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

Javad Karimi 1, Aziz Kharazi-pakdel 2, Toyoshi Yoshiga 3

1.Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad , Iran ;2. Department of Plant Protection , Campus of Agriculture and Natural Resources , University of Tehran , Karaj , Iran ;3. Lab.Nematology , Department of Applied Biological Sciences , Saga university , Saga , 840-8502 , Japan

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 sequenc 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 pathogenecity of thise entomopathogen.

Key words: Entomopathogenic Nematodes, *Steinernem*a *feltiae*, *Xenorhabdus bovienii*, Iran

Introduction

Entomopathogenic nematodes (EPNs) are suitable candidate for using in insect pest management.Severel 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 suveys , 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 (IJs) 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 ligh 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 was 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.Screeing were conducted as describedin 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 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 25Ċ (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 IJss 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 idenditical 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 islates 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.

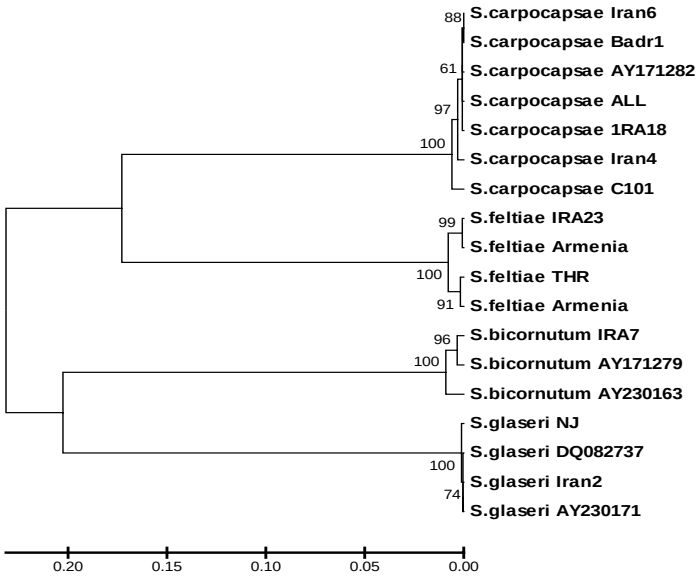

Fig.1.Phylogenetic analysis of Steinernematid species /isolates based on ITS sequences. The dendrogram was constructed by the maximum parsimony method and Kimura-2 parameter with 1000 resamplings values of bootstrap .

Cross Hybridization Results

Males and females of *Steinernema* sp. THR did interbreed with *S.feltiae* Belgium strain . In the control tretments, 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 lenght. 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 idendity 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 pathogenecity.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.

Acknowledgements

This work was supported in part by Grants-in-Aid from the University of Tehran.The scholarship by the Ministry of Science; Research & Technology of Iran to J.Karimi for a short-term scientific mission to the Saga University of Japan is gratefully acknowledged. We are grateful to Minshad A. Ansari, E.Kondo, R.Kuwata, R .Tanaka and Y .Yoshie for helping us in this study.

References

Akhurst, R.J. 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes Neoplectana and *Heterorhabditis*. Journal of General Microbiology 121, 303–309.

Adams, B. J. & Nguyen, K. B. 2002 Taxonomy and Systematics. In: Entomopathogenic Nematology. (Gaugler, R. ed.), CABI Publishing, Wallingford, 1-34.

Hominick, W. M. 2002. Biogeography. In: Entomopathogenic Nematology. (Gaugler, R. ed.), CABI Publishing, Wallingford, 115- 143.

Kaya, H.K. & Stock, P. 1997. Techniques in insect nematology.In: Lawrence, A.L. (Ed.). Manual of techniques in insect pathology. San Diego, CA, USA, Academic Press, pp. 282-324.

Kuwata, R., Shigematsu, M., Yoshiga, T., Yoshida, M. and Kondo, E.2006. Intraspecific variations and phylogenetic relationships of steinernematids isolated from Japan based on the sequences of the ITS region of the nuclear rRNA gene and the partial mitochondrial COI gene. Japanese Journal of Nematology. 36(1):11-21.

Nguyen, K. B., Maruniak, J. and Adams, B. J. 2001. Diagnostic and phylogenetic utility of the rDNA internal transcribed spacer sequences of *Steinernema*. Journal of Nematology 33, 73-82.

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.

Stock, S. P., Campbell, J. F. and Nadler, S. A. 2001. Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. Journal of Parasitology 87, 877-889.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25, 4876-4882.

Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599.