Natural Enemies of the Sugar Beet Army Worm, *Spodoptera exigua* (Lepidoptera: Noctuidae) in Northeast Iran

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NATURAL ENEMIES OF THE SUGAR BEET ARMY WORM, *SPODOPTERA EXIGUA* (LEPIDOPTERA: NOCTUIDAE) IN NORTHEAST IRAN[1]

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ABSTRACT: During 2014-2015, a survey was carried out to determine the natural enemies of the sugar beet armyworm, *Spodoptera exigua* (Hübner) in the Mashhad region of northeastern Iran. Through extensive sampling, different larval stages of *S. exigua* with possible signs or symptoms of infection were collected from beet fields, kept in laboratory conditions, fed with fresh beet leaves, and checked daily until the emergence of parasitoids. Herein, 12 species of parasitoids, a virus: *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV), an entomopathogenic fungus: *Beauveria varroae,* and an entomophilic nematode, *Pristionchus pacificus* are reported. The parasitoids were *Meteorus rubens*, *Microplitis rufiventris*, *Cotesia* sp., *Chelonus* (*Chelonus*) *annulipes* (Braconidae), *Euplectrus* sp. (Eulophidae), *Sinophorus* nr. *xanthostomus, Anomalon* sp., *Diadegma pulchripes*, *Temelucha* sp. (Ichenumonidae), two species of *Drino* genus and *Exorista* sp. (Tachinidae). The body tissues of the virus-infected larvae were liquefied, swollen, and glossy with a very thin cuticle. SEM photographs of viral occlusion bodies (OBs) showed polyhedral and irregular shapes. A cross-section of an OB indicated that each virion contained multiple nucleocapsids arranged randomly within the occlusion matrix. Genetic analysis based on *lef-8* and *Polyhedrin* gene sequences confirmed the identity of the Iranian strain as SeMNPV. The virus was transmitted vertically and horizontally. Another pathogen was *Beauveria varroae,* isolated from a lepidopteran insect for the first time; *S. exigua* was indicated as a new host for this fungus. Both pathogens are herein introduced as new records for Iran microflora. The results of the current work highlight the diversity of natural enemies on the sugar beet armyworm in Iran.

KEYWORDS: Baculovirus, Entomopathogenic fungi, Entomophilic nematode, Parasitoids

INTRODUCTION

The sugar beet armyworm, *Spodoptera exigua* Hübner, 1808 (Lepidoptera: Noctuidae), is a major pest of many agricultural crops, including sugar beet, soybean, sweet pepper, tomato and melon (Dai et al., 2000). This pest is native to Asia but has been recorded in different regions throughout the world, such as Australia, Europe and North America (Pathak and Khan, 1975). Its larvae feed on the foliage of plants and the upper portion of beet roots and can completely defoliate small plants, frequently causing economic damage. Due to the problem of resistance to insecticides, the adverse effects of insecticides on the environment (Asi et al., 2013), their negative effects on wildlife, and natural enemies (Ordóñez-García et al., 2015), biological control as a pest management strategy is desirable. Most biocontrol agents are host specific, and the probability of pest resistance problems is low (Ahmad and Arif, 2010).

Various species of natural enemies are active on different life stages of *S. exigua*, and a number of them play a significant role in biological control. Parasitoids of the *Spodoptera* genus belong to the families of Ichneumonidae,

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Braconidae, Eulophidae, and Tachinidae (Rios-Velasco et al., 2011). Entomopathogenic bacteria, viruses, nematodes, and fungi have been introduced as natural pathogens of *Spodoptera* larvae (Molina-Ochoa et al., 2003; Hajek et al., 2007; Rios-Velasco et al., 2011).

Various populations of *S. exigua* are infected by different species/strains of baculoviruses (Virto et al., 2014). Cheng et al. (2000) characterized an ascovirus isolate from *S. exigua* in Indonesia, and two isolates (SeIV-1, SeIV-2) from Iflaviridae were isolated and identified from *S. exigua* larvae by Millán-Leiva et al. (2012).

Among baculoviruses, *S. exigua multiple nucleopolyhedrovirus* (SeMNPV) was isolated from *S. exigua* larvae (Jakubowsaka et al., 2005; Virto et al., 2013). SeMNPV is a specific pathogen of *S. exigua* and has the potential to be used as a biocontrol agent (Virto et al., 2014). SeMNPV produces epizooty in larval populations. A number of biopesticides are formulated for use against this pest such as SPOD-X® (Certis, USA), SPEXIT® (Andermatt Bio-control, Switzerland), and VIR-EX® (Biocolor, Spain) (Virto et al., 2014). The virus spreads by vertical and horizontal transmission among individuals of a population; this trait increases the mortality rate of the pest larvae (Kukan, 1999).

The present survey investigated the natural enemies of *S. exigua* in Razavi Khorasan province, Iran. Twelve species of parasitoids, a virus isolate, an entomopathogenic fungus, and a nematode were isolated from infected *S. exigua* larvae and pupae. Parasitoids were identified using morphological and molecular techniques. The nematode was identified using DNA sequences of the internal transcribed spacer (ITS) region. For viral identification, Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and DNA sequence analyses of *late expression factor* 8 (*lef*-8) and *polyhedrin* genes were employed. The entomopathogenic fungus was identified using standard methods and analysis of the DNA sequence of the ITS gene.

MATERIALS AND METHODS

Insect collection

Spodoptera exiguae larvae were collected from sugar beet fields mostly from the northern part of Razavi Khorasan province (36.2980°N 59.6057°E), northeastern Iran. Larvae were reared in the laboratory in cylindrical containers (10 cm diameter × 5 cm height), at 25±1°C, with a photoperiod of 16L:8D, 60±5% RH. Fresh beet leaves were provided for food. Screening was carried out daily for symptoms of disease and evidence of parasitism.

Collection and identification of parasitoids

Suspected parasitized larvae were isolated in petri dishes (9 cm diameter) and checked daily for emergence. Ectoparasitised larvae were transferred to a container, kept at room temperature for 2-3 weeks and monitored daily for adult parasitoid emergence. Pupae were screened daily for parasitoids. All parasitoids were preserved in 96% ethanol for future study.

Microscopic slides of parasitoids were prepared using Hoyer's medium (Rosen and DeBach, 1979). The identities of the Braconidae, Ichneumonidae, Eulophidae, and Tachinidae parasitoids were confirmed by Samira Farahani (Research Institute of Forests and Rangelands, Tehran, Iran), Kees Zwakhals (CM Arkel, Netherlands), Zoya Yefremova (Tel-Aviv University, Israel), and Ebrahim Gilasian (Iranian Research Institute of Plant Protection, Tehran, Iran), respectively.

The following protocol was used for DNA bar-coding. DNA was extracted using the 5% Chelex®100 solution (Karimi and Darsouei, 2014). The cytochrome oxidase subunit I gene was amplified using LCO1490 and HCO2198 primers (Folmer et al., 1994), and PCR products were sequenced using macrogen sequence facilities. Sequences were checked using Nblast analysis, and then the consensus sequence was prepared and submitted to the GenBank.

Isolation of entomopathogenic virus

Moribund larvae by color change, enlarged body, reduced feeding, and upward tendency were isolated as a viral infection, and Koch's postulate was used to confirm infection (Vega and Kaya, 2012). After confirmation, a viral inoculum was used to treat the larvae and prepare stock culture. To investigate possible vertical transmission, batches were held in a separate container to record mortality with associated virus symptoms.

Purification of viral occlusion bodies (OBs)

Three different larval batches were found to have a virus infection and occlusion bodies from three isolates that were purified prior to DNA extraction. In the extraction process, infected larvae were homogenized in a buffer (1% ascorbic acid; 2% sodium dodecyl sulphate SDS; 0.01 M Tris–HCl; 1 mM EDTA, pH 7.8) and stored at room temperature for 1 h; the homogenate was then centrifuged at 6000 rpm for 5 min to remove larval particles. The supernatant was transferred to a new microtube and centrifuged at 14000 rpm for 15 min to collect the pellets of the occlusion bodies. The pellets were eluted in TE buffer (0.01 M Tris–HCl; 1 mM EDTA; pH 7.8) and centrifuged at 14000 rpm for 15 min. Finally, the pellets were washed in TE buffer containing 0.1% SDS and 0.1 M NaCl and centrifuged at 14000 rpm for 15 min (Alexandre et al., 2010). Occlusion bodies were stored at -20°C.

Purification of DNA from OBs

To extract DNA from OBs, the virions were dissolved in 1 ml alkaline solution (0.1 M Na_2CO_3, 0.17 M NaCl, 0.01 M EDTA, pH 10.9) and kept at 37°C for 30 min. Then, 1% SDS (200 µl) and 0.5 mg/ml proteinase K (20 µl) were added and kept at 37°C overnight. After proteins were removed using the phenol:chloroform:isoamylalcohol (25:24:1) method, the supernatant was transferred to a clean microtube. For precipitation of DNA, 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol were added to the aqueous phase and

then incubated overnight at 20°C. Finally, DNA was precipitated by centrifugation at 14000 rpm for 10 min and washed in 70% (v/v) ethanol. The viral DNA was dried at room temperature, suspended in sterile water, and stored at -20°C (Alexandre et al., 2010).

Scanning Electron Microscopy (SEM)

The morphology of OBs extracted from the individual larva was investigated using SEM. The suspension was fixed with 2.5% (v/v) glutaraldehyde in 0.05 M phosphate buffer for 24 h and then in 2% (v/v) aqueous osmium tetroxide for 2 h. Samples were dehydrated by washing in a serial alcohol solution (25%, 50%, 75%, 95%, 100%) and dried. The sample was gold coated using SC7620 and observed under Leo 1450VP. Images were obtained at different magnifications (Kumar et al., 2011).

Transmission electron microscopy (TEM)

OB pellets were fixed in 2.5% (v/v) glutaraldehyde in a 0.05 M phosphate buffer (pH 7.2) for 24 h and then fixed in 0.5% (v/v) aqueous osmium tetroxide for 2 h. Samples were dehydrated in a series of alcohol concentrations. Resin was added and ultra-sections with a thickness of 50-70 nm were prepared (Kumar et al., 2011). Sections were stained with uranyl acetate, stained with 4% lead citrate, and observed under TEM (Leo 912 AB). Ten OBs and nucleocapsids were measured for the morphometrics of the viral particles.

Virion characterization

Two genes, i.e. *polyhedrin* and *lef*-8*,* were amplified and sequenced. To amplify the *polyhedrin* gene, the primer set including prPH-1 (TGTAAAACGACG GCCAGT NRCNGARGAYCCNTT) and prPH-2 (CAGGAAACAGCTATGAC CDGGNGCRAAYTCYTT) were used (Lang et al., 2004). Those degenerated primers amplify a fragment with 686 bps corresponding to 42075-41389 bps in the AcMNPV genome. Moreover, to amplify the *lef*-8 gene, prL8-1 (CAGG AAACAGCTATGACCCAYGGHGARATGAC) and prL8-2 (CAGGAAACAG CTATGACCAYRTASGGRTCYTCSGC) primers were used, which amplify a fragment approximately 700 bps (Lang et al., 2004).

The PCR products were sequenced by Macrogen Co. (Seoul, Korea). The sequence chromatograms were checked using Bioedit software (Hall, 1999), and the database was compared using Nblast software in Genbank. Consensus sequences were submitted to GenBank. Seven sequences of the *lef*-8 and *polyhedrin* genes were retrieved from GenBank and aligned with the current Iranian isolate using Clustal X software (ver. 2) (Larkin et al., 2007) with default parameters. The MEGA 5 program (Tamura et al., 2011) with the K2P model (Kimura, 1980) was used to calculate distances between nucleotide sequences. Phylogenetic analyses were performed using the neighbor-joining method (Saitou and Nei, 1987) with 10000 Bootstrap replications (Felsenstein, 1985) in MEGA 5.

Isolation of entomopathogenic fungus

A fifth instar *S. exigua* larva cadaver infected with an entomopathogenic fungus was transferred to a moist Petri dish for fungal growth. Part of the cadaver was sterilized in 70% ethanol, plated on Potato Dextrose Agar (PDA) medium, and stored at 28±1°C for 10 days. To confirm Koch's postulate, a suspension with 10^7 conidia/ml was used for treatment of fifth instar larvae. Samples were kept at 28±1°C and 70±5% humidity for 7 days (Lacey, 2012).

Scanning Electron Microscopy (SEM)

For SEM studies, the fungal spores were fixed at room temperature using 2.5% glutaraldehyde for 12 hours, then washed with phosphate buffer and treated with 2% osmium tetroxide for 2 hours. The fixed material was washed in distilled water and dehydrated in an acetone series. The sample was gold coated using SC7620 and observed under Leo 1450VP; images were provided at various magnifications (Liu et al., 2001). To morphologically identify and compare the Iranian isolate with voucher samples (Rehner et al., 2011), microscopic slides were provided using Lactophenol Cotton Blue solution for staining (Leck, 1999).

Molecular identification of entomopathogenic fungus

To extract DNA, the fungal mycelium was produced on Potato Dextrose Broth (PDB) and shaken for 2 weeks at room temperature. Genomic DNA was extracted with the CTAB method (Black and Duteau, 1997). ITS region primers were amplified and sequenced using ITS4 and ITS5 (White et al., 1990).

Thirty-seven sequences from the ITS gene of the *Beauveria* genus were retrieved from GenBank and aligned with the sequence of the Iranian isolate using Clustal X software (ver. 2) (Larkin et al., 2007) and default parameters. The ITS sequences of *Isaria cicadae* (HQ880826) and *Cordyceps militaris* (HQ880829) were used as outgroups. The MEGA 5 program (Tamura et al., 2011) and K2P model (Kimura, 1980) were used to calculate inter- and intra-nucleotide distances between *Beauveria*. Phylogenetic analyses were performed using the neighbor-joining method (Saitou and Nei, 1987) with 10000 Bootstrap replications (Felsenstein, 1985) in MEGA 5.

Isolation of the entomopathogenic nematode

Entomopathogenic and entomophilic nematodes recovered from *S. exigua* larvae were characterized using molecular techniques. The ITS region was amplified using 18S and 28S primers (Vrain et al., 1992) and used for future analyses as described in Darsouei et al. (2015).

RESULTS

Natural enemies of *S. exigua* larvae were collected and identified in the current work. In total, 4350 larvae were collected from 10 different fields in northeastern Iran. From the collected larvae, 50 were found to be parasitized, 26 were

infected with NPV, and one larva was infected with *B. varroae*. The nematode *Pristionchus pacificus* was found in the hemolymphs of four dissected larvae (Table 1).

Table 1. Natural enemies of *Spodoptera exigua* larvae collected in sugar beet fields in Razavi Khorasan province, Iran, 2015.

Date	Larvae (n)	Parasitized (n)	NPV	*Beauveria varroae*	*Pristionchus pacificus*
9 June	200	1	0	0	0
16 June	250	4	5	0	0
23 June	350	0	0	0	0
30 June	350	4	4	1	0
7 July	500	3	0	0	4
14 July	500	4	3	0	0
21 July	500	8	7	0	0
28 July	500	11	6	0	0
5 August	400	10	1	0	0
12 August	400	5	0	0	0
19 August	400	0	0	0	0
Total	4350	50	26	1	4

Parasitoids

Twelve parasitoids from the larval and pupal stages of *S. exigua* were collected, namely the braconids *Meteorus rubens* Nees, 1811 (14 ♂, 18 ♀), *Microplitis rufiventris* Kokujev, 1914 (4 ♂, 5 ♀), *Cotesia* sp. (1 ♂, 21 ♀), and *Chelonus* (*Chelonus*) *annulipes* Wesmael, 1835 (7 ♂, 11 ♀), the ichneumonids *Sinophorus* nr. *xanthostomus* Gravenhorst, 1829 (4 ♀), *Anomalon* sp. (1 ♂, 2 ♀), *Diadegma pulchripes* Kokujev 1915 (1 ♂, 3 ♀), and *Ectomyelois* sp. (2 ♂, 1 ♀), the eulophid *Euplectrus* sp. (8 ♂, 24 ♀), two unknown *Drino* spp. (1 ♂, 1 ♀), and *Exorista* sp. (1 ♂, 1 ♀) (Tachindae).

Meteorus rubens and *Cotesia* sp. are gregarious larval endoparasitoids, *Microplitis* sp. is a solitary larval endoparasitoid, and *Euplectrus* sp. is a gregarious ectoparasitoid of the larvae. *Sinophorus* nr. *xanthostomus*, *Anomalon* sp., *Diadegma pulchripes* and *Ectomyelois* sp., and *Chelonus* (*Chelonus*) *annulipes, Drino* sp. and *Exorista* sp. are endoparasitoids.

Virus

The virus-infected larvae of *S. exigua* were characterized with changed color and shape (Fig. 1); they were swollen and shiny with negative geotropism. Dissection of the infected larvae showed that their body tissues were liquefied with a very thin cuticle. Due to cuticle rupture, the larval hemolymphs spread on the leaf and the occlusion bodies were released in the emerged liquid, which accelerated horizontal transfer of the pathogen. The results indicated a high rate of vertical transmission, estimated at almost 100%; all hatched eggs died in less than five days. The results of the preliminary bioassay demonstrated that the virus strain was highly virulent. This confirmed the high potential of the SeMNPV YV isolate to transfer vertically (data not shown).

Fig. 1. Comparison of healthy (left) and MNPV infected larva (right) of *Spodoptera exigua*. Healthy larvae showing greenish color and being able to chew hole in sugar beet leaf. The infected larva showing yellowish color.

SEM

Scanning Electron Microscopy images of the virus particles indicated that OBs were polyhedral and irregular in shape and their size was 1-1.375 μm in diameter (average: 1.21 ± 0.05 μm, n=10) (Fig. 2).

TEM

Cross-sections from the OB of the virus-infected larvae of *Spodoptera exigua* indicated the virion contained multiple nucleocapsids arranged randomly within the occlusion matrix (Fig. 3).

Molecular characterization

The partial sequence of the *lef*-8 gene for the Iranian strain (accession number KT956220) was 724 nucleotides in length. NBLAST analysis based on the *lef*-8 sequence gene attributed 98% similarities of the Iranian virus strain with *Spodoptera exigua multiple nucleopolyhedrovirus* (HT-SeG26). The multiple alignment of this gene for 8 taxa indicated that 714 sites were conserved among 724 bps; therefore, 10 sites were variable and a single site was parsimony-informative. The reconstructed phylogenetic tree based on the *lef*-8 sequence, using the neighbor-joining method, showed that the Iranian strain was placed in a unique clade, namely III, while HT-SeG25, HT-SeSp2A, VT-SeA12, HT-SeG26, VT-SeAl1, VT-SeOx4, and HT-SeG24 strains were placed in two sub-

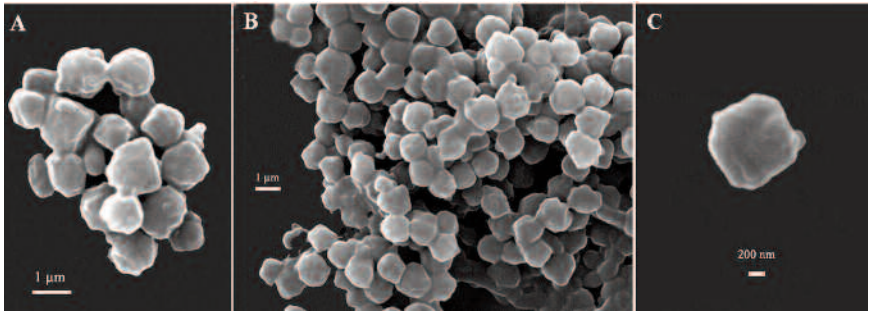

Fig. 2. Scanning Electron Micrographs of polyhedral OBs extracted from infected larvae of *Spodoptera exigua* with a baculovirus (A, Magnification = 30000; B, Magnification = 20000; and C, Magnification = 50000)

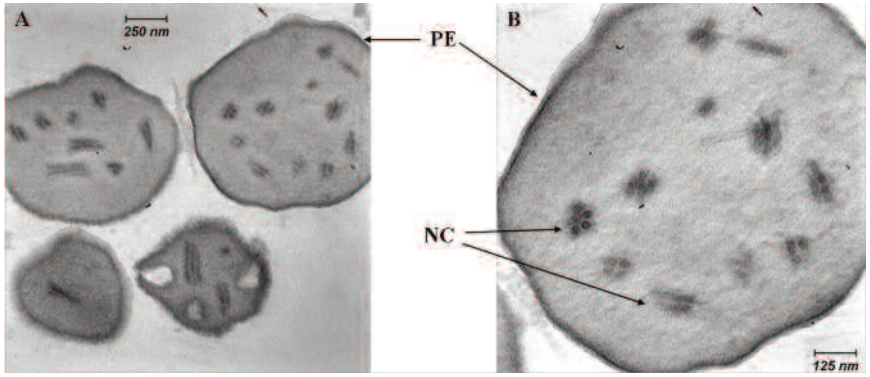

Fig. 3. Transmission electron micrographs from cross section of OBs obtained from the infected larvae of *Spodoptera exigua* by a baculovirus (NC, nucleocapsids; PE, polyhedral envelope).

clades as clades I and II (Fig. 4-A). Multiple alignments of the *lef*-8 gene showed six single nucleotides inserted in the Iranian strain from site 141 to site 146 (ACGACG). When this insertion was compared within the complete genome sequence of the HT-SeG25, HT-SeSp2A, VT-SeA12, HT-SeG26, VT-SeAl1, VT-SeOx4, and HT-SeG24 strains, it was contributed from site 109476 to 109482 nucleotides (data not shown). Mean interspecific distance of *lef*-8 sequences was 0.004 (range 0.00–0.015), which was calculated according to the K2P model. There were differences of 0.014 and 0.015 nucleotides between the Iranian isolate and subclades 2 and 1, respectively.

The partial sequence of the *polyhedrin* gene of the virus isolate (405 bps in length) was submitted to GenBank (accession number KT956221). NBLAST analysis based on the *polyhedrin* sequence for the Iranian strain attributed 100% of similarities with *Spodoptera exigua multiple nucleopolyhedrovirus* (HT-SeG26 strain; accession number HG425348). Multiple alignment of a 405-bps segment of this gene for eight taxa indicated that 406 sites were conserved, one site was variable, and one site was parsimony-informative. The reconstructed phylogenetic tree based on the *polyhedrin* sequence using the neighbor-joining method showed that the Iranian strain was placed in a single clade with VT-SeA12, HT-SeG24, HT-SeG26, VT-SeOx4, and VT-SeAl1 strains (clade II) and two strains, HT-SeG25 and HT-SeSp2A, were placed in another clade (clade I) (Fig. 4-B).

Fig. 4. Phylogenetic relationships between Iranian (▲) and other strain of *Spodoptera exigua multiple nucleopolyhedrovirus* by neighbor joining method (A) *lef*-8 gene, (B) *polyhedrin* gene.

The mean interspecific distance of the *polyhedrin* gene sequences between eight strains was 0.001 (range 0.00–0.002), calculated according to the K2P model. There was no intra-nucleotide difference between the Iranian strain and VT-SeA12, HT-SeG24, HT-SeG26, or VT-SeOx4 strains, while 0.001 difference was observed between the Iranian strain and three others, namely VT-SeAl1, HT-SeG25, and HT-SeSp2A.

Entomopathogenic fungi

An isolate of *Beauveria varroae* (Rehner and Humber, 2005) was isolated from the *S. exigua* larvae. The fungus was isolated and characterized using molecular techniques and morphological methods. This is the first record of it as a natural pathogen on lepidopterans and a new record for Iranian mycobiota.

Morphological identification

The fungal colony on the PDA was 25–29 mm in diameter after 10 days of incubation at 28ºC. The conidia aggregated as a white mass. The hyphae were 1-2 µm in width. The wall was smooth and branched. The conidia were globose, subglobose, or broadly ellipsoid in shape, 1.62 ± 0.09 and 1.93 ± 0.07 µm in height and width, respectively (Fig. 5).

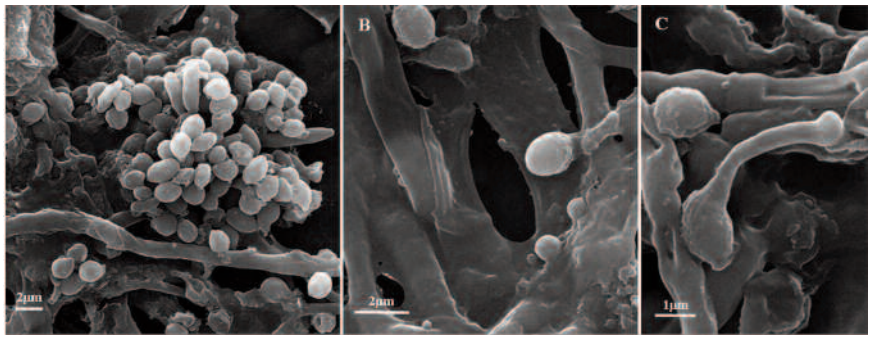

Fig. 5. Scanning Electron Micrographs of spore of *Beauveria varroae.* A) arrangement of conidia on conidiogenous cell, B) conidia, C) appressorium and germ tube.

Molecular identification

The partial sequence of the ITS region for the Iranian isolate of *B. varroae* (550 bps) was submitted to GenBank (accession number KU058600). NBLAST analysis attributed 99% of similarity of the Iranian isolate with the French isolate of *B. varroae* (strain ARSEF 8259, HQ880801). The multiple alignment of a 446-bps segment of the ITS gene for 20 taxa indicated that 402 sites were conserved, 44 sites were variable, and 37 sites were parsimony-informative. The phylogenetic tree reconstructed based on DNA sequences of this gene using the neighbor-joining method indicated that the Iranian isolate was placed in a single clade with other isolates of *B. varroae* with 99% bootstraps (Fig. 6). The

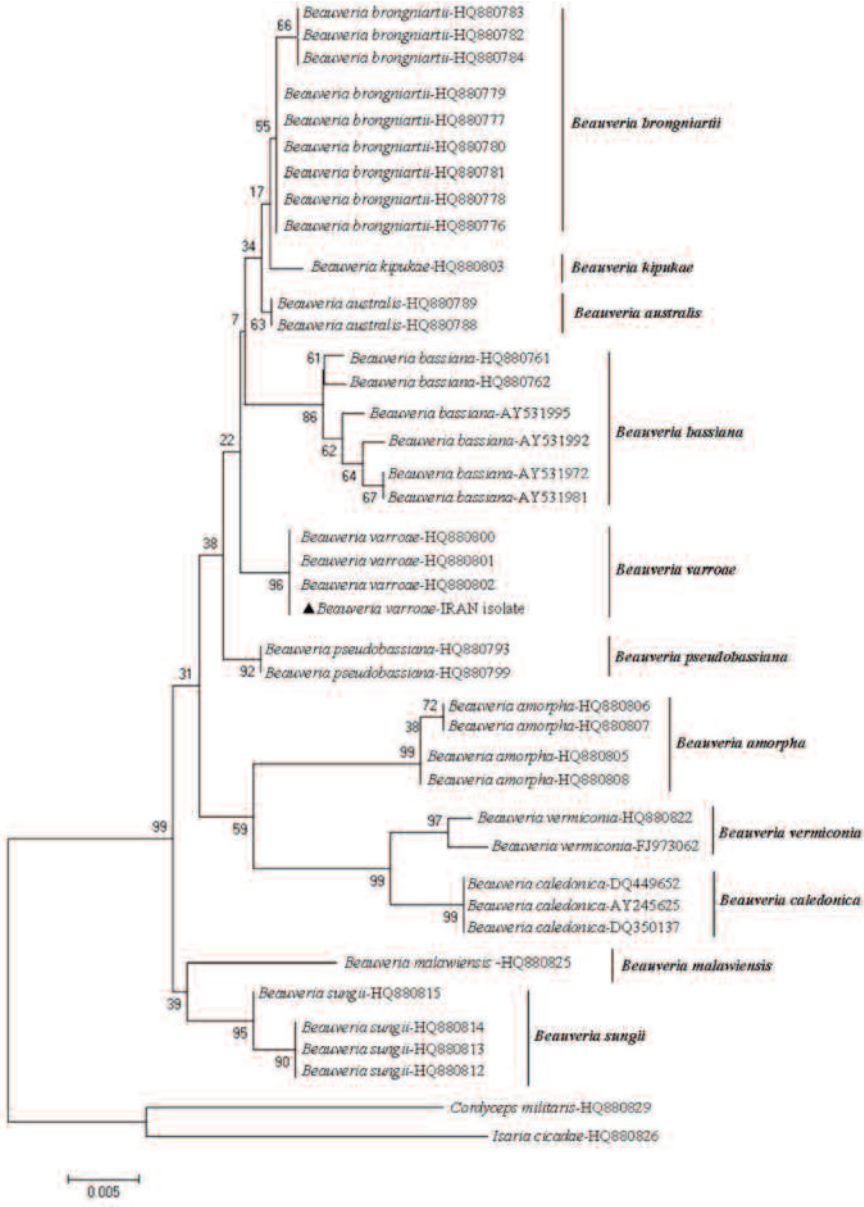

Fig. 6. Phylogenetic relationships between Iranian (▲) and other isolates of *Beauveria* genus by neighbor joining method based on ITS rDNA sequences data. *Isaria cicadae* and *Cordyceps militaris* were used as outgroups.

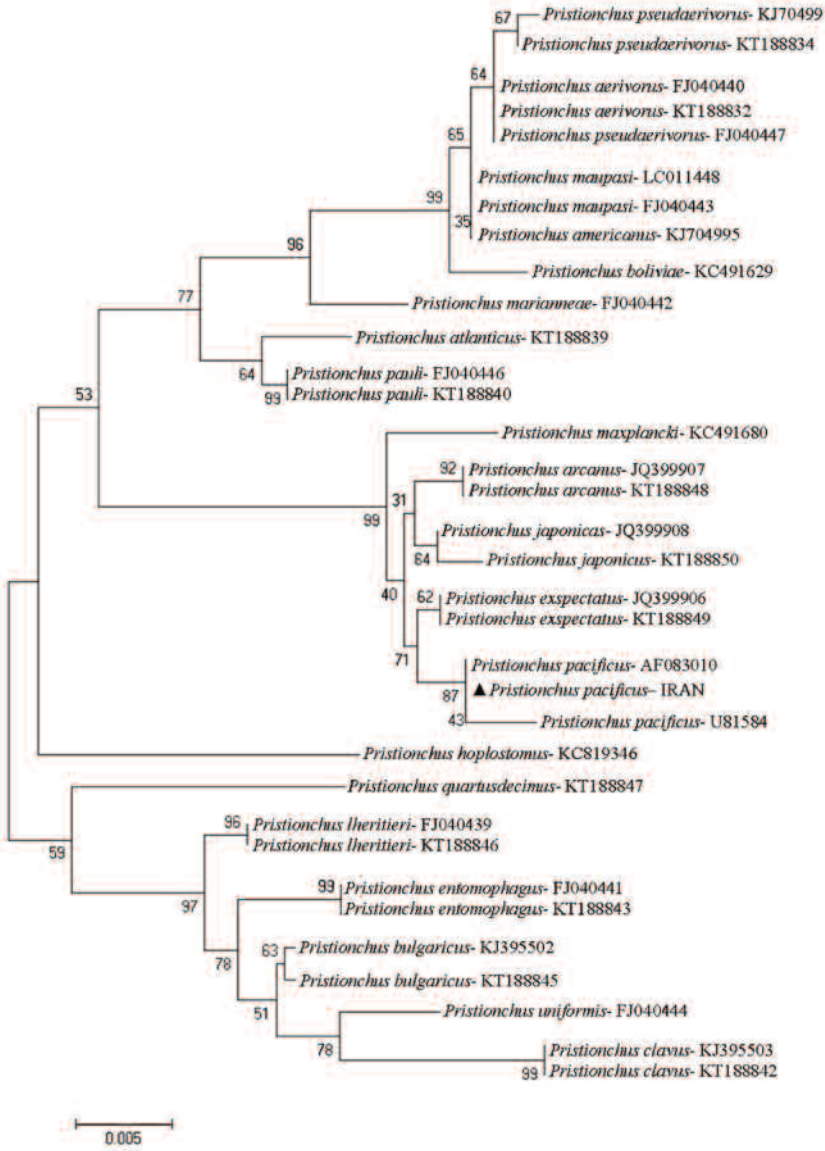

Fig. 7. Phylogenetic relationships between Iranian (▲) and other isolates of *Pristionchus* genus by neighbor joining method based on ITS rDNA sequences data.

mean interspecific distance between ITS DNA sequences of the analyzed samples was 0.017 (range 0.00–0.043) as calculated using the K2P model. There were no intranucleotide differences between the Iranian isolate and other *B. varroae* isolates. The *B. bassiana* clade was a sister group with *B. varroae*.

Entomophilic nematode

Spodoptera exigua larvae were dissected and four were found to be infected in the hemolymph with different life stages of a nematode. For identification, a fragment 860 bps in length of 18S gene was sequenced and submitted to GenBank (accession number 1914868). NBLAST analysis matched with 99% similarity to *Pristionchus pacificus* Sommer, Carta, Kim, Sternberg, 1996 (Rhabditida: Neodiplogasteridae). The Iranian isolate with another isolate *of P. pacificus* was placed in one subclade with 87% bootstraps, and the interspecific distance was 0-0.006 (Fig. 7).

DISCUSSION

Spodoptera exigua is an important destructive pest of the sugar beet in Iran, and in the last decade the population density of this species has increased markedly (Khanjani, 2005). The use of chemical insecticides for the control of lepidopterans has resulted in the development of resistance in several species of *Spodoptera* (Caccia et al., 2013) and thus, alternative methods such as biocontrol could be considered in an integrated management program for this pest.

Parasitoids and pathogens have been reported as natural biocontrol agents on *Spodoptera* species that include Braconidae, Ichneumonidae, Eulophidae, and Tachinidae (Rios-Velasco et al., 2011), Baculovirus (Escribano et al., 1999; Smits et al., 1988), fungus (Malarvannan et al., 2010; Asi et al., 2013), *Bacillus thuringiensis* (Romeis et al., 2006), *Heterorhabditis* spp. and *Steinernema* spp. (Caccia et al., 2013). In the current study, three entomopathogens, multiple nucleopolyhedrovirus (SeMNPV), *B. varroae,* and *P. pacificus,* were isolated from *S. exigua* larvae.

Previously, several species of parasitoid wasps including *Euplectrus flavipes* (Eulopidae), *Cuyptus inculcator* L. (Ichneumonidae), *Barylyapa* sp., *Chleonus inanitus* L. (Braconidae) and *Meteorus meseoptunicus* F. (Braconidae) and SeMNVP have been reported from Iran for *S. exigua* larvae (Khanjani, 2005).

Baculovirus has the potential to be a biocontrol agent of lepidopteran pests because of its high pathogenicity, environmental safety, and developed mass rearing methods. This virus could be effectively used as a potential biocontrol agent of *S. exigua* larvae in the field and in greenhouses (Caballero et al., 1992). The virus has been reported in different regions, including North America (Hunter and Hall, 1968), the Netherlands (Vlak et al., 1981), Spain (Caballero et al., 1992), and China, Japan, India, and New Zealand (Rao et al., 1992). Narayanan (1985) reported the occurrence of a granulosis virus in dead *S. litura* larvae.

SeMNPV belongs to the II clade of baculovirus and is specific to *S. exigua* (Gelernter and Federici, 1986; Vlak et al., 1981; Simon et al., 2004; Berretta et al., 2006). The complete genome sequences of this virus provide the entire genome sequencing of SeMNPV in seven populations from Europe (VT-SeAL1, VT-SeAL2, VT-SeOx4, HT-SeG24, HT-SeG25, HT-SeG26 and HT-SeSP2) (Theze et al*.,* 2014). Among them, two isolates, HT-SeG25 and HT-SeG26, had horizontal transmission, and VT-SeAL1, VT-SeAL2, and VT-SeOx4 transferred vertically.

The Iranian isolate of SeMNPV (YV isolate) transfers both vertically and horizontally, which explains why it displayed high virulence. The intra- and inter-transmission of the baculovirus among individuals of a population is essential to understanding the biology and virulence of the virus (Virto et al., 2014). When the horizontal and vertical transmission rate was increased, the efficacy of the pathogen as a biocontrol agent increased (Vega and Kaya, 2012). Previously, vertical transmission of the SeMNPV was reported by Bianchi et al. **(**2001) and Smits et al. (1988).

Beauveria varroae was identified as a natural pathogen of *S. exigua*. Previously, *B. varroae* was isolated from *Varroa destructor* (Acari: Varroidae) in France and was subsequently identified as a new pathogen of *Larinus* sp. (Coleoptera: Curculionidae) in Switzerland (Rehner et al., 2011). The present authors believe this is the first record of *B. varroae* occurring as an entomopathogen of a lepidopteran. Species delimitation of the *Beauveria* genus based on morphological characters is challenging. Distinguishing between *Beauveria bassiana* Balsamo 1835 and *B. australis* Rehner and R.A. Humber 1988, *B. kipukae* Rehner and Humber 2002, *B. pseudobassiana* Rehner and Humber 1991 and *B. varroae* has been achieved using molecular taxonomic techniques (Rehner et al., 2011).

Beauveria varroae produces globose/subglobose/broadly ellipsoid conidia that are the same as *B. australis*, *B. kipukae*, *B. pseudobassiana,* and *B. bassiana*, and the identification of this species is not possible using morphological characteristics (Rehner et al., 2011). Molecular analyses using DNA sequences of the ITS gene have resolved the taxonomy of this species complex.

Several previous studies recovered entomopathogenic fungus from *Spodoptera*. Zaz and Kushwaha (1983) reported *Beauveria bassiana* on *Spodoptera litura* Fabricius. *Metarhizium rileyi* Farlow, *Beauveria bassiana*, *Nomuraea rileyi* Farlow, and *nucleopolyhedrovirus* was isolated from *S. frugiperda* in Mexico (Ordóñez-García et al., 2015). *Spodoptera litura* was introduced as a host of *Metarhizium anisopliae* Metchnikoff and *Micrococcus* sp. (Kore and Bhide, 1978; Zaz and Kushwaha, 1983).

In this study, an entomophilous nematode, *P. pacificus,* was recovered from *S. exigua*. Previously, *S. litura* was recorded as a host of other entomopathogenic nematodes including *Steinernema feltiae* and mermitid nematodes, *Ovomermis albicans* Siebold 1842, *Hexamermis* sp., and *Pentatomermis* sp. More-

over, infection of *S. litura* larvae by *Steinernema* sp. was reported in Japan (Kondo and Ishibashi, 1984; Bhatnagar et al., 1985).

In the current study, parasitic species of Braconidae, Icheneumonidae, Eulopidae, and Tachinidae were the most prevalent parasitoids of *Spodoptera*. Rao et al. (1992) identified 71 species of Hymenoptera and Diptera parasitoids on *S. litura* (in India), *Apanteles colemani* Viereck 1912, *Apanteles plutellae* Kurd 1912, *Bracon brevicornis* Wesmael 1838, *Microplitis demolitor* Wilkinson 1934, *Microplitis pallidipes* Szepligeti 1902, (Braconidae), and *Diadegma* sp., *Charops obtusus* Morley 1913, and *Ichneumon* sp., (Ichneumonidae), and *Actia nigritula* Malloch 1930 and *Peribaea orbata* Wideman (Tachinidae) as parasitoids of larvae and *Brachymeria* sp. (Chalcididae), and *Sarcophaga albiceps* Meigen 1826 (Sarcophagidae) as pupal parasitoids on *S. litura*.

Gabriela Murua et al. (2009) identified parasitoids of *S. frugiperda* Smith 1797 larvae in Argentina, including *Campoletis grioti* Blanchard 1946 (Ichneumonidae), *Chelonus insularis* Cresson 1865 (Braconidae), *Archytas marmoratus* Townsend 1915, *Incamyia chilensis* Aldrich 1928 (Tachinidae), and *Euplectrus platyhypenae* Howard 1885 (Eulophidae).

Ordóñez-García et al. (2015) investigated the natural enemies of *S. frugiperda* in Chihuahua, Mexico and found *Chelonus insularis* Cresson 1865 and *Meteorus arizonensis* Muesebeck 1923 (Braconidae); *Campoletis sonorensis* Cameron 1886, *C.s flavicincta* Ashmead 1980, *Pristomerus* sp. (Ichneumonidae); *Euplectrus platyhypenae* Howard 1885 (Eulophidae); and *Lespesia* sp. and *Archytas marmoratus* Townsend 1915 (Tachinidae).

Species of Formicidae, Carabidae, Chrysoplidae, Miridae, and Tettigonidae were introduced as predators of *S. exigua* (Rao et al. 1992). *Serratia marcescens,* an entomophatogenic bacterium, was reported on *Helicoverpa armigera* Hübner 1808 and *S. litura* in India (Ansari et al., 1987). The Microsporidia *Nosema carpocapse* Paillot 1938 was found on *S. litura* larvae in New Zealand, India, Japan, and China (Rao et al., 1992).

The comparison of the current results with similar surveys done in other regions showed a similar diversity of natural enemies on *Spodoptera* larvae and pupae that hold potential as biocontrol agents for the integrated management of this pest. The YVMNPV seems to have the best potential for biocontrol of *S. exigua* if it can be produced in large quantities for field dissemination.

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