E-1 DETERMINING INDICATORS FOR SUSTAINABLE MANAGEMENT OF TROPICAL FOREST

(3) Study on genetic relationship between individuals in tropical rainforest.

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Abstract

Microsatellite markers were developed from *Shorea curtisii* using. Analysis of 40 individuals of *S.curtisii* from natural forest in Malaysia revealed that all SSR loci were polymorphic. Four SSR markers, *Shc*01, *Shc*04, *Shc*07, and *Shc*09 showed highly polymorphic. We have also compared the genetic diversity between three populations with the different generattions with DBH>5cm, 1cm>DBH>5cm and seedlings of 1 year old. The genetic diversity of seedling population was not high comaring to those of the other generations. The limited number of parent trees, therefore, might be contributed to the next generations. We have also tested the applicability of these SSR primers to other Dipterocarpaceae species using PCR amplification. Since the flanking region sequences of the *S.curtisii* SSR were well conserved within this family, the SSR primers for *S.curtisii* can be applied to almost all Dipterocarpaceae species. The average of outcrossing rate in the trees of natural forest was 86.4 % and showed similar tendency of the other dipterocarps species. We also investigated five individuals in logged forest to clarify the genetic diversity and inbreeding rate. The inbreeding rate was quite different in each mother tree and ranged from 21.2 to 85.7 %. with the average of 53.6 %. The inbreeding rate between natural and logged forests was also quite different.

Key Words Dipterocarpaceae, microsatellites, *Shorea curtisii*, Genetic diversity, outcrossing rate

introduction

Shorea curtisii is a species of the Dipterocarpaceae: a family that includes major component species of the tropical forests of South East Asia. Although the family is important in tropical forestry and ecosystems, genetic information about member species is limited. Therefore, to determine genetic diversity and structure in *S.curtisii* and related species, we planned to develop a series of SSR markers.

Several improvements in methodology have been reported recently for efficient development of SSR markers (Ostrander *et al.*, 1992; Karagyozov *et al.*, 1993; Lyall *et al.*, 1993; Cifarelli *et al.*, 1995; Kirkpatrick *et al.*, 1995; Takahashi *et al.*, 1996), one of which is the vectorette PCR strategy (Lench *et al.*, 1994). The procedure relies on PCR amplification using a vectorette specific primer in combination with anchored dinucleotide repeat primers. in this case, we used genomic library DNA sequences as templates. Anchor primers consist of tandem repeat sequences and possible 3' anchor nucleotide sequences. If an SSR region occurs in the insert DNA, it can be amplified when the 3' nucleotide sequence flanking the SSR locus matches the anchor nucleotide of the primer. This allows specific SSR primers to

be designed, which can be used to re-amplify the vectorette libraries in combination with the other side of the vectorette primer.

In this study, SSR markers of *S.curtisii* were developed using a vectorette PCR method and a general method involving screening by colony hybridization. The applicability of these primers to other species of the Dipterocarpaceae was also examined.

Materials and Methods

Plant materials

Leaves of *S.curtisii* were collected at the research plot at Semangkok Forest Reserve in Serangor, Malaysia (Niiyama et al. in press). The leaves of various other Dipterocarpaceae species were collected from the aboretum of the Forest Research institute of Malaysia (FRIM). Total DNA was extracted from leaves of each individual by a slightly modified CTAB method (Tsumura *et al.*, 1996).

Study plots

Research plots have been constructed in Semangok forest reserve. This forest is a typical hill forest in Peninsula Malaysia, and *Shorea curtisii* is dominat species in this reigion. Two kinds of research plots have been constructed in this forest, which are 6 ha natural and 4 ha logged forests. Selective cutting was conducted in 1988 and the proportion was 30 % of the total wood volume of *S. curtisii*. Species identification and locations of all trees more than 5 cm diameter of breath hight (DBH 1.2m) in both plots have been confirmed. We collected leaves or inner barks from individuals of *S. curtisii* with 20 cm DBH in the both plots (Niiyama et al. 1993). General flowering have occurred at this forest in 1998, and *S. curtisii* also had a lot of fruits on August to September in this year. We also collected the seeds from ten and five individuals of natural and logged plots, respectively to survey the inbreeding rate in each tree.

Screening the Genomic DNA library

The genomic DNA samples were digested with *Sau*3A I, *Hae*III, *Alu*I, and *Rsa*I, and fragments of 300 to 500bp were fractionated. The DNA fragments were ligated into pUC18, and the ligation mixtures were then transformed into *E.coli*, JM109. Colony hybridization was carried out using a DIG detection kit (Boehringer Co. Ltd.) according to the manufacturer's instructions, except that 6×SSC was used in the hybridization buffer. Two oligonucleotides, (CA)15 and (CT)15, were used as probes to survey SSR sequences. The hybridization temperature was 64º C for each probe. Positive clones were isolated and were subjected to DNA sequencing, using an ABI 377 automatic sequencer, according to the manufacturer's instructions (Perkin-Elmer ABI Co. Ltd.).

Vectorette PCR

This procedure was mainly based on the protocol described by Lench et al (1994). Genomic DNA of *S.curtisii* was digested with a mixture of restriction enzymes including *Eco*RI, *Eco*RV, *Xba*I, and *Xho*I. Digested fragments between 500bp and 2500bp in size were fractionated. Fragments were then repaired by the Klenow method to make the ends blunt, and were ligated to pUC18. The ligation mixtures were transformed into *E.coli* JM109. The transformants were incubated on LB agar plates overnight at 37º C, after which five ml of LB medium was added to the surface of each plate. The plates were shaken gently for 30 min to recover cell suspensions. The cell suspensions were incubated at 37º C for a further 30 min. The DNA mixture of this genomic DNA library was prepared from the cell suspensions by the alkaline lysis procedure.

Vectorette PCR was performed in 25ul reaction volumes containing 10ng DNA from the genomic DNA library, and 0.128uM universal or reversal vectorette primer for pUC18 (primer 1; CCCAG TCAGC AGCTT GT or primer 3; GGAAA CAGCT ATGAC CATG, Nippon Gene Co.) in combination with each one (separately) of the 12 anchored dinucleotide repeat primers; (CT)10A, (CT)10T, (CT)10G, (CT)10CA, (CT)10CG, (CT)10CC, (CA)10A, (CA)10T, (CA)10G, (CA)10CT, (CA)10CG, (CA)10CC. Reaction mixtures were denatured at 94º C for 3min, followed by 34 cycles of amplification consisting of: 94º C for 30s, 55º C for 30s, and 72º C for 30s. Amplified fragments were then fractionated in 2% agarose gel. When a single fragment was observed, the PCR products were purified by Suprec-02 columns (TAKARA Co). When multiple fragments were observed, individual fragments were picked up from the gel by pipette tip. The tips were washed in PCR reaction mixture directly, and a single fragment was obtained through one more PCR cycle. The isolated fragments, including SSR flanking regions were sequenced. Specific upward primers were designed for the flanking regions of each SSR based on the sequence data with the aid of the Oligo program (version 4.0 National Bioscience). Using the forward primers in combination with the other side of the vectorette primers, vectorette libraries were re-amplified by PCR. Amplified fragments were sequenced, and downward primers were also designed.

***SSR polymorphism of* S.curtisii**

PCR was performed in 25ul reaction volumes containing 25ng genomic DNA. The PCR annealing temperature was 50 or 55º C, as appropriate for each pair of primers. Amplified fragments were fractionated on 3.5% agarose gels, and were sequenced to investigate their polymorphism.

Application of SSR primers to other Dipterocarp species

The PCR was carried out using seven primer pairs: *Shc*01, 04, 07, 08, 09, 11, and *Shc*14, using the same conditions as for *S.curtisii* DNA, except that the annealing temperature was set at 50º C for three cycles for each locus. All amplified fragments of *Shc*07 and *Shc*11 were sequenced, and randomly selected fragments obtained using the other five SSR loci were sequenced to confirm the SSR sequences in the various species.

Results and Discussion

Analysis of polymorphism of the SSRs

Length polymorphism of 12 SSR loci among 32 *S.curtisii* specimens was investigated using sequence analysis. Eleven SSR loci were polymorphic, but *Shc*15 was monomorphic. *Shc* 08 seemed to show more than three alleles on 3.5% agarose gels, but their sequence data were not sufficiently clear, to allow its allele number to be determined exactly. These results are summarized in Table 1.

For simple repeat loci such as *Shc*04 and *Shc*09, allele polymorphism was found to depend mainly on differences in the number of CT repeats. However, in some cases, short compound repeats were found in the SSRs, especially at the *Shc*11, *Shc*13, *Shc*14, and *Shc*17 loci. The allelic polymorphism at these loci was found to depend on insertions or deletions in the flanking region. *Shc*01 and *Shc*07 were also compound repeats, and highly polymorphic. Their polymorphism depended not only on differences in the repeat number of each repeat unit, but also on the combination of different repeat units. Therefore, we observed many alleles per locus for *Shc*01 and *Shc*07.

In this study, eleven polymorphic SSR markers were developed using two different strategies. Comparing the two strategies, a vectorette PCR approach may generally be a more

useful strategy than the commonly used method, such as screening by colony hybridization, because it allows more rapid surveys of numerous clones. However, as shown in Table 1, some SSR loci isolated by the vectorette PCR method included short or compound repeats. Using the commonly used, SSR loci which have short or compound repeats can be discarded before primers are designed, but for the vectorette primer method, it was necessary to design forward primers for all possible SSR loci to obtain SSR core sequences. This problem could be overcome by optimization of PCR conditions.

The CT/AG motif in *Shorea curtisii* is as abundant as it is in other plants (Lagercrantz *et al.*, 1993; Wang *et al.*, 1994). Simple CA repeats, known to be common in animals, were not isolated in this study.

The allelic polymorphism of *Shc*04 and 09, which have simple repeats, depends mainly on differences in CT repeat number, but the polymorphism of alleles involving compound repeat SSR loci is much more complex (Table 1). Such complexity may lead to errors in designating genotypes. in other words, even if amplified fragments are the same in size, they may not always have the same sequences. The complex compound repeats like *Shc*01 and *Shc*07 were found to be highly polymorphic, but it may be better to develop SSR markers only for simple repeat loci to limit errors in genotype identification.

The frequency of dinucleotide repeats in the genome of several woody plants has been assessed, the estimates ranging from one repeat every 64 to 1105 kbp (Condit & Hubbell 1991). in our study, two SSR loci were isolated out of 6000 clones from a genomic DNA library in which the average insert size was 400bp, and eleven SSR loci were isolated out of 104 clones from a genomic library with an average insert size of 1500bp. Thus, according to these results, although the frequency of the dinucleotide repeats in the *Shorea* genome could not be determined exactly, they seem to occur approximately once every 1200 kb. in *S.curtisii*, therefore, dinucleotide repeats are apparently more widely separated than in most other species.

The SSR primers developed for *S.curtisii* analysis are also useful for analyzing other Dipterocarp species, therefore they are potentially powerful tools in genetic analysis of the tropical forest. The results also indicate that affinities among these species are relatively close.

Conservation of SSR loci within Dipterocarpaceae

The primers developed for analyzing seven of the SSR loci were used to assess conservation of the loci among 30 species from 10 genera of the Dipterocarpaceae. Within the genus *Shorea,* all seven SSRs were well conserved (Table 2), but *Shc08* amplification was weak for *S.macroptera* and *S.singkawang*, and multiple amplification products were obtained from *S.lepidota, S.macrophylla, S.ovalis*, and *S.scaberrima* DNA using *Shc*01 primers, from *S.scaberrima* DNA using *Shc*07 primers, and from *S.lepidota* DNA using *Shc*09 primers. For species in the genus *Hopea*, *Shc*01, 07, 11, and 14 were well amplified by PCR. However, *Shc*04, 08 and 09 were all amplified weakly from the DNA of certain *Hopea* species: *Shc*04 from *H.latifolia*, *H.nervosa*, *H.sangal*, and *H.subalata* DNA, *Shc*08 from *H.latifolia, H.odorata, H.subalata*, and *H.wightiana* DNA, and *Shc*09 from *H.dyeri* DNA. All loci in *Neobalanocarpus heimii, Parashorea lucid* and *Dryobalanops aromatica*, were well amplified except *Shc*04 in *Parashorea*. Among the other seven species (*Dipterocarpus baudii, D. kerii, D. oblongifolius, Anisoptera oblonga, Vatica odorata, Cotylelobium malayanum*, and *Upuna bornensis*), the sequences of the flanking SSR region were found to be better conserved than they were in *Shorea* and *Hopea*.

*Shc*07 and 11 were selected from the seven loci, for sequence analysis of the 30 test species. *Shc07* was chosen because the PCR amplification lengths for this marker

differed on 3% agarose gels: *Hopea* species, in particular, giving longer lengths than other genera. *Shc*11 was selected because this locus was well amplified in all species except for *Upuna bornensis*. We observed some base substitutions between species at this locus, but the locus was well conserved within the family.

Recently, a molecular phylogeny of the same 30 species of Dipterocarpaceae was constructed using PCR-RFLP analysis of chloroplast genes by Tsumura et al. (1996). According to the molecular phylogeny, there are two major groups of Dipterocarpaceae; Shoreae which includes *Shorea, Hopea, Parashorea, Neobalanocarpus,* and *Dryobalanops*, and Dipterocarpeae which includes *Dipterocaropus, Anisoptera, Upuna,* and *Cotylelobium*. The molecular phylogeny analysis also revealed close affinities among *Shorea, Hopea, Parashorea, Neobalanocarpus*, and *Dryobalanops*.

PCR Amplification patterns of these 30 species, using the seven SSR loci markers developed for *Shorea curtisii,* do not show clear correlations with the molecular phylogeny. However, the amplification patterns of *Shc*04, *Shc*08, and *Shc*09 suggest that the sequences of the flanking regions of these SSR loci are not well conserved in relatively distant species. These loci were well amplified in Shorea, but not in several species from other genera.

The average size of *Shc*07 amplification length also seems to correlate well with the molecular phylogeny. Based on the sequence data for *Shc*07, the average amplification length is 200 bp in *Hopea*, 130bp in *Neobalanocarpus heim*, 168bp in *Shorea* and *Parashorea lucida*, and 143bp in the other seven species included in the tribe Dipterocarpeae. According to the sequence data, these differences mainly depend on CATA repeats in core sequences. in the tribe Dipterocarpeae, CATA repeats do not seem to exist.

In contrast, although base substitutions in *Shc*11 were detected in several species, it is not clear whether or not this reflects genetic distance.

Further study is required in order to evaluate the generality of the conservation phenomenon, and is likely to lead to greater understanding of the evolution of SSR markers during speciation, and the genetic mechanisms involved.

Genetic diversity of mother trees in both plots

The genotypes of three microsatellite loci for all individuals were determined, and the genetic parameters were estimated (Table 3). The *He* is ranged from 0.663 for seedling population of natural forest to 0.865 for sapling population of logged forest. The smallest *Na* was 6.00 for mature tree population for logged forest and the highest one was 11.50 for sapling population of natural forest. There were some differences between mature tree, sapling and seedling, but these parameters were affected by investigating population size. Thus, we compared the unbiased *He* and *Ne* between the categories, which are not affected by the population size. The five populations of the unbiased *He* were similar, 0.769 to 0.786, and the Ne between them were also very similar, 4.33-4.59. Consequently, there were no significant difference between them. The area of study sites were 6 ha for natural forest and 4 ha for logged forest, therefore, we can estimate the genetic diversity per area but the number of alleles per ha was also very similar between natural and logged forests.

Gene flow and outcrossing rate

In 1998, general flowering had occurred in this region, and S. curtisii also had lots of fruits in this time. We collected seeds from each individuals of natural and logged plot to survey the mating system and the genetic diversity of next generation. The number of detected alleles in natural plot for each mother tree in three loci was ranged from 16 to 22. The inbreeding rate also had quite wide range, 0.0 - 38.7 % and its average was 13.6.(Table 4) The average inbreeding rate is similar as other reports. Murawski and Bawa (1994) reported that the

outcrossing rate of *Stemonoporus oblongifolious* which is another genus of Dipterocarpacea was 84 % using isozyme data. Kitamura et al also estimated the outcrossing rate of *Dryobanops aromatica* in primary and secondary forests and the estimates was ranged from 79.4 to 85.6 %. Our estimates was 86.4 % as the average and showed similar tendency of the other dipterocarps species (Table 4).

We also investigated five individuals in logged forest to clarify the genetic diversity and inbreeding rate. The number of detected alleles was 17 except for No. 7 tree which was only 11. The inbreeding rate was quite different in each mother tree and ranged from 21.2 to 85.7 %. with the average of 53.6 %. The inbreeding rate between natural and logged forests was also quite different, and the inbreeding rate was about 40 % high at the average values. The inbreeding rate is generally affected by self-incompatibility, biparental inbreeding and apomixis. Self-incompatibility was reported in some diptercarps species such as *S. hemsleyana, S.,acroptera, S. lepidota, S. acuminata, S. macroptera, S. aplendida, D. oblongifolius* and inferred to five dipterocarps species (Chan 1981). Apomixis also occurred in some dipterocarps species such as *S. agami, S. ovalis , S. sericea* and inferred to *S. macroptera, S. resinosa* and three *Hopea* species (Kaur et al. 1978). But there is no evidence of self-incompatibility and apomixis in *S. curtisii*.

The outcrossing rate is also influenced by the pollinator behavior, flowering density, the spatial positioning of flower and the density of mature trees. In 1998, mass-flowing, following by mast fruiting had occurred in the Semangok region. *S. curtisii* also had lots of fruits in this period, therefore, the flowering density was quite high in each tree and we don't think the spatial positioning of flower affected the outcrossing rate in this study because each tree had lots of flowers in a whole branches. However, the density of mature tree is six times different between natural and logged plots, which are 31.5 trees per ha and 5 trees per ha (DBH>30cm), respectively. The pollinator of *S. curtisii* thought to be small beetle and thrips which are not long distant pollinator. Their behavior range is not so wide comparing to *Apis*, small social bee, *Xylocopa, Amegilla*, *Halictidae* and *Megachille*. Momose et al. (1998) reported that characteristics of he pollinator community in a lowland dipterocarps forest at Lambir. According to their results, the main pollinator of dipterocarps are *Apis* in *Dipterocarpus* and *Dryobalanops* species and Chrysomelidae and Curculionidae in many *Shorea* species. Sakai et al. (1999) also reported beetle pollination of *S. parvifolia* at Lambir, and thrip also visited the flowers but the density of thrip was much lower than that at Pasoh of Peninsula Malaysia. The density at Pasoh (2.4 thirps per flower) was eight times higher than that of Lambir (0.31) in six *Shorea* species sect. *Mutica* (Appanah and Chan 1981). *S. curtisii* also is included same sect. *Mutica* and flower shape also very similar, therefore, the pollinator of this species must be a kind of thrip or small beetle. If the main pollinator was thrip, their move to tree to tree by wind. They can not move to the long distant mother tree exactly. In our study, the most important factor affected the inbreeding rates of *S. curtisii* between natural and logged forests might be the density of mature trees. Murawski and Hamlick (1992) reported that the outcrossing rate of *Cavanillesia platanifolia* associate positively with flowering-tree density. They compared the outcossing rate between three different flowering-tree densities, and the outcrossing rate was reduced depending on their flowering-tree density. Unfortunately, they don't discuss the pollinator of this species. The behavior and quantity of pollinator is one of the most important factor to the outcrossing rate. Kitamura et al. (1994) compared outcrossing rate between primary and secondary forests for two years in *D. aromatica*. The flowering density of primary and secondary (2nd year) forests were 14.0 and 6.7 (6.7) tree per ha, respectively and the outcrossing rates were 0.856 and 0.794 (0.787), respectively. But there were no significant different statistically. The pollinator of *D. aromatica* is *Apis dorsata* in Lambir (Momose et al. 1998), therefore, the

outcrossing rates between primary and secondary forests is not significant difference as the behavior range is rather wider than that of thrip or beetle. The reason of the difference in our study is the density of flowering-tree and the behavior of pollinator.

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Research Output

Publications

Ujino, T., T. Kawahara, Y. Tsumura, T. Nagamitsu, Wickneswari R. and H. Yoshimaru (1998) Development and polymorphism of simple sequence repeat DNA markers for Shorea curtisii and other Dipterocarpaceae species. *Heredity* 81:422-428

Kajita, T.,K.Kamiya, K. Nakamura, R. Wickneswari, H. Yoshimaru, Y.Tsumura, H.Tachida and T. Yamazaki (1998) Molecular phylogeny of Dipterocarpaceae in Southeast Asia based on nucleotide sequence of matK, trnL intron and trnL-trnF intergenic spacer region in chloroplast DNA. *Molecular Phylogenetic Evolution* (in press)

Tsumura, Y., Kawahara, T., Wiekneswari, R. and Yoshimura, H. (1996). Molecular ohirogeny of dipterocarpaceae in Southeast Asia using RFLP of PCR-amplified chloroplast genes. *Theor Appl Genet* **93**: 22-29.

References

Appanah, S. 1981. Pollination in Malaysian primary forests. *Malay. Forest.* 44:37-42.

Appanah, S., and M. R. A. Manaf 1990. Smaller trees can fruit in logged dipterocarp forests. *J. Trop. For. Sci.* 3: 80-87

Bawa, K. S. 1992. Mating system, genetic differentiation and speciation in tropical rain forest plants. *Biotropica* 24: 250-255.

Chan, H. T. 1981. Reproductive biology of some Malaysian dipterocarps III. Breeding systems. *Malay. Forest.* 44: 28-36.

Charlesworth, B., Sniegowaski, P. and Stephan, W. 1994. The evolutionary dynamics of repetitve DNA in eukaryotes. *Nature*, **371**, 215-220.

Chase, M., Kesseli, M. R. and Bawa, K. S. 1996a. Microsatellite markers for population and conservation genetics of tropical trees. *Am. J. Bot.*, **83**, 51-57.

Chase, M., Kesseli, M. R. and Bawa, K. S. 1996b. Distant gene flow in tropical trees. *Nature* **383**, 398-399

Cifarelli, R. A., Gallitelli, M. and Cellini, F. 1995. Random amplified hybridization microsatellites(RAHM): isolation of a new class of microsatellite-containing DNA Clones. *Nucleic Acid Research* , **23**, 3802-3803.

Condit, R, and Hubbell, S. P. 1991. Abundance and DNA sequence of two-base repeat regions in tropical tree genomes. *Genome*, **34**, 66-71.

Dayanandan, S., Bawa, K. S. and Kesseli, R. 1997. Conservation of microsatellites among tropical trees (Leguminosae). *Am. J. Bot.*, **84**, 1658-1663.

Dow, B. D., Ashley, M. V. and Howe, H. F. 1995. Characterization of highly variable (GA/CT)n microsatellites in the bur oak, *Quercus macrocarpa*. *Theor. Appl. Genet.*, **91**, 137-141.

Echt, C. S., May-Marquqrdt, P., Hseih, M. and Zahorchak, R. 1996. Characterization of

microsatellite markers in eastern white pine. *Genome*, **39**, 1102-1108.
Ellegren, H., Moore, S., Robinson, N., Byrne, K., Ward, W. AND Sheldon, B. C. 1997. Microsatellite evolution - a reciprocal study of repeat length at homologous loci in cattle and sheep. *Mol. Biol. Evol.*, **14**, 854-860.
Goldstein, D. B. and Pollock, D. D. 1997. Launching microsatellites: A review of mutation processes and methods of phylogenetic inference. *J. Hered.,* **88**, 335-342.
Jarne, P. and Lagoda, P. J. L. 1996. Microsatellite, from molecules to populations and back. *Trends. Ecol. Evol.*, **11**, 424-429.
Kajis, J. M. H., Fowler, J. C. S. and Thomas, M. R. 1995. An evaluation of sequence tagged microsatellite site markers for genetic analaysis *Citrus* and related species. *Genome*, **38**, 349-355.
Kajita, T., Kamiya, K., Nakamura, K., Wickneswari, R., Yoshimaru, H., Tsumura, Y., Tachida, H. and Yamazaki, T. 1998. Molecular phylogeny of Dipterocarpaceae in Southeast Asia based on nucleotide sequence of *mat*K, *trn*L intron and *trn*L-*trn*F intergenic spacer region in chloroplast DNA. *Mol. Phyl. Evol.*, (in press).
Karagyozov, L., Kalcheva, I. D. and Chapman, V. M. 1993. Construction of random small-insert genomic libraries highly enriched for simple sequence repeats. *Nucl. Acids Res.*, **21**, 3911-3912.
Kirkpatrick, B.W., Bradshaw, M., Barendse, W. and Dentine, M. R. 1995. Development of bovine microsatellite markers from a microsatellite-enriched library. *Mammalian Genome*, **6**, 526-528.
Kostia, S., Varvio, S. L. ,Vakkai, P., and Pulkkinen, P. 1995. Microsatellite sequences in a conifer, *Pinus sylvestris*. *Genome*, **38**, 1224-1248.
Kimura, M. and Crow, J. F. 1964. The number of alleles that can be maintained in a finite population. *Genetics* 49: 725-738.
Kitamura, K., M. Y. B. A. Rahman, Y. Ochiai and H. Yoshimaru 1994. Estimating of the outcrossing rate on Dryobalanops aromatica Gaertn. f. in primary and secondary forests in Brunei, Borneo, Southeast Asia. *Plant Species Biol.* 9: 37-41.
Kaur, A., C. O. Ha, K. Jong, V. E. Sands, H. T. Chan, E. Soepadmo and P. S. Ashton 1978. Apomixis may be widespread among trees of the climax rain forest. *Nature* 271: 440-442.
Lagercrantz, U., Ellegren, H., and Andersson, L. 1993. The abundance of various polymorphic microsatellite motifs dffers between plants and vertebrates. *Nucl. Acids Res.*, **21**, 1111-1115.
Lench, N. J., Norris, A., Bailey, A., Booth, A. and Markham, A. F. 1996. Vectorette PCR isolation of microsatellite repeat sequence using anochored dinucleotide repeat primers. *Nucl. Acids Res.*, **24**, 2190-2191.
Levine, I., Cheng, H. H., Bacter, J. C. and Hillel, J. 1995. Turkey microsatellite DNA loci amplified by chicken specific primers. *Anim. Genet.*, **26**, 107-110
Lyall, J. E. W., Brown, G. M., Furlong, R. A., Ferguson-Smith, M. A., and Affara, N. A. 1993. A method for creating chromosome-specific plasmid libraries enriched in clones containing [CA]n microsatellite repeat sequences directly from flow-sorted chromosome. *Nucl. Acids Res.*, **21**, 4641-4642.
Momose, K., T. Yumoto, T. Nagamitsu, M. Kato, H. Nagamasu, S. Sakai, R. D. Harrison, T. Itioka, A. A. Hamid and T. Inoue 1998. Pollination biology in a lowland dipterocarp forest in Sarawak, Malaysia. I. Characteristics of the plant-pollinator community in a lowland dipterocarp forest. *Am. J. Bot.* 85: 1477-1501.
Moore, S. S., Sargeant, L. L., King, T. J., Matick, J. S., Georges, M. and Hetzel, D. J. 1991. The conservation of dinucleotide microsatellites among mammalian genomes allows

the use of heterologous PCR primer pairs in closely related species. *Genomics*, **10**, 654-660.

Murawski, D. A. and J. L. Hamrick 1991. The effect of the density of flowering individuals on the mating systems of nine tropical tree species. *Heredity* 67: 167-174.

Murawski, D. A. and J. L. Hamrick 1994. Genetic structure and mating system of Stemonoporus oblongifolius (Dipterocarpaceae) in Sri Lanka. *Am. J. Bot.* 81: 155-160.

Murawski, D. A. and J. L. Hamrick 1992. The mating system of Cavanillesia platanifolia under extremes of flowereing-tree density: a test of predictions. *Biotropica* 24: 99-101.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics,* 89, 583-590.

Nei, M. 1987. Molecular Evolutionary Genetics. pp.521, Columbia University Press, New York.

Niiyama, K. Abd. Rahman K., Iida, S., Kimura, K. and Appanah S. (1993) introduction of Ecological Studies in A Hill Dipterocarp Forest in Peninsula Malaysia. Japanese Journal of Forest Environment 35 : 25-29.

Ostrander, E. A., Jong, P. M., Rine, J., and Duyk, G. 1992. Construction of small-insert genomic DNA libraries highly enriched for microsatellite repeat sequences. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 3419-3423.

Sakai, S., K. Momose, T. Yumoto, M. Kato and T. Inoue 1999. Beetle pollination of Shorea parvifolia (section Mutica, Dipterocarpaceae) in Sarawak, Malaysia. *Am. J. Bot.* 86: 62-69.

Schlotterer, C., Amos, B. and Tautz, D. 1991. Conservation of polymorphic simple sequence loci in cetecean species. *Nature*, **354**, 63-65.

Smith, D. N. and Devey, M. E. 1994. Occurrence and inheritance of microsatellite in *Pinus radiata*. *Genome*, **37**, 977-983.

Takahashi, H., Nirawasa, N. and Furukawa, T. 1996. An efficient method to clone chicken microsatellite repeat sequence. *Jpn. Poultry Sci.*, **33**, 292-299.

Tsumura, Y., Kawahara, T., Wiekneswari, R. and Yoshimura, H. 1996. Molecular phylogeny of dipterocarpaceae in Southeast Asia using RFLP of PCR-amplified chloroplast genes. *Theor. Appl. Genet.*, **93**, 22-29.

Ujino, T., T. Kawahara, Y. Tsumura, T. Nagamitsu, Wickneswari R. and H. Yoshimaru (1998) Development and polymorphism of simple sequence repeat DNA markers for *Shorea curtisii* and other Dipterocarpaceae species. *Heredity* 81:422-428

Ven, W. T. G Van De and Mcnicol, R. J. 1996. Microsatellites as DNA markers in Sitka spruce. *Theor Appl Genet* **93**: 613-617.

Wang, Z., Weber, J. L., Zhong, G. and Tanksley, S. D. 1994. Survey of plant short tandem DNA repeats. *Theor. Appl. Genet.*, **88**, 1-6.

White, G. and Powell, W. 1997. Isolation and characterization of microsatellite loci in *Swietenia humilis* (Meliaceae) : an endangered tropical hardwood species. *Mol. Ecol.*, **6**, 851-860.

Wu, K. and Tanksley, S. D. 1993. Aboundance, polymorphism and genetic mapping of microsatellites in rice . *Mol. Gen. Genet.*, **241**, 225-235.

Table 1. SSR allele number, and observed allele types based on sequence data.

Locus	Core sequence*	Number of allele	Heterozygosity (H_e)
*Shc*01	$(CT)_n(CA)_mCT(CA)_lCTCA$	20	0.922
*Shc*02	$(CT)_2CA(CT)_nGC(AT)_2$	2	0.180
*Shc*03	$(CT)_n$	3	0.591
*Shc*04	$(CT)_n$	12	0.844
*Shc*07	$(CT)_nCA(CT)_mCACCC(CTCA)_lCT(CA)_k$	11	0.810
*Shc*09	$(CT)_n$	9	0.818
*Shc*11	$(CT)_m(A/T)T(CT)_n$	4	0.640
*Shc*17	$(CT)_5AT(CT)_n$	2	0.304
	Average	7.9	0.639

Table 2, Application of SSR primers developed for *Shorea curtissi* to other Dipterocarpaceae species.

Species	Locus						
	*Shc*01	*Shc*04	*Shc*07	*Shc*08	*Shc*09	*Shc*11	*Shc*14
Anisoptera oblonga	S	W	S	W	S	S	M
Cotylelobium malayanum	S	W	W	W	W	S	S
Dipterocarpus baudii	S	W	S	W	W	S	S
Dipterocarpus kerrii	S	W	M	W	W	S	S
Dipterocarpus oblongifolius	S	W	S	W	S	S	S
Hopea dyeri	S	S	S	S	W	S	S
Hopea latifolia	M	W	S	W	S	S	S
Hopea nervosa	S	W	S	S	-	S	S
Hopea odorata	S	S	S	W	S	S	S
Hopea sangal	S	W	S	S	S	S	S
Hopea subalata	S	W	S	W	S	S	S
Hopea wightiana	S	S	S	W	S	S	S
Neobalanocarpus heimii	S	S	S	S	S	S	S
Shorea atrinervosa	S	S	S	S	S	S	S
Shorea bracteolata	S	S	S	S	S	S	S
Shorea kunstleri	S	S	S	S	S	S	S
Shorea lepidota	M	S	S	S	M	S	S
Shorea macrophylla	M	S	S	S	S	S	S
Shorea macroptera	S	S	S	W	S	S	S
Shorea multiflora	S	S	S	S	S	S	S
Shorea ovalis	M	S	S	S	S	S	S
Shorea parvifolia	S	S	S	S	S	S	S
Shorea scaberrima	M	S	M	S	S	S	S

Shorea singkawang	S	S	S	W	S	S	S
Parashorea lucida	S	-	S	S	S	S	S
Vatica odorata	S	W	S	S	S	S	S
Upuna bornensis	M	-	S	W	S	W	M

[a] S: strong amplification, W: weak amplification, -: amplification failed M: multiple bands amplified.

Table 3 Genetic diversity between natural and lohhed forests.

Population	n	*He*	unbiased *He*	*Na*	*Ne*
Natural DBH>30cm	45	0.800	0.786	9.00	4.63
Natural sapling	171	0.739	0.782	11.50	4.59
Natural seedling	58	0.663	0.769	7.25	4.33
Logged DBH>30cm	10	0.850	0.779	6.00	4.52
Logged sapling	26	0.865	0.768	7.75	4.44

Table 4 Comparison of outcrossing rate and heterozygosity between natural and logged forests.

	No. of investigated mother trees	No. of analyzed seeds	No of detected alleles	Outcrossing rate (%)	*He*
Natural Forest	10	326	31	86.4	0.761
Logged Forest	5	169	26	46.4	0.736