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ORIGINAL RESEARCH PAPER

Isolation, characterization and virulence of entomopathogenic fungi from *Gryllotalpa gryllotalpa* (Orthoptera: Gryllotalpidae)

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Abstract Mole crickets are significant pests of turf, vegetable and some tree seedlings worldwide, and it is costly to control them. In this study, we obtained 15 fungal isolates from *Gryllotalpa gryllotalpa* L. (Orthoptera: Gryllotalpidae) and compared the efficacy of these isolates against this pest with the aim of identifying their biocontrol potential. The fungal isolates were identified based on their morphological and molecular characteristics, using *ITS*, *EF1-α*, *Bloc*, *RPB1*, *RPB2* and *β-Tubulin* gene sequencing. Consequently, the isolates were identified as *Beauveria bassiana* (Balsamo) Vuillemin (Gg-1), *Clonostachys* sp. (Gg-2, Gg-3, Gg-5, Gg-6, Gg-8 and Gg-13), *Bionectria* sp. (Gg-4), *Metarhizium anisopliae* (Metschnikoff) Sorokin (Gg-7, Gg-12 and Gg-14), *Clonostachys rogersoniana* (Schroers) (Gg-9 and Gg-15), *Myriodontium* sp. (Gg-10) and *Myriodontium keratinophilum* (Samson and Polon.) (Gg-11). These isolates caused mortalities ranging from 0 to 87 %, with the most virulent fungus being *M. anisopliae* Gg-12, which caused 87 % mortality within 15 days post inoculation using a conidial concentration of 1×10^7 conidia ml^{-1}. Concentration–response test was conducted to determine the LC_{50} value of *M. anisopliae* Gg-12, and it was calculated as 1.069×10^6 conidia ml^{-1}. As a result, *M. anisopliae* Gg-12 can be further investigated in terms of controlling mole crickets.

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Keywords Mole crickets · Entomopathogenic fungi · Microbial control · *Metarhizium anisopliae*

Introduction

The mole cricket, *Gryllotalpa gryllotalpa* L. (Orthoptera: Gryllotalpidae), is a major pests of turf, vegetable and some tree seedlings in Turkey and many other parts of the world (Frank and Parkman 1999; The Ministry of Agriculture of Turkey 2008). It feeds on the roots of a wide range of plants, resulting in plant stress and even death (Thompson 2003). Feeding damage can also provide a route of entry for plant pathogenic fungi and bacteria. Current control of this pest still depends upon the use of chemical pesticides, which often have to be applied at relatively high rates to reach the insects that live under the ground (Thompson and Brandenburg 2005).

There is increasing attention on the use of entomopathogenic fungi to control insect pest populations, because they offer an environmentally safe alternative to conventional chemical pesticides, many of which have been withdrawn due to the damage they cause to the environment and human health (Khetan 2001). Entomopathogenic fungi are widely reported as pathogenic to a number of subterranean pests (Ansari et al. 2009; Ansari and Butt 2012, 2013; Talwar 2005).

To our knowledge, there have been no reports on natural infections of *G. gryllotalpa* by entomopathogenic fungi. However, Thompson and Brandenburg (2005) evaluated three different isolates of *B. bassiana*, including the commercial product BotaniGard. Thompson and Bradenburg (2006) also tested the effectiveness of *B. bassiana* against mole crickets in combination with imidacloprid. Also, Thompson et al. (2007) determined the behavioral response

of mole crickets in soils treated with *B. bassiana*. Finally, Xia et al. (2000) tested three commercial bioproducts of *B. bassiana* against mole crickets. Herein, we report for the first time natural fungal infections of *G. gryllotalpa*, and evaluate these fungi against this pest under laboratory conditions in order to determine the biocontrol potential of the isolates.

Materials and methods

Isolation of fungi

Mole cricket nymphs and adults were collected from soils of Trabzon, Turkey between April and May of 2010 and 2011. After collection, they were incubated in ventilated plastic boxes (30 mm^3) and fed on fresh slices of potato under 12:12 photoperiod at room temperature. The insects were checked once a week, and those that had died or exhibited mycosis were transferred to a moist chamber for 1 week to stimulate fungal sporulation. Fungi were isolated by cutting out portions of mycosed cadavers and placing these onto Potato Dextrose Agar + 1 % yeast extract (PDAY) medium containing 50 µg/ml ampicillin and 50 µg/ml tetracycline (AppliChem, Germany) to avoid growing bacteria. The petri dishes were incubated for 2 weeks at 28 °C for further growth and sporulation. Each isolate was subcultured from a single colony to obtain pure cultures. To reach the aim, a conidial suspension of 1×10^6 ml^{-1} was prepared and 100 µl from this suspension was plated on PDAY and incubated at 28 °C for 1 week under 12:12 photoperiod. After growing, single colonies were cut out as plugs and transferred individually to fresh PDAY plates. The inoculated plates were incubated at 28 °C for 2–3 weeks until they were fully overgrown. Single colony spore suspensions were stored in sterile 20 % glycerol at −80 °C.

Morphological identification

The fungal isolates were initially sent to Dr. Richard Humber for morphological characterization (Humber 1997). The fungal cultures were deposited in the USDA-ARS Collection of Entomopathogenic Fungi (ARSEF; Ithaca, NY, USA) and the Microbiology Laboratory at Karadeniz Technical University (Trabzon, Turkey).

Molecular characterisation of the isolates

Sequencing of the *ITS1-5.8S-ITS2* gene region and the partial sequencing of *EF1-α*, *Bloc*, *RPB1*, *RPB2* and *β-Tubulin* genes was conducted to confirm the identity of the isolates. Briefly, fungal conidia were inoculated into 100 ml flasks including 50 ml potato dextrose broth (PDB medium) (Difco, NJ, USA) and the cultures were incubated at 25 °C in a rotary shaker (GFL 3031) at 230 rpm for 1 week. After growing, mycelial mass was harvested on filter paper, frozen in liquid nitrogen, then crushed. Genomic DNA was extracted using the DNeasy Plant mini Kit (Qiagen, Hilden Germany) and Nucleospin Plant Kit (Clontech, CA, USA) from 50 mg of crushed mycelium. Genomic DNA was stored at −20 °C for later use.

The full length of the *ITS1-5.8S-ITS2* gene for each isolate was amplified using the primer pair of ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) as forward primer and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as reverse primer (White et al. 1990) in a 50 µl reaction volume containing 5 µl 10 × *Taq* DNA polymerase reaction buffer, 200 µM of each dNTPs, 0.05 nmol of each opposing amplification primer, 2.5 unit *Taq* DNA polymerase (Promega, CA, USA) and 50 ng genomic DNA. PCR conditions were as follows: initial denaturation at 95 °C for 4 min; 35 cycles of 95 °C for 1 min, annealing at 58 °C for 55 s and extension at 72 °C for 2 min; and final extension at 72 °C for 10 min.

In addition, an approximately 1200 bp segment of *tef* (translation elongation factor1-α), 1500 bp segment of *Bloc* (the nuclear intergenic region), 700 bp segment of *RBP1* (RNA polymerase II largest subunit) and 1200 bp segment of *RBP2* (RNA polymerase second largest subunit) were amplified and sequenced for further characterization of *Beauveria* isolates according to the study of Rehner et al. (2011). PCR conditions were adapted essentially as described earlier (Rehner and Buckley 2005; Rehner et al. 2011). Similarly, for the genus of *Metarhizium*, the same gene regions used for *Beauveria* genus were amplified and sequenced, except that 1300 bp segments of the *β-Tubulin* gene were amplified instead of the *Bloc* gene (Bischoff et al. 2009). PCR conditions were adapted according to the study of Rehner and Buckley (2005) and Rehner et al. (2011). The primers used in this study are given in Table 1. GenBank accession numbers for the isolates are given in Table 2.

PCR products were loaded on 1.0 % agarose gels that contained ethidium bromide and scanned under UV light for visualization. PCR products were purified using QIAquick PCR purification kit (Qiagen, Hilden Germany) and then sent to MACROGEN (The Netherlands) for sequencing. Obtained sequences were subjected to BLAST searches using the NCBI GenBank database and phylogenetic analysis to compare with known species (Altschul et al. 1990; Benson et al. 2012). *ITS1-5.8S-ITS2*, *tef*, *bloc*, *RPB1* and *RPB2* gene sequences of our isolates and the sequences from the study of Rehner et al. (2011) were included for comparison of *Beauveria* isolates. For *Metarhizium* isolates, *tef*, *RPB1*, *RPB2* and *β-Tubulin* gene

Table 1 Primers used in this study and their sequences

Gene	Primer name	Sequence	Reference
ITS1-5.8S-ITS2	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	White et al. (1990)
	ITS5	5′-GGAAGTAAAAGTCGTAACAAGG-3′	
Tef	EF1T	5′-ATGGGTAAGGARGACAAGAC-3′	Rehner and Buckley (2005)
	1567R	5′-ACHGTRCCRATACCACCSATCTT-3′	
Bloc	B5.1F	5′-CGACCCGGCCAACTACTTTGA-3′	Rehner et al. (2006)
	B3.1R	5′-GTCTTCCAGTACCACTACGCC-3′	
RPB1	RPB1Af	5′-GARTGYCCDGGDCAYTTYGG-3′	Stiller and Hall (1997)
	RPB1C	5′-CCNGCDATNTCRTTRTCCATRTA-3′	
RPB2	Frpb2-5F	5′-GAYGAYMGWGATCAYTTYGG-3′	Liu and Whelen (1999)
	RPB2-7cR	5′-CCCATRGCTTGYTTRCCCAT-3′	
β-Tubulin	T1	5′-AACATGCGTGAGATTGTAAGT-3′	O'Donnell and Cigelnik (1997)
	T2	5′-TCTGGATGTTGTTGGGAATCC-3′	

Table 2 The fungal isolates used in this study, their source and GenBank accession numbers

Isolate	Source	Species	ARSEF No	GenBank accession numbers				
				ITS	*Bloc*	*Tef*	*Rpb1*	*β-Tubulin*
Gg-1	Adult	*Beauveria bassiana*	11,688	KM215661	KM215673	KM215654	KM215669	–
Gg-2	Adult	*Clonostachys* sp.	11,689	KM215678	–	–	–	–
Gg-3	Adult	*Clonostachys* sp.	11,690	KM215679	–	–	–	–
Gg-4	Adult	*Bionectria* sp.	11,691	KM215674	–	–	–	–
Gg-5	Adult	*Clonostachys* sp.	11,692	KM215680	–	–	–	–
Gg-6	Adult	*Clonostachys* sp.	11,693	KM215681	–	–	–	–
Gg-7	Adult	*Metarhizium anisopliae*	11,694	KM215664	–	KM215657	KM215672	KM215660
Gg-8	Adult	*Clonostachys* sp.	11,695	KM215682	–	–	–	–
Gg-9	Adult	*Clonostachys rogersoniana*	11,696	KM215676	–	–	–	–
Gg-10	Nymph	*Myriodontium* sp.	11,697	KM215684	–	–	–	–
Gg-11	Nymph	*Myriodontium keratinophilum*	11,698	KM215683	–	–	–	–
Gg-12	Adult	*Metarhizium anisopliae*	_	KM215662	–	KM215655	KM215670	KM215658
Gg-13	Nymph	*Clonostachys* sp.	11,700	KM215677	–	–	–	–
Gg-14	Nymph	*Metarhizium anisopliae*	_	KM215663	–	KM215656	KM215671	KM215659
Gg-15	Nymph	*Clonostachys rogersoniana*	11,702	KM215675	–	–	–	–

sequences of *Metarhizium* isolates were compared with previously published sequences according to the study of Bischoff et al. (2009). *ITS1-5.8S-ITS2* sequences of *Clonostachys* and *Bionectria* isolates were compared to other *Clonostachys* and *Bionectria* isolates discussed by Schroers (2001). Finally, *ITS1-5.8S-ITS2* sequences of *Myriodontium* isolates were compared with other isolates using BLAST searches in NCBI GenBank database (Altschul et al. 1990; Benson et al. 2012).

Experimental infection

To determine the pathogenicity of the fungal isolates, bioassay experiments were conducted against the third instar of *G. gryllotalpa* nymphs that were collected from the field. Conidial suspensions were prepared from 4-week-old cultures that were grown on PDAY. Conidia were harvested from plates by adding 10 ml of sterile 0.01 % Tween 80 (Applichem, Darmstadt, Germany) and the conidia were taken from the agar surface using a sterile cell scraper. The conidial suspensions were filtered through two layers of sterile muslin into sterile 50-ml tubes (Becton–Dickinson Falkon). Conidia concentrations were determined using a Neubauer haemocytometer and adjusted to 1×10^7 conidia ml^{-1}. The germination of conidia was observed by inoculating the conidia on PDAY and counting the number that had germinated after 24 h of incubation at 25 °C. Conidia were considered to have germinated if

the germ tubes were equal to or greater than the conidium width.

For the pathogenicity test, *Gryllotalpa gryllotalpa* nymphs were collected from damaged farmland in the vicinity of Trabzon, Turkey. Nymphs were maintained in plastic boxes (30 mm^3) and fed on fresh slices of potato at room temperature until bioassays were performed. We waited approximately 1 week so that nymphs were adapted to laboratory conditions. There were ten nymphs for each treatment; treatments were replicated three times and the experiment was repeated three times. Nymphs were inoculated by dipping into 10 ml of 1×10^7 ml^{-1} conidial suspension prepared from different cultures at different times for 4–5 s; they were then transferred to plastic containers (12 cm wide and 8 cm deep) containing sterile soil. Control nymphs were exposed to 0.01 % aqueous Tween 80 only. Each box containing ten nymphs was incubated at 20 °C under 12:12 photoperiod, and examined at 15 days post inoculation. Dead insects were surface sterilized by dipping into 2 % sodium hypochlorite solution for 3 min, followed by 70 % ethanol for 3 min, and then washed three times in sterile distilled water before being placed in a moisture chamber to stimulate external mycosis from the cadaver (Sevim et al. 2010a).

Concentration–response test

Concentration–response studies were conducted using *Metarhizium anisopliae* Gg-12, which was the most pathogenic isolate identified from the bioassays mentioned above.

Healthy third instar nymphs were collected from the farmland as described in the collection of nymphs, and were used for the concentration–response test. The nymphs were treated with four different concentrations of conidial suspension (1×10^6, 1×10^7, 1×10^8 and 1×10^9 conidia ml^{-1}) and the control group was treated with sterile water supplemented with 0.01 % Tween 80. For each replicate, 30 nymphs were used and the whole experiment was repeated three times. Nymphs were treated by dipping into 10 ml of each conidial suspension for 4–5 s, and they were then put into a plastic box (30 cm^3) together, and fresh potato slices were provided as the food. After that, boxes were incubated at 20 °C temperature under 12:12 photoperiod. Mortality of nymphs was checked daily for 15 days.

Data analysis

DNA sequences of the isolates were edited and aligned with Clustal W packed in Bioedit (version 7.09) (Hall 1990; Thompson et al. 1994). For *Beauveria* isolates, sequences of *ITS1-5.8S-ITS2*, *tef*, *bloc*, *RPB1* and *RPB2* gene regions were combined using a text editor. For *Metarhizium* isolates, the partial sequences of *tef*, *RPB1*, *RPB2* and *β-Tubulin* gene regions were combined and used for pyhlogenetic analysis. Genomic analyses using the neighbor joining were performed using the *p*-distance model with MEGA 6.0 software (Saitou and Nei 1987; Tamura et al. 2013). The reliability of the dendrogram was tested by bootstrap analysis with 1.000 pseudoreplicates using MEGA 6.0 (Felsenstein 1985; Tamura et al. 2013).

Mortality data were corrected according to Abbott's formula (Abbott 1925) and percent mycosis values were calculated based on the mycelia growth outside cadaver. The data were subjected to analysis of variance (ANOVA) followed by post hoc Tukey's tests to identify significant differences among fungal isolates and compare test isolates with each other and the control group with respect to mortality and mycosis ($p < 0.05$). Before performing the ANOVA, all data sets were tested for homogeneity of variance using Levene's statistic and all percentage data were subjected to arcsin transformation. The LC_{50} value of *M. anisopliae* Gg-12 was determined by probit analysis. Computations for all experiments were performed using SPSS 16.0.

Results

Isolation and morphological identification of fungi

A total of 15 fungal isolates were obtained from the field-collected *G. gryllotalpa* nymphs. The infected individuals were recognized according to the mycelia growth outside cadaver. The isolates were morphologically identified as *Beauveria bassiana* (×1), *Clonostachys rosea* (×9), *Metarhizium anisopliae* (×3) and *Myriodontium keratinophilum* (×2) (Table 2).

Molecular characterization

For further characterization of *Beauveria* isolates, the *ITS1-5.8S-ITS2* and partial sequence of *EF1-α*, *RPB1*, *RPB2* and *Bloc* gene regions were determined and used for genomic analysis. Based on the genomic analysis, one isolate was found to be identical to *B. bassiana* according to the study of Rehner et al. (2011) (Fig. 1).

For *Metarhizium* isolates, the partial sequences of *tef*, *RPB1*, *RPB2* and *β-Tubulin* gene regions were determined and used for genomic analysis. According to this analysis, three isolates (Gg-7, Gg12 and, Gg14) were found to be identical to *M. anisopliae* as described by Bischoof et al. (2009) (Fig. 2).

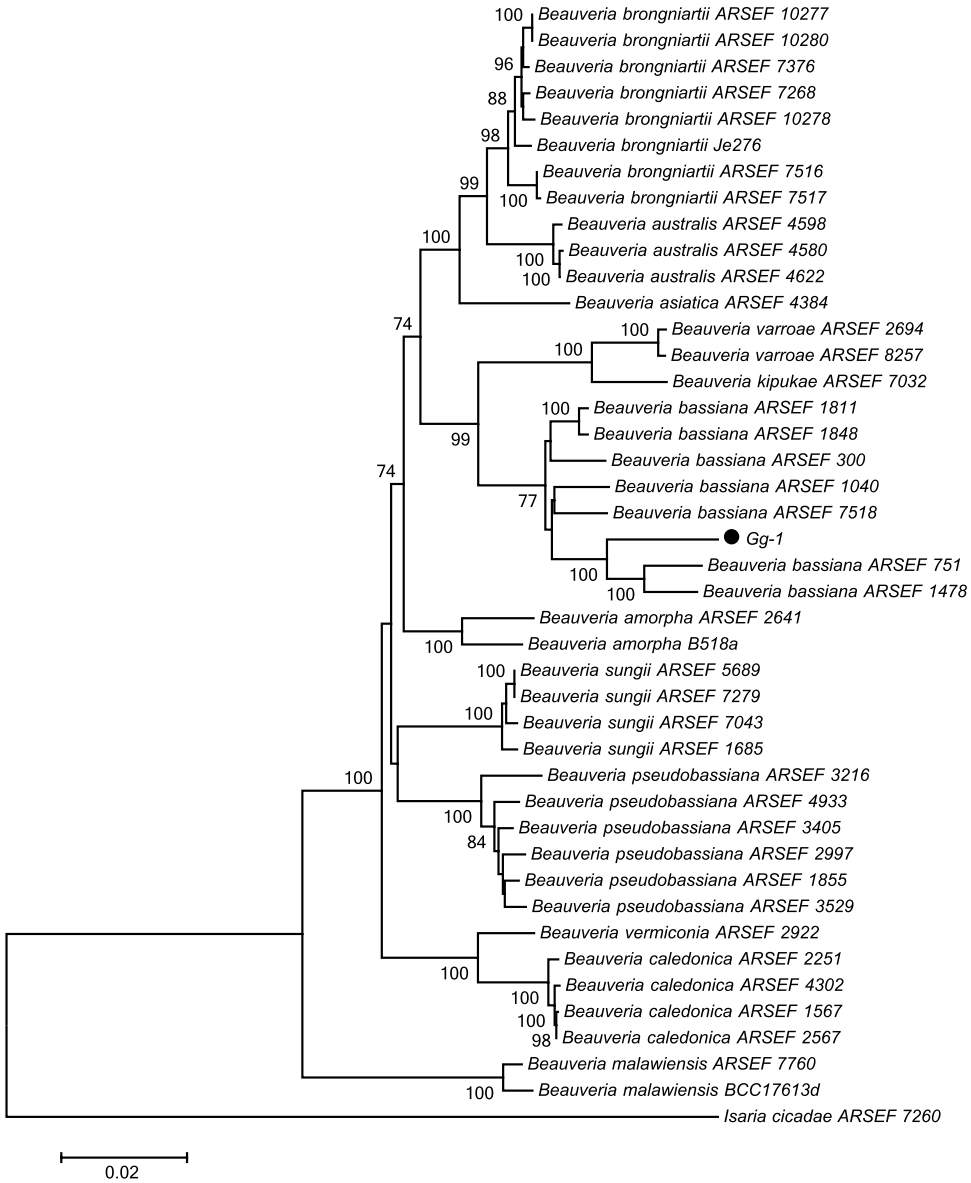

Fig. 1 Taxonomic position of *Beauveria bassiana* Gg-1 within *Beauveria* genus, based on the combined data from the *ITS* and partial sequence of *EF1-α*, *RPB1*, *RPB2* and *Bloc* gene regions. The reference strains were taken from the study of Rehner et al. (2011). The dendrogram was constructed by using the Neighbor-Joining analysis with *p*-distance model in MEGA 6.0. Bootstrap values shown next to nodes are based on 1000 replicates. Bootstrap values C ≥ 70 % are labeled. Gg isolates are indicated with *black dots*. The *scale* on the *bottom* of the dendrogram shows the degree of dissimilarity

To confirm the morphological identification of *Clonostachys* and *Bionectria* isolates, an approximately 510 bp fragment of the *ITS1-5.8S-ITS2* region was sequenced and used for pyhlogenetic analysis to compare with the reference strains from the study of Schroers (2001). Based on the pyhlogenetic analysis, two isolates (Gg-9 and Gg-15) were found to be identical to *C. rogersoniana*. Other isolates (Gg-2, Gg-3, Gg4, Gg-5, Gg-6, Gg-8 and, Gg-13) couldn't be identified at the species level. Therefore, they were identified as *Clonostachys* sp., except for Gg-4, which was identified as *Bionectria* sp. (Fig. 3).

Finally, we couldn't compare *Myriodontium* isolates with known reference isolates since there was no authoritative paper. Therefore, we conducted the BLAST searches using GenBank to find the exact identities of these isolates. Based on the BLAST searches of the *ITS1-5.8S-ITS2* sequences, Gg-10 showed 100 % homology with *Myriodontium* sp. NCP02/09 and Gg-11 showed 100 % homology with *M. keratinophilum* isolate DUMC 134.08.

Experimental infection

Based on total exposed insects, mortalities caused by the different fungal isolates ranged from 0 to 87 %, with control mortality being 10 % (Fig. 4). Mortalities by the pathogenic isolates Gg-1, Gg-2, Gg-7, Gg-11 and Gg-12 were significantly greater than in control group within 15 days post inoculation ($F = 45.389$; $df = 15, 32$; $p < 0.05$) (Fig. 4). The most virulent isolate was *M. anisopliae* Gg-12, which caused 87 % mortality within 15 days. The success of isolates Gg-1, Gg-2, Gg-4, Gg-7, Gg-10, Gg-11 and Gg-12 in growth and sporulation on cadavers

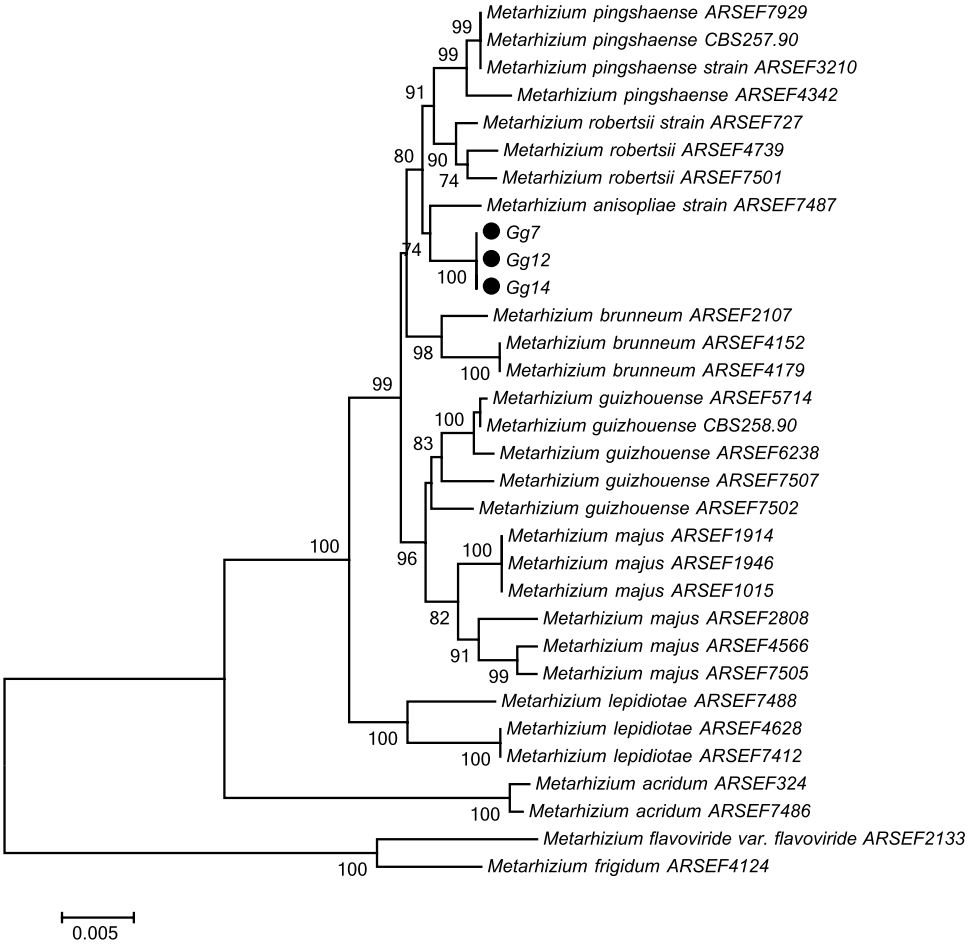

Fig. 2 Taxonomic position of *Metarhizium* isolates within *Metarhizium* genus based on the combined data from the partial sequence of *EF1-α*, *RPB1*, *RPB2* and *β-Tubulin* gene regions. The reference strains were taken from the study of Bischoff et al. (2009). The dendrogram was constructed by using the Neighbor-Joining analysis with *p*-distance model in MEGA 6.0. Bootstrap values shown next to nodes are based on 1000 replicates. Bootstrap values C ≥ 70 % are labeled. Gg isolates are indicated with *black dots*. The *scale* on the *bottom* of the dendrogram shows the degree of dissimilarity

was greater than for the control group ($F = 16.225$; $df = 15, 32$; $p < 0.05$). Isolates displayed different mycosis values in comparison with each other ($F = 12.035$; $df = 15, 32$; $p < 0.05$). The highest mycosis value was also obtained from *M. anisopliae* isolate Gg-12, at 57 % (Fig. 4).

Concentration–response test

In the concentration–response test, *M. anisopliae* Gg-12 showed 100 % mortality rate against *G. gryllotalpa* nymphs within 7 days after application of the conidial concentration of 1×10^8 conidia ml^{-1}. The LC_{50} of this fungus against the nymphs within 15 days after treatment was determined to be 1.069×10^6 conidia ml^{-1} with 95 % fiducial limits of 6.76×10^3–3.3×10^6 (intercept slope with standard error; 1.28 ± 0.511, χ^2 0.141 and *df* 2).

Discussion

This study showed that the mole cricket is susceptible to a range of soil-borne fungi; in particular, entomopathogens like *M. anisopliae* and *B. bassiana*, which are often reported as infecting disparate insect hosts (Sevim et al. 2010a, b, c, 2012a, 2013; Tanyeli et al. 2010; Zimmermann 2007a, b). *B. bassiana* and *M. anisopliae* are often isolated from insects (Kocacevik et al. 2015; Mascarin et al. 2013; Sevim et al. 2010a), *Clonostachys* sp. occasionally (Toledo et al. 2006), but this is the first time *M. keratinophilum* has been isolated from an insect. *M. keratinophilum* is a keratinophilic fungus that can cause infections in humans (Samson and Polonelli 1978).

In previous studies, some scientists reported that *B. bassiana* is a natural fungal pathogen for many different insect species in almost all insect orders including Orthoptera (Er et al. 2007; Goettel et al. 1990; Lomer et al. 2001; Sevim et al. 2010b; Thompson and Brandenburg 2006). Strains of *B. bassiana* that were isolated from a different location and host insect vary in terms of virulence against insects (Muro et al. 2003; Takatsuka 2007), and *B. bassiana* is the most common entomopathogenic fungi in agricultural fields (Meyling and Eilenberg 2006, 2007). There is a limited understanding of the biological control of *G. gryllotalpa* by application of *B. bassiana*. An example includes the application of either *B. bassiana* alone or *B.*

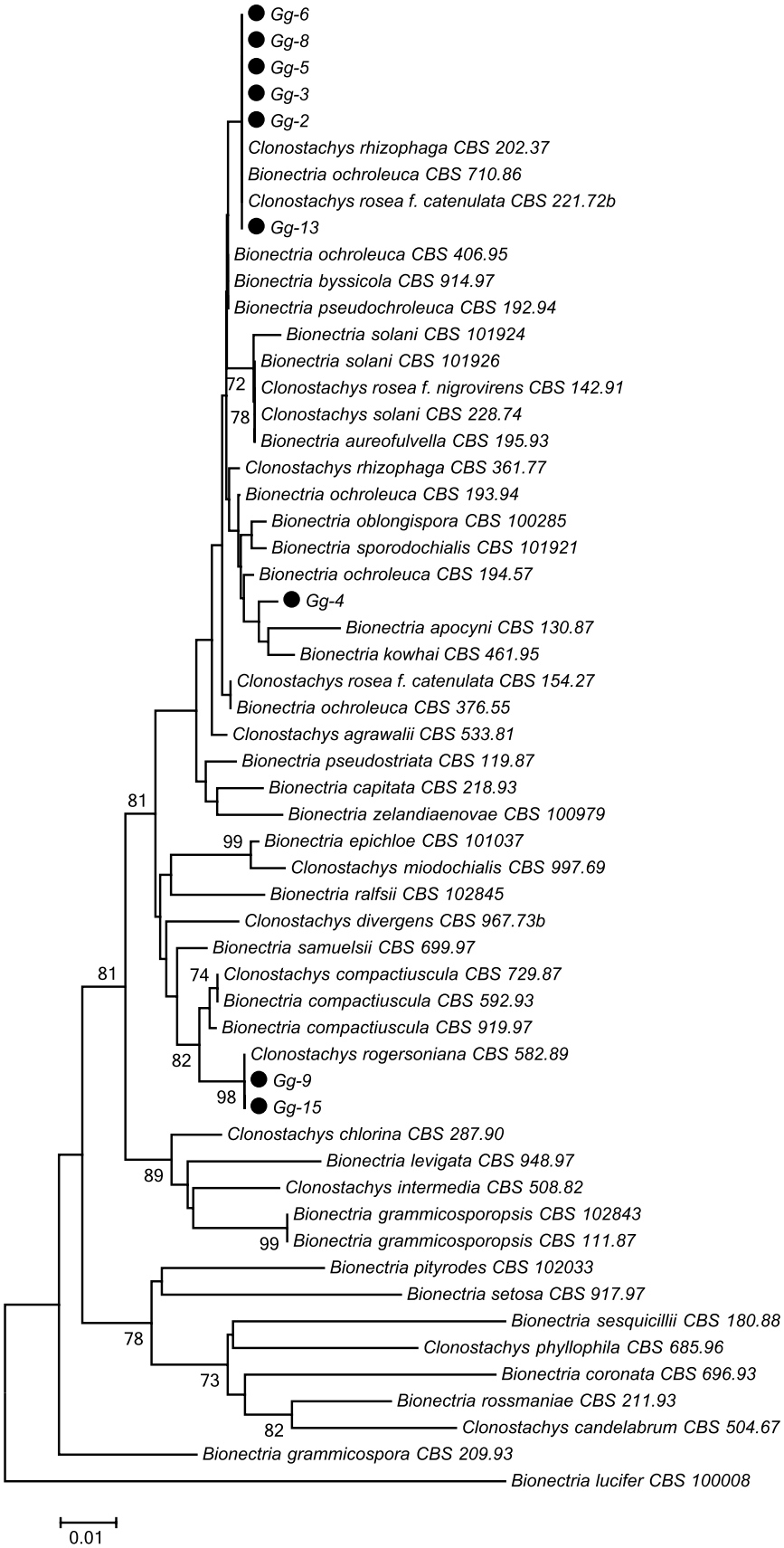

Fig. 3 Taxonomic position of *Clonostachys* and *Bionectria* isolates within the genus of *Bionectria* based on the *ITS* sequences. The reference strains were taken from the study of Schroers (2001). The dendrogram was constructed by using the Neighbor-Joining analysis with *p*-distance model in MEGA 6.0. Bootstrap values shown next to nodes are based on 1000 replicates. Bootstrap values C $\geq$ 70 % are labeled. Gg isolates are indicated with *black dots*. The *scale* on the *bottom* of the dendrogram shows the degree of dissimilarity

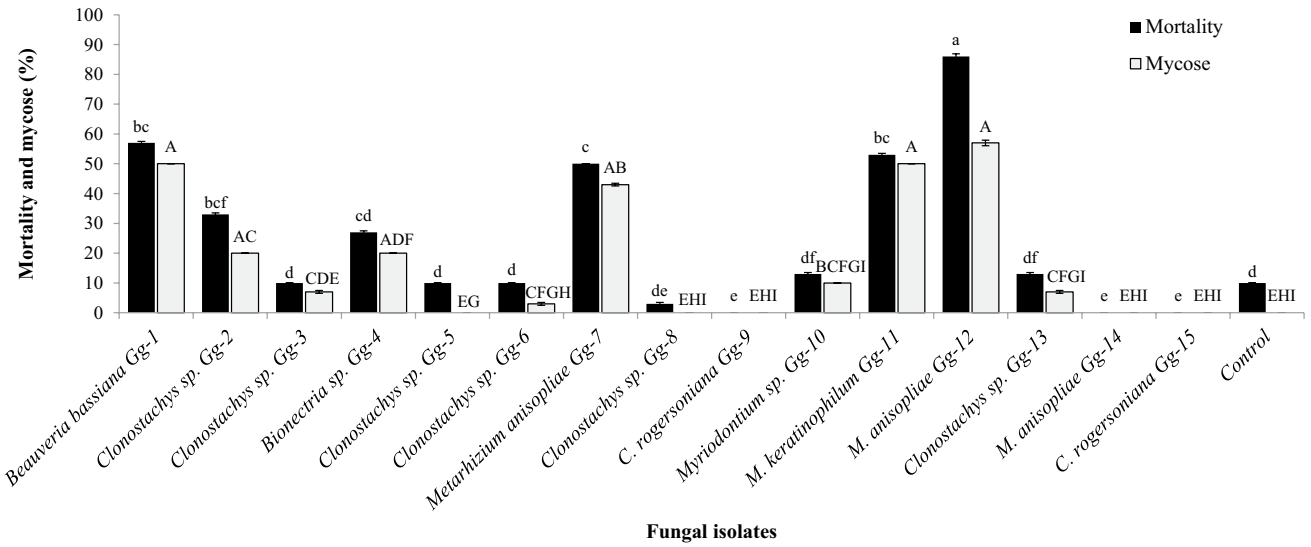

Fig. 4 Mortality of *G. gryllotalpa* nymphs after application of 15 entomopathogenic fungal isolates within 15 days after application of 1×10^7 conidia ml^{-1}. Mortality data were corrected according to Abbott's formula (Abbott 1925). Different *uppercase* and *lowercase letters* represent statistically significant differences among treatments with respect to mortality and mycosis according to Tukey's test ($p < 0.05$). *Bars* show standard deviation

bassiana with imidacloprid to *G. gryllotalpa* in a turfgrass field. However, none of the treatments caused an acceptable mortality rate (Hertl and Brandenburg 1998). In this study, we isolated *B. bassiana* from *G. gryllotalpa* and determined that it had 57 % insecticidal activity on the pest within 15 days. It has also 50 % mycosis rate. In the study of Thompson and Bradenburg (2005), three different strains of *B. bassiana*, including one isolated from the southern corn rootworm (BotaniGard), one from darkling beetles (DB-2), and the other from soil (10–22), were evaluated against *G. gryllotalpa*. Thompson (2003) showed that *B. bassiana* isolates DB-2 and 10–22 achieved 46.7 % mortality value with conidial concentration of 1×10^7 conidia ml^{-1} within 16 days. While the concentration was 1×10^8 conidia ml^{-1}, the mortality rate was 80 % with DB-2 and 60 % with 10–22. These results indicated that *B. bassiana* Gg-1 obtained from this study seems to be more effective than other isolates in previous studies against *G. gryllotalpa*. These differences observed in pathogenicity tests may be related to host species in which fungi were obtained or the test conditions.

Up to now, the species and strains of *Clonostachys* sp. have been isolated from different plants parts and they were cultivated from grassland, woodland, forest, freshwater and coastal soils, except for *C. rosea*, which was reported from adult females of *Oncometopia tucumana* Schröder (Hemiptera: Cicadellidae) (Sutton et al. 1997; Ten Hoopen et al. 2010; Toledo et al. 2006; Zhang et al. 2008). The present study showed the second determination of *Clonostachys* sp. from an arthropoda, although *Clonostachys* sp. is not a common fungal species among insects. Insecticidal effects of *Clonostachys* isolates on *G. gryllotalpa* nymphs varied from 0 to 33 % depending on the isolate. While the mortality rate was 33 % with Gg-2 and 27 % with Gg-4 within 15 days after application, the fungal growth was observed as 20 % in dead insects for both isolates. Toledo et al. (2006) showed that insecticidal effects of different *Clonostachys rosea* isolates were 82.5 and 45.5 % on *O. tucumana* and *Sonesimia grossa* Signoret (Hemiptera: Cicadellidae) within 14 days after inoculation, respectively. However, mycosis rates obtained in those applications were found to be only 12.5 and 11.8 %, respectively. All these may suggest that *Clonostachys* sp. is not common in insects, and is possibly an insect pathogen. However, more detailed bioassay experiments, such as calculation of LC_{50} and LT_{50} values, are needed to prove this suggestion.

Metarhizium anisopliae is a widely distributed soil-inhabiting fungus (Goettel et al. 2005; Lacey et al. 2001; Zimmermann 2007b). So far, the strains of *M. anisopliae* have been isolated from many different species of arthropod (ARSEF, Ithaca, NY, USA; Luz et al. 1998). We also isolated a *M. anisopliae* isolate (Gg-12) from *G. gryllotalpa*, and determined that it has significant mortality (87 %) and mycosis rates (57 %) against the pest. *M. anisopliae* has been reported to be effective in the suppression of soil borne pests like termites, crickets, locusts, brown plant hopper in rice, pyrilla, spittle bug in sugarcane and root grubs (Zimmermann 2007b). That means that this isolate could be further investigated as a possible biocontrol agent against *G. gryllotalpa*.

Ansari et al. (2009) tested the effectiveness of ten virulent strains of entomopathogenic fungus *M. anisopliae* against *Agriotes lineatus* L. (Coleoptera: Elateridae) larvae. In that study, *A. lineatus* larvae treated with the isolates of V1002 and LRC181A caused 90 and 100 % mortality at 3 weeks post-inoculation, respectively. Due to *A. lineatus* being a root pest like *G. gryllotalpa*, *M. anisopliae* Gg-12 can be also effective against this pest. Moreover, fungi take a long time to kill the insect; meanwhile, damage is being done to the crop. Therefore, a faster controlling method is sometimes needed or the efficacy of the fungus could be improved by using low dose chemicals, certain botanicals or entomopathogenic nematodes. Because *M. anisopliae* is mainly from soil, the inclusion of spinosad or other insecticide treatments could extend the total control period provided by one application (Ericsson et al. 2007). Additionally, *M. anisopliae* Gg-12 was directly isolated from *G. gryllotalpa*, and this could be an important advantage in terms of biocontrol, as some studies have shown that entomopathogenic fungi may have ecological compatibility with the pest species, geographic location or habitat type (Bidochka et al. 2001; Sevim et al. 2010a, b). Furthermore, the isolate Gg-12 showed the highest mycosis value (57 %) on dead cadavers. This is also an important advantage, since sporulation on the host is essential for spread of fungi in the farmland.

Although there is a limited information about *M. keratinophilum* in the literature, there have been some studies showing its occurrence in natural materials and mammals. Samson and Polonelli (1978) showed that natural sources of *M. keratinophilum* are soil and different parts of mammals such as penis of a bull and keratinous substrates. In addition, this fungus has not been seen around the world since 1979 (Humber, personal communication). In this study, we obtained two *Myriodontium* isolates from *G. gryllotalpa* nymphs, and one of these (Gg-11) caused significant mortality (53 %) and mycosis (50 %) values against *G. grylloyalpa* nymphs based on preliminary pathogenicity tests. We propose that this fungus might be a potential insect pathogen.

In conclusion, we have shown that *M. anisopliae* Gg-12 has a good potential for further investigation to develop an effective biological control agent against *G. gryllotalpa*. The next step is to assess commercial productivity of the isolate Gg-12. It is notable to say that this study has been performed under laboratory conditions. However, field conditions have external factors such as UV, humidity and temperature. Therefore, the isolate should also be examined in the field to draw a broader conclusion. The suitability of the fungus for mass production and the stability of a formulated or unformulated product containing the fungus under various conditions should be also warranted.

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