The genetic diversity of *angsana* (*Pterocarpus indicus*) in Purwodadi Botanical Garden Indonesia revealed by rDNA ITS

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**Abstract.** *Pterocarpus indicus* or *angsana* is one of the tropical tree species that produce redwood. It has been listed as an endangered species by IUCN since 2018, as its natural population number has declined and possibly extinct in some countries. Therefore, conservation efforts to protect this species must be carried out based on the appropriate conservation strategy. This study aimed to assess molecular characters of *Pterocarpus* species in Purwodadi Botanical Garden using rDNA ITS (internal transcribed spacer) and its association with morphological characters. Information on genetic and morphological characters will precisely identify this species so that conservation strategy can be appropriately planned. Leaf samples of eighteen *P. indicus* trees were collected from Purwodadi Botanical Garden (PBG) and used in this study. Twelve specimens that provide clear DNA sequence were genetically assessed. The results showed that *P. echinatus* exhibited rDNA ITS character similar to that *P. indicus*, while *P. indicus* specimens from Morotai were very different from other *P. indicus* specimens. Morotai specimens showed 89% similarity to several *Pterocarpus* species, including *P. acapulcensis*, *P. rohrii* and *P. indicus*. The morphological characters were assessed along with molecular characters. The impacts of conservation strategies are discussed in this paper.

1. **Introduction**

*Pterocarpus indicus* is one of the tree species producing redwood. It is also known as *rosewood* or *angsana*. This tree is native to several tropical countries, such as Malaysia, Papua New Guinea, Philippines, Cambodia, Thailand, Vietnam, East Timor, Solomon Islands, and Indonesia, and also found in tropical parts of China, Japan, and Australia [1]. The trees were highly harvested for their high-quality timber and used for construction, furniture, and musical instrument [2]. It also provides material for traditional medicine practices to cure several diseases. The *P. indicus* populations are seriously threatened and extinct in Vietnam, Sri Lanka, and possibly Peninsular Malaysia [3]. Despite being listed as vulnerable species in IUCN Red List in 1998 [1], the natural population of *P. indicus* is continuously scattered and declining. Based on further assessment, it was listed as an endangered tree species in 2018 [3].

Natural population of *P. indicus* in Indonesia were distributed in Sumatera, Java, Kalimantan, Sulawesi, Maluku and Lesser Sunda Islands. The number of redwood/angsana trees in their natural
The population is decreased as overexploitation for fulfilling the demand of the timber, illegal logging, and land conversion [3]. The low germination rate, low fruit number, and the low number of fruit-bearing trees in the natural population might cause this species's low natural regeneration rate.

Conservation of *angsana* in Indonesia has been initiated by the Center of Forest Biotechnology and Tree Improvement (CFBTI). The ex-situ conservation plot has been established in Gunung Kidul [4] using material collected during exploration in the natural populations in the Lesser Sunda Islands (Sumbawa, Timor, Flores) and Seram Island [5]. The exploration of the material genetic of *angsana* in Sumbawa has found that *angsana* trees produce bristles-covered surface fruits [6] similar to fruits of *angsana* trees in Flores. In contrast, trees in Seram produce smooth surface fruits. A genetic variation study on genetic material collected from Maluku, Timor, and Flores using Random Amplification of Polymorphic DNA (RAPD) markers has revealed that the population of Flores island was genetically distanced from Maluku and Timor population [7]. However, there is no report on the genetic variation of *angsana* from the Sumbawa population based on these markers.

According to International Legume Database & Information Service (ILDIS), Pterocarpus has 35 species [8], mainly are distributed in Asia then Africa [9], but only one species (*P. indicus*) has been reported present in Indonesia. However, various morphological characters have been observed [2, 10]. Proper conservation strategies of *angsana* require proper species identification, especially when they are showing high variation morphologically and genetically. Molecular tools such as DNA barcoding markers have been utilized to identify forest tree species [11-13]. DNA barcoding markers generally applied for plant or forest trees were matK, rbcL, trnL-trnF, trnH-psbA, and ITS [12, 14-17]. A combination of at least two markers provides a higher success rate in identification and a high discrimination rate between species [18], but the application of a single ITS marker has been proven to have sufficient discrimination power for rosewood/*Dalbergia* species [18,19] and also for other species [20].

Even though many studies involve barcoding markers conducted on rosewood species, including *Pterocarpus* [17, 19, 21], the barcoding sequence of Indonesian redwood species has not been available publicly. Therefore, we conducted this study to obtain the rDNA ITS character of *Pterocarpus* species in Indonesia. We are targeting the Purwodadi Botanical Garden (PBG) collection of *Pterocarpus* species in this study as a taxonomist has properly identified based on morphological characters. The molecular characterization of *Pterocarpus* species will provide important information in species identification, genetic diversity of *Pterocarpus*, and as references for further study using more samples collected from the natural population in Indonesia, and the conservation strategy can be planned according to this information.

### 2. Materials and Methods

**2.1. Plant materials, morphological observation, and DNA extraction**

Leaves samples were collected from 18 trees of *Pterocarpus* species from Purwodadi Botanical Garden (PBG), random collections of *angsana* trees (unknown species) from nearby areas (roadside trees in Cangar, Batu, and Malang) were also conducted and used as references. Species identification of these additional collections was conducted based on the morphological character of leaves and bark. The total samples used in this study were 21 trees (Table 1).

Morphological characters of leaf, bark, fruit, and pod were observed based on identification by Baker [22]. In addition, the observations of morphological characters were conducted on two species of *Pterocarpus*, i.e., *P. indicus* and *P. echinatus* in PBG. The DNA extraction, purification, and amplification were conducted in the Molecular Genetic Laboratory of CFBTI in Yogyakarta. The total genomic DNA was extracted from dried leaves specimen of *angsana* using a modified Cetyl Trimethyl Ammonium Bromide (CTAB) method [23]. The total DNAs were diluted ten times using sterile water and used as a PCR reaction template to amplify the ITS region.
2.2. PCR amplification and sequencing of internal transcribed spacer DNA

Amplification of internal transcribed spacer (ITS) region to target sequence of ITS1, 5.8s, and ITS2 were conducted using a combination of ITS5p primer (5' CCT TAT CAY TTA GAG GGA G 3') [20] and ITS4 primer (5' TCC TCC GCT TAT TGA TAT GC 3') [24]. Another combination of ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 primer (5' TCC TCC GCT TAT TGA TAT GC 3') [24] was used for a small number of samples. The amplification was performed in 50 µl of PCR mix containing 10 µl of genomic DNA, 0.5 µM of each primer, and 25 µl of 2x My Taq HS Red Mix (Bioline), 0.2 µg of bovine serum albumin (BSA), and sterile water. In addition, DNA amplification was performed in a ProFlex PCR system (Applied Biosystems) with a program similar to a previous study conducted by [15]. The electrophoresis and sequencing process was also conducted similar to [15].

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 [25] on Bioedit software version 7.0.5.3 [26].

Table 1. The genetic materials collection of *angsana* from Purwodadi Botanical Garden and nearby areas for genetic diversity assessment and their location.

| Specimen ID | Collection ID | Origin   | GPS coordinate | Species       |
|-------------|---------------|----------|----------------|---------------|
| P1          | KRP XIII.F.37 | Taiwan   | 07’47’780      | 112’44’287    | *Pterocarpus indicus* |
| P2          | KRP XIII.F.37 | Taiwan   | 07’47’779      | 112’44’290    | *P. indicus*          |
| P3          | KRP XIII.E1.10 | Taiwan   | 07’47’792      | 112’44’326    | *P. indicus*          |
| P4          | KRP XIII.E1.10 | Taiwan   | 07’47’795      | 112’44’326    | *P. indicus*          |
| P5          | KRP XIII.E1.10 | Taiwan   | 07’47’795      | 112’44’326    | *P. indicus*          |
| P6          | KRP XIII.E1.10 | Taiwan   | 07’47’792      | 112’44’325    | *P. indicus*          |
| P7          | KRP XIII.H.9  | Tropical Asia | 07’47’780    | 112’44’287    | *P. indicus*          |
| P8          | KRP-non collection | Unknown | 07’47’779      | 112’44’290    | *P. indicus*          |
| P9          | KRP XIII.H.53 | Tropical Asia | 07’47’792    | 112’44’326    | *P. indicus*          |
| P10         | KRP XIII.H.53a | Tropical Asia | 07’47’795   | 112’44’326    | *P. indicus*          |
| P11         | KRP XIII.H.54 | Tropical Asia | 07’47’795    | 112’44’326    | *P. indicus*          |
| P12         | KRP XIII.H.54 | Tropical Asia | 07’47’792    | 112’44’325    | *P. indicus*          |
| P13         | KRP XIII.I.23 | Morotai   | 07’47’780      | 112’44’287    | *P. indicus*          |
| P14         | KRP XIII.I.23 | Morotai   | 07’47’779      | 112’44’290    | *P. indicus*          |
| P15         | KRP XIII.I.23 | Morotai   | 07’47’792      | 112’44’326    | *P. indicus*          |
| P16         | KRP XIII.I.23 | Morotai   | 07’47’795      | 112’44’326    | *P. indicus*          |
| P17         | M17           | Malang, roadside tree | n/a    | n/a           | unknown                |
| P18         | KRP XIII.B.2  | Unknown   | 07’47’793      | 112’44’395    | *P. echinatus*        |
| P19         | KRP XIII.B.2  | Unknown   | 07’47’792      | 112’44’397    | *P. echinatus*        |
| P20         | n/a           | Batu, Malang roadside tree | 07’49’386 | 112’31’678'' | unknown                |
| P21         | n/a           | Cangar, roadside tree | 07’46’901 | 112’31’455    | unknown                |
2.3. Phylogeny analysis

The phylogeny analysis was aimed to verify the identity of the species. DNA sequences retrieved from GenBank nucleotide databases with the highest similarity revealed by BLAST online software and an outgroup sequence from a more distantly related taxon were included in the analysis (Table 2). Pairwise distance to calculate the genetic distance within and between species of *Pterocarpus* was created using Mega7 [27]. The phylogenetic trees were constructed using the Maximum Likelihood and Maximum Parsimony method based on the Tamura-Nei model [28] with 500 bootstraps in Mega7.

Table 2. DNA sequences retrieved from GenBank nucleotide databases included in Phylogeny analysis of *Pterocarpus*.

| Accession number | Species       | Specimen ID    | Origin   | Sequence length (bp) |
|------------------|---------------|----------------|----------|----------------------|
| MH245214         | *Pterocarpus* | IFGTB-PS-002   | India    | 747                  |
| MH245215         | *P. santalinus* | IFGTB-PS-003  | India    | 776                  |
| MH245225         | *P. marsupium* | IFGTB-PM-009   | India    | 682                  |
| MH245226         | *P. indicus*  | IFGTB-PI-001   | India    | 711                  |
| AF269175         | *P. acapulcensis* | Lavin 5325         | Mexico   | 637                  |
| AF269176         | *P. macrocarpus* | Lavin 721        | Puerto Rico | 624                  |
| MH487680         | *P. indicus*  | P642           | unknown  | 709                  |
| EF451061         | *P. rohrii*   | C.A. Sothers 1025 | Brazil   | 608                  |
| KY829140         | *P. indicus*  | CAFW20667      | Malaysia | 332                  |
| KY829141         | *P. indicus*  | CAFW23342      | Indonesia | 332                  |
| KY829139         | *P. angolensis* | CAFW18219       | Anggola  | 332                  |
| MT093373         | *Pericopsis mooniana* | M21           | Indonesia | 636                  |

3. Results and Discussion

3.1. Morphological character of *Pterocarpus* species

According to Baker's description of this genus, the morphological character of bark and leaves of two species *Pterocarpus* (*P. indicus* and *P. echinatus*), grew in PBG [29]. However, flowers and pods were unavailable during the examination. Therefore, those two characters were not examined and described.

*Pterocarpus indicus* (Figure 1. A, B), bark, have grooved or slightly grooved bark, bole straight, a large tree with height up to 30 m. Leaves are composite (imparipinnate). The number of the leaflet are 6-8-10-12, ovate, petiolus communis bases are pelvinus, thin, the color in adaxial of the leaf is dark green, light green in abaxial, acuminate at the tip, rotundate at the base, wavy at the leaflet edge (*margo folii*), primary veins are thin with light green color, secondary veins are alternate with each other, width: length ratio is 1:2, rachis color is dark or light brown. Species from Morotai (Figure 1. B) have a different character in bark and leaves. The bark of species from Morotai is glabrous or not grooved. The leaves are slightly wavy leaves, and primary veins are slightly stand out. Rachis's color is dark brown.

*Pterocarpus echinatus* (Figure 1. C), the bark is slightly peeled, trees are around 40–50 ft. high with slender woody stems and densely branchlets. Leaves are composite, imparipinnate. Thin pelvinus at the base of petiolus communis, petioles around 1-2 in., number of leaflet 5-6-8-10 stalked alternately, around six in. long, ovate or oblong leaflets around 2-4 in. long, bases are rounded or rotundate. Tips are subacute or acuminate, wavy margin, glabrous upper surface, finely pubescent below, rachis slightly grooved, light brown, dark green in adaxial and abaxial, but adaxial part is darker than the abaxial part.
A previous study on *Pterocarpus* morphological characters was conducted on the pod of trees collected during exploration of material genetic from several areas in Indonesia, such as Sumbawa Island (Dompu and Bima) [6], Timor Island (Kupang, Soe-South Timor Tengah and Kefamenanu-North Timor Tengah Regency), Flores Island (Ngada and East Manggarai) [7] and Seram Island (West Seram) [10]. The assessment of the morphological characters of collected pods revealed two different types of pods, i.e., bristles covered surface and smooth surface. The differences in pods' surface may be related to the genetic character of *Pterocarpus*, as reported in a study on genetic variation of *P. indicus* based on Random Amplified Polymorphism DNA (RAPD) [7]. However, the type of pods or fruit of *Pterocarpus* specimen observed in this study were unknown as they were unavailable during the study, therefore the correlation between pods type (smooth surface and bristle surface pods) and genetic character of the trees was not observed.

3.2. Amplification of rDNA ITS

The amplification using ITS5p/ITS4 primer set in the PCR was successfully produced rDNA ITS amplicon for most of the *P. indicus/angsana* specimen, except for specimen no P6. The amplicons length produced from *angsana* specimens was approximately 750bp (Figure 3). Amplification of ITS region from the plant often failed to produce amplicon due to contamination of inhabiting fungal leaves [18]. The failure of ITS amplification causing this markerless favorable for plant barcoding. In this study, instead of using a universal ITS primer pair, we opted to use a combination of ITS primer that explicitly targets plant, i.e., ITS5p [20], and a universal ITS primer, i.e., ITS4 [24]. The application of this combination of forward and reverse primer was aimed to amplify rDNA ITS of plants and reduced the chance of fungal DNA being amplified during PCR. In this study, the successful level of amplification using ITS5p/ITS4 primers was 95.2%, with only one specimen failed to be amplified. All of the successful specimens produce a strong and clear single band.

**Figure 1.** *Pterocarpus indicus* (A, B) and *P. echinatus* growing in Purwodadi Botanical Garden and their close-up leaves in the insert pictures.
Studies to compared the success level between barcoding primers (rbcL, matK, ITS) have been conducted and were reported different results. The success level of ITS primer for producing DNA amplicon by PCR were lower than other barcoding primers in tree species [14] and also in Dalbergia species [18], while in meranti and rattan, the PCR success level of ITS was higher than other primers [30]. A study in Aquilaria species using eight barcoding primers, including rbcL, matK, trnL- trnF and ITS, successfully (100%) amplified all of the targeted DNA regions [31]. Other studies found that the success level of ITS in gymnospermae was 88% [32] and 100% in Pericopsis mooniana [15]. The differences in the success level of ITS PCR might be correlated with tree species, specimen type as DNA source, DNA quality and quantity of each specimen, and fungal contamination [19, 20, 33].

3.3. rDNA ITS characterization of Pterocarpus

Twenty DNA amplicons of angsana obtained from the previous step were processed for sequencing, but only 15 out of 20 specimens (75%) producing clear nucleotide sequences length between 510 to 658 bp. In contrast, six specimens were failed to produce a clear nucleotide sequence (Table 1). Five out of six failed specimen (P1-P5) produced a noisy sequence chromatogram. Therefore these specimens can not be appropriately identified, while a specimen (P20) produces a slightly noisy sequence chromatogram. All of these six specimens were not included in the phylogeny analysis.

A blast matching search was conducted on all 15 specimen sequences to estimate the identity of the specimens (Table 3). The DNA sequence of ten specimens (P7-P12, P18-P21) were up to 99% similar to several species of Pterocarpus, including P.indicus and P. macrocarpus, while four specimens of Morotai collection (P13-P16) showing DNA sequence that only 89% similar to P. indicus. One additional specimen was collected from a roadside tree in Malang (P17), showing a DNA sequence that is 99% similar to Gliricidia sepium. This specimen was not included in the phylogeny analysis.

The sequencing success level of ITS DNA amplicon of tree species in the previous studies were varied from as high as 100% for Aquilaria species [31], around 80% for Dalbergia species [18, 19], 74% for Pericopsis mooniana, and 62% success level for various tree species [14]. In the evaluation studies of ITS as barcoding primer in Fabaceae species, including Pterocarpus, the success level of sequencing was lower than rbcL and matK primers [18, 19]. However, despite these low success levels of sequencing using ITS primer in Fabaceae species, the success level of species identification was higher than other primers [19]. Therefore, ITS primer was considered a suitable primer for identifying species [20, 32].
Table 3. The amplification and sequencing product of 20 Pterocarpus/angsana specimens collected from PBG and nearby area and their accession number in Genebank database.

| Sample ID | rDNA Amplicon | Sequencing chromatogram | Sequence length | Blast matching results | Genebank Assesion No |
|-----------|---------------|--------------------------|----------------|-----------------------|---------------------|
| P1*       | present       | noisy                    | n/a            | n/a                   | n/a                 |
| P2*       | present       | noisy                    | n/a            | n/a                   | n/a                 |
| P3*       | present       | noisy                    | n/a            | n/a                   | n/a                 |
| P4*       | present       | noisy                    | n/a            | n/a                   | n/a                 |
| P5*       | present       | noisy                    | n/a            | n/a                   | n/a                 |
| P7        | present       | clear                    | 658            | 100% similar to several P. indicus including MH487679 | MW826113 |
| P8        | present       | clear                    | 658            | Up to 99% similar to several P. indicus including MH487679 | MW826114 |
| P9        | present       | clear                    | 658            | similar to P8         | MW826115 |
| P10       | present       | clear                    | 658            | similar to P8         | MW826116 |
| P11       | present       | clear                    | 658            | similar to P8         | MW826117 |
| P12       | present       | clear                    | 659            | similar to P8         | MW826118 |
| P13       | present       | clear                    | 543            | Up to 89% similar to several Pterocarpus species including P. indicus MH487679 | MW826119 |
| P14       | present       | clear                    | 541            | similar to P13        | MW826120 |
| P15       | present       | clear                    | 541            | similar to P13        | MW826121 |
| P16       | present       | clear                    | 512            | similar to P13        | MW826122 |
| P17*      | present       | clear                    | 510            | Up to 99% similar to several Gliricium sepidum | MT093370 |
| P18       | present       | clear                    | 658            | similar to P8         | MW826123 |
| P19       | present       | clear                    |                | similar to P8         | MT093371 |
| P20*      | present       | A bit noisy              | 651            | similar to P8         | n/a                 |
| P21       | present       | clear                    | 649            | similar to P8         | MW826124 |

Remark: sample with an asterisk (*) were not included in the phylogeny analysis.

3.4. Phylogeny of Pterocarpus based on ITS DNA
Phylogeny analysis of 15 specimen Pterocarpus and nucleotide sequence references retrieved from Genbank (Table 2) were conducted using Maximum Likelihood (Figure 3) dan Maximum Parsimony (Figure were not shown) and involved 500 bootstrapping tests. The phylogeny tree was constructed using the full ITS region (ITS1, 5.8s, and ITS2) and also part of ITS (ITS2). Three additional sequences (ITS2) of Pterocarpus species retrieved from Genbank (KY829139, KY829140, KY829141) were included in the phylogeny analysis of ITS2 but not included in the analysis of the full ITS region. The phylogeny tree of the ITS2 sequence was constructed using the Maximum Likelihood method that involved the 500 bootstrapping test (Figure 4).
Figure 3. Molecular phylogenetic analysis *Pterocarpus* species in PBG based on full ITS (ITS1, 5.8s, and ITS2) sequences by Maximum Likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches, and the tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 679 positions in the final dataset and involved 22 nucleotide sequences. *Pericopsis mooniana* (MT093373) was used as an outgroup.

Phylogeny trees constructed by Maximum Likelihood and Maximum Parsimony showed similar results, separating four specimens of *P. indicus* of Morotai from other specimens. Phylogeny trees (Figure 3, 4) constructed using full ITS region (Figure 3) and partial ITS (Figure 4), we also show similar results. The sequence of partial ITS, i.e., ITS2, shows a high discriminating level between two groups of *Pterocarpus*. A study on the application of ITS2 primer to identify medicinal plants revealed 92.7% of the successful level for identification.
Figure 4. Molecular phylogenetic analysis of *Pterocarpus* species in PBG based on ITS2 sequences by Maximum Likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches, and the tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 347 positions in the final dataset and involved 25 nucleotide sequences. *Pericopsis mooniana* (MT093373) was used as an outgroup.

3.5. Genetic diversity of angsana and the impact on the conservation strategy
The results of this study show that 12 specimens of *P. indicus* of PBG show different genetic characters. Eight specimens belong to the *P. indicus* group, which is also closely related to several *Pterocarpus* species, such as *P. macrocarpus* and *P. angolensis*. Four specimens (P13-P16) that were identified as *P. indicus* showed high differences in the ITS sequences of *P. indicus*. Their ITS sequences were less than 90% similar to *P. indicus* and also around 89% similar to *P. rohrii* and *P. acapulcensis*. Therefore, we assumed that the specimen of Morotai may belong to the unknown species of *Pterocarpus* (*Pterocarpus* sp.).

Further studies are needed to observe these specimens to properly identify this group and visit the natural population for genetic material collection and biological and distribution studies. Public databases such as Genbank have a limited collection of ITS region sequences of all *Pterocarpus* species. This resulted in the identification of Morotai specimens being unsuccessful. The success rate of species identification depends on the availability of sequence references in the public database [20]. In Genbank, many DNA sequences of the *Pterocarpus* species were submitted, but most were DNA sequences of other barcode regions such as trnL, matK, and rbcL.

Two specimens identified as *P. echinatus* (P18, P19) were clustered in the same group as *P. indicus*. For conservation purposes, these groups may be treated as the same species. The morphological character examination of *P. indicus* and *P. echinatus* leaf and bark revealed that these two groups shared similar characteristics. At the same time, *Pterocarpus sp.* of Morotai shows a slightly different character of leaves and bark.
This study observed low genetic variation based on ITS sequences existed among individuals within *P. indicus* and within *Pterocarpus* species of Morotai. A previous study on the genetic variation of *P. indicus* using RAPD marker [7] found low genetic variation within the population. The same study also detected slightly higher genetic variation between certain populations of *P. indicus* on Flores and Timor. Therefore, the high genetic variation between the Morotai and other populations can be caused by different species growing in different islands.

Based on the results of this study, we propose further studies involving a wider population in Indonesia to obtain information on the species identification and genetic and morphological variation of *Pterocarpus*. In addition, conservation efforts such as ex-situ or in-situ conservation should involve a wider population of *Pterocarpus* and pay attention to the distribution of species, geographic distribution, and genetic information of each species.

4. Conclusion

Characterization of the rDNA ITS sequence of *P. indicus* in PBG revealed low genetic variation between individuals. A further study on *P. indicus* specimens from the Morotai population might lead to the identification of another species of *Pterocarpus* species in Indonesia.

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**Acknowledgments**

This study was funded by DIPA through the Ministry of Environmental and Forestry. We thank Wahyunisari for her assistance in sample collection and DNA extraction. We also thank Purwodadi Botanical Garden for providing genetic material for this study. All authors contributed equally to this study.

**Authors’ contribution**

SAD contributed to the material collection and preparing the manuscript, MQ contributed to DNA analysis and preparing the manuscript, IP contributed to the material collection, DNA analysis and preparing the manuscript, and AYPBCW was the research project coordinator who also contributed to preparing the manuscript.