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ISOLATION AND CHARACTERIZATION OF *Isaria farinosa* FROM IRAQI GARA MOUNTAIN SOIL USING MORPHOLOGICAL AND MOLECULAR APPROACHES

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ABSTRACT

***Isaria farinosa,* formerly known as *Paecilomyces farinosus*, have a worldwide distribution and a relatively wide host range. The aim of this work is to isolate and identify *Isaria* fungus from soil of Gara mountain using molecular analysis and morphological characteristics. *Isaria* species were isolated from soil samples collected at an insect overwintering sites in Gara mountain, Kurdistan Region, Iraq using selective medium as an Oat culture modified with CTAB and cyclohexamide. Results demonstrated that conidia of the isolated species are globose to subglobose to ellipsoidal in shape, 2-2.3 x 1-1.3 um in size, and hyaline with smooth walls. Besides, PCR based method revealed that the sequences of the isolated fungus has high level (99.8%) of sequence homology to the fungus *I. farinosa.* The present study provides better insight into the *Isaria* species and related entomopathogenic fungi in terms of isolation, morphology and molecular identification.**

***KEYWORD**:* Fungal species, ITS, Kurdistan region, Morphology, rDNA

INTRODUCTION

For more than 30 years, the entomopathogenic fungus *Isaria farinosa* (Holmsk.) Fr. 1832 (Hypocreales: Cordycipitaceae) was known as *Paecilomyces farinosus* and then the genus was transferred to *Isaria* (Samson 1974; Sung et al. 2007). Originally, *Isaria* was considered a subsection within the genus *Paecilomyces* sensu. Samson (1974), who divided this genus into two sections: section Paecilomyces (thermophilic) and section Isarioidea (mesophilic). However, this distinction was based on morphological characteristics that may be highly subjective and lead to ambiguous identifications at the species level. Formal conservation of the generic name *Isaria* was officially accepted in 2005 (Gams et al. 2005). Molecular phylogenetic studies have resurrected the genus *Isaria* (Luangsa-ard et al. 2005; Sung et al. 2007). The polyphyletic nature of the genus *Paecilomyces* (i.e., including the sect. Isarioidea) has been demonstrated several times previously by analyses of the large and small subunit rRNA genes (Obornik et al. 2001; Luangsa-ard et al. 2004). However, using the b-tubulin gene and the nuclear ribosomal internal transcribed spacer (ITS) region, Luangsa-ard et al. (2005) investigated the phylogenetic relationships of Paecilomyces sect. Isarioidea species, and established the existence of a monophyletic group named 'Isaria clade'. A recent taxonomic treatment based on phylogeny, *Isaria* is asexual genus linked to *Cordyceps* Fr. (Family: Cordycipitaceae: Hypocreales) (Shreshtha et al. 2017).

Species of the genus *Isaria* are insect's fungal pathogen with a worldwide distribution (Gams et al. 2005). More than 1000 isolates of *Isaria* from the Australia, United States, Africa, Europe, and Asia were found in the (ARSEF) collection catalogue (2021). According to Domsch et al. (1980), the fungus *I. farinosa* is extensively distributed in temperate and tropical zones and has been isolated mostly from Lepidoptera insects, air, water, plants, and forest soils containing various trees such as poplar, pine, Acacia and oak. Furthermore, *Isaria* strains can infect insects at different developmental stages in many orders (Zimmermann, 2008; Gallou et al.

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2016; Da Silva-lopez et al. 2019). This fungus, like many other EPF, was isolated using the Galleria bait method (Zimmermann 1986) or alternative selective media (Shin et al. 2010; Niu et al. 2019).

In Iraq, according to the previous studies, *Isaria farinosa* was isolated from Gara Mountain soils and identified as *Paecilomyces farinosus* (Assaf et al, 2011) depending on its cultural and morphological characteristics. Many other entomopathogenic fungi were isolated from Gara mountain soil as *Isaria javanica* (Hassan et al. 2012), *Beauveria bassiana* and *B.brongniartii* (Assaf et al. 2011). More recently, *B. varroae* and *B. pseudobassiana* were identified morphologically and also based on ITS-rDNA analysis added to the genus *Beauveria* in Iraq (Hassan et al. 2019; 2020).

For a biological control program to be successful, knowledge of the exact identities of the pest and biological control agent species is crucial. For this reason, the *Isaria* isolates were characterized morphologically. However, the change in status of the genus *Isaria* pointed out the need to use molecular methods (Luangsa-ard et al. 2005; Sung et al. 2007) to characterize *Isaria* isolates. Therefore, the aim of this study was to obtain morphological and molecular characterizations of the *Isaria* isolates from soils of Gara mountain in Kurdistan Region of Iraq.

MATERIALS AND METHODS

Isolation

To isolate the fungus, twenty soil samples (about 500 g each) from a depth of 0-10cm were collected under different plant types that are regarded as hibernation sites for many insects (especially sunn pest *Eurygaster integriceps*) at Gara mountain - Duhok province, Kurdistan region, Iraq during May to July 2016. A selective medium based on Oat Meal Agar (OMA), 0.6g/l Cetyltrimethyl Ammonium Bromide (CTAB) and 0.25 g/l cycloheximide was used for isolation (Posadas et al., 2012). The plates were incubated at 25˚C for two weeks. Fungi growing were transferred on standard PDA medium ((Himedia laboratories Pvt. Ltd.India) for identification. Isolates recovered from single conidia were grown as mycelia in 250-ml conical flasks containing 100 ml of potato dextrose broth. Cultures were then shaken and incubated at 25˚C for seven days in darkness. The mycelial growth was filtered from liquid culture under aseptic conditions and then frozen at ¯20˚C to be used for molecular identification.

Morphological observation

To produce monosporic cultures, conidial suspension of 1 × 10 conidia/ ml was 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. The identification was based on macroscopic and the microscopic characteristics following an identification key (Samson, 1974; Domsch et al.1980). 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.

DNA extraction

For molecular identification, after the fungi being isolated and cultured on potato dextrose broth about seven days at 25°C. The mycelia mat for each isolate was pelleted from broth and frozen at –20°C. The extraction of DNA was then carried out according to a commercial animal and fungi DNA preparation kit protocol (Jena Bioscience, Germany). The purity and concentration of the extracted DNA was measured by Nanodrop 2000 spectrophotometer.

Polymerase chain reaction

Genomic DNA was used as template for PCR amplification of ribosomal DNA genes and internal transcribed spacer regions (ITS4 and ITS5) using universal primers (TCCTCCGCTTATTGATATGC) and (GGAAGTAAAAGTCGTAACAAGG) (White et al. 1990). The PCR reactions were done in a final volume of 50 µl as 25µl of ready to use 2x Taq PCR Master Mix (Jena Bioscience, Germany), 2 µl of each reverse and forward primer (20 pml), 2 µl of genomic DNA (30–100 ng/ µl) and 19 µl of RNase-free water. A GeneAmp PCR System 9700 thermocycler (Applied Biosystems) used for amplification according to a program as follow: 95 ˚C 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 3µl of EvaGreen® Fluorescent Gel Stain (Jena Bioscience, Germany). The electrophoresis was done at 100V/ cm gel a voltage source (80 V) for 40 minutes.

Sequencing and similarity index

The amplified PCR product was sequenced by the Macrogen Company, Korea. The sequence was checked using BioEdit software (Hall et al, 2011). The sequence was then submitted to the

National Center for Biotechnology Information (NCBI) database under the GenBank accession no. MH374539. The similarity of the partial sequence of the ribosomal DNA gene was identified by blasting against the homologous sequences of fungal species deposited in GenBank within the NCBI database.

RESULTS AND DISCUSSION

Isolation:

Different soil borne fungi as 72 isolates were obtained from soil samples of Gara Mountain, Duhok, Kurdistan, Iraq. Based on microscopic observations, the isolates displayed the typical morphological characteristics found in 13 genera of fungi (Fig. 1). From them, the occurrence percent of entomopathogenic fungi related to genera *Beauveria* and *Isaria* were 29.34 and 2.33%, respectively.

Growth medium amended with CTAB was effective for isolation of several entomopathogenic fungi and agreed with the finding of Posadas et al. (2012) who found that CTAB (0.6g/L) addition to oat medium give more chance for recovery of *B. bassiana* and other entomopathogenic fungi. The hypocrlean entomopathogenic fungi grow relatively slowly on isolation media in comparison to the ubiquitous saprophytic soil fungi. Thus, the content of media for the isolation of specific entomopathogenic fungus from soil should have both a nutrient source for its growth and antimicrobial agent (antibiotic and fungicides) in suitable concentrations to inhibit the growth of non- target saprophytic fungi and allow to target fungus to grow (Luz et al., 2007).The species richness of insects, especially sunn pest, in the Gara Mountain under the plants undoubtedly affects the species diversity of their natural enemies, including the entomopathogenic fungi. Assaf (2007) reported that the highest number of hibernated sunn pest individuals was under the *Astragulus* spp plants as 36.33 individuals and recorded that 33.33% of their death was due to the effect of entomopathogenic fungi.

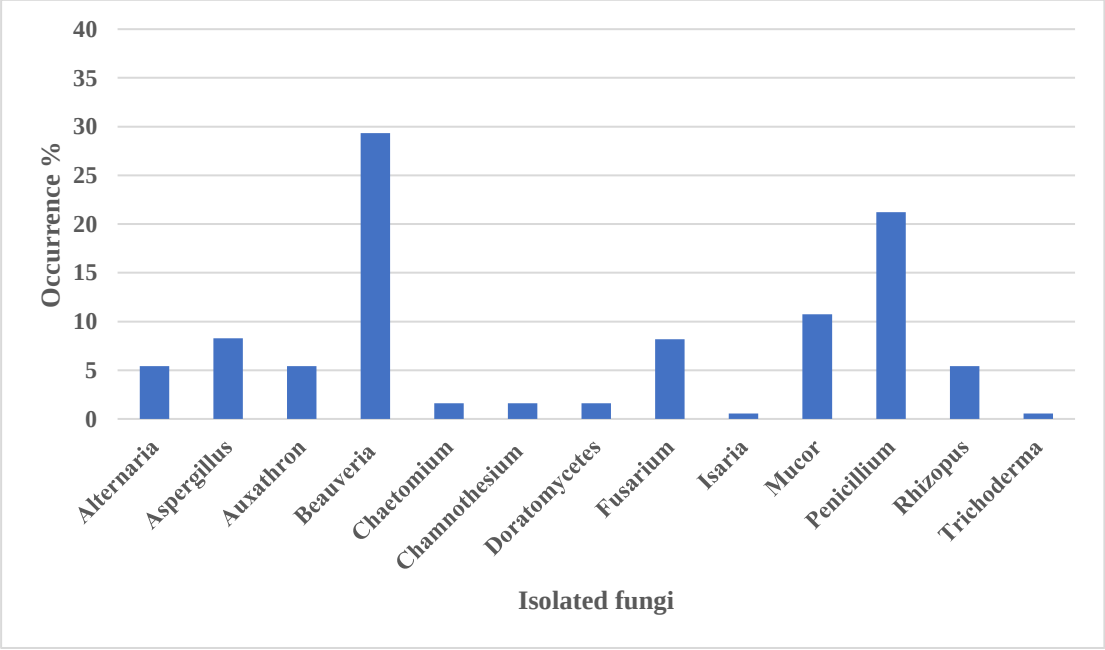

Fig. (1): Occurrence percentage of isolated fungi from the Gara Mountain soil samples.

Morphological observation:

Based on microscopic analysis, 2.33 % of the isolates share the same morphological characteristics as *Isaria* species which described before (Luangsa-ard et al. 2005). On PDA, the colony grow quickly, reaching 40-45mm in 7 days incubating at 25°C. The mycelium is floccose, white at first, changing cream or yellow and powdery during conidiation. The reverse side of the colony is yellow or creamy. Conidiophores can grow from aerial hyphae side branches or straight from submerged mycelia. Conidiogenous cells are flask-shaped with smooth walls that measure 2-3 x 1-1.4 um. Conidia are globose to subglobose to ellipsoidal in shape, 2-2.3 x 1-1.3 um in size, and hyaline with smooth walls (Fig.2).

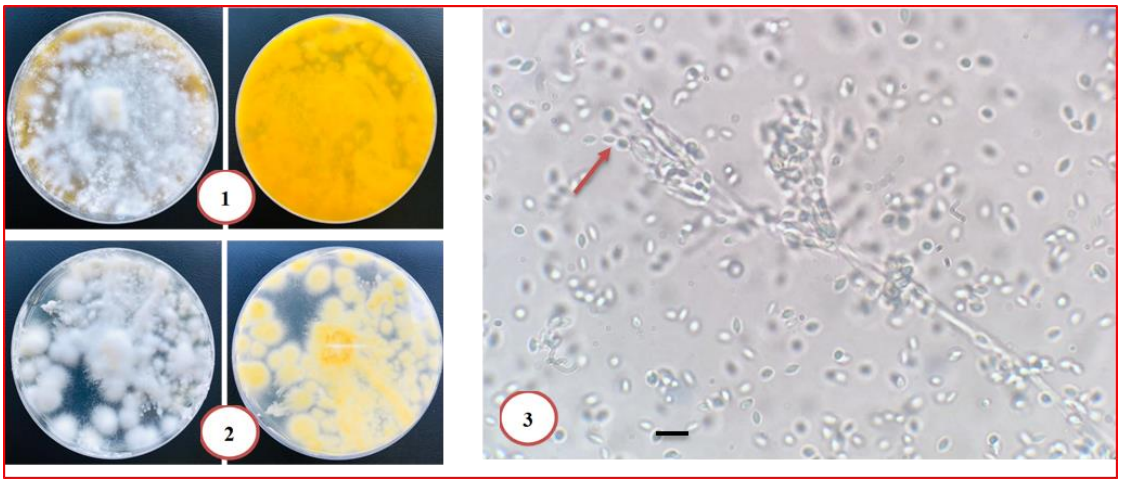

Fig (2): *Isaria* (*Cordyceps*) *farinosa* (isolate B10) grown on CA (carrot agar) and PDA
1- The colony and aerial hyphae (top and bottom views) on CA; 2- The colony and aerial hyphae (top and bottom views) on PDA; 3- conidia and conidiogenous cells of *I. farinosa* and individual conidia, scale bars=5 m.

Samson (1974) and Domsch et al. (1980) distinguished *Isaria farinosa* from other species related to *Isaria* genus that their conidia are ellipsoidal to fusiform, and its colonies are white to yellow. Fast growing, producing white colonies first, then yellow or cream colonies with floccose aerial mycelium and occasionally yellowish synnemata. Kleespies et al. (1989) reported that *I. farinosa* occurrence in natural habitats is more often than in agricultural soils. Gallou et al. (2016) who study the morphological features of six isolates of *Isaria*, mentioned that the conidia have an ellipsoidal to fusiform-elliptical shape with overall dimensions of 3.48-4.92 x1.54-2.11 mm. The phialides of the isolates were characterized by a wide globose basal portion with a long distal neck, and overall dimensions of 4.81-5.73 x 1.86-2.39 mm.

Similarity index

Results of PCR, sequencing and NCBI blasting indicated that the DNA fragment with length 588bp of ribosomal DNA genes including partial sequence of 18S rDNA (small subunit) and 28S rDNA (large subunit); and complete sequence of ITS4 (internal transcribed spacer 1), 5.8S ribosomal DNA gene, and ITS5 (internal transcribed spacer 2) of the isolated *Isaria* from Iraqi Gara mountain soil has 99.8% pairwise identity to the *Isaria farinose* strains which represented in different geographic locations: Turkey, Switzerland, Denmark, Canada and China (Table1). The sequence of the isolated species of our study is published in the NCBI database under the GenBank accession No. MH374539.

Table (1): Similarity index% of the partial sequence of the ribosomal DNA gene of the present study and sequences of isolates from the NCBI database

Species	GenBank Numbers	isolate number	Isolate from source	Country	Similarity %
Isaria farinose	**MH374539**	**B10**	**Soil**	**This study**	
Cordyceps farinose	MH864784	CBS 127996		Switzerland	99.8
Isaria farinose	MH191137	N42/1	Sunn pest	Turkey	99.8
Isaria farinose	MH191129	N16	Sunn pest	Turkey	99.8
Isaria farinose	GU354353	KVL 07-47	Soil	Denmark	99.8
Isaria farinose	DQ888729	----	Soil	Canada	99.8
Isaria farinose	HQ711849	GZUIFR-XS.3		China	99.8
Isaria farinose	MH191127	DIKA12	Sunn pest	Turkey	99.8
Isaria farinose	MH191123	KIR1	Sunn pest	Turkey	99.8
Isaria farinose	MH191136	N41	Sunn pest	Turkey	99.8
Isaria farinose	MH191133	N21/1	Sunn pest	Turkey	99.8

Luangsa-ard et al. (2005) examined the phylogenetic relations of *Paecilomyces* sect. Isarioidea species using the b-tubulin gene and the internal transcribed spacer (ITS) rDNA. They reported that the section is polyphyletic within the Hypocreales (Ascomycota), and a monophyletic group known as the *Isaria* clade, which contains *I. farinosa* and *I. fumosorosea*. D'Alesssandra et

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al. (2013) who studied the identification of *Isaria* spp from Argentina, Mexico, and Brazil, mentioned that the molecular identification by using ITS1-5.8-ITS2 gene, was helpful in analyzing isolates of *Isaria farinosa*. They placed this species within the family Cordycipitaceae polyphyletic, the genus *Isaria* was confirmed, Isaria species were related to anamorphic species of *Beauveria*, *Simplicillium* and *Lecanicillium*, and to teleomorphic Cordyceps and *Torrubiella*.

CONCLUSIONS

Morphological and molecular analyses of our study concluded that the isolated fungi from Gara Mountain soils In Iraq has 99.8% pairwise identity to the *Isaria farinosa* strain. Nevertheless, for accurate identification of fungal species at molecular level, it is highly recommended for future studies to use the complete sequences of ribosomal DNA genes including 18S rDNA, ITS1, 5.8S rDNA, ITS2 and 28S rDNA.

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