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Short note

Report of entomopathogenic nematode, *Steinernema glaseri*, from Iran

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Entomopathogenic nematodes have been used successfully to control various economically important insects (Klein, 1990; Shapiro-Ilan *et al.*, 2002). White grub, *Polyphylla olivieri* (Coleoptera, Melolonthidae), is a serious pest in different regions of Iran and is characterized by its polyphagous feeding behaviour. In recent years, several chemical pesticides have been used in control programmes against this grub. The interest in biological control of white grub increased during the last few years. Different insect pathogens attack this melolonthid beetle in Iran (*e.g.*, *Beauveria bassiana* and *Metarhizium anisopliae*). Entomopathogenic nematodes (EPN) are suitable candidates for use to control this group. Several EPN cultures were isolated during the survey in Iran. The internal transcribed spacer (ITS) region of the rRNA gene has proven to be useful for not only identifying but also inferring the phylogenetic relationships of steinernematid species (Nguyen *et al.*, 2001; Stock *et al.*, 2001; Spiridonov *et al.*, 2004). Bacteria in the genera *Photorhabdus* and *Xenorhabdus* are 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 of the symbiotic bacteria (Rainey *et al.*, 1995; Suzuki *et al.*, 1996).

Larval stages of the white grub, *Polyphylla olivieri* were collected from different sites in Tehran province of Iran in 2005-2006. The larvae were reared in laboratory conditions. Larvae with symptoms of infection by EPNs were transferred to White trap. Isolated infective juveniles were used for morphological and molecular characterization. For molecular studies single female was crushed in 50 µl worm lysis buffer in a sterilized 500 µl microcentrifuge tube. The tube was incubated at 65°C for 1 h, followed by 95°C for 15 min. After centrifugation at 4000 *g* for 2 min the supernatant was collected as template DNA and stored at -30°C for further use. The ITS region from each isolate was PCR amplified in a sterile 0.5 ml tube using the primers described by Vrain *et al.* (1992). The PCR conditions were as described by Hominick *et al.* (1997). 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 (AppliedBiosystems, Foster City, CA,USA) with one of the primers used for PCR and internal primers, KN58 and KNRV, described in Nguyen *et al.* (2001), under the conditions recommended by the manufacturer. Sequences of both strands of DNA were determined by ABI Prism® 310 Genetic Analyzer. DNASTAR (Lasergene USA) was used for sequence editing and verifying basecalls. 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 (Promega, Leiden, The Netherlands). Several clones were isolated using blue/white selection and submitted to PCR with vector primers. After confirmation, each clone was inoculated into 5 ml LB/AMP tubes. Overnight cultures were used for plasmid extraction using 10-min miniprep method after a modification. From each strain three clones were selected and sequenced in both directions using two vector primers, one internal forward primer and one internal reverse primer with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's

instructions. Sequences of studied samples were optimized manually in MacClade 4.0 (Maddison & Maddison, 2002). Multiple-sequence alignments were created with 15.0 of gap opening penalties and 6.66 of gap extension penalties using CLUSTAL X version 1.81 (Thompson *et al.*, 1997). Previously published ITS sequences from *S. glaseri* (AF122015, AY230171, DQ082737 and FJ463939) were used as standards for comparing the sequence of nematode isolate.

Crossbreeding tests with *S. glaseri* (NC Strain) and *S. arenarium* were carried out on *G. mellonella* hemolymph according to the method described by Nguyen & Duncan (2002).

Symbiotic bacteria were isolated from surface-sterilized infective juveniles. Infective juveniles were immersed in 0.1% Merthiolate solution for 2 h, washed three times in sterile saline and crushed with the end of a Pipetman (Gilson) 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 NBTA and McConkey plates (Akhurst, 1980). Single colonies were successively extracted and streaked on a new NBTA plate until no contamination was identified. Bacterial colonies maintained on the NBTA plates were used for further study Overnight cultures of symbiotic bacteria was used for extraction of genomic DNA. After lysing, DNA was purified by phenol/chloroform extraction, precipitated and used for PCR amplification of 16S rRNA gene (Kuwata *et al.*, 2006). PCR amplification was performed in a Biometra thermal cycler with the use of Takara ExTaq (Takara Co., Japan). The nucleotide sequences of 16S was determined directly from the PCR fragments. PCR primers and sequencing primers used in this study were based on Fischer-Le Saux *et al.* (1999). Sequencing was carried out using an ABI Prism BigDye Terminator Cycle Sequencing Kit and a DNA sequencer (Perkin-Elmer) according to the manufacturer's instructions.

The isolated nematode showed the specific developmental characters of *Steinernema*. Morphological examination indicated that most of the characters of *Steinernema* sp. (Iran 2) resembles this of *S. glaseri*. Key diagnostic features of the third-stage infective juveniles (IJs) and males were identical to those of *S. glaseri*. Phylogenetic analysis of ITS rDNA sequence data placed this species in a clade with other isolates of *S. glaseri*. The DNA sequences were deposited in the NCBI GenBank with accession number EU048543.

Males and females of *Steinernema* sp. Iran 2 did not interbreed with *S. arenarium*, but crossing with *S. glaseri* strain was successful. In the control treatments, males and females of isolated nematode mated and produced offspring.

Colonies of symbiotic bacteria associated with *Steinernema* sp. Iran 2 on NBTA and McConkey were similar to those found for *Xenorhabdus poinari*. Almost complete 16S rDNA sequences (1500 bp) were obtained for Iran 2 strain. The sequence that is available with the NCBI accession numbers EU250472, shares a high sequence similarity with *X. poinarii* – symbionts of *S. glaseri*.

The present investigation clearly demonstrated the presence of *S. glaseri* in Iran and its role as natural pathogen of local populations of the white grub, *Polyphylla olivieri*. Several other isolates of steinernematids and heterorhabditids were isolated from the white grub in Iran and further study is planned to compare the infectivity of different strains against this pest.

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