Isolation, molecular characterization of indigenous *Beauveria bassiana* isolate, using ITS-5.8 s rDNA region and its efficacy against the greatest wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) as a model insect

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**Abstract**

The entomopathogenic fungi (EPF), as a biological control agent, can provide an alternative option for high-risk insecticides. *Beauveria bassiana* is one of the most potential EPF of pest control all over the world. The EPF isolate Y-F_ITS1 was isolated from Egyptian fauna on *Beauveria*-specific selective medium (BS medium). Molecular screening of the *B. bassiana* isolate, using PCR amplification with *B. bassiana*-specific primers and nucleotide sequence analysis of the internal transcribed spacer (ITS) region, confirmed the isolate as *B. bassiana* (accession no. MK773644.1). Efficacy of the isolate Y-F_ITS1 was examined against fourth instar larvae of *Galleria mellonella* L. as a model insect using concentrations from $10^4$ to $10^7$ spores ml$^{-1}$ in a contact toxicity assay under laboratory conditions. The pathogenicity experiment showed that all the tested concentrations caused mortalities ranged from 75 to 98.33%, within 5 days posttreatment with cumulative mortalities, reached 100%, before 7 days with typical symptoms of infection and sporulation. Further investigations are needed to prove its efficacy against different economic pests as a credible candidate of integrated pest management (IPM) program.

**Keywords:** Biological control, Entomopathogenic fungi, *Beauveria bassiana*, ITS, *Galleria mellonella*

**Background**

There is an increasing expectancy worldwide in developing microbial bio-pesticides as a considerable component of integrated pest management programs (IPM) and stimulated necessities for chemical-free pesticides (Ravensberg 2015). The entomopathogenic fungi (EPF) fulfilled the criteria as a promising natural mortality factor of insects (Boomsma et al. 2014). *Beauveria* spp. based products proved their virulence and represent almost about 40% of the total myco-insecticides (Faria and Wraight 2007). Quite recently, considerable attention has been paid to the use of DNA barcoding, using the internal transcribed spacer (ITS), as a short gene sequence was taken from a standardized portion of the genome and can be recovered and characterized as a unique identification marker for all biological identifications and diagnostic species including fungi (Hebert and Barrett 2005; and Pečnikar and Buzan 2014). Nearest neighbor algorithms are usually used to assign an unknown sample to a known species by finding the closest sequence to the sample sequence in the international database (Saitou and Nei 1987). Basic Local Alignment Search Tool (BLAST) is a common matching tool,

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provided through the National Center of Biotechnology Information (NCBI) that searches for correspondence between a query sequence and a sequence library (Altschul 1990). The main target of this study was to identify a new EPF isolate from Egyptian fauna and evaluate its efficacy against the greatest wax moth, Galleria mellonella L., as a model insect under laboratory conditions.

Materials and methods

Sample collection
Various clay soil samples from different clover farm locations at Dakahlia Governorate, Egypt, were collected by gathering the topsoil down to 40 cm depth, using a metal shovel in late winter 2017. The samples of each site were placed in sterile plastic bags, transferred to the laboratory, and stored at 4–8 °C until used (Chandler et al. 1997).

Insect bait method
Insect bait technique recommended by (Zimmermann 1986) was chosen to screen and isolate the indigenous species of EPF, using larvae of the wax moth, G. mellonella. Larvae were treated by warm water to prevent extensive webbing in the soil (Meyling and Eilenberg 2006). Soil samples were moistened and placed in petri dishes. Twenty medium-sized larvae were used for each soil sample. Samples were incubated at 20 ± 2 °C in the dark and inverted every day. Soil samples were examined after 5 days; dead bait larvae were collected and surface sterilized with 1% Na-hypochlorite to prevent external saprophytic fungi from growing on the dead cadaver. Dead larvae were kept in petri dish lined with a single layer of wet filter paper until signs of muscardine were observed. The fungal spore was cultivated on Sabouraud dextrose yeast agar (SDAY) medium. The petri dishes (5 cm × 1 cm) were incubated at 25 °C for 3–7 days. For extra purification, single spore cultures were plated out from multisporal cultures. Fungal strain exhibiting good growth and spore production traits was selected, purified, and identified according to microscopic observations following the taxonomic keys, using color atlas of pathogenic fungi for Beauveria genus (Frey et al. 1979; Webster and Weber 2007).

Molecular identification of fungal isolate
Mycelia and conidia from the isolated fungal strain were inoculated, and a single spore colony was grown on SDAY and incubated on a shaker (150 rpm) at 20 °C for 5–7 days. DNA extraction was done, following the manufacturer’s protocol, using Animal and Fungi DNA Preparation Kit (Jena Bioscience, Germany). Thermo Scientific NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, MA, USA) determined the quantity and quality of DNA. For the ITS1-5.8S-ITS2, PCR amplification and sequencing were performed. ITS1F (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4R (5′-TCC TCC GCT TAT TGA TAT GC-3’) primers were used to amplify the partial gene region. Using ABI Prism Dye Terminator Cycle Sequencing Core Kit (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA), amplicons were sequenced with 310 Automated DNA Sequencer (Company, country). A BLASTN search was conducted on the NCBI database to classify associated sequences. The BioEdit 4.8.9 software alignment editor (Hall 1999) was used and the alignment was corrected manually. CLUSTAL-X was used to perform multiple sequences alignment. A phylogenetic tree was reconstructed using MEGA ver. 7.0 by using Maximum Likelihood method based on the Jukes-Cantor model. Branch support was estimated with 1000 bootstrap replicates under appropriate substitution models. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer phylogenetic tree.

Efficacy of the fungal isolate against G. mellonella
Fourth instar larvae of G. mellonella were used in the present experiment. For treatment experiments and control, 30 larvae (10 larvae per replicate) were placed in plastic boxes (25 × 25 × 20 cm; length × width × height). All boxes were provided with a sterile wax for nutrition. To determine the LC50 and LC90, 5 spore concentrations of the fungal suspension were used (viz.107, 106, 105, 104, and 103 spores ml−1). For each concentration, 30 larvae were placed on a folded Whatman No. 1 filter paper in a glass funnel, and then 30 ml of the fungal suspensions were added; sterile distilled water mixed with 0.1% Tween 20 was used as a control treatment. The treated insects were left for 30 s to dry and then transferred to the plastic boxes lined with a filter paper to remove excess humidity. All treatments and the control were incubated at 25 °C. The whole experiment was repeated 3 times. The mortality rate was recorded daily for 6 days.

Statistical analysis
The mortality percentage caused by EPF was corrected according to Abbott’s formula (Abbott 1925). Percentage mortality was calculated according to Feng et al. (1992). The lethal time LT50 and LT90 values, that were required to kill 50 and 90% respectively, were estimated according to Finney (1971), using the “LdPLine” software to the [http://embakr.tripod.com/ldp-line/ldpline.html].

Results and discussion

Isolation and morphological identification
After 5 days of soil sample examination, the dead bait larvae of G. mellonella were separated, sterilized, and
kept in a petri dish until infection symptoms appear. The infection test showed that the dead bait larvae suffered infection symptoms, feed less actively, slow motion, and sluggishness. White mycelium grew in membranes and inter-segmental membranes of dead larvae after 5 days or longer. Morphological identification indicated that the strain Y-F_ITS1 was B. bassiana. The samples collected fungal species on selective BS medium showed morphological and cultural characteristics similar to Beauveria species. Colonies were lightly raised, round, with white powdery surface, and lightly downy with circular rings. White powdery translucent mycelium showed a radial growth. Conidiospores were densely clustered in whorls, one celled, smooth, hyaline, and short. The isolation of EPF, using Galleria bait method, proved to be an effective technique for screening indigenous species than common plating on media. Keller et al. (2003) and Meyling and Eilenberg (2006) mentioned that isolation and identification, using baiting soil samples with larvae of G. mellonella, could be used on a broad range for indigenous isolates present in soil. Identification based on morphological characteristics could not identify correctly down to the species level; thus, the genetic diversity of Beauveria species were detected, using ITS and additional loci such as EF-α or mt intergenic regions (Aquino de Muro et al. 2005; Rehner and Buckley 2005; Ghikas et al. 2010; Poeaim et al. 2014; and Chen et al. 2018).

**Molecular characterization**

A total of 1597 bp was deposited under accession no. MK773644.1 in the GenBank with 73.48% as GC content of the present sordariomycetes isolate for the ITS1-5.8S-ITS2 gene region. A unique genetic sequence was revealed by a parallel comparison of the isolated gDNA sequence of the present isolate with a range of other genotypes belonged to Hypocreomycetidae subclass. Calculating the percentage of identity between this novel sequences and the others from GenBank revealed a high degree of resemblance (> 90%). Phylogenetic analyses were conducted based on the alignment of partial and complete sequences of ITS1-5.8S-ITS2 for 24 taxa using Maximum Likelihood method based on the Jukes-Cantor model representing 2 orders, Hypocreales and Glomerellales (Table 1 and Fig. 1).

| Table 1 | Species used in the phylogenetic analysis of ITS1 gene region of the present Beauveria bassiana |
|---------|------------------------------------------------------------------------------------------|
| Name of isolates | Order/family | Isolate source | Accession no. | GC content % | Identity % |
| Beauveria bassiana | Hypocreales/Cordycipitaceae | Argania spinosa forest soil | KT378247.1 | 56.52 | 99.61 |
| Beauveria bassiana | Hypocreales/Cordycipitaceae | Galleria mellonella | KU523254.1 | 55.82 | 99.42 |
| Beauveria bassiana | Hypocreales/Cordycipitaceae | Coffea Arabica | DQ682566.1 | 55.68 | 99.42 |
| Beauveria pseudobas | Hypocreales/Cordycipitaceae | Agricultural soil | KC355185.1 | 56.36 | 99.41 |
| Beauveria bassiana | Hypocreales/Cordycipitaceae | Farm soil | MH233319.1 | 56.57 | 99.41 |
| Isaria fanniae | Hypocreales/Cordycipitaceae | Bombyx mori | JF429897.1 | 50.21 | 99.31 |
| Beauveria bassiana | Hypocreales/Cordycipitaceae | Ant | KX553851.1 | 54.46 | 99.23 |
| Beauveria bassiana | Hypocreales/Cordycipitaceae | Cycloneda sanguinea | KF308683.1 | 55.82 | 99.23 |
| Cordyceps bassiana | Hypocreales/Cordycipitaceae | Coffea arabica | EF672309.1 | 55.78 | 99.23 |
| Cordyceps sp. | Hypocreales/Cordycipitaceae | Miscanthus giganteus | HQ630968.1 | 55.24 | 99.23 |
| Beauveria sp. | Hypocreales/Cordycipitaceae | Marine sponge | JQ022139.1 | 54.99 | 99.22 |
| Beauveria bassiana | Hypocreales/Cordycipitaceae | – | MH233302.1 | 56.38 | 98.85 |
| Beauveria bassiana | Hypocreales/Cordycipitaceae | Hypohenemus hampei | MK049981.1 | 55.96 | 98.85 |
| Beauveria sp. | Hypocreales/Cordycipitaceae | – | JQ411373.1 | 54.72 | 97.97 |
| Beauveria bassiana | Hypocreales/Cordycipitaceae | Punjab soil | MG670098.1 | 55.22 | 97.78 |
| Lecanicillium sp. | Hypocreales/Cordycipitaceae | Ophiocordyceps sinensis | JF994475.1 | 51.24 | 94.51 |
| Fusarium proliferatum | Hypocreales/Nectriaceae | – | LT841264.1 | 51.82 | 93.21 |
| Fusarium solani | Hypocreales/Nectriaceae | – | FJ345352.1 | 52.03 | 92.26 |
| Fusarium oxysporum | Hypocreales/Nectriaceae | – | LT841236.1 | 51.65 | 92.87 |
| Epichloë festucae | Hypocreales/Clavicipitaceae | – | KU710346.2 | 52.67 | 93.18 |
| Metarhizium anisopliae | Hypocreales/Clavicipitaceae | – | AF218207.1 | 51.10 | 92.10 |
| Clonostachys rosea | Hypocreales/Bionectriaceae | – | MH047188.1 | 50.38 | 92.80 |
| Acremonium sp. | Hypocreales/Incertae sedis | – | GQ867783.1 | 52.20 | 92.27 |
| Colletotrichum graminicola | Glomerellales/Glomerellaceae | – | XR 001139481.1 | 51.68 | 92.30 |
Comparison of the nucleotide sequences and divergence showed that the ITS1-5.8S-ITS2 of this isolate revealed sequence identities of 92.27–99.61% with members of the order Hypocreomycetidae and 92.30% of the Glomerellales (Table 1). Among Hypocreomycetidae, the present isolate showed 94.51–99.61% sequence identity with taxa under Cordycipitaceae, 92.10–93.18% with Clavicipitaceae, 92.26–93.21% with Nectriaceae, 92.27% with Incertae sedis, and 92.80% with Bionectriaceae. Among Cordycipitaceae, the maximum sequence identity (99.61%) with lowest divergent value was recorded between the present isolate and \textit{B. bassiana} (gb KT378247.1), followed by \textit{B. bassiana} (99.42%, gb

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**Fig. 1** Sequence alignment of ITS1 gene region of the present \textit{Beauveria bassiana} with the most closely related species. Only variable sites are shown. (.) represents bases identical to those of the first sequence and (-) indicates gaps.
KU523254.1, DQ682566.1), B. pseudobassiana (99.42%, gb KC355185.1), B. bassiana (99.41%, MH233319.1), Isaria farinose (99.31%, gb JF429897.1), B. bassiana (99.23%, gb KX553851.1, KF308683.1), Cordyceps bassiana (99.23%, gb EF672309.1), Cordyceps sp. (99.23%, gb HQ630968.1), Beauveria sp. (99.22%, gb JQ922139.1), B. bassiana (98.85%, gb MH233302.1, MK049981.1), Beauveria sp. (97.97%, gb JQ411373.1), B. bassiana (97.78%, gb MG670098.1), and Lecanicillium sp. (94.51%, gb JF794475.1).

The ME tree showed a cluster that contained all of the sordariomycetes taxa, which was clearly divided into 2 distinct clades (Fig. 1). The first clade divided into 2 subclades, one clustered all the contained Cordycipitaceae taxa, while the other contained other taxa of the Clavicipitaceae, Nectriaceae, Incertae sedis, and Bionectriaceae. The second clade, represented by Colletotrichum graminicola belonged to the Glomerellaceae within the Glomerellales. The ME tree showed a well-resolved distinct clade for the present isolate with members belonging to the family Cordycipitaceae and deeply embedded within the genus Beauveria with close relationship to the previously described B. bassiana (gb KT378247.1, KU523254.1, DQ682566.1, MH233319.1, KX553851.1, KF308683.1, MH233302.1, MK049981.1, and MG670098.1) as a more related sister taxa (Fig. 2). In the present study, the internal transcribed spacer region (ITS1-5.8S-ITS2) was selected in agreement with White et al. (1990), Curran et al. (1994), and Zare and Gams (2001) who reported that the nuclear ITS1-5.8S-ITS2 region was identified as a useful tool for providing molecular sequence data suitable for establishing fungal phylogenies. Herein, the selected gene region was amplified by PCR, using the ITS1F/4R primers as recommended by White et al. (1990) who considered them as universal primers for fungal DNA barcode. The ML analysis of the DNA-ITS region formed a highly supported clade together with other isolates retrieved from the GenBank. The clustering of the recovered B. bassiana isolate showed the closest relationship with different isolates of B. bassiana, B. pseudobassiana, and Beauveria sp. from other regions. The obtained phylogeny supports the monophyly of the Beauveria group with low variation observed among these ITS regions, which agree with Coates et al. (2002), followed by Ghikas et al. (2010) who supported the observation that other genetic variant are likely to occur more rapidly than mutations detected in ITS regions. The presence of B. pseudobassiana in the present

![Fig. 2 Molecular phylogenetic analysis by Maximum Likelihood method based on the Jukes-Cantor model. The tree with the highest log likelihood (−3386.08) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved the nucleotide sequences of 25 species. There were 545 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.](image_url)
phylogeny with B. bassiana assured the hypothesis of Rehner et al. (2011) that although they comprise morphospecies group, the phylogenetic analysis demonstrated that both of them are distantly related. In addition, Entz et al. (2005), Becerra et al. (2007), Freed et al. (2011), and Muthabathula et al. (2019) supposed that the B. bassiana and Cordyceps militaris are closely related, which share the same clade in agreement with the present results.

**Efficacy of the B. bassiana strain against G. mellonella**

As shown in Table 2, G. mellonella larvae exhibited considerable linear mortality percentages when inoculated with serial concentrations (viz. $10^5$, $10^6$, $10^7$, and $10^8$ spores ml$^{-1}$) of the indigenous B. bassiana strain Y-F_ITS1 that was isolated from Egyptian fauna. The highest concentration ($1 \times 10^7$ spores ml$^{-1}$) showed mortality percentages ranging from 8.33% to 98.33% after 24 and 120 h, respectively, while the least concentration ($1 \times 10^5$ spores ml$^{-1}$) showed mortality percentages ranged from 0.8% to 75% after 24 and 120 h, respectively. It was also noticed that all the concentrations used in this investigation resulted in 100% mortality of the treated larvae within 7 days. Typical symptoms could be developed on the infected larvae when kept under high humidity in petri dishes within 15 days with a complete sporulation. Concerning the lethal time $LT_{50}$ and $LT_{90}$ values (calculated for the mortality ranged between 26.6–80%) were 3.58 days and 5.91 days, respectively for G. mellonella larvae inoculated with the concentration $1 \times 10^5$ spores ml$^{-1}$. The obtained results using different concentration of fungal spores indicated that the mortality rate was directionally proportional to spore concentration and exposure times. These results are in line with Fuguet and Vey (2004) and Shoukry et al. (2019). The highest virulence of this indigenous isolate advocates that it could be part of the biological control systems of IPM programs for the control of insect pests and offers the advantage of no pollution, safety, and avoiding pesticide resistance. Further research is needed to examine its efficacy and large-scale application as a biological control agent of economic pests in agriculture.

### Table 2 Efficacy of the B. bassiana strain (Y-F_ITS1) against fourth instar larvae of G. mellonella

| Concentrations (spores ml$^{-1}$) | 24 hours Mortality (%) | 48 hours Mortality (%) | 72 hours Mortality (%) | 96 hours Mortality (%) | 120 hours Mortality (%) | $LT_{50}$ (days) | $LT_{90}$ (days) |
|-----------------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-----------------|-----------------|
| $1 \times 10^7$                   | 0.8 ± 0.5               | 3.3 ± 0.5               | 6.6 ± 0.5               | 50 ± 1                  | 75 ± 1                  | –               | –               |
| $1 \times 10^5$                   | 1.66 ± 0.5              | 5.8 ± 0.5               | 26.6 ± 0.5              | 68.3 ± 0.5              | 80 ± 1                  | 3.58            | 5.91            |
| $1 \times 10^6$                   | 1.66 ± 0.5              | 10 ± 1                  | 35 ± 1                  | 83 ± 1                  | 93.6 ± 1.5             | –               | –               |
| $1 \times 10^7$                   | 8.33 ± 1                | 26.6 ± 0.15             | 51.66 ± 0.5             | 90 ± 1                  | 98.3 ± 0.5             | –               | –               |

### Conclusion

The local fungal isolate of this study, identified as Y-F_ITS1, using ITS-5.8s rDNA region deposited in GenBank, showed a significant pathogenicity towards G. mellonella, as a model insect. Further studies are needed to evaluate its efficacy as a biological control agent against economic pests in Egypt.

**Abbreviations**

- IPM: Integrated pest management; B. bassiana: Beauveria bassiana; BS medium: Beauveria-specific selective medium; ITS: Internal transcribed spacer; BLAST: Basic local alignment search tool; NCBI: National Center of Biotechnology Information; G. mellonella: Galleria mellonella; (SDAY) medium: Sabouraud dextrose yeast agar; LC50: Median lethal concentration; LT50: Median lethal time; B. pseudobassiana: Beauveria pseudobassiana

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**Authors’ contributions**

All authors contributed significantly to this research and preparation of the manuscript. YF carried out the experiments. YF and RSY analyzed the data; YF was involved in the conception and design of the study. YF, methodology and RSY and YF, writing and editing. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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