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SCIENTIFIC (SHORT) NOTE

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Morpho-molecular characterization of two Syrian soil-sourced isolates of *Beauveria* (Bals.) Vuill.

Lobna Rajab[1*], Mohammad Ahmad[1] and Ibtisam Gazal[1]

Abstract

Background The genus *Beauveria* (Bals.) Vuill. includes many species, some of which are limited to specific regions while others are distributed worldwide. The diversity of *Beauveria* species is poorly investigated in Syria and most studies lack proper diagnosis of species. Entomopathogenic isolates of this genus were obtained using the *Galleria* Bait Method. This study aimed to identify these isolates based on morphological characterizations combined with molecular data, using nuclear ribosomal internal transcribed spacer (ITS) and elongation factor 1-alpha (EF1-α) sequences. The diversity of this genus in Syria has also been evaluated using a phylogenetic analysis of available ITS sequences of Syrian isolates in the GenBank.

Results Two entomopathogenic isolates, B195 and B243, were detected in the soil of agro-ecosystems in the Syrian coastal region. Morphological and molecular information revealed that these two isolates belong to *Beauveria bassiana* (Bals.) Vuill. (Hypocreales: Cordycipitaceae) with 514 bp and 284 bp for the sequences of each isolate for ITS and EF1-α, respectively. Pathogenicity test showed 100% mortality of *Galleria mellonella* L. larvae 2–3 days post-fungal exposure for both isolates. The phylogenetic tree showed that all Syrian sequences of *Beauveria* clustered within the species *B. bassiana*, with a considerable intraspecific diversity, except for two isolates previously identified as *B. bassiana*, which are closely related to *Beuveria pseudobassiana* S.A. Rehner and Humber.

Conclusions This study presents a morpho-molecular characterization of two Syrian soil-sourced *B. bassiana* isolates highly pathogenic to *G. mellonella* larvae and clarifies their phylogenetic placement. Depending on our findings, further exploration studies of the genus *Beauveria* in Syria are still needed to better our understanding of the diversity and distribution of this entomopathogen in Syria.

Keywords *Beauveria bassiana*, Morpho-molecular characterization, *Galleria* bait method, Phylogenetic analysis, Syria

Background

Beauveria (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) comprises more than 20 entomopathogenic species that cause diseases in several invertebrate member groups (Rehner et al. 2011). The number of *Beauveria* species has steadily increased in recent years with the advent of molecular techniques (Wang et al. 2022). Around the world, this fungus has been isolated from the host's cadavers, soils (Inglis et al. 2001), and plant tissues (Ownley et al. 2010). Soils are the traditional environment to isolate entomopathogenic hypocrealean fungi, including *Beauveria* species (Hajek 1997), because they spend a part of their life cycle in the soil when the host is absent and form soil-borne molds (Rehner 2005).

In Syria, there is a serious lack of information about entomopathogenic fungi (EPFs) regarding their diversity, geographical distribution, host range, and optimum

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conditions to cause natural epizootics. The greatest number of local studies depended on the zigzag pattern of conidiophores and the spherical to semi-round spores to identify the fungus to species level, which represent, according to Rehner (2005) and Imoulan et al. (2017), general characteristics of the genus *Beauveria*. The accurate identification of most *Beauveria* isolates to species level requires molecular analyses due to the fact that most *Beauveria* species are morphologically indistinguishable and lack major variation (Al Khoury et al. 2021). Several molecular markers are used to identify the fungal isolates and strains in this genus, using in most cases multiple gene regions to support the result, among which the nuclear ribosomal internal transcribed spacer region (ITS), the translation elongation factor 1-alpha (EF1-α) gene, and the Bloc nuclear intergenic region (Bloc) are the most common loci for molecular diagnosis of *Beauveria* species (Wang et al. 2022).

There is a lack of information about the diversity of *Beauveria* species in Syria. Hence, this study aimed to assess the morphological and molecular characterization of *Beauveria* isolates from soil samples of the coastal region of Syria for the development of bio-pesticides, in addition to investigating the phylogenetic placement of the isolates with regard to the available Syrian sequences of *Beauveria* species.

Methods

Isolation

Two *Beauveria* isolates were obtained from local soils using larvae of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), as a bait (Galleria Bait Method) (Meyling 2007); B195, which was isolated in 2018 from olive orchard soil (*Olea europea* L.: Oleaceae), (98 m above sea level), at Al-Shabatliyah, Latakia, Syria (35°41′ 10.6′′ N, 35°49′ 36.6′′ E), and B243, isolated in 2018 from Avocado tree soil (*Persea americana* Mill.: Lauraceae), (265 m above sea level), at Farsh village, Baniyas, Tartus, Syria (35°11′ 21.5′′ N, 36°00′19.9′′ E).

Larvae cadavers covered with fungal outgrowth were used immediately to isolate and purify the fungi using Potato Dextrose Agar medium (PDA; Titan Biotech LTD.) to which the antibiotic amoxicillin was added, whereas the cadavers without fungal outgrowth were surface-sterilized with 2% *w*/*v* sodium hypochlorite for 1 min, washed with sterile distilled water, and incubated on PDA plates at 25 ± 1 °C.

After purification of the fungal isolates, the last two larval instars of *G. mellonella* were used to test the fungal pathogenicity. Larvae were directly rolled on the surface of 14-day fungal cultures and left on these plates for about 30 min (or on PDA plates without any fungal growth for control treatment) and then kept in darkness in sterile Petri dishes with moist filter paper at 25 ± 1 °C with daily monitoring (Shin et al. 2013). Five replicates (5–6 larvae each) were prepared, and mortality (%) was recorded daily. *Galleria mellonella* was reared on an artificial diet (modified of diet B of Lee et al. (2007); composited from honey, malt, wheat bran, glycerin, multi vitamin, yeast, water, and pollen grains) to get the wanted larval stages of *G. mellonella* for the pathogenicity test. The most appropriate larval instar is one to two days after molting into the last larval instar (approximately four week old larvae) to avoid pupating before the end of the experiment (Lacey 2012).

Morphological characterization

Features of conidia, conidiophores, conidiogenous cells, and blastospores were characterized under 600X and 1000X magnification, in addition to features of the fungal cultures grown on PDA, including appearance, texture, elevation, color, and growth diameter at day 14. Conidiogenous cells and conidia (at least 100 units each) were measured on the 7th day from PDA cultures, whereas blastospores were measured on the 2nd and 3rd days from Potato Dextrose Broth (PDB: HIMEDIA®, Pvt. Ltd. India) cultures incubated with stirring at 25 ± 2 °C on a magnetic stirrer (LED digital 7′′ hotplate magnetic stirrer (DALB), MS7-H550-S, USA). Morphological studies were performed based on Inglis et al. (2012).

Molecular characterization

The identification of the EPFs isolates was confirmed molecularly. Two nuclear regions were amplified, the ITS region (the nuclear ribosomal internal transcribed spacer region) and EF1-α region (the translation elongation factor 1-alpha). DNA extraction was performed using the modified CTAB method (cetyltrimethylammonium bromide) (Murray and Thompson 1980) as detailed in Habib et al. (2021). DNA quantity and integrity were examined using Nanodrop spectrophotometer (Shimadzu, Japan) and by electrophoretic separation on 1% agarose gel using a standard 1-kb DNA Ladder (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Polymerase chain reaction amplification and sequencing were performed as described by Rajab et al. (2023). The primers used for PCR amplification were ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAAC AAGG-3′) primers for amplification of ITS region (White et al. 1990) and EF1-986R (5′ -TA CTTGAA GGA ACC CTT ACC-3′) and EF1-728F (5′ -CA TCG AGA AGT TCGAGA AGG-3′) primers for amplification of EF1-α region (Carbone and Kohn 1999). The protocol thermocycler for the PCR amplification of the EF1-α region was as follows: one cycle at 95 °C for 5 min (initial denaturation), 30 cycles at 95 °C for 1 min, followed by 57 °C for 75

s, and 72 °C for 1 min for each of denaturation, annealing, and extension, respectively. The final extension was 72 °C for 10 min. The PCR conditions for the ITS region were as follows: one cycle at 95 °C for 5 min, 25 cycles at 95 °C for 1 min, followed by 58 °C for 1 min, 72 °C for 1 min, then 75 °C for 7 min. The kit of QIAquick PCR amplification (Qiagen) was used to purify the PCR products, and Sanger sequencing was used to sequence them. The obtained sequences were subjected to BLASTn analyses to compare with accessions in the GenBank database to detect the most likely taxonomic identification.

Phylogenetic analysis

A phylogenetic analysis was performed to show the relationships between the sequences obtained in this study and the available Syrian sequences of *Beauveria* species in GenBank. The largest number of *Beauveria* sequences from Syria was analyzed depending on the ITS region, so the phylogenetic analysis was run using only ITS sequences. In addition to the Syrian sequences, sequences representing 3 recognized isolates of *B. bassiana* and 12 *Beauveria* species were used to build the phylogenetic tree. The tree was rooted using *Cordyceps cicadae* (Miq.) Massee (Hypocreales: Cordycipitaceae) as an out-group taxon (Additional file 1: Table S1). The alignment for the ITS sequences was implemented in the MEGA 11 software, using MUSCLE (Edgar 2004) along with the reference sequences. Maximum likelihood analysis (Kumar et al. 2018) was used to construct the phylogenetic tree with 1000 bootstrap replications, removing gaps, and the General Time Reversible Model substitution model Gamma distributed (Nei and Kumar 2000).

Results

Isolation and characterization

The two *Beauveria* isolates, B195 and B243, were obtained from all larvae used in the trap. Most of the dead larvae of *G. mellonella* were covered with white fungal structures (Fig. 1A). In some cases, hyphal growth radiated out from the cadavers and spread into the surrounding environment (Fig. 1B). Both B195 and B243 isolates showed 100% mortality in the result of the pathogenicity test, which was performed using *G. mellonella* as a model insect, after 2–3 days post-treatment. The fungus outgrew and built up biomass on dead larvae 4–6 days later (Fig. 1C and D).

The EPF isolates were initially identified as more related to the species *B. bassiana* based on morphological characters and further confirmed by molecular identification using ITS and EF1-α regions. Morphological features were similar between the two isolates but showed a few variations in the characteristics of colonies and conidia within the isolate with every re-culturing. Conidia averaged 1.95 × 1.25 μm (1.8–2.9 × 0.9–1.7 μm), mostly taking a spherical shape and sometimes forming ellipsoidal one. Conidia were one-celled, hyaline, and placed alternately on the zigzag pattern conidiophore, which in turn was toothed (denticulate), formed from the top of subglobose to flask-like conidiogenous cells (2.5–5 μm wide and 3–6 μm length) (Fig. 2B and C). Blastospores were oblong to cylindrical, one-celled, single, or in short chains, averaging 3.23 μm (1.87–5.18 μm length) (Fig. 2D, E, F).

Colonies growth was slow, as the radial growth of fungal mycelia ranged between 53.6 and 60.2 mm at 25 °C after 14 days of incubation. In most cultures, the fungus tends to form many small colonies on the plate due to the ease with which the spores detach from the conidiophores, spread, and form a new colony. Colonies were semi-rounded, white or off-white on the upper side and yellowish on the reverse side, cottony or woolly in texture, which turned powdery with dense sporulation. The elevation varied from a flat surface in some colonies to aerial, erect fascicles of hyphae in others (Fig. 2A).

Sequences of ITS and EF1-α (514 bp and 284 bp, respectively, for both isolates) were deposited on the portal of the National Center for Biotechnology Information (NCBI) with the accession numbers, respectively, OM302229 and OP573422 for the isolate B195, and OM302230 and OP573423 for the isolate B243.

Sequences were subjected to BLASTn analyses and possessed 100% identity with the ITS sequences of *B. bassiana*. The isolate B195 possessed 100% identity with the soil-sourced Lebanese isolate BbL_1, accession number MT533246; the soil-sourced Iranian isolate SHU.M.161, accession number KU158472; the soil-sourced Turkish isolate BbMg-5, accession number MW255014; and the endophytic Syrian strain BNE19, accession number OM302227; and 99.81% with the endophytic Syrian strains (the strain BNE20, accession number OM302228; and the strain BNE14, accession number OM302224).

The isolate B243 possessed 100% identity with the soil-sourced Iranian isolate SHU.M.111, accession number KU158450; the endophytic Syrian strains (the strain BNE16, accession number OM302225; and the strain BNE18, accession number OM302226); and the Kenyan isolate IMI 386701, accession number AJ560668.

EF1-α sequences, for both isolates, possessed 98.94% identity with the Turkish strain of *B. bassiana*, Ya2, accession number MK550628; the isolate 1811, accession number AY531901; and 98.59% with the endophytic Syrian strains (the strain BNE10, accession number OP573414; BNE16, accession number OP573418; and the strain BNE18, accession number OP573419); the strain ARSEF 5987, accession number KJ500423; and the strain ARSEF 7247, accession number AY883706.

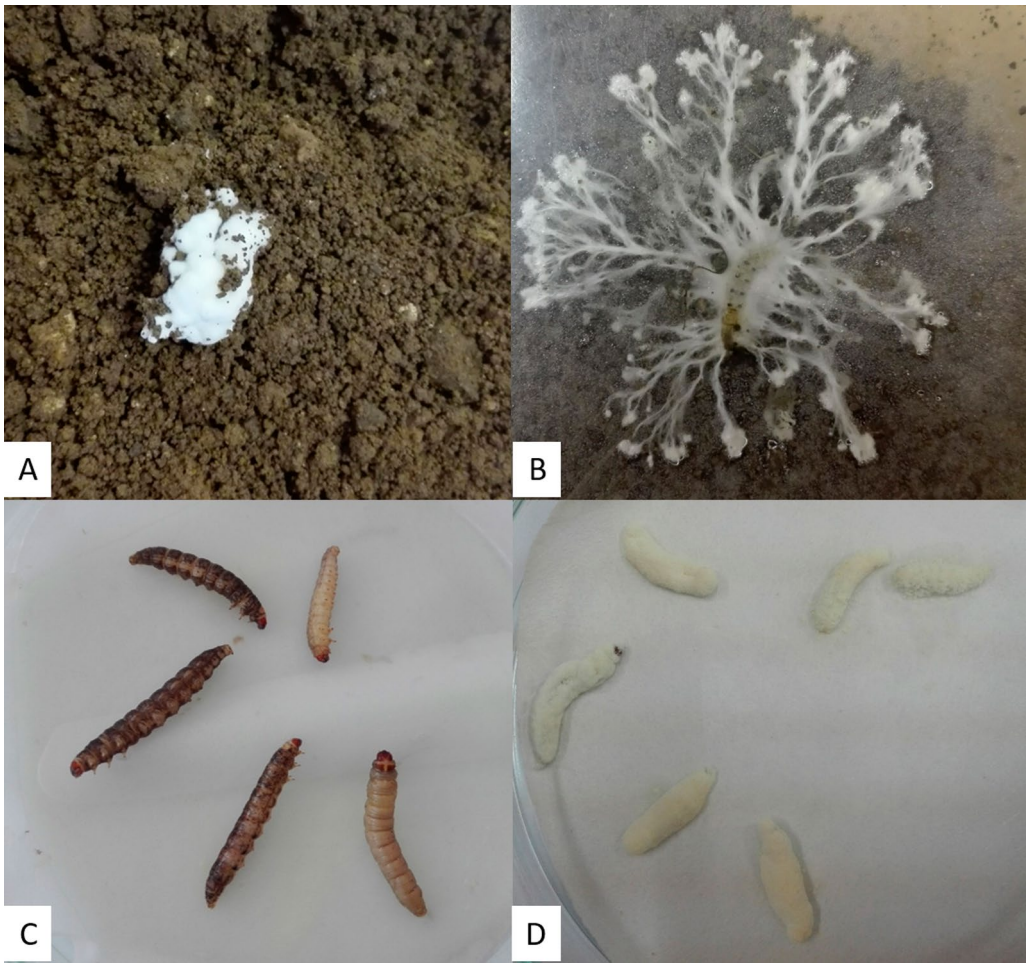

Fig. 1 *Galleria mellonella* larvae infected by the fungus *Beauveria bassiana*. **A**: Cadavers covered with fungal mycelium and conidia in the soil trap method. **B**: Spread of fungal hyphae far beyond cadavers through the surrounding soil in the trap method. **C**: Dead *G. mellonella* larvae after 2–3 days post-pathogenicity test with melanin areas. **D**: Dead larvae covered with the fungal growth in full sporulation 4–6 days after pathogenicity test

Phylogenetic analysis

The phylogenetic tree (Fig. 3) showed that all Syrian isolates and strains (21 in total; including the current study isolates) are related to each other and to the three recognized isolates of *B. bassiana* (GHA, 1811, and ARSEF 1564), and split from the other *Beauveria* species, except the isolates IMI 319043 and IMI 319361, which were more closely related to the isolate ARSEF 7242 representing *Beauveria pseudobassiana* S.A. Rehner & Humber. Because of that, their sequences (accession numbers, respectively, EU086423 and EU086432) were subjected to BLASTn analyses and compared to accessions in the GenBank database and possessed 100% identity with the ITS sequences of *B. pseudobassiana* (the isolate SUAn12, accession number MT241786; the isolate SUAb28, accession number MT239436; the isolate SUAf76, accession number MT239435; the isolate C5, accession number MK142275; and the strain NREP099, accession number MK490877).

The pattern of branching reflected that the isolate IMI 391362 was more closely related to isolate ARSEF 1564 and less closely related to the other Syrian isolates. The different numbers of clades identified reflected a substantial degree of diversity. The isolate B243 clustered with the endophytic strains BNE10, BNE16, and BNE18 as a monophyletic group and clustered with the strain BNE20 as a paraphyletic group. The isolate B195 clustered with the strain BNE19 and the isolate bbph2 as a monophyletic group and with the isolates SPT2-321/1 and 1811 as a paraphyletic group. The isolates/strains BNE11, BNE14, and SPT3-372 clustered with the commercial strain GHA as a monophyletic group (Fig. 3).

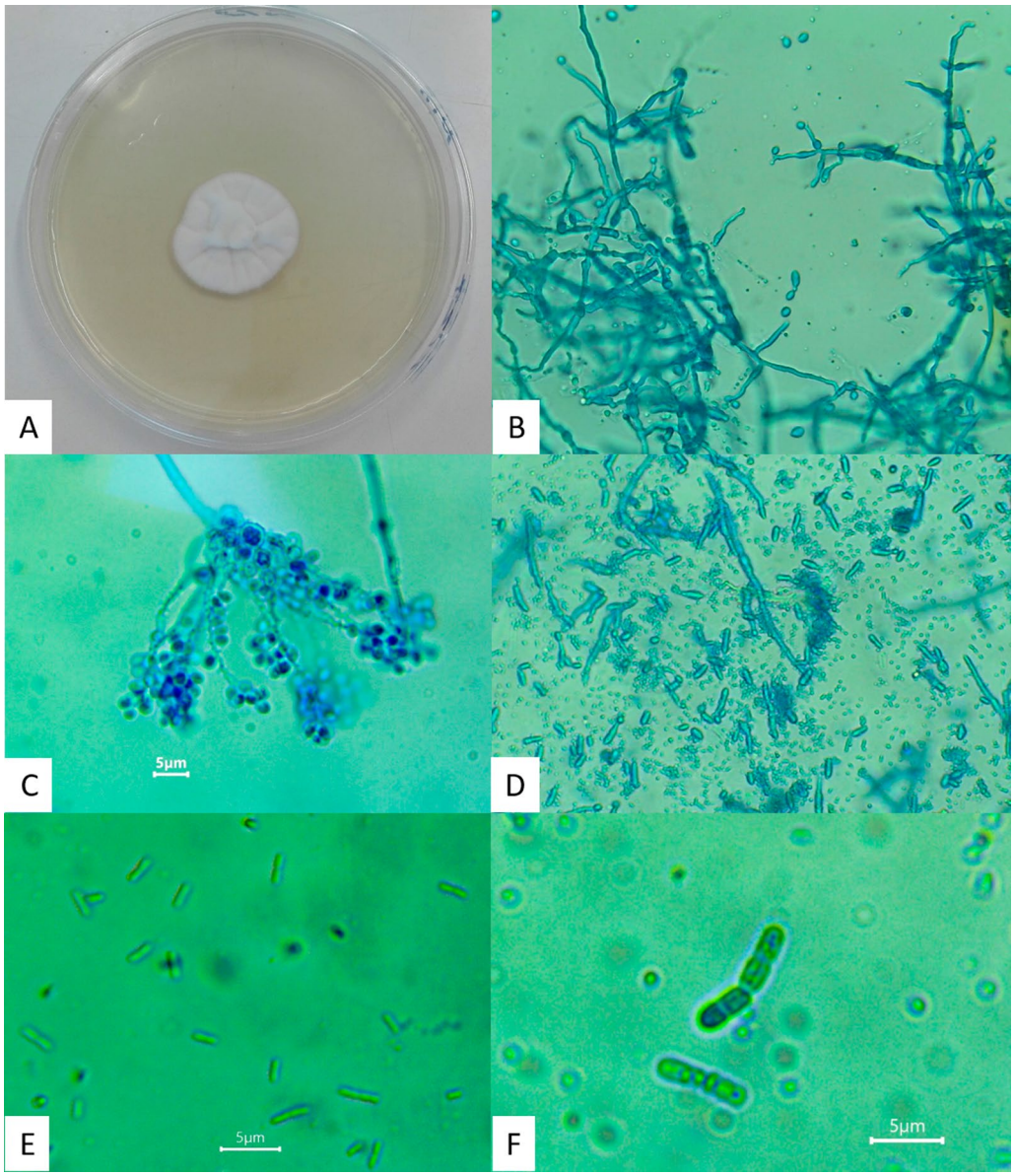

Fig. 2 Morphological characters of the fungus *Beauveria bassiana*. **A**: Fungal colony on PDA on day 10. **B**: Hyphae and conidiogenous cells at the day 4th (60X). **C**: Conidia and toothed conidiophores (100X). **D**, **E**, and **F**: Oblong to cylindrical blastospores (100X)

Discussion

The entomopathogenic *Beauveria* isolates, B195 and B243, were characterized using morphological and molecular characteristics from Syrian soils. Two gene sequences, ITS and EF1-α, strongly support that these isolates were distinct within the species *B. bassiana*. The morphological study showed a high degree of similarities between the two isolates in their microscopic traits such as shape and size of conidia, blastospores, and conidiogenous cells as well as traits specific to fungal cultures on PDA such as growth pattern, appearance, elevation, and color.

These morphological features were the main traits responsible for species recognition in *Beauveria* until the beginning of using molecular techniques in the identification of EPFs in 1990 (Imoulan et al. 2017), and the changes in microscopic and cultural characteristics within the species after culturing and re-culturing and with every passing in insects increase the misidentification to species level based on morphological features.

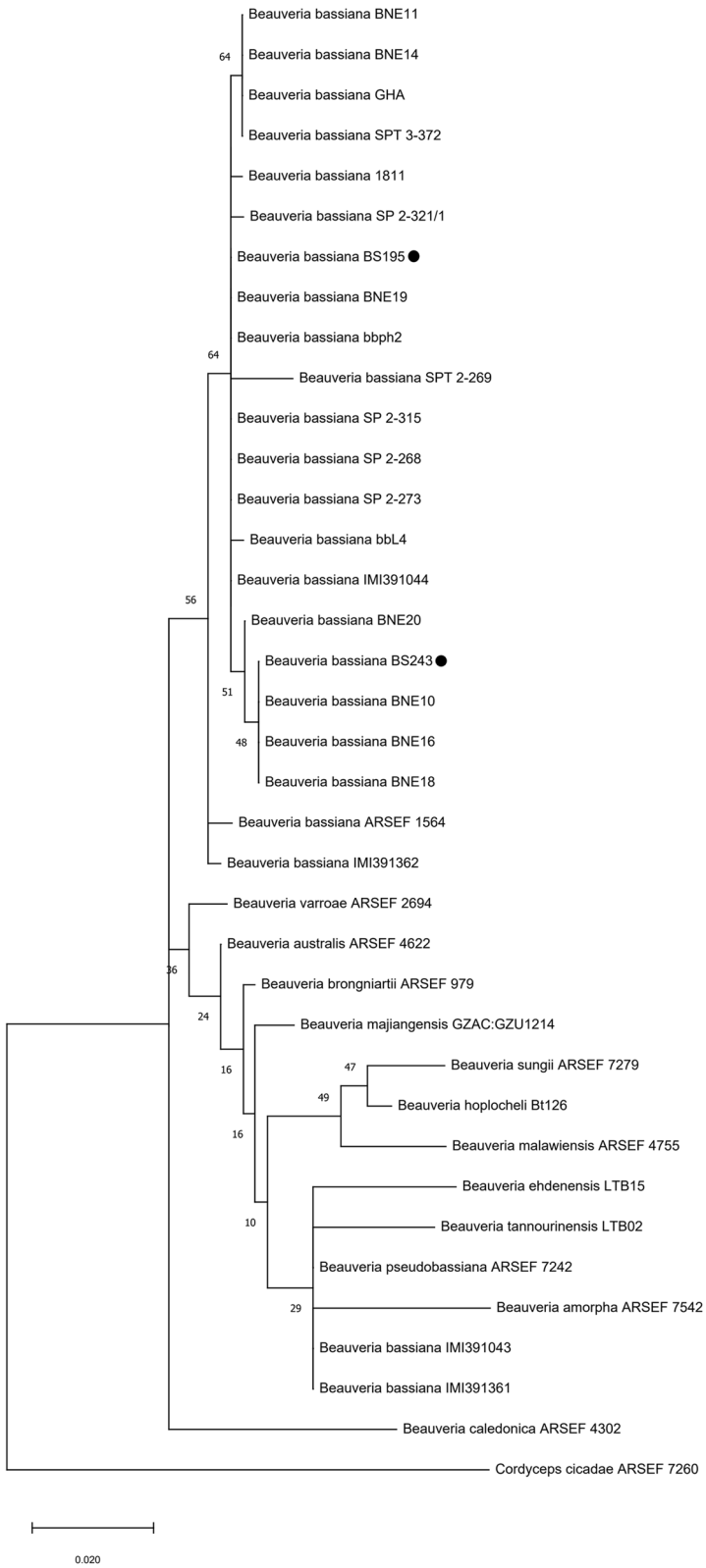

Fig. 3 Phylogenetic tree of ITS for the Syrian *Beauveria bassiana* isolates (the pointed isolates represent the current study sequences) and sequences of three recognized *B. bassiana* isolates and 12 other *Beauveria* species. The phylogenetic tree was constructed using the maximum likelihood method and the general time reversible model. *Cordyceps cicadae* was displayed as an out-group taxon

The ITS and EF1-α regions are extensively used to identify *Beauveria* species worldwide and have contributed for a long time to understanding interspecific variation. The majority of recent taxonomic investigations, however, revealed that the ITS region is limited in resolving some species of the genus *Beauveria* (Al Khoury et al. 2021), and most of the recent studies strongly support the theory that recommends multilocus genes to diagnose *Beauveria* species (Chaithra et al. 2022). Due to our phylogenetic analysis results, which showed that two Syrian isolates (IMI319043 and IMI319361) were more closely related to the species *B. pseudobassiana* and more distantly related to *B. bassiana*, it was believed that it might be necessary to use more than one gene region to identify the *Beauveria* species to avoid misidentification of species. According to Imoulan (2017), there are 17 species of *Beauveria* identified so far based on multilocus analyses.

During the last decade, some limited surveys were carried out in Syria aiming to isolate the fungus *Beauveria* from the soils, insects, or plants in different regions of Syria (Alali et al. 2019; Rajab et al. 2023). The notable dominance of the species *B. bassiana* in these surveys is not surprising considering the wide geographic and host range of this species. However, the low number of *Beauveria* species reported from Syria is questionable, especially taking into account the diversity in climatic regions and the geographic nature of this country. This could be partially explained by possible misidentifications in some studies or the absence of extensive bio-exploration surveys. Therefore, more extensive surveys using reliable identification techniques (multigenes) are needed to explore the complete diversity of the genus *Beauveria* in Syria.

Various studies focused on the use of *B. bassiana* in the biocontrol of locally recurring pests such as *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) (Rajab 2017), *Tetranychus urticae* Koch (Acari: Tetranychidae) (Ahmad et al. 2018), *Rhynchophorus ferruginus* Olivier (Coleoptera: Curculionidae) (Kadour et al. 2014) and *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) (Trissi et al. 2019). However, it is notable that most of these studies were restricted to laboratory experiments. Therefore, future research using this entomopathogen should focus on field application in greenhouses and open fields. Moreover, the efficacy of native isolates (such as those obtained in this study) should be evaluated against newly introduced (invasive) pests in the country such as the fall armyworm *Spodoptera frugiperda* Smith & Abbot (Lepidoptera: Noctuidae) (Heinoun et al. 2021), and the tomato red spider mite, *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae) (Dayoub et al. 2022), which proved challenging to control using traditional methods.

Conclusion

Global research on the diversity and distribution of the EPFs, genus *Beauveria*, has been significantly influenced by the rapid development of the molecular techniques because most *Beauveria* species lack distinct morphological characters. In Syria, the diversity of *Beauveria* species is not well explored. This study provided a morphological and molecular characterization of two *B. bassiana* isolates collected from soil samples of the coastal region of Syria with a high pathogenicity against *G. mellonella* larvae. The phylogenetic analysis illustrated poor diversity of this genus in Syria, but a considerable intraspecific diversity of *B. bassiana*. Due to the findings of this study, further phylogenetic explorations of the genus *Beauveria* in Syria should be investigated.

Abbreviations

BLASTn	Basic local alignment search tool
Bloc	The Bloc nuclear intergenic region
CTAB	Cetyltrimethylammonium bromide
EF1-α	The translation elongation factor 1-alpha gene
EPFs	Entomopathogenic fungi
ITS	The nuclear ribosomal internal transcribed spacer region
NCBI	The portal of the National Center for Biotechnology Information
PDA	Potato dextrose agar medium

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-024-00772-w.

Additional file 1. A list of sequences representing the isolates of *Beauveria* species used to analyze the phylogenetic position of Syrian *Beauveria* isolates with information on their origin, host/source, country, and ITS GenBank accession numbers.

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Author contributions

LR conceived and designed the study, performed the laboratory work, analyzed and interpreted the data, and drafted the work. MA and IG made contributions to reviewing and editing the paper.

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Availability of data and materials

All data generated or analyzed during this study are included in the text.

Declarations

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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