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Abstract. The endophytes are microorganisms that colonize plant tissue without causing infections. One of endophytic microorganisms, fungi, comprises a reliable source of genetic diversity and has been applied in pharmacology. In this study, the endophytic fungi was isolated from fruit and seed of *Syzygium cumini* and identified based on ITS-rDNA. Five fungal isolates were obtained from fruit and seed of Jambolana, two isolates from fruit and three isolates from seed. Based on ITS-rDNA analysis showed that that endophytic fungus isolates from jambolana (*S. cumini* L.) Skeels was identified as *Neofusicoccum parvum* (F1 isolates), *Pestalotiopsis vismiae* (F2 isolates), *Phomopsis* sp. (S1 and S3 isolates), *Colletotrichum fructicola* (S2 isolates). All the isolates are belonged to Ascomycota.

Keywords: Endophytic, fungi, identification, ITS-rDNA, Jambolana

1. Introduction

Endophytic fungi colonize plant tissues internally without causing apparent symptoms of infections [1,2]. They have been found in many different plants, one of them is Jambolana (*S. cumini* L.) Skeels. Endophytic microorganism comprises a reliable source of genetic diversity, a source of organic compounds and has been applied in pharmacology [3]. One strategy to optimize the benefits of endophytic fungi is isolation of this fungus from the host plant [4]. Jambolana (*S. cumini* L.) Skeels is a plant that has many benefits. All organs of jambolana can be used as a medicinal plant. The fruit of jambolana is used as an antioxidant [5], anticancer [6], hyperlipidemia [7]. Other organs can be used as a medicinal plant is seed. The benefits of jambolana seed are used as antidiabetic, antioxidants [8], anti-inflammatory [9]. Morphological identification of endophytic fungi has been done. However, morphological identification has disadvantages including a long time needed and error resulted in closely related species. Therefore, the identification of molecular must be done. Molecular identification of species takes quick and precise results and accurate identification. Molecular identification in this study used the identifier code Internal transcribed spacer region (ITS) of ribosomal DNA. According to Vicente [10], the selection of ribosomal DNA for the purpose of identification of an organism based on: (i) the nature of the homologous on a variety of different organisms, (ii) there are many in the cell, (iii) the sequence range from 500-800 bp to allow a statistical test to see the difference each other. In this study, we characterized endophytic fungi from healthy Jambolana (*S. cumini* L.) Skeels in Dampit, Malang by morphology and molecular analysis.

2. Methods

2.1. Plant materials

Matured *S. cumini* fruits were collected as plant material on December 2017 in Dampit region of Malang, East Java, Indonesia. After selection, the fruit was excised with a sterile scalpel and placed in sterile plastic bags, storage at 4 °C until it was used. Fruit and seed of *S. cumini* were used for further investigation of endophytic fungi.

2.2. Isolation of fungal endophyte

Fungal endophytes from the fruit of *S. cumini* were isolated using a direct inoculation modified method by Ma [11]. Healthy tissues of fruit were cut into 1 cm. For surface sterilization, the sample was washed with running water for 10 mins, soaked in 70% alcohol for 2 mins, soaked twice for a minute in distilled water, rinsed with 53% NaOCl for 5 mins, and again soaked twice for a minute in distilled water. The result of the last soaking distilled water was taken 0.1 mL and poured into a petri dish containing PDA medium as a control. Four pieces from fruit were placed on PDA medium supplemented with streptomycin as an antibiotic and incubated at room temperature for 3 – 7 days. The isolation of endophytic fungi in seed was based on the dilution method [12]. The healthy tissues of *S. cumini* seed were weighed 2.5 g, washed with running water for 10 min, soaked in 70% alcohol for 2 mins, soaked twice for a minute in distilled water, soaked in 53% NaOCl for 5 mins, soaked twice for a minute in distilled water. The result of the last soaking distilled water was taken 0.1 mL and poured into a petri dish containing PDA medium as a control. Seeds sterile were pounded in mortal sterile and put into 225 mL of PDB medium. The medium was shaken at room temperature for 72 hrs. After incubation, the PDB medium was taken 0.3 mL and poured on PDA plate. The medium was incubated at room temperature for 3 – 7 days.

2.3. Identification of endophytic fungi

2.3.1. Morphological examination. The fungal morphology was identified based on a microscopic and macroscopic characteristic. Microscopic identification was based on slide culture method [13]. PDA medium was cut 5 mm square; the square agar was picked up carefully; this agar was transferred to the centre of the slide, the four sides of the agar square were inoculated with spores or mycelial fragments of the fungus to be examined, the cover glass was placed on the petri dish, incubated at room temperature for 48 hrs. After incubation, the cover glass was taken care and poured with Lactophenol Cotton Blue (LCB). Macroscopic and microscopic identification was based on an identification guide book by Barnet and Hunter [14]. It included hyphae, hyphae form, conidia shape, spore size, colonies upper surface, reverse surface, type of concentric, hyphae color, type of hyphae, hyphae shape.

2.3.2. Molecular examination. Total of genomic DNA was extracted directly from pure cultures following Doyle & Doyle [15] with modifications. 100 mg of mycelium were moved in the 2 mL tube, 1000 mL 2X CTAB buffer added. The mycelium was incubated at 65 ºC for 60 mins, added with 900 mL (24:1) chloroform: isoamyl alcohol, incubated at room temperature for an hour, centrifuged at 13000 rpm for 10 mins. After centrifuge, the supernatant was moved in 1.5 mL tube, chloroform: isoamyl alcohol (24:1) 1x volume was added, centrifuged at 13000 rpm for 10 mins. After centrifugated, the supernatant was added with Isopropanol 2/3 volume of supernatant. The reaction mixture was incubated at -4 ºC for overnight, centrifuged at 13000 rpm for 10 minutes, discarded the supernatant. The pellets were washed with 500 mL of absolute ethanol, centrifuged at 13000 rpm for 5 minutes, discard supernatant. The pellets were dried at 25 °C in the oven. The pellets were added with 50µL TE Buffer and Kept at -4 ºC. The quality of the DNA was determined by gel electrophoresis in 1% (v/v) agarose gel. The DNA concentration and purity were determined by Nanodrops AE-Nano200 Nucleic Acid Analyzer version 2.0. The concentration was recorded in ng/IL and the purity of DNA was based on the ratio of optical density (OD) at the wavelength of 260 and 280 nm. Polymerase Chain

Reaction (PCR) amplification was used to amplify the ITS rDNA: ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) [16], the composition of PCR amplification, DNA template (1 mL), forward primer (1 mL), reverse primer (1 mL), PCR Mix Sigma Readmix™ REDTaq™ (7 mL), aqua bidest (15 mL). Cycling reactions were run on My Cycler™ Cycler BIO-RAD thermocycler with the following protocol, Pradenaturasi at 95 ºC 15', denaturation at 95 °C 1', annealing at 56 °C 30", elongation 72°C 1' 35 cycles, post elongation 72 ºC 10'. A sequence of the PCR products was performed by the service of 1st BASE sequence DNA Sequencing Services Singapore.

2.3.3. Phylogenetic Analysis. The sequences were matched in the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) [17]. The ITS sequence was contig with Bioedit version 7.2.5., aligned with the ClustalX program. FASTA alignment results were imported to MEGA 5.0 program for the reconstruction of the phylogenetic relationship of the endophytic fungi. The Neighbor-Joining (NJ) phylogenetic tree was constructed from an evolutionary distance by MEGA 5.0 software. The bootstrap was 1000 replications to assess the reliable level to the nodes tree.

3. Results and Discussion

Endophytic fungi were isolated from fruits and seed of jambolana (*S. cumini* L.) Skeels. Based on the purification step, a total of two isolates of endophytic fungi from fruit organ (code of isolates are F1 and F2) and three isolates from seed organ (code of isolates were S1, S2 and S3). The description of cultural and morphological characteristics of fungal endophytes, microphotographs of morphological structure of the species are shown in Table 1, Figure 1.

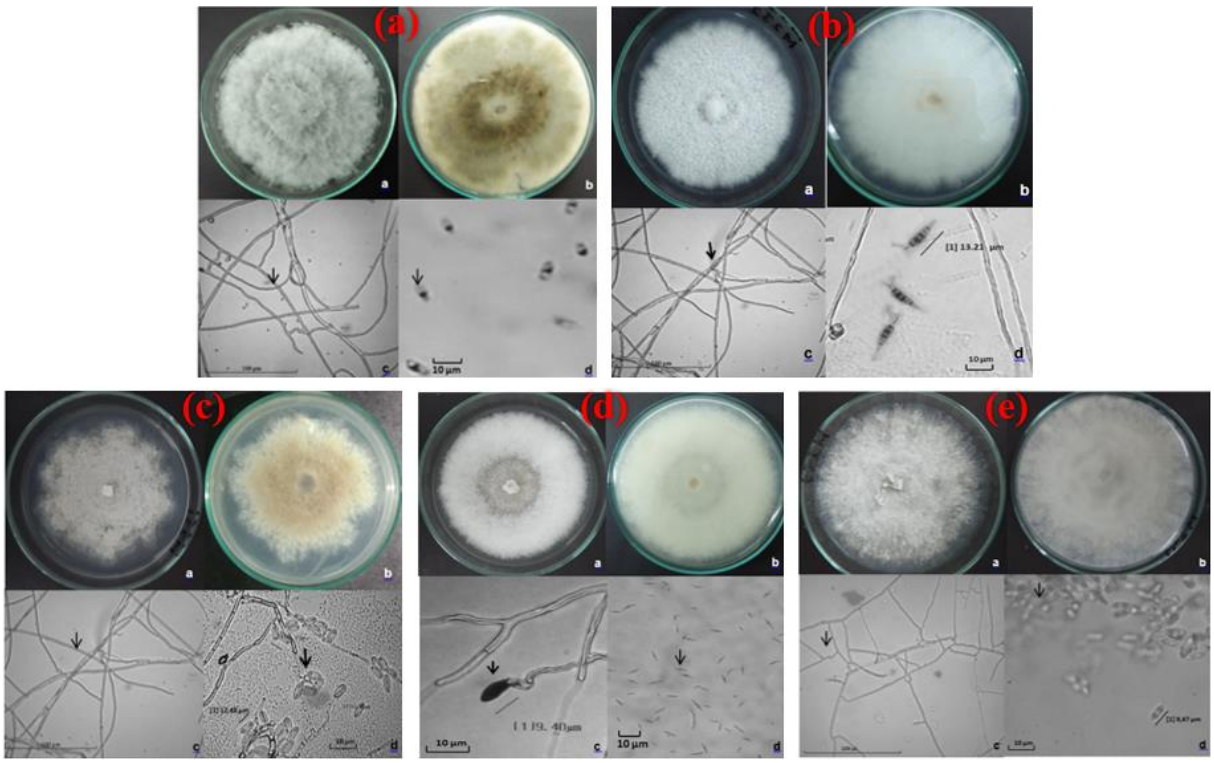

Figure 1. The morphology (colony appearance, hypha and conidia) of endophytic fungi from jambolana (*S. cumini* L.) Skeels. Fungal isolates code F1 (A), F2 (B), S1 (C), S2 (D), and S3 (E). Colonies on PDA after 7 days at 25 ºC each isolate, upper, and reverse side (a-b), generative hyphae (c) and conidia each isolates(d) (scale bars 100 µm (c) and 10 µm (d))

The colony of endophytic fungi from jambolana (*S. cumini* L.) Skeels growth moderately fast at 25 ºC. F1 isolates the most fast growing culture among other isolates. The fungal colony was whitish to blackish gray (F1 isolates), brown (S3 isolates) or changes to orange for old mycelium (S2 isolates). Textures were smooth and rough, cottony mycelium. Conidia varied in size and shape, a cylindrical and elliptical shape, the largest conidia was 13-16 µm in size (F2 isolates).

Table 1. Cultural characteristics of fungal strains from jambolana (*S. cumini* L.) Skeels

Characters	**Fruit**		**Seed**		
Morphology	**F1 isolates**	**F2 isolates**	**S1 isolates**	**S2 isolates**	**S3 isolates**
Colonies texture upper surface	The texture of smooth colonies looks like cotton,	Rough surface texture, aerial mycelia looks like cotton,	Rough surface texture, the mycelium is thin,	Smooth surface texture, looks like cotton,	Average surface texture, the mycelium is thin,
Colonies color upper surface	colored light gray -blackish gray.	white, on the sixth day of mycelium turns into amber.	light brown to dark brown.	white color, on the tenth-day changes to orange.	white colony color changes to brown.
Colonies color reverse side	brown - changes to dark gray.	white turns into a yellowish brown color.	beige on white	white turns into light brown color	white color to yellowish
Size of culture colonies	9 cm on the fifth day	6.5 cm on the seventh day	5.3 cm on the seventh day	6 cm on the seventh day	6 cm on the seventh day
Concentric circles	shown	-	shown	shown	shown
Hyphae	Insulated, branched	Not insulated, branched	Insulated, branched	Insulated, branched Appressorium with the blunt end.	Insulated, branched
Conidia	8-11 µm. elliptical shape, rounded apex flat, no insulation	13-16 µm, an elliptical shape, has 2 setulae and 1 pedestal.	α-conidia 7-15 µm, the elliptical shape, the blunt end, hyaline	3-10 µm, cylindrical shape with a blunt tip, hyaline.	7-15 µm, the elliptical shape, the blunt end, hyaline

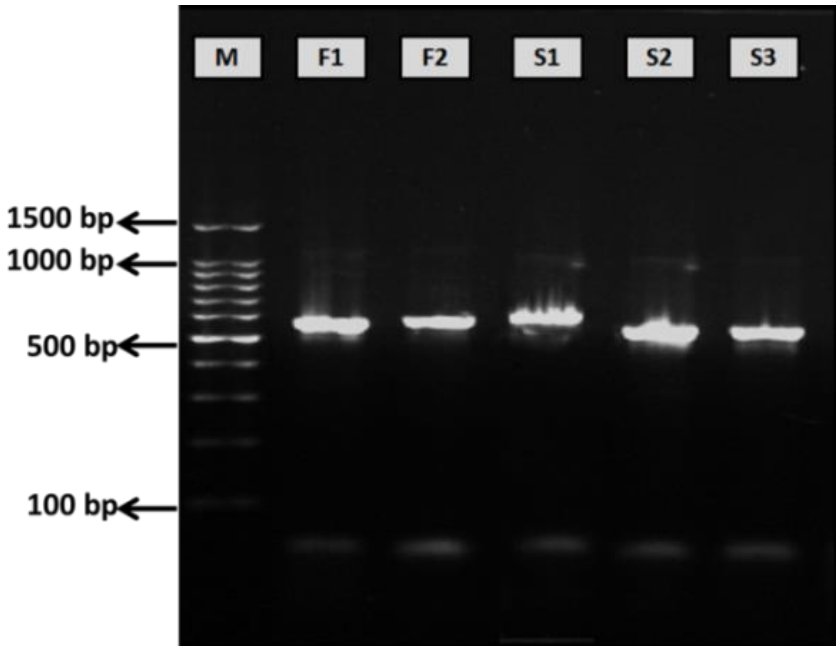

Figure 2. The visualization of amplification PCR (100 volts, 30 mins). (M) marker, (F1) F1 isolates, (F2) F2 isolates, (S1) S1 isolates, (S2) S2 isolates, (S3) S3 isolates

The high-quality DNA extracted from endophytic fungi used ITS1 and ITS4 as a primer for PCR. The genomic DNA of the fungi that successfully amplified F1 and F2 isolates had a length of 550 bp; S1 isolate had a length of 600 bp, while the S2 and S3 isolate had a length of 500 bp (Figure 2). According to Porter [18], the ITS region in the fungi kingdom had an average length of 500-600 bp to Ascomycetes and Basidiomycetes.

Table 2. NCBI Blast results

Isolates Code	Species	Identity (%)	Sequence ID
F1	*Neofusicoccum parvum*	99	KY111851.1
F2	*Pestalotiopsis vismiae*	99	KM513583.1
S1	*Phomopsis* sp.	99	GU066650.1
S2	*Colletotrichum fructicola*	99	MF543120.1
S3	*Phomopsis* sp.	97	GU066650.1

The results were in F1, F2, S1, S2 with > 98% similarity or "maximum identity", but in S3 isolate was 97% (Table 2). F1 Isolate was 99% identical to a *Neofusicoccum parvum* (KY111851.1), F2 isolate was 99% identical to a *Pestalotiopsis vismiae (*KM513583.1), S1 isolate was 99% identical to a *Phomopsis* sp. (GU066650.1), S2 isolate was 99% identical to a *Colletotrichum fructicola* (MF543120.1), and S3 isolate was 97% identical to a *Phomopsis* sp. (GU066650.1).

The five isolates were found as endophytic fungi in Jambolana, but in other plants as pathogenic or saprophytic fungi. Some of the same species fungi in host plant can be endophytic, pathogenic, or saprophytic fungi [19]. *Neofusicocccum parvum* was found in *Artemisia madagascariense* leaves [20] and found in *Artemisia thuscula* stem as endophytic fungi, but in *Vitis vinifera* leaves as pathogenic fungi [21]. *Pestalotiopsis vismiae* was found in *Pinus armandi* bark as endophytic fungi [22], but found in *Leucospermum* sp. leaves as pathogenic fungi [23]. *Colletotrichum fructicola* was found in *Pennisetum purpureum* leaves as endophytic fungi [24] but found in *Capsicum annuum* fruit as

pathogenic fungi [25]. *Phomopsis vexans* was found in *Solanum xanthocarpum* leaves as endophytic fungi, but in *Solanum melongena* as pathogenic fungi [26].

Endophytic fungi were beneficial to host plants. First, some endophytic fungi could increase the growth of their host plant through the hormones produced [27]. Second, some endophytic fungi would produce different bioactive compounds to increase the resistance to biotic and abiotic stresses of their host plants [28]. Thirds, some endophytic fungi could promote the accumulation of secondary metabolites (including essential medicinal components or drugs) produced initially by host plants [29].

Phylogenetic analysis based on the ITS region identified four genera belonging Ascomycota, with Pestalotiopsis and Phomopsis as the most prevalent endophytes. The results showed five different species of fungi were successfully identified and was confirmed through neighbor-joining tree using Molecular Evolutionary Genetics Analysis (MEGA) (Figure 3). All the isolates from fruit and seed host plants could be classified as Ascomycota. Based on the results of phylogenetic tree reconstruction, the obtained four clades in the fifth there clade C isolates of endophytic fungi isolated from fruits and seeds of jambolanana - F1 isolates were derived from the Botryosphaeriaceae family. Bootstrapping value F1 isolates was 99% so categorized branching phylogenetic tree is stable and will not change. F2 isolates were derived from the Sporocadaceae family, bootstrapping value F2 isolates was 95%. S2 isolates were derived from the from Glomerellaceae family, bootstrapping value S2 isolates was 100%. S1 and S3 isolates were derived from the from Diaporthaceae family, bootstrapping value S2 isolates was 100%.

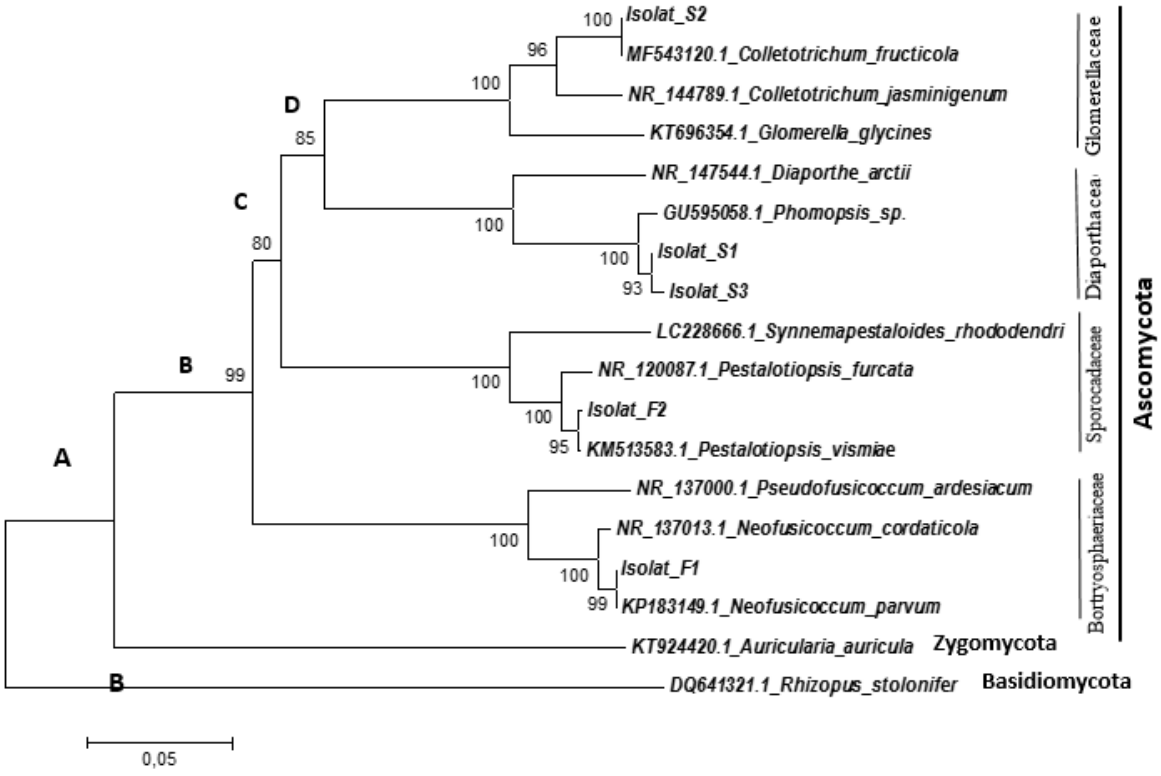

Figure 3. The reconstruction of the phylogenetic trees of endophytic fungi isolated from fruits and seeds jambolana (*S. cumini* L.) Skeels from Neighbor-Joining analysis. Bootstrap values from 1000 replications as numbers above the nodes.

However, molecular analysis alone also has limitations, such as the problem of over-isolating fast growing fungal species at the expense of slow-growing taxa, nor isolating species that will not grow in culture [30]. The use of ITS sequences also has limitations in phylogenetic analysis because the

noncoding ITS sequence is fast evolving with many variable characters, it is usually difficult to achieve a perfect sequence alignment at high taxonomic levels. The examples are S1 species and S3 species that have differences not only in BLAST value but also on morphology characterization. However, in the reconstruction of phylogenetic, both species were in the same branch. Further studies using different gene sequences can be conducted to resolve this type of difficulties in the fungi phylogenetic analysis.

4. Conclusion

ITS-rDNA sequence analysis showed that endophytic fungi isolates from jambolana (*S. cumini* L.) Skeels were identified to be *Neofusicoccum parvum* (F1 isolates), *Pestalotiopsis vismiae* (F2 isolates), *Phomopsis* sp. (S1 and S3 isolates), *Colletotrichum fructicola* (S2 isolates).

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