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Variability study of entomopathogenic nematode populations (Heterorhabditidae) from Argentina

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Abstract

Entomopathogenic nematodes (EPN) belonging to the Heterorhabditidae family are lethal parasites of soil-dwelling insects. Two species were reported in Argentina: *Heterorhabditis argentinensis* and *Heterorhabditis bacteriophora* characterized mainly by morphometric features. In this work a comparative and phylogenetic study between five *Heterorhabditis* populations from Argentina was conducted to analyze the variability between strains and to evaluate the taxonomic position of *Heterorhabditis argentinensis*. The PCA analyses of morphometric characters separated the larger juvenile, female and male *H. argentinensis* from *H. bacteriophora* populations. The juvenile (IJs) stage provided the clearest separation of *Heterorhabditis* populations presenting the least variability between strains. The variable L and MBW were highly related to *H. argentinensis* IJs. Three groups were separated by this stage considering PC1 and PC2: one formed by *H. bacteriophora* OLI, RIV and RN strains, (isolates from Córdoba and Río Negro province), one for *H. bacteriophora* VELI strain (Buenos Aires province) and one for *H. argentinensis* (Santa Fe province). *Heterorhabditis bacteriophora* VELI and *H. argentinensis* isolated from regions with more rainfalls and humidity presented larger values for morphometric features. Molecular analyses showed the Argentinian populations (*H. bacteriophora* VELI strain and *H. argentinensis*), forming a same clade, with six other *H. bacteriophora* populations (not from Argentina) with a genetic similarity between them of 99%. *Heterorhabditis argentinensis* presented one unique nucleotide that was not present in any of the other species of the clade. Considering the results of this study *H. argentinensis* would be conspecific to *H. bacteriophora,* constituting a strain with a great morphometric variation where the host and climatic conditions could have influenced on the measurements.

Keywords: entomonematodes, Heterorhabditidae, variability, strains, Argentina.

Estudo da variabilidade entomopatogênicos nematóides populações (Heterorhabditidae) da Argentina

Resumo

Nematóides entomopatogênicos (EPN) pertencentes à família Heterorhabditidae são parasitas letais de insetos que vivem no solo. Duas espécies foram relatados na Argentina: *Heterorhabditis argentinensis* e *Heterorhabditis bacteriophora*, caracterizada principalmente por características morfométricas. Neste trabalho um estudo comparativo e filogenética entre cinco populações do *Heterorhabditis* da Argentina foi conduzido para analisar a variabilidade entre as linhagens e avaliar a posição taxonômica das *Heterorhabditis argentinensis*. Características morfométricas de *Heterorhabditis bacteriophora* VELI e *H. argentinensis* isoladas de regiões com mais chuvas e umidade apresentaram dimensões maiores. Analisa o PCA de personagens morfométricas separou a maior juvenil, feminino e masculino *H. argentinensis* de *H. bacteriophora* populações. A fase juvenil (JIs) fornece a mais clara separação de populações *Heterorhabditis* apresentando a menor variação entre as cepas. A L variável e MBW foram altamente relacionada com *H. argentinensis* JIs. Três grupos foram separados por esta fase considerando PC1 e PC2: um formado por *H. bacteriophora* OLI, RIV e estirpes RN, (isolados de Córdoba e província de Rio Negro), um para a estirpe *H. bacteriophora* VELI (província de Buenos Aires) e um para *H. argentinensis* (província de Santa Fe). *Heterorhabditis bacteriophora* VELI e *H. argentinensis* isolado a partir de regiões com mais chuvas e umidade apresentaram maiores valores para as características morfométricas. A análise molecular mostrou as populações da Argentina (estirpe *H. bacteriophora VELI* e *H. argentinensis*), formando um mesmo subtipo, com seis outras populações *H. bacteriophora* (não da Argentina),

com uma similaridade genética entre eles de 99%. *Heterorhabditis argentinensis* apresentado um único nucleótido que não estava presente em nenhum dos outros espécies do clado. Considerando os resultados deste estudo *H. argentinensis* seria conspecific a *H. bacteriophora*, constituindo uma estirpe com uma grande variação morfométrica onde o anfitrião e as condições climáticas podem ter influenciado nas medições.

Palavras-chave: entomonematodes, Heterorhabditidae, variabilidade, cepas, Argentina.

1. Introduction

Entomopathogenic nematodes (Heterorhabditidae), are generalized consumers of insects in soil food webs that occur widely in natural and agricultural ecosystems. Their associations with symbiotic pathogenic bacteria make them highly virulent, constituting an alternative for the control of insect pests. The third juvenile stage of entomopathogenic nematodes is referred to as the "infective juvenile" or "dauer" stage and is the only free-living stage. They are capable of surviving in the soil without nourishment for prolonged periods up it locates, attacks, and infects an insect host. Once inside the host hemocoel, the IJ releases symbiotic bacteria and kills the host by a combination of toxins and septicemia. Soon after, the IJs begin to feed on the bacteria and develop to reproductive stages. Infective juveniles of the genus *Heterorhabditis* present a first generation of adult hermaphrodites and generally, a second generation with males and true females. Finally, IJs leave the cadavers in response to declining nutrients. Thus, a single IJ has the potential to colonise a new habitat (Boff et al., 2000).

Two species of heterorhabditid nematodes were cited for Argentina: *Heterorhabditis argentinensis* Stock, 1993 in Santa Fe and La Pampa provinces, and *Heterorhabditis bacteriophora* Poinar, 1976, reported in Buenos Aires, Cordoba, La Pampa, Neuquén, Río Negro, Mendoza and Santa Fe provinces (Stock, 1993, 1995; Doucet and Bertolotti, 1996; Giayetto and Cichón, 2006; Del Valle et al., 2013). These strains were characterized mainly by morphological and morphometric features. In this way, *Heterorhabditis argentinensis* was reported and characterized at first, by the large size of the adult males and first-generation females, the longer tail, and the peloderan bursa with 9 pairs of genital papillae in the arrangement 1, 2, 1, 2, 3 (Stock, 1993). However, Adams et al. (1998) considered *H. argentinensis* and *H. bacteriophora* species as sister taxa and possibly even conspecific by PCR amplification of the ITS-1 region. Even, Nguyen (2016) mentioned the arrangement of the last three genital papillae of the bursa, as highly variable in strains of Argentina.

The nematode stage and main traits that should be used for *Heterorhabditis* species distinctions is a matter of discussion. Several authors considered morphological and morphometric features of males and IJs as the most suitable for the distinction among heterorhabditid populations (Adams et al., 1998; Dolinski et al., 2008). Body (L) and tail (T) length in IJs, plus body (L) and reflection of testis in males were used to identify *Heterorhabditis* species (Hominick et al., 1997). However, Phan et al. (2003) suggest length in IJs, and spicule and gubernaculum length and shape in males as ones that should be considered.

Stock and Mrácek (2000), suggested that geographical origin and habitat can influence morphometric data. Intraspecific morphometric variations were observed among strains of the entomopathogenic nematode *Steinernema feltiae* from UK, Pakistan, Rioja and Catalonia (Spain). Also, differences were observed for IJs and males from different infected hosts (Campos Herrera et al., 2006).

Considering the number of *Heterorhabditis* populations isolated from different regions from Argentina, the lack of variability studies for this region and the discussion about the position of *H. argentinensis*, the aims of this work was to analyze morphometric variability between Argentinian strains of *Heterorhabditis* spp. and to evaluate the taxonomic position of *H. argentinensis*.

2. Material and Methods

2.1. Morphometric study of Heterorhabditis spp. populations

Five populations of *Heterorhabditis* spp. isolated from Argentina were considered. Measurements of morphometric features were taken from the literature: *Heterorhabditis bacteriophora* RIV from Río Cuarto city, Córdoba province (33°08′00″S; 64°21′00″W) OLI strain, from Oliva, Córdoba province (32°02′00″S; 63°34′00″ W), (Doucet and Bertolotti, 1996; Doucet et al., 1996), RN strain from Río Negro province (38°56′00″S 68°01′00″W) (Doucet and Bertolotti, 1996), VELI strain from Villa Elisa, Buenos Aires province (34°51'12" S, 58°04'45"W) (Salas et al., 2013), and *H. argentinensis* isolated from Rafaela, Santa Fe province (31°16′00″S 61°29′00″W) (Stock, 1993). Data of the climatic conditions for regions where nematodes were isolated, are shown in Table 1.

Principal component analyses (PCA) were performed on the morphometric variables representing data of hermaphroditic females, amphimictic females, males and infective juveniles of the *Heterorhabditis* species, to examine the general grouping of all individuals with Infostat statistical program (version 2014). Six variables were considered for hermaphroditic females: L, MBW, NR, EP, ES, TL, and V; eight for amphimictic females: L, MBW, NR, EP, ES, TL, ABW and V; nine for males: L, MBW, NR, EP, ES, TRL, TL, SpL and GuL; and five for infective juveniles: L, MBW, EP, ES and TL (as shown in Tables 2-5).

2.2. Molecular-genetic analyses

The genetic variability among Argentinian populations was analyzed. *Heterorhabditis argentinensis* was molecularly characterized by DNA sequences of the ITS1 region (Adams et al., 1998), so this nucleotide sequence was

Table 1. Climatic conditions of origin regions of entomopathogenic nematodes strains considered in this study (H. b: *H. bacteriophora*).

	H.b. VELI	**H.b. OLI**	**H.b. RIV**	**H.b. RN**	***H. argentinensis***
Origin	Villa Elisa	Oliva	Río Cuarto	Río Negro valley	Rafaela
Coordinates	34°51'12" S, 58°04'45" W	32°02′00″S; 63°34′00″W	33°08′00″S; 64°21′00″W	38°56′00″S; 68°01′00″W	31°16′00″S 61°29′00″W
Geography	Plain	Plain	Plain	Plain	Plain
Clime	temperate and humid	temperate and semi arid	temperate and semi arid	temperate and dry	temperate and humid
Mean annual max. temp. (°C)	11	10	10	8	12
Mean annual min. temp. (°C)	21	24	22	21	25
Mean annual temp. (°C)	15.8	16.9	16.8	14.1	18
Total annual rainfall (mm)	1007	711	846	213	959
Humidity (%)	77	67	68	56	76

Table 2. Morphometrics (mean and range) of hermaproditic females of *Heterorhabditis* isolates provided by the bibliography. All measurements are in µm (H. b: *H. bacteriophora*; NA: not available).

Characters	**Hermaprhroditic females**				
	H.b. VELI	**H.b. OLI**	**H.b. RIV**	**H.b. RN**	***H. argentinensis***
L	2,835.46 ± 482.72 (2,160-3,840)	4,800 ± 550 (3,900-5,800)	5,010 ± 410 (4,200-5,600)	3,460 ± 780 (2,400-4,800)	6,500 (5,000-7,500)
MBW	117.64 ± 14.32 (97.4-138.65)	215.7 ± 27.64 (177.5-255)	202.6 ± 16.99 (175-242)	185.75 ± 24.03 (145-222.5)	360 (250-275)
VBW	123.60 ± 17.54 (109.04-153.12)	NA	NA	NA	NA
STL	8.91 ± 2.10 (6.25-11.6)	11.13 ± 1.9 (7.5-15)	NA	NA	13 (10.0-16.0)
STW	8.16 ± 1.34 (5.87-9.28)	11.13 ± 1.89 (7.5-12.5)	NA	NA	10 (6.0-12)
NR	88.95 ± 1.34 (69.6-118.7)	77.88 ± 7.71 (67.5-92.5)	138.86 ± 8.37 (125-152)	136.41 ± 17.26 (107.5-167.5)	160 (132-196)
EP	157.65 ± 19.89 (132.24-200)	194.38 ± 15.53 (175-225)	189.93 ± 13.65 (163-225)	175.58 ± 23.27 (142.5-212.5)	294 (250-340)
ES	152.58 ± 16.41 (132.22-181.25)	183.7 ± 14.06 (162.5-207)	199.03 ± 8.25 (180-220)	189.41 ± 23.83 (155-225)	274 (235-300)
TL	47.87 ± 9.46 (37.5-51.04)	81 ± 7.09 (70-95)	72.8 ± 7.09 (50-87)	61.16 ± 8.97 (47.5-87.5)	118 (100-140)
ABW	52.41 ± 17.80 (37.12-63.45)	48.5 ± 6.24 (35.5-59)	53.3 ± 3.66 (45-62)	35.16 ± 9.3 (22.5-55)	86 (70-120)
V	42.15 ± 12.79 (39.86-47)	41.83 ± 2.1 (36.1-45.1)	40.53 ± 1.54 (35-45)	45.8 ± 2.96 (39.53-50.9)	44.5 (40-50)

considered as limit length, of the segments of analyzed species. A set of 37 homologous sequences recovered from GenBank were aligned with other ITS1 rDNA for bioinformatic analyses. Longer sequences were shortened in order to have a common informative genome segment.

Among five isolates compared in the morphometric study, only *H. bacteriophora* (VELI strain) and *H. argentinensis* were included in the phylogenetic analysis because there are no records of sequences for ITS-1 region in the Genbank for the rest. The evolutionary history was inferred by means of the neighbor-joining method (Saitou and Nei, 1987). The evolutionary distances were computed by means of the maximum-composite-likelihood method (Tamura et al., 2004) and the rate of variation among loci modeled with

Table 3. Morphometrics (mean and range) of amphimictic females of *Heterorhabditis* isolates provided by the bibliography. All measurements are in µm (H. b: *H. bacteriophora*; NA: not available).

Characters	Amphimictic females				
	H.b. VELI	**H.b. OLI**	**H.b. RIV**	**H.b. RN**	***H. argentinensis***
L	1,646.34 ± 282.42	2,220 ± 210	2,140 ± 170	1,830 ± 120	3,000
	(1,251-2,286)	(1,800-2,600)	(1,800-2,400)	(1,650-2,150)	(2,000-3,500)
MBW	97.08 ± 11.89	114.13 ± 9.81	123.46 ± 13.39	112.9 ± 8.2	130
	(78.8-113.68)	(97.5-133)	(100-162)	(95-127.5)	(90-180)
STL	7.76 ± 1.36	7.88 ± 1.47	NA	NA	9.5
	(6.96-11.6)	(5.0-10.0)			(7-12)
STW	7.22 ± 0.69	8.25 ± 1.43	NA	NA	8
	(6.96-9.28)	(5.0-10.0)			(5-10)
NR	73.69 ± 13.56	65 ± 7.43	93.23 ± 5.2	95.75 ± 6.47	114
	(58-104.4)	(55-87.5)	(83-102)	(82.5-107.5)	(88-140)
EP	117.18 ± 20.88	165 ± 7.4	149.76 ± 8.33	131.25 ± 8.34	203
	(83.52-173.32)	(152.5-175)	(122-162)	(117.5-150)	(105-240)
ES	123.13 ± 10.04	137.25 ± 6.48	133.63 ± 6.77	135.83 ± 5.62	180
	(104.4-141.52)	(125-148)	(108-145)	(125-150)	(162-200)
TL	41.58 ± 0.39	71.5 ± 4.32	54.46 ± 4.83	67.41 ± 4.61	93
	(20.88-53.36)	(62.5-80.0)	(40-65)	(60-77.5)	(75-108)
ABW	48.36 ± 12.30	NA	27.7 ± 3.79	26.25 ± 2.6	45
	(27.84-69.6)		(23-40)	(22.5-32.5)	(33-55)
V	45.61 ± 2.69	49.88 ± 5.2	46.73 ± 2.37	46.51 ± 1.52	45
	(39.2-49.68)	(40.7-69.7)	(41-50)	(42.7-48.9)	(42-48)

a gamma distribution (shape parameter = 2.25). These evolutionary analyses were carried out by the MEGA5 software (Tamura et al., 2011).

3. Results

3.1. PCA analysis

Morphometric variation was greatest between *H. argentinensis* and all other *H. bacteriophora* isolates. *Heterorhabditis argentinensis* was separated from the rest of *H. bacteriophora* populations for juveniles, males, amphimictic and hermaphroditic females (see Figure 1 A-D).

An accumulated variability of 91% was reached in hermaphroditic females by the PC1 (76%) and PC2 (15%). Except for NR and V, all variables showed positive correlation between them and were responsible of the great variability of the PC1. *Heterorhabditis argentinensis* hermaphroditic females differed from the other *H. bacteriophora* populations due to larger dimensions. *Heterorhabditis bacteriophora* OLI and RIV strains were more similar than the other *H. bacteriophora* strains. The variable V was responsible to the major variability of the PC2 (see Figure 1A).

As well as hermaphrodites, all morphometric characters for amphimictic females, except V and NR, explained the PC1 variability (72.8% of the total) and had a positive and high correlation. The variable V was correlated with PC2 and separated one of isolates of *H. bacteriophora* (OLI). RN and RIV strains of *H. bacteriophora* were most similar. *Heterorhabditis argentinensis* was separated from the rest of populations (see Figure 1B).

Males showed the greatest morphometric differences, being the five strains individually separated by PCA analysis. Results of the first two principal components for males explained 68.9%. The PC1 separated *H. argentinensis* from the other *H. bacteriophora* populations by MBW, TL, L and EP variables. L, MBW, EP, ES, TL and GuL were responsible of the major variability in the PC1, while TRL and SpL defined PC2. *Heterorhabditis bacteriophora* VELI strain was separated from the others by a high value of TRL (see Figure 1C).

The infective juvenile was the stage with lower variability between strains and the best to discriminate Argentinian populations. The principal components 1 and 2 accounted for 48% (PC1) and 28% (PC2) respectively of the total variation of juveniles (76.3%). Three groups were evident, one formed by *H. bacteriophora* OLI, RIV and RN strains, one for *H. bacteriophora* VELI strain, and one for *H. argentinensis* (see Figure 1D). The variable L and MBW were highly related to *H. argentinensis,* while ES and TL separated *H. bacteriophora* (OLI, RIV, RN strains) and EP *H. bacteriophora* VELI strain.

3.2. Phylogenetic analysis

In our phylogenetic tree, *Heterorhabditis* spp. formed three separate clades. The bioinformatic study placed *H. argentinensis* as a member of the clade B (100% support of the most ancestral node), related to six populations of *H. bacteriophora* (among them the argentine VELI strain), five populations of *H. georgiana*, one isolate of *H. zealandica*, and two isolates of *Heterorhabditis* sp.

Table 4. Morphometrics (mean and range) of males of *Heterorhabditis* isolates provided by the bibliography. All measurements are in µm (H. b: *H. bacteriophora*; NA: not available).

Characters	Males				
	H.b. VELI	**H.b. OLI**	**H.b. RIV**	**H.b. RN**	***H. argentinensis***
L	822.46 ± 83.16 (711-972)	910 ± 60 (800-1005)	845 ± 57 (700-940)	938 ± 44 (850-1,003)	1,500 (1,000-2,000)
MBW	46.89 ± 6.61 (42.3-62.6)	48.05 ± 3.86 (40-57)	44.5 ± 2.7 (37-47)	43.56 ± 1.63 (40-47)	56 (42-70)
STL	5.22 ± 1.71 (2.35-4.28)	7.15 ± 0.93 (6-9)	6 ± 0.6 (5-7)	NA	5 (3.5-6.0)
STW	4.264 ± 1.15 (2.35-6.9)	5.25 ± 0.55 (4-6)	5.05 ± 0.51 (4-6)	NA	4 (2.5-5.0)
NR	66.39 ± 10.21 (39.9-81.2)	51.9 ± 4.45 (45-62)	77.25 ± 3.43 (70-85)	77.46 ± 3.78 (70-85)	72 (64-82)
EP	110.17 ± 22.31 (95.12-134.5)	125.9 ± 7.6 (113-140)	129.15 ± 4.57 (120-137)	88.36 ± 4.52 (78-98)	157 (145-170)
ES	116.07 ± 12.40 (104.4-141.52)	101.4 ± 4.69 (92.5-107.5)	102.3 ± 3.34 (95-110)	101.9 ± 3.06 (94-107)	113 103-120
TRL	200.78 ± 77.98 (103.4-229.68)	96 (54-210)	88.7 ± 11.5 (75-115)	97.23 (80-114)	133 (100-194)
TL	27.95 ± 8.47 (22.0-41.76)	28.9 ± 2.27 (24-33)	24.35 ± 1.56 (20-27)	30.33 ± 2.03 (26-35)	37 (28-49)
ABW	36.91 ± 10.97 (27-8-44.08)	NA	NA	20.2 ± 1.42 (18-25)	24 (21-30)
SpL	45.00 ± 4.76 (34.8-48.7)	46.8 ± 2.86 (40-51)	43.2 ± 1.96 (39-47)	48.03 ± 2.1 (45-53)	46 (42-49)
SpW	4.64 ± 0.66 (3.48-5.8)	NA	NA	NA	NA
GuL	22.5 ± 6.49 (18.5-21.1)	22.9 ± 2.22 (19-27)	20.6 ± 1.35 (18-24)	19.2 ± 2.32 (14-23)	23 (20-26)
GuW	5.21 ± 1.37 (3.94-6.96)	NA	NA	NA	NA
GS (GuL/SpL)	0.53 ± 0.13 (0.48-0.55)	NA	NA	NA	NA
SW (SpL/ABW)	10.28 ± 2.09 (7.29-14.0)	NA	NA	NA	NA
E (EP/TL)	4.32 ± 3.11 (2.96-5.65)	NA	NA	NA	NA
D (EP/ES)	1.11 ± 0.18 (0.89-1.17)	NA	NA	NA	NA
L/TL	35.62 ± 19.58 (21.36-44.18)	NA	NA	NA	NA
L/MBW	19.31 ± 3.64 (13.5-22.97)	NA	NA	NA	NA
MBW/TL	1.57 ± 1.09 (1.1-2.7)	NA	NA	NA	NA

(see Figure 2; Table 6). Within the above set of sequences, the genetic similarities between *H. argentinensis* and the other members of the clade B varied between 98.3 and 100%; and this isolate proved to be included in a subgroup together with five isolates of *H. bacteriophora*, where the similarities were between 99.4% and 99.7%.

In clade B, *H. bacteriophora* HP88, N-KMD7, and 190-C strains had identical nucleotide sequences. Similarly, the strains of *H. georgiana* N-SPCM3, N-GPS29, N-KMD82 and *H. zealandica* NZH3 contained no differences (Table 6). However, the Argentinian *H. bacteriophora* VELI strain, differed from the above three H. *bacteriophora* populations

Table 5. Morphometrics (mean and range) of infective juveniles of *Heterorhabditis* isolates provided by the bibliography. All measurements are in µm (H. b: *H. bacteriophora* ; NA: not available).

Characters	Infective juveniles				
	H.b. VELI	H.b. OLI	H.b. RIV	H.b. RN	*H. argentinensis*
L	616.75 ± 60.95 (505.04-675.12)	540 ± 0.03 (490-610)	590 ± 29.57 (540-640)	603 ± 19.67 (560-640)	657 (610-710)
MBW	25.04 ± 1.6 (23.2-27.84)	23 ± 1.03 (22-45)	25.45 ± 2.35 (22-29)	23 ± 0.78 (22-25)	31 (24-38)
EP	100.11 ± 12.34 (78.8-109.04)	93.95 ± 2.96 (87-101)	100.4 ± 3.74 (96-110)	96.76 ± 4.93 (90-112)	95 (82-116)
ES	101.93 ± 14.46 (74.24-113.68)	112 ± 4.68 (103-119)	121.4 ± 4.92 (110-130)	116.3 ± 4.16 (108-122)	107 (68-122)
TL	38.54 ± 14.3 (32.16-51.4)	89.6 ± 10.22 (72-105)	93.4 ± 4 (85-100)	101.5 ± 6.72 (88-113)	84 (70-105)
ABW	18.63 ± 3.25 (15.08-27.84)	NA	15.9 ± 0.75 (14-17)	15.25 ± 1.2 (13-17)	NA
Ratio a (L/MBW)	23.76 ± 3.03 (22.2-26.2)	23.44 ± 1.01 (20.4-25)	23.6 ± 1.5 (20-26)	26.24 ± 1.16 (23.75-28.63)	NA
Ratio b (L/ES)	6.19 ±1.2 (5.3-8.8)	4.77 ± 0.24 (4.3-5.2)	4.92 ± 0.3 (4.5-5.8)	5.18 ± 0.17 (4.9-5.6)	NA
Ratio c (L/TL)	9.87 ± 3.63 (6.2-14.4)	6.07 ± 0.79 (4.9-7.6)	6.39 ± 0.19 (6.1-6.8)	5.95 ± 0.37 (5.22-6.66)	NA
Ratio d (EP/ES)	0.99 ± 0.14 (0.62-1.07)	0.84 ± 0.02 (0.8-0.88)	0.82 ± 0.05 (0.75-0.9)	0.83 ± 0.03 (0.77-0.94)	NA
Ratio e (EP/TL)	1.54 ± 0.39 (1.9-2.1)	1.0 ± 0.14 (0.8-1.3)	1.07 ± 0.06 (0.98-1.2)	0.95 ± 0.07 (0.82-1.1)	NA

in one nucleotide at position 176 (a T *vs*. C transition), whereas *H. argentinensis* did so with respect to an insertion of a C at position 348. The latter species also exhibited variation at three positions with respect to *H. bacteriophora* N-KMD6 (at nucleotides 97, 176, and 231) and to *H. bacteriophora* 51-C (at nucleotides 176, 295, and 310). Greater differences were observed between VELI strain and *H. georgiana* populations (between 4 and 5 different nucleotides; Table 6).

The clade A (100% support of the most ancestral node) comprised all species isolated from the Southern Asia and Indian-Ocean region: five isolates of *H. indica*, one isolate of *H. gerrardi*, and two *Heterorhabditis sp*.

The remaining group, clade C (100% support of the most ancestral node), comprised five species: *H. marelatus*, *H. safricana, H. atacamensis, H. downesi*, and *H. megidis.* Also, this clade was composed of five subgroups (51-100% support; see Figure 2). Length of expected amplicon for the species analyzed in this phylogenetic study (without considering primers used for each case) varied between 829 and 830 bp in clade A, 861-862 bp in clade B, and 836- 851 bp in clade C. These results are in accordance with the similarity levels data obtained within clades by phylogenetic inference, indicating a major homogeneity between *H. bacteriophora* populations.

4. Discussion

Two species of heterorhabditid nematodes were reported for Argentina: six isolates of *H. argentinensis* in Santa Fe and La Pampa provinces (Stock, 1993, 1995), and more than 20 populations of *Heterorhabditis bacteriophora* reported in the central-pampean region, Cuyo and Patagonia (Doucet and Bertolotti, 1996; Giayetto and Cichón, 2006; Del Valle et al., 2013).

Studies on the morphometric variability are useful to provide valuable information about geographical and ecological requirements for EPN. Morphometric differences can be observed in nematode strains isolated from different sites and hosts (Campos Herrera et al., 2006).

In our study, morphometric variations were observed according to Nguyen (2016) which considered some morphological features as highly variables for Argentinian strains. The greatest differences were registered between *H. argentinensis* and all other *H. bacteriophora* populations, unlike between *H. bacteriophora* strains from different regions.

Results of PCA analysis for morphometric characters separated *H. argentinensis* from the rest of *H. bacteriophora* strains from Argentina, considering the four nematode stages. The infective juvenile was a stage with high weight in the separation between *Heterorhabditis* populations. Three groups were separated considering PC1 and PC2

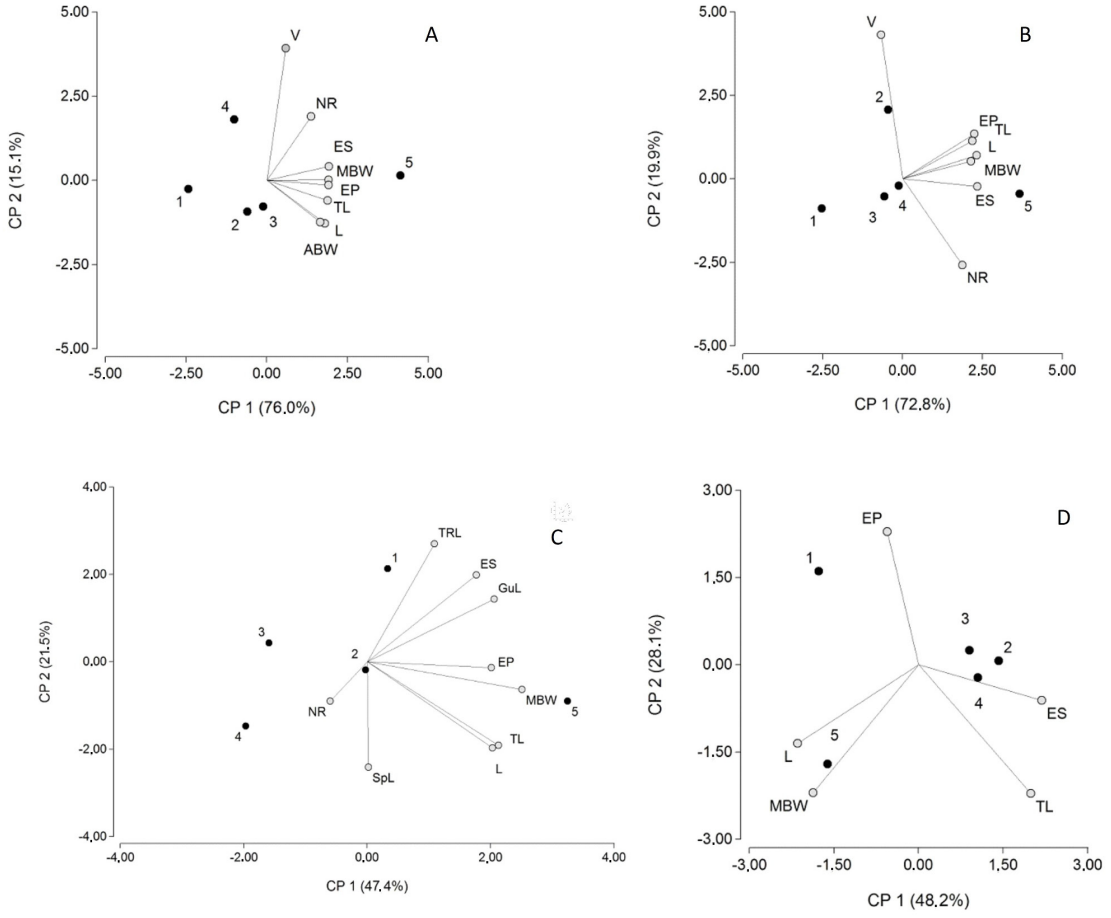

Figure 1. Principal component analysis of *Heterorhabditis* spp., based on mean values of morphometric characters for all nematode stages. (A) =Hermaphroditic females, (B) =Amphimictic females, (C) =Males, (D) =Infective juveniles. (*H. bacteriophora* strains: 1. VELI, 2. OLI, 3. RIV, 4. RN; 5: *H. argentinensis*).

for this stage; one formed by *H. bacteriophora* OLI, RIV and RN strains, one for *H. bacteriophora* VELI strain and one for *H. argentinensis*. The variable L was negatively correlated with TL for juveniles, showing that longer specimens had shorter tails. Measurements of males and infective juveniles were suggested for several authors as the most suitable for the distinction among heterorhabditid populations (Adams et al., 1998; Dolinski et al., 2008) although in our study males were not as good as juveniles.

Morphometric characteristics of *H. bacteriophora* strain VELI, were slightly larger respect to other Argentinian isolates of the same species, which was observed always separated from the rest of populations by PCA analysis. However, these variations were not as considerable as in *H. argentinensis*.

Even though morphometric study separated *H. argentinensis* from the rest of *H. bacteriophora* isolates, the phylogenetic analysis placed in the same clade *H. argentinensis* and six populations of *H. bacteriophora*. The genetic similarity between *H. bacteriophora* populations and *H. argentinensis* was over 99%. *Heterorhabditis bacteriophora* VELI strain and *H. argentinensis* had the same number of nucleotide-sequence variations with respect to the rest of the *H. bacteriophora* isolates analyzed in our study. The Argentinian isolations exhibited two differences in nucleotide sequence: a T *vs*. C at position 176 of ITS1-rDNA (constituting a new variation in coding sequence) in *H. bacteriophora* (VELI strain) and a C inserted at position 348 in *Heterorhabditis argentinensis*. These nucleotide variations were not present in the rest of *H. bacteriophora* member of the clade B. Results obtained in our study are according to Adams et al. (1998) and Phan et al. (2003) who analyzing DNA sequences of the ITS1 of the *Heterorhabditis* genus, showed the type strain *H. bacteriophora* (HB1) differing from *H. argentinensis* in a single transversion (G *vs*. C) at position 620 (Adams et al., 1998; Phan et al., 2003) and in a single insertion of a C at position 348 as we mentioned (Phan et al., 2003). Therefore, by the results obtained in our study, *H. argentinensis* would be a sister taxa of *H. bacteriophora*, as was considered by Adams et al. (1998).

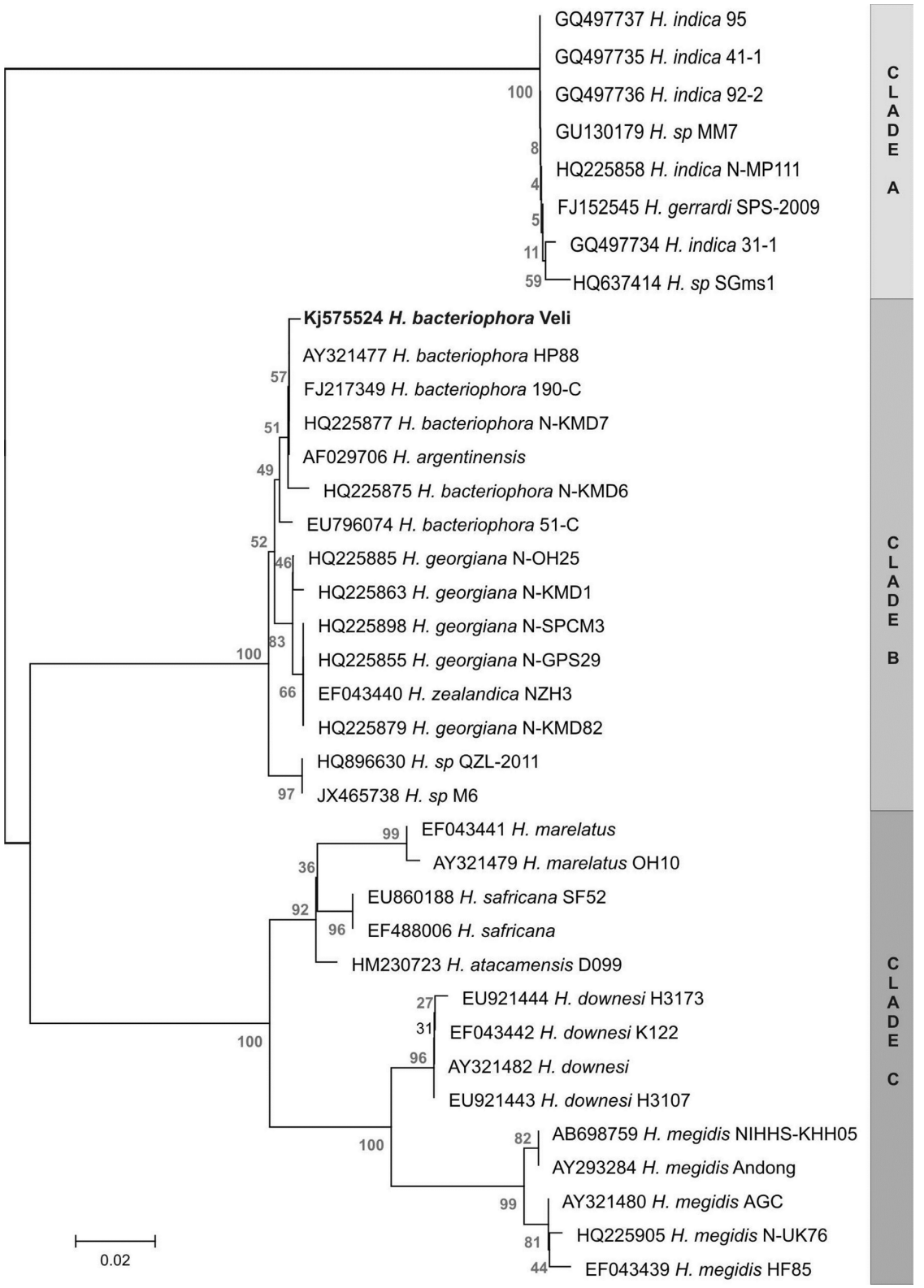

Figure 2. Evolutionary relationships among *Heterorhabditis* taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches, Felsenstein (1985). Only node consistencies above 40% are shown. The tree is drawn to scale, with branch lengths in the same proportions as the evolutionary distances used to infer the phylogenetic tree.

Table 6. Nucleotide differences between populations of *Heterorhabditis* spp. within the Clade B.

Alignment relative position	HQ225898_*H. georgiana*_N-SPCM3	HQ225855_*H. georgiana*_N-GPS29	EF043440_*H. zealandica*_NZH3	HQ225879_*H. georgiana*_N-KMD82	HQ225885_*H. georgiana*_N-OH25	HQ225863_*H. georgiana*_N-KMD1	AY321477_*H. bacteriophora*_HP88	HQ225877_*H. bacteriophora*_N-KMD7	FJ217349_*H. bacteriophora*_190-C	AF029706_*H. argentinensis*	KJ575524_*H. bacteriophora*_VELI	HQ225875_*H. bacteriophora*_N-KMD6	EU796074_*H. bacteriophora*_51-C	HQ896630_*Heterorhabditis* sp_QZL-2011	JX465738_*Heterorhabditis* sp_M6
5	T	T	T	T	G	G	G	G	G	G	G	G	G	G	G
97	T	T	T	T	T	T	T	T	T	T	T	C	T	T	T
176	T	T	T	T	T	T	T	T	T	T	C	T	T	T	T
204	G	G	G	G	G	G	C	C	C	C	C	C	C	C	C
214	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C
225	A	A	A	A	A	G	A	A	A	A	A	A	A	A	A
229	A	A	A	A	A	A	A	A	A	A	A	A	A	T	T
230	C	C	C	C	C	C	C	C	C	C	C	C	C	A	A
231	A	A	A	A	A	A	A	A	A	A	A	G	A	A	A
233	T	T	T	T	T	T	A	A	A	A	A	A	A	C	C
295	C	C	C	C	C	C	G	G	G	G	G	G	C	C	C
310	C	C	C	C	C	C	C	C	C	C	C	C	A	C	C
348	--	--	--	--	--	--	--	--	--	C	--	--	--	--	--

Morphometry of the parasites can be influenced by nutritional conditions inside the host, and environmental factors (Phan et al., 2003; Canto-Silva, et al., 2005). Stock and Mrácek (2000), mentioned that the geographical origin and habitat can influence on morphometric data, so even values obtained from a same host can change based on abiotic factors and rearing conditions. Boff et al. (2000) observed that a larger body size of the host and a lower dose of infection increased the size of IJs of *Heterorhabditis megidis*. These variations could be produced because measurements often are taken from the progeny of a few soil-baited insect hosts, so is unlikely that they represent the range of variation present in the population (Adams et al., 1998).

In Argentina, *Heterorhabditis* spp. populations were isolated from different locations and hosts. *Heterorhabditis argentinensis* was found parasitizing the alfalfa weevil *Graphognathus* sp. (Coleoptera: Curculionidae) (Stock, 1993), from Santa Fe province, *H. bacteriophora* RIV strain from *Heliothis* sp. (Lepidoptera: Noctuidae) from Cordoba province, and both were later maintained in the laboratory in *Galleria mellonella* larvae (Lepidoptera) for the identification; meanwhile *Heterorhabditis bacteriophora* OLI strain, from Córdoba province, RN strain from Río Negro province and VELI strain from Buenos Aires province were isolated directly from field, in *Galleria mellonella* baits, leaving the natural hosts unknown (Aguera de Doucet and Doucet, 1986; Stock, 1995; Doucet and Bertolotti, 1996; Giayetto and Cichon, 2006). Geographically, Argentina is divided in different regions determined by a homogeneous relief and climate. Rainfalls decrease from east to west, from Mesopotamia to the mountains by the rainfall regime of the Atlantic. From there we can find humid climates, with over 800 mm per year, less than 400 mm dry climates and arid and semiarid in the transition zone between both (Bianchi and Cravero, 2010). Buenos Aires and Santa Fe provinces, where *H. bacteriophora* VELI and *H. argentinensis* strains were isolated, present more rainfalls and humidity conditions, respect the other sites. These requirements could have influenced on the larger dimensions observed in *H. bacteriophora* VELI and *H. argentinensis* populations, as the conditions of the host at the time to be isolated from the field.

In the same way, the average annual temperature declines in the plains of central and northeastern of Argentina with increasing latitude. The"Río Negro" river where the upper Valley of Río Negro province is located, and where *H. bacteriophora* RN strain was isolated, born at the eastern end of the province of Neuquen, and flows to the Río Negro territory in southeast direction to reach the Atlantic Ocean, being in its last leg the natural boundary between the provinces of Rio Negro and

Buenos Aires. The presence of mountains and plateaus in the west and south of the"Río Negro" river, deflects the isotherms, which take a parallel course to the mountain, producing a decreasing of the temperatures to the south of the country. According to this parameter, in Argentina can be distinguished subtropical climates, with annual average temperatures above 18 °C, temperate, ranging between 18 °C and 12 °C, and cold climates, less than 12 °C (Bianchi and Cravero, 2010). While all Argentinian strains were isolated from temperate climates, the average annual temperature for Santa Fe province (18 °C) is at the limit between a mild and subtropical climate, which also could have influenced the largest morphometry reached by *H. argentinensis*.

Considering the results of this study, morphometric variations are present between Argentinian populations of *Heterorhabditis* spp. isolated from different regions. In this way, *Heterorhabditis argentinensis* would be conspecific to *H. bacteriophora,* constituting a strain with a great morphometric variation where the host and climatic conditions could have influenced on the measurements.

This work constituted the first comparative and phylogenetic study of heterorhabditid populations from Argentina.

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