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First Report of *Heterorhabditis amazonensis* from Venezuela and Characterization of Three Populations

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Abstract: During a survey in western Venezuela in 2011, three new populations of *Heterorhabditis amazonensis* (LPV081, LPV156, and LPV498) were isolated. Some differences were found in terms of morphometry compared with the original description; however, the distance from the anterior end to the excretory pore is the most variable character; significantly shorter in all infective juveniles and in other developmental stages depending on the population. According to a Principal Component Analysis, LPV498 possesses more differences in morphometric characteristics and can be separated from the other two. Those intraspecific differences could be attributed to the geographic origin of the nematode. Molecular studies of ITS regions demonstrated that the sequences of the Venezuelan strains were identical to those of the type species originally isolated in the Brazilian Amazonian forest. This is an interesting fact because in several studies on heterorhabditids, intraspecific variability has been recorded. Herein, we present the first report of *H. amazonensis* in Venezuela and the characterization of three populations of this species.
Key words: biogeography, entomopathogenic nematode, *Heterorhabditis amazonensis*, morphology, Venezuela.

Entomopathogenic nematodes (EPNs) of the genus *Heterorhabditis* and *Steinernema* have been used as biological control agents for many years because of their effectiveness with many insects. Both nematode genera reveal differences in host range, field performance, and environmental tolerance. For these reasons, many surveys have been done and are currently in progress around the globe to find indigenous EPNs capable to become biological control agents in local pest management programs (Burnell and Stock, 2000).

The genus *Heterorhabditis* comprises 18 species and despite their worldwide distribution, its diversity is lower than *Steinernema* with 95 recognized species up to date. In Venezuela, 29 *Heterorhabditis* isolates have been isolated, belonging to the species *H. amazonensis* Andaló et al., 2006 and *Heterorhabditis indica* Poinar et al., 1992 (San-Blas et al., 2015).

Heterorhabditis amazonensis, was described by Andaló et al., in 2006 by baiting of soil samples in the northern forest of Amazonas state, near the city of Benjamin Constant, Brazil. Extensive sampling in the same country has demonstrated the presence of *H. amazonensis* in many other regions besides the Amazonian area (Andaló et al., 2009).

Steinernematid and heterorhabditid strains were isolated in western Venezuela in a survey carried out in 2011. Three species of the heterorhabditids as populations of *H. amazonensis*. In this paper, we present the first taxonomical and molecular report of *H. amazonensis* in Venezuela.

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Materials and Methods

Sampling procedure: Three *H. amazonensis* populations were isolated from composite soil samples (seven sub samples, *ca* 1 kg each) collected from (i) LPV-081 at a Plantain-maize field close to Santa Barbara (Zulia State), 9° 06′ 52′′N; 71° 28′ 54′′ W; (ii) LPV-156 at a cultivated grassland close to Bobures town (Zulia State), 9° 13′ 49′′ N; 71° 10′ 42′′ W; and (iii) LPV-498 at a natural grassland close to Barinas City (Barinas State), 8° 33′ 18′′ N; 70° 23′ 35′′ W. *Galleria mellonella* L. was used as insect bait (Bedding and Akhurst, 1975). Soil samples with the insect larvae were kept at 25°C in darkness and after 7 d the dead larvae were removed daily (up to 14 d), and placed in White traps (White, 1927). Emerging infective juveniles (IJs) from the traps were stored at 20°C for further studies.

Morphological and morphometrical characterization: Ten *G. mellonella* were exposed to IJs (100 IJs per *G. mellonella*) in a 9.0-cm petri dish lined with a moistened filter paper and kept in the dark at 25°C. First and second generation males and females were collected at the 4th and 7th d, respectively, by dissecting the dead *G. mellonella* cadavers in Ringer's solution. To obtain IJs, dead *G. mellonella* were placed in White traps and nematodes collected within the first week.

Twenty hermaphrodites, males and females were heat killed at 60°C for 5 min and fixed with TAF (7 ml formalin, 2 ml triethanolamine, 91 ml distilled water) (Courtney et al., 1955) for light microscope observations on permanent slides. Fixed nematodes were processed with glycerol by slow evaporation, mounted with a piece of Parafilm® (Bemis, USA) used as cover glass support and the slides were sealed with nail polish. Infective juveniles were mounted in Ringer's solution on a glass slide using little pieces of paper as cover glass support, and measured. The tail region with bursa of 20 males per strain was stained with acid fuchsin (Nguyen, 2007). Measurements and observations were done using a Leica DM2500 compound microscope (Leica Microsystems, Germany) fitted with

a differential interference contrast system. These data were compared with the original description (Andaló et al., 2006).

Statistical analysis: Measurements of hermaphrodites, males, females, and IJs were compared between populations. All data were checked for normality using the probability plot function in Minitab (Minitab Inc., USA). Values calculated in percentage were angular transformed when the values were only possible between 0% and 100% (i.e., relative position of the vulvae [%V] in hermaphrodites and females, hyaline portion of IJ tails [%H]). The rest of the percentage values (%D, %E, etc.) were transformed in proportions and evaluated as such (all percentage data were displayed in figures untransformed). Analysis of variance tests were performed to observe differences between the measured characters related to the populations. Principal components analysis was also done for IJs measurements using total length (L), W, excretory pore (EP), nerve ring (NR), esophagus (ES), tail length T, and anal body width (ABW) (description in Table 1). Ratios were not used to avoid over-representation of the variables in order to observe if the studied populations were different from each other in terms of their entire morphometry data.

Molecular characterization: DNA was extracted from single hermaphrodites (Li et al., 2012). Each specimen was transferred into a sterile Eppendorf tube (Eppendorf, Germany) (250 μl) with 20 μl of extraction buffer (17.7 μl of ddH2O, 2 μl of 10 × PCR buffer, 0.2 μl of 1% tween, and 0.1 μl of proteinase K). Buffer and nematode were frozen at −20°C for 20 min and then immediately incubated at 65°C for 1 hr, followed by 10 min at 95°C. The lysates were cooled on ice, centrifuged (2 min, 9,000 g) and 1 μl of supernatant used for PCR. A fragment of rDNA containing the internal transcribed spacer regions (ITS1, 5.8S, ITS2) and flanking regions of 18S and 28S rDNA was amplified using primers 18S: 5′-TTGATTACGTCCCTG CCCTTT-3′ (forward), and 28S: 5′-TTTCACTCGCC GTTACTAAGG-3′ (reverse) (Vrain et al., 1992). The PCR master mix consisted of ddH2O 7.25 μl, 10 × PCR buffer 1.25 μl, dNTPs 1 μl, 0.75 μl of each forward and reverse primers, polymerase 0.1 and 1 μl of DNA-extract. The PCR profile consisted of one cycle of 94°C for 7 min followed by 35 cycles of 94°C for 60 sec, 45°C for 60 sec, 72°C for 60 sec and a final extension at 72 °C for 7 min (Nguyen, 2007). PCR was followed by electrophoresis (45 min, 120 V) of 2 μl of PCR product in a 1% TAE buffered agarose gel stained with ethidium bromide (20 μl ETB per 100 ml of gel).

The PCR products were sequenced by Macrogen Inc. (Korea) and deposited in GenBank under accession numbers KM211575 (LPV081), KM211576 (LPV156), and KM211577 (LPV498). The sequences were edited and compared with those deposited in GenBank by means of a Basic Local Alignment Search Tool of the National Centre for Biotechnology Information (NCBI). An alignment of our samples together with other sequences of the species of *Heterorhabditis* was produced by default ClustalW parameters in MEGA 6.0 (Tamura et al., 2013) and optimized manually in BioEdit (Hall, 1999). Pairwise distances were computed using MEGA 6.0 (Tamura et al., 2013). Codon positions included were 1st + 2nd + 3rd + Noncoding.

Table 1. Morphometrics of *Heterorhabditis amazonensis* Andaló et al., 2006 (LPV081 strain).

Character	Developmental stage			
	Hermaphrodite	Male	Female	Infective juvenile
N	20	20	20	20
Total length (L)	4,118 ± 235 (3,562–4,980)	798 ± 128 (719–905)	1,674 ± 194 (1,376–1,963)	503 ± 225 (454–549)
a (L/W)	-	-	-	20 ± 3 (17–22)
b (L/ES)	-	-	-	4.4 ± 0.4 (3.9–5.3)
c (L/T)	-	-	-	5.1 ± 0.6 (4.8–5.9)
Vulva position (%V)	44 ± 4 (39–49)	-	48 ± 5 (44–53)	-
Maximum body diameter (W)	223 ± 12 (189–279)	46 ± 10 (40–50)	126 ± 18 (101–156)	24 ± 6 (24–25)
Excretory pore (EP)	163 ± 5 (140–174)	93 ± 4 (89–96)	121 ± 4 (115–126)	92 ± 7 (85–98)
Nerve ring (NR)	133 ± 10 (122–149)	81 ± 6 (71–87)	103 ± 6 (90–113)	77 ± 4 (72–88)
Pharynx (ES)	196 ± 12 (179–207)	107 ± 12 (101–113)	144 ± 14 (126–156)	114 ± 13 (104–122)
Testis reflexion	-	48 ± 5 (34–38)	-	-
Tail length (T)	120 ± 7 (105–41)	34 ± 3 (31–38)	91 ± 8 (87–97)	97 ± 7 (89–105)
Tail without sheath (TWS)	-	-	-	71 ± 4 (65–79)
Anal body diameter (ABD)	55 ± 6 (47–62)	26 ± 2 (23–28)	32 ± 3 (28–35)	15 ± 1.7 (14–16)
Spicule length (SL)	-	42 ± 4 (39–44)	-	-
Gubernaculum length (GL)	-	21 ± 2 (18–22)	-	-
D% = (EP/ES) × 100	-	86 ± 7 (82–90)	-	81 ± 6 (73–87)
E% = (EP/T) × 100	-	-	-	95 ± 11 (86–104)
SW% = (SL/ABD) × 100	-	162 ± 24 (144–184)	-	-
GS% = (GL/SL) × 100	-	51 ± 2 (46–54)	-	-
H% = (H/T) × 100	-	-	-	26 ± 4 (20–30)

All measurements are in μm and in the form: mean ± SD (range).

TABLE 2. Morphometrics of *Heterorhabditis amazonensis* Andaló et al., 2006 (LPV156 strain).

Character	Developmental stage			
	Hermaphrodite	Male	Female	Infective juvenile
N	20	20	20	20
Total length (L)	3,811 ± 278 (3,126–4,622)	788 ± 144 (687–873)	1,599 ± 151 (1,137–1,859)	497 ± 148 (462–540)
a (L/W)	-	-	-	21 ± 2 (19–24)
b (L/ES)	-	-	-	4.6 ± 0.7 (4.3–4.9)
c (L/T)	-	-	-	5 ± 0.5 (4.1–5.6)
Vulva position (%V)	43 ± 5 (36–47)	-	48 ± 5 (44–57)	-
Maximum body diameter (W)	210 ± 23 (184–278)	45 ± 8 (43–50)	123 ± 13 (90–145)	22 ± 3 (21–25)
Excretory pore (EP)	171 ± 13 (152–188)	87 ± 6 (81–90)	80 ± 4 (72–102)	91 ± 9 (85–98)
Nerve ring (NR)	129 ± 11 (121–138)	74 ± 7 (69–80)	69 ± 7 (57–95)	78 ± 11 (73–81)
Pharynx (ES)	199 ± 12 (184–220)	105 ± 16 (101–110)	118 ± 16 (104–150)	105 ± 15 (94–109)
Testis reflexion	-	40 ± 6 (37–43)	-	-
Tail length (T)	126 ± 6 (110–148)	36 ± 5 (33–39)	86 ± 6 (75–117)	97 ± 4 (86–110)
Tail without sheath (TWS)	-	-	-	63 ± 4 (54–72)
Anal body diameter (ABD)	53 ± 7 (41–71)	27 ± 6 (24–30)	31 ± 3 (25–40)	14 ± 2.5 (13–18)
Spicule length (SL)	-	42 ± 4 (40–46)	-	-
Gubernaculum length (GL)	-	21 ± 2 (19–22)	-	-
D% = (EP/ES) × 100	-	82 ± 9 (74–87)	-	87 ± 6 (78–97)
E% = (EP/T) × 100	-	-	-	94 ± 5 (86–99)
SW% = (SL/ABD) × 100	-	156 ± 37 (131–191)	-	-
GS% = (GL/SL) × 100	-	49 ± 5 (45–54)	-	-
H% = (H/T) × 100	-	-	-	32 ± 4 (25–38)

All measurements are in μm and in the form: mean ± SD (range).

The phylogenetic trees were obtained by Bayesian inference (BI) and maximum likelihood (ML). All characters were treated as equally weighted and gaps as missing data. *Caenorhabditis elegans* was used as an outgroup taxon and to root the trees. Bayesian phylogenetic reconstruction was performed using MrBayes 3.1.1. (Huelsenbeck and Ronquist, 2001). The best-fit model was identified as the GTR + G model test using the MrModeltest 2.0 program (Nylander, 2004). Metropolis-coupled Markov chains Monte Carlo generations were run for 10,000,000 cycles and one tree was retained every 1,000 generations and a burn-in of 3,000 generations (Huelsenbeck and Ronquist, 2001). Maximum likelihood was implemented in MEGA 6.0 (Tamura et al., 2013) using Tamura 3-parameter model (Tamura, 1992) that was selected as the best-fit model using the same program. Initial trees for the heuristic search were obtained by applying the neighbor–joining

TABLE 3. Morphometrics of *Heterorhabditis amazonensis* Andaló et al., 2006 (LPV498 strain).

Character	Developmental stage			
	Hermaphrodite	Male	Female	Infective juvenile
N	20	20	20	20
Total length (L)	3,942 ± 210 (3,389–5,119)	832 ± 160 (669–977)	1,428 ± 180 (1,231–1,661)	542 ± 280 (514–582)
a (L/W)	-	-	-	21 ± 3 (19–23)
b (L/ES)	-	-	-	5.4 ± 0.5 (4.9–6.2)
c (L/T)	-	-	-	6.1 ± 0.8 (5.1–7.2)
Vulva position (%V)	44 ± 2 (40–51)	-	51 ± 2 (46–56)	-
Maximum body diameter (W)	215 ± 10 (181–284)	47 ± 10 (43–54)	101 ± 10 (92–118)	26 ± 4 (22–27)
Excretory pore (EP)	170 ± 6 (142–201)	96 ± 6 (84–101)	91 ± 5 (86–99)	78 ± 5 (66–97)
Nerve ring (NR)	118 ± 8 (94–138)	80 ± 6 (71–88)	72 ± 6 (63–78)	67 ± 2 (58–78)
Pharynx (ES)	200 ± 10 (162–231)	110 ± 10 (100–117)	120 ± 10 (109–140)	99 ± 8 (88–107)
Testis reflexion	-	38 ± 4 (34–44)	-	-
Tail length (T)	130 ± 8 (101–165)	36 ± 2 (28–40)	81 ± 5 (63–97)	87 ± 6 (81–103)
Tail without sheath (TWS)	-	-	-	67 ± 4 (51–84)
Anal body diameter (ABD)	57 ± 5 (41–79)	29 ± 3 (26–37)	28 ± 3 (25–31)	15 ± 1.5 (14–17)
Spicule length (SL)	-	41 ± 2 (36–45)	-	-
Gubernaculum length (GL)	-	21 ± 2 (18–25)	-	-
D% = (EP/ES) × 100	-	87 ± 5 (79–95)	-	78 ± 4 (66–91)
E% = (EP/T) × 100	-	-	-	86 ± 5 (63–103)
SW% = (SL/ABD) × 100	-	140 ± 20 (115–163)	-	-
GS% = (GL/SL) × 100	-	50 ± 3 (44–60)	-	-
H% = (H/T) × 100	-	-	-	27 ± 2 (21–36)

All measurements are in μm and in the form: mean ± SD (range).

method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites. Branch support was estimated by bootstrap analysis (1,000 replicates).

Results

Morphometric characterization: In hermaphrodites, all measurements were similar except for the distance from the anterior end to NR (Tables 1–3) (Fig. 1A), which in the LPV-489 population was significantly smaller (118.31 ± 13.72 µm; $p \leq 0.001$; $F = 14.52$; $\alpha = 0.05$) than the other two. Females showed differences in some characteristics (Tables 1–4) (Fig. 1B,C), such as L ($P \leq 0.001$; $F = 11.2$; $\alpha = 0.05$), the distance from the anterior end to EP ($P \leq 0.001$; $F = 254.6$; $\alpha = 0.05$), NR ($P \leq 0.001$; $F = 118.4$; $\alpha = 0.05$), the total length of the ES ($P \leq 0.001$; $F = 59.1$; $\alpha = 0.05$), and the relative position of the vulva (V) ($P = 0.004$; $F = 6.21$; $\alpha = 0.05$). Males were different in the following characteristics (Tables 1–3) (Fig. 1D): EP ($p \leq 0.001$; $F = 31.4$; $\alpha = 0.05$), NR ($P \leq 0.001$; $F = 16.3$; $\alpha = 0.05$), ES ($P = 0.002$; $F = 7.19$; $\alpha = 0.05$), %D ($P \leq 0.001$; $F = 15.7$; $\alpha = 0.05$), and %SW ($P \leq 0.001$; $F = 15.5$; $\alpha = 0.05$).

The most extended differences among the three populations occurred in the IJs stages (Tables 1–4) (Fig. 2): L ($P \leq 0.001$; $F = 37.24$; $\alpha = 0.05$), EP ($P \leq 0.001$; $F = 22.9$; $\alpha = 0.05$), NR ($P \leq 0.001$; $F = 20.7$; $\alpha = 0.05$), ES ($P \leq 0.001$; $F = 37.3$; $\alpha = 0.05$), T ($P \leq 0.001$; $F = 22.6$; $\alpha = 0.05$); the ratios a ($P \leq 0.001$; $F = 9.98$; $\alpha = 0.05$), b ($P \leq 0.001$; $F = 50.5$; $\alpha = 0.05$), and c ($P \leq 0.001$; $F = 39.5$; $\alpha = 0.05$); and the value of %D ($P \leq 0.001$; $F = 15.6$; $\alpha = 0.05$), %E ($P \leq 0.001$; $F = 39.3$; $\alpha = 0.05$), and %H ($P \leq 0.001$; $F = 14.95$; $\alpha = 0.05$).

PCA results (Fig. 3) demonstrated that the population LPV-498 was different in terms of their morphometry from the other two studied populations. The first principal component had variance (eigenvalue) of 3.67, accounted for 52.4% and was represented by NR (0.47), ES (0.46), ES (0.38), and T (0.36). The second principal component had variance of 1.3, accounted for 18.3%, and was represented by ABW (0.69), ES (0.44), W (0.39), and L (0.31).

Molecular characterization and phylogenetic analysis

Sequencing of the PCR products of all three populations produced identical sequences within them and with the reference sequence of *H. amazonensis*

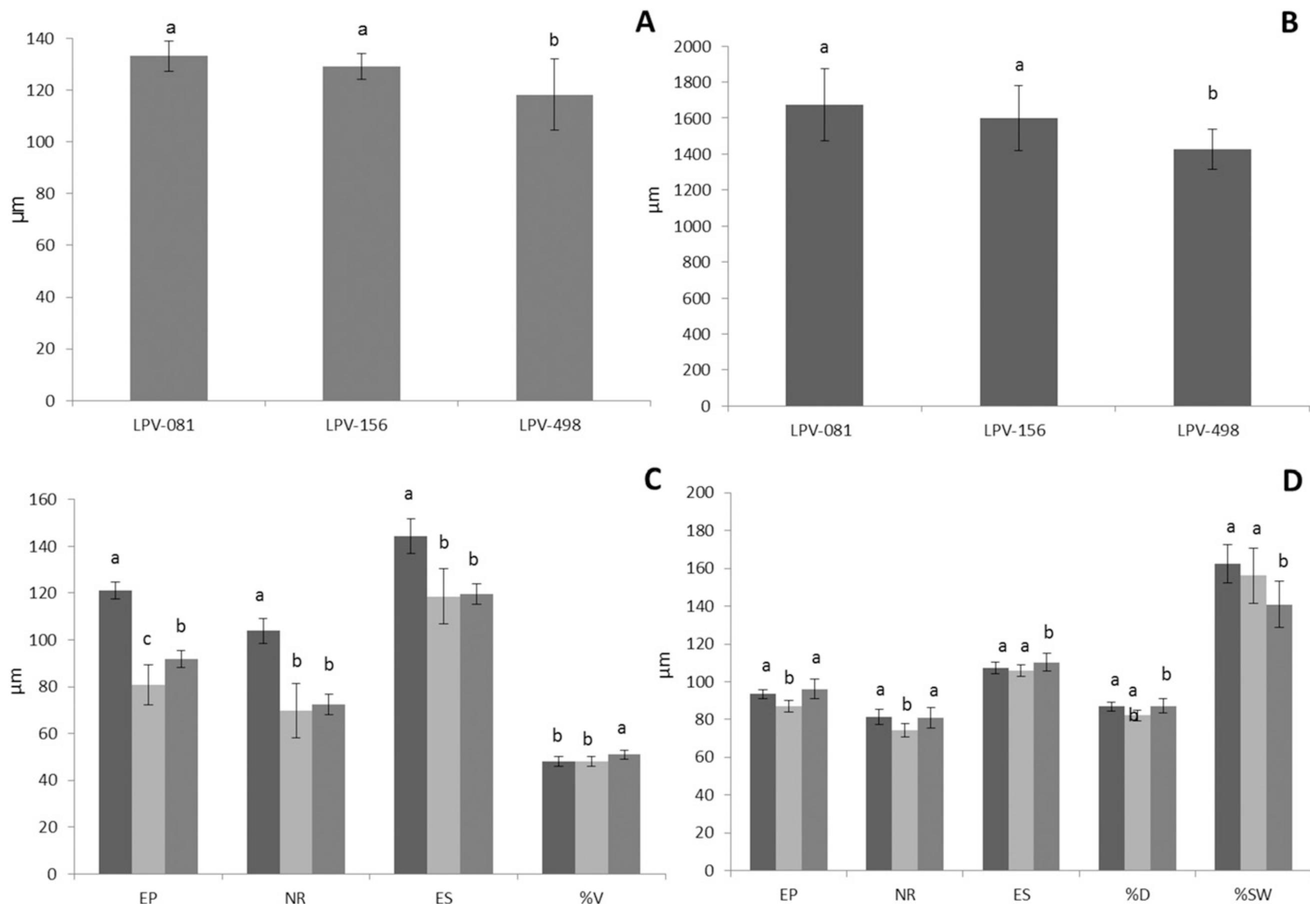

Fig. 1. Morphometrical differences between three *Heterorhabditis amazonensis* Andaló et al., 2006 populations: LPV081 (dark gray), LPV156 (light gray), and LPV498 (medium gray). A. Distance from anterior end to nerve ring (NR) in hermaphrodites. B. Total length of females. C. Distance from anterior end to excretory pore (EP), distance from anterior end to NR, pharynx length (ES), relative position of the vulva (%V) of females. D. Distance from anterior end to EP, distance from anterior end to NR, ES, in males. Different letters indicate significant differences ($p < 0.05$).

TABLE 4. Comparative morphometrics (μm) of infective juveniles of *Heterorhabditis amazonensis* Andaló et al., 2006 isolated in Brazil and Venezuela.

Character	Andaló et al., 2006	Andaló et al., 2009		Present study		
	Type population (Brazil)	GL population (Brazil)	MG population (Brazil)	LPV081 (Venezuela)	LPV156 (Venezuela)	LPV498 (Venezuela)
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)
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)
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)
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)
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)
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)
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)
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)
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)
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)
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)
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)

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, ABD = anal body width, D (%) = (EP/ES), E (%) = (EP/T).

(DQ665222) (Table 5). Both phylogenetic analyses produced the same topology represented by the BI tree (Fig. 4). The analyses showed a well-supported monophyletic group of *H. amazonensis* Venezuelan strains with the Brazilian population (Fig. 4).

DISCUSSION

Until now, *H. amazonensis* had been reported only in Brazil, and thus finding of this species in Venezuela extends its known distribution. The nematode was originally found in an undisturbed soil from a forest in the Brazilian Amazonas State, but in Venezuela these populations have been isolated in various agricultural systems (see sample procedure).

The three populations showed some differences compared with the original description in terms of morphometry; however, the distance from the anterior end to the excretory pore was the most variable character; significantly shorter in all IJs and in other developmental stages depending on the population (Tables 1–4). According to the statistical analysis, the LPV498 population possesses more differences in morphometric characteristics and can be separated from the other studied populations found in Venezuela (Fig. 3). The comparison of the IJ morphometrics of the Venezuelan populations against other three Brazilian isolates (Table 4) (Andaló et al., 2006; 2009) reveal some differences but molecularly remain identical. Those intraspecific differences have been attributed to the geographic origin of the nematodes under study, different environmental conditions and host interactions (Stock et al., 2000); similar results have been reported in other steinernematid species (Poinar, 1992; Stock et al., 1997). The differences in morphometric characteristics of LPV-498 could be explained by geographic isolation resulting from the Andean mountains.

Our results indicate that *H. amazonensis* is a more widely distributed species than previously acknowledged, opening to the possibility of further extension of its habitat, as research programs extend across the tropical and subtropical Latin American countries. The type locality in Brazil is part of the Amazonian forest. However, the locality of the Venezuelan sites suggests that the species could be distributed across the Amazonian rainforest, the Colombian and Venezuelan Llanos (tropical grassland plain), and the Andes mountains, all ecosystems with enormous differences. Thus, we may think that *H. amazonensis* is not limited to any particular habitat.

The fact that the ITS of the tested strains of *H. amazonensis* were identical to the reference sequence of the geographically distant isolate is surprising. The genus *Heterorhabditis* is evolutionary younger in comparison to *Steinernema* (Adams et al., 2007) and thus it has

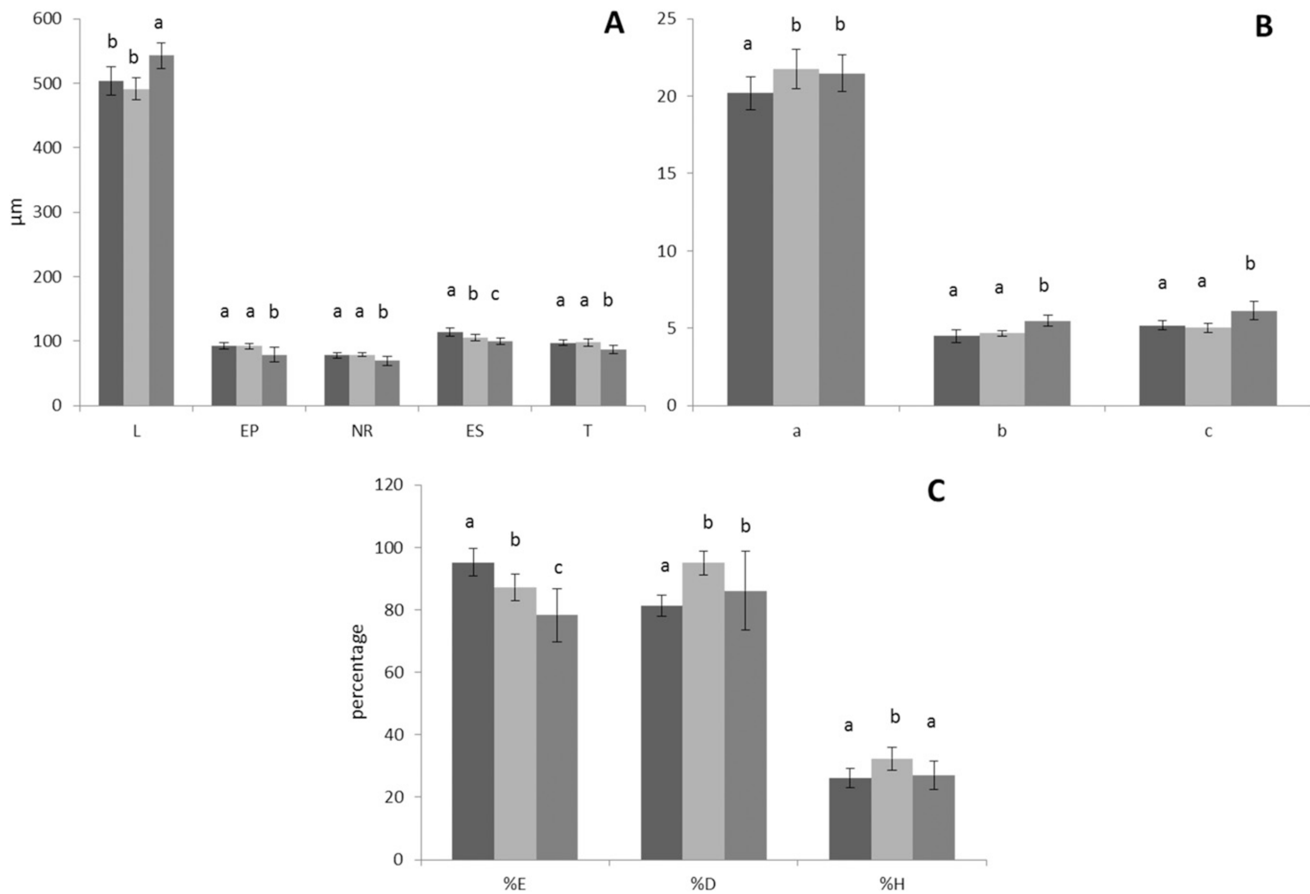

Fig. 2. Morphometrical differences between infective juveniles of 3 *Heterorhabditis amazonensis* Andaló et al., 2006 populations: LPV081 (dark gray), LPV156 (light gray), and LPV498 (medium gray). A. Total length (L), distance from anterior end to excretory pore (EP), distance from anterior end to nerve ring (NR), pharynx length (ES), tail length (T). B. a, b, and c ratios. C. E%, D%, and hyaline portion (%H). Different letters indicate significant differences ($p < 0.05$).

a lower intraspecific molecular variability. However, in several studies, some intraspecific variability has been recorded. For instance, Maneesakorn et al. (2011) observed intraspecific variability in the ITS sequence in *Heterorhabditis bacteriophora* Poinar, 1976 and *Heterorhabditis megidis* Poinar et al., 1987. Similarly, Li et al. (2012) reported

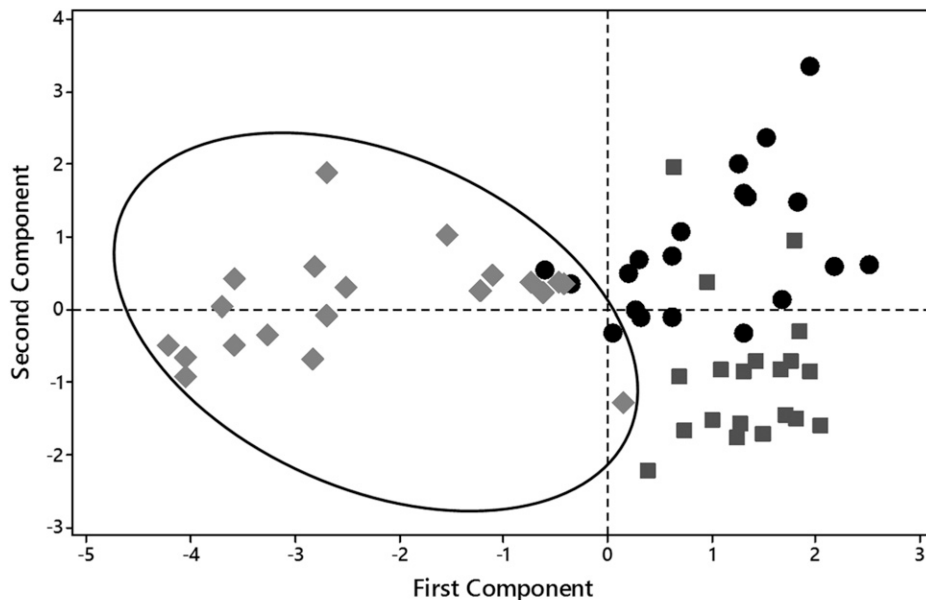

Fig. 3. PC1 to PC2 case score plots of morphometric values of three *Heterorhabditis amazonensis* Andaló et al., 2006 populations: LPV081 (black circles), LPV156 (dark gray squares), and LPV498 (light gray diamonds).

Table 5. Pairwise distances of the ITS regions of the rDNA between species of *Heterorhabditis*.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	KM211575 *H. amazonensis* LPV081		0	0	0	13	20	19	17	81	80	82	160	196	186	172	169	173	169	169	174
2	KM211576 *H. amazonensis* LPV156	100		0	0	13	20	19	17	81	80	82	160	196	186	172	169	173	169	169	174
3	KM211577 *H. amazonensis* LPV498	100	100		0	13	20	19	17	81	80	82	160	196	186	172	169	173	169	169	174
4	DQ665222 *H. amazonensis*	100	100	100		13	21	24	20	85	82	83	160	206	192	178	176	183	175	175	181
5	AF548768 *H. baujardi*	98.3	98.3	98.3	98.4		23	23	16	81	80	82	159	198	186	173	171	174	170	170	175
6	AY321478 *H. mexicana*	97.4	97.4	97.4	97.9	97.1		11	14	88	88	89	167	215	196	186	185	188	186	186	192
7	EF043443 *H. tayserae*	97.5	97.5	97.5	97.7	97.1	98.9		20	91	91	92	163	215	192	183	184	185	188	188	193
8	DQ372922 *H. floridensis*	97.8	97.8	97.8	98.1	98	98.6	98.1		90	88	89	164	211	195	183	182	189	180	180	186
9	JN620538 *H. noenieputensis*	89.1	89.1	89.1	91.6	89.4	91	91.1	91.2		12	11	155	204	189	175	176	175	172	174	171
10	FJ152545 *H. gerrardi*	89.2	89.2	89.2	90.4	89.5	89.7	89.3	89.7	98.6		3	153	198	186	172	171	170	170	172	170
11	AY321483 *H. indica*	88.9	88.9	88.9	91.5	89.3	90.9	90.6	90.9	98.9	99.6		154	203	188	174	174	173	174	176	173
12	HM230723 *H. atacamensis*	77.6	77.6	77.6	77.7	77.7	76.6	77.2	77	77.8	78.1	77.9		71	48	26	22	13	142	134	141
13	AY321481 *H. zaelandica*	73.6	73.6	73.6	79.1	74.2	78.1	78.1	78.5	78.8	76.1	78.9	90.2		100	79	78	83	187	184	190
14	AY321480 *H. megidis*	74.7	74.7	74.7	80.3	75.5	79.8	80.2	79.9	80.3	77.6	80.3	93.4	89.8		36	56	52	165	159	166
15	AY321482 *H. downesi*	76.5	76.5	76.5	81.7	77.1	80.9	81.2	81.2	81.7	79.2	81.8	96.4	92	96.3		36	31	153	146	154
16	AY321479 *H. marelatus*	77.1	77.1	77.1	82	77.6	81.1	81.2	81.4	81.7	79.4	81.9	97	92.1	94.3	96.4		25	151	144	153
17	EF488006 *H. safricana*	76.5	76.5	76.5	82	77.2	80.8	81.9	81.5	82.6	79.5	82	98.2	91.6	94.7	96.9	97.5		152	145	153
18	EU099032 *H. georgiana*	77.5	77.5	77.5	82.4	78.1	81.3	81.1	81.9	82.4	79.9	82.2	80.2	81.1	83.1	84.3	84.6	84.5		21	21
19	AY321477 *H. bacteriophora*	77.6	77.6	77.6	82.4	78.1	81.3	81.1	81.9	82.2	79.7	82	81.4	81.4	83.7	85.1	85.4	85.2	97.9		21
20	HQ896630 *H. beicherriana*	77	77	77	81.3	77.6	80.2	80.1	80.8	82	79.4	81.8	80.4	80.2	82.6	83.8	84	84	97.9	97.9	

Below diagonal: percentage similarity, above diagonal: total character differences.

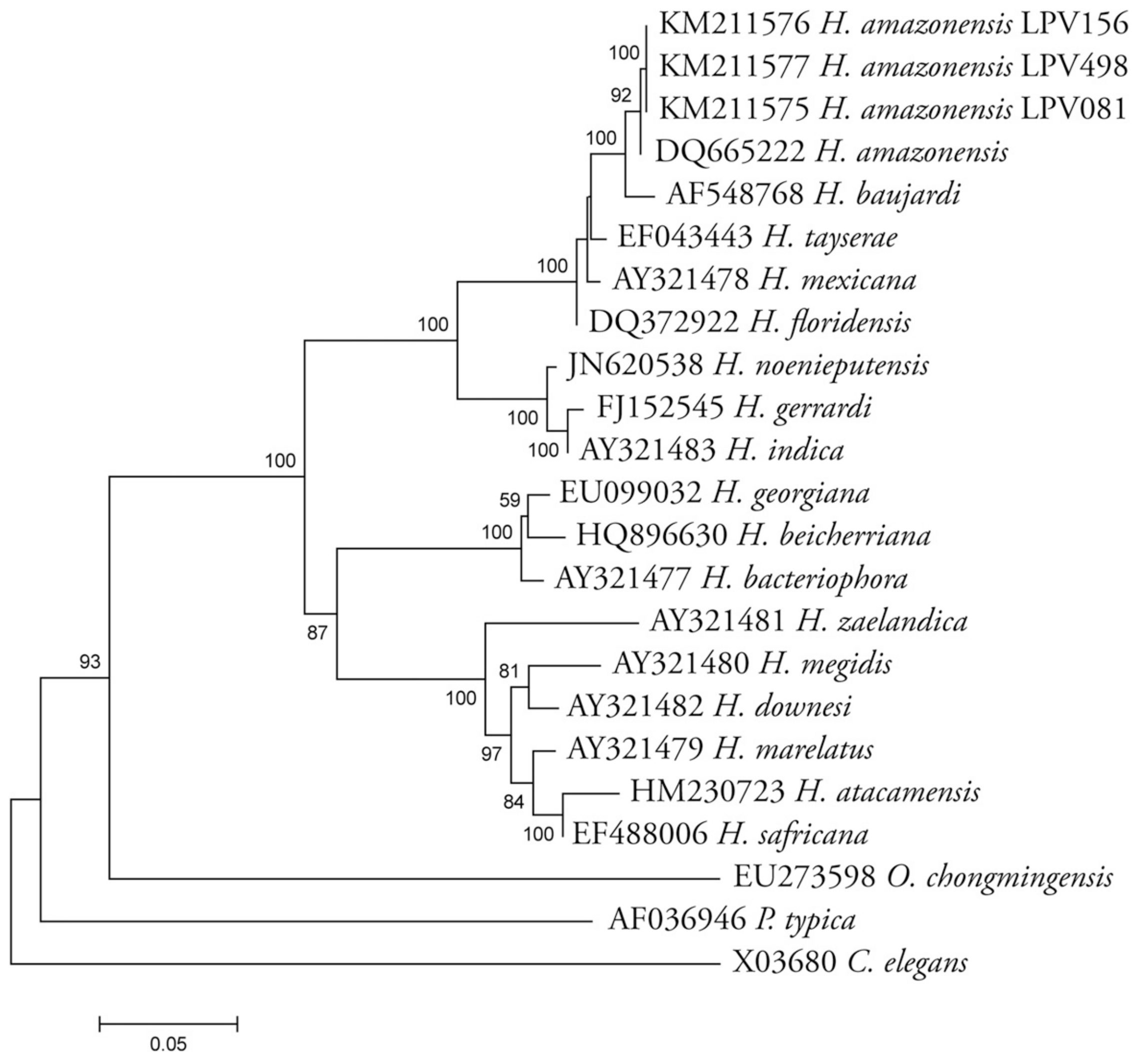

Fig. 4. Phylogenetic relationships of Venezuelan *Heterorhabditis amazonensis* populations based on analysis of ITS rDNA regions as inferred from Bayesian and maximum likelihood (ML) analyses. *Oscheius chongmingensis*, *Pellioditis typica*, and *Caenorhabditis elegans* were used as outgroup taxa. Support values are given next to the nodes in the form: Bayesian inference (BI) posterior probability/ML bootstrap value.

Chinese (GenBank number HQ896630) and Martinique (JX465738) populations of *Heterorhabditis beicherriana* that differ by 5 bp in the ITS sequence. This difference could be due to the continuous range of *H. amazonensis* that does not restrict the gene flow among the populations. Therefore, more populations of *H. amazonensis* and others species of this genus must be analyze, to support low intraspecific variation of ITS among populations of the same species, but geographically separated. Finally, the inclusion of mitochondrial DNA markers could lead to find lineages among distant populations of the same species with similarities on nuclear ITS marker.

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