The molecular characterization of Indonesian Gonystylus species

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Abstract. The proper identification of a species is a very important factor for establishment of management and conservation strategies. This study aimed to confirm the identification of Gonystylus spp. using DNA markers. Twenty-four leaf samples of nine species of ramin were extracted using the modified CTAB method to obtain the template DNAs. Four primers were used to amplify ITS ribosomal DNA (partial of ITS2) and three chloroplast non-coding regions sequences. DNA sequences were analyzed using BLAST online software to identify the species. The phylogenetic tree was constructed to confirm species relationship. Based on the DNA markers, one sample of Aquilaria was misidentified as Gonystylus, and the other 23 samples were clustered into seven groups i.e. G. bancanus, G. brunescens, G. stenosepalus, G. forbesii, G. consanguineus, G. lucidulus and an unspecified Gonystylus. The clustering of those samples based on molecular characters was in disagreement with clustering based on morphological characters. This finding suggests that molecular characterization should be conducted to confirm the identification of species prior to establishing conservation areas.

1. Introduction
Gonystylus species also known as ramin is tropical hardwood tree that mostly grows in peat-swamp forests of Indonesia and Malaysia. Approximately about 30 species are distributed throughout the Indonesia, Malaysia, the Philippines, Papua New Guinea, Singapore and Brunei Darussalam. In Indonesia, the majority of species was found in Borneo Island. Ramin is economically valuable to supply international markets for timber and processed wood products, thus it has been heavily harvested in all over natural distribution area [1]. Prior to 2000s, the declining of ramin population in Indonesia was due to heavy exploitation but after 2000s it was due to the slow regeneration, slow rate of growth and habitat degradation due to land conversion for agricultural and infrastructure [1]. The remainings of Indonesian ramin are found in national park and protected areas that provide vital habitat for many other threatened species [3]. Being listed on Appendix III has provided important enforcement benefits for ramin in a number of areas, but these benefits are being challenged by illegal smuggling and laundering activities causing the protection effort powerless. Therefore, conservation effort is urgently required as well as other effort such as proposing ramin to be listed on the appendix.
II [3]. Since 2005, ramin species from Indonesia has been listed on Appendix II and become conservation priority by establishing ex situ seed and planting material [1].

Exploration and collection of trees and seedlings from native forest for conservation activities were often conducted when trees were lacking of important characters for species identification such as flower and fruit. Other morphological characters such as leaf, branch and stem form vary with environmental condition, therefore the accurate species identification was challenging especially for non-taxonomists. Molecular data are often considered to be more reliable than morphological data when inferring phylogenetic relationships at lower taxonomic levels (reference/s). Recently, diverged taxa in which morphological characters are prone to phenotypic plasticity, molecular characters, such as DNA sequences, may provide greater resolution of phylogenetic relationships [4]. The internal transcribed spacers (ITS) of nuclear ribosomal DNA are frequently used for phylogenetic analysis at species level [5]. Although nuclear ribosomal DNA is multicopy in large arrays of repeats, it is useful for phylogenetics as the copies are usually highly homogenous due to concerted evolution. Non-coding region has been used for elucidating phylogenetic relationship of different taxa and sequence variations. Compared with coding regions, non-coding regions may provide more informative characters in phylogenetic studies at species level because of their high variability due to the lack of functional constrains. The present study uses DNA sequences from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) and three non-coding regions of chloroplast DNA (cpDNA) to confirm the identification of nine Gonystylus species that previously identified based on morphological characters.

2. Material and Methods

2.1. Plant materials and DNA extraction
Nine Gonystylus species were identified by local taxonomist using morphological characters of leaves and tree trunk (Table 1). In order to identify sequence variation within species, between 2 to 5 leaf samples were selected among the species. The total genomic DNA was extracted using a modified Cetyl Trimethyl Ammonium Bromide (CTAB) method [6]. The DNA was purified using GENECLEAN III (BIO101), diluted to 2.5ng/µl and used as a template for PCR reaction to amplify ITS and cpDNA region.

| No | Species identity Based on morphological characters | Population | Tree Code |
|----|------------------------------------------|------------|-----------|
| 1  | G. macrophyllus A. shaw                   | Jawa/Bodogol |           |
| 2  | G. macrophyllus A. shaw                   | Jawa/Bodogol |           |
| 3  | G. macrophyllus A. shaw                   | Jawa       |           |
| 4  | G. macrophyllus A. shaw                   | Jawa       |           |
| 5  | G. velutinus A. Shaw                      | KRB        |           |
| 6  | G. velutinus A. Shaw                      | KRB        |           |
| 7  | G. velutinus A. Shaw                      | unknown    | WR        |
| 8  | G. velutinus A. Shaw                      | unknown    | WR        |
| 9  | G. keithii A. Shaw                        | CNV 7      | No. 18    |
| 10 | G. malacensis                             | unknown    | 2         |
| 11 | Gonystylus sp                             | Plot CNV 9 | No. 82    |
| 12 | Gonystylus sp                             | unknown    |           |
| No | Species identity Based on morphological characters | Population | Tree Code |
|----|---------------------------------------------------|------------|-----------|
| 13 | *Gonystylus* sp | unknown |            |
| 14 | *G. brunescens* | unknown | BK 1195   |
| 15 | *G. brunescens* | unknown | BK 153    |
| 16 | *G. brunescens* | unknown | A14-A15.48|
| 17 | *G. brunescens* | unknown | A17-A18.65|
| 18 | *G. brunescens* | unknown |            |
| 19 | *G. consanguineus* | unknown | WR        |
| 20 | *G. consanguineus* | unknown | WR        |
| 21 | *G. bancanus* | unknown |            |
| 22 | *G. bancanus* | unknown |            |
| 23 | *G. bancanus* | unknown |            |
| 24 | *G. bancanus* | unknown |            |

2.2. PCR Amplification and sequencing of internal transcribed spacer and chloroplast DNA

Amplification of partial of internal transcribed spacer (ITS) region targeted sequence of 5.8s to ITS2 was conducted using combination of ITS3 (5’ GCA TCG ATG AAG AAC GCA GC 3’) and ITS4 primer (5’ TCC TCC GCT TAT TGA TAT GC 3’). Amplification of chloroplast DNA (cpDNA) targeted three chloroplast non coding regions (the *trnL* intron, the intergenic spacer between *trnL - trnF*, and *trnD - trnY*) [7]. The amplification reaction was performed in a total volume of 20 µl containing 4 ng of genomic DNA, 0.25 µM of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl₂, 200 mM of each dNTP, and 0.25 unit/10µl Ex Taq DNA polymerase. DNA amplification was performed with a Gene Amp PCR System Model 9600 (Perkin-Elmer) and programmed as follows: 95°C for 90s, 30 cycles of 30s at 94°C, 30s at 55°C, and 90s at 72°C, followed by 60s at 72°C. The PCR product was separated by electrophoresis in 1.5% agarose gel and the target fractions were excised from the gel. DNA amplicon was recovered from the gel particles and purified using QIAEX II Gel Extraction (QIAGEN), then sequenced in a reaction with Thermo Sequenase Cycle Sequencing Kit (ThermoFisher) using ABI 3100 DNA Sequencer (Applied Biosystem).

DNA sequence chromatograms were viewed and edited to remove poor quality sequences at each end using Chromas software (http://technelysium.com.au/wp/chromas/). A sequence with high similarity was retrieved from public DNA databases i.e. GenBank [8] using BLAST. Sequences were grouped according to BLAST search results and included in a phylogenetic analysis with several sequences of the best matches retrieved by the BLAST searches to verify the identity. Sequences were aligned using ClustalW [9] with full multiple alignment and 1000 bootstraps in BioEdit 7.0.9.0 [10] prior to phylogenetic analysis. One sequence from a more distantly related taxon was included as outgroup in each analysis. The phylogenetic trees were inferred for each group using the Maximum Likelihood method based on the Tamura-Nei model [11] and were performed using 1000 bootstraps in Mega7 [12]. Each tree was rooted using the outgroup taxon. The trees with the highest log likelihood are shown and the percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.
3. Result and discussion

3.1. Results

3.1.1. ITS DNA Sequence of Gonystylus

Sequencings of partial ITS region from 24 individuals were produced between 221bp to 384bp of nucleotide and mostly identic to Gonystylus species, except for individual 7 (Table 2). The individual was morphologically recognized as G. velutinus; however the ITS DNA marker shows that it is 99% identic to Aquilaria species. Based on ITS DNA sequences, only 10 out of 24 individuals were identic (with 98%-100% of similarity) to species level i.e. G. stenosepalus (individual 5, 6), G. spectabilis (individual 8), G. forbesii (individual 9, 10, 18), G. affinis (individual 11, 12, 15) and G. bancanus (individual 24). The 23 remaining samples were identic to unspecified Gonystylus species. Individual 1, 2, 3, 4 were closely related (95%) to G. maingayi and also G. bancanus and G. eximius, individual 13, 14, 16 were closely related (92%-95%) to G. affinis, G. eximius and G. brunnescens, individual 17 and 19 were closely related (95%-96%) to G. stenosepalus, while individual 20, 21, 22 and 23 were closely related (93%) to G. bancanus and 91% identic to G. affinis. DNA sequence reference of ITS region for G. macrophylus was unavailable, therefore no matching sequences were found between Gonystylus species examined in this study to any G. macrophylus.

3.1.2. cpDNA Sequence of Gonystylus

Sequencing of trnL intron region has produced 500-536 bp nucleotides (data not shown). The sequence references of this region for Gonystylus in Genbank is very limited, currently only three submissions available i.e. one nucleotide sequence of G. macrophylus and two nucleotide sequences of G. bancanus. This limitation has caused the identification at species level of Gonystylus in this study unmanageable. Most of the samples were closely related (99%-100%) to G. macrophylus and G. bancanus, except individual 7 that was closely related (up to 99%) to Aquilaria spp, while one individual (6) was not sequenced.

Sequencing of trnL – trnF intergenic spacer region has produced 414-434 bp nucleotides (data not shown). The sequence references of this region for Gonystylus in Genbank are also limited, with only five submissions available i.e. one nucleotide sequence of G. macrophylus and four nucleotide sequences of G. bancanus. This limitation has also caused the identification at species level of Gonystylus based on DNA character of this region unmanageable. Similar with trnL intron, most of the samples were closely related (99%-100%) to G. macrophylus and G. bancanus, except individual 7 that was closely related (87%) to Aquilaria spp.

Sequencing of trnD – trnY region has produced 326-506 bp nucleotides (data not shown). Due to the lack of sequence references of this region for Gonystylus in Genbank (only one submission available i.e. G. bancanus), the identification at species level of Gonystylus based on trnD-trnY region was also unmanageable. Most of the samples were closely related (99%-100%) to G. bancanus, except 7 that was 100% identic to Aquilaria malaccensis.

| Individual | Sequence Length (bp) | Identity | Accession no of highest similar reference |
|------------|----------------------|----------|----------------------------------------|
| 1          | 252                  | 221/224 (99%) identic to G. maingayi and up to 239/252 (95%) identic to other Gonystylus species including G. bancanus and G. eximius | KU991097 |
| Individual | Sequence Length (bp) | Identity | Accession no of highest similar reference |
|------------|----------------------|----------|------------------------------------------|
| 2          | 363                  | 323/326 (99%) identic to *G. maingayi* and up to 346/363 (95%) identic to other *Gonystylus* species including *G. bancanus* and *G. eximius* | KU991097 |
| 3          | 363                  | 319/322 (99%) identic to *G. maingayi* and up to 317/322 (98%) identic to other *Gonystylus* species including *G. brunnescens* | KU991097 |
| 4          | 361                  | 319/322 (99%) identic to *G. maingayi* and up to 317/322 (98%) identic to other *Gonystylus* species including *G. brunnescens* | KU991097 |
| 5          | 289                  | Up to 288/289 (99%) identic to *G. stenosepalus* and up to 286/289 (99%) identic to other *Gonystylus* species including *G. brunnescens* and *G. eximius* | KU991091 |
| 6          | 221                  | 221/221 (100%) similar to *G. stenosepalus* and up to 220/221 (99%) similar to other *Gonystylus* species including *G. brunnescens* and *G. eximius* | KU991091 |
| 7          | 384                  | 346/349 (99%) identic to *Aquilaria beccariana* and *A. malaccensis*, up to 345/348 (99%) identic to other *Aquilaria* species | KT779116 / KT347174 |
| 8          | 288                  | 285/288 (99%) identic to *G. spectabilis* and, up to 285/288 (99%) identic to other *Gonystylus* species, including *G. brunnescens* and *G. eximius* | KU991106 |
| 9          | 290                  | 286/290 (99%) identic to *G. forbesii* and, up to 284/290 (98%) identic to other *Gonystylus* species including *G. spectabilis* and *G. stenosepalus* | KU991065 |
| 10         | 289                  | 288/290 (99%) identic to *G. forbesii* and, up to 286/290 (99%) identic to other *Gonystylus* species including *G. spectabilis* and *G. stenosepalus* | KU991065 |
| 11         | 288                  | 286/291 (98%) identic to *G. affinis* and up to 282/291 (97%) identic to other *Gonystylus* species including *G. eximius* and *G. areolatus* | KU991087 |
| 12         | 283                  | 280/287 (98%) identic to *G. affinis* and up to 276/287 (96%) identic to other *Gonystylus* species including *G. eximius* and *G. areolatus* | KU991087 |
| 13         | 281                  | 274/285 (96%) identic to *G. affinis* and up to 270/285 (95%) identic to other *Gonystylus* species including *G. eximius* and *G. stenosepalus* | KU991087 |
| 14         | 368                  | 319/322 (99%) identic to *G. affinis* and up to 349/368 (95%) identic to other *Gonystylus* species including *G. bancanus* and *G. eximius* | KU991087 |
| 15         | 283                  | 280/285 (98%) identic to *G. affinis* and up to 276/285 (97%) identic to other *Gonystylus* species including *G. eximius* and *G. areolatus* | KU991087 |
| 16         | 364                  | 308/319 (97%) identic to *G. affinis* and up to 344/372 (92%) identic to other *Gonystylus* species including *G. eximius* and *G. brunnescens* | KU991087 |
| 17         | 370                  | 320/320 (100%) identic to *G. stenosepalus* and up to 354/370 (96%) identic to other *Gonystylus* species including *G. eximius* | KU991091 |
| Individual | Sequence Length (bp) | Identity | Accession no of highest similar reference |
|------------|---------------------|----------|-----------------------------------------|
| 18         | 285                 | 279/283 (99%) identic to *G. forbesii* and, up to 97.88% identic to other *Gonystylus* species including *G. spectabilis* and *G. stenosepalus* | KU991065 |
| 19         | 304                 | up to 290/311 (93%) identic to *G. stenosepalus* and other *Gonystylus* species including *G. forbesii* and *G. affinis* | KU991091 |
| 20         | 373                 | up to 359/373 (96%) identic to *G. bancanus* and 323/323 (100%) identic to *G. stenosepalus* | KY235216/ KU991091 |
| 21         | 277                 | Up to 260/276 (94%) identic to *G. bancanus* and up to 264/288 (92%) identic to *G. consanguineus* | KU244107 |
| 22         | 304                 | Up to 286/310 (92%) identic to *G. bancanus* and up to 283/311 (91%) identic to *G. spectabilis* | KU244107 |
| 23         | 296                 | 282/303 (93%) identic to *G. bancanus* and up to 277/304 (91%) identic to *G. affinis* | MH432153 |
| 24         | 287                 | 284/289 (98%) identic to *G. bancanus* and, up to 276/290 (95%) identic to *G. affinis* | MH432153 |

### 3.1.3. Phylogeny of *Gonystylus* based on partial ITS region

A phylogeny tree to observe genetic relationship of 21 samples was constructed (Figure 1), which showed the tree with the highest log likelihood (-1033.8668). The analysis involved 40 nucleotide sequences and a total of 270 positions in the final dataset. Individual no 1, 22 and 23 were not included in this analysis as their sequence chromatograms were noisy and illegible. Several closely related sequences such as *G. maingayi*, *G. bancanus*, *G. spectabilis*, *G. forbesii*, *G. affinis*, *G. stenosepalus G. consanguineous*, *G. areolatus*, *G. eximius*, *G. brunnescens*, *A. malaccensis*, *A. hirta* and *A. microcarpa* were used as references. One reference from distantly related taxon as outgroup (*Dirca palustris*) was also included in the alignment and phylogeny analysis.

Based on dendogram of 338 bp nucleotide from 41 nucleotide sequences, 23 individuals of *Gonystylus* sp were clustered into 7 group. A group consists of individual 1, 2, 3, and 4 is closely related to *G. maingayi*. These samples were recognised as *G. macrophyllus* but ITS sequence of this species is currently unavailable in Genbank. Another group (individual 6, 15, 19, 21) is also closely related to *G. maingayi*, but the variation in this group is too wide with more than 5 nucleotides differences. A group consists of individual 5, 17, 20 and 8 is closely related to *G. stenosepalus* although individual no 8 has more variation than any other member of this group.
Figure 1. Dendrogram of *Gonystylus* and *Aquilaria* constructed using Mega7 based on partial 5,8s and ITS-2 DNA sequence with *Dirca palustris* (GQ471037) used as an outgroup.

3.1.4. Phylogeny of *Gonystylus* based on partial cp DNA region
Phylogeny analysis was conducted based on three different regions (the trnL intron, the intergenic spacer between trnL - trnF, and trnD - trnY), but only dendogram of the trnL intron and intergenic spacer between trnL – trnF are shown here (Figure 2 and 3). The sequence reference of those three partial DNA region of *Gonystylus* are limited to only two species i.e *G. bancanus* and *G. macrophyllus*, therefore the individual samples were unidentified. The DNA characters between individual samples based on those three regions were also similar. Characters of trnL intron DNA sequence of 23 *Gonystylus* individuals were uniforms except for individual no 8 (Figure 2), in a tree with the highest log likelihood (-996.5002). The analysis involved 30 nucleotide sequences and a total of 538 positions in the final dataset. DNA sequence of intergenic spacer between trnL - trnF has clustered 23 individuals of *Gonystylus* species into four groups, again individual no 8 is solely excluded from other groups (Figure 3), in a tree with the highest log likelihood (-884.67). The analysis
involved 37 nucleotide sequences and a total of 350 positions in the final dataset. Meanwhile, DNA sequence of trnD – trnY between 23 *Gonystylus* individuals were varied into two big groups (data not shown).

**Figure 2.** Dendrogram of *Gonystylus* and *Aquilaria* constructed using Mega7 based on trnL intron DNA sequence with *Dirca palustris* (GQ471060) used as an outgroup.
Figure 3. Dendrogram of *Gonystylus* and *Aquilaria* constructed using Mega7 based on intergenic spacer between trnL – trnF DNA sequence with *Dirca palustris* (GQ471060) used as an outgroup.
3.1.5. Phylogeny of Gonystylus based on combination of ITS and partial cp DNA region

Multigene phylogeny analysis was conducted based on the combination ITS and partial cp DNA region (the trnL intron, the intergenic spacer between trnL - trnF, and trnD - trnY) using data of 24 individuals (Figure 4). The analysis involved 41 nucleotide sequences and a total of 1742 positions in the final dataset. As the sequences of three cp DNA region were showing small variation, the multigene phylogeny tree of Gonystylus is similar with phylogeny tree based on ITS region. The 23 of Gonystylus individuals were clustered into eight groups i.e. an individual that closely related to G. lucidulus, a group that closely related to G. stenosepalus, a group close to G. bancanus, a group close to G. maingayi, a group close to G. forbesii and also three clusters of individual that did not closely relate to any specific Gonystylus. One individual of Aquilaria identified in this study related closely to Aquilaria group i.e A. beccanesis, A. malaccensis and also A.microcarpa.

3.2. Discussion

Phylogeny of family Thymelaceae based on barcoding using molecular characters has been studied globally [13], [14] especially on genus that are commercially valuable such as Aquilaria, Gyrinops and Gonystylus [5], [15], [16]. Most of these studies were using ribosomal DNA sequence of ITS region, non-coding cp DNA such as intergenic spacer and, trnL-trnF region, and coding cp DNA such as matK and rbcL. The phylogeny in study was constructed based on DNA sequence of ITS region and three partial cp DNA. ITS sequence was able to discriminate groups of Gonystylus. This finding is in corresponding with other studies that suggest ITS to be incorporated into other barcodes for plant [15], [17], following suggestion on the use of rbcL and matK as plant barcodes [18]. This study also found that the non-coding cp DNA (trnL intron, intergenic spacer between trnL - trnF, and trnD - trnY) was less powerful compare to ITS in order to discriminate the species of Gonystylus in corresponding with previous studies on Thymelaceae [15], [16].

The use of four barcodes in this study was also proven to discriminate one individual of Aquilaria from other species of Gonystylus. These two genus of Thymelaceae have similar phenotypic characters and the morphological identification of this family was mainly relied on fruit and flower that not always available throughout the year [16], [19] thus only skillfull and experienced taxonomist can properly identify those species based on morphological characters. Previous studies also found that molecular identification revealed unmatched identification with morphological observation of trees especially when flower and fruit are not available [20]–[22]. Thus, the proper identification using DNA barcoding should be conducted prior to collection of material genetic of forest tree in natural population.

The application of ITS and cpDNA as barcoding in this study could not successfully identify individual of Gonystylus up to species level, due to the limitation of DNA sequence of this genus in public database, although plenty of studies on this genus and other species of Thymelaceae have been conducted. The studies of molecular characters of this family were mostly only limited to specific species and involved one to three selected barcoding. Although ITS was proven to have better discrimination on woody trees, the comprehensive study involved ITS along other barcodes for Gonystylus and other Thymelaceae are still limited. Therefore, the result of this study could provide more information of Gonystylus DNA sequences of ITS region and cp DNA. Further study on identification of threatened species especially Gonystylus species using comprehensive plant barcodes along with morphological description of the trees is still required.
Figure 4. Dendrogram of *Gonystylus* and *Aquilaria* constructed using Bioedit based on a combination of ITS and three regions of chloroplast non coding (the trnL intron, the intergenic spacer between trnL-trnF, and trnD-trnY) DNA sequences. *Dirca palustris* was used as an outgroup.

4. Conclusion

Application of molecular markers confirms that 23 individuals used in this study were identified as *Gonystylus* species while one individual was identified as *Aquilaria*. ITS marker has strong ability to discriminate *Gonystylus* species compared to chloroplast non coding markers. The 23 of *Gonystylus* individuals were clustered into eight groups i.e. an individual that closely related to *G. lucidulus*, a group that closely related to *G. stenosepalus*, a group close to *G. bancanus*, a group close to *G. maingayi*, a group close to *G. forbesii* and also three clusters of individual that did not closely relate to any specific *Gonystylus*. Clustering of individuals based on molecular characters was in disagreement with clustering based on morphological characters. This finding suggests that molecular
characterization should be conducted to confirm the identification of species prior to establishing conservation areas

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References
[1] Barstow M 2018 Gonystylus bancanus IUCN Publication
[2] IUCN 2004 IUCN Red List of threatened species: a global species assessment IUCN Publication Gland Switzerland Cambridge
[3] SSN 2004 Ramin Washington
[4] Ghorbani A, Saeedi Y and de Boer H J 2017 Unidentifiable by morphology: DNA barcoding of plant material in local markets in Iran PLoS One 12 4 e0175722
[5] Tanaka S and Ito M 2019 DNA barcoding for identification of agarwood source species using trnL-trnF and matK DNA sequences J. Nat. Med.
[6] Shiraishi S and Watanabe A 1995 Identification of chloroplast genome between Pinus densiflora Sieb et Zucc and P thunbergii Parl based on the polymorphism in rbcL gene J Japanese For Soc /Nihon Ringakkai Shi 77 5 pp 429–436
[7] Widyatmoko A Y P B C and Shiraishi S 2013 Geographic Variation of Chloroplast DNA Haplotypes in Acacia auricocarpa A Cunn ex Benth Indones. J. For. Res. 10 1 pp 43–56
[8] Benson D A, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman D J, Ostij J, Sayers E W. 2013 GenBank Nucleic Acids Res. 41 pp D36–D42
[9] Thompson J D, Higgins D G and Gibson T J 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice Nucl. Acid Res. 22 pp 4673-4680
[10] Hall T A 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT Nucl. Acids Symp. Ser. 41 pp 95-98
[11] Tamura K and Nei M 1993 Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees Mol. Biol. Evol. 10 3 pp 512–526
[12] Kumar S, Stecher G and Tamura K 2016 MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets Mol. Biol. Evol. 33 7 pp 1870-1874
[13] Van Der Bank M, Fay M F and Chase M W 2002 Molecular phylogenetics of Thymelaeaceae with particular reference to African and Australian genera Taxon 51 2 pp 329–339
[14] Eurlings M C and Gravendeel B 2005 TrnL-trnF sequence data imply paraphyly of Aquilaria and Gyrinops (Thymelaeaceae) and provide new perspectives for agarwood identification Plant Syst. Evol. 254 1–2 pp 1–12
[15] Lee S Y, Ng W L, Mahat M N, Nazre M and Mohamed R 2016 DNA barcoding of the endangered aquilaria (Thymelaeaceae) and its application in species authentication of agarwood products traded in the market PLoS One 11 4 e0154631
[16] Farah A H, Lee S Y, Gao Z, Yao T L, Madon M and Mohamed R 2018 Genome size molecular phylogeny and evolutionary history of the tribe aquilarieae (Thymelaeaceae) the natural source of agarwood Front Plant Sci. 9 May pp 1–12
[17] China Plant BOL group 2011 Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants Proc. Natl. Acad. Sci. 108 49 pp 19641–19646
[18] Hollingsworth P M 2011 Refining the DNA barcode for land plants Proc. Natl. Acad. Sci. 108 49 pp 19451 – 19452
[19] Wardhani M, Yafid B, Komar T E, Nurjanah S and Rosita D T 2010 Eds Gonystylus Spp (Ramin ): Population Status Genetics and Gene Conservation Bogor: Indonesia’s Work
 Programme for 2008 ITTO CITES Project

[20] Palandačić A, Naseka A, Ramler D and Ahnelt H 2017 Contrasting morphology with molecular data: an approach to revision of species complexes based on the example of European Phoxinus (Cyprinidae) BMC Evol. Biol. 17 184

[21] Handayani R 2016 Akurasi DNA barcode untuk identifikasi jenis Dipterokarpa langka di Hutan Harapan Jambi Institut Pertanian Bogor

[22] Rangkuti A B 2016 Aplikasi DNA barcode untuk identifikasi jenis meranti dan rotan Institut Pertanian Bogor