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Contrasting cpDNA Variation in Two Indonesian Endemic Lowland Dipterocarp Species and Implications for their Conservation

[1]Henti Hendalastuti Rachmat, [2]Koichi Kamiya and [2]Ko Harada
[1]The United Graduate School of Agricultural Science,
[2]Faculty of Agriculture, Ehime University 3-5-7 Tarumi, Matsuyama-Shi 790-8566, Japan

Abstract: *Shorea javanica* (Dipterocarpaceae) is an economically important dammar-producing tree, endemic to the tropical lowland forests of Sumatra and Java, Indonesia. However, its total population size is limited and this species is endangered. *Shorea selanica* is one of the very limited numbers of species in genus *Shorea* (Dipterocarpaceae) that grows in Wallacean. This species can be found only in the central part of the Moluccas, eastern Indonesia. Six populations (77 individuals in total) were sampled for *S. javanica* while three populations (27 individuals in total) were sampled for *S. selanica*. To determine genetic variation and population structure, three non-coding chloroplast DNA regions of *trnL-trnF*, *psbC-trn*S, *trn*S-*trnf*M and two non-coding chloroplast DNA regions of *trn*T-*trn*L, *trn*L-*trn*F were sequenced from *S. javanica and S. selanica*, respectively. There was no variation in the chloroplast DNA regions from *S. javanica*, except for one unique indel polymorphism. Nucleotide diversity within *S. selanica* populations ranged from 0 (Seram) to 0.00044 (Buru), with a pooled value of 0.00041. *S. javanica* was determined as having no population structure while high levels of genetic differentiation was found among populations of *S. selanica* (F_{ST} = 0.702). Different pattern of population structure among the two species in this study suggested the need for distinct management and conservation strategies for each species. For *S. javanica*, connectivity within and among populations, including augmentation of population size across the species range, should be promoted. For *S. selanica,* an *in situ* conservation plan that defines core areas completely free from perturbation within each population is necessary.

Key words: *Shorea javanica*, *Shorea selanica*, dipterocarpaceae, cpDNA, genetic variation

INTRODUCTION

Dipterocarpaceae (dipterocarps) is the dominant tree family in the rain forests of Southeast Asia. Within the region, this family consists of 10 genera with more than 380 species. Dipterocarps show a high rate of endemicity and more than half of the species have a narrow distribution range (Ashton, 1982). Conservation of dipterocarps was not previously seen important because the family still can be found common and abundant in the nature and none of the species was presumed threatened (Lee *et al*., 2006). However, the high rates of forest cover loss and modification in Southeast Asian rainforests is leading directly to the endangerment and local extinction of many plant and animal species, including many ecologically and economically important species of Dipterocarpaceae (Sodhi *et al*., 2010). Up to 42% of flora and fauna in Southeast Asia can be lost if the current rates of habitat alteration continue (Sodhi *et al*., 2004). Due to these imperiling conditions, there is now increased concern for the conservation of dipterocarps.

Shorea javanica is one of lowlands dipterocarps species endemic to Indonesia. Habitat loss and forest fragmentation has threatened this species with extinction. *S. javanica* produces clear resin known as dammar mata kucing or cat eye resin. The dammar resin has been traded internationally for centuries. Its economical importance had triggered the community to begin domesticating damar from the nearby natural forest more than a century ago and their unique agroforestry system known as repong dammar. *S. javanica* requires fertile volcanic soils to grow (Torquebiau, 1984) and its distribution is limited to the western coast of Sumatra and southern coast of western Java (Newman *et al*., 1996, 1998). In Sumatra, *S. javanica* is assumed to have been predominant in a narrow coastal strip of lowland forest adjacent to the Bukit Barisan mountain belt which features higher elevation and more undulating terrain. However, recent field surveys suggested that its distribution might become smaller, more fragmented and patchy due to extensive deforestation in this region. At present, most of its populations are maintained in repong dammar and the

Corresponding Author: Ko Harada, Faculty of Agriculture, Ehime University 3-5-7 Tarumi, Matsuyama-Shi 790-8566, Japan
Tel: + 81-89-946-9870 Fax +81-89-946-9870

remnant natural population can only be found in Bukit Barisan Selatan National Park. Central Java is one of its natural distribution for this species (Newman *et al*., 1996, 1998), but current field sampling found that only a single mature tree grows in Sancang Nature Reserve (southern coast of West Java). In addition, there is several trees collection in Bogor Botanical Garden.

Shorea selanica is listed as a critically endangered species on the IUCN Red List (www.iucnredlist.org). *S. selanica* grows only in the central part of the Molucca Archipelago, eastern Indonesia which includes the islands of Buru, Sula, Obi, Seram and Ambon. It is locally gregarious and dominant in semi-evergreen lowland forests on well-drained land with fertile soils that sometimes overlying limestone (Newman *et al*., 1998). *S. selanica* is also an important timber source. It can reach 50 cm in diameter at breast height in less than 30 years which has made it one species with good potential for plantations and reforestation (Subiakto *et al*., 2001). Although intensive studies have been carried out regarding its silviculture and it has also been widely planted for experimental and reforestation objectives, no genetic information for this species is available so far.

Deforestation in Indonesia has been taking place for a long time. The Indonesian Deforestation Model predicts that Indonesian forests will continue to decline and that Sumatra and the Moluccas could lose all but 25-30% of their natural forest cover (Nasendi, 2001). According to that model, effective *in situ* and *ex situ* conservation strategies are required to conserve existing genetic resources. A better understanding of diversity and the mechanisms maintaining diversity may be helpful for developing effective strategies to conserve genetic resources. Molecular marker-based genetic studies are essential to determine appropriate conservation management for both endemic and more common widespread species. This study is the first investigation of chloroplast DNA variation of two important lowland dipterocarp species endemic to Indonesia, *Shorea javanica* and *Shorea selanica*. Advancing our understanding of the genetic aspect across a broader range of dipterocarps will support improved management and restoration of dipterocarp forests.

MATERIALS AND METHODS

Plant materials: Leaf samples of *S. javanica* were collected from five Sumatran populations and one pooled Java population to represent the current geographical distribution of this species. In Sumatra, 15 adult trees (for each individual sampled minimum diameter at breast height (dbh) was 30 cm and minimum distance between individuals sampled was 50 m) were sampled from each population, originating from four repong dammar populations and one remnant natural population in Kubu Perahu, Bukit Barisan Selatan National Park. Because only one remaining tree was found in Sancang Nature Reserve, samples were also taken from a tree in Bogor Botanical Garden in Java, (exact sampling site is not known; personal communication). Therefore, these samples were pooled together as the Java population. A total of 77 adult trees were sampled from seven different geographic locations for *S. javanica*. For *S. selanica*, leaf samples were collected from three populations in the Moluccas, eastern Indonesia. Leaf samples were taken from adult trees at minimum dbh of 30 cm and from individuals spaced at a minimum distance of at least 50 m. Nine individuals were sampled from the Sula Islands, 10 individuals from Seram Island and eight individuals from Buru Island. In total, 27 individuals of *S. selanica* were analyzed in this study. Sampling location and collected individuals are described in Fig. 1 and Table 1. Sampled leaves were dried with silica gel and subsequently used for DNA extraction.

Loci studied: Eight chloroplast DNA regions (cpDNA) were tested from *S. javanica* (*trn*L-*trn*F, *trn*H-*trn*K, *psb*C-*trn*S, *pet*B, *trn*S-*trn*T, *pet*L-*psb*E, *trn*S-*trn*fM and *trn*T-*trn*L) and five cpDNA from *S. selanica* (*trn*L-*trn*F, *trn*H-*trn*K, *psb*C-*trn*S, *trn*S-*trn*fM and *trn*T-*trn*L). For

Table 1: Genetic variation and population differentiation of the two Indonesian endemic lowland dipterocarp species, *S. javanica* and *S. selanica*, as inferred by non-coding chloroplast DNA region sequences

Species	Population	N	S	Hd	π	Tajima's D	F_{ST}
S. javanica	a. Pahmungan (Pa)	15	0	0.000	0.00000	na	
	b. Gunung Kemala (Gk)	15	0	0.000	0.00000	na	
	c. Kubu Perahu (Kp)	15	0	0.000	0.00000	na	
	d. Bengkunat (Be)	15	0	0.000	0.00000	na	
	e. Oku (Ok)	15	0	0.000	0.00000	na	
	f, g. Java (Bg, Sc)	2	0	0.000	0.00000	na	
	Total (a-f)	77	0	0.000	0.00000	na	na
S. selanica	h. Buru	8	2	0.714	0.00044	0.41421	
	i. Seram	10	0	0.000	0.00000	na	
	j. Sula	9	1	0.556	0.00029	1.40117	
	Total (h-j)	27	3	0.798	0.00041	0.06046	0.70222***

N: No. of individuals analyzed per population, S: No. of segregating sites in nucleotide sequences, Hd: Haplotype diversity, π: Nucleotide diversity, na: Not available, F_{ST}: Population differentiation, ***$p<0.0001$

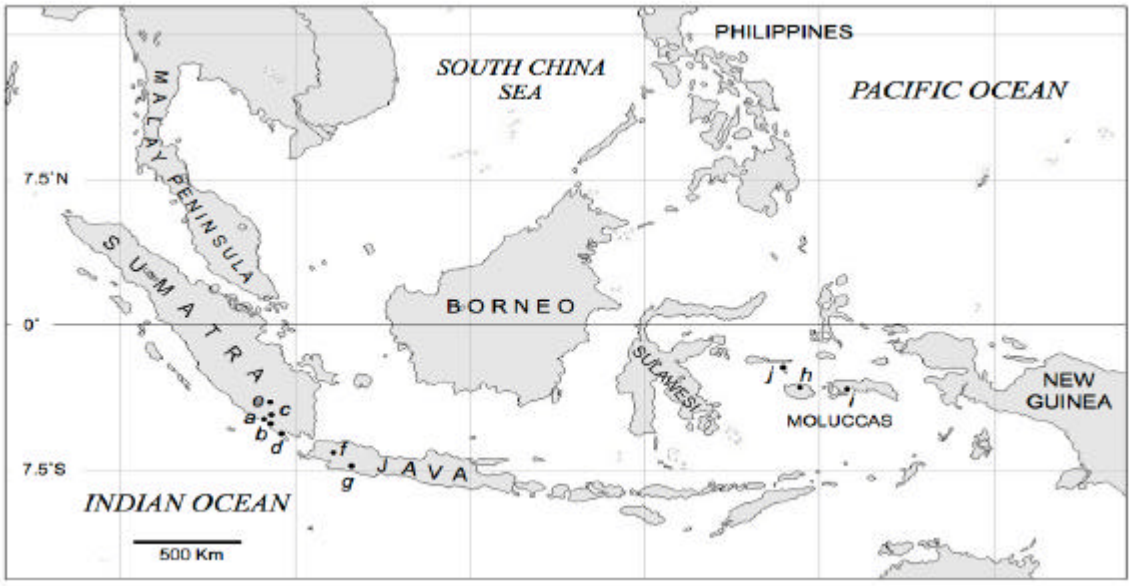

Fig. 1: Sampling location for *S. javanica* and *S. selanica*, (a, b, c, d, e, f, g): Sampling sites for *S. javanica*; (h, i, j): Sampling sites for *S. selanica*

S. javanica, three cpDNA regions could be amplified successfully by PCR using the universal primers described by Taberlet *et al.* (1991) for *trnL-trnF* and those described by Demesure *et al.* (1995) for *psb*C-*trn*S and *trn*S-*trnf*M. For *S. selanica* two cpDNA intergenic spacers, *trn*T-*trn*L and *trn*L-*trn*F (Taberlet *et al*., 1991) were amplified.

DNA isolation, amplification and sequencing: Genomic DNA was isolated from adult leaves using a modified CTAB method (Murray and Thompson, 1980). Polymerase Chain Reaction (PCR) amplifications for *S. javanica* samples were performed in a volume of 20 μL containing 10 ng of genomic DNA, 5 pmol of each forward and backward primer and 10 μL of Go *Taq*® Hot Start Colourless Master Mix (Promega, Madison, WI, USA) according to the manufacturer's instructions. Initial denaturation was performed at 95°C for 2 min, followed by 30-35 cycles of denaturation at 95°C for 1 min, annealing at 52-62°C and polymerization at 72°C for 2 min and final extension at 72°C for 7 min. PCR amplifications for *S.selanica* samples were performed in a volume of 25 μL containing 10 ng of genomic DNA, 5 pmol of each forward and backward primer, 10x buffer KOD-Plus, 2 mM dNTPs, 25 mM $MgSO_4$, KOD Plus DNA polymerase and nuclease free water following the manufacturer's instruction (TOYOBO Co., Osaka, Japan). A touchdown PCR program was applied at 94°C for 2 min; 4 cycles at 94°C for 45 sec, 59°C for 45 sec (decreasing 1 degree each cycle) and at 68°C for 2.5 min; then 26 cycles at 94°C for 45 sec, 55°C for 45 sec and 68°C for 2.5 min and holding at 68°C for 30 min. Prior to sequencing, the PCR products were purified using rAPid Alkaline Phosphatase™ (Roche, Germany) and Exonuclease I (New England Biolabs, Ipswich, MA, USA). Purified products were directly sequenced on both strands using an ABI Prism 3100 automatic sequencer (Applied Biosystems, Foster City, CA, USA).

Data analyses: DNA sequences were checked visually and then forward and reverse traces were assembled using the ATGC program (Genetyx Corporation, Japan). To assess levels of nucleotide polymorphism, nucleotide diversity (π) (Nei, 1987) and Haplotype Diversity (Hd) (Nei, 1987) for each of the investigated loci were estimated. To test for deviations from selective neutrality and other assumptions (e.g., constant population size with no migration), Tajima's D (Tajima, 1989) test was performed for individual loci. Population structure was examined using ARLEQUIN ver. 3.5 (Excoffier and Schneider, 2005). Genetic differentiation, F_{ST} of nuclear loci was estimated through analysis of molecular variance (AMOVA). Pairwise F_{ST} was also calculated to determine population structure between pairs of populations (Gaggiotti and Excoffier, 2000).

RESULTS AND DISCUSSION

Genetic diversity and population structuring: In *S. javanica*, sequences of 953, 1510 and 1475 bp (3938 bp in total) were determined, excluding alignment gaps, for three non-coding regions of cpDNA, *trn*L-*trn*F, *psb*C-*trn*S and *trn*S-*trn*fM, respectively. There were no variants except for single occurrence of a 6-bp indel (TTTTTA) at the *psb*C-*trn*S region in four of the 15 individuals from the Gk population. Considering the highly specific habitat and very restricted distribution of *S. javanica*, it is expected that populations would show low levels of genetic variation as compared to other dipterocarp species with wider distributions. This is indeed the case for *S. javanica*, both at the population and species level. There were no variants in three cpDNA regions among the 77 individuals analyzed in this study. It could be that the low level of genetic variation is a specific characteristic of the cpDNA regions examined in this study. However, considerable numbers of substitutions were previously observed within and between *Shorea* species in the *trn*L-*trn*F and *psb*C-*trn*S regions (Tsumura *et al*., 2011).

In *S. selanica*, the alignment lengths for non-coding chloroplast DNA regions *trn*T-*trn*L and *trn*L-*trn*F were 1010 and 951 bp, respectively (1961 bp in total). Haplotype diversity for the pooled population (Hd) was 0.798 and population nucleotide diversities ranged from 0 (Seram) to 0.00044 (Buru), with 0.00041 for pooled populations (Table 1). This value is lower than that reported for cpDNA nucleotide diversity in the common species *Shorea curtisii* (π_{sil} = 0.00155; Kamiya *et al*., 2012) from the Malay Peninsula and Borneo.

The study result suggests that the extremely low level of genetic variation in the cpDNA regions examined here is not specific to the studied loci, but specific to *S. javanica* as other *Shorea* species maintain some degree of intrapopulation genetic variation. Three nucleotide substitutions were also found at the same loci (*trn*L-*trn*F) in lowland endemic *S. selanica* in this study. The low level of genetic variation in two endemic lowland dipterocarps in this study is congruent with that of Hamrick and Godt (1996) which stating that endemic species tend to have low levels of genetic diversity due to their small population sizes. Overall, the result of this study also supports the general expectation of reduced genetic diversity in rare endemic species and showed that genetic diversity in rare endemic species and showed that species with a narrow geographic distribution harbor less genetic variation than do widespread species (Hamrick *et al*., 1992).

Table 2: Pairwise F_{ST} among populations of *S. selanica* as inferred by non-coding chloroplast DNA region sequences

Population	Buru	Seram	Sula
Buru	0.000		
Seram	0.907***	0.000	
Sula	0.462***	0.394***	0.000

F_{ST}, population differentiation; ***p<0.0001

While nucleotide substitution was only detected in *S. selanica*, the level of genetic differentiation between populations (F_{ST}) could only be estimated for this species. Significant differentiation was found among populations (F_{ST} = 0.702) (Table 1). Pairwise F_{ST} values between sampled populations indicated significant population differentiation for all pairs of populations, with values ranging from 0.39 (Seram-Sula) to 0.90 (Seram-Buru) (Table 2). The chloroplast genome is primarily maternally inherited in angiosperms and is considered a single, non-recombining unit of inheritance which is structurally stable (Olmstead and Palmer, 1994; Birky Jr, 1995). The level of differentiation observed in the chloroplast genome of *S. selanica* is most probably due to the separation and isolation of populations over an extended period of time during the evolutionary history of this species. The genetic structuring of populations of organism and ultimately the establishment of independent evolutionary lineages, is strongly influenced by patterns of genetic exchange (gene flow) within and between populations. Seed dispersal for *Shore*a species is facilitated by wind or gravity. Distances for seed dispersal can be up to 500 m, but under forest conditions, more than half of the mature seeds will land within 50 m of the parent trees (Chan and Appanah, 1980; Takeuchi *et al*., 2004). Furthermore, seed dispersal is limited even for species with comparatively light seeds and long-winged fruits (Fukue *et al*., 2007). Disjunction of the distribution of the species would lead to the evolution of different lineages among separated islands through the effects mutation and drift. The absence of permanent land bridges among islands (De Jong, 1998) strengthens the isolation process and limits gene flow between populations.

The pairwise F_{ST} values describe the occurrence of population structuring in *S. selanica*, in which populations have become differentiated from each other. The Moluccas which are the islands where *S. selanic*a grows naturally, have a distinctive geological history. It should be noted that the absence of permanent land bridges among these islands since the land emerged (De Jong, 1998) could have restricted historical gene flow and increased genetic differentiation among populations. For example, the pairwise F_{ST} value between the extant adjacent islands Buru and Seram was quite high (F_{ST} = 0.90 for cpDNA). While there is close stratigraphic similarity

between Seram and Buru, the two islands show very different structural styles (Charlton, 2000). At present, although Buru and Seram are located close to each other; however, 5 mya, Buru was located farther to the north of Seram island (Hall, 1996), too distant to permit intensive gene flow.

Implications for conservation: An understanding of the partitioning the genetic diversity within and between populations (known as genetic structure) is important for developing a conservation strategy for endangered species, especially if not all populations can be protected. Species with low levels of population structure could be simplified, as the loss of single population may have little impact on the species-wide genetic diversity. In contrast, for species with a high level of population structure, the loss of a single population might significantly reduce overall genetic diversity. The uniformity of nucleotide sequences across individuals and populations of *S. javanica* in this study revealed that no population differentiation could be detected within this species based on cpDNA variation, suggested that each population (from either repong dammar or natural stands) harbored similar genetic characteristics. However, modern selection pressure from farmers preferentially collecting and planting seeds from high-dammar-producing trees, together with the declining population sizes, could reduce inter and intra-population gene flow and may generate systematic genetic erosion. In contrast to *S. javanica*, *S. selanica* exhibits distinct genetic structure among populations, indicating that they are genetically differentiated. Different pattern of population structuring between the two endemic lowland dipterocarps in this study suggested the need for different management and conservation strategies for each species.

For *S. javanica*, connectivity within and among populations, including augmentation of population sizes across the species range should be promoted. Even though repong dammar have been established primarily for dammar production, they have also been managed for adaption to the local environmental conditions. Maintenance of these plantations can preserve both biological and genetic diversity and enable them to serve as refugia for biodiversity. Thus, maintenance of repong dammar should be a primary focus and conversion to other land uses, especially monoculture-based systems, should be avoided. Conserving *S. javanica* populations in native habitat is also essential.

The appropriate conservation strategy for *S. selanica* would require a combination of different approaches. The first would consist of an *in situ* conservation plan that would define core areas completely free from perturbation in each population. The second strategy would employ *ex situ* conservation to maintain genetic variation by pooling populations from various islands. In the short term, recovery of as many seeds as possible spanning their entire distribution will be critical to obtain a good representation of each specie's genetic diversity.

CONCLUSION

This study was carried out to determine genetic variation of two lowland dipterocarp species endemic to Indonesia, *S. javanica* (Sumatran and Java endemic) and *S. selanica* (Moluccan endemic) inferred from non-coding chloroplast DNA regions. The result showed extremely no variation for *S. javanica* while low level of genetic variation was attributed to *S. selanica*. In contrast with no population structuring of *S. javanica*, *S. selanica* exhibited high levels of genetic differentiation among populations. According to different pattern of population structuring for the two species determined in this study, distinct management and conservation strategies are required for each species. Connectivity within and among populations, including augmentation of population size across the species range should be promoted for *S. javanica* while an *in situ* conservation plan that defines core areas completely free from perturbation within each population is necessary for *S. selanica*.

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