Challenges in Molecular Identification of Endomycorrhizal Fungi from Rhizosphere of Cashew Plant

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Abstract. Molecular identification of endomycorrhiza through DNA analysis is needed to complement identification based on morphological characters. This research aimed to study molecular DNA techniques to identify endomycorrhiza in the rhizosphere of cashew plants (Anacardium occidentale L.). DNA extraction was carried out using CTAB buffer from cashew roots, soil and spores and PCR was carried out with specific primers for mycorrhiza. The primers used were the primer pairs of SSU-Glom1 and NDL22 or LR4 + 2 and SSUmCf-LSUmBr. The results showed that DNA extracted from endomycorrhizal spores has the potential for molecular identification of endomycorrhiza, however it needs high amount of spores to obtain high quality and quantity DNA. DNA samples from cashew roots and cashew rhizosphere soil showed no PCR product and could not be used to identify endomycorrhiza molecularly. Optimization of PCR conditions and primer selection are needed to obtain better PCR products.

1. Introduction

Identification of endomycorrhiza is commonly conducted using classical method based on differences in morphological characteristics of spores, germination of hyphae and sporulation of endomycorrhizal fungi. Identification of endomycorrhizal species carried out based on differences in character and spore morphology has several disadvantages such as the presence of spore morphological dimorphism which causes identification errors [1]. In addition, mycorrhizal sporulation is highly dependent on environmental factors, seasons, host plants and can change over time [2] making it difficult to provide spores for identification.

The use of spores found in the soil which is in the resting stage for analysis of mycorrhizal community cannot show whether mycorrhiza is active in plant roots [3]. On the other hand, identification of arbuscular mycorrhiza to species level is difficult to do based on morphology in plants, because of the lack of informative characteristics that can be used in diagnosing species [4]. Molecular method is an alternative technique in the identification of mycorrhizal species because identification can be done from a small portion of materials and is more accurate because it can identify species and can even distinguish individuals. The DNA fragments commonly used are ribosomal DNA (rDNA) genes. Ribosomal DNA is a group of genes that encode RNA consisting of small sub-units (SSUs), 5.8S and large sub-units [4].

Mycorrhizal diversity is very important in maintaining ecosystem productivity and stability. The increasing diversity of mycorrhizal species causes changes in plant community structure which ultimately increases productivity [5]. Information on the diversity of mycorrhizal species that colonize cashew plants in Bali, Indonesia is important. Cashew plants (Anacardium occidentale L.) are one of the superior plants planted by farmers in dry land. Cashew plants are resistant to drought and are
capable of symbiotic with endomycorrhiza [6]. Endomycorrhizal exploration in the rhizosphere of cashew plants in Karangasem Bali, Indonesia obtained five genera including Glomus, Gigaspora, Acaulospora, Sclerocystis and Scutelospora genera [7]. To further explore the mycorrhizal diversity and to confirm the species name, molecular identification is needed. The objectives of this research were to study molecular identification technique of endomycorrhiza from rhizosphere of cashew plants in Karangasem Bali, Indonesia and to analyze the challenges of the technique.

2. Materials and Methods

2.1. Sample Collection
Samples were collected from cashew plantations in Abang, Kubu and Sukadana Villages, Karangasem Regency, Bali, Indonesia. Young roots from cashew plants were collected and 1000 grams of soil was taken in the rhizosphere for extraction of spores.

2.2. DNA Extraction
As much as 0.1 g of roots were ground with mortar and pestle, and 1 ml of CTAB buffer [8] was added and, incubated at 60°C for 30 minutes. After centrifugation for 5 minutes at 14,000 rpm, the supernatant was transferred to a new tube and added with 1 ml of chloroform:isoamylalcohol (24:1) and centrifuged for 5 minutes at 14,000 rpm. The supernatant was transferred to a new tube and 2/3 volumes of cold isopropanol was added. The sample was then incubated at -20°C for 1 hour, and centrifuged 3 minutes at 14,000 rpm. Pellet was washed with 70% ethanol and then air dried. As much as 100 mL of sterile water was added to dissolve the DNA pellets. Then added 3µl of RNase (RNase concentration of 1mg/ml) and incubated at 37oC for 30 minutes.

The same method was carried out for soil samples and endomycorrhizal spores. DNA was visualized by electrophoresis on 0.8% agarose gel in TAE buffer, for 30 minutes. The gel is then stained with ethidium bromide and observed with UV transiluminator.

2.3. PCR and Visualization of PCR Products
PCR was carried out based on Krüger, et al.[9] and Stockinger [10]. The primers used were primer pairs of SSU-Glom1 and NDL22 or LR4 + 2 [10] and SSUmCf-LSUmBr[10]. The DNA concentration used in the PCR reaction was 25 ng. The total reaction volume was 20µl, which consists of 1 U taq polymerase (BD Bioscience), 1 x buffer polymerase (BD bioscience), 1.5 mM MgCl2, 200µM dNTP and 0.5 µM of each primer and H2O until it reaches a volume of 20 µl. PCR was carried out in the following cycles: initial denaturation for 5 minutes at 95oC, followed by 40 cycles consisting of 1 minute denaturation at 95°C, annealing at 60°C for 1 minute, and 2 minutes elongation at 72°C, ended with 1 final elongation cycle of 10 minutes at 72oC.

The PCR product was electrophoresed with 1% agarose gel in TAE buffer for 45 minutes at 100V. Visualization with UV transiluminator was carried out after staining with ethidium bromide.

3. Result and Discussion
The results showed that DNA extraction carried out from 0.1 g of root and 0.1 g of soil resulted in DNA bands in both types of samples. The results of DNA extraction are presented in Figure 1. PCR was carried out based on Kruger et al.[9]. Using DNA extracted from root and soil, no PCR product was obtained (data not shown). This failure can be caused by several factors, among others: (1) root does infected by mycorrhiza, therefore, there was no mycorrhizal DNA in the samples or rhizosphere soil used as source of DNA extraction did not contain mycorrhizal spores, (2) high contamination in DNA solution, (3) the PCR program is not appropriate, therefore optimization must be done by modifying the PCR component, temperature and the PCR cycle, (4) the primers used were too specific. Brundrett at al [11] stated that mycorrhizal colonization in roots is strongly influenced by environmental factors such as season, P content in the soil and the ability of mycorrhizal spores to colonize the host. According to Gollotte et al [12], modifications in DNA extraction can be made by increasing the concentration of 2-mercaptoethanol and EDTA to avoid the brown color of the soil which was still carried away during DNA isolation.
Figure 1. Results of DNA extraction from cashew root samples (top figure) and from soil samples (bottom figure). MW=molecular weight

To solve the above problems, spores were isolated from soil. Isolation of spores was carried out by multilevel filtration and spores were observed under a monocular microscope (Figure 2a). Then the spores were collected using pipette one by one and transferred in micro tubes for DNA extraction. Result of DNA extraction from spores was presented in Figure 2b. PCR was carried out with DNA template extracted from a mixture of mycorrhizal spores. PCR was performed with SSU-Glom1 and NDL22 primers. The PCR results are shown in Figure 3.

Figure 2. Mycorrhizal spores observed under microscope. Arrows show the spores isolated for DNA extraction (a), DNA extracted from spores (b). Lambda DNA with known concentrations was used for comparison of DNA quantity.

Figure 3. PCR products using DNA extracted from spores as DNA template. As size comparison, 100 bp ladder was used. Letters a and b are PCR products produced by amplification with SSU-Glom1 and NDL22 primers.

Based on Figure 3, it can be seen that when DNA from mycorrhizal spores were used as DNA template, two samples produced visible bands of amplification with sizes of approximately 220 bp and 345 bp. However, there were also unspecific PCR products since there were multiple bands.
produced. The strongest band was then extracted and purified from the gel. The purified PCR product was sent to 1st BASE through PT Genetika Science Indonesia. Due to low concentration of PCR product, the sequencing failed to reveal readable DNA base sequence. These results indicated that DNA extracted from root or soil was not suitable as template DNA. DNA extracted from mycorrhizal spores was better used as template DNA, however high amount of spore is needed to obtain high quality and quantity of DNA. The PCR conditions both PCR reaction and cycles also need to be optimized to get stronger PCR band. The choice of primer pairs need to be further considered. The used of universal primers for fungi may resulted in better products.

4. Conclusion
Molecular identification of endomycorrhiza failed to be done using DNA template extracted from root or rhizosphere soil of cashew plants. DNA should be extracted from endomycorrhiza spores, however large quantity of spores is needed. Optimization of PCR reaction and cycles as well as primer choices also need to be further studied.

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