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Genetic Relationships of Mulberry (*Morus l.)* Using Internal Transcribed Spacer (*ITS*) Markers

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Abstract

Mulberry, belonging to the family Moraceae, and genus Morus. Thirty-nine accessions, which belong to 9 species of Morus and 2 species as outgroup (*Ficus adhatodifolia, Broussonetia papyrifera*) were examined using ITS markers in this study. Genetic variation and phylogenetic relationships among 19 samples from West Sumatra were assessed using the internal transcribed spacer region of nuclear ribosomal DNA (nrDNA ITS). The estimated genetic diversity revealed low genetic variation. Phylogenetic relationships and intraspecific divergence were inferred by Neighbor Joining (NJ) analysis was generally resolved. *M. alba* accessions consistently had one lineage, which indicates the absence of distant geographic isolation and genetic divergence between China accessions and those from other regions. Phylogenetic analysis showed that the monophyletic topology forms a cluster. From the results of this study, it can be concluded that *M. alba* which is spread in Indonesia comes from the same ancestor. The Reconstruction of phylogenetic tree using the Neighbor-Joining (NJ) method 1000x showed that the ITS region was successfully to predict phylogenetic relationships genus Morus.

1. Introduction

Mulberry is a typical East Asian species distributed in the tropical, subtropical, and temperate region of the world. It is a small to medium-sized dioecious, occasionally monoecious, perennial, wind-pollinated, an outbreeding heterogeneous tree with a wide range of distribution from tropical and subtropical to temperate zones in Asia, Europe, North America, Africa, and South America (Kafkas et al, 2008). M. alba is widely believed originated on the low slopes of the Himalayas

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bordering China and India (Awashi et al, 2004). It is an economically important plant used for sericulture and extensively cultivated in the East, Central, and South Asia. Like most other edible fruits, this plant plays an important role as a source of household income (Navia et al, 2020; Suwardi et al, 2020).

ITS is a ribosomal gene that locates between 18S and 26S rRNA genes and includes two fragments separated by the 5.8S rRNA. Because ITS is not included in the mature ribosome and therefore subjected to lower selection pressure, its evolution is relatively faster. However, the length of ITS is relatively conservative among Angiosperms, and it applies to the phylogenetic analysis of closely related species (Kress, 2005). Quanliang and Weiguo (2001) reported the ITS sequence of M. mongolica with a full length of 558 bp and surveyed the prospect of applying the sequences in phylogenetic analysis from Morus (Quanliang and Weiguo, 2001). Weiguo et al (2004) analyzed 13 ITS sequences from Morus. After using Broussonetia as an outgroup and applying the clustering analysis method, they found that Broussonetia and Morus formed independent clusters, and this demonstrated that Morus is monophyletic. The M. mongolica clustered into a single branch and stayed far from others, while M. alba was the species that is evoluted (Weiguo et al, 2004). However, the significance of these studies of Morus ITS sequences is still limited due to the insufficiency in the amount and type of the testing materials. Therefore, in this study, the authors collected 38 different types of Morus from different sources of the world and downloaded the ITS sequences of Morus (Moraceae) from GenBank. The sequence analysis of ITS in this study provides molecular for analyzing their genetic relationships.

2. Materials and Methods

2.1 Study Area

A total of 19 accessions of Morus alba were collected at West Sumatra from the following four districts: 5 from Dharmasraya, 5 from Padang Panjang, 4 from Agam, 5 from Padang (Figure 1). The ITS spacer region of 19 M. alba accessions was sequenced in this study, and 19 additional species sequences such as Morus australis, Morus macroura, Morus mongolica, Morus celtidifolia, Morus nigra, Morus rubra, Morus cathayana, and Morus yunnanensis (from Thailand, China, India, America, Europe) were obtained from GenBank (National Center for Biotechnology Information: NCBI). The sample code, location, haplotype, and accessions number of all accessions are listed in Table 1. Ficus adhatodifolia and Broussonetia papyrifera were used as an outgroup for phylogenetic analysis (Chen et al, 2010; Zeng et al, 2015).

Table 1
List of Morus alba L. samples used in the present study with sample code/GenBank GenBank accessions, locality, and individual haplotypes

Specimen	**Sample's code/ GenBank accessions**	**Locations**	**Sources**
Morus alba	DMR_01	Dharmasraya	Present Study
Morus alba	DMR_03	Dharmasraya	Present Study
Morus alba	DMR_04	Dharmasraya	Present Study
Morus alba	DMR_05	Dharmasraya	Present Study
Morus alba	PP_06	Padang Panjang	Present Study
Morus alba	PP_07	Padang Panjang	Present Study
Morus alba	PP_08	Padang Panjang	Present Study

Morus alba	PP_09	Padang Panjang	Present Study
Morus alba	PP_10	Padang Panjang	Present Study
Morus alba	AG_11	Agam	Present Study
Morus alba	AG_12	Agam	Present Study
Morus alba	AG_13	Agam	Present Study
Morus alba	AG_14	Agam	Present Study
Morus alba	PD_15	Padang	Present Study
Morus alba	PD_16	Padang	Present Study
Morus alba	PD_17	Padang	Present Study
Morus alba	PD_18	Padang	Present Study
Morus alba	PD_19	Padang	Present Study
Morus alba	HQ144172	Amerika	Nikaido (2010)
Morus alba	MH187221	Thailand	Tanruean and Poolprasert (2018)
Morus alba	FJ980402	China	Chen and Han (2009)
Morus alba	FJ599759	Europe	Daniel and Knoess (2008)
Morus australis	MH710938	China	Xu et al. (2010)
Morus australis	MH711089	China	Xu et al. (2010)
Morus macroura	AM042000	India	Bhattacharya (2005)
Morus macroura	HM747170	America	Nepal and Ferguson (2012)
Morus mongolica	KF784880	China	Zeng et al. (2013)
Morus mongolica	KF784879	China	Zeng et al.(2013)
Morus mongolica	MH357905	China	Wu et al. (2018)
Morus mongolica	HM747173	America	Nepal and Ferguson (2012)
Morus celtidifolia	HQ144186	America	Nikaido (2010)
Morus nigra	KT002542	China	Chen and Liu (2016)
Morus rubra	HM747165	America	Nepal and Ferguson (2012)
Morus rubra	KF672603	America	Nepal and Wichern (2013)
Morus rubra	HQ144179	America	Nikaido (2010)
Morus cathayana	MH710939	China	Xu et al. (2010)
Morus yunnanensis	KJ605416	China	Chen (2014)

2.2 Procedures

The procedure of study contains a few stages, such as: DNA extraction, PCR amplification, and sequencing.

2.3 DNA extractions

Total genomic DNAs were extracted from fresh leaves using CTAB (cetyl trimethyl ammonium bromide) method (Doyle and Doyle, 1987) which had been modified. The modifications made were grinding the sample using liquid nitrogen, replacing the CIAA solution (Chloroform; Isoamyl; Alcohol) to chloroform and washing it twice, substituting isopropanol with cold alcohol.

In the DNA extraction procedure, 2 grams of dried mulberry leaf samples were crushed with liquid nitrogen to assist in the mechanical destruction of the cell wall. After the refinement is

inserted into 50 ml microtube, then 20 ml of 2x CTAB is added to free DNA and remove contaminants other than DNA (DNA purification). Subsequently incubated in a water bath at 60oC for 1 hour and every 10 minutes the tube was reversed. After incubation, 20 ml of chloroform was added, then homogenized using vortex for 15 seconds and centrifuged at 13,000 rpm for 5 minutes. After two phases are formed, namely the pellet and the supernatant, the supernatant at the top is taken and transferred into a new Eppendorf tube.

The DNA purification stage is followed by DNA precipitation, 96% cold alcohol (2/3 volume of the supernatant) is added and then homogenized by turning the tube back and forth until DNA strands are formed. The sample is then allowed to settle overnight in a refrigerator at 4oC. After being deposited overnight, then centrifuged at 12,000 rpm for 5 minutes, the supernatant phase was discarded, the pellet phase or DNA sediment formed was then washed with 70% EtOH and centrifuged again at 12,000 rpm for 5 minutes. The pellets were dried with the addition of TE buffer ± 100 µL and stored at 20oC as a DNA stock solution.

2.4 PCR amplification

Amplification using primers recommendation from (Syamsuardi et al, 2018) with the modification of temperature optimization PCR amplification using ITS primer (Muellner et al, 2003) eiguo et al (2004). The total DNA of the purified M. alba was used as a DNA template for the amplification process. Amplification aims to multiply the DNA of the ITS area. Amplification was performed using the PCR method, namely PCR Sensoquest. Previously, the resultant DNA concentration was matched to be used. The results of the concentration test with Biostep were used as a diluent of isolated DNA. In PCR amplification, DNA with a concentration of about 30 ng/µL was used.

The composition for one PCR reaction consists of 11 µl of Master Mix (MyTaq Red Mix), 9 µl ddH2O, 3 µl of isolated DNA, and 1 µl each of forward and reverse primers (PCR volume composition can be changed, adjusted to the conditions of the tape during documentation). DNA amplification was performed using a PCR thermal cycle tool. To amplify the ITS area, the PCR process was carried out as many as 30 cycles with conditions for each cycle, namely 3 minutes of the pre-saturation stage at 94oC, 1 minute of denaturation stage at 94oC, 1 minute of primary attachment stage at 55oC, 90 seconds of synthesis stage at 72oC, 5 minutes the next elongation stage is at 72oC, and the storage stage is at 4oC (Modification of Muellener et al, 2003).

Sequencing

The PCR product was then sent to the 1st Base Sequencing Service Laboratory in Malaysia for sequencing.

Data analysis

Sequencing data were aligned using the MEGA ver.7 application (Molecular Evolutionary Genetics Analysis) (Tamura et al. 2013). Phylogenetic trees were made based on Neighbor-joining algorithms (Saitou and Nei, 1989).

3. Results and Discussions

Data Matrices— Total DNA in genus Morus samples were successfully isolated. A total of 38 nrITS sequences from genus Morus were aligned with a length of 708 bp. BLAST of the ITS gene

sequence showed the identity of nine populations at 94% with the query cover 99%. Sequence characteristics and nucleotide polymorphisms were estimated including the total number of sites, 69.3 % conserved sites, 30.6 % variable sites, 12 % parsimony-informative sites, and 18.6% singleton site.

The results of BLAST show that M. alba in West Sumatra has homology or is identical to M. alba from China, Thailand, America, and Europe, M. australis from China, and M. Mongolica from America with a value of 99.86%. Nepal and Ferguson (2012) reported that these three species have the same similarities due to the relationship between the Morus group in Asia, while the similarities between M. alba and M. rubra have a low level of similarity, namely ± 94.20%, this is caused by M. rubra is a species native to America, and is not included in the sister group of M. alba (Nepal and Ferguson (2012).

Based on the 19 sample sequences studied and 19 sequences obtained from the analyzed genbank, 708 characters were obtained, where there were 491 conservative characters (conserved region) and 217 variable characters. The ITS region sequence has a conserved region, namely an area that does not experience mutations or changes in nucleotide bases. The more conserved regions in the sequence, the less genetic variation will appear. According to Ekasari (2012) the ITS marker has superior characteristics as well as having a high copy of the core genome with a length of approximately 680 to 710 bp.

The strengths and existence of the ITS area have been proven by several studies that have been conducted by several previous researchers, including; The pattern of genetic diversity in the invasive wild radish species (Pastinaca sativa) proves that wild or cultivated radishes have an amplification of 483 bp (Johesh et al, 2015). Chen et al (2010) analyzed the determination of the origin and evolution of Morus (Moraceae) with the ITS marker having a base length sequence of 576 bp.

3.1 Phylogenetic analysis

Based on the NJ method, the results of a phylogenetic tree that classified 38 sequences into one cluster and one outgroup cluster with the 1000x bootstrap indicating that genus Morus was monophyletic (Figure 1).

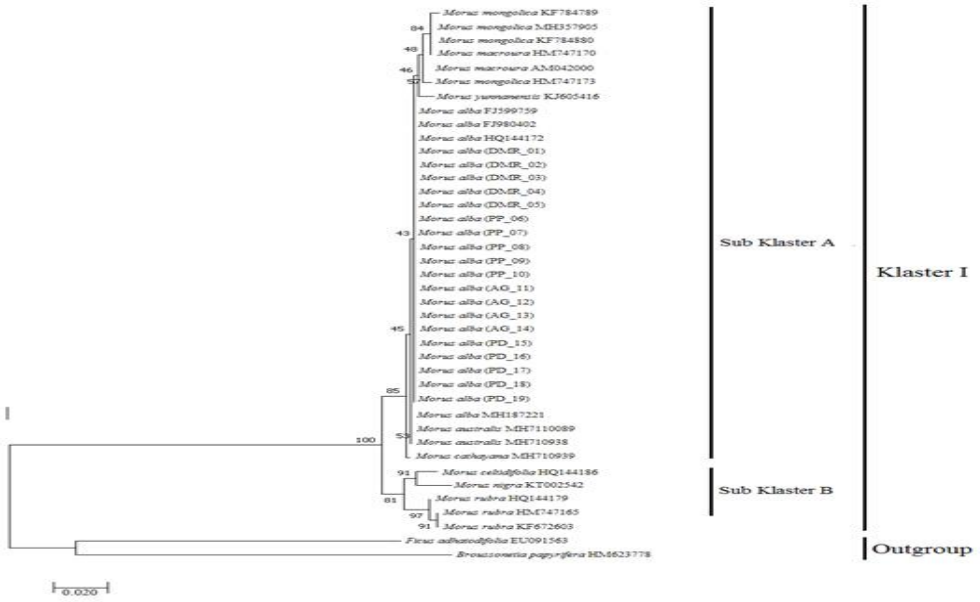


Figure 1. Phylogenetic tree of the genus Morus based on ITS marker suitable for neighbor joining (NJ) with 1000x bootstrap

3.2 Discussions

The strengths and existence of the ITS area have been proven by several studies that have been conducted by several previous researchers, including; The pattern of genetic diversity in the invasive wild radish species (Pastinaca sativa) proves that wild or cultivated radishes have an amplification of 483 bp (Johesh et al, 2015). Chen et al (2010) analyzed the determination of the origin and evolution of Morus (Moraceae) with the ITS marker having a base length sequence of 576 bp.

The phylogenetic trees of M. alba samples with other species from genbank data can be seen in Figure 1 which forms a cluster or monophyletic which is part of the genus Morus based on ITS markers. This is in accordance with the statement of Nepal and Ferguson (2012) that most of the Morus group forms a monophyletic which corresponds to the genus Morus subgenus Morus sensu Leroy (1949).

Based on the ITS markers, it can be seen that each population has clear cluster separation with its relatives between the Morus genus in West Sumatra, China, India, Thailand, America and Europe from the formed phylogenetic trees. In this cluster there are 2 sub-clusters, namely sub-cluster A and sub-cluster B, where the separation of sub-cluster A and sub-cluster B is supported by the statement of (Nepal and Ferguson (2012) who reported that in the Morus group, most of the genus Morus originated from Asia. form a clad, as well as the genus Morus from America.

Sub-cluster A with a bootstrap value of 85% consists of the population of M. alba found in West Sumatra, Thailand, China, Europe, America, and the population of M. australis from China. The presence of M. alba populations in America and Europe indicates that this species has traits that are able to adapt to new environments. According to Wunderlin (1997) M. alba is a native species from China, then became invasive in all areas where M. rubra (a species native to America) is present.

The M. australis population in China also has the same similarities with the M. alba population, as well as the M. cathayana which originated from China as well. According to Zeng et al (2015) sorted species names and found that all of them were synonymous with subspecies / varieties M. alba, namely M. alba, M. australis, M. cathayana, M. macroura, M. mongolica, M. nigra, M. notabilis, M. serrata, M. celtidifolia, M. insignis, M. microphylla, M. rubra, M. mesozygia, M. bombycis, M. wittiorum, and M. trilobata (Zhou and Gilbert, 2003; Nepal, 2008; Nepal and Ferguson, 2012).

The M. macroura population in India is grouped with the M. mongolica population in China with a bootstrap value of 84%, and at a bootstrap value of 57% it is also closely related to the M. yumanensis population. The M. cathayana population in China is also clustered with the previous population which is supported by a bootstrap rate of 53%. This grouping corresponds to the Morus cluster from Asia reported by Nepal and Ferguson (2012).

Sub-cluster B describes the presence of a population from the American clade, this is consistent with reports from Nepal and Ferguson (2012) that M. rubra from America has a bootstrap value of 97%. Meanwhile, the other group is M. rubra from America. In a molecular phylogenetic study of the genus Morus, Nepal and Ferguson (2012), it was stated that M. rubra formed a single land which was supported by the presence of a species native to North America, namely M. celtidifolia Kunth, and also with the ITS marker show that M. alba is separated from M. rubra because this species is a mulberry plant native to America or Canada which is endangered due to the presence of invasive M.alba which has a fast growth pattern so it can dominates an area it occupies, and the breeding pattern is also faster than that of M. rubra (Burgess et al, 2005; Nepal, 2008).

The distribution of clusters on the phylogenetic tree where the distribution separation based on the genus Morus originates from Asia and also from America, so it can be stated that most haplotypes cluster in the Asian region of China. This is because the Morus group is included in the Himalayan mulberry originating from the Himalayas bordering India and China (Awashi et al. 2004), until now the distribution of mullberry is suitable and supportive in tropical as well as sub-tropical areas from an altitude of 0–4000 masl. The genus Morus has the characteristics to survive or excel, namely producing lots of leaves, quality, good root development, growth by means of cuttings.

The phenomena of current research confirms that the mulberry samples in West Sumatra have lineages originating from China, it is proven that the distribution of M. alba/ mulberry species obtained is similar to those in China. The sequence divergence value shows a genetic relationship between M. alba populations, where the lower the sequence divergence value, the closer the relationship between these populations.

4. Conclusion

The result from current study indicated that ITS analysis of mulberry accessions for reconstructing the phylogenetic tree of the genus Morus, we suggest that the genus Morus can be classified into nine species and that ITS might to cluster of species in this genus.

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