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Molecular characterization and evaluation of indigenous entomopathogenic fungal isolates against Sorghum Chafer, *Pachnoda interrupta* (Olivier) in Ethiopia

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Sorghum chafer, *Pachnoda interrupta (*Olivier*)* is a damaging pest of sorghum, other cereals and horticultural crops in Africa which results in complete crop loss. Currently, the management strategies rely heavily on chemical pesticides, which do not provide effective control. There is evidence showing that microbial biocontrol agents have the potential to control *P. interrupta*. In this study, the entomopathogenic fungi isolated from soils and insects in Ethiopia were identified using morphological and cultural characteristics. These were then evaluated for viability and virulence against *Galleria mellonella* (Fabricius) (Lepidoptera, Pyralidae) larvae and *P. interrupta* adults. Firstly, 116 *Metarhizium* spp. and *Beauveria* spp. were considered. The isolates were evaluated based on high viability as measured by percentage germination on SDA media. Only 56 isolates had greater than 70% viability. Secondly, these were further evaluated using conidial suspensions applied to *G. mellonella* larvae at a concentration of 1×10^{8}conidia/ml. Four *Beauveria* spp. and three *Metarhizium* spp. isolates which caused over 60% mortality were selected for final virulence assay against adults of *P. interrupta*. The selected isolates were further confirmed by PCR amplification of ITS4 and ITS5 gene regions and *chi*1 and *chi*4 primers. Finally, the seven isolates were evaluated for virulence against *P. interrupta* at a dose of 1 mg dry conidia/10 beetles under laboratory condition. Mortality of *P. interrupta* ranged from 14% for isolate 9604 to 82% for isolate PPRC51. Dose-response tests showed that the LD_{50} of PPRC2 (0.62 mg/10 beetles) and PPRC51 (0.55 mg/10 beetles) isolates were not significantly different from each other. The results demonstrated the high potential of the two isolates (PPRC51 and PPRC2) as microbial biocontrol agents. However, field evaluation of the isolates should be performed for their development into a mycopesticide against *P. interrupta*.

Key words: *Pachnoda interrupta*, *Metarhizium anisopliae, Beauveria bassiana,* bioassay, ITS4, ITS5.

INTRODUCTION

Sorghum chafer, *Pachnoda interrupta* (Olivier) (Coleoptera: Scarabaeidae) is one of the most destructive polyphagous insect pests of sorghum and over 35 other important crops in Africa in general and in Ethiopia in particular (*Grunshaw, 1992; Hiwot,* 2000). Some of the crops and host species damaged by the adults of *P. interrupta* recorded in Ethiopia, Eritrea, Mali, Cameroon, Nigeria, and Somalia include cereals such as sorghum, pearl millet, rice, maize, ornamentals such as roses, vegetables such as cucumber and okra, oil crops such as sunflower, niger seed and sesame; fruits such as guava, banana, mango and papaya and other tree plants such as *Acacia* spp. (*Andemeskel, 1987; Grunshaw*, 1992; Troure and Yehouienou, 1995; Jago, 1995; Ratnadas and Ajayi, 1995; MOA and EARO, 1999; Sastawa and Lale, 2000; Hiwot, 2000). *P. interrupta* out-breaks cover wide geographic areas (MOA and EARO, 1999; Yeraswork, 2000; Asmare and Yeshitila, 2014) and completely destroy sorghum fields at the milk stage (Tsedeke, 1988). Grain abortion and panicle sterility caused by the pest can result in 100% yield loss even on insecticide treated sorghum fields (Yitbarek and Hiwot, 2000; Yeraswork, 2000).

Control of *P. interrupta* mainly depends on the use of insecticides (Seneshaw, 2001) which incurs high costs to small farmers and entails environmental, human and animal health related hazards. Moreover, controlling adult beetles through application of insecticides on scattered sorghum fields does not provide long-lasting control because of continuous re-infestation (*Seneshaw and Mulugeta, 2002; Yitbarek,* 2008). Thus, alternative control methods that can contribute to integrated management of *P. interrupta* need to be sought. One of the possible methods is to develop efficient biological control agents that can control larvae and adults at the breeding sites (*Seneshaw and Mulugeta*, 2002). Biological control agents, which include naturally occurring microbial bio-control agents, have great potential in controlling pest populations with little or no detrimental effects on human health and the environment *(Khan et al.,* 2012). Among them, the entomopathogenic fungi (EPF) are most preferred because of ease of production and application, mode of action that does not need ingestion of the entomopathogen by the target pest (Butt, 2002; Wang and St. Leger, 2007; Thomas and Read, 2007). EPF are especially important when used within IPM programs as they are compatible with pesticides (Lacey and Goettel, 1995; *Wraight et al.,* 2007). These fungi have also restricted host ranges and thus cause little or no harm to non-target organisms such as parasitoids and predators (Goettel and Hajek, 2001; *Vestergaard et al.,* 2003, Hajek and Goettel, 2007).

Developing microbial bio-control agents in modern scientific studies requires identification of selected isolates. Characterization and analysis of genetic traits of fungi and other micro-organisms is facilitated by accurate and powerful molecular tools (*Inglis et al., 2012).* Moreover, molecular identification of potential EPF is gaining acceptance as an important first step for successful development of myco-insecticides (*Islam et al., 2014).* Particularly, molecular tools such as PCR based analysis of DNA are used as standard procedures for identification and phylogenetic comparisons between EPF (*Jensen et al.,* 2001; *Destefano et al.,* 2004) and as molecular markers for species identification (*Driver et al.,* 2000; *Entz et al.,* 2005; *Islam et al.,* 2014). The objective of this study was, therefore, to identify and evaluate the potentials of native entomopathogenic fungi for the development of myco-insecticides against *P. interrupta.*

MATERIALS AND METHODS

Isolation and sources of EPF

All the EPF were isolated from soils and infected insects. The source institutions, places of collection, habitats/ host genera and germination of isolates used for the experiments are presented in Table 1.

Initial identification

The key for the identification of major genera of fungi described by Humber (2005) was used to initially identify all the 116 isolates. Only those isolates identified as belonging to the genera *Beauveria* or *Metarhizum* were screened for viability and pathogenecity against larvae of *G. mellonella*.

Screening of isolates

Screening for viability

All isolates initially identified as belonging to either *Beauveria* or *Metarhizium* (101 isolates), were subjected to germination test to select the ones with reasonable viability. Spores were harvested from the surfaces of media using sterile metal spatula and added to test tubes containing 10 ml of sterilized Tween 80 (0.01%) to make a stock suspension. The concentration of the stock suspension was adjusted to 3×10^6 conidia/ml using an improved neubaour heamocytometer and 100 µl of the suspension was then spread plated on SDA media in 90 mm diameter Petri-dishes. After 24 h of incubation at 25°C, a sterile cover slip was put on each Petri-dish and percent germination was determined by counting at least 300 conidia under a compound microscope at 400 X magnification. A conidium was declared germinated if it showed a growth as big as its size. Three replicate Petri-dishes were used for each isolate.

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Table 1. Source (host/habitat), place of collection, genera and germination of isolates selected from the screening for viability and used for bioassay on *G. mellonella* larvae.

No.	Isolate code	Host/Habitat	Place of collection	Genera	Source institution	% germination
1	PPRC 2	*P. interrupta*	Ashan	Metarhizium	PPRC Ambo	93
2	9604	Aceraea acerata	Bugae (Arbaminch)	Beauveria	PPRC Ambo	99
3	MP3POST(B)	Soil	Metahara	Beauveria	PPRC Ambo	98.5
4	PPRC 51	*P. interrupta*	Shewa robit	Metarhizium	PPRC Ambo	96
5	DLCO 131	N/A*	N/A	Metarhizium	DLCO-EA	99
6	MELKE 29	Soil	Melke/Tokekutaye	Beauveria	PPRC Ambo	88.3
7	MELKE 36	Soil	Melke/Tokekutaye	Beauveria	PPRC Ambo	96.4
8	9609	*Blosyus rugulosus*	Mugundo (Dilla road)	Beauveria	PPRC Ambo	72
9	MELKE 4	Soil	Melke/Tokekutaye	Beauveria	PPRC Ambo	78.2
10	MELKE 11	Soil	Melke /Tokekutaye	Beauveria	PPRC Ambo	76.4
11	DLCO 54	Grasshopper	Wikro	Beuveria	DLCO –EA	72.1
12	PPRC 56	*P. interrupta*	Berbere	Beauveria	PPRC Ambo	79
13	9605	N/A	N/A	Beauveria	PPRC Ambo	70.4
14	DLCO 26	Grasshopper	Harmokale	Metarhizium	DLCO-EA	71.2
15	PPRC 27	*P. interrupta*	Sefi beret	metarhizium	PPRC Ambo	73
16	DLCO 31	Grasshopper	Biyo	Metarhizium	DLCO-EA	70.5
17	Ke 43,s/no 03	Soil	Wenji	Beauveria	PPRC Ambo	79.4
18	PPRC 21	*P. interrupta*	Berbere	Metarhizium	PPRC Ambo	72.3
19	PPRC 72	N/A	N/A	Metarhizium	PPRC Ambo	70.6
20	DLCO 137	Soil	Wondogenet	Metarhizium	DLCO-EA	70.8
21	DLCO 78	Grasshopper	Maitimket	Beauveria	DLCO-EA	75.1
22	PPRC 67	*P. interrupta*	Kewet	Metarhizium	PPRC Ambo	76.4
23	**9515	Spider	Awassa	Beauveria	PPRC Ambo	73.9
24	DLCO 81	N/A	N/A	Metarhizium	DLCO-EA	70.9
25	F/no 1261A	Soil	Fincha	Beauveria	PPRC Ambo	81.2
26	DLCO 8	*P. interrupta*	Shewarobit	Beauveria	DLCO-EA	73
27	PPRC 46	*P. interrupta*	Dedeaa	Metarhizium	PPRC Ambo	70.5
28	DLCO 66	Grasshopper	Ibab	Beauveria	DLCO-EA	74.3
29	DLCO 44	Grasshopper	Wikro	Beauveria	DLCO-EA	70.6
30	PPRC 19	*P. interrupta*	Rufea kure	Metarhizium	PPRC Ambo	83.4
31	DLCO 46	N/A	N/A	Beauveria	DLCO-EA	71.3
32	MELKE 19	Soil	Melke/Tokekutaye	Beauveria	PPRC Ambo	83.8
33	DLCO 6	Soil	Shewarobit	Beauveria	DLCO-EA	80.1
34	DLCO 73	Grasshopper	Fura	Beauveria	DLCO-EA	70.4
35	DLCO 93	Grasshopper	Shelle	Beauveria	DLCO-EA	71.3
36	F/no 17A	Soil	Fincha	Beauveria	PPRC Ambo	85.3
37	DLCO 56	Grasshopper	Fura	Beauveria	DLCO-EA	72.3
38	PPRC 29	*P. interrupta*	Gobenaytu	Metarhizium	PPRC Ambo	84.3
39	MP3POST(M)	Soil	Metahara	Metarhizium	PPRC Ambo	77.4
40	PPRC 6	*P. interrupta*	Kewet	Metarhizium	PPRC Ambo	79.5
41	EE	Crustacean	Alamata	Metarhizium	PPRC Ambo	70.5
42	9505	N/A	N/A	Beauveria	PPRC Ambo	71.3
43	DLCO 135	Grasshopper	Ziway	Metarhizium	DLCO-EA	73.8
44	**MM	N/A	N/A	Metarhizium	PPRC Ambo	70.4
45	Ke 42 s/no 06	Soil	Fincha	Beauveria	PPRC Ambo	89.3
46	M2E	N/A	N/A	Metarhizium	PPRC Ambo	70.1
47	ICIPE 30	Busseola fusca	Kendu bay(Kenya)	Metarhizium	PPRC Ambo	74.2
48	PPRC 61	*P. interrupta*	Awaketu	Metarhizium	PPRC Ambo	79.8
49	**DLCO 65	Grasshopper	Kobo	Beauveria	DLCO-EA	70.5
50	**DLCO 61	Grasshopper	Maitimket	Beauveria	DLCO-EA	72.4

Table 1. Contd.

51	**DLCO 105	Grasshopper	Shelle	Beauveria	DLCO-EA	70.4
52	**DLCO 39	Grasshopper	Qoriso	metarhizium	DLCO-EA	70.6
53	**DLCO 48	Grasshopper	Wikro	Beauveria	DLCO-EA	70.5
54	**DLCO 88	Grasshopper	Qoriso	Beauveria	DLCO-EA	70.8
55	**PPRC 14	*P. interrupta*	Dedeaa	Metarhizium	PPRC Ambo	81.2
56	B42,3c	Soil	Fincha	Beauveria	PPRC Ambo	86.2

Screening against G. mellonella

The isolates selected from screening for viability were further screened for pathogenicity against *G. mellonella* larvae. To obtain larvae, adult moths were collected in 500 ml flasks containing folded tissue paper impregnated with water and honey. When eggs were laid, the tissue paper was removed from the flasks and put in plastic rearing boxes containing 180, 50 and 180 g of honey, wheat bran and glycerol, respectively as feed for the larvae. The boxes were incubated in the dark at 20°C for four weeks. The resulting fourth to fifth instar larvae were used for the bioassays.

Stock suspensions were prepared from the respective isolates as for the initial screening and spore concentration was adjusted to 1×10^8 conidia/ml. The *Metarhizium* spp. isolates were bio-assayed in two separate assays, while the *Beauveria* spp. isolates were bio-assayed in three separate assays. For each isolate, 10 larvae were immersed in 10 ml conidial suspension for 10 s in a sterile beaker after which the contents of the beaker were passed through a sterile muslin cloth. The larvae were then transferred into 55 mm diameter sterile plastic Petri-dishes containing filter paper and incubated at room temperature (22-26°C). The control was treated with 10 ml solution of Tween 80(0.01%). A completely randomized design (CRD) with four replications was used for the experiments.

Mortality of the larvae was assessed every day for 10 days. Dead larvae were surface sterilized by briefly immersing in 70% of alcohol and quickly rinsing with sterile distilled water twice. Finally the larvae were transferred to a sterile Petri-dish containing wet filter paper, sealed with parafilm and incubated at room temperature to check for mycosis.

Screening against *P. interrupta*

Three *Metarhizium anisopliae* and four *Beauveria bassiana* isolates which caused 60% or more larval mortality were selected from the screening against *G. mellonella* for final bio-assay against *P. interrupta*. Adult beetles were collected during the mating season of 2013 from breeding areas around Mendubo village (10°50'N, 040°05'E; altitude 1206 m.a.s.l.) in Oromia zone of Amhara Regional State in Ethiopia and kept in plastic baskets containing moistened sterile soil collected from the same area. The baskets had side openings for aeration and the tops were covered with muslin cloth to prevent beetles from escaping. Collected beetles were fed with slices of ripe banana and observed for any natural infection for 10 days before being used for bio-assays.

Conidia of the selected isolates were re-isolated from sporulating cadavers of *G. mellonella* larvae and grown on SDA media at 25°C. After 2 to 3 weeks of incubation, conidia were harvested with sterile metal spatula and collected in sterile Petri-dishes. To remove excess moisture, the conidia were oven-dried at 30°C overnight before the bioassays. The number of spores/mg of the isolates were 3.03×10^8 , 2.9×10^8, 3.5×10^8, 2.9×10^8 , 2.0×10^8, 1.4×10^8, 1.7×10^8 and 3.9×10^8 for isolates 9604, 9609, MP3POST, Melke36, DLCO131, Green Muscle, PPRC51 and PPRC2, respectively. Ten beetles were put in sterile 300 ml plastic tubs with perforated lids. One miligram spores of each isolate were applied on top of the beetles in each of the plastic tubs and the beetles were allowed to move in the tubs for 30 min. The beetles were then transferred to 120 mm diameter plastic Petri-dishes containing moist filter paper and incubated at room temperature for 10 days. During incubation, beetles were fed with slices of ripe banana changed every other day. To provide adequate moisture, 1 ml of sterile distilled water was added to the Petri-dishes every day. Mortality was assessed every day and dead beetles were removed and surface sterilized with 70% ethanol and rinsed thrice in sterile distilled water. The surface sterilized beetles were then transferred to sterile Petri-dishes containing moist filter paper and incubated at 25°C to check for sporulation and to confirm death due to fungal infection. The experimental design was a completely randomized design (CRD) with four replications. The control was treated with the respective spores killed at 80°C in an oven for 48 h. The bioassay was repeated after 75 days using beetles from the October 2013 population from Rassa area (09° 57' N and 040° 04' E) and freshly sub-cultured and harvested spores as in the first bio-assay. Mean percentage mortality from the two experiments were used as measures of virulence. Comparison among selected most virulent isolates was done using their respective LT_{50} and LT_{90} values. The commercialized myco-insecticide Green Muscle containing *M. anisopliae* spores as an active ingredient was used as a standard in the two bioassays.

Dose-response test

A dose-response bioassay was conducted on the three promising isolates (PPRC51, PPRC2 and MP3POST) obtained from the screening against *P. interrupta*. To determine the range of conidial doses used for the test, initial bioassay experiments were conducted with six different doses (0.05, 0.1, 0.25, 0.5, 0.75 and 1 mg/10 beetles). The two lower doses (0.05 and 0.1 mg/10 beetles) did not result in any mortality within 10 days and therefore, excluded from the dose range. Thus, only four doses were used for the final dose-response tests using probit analysis: Finney (1964). Dry conidia were prepared for the evaluation against *P. interrupta* and weighed on a sensitive balance (Adventure ™ USA). All the procedures used for the screening against *P. interrupta* were repeated exactly except that four different doses were applied.

Molecular characterization

The characterization of the selected seven isolates obtained from the screening against *P. interrupta* was done at molecular level using the internal transcribed spacer (ITS) gene region (ITS4 and ITS5) and Chitinase (chi1 and chi4) primers.

DNA extractions

EPF isolates from Ethiopia were cultured on (SDA) media. For each

fungal isolate, 50 to 100 mg of the mycelia was scrapped and DNA were extracted using the Isolate II Plant DNA Kit (Bioline), following the manufacturer's protocol. The resultant DNA was eluted in 50 µL of the elution buffer and stored at -20ºC, until further processing. The extracted DNA quality was then checked using a Nanodrop 2000/2000c Spectrophotometer.

PCR amplification of the ITS4 and ITS5 gene region

Amplifications were carried out for the rDNA region of the fungal isolates using the ITS primers (White et al., 1990). The PCR was carried out in a total volume of 30 µL containing 0.2 µM of each primer (ITS 5; 5'GGA AGT- AAA- AGT- CGT- AAC- AAG -G 3' and ITS 4; 5' TCC- TCC- GCT -TAT -TGA –TAT- GC 3', respectively), 5X My *Taq* Reaction Buffer (Bioline), 1.25 mM MgCl2, 1 unit My *Taq* DNA polymerase (Bioline) and 3 µL of genomic DNA template. Typical cycle conditions were as follows: Initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 40 s, and primer elongation at 72°C for 1 min followed by a final extension at 72°C for 10 min done in an Arktik programmed thermal cycler.

PCR amplification using chi 1 and chi 4 primers

The results from the ITS gene region amplification were also confirmed by amplifying the chitinase gene region using redesigned c*hi* 1 and *chi* 4 primers. In this regard, markers were designed from the chitinase gene to discriminate between the two species through gel electrophoresis. The PCR was carried out in a total volume of 30 µl containing 0.2 µM of each specific primer, 5X My *Taq* Reaction Buffer (Bioline), 1 unit My *Taq* DNA polymerase (Bioline) and 3 µL of DNA template. *Chi* 1 primer set gave a target region of 800 bp, while the *Chi* 4 primer set had a target size of approximately 1000 bp. Typical cycle conditions were as follows: Initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 15 s, specific annealing for 15 s, and primer elongation at 72°C for 10 s followed by a final extension at 72°C for 10 min that was done in an Arktik programmed thermal cycler.

Detection and analysis of the PCR products

The amplified PCR products were resolved through a 1% agarose gel stained with ethidiumbromide (10 mg/ml) and subjected to electrophoresis set at 70 volts for 1 h (Bio-Rad model 200/2-0 power supply and wide mini-sub cell GT horizontal 56 electrophoresis system, Bio-Rad laboratories, Inc., USA), followed by visualization of the DNA under UV-illumination. The gel photo was analyzed and documented using the KETA GL imaging system from Wealtec Corp.

Gel extraction and purifications

All successfully amplified PCR products for each of the targeted gene regions were excised and purified using the Isolate II PCR and Gel Kit from Bioline following the manufacturer's instructions. A total of 17 purified PCR products were sent to Macrogen Inc, Europe Laboratories, the Netherlands for bi-directional sequencing.

Sequencing data analysis

This was done using bioinformatics tools and software. The sequences obtained were edited by Chromas Lite version 2.1.1 software and the consensus sequences from both the forward and reverse strands generated. For conclusive identification, the consensus reads generated were queried through BLASTN, at the GenBank data base hosted by the National Centre of Biotechnology Information USA (NCBI). This was also to check for similarity with organisms already identified. Furthermore, the consensus sequences generated were multi-aligned using Clustal X (version 2.1). The multiple alignments created were used to generate a phylogenetic tree by use of Mega software (version 6.06).

Statistical analysis

Mortality data were corrected using Abbot's formula (Abbott, 1925), arcsine transformed and subjected to the ANOVA procedure of SAS version 9.0. Percentage viability data were also analyzed in the same way. Means were separated using LSD and Tukey's Honestly Significant Difference (HSD) for screening experiments against *G. mellonella* and *P. interrupta,* respectively. LT_{50}, LT_{90} and $LD_{50,}$ values were estimated with probit analysis for correlated data followed by ANOVA and mean separation with LSD. Probit analyses were done using SPSS version 17.

RESULTS

Initial identification

Out of 116 isolates collected from the different sources, 101 isolates were preliminarily identified using morphological and cultural characteristics as *Metarhizium* spp. or *Beauveria* spp. Fifteen of the isolates were identified as not belonging to the two genera.

Screening of isolates

Viability of isolates

The germination percent of the 101 isolates evaluated for viability ranged from 0 to 99 (data not shown). The isolates with greater than 70% germination were considered sufficiently viable and selected for further screening (56 isolates) against *G. mellonella* (Table 1).

Effects of fungal isolates on G. mellonella

Corrected percent mortality of *G. mellonella* larvae due to fungal isolates significantly varied among isolates of each of *Beauveria* spp. and *Metarhizium* spp. (Tables 3 and 4). The *Beauveria* spp. isolates caused between 0 and 63%, 0 and 78%, and 0 and 73% mortality in the first, second and third bioassays, respectively. Similarly, the *Metarhizium* spp. isolates caused 0 to 83% and 0 to 67% mortality in the first and second bioassays, respectively.

Effects of fungal isolates on P. interrupta

There was a highly significant variation among the

Table 2. Characterization of the entomopathogenic fungi selected from Ethiopia.

Sample name	Synonymous isolate from NCBI	Accession no.	% identity
BL2 (PPRC51)	*Metarhizium anisopliae*	JF495775.1	99
BL3 (PPRC2)	*Metarhizium anisopliae*	FJ545313.1	99
BL4 (MP3POST)	*Beauveria bassiana*	KC461112.1	100
BL5 (Melke 36)	*Beauveria bassiana*	KC461112.1	100
BL6 (9609)	*Beauveria bassiana*	KC461112.1	100
BL7 (9604)	*Beauveria bassiana*	GU233705.1	99
BL8 (DLCO131)	*Metarhizium anisopliae*	JN256674.1	99

Table 3. Mortality of *G. mellonella* larvae caused by *Beauveria* spp. isolates from Ethiopia in three different bioassays.

First assay		Second assay		Third assay	
Isolate	**%Mortality ± SE**	**Isolate**	**%Mortality ± SE**	**Isolate**	**% Mortality ± SE**
9605	37.05±16.22^{abc}	9505	0.03±0^{d}	9615	23.33±4.92^{bcd}
DLCO6	16.68±10.65^{bc}	9604*	77.78±13.75^{a}	DLCO31	33.33±15.00^{ab}
DLCO8	16.68±11.07^{bc}	9609*	60±18.20^{ab}	DLCO54	45.83±4.83^{abcd}
F/2NO17A	10.01±7.57^{c}	DLCO135	0.03±0.00^{d}	DLCO56	10.01±7.57^{d}
F/2NO1261	16.68±11.07^{bc}	DLCO44	13.34±9.34^{dc}	DLCO81	16.68±9.70^{dc}
Ke43S/NO	26.68±13.18^{abc}	DLCO46	33.33±9.34^{bc}	DLCO93	10.01±7.57^{d}
MELKE29	63.33±3.93^{a}	DLCO61	13.33±2.71^{dc}	MELKE11	53.33±13.06^{abc}
MELKE36*	63.33±7.22^{a}	DLCO73	10.01±7.57^{dc}	MELKE19	33.33±4.22^{abcd}
MELKE4	59.26±15.77^{ab}	DLCO78	29.17±7.08^{bc}	MP3POST*	73.33±13.18^{a}
Ke42S/N0	0.19±0^{c}	PPRC56	40±9.96bc		

*Isolates selected for further screening.

isolates (P = 0.0003, df = 7, 16, F = 8.01) in causing mortality to *P. interrupta* (Figure 2). The lowest mean percent mortality was caused by the *B. bassiana* isolate 9604 (14.08%) which was not significantly different from *B. bassiana* isolate 9609 (27.97%), Melke36 (15.75%), *M. anisopliae* isolates DLCO131 (25%) and Green Muscle (20.38%). The highest mortality to *P. interrupta* was caused by PPRC51 (82.40%) which did not significantly differ from the *B. bassiana* isolate MP3POST and the *M. anisopliae* isolate PPRC2 which caused 79.63 and 77.14% mortality, respectively. Based on the results of the virulence assays, two *M. anisopliae* isolates (PPRC51 and PPRC2) and one *B. bassiana* isolate (MP3POST) which caused over 75% mortality to *P. interrupta* adults were selected. The time taken by the three selected isolates to cause death to 50 and 90% of the experimental insects (LT_{50} and LT_{90} days) is shown in Table 4. The LT_{50} (days) of the selected isolates varied significantly (P = 0.0087, df = 2, 6, F = 11.6) with the lowest (5.33 days) recorded from PPRC51 followed by MP3POST (5.7 days). The highest (6.92 days) was recorded due to PPRC2 which was significantly different from both isolates. However, there was no significant difference (P = 0.057, df = 2, 6, F = 4. 78) in the LT_{90} (days) of the three isolates. Figure 1 shows growth and sporulation of PPRC51 and PPRC2 isolates on *P. interrupta* adults during the bio-assays.

Dose-response test

Mortality of *P. interrupta* adults at different doses of the selected *M. anisopliae* and *B. bassiana* isolates is shown in Figure 3. There were no significant mortality differences within each dose except for the dose of 0.5 mg in which the *B. bassiana* isolate MP3POST showed significantly lower mortality (P = 0.039, F = 5.78, df = 2 , 6) than the two *M. anisopliae* isolates. The mean LD_{50} of the three isolates also varied significantly (P = 0.025, F = 7.12, df = 2,6) among the isolates with isolate MP3POST requiring higher dose (0.8 mg/10 beetles) to kill 50% of the test insects than PPRC2 (0.62 mg/10 beetles) and PPRC51 (0.55 mg/10 beetles) isolates, which were not significantly different from each other (Table 5). There were no significant differences in the mean LD_{90} among all the three isolates (P = 0.41, F = 1.04, df = 2, 6).

Molecular identification

The internal transcribed spacer region (ITS) was successfully amplified in all of the isolates as shown in

Table 4. Mortality of *G. mellonella* larvae caused by *Metarhizium* spp. isolates from Ethiopia in two different bioassay.

First assay		Second assay	
Isolate	**% Mortality±SE**	**Isolate**	**% Mortality±SE**
EE	3.72±6.19^{bc}	DLCO137	25.93±13.70^{b}
M2E	0.03±0.00^{c}	DLCO26	37.04±5.87^{ab}
ECIPE30	0.03±0.00^{c}	DLCO131*	66.67±8.07^{a}
MP3POSTM	12.52±12.29^{bc}	DLCO66	13.34±9.34^{b}
PPRC2*	83.33±11.07^{a}	PPRC19	13.34±8.55^{b}
PPRC21	25±0.00^{b}	PPRC27	36.67±3.93^{ab}
PPRC46	14.81±2.89^{bc}	PPRC29	6.68±8.55^{b}
PPRC67	22.23±12.02^{bc}	PPRC51*	66.67±14.70^{a}
PPRC72	23.82±12.15^{bc}	PPRC6	7.42±9.08^{b}

*Isolates selected for further screening.

Figure 1. Pachnoda interrupta adults infected with M. anisopliae isolates PPRC51 and PPRC2.

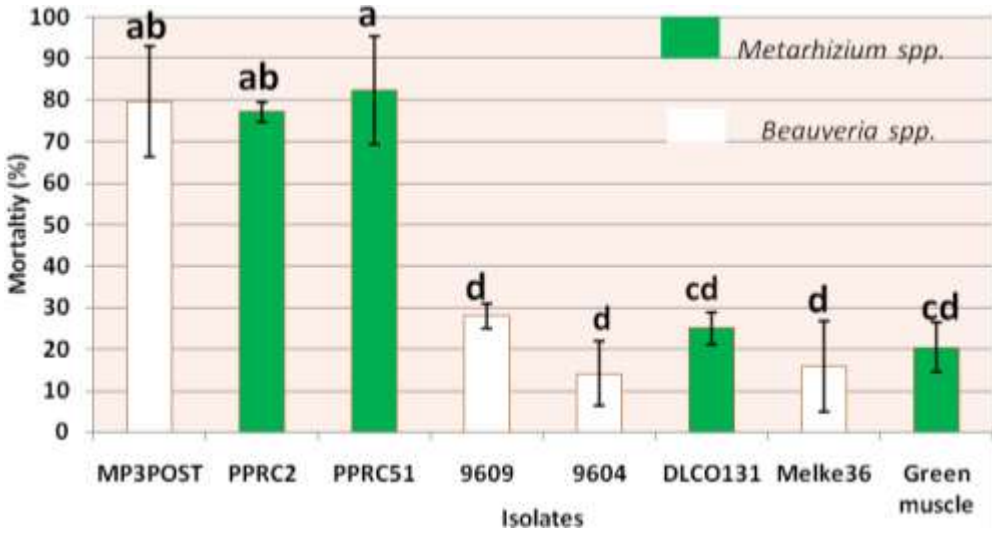

Figure 2. Mortality ± SE of P.interrupta due toB.bassiana and M. anisopliae isolates from Ethiopia. Bars containing similar letters are not significantly different according to Tukey's HSD test (α =0.05,df = 7, 16, P = 0.0003, F = 8.01).

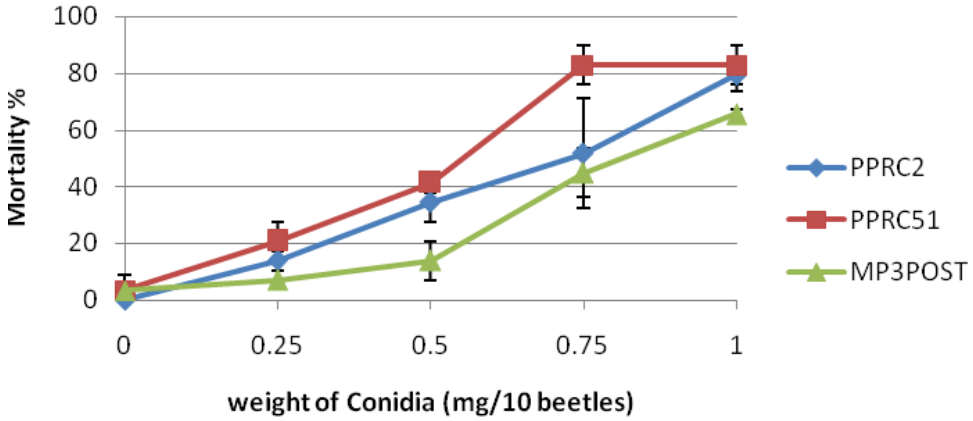

Figure 3. Mean percentage mortality ± SE caused by the selected isolates of B. bassiana and M. anisopliae on P. interrupta adults at different doses per 10 beetles (10 days after application).

Table 5. Mean LT_{50} and LT_{90} (days ± SE) of the three selected isolates on *P. interrupta* adults 10 days post treatment.

Isolate	LT_{50} ± SE	LT_{90} ± SE	Slope ±SE
PPRC2	6.92±0.21[a]	16.14±1.17[a]	3.56±0.32[a]
MP3POST	5.7±0.25[b]	12.59±1.35[ab]	4.38±0.59[a]
PPRC51	5.33±0.27[b]	11.63±0.59[a]	3.85±0.34[a]

Means with similar letters in the same column are not significantly different according to LSD test at α=0.05.

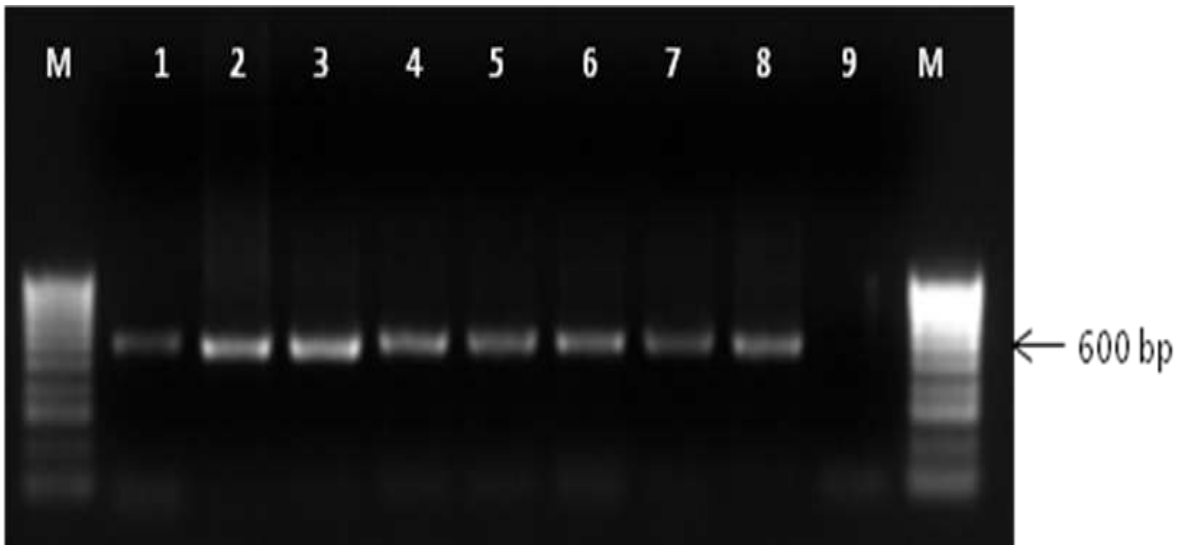

Figure 4. 1% agarose gel electrophoresis for fungal isolates from Ethiopia using ITS primers. M = 1KB DNA ladder, Lane 1 = IC279, Lane 2 = PPRC 51, Lane 3 =PPRC2. Lane 4 = MP3POST, Lane 5 = Melke36, Lane 6 = 9609, Lane 7 = 9604,Lane 8 = DLCO131, Lane 9 = Negative control.

Figure 4. Figures 5 and 6 show the amplification of the two fungal genera *Metarhizium* and *Beauveria*, respectively using c*hi*1 and *chi*4 genes. The BLAST searches corresponded to sequences registered under

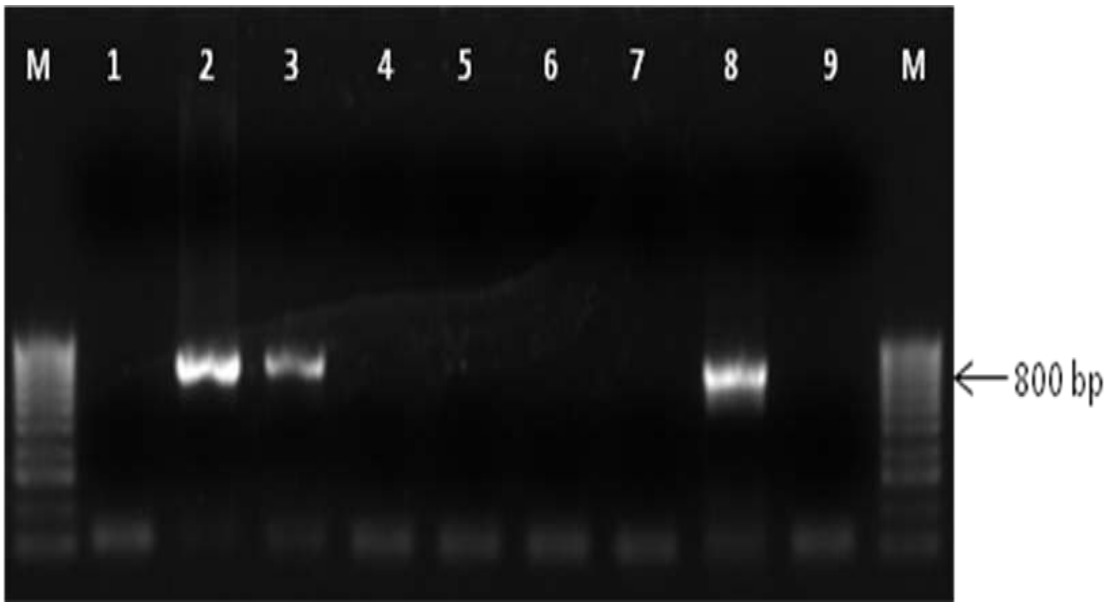

Figure 5. *1% agarose gel electrophoresis for fungal isolates from Ethiopia, using chi 1 primers. M= 1Kb DNA ladder, Lane 1 = IC279, Lane 2 = PPRC51, Lane 3 = PPRC2, Lane 4 = MP3POST Lane 5 = Melke36, Lane 6 = 9609, Lane 7=9604, Lane 8=DLCO131, Lane 9 = Negative control.*

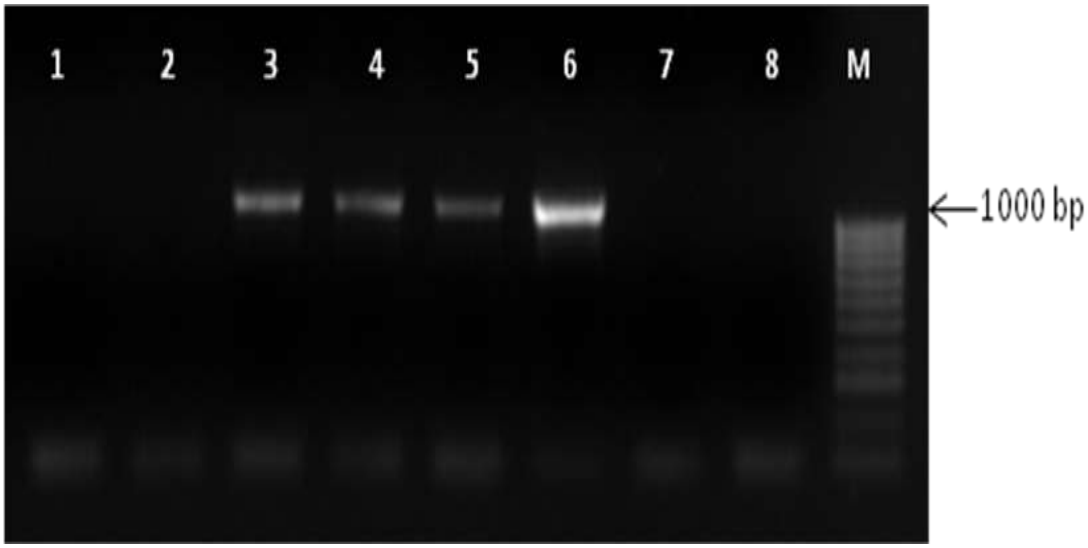

Figure 6. 1% agarose gel electrophoresis for fungal isolates from Ethiopia using chi4 primers. M = 1Kb DNA ladder, Lane 1 = PPRC 51, Lane 2 = PPRC2, Lane 3 = MP3POST, Lane 4 = Melke36, Lane 5 = 9609, Lane 6 = 9604, Lane 7 = DLCO131, Lane 8 = Negative control.

M. anisopliae and *B. bassiana* and were provided with accession numbers as depicted in Table 2 which confirms the identities of the 7 isolates. Three of the samples were linked to *M. anisopliae* and four to *B. bassiana* in the GenBank database.

The sequences from the ITS gene region were used to generate a phylogenetic tree (Figure 7). The evolutionary history was inferred using the Neighbor joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.19117607 is shown in Figure 4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base

Table 6. Mean LD_{50} and LD_{90} (mg/10 beetles ± SE) of the three selected isolates on *P. interrupta* adults 10 days post treatment and slope of the regression line ± SE.

Isolate	LD_{50} ±SE	LD_{90} ± SE	Slope ± SE
PPRC2	0.62±0.075[b]	1.72±0.21[a]	2.89±0.18[a]
PPRC51	0.55±0.029[b]	1.83±0.17[a]	2.53±0.33[a]
MP3POST	0.8±0.029[a]	2.22±0.34[a]	3.13±0.48[a]

Means with similar letters in the same column are not significantly different according to LSD test at α=0.05.

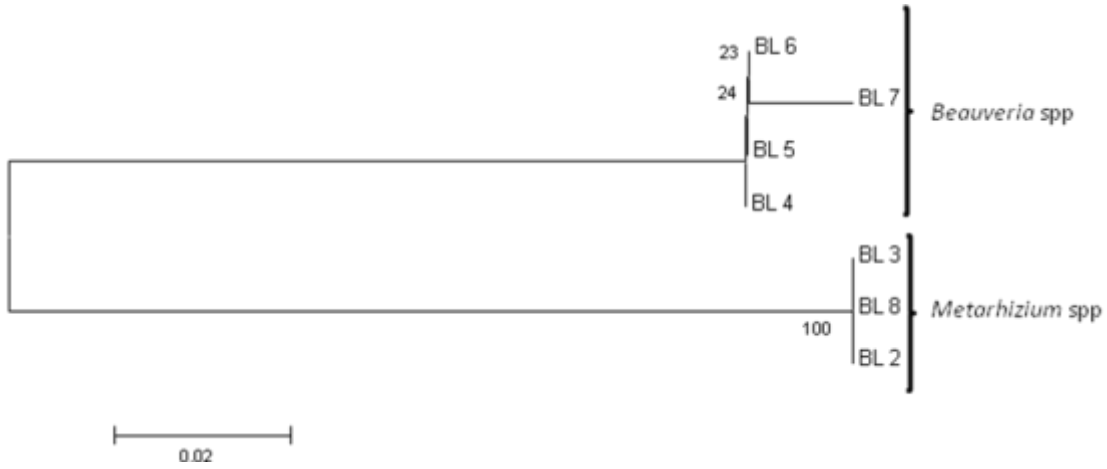

Figure 7. Phylogenetic tree showing evolutionary relationships of entomopathogenic fungi from Ethiopia resulting from sequences of the internal transcribed spacer (ITS4 and ITS5) gene region. BL2 = PPRC51, BBL3 = PPRC2, BL4 = MP3POST, BL5 = Melke36, BL6 = 9609, BL7 = 9604.

substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Non coding. All positions containing gaps and missing data were eliminated. There were a total of 518 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (*Tamura et al., 2013).* The tree clustered the *Metarhizium* and *Beauveria* species into two clusters, as expected.

DISCUSSION

Fungi can be identified using morphological characteristics but this method is not adequate due to ambiguous descriptions and limited availability of morphological keys (*Fernandes et al., 2010*). However, the method can be used to minimize time and resource expenses when dealing with large number of isolates as a preliminary identification tool as employed in this study. The finally selected 7 entomopathogenic fungi from Ethiopia screened against *P. interrupta,* were identified as *M. anisopliae* and *B. bassiana* by the PCR amplification of the ITS regions of rDNA and chitinase genes. The ITS has been widely and effectively used to amplify DNA from a wide range of fungi (Kendall and Rygiewicz, 2005). This region is highly conserved and is commonly used as a sole tool or supplemented with other universal sequences (e.g. tubulin and actin) for identification, characterization and phylogenetic analysis of fungal isolates (*Balazy et al., 2008).* The ITS region has been used in other studies for successful identification of entomopathogenic fungi (*Islam et al., 2014*). The results from amplification of the ITS region from the current study were confirmed with amplification with the chitinase genes. This functional gene confirmed the identity of the Ethiopian isolates as belonging to *M. anisopliae* and *B. bassiana*, as expected. Similarly, Chitinase genes have been used in other studies as molecular markers for *M. anisopliae* identification and characterisation (*Bogo et al., 1998*; *Kang et al., 1999*; *Enkerli et al., 2009).*

Merid et al. (2016) have recently reported the use of *Metarhizium* spp. for field control of *P. interrupta* which showed up to 71% mortality within 15 days of infection. Researches on other scarab beetles (*Lacey et al., 1994;* Klein and Lacey, 1999; *Cuthbertson et al., 2012*) have also shown the potential of these fungi for control of coleopterous insects. This current study has demonstrated the importance of these entomopathogenic fungi as potential microbial biocontrol agents for *P. interrupta*. Among the seven isolates, the *M. anisopliae* isolate PPRC51 caused 82.40 % mean mortality on field

collected *P. interrupta* adults within 10 days post application and had the shortest LT_{50} (5.33 days). The *B. bassiana* isolate MP3POST and *M. anisopliae* isolate PPRC2 also caused 79.67 and 77.14% mortality, respectively. In a similar study, *Lacey et al. (1994)* observed 100% mortality of the Japanese beetle, P*. japonica* adults within 8 and 9 days after application of *M. anisopliae* and *B. bassiana* dry conidia, respectively, using similar doses *Gindin et al.* (2006) also reported 85% mortality of the red palm weevil, *Rhynchophorus ferrugineus*, two weeks after contact with dry conidia of *M. anisopliae* indicating the potential of the fungi for biocontrol of coleopterous insects. Although isolate MP3POST needed significantly higher LD_{50} than PPRC2 and PPRC51, its LT_{50} was not significantly different from that of PPRC51.

Conclusion

The findings of this study have indicated the potential use of the indigenous EPF isolates against *P. interrupta*. The isolates PPRC51, PPRC2 and MP3POST are as found the potential candidates for development of myco-insecticide against *P. interrupta* as a component of an integrated management strategy of the pest. However, field studies using appropriate formulation under high insect population conditions and more research on mass production characteristics and shelf life of the isolates are needed.

Conflict of Interests

The authors have not declared any conflict of interests.

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