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# A new entomopathogenic nematode species and its association with a facultative necromenic nematode

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**Summary**

This is the report of mixed infection of an entomopathogenic nematode and a free-living bacteriophage nematode both invading *Galleria mellonella* larvae, apparently showing strong intraguild competition. The entomopathogenic nematodes isolated in this study were classified as *Heterorhabditis kankabi* n. sp., and *Metarhabditis rainai* through light and electronic microscopy, sequencing of ITS and 28S ribosomal DNA regions, and phylogenetic analyses. The bacteriophage nematode *M. rainai* was recognized as a facultative necromenic nematode, as it consumed the food resources obtained by entomopathogenic nematodes, directly affecting the life cycle of the later by stopping their development and forcing them to leave *G. mellonella* in search of new hosts.

**Keywords:** Bacteriophage, Entomopathogenic nematodes, *Galleria mellonella*, *Heterorhabditis kankabi* n. sp., *Metarhabditis rainai*

## Introduction

Entomopathogenic nematodes (EPNs) are successfully used in agricultural crops to control a wide variety of insect pests (Thanwisai *et al.,* 2022). Currently, eight families that cause diseases to their arthropod hosts have been determined, namely: Mermithidae, Tetradonematidae, Allantonematidae, Phaenopsitylenchidae, Sphaerulariidae, Rhabditidae, Steinernematidae and Heterorhabditidae (Othman *et al.,* 2022). Among these families, the Steinernematidae and Heterorhabditidae (Rhabditida) are the most interesting families due to their roles as entomopathogens. To date, around 100 species of *Steinernema* and 21 species of *Heterorhabditis* have been described around the world, of which 15 were described in Latin America (12 *Steinernema* and 3 *Heterorhabditis*) (Sadhaus, 2011; Shapiro-Ilan *et al.,* 2018; Dhakal *et al.,* 2020). The entomopathogenic mode of action of *Heterorhabditis* is based on the symbiotic interaction of the nematode with the bacterium *Photorhabdus* spp. (Gaugler, 2002; Karimi *et al.,* 2011). The pathogenic cycle of these nematodes begins when the free-living infective juveniles (IJs) manage to penetrate the hosts through natural openings such as mouth, anus or spiracles (Kaya & Gaugler, 1993). Once the nematode enters the host's body and reaches the hemocoel, it releases its symbiotic bacterium. The bacterium produces antibiotics to prevent another pathogenic organism from general septicaemia of the host and protects the cadaver from saprophytic activity, while the bacterium multiplies and creates

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the ideal environment for the reproduction and feeding of the nematode (Gaugler, 2002; Kary, 2009; Vázquez, 2014). The bacterium spreads rapidly until killing the insect host by septicaemia within 24 to 48 hours. Within the host, *Heterorhabditis* produces the first generation of hermaphrodites followed by amphimictic generations. Once the food resources from the host are exhausted, IJ stages emerge in search for a new host (Rakubu *et al.,* 2024). However, it is possible that some soil organisms are resistant to compounds that prevent scavenging activity including certain free-living or facultative necromenic nematodes. These bacteriophage nematodes can compete for insect carcasses (Campos-Herrera *et al.,* 2012), being able to show a necromenic behavior, term Greek that means wait inside the body until the decay to benefit from the resources previously obtained by other species (Trejo-Meléndez *et al*., 2024).

The original goal of the present study was to present the description of a new species of *Heterorhabditis*. However, after repeatedly trying to find all the life stages of the new species, we found no males of the new species of *Heterorhabditis*, but only of the necromenic *Metarhabditis rainai*. Therefore, male morphology is not included in the description of the new species. So, an approach similar to that proposed by Sharkey *et al.,* (2021) was followed, as they described more than 400 new species of wasps using DNA sequences was used directly as the main diagnostic character but supported by morphometric characters.

**Material and Methods**

*Soil samples:*

In a previous study in the state of Yucatan, Mexico, two EPN species were detected by ribosomal gene sequencing: *Heterorhabditis indica* and a putative new species of *Heterorhabditis* sp. (Ávila-López *et al.,* 2021). New samples from Ticuch, Yucatan, the original location of the putative new species, were required to try to recover all the larval and mature forms of the nematode. Several soil samples were collected from sour orange (*Citrus aurantium*) and sweet orange (*Citrus sinensis*) fields in Ticuch, from October to December 2021.

A total of 20 soil samples from 10 sampling points from one agricultural site were collected. At each sampling point, 2 composite soil samples were obtained; these samples were placed in transparent plastic bags (300 – 500 g per bag) and carried to the Laboratorio de Patología Acuática del Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, (Cinvestav, Mérida) where each sample was allocated in 1 L plastic flask.

Laboratory-reared *Galleria mellonella* larvae were used as insect baits to isolate EPNs from these soil samples (Bedding & Akhurst, 1975). Five *G. mellonella* larvae per flask were used, incubated for five days at room temperature. The infected *G. mellonella* (brick colored) were placed in moist chambers until the emergence of infective juveniles (Kaya & Stock, 1997). The infective juveniles were collected and cleaned by sedimentation techniques in White traps (White, 1927). Additionally, the same infected *G. mellonella* larvae were dissected and the remaining nematodes were fixed in 70 % ethanol for morphological measurements, in glutaraldehyde for electron microscopy and 96 % ethanol for molecular characterization.

*Morphological identification of nematodes:*

The identification of males, females, and juveniles was performed based on the morphology and morphometric characters based on recommendations of Hominick *et al.* (1997); Nguyen and Hunt (2016) as well as in proportions and keys used for the identification of nematodes (Table S1) (Nguyen, 1996; Poinar, 1990). Subsequently, images from a light microscope (Olympus®, Japan) were used to identify the different structures of the nematodes. These structures were measured using the ImageJ software (Abramoff *et al.*, 2004) which was calibrated with the micrometer of the microscope.

*Scanning electron microscopy:*

Preparation of EPNs for scanning electron microscopy at critical point drying included washing samples with 0.1 M cacodylate buffer and phosphates (1X) pH 7.2 – 7.4, for 24 hours. Subsequently, samples were fixed with 2.5 – 3 % glutaraldehyde for at least one hour at 25°C. Then, three washes were performed with cacodylate, as well as a second fixation with osmium tetroxide (2 h) and buffer (Dulbecco's Phosphate-Buffered Saline, Gibco TM). Samples were washed three times with distilled water for subsequent dehydration with a series of ethanol concentrations (30, 50, 70, 90, 95, and 100 %) for 15 minutes each (Nguyen & Smart, 1996). At the end, samples were mounted and observed using a scanning electron microscope Hitachi Stereoscan SU1510 SEM (Hitachi Ltd., Chiyoda, Tokyo, Japan) working at Instituto de Biología, Universidad Nacional Autónoma de México (UNAM).

*Molecular characterization:*

DNeasy Blood and Tissue kit (Qiagen™) was used for the DNA extraction, following the manufacturer's instructions. Three females, two infective juveniles and five male specimens were taken at random from the total number of nematodes obtained. Amplification of the 28S and ITS ribosomal genes was performed by PCR, using the GoTaq® DNA Polymerase. Primers used in the amplification reactions for each gene, as well as the concentrations of the reagents and the amplification conditions of the PCR can be consulted in Ávila-Lopez *et al.* (2021). PCR products were visualized by electrophoresis on a 1 % agarose gel using 1X TAE buffer at 85 V for 45 min in a BioRad Sub-Cell®GT agarose gel electrophoresis system using a Promega® DNA leader of 1KB molecular weight as a reference, in a BioDoc-It® Imager. For sequencing, in addition to the external primers, the internal primers BD3 5'-GAACATCGACATCTTGAACG-3', and BD4 5'-ATAAGCCGACCCTCGGC-3' (Hernández-Mena *et al*., 2014) for ITS, along with 503 5'-CCTTGGTCCGTGTTTCAAGACG-3' (Stock

Table 1. Genetic distances between *Heterorhabditis kaankabi* n. sp. and the *Heterorhabditis* species in the 28S and ITS ribosomal gene sequences. .

| **Species** | *Heterorhabditis kankabi* n. sp. | | | |
|---|---|---|---|---|
| | **28S** | | **ITS** | |
| | ***p*** | **N** | ***p*** | **N** |
| *Heterorhabditis taysearae* | na | na | 1.22 | 11 |
| *Heterorhabditis mexicana* | 0.67 | 6 | 1.22 | 11 |
| *Heterorhabditis floridensis* | 0.67 | 6 | 0.66 | 6 |
| *Heterorhabditis baujardi* | 0.67 | 6 | 1.37 | 11 |
| *Heterorhabditis amazonensis* | 0.79 | 7 | 1.66 | 15 |
| *Heterorhabditis noenieputensis* | 2.92 | 26 | 9.43 | 83 |
| *Heterorhabditis indica* | 2.92 | 27 | 9.01 | 79 |
| *Heterorhabditis zealandica* | 5.77 | 51 | 24.66 | 215 |
| *Heterorhabditis megidis* | 6.00 | 53 | 22.45 | 194 |
| *Heterorhabditis downesi* | na | na | 21.69 | 187 |
| *Heterorhabditis safricana* | 5.20 | 46 | 22.03 | 191 |
| *Heterorhabditis marelatus* | 5.20 | 46 | 21.91 | 190 |
| *Heterorhabditis atacamensis* | 4.88 | 42 | 20.65 | 179 |
| *Heterorhabditis bacteriophora* | 4.68 | 42 | 21.81 | 193 |
| *Heterorhabditis georgiana* | 5.10 | 45 | 22.03 | 195 |
| *Heterorhabditis beicherriana* | 4.95 | 43 | 22.03 | 195 |

*p*= uncorrected *p* distances, N= number of different nucleotides (na= sequences not available in Genbank)

*et al*., 2001) and 504 5'-CGTCTTGAAACACGGACTAAGG-3' (García-Varela & Nadler, 2005) for 28S were used. PCR products were sequenced using an-ABI 3500xL Genetic Analyser (Applied Biosystems, Waltham, Massachusetts, USA) at the Laboratorio de Secuenciación Genómica de la Biodiversidad, de la Universidad Nacional Autónoma de México. Subsequently, the consensus sequences of ITS and 28S were obtained from resulting sequences of each primer using Geneious Pro 4.8.4. (Biomatters Ltd., Auckland, New Zealand). The consensus sequences from each specimen were deposited at the GenBank.

*Phylogenetic analysis:*

Sequences generated in this study were aligned with sequences available in the GenBank for other species of *Heterorhabditis* and *Metarhabditis*. Three datasets were generated in Mesquite 3.62 (https://www.mesquiteproject.org/) to determine the phylogenetic relationship: two datasets (ITS and 28S) for *Heterorhabditis* and a single one (28S) for *Metarhabditis* (no ITS rDNA was amplified). The alignment of each data set was performed with ClustalW (Thompson *et al.,* 1994), implemented on the website http://www.genome.jp/tools/clustalw/, with the approach 'SLOW/ACCURATE' and weight matrix 'CLUSTALW (FOR DNA)'. The nucleotide substitution model was estimated in jModelTest v2 (Darriba *et al.,* 2012). Phylogenetic analyses were performed using the Maximum Likelihood (ML) method through the RAxML v. 7.0.4 software (Stamatakis, 2006) where 1000 bootstrap (Bt) repetitions were also made to know the support value of the clades of the trees obtained with each data set. The ML trees were visualised in FigTree v.1.4.3. (Rambaut, 2016). Molecular variation was estimated using uncorrected *p* distances and the number of different nucleotides with the software MEGA v.6 (Tamura *et al.,* 2013).

**Ethical Approval and/or Informed Consent**

The authors declare that they have complied with all applicable ethical standards.

**Results**

After infection of *G. mellonella* larvae from soil samples, several life stages of the nematodes were observed. Interestingly, molecular data indicated the presence of two different nematodes that were infecting the same individual of *G. mellonella*, simultaneously. Juveniles and amphimictic females were identified within the genus *Heterorhabditis* while males were identified within the species *Metarhabditis rainai*. This result was particularly interesting as there are few records about this interaction between different nematodes under conditions that are not experimental. The native *Heterorhabditis* isolated exhibited low variation of sequence in 28S and high variation in ITS (Table 1) in comparison to other nematodes described, which indicated that those sequences belonged to a new species, as follows:

*Taxonomic description*:
Family: Heterorhabditidae, Poinar 1976.
Genus: *Heterorhabditis,* Poinar 1976.
*Heterorhabditis kankabi* n. sp. Ávila-López, Hernández-Mena, Ibarra and Vidal-Martínez, (2023) (Figs. 1, 2).

*Morphological diagnosis:*
Amphimictic female (Table S1): Measurements based on 10 specimens. Females with an average length of 1028 ± 87 (880 − 1175) µm, 63 ± 10 (50 − 80) µm in width and 199 ± 11 (182 −220) µm in length of the oesophagus. Immature females exhibited oocytes, gravid females had embryonated eggs and larvae. Annulation of cuticle under scanning electron microscope, truncated head, labial region with six separate conical lips, each lip with a terminal labial papilla, with very small double cephalic papillae at the base of lips observed by scanning electron microscopy. In apical view, the mouth was hexagonal in shape (Fig. 1). Amphidial opening circular. Funnel-shaped tubular stoma, longer than wide with cheilorhabdions. Muscular oesophagus with cylindrical corpus. Long and visible isthmus. Nerve ring in the distal part of the isthmus

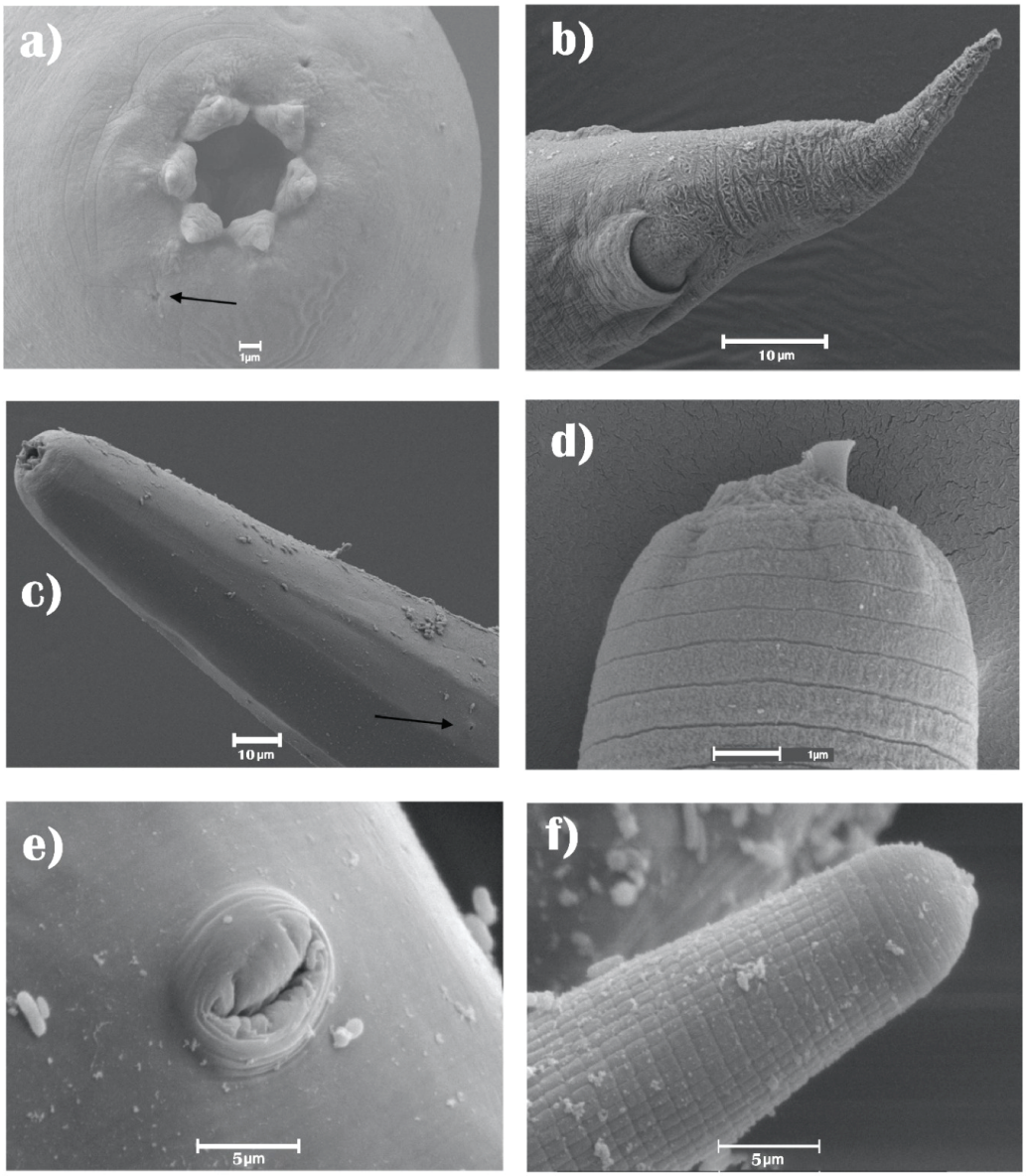


Fig. 1. *Heterorhabditis kankabi* n. sp. SEM microphotographs. A, Head of an amfimictic female with six labial papillae and two amphids (magnification 5,000x); B, Posterior region of a hermaphrodite nematode showing anal area and tip of the tail (magnification 2,500x); C, Anterior region of the nematode where the excretory pore is observed in a hermaphroditic specimen (magnification 1,000x); D, infective juvenile with larval tooth and striated cuticle of the second instar (magnification 5,000x); E, Protruding vulvar region located in the middle part of the hermaphrodite specimen (magnification 5,000x); F, Anterior region of an ensheathed nematode showing the body with tessellate pattern (magnification 5,000x).

(Fig. 2). Prominent basal bulb with pumping chamber. Excretory pore at the level of the basal bulb base. Prominent intestinal cells, especially between the basal bulb and the anterior ovary, and between the rectum and the posterior ovary. Defined intestinal lumen. Reproductive system exhibited two amphiphilic ovaries. Vulva located passing the anterior half of the body (V = 61 ± 7 [50 − 80 %]). Phasmids were not observed. Conoid tail.

*Hermaphrodite female*:
The fixed hermaphrodite females acquired a 'C' shape. Robust body, with a mean body length of 2893 ± 597 (2200−3750) µm and a width of 146 ± 27 (100 − 125) µm. The excretory pore had a mean distance to the anterior end of 205 ± 33 (142 − 242) µm, located at the level of the basal bulb base. Elliptic and protuberant vulva, with distance to the anterior end of 50 ± 5 (42 − 61) µm. Phasmids were not observed. Near the posterior end, the tail widened and reduced in size finishing in a sharp tip. Anal protrusion present.

*Infective juvenile*:
Elongated body, 545 ± 95 (420 − 685) µm in length and width of 26 ± 4 (20 − 35) µm. Cephalic region without papillae and small amphid. Labial region with the presence of a dorsal tooth (used to break the host's cuticle), with seven transverse rings, followed by a cuticle with the appearance of a cob. Longitudinal fissures from the excretory pore. Small oesophagus and intestine. Excretory pore at the level of the basal bulb base. Phasmids were not observed. Long and conoid tail.

*Taxonomic summary:*
*Type host*: *Galleria mellonella (*Linnaeus, 1756) wax moth (Lepidoptera: Pyralidae).
*Type locality*:
Ticuch, Yucatán, México (20°42'5.82" N, 88°6'39.07" O).
*Other localities*:
Dzan, Yucatán, México (20°22'36.1" N, 89°27'2.6" O).
*Type material*: Holotype CNHE 11662; Paratypes CNHE 11663-11666 (n=5).

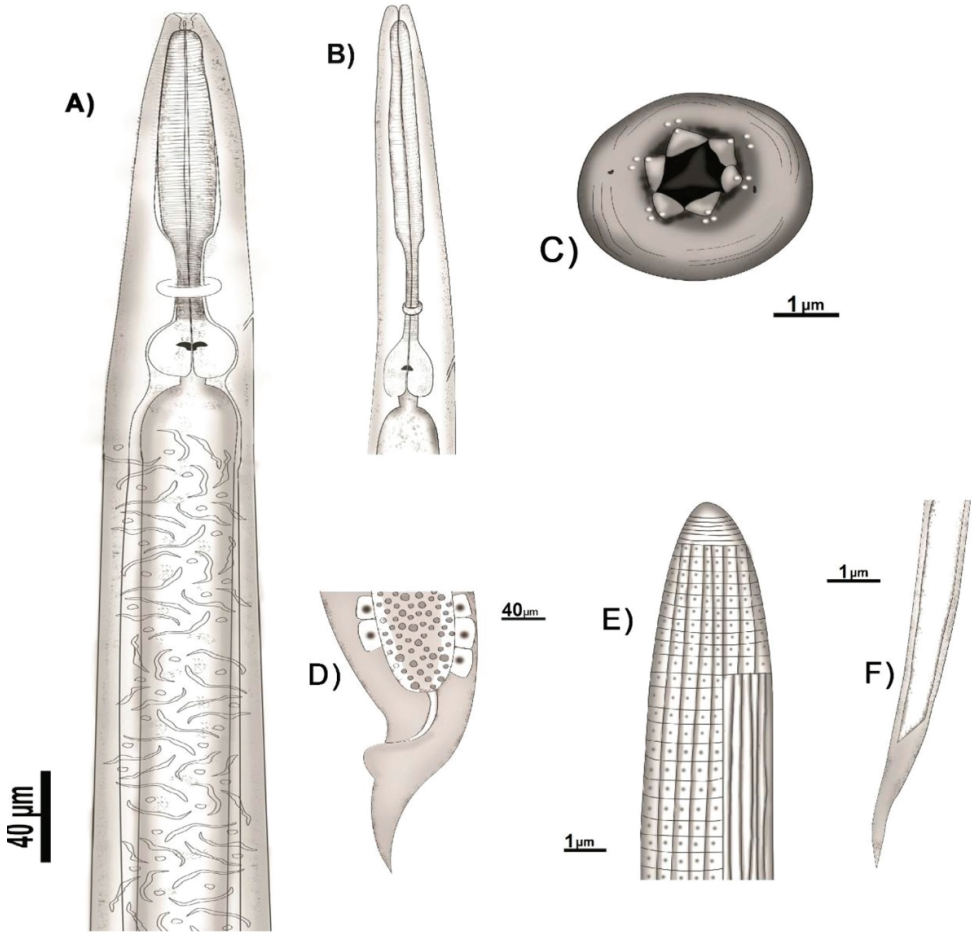


Fig. 2. Schematic details of *Heterorhabditis kankabi* n. sp. A) anterior region of a hermaphrodite female; B) anterior region of an amphimictic female; C) apical view of a hermaphrodite female; D) tail of a hermaphrodite female; E) anterior region of an infective juvenile; F) tail of infective juvenile.

*Representative DNA sequences GenBank accession numbers*: 28S rDNA (OQ971709 to OQ971713); ITS rDNA (OQ971701 to OQ971705).

*Etymology*:
Word in Mayan that means yellow down (K'an: yellow Kab: below) referring to a type of soil in the Yucatán Peninsula where this species was isolated.

*Remarks:*
The hermaphroditic females exhibited a protruding vulvar morphology very similar to that of *Heterorhabditis mexicana*. However, *H. mexicana* has larger body length, i.e., 3,516 ± 543 (2,440 − 4,606) µm compared to the description of the native nematode *H. kankabi* n. sp., i.e., 2,893 ± 597 (2,200 − 3,750) µm. The morphometric characters of the new species were similar to those of *H. bacteriophora*, *H. baujardi*, *H. floridensis*, and *H. indica*. The body length of the infective juvenile of *Heterorhabditis kankabi* n. sp. was similar to that of *H. indica*, *H. noenieputensis*, *H. baujardi*, and *H. sonorensis (*Table S2). However, the diameter of the body at the level of the anal zone was only like that of *H. taysearae* and *H. sonorensis* (Table S2). The infective juvenile had an EP (distance from the anterior part to the excretory pore), ES (distance from the anterior part to the end of the pharynx), length of the tail without sheath and a total body length/greatest body width value which is in line with the data reported for *H. taysearae* (Table S2).
The length of the hermaphroditic female of *H. kankabi* was 2,893 ± 597 (2,200 − 3,750) µm, and the length of the amphimictic female 1,028 ± 87 (880 − 1,175) µm, similar to that found in female specimens of *H. baujardi*. The oesophagus of the native nematode was longer than that of other reported species (132 ± 12 [110 − 145] µm). The only similar species was *H. baujardi* (116 ± 10 [105 − 132] µm). For H. *kankabi* n. sp., the only morphological variability found in comparison with other described species was the protruding and globose vulva.

*Phylogenetic analysis of Heterorhabditis kankabi n. sp.:*
Five sequences of the 28S gene were obtained from different stages of development of the new species showing, as expected, the same sequence. The phylogenetic analysis was performed with an aligned matrix of 935 bp length and containing representatives of 16 species of *Heterorhabditis*, while the substitution model estimated for this matrix was the GTR. The tree obtained had a likelihood value of −2090.969312. In the tree, the species were distributed in 4 main clades with high support values (Fig. 3), which we named: Mexicana (Bt= 98), Indica (Bt= 100), Megidis (Bt= 100) and Bacteriophora (Bt= 100). In the Mexicana clade, *H. kankabi* n. sp., *H. floridensis*, *H. baujardi*, *H. amazonensis* and *H. mexicana* were nested; in particular, the new species was grouped as the sister species of *H. mexicana* (Bt= 79). The genetic distance between the sister species *H. kankabi* n. sp. and *H. mexicana* was 0.67 %, and between *H. kankabi* n. sp. and the other species of the Mexicana clade was 0.67 to 0.79 %. The genetic distances with the species distributed in the other three major clades were as follows: 2.97 % with the Indica clade; from 4.88 to 6 % with the Megidis clade; and from 4.68 to 5.1 % with the Bacteriophaga clade (Table 1).
Five ITS sequences were obtained from the same specimens

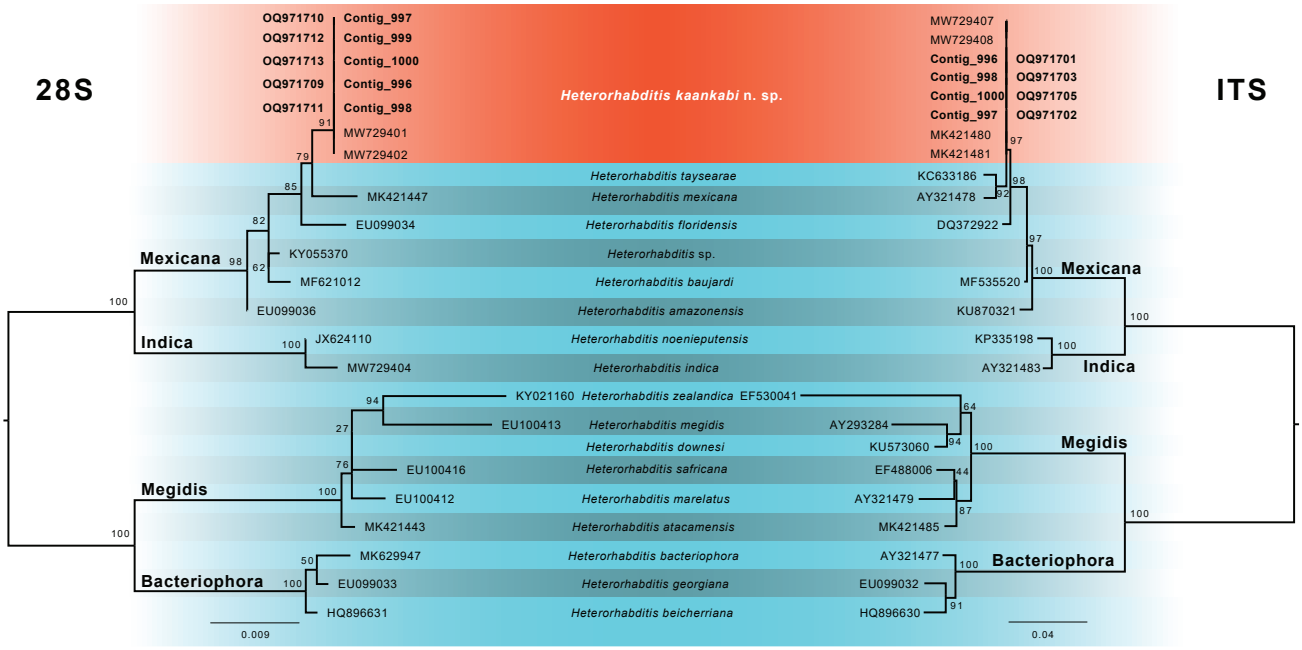


Fig. 3. Phylogenetic relationships of species of the genus *Heterorhabditis* using the maximum likelihood method to generate the phylogenetic trees. The tree shows ITS on the right side and the 28S on the left side.

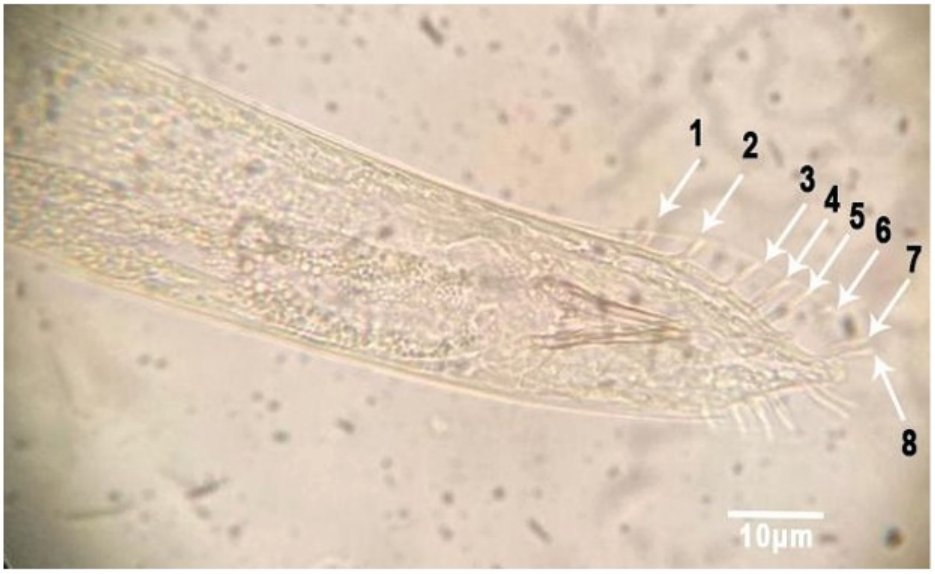


Fig. 4. Clear field micrography of *Metarhabditis rainai* male ventral view showing eight bursal papillae.

used for the 28S sequencing all showing, as expected, the same sequence. The phylogenetic analysis was performed with an aligned matrix of 1041 bp length and containing representatives of 17 species of *Heterorhabditis*; the substitution model estimated for this matrix was the GTR+GAMMA+I. The tree obtained had a likelihood value of −4155.806221. In the ITS tree, the same 4 main clades that were obtained with 28S were also observed (Fig. 3) with high support values: Mexicana (Bt= 100), Indica (Bt= 100), Megidis (Bt= 100) and Bacteriophora (Bt= 100). In the clade Mexicana, *H. kankabi* n. sp., *H. floridensis*, *H. baujardi*, *H. amazonensis*, *H. mexicana* and additionally *H. taysearae* were nested. *Heterorhabditis kankabi* n. sp. was grouped as the sister species of the clade formed by *H. mexicana* + *H. taysearae* with high support values (Bt=97). The interspecific genetic distances were greater than with 28S sequences: between *H. kankabi* n. sp. with *H. mexicana* and *H. taysearae* it was 1.22 %, while with the other species of the Mexicana clade it was 0.66 to 1.66 %. The distances between the new species with the species of the other three major clades were as follows: from 9.01 to 9.43 % with the Indica clade; from 20.65 to 24.66 % with the Megidis clade; and from 21.81 to 22.03 % with the Bacteriophaga clade (Table 1).

*Association to a facultative necromenic nematode.*

As mentioned above, *G. mellonella* larvae exposed to the field collected soil samples showed the presence of two nematode species. One was described above as a new species of *Heterorhabditis*. The second was found by serendipity, when putative males of *H. kankabi* were processed to obtain DNA and its 28S gene was sequenced. Once these sequences were compared in the GenBank, it was clear that those males belonged to the species *Metarhabditis rainai* (Carta & Osbrink, 2005) Sudhaus 2011. The presence of this facultative necromenic nematode may explain the absence of amphimictic males in the larval cadavers, as *M. rainai* competed for food sources with *H. kankabi*, preventing the successful development of the necessary second amphimictic generation of *H. kankabi*.

Five sequences of the 28S gene were obtained from males of *M. rainai* (GenBank accession numbers OQ954515 to OQ954519) which were previously photographed and measured (see below). As expected, the five sequences were identical to each other which were aligned with the 28S rRNA genes from representative 23 species from different genera of Rhabditidae (Fig. 4). The estimated evolution model was the GTR+GAMMA+I, with a value of the tree of ln= −6334.028607. Sequences from our samples were nested in the same clade with other *M. rainai* specimens previously sequenced and available in the GenBank, where they were also grouped as the sister species of the clade made up of *M. blumi* + *M. amsactae* (Fig. 4). The intraspecific genetic distances in the *M. rainai* clade were null, while with respect to *M. blumi* and *M. amsactae* it was 19.33 and 18.27 %, respectively.

Identification of *M. rainai* was corroborated by the morphometrics of 10 males, which showed 812 ± 105 (650−980) µm body length, 52 ± 7 (40 − 62) µm wide and pharynx length 169 ± 19 (140 − 197) µm. Stoma length 13 ± 1 (12 – 15) µm and stoma width 2.5 ± 0.1 (1.9 – 3.2) µm. Pharynx with cylindrical corpus and large isthmus. Nerve ring is near the basal bulb. Excretory pore in the same position of basal bulb 184 ± 11 (162 − 202) µm. A single reflexed testis. Spicules paired short and separated, 39 ± 3 (32 – 47) µm length. Gubernaculum slightly curved ventrally, shape of D 21 ± 7 (15 − 30) µm. Male specimens of *M. rainai* had an open, oval-shaped peloderan bursa and eight genital papillae in the bursa. The papillae pattern was 1+2+3+2 (Fig. 5). In addition, it had a pair of very small adcloacal papillae below the spicule, just between papillae seven and eight in superficial ventral view (Fig. 6).

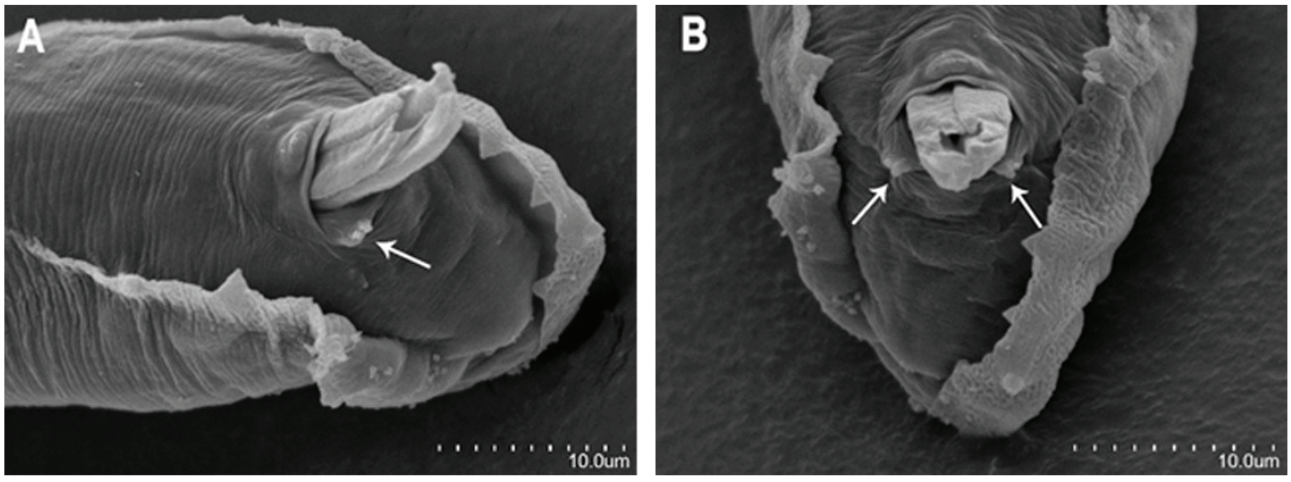


Fig. 5. *Metarhabditis rainai* SEM microphotographs of male. A, posterior region lateral view bursal papillae with small adcloacal papillae, B, ventral view showing two pairs of small papillae on posterior edge of cloaca and spicules.

In general, Body length of the holotype was larger than that of the male specimens isolated in Ticuch, but the male nematode type was similar to that of the native specimens. The spicule length of the type specimens was larger than that of the native spedimens. Type and native specimens shared eight pairs of stalked papillae in their caudal wings, but the native specimens differed by exhibiting a pair of bifurcated papillary appendages on the sides of the spicule (Fig. 6).

*Taxonomic coments of Metarhabditis rainai male:*

The male specimens of *M. rainai* found in this study had some differences with those originally described by Carta and Osbrink

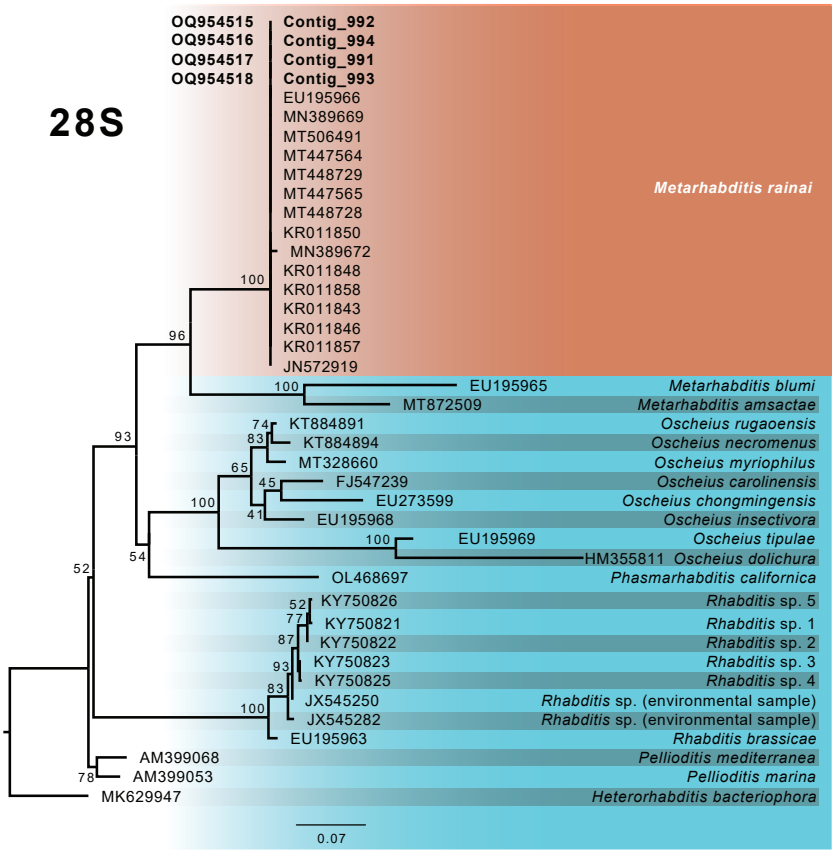


Fig. 6. Phylogenetic relationships of species of the genus *Metarhabditis* using the maximum likelihood method to generate the phylogenetic trees.

(2005). The original species described has a body length of 1001 ± 83 (860 − 1169) µm, while our specimens had a body length of i.e.,812 ± 105 (650 – 980) µm. However, the diameter of the male nematode of the original description of *M. rainai* was 60 ± 9 (48 − 76) µm, similar to that of the specimens isolated from Ticuch, i.e., 52 ± 7 (40 − 62) µm. The spicule length of the original description (49 ± 1 [46 − 52] µm) was larger than that of our specimens, i.e., 39 ± 3 (32 − 47) µm. The male specimens of the native species had caudal wings with eight pairs of stalked papillae, sharing the same papillary pattern as the original description (1−2−3−2). However, our specimens of *M. rainai* differed from those of the original description by exhibiting a pair of bifurcated papillary adcloacales on the sides of the spicule (Fig. 5). *Metarhabditis rainai* taxonomic identification was corroborated and reinforced by Five sequences analysis of the 28S gene obtained from males. The sequences length was of 850 to 854 bp. The aligned array was 954 bp long and had representatives of 23 species from different genera of Rhabditidae (Fig. 6). The estimated evolution model was the GTR+GAMMA+I. The value of the tree was ln= −6334.028607. Our specimens were nested in the same clade with other *M. rainai* specimens previously sequenced and available in the Genbank, where they were also grouped as the sister species of the clade made up of *M. blumi* + *M. amsactae* (Fig. 6). The intraspecific genetic distances in *M. rainai* were null, while with respect to *M. blumi* and *M. amsactae* it was 19.33 and 18.27 %, respectively. Unfortunately, we were unable to successfully sequence the ITS ribosomal gene for *M. rainai*.

**Discussion**

In the present study, two species of nematodes were isolated in citrus crop soil samples from Ticuch, Yucatán, Mexico. When larvae of *G. mellonella* were exposed to those samples, several stages of development were found within the same carcass, all of which belonged to a species of EPN, with the exception of the males, which belonged to a facultative necromenic nematode (FNN) species.

The EPN was described and classified as *Heterorhabditis kankabi* n. sp. and the FNN was identified as *Metarhabditis rainai*. The two species of nematodes were identified by molecular characterization of DNA sequences from the ribosomal region (28S and ITS), complementing the morphology of hermaphrodites, infective juveniles, and amphimictic females, in the case of *H. kankabi* n. sp., and the description of the male specimens in the case of *M. rainai* which was classified as an FNN, because it competed for food resources previously obtained by another species, in this case, against *H. kankabi* n. sp. Similar results of necromeny have been observed before (Campos-Herrera *et al.*, 2012; Dichusa *et al.,* 2021).

Regarding the description of the new species without male specimens, it is well known that for most groups of nematodes, males are mainly used to distinguish between species, since this stage is the one that presents qualitative morphological differences between species. This is the case of *Heterhorabditis*, where the morphological characters of the males have been the main resource to describe new species since the females or the larvae show little or no morphological difference with their congeners. In general, the differences in females and larvae are limited to differences in measurement ranges, but these ranges can generate confusion since several species may have overlapping ranges, which is why we think it is preferable to use some difference. qualitative to diagnose and differentiate the species of this group of nematodes. The male-based taxonomy may be justified because it can provide these qualitative differences like spicule shape and length, caudal wings, pairs of stalked papillae and gubernaculum shape, but it may also have its limitations, since some observable differences may be phenotypic variability, which has led to the recent synonymy of some species of the genus (Borkent, 2021). Even so, we believe that what is ideal in good taxonomic practices is to have the largest number of stages of the life cycle in order to have data comparable with other descriptions, however, this is not always possible.

In our case, our multiple failed attempts to recover the males of the new species led us to a dead end, so we had to reconsider our options to describe the new taxon. We had to return to the collection sites several times to collect soil samples and repeatedly bait the soil with insect traps (*G. mellonella*) to obtain the EPNs, almost always resulting in failure. Additionally, every time we returned to the localities, we realized with some concern that the environment is changing rapidly due to the urban expansion of human communities, in addition to the fact that it was increasingly difficult to access the places of land collection due to the interest's personnel of the owners of the crops, and although we have tried to recover ENPs from nearby places, to date we have not been able to do so. When we finally managed to obtain infections with EPNs, we were surprised that not all stages of development belonged to the same species and genus of nematode. Therefore, after considering that we have already made multiple unsuccessful efforts to recover the males and that access to crops is becoming complicated due to social issues and because it is rapidly being transformed by the urban environment, we have decided to make the description of the new species without the males.

On the one hand, we chose to use the life stages that we confirmed belong to the new species. The description of the new species based on females and larvae has not been easy, since as we mentioned above, there is not much morphological difference between one species and another in these stages, however, we have managed to rescue that the new species has a vulva slightly protruding and globose. Regarding the rest of the characters and morphometric measurements, it is difficult to find accurate differences, since there are no papillae or ornamentations that characterize the new species or ranges of measurements that do not overlap with other species. Therefore, practically these stages are not diagnosable.

On the other hand, we also resort to the use of DNA sequences to

make the diagnosis of the new species. The use of DNA sequences for the description of species is not new since they are generally used to confirm the differences between species, reinforcing them with the use of genetic distances and with phylogenetic analyses (Nguyen *et al*., 2004; 2006) but rarely have been used explicitly in the diagnosis of the species described. However, although the use of these sequences as part of the diagnosis of species is not a new approach (Hibbett *et al*., 2011; Renner, 2016; Fernandez-Triana, 2022), a stir has recently been generated because Sharkey *et al*. (2021) chose to describe many new species based almost exclusively on DNA barcodes, which are used explicitly as the diagnosis of such species. This type of approach is known as "Turbo taxonomy", which can be briefly defined as the rapid description of many species in "fast" documents (Butcher *et al*., 2012; Fernandez-Triana, 2022). Although the approach of Sharkey *et al*. (2021) has already been extensively discussed in several articles (e.g. Fernandez-Triana, 2022), where there are both positive and negative comments about this way of doing taxonomy, we particularly think that it is a good approximation, when the description of certain groups of species is difficult, for example: when there is a very diverse group and a large number of species have to be described in a short time; when no significant differences in morphology are found (in the case of cryptic species); when there is large phenotypic variation; or as in our case, where not all the stages are available to make an adequate morphological diagnosis.

Regardless, to describe the new *Heterorhabditis* species, we are not performing turbo taxonomy, as we do not intend to describe a large number of species in this article, and we also use morphological characteristics in the description of the new species. What we do rescue from the study by Sharkey *et al.* (2021) is the explicit use of sequences in diagnoses. In the present work, in the absence of males to find robust differences, we have included the sequences of the ribosomal region of genomic DNA as part of the diagnosis of the new species, additionally to the morphometrics of hermaphroditic and amphimictic females as well as that of infective juveniles. Unlike Sharkey *et al.* (2021) approach, instead of using the cytochrome oxidase subunit I, which is the preferred "DNA Barcoding" gene for animal species, we used two molecular ribosomal markers (ITS and 28S) which have been widely used in this group of nematodes, as they have proven to be very effective to differentiate species (Subkrasae *et al.,* 2022). Besides, the sequence analyses also added information from the phylogenetic analyses and the genetic distances between the species of the genus *Heterorhabditis*.

In fact, the phylogenetic affinities found with *H. kankabi* n. sp. indicate its association within a clade where most of the species have been described in the Americas. Still, the species in this clade can be clearly discriminated in terms of genetic differentiation of their 28S and ITS sequences, showing more difference with the later one. The difference between regions can be attributed to the fact that the 28S gene has a slower nucleotide substitution rate as compared to the ITS region (Sharkey *et al.,* 2021). Still, both markers presented differences that are reliable for discriminating between closely related species because the DNA is not directly affected by intraspecific phenotypic variation or the environment. Therefore, DNA sequences were useful in the diagnosis of the new species because they represented more reliable evidence to discriminate it when compared with all other described species in the genus.

In regard with the association of *H. kankabi* n. sp. with *M. rainai*, first of all, the later was positively identified by sequence comparison of the 28S gene with others species of *Metarhabditis*. However, some morphometric features comparison of males showed some few differences which may not be significant to suspect a misidentification. However, a possible explanation for the lack of males of *H. kankabi* n. sp. is that the mixed infection with *M. rainai* in *G. mellonella* larvae, caused strong intraguild competition which in turn affected the development of males of the new species. Perhaps, in other circumstances of high concentration of food, a generation of males and females of the EPN could be expected (Strauch & Ehlers, 1998).

Conversely, no other stages but males of *M. rainai* were found in the samples used for morphological and molecular identification. Certainly, a quantitative experiment would be necessary to perform to detect the numbers of the nematodes in both groups, as it is unknown to what extent *M. rainai* specimens displaced the progeny of *H. kankabi* n. sp., given that only *M. rainai* males were found when dissecting the corpse of infected *G. mellonella* larvae. Campos-Herrera *et al.* (2012) described a set of probes that allowed the quantification of FNN in combination with EPN. The authors performed mixed infections of *Acrobeloides maximum* and *M. rainai* with *Steinernema diaprepesi*, *Steinernema riobrave* and *Heterorhabditis* sp. on larvae of the weevil *Diaprepes abbreviatus*. Among the results, it was found that both strains of *A. maximum* reproduced 25.64 % more than *M. rainai* inside the carcasses. In contrast, the reproduction of *S. riobrave* decreased when combined with *A. maximum*. Similarly, Duncan *et al.* (2003) determined the extent to which FNN managed to displace EPN within the hosts. These authors reported alterations in the reproduction of *S. riobrave* when combined with *Pellioditis* sp., as a decrease of 84 % in infective juveniles that emerged from the carcasses of *D. abbreviatus* was observed.

In the case of *Heterorhabditis*, the infective juveniles that manage to enter the host becomes adult hermaphrodites, who keep the eggs that hatch inside the adult uterus and give rise to the first generation of juveniles. These juveniles develop and feed on the hermaphrodite adult until they deplete the tissues (*endotokia matricida*) (Luc *et al.,* 1979). After the hermaphrodites died, the infective juveniles emerge. These infective juveniles become an amphimictic generation of males and females, unless they are interrupted in the infective juvenile stage due to low food concentration. Interestingly, when infective juveniles of *Heterorhabditis* spp. were inoculated in liquid culture media with *Photorhabdus luminescens*, and compared with 24 h-fasted infective juveniles, in the first treat-

ment, nematodes developed into 30 % amphimictic females, 38 % males, and 32 % hermaphrodites, while the second tratmenet developed into 53.3 % infective juveniles, 40 % hermaphrodites, and only 6.6 % amphimictic adults (Strauch *et al.,* 1994). These results indicate that the outcome of a second amphimictic generation depends on the availability of food.

All these findings led us to wonder whether all EPN species could be susceptible to mixed infection with FNN, given that the strategy they use—i.e., pausing their development to search for other hosts—may influence the persistence and efficacy of the EPN used in the biological control of insect pests.

## Conclusions

DNA sequencing was useful to diagnose and detect a new *Heterorhabditis* species, *H. kankabi* n. sp., especially under the absence of males, which limited the use of morphological characters to the remaining three developmental stages.

The absence of *H. kankabi* males may be explained by the intraguild competition between *H. kankabi* n. sp. and *M. rainai*. In this work we classified *M. rainai* as a facultative necromenic nematode for competing for food sources of a carcass parasitized by an EPN.

## Conflict of Interests

The authors declare that they have no competing interests.

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## References

Abràmoff, M.D., Magalhães, P.J., Ram, S.J. (2004): Image processing with ImageJ. *Biophotonics Int.*, 11(7): 36 – 42

Ávila-López, M.B., García-Maldonado, J.Q., Estrada-Medina, H., Hernández-Mena, D.I., Cerqueda-García, D., Vidal-Martínez, V.M. (2021): First record of entomopathogenic nematodes from Yucatán State, México and their infectivity capacity against *Aedes aegypti*. *PeerJ*. DOI:10.7717/peerj.11633

Bedding, R.A., Akhurst, R.J. (1975): A simple technique for the detection of insect parasitic rhabditidae nematodes in soil. *Nematologica,* 21: 109 – 110. DOI: 10.1163/187529275X00419

Borkent, A. (2021): Diagnosing diagnoses - can we improve our taxonomy? *Zookeys.* 1071: 43 – 48. DOI: 10.3897/zookeys.1071.72904.

Carta, L.K., Osbrink, W. (2005): *Rhabditis rainai* n. sp. (Nematoda: Rhabditida) associated with the Formosan subterranean termite, *Coptotermes formosanus* (Isoptera: Rhinotermitidae). *Nematology,* 7: 863 – 879. DOI: 10.1163/156854105776186299.

Campos–Herrera, R., El–Borai, F.E., Duncan, L.W. (2012): Wide interguild relationships among entomopathogenic and free–living nematodes in soil as measured by real time qPCR. *J Invertebr Pathol,* 111: 126 – 135. DOI: 10.1016/j.jip.2012.07.006

Dhakal, M., Nguyen, K.B., Hunt, D.J., Ehlers, R.U., Spiridonov, S.E., Subbotin, S.A. (2020): Molecular identification, phylogeny and phylogeography of the entomopathogenic nematodes of the genus *Heterorhabditis* Poinar, 1976: a multigene approach. *Nematology,* 23(4): 451 – 466. DOI: 10.1163/15685411-bja10052

Dichusa, C.A., Ramos, R., Aryal, S., Sumaya, N.P., Sumaya, N.H. (2021): Survey and identification of entomopathogenic nematodes in the province of Cotabato, Philippines, for biocontrol potential against the tobacco cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). *Egypt. J. Biol. Pest Control,* 31: 60. DOI: 10.1186/s41938-021-00390-w

Duncan, L.W., Dunn, D.C., Bague, G., Nguyen, K. (2003): Competition between entomopathogenic and free-living bactivorous nematodes in larvae of the weevil *Diaprepes abbreviatus*. *J. Nematol,* 35: 187 – 193

Fernandez-Triana, J.L. (2022): Turbo taxonomy approaches: lessons from the past and recommendations for the future based on the experience with Braconidae (Hymenoptera) parasitoid wasps. *Zookeys*, 1087: 199 – 220. DOI: 10.3897/zookeys.1087.76720

Gaugler, R. (2002): *Entomopathogenic nematology*. Rutgers University, New Brunswick, New Jersey, USA. 388 pp.

Goodrich-Blair, H., Clarke, D.J. (2007): Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. *Mol Microbiol,* 64: 260 – 268. DOI: 10.1111/j.1365-2958.2007.05671.x

Hominick, W.M., Briscoe, B.R., Del Pino, F.G., Heng, J., Hunt, D.J., Kozodoy, E., Mracek, Z., Nguyen, K.B., Reid, A.P., Spiridonov, S., Stock, P., Sturhan, D., Waturu, C., Yoshida, M. (1997): Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. *J Helminthol,* 71: 271 – 298. DOI: 10.1017/s0022149x00016096

Karimi, J., Kharazi-Pakdel, A., Yoshiga, T., Koohi-Habibi, M., Hassani-Kakhki M. (2011): Characterization of *Xenorhabdus* (γ-Proteobacteria) strains associated bacteria with the *Steinernema* (Nematoda: Steinernematidae) isolates from Iran. *J. Entomol. Soc. Iran,* 31(1): 57 – 69

Kary, N.E., Niknam, G., Griffin, C.T., Mohammadi, S.A., Moghaddam, M. (2009): A survey of entomopathogenic nematodes of the fam-

ilies Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in the north-west of Iran. *Nematology*, 11: 107 – 116

Kaya, H.K., Gaugler, R. (1993): Entomopathogenic nematodes. *Annu. Rev. Entomol,* 38: 181 – 206

Kaya, H.K., Stock, S.P. (1997): Techniques in insect nematology. In *Manual of techniques in insect pathology*. Academic Press, pp. 281 – 324. DOI: 10.1016/B978-012432555-5/50016-6

Malan, A.P., Ferreira, T. (2017). Entomopathogenic Nematodes. In: Fourie, H., Spaull, V., Jones, R., Daneel, M., De Waele, D. (Eds) *Nematology in South Africa: A View from the 21st Century*. Springer, Cham, pp. 459–480. DOI: 10.1007/978-3-319-44210-5_22

Nguyen, K.B., Smart, Jr. G.C. (1996): Identification of entomopathogenic nematodes in the *Steinernematidae* and *Heterorhabditidae* (Nematoda: Rhabditida). *J Nematol*, 28(3): 286 – 300

Nguyen, N.C., Shapiro, I., Stuart, D.L., Mccoy, R.J., James, R.R., Adams, B.J. (2004): *Heterorhabditis mexicana* n. sp. (Rhabditida: Heterorhabditidae) from Tamaulipas, Mexico and morphological studies of the bursa of *Heterorhabditis* spp. *Nematology*, 6: 231 – 244. DOI: 10.1163/1568541041218031

Nguyen, K.B., Hunt, D.J. (2016): Heterorhabditidae: species descriptions. In *Advances in entomopathogenic nematode taxonomy and phylogeny*. Leiden, The Netherlands: Brill. DOI:10.1163/9789004285347_006

Nguyen, K.B., Gozel, U., Koppenhöfer, H., Adams, B. (2006): *Heterorhabditis floridensis* n. sp (Rhabditida: Heterorhabditidae) from Florida. *Zootaxa*. DOI: 10.11646/zootaxa.1177.1.1

Othman, N.S., Al-Hakeem, A.M., Al-Taee, H.H. (2022): Entomopathogenic Nematodes: A review. *Texas J. Agric. Biol. Sci*, 6: 49 – 54

Örley, L. (1880): Az Anguillulidák magánrajza. (Monographie der Anguilluliden). *Természetraji Füz*., 4: 16 – 150 (In Hungarian)

Poinar, G.O. Jr. (1976): Description and biology of a new insect parasitic rhabditoid, *Heterorhabditis bacteriophora* n. gen., n. sp. (Rhabditida: Heterorhabditidae n. fam.). *Nematologica*, 21(4): 463 – 470

Poinar, G.O. Jr. (1990): Taxonomy and biology of Steinernematidae and Heterorhabitidae. In: Gaugler, R., Kaya, H.K. (Eds) *Entomopathogenic Nematodes in Biological Control*. CRC Press. Boca Raton, Fla. p. 23 – 61

Rakubu, I.L., Katumanyane, A., Hurley, B.P. (2024): Screening five local entomopathogenic nematode species for their virulence against pupae of the Eucalyptus snout beetle*, Gonipterus* sp. n. 2, under laboratory conditions. *Crop Protect*, 176: 106500. DOI: 10.1016/j.cropro.2023.106500

Shapiro-Ilan, D.I., Hiltpold, I., Lewis, E.E. (2018): Nematodes. In: Hajek, A.E., Shapiro-Ilan, D.I. (Eds) *Ecology of Invertebrate Diseases*. Wiley, Oxford, p. 415 – 468

Sharkey, M.J., Janzen, D.H., Hallwachs, W., Chapman, E.G., Smith, M.A., Dapkey, T., Brown, A., Ratnasingham, S., Naik, S., Manjunath, R., et al. (2021): Minimalist revision and description of 403 new species in 11 subfamilies of Costa Rican braconid parasitoid wasps, including host records for 219 species. *ZooKeys,* 1013: 1 – 665. DOI: 10.3897/zookeys.1013.55600.

Stock, S.P. (2015): Diversity, biology and evolutionary relationships. In: Campos-Herrera, R. (Ed) *Nematode Pathogenesis of Insects and Other Pests. Series: Sustainability in Plant and Crop Protection (Ciancio, A., Series Ed)*. Springer International Publishing Switzerland, p. 3 – 27. DOI: 10.1007/978-3-319-18266-7_1

Strauch, O., Stoessel, S., Ehlers, R.U. (1994): Culture conditions define automictic or amphimictic reproduction in entomopathogenic rhabditid nematodes of the genus *Heterorhabditis*. *Fundam Appl Nematol*, 17(6): 575 – 582

Strauch, O., Ehlers, R.U. (1998): Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. *Appl Microbiol Biotechnol*, 50: 369 – 374. DOI: 10.1007/s002530051306

Subkrasae, C., Ardpairin, J., Dumidae, A., Janthu, P., Meesil, W., Muangpat, P., Tandhavanant, S., Thanwisai, A., Vitta, A. (2022): Molecular identification and phylogeny of *Steinernema* and *Heterorhabditis* nematodes and their efficacy in controlling the larvae of *Aedes aegypti*, a major vector of the dengue virus. *Acta Trop*, 228: 106318. DOI: 10.1016/j.actatropica.2022.106318

Sudhaus, W. (2011): Phylogenetic systematisation and catalogue of paraphyletic "Rhabditidae" (Secernentea, Nematoda). *J. Nematode Morphol. Syst*, 14: 113 – 178

Tahseen, Q., Hussain, A., Tomar, V., Shah, A.A., Jairajpuri, M.S. (2004): Description of *Metarhabditis andrassyana* gen. n., sp. n. (Nematoda: Rhabditidae) from India. *Int J Nematol*, 14: 163 – 168

Thanwisai, A., Muangpat, P., Meesil, W., Janthu, P., Dumidae, A., Subkrasae, C., Ardpairin, J., Tandhavanant, S., Yoshino, T.P., Vitta, A. (2022): Entomopathogenic Nematodes and Their Symbiotic Bacteria from the National Parks of Thailand and Larvicidal Property of Symbiotic Bacteria against *Aedes aegypti* and *Culex quinquefasciatus*. *Biology*, 11: 1658. DOI: 10.3390/biology11111658.

Trejo-Meléndez, V.J., Ibarra-Rendón, J., Contreras-Garduño, J. (2024): The evolution of entomopathogeny in nematodes. *Ecol Evol*, DOI: 10.1002/ece3.10966.

Vázquez-Montoya, E.L. (2014): *Characterization of entomopathogenic nematodes isolated from Guasave valley, Sinaloa, México*. Master's Thesis. Instituto Politécnico Nacional, Unidad Sinaloa, México, pp 103. (In Spanish)

White, G.F. (1927): A method for obtaining infective nematode larvae from cultures. *Science,* 66(1709): 302 – 303. DOI: 10.1126/science.66.1709.302-a