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Molecular detection of the entomopathogenic fungus *Beauveria bassiana* from soils of coffee growing areas in Ethiopia using rDNA-ITS

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ABSTRACT

Objective: This study molecularly characterized the entomopathogenic fungus using ITS gene with the aim of using as a source for future control of the coffee berry borer

Methodology and results: Soil samples were collected from three localities in Ethiopia. For each soil sample, ten *Galleria* larvae were used as bait for trapping entomopathogenic fungi. The total number of *Beauveria* spp. trapped from the different soil samples were 53% out of 300 *Galleria* larvae tested, in which 26.7%, 18.7% and 7.7% were from Belete chaka, Geruke and Choche farm, respectively. The amplification of the internal transcribed spacer (ITS) region of *Beauveria* spp. produced single fragments of about 560 bp for all tested isolates. Further, eight randomly selected and sequenced isolates revealed 98-100% sequence similarity and shared an overall intra-sequence similarity value of 99% among our isolates. The maximum likelihood (ML) analysis of the ITS region of *B. bassiana* formed a highly supported clade together with other isolates retrieved from GenBank. The highest evolutionary divergence estimate between sequences of Ethiopian isolates was 1%. The rDNA-ITS analysis, supported the species identification based on cultural and morphological traits and confirmed and characterized the isolates as *B. bassiana*.

Conclusion and application of results: The identification based on molecular tool helps to maintain a pure culture of the species. This result will assist for future application as a biocontrol of the fungal isolate against the coffee bean borer.

Keywords: Biocontrol, coffee, Ethiopia, Galleria baiting, ITS gene, *Beauveria bassiana*, molecular characterization

INTRODUCTION

Ethiopia is believed to be the country of origin of Arabica coffee (*Coffea arabica*) that supplies coffee beans to the world. In Ethiopia, coffee represents the major source of revenue for foreign exchange. Nevertheless, the industry is facing a great challenge by the coffee pest, coffee berry borer *(Hypothenemus hampei* Ferrari), that causes direct damage to the beans. Yield losses of 30–35% with 100%-perforated berries at harvest time were reported (Vega *et al.,* 2009). Moreover, it was

reported that *H. hampei* serves as a vector for *Aspergillus ochraceus* (Vega *et al*., 2009) that produces the toxin Ochratoxin A, contaminating green coffee beans, roasted coffee, and coffee brews, including instant coffee. For control of *H. hampei* insecticides such as endosulfan were considered to be effective if applied when the female is in the entry tunnel just before penetrating the endosperm (Brun *et al.,* 1989). However, continuous and indiscriminate use of synthetic pesticides has led to the development of resistant strains, the presence of toxic residues in beans and environmental problems. Consequently, there is a high demand for effective and environmentally friendly methods with which *H. hampei* could be managed. One alternative to control this insect pest is seen in microbial agents with high specificity and efficiency in reducing its ability to cause injury to coffee beans. Hence, the entomopathogenic fungus *Beauveria bassiana* is seen as a promising candidate for insect control due to its high specificity and pathogenicity (Fernando *et al*., 2009). This fungus colonizes the insect body but also establishes endophytically in the plant tissue (Biswas*et al*., 2013). Therefore, there is considerable interest in the development of this fungus for the control of *H. hampei* as an alternative to insecticide treatment (Ludwig and Oetting, 2002). Several isolates of *B. bassiana* have been formulated and registered as commercial products against a wide range of insect pests elsewhere (Masoud *et al.,* 2013). In this regard, accurate identification of the fungal species is of paramount importance for its use as a biopesticide. Identification of *Beauveria* spp. based on morphological characteristics has always been problematic, due to large heterogenicity of the spherical conidia (Glare and Inwood, 1998). Therefore, molecular techniques have been developed and used to assist these limitations in the identification (De Muro *et al*., 2005). Previous works based on DNA-PCR method indicated that *B. bassiana* contains a wide genetic diversity (Muro *et al*., 2005). This method provides little phylogenetic information and cannot be used to compare data between studies (Rehner and Buckley, 2003). However, sequences from specific targets in the DNA provide tools for explicit comparison between isolates across studies. For entomopathogenic fungi, attempts were made to analyze the sequence of the ITS region using general fungal primer sets (de Muro *et al*., 2005). Hence, sequencing of ITS regions can be effective to detect and differentiate entomopathogenic fungi (Bidochka *et al*., 2002). Ethiopia is such rich in flora and fauna comprising different agro-ecologies. Despite this fact and considering the cosmopolitan nature of entomopathogenic fungi, only little is known about the diversity and identity of *Beauveria* isolates in Ethiopia. So far, only few indigenous *Beauveria* isolates have been detected in Ethiopia from insect cadavers, collected mainly from locust (Senshaw, 1998), tsetse flies breeding areas (Mamuye, 1999; Seneshaw *et al*.,2003) as well as termite cadavers and termite mound material (Haimanot, 2002). Therefore, the objective of this study was to collect ecologically adapted *Beauveria* isolates from soil samples of three coffee growing areas in the southwest part of Ethiopia using the baiting method and molecularly characterize those isolates as a source for future control of the coffee berry borer.

MATERIAL AND METHODS

Soil sample collection: Soil samples were collected from three different localities viz. Belete chaka, Choche and Geruke (Table 1 and Fig. 1) using core sampling techniques described by Amy *et al*. (2009). At each of the three localities, ten soil samples at five random points that are 5 m apart in transect were taken by digging (15 cm diameter, 10-15 cm depth). From each five sampling places, about 1.5 kg of soil was combined together (pooled) and mixed. From the mixed soil, 1 kg was kept in sterile plastic bag and moisturized as per required with sterile water to make sure that the only source of the microbes are from the soil and avoid any antagonist effect and packed. In the Mycology lab, Addis Ababa University, the aggregated soils were mechanically crushed, and sieved using a 20 mm aperture sieve.

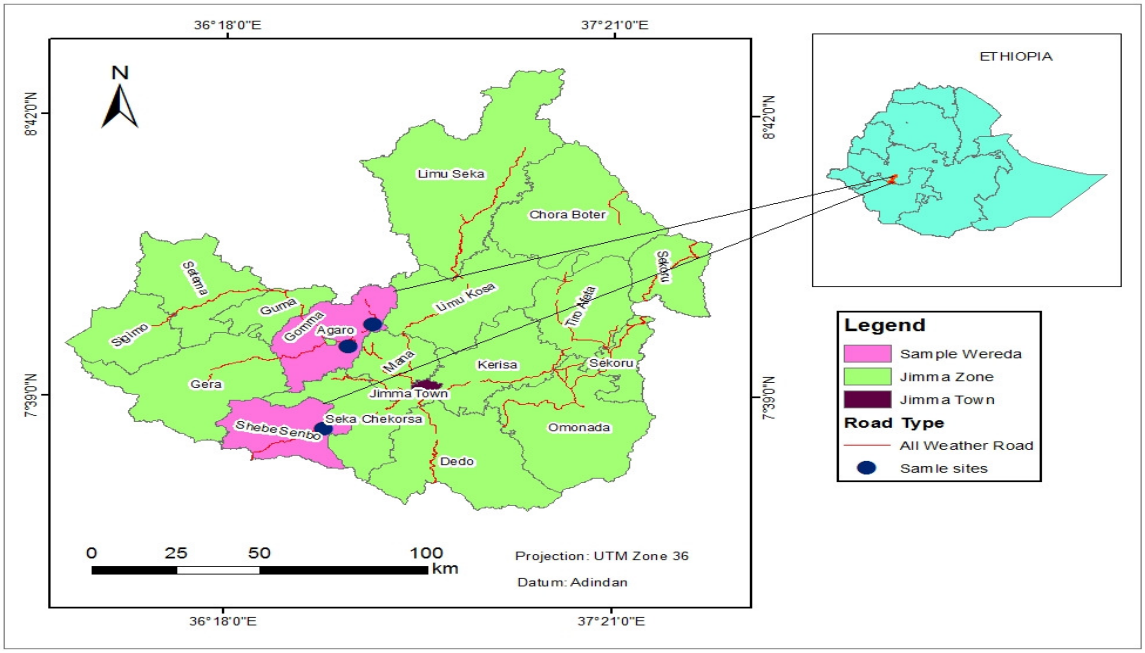

Figure 1: Map of soil samples collected from different localities of Jimma zone.

***Galleria mellonella* (Greater Wax Moth) rearing and baiting:** At the Ambo plant protection agricultural research institute, *G. mellonella* was reared according to the method described by Meyling (2007). Male and female adult *G. mellonella* were kept in 500 ml flasks with a lid. Strip folded paper was placed in the flask to facilitate mating and egg laying. Each folded paper with the eggs attached was carefully transferred into a container with a mixture of sterile wheat bran, glycerol and honey as food source for hatching larvae and kept in an incubator at 35°C. When larval growth reached about 2.5 cm in length within 4-5 weeks after hatching, larvae were used for baiting. The baiting process was done following the method described by Meyling and Eilenberg (2006). Larvae were heat treated in warm water to prevent extensive webbing in the baiting test soil. Therefore, a 1000 ml beaker filled with 500 ml of sterile water was placed in a water bath at 56°C. Larvae from the rearing containers were collected in a box and at once poured into the beaker with 56°C warm water for 10 seconds. The beaker was emptied through a sterile sieve of 20 mm aperture and then the larvae were cooled by immersing in sterile cold water for 30 seconds and placed on dry sterile tissue paper in the dark for 3-5 h until they have recovered from the treatment.

Table 1: Frequency of occurrence of Beauveria spp. Geographical location and habitat of soil samples collected from Jimma Zone.

Locality	Location	Habitat	Occurrence (%)
Belete chaka	N7°31'53", E36°33'46"	Natural forest	80
Choche	N7°55'20", E36°4'42"	Cultivated land	23
Geruke	N7°50'23", E36°37'53"	Open grazing area	56

Inoculation and isolation: About 1 kg sieved soil was placed on a disinfected tray and moisturized with sterile water and then filled into a 1.5 litre glass container with a screw cap leaving some space on the top to inoculate *Galleria* larvae. For each sample, ten glass containers were used and 10 larvae were inoculated per container. Sterile soil was used as control. Containers were incubated at 25 ± 5°C in the dark. Dead larvae were

removed every 3 days from the glass containers. The moisture level of inoculated soil was maintained by gentle moistening with sterile water each time following the inspection of the dead larvae. The dead larvae were surface sterilized in 0.5% sodium hypochlorite for 3 min, rinsed in sterile distilled water for 2 min, immersed in 70% ethanol for 2 min and finally rinsed three times in sterile distilled water. After surface sterilization, the cadavers were placed on a sterile plate that contained sterile Whatman filter paper No. 1 (Macherey-Nagel, Duren, Germany) moistened with sterile water and incubated at 25 ± 5°C in the dark. *Beauveria* sporulated on the cadaver were then subcultured on potato dextrose agar (PDA) and pure cultures were preserved on 3% glycerol PDA slants in the refrigerator at 4°C until transported to the Julius Kühn-Institut, Münster, Germany, for molecular characterization.

DNA extraction and purification: From the preserved slants, fungal spores were placed on PDA. After 20 days, a sterile spatula was used to transfer the spores into 50 ml of potato dextrose broth in a 100 ml flask and allowed to grow at 26.5°C at room temperature for four days on a shaker at 300 rpm. The grown mycelium was then harvested by filtering through a sterile Whatman No. 1 filter paper and allowed to dry for 5 min in a microbiological safety hood. One gram of mycelium was then transferred into a 2 ml sterile Eppendorf tube and then incubated in a freezer at -80°C for 20 min. About 300 g crashed and sterilized pieces of glass were added to the mycelium and mixed with the mycelium by thoroughly stirring with a sterile spatula. Next, 100 µl of DNA extraction buffer (Tris-HCl, 1M; EDTA, 0.5 mM pH 8.0; KCl, 25 mM and SDS, 0.5%) was added and vortexed for 5 min and centrifuged at 13,000 rpm for 1 min. The supernatant was transferred to a new Eppendorf tube into which 3 µl of proteinase K was added. The mixture was then incubated at 64°C for 1 hr followed by 96°C for 15 min. The mixture was then centrifuged at 13,000 g for 1 min and the supernatant transferred to a new Eppendorf tube in which 300 µl of cold isopropanol was added, mixed and centrifuged at 13,000 g for 20 min at 4°C. The supernatant was carefully discarded and the pellet washed with 40 µl of 70% ethanol and air-dried at 37°C for 15 min. Finally, the DNA was resuspended in 50 µl of nuclease free water and stored at -20°C.

PCR amplification: The rDNA regions selected for PCR cloning were ITS1, 5.8S gene and ITS2. Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the target regions (White *et al.,* 1990). The PCR amplification reaction was conducted in a total volume of 25 µl. The mixture contained 13.5 µl of water, 2.5 µl buffer, 3.0 µL of dNTP, 1 µl of each primer, 1µl Taq polymerase, and 3.0 µl genomic DNA. PCR thermocycler settings were 4 min initial denature at 94°C followed by 35 cycles of 30 sec denaturation at 94°C, 30 second annealing at 58°C and 1 min at 72°C for extension. The PCR product was assayed by electrophoresis on a 1.5 % agarose gel run with TBE buffer at 100 mV and stained with 0.0001% Ethidium bromide. The gel was photographed under UV light.

DNA Sequencing: The amplified PCR product was sequenced. Prior to sequencing, the amplicon was purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instruction. For sequencing, 5 µl of the PCR products and 5 µl of 10 pmole/µl of the forward primer were mixed. Eight isolates were randomly selected and sequenced in one direction. Sequencing was performed at the Macrogene sequencing facility service (Amsterdam, The Netherlands). Nucleotide sequences were deposited in GenBank nucleotide sequence databases (Table 3).

Table 2. *Beauveria bassiana* isolates from Ethiopia used for molecular analysis in this study

Locality	Isolate code	Accession
Belete chaka	B5B	KR337495
	B7E	KR337496
	B2J	KR337494
	B6F	KR337499
	B7A	KR337493
Geruke	G5I	KR337500
Choche	C3C	KR337498
	C1C	KR337497

Sequence alignment: All raw sequence chromatograms obtained in this study were edited in Chromas Lite version 2.1 (Technelysium Pty Ltd., 2012) to exclude incorrect base calls before multiple alignment was performed.

Consensus quality sequences were subjected to BLAST engine (Altschul *et al*., 1990) for sequence similarity search in GenBank, NCBI database (Table 4). All the new sequences and sequences from the GenBank were then aligned using the default parameters of ClustalX version 2.1 (Larkin *et al*., 2007).

Phylogenetic analysis: Newly obtained sequences of rDNA-ITS gene plus related published sequences from GenBank were used to reconstruct the phylogenetic trees. *Cordyceps militaris* (KM197165) from Taiwan was chosen as out-group taxa. Pair wise distance between our isolates and *B. bassiana* sequences in GenBank from China (KU158450), Kenya (AJ560666), India (KU363833), Switzerland (KT583186), Turkey (KP862983), Thailand (EU573330), and Portugal (LT220530) together with other fungal species from Greece (*L. lecanii,* EF513006), Portugal (*B. pseudobassiana*, LT220534), and Thailand (*B. brongniarti,* EU573325) were analysed using MEGA5 (Tamura *et al*., 2011). The phylogenetic analysis was carried out with Maximum Likelihood (ML). ML analysis was performed using heuristics searches with Nearest-Neighbour-Interchange (NNI) branch swapping filter. Gaps and missing data were also considered in the Tamura-Nie Model. The support for each branch was estimated using the bootstrap (bs) method with heuristics search and 1500 replicates in MEGA5 analysis (Tamura *et al*., 2011).

Table 3. List of GenBank accession numbers for reference sequences used for phylogenetic analysis

Species	Accession Nr.	Origin of country	Reference
Beauveria bassiana	KU158450	China	Masoudi *et al*., 2015
Beauveria bassiana	AJ560666	Kenya	Aquino de Muro, *et al*., 2003
Beauveria bassiana	KU363833	India	Chellappa *et al*., 2015
Beauveria bassiana	KT583186	Switzerland	Laurent *et al*., 2015
Beauveria bassiana	KU158436	China	Masoudi *et al*.,2015
Beauveria bassiana	KP862983	Turkey	Keskin *et al*., 2015
Beauveria bassiana	EU573330	Thailand	Aung *et al*., 2008
Beauveria bassiana	KJ152166	China	Li *et al*, 2014
Beauveria bassiana	LT220530	Portugal	Sharma and Marques, 2016
Lecanicillium lecanii	EF512980	Greece	Kouvelis *et al*., 2007
Lecanicillium lecanii	EF513006	Greece	Kouvelis *et al*., 2007
Beauveria brongniartii	HM595509	China	Yuan et al., 2010
Beauveria brongniartii	EU573310	Thailand	Aung *et al*., 2008
Beauveria brongniartii	EU573318	Thailand	Aung *et al*., 2008
Beauveria brongniartii	EU573325	Thailand	Aung *et al*., 2008
Beauveria pseudobassiana	LT220535	Portugal	Sharma and Marques, 2016
Beauveria pseudobassiana	LT220534	Portugal	Sharma and Marques, 2016
Beauveria pseudobassiana	KT368174	Slovakia	Schemmer *et al*., 2015
Beauveria pseudobassiana	KT368173	Slovakia	Schemmer*et al*., 2015
Cordyceps militaris	KM197165	Taiwan	Wu, 2014

RESULTS

Isolation of entomopathogenic fungi by baiting with 100 *Galleria* larvae from soil samples of the three localities are shown on (Table 2). Accordingly, entomopathogenic fungi occurred the highest (80%) on the natural forest and lowest (23%) on the cultivated land.

ITS amplification: The amplification of the ITS region produced a single fragment of approximately 560 bp long (Fig. 2). All 36 isolates tested gave the same fragment size, of which eight were randomly selected and sequenced. A BLAST search at NCBI confirmed the species identity of the Ethiopian isolates in which each isolate could reveal 98-100% sequence similarity with one or more previously published isolates of *B. bassiana*. Moreover, both sequence and phylogenetic analysis confirmed that the Ethiopian isolates represent the species *B. bassiana.*

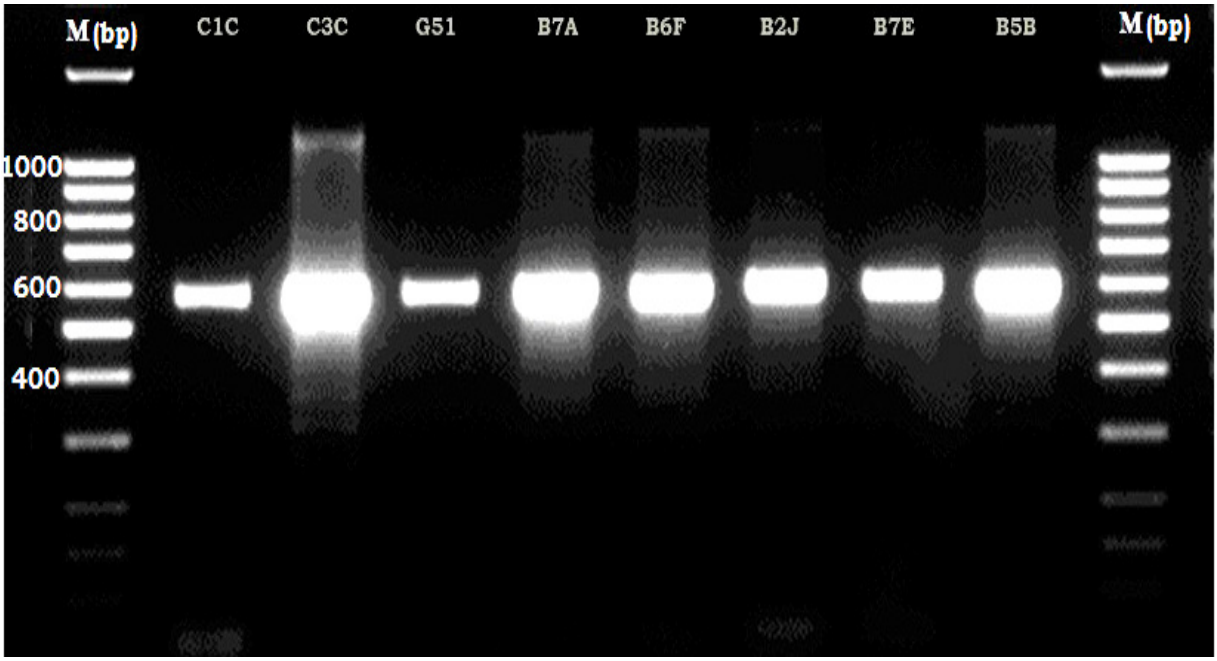

Figure 2: PCR products amplified using ITS primers (ITS1 and ITS4) from eight representative specimens of *Beauveria bassiana* isolates from Ethiopia. M: 5000 bp ladder.

Sequence analysis: Following a manual trimming of nucleotides at both ends, CLUSTALX multiple sequence alignment of all trimmed ITS rDNA sequences including the outgroup taxa, yielded a consensus length of 512 bp, of which 36 (7% of all sites) parsimony - informative and 78 (15%) were variable. An estimate of the evolutionary divergence between sequences among Ethiopian isolates revealed identical intra-sequence (similarity value of 100%) except between isolate C3C and G5I, which resulted in a 1% sequence divergence (Table 4). Similarly, the sequence similarity among all isolates of *B. bassiana* sequences aligned in the sequence analyzed ranged between 99 and 100%. In comparisons, the highest sequence disparity of our isolates from other fungal species was 4% for *B. brongniarti* or *L. lecanii* and 5% for *B. pseudobassi.* Moreover, sequence divergence between in-group and outgroup taxa ranged from 9 to 10% at an overall distance of 0.015 ± 0.007. Estimated average nucleotide composition among *Beauveria* spp. was 23.6% (A), 18.9% (T), 31.9% (C), 25.6% (G), 42.5% (A+T), and 57.5% (G+C). The Ethiopian isolate C3C differed in AT richness (43%) from the rest of the Ethiopian isolates (42%), but was similar to the other *Beauveria* species analyzed here.

Table 4: Sequence similarity (%) of ITS region of *Beauveria* isolates from Ethiopia and selected fungal isolates.

		Fungal isolates																		
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1	LT220530 *Beauveria bassiana*	-																		
2	EU573330 *Beauveria bassiana*	100	-																	
3	KU363833 *Beauveria bassiana*	100	100	-																
4	C3C (KR337498)	100	100	100	-															
5	B2J (KR337494)	99	100	100	99	-														
6	C1C (KR337497)	99	100	100	99	100	-													
7	B5B (KR337495)	99	100	100	99	100	100	-												
8	B6F (KR337499)	100	100	100	99	100	100	100	-											
9	B7A (KR337496)	100	100	100	99	100	100	100	100	-										
10	B7E (KR337500)	100	100	100	99	100	100	100	100	100	-									
11	G5I (KR337493)	100	100	100	99	100	100	100	100	100	100	-								
12	KU158450 *Beauveria bassiana*	100	100	100	99	100	100	100	100	100	100	100	-							
13	AJ560666 *Beauveria bassiana*	100	100	100	99	100	100	100	100	100	100	100	100	-						
14	KP862983 *Beauveria bassiana*	100	100	100	100	100	100	100	100	100	100	100	100	100	-					
15	KT583186 *Beauveria bassiana*	100	100	100	100	100	100	100	100	100	100	100	100	100	100	-				
16	EF513006 *Lecanicillium lecanii*	96	96	96	97	96	96	96	96	96	96	96	96	96	96	96	-			
17	LT220534 *Beauveria pseudobassiana*	96	96	96	96	95	95	95	96	96	96	96	96	96	96	96	98	-		
18	EU573325 *Beauveria brongniartii*	96	96	96	97	96	96	96	96	96	96	96	96	96	96	96	98	98	-	
19	KM197165 *Cordyceps militaris*	91	91	91	91	91	91	91	91	91	91	91	91	91	91	91	90	90	90	-

Phylogenetic analysis: The evolutionary history of 28 sequenced isolates was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Fig. 3). Based on default parameters, the best-fit sequence evolution model was T92+G representing the model Tamura 3-parameter (Nei and Kumar, 2000). The base frequency varied in that both A and T had 0.21 while C and G on the other hand had both 0.29. The estimated gamma distribution shape parameter was 0.16 and the phylogenetic tree had a –ln likelihood of 950.4964. The phylogenetic tree constructed from the rDNA-ITS sequences using the ML methods placed all the Ethiopian isolates in a strongly supported clade with bootstrap values of 76% that were clearly separated from other *Beauveria* and fungal species. In addition, both MP and ML trees consistently placed isolate C3C as the basal taxon to all *B. bassiana* isolates. Hence, the ML consensus tree formed three distinct clades of the Ethiopian *B. bassiana* isolates together with the isolates retrieved from the GenBank except *C. militaris*: (i) *B. bassiana*, (ii) *B. brongniartii* and *L. lecanii* and (iii) *Beauveria pseudobassiana.*

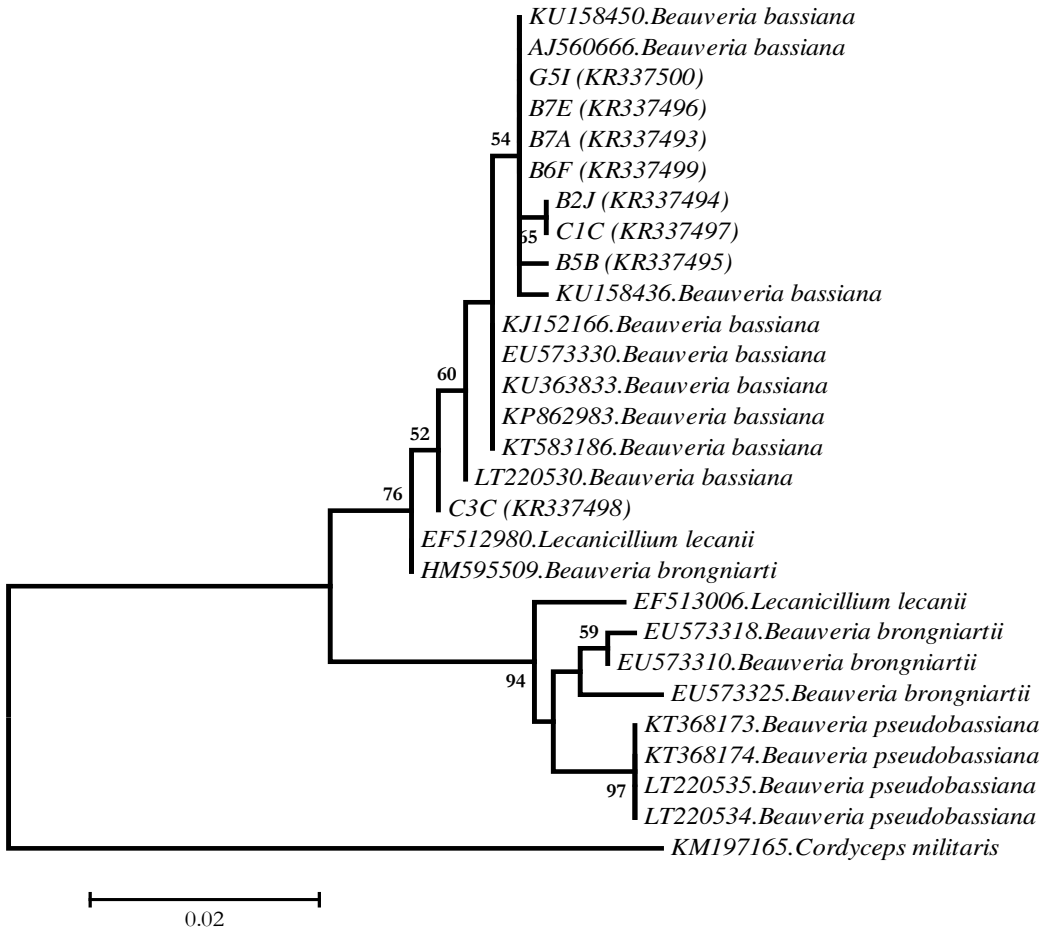

Figure 3: Maximum likelihood analysis of the ITS region of Ethiopia isolates of *Beauveria bassiana* and other related sequences from the GenBank. Sequences from GeneBank are given with their corresponding accession number. The analysis was performed using 1000 bootstrap replicates. Corresponding bootstrap support more than 50% are given for each appropriate clade. Whereas sequences for Ethiopian isolates are given by, codes (B5B, B7E, B2J, B6F, B7A, G5I, C3C and C1C) and their corresponding accession number in parenthesis. *Cordyceps militaris* has been used as an outgroup taxa.

DISCUSSION

The genus *Beauveria* comprises entomopathogenic fungi known to provide effective insect control due to the activity of their pathogenicity. As entomopathogenic fungi are common in soil, isolation of new fungal strains by baiting with *Galleria* larvae is a common practice. The biocontrol potential of *B. bassiana* is well documented (Quesada-Moraga *et al*., 2007). In the present study, the occurrence of *B. bassiana* associated with coffee growing areas was evaluated. Entomopathogenic fungi were isolated from soil samples of the localities *Belete chaka*, *Geruke* and *Choche* using *Galleria* larvae. The trapping frequency of *B. bassiana* isolation varied among localities *Belete chaka* being the highest trapped 80% followed by Geruke, which is 56% as compared to *Choche* that was only 23% (Table 2). The number of trapped *Beauveria* isolate from these localities was high. This might be due to the core soil sampling method and number of larvae used (10) per treatment. Using the same soil sampling method from five pooled soil samples Quesada-Moraga *et al.* (2007) reported that of the 244 soil samples, 104 yielded *B. bassiana* (42.6 %)and 53 samples (21.7%) harboured both species (*B. bassiana and M. anisopliae*). On the contrary, from single soil sample Bing and Xing (2008) reported that *B. bassiana* and *M. anisopliae* were isolated from 171 (20.4%) *B. bassiana* and from 24 (3%) *M. anisopliae*. The difference in isolation frequency of *Beauveria bassiana* could be because soil samples pooled by core sampling increase the chance of mixing fungal propagules of different species in the soil sample and exposed *Galleria* larvae than using single soil sample. Using more numbers of trapping larvae (10) would also increase the chance of infection than using small numbers (3) per treatment Bing and Xing (2008). The low baiting of *B. bassiana* from *Choche* could be due to the intensive agricultural practice in this region. The farmed soil is routinely exposed to plant protection agents including fungicides and insecticides. Fungicides used by the farmers for fungal disease control could have directly affected *B. bassiana* in the soil while applications of insecticides could have reduced their natural host. In this regard, Vänninen (1996) highlighted the fact that *B. bassiana* requires frequent serial passage through insect hosts to survive. Scarcity of host insects in heavily cultivated and insecticide treated areas subsequently will put *B. bassiana* at a disadvantage in those soils. Similarly, Vänninen *et al*. (2000) confirmed that *B. bassiana* requires host insects for survival and long-term persistence in the soil. The higher frequency of *B. bassiana* isolation from *Belete chaka* could be related to an intact forest ecosystem with a high diversity of plants and insect hosts with the latter serving as the host for *B. bassiana*. Studies from tropical and temperate forests of Mexico and worldwide (Wongsa *et al*., 2005) confirmed the natural forest areas harbour a diverse spectrum of entomopathogenic fungi. The third location *Geruke* is characterized by natural vegetation serving as grazing area for cattle, sheep and goats. Their fecal matter harbours a diverse spectrum of insects providing natural hosts for entomopathogenic fungi including *B. bassiana*. Overall, those results confirm the importance of natural habitats as reservoirs for natural enemies as potential biological control agents.

Molecular analysis: Traditionally, the identification of entomopathogenic fungi relies on fungal isolation and a highly skilled expert in fungal identification. In comparison, modern DNA based molecular diagnostics are fast, independent of fungal culturing and often more accurate. Therefore, the feasibility of using the ITS region for the detection and identification of *Beauveria* spp. was evaluated. The amplification of the ITS region produced a fragment size of approximately 560 bp which is in agreement with that report by De Muro *et al*. (2005). Similar, Carneiro *et. al*. (2008) reported a fragment length of 570 bp from the ITS-rDNA region for 24 *B. bassiana* isolates. Hence, the size of the PCR product already allows discriminating *B. bassiana* from other species. However, the presence of unusual PCR size polymorphism even within isolates of the same species has been reported from other organisms such as nematodes (Blok *et al*., 2002) and might occur for fungi. Furthermore, a low level of sequence variation is commonly detected within ITS regions (De Muro *et al.* 2003). Thus, the mere use of the PCR fragment size is not conclusive for correct species identification. The ITS primers utilized in this study amplified a fragment with approximately 560 bp for all fungal isolates. Analysis at nucleotide level provided more information and the ITS sequences from all isolates suggested that the isolates examined could have a common genetic basis with low variation at conserved regions. There was only 1% sequence disparity among the Ethiopian isolates of *B. bassiana*. Sequence disparity was also reported by Carneiro *et al*. (2008) who found four variations in nucleotide positions out of 571 nucleotides between *B. bassiana* isolates. In the present study, the eight randomly sequenced isolates of *B. bassiana* were collected from different habitats within a relatively close geographical origin. Therefore, the level of sequence disparity observed between those Ethiopian isolates is considered realistic and supports the hypothesis that the

overall sequence variability within *Beauveria* isolates ranges between 1-4%. The ML analysis of the DNA-ITS region formed a highly supported clade together with other isolates retrieved from GenBank. The clustering of the *B. bassiana* isolates from Ethiopia showed the closest relationship with different isolates of *B. bassiana* from other regions. Except for isolate C3C, all Ethiopian isolates appeared between isolates from Kenya and China. The analyses of these isolates indicated intraspecific variation amongst Ethiopian isolates. Isolate C3C showed distant relation to the rest of the Ethiopian isolates but closely related to Portuguese isolate (LT220530) isolated from Vineyard soils. However, Carneiro *et al*. (2008) obtained *B. bassiana* that clustered based on the origin of the isolates All the *B. bassiana* isolates are genetically similar and quite distant from the out-group fungal isolate *Cordyceps militaris*.

CONLCLUSION AND APPLICATION OF RESULTS

In conclusion, the species identification of entomopathogenic fungi using molecular diagnostics is reliable which can be effectively used in parallel with classical identification methods based on morphology and culture characteristics. This study is the first report of molecular characterization of *B. bassiana* isolated from coffee growing regions in Ethiopia.

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REFERENCE

Altschul SF, Gish W, Miller W, Myers E, Lipman DJ, 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403-410.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25: 3389-3402.

Amy RT, Jessica LM, Shannon UM, Thomas JD, Richard CF, Michael M, Sadia S, Dieshia R, Kirby, CS, 2009. Isolation of entomopathogenic fungi from soils and *Ixodes scapularis* (Acari: Ixodidae) ticks: Prevalence and methods. Journal of Medical Entomology 46: 557–565.

Bidochka MJ, Menzies FV, Kamp AM, 2002. Genetic groups of the insect-pathogenic fungus *Beauveria bassiana* are associated with habitat and thermal growth preferences. Archives in Microbiology 178: 531-537.

Bing DS, Xing ZL, 2008.Occurrence and diversity of insect-associated fungi in natural soils in China. Applied Soil Ecology 39: 100-108.

Biswas C, Dey P, Satpathy S, Satya P, Mahapatra, BS, 2013. Endophytic colonization of white jute (*Corchorus capsularis*) plants by different *Beauveria bassiana* strains for managing stem weevil (*Apioncorchori*). Phytoparasitica 41: 17-21.

Blok V, Wishart JW, Fargette MF, Berthier KB, Philips MS, 2002. Mitochondrial DNA differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. Nematology 4: 773-781.

Vänninen I, 1996. Distribution and occurrence of four entomopathogenic fungi in Finland: effect of geographical location, habitat type and soil type. Mycological Research 100: 93-101.

Brun LO, Marcillaud C, Gaudichon V, Suckling DM, 1989. Endosulfan resistance in *Hypothenemus hampei* (Coleoptera: Scolytidae) in New Caledonia. Journal of Economic Entomology 82: 1311–1316.

Carneiro AA, Gomes EA, Guimarães CT, Fernandes FT, Carneiro NP, Cruz I, 2008.Molecular characterization and pathogenicity of isolates of *Beauveria* spp. to fall armyworm. Pesquisa Agropecuaria Brasíleira 43: 513-520.

De Muro MA, Elliott S, Moore D, Parker BL, Skinner M, Reid W, Bouhssini EL, 2005. Molecular characterization of *Beauveria bassiana* isolates obtained from overwintering sites of Sunn pests (*Eurygaster* and *Aelia* species). Mycological Research 109: 294-306.

De Muro, MA, Mehta S, Moore D, 2003.The use of amplified fragment length polymorphism for molecular analysis of *Beauveria bassiana* isolates from Kenya and other countries, and their correlation with host and geographical origin. FEMS Microbiology Letters 229: 249-257.

Fernando EV, Francisco I, Alfredo C, Juliana J, 2009. The coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae): a short

review, with recent findings and future research directions. *Terrestrial Arthropod Reviews* 2,129–147.

Glare TR, Inwood AJ, 1998. Morphological and genetic characterization of *Beauveria* spp. from New Zealand. Mycological Research 102: 250-256.

Haimanot A, 2002. Potential of entomopathogenic fungi for the control of *Macrotermes subhyalinus* (Isoptera: Termitidae). Ph.D thesis, University of Hannover, Hannover, Germany.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948.

Latifian M, Rad, B., Amani. M. and Rahkhodaei, E.2013.Mass production of entomopathogenic fungi *Beauveria bassiana* (Balsamo) by using agricultural products based on liquid- solid diphasic method for date palm pest control. Int. J. of Agri. and Cro.Sci.5: 2337-2341.

Mamuye H, 1999. Pathogenicity of Ethiopian isolates of entomopathogenic fungi against *Glossina morsitans morsitans*, MSc. Thesis, Addis Ababa University School of Graduate Studies, Addis Ababa, Ethiopia, 1999.

Meyling NV, 2004. Methods for isolation of entomopathogenic fungi from soil environment. Laboratory manual. Department of Ecology, Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark.

Meyling NV, Eilenberg J, 2006. Occurrence and distribution of soil borne entomopathogenic fungi within a single organic agroecosystem. Agriculture Ecosystems and Environment 113: 336-341.

Quesada-Moraga E, Navas-Cortés JA, Elizabeth AA, Maranhao A, Cándido SÁ, 2007. Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. Mycological Research 111: 947-966.

Rehner SA, Buckley EP, 2003. Isolation and characterization of microsatellite loci from the entomopathogenic fungus *Beauveria bassiana* (Ascomycota: Hypocreales). Molecular Ecology 3: 409-411.

Seneshaw A, Seyum E, Abate D, 2003. Evaluation of Ethiopian isolates of entomopathogenic fungi as potential biological control agents of the desert locust (*Schistocerca gregaria*) pest management. Addis Ababa University, College of Natural Sciences, 1-73.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance and Maximum Parsimony methods. Mol.r Bio and Evol. 28: 2731-2739.

Vega FE, Francisco I, Alfredo C, Juliana J, 2009.The coffee berry borer, *Hypothenemus hampei*(Ferrari) (Coleoptera: Curculionidae): A short review, with recent findings and future research directions. Terrestrial Arthropod Reviews 2, 129-147.

Vänninen I, Tyni-Juslin J, Hokkanen H, 2000.Persistence of augmented *Metarhizium anisopliae* and *Beauveria bassiana* in Finnish agricultural soils. Biocontrol 45: 201-222.

Wongsa P, Tasanatai K, Watts P, Hywel-Jones N, 2005. Isolation and *in vitro* cultivation of the insect pathogenic fungus *Cordyceps unilateralis*. Mycological Research 109: 936-940.