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Isolation and identification of fungi associated with *Falcataria moluccana* gall-rust in Kediri, East Java, Indonesia

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Abstract. *Istikorini Y, Cahyani NN, Haneda NF, Shabrina H, Nugroho A, Indriani F, Siregar UJ. 2022. Isolation and identification of fungi associated with* Falcataria moluccana *gall-rust in Kediri, East Java, Indonesia. Biodiversitas 23: 6171-6179.* Gall-rust disease caused by *Uromycladium falcatariae* is the most important disease of sengon in Indonesia. Plant pathogenic parasitism as a biological control mechanism is usually associated with fungal biocontrol agents. This study aimed to isolate and identify the fungi associated with the sengon gall rust from the Forest Management Unit of Jatirejo, Indonesian State Forest Company, Kediri. Fungi were isolated from the black or gray-green patches of gall-rust samples on Potato Dextrose Agar (PDA) plates. The fungi were identified using macroscopic and microscopic examinations. The molecular identification of fungal DNA was amplified by PCR using a specific internal transcribed spacer primer (ITS1/ITS4). The PCR products were sequenced and compared with the other related sequences in GenBank (NCBI). DNA barcode identification results showed 9 isolates containing of 8 species, namely *Fusarium oxysporum, F. solani, F. chlamydosporum, F. circinatum, F. incarnatum, F. verticillioides, Lasiodiplodia theobromae*, and *Pestalotiopsis palmarum*. This result showed that the *Fusarium* was the most fungi commonly found fungi on the gall's rust teliospore surface.

Keywords: DNA barcoding, *Falcataria moluccana,* internal transcribed spacer, *Uromycladium falcatariae*

INTRODUCTION

Sengon (*Falcataria moluccana* (Miq.) Barneby & Grimes) is a fast-growing legume tree species with natural distribution in Indonesia, involving Moluccas, Papua, South Sulawesi, and Java (Krisnawati et al. 2011; Nuroniah et al. 2021). Sengon timber ranked in the top five of Indonesia's log production in 2017 (BPS 2018) and contributed 54,87% of the total logs produced in Java Island (BPS 2019). The tree can live in various soil conditions, in both fertile and marginal land (Widyastuti et al. 2013). One main obstacle to the cultivation of sengon is the presence of pests and diseases. Sengon plantation, especially in the monoculture system, is generally susceptible to pests and diseases such as stem borers, dumping off, and gall-rust (Siregar and Olivia 2012).

Gall-rust is a disease that massively attracts the attention of farmers because it causes considerable losses. The status of gall rust disease incidence and severity in sengon plants were widespread and very severe, of which could reach 100% (Darwiati and Anggraeni 2018; Firdaus et al. 2019; Rahayu et al. 2020; Syarifuddin et al. 2021). A molecular study identified the gall rust pathogen in sengon and changed the name from *Uromycladium tepperianum* to *U. falcatariae* (Doungsa-ard et al. 2015; Doungsa-ard et al. 2018; Lelana et al. 2022). *Uromycladium falcatariae* is an obligate parasite that can grow and multiply if it remains in contact with its host plant. *Uromycladium falcatariae* requires an entire year to complete a life cycle (monocyclic pathogens) and can cause only one series of infections per year. In monocyclic diseases, the inoculum builds up from one year to the next (Agrios 1997; Anggraeni and Lelana 2010). Gall-rust disease attacks sengon plants during all growth stages, starting from seedlings, young, and mature plants (Rahayu et al. 2009). The infection of gall-rust in the sengon plant can inhibit plant growth, promote deformities, and could kill the plant, especially at a young age. *Uromycladium falcatariae* reduces sengon productivity by causing the death of young trees and decreasing the price of sengon wood when sold (Baskorowati 2014). Gall-rust spreads rapidly, affecting wood productivity (Rahmawati et al. 2019). The spread of the disease was faster in foggy areas, highland areas, or stands that received less sunlight. The relative humidity and wind speed were two dominant environmental conditions supporting gall rust disease development (Rahayu et al. 2018).

Various control methods, such as cultural, biological, physical, and chemical approaches, have been tried to control gall rust disease. However, none is effective. Reducing the initial inoculum is considered the most effective for managing monocyclic pathogens. Control measures such as the removal of pruning debris will reduce the initial inoculum (Agrios 1997). Chemical pesticides to control gall rust, such as applying lime sulfur and copper-based fungicide, have been used to protect plant surfaces from infection and eradicate a pathogen that has already

infected a plant (Agrios 1997; Anggraeni et al. 2010, Lelana et al. 2014). Integrating various physical, chemical, and biological control methods seems promising in reducing gall rust disease infestation, although in a large infected area, the effectiveness and economic aspect are questionable. Some small-cale measures applied to sengon seedlings, including selecting nursery locations, using biological agents *Trichoderma* sp., and regular spraying with fungicides were shown effectively prevent the seedling from gall rust disease infection. Further treatment using organic and chemical fertilization, pruning, and chemical fungicide (a mixture of azoxystrobin and difenoconazole) application at the sapling level could also reduce gall rust disease infestation (Lelana et al. 2021).

Biological control of gall-rust diseases needs to be developed to complement the existing management. Control by utilizing microbes is expected to be environmentally safe and cost less than chemicals. To explore the potential of fungi as biological agents controlling gall-rust disease, it is necessary to identify them at the morphological and molecular levels. Morphological identification cannot describe phylogeny up to the species level. DNA barcoding technique can identify and distinguish up to species to species to subspecies level (Hajibabaei et al. 2006). DNA Barcoding utilizes short-sized standard DNA regions as markers for rapid and accurate species identification (Valentini 2009). The object of this study was to isolate and identify fungi associated with the gall rust of sengon plant from the Forest Management Unit of Jatirejo, Indonesian State Forest Company, Kediri, Indonesia.

MATERIALS AND METHODS

Study area

A total of five-gall-rust samples were collected from the Forest Management Unit of Jatirejo, Indonesian State Forest Company, Kediri Regency, Indonesia. The plot was stationed at 461 m asl (meter above sea level) with climate type C based on Schmidt and Ferquson, which has an average rainfall of 2301 mm/year (Figure 1). The type of soil was latosol.

medium for fungal isolation and morphological identification, while PDB was used for molecular identification. PDA (20 g of dextrose, 20 g of potato, and 15 g of agar) and PDB (20 g of dextrose and 20 g of potato) were prepared by dissolving all the ingredients in 1000 ml distilled water. Media was poured into Erlenmeyer and put in an autoclave for sterilization (121°C for 15 minutes).

Isolation of fungi

Fungi were isolated from old gall-rust sengon with black or gray-green spots (with teliospore dark brown), according to Yusuf et al. (2014). The hyphae growing as a spot-on gall-rust were picked and placed on PDA medium containing chloramphenicol. The plates were incubated at room temperature for one week. Then fungal hyphae were transferred to a new PDA to obtain pure cultures.

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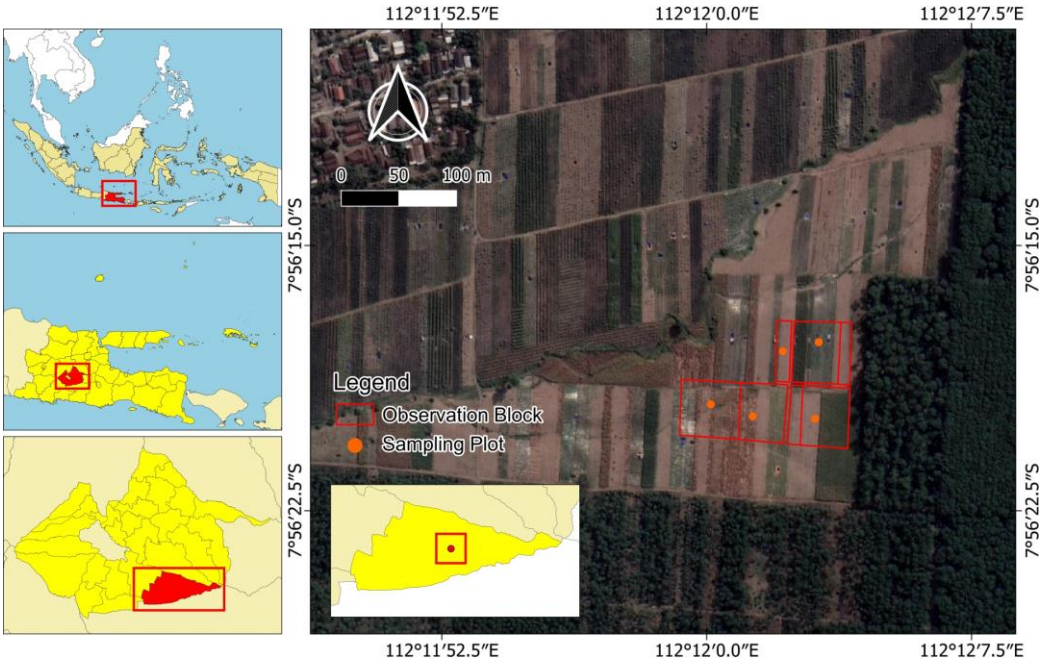

Figure 1. Location of the sampling sites of gall-rust in Forest Management Unit of Jatirejo, Indonesian State Forest Company, Kediri, Indonesia

Isolation of fungi

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Morphological identification of fungi

The fungal morphology was identified based on color, colony characteristics, and microscopic shape. Microscopic features, such as hyphae, conidia, and conidiophores, were observed by a compound microscope with a digital camera. The identification was carried out according to Barnet and Hunter (1998) and Watanabe (2002).

Molecular identification of fungi

DNA isolation

The fungal disc was cut using a sterile cork borer and placed in PDB media, and shook in a shaker for nine days at 18 rpm speeds. The isolate grown on PDB media was then filtered using filter paper. Biomass in the form of fungal mycelia was harvested for DNA isolation process. The DNA isolation of fungal mycelia was carried out using the method of Doyle (1991) with modifications. Each fungal mycelia (0,3 g) were ground with liquid nitrogen using a mortar and pestle and then placed into a 2 mL tube, and 500 µL CTAB extraction buffer, which contains 2% CTAB, 1.4 M NaCl; 0.2% 2 mercaptoethanols, 20 mM EDTA; 100 mM Tris-HCl, pH 8.0 and homogenized. The sample was incubated for two hours at 65°C. The samples were then cooled to room temperature before adding 500 µL chloroform-isoamyl alcohol (24: 1) mixture, mixed, and centrifuged at 10 000 rpm for 10 minutes to obtain clear supernatant. The chloroform: isoamyl alcohol purification was performed twice. The supernatant obtained from the last purification was then transferred to a new tube. After that, the isopropanol with equal volume as the supernatant was added into the tube, as well as a quarter volume of supernatant of 5 M NaCl, then mixed gently. The samples were then incubated at -20 ℃ for 24 h. The mixture was centrifuged at 10000 rpm for 10 minutes to obtain DNA pellets. The DNA pellet was rinsed by adding 500 µL 70% ethanol to pellet and centrifuged at 10 000 rpm for 10 minutes, then the liquid part was discarded, and tube containing pellet dried in a desiccator for approximately 15 minutes. DNA was resuspended in 50 µL TE buffer (10mM Tris; 0.1 mM EDTA). The isolated DNA was analyzed by 1% agarose gel (stained with 1 µL GELRED Safe DNA stain) electrophoresis with 1× TAE (Tris, Acetic acid, EDTA) buffer. The electrophoresis process was performed at 100 volts for 30 minutes. The gel was then illuminated using UV Transilluminator to visualize DNA.

Polymerase chain reaction

Polymerase Chain Reaction (PCR) was performed using MyTaq™ DNA Polymerase (Bioline, London, UK). The primers used were ITS 1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Ben et al. 2021). The PCR solutions contained 1 µL of DNA sample as a template, 1 µL of each primer with 10 µM concentration, 12.5 µL MyTaq DNA polymerase master mix, and nuclease-free water to make a final the volume of 25 µL. The PCR was performed using Applied Biosystems™ Veriti™ DX 96 thermal cycler (Applied biosystem, Waltham, USA) with the following protocol: one cycle of denaturation at a temperature of 95°C for 5 minutes; 35 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds; and one cycle of final extension at temperature 72°C for 7 minutes. The PCR result was analyzed by electrophoresis in 2 % agarose gel.

DNA sequencing and analysis

The PCR products obtained was sent for sequencing to the 1st Base Laboratory Singapore branch. The raw data then go through quality control using Codon Code aligner (https://www.codoncode.com/aligner/) to remove ambiguous and low-quality bases and combine the forward and reverse sequences. The full sequences were then aligned with the sequence from the database using Basic Local Alignment Search Tool (BLAST) menu in the National Center of Biotechnology Information (NCBI) using nucleotide blast (blastn) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to identify the samples. The phylogenetic tree was constructed using MEGA X software (https://www.megasoftware.net/) with the ClustalW method (Kumar et al. 2018) using the homologue sequences from the database. The tree was constructed using the Kimura-2 Parameter model (Kimura 1980) with Gamma distribution (K2+G). The sequence of Halophytophthora exoprolifera (Phytiaceae, Oomycete) was also used from NCBI with accession number MN565893.1 as outgroup species.

RESULTS AND DISCUSSION

Isolation of fungi

The result showed that gall rust pathogen isolated from sengon was *U. falcatariae*. Galls rust on swollen distorted stems containing teliospores on its surface (Figure 2.A-B). Fungi associated with gall rust were isolated from old gall rust with dark brown teliospore of sengon. Teliospores of *U. falcatariae* were collected from gall rust, placed at the center of the glass slide with one drop of water and the cover glass placed over the specimen, and viewed with a compound microscope. Teliospores of *U. falcatariae* were globose or subglobose, and yellowish-brown in color (Figure 2.A-C).

Morphological identification of fungi

The macroscopic identification was performed by comparing fungal colony color and shape characteristics. The results of fungal isolation found 9 isolates with different morphological characteristics. Some isolates had

white colony color, accompanied by pink or purple; one had a black color (Table 1 and Figure 3).

Table 1. Colony characteristics of fungal isolates associated with sengon gall rust

Isolates	Surface	Reverse	Texture	Patterns
Fusarium oxysporum MP 1	White	Orange	Powdery	Flowery
F. oxysporum MP 3	White	Purple	Cotton	Radiate
F. chlamydosporum MP 6	White	Yellowish	Powdery	Zonate
F.circinatum MP 7	White	Orange	Cotton	Radiate
F. solani MP 8	White	White	Cotton	Radiate
Lasiodiplodia theobromae MP 9	Black	Black	Moss	Radiate
F. verticilliodes MP 10	White	White	Cotton	Radiate
F. incarnatum MP 11	White	Orange	Powdery	Radiate
Pestalotiopsis palmarum MP 12	White	White	Cotton	Zonate

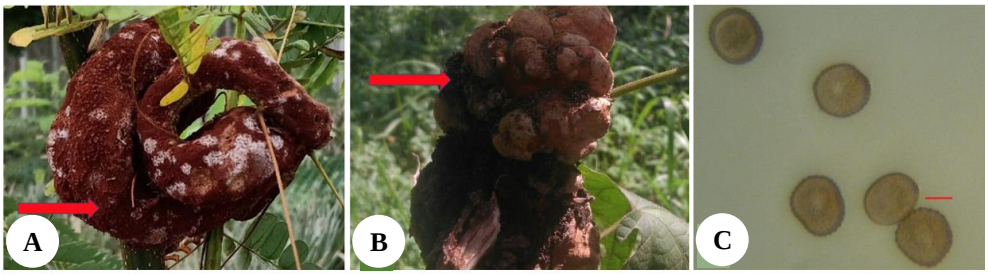

Figure 2. A. Gall-rust on the stem with red-brown teliospores on its surface, B. Old gall-rust become grey or black, C. Teliospores of *Uromycladium falcatariae.* Scale bars = 10 µm

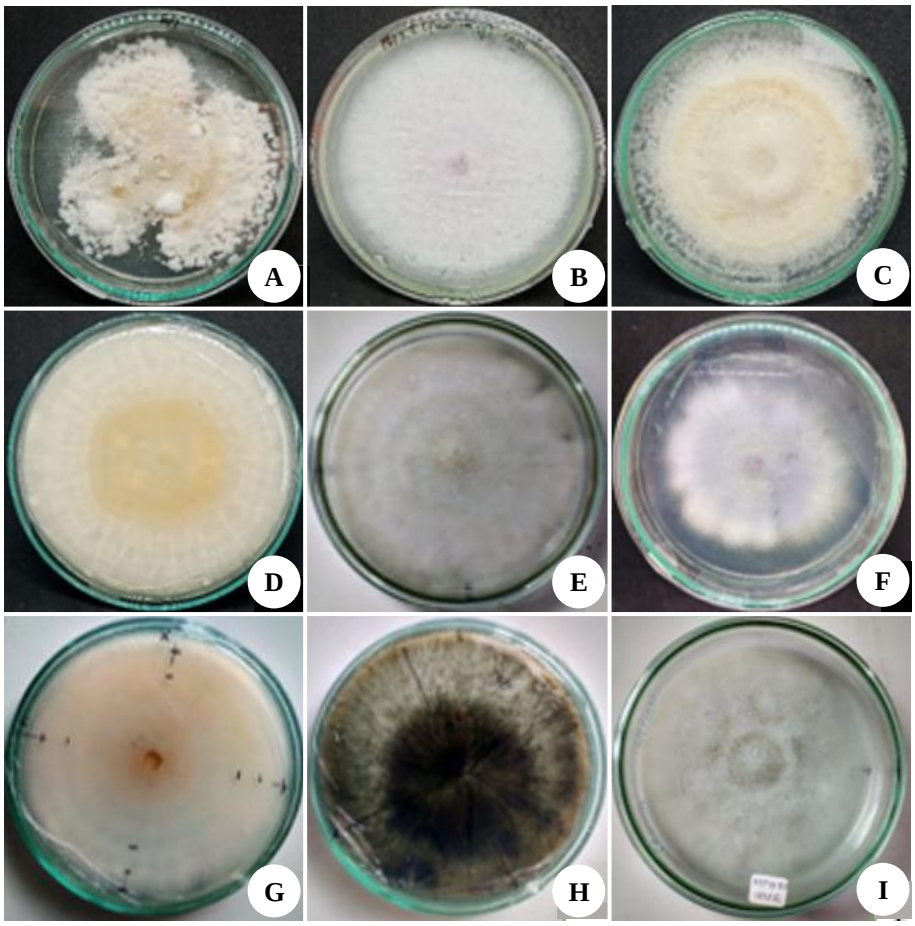

Figure 3. The cultural characteristics of fungi associated with gall-rust**:** A. *Fusarium oxysporum* MP1, B. *F. oxysporum* MP3, C. *F. chlamydosporum* MP6, D. *F. circinatum* MP7, E. *F. solani* MP8, F. *F. verticillioides* MP10, G. *F. incarnatum* MP11, H. *Lasiodiplodia theobromae* MP9, I. *Pestalotiopsis palmarum*

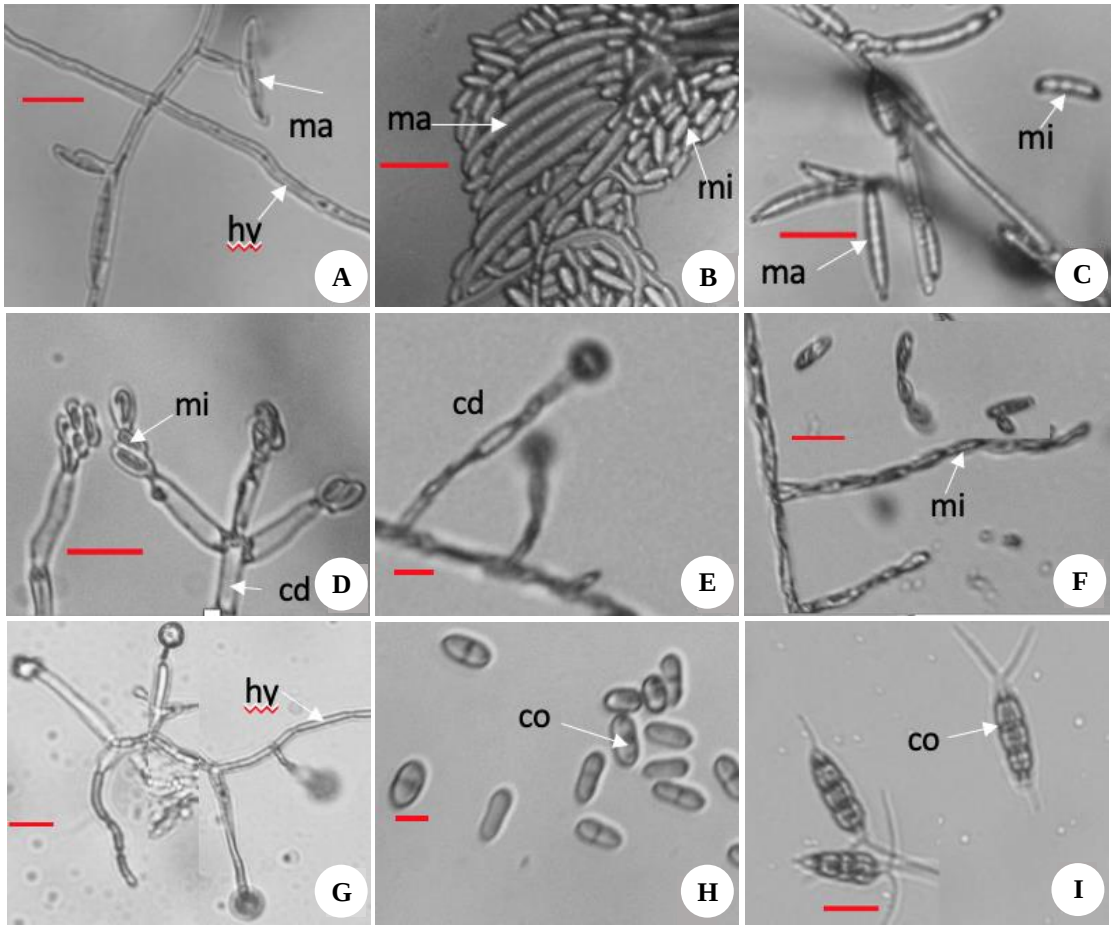

Figure 4. Microscopic morphology of 9 isolates. A. *Fusarium oxysporum* MP1, B. *F. oxysporum* MP3, C. *F. chlamydosporum* MP6, D. *F. circinatum* MP7, E. *F. solani* MP8, F. *F. verticillioides* MP10, G. *F. incarnatum* MP11, H. *Lasiodiplodia theobromae* MP9, I. *Pestalotiopsis palmarum* MP12. Scale bars =10 µm. hy: hypha, cd: conidiophore, mi: microconidia, ma: macroconidia, co: conidia

The results of cultural characteristics and microscopic identification (Figure 3 and 4) showed that from the nine isolates of fungi, seven isolates belonged to the genus *Fusarium* (MP1, MP3, MP6, MP7, MP8, MP10, and MP11), one isolate of *L. theobromae* (MP9), and one isolate of *P. palmarum* (MP12). Most *Fusarium* sp. had white colored colonies and having purple, pink, or yellowish colors in the center. The colony of *Lasiodiplodia* began to grow white-colored mycelium and then turned grey and black. *Pestalotiopsis palmarum* MP12 colonies had white color, cotton textured, a circle pattern with thicker mycelium, and a circle with thinner mycelium (Figure 3).

Fusarium had simple, septate and hyaline conidiophores. *Fusarium* produced both macroconidia and microconidia. Macroconidia had several-celled, boat-shaped and found only in *F. oxysporum* MP1, MP3, and MP6 isolates, while microconidia were ellipsoidal, and 1-celled. Chlamydospores were not found in all *Fusarium* species (4a-g). Conidia of *L. theobromae* MP9 were ellipsoid with a septum in the middle (Figure 4.H). *Pestalotiopsis palmarum* MP12 had dark brown, fusiform conidia consisting of with 4-5 cells with straight or curved cell edges. Both ends of the conidia had 1-2 apical appendages or hairs (Figure 4.I).

Molecular identification of fungi

The molecular identification results using DNA sequence are shown in Table 2. The amount of nucleotide bases covered in the BLAST analysis was displayed in the query cover. Molecular identification results showed that MP12 sample showed 100% query cover and 99.81% similarity with *P. palmarum*, and MP3 sample showed 100% query cover and 99.10% similarity with *F. oxysporum*. Some samples had <90% identity and <90% query cover, which may be caused by the length differences in the samples and database sequences.

The phylogenetic tree was constructed using respective sequences from isolates in the GeneBank database due to low % identity in most of the identified species. The results of the phylogenetic tree analysis formed groups according to their respective genera. This shows a positive result because the fungus has been grouped by species.

The phylogenetic tree analysis formed the species into groups according to their respective genera, as shown in Figure 5 and Table 2. This shows a positive result because species have grouped the fungus.

Table 2. Results of fungal molecular identification

Isolates	Species	Query cover	% Identity	Accession No.	GenBank isolates
MP1	*Fusarium oxysporum*	85%	88.10%	KU671036.1	FLS58
MP3	*F. oxysporum*	100%	99.10%	MT448908.1	TWD26
MP6	*F. chlamydosporum*	94%	92.86%	MN871803.1	DFRP8
MP7	*F. circinatum*	80%	84.87%	MN326463.1	FCVF
MP8	*F. solani*	87%	96.01%	MW326307.1	Rh_231
MP9	*Lasiodiplodia theobromae*	91%	93.94%	MW254946.1	AC_16L
MP10	*F. verticillioides*	89%	98.83%	MW928602.1	QG7
MP11	*F. incarnatum*	92%	96.63%	MT560229.1	CBB-3
MP12	*Pestalotiopsis palmarum*	100%	99.81%	GQ888738.1	CLB5

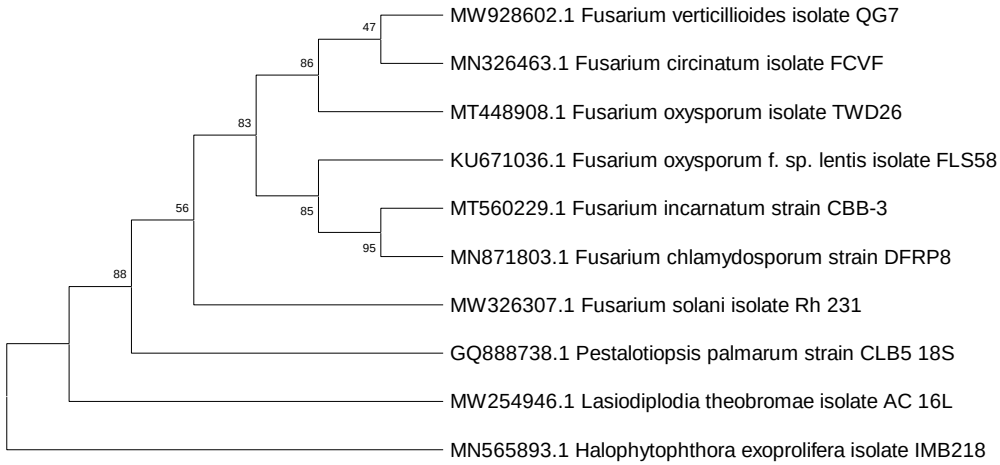

Figure 5. Phylogeny tree of 9 fungi species associated with gall-rust in *Falcataria moluccana*, based on ITS locus with Maximum Likelihood method and bootstrap test 1000, using the Kimura-2 Parameter model with Gamma distribution

Discussion

Nine fungal isolates, namely *F. oxysporum* (MP1 and MP3), *F. chlamydosporum* (MP6), *F. circinatum* (MP7), *F. solani* (MP8), *F. verticillioides* (MP10), *F. incarnatum* (MP11), *L. theobromae* (MP9), and *P. palmarum* (MP12) belonging to three different genera were found associated with gall-rust disease of sengon. Most of the fungi belonged to the genus *Fusarium.*

Macroscopic observations showed a similarity in the morphology of colony colors in a large number of *Fusarium* isolates spp. Most isolates of *Fusarium* spp. had white colonies with purple or pink color centers. *Fusarium* spp. has a wide variety, both in the same medium and in different mediums (Leslie and Summerell 2003). According to Mekuria and Alemu (2020), the colony of *Fusarium* species on PDA media showed white, creamy white, dull pink, and pink coloration. *Fusarium* spp forms three types of asexual spores: microconidium, macroconidium, and chlamydospore (Leslie and Summerell 2003; Sutejo et al. 2008).

Identification of fungi by morphological observations is not accurate enough to classify *Fusarium* spp. as this fungus has a high variation in the same and different mediums (Leslie and Summerell 2003). Morphological-based identification sometimes has its limitation in determining the identity of fungal species or even at the lower level due to some reasons, i.e., hybridization between species, convergent evolution, and cryptic speciation (Raja et al. 2017). Compared to other eukaryotes kingdoms, the simple features of fungi, and in some fungi only limited in vegetative structures, made the identification process harder, sometimes not even giving accurate identifications (Lücking et al. 2020). The identification process also requires extensive necessary resources and taxonomy knowledge (Nilsson 2011). However, we cannot say that the identification based on morphological features is incompetent. At least the morphological features could become the first guide or hint in determining the species identity.

To confirm the identification based on the morphological features, further molecular identification using DNA is essential to support the identification. However, DNA-based identification was not flawless in fungal identification. Since the identification relied solely on comparing the sample sequence and the sequences deposited in the database, problems with the correctness of the database could lead to the wrong conclusion (Xu 2016). This issue was resolved by GenBank by re-annotating and

formatting the curated database (Schoch et al. 2014). One of the advantages of utilizing DNA barcoding in fungal identification was that the identification process could be conducted at any of the fungal phases. Therefore, the identification could be performed even though the fungi specimen did not have a complete phase (Aslam et al. 2017).

Further molecular identification using DNA is necessary to confirm identity-based on morphological features. Phylogeny describes a kinship based on the composition of DNA sequences and is used to estimate evolution that has occurred in the past (Handayani 2016). The phylogeny analysis was compiled using the Maximum likelihood method of the Kimura 2-parameter model with 1000x bootstrap. The bootstrap value on the phylogeny tree has a scale of 1-100% and indicates the phylogenetic branch's stability. The higher the bootstrap value, the more stable and accurate the branching position of the phylogeny tree (Putri 2015). Results showed that the phylogeny tree had a bootstrap value exceeding 50. A bootstrap value above 50 indicates that the construction of the phylogeny tree is quite good and consistent (Robbani 2016). In addition, based on the phylogenetic tree, the same *Fusarium* species occurred on the same branch. This result shows that the markers used are effective in classifying Fusarium species. The best model analysis gave Kimura-2 parameter with a gamma distribution as the best model. The parameter was best used in species with low genetic distance (Hebert et al. 2003). Since all of our samples were members of the Ascomycetes phylum, with the majority coming from *Fusarium* genera, the genetic distance most likely was low. Hence the Kimura-2 parameter was the correct parameter to be applied.

Fusarium, *Pestalotiopsis*, and *Lasiodiplodia* are all members of the Ascomycota phylum, many species of which are pathogens, especially in plants (Berbee 2001). However, at the species level, the placement of *P. palmarum* in the phylogenetic tree was mixed with all the *Fusarium* species found in the present research. Even though *Fusarium* and *Pestaloptiosis* are from the same class (Class: Sordariomycetes), species from both genera did not group into the same group in another study by Giraldo-Arias et al. (2018). The mixed species phenomenon might be because, in calculating the distance, the K2P model performed poorly at the species level, even though the difference with the best model was minimal (Collins et al. 2012).

Fungi associated with gall rust that are isolated have the potential to cause plant diseases and antagonistic agents. The *F. oxysporum* is a fungus that causes various diseases, e.g. wilt disease in some plants. In the present research, the most commonly found fungus on the gall rust teliospore surface was *Fusarium*. *F. circinatum was* reported as a pitch canker on pine, which causes extensive tree death, decreased growth, and reduced wood quality. This fungus also infects pinecones and causes damping (premature death caused by fungal infection in moist conditions) in plant seeds. *F. incarnatum* was reported to cause root rot in watermelon plants in Georgia (Li and Ji 2015) and China (Li et al. 2018). According to Anggraeni and Lelana (2011), *Lasiodiplodia* spp. is a pathogen that causes leaf spot disease in a number of forestry plants. *L. theobromae* caused various diseases, such as leaf spots, root rot, fruit rot, and the death of plant shoots (Espinoza et al. 2008). The infected plant was diverse, ranging from fruit trees to crops and forestry plants. In the case of forestry plants, this fungal caused plant shoot death in Jabon seedlings in Bogor to reach 15% (Falah et al. 2015).

Non-pathogenic *F. oxysporum* isolates CAV 255, and CAV 241 reduced the incidence of fusarium wilt diseases in bananas with efficacy of 87.4% and 75.0% (Nel et al. 2006). Based on research by Isniah and Widodo (2015), non-pathogenic *F. oxysporum* decreased fusarium base rot disease in red onion with efficacy up to 50% and higher than the fungicide treatment with the active ingredient benomyl. As stated by Mathivanan and Murugesan (2000), *F. clamydiosporum* can be used as a biocontrol agent for gall-rust disease on peanuts plant caused by *Puccinia arachidis*. *F. verticilioides* is often associated with symptomatic and asymptomatic plants as a primary disease agent, secondary invader, or endophytic fungus (Pampile and Azevedo 2002). *Lasiodiplodia theobromae* is known as the endophytic fungi. Many studies have shown that various fungi can synthesize bioactive products, and *L. theobromae* is one of the fungi with a high ability to synthesize bioactive products (Salvatore et al. 2020). *Pestalotiopsis palmarum* is an endophytic fungus associated with the medicinal plant *Sinomenium acutum* (Thunb.) Rehd et Wils. Other study showed ethyl acetate extract from the *P. palmarum*, sinopestalotiollides A-D, showed weak to potent cytotoxicity against three human tumor cells (Xiao et al. 2018). It is necessary to performed pathogenicity test to know the ability of fungus identified in this research as a mycoparasite against the fungus that causing gall rust (*U. falcatarium*).

In conclusion total of 9 fungal isolates were isolated from the gall-rust body that were characterized by the presence of gray-greenish or blackish fungal hyphae growth. All isolates were identified at the macroscopic and microscopic levels. DNA barcode identification results showed 9 isolates consisting of 8 species of fungi and identified as *F. oxysporum, F. solani, F. chlamydosporum, F. circinatum, F. incarnatum, F verticillioides, L. theobromae*, and *P. palmarum*. The present investigation showed that *Fusarium* was the most commonly found fungus on the gall's rust teliospore surface.

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