Identification and characterization of an isolate of *Mucor irregularis* as a potential bio-control agent for brown planthopper in rice (*Oryza sativa* L.)

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Abstract

Brown Pant Hopper (BPH) (*Nilaparvata lugens*) is considered as the number one pest in rice cultivation of Sri Lanka. To manage BPH problem in rice, host plant resistance and chemical control measures are commonly applied. Alternative approaches to control BPH are needed since BPH has developed resistance against recommended chemicals. In this study a naturally occurring fungus over the body of BPH, was isolated from the fields of the Rice Research and Development Institute, Batalagoda during 2018/19 *Maha* season. Its effectiveness in causing mortality in BPH was evaluated. The isolated fungus was named as BG-01 and characterized at morphological and molecular levels. These analyses show that the BG-01 isolate is identical to *Mucor irregularis*, which has already been identified as an entomopathogenic fungus in other countries. The results show that the fungus has the ability to grow on BPH causing 100% mortality to the insect with time. With the available records in the country, this is the first report on identification of *M. irregularis* species as a natural biocontrol agent for BPH. The sequence data belong to the ITS region of the isolated fungus was deposited in the GenBank® with the accession number MT459452. Isolated fungus BG-01 has the potential to use as a biocontrol agent for BPH control in Sri Lanka.

Keywords: Bio-control agent, Brown plant hopper, *Mucor irregularis*, Resistance, Rice

Introduction

Rice crop is cultivated throughout Sri Lanka in two seasons; *Maha*-the major rainy season and *Yala*-the dry season. Though the estimated paddy yield is above 10 t ha^{-1} (i.e., Var. Bg 374), the actual national average paddy yield is 4.8 t ha^{-1} during 2019 (Department of Census and Statistics, 2020). Low yields of paddy in Sri Lanka is caused by a number of abiotic stresses such as drought, floods and high temperature injury, and biotic stresses such as pests, diseases and weeds infestation (www.fao.org). Among the pests, Brown Plant Hopper (BPH) (*Nilaparvata lugens* (Stål) (Homoptera: Delphacidae) is considered as the number one pest problem in rice cultivation in Sri Lanka (Pushpakumari *et al.*, 2017). BPH occur in most of the tropical countries in Asia and the South Pacific regions including Sri Lanka (Anon, 1979). BPH also acts as a vector of both ragged-stunt and grassy-stunt viruses (Hibino, 1996). BPH exclusively feeds on rice (*Oryza* spp.) cultivars (Wilson and Claridge, 1991). The insect is a phloem feeder while adults and nymphs suck the plant sap from the sheaths at the base of the tillers of rice crop at all stages of plant growth (Wilson and Claridge, 1991). Due to severe infestations of BPH, rice plants turn yellow and dry up rapidly causing 'hopper burn', which is irreversible (Bottrell and Schoenly, 2012). Host plant resistance has been focused by many countries, including Sri Lanka to combat the BPH outbreaks (Fujita *et al.*, 2013). However, BPH has the ability to adapt rapidly to resistant varieties (Roderick, 1994). Under present situation almost all of the rice varieties seem to be susceptible to BPH (Personnel communications, Entomologist, RRDI, Batalagoda).

BPH is gaining resistance to some of the insecticides due to inappropriate and over-applications of them to control BPH (Rombach and Gallagher, 1994, Hemachandra, 2020). Thus, biological control can be used to minimize the BPH damage of rice cultivations in a more environmental friendly way. Entomopathogenic organisms such as fungi, bacteria, viruses, protozoa, and nematodes can be used to control pests (Kachhawa, 2017). Several insect pathogens have been formulated as microbial insecticides for BPH in rice (Rombach, 1987). Upadyay *et al.* (2001) described about important entomopathogenic fungi, their utilization in pest management and commercial availability of mycoinsecticides. Entomopathogenic fungi have evolved a series of strategies to counter insect immune defense such as penetration, growth, and proliferation within the body of the host and interaction with insect defense mechanism and re-emergence on the carcass (Thomas *et al.*, 1996). Entomopathogenic fungi, *Verticillium lecanii*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Mucor* species have been studied intensively as important epizootics of aphids and other agricultural pests (Roberts and St. Leger, 2004; Li and Sheng, 2007; Raja *et al.*, 2017). In this study, a fungal species naturally isolated from BPH associated with rice was tested for its ability to use as a biological control agent for BPH. Morphological and molecular characterization of the isolated fungus was carried out.

Materials and Methods

Isolation of the fungus

A few adult BPH insects (live and dead), with a fungal growth on their bodies, were collected using an aspirator from the rice field of the Rice Research and Development Institute (RRDI), Batalagoda, Sri Lanka during 2018/19 *Maha* season. Collected BPH were surface sterilized with 70% ethanol followed by washing with sterilized distilled water and dried on sterilized filter papers. The fungus found on the sterilized BPH were cultured on Potato Dextrose Agar (PDA) medium (keeping one insect per plate) and incubated at the culture facility of the Plant Pathology Division, RRDI under room temperature (28-30 °C). Growing edges of the cultures were selected and sub-cultured to obtain pure cultures. Pure cultures with fruiting structures were maintained on PDA culture plates (9 cm diameter) and culture slants (PDA) were also prepared and maintained for further use. All the cultures were morphologically similar to each other and one pure culture designated as BG-01, was selected for further studies.

Inoculation of isolated fungus to BPH

Visible fruiting structures of the isolated fungus was separated from 10 days old cultures and spread on sterilized filter papers. Then, the filter papers containing the fruiting structures were kept inside of sterilized petri dishes (9 cm diameter). Collected live adult BPH (10-20 days old), which did not show the presence of fungal growth, were kept in petri-dishes (10 BPH/ petri-dish). Two pieces of fresh leaf sheaths (around 2-3 cm length) of rice plants (var. Bg 352, around 10 weeks old) were also kept in these petri dishes for

BPH to feed on. For the control treatment, the same procedure was followed without fungal fruiting structure. The experiment was arranged in Completely Randomized Design (CRD) with six replicates. The petri-dishes covered with lids were maintained in an open space of the Plant Pathology laboratory of the RRDI under room temperature (28-30 °C). The mortalities of adult BPH were counted every day up to 10 days from the beginning of this step of the experiment. Only those dead adults in treated petri-dishes with external fungal growth on their bodies were considered to have died from fungal infection. The experiment was repeated as follows. External growth of fungal mycelia from a few adult BPH carcasses of treated petri dishes were re-isolated and cultured on PDA medium. The presence of the same fungus, which was used for the infection, was confirmed through visual and microscopic observations. Then, this re-isolated fungus was re-inoculated to live BPH following the same procedure and mortalities of adults were checked daily up to 10 days as described above.

Morphological and molecular identification of the fungus

Growth and culture characteristics of the isolated fungus (BG-01) were observed and recorded as described by Harold (1978). Microscopic observations were carried out by slide culture technique using a light microscope (Model: OPTIKA B383PLi, OPTIKASrl, Ponteranica, Italy). A Luria Broth (LB) culture (50 mL) was prepared in a 250 mL erlenmeyer flask aseptically. A mycelial disk was taken using a cork borer from a 7 day old actively growing selected pure fungal culture (BG-01 isolate) and was kept in Luria Broth under aseptic conditions. BG-01 isolate inoculated LB containing flask was incubated in an orbital shaker at 50 rpm for 7 days at 28 °C. Total genomic DNA was extracted from the mycelia of the 7 day old broth culture using a modified cetyl trimethyl ammonium bromide (CTAB) extraction method. Quality of the isolated DNA was measured electrophoresing 3 µL of isolated DNA after mixing with 2 µL of 6X gel loading buffer (Promega, USA) in 1% of Agarose gel containing ethidium bromide (0.2 µg mL^{-1}) in 1× TAE buffer (1M Tris HCl pH 8.0, 1M acetic acid, and 0.5 M ethylene diamine tetra acetic acid (EDTA), pH 8.0) at 80 V for 20 min. Quantity of DNA was also measured using a spectrophotometer (Model: Genova Nano, Jenway, UK) (Data are not shown). A

Polymerase Chain Reaction (PCR) was performed with the isolated DNA of the fungus using the universal primers; ITS -1 (Forward): 5'- TCCGTAGGTGAACCTGCGG-3' and ITS- 4 (Reverse): 5'- TCCTCCGCTTATTGATATGC - 3' (White *et al*., 1990). The PCR mix in a total volume of 15 µL consisted of 1µL of the template DNA (100 ng DNA) with 1.0 µL of each primer (10 mM), 1.0 of 10 mM dNTP and 0.25 µl of *Taq* DNA polymerase (5000 U/ µL) (Promega, USA), 2 µL of 5x PCR Buffer with loading dye (Promega, USA), 1.5µL of 25 mM MgCl2 and sterilized distilled water to mark up the volume. The PCR cycle consisted of an initial denaturation of 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C, with a final extension of 10 min at 72 °C followed by termination of the cycle at 4 °C. The PCR was carried out in ABS Verity thermal cycler (Applied Bio Systems, USA). Amplification of PCR product was checked through electroporation in 1.0% Agarose gel (w/v) in 1XTAE as above, and viewing on a UV transillumінator (Model: UPV, Ultra-Violet Products, Cambridge, USA) (Data are not shown). The PCR product of the sample was sequenced using Sanger method (Macrogen, Korea). Sequence data were assembled and the sequence was checked in BLAST (NCBI) for homologies. Similar sequences were compared with the sequence data obtained in this study using BioEdit (Version 7.2.5.). A phylogenetic relationship of the isolated fungus with similar matches found on the NCBI was developed using MEGA six Software.

Results and Discussion

Fungal growth on BPH carcasses was seen covering the whole body in whitish to yellow in color (Plate 1).

Plate 1. Fungal growth observed on BPH carcasses collected from the field of the RRDI, Batalagoda during 2018/19 *Maha* season

Fungal morphology

The diameter of the colony of the fungus, which was cultured on PDA medium in petri dishes, till the fruiting structures were produced, was measured 1 day, 3 days, 5 days, 7 days, 9 days, 11 days, and 13 days post culturing up to the maximum growth of the fungus in the petri dish was completed. The growth rates of isolated and re-isolated fungus (Figure 1) showed a normal growth pattern as of many other fungi found in the natural environment.

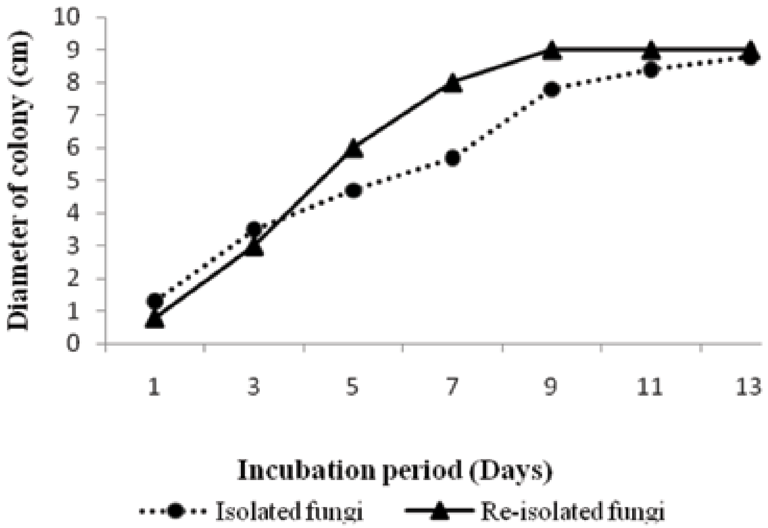


Figure 1. Growth rate of isolated and re-isolated fungus BG-01. The diameter of the colony (cm) was measured up to the maximum growth

Isolated fungal colony on PDA reached a diameter of 9 cm after 13 days (Plate 2). At the beginning, it was presented as white mycelia, which later turned in to yellowish to green color and bore fruiting structures with white mycelia. At the maximum growth of the fungus, the upper side of the culture was in dark green color and the reverse yellowish (Plate 3). Spore formation on culture plates was observable around 8 days post culturing. Microscopic observations of the isolated fungus (BG-01) is also shown in plates 2 & 3.

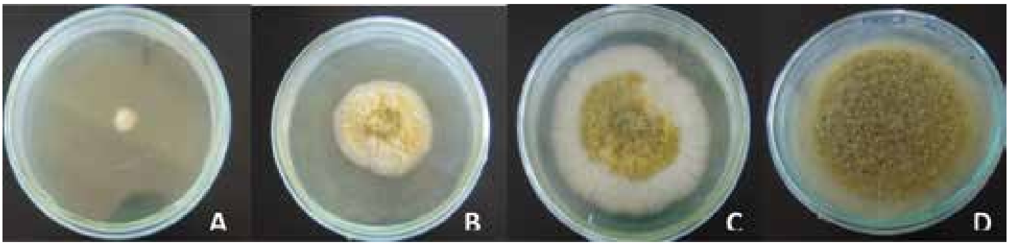


Plate 2: Growth pattern of fungal culture isolated from BPH; A = One day old, B = Four day old, C = Nine day old, and D = 14 day old

Plate 3: View of the fully grown fungal culture (BG-01) isolated from BPH; A and B =Upper side and Reverse side view of cultures, and microscopic observation of isolated fungus BG-01 (Magnification 10×40) through the light microscopy (C)

Sporangiophores were hyaline and branches were ending in a sporangium. Sporangia were spherical. Sporangiospores were light yellow in color and observed as sub-spherical to ellipsoidal. The culture characteristics and microscopic observations of re-isolated fungus were also found to be similar to those of the initially isolated fungus (Data are not shown).

Morphological characteristics of the fungus (BG-01) isolated from BPH grown on PDA medium are presented in Table 1.

Table 1. Morphological characteristics of the isolated fungus (BG-01)

No. of Days	Color of the culture		Colony characters*		
	Upper	**Reverse**	**Configuration**	**Margin**	**Elevation**
1	White mycelium with raised center	White	Round	Wavy	Drop-like
2	White mycelium with yellowish raised center	White	Round	Wavy	Drop-like
3	White mycelium with yellowish raised center	White	L-form	Wavy	Drop-like
4-5	White mycelium with yellowish raised center	White	L-form	Wavy	Convex
6 -7	White broad outer margin with yellowish center	White	L-form	Wavy	Convex
8-10	White broad outer margin with yellowish green center	White	L-form	Wavy	Convex
11-14	White thinner outer margin with greenish color center	White	L-form	Wavy	Convex

**Colony characters were recorded according to the method described by Harold (1978)*

Fungal identification is basically done observing their morphological characteristics. The fungus isolated from BPH in this study showed morphological characters similar to a *Mucor* species. Nguyen *et al.* (2016) reported about characterization of two new Mucoralean species (*M. irregularis* and *Mucor fragilis*) isolated from gut of soldier fly larva in Korea. In this study, the colony morphology and culture characteristics of *M. irregularis* on PDA medium have shown that the culture has attained the diameter of 62~64 mm after 2 days at 28-30 ^{o}C and the colour of the colonies was cotton yellow on the upper side and pale yellow on the reverse side. In the present study, the upper side of the culture was dark green colour and the reverse the yellowish while the cultures took 7-day to grow 60 mm at 28-30 ^{o}C. Nguyen *et al.* (2016) also reported sporangiosphores of *M. irregularis* mostly sympodially branched, ellipsoidal and sporangia were globose to sub-globose, light yellow and were multi-spored. Comparing the microscopic observations of *M. irregularis* on Malt Extract Agar (MEA) medium in previous studies done by Nguyen *et al*. (2016) and Lu *et al*. (2013) have shown that present isolate (BG-01) was resembling *M. irregularis*.

Infection study

The mortality of BPH with time, after exposure to the isolated fungal fruiting structures, are shown in Figure 2. Mortality of BPH in treated petri dishes was higher than that of non- treated petri-dishes throughout the observation period. Significantly higher numbers of mortalities were reported from BPH treated with BG-01 fungal isolate, compared to the control treatment. Dead adults in treated petri-dishes showed external fungal growth on their bodies after 3-4 days of death and no fungal growth was observed on the carcasses with in the control treatment. The mortalities of the BPH with time, after exposure to re-isolated fungal fruiting structures are shown in Figure 3. In this experiment, a higher mortality of BPH was also observed in fungal spores treated petri-dishes than that of the non-treated in all the counts.

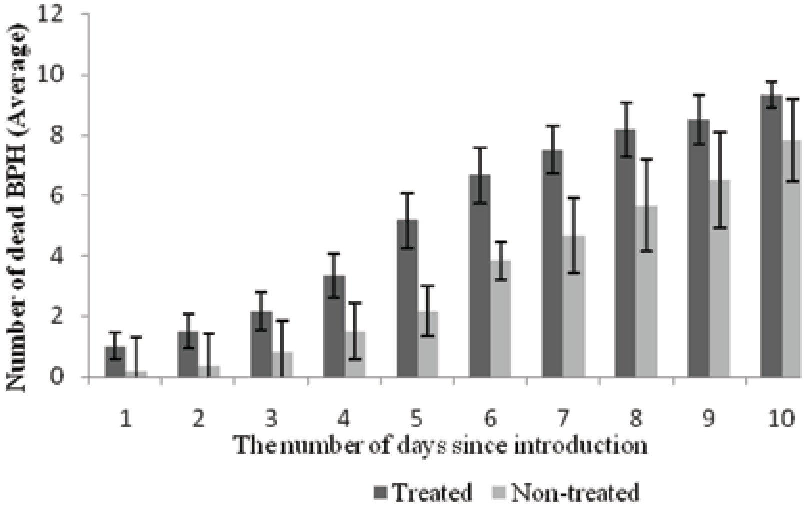


Figure 2. Mortalities of BPH in treated (exposed to the fruiting structures of BG-01) and in untreated control (not exposed to the fungal fruiting structures). Error bars represent the standard error (SE) for the means of six replicates

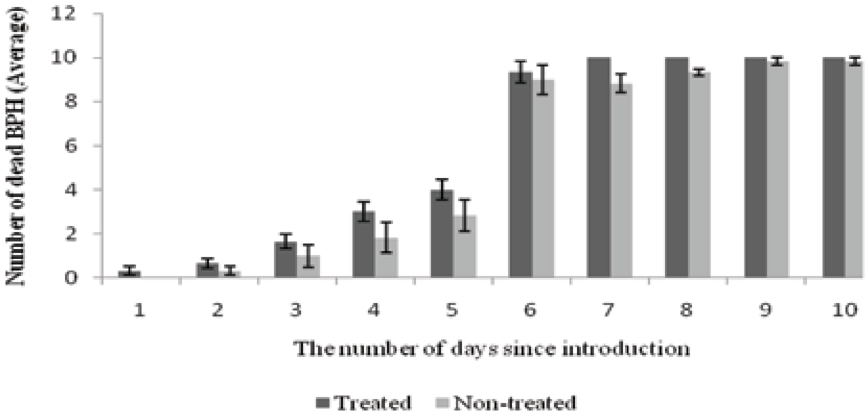


Figure 3. Mortalities of BPH in treated (exposed to the re-isolated fungal fruiting structures of BG-01) and in untreated control (not exposed to the re-isolated fungal fruiting structures). Error bars represent the standard error for the means of six replicates

Molecular characterization of the fungus

PCR with ITS 1 and ITS 4 produced a band around 600 bp (Figure 4).

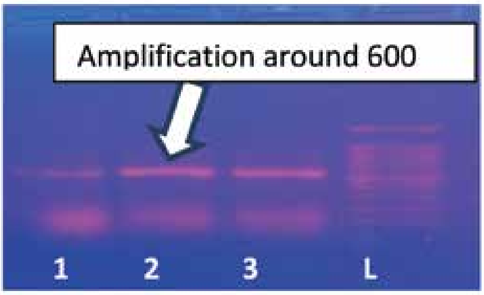


Figure 4. PCR amplification of BG-01 isolate with ITS 1 and ITS 4 universal primers Lane 1 - 3 = Isolated DNA of three samples of BG-01 fungal isolate, L = 100 bp ladder

The PCR product of BG-01 fungal isolate, sequenced and assembled, gave 539 nt sequence. The sequence data of BG-01 isolate was deposited at the NCBI GenBank® with the accession number MT459452. The NCBI Blast searches revealed that the sequence data of MT459452, was mostly matching with sequence data of *M. irregularis* isolates available in the GenBank® with 99-100% query coverage and around 99-100% identity (Table 2). It also gave above 99% identity with 100% query coverage with a Rhizomucor species available in the GenBanK®. However, the sequence data of BG-01 isolate gave less than 87.6% and query coverage of 26% with the Metarhizium spp. available in the

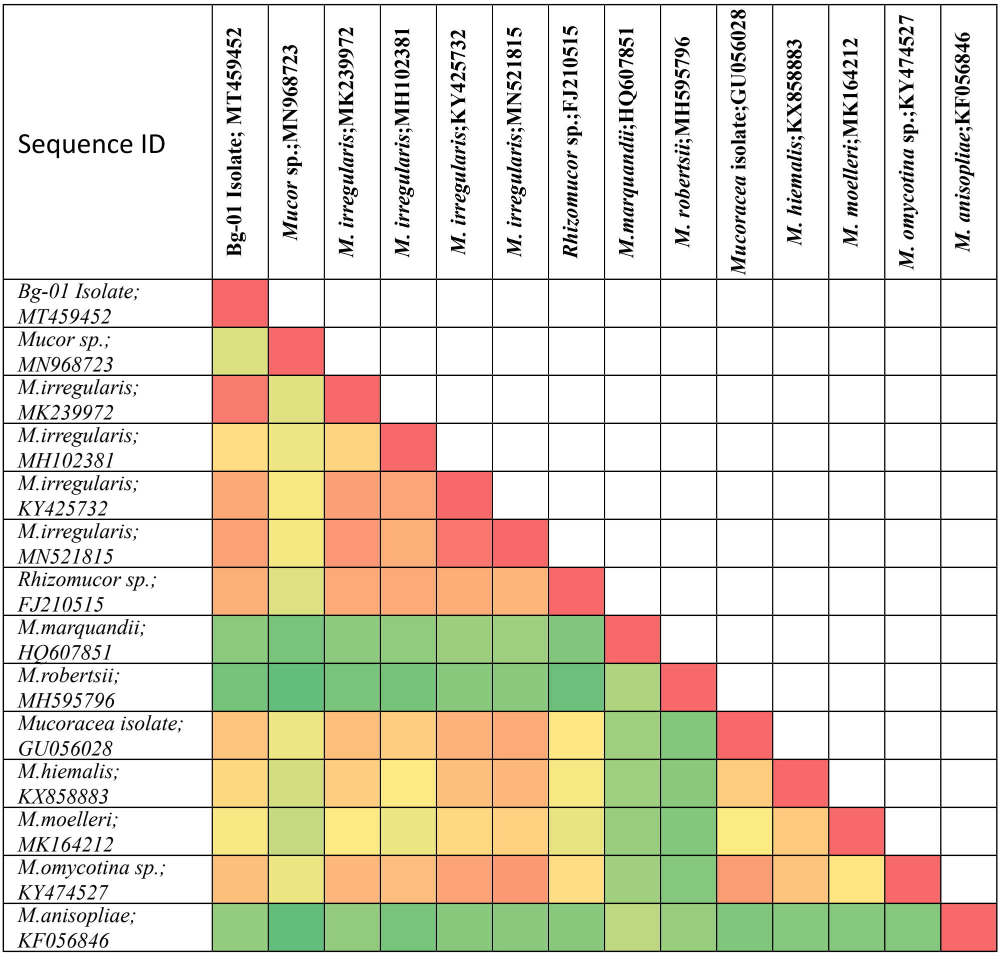
GenBank®. It can be concluded that the sequence data of BG-01 isolate; The MT459452 belongs to internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2 as the similar matching sequences, which also belongs to this region of fungi (Table 2). Of these most matching sequences, some of them have been reported as endophytes while some as biocontrol agents. *Mucor irregularis*-The MK239972 is found to be an endophytic fungi, which has nematcidal properties (Bechem *et al*., 2018). The *M. irregularis*-MH102381 has been identified as an endophyte of Drosera (Source: NCBI). *Mucor* sp. - MN521815 has been identified as an effective bio-control agent for black rot in sweet potato (Source: NCBI). *Rhizomucor* sp. - FJ210515 has been identified as a fungal-fungal association that affects the assembly of endophyte communities in maize (*Zea mays*). Uncultured Mucoraceae isolate - GU056028 has been defined under highly specific fungal culture in plant-ants. *Mucor hiemalis* - KX858883 has been defined under anti-microbial and insecticidal compounds from entomopathogens. *Mucor moelleri* - MK164212 has been defined under the presence and distribution of insect-associated and entomopathogenic fungi in a temperate pine forest soil (Deaver *et al.,* 2019).

Table 2. Selected most matching sequences available in the gene bank for the sequence of BG-01 isolate; MT459452

Description	Query Cover	E value	Identity (%)	Accession No.
M.irregularis	100%	0.0	100.00	MK239972
M.irregularis	100%	0.0	100.00	MH102381
M.irregularis	100%	0.0	100.00	KY425732
Mucor sp.	100%	0.0	99.63%	MN521815
M.irregularis	100%	0.0	96.49	MH766398
Mucor sp.	100%	0.0	100.00	MN968723
M.irregularis	100%	0.0	100.00	JN206155
M.irregularis	100%	0.0	99.26	MG269828
Metarhizium marquandii	26%	7e-44	87.59	HQ607851
M. robertsii	26%	3e-42	86.90	MH595796

The sequence data comparisons with the sequence data available at the NCBI GenBank® using the Clustal W programme of the BioEdit (version. 7.2.5) also revealed that the query sequence of this study, Bg-01 Isolate - MT459452 was mostly matching with *M. irregularis* species and *Rhizomucor* species and less with *Metarhizium* species (Figure 5).

The phylogenetic relationship of internal transcribed spacer sequence data of BG-01 isolates- MT459452 with the similar other sequence data also showed that BG-01 isolate was grouping with *M. irregularis* species, MN908723, MK239972, MH102381, KY425732, MN521815 and *Rhizomucor*- FJ210515 in one cluster. Some of these are entomopathogenic in nature as described above (Figure 6).



Scale	10	20	30	40	50	60	70	80	90	100
Colour										

Figure 5. Genetic identity matrix (%) of multiple alignment of the nucleotide sequences BG-01 isolate; MT459452 with matching sequences of NCBI database in Clustal W of bioedit (Version 7.2.5)

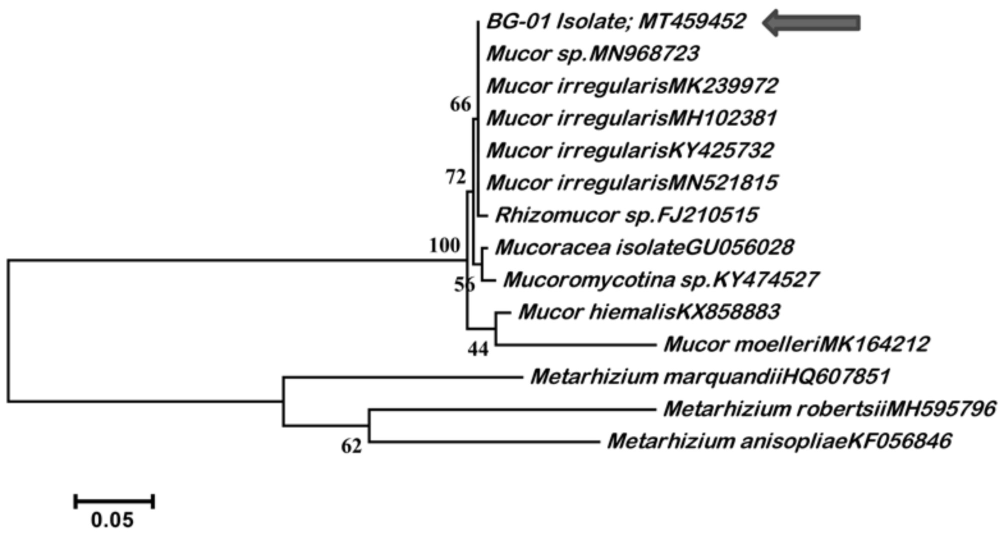


Figure 6. Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993). Evolutionary analyses were conducted in MEGA 6 (Tamura *et al.*, 2013)

Samson *et al.* (2010) described that the unique characteristics of fungi make it difficult for morphology-based identification and classification. Use of ITS region has been designated as primary DNA barcode for fungal kingdom and it is widely used for taxonomic purposes (Fajarningsih, 2016). The ITS region shows higher degree of variations compared to the other regions of rDNA (SSU and LSU) and therefore, the ITS region has been designated as the DNA barcode for fungal kingdom (Bellemain *et al*., 2010). The Internal Transcribed Spacer (ITS) region of the nuclear DNA (rDNA) has been the most sequenced region to identify fungal taxonomy at species level, and even within species (Nilsson *et al*., 2009). In this study, the ITS region of the isolated fungus BG-01 was amplified using a universal primer set. The sequence data of the internal transcribed spacer area of the fungal genome of BG-10 isolate (MTMT 459452) showed that the isolated fungal species were matching with *M. irregularis.* A very little information has been documented regarding *M. Irregularis* as an entomopthaogenic agent. In a previous study, Raja *et al*. (2017) reported two *Mucor* species isolated from the soils of Assam, North India using *Galleria mellonella* for trapping the soil borne entomopathogenic fungi. Bibbs *et al*. (2013) reported that the fungus *Mucor fragilis* isolated from a population of adult reproductive female brown widow spiders, *Latrodectus geometricus* Koch (Araneae: Theridiidae) infects and kills the *L. metricus* in North Central Florida. Some of the matching fungal sequences found on the GenBank® with the sequence data of this study are found to be from the fungus of entomopathogenic nature.

Results of this study support the hypothesis that isolated *M. irregularis* was responsible for the deaths observed in the BPH and this fungal species is pathogenic to BPH. The fungus (BG-01) was able to develop on BPH and kill the BPH under *in-vitro* condition and this information indicates that it is a natural entomopathogen. Further, the evidence presented in this study, indicates that the potentiality for developing a mycoinsecticide for the bio-control of BPH using the *M. irregularis* species isolated in this study. Meanwhile, studies are also needed to investigate the effect of this isolate on BPH, other insects and natural enemies under field conditions before scaling up on commercial level application of the technology.

Conclusion

The isolated fungus of the present study was identified as *Mucor irregularis* through morphological and molecular studies. The laboratory studies have shown that the tested isolate (BG-01) has the ability to grow on BPH causing significant mortalities to the insect. According to the available records of the country, this is the first finding of *M. irregularis* as a natural biocontrol agent for BPH in Sri Lanka and outside the country. These findings show the possibility of developing a myco-insecticide to control BPH as a new natural entomopathogen and it provides an environmental friendly solution to control BPH in rice cultivations in Sri Lanka and throughout the world.

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