

BEAUVERIA PSEUDOBASSIANA REHNER AND HUMBER, 2011 A NEW ENTOMOPATHOGENIC FUNGUS FROM GARA MOUNTAIN, IRAQ

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ABSTRACT

There is a growing interest in the exploitation of naturally occurring entomopathogenic microorganisms for the control of pests. *Beauveria* spp is an important entomopathogenic fungi that used as a biocontrol agent of insect pests. The species *Beauveria pseudobassiana* Rehner and Humber, 2011 was isolated from soil samples collected from the hibernation site of sunn pest *Eurygaster integriceps* Puton (The most important insect attacks wheat plants in Iraq) at Gara Mountain, Iraq by using selective medium based on oat meal agar amended with CTAB and cyclohexamide. The isolated species was identified depending on both morphological characteristics and molecular data based on ITS-rDNA region. Using blast search, the sequences exhibited high sequence homology (99%) to the fungus *B. pseudobassiana* that recorded for the first time in Iraq.

Keyword: *Beauveria pseudobassiana*, Iraq, Kurdistan region, new record.

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INTRODUCTION

The genus *Beauveria* Vuill. 1912 (Cordycipitaceae: Hypocreales) is a cosmopolitan entomopathogenic fungus recorded in various kinds of habitats and ecosystems including insects, soil and plants (Meyling and Eilenberg, 2007; Medo *et al.*, 2016; Imoulan *et al.*, 2017) and used for control of many species of insects related to different orders (Roy *et al.*, 2010). The isolation of *Beauveria* species from soil are commonly based either on using *Galleria* bait method (larvae of *Galleria mellonella* L., 1758, Lepidoptera: Pyralidae), or by, the use of selective media (Meyling *et al.*, 2009; Medo and Cagáñ, 2011; Posadas *et al.*, 2012). Recent phylogenetic studies indicated that *Beauveria* showed a lot of genetic diversity and a complex that consists of a number of cryptic species. Currently, there are totally 21 species included in the genus. Based on the recognition of 12 species by Rehner *et al.* (2011), i.e., *B. bassiana*, *B. brongniartii*, *B. caledonica*, *B. amorpha*, *B. asiatica*, *B. australis*, *B. kipukae*, *B. pseudobassiana*, *B. varroae*, *B. sungii*, *B. malawiensis* and *B. vermiconia*, some 9 more species have been described, these include, *B. lii* (Zhang *et al.*, 2012), *B. sinensis* (Chen *et al.*, 2013), *B. rudraprayagi* (Agrawal *et al.*, 2014) and *B. hoplocheli* (Robène *et al.*, 2015), *B. gryllotalpidicola* and *B. loeiensis* (Ariyawansa *et al.*, 2015). *B. medogensis* (Imoulan *et al.*, 2016b), *B. locustipuila* (Kepler *et al.*, 2017) and *B. majiangensis* (Chen *et al.*, 2018). Therefore, species differentiation using general phylogenetic

markers as intergenic spacer (ITS) region of rDNA as DNA barcode has been successfully employed for discrimination of very closely related species (Rehner and Buckley, 2005; Tu and Krischner, 2014; Imoulan *et al.* 2016a). Several studies have revealed that *B. pseudobassiana* is a promising entomopathogen and might be used as a pesticide for controlling a wide spectrum of insects. Isolates from *B. pseudobassiana* have been reported to be virulent to pine weevil *Pissodes nemorensis* (Romon *et al.*, 2017) and showed a high virulent against males and females of *Monochamus galloprovincialis*, the vector of pine wilt disease (Alvarez-Baz *et al.*, 2015). In Turkey, Kocacevik (2015) showed the efficacy of *B. pseudobassiana* as a promising bicontrol agent against bark beetle *dendroctonus micans* (Coleoptera: Curculionidae) and used in Italy as a biological insecticide in protein bait sprays to control the Mediterranean fruit fly *Ceratittis capitata* (Bedini *et al.*, 2018).

In Iraq and according to the previous studies of entomopathogenic fungi isolated from soils at Gara Mountain/ Iraq, *Beauveria* species were recorded as *B. bassiana* and *B. brongniartii* (Assaf, 2007; Assaf *et al.*, 2011) but they were only identified depending on their morphological characteristics. More recently, *B. varroae* was identified based on morphological characteristics and the analysis of ITS-rDNA added to the genus in Iraq (Hassan *et al.*, 2019). The present study aims to identify *Beauveria* isolates obtained from Gara mountain soils based on both morphological and molecular analysis.

MATERIALS AND METHODS

Isolation: Soil samples (about 500 g each) were collected from a depth of 0-10cm beneath surface litter under the plants that regards as most suitable hibernation sites for sunn pest from Gara Mountain (2066m above sea level, 37.0111° N, 43.3670° E). Isolation method of *Beauveria* species from soil was described in details in Hassan *et al.* (2019). The medium used basically composed of oat meal agar amended with 0.6g/l cetyltrimethyl ammonium bromide (CTAB) as described by Pasadas *et al.* (2012) with a modification by addition of 0.25 g/l cyclohexamide.

Morphological observation: To produce monosporic cultures, conidial suspension of 1×10^8 conidia/ml were prepared from fungal cultures grown on PDA plates for two weeks. The single colony reproduced from single conidia was transferred into a new PDA dish and incubated at 25 °C. Microscopic measurements of conidia were taken from slide-cultures produced by inoculation a small amount of mycelium on a drop of methylene blue stain and overlaid by a cover slip. Measurements were performed with graticule lens.

Genomic DNA extraction, PCR and sequencing: Establishment of monosporic cultures and microscopic measurements of conidia were mentioned in Hassan *et al.* (2019). The extraction was done according to a commercial animal and fungi DNA preparation kit protocol (Jena Bioscience, Germany).

Genomic DNA was used as template for PCR amplification of its stander for ITS region using universal primers ITS5/ITS4 (White *et al.*, 1990). The PCR reactions were performed in a final volume of 50µl containing 25µl 2x Taq PCR Master Mix, 2µl of each reverse and forward primer (20 pml), 2µl of genomic DNA (30-100 ng/ µl) and 19µl of RNase- Free water. Amplification was performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems) according to a program as follow: 95C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s and final extension step of 72°C for 10 min.

Amplified PCR products were visualized by 1% agarose gel electrophoresis stained with 3 µl of EvaGreen® Fluorescent Gel Stain (Jena Bioscience, Germany). The electrophoresis was done at 100V/cm gel a voltage source (80V) for 40min., photography and visualization of bands were carried out using a trans-illuminator equipped with a digital camera.

Phylogentic and Data analysis: The resulting sequences were checked and aligned using BioEdit sequence alignment editor 7.0.0 (Isis Pharmaceuticals, Inc., Carlsbad, CA, USA). The similarity of the sequence with

homologous sequences deposited in GenBank was calculated using the “BLAST” tool on the National Center for Biotechnology Information (NCBI) website. Alignment of selected sequences was done with clustalW. All sequences generated in this study were submitted to GenBank. The phylogenetic tree was constructed using the Neighbor-Joining method with Jukes-Cantor model in MEGA7. Branch support was estimated by bootstrap analysis with 1000 replicates.

RESULTS

Morphological observation: Based on microscopic observation, samples isolated from Gara Mountain soil display the typical morphological characteristics found in species of *Beauveria* that described elsewhere (Rehner *et al.*, 2005). The colonies had the appearance of dense clusters of globose spherical conidiogenous cells, with apical denticulate rachis, which give them a zigzag appearance giving rise to sessile, hyaline smooth conidia.

Sequencing of ITS and phylogenetic analysis: The results of rDNA-ITS sequencing of the *Beauveria* isolates showed 600bp of special DNA fragments sequenced.

Using BLAST search, to compare the resulting sequences with sequences of rDNA accessed in Genbank, phylogenetic analysis showed that the obtained sequences shares 99% homology to *Beauveria pseudobassiana* strains: Turkish isolates (MH185843, MH185848, MH185847, MH259852, MH185849, MH185845) and Mexico isolates (KC355187 and KC355186) (Figure 1). Together, morphological identification and molecular identification showed that *Beauveria* isolate is *Beauveria pseudobassiana* (Genbank accession No. MH374534).

Taxonomy

Description of *Beauveria pseudobassiana* as a new record for Iraq: *Beauveria pseudobassiana* S.A. Rehner and R.A. Humber, Mycologia 103: 1068(2011) (Fig.2:1-4).

Colony growth and appearance on potato dextrose agar attaining a diameter of 28 mm after 10 days at 25°C. Surface mycelium velutinous to cottony, closely appressed to agar surface; margin white with colony interior white or changing to yellowish white (Figure. 2) to pale yellow. Conidia aggregated as spherical clusters, white in mass. Colony reverses either uncolored or yellowish white. Vegetative hyphae septate, branched, hyaline, smooth-walled, 1–2.2 µm wide. Conidiogenous cells solitary but usually in dense lateral clusters, base subspherical to ampulliform and 3–6 µm wide. Conidia 2–2.5 x 1.3–2.2 µm, primarily subglobose or broadly ellipsoid, hyaline, aseptate, walls thin and smooth.

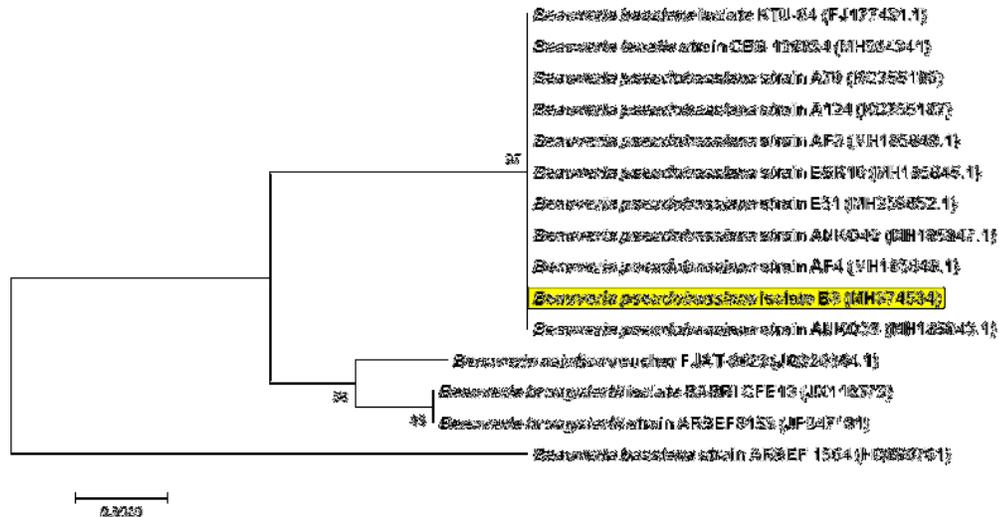


Figure 1. Phylogenetic tree of *Beauveria pseudobassiana* based on Neighbor-Joining analysis with 1000 bootstrap replicates of ITS-rDNA sequences of the new strain from Iraq (in yellow) and related *Beauveria* species from GenBank. GenBank accession numbers provided behind the species names.



Figure 2. *Beauveria pseudobassiana* (isolate B3) grown on PDA.

1-Colony and aerial hyphae top view; 2- Colony and aerial hyphae bottom view; 3- conidia clusters (zigzag shape) scale bars=5 μm.

Material examined: Iraq, Kurdistan region, Duhok; from soil samples collected under *Rhus coriaria* trees, Gara mountain, 10 June 2016, using selective media based on oat amended with CTAB and cyclohexamide. Living cultures in sterile water were deposited in Mycology bank, Department of plant protection, College of

Agricultural Engineering Sciences, University of Duhok, (BEG22), F.R. Hassan (Genbank MH374534).

DISCUSSION

The study of entomopathogenic fungi in Iraq is less developed with few works being published that they depend only on morphological characteristics for identification. The new species record for Iraq reported

that *Beauveria pseudobassiana* occurs in Gara Mountain soil along with other entomopathogenic fungi as *B. bassiana*, *B. brongniartii*, *Paecilomyces farinosus* (Assaf *et al.*, 2011), *Isaria javanica* (Hassan *et al.*, 2012) *Metarhizium anisopliae* (Abdullah *et al.*, 2015) and *B. varroae* (Hassan *et al.*, 2019).

B. pseudobassiana conidia are generally smaller than *B. bassiana* but it is indistinguishable from *B. varroae*, *B. kipukae* and *B. australis*, which also have globose/ subglobose/broadly ellipsoid conidia. Recently *B. pseudobassiana* is introduced by Rehner *et al.* (2011) as phylogenetically distant from *B. bassiana* but phenotypically is similar. Therefore their distribution and abundance might be not accurate and probably was identified in previous works as *B. bassiana*. Medo and Cagáň (2011), reported *B. bassiana* along with *B. pseudobassiana* as a common soil borne entomopathogens in Slovakia. They furthermore stated that *B. bassiana* predominate in arable soil whereas *B. pseudobassiana* was more abundant in forest soil. This is in line with our finding. The prevalence of *B. pseudobassiana* on hard tick, *Ixodes ricinus* (Acari: Ixodidae) was reported by Munteanu *et al.* (2014) from Moldova. The recovery percentage of *B. pseudobassiana* isolates from agricultural soil in Mexico was low comparable to high recovery percentage for *B. bassiana* as reported by Perez-Gonzales *et al.* (2014). Populations from hibernating pupae of *Cameraria ohridella* were found naturally infected with *B. pseudobassiana* in Slovakia (Schemmer *et al.*, 2016). *B. pseudobassiana* was reported among the entomopathogens of *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) in Turkey (Yucel *et al.*, 2018).

The result of our study provides more information on the occurrence of indigenous *Beauveria* species in hibernation sites of sunn pest *Eurygaster integriceps* (Puton) in Iraq and the necessity to test their efficacy against insect pests.

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