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Molecular Identification of Endophytic Fungi from Local Rice and Growth Test on Several Types of Culture Media

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ABSTRACT

Local rice is rice that has been cultivated for generations by the community and commonly cultivated without using chemical inputs. Endophytic fungi are fungi that live in the plant tissue and does not cause disease symptoms in the host plants. This study aimed to molecular identifying isolates of MDTA and MDTB endophytic fungi which have been isolated from the local Pulu Mandoti rice plant tissue and growth test on the four types of culture media those were synthetic PDA, natural PDA, MPA, and MEA. The fungi DNA isolation using DNesay Kit. DNA sequencing analysis using the mega BLAST program showed that the MDTB fungus has similarities to *Podoscypha bolleana* strain 32034 no accession JQ675334 and *Podoscypha bolleana* strain 32032 no accession JQ675332, whereas the MDTA fungus has similarities to Coprinopsis cinerea A2S3-5 isolate and *Coprinopsis cinerea* strain CNRMA / F 07-32. The best culture media and sporulation of endophytic fungi is MPA media. This research is the first study to molecular identifying with endophytic fungi from local rice and viability test on the four types of culture media. The results of this study contribute to the diversity of local rice endophytic fungi in Sulawesi.

Keywords:
Coprinopsis cinere; culture media; Podoscypha bolleana; Sporulation

1. Introduction

Endophytic fungi are microorganisms that live in plant tissues without causing symptoms of damage to plants (Hilarino *et al.*, 2011; Afandhi *et al.*, 2018). Endophytic fungi have been isolated from several types of plants such as: 1) Apple (*Malus domestica*) (Afandhi et al., 2018); 2) Orchid (*Cymbidium aloifolim* L) (Shubha & Srinivas, 2017); 3) medicinal plant (*Hedychium flavescens* and *Hedychium coronarium*) (Uzma, Konappa, & Chowdappa, 2016); 4) medicinal plant (*Asclepias sinaica*) (Fouda, Hassan, Eid, & Ewais, 2015); 5) Medicinal Plant (*Adhatoda vasica* Nees, *Coleus aromaticus* Benth,

Costus igneus N.E.Br and *Lawsonia inermis* Linn) (Amirita *et al*., 2012); 6) Wild rubber trees (*Hevea brasiliensis*) (Gazis & Chaverri, 2010).

Endophytic fungi had an important role in agriculture such as stimulating or increasing plant growth, increasing plant resistance to pests, diseases, and nematodes, increasing plant resistance to drought stress. Some research results on the important role of endophytic fungi in agriculture are: 1) biocontrol agents for blast disease (Sucipto, Munif, Suryadi, & Tondok, 2015); 2) spur plant growth (Saylendra & Firnia, 2013) ; 3) biocontrol agent (Suciatmih, Yuliar, & Supriyati, 2011); 4) increase resistance to pathogenic pests (G, A, & Kannan, 2015); 5) increasing plant resistance to drought stress(Shukla, Awasthi, Rawat, & Kumar, 2012); 6) protect host plants from pest attacks (Faeth, 2002).

Exploration of endophytic fungi has been carried out on several types of plants to obtain a collection of endophytic fungi that can be utilized in agricultural fields. Local rice plants are one of the plants that had the potential of endophytic fungi that need to be explored in depth because they have some potential. According to Sitaresmi, Wening, Rakhmi, Yunani, & Susanto (2013), local rice naturally has resistance to pests and diseases, abiotic stress tolerant and has good rice quality and flavor favored by consumers in every location where the rice plant was cultivated.

Regional development centers of local rice in South Sulawesi are generally located in the mountainous area with an altitude 1000 m above sea level such us Tanah Toraja, Enrekang, and Luwu. Local rice varieties developed in Toraja are Pare Lalodo; Rogon; Pare Lea; Pare Kobo; Pare Ra'rari, Pare Ambo, Pare Tallang, Pare Bau; Pare Birrang and Pare Bumbungan (Juhriah, A.Masniawati, Tabaru, & Astuti, 2013). While in Enrekang local rice was developed; Pare Salle; pare Pulu Lotong, pare Pinjan, pare Pulu Mandoti, pare Lambau, pare Pallan, Pare Solo, pare Mansur and pare Kamida (Maulana, Kuswinanti, Sennang, & Syaiful, 2014). Luwu has 5 local rice varieties, namely: Tarone, Dambo, Kamba, Mandi, and Remaja.

Exploration and identification of endophytic fungi are very important because endophytic fungi have an important role in the ecosystem. Besides that, it can be used as information and as a basis for developing the potential of endophytic fungi. Some endophytic fungi have been isolated from rice plants and morphologically identified, namely: 1) *Fusarium* sp, *Cladosporium* sp, *Phoma* sp, *Penicillium* sp (Sucipto, Munif, & Tondok, 2016); 2) *Aspergillus* sp1, *Aspergillus* sp2, *Aspergillus candidus* and *Acremonium* (Syamsia, 2016); 3) *Nigrospora* sp. 3, *Penicillium*, *Trichoderma* sp. 2, *Nigrospora* sp. 4, *Verticillium*, and brown sterile hyphae 2 (Asiah, Wiyono, & Triwidodo, 2011).

Identification of fungi morphologically based on colonies and spores often less certainty of the identity of isolates. This is because the morphology endophytic fungi can be changed, other than that some endophytic fungus has very slow growth and frequent sporulation (Hyde dan Soytong 2008);(Legiastuti & Aminingsih, 2012)). According to (Diaz, Hennell, & Sucher, 2013), one solution to anticipate identification deficiencies morphologically is through molecular identification.

Molecular identification of endophytic fungi using comparative analysis of ribosome DNA sequences, especially the Internal transcribed spacer (ITS) region using primary ITS2, ITS4 and ITS5 (Y.W. *et al.,* 2009); primary ITS1 and ITS4 (Fernandes, Pereira, Silva, Bento, & Queiroz, 2015) and primary ITS5 and ITS4 (Rakhmana, Rahayu, Ardhi, & Wahyu, 2017); primary ITS1 and ITS2 (Alwakeel, 2013)

The types of culture media that can be used for fungi were Potato Dextrose Agar (PDA), Carrot Agar Potato (PCA), Sabouraud Dextrose Agar (SDA), Czapex Dox Agar (CDA), Corn Meal Agar (CMA), Nutrient Agar (NA), Malt Extract Agar (MEA) (Taurisia, Provorini, & Nurantoro, 2000).

This study aimed to identify endophytic fungi isolates from Enrekang local rice plants and test the ability of fungi to grow on several types of media. The molecular identification of endophytic fungi from Enrekang local rice has never been done before, so this is the first study. In addition, growth test of local rice in several types of media. The results of this study will provide the benefits of the diversity of endophytic fungi from local rice and further research for the development of potential endophytic fungi as PGPF in increasing agricultural production.

2. Materials and Method

2.1 Rejuvenation of Isolates and Endophytic Fungi DNA Extraction

Two endophytic fungi isolate that were isolated from the roots and stems of Pulu Mandoti rice namely MDTB and MDTA were re-grown on PDA media. Mycelia of fungi isolates was harvested and extracted using DNesay DNA DNA kits from Qiagen. Miselia of fungi was taken and placed in mortal then crushed until smooth. Mycelia was inserted into the microtube that has been filled with 400 µl *buffer* AP1, 40 µl PVP 26% and 4 µl RNase A stock (100 mg/ml) in the vortex then incubated in a water bath at 65 ° C for 30 minutes. The solution was then centrifuged.

2.2 Fungi DNA amplification

Endophytic fungi DNA amplification using primers ITS1 and ITS4. The amplification started with the manufacture of PCR mix consisting of hotstar mix PCR (Qiagen), Primer ITS 1 and ITS 4, DNA working and DDh_2O. The PCR mix solution was then inserted into the PCR machine for DNA amplification in vitro.

The PCR solution was inserted into the PCR machine and the amplification process started with an initial denaturation at 95°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 55.4°C for 1 minute and the final extension of 72°C for 10 minutes. The denaturation stage until the extension was repeated 35 times. The DNA amplification process lasts for ± 2 hours 16 minutes.

2.3 Separation Process of DNA Amplification Results

DNA amplification product separation was carried out using a horizontal electrophoresis method. This method was used 2% agarose and Tris Acetate EDTA (TAE) buffer 1 x. The results of separation then inserted in geldoc to see the results of

separation used UV transuminator and documented. Separation DNA amplification product was repaired to determine whether the amplification process was successful or not and determine the size of the amplification product.

2.4 DNA Sequencing Analysis

PCR products that were successfully amplified from MDTB and MDTA endophytic fungi isolates were sent to Genetic science, Jakarta for sequencing. The sequencing results were then analyzed using the Basic Local Alignment Search Tool (BLAST)

2.5 Growth Test on Four Types of Culture Media

Growth test of both endophytic fungi isolates on four types of culture media was carried out by growing each isolate in synthetic Potato Dextrose Agar (PDA) media, natural PDA, Malt Extract Agar (MEA) and Malt Peptone Agar (MPA). Endophytic fungus mycelium of two isolates were grown on media, Synthetic PDA (synthetic PDA), natural PDA (made from potato extract), MEA (15 g malt extract, 16 g agar / L sterile water), MPA (15 g malt extract, 20 g glucose, 5 g peptone and 16 agar / L sterile water). The isolates were incubated at room temperature. The growth of two isolates was measured based on the colony diameter until the 7th day (Rahim et al. 2015)

3. Results and Discussion

3.1 Molecular Identification

The two of endophytic fungi isolates had different morphological characters, MDTB isolates were gray, the upper surface of the colony was compact and thick, the colony grows was very slow, small diameter (± 1.25 cm), the colony reverse was rather creamy. MDTB isolate was blackish white, smooth upper surface, the colony growth was very fast, large diameter (± 8.89 cm), colony reverse was white, the two isolates have concentric zones types.

Isolates DNA endophytic fungi were successfully amplified using primers pairs of ITS1 and ITS4 at an annealing temperature of 55.4 ° C. The formation of DNA bands on an agarose gel after electrophoresis showed the PCR amplification process was successful. The success of PCR amplification was determined by the primer pairs and the corresponding annealing temperature in each fungus. This result was similar to the (Sibero et al., 2018), who succeeded in clarifying endophytic fungi R3 isolates isolated from coastal plants *Hydnophytum formicarum* from Sorong using primers pairs of ITS1 and ITS4. Likewise, the research of (Rahayu, Saryono, & Nugroho, 2015), succeeded in amplifying the DNA PCR of endophytic fungi from LBKURCC69 isolates that had been isolated from dahlia bulbs in the area ITS-1 and ITS-2 rDNA and primer pairs of ITS4 and ITS5. Research (Alwakeel 2013) using primer pairs of ITS1 and ITS 2.

The results of electrophoresis analysis on the results of PCR produce a single band for each DNA amplification (Fig. 1). The size of the molecular weight of the two isolates was 700 bp. This result was different from the DNA band size of two endophytic fungi isolates isolated from dahlia plants, which were only 583 and 537 bp in size (Rakhmana

et al., 2017), as well as the DNA size of endophytic fungi isolates from srikaya plants was 600 bp (Yunianto et al. 2012).

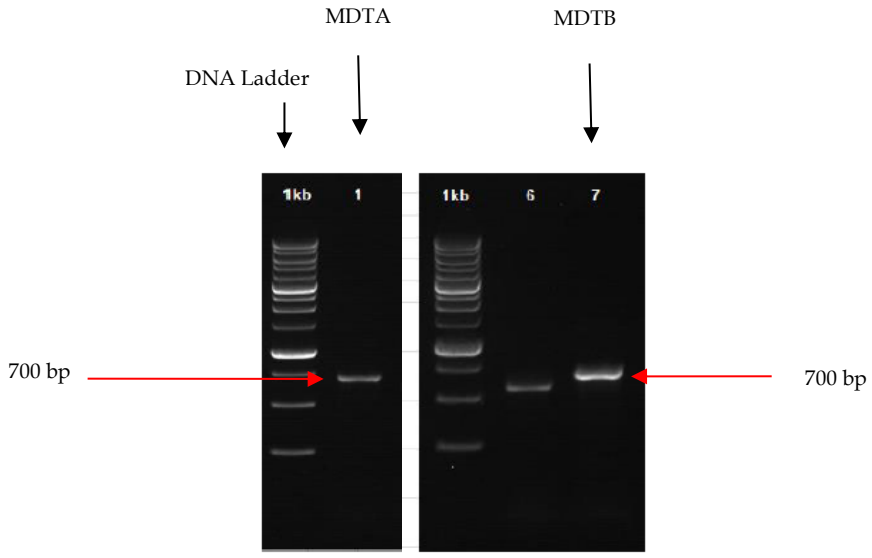


Fig. 1 The results of DNA amplification MDTB endophytic fungi isolates (1) and MDTA (7) using primers pairs of ITS1 and ITS4, DNA ladder (1kb).

Determining the identity of fungi isolates conducted based on sequencing data of text files compared with the closest species sequence homologies in the sequence data in the database / NCBI GenBank (Hall, 2004; Nuryadi et al., 2016). The homology Blast results on DDBJ / NCBI conducted on 2 samples of endophytic fungi isolates were presented in Table 2 and Tabel 3

Table 2. The strains on NCBI GenBank Database having a close kinship to MDTB

Species	Strain	GenBank access code	Reference	Score	Query cover	Max identity
Podoscypha bolleana	32032	JQ675332.1	Langer, E (2012)	1055	97%	94,29%
Podoscypha bolleana	32034	JQ675334.1	Langer, E (2012)	1055	97%	94,29%

Table 3. The strains on NCBI GenBank Database having a close kinship to MDTA

Species	Strain	GenBank acces code	Reference	Score	Query cover	Max identity
Coprinopsis cinerea	HN08	JQ796875.1	Zhang,Y. and Li, D. C (2012)	1306	98%	99,71%
Coprinopsis cinerea	A2S3-5	KJ780765.1	(Teh Li & Latiffah, 2014)	1317	98%	99,71%

The results of identification using DNA barcoding proved that endophytic fungi from local Sulawesi Selatan rice with sample code 1 were homologs of *Podoscypha bolleana* strain 32034 no accession JQ675334 and *Podoscypha bolleana* strain 32032 no accession JQ675332 with 100% similarity level and sample code 8 was a homolog of *Coprinopsis cinerea* isolate A2S3-5 with 99% similarity level and *Coprinopsis cinerea* CNRMA / F 07-32 strain with 100% similarity level. Research (Sibero et al., 2018) proved that RS3 fungus was a homolog of Annulohypoxylon stygium DR47 strain with 99% similarity level, with accession number MG605083.1. Research

by (Nuryadi et al., 2016), succeeded in identifying molecular fungi from dahlia tubers using ITS molecular markers, there were 4 isolates as *Lasiodiplodia* genus, 4 isolates as *Didymellaceae* family, 11 isolates were identified as *Phomopsis* genus, 5 isolates as *Colletotrichum* genus, 1 isolates as *Nemania* genus, and 1 isolate as Xylaria genus. *Coprinopsis cinerea* is a basidiomycete fungus that is used for many basic studies, including research into the fungus development stage. *C. cinerea* fungus was easy to maintain, has a short life cycle and can be induced to develop fruit bodies in the laboratory and only takes 2 weeks to produce a ripe fruit body.

The sequence results created in the FASTA format for phylogenetic tree construction using MEGA7. The tree was constructed using UPGMA models and Kimura-2-Parameter genetic distances. In the process of making the tree, *Podoscypha bolleana* Strain 32032, *P. bolleana* Strain 32034 having a close kinship to MDTA (Table 2.) and *Coprinopsis cinerea*Strain HN08 and C. *cinerea* A2S3-5 having a close kinship to MDTB (Table 3.).

Dendogram based on the similarity value of the strain obtained from Gen-Bank. Each morphotype was grouped together and separated from each other. The phylogenetic tree produced (Fig 2). The tree showed that the position of the sample with its relative species. The position of MDTA with MDTB in the phylogenetic tree lies in one cluster so it showed that they were closely related. The genetic distances between MDTA and MDTB samples were calculated using the Kimura-2-Parameter method in MEGA7 according to (Kumar, Stecher, & Tamura, 2016). The results showed that the genetic distances between MDTA and MDTB with the highest boostrap value at 93%. These results indicate them likely that MDTA and MDTB endophytic fungi are closely related and even tend to be subspecies

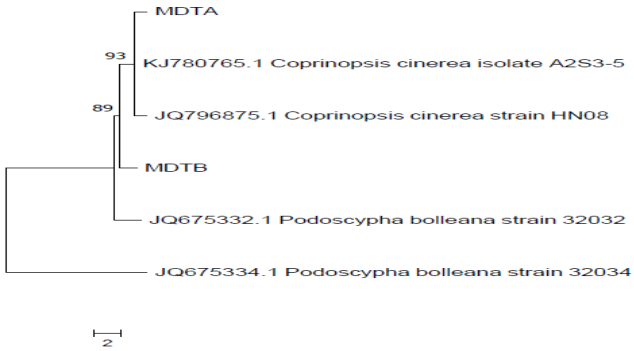


Fig 2. Phylogenetic trees of MDTB and MDTA isolates

3.2 The Growth of endophytic fungi in four type of media

The growth of the two endophytic fungi isolates in four types of media showed that the diameter of the colonies, the color of the upper surface, the upper surface and zoning were basically the same for all types of media, except for texture and sporulation there were differences in MEA media, the texture of the two isolates very

thin in the MEA medium and sporulate sporulation was very poor (Table 5 and Table 6).

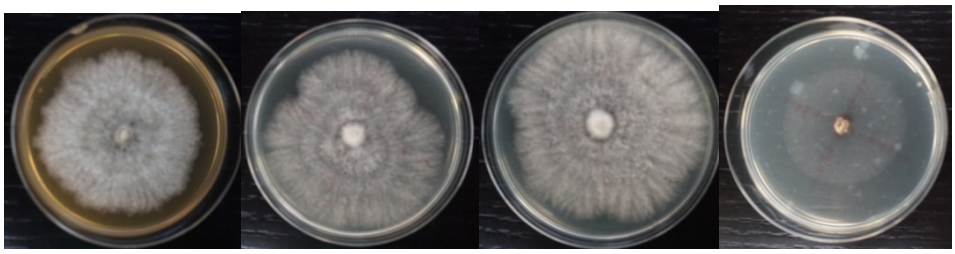
Table 5. Growth of MDTB isolates in four media

Media	Colony	Colony character		Colony Reverse	Zonation	Sporulation
	Diameter (cm)	Texture	Upper Surface Color			
Synthetic PDA	2.23	Thick compact	Gray	Cream	Concentric zones	Average
Natural PDA	2.33	Thick compact	Gray	Cream	Concentric zones	Average
MPA	2.30	Thick compact	Gray	Cream	Concentric zones	Much
MEA	2.02	Thin growth	Gray	Cream	Concentric zones	Little/ Less

Table 6. Growth of MDTA isolates on four media

Media	Colony	Colony character		Colony Reverse	Zonation	Sporulation
	Diameter (cm)	Texture	Upper Surface Color			
Synthetic PDA	8.25	Furry	Blackish white	White	Concentric zones	Average
Natural PDA	8.43	Furry	Blackish white	White	Concentric zones	Average
MPA	8.89	Furry	Blackish white	White	Concentric zones	Much
MEA	6.34	Thin growth	Blackish white	White	Concentric zones	Little/ Less

The growth of MDTB endophytic fungi isolates in four types of media namely synthetic PDA, natural PDA, MPa and MEA (Fig. 3). Both endophytic fungi isolates have the ability to grow well in all three types of media, namely PDA Synthetic, natural PDA and MPA. The media suitable for sporulation was MPA media. The result of this study was similar to research (Rahim, Kuswinanti, Asrul, & Rasyid, 2015) who was found MPA media as the best medium for foliage fungus growth. However, different research result (Devi, Misra, Saha, Devi, & Sinha, 2018) was getting the best media for fungi sporulation were MEA and OMA.



Synthetic PDA Natural PDA MPA MEA

Fig. 3 The growth of MDTB endophytic fungi isolates in four types of media: Synthetic PDA (A), Natural PDA (B), MPA (C) and MEA (D)

In general, natural PDA media and synthetic PDA were still suitable for growth and sporulation of endophytic fungi. The result of the study (Noerfitryani & Hamzah, 2017), who use PDA media for the growth of *Fusarium*, *Aspergillus* and *Trichoderma* fungi. According to (Devi et al., 2018) PDA media was a medium commonly used for fungus growth because the formulation was simple and has the ability to support the growth of mycelia in almost all types of fungi. Likewise, research (Aini & Rahayu, 2015) showed that PDA media provide the best growth of fungi *Candida albicans* and *Aspergillus niger* than alternative media.

4. Conclusion

The MDTB fungus has similarities to *Podoscypha bolleana* strain 32034 no accession JQ675334 and *Podoscypha bolleana* strain 32032 no accession JQ675332, whereas the MDTA fungus has similarities to Coprinopsis cinerea A2S3-5 isolate and *Coprinopsis cinerea* strain CNRMA/F 07-32. The best culture media and sporulation of endophytic fungi is MPA media.

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