18   | 20-27   | –       | 41-48   | 30-38   | 63-78   | India     | Vantahhipqua et al. (2018) |
| as *H. somsockae*        | 2159-2666 | 117-194 | 143-156  | 90-112   | 128-144  | 41-80   | –       | –       | –       | 2.9*    | 36-51   | 21-35   | 104-111  | Thailand  | Maneesakorn et al. (2015) |
| *H. beicheniana*         | 1581-3026 | 125-218 | 95-165   | 59-138   | 105-186  | 68-105  | 10-18   | 10-23   | 19-34   | 1.6-2.4 | 41-49   | 35-81   | 88-98   | China     | Li et al. (2012) |
| *H. downesi*             | 1231-2728 | 74-131  | 99-126   | 117-151  | 111-155  | 70-122  | –       | –       | –       | 2.5*    | 47-60   | 25-38   | –        | Ireland   | Stock et al. (2002) |
| *H. egyptii*             | 1050-1420 | 56-84   | 69-106   | 69-94    | 106-125  | 56-78   | 17.5**  | 14.4**  | 22.2**  | 3.1**   | 44-51   | 19-27   | 78**    | Egypt     | Abd–Elgawad and Ameen (2005) |
| *H. floridensis*         | 2054-2548 | 120-156 | 110-168  | 86-122   | 126-178  | 69-87   | –       | –       | –       | –       | 44-50   | 32-42   | –        | USA       | Nguyen et al. (2006) |
| *H. georgiana*           | 1640-2779 | 101-188 | 111-177  | 96-162   | 136-219  | 62-88   | –       | –       | –       | 1.5*    | 46-53   | 42*     | –        | USA       | Nguyen et al. (2008) |
| *H. hamblietoni*         | 600-1200  | 70-100  | 80-90    | 70-80    | –       | –       | –       | –       | –       | 5.0-5.8*| –       | –        | Brazil    | Pereira (1937) |
| *H. hopthai*             | 2826-3983 | 148*    | 161*     | 219*     | 28*      | 13-19   | 47-67   | 0.8*    | 43-49   | 33*     | 92*     | –        | New Jersey | Turco (1970) |
| *H. indica*              | 1200-1800 | 76-113  | 118-138  | 88-96    | 120-139  | 66-88   | –       | –       | –       | 40-53   | 22-32   | –        | India     | Poinar et al. (1992) |
| *H. indicus*             | 1713-2242 | 110-156 | 135-172  | 77-92    | 120-138  | 61-83   | 13-17   | 11-18   | 22-36   | 1.9-2.9 | 44-50   | 27-33   | 102-128  | India     | Kajd et al. (2020) |
| Species                        | Sample Size | Country       | Reference         |
|-------------------------------|-------------|---------------|-------------------|
| H. hawaiiensis                | 1994        | USA           | Bhat et al. (2021b) |
| H. pakistanense               | 1996        | China         | Liu (1994)        |
| H. marelatus                  | 1996        | Australia     | Plichta et al. (2009) |
| H. havaileenae                | 1994        | USA           | Gardner et al. (1994) |
| H. pakistanense               | 1997        | Pakistan      | Shahtina et al. (2017) |
| H. marelatus                  | 1996        | USA           | Liu and Berry (1996) |
| H. heliaculus                 | 1997        | USA           | Stock (1997)      |
| H. megiis                     | 1996        | USA           | Stock et al. (1996) |
| H. mexicana                   | 2004        | Mexico        | Nguyen et al. (2004) |
| H. noeniputensis              | 2004        | S. Africa     | Malan et al. (2014) |
| H. poinari                    | 1997        | Rwanda        | Kakulja and Mikaia (1997) |
| H. ruandica                   | 2008        | Rwanda        | Malan et al. (2014) |
| H. safricana                  | 1997        | S. Africa     | Malan et al. (2008) |
| H. tayseareae                 | 1997        | Egypt         | Shamseldiane et al. (1996) |
| H. sonorensis                 | 2009        | Mexico        | Stock et al. (2009) |
| H. zealandica                 | 1990        | N. Zealand    | Poinar (1990) |
| H. heliotidis                 | 1979        | N. Zealand    | Wouts (1979) |
| H. zacetaneica                | 1979        | Mexico        | This study         |

Note: All measurements are in µm (except ratios and percentages). *Synonymized species. H. bacteriophora (Syn.: H. argentinensis); H. baujardi (Syn.: H. somssockae); H. indica (Syn.: H. brevicaudis, H. gerrardi, H. havaileenae, and H. pakistanense); H. marelatus (Syn.: H. heliaculus); H. tayseareae (Syn.: H. sonorensis); and H. zealandica (Syn.: H. heliotidis apud Wouts, 1979 nec Khan, Brooks & Hirschmann, 1976). **H. heliotidis apud Khan et al., 1976 (=Chromonema heliotidis) is declared herein species inquirendae as the male morphology of H. bacteriophora and H. heliotidis differ. **Re-instated as valid species herein based on morphological evidence. H. egyptii and H. hambletoni (=Rhabditis hambletoni) are declared herein valid species as their original descriptions provide all the diagnostic characters to differentiate them morphologically from all species of the genus. **Species inquirendae. H. hoptha (=Neoaplectana hoptha) and H. poinari are maintained as species inquirendae because their original descriptions do not include important diagnostic characters to fully differentiate them from the other species of the genus. **Data calculated from the drawings provided in the original publication. **Data not provided in the original publication.
Table 6. Comparative morphometrics of infective juveniles of *Heterorhabditis ruandica* n. sp., *H. zacatecana* n. sp., and of different closely related *Heterorhabditis* species.

| Species               | L    | BD   | EP   | NR   | NL   | T    | a    | b    | C    | c'   | D%   | E%   | Country      | Reference          |
|-----------------------|------|------|------|------|------|------|------|------|------|------|------|------|-------------|--------------------|
| *H. amazonensis*      | 567  | 124  | 216  | 202  | 207  | 24-29| 4.4-5.5| 5.1-6.1| 7.3*  | 83-92| 89-109| Brazil| Andaló et al. (2006) |
| *H. atacamensis*      | 578  | 124  | 234  | 207  | 208  | 24-29| 4.4-5.7| 5.7-7.1| 5.7*  | 79-94| 149-182| Chile| Edgington et al. (2011) |
| *H. bacteriophora*    | 512  | 19-25| 21-25| 100  | 103  | 17-30| 4.0-5.1| 5.7-7.0| 6.0*  | 76-92| 103-130| Australia| Polantar (1976) |
| *H. baujardi*         | 530  | 22-30| 93-108| 80-90| 110-130| 23*  | 4.4*  | 5.7*  | 4.8*  | 81*  | 106*  | Argentina| Agüera de Doucet and Doucet (1986) |
| *H. brevicaudis*      | 537  | 19-22| 120  | 87-93| 72-93| 23-31| 3.3-3.6| 3.6-4.6| 7.0*  | 73-88| 87-105| Australia| Sagun et al. (2015) |
| *H. bushi*            | 474  | 110-127| 61-90| 90-115| 57-90| 19-25| 4.7-6.1| 5.5-9.3| 3.4-7.5| 105-139| 131-211| India      | Bhat et al. (2019a) |
| *H. downesi*          | 453  | 129-167| 72-102| 50-74| 83-106| 47-89| 19-29| 4.9-7.4| 6.0-12| 3.7-6.5| 78-107| 105-189| India      | Rana et al. (2020) |
| *H. egyptii*          | 610  | 24-38| 68-112| 82-116| 101-150| 70-105| 18.3* | 3.7*  | 6.5*  | 4.3*  | 80*  | 141* | Argentina| Stock (1993) |
| *H. heliothidis*      | 619  | 23-29| 112* | 108* | 130-139| 104-112| 22-28| 4.6-5.4| 5.8-6.3| 6.0*  | 83*  | 97*  | USA       | Khan et al. (1976) |
| *H. indica*           | 497  | 18-22| 91-103| 75-86| 107-120| 83-97| 26-30| 4.5-5.1| 6.6-7 | 7.2*  | 78-88| 98-114| Vietnam   | Phan et al. (2003) |
| *H. latemana*         | 525  | 18-25| 88-96| 68-85| 98-120| 95-108| 24-32| 4.6-5.9| 5.2-6.1| –     | 74-86| 89-92| India     | Variahlimpuia et al. (2018) |
| *H. longispina*       | 502  | 25-56| 81-91| 78-94| 106-117| 91-131| 23-27| 5-5   | 4-6   | 8.0*  | 76-87| 64-95| Thailand  | Maneesakom et al. (2015) |
| *H. longiurina*       | 566  | 21-25| 100-122| 85-106| 118-146| 80-111| 24-29| 4.2-4.9| 5.9-6.8| 6.0-7.4| 80-93| 103-121| China     | Li et al. (2012) |
| *H. lowi*             | 589  | 15-22| 96-128| 96-105| 110-124| 62-74| 29-42| 4.4-5.3| 8.5-10.5| 4.4*  | 76-96| 160-180| Ireland   | Stock et al., 2002 |
| *H. oryzae*           | 484  | 18-23| 81-94| 78-100| 100-119| 53-75| 20-27| 4.2-5.2| 6.8-9.1| 6.9*  | 74-82| 100-170| Egypt     | Abd-Elgawad and Ameen (2005) |
| *H. floridensis*      | 554  | 19-23| 101-122| 68-107| 123-142| 91-113| 25-32| 3.9-4.9| 5.3-6.6| 7.2*  | 71-90| 95-134| USA       | Nguyen et al. (2006) |
| *H. georgiana*        | 547  | 17-26| 97-113| 74-94| 110-139| 86-108| 23-34| 4.1-5.3| 5.5-6.9| 6.8*  | 70-93| 106* | USA       | Nguyen et al. (2008) |
| *H. hambetton*        | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | Brazil     | Pereira (1937) |
| *H. hoptha*           | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | USA        | Turco (1970) |
| *H. indica*           | 479  | 19-22| 88-107| 72-85| 109-123| 93-109| 25-27| 4.3-4.8| 4.5-5.6| –     | 79-90| 83-103| India     | Poinar et al. (1992) |
| *H. jordanii*         | 511  | 21-24| 92-108| 63-73| 86-103| 24-34| 22-25| 5-6   | 4.6-5.4| 2.8-5.2| 77-96| 100-118| India     | Kajol et al. (2020) |
| *H. koreanensis*      | 516  | 21-25| 98-123| 82-101| 102-129| 80-112| 24-27| 4.5-5.4| 4.9-5.7| 5.6-8.1| 83-97| 93-136| India     | Phan et al. (2021b) |
| *H. brevicaudis*      | 528  | 20-24| 104-116| 96-104| 120-136| 68-80| –    | –    | 6.6-8.6| 6.3*  | 81*  | 150-180| China     | Liu (1994) |
| *H. gerrardi*         | 551  | 18-29| 92-111| 81-105| 110-130| 76-141| 23-32| 16-23| 11-21| 6.8*  | 73-92| 73-138| Australia | Plichta et al. (2009) |
| *H. hawaiensis*       | 506  | 21-28| 116-175| 79-103| 115-181| 82-108| –    | –    | –    | 6.0*  | 77*  | 88*  | USA       | Gardner et al. (1994) |
| *H. pakistanense*     | 558  | 19-23| 95-106| 73-90| 113-125| 95-110| 25-29| 4.7-5.3| 5.4-6.2| 5.4*  | 78-97| 95-107| Pakistan  | Shahna et al. (2017) |
| Species                  | Length (µm) | Width (µm) | Length (µm) | Width (µm) | USA   | Reference                      |
|-------------------------|-------------|------------|-------------|------------|-------|--------------------------------|
| H. marelatus            | 588-700     | 24-32      | 81-113      | 83-113     | 21-129| Machado et al. (1996)          |
| as H. hepialus¹         | 540-600     | 34-39      | 84-112      | 80-101     | 49-60 | Liu and Berry (1996)           |
| H. megidis              | 736-800     | 27-32      | 123-142     | 104-115    | 112-128| USA                            |
| H. mexicana             | 530-620     | 20-24      | 83-109      | 74-88      | 91-106| Mexico                         |
| H. noenieputensis       | 484-578     | 21-25      | 88-105      | 69-96      | 79-115| USA                            |
| H. poinari²             | 350-410     | 18-22      | -           | -          | 15-22 | Kakula and Mikaia (1997)       |
| H. ruandica             | 496-591     | 18-27      | 70-89       | 52-64      | 49-64 | Rwanda                         |
| H. safricana            | 550-676     | 19-23      | 103-122     | 86-101     | 125-141| S. Africa                      |
| H. taysearae            | 332-499     | 17-23      | 74-113      | 58-87      | 96-130| Egypt                          |
| as H. sonorensis³       | 495-570     | 19-32      | 97-116      | 87-98      | 110-131| Mexico                         |
| H. zealandica           | 570-740     | 22-30      | 94-123      | 90-107     | 135-147| USA                            |
| as H. heliothidis        | 570-740     | 22-30      | 94-123      | 90-107     | 135-147| N. Zealand                     |
| H. zacatecana            | 493-578     | 23-27      | 72-99       | 69-72      | 96-124| Mexico                         |

Note: All measurements are in µm (except ratios and percentages). ¹Synonymized species. H. bacteriophora (Syn.: H. argentinensis); H. baujardi (Syn.: H. somsookae); H. indica (Syn.: H. brevicaudis, H. gerrardi, H. hawaiiensis, and H. pakistănense); H. marelatus (Syn.: H. hepialus); H. taysearae (Syn.: H. sonorensis); and H. zealandica (Syn.: H. heliothidis apud Wouts, 1979 nec Khan, Brooks & Hirschmann, 1976). ²H. heliothidis apud Khan et al., 1976 (=Chromonema heliothidis) is declared herein species inquirenda as the male morphology of H. bacteriophora and H. heliothidis differ. ³Re-instated as valid species herein based on morphological evidence. H. egyptii and H. hambletoni (=Rhabditis hambletoni) are declared herein valid species as their original descriptions provide all the diagnostic characters to differentiate them morphologically from all species of the genus. ⁴Species inquirenda. H. hopth (= Neaplectana hoptha) and H. poinari are maintained as species inquirenda because their original descriptions do not include important diagnostic characters to fully differentiate them from the other species of the genus. *Data calculated from the drawings provided in the original publication. **Data calculated from other measurements provided in the original publication. –Data not provided in the original publication.
**Type species of the genus**

*Heterorhabditis bacteriophora* Poinar, 1976

= *H. argentinensis* Stock, 1993. Synonymized by Hominick (2002) based on molecular evidence provided by Adams et al. (1998). Synonymization status is supported by molecular data of Phan et al. (2003) and Achinelly et al. (2017).

**Other species of the genus**

*H. amazonensis* Andaló, Nguyen & Moino, 2006

*H. atacamensis* Edgington, Buddle, Moore, France, Merino & Hunt, 2011

*H. baujardi* Phan, Subbotin, Nguyen & Moens, 2003

= *H. somsookae* Maneesakorn, An, Grewal & Chandrapatya, 2015. Synonymized by Hunt and Nguyen (2016) based on the minor ITS sequence divergencies between *H. baujardi* and *H. somsookae*. Synonymisation status is further supported by the molecular data analyses carried out by Dhakal et al. (2020).

*H. beicherriana* Li, Liu, Nermit, Půža & Mráček, 2012

*H. egyptii* Abd-Elgawad & Ameen, 2005. This species was declared *species inquirenda* by Nguyen and Hunt (2007) but considered valid by Sudhaus (2011). As this species was described showing all diagnostic characters of adults and larvae stages, and it is morphologically distinct from all the other valid species, this species is also considered valid herein. The lack of molecular data, however, impairs its inclusion in future phylogenetic studies. Nevertheless, new species description should contrast morphological characters with this species.

*H. downesi* Stock, Griffin & Burnell, 2002

*H. floridensis* Nguyen, Gozel, Köppenhöfer & Adams, 2006

*H. georgiana* Nguyen, Shapiro-Ilan & Mbata, 2008

*H. hambletoni* (Pereira, 1937) Poinar, 1976

= *Rhabditis hambletoni* Pereira, 1937. This species was described showing all diagnostic characters of adults and larvae stages. It was transferred to the genus *Heterorhabditis* by Poinar (1976). As this species was described showing all diagnostic characters of adults and larvae stages, and it is morphologically distinct from all the other valid species, this species is considered valid herein. The lack of molecular data, however, impairs its inclusion in future phylogenetic studies. Nevertheless, new species description should contrast morphological characters with this species.

*H. indica* Poinar, Karunakar & David, 1992

= *Heterorhabditis brevicaudis* Liu, 1994. Several important diagnostic characters are missing and no molecular data are provided in the description of this species, although, it appears to be morphologically different from *H. downesi*, *H. baujardi*, and *H. mexicana* (Stock et al., 2002; Phan et al., 2003; Nguyen et al., 2004). Perhaps due to this reason, it was declared *species inquirenda* by Nguyen and Hunt (2007). A nematode population that shares several morphological characters with the original population used to describe the species was characterized more recently (Hsieh et al., 2009). ITS sequences are almost identical to the sequences of *H. indica*, justifying its synonymization (Hunt and Nguyen, 2016; Dhakal et al., 2020).

= *Heterorhabditis hawaiiensis* Gardner, Stock & Kaya, 1994. Not formally synonymized. However, synonymization status is supported by molecular data of Adams et al. (1998), Liu et al. (1999), and Phan et al. (2003), and multivariate analyses based on morphological characters of Stock and Kaya. (1996).

= *Heterorhabditis gerrardi* Plichta, Joyce, Clarke, Waterfield & Stock, 2009. Synonymized by Hunt and Nguyen (2016) based on the absence of ITS sequence divergencies. Synonymisation status is supported by further molecular data analyses carried out by Dhakal et al. (2020).

= *Heterorhabditis pakistanensis* Shahina, Tabassum, Salma, Mehreen & Knoetze, 2016. Synonymized by Hunt and Nguyen (2016) based on the minor ITS sequence divergencies between *Heterorhabditis pakistanensis* and *H. indica*. Synonymisation status is further supported by molecular data analyses carried out by Dhakal et al. (2020).

*H. marelatus* Liu & Berry, 1996

= *Heterorhabditis hepialius* Stock, Strong & Gardner, 1996. Synonymized by Stock (1997) based on morphological and morphometric analyses and cross-breeding tests. Synonymization status is further supported by molecular data of Adams et al. (1998) and Liu et al. (1999).

*H. meigidis* Poinar, Jackson & Klein, 1987

*H. mexicana* Nguyen, Shapiro-Ilan, Stuart, McCoy, James & Adams, 2004

*H. ruandica* n. sp.

*H. noenieputensis* Malan, Knoetze & Tiedt, 2014

*H. safricana* Malan, Nguyen, De Waal & Tiedt, 2008

*H. taysearae* Shamseldain, Abou El-Sooud, Abd-Elgawad & Saleh, 1996

= *Heterorhabditis sonorensis* Stock, Rivera-Orduño & Flores-Lara, 2009. Synonymized by Hunt and Nguyen (2016) based on the minor ITS sequence divergencies between *H. taysearae* and *H. sonorensis*. Synonymisation status is further supported by molecular data analyses carried out by Dhakal et al. (2020).
Multi-locus phylogenetic analyses uncover species boundaries: Machado et al.

H. zacatecana n. sp.
H. zealandica Poinar, 1990
= Heterorhabditis heliothidis apud Wouts, 1979 nec Khan, Brooks & Hirschmann, 1976. This species was reclassified as H. zealandica by Poinar (1990) as it is morphologically different from Heterorhabditis heliothidis apud (Khan et al., 1976).

Species inquirendae

H. hoptha (Turco, 1970) Poinar, 1979.
= Neoapectana hoptha Turco, 1970
This species was poorly described. The original description lacks differentiated description of all diagnostic characters of adult and larval stages. According to this, this species should remain on the list of species inquirendae.

H. poinari Kakuliya and Mikaia, 1997. This species was poorly described. The original description lacks differentiated description of all diagnostic characters of adult and larval stages. According to this, this species should remain on the list of species inquirendae.

H heliothidis (Khan, Brooks & Hirschmann, 1976) Poinar, Thomas & Hess, 1977.
= Chromonema heliothidis (Khan, Brooks & Hirschmann, 1976)
This species was synonymized by Akhurst 1987 based on differential electrophoretic patterns of nematode lysates. However, the original description of H. bacteriophora carried out by Poinar (1976) shows males with very anterior GP1, while in its synonymized species H. heliothidis (Khan, Brooks & Hirschmann, 1976) Poinar, Thomas & Hess, 1977 the GP1 appears more posterior (Khan et al., 1976; Poinar, 1976). Probably both species are not conspecific. We therefore declare H. heliothidis (Khan, Brooks & Hirschmann, 1976) Poinar, Thomas & Hess, 1977 species inquirenda.

Nomina nuda

H. downesi Hass et al. 2001 nec H. downesi Stock, Griffin & Burnell, 2002
H. minutus Prabhuraj, Viraktamath & Kumar, 2002.

Conclusions

The results of our study uncover the low levels of interspecific variation in some regions of the rRNA genes, especially in the D2–D3 expansion segments of the 28S rRNA, and also uncover the almost absent intraspecific variation of these sequences in the nematodes of the “Bacteriophora-group”. Mitochondrial genes such as COI provide better phylogenetic resolutive power, even at the population level, highlighting their great potential for the taxonomic characterization of closely related species of the genus Heterorhabditis. The threshold for species delimitation using COI sequences has been proposed to be around 94% (Pentinsaari et al., 2016). Using this threshold, we can clearly assign the Mexican and the Rwandan nematodes to new taxa within the “Bacteriophora group”. However, the sequence similarity scores of the Mexican and the Rwandan nematodes is between 97.6% and 98.2%. These scores are higher than the proposed 94% threshold but are consistent across nematode isolates and significantly lower than the intraspecific variations, prompting the question of whether the Rwandan and the Mexican nematodes should be classified into two different species, or into the same. Based on the results of the self-crossing and cross-hybridization experiments, and on the evident morphological and morphometric differences between these two groups of nematodes, we conclude that they indeed represent two distinct biological species. Thus, the boundary that delimits species in the genus Heterorhabditis is around 97% to 98% sequence similarity in the COI genomic sequence, and the Rwandan and the Mexican nematodes represent two new species, Heterorhabditis ruandica n. sp and H. zacatecana n. sp.

Supplementary Material

Supplementary figures and tables can be retrieved from: https://doi.org/10.5281/zenodo.5614704

Conflicts of interest

The authors declare no competing interests.

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