Steinernema Feltiae- Xenorhabdus Bovienii: More Information about this Bactohelminthic Complex from Iran

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Abstract

**Background:** Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae that are symbiotically associated with *Xenorhabdus* and *Photorhabdus* bacteria are one of the effective biological control agents of insect pests. Native isolates can probably be more efficacious to control insect pests than exotic ones due to their adaptability to indigenous environmental conditions.

**Results:** In this study, *Steinernema feltiae* isolate FUM221 was recovered from soil samples collected from the Fandoghloo pasture, Ardabil province, Iran. Morphological investigations of the first and second-generation adults, infective juveniles, and molecular characterizations were given based on ITS and 18S rDNA genes. Besides, molecular analysis based on the 16S rRNA region and phenetic data recognized *Xenorhabdus bovienii* as its symbiont bacterium. The scanning electron microscopy (SEM) images verified the identification of this isolate.

**Conclusion:** The molecular characterization using two loci and phylogenetic analyses provided more evidence for the classification of this steinernematid and its difference of the same species from other countries. Moreover, molecular and phenetic characterizations of its symbiotic bacterium were provided with low variations compared to other isolates. Herein, the comprehensive taxonomic data of this steinernematid from Iran is presented.

**Background**

Entomopathogenic nematodes (EPNs) are highly pathogenic to the wide array of insect pests in the foliar environment, cryptic, and especially soil-dwelling habitats (Kaya & Gaugler, 1993; Kaya et al., 2006; Malan & Ferreira, 2017; Askary & Abd-Elgawad, 2017). They mostly belong to Steinernematidae and Heterorhabditidae families that are symbiotically associated with the entomopathogenic bacteria, *Xenorhabdus* and *Photorhabdus*, respectively (Boemare & Akhurst, 1988; Boemare, 2002; Malan & Ferreira, 2017). Studies show that the bacteria in the *Photorhabdus*-heterorhabditis combination play a principal role in suppressing the immune system and killing the host, causing addiction and sepsis, whereas, in the *Xenorhabdus*-Steinernema combination, nematodes have a more efficient role in the pathogenicity of the nematode-bacterial complex. (Lewis & Clarke, 2012; Lu et al., 2017; Shapiro-Ilan et al., 2018; 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 biocontrol agents against native pests with the lowest adverse effects on non-target organisms. (Quiet et al., 2004; Ehlers, 2005; Stokwe et al., 2011; Torrini et al., 2014; Lulamba & Serepa-Dlamini, 2020). To date, about 100 species of *Steinernema* and 21 species of *Heterorhabditis* have been identified worldwide and the majority of species have been collected from Asia (Lewis & Clarke, 2012; Shapiro-Ilan et al., 2017, 2018; Didiza et al. 2021). The application of EPNs began in the 1980s, in the
Recent two centuries, intensive researches were conducted in different fields on EPNs that led to monumental results of 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 at least 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 & Razia, 2021).

In Iran, isolation, identification, and characterization of EPNs have been started since 2000 (Parvizi, 2000; Karimi et al., 2010) and a few species of *Steinernema* and *Heterorhabditis* were isolated until now. Species from *Steinernema* include *Steinernema carpocapsae*, *Steinernema feltiae*, *Steinernema glaseri*, *Steinernema monticolum*, *Steinernema bicomutum*, *Steinernema arasbaranense*. From *Heterorhabditis* genus, just *Heterorhabditis bacteriophora* has been identified so far (Karimi et al., 2010; Nikdel et al., 2010, 2011; Karimi & Salari, 2015; Seddighi et al., 2016; Abdolmaleki et al., 2016; Salari et al., 2019; Ebrahimi et al., 2019; Karimi & Hassani-kakhki, 2021).

This study is the first documented record for the presentation of the 18S tree regarding *Steinernema feltiae* to accreditation for identification of this species. The purpose of the present research was the comprehensive characterization of a new native isolate of EPN and its symbiont bacterium according to morphological and molecular approaches, while there's no sufficient information about that.

**Methods**

**Soil sampling and isolation of entomopathogenic nematodes**

Soil samples were collected from different ecosystems including gardens, pasturelands, strands, parks and natural undisturbed soils in ten different locations of Ardabil city, Ardabil province, Iran. Moisstsoil samples were taken from up to a depth of 15-20 cm, by a hand spade, and then transported to the laboratory where the samples were placed in the plastic containers (300 ml) with lids. The collected soil samples were inoculated by ten last instar larvae of the greater wax moth, *Galleria mellonella* L., with more than ten replicates for each soil sample. The containers with the larvae were maintained under laboratory conditions (25±2 °C) for 10 days.

Sample containers were checked out daily for larval death according to color change due to infection. (Bedding & Akhurst, 1975). Based on the White-trap method, dead larvae were collected and cultured individually (White, 1927). Infective juveniles (IJs) emerging from the cadavers of *G. mellonella* were collected and stored at 8–10 °C. To confirm the pathogenicity of collected nematodes, last instar larvae of *G. mellonella* were inoculated by about 100 emerged IJs/larva to observe same previous symptoms (Koch's postulates) (Kaya & Stock, 1997).

**Light microscopy**
For morphological characterization, ten *G. mellonella* have been exposed to the nematode infective juveniles in a Petri dish (10 cm) lined with moistened filter papers. The petri dish was maintained at room temperature (25 °C). After the insects died, first and second-generations of adult nematodes were obtained by dissecting infected insects in 2 to 3 and 4 to 6 days post-infection, respectively. More than 30 adults and infective juveniles (IJs) were randomly selected from *G. mellonella* cadavers, fixed using hot (80°C) 4% formaldehyde solution, and transferred to anhydrous glycerin for mounting (Ryss, 2017). Fixed nematodes were mounted on a glass slide using cover glass and glass rod that preventing the flattening. Measurements and microphotographs were taken using an ocular.

**Morphological characterization of EPN**

Complementary morphological characterization of the nematode include infective juveniles and the first generation of adults (both males and females) was conducted by using a scanning electron microscope (SEM) according to the suggested protocols. (Ye et al., 2010; Nikdel & Niknam, 2015).

1) Each sample was rinsed with 0.1 M sodium cacodylate three times each for 15.

2) They were fixed in 3% glutaraldehyde with 0.1 M sodium cacodylate at pH 7.2 for 24 hours at 4°C in the dark condition (wrapped in an aluminum foil).

3) Samples were rinsed with 0.1 M sodium cacodylate three times.

4) They were postfixed with 2% osmium tetroxide solution for 12 hours at 25°C (room temperature).

5) Samples were rinsed with 0.1 M sodium cacodylate three times.

6) Each sample was dehydrated through a graded ethanol series with 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).

8) Samples were mounted on aluminum SEM stubs, and gold-coated by mini sputter coater SC7620 (Quorum Technologies, UK). The SEM images of the species were obtained with LEO 1450VP scanning electron microscope (LEO Co. Ltd., Germany).

**Molecular characterization of EPN**

**DNA extraction**

DNA content of nematode was extracted using the 5% Chelex®100 solution (SIGMA, Bio-Rad Laboratories, Inc., USA). An individual female was transferred into the 1.5 ml microtube. The sample was crushed using a micro pestle in 50 μl Chelex solution and 2 μl Proteinase K. Subsequently the microtube was incubated at 60°C for 3 h, followed by heating at 95°C for 10 min on a hotplate (thermo-block). Then it was centrifuged at 13,000 rpm for 3 min, the supernatant was transferred to another 1.5 ml microtube and stored at −20°C to use for the subsequent step.
**Amplification of ITS and 18S genes**

The primer sets of TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-ATATGCTTAAGTTTCAGCGgt-3′) were used for amplification of the internal transcribed spacer (ITS) region (Joyce et al., 1994). The 18S gene was amplified using forward primer (5′-AAAGATTAAGCCATGCATG-3′) and reverse primer (5′-CATTCTTGCAATGCTTTCG-3′) (Nguyen & Hunt, 2007). The PCR solution had a final volume of 25 μl comprising 6.5 μl sterilized water, 12.5 μl 2X Taq PreMix, 1 μl of each forward and reverse primers, and 4 μl genomic DNA. The PCR reactions were carried out in a thermo-cycle (A300 Fast Thermal Cycler, Hangzhou Long gene Scientific Instruments Co., Ltd. China). The PCR run was stared by the initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. Then after, all PCR products were electrophoresed on 1% agarose gels for 40 min with 10X TBE buffer 5% and subsequently, green-viewer was added to stain the gel. Eventually, 2 μl of DNA ladder and 3 μl of the PCR products were loaded into each well of the gel. The size of amplified products was determined by comparing them to 100-bp molecular DNA ladder (Pars Tous, Iran).

**ITS rDNA and 18S rDNA characterization**

The sequencing of the PCR products was employed by Macrogen Co. in the Seoul, Korea. Afterward, the quality of chromatograms were checked and the consensus sequence was created by the BioEdit software (Hall, 1999). Then, the DNA sequence was blasted against in the NCBI database. Thirteen sequences of the ITS region and seventeen sequences of 18S gene were retrieved from authentic and verified DNA sequences from peer-reviewed articles and aligned together with the sequences from this study by Clustal W with default parameters. The Molecular Evolutionary Genetics Analysis (MEGA) (Version 7) software (Kumar et al., 2016) and Kimura 2- model (Kimura, 1980) was used to calculate the nucleotide distances. Phylogenetic analyses were performed using neighbor Joining method (Saitou & Nei, 1987) with 10000 replications of bootstrap (Felsenstein, 1985).

**Isolation of bacterial strain**

The bacteria were obtained from 100-150 of new emerged infective juveniles nematode (IJs) of the fifth instar larvae of *Galleria mellonella*. IJs nematodes were collected in a 1.5 ml micro-tube. The infective larvae were washed three times by sterilized water for about 2 minutes. Then, they were sterilized using sodium hypochlorite (NaOCl) 10% (V/V) for 10 min. After that, they were centrifuged at 8000 speed and IJs precipitated in the bottom of the tube. The IJs were washed two times with sterilized water to remove any remained of sodium hypochlorite and then were crushed using a modified pipette in 10μl deionized water. At the end step, the suspension was cultured on 9 mm Petri-dishes containing NBTA medium (Nutrient agar, 0.025% bromothymol blue, and 0.004% triphenyltetrazoliumchloride (TTC)). The plates were kept at 28°C ± 2 for 48 hours in a dark condition (Akhurst, 1986). Target bacterium was sub cultured to obtain the pure bacterial clones.

**DNA extraction, PCR, and DNA sequencing**
Bacterial DNA was extracted from a 2-day-old culture using boiling-based PCR cloning (Mcpherson & Møller, 2006). The clone was diluted 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 containing 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, 1µl of each primer (2 µl), 6.5µl deionized water (dH2O) and 4µl DNA template. The PCR reaction of the 16S rDNA gene was performed as follow:

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. In the end, the PCR product was electrophoresed for 40 minutes on 1% agarose gels with 10X TBE buffer 5% and green-viewer (SYBR) was used to stain the gel by loading 3µl from the PCR product. The size of the amplified product was identified by comparing it with the 100-bp molecular DNA ladder (Pars Tous, Iran). The sequencing of the PCR products was employed by Macrogen Co. in the Seoul, Korea. The gene sequence was deposited in the Genbank using Bankit software.

1.3. Alignment and phylogenetic analyses

The 16S rRNA gene sequence obtained from the isolated bacterial strain was edited using the BioEdit program and saved as Fasta form (Hall, 1999) and then nucleotide comparison was done using the BLAST available on the National Center for Biotechnology Information (NCBI). twelve taxa of Xenorhabdus species and one species of Photorhabdus luminescens subsp. Kleinii strain KMD37 (accession number of HM072284) were downloaded from GenBank to investigate and compared with sequences of the studied isolate. The MEGA7 (Kumar et al., 2016) was used to construct the phylogenetic tree and to carry out multiple sequence alignment. The module of neighbor-joining (NJ) method implemented in MEGA7 was used to accomplish a phylogenetic analysis of trimmed alignments (Saitou & Nei, 1987).

The evolutionary distances was estimated by the neighbour-joining method (NJ) and the Kimura 2-parameter model to determine the number of base compositions per site in units (Kumar et al., 2016). The 10000 bootstrap replications were performed to assess the tree topology and more than 70% of bootstrap values were considered as nodes (Felsenstein, 1985). Photorhabdus luminescens subsp. Kleinii strain KMD37 with accession number of HM072284 was used as the out-group.

1.4. Bacteria phenotypic characterization

Few significant phenotypic characterizations of the associated bacterium were investigated in the present study by following (Akhurst & Boemare, 1988) and (Tailliez et al., 2010). The bacteria were inoculated in different agar 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. After 48 h of bacterial growth, a single clone of bacteria was cultured on two mediums including NBTA and NA to record the ability of associated bacteria to absorb dye after 48
hours (Akhurst, 1986). An antibiotic test was done to evaluate the resistance of bacteria through bacteria culture on the NA medium. Suspension of 1% tetracycline was prepared and disc (0.5 in diameter) of sterilized filter papers was submerged in the suspension of tetracycline and then placed on the center of bacteria culture and incubated at 28 ± 1°C for 48 hours (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. To examine the activity of bacterium on the Lecitinase, 2g of NA (2%) and 10 ml of fresh egg yolk (10%) were mixed with 100 cm³ of sterilized water. Then, the mixture was poured into the sterilized Petri dishes. Afterwards, the bacterium was streaked on the medium culture. Finally, the plate was incubated at 28 ± 1°C for 48 hours. Semi-solid NA and NB were prepared for movement test, bacterial suspension of associated symbiont bacterium was formulated. Then, 0.5 cm of sterilized filter paper disc was made and submerged into the bacterial suspension. At the end step, the disc was placed in the center of medium culture. The sample was incubated at 28±1°C for 48 hours. The growth of bacteria 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).

**Results**

The nematodes were collected by baiting with *G. mellonella* larvae from the soil sample taken from the pasture lands in Fandoghloo, Ardabil city, Ardabil province of Iran with geographical position 38.3822° N, 48.5550° E. The natural host remains unknown.

The studied isolate of *Steinernema* was identified and characterized according to the both ITS and 18S genes of rDNA. The infected nematodes were recognizable via some changes in appearance such as color and shape. In addition, the molecular recognition method was also carried out for the isolated species from the soil sample. The sequence data and DNA sequence were blasted on the NCBI database in GenBank. The result of the blast showed that the determined isolated species belong to the *Steinernema* species.

**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.

**Infective juvenile**

Body slender, habitus moderately curved ventrally upon heat killed (Figure 1A). Mouth and anus closed (Figure 1B). Head broad, offset from the body, labial papilla were not observed, transverse slit-like amphidial apertures posterior to labial disc but at the level of four distinct cephalic papillae. Pharynx long, narrow, isthmus distinct, surrounded by nerve ring, basal bulb elongate and valvate. Cardia prominent. Secretory-excretory pore at mid pharynx level (Figure 1C). Lateral fields begin anteriorly with one line followed by two additional lines to form two ridges (Figure 1D). Near the level of the secretory-excretory pore, two ridges are separated into four, which increases the number of ridges to eight, the maximum number in the lateral fields (Figure 1E). The equally developed eight ridges (i.e., 9 lines or incisures) pattern extends posteriorly close to three or four annules before the anus. The number of ridges reduced to six then to four. Near the phasmid, the four ridges change to two ridges. Based on the above descriptions, the formula for the arrangement of ridges from head to tail is 2, 8, 6, 4, 2 (Figure 1F). The section of eight ridges is the longest part (compare with portions with 2, 6, 4 ridges) of the lateral fields. In the portion with six ridges, the middle two are indistinct. Tail conical and often bent towards the ventral side, tail terminus finely rounded, hyaline tail portion distinct.

**Female**

Body robust, habitus C-shaped (Figure 2A). Cuticle appearing smooth under light microscopy, lateral fields were not observed. Head widely rounded, six labial papillae and also four cephalic papillae visible under SEM (Figure 2B). Amphids imperceptible. Buccal cavity funnel- or cupshaped, stoma shallow. Pharynx with cylindrical procorpus, metacorpus slightly swollen and nonvalvate, isthmus distinct, basal bulb pyriform and valvate. Nerve ring in isthmus portion (Figure Drawing). deirids visible. Secretory-excretory pore usually at mid-pharynx level and excretory duct cuticularized (Figure 2C). Cardia prominent protruding into the intestinal lumen. Genital system didelphic, reflexed, filled with eggs, vulva a median transverse slit (Figure 2D), protruding from the body, vagina short, oblique with muscular walls. Tail dome-shaped, shorter than anal body diameter, with one terminal peg (Figures 2E) and anus with a wide slit (Figure 2F). The second generation of female was similar to the first generation but smaller.

**Male**

Body ventrally curved, habitus C- or J-shaped, much smaller and more slender than female (Figure 3A). Under light microscope, cuticle smooth, lateral fields not observed. Head rounded, slightly depressed from the body. Six pointed labial papillae and four cephalic papillae visible under SEM (Figure 3A). Amphids imperceptible. Buccal cavity funnel- or cupshaped, stoma shallow. Pharynx muscular, procorpus cylindrical, metacorpus slightly swollen, nonvalvate, isthmus distinct, basal bulb pyriform and valvate. Nerve ring in isthmus portion. Cardia prominent protruding into the intestinal lumen, deirids not seen. Secretory-excretory pore at the middle of pharynx (Figure 3B) excretory duct cuticularized. Testis
monorchic, reflexed, with a short reflection. 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, dorsal lobe well curved, terminating at spicule tip, lateral lobe prominent, terminating at spicule tip,
ventral lobe enlarged anteriorly at dorsal and ventral side to form prominent apex and rostrum,
terminating posterior to rounded spicule tip, velum large, not covering spicule tip. Gubernaculum
approximately 75% of spicule length, boat- shaped in lateral view, swollen at middle, with slightly
ventrally curved knob at proximal end, in ventral view, cuneus short, pointed posteriorly, wing of corpus
expanding laterally. There are 11 pairs of papillae (Figure 3E). Tail conoid with mucron that always
present. Phasmids imperceptible. Male, second generation Similar to first generation male but more
slender. Testis flexure extending more posteriorly.

Analysis the ITS sequence for Steinernama species

The length of the ITS gene for the FUM221 isolate was 1012 bp. The sequence showed 100% of
similarities and 99% of query coverage with Steinernama feltiae from Turkey and Belgium countries with
accession numbers MN861044 and JF728859, respectively. The multiple alignments of a 819 bps
segment of ITS gene for 13 taxa (the new isolate in this study with 11 taxa of Steinernema and a species
of Heterorhabditis as outgroup) indicated that 238 sites were conserved, 532 sites were variable, 168
sites were parsimony informative and 347 sites were singleton. The phylogenetic tree reconstructed
based on ITS sequences, using neighbor-joining analysis and 10000 bootstrap replicates indicated that
the FUM221 isolate forms a monophyletic group with other Steinernama feltiae isolates (Figure 4). The
mean inter-specific distance of ITS sequences was 0.26 ranged from 0.00 – 0.36 , which was calculated
by the Kimura 2- parameter model (Table 2).

Analysis of the 18S sequence of Steinernama species

BLAST analysis, based on the 18S gene for FUM221 isolate, showed that the similarity was 99.51% and
the query coverage was 100% with Steinernema sp. (MH084672) from the United Kingdom. The length of
the 18S gene for this isolate was 935 bp. The multiple alignments of a 781 bps segment of 18S region
for 17 taxa (the new isolate in this study with 15 taxa of Steinernema and a species of Heterorhabditis as outgroup),
illustrated that 580 sites were conserved, 201 sites were variable, 99 sites were singleton and
102 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, FUM221 isolate of Steinernama feltiae was
placed in the same clade with isolates of Steinernema (Figure 5). The mean inter-specific distance among
Steinernama feltiae FUM221 isolate and other isolates of Steinernama feltiae was 0.09 (range 0.00–
0.14), which were calculated from the 18S gene using the Kimura 2- parameter model (Table 3).

Phylogenetic analyses 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 the present study showed that
100% similarity and 98% of query coverage with X. bovienii (KJ413078.1) from Russia. The multiple
alignments of the 1053 bps segment of this gene for 14 taxa showed that 984 sites were conserved, 65 sites were variable, 38 sites were parsimony informative and 27 sites were singleton. The phylogenetic tree reconstructed based on 16S rRNA sequences, using neighbor-joining analysis with 10000 bootstraps showed that the isolate of bacterium (FUM221) forms a monophyletic group with other Xenorhabdus strains (Figure 6). The main inter-specific distance of 16S rDNA sequences was 0.02 (range 0.00-0.03), which was calculated using the Kimura 2-parameter model (table 4).

**Phenotypic characterization**

The bacterium 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 of this bacterium were shown, phase I was round and glossy, while phase II was mucoid. The isolate was gram-negative and the colonies did not show catalase activity when was examined using hydrogen peroxide, and they were rod shape 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. At the end, after 24h from injection by bacterial suspension, the *G. mellonella* larvae were dead compared with the larvae that were injected with sterilized water. These biochemical testes 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 as 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 north-west of Iran that 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 location usually experience the temperature below of 8°C that is 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 area (Hazir et al. 2003b; Eivazian Kary et al., 2009; Yuksel and Canhilal, 2019). Similarly, *S. feltiae* was reported from several provinces such as Tehran, Mazandaran, East Azerbaijan, Ardabil, Kurdistan in 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 growth and establishment of EPNs (Campos-Herrera et al., 2008). This low recovery rate might be due to use *Galleria mellonella* as the sole host to trap insect. As reported previously, *Galleria mellonella* would not be a suitable host for all EPN species/strains (Spiridonov & Moens, 1999). In addition, the room temperature for baiting the soil samples might be another factor for
low recovery rate. However, such a low recovery rate was observed in another investigation performed from 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/or isolates of EPNs is important to the success of biological control programs and their commercialization due to the adaptability of nematode isolates to native environmental conditions (Stock, 2009). Currently, the classical and molecular methods are used for species identification. In classical methods, some structures such as oral cavity, lips, esophagus, intestine, reproductive system, sensory organs, and tail are measured by light microscopy. This method could be 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 phylogenetically 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 characters such as 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). 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 that could be another reason for shorter morphometric values. The difference of FUM 221 strain can be due to the intraspecific variability (Stock et al., 1999).

Molecular methods are very helpful and used as a complementary method to unravel the problems such as identifying members of a species and distinguishing species with similar morphological traits (Stock and Reid, 2004). These methods are not only substantial for the differentiation of nematode species but are also valuable for estimating phylogenetical 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; Szalanski 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). It seems that 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 an important role in the identification of unknown species of nematodes (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 identification 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.
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 sequences of the 16S ribosomal RNA gene (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 characteristics and sequences in particular should be evaluate in the future.

**Conclusions**

As referred before, this study is the first documented record about the presentation of 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 candid for the commercialization of EPNs in these districts. Further studies are suggested to evaluate the potential effectiveness of symbiotic bacterial 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.

**Abbreviations**

**ABD**: Anal body diam

**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

**NB**: Nutrient agar
**NBTA:** Bromothymol blue-triphenyltetrazolium chloride agar

**NR:** Distance from anterior end to nerve ring

**Ph:** *Photorhabdus*

**Sd:** Standard deviation

**SEM:** scanning electron microscopy

**SP:** Spicule length

**SW:** Spicule width

**T:** Tail length

**V:** Vulva

**W:** greatest body diam

**X:** *Xenorhabdus*

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

This study does not contain any individual person's data.

### Availability of data and materials

All data generated or analysed during this study are included in this published article.

### Competing interests

The authors declare that they have no competing interests.

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### Authors’ contributions
JK designed the project. KK collected, isolated, and characterized the sample. KK and JK analyzed data. KK and JK wrote the preliminary manuscript. All authors read and approved the manuscript.

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Tables

**Table 1.** Morphometric characters of Steinemema 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 (772-1480) | 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 (82.5-6825) | 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 (190.0-293.0) | 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 (43.2-774.4) | 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 (48.7-73.4) | - | - |

**Table 2.** Pairwise comparison on the number of nucleotide differences among some of *Steinernema* species and *Steinernema* isolate FUM221 based on ITS rDNA sequences
Table 3. Pairwise comparison on the number of nucleotide differences among some of *Steinernema* species and *Steinernema* isolate FUM221 based on 18S rDNA sequences

Table 4. Pairwise comparison on the number of nucleotide differences among some of *Xenorhabdus* species and *Xenorhabdus* isolate FUM221 based on 16S rDNA sequences

Table 5. General biochemical characteristics of symbiotic bacteria isolated from FUM221 *Steinernema feltiae*
| Biochemical characteristics                  | Steinernema feltiae 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                  | +                           |

**Figures**

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.
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,

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.

Figure 4
Phylogenetic relationship of *Steinernema feltiae* isolateFUM221 with other *Steinernema* species based on ITS-rDNA region. Heterorhabditis bacteriophora as outgroup. Using neighbor-joining analysis in the bootstrap test (10000 replicates) under Kimura 2- model.

Figure 5
Phylogenetic relationship of *Steinernema feltiae* isolate FUM221 with other *Steinernema* species based on 18S region. Heterorhabditis bacteriophora as outgroup. Using neighbor-joining analysis in the bootstrap test (10000 replicates) under Kimura 2- model.

**Figure 6**

Phylogenetic relationship of *Xenorhabdus bovienii* isolate FUM221 with other *Xenorhabdus* species based on 16S rRNA region. Photorhabdus luminescens as outgroup. Using neighbor-joining analysis in
the bootstrap test (10000 replicates) under Kimura 2- model.

**Figure 7**

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