Introduction of insect pathogenic nematode, *Steinernema feltiae* from Iran

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Abstract: A survey of entomopathogenic nematodes was conducted in the Iran. Out of a total of 194 soil samples, 9 were positive for an isolate of entomopathogenic nematodes from *Steinernema* genus. Morphological and molecular studies as well cross hybridization tests were undertaken to characterization. This isolate identified as *Steinernema feltiae*. ITS sequences confirmed this. 16S rRNA sequence and phenotypic characters of its symbiotic bacterium were determined and used for identification. In phylogenetic analysis, relationship among different isolates of native *Steinernema* were indicated. Laboratory test on *Spodoptera exigua* showed high pathogenicity of this entomopathogen.

Key words: Entomopathogenic Nematodes, *Steinernema feltiae*, *Xenorhabdus bovienii*, Iran

Introduction

Entomopathogenic nematodes (EPNs) are suitable candidate for using in insect pest management. Several examples from successful using of EPNs present. It has been demonstrated that for routine identification of EPNs, DNA based diagnostics are quicker than the traditional strategy using morphology and morphometrics (Poewer et al., 1997). Sequences of the ITS region of *Steinernema* species have been used by different authors in taxonomic and phylogenetic studies (Stock et al., 2001; Nguyen & Duncan, 2002; Nguyen & Adams, 2003; Kuwata et al., 2006).

During this 5 years, a few studies have conducted to characterize EPNs from Iran. After Tanha Maafi *et al.* (2003) and Parvizi, author introduced some isolates and their symbionts. Eivazian *et al.* also studied their distribution in East Azerbaijan. In these surveys, species of *S.carpocapsae*, *S.biocornotum*, *Heterorhabditis bacteriophora* and recently *S.glaseri* were identified from different locations.

Material and methods

Entomopathogenic Collection

Entomopathogenic nematodes was isolated using white trap from soil extracted from Tehran province, Iran. The nematodes were maintained on last instar larvae of the greater wax moth *Galleria mellonella*. The third stage infective juveniles (IJ) emerging from host insect cadavers were washed off and stored in distilled water at 15°C until use (Kaya and Stock, 1997).

Nematode isolation, DNA extraction and sequencing ITS region

Isolated IJs were prepared for light microscopy examination. EPNs DNA was extracted from a first generation adult. The nematode was crushed in 50 μl worm lysis buffer in a sterilized 0.5
ml microcentrifuge tube. After heat treatment and centrifugation, the supernatant containing nematode DNA was collected and stored at –30°C. In PCR, ITS regions were amplified in a 50 μl reaction using Takara ExTaq® as described in Kuwata et al., (2006). After amplification, PCR product was loaded by electrophoresis and purified with a Gel-M Gel Extraction system (Viogene). Sequencing reactions were performed by using an ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences of double-stranded PCR products were carried out by ABI Prism® 310 Genetic Analyzer.

**Cloning and sequencing**

Initial direct sequencing showed ambiguous positions and multiple peaks, so ITS product were cloned and resequenced. Pure PCR products, cloned into the pGEM-T Easy vector and transformed into XL-10 Competent Cells. Screening were conducted as described in Spiridonov et al. (2004). From each strain three clones were selected and sequenced in both directions. Sequence obtained during this study is deposited in GenBank (FJ657532). Multiple-sequence alignments were created using CLUSTAL X version 1.81 (Thompson et al., 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura, Dudley, Nei, and Kumar 2007).

**Cross-breeding tests**

Crossbreeding tests with *S.feltiae* (Belgium strain) was carried out on *G.mellonella* hemolymph according to the method described by Nguyen & Duncan (2002).

**Insecticidal Bioassay**

Pathogenicity *S.feltiae* THR strain was studied in the laboratory. The concentrations were 10, 50, and 100 IJs/larva. Fourth host larvae of *Spodoptera exigua* were selected as test host. 30 larvae were used in each treatment. In insecticide assay 9-cm petri dishes were lied with filter paper Whatman n. 1. 0.5 ml of nematode suspension was applied in each petri dish. Two hours later, after application, host larvae were individually placed into a container. The larval mortality was assessed after 24 h. Statistical analysis was performed using the test of least significant difference (LSD) at the 5% level (SAS, 1989).

For comparing, another isolates of *S.feltiae*, Belgian isolate, kindly provided by M. Moens and M.A. Ansari (Ghent university) was used. This isolate cultured at *G.mellonella* larvae at 25°C (Kaya & Stock, 1997). Experiments were conducted at room temperature. In control treatments only DW were applied. Percentage mortality after 48 h analysed using ANOVA and Tukey’s test for separation of means. Differences among mean mortality considered significant at p<0.05.

**Isolation of symbiotic bacteria and sequencing of 16S rRNA gene**

Symbiotic bacteria were isolated from surface-sterilized IJs using 0.1% merthiolate solution. After crashing IJs, 0.5 ml of LB broth was added to the suspension and the suspension was spread on an NBTA plate (Akhurst, 1980). Single colonies were successively extracted. The 16S rDNA fragment was amplified by PCR from bacterial cultures and from total DNA isolated from adult and juvenile stages of nematode. PCR and purification conditions were those described by Kuwata et al (2006), using Fischer-Le Saux et al. (1999) primers. Purified PCR products were sequenced directly as described earlier.

**Results and discussion**

The isolated nematode showed the specific developmental characters of *Steinernema*. Morphological examination indicated *Steinernema* sp. (THR) resembles most *S.feltiae* characters. Key diagnostic traits of the IJs and males were identical to *S.feltiae*. Phylogenetic analysis of ITS rDNA sequence data placed this species in a clade with other isolates of
S. feltiae. The partial 18S, 5.8S gene sequence and 28S portion show little variation among different isolates. The ITS1 and ITS2 regions are much more variable and provide most of the base differences for species diagnosis (Adams et al., 1998; Nguyen et al., 2001). In the phylogenetic analysis of the ITS sequences, all Iranian isolates of EPNs were categorized tentatively into four different clades. These clusters were supported by high bootstrap numbers. The isolate from of S. feltiae aligned clearly with those of the other isolates of S. feltiae. The nematode isolate clustered with other isolates of S. feltiae that was supported highly. Phylogenetic cladogram showed symbiont of this nematode made a clade with other strains of X. bovienii. Bootstrap values (1000 re-samplings) of up to 83% each confirm the statistical significance of the branching point of the Xenorhabdus strain.

Fig. 1. Phylogenetic analysis of Steinernematid species/isolates based on ITS sequences. The dendrogram was constructed by the maximum parsimony method and Kimura-2 parameter with 1000 resamplings values of bootstrap.

Cross Hybridization Results
Males and females of Steinernema sp. THR did interbreed with S. feltiae Belgium strain. In the control treatments, males and females of isolated nematode mated and produced offspring. This study is the first record of bact-helminthic complex of the S. feltiae –X. bovienii from Iran. The sequences analysis of the ITS and 16S regions confirmed this.

Insecticide survey
The host mortality was 74.2–100%. Ranges of LT50 values were from 23.6 to 37.1 h. Pathogenicity determination of this isolate showed that it is highly pathogenic to S. exigua. It was indicated that S. feltiae could cause 100% mortality at a concentration of 50 IJs/larva S. exigua. Comparing to Belgian isolate, Iranian isolate caused higher mortality rates. These showed this nematode might be useful as a potential biocontrol candidate.

Symbiotic Bacteria
Colonies of symbiotic bacteria associated with Steinernema sp. THR on NBTA were similar to those found for Xenorhabdus spp. Almost complete 16S rDNA sequences were generated from X. bovienii, 1502 bp in length. This strain sharing high sequence similarity related to
*X.bovienii*, symbiont of *S.feltiae*. nBLAST search showed that 16S sequence of the bacterium has high identity with these sequences in other *X.bovienii* strains. Homology matrix analysis showed 97-99% similarity with other strains of *X.bovienii*. The bacterial sequences from with *Steinernema* sp. THR isolate was identical. 16S sequences aligned clearly, and without gaps, with those of the other *X.bovienii* species.

The present investigation clearly demonstrated the presence of *S.feltiae* in Iran. An important step towards achieving an effective EPNs for pest control is to seek naturally occurring endemic EPNs isolates. So introduction of endemic isolates of EPNs are important for this. Especially because the mentioned white grub is not easy to control using chemical pesticides. In addition to *S.feltiae* isolate THR, some other isolates from other steinernematid and heterorhabditid were isolated from the white grub. Among them THR isolate of *S.feltiae* had the moderate pathogenicity. Furture survey for characterization of more virulent strains of EPNs as well other insect pathogens and their screening will provide more information about natural biocontrol agents. This is the first report of this species from Iran.

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**References**


Spiridonov, S. E., Reid, A. P., Podrucka, K., Subbotin, S. A. and Moens, M. 2004. Phylogenetic relationships within the genus *Steinernema* (Nematoda: Rhabditida) as inferred from analyses of sequences of the ITS1-5.8S-ITS2 region of rDNA and morphological features. Nematology 6, 547-566.
