

Chloroplast DNA variation of *Shorea acuminata* Dyer in Eastern Sumatra assessed by microsatellite markers

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ABSTRACT

Zulfahmi, Siregar IZ, Siregar UJ (2010) Chloroplast DNA variation of *Shorea acuminata* Dyer in Sumatra assessed by microsatellite markers. *Biodiversitas* 11: 107-111. *Shorea acuminata* Dyer is member of the Dipterocarpaceae family. It is ecologically and commercially important in the Indonesian region. In the present study, chloroplast microsatellites (cpSSRs) were used to study the distribution of chloroplast DNA haplotypes and to assess the variation within and among populations of *S. acuminata* from Riau and Jambi provinces eastern part of Sumatra. Based on chloroplast microsatellite analysis, six haplotypes were observed for *S. acuminata*, namely haplotype P, Q, R, S, T, and U, respectively. The high haplotype variation was detected in Bukit Barisan National Park (TNBT) population (five haplotypes); it may be due to TNBT population status as national parks (conservation area) under government protection. The value of genetic differentiation measured for *S. acuminata* was $G_{st} = 0.150$. The G_{st} values in this study is lower than the mean G_{st} value estimated in angiosperms plant for maternally inherited. Information on the status of genetic variation of the species in this study could be used as scientific consideration in formulating appropriate strategies for conservation and sustainable utilization of genetic resources.

Key words: Dipterocarpaceae, *Shorea acuminata*, chloroplast microsatellites, genetic variation.

Indonesian tropical rain forest is rich in genetic resources. Rapid destruction of forests due to illegal logging, forest fires, and over exploitation has been threatening the existence of genetic resources in forest ecosystem. We must consider how to conserve the genetic diversity of the tropical rain forest. For this purpose, we started a study of genetic variation in forest trees (*Shorea acuminata*) which is one of high quality timbers in Indonesia.

Shorea acuminata Dyer is a member of the genus *Shorea* (section *Mutica*) in the Dipterocarpaceae family. This tree is distributed in a mixed dipterocarp forest in Malaysia, Sumatra and Lingga (Ashton 1982). In Indonesia, it is known locally as meranti bunga, and belongs to the light red meranti timber group (Newman et al. 1996). Meranti bunga is an important timber because of its economic value. The hard wood of meranti bunga is suitable for heavy construction, to make poles, flooring, furniture, window panels, and doors. Meranti bunga is one of the fast growing species among other dipterocarps for planting as quality timber in Indonesia (Soekotjo and Wardhana 2005).

Genetic variation is the fundamental requirement for the maintenance and long term stability of forest ecosystem since the amount and pattern of genetic variation would determine the ability of forest tree species to adapt on

variable environment condition. Information on the genetics of species would be useful in designing appropriate tree breeding program and conservation of genetic resources.

One of the genetic resources conservation objectives is to prevent the species extinction. Genetic information as life history (evolution) and population structure knowledge of plants are important for development of sound genetic conservation strategies. Therefore, there are needs to know status of variability of chloroplast DNA, mitochondria and nuclear genome of plants. Studies on genetic variation of *S. acuminata* have been conducted based on various markers targeted at nuclear DNA such as RAPDs, (Harada et al. 1994), microsatellites (Takeuchi et al. 2004; Tani et al. 2009), AFLPs (Cao et al. 2006, 2009), DNA sequencing (Ishiyama et al. 2003; Kamiya et al. 2005; Inomata et al. 2008).

In this study, we analyzed the chloroplast genome using the microsatellite markers. We choose the chloroplast genome due to its uniparental inheritance, the absence of recombination, and slow mutation rates (Provan et al. 2001). The objectives of this research were to determine distribution of chloroplast DNA haplotypes and to estimate the genetic diversity of chloroplast DNA within and among populations of *S. acuminata*.

MATERIALS AND METHODS

Sample collection

Leaf tissues from seedlings or poles or trees of the *Shorea acuminata* were harvested from natural populations in Sumatra. The number of individuals collected per population is showed in Table 1. Field distance among individuals was kept at around 150 m. The samples collected in the fields were stored in plastic packages containing silica gel with ratio leaf to silica (1:5 g), subsequently stored in a freezer at temperature -60°C until DNA extraction was performed.

DNA extraction and PCR-cpSSR

Total DNA was extracted from dry leaf tissue (2 cm²) using the Dneasy 96 Plant DNA isolation Kit (Qiagen, Hilden). The extraction was done following the manufacturer's instructions. The quality of DNA isolation results was visualized in 0.8% (w/v) agarose gels. Electrophoresis was performed using 1X Tris-acetate (TAE) buffer for about 30-80 minutes at 100-150 V. The quality of DNA was examined in comparison to a Molecular Weight Standard (Lambda DNA Marker, Roche Mannheim). Ten universal primers, namely consensus chloroplast microsatellite primers (*ccmp*) *ccmp1* to *ccmp10* (Weising and Gardener 1999) were tested in order to analyze the chloroplast microsatellite genome. The amplification of cpSSRs was performed using fluorescence dyed forward primers (Metabion) for genotyping purpose, namely 6-FAM/Blue (*ccmp2*, *ccmp4*, *ccmp6* and *ccmp9*), HEX/Green (*ccmp1*, *ccmp3*, *ccmp7* and *ccmp10*) and NED/yellow (*ccmp5* and *ccmp8*).

The PCR procedure was according to Indrioko (2005) using the following reaction conditions: initial denaturation for 15 minutes at 95°C, followed 35 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 50°C, extension for 1 minute at 72°C and final extension for 10 minutes at 72°C. Reaction mix (15 µl) of PCR reagents was prepared as follows: 2.0 µl template DNA (5-10 ng), 1.8 µl forward primers (5 pmol/µl) and reverse primers (5 pmol/µl), 1.9 µl Distillated water, and 7.5 µl HotStarTaq Master Mix Kit (Qiagen, Hilden). PCR products were separated on 2.0% (w/v) agarose gels and quality of DNA was examined in comparison to a Molecular Weight Standard XIV (100 bps ladder) DNA Marker (Roche Mannheim). The gel was stained in ethidium bromide solution for about 20 minutes at room temperature; the banding patterns of gel were observed under UV light apparatus in the dark room and documented using a digital camera.

Genotyping of PCR products

The reagents for genotyping (96 probes) were composed of: 1152 µl HiDi Formamide (Applied Biosystem) and 1.5 µl GS 500 ROX™ (Applied Biosystems). The reagents mix was distributed equally into 96 samples tubes (12 µl each sample), and then 2 µl of the amplification product of each sample was added to the tubes. The samples were denaturated for 2 minutes at 90°C, subsequently stored on ice for about 5 minutes before capillary electrophoresis.

The separation was done by capillary electrophoresis on an automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with polymer 3100 POP-4™ (Applied Biosystems). The length of electrophoresis products expressed in base pairs was measured with the help of the internal size standard GS500 ROX™ (Applied Biosystems). Individual alleles were analyzed using GeneScan Version 3.7 (Applied Biosystems) and Genotyper Version 3.7 NT (Applied Biosystems).

Data analysis

Haplotypes were inferred as combination from individual alleles sizes found at each locus. In analyzing fragment patterns of cpSSR, the fragments are coded with 1 and 0 indicating the presence or absence of fragments. Haplotype frequencies and population structure was calculated using the POPGEN Software Version 32 (Yeh et al. 1999). UPGMA dendrogram analysis based on Nei's genetic distance (1972) was calculated with NTSYSpc Software Version 2.0 (Rohlf 1998).

RESULTS AND DISCUSSION

cpSSR haplotypes

The ten chloroplast microsatellite (cpSSR) primers *ccmp1-ccmp10* (Weising and Gardener 1999) were tested initially in two samples per population. Out of ten chloroplast microsatellites primers used, five primers (*ccmp4*, *ccmp5*, *ccmp7*, *ccmp8* and *ccmp9*) showed no amplification products, whereas five primers (*ccmp1*, *ccmp2*, *ccmp3*, *ccmp6* and *ccmp10*) were successful to be amplified in all DNA samples. Out of these five primers, three primers (*ccmp1*, *ccmp2* and *ccmp10*) showed monomorphic patterns with fragment sizes of 113 bp, 150 bp and 101 bp, respectively. At *ccmp3* and *ccmp6* primers showed polymorphic patterns. Amplification products of *ccmp3* revealed presences of four length variants (100 bp, 101 bp, 102 bp and 104 bp), while the products of *ccmp6* showed only two length variants (97 bp and 98 bp). In total, there were observed six haplotypes (Table 2).

Table 1. The number of individuals (N), approximate latitude and longitude, and haplotype frequency of *S. acuminata* per population.

Province	District	Population name	N	Longitude	Latitude
Jambi	Tebo	Pasir Mayang	6	101°48'57"-101°49'17"E	00°52'32"-01°54'17"S
Riau	Pelalawan	Nanjak Makmur	7	101°30'37"- 103°21'36"E	00°46'24"-00°24'34"S
Riau	Indragiri Hulu	Bukit Tigapuluh National Park (TNBT)	7	102°13'-102°45'E	00°40'-01°30'S
Total			20		

Table 2. Definition of haplotypes and fragment sizes of cpSSRs

Haplotypes	Fragment amplification sizes of cpSSR (bp)	
	<i>ccmp3</i>	<i>ccmp6</i>
P	101	97
Q	101	98
R	100	98
S	102	97
T	104	97
U	100	97

cpSSR haplotypes variation

Based on *Genescan* results, there were six haplotypes observed for *S. acuminata*, namely P, Q, R, S, T and U, respectively. Details of haplotype frequencies in *S. acuminata* are shown in Table 3. Haplotype P, Q and T are only observed in TNBT population, haplotype R in TNBT and Nanjak Makmur populations, Haplotype S in Pasir Mayang, TNBT and Nanjak Makmur populations, whereas haplotype U is only found in Pasir Mayang population. The high haplotype variation was detected in TNBT population (five haplotypes); it may be due to TNBT population status as national parks (conservation area) under government protection.

In general, the number of haplotype variation is low in this study. The low chloroplast DNA haplotypes is closely related to slow mutation rates (Provan et al. 2001). A low number of haplotypes was also observed in *Populus tremula* (six haplotypes, Salvini et al. 2001), wild Grapevine (five haplotypes, Grassi et al. 2006), *Fraxinus excelsior* (six haplotypes, Hebel et al. 2006), *Fraxinus ornus* (four haplotypes, Heuertz et al. 2006), and *Hagenia abyssinica* (six haplotypes, Ayele et al. 2009). On the other hand, high chloroplast DNA haplotype variation was observed in Oak (Deguilloux et al. 2004b), *Fraxinus excelsior* L. (12 haplotypes, Heuertz et al. 2004), *Fagus sylvatica* (14 haplotypes, Vettori et al. 2004), *Ulex* (47 haplotypes, Cubas et al. 2005), *Fraxinus angustifolia* (13 haplotypes, Heuertz et al. 2006), *S. boivinii* (14 haplotypes, Pardo et al. 2008), and *S. genistoides* (30 haplotypes, Pardo et al. 2008).

Genetic variation within and among populations

Values of genetic differentiation measured for *S. acuminata* was $G_{st} = 0,150$ ($H_s = 0,203$; $H_t = 0,239$). This value indicated that moderate gene flow among populations of *S. acuminata*, which is explained by restricted seed dispersal due to relatively heavy seeds and dispersed by wind or gravity. Although seed of *S. acuminata* dispersal

distances can be up to 500 m or even further, more than half of the mature seeds land within 50 m of the parent tree under forest conditions (Takeuchi et al. 2004).

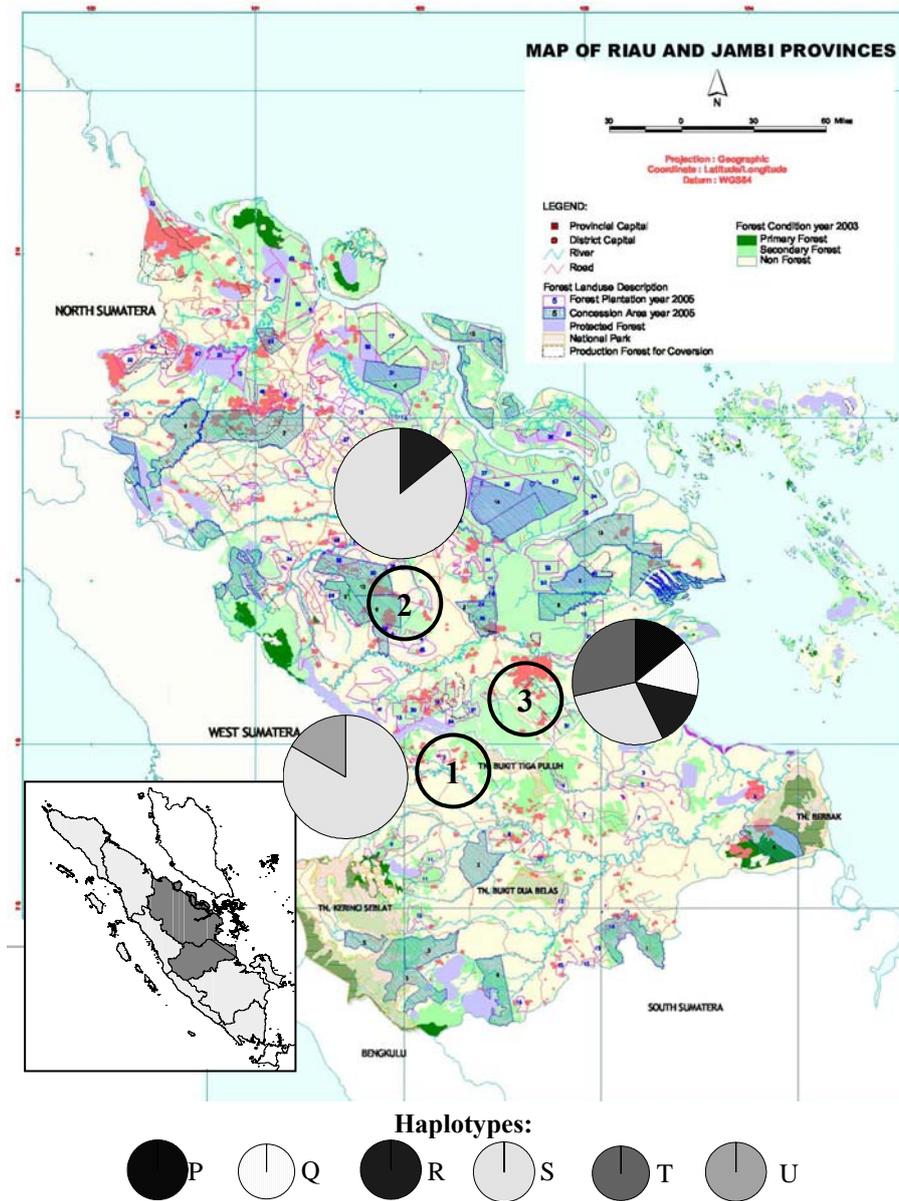
The G_{st} values in this study is lower than the mean G_{st} value estimated in angiosperm plants for maternally inherited DNA ($G_{st} = 0.637$, Petit et al. 2005) and another study for some species such as *Arabis holboellii* ($G_{st} = 0.20$, Dobes et al. 2004); *Fagus sylvatica* ($G_{st} = 0.855$, Vettori et al. 2004); wild Grapevine ($G_{st} = 0.631$, Grassi et al. 2006); *Fraxinus ornus* ($G_{st} = 0.983$, Heuertz et al. 2006); *Fraxinus angustifolia* ($G_{st} = 0.964$, Heuertz et al. 2006); and *Hagenia abyssinica* ($G_{st} = 0.899$, Ayele et al. 2009). The low of G_{st} estimated based on chloroplast microsatellite markers were also obtained in *Populus tremula* L. ($G_{st} = 0.07$, Salvini et al. 2001); *Cunninghamia lanceolata* ($G_{st} = 0.017$, Hwang et al. 2003); *Cunninghamia konishii* ($G_{st} = 0.073$, Hwang et al. 2003); and *Magnolia stellata* ($G_{st} = 0.137$, Setsuko et al. 2007).

The geographic distribution of haplotypes for *S. acuminata* does not correspond with geographical separation among populations (Figure 1). In *S. acuminata*, six haplotypes (P, Q, R, S, T, and U) were found. Five out of the six haplotypes were found in TNBT population, two haplotypes in Pasir Mayang and Nanjak Makmur populations. This result also showed that haplotype P, Q, and T was specifically in TNBT population and haplotype U was specifically in Pasir Mayang population. The specific haplotype may be useful for the detection of species geographical origin. This study was first method to explore the possibility of using molecular marker as tool to prove the geographical origin of the individual trees. The DNA method to detect the geographic origin of species has been developed in Oak species (Deguilloux et al. 2002; 2003; 2004a) and Dipterocarps species (Finkeldey et al. 2007; Indrioko 2007; Lee and Tnah 2007; Nuroniah 2009; Finkeldey et al. 2010).

Genetic distance indicates the genetic relationship among populations. Genetic distances among populations, namely TNBT and Pasir Mayang, Nanjak Makmur and Pasir Mayang, and Nanjak Makmur and TNBT was 0.0942, 0.0129, and 0.0791, respectively. UPGMA dendograms (Figure 2) based on Nei's genetic distance (1972) showed that *S. acuminata* was divided into two clusters with Pasir Mayang and Nanjak Makmur populations forming first a cluster, and TNBT population forming a second cluster. TNBT population was separated from another population due to high haplotype diversity within population.

Table 3. The number of individuals (N), approximate latitude and longitude, and haplotype frequency of *S. acuminata* per population.

Province	District	Population name	N	Haplotypes					
				P	Q	R	S	T	U
Jambi	Tebo	Pasir Mayang	6	0.000	0.000	0.000	0.833	0.000	0.167
Riau	Pelalawan	Nanjak Makmur	7	0.000	0.000	0.143	0.857	0.000	0.000
Riau	Indragiri Hulu	Bukit Tigapuluh National Park (TNBT)	7	0.143	0.143	0.143	0.286	0.286	0.000
		Total	20						



Implications on conservation of genetic resources of *Shorea acuminata* in Sumatra

One of the goals of the conservation of genetic resources is to prevent species extinction. Genetic conservation of *S. acuminata* can be made either *in situ* or *ex situ*. *In situ* conservation requires a large population size, whereas *ex situ* conservation requires collection from represented individuals so that genetic diversity within population is maintained.

Based on differentiation values of *S. acuminata* ($G_{st} = 0.150$) showed that moderate level of genetic diversity partitioned between populations. The most important conservation objective is preservation of maximum number alleles of target species. Therefore, if *in situ* conservation and sampling for *ex situ* conservation of *S. acuminata* will be established, we recommend that *S. acuminata* conservation could be implemented in TNBT population for Sumatra island due to high haplotype diversity and habitat status of this population which is found in a national park.

CONCLUSION

The six haplotypes were observed for *S. acuminata* populations in Sumatra, namely P, Q, R, S, T and U, respectively in which TNBT population possessed high haplotype diversity. The genetic differentiation in the three studied populations was moderate ($G_{st} = 0.150$). It was suggested to use the available information as scientific consideration in formulating genetic conservation strategies of the species.

Figure 1. Geographical distribution of haplotypes chloroplast microsatellite in *S. acuminata*. Note: 1. Pasir Mayang, 2. Nanjak Makmur, 3. Bukit Tigapuluh National Park (TNBT).

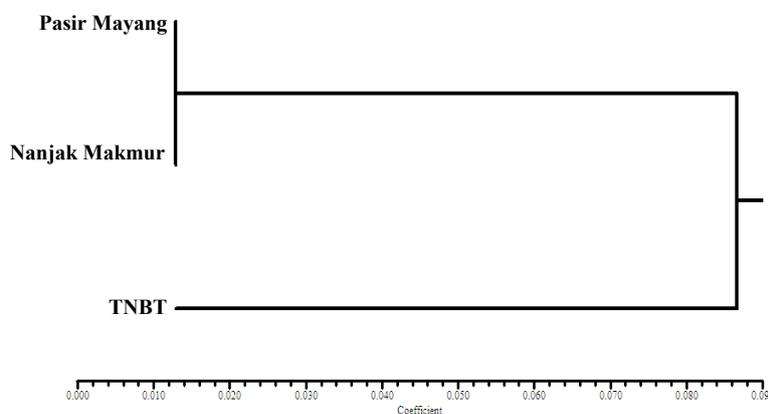


Figure 2. Dendrogram of UPGMA cluster analysis on *S. acuminata* based on Nei's genetic distance (1972).

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