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Genetic variability of *Beauveria bassiana* and *Metarhizium anisopliae* var. *anisopliae* isolates obtained from the Eastern Black Sea Region of Turkey

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Abstract: *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* var. *anisopliae* (Metschnikoff) Sorokin are the most common entomopathogenic fungi used in microbial control programs all over the world. Assessments of the genetic variability of these 2 important species are useful for the development of effective biocontrol strategies and for evaluating the impact of artificial epizootics. In this study, the genetic diversity of 13 *B. bassiana* and 33 *M. anisopliae* var. *anisopliae* strains isolated from the hazelnut-growing region of Turkey was determined by using the amplified fragment length polymorphism (AFLP) and α- and β-isoenzyme analyses. Cluster analysis of AFLP data clearly separated both *B. bassiana* and *M. anisopliae* var. *anisopliae* strains into 3 and 4 different groups, respectively. While α- and β-esterase banding patterns clearly separated *M. anisopliae* var. *anisopliae* strains, it did not give enough information about *B. bassiana* strains. We also assessed the growing ability of all isolates at different temperatures (8, 16, 25, and 37 °C) and UV exposures (30 and 60 min), and virulence against *Tenebrio molitor*. These results indicated that there is a significant variability within the *B. bassiana* and *M. anisopliae* var. *anisopliae* populations in this region. Although the diversity of *B. bassiana* isolates is associated with geographic location, it is not associated with habitat type. There is also no association amongst *B. bassiana* isolates in terms of the ability to grow at different temperatures and UV exposures, or virulence against *T. molitor*. The diversity of *M. anisopliae* var. *anisopliae* strains is neither associated with habitat type nor geographic location, and there is no association amongst *M. anisopliae* var. *anisopliae* strains in terms of the ability to grow at different temperatures (except for 16 °C) and UV exposure, or virulence against *T. molitor*. The data presented here might be useful for controlling some hazelnut pests in this region.

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, genetic variability, hazelnut, geographic location

Türkiye'nin Doğu Karadeniz Bölgesi'nden elde edilen *Metarhizium anisopliae* var. *anisopliae* ve *Beauveria bassiana* izolatlarının genetik çeşitliliği

Özet: *Beauveria bassiana* (Balsamo) Vuillemin ve *Metarhizium anisopliae* var. *anisopliae* (Metschnikoff) Sorokin dünyadaki mikrobiyal kontrol programlarında kullanılan en yaygın entomopatojenik funguslardır. Bu 2 önemli türün genetik çeşitliliğinin değerlendirilmesi etkili biyolojik mücadele stratejilerinin geliştirilmesi ve yapay epizootiklerin etkisinin değerlendirilmesinde faydalıdır. Bu çalışmada, Türkiye'nin Doğu Karadeniz Bölgesi'nden elde edilen 13 adet *B. bassiana* ve 33 adet *M. anisopliae* var. *anisopliae* suşu içerisindeki genetik çeşitlilik Çoğaltılmış Parça Uzunluk

Polimorfizm (ÇPUP) tekniği ve izoenzim analizi kullanarak belirlenmiştir. ÇPUP verilerinin küme analizi hem *B. bassiana* hem de *M. anisopliae* var. *anisopliae* suşlarını sırasıyla 3 ve 4 farklı gruba ayırmıştır. α ve β- esteraz band paterni *M. anisopliae* var. *anisopliae* izolatlarını açıkça ayırmasına rağmen, *B. bassiana* izolatları için yeterli bilgi vermemiştir. Ayrıca, bu çalışmada bütün izolatların farklı sıcaklıklarda (8, 16, 25, 37 °C), UV muamelesinde (30 ve 60 dak.) ve *Tenebrio molitor*'a karşı virulansı belirlenmiştir. Bu sonuçlar, bu bölgedeki *B. bassiana* ve *M. anisopliae* var. *anisopliae* populasyonlarında önemli bir genetik çeşitliliğin olduğunu göstermektedir. *B. bassiana* suşlarının çeşitliliği coğrafik konumla ilişkili görülmesine rağmen habitat tipi ile ilişkili değildir. *B. bassiana* izolatları arasında farklı sıcaklıklarda, UV muamelesinde ve patojenite bakımından da bir ilişki tespit edilmemiştir. *M. anisopliae* var. *anisopliae* suşlarının çeşitliliği ise ne coğrafik konumla ne de habitat tipi ile ilişkilidir. *M. anisopliae* var. *anisopliae* izolatları arasında da farklı sıcaklıklarda (16 °C hariç), UV muamelesinde ve patojenite bakımından bir ilişki tespit edilmemiştir. Burada verilen bilgiler bu bölgedeki fındık zararlıları ile mücadelede faydalı olabilir.

Anahtar sözcükler: *Beauveria bassiana*, *Metarhizium anisopliae* var. *anisopliae*, genetik çeşitlilik, fındık, coğrafik lokalite

Introduction

The anamorphic entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin from the order Hypocreales (Ascomycota) have a worldwide distribution as members of the natural soil flora (1). These species infect a wide range of insect hosts; however, the host range of *M. anisopliae* is more restricted than that of *B. bassiana* (2,3). Much effort has been put into research on the development of *B. bassiana* and *M. anisopliae* as biological control agents (for inundation and inoculation biological control) to be applied in agriculture and forestry in temperate regions. However, this bulk of knowledge is in striking contrast to the lack of research into the fundamental ecology of these fungi in terrestrial ecosystems, including agroecosystems. To understand the ecology of indigenous fungal populations, studies must be carried out on isolates collected at a local scale and in different spatial compartments of the ecosystem. These isolates will represent genotypes that potentially interact with host populations, with each other, and with the environment under field conditions (4).

In Turkey, a total of 16 pest species can be considered as economically important hazelnut pests (5). Among these, some hazelnut pests such as *Melolontha melolontha*, *Curculio nucum*, *Xyleborus dispar*, and *Palomena prasina* have soil-borne phases in their life cycles. Considering the life cycle of the hazelnut pests and the climate in the Eastern Black Sea Region, it is believed that utilization of entomopathogenic fungi is the best approach to control the pest species that have soil-borne phases in their life cycles (6). We previously showed that entomopathogenic fungi have a good potential to control *Melolontha melolontha* L. (Coleoptera: Scarabaeidae) in the Eastern Black Sea Region of Turkey (6).

A variety of different genetic markers have been proposed to assess genetic variability as a complementary strategy to more traditional approaches in genetic resources management. A number of markers, such as RFLPs, RAPDs, AFLPs, DNA barcoding, and microsatellites, are now available to detect polymorphisms in nuclear DNA. Among these techniques, AFLP methods can rapidly generate hundreds of highly replicable markers from DNA; thus, they allow high-resolution genotyping of fingerprinting quality, and AFLP markers have found the widest application in analyses of genetic variation below the species level (7). Alternatively, isoenzymes can be used to detect polymorphism in strains of the 2 most common entomopathogenic fungi, *B. bassiana* (8-10) and *M. anisopliae* (11,12).

Several studies have been performed to determine genetic diversity within *B. bassiana* strains isolated from different (major) geographic regions and host species (13-17). However, very few studies have been carried out on the genetic diversity of *M. anisopliae* at a local or regional scale. Most of the knowledge has been generated in Canada on *M. anisopliae* isolates from different agricultural and forest habitats (18-21). Therefore, specific genetic studies of local populations of *M. anisopliae* still need to be conducted (4).

In selecting efficacious genotypes for microbial control programs and for understanding factors that modulate epizootics, it is important to have an understanding of the population genetics represented

within a particular ecosystem (19). To date, there have been no studies about the genetic diversity of populations of *B. bassiana* and *M. anisopliae* var. *anisopliae* isolates from Turkey. In our previous study, we determined that *Metarhizium anisopliae* and *Beauveria bassiana* could be found in hazelnut gardens and hypothesized that these fungi might be adapted to certain climatic conditions and habitat types (hazelnut) in the Eastern Black Sea Region of Turkey (6). In this study, we used AFLP and α- and β-esterase isoenzyme analyses to determine the genetic variability of *B. bassiana* and *M. anisopliae* var. *anisopliae* isolates from this region. This information might be useful in the selection of suitable biological control agents for microbial control programs and pest management strategies of hazelnut pests in this region.

Materials and methods

Description of study area

The Eastern Black Sea Region is located in the northeastern part of Turkey. The region can be separated into 2 main parts according to their climatic conditions. Ordu, Giresun, Trabzon, Rize, and Artvin constitute the coastal part of the region, while Gümüşhane and Bayburt constitute the inner part of the region (Figure 1). The inner and coastal parts show different environmental characteristics. The climate of the coastal part (Ordu, Giresun, Trabzon, Rize, and Artvin) is humid and that of the inner part (Bayburt and Gümüşhane) is continental. In the humid locations, annual temperatures are lower and rainfall is higher. However, the continental locations have a strong continental climate (very hot summer and very cold winter) with minimal rain in summer. Quantitative descriptions of the climatic conditions (annual temperatures, mean temperatures of the coldest and warmest months, and total annual rainfall) are given in Table 1 (22).

Moreover, hazelnuts are mostly grown in the coastal part of the region (especially in Trabzon, Giresun, and Ordu) and there are no hazelnut plantations in the inner part because of the climatic conditions.

DNA extraction

In this study, a total of 13 *B. bassiana* and 33 *M. anisopliae* var. *anisopliae* strains isolated from different parts of the hazelnut-growing region of Turkey were used. Detailed information about the isolates is given in Table 2. All isolates were identified in our previous study based on their morphological and molecular characteristics (6). All isolates were both stocked at –80 °C and maintained at 4 °C as cultures on potato dextrose agar (PDA) medium (Difco, USA). The isolates were propagated from a single conidium for DNA isolation. To achieve this,

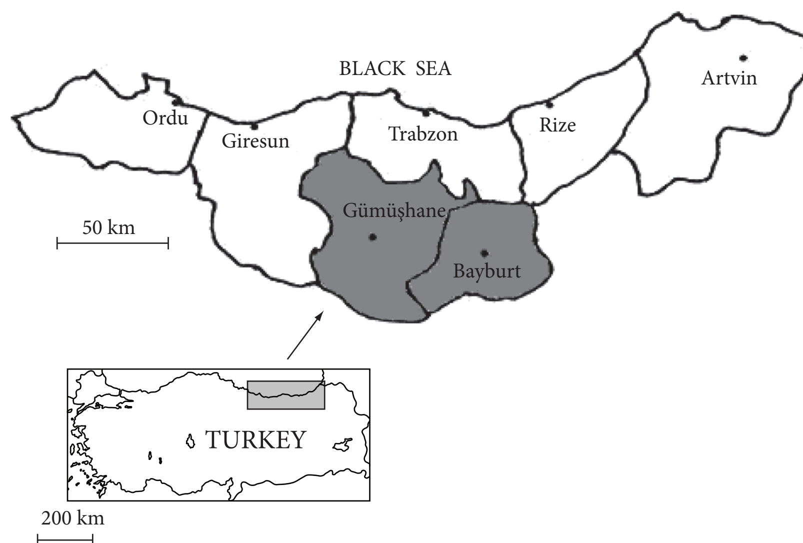

Figure 1. A map of the study area. Gray areas show the inner parts of the Eastern Black Sea Region (Gümüşhane and Bayburt) and white areas show the coastal parts of the region (Artvin, Rize, Trabzon, Giresun, and Ordu).

Table 1. Sampling locations and climatic characteristics of the Eastern Black Sea Region of Turkey (22).

Location	Total rainfall (mm)	Main temperatures (°C)			Aridity index*
		Annual	Warmest month	Coldest month	
Artvin	726.2	12.3	40.8	–9.6	61
Rize	2292.9	14.7	34.4	–4.6	77
Trabzon	897.3	15	37	–4	73
Giresun	1264	14.8	36	–3	71
Ordu	1018.5	14.7	37.1	–4	68
Gümüşhane	451.1	9.7	41	–21.6	67
Bayburt	543.7	7	19	–5.5	15.04

*Numerical indicator of the degree of dryness of the climate at a given location

100 µL of conidial suspensions (1×10^5 mL^{-1}) were plated on PDA medium and incubated at 25 °C for 3-4 days. At the end of the growth period, a single colony was transferred onto another PDA agar medium and incubated at 25 °C, and the resultant mycelium was used to inoculate flasks containing 75 mL of potato dextrose broth (PDB) liquid medium and incubated with shaking (120 rpm) at 25 °C for 1 week. After the incubation period, the mycelium was collected using filter paper, and then the samples were ground in liquid nitrogen using a mortar and pestle. The remaining mycelium was stored at –80 °C. Approximately 50 mg of crushed mycelium was used for DNA extraction, and the rest of the sample was stored at –20 °C until needed. DNA extraction was done using the DNeasy Plant Mini Kit (QIAGEN) and the NucleoSpin Plant Kit (Clontech) according to the manufacturers' recommendations. The extracted DNA was stored at –20 °C until use.

Esterase analyses

For protein extraction, the mycelium of each strain was transferred to a liquid culture medium (10 g of dextrose, 2.5 g of peptone, and 0.4 g of yeast extract in a 75-mL flask) and incubated at 28 °C for 7 days. The mycelium was harvested using filter paper. The protein extraction was carried out in liquid nitrogen by crushing with a mortar and pestle. The final extract was dissolved in a Tris-glycine buffer and centrifuged to clarify it. The protein concentration was determined utilizing a Bradford assay (23). From each sample, 100 µg of protein was loaded on a native-PAGE. The resolving gel was prepared by mixing 5.9 mL of distilled water and 2.4 mL of 30% acrylamide Bis (29:1), 2.6 mL of 1.5 M Tris-hydrochloric acid (pH 8.8), 100 µL of 10% ammonium persulfate, and 4 µL of TEMED. The stacking gel was prepared by mixing 6.2 mL of distilled water and 1.3 mL of 30% acrylamide Bis (19:1), 2.5 mL of 0.5 M Tris-hydrochloric acid (pH 6.8), 50 µL of 10% APS, and 15 µL of TEMED. The gel was run for 1.3 h at a constant 80 V with a running buffer (0.031 M Tris and 0.25 M glycine at pH 8.6). After the electrophoresis, the gels were washed in 0.05 M phosphate buffer (pH 7.2) and then stained with the staining mixture (100 mL of 0.05 M phosphate buffer at pH 7.2, 10 mg each of α- and β-naphthyl acetate dissolved in 1 mL of acetone, 50 mg of Fast Blue RR, and 4 mL of 4% formaldehyde) for 30 min at 37 °C.

Amplified fragment length polymorphism

AFLP analysis was carried out according to the method of Vos et al. (24) with minor modifications: 500 ng of genomic DNA was digested with *Eco*RI (10 U mL^{-1}) and *Mse*I (10 U mL^{-1}) restriction enzymes in restriction ligation buffer (100 mM Tris-acetate at pH 7.5, 100 mM $Mg(Ac)_2$, 500 mM KAc, 50 mM dithiothreitol). Following the complete digestion, adapters were ligated in the same reaction mixture for 4 h at 37 °C adding T4 ligase (1 U mL^{-1}). At the end of the incubation period, 150 µL of highly purified water was added and the product was stored at –20 °C until

Table 2. *B. bassiana* and *M. anisopliae* var. *anisopliae* isolates used in this study and their locality, source, and vegetation.

No	Isolates	Species	Locality		Source	Vegetation	Habitat type
			City	County			
1	KTU-2	*Metarhizium anisopliae* var. *anisopliae*	Rize	Ardeşen	Soil	Tea	1[a]
2	KTU-3	*Metarhizium anisopliae* var. *anisopliae*	Rize	Derepazarı	Soil	Tea	1
3	KTU-4	*Metarhizium anisopliae* var. *anisopliae*	Rize	Derepazarı	Soil	Tea	1
4	KTU-6	*Metarhizium anisopliae* var. *anisopliae*	Rize	Derepazarı	Soil	Tea	1
5	KTU-10	*Metarhizium anisopliae* var. *anisopliae*	Ordu	Center	Soil	Hazelnut	1
6	KTU-12	*Metarhizium anisopliae* var. *anisopliae*	Ordu	Perşembe	Soil	Hazelnut	1
7	KTU-14	*Metarhizium anisopliae* var. *anisopliae*	Trabzon	Sürmene	Soil	Pine, chestnut	2[b]
8	KTU-15	*Metarhizium anisopliae* var. *anisopliae*	Artvin	Borçka	Soil	Hazelnut, walnut	1
9	KTU-18	*Metarhizium anisopliae* var. *anisopliae*	Artvin	Murgul	Soil	Grass	2
10	KTU-19	*Metarhizium anisopliae* var. *anisopliae*	Artvin	Murgul	Soil	Alder	2
11	KTU-20	*Metarhizium anisopliae* var. *anisopliae*	Artvin	Camili	Soil	Corn	1
12	KTU-21	*Metarhizium anisopliae* var. *anisopliae*	Artvin	Borçka	Soil	Hazelnut	1
13	KTU-26	*Metarhizium anisopliae* var. *anisopliae*	Rize	Kalkandere	Soil	Meadow	2
14	KTU-27	*Metarhizium anisopliae* var. *anisopliae*	Rize	İkizdere	Soil	Tea	1
15	KTU-28	*Metarhizium anisopliae* var. *anisopliae*	Rize	İkizdere	Soil	Alder	2
16	KTU-29	*Metarhizium anisopliae* var. *anisopliae*	Rize	Güneysu	Soil	Tea	1
17	KTU-31	*Metarhizium anisopliae* var. *anisopliae*	Ordu	Bolaman	Soil	Meadow	2
18	KTU-32	*Metarhizium anisopliae* var. *anisopliae*	Ordu	Bolaman	Soil	Hazelnut	1
19	KTU-34	*Metarhizium anisopliae* var. *anisopliae*	Ordu	Fatsa	Soil	Meadow	2
20	KTU-37	*Metarhizium anisopliae* var. *anisopliae*	Giresun	Görele	Soil	Hazelnut	1
21	KTU-39	*Metarhizium anisopliae* var. *anisopliae*	Ordu	Ulubey	Soil	Hazelnut	1
22	KTU-40	*Metarhizium anisopliae* var. *anisopliae*	Trabzon	Akçaabat	Soil	Cabbage	1
23	KTU-41	*Metarhizium anisopliae* var. *anisopliae*	Rize	Kalkandere	Soil	Tea	1
24	KTU-44	*Metarhizium anisopliae* var. *anisopliae*	Trabzon	Of	Soil	Hazelnut	1
25	KTU-45	*Metarhizium anisopliae* var. *anisopliae*	Trabzon	Of	Soil	Hazelnut	1
26	KTU-46	*Metarhizium anisopliae* var. *anisopliae*	Rize	Güneysu	Soil	Tea	1
27	KTU-47	*Metarhizium anisopliae* var. *anisopliae*	Rize	Güneysu	Soil	Tea	1
28	KTU-48	*Metarhizium anisopliae* var. *anisopliae*	Trabzon	Of	Soil	Tea	1
29	KTU-49	*Metarhizium anisopliae* var. *anisopliae*	Rize	Hemşin	Soil	Tea	1
30	KTU-51	*Metarhizium anisopliae* var. *anisopliae*	Gümüşhane	Center	Soil	Apple	1
31	KTU-54	*Metarhizium anisopliae* var. *anisopliae*	Bayburt	Çalıdere	Soil	Poplar	2
32	KTU-58	*Metarhizium anisopliae* var. *anisopliae*	Bayburt	Center	Soil	Vegetable	1
33	KTU-60	*Metarhizium anisopliae* var. *anisopliae*	Gümüşhane	Center	Soil	Vegetable	1
34	KTU-7	*Beauveria bassiana*	Trabzon	Yomra	Soil	Hazelnut	1
35	KTU-8	*Beauveria bassiana*	Ordu	Bolaman	Soil	Meadow	2
36	KTU-57	*Beauveria bassiana*	Gümüşhane	Center	Insect	Meadow	2
37	KTU-17	*Beauveria bassiana*	Artvin	Borçka	Soil	Hazelnut	1
38	KTU-22	*Beauveria bassiana*	Artvin	Borçka	Soil	Cabbage	1
39	KTU-23	*Beauveria bassiana*	Rize	Hemşin	Soil	Tea	1
40	KTU-25	*Beauveria bassiana*	Ordu	Ünye	Soil	Hazelnut	1
41	KTU-38	*Beauveria bassiana*	Ordu	Ulubey	Soil	Hazelnut	1
42	KTU-50	*Beauveria bassiana*	Gümüşhane	Center	Soil	Apple	1
43	KTU-52	*Beauveria bassiana*	Gümüşhane	Center	Soil	Vegetable	1
44	KTU-56	*Beauveria bassiana*	Gümüşhane	Center	Soil	Apple	1
45	KTU-59	*Beauveria bassiana*	Gümüşhane	Center	Soil	Apple	1
46	KTU-62	*Beauveria bassiana*	Bayburt	Center	Soil	Poplar	2

[a]Agricultural habitat
[b]Nonagricultural habitat

use. The diluted ligation product was used as complete DNA in the preamplification step (nonselective amplification). In the preamplification (nonselective) step, amplification was performed with primers *Eco*RI (5'-GACTGCGTACCAATTCNNN-3') and *Mse*I (5'-GATGAGTCCTGAGTAANNN-'3), in the following PCR conditions: 5 µL of 10× PCR buffer (100 mM Tris-HCl at pH 8.3, 25 mM $MgCl_2$, and 500 mM KCl), 2 µL of 5 mM dNTP, 1.5 µL of *Eco*RI primer (50 ng μL^{-1}), 1.5 µL of *Mse*I primer (50 ng μL^{-1}), 0.1 µL of Taq polymerase, and 29.9 µL of highly purified water. Twenty cycles were conducted in the thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) after denaturation at 94 °C for 30 min, 56 °C for 60 min, and 72 °C for 60 min. The selective amplification profiles were obtained with *Eco*RI-AG and *Mse*I-C primers for *B. bassiana* isolates and with *Eco*RI-AC/*Mse*I-AA for *M. anisopliae* var. *anisopliae* isolates. Both primers were shown to be informative and polymorphic (data not shown). The forward primer *Eco*RI with selective nucleotides was radio-labeled with γ-[33_p]-ATP (Amersham Biosciences Europe, Roosendaal, the Netherlands) and used in a PCR reaction with the following conditions: 2 µL of 10× PCR buffer, 0.4 µL of dNTP, 0.5 µL of *Eco*RI primer, 0.5 µL of *Mse*I primer, 0.04 µL of Taq polymerase, and 11.46 µL of highly purified water. The PCR program consisted of 13 cycles of 30 s at 94 °C, 30 s at 65 °C to 56 °C, $\Delta T = 0.7$ °C, and 60 s at 72 °C, followed by 18 cycles of 30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C. After amplification, an equal volume of formamide loading dye (approximately 20 µL) was added to the PCR products to stop the reaction. Before loading, PCR products were denatured by heating at 95 °C for 5 min. The denatured fragments were separated by electrophoresis at 100 V for 2.5 h on a 5% denaturating polyacrylamide gel using 1× TBE buffer, and the bands were visualized using autoradiography.

Evaluation of growth at different temperatures and UV exposures

The fungi were grown on PDA medium (Difco) in an incubator at 25 °C and under a 12:12 photoperiod. Conidia were harvested from 4-week-old cultures by adding 10 mL of sterile 0.01% Tween 80 (AppliChem, Germany) to the petri dishes. The conidial suspension was filtered through 2 layers of cheesecloth into a sterile 50-mL plastic universal bottle (Sterilin), which was then shaken for 5 min using a vortex. The concentration of conidial suspension was subsequently adjusted to 1×10^5 conidia mL^{-1} using a Neubauer hemocytometer. Fungal growth at different temperatures and UV exposures was determined according to the method described by Bidochka et al. (18) with small modifications: 96-well, flat-bottomed cell culture plates were filled with 100 µL of PDA and each well was inoculated with 3 µL of 1×10^5 mL^{-1} conidial suspension in 0.01% Tween 80. Fungal growth was evaluated at 8, 16, 25, and 37 °C. Criteria from Bidochka et al. (18) were adapted for indication of positive growth: at 8 °C, $OD_{630\ nm} > 0.15$ after 14 days; at 16 °C, $OD_{630\ nm} > 0.50$ after 5 days; at 25 °C, $OD_{630\ nm} > 0.50$ after 2 days; and at 37 °C, $OD_{630\ nm} > 0.25$ after 3 days. UV resistance of fungal isolates was tested by exposing fungal conidia to UV radiation (306 nm wavelength) for 30 and 60 min. After 2 days of incubation at 25 °C, $OD_{630\ nm} > 0.25$ was chosen as a positive indicator (18).

Bioassay

For the bioassay experiments, 4-5 instars of *Tenebrio molitor* larvae were used. The larvae were obtained from a laboratory culture and were stored in plastic boxes with old bread at room temperature and a 12:12 photoperiod until the bioassay was performed. Ten *T. molitor* larvae were put on the conidia-developing plate for 10-15 s, where they came in contact with the conidia. Plates without fungi were used as the control group. Following this, larvae were transferred to plastic petri dishes (35 mm) lined with moist filter paper, and were kept for 10 days at room temperature. Old bread was provided as food. The mortality counts of the larvae were recorded on day 10, and all dead larvae were removed from the petri dishes after every counting. All experiments were repeated 3 times.

Data analysis

The esterase zymogram and DNA fingerprint (AFLP analysis) were visually scored for the presence (1) or absence (0) of electrophoretic bands for each isolate to generate a binary matrix. The resulting data matrices were analyzed using Fingerprint Analysis with Missing Data version 1.1β (25) and TREECON version 1.3b (26). Biochemical or genetic similarities were calculated based on Jaccard's coefficient (27) and unbiased genetic distances (28). The dendrograms

were generated with the help of TREECON, using the unweighted pair group method with arithmetic mean (UPGMA) (29). Reliability of the dendrograms was tested by bootstrap analysis with 1000 replicates using TREECON.

The mortality data obtained from the laboratory bioassay was corrected according to Abbott's formula (30). Obtained data were subjected to chi-square tests for the assessment of differences between the clusters with respect to growth at different temperatures and UV exposures, habitat type, geographic location, and virulence data. All statistical analyses were done using SPSS 15.0.

Results

We conducted AFLP and α- and β-esterase analyses to determine genetic variability within 13 *B. bassiana* and 33 *M. anisopliae* var. *anisopliae* isolates from the hazelnut-growing region of Turkey. For *B. bassiana* strains, although α- and β-esterase band patterns did not reveal significant variability, AFLP revealed high genetic variability. Alpha- and β-esterase analyses produced a total of 2 clear bands on the esterase gel (Figure 2). Although the first band was the same for all isolates, the second band was determined for cluster 2 of AFLP, including the isolates KTU-59, KTU-56, KTU-62, and KTU-50 and KTU-57 and for only one isolate (KTU-7) of cluster 1 of AFLP (Figure 2). In addition, we used 1 primer combination (*Eco*RI-AG/*Mse*I-C) for AFLP analysis and obtained a total of 163 fragments. Of those fragments, 97 were polymorphic, resulting in a polymorphism level of 59.5%. A similarity value was constructed to estimate the level of DNA polymorphism among the 13 populations. The calculation of similarity values was based on the presence or absence of discrete characters (AFLP fragments) from paired samples. According to Jaccard's similarity coefficient, the similarity values ranged from 98% to 26%. The highest genetic similarity (98%) was found between KTU-22 and KTU-8 and between KTU-23 and KTU-8. The lowest genetic similarity (26%) was found between the KTU-38 and KTU-57 isolates. Based on cluster analysis of the obtained fragments from AFLP, *B. bassiana* strains fall into 3 distinct clusters (Figure 2). The first cluster consists of 6 isolates that were, except for KTU-52, isolated mainly from agricultural fields in coastal parts of the region. The second group consists of 5 isolates that were isolated from nonagricultural and agricultural fields in the inner parts of the region. The third cluster, very different from the other 2, consists of 2 isolates that were isolated from agricultural fields in the coastal region of Ordu. Each of the 3 clusters was supported by a bootstrap value of 100% (Figure 2).

For *M. anisopliae* var. *anisopliae* isolates, the esterase banding pattern varied depending on the isolates (Figure 3). We used 1 primer combination (*Eco*RI-AC/*Mse*I-AA) and obtained a total of

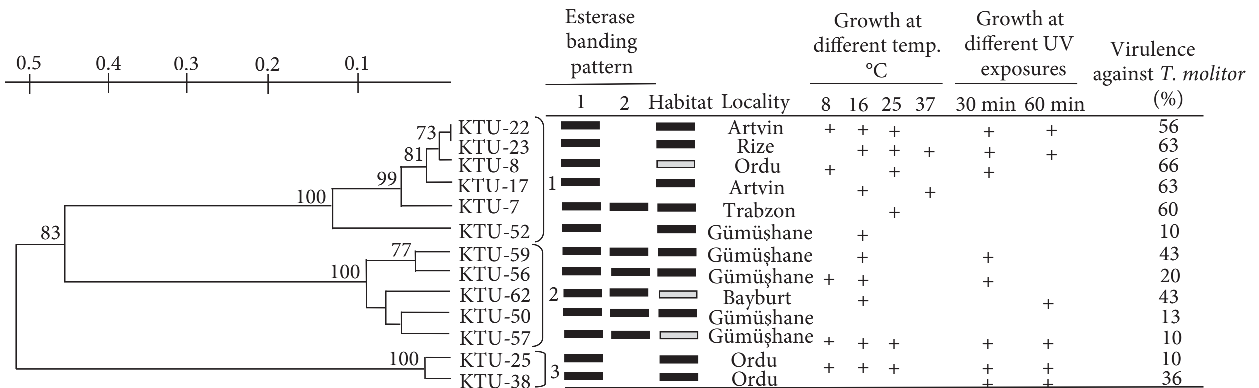

Figure 2. A dendrogram showing the genetic relationship among *Beauveria bassiana* strains based on AFLP analysis, using the UPGMA clustering method. A bootstrap analysis was carried out to test the reliability of the clusters based on 1000 replicates. The scale on the top of the dendrogram shows the degree of dissimilarity. Only bootstrap values >70% are labeled. Black bars show agricultural habitats and gray bars show nonagricultural habitats. For the temperature and UV experiments, + shows positive growth.

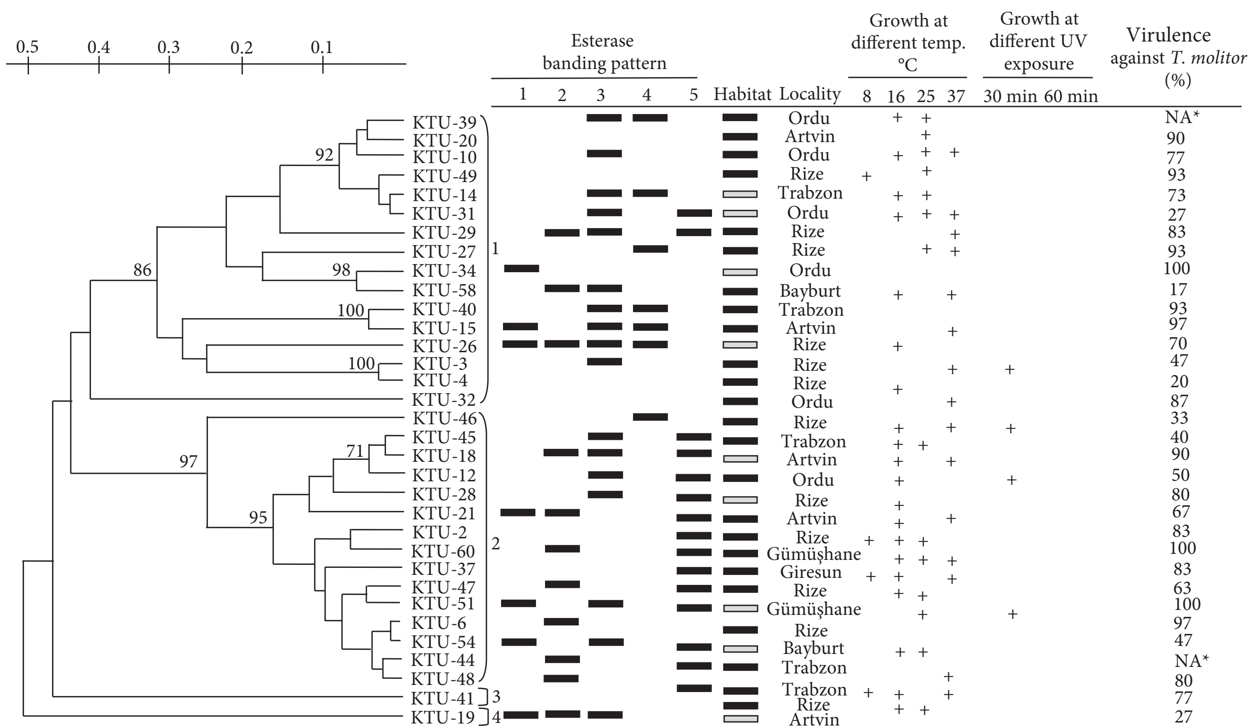

Figure 3. A dendrogram showing the genetic relationship among *M. anisopliae* var. *anisopliae* strains based on AFLP analysis, using the UPGMA clustering method. A bootstrap analysis was carried out to test the reliability of the clusters based on 1000 replicates. The scale on the top of the dendrogram shows the degree of dissimilarity. Only bootstrap values >70% are labeled. Black bars show agricultural habitats and gray bars show nonagricultural habitats. For the temperature and UV experiments, + shows positive growth. *NA: not available.

115 fragments. From those, 91 fragments were polymorphic, resulting in a polymorphism level of 79.1%. The similarity values, calculated according to Jaccard's similarity coefficient, ranged from 95% to 22%. The highest genetic similarity (95%) was found between KTU-3 and KTU-4, KTU-18 and KTU-12, KTU-14 and KTU-31, and KTU-18 and KTU-45. The lowest genetic similarity (22%) was found between KTU-32 and KTU-41. Based on cluster analysis of the obtained fragments of AFLP, *M. anisopliae* var. *anisopliae* strains fall into 4 distinct clusters (Figure 3). Cluster 1 consists of 16 isolates that were isolated from different habitats and vicinities, supported by a bootstrap value of 86%. Cluster 2 consists of 15 isolates that were isolated from different habitats and vicinities, supported by a bootstrap value of 97%. Subclusters exist within cluster 1 and cluster 2, but the clustering pattern did not reveal any relationship to geographic locations and habitat types as all isolates grouped randomly. Both cluster 3 and cluster 4 consist of single isolates: KTU-41 and KTU-19, respectively (Figure 3).

As soon as different clusters were identified for both *B. bassiana* and *M. anisopliae* var. *anisopliae* isolates, they were analyzed for associations with the habitat and geographic location from which the isolates were obtained. For *B. bassiana* isolates, there was no association among clusters with respect to the habitat type (chi-square = 4.409, df = 2, P > 0.05). However, in terms of geographic locations, the isolates from the coastal part were associated with clusters 1 and 3, while isolates from the inner part were associated with cluster 2 (chi-square = 9.647, df = 2, P < 0.05). For *M. anisopliae* var. *anisopliae* isolates, only clusters 1 and 2 were compared, since both clusters 3 and 4 consisted of only one isolate. There was no association between clusters 1 and 2 with respect to habitat (chi-square = 0.11, df = 1, P > 0.05) or geographic location (chi-square = 1.302, df = 1, P > 0.05).

Moreover, the growth ability of the isolates at different temperatures (8, 16, 25, and 37 °C) and their resilience to different UV exposures (30 and 60 min) were evaluated. For *B. bassiana* isolates, there was no association among clusters with respect to the ability to grow at 8 °C (chi-square = 1.107, df = 2, P > 0.05), 16 °C (chi-square = 0.638, df = 2, P > 0.05), 25 °C (chi-square = 2.404, df = 2, P > 0.05), and 37 °C (chi-square = 2.758, df = 2, P > 0.05). There was also no association among clusters with respect to the ability to grow at different UV exposures of 30 min (chi-square = 2.136, df = 2, P > 0.05) and 60 min (chi-square = 2.297, df = 2, P > 0.05). For *M. anisopliae* var. *anisopliae* isolates, there was no association among clusters with respect to the ability to grow at 8 °C (chi-square = 1.302, df = 1, P > 0.05), 25 °C (chi-square = 0.45, df = 2, P > 0.05), and 37 °C (chi-square = 0.034, df = 1, P > 0.05), but cluster 2 grew significantly better than isolates from cluster 1 at 16 °C (chi-square = 4.288, df = 1, P < 0.05). There was also no association among groups with respect to the ability to grow at the different UV exposures of 30 min (chi-square = 1.302, df = 1, P > 0.05) and 60 min (chi-square = 2.297, df = 2, P > 0.05).

Virulence values of *B. bassiana* isolates against *T. molitor* ranged from 10% to 66%, and there was no association among *B. bassiana* isolates in terms of virulence against *T. molitor* (chi-square = 18.489, df = 16, P > 0.05). Virulence values of *M. anisopliae* var. *anisopliae* isolates ranged from 17% to 100%, and there was also no association among *M. anisopliae* var. *anisopliae* isolates in terms of virulence against *T. molitor* (chi-square = 17.653, df = 18, P > 0.05).

Discussion

This study provides general information about the genetic diversity of entomopathogenic fungi *B. bassiana* and *M. anisopliae* var. *anisopliae* strains in the hazelnut-growing region of Turkey and their associations regarding habitat type, geographic location, growth at different temperatures and UV exposures, and virulence against *T. molitor*. Considering the variability observed from AFLP and esterase analysis, it is possible to say that populations of *B. bassiana* and *M. anisopliae* var. *anisopliae* comprise different populations belonging to different clones.

The findings presented here utilizing AFLP and esterase banding patterns related to the great diversity in *B. bassiana* isolates support many studies that have already been published using the same or different techniques, such as AFLP, RAPD, RFLP, and ISSR-PCR isoenzymes (10,15,17,31). In the present study, we found that the diversity within *B. bassiana* isolates is related to geographic location. Although isolates within cluster 1 and cluster 3 were isolated from mainly coastal parts (Artvin, Rize, Trabzon, Giresun, and Ordu), isolates from cluster 2 were isolated from inner parts (Gümüşhane and Bayburt) of the region. Knowing the different environmental characteristics of the inner and coastal parts of the region, it may be possible to indicate that some *B. bassiana* isolates from this region have adapted to certain climatic conditions. Furthermore, considering the distribution of hazelnut plantations within the region (hazelnut is mostly grown in the coastal part of the region), it might be possible to say that *B. bassiana* strains that have adapted to the coastal parts of the region might be used to control some important hazelnut pests. These results do not agree with those given by Bidochka et al. (32). They found distinct groups within *B. bassiana* populations with respect to habitat type and various temperatures: 1 genetic group associated with agricultural habitats, 2 groups with forest habitats, and 1 group (3 isolates) in the Canadian Arctic.

Although many studies about the genetic diversity of *B. bassiana* isolates exist, there are few studies about *M. anisopliae* var. *anisopliae* isolates and most of them are about Canadian isolates (18-21,33,34). In the present study, high genetic variability was found among *M. anisopliae* var. *anisopliae* isolates, and the determined clusters are not associated with respect to habitat type, geographic location, growth at different temperatures, UV resistance, or virulence against *T. molitor*. Bidochka et al. (18) illustrated that there is a clear association within *M. anisopliae* isolates in Ontario, Canada. They determined 2 groups, the first associated with agricultural habitats and the second associated with forested habitats. These results are not similar to our results. On the other hand, recently, Inglis et al. (19) found very minimal genetic variability among the 63 isolates of *M. anisopliae* var. *anisopliae* that were isolated throughout southwestern British Columbia, and this result is inconsistent with the results of Bidochka et al. (18).

We also tested *B. bassiana* and *M. anisopliae* var. *anisopliae* isolates in terms of their ability to grow at different temperatures and UV exposures, which are important components influencing the population structure of these species (18,32). Normally, we would expect to find an association between *B. bassiana* isolates with regard to growth at different temperatures and UV exposures, but we did not determine any such association. However, Bidochka et al. (32) demonstrated that certain associations could be found between groups of *B. bassiana* isolates with regard to growth at different temperatures and UV resiliencies. The reason for this might be that different isolates were used in both studies. This kind of association was also found for *M. anisopliae* isolates from Canada (32). In this study, although we did not determine any growth associations at 8, 25, and 37 °C, we found that cluster 2 can grow better than cluster 1 at 16 °C.

We also determined the virulence of both *B. bassiana* and *M. anisopliae* var. *anisopliae* isolates against *T. molitor*. Although there is much evidence indicating that *B. bassiana* and *M. anisopliae* genotypes might be related to the host species, we did not determine any such association in this study (8,12,17,21,34-37). However, *M. anisopliae* var. *anisopliae* isolates seemed to be more virulent than *B. bassiana* isolates in this study.

In conclusion, based on esterase and AFLP data, it might be possible to say that some *B. bassiana* strains from the Eastern Black Sea Region of Turkey are adapted to certain climates in this region, and the data presented here could be useful for isolate selection to control some hazelnut and forest pests in this region. The genetic variability determined in this study within *M. anisopliae* var. *anisopliae* populations might be explained by adaptation to different pest species in the region. However, this needs to be investigated. Consequently, we showed that geographic location at the local scale could have an effect on determination of the diversity of distribution of *B. bassiana* populations in the Eastern Black Sea Region. Future studies should include clarification of some associations between different isolates, especially for *M. anisopliae* var. *anisopliae* isolates.

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