Clarification of Sumatran Mulberry (*Morus macroura* var. *macroura*, Moraceae) from West Sumatra, Indonesia using Nucleus Ribosomal ITS (*Internal Transcribed Spacer*) Gene

Erma Nuratika, Nindi Aseny, Syamsuardi, Nurainas, Fitmawati¹, Friardi²

**ABSTRACT**

**Background:** *Morus macroura* Miq. is a potential tree species belonging to the Mulberry group, and consider as flora identity of West Sumatra Province with local name is Andalas. This germplasm tropical tree species utilize widely by local West Sumatran people for various purposes such as wood material to construct the traditional house of ethnic Minangkabau and medicinal substance for multiple diseases. However, the population numbers of this species were decreased and rare due to overexploitation without effort for planting. Based on this reason, it is imperative to conserve and utilize sustainable Mulberry. There is a highlight confusion to clarify that *M. macroura* from Sumatra population similar to Himalayan Mulberry. For this reason, it is essential to define the crucial local *M. macroura* from Sumatra (Sumatran Mulberry) for its conservation and sustainable utilization.

**Method:** In this study, the leaves from seven individuals of *M. macroura* collected from West Sumatra were used for DNA extraction and processed to the next step of the analysis. Four individuals of Himalayan Mulberry and both of two out-group, White Mulberry (*M. alba*), and Black Mulberry (*M. nigra*) were used in this study. Polymerase chain reaction (PCR) amplification was performed using two ITS markers (ITS5-F and ITS4-R) with an annealing temperature of 55°C. The PCR products were purified and sequenced by Macrogen USA DNA Sequencing. The phylogenetic relationship between seven accessions and two of out-group (*M. alba* and *M. nigra*) were analyzed using the MEGA6 program.

**Result:** The result indicated that all of the accessions *M. macroura* from Sumatra were grouped into the same cluster and separated to the Himalayan Mulberry group. The differentiation between Sumatran Mulberry and other Mulberry reflected in morphological and haplotype differentiation indicated that the Sumatran Mulberry as the different group and suggested as a variety with scientific name is *M. macroura* Miq. var. *macroura*. This clarification is useful for conservation and sustainable utilization of this variety, a specific Mulberry from Sumatra.

**Key words:** Differentiation, Haplotype, ITS gene, *M. macroura* var. *Macroura*, Sumatran mulberry

**INTRODUCTION**

*M. macroura* Miq. is one of the species in the Mulberry Group (Genus *Morus* spp.), that belongs to the family Moraceae in order Rosales (The Plant List, 2013). Although seventeen species of *Morus* have the scientific name with the accepted status, more than 85 name species still have the unresolved rank (The Plant List, 2013) suggested that the taxonomic status of this group was still unclear and disputed (Nepal, 2008). Like other species in Mulberry group, *M. macroura* widely distributed from South-East Asia, such as Indonesia, Thailand, Myanmar, Vietnam, Laos and Nepal, to East Asia (China) and South Asia (India) (GBIF, 2019). In Indonesia, Sumatra Island, especially the West Sumatra region, is the central distribution of the species (*Syamsuardi et al.*, 2015; GBIF, 2019). The scientific name *M. macroura* was published by Miquel (1862) based on the herbarium specimen collected from Batang Barus, North Sumatra. But the centre distribution of the populations of this species is the West Sumatra region that growing at a humid habitat near the riverside in the secondary and primary tropical forest (Jawati 2006; Syamsuardi, 2015). The *M. macroura* population from Sumatra (hereafter is Sumatran Mulberry) is an essential economic woody plant. This tree has a very significant impact on local people (ethnic Minangkabau) due to its economic value as the wood material for building house mainly traditional big house (*Yusfita*, 2008), for medicinal various disease and resource of modern medicine (*Arbain*, 2012; *Farrag et al.*, 2017). The potential of this plant can be
developed as well as bioprospecting of the others Mulberry (Song et al., 2019). However, the individuals or the populations this wild tree species significantly decreased due to overexploitation and reproductive problems without effort to plant develop the number of population (Syamsuardi, 2015). Based on these facts and it is imperative to build conservation strategy for sustainable utilization. The fundamental approach in the bioprospection of this tree species is precise and accurate information about the source of the material used for its sustainable utilization and conservation.

Further, M. macroura is known locally as Andalas and is famous for its strength and resistance towards termites. Andalas also is another name of the Sumatra island and is used as the name of a government university in West Sumatra (Arbain, 2012). This plant decided as an identity flora of West Sumatera province as unique flora and closely related to the living and culture of local people (Minangkabau ethnic). However, some information mentioned the Andalas tree is the same as Himalayan Mulberry. So, the utilization and conservation of this local Mulberry will be not accurate and uncertain. It is imperative to clarify the classification and identification of this group. The molecular marker is a useful and precise tool to resolve this taxonomic problem. ITS is one of the molecular markers applied to clarify the taxonomic issue.

The ITS marker has widely conducted in the phylogenetic relationship and evolution of plant species (Zang et al., 2019). This marker has proved as the best marker to clarified more than 6600 samples of the plants (Chen et al., 2010; Saddhe and Kumar, 2018). This marker also used to analyze the infraspecific tax, landrace and a unique population of the tropical plant (Huda et al., 2019) and authentication of medicinal plants (Hao et al., 2014; Kim et al., 2016;). It is also used to identify the material resources from mixed traditional medicine or adulterants (Zhou et al., 2014; Doh et al., 2017). This study clarified diagnostic molecular characteristics. It proved the uniqueness of Sumatran Mulberry and its differentiation to Himalayan Mulberry and other closely related species of Mulberry Group using the ITS molecular marker.

**MATERIALS AND METHODS**

In this study, the leaves from seven individuals of M. macroura collected from West Sumatra were used for DNA extraction and processed to the next step of the analysis. The detail collection site of the samples is presented in Fig 1. Four individuals of Himalayan Mulberry and both two Outgroup, White Mulberry (M. alba) and Black Mulberry (M. nigra) used this study. Total DNA was extracted from young leaves samples using the CTAB (Doyle and Doyle, 1987). The little modification procedure conducted and followed by run electrophoresis using a voltage of 100 volts for 45 minutes. Polymerase chain reaction (PCR) amplification was performed using ITS markers (Primer ITS5-F; Sequence (5’-3’): GGAAGTAAAAAGT-CGTACAAGG; Primer ITS4-R: Sequence (5’-3’): TCCCTCCGCTTA-TTGATATGC) from Muellner et al., (2003) with an annealing temperature of 55°C. The PCR products were purified and sequenced by Macrogen USA DNA Sequencing.

The SegMan program in DNA STAR used to contig the sequence obtained in the form of the forward and the reverse (Burland, 2000). The alignment of the sequences conducted using ClustalX (Thompson et al., 1997) and then edited using the BioEdit program (Hall, 1999). Polymorphism sequence analysis was performed using the DNA Sequence Polymorphism 5.10 program (Rozas et al., 2003) to see changes in nucleotide bases (haplotypes). The phylogenetic relationship was analyzed using the MEGA6 program. The additional four DNA sequences of Himalayan Mulberry and those of the sequence of M. alba and M. nigra obtained from NCBI used in the analyzed (Table 1).

**RESULTS AND DISCUSSION**

Based on the observation in the natural habitat of population M. macroura we noted the individuals or populations tend to decrease. This plant distributed at the humid habitat of secondary tropical forests in West Sumatra with an altitude of more than 800 m up to sea level (see Fig 1). For identification of Mulberry group using leaves characters is complicated due to wide variation not only between species (Nepal, 2008) but also within species. Leaves shape variation within species also was detected in one population of M. macroura in the Sumatra population (Syamsuardi, 2015). Analysis of morphological on the character generative indicated that individuals from West Sumatra (Sumatran mulberry) differed from Himalayan Mulberry and closed related species. The fruit type of Sumatran Mulberry is dried drupe and not edible. In the case of Himalayan Mulberry, the fruit type is berry and edible (Table 2). Based on these facts suggested that Sumatran Mulberry was not the same with Himalayan Mulberry and Sumatran Mulberry different taxa with Himalayan Mulberry. The consequence of this is that the Sumatran Mulberry was still the same species to Himalayan mulberry but suggested to separate Sumatran Mulberry. Based on Article 46.1 of the International Code of Nomenclature Botany (Turland et al., 2018), the legitimate scientific name of Sumatran Mulberry with holotype from Sumatra was M. macroura var. macroura. The results of previous studies also revealed the morphological differences between cultivated variety from the different geographical regions (Moawed, 2015; Strbanovic et al., 2015) and between the wild infra-specific taxa (Tripathy et al., 2016; Huda et al., 2019) were detected.

Furthermore, molecular evidence is a significant character to support the stable identification of a taxon and classification of the taxonomic group. The haplotype is a set of DNA that can be used to identify differentiation between accessions (Huda et al., 2019) or infraspecific and species. In this study, the haplotype analysis was carried out on eleven sequences that consisting of seven sequences obtained from this study and four other sequences of DNA
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**Fig 1:** Collection site of eight individuals of *M. macroura* in West Sumatra. The coordinate of the location presented on the left side of the map.

**Table 1:** Genbank data used in data analysis.

| Sample          | Accession Version | Definition                                                                 |
|-----------------|-------------------|---------------------------------------------------------------------------|
| *M. macroura*   | AM042000.1        | *M. macroura* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial). |
| *M. macroura*   | AY345147.1        | *M. macroura* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. |
| *M. macroura*   | HM747170.1        | *M. macroura* voucher MO: Dao 90-272 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence and internal transcribed spacer 2, partial sequence. |
| *M. macroura*   | AB604280.1        | *M. macroura* genes for ITS1, 5.8S rRNA, ITS2, complete sequence, specimen_voucher: XNS0099. |
| *M. alba*       | AB604289.1        | *M. alba* genes for ITS1, 5.8S rRNA, ITS2, complete sequence, specimen_voucher: XNS0256. |
| *M. nigra*      | KT002542.1        | *M. nigra* voucher XNS0709 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. |

(Source: [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/))

**Table 2:** The morphological differentiation of Sumatran Mulberry compared to Himalayan mulberry and closed related species.

| Characteristics                      | *M. macroura* | *M. alba*** | *M. nigra* |
|--------------------------------------|---------------|-------------|------------|
|                                      | Sumatran mulberry | Himalayan mulberry* | Small tree | Small tree |
| Habit                                | Tree          | Tree        | Small tree | Small tree |
| Colour of the stem                   | Brown         | Gray        | Brownish green | Green grey |
| Leaves character                     | Acuminate, obtuse, serrulate or serrate, ovate or cordate | Acute or acuminate, rounded or cordate, serrate, ovate or cordate | Acute, cordate, serrate, cordate | Acute or acuminate, cordate, serrate, ovate |
| Apice, base, margin and shape        | Long catkin   | Long catkin | Short catkin | Short catkin |
| Inflorescence type                   | Long catkin   | Long catkin | Short catkin | Short catkin |
| Sexual kind of flowers               | Dioecus       | Dioecus     | Subdioecus | Subdioecus |
| Fruit                                | Drupa and dry | Berry and fleshy | Berry and fleshy | Berry and fleshy |
| Edibility                            | Not edible and green | Edible with purple colour | Edible with white or dark red, black colour | Edible with purple or black colour |

Note: some information from *Nepal (2008)*, **Rao, et al. (2011)**
collected from Genbank Data (NCBI, 2019). From the sequential analysis using the DNA Sequence Polymorphism program was obtained three Haplotypes (Table 3). This evidences suggested that three diagnostic molecular characters were detected and can be applied to distinguish a taxonomic group.

All of the accesses from West Sumatra (Sumatran Mulberry) have a similar haplotype (H01). While the Himalayan Mulberry from India has a different haplotype (H02) and Himalayan Mulberry from the USA possessed a different haplotype (H03). In the case of an unusual haplotype pattern of Himalayan Mulberry from China, we assumed that the collected sample did not originate from China. Based on this fact, the Sumatran Mulberry differed from Himalayan Mulberry and other closed related species. The molecular evidence supported the morphological evidence from generative organs and reflected the uniqueness of Sumatran Mulberry. This evidence is very important to support this tree Mulberry from West Sumatra (Sumatran Mulberry, local name: Andalas) is the identity flora of West Sumatra Province.

Moreover, the analysis of haplotype diversity of this taxa is fundamental to obtain for conservation and sustainable utilization. The high Haplotype Diversity (0.582) was detected in M. macroura (Sumatran and Himalayan Mulberry). According to Nei and Tajima (1981), the haplotype diversity value range is 0 to 1. Haplotype diversity was high if the value was > 0.5 and will be said low if it is <0.5. The high value of haplotype diversity in the results of the analysis was due to different distances and geographic of the sampling site.

Moreover, the genetic distance between thirteen Operational Taxonomic Unit’s (OTU’s) or accesses reflected the relationship between them. Here, we analyzed the genetic distance between Sumatran Mulberry, Himalayan Mulberry and both of two out-group, M. alba and M. nigra (Table 4). The results indicated that the highest average distance was detected between Sumatran Mulberry and M. nigra (0.460 ± 0.1), followed by between Sumatran Mulberry and Himalayan Mulberry (0.449 ±0.02), Sumatran Mulberry and M. alba (0.449±0.1). While, the average of the genetic distance of Himalayan Mulberry and M. nigra (0.029±0), Himalayan Mulberry and M. alba (0.002±0) and M. alba and M. nigra (0.027) were slightly similar.

Moreover, Morus has a different morphological character in genetic analysis. These facts also supported the differentiation of Sumatran Mulberry.

Further, the differentiation of Sumatran Mulberry was shown in the result of the topology of the tree constructed from the sequence of ITS data (Fig 2). Analysis of the phylogenetic relationship between thirteen accesses that consist of seven accessions of M. macroura from West

| Accession Code | Location | ITS | Haplotype |
|---------------|----------|-----|-----------|
| MM1           | West Sumatra | 45  | A         |
| MM2           | West Sumatra | 559 | G         |
| MM3           | West Sumatra |     |           |
| MM4           | West Sumatra |     |           |
| MM5           | West Sumatra |     |           |
| MM6           | West Sumatra |     |           |
| MM7           | West Sumatra |     |           |
| HM1           | China      |     |           |
| HM2           | India      |     |           |
| HM3           | USA        |     |           |
| HM4           | Asia       |     |           |

Note: MM= M. macroura (Sumatran Mulberry), HM1, HM2, HM3 and HM4 = M. macroura (Himalayan Mulberry from China, India, USA and Asia, respectively).

Table 4: Population genetic distance M. macroura and outgroup.

| OTU's | MM5 | MM6 | MM7 | MM3 | MM1 | MM2 | MM4 | HM3 | HM2 | HM1 | HM4 | MA | MN |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|----|
| MM5   | 1.708 | 1.966 | 2.046 | 1.956 | 2.030 | 1.977 | 1.856 | 1.856 | 1.887 | 1.887 | 1.887 | 1.809 |
| MM6   | 1.942 | 1.010 | 1.016 | 0.182 | 0.175 | 0.178 | 0.169 | 0.173 | 0.167 | 0.167 | 0.167 | 0.184 |
| MM7   | 0.942 | 0.102 | 0.101 | 0.011 | 0.002 | 0.009 | 0.025 | 0.029 | 0.023 | 0.023 | 0.023 | 0.047 |
| MM3   | 0.178 | 0.182 | 0.178 | 0.111 | 0.18 | 0.178 | 0.025 | 0.029 | 0.025 | 0.025 | 0.025 | 0.049 |
| MM1   | 0.178 | 0.102 | 0.101 | 0.011 | 0.002 | 0.009 | 0.025 | 0.029 | 0.023 | 0.023 | 0.023 | 0.049 |
| MM2   | 0.178 | 0.102 | 0.101 | 0.011 | 0.002 | 0.009 | 0.025 | 0.029 | 0.023 | 0.023 | 0.023 | 0.049 |
| MM4   | 0.178 | 0.102 | 0.101 | 0.011 | 0.002 | 0.009 | 0.025 | 0.029 | 0.023 | 0.023 | 0.023 | 0.049 |
| HM3   | 0.178 | 0.102 | 0.101 | 0.011 | 0.002 | 0.009 | 0.025 | 0.029 | 0.023 | 0.023 | 0.023 | 0.049 |
| HM2   | 0.178 | 0.102 | 0.101 | 0.011 | 0.002 | 0.009 | 0.025 | 0.029 | 0.023 | 0.023 | 0.023 | 0.049 |
| HM1   | 0.178 | 0.102 | 0.101 | 0.011 | 0.002 | 0.009 | 0.025 | 0.029 | 0.023 | 0.023 | 0.023 | 0.049 |
| HM4   | 0.178 | 0.102 | 0.101 | 0.011 | 0.002 | 0.009 | 0.025 | 0.029 | 0.023 | 0.023 | 0.023 | 0.049 |
| MA    | 0.178 | 0.102 | 0.101 | 0.011 | 0.002 | 0.009 | 0.025 | 0.029 | 0.023 | 0.023 | 0.023 | 0.049 |
| MN    | 0.178 | 0.102 | 0.101 | 0.011 | 0.002 | 0.009 | 0.025 | 0.029 | 0.023 | 0.023 | 0.023 | 0.049 |

Note: MM= M. macroura (Sumatran Mulberry); HM1, HM2, HM3 and HM4= M. macroura (Himalayan Mulberry from China, India, USA and Asia, respectively); MA= M. Alba and MN= M. nigra.
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**CONCLUSION**

Based on analyzed eleven accessions of *M. macroura* from West Sumatra using *Internal Transcribed Spacer* (ITS) markers. We concluded that the population *M. macroura* from Sumatra was grouped into the same cluster and separated into the Himalayan Mulberry group. The differentiation between Sumatran Mulberry and other mulberry was reflected from morphological and haplotype differentiation. This clarification is useful for the conservation and sustainable utilization of *M. macroura* specific taxa from Sumatra (Sumatran Mulberry).

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