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Morphology and molecular study of an entomopathogenic nematode, *Steinernema bicornutum* (Nematoda, Rhabditida, Steinernematidae) from Iran

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Abstract

An entomopathogenic nematode was isolated by *Galleria* baiting technique from soil samples collected near Marand, East Azarbaijan province, North-west of Iran during 2003. Based on morphological and morphometric characters, as well as molecular data it was identified as *Steinernema bicornutum* Tallosi, Peters & Ehlers, 1995. Morphometrics comparison of the isolate with the type species showed no obvious differences between them. Differences in ITS-rDNA PCR-RFLP were found between the Iranian isolate, i.e. IRA7, with the type (Yugoslavian) isolate. The RFLP analysis was able to distinguish Iranian isolate from the type population of *S. bicornutum* in the *Hinf*I (four restriction sites in the type *vs* three restriction sites in the IRA7), *Hin*6I (550, 250 and 250 bp in the studied isolate *vs* 541 and 474 in the type isolate) and *Alu*I profiles (715, 177 and 123 in the type and 710 and 220 in the IRA7). These molecular differences are considered as intraspecific variations, and the described population from Iran is another isolate of *S. bicornutum*.

Key words: entomopathogenic nematodes, *Galleria*, Iran, ITS-rDNA, Marand, *Steinernema bicornutum*

چکیده

یک گونه نماتد بیمارگر حشرات، با استفاده از روش تله‌گذاری خاک با لارو پروانه‌ی موم‌خوار (*Galleria mellonella* (L.)) از نمونه خاک‌های جمع‌آوری‌شده از حومه‌ی مرند، استان آذربایجان شرقی، شمال غرب ایران جداسازی گردید. این گونه بر اساس صفات ریخت‌شناسی، داده‌های ریخت‌سنجی و نیز بررسی‌های مولکولی به عنوان *Steinernema bicornutum* Tallosi, Peters & Ehlers, 1995 شناسایی شد. در مقایسه‌های ریخت‌شناسی، تفاوت آشکاری بین جدایه‌ی مورد مطالعه و جدایه‌ی تیپ مشخص نشد. تجزیه و تحلیل الگوی PCR-RFLP ناحیه‌ی ITS-rDNA جدایه‌ی ایرانی – که با نام IRA7 معرفی می‌گردد – و مقایسه‌ی آن با جدایه‌ی تیپ (یوگسلاویایی) نشان داد که این دو جدایه با هم اختلاف دارند. ناحیه‌ی ITS-rDNA جدایه‌ی ایرانی برای آنزیم *Hinf*I دارای سه جایگاه برشی بوده ولی جدایه‌ی تیپ از چهار جایگاه برشی برخوردار می‌باشد. الگوی *Hin*6I جدایه‌ی تیپ شامل دو قطعه‌ی ٥٤١ و ٤٧٤ جفت باز بوده، درحالی‌که جدایه‌ی IRA7 شامل یک قطعه‌ی ٥٥٠ و دو قطعه‌ی تقریبی ٢٥٠ جفت باز می‌باشد. در مورد آنزیم *Alu*I نیز جدایه‌ی تیپ دارای سه قطعه‌ی ٧١٥، ١٧٧ و ١٢٣ جفت باز، ولی جدایه‌ی IRA7 شامل دو قطعه‌ی ٧١٠ و ٢٢٠ جفت باز است. این تفاوت‌های مولکولی به عنوان اختلافات درون‌گونه‌ای در نظر گرفته می‌شود و جمعیت توصیف‌شده از ایران جدایه‌ی دیگری از نماتد *S. bicornutum* می‌باشد.

واژگان کلیدی: نماتدهای بیمارگر حشرات، *Galleria*، ایران، ITS-rDNA، مرند، *Steinernema bicornutum*

Introduction

Entomopathogenic nematodes (EPNs) of the genus *Steinernema* Travassos are frequently used as biological control agents of several insect pests (Gauglar & Kaya, 1990).

They are obligate pathogens of insects. The *Steinernema* harbors bacterial symbiont, *Xenorhabdus* Thomas & Poinar, that kills the insect host and digests tissues, providing suitable conditions for growth and development within the cadaver (Boemare *et al*., 1993; Forst & Clarke, 2002). The only free living stage is the third stage infective juvenile (IJ), which carries cells of the bacterial symbionts in its intestine. Symbiotic bacteria play an important role in the pathogenicity of the nematode-bacterium complex. Once the IJ finds a suitable host, enters through natural openings and penetrates into hemocoel, where the symbiotic bacteria are released. They kill the host within 48 h by septicaemia (Adams & Nguyen, 2002).

The *Steinernema bicornutum* Tallosi, Peters & Ehlers, 1995 was first isolated from Vojvodina (Northern Yugoslavia) (Tallosi *et al*., 1995). The peculiarity of this species is the presence of two horn-like appendages on the anterior end of infective juveniles. In a survey on EPNs in north-west of Iran, specimens with morphology similar to *S. bicornutum*, were isolated using *Galleria* baiting technique. After morphological and molecular study and comparison with existing data, this nematode was recognized as *S. bicornutum*. In this article we present morphometrics and ITS-rDNA profiles of the isolate.

Material and methods

Soil samples were collected from an alfalfa field around Marand (N = 38º 26' 6.36", E = 45º 44' 29.69") in north-west of Iran during 2003 and the nematodes were isolated using insect baiting method (Bedding & Akhurst, 1975).

Morphological study

For light microscopy, nematodes were examined live or heat killed in 60ºC Ringer's solution. All nematodes used in this study were reared in *Galleria mellonella* (L.) larvae. Twenty *G. mellonella* larvae were exposed to about 1000 infective juveniles in a Petri dish lined with two moistened filter papers at room temperature (25 ± 3ºC). For isolating mature males of the first generations, the infected larvae were dissected in Ringer's solution three days after infection. The collected nematodes were killed using hot (50-60ºC) Ringer's solution. Heat killed nematodes were placed in triethanolamin-formalin (TAF) fixative (Kaya & Stock, 1997) and processed to anhydrous glycerine for mounting by a slow evaporation method. The nematodes were mounted on microscopic slides. Morphologic and morphometric studies were made using an Olympus BX41 microscope equipped with interference contrast

and a drawing tube. Additionally, morphological features of males and IJs were examined using scanning electron microscopy (LEO 44oi). For this purpose, specimens were processed following protocols described by Nguyen & Smart (1995).

Molecular study

DNA was extracted from a single IJ with the following method. The nematode was crushed in 15 µl 1× PCR buffer and transferred to a precooled sterilized 0.2 ml tube containing 10 µl of the same buffer. The tube was incubated at -70ºC for 15 min and thawed at 60ºC, then inoculated with 2 µl of 60 µg ml^{-1} proteinase K. The tube was incubated at 65ºC for 2 h, and then heated at 95ºC for 15 min. After centrifugation at 16000 g for 15 min, the supernatant containing nematode DNA was collected and stored at -70ºC until use (Eivazian Kary *et al*., 2009).

The ITS-rDNA was amplified by PCR in a 50 µl reaction containing: 15 µl of worm lysis mix, 5 µl of 10× PCR buffer, 2 µl of dNTP mix, 1.2 unit of *Taq* DNA polymerase,1 µl of each primer and double distilled water to final volume. The forward primer TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and the reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') were used in the PCR reaction for amplification of the complete ITS region (Joyce *et al*., 1994).

Amplified products were digested with restriction endonucleases (*Hind*III, *Eco*RI, *Bam*HI, *Sa*II, *Mva*I, *Mbo*I, *Pst*I, *Rsa*I, *Kpn*I, *Msp*I, *Alu*I, *Hin*6I, *Xba*I, *Bsu*RI, *Pvu*II, *Hinf*I) according to the manufacture's instruction using 6 µl PCR product in a 12.5 volume. The entire digest was loaded on a 1.8% agarose gel and run at 8 v/cm for 3 h., stained with ethidium bromide, visualised and photographed under UV.

In order to compare the results of PCR-RFLP profile with the type nematode, ITS-rDNA sequence of type isolate (AF121048) was restricted using Restriction Mapper.

Results

The morphometrical data of the two *S. bicornutum* isolates are presented in the table 1 and 2. The values for type specimens of the species were obtained from original description (Tallosi *et al*., 1995).

Morphology

Males (first generation): body curved posteriorly, J-shaped when heat-killed (fig. 1A). Cuticle smooth under light microscopy, but with fine transverse striae visible under SEM.

Lateral fields and phasmids inconspicuous. Head truncate to slightly rounded, continuous with body. Six lip amalgamated but tips distinct, and with one labial papilla on each. Four cephalic papillae. Amphids small, located behind lateral labial papillae. Stoma reduced, short and wide. Testis single and reflexed. Anterior to the cloacal opening a row of six pairs of genital papillae visible in ventro-lateral position and a single ventral precloacal papilla. Three pairs posterior to the gubernaculum and another three pairs surrounding the tail tip. Spicules paired and light brown in color. Spicules (fig. 1B, C) curved; capitulum distinct and well separated from lamina, bowel-shaped, proximally broad and flattened; calamus with a small pointed protrusion. From this point a distinct velum extends almost to the proximal end of the lamina (type I). In another type of spicules (fig. 1C) capitulum less separated from lamina and not much broader. Gubernaculum spindle-shaped, flattened or convex, much variable in shape and size (fig. 1B, C). Tails short, round and without a mucron. Tail in second generation males longer and usually with a small mucron.

Females: body C-shaped upon heat-killed (fig. 1D). Cuticle smooth, head rounded, not offset from rest of the body (fig. 1E). Each of the six lips bearing a single labial papilla. Outer circle of four cephalic papillae further back on the head. Stoma short and wide. Esophagus muscular and almost cylindrical, but widening towards the basal end. Metacorpus nonvalvate; isthmus much thinner than metacorpus; nerve ring surrounding esophagus between isthmus and basal bulb, basal bulb slightly broader than or as wide as metacorpus. Excretory pore slightly anterior to isthmus, almost at the middle of the esophagus. Gonads amphidelphic with opposed reflexed ovaries. Vulva, a transverse slit protruding only in second generation (fig. 1D), and never in first generation females. Tail of first generation short, blunt, with a short wedge-shaped projection and always with mucron (fig. 1F).

Infective juveniles: body slender, tapering regularly from base of oesophagus to anterior end and from anus to terminus. Lip region smooth, mouth closed. Lips indistinct, with six labial papillae. Head hemispherical, labial region bearing a hornlike structure composed of two protuberances, which possibly are laterally protruding labial papillae, located on an oval ring around the oral opening (fig. 2A, B). The third stage infective juvenile is generally ensheathed by the second stage juvenile cuticle. Amphidial openings lateral, a little further back from the lip region, almost covered by the two horns. Four distinct cephalic papillae arranged medially on the head at a 45 degrees angle to the hornlike structure (fig. 2A, B). Excretory pore situated half-way between head and bulbus. Oesophagus and intestine lumen collapsed. In the anterior portion of the intestine, a pouch containing cells of the symbiotic

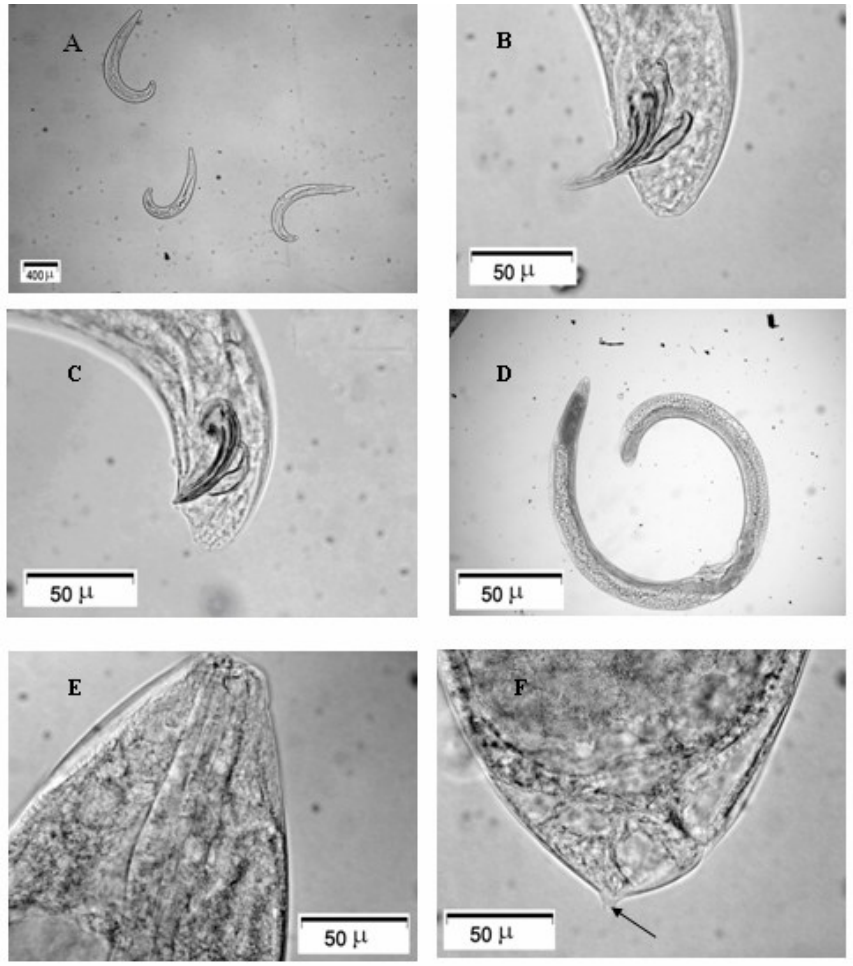

Figure 1. *Steinernema bicornutum*. A, body shape in heat killed males; B, type I spicule; C, type II spicule; D, body shape in heat killed females; E, Anterior region of female; F, posterior region of female, mucron (arrow).

bacterium, probably *Xenorhahdus* sp. Body slender with transverse annulations. Lateral fields with eight ridges (fig. 2C) of which the submarginal ridges are less distinct than the others. Tail constricted with a pointed end (fig. 2D).

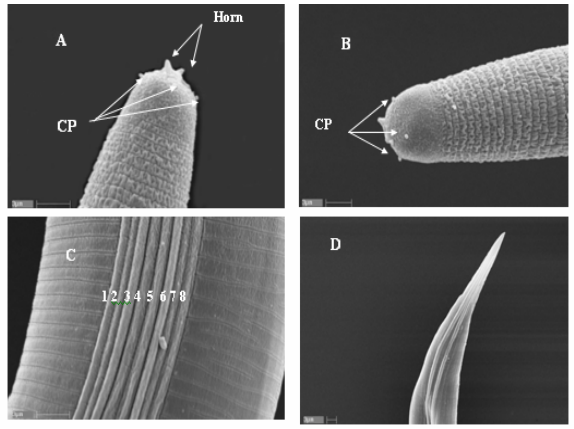

Figure 2. *Steinernema bicornutum* infective juvenile. A-B, head region showing horn-like structure and cephalic papilla (CP); C, lateral fields showing eight ridges; D, tail.

PCR-RFLP pattern

PCR-RFLP patterns comparison between type and Iranian population of *S. bicornutum*, i.e. IRA7, revealed differences in *Hinf*I and *Hin*6I profiles. *Steinernema bicornutum* IRA7 showed four bands (380, 370, 180 and 110 bp.) of *Hinf*I profile whereas *Hinf*I profile of type isolate had five bands (table 3). In treatment with *Hin*6I, type isolate showed one restriction site resulted in two 541 and 474 bp (table 3), but IRA7 had one more restriction site and yielded three bands including 550, and two 250 bp (in the form of more or less two overlapping fragments) (fig. 3). In the case of *Alu*I, type isolate showed two restriction sites resulted in three 715,177 and 123 (table 3), but Iranian isolate had two detectable bands including 710 and 220.

Discussion

Based on morphometrical data (tables 1 and 2), two isolates showed similar range and therefore the studied isolate could be considered as *S. bicornutum*. There are a few reported data on the morphology and molecular intraspecific variations of EPNs. Szalanski *et al*. (2000) reported similar sequence for rDNA ITS1 and mtDNA 16S-COII in three *Steinernema feltiae*. Yoshida (2003) showed differences in the populations of *S. feltiae* and *S. kraussei* from Japan. He identified two variants in excretory pore location in comparison of Japanese

Table 1. Comparative morphometrics of infective juveniles of *S. bicornutum* of type culture and Iranian isolate; mean value ± standard deviation (range).

	L	W	AbW	T	Es	EP	NR	a	b	c	d	e
IRA7	723 ± 42	31 ± 3	17.3 ± 1.4	62 ± 3	121 ± 6	56.4 ± 2.9	85.5 ± 8.4	23.6 ± 2	5.96 ± 0.57	11.76 ± 0.69	46.8 ± 4.3	92.9 ± 6.7
	638-797	26-40	15-21.2	51.9-67.5	107-131	50-60	68-102	18.3-26.4	5.1-6.94	9.87-12.64	40.4-55.2	80-109.6
Type isolate	796	29	*	72	124	61	92	27	6.2	10.7	50	84
	648-873	25-33		63-78	113-135	53-65	88-100	23-29	5.6-6.9	9.7-12	40-60	80-100

* Data not available.

Table 2. Comparative morphometrics of first generation males of *S. bicornutum* of type culture and Iranian isolate; mean value ± standard deviation (range).

	L	W	AbW	T	Es	EP	NR	SpL	SpW	Gl	SW	GS	d
IRA7	1177 ± 258	104 ± 38	38 ± 7	21 ± 3	132 ± 24	82 ± 9	93 ± 14	64 ± 8	10.1 ± 1.8	37 ± 4	1.7 ± 0.2	58 ± 7.3	61 ± 5
	843-1681	62-168	28-50	15-26	71-166	72-101	64-109	55-81	8-13	31-44	1.3-2.1	51-79	53-66
Type isolate	1353 ± 150	109	*	*	*	*	*	65	*	48	2.22	72	52
	945-1539												

* Data not available.

Table 3. Size and number of fragments for each restriction enzyme (in descending order) of ITS sequences of type isolate of *S. bicornutum* (AF121048) cut by 16 identifying enzymes.

*Hind*III	*Eco*RI	*Bam*HI	*Sa*lI	*Mva*I	*Mbo*I	*Pst*I	*Rsa*I	*Kpn*I	*Msp*I	*Alu*I	*Hin*6I	*Xba*I	*Bsu*RI	*Pvu*II	*Hinf*I
		-	-	-	531	-	806	-	896	715	541	-	-	715	378
					164		207		97	177	474			300	377
					162		2		22	123					167
					142										62
					16										31

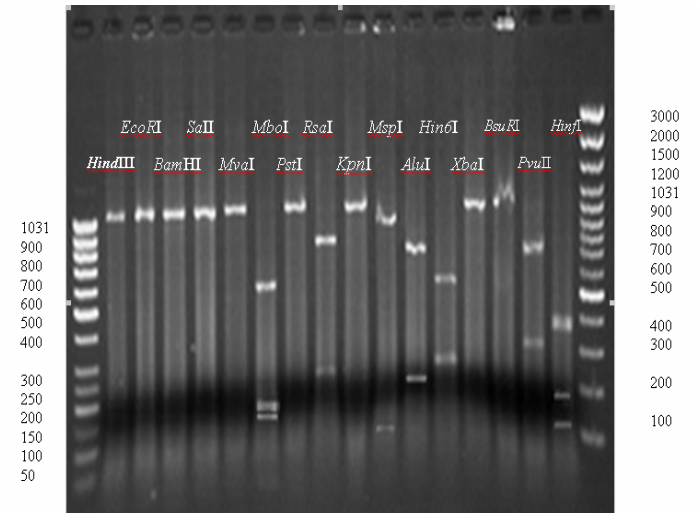

Figure 3. RFLP yielded by digestion of PCR amplified product of ITS-rDNA of *S. bicornutum* IRA7 with 16 different restriction enzymes (upper rows). Right and left columns are 100 and 50 molecular weight markers, respectively.

S. feltiae with some foreign isolates of the species. The molecular analysis showed different restriction patterns for the Japanese isolates of *S. feltiae* compared with European isolates. In the case of *S. kraussei*, Japanese isolate had a longer mucron and clear difference in RFLP pattern of ITS region of rDNA compared with UK isolate. Spiridonov *et al*. (2004) reported intraspecific variability for ITS sequences of 11 isolates from *S. feltiae* ranging from 0% to 2.4%. They also indicated sequences differences among 13 *S. kraussei* isolates up to 2.8%. Kuwata *et al*. (2006) compared the intraspecific variations of steinernematid species isolated from Japan based on the DNA sequences of their ITS and COI regions and identified intraspecific differences between the studied isolates of *Steinernema*. In the ITS region, the intraspecific variations of the sequences ranged from 0% to 0.7%. On the other hand, the differences in the COI region were 5% and 7.8%, respectively. Their results indicated that the intraspecific divergence of the ITS region is less variable than the COI region.

Ivanova & Spiridonov (2003) reported infective juvenile and spicule length differences between Krasnodar isolate of *S. bicornutum* and type isolate. The two populations were distinguished based on their RFLP pattern of *Cfo*I. Sequencing of long fragment of ribosomal

gene (ITS1 + 5.8S + ITS2) revealed differences in 18 pairs of nucleotides in fragment of 715 bp between Krasnodar and type isolates.

Based on primary experiment, infective juveniles of *S. bicornutum* IRA7 were found to be highly invasive against *G. mellonella* at 24ºC than 20ºC that normally considered as an appropriate temperature for *Steinernema.* According to available literature, this is the first report of *S. bicornutum* from Iran. In addition, according to our results, RFLP pattern for two enzymes including *Hinf*I and *Hin*6I could be used successfully for differentiating Iranian *S. bicornutum* isolate from at least type strain, but more isolates of this species from different countries must be studied to document a definitive statement on intraspecific variability within *S. bicornutum.*

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