Genetic diversity of Shorea producing tengkawang populations in Kalimantan based on SSR markers

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Abstract. Shorea producing tengkawang grows naturally in Indonesia, however several species are now listed in the IUCN as endangered species. Their presences in natural forests are becoming scarce so that it requires conservation efforts such as the construction of ex-situ conservation plots. This research aims to obtain data and information about the genetic diversity structure of several species of Shorea producing tengkawang. The material used was leaf samples from 11 (eleven) populations consisting of 4 (four) species of Shorea producing tengkawang. The method used 4 pairs of microsatellite DNA markers (SSR), and data analysis used POPGENE and GenAleX. The results show various genetic distances between populations, ranging from 0.028 to 1.510 with the value of genetic diversity in the population larger (72%) than among the populations (28%). Genetic diversity in the population ranges from 0.5269 - 0.8244. Cluster analysis shows that the existing Shorea producing Tengkawang population does not form a specific grouping. This genetic diversity information can be used as the basic data for the development of Shorea producing tengkawang ex-situ conservation plots.

1. Introduction
Indonesian forest, particularly in Sumatra and Kalimantan, are dominated by Dipterocarps (Dipterocarpaceae). Many of Dipterocarps species are known for its important role both from an ecological and an economic perspective. The genus Shorea is probably the biggest genus of Dipterocarps and distributed widely in Southeast Asia. Shorea species has good quality of wood which is used for veneer, plywood, house construction, boats and others; and also produce non-timber forest products known as tengkawang. The Shoreaproducing tengkawang species such as S. pinanga, S. stenoptera, S. macrophylla and S. seminis are known for their high-value wood and non-wood products. These Shorea seed (illipe nut) contains tengkawang oil usually used as a raw material and supporting material for the manufactures of food, pharmaceuticals and cosmetics [1];[2];[3];[4];[5];[6];[7].

The high economic value for its wood and non-wood products makes these species much wanted by consumers which lead to over-exploitation in natural plantation, together with wood/seed harvesting and forest fragmentation resulting in the declining of its genetic diversity, changing of inter-population structure, increasing inbreeding and genetic drift. In addition, these species are threatened by looting, illegal logging and forest fires. The existence of those species in their natural habitat was decreasing; therefore, these species are protected under Indonesian Government Regulation No. 7/1999 and Ministerial Decree No. 692/Kpts-II/1998. IUCN also stated that these species are now rare, critically endangered and vulnerable. Hence the strategic action to conserve the genetic diversity of tengkawang species through either in situ or ex situ conservation and plant breeding is needed. Shorea material genetic collection is previously done by ITTO Project PD 586/10 Rev.1 (F)
on conservation of tengkawang genetic diversity and property sustenance of its local community in Kalimantan. The material genetics have been used for establishing ex-situ conservation plot in Mulawarman University Botanical Garden (Kebun Raya UnMul Samarinda/KRUS), Samarinda, East Kalimantan.

In general, plant breeding aims to improve the genetic quality through the selection process and the hybridization. This is the reason why the information of genetic variation, kinship and genetic distance between species will fundamentally determine the success of the plant breeding efforts. Studies on genetic diversity of Shorea as a genus to support conservation and plant breeding efforts have been conducted using various methods but genetic aspect of the Shorea producing tengkawang such as genetic diversity, kinship among species (phylogeny) and the genetic distance between species is not widely available. In addition, these data will also important for the implementation of development conservation of Shorea producing tengkawang both in situ and ex situ. Method for estimating genetic diversity is to measure genetic variation using molecular genetic markers. However, deoxyribonucleic acid (DNA) variation that resides within the non-coding genomics regions or does not cause an amendment within the organic compound is unlikely to own any vital contribution to adaptation. Many genetic markers belong to alleged anonymous DNA marker kind like microsatellites or simple sequence repeats (SSRs).

Microsatellites (Simple Sequence Repeats- SSRs) consist of segments of DNA containing numerous tandem repeats of a short “motif” sequence, usually of one to six bases (e.g. AGAGAG...) largely distributed over the entire genome. They are amplified, by PCR reaction, using primers designed to match unique sequences flanking the tandem repeat. Advantages of microsatellites are their abundance, high degree of polymorphism, multi-allelic and co-dominant nature, basis on PCR reactions (requiring only small amounts of DNA and no radioactive labels), and that they can be shared among laboratories by exchanging primer DNA sequences [8];[9];[10];[11];[12].

The aim of this research was to obtain data and information on the genetic diversity of several species of Shorea producing tengkawang from Kalimantan using SSR markers; those have been planted for ex-situ conservation plot of tengkawang in KRUS. The results of this research can also be used to support the conservation strategies of these species.

2. Experimental Methods

2.1. Description of sampling

Leaf samples were taken from the nursery randomly and consist of 4 species of Shorea producing tengkawang; i.e. S. pinanga, S. macrophylla, S. stenoptera and S. seminis from 11 populations with 20 samples from each population.

2.2. DNA Extraction and PCR

Total DNA samples were extracted using a modified CTAB method [13]. The total PCR volume was 10 µL, consist of 10 × EDTA buffer, 25 mM MgCl2, 2.5 mM dNTPs, 10 µM microsatellite primer, 5 U AmpliTaq Gold polymerase (Applied Biosystems) and 10 ng/µL DNA solution.

PCR was performed using GeneAmp 9700 thermocycler (Applied Biosystems). Preheating temperature of 94°C for 10 minutes, followed by 10 cycles with 3-stage temperature i.e. DNA denaturation at 94°C for 30 seconds, DNA annealing at 60°C - 50°C for 30 seconds and DNA elongation at 72°C for 1 minute. The process followed by another 20 cycles consisting of denaturation and elongation as above and annealing at 50°C for 30 seconds. PCR cycle ended at 72°C for 1 minute to complete the elongation process. Electrophoresis was performed using gene analyzer machine ABI 3100 Avant (Applied Biosystems). This study used three microsatellite markers developed from Shoreacurtisii[14] as on the Table 1.
### Table 1. Sequence and repeated motifs of microsatellite on S. curtisii[14]

| Locus | Sequences (5' - 3') | Repeated Motifs | T. ann (°C) | N_a | H_e  |
|-------|---------------------|----------------|------------|-----|------|
| Shc-02 | CACGC TTCCC CAATC TG TCAAGA GCAGA ATCCA G CTTAT GAGAT CAATT TGACA G | (CT)₂CA(CA)₅ CTCA | 54 | 2 | 0.180 |
| Shc-07 | ATGTC CATGT TTGAG TG CATGG ACATA AGTGG ATG | (CT)₃CA(CA)₅CACCC (CTCA)₃ CT(CA)₁₀ | 54 | 11 | 0.810 |
| Shc-09 | TTTCT GTATC CGTGT GTTG GCGATT AAGCG GACCT CAG | (CT)₁₂ | 54 | 9 | 0.818 |

Note: T. ann: annealing temperature; N_a: number of detected alleles; H_e: expected heterozygosity

2.3. Data Analysis

Genetic diversity parameters inside population such as number of detected alleles (N_a), observed heterozygosity (H_o), and expected heterozygosity (H_e) were analyzed using GenAleX 6.4[15]. Principle coordinates analysis (PCoA) illustrates the close genetically linked to the geographical position. In addition, the effects of regions between populations and in the populations that contribute to differences in genetic diversity were determined using Analysis of Molecular Variance (AMOVA). POPGENE 1:32 programme [16] was used to calculate the value of genetic diversity and genetic distance based on gene-diversity and genetic distance by exploiting differences in allele frequency among individuals and populations. Cluster analysis was performed using the UPGMA (Unweighted Pair Group Method with Arithmetic Averaging) method to classify the population within a range based on the concept of genetic distance. The results are displayed in the form of cluster analysis dendrogram kinship between populations.

3. Result and Discussion

3.1. Results

Twenty (20) individuals samples for each population was taken from the Dipterocarp Ecosystem Research and Development Center nurseries. This research used three (3) microsatellite markers as shown in Table 1. In terms of polymorphisms marker, the results were quite expected for microsatellite loci, which have demonstrated high polymorphism (100 %) in all three (3) loci used in this research. Total number of alleles was 188 with the size of detected allele were between 86 – 208 for all 3 loci, while the number of detected alleles varied from 12 (S.stenopteraHaurbentes A) to 22 (S. stenopteraKetapang). These amounts of allele were suitable to be applied for studying population genetics and genetic diversity of these species.

Basic statistics were calculated to determine number of detected alleles, the mean observed heterozygosity (H_o), Nei’s expected heterozygosity (H_e), and the number of alleles for each populations. The genetic diversity parameters for each population were shown in Table 2. The mean of observed heterozygosity value was 0.699 and varied between 0.267 (S.pinangaKetapang) to 0.817 (S. stenopteraSintang); while the mean of expected heterozygosity was 0.672 ranged from 0.543 (S.pinangaKetapang) to 0.801 (S. stenopteraKetapang). The value of expected heterozygosity illustrates the genetic diversity of a population.
Table 2. Genetic diversity parameter in each population of Shorea producing tengkawang

| Population                | N  | Na    | Ho       | He       | Σ Allele |
|---------------------------|----|-------|----------|----------|----------|
| S. stenoptera Ketapang    | 20 | 8.3 ± 0.9 | 0.633 ± 0.23 | 0.801 ± 0.01 | 22       |
| S. pinanga Sanggau        | 20 | 6.7 ± 0.3 | 0.817 ± 0.04 | 0.763 ± 0.03 | 17       |
| S. stenoptera Sanggau     | 20 | 7.0 ± 1.2 | 0.783 ± 0.04 | 0.717 ± 0.01 | 17       |
| S. macrophylla Sanggau    | 20 | 6.0 ± 0.0 | 0.750 ± 0.10 | 0.713 ± 0.06 | 15       |
| S. stenoptera Sintang     | 20 | 6.0 ± 0.0 | 0.817 ± 0.06 | 0.726 ± 0.01 | 18       |
| S. macrophylla Haurbentes| 20 | 5.3 ± 1.3 | 0.633 ± 0.16 | 0.564 ± 0.09 | 15       |
| S. pinanga Ketapang       | 20 | 7.0 ± 2.6 | 0.267 ± 0.14 | 0.543 ± 0.22 | 19       |
| S. stenoptera Sungai Buaya| 20 | 7.7 ± 2.4 | 0.550 ± 0.20 | 0.580 ± 0.22 | 20       |
| S. stenoptera Haurbentes A| 20 | 5.7 ± 0.9 | 0.650 ± 0.05 | 0.662 ± 0.07 | 12       |
| S. stenoptera Haurbentes B| 20 | 7.3 ± 1.9 | 0.750 ± 0.15 | 0.703 ± 0.06 | 19       |
| S. seminis Bengkayang      | 20 | 6.7 ± 1.5 | 0.717 ± 0.04 | 0.622 ± 0.07 | 14       |

Note: N: number of samples; Na: number of detected alleles; Ho: Observed Heterozygozyity; He: Expected heterozygosuty

Table 3. Genetic distance/diversity among populations of Shorea producing tengkawang using microsatellite marker

|     | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1   | **** |     |     |     |     |     |     |     |     |     |     |
| 2   | 0.3108 | **** |     |     |     |     |     |     |     |     |     |
| 3   | 0.4685 | 0.2623 | **** |     |     |     |     |     |     |     |     |
| 4   | 0.3301 | 0.3962 | 0.2343 | **** |     |     |     |     |     |     |     |
| 5   | 0.4171 | 0.3893 | 0.2292 | 0.0756 | **** |     |     |     |     |     |     |
| 6   | 0.5057 | 0.5205 | 0.3087 | 0.1081 | 0.0362 | **** |     |     |     |     |     |
| 7   | 0.8647 | 1.3216 | 0.8149 | 1.1735 | 1.3572 | 1.2326 | **** |     |     |     |     |
| 8   | 0.6152 | 0.8741 | 0.5192 | 0.5941 | 0.7422 | 0.7152 | 0.1547 | **** |     |     |     |
| 9   | 0.5543 | 1.1553 | 0.9446 | 0.6872 | 0.597 | 0.7194 | 0.6394 | 0.5351 | **** |     |     |
| 10  | 0.4381 | 0.7217 | 0.5298 | 0.2024 | 0.3683 | 0.2892 | 0.5681 | 0.3562 | 0.521 | **** |     |
| 11  | 1.1358 | 1.6526 | 1.4048 | 1.4527 | 1.6319 | 1.4176 | 0.565 | 0.6057 | 0.9271 | 0.9111 | **** |

NOTE: 1. S. stenoptera Ketapang; 2. S. pinanga Sanggau; 3. S. stenoptera Sanggau; 4. S. macrophylla Sanggau; 5. S. stenoptera Sintang; 6. S. macrophylla Haurbentes; 7. S. pinanga Ketapang; 8. S. stenoptera Sungai Buaya; 9. S. stenoptera Haurbentes A; 10. S. stenoptera Haurbentes B; 11. S. seminis Bengkayang

Microsatellite marker can also be used to observe the genetic distance among populations. Table 3 revealed the genetic distance of eleven (11) populations of Shorea producing tengkawang. The average of genetic distance among eleven populations was 0.8245. Based on three (3) microsatellite markers used in the research, it showed that the longest genetic distance was between population of S. seminis from Bengkayang and S. pinanga from Sanggau (1.6526), while the shortest genetic distance was between population of S. macrophylla from Sanggau and S. stenoptera from Sintang (0.0362).

To assess the overall distribution of diversity within and among population, an Analysis of Molecular Variance (AMOVA) was performed from the distance matrix. Amova was used to calculate the level of genetic differentiation among different populations. The Amova presented that 72% of the genetic variance was found within populations and 28% among populations showing significant difference genetic (P value < 0.001) as shown in Table 4.
Table 4. Analysis molecular variance of Shorea producing tengkawang populations

| Source        | df | SS       | MS    | %   | P - value |
|---------------|----|----------|-------|-----|-----------|
| Among Pops    | 10 | 188.505  | 18.850| 28% | *         |
| Within Pops   | 209| 445.100  | 2.130 | 72% | **        |
| Total         | 219| 633.605  |       | 100%|           |

The UPGMA cluster analysis based on Nei’s unbiased genetic distance showed that two (2) grouping could be recognized from the dendogram. The first group consisted of *S. stenoptera* (Ketapang, Sanggau, Sintang and Haerbentes A), *S. pinanga* from Sanggau and *S. macrophylla* (Sanggau and Haerbentes). The second group consisted of *S. pinanga* from Ketapang, *S. stenoptera* (Sungai Buaya and Haerbentes B) and *S. seminis* from Bengkayang. However, although the dendogram showed two divided groups, there is no clear grouping fragmentation for both population which were geographically close and for the same species population. The UPGMA result was supported by the principal coordinates analysis (PCoA) which revealed an unclear separation between those 11 populations of shorea producing tengkawang (Fig. 2).

Figure 1. Cluster analysis between populations of Shorea tengkawang based on [17] using UPGMA method.

Principal Coordinates (PCoA)
Figure 2. Principal Coordinat Analysis (PCoA) of Shorea producing Tengkawang using three microsatellite markers

3.2. Discussion

Microsatellite markers (SSR)

Microsatellite markers (SSR) used in this research were the markers developed for Shorea curtisii[14]. The markers were screened from and can be used for several species of Shorea producing tengkawang[18]. All the markers were polymorphic and a total of 188 alleles were detected across the 3 loci. Selection of microsatellites with a range of polymorphism is an important thing to do before conducting a analysis using several samples. The reason is because selecting suitable markers reduced the risk of overestimating genetic variability, which might occur with the selective use of highly polymorphic loci. Microsatellite markers should preferably have at least 4 alleles to be useful for the evaluation of genetic diversity as per the standard selection of microsatellites loci and 3 alleles per locus to evaluate genetic diversity [19], [20].

Genetic Heterozygosity

Genetic variability is also measured as the amount of actual or potential heterozygosity, which is presented in Table 2. The mean of observed heterozygosity value was 0.699 while the mean of expected heterozygosity was 0.672. However, expected heterozygosity was lower than the observed heterozygosity at all species in all populations except for four (4) populations of S. stenoptera and population of S. pinanga. High heterozygosity means lots of genetic variability. Low heterozygosity means little genetic variability.

In the case of S. stenoptera and S. pinanga, there was a discrepancy toward the Hardy Weinberg principle. This discrepancy is probably due to genetic drift, gene flow, population bottleneck and inbreeding. The difference between the observed and expected heterozygosity can be attributed to the non-random mating among the individuals of the population. In addition, levels of heterozygosity are directly linked to fitness at individual level and this leads to the expectation that at population level, mean fitness and level of heterozygosity are correlated [27]. A large number of plant species also confirm these expectations; long-lived, woody perennial plants with wide geographic ranges, particularly those with a boreal temperate distribution, generally have high levels of genetic diversity (heterozygosity) and a large fraction of polymorphic loci with many alleles per locus, i.e. allelic richness. The genetic diversity values of several Shorea producing tengkawang species from eleven (11) populations used in this study were relatively high, and these indicate that the populations are suitable as the sources for establishing ex situ conservation plots. The genetic materials collected can represent the genetic diversity of the population.

Genetic Relationship among Population

The average value of genetic distance between two populations or more illustrates the genetic diversity among populations. Table 3 revealed the genetic distance from eleven (11) population of Shorea producing tengkawang with genetic distance average among eleven populations of 0.8245. The longest genetic distance was between population of S. seminis from Bengkayang and S. pinanga from Sanggau (1.6526), while the shortest genetic distance was between population of S. macrophylla from Sanggau and S. stenoptera from Sintang (0.0362).
The level of genetic diversity maintained in a population is determined by the combined action of mutation, gene flow, genetic drift and selection. Populations which are genetically isolated will have differentiation in the allele frequencies due to mutation and genetic drift. When the separation time happens in a long time, the differences in allele frequencies should also increase, until each populations has completely fixed separate alleles. On the other hand, when there is no genetically isolation, the values of genetic distance could show population structure in which there are random breeding and low gene flow. Forest trees with wide geographic distributions, large population sizes and high outcrossing rates turns out to have long generation times and have extensive gene flow over large geographic distance through both pollen and seeds.

The result of Amova analysis showed that 72% of the genetic variance was found within populations and 28% among populations (Table 4). Long-lived woody perennials maintain higher levels of genetic diversity within populations compared to other plant types [28]. In addition, forest trees are often wind pollinated and many have adaptations that allow seed dispersal to occur over great distances. This can result in lower levels of genetic differentiation among populations compared to other plant species. On average, up to (and sometimes exceeding) 90% of all genetic variation in widespread forest trees can be found within local populations, with the remaining variation distributed among populations across its range.

The characterization of genetic diversity pattern at intra- and inter-population levels is a fundamental requirement for the establishment of programs aimed at conservation of forest genetic resources. Population genetic diversity has a substantial effect on both individual fitness and population adaptive capacity, thereby playing a vital role in ensuring species capability to withstand various biotic and abiotic stressors and evolve under altered environmental conditions [29];[30];[31]. The high level of genetic variation would work as a buffer to both abiotic or biotic environmental changes, and can perhaps be one reason for the success (in evolutionary terms) of many forest trees. Such environmental changes can include either abiotic factors, such as changes in climatic conditions, or biotic factors, for instance the introduction of novel pests or pathogens[32].

Genetic distance and cluster analysis showed that the average genetic distance among population is quite large, including the genetic distance among population for the same species. There is no clear group separation between populations and between regions, however there is a tendency for population of the same species to form the same cluster/group. From these results, in the framework of developing ex-situ conservation plots of Shorea Tengkawang species, several things to note are: 1) Separate populations for the same species, and 2) Populations with different species should be separated with a sufficient distance so that crossing is not possible, considering that there is a possibility for hybridization between species.

In terms of conservation strategy of Shorea Tengkawang species, it should be known that the population with large potential numbers of individual with high genetic diversity is needed to be protected. In addition, for each species, selection of several populations as in-situ conservation plots should also be conducted. Each province is expected to have in-situ conservation plots for each species of Shorea Tengkawang.

4. Conclusion
The genetic diversity of Shorea spp. producing tengkawang collected from native forest highly determines the success of developing ex-situ conservation plots of that species. The high genetic diversity of seedlings collected and planted in ex-situ conservations plots in KRUS indicates that those genetic materials have represented the genetic diversity of the natural population. It is then necessary to build a proper design of ex-situ conservation plot to follow up the result of genetic diversity, genetic distance and group division from populations and species that have been collected. Information
obtained from this study is very useful in compiling conservation and development strategy of Shorea spp producing tengkawang, especially those in Kalimantan.

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