MICROSATELLITE ANALYSIS OF THE BREEDING SYSTEM AND SEED DISPERSAL IN 
SHOREA LEPROSULA (DIPTEROCARPACEAE)

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To understand the breeding system and seed dispersal in Shorea leprosula (Dipterocarpaceae) in Peninsular 
Malaysia, a microsatellite analysis was conducted of embryos of immature and mature fruits fallen in litter 
traps under the crowns of five trees. Outcrossed and selfed progeny mothered by the trees and those dispersed 
from other trees were distinguished by genotypes of three polymorphic microsatellite loci. The mean outcrossing 
rate of mature fruit embryos in S. leprosula pollinated by thrips (0.91) was not lower than those previously 
reported from Shorea species pollinated by bees, even though thrips seem to be less efficient pollinators than 
bees. Although four of the five trees showed high and stable outcrossing rates during fruit maturation, the 
outcrossing rate increased in one tree with highly selfed embryos of immature fruits. These results suggest 
that inbreeding depression during fruit maturation as well as self-incompatibility reduce the proportion of 
inbred embryos. The proportion of fruits dispersed from neighbor trees in fruits trapped under a tree crown 
had a mean value of 0.20 and was lowest under the tree with highly selfed embryos of immature fruits. This 
low fraction of dispersed fruits under this tree suggests long distances from this tree to reproductive neighbors, 
which may reduce cross pollination.

Keywords: fruit abortion, Malaysia, outcrossing rate, Pasoh, SSR.

Introduction

The genus Shorea (Dipterocarpaceae) contains about 350 spe-
cies, which are dominant emergent trees in tropical Asia (Ashton 
1982). Pollinators of Shorea are known to be thrips, beetles, 
and bees. Appanah and Chan (1981) showed that species of 
Shorea section Mutica were pollinated by thrips in Peninsular 
Malaysia. However, chrysomelid beetles pollinated these species 
in Sarawak (Sakai et al. 1998). Honeybees and small social bees 
were pollinators of three species of Shorea in rain forests of 
Sri Lanka (Dayanandan et al. 1990). In dry, deciduous forests in 
Thailand, stingless bees pollinated Shorea siamensis (Ghazoul 
et al. 1998). In spite of the diversity of these insect pollinators, 
matings systems have been investigated only in bee-pollinated 
species. The outcrossing rates of the three Shorea species in Sri 
Lanka ranged from 0.54 to 0.87 (Murawski et al. 1994a, 
1994b). Because thrips and beetles seem to be less effective pol-
linators than bees, a lower outcrossing rate would be expected 
in species of Shorea section Mutica in Malaysia. Some isozyme 
analyses of thrips-pollinated Shorea populations indicated that 
genetic variation was so high that random mating could be 
assumed (Gan et al. 1977, 1981). However, the outcrossing rates of 
these populations had not been reported.

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Manuscript received March 2000; revised manuscript received July 2000.
curtissii, and the markers can be applied to most species of the genus Shorea (Ujino et al. 1998). In this study, we investigated the breeding system and seed dispersal using microsatellite analysis of embryos of immature and mature fruits caught in litter traps under crowns of a thrips-pollinated species of Shorea section Mutica, Shorea leprosula.

Material and Methods

This study was conducted in Pasoh Forest Reserve in Malaysia (2°58’N, 102°18’E; 75–150 m above sea level). The dominant vegetation type in the reserve is lowland dipterocarp forest, characterized by a high proportion of Dipterocarpaceae. Shorea leprosula Miq. is a common species in the study site, and 254 trees of this species with ≥10 cm diameter at breast height were found in the 50-ha plot near the study site (Kochummen 1997). Shorea leprosula is pollinated by thrips (Appanah and Chan 1981), and its winged fruits are wind dispersed by autogyration (Ashton 1982).

Fruit Maturation

We investigated five emergent trees (trees 117, 196, 226, 241, and 256) of S. leprosula in 1996 (fig. 1). These trees bloomed in early June 1996. Under the crowns of the five trees, we set five litter traps made of fine nylon mesh (circles with 1 m diameter) for each tree. Every week from June 10, 1996, we counted fruits caught in the seed traps and randomly selected 20 fruits for measurement of dry weights. For the genetic analysis, we collected progeny in different maturation stages. Immature fruits were collected from the litter traps from July 22 to August 5, and mature ones were collected from September 4 to September 23. In order to examine the genotypes of the five trees from which the fruits were collected, inner bark of the trees was sampled.

Microsatellite Genotyping

DNA was extracted from embryos of the collected fruits using the modified CTAB method (Murray and Thompson 1980). Although each flower of S. leprosula has six ovules, each collected fruit has a single embryo (Swarupanandan 1986). DNA of the sampled inner bark was also extracted by the same method. Nine microsatellite markers developed in Shorea curtissii were examined by PCR amplification using assigned primer sets (Ujino et al. 1998). The reaction mixtures (20 μL) contained 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM MgCl2, 0.16 mM each dNTP, 160 nM fluorescently labeled forward primer and nonlabeled reverse primer, 10 ng of template DNA, and 0.6 units of Taq polymerase (Gibco BRL). PCR amplification consisted of 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 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 9600 (Perkin-Elmer ABI, Foster City, Calif.). Amplified DNA fragments were analyzed by an ABI 310 Genetic Analyzer, and each fragment size was determined by the GeneScan program (Perkin-Elmer ABI). Three microsatellite loci, Shc03, Shc07, and Shec09, were successfully amplified from the extracted DNA of S. leprosula, and genotypes of the three loci were determined.

Fig. 1 Map of the study site in Pasoh Forest Reserve and the locations of five investigated trees of Shorea leprosula (closed circles).

Data Analysis

Multilocus genotypes of each tree from which fruits were collected (called the “focal trees” below) were determined from inner bark samples. Otherwise, the genotype of a focal tree was inferred from the genotype of embryos in the collected fruits. If the estimated frequency of a single allele was significantly greater than 0.5 in collected samples (χ² test), the focal tree was considered to be homozygous. Otherwise, it was considered to be heterozygous. The homozygous locus of the focal tree was assumed to contain the most frequent allele, and the heterozygous locus was assumed to contain the two most frequent alleles in the sampled embryos. Embryos that did not share any alleles with a focal tree in at least one locus were regarded as progeny dispersed from trees other than the focal tree. Among the offspring of a focal tree, embryos that had an allele that was not present in the focal tree in at least one locus were classified as outcrossed progeny. Embryos that had only alleles of the focal tree in all loci were classified as selfed progeny.

The outcrossing rate was defined as the proportion of outcrossed progeny in offspring mothered by a focal tree. The dispersal rate was defined as the proportion of progeny dispersed from trees other than the focal tree in offspring collected from the litter traps under the focal tree. Differences in both rates between fruit maturation stages and among focal trees were tested by χ² test or Fisher’s exact probability test.

Results

Fruit Maturation

Flowers of five Shorea leprosula trees (117, 196, 226, 241, and 256) were observed on June 10, 1996. On June 16, flowers were found on only two trees (196 and 226), and on June 24, no flowers were observed. After the end of flowering, the num-
Fig. 2 Temporal changes in (a) the number of fruits fallen in a litter trap and (b) the dry weight of sampled fruits of *Shorea leprosula*. Five trees are shown by different symbols. The two shaded areas show the periods when immature fruits (left: from July 22 to August 5) and mature fruits (right: from September 4 to September 23) were collected.

**Microsatellite Genotyping**

The three microsatellite loci were highly polymorphic, and a total of 41 alleles was detected from progeny samples (table 1). The mean expected heterozygosity was 0.664, which is similar to that observed in *Shorea curtisii* (Ujino et al. 1998). There were no significant deviations from Hardy-Weinberg expectations for the $F_{IS}$ values in any of the loci. The paternity exclusion rate for the three loci was 0.927.

**Outcrossing and Dispersal Rates**

Multilocus genotypes of two trees (226 and 241) were determined from DNA of inner bark samples, although those of the remaining trees (117, 196, and 236) were not determined because the expected microsatellites could not be amplified from these samples. Polyphenols and many other secondary metabolites in the inner barks were likely to obstruct DNA isolation by the standard CTAB method. All five of the trees were regarded to have the multilocus genotypes shown in table 2. In trees 226 and 241, all the inferred genotypes, except for a genotype in *Shc09* of tree 241 (177/185), were identical to the genotypes determined from DNA of inner bark samples. Based on these tree genotypes, the outcrossing rate and the dispersal rate were obtained.

The mean outcrossing rate of the five trees in the mature fruit stage was 0.91 (table 2). The outcrossing rate in the total samples of the immature fruits did not differ from that of the mature fruits ($\chi^2 = 1.55, df = 1, P = 0.213$). The outcrossing rates were different among the five trees ($\chi^2 = 20.16, df = 4, P < 0.001$), and the outcrossing rate in tree 226 was lowest. In this tree, the outcrossing rate significantly increased from 0.27 in the immature fruit stage to 0.77 in the mature fruit stage (Fisher's exact probability test: $P = 0.004$).

The mean dispersal rate in the mature fruit stage was 0.20 (table 2). The dispersal rate in the total samples of immature fruits was not different from that of the mature fruits ($\chi^2 = 0.16, df = 1, P = 0.691$). The dispersal rates differed among the five trees ($\chi^2 = 16.18, df = 4, P = 0.003$), and the dispersal rate in tree 226 was lowest. There were no differences in the dispersal rates in any trees between the immature and mature fruit stages (Fisher's exact probability test: $P > 0.069$).

**Discussion**

The outcrossing rates of mature fruit embryos of *Shorea leprosula* ranged from 0.77 to 1.00, which are similar to multilocus outcrossing estimates for two *Shorea* species, *Shorea congestiflora* (0.87) and *Shorea megistophylla* (0.71–0.87), but are higher than those of *Shorea trapezifolia* (0.54–0.62) (Murawski et al. 1994a, 1994b). Honeybees and small social bees pollinate these *Shorea* species, whereas thrips are pollinators.

**Table 1**

<table>
<thead>
<tr>
<th>Locus</th>
<th>No. of alleles</th>
<th>Heterozygosity</th>
<th>Fixation index</th>
<th>Paternity exclusion rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shc03</td>
<td>317</td>
<td>5</td>
<td>0.568</td>
<td>0.548</td>
</tr>
<tr>
<td>Shc07</td>
<td>336</td>
<td>25</td>
<td>0.795</td>
<td>0.889</td>
</tr>
<tr>
<td>Shc09</td>
<td>293</td>
<td>11</td>
<td>0.614</td>
<td>0.555</td>
</tr>
<tr>
<td>Mean</td>
<td>14</td>
<td>11</td>
<td>0.659</td>
<td>0.664</td>
</tr>
<tr>
<td>Multiple loci</td>
<td>14</td>
<td>11</td>
<td>0.659</td>
<td>0.664</td>
</tr>
</tbody>
</table>
of *Shorea leprosula* in the study site (Appanah and Chan 1981; Dayanandan et al. 1990). Thrips seem to be less effective pollinators than bees based on the expected differences in flight distance, frequency of movement between flowers, and pollen load on individuals. However, the outcrossing rate of thrips-pollinated *S. leprosula* was equal to or higher than those of the bee-pollinated species. This result suggests that the mating opportunity of reproductive trees and/or postpollination selection against selfing are high in *S. leprosula*. The relatively high density of large trees distributed randomly or regularly in the adjacent 50-ha plot (154 trees with >30 cm diameter at breast height) provides high mating opportunity of most adult trees (Okuda et al. 1997). The mass flowering in the study year (a general flowering event after a 7-yr interval) may also have increased the mating opportunity of *S. leprosula* (Yasuda et al. 1999).

Postpollination selection against inbreeding consists of self-incompatibility in the fertilization process and inbreeding depression during fruit maturation. Self-incompatibility is further divided into pre- and postfertilization self-incompatibility. Postfertilization self-incompatibility, which is often called “late-acting self-incompatibility,” has been observed in diverse tropical plants (Seavey and Bawa 1986). However, late-acting self-incompatibility is difficult to distinguish from inbreeding depression in the early stage of embryo development. In the genus *Shorea* section *Mutica*, previously conducted pollination experiments revealed that nearly all (>0.97) fruits in a self-pollination treatment were aborted within 30 d after pollination, while fruit sets in a cross-pollination treatment ranged from 0.02 to 0.42 in this period (Chan 1981; Sakai et al. 1998). These results suggest that self-incompatibility and/or inbreeding depression acting within a month after pollination nearly exclude selfed progeny. In this study, the mean outcrossing rate 40–50 d after flowering was high (0.77) and was not different from that in the mature fruit stage (0.91). The present result also supports the hypothesis that there is comprehensive selection against inbreeding within a month after flowering.

In contrast to this overall trend, the outcrossing rate in one of the investigated trees (226) increased during fruit maturation, which followed a month after flowering. This result suggests the presence of inbreeding depression during the latter part of fruit maturation, which was not clearly demonstrated by the previous pollination experiments because few fruits in the self-pollination treatments remained in this period. Furthermore, this result indicates that the outcrossing rates in the mother trees vary more in the immature fruit stage than in the mature fruit stage, which is probably due to variation in the frequency of cross pollination and/or the intensity of self-incompatibility. Such variation in the outcrossing rates among mother trees has been observed in *Shorea* species in Sri Lanka (Murawski et al. 1994a). Murawski et al. (1994b) showed that a part of this variation is due to the distances between neighboring reproductive trees, which affect the frequency of cross pollination (Ghazoul et al. 1998). In this study, the distances from the investigated trees to reproductive neighbors were not measured. However, the proportion of trapped fruits that were dispersed from other trees (the dispersal rate) may provide an estimate of the distances to reproductive neighbors because these fruits are wind dispersed by autogamy. The largest distances from tree 226 to reproductive neighbors estimated from the lowest dispersal rate may cause the lowest outcrossing rate due to the reduced frequency of cross pollination.

**Acknowledgments**

We thank Dr. Akihiro Konuma (National Institute of Environmental Science) and the staff of the Forest Research Institute Malaysia for helping in the sample collection. This study is part of a joint research project between the Forest Research Institute of Malaysia, Universiti Putera Malaysia, and the National Institute for Environmental Studies, Japan (grant E-2 from the Global Environment Research Program, Japan Environment Agency). The Japan Science and Technology Corporation supported the research work of T. Nagamitsu and S. Ichikawa.

**Literature Cited**


