STUDIES OF TWO NEW POPULATIONS OF *HETERORHABDITIS AMAZONENSIS* (RHABDITIDA: HETERORHABDITIDAE)

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ABSTRACT

Andaló, V., G. F. Moreira, and A. Moino, Jr. 2009. Studies of two new populations of *Heterorhabditis amazonensis* (Rhabditida: Heterorhabditidae). Nematropica 39:199-211.

In a survey in Lavras, Minas Gerais state, Brazil, two populations of the genus *Heterorhabditis* were obtained from soil by the insect baiting technique. Light and scanning electron microscopy and DNA characterization were used for identification. Both populations can invade and develop in *Galleria mellonella* larvae and are morphologically similar to *H. indica, H. baujardi, H. amazonensis* and *H. mexicana*. Observation of scanning electron photographs indicates that morphometrics of both populations are similar to that in *H. amazonensis*. Similarities were found in the molecular sequence of the LSU D2D3 region. Morphological and molecular studies of the two populations showed that these nematodes are isolates of *H. amazonensis*.

Key words: description, entomopathogenic nematode, morphology, morphometrics, SEM, taxonomy, biological control.

RESUMO

Andaló, V., G. F. Moreira, and A. Moino, Jr. 2009. Estudos de duas novas populações de *Heterorhabditis amazonensis* (Rhabditida: Heterorhabditidae). Nematropica 39:199-211.

Em um levantamento realizado em Lavras, Minas Gerais, Brasil, duas populações do gênero *Heterorhabditis* foram obtidas do solo através da técnica de inseto-isca. Microscópio óptico de luz e eletrônico de varredura e caracterização molecular foram usados para a identificação. Ambas as populações mostraram-se patogênicas a larvas de *Galleria mellonella* e foram caracterizadas como morfologicamente similares a *H. indica*, *H. baujardi, H. amazonensis* and *H. mexicana*, podendo ser diferenciadas dessas espécies por características do juvenil infectivo, macho e fêmea. Foram observadas nas fotos de microscopia eletrônica estruturas padrão para as duas populações semelhantes às encontradas em *H. amazonensis*, e também os dados moleculares mostraram a mesma composição de nucleotídeos. Estudos morfológicos e moleculares dessas populações mostraram que os nematóides são a mesma espécie que *H. amazonensis*.

Palavras chave: descrição, nematóides entomopatogênicos, morfologia, morfometria, MEV, taxonomia, controle biológico.

INTRODUCTION

Insect management is traditionally carried out using chemical pesticides. Negative aspects related to food safety, pollution of the soil and water, pest resistance to insecticides, effect on non-target insects and the increase of environmental degradation have increased the search for different methods to control pests. Entomopathogenic nematodes (EPN) (Rhabditida: Steinernematidae, Heterorhabditidae) have been shown to possess a combination of attributes that could be

used as alternatives to chemical control of insects. The mutualistic association with specific bacteria, *Xenorhabdus* (Poinar and Thomas, 1979) and *Photorhabdus* (Thomas and Poinar, 1979), with nematodes of the genus *Steinernema* (Travassos, 1927) and *Heterorhabditis* (Poinar, 1975) respectively, confers to these organisms pathogenic potential to control insects (Adams and Nguyen, 2002).

The discovery of new species or populations of EPN can expand or improve the use of these organisms in programs of biological control programs. The variability found in native nematodes was allowed for better adapted populations for control in specific climatic conditions and to local insect population.

The purpose of this study was to isolate EPN from soil samples collected at Universidade Federal de Lavras, Minas Gerais state, Brazil, and to characterize the populations through the use of morphological, molecular techniques and determine their pathogenicity.

MATERIAL AND METHODS

Insect's hosts used to isolate entomopathogenic nematodes

Entomopathogenic nematodes populations were obtained from soil by the insect baiting technique (Kaya and Stock 1997; Stock 1998). Larvae of the last instar of *Galleria mellonella* (Linneaus, 1758) (Lepidoptera: Pyralidae) and *Tenebrio molitor* Linneaus, 1785 (Coleoptera: Tenebrionidae) were used as bait. *Galleria mellonella*, for maintaining nematode populations, was reared using the combination of the methods described by Dutky *et al.* (1964) and Parra (1998). *Tenebrio molitor* was reared in plastic trays, using a diet with wheat flour mixed with pieces of *Sechium edule* (Jacq.) SW or *Daucus carota* L., described by Parra (1998).

Sampling and processing soil samples

The soil samples for isolation of EPN were collected from 22 sites of agricultural and natural areas near Universidade Federal de Lavras, Brazil. An each site, an area of about 4 m^2 was randomly selected and 4 soil samples of about 500 g from15-20 cm deep were removed by a shovel. Those soil samples were mixed well and keep in a plastic bag labeled with date, associated crop or nature vegetation, and geographic coordinates of the site. All samples collected were transported to a laboratory where 200g of soil from each sample was transferred to each of 8 plastic containers (14 × 14 × 7 cm). Soil in each container was then moistened with sterile distilled water (15% v/w).

Five last instar *G. mellonella* larvae were added to 4 containers and 5 last instar *T. molitor* larvae were added to the other 4. The containers were closed and inverted to keep the bait at the bottom and, then, they were incubated at 25 ± 2°C in the dark for seven days. The dead larvae showing infection symptoms were washed with sodium hypochlorite 2% and placed in a dry chamber (9-cm Petri dish with filter paper) for five more days (Molina and López, 2001), under the same conditions. At this time the dead larvae were placed in a White trap (White, 1927) to collect infective juveniles (IJ) of the nematodes.

Experiments using Koch's postulates for each nematode population collected were conducted (Alves, 1998) to prove the parasitism for the entomopathogen. The nematodes populations isolated were multiplied in *G. mellonella* larvae (Woodring & Kaya (1988) and kept in a refrigerator at 8°C.

Pathogenicity test

The pathogenicity test was conducted on each population by applying a 1 ml

suspension of 200 IJ on 10 *G. mellonella*, *Diabrotica speciosa* (Germar, 1824) (Coleoptera: Chrysomelidae), and *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae) larvae in a Petri dish lined with filter paper on the bottom. The experiments were replicated three times. Sterile distilled water without nematodes was used for control. The dishes were kept in climatized chamber at 25 ± 2°C. Insects were observed and larvae mortality was verified after 5 days, observing symptomatology characteristic by EPN infection.

To confirm the insects death caused by nematodes of *Heterorhabditis* genus, a *G. mellonella* larva and a IJ were placed in an Eppendorf® tube containing a piece of humidified paper with 70µl of distilled water to conduct a one by one (1:1) assay (Glazer and Lewis, 2000). There were 20 replications for each population. After five days the insect dead larvae with symptoms were dissected to confirm the formation of nematode hermaphroditic females of the first generation.

Data analysis

Statistical analyses were done with SISVAR software (InstallShield Corporation, Inc., Lavras, MG, Brazil) (Ferreira, 2003). Pathogenicity tests results were statistically analyzed using ANOVA and means separated by Tukey ($P > 0.05$).

Morphometrics analysis of entomopathogenic nematodes

Nematodes populations were compared to other genetically close species through measurements of different body structures. Ten larvae of *G. mellonella* were exposed to approximately 300 IJ in two filter papers on the bottom of a 9 cm diam Petri dish. The first generation hermaphrodites and the second generation males and females were obtained by dissection of infected insects at 4-5 days and 7-8 days, respectively, after the insect death (Nguyen *et al.*, 2006). The IJ were obtained in the two first days after its emergence from the dead insect, as suggested for Nguyen and Smart (1995a).

Twenty specimens of each stage (infective juvenile, hermaphrodite first generation, female second generation and male) were observed alive. Additional nematodes of different stages were killed and fixed in triethanolamine formalin (TAF) and processed in glycerin using the methodology described by Seinhorst (1959). The specimens were mounted on slides and the nematodes observed using light microscopy (Olympus CX31, magnification of 10×, 20× and 40×).

Table 1. Pathogenicity of *Heterorhabditis* spp. to three selected insects.

	Heterorhabditis sp. GL	*Heterorhabditis* sp. SG	Control	CV
Insect	Mortality (%)			
Galleria mellonella	73.3 ± 15.2 ab	80.0 ± 17.3 b	3.3 ± 5.7 a	37.68
Diabrotica speciosa	70.0 ± 10.0 b	80.0 ± 10.0 b	0.0 ± 0.0 a	11.55
Spodoptera frugiperda	76.7 ± 5.8 b	80.0 ± 10.0 b	0.0 ± 0.0 a	12.77

Means followed by the same letter within a row are not significantly different by Tukey test ($P < 0.05$).
CV (%) = Coefficient of variation.

Table 2. Morphometrics (μm) of *Heterorhabditis* isolate GL. Measurements are in the form: mean ± sd (range) (n = 20).

Character	Male	Hermaphrodite	Female	Infective juvenile
L	739.2 ± 23.4 (694-790)	4001.6 ± 290.3 (3279-4574)	1872.8 ± 129.4 (1672-2180)	598 ± 12.7 (567-618)
W	35.4 ± 4 (28-42)	185.8 ± 9.3 (176-206)	116.4 ± 10.5 (98-141)	23 ± 0.9 (21-24)
NR	68.2 ± 4.7 (60-81)	129.9 ± 9.1 (112-150)	88.2 ± 9.3 (70-102)	92.9 ± 6.1 (83-104)
EP	83.6 ± 5.2 (73-98)	154.4 ± 14.7 (129-180)	114.8 ± 9.4 (103-138)	101.6 ± 6.4 (91-112)
ES	97.3 ± 4 (91-107)	178.6 ± 9 (163-192)	127.9 ± 9.1 (117-154)	117.7 ± 5.8 (111-128)
T	39 ± 2 (34-42)	124.4 ± 9.2 (111-143)	76.2 ± 6.4 (62-83)	112.9 ± 3.2 (106-119)
ABW	22.9 ± 1.9 (20-26)	52.5 ± 3.8 (47-57)	34.2 ± 2.5 (29-39)	15.9 ± 0.8 (15-18)
Hyaline				46.7 ± 3.7 (39-54)
D%	85.8 ± 3.2 (77.9-91.6)			86.1 ± 2.2 (81-90)
E%				89.9 ± 5.9 (81-99)
H/T%				41.4 ± 3.2 (34-46)
a				26 ± 1 (24.4-28.5)
b				5.1 ± 0.3 (4.6-5.5)
c				5.3 ± 0.1 (5-5.5)
STW		8.3 ± 0.6 (8.1-9.8)	6.9 ± 0.7 (6.5-8.1)	

L = body length; W = body width; NR = distance from anterior end to nerve ring; EP = distance from anterior end to excretory pore; ES = distance from anterior end to end of pharynx; T = tail length; ABW = anal body width; Hyaline = portion between inner and outer cuticle; D(%) = (EP/ES); E(%) = (EP/T); H/T(%) = (Hyaline/T); a = (L/W); b = (L/ES); c = (L/T); STW = stoma width; STL = stoma length; V = distance from anterior end to vulva; V(%) = V/L × 100; SP = spicule length; SW = spicule width; GU = gubernaculum length; SW(%) = SP/ABW; GS(%) = GU/SP and TR = testis reflexion.

Table 2. (Continued) Morphometrics (µm) of *Heterorhabditis* isolate GL. Measurements are in the form: mean ± sd (range) (n = 20).

Character	Male	Hermaphrodite	Female	Infective juvenile
STL		9.4 ± 0.9	7.3 ± 0.8	
		(8.1-11,4)	(6.5-8.1)	
V		1697.8 ± 134.4	892.3 ± 73.3	
		(1377-1934)	(803-1066)	
V%		42.4 ± 1.6	47.6 ± 1.8	
		(39.6-46.1)	(44-51)	
SP	36.5 ± 2.1			
	(33-41)			
SW	6.3 ± 0.6			
	(4.9-6.6)			
GU	18.2 ± 1.7			
	(16-21)			
SW%	160.6 ± 14.7			
	(138-178)			
GS%	49.8 ± 4.1			
	(43.2-54.1)			
TR	65.4 ± 8.2			
	(50-81)			

L = body length; W = body width; NR = distance from anterior end to nerve ring; EP = distance from anterior end to excretory pore; ES = distance from anterior end to end of pharynx; T = tail length; ABW = anal body width; Hyaline = portion between inner and outer cuticle; D(%) = (EP/ES); E(%) = (EP/T); H/T(%) = (Hyaline/T); a = (L/W); b = (L/ES); c = (L/T); STW = stoma width; STL = stoma length; V = distance from anterior end to vulva; V(%) = V/L × 100; SP = spicule length; SW = spicule width; GU = gubernaculum length; SW(%) = SP/ABW; GS(%) = GU/SP and TR = testis reflexion.

Scanning electron microscopy

Adults nematodes and IJ were fixed in 3% glutaraldehyde buffered with 0,1M sodium cacodylate, pH 7,2 for 24h at 8°C. The nematodes were post-fixed with 2% osmium tetroxide solution for 12h at 25°C, dehydrated in ethanol series, critical point dried with CO_2, mounted on SEM stubs and coated with gold (Nguyen and Smart, 1995b).

Molecular characterization

DNA was extracted from a single hermaphroditic female as described by Nguyen *et al.*, 2004, 2006) and the LSU D2D3 expansion regions were used. The method for working with this new locus was as reported by Nguyen *et al.* (2007).All PCR reactions were conducted in a Thermocycler PTC-200 (MJ Research, Inc., Watham, MA, USA) with the following cycle profile:

Table 3. Morphometrics (µm) of *Heterorhabditis* isolate SG. Measurements are in the form: mean ± sd (range) (n = 20).

Character	Male	Hermaphrodite	Female	Infective juvenile
L	820.4 ± 32.7 (771-879)	4520.5 ± 205.3 (3984-4803)	2318.8 ± 126.6 (2016-2492)	506 ± 19.7 (465-541)
W	44.2 ± 2.7 (41-49)	184.5 ± 8.8 (167-200)	155.9 ± 9.3 (135-172)	22.9 ± 1.7 (21-26)
NR	76.9 ± 5.4 (70-89)	125.2 ± 8.5 (111-140)	102.5 ± 5.5 (96-114)	86.1 ± 11.4 (74-106)
EP	95.5 ± 4 (91-104)	162.6 ± 10.4 (138-179)	131.1 ± 8.1 (114-143)	103 ± 11.4 (86-124)
ES	107.7 ± 3.7 (102-114)	177.7 ± 8.1 (163-193)	146.6 ± 8.7 (124-163)	115 ± 10.5 (99-135)
T	42.4 ± 3 (37-47)	111.8 ± 11.3 (96-130)	83.1 ± 6 (73-98)	98.6 ± 6.2 (89-112)
ABW	25.6 ± 1.6 (23-28)	47.7 ± 3.9 (41-55)	38.7 ± 4.1 (33-46)	16.2 ± 1.7 (13-20)
Hyaline				52.4 ± 7 (42-67)
D%	88.7 ± 3.1 (80-93)			89.3 ± 2.7 (85-94)
E%				104.1 ± 9.9 (87-120)
H/T%				53.2 ± 6.9 (44-68)
a				22.2 ± 1.6 (20-25)
b				4.4 ± 0.4 (3.8-5)
c				5.1 ± 0.3 (4.6-5.7)
STW		10.2 ± 0.7	7.8 ± 0.7	

L = body length; W = body width; NR = distance from anterior end to nerve ring; EP = distance from anterior end to excretory pore; ES = distance from anterior end to end of pharynx; T = tail length; ABW = anal body width; Hyaline = portion between inner and outer cuticle; D(%) = (EP/ES); E(%) = (EP/T); H/(%) = (Hyaline/T); a = (L/W); b = (L/ES); c = (L/T); STW = stoma width; STL = stoma length; V = distance from anterior end to vulva; V(%) = V/L × 100; SP = spicule length; SW = spicule width; GU = gubernaculum length; SW(%) = SP/ABW; GS(%) = GU/SP and TR = testis reflexion.

Table 3. (Continued) Morphometrics (µm) of *Heterorhabditis* isolate SG. Measurements are in the form: mean ± sd (range) (n = 20).

Character	Male	Hermaphrodite	Female	Infective juvenile
		(9.8-11.4)	(6.5-8.1)	
STL		10.5 ± 0.8	8.3 ± 0.5	
		(9.8-11.4)	(8.1-9.8)	
V		1919.7 ± 128.2	1090.3 ± 73.7	
		(1640-2148)	(967-1246)	
V%		42.4 ± 1.7	47 ± 1.6	
		(39-45)	(44-50)	
SP	35.2 ± 2.5			
	(31-39)			
SW	7.1 ± 0.8			
	(6.5-8.1)			
GU	19 ± 1.8			
	(15-21)			
SW%	138.5 ± 15.2			
	(111-163)			
GS%	53.9 ± 4.1			
	(47-64)			
TR	87.9 ± 10.5			
	(70-99)			

L = body length; W = body width; NR = distance from anterior end to nerve ring; EP = distance from anterior end to excretory pore; ES = distance from anterior end to end of pharynx; T = tail length; ABW = anal body width; Hyaline = portion between inner and outer cuticle; D(%) = (EP/ES); E(%) = (EP/T); H/(%) = (Hyaline/T); a = (L/W); b = (L/ES); c = (L/T); STW = stoma width; STL = stoma length; V = distance from anterior end to vulva; V(%) = V/L × 100; SP = spicule length; SW = spicule width; GU = gubernaculum length; SW(%) = SP/ABW; GS(%) = GU/SP and TR = testis reflexion.

1 cycle of 94°C for 7 min followed by 35 cycles at 94°C for 1 min; 45°C for 1 min; 72°C for 1 min and the last step was 72°C for 10 min (Nguyen *et al.*, 2004). Sequences of the complete D2D3 array were aligned to previously published sequences of the D2D3 region (Nguyen *et al.*, 2006) using the profile alignment option of Clustal X (Thompson *et al.*, 1997), then optimized manually in MacClade 4.05 (Maddison and Maddison, 2002).

RESULTS AND DISCUSSION

Sampling entomopathogenic nematodes

Twenty-two soil samples were collected and in two of them EPN were found to have populations of EPN. The two samples, garlic (latitude 21°13'49.43"S, longitude 44°58'29.45"W) and sorghum (latitude 21°13'23.54"S, longitude 44°58'26.09"W) fields, were

Table 4. Comparative morphometrics (µm) of male of *Heterorhabditis* isolate GL, *Heterorhabditis* isolate SG and related species.

Character	AM Andaló *et al.*, 2006	MX Nguyen *et al.*, 2004	BJ Phan *et al.*, 2003	GL Present study	SG Present study
L	752 ± 43 (692-826)	686 ± 38 (614-801)	889 ± 45 (818-970)	739.2 ± 23.4 (694-790)	820.4 ± 32.7 (771-879)
W	41 ± 2.3 (36-43)	42 ± 3 (38-47)	49 ± 2 (43-53)	35.4 ± 4 (28-42)	44.2 ± 2.7 (41-49)
NR	79 ± 5 (71-88)	71 ± 6 (61-83)	65 ± 7 (54-77)	68.2 ± 4.7 (60-81)	76.9 ± 5.4 (70-89)
EP	109 ± 6 (96-116)	124 ± 10 (108-145)	81 ± 7 (71-93)	83.6 ± 5.2 (73-98)	95.5 ± 4 (91-104)
ES	105 ± 5 (97-114)	96 ± 5 (89-108)	116 ± 10 (105-132)	97.3 ± 4 (91-107)	107.7 ± 3.7 (102-114)
T	33 ± 2.7 (29-41)	27 ± 4 (21-36)	33 ± 3 (28-38)	39 ± 2 (34-42)	42.4 ± 3 (37-47)
ABW	27 ± 2.6 (23-33)	24 ± 1.3 (23-27)	22 ± 1 (20-24)	22.9 ± 1.9 (20-26)	25.6 ± 1.6 (23-28)
SP	41 ± 2.9 (35-45)	41 ± 3.8 (30-47)	40 ± 3 (33-45)	36.5 ± 2.1 (33-41)	35.2 ± 2.5 (31-39)
GU	21 ± 1.5 (19-23)	23 ± 3 (18-32)	20 ± 1.5 (18-22)	18.2 ± 1.7 (16-21)	19 ± 1.8 (15-21)
D%	103 ± 3.7 103 ± 3.7	129 ± 9 (114-149)	70 —	85.8 ± 3.2 (77.9-91.6)	88.7 ± 3.1 (80-93)
SW%	152 ± 20 (120-187)	167 ± 2 (130-196)	182 ± 18 (138-208)	160.6 ± 14.7 (138-178)	138.5 ± 15.2 (111-163)
GS%	51 ± 3.2 (44-56)	56 ± 7 (43-70)	50 ± 5 (44-61)	49.8 ± 4.1 (43.2-54.1)	53.9 ± 4.1 (47-64)

L = body length; W = body width; NR = distance from anterior end to nerve ring; EP = distance from anterior end to excretory pore; ES = distance from anterior end to end of pharynx; T = tail length; ABW = anal body width; SP = spicule length; GU = gubernaculum length; D(%) = (EP/ES); SW(%) = SP/ABW and GS(%) = GU/SP.
AM = *Heterorhabditis amazonensis*; MX = *Heterorhabditis mexicana*; BJ = *Heterorhabditis baujardi*; GL = *Heterorhabdtis* sp. GL; SG = *Heterorhabditis* sp. SG.

collected at Universidade Federal de Lavras (UFLA). The symptomatology shown by *G. mellonella* larvae was characteristic of infection for nematodes of *Heterorhabditis* genus, with a dark red coloration. According to Poinar and Thomas (1984), insects parasitized by heterorhabditids present a red coloration due to the presence of the symbiotic bacterium. The populations were named in this work as *Heterorhabditis* sp. GL and *Heterorhabditis* sp. SG.

Table 5. Comparative morphometrics (µm) of infective juveniles of *Heterorhabditis* isolate GL, *Heterorhabditis* isolate SG and related species.

Character	AM Andaló *et al.*, 2006	MX Nguyen *et al.*, 2004	BJ Phan *et al.*, 2003	GL Present study	SG Present study
L	589 ± 12 (567-612)	578 ± 23 (530-620)	551 ± 27 (497-595)	598 ± 12.7 (567-618)	506 ± 19.7 (465-541)
a	26 ± 1.3 (24-29)	25.8 (23.6-28.4)	28 ± 1 (26-30)	26 ± 1 (24.4-28.5)	22.2 ± 1.6 (20-25)
b	4.9 ± 0.3 (4,4-5,5)	4,6 (4.2-5.1)	4.8 ± 0.2 (4.5-5.1)	5.1 ± 0.3 (4.6-5.5)	4.4 ± 0.4 (3.8-5)
c	5.5 ± 0.2 (5.1-6.1)	5,9 (5.5-6.3)	6 ± 0.3 (6-6.7)	5.3 ± 0.1 (5-5.5)	5.1 ± 0.3 (4.6-5.7)
W	23 ± 1.2 (20-24)	23 ± 0.8 (20-24)	20 ± 2 (18-22)	23 ± 0.9 (21-24)	22.9 ± 1.7 (21-26)
NR	85 ± 4.9 (76-93)	81 ± 4.2 (74-88)	81 ± 3 (75-86)	92.9 ± 6.1 (83-104)	86.1 ± 11.4 (74-106)
EP	107 ± 6.1 (89-115)	102 ± 5.2 (83-109)	97 ± 3 (91-103)	101.6 ± 6.4 (91-112)	103 ± 11.4 (86-124)
ES	121 ± 6.6 (107-132)	122 ± 27 (104-142)	115 ± 3 (107-120)	117.7 ± 5.8 (111-128)	115 ± 10.5 (99-135)
T	107 ± 4.7 (98-115)	99 ± 4.2 (91-106)	90 ± 4 (83-97)	112.9 ± 3.2 (106-119)	98.6 ± 6.2 (89-112)
ABW	14 ± 1.4 (13-17)	15 ± 1.2 (12-17)	13 ± 0.7 (11-14)	15.9 ± 0.8 (15-18)	16.2 ± 1.7 (13-20)
D%	88 ± 2.7 (83-92)	81 ± 3 (72-86)	84 ± 3 (78-88)	86.1 ± 2.2 (81-90)	89.3 ± 2.7 (85-94)
E%	100 ± 6 (89-109)	104 ± 5.2 (87-111)	108 ± 4 (98-114)	89.9 ± 5.9 (81-99)	104.1 ± 9.9 (87-120)

L = body length; a = (L/W); b = (L/ES); c = (L/T); W = body width; NR = distance from anterior end to nerve ring; EP = distance from anterior end to excretory pore; ES = distance from anterior end to end of pharynx; T = tail length; ABW = anal body width; D(%) = (EP/ES); E (%) = (EP/T).
AM = *Heterorhabditis amazonensis*; MX = *Heteorhabditis mexicana*; BJ = *Heterorhabditis baujardi*; GL = *Heterorhabdtis* sp. GL; SG = *Heterorhabditis* sp. SG.

One of the favorable conditions that may have facilitated the occurrence of EPN in the garlic field was the humidity, due to constant irrigation in this area. The soil sample collected of sorghum field may have been favorable to the presence of EPN due the hot and humid period of the year. Some other factors such as competitor, contaminates and antagonistic organisms can negatively influence the infectivity and the pathogenicity of nema-

Table 6. Sequence length (base pairs = bp) and composition of D2D3 region of species of *Heterorhabditis*.

Species	A	C	G	T	Sites (bp)
H. amazonensis	0.26087	0.19398	0.29208	0.25307	897
H. georgiana	0.25618	0.19663	0.29326	0.25393	890
H. bacteriophora	0.25506	0.19888	0.29438	0.25168	890
H. floridensis	0.25558	0.19643	0.29464	0.25335	896
H. indica	0.25864	0.19398	0.29208	0.25530	897
H. marelatus	0.25814	0.19529	0.29630	0.25028	891
H. megidis	0.25084	0.20269	0.30347	0.24300	893
H. mexicana	0.25475	0.19665	0.29609	0.25251	895
H. zealandica	0.25140	0.19753	0.29966	0.25140	891

todes to the insects. These factors may have hindered the attainment of other populations.

Pathogenicity test

Infective juveniles of both populations of nematodes as *Heterorhabditis* sp. GL and *Heterorhabditis* sp. SG infected *G. mellonella*, *D. speciosa* and *S. frugiperda* larvae and no difference of virulence was found between the GL and SB populations. *Heterorhabditis* sp. GL caused 73.3, 70 and 76% mortality of *G. mellonella*, *D. speciosa* and *S. frugiperda* larvae, respectively. *Heterorhabditis* sp. SG caused 80% mortality to the insects. In the control, it was observed mortality only of *G. mellonella* larvae (3.33%) (Table 1).

In the one by one (1:1) assay, it was possible to observe hermaphroditic females in all dead larvae, confirming that both populations belong to *Heterorhabditis* genus.

Morphometric analysis of entomopathogenic nematodes

Morphological studies show that the isolate *Heterorhabditis* sp. GL belongs to *Heterorhabditis indica* group (*Heterorhabditis indica* Poinar, Karunakar and David, 1992, *Heterorhabditis baujardi* Phan, Subbotin, Nguyen and Moens, 2003, *Heterorhabditis amazonensis* Andaló, Nguyen and Moino, 2006 and *Heterorhabditis mexicana* Nguyen *et al.*, 2004) but it is closer to *H. baujardi* and *H. amazonensis*. Both populations differ from the other members of the "*H.* indica group" in some aspects.

Heterorhabditis isolate GL differs in characters as body length; distance of anterior end to nervous ring; tail length and E% for IJ and, for males also the tail length and gubernaculum size. Males are bigger than males of *H. mexicana*, but are smaller than the others from "*H.* indica group". However, the length of infective juveniles shows that they are bigger than all the others in this group (Tables 2, 4 and 5).

Infective juveniles of *Heterorhabditis* isolate SG differs from the others in the values a, b and c; body length is the smallest found in "*H.* indica group". For males differences are observed in tail length, D% and SW%. Males of this isolate are smaller than *H. baujardi*, but bigger than the others (Tables 3, 4 and 5).

Description on scanning electron microscopy and light microscopy

SEM and light microscopy photographs show that morphological structures of *Heterorhabditis* isolate GL and *Heterorhabditis* isolate

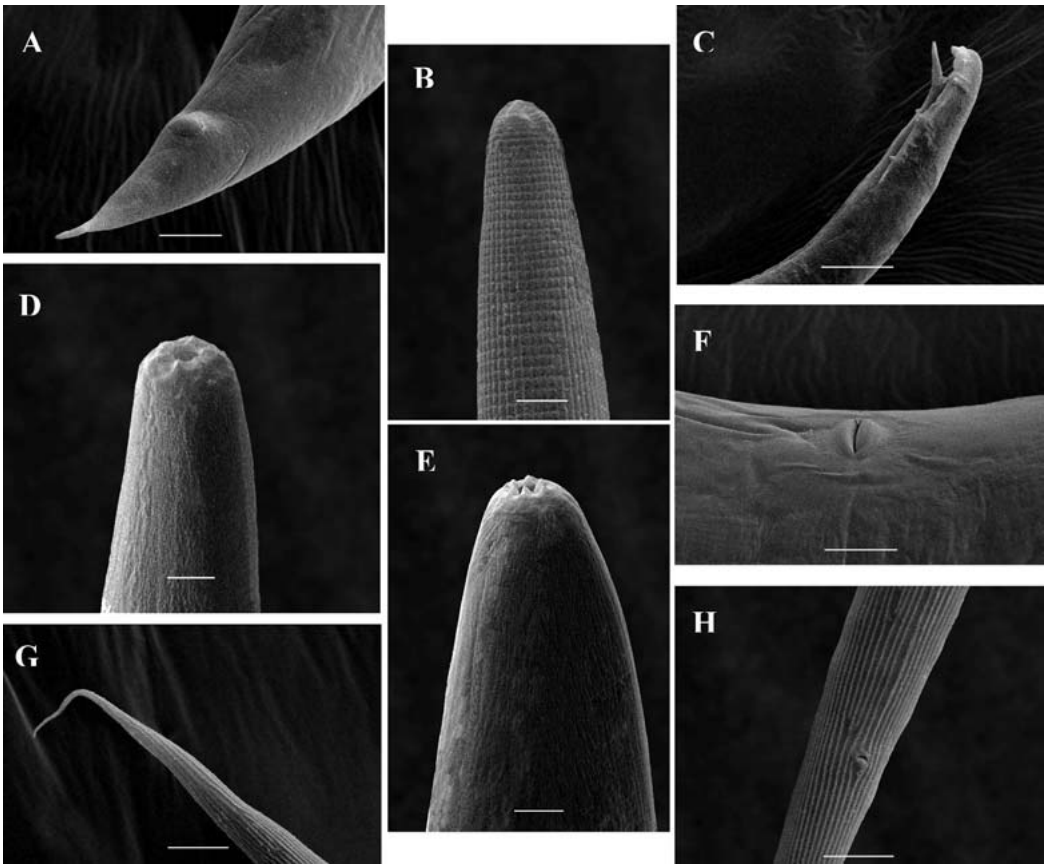

Fig. 1. Scanning electron microscopy photographs of *Heterorhabditis* sp. GL. A: Posterior region of hermaphoditic female; B: Anterior region of infective juvenile. C-D: Male; C: Posterior region; D: Anterior region. E-F: Hermaphoditic female; E: Anterior region; F: Vulva. G-H: Infective juvenile; G: Posterior region; H: Anus. Scale bars: A, F = 20 µm; E, G, H = 10 µm; B, D = 4 µm; C = 30 µm.

SG are similar to structures found in *H. amazonensis*. Light microscopy shows the males of both the GL and SG populations curve ventrally when killed by gentle heat. With SEM, the male head region is truncated and slightly swollen (Figs. 1B and 2 B). The anterior end has six labial papillae and cephalic papillae were not observed on the anterior end (Figs. 1D and 2B). The spicules were paired and separate. The bursa has 7 to 8 pairs of papillae with two pairs in the terminal group. The tail is conoid and slightly curved ventrally (Figs. 1C and 2G).

Hermaphroditic females are C-shaped after killing with gentle heat. Six labial papillae were observed in face view (Figs. 1E and 2 B). The body is robust, with many eggs in young females and with many eggs and juveniles in mature females. Lateral field and phasmids were not observed. Vulva is a transverse slit, located in a protuberant region slightly anterior to mid-body. In ventral view, it has elliptical form, surrounded by a unique pattern in form of a rough area radiating from vulva opening. Close to the vulva opening many wart-like

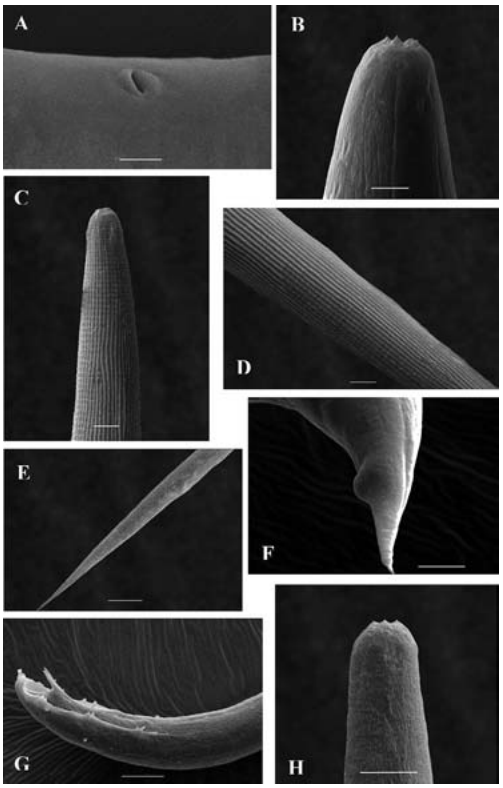

Fig. 2. Scanning electron microscopy photographs of *Heterorhabditis* sp. SG. A-B: Hermaphoditic female; A: Vulva; B: Anterior region. C-E: Infective juvenile; C: Anterior region; D: Tesselate pattern; E: Tail. F: Posterior region of hermaphroditic female. G-H: Male; G: Posterior region; H: Anterior region. Scale bars: A, E, F, G = 20 µm; B, H = 10 µm; C, D = 4 µm.

structures are present, characteristic of *H. amazonensis* and different from other species of *Heterorhabditis* (Figs. 1F and 2A). The tail is conoid with a pointed terminus. Post-anal swelling is present (Figs. 1A and 2F). Amphimictic females are similar to hermaphroditic, however they are smaller. Exudates or coupling plug were observed covered the vulva of amphimictic females.

Infective juvenile with a sheath (second stage-cuticle) present, however many infective juveniles (IJ) lost their sheath in storage. The anterior part of the IJ body has tessellate pattern and the posterior part has longitudinal ridges (Figs. 1B and 2C, D). The tail is long and pointed (Figs. 1G and 2E). The excretory pore is located posterior to the nerve ring just anterior to the base of the esophagus.

Molecular characterization

The D2D3 expansion regions of the new isolates, flanked by the primer D2F and 536, are characterized by the sequence length (897 bp) and nucleotide usage composition (A = 0.26087, C = 0.19398, G = 0.29208, T = 0.25307). The closest species is *H. indica* which has a similar sequence length to the new isolates (897 bp) and the most divergent taxon are *H. bacteriophora* and *H. georgiana* (890 bp) which is seven bp smaller than that of the new isolates (Table 6). It was observed that *Heterorhabditis* isolate GL and *Heterorhabditis* isolate SG have sequences identical to those of *H. amazonensis*, characterized by the same composition of nucleotides (Andaló *et al.*, 2006). It is inferred from the above molecular data, that both *Heterorhabditis* isolates (GL and SG) obtained from Lavras, MG, Brazil, are isolates of the species *H. amazonensis*.

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