

Molecular Identification of Fungal Species Causing Brown Circular Leaf Spot Disease in Seedlings of Siamese Rosewood (*Dalbergia cochinchinensis* Pierre ex Laness)

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ABSTRACT

Morphological and pathological features of fungi isolated from leaves of *Dalbergia cochinchinensis* Pierre ex Laness seedlings (Siamese rosewood) with brown circular leaf spot disease have previously been reported. According to morphology, there are different fungi that cause this disease but information is only available on the fungi genera e.g. *Curvularia* sp., *Alternaria* sp., and *Colletotrichum* sp. including an unknown rust fungus. In order to identify the species of these fungi, molecular identification using information derived from the amplification of internal transcribed spacer sequences (ITS) via ITS1, ITS4 and ITS5 primers and phylogenetic analysis was performed. According to the results obtained from the analysis using Neighbor-joining method, the isolated fungal species from the diseased leaves were revealed as *Alternaria alternata*, *Colletotrichum siamense*, *Curvularia aerea* and *Maravalia pterocarp*, with supportive bootstrap scores. This research reports the occurrence of the fungal species on this plant to provide additional data for controlling the causal agents of the disease in a more specific and targeted manner.

Keywords: Economic plant; Leaf spot; Plant pathogenic fungi; Rosewood; Seedlings

1. Introduction

The economic plant known as Siam rosewood (*D. cochinchinensis*) in Thailand is highly valuable, at approximately 250,000 Baht per cubic meter and is also included in the IUCN Red List of threatened species [1]. Seedlings of this plant found with severe brown circular leaf spot disease during the seedling stage were collected from two nurseries, Mahasarakham Forest Nursery Center and Khon Kaen Forest Nursery Station. It has been reported that this disease is caused by multiple fungi, morphologically identified as *Curvularia* sp., *Alternaria* sp., *Colletotrichum* sp., and an unknown rust fungus [2]. They are common plant pathogenic fungi able to cause diseases in many plants. *Curvularia* is a fungal genus consisting of more than 40 species, most being phytopathogenic, and are able to bring significant losses to agricultural operations [3]. Many of the fungi in this genus are reported as causes of foliar diseases; for example, in China, *C. microspore* is a new species causing leaf diseases of *Hippeastrum striatum* [4], *C. eragrostidis*, *C. geniculata*, *C. hawaiiensis*, *C. aerea* and *C. lunata* are causing rice leaf spots in Malaysia [5], *C. verruculosa* is causing leaf spots on *Cynodon* sp. in Hubei, China [6] and leaf spots on lettuce are being caused by *C. aerea* [7] as well as many *Curvularia* species causing leaf blight and spots in oil palm seedlings in Thailand [8]. Another common fungal leaf-disease relating agent is *Alternaria*, which can cause leaf spot and blight diseases in various plants e. g. *A. alternate*, *A. brassicicola* and *A. brassicae* [9-13]. *Colletotrichum* species are also significant pathogenic fungi leading to major damage in different plants e. g. citrus, tulip and rubber trees [14-16]. Apart from these common fungi, rusts such as basidiomycetes are reported as another prominent phytopathogen e.g. *Austropuccinia psidii* causing leaf spot rust on *Rhodamnia rubescens* and *Eugenia reinwardtiana*, *Thekopsora minima* on blueberry, *Puccinia*

pelargonii-zonalis on geranium, and *P. arachidis* on *Arachis hypogaea* [17-19].

Seedlings are vulnerable to infection because of the environment for nursing the seedlings. They are usually maintained in moist conditions and are put close to the ground where there are easily exposed to pathogens [20-21]. In these two nurseries, the plant seedlings were found to be dramatically invaded by the fungi on their leaves, resulting in poor quality of the seedlings before being distributed to farmers. Although the pathological aspects have been studied, the species identification of these fungi was still left to question. According to the morphology of the fungi reported in a previous study [2], the species was unable to be identified. Thus, this gap in knowledge left unfilled by the previous study leads to the purpose of this study, aiming to decipher the species identity of the unidentified fungi, seeking better plant management strategies and control methods that are more precise and possibly species specific.

2. Materials and Methods

2.1 Fungal isolation and genomic DNA extraction

The fungi were isolated and cultured in synthetic media according to previously used methods [2]. Spores of the rust fungus, taken directly from diseased leaves, were briefly washed in 95% ethanol and passed through sterile distilled water 3 times, and fresh fungal mycelia, approximately 1 g were also taken from the culture for genomic DNA extraction via a previously reported method [22]. Briefly, the fungal mycelia were cultured for 7-10 days on potato dextrose agar with cellophane paper and the mycelia on the surface of the paper were scrapped out before the addition of liquid nitrogen. The mycelia were finely ground with cetyltrimethyl ammonium bromide (CTAB) lysis buffer (600 μ l), and then transferred into new Eppendorf tubes. The tubes were incubated at 60°C for 60 min and then 700 μ l of chloroform: isoamyl (24:1) was added

before centrifugation at 12,000 rpm for 5 min at 4°C. In new tubes, only supernatants were collected and combined with isopropanol, 0.7 volume of the supernatant. The tubes were gently mixed then stored at -20°C for 20 min before 5-20 min of centrifugation at 12,000 rpm at 4°C in order to get DNA pellets. Ethanol (500 µl, 70%) was added to the tubes to clean the precipitated pellets again and left at room temperature until dry. Finally, TE buffer made up of Tris-HCl (10 mM) and EDTA (1 mM), 40 µl was added to dissolve the dried pellets and was then stored at -20°C for further use.

2.2 PCR amplification

To amplify the informative region for species identification, different primers were used, primers for general fungal identification ITS1-TCCGTAGGTGAACCTGCGG, ITS4-TCCTCCGCTTATTGATATGC and ITS5-GGAAGTAAAAGTCGTAACAAGG [23] for *Curvularia* sp., *Alternaria* sp., and *Colletotrichum* sp. isolates, and primers specific to rust fungi ITS1rustF10d-TGAACCTGCAGCAGGATCATTA, ITS1rustR3C-TGAGAGCCTAGAGATCCATTGTTA [24] for the unknown rust isolates because the ITS primers were unsuccessful. Before performing PCR, the extracted genomic DNA was diluted 10-fold with autoclaved dH₂O. The 25-µl PCR reaction was composed of 5 µM of each 20 mM primer solution, 2.5 ul of 20mM dNTP, 2.5 ul of 10x Immo buffer, 0.75 ul of 50 mM MgCl₂, 0.15 ul of *Taq* DNA polymerase, 2 ul of diluted genomic DNA and 14.6 ul of dH₂O. The PCR conditions were set as follows: 95°C for 7 min then 35 cycles of 95°C for 1 min, 55°C for 1.5 min then 72°C for 2 min and final extension at 72°C for 10 min. Electrophoresis with 1.2% agarose in TBE buffer (0.01 M EDTA, 0.9 M boric acid, 1 M Tris-base, pH 8.3) was done for 1h to investigate the successful PCR yields then stained with Redsafe™ Nucleic acid staining solution and

visualized under UV light. Samples with products were in-gel purified and sequenced at 1st BASE, Apical Scientific Sdn Bhd, Selangor, Malaysia.

2.3 Phylogenetic analysis

Prior to phylogenetic analysis, DNA sequences were trimmed and evaluated from unclear chromatograms on Sequence Scanner Software v2.0. According to BlastN search, fungal DNA sequences of Internal transcribed Spacer (ITS) regions were selected from GenBank (www.ncbi.nlm.nih.gov) in order to construct the phylogenetic tree (Table 1). The sequence alignment of the sequences from the database with the ones from this study was performed using ClustalW and edited manually using MEGA 6.06 [25]. The analysis parameters of Neighbor-joining methods with 1,000 bootstrap replicates were as follows: Jukes-Cantor method was defaulted for processing the distances in the units of the number of base substitutions per site. To model the alteration rate of sites, gamma distribution was set for the calculation and the elimination of gaps was applied to all positions. The same software was also used to visualize the resulting trees. Bootstrap scores shown on tree nodes greater than 50 were considered as being supportive [26].

Table 1. Sequences of fungal species and their accession numbers from GenBank.

Fungal species	Accession no.	References
<i>Alternaria alternata</i>	KP124317	[27]
	KP124318	
	KP124322 KP124370	
<i>Alternaria brassicae</i>	KP115600	[27]
	KP115601	
	KP115602	
<i>Alternaria brassicicola</i>	JX499031	[28]
	JX648198	
	JX499030	
	JF417686	[29]
<i>Alternaria cassiae</i>	KJ718135	[30]
	KJ718136	
	KJ718137	
	KJ718138	

Fungal species	Accession no.	References	Fungal species	Accession no.	References
<i>Alternaria passiflorae</i>	KJ718209	[30]	<i>Melampsora larici-populina</i>	DQ228110	[48]
<i>Alternaria</i> sp.	This study	<i>Alternaria</i> 001, 002, 003	<i>Marvalia pterocarpi</i>	KU301795	[49]
<i>Colletotrichum kahawae</i>	KC735140 KC735141 KC735142 KC790932	[31]	Rust	This study	Rust1, 2, 3
<i>Colletotrichum siamense</i>	KP703383 KP703384 KP703386 KP703390	[32]			
<i>Colletotrichum gloeosporioides</i>	JX010151 JX010152 JX010153	[33]			
<i>Colletotrichum gigasporum</i>	KF687723 KF687724 KF687725 KF687726	[34]			
<i>Colletotrichum acutatum</i>	JQ948395 JQ948396 JQ948397 JQ948398	[35]			
<i>Colletotrichum cereal</i>	JX625161	[36]			
<i>Colletotrichum</i> sp.	This study	Collet 1, 2, 3			
<i>Curvularia aerea</i>	KF941270 KP340066 KP340067	[37] [38]			
<i>Curvularia affinis</i>	JX966602 JX966610 KR012898 KP340047	[39] [40] [38]			
<i>Curvularia eragrostidis</i>	KP131873	[41]			
<i>Curvularia geniculata</i>	KP131874 KP131875	[41]			
<i>Curvularia lunata</i>	JX960584 JX960585 JX960594	[42]			
<i>Curvularia oryzae</i>	KP400650 MH857148 MH859835	[43]			
<i>Curvularia</i> sp.	This study	Cur 1, 2, 3			
<i>Puccinia coronata</i>	GU598112 GU598111 GU598110 HM131335	[44] [45] [46]			
<i>Puccinia monoica</i>	AF182994 AF182996 AF182997 AF182993	[46]			
<i>Puccinia graminis</i>	HQ012445 HQ012444 HQ012440 HQ012441	[47]			

3. Results and Discussion

The Neighbor-Joining analysis revealed these 3 *Curvularia* isolates derived from the seedlings were *Curvularia aerea* (Fig. 1). The total branch length was 0.20514756. The supportive bootstrap scores above 50 are shown at the tree nodes. In the analysis, there were 19 nucleotide sequences with a total of 638 positions in the final dataset. Among *Curvularia* species, the consensus outcome from the analysis was highly supported by the bootstrap at 81. It can be concluded that the fungus is *Curvularia aerea*.

As illustrated in Fig. 2, the phylogenetic inference using the Neighbor-Joining method with the total branch length of 0.09244556 revealed the species of *Alternaria* isolates 001, 002, and 003 as *Alternaria alternata* supported by the high bootstrap score of 100. This analysis involved 20 nucleotide sequences. There were 481 informative positions total in the final dataset included in the analysis.

The identification of *Colletotrichum* isolates was suggested by Neighbor-Joining analysis and yielded the phylogenetic tree with the total branch length of 0.17737448. It suggested the *Colletotrichum* isolates Collet 1, 2, and 3 were clustered inside *Colletotrichum siamense* branch with a supportive bootstrap score of 84. For the final dataset put in the analysis, there were 23 nucleotide sequences with a total of 622 positions (Fig. 3).

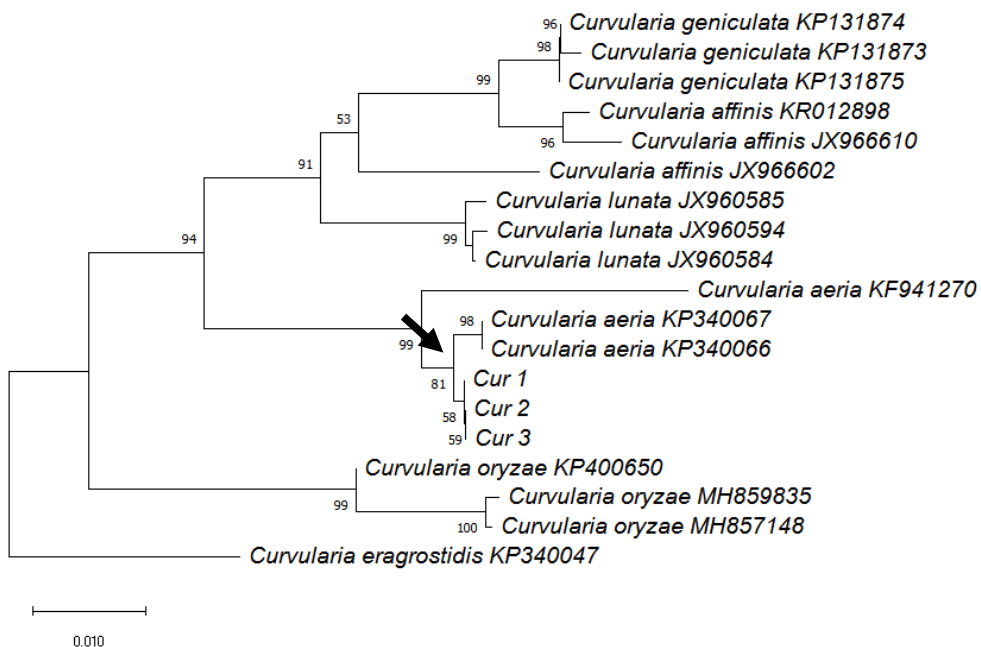


Fig. 1. The phylogenetic tree for *Curvularia* isolates using the Neighbor-Joining method with bootstrap test of 1000 replicates suggested that the fungal isolates were *Curvularia aerea*. The inference was supported by the bootstrap score of 81 (arrowhead).

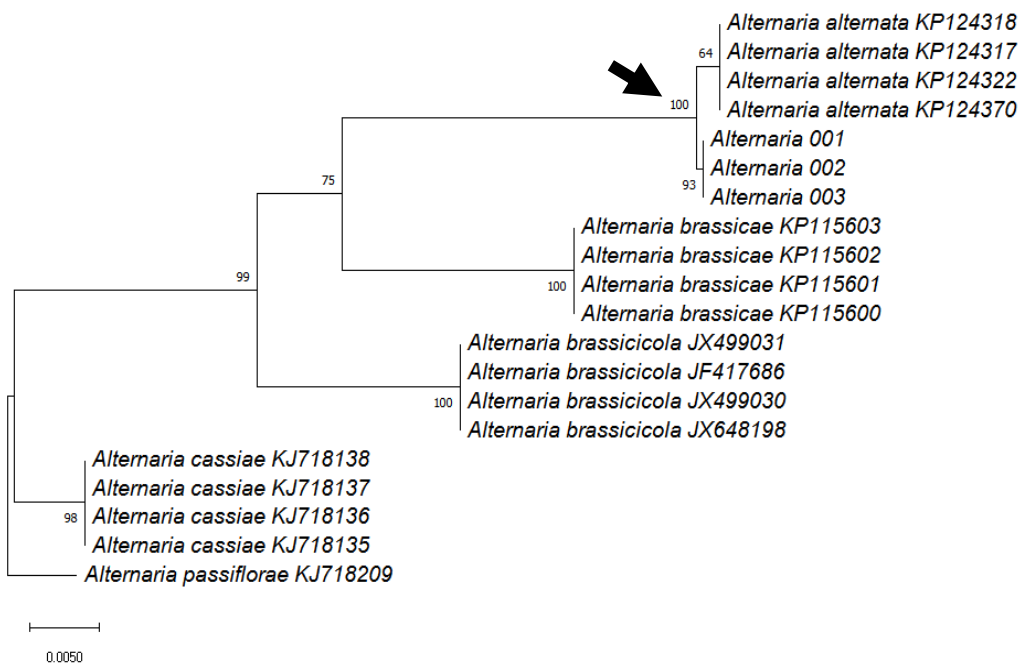


Fig. 2. The phylogenetic tree for *Alternaria* isolates using the Neighbor-Joining method with bootstrap test of 1000 replicates suggested that the fungal isolates were *Alternaria alternata*. The inference was supported by the bootstrap score of 100 (arrow head).

The unknown rust fungus was identified by the Neighbor-Joining inference with a highly supportive bootstrap score of 100 concluding that the rust fungus found on the plant leaves was *Maravalia pterocarpi*. The total length of the tree was 4.09256894. The total positions of this dataset were 216

from selected 16 nucleotide sequences (Fig. 4). *Melampsora larici-populina* was used as a root of the tree of rust fungi because it is a close relative to other members in Pucciniales including *Puccinia* and *Maravalia* [50].

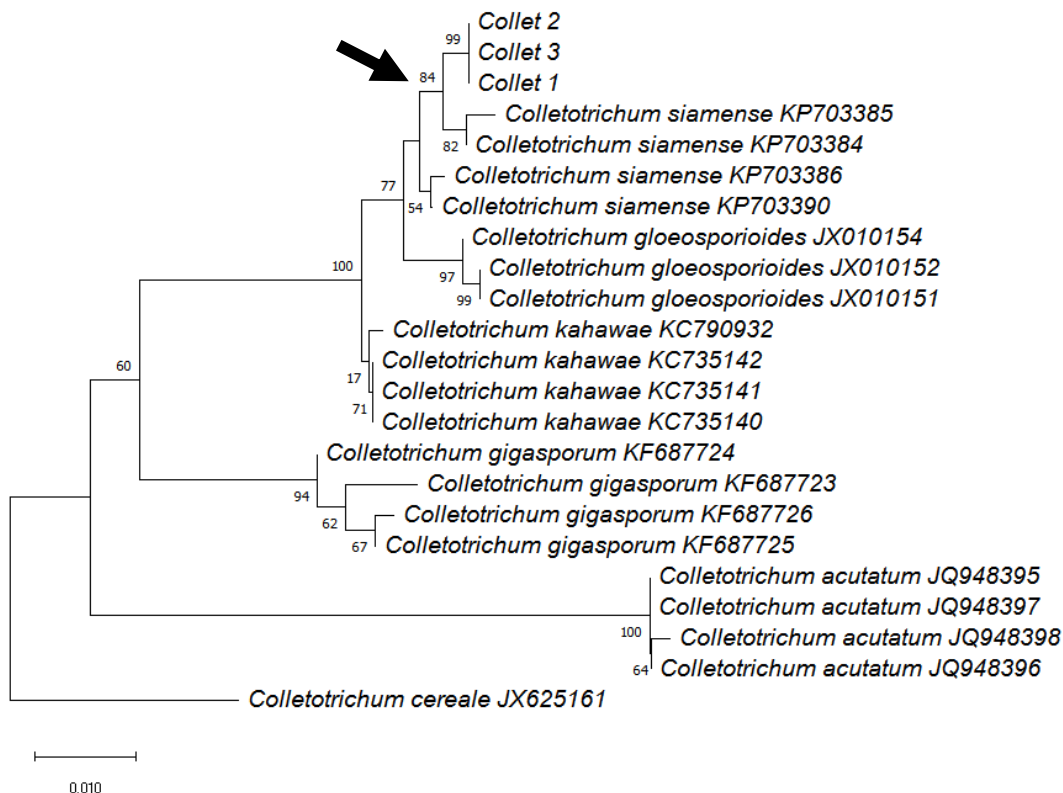


Fig. 3. The phylogenetic tree for *Colletotrichum* isolates using the Neighbor-Joining method with bootstrap test of 1000 replicates suggested that the fungal isolates were *Colletotrichum siamense*. The inference was supported by the bootstrap score of 84 (arrowhead).

There are a large number of *Alternaria*, *Curvularia*, and *Collectotrichum* species reported as plant pathogenic fungi causing leaf spot disease in various host plants as previously mentioned [3-13]. Exclusively in the *Dalbergia* genus, *A. alternata* and *C. gloeosporioides* were found to be the causal agents of leaf blight and leaf anthracnose on *D. sissoo* Roxb in India and Bangladesh [51-52]. Other *Alternaria* species, *A. porri* and *C. lunata*, have also

been recorded from different parts of *D. sissoo* in Pakistan [53]. Similarly, in this study, it was molecularly proven that *A. alternata*, *C. siamense*, and *C. aeria* are the fungi significantly causing the serious leaf spot disease on leaves of the plant. Additionally, the rust fungus identified as *M. pterocarpi* is the first report using molecular methodology for identification. This fungus is also a significant risk to seedlings as a plant pathogenic agent e.g. leave spot

diseases of another *Dalbergia* species (*D. tonkinensis*) in China [54]. It can be said that these are common fungal pathogenic agents

leading to the damage of this valuable plant, both in terms of plant survival rates and wood quality.

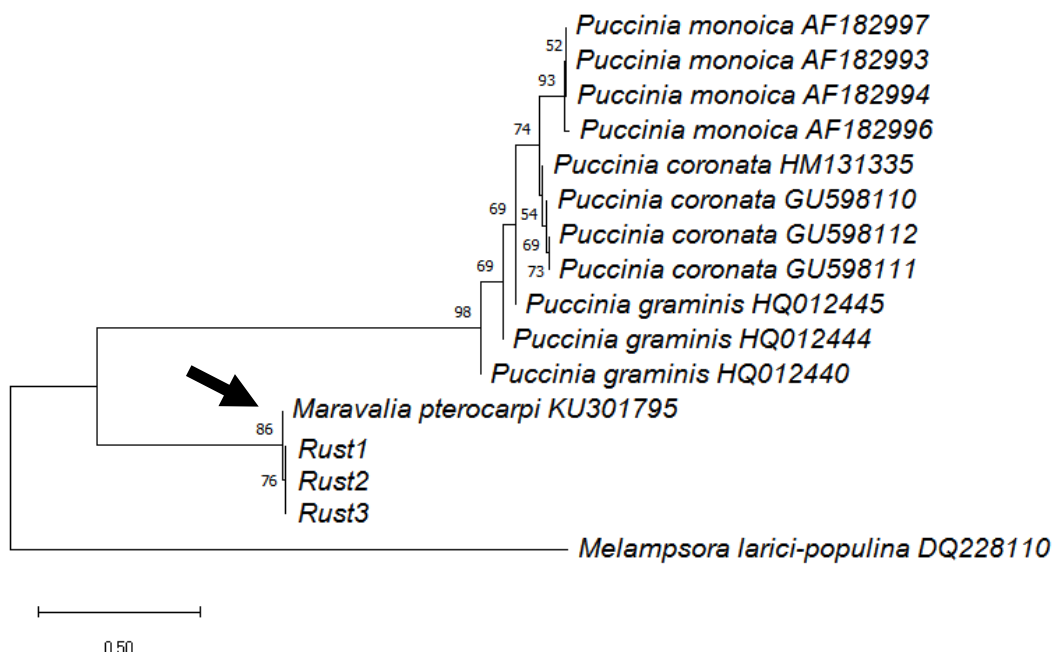


Fig. 4. The phylogenetic tree for the rust isolates using the Neighbor-Joining method with bootstrap test of 1000 replicates suggested that the fungal isolates were *Maravalia pterocarpi*. The inference was supported by the bootstrap score at 86 (arrowhead).

4. Conclusion

The brown circular leaf spot disease in Siamese rosewood seedlings is caused by a combination of fungi in one spot and the rust fungus, *M. pterocarpi*. It is very severe in seedlings and leads to low plant quality which could further bring about a reduction in the survival rate when planted in the field. Thus, in further studies, specific management of the disease caused by the fungi should be considered to overcome the disease.

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