Pathogenicity of native isolates of entomopathogenic fungi Beauveria *and* Metharizium *genera on* Microcerotermes diversus *(Blattodea: Termitidae) in the laboratory*

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ORIGINAL RESEARCH ARTICLE

Pathogenicity of native isolates of entomopathogenic fungi *Beauveria* and *Metharizium* genera on *Microcerotermes diversus* (Blattodea: Termitidae) in the laboratory

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Abstract

Microcerotermes diversus Silvestri (Blattodea: Termitidae) is a worldwide destructive termite whose control by conventional methods is often difficult. Biological control using entomopathogenic fungi could be an alternative management strategy. Two species of entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*, isolated from natural habitats of Mashhad and Lahijan regions, Iran. The fungi were characterized based on sequences of ITS gene as well as classic data. Then, the infectivity of both isolates of *M. anisopliae* and *B. bassiana* in different concentrations (1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml) were evaluated under laboratory conditions by two methods, including spray and pipetting against termite, *M. diversus*. Both entomopathogenic fungi species were capable of infecting and killing *M. divesrus*. In the pipetting method, the LC_{50} value for *B. bassiana* and *M. anisopliae* calculated 8.03×10^5 (conidia/ml) and 1.03×10^6 (conidia/ml), respectively. But in the spray method, the effect of *M. anisopliae* on *M. diversus* was more than *B. bassiana*. The LC50 value in the spray method for *B. bassiana* and *M. anisopliae* was 3.52×10^7 (conidia/ml) and 1.65×10^6 (conidia/ml). The mortality caused by the fungus was dose-dependent, with the highest mortality recorded at the highest concentration. In the pipetting method, the mortality rate for *B. bassiana* and *M. anisopliae* was 0 to 97.5% and 0 to 100% at 8 day post infection. In the spray method, the mortality percentage for *B. bassiana* was from 2.5 to 72.5% and for *M. anisopliae* was 0 to100% by 4 days post-infection. The results of this study indicated that *B. bassiana* and *M. anisopliae* are potentially useful biological control agents for *M. diversus*. Future studies with field trails will provide a confident approach toward termite management.

Keywords Entomopathogenic fungi · *Beauveria bassiana* · Insect pathology · *Metarhizium anisopliae* · Termites

Introduction

Termites (Isoptera) are critical foresty, agriculture, and household pests (Balachander et al. 2009). They damage dead woody materials (Balachander et al. 2009). Sometimes, their activity can cause severe economic damage (Rath 2000). Prevention of termite damage is complicated because their population is very high, and their cryptic behavior.

The important genera of termites in Iran are *Amitermes*, *Microcerotermes* (Termitidae), *Anacanthotermes* (Hodotermitidae), and *Psammotermes* (Rhinotermitidae) (Cheraghi et al. 2013). Previously, it has been indicated that *Microcerotermes diversus* is the most destructive termite in middle East on date palms in Iran, Iraq and Saudi Arabia (Cheraghi et al. 2013).

Different methods were applied to control termites, such as physical, cultural, chemical, and biological methods (Ravindran et al. 2015). Various organochlorine/cyclodiene insecticides such as Aldrin, Dieldrin, Chlordane, and Heptachlor are used to control termites. But some of them are now banned because of their bioaccumulation and the effect on non-target insects (Singha et al. 2011). The use of some organophosphorus insecticides has limitations. Chlorpyriphos is an organophosphosphate compound that is expensive and toxic to the environment (Arora and Arora 1995). Logan et al. (1990) suggested using non-chemical for termite control.

Biological control with entomopathogenic fungi protects non-target organisms (Lacey and Kaya, 2000), natural enemies, and is safe for the environment (Pell et al. 2001).

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Various groups of microorganisms, including entomopathogens with biological potential, are associated with termite nests (Milner et al. 1998). The termites live in habitats with high humidity habitats as suitable niches for fungal infection (Vargo et al. 2003. However, there are few limitations in the application of entomopathogenic fungi for the control of termites. The termites remove fungal conidial by grooming. In addition, termites remove infected termites of the colony (Culliney and Grace 2000). This social behavior might lead to increased resistance to pathogens in their colony (Yanagawa and Shimizu, 2005).

Numerous studies have been performed on the use of the fungi to control termites (Lacey and Kaya 2000; Wright et al. 2005). Milner (2000) evaluated the effect of *M. anisopliae* on termites. Wright et al. (2005) evaluated the impact of *M. anisopliae* on *Coptotermes formosanus*. Yanagawa and Shimizu (2007) determined the resistance of *C. formosanus* against *M. ansiopliae* by grooming. In another work, *Paecilomyces* sp. was used for the control of subterranean termites (Eilenberg et al. 2001).

It had been confirmed that *M. diversus* is the most crucial termite in Ahwaz (Habibpour 1994, 2006). In Iran, in performed studies on the control of termites, Ghayourfar and Mohammadpour (2009) used Acid boric and Hexaflumuron to control subterranean termite in date palm orchards. In 2012, Horizontal transmission of the *M. anisopliae* in *M. diversus* was evaluated by Cheraghi et al. (2012). Rahimzadeh et al. (2012) evaluated the pathogenicity of *M. anisopliae* on *Microcerotermes gabrielis* in the laboratory.

The objectives of this research were isolation and characterization of native entomopathogenic fungi from selected regions of Iran and then survey on their pathogenecity on *Microcerotermes diversus*.

Materials and methods

Insect

Insects collection

Thorough 2018–2019, different termite casts, *M. diversus* were collected from palm orchards in Ahwaz (Khuzestan province 31.4360° N, 49.0413° E). At first, wood damaged with termites in palm orchards of the Ahwaz region were collected, then transferred to the laboratory and cultured on pieces of wood in the containers with 15 cm diameters at 28 ± 1 °C and 75 ± 5% RH.

Termite identification

A molecular approach was used initially for termite identification, which complemented the classic method. For DNA extraction, an individual worker cast was used. DNA was extracted using a 5% Chelex®100 solution (Karimi and Darsouei 2014). Cytochrome oxidase subunit I gene was amplified using C1J-1718 (forward) and C1N-2191 (reverse) primers as described by Simon et al. (1994). The PCR product was sequenced by Macrogen Company (South Korea, Seoul). The obtained sequence was checked using Nblast software for quality, assembled, and then submitted in the GenBank. For phylogenic analysis, 37 valid DNA sequences were provided form the GenBank. The sequences were aligned in Clustal X (ver. 2) (Larkin et al. 2007). The phylogenetic tree was reconstructed using MEGA7 (Kumar et al. 2016) with 10,000 bootstraps (Felsenstein 1985) and neighbor-joining method (Saitou and Nei 1987).

Complementary identification of termite has done using the classic approach. A morphological key was used to identify termite species.

Entomopathogenic fungus

Isolation of entomopathogenic fungi

The soil samples were collected from two regions in Iran. The first region, including Mashhad (Razavi Khorasan, Iran, 36.2980° N, 59.6057° E) in NorthEast of Iran. The second region of sampling was the North part (Lahijan, Guilan, 37.2071° N, 50.0034° E(. Different regions have sampled via soil sampling then surveyed by baiting technique using *Galleria mellonella* larvae. The soil samples were surveyed every 48 h. Dead *G. mellonella* larvae were placed into the traps at 25 ± 1 °C, in the dark till the growth of the entomopathogenic fungi. Then for the purification, some insect cadavers were sterilized in 70% ethanol and cultivated on PDA (Potato Dextrose Agar) medium and kept at 28 ± 1 °C for the future test (Darsouei et al. 2018).

Characterization of entomopathogenic fungi

For molecular identification, the fungal isolates were cultured on 20 ml liquid culture (Potato Dextrose Broth) and shaked for two weeks at room temperature. Then the mycelia were harvested, eluted, and transferred into a sterile 1.5-ml microtube. Genomic DNA was extracted with Pars Tous Kit (Cat Number A101211; http://www.parstous.com). Then ITS region was amplified and sequenced by using ITS4 and ITS5 primers (White et al. 1990).

A number of valid DNA sequences of ITS gene *Beauveria* and *Metarhizium* genus were retrieved from the GenBank and aligned together with corresponded isolates/species of the Iranian isolate sequence using Clustal W software (ver. 2). The nucleotide distances were calculated with the K2P model (Kimura 1980) using the MEGA 7 software (Kumar et al. 2016). The phylogenetic tree was reconstructed in MEGA 7

with the neighbor-joining method (Saitou and Nei 1987) and 10,000 replications of bootstrap (Felsenstein 1985).

Laboratory bioassays

Spray method

For determination the efficacy of the fungal isolates against termite, at first, the termites were sterilized with 1% $NaClO_4$, washed with sterilized water twice then, transferred into the moistened filter paper in the petri dish (diameter 9 cm). Five concentrations of two isolates of entomopathogenic fungi resulted from sampling, which were assigned as Iran111 and Iran112, were prepared. An initial dose-response assay was done to measure the doses rang. Then, the applied concentrations were 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml. The suspension was sprayed on termites and incubated in germinator at 28 ± 1 °C and 80% RH. There were ten termites on each petri dish. The control termites were treated with distilled water and 0.2% Tween 20. Each treatment had five replications. For the mortality rate, the Petri dishes were checked daily and dead individuals transferred on moisturized filter paper to confirm the Koch's postulate to monitor the fungal hypha's emergence. At last, the percentage of mortality was calculated.

Pipetting technique

Four concentrations of *B. bassiana* and *M. anisopliae*, including 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml were prepared in sterile distilled water and 0.2% Tween 20. The fungal isolates were applied by pipetting 1 ml of conidial suspensions on filter paper in a Petri dish. Then five adult termites in each treatment were placed on inoculated filter paper. Each treatment had four replications. Control treatments were treated with 1 ml, 0.2% Tween 20. For mortality determination, the Petri dishes were checked daily and dead individuals transferred on moisturized filter paper to confirm the Koch's postulate to monitor fungal hypha's emergence. At last, the percentage of mortality was calculated.

Statistical analysis

Statistical analyses for all bioassays (larvae and adult insects) were conducted with the SAS software (SAS Institute 1989). Cumulative mortalities were corrected for control mortality (Abbott 1925). One-way analysis of variance (ANOVA) was used to determine the effect on mortality across fungal species and the effect on mortality across spore concentration. The influences of fungal species and different concentrations on the mortality rate of *M. diversus* were analyzed by two-way full factorial ANOVA (fungal species * different concentrations). Then a slicing test was used to measure the significant difference between means. Before ANOVA analysis, data were assessed for normality and homogeneity of variance (SAS Institute 1989). All graphic data indicate the mean ± standard error (SE) of the mean in each treatment.

Results

Termite

A partial sequence of the COI gene for collected termite population from Ahwaz was submitted into the GenBank. This is the first DNA sequence for this termite species. Due to the absence of DNA sequence from this species, we selected some DNA for the nearest species and genus within the corresponded subfamily and tribe. The NBlast analysis was indicated that Iranian isolate had %100 similarity with *Microcerotermes* sp. TB-2017 isolate (accession number KY224717). In phylogenetic analysis, the Iranian isolate was placed in a clade with other isolates of *Microcerotermes* sp. with %100 bootstrap (Fig. 1). The mean nucleotide distances were 0.116 (range 0.00–0.156), which was calculated using the K2P model. Here, 787 nucleotides were aligned that 517 sites were conserved, 270 sites were variable, and 241 sites were parsimony-informative. The parallel classic identification was confirmed the species identify as *M. diversus*.

Entomopathogenic fungi

Morphological characterization

The results of sampling led to the isolation of nine isolates from two fungal species. The initial morphological and morphometric measurements showed high similarities of corresponded isolates of each species. The *Beauveria* isolates named Iran111, Iran113, Iran114, Iran 115, and Iran 116, collected from Mashhad and assigned as *Beauveria bassiana* based on the morphology and morphometry of conidia. The other four isolates, Iran121, Iran 122, Iran 123, and Iran 124 were isolated from Lahijan and characterized based on the morphology and micrometry of conidia as *Metarhizium anisopliae.*

For *B. bassiana*, the conidia aggregated as a white mass and were globose, subglobose, or broadly ellipsoid in the chape. Their diameter was 3.5 ± 0.25 μm. Initially, conidia in *M. anisopliae* were white. After 5–7 days, their color change to the green and were oval in shape. Their length was 8.35 ± 0.05 μm and adhered together like a chain. Due to the high similarities of each species' isolates, we did a preliminary assay on the termite to select the virulent isolate, and this isolates considered for future tests.

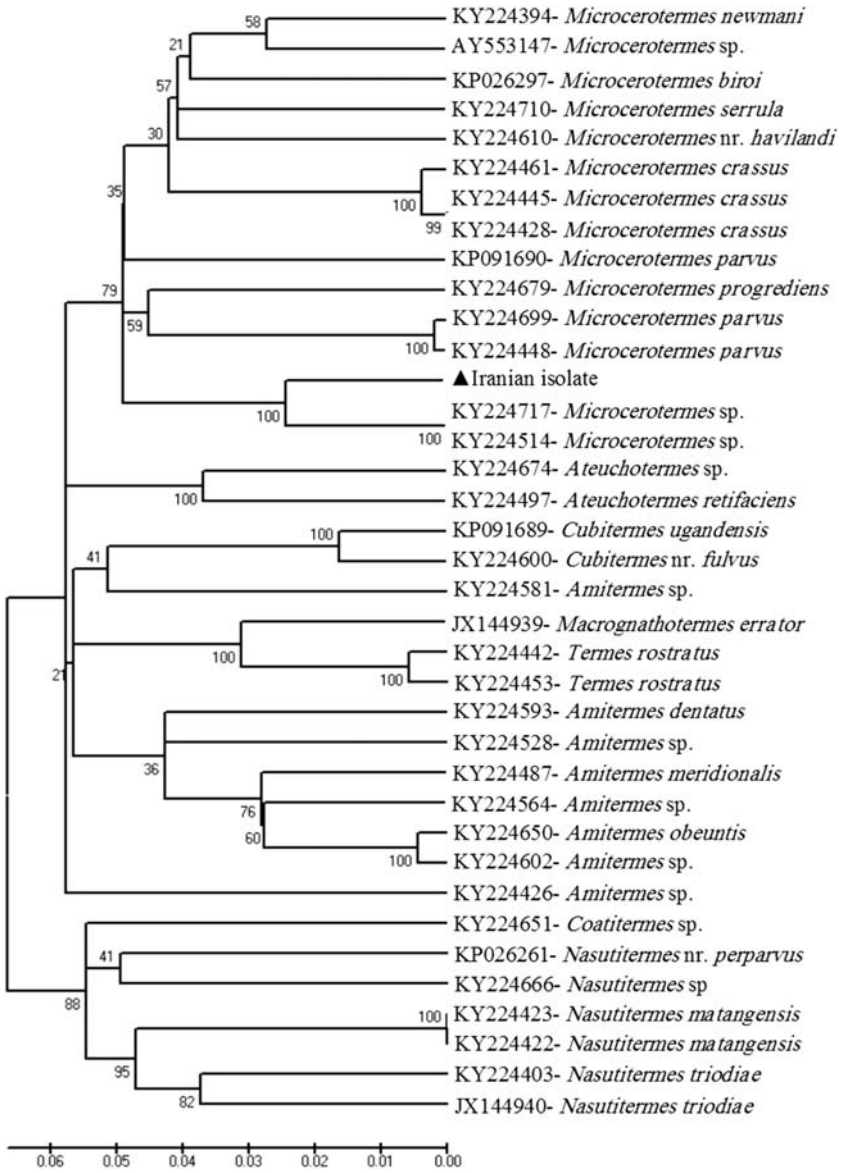

Fig. 1 Phylogenetic analysis of the Iranian termite population of *Microcerotermes* based on the Cytochrome Oxidase subunit I gene. Iranian isolate indicated with a (▲) symbol. The tree reconstructed using MEGA 7 with the NJ approach

Molecular characterization

The partial sequence of the ITS region for tow isolates, Iran 111 of *B. bassiana* and Iran 121 of *M. anisopliae* were submitted to the GenBank with accession numbers MK942398 and MK942399, respectively. For the Iran111 isolate, NBLAST analysis indicated 98.6% similarities with the *B. bassiana* isolate FAFU-13 (accession numbers MG844430). Nblast analysis of Iran112 isolate revealed %99.1 similarity with *M. anisopliae* isolate PAL-M02 with accession numbers JN713140.

Phylogenetic analysis of *B. bassiana* Here, 550 nucleotides of the ITS gene for 36 taxa of *Beauveria* genus were aligned. The results were indicated that 490 sites were conserved, 59 sites were variable, and 45 sites were parsimony-informative. In the reconstructed phylogenetic tree, Iran111 isolate was placed in a single clade with *B. bassiana* isolates with 99% bootstraps (Fig. 2). The mean nucleotide distances were 0.033 (range 0.00–0.044), which was calculated using the K2P model. The nucleotide distances between Iran111 isolate and other isolates of *B. bassiana* were from 0 to 0.011.

Phylogenetic analysis of *M. anisopliae* For survey the phylogenetic relationship between Iran112 isolate and other species of *Metarhizium* genus, 479 nucleotides of the ITS gene for 41 valid taxa were aligned. Here, 253 sites were conserved, 222 sites were variable, and 179 sites were parsimony-informative. In the phylogenetic tree, Iran112 isolate was placed in a clade with other isolates of *M. anisopliae* with 100% bootstraps (Fig. 3). The average of nucleotide distances was 0.151 (range 0.00–0.302) which calculated using the K2P model. The intra-

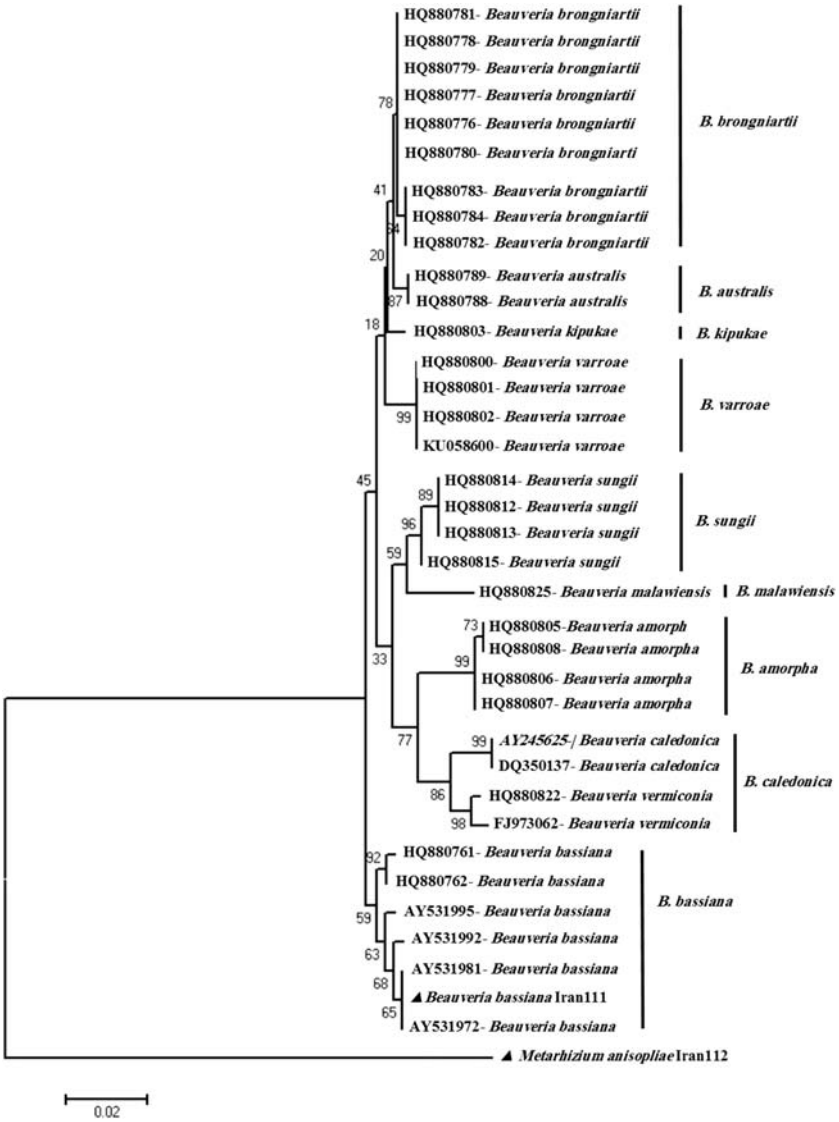

Fig. 2 Phylogenetic analysis showing the relationship among Iranian (▲) and other isolates of *Beauveria* genus based on the neighbor joining method using ITS rDNA sequences data. The DNA sequence for ITS gene of *Metarhizium anisopliae* was used as an outgroup

nucleotide distances between Iran112 isolate and other isolates of *M. anisopliae* were from 0 to 0.003.

Mortality of *M. divesrus* adult treated with entomopathogenic fungi under laboratory experiment

Calculated LC_{50} value (conidia/ ml^{-1}) for *Beauveria bassiana* and *Metarhizium anisopliae* on an adult termite, *Microcerotermes diversus* using two methods, has been indicated in Table 1.

Mortality of *M. divesrus* caused by spraying of fungi

Overall, this bioassay demonstrated significant main effects on *M. divesrus* mortality for both fungal species (F = 18.84; DF = 36, 1; $P = 0.0001$) and spore concentration level (F = 38.01; DF = 36, 5; P = 0.0001) with a significant interaction effect (F = 5.51; DF = 36, 5; $P = 0.0007$) (Fig. 4). Across the both species, significant differences in *M. divesrus* mortality occurred when *M. divesrus* were treated with either spore concentration (conidia/ ml^{-1}): 1×10^5 (F = 0.00; DF = 6, 1; $P = 1.00$), 1×10^6 (F = 5.76; DF = 6, 1; $P = 0.05$), 1×10^7 (F = 51.13; DF = 6, 1; $P = 0.0004$) and 1×10^8 (F = 1.28; DF = 6, 1; $P = 0.3006$). For each species used with 1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 (conidia/ml) concentrations: *B. bassiana* (F = 6.99; DF = 18, 5; $P = 0.00009$) and *M. anisopliae* (F = 6.99; DF = 18, 5; P = 0.3006) (Table 2). The *M. anisopliae* caused the equal cumulative mortality (100%) followed by *B. bassiana* (97.5%) at the highest concentration (Fig. 5).

Mortality of *M. divesrus* using the pipetting technique

In the laboratory assay using the pipetting technique, the LC_{50} was 8×10^5 and 1×10^6 conidia ml^{-1} for *B. bassiana* and

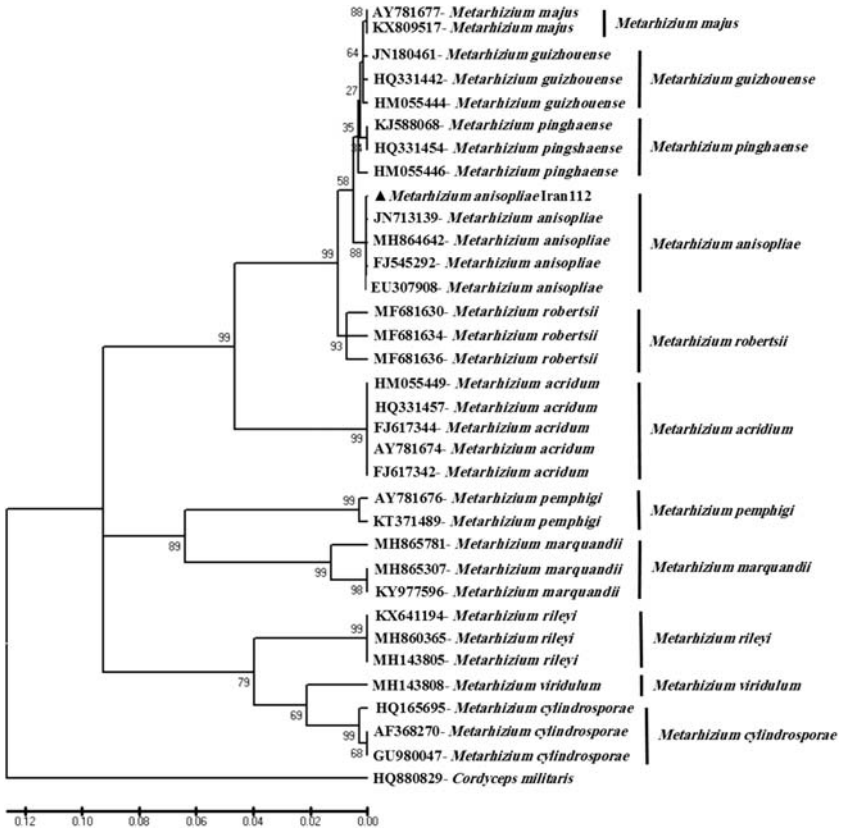

Fig. 3 Phylogenetic analysis between Iranian (▲) and other isolates of *Metarhizium* genus by neighbor joining method based on ITS rDNA sequences data. *Cordyceps militaris* (HQ880829) used as an outgroup

M. anisopliae, respectively (Table 1). Both tested entomopathogenic fungi species were capable of infecting and killing *M. divesrus*. Mortality started to appear between 96 and 48 h after treatment for *B. bassiana* and *M. anisopliae*, respectively. The cumulative mortalities increased through time for both species (Table 3). However, variations were not observed among the tested species. The *M. anisopliae* caused equal cumulative mortality (100%), followed by *B. bassiana* (97.5%) at the highest concentration (Fig. 6).

Overall this bioassay study demonstrated no significant main effects on *M. divesrus* mortality for both species (F = 0.38; DF = 36, 1; $P = 0.5413$) and spore concentration level (F = 71.64; DF = 36, 5; $P = 0.0001$) with a significant interaction effect (F = 2.41; DF = 36, 5; $P = 0.0554$) (Fig. 7). Across the both species, significant differences in *M. divesrus* mortality occurred when *M. divesrus* were treated with either spore concentration: 1×10^4 (F = 1.00; DF = 6, 1; $P = 0.3559$), 1×10^5 (F = 0.27; DF = 6, 1; $P = 0.6202$), 1×10^6 (F = 3.67; DF = 6, 1; $P = 0.1040$), 1×10^7 (F = 2.40; DF = 6,1; $P = 0.1723$) and 1×10^8 (conidia/ ml^{-1}) (F = 1.00; DF = 6,1; P = 0.3559).

Generally, there was no difference between mortality rates caused by *B. bassiana* and *M. anisopliae* when applied 1×10^4, 1×10^5, 1×10^6, $1\times 10^{7,}$ and 1×10^8 (conidia/ml) concentrations: *B. bassiana* (F = 33.60; DF = 18, 5; P = 0.0001) and *M. anisopliae* (F = 40.17; DF = 18, 5; P = 0.0001). The mortality caused by the fungus was dose-dependent with the highest mortality recorded with highest concentration. Mean levels of mortality in fungus treated groups ranged from 7.5%

Table 1 Calculated LC_{50} value (conidia/ ml^{-1}) for *Beauveria bassiana* and *Metarhizium anisopliae* on an adult termite, *Microcerotermes diversus* using two methods, pipetting after eight days and spraying after four days

EPF species	Method of use	n	LC_{50} (95% FL[a])	Intercept± SE	Slop± SE [b]	X^2 (DF = 2)	*P* value
B. bassiana	Pipette	40	8.03×10^5 (2.71×10^5–2.31×10^6)	−4.91 ± 0.59	0.83 ± 0.10	3.12	0.6
	Spray	40	3.52×10^7(1.12×10^7–2.28×10^8)	−7.51 ± 0.09	0.99 ± 0.15	4.14	0.4
M. anisopliae	Pipette	40	1.03×10^6 (2.75×10^5–4.47×10^6)	−7.49 ± 0.91	1.25 ± 0.15	11.97	0.9
	Spray	40	1.65×10^6 (1.08×10^6–2.50×10^6)	−10.97 ± 1.55	1.76 ± 0.25	040	0.3

[a] Fiducial limits 95%: (Lower limit–upper limit)

[b] In linear regression analyses, all slope coefficients were significantly different from zero ($p < 0.001$)

[c] Since significance level is greater than 0.15, no heterogeneity factor was used in the calculation of fiducial limit

Table 2 Efficacy of entomopathogenic fungi, *Beauveria bassiana,* and *Metarhizium anisopliae* on adult termite of *Microcerotermes diversus* when spray technique under laboratory conditions

Spray method							
days after treatment % Mortality (± SE)	2	4	6	8	10	12	14
Fungal species: *B.bassiana*							
Control	0 ± 0.00	0 ± 0.00 Ab	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
1× 10^4 conidia ml^{-1}	0 ± 0.00	0 ± 0.00 Ab	2.5 ± 2.5	7.5 ± 4.78	7.5 ± 4.78	7.5 ± 4.78	7.5 ± 4.78
1× 10^5	2.5 ± 2.5	2.5 ± 2.5 Ab	17.5 ± 8.53	32.5 ± 11.08	47.5 ± 18.87	47.5 ± 18.87	47.5 ± 18.87
1× 10^6	0 ± 0.00	5 ± 5 Ab	10 ± 10	40 ± 9.12	90 ± 10	100 ± 0.00	100 ± 0.00
1× 10^7	0 ± 0.00	22.5 ± 8.53 Bb	77.5 ± 11.08	87.5 ± 12.5	100 ± 0.00	100 ± 0.00	100 ± 0.00
1× 10^8	2.5 ± 2.5	72.5 ± 24.28 Aa	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
Fungal species: *M.anisopliae*							
Control	0 ± 0.00	0 ± 0.00 Ac	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
1× 10^4 conidia ml^{-1}	0 ± 0.00	0 ± 0.00 Ac	0 ± 0.00	0 ± 0.00	0 ± 0.00	2.5 ± 2.5	2.5 ± 2.5
1× 10^5	0 ± 0.00	2.5 ± 2.5 Ac	35.5 ± 12.5	60 ± 17.79	70 ± 23.80	97.5 ± 2.5	97.5 ± 2.5
1× 10^6	5 ± 0.00	32.5 ± 10.30 Ab	67.5 ± 13.14	95 ± 5	100 ± 0.00	100 ± 0.00	100 ± 0.00
1× 10^7	62.5 ± 8.53	92.5 ± 4.78 Aa	97.5 ± 2.5	97.5 ± 2.5	97.5 ± 23.80	97.5 ± 2.5	97.5 ± 2.5
1× 10^8	95 ± 5	100 ± 0.00 Aa	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00

Mean values within collected data-mortality (After four days for adult) bearing the same upper case letter showing no significant difference among fungal species while the same lower case indicated no significant difference between fungal concentrations (Least significant difference test, $P > 0.05$). The total numbers of replicates were 4 per treatment, and the experiment repeated twice

to 97.5% for *B. bassiana* and 0.00% to 100% for *M. anisopliae* (low dose to high dose, respectively) (Table 3).

Discussion

This study identified virulent isolates of entomopathogenic fungi that could use as promising candidates for termites control. Here, in the spray method, for four-day post-infection, maximum mortality in treated termites with *B. bassiana* was 72.5 ± 24.28%, while, for *M. anisoliae*, the percentage mortality reached 100. Thus, *M. anisopliae* elicited quicker mortality. This result was according to an earlier study (Lai et al. 1982; Wells et al. 1995; Milner et al. 1998; Dong et al. 2009; Pik-Kheng et al. 2009; Singha et al. 2011). But in the study of Neves and Alves (2000), there was no difference between *M. anisoliae* and *B. bassiana* against *Cornitermes cumulans* (Termitidae: Isoptera) (Neves and Alves 2000). Also, the virulence of *B. bassiana* against *Heterotermes tenuis* (Termitidae: Isoptera) was more than those of *M. ansiopliae* (Almeida et al. 1997). Toumanoff and Rombaut (1965) indicated 100% mortality in *Reticulitermes santonensis* (de

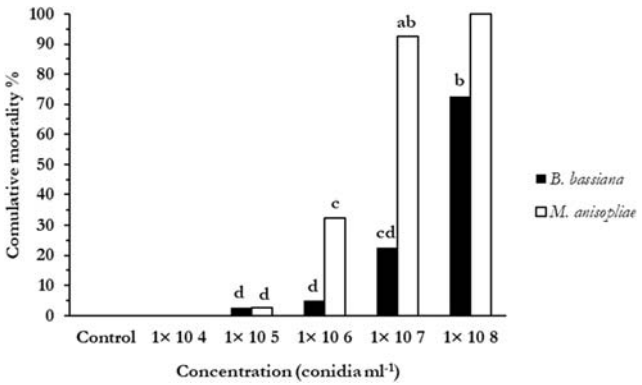

Fig. 4 Mortality of adult stage of *Microcerotermes diversus* infected by spraying *Beauveria bassiana* and *Metarhizium anisopliae* in six different concentrations (0, 1 × 10^4, 1 × 10^5, 1 × 10^6, 1 × 10^7 and 1 × 10^8 conidia ml^{-1}) (after four days) under laboratory conditions. Different letters indicate significant differences between treatments. Data presented as mean (± SEM)

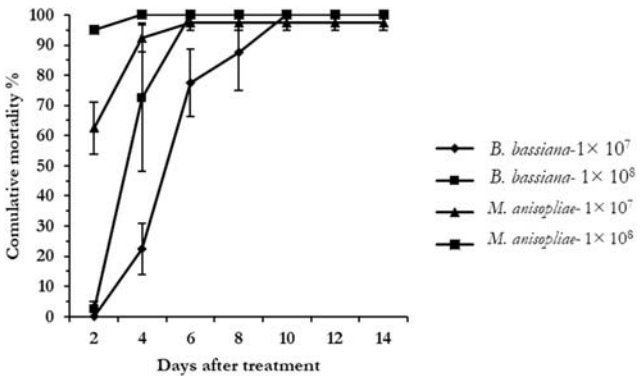

Fig. 5 Cumulative mortality of *Microcerotermes divesrus* adult, 1–14 days post spraying of *Beauveria bassiana* and *Metarhizium anisopliae* at 1× 10^7 and 1× 10^8 (conidia/ ml^{-1}) concentrations under laboratory conditions

Table 3 Pathogenicity of two isolates of *Beauveria bassiana* and *Metarhizium anisopliae* on adult termite, *Microcerotermes diversus* following exposure to various conidia concentrations with pipetting technique under laboratory conditions

Pipette method							
Days after treatment % Mortality (± SE)	2	4	6	8	10	12	14
Fungal species: *B. bassiana*							
Control	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00 Ad	0 ± 0.00	0 ± 0.00	0 ± 0.00
1×10^4 conidia ml^{-1}	0 ± 0.00	0 ± 0.00	0 ± 0.00	2.5 ± 2.5 Ad	2.5 ± 2.5	2.5 ± 2.5	2.5 ± 2.5
1×10^5	0 ± 0.00	12.5 ± 6.29	17.5 ± 4.78	27.5 ± 7.5 Ac	32.5 ± 4.78	32.5 ± 4.78	35 ± 6.45
1×10^6	0 ± 0.00	10 ± 5.77	32.5 ± 4.78	57.5 ± 7.5 Ab	87.5 ± 4.78	95 ± 5	95 ± 5
1×10^7	0 ± 0.00	7.5 ± 4.78	57.5 ± 19.31	75 ± 12.58 Ab	75 ± 12.58	75 ± 12.58	75 ± 12.58
1×10^8	2.5 ± 2.5	57.5 ± 10.30	97.5 ± 2.5	97.5 ± 2.5 Aa	97.5 ± 2.5	97.5 ± 2.5	97.5 ± 2.5
Fungal species: *M. anisopliae*							
Control	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00 Ac	0 ± 0.00	0 ± 0.00	0 ± 0.00
1×10^4 conidia ml^{-1}	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00 Ac	0 ± 0.00	0 ± 0.00	0 ± 0.00
1×10^5	0 ± 0.00	0 ± 0.00	2.5 ± 2.5	20 ± 12.24 Ac	37.5 ± 16.52	52.5 ± 7.96	60 ± 21.60
1×10^6	0 ± 0.00	2.5 ± 2.5	2.5 ± 2.5	30 ± 12.24 Ab	35 ± 20.61	82.5 ± 10.30	90 ± 10
1×10^7	0 ± 0.00	10 ± 7.07	57.5 ± 21.74	95 ± 2.88 Aa	100 ± 0.00	100 ± 0.00	100 ± 0.00
1×10^8	22.5 ± 8.53	70 ± 15.81	87.5 ± 12.5	100 ± 0.00 Aa	100 ± 0.00	100 ± 0.00	100 ± 0.00

Mean values within collected data-mortality (After eight days for adult) bearing the same upper case letter showing no significant difference among fungal species while the same lower case indicated no significant difference between fungal concentrations (Least significant difference test, $P > 0.05$). The total numbers of replicates were 4 per treatment

Feytaud) (Rhinotermitidae) within 5–6 days of exposure to B. *bassiana* and *M. anisopliae.*

Recent reports have suggested that naturally, the potential of *M. anisopliae* for control of termites is more than other entomopathogenic fungi (Kramm et al. 1992; Rath 2010; Cheraghi et al. 2013). Sajap and Kaur (1990) indicated that *Coptotermes curvignathus* (Holmgren) (Rhinotermitidae) is highly susceptible to infection by *M. anisoplia* and termites died within 36–48 h post-infection. In another study, 100% mortality was recorded for termites that expose to *M. anisopliae* conidia (Zoberi 1995). In fungal infection, there is a correlation between dose and time. In termite treated with the higher concentration, the required time to reach the LT50 is less than the lower concentration (Singha et al. 2011). In this research, the obtained results were, according to Singha et al. (2011).

In addition to the spray method, the bait method with entomopathogenic fungus is a suitable method for controlling pest termites (Cheraghi et al. 2013). For effective control of termites using fungal infection-bait, the sufficient spore inoculum should be delivered to termites and transfer from them to nestmates without stimulating colony-defensive behaviors. Grace (1993) indicated termites exposed to baits accumulated

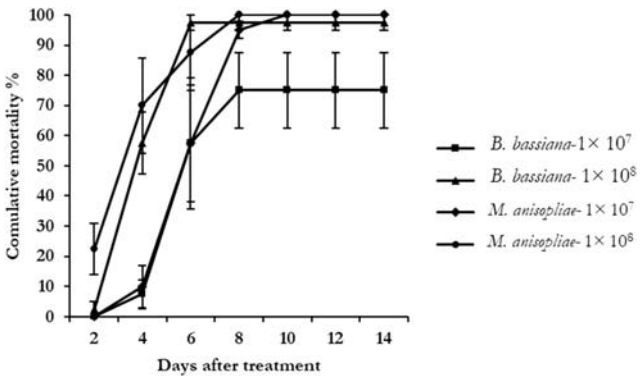

Fig. 6 Cumulative mortality of *Microcerotermes divesrus* adult, 1–14 days post treatment of *Beauveria bassiana* and *Metarhizium anisopliae* at 1×10^7 and 1×10^8 (conidia/ ml^{-1}) concentration using pipetting technique under laboratory conditions

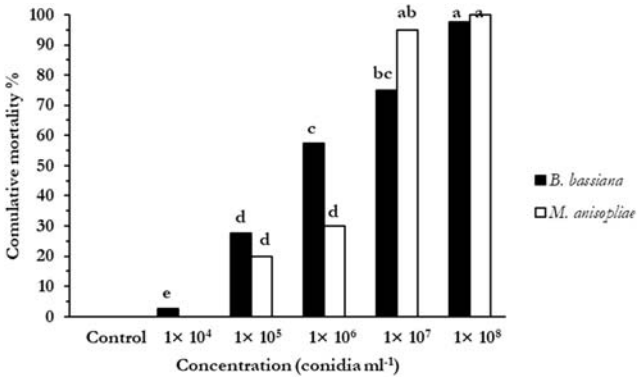

Fig. 7 Mortality of adult stage of *Microcerotermes diversus* after treatment with *Beauveria bassiana* and *Metarhizium anisopliae* (after eight days) in six different concentrations (0, 1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia ml^{-1}) using pipetting technique under laboratory conditions. Different letters indicate significant differences between treatments. Data are presented as mean (± SEM)

spore were able to transmit pathogens to other colony individuals.

Also, the use of some pesticide such as Imidacloprid in sublethal doses and a fungal pathogen can increase the fungi (Ramakrishnan et al. 1999). Ramakrishnan et al. (1999) indicated which the susceptibility of *Reticulitermes falvipes* (Termitidae: Isoptera) increase when *M. anisopliae* and imidacloprid combine.

Some components in entomopathogenic fungi such as beauverin in *B. bassiana* (Hamill et al. 1969) and destruxin in *M. anisopliae* (Pais et al. 1981) are introduced as candidates for mortality factors of the fungal disease in the insect virulence (Jones et al. 1996).

The control of *M. diversus* in Khuzestan is very important (Ghayourfar 2005) because Khuzestan is the warmest province in Iran and a suitable climate for the growth and development of termites. For successful control of termites with entomopathogenic fungi, the horizontal transmission between infected and uninfected individuals is critical that can occur through direct contact between the different individual (Vega et al. 2000; Cheraghi et al. 2012).

While workers mechanically transfer the infected termites to outside of the nest (Cheraghi et al. 2012). The worker's behavior against infected termites by *B. bassiana* and *M. anisopliae* are different. The termite cadavers killed by *B. bassiana* emit from the colony while cadavers were killed by *M. anisopliae* buried in the substrate (Kramm et al. 1982; Zoberi and Grace 1990). This is a defensive mechanism for preventing form spread infection. Also, termites can escape from entomopathogenic fungi by behavioral avoidance, such as grooming. This response decreases the efficiency of entomopathogenic fungi in the field (Chouvenc et al. 2008). According to Fernandes (1991) and Milner and Staples 1996, injection of a large number of conidia directly into the termite nest has had the greatest success in the field studies. Grace and Zoberi performed the study (Grace and Zoberi 1992), determined that living *R. flavipes* workers infected with *B. bassiana* effectively spread fungal infection in nest termites, while fungus-killed workers were not suitable for spread fungal spores. Similarly, Kramm et al. (1982) indicated *Reticulitermes* sp. workers infected with *M.anisopliae* transferred fungal conidia to healthy termites through grooming. In contrast, termites killed by the fungus were less effective in spreading the disease to pathogenicity because they were avoided by healthy individuals.

To date, many fungal strains used for termite control, but few strains were effective under suitable conditions. The termites showed a degree of resistance to fungal pathogens (Burges 1981). Moreover, Almeida et al. (1997) indicated that the virulence of isolated *B. bassiana* from termites was more than that *B. bassiana* from soil samples. Thus, in future studies, addressing suitable ways of incorporating fungal entomopathogens within the termite management strategy has top priority.

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Compliance with ethical standards

Conflict of interest The authors confirm they don't have any conflict of interest.

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