Molecular identification of rust disease on Acacia mangium collected from West Java, Indonesia

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Abstract. Acacia mangium is one of wood producing species widely planted in industrial and small scale forest. The only rust disease that has been reported on A. mangium is the phyllose rust caused by Endoraecium digitatum (syn. Atelocauda digitata). Some revision had been made for E. digitatum and some new species had been proposed on acacias. However, the information for Indonesian isolates is still limited. We found rust disease on A. mangium plantation in Tasikmalaya, West Java that had not been reported previously. Morphological identification is quite difficult to determine the species of those rust pathogen. Therefore, a molecular approach is needed. This study was aimed to identify the rust disease of A. mangium based on sequence data of internal transcribed spacer rDNA. The DNA samples of rust pathogen were isolate using CTAB methods followed with ITS amplification using specific rust primer and sequenced. BLAST analysis based on ITS fragments showed that rust pathogen has 98% identity to the E. auriculiforme. Meanwhile phylogenetic analysis showed that the rust pathogen on A. mangium was more closely related to the E. auriculiforme.

1. Introduction

Acacia mangium Willd., A. crassicarpa Cunn. ex Benth., A. aulacocarpa Cunn. ex Benth., and A. auriculiformis Cunn. ex Benth., are major tropical acacias species in South East Asia forest plantation [1]. Among those species, A. mangium is the most dominant in plantation forest in Indonesia. Acacia mangium is a member of Fabaceae family occurring naturally in northern Australia, eastern part of Indonesia, and Papua New Guinea. The tree is preferred as one of fast-growing tree species planted in industrial plantation. In Indonesia, A. mangium plantations with an area of around 1.3 million ha have been established for pulpwood production [2, 3]. Acacia mangium is also one of dominant species in smallholder plantation. Based on agricultural census in 2013, the population of A. mangium in smallholder plantation was about 7% [4].

Most of plantation forest area in Indonesia is in central and western part. Although A. mangium is indigenous plant in Indonesia, ecologically this tree is exotic. In their native habitats, there are no serious diseases have been reported for tropical acacias [5, 6]. However, in the area outside their native habitat, several serious diseases of this trees have been reported. Some of the diseases are root rot caused by Ganoderma sp. and Phellinus spp.; pink disease caused by Corticium salmonicolor;
phyllode rust caused by *Endoraecium digitatum*; heart rot caused by *Phellinus noxius*; and Ceratocystis wilt [7, 8, 9, 10].

For the rust disease, the only one that has been reported on *A. mangium* in Indonesia was the phyllode rust caused by *Endoraecium digitatum* (syn. *Atelocauda digitata*). Previously, *E. digitatum* was considered as a single species with a wide host and geographic distribution. The host range of tropical and subtropical acacias including *A. auriculiformis*, *A. aulacocarpa*, *A. crassicarpa*, *A. leptocarpa*, *A. koa*, *A. mearnsii*, *A. polystachya*, and *A. mangium*, [8]. Disease distribution involved Australia [11], Hawaii [12], Malaysia [6], Indonesia, Papua New Guinea, and China [7, 8]. Specimens of *E. digitatum* from Australia and Hawaii are considered a complex species consisted of some morphologically distinct species [13]. There were *E. parvum* R. Berndt, *E. kauaiianum* R. Berndt, *E. phyllodiorum* (McAlp.) R. Berndt, *E. walkerianum* R. Berndt, and *E. violae-faustae* R. Berndt. However, the morphological and molecular information for acacias rust in Indonesia were still limited. Therefore, this study provides the molecular identification of rust disease from *A. mangium* collected from West Java based on internal transcribed spacer sequence data.

2. Methods

2.1. Sample collection

The samples of rust from *A. mangium* used in this study were collected from Tasikmalaya District, Province of West Java. Field samples were kept in individual plastic bags and maintained in a freezer until further use.

2.2. Symptom and morphological analysis

Disease symptom was observed directly from infected trees. Fungi spores from 5 collected sample were observed under a microscope and spores were collected from galls using soft brush. Spores were then placed in microscope slide glass containing water. A 50% glycerol was added and covered with cover glass. Spore dimensions were determined by measuring the length and width of spores. Measurement were carried out for 20 spores each samples.

2.3. Molecular identification

2.3.1. DNA isolation. The fungus DNA was extracted from the spores using cetyltrimethylammonium bromide (CTAB) method [14] with some modifications. As many as 1 mg of spore was homogenized in a mortar in the presence of 500 μL of (CTAB) extraction buffer. The sample was then transferred into a fresh tube. The tube was vortexed and incubated for 2 hr at 65 °C. After incubation, an equal volume of chloroform-isoamyl alcohol (24:1) was added into the tube. The tube was then vortexed and then centrifuged for 15 min at 11,000 rpm. The supernatant was transferred into a fresh tube. Precipitation DNA was then carried out by adding 2.5 × volume of absolute ethanol and a 1/10 volume of 3 M sodium acetate and incubating for 2 hr at -20 °C. The tube was centrifuged for 15 min at 14 000 rpm and the precipitate was rinsed with 500 μL 70% ethanol. DNA was finally resuspended in 100 μL Tris-EDTA (TE) buffer.

2.3.2. PCR amplification and nucleotide sequencing. The ITS region was amplified using polymerase chain reaction with rust specific primer ITS1rustR3c and ITS1rustFl0d [15]. The reactions were performed with Phusion High Fidelity DNA Polymerase Master Mix (Thermo Scientific). Final concentration included 1 × GC Buffer, 200 μM of each dNTPs, 0.5 μM of each primer, 0.02 Unit/μL of Phusion DNA Polymerase, and 100 ng of DNA template. The conditions of cycling were an initial denaturation at 98 °C for 30 sec; 30 cycles of denaturation at 98 °C for 10 sec, annealing at 58 °C for 20 sec, polymerization at 72 °C for 30 sec; and final polymerization at 72 °C for 5 min. The amplified products were sent to Firtasbase (Singapore) for sequencing.
2.3.3. Agarose gel electrophoresis. To confirm the product, the amplified DNA were visualized on 1% agarose gel (w/v). Agarose gel was prepared using 1 x TAE buffer and stained with SYBR® safe (Invitrogen). DNA sample was loaded into agarose well using 6X gel loading dye. The electrophoresis run on 100 V for 30 min and the DNA then visualized under UV visible light.

Table 1. List of species included in the research.

| No. | Species                     | Host          | Origin                               | ITS GenBank accesion no |
|-----|-----------------------------|---------------|--------------------------------------|-------------------------|
| 1   | *Endoraecium* sp.           | *A. mangium*  | Indonesia: Tasikmalaya, West Java,   | -                       |
| 2   | *E. auriculiforme* BRIP 56549 | *A. auriculiformis* | Australia: Darwin, Northern Territory | KJ862356               |
| 3   | *E. auriculiforme* BRIP 55609 | *A. auriculiformis* | Australia: Darwin, Northern Territory | KJ862353               |
| 4   | *E. phyllodiorum* BRIP 57590 | *A. aulacocarpa* | Australia: Tibrogargan, Queensland   | KJ862382               |
| 5   | *E. violae-faustiae* BRIP 56547 | *A. difficillis* | Australia: Darwin, Northern Territory | KJ862401               |
| 6   | *E. violae-faustiae* BRIP 55601 | *A. aulacocarpa* | Australia: Julatten, Queensland      | KJ862393               |
| 7   | *E. parvum* BRIP 53616      | *A. leiocalyx* | Australia: Carnarvon, Queensland     | KJ862375               |
| 8   | *E. tropicum* BRIP 56557    | *A. tropica*  | Australia: Gregory, Northern Territory | KJ862392               |
| 9   | *E. peggii* BRIP 55631      | *A. holosericea* | Australia: Dimbulah, Queensland      | KJ862362               |
| 10  | *E. tierneyi* BRIP 27887    | *A. harpophylla* | Australia: Springsure, Queensland    | KJ862353               |
| 11  | *E. irroratum* BRIP 55671   | *A. irrorata*  | Australia: Warrumbungle, New South Wales | KJ862364              |
| 12  | *Uromycladium tepperianum* BRIP 56962 | *A. saligna* | Perth, Western Australia           | KJ632996               |

2.3.4. Homology and phylogenetic analysis. Homology analysis was performed using BLAST2 (basic local alignment search tool) program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Analysis of phylogenetic was performed with several species of *Endoraecium* from GenBank (Table 1). Multiple sequence alignments of ITS were performed with MAFFT 7.311 [16]. Phylogenetic tree was constructed with PAUP 4.0a150 [17], using a maximum likelihood (ML) criterion [18].
3. Results and discussion

3.1. Incidence of rust disease on A. mangium
Rust diseases were found on 1 yr old A. mangium plantation in Tasikmalaya, West Java. The disease bears gall formation on phyllodes and shoot tip (Fig. 1a). Previously, this rust disease was identified as *Atelocauda digitate* (syn. *Endoraecium digitatum*). This rust was macrocyclic which produced 5 spore types. There were uredospores, teliospores, basidiospores, pycniospores and aeciospores. Infected phyllodes may produce range of symptom, from proliferation of cell in the form of bullate (blistery-like) swellings or galls to suffer malformation on their phyllodes, shoot tips, and fruits [8]. The gall produce urediniospores in the surface, brown in color and become almost black. The Urediniospores were oval to fusiform, obovoid, apex acute, yellowish brown, 31–42 × 15–19 μm (Fig. 1b).

In Indonesia, the occurrence of *E. digitatum* on A. mangium have only been recorded in Sumatera and Kalimantan. Meanwhile in Java, the occurrence of *E. digitatum* have been recorded only in *A. auriculiformis* [7, 8]. Our report showed that the rust disease was also occurred in A. mangium plantation in Java.

![Figure 1: Gall rust disease on A. mangium (a) and Uredospores of Endoraecium sp. from A. mangium (bar = 20 μm) (b).](image1)

3.2. BLAST and phylogenetic analysis
Fungi amplification using PCR method produced good DNA quality and quantity (Fig. 2). The specific primers amplify ITS1 region with the size about 250 bp. The ITS1 nucleotide fragments of A. mangium gall rust pathogen from West Java, Indonesia was aligned to the database GenBank using BLAST program. The highest identities for BLAST search were *Endoraecium auriculiforme* with accession number KJ862353 and KJ862354 (Table 2). Both of them resulted in 98% identity (query cover = 100%).

![Figure 2: The results of ITS1 fragment amplification of acacia rust disease.](image2)
Table 2. BLAST alignment of the ITS fragment rust pathogen with GenBank database.

| Acession                        | Identities (%) | Query cover (%) | E-value |
|---------------------------------|---------------|----------------|---------|
| Endoraecium auriculiforme BRIP 56550 | 98            | 100            | 5e-113  |
| Endoraecium auriculiforme BRIP 55609 | 98            | 100            | 5e-113  |
| Endoraecium peggii BRIP 58324    | 96            | 100            | 3e-105  |
| Endoraecium phyllodiorum BRIP 57580 | 89            | 100            | 2e-77   |
| Endoraecium disparrimum BRIP 55632 | 87            | 100            | 1e-69   |
| Endoraecium violae-faustiae BRIP 55611 | 85            | 100            | 2e-62   |

Phylogenetic trees were constructed using fragments of ITS. The non-homologous areas were eliminated in phylogenetic tree construction. Phylogenetic trees which are quite congruent in branching and grouping taxa are produced by maximum likelihood (Fig. 3). The rust on A. mangium from Java was on the same clade with E. auriculiforme and E. peggii. These 2 species had hosts that are in the same subclade, A. auriculiformis group. This group forms a different clade with E. phyllodiorum and E. violae-faustiae which had different subclade host (A. aulococarpa group).

**Figure 3.** Phylogenetic tree obtained from maximum likelihood search of ITS1 gene region. Host clades and sections are mapped on the tree.

The fungi *E. digitatum* has a wide range of hosts and varied morphological forms, therefore it is indicated as a complex species. *E. digitatum* are divided into 5 new species based on its morphology and host range, namely *E. kauaiianum*, *E. phyllodiorum*, *E. violae-faustae*, *E. parvum*, and *E. walkerianum*. Among these species, the only rust fungi known on *A. mangium* were *E. parvum* and *E.*
The gall rust diseases that were found on 1 year-old A. mangium plantation in Tasikmalaya, West Java was not E. digitatum as described from the revision proposed for E. digitatum. However, the BLAST search and phylogenetic data is not enough to conclude the exact name for the species. It could be E. auriculiforme as on A. auriculiformis or a new species as described by McTaggart et al. [19]. A comparison study with rust on A. auriculiformis from Java and A. mangium from outside Java is further needed to confirm the species.

4. Conclusion
The gall rust diseases that were found on 1 yr-old A. mangium plantation in Tasikmalaya, West Java was not E. digitatum as described from the revision proposed for E. digitatum. However, the BLAST search and phylogenetic data is not enough to conclude the exact name for this species. It could be E. auriculiforme as on A. auriculiformis, E. violae, E. auriculiforme, and E. phyllophorum [13]. The known host of E. digitatum was A. notabilis in Botrycephalae subclade [13]. Meanwhile the 5 new species were included in Juliflorae subclade. McTaggart et al [19] in his further study based on morphology, molecular data, and host taxonomy proposed the revision for E. parvum, E. digitatum, E. violae-faustiae, and E. phyllophorum. Nine new species of Endoraecium from Australia are then described. The rusts on A. auriculiformis was then identified as E. auriculiforme, meanwhile the rust on A. mangium was still unknown.

Rust fungi on A. mangium from Java is in the same clade with E. auriculiforme. BLAST search and phylogenetic data confirmed that E. digitatum was not the rust on A. mangium from Java but it is still not enough data to conclude the exact name for this species. It could be E. auriculiforme as on A. auriculiformis or a new species as described by McTaggart et al. [19]. A comparison study with rust on A. auriculiformis from Java and A. mangium from outside Java is further needed to confirm the species.

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