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Morphological and Molecular Characterization of Entomopathogenic Fungi *Beauveria* spp. Bengkulu Isolate

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ABSTRACT

Beuveria is a genus of entomopathogenic fungi that plays an important role in controlling agricultural pests. Pest control with entomopathogenic fungi is an alternative to reduce influence the use of chemical insecticides. The diversity of entomopathogenic fungi intraspecies is by the genetic diversity of isolates, a high diversity of isolates can result diversity of fungus virulence. The purpose of this study was to perform the characterization molecular of entomopathogenic fungi *Beauveria* spp. Bengkulu isolate. Polymerase chain reaction (PCR) was performed for molecular identification *Beauveria* isolates were obtained from infected larvae in the field (1. Isolate from Coleoptera in Kepahiang, Bengkulu (BBC), 2. Isolate from Hemiptera: Pentatomidae in Rejang Lebong, Bengkulu (BBL), and 3. Isolate from Hemiptera: Alydidae in Taba Mulan, Merigi District, Kepahiang, Bengkulu (MT). The results of the amplification of the entomopathogen *Beauveria* samples produce DNA bands around ±580 bp. The DNA sequence analysis of ITS 5F and ITS 4R primers showed that the isolates BBC, MT and BBL have fairly high similarity with the *Beauveria bassiana* isolate 1397, *Beauveria bassiana* isolate SBI TNSPI, *Beauveria bassiana* strain B-Bug, *Beauveria bassiana* voucher TSJBB, *Beauveria bassiana* isolate SASRI C2, *Beauveria bassiana* isolate IMI 382764, *Beauveria bassiana* BCRC:FU31669, *Beauveria bassiana* isolate B098, *Beauveria bassiana* isolate INRS-CFL, *Beauveria* sp. JS-2009a isolate B4B, *Beauveria bassiana* isolate KACC with the homology of 99.83%, 99.83%, and 98.61%.

1. Introduction

Beuveria is a genus of entomopathogenic fungi that plays an important role in controlling agricultural pests. Pest control with entomopathogenic fungi is an alternative to reduce the use of chemical insecticides. Entomopathogenic fungi are important to control pest insects because they can cause epizootics and spread in ideal environmental conditions. *Beauveria* can infect pests from various orders including Coleoptera, Diptera, Embioptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Mantodea, Neuroptera, Orthoptera, Siphonaptera, and Thysanoptera (Chen *et al*. 2017; Kepler *et al*. 2017).

Chen *et al*. (2018) reported that eleven species of *Beauveria* have infected the order Coleoptera, including *B. bassiana* Vuill, *B. brongniartii* (Sacc.) Petch, *B. amorpha* (Höhn.), *B. asiatica* S.A. Rehner & Humber, *B. caledonica* Bissett & Widden, *B. malawiensis* S.A. Rehner & Aquino de Muro, *B. pseudobassiana* S.A. Rehner & Humber, *B. sungii*

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S.A. Rehner & Humber, *B. varroae* S.A. Rehner & Humber, *B. lii* Sheng L. Zhang & B. Huang, *B. hoplocheli* I. Robène-Soustrade & S. Nibouche, dan *Beauveria majiangensis*.

Beuveria bassiana Vuill. is the most widely used and commercially available species as a mycoinsecticide to control many agricultural pests (Zimmermann 2007), and *B. brongniartii* (Sacc.) Petch is used for control the ground beetles (Robène-Soustrade *et al*. 2015). Mycoinsecticides and mycoacaricides with the most formulations were *B. bassiana* (33.9%) and *B. brongniartii* (4.1%). Other species of the genus *Beauveria* have also been shown to have great potential for use in the management of various insect pests (Imoulan *et al*. 2016a). Apriyanto and Nadrawati (2019) reported that the mortality of *Hyphotenemus hampei* Ferrari was higher 80.6% with the application of *B. bassiana* isolate Bengkulu was isolated from *Cylas formicarius* compared to isolated from *Nezara viridula* (76.7%).

Intraspecies diversity of entomopathogenic fungi is influenced by the genetic diversity of isolates, and high diversity of isolates can result in fungal virulence diversity. *B. bassiana* isolates from Bengkulu used for pest control have only been identified based on their morphological characteristics. Morphological characters have weaknesses because they only show dominant and recessive inheritance traits, the level of polymorphism are small and strongly influenced by the environment. Similarity at the phenotype does not necessarily indicate similarity at the DNA level. Differences between species relate to DNA sequences. Based on this, morphologically identified *B. bassiana* needs to be equipped with molecular identification data. The research aim to find the characterization molecular of entomopathogenic fungi *Beauveria* spp. Isolate Bengkulu. The results of this study are expected to provide information on the genetic diversity of *Beauveria* spp. Bengkulu isolates and their similar to the *Beauveria* species.

2. Materials and Methods

2.1. Research Sites

The *Beauveria* isolate used was obtained from infected larvae in the field and cultured on Potato dextrose Agar (PDA) media: 1. Isolate from Coleoptera: Brentidae (Kampung Bogor, Kepahiang District, Kepahiang Regency with coordinates 3°37'16.56512"S 102°35'19,09133E, Bengkulu, an altitude of 659 m above sea level 2. Isolate from Hemiptera: Pentatomidae (Pall V, Bermani Ulu Raya District, Rejang Lebong Regency with coordinates 3°27'19,09008" S 102°37'12.02304E, with an altitude of 800 m asl.), and 3. Isolate from Hemiptera: Alydidae (Taba Mulan, Merigi District, Kepahiang Regency, Bengkulu, Indonesia), with coordinates 3°29'52,15229"S 102°30'23,7793E, with an altitude of 610 m above sea level.

Morphological identification of entomopathogenic fungi was carried out at the Plant Protection Laboratory, Faculty of Agriculture, Bengkulu University, and molecular identification at the Molecular Biology Laboratory, Faculty of Mathematics and Natural Sciences, Bengkulu University. This research was conducted from January to July 2024.

2.2. Morphological Characterization of *Beauveria* spp. Isolates

Morphological identification of the fungus observing at the morphological characters possessed by each isolate (Humber 1997). Morphological observations were carried out macroscopically by observing the growth of fungal isolate colonies on Potato Dextrose Agar (PDA) media in Petri dishes which is including colony color, colony shape, colony texture, and colony edge shape. Observations with an optical microscope included the shape of conidia and hyphae.

2.3. Molecular Characterization

2.3.1. DNA Extraction and Sequencing

Entomopathogenic fungi were isolated from infected larvae and cultured on Potato dextrose Agar (PDA) media. Pure cultures were transferred to PDA media and incubated at 25°C for 1-2 weeks, then all mycelia from each sample were frozen in liquid nitrogen before being crushed with a mortar and pestle. About 50 mg of crushed mycelium was used for DNA extraction.

DNA extraction was carried out with Cetyl-Trimethyl Ammonium Bromide (CTAB) (Doyle & Doyle 1990). Isolated DNA was stored at -20°C until use. The fungal isolate sequences were amplified using ITS5 (5-GGAAGTAAAAGTCGTAACAAGG-3) as the forward primer and ITS4 (5-TCCTCCGCTTATTGATATGC-3) as the reverse primer (White et al. 1990). The PCR reaction was carried out in a volume of 40 µl, consisting of 4 µl template, 2 µl of each primer, 4 µl of 10 × Taq DNA polymerase buffer, 4 µl dNTP, 1 µl Taq DNA polymerase, and 23 µl sterile distilled water. The PCR conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles

of 95°C for 1 minute, 55°C for 55 seconds and 72°C for 2 minutes; and extension at 72°C for 10 min. PCR conditions were adapted according to the study of Rehner and Buckley (2005). The PCR products were separated on 1.0 % agarose gel, stained with ethidium bromide, and viewed under UV light. After being purified, the PCR product was then sequenced. Sequencing of amplified DNA fragments was carried out by 1st BASE Singapore through PT. Genetics Science. The sequences obtained were subjected to a BLAST search using the NCBI GenBank database for comparison with other Beauveria species. Finally, these sequences were used for genomic analysis using the representative sequences described by Rehner and Buckley (2005) to determine the taxonomic position of the Beauveria isolates of Bengkulu.

2.4. Data Analysis

The nucleotide sequence of the sample *Beauveria* spp. (BBC, MT, and BBL) were compared with the nucleotide sequences of other *Beauveria* species published on the National Center for Biotechnology Information (NCBI) Gen Bank website through the BLAST (Basic Local Alignment Search Tools) program. Nucleotide sequence data that have similarities were analyzed using the alignment program and Clustal W with the Bioedit ver 7.1.7 program to determine the nucleotide homology of the sample. Phylogenetic analysis was carried out based on the Neighbor-Joining, Bootstrap 1000× approach with the MEGA-6 program (Tamura *et al*. 2011).

3. Results

Genome amplification of the entomopathogenic fungus *Beauveria* spp. using the PCR method with ITS 5F and ITS 4R primers resulted in DNA fragments measuring about ± 580 bp (Figure 1). DNA sequencing of *Beauveria* spp. carried out to determine the percentage of similarity of isolates based on ITS 5F and ITS 4R primers. The identity of a gene whose sequence is known is determined by comparing it with the sequence data contained in the Gen Bank, the results of the alignment analysis using bio edit software showed that the isolates BBC, MT and BBL have fairly high similarity with the *Beauveria bassiana* isolate 1397, *Beauveria bassiana* isolate SBI TNSPI, *Beauveria bassiana* strain B-Bug, *Beauveria bassiana* voucher TSJBB, *Beauveria bassiana* isolate SASRI C2, *Beauveria bassiana* isolate IMI 382764, *Beauveria bassiana* BCRC:FU31669, *Beauveria bassiana* isolate B098, *Beauveria bassiana* isolate INRS-CFL, *Beauveria* sp. JS-2009a isolate B4B, *Beauveria bassiana* isolate KACC with the homology of 99.83%, 99.83%, and 98.61%. (Table 1).

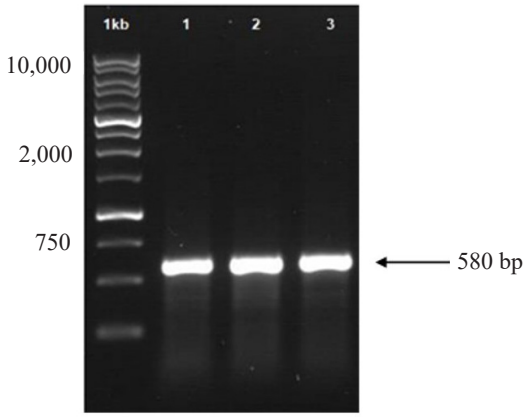

Figure 1. PCR amplification of the gene for the entomopathogenic fungus *Beauveria* spp. using ITS 4R primer and ITS 5F primer; M = marker 1 Kb ladder; 1= BBC isolate, 2= BBL isolate, and 3 = MT isolate

Table 1. Alignment results of ITS Isolate BBC, BBL, and MT gene sequences against data available at NCBI (BLASTX)

Isolate name	Description	Max score	Query cover (%)	E value	Identity (%)	Accession number
BBC	*Beauveria bassiana* isolate 1397	1053	100	0.0	99.83	MZ151180.1
	Beauveria bassiana isolate SBI_TNSPI	1053	100	0.0	99.83	PQ237728.1
	Beauveria bassiana strain B-Bug	1053	100	0.0	99.83	MK862359.1
	Beauveria bassiana voucher TSJBB	1053	100	0.0	99.83	KF937310.1
MT	*Beauveria bassiana isolate* SBI_TNSPI	1053	100	0.0	99.83	PQ237728.1
	Beauveria bassiana isolate SASRI C2	1053	100	0.0	99.83	JX110371.1
	Beauveria bassiana isolate IMI 382764	1053	100	0.0	99.83	AJ560683.1
	Beauveria bassiana BCRC:FU31669	1053	100	0.0	99.83	LC768985.1
BBL	*Beauveria bassiana isolate* B098	935	100	0.0	99.61	ON417453.1
	Beauveria bassiana isolate INRS-CFL	935	100	0.0	99.61	EU334674.1
	Beauveria sp. JS-2009a isolate B4B	935	100	0.0	99.61	GQ354257.1
	Beauveria bassiana isolate KACC	935	100	0.0	99.61	MG833297.1

3.1. Phylogenetic Tree

Nucleotide sequence phylogeny tree construction using the Neighbor-Joining (NJ) method. The construction of the phylogeny tree shows the formation of clusters. BBC, MT and BBL isolate, were in the same cluster with *Beauveria bassiana* isolate 1397, *Beauveria bassiana* isolate SBI TNSPI, *Beauveria bassiana* strain B-Bug, *Beauveria bassiana* voucher TSJBB, *Beauveria bassiana* isolate SBI_TNSPI, *Beauveria bassiana* isolate SASRI C2, *Beauveria bassiana* isolate IMI 382764, *Beauveria bassiana* BCRC:FU31669, *Beauveria bassiana* isolate B098, *Beauveria bassiana* isolate INRS-CFL, *Beauveria* sp. JS-2009a isolate B4B, *Beauveria bassiana* isolate KACC, while *Akanthomyces aculeatus* strain TS772 was in separate clusters (out-group) (Figure 2).

The three isolates from Bengkulu were identified as *Beauveria* sp. The main characteristic of the fungus *Beauveria* sp. is conidiophores branched with a zig-zag and conidia are formed at the ends. Single-celled conidia are round or oval, hyaline. The hyphae are insulated and branched, and the mycelia are white or pale yellow, in the form of fine threads, and look like cotton or chalk.

4. Discussion

The ITS area was tested to differentiate isolates of entomopathogenic fungi of insect origin because according to Schoch *et al*. (2012) ITS is an area proposed for barcoding fungi. This region has a high level of variation (less conserved) compared to the small subunit and large subunit rDNA regions (Iwen *et al*. 2002). The *B. bassiana* culture amplicon using ITS4/ITS5 is approximately ±580 bp (Figure 1). The research results of Sevim *et al*. (2010) reported that the DNA band size of *Beauveria* spp. around 567 bp. This is in accordance with Rosmeita and Sari (2019) Primers ITS1/ITS4 has successfully amplified the internal transcribed spacer region from *B. bassiana* at the size around 600 bp.

Molecular identification using ITS1/ITS4 and ITS5/ITS4 markers of three fungal cultures associated with dead insects, showed that culture 1 = BBC isolate, 2 = BBL isolate, and 3 = MT isolate was *Beauveria* sp. (Figure 1). In general, the molecular identification results are supported by the morphological identification results (Figures 3 and 4). Phylogenetic analysis of the sequences of the three isolates was *Beauveria bassiana* with a fairly large bootstrap value of 99% (Figure 2). However, ITS4 and ITS5 cannot separate other species which are *Beauveria*. According to Rehner and Buckley (2005), the elongation factor 1 alpha (EF1-a) gene is much more informative for determining kinship relationships in the genus *Beauveria* compared to ITS. Intersimple sequence repeat (ISSR) can also be used to investigate genetic diversity in the fungi *Beauveria* spp. Several fungi of the *Beauveria* genus that have been successfully identified using ISSR are *B. bassiana*, *B. brogniartii*, *B. amorpha*, and *B. velata* which were

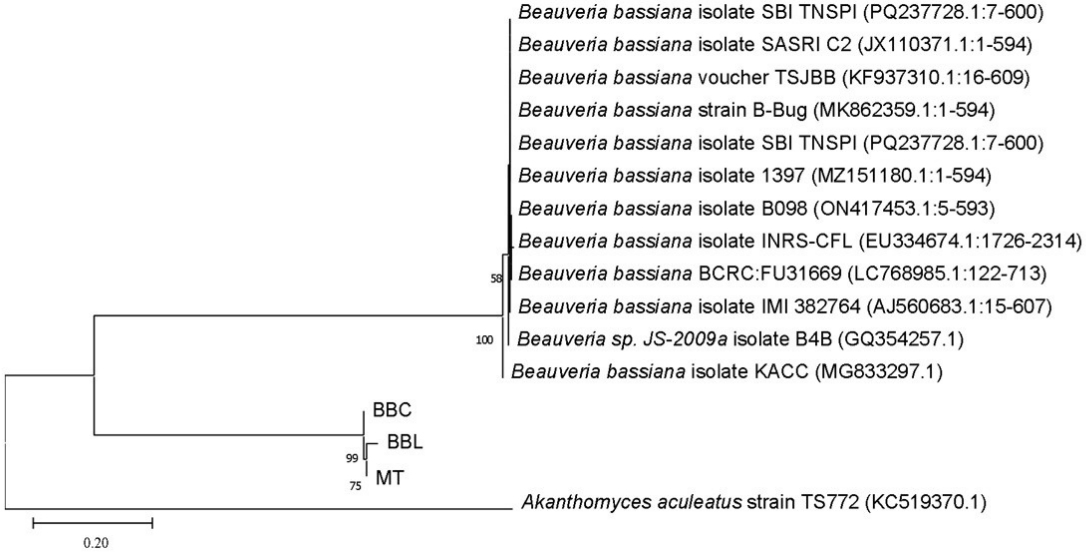

Figure 2. Phylogenetic tree depicting the proximity of isolates, BBC, BBL, and MT to other entomopathogenic fungi in one clade. Construction based on the Neighbor-Joining Tree method with a bootstrap value of 1000× replications

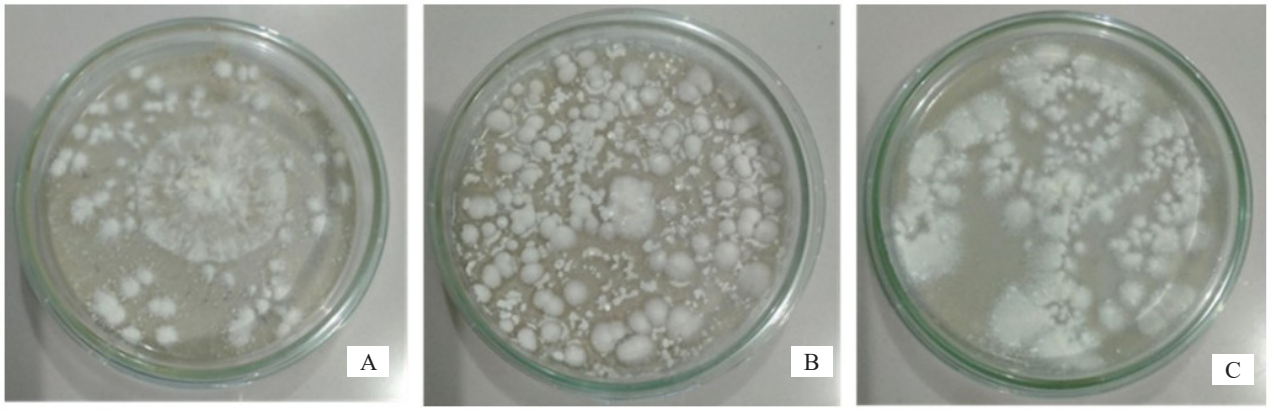

Figure 3. Colonies of *Beauveria* sp. Bengkulu isolate, (A) Isolate BBC: from Coleoptera: Brentidae in Kepahiang, Bengkulu, (B) Isolate BBL: from Hemiptera: Pentatomidae in Rejang Lebong, and (C) Isolate MT: from Hemiptera: Alydidae in Taba Mulan, Merigi District, Kepahiang, Bengkulu

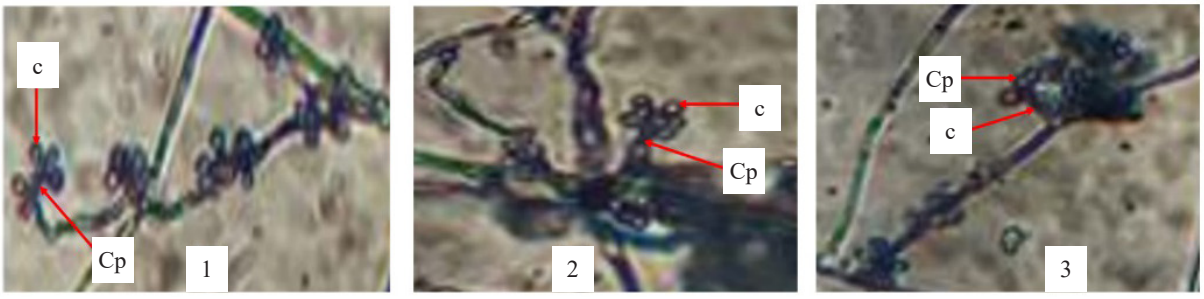

Figure 4. Conidiophore (Cp) and conidia (c) *Beauveria* sp. Bengkulu isolates using an Olympus BX51 microscope with magnification using bar scale instead of 40× (40×10). 1. Isolate BBC: from Coleoptera: Brentidae in Kepahiang, Bengkulu, 2. Isolate BBL: from Hemiptera: Pentatomidae in Rejang Lebong, and 3. Isolate MT: from Hemiptera: Alydidae in Taba Mulan, Merigi District, Kepahiang, Bengkulu

isolated from different host insects. Studies show that ISSR can be used as a powerful molecular marker for entomopathogenic fungi (Wang *et al*. 2005).

Microscopic observation of the culture morphology of the three isolates showed a picture of *Beauveria*, with oval, slightly rounded conidia, conidia attached to the tips and sides of the conidiophores (Figures 4). *Beauveria* is a cosmopolitan species of arthropods, in the form of asexual reproductive structures and is pathogenic to insects. *Beauveria* plays an important role in the biological control of insect pests on plants or known as mycoinsects, for example *B. bassiana* and *B. brogniarti* (Rehner *et al*. 2011).

Based on the morphological characteristics of *Beauveria* sp. is *Beauveria bassiana*. The main characteristic of the fungus *Beauveria bassiana* is conidiophores branched with a zig-zag and conidia are formed at the ends (Figure 4). Single-celled conidia are round or oval, hyaline, and appear at each branching end of the conidiophores. The hyphae are insulated and branched, and the mycelia are white or pale yellow, in the form of fine threads, and look like cotton or chalk (Chen *et al*. 2013).

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