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Genetic diversity of *Falcataria moluccana* and its relationship to the resistance of gall rust disease

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Abstract. *Lelana Ne, Wiyono S, Giyanto, Siregar IZ. 2018. Genetic diversity of* Falcataria moluccana *and its relationship to the resistance of gall rust disease. Biodiversitas 19: 12-17.* The use of cultivars that are resistant to a particular disease is one strategy that could mitigate the incidence of gall rust disease on *Falcataria moluccana*. Previous studies on the genetic diversity of *F. moluccana* did not attempt to link that genetic diversity to gall rust disease resistance. This research was carried out using RAPD analysis to determine the preliminary information on the association between different markers and the resistance to gall rust disease. The analysis evaluated a total of 20 pairs of healthy and infected *F. moluccana* trees that were classified based on their disease severity level. The RAPD primers used in this study were as follows: OPA-05, OPA-08, OPA-10, OPA-13, OPA-18, OPB-07, OPD-13, OPF-02, and OPG-05. The results showed that each RAPD primer produced a varying number of polymorphic bands, ranging from 3 to 12 bands, with a total of 80 polymorphic bands. Despite the number of loci analyzed, however, no specific polymorphic bands were found that could distinguish between healthy and diseased trees. This was supported by principal component analysis, which showed that healthy and diseased populations were not distributed separately. The structure analysis also showed that the healthy and diseased populations were not different.

Keywords: Diseased, healthy, pairing, polymorphic band, RAPD

INTRODUCTION

Falcataria moluccana (Miq.) Barneby & J. W. Grimes, also known as sengon in Indonesia, is native to Indonesia, Papua New Guinea, and the Solomon Islands (Soerianegara and Lemmens 1993). Since the 1870s, *Falcataria moluccana* has spread throughout Southeast Asia from Burma to the Philippines (NAS 1979). Currently, *F. moluccana* is the most prevalent tree found in small-scale community (private) forests and its population is increasing. Based on the results of the agricultural census of 2013, the population of *F. moluccana* increased almost five times since 2003 (BPS 2013). Its rapid growth rate and stable market availability is the reason why this species is preferred by farmers. *F. moluccana* is widely grown as a shade tree for reforestation, afforestation, and wood production (Soerianegara and Lemmens 1993). The wood of *F. moluccana* can be used for various purposes, such as veneer, plywood, light construction, lightweight packaging material, toys, firewood, and pulp (Soerianegara and Lemmens 1993; Krisnawati et al. 2011). Recently, the use of this wood for bare-core industry has been preferred because of demands for exports (BPS 2014).

Various pests and diseases are known to be associated with *F. moluccana* trees. Of these, the most recent major disease attacking the *F. moluccana* plantation is gall rust disease. The gall rust pathogen was first identified as the fungus *Uromycladium tepperianum* (Sacc.) McAlpine (Rahayu et al. 2010). In 2015, Doungsa-ard et al. (2015) proposed a new name for this fungus as *Uromycladium falcatarium.* This disease has become a serious threat to *F. moluccana* plantations in Indonesia. The disease not only causes significant economic losses, but also makes farmers worried. In Indonesia, gall rust disease was first reported on Seram Island, Moluccas (Maluku). In Java, the disease was first detected in Eastern Java in 2004. Since then, the disease has spread throughout Java (Anggraeni 2008).

In addition to environmental factors and pathogenic virulence, the resistance or susceptibility of plants to pathogens is also important in influencing the epidemic rate of the disease (Burdon and Thrall 2008). Susceptible plants will support the spread of the disease, while plants that show high resistance to the pathogens will inhibit the spread of the disease. Therefore, the use of resistant genotypes is often proposed as the first line of defense for controlling diseases.

Identifying molecular markers associated with host resistance to disease is typically the first step applied in the breeding strategy for resistant varieties for plant disease control. Some markers, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), microsatellites, single nucleotide polymorphism (SNP), and next-generation sequencing (NGS), have been widely used in studies related to plant resistance (Zhang et al. 2013; Asad et al. 2015; Klosterman

et al. 2016; Quesada-Ocampo et al. 2016; Vaghefi et al. 2016).

Despite the rapid development of genetic markers, information concerning the resistance of a variety or the provenance of *F. moluccana* to gall rust disease is still limited. Therefore, studies that identify molecular markers related to the resistance of *F. moluccana* to gall rust disease are indispensable. As a first step, RAPD is suitable for this study. RAPD is a molecular marker based on the amplification of random segment of DNA with single primers of arbitrary nucleotide sequences (William et al. 1990). The major advantages of this technique are that there is no requirement for a DNA sequence, it is relatively quick and easy to perform, and it is efficient to screen DNA polymorphisms at a very large number of loci (William et al. 1990; Kumari and Thakur 2014). Unfortunately, this technique has some limitations, such as low reproducibility and it only produces dominant markers. Several studies using RAPD to identify molecular markers linked to plant resistance genes have formed the foundation of plant breeding programs. Mumtaz et al. (2009) and Dhillon and Dhaliwal (2011) used RAPD markers to identify the presence of resistance genes against rust in wheat. Meanwhile, Salah et al. (2016) used RAPD markers to identify genes related to maize resistance against stalk rot disease caused by *Fusarium moniliforme*. Thus, this study aimed to identify molecular markers related to the resistance of *F. moluccana* against gall rust disease using RAPD markers.

MATERIALS AND METHODS

Study area

The study area covered 10 districts distributed among three provinces in Java, Indonesia. The districts were Bogor, Cianjur, Sukabumi, and Majalengka (West Java Province); Batang, Purbalingga, Banjarnegara, Salatiga, and Boyolali (Central Java Province); and Lumajang (East Java Province) (Figure 1). The number of samples collected from each district and their geographical locations are presented in Table 1.

Table 1. List of *Falcataria moluccana* leaves samples that were collected from various locations in Java, Indonesia

Location	Latitude	Longitude	Healthy plant code	Infected plant code
Bogor	-6.66196	106.79884	H01	D01
Bogor	-6.66721	106.80596	H02	D02
Bogor	-6.66721	106.80596	H03	D03
Bogor	-6.53107	106.42696	H04	D04
Cianjur	-7.10665	107.09946	H05	D05
Cianjur	-6.99676	107.12364	H06	D06
Cianjur	-6.93218	107.12387	H07	D07
Majalengka	-7.06058	108.39017	H08	D08
Majalengka	-6.99158	108.30591	H09	D09
Sukabumi	-7.02644	106.80064	H10	D10
Purbalingga	-7.22007	109.33750	H11	D11
Purbalingga	-7.27352	109.34550	H12	D12
Boyolali	-7.55987	110.52350	H13	D13
Banjarnegara	-7.30036	109.80983	H14	D14
Batang	-7.05156	109.78780	H15	D15
Batang	-7.30530	109.78454	H16	D16
Batang	-7.08680	109.76557	H17	D17
Salatiga	-7.36890	110.45323	H18	D18
Lumajang	-8.12772	113.14735	H19	D19
Boyolali	-7.56382	110.53833	H20	D20
Boyolali	-7.56382	110.53833	H21	D21

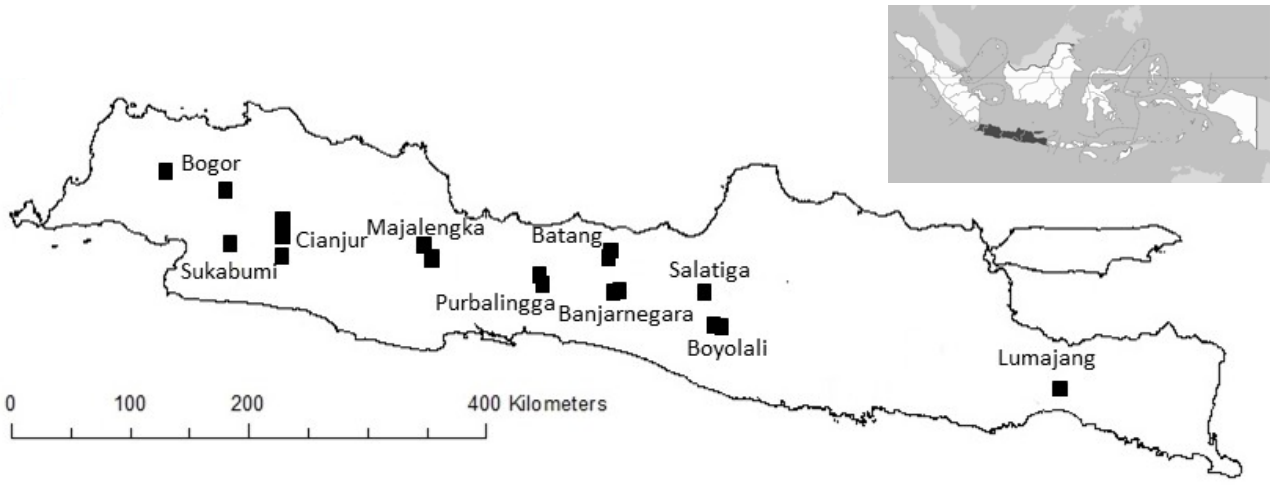

Figure 1. Location of sample collection covered 10 districts distributed among three provinces in Java, Indonesia. The black square (■) indicated location of sample collection

Procedures

Sample collection

Forty-two *F. moluccana* were sampled from various locations on a *F. moluccana* plantation (Table 1). To analyze *F. moluccana* resistance, sampling was carried out using a pairing method (Figure 2). Samples were taken from healthy and diseased trees that were located side-by-side. Healthy trees were categorized as trees with no disease or low severity of disease (severity score: 0 or 1). Meanwhile, infected trees were categorized as trees with high disease severity (severity score: 4 or 5). The criteria for the disease severity score were as follows: 0: There was no disease recorded; 1: There were up to 20% gall on the leaves, twigs, or branches; 2: There were 21-50% of galls on the leaves, twigs, or branches; 3: There were more than 50% of galls on the leaves, twigs, or branches, and one or more on the top trunk; 4: There were up to two galls on the branch-free trunk, and 5: There were three galls or more on the branch-free trunk.

We only sampled one sample or some pairs of samples in one population. There were two considerations for this sampling. First, it was difficult to find many health-diseased pairs of trees in one location. Therefore, we can only find one or some pair samples in one population. Second, the population of *F. moluccana* in Java was not structured. Suharyanto et al. (2002) used the RAPD marker approach and found that Java provenances of *F. moluccana* were not genetically differentiated. Similar results were also reported by Seido and Widyatmoko (1993). By using isozyme analysis, they reported that Java provenances of *F. moluccana* possess a similar genetic composition. Samples of *F. moluccana* were taken from young leaves. The collected samples were placed into individual plastic bags with silica gel (1: 5 v v^{-1}) and kept in an icebox for transport to the laboratory.

DNA extraction

DNA was isolated from leaves using the CTAB method of Doyle and Doyle (1987) with some modifications. Using liquid nitrogen, 1 mg of leaves was ground using a mortar into a fine powder. The powder was then transferred into a fresh tube containing 500 µL of cetyltrimethylammonium bromide (CTAB) extraction buffer [1.4 M NaCl; 100 mM Tris-HCl, pH 8.0; 20 mM EDTA; 2% CTAB; 2% polyvinylpyrrolidone (PVP); 1% β-mercaptoetanol] preheated to 65°&. The sample was then vortexed and incubated for 2 h at 65°&. The mixture of chloroform-isoamyl alcohol (24: 1) with the same volume was then added and vortexed. The tube was centrifuged for 15 min at 11,000 RPM. The supernatant was transferred into a fresh tube, and 2.5× volumes of absolute ethanol and a 1/10 volume of 3 M sodium acetate were added. The tube was then incubated overnight at -20°& to precipitate the DNA. The DNA was collected by centrifugation for 15 min at 14,000 RPM and rinsed with 500 µL ice-cold 70% ethanol. Finally, the DNA was resuspended in 100 µL of Tris-EDTA (TE).

RAPD amplification

The RAPD reaction was performed using nine primers (Operon Technology) (Table 2). The reaction was performed using Dream Taq Green PCR Master Mix (Thermo Scientific). The reaction mixture consisted of 200 µM of each dNTP, 1× GC Buffer, 0.5 µM of each primer, 0.02 unit μl^{-1} of Dream Taq DNA Polymerase, and 100 ng of the DNA template. The reaction conditions were as follows: pre denaturation at 94°& for 5 min; 45 cycles of denaturation at 94°& for 1 min, annealing at 34°& for 1 min, and polymerization at 72°& for 1 min; and a final polymerization at 72°& for 5 min. The resulting PCR product was then visualized using electrophoresis of a 1% (w v^{-1}) agarose gel stained with SYBR®SAFE (Invitrogen) in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 100 V for 40 min.

Data analysis

The DNA bands were visualized using electrophoresis, scored, and translated into binary data. Scoring was completed manually. If the DNA band appeared in the gel, it was scored as 1. If the DNA band did not appear, it was scored as 0. The genetic diversity and principal component analysis (PCoA) was carried out using the Genalex 6.5 program (Peakall and Smouse 2012). A structure analysis was performed using STRUCTURE version 2.3.4 software (Hubisz et al. 2009).

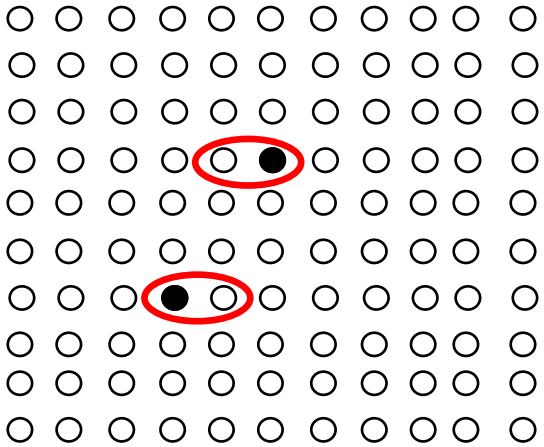

Figure 2. Pairing method by taking healthy and diseased trees side by side

Table 2. The list of RAPD primers

Primers	Sequence
OPA-05	AGGGGTCTTG
OPA-08	GTGACGTAGG
OPA-10	GTGATCGCAG
OPA-13	CAGCACCCAC
OPA-19	CAAACGTCGG
OPB-07	GGTGACGCAG
OPD-18	GAGAGCCAAC
OPF-02	GAGGATCCCT
OPG-05	CTGAGACGGA

RESULTS AND DISCUSSION

Genetic diversity of *Falcataria moluccana*

A total of nine RAPD primers were used to analyze two populations of *F. moluccana*, which included a healthy and diseased population grouped by their severity of gall rust disease. All primers used produced polymorphic bands. An example of the RAPD amplification results using OPA-05 and OPD-18 primers is presented in Figure 3. Each primer produced a varying number of polymorphic bands, ranging from 3-12 bands. The total number of polymorphic bands produced were 80 bands. Most polymorphic bands were produced by the OPA-19, OPD-18, and OPG-05 primers, while the fewest bands were produced with the OPA-05 primer.

The genetic diversity value of the *F. moluccana* plants is presented in Table 3. The value of genetic diversity in the infected tree's population was 0.299. This value was higher than the healthy tree population, which was 0.262. We expected that the genetic diversity of healthy tree populations was higher than diseased tree populations. Commonly, host populations with high genetic diversity suffer less from pathogens (Lively 2010). Meanwhile, another study found that the host's genetic diversity for resistance to infection may depend on the level of genetic diversity in the parasite population (Ganz and Ebert 2010). Therefore, there may not be a definite pattern for genetic diversity values for *F. moluccana* in relation to gall rust resistance, and the pattern of values may change depending on the gall rust pathogen population.

The values for genetic diversity were similar to those obtained in a previous study by Siregar and Olivia (2013). Using RAPD markers, they reported that the genetic diversity for some provenances of *F. moluccana* in Java varied from 0.133-0.295 with an average score of 0.235. The value of genetic diversity from their study was largely contributed within the population and this was about 82%. Meanwhile, the variation among populations was only 18%. The distribution of the *F. moluccana* population in Java was random and revealed no relationship between genetic and geographic distance. Similar results were also obtained by Suharyanto et al. (2002), who used RAPD markers as well. They reported that populations in Java were not genetically differentiated. They also reported lower genetic diversity for *F. moluccana* populations in Java compared to populations outside Java. Several studies discussed the history of the spread of *F. moluccana* in Java. According to the Heyne (1987), *F. moluccana* was first introduced in Java by Teysmann from Banda Island, Moluccas. It was planted in Bogor Botanical Garden, and since 1871, *F. moluccana* was planted throughout Java. Another study showed that populations in Java may not have been introduced from only one provenance, but also from some provenances in Papua and Moluccas (Suharyanto et al. 2002).

Our RAPD analysis results showed that no specific bands can distinguish between healthy and diseased populations. This was supported by PCoA analysis, which showed that healthy and diseased populations were not distributed separately (Figure 4). This result was also confirmed by structure analysis. Structure analysis is a model-based clustering method using multi-locus genotype data to infer population structures and assign individuals to populations (Hubisz et al. 2009). The results of our structure analysis are presented in Figure 5. The calculation of delta K values of 1 to 10 showed that K: 3 (Delta K: 146.37) had the highest score. Thus, three genetic groups were resolved. The results of this structure analysis showed unclear separation between the healthy and diseased populations.

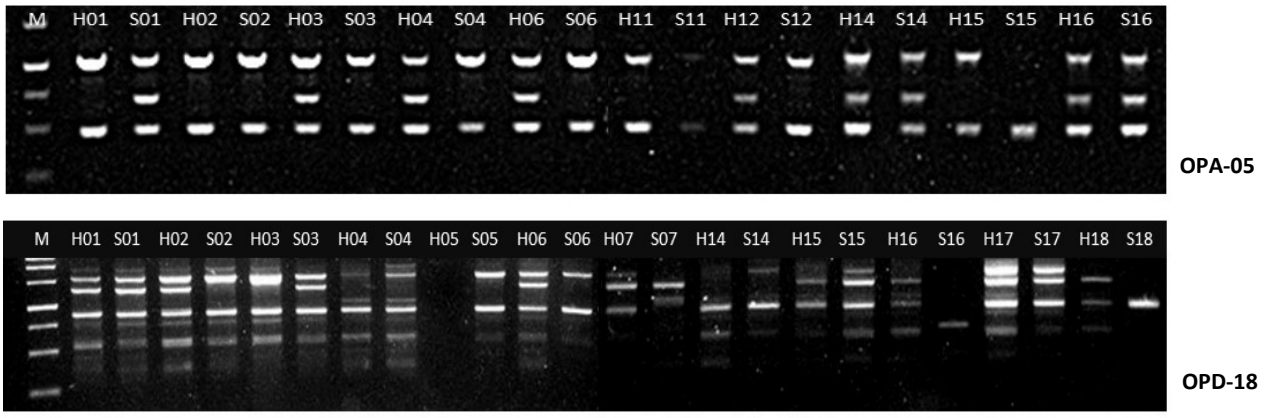

Figure 3. RAPD amplification results using OPA-05 and OPD-18 primers

Table 3. The genetic diversity of *F. moluccana* trees from healthy and diseased populations in Java, Indonesia

Population	N	Na	Ne	I	he	P (%)
Health	21	1.815 ± 0.064	1.428 ± 0.037	0.406 ± 0.025	0.262 ± 0.018	90.12
Diseased	21	1.827 ± 0.063	1.502 ± 0.038	0.454 ± 0.023	0.299 ± 0.018	91.36

Note: N: no. of samples, Na: no. of different allele, Ne: no. of effective allele, he: genetic diversity, P: percentage of polymorphic loci

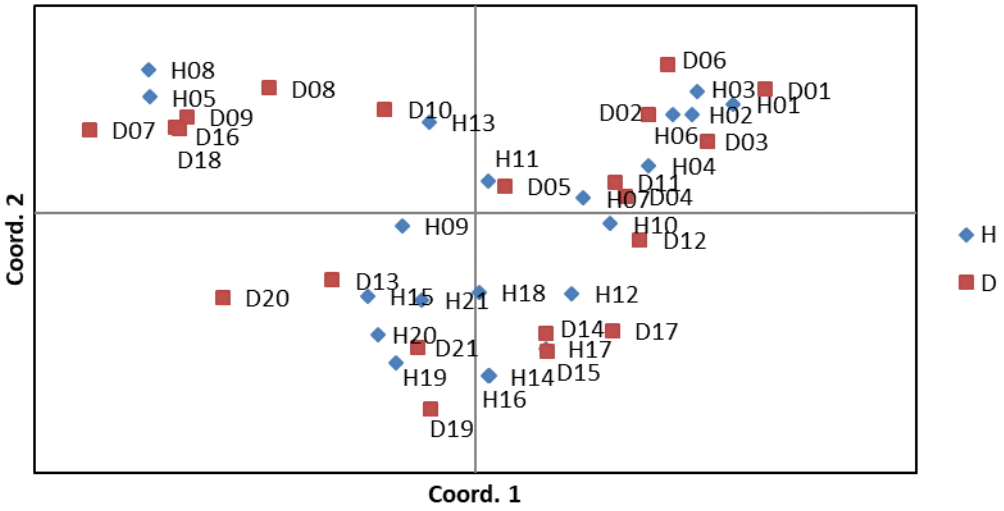

Figure 4 Principal coordinate analysis of individual sengon from Java, Indonesia. The first and second coordinate contributed 20.93 and 8.87% for the variation respectively. The blue (◆) marker indicated healthy population and the orange (■) marker indicated diseased population

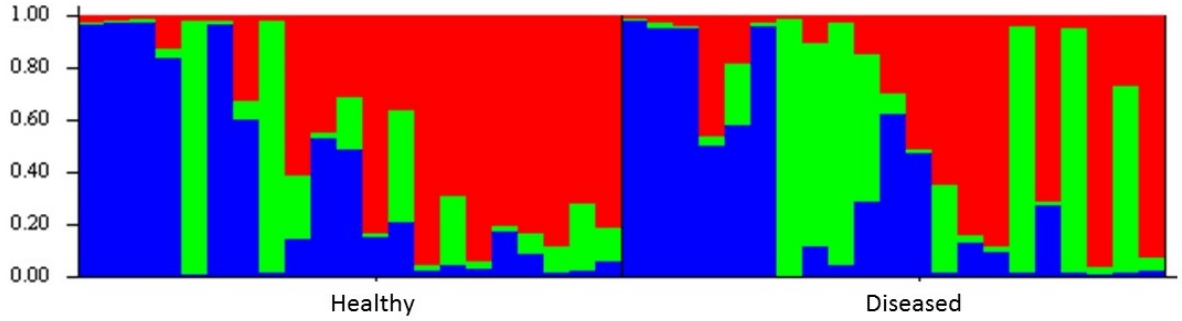

Figure 5. STRUCTURE analysis results from healthy and diseased *F. moluccana* (21 samples each) with K: 3 clusters

The absence of specific bands that can distinguish between healthy and diseased populations may due to two possible reasons. First, the markers used cannot distinguish between healthy and diseased populations. The primers used may be ineffective for this purpose or the number of loci may be too few. Second, field observations may not make clear classifications between resistant and susceptible trees. Some susceptible plants may remain free from infection or symptoms and, thus, appear resistant. The apparent resistance to diseases in plants known to be susceptible is generally a result of disease tolerance (Agrios 2005). The *F. moluccana* that were free of gall rust disease might not have true resistance, but only have apparent resistance. This result is in contrast to the study conducted by Mumtaz et al. (2009), Dhillon and Dhaliwal (2011), and Salah et al. (2016). These three studies used two distinct varieties with clear phenotypes related to disease resistance or susceptibility, so some polymorphic bands could be identified as candidate genes for plant resistance.

Implication for gall rust disease control strategies

The findings of this research could be used as a basis for improving plant disease management in the future. The epidemic of gall rust disease that has spread quickly in almost all of the *F. moluccana* plantations from East Java to West Java is possibly related to the genetic diversity of the *F. moluccana* among the populations in Java. Suharyanto et al. (2002) and Seido and Widyatmoko (1993) reported *F. moluccana* populations were not genetically differentiated in Java. Rust fungi are obligate pathogens well-known for their ability to undergo long-distance dispersals (Helfer 2014). Improving *F. moluccana* resistance to gall rust disease could be the key to curbing future epidemics of gall rust disease.

In conclusion, based on the results of our RAPD analysis, the number of polymorphic bands produced from each primer varied from 3-12 bands, and the total number of polymorphic bands produced was 80 bands. However, there were no specific polymorphic bands found that

distinguish healthily versus diseased *F. moluccana* trees. The resistance to gall rust disease shown by *F. moluccana* on plantations is probably due to the tolerance of the disease. In the future, genetic diversity studies of *F. moluccana* related to its resistance to gall rust disease needs to include the provenance from its center of origin because no reports of gall rust infestations have been reported for this region, except in Moluccas.

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