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First report of an entomopathogenic nematode, *Steinernema kraussei* (Rhabditida, Steinernematidae) from Iran

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Abstract

During conduction a survey of entomopathogenic nematodes throughout Arasbaran forests and rangelands, north west of Iran in 2007-2008, an entomopathogenic nematode was isolated using *Galleria* baiting method from soil samples collected from rangelands, near Chichakloo, Varzeghan, East Azarbaijan. It was identified as *Steinernema kraussei* (Steiner, 1923) Travassos, 1927 based on morphology and morphometric characters, cross breeding test, as well as molecular data. This species can be separated from other members of the genus by medium body length of infective juvenile (818 µm), lateral field with eight ridges in which the central pair is less distinct than others, head smooth, long cephalic papillae, secretory-excretory pore located at the level of middle of esophagus and anterior to the nerve ring; broad, slightly yellowish and short spicules, low D% value and tail with fine mucron in males. The analysis of ITS-rDNA sequence placed Iranian population of *S. kraussei* in the '*feltiae - kraussei - oregonense*' group in the clade that containing different isolates of the species. It has some morphological and morphometric differences such as long cephalic papillae of IJ, more curvature of spicules and absence of mucron in some males as compared with type species. The mentioned differences are considered as intraspecific variations, and the described population from Iran is another isolate of *S. kraussei*. This is the first record of *S. kraussei* from Iran.

Key words: Arasbaran, Iran, entomopathogenic nematodes, morphology, morphometrics, *Steinernema kraussei*

چکیده

در طی نمونه‌برداری از خاک‌های جنگل‌ها و مراتع ارسباران در شمال غرب ایران که با هدف جمع‌آوری و شناسایی نماتدهای بیمارگر حشرات در سال‌های ۱۳۸۷-۱۳۸۶ انجام گرفت، گونه‌ای نماتد بیمارگر حشرات به روش طعمه‌گذاری با لاروهای سن آخر پروانه‌ی موم‌خوار از نمونه‌ی خاک مراتع چیچکلو واقع در اطراف شهرستان ورزقان استان آذربایجان شرقی، جداسازی شد. این نماتد که بر اساس صفات ریخت‌شناسی، داده‌های ریخت‌سنجی، آزمون دگرآمیزی و نیز بررسی‌های مولکولی به عنوان گونه‌ی *Steinernema kraussei* (Steiner, 1923) Travassos, 1927 شناسایی گردید، با داشتن میانگین طول بدن حدود ۸۱۸ میکرومتر، هشت شیار طولی سطح جانبی با دو شیار وسطی کمتر برآمده، پاپیل‌های بلند سری، منفذ دفعی- ترشحی در ناحیه‌ی وسط مری و جلوتر از حلقه‌ی عصبی در لاروهای آلوده‌کننده و آلت نرینه‌ی پهن و کوتاه به رنگ مایل به زرد، نسبت کم D% و دم دارای موکرون ظریف در افراد نر، از دیگر اعضای این جنس متمایز می‌شود. بررسی توالی ناحیه‌ی ITS-rDNA نیز جدایه‌ی ارسباران را در گروه '*feltiae - kraussei - oregonense*' شامل جدایه‌های مختلف گونه‌ی *S. kraussei* قرار داد. این گونه با دارا بودن برخی تفاوت‌های ریخت‌شناسی و ریخت‌سنجی از قبیل پاپیل‌های بلند در سر لاروهای آلوده‌کننده، آلت نرینه خمیده‌تر و فقدان موکرون در برخی افراد نر، از گونه‌ی تیپ متمایز است. با این حال این تفاوت‌ها به‌عنوان اختلاف‌های درون‌گونه‌ای محسوب شده و جمعیت ایرانی، جدایه‌ای دیگر از گونه‌ی *S. kraussei* می‌باشد که بر اساس منابع موجود برای اولین‌بار از ایران گزارش می‌گردد.

واژگان کلیدی: ارسباران، ایران، نماتدهای بیمارگر حشرات، ریخت‌شناسی، ریخت‌سنجی، *Steinernema kraussei*

Introduction

Entomopathogenic nematodes (EPNs) of the genus *Steinernema* Travassos, 1927 are cosmopolitan, having been isolated from all continents except Antarctica (Gaugler & Kaya, 1990; Griffin *et al*., 1990). The species of *Steinernema* are frequently used as biological control agents of several insect pests (Gaugler & Kaya, 1990). They are obligate pathogens of insects. *Steinernema* harbors bacterial symbiont, *Xenorhabdus* (Thomas & Poinar, 1979), which kills the insect host and digests tissues, providing suitable conditions for growth and development of the nematode within the cadaver (Boemare *et al*., 1993; Forst & Clarke, 2002). The only free living stage is the third-stage infective juvenile (IJ) that carries cells of the bacterial symbionts in its intestine. Symbiotic bacteria play an important role in the pathogenecity of the nematode-bacterium relationship. Once the infective juvenile finds a suitable host, enters it through natural openings and penetrates into hemocoel. In the hemocoel, IJ releases the symbiotic bacteria that kill the host within 48 h. by septicemia (Adams & Nguyen, 2002). Collectively, nearly 36 valid species of the genus *Steinernema* have been described worldwide and these are divided into five groups according to their morphology and molecular characteristics (Nguyen, 2005; Spiridonov *et al*., 2004). *Steinernema kraussei* (Steiner, 1923) Travassos, 1927 is the first entomopathogenic nematode that was isolated from infected sawflies in Germany by Steiner in 1923 (Stock *et al*., 2001).

A survey on entomopathogenic nematodes was conducted in the Arasbaran forests and rangelands, near Chichakloo, Varzeghan, north west of Iran during 2008. As a result, some species of *Steinernema* were identified. One isolate of the genus was identified as *S. kraussei* based on morphological and molecular information. In this article we report it for the first time from Iran and discuss its morphology, morphometrics and ITS (internal transcribed spacer) - rDNA profiles.

Materials and methods

Steinernema sp. IRAZ20 was recovered from soil samples collected from rangelands, near Chichakloo, Varzeghan, north west of Iran (from the rhizospher of an oak tree, *Quercus macrantera* Fish & Meyer dominated forest habitat, longitude 46° 45' 45" E, latitude 38° 55' 20" N, altitude 2121 m a.s.l., annual average of 12°C temperature, precipitation 415 mm/year) using *Galleria mellonella* L. baiting method described by Bedding & Akhurst (1975). Infective juveniles (IJs) were collected from *G. mellonella* cadavers, using the method of White (1927) and stored in aerated water at 7°C. All nematodes used in this study were reared

in *G. mellonella* larvae. Twenty *G. mellonella* larvae were exposed to IJs suspension (about 1000 IJ/ml) in a Petri dish lined with two moistened filter papers at laboratory temperature (23 ± 3ºC).

Morphological and morphometrical characterization

Light microscopy - First generation males and females were collected from *Galleria* cadavers (dissected out in Ringer's solution) 4-5 days post inoculation. Infective juveniles and second generation adults were collected during the week after their first emergence from *Galleria* cadavers and killed using hot (50-60°C) Ringer's solution (Nguyen & Smart, 1990). Dead nematodes were fixed in triethanolamine formalin (TAF) and processed to anhydrous glycerine by a slow evaporation method (Woodring & Kaya, 1988) and mounted on microscopic slides. Morphological and morphometrical studies were made using an Olympus BX41 microscope equipped with an interference contrast and a digital camera. Image tool software was used for obtaining quantitative measurements.

The following abbreviations regarding morphometrical measurements have been used in the text or tables: n = number of specimens measured; L = body length; ABW = anal body width; EP = excretory pore position; ES = oesophagus length; GL = gubernaculum length; MBW = maximum body width; NR = nerve ring position; T = tail length; SL = spicules length; SPW = spicules width; ratio a = L/MBW; ratio b = L/ES; ratio c = L/T; ratio d = EP/ES × 100; ratio e = EP/T × 100; GS = GL/SL × 100; SW = SL/ABW. For analysis of morphometric variables on males and infective juveniles, Microsoft Excel software was used.

Scanning electron microscopy - Scanning electron microscopy (SEM) was carried out using Nguyen & Smart (1995) method. First generation adults were dissected from *G. mellonella* larvae in Ringer's solution. They were rinsed for 5 min each in Ringer's solution three times. Adults and IJs were fixed in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.2 for 24 hours at 8°C. They were post-fixed with 2% osmium tetroxide (OsO4) solution for 12 hours at 25°C, dehydrated in a graded ethanol series (5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and 100%), mounted on aluminium SEM stubs, coated with gold powder (200 nm thicknese) and studied using a LEO 44oi scanning electron microscope.

DNA extraction, sequencing and sequence alignment - Total genomic DNA was extracted from single IJ as described by Phan *et al*. (2001), with some modifications. Extracted DNA was used in polymerase chain reaction. The forward and reverse primers were used in PCR

reaction for amplification of the complete ITS-rDNA region (Vrain *et al*., 1992). Primers were synthesized by Sinagene Company (Iran). Amplifications were carried out in a 50 µl volume, containing 50 mM KCl, 10 mM Tris (pH 8.4), 1.5 mM MgCl2, 0.1% Triton × 100, 0.2 mM of each dNTP, 0.4 mM of each primer, 10 µl of nematode lysate and 0.25 µl of *Taq* DNA polymerase (1.25 unit). Amplification were carried out using Biometra thermocycler with heated lid pre-set at 95ºC and subjected to the following cycling profile: one cycle of 94ºC for 5 min followed by 35 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 1.5 min. A final step of 5 min at 72ºC was also included to ensure all of the final amplification products are full length. Amplification products were purified with a QIAGEN PCR purification kit. Purified DNA was sequenced in IBMP-CNRS, France.

The ITS sequence of studied isolate IRAZ20 (GenBank accession number FJ860038) was aligned using the default option of Clustal X (Thompson *et al*., 1997) with ITS sequences of 22 species of *Steinernema* which have been deposited in GenBank and *Caenorhabditis elegans* (Maupas) as the outgroup taxon (X03680). Molecular phylogenetic relationships were obtained by equally weighted maximum parsimony (MP) and maximum likelihood (ML) using PAUP 4.0b8 (Swofford, 2002).

Cross breeding test - To further confirm the identity, reproductive compatibility of *S. kraussei* was tested using the *Steinernema feltiae* (Filipjev). The third stage juveniles produced by first generation adults were surface sterilised in 0.4% hyamine and washed four times in sterile distilled water. Single juveniles were transferred to hanging drops of haemolymph according to procedures described by Kaya & Stock (1997). A total of 20 pairs / nematode combination were established by transferring one male into a drop containing a morphological female once they could be differentiated for each of the tested combinations. Controls consisted of hanging drops with adults of the same species.

Results

The measurements of the morphometric characters of Iranian population and type isolate of *S. kraussei* are shown in table 1.

Description

Males - Body curved posteriorly, J-shaped upon heat-killed (fig. 2, A). Head truncate, slightly offset from the rest of body, fused lips bearing two circles of papillae, six inner-labial papillae and four outer-cephalic papillae (fig. 3, A). Pore-like amphid openings located laterally near

the cephalic papillae circle. Cuticle smooth with slight annulation visible under SEM. Tail tip rounded with terminal fine mucron. Eleven pairs of genital papillae present, of these five pairs are preanal and situated subventrally in two fine rows, three pairs adanal in lateral to dorso-lateral position, three pairs postanal at the tail tip and one single papilla just in front of cloacal

Table 1. Morphometric characters of Iranian population and type isolate of *S. kraussei*. Measurements are in micrometer and in the form: mean ± SD. See text for the abbreviations.

Character	Infective juvenile		First generation male	
	Iranian population	Type isolate	Iranian population	Type isolate
n	25	10	20	10
L	818.6 ± 37.6 (742-897)	951 (797-1102)	1367 ± 248.5 (1005-1807)	1400 (1200-1600)
MBW	42.4 ± 4.7 (37-54)	33 (30-36)	137 ± 25.8 (103-195)	128 (110-144)
EP	52.6 ± 1.7 (50-56)	63 (56-66)	91 ± 13.9 (95-120)	81 (73-99)
NR	90 ± 3.1 (87-93)	105 ± 4.1 (99-111)	*	*
ES	114 ± 5.1 (106-127)	134 (119-145)	143 ± 14.2 (111-163)	152 (139-178)
Testis flexure	-	-	389.3 ± 20.4 (364-413)	*
Tail	81.7 ± 6.5 (72-89)	79 (69-86)	32.3 ± 4.3 (25-41)	39 (36-44)
ABW	21.9 ± 1.1 (20-24)	20 (19-22)	44.6 ± 6.4 (34-58)	*
Mucron length	-	-	5.5 ± 0.3 (5.2-5.8)	*
Spicule length	-	-	56.4 ± 3.9 (49-63)	49 (60-65)
Spicule width	-	-	11 ± 0.7 (9-14)	*
Gubern. length	-	-	35.2 ± 2.6 (32-40)	33 (29-37)
a	19.9 ± 1.6 (18-23)	29 *	10 ± 0.7 (8-10.7)	10.9 *
b	7.2 ± 0.5 (6-7.7)	7.1 *	9.5 ± 1.3 (7.6-11)	9.2 *
c	10.1 ± 1 (8-11)	12.1 *	42.2 ± 5.3 (31-50)	35.9 *
D%	46.3 ± 1.7 (43-48)	47 *	63.9 ± 6.4 (56-73)	53 *
E%	64.8 ± 4.5 (60-72)	80 *	290.7 ± 33.3 (247-322)	227 *
SW	-	-	128.2 ± 15.9 (98-140)	110 *
GS	-	-	62.7 ± 4.3 (55-70)	68 *

* Data not available

opening (fig. 3, B). Mouth opening triangular. Esophagus muscular with a characteristic rhabditoid cylindrical corpus, subdivided into procorpus and slightly enlarged metacorpus, isthmus narrow and distinct, basal bulb enlarged and valvate (fig. 2, B). Secretory-excretory pore always anterior to nerve ring situated at the level of metacorpal portion (fig. 2, B). Testis monorchic and reflexed, spicules shape variable but a well curved spicules is more common, with head variable in shape, velum present, extending about two-third of blade length, spicules tip with an aperture, similar to that is present in *Steinernema glaseri* (Steiner), and a bluntly rounded or flattened terminus. In lateral view, the gubernaculum tapering anteriorly to a ventrally curved end, in ventral view, cuneus short, Y-shaped, pointed posteriorly and not reaching the end of corpus. Second generation males are smaller than the first generation ones.

Females - Body C-shaped when heat-killed. Head rounded, continues with the rest of body. Cuticle slightly annulated (fig. 3, C). Each of the six lips bearing a single labial papilla. The arrangement of lips, papillae and alimentary tract almost similar to that of males. Secretory-excretory pore opening slightly anterior to isthmus, near the middle of the esophagus (fig. 3, D). Tail pointed in young females of the first generation, with a postanal swelling, but blunt and with a short spine in mature females, rarely without a spine. Moderately elevated vulva situated closely behind midbody, gonads paired and reflexed. Second generation females are smaller, tail always pointed, rarely with postanal swelling.

Infective juveniles - Body thin, tapering regularly from base of oesophagus to anterior end and from anus to tail. Second stage cuticle sometimes present as a sheath around the body of IJ. Head with four relatively long cephalic papillae that located on a circle around the oral opening, labial papillae indistinct (fig. 1, A-B). Amphidial openings at the level of cephalic papillae. Oral opening closed, esophagus collapsed, basal bulb more elongated than that of the adults. Secretory-excretory pore near mid-esophagus (approximately 46% of esophagus length from anterior end). Lateral fields with eight ridges (fig. 1, C). The central pair less distinct than the others and some of the change merges occasionally along the length of lateral field. Tail pointed without any constriction or spike-like structure.

Diagnostic characters - The *S. kraussei* is characterized by medium body length of infective juveniles (averaging below 1000 μm). Lateral fields with eight ridges, but the central pair is less prominent, head smooth, lacking horn-like structures, secretory-excretory pore located at the level of middle of pharynx. Secretory-excretory pore of adults is situated far in front of the nerve ring. This nematode is morphologically similar to *S. feltiae*, *S. diaprepesi* and *S. glaseri*.

S. kraussei can be distinguished from *S. feltiae* with shorter IJ body length (818 *vs.* 880 µm), less prominent central pair of lateral lines and more anterior secretory-excretory pore of the IJ (52 *vs.* 62 µm), by the presence of a short tail mucron (never more than 5.5 µm) in the adults of the first and long mucron in the second generation adults, by the size, shape and colour of the spicules which are brown-orange and possess a wider velum. The species can be separated from *S. diaprepesi* by presence of a mucron on the male tail, the much shorter spicule length of males (56.4 *vs.* 79 µm), smaller mean body length of IJ (818 *vs.* 1002 µm). Finally, it differs from *S. glaseri* as follows: in *S. kraussei* secretory-excretory pore of males is close to mid-esophagus, while in *S. glaseri* is close to the nerve ring, male tail with fine mucron which is absent in *S. glaseri*, spicule tip pointed without hook-like structure which is present in *S. glaseri*, length to width of manubrium (1 to 1 *vs.* 1.5 to 1), rostrum usually developed, while never present in *S. glaseri*. Infective juveniles of *S. kraussei* belong to the group of species characterized by medium body length, averaging below 1000 micrometers but *S. glaseri* belong to the group of species characterized by long body length (averaging above 1000 micrometers). Iranian isolate is separated from other isolates of *S. kraussei* by having long cephalic papillae in infective juveniles.

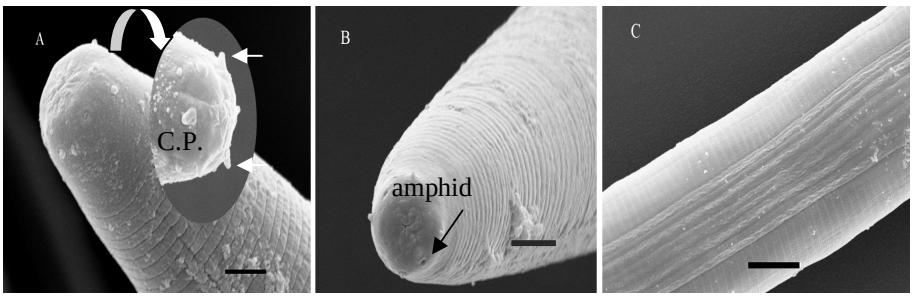

Figure 1. Infective juvenile. A) Head region showing cephalic papillae (C.P.); B) amphids and closed mouth opening; C) lateral field. Scale bars: A, B and C = 3 µm.

Molecular characterisation

The sequence of ITS-rDNA regions of *S. kraussei* IRAZ20 (FJ860038), including primers TW81 and AB28 can be recognized by its length of 781 base pairs (bp). Phylogenetic

analysis of ITS regions showed that the Iranian isolate is closely related to the *S. kraussei* isolate C46 (EU914856) from Slovenia (fig. 4). The species in '*feltiae - kraussei - oregonense*' group (*S. cholashanense*, *S. feltiae*, *S. texanum*, *S. kraussei*, *S. kushidai*, *S. monticolum* and *S. oregonense*) form a monophyletic group with high bootstrap support (95%) and Iranian isolate of *S. kraussei* does cluster with the mentioned group.

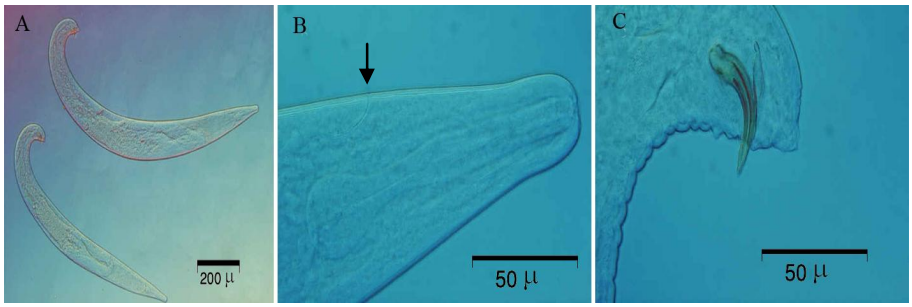

Figure 2. Male. A) Body shape; B) head region, secretory-excretory pore (arrow) and esophagus; C) spicules and gubernaculum in first generation.

Discussion

The morphometric data of the Iranian population and type species of *S. kraussei* are presented in the table 1. The values for type specimens of the species were obtained from redescription of its topotype (Mracek, 1994; Nguyen, 2005). Based on comparison of morphological and morphometric data, the two isolates were found similar and they could be considered as conspecies. The differences were also obtained between them in the average body length, labial papillae length, ratio E% and NR of infective juveniles as well as spicules shape, size and color, and ratio E% of males. Molecular studies revealed that Iranian population is an isolate of *S. kraussei* and most of its morphologic and morphometric characters are also similar to the mentioned species. Therefore, the differences are considered as intraspecific variations.

There are very rare reported data on the intraspecific morphological variations of *S. kraussei*, however, some molecular studies are recently available. Yoshida (2003) showed differences in the populations of *S. feltiae* and *S. kraussei* from Japan. He reported that Japanese isolate of *S. kraussei*, had a longer mucron and clear difference in RFLP pattern of

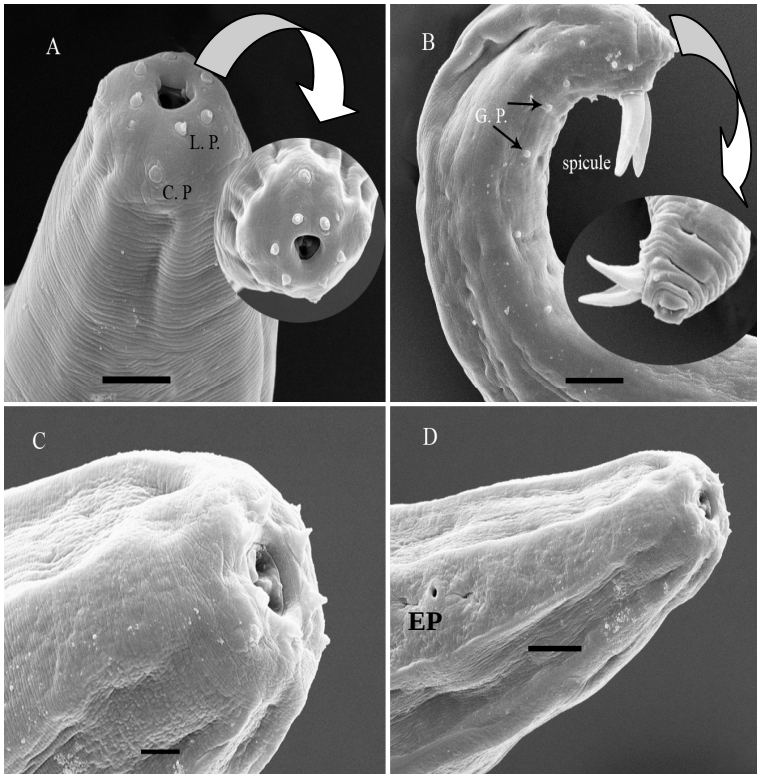

Figure 3. A) Labial papillae (L.P.), cephalic papillae (C.P.) and mouth opening in first generation male; B) arrangement of genital papillae and spicules; C-D) head region and secretory-excretory pore (EP) of first generation female. Scale bars: A, B, C and D=10 µm.

ITS rDNA compared with UK isolate. Iranian isolate has also long mucron in the second generation males. Spiridonov *et al.* (2004) indicated sequence differences between 13 *S. kraussei* isolates from Germany, Russia, UK, Belgium, Iceland, Scotland and Switzerland, usually varied between 1-11 bp (up to 1%) but reached 21 bp (2.8%) between the UK (B2) and the Moscow isolates. The sequence divergence of *S. affine* ranged from 0.2-0.6% (2-5 bp); however, the difference between sequences of *S. carpocapsae* strains from Europe and USA was 3 bp (0.4%). Therefore, the intraspecific variability of *S. kraussei* ITS sequences range is more than other studied species. The sequence length of ITS region of the Iranian isolate is longer than that of closely related isolate, *S. kraussei* C46 (781 *vs.* 766 bp).

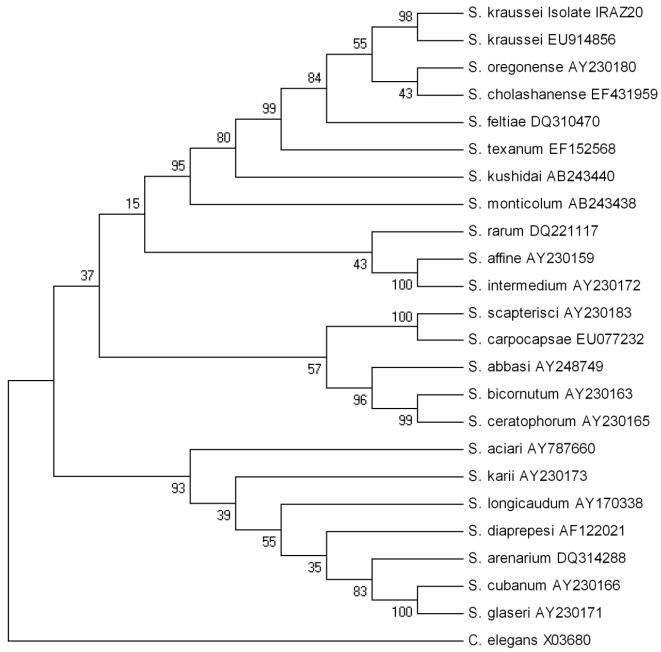

Figure 4. Phylogenetic relationships of *S. kraussei* IRAZ20 (Iranian isolate) with other 22 species of *Steinernema* based on analysis of ITS-rDNA regions. The species in '*feltiae* - *kraussei* - *oregonense*' group form a monophyletic group with high bootstrap support (95%) and *S. kraussei* IRAZ20 does clustering with that group. Numbers at the nodes represent bootstrap values.

The phylogenetic relationships between 22 species of *Steinernema* inferred from the ITS-rDNA sequences are presented in figure 4. In this consensus tree, *S. kraussei* IRAZ20 is located inside the species belonging to the '*feltiae* - *kraussei* - *oregonense*' group and forms a monophyletic group with *S. cholashanense, S. kraussei* C46, and *S. oregonense,* as well as sister group for other species of the '*feltiae* - *kraussei* - *oregonense*' group including *S. feltiae, S. texanum, S. kushidai, S. monticolum.* Bootstrap support in this clade is from low (43) to high (99). However they can be differentiated by pairwise distances. Iranian isolate of *S. kraussei* in our sampling was mainly obtained from the rangelands of Arasbaran region. Similarly, it has been reported from alpine grasslands in Switzerland (Steiner, 1996), Scotland (Gwynn & Richardson, 1996), and from alpine meadows in Bulgaria (Shishinova *et al*.,

2000). The Iceland isolate of *S. kraussei* was obtained from a swampy, treeless, area in a volcanic valley. The distribution of *S. kraussei* in woodland habitats is almost a rule in lowland parts of Europe, although this species can also be commonly found outside woodlands at high altitudes and latitudes. On the other hand, habitat preference patterns of the species are variable. Based on the available literature, this is the first record of *S. kraussei* from Iran.

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