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Research Article

Isolation of entomopathogenic fungi from cultivated and uncultivated soils and evaluation of virulence against cowpea beetle, *Callosobruchus maculatus* (F.) (Coleoptera:Chrysomelidae)

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Abstract

We aimed to explore the entomopathogenic fungi (EPF) from the different ecosystems including forests, gardens, fields, and rangeland soils in Kermanshah province, Iran. Trials were carried out on morphological, molecular characterization, diversity indices, and virulence assessments of indigenous EPF from 41 sampling sites of various localities. Using the *Ephestia kuehniella* (Zeller) as host bait, 114 fungal isolates were recovered-i.e., 39 from forests, 38 from fields, 22 from rangelands, and 15 from garden soils. Based on morphological features and the sequence analysis of the internal transcribed spacer (ITS) of the *ribosomal* DNA, the recovered entomopathogenic fungi were identified as *Alternaria chlamydosporigena*, *Aspergillus nomius*, *Beauveria bassiana*, *B. pseudobassiana*, *B. brongniartii*, *Chaetomium elatum*, *Fusarium equiseti*, *F. oxysporum*, *Fusarium* sp., *Meyerozyma guilliermondii*, *Paramyrothecium roridum*, *Penicillium sizovae*, *P. solitum* and *Penicillium* sp.. Higher species richness was found in the oak forest soils compared with fields, gardens, and rangelands. Additionally, the oak forest soils had high values of diversity indices, i.e., Simpson Ds (0.97), Shannon (3.30), Equitability (0.69), and Fisher's alpha (25.8). The dominance index was higher in the rangelands compared with the others. Following the priliminary assays, the insecticidal activity of three selected EPF isolates, including *Beauveria brongniartii*, *B. bassiana*, and *B. pseudobassiana* was tested against adults of the cowpea beetle, *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae: Bruchinae) as host. Hypervirulent strains caused high mortality considered as promising effective biocontrol agents in insect pest management programs.

Key words: Molecular identification, Fungal biodiversity, Local entomopathogenic fungi, virulence, Iran

جداسازی قارچ‌های بیمارگر حشرات از خاک‌های زراعی و بکر و ارزیابی بیمارگری آنها روی سوسک لوبیا *Callosobruchus maculatus* (Coleoptera:Chrysomelidae)

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چکیده

هدف این مطالعه بررسی قارچ‌های بیماری‌زای حشرات مرتبط با مناطق مختلف اکولوژیکی از جمله جنگل‌ها، باغ‌ها، مزارع و خاک‌های مرتعی استان کرمانشاه بود. برای ارزیابی‌های مورفولوژیکی، مولکولی، شاخص تنوع و ارزیابی بیماری‌زایی قارچ‌های بیماری‌زای حشرات بومی، نمونه برداری از ۴۱ منطقه مختلف انجام شد. با استفاده از روش طعمه‌گذاری با استفاده از لاروهای *Ephestia kuehniella* (Zeller)، 114جدایه قارچ که ۳۹ جدایه از جنگل‌ها، ۳۸ جدایه از مزارع، ۲۲ جدایه از مراتع و ۱۵ جدایه از خاک‌های باغی بودند، بدست آمد. بر اساس ویژگی‌های مورفولوژیکی و مطالعه توالی‌های نسخه برداری

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شده داخلی DNA (ITS) ریبوزومی، قارچ‌های بدست آمده شامل گونه‌های *Alternaria chlamydosporigen*، *Chaetomium elatum*، *B. brongniartium*، *B. pseudobassiana*، *Beauveria bassiana*، *Aspergillus nomius*، *Fusarium equiseti*، *F. oxysporum*، *Fusarium* sp.، *Meyerozyma guilliermondii*، *Paramyrothecium roridum*، *Penicillium* sp.، *Penicillium sizovae* و *P. solitum* بودند. نتایج حاصله از این مطالعه نشان داد غنای گونه‌ای در خاک‌های جنگل بلوط بیشتر از مناطق دیگر و خاک‌های زراعی، باغی و مرتعی به ترتیب در ردیف‌های بعدی قرار داشتند. علاوه بر این، خاک‌های جنگلی بلوط دارای مقادیر بالایی از شاخص‌های تنوع بودند، که شاخص سیمپسون ۰/۹۷، شاخص شانون ۳،۳۰، شاخص تعادل ۰/۶۹ و فیشر آلفا ۲۵،۸. شاخص دومینانس در مراتع نسبت به سایرین بیشتر بود. پس از سنجش اولیه، فعالیت حشره‌کشی سه جدایه منتخب از قارچ‌های بیمارگر شامل، *Beauveria brongniartii*، *B. bassiana* و *B. pseudobassiana* روی حشره بالغ سوسک لوبیا چشم بلبلی، *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae) به‌عنوان میزبان جدید مورد آزمایش قرار گرفت. از نظر شاخص حشره‌کشی جدایه‌های مورد مطالعه همگی بیمارگری بالایی روی حشرات بالغ سوسک لوبیا نشان دادند. مرگ و میر حشرات مورد آزمایش وابسته به دوز بود. نتایج این مطالعه نشان داد کاربرد این قارچ‌های بومی به عنوان عوامل کنترل زیستی موثر برای برنامه های مدیریت آفات امیدوار کننده هستند.

واژه‌های کلیدی: شناسایی مولکولی، تنوع زیستی قارچ‌ها، قارچ‌های بومی بیمارگر حشرات، بیمارگری، ایران

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Introduction

Microbial organisms like soil-inhabiting fungi have an essential role in the soil functions and provide ecosystem services, including crucial pest control (Sommermann *et al*., 2018). The soil habitat constitutes an essential reservoir for diverse entomopathogenic fungi (EPF) (Keller *et al*., 1989). The entomopathogenic fungi (EPF) within the order Hypocreales (Ascomycota) are phylogenetically related to phytopathogens (Hu *et al*., 2014). Furthermore, EPF are taxonomically diverse and differ in their host specificity, genomic features, life strategies, and infection cycles (Shang *et al*., 2015). Soil is considered the best reservoir of fungi due to the protection of EPF from environmental stress factors such as UV radiation (Keller *et al.*, 1989; Jaronski, 2010). *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) (Mantzoukas *et al*., 2020) and *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) larvae (Kurtuluş *et al.*, 2020) are both prevalent host baits for detecting and trapping EPF from the soil.

Identifying EPF based on molecular techniques is necessary to precise identification and proper selection of isolates and species for successful identification of potential biocontrol agents. (Gebremariam *et al*., 2021). The internal transcribed spacer ITS1-5.8S-ITS4 region of ribosomal DNA is the most widely used for identifying EPF species (i.e., *Beauveria* spp.) (Gardes & Bruns 1993, Barra *et al*., 2013). Earlier studies have shown that diversity and activity were more greatly affected in cultivated than in natural ecosystems (Meyling & Eilenberg, 2007; Sun & Liu, 2008). Furthermore, information concerning indigenous species' diversity and dispersal patterns is imperative for determining their abundance, conservation status, and environmental requirements (Evangelista *et al*., 2018). One of the primarily

cosmopolitan insect pests of beans (cowpea, beans, peas, gram, broad bean, and lentil) is the cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae: Bruchinae) (Singh & Emden, 1979). *Callosobruchus maculatus* not only causes significant quantitative and qualitative losses by feeding and feces contamination in storage facilities (Hagstrum *et al.*, 2012), but also has been shown *resistance* to commercial *pesticides* (Kang *et al*., 2013).

Using EPF for management of destructive insects requires the selection of the most effective isolates (Lord, 2001; Dal Bello *et al.*, 2018). Indigenous strains of EPF have ecological compatibility with insect pests, positive effects on the local environment, lessened pesticide residues in food, reduced risk of significant impact on non-target organisms, and increased biodiversity in managed ecosystems (Inglis *et al.*, 2001; Pourian & Alizadeh, 2021). Some species of Hypocrealean EPF, including *Beauveria bassiana* (Bals.) Vuill. (Cordycipitaceae), and *Metarhizium anisopliae* (Metschinkoff) Sorokin (Clavicipitaceae), have been widely used for management of different insect pests including *C. maculatus* (Cherry *et al.*, 2005; Khoobdel *et al*., 2019; Pourian & Alizadeh, 2021). Biotic and non-biotic factors such as plant community, microbiome community, temperature, vegetation, rainfall, soil texture, soil organic matter, moisture content, and host availability significantly influence the distribution and abundance of EPF in the soil (Burgess & Summerell, 1992), as well geographic regions in different areas through adjusting to particular environmental parameters (Meyling *et al.*, 2009). These factors can affect the efficacy and persistence of EPF treatments against pests (Jaronski, 2010). Therefore, a more thorough understanding of EPF biodiversity is needed to find the optimal conditions for release into the agroecosystem for pest biological control purposes (Lacey *et al.*, 2015). Besides possessing considerable genetic diversity, indigenous isolates of EPF from different localities have been shown to have ample reservoirs of potential biocontrol tools upon damaging insects within the *agroecosystems* (Inglis *et al*., 2000; Gulsar Banu *et al*., 2004). Hence, we hypothesized that trapped EPF from the different ecological zones with high diversity indices possess an acceptable potential for control of key insect pests such as stored product beetles when utilized in a biological control program. The present study aimed to isolate and identify EPF isolates from the soil of four different geographical locations as well as estimate their species diversity and in vivo virulence against *C. maculatus.*

Materials and methods

Soil sampling

Forty one soil samples were collected from cultivated and uncultivated ecosystems categorized into four areas: rangeland (14 regions), field crop (13 regions), oak forest (eight regions), and garden (six regions) to survey for EPF in Kermanshah province, West Iran from July 2017 to April 2018 (Table 1, Fig. 1). These various sites (n=41) were chosen to represent soil type and climate variations (Parsa & Maleki, 1978). According to Booth (1971) all

instruments were first sprayed and/or dipped in fresh 10% sodium hypochlorite solution (30-40 s) and then rinsed in pure water before starting each sampling at any point. At each site, samples containing ten soil sub-samples were randomly taken at 0-15 cm depth and mixed to prepare an approximately 2 kg homogeneous sample. Soil samples were stored in 20×25 cm sterile sealed bags (Badook®), returned to the laboratory and kept at 4°C until processing up to 24 h. Finally, ten replications (200 g) from each combined soil samples were used for trapping entomopathogenic fungi. The sites' geographic attributes, including height, latitude, and longitude, were recorded using GPSMAP device model 76CSx. The sampled soils were analyzed for physicochemical parameters including $CaCO_3$ content (Loeppert & Suarez, 1996), electrical conductivity (EC), organic carbon (Walkley & Black, 1934), pH (Thomas, 1996), and texture (Gee & Bauder, 1986).

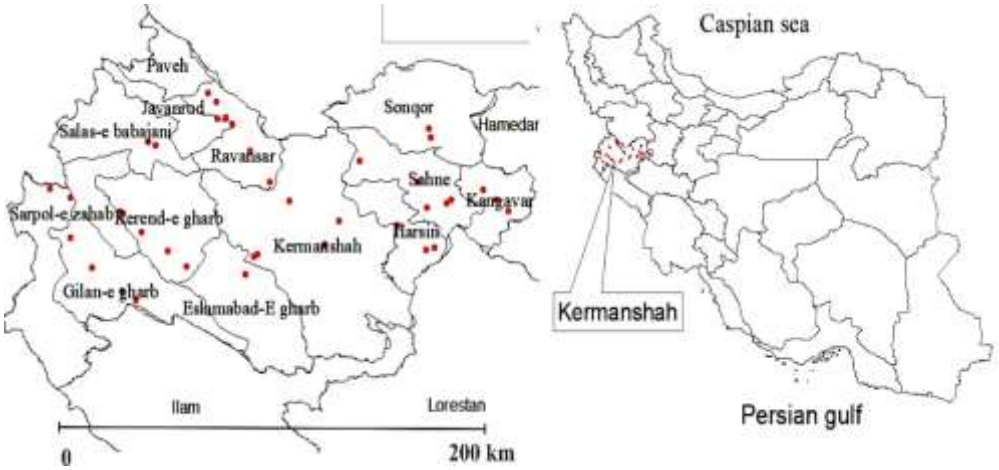

Fig. 1. Location of sampling sites for the isolation of entomopathogenic fungi in Kermanshah province, West Iran.

Isolation, identification and pathogenicity test of the isolates

The Mediterranean flour moth, *Ephestia kuehniella* (Zeller), (Lepidoptera: Pyralidae) was reared according to Kurtuluş *et al.* (2020), and fifth instar larvae were incorporated as bait (Alali *et al.*, 2019). Ten sub-samples of 200 g from each combined soil sample were poured into the autoclaved 200mLheat-resistant plastic container (8 cm dia). Ten healthy fifth instar larvae of *E. kuehniella* were added to the beakers (500 mL) and were incubated in complete darkness at 25°C and 75% humidity for one week (Zimmermann, 1986). Dead larvae were surface sterillized, and then placed on either potato dextrose agar, Sabouraud dextrose agar (SDA), malt agar (MEA) or carnation leaf agar (CLA)**.** After the fungal growth on the media plates and purification, the fungi were identified using taxonomic keys (Humber, 1997). At least 50 fungal isolates were measured using BioloMICS Measure software and photographed using an Olympus Optical microscope (Model BX51)**.** Afterward, five serial *in vivo* passages through *E. kuehniella* larvae (5^{th} instar) were conducted to demonstrate the potential pathogenicity of all recovered strains (Schleif, 2012).

After passaging, dead larvae were chosen gently and placed into a sterile Petri dish and kept under controlled conditions (25±2 °C, 65±10% R.H. and 12:12 h L: D (to provoke cadaver-derived mycosis two weeks after inoculation.

Molecular identification and phylogenetic analysis

Based on Gardes & Bruns (1993) method, whole genomic DNA was derived utilizing CTAB (Cetyltrimethyl ammonium bromide) and a genomic DNA purification Kit (Sinagen co., Iran) according to the manufacturer's instructions. The ITS1+5.8S+ITS2 of the ribosomal RNA were amplified by using *the universal primers* ITS1 (5´-TCC GTA GGT GAA CCT GCG G-3´), and ITS4 (5´-TCCTCC GCT TAT TGA TAT G-3') (White *et al.,* 1990). Refined PCR products were sequenced in forwarding adjustment using the corresponding primers as in the PCR protocol. The edited sequences were deposited at GenBank using Bankit software. The multiple alignments of edited sequences and sequences from previously published studies (Table 1) were conducted with ClustalW software (http://www.clustal. Org/download), subsequently adjusted manually using BioEdit software. Inadequately aligned wards were excluded using Gblocks software ver. 0.91b under the less rigorous parameters chosen (Castresana, 2000). Phylogenetic trees were inferred using MEGA 7 software (Guindon *et al.,* 2010). Tree topology reliability was estimated with the bootstrap technique applying 1000 replications (Felsenstein, 1985).

Fungal species richness using ecological indices

The diversity of collected EPF in different regions, altitudes, and soil types were measured using the Simpson's diversity index (d_s) and the Shannon–Wiener index (H') (Pielou, 1975). Simpson's diversity index (1-D) was calculated as 1- [$D = \sum_{i=1}^{n}(ni/n)^2$. It measures the 'evenness' of the regions from 0 to 1. The Shannon Wiener Index (H', log-based) was calculated as $\acute{H} = -\sum_{i=1}^{n} pi \ln pi$ and (p_i=n_i/n), where n_i is the number of distinct species (i) and (n) is the abundance of each species in each region. The H'value varies from 0 for communities with only a single taxon to high values for communities with many taxa, each with few individuals. Pielou's evenness (Equitability) was estimated by Shannon diversity divided by the logarithm of the number of taxa (E=H'/H_{max}), where H' is the Shannon–Wiener index and H_{max} is equal to ln(n_i). The Dominance (D) is equal to the 1-Simpson index. Ranges from 0 (all taxa are equally present) to 1 (one taxon dominates the community completely). Fisher's alpha is a diversity index, which was defined implicitly by the formula S=a×ln(1+n/a) where S is a number of taxa (richness), n is a number of individuals, and a is the Fisher's alpha.

Bioassays

There were 75 isolates obtained from 142 that showed pathogenic activity against *E. kuehniella* larvae. According to a discriminatory concentration time-mortality screening test, only three indigenous hypocralean isolates, including *B. bassiana* (strain RUF-Bb3), *B. brongniartii* (Saccardo) Petch (strain RUF-Bt1), and *B. pseudobassiana* (Bals.) Vuill (strain

RUF-Bp2), were chosen among identified species with acceptable mortality and producing the lowest LT_{50} values (Mehrmoradi, Jamali, Pourian, unpublished data). Selection of these selected hypocralean strains were judged based on host specificity and safety principles (Zimmermann, 2007b). Firstly, a cowpea beetle, *C. maculatus* colony was reared on a healthy, sterilized, adapted local cowpeas variety (*Vigna unguiculata* (L.) var. Parastoo) in the laboratory based on the methodology described by Khoobdel *et al.*, (2019). Then, 50 mL of fresh conidial suspensions (10^6 conidia mL^{-1}) were supplied for each established isolate and poured into a series of Petri dishes (14 cm in dia.) after full sporulation in media in a sterile circumstance. For host passages, ca. 50 healthy adult of *C. maculatus* were dipped to these Petri dishes for 20-30 second, then air dried and put into the sterile Petri dishes (5 cm dia). Assays chambers were incubated in complete darkness at 25°C and 75% humidity for one week. Individuals were considered dead if no movement was observed and mycoszied. In the same way, following an additional serial passage of each chosen *Beauveria* species through the adults of the cowpea beetle, then the produced conidia on cadavers were cultured on potato dextrose agar (PDA) and incubated up to 16 days under controlled conditions (25±2 °C, 65±10% R.H. and 12:12 h L: D. After 2 weeks, fully sporulated conidia on PDA were harvested and suspended in 20-ml of sterile distilled water containing autoclaved 0.02% Tween® 80. Conidial concentrations were calculated using a Neubauer hemocytometer. Like Pourian *et al*. (2008), germination percentage was calculated by counting 100 conidia on each PDA plate following 20 hours of incubation and then 48 hours (for slow-germinated but viable conidia) at 25±2°C. The fresh conidial germination rate of > 85-90% on PDA was assessed after a 20 h incubation at 25±2°C.

Following the bracketing tests, we selected concentrations of 2.04× (10^4, 10^5, 10^6, and 10^7) conidia mL^{-1} for *B. bassiana*, 1.5× (10^4, 10^5, 10^6 and 10^7) conidia mL^{-1} for *B. brongniartii*, and 1.4× (10^4, 10^5, 10^6 and 10^7) conidia mL^{-1} for *B. pseudobassiana*. Four ventilated Petri dishes (14 cm in diam.) were used as bioassay chambers for each dose rate. Replications of sixty adults of *C. maculatus* (five replicates of 12 individuals) were placed separately in thin mesh and treated with each isolate by direct immersion in the conidial suspension for 10 seconds (Kalvnadi *et al.*, 2018). An additional sixty adults were immersed in sterile distilled water containing 0.02% Tween 80 as a control. Treated adult beetles for each isolate were gently transferred to clear Petri dishes (14cm diam.) in five groups of 12, with each dish containing 10 g of untreated sterile seeds as food and incubated in a controlled chamber (25±2 °C, 60±5% RH, and 12:12 h L: D). Mortality was recorded daily for up to ten days post-treatment. For each Beauveria isolate, values of LC_{50}, LT_{50} and toxicity index results (Sun, 1950, Kaur & Padmaja, 2008a) were incorporated for select fast and hypervirulent strains. The entire bioassay experiment was replicated twice.

Statistical analysis

Ten days of cumulative mortality of the corresponding assay were corrected for control mortality using Schneider Orelli's formula (Püntener, 1981). Mortality results were subjected to probit analysis for estimation of lethal median time (LT_{50}) and concentration (LC_{50}) using the PoloPlus ver.2.0 software program (LeOra Software, 2018). The toxicity index was calculated by dividing the LT_{50} of each control with that of the treatment (Sun, 1950). Different strains' virulence potency was categorized based on an arbitrary rating (Kaur & Padmaja, 2008b). Diversity indices were calculated by software's Stats Direct *ver 3.2* and SDR (Species Diversity Richness) software package *ver.* 4.0 with 10000 bootstraps (Freemantle, 2000). Resampling was conducted to compare the efficiency of indices using bootstrap. A 95% bootstrap confidence interval of diversity indices generated by SDR software was used to make comparisons. If those intervals overlap, they assume that the difference between areas is not statistically significant at a level of 0.05 (MacGregor-Fors & Payton, 2013). All other computations, if needed, were made using SYSTAT *ver* 13.2 (SYSTAT Software, San Jose, CA). To examine the relationship between EFPs and environmental variables with a unimodal response, detrended correspondence analysis (DCA) was done to define the range of the ecological gradient (Gauch & Wentworth, 1976). Before starting canonical correspondence analysis (CCA), the ordination analyses were done in CANOCO for Windows *ver. 4.5* (Ter Braak & Smilauer, 2002).

Results

Sampling

Of recovered strains from four different ecological zone, 15 isolates were obtained from garden soils (six regions), 39 isolates from oak forest soils (eight regions), 38 isolates from field soils (13 regions), and 22 isolates from rangeland soils (14 regions). Among the identified genera, *Fusarium* (40.3.%) represented the dominant genus, followed by *Aspergillus* (24.6%), *Penicillum* (20.17%), and *Beauveria* (11.4%). Other fungi were *Meyerozyma* at 0.88%, *Alternaria* at 0.87%, *Chaetomium* at 0.87%, and *Paramyrothecium* at 0.87% with less frequency. Putative EPF were found in 17.07% of the soil samples (7 from 41 samples). Among the putative EPF, *B. bassiana* and *B. pseudobassiana* were the most common species. The frequency of *Beauveria* in forest soils was more than other soils and with a ratio of 23%. About 50% of forest soils had *Beauveria* fungus. This genus was not isolated in orchard soils.

Molecular study

The ITS1-5.8s-ITS2 region of genomic DNAs was successfully amplified using ITS4 and ITS1 universal primers from 20 representative isolates. Results of the sequencing and search for similar ITS1-5.8s-ITS2 sequences in the GenBank DNA database using Blast program (http://blast.ncbi.nlm.nih. gov/blast.cgi) produced significant alignments with the ITS sequences of *Aspergillus nomius* Kurtzman, B.W. Horn & Hesselt (Ascomycota;

Trichocomaceae) from Egypt (KX431672), *Fusarium oxysporum* (Schlechtendal) (Hypocreales: Nectriaceae) from China (AB470914), *F. equiseti* from China (KR709055), *Fusarium* sp. from China (MG211065), *Penicillim solitum* Thom (Ascomycota: Trichocomaceae) from Russia (MH860761), *P. sizovae* from Portugal (MH858522), *Penicillium* sp. from Mexico (KT354994), *Alternaria chlamydosporigena* Woudenb. & Crous 2013 (Ascomycota: Pleosporaceae) from Spain (KX343144), *Meyerozyma guilliermondii* from Netherlands (MK394108), *Paramyrothecium roridum* (Tode) L. Lombard & Crous (Hypocreales: Stachybotryaceae), from China (MK696385), *Chaetomium elatum* Kunze (Ascomycota: Chaetomiaceae) from Germany (MH871792), *Beauveria bassiana* from Spain and Turkey (KC753395, KP862970), *B. pseudobassiana* from USA and Mexico (MF872417, MK142275) and *B. brongniartii* (ZM154107) deposited in GenBank. The sequence analysis of these isolates using the BLAST search tools showed that our isolates' homology with other validated isolates in the GeneBank from other countries was 98 to 100. The accession number of the sequenced isolates are listed in Table 1.

Table 1 GenBank accession numbers of ITS regions of entomopathogenic species used for phylogenetic studies and origin of these species

Species Name	Country	Source	Isolation number	GenBank (ITS)	Reference
Alternaria chlamydosporigena	Netherlands		CBS 125822	MH863792	(Vu *et al*. 2019)
Alternaria chlamydosporigena	Netherlands		CBS 719.71	MH860308	(Vu *et al*. 2019)
Alternaria chlamydosporigena	Iran-Paveh	Rangeland	RUF-Ac1 (IRAN 3345C)	MK651115	This study
Aspergillus nomius	Netherlands		DTO_246B2	KJ775515	(Visagie *et al*. 2014)
Aspergillus nomius	Netherlands		DTO_246A1	KJ775514	(Visagie *et al*. 2014)
Aspergillus nomius	Eslamabad-e Gharb	Rangeland	RUF-An1 (IRAN 3301C)	MK450362	This study
Beauveria brongniartii	Netherlands		CBS 129099	MH865206	(Vu *et al*., 2019)
Beauveria brongniartii	Netherlands		CBS 126934	MH864341	(Vu *et al*., 2019)
Beauveria pseudobassiana	USA		ARSEF 7242	HQ880793	(Rehner *et al*., 2011)
Beauveria pseudobassiana	USA		ARSEF 6229	HQ880799	(Rehner *et al*., 2011)
Beauveria bassiana	Netherlands		CBS 120.50	MH856568	(Vu *et al*., 2019)
Beauveria bassiana	Netherlands		CBS 212.61	MH858026	(Vu *et al*., 2019)
Beauveria brongniartii	Iran-Harsin	Forest	RUF-Bt1 (IRAN 3297C)	MK459301	This study
Beauveria pseudobassiana	Iran-Harsin	Field Crop	RUF-Bp1 (IRAN 3295C)	MK459303	This study
Beauveria pseudobassiana	Iran-Harsin	Forest	RUF-Bp2 (IRAN 3296C)	MK459304	This study
Beauveria pseudobassiana	Iran-Gilan-e-Gharb	Forest	RUF-Bp3 (IRAN 3334C)	MK651216	This study
Beauveria pseudobassiana	Iran-Gilan-e-Gharb	Forest	RUF-Bp4 (IRAN 3335C)	MK651215	This study
Beauveria bassiana	Iran-Harsin	Field Crop	RUF-Bb1 (IRAN 3336C)	MK651117	This study
Beauveria bassiana	Iran-Eslamabad-e Gharb	Rangeland	RUF-Bb2 (IRAN 3337C)	MK651127	This study
Beauveria bassiana	Iran-Harsin	Forest	RUF-Bb3 (IRAN 3338C)	MK651136	This study
Chaetomium elatum	Netherlands		CBS 126656	MH864188	(Vu *et al.*, 2019)
Chaetomium elatum	Netherlands		CBS 393.67	MH859003	(Vu *et al.*, 2019)
Chaetomium elatum	Iran-Harsin	Forest	RUF-Ce1 (IRAN 3298C)	MK459302	This study
Embellisia telluster	USA		-	JN383494	(Lawrence *et al*., 2012)
Fusarium equiseti	Netherlands		CBS 126202	MH864013	(Vu *et al.*, 2019)
Fusarium equiseti	Netherlands		CBS 307.94	MH862468	(Vu *et al*., 2019)
Fusarium oxysporum	Netherlands		d22	GQ922565	(Geydan *et al*., 2012)
Fusarium oxysporum	Netherlands		d14	GQ922564	(Geydan *et al*., 2012)
Fusarium tricinctum	Netherlands		d17	GQ922562	(Geydan *et al*., 2012)
Fusarium chlamydosporum	Netherlands		CBS 677.77	MH861111	(Vu *et al*., 2019)
Fusarium equiseti	Iran-Qasr-e Shirin	Field Crop	RUF-Fe1 (IRAN 3340C)	MK651237	This study
Fusarium oxysporum	Iran-Sahneh	Forest	RUF-Fo1 (IRAN 3341C)	MK651259	This study
Fusarium sp.	Iran-Sahneh	Garden	RUF-Fs1 (IRAN 3342C)	MK651505	This study
Meyerozyma guilliermondii	Netherlands		CBS_8105	KP109752	(Corte *et al*., 2015)
Meyerozyma guilliermondii	Netherlands		CBS_7369	KP109751	(Corte *et al.*, 2015)
Meyerozyma guilliermondii	Iran-Sahneh	Forest	RUF-Mg1 (IRAN 3343C)	MK651508	This study
Penicillium solitum	Netherlands		CBS 500.73	MH860761	(Vu *et al*., 2019)
Penicillium solitum	Netherlands		CBS 487.75	MH860945	(Vu *et al.*, 2019)
Penicillium sizovae	Netherlands		CBS 413.69	MH859338	(Vu *et al.*, 2019)
Penicillium sizovae	Netherlands		CBS 115968	GU944585	(Houbraken *et al.*, 2010)
Penicillium roqueforti	Netherlands		CBS 280.67	MH858967	(Vu *et al*., 2019)
Penicillium carneum	Netherlands		CBS 112297	HQ442338	(Houbraken *et al.*, 2010)
Penicillium solitum	Iran-Kerend-e Gharb	Rangeland	RUF-Pso1 (IRAN 3302C)	MK461564	This study
Penicillium sp.	Iran-Gilan-e-Gharb	Forest	RUF-Psp1 (IRAN 3300C)	MK459305	This study
Penicillium sizovae	Iran-Sahneh	Garden	RUF-Ps1 (IRAN 3299C)	MK459306	This study
Penicillium sizovae	Iran-Qasr-e Shirin	Field Crop	RUF-Ps2 (IRAN 3339C)	MK459319	This study
Paramyrothecium roridum	Netherlands		CBS 212.95	KU846299	(Lombard *et al.* 2016)
Paramyrothecium roridum	Netherlands		CBS 357.89	KU846300	(Lombard *et al.* 2016)
Paramyrothecium roridum	Kermanshah	Field Crop	RUF-Pr1 (IRAN 3344C)	MK651513	This study
Filobasidium floriforme	Netherlands		CBS:6242	KY103419	(Vu *et al.* 2019)

Phylogenetic analysis

Phylogenetic tree analyses based on ITS regions allowed us to establish the precise taxonomic placement of each species. The phylogenetic reconstruction of 52 sequences of 560 bp covering the ITS1 + 5.8S + ITS2 regions was inferred using the cladistic (maximum parsimony) and the distance (neighbor-joining) methods. The final aligned data matrix comprised 458 characters, including the alignment gaps, of which 210 were parsimony-informative, 228 variables, and 230 were conserved. The ITS phylogenetic trees inferred by both cladistic and distance methods showed the same topology, although there were differences in percent bootstrapping.

The entirety of the optimal tree branch length in the neighbor-joining method was 0.75 (data not shown). In the maximum parsimony analysis (Fig. 2), the tree length was 511 with a retention index (RI) = 0.94, consistency index (CI) = 0.72, iCI = 0.70 for parsimony informative sites, a RCI = 0.67 for all sites, and iRI = 0.94. With this, 39 trees were retained. Our isolates were clustered in a distinct monophyletic clade through reliable strains of other countries besides a high bootstrap value (96-100%). These results fortify the BLAST-based identification.

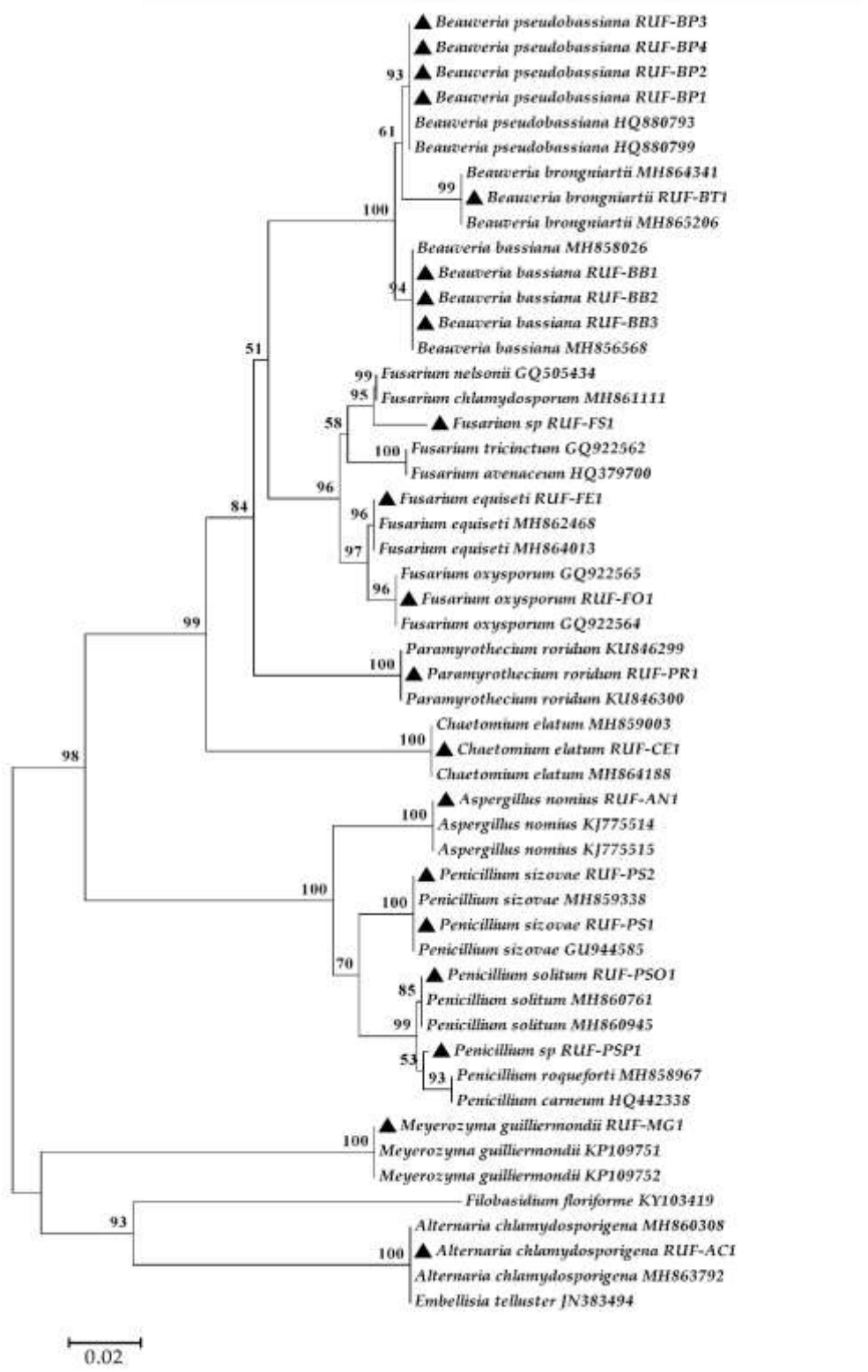

Fig. 2. The phylogenetic position of the isolates identified based on a comparison of the nucleotide sequence of the ITS regions. The numbers on the branches indicate the percentage of the values of the bootstrap with 1000 repetitions. The triangles refer to indigenous entomopathogenic isolates in Iran.

Pathogenicity assessment

Based on the pathogenicity screening test, *Alternaria chlamydosporigena*, *Aspergillus nomius*, *Chaetomium elatum*, *Fusarium equiseti*, *F. oxysporum*, an unidentified *Fusarium* sp., *Meyerozyma guilliermondii*, *Paramyrothecium roridum*, an unidentified *Penicillium* sp., *Penicillium sizovae*, and *P. solitum*, *Beauveria bassiana*, *B. pseudobassiana* and *B. brongniartii* all showed entomopathogenic activity against *E. kuehniella* larvae. With the exception to isolates within the genus *Beauveria*, the other isolates are not well-known insect pathogens, and some of these are plant pathogens but can infect *E. kuehniella* larvae. These isolates are grouped into opportunistic pathogens (Sun & Liu, 2008). A total of three species of *Beauveria* isolates showed high efficiency and rapid killing activity to cowpea beetles (Table 3).

Relationships between *Beauveria* species and environmental parameters

The relation between *Beauveria* species and environmental parameters was analyzed by canonical correspondence analysis (CCA) (Fig. 3). Gradient judgment conferred a length of 7 for DCA; hence, the unimodal approach was conducted. Results revealed that *the environmental parameters influenced Beauveria species*. The correlation between environmental parameters and species was 75% for the first (eigenvalue = 0.34) and 69% for the second axis (eigenvalue = 0.13). Electrical conductivity (EC), sand, and $CaCO_3$ were negatively correlated with the first CCA axis, organic carbon, silt, clay, elevation, and pH correlated positively (Fig. 3). Additionally, the *Beauveria* on the second axis negatively correlates with silt and EC and positively correlates with soil carbon, clay, pH, sand, $CaCO_3$, and elevation. In canonical correspondence analysis, arrow length indicates the importance of variables on species. *Beauveria* was most numerous in soil with very low EC and silt, low pH, intermediate clay, sand, soil carbon, and $CaCO_3$. For the elevation, *Beauveria* was found at high altitudes.

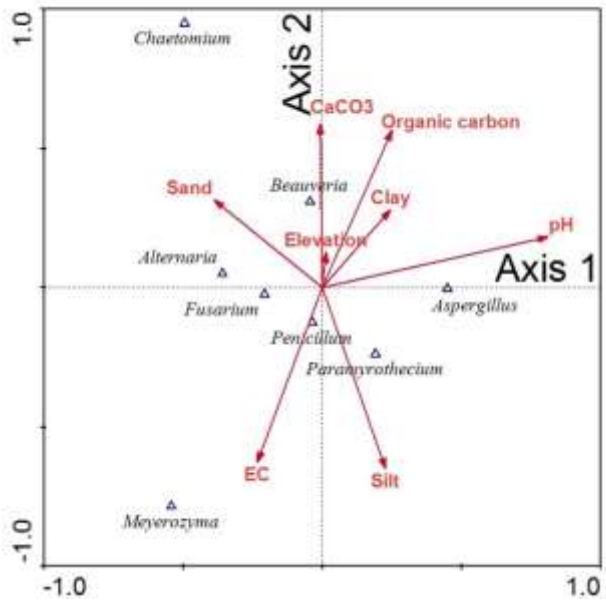

Fig. 3. Correspondence analysis (CA) of the entomopathogenic species communities found in different parts of Kermanshah province, Iran. The eigenvalues of the first and second axes in the two dimensional ordination diagrams are as: CA1= 0.34 and CA2 =0.13.

The diversity of entomopathogenic fungi in different altitudes and soil types

The diversity of fungi was observed at the total number of taxa (richness=S), number of isolates (abundance), and differed among the four geographical sites. There was a higher species richness and abundance in forest soil (S=31 and n= 60 individuals) to other regions (Table 2). The Simpson's, Shannon–Wiener, Dominance, Equitability, and Fisher alpha of fungi isolated were estimated (Table 2). The highest and lowest diversity index with a confidence interval of 95% was found in the forest and garden soils, respectively: (Simpson D_s: 0.97 vs. 0.89), Shannon–Wiener index (3.30 vs. 2.45), and Equitability (0.969 vs. 0.911). Both forest and field soils had a higher value of Fisher alpha index than rangeland and garden. Only rangeland (0.1) and garden (0.09) had a greater dominance index (Table 2).

Aggressivity assessment of selected isolates of *Beauveria*

Estimation of LC_{50} and LT_{50} values of three *Beauveria* isolates (*B. brongniartii*, *B. bassiana,* and *B. pseudobassiana*) to cowpea beetle is shown in Table 3. Results of the lethal dose ratio (LDR) test (95% CL) showed no differences in *C. maculatus* mortalities in treated adults with three strains (Table 3). According to LC_{50} comparisons, the insecticidal activity of selected fungal species for *C. maculatus* was 1.14 (*B. bassiana*), 3.86 (*B. brongniartii*) and, 0.197 (*B. pseudobassiana*), respectively. The responses of insects to selected isolates were dose-dependent. In the cowpea beetle, the cumulative mortality percentage produced by higher dose rates of *B. bassiana*, *B. brongniartii,* and *B. pseudobassiana* was 88.83± 4.40, 96.15± 3.85, and 72.8± 3.30. The LT_{50}-values of selected isolates are listed in Table 3 for beetles. No significant differences were found among isolates based on 95% confidence intervals (CI) (Table 3). The toxicity index (Sun, 1950) illustrates the differences in insects' survival in the tested isolates. In cowpea weevils, the toxicity index of *B. bassiana*, *B. brongniartii*, and *B. pseudobassiana* were 3.30, 3.88, 3.20-fold higher than control, respectively. The results indicated that all selected fungi were more effective to the beetles following the assay period.

Table 2 Analysis of the diversity of fungal isolates in different soil types of Kermanshah province, West Iran

Parameters	Forest	Rangeland	Fields	Garden	Total
Total number of isolates (abundance or n)	39	22	38	15	114
Total number of taxa (richness or S)	31	15	23	13	44
Estimated total number of classes	37	20	66	18	18
Simpson D_s (Hurlburt PIE) (95% CI)	0.97 (0.96-0.98)	0.92 (0.91-0.98)	0.93 (0.92-0.96)	0.89 (0.883-0.89)	0.96 (0.918-0.932)
Dominance D (95% CI)	0.042 (0.04-0.08)	0.10 (0.075-0.11)	0.06 (0.04-0.07)	0.09 (0.06-0.13)	0.044 (0.04-0.06)
Shannon Wiener Index (H', log-based) (95% CI)	3.30 (3.17-3.43)	2.49 (2.26-2.72)	2.92 (2.71-3.14)	2.45 (2.44-2.66)	3.44 (3.31-3.57)
Equitability (j=evenness) (95% CI)	0.96 (0.90-0.97)	0.92 (0.89-0.96)	0.92 (0.85-0.97)	0.91 (0.86-0.95)	0.91 (0.88-0.93)
Fisher alpha (95% CI)	25.8 (13.6-25.8)	11.4 (11.4-30.35)	24.67 (11.9-36.3)	14.56 (9.33-49.9)	20.97 (13.1-20.97)

Diversity indices calculated by software's Stats Direct version 3.2 and SDR version 4 with 10000 bootstraps.
CI: Confidence Interval; 95% bootstrap confidence interval of diversity indices. If the CI includes overlap, there is no difference at a significance level of 0.05.

Table 3. Regression statistics for the estimation of LC_{50} (lethal concentration) and LT_{50} (days) values (with 95% CI) of different indigenous entomopathogenic fungi isolates against adults of *Callosobruchus maculatus* (n=262-300 individual for each isolate) via immersion bioassay ten days after inoculation.

Fungi	LC_{50} (conidia/mL) (95% CL)	χ^2 (df=18)	Slope (±SE)	P-Value	LDR (95% CL)	LT_{50} (days) (95% CL)	Slope (±SE)	χ^2 (df=18)	Toxicity index	virulence
Beauveria bassiana	1.56×10^5 ($2.8 \times 10^4 - 4.6 \times 10^5$)	3.84	0.481±0.09	0.91	-	4.85 (4.41-5.32)	3.51±0.27	7.11	3.30	H
Beauveria brongniartii	4.04×10^4 ($8.2 \times 10^3 - 1.07 \times 10^5$)	8.9	0.482±0.09	0.58	3.86 (0.68-21.4)	3.23 (2.81-3.56)	3.48±0.29	3.24	4.95	H
Beauveria pseudobassiana	7.9×10^5 ($1.7 \times 10^5 - 3.2 \times 10^6$)	3.11	0.372±0.103	0.84	0.197 (0.026-1.50)	5.06 (4.53-5.66)	2.51±0.24	3.69	3.16	H
control	-	-	-		-	16.01 (12.1-20.11)	2.44 ± 0.55	3.70	1	-

LDR (Lethal dose ratio), *If the 95% CL includes 1, then the LC50s are not significantly different (Robertson et al., 2017).
CI: confidence limit (CL).
χ^2: chi-squared goodness of fit test
Toxicity index: LT_{50} of control / LT_{50} of treatment (Sun 1950). H: highly virulent (LT_{50} between 4.5 - 5.5 d) M: moderately virulent (LT_{50} between 5.6 - 5.9 d), and less virulent or L when LT50>5.9 d (Kaur and Padmaja

Discussion

This study is the first detailed investigation of indigenous EPF in Kermanshah province, Iran, with molecular characterization for identification, diversity assessment, and species bio-efficacy assays. Identifying and classifying fungi based on morphological traits and nucleic acid sequence analysis is a frequently adopted technique that compares the association between homologous molecules through determining the composition of nucleotide sequences in the basic structure of nucleic acid (Diaz *et al.*, 2012). Concerning EPF isolation from the soil, the antibiotic-based isolation platforms reduce fungi yield and their pathogenicity potency compared to the insect-bait method (Kim *et al.,* 2018). Therefore, earlier studies preferred the insect-bait-methods by applying the greater wax moth larvae (*Galleria mellonella*), mealworms (*Tenebrio molitor*), and *E. kuehniella* for EPF screening in soil samples (Barra *et al.,* 2013; Kim *et al.,* 2018; Chang *et al.,* 2021). Insect-bait methods increase the number of indigenous isolated fungi from samples at the same time maintaining their fitness and pathogenicity(Chang *et al.,* 2021).

In the current study, the trapped-EPF was more abundant under forest trees soils (34.21% of the recorded isolates) than under each field (33.3%), rangelands (19.29%), and fruit trees (13.15%). Herein, well-known entomopathogenic fungi within *Beauveria* were isolated from rangeland, field crop, and forest soils with frequency 4%, 8%, and 24%, respectively. Fifty percent of forest soils had *Beauveria* fungus; but its species has not been detected in orchard soils. In previous studies, tropical forests had a rich and varied insect pathogenic fungal species, and *Beauveria* was the dominant fungus found in soil (Evans, 1982). In our study, the most obtained isolates were placed in Hypocreales (60%) and Eurotiales (25%), followed by *Pleosporales*, *Saccharomycetales*, and *Sordariales* orders with lower frequency. Like our study, prior research on the biodiversity of EPF pointed that the *Hypocerales* and *Eurotiales* species had been the most frequent order (Sookar *et al.,* 2008; Barra *et al.,* 2013; NouriAiin *et al.,* 2014).

Among the opportunistic fungi, *A. nomius*, *F. oxysporum*, an unidentified *Fusarium* sp., *P. roridum*, *C. elatum*, and *M. guilliermondii*, resulted in the highest *E. kuehniella* mortality in the preliminary pathogenicity test. In this study, the most common opportunistic fungus isolated from the soil was *Fusarium* spp. *Fusarium* species represent essential functions in soil and plants as endophytes (Dababat & Sikora, 2007), pathogens, and saprotrophs (Sharma *et al.,* 2018). However, in most studies, these fungi are considered opportunistic and likely attack injured or weakened insects. Previous studies also revealed that some of these opportunistic fungi were highly pathogenic to insects (Sun & Liu, 2008).

Little is known about the influence of environmental parameters on the distribution of *Beauveria* under natural and agricultural soil conditions. Sun &Liu (2008) showed that *B. bassiana* was frequently found in colder regions, while *Metarhizium* was the most common fungus in warmer regions in China (Sun & Liu, 2008). Kermanshah province (West Iran) is

a mountainous area situated at 1,200 m altitude with a sei-cold climate. Our results correspond well with previous investigations (Sun & Liu, 2008). Meyling & Eilenberg (2007) showed that *M. anisopliae* was rarely found in agricultural soils. These may be returned to the mild thermophile ecology of *M. anisopliae* in soils (Quesada-Moraga *et al.,* 2007 a). Shin *et al.,* (2013) showed that *Beauveria* species distributed in natural habitats more often than in agricultural habitats. Zimmermann (2007a), demonstrated that *B. bassiana* is cosmopolitan while others are restricted due to adaptation to the particular climatic region and specific soil environmental conditions. In our study, all the soils sampled were alkaline, primarily in the range of 7 to 8.19. *Beauveria bassiana* was found in soils with huge clay content, high pH, and enriched organic matter. Our results showed that the *Beauveria* species are soil-habiting, with characteristics including low EC and silt, low pH, intermediate clay, sand, soil carbon, and $CaCO_3$. Higher occurrence of *B. bassiana* was found in soils with a high content of organic matter and minerals in soils of fine texture.

Assessment of novel EPF strains against insect pests is essential in selecting virulent strains before large-scale applications are possible (Barra *et al.,* 2013; Wakil *et al.,* 2013). Consistent with da Silva Santos *et al.* (2020) in our study, *Fusarium* spp., strains can lead to various mortality (≈20-80%) against cowpea beetles (Unpublished data). Although the genus *Fusarium* infects insect pests, most species of this genus are not specialized and can damage the plants; therefore, more data is needed to prove their safety, and host specificity (Chang *et al.,* 2021). *Paramyrothecium roridum* has been considered an important plant pathogen (Chen *et al.,* 2018), and recently it was reported as pathogenic against the squash beetle *Epilachna chrysomelina* (F.) (Hassan *et al.,* 2021). Similar to Hassan *et al.,* (2021), in our study, the pathogenicity of *P. roridum* lineages to *E. kuehniella* larvae and *C. maculatus* adults was demonstrated. Therefore, this species has potential for application in stored pest biological control programs.

The diverse genus *Chaetomium* with word wide distribution is well known as coprophilous, seed, and soil fungi (Abdel-Azeem, 2020). Furthermore, *Chaetomium* taxa are considered as an antagonist of plant fungal pathogens (Abdel-Azeem, 2020), antibitosis against nematodes ,*Meloidogyne incognita* (Kofold & White) Chitwood (Rhabditida: Meloidogynidae), and insect pests control (Moya *et al.,* 2020). For the first time, our result has shown that *C. elatum* isolates (RUF-CE1) are pathogenic to both *E. kuehniella* larvae and *C. maculatus* adults.

All the *Beauveria* isolates were found pathogenic to tested insects. Considering host specificity, at least the three diverse species of *Beauveria*, were categorized as hypervirulent to cowpea beetle adults by using comparative virulence assays and arbitrary rating results (Kaur & Padmaja, 2008 b). Similar results were noted in other studies involving treating beetles with EPF (Cherry *et al.,* 2005; Barra *et al.,* 2013; Khoobdel *et al.,* 2019). Application of native and eco-friendly endemic insect natural enemies can be one of the best options for

controlling local insect pests (Pourian & Alizadeh, 2021). Genus *Beauveria* has a generalist nature and contains a different assemblage of genotypes as well some of its isolates have shown host-specificity to insects (Uma Devi *et al.*, 2008). *Beauveria* species were shown to cause a pretty different susceptibility in time-mortality tests and caused impressive cumulative mortality over time. Different virulence of *Beauveria* strains has been well documented in insect species (Uma Devi *et al.*, 2008; Barra *et al.*, 2013). Much of the variation in virulence may be linked to variable ecological habitats or the origin of the strain (Cherry *et al.*, 2005; Meyling & Eilenberg, 2007; Quesada-Moraga *et al.*, 2007b; Sun & Liu 2008; Herrero *et al.*, 2012; Kalvnadi *et al.*, 2018). All three virulent strains of *Beauveria* to *C. maculatus*, as novel host from different phylogenetic taxon were recovered from forest soil habitats. Forest terrestrial ecosystems describe some of the most significant and vital microbiomes on Earth, considering a massive proportion of the global fungal diversity (Willis, 2018). Entomopathogenic fungi associated with the forest soil environment play a notable role in forest plant community health and productivity (Shi *et al.*, 2014). In dose-response applications, the chosen fungal species had an acceptable insecticidal activity against *C. maculatus* adults (ca. 88-96%). Other researchers have documented the dose-response mortality of *Beauveria* sp., to insect pests using both dry conidia and conidial suspensions (Batta & Kavallieratos, 2018; Kalvnadi *et al.*, 2018; Khoobdel *et al.*, 2019).

Conclusion

The selection of locally adapted virulent strains in agroecosystems is the initial step in commercialization and large-scale application. Our study highlights the alterations in fungal diversity along with various virulence, through altitude and soil composition in four ecological zones. Collecting small samples of host-specific hypocrealean fungus revealed that, despite previous studies, *E. kuehniella* larvae are not a suitable option for isolating pathogenic fungi from soil, and the probability of underestimation in diversity increases. Moreover, few recovered indigenous EPF isolates are an effective for insect biocontrol programs; however, sizeable genotypic variability could be expected.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Reference Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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