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Comparison of the fine-scale genetic structure of three dipterocarp species

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We investigated the fine-scale genetic structure of three tropical-rainforest trees, *Hopea dryobalanoides*, *Shorea parvifolia* and *S. acuminata* (Dipterocarpaceae), in Peninsular Malaysia, all of which cooccurred within a 6-ha plot in Pasoh Forest Reserve. A significant genetic structure was found in *H. dryobalanoides*, weaker (but still significant) genetic structure in *S. parvifolia* and nonsignificant structure in *S. acuminata*. Seeds of all three species are wind dispersed, and their flowers are thought to be insect pollinated. The most obvious difference among these species is their height: *S. parvifolia* and *S. acuminata* are canopy species, whereas *H. dryobalanoides* is a subcanopy species. Clear differences were also found among these species in their range of seed dispersal, which depends on the height of the release point; so taller trees disperse their seed more extensively. The estimates of seed dispersal area were consistent with the degree of genetic structure found in the three species. Therefore, tree height probably had a strong influence on the fine-scale genetic structure of the three species.
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Introduction

Ecological geneticists have intensively discussed the establishment of the spatial genetic structure in plant populations and the hypothesis that limited gene dispersal should lead to genetic differentiation within a continuous population (Nason *et al*, 1997). In such cases, mating does not occur randomly at the whole-population scale, but is affected by the distance between individuals, according to the isolation-by-distance model (Wright, 1943). Limited gene dispersal can produce the fine-scale genetic structure even within a random mating population. The studies on the spatial genetic structure in temperate and tropical trees have detected a positive relationship between genetic relatedness and spatial distance, which could be explained by limited gene dispersal or the involvement, to some degree, of vegetative reproduction (Berg and Hamrick, 1995; Boshier *et al*, 1995; Alvarez-Buylla *et al*, 1996; Ueno *et al*, 2000). On the other hand, some studies have reported that several species have weak spatial genetic structure, due to extensive seed or pollen dispersal (Hamrick *et al*, 1993; Geburek and Trippknoeles, 1994; Doligez and Joly,

1997). The genetic structure is also influenced by gene dispersal mechanisms; the genetic structure of wind-dispersed species tends to be greater than that of animal-dispersed species (Hamrick *et al*, 1993, Loiselle *et al*, 1995, Hamrick and Nason, 1996). Thus, knowledge of the extent of gene dispersal is crucial to understanding genetic structure within plant populations.

Hopea dryobalanoides Miq. (Dipterocarpaceae) is a subcanopy tree widely distributed in Malaya, Sumatra and Borneo (Ashton, 1982). It has hermaphrodite flowers and is probably fly pollinated (Appanah, 1987). *Shorea parvifolia* Dyer and *S. acuminata* Dyer are canopy or emergent trees found in Malaya, Sumatra and Borneo (Ashton, 1982). Both *S. parvifolia* and *S. acuminata* have hermaphrodite flowers, and the main flower visitors are thrips and/or small beetles (Appanah, 1981). Seeds of all three species are dispersed by wind or gravity. These three species occur in sympatry in lowland dipterocarp forests and there is little difference in their density. The major difference among these three species is their height. The main objectives of this study were to compare the spatial genetic structure of the three dipterocarp species, and to evaluate the effect of seed dispersal distance on their genetic structure.

Materials and methods

Study site and sample collection
This study was conducted in Pasoh Forest Reserve in Peninsular Malaysia (2°58′N, 102°18′E, 75–150 m above sea level). The reserve is a lowland dipterocarp forest, characterized by high proportions of Dipterocarpaceae

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(Manokaran *et al*, 1992). A permanent 6-ha ecological research plot (200–300 m) was established in 1994, where the location and species of all individual trees, with a diameter at breast height (dbh) greater than 5 cm, have been recorded at least three times every 2 years to monitor growth, mortality and recruitment for population dynamic study.

In this study, leaves or inner bark samples were collected from all mapped individuals of *H. dryobalanoides* (64 trees), *S. parvifolia* (50 trees) and *S. acuminata* (46 trees) in the plot (Figure 1).

DNA extraction and microsatellite analysis

Total DNA was extracted from leaves or inner bark samples of each individual using a modified CTAB

method (Tsumura *et al*, 1996). Following crude DNA extraction, these DNA samples were purified with a FastDNA Kit (Bio 101, Inc.).

Nine primer pairs developed in *S. curtisii* (Ujino *et al*, 1998) and six developed in *Neobalanocarpus heimii* (Iwata *et al*, 2000) were tested, and suitable primer pairs were selected. Five primer pairs (*Shc*02, *Shc*09, *Nhe*004, *Nhe*005 and *Nhe*018) were used in *H. dryobalanoides*, four (*Shc*02, *Shc*03, *Shc*04 and *Nhe*05) in *S. parvifolia* and four (*Shc*03, *Shc*04, *Shc*09 and *Nhe*005) in *S. acuminata*. PCR reaction mixtures (10 μl) contained $1 \times$ PCR Buffer (Gibco BRL Co Ltd), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 200 nM of each primer (one of each pair was fluorescently labeled), 1 ng of template DNA and 0.25–0.5 U of *Taq* polymerase (Gibco BRL Co Ltd). PCR amplification was carried out for 3 min at 94°C, followed by 30 cycles of 30 s at 94°C,
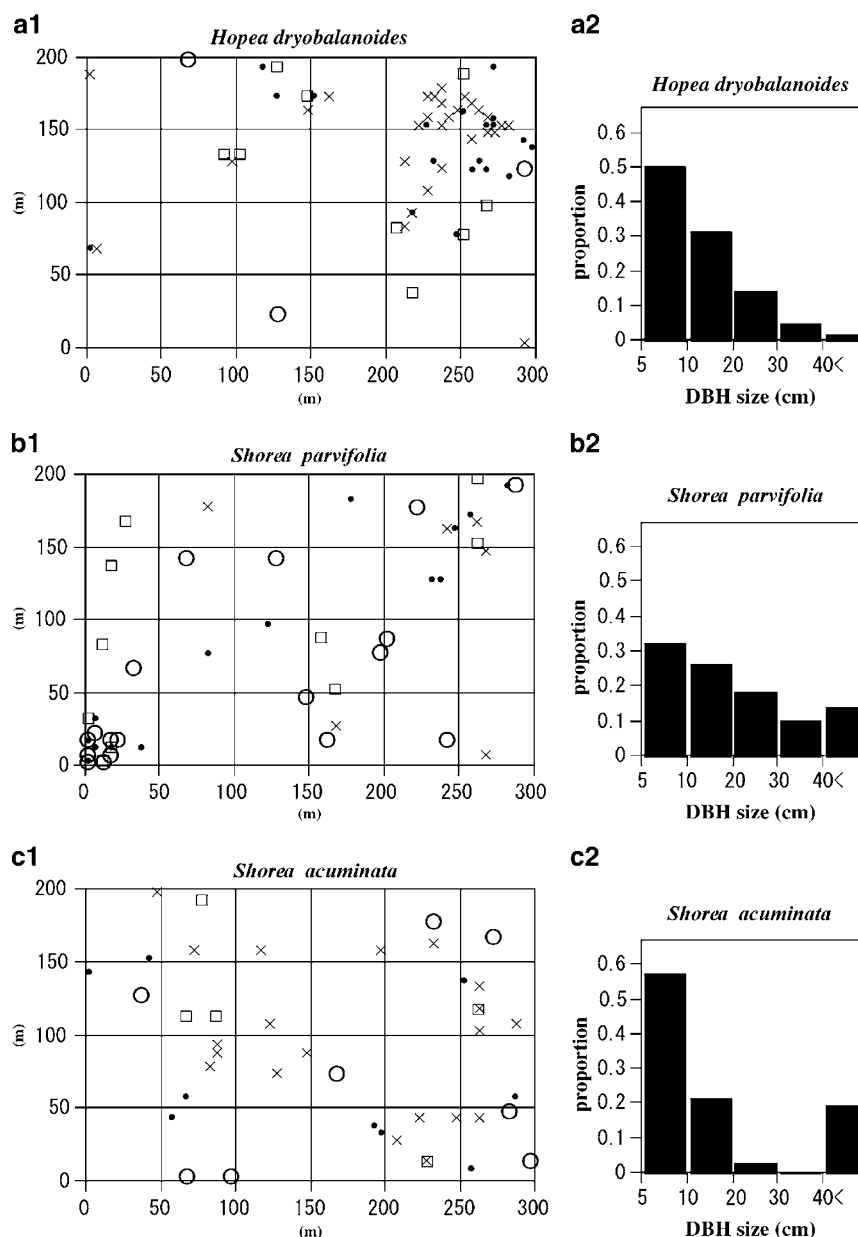
Figure 1 Spatial distribution of (**a-1**) *H. dryobalanoides*, (**b-1**) *S. parvifolia* and (**c-1**) *S. acuminata* in the 6-ha plot. The unfilled circles indicate trees with dbh >30 cm, squares are 20–30 cm, filled circles are 10–20 cm and crosses are 5–10 cm. dbh frequency classes are also shown for (**a-2**) *H. dryobalanoides*, (**b-2**) *S. parvifolia* and (**c-2**) *S. acuminata*.

30 s at the optimized annealing temperature and 30 s at 72°C, with a final 3 min incubation at 72°C, using a GeneAmp™ PCR System Model 9700 (PE Applied Biosystems). Genotypes were determined using an ABI 310 Genetic Analyzer and GeneScan™ software Ver. 2.0.1 (PE Applied Biosystems).

Data analysis

For each locus, the allele frequency and observed heterozygosity (H_o) were calculated. In addition, the expected heterozygosity (H_e), effective number of alleles [$N_e = 1/(1–H_e)$] and fixation index (F_{IS}) were estimated according to Nei (1987), and deviations from Hardy–Weinberg equilibrium were tested by the method of Li and Horvitz (1953).

Genetic relatedness between all individuals of each species was calculated using Relatedness Software, version 5.0.8 (Goodnight, 2001) based on Queller and Goodnight (1989). The spatial distances were also calculated between pairs of all individuals of each species. The statistical test for correlation between the genetic relatedness and spatial distances among all individuals was performed using The R Package for Multivariate and Spatial Analysis version 4.0 (Casgrain and Legendre, 2000) with 5000 randomizations, which is a realistic minimum for establishing a significance level of about 0.01 (Manly, 1997). Genetic clustering would be expected when this correlation is significant.

Morishita's index of dispersion (I_δ) was calculated to assess the spatial distribution of individuals of the three dipterocarp species (Morishita, 1959). If individuals are randomly distributed over the plot, $I_\delta = 1$; if individuals are patchily distributed, $I_\delta > 1$; and if individuals are uniformly distributed, $I_\delta < 1$. The significance of the departure from randomness was tested using F-statistics.

Estimation of seed dispersal distance

Generally, the seed dispersal distance of wind-dispersed species is determined by three parameters: wind speed, tree height and terminal velocity of the seed (Green and Johnson, 1989)

$$X = \sum \frac{u(H)}{F(H)}$$

where $u(H)$ is wind speed at height H meters and $F(H)$ is terminal velocity of the seed at height H meters. The measured terminal velocities of falling *S. parvifolia* and *S. acuminata* seeds were used as the $F(H)$ values, while the measured terminal velocity of *H. mengarawan* seeds was used for *H. dryobalanoides*, two related species with similar seed form (Ashton, 1982).

Wind speed varies depending on the vertical distance from the ground, so when estimating seed dispersal distances the vertical variation in wind speed was expressed as a function of height, using wind speed data collected at Pasoh (not shown):

$$fu(H) = u_{53}(0.45H - 1.385)$$

$$(40 < \text{H } 53)$$

$$fu(H) = u_{40} \exp^{(-0.04(40-H))} 0$$

$(0 < \text{H } 40)$ where $fu(H)$ is wind speed at height H meters, while u_{40} and u_{53} are the wind speeds at 40 and 53 m, respectively.

Results

Spatial distribution patterns of individuals

Individuals of all three dipterocarp species examined in this study exhibited clumped distributions at all quadrat sizes, according to Morishita's index of dispersion (Table 1). For *H. dryobalanoides* and *S. parvifolia*, clumping was strong and significant, but although there were indications of a clumped distribution in *S. acuminta*, it was not statistically significant (Table 1).

Genetic diversity

In *H. dryobalanoides*, PCR products were successfully detected for all individuals at five loci: *Shc*02, *Shc*09, *Nhe*04, *Nhe*05 and *Nhe*18. The expected heterozygosity (H_e) ranged from 0.54 to 0.76, with the number of alleles ranging from three to nine (Table 2a). For *S. parvifolia*, PCR products were obtained for all individuals at five microsatellite loci: *Shc*02, *Shc*03, *Shc*04, *Shc*09 and *Nhe*04. The expected heterozygosity (H_e) ranged from 0.33 to 0.85, with the number of alleles ranging from four to 18 (Table 2b). For *S. acuminata,* PCR products were successfully detected for all individuals at four microsite loci: *Shc*03, *Shc*04, *Shc*09 and *Nhe*04. The expected heterozygosity (H_e) ranged from 0.42 to 0.79, with the number of alleles ranging from three to 10 (Table 2c).

Significant deviations from Hardy–Weinberg equilibrium were found at three loci (*Nhe*04, *Nhe*05 and *Shc*09) in *H. dryobalanoides*, at two loci (*Shc*04 and *Shc*09) in *S. parvifolia* and at one locus (*Shc*03) in *S. acuminata*. The deviations may have resulted from bias due to mis-amplification of loci caused by null alleles, leading to an apparent excess of homozygosity (Marshall *et al*, 1998).

Spatial genetic structure

The relationship between spatial distance and genetic relatedness is shown in Figure 2. When strong genetic structure within populations is present, a negative correlation with high *r*-values would be expected between these parameters. Significant, negative correlation was detected in *H. dryobalanoides* ($r = -0.301$, $P < 0.01$; Figure 2a) and *S. parvifolia* ($r = -0.149$, $P < 0.05$; Figure 2b), but the correlation was not significant in *S. acuminata* ($r = -0.050$, $P > 0.05$; Figure 2c). The results indicate that the level of genetic structure differed among the three species at the study site.

Seed dispersal of three dipterocarps

The estimated seed-dispersal distances for the three dipterocarp species differed (Figure 3), and corresponded with the order of degree of genetic structuring detected in them. The seed-dispersal distance would strongly correlate with the degree of genetic structures of the three species.

Discussion

A significant genetic structure was found in *H. dryobalanoides* and *S. parvifolia*, but not in *S. acuminata*. Notably, the genetic structure of *H. dryobalanoides* was greater than that of the two *Shorea* species (Figure 2). Since the study site appears to have no clear microhabitat heterogeneity, the difference in the degree of genetic structure among

a *Hopea dryobalanoides*

b *Shorea parvifolia*

c *Shorea acuminata*
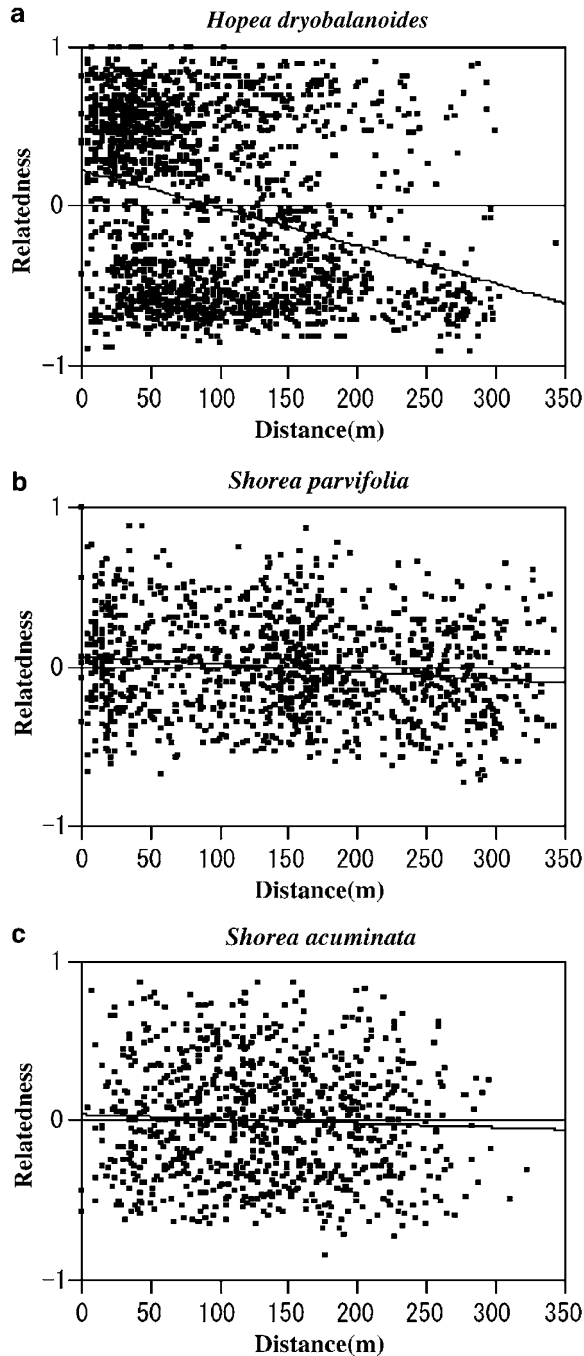
Figure 2 Relationship between spatial distance and relatedness for: (a) *H. dryobalanoides*, (b) *S. parvifolia* and (c) *S. acuminata*. The correlation is significant and negative in *H. dryobalanoides* and *S. parvifolia* (*H. dryobalanoides*, $r = -0.301$, $P < 0.01$; *S. parvifolia*, $r = -0.149$, $P < 0.05$; 5000 permutations, Mantel test (Mantel, 1967)), but it is not statistically significant in *S. acuminata* ($r = -0.0504$, $P > 0.05$, 5000 permutations, Mantel test).

these species is probably due to differences in gene dispersal, rather than selection.

The seeds of all three species are dispersed by wind or gravity, so the most obvious differences with respect to seed dispersal among these species are related to tree height. *S. parvifolia* and *S. acuminata* are canopy species, whereas *H. dryobalanoides* is a subcanopy species and the adult *Shorea* trees are 20 m or more taller on average than

a *Hopea sp.*

b *Shorea parvifolia*

c *Shorea acuminata*
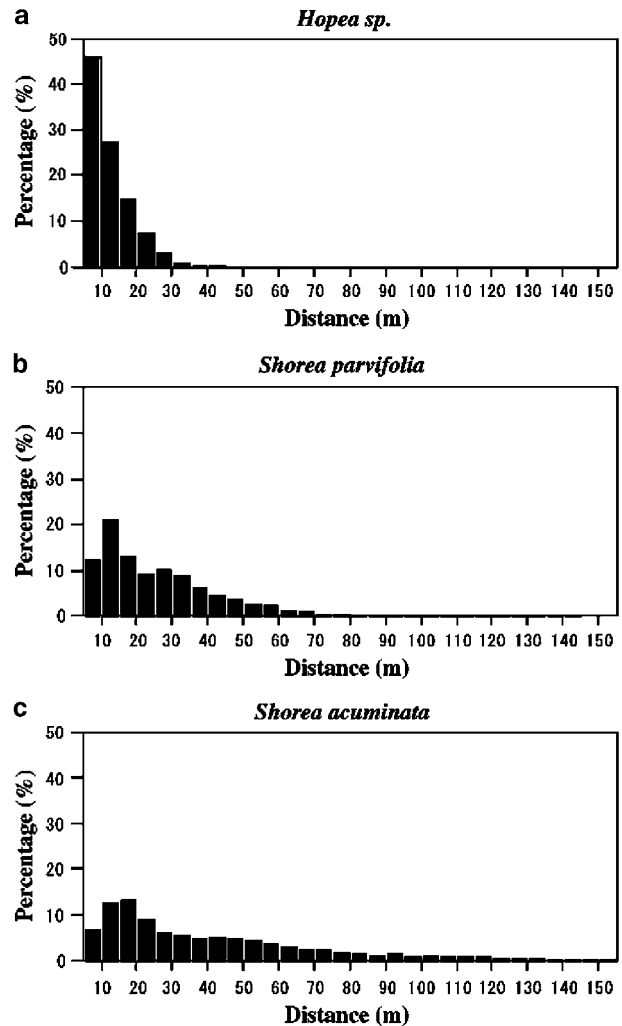
Figure 3 Estimated seed dispersal distance of: (a) *Hopea* sp (b) *S. parvifolia* and (c) *S. acuminata*. The horizontal axis shows the estimated seed dispersal distances, while the vertical axis is the frequency of each class as a proportion of the total. The maximum seed dispersal distance was about 500 m in *S. acuminata* (not shown).

the *Hopea* trees. This suggests that the seed shadows differ among these species since it depends on the height of the release point; taller trees can disperse their seed more extensively. The seed dispersal distance for three dipterocarps clearly indicates the difference of the seed shadows, which may be a major contributory factor to differences of the spatial genetic structure among the species.

Generally, the main causes of clumped distribution patterns within a forest are related to seed dispersal patterns and/or habitat heterogeneity (Manabe and Yamamoto, 1997). The results of the spatial distribution pattern analysis of these three dipterocarp species were consistent with the estimates of their seed dispersal distances (Table 1). Clumping may affect pollinator behavior, resulting in nearest-neighbor pollination or inbreeding (Levin, 1984), and it tends to promote the genetic structure. A simulation study has also shown that the presence of strong clumping leads to a slight increase in spatial autocorrelation, caused by limited dispersal of seed and pollen (Doligez et al, 1998).

The genetic structure of adult trees (dbh >30 cm) of *N. heimii* in a 42-ha plot (Konuma *et al*, 2000) was also significant, with a negative correlation between relatedness and spatial distance ($r = -0.228$). Thus, it seems that the degree of genetic structure in *N. heimii* is stronger than that of the two *Shorea* spp but weaker than that of *H. dryobalanoides*, because *N. heimii* is also an emergent tree, and seed dispersal distance is limited to a radius of around 20 m (Konuma *et al*, 2000). Boshier *et al* (1995) reported that *Cordia alliodora*, a neotropical tree with a range of seed dispersal limited to ca. 20 m, showed a positive relationship between genetic relatedness and spatial proximity. Another study by Hamrick *et al* (1993) showed that the genetic structure of wind-dispersed species was stronger than that of animal-dispersed species. Thus, all of these studies showed that the seed dispersal distance affects the spatial genetic structure, which is consistent with our results.

Pollen flow is another important factor that affects the genetic structure (Loveless and Hamrick, 1984), since extensive pollen flow results in weak genetic structure. This has already been confirmed for several tropical species (Doligez and Joly, 1997; Konuma *et al*, 2000). Unfortunately, the pollen flow distances of the three species analyzed in the present study are not known. The only relevant reports so far suggest that the main pollinators are thrips and/or small beetles in *Shorea* spp, and likely flies in *H. dryobalanoides* (Appanah, 1987). To assess the effect of pollen flow on the genetic structure of these three species, we need to estimate the pollen flow distances when flowering occurs. Finally, we would understand how the contribute to pollen flow and seed dispersal for the spatial genetic structure of each species in this plot.

We also found that the genetic structure of *S. parvifolia* was stronger than that of *S. acuminata*, which may be due to differences in self-compatibility between the two species, since self-incompatibility has been reported in *S. acuminata* (Chan, 1981). The breeding system is an important factor affecting the genetic structure in plant populations (Nason *et al*, 1997), and outcrossing would tend to decrease the genetic structure (Loveless and Hamrick, 1984). To assess the effect of self-incompatibility on the genetic structures in these three species, it will be necessary to investigate their breeding systems, especially their respective degrees of self-incompatibility.

Tropical forests in Peninsular Malaysia have become increasingly affected by human activity, especially selective logging and issues related to the conservation and sustainable use of the tropical forests are increasing in importance (Ratnam *et al*, 1999). However, there is a scarcity of relevant data for developing sustainable forest management guidelines and effective conservation strategies such as selective logging. Obayashi *et al* (2002) reported that there has been a considerable increase in the selfing rate in a logged forest of *S. curtisii*, although there was no difference in the genetic diversity of adult trees between the primary and the logged forest. In other *Shorea* sp, there have been suggestions that inbreeding is increased in logged forest (Murawski *et al*, 1994). In this study, we found that each species has a different level of within-population genetic structure. If a population with strong genetic structure is conducted selective logging without consideration of its structure, probability of mating with relatives may increase. Studies on genetic structure provide not only fundamental data on forest genetics but also essential information for understanding tropical trees and developing more effective and sensitive ways to exploit them.

Table 2 Profiles of microsatellite loci detected in (a) *H. dryobalanoides*, 64 trees, (b) *S. parvifolia*, 50 trees, and (c) *S. acuminata*, 46 trees

Locus	N_a	H_o	H_e	F_{IS}
(a) H. dryobalanoides				
*Nhe*04	4	0.60	0.67	0.10**
*Nhe*05	3	0.67	0.54	−0.24**
*Nhe*18	9	0.69	0.76	0.09
*Shc*02	7	0.70	0.75	0.07
*Shc*09	5	0.56	0.67	0.15**
(b) S. parvifolia				
*Nhe*04	4	0.38	0.46	0.19
*Shc*02	6	0.34	0.33	−0.03
*Shc*03	5	0.38	0.47	0.19
*Shc*04	14	0.48	0.85	0.43**
*Shc*09	18	0.60	0.84	0.28**
(c) S. acuminata				
*Nhe*04	4	0.33	0.42	0.21
*Shc*03	3	0.33	0.55	0.39**
*Shc*04	10	0.66	0.79	0.17
*Shc*09	10	0.76	0.76	0.00

The number of alleles detected (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), and fixation index (F_{IS}) are shown. The deviations from Hardy–Weinberg equilibrium were assessed using a χ^2 goodness-of-fit test.
**$P < 0.01$.

Table 1 Values of Morishita's index of distribution (I_δ) for *H. dryobalanoides*, *S. parvifolia*, and *S. acuminata* (dbh ⩽5 cm)

Quadrat size (m²)	Number of quadrat in 6 ha	Hopea dryobalanoides		Shorea parvifolia		Shorea acuminata	
		I_δ	F	I_δ	F	I_δ	F
150 × 100	4	1.88	19.38***	1.33	6.62***	0.97	0.48
100 × 100	6	2.53	20.31***	1.69	8.02***	1.01	1.09
50 × 50	24	2.64	5.5***	4.24	8.17***	1.22	1.44
25 × 25	96	4.86	3.56***	9.41	5.52***	1.15	1.07

If individuals are randomly distributed over the plot, $I_\delta = 1$; if individuals are patchily distributed, $I_\delta > 1$; and if individuals are uniformly distributed, $I_\delta < 1$.
The significance was tested by calculating F-statistics.
***$P < 0.001$.

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