DNA barcoding of *Pericopsis mooniana* from two different populations in Indonesia based on rDNA ITS (Internal Transcribed Spacer)

I Prihatini1,*, A Y P B C Widyatmoko1, I L G Nurtjahjaningsih1, V Yuskianti2, and S A Danarto3

1Center for Forest Biotechnology and Tree Improvement, Sleman, Indonesia
2Forest Products Research and Development Center, Ministry of Environment and Forestry, Bogor, 16610, Indonesia
3Research Center for Plant Conservation and Botanic Garden Purwodadi, Pasuruan

*E-mail: istiana.prihatini@biotifor.or.id

**Abstract.** A DNA barcoding is a taxonomy method that provide fast and accurate species identification, thus it can be applied to anticipate when a species can not be identified morphologically. This study aimed to assess molecular characteristics of *Pericopsis mooniana* from two wild populations in Indonesia using a barcoding marker rDNA ITS (Internal Transcribed Spacer) as additional information for establishing conservation strategy of this species. Leaf samples of *P. mooniana* were collected from natural forests at South East Sulawesi and South Kalimantan, and a germplasm orchard at Purwodadi Botanic Garden. Molecular characteristic was evaluated based on a pair of ITS primers that produce between 634-641 bp nucleotide. The sequence data were analyzed by Bioedit version 7.0.5.3 and Mega 7 software. Results showed that *P. mooniana* from South East Sulawesi and South Kalimantan were clustered together, while *P. mooniana* from Purwodadi Botanic Garden were separated with only one base difference from those two population. In conclusion, ITS barcoding in this study shows a differences between *P. mooniana* collected from South East Sulawesi and South Kalimantan with *P. mooniana* that originally from Papua. A further study using more barcoding marker and more population could be approached to obtain more information on molecular characters of *P. mooniana* populations that will useful for establishing conservation and tree breeding program of this species.

1. **Introduction**

*Pericopsis mooniana* is one of Fabaceae species that categorized as a vulnerable economical tree species (Vulnerable Alcd) by the IUCN Red List of Threatened Species, since 1998 [1]. The genus Pericopsis consist of four species, while three species distributed in tropical Africa [2,3]. *Pericopsis mooniana* is the only species of Pericopsis that naturally distributed in Southeast Asia, Micronesia, and New Guinea. In Indonesia, it is distributed in Java, Sumatra, Kalimantan, Sulawesi and Papua. Recently, only small number of stands remain in South East (SE) Sulawesi (Lamedai Nature Reserve and Tanggeletada) [4], in Pulau Laut South (S) Kalimantan [5], Jambi [6] and Papua [7,8], where illegal logging and land conversion are the most common causes threatening natural species distribution [5]. This species tends to survive in the clusters and continuous forests [9]. The populations of *P. mooniana* had been fragmented by human activities such as overexploitation, and land conversion to
agriculture and settlement. Low natural regeneration and lack of replanting also causing this species to decline thus encouraged the Indonesian Government to establish a conservation strategy to restore the natural habitat. The conservation strategy has been initiated by assigned Lamedai Nature Preserve as conservation areas since 1974 and many studies on several aspects to provide information for establishing conservation and plantation site to protect this species have been conducted. Studies on cultivation techniques and seed germination are developed to increase the survival rate in the field [4,10] and the prospect of planting this species in rehabilitating ex-mine program [11,12]. Wood properties study on *P. mooniana* plantation revealed that breeding program for wood quality is required to promote the establishing plantation [13].

Ministry of Environment and Forestry through Center for Forest Biotecnology and Tree Improvement (CFBTI) has collected seed and seedling of *P. mooniana* from its remaining natural population in SE Sulawesi and S Kalimantan. These genetic materials were used to establish an ex-situ conservation plot in Java island and to provide materials for tree breeding program of this species in the near future. Conservation genetic should cover large genetic variation as possible and therefore studies on genetic diversity of these genetic materials prior to establish the ex-situ conservation plot were conducted. The observation on morphological characters such height and diameter growth on seedling of *P. mooniana* (collected from SE Sulawesi) in nursery shows high variability [14]. This finding was supported by an examination of genetic diversity using molecular marker (Random Amplification of Polymorphic DNA, RAPD) that has been applied on genetic material from SE Sulawesi [9]. Both studies show high genetic diversity of SE Sulawesi population, but currently no published data for genetic diversity of S. Kalimantan population. Despite of the decreasing of the number of population and the number of tree in each population, the previous studies revealed that material genetic collected from SE. Sulawesi population are important and optimum for establishing conservation plot of this species. The molecular genetic study also reported that genetic diversity in Lamedai Nature Preserve had the highest value among the tested populations. The genetic relationship analysis also indicated that Lamedai Nature Preserve represented a large continuous forest in the ancient period [9].

Establishing conservation planning required information on species taxonomy, species biology, species distribution, and current number in wild population [15]. Proper and accurate identification of species are important for recording the ecological data [16], especially for samples in mixed-species environment. The germplasm orchard of this species had been established to keep the genetic resources. Therefore, barcoding study was conducted to confirm the species identity and to observe the taxonomical position of *P. mooniana* individual collected from different population. Barcoding of plants have been conducted since a long time ago until recently, and no universal barcoding are available for plants [17], therefore it is still challenging. Despite of its economical value and massive studies on this vulnerable species, the genetic information based on DNA barcoding of this species is currently very limited. Currently, only one sequence ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit (rbcL) gene of *P. mooniana* was submitted in public database i.e. Genbank. Taxonomist classified plants based on morphological characters, such as leaves, bark, flowers and fruits. However, the characteristics might difficult when flowers and fruits are absent. DNA barcode are used to identify unknown species when morphological characters are incomplete [18]. The DNA barcoding is a useful tool for species identification, a dataset of DNA barcoding provide a foundation for bioforensic and provide information to support regulatory enforcement [17]. This study aimed to assess molecular characteristics of *Pericopsis mooniana* from two wild populations in Indonesia using a barcoding marker rDNA ITS (Internal Transcribed Spacer) as additional information for establishing conservation strategy of this species.
2. Materials and Methods

2.1. Materials

2.1.1. Plant materials, morphological observation and DNA extraction

Leaf samples were collected from 19 *P. mooniana* trees from natural population in South (S) Kalimantan (9 trees) and South East (SE) Sulawesi (10 trees). Two samples of *Pericopsis* from Purwodadi Botanic Garden (PBG) and two closely related species (*Pterocarpus* sp.) were included in this study (Table 1). Morphological characters of bark, leaf, inflorescence and pods were observed on *P. mooniana* collection in PBG (Purwodadi Botanic Garden). The DNA extraction, purification and DNA amplification were conducted in Molecular Genetic Laboratory CFBTI. The total genomic DNA was extracted from leaf samples using a modified Cetyl Trimethyl Ammonium Bromide (CTAB) method [19]. The total DNAs were diluted 10 times and use as a template for PCR reaction to amplify ITS.

**Table 1.** List of individual samples used in *Pericopsis mooniana* molecular study

| Sample Codes | Origins         | Habitats          | Species ID         |
|--------------|-----------------|-------------------|-------------------|
| K1           | South Kalimantan| Inhutani mess     | *P. mooniana*     |
| K4           | S Kalimantan    | Local community garden | *P. mooniana* |
| K5           | S Kalimantan    | Seed orchard      | *P. mooniana*     |
| K37          | S Kalimantan    | arboretum         | *P. mooniana*     |
| K38          | S Kalimantan    | arboretum         | *P. mooniana*     |
| K39          | S Kalimantan    | arboretum         | *P. mooniana*     |
| K40          | S Kalimantan    | Stagen            | *P. mooniana*     |
| K41          | S Kalimantan    | Stagen            | *P. mooniana*     |
| K42          | S Kalimantan    | Stagen            | *P. mooniana*     |
| S10          | South East Sulawesi | Bali Jaya     | *P. mooniana*     |
| S11          | SE Sulawesi     | Bali Jaya         | *P. mooniana*     |
| S12          | SE Sulawesi     | Bali Jaya         | *P. mooniana*     |
| S106         | SE Sulawesi     | Lamedai           | *P. mooniana*     |
| S107         | SE Sulawesi     | Lamedai           | *P. mooniana*     |
| S110         | SE Sulawesi     | Lamedai           | *P. mooniana*     |
| S131         | SE Sulawesi     | Lamedai           | *P. mooniana*     |
| S132         | SE Sulawesi     | Lamedai           | *P. mooniana*     |
| S50          | SE Sulawesi     | Tantetada         | *P. mooniana*     |
| S51          | SE Sulawesi     | Tantetada         | *P. mooniana*     |
| M18          | Unknown         | Puwodadi Botanic Garden | *Pterocarpus echinatus* |
| M19          | Unknown         | Puwodadi Botanic Garden | *P. echinatus* |
| M20          | Papua           | Puwodadi Botanic Garden | *P. mooniana* |
| M21          | Papua           | Puwodadi Botanic Garden | *P. mooniana* |

2.2. Methods

2.2.1. PCR Amplification and sequencing of internal transcribed spacer DNA

Amplification of partial of internal transcribed spacer (ITS) region targeted sequence of ITS1, 5.8s and ITS2 were conducted using combination of ITS1 (5’TCC GTA GGT AAG CCT CGG G 3’) and ITS4
primer (5’ TCC TCC GCT TAT TGA TAT GC 3’) [20]. The amplification reaction was performed in a total volume of 50 µl containing 10 µl of genomic DNA (final concentration was varying between 2.2 ng to 16 ng per µl), 0.5 µM of each primer, and 25 µl of 2x My Taq HS Red Mix (Bioline), 0.2µg of bovine serum albumin (BSA), and sterile water. DNA amplification was performed in a ProFlex PCR system (Applied Biosystem) with a program as follows: 94°C for 5 minutes, 30 cycles of 30 s at 94°C, 30 s at 55°C, and another 60 s at 72°C, followed by 7 minutes at 72°C. The PCR products were separated by electrophoresis in 1.5% agarose gel. Positive amplification products were sent to the 1st Base for sequencing following DNA purification and DNA extraction from gel.

DNA sequence chromatograms obtained from the 1st Base were viewed and edited to remove poor quality sequences at each end using Chromas software (http://technelysium.com.au/wp/chromas/). DNA sequence with good quality chromatograms was aligned using ClustalW [21] on Bioedit software version 7.0.5.3 [22]. DNA sequence with high similarity revealed by BLAST was retrieved from public DNA databases i.e. GenBank and were included in phylogeny analysis to verify the identity of species. One sequence from a more distant related taxon was included as out group in the analysis. Pairwise distance to calculate the genetic distance within and between species of Pericopsis was created using Mega7 [23]. The phylogenetic trees were constructed using the Maximum Likelihood method based on the Tamura-Nei model [24] with 500 bootstraps in Mega7.

3. Results and Discussion

3.1. Results

3.1.1. Morphological character of Pericopsis mooniana

**Bark.** Medium sizes to large trees with height up to 24 m (Figure 1A), grooved skin bark, bark soft and thin, bole straight or twisted, slightly grooved in the bark. **Leaf.** Leaf composite (imparipinnate), there are pelvinus at the base of the petiolus communis, petiolus communis is thin, number of leaflet is 6-8-10, ovate to elliptical, alternate or rarely sub opposite, glabrous, the color in adaxial of leaf is dark green, rough surface like parchment, bright green abaxial, there is pelvinus at the base, acute to broadly acuminate at the tip, rounded at the base, wavy at the leaflet edge (margo folii), primary vein protude at the abaxial part, secondary vein alternate with each other, length: width ratio is 3:1, has stipules, there is a thick pelvinus at the base, rachis and petiol slightly grooved (Figure 1B).

![Figure 1. A. Pericopsis mooniana that observed in PBG; B. Leaf composite of P. mooniana with ovate shape and dark green in colour.](image-url)
Pod. Pod-shaped fruit, long stalked, rounded at the base, flat linear to oblong, tapered at the tip, dark brown, hard, pod have 1-4 spaces, compressed ovate-orbicular, skin pod will break when ripe and seed will spread in surrounding of trees. *P. mooniana* has hard seed with dark brown colour.

Inflorescense. We were observed of inflorescense on *P. mooniana* refer to publication by Bentham and Hooker [25] and Knaap-van Meeuwen [26]. Inflorescence racemose or paniculate, flower cream or white or purple, in axillary racemes or terminal panicles, bract and bracteoles minute, calyx large, campanulate, cleft to below the middle, lobes sub-equal or upper 2 shorter, sub connate, caducous, standard broad to orbicular, wings and keel petals distinctly eared, wings falcate to obovate, keel incurved, blunt, petals free, stamens free, apically recurved, anthers versatile, ovary stalked, oblong, villous, encircled by conspicuous disk, few-ovuled, style long, filiform, awl-shaped, apically involute, stigma terminal, introrse.

3.1.2. DNA ITS sequences

Twenty three individual of *P. mooniana* were amplified by a set of primer (ITS1/ITS4) shown by clear strong bands around size 870bp (Figure 2). However, in the further analysis using ITS4 for DNA sequencing, only 17 individual were produced good quality product shown by the clean and clear chromatogram, while the chromatogram of the other four samples (S11, S13, S106, S110, and S50) were noisy and illegible. Only good quality sequences were used for further analysis (phylogenetic study). The ITS DNA sequences were started from 5’GATCATTG to CAGGCGGG 3’ with total length from 634 to 650 bp. All of these good quality sequences were submitted to GenBank (Table 2).

![Figure 2. Purified amplicon of ITS DNA of 23 individual of *Pericopsis* that selected for DNA sequencing are marked with red squares. LD = DNA ladder](image)

| Individual | Band Length (bp) | Sequence chromatogram | Best match in BLAST | Genbank Accession No |
|------------|-----------------|-----------------------|---------------------|---------------------|
| K1         | 634             | Clear                 | 573/651(88%) similar to *Pericopsis laxiflora* (KX057892) and 589/678(87%) to *P. angolensis* (KX584401) | MT093361 |
| K4         | 634             | Clear                 | 573/651(88%) similar to *P. laxiflora* (KX057892) and 589/678(87%) to *P. angolensis* (KX584401) | MT093362 |
| K5         | 634             | Clear                 | 573/651(88%) similar to *P. laxiflora* (KX057892) and 589/678(87%) to *P. angolensis* (KX584401) | MT093363 |
| K37        | 639             | Clear                 | 578/656(88%) similar to *P. laxiflora* (KX057892) and 594/683(87%) to *P. angolensis* (KX584401) | MT093364 |
| K38        | 639             | Clear                 | 578/656(88%) similar to *P. laxiflora* (KX057892) and 594/683(87%) to *P. angolensis* | MT093365 |
| Individual | Band Length (bp) | Sequence chromatogram | Best match in BLAST | Genbank Accession No |
|------------|-----------------|-----------------------|---------------------|---------------------|
| K39        | 638             | Clear                 | 577/656(88%) similar to *P. laxiflora* (KX057892) and 592/682(87%) to *P. angolensis* (KX584402) | MT093366            |
| K40        | 638             | Clear                 | 577/656(88%) similar to *P. laxiflora* (KX057892) and 592/682(87%) to *P. angolensis* (KX584402) | MT093367            |
| K41        | 639             | Clear                 | 578/656(88%) similar to *P. laxiflora* (KX057892) and 594/683(87%) to *P. angolensis* (KX584401) | MT093368            |
| K42        | 638             | Clear                 | 577/656(88%) similar to *P. laxiflora* (KX057892) and 592/682(87%) to *P. angolensis* (KX584402) | MT093369            |
| S10        | 639             | Clear                 | 578/656(88%) similar to *P. laxiflora* (KX057892) and 594/683(87%) to *P. angolensis* (KX584401) | MT093355            |
| S11        | unknown         | Illegible             | -                   | -                   |
| S12        | 639             | Clear                 | 578/656(88%) similar to *P. laxiflora* (KX057892) and 594/683(87%) to *P. angolensis* (KX584401) | MT093356            |
| S106       | unknown         | Illegible             | -                   | -                   |
| S107       | 641             | Clear                 | 578/656(88%) similar to *P. laxiflora* (KX057892) and 594/683(87%) to *P. angolensis* (KX584401) | MT093358            |
| S110       | unknown         | Illegible             | -                   | -                   |
| S131       | 640             | Clear                 | 578/657(88%) similar to *P. laxiflora* (KX057892) and 594/682(87%) to *P. angolensis* (KX584401) | MT093359            |
| S132       | 640             | Clear                 | 578/657(88%) similar to *P. laxiflora* (KX057892) and 594/682(87%) to *P. angolensis* (KX584401) | MT093360            |
| S50        | unknown         | Illegible             | -                   | -                   |
| S51        | 639             | Clear                 | 578/656(88%) similar to *P. laxiflora* (KX057892) and 594/683(87%) to *P. angolensis* (KX584401) | MT093357            |
| M18        | 578             | Clear                 | 578/578(100%) match to *Pterocarpus indicus* (MH487680) and 577/578(99%) to *P. macrocarpus* (MH487681) | MT127631            |
| M19        | 578             | Clear                 | 578/578(100%) match to *P. indicus* (MH487679) and 577/578(99%) to *P. macrocarpus* (MH487681) | MT093371            |
Sequencing of ITS region from 17 individuals produced between 634 bp to 650 bp of nucleotide and mostly 88% were similar to *Pericopsis* species *P. laxiflora* and *P. angolensis* (Table 2). There was no ITS DNA sequences of *P. mooniana* available in GenBank as a sequence reference to this study. This is the first study of *P. mooniana* barcoding based on ITS region and the first submission to GenBank. The individual M18 and M19 were 99% related to *Pterocarpus* species (*P. indicus* and *P. macrocarpus*). These two collection of PBG were identified as *P. echinatus* based on their morphological characters.

Based on their DNA ITS sequence, *P. mooniana* between SE Sulawesi and S. Kalimantan showed no significant differences (genetic distance= 0) calculated using Mega7, with only one nucleotide difference (an insertion of G base in 512 base position) for three individual from Lamedai S.E. Sulawesi. Population from Papua with only two individual shows small distance (0.003) to *P. mooniana* from both population, S.E. Sulawesi, and South Kalimantan with two insertion of G bases in 188 and 512 base position (data not shown).

### Table 2: ITS Sequences and BLAST Matches in Pericopsis mooniana

| Individual | Band Length (bp) | Sequence chromatogram | Best match in BLAST | Genbank Accession No |
|------------|------------------|------------------------|----------------------|----------------------|
| M20        | 640              | Clear                  | 579/656(88%) similar to *P. laxiflora* (KX584403) and 594/682(87%) to *P. angolensis* (KX584402) | MT093372 |
| M21        | 640              | Clear                  | 579/656(88%) similar to *P. laxiflora* (KX584403) and 594/682(87%) to *P. angolensis* (KX584402) | MT093373 |

3.1.3. Phylogeny of Pericopsis based on ITS DNA

Phylogeny analysis of *P. mooniana* in this current study (Figure 3) shows that DNA ITS sequences of S.E. Sulawesi population and S. Kalimantan population clustered together despite of a single nucleotide difference on individual S107, S131, and S132. Two individuals of *P. mooniana* from Papua population (M20 and M21) that planted in PBG were a separate cluster of SE Sulawesi population and S Kalimantan population. The reference sequences of other Pericopsis species (*P. laxifolia* and *P. angolensis*), were clustered together but separated from cluster *P. mooniana* of this study. The DNA ITS sequences of *P. elata* was not available from NCBI database (Genbank). Genetic distance of *P. mooniana* calculated based on pairwise distance matrices was 0.116 when paired with *P. laxifolia*, and 0.088 when paired with *P. angolensis*, while genetic distance between *P. laxifolia* and *P. angolensis* was 0.060.
Figure 3. Dendrogram of *Pericopsis* based on ITS DNA sequence constructed using the Maximum Likelihood method in Mega7 with 1000 bootstrapping. The analysis involved 29 nucleotide sequences with a total of 693 positions in the final dataset. The tree with the highest likelihood is shown and drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together is shown next to the branches and *Dalbergia assamica* (KM521355) used as an outgroup.

Two individual of *Pterocarpus* from PBG collection also included in this study. Based on the morphological character, these two trees were identified as *P. echinatus* that belong to Fabaceae, the same family as *Pericopsis*. In the phylogeny dendrogram these individual were clustered with two different species i.e. *Pterocarpus indicus* and *P. macrocarpus*. The cluster of *Pterocarpus* was separated with cluster of *Pericopsis* with genetic distance measured at 0.320.

3.2. Discussion

Numerous barcoding studies have been conducting on Fabaceae species using various primer such as rbcL, matK, and trnH-psbA based on a single gene/DNA region [27] and multi gene combinations between different primers [28], but no ITS sequences of *P. mooniana* are reported from the previous studies. Application of a DNA barcoding marker (ITS) in this study was successfully amplified all of the samples studied (*P. mooniana* and *Pterocarpus* sp.) and the sequencing process was successfully recovered from 74% of the total samples. A study conducted on Shorea and rattan also obtained high number of amplification and sequences product using ITS marker [29]. This result showed that ITS primer is an easy marker to be applied on *P. mooniana* and *Pterocarpus* sp. (used as a reference species).

The difference on nucleotide sequences of DNA ITS region between individual of *P. mooniana* found in this study is a good sign to prove that ITS marker is a powerful tool for identification of *P. mooniana*. This study also found that barcoding based on DNA ITS able to discriminate *P. mooniana* from other species of *Pericopsis* (*P. laxifolia* and *P. angolensis*), although the differentiation with *P. elata* remain unknown as no ITS sequence of *P. elata* available. This study is
the first to provide information on ITS sequences of *P. mooniana* on Genbank database. A barcoding study on flowering plant in Sumatra involving more than 2500 samples, was able to generate barcoding information of several individual of *P. mooniana* that was found in Jambi during the study [6], but unfortunately no ITS marker used in this study.

Contrary to *Pericopsis*, the discrimination power of ITS for *Pterocarpus* is low as this primer can not differentiate each species under genus of *Pterocarpus*. The limited number of each *Pterocarpus* species used in this study might be one of the factor that reducing the power of discrimination. Small number of *Pterocarpus* included in this analysis was used as a sequence reference from closely related genera under Faboidae subfamily. *Pericopsis mooniana* and *Pterocarpus indicus* have been selected by CFBTI as priority species for establishing ex situ conservation plot to provide material for tree breeding program in the near future. Therefore, barcoding study that focus on *Pterocarpus indicus* is required. This study should involve more individual collected from wild population along with the application of other barcoding markers such as chloroplast DNA marker, rbcL and matK, but combination of rbcL and matK has proven to improve the discrimination rate [6].

Morphological observation of *P. mooniana* from SE Sulawesi and Papua planted in PBG revealed that SE Sulawesi has stiff leaflets like parchment with ratio of length : width is 3:1. Edge of leaflet is wavy rarely flat. Adaxial part of leaflets has dark green coloured. Pelvinus on leaflet is slightly thicker. While, *P. mooniana* from Papua has 5-10 leaflets, rotundate to slightly emarginate at base of leaflet, leaflet like membraneceous rarely stiff, ratio of length : width is 2:1, leaflets edge commonly flat rarely wavy, the colour of adaxial part of leaflets is light green rarely dark green. Unfortunately, the *P. mooniana* from SE Sulawesi planted in PBG was not included in DNA ITS characterization. Another genetic characterization study that involved the SE Sulawesi planted in PBG, collection from wild population in Papua along with Jambi population might be reveal differentiation in their genetic characters as well.

The identification of plant species based on morphological characters is very challenging [30], as phenotypic character often resulted from a combination between genetic factors as well as environmental factors [31,32], therefore the ecological dan geographical variation can lead into variation of phenotypic character. This variation will causing the difficulty and uncertainty in identifying the species especially when collecting seedling from multispecies population [15] or collection was conducted by different person with different level of expertise [17], while misidentification of plant species will have serious implication for conservation effort and breeding program of those species [15]. The problem with species identification also occur in bioforensic when wood was only the material left for identification [33]. DNA barcoding has been proved to the misidentification applying barcoding marker for identification, as proven in previous studies that molecular identification will solve these problem and often found that plant species was inappropriately identified by morphological characters [6]. DNA barcoding also helps the rediscovery of extinct species [34]. Despite of many studies conducted to generate DNA barcoding of different species, currently no universal barcoding marker is available for all different plant species [17,35,36]. Therefore barcoding study always challenging and requires proper selection of marker.

4. Conclusions

Barcoding of rDNA ITS of *Pericopsis mooniana* was generated in this study and produced between 634-641 bp nucleotides. Genetic distance between *P. mooniana* with *P. laxifolia* and with *P. angolensis* were 0.116 and 0.088 respectively, while genetic distance between *P. laxifolia* with *P. angolensis* was 0.060. Barcoding of rDNA ITS is a powerful tool for discrimination of *Pericopsis* spp. but not for *Pterocarpus* spp. The difference in morphological characters observed on *P. mooniana* different population planted in PBG, proved that morphological identification of *P. mooniana* need to be conducted properly, and identity confirmation can be conducted using rDNA ITS marker.
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