Molecular characterization of andalas tree dioecious plant [Morus macroura Miq.] using SRAP marker

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Abstract. Sumatran Mulberry [Morus macroura var. Macroura] is the mascot identity flora of West Sumatra. Clarification of individual sex and genetic variation is necessary for its conservation. The SRAP [Sequence Related Amplified Polymorphism] marker system is one of the powerful molecular tools for clarification of individual sex and estimating its genetic diversity of plant species. The purpose of this study was to examine the male and female individuals in Andalas [Morus macroura] trees and to analyze their genetic variation. The fifteen individuals [accessions] of Morus macroura from three locations in West Sumatra [Limau Manis, Padang, Lubuak Gadang, South Solok and Andaleh, Tanah Datar] were collected to provide the DNA samples. A combination of seven primers was used to amplify the fifteen DNA samples. Specific bands for the sex were examined in selected individuals, and Genetic variation with four parameters: Number of allele [na], Number of effective allele [ne], Gene Diversity [h]; Shannon's Information Index [I] were analyzed. The PCR Test indicated that Fifty fragments were obtained from the amplification of DNA samples using seven combinations of primers. The most number of fragments were found in the combination of primer B and E. The specific band at The 20 bp and 30 fragments were useful to distinguish the sex of the examined individual trees. The genetic variation of Morus macroura was moderate low [na=1.540; ne=1.273; h=0.168; and I=0.259]. Genetic diversity of Limau Manis Population [h=0.241] was higher than Lubuak Gadang [h=0.116] and Andaleh population [h=0.148].

Keywords: genetic diversity, molecular marker, sex determination, sumatran mulberry

1. Introduction
Sumatran Mulberry [Morus macroura var. Macroura] is the mascot [identity] flora of West Sumatera and the local name is Andalas tree. The decline in the population of Andalas trees was probably caused by exploitation without conservation efforts [1]. There is difficulty in identifying male and female individuals morphologically if there are no flower and fruit organs [2]. The reproductive biology of the Andalas tree is dioecious with male and female flowers found in different individuals. The phenology of flowering time is not uniform [1], as well as the female flowers of the Andalas tree which have apomixis properties [3]. This causes Andalas tree saplings to be very rarely found in nature.

Efforts to use Andalas trees sustainably by applying conservation principles, both ex-situ and in-situ have the potential to be further developed to produce quality seedlings [1]. Then, consider aspects of genetic diversity and select individuals or populations that have high genetic variation and need seeds that have information on male or female sex [1]. This study is to make it easier for researchers to distinguish male and female individuals on the Andalas tree, which is a dioecious plant. Information
about the sex of individual Andalas trees is very important to find out their genetic variations related to their adaptability to cope with environmental changes in nature.

The SRAP [Sequence Related Amplified Polymorphism] marker system has become a popular marker for genetic diversity [4] and to be related to plant evolution processes that occur naturally and in evolution directly. SRAP is commonly used to limit and test variations within and between individual samples [5] and identify the sex of a species [6]. Therefore, a study was conducted for the molecular characterization of plants dioecious of Andalas tree [Morus macroura Miq.] using Sequence Related Amplified Polymorphism or better known as SRAP.

2. Materials and methods

2.1. Sampling
Collecting five individual males and five females [6] samples of plants Morus macroura Miq. [samples of fresh young leaves] were carried out in three areas in West Sumatra, namely, 1) Limau Manis, Padang, 2) Lubuak Gadang, South Solok, and 3) Andaleh, Tanah Datar [shown in Figure 1.]. The collected samples were then put into a tea bag, then stored in a plastic bag filled with silica gel as a preservative in the field. Then the sample is taken for DNA extraction.

![Sampling locations](image)

**Figure 1.** Sampling locations for Morus macroura from three districts in West Sumatra. Limau Manis, Padang [-0.91083, 100.45916], Lubuak Gadang, South Solok [-1.63826, 101.26067] and Andaleh, Tanah Datar [-0.44600, 100.44444].

2.2. Genomic DNA extraction
10 grams of Morus macroura leaf were used as DNA extraction samples. Total genomic DNA was extracted using Kit Bioline Plant Genomic DNA Purification Extraction with a small modification. The quality of DNA was tested by electrophoresis using 2% agarose while DNA quantity was measured using a NanoDrop Spectrophotometer [ThermoScientific, USA].

2.3. The primer screening
A combination of SRAP primers was tested using the BSA [Bulked Segregant Analysis] strategy [7] using individual DNA samples. Bulked DNA was constructed by mixing each genomic DNA [50 ng / μl] from the selected samples in one tube. Mixed PCR and amplification processes were performed using the standard SRAP protocol [4].
2.4. The PCR procedure
SRAP marker was used to amplified genomic DNA of each sample using a selected primer that already screening with the BSA method. Primer lists and combination that used in this study following the methods which was described in the previous study [8] with the amplification program was: 5 min of initial denaturation at 94°C, 5 cycles of 1 min denaturation at 94°C, 1 min of annealing at 35°C and 1 min elongation at 72°C, followed by 35 cycles with an annealing temperature of 50°C, and a final elongation step of 8 min at 72°C.

2.5. Data analysis
Band measurements were carried out using the PyElph1.4 program. SRAP fragments were scored for presence or absence and converted into a spreadsheet as codes 0 and 1. Total genetic diversity [HT], diversity in population [HS], genetic differentiation [GST], and gene flow [NM] and genetic variation with four parameters: number of alleles [na], number of effective alleles [ne], diversity of genes [h]; Shannon [I] diversity index was calculated using the features provided by the Poptgene software package [version 3.2] [9]. The analysis was continued with clustering and ordination using the Past4.03 program.

3. Results and discussions
Screening of 16 combination primers was carried out using the BSA method [Table 1]. The primers combination E produces four fragments, followed by the primer combination K and H with three fragments, then B, C, J, and O with two fragments, while another nine primers combinations [A, D, F, L, M, N, P] does not generate any fragments. The primers combination O and J has a less clear band, so it was not continued for analysis. SO from the BSA screening method, seven primer combinations were selected, namely B, C, E, G, H, I, and K.

Of the 15 samples, 10 samples had been identified as 5 male individuals and 5 female individuals. 3 male samples and 3 female samples came from Limau Manis, Padang while the other 2 samples for each sex came from Andaleh, Tanah Datar. PCR amplification was performed on 15 samples from 3 populations using 7 combination primers. A total of 50 fragments was produced with a range of 3-11 fragments for each individual. The fragment size range was 20-280 bp for the entire individual. Primers B and E produced the most fragments [11 fragments] followed by fragments C [9 fragments], H [8 fragments] I and K [4 fragments], and G produced the least fragments [3 fragments]. The longest, shortest, and most common alleles are found in the primer's combination E.

Fragment analysis to identify sex in 10 samples obtained 1 specific band that differentiates between male and female individuals using primer combination E. In 5 female individuals, 4 of them have a specific band at a size of 30 bp. Whereas male individuals have a specific band at a size of 20 bp. The discovery of this specific band is called the unique band and can be a distinguishing indicator for sex identification.

| Forward [5'-3'] | Reverse [5'-3'] | ID | Fragment no in BSA | Fragment no in individual plant | Fragment size range [bp] |
|----------------|----------------|----|-------------------|---------------------------------|-------------------------|
| TGAGTCCAAAAC   | CCGATA         | A  | 0                 | 11                              | 30-130                  |
| GACTGCATCGA     | ATTATAT        | B  | 2                 | 9                               | 30-120                  |
| GACTGCATCGA     | ATTTGCGA       | C  | 2                 |                                 |                         |
| GACTGCATCGA     | ATTGAC         | D  | 1                 |                                 |                         |
| GACTGCATCGA     | ATTTG           |    |                    |                                 |                         |
Table 2. Genetic variation of *Morus macroura* from three populations

|                | Limau Manis | Lubuak Gadang | Andaleh | All populations |
|----------------|-------------|---------------|---------|-----------------|
| na             | 1.88        | 1.32          | 1.42    | 1.540           |
| ne             | 1.3769      | 1.1953        | 1.2475  | 1.273           |
| h              | 0.2408      | 0.1542        | 0.148   | 0.168           |
| I              | 0.3816      | 0.2079        | 0.2644  | 0.259           |

na = number of alleles; ne = Number of effective alleles; h = genetic diversity; I = Shannon’s diversity index; SD * = Standard of deviation.

Genetic variation in three *Morus macroura* populations in West Sumatra is low. In each population, the value of genetic diversity in the population originating from Limau Manis \( h = 0.241 \) then Andaleh \( h = 0.148 \), and the lowest from Lubuak Gadang \( h = 0.116 \). The number of samples in a population can influence genetic variation. The Limau Manis population has the largest number of samples, namely 7 individuals out of a total of 15 individuals.

Table 3. Total genetic diversity of *Morus macroura* populations

|                  | \( H_T \) | \( H_S \) | \( G_{ST} \) | \( N_{M*} \) |
|------------------|----------|----------|--------------|-------------|
|                  | 0.2143   | 0.1667   | 0.2222       | 1.7504      |

\( H_T \) = total genetic diversity; \( H_S \) = genetic diversity within populations; \( G_{ST} \) = coefficient of genetic differentiation; \( N_{M*} \) = gene flow among populations; \( [N_{M*} = 0.5 \cdot (1 - GST) / GST] \).
The $H_T$ and $H_S$ values of *Morus macroura* was presented in Table 3. The total value of $H_T$ obtained is 0.2143 and $H_S$ is 0.1667. These results indicate that the total genetic diversity value is higher than the genetic diversity within populations. In other words, genetic variation remains among the population sample tested. The speciation process *Morus macroura* population is relatively high because of the value of $N_M[1.7504]$, which indicates the level of gene flow between populations. The $G_{ST}$ value of the three *Morus macroura* populations was 0.2222. This suggests that the genetic differentiation of *Morus macroura* at the three target sites was low.

The 15 individuals of *Morus macroura* that were analyzed from three different populations had a close genetic distance [Figure 2.] Individuals 1 and 3 from Padang were close to individuals 7 and 8 from South Solok. Likewise, individuals 9 and 10 originating from South Solok are in the same sub-cluster as individuals 11 and 12 originating from Tanah Datar. Individuals 13 and 14 originating from Tanah Datar were in the same sub-cluster as individuals 15 from Padang. With close genetic distance, it is assumed that 15 individuals came from Tanah Datar cultivated in Padang.

In Figure 4. Shows the ordination of the genetic variation of 15 individuals of *Morus macroura*. It can be seen that individuals 1, 3, 4, and 5 originating from Padang are in the same coordinate as individuals 7 from South Solok. Individuals 9 and 10 are also at the same coordinates as individuals 11 and 12. As well as individuals 13 and 14 who come from the same population as individuals 11 and 12 clusters at coordinate B with individual 15. This strengthens the clustering results [Figure 3.] that of the three populations *Morus macroura* had a low level of genetic variation and consistency of alleles. Allele consistency describes the origin of an individual even though it has been in a new population.

![Figure 2. The cluster of 15 individual samples *Morus macroura* using the UPGMA method based Jaccard.](image)

1= *Morus macroura* _Padang_Female_ 01, 2= *Morus macroura* _Padang_Female_ 02, 3= *Morus macroura* _Padang_Female_ 03, 4= *Morus macroura* _Padang_Male_ 01, 5= *Morus macroura* _Padang_Male_ 02, 6= *Morus macroura* _Padang, 7= *Morus macroura* _South Selatan_01, 8= *Morus macroura* _South Selatan_02, 9= *Morus macroura* _South Selatan_03, 10= *Morus macroura* _South Selatan_04, 11= *Morus macroura* _Tanah Datar_Male_01, 12= *Morus macroura* _Tanah Datar_Male_02, 13= *Morus macroura* _Tanah Datar_Female_01, 14= *Morus macroura* _Tanah Datar_Female_02, 15= *Morus macroura* _Padang_Male_03.
Figure 3. Ordination of *Morus macroura* using Principal Component Analysis [PCA] method

1 = *Morus macroura* Padang_Female 01, 2 = *Morus macroura* Padang_Female 02, 3 = *Morus macroura* Padang_Female 03, 4 = *Morus macroura* Padang_Male 01, 5 = *Morus macroura* Padang_Male 02, 6 = *Morus macroura* Padang, 7 = *Morus macroura* South_Selatan_01, 8 = *Morus macroura* South_Selatan_02, 9 = *Morus macroura* South_Selatan_03, 10 = *Morus macroura* South_Selatan_04, 11 = *Morus macroura* Tanah_Datar_Male_01, 12 = *Morus macroura* Tanah_Datar_Male_02, 13 = *Morus macroura* Tanah_Datar_Female_01, 14 = *Morus macroura* Tanah_Datar_Female_02, 15 = *Morus macroura* Padang_Male_03

There is a mechanism for determining sex in seed plants, namely by looking at heteromorphic sex chromosomes, single major gene control of sex determination [10], and hormonal regulation [11]. In 10 individuals of *Morus macroura* in West Sumatra, which consisted of 5 males and 5 females, two unique bands of 50 fragments were found. One fragment identifies a male individual and the other identifies a female individual. Further tests in determining sex can be carried out by sequencing analysis and hormonal regulation. Small differences in the sex chromosomes of seed plants can be influenced by the plant's evolutionary pathway [12]. Identification of sexual differences using the SRAP fragment can be followed up with SCAR to verify the target fragment because SCAR is a stable marker. The use of reliable primary pairs of D0f and D0r for gender identification and is expected to save time and reduce costs in the nursery [6].

*Morus macroura* found in Padang, South Solok, and Tanah Datar has low genetic variation but has a very wide distribution. It is assumed that *Morus macroura* in West Sumatra comes from the same parent even though it is in a different location. SRAP markers can limit and test variations within and between individual samples and can detect plant evolutionary processes that occur naturally and in evolution directly [6]. Genetic characterization is one of the key successes to crop breeding programs. Knowledge of the genetic variation between the different accessions supplying this diversity can greatly assist the development of efficient germplasm-management and utilization strategies [13].

4. Conclusion

PCR-based SRAP marker technique was successfully used to assess genetic differentiation among *Morus macroura*. There are distinguishing characters in the male and female bands from the PCR results with the combination primer E. Genetic variability in 3 populations of *Morus macroura* in West Sumatra is classified as low variability.
Acknowledgment
This study is funded by the Ministry of Research and Technology of the Republic of Indonesia through research funding for Master's Thesis [LPPM Contract No: T / 32 / UN.16.17 / PT.01.03 / PTM-Pangan / 2020].

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