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Steinernema feltiae- Xenorhabdus bovienii: more information on this bactohelminthic complex from Iran

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Abstract
Two families of entomopathogenic nematodes (EPNs), Steinernematidae and Heterorhabditidae, symbiotically associated with *Xenorhabdus* and *Photorhabdus* bacteria, are effective biological control agents of insect pests. Native isolates are likely to be better candidates for insect pest control than exotic specimens due to their adaptation to native environmental conditions. In this study, Steinernema feltiae isolate FUM221, was recovered from soil samples collected from the Ardabil Province, Iran. Morphological and morphometric investigations of the first and second-generation adults, infective juveniles, and molecular characterizations were conducted based on ITS and 18S rDNA genes. Molecular analysis based on the 16S rRNA region and phenetic data revealed *Xenorhabdus bovienii* as this isolate symbiont bacterium. The scanning electron microscopy (SEM) verified the identification of this isolate. The molecular characterization using two loci and phylogenetic analyses provided more evidence on the classification of this steinernematid and its distinction from same species from other countries. Moreover, molecular and phenetic characterizations of its symbiotic bacterium indicated minor variations compared to other isolates. Herein, the comprehensive taxonomic data of this steinernematid and its symbiont bacterium, is presented.

Key words: *Characterization; Entomopathogenic nematodes; Steinernema; Survey; Taxon, Xenorhabdus.*

INTRODUCTION

Entomopathogenic nematodes (EPNs) are highly pathogenic to the wide array of insect pests in the foliar environment, cryptic, and especially soil-dwelling habitats (Kaya and Gaugler, 1993; Kaya et al., 2006; Malan and Ferreira, 2017; Askary and Abd-Elgawad, 2017). They mostly belong to the families Steinernematidae and Heterorhabditidae that are symbiotically associated with the entomopathogenic bacteria, *Xenorhabdus* and *Photorhabdus*, respectively (Boemare and Akhurst, 1988; Boemare, 2002; Malan and Ferreira, 2017). Studies show that the bacteria in the *Photorhabdus-Heterorhabditis* association play a principal role in suppressing the immune system and killing the host, causing addiction and sepsis, whereas, in the *Xenorhabdus-Steinernema*, nematodes have a more efficient role in the pathogenicity of the complex (Lewis and Clarke, 2012; Lu et al., 2017; Shapiro-Ilan et al., 2018;

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Koppenhöfer et al., 2020). Nineteen species of *Photorhabdus* and twenty-six species of *Xenorhabdus* have been identified so far (Koppenhöfer et al., 2020).

Efforts to discover new indigenous species/strains of EPNs are necessary because they have adapted climatically as agents that able to regulate native pests (Qiuet et al., 2004; Ehlers, 2005; Stokwe et al., 2011; Torrini et al., 2014; Lulamba and Serepa-Dlamini, 2020). To date, about 21 species of *Heterorhabditis* and 100 species of *Steinernema* have been identified worldwide and the majority of species have been collected from Asia (Lewis and Clarke, 2012; Shapiro-Ilan et al., 2017, 2018; Didiza et al. 2021). The application of EPNs has begun in the 1980s, in the recent two decades, intensive researches were conducted in different fields on EPNs that led to effective results in their taxonomy and commercialization (Koppenhöfer et al., 2020). Currently, they are used commercially as biological control agents on numerous economically important insect pests (Shapiro-Ilan et al., 2002; Azazy et al., 2018; Koppenhöfer et al., 2020). Heretofore leastwise five *Heterorhabditis* species and eight *Steinernema* species have been commercialized (Shapiro-Ilan et al., 2002; Piedra Buena et al., 2015; Azazy et al., 2018; Koppenhöfer et al., 2020; Sivaramakrishnan and Razia, 2021).

In Iran, isolation, identification, and characterization of EPNs have begun since 2000 (Parvizi, 2000; Karimi et al., 2010) and some *Steinernema* and *Heterorhabditis* species have been isolated so far. The collected species of *Steinernema* include *Steinernema feltiae* (Filipjev, 1934) Wouts, Mraček, Gerdin and Bedding, 1982, *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mraček, Gerdin and Bedding, 1982, *Steinernema glaseri* (Steiner, 1929) Wouts, Mraček, Gerdin and Bedding, 1982, *Steinernema bicornutum* Tallosi, Peters and Ehlers, 1995, *Steinernema arasbaranense* Nikdel, Niknam and Ye, 2011, and *Steinernema kraussei* (Steiner, 1923) Travassos, 1927. *Heterorhabditis bacteriophora* is the only species in the *Heterorhabditis* genus that has been identified in Iran until now (Karimi et al., 2010; Nikdel et al., 2010, 2011; Karimi and Salari, 2015; Seddighi et al., 2016; Abdolmaleki et al., 2016; Salari et al., 2019; Ebrahimi et al., 2019; Karimi and Hassani- kakhki, 2021).

This study is the first documented record for the presentation of the 18S tree related to *Steinernema feltiae* to accreditation to identification of this species. The purpose of the current research was the comprehensive characterization of a new native isolate of EPN and its symbiont bacterium by means of morphological and molecular approaches.

MATERIAL AND METHODS

Sampling from soils and entomopathogenic nematodes isolation

The soil samples were gathered from various ecosystems including gardens, pasturelands, strands, parks, and natural undisturbed soils of Ardabil city, Ardabil Province, Iran. The samples were taken randomly from the fertile moisture soils up to a depth of 15-20 cm during late autumn 2018. After transporting to the laboratory, the soils from each site were distributed in a few plastic containers (300 ml) with lids. A routine baiting technique was applied in each container using 10 last instar larvae of the greater wax moth, *Galleria mellonella* Linnaeus, 1758. They were maintained under laboratory conditions (25±2 °C) for 10 days and checked out for dead *Galleria* larvae, considering the noticeable color change (Bedding and Akhurst, 1975). Then the cadavers were retrieved and placed in white traps individually (White, 1927). Finally, to ensure the pathogenicity of emerged nematodes, they were investigated to Koch's postulates (Kaya and Stock, 1997). The collected infective juveniles were stored at 8–10 °C for long-term use.

Light Microscopy

For morphological characterization, all developmental stages of the nematodes were obtained from more than ten *G. mellonella* larvae that were infected by IJ nematodes. *G. mellonella* larvae were placed in a petri dish (10 cm) covered with wet filter papers in its bed at room temperature (25 ºC). Adults of first and second-generation nematodes were harvested by dissecting infected cadavers of *G. mellonella* larvae approx 2-3 and 4-6 days after inoculation of insects, respectively. About 30 specimens of different stages were fixed utilizing hot (80 °C) 4% formaldehyde and transferred to pure glycerin for mounting (Ryss,

2017). Finally, the slides of fixed nematodes were prepared, and morphometric and morphological parameters were performed using an Olympus light microscope CH-2.

Morphological characterization of EPN

Complementary morphological characterization of the infective juveniles and the first generation of adults (both males and females) was conducted by using a scanning electron microscope (SEM) following the procedures in Ye et al., 2010; Nikdel and Niknam, 2015.

1) Each sample was rinsed three times with 0.1 M sodium cacodylate each for 15 min.
2) All nematodes were fixed in 3% glutaraldehyde with 0.1 M sodium cacodylate at pH 7.2 for 24 hours at 4 ºC in the dark condition (wrapped in an aluminum foil).
3) Samples were rinsed three times with 0.1 M sodium cacodylate.
4) The specimens were postfixed with 2% osmium tetroxide solution for 12 hours at 25 ºC (room temperature).
5) They were rinsed with 0.1 M sodium cacodylate three times.
6) Each sample was dehydrated in a graded ethanol series of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, and 100%, each for 20 min, on the dried ice; (dehydrated with 100% ethanol 3 times at room temperature).
7) Samples were mounted on aluminum SEM stubs, and coated with gold. The SEM images were obtained with LEO 1450VP scanning electron microscope (LEO Co. Ltd., Germany).

Molecular characterization of EPN

Extraction of DNA

Genomic DNA contents of nematode was extracted using an individual female in the 5% Chelex®100 solution (SIGMA, Bio-Rad Laboratories, Inc., USA). A single female was picked up by an eyelash, transferred into a 1.5 ml Eppendorf tube, and then was crushed in 50 µl of Chelex and 2 µl of Proteinase K (www.parstous.com) by a micro pestle. The Eppendorf tube was incubated at 60 °C for 3 h, then heating for 10 min at 95 °C utilizing a thermos-block. Finally, the specimen was centrifuged at 13000 rpm for 3 min, then extracted DNA was collected and stored at -20 °C until use.

ITS and 18S genes amplification

A molecular approach was utilized for the characterization of the isolate. For this purpose, the primer sets of TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) were used for amplification of the internal transcribed spacer (ITS) region (Joyce et al., 1994). Also, in, the other fragment of rDNA containing the 18S gene was amplified using forward primer (5′-AAAGATTAAGCCATGCATG-3′) and reverse primer (5′-CATTCTTGGCAAATGCTTTCG-3′) (Blaxter et al., 1998).

The PCR mixture with a final volume of 25 µl comprising 12.5 µl 2X Taq PreMix, 6.5 µl distilled water, 1 µl of each primer (forward and reverse), and 4 µl genomic DNA, was carried out in a thermo-cycle for amplification of DNA template. The PCR cycling process were started with the initial denaturation at 94 °C for 4 min, then 35 cycles of 94 °C for 1 min (denaturation stage), 55 °C for 1 min (annealing stage), and 72 °C for 2 min (extension stage); in the end, a post-amplification extension at 72 °C for 10 min. Amplified PCR product was loaded into 1% agarose gel. The PCR product was electrophoresed at 80 V for 40 min with 10X TBE buffer 5% and a green-viewer for staining the gel. The ladder was used to determine the PCR product size.

ITS rDNA and 18S rDNA characterization

The PCR products sequencing was employed by Macrogen Co. in Seoul, Korea. Before creating the consensus sequence by the BioEdit software (Hall, 1999), the quality of chromatograms was checked, and the bad peaks from the begining and the end of them (the attachment site of the tag polymerase enzyme) were omitted. Then, the DNA sequence was blasted against the NCBI database and compared with the other presented sequences in the GenBank. Thirty-three sequences of the ITS region and sixteen

sequences of the 18S gene of *Steinernema* species, were retrieved from corresponding published gene sequences and aligned using ClustalX.

Isolation of the bacterial strain

The bacteria were extracted from more than 100 infective juvenile nematode (IJs) that newly emerged from the fifth instar larvae of *Galleria mellonella*. After the emerged IJ nematodes were collected in a 1.5 ml micro-tube, they were washed three times with deionized water for 2 min. Then, they were immersed in sodium hypochlorite (NaOCl) 10% (V/V) for 10 min and centrifuged at 8000 speed to precipitate the IJs in the bottom of the tube. To remove remaining sodium hypochlorite, IJ nematodes were washed two times with deionized water and were crushed in 10µl of deionized water. At final step, 100 µl of the target bacterium suspension was streaked onto 9 mm Petri-dishes containing NBTA medium (Nutrient agar, 0.025% bromothymol blue, and 0.004% triphenyltetrazoliumchchloride (TTC)). The plates were incubated at 28 ºC ± 2 for 48 hours in dark condition (Akhurst, 1986). The bacterium was sub-cultured several times to obtain the pure bacterial clones.

DNA extraction, PCR, and characterization

DNA content of bacteria was extracted from a 2-day-old culture using boiling-based PCR cloning (Mcpherson and Møller, 2006). The clone was solved to 20 µl by the sterilized water and boiled for 10 minutes, then it was used as a template for PCR reaction. The universal bacterial primers of the 16S rDNA gene fragment including 27F (Forward primer 5'- AGAGTTTGATCCTGGCTCAG -3') and 1492R (reverse primer 5'-TACGGCTACCTTGTTACGA-3') were used for the amplification process (Heuer et al., 1997). The 25µl reaction mixture consisted of 12.5µl Master Mix, 6.5µl deionized water (dH2O), 1µl of each primer (2 µl), and 4µl of DNA template. The PCR reaction of the 16S rDNA gene was performed as follows: initial denaturation at 95 °C for 10 min, 35 cycles for 1 min at 94 °C, 1min at 56.5 °C, 2 min at 72 °C, followed by the final extension at 72 °C for 8 min. Eventually, the PCR product was electrophoresed at 80 V for 40 min with 10X TBE buffer 5% and a green-viewer for staining the gel. The sequencing of the PCR product was employed by Macrogen Co. in Seoul, Korea. The 16S rRNA gene sequence was edited using the BioEdit program and saved as Fasta format (Hall, 1999). The nucleotide comparison was conducted using the BLAST available on the National Center for Biotechnology Information (NCBI). Thirteen taxa of *Xenorhabdus* species and one species of *Photorhabdus luminescens* subsp. Kleinii strain KMD37 (HM072284) were used to compare with sequences of the studied isolate. The gene sequence was deposited in the Genbank using BankIt software.

Bacteria phenotypic characterization

Some significant phenotypic characterizations were investigated in the present study of the symbiotic bacterium according to Akhurst and Boemare, 1988 and Tailliez et al., 2010. The bacterium was inoculated in different media such as Nutrient Broth (NB), Nutrient Agar (NA), and bromothymol blue and 2, 3, 5- triphenyltetrazolium chloride (NBTA) and then incubated at 28 ± 2 °C for 48h to evaluate some characters such as size, shape, and color. A single clone of the bacterium was cultured on NBTA and NA to investigate the ability of the associated bacterium to absorb dye after 48 hours (Akhurst, 1986). An antibiotic test was carried out to assess the resistance of bacteria through bacteria culture on the NA medium. For this purpose, a disc (0.5 in diameter) of sterilized filter papers was immersed in the suspension of 1% tetracycline. Then it was placed in the center of the bacterial culture and incubated at 28±1°C for 48 h (Kazmierczak et al., 2016). The catalase test was carried out using 5 µl 3% (v/v) drops of H2O2 on a glass slide. Then, a single clone of the bacterium from the pure culture was added to the medium using a sterile plastic loop. The activity of bacterium on the Lecitinase was considered by 2g of NA (2%) and 10 ml of fresh egg yolk mixed with 100 cm3 of sterilized water. Then the bacterium was streaked on the medium culture containing the above mixture. Finally, the petri dish was incubated at 28 ± 1°C for 48 hours. To examine the movement activity of the bacterium, a semi-solid medium containing NA and NB was prepared. Then, a sterilized filter paper disc (0.5 in diameter) was immersed in the

formulated bacterial suspension and placed in the center of the medium culture. After incubation at 28±1°C for 48 hours, the bacterial growth around the disc is evidence of motility. To determine the lipase activity, the medium containing 2 grams of agar (2%), 100 µl Tween (0.1 v/v) was prepared in 100 cc of water. The bacterium was cultured on the medium. Then, the plate was incubated at 28±1°C and bacterial activity was recorded after 48 hours. The precipitate around the edge of the bacterial colony was an indication of lipase activity.

Furthermore, the pathogenicity of the bacterium was evaluated against the last instar larvae of *G. mellonella* according to the method of Peel et al. (1999).

Phylogenetic analysis

The authentic and verified sequences were retrieved from peer-reviewed articles and aligned using ClustalX. The alignment file was edited in MEGA 7 manually (Kumar et al., 2016). The number of base differences per site and pairwise distances were computed using Geneious, and MEGA 7.0, respectively (Kumar et al., 2016).

The best fit model was identified under the GTR + I + G (for both genes) and HKY + I + G (for bacterium sequence) model using the MrModeltest 2 (Nylander, 2004). The number of generations was started from two million using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) in the Bayesian analysis. The Markov chain Monte Carlo (MCMC) chains were sampled every 100 generations (Larget and Simon, 1999) and estimated the posterior probabilities (PP) of the phylogenetic trees using the 50% majority rule. At the last step, the burn-in step was set at 25% of the converged runs. The Dendroscope V.3.5.7 (Huson and Scornavacca, 2016) and CorelDRAW version 2020 software were used to visualize the output file of the phylogenetic program and resized the tree, respectively.

RESULTS

The Entomopathogenic nematodes were gathered by baiting with *G. mellonella* larvae from the soil sample taken from the pasture lands in Fandoghloo, Ardabil city, Ardabil Province, Iran with the geographical position 38.3822° N, 48.5550° E. There is no knowledge of natural hosts of this nematode species/ the collected nematodes.

Both classic (morphological and morphometric analysis) and molecular (two loci containing ITS and 18S genes of rDNA, and also phylogenetic analysis) methods were used for identification and characterization of the studied isolate. The infected insects were recognized through change in color to brown and shape. The sequences were blasted on the NCBI database in GenBank. The resulting blast showed that the isolated species belong to the *Steinernema* genus. The partial sequences of this species were deposited in the GenBank by BankIt software. The sequences were submitted under the accession numbers MZ540323 (ITS), MZ540331 (18S), and MZ540333 (16S) for the nematode and its symbiont bacterium, respectively.

Morphological characteristics

Measurement

The measurements of IJ, male and female adults including body length (L), width (W), tail length (T), anal body diameter (ABD), distance from anterior end to excretory pore (EP), distance from anterior end to nerve ring (NR), distance from anterior end to end of esophagus (ES), spicule length (SL), gubernaculum length (GL), vulva (V), spicule width (SW), gubernaculum width (GW), and Standard deviation (Sd) are presented in Table 1. The morphological characterizations were similar to those described by Filipjev, 1934 and then Wouts, Mraček, Gerdin and Bedding, 1982. Briefly:

Infective juvenile

Body slender (Figure 1, A), mouth and anus closed (Figure 1, B). Head offset, labial papilla was not observed. Pharynx long and narrow, isthmus surrounded by nerve ring, basal bulb elongates. Secretory-excretory pore at mid pharynx level (Figure 1, C). Lateral fields distinct and begin with one line and then two additional lines to form two ridges (Figure 1, D). The maximum number of ridges, in the longest part,

Table 1. Morphometric characters of *Steinernema feltiae* FUM221. All measurements are in μm and in the form: mean ± sd (range).

Character	First generation male	female	Second generation male	female	Infective juvenile
n	18	26	12	14	32
L	1128.3 ± 139.5 (870-1330)	3730.6 ± 1204.8 (1170-5550)	1188.3 ± 205.1 (772.5-1480)	4687.5 ± 1035.4 (3150-6825)	562.5 ± 76.0 (417.5-700)
W	109.1 ± 22.1 (64-138)	214.8 ± 52.0 (92-360)	127.4 ± 14.7 (105-145)	267.5 ± 28.7 (212.5-307.5)	38.5 ± 9.4 (24-56)
V	-	1935.4 ± 607.3 (550-3125)	-	2425 ± 534.6 (1650-3375)	-
EP	78.9 ± 9.3 (60-92)	85.0 ± 25.5 (40-129)	84.5 ± 11.4 (61-98)	885.7 ± 317.1 (82.5-1270)	44.4 ± 8.8 (24-56)
NR	89.5 ± 6.4 (76-100)	106.7 ± 10.0 (87-128)	84.6 ± 8.7 (72-98)	103.5 ± 12.0 (84-120)	57.4 ± 10.4 (26-73)
Neck	147 ± 7.4 (127-156)	179.6 ± 14.8 (157-203)	143.7 ± 8.5 (129-155)	179.7 ± 17.0 (160-212)	88.8 ± 12.8 (51-111)
ES	137.9 ± 8.1 118-150	165.3 ± 14.9 (135-194)	136.1 ± 9.3 (123-152)	164.4 ± 15.7 (144-196)	86.9 ± 11.2 59-108
T	31.9 ± 4.8 (23-44)	42.0 ± 12.8 (20-70)	46.4 ± 16.3 (25-75)	42.8 ± 15.7 (24-83)	43.0 ± 8.8 (18-58)
ABD	35.9 ± 3.2 (30-42)	59.7 ± 25.6 (22-111)	45.6 ± 11.4 (25-65)	67 ± 13.3 (40-85)	17.8 ± 3.8 (8-25)
SL	69 ± 5.2 (59-77)	-	73 ± 5.2 (64-81)	-	-
SW	11.9 ± 2.2 (8-18)	-	12.1 ± 2.5 (8-16)	-	-
GL	45.5 ± 8.0 (31-65)	-	45.9 ± 5.9 (36-55)	-	-
GW	7.1 ± 1.1 (6-9)	-	7.1 ± 0.5 (6-8)	-	-
a	10.5 ± 1.6 (8.4-14.8)	17.4 ± 5.5 (9.6-37.0)	9.4 ± 1.6 (7.4-12.6)	17.6 ± 3.8 (11.9-26.1)	15.3 ± 3.7 (10.0-24.1)
b	8.2 ± 0.9 (6.8-9.9)	22.4 ± 7.5 (7.7-38.3)	8.7 ± 1.6 (6.2-11.3)	28.3 ± 4.3 (21.9-35.4)	6.4 ± 0.6 (5.4-8.2)
c	36.3 ± 5.4 (28.8-45.7)	97.7 ± 44.8 (190.0-29.3)	27.8 ± 9.0 (18.1-49.6)	122.0 ± 37.1 (77.4-204.2)	13.2 ± 2.6 (11.3-23.2)
c´	0.9 ± 0.1 (0.8-1.0)	0.8 ± 0.4 (0.5-1.9)	1.0 ± 0.3 (0.7-1.7)	0.7 ± 0.5 (0.4-2.1)	2.4 ± 0.4 (1.7-3.2)
D%	56.8 ± 8.2 (41.1-68.7)	51.5 ± 14.5 (26.3-71.3)	61.8 ± 8.5 (43.6-72.0)	529.3 ± 189.2 (53.2-774.4)	52.5 ± 8.5 (41.7-69.1)
E%	248.9 ± 24.4 (206.8-296.7)	225 ± 118.2 (108.1-645)	200.9 ± 75.4 (130.7-320)	2268.0 ± 1095.8 (250-4233.3)	114.1 ± 30.2 (86.7-180.6)
SW%	193.9 ± 21.8 (163.9-250)	-	169 ± 42.9 (115.4-284)	-	-
GS%	65.5 ± 9.3 (52.5-92.9)	-	63.1 ± 8.0 (48-73.4)	-	-

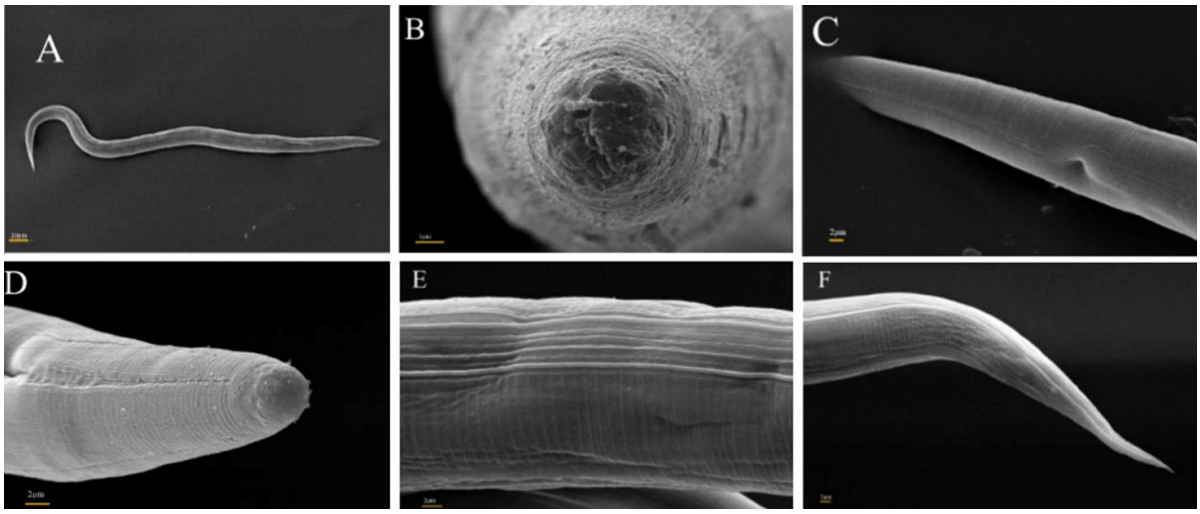

FIGURE 1. Scanning electron microscopy (SEM) photographs of infective juveniles of *Steinernema feltiae* isolate FUM221: A, An overview of larval body; B, head showing closed mouth; C, Secretory-excretory pore; D, lateral fields with one incisure and changes to two ridges; E, lateral field showing eight ridges; F, tail showing reduced number of ridges to four then two at the end of lateral fields. Scale bars: A= 20µm, B= 1 µm, C, D, F= 2 µm.

is eight in the lateral fields. (Figure 1, E). The formula for the arrangement of ridges from head to tail is 2, 8, 6, 4, 2 (Figure 1, F).

Female

Body robust and C-shaped (Figure 2, A). Lateral fields were not observed. Head widely rounded, six labial papillae and four cephalic papillae are visible. (Figure 2, B). Pharynx with cylindrical procorpus, metacorpus slightly swollen, basal bulb pyriform. Secretory-excretory pore at the middle of the pharynx and excretory duct cuticularized (Figure 2, C). Genital system filled with eggs, vulva a median transverse slit (Figure 2, D), protruding from the body, vagina short, oblique with muscular walls. Tail shorter than anal body diameter, with one terminal peg (Figures 2, E) and anus with a wide slit (Figure 2, F). The second generation of female was similar to the first generation.

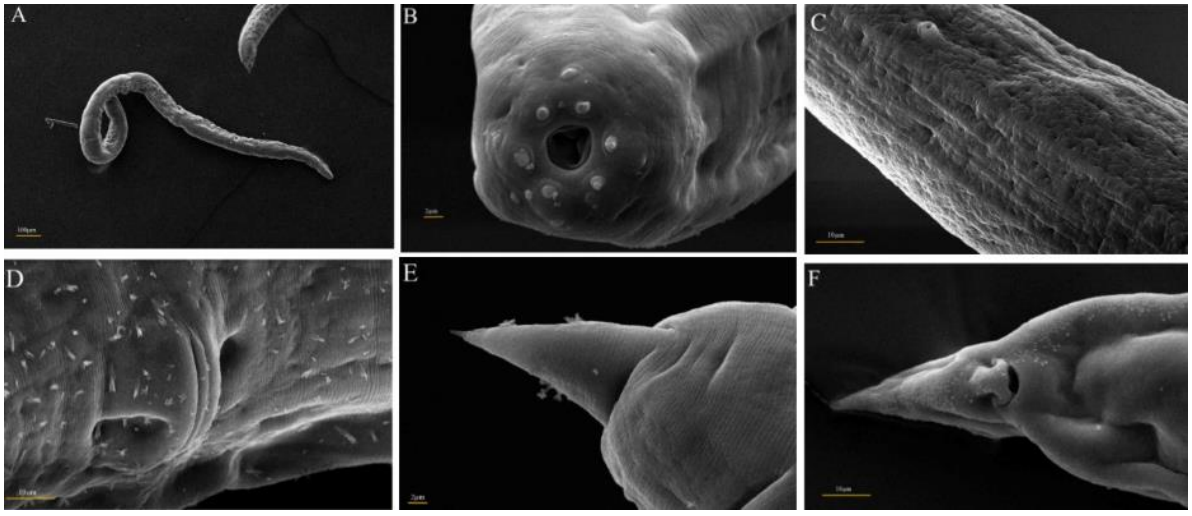

FIGURE 2.. Scanning electron microscopy of first generation females of *Steinernema feltiae* isolate FUM221: A, An overview of female body; B, labial and cephalic papillae; C, Secretory-excretory pore; D, vulva; E, Tail; F, Anus. Scale bars: A= 100 µm, B, E= 2 µm, C , D, F= 10 µm.

Male

Cuticle smooth, Body J-shaped, much smaller and slender than female (Figure 3, A). Head slightly depressed from the body. Lateral fields absent. Anterior region similar to female. Six pointed labial papillae and four cephalic papillae (Figure 3, A). Isthmus distinct, basal bulb pyriform and valvate. Nerve ring in isthmus portion. Deirids not seen. Secretory-excretory pore at the middle of pharynx (Figure 3, B). Testis monarchic and reflexed. Spicules paired, slightly brownish in color, strongly curved, head (manubrium) width is approximately equal to length (Figures 3, C, D), blade arcuate with a straight tip. Gubernaculum approximately three-quarters of spicule length, boat- shaped in lateral view, cuneus short, pointed posteriorly, wing of corpus expanding laterally. There are 11 pairs of papillae (Figure 3, E). Tail conoid with mucron. phasmids imperceptible.

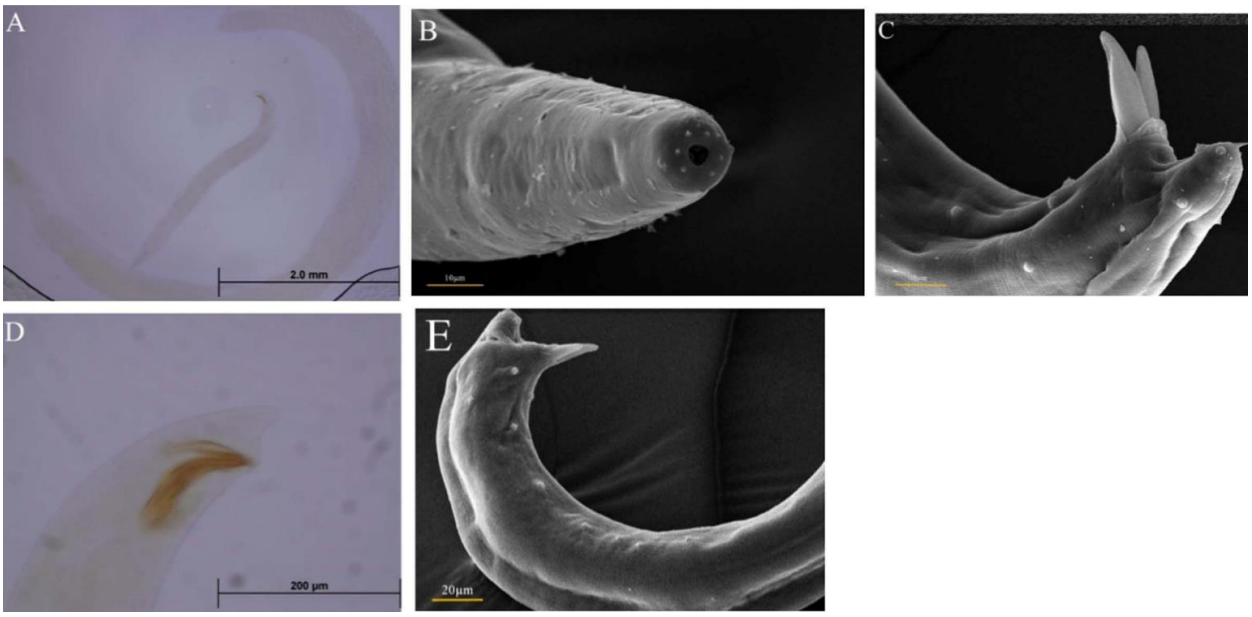

FIGURE 3. Scanning electron microscopy (SEM) (B, C, E) and light microscope (A, D): A, Comparison of male and female body size; B, Labial and cephalic papillae and secretory-excretory pore; C&D, Spicule and gubernaculum; E, Number and distribution of genital papillae in first generation male. Scale bars: A= 2 mm, B, C= 10 µm, D= 200 µm, E= 20 µm.

The ITS sequence analysis of *Steinernema* species

The ITS gene length for the FUM221 isolate was 1012 bp after alignment. The sequence revealed 100% similarities and 99% query coverage with *Steinernama feltiae* from Turkey and Belgium (accession numbers MN861044 and JF728859). The multiple alignments of 1012 bps segment of ITS gene for 33 taxa (the new isolate in this study with 31 taxa of *Steinernema* and a species of *Caenorhabditis elegans* as an outgroup) indicated that 697 sites were variable, 165 sites were conserved, 478 sites were parsimony informative, and 197 sites were singleton. The phylogenetic analysis indicated that the FUM221 isolate forms a monophyletic group with other *Steinernama feltiae* isolates (Figure 4). The overall average distance of ITS sequences was 0.22 ranging from 0.00 to 0.46, calculated by the Kimura 2- parameter model (Table 2).

TABLE 2. The number of bases which are not identical (upper triangle), and pairwise comparison on the number of nucleotide differences (lower triangle) among some of *Steinernema* species and *Steinernema* isolate FUM221 based on ITS rDNA sequences.

	Species	Acc. no.	1	2	3	4	5	6	7	8	9	10	11	12
1	***S. feltiae***	**MZ540323**	**-**	**1**	**13**	**44**	**66**	**86**	**69**	**61**	**175**	**102**	**133**	**98**
2	***S. feltiae***	AF121050	**0.00**	-	12	45	67	85	70	62	175	103	132	97
3	***S. feltiae***	EU200355	**0.00**	0.00	-	52	73	92	75	68	182	109	139	104
4	***S. ichnusae***	EU421129	**0.03**	0.03	0.03	-	53	76	54	44	170	87	120	90
5	***S. citrae***	EU740970	**0.05**	0.05	0.05	0.04	-	84	26	51	176	90	134	88
6	***S. jollieti***	AY171265	**0.06**	0.06	0.06	0.05	0.06	-	87	79	169	100	129	96
7	***S. nguyeni***	KP325084	**0.04**	0.04	0.04	0.03	0.02	0.05	-	55	182	91	138	93
8	***S. litorale***	AB243441	**0.04**	0.04	0.04	0.03	0.05	0.05	0.04	-	175	98	132	100
9	***S. kushidai***	AB243440	**0.15**	0.15	0.15	0.14	0.14	0.13	0.14	0.15	-	173	164	178
10	***S. texanum***	EF152568	**0.09**	0.09	0.09	0.07	0.09	0.07	0.08	0.09	0.13	-	121	96
11	***S. sangi***	AY355441	**0.10**	0.10	0.10	0.09	0.10	0.09	0.10	0.10	0.13	0.09	-	124
12	***S. sandneri***	MW078536	**0.15**	0.15	0.15	0.15	0.14	0.13	0.14	0.14	0.15	0.14	0.33	-

The 18S sequence analysis of *Steinernema* species

Based on the 18S gene of FUM221 isolate, BLAST analysis revealed 99.51% and 100% similarity and query coverage (respectively) to *Steinernema* sp. (MH084672) from the United Kingdom. The product length of the 18S gene amplified in this study was 840 bps. The multiple alignments of a 1654 bps segment of the18S region for 16 taxa (the new isolate in this study with 14 taxa of *Steinernema* and a species of *Heterorhabditis* as an outgroup) demonstrated that 1366 sites were conserved, 285 sites were variable, 136 sites were singleton, and 149 sites were parsimony informative. The obtained results of the 18S regions were similar to those that resulted from the ITS gene. In the phylogenetic analysis, the FUM221 isolate of *Steinernama feltiae* was placed in the same clade as isolates of *Steinernema* (Figure 5). The overall average was 0.09 (range 0.00–0.14), which were calculated from the 18S gene using the Kimura 2- parameter model (Table 3).

Phylogenetic analysis of symbiont bacteria

The length of the 16S rRNA gene for the bacterium isolate was 1458 bps. The BLAST analysis using the 16S rDNA sequences of the symbiont bacteria of *Xenorhabdus* isolate of this study showed 100% similarity and 98% of query coverage with *X. bovienii* (KJ413078) from Russia. The multiple alignments of the 1413 bps segment of this gene for 14 taxa revealed 110 sites were variable, 1297 sites were conserved, 64 sites were parsimony informative, and 46 sites were singleton. The phylogenetic analysis based on 16S rRNA sequences demonstrated that the isolate of bacterium (FUM221) forms a monophyletic group with other *Xenorhabdus* strains (Figure 6). The overall average distance of 16S rDNA sequences was 0.02 (range 0.00-0.03), calculated by the Kimura 2-parameter model (Table 4).

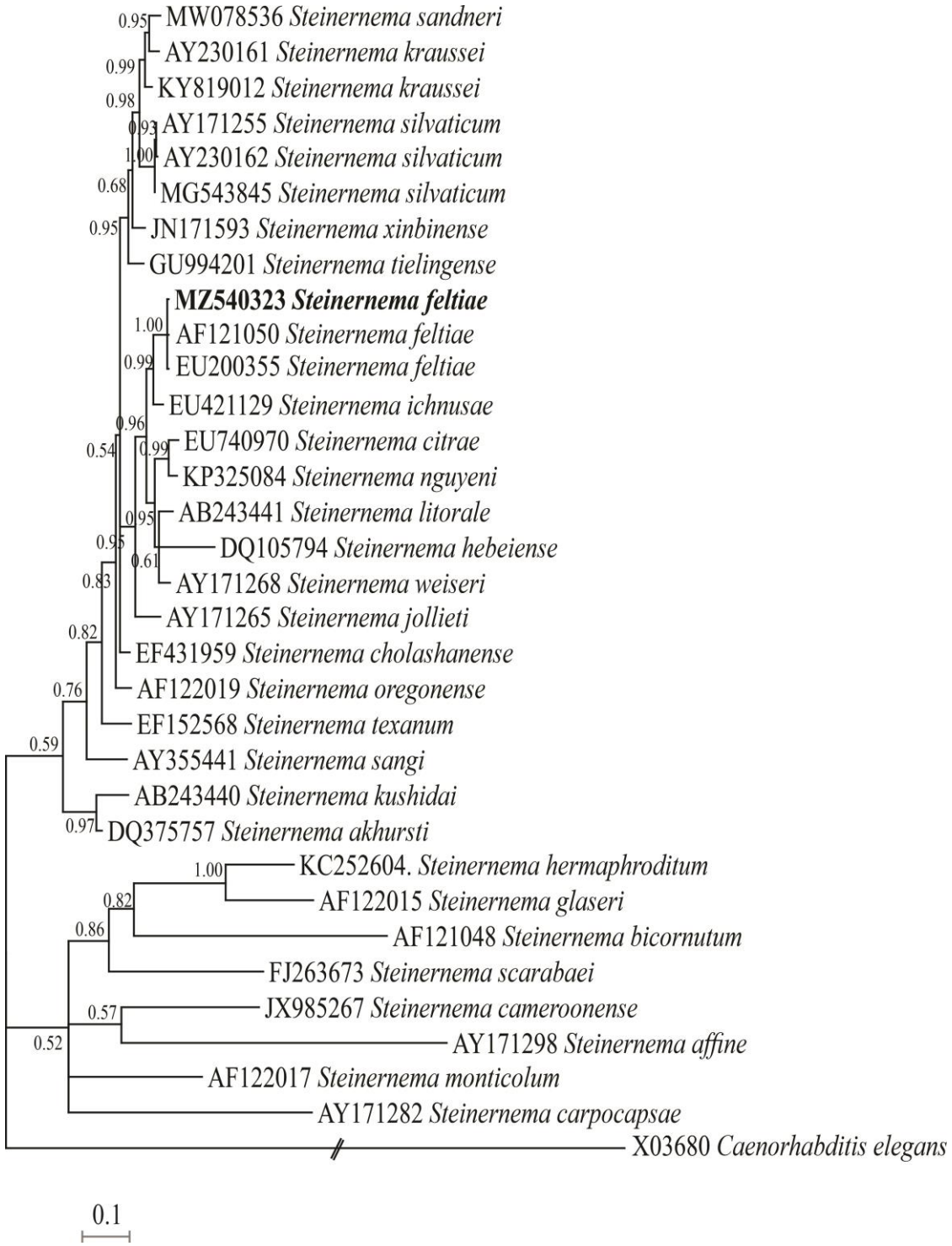

FIGURE I. Phylogenetic relationship the Iranian strain of *Steinernema feltiae* isolate FUM221 with other *Steinernema* species as inferred from Bayesian analysis of sequences of the Internal Transcribed Spacer (ITS rDNA region) under GTR + I + G model. Bayesian posterior probability amounts equal to or more than 0.50 are given for appropriate clades. The scale bar shows the number of substitutions per site.

TABLE 3. The number of bases which are not identical (upper triangle), and pairwise comparison on the number of nucleotide differences (lower triangle) among some of *Steinernema* species and *Steinernema* isolate FUM221 based on 18S rDNA sequences.

	Species	Acc. no.	1	2	3	4	5	6	7	8	9	10	11	12
1	***S. feltiae***	**MZ540331**	**-**	**2**	**2**	**1**	**3**	**2**	**2**	**55**	**17**	**84**	**90**	**107**
2	***S. feltiae***	FJ040418	**0.00**	-	3	3	4	4	3	80	14	84	121	118
3	***S. feltiae***	LN611147	**0.00**	0.00	-	2	3	4	0	76	14	85	106	103
4	***S. feltiae***	FJ040419	**0.00**	0.00	0.00	-	3	4	2	79	13	84	120	117
5	***S. feltiae***	FJ040417	**0.00**	0.00	0.00	0.00	-	5	3	80	15	85	121	118
6	***S. feltiae***	KJ636413	**0.00**	0.00	0.00	0.00	0.00	-	4	81	18	90	120	136
7	***S. feltiae***	LN611148	**0.00**	0.00	0.00	0.00	0.00	0.00	-	76	14	85	106	103
8	***S. scarabaei***	FJ040424	**0.06**	0.06	0.06	0.06	0.06	0.06	0.06	-	54	91	138	135
9	***S. akhursti***	KT878310	**0.01**	0.01	0.01	0.01	0.01	0.01	0.01	0.05	-	92	96	110
10	***S. karii***	AJ417021	**0.09**	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.10	-	121	138
11	***S. carpocapsae***	LN624756	**0.11**	0.11	0.11	0.11	0.11	0.11	0.11	0.12	0.11	0.14	-	4
12	***S. carpocapsae***	AF036604	**0.11**	0.11	0.10	0.11	0.11	0.11	0.10	0.12	0.11	0.13	0.01	-

Phenotypic characterization

The bacterial isolate of the present study could produce pigments, and the isolate was blue to greenish to blue on NBTA media. During the incubation, two phases were recognizable, phase I (round and glossy) and phase II (mucoid). The isolate was gram-negative to the colonies did not show catalase activity on hydrogen peroxide, and they were rod-shaped with wide variable cell length. The colonies could grow and absorbed dye on NBTA and NA medium. The isolate was also motile, and it had a growth inhibition zone with tetracycline. In the end, 24h post-injection by bacterial suspension, the *G. mellonella* larvae were dead compared with the larvae injected with sterilized water. These biochemical tests and others for Iranian bacterial isolate are shown in table 5, and the reaction of the bacterium is presented in figure 7.

DISCUSSION

Iran has a variety of climatic zones, which makes it appropriate for a wide diversity of plants and insects, and could be considered a shelter for several different species and strains of EPNs. As indicated in the present study, the new native isolate of entomopathogenic nematode, *Steinernema feltiae*, which has a mutualistic relationship with *Xenorhabdus bovienii*, was recovered from the Ardabil province of Iran. The northwest of Iran which shares a border with Turkey is a mountainous area with a cool continental climate in which the annual absolute temperature can vary from −38.5 to 44 °C. The out-of-range of 40 °C and 8 °C could be fatal for most EPN populations (Griffin, 1993; Grewal et al., 1994), and studied locations usually experience a temperature below 8 °C which is a limiting factor for EPNs.

Previously, *S. feltiae* was recovered from the coast of the Black Sea and Ankara (Özer et al. 1995; Susurluk et al., 2002; Hazir et al., 2003a), and in some cases, it was the most common species in those

TABLE 4. The number of bases which are not identical (upper triangle), and pairwise comparison on the number of nucleotide differences (lower triangle) among some of *Xenorhabdus* species and *Xenorhabdus* isolate FUM221 based on 16S rRNA sequences.

	Species	Acc. no.	1	2	3	4	5	6	7	8	9	10	11	12
1	***X. bovienii***	MZ540333	-	2	3	2	2	2	3	3	4	31	32	32
2	***X. bovienii***	KJ413082	0.00	-	2	0	3	0	1	2	4	43	54	43
3	***X. bovienii***	NR119151	0.00	0.00	-	2	5	2	3	4	6	44	55	44
4	***X. bovienii***	HM140697	0.00	0.00	0.00	-	3	0	1	2	4	43	54	43
5	***X. bovienii***	KJ413070	0.00	0.00	0.00	0.00	-	3	2	2	3	45	55	44
6	***X. bovienii***	KJ413065	0.00	0.00	0.00	0.00	0.00	-	1	2	4	43	54	43
7	***X. bovienii***	MG995576	0.00	0.00	0.00	0.00	0.00	0.00	-	1	3	43	55	44
8	***X. bovienii***	KJ413083	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	3	44	55	45
9	***X. bovienii***	KU312061	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	43	53	44
10	***X. nematophila***	AY286478	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	-	51	46
11	***X. magdalenensis***	NR109326	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	-	39
12	***X. szentirmaii***	DQ211712	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.02	-

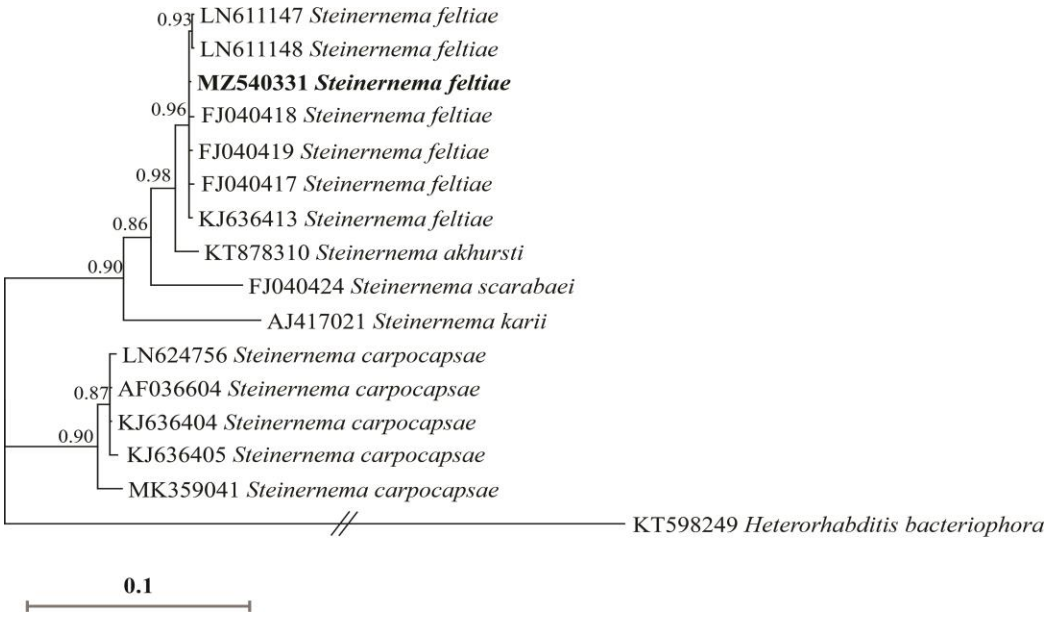

FIGURE 5. Phylogenetic relationship of *Steinernema feltiae* isolate FUM221 with other *Steinernema* species as inferred from Bayesian analysis of sequences of the small subunit (SSU rDNA region) under GTR + I + G model. Bayesian posterior probability amounts equal to or more than 0.50 are given for appropriate clades. The scale bar shows the number of substitutions per site.

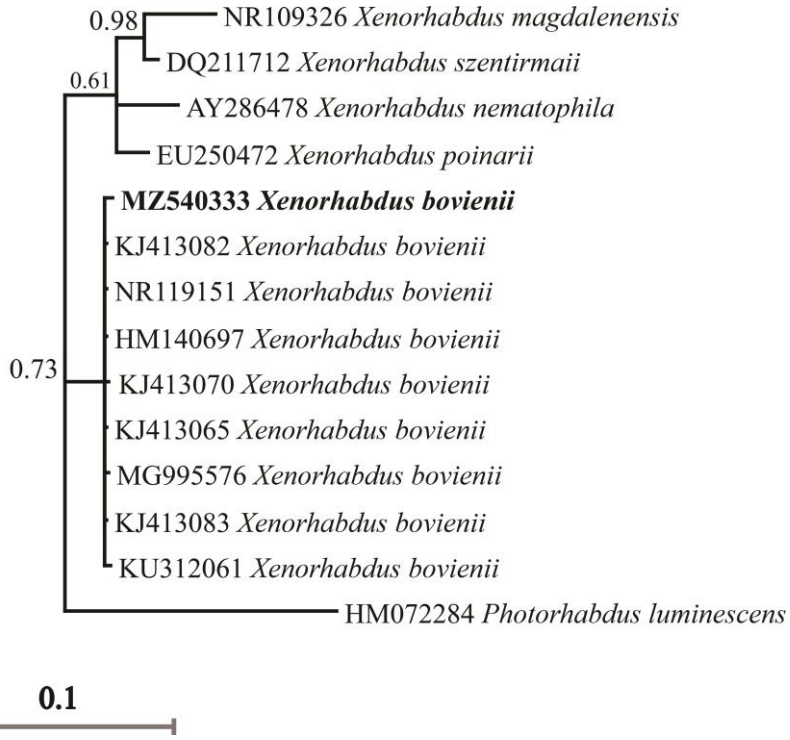

FIGURE 6. Phylogenetic relationship of *Xenorhabdus bovienii* isolate FUM221 with other *Xenorhabdus* species as inferred from Bayesian analysis of sequences of the 16S rRNA region under HKY + I + G model. Bayesian posterior probability amounts equal to or more than 0.50 are given for appropriate clades. The scale bar shows the number of substitutions per site.

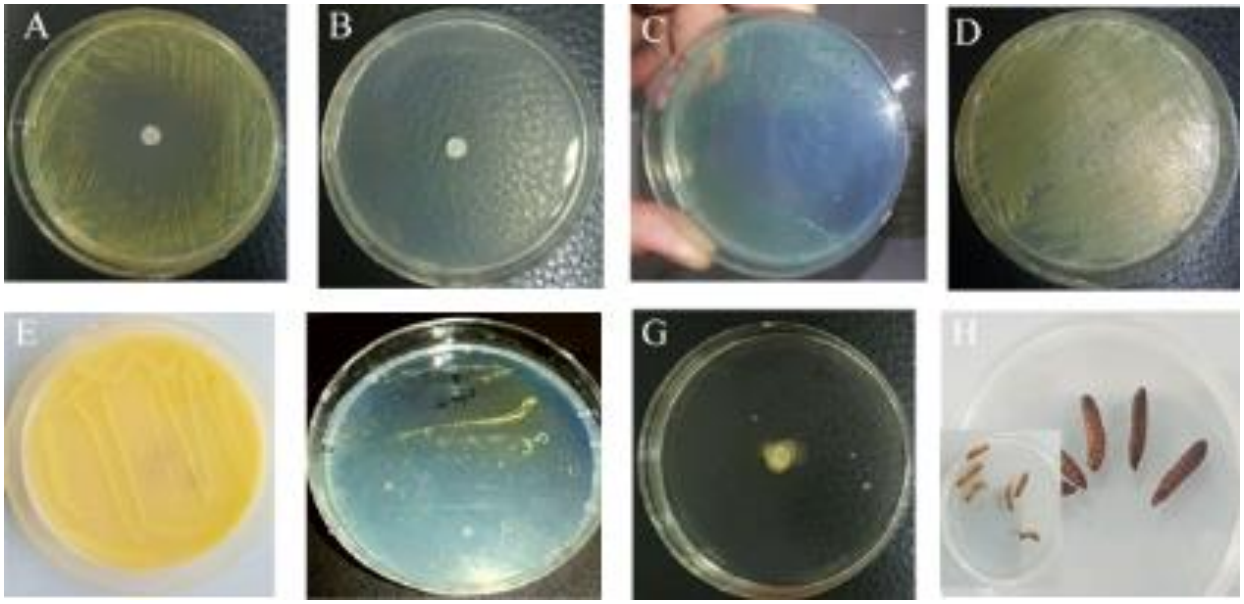

FIGURE 7. Biochemical tests. A and B, Antibiotic and control; C, dye absorption on NBTA; D dye absorption on NA; E, Lesitinase; F, Lipase; G, Motility; H, Mortality, and control.

TABLE 5. General biochemical characteristics of symbiotic bacteria isolated from Steinernema feltiae FUM221.

Biochemical characteristics	*Xenorhabdus bovienii* FUM221
Gram staining	-
Bromothymol blue from NBTA [pigmentation]	Greenish to blue
Pigmentation (nutrient agar)	Yellow
Motility	+
Tetracycline resistance	week
Catalase	_
Lecitinase	+
Lipase	+
Mortality on *G. mellonella*	+

areas (Hazir et al. 2003b; Eivazian Kary et al., 2009; Yuksel and Canhilal, 2019). Similarly, *S. feltiae* was reported in several provinces such as Tehran, Mazandaran, East Azerbaijan, Ardabil, and Kurdistan, Iran (Tanha Ma'afi et al., 2006; Eivazian Kary et al., 2009; Karimi et al., 2009; Nikdel and Niknam, 2015).
In this study, just one species was recovered from the pasture with adequate moisture and herbage, a favorable environment for the growth and establishment of EPNs (Campos-Herrera et al., 2008). This low recovery rate might be due to the use of *Galleria mellonella* as the sole host to trap the insect. As reported previously, *Galleria mellonella* would not be a suitable host for all EPN species/strains (Spiridonov and Moens, 1999). In addition, the room temperature for baiting the soil samples might be another factor for the low recovery rate. However, such a low recovery rate was observed in another investigation performed in different districts of the world and it is not uncommon (Choo et al., 1995; Rosa et al., 2000; Hazir et al., 2003a).

Accurate diagnosis of novel species and isolates of EPNs is necessary to the success of biological control programs due to the adaptability of nematode isolates to native environmental conditions (Stock, 2009). Currently, classical and molecular methods are used for species identification. In classical methods, some structures such as the oral cavity, lips, esophagus, intestine, reproductive system, sensory organs, and tail are measured by light microscopy. This method could be a difficult and sometimes dubious process (Dorris et al., 1999; Abebe et al., 2011). It seems that classical methods are not used optimally in the identification or classification of pathogenic nematodes because of diversity reduction in morphological characteristics (Campos-Herrera et al 2012). In addition, these traits are only suitable for identification but not phylogenetic studies and intraspecific morphometrics variability could be observed within the strains (Yoshida, 2003; Nikdel and Niknam, 2015) and with the original descriptions (Poinar, 1990).

In the present study, some characteristics like the body length of IJs were relatively less than those described *S. feltiae* originally. Some other researchers had the same results (Campos-Herrera et al., 2006; Majić et al., 2018; Flores et al., 2021). The research on morphological characters showed there are up to 14 similarity degrees of body length with a maximum of 28 populations in the same homogenous group (Clausi et al., 2020). On the other hand, it has been suggested that IJs body length is the longest when EPNs are raised at 8 °C, and this character could be limited at higher room temperature conditions (Hazir et al., 2001). In our study, the strain was reared at room temperature could be another reason for shorter morphometric values. The difference in the FUM221 isolate can be due to intraspecific variability (Stock et al., 1999).

Molecular methods are very beneficial and used as a complementary method to unravel the problems like identifying the members of one species and recognizing species with similar morphological traits (Stock and Reid, 2004). These methods are not only substantial for the identification of nematode species but are also beneficial for estimating phylogenetic relationships at different levels of classification (Dorris et al., 1999; Blaxter 2003; Stock, 2009).

The ITS region is a fundamental marker in the separation of species (Adams et al., 1998; Szalanaski et al., 2000; Nguyen et al., 2001). Accordingly, many researchers used this region for the identification of *Steinernema feltiae* species (Nikdel and Niknam, 2015; Tumialis et al., 2016; Majić et al. 2018; Flores et al., 2021). The ITS region is variable between species groups of *Steinernema* and could be observed among individuals of the same species as well. Hence, this region can not be a suitable marker for the distinction of all *Steinernema* species (Stock, 2009). The 18S rDNA has a significant role in the identification of unknown nematode species (Blaxter et al., 1998). Due to its conserved nature, this subunit evolves slowly and uses for classification (Stock and Hunt, 2005). Many researchers have used the ITS region for the recognition of *S. feltiae*, but very little research has been conducted on the 18S rDNA for *S. feltiae*. In the present study, identification of this species was carried out based on ITS and 18S rDNA regions.

Briefly, although molecular analyses are an essential tool for the identification of species, the studies showed that morphological and biological researches on the variability of *S. feltiae* strains are incredibly vital and provide crucial information for species identification. For a comprehensive species characterization, morphological (infective juveniles, spicule and gubernaculum shapes for males), biological (time to achieve adult stage, reproduction, and progeny), and molecular research must be carried out simultaneously (Clausi et al., 2020).

The EPN species have mutual relationships with an exclusive bacterial species. However, few species, particularly *Xenorhabdus* spp. are associated with more than one EPN species. Isolation and identification of the symbiotic bacterium of the EPNs are necessary to prove the exact nematode species. In this study, *Xenorhabdus bovienii* was isolated from *S. feltiae*. The bacterial isolates were characterized by their phenetic characters and 16S ribosomal RNA gene sequences (Agazadeh et al., 2010; Karimi et al., 2011). The isolation of this symbiotic bacterium is inevitable for completing phylogeny and clarifying the ambiguous aspects of its characteristics. Because of the variation in different isolates, the present data for chemical characters, in particular, should be evaluated in the future.

CONCLUSION

As referred before, this study is the first documented record about the presentation of the 18S tree for *Steinernema feltiae* species to accreditation for identification of this species. Also, due to the adaptation of this species to cold climate conditions, it can be suitable to introduce as an option for pest control in cool regions like the west and northwest of Iran. So, this strain can be a candidate for the commercialization of EPNs in these districts. Further studies are suggested to evaluate the potential effectiveness of symbiont bacteria of this EPN species as a new strain with the possibility of having novel metabolites and toxins about its beneficial effects in agriculture and medicine.

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LIST OF ABBREVIATIONS

ABD: Anal body diameter
EP: Distance from anterior end to excretory pore
ES: Distance from anterior end to end of esophagus

EPNs: Entomopathogenic nematodes
GL: Gubernaculum length
GW: Gubernaculum width
ITS: internal transcribed spacer
L: body length
Mega: Molecular evolutionary genetics analysis
n: number of specimens analyzed
NA: Nutrient agar
NBTA: Nutrient Bromothymol blue-triphenyltetrazolium chloride agar
NR: Distance from anterior end to nerve ring
Ph: *Photorhabdus*
PP: posterior probabilities
Sd: Standard deviation
SEM: scanning electron microscopy
SP: Spicule length
SW: Spicule width
T: Tail length
V: Vulva
W: greatest body diam
X: *Xenorahbdus*

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