Morphological and Molecular Characterization of Natural Entomopathogenic Fungi

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ABSTRACT— ***In this study, entomopathogenic fungi were isolated from soil samples collected from the central villages of Kahramanmaras wheat fields and Baskonus forests. Development characteristics of isolates on artificial medium, microscopic examination of the morphological structures and molecular characterization were performed. Observed sequence results were compared with database sequence using Blast and phylogenetic tree was drawn. Beauveria bassiana (14), Fusarium oxysporum (8), F. solani (1), Aspergillus flavus (4) and Penicillium sp. (1) were identified as entomopathogenic fungi species in given area.***

Keywords— entomopathogenic fungi, soil, molecular identification, *Beauveria bassiana*

1. INTRODUCTION

Chemical pesticides have been used by most of the growers for decades to control economically important pest insects, but their side effects on environment and non-target organisms have forced the industry and scientists to develop alternative control methods. This has induced great interest in finding and developing pathogens for microbial control. Most of these studies have focused on the use of entomopathogenic fungi which have several advantages over other entomopathogens due to broad host range, a large number of species, mode of action, easy to identify from the outside and widely observed. They are considered to be safe for environment, humans and other non-target organisms or beneficial insects in pest management [1,2].

The soil environment is an important reservoir and an excellent environmental shelter for entomopathogenic fungi due to providing protection from UV radiation and other abiotic and biotic factors3. Many fungal species belonging to the genera *Beauveria*, *Conidiobolus*, *Metarhizium* and I*saria* (=*Paecilomyces*) are generally found in soil [3,4]. The isolation and characterization of local entomopathogenic fungi species are essential for understanding fungal biodiversity in a specific region and important to provide potential biological control agents for pest control [5,6]. There is some hesitation about using exotic strains of fungi for pest control in a different country. These strains could be ineffective due to strain and different environmental conditions or may disrupt local ecology [7]. Previous studies have been reported entomopathogenic fungi in differing climatic conditions, habitats, cropping systems and soil types in Canada, Denmark, Finland, Palestine, Spain and Switzerland [5-10] and in Turkey [11-14].

Several methods have been used to identify a species of entomopathogenic fungi. Hyphomycetes are classified by the morphological characteristics of spores, conidiogenous cells and colonies, their hosts, pathogenicity and growth or nutrient requirements [15]. Obviously, it is not quite possible to distinguish fungal isolates using only morphological characters. In recent years, molecular identification techniques are required as well as the traditional morphological characteristics. Different molecular techniques were used to identify a species of entomopathogenic fungi [11,16-19].

In order to find a native fungal biocontrol agents against the insect pests, İzgi and Güven [14] and Koz and Güven [13] isolated insect pathogenic fungi from the soils in Kahramanmaraş province. In this study, we identified these fungal species using a combination of classical and molecular techniques.

2. MATERIALS AND METHODS

2.1 Fungal isolates

Entomopathogenic fungi were isolated with "*Galleria* bait insect" method[20] from soil samples in Kahramanmaraş province [13,14]. Eighteen isolates obtained from soils at wheat fields[13] and ten isolates obtained from soils at Başkonuş forestland [14] (Total 28 isolates) were used for molecular and morphological characterization. All isolates used during this study, and their geographic origin, are summarized in Table 1.

Table 1: Fungal species isolated from soils in Kahramanmaraş in Turkey showing locality, vegetation, sampling date and gene accession numbers

No	Isolate	Species [a]	Gene accession number	Vegetation /Location	Date
1.	Aksu.2010.C	*Aspergillus flavus*	KJ672508	Wheat fields / Aksu	2010
2.	Aksu.2010.F	*Beauveria bassiana*	-	Wheat fields / Aksu	2010
3.	Aksu.2011.2A	*Fusarium oxysporum*	KJ874344	Wheat fields / Aksu	2011
4.	Aksu.2011.2C	*Beauveria bassiana*	-	Wheat fields / Aksu	2011
5.	Bk.2010.1A	*Beauveria* sp.	-	Forestland/Başkonuş	2010
6.	Bk.2010.2C	*Beauveria bassiana*	-	Forestland/Başkonuş	2010
7.	Bk.2010.3A	*Aspergillus flavus*	-	Forestland/Başkonuş	2010
8.	Bk.2011.1B	*Fusarium oxysporum*	-	Forestland/Başkonuş	2010
9.	Bk.2011.1C	*Beauveria bassiana*	-	Forestland/Başkonuş	2011
10.	Bk.2011.2B	*Aspergillus flavus*	-	Forestland/Başkonuş	2011
11.	Bk.2011.2D	*Fusarium oxysporum*	-	Forestland/Başkonuş	2011
12.	Bk.2011.2K	*Beauveria bassiana*	-	Forestland/Başkonuş	2011
13.	Bk.2011.3A	*Fusarium oxysporum*	-	Forestland/Başkonuş	2011
14.	Bk.2011.3E	*Beauveria bassiana*	-	Forestland/Başkonuş	2011
15.	Kapıçam.2010.C	*Beauveria bassiana*	-	Wheat fields / Kapıçam	2010
16.	Kapıçam.2010.E	*Beauveria bassiana*	-	Wheat fields / Kapıçam	2010
17.	Kapıçam.2011.1A	*Beauveria bassiana*	-	Wheat fields / Kapıçam	2011
18.	Kılavuzlu.2010.B	*Beauveria bassiana*	-	Wheat fields / Kılavuzlu	2010
19.	Kılavuzlu.2010.C	*Aspergillus flavus*	-	Wheat fields / Kılavuzlu	2010
20.	Kılavuzlu.2011.2A	*Penicillium* sp.	KJ874346	Wheat fields / Kılavuzlu	2011
21.	Kılavuzlu.2011.2B	*Fusarium oxysporum.*	-	Wheat fields / Kılavuzlu	2011
22.	Kılılı.2010.A	*Fusarium oxysporum*	-	Wheat fields / Kılılı	2010
23.	Kılılı.2010.B	*Fusarium solani*	KJ874345	Wheat fields / Kılılı	2010
24.	Kılılı.2011.2A	*Fusarium. solani*	-	Wheat fields / Kılılı	2011
25.	Kürtül.2010.C	*Beauveria bassiana*	-	Wheat fields / Kürtül	2010
26.	Kürtül.2011.2A	*Fusarium* sp.	-	Wheat fields / Kürtül	2011
27.	Önsen.2010.B	*Beauveria bassiana*	KJ874347	Wheat fields / Önsen	2010
28.	Önsen.2011.2B	*Beauveria bassiana*	-	Wheat fields / Önsen	2011

[a] The species name confirmed by sequence analysis.

2.2 Maintenance of cultures

Entomopathogenic fungi spores from the insects cadavers were transferred on potatoes dextrose agar with 0.2% yeast extract (PDAY) to which 40 lg ml-1 tetracycline were added to prevent bacterial growth. Pure cultures on PDAY medium without antibiotic were maintained at 23±2 ^{o}C under dark conditions at 75% RH.

2.3 Morphological identification

For the preliminary identification of the fungal isolates, the appearance of infected larvae, colony size and morphology, spore sizes and shape were determined at 23±2 ^{o}C under dark conditions at 75% RH. For comparisons of in vitro growth, surface cultures of all the isolates were prepared on PDA. The plates (3 plates/ strain) were inoculated with stripe-plate technique and 100 individual spores were counted each time to calculate percentage germination in each plate. For colony size, fungal spores were inoculated (5 sites/plate) using sterile needle loop and replicated three times for each isolate. Plates were examined at 16, 24, and 36 h, and daily thereafter. Slide cultures were prepared for light microscopy as described by Goettel and Inglis[21]. Spore and conidiophores sizes were measured with a microscope (Nikon Eclipse 80İ) and identified according to the key explained by Humber [22].

2.4 DNA extraction

For DNA preparation, inoculated spores in PDA were collected in mycelium stage to inoculate flasks containing 50 ml of Sabouraud dextrose broth (Merck). Cultures were shaken at 250 rpm at 26 ^{o}C for 5-7 days; cells were collected by centrifugation (4200 rpm for 5 min) and stored at -20^{o}C until use. DNA was extracted from 50 mg mycelium preparations using a modified CTAB protocol as described briefly. Mycelium cells were crushed with mortar into liquid nitrogen and suspended in 700 µl CTAB isolation buffer (0.1 M TrisHCl, pH 8.0; 1.4 M NaCl; 0.02 M EDTA; %2 CTAB) and incubated at 70 ^{o}C for 1 h. In each tube, 500 µl chloroform was added and the suspension was incubated for 5 min at 70 ^{o}C and centrifuged at 10000 g for 10 min. The supernatant was extracted with an equal volume of chloroform/isoamylalcohol (24:1), and 300 µl isopropanol was added to precipitate the nucleic acids. The pellets were obtained by centrifugation at 10000 g for 10 min and washed with 70% sterile ethanol, dried and resuspended in 50 µl of TE buffer (1mM TrisHCl, pH 8.0; 0.1 mM EDTA). All samples were stored at -20 ^{o}C for further study. The extracted DNA was assessed on 1% TAE (Tris/Acetic Acid/EDTA) agarose gel stained with ethidium bromide [23].

2.5 Sequencing

PCR amplification of ITS1-5.8S-ITS2 and 18S genes of fungal isolates was performed with universal primers (White et al. 1990). ITS1 (5'- TCCGTAGGTGAACCTGCGG -3') as forward primer, ITS4 (5'-TCCTC CGCTTATTGATATGC-3') as reverse primer and NS3 (5'- GCAAGTCTGGTGCCAGCAGCC-3') as forward primer, NS6 (5'- GCATCACAGACCTGTTATTGCCTC -3') as reverse primer were used. PCR amplifications were performed in a total volume of 50 µl which included 5 µl 10X Taq-DNA polymerase reaction buffer, 1 µl (1 mM) dNTPs, 1 µl (20 pmol) each of the opposing amplification primers, 0.5 µl (5 u/ µl) Taq-DNA polymerase, and 50 ng genomic DNA and sdH2O. The PCR program involved 40 cycles of 95 ^{o}C for 30 sec, 65 ^{o}C for 30 sec, and 72 ^{o}C for 1 min. PCR products were separated on 1.0% agarose gels, stained with ethidium bromide and viewed under UV light. After the amplification, PCR products were purified with QIAquick PCR purification kit (QIAGEN). Amplification products were extracted from agarose gels with the QiaxII gel extraction kit (Qiagen) and sequenced (Applied Biosystems 3130xl Genetic Analyzers).

2.6 Data analysis

For the morphological characterization of fungi, measurements were subjected to ANOVA and differences between the means were determined by Tukey's multiple comparison test (<0.05). Data analysis was performed using MINITAB statistical software[24].

Cluster analyses of the sequences were performed using the Clone Manager 9 software. Alignment gaps were treated as missing data. Sequences were compared with the NCBI GenBank accessions using BLAST to confirm identifications of isolated strains. Then phylogenetic tree of the species are plotted using Mega 4.1 program. Some of the obtained sequences are available in the GenBankTM (Table 1).

3. RESULTS

3.1 Morphological Characterization

Based on the morphological characteristics, four different species were identified within the 28 strains examined and these species were confirmed by molecular technique. Germination rate hyphal growth and colony size of obtained entomopathogenic fungi and their species name are given in Table 2 and 3.

The germination of the most of Beauveria bassiana (Balsamo) Vuillemin isolates were observed within 16 hours and completed within 24 hours. All B. bassiana isolates completed colony growth in 5 days and colony sizes were differed significantly within days (For 2nd day F= 2.86, P =0.006; for 3rd day, F= 4.41, P =0.00; for 5th day, F= 3.02, P =0.004) (Table 2). Colonies on PDA at 5th d at 23 ^{o}C were approximately 18–23 mm diam, feathery, lanose, and velvety to dusty appearance. At the beginning, colonies were white and later becoming yellowish white. Underside of the colonies was pale to yellowish white and no odor was observed. Hyphae were hyaline, smooth-walled, septate. Conidiogenous cells were solitary but generally in dense clusters. The base of conidiogenous cells subspherical to ampulliform, 1.7±0.3 x 5.9±1.2 µm in size and apex with denticulate rachis, produced laterally. From each of these denticulate rachis, hyaline,

aseptate, smooth wall, globose, subglobose or broadly ellipsoid, average 1,6±0,3 x 1,8±0,3 µm in size conidia were produced.

Table 2: Germination rate, hyphal growth and colony size of *Beauveria bassiana* isolates

Isolate	Species [a]	Germination rate (h)	Hyphal growth (h)	Colony size		
				2. day (mm)*	3. day (mm)*	5. day (mm)*
Aksu.2010.F	*Beauveria bassiana*	16	24	5.2 ±1.1 *a*	11±0.7 *a*	21.4±2.6 *ab*
Aksu.2011.2C	*Beauveria bassiana*	16	36	4.8±0.8 *a*	12.6±1.7 *a*	20.6±0.9 *ab*
Bk.2010.1A	*Beauveria* sp.	16	36	6.5±1.3 *ab*	14.4±1.5 *b*	19.6±2.9 *ab*
Bk.2010.2C	*Beauveria bassiana*	16	24	6.6 ±1.1 *ab*	10±1.2 *a*	18.2± 2.8 *ab*
Bk.2011.1C	*Beauveria bassiana*	16	24	6.8 ±1.6 *ab*	12.2±2.8 *ab*	23.4±4.4 *b*
Bk.2011.2K	*Beauveria bassiana*	16	24	5.2 ±0.4 *a*	11.4±1.1 *ab*	23±1.6 *ab*
Bk2011.3E	*Beauveria bassiana*	16	24	7± 1.6 *ab*	11.6±1.5 *ab*	21±4.1 *ab*
Kapıçam2010.C	*Beauveria bassiana*	16	24	7.8±1.3 *b*	10.6±0.9 *a*	19.6±1.1 *ab*
Kapıçam2010.E	*Beauveria bassiana*	16	24	5.6±0.5 *ab*	10.8±0.8 *a*	19±2.9 *ab*
Kapıçam2011.1A	*Beauveria bassiana*	16	36	4.4±0.5 *a*	14±1.6 *b*	21±1.6 *ab*
Kılavuzlu2010.B	*Beauveria bassiana*	16	36	5 ± 0.7 *a*	12.2± 1.1 *a*	22.8±3.1 *ab*
Kürtül. 2010.C	*Beauveria bassiana*	16	24	6.4 ±1.1 *ab*	12.2 ± 1.6 *a*	22±2.1 *ab*
Önsen.2010.B	*Beauveria bassiana*	16	24	6.4 ±1.1 *ab*	10 ±0.7 *a*	21.8 ±3.8 *ab*
Önsen.2011. 2B	*Beauveria bassiana*	16	24	5.8 ±0.8 *ab*	9.6±1.5 *a*	18±2.9 *a*

* Values followed by the same italic letter are not significantly different (Tukey test, n=14, P>0.05)

[a] The species name confirmed by sequence analysis.

Spore germinations of the fungus isolates identified as *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen and *Fusarium solani* (Mart.) Sacc. were observed in 16 h and completed within 24 h. In 3rd day, all isolates completed colony growth and colony sizes were differed significantly within days (For 2nd day F= 17.89, P =0.000; for 3rd day, F= 31.71, P =0.000) (Table 3). The aerial mycelium of F. oxysporum were first appears white in color and afterward may change to colors from violet to dark purple. Underside of the colony was reddish to dark purple. Microconidia are generally one celled, aseptate, cylindrical or comma shaped and 2.7±0.5 x 8.6±1.3 µm in size. Macroconidia were fusiform, generally two to three celled, gradually pointed and 15.1±7.4 x 7.6±7.4 µm in size. The aerial mycelium of *F. solani* was sparse to abundant, white cream in color. Long, slender phialides in aerial mycelium of *F. solani* was easily distinguished from the short and plump shaped phialides of *F. oxysporum*. Macroconidia were slightly curved with a slightly blunted apical end and had two to three septa and 3.5±0.5 x 15.7±2.5 µm in size. Microconidia were oval to kidney shaped, one or two celled and 2.8±0.4 x 9.1±1.65µm in size.

Table 3: Germination rate, hyphal growth and colony size of *Fusarium oxysporum* and *Fusarium solani* isolates

Isolate	Species [a]	Germination rate (h)	Hyphal growth (h)	Colony size	
				2. day (mm)*	3. day (mm)*
Kılılı.2010.A	*Fusarium oxysporum*	16	24	12.0±2.0 a	22.8±0.8 a
Aksu.2011.2A	*Fusarium oxysporum*	16	24	16.4±1.7 b	30.2±2.3 b
Bk.2011.1B	*Fusarium oxysporum*	16	24	19.8±1.5 b	32.2±3.0 b
Bk.2011.2D	*Fusarium oxysporum*	16	24	13.6±2.5 a	23.2±3.0 a
Bk.2011.3A	*Fusarium oxysporum*	16	24	18.6±1.1 b	33.2±1.9 b
Kılavuzlu2011.2B	*Fusarium oxysporum*	16	24	18.6±1.3 b	34.6±2.4 b
Kılılı.2010.B	*Fusarium solani*	16	24	15.2±2.8 ab	25.0±1.6 a
Kılılı.2011.2A	*Fusarium. solani*	16	24	11.2±1.1 a	28.8±1.3 ab
Kürtül.2011.2A	*Fusarium* sp.	16	24	24.2± 2.7 c	39.8±3.2 c

* Values followed by the same italic letter are not significantly different (Tukey test, n=9, P>0.05)

[a] The species name confirmed by sequence analysis.

The spores of *Aspergillus flavus* Link isolates germinated within 14 h and spore production were observed within 48 h. Colonies on PDA reached 12-16 mm after 3 days, colony color was green and reverse hyaline. Conidia were 4.5±0.6 x 4.9±0.6 µm in size, globose to subglobose, pale green and roughened.

Germination of spores and spore formation in *Penicillium* sp. were first observed within 16 h and 48 h, respectively. Colonies were in shades of green, had dense conidiophores, grew very fast and covered entire plates within 3 days. Conidia were 2.1±0.5 x 4.9±0.6 µm in size, globose and plain.

3.2 DNA sequence analyses

Partial sequences of the 18S rDNA and ITS1-5.8s-ITS2 region were successfully amplified and some of the obtained sequences were submitted to GenBank. To confirm identifications of the *A. flavus* isolates (Aksu.2010.C, Bk.2010.3A, Bk. 2011.2B) NS3 and NS6 primers were used to sequence 18S gene region. For Kılavuzlu.2010.C isolate, ITS1 and ITS4 were used to sequence ITS1-5.8S-ITS2 gene region. Isolates morphologically identified as *F. oxysporum* (Aksu.2011.2A, BK.2011.1B, BK.2011.2D, BK.2011.3A, Kılavuzlu.2011.2B Kılılı.2010.A, Kılılı.2010.2A) and Kılılı.2011.2A isolate identified as *F. solani* were sequenced with NS3 and NS6 primers. Single isolate of *Penicillium* sp. (Kılavuzlu.2011.2A) was confirmed with NS3 and NS6 primers.

To confirm the morphological identification of *B. bassiana* isolates (Aksu.2010.F, Aksu.2011.2C, Bk.2010.1A, BK.2010.2C, Bk.2011.1C, BK.2011.2K, BK.2011.3E Kapıçam.2010.E, Kapıçam.2010.C, Kapıçam.2011.1A Önsen.2010.B, and Önsen.2011.2B) NS3 and NS6 primers and for Kılavuzlu.2010.B and Kürtül.2010.C isolates ITS1 and ITS4 primers were used to sequence gene regions.

Finally, 900 bp fragment of the sequences were used to define genetic similarity of the isolates with Mega 4.1 program by NJ (Neighbour-joining) analysis. Based on 18S gene region, isolates obtained from the same location were not observed in same group. After analysis, Boot Strap value for Bk.2010.1A and Önsen.2010B were very high (92%). Aksu.2010F, Önsen 2011.2B, Bk.2010.2C, Aksu.2011.2C, Bk.2011.1C and Bk.2011.2K group is separated from the other group with 86% Boot Strap value (Figure 1).

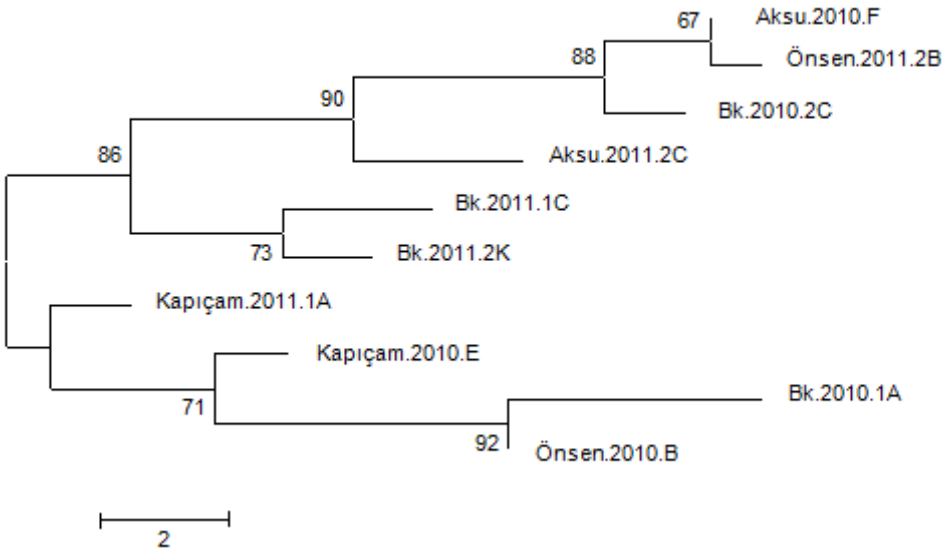

Figure 1: Neighbour-joining tree of 10 *Beauveria bassiana* isolates based on 18S gene region.

Aspergillus flavus isolates obtained from the same location were not observed in same group. Kılavuzlu.2010.C with Bk.2011.2B and Bk.2010.3A with Aksu.2010.C formed two separate groups. Boot Strap value for Bk.2010.3A and Aksu.2010.C with 83% was very high (Figure 2)

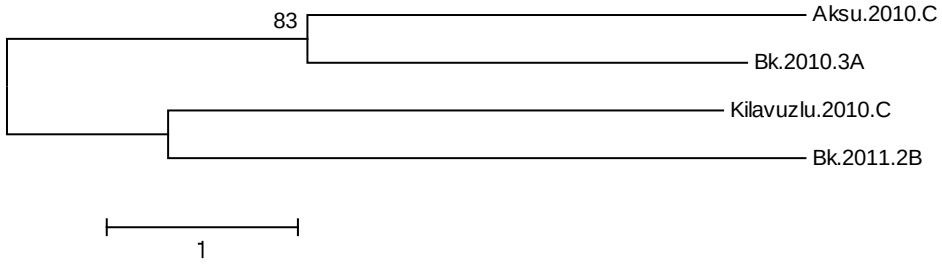

Figure 2: Neighbour-joining tree of 4 *Aspergillus flavus* isolates based on 18S gene region.

Fusarium oxysporum and *F. solani* isolates collected from the same locations, based on 18S gene region were not observed in same group. Bk.2011.1B isolate was located in separate group (Figure 3).

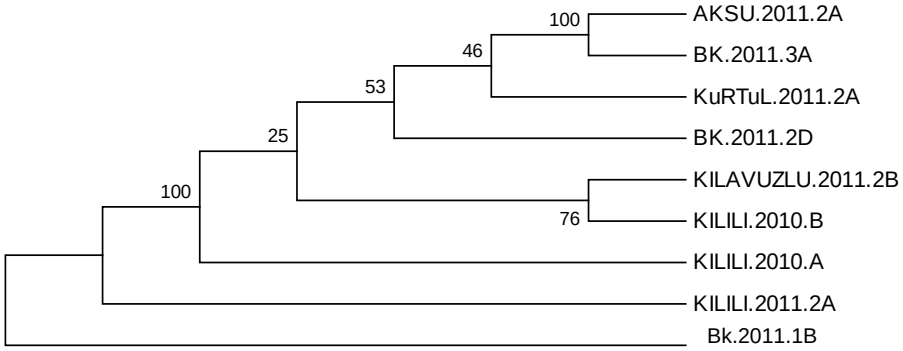

Figure 3: Neighbour-joining tree of *F. oxysporum* and *F. solani* isolates based on 18S gene region

4. DISCUSSION

This study provides general information on morphological and molecular identification of entomopathogenic fungi collected from soils in Kahramanmaraş province. Interest in finding effective and reliable biological control agents for insect pest induces more research to isolate local entomopathogens. Previously, some studies have been conducted to isolate and identify local entomopathogenic fungi in Kahramanmaraş [13,14,23]. They mostly found *Beauveria* sp., *Peacilomyces* sp. and *Metarhizium* sp. and identified them based on their morphological characteristics. Sevim et al. [11] isolated 62 entomopathogenic fungi from hazelnut-growing region of Turkey based on morphology, ITS sequence and partial sequencing of the 18S (SSU rDNA) genes. They mostly identified *M. anisopliae* var. *anisopliae, B. bassiana, B.* cf. *bassiana, I. fumosorosea* and *Evlachovaea* sp..

The entomopathogenic fungi used in this study were selected based on their pathogenicity to *Galleria mellonella* L. larvae [13] and to represent fungal species isolated from each sampled regions. Most of the entomopathogenic fungi belonging to *Beauveria, Aspergillus* and *Fusarium* genus were chosen for molecular identification. *Metarhizium* was not found in these studies. We identified *B. bassiana* (13), *A. flavus* (4), *F. oxysporum* (8*), F. solani* (1), and *Penicillium* sp. (1). We were unable to identify the *Beauveria* (Bk.2010.1A) strain up to the species level.

In current study, most of the isolated fungi were determined as opportunistic pathogen such as *A. flavus, F. oxysporum* and *F. solani.* Previous studies also showed that both insect pathogenic fungi and opportunistic pathogenic fungi frequently occurred in China [26,27] and in the Palestinian area [8]. They also showed that some of these opportunistic pathogen species were highly pathogenic to insects [26]. However, Koz & Güven [13] showed week pathogenicity of *A. flavus* on *G. mellonella* larvae. These fast growing opportunistic fungi in soil habitat may infect injured or weakened insects [26].

Identification and detailed description of the fungal strain as an active ingredient of biopesticide is required environmental and epizootiologial information. In this study, we conducted a morphological and molecular study of entomopathogenic fungi isolated from the soils in Kahramanmaraş province in Turkey. Additional research is required to test pathogenicity of these fungi to other pests and to determine the effectiveness in the field conditions.

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