Original Article

First report and biological characteristics of *Heterorhabditis amazonensis* in the state of Paraná, Brazil

Primeiro registro e caracterização biológica de *Heterorhabditis amazonensis* no estado do Paraná, Brasil

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Abstract

Entomopathogenic nematodes (EPNs) from Heterorhabditidae and Steinernematidae families are extensively used to control insect pests. In Brazil, however, relatively few studies have identified and characterized these entomopathogens. The objective of this study was to identify and characterize an EPN isolate obtained from soil samples collected in the state of Paraná, Brazil. An isolate (UEL 08) of *Heterorhabditis* was detected in a soil sample collected from a pasture area cultivated with Brachiaria grass in Londrina, state of Paraná, Brazil (23°34′311″S, 050°58′298″W), using the insect-baiting technique with *Galleria mellonella* larvae as hosts. The nematode was identified through morphometric studies and molecular analyses based on amplification of the rDNA ITS region. Although we identified certain morphometric differences compared with the original description, the molecular data indicated that the ITS sequence obtained for the UEL 08 isolate is identical to the reference sequence of *H. amazonensis* (DQ665222) and presented 100% similarity. Thus, the findings of our morphological and molecular studies confirmed that the isolated nematode is *H. amazonensis*, which is the first time this species has been registered in Paraná. Study of the biological characteristics of *H. amazonensis* (UEL 08) revealed that the isolate has two distinct life cycles – one short (216 h) and the other long (288 h) – and produces two generations in both cycles. We observed that *H. amazonensis* (UEL 8) was pathogenic and virulent to the three evaluated hosts, although with different virulence against these hosts. The larvae of *G. mellonella* and *Alphitobius diaperinus* were more susceptible than adult *Dichelops* (*Diacereus*) *melacanthus*, with 100%, 85%, and 46% mortality, respectively. Furthermore, an *in vivo* production assay revealed a mean daily yield of 3.4×10^3 infective juveniles/g host larvae.

Keywords: entomopathogen, morphometry, taxonomy, molecular analysis, biological control.

Resumo

Nematoides entomopatogênicos (NEP) das famílias Heterorhabditidae e Steinernematidae são amplamente utilizados no controle de insetos-pragas. No Brasil, os estudos relacionados a caracterização e identificação destes entomopatógenos são recentes e escassos. Nesse sentido, o objetivo deste estudo foi isolar NEP de amostras de solos coletadas em diferentes áreas no estado do Paraná, Brasil. Um isolado *Heterorhabditis* (UEL 08) detectado em amostra de solo em área de pastagem cultivada com braquiária, localizada em Londrina, Paraná, Brasil (23°34′311″S, 050°58′298″W), utilizando o método de "inseto-isca" com lagartas de *Galleria mellonella*. Para a identificação foram realizados estudos de morfometria e identificação molecular a partir da amplificação da região ITS. Algumas diferenças foram encontradas em termos de morfometria em comparação com a descrição original, entretanto, os dados moleculares demonstraram que a sequência obtida para o isolado UEL 08 é idêntica à sequência de referência de *H. amazonensis* (DQ665222), com a qual apresentou 100% de similaridade. Os estudos das características biológicas de *H. amazonensis* (UEL 08) revelaram que o isolado tem dois ciclos de vida distintos, um curto (216 h) e outro longo (288 h), sendo que ocorrem duas gerações em ambos os ciclos. O isolado UEL 08 *H. amazonensis* foi patogénico e virulento sobre os três hospedeiros avaliados. Notadamente, as larvas de *G. mellonella* e *Alphitobius diaperinus* foram consideradas mais susceptíveis do que os adultos do percevejo *Dichelops (Diacereus) melacanthus*, com percentagens de mortalidade de 100%, 85% e 46% de mortalidade, respectivamente. O ensaio de produção *in vivo* revelou um rendimento médio diário de $3,4 \times 10^3$ juvenis infectantes/g de larva hospedeira.

Palavras-chave: entomopatógeno, morfometria, taxonomia, análise molecular, controle biológico.

Introduction

Entomopathogenic nematodes (EPNs) are among the most numerous organisms on the planet and are distributed in a diverse range of different soil types, geographical areas, and environments (Lewis et al., 2006). EPNs of Heterorhabditidae and Steinernematidae families infect and kill insects in association with symbiotic bacteria in the genera *Photorhabdus* (for *Heterorhabditis*) and *Xenorhabdus* (for *Steinernema*) and have excellent potential as biological control agents (Grewal et al., 2001).

The nematode genus *Heterorhabditis* includes 20 known species (Shapiro-Ilan et al., 2017), and in Brazil, the 27 *Heterorhabditis* isolates reported to date belong to the species *H. amazonensis*, *H. indica*, *H. baujardi*, and *H. bacteriophora* (Dolinski et al., 2008; Dolinski et al., 2017).

Heterorhabditis amazonensis was first isolated using the insect-trap or insect-bait method (Bedding and Akhurst, 1975) from soil samples collected close to the city of Benjamin Constant in the northern region of the state of Amazonas, Brazil (Andaló et al., 2006). Other studies which EPNs have isolated in Brazil have indicated the presence of *H. amazonensis* in other regions (Andaló et al., 2009a).

The discovery of new species or populations of EPN may expand or enhance the use of these organisms in biological control programs. Moreover, the variability detected among populations of native nematode species facilitates better adaptation to climatic conditions and more effective control of insect populations (Dolinski and Moino-Junior, 2006; Andaló et al., 2009a).

Thus, the objective of this study was to isolate EPNs from soil samples collected from different areas in Londrina, Paraná, Brazil, and characterize these isolates based on morphological, molecular, and biological analyses.

Materials and Methods

Collecting and processing of soil samples

Sixty soil samples were collected in different areas in several municipalities in the state of Paraná (Figure 1). To isolate nematodes, we used insect bait according to the methodology described by Bedding and Akhurst (1975).

Dead larvae with symptoms of infection were washed with Ringer's solution, transferred to a dry chamber (9-cm diameter Petri dish containing a filter paper), and maintained in a climatic chamber at 25 ± 1 °C, without photoperiod control for 5 days. Thereafter, the larval cadavers were placed in a White trap (White, 1927) for emergence and collection of infective nematode juveniles (IJs).

Morphological and morphometric studies

For morphological studies, the nematodes were multiplied *in vivo* in *G. mellonella* larvae. The first-generation females (hermaphrodites) were obtained by dissecting the infected larvae 5–6 days after the larvae died. Second-generation males and females (amphimictic) were obtained after 7–8 days. The IJs were collected after emergence from the insect cadavers.

Light microscopy analysis

For light microscopy analysis, 25 specimens from different nematode stages (hermaphrodites, females, males, and IJs) were observed. The IJs were observed alive. Hermaphrodites as well as second-generation males and females were killed and fixed in TAF (7 mL of formalin, 2 mL of triethanolamine, and 91 mL of distilled water) (Courtney et al., 1955). In addition, hermaphrodites and second-generation females were fixed in lactophenol (Franklin and Goodey, 1949) so that morphological

Figure 1. Map of Paraná state and soil collection locations.

structures such as the esophagus, nerve ring, and excretory pore were easier to observe.

The fixed specimens were mounted on coverslip holders to avoid flattening the nematodes. The slides and nematodes were observed using a Motic BA 310 light microscope (with ×4, ×10, and ×40 objectives). Observations were compared with those of the original description (Andaló et al., 2006) and *H. amazonensis* populations described in two further studies (Andaló et al., 2009a; Morales et al., 2016).

Molecular identification

For molecular analysis of the UEL 08 isolate, DNA was extracted from IJs using a NucleoSpin® Tissue kit (Macherey-Nagel), following the manufacturer's protocol. A fragment of the rDNA containing the ITS1, 5.8S, and ITS2 regions was amplified via PCR using the primers 18S: 5′-TTGATTACGTCCCTGCCCTTT-3′ (forward) and 26S: 5′ TTTCACTCGCCGTTACTAAGG-3′ (reverse) (Vrain et al., 1992).

The PCR reaction mixtures were prepared in a final volume of 25 µL containing 1× PCR Master Mix (Promega), 0.5 µM of each primer, and approximately 10 ng of template DNA. Amplifications were carried out in an MJ Research PTC-100 thermocycler, with an initial denaturation step of 5 min at 94 °C, followed by 35 cycles at 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. After purifying the PCR products with 0.5 µL of the enzyme provided with an Illustra Exo-Star 1-Step PCR Clean Up Kit (Thermo Fisher Scientific, Waltham, MA, USA), the DNA fragments were sequenced bidirectionally using a BigDye™ Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Carlsbad, CA, USA), following the manufacturer's specifications. The base sequence was determined using an ABI-PRISM 3500 XL automatic sequencer (Applied Biosystems) and deposited in the GenBank database under accession number MK262740.

The sequence obtained for the UEL 08 isolate was edited and aligned with sequences of the same segment from other *Heterorhabditis* isolates in the GenBank database (the accession numbers of which are shown in Figure 2) using MEGA 5.0 software (Tamura et al., 2011). The same software was also used to calculate pairwise distances and conduct the following phylogenetic analyses. The phylogenetic signal of the data was analyzed using the maximum parsimony method, based on MP trees inferred from 1000 repetitions, with a subtree pruning and regrafting (SPR) algorithm at research level 1, in which the initial trees were obtained by adding sequences at random (10 replicates). Phylogenetic relationships were examined using the maximum likelihood (ML) method. The ML tree was inferred from an initial neighbor-joining tree, including a heuristic search of the tree space at each tree bisection-reconnection (TBR) branch exchange. All analyses were conducted with 1000 bootstrap repetitions.

Life cycle

The life cycle of the UEL 08 isolate was examined using the methodology adapted from Andaló et al. (2009b). Nematodes were multiplied in *G. mellonella* larvae, and for inoculation, we used 400 IJs/larvae (short cycle) or 10 IJs/larvae (long cycle). Each treatment consisted of 20 repetitions, with a Petri dish containing 10 larvae.
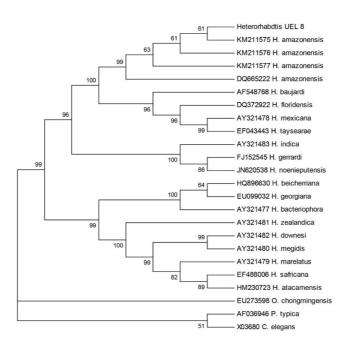

Figure 2. Phylogenetic relationships of *Heterorhabditis amazonensis* (UEL 08) infective juveniles based on analysis of the ITS regions of the rDNA gene inferred by maximum parsimony analysis. *Oscheius chongmingensis, Pellioditis typica,* and *Caenorhabditis elegans* were used as an outgroup.

The plates were incubated 24 ± 1 °C for 48 h without photoperiod control. The nematode development was evaluated at 24-h intervals by dissecting 10 larvae from each treatment under a stereoscopic microscope. Development was observed until the nematodes had completed their life cycle and all available nutrients in the larvae had been exhausted.

Pathogenicity and virulence test

The pathogenicity and virulence of *H. amazonensis* (UEL 08) was evaluated in the final-instar larvae of *G. mellonella*, larvae of the lesser mealworm *Alphitobius diaperinus* (Panzer 1797) (Coleoptera: Tenebrionidae), and adults of the green belly stink bug *Dichelops* (*Diceraeus)* *melacanthus* (Dallas, 1851) (Hemiptera: Pentatomidae).

In the experiments using *G. mellonella* and *D. melacanthus*, five repetitions were performed, each of which consisted of a glass Petri dish (9 cm diameter) containing two filter papers and 10 moth larvae (final instar) or adult stink bugs. Nematodes were then applied at a concentration of 100 IJs/cm². In the control treatment, 2 mL of distilled water was applied instead of nematodes.

For the larvae of lesser mealworm, the experiment consisted of four repetitions with twelve insects, and the insects were individualized in 12-well tissue culture plate because the larvae have a cannibalistic habit. The EPN isolate was applied at a concentration of 100 IJs/cm².

Each of the three insects were incubated at 25 ± 1°C without photoperiod control. The mortality was checked after 5 days. Dead insects with symptoms of infection (dark brown coloring) were dissected to confirm death attributable to the nematode. The experimental design was entirely randomized, and data were assessed for normality (Shapiro–Wilk test) and homoscedasticity (Hartley test). Means were compared with Tukey's test (p ≤ 0.05) using the statistical program SISVAR (Ferreira, 2011).

In vivo *production of* H. amazonensis *(UEL 08) in* G. mellonella *larvae*

To examine *in vivo* production, we used the methodology of Guide et al. (2016). The variables evaluated were daily production (total number of IJs emerged in 1 day), accumulated production (total number of IJs that emerged until the exhaustion of larval reserves) (Molina et al., 2004), average total production per larvae, and average production per gram of larvae. The experimental design was entirely randomized, and the daily production data were subjected to regression analysis using the statistical program SISVAR (Ferreira, 2011) to determine the production over time.

Results and Discussion

Sampling and processing of soil samples

Of the 60 samples collected, only one sample was found to contain nematodes. This sample was collected in an area of pasture cultivated with Brachiaria grass in Londrina, Paraná (23°34′311″S, 050°58′298″W). The *G. mellonella* larvae killed by the isolate showed symptoms characteristic of infection by nematodes of the genus *Heterorhabditis*, exhibiting a dark red color. Insects parasitized by heterorhabditids develop this coloration due to the presence of the symbiotic bacterium *Photorhabdus* sp. (Poinar, 1990). The isolate obtained in the present study was designated *Heterorhabditis* sp. (UEL 08).

Morphometric characterization

Heterorhabditis sp. (UEL 08) was characterized by a combination of morphological and morphometric characters exhibited by 25 specimens, including males, females, and IJs (Table 1).

Comparison of the morphological characteristics of the UEL 08 isolate with those of previously described *H. amazonensis* populations revealed differences, including those relating to total body length, widest body diameter, tail length, and body diameter in the anal region for males (Table 2), and esophageal length for infective juveniles (Table 3). In contrast, the distance from the anterior end to the excretory pore, tail length, and E% (distance from the anterior end to the excretory pore/tail length) were found to be like those of IJ specimens of the Type population of *H. amazonensis* (Table 3).

These intraspecific differences could be attributed to the geographical origin of the nematode under study, different environmental conditions, or interactions with the host (Stock et al., 2000). This is consistent with the findings of Morales et al. (2016), who observed differences between Venezuelan populations of *H. amazonensis*, and Poinar (1993) and Stock et al. (1996), who reported intraspecific differences among species of Steinernematidae. Achinelly et al. (2017), evaluating the morphometric characteristics of *Heterorhabditis bacteriophora* VELI and *H. argentinensis* isolated from different regions of Argentina, observed that the isolates obtained in regions with more rainfall and humidity had larger dimensions. According to these authors, the juvenile phase (JIs) has the least variability and is the best option for the morphological comparison of different *Heterorhabditis* populations.

Molecular identification and phylogenetic analysis

Molecular analysis of a stretch of the ribosomal gene containing the ITS1, 5.8S, and ITS2 regions revealed a sequence of 962 base pairs (bp), including 214 polymorphic sites (292 singletons). Amplification and sequencing of part of the rDNA gene (ITS1, 5.8S, 1ITS2) from isolate UEL 08 revealed that the sequence is identical to the reference sequence of *H. amazonensis* (DQ665222) and had 100% of similarity (Table 4). In the phylogenetic analysis, the topology represented by a Maximum Parsimony tree revealed a well-supported (100% bootstrap) monophyletic group, formed by *H. amazonensis* UEL 08 together with the other Brazilian and Venezuelan isolates of *H. amazonensis* and the *Heterorhabditis* species *H. baujardi, H. floridensis, H. mexicana,* and *H. taysearae* (Figure 2).

Sequencing of the ITS gene decisively identified the UEL 08 isolate as belonging to the species *H. amazonensis*. Accordingly, further studies examining previously identified populations of *H. amazonensis* in Brazil would be interesting to verify several inferences relating to the distribution of this species in Brazil and the relationships between these populations. Furthermore, the sequences of other

Table 1. Morphometry of *Heterorhabditis amazonensis* UEL 08. Measurements are in μm and data are presented as the means ± standard deviation (variation) (N = 25).

Character	Males	Hermaphrodites (1st generation)	Females (2nd generation)	Infective Juveniles
N	25	25	25	25
L	890 ± 33.3 (825–936)	3,933 ± 408.4 (3,384–4,807)	1,988 ± 132.0 (1,794–2,285)	554 ± 35.5 (508–634)
A				23 ± 3.0 (19–28)
B				4.3 ± 0.4 (3.6–5.6)
C				5.1 ± 0,3 (4.5–5.8)
V		44 ± 3.3 (37–53)	48 ± 2.9 (43–55)	
W	66 ± 6.6 (63–79)	247 ± 26.4 (206–307)	134.8 ± 12.7 (112–158)	24 ± 3.7 (20–32)
Stoma length		11.2 ± 2.0 (8–16)	11.8 ± 1.3 (8–14)	
Stoma diam.		11.7 ± 2.0 (8–16)	11.8 ± 1.1 (8–14)	
EP	91 ± 9.0 (68–104)	144 ± 15.3 (104–164)	116 ± 12.5 (92–140)	106 ± 10.2 (92–128)
NR	67 ± 10.1 (44–84)	107 ± 14.1 (84–136)	89 ± 9.0 (68–104)	83 ± 9.5 (72–104)
ES	112 ± 3.9 (108–120)	179 ± 17.3 (160–216)	143 ± 10.6 (124–160)	130 ± 10.8 (100 ± 144)
Testis reflexion	67 ± 13.2 (44–88)			
Tail length with sheath (T)	62 ± 10.8 (36–80)	116 ± 19.7 (88–164)	87 ± 12.2 (72–116)	108 ± 8.4 (96–128)
Tail length without sheath				75 ± 7.9 (60–92)
Anal body diam. (ABD)	39 ± 3.9 (32–48)	57 ± 15.8 (40–96)	34 ± 4.0 (28–48)	16 ± 3.5 (12–24)
Spicule length (SP)	43 ± 4.9 (36–52)			
Gubernaculum length (GU)	22 ± 3.1 (16–28)			
D% = EP/ES × 100	81 ± 8.1 (61–96)			82 ± 5.7 (71–92)
E% = EP/T × 100				99 ± 11.1 (86–129)
SW% = SP/ABD × 100	110 ± 17.0 (82–144)			
GS% = GU/SP × 100	52 ± 8.2 (42–67)			
Hyaline/tail × 100				69.8 ± 4.6 (63–77)

L = total body length; NR = distance from the anterior end to the nerve ring; W = Maximum body diameter; EP = distance from the anterior end to the excretory pore; ES = length of the esophagus; V = distance from the vulva to the anterior extremity; A = L/W; B = L/ES; C = L/T.

genes could be examined to assess the genetic variability between *H. amazonensis* populations.

In conclusion, the combination of molecular and morphological data confirmed that the nematode *Heterorhabditis* UEL 08 obtained from Londrina, PR, Brazil, is an isolate of the species *H. amazonensis*, and this is the first time that this species has been registered in Paraná.

A work by Foelkel et al. (2017) to isolate and identify NEPs in the same state (Paraná) found only nematodes of the genus *Oscheius* in an apple orchard of Porto Amazonas County.

Life cycle

The life cycle of *H. amazonensis* (UEL 08) includes three phases of development: egg, juvenile (differentiated into four stages – J1, J2, J3, and J4), and adult. In the adult phase, the first generation is composed of hermaphrodite females and the second generation by males and amphibiotic females, similar to that observed for other *Heterorhabditis* species (Adams and Nguyen, 2002; Molina et al., 2005) and for *H. amazonensis* RSC 05 (Andaló et al., 2009b).

During the initial 96 h of the life cycle, we observed that the juvenile stages of the long and short cycles were similar. Thereafter, long cycle individuals had delayed development compared with those of the short cycle, which was also observed by Molina et al. (2005) and Andaló et al. (2009b). In both cycles, it was still possible to distinguish hermaphrodite females (first generation) and second-generation males and females (Table 5).

The total durations of the short and long cycles were 216 h (9 days) and 288 h (12 days) (Table 5), respectively. In both cycles, the production of two generations was observed, which differs from the findings of Andaló et al. (2009b), who, also using final-instar larvae of *G. mellonella*,

Table 2. Comparative morphometry (μm) of the male *Heterorhabditis amazonensis* isolated in Brazil (Andaló et al., 2006, 2009b and Venezuela (Morales et al., 2016).

Character	Andaló et al. (2006)	Andaló et al. (2009b)		Morales et al. (2016)			Present Study
		GL population	SG population	LPV081	LPV156	LPV498	UEL 08
	Type population (Brazil)	(Brazil)	(Brazil)	(Venezuela)	(Venezuela)	(Venezuela)	(Brazil)
L	752 ± 43 (692–826)	739.2 ± 23.4 (694–790)	820.4 ± 32.7 (771–879)	798 ± 128 (719–905)	788 ± 144 (687–873)	832 ± 160 (669–977)	890 ± 33.3 (825–936)
W	41 ± 2.3 (36–43)	35.4 ± 4 (28–42)	44.2 ± 2.7 (41–49)	46 ± 10 (40–50)	45 ± 8 (43–50)	47 ± 10 (43–54)	66 ± 6.6 (63–79)
EP	109 ± 6 (96–116)	83.6 ± 5.2 (73–98)	95.5 ± 4 (91–104)	93 ± 4 (89–96)	87 ± 6 (81–90)	96 ± 6 (84–101)	91 ± 9 (68–104)
NR	79 ± 5 (71–88)	68.2 ± 4.7 (60–81)	76.9 ± 5.4 (70–89)	81 ± 6 (71–87)	74 ± 7 (69–80)	80 ± 6 (71–88)	67 ± 10.1 (44–84)
ES	105 ± 5 (97–114)	97.3 ± 4 (91–107)	107.7 ± 3.7 (102–114)	107 ± 12 (101–113)	105 ± 16 (101–110)	110 ± 10 (100–117)	112 ± 3.9 (108–120)
T	33 ± 2.7 (29–41)	39 ± 2 (34–42)	42.4 ± 3 (37–47)	34 ± 3 (31–38)	36 ± 5 (33–39)	36 ± 2 (28–40)	62 ± 10.8 (36–82)
ABD	27 ± 2.6 (23–33)	22.9 ± 1.9 (20–26)	25.6 ± 1.6 (23–28)	26 ± 2 (23–28)	27 ± 6 (24–30)	29 ± 3 (26–37)	39 ± 3.9 (32–48)
SP	41 ± 2.9 (35–45)	36.5 ± 2.1 (33–41)	35.2 ± 2.5 (31–39)	42 ± 4 (39–44)	42 ± 4 (40–46)	41 ± 2 (36–45)	43 ± 4.9 (36–52)
GU	21 ± 1.5 (19–23)	18.2 ± 1.7 (16–21)	19 ± 1.8 (15–21)	21 ± 2 (18–22)	21 ± 2 (19–22)	21 ± 2 (18–25)	22 ± 3.1 (16–28)
D%	103 ± 3.7 (95–109)	85.8 ± 3.2 (77.9–91.6)	88.7 ± 3.1 (80–93)	86 ± 7 (82–90)	82 ± 9 (74–87)	87 ± 5 (79–95)	81 ± 8.1 (61–96)
SW%	152 ± 20 (120–187)	90 ± 5.9 (81–99)	138.5 ± 15.2 (111–163)	162 ± 24 (144–184)	156 ± 37 (131–191)	140 ± 20 (115–163)	110 ± 17 (82–144)
GS%	51 ± 32 (44–56)	49.8 ± 4.1 (43.2–54.1)	53.9 ± 4.1 (47–64)	51 ± 2 (46–54)	49 ± 5 (45–54)	50 ± 3 (44–60)	52 ± 8.2 (42–67)

L = body length; W = body width; NR = distance from the anterior end to the nerve ring; EP = distance from the anterior end to the excretory pore; ES = distance from the anterior end to the end of the pharynx; T = tail length; ABD = body width in the anal region; GU = gubernaculum length; SP = spicule length; D% = (EP/ES); SW% = (SP/ABD); GS% = GU/SP.

observed the production of three generations in the short cycle of *H. amazonensis* (RSC 05). Consistent with the findings of the present study, Poinar (1976) found that the life cycle of *H. bacteriophora* is characterized by a short and long cycle with two generations.

The life cycle of *H. amazonensis* (UEL 08) differs from that observed by Molina et al. (2005) and Andaló et al. (2009b) for *H. amazonensis* (JMP4) and (RSC 05), respectively, in the time required for cycle completion and duration of the different developmental stage, which were shorter in the present study than those reported by these authors.

According to Adams and Nguyen (2002), the life cycles of EPNs, as well as the number of generations produced, can vary depending on the availability of food and the body size of host insects, given that larger insects (and correspondingly larger nutrient reserves) favor longer cycles and a more generations.

It is evident that variability can occur between nematode species of the same genus and even between isolates of the same species. Grewal et al. (1994) suggested that environmental factors such as temperature, aeration, and humidity may also affect the length of the life cycle. The optimal temperature is related to the climate of the nematode's region of origin (Grewal et al., 1994), whereas aeration is necessary for nematode development (Burman and Pye, 1980). In addition, according to Woodring and Kaya (1988), humidity is another essential component, as high humidity must be maintained throughout the development cycle to prevent host insect desiccation.

Pathogenicity and virulence test

Infectious juveniles of *H. amazonensis* (UEL 08) were found to be pathogenic to *G. mellonella* and *A. diaperinus* larvae, as well as *D. melacanthus* adults. We observed a difference in nematode virulence against the different hosts, with mortality rates of 100%, 85%, and 46%, respectively (Table 6). The findings of other studies (Molina et al.,

Table 3. Comparative morphometry (µm) of infective juveniles of *Heterorhabditis amazonensis* isolated in Brazil (Andaló et al., 2006, 2009b) and Venezuela (Morales et al., 2016).

| Character | Andaló et al. (2006) | Andaló et al. (2009b) | | Morales et al. (2016) | | | Present Study |
| | | GL population | SG population | LPV081 | LPV156 | LPV498 | UEL 08 |
	Type population (Brazil)	(Brazil)	(Brazil)	(Venezuela)	(Venezuela)	(Venezuela)	(Brazil)
L	589 ± 12 (567–612)	598 ± 12.7 (567–618)	506 ± 19.7 (465–541)	503 ± 225 (454–549)	497 ± 148 (462–540)	542 ± 280 (514–582)	554 ± 35.5 (508–634)
A	26 ± 1.3 (24–29)	26 ± 1 (24.4–28.5)	22.2 ± 1.6 (20–25)	20 ± 3 (17–22)	21 ± 2 (19–24)	21 ± 3 (19–23)	23 ± 3 (19–28)
B	4.9 ± 0.3 (4.4–5.5)	5.1 ± 0.3 (4.6–5.5)	4.4 ± 0.4 (3.8–5)	4.4 ± 0.4 (3.9–5.3)	4.6 ± 0.7 (4.3–4.9)	5.4 ± 0.5 (4.9–6.2)	4.3 ± 0.4 (3.6–5.6)
C	5.5 ± 0.2 (5.1–6.1)	5.3 ± 0.1 (5–5.5)	5.1 ± 0.3 (4.6–5.7)	5.1 ± 0.6 (4.8–5.9)	5 ± 0.5 (4.1–5.6)	6.1 ± 0.8 (5.1–7.2)	5.1 ± 0.3 (4.5–5.8)
W	23 ± 1.2 (20–24)	23 ± 0.9 (21–24)	22 ± 1.7 (21–26)	24 ± 6 (24–25)	22 ± 3 (21–25)	26 ± 4 (22–27)	24 ± 3.7 (20–32)
EP	107 ± 6.1 (89–115)	102 ± 6.4 (91–112)	103 ± 11.4 (86–124)	92 ± 7 (85–98)	91 ± 9 (85–98)	78 ± 5 (66–97)	106 ± 10.2 (92–128)
NR	85 ± 4.9 (76–93)	92.9 ± 6.1 (83–104)	86 ± 11.4 (74–106)	77 ± 4 (72–88)	78 ± 11 (73–81)	67 ± 2 (58–78)	83 ± 9.5 (72–104)
ES	121 ± 6.6 (107–132)	118 ± 5.8 (111–128)	115 ± 10.5 (99–135)	114 ± 13 (104–122)	105 ± 15 (94–109)	99 ± 8 (88–107)	130 ± 10.8 (100–144)
T	107 ± 4.7 (98–115)	113 ± 3.2 (106–119)	99 ± 6.2 (89–112)	97 ± 7 (89–105)	97 ± 4 (86–110)	87 ± 6 (81–103)	108 ± 8.4 (96–128)
ABD	14 ± 1.4 (13–17)	16 ± 0.8 (15–18)	16 ± 1.7 (13–20)	15 ± 1.7 (14–16)	14 ± 2.5 (13–18)	15 ± 1.5 (14–17)	16 ± 3.5 (12–24)
D%	88 ± 2.7 (83–92)	86 ± 2.2 (81–90)	89 ± 2.7 (85–94)	81 ± 6 (73–87)	87 ± 6 (78–97)	78 ± 4 (66–91)	82 ± 5.7 (71–92)
E%	100 ± 6.0 (89–109)	90 ± 5.9 (81–99)	104 ± 9.9 (87–120)	95 ± 11 (86–104)	94 ± 5 (86–99)	86 ± 5 (63–103)	99 ± 11.1 (86–129)

L = body length; A = (L/W); B = (L/ES); C = (L/T); W= body width; NR = distance from the anterior end to the nerve ring; EP = distance from the anterior end to the excretory pore; ES = distance from the anterior end to the end of the pharynx; T = tail length; ABD = body width in the anal region; D% = (EP/ES); E% = (EP/T).

2005; Andaló et al., 2009a) have consistently indicated that isolates of the genus *Heterorhabditis* are pathogenic to *G. mellonella* larvae and corroborate our results obtained for *H. amazonensis* (UEL 08).

The larvae of lesser mealworm *A. diaperinus* are also susceptible to *H. amazonensis* (UEL 08) (Table 6), which is consistent with the findings of other studies carried out under similar conditions that have demonstrated the susceptibility of this insect to EPNs in the genera *Heterorhabditis* and *Steinernema* (Pezowicz, 2003; Alves et al., 2005; Alves et al., 2012). However, adults of the stink bug *D. melacanthus* were found to be less susceptible to the UEL 08 isolate; a finding that differs from that reported by Guide et al. (2015), who evaluated different *Heterorhabditis* isolates from *D. melacanthus* adults, obtaining mortalities of up to 76%.

Such differences in the virulence of different isolates, even those of the same species, can be explained by several factors. Different isolates may have adapted to different climatic conditions or even developed local host specificities (Alves et al., 2009). According to Doucet et al. (1999), a variation in susceptibility is to be expected, given that different isolates have co-evolved with different host species, and therefore have specificities that render them more or less virulent to certain insects, which may explain the differences observed in the present study.

In Vivo Production of Heterorhabditis amazonensis *(UEL 8) in* G. mellonella *Larvae*

The production of *H. amazonensis* IJs (UEL 08) in *G. mellonella* larvae occurred over a period of 8 days, with the highest yields observed on the first (1.1×10^6 IJs) and second (8.0×10^5 IJs) days (Figure 3), representing 38% and 27%, respectively, of the total accumulated production (2.9×10^6 IJs). Furthermore, the average production per larvae was 5.8×10^4 IJs/larvae and the production per gram of larvae was 3.4×10^3 IJs. Notably, these values are lower than those reported by Guide et al. (2016), who founded 7×10^4 and 7.2×10^4 IJs/g larvae for *Heterorhabditis*

Table 4. Paired distances of the ITS (Internal Transcribed Spacer) regions of the rDNA between *Heterorhabditis* species.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	MK262740 *H. amazonensis* UEL 08		99	100	100	100	88.3	88.9	88.2	87.9	87.2	87.0	87.7	88.0	88.1	92.4	92.4	92.2	95.9	95.4	95.6	96.2	85.9	88.2	85.7
2	DQ665222 *H. amazonensis*	1		100	100	100	88.3	88.9	88.2	87.8	87.2	87.0	87.7	87.9	88.0	92.4	92.3	92.2	96.0	95.4	95.5	96.2	86.0	88.2	85.4
3	KM211575 *H. amazonensis* LPV081	0	0		100	100	88.5	86.0	88.2	88.1	87.3	87.5	87.7	88.0	88.2	92.5	92.5	92.3	96.0	95.4	95.7	96.2	86.4	89.3	86.6
4	KM211576 *H. amazonensis* LPV156	0	0	0		100	88.5	86.0	88.2	88.1	87.3	87.5	87.7	88.0	88.2	92.5	92.5	92.3	96.0	95.4	95.7	96.2	86.4	89.3	86.6
5	KM211577 *H. amazonensis* LPV498	0	0	0	0		88.5	86.0	88.2	88.1	87.3	87.5	87.7	88.0	88.2	92.5	92.5	92.3	96.0	95.4	95.7	96.2	86.4	89.3	86.6
6	EF488006 *H. safricana*	172	171	163	163	163		96.0	95.3	94.6	92.1	91.4	88.9	89.0	88.6	89.0	89.2	88.7	88.2	88.3	88.5	88.4	85.6	87.6	84.5
7	HM230723 *H. atacamensis*	151	151	150	150	150	13		95.4	95.1	92.5	91.7	89.6	89.6	88.9	89.5	89.8	89.3	88.9	89.0	89.2	88.9	86.1	88.7	85.5
8	AY321479 *H. marelatus*	170	169	161	161	161	24	21		94.3	92.3	91.2	88.6	88.6	88.3	88.8	88.9	88.5	88.1	88.0	88.2	88.1	85.4	87.8	83.9
9	AY321482 *H. downesi*	169	168	161	161	161	31	27	37		93.9	91.3	89.2	89.1	88.6	88.7	89.0	88.5	87.8	87.8	88.1	88.0	85.5	87.6	84.6
10	AY321480 *H. megidis*	178	177	170	170	170	58	55	63	41		90.3	88.1	88.3	88.1	88.0	88.3	87.9	87.4	87.5	87.8	87.6	85.0	87.5	84.4
11	AY321481 *H. zealandica*	195	195	185	185	185	83	71	78	81	104		87.7	87.6	87.3	87.6	87.9	87.5	87.1	87.0	87.2	87.5	84.9	87.6	83.6
12	AY321477 *H. bacteriophora*	162	161	155	155	155	138	121	136	140	152	175		94.1	93.9	88.2	88.1	87.9	87.8	87.5	87.1	87.9	84.8	88.0	84.1
13	HQ896630 *H. beicherriana*	162	161	154	154	154	133	116	132	136	152	170	39		93.7	88.4	88.5	88.1	87.8	87.4	87.4	88.0	84.7	87.9	84.3
14	EU099032 *H. georgiana*	161	160	154	154	154	142	127	142	141	154	175	45	43		88.6	89.0	88.7	88.1	87.9	87.7	88.2	84.9	87.8	84.5
15	AY321483 *H. indica*	70	69	68	68	68	160	139	164	162	173	191	146	141	144		96.7	95.7	92.2	92.1	91.9	92.3	85.4	88.2	85.6

Below the diagonal: total base pair differences; above the diagonal: percentage similarity.

Table 4. Continued...

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
16 FJ152545 *H. gerrardi*	67	67	65	65	65	154	135	158	157	169	183	148	141	139	12		96.6	92.0	91.8	91.6	92.4	85.8	89.0	86.3
17 JN620538 *H. noenieputensis*	73	72	71	71	71	164	143	168	165	177	194	156	148	145	20	12		92.1	91.7	91.4	92.1	85.6	88.3	85.4
18 DQ372922 *H. floridensis*	18	17	17	17	17	178	157	177	175	183	202	168	169	165	81	78	80		96.0	95.9	96.2	86.4	88.1	85.4
19 AY321478 *H. mexicana*	22	21	20	20	20	176	155	175	173	179	200	170	172	169	82	80	84	14		96.6	95.4	86.4	88.1	85.5
20 EF043443 *H. taysearae*	21	21	19	19	19	174	152	175	171	177	202	175	172	171	84	81	85	16	11		95.5	86.2	88.0	85.7
21 AF548768 *H. baujardi*	15	15	15	15	15	166	150	165	164	174	186	160	161	156	76	70	77	16	23	23		86.6	89.3	86.5
22 EU273598 *O. chongmingensis*	283	281	263	263	263	309	277	301	302	306	325	316	320	317	290	271	284	280	283	283	268		87.4	85.0
23 AF036946 *P. typica*	178	178	146	146	146	195	157	192	195	204	195	192	194	192	177	156	177	179	178	180	154	218		87.3
24 X03680 *C. elegans*	281	284	250	250	250	297	245	295	293	308	313	313	318	317	288	257	289	279	281	281	252	388	213	

Below the diagonal: total base pair differences; above the diagonal: percentage similarity.

Table 5. Life cycle: duration of the developmental stages of *Heterorhabditis amazonensis* (UEL 08) in larvae of *Galleria mellonella.* Short life cycle nematode concentration used for insect inoculation was 400 IJ/larvae; Long life cycle: nematode concentration used for insect inoculation was 10 IJ/larvae.

Developmental stages	Short life cycle (Time in hours)*		Long life cycle (Time in hours)	
	1st generation	2nd generation	1st generation	2nd generation
J_4	0–24		0–24*	
Hermaphrodites	48		48	
Hermaphrodites + eggs / J_1	72		72	
Hermaphrodites + J_2	96		96	
Males and females		144		144
Females + J_2		192		216
J_3 or IJs		216		288

*Times presented are approximate and include the time spent in dissecting the larvae.

Table 6. Virulence of *Heterorhabditis amazonensis* (UEL 08) applied at a concentration of 100 JIs / cm² under three different insects.

Treatment	*H. amazonensis* (UEL 8)	Control
	Mortality (%) ± SD	
Galleria mellonella	100.0 ± 0.0 A*	0.0 ± 0.0
Alphitobius diaperinus	85.0 ± 8.0 A	0.0 ± 0.0
Dichelops melacanthus	46.0 ± 17.3 B	0.0 ± 0.0
CV (%) = 13.15		

*Means followed by a distinct letter in the column differ from one another by Tukey test (p= 0.05).
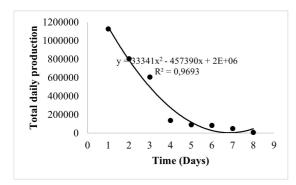

Figure 3. Daily production of infective juveniles of *Heterorhabditis amazonensis* (UEL 08) in 50 *G. mellonella* larvae under controlled conditions (24 ± 1 °C and without photoperiod).

sp. (NEPET 11) and *H. amazonensis* (RSC 05), respectively. Also, Costa et al. (2007) obtained a mean value of 1.6 × 10⁵ IJs/g for *H. riobraves*. Finally, Bortoluzzi et al. (2013) observed 2.2 × 10⁶ IJs/g for the two H*eterorhabditis* isolates IBCBn-24 and IBCBn-40.

Several factors can influence the *in vivo* production of EPNs, and these factors may interact in unpredictable ways (Zervos et al., 1991; Shapiro-Ilan et al., 2004). Differences in infectivity and production of nematode species can be greater or smaller, even in a host considered susceptible, as in the case of *G. mellonella* (Ozer and Unlu,

2003). In addition, the availability of food may influence the permanence of the IJs within the host insect and in the development of new generations (Ehlers, 2001). According to Boff et al. (2000), the rate of metabolism in the processing of host tissue by symbiont bacteria differs among nematode species and may also influence their survival and reproduction, thus affecting production.

IJ concentration-dependent factors also play an important role in entomopathogenic nematode production and can act directly by affecting the number of IJs produced by infected cadavers, or indirectly by influencing the longevity of juveniles (Selvan et al., 1993; Zervos et al., 1991). According to Poinar (1979), the nematodes production in the larvae of *G. mellonella* ranges from between 30,000 to 50,000 IJs/larvae but can also reach 2 × 10⁵ (Dutky et al., 1964; Gaugler and RiChou, 2002). These values are comparable to those obtained for the average production of *H. amazonensis* (UEL 08) in *G. mellonella*, thereby indicating the potential utility of this isolate in pest control programs.

Thus, we established that the nematode *Heterorhabditis* UEL 08, isolated from soil in Londrina, PR, Brazil, is an isolate of the species *H. amazonensis*, and this is the first register of this nematode in the state of Paraná. Furthermore, we determined that the UEL 08 isolate has two life cycles (short and long) of different duration, which are influenced by the concentration of IJs used. The IJs of this isolate were found to be pathogenic to the larvae of *G. mellonella* and *A. diaperinus* and adults of *D. melacanthus.* The larvae of

G. mellonella were observed to be the most susceptible hosts. The production of IJs in *G. mellonella* extends over a period of 8 days, with production peaking on the first and second days.

Acknowledgements

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