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POPULATION GENETICS OF THE CRITICALLY ENDANGERED SPECIES *Dipterocarpus littoralis* BLUME (DIPTEROCARPACEAE) ENDEMIC IN NUSAKAMBANGAN ISLAND, INDONESIA

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ABSTRACT

Dipterocarpus littoralis Blume is a critically endangered dipterocarp species found only in Nusakambangan Island, Central Java, Indonesia. Patterns of genetic diversity and population genetic structure of adults and saplings in two extant populations (Kali Jati and Solok Besek) were estimated using ten microsatellite markers. A total of 39 alleles were found, with two and four alleles being unique in adult and sapling populations, respectively. Allelic richness and heterozygosity were similar between adult (Ar = 3.00; H_e = 0.423) and sapling (Ar = 3.25; H_e = 0.441) populations. Inbreeding coefficients in saplings were positive in both populations and statistically significant in Kali Jati, while those in adult populations were not significantly different from zero, indicating excessive inbreeding and selfing in the sapling populations. Genetic differentiation of the sapling populations (F_{ST} = 0.036) was slightly lower than in the adult populations (0.050), but only significantly so for saplings. This study revealed that *D. littoralis* has low genetic diversity in both adults and saplings. Similarly low values in allelic richness and heterozygosity suggest that reductions of population size have been ongoing for long periods in this species. Significant genetic differentiation between sapling populations but not adult populations indicates that recent fragmentation is further accelerating the isolation process.

Keywords: *Dipterocarpus littoralis*, microsatellite, genetic diversity, genetic differentiation

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INTRODUCTION

In tropical regions, recent expansion of human populations and their activities have caused rapid loss of forest cover (Hansen *et al.* 2010) and forest degradation (Sasaki & Putz 2009). Degradation of forests may turn common species into rare and endangered. Tropical trees are thought to be particularly vulnerable to the effects of habitat degradation due to their demographic and reproductive characteristics, including low density of occurrence, high rate of outcrossing (Cascante *et al.* 2002, Lowe *et al.* 2005) and intimate interactions with pollinators and seed dispersers (Didham *et al.* 1996, Dick *et al.* 2003, Ward *et al.* 2005). As rare and endangered species are more often vulnerable, efforts to learn more about the ecological and genetic process of extinction are crucial for protecting and managing remnant populations of threatened plant species in altered environments (Furches *et al.* 2009).

A substantial amount of genetic variation within a species guarantees its evolutionary potential under global environmental change, and information about the spatial distribution of such variation is important for formulating effective strategies to maintain maximum genetic variation (Falk & Holsinger 1991, Groom *et al.* 2006). Remnant populations can be predicted to have a greater chance of deterioration in genetic variations, and populations tend to become more strongly isolated by founder effects, random genetic drift and limited biparental gene flow (Templeton *et al.* 1990, Ellstrand & Elam 1993, Young *et al.* 1996). As results, this may lead to reduced population viability and finally extinction (Godt & Hamrick 1998, Thomas *et al.* 2004, Kramer & Havens 2009). Studies of remnant populations have therefore, gained much attention in conservation biology.

Dipterocarpus littoralis Blume (locally known as pelahlar) is a member of the Dipterocarpaceae family and endemic to Nusakambangan Island, Central Java, Indonesia (Ashton 1982). This tree species has been critically endangered under subcategory B1+2c, C2a in the Red List of Threatened Species (IUCN) since 1997 and is also included nationally on the list of priority species for the 2008-2018 Indonesia conservation action (Minister of Forestry Decree P.57/Menhut-II/2008). *Dipterocarpus littoralis* is currently restricted to the western part of Nusakambangan Natural Reserve, which has an area of 625 ha (Staatsblad van Nederlandsch-Indie 1937, Abdiyani 2008). The species is often found along rivers because it prefers moist habitats (Silvagama 2000) and grows approximately an altitude of 1-100 m above sea level (Wardani 2006).

Like many other tropical forest species, *D. littoralis* is currently highly vulnerable due to forest destruction and illegal logging increasingly isolating populations from each other (Yulita & Partomihardjo 2011). So far, little is known about the genetic risk of these small remnant patches, although knowledge of genetic variation and population differentiation of *D. littoralis* is important for conservation. To facilitate efforts to conserve the remnant *D. littoralis* populations, this study evaluated genetic variation and population genetic structure in two small remnant patches. Specifically, the study asked these questions: (1) Does *D. littoralis* have lower genetic diversity when compared to common species?; (2) Are there any differences in the extent of genetic

diversity between adult and sapling populations?; (3) Does *D. littoralis* have high genetic differentiation between populations?.

MATERIALS AND METHODS

Plant Materials

Sampling of *D. littoralis* populations was conducted in two areas, Kali Jati and Solok Besek, in the western part of Nusakambangan Natural Reserve (Fig. 1). The two populations are separated from each other by a distance of 1.4 km. In each population, all adult trees with diameter at breast height (dbh) > 20 cm were sampled (N = 11 at Kali Jati and N = 7 at Solok Besek). Leaves from 2-5 saplings (1.3 m to 1.6 m tall) under each adult tree were collected. Finally, 53 and 18 saplings were collected in Kali Jati and Solok Besek, respectively. Plant materials were dried with silica gel in the field and stored in a freezer at -80°C. They were subsequently used for DNA extraction.

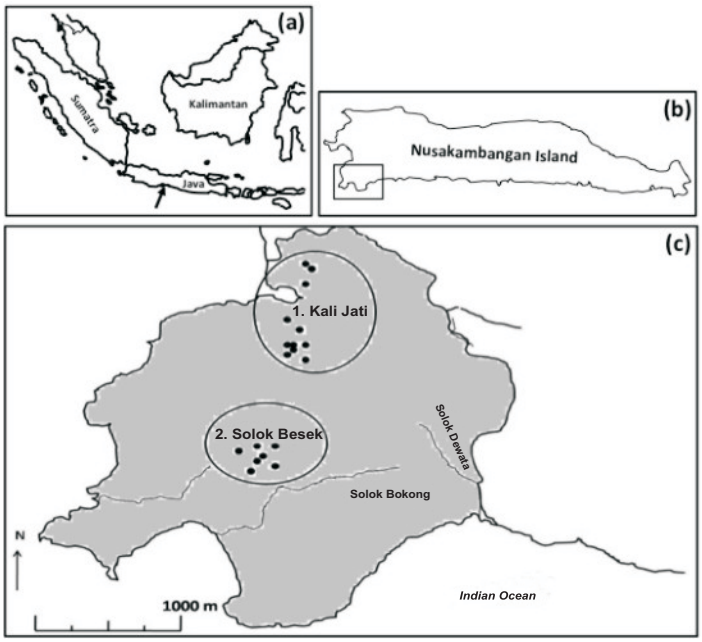

Figure 1. Location of the two *D. littoralis* populations examined in this study.(a) Map of Indonesia showing that Nusakambangan Island is located in the southern part of Java Island (shown by an arrow). (b) Map of Nusakambangan Island that shows western part of Nusakambangan Natural Reserve location in small frame. (c) Collections sites in western part of Nusakambangan Natural Reserve. Dots show adult sampling sites. The area of Kali Jati is ca. 21 ha, and that of Solok Besek is ca. 18ha.

Microsatellite Genotyping

Silica gel-dried leaves were ground to a fine powder using a Tissue Lyser II (QIAGEN). Total genomic DNA was extracted from each sampled tree using modified CTAB method (Doyle & Doyle 1990). Ten microsatellite loci that had been developed for other dipterocarp species were utilized for this study [DT07, DT09, DT18, DT20, DT29, DT35 and DT39 in Isagi *et al.* (2002), Shc07 in Ujino *et al.* (1998), Tum1406G13 in Ohtani *et al.* (2012), and DL(GT)202 in Terauchi (1994)]. The forward primer of each marker was labeled with either 6-FAM, VIC, NED, or PET phosphoramidite (Applied Biosystems). Details of the markers used in this study are shown in Table 1.

A Type-it Microsatellite PCR kit (QIAGEN) was used for amplification of microsatellite loci. Multiplex PCR amplification was performed in a volume of 10 µl, containing 5 µl of 2 × Type-it Multiplex PCR Master mix, 1 µl primer mix (2µM each), 3 µlRNase-free water and 1 µl genomic DNA (~40 ng), using a thermal cycler (Applied Biosystems 2720) under the following conditions: initial denaturing at 95°C for 5 min, then 31 cycles of denaturing at 95°C for 30 s, annealing for 1 min 30 s and extension at 72°C for 30 s, followed by a final incubation at 60°C for 30 min. Annealing temperatures were 49°C for Shc07 54°C for Tum1406G13 and DL(GT)202 and 58°C for DT07, DT09, DT18, DT20, DT29, DT35 and DT39. Fragment sizes were scored using an ABI PRISM^{TM} 310 Genetic Analyzer (Applied Biosystems) and visualized using GeneMapper 3.0 software (Applied Biosystems).

Data analysis

Basic statistics of genetic diversity, including number of alleles per locus (*Na*), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (*F*) and significant deviation from HWE, were calculated using GenAlEx software version 6.41 (Peakall & Smouse 2006). Allelic richness (*Ar*) was calculated using FSTAT Version 2.9.3.2 (Goudet 1995, 2001). Pairwise F_{ST} was calculated in adults and saplings to determine the level of population differentiation using ARLEQUIN Version 3.5.1.2 (Excoffier & Lischer 2010).

RESULTS AND DISCUSSIONS

Low Genetic Diversity in *D. littoralis*

This study demonstrated that seven of the primer pairs developed for *Dipterocarpus tempehes*, one developed for *Shorea curtisii*, one developed for *Shorea leprosula* and one developed for *Dryobalanops lanceolata* could be successfully used to estimate the genetic variation for *D. littoralis*. Primer information and the results of genetic analysis are summarized in Table 1. Very small number of alleles (two to six) and associated low level of heterozygosity were detected in all microsatellite loci.

The primer pairs developed by Isagi *et al.* (2002) for *D. tempehes* were also successfully used for the species *D. crinitus* and *D. globosus* (Harata *et al.* 2012),

Table 1. Primer information for 10 microsatellite loci used in this study

Locus	Length (bp)	Annealing temperature	Number of alleles	H_e	F	HWE test	Ref.
DT07	75-85	58 °C	3	0.235	-0.053	ns	1
DT09	155-193	58 °C	7	0.811	0.045	ns	1
DT 18	134-139	58 °C	2	0.033	-0.017	ns	1
DT20	234-238	58 °C	2	0.342	-0.084	ns	1
DT29	215-227	58 °C	5	0.655	0.262	***	1
DT35	357-369	58 °C	5	0.663	0.170	ns	1
DT39	172-190	58 °C	8	0.681	0.076	ns	1
Shc 07	155-161	49 °C	3	0.616	-0.058	ns	2
Tum1406G13	350-364	54 °C	2	0.383	0.062	ns	3
DL(GT)202	205-217	54 °C	2	0.145	0.070	ns	4

H_e= Nei's expected heterozygosity; F= Fixation index; ns= not significant; ***= $P< 0.001$;
Ref. 1= Isagi *et al.* (2002); 2= Ujino *et al.* (1998); 3= Ohtani *et al.*(2012); 4= Terauchi (1994).

Table 2. Comparisons of locus heterozygosity in four *Dipterocarpus* species*

Locus	Number of samples				Number of alleles				H_O				H_E			
	D. crinitus	*D. globosus*	*D. littoralis*	*D. tempehes*	*D. crinitus*	*D. globosus*	*D. littoralis*	*D. tempehes*	*D. crinitus*	*D. globosus*	*D. littoralis*	*D. tempehes*	*D. crinitus*	*D. globosus*	*D. littoralis*	*D. tempehes*
DT07	23	-	18	34	3	-	2	10	0.522	-	0.278	0.727	0.567	-	0.239	0.834
DT09	23	289	18	34	9	49	6	7	0.609	0.903	0.889	0.667	0.688	0.926	0.807	0.762
DT18	-	289	18	34	-	26	2	14	-	0.830	0.056	0.485	-	0.921	0.054	0.807
DT20	23	289	18	34	6	14	2	13	0.870	0.619	0.444	0.818	0.768	0.691	0.401	0.836
DT29	23	289	18	34	10	29	5	8	0.870	0.678	0.444	0.455	0.864	0.725	0.613	0.565
DT35	-	289	18	34	-	30	5	11	-	0.896	0.556	0.848	-	0.928	0.664	0.846
DT39	23	289	18	34	11	24	6	11	0.957	0.858	0.722	0.848	0.878	0.867	0.556	0.862
Average					7.8	28.7	4	10.6	0.766	0.797	0.484	0.693	0.753	0.843	0.476	0.787
Total					39	172	28	74								

* *D. crinitus* and *D. globosus* from Harata *et al.* (2012); *D. littoralis*, this study; *D. tempehes* from Isagi *et al.* (2002).

suggesting that sequences of flanking regions of these microsatellite regions are well-conserved across many dipterocarp species (Ng *et al.* 2004). The genetic variation found in *D. littoralis* was compared with that of other congeneric species, which are studied using the same primer pairs (Table 2). Results showed that genetic variation in *D. littoralis* was much lower than the other species. The average number of alleles per locus as observed and expected heterozygosities were low in *D. littoralis*. Although the number of alleles increased with sample size, the observed number of alleles in *D. littoralis* populations seems to be extremely small in most of the loci. It is possible that this is due to the presence of null alleles. Presence of null alleles will increase the number of individuals with apparent homozygotes, and samples with no amplification will be observed if these are null homozygotes. However, we successfully amplified microsatellite regions for all samples. Except for DT29, no deviation from HWE was detected at any studied locus (Table 1). Therefore, presence of null alleles could not explain the low level of microsatellite variability across loci in *D. littoralis*.

Our study also showed that allelic diversity and heterozygosity in *D. littoralis* was lower in comparison with other genera of Dipterocarpaceae, such as *Neobalanocarpus heimii* (Konuma *et al.* 2000), *Shorea curtisii* (Ujino *et al.* 1998, Obayashi *et al.* 2002, Ng *et al.* 2006, Harata *et al.* 2012), *S. leprosula* (Nagamitsu *et al.* 2001, Ng *et al.* 2004, Fukue *et al.* 2007), *S. lumutensis* (Lee *et al.* 2006), *S. macroptera* (Ng *et al.* 2006), and *S. ovalis* (Ng *et al.* 2004). Comparatively low microsatellite variation has been found in the endemic dipterocarp species *S. javanica*in Sumatra, Indonesia (Rachmat *et al.* 2012). The low level of genetic diversity in *D. littoralis* corresponds to a pattern often found in endangered endemic plants (Gitzendanner & Soltis 2000). This pattern seems to be not always holding, however, at least in some dipterocarp species. For example, there were no notable differences in heterozygosity among rare and common dipterocarp species in Northern Borneo (Harata *et al.*2012), and a surprisingly large amount of genetic variation was found in the endemic and rare species *S. lumutensis* (Lee *et al.* 2006).

Comparison between Adult and Sapling Populations

In this study a total of 39 alleles were detected across 10 microsatellite loci, and 35 and 37 alleles were found in adults and saplings, respectively. Thirty-three alleles were found in both populations, while 2 were unique in adults and 4 were unique in saplings. Unique alleles found in saplings may be derived from absent adult trees, which were lost through logging or other causes. It is also possible that we were unable to make a complete inventory of all remaining adult trees in this area.

No difference between Kali Jati and Solok Besek populations was observed in levels of genetic variation in terms of number of alleles, allelic richness, and heterozygosity, within either adults or saplings or between adults and saplings for each population (Table 3). Previous population genetic studies of *Shorea leprosula* (Lee *et al.* 2000), *Primula vulgaris* (Van Geert *et al.* 2008), *Prunus africana* (Farwig *et al.* 2008), and *Vateriopsis seychellarum* (Finger *et al.* 2012) showed that the level of genetic variation was higher in adults than in saplings. This is possibly due to limited pollen and seed dispersal, as in many dipterocarp species (e.g. Osada *et* al. 2001, Kettle *et al.*

Table 3. Summary of genetic variation in adult and sapling populations

Population	*N*	*N*a	*A*r	H_o	H_e	*F*
Adults						
Kali Jati	11	2.90	2.71	0.382	0.393	-0. 005 ns
Solok Besek	7	3.30	3.30	0.586	0.454	-0.248 ns
Grand mean	9	3.10	3.00	0.484	0.423	-0.133
Saplings						
Kali Jati	53	3.70	3.31	0.415	0.456	0.068 **
Solok Besek	18	3.20	3.20	0.411	0.426	0.004 ns
Grand mean	36	3.45	3.25	0.413	0.441	0.038

Number of samples (*N*), mean number of alleles per population (*N*a), allelic richness (*A*r), mean observed heterozygosity (H_o), Nei's mean expected heterozygosity (H_e), and mean Fixation index (*F*) for the ten loci in two populations for adults and saplings of *D. littoralis*

2011, Finger *et al.* 2012). The equivalent level of genetic variation in adults and saplings may be explained by the limited number of samplings, even though all known remaining adults in this area were collected.

The inbreeding coefficient *F* was higher in saplings than in adults (Table 3). Values in adults were negative, but not significantly different from zero in both Kali Jati and Solok Besek, indicating no deviation from Hardy-Weinberg equilibrium. In contrast, values in sapling populations were both positive and significantly different from zero in Kali Jati ($P < 0.05$), suggesting frequent inbreeding and self-fertilization (Table 3). Relatively high inbreeding coefficients in seedling and sapling stages compared to adult stages have been found in many studies (e.g. Lee *et al.* 2000, Michalski & Durka 2007, Van Geert *et al.* 2008, Farwig *et al.* 2008), which is not surprising given that heterosis and inbreeding depression result in higher survival rates of heterozygotes (Alvarez-Buylla *et al.* 1996). However, since we collected 2-5 saplings aggregated around each mother trees, it is possible that positive values of *F* could be the result of collecting the cohort of half sibs and selfed progenies.

A small but significant F_{ST} (0.036, permutation test $P< 0.05$) was found across all loci between Kali Jati and Solok Besek sapling populations. The value for the adult populations (F_{ST} = 0.050) was however, not significant. The significant genetic differentiation in the sapling stage implies that gene dispersal has been comparatively limited between the two currently fragmented populations, since known pollinators (including thrips, beetles and honey bees) are not thought to cover large distances (Chan & Appanah 1980, Momose *et al.* 1998, Sakai *et al.* 1999a). No genetic differentiation between adult populations suggests that these two populations formerly formed parts of one panmictic population. Overlapping generations in adult populations also lead to little or no genetic differentiation among populations (Kalisz *et al.* 2001, Chung *et al.* 2003, Jones & Hubbell 2006, Van Geert *et al.* 2008). Many

dipterocarp species only flower every 2-10 years in immense synchronized flowerings across diverse plant families called general flowerings (Sakai 2002). However, even in the general flowering period not all conspecifics flower together, with flowering trees probably not exceeding 50% of the total (Sakai *et al.* 1999b). If this is also the case for *D. littoralis* in this region, effective population size of an age class may be much smaller than its actual size and could have accelerated the reduction of genetic variation in the sapling stage.

Implications for Conservation and Management

The pattern of population structure in *D. littoralis* has important conservation implications. For *D. littoralis*, the low genetic diversity associated with declining populations and the significance of genetic differentiation in sapling populations have been a consequence of geographical and topological isolation, limited gene flow, and habitat fragmentation. We propose that efforts toward conservation management should be aimed at preserving and increasing the size of current populations. Considering the significant genetic differentiation among populations present in sapling populations, extinction of any population may lead to a considerable loss of genetic variation. Thus, management schemes should involve all populations simultaneously.

The outcome of our study indicates urgency for both *in situ* and *ex situ* conservation. For *D. littoralis*, the latter approach must entail the collection from representative samples of individuals from all populations to ensure the availability of genetic resources for future use in programs of reintroduction or reinforcement. Like other dipterocarp species, *D. littoralis* seeds are recalcitrant and cannot easily be stored in conventional seed banks. *Ex situ* conservation will thus only be achieved through nursery grown seedling banks. Furthermore, new population should be also established at strategic locations on the Nusakambangan Island by transplanting seedlings taken from a mixture of the two extant populations. Such locations should consider the ecological context of the relatively short gene dispersal distances (mostly <50 m) and should thus be located close to and between remaining populations to enhance connectivity of existing populations (Finger *et al.* 2012).

Finally, further investigations should concentrate on life history characteristics, mechanisms for pollen/seed dispersal, seed germination, vegetative propagation and impacts from herbivores and pathogens. Those studies will be a principal step toward deepening our understanding of the causes for rarity when devising suitable conservation guidelines.

CONCLUSIONS

This study revealed that *D. littoralis* has low genetic diversity in both adults and saplings. Similarly low values in allele richness and heterozygosity suggest that reductions of population size have been ongoing for long periods in this species. Significant genetic differentiation between sapling populations but not in adult

populations indicates that fragmentation is further accelerating the isolation process. This knowledge is essential for developing management strategies for conservation of this rare endemic dipterocarp species.

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