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Identification of Three Isolate Fungal To Produce Agarwood Sapwood on *Gyrinops Versteegii (Gilg.)* Domke Plant By Molecular Analysis

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Abstract
Agarwood sapwood is one of the non-timber forest products (NTFPs) that have economic value and as an export commodity. This product usually used as a raw material for cosmetics, perfume, incense, and medicines. The production of agarwood sapwood influenced by the type of agarwood-producing plants, the type of microbial induction, and environmental factors. One of the microbial inducing agarwood-producing plants is fungi. Several studies suggested that Fusarium, Rhizopus, and Trichoderma fungi can assist in the formation of agarwood sapwood in the Gyrinops versteegii plant, but the species of fungi is unknown. This study aims to identify three isolates that produce agarwood sapwood on the Gyrinops versteegii plant in Bali. The fungi Identification is using molecular analysis. Fungi isolates were analyzed based on internal transcribed spacer (ITS) sequences in the ribosome DNA area by PCR technique using universal primers ITS-1 and ITS-4. The results showed that the fungi species helped in the formation of agarwood sapwood were: Fusarium solani, Rhizopus microsporus, and Trichoderma harzianum.

Keyword: fungi species, agarwood sapwood, Gyrinops versteegii, PCR

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I. INTRODUCTION

Agarwood sapwood is a product in the form of solid mass brownish-black color to black color with fragrant scent found in the woods or roots of host tree plants (example *Aquilaria sp*). The wood part has undergone a process of physical and chemical changes due to infection by specific fungi **[5].**

Agarwood is one of the non-timber forest product commodities (NTFPs) with high economic value and an export commodity, as well as a good source of income for the community **[11].** Agarwood which has been exported to Saudi Arabia from January to June 2016 was more than 10 tons with a value of more than IDR 28 billion. Furthermore, PT Idaman Polanusa recorded an agreement to supply 100 tons of agarwood for a year to Saudi Arabia **[13].** Furthermore, the government has declared that agarwood to be a national non-timber forest product commodity to be developed on a broader scale **[16]**. The high number of agarwood sapwood demand from abroad is due to the many benefits of agarwood such as perfumes, cosmetics, medicines, and religious ritual purposes **[1]**.

The production of agarwood sapwood is influenced by various factors, such as the genetics of agarwood-producing trees, microbial inductions, the environment, and the duration of the sapwood formation process. Agarwood sapwood can occur when certain pathogens infect agarwood-producing trees. The response of trees to the pathogen attack is the production of secondary metabolites or resin compounds in which produce fragrance when agarwood sapwood is burned **[18]**.. The results of Mega and Phabiola's research (2010), revealed that the fungi species *Fusarium sp* and *Rhizopus sp*. causes the formation of agarwood sapwood in the *Gyrinops versteegii* plant. Putri *et al*. (2016), showed that agarwood-producing plants inoculated with fungi produced 1.1% higher resin content than non-inoculated plants. Furthermore, the study results of Budi *et al.* (2010) showed that four Fusarium species that induced *Aquilaria spp* plants, namely: *F. Solani* (Mart) Appel & Wollenw, *F. Lateritium* Ness, *F. Tricinotium* (Corda) and *F. moniliformae* Sheldon. Sangareswari *et al* (2016) reported that several fungi species naturally infecting agarwood-producing plants at various places in India such as Aspergillus, Lasiodiploidia, Chaetomium, Fusarium, and Penicillium. Furthermore, Mega *et al* (2015) found that the liquid inoculant mixture of *Fusarium solani* and *Rhizopus sp*. successfully inoculated the agarwood plant (*Gyrinops versteegii*) for 16 months and produced agarwood sapwood with a resin content of 13.58%. Chhipa and Kaushik (2017) revealed that Trichoderma fungi were found dominant in the *Aquilaria malacensis* stem. Mega and Nuarsa (2018) study showed that three types of fungi can help in the formation of agarwood sapwood, namely *Fusarium solani*, *Rhizopus sp*., and *Trichoderma sp*. This study used two indicators, such as

the color of the wood (changing from white to brown to blackish-brown) and resin levels (5.24% (Rhizopus fungi), 5.31% (Fusarium fungi), 7.92% (Trichoderma fungi)). The three fungi isolates were observed macroscopically, so it needs further identification by another method. This study will use the molecular analysis method by using the DNA approach of each fungus. This analysis will show the species of fungi isolates.
Based on no certainty and exact species of fungi isolates on agarwood sapwood *Gyrinops versteegii plant*, it is necessary to research the identification of fungi isolates on agarwood sapwood molecularly.

II. RESEARCH METHOD

2.1 Tools and Materials

The tools used in this study were erlenmeyer, scales, bunsen lamps, petri dishes, micropipettes, autoclaves, laminar airflow, scissors, stoves, pans, spoons, filters, measuring cups, plastic bags, masks, labels, stationery, ruler, camera, laptop, oose needle, cork borer, microscope, optic lab, PCR (Polymerase Chain Reaction), Eppendorf, vortex, centrifugation, electrophoresis, and ultraviolet transilluminator.

Materials used in this study were isolated fungi *Fusarium sp*. (isolate-1), fungi *Rhizopus sp*. (isolate-2), and *Trichoderma sp*. (isolate-3). Isolate-1 and isolate-2 isolated on agarwood trees in Pupuan District, Tabanan Regency. However, isolate-3 isolated at a garden area in Baturiti District, Tabanan Regency. Also, another supporting material was used such as distilled water, 70% alcohol, 0.5% Clorox, PDA media, NA media (nutrient agar), liquid nitrogen, CTAB buffer, TE buffer, ITS1 primer, ITS4 primer, mercaptoethanol, sodium acetate, isoamyl alcohol, and ethanol (70%).

2.2 Molecular Identification

DNA extraction follows the procedure of Doyle and Doyle (1987). Samples of 0.2 g of mycelium fungi (pathogens) were crushed with liquid nitrogen and pathogenic fungal powders were inserted into the Eppendorf tube. Next, 500 µL of CTAB buffer and 50 µL β-mercaptoethanol was added, then mixed until homogeneous with the vortex. For lysis of cell walls, heat the lysis sample with a temperature of 70ºC for 60 minutes, every 10 minutes, swing the lysis sample back and forth to speed up the lysis process. Then cooled it down to room temperature. Subsequently, 500 µL of isoamyl alcohol (24: 1) chloroform added to the tube and then mixed until homogeneous with vortex and centrifuged at 12,000 rpm for 15 minutes. The obtained supernatant then transferred to a new Eppendorf tube by adding 500 µL sodium acetate mixed to homogeneous with vortex and centrifuged again at 12,000 rpm for 10 minutes. The supernatant transferred to an Eppendorf tube then added sodium acetate and isopropanol each 500 µL, mixed until homogeneous with vortex and centrifuged again at 12,000 rpm for 10 minutes. The tube was shaken slowly to bind the DNA then incubated at -20ºC for 30 minutes. The DNA strands result were deposited for sedimentation by centrifugation for 10 minutes. The supernatant removed, washed the pellet with ethanol (70%) then centrifuged at 8,000 rpm for 5 minutes. Discard the ethanol then dried the pellets. The pellets were resuspended with 50 µL of the TE buffer and stored it at -20ºC for further use at the DNA amplification process.

2.3 DNA Amplification

DNA amplification was performed on a Thermo Cycle PCR machine. Amplification was performed using universal primers to detect the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA), namely forward primer ITS1 (5'- CTTGGTCATTTAGGAAGTAA-3 ') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') with the target size amplification was 490 bp (Doyle & Doyle, 1987).
DNA amplification reactions carried out with a total volume of 25 µL consisting of 1 µL DNA, 2.5 µL buffer 10 x and Mg2 +, 0.5 µL dNTP 10 mM, 1 µL each primer, 12.5 µL Taq DNA (10 units / µL), and 9.5 µL H2O. The amplification conditions divided into several stages, namely 94 ºC pra-denaturation for 3 minutes, followed by 30 amplification cycles, each cycle consisting of DNA / DNA 94 ºC denaturation separation for 1 minute, primary attachment/annealing 45 ºC for 1 minute, DNA synthesis 72 ºC for 2 minutes. Especially for the last cycle plus the synthesis stage for 10 minutes, then the cycle will end at 4ºC.

2.4 DNA Electrophoresis

The amplified product analyzed using Blued electrophoresis with 1% agarose gel (0.5xTris-Borate EDTA / TBE). Electrophoresis carried out at 100 volts for 28 minutes, then agarose gel incubated in dye-containing ethidium bromide (1%) for 15 minutes, then washed with H2O for 10 minutes. Electrophoresis results visualized with an ultraviolet transilluminator. DNA bands formed on the results of the electrophoresis documented with a digital camera.

2.5 Analysis of DNA Sequences

The amplification product delivered to the 1st Base (Malaysia) for nucleotide tracking. The sequencing results analyzed using the basic local alignment search tool (BLAST) program with an optimization program to obtain DNA base sequences that have homology with DNA sequences published at the National Center for

Biotechnology Information (NCBI) website. The nucleotide sequences result then analyzed using *ClustalW* multiple alignments in the Bioedit sequence alignment editor software version-7.0.5. Homology results close to 100% similarity categorized as the same species as the sample species.

III. RESULT AND DISCUSSION

3.1 Fusarium solani

DNA fragments of 650 pb were successfully amplified from 3 fungal samples using an ITS1 / ITS4 universal primer (Figure 1). The amplified DNA sample and then used for the sequencing stage to confirm the fungal species. Sequencing analysis confirmed that the identity of the fungus sample 1 was *Fusarium solani* with 95-97% homology in succession to several *Fusarium solani* isolates in genebank (Table 1).

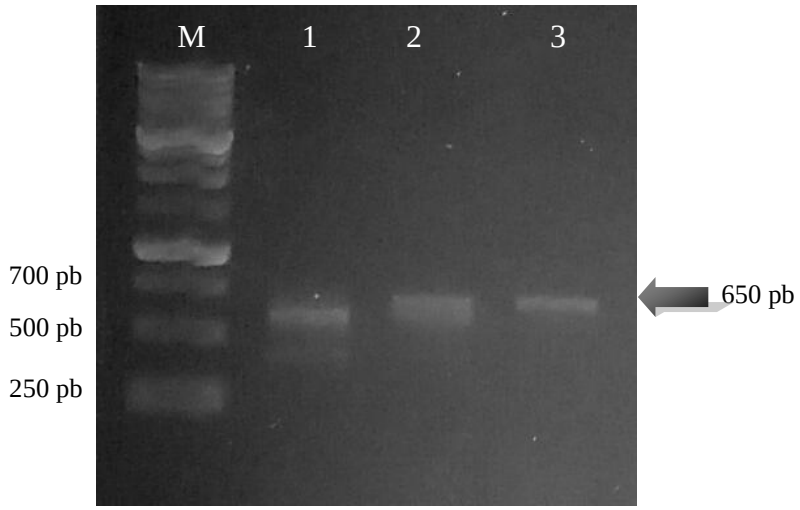
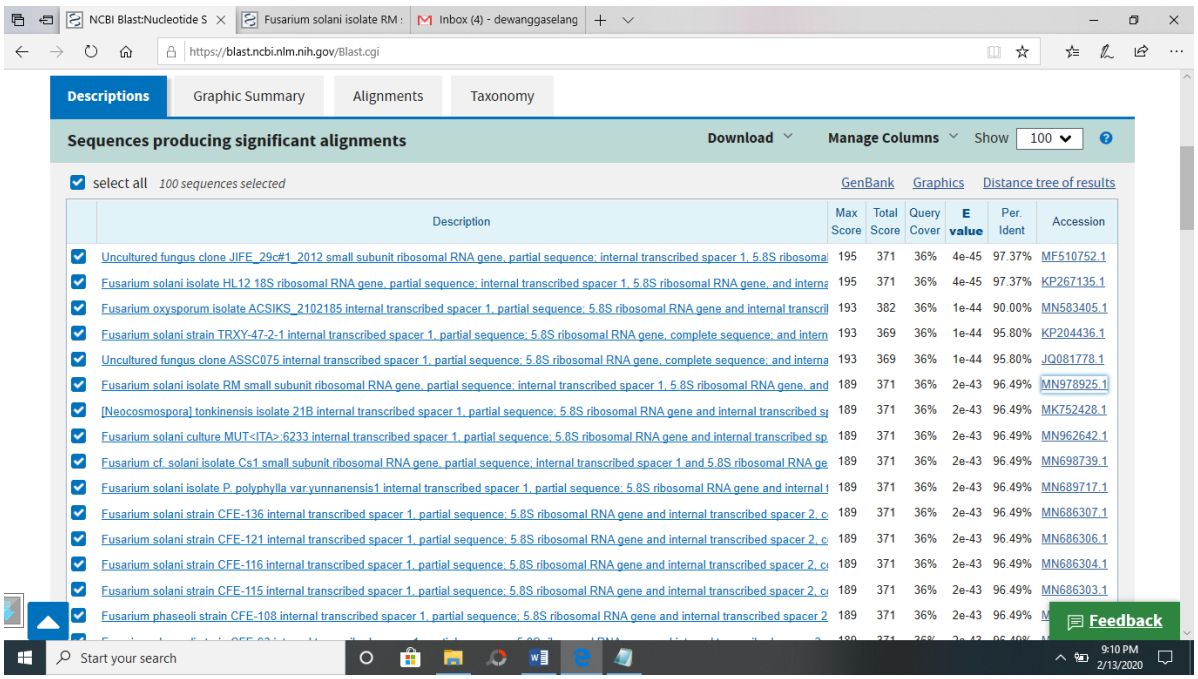


Figure 1. Visualization of Fungal DNA results from amplification using universal ITS1 / ITS4 primers on 1% agarose gel. M: DNA marker (1kb ladder); Sample no. 1 (isolate 1), 2 (isolate 2) and 3 (isolate 3).



https://blast.ncbi.nlm.nih.gov/Blast.cg

Table 1. Homology (%) of the nucleotide sequence of *Fusarium solani* isolates with several isolates that have been reported in GenBank

Sikuen	Fusarium _solani	KP267135 _CHI	MN978925 _PAK	MN962642 _ITA	MN698739 _IND	MN686302 _IND	MN658459 _CHI	MN653387 _RUS	MN637860 _CHI	MN634542 _SOA	AF130378 _Fusarium _dimerum
Fusarium_solani	ID	95%	96%	95%	96%	97%	97%	95%	95%	95%	44%
KP267135_CHI	95%	ID	96%	100%	97%	97%	97%	100%	100%	100%	44%
MN978925_PAK	96%	96%	ID	97%	97%	97%	97%	97%	97%	97%	44%
MN962642_ITA	95%	100%	97%	ID	98%	97%	97%	100%	100%	100%	44%
MN698739_IND	96%	97%	97%	98%	ID	99%	99%	98%	98%	98%	44%
MN686302_IND	97%	97%	97%	97%	99%	ID	100%	97%	97%	97%	44%
MN658459_CHI	97%	97%	97%	97%	99%	100%	ID	97%	97%	97%	44%
MN653387_RUS	95%	100%	97%	100%	98%	97%	97%	ID	100%	100%	44%
MN637860_CHI	95%	100%	97%	100%	98%	97%	97%	100%	ID	100%	44%
MN634542_SOA	95%	100%	97%	100%	98%	97%	97%	100%	100%	ID	44%
AF130378_ Fusarium_ dimerum	44%	44%	44%	44%	44%	44%	44%	44%	44%	44%	ID

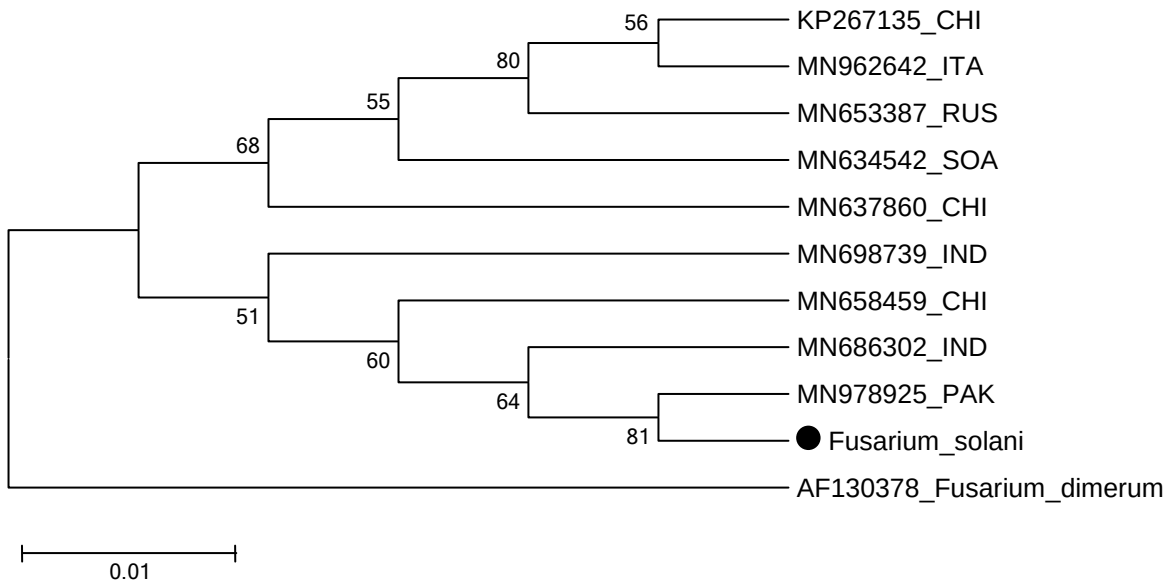


Figure 2. Phylogenetic analysis of *Fusarium solani* isolates based on the alignment of the partial nucleotide sequences of DNA-A using Mega 6.06 (Neighbor-Joining Algorithm with 1,000 bootstraps

The phylogenetic analysis result showed that *Fusarium solani* isolates formed two-groups. *Fusarium solani* isolates from Bali, formed a group with 4 isolates from genebank, such as Indian isolates (MN698739), Chinese isolates (MN658459), Indian isolates (MN686302), and Pakistan isolates (MN978925). And the other group consists of 5 isolates from genebank, such as Chinese isolates (KP267135), Italian isolates (MN962642), Russian isolates (MN653387), South African isolates (MN634542), and Chinese isolates (MN637860). *Fusarium dimerum* isolate (AF130378) used as a comparison and categorized as an outgroup isolate (Figure 2). Nugraheni *et al* (2015) revealed that the three fungi isolate induced *Gyrinops versteegii* in West Nusa Tenggara, with PCR molecular identification (primary ITS) belonged to the *Fusarium solani* species. *Fusarium solani* was also found to be associated with Aquilaria spp. in Nunukan Regency, North Kalimantan **[10]**.

3.2 Rhizopus microsporus

DNA fragments of 650 bp were successfully amplified from 3 fungal samples using an ITS1 / ITS4 universal primer (Figure 1). The amplified DNA sample used for the phase of sequencing to ascertain the fungus species. Sequencing analysis confirmed that the fungus that sampled 2 was *Rhizopus microsporus* with 98-100% homology in succession to several *Rhizopus microsporus* isolates in genebank (Table 2).

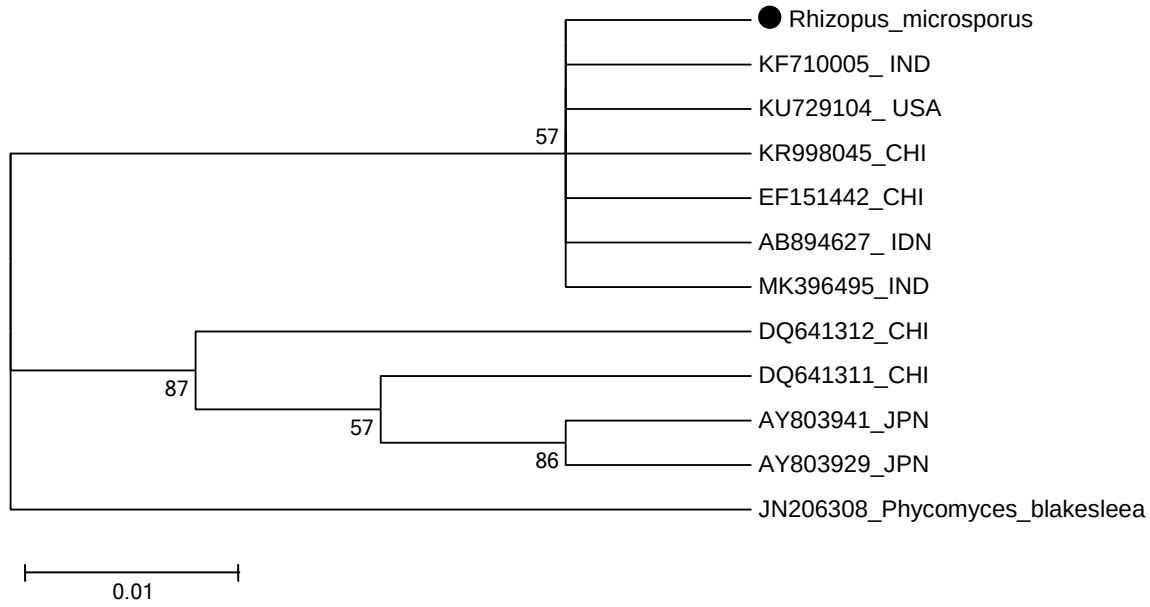
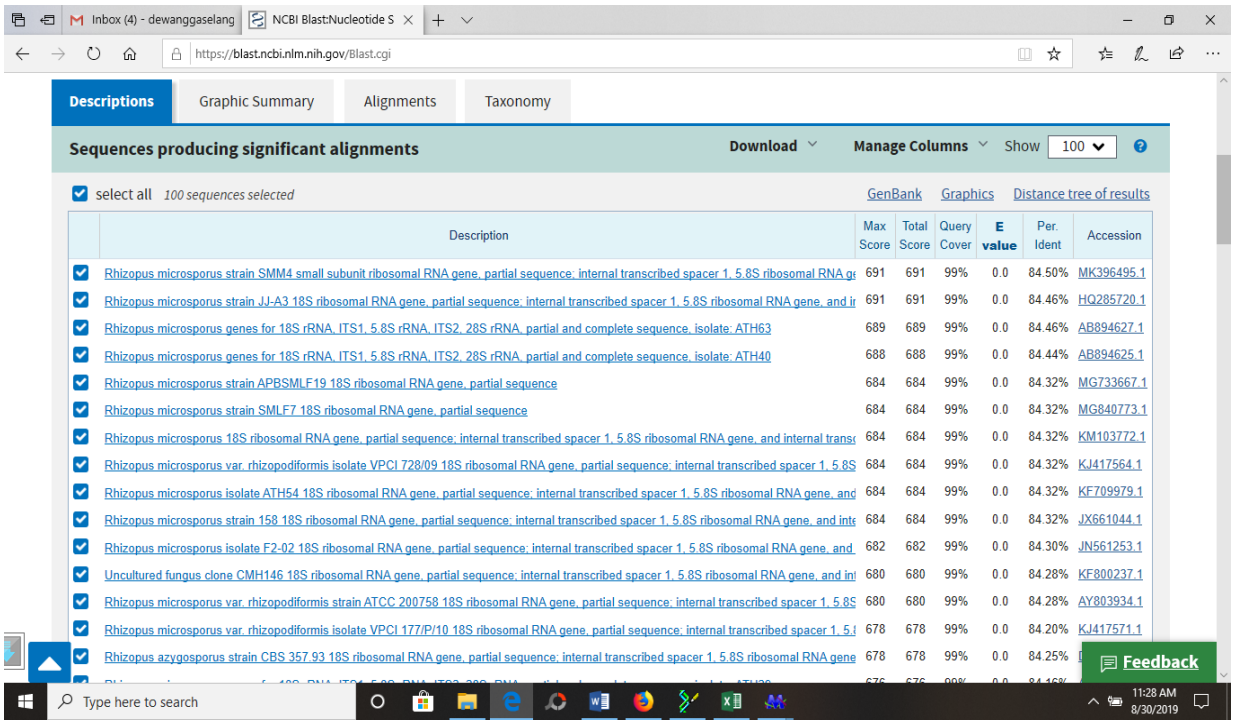


Figure 3. Phylogenetic analysis of *Rhizopus microsporus* isolates based on the alignment of the partial nucleotide sequences of DNA-A using Mega 6.06 (Algorithm Neighbor-Joining with 1,000 bootstraps

Further phylogenetic analysis showed that isolates of *Rhizopus microsporus* formed two groups. *Rhizopus microsporus* isolates from Bali categorize as a group with 6 other isolates from genebank, such as Indian isolates (KF710005), United States isolates (KU729104), Chinese isolates (KR998045), Chinese isolates (EF151442), Indonesian isolates (AB894627), and Indian isolates (MK396495). While the second group consisted of 4 isolates from genebank including Chinese isolates (DQ641312), Chinese isolates (DQ641311), Japanese isolates (AY803941), and Japanese isolates (AY803929). As outgroup isolates, *Phycomyces_blakesleeanus* (JN206308) isolates used as a comparison (Figure 3). Schwertz *et al*. (1997) found the *Rhizopus microsporus var. oligosporus* as a fermentation agent in tempeh in Bali. Dewi and Aziz (2011) revealed that the results of isolation of fungi in fermented soybean patty at Central Java were *Rhizopus microsporus*, while fungi *Rhizopus sp*. could infect agarwood-producing plants from *Gyrinops versteegii* in Tabanan **[6]**.



Source: https://blast.ncbi.nlm.nih.gov/Blast.cgi

Table 2. Homology (%) of nucleotide sequences of *Rhizopus microsporus* isolates with several isolates that have been reported in GenBank

Sikuen	R_oligosporus	KU729104_USA	MK396495_IND	AB894627_IDN	EF151442_CHI	KF710005_IND	KR998045_CHI	DQ641312_CHI	AY803941_JPN	AY803929_JPN	DQ641311_CHI	JN206308_P_blakesleeanus
R_oligosporus_R	ID	100%	100%	100%	100%	100%	100%	99%	99%	99%	98%	57%
KU729104_USA	100%	ID	100%	100%	100%	100%	100%	99%	99%	99%	98%	57%
MK396495_IND	100%	100%	ID	100%	100%	100%	100%	99%	99%	99%	98%	57%
AB894627_IDN	100%	100%	100%	ID	100%	100%	100%	99%	99%	99%	98%	57%
EF151442_CHI	100%	100%	100%	100%	ID	100%	100%	99%	99%	99%	98%	57%
KF710005_IND	100%	100%	100%	100%	100%	ID	100%	99%	99%	99%	98%	57%
KR998045_CHI	100%	100%	100%	100%	100%	100%	ID	99%	99%	99%	98%	57%
DQ641312_CHI	99%	99%	99%	99%	99%	99%	99%	ID	100%	99%	99%	56%
AY803941_JPN	99%	99%	99%	99%	99%	99%	99%	100%	ID	100%	100%	57%
AY803929_JPN	99%	99%	99%	99%	99%	99%	99%	99%	100%	ID	99%	56%
DQ641311_CHI	98%	98%	98%	98%	98%	98%	98%	99%	100%	99%	ID	57%
JN206308_P_blakesleeanus	57%	57%	57%	57%	57%	57%	57%	56%	57%	56%	57%	ID

3.3 Trichoderma harzianum

DNA fragments of 650 bp were successfully amplified from 3 fungal samples using an ITS1 / ITS4 universal primer (Figure 1). The amplified DNA sample is then used for the phase of sequencing to ascertain the fungus species. Sequencing analysis confirmed that the fungi sampled 3 were *Trichoderma harzianum* with 98-99% homology in a succession of several *Trichoderma harzianum* isolates in genebank (Table 3).

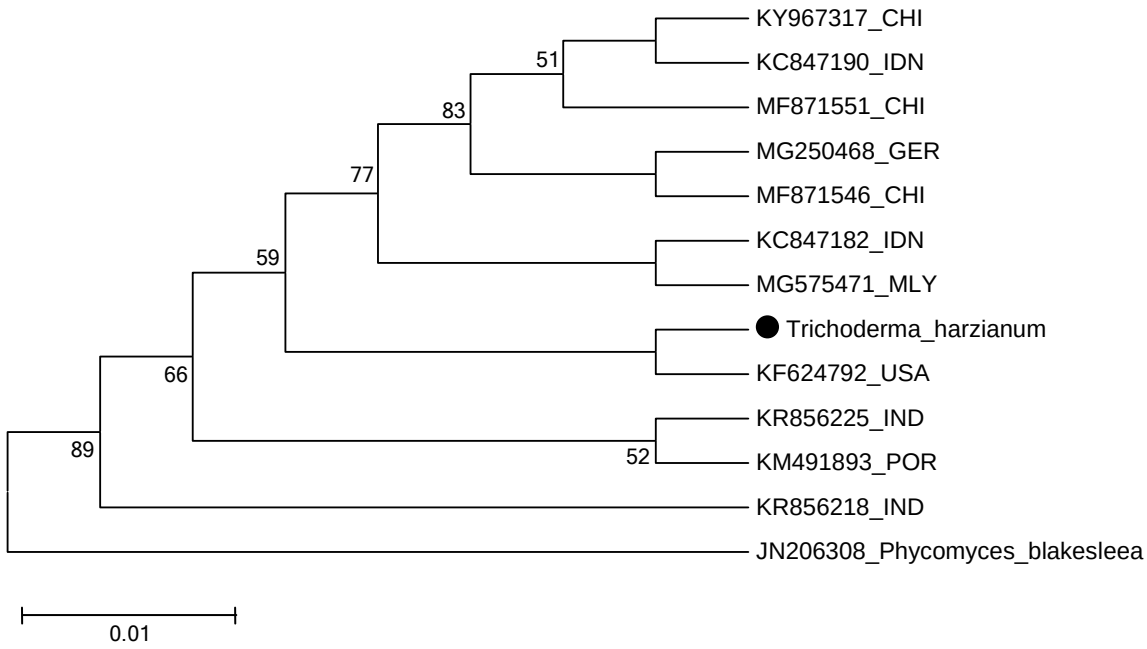


Figure 4. Phylogenetic analysis of *Trichoderma harzianum* isolates based on an alignment of the partial nucleotide sequences of DNA-A using Mega 6.06 (Algorithm Neighbor-Joining with 1,000 bootstraps replicates).

The phylogenetic analysis showed that *Trichoderma harzianum* isolates formed two groups. *Trichoderma harzianum* isolates from Bali formed a group with several isolates from genebank including isolates from Indonesia, isolates from India, isolates from Malaysia, isolates from China, several isolates outside of Asia such as Portugal isolates, and the United States. As outgroup isolates, *Phycomyces_blakesleeanus* (JN206308) isolates used as a comparison (Figure 4). Based on the morphology, three isolates from Batu Organic Farm Soil, BPTP Ngijo, and Blitar Cocoa Plantation Soils were fungi antagonists of *T. harzianum* but after tracing DNA genetic similarities as a result of molecular analysis by PCR technique on GeneBank it was found that three isolates were *T. asperellum* **[14]**.

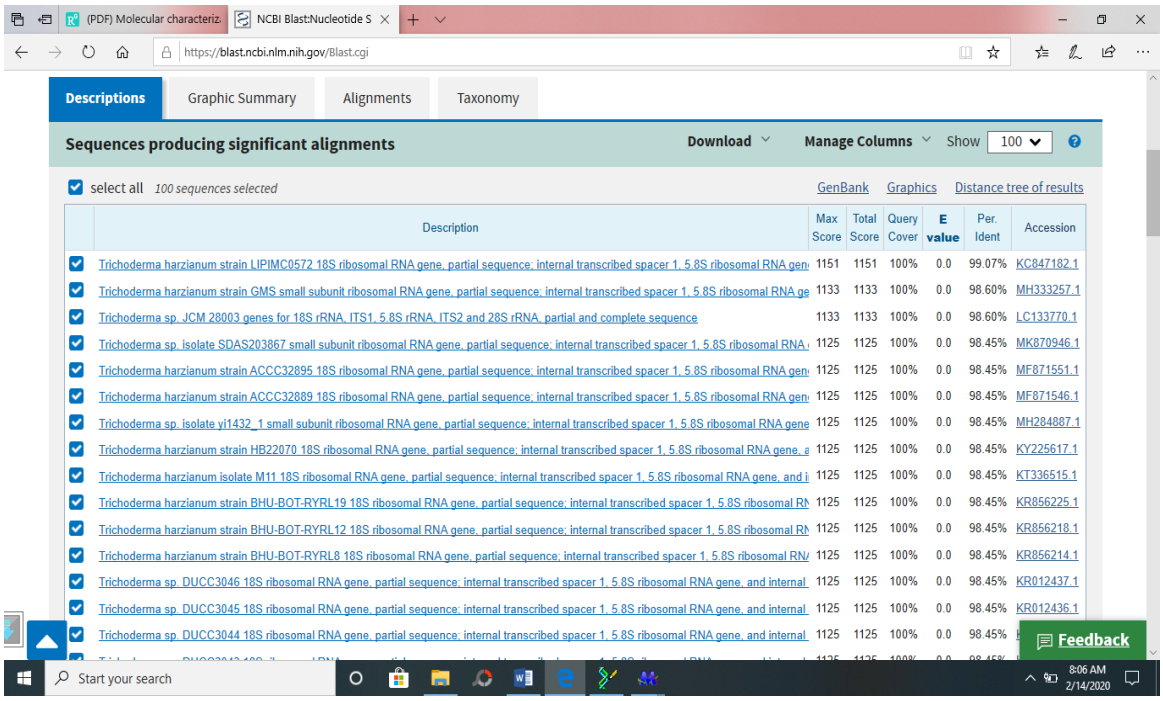


Table 3. Homology (%) of nucleotide sequences of *Trichoderma harzianum* isolates with several isolates that have been reported in GenBank

Sikuen	T_harzianum	KC847182_IDN	KR856225_IND	MF871551_CHI	MF871546_CHI	KR856218_IND	KM491893_POR	MG575471_MLY	KY967317_CHI	MG250468_GER	KC847190_IDN	KF624792_USA	JN206308_P_blakesleeanus
Trichoderma_harzianum	ID	99%	99%	99%	99%	99%	99%	98%	98%	98%	99%	99%	51%
KC847182_IDN	99%	ID	100%	100%	100%	100%	100%	99%	99%	99%	100%	100%	52%
KR856225_IND	99%	100%	ID	100%	100%	100%	100%	100%	100%	100%	99%	100%	51%
MF871551_CHI	99%	100%	100%	ID	100%	100%	100%	100%	100%	100%	99%	100%	51%
MF871546_CHI	99%	100%	100%	100%	ID	100%	100%	100%	100%	100%	99%	100%	51%
KR856218_IND	99%	100%	100%	100%	100%	ID	100%	100%	100%	100%	99%	100%	51%
KM491893_POR	99%	100%	100%	100%	100%	100%	ID	100%	100%	100%	99%	100%	51%
MG575471_MLY	98%	99%	100%	100%	100%	100%	100%	ID	100%	99%	99%	100%	51%
KY967317_CHI	98%	99%	100%	100%	100%	100%	100%	100%	ID	100%	99%	100%	51%
MG250468_GER	98%	99%	100%	100%	100%	100%	100%	99%	100%	ID	99%	100%	51%
KC847190_IDN	99%	100%	99%	99%	99%	99%	99%	99%	99%	99%	ID	99%	51%
KF624792_USA	99%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	ID	51%
JN206308_P_blakesleeanus	51%	52%	51%	51%	51%	51%	51%	51%	51%	51%	51%	51%	ID

IV. CONCLUSION

Three fungal isolates of agarwood sapwood on *Gyrinops versteegii* plant in Tabanan Regency, Provincy of Bali belong to the species: *Fusarium solani*, *Rhizopus microsporus*, and *Trichoderma harzianum.*

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