MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF A NEW ISOLATE OF *HETERORHABDITIS BACTERIOPHORA* POINAR, 1976 FROM EAST AZARBAIJAN, IRAN

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Summary. *Heterorhabditis bacteriophora*, strain Azarbaijan (IRAZ8), was collected from a natural rangeland in East Azarbaijan province, Iran. Morphometric and morphological characters of the new isolate were compared with those published for the type species and of other isolates obtained from various regions of Iran. The results showed that the characteristics of infective juveniles and amphimictic females of IRAZ8 are similar to those of other isolates with some minor differences. However, hermaphroditic females have a peculiar cuticular pattern surrounding the vulva, forming a number of transverse ridges separated by grooves and protruding outward with distinct crescent patterns and hypertrophied vulval lips. In the males, the proximal portion of the gubernaculum is noticeably curved and the eighth pair of bursal papillae is shorter. Molecular studies of the rDNA ITS region showed that the Iranian isolate is closely related to the other described isolates of *H. bacteriophora* in GenBank and that they form a monophyletic group with a high bootstrap support.

Key words: Description, entomopathogenic nematodes, morphology.

Entomopathogenic nematodes (EPNs) are important biological control agents of a variety of economically important pests (Shapiro-Ilan *et al*., 2002; Klein, 1990). At present, more than 63 species of *Steinernema* and 16 species of *Heterorhabditis* have been reported (Nguyen and Hunt, 2007) and the number of nominal species is increasing all the time. *Heterorhabditis bacteriophora* Poinar, 1976 has been collected from many places all over the world (Hominick, 2002). This species was originally described from the body cavity of *Heliothis punctigera* Hall (Noctuidae: Lepidoptera), from Brecon, South Australia (Poinar, 1976). The surveys conducted for *Heterorhabditids* identification in different parts of the world have revealed its global distribution (Hominick *et al*., 1997; Stock *et al*., 2001). In Iran, a survey on entomopathogenic nematodes was conducted in the natural forests and rangelands, in the north-west of the country, in 2007. Of the 691 soil samples collected, nine (1.3%) contained specimens of *Heterorhabditis* (Nikdel *et al*., 2010). Morphological and molecular studies revealed that all the isolates belonged to *H. bacteriophora*. Among them, isolate IRAZ8, despite having molecular and morphological similarity with the other isolates, differs in some morphometric characters. Therefore, we characterized the isolate molecularly and morphologically.

MATERIAL AND METHODS

Nematode population

Heterorhabditis bacteriophora IRAZ8 was recovered from soil samples collected from Shirin Boolag rangelands, near Kaleibar town, East Azarbaijan province, in north-west Iran (longitude 46°45,45-E, latitude 38°55,20-N, altitude 1621 m a.s.l., annual average temperature of 12 °C, precipitation 415 mm/year) using the *Galleria mellonella* L. baiting method described by Bedding and Akhurst (1975). Infective juveniles (IJs) were collected from *Galleria* cadavers using White's (1927) method and stored in aerated water at 7 °C. All nematode specimens used in this study were reared in *G. mellonella* larvae.

Morphological characterization

Light microscopy. For morphometrical studies, ten *G. mellonella* were exposed to about 500 infective juveniles in a Petri dish (90 mm diameter × 10 mm height) lined with two moistened filter papers. The Petri dishes were kept at room temperature (25 °C). First and second generation adults were collected from *Galleria* cadavers (dissected out in Ringer's solution) two and three days after inoculation, respectively. Infective juveniles were collected during the week after their first emergence from the *Galleria* cadavers and killed using hot (50-60 °C) Ringer's solution (Nguyen and Smart, 1990). Dead nematodes were fixed in tri-ethanolamine formalin (TAF), processed to anhydrous glycerin by the slow evaporation method (Woodring and Kaya, 1988) and mounted on microscope slides. Cover glass supports were used in all cases to avoid flattening the nematode specimens. Morphological and morphometrical studies were made using an Olympus BX41 microscope equipped with interference contrast and a digital camera. Image tool software was used to obtain quantitative measurements.

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Scanning electron microscopy. Scanning electron microscopy (SEM) studies were conducted using Nguyen and Smart's (1995) method with minor modifications. First generation adults were dissected from *G. mellonella* larvae in Ringer's solution. Infective juveniles and adult nematodes were rinsed for 5 minutes in Ringer's solution three times. They were fixed in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.2 for 24 hours at 8 °C, post-fixed with 2% osmium tetroxide (OsO4) solution for 12 hours at 25 °C, dehydrated in a graded ethanol series, mounted on aluminium SEM stubs, coated with gold powder (200 nm thickness) and studied using a LEO 44oi scanning electron microscope.

Molecular characterization

Extraction of DNA. Total genomic DNA extraction from single infective stages and ITS-rDNA amplification were done as described by Phan *et al.* (2001). Amplified products were purified with a QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA). Purified DNA was sequenced on automated sequencers in the Institut de Biologie Moléculaire des Plantes - Centre National de la Recherche Scientifique (IBMP-CNRS), Strasbourg, France.

Phylogenetic relationships. The ITS sequences of the isolate IRAZ8 (FJ860046) were aligned with ITS sequences of other isolates of *H. bacteriophora* obtained from GenBank. Molecular phylogenetic relationships were obtained by equally weighted maximum parsimony (MP) using PAUP, 4.0b8 (Swofford, 1998). MP was performed with a heuristic search with the following setting: one hundred replicates of random taxon addition (RTA), tree-bisection-reconnection (TBR) branch swapping, multiple trees retained, no steepest descent and accelerated transformation. All data were assumed to be unordered, all characters were treated as equally weighted, and gaps were treated as missing data (Posada and Crandall, 1998). Bootstrap analysis with 1000 replicates was conducted as a measure of support for individual clades for the MP tree (Phan *et al*., 2003).

The DNA sequences were edited with Chromas 2.01 and aligned using Clustal X 1.64 (Thompson *et al*., 1997) with the ITS-rDNA sequences of other described *Heterorhabditis* species and eight isolates of *H. bacteriophora*, including strains IRAZ8 (FJ860046); HP88 (EF043438); 63-C (EU848594); Muaggar (EU200360); J172 (EU716335); SF179 (FJ791248); X7 (EU435140) and IRA10 (EU598227), obtained from GenBank.

RESULTS

Description

Measurements. See Table I.

Male. Anterior region similar to females, but smaller. Testis single and reflexed leading into a seminal vesicle containing spermatozoa; vas deferens well developed. Spicules paired and separate; shape of capitulum variable, from pointed to flat. Gubernaculum with the proximal portion noticeably ventrally curved in 80% of the specimens observed (97 out of 120 and 7 out of 10, fixed and live specimens, respectively) (Fig. 3 B). Bursa peloderan, open, supported normally by nine pairs of papillae (Fig. 3 C, D), bursal papillae with the formula 1, 2, 3, 3 (one single papilla and three groups with 2, 3, and 3 papillae with variations in the middle one), often in the two terminal groups of three papillae, the median one shorter than others (Fig. 3 D) as seen in *Heterorhabditis baujardi* Phan, Subbotin, Nguyen *et* Moens.

First generation (hermaphroditic) female. Head slightly rounded. Face view similar to other *Heterorhabditis* species, four cephalic and six labial papillae prominent (Fig. 2 A). Amphids small and located posterior to labial papillae. Under a compound microscope, cheilorhabdions seen as lightly refractile areas lining anterior (non-collapsed) portion of stoma. Oseophageal basal bulb often surrounded by anterior portion of intestine. Nerve ring surrounding isthmus just anterior to basal bulb. Excretory pore usually posterior to the basal bulb. Intestine with few large cells. Lateral fields not seen, phasmids inconspicuous. Gonads amphidelphic and reflexed. Vulval opening near mid-body, a transverse slit, vulval lips hypertrophied, cuticle anterior and posterior to vulva thickened, forming a number of transverse ridges separated by grooves and protruding outward with distinct crescent patterns, epiptygma present in the anterior part of the vulva (Fig. 2 B). Tail relatively short with pointed terminus (Fig. 2 C).

Second generation (amphimictic) females. Similar to hermaphroditic females but with different shape of head and vulva region. Head rounded, lip region often faintly set off; vulva non-functional for oviposition, protruding outward, often covered with a hardened deposit (Fig. 2 D, E). Anal region slightly protruding.

Infective juvenile. Body elongate, sheath (second-stage cuticle) present immediately after harvesting (Fig. 1 A-E), but many infective juveniles losing the sheath in storage (Fig. 1 F). Ensheathed infective juveniles with body length 530±10 µm close to that of *H. indica*. Mouth and anus closed, pharynx and intestine collapsed. In the ensheathed IJs cuticle on dorsal side of nearly the first one-third of body ornamented with tessellate pattern, posterior part with longitudinal striae. Body of exsheathed IJs annulated, without longitudinal striae, lateral fields at the middle of the body with two prominent ridges (Fig. 1 F). Labial region with a dorsal tooth, labial and cephalic papillae inconspicuous (Fig. 1 A). Amphids prominent, pore-like. Excretory pore posterior to nerve ring but located just anterior to base of pharynx, excretory duct pronounced, well cuticularized except in anterior part, or 4-6 µm from excretory pore.

Hemizonid, when observed, 3-4 annules anterior to excretory pore. Pharynx typical for *Heterorhabditis*. Phasmids not seen under light microscope. Tail elongate conoid with pointed terminus (Fig. 1 C). Tail length without sheath 86 µm or about 61% of ensheathed tail.

Morphometrical and morphological comparisons with other isolates. Morphometrics and morphological characters (Table II) of *H. bacteriophora* IRAZ8 are similar to the other Iranian isolates except for the following: noticeable curving in the proximal portion of gubernaculum (Fig. 3 B) and the shortness of the eighth pair of

Table I. Morphometrics of *Heterorhabditis bacteriophora* isolate IRAZ8. Measurements are in microns and in the form: mean ± standard deviation (range).

Character	First generation	Second generation		Infective juvenile
	Hermaphroditic Female	Female	Male	
n	20	20	22	25
L	4519±333 (3943-4900)	2432±155 (2271-2500)	892±63 (780-995)	530±10 (490-578)
a	23±2 (22-25)	15±1 (14-17)	18±1 (14-18)	19±1.3 (16-21)
b	25±1 (25-27)	11±1 (10-13)	8±1 (7-9)	4±0.2 (4-4.7)
c	228±14 (220-242)	43±2 (38-46)	24±5 (21-39)	6±0.3 (5.5-6.5)
V	43±2 (41-46)	45±1 (44-46)		
Body diameter (W)	278±30 (235-335)	170±6 (157-183)	58±6 (50-72)	28±2 (24-31)
Excretory pore (EP)	224±20 (202-253)	135±5 (127-143)	142±8 (128-163)	96±5 (90-101)
Nerve ring	153±17 (140-172)	96±4 (78-109)	112±3 (108-118)	83±5 (77-89)
Pharynx length (ES)	204±11 (186-213)	138±9 (126-146)	159±28 (129-197)	124±6 (112-135)
Tail length (T)	77±10 (61-94)	65±3 (61-69)	38±5 (25-43)	86±4 (75-93)
Anal body diameter (ABD)	57±6 (50-68)	28±4 (27-30)	26±3 (17-30)	17.2±0.8 (16-19)
Spicule length (SP)	-	-	39.6±3.5 (33-46)	-
Spicule width	-	-	6±1 (5-8)	-
Gubernaculum length (G)	-	-	20±2 (16-25)	-
D%	45±6 (42-52)	38±4 (36-43)	127±7 (117-143)	78.7±5.3 (73-85)
E%	412±35 (382-451)	148±21 (129-156)	383±50 (360-570)	99.8±7 (85.8-124)
SW%	-	-	157±24 (122-203)	-
GS%	-	-	49±4.7 (39-57)	-

Abbreviations: D% (= EP/ES × 100), E% (= EP/T × 100), SW (= SP/ABD) × 100, GS (= G/SP × 100)
a = L/W, b = L/ES, c = L/T, V= distance from anterior end to vulva/body length × 100.
n = number of specimens measured

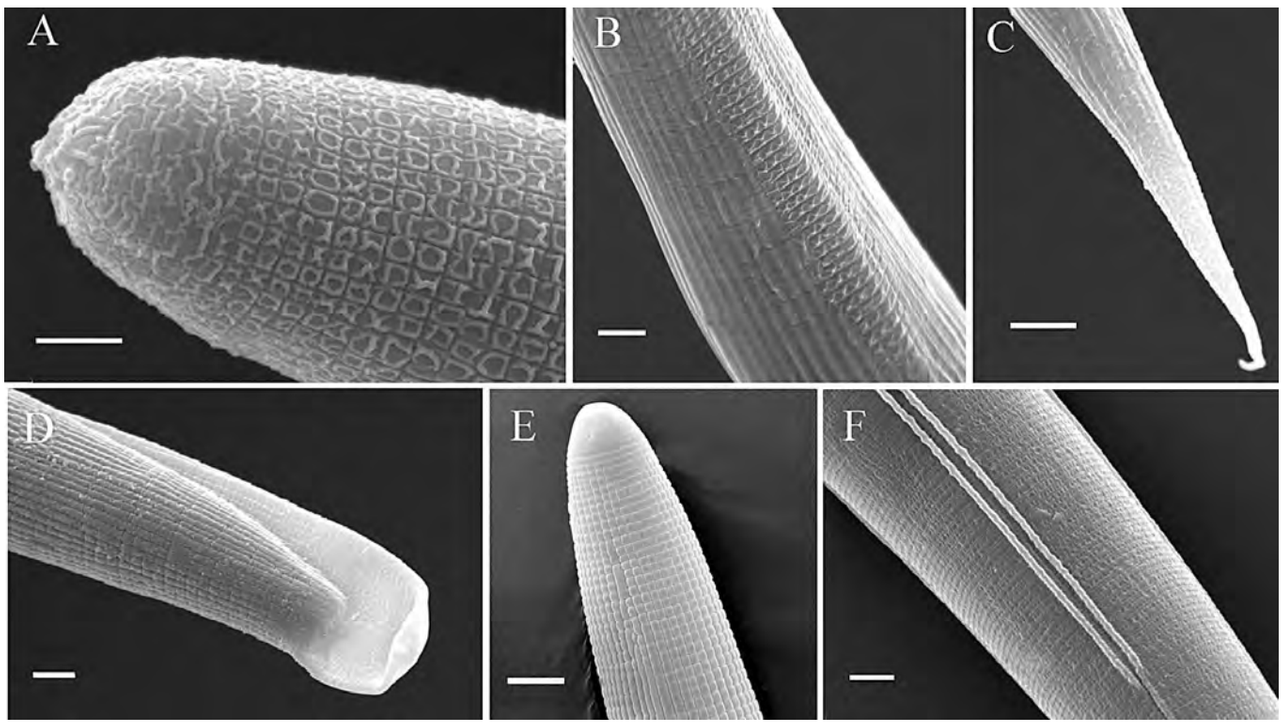

Fig. 1. SEM of ensheathed (A-E) and exsheathed (F) infective juveniles of *Heterorhabditis bacteriophora* IRAZ8 isolate. A, anterior region showing smooth head and dorsal tooth and tessellate pattern on cuticle. B, middle part with longitudinal ridges and continuing of tessellate pattern. C, pointed tail. D, E, anterior region showing smooth head and annulated body with tessellate pattern and without longitudinal ridges. F, lateral field at the middle of the body with two ridges. Scale bar (in µm): A = 1, B = 2, C = 3, D = 3, E = 4, F = 2.

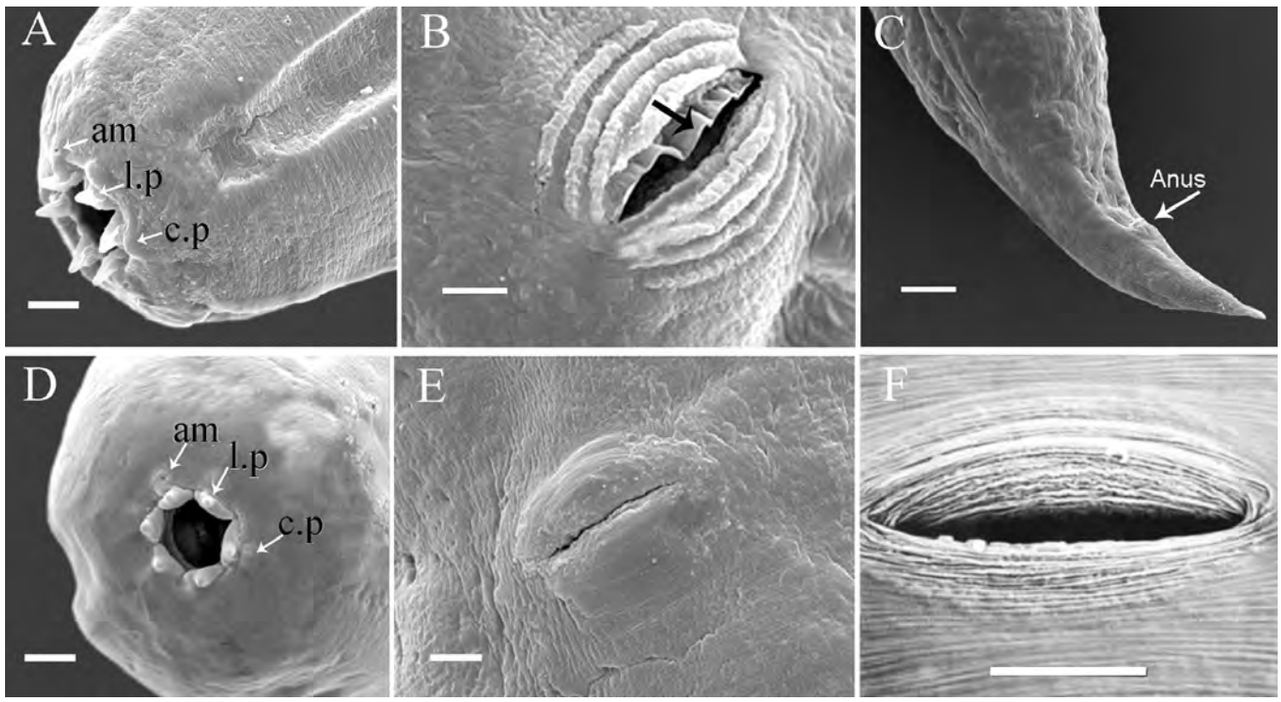

Fig. 2. SEM photographs of females of *H. bacteriophora* IRAZ8 isolate (A-E) and most common isolates of *H. bacteriophora* (F). A, en-face view of a hermaphroditic female showing 6 lips with labial papillae on top (l.p), two pore-like amphids (am) and cephalic papillae (c.p). B, vulval pattern and epiptygma (arrowed) of hermaphroditic female. C, Tail shape of hermaphroditic female and anus. D, en-face view of an amphimictic female showing 6 prominent lips, 6 labial papillae (l.p) on top, amphid (am), and cephalic papillae (c.p). E, vulval pattern of amphimictic female. F, vulval region of hermaphroditic females in the other reported isolates of *H. bacteriophora*. Scale bar (in µm): A = 4, B = 3, C = 8, D = 3, E = 5, F = 8.6.

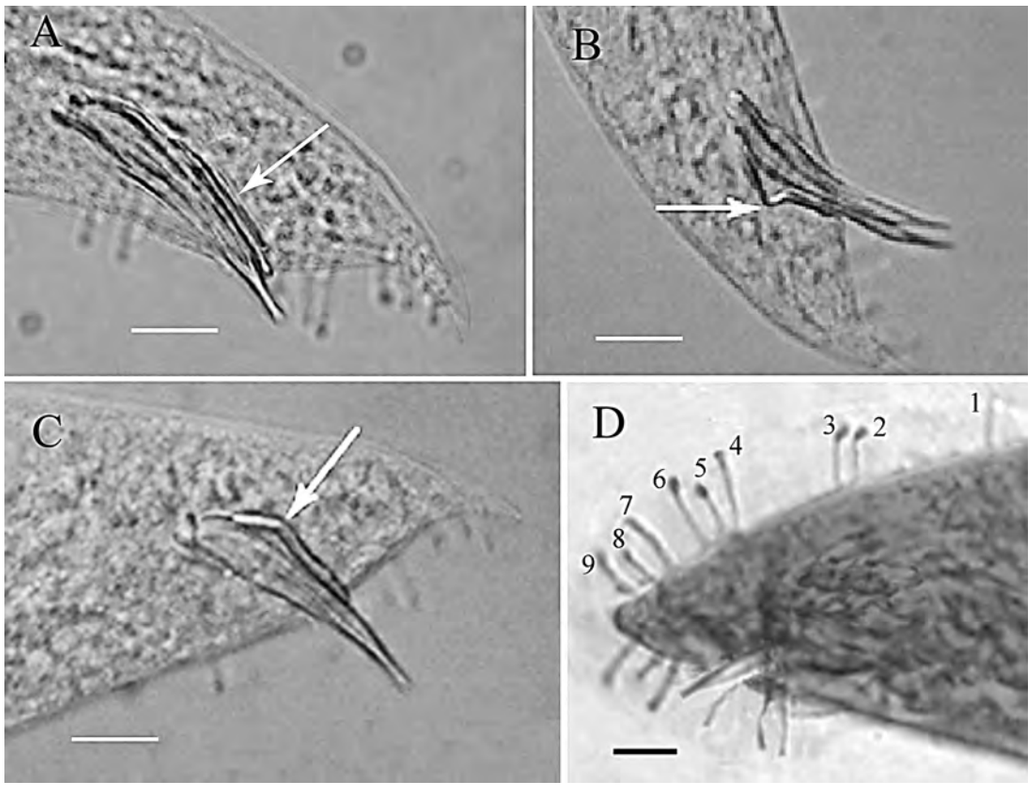

Fig. 3. Light microscopy of males of Iranian isolate of *H. bacteriophora*. A, spicules and normal gubernaculum (arrowed) in other Iranian isolates. B and C, spicules and proximal curvature of gubernaculum (arrowed) of IRAZ8 isolate. D, spicules and arrangement of genital papillae of IRAZ8 isolate. Scale bar (in µm): A = 10, B = 14, C = 12, D = 20.

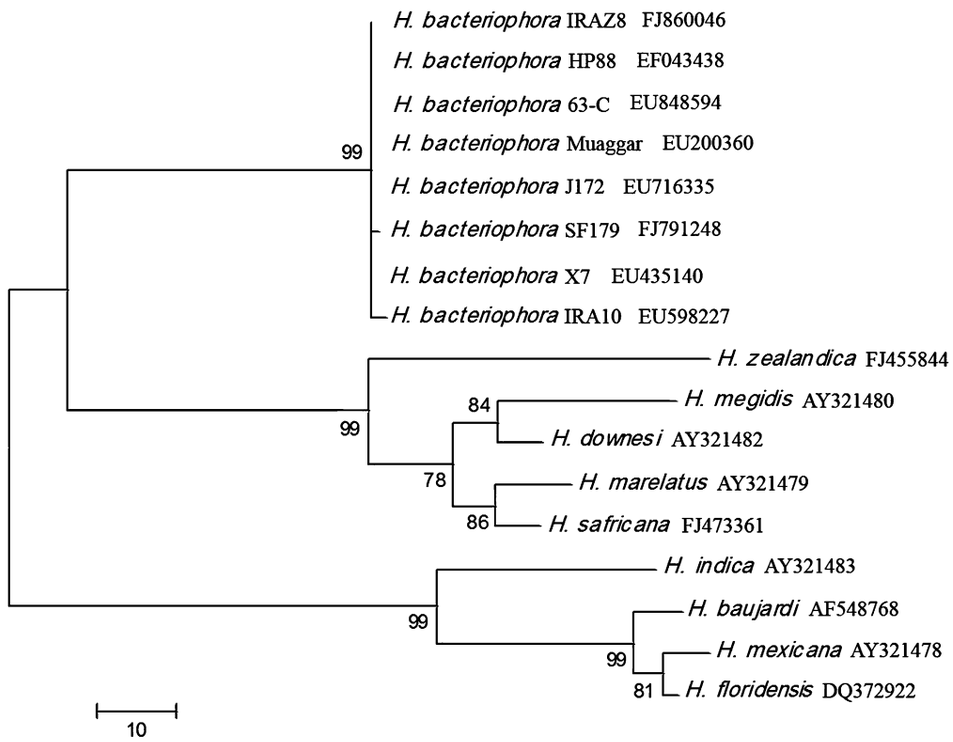

Fig. 4. Phylogenetic relationships between ten species of *Heterorhabditis* and eight isolates of *H. bacteriophora* with bootstrap analysis of ITS regions. The eight strains of *H. bacteriophora* form a monophyletic group. Numbers at the nodes represent bootstrap proportion.

Table II. Comparative morphometrics of Iranian isolates and type species of *Heterorhabditis bacteriophora*. Measurements are in microns and in the form: mean ± standard deviation (range).

		Infective juvenile								Male (Second generation)		
Character	n	L	EP	ES	T	ABW	A	B	c	D%	E%	SL	SW	GS
Type species*	25	588 (512-671)	103 (87-110)	125 (100-139)	98 (83-112)	10±1 (9-13)	25 (17-30)	4.5 (4-5.1)	6.2 (5.5-7)	84 (76-92)	112.0 (103-130)	40.0 (36-44)	128.0 (120-134)	48±10 (45-51)
IRAZ1	28	564±25.1* (517-607.7)	101±9.2 78.8-123.1)	127.8±7.2 (111-142)	95.7±9.6 (88-108)	16±2 (11-19)	22.9±3.5 (20-29)	4.4±0.3 (4-5.4)	6±0.3 (5.1-7.2)	79.5±11.4 (61-92.5)	108.7±8.3 (82.4-133)	34.7±8.7 (32.7-39)	164.7±43 (128-184)	52±3.5 (46.4-57)
IRAZ2	27	604.3±37.4 (549-659)	105.1±7.7 (93-122)	129.7±5.7 (119-145)	95±6.7 (84-108)	17±1.4 (15-20.3)	22.4±1.5 (20-25.6)	4.6±0.3 (4.3-5.4)	6.3±0.4 (5.7-7.6)	81±4.1 (74.7-91)	110±9.2 (95-129)	42±2.4 (37.5-45)	189±27 (140-251)	48.3±7.3 (36-57)
IRAZ4	20	537.8±49.5 (450-635)	104±9.6 (93-111)	124.9±4.9 (119-133)	93.4±3.3 (88-97)	15.2±1.8 (13-17.5)	19.4±2.1 (16.4-24)	4.2±0.4 (3.5-4.8)	5.9±0.4 (5.4-6.5)	59.5±40.2 (51-74.4)	97.8±7.5 (89-106)	43.2±2.3 (38.5-46)	194.7±25 (130-244)	49.6±5 (41-60)
IRAZ5	27	569.8±25.1 (497-618)	93±8.4 (73-105)	109.3±15 (78-131)	84.2±9.2 (66-100)	14.3±1.9 (11.3-20)	23.3±1.6 (21-28.5)	5.2±0.8 (4.2-6.8)	6.7±0.9 (5.5-9)	85.1±9.6 (72-107)	110.4±17.3 (89-136)	43.1±2.3 (38.8-46)	120±19 (91-143)	52±12.5 (48-60)
IRAZ6	26	577±18.5 (543-606)	101.8±4.5 (94-110)	124.5±5.2 (115-137)	95.5±5.6 (82-109)	13.6±0.9 (11-15.6)	25.5±1.3 (23.5-28)	4.6±0.2 (4.1-5)	6±0.4 (5.2-6.7)	82.2±4.3 (74.6-92)	107.6±8.6 (90-125)	44.6±2 (42-48.8)	197±16.7 (171-229)	53±4.7 (44-66.7)
IRAZ8	25	530±10 (490-578)	96±5 (90-101)	124±6 (115-135)	97±4.5 (90-106)	17.2±0.8 (16-19)	19±1.3 (16-21)	4±0.2 (4-4.7)	5.5±0.3 (4.9-5.9)	78.7±5.3 (73-85)	99.8±7 (85.8-124)	39.6±3.5 (33-46)	157±24 (122-203)	49±4.7 (39-57)
IRAZ10	22	582.1±31 (514-633)	100±1 (99-101)	130.7±7.1 (115-140)	96.4±3.3 (92-104)	16.6±1.2 (14-18.8)	20.6±1.2 (18-23.3)	4.5±0.3 (3.7-5.1)	6±0.3 (5.5-6.4)	76.5±2 (74.2-78)	103.8±1.6 (102-105.3)	40±1.8 (36-42)	155±22 (125-190)	55.5±1.5 (53.6-57)
IRAZ16	17	598±20.1 (544-621)	102.4±4.3 (96-108)	128±8.4 (112-137)	88.9±6.1 (81-101)	17.1±1 (15.7-19)	21.9±1.8 (18-25.2)	4.6±0.3 (4.2-5.3)	6.8±0.6 (6-7.4)	76.7±5.4 (71.6-82)	119.4±19.7 (105-133)	45±3 (40-48)	181±24 (147-214)	56±4 (51-62)
IRAZ17	22	555.8±34.6 (502-632)	98±4.6 (89-101)	128±5.5 (119-136)	92.2±4.9 (84-99)	16.7±1.2 (15-19)	20.7±1.6 (17-23.5)	4.4±0.3 (3.9-4.8)	6±0.4 (5.4-6.8)	75.5±4.8 (70-82.1)	102.6±7.8 (91.3-108)	44±1.9 (41-49)	182.4±13 (159-202)	53.6±3.6 (47-60.4)

*Data of type species *H. bacteriophora* from Poinar (1976).

bursal papillae in the male; pattern of vulval surround in the hermaphroditic females that is distinct and different from that of other described isolates (Fig. 2 B).

Molecular characterization

DNA characterization and Phytogenetic relationships. The partial 18S and 5.8S gene sequences showed little variation among the different isolates. The ITS1 and ITS2 regions are much more variable and provide most of the basic differences for species diagnosis (Nguyen *et al*., 2001). The sequence lengths, flanked by the two primers TW81 and AB28, of the ITS regions of *H. bacteriophora* strain IRAZ8 are 784 base pairs (bp) and, in the multiple alignment, aligned clearly without gaps with SF179, X7, 63-C and Muggar and with two gaps and two transitions with IRA10 as the most divergent isolate with the same length.

The phylogenetic relationships among the ten *Heterorhabditis* species and eight isolates of *H. bacteriophora* are presented in Fig. 4. The eight strains of *H. bacteriophora* compared form a monophyletic group by analysis of the ITS region. In this clade, the six strains IRAZ8, HP88, 63-C, Muggar, J172 and X7 compose a sister group to strains SF179 and IRA10. This topology shows that isolate IRA10 is genetically different from other strains of *H. bacteriophora*, but the difference is not enough to erect a new species.

DISCUSSION

There are only a few reports on the intraspecific morphological and molecular variations of *H. bacteriophora*. Spiridonov *et al*. (2004) reported that sequence differences between thirteen *H. bacteriophora* isolates from Germany, Russia, UK, Belgium, Iceland, Scotland and Switzerland usually varied between one and eleven bp (up to 1%) but reached 21 bp (2.8%) between the UK (B2) and the Moscow isolates. Also, Doucet *et al*. (1996) described for the first time a new isolate of *H. bacteriophora* with hermaphroditic female in the second and third generations from Argentina. In the first generation females and males of the Iranian isolate (IRAZ8), the specific patterns of vulval lip ornamentation (Fig. 2 B), the gubernaculum with the proximal portion distinctly ventrally curved (Fig. 3 B) and the shorter median pair in the terminal group of bursal papillae (Fig. 3 D) can be considered as intraspecific variations and therefore as a character that discriminate this isolate from others.

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