A new record of insect pathogen on larva of Rosaceous longhorn beetle, *Osphranteria coerulescens* (Coleoptera: Cerambycidae)

Mehrdad Mohammadyani[1], Javad Karimi[1*], Gholam Hossein Moravej[2]

1. Biocontrol and Insect Pathology Laboratory, Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.
2. Plant Protection Department, Ferdowsi University of Mashhad, Mashhad, Khorasan Razavi, Iran.

ABSTRACT

The Rosaceous longhorn beetle (RLHB), *Osphranteria coerulescens* Redtenbacher (Coleoptera: Cerambycidae) is a serious wood borer insect that commonly occurs on fruit trees in Iran. During an ongoing project on natural enemies of *O. coerulescens* in Mashhad region, North East of Iran, two isolates of entomopathogenic fungi (EPF) were isolated as natural pathogens on the larvae. The initial species identity of the isolated EPFs was determined based on classic methods. For the molecular, ITS gene sequences were used for inferring phylogenetic relationship. The results confirmed the species identity as *Beauveria bassiana sensu lato* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) and *Metarhizium anisopliae sensu lato* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae). This is the first report about natural incidence of fungal infection on the *O. coerulescens*.

Key words: *Beauveria bassiana;* Entomopathogenic fungus; Insect pathology; Iran; ITS sequence; *Metarhizium anisopliae; Osphranteria coerulescens.*

Introduction

The Rosaceous longhorn beetle (RLHB), *Osphranteria coerulescens* Redtenbacher (Coleoptera: Cerambycidae) is a serious wood borer treat that commonly occurs on fruit trees of family Rosaceae such as apricot, cherry, peach, plum, and other stone fruits (Esmaeili, 1983). The beetle populations can reach to high density in the cold regions due to the insect's reproductive capacity and tolerance of cold weather for long time (Behdad, 1974). Cryptic habitat of the destructive stage of the pest caused the insect past has propelled it as a serious threat to their host trees.

However, there are several species of natural enemies with various efficacy degrees on different life stages including larva and egg (Rajabi, 1967). Among insect pathogens, while there is a scare report about efficacy of this group on the immature stage of the pest, there is not any record of natural occurrence of insect pathogens among the pest populations. Due to cryptic habitat of destructive stage of wood borer insect, the insect pathogens that could persist and apply in those niches are desirable (Hajek and Bauer, 2007).

The entomopatogenic fungi, especially Hypocreales, have been often used against numerous soil insect populations in biological control program (Keller and Zimmerman, 1989). They are cosmopolitan as a ubiquitous group of natural enemies of arthropods that exhibit a great diversity worldwide (Reineke *et al*., 2013). They can be isolated and developed relatively easily in affordable and available media, since because they are facultative pathogens (Goettel and Inglis, 1997; Inglis *et al*., 2012).

Among the entomopathogenic fungi, which have been developed in the natural regulation of insect pests, the anamorphic taxa *Beauveria bassiana sensu lato* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) and *Metarhizium*

* Corresponding author E-mail: jkb@ferdowsi.um.ac.ir

anisopliae sensu lato (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) are most widely studied taxa (Castrillo *et al*., 2008; Driver *et al*., 2000; Jackson *et al*., 2010; Jaronski, 2009). Both fungal species play a unique role in microbial control with highly virulence on insect pests (Chandler *et al*., 1996; Goettel *et al*., 2000; Lin *et al*., 2011).

The isolation and characterization of native entomopathogenic fungi from different components agroecosystem is essential for gaining understanding of fungal biodiversity in different regions (Shin *et al*., 2013). Furthermore, awareness of distribution and abundance of entomopathogenic fungi provide optimal conditions for their use in biological control program as pest control agents. So, the objective of this study was to isolate and identify native isolates of entomopathogenic fungi as natural pathogens from *O. coerulescens* larvae.

Materials and methods
Sampling for detection of infected larvae
For detection of entomopatogenic fungi, the larvae of *O.coerulescens* were collected during 2014- 2015 from Astan Qods orchards, Mashhad, Khorasan-e-Razavi province, Iran. For this purpose, the infected larvae were collected from galleries and placed individually in sterilized glass Petri dishes for sporulation at control conditions [25 ± 2˚C, 60% ± 5% RH with a photoperiod of 16:8 h (L: D)]. After one week, the cadavers were surface sterilized by 70% ethanol (30 sec each) and placed in sterilized glass Petri dishes to satisfy Koch's postulate (see Kumar *et al*., 2015). Those larvae washed and incubated were transferred to Petri dishes containing containing moist cotton and Whatman No. 1 filter

paper for sporulation of the fungi in control condition (described above). The Petri dishes checked daily for presence of fungal growth on dead larvae, usually two weeks (White, 1927).

Isolation and purification of entomopathogenic fungi
Detection of fungal pathogens was conducted by isolation of entomopathogenic fungi from sporulated larvae. Each cadaver was washed and surface sterilized by 3% sodium hypochlorite solution (1 min each). Those cadavers were removed and cultured onto Petri dishes containing Potato Dextrose Agar plus 1% peptone (PPDA). All Petri dishes were incubated in control condition described above.

Morphological Characterization
The morphological identification of entomopathogenic fungi was carried out based on description method of Humber (2012b). Various futures of putative entomopathogenic fungi such as macro and microscopy type were investigated. In the macroscopic type, it was focused on fungal conidium color, shape and mode of growth in culture medium. For microscopic futures, each isolate mounted on the glass slide, stained by Methylene blue and covered. The isolates were examined with microscope (Olympus) attached with a digital camera and photographs of conidia and conidiophores were taken. Different morphometric characters were recorded and used for possible position of the entomopathogenic fungi according to Humber's key (Humber, 2012 b).

Molecular Characterization
DNA extraction
For DNA extraction, each isolate was

cultured onto PDA in 9 cm Petri dishe and incubated at controlled conditions (described above). The liquid media containing PDB (200 g potato and 10 g dextrose in one l water plus 0.035 g streptomycin) were distributed in Erlenmeyer flask (250 ml capacity) and then inoculated with fungal isolates selected above. All inoculated Erlenmeyer flasks were placed on a shaker at 120 r-p-m and 25˚C until growth of mycelium, usually for 10 days. The mycelium was harvested, rinsed with sterile distilled water and overnight at on sterile whatman No. 1 filter paper under sterile microbiological hood. Dry mycelia were transferred into a sterile 2 ml Eppendorf tube and stored in 4˚C until required. DNA was extracted following the technique of Perez-González *et al*. (2014) with slight modifications. Briefly, the Eppendorf tubes were left in liquid nitrogen for five minutes and frozen mycelium were crushed using a pellet pestle rod. The QIAGEN DNeasy® kit (Venlo, the Netherlands) were used for DNA extraction following the manufacturer's instructions and extracted DNA was stored at -18˚C until use.

PCR and Molecular sequencing
The ITS region from both isolates were PCR amplified using universal primer with the following pair of primers: ITS4 (5´ - TCCTCCGCTTATTGATATGC-3´) and ITS5 (5´ - GGAAGTAAAAGTCGTAACAAGG-3´) (White *et al.*, 1990). The PCR reaction mixture consist of 200 mmole for each dNTP, 2.5 units DNA polymerase, 5 ml 10X polymerase buffer, and sterile Millipure water to 50 ml. In control tube containing all above material and sterile distilled water without DNA were used in the experiment. PCR amplification were carried out using a Biometra thermal cycler with a program comprising an initial denaturation on for 2 min at 94˚C followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, extension step at 72 °C for 1 min and secondary extension at 72 °C for 10 min as final step of PCR amplification. For visualization of PCR products, electrophoresed was done with 3.0% agarose gel in TBE buffer and subsequently the gels stained with Green viewer (SYBR). For the direct sequencing of double strand all PCR products were sent to the Macrogen sequencing service facilities (Macrogen Inc., Seoul, Korea). The sequences chromatograms were assemble using Bioedit software (Hall, 1999). Then, DNA sequences were placed in BLAST (Basic Local Alignment Search Tool) from NCBI and sequences of other fungi were retrieved from Genbank. The other sequences deposited in GenBank that had significant homology compared with multiple sequences using the nBLAST. The Neighbor-joining method was performed for reconstruction of phylogenic tree using Molecular Evolutionary Genetic Analysis (MEGA5) software (Tamura *et al*., 2011). Multiple alignments were performed by Clustal W software and bootstrapping analysis was carried out with 1000 replicates sampling of the data.

Results
Morphological Characterization
The morphological identification of isolates was first carried out based on macroscopic features; color of mycelium for each isolates was initially white in mass but changed gradually to green for *Metarhizium anisopliae* after

sporulation (Fig. 1a.). In the microscopic future focused on mean conidia size following the technique of Hamber (2012 b). Briefly, the mean conidia size from 20 slides of each isolates were examined which for *Beauveria* isolates was 3.5 ± 0.25 μm in length and 2.35 ± 0.14 μm in 250 width. Also, other diagnostic microscopic characters for *Beauveria* isolates such as conidiogenous were examined. The conidiogenous of *Beauveria* found usually densely clustered with swollen bases and typical dense of conidia was ball shaped (Fig. 2a. and Fig. 2b.). For the diagnostic characters of *Metarhizium* isolates mean conidia size was 8.35 ± 0.05 μm in length and 2.89 ± 0.19 μm in width. The conidia produced long parallel chain which showing side-by-side patterns and often adhere to chain, laterally (Fig. 1b.). Furthermore, the *Metarhizium* isolates were sent confirmed by Prof R. Zare (Iranian Research Institute of Plant Protection).
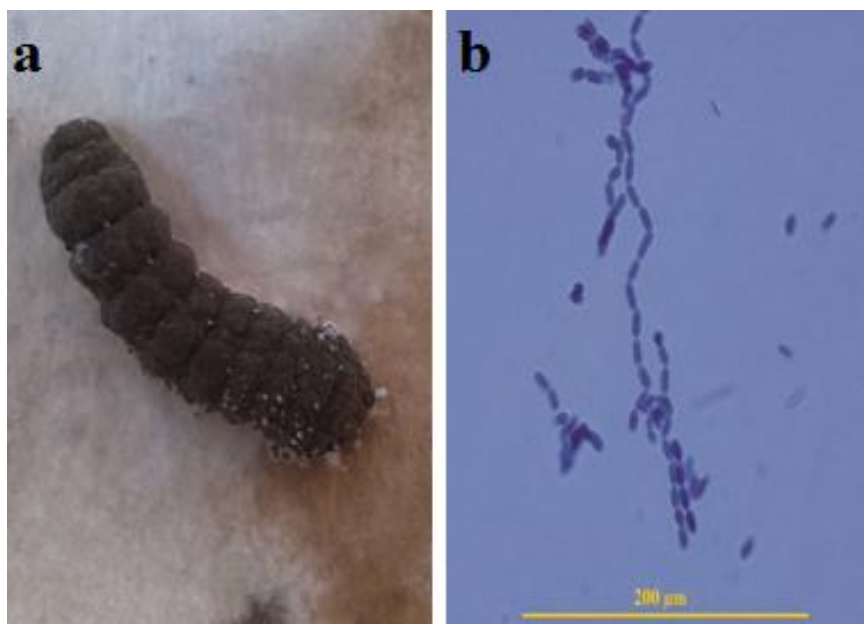

Figure 1. Macroscopic (a) and microscopic (b) morphology feature of *Metarhizium anisopliae*. Colony (a), and conidia (b) of *M. anisopliae* grown on cadaver of *Osphranteria coerulescens* larvae.

Phylogenetic Analysis

Genetic analysis of the isolated fungi was conducted using internal transcribed spacer region (ITS) including ITS4 and ITS5 and for both isolated species the phylogenetic tree were reconstruct by ITS sequences. The ITS sequence data generated for *B. bassiana* and *M. anisopliae* isolates were deposited in GenBank with accession numbers KP213287 and KP213288, respectively. Both isolated species had 550 bps length of ITS, approximately. The result of BLAST search for ITS sequences indicated that most similar Genbank sequences have highest similarity to *B. bassiana* (FUM 01) (Fig. 4) and phylogenetic relation of *B. bassiana* was confirmed by genetic analysis. Also, the ITS Gene-based phylogenetic tree obtained by neighbor joining method revealed two major groups (Fig. 4). In the *Metarhizium* (FUM 02) phylogenic analysis, BLAST analysis based on ITS showed 99% similarity with Genbank sequences (Fig. 3). Also, the ITS Gene-based phylogenetic tree obtained by neighbor joining method revealed two major groups (Fig. 3). These species

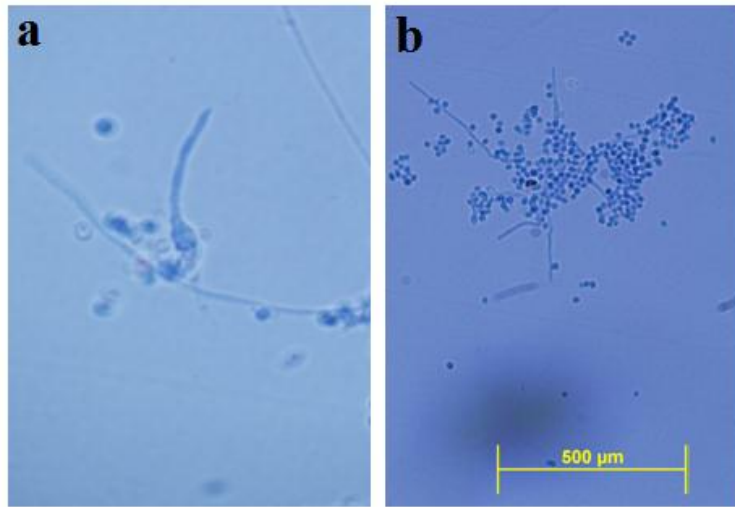indicated 99% bootstrap support for the inclusion in similar clade within phylogenetic analyses (Fig. 3).

Figure 2 The microscopic morphology feature (a,b) of *Beauveria bassiana*. Conidiogenous (a), and conidia (b) of *B. bassiana* grown in PDA medium.
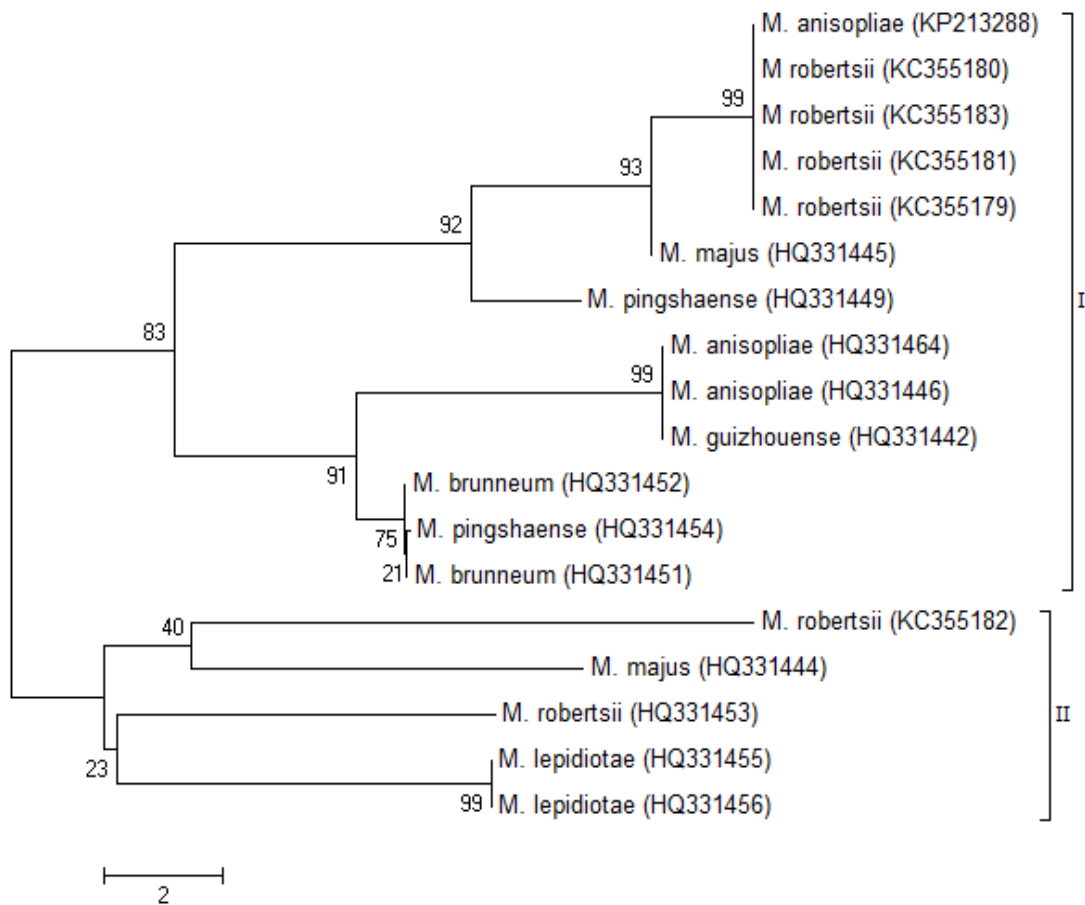

Figure 3 Phylogenetic relationships *Metarhizium*, based on ITS sequences of *Metarhizium* isolates using neighbor-joining method. The reliability of fungal species in phylogenetic tree showed by bootstrap analysis with 10,000 replicates in nodes of tree.

Figure 4. Phylogenetic relationships *Beauveria*, based on ITS sequences of *Beauveria* isolates using neighbor-joining method. The reliability of fungal species in phylogenetic tree showed by bootstrap analysis with 10,000 replicates in nodes of tree.
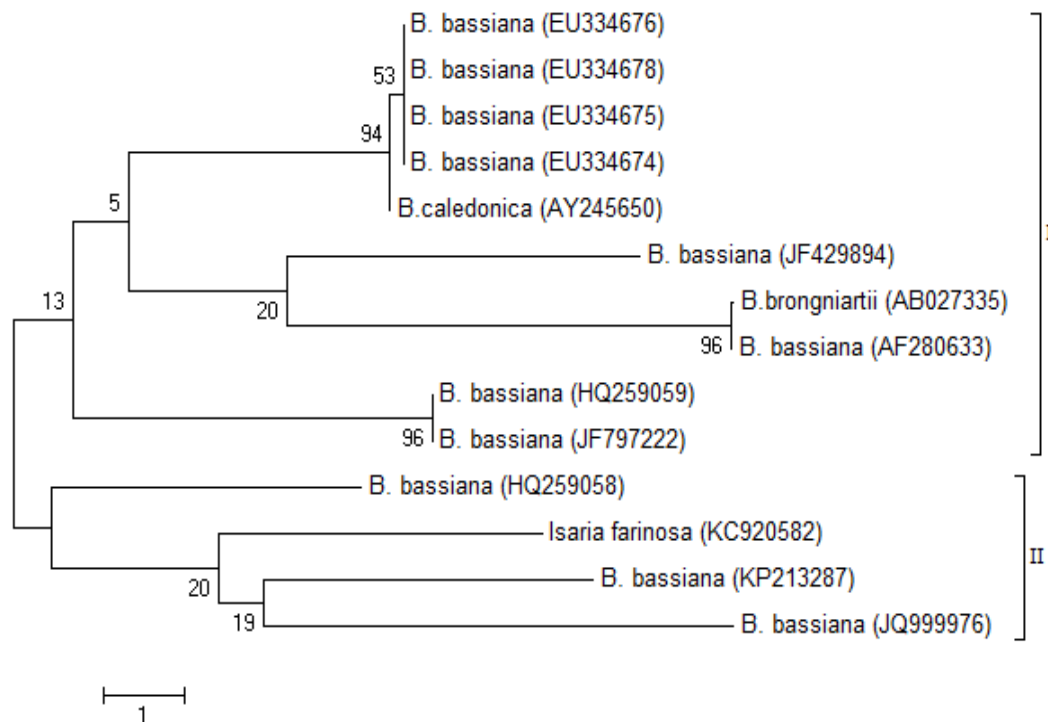
Discussion

This study revealed that the crop environments pose as best agroecosystem harbor for diverse entomopatogenic soil fungi community. Steinwender *et al.* (2014) stated the entomopathogenic fungi harbored in various environments and their habited as well as diversity of entomopathogenic *Metarhizium* community. Recently, our survey on natural enemies of *O. coerulescens* indicated occurrence of two isolates of entomopathogenic fungi on the pest larvae in sequential sampling of fruit orchards in Mashhad, Razavi Khorasan province, North Eastern Iran (36˚4′ N, 59˚7′ E). The genetic analysis of ITS region revealed the presence of two entomopatogenic fungi, *B. bassiana* and *M. anisopliae*, that being the most abundant. Pérez-González *et al.* (2014) stated genus *Beauveria* can survive in different hosts, while the other genus of

entomopatogenic fungus is being specialized to specific hosts, also in our sampling in the case of *O. coerulescens* we found that *Beauveria* genus was abundant compared to *Metarhizium*. In recent years, several researches lead to report the species belonging to *Beauveria* and *Metarhizium* indigenous to different conditions in several countries (Bidochka *et al.*, 1998; Keller *et al.*, 2003; Meyling and Eilenberg 2007; Quesada-Moraga *et al.*, 2007). The *B. bassiana* and *M. anisopliae* are two most commonly species made known in many countries; introduced them as best biocontrol agents for their use (Shin *et al.*, 2013; Patricia *et al.*, 2008). Using morphological characteristics in distinguishing of entomopathogenic species exclusively is not sufficient (Driver *et al.*, 2000) Most researchers concluded that combined molecular approaches with morphological characters could be

arranged to provide new insight into characterization of entomopatogenic fungus (Driver *et al*., 2000). In present work, Both isolates were identified initially based on microscopic and macroscopic futures following described method by Humber (Humber, 2012 b). Riba *et al*. (1986) compared mean of conidia size for *M. anisopliae* identification and relationship with their virulence against European corn borer. Pérez-González *et al*. (2014) also investigated specific diversity of the entomopathogenic fungi using morphological method that described by Humber (Humber, 2012 b). However, applying classical and morphological methods due to the lack of consistent and definite morphological characteristics for identification is not sufficient and should always be complemented with other methods (Borman *et al*., 2006). For this reason, using of molecular genetics techniques are essential to monitor of entomopathogens the environment (Oliveira *et al*., 2011). Furthermore, PCR-based techniques provide highly specific approach for identification of different targeted fungal species (Reineke *et al*., 2014). Our result based on ITS sequencing suggested that obtained species from *O. coerulescens* were closely related to other sequences obtained from GenBank. In the *Beauveria* phylogenetic analysis, our results identified confidently at the species level but in *Metarhizium* analysis identification of species was not clear. Based on the molecular sequencing data of *B. bassiana*, high similarity observed with other *Beauveria* strains in GenBank; nevertheless, it but can be distinguished through morphological future. In this study, *M. anisopliae* has high resemblance to *M. robertsii;*

however, it can be distinguished with some morphological characteristics such as conidia size and shape, conidiogenous cells (phialides) and other future (after Prof. R. Zare). Aquino-Demuro *et al*., 2004 investigated molecular characterization of *Beauveria bassiana* from Sunn pests and based on ITS sequencing showed isolated species were very closely related. The present study demonstrates that natural entomopatogenic fungi are occurred in the cryptic environments of fruit orchard. Meyling *et al*. (2009) state *B. bassiana* are frequently isolated from agricultural soil while several other fungus species distributed in soil from orchard. Furthermore, Steinwender *et al*. (2014) found that *Metarhizium* spp. play important role in infecting of insect in the below-ground environment, while *Beauveria* spp. are often occurring above ground. In this study, both identified isolates were obtained from *O. coerulescens* larvae. The cryptic environment of the agroecosystem included many natural fungal isolates that play essential role as most attractive biological agents for IPM purposes around the world. Consider to diversity of native entomopathogenic fungi as natural occurrence of some insect is essential and providing a great potential in biological control sciences for pest management (Shin *et al*., 2013). Thus, it will be interesting to isolate and identify other strains of entomopatogenic fungi and compare their abilities such as virulence, germination or penetration. Also, assessment of synergistic interaction between the combined applications of entomopathogenic fungus with other soil pathogens is useful in future studies that lead to improve overall efficacy in the management of pests.

REFERENCES

Aquino Demuro, M., Elliotte, S., Moore, D., Parker, B., Skinner, M., Reid, W. and El bouhssini, M. 2004. Molecular characterisation of *Beauveria bassiana* isolates obtained from overwintering sites of Sunn Pests (Eurygaster and Aelia species). Mycological Research, 109: 294-306.

Behdad, E. 1984. Pests of fruit trees in Iran. Tehran, Iran, Plant Pests and Diseases Research   Institute, 254-259.

Bidochka, M. J. 2001. Monitoring the fate of biocontrol fungi. In Butt, T. M., Jackson, C. W. and   Magan, N. (Eds) *Fungi as biocontrol agents: Progress, problems and potential.*   Wallingford, UK: CABI Publishing. pp. 193-218.

Borman, A., Szekely, A., Campbell, C. and Johnson, E. 2006. Evaluation of the viability of   pathogenic filamentous fungi after prolonged storage in sterile water and review of recent   published   studies   on   storage   methods. Mycopathologia, 161: 361–368.

Castrillo, L. A., Ugine, T. A., Filotas, M. J., Sanderson, J. P., Vandenberg, J. D. and Wraight, S.   P. 2008. Molecular characterization and comparative virulence of *Beauveria bassiana*   isolates   (Ascomycota: Hypocreales) associated with   the greenhouse shorefly,   *Scatella tenuicosta* (Diptera: Ephydrididae). Biological Control, 45: 154-162.

Chandler, D., Hey, D. and Reid, A.P. 1996. Sampling and occurrence of entomopathogenic fungi   and nematodes in UK soils.   Applied   Soil Ecology, 5: 133-141.

Driver, F., Milner, R. J. and Trueman, J. W. H. 2000. A taxonomic revision of *Metarhizium* based on phylogenetic analysis of rDNA sequence data. Mycological Research, 104: 134–150.

Esmaeili, M. 1983. Important pests of fruit trees. Tehran, Iran, Sepehr Publishing Center, 176-   180.

Goettel, M. S. and Inglis, G. D. 1997. Fungi: Hyphomycetes. In . Lacey, L. (Eds) Manual of   techniques in insect pathology. San Diego, CA: Academic Press, pp. 213–249.

Goettel, M. S., Inglis, G. D. and Wraight, S.P. 2000. Fungi. In: Lacey, L. A. and Kaya, H. K.   (Eds) Field Manual of Techniques in Invertebrate Pathology. Kluwer Academic Press,   Dordrecht, the Netherlands, pp. 255–282.

Hall, T. A. 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Research,41: 95–98.

Humber, R. A. 2012b. Identification of entomopathogenic fungi. In Lacey L. A. (Eds) Manual of   Techniques in Invertebrate   Pathology.   Academic   Press, Amsterdam, the Netherlands, pp   151–187.

Inglis, G. D., Enkerli, J. and Goettel, M. S. 2012. Laboratory techniques used for entomopathogenic fungi: Hypocreales. In: Lacey, L. A. (Eds) Manual of Techniques in Invertebrate Pathology, 2 edition. Academic Press, Elsevier Science, pp. 18-53.

Jackson, M. A., Dunlap, C. A. and Jaronski, S. T. 2010. Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. BioControl, 55: 129–145.

Jaronski, S. 2009. Ecological factors in the inundative use of fungal Entomopathogens.   BioControl, 55:159–185.

Keller, S. and Zimmerman, G. 1989. Mycopathogens of soil insects. In: Wilding, N.,

Collins, N.   M., Hammond, P. M. and Webbe, J. F. (Eds) *Insect-Fungus Interactions. Academic Press, London, UK*, pp. 239-270.

Keller, S., Kessler, P. and Schweizer, C . 2003. Distribution of insect pathogenic soil fungi in Switezerland with special reference to *Beauveria brongniartii* and *Metarhizium   anisopliae*. Biocontrol, 48: 307–319.

Lin, L., wang, F. and Wei, D. 2011. Chlorimuron ethyl as a new selectable marker for disrupting   genes in the insect-pathogenic fungus *Metarhizium robertsii*. Journal of Microbiological   Methods, 87: 241–243.

Meyling, N. V. and Eilenberg, J. 2007. Ecology of the entomopathogenic *Beauveria bassiana*   and *Metarhizium anisopliae* in temperate agroecosystems: conservation biological control. Biological Control, 43: 145–155.

Meyling, N. V., Lubeck, M., Buckley, E. P., Eilenberg, J. and Rehner, S. A., 2009. Community   composition, host range and genetic structure of the fungal entomopathogen *Beauveria* in adjoining agricultural and seminatural habitats. Mycological Research, 18: 1282–1293.

Oliveira, I., Pereira, J. A., Bento, A. and Baptista, P. 2010. Viability of *Beauveria bassiana*   isolates after storage under   several   preservation   methods. Annals of Microbiology,   61:339-344

Patricia, H. S., Pedro, M. O. J. N., Talita, M. A., Daniele, S., Luis, F. A. A. & and, Fungaro, M.   H. P. 2008. Selection of *Beauveria   bassiana* isolates to control *Alphitobius diaperinus*.   Journal of Invertebrate Pathology, 97: 83–90.

Pérez-González, V. H., Guzmán-Franco, A. W., Alatorre-Rosas, R., Hernández-López, J.,   Hernández-López, A., Carrillo-Benítez, M. G. and Baverstock, J. 2014. Specific diversity   of the entomopathogenic fungi *Beauveria* and *Metarhizium*   in Mexican agricultural soils. Journal   of Invertebrate Pathology, 119: 54–61.

Quesada-Moraga, E., Navas-Cortés, J. A., Maranhao, E. A. A., Ortiz-Urquiza, A. and Santiago- Álvarez, C., 2007. Factors affecting the occurrence and distribution of entomopathogenic   fungi in natural and cultivated soils. Mycological Research, 111: 947– 966.

Reineke, A., Bischoff-Schaefer, M., Rondot, Y., Galidevara, S., Hirsch, J. and Uma Devi, K. 2014. Microsatellite markers to monitor a commercialized isolate of the   entomopathogenic fungus *Beauveria bassiana* in different environments: Technical   validation and first applications. Biological Control, 70: 1–8.

Riba, G., Bouvier-Fourcade, I. and Caudal, A. 1986. Isoenzymes polymorphism in *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) entomogenous fungi. Mycopathologia, 96: 161-169.

Senthil kumar, C. M., Jacob, T. K., Devasahayam, S., D silvia, S. and Krishna Kumar, N. K. 2015. Isolation and characterization of a *Lecanicillium psalliotae* isolate infecting   cardamom thrips (Sciothrips cardamomi) in India. BioControl,. 60: 363-373.

Shin, T. Y., Lee, W. W., Hyun Ko, S., Choi, J. B., Bae, S. M., Choi, J. Y., Lee, K. S., Je, Y. H.,   Jin, B. R. and Woo, D. S. 2013. Distribution and characterization of entomopathogenic fungi   from Korean soils. Biocontrol Science and Technology, 3: 288-304.

Steinwender, B. M., Enkerli, J., Widmer, F., Eilenberg, J., Thorup-Kristensen, K. and Meyling   N. V. 2014. Molecular diversity of the entomopathogenic fungal

Metarhizium community   within an agroecosystem. Journal of Invertebrate Pathology, 123: 6-12.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5:   molecular evolutionary genetics analysis using maximum likelihood, evolutionary   distance, and maximum parsimony methods. Molecular Biology and Evolution, 28: 2731-   2739.

White, G. F. 1927. A method for obtining infective nematode from cultures. Sciences, 66: 302-303.

White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of   fungal ribosomal RNA genes for phylogenetics. In Innis, M. A, Gelfand, D. H, Sninsky,   J. J, White, T. J. (Eds) PCR protocols: a guide to methods and   applications.   Academic Press, San Diego,USA, pp 315-322.