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Introduction of Steinernema Carpocapsae Weiser, 1955 (Rhabditida: Steinernematidae) From Natural Population of White Grub, Polyphylla Olivieri (Coleoptera: Melolonthidae) From Iran

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Abstract: White grub, *Polyphylla olivieri* Cast. (Col., Melolonthidae) is major pest of the Iran. In a survey for collecting natural entomopathogenic nematodes associated with this melolonthid, two isolates of *Steinernema* spp. were collected as natural pathogens in larval populations of the white grub in the Tehran province of Iran. Morphological characters identified those as members of "*carpocapsae* "group. Morphological and molecular characters using ITS sequences as well cross hybridization tests confirmed those as *S.carpocapsae*.16S rRNA sequences and phenotypic characters of symbiotic bacteria were determined and used for identification. Phylogenetic analysis for studying relationship was performed. This is the first information about this pathogen of *P.olivieri* as well new information about symbiotic bacteriua associated with Iranian entomopathogenic nematodes.

Key Words: Entomopathogenic Nematodes, *Steinernema carpocapsae*, *Xenorhabdus nematophila*, white grub, *Polyphylla olivieri*, Iran.

Introduction

Entomopathogenic nematodes (EPNs) are among one of the best biocontrol agents to control various economically important insects, successfully (Klein, 1990; Shapiro-Ilan *et al*., 2002). Many surveys have been conducted all over the world in order to collect EPNs that may have potential in management of economically important insect pests (Hominick, 2002). White grubs, the root-feeding larvae of scarab beetles, cause significant damage to many agricultural and horticultural plants. In the Iran, larvae *Polyphylla olivieri* Cast. (Col., Melolonthidae) is major pest of throughout much of the provinces.This melolonthid is a serious pest in different agroecosystems The white grub has a long life cycle with adults emerging in June to lay eggs in the soil near the roots of the host plants of the larvae. By

late summer, most larvae have developed into the third instar. Overwintering stage is larvae. The extensive feeding activity of the larger larvae can kill large areas of host plant especially on some hosts like cherry. *P. olivieri* complete its generation through three years, one year for each larval stage.Host range of this pest is wide (Radjabi,1991). Considering its cryptic habitates, chemical control doesn't useful everywhere. During past decade, several reports presented about efficiency of EPNs against different white grub species in Europe and USA.As preliminary step in organizing a biocontrol plan, characterization of natural EPNs associated with this insect was addressed in this study. In few studies about EPNs in Iran Parvizi (2001) reported occurrence of some isolates from West Azerbaijan, following by Tanha Ma'afi *et al* (2006) and recently by Eivazian Kari *et al* (2009) who introduced some species from North West of Iran. For identify associated EPNs, emphasis was on molecular methods. 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, Stock *et al*., 2009). Sequences of the ITS region of *Steinernema* species have been used by different authors in taxonomic and phylogenetic studies (Nguyen *et al*., 2001; Stock *et al*., 2001; Nguyen &Duncan, 2002; Nguyen & Adams, 2003). Bacteria in the genera *Photorhabdus* and *Xenorhabdus* are considered the main symbionts of EPNs and to be the species primarily responsible for the death of the insect hosts and the growth of the nematodes within the insect cadaver.Comparison of 16S rRNA gene sequences has proved extremely useful for species identification and phylogenetic reconstruction for symbiotic bacteria (Rainey *et al* 1995; Suzuki *et al*., 1996). We here present the relationships of Iranian isolates of *S.carpocapsae* with other steinernematids based on the analyses of the complete ITS-rDNA sequences.Morever, symbiotic bacteria related as symbiont with this nematodes characterized.

Material and Methods

Collection of Polyphylla olivieri

1180 Second and third instar larvae of the white grub *Polyphylla olivieri* were collected during 2005-2006 from different fruit charden at the Karaj, Shahryar, Hashtgerd, Lavasan, Varamin and Chalous road towns, Tehran province, Iran. Larvae were kept individually in dish-pans at 25±2 Ċ at mixture of organic compost and loamy sand with pieces of carrot as food. Grub with EPNs symptoms transferred to the White trap. The emerging infective juveniles (IJs) from infected white grub were harvested from the traps and stored in tap water at 10 Ċ (Kaya and Stock, 1997). For light microscopy, IJs collected during a week after their first emergence from the insect cadavers; adults of the first generation were dissected from the cadavers (Nguyen & Smart, 1995). Microscopic slides prepared Seinhorst's (1959) rapid method as modified by De Grisse (1969). All measurements were made using an Olympus BX50 light Microscope, based on Kaya &Stock (1997). The following characters were measured: total body length; anal body diam, maximum body diam.; excretory pore position; distance from anterior end to base of pharynx; gubernaculum length; spicule length, gubernaculum length divided by spicule length (%); distance from anterior end to nerve ring position; ratio a (Length divided by width.); ratio b (Length divided by distance from head to pharynx base); ratio c *(*Length divided by tail length); ratio D (Distance from head to excretory pore divided by distance from head to pharynx base); ratio E; spicule length divided by anal body diam.and tail length.

Nematode isolation and sequencing ITS region

Genomic DNA was extracted from individual nematodes using Qiagen kit. The ITS regions of rDNA were amplified using the methods reported by Nguyen *et al*. (2001).All PCR reactions were run in a PTC-200 Peltier Thermo Cycler (MJ Research, Inc., Waltham, MA). Initial direct sequencing showed ambiguous positions and multiple peaks, so ITS product were cloned and resequenced. Ligation and transformation were based on Spiridonov *et al*.(2004). DNA sequences were determined from both strands and from multiple, independently amplified templates. Sequence obtained during this study is deposited in GenBank under accession No. EU122951 and EU077232. Multiple-sequence alignments were created with the default parameters of Clustal X (Chenna *et al*., 2003). The DNA sequences were aligned using Clustal X 1.64 (Thompson *et al*., 1997) with the ITS1-5.8S-ITS2 for Sequences of other *Steinernema* species obtained from GenBank, except of *S.glaseri*, which obtained during a study parallel to this. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura, Dudley, Nei, and Kumar 2007). To establish a root for an analysis of nematodes, the sequences of the ITS region of *Pratylenchus coffeae* (AY561436) was aligned as the out group.

Cross-breeding tests

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

Isolation of symbiotic bacteria

Symbiotic bacteria were isolated from surface-sterilized IJs. Infective juveniles were immersed in 0.1% Merthiolate solution, washed three times in sterile saline and crushed tip in a small amount of sterile saline to release the bacteria from the nematode intestine. About 0.5 ml of LB broth was added to the suspension and the suspension was spread on an NBTA plates (Akhurst, 1980). Single colonies were successively extracted and streaked on a new NBTA plate until no contamination was identified.

Sequencing of 16S rRNA gene of symbiotic bacteria

The 16S rDNA fragment was amplified by PCR from bacterial cultures and from total DNA isolated from adult and juvenile stages of nematode. DNA amplification were repeated three times. DNA from *Escherichia coli* was used as positive control for PCR. Fischer-Le Saux *et al.* (1999) primers (16S-F and 16S-R) were used for PCR and sequencing as described by Kuwata *et al* (2006). The DNA sequences were deposited in the gene bank.

Results

Out of a total 11800 soil samples 51 were positive for entomopathogenic nematodes (7/3%) with 33 (2.7%) containing *Steinernema* and 18 (1/5%) *Heterorhabditis* isolates. Morphological examination indicated *Steinernema* sp. resembles most "*carpocapsae"* group characters.Key diagnostic traits of the third-stage IJs and males were idenditical to a

member of "*carpocapsae*" group. The total lengths of the IJs of both isolats were within the characteristics of the species (586-590 µm. Greates width were 24 µm. EP, NR and ES were 41.1, 81.8 and 125.6 µm. Tail length were between 45-49 µm.Lenght and greatest width for first generation males of both isolates were 1360 and 105.8 µm, respectively. This value is considered to be the limit the species. Morphometric characteristic and indexes on these two strains were within the limits, described in the literature for *S.carpocapsae* (Nguyen & Smart, 1995). (Poinar, 1986).

Phylogenetic analysis of ITS rDNA sequence data placed this species in a clade with other isolates of *S.carpocapsae* (Figure 1).

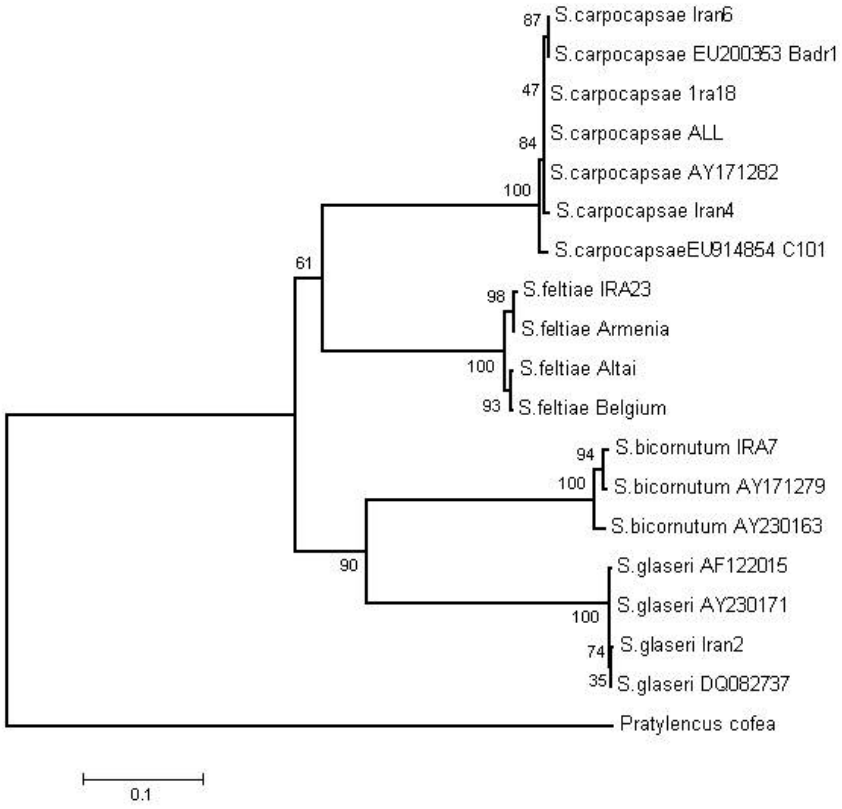

Figure 1. Phylogenetic relationship among Iranian steinernematid species based on the sequences of the ITS region by the neighbor-joining method. Bar represents 0.1 substitutions per nucleotide position.

The partial 18S, 5.8S gene sequence and 28S portions showed little variation among different isolates. The ITS1 and ITS2 regions are much more variable and provide most of the base differences for species diagnosis (Nguyen *et al*., 2001). The isolates from of *S.carpocapsae* aligned clearly, and without gaps, with those of the other of *S.carpocapsae* isolates.In phylogram based on ITS sequences, Iran6 isolates was near to an isolate from

Jordan, with less distance to another Iranian isolate, IRA18.The second isolate,Iran 6 was nearest to an isolate from China, C101.

Males and females of both *Steinernema* sp. Iran4 and Iran6 isolates did interbreed with *S.carpocapsae* ALL strain. In the control treatments, males and females of isolated nematode mated and produced offspring. Cross hybridization test showed that the male and female of the two strains mated andoffspring developed indicating that the two isolates are in the same species.

Symbiotic Bacteria

Colonies of symbiotic bacteria associated with both isolates on NBTA was similar to those found for *Xenorhabdus* spp.Almost complete 16S rDNA sequences were generated from *X.nematophila*, 1502bp in lenght. DNA sequence had sharing high similarity related to *X.nematophila*, symbiont of *S.carpocapsae*. nBLAST search showed that 16S sequence of the bacteria has high identity with these sequences in other *X.nematophila* strains. Homology matrix analysis showed 97-99 % similarity with other strains of *X.nemtophila*. Percentage similarity of 16S sequences with Breton strain (DQ282116) was 99% and with DSM 3370 strain (X82251) was 98% (Figure 2). The bacterial sequences from with both strains of *Steinernema* were identical with *Xenorhabdus* species. 16S sequences aligned clearly, and without gaps, with those of the other *X.nematophila* species.

The present study provides the data about symbiotic bacterium of genus *Xenorhabdus* from Iran. The nematode was isolated from a dangerous white grub. The present investigation demonstrated the presence of *S.carpocapsae* in natural population of the white grub, *P. olivieri*. An important step towards achieving an effective EPNs nematode for pest control is to seek naturally occurring endemic EPN isolates. So introduction of endemic isolates of EPNs are important for this. In this study EPN were recovered at a low rate (2.7%) of EPNs. Eivazian reprted that 3% of sampling sites were positive for *Steinernema* isolates (Eivazian *et al*., 2009).

In addition to *S.carpocapsae* isolates, some other isolates from other steinernematid and heterorhabditid were isolated from the white grub. Among them isolate of *S.carpocapsae* had the lower 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. It's predicted more EPNs species may be discovered in future surveys adding significant data to the biodiversity of this biocontrol agents. Untill today several species of EPNs including *Heterorhabditis bacteriophora*, *S.carpocapsae*, *S.feltiae*, *S.biocornotum* as well *S.glaseri* reported as natural fauna of Iran(Eivazian *et al*., 2009,Tanha Ma'afi *et al*., Karimi *et al*., in press).

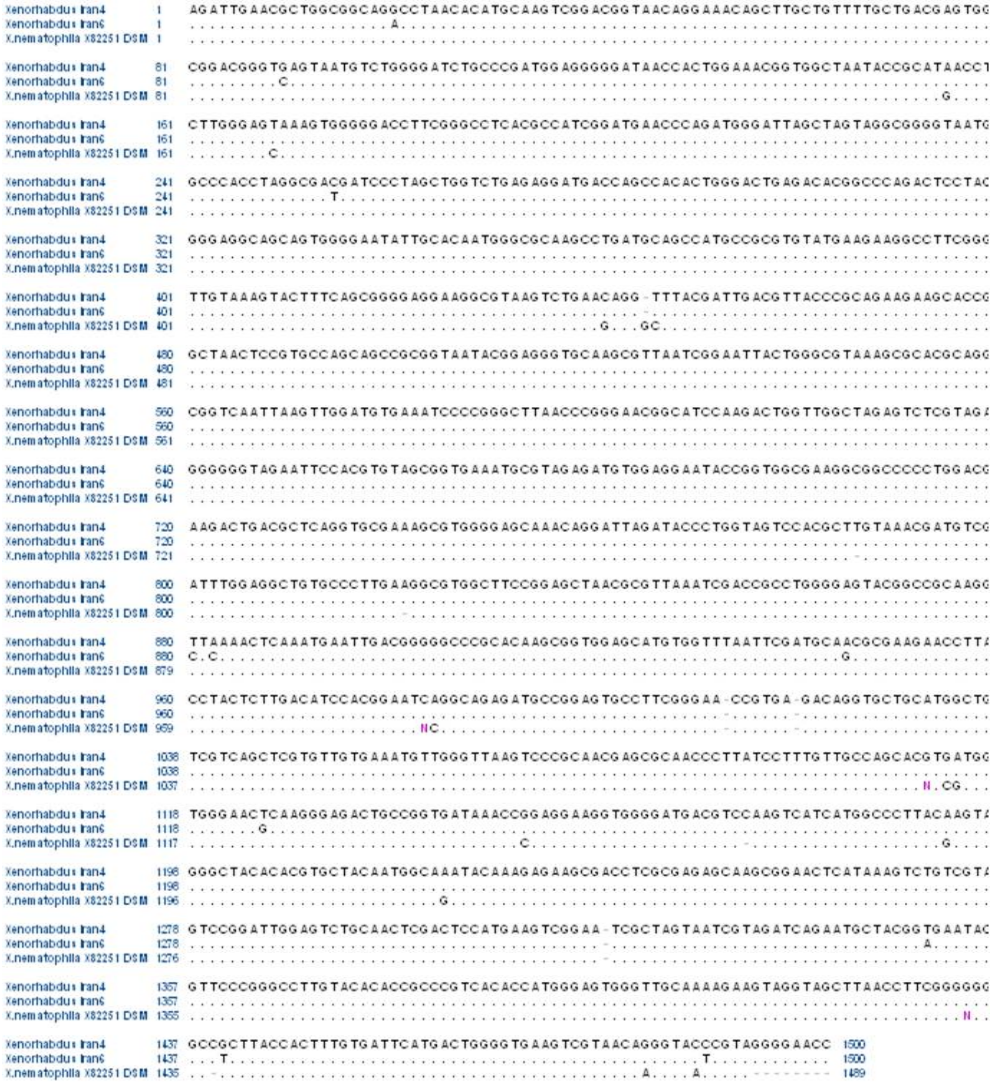

Figure 2. Alignment of the 16S rRNA of Iranian strains of *Xenorhabdus* with the sequence of *X.nematophila* DSM 3370 (X82251).

Among EPNs, *S.carpocapsae* is a cosmopolite species recorded from Asia, America and Europe. Description of nematodes is basically founded on morphological characters, which are not readily applicable to nematode identification primarily because of overlapping morphometrics and similar morphology (Poinar, 1990; Hominick, 2002). In adition to taxonomic characterization of above bacto-helminthic complex, pathogenecity of these nematodes has studied that their results will publish in future. By gathering all information, it's promising to provide a non-chemical method in management of this white

grub and reducing its damage in this area. This will be particularly based on some more pathogenic species like *S.glaseri*.

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