Genetic variation of the genus *Shorea* (Dipterocarpaceae) in Indonesia

Dissertation

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1 Background and objectives of the study

1.1 Background

The analysis of genetic diversity is a primary concern of population genetics. Genetic variation is an important attribute of forest tree populations enabling them to survive spatial and temporal variations of environmental conditions. High levels of genetic variability permit a sustained adaptive response to environmental change, lowering the extinction risk. The genetic variation and its structure within and between populations are also important for the conservation and management of genetic resources and for applications in breeding and silviculture (Brown 1978, Hattemer 1987, 1988, 1990; Ziehe et al. 1989; Finkeldey 1993; Gregorius 1994). The determinants of genetic variation are the sexual system, gene flow, mating system, selection, mutation, migration and drift.

Tropical forests in Asia are a centre of global biological diversity. Many of them are dominated by dipterocarps (Dipterocarpaceae), which is one of the most important tree families in Asian tropical rain forests both from an ecologic and economic perspective, and a species-rich tree family with more than 450 species in 13 genera in Asia (Ashton 1982; Londoño et al. 1995; Morton 1995). Indonesia, in particular Sumatra and Kalimantan (Borneo), is the region of the highest species diversity of Dipterocarpaceae. In the past half-century, dipterocarps are heavily affected by deforestation, forest fragmentation and non-sustainable forest management due to the exploitation for their valuable timber. Several species are threatened, even coming to the brink of extinction (Korsgaard 1985). Information on the phylogeny of Indonesian dipterocarps is scarce. Genetic variation and structure of dipterocarp species based on DNA markers have also been rarely investigated. It is important to obtain knowledge of phylogenetic relationships and the amount and spatial distribution of genetic diversity in order to provide information for the development of strategies for the conservation and sustainable utilization of dipterocarps.

1.2 Objectives of the study

The research program was focused on the following objectives:

(1) to study variation patterns at Amplified Fragment Length Polymorphisms (AFLPs) among dipterocarp species, in order to test its relation to molecular phylogenies and taxonomic subdivisions (see part 1);

(2) to analyse genetic diversity within and among populations of the important and widespread species *Shorea leprosula* Miq. and *S. parvifolia* Dyer (Dipterocarpaceae) in Indonesia using AFLP markers, in order to develop strategies for conservation of genetic resources of the two species, and to contribute to the development of molecular tools for identification of the origin of dipterocarp wood (see part 2);
(3) to analyse genetic variation in nine *Shorea* species in Indonesia using AFLP markers, in order to compare levels of diversity among species and regions, to test the usefulness of the AFLP technique for species identification, and to detect individual AFLP fragments with strong differentiation among species (see part 3).

2 Dipterocarpaceae and the genus *Shorea*

2.1 Traditional taxonomy and natural distribution

The Dipterocarpaceae family comprises three subfamilies with about 510 species in 17 genera according to recent classifications by Ashton (1982), Londoño et al. (1995) and Morton (1995): Dipterocarpoideae from tropical Asia with approximately 470 species in 13 genera, distributed in the Seychelles, Sri Lanka and India northeastwards to southern China and the Batan Islands, and southeastwards to New Guinea and D'Entrecasteaux Island; Monotoideae from Africa with 39 species in two genera and the new monotypic genus *Pseudomonotes* from Colombia in South America, and the monotypic subfamily Pakaraimoideae from Guyana in South America. The Asian dipterocarps (subfamily Dipterocarpoideae) are further divided into two tribes, Dipterocarpeae and Shoreae (Brandis 1895), with haploid chromosome numbers of 11 and 7, respectively. The tribe Shoreae is richer in species than Dipterocarpeae due to species richness in the genera *Shorea* and *Hopea*. *Shorea* is the largest and economically most important genus of Dipterocarpaceae, encompasses about 200 species in 11 sections, of which 163 species are distributed in Malesia, mostly in Indonesia, in particular on Sumatra and Borneo (Kalimantan), while genus *Hopea* comprises more than 100 species.

2.2 Ecological and economical significance

The tropical rainforests in Southeast Asia are characterized by a high species diversity of trees (Whitmore 1984). The extreme floristic richness is largely due to the co-occurrence of a great number of species within the same community (Whitmore 1998). Dipterocarpaceae species are distributed mainly in Southeast Asia, confined to tropical climates with a mean annual rainfall exceeding 1000 mm, and/or a dry season of less than six months. Most species do not

occur above an elevation of about 1000 m where an important floristic transition occurs. Many dipterocarps are common and emergent trees, dominating the forest canopy in lowland Southeast Asian tropical forests and usually grow in mixed stands (Ashton 1988). The family is one of the region's most important tree families, being represented by an extreme richness of species in many tropical rain forests (Fedorov 1966; Ashton 1969; Whitmore 1984). Many different species of dipterocarps can be found on entirely different site types and in nearly all of the different forest types of Southeast Asia (Lamprecht 1989). The highest species diversity of dipterocarps is observed in evergreen rainforests in Peninsular Malaysia, Sumatra and Borneo (Ashton 1982; Symington 1943; Whitten et al. 1987). In particular, Borneo is the main centre of dipterocarps with the highest number of endemic species (155 species). Approximately one quarter of all trees in most lowland forests of Borneo are dipterocarps (Ashton 1982; Sist 1996; Slik et al. 2003). The greatest richness in terms of abundance of species is attained in the emergent stratum in Sarawak and Brunei, Northwest Kalimantan (Whitmore 1975). Dipterocarpaceae show a high rate of endemicity in the non-seasonal humid tropics that is not reflected in the more seasonal parts of their range. This may be ascribed to their poor fruit dispersal in a windless climate, allowing easy isolation by natural barriers such as quite small river valleys (Ashton 1969) and rapid edaphic specialisation.

The symbiosis with ectomycorrhiza improves the physiological adaptability and is important for the growth of dipterocarps, especially in nutrient poor conditions (Smits 1994). Thus, the inoculation of ectomycorrhiza is crucial for the establishment of dipterocarp plantations. Silvicultural treatment, such as liberation thinning, also increases the periodic annual diameter increment (PAI) in larger trees (diameter at breast height / DBH > 20 cm) of the Red Meranti group and smaller trees (DBH = 10-20 cm) of other *Shorea* spp. and some *Hopea* spp. (Kammesheidt et al. 2003).

Dipterocarpaceae are the main timber family in the tropical forests of Southeast Asia (Ashton 1982). More than 70% of the world's demand for plywood made from hardwoods has been supplied by Indonesia, principally from dipterocarp species (Choong and Achmadi 1996). Vernacular names like meranti for timber of many *Shorea* species are usually used for timber trading purposes. Symington (1943) used vernacular names to divide *Shorea* into four groups, namely Balau (Selangan Batu), White Meranti (Meranti Pa'ang), Yellow Meranti (Meranti Damar Hitam) and Red Meranti. Newman et al. (1996a, 1996b, 1998a, 1998b, 1998c) described the other common timber names of Indonesian dipterocarps related to the genus, namely Giam / Merawan (*Hopea*), Keruing (*Dipterocarpus*), Kapur (*Dryobalanops*),

Mersawa (*Anisoptera*), White Seraya (*Parashorea*), and Resak (*Vatica*, *Cotylelobium*, and *Upuna*).

The various wood varieties of dipterocarps are used for veneers and for outdoor and indoor construction (Lamprecht 1989). Additionally, resin is also obtained from various species. Several dipterocarp species, mainly of the genus *Shorea*, produce a nut with an edible fat, identical to that of cocoa, and an excellent substitute for cocoa butter in the manufacture of chocolate and cosmetics (Seibert 1996).

2.3 Reproductive biology

Outcrossing is predominant in many tropical tree species, and the average outcrossing rate is more than 80% (Nason and Hamrick 1996). Outcrossing rates may be strongly influenced by flowering-tree density (Murawski and Hamrick 1992; Murawski et al. 1994) and the types and behaviors of pollinators governing the pollen movement (Ghazoul et al. 1998).

Dipterocarpaceae have an outcrossing habit, and some species have a considerable degree of self-incompatibility (Chan 1981). The flowers of dipterocarps are hermaphroditic and pollinated by a variety of insects as vectors (Appanah 1981; Appanah and Chan 1981; Corlett 2004; Momose et al. 1998; Sakai et al. 1999). One of the most important characteristics of the family in the non-seasonal zone is its flowering behavior. Flowering is sporadic throughout the year (Ashton 1988) and gregarious at intervals of two to five years (reviewed in Soerianegara and Lemmens 1993). Observations in Kalimantan (Borneo) and Peninsular Malaysia revealed that most dipterocarps flower in March-April and the fruits of most species matured and fell in September-October (Chan and Appanah 1980; Wood 1956).

Seed dispersal of dipterocarps can be divided as follows (Ashton 1982; Murawski and Bawa 1994; Suzuki and Ashton 1996): (1) by wind in most species which have wing-like structure (aliform) sepals; (2) by water in many species which have short sepals and grow in swamps or on river banks, (3) by falling on the ground in species without wing-like sepals. Seeds disseminated by water can potentially disperse over longer distances than by wind.

The frequency of natural hybridization among Dipterocarpaceae species is unknown, but interspecific hybrids have been reported in several studies (Bawa 1998). Hybrids between *Shorea leprosula* and *S. curtisii* have been reported at several locations in Peninsular Malaysia and Singapore (Chan and Appanah 1980; Ashton 1982). The successful fruit formation resulting from a cross between *Shorea splendida* and *S. stenoptera* indicated the potential for natural hybridization among closely related species (Chan 1981). Putative hybridization between *Shorea acuminata* and *S. leprosula* was suggested by an investigation of nucleotide sequences in the *GapC* region (Ishiyama et al. 2003). Most dipterocarps are

diploid, but polyploidy has been reported for several species indicating the possibility of hybridization. Triploid trees were found in *Hopea beccariana*, *H. latifolia*, *H. subalata*, *H. odorata* and *Shorea resinosa*. *Hopea nutans* and *Shorea ovalis* ssp. *sericea* are tetraploid species (Ashton 1982; Jong and Kaur 1979; Kaur et al. 1978; Somego 1978).

3 Genetic markers

3.1 AFLPs

Amplified fragment length polymorphisms (AFLPs) are polymerase chain reaction (PCR)-based markers for the rapid screening of genetic diversity (Mueller and Wolfenbarger 1999). AFLP is a DNA fingerprinting technique which detects DNA restriction fragments by means of PCR amplification, and can be thought of as a combination of RFLP (restriction fragment length polymorphism) and PCR technology. The AFLP technique usually consists of the following steps (Vos et al. 1995):

(1) The restriction of the DNA with two restriction enzymes;

A DNA sample is simultaneously digested with two restriction enzymes (e.g. *Eco*RI, a 6-base, or rare cutter, and *Mse*I, a 4-base, or frequent cutter).

(2) The ligation of adaptors;

Two short, synthetic, double-stranded (ds) adaptor sequences, each with overhanging bases complementary to those produced by one of the restriction enzymes, are ligated to the ends of the restriction fragments to generate template DNA for PCR amplification.

(3) PCR amplification;

A subset of the restriction fragments is amplified using two primers which are complementary to the adaptors and restriction site sequences, and extended at their 3' ends by 1 to 3 "selective" nucleotides. The PCR amplification is performed in two steps: (1) the preselective amplification with a primer combination, each possessing one selective nucleotide; (2) the selective amplification with a primer combination, each having three selective nucleotides.

(4) Electrophoresis and detection.

The amplified restriction fragments are electrophoretically separated on denaturing polyacrylamide gels (or in an automated gene sequencer) and detected by means of a radioactive (or fluorescent) label on one of the PCR primers

Restriction fragment patterns generated by means of the AFLP technique are known as AFLP fingerprints. These AFLP fingerprints are a rich source of restriction fragment polymorphisms, termed AFLP markers. A typical AFLP fingerprint contains between 50 and

100 amplified fragments, of which up to 80% may serve as genetic markers (http://www.keygene.com/html/aflp.htm). The AFLP marker technique is a very reliable and robust technique, because it makes use of stringent PCR conditions, and is unaffected by small variations in amplification parameters (e.g. thermal cyclers, template concentration, PCR cycle profile). The major advantages of the AFLP technique include the large numbers of genome-wide polymorphic markers, a high reproducibility due to high PCR annealing temperatures, and relative economy on a per marker basis. Moreover, the AFLP technique requires no sequence information or probe collections prior to the generation of AFLP fingerprints. Due to the ligation of synthetic adaptors with known sequences, this technique is applicable to any species. Segregation of AFLP markers can be tested in half-sib (single tree progenies) and full-sib families. Generally AFLP markers segregate in a Mendelian fashion (Vos et al. 1995; Maughan et al. 1996; Liu et al. 1998), indicating that they are unique DNA fragments (http://www.keygene.com/html/aflp.htm), and can be used for population genetics and QTL analyses (Mueller and Wolfenbarger 1999).

The AFLP marker technique is a very effective, fast and reliable tool to assess genetic variation compared to other markers. Many studies have applied this technique not only to DNA fingerprinting and genetic diversity studies, but also to genome mapping, phylogenetic studies and parentage analysis (Mueller and Wolfenbarger 1999).

3.2 Interpretation of AFLP fingerprints

AFLP polymorphisms are displayed in the form of the presence and absence of bands, and correspond to single-nucleotide polymorphisms in restriction sites and primer binding sites (selective bases), insertions or deletions. AFLP variation are considered to be bi-allelic. Each AFLP band is assumed to correspond to a dominant allele at a single locus.

In this study, AFLP fragments were separated in a capillary sequences (ABI 3100). Only well scorable and reproducible fragment sizes of the total AFLP fingerprint patterns were manually selected and scored as present (1) or absent (0) in each sample. Results were converted into a binary character matrix for further analyses. The software packages GeneScan 3.7 and Genotyper 3.7 (Applied Biosystems) were used for the analysis of raw data.

3.3 Chloroplast DNA markers (PCR-RFLPs and cpSSRs)

The development of higher plants is dependent on a coordinated expression of three distinct genomes: the chloroplast (cp), mitochondrial (mt) and nuclear genome. In comparison to the nuclear genome, cpDNA is small [120-217 kilobase-pairs (kb)] and always circular (Palmer 1990). CpDNA is generally uniparentally inherited (maternal in angiosperms, paternal in

gymnosperms), shows no recombination, and changes only by occasional mutations. Therefore, inheritance of cpDNA follows a parental line over generations (Chesnoy and Thomas 1971; Corriveau and Coleman 1988). CpDNA can be readily detected because of its occurrence in multiple copies per cell. The cpDNA molecule is smaller and structurally simpler than nuclear DNAs, allowing straightforward molecular interpretations of polymorphisms (e.g. Palmer et al. 1988).

Two cpDNA markers are used in this study, namely PCR-RFLP and cpSSRs, to detect the patterns of cpDNA polymorphisms in different *Shorea* species in order to distinguish them.

PCR-RFLPs

The PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism) technique has been particularly widely applied to study polymorphisms of cpDNA (e.g. Tsumura et al. 1996), and employs universal cpDNA primers (Taberlet et al. 1991; Dumolin-Lapegue et al. 1997). Certain primers selectively amplify DNA of the chloroplasts. The fragments produced by PCR are "digested" by restriction enzymes. This technique has gained popularity for its easy accessibility and rapidity.

cpSSRs

Microsatellites were first developed for use in the human genome (Weber and May 1989) and were later found to be abundant in plants (Morgante and Olivieri 1993). Chloroplast microsatellites are a powerful derivative of PCR technology. These markers are also known as simple sequence repeats in chloroplast DNA (cpSSRs). Usually they consist of mononucleotide repeats. CpSSRs are highly polymorphic markers and have a very high discrimination power for species and for regions within species (Weising and Gardner 1999; Vendramin et al. 1996).

4 Quantification of genetic variation

4.1 Basic genetic parameters

AFLP markers are dominant markers. At Hardy-Weinberg equilibrium, the relative frequency (p_0) of the recessive allele (scored as 0) and the relative frequency (p_1) of the dominant allele (scored as 1) at a locus are calculated:

$$p_0 = \sqrt{\frac{n_0}{N}}$$

$$p_1 = 1 - p_0$$

where n_0: the number of samples showing the absence of the band (null allele);

N: the total number of samples.

4.2 Measurement of genetic variation within populations

Genetic variation within a population was characterized by the following measures:

- The percentage of polymorphic loci (PPL)

$$PPL = \frac{N_P}{N_P + N_M} * 100$$

where N_P: the number of polymorphic loci;

N_M: the number of monomorphic loci.

PPL was calculated regardless of allele frequencies for AFLPs. If one locus has two phenotypes, namely presence (1) and absence (0) of a band, then this locus is considered polymorphic.

- observed number of alleles per locus n_a

$$n_a = \sum n \Big/ L$$

where $\sum n$: the total number of observed alleles at all loci;

L: the number of observed loci.

- effective number of alleles per locus n_e (Crow and Kimura 1970; Hartl and Clark 1989)

the effective number of alleles at the locus A is calculated as follows:

$$v_{(a)} = 1 \Big/ \sum_i p_{i(a)}^2$$

where p_i**:** the frequency of the *i*th allele at locus A.

Then, the effective number of alleles over all loci (gene pool) studied are computed as follows:

$$n_e = \bar{v} = \left[\frac{1}{L} \sum_a \frac{1}{v_{(a)}} \right]^{-1}$$

where L**:** the total number of loci studied.

- expected heterozygosity (gene diversity H_e; Nei 1973)

$$H_e = \frac{1 - \sum p_i^2}{L}$$

where p_i: the frequency of the *i*-th allele at one locus;

L: the total number of loci studied.

- Shannon's information index (Lewontin 1972)

$$I = -\sum p_i \log_2 p_i$$

where p_i: the frequency of the presence or absence of a given AFLP phenotype (band).

4.3 Measurement of genetic variation among populations

- Nei's (1978) unbiased estimate of genetic distance

$$\hat{D} = -\ln\left[\hat{G}_{xy} \Big/ \sqrt{\hat{G}_x \hat{G}_y}\right]$$

where $\hat{G}_x$: the averages of $(2n_x J_x - 1)/(2n_x - 1)$ over the r loci studied;

$\hat{G}_y$: the averages of $(2n_y J_y - 1)/(2n_y - 1)$ over the r loci studied;

$\hat{G}_{xy} = J_{xy}$;

n_x: the numbers of individuals sampled from population X;

n_y: the numbers of individuals sampled from population Y;

J_x: the averages of $\sum x_i^2$ over the r loci studied;

J_y: the averages of $\sum y_i^2$ over the r loci studied;

J_{xy}: the averages of $\sum x_i y_i$ over the r loci studied;

x_i: the corresponding sample allele frequencies;

y_i: the corresponding sample allele frequencies.

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- the relative magnitude of genetic differentiation among populations (Nei 1987)

$$G_{st} = \frac{D_{st}}{H_s + D_{st}} = \frac{D_{st}}{H_t}$$

where D_{st} : the genetic diversity among populations;

H_s : arithmetic mean of gene diversities within populations;

H_t : total gene diversity within the pooled material.

- **gene flow (McDermott and McDonald (1993)**

$$N_m = \frac{0.5(1-G_{st})}{G_{st}}$$

where N_m : the number of migrants per generation.

5 Summary of results

5.1 Genetic variation at AFLPs for the Dipterocarpaceae and its relation to molecular phylogenies and taxonomic subdivisions (see part 1)

The diversity center of the tropical tree family Dipterocarpaceae is located in Southeast-Asia. A total of 81 samples of dipterocarp trees from Indonesia belonging to 54 species in nine genera were investigated at 125 AFLP loci in order to assess genetic differentiation among species. The resultant UPGMA tree clearly separated all investigated dipterocarps into two major groups corresponding to tribe Dipterocarpeae with base chromosome number of $x = 11$ containing genera *Dipterocarpus*, *Anisoptera*, *Cotylelobium, Upuna*, and *Vatica*, and to tribe Shoreae with $x = 7$ containing genera *Hopea*, *Parashorea*, *Shorea* and *Dryobalanops*. Genus *Dryobalanops* grouped together with *Shorea virescens* basal to other members of tribe Shoreae. Evidence from chloroplast DNA, caryological and morphological characters also suggested an intermediate position of this genus between Dipterocarpeae and Shoreae. The results of the present study using the AFLP marker technique are in accordance with the topology of molecular phylogenetic trees derived from PCR-RFLP analysis of chloroplast DNA and with the classification based on caryological and morphological characters (with a few exceptions) and generally support the traditional taxonomic assessments.

Despite the generally high within-species varaition of AFLPs, this marker system seems to be appropriate for detection of taxonomic relationships within dipterocarps. The amount of within-species variation and the power of AFLPs to discriminate between *Shorea* species is analysed and discussed in detail in part 3.

5.2 Genetic diversity within and among populations of *Shorea leprosula* Miq. and *S. parvifolia* Dyer (Dipterocarpaceae) in Indonesia detected by AFLPs (see part 2)

Shorea leprosula Miq. and *S. parvifolia* Dyer are economically and ecologically very important emergent tree species of lowland tropical rain forests in southeast Asia. Amplified fragment length polymorphisms (AFLPs) were used to assess the genetic diversity in these two species from Indonesia, particularly on Sumatra and Borneo. A total of 268 individuals were analysed at 56 AFLP loci, including 133 individuals from seven populations of *S. leprosula* and 135 individuals from six populations of *S. parvifolia*. The results of this study indicated that *S. leprosula* is genetically more variable than *S. parvifolia*. At the population level, a higher level of genetic diversity was revealed for *S. leprosula* with a percentage of polymorphic loci (PPL_p) of 53.32% and an expected heterozygosity (H_{ep}) of 0.16 in comparison with *S. parvifolia* showing PPL_p of 51.79% and H_{ep} of 0.14. At the species level, *S. leprosula* showed a percentage of polymorphic loci (PPL_s) of 92.86% and an expected heterozygosity (H_{es}) of 0.21, while *S. parvifolia* showed PPL_s of 85.71% and H_{es} of 0.21. Genetic differentiation (G_{st}) calculated on the basis of estimated allele frequencies indicated that 25% and 31% of total genetic diversity in *S. leprosula* and *S. parvifolia*, respectively, were attributed to the differences among populations. Likewise, AMOVA analysis at two hierarchical levels exhibited that most genetic variation resided within populations with a proportion of 70.2% for *S. leprosula* and 66.2% for *S. parvifolia*. The AMOVA at three hierarchical levels based on the complete data set revealed that the genetic difference between the two species was remarkably higher with a proportion of 44.1% than differences within and among populations (38.1% and 17.8%, respectively). The genetic differentiation between islands was significant for *S. leprosula* but not for *S. parvifolia*. The observed genetic diversity within populations and genetic differentiation among populations agreed with the life history traits of *Shorea* species. Some specific AFLP markers were found, which show high frequency differences between species, between islands and among regions, and can serve as diagnostic markers for the identification of wood of different species, from different islands and regions.

5.3 Genetic variation in nine *Shorea* species in Indonesia revealed by AFLPs (see part 3)

Shorea is the largest and most important genus of the Dipterocarpaceae. The genetic variation in nine *Shorea* species from two different locations, namely Nanjak Makmur on Sumatra and

Sumalindo on Kalimantan (Borneo) in Indonesia were evaluated using AFLP markers. A total of 274 trees were investigated at 85 polymorphic AFLP loci, including 141 individuals of six species from Nanjak Makmur Sumatra and 133 individuals of five species from Sumalindo Borneo. The results showed similar levels of mean genetic variation for species from Nanjak Makmur Sumatra and from Sumalindo Borneo ($\overline{H_e}$ = 0.138 for Sumatra; $\overline{H_e}$ = 0.129 for Borneo). *S. blumutensis* and *S. dasyphylla* from Nanjak Makmur Sumatra possessed the highest genetic diversity with H_e of 0.165 and H_e of 0.164, respectively. *S. acuminata* from Nanjak Makmur Sumatra harboured the lowest genetic diversity with H_e of 0.100. The hypothesis that widespread species (e.g. *S. leprosula* and *S. parvifolia*) show a higher level of genetic variation than rare species (*S. blumutensis*, *S. dasyphylla*) is rejected. AMOVA analysis revealed that the genetic variation was mainly found among species both in Nanjak Makmur Sumatra (57.7%) and in Sumalindo Borneo (56.3%). Surprisingly, the UPGMA dendrogram of all samples revealed an almost complete separation of clusters according to species affiliation. Thus, AFLP markers proved appropriate to dissection of phylogenetical relationships among *Shorea* species. Species-specific markers with high frequencies (> 80%) have been detected in two species (*S. platyclados* and *S. johorensis*). Several other markers showed high frequency differences among species, and between regions within species (for *S. leprosula* and *S. parvifolia* that are represented in both regions). The homology of equal-sized AFLP fragments has to be confirmed by sequencing. Sequence information can be used to develop specific PCR markers for wood identification purposes.

In conclusion, AFLPs proved to be appropriate markers to reveal phylogenetic relationships. Genome wide variation patterns at AFLPs are largely congruent with taxonomic classifications and phylogenies derived from cpDNA markers (sequence data, PCR-RFLPs) (part 1). Despite the relatively high amount of within-species variation, species can be clearly distinguished at AFLP markers (part 2 and part 3). Analysis of nine *Shorea* species revealed that more than 50% of the total variation was among species. In most cases, species identification was unambiguous at AFLP markers. In addition, significant differentiation among populations from different geographical regions was detected for the widespread species *S. leprosula* and S. *parvifolia* (part 2). Thus AFLPs can be considered as a very powerful tool to differentiate between populations of one species, but also among different dipterocarp species (see also part 1). Specific AFLP markers with high discriminative power among regions or among species can be selected and characterised more closely by sequence analysis.

Similar levels of variation were detected for rare and widespread *Shorea* species (part 2 and part 3). The exact reasons for the rather low variance of diversity estimates are unknown, but it is unlikely that genetic drift had a strong impact on genetic structures in the recent past of some populations, but not in others. Thus, the result confirmed previous findings that dipterocarps, like most other tropical trees, are able to avoid very low effective population sizes even if they occur in low density.

The results of these studies can be applied within the context of the development of strategies for the conservation of genetic resources of dipterocarps, and as a basis for the development of molecular tools to identify the origin of dipterocarp wood.

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Part 1

Genetic variation at AFLPs for the Dipterocarpaceae and its relation to molecular phylogenies and taxonomic subdivisions

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Abstract: Genetic differentiation was investigated among 54 Indonesian species of Dipterocarpaceae, a dominant tree family in Asian tropical rainforests, using AFLP markers. The resultant UPGMA tree clearly separated all investigated dipterocarps into two major groups corresponding to tribe Dipterocarpeae and to tribe Shoreae. These results are in accordance with the topology of molecular phylogenetic trees derived from PCR-RFLP analysis of chloroplast DNA, and generally support the traditional taxonomic assessments. The possibility of interspecific hybridization is also discussed.

Key words: AFLPs, chloroplast DNA, Dipterocarpaceae, PCR-RFLPs, phylogeny, tropical rainforests.

Dipterocarpaceae are an important pantropical tree family. It comprises three subfamilies according to recent classifications by Ashton (1982), Londoño et al. (1995) and Morton (1995): Dipterocarpoideae from tropical Asia with approximately 470 species in 13 genera, Monotoideae with 39 species in two genera from Africa and the new monotypic genus *Pseudomonotes* from Colombia in South America, and the monotypic subfamily Pakaraimoideae from Guyana in South America. Many tropical forests in Asia are dominated by dipterocarps (Dipterocarpoideae), and can be regarded as hot spots of global biological diversity. The timber industry of many Southeast-Asian countries critically depends on wood of dipterocarps. Indonesia, in particular Sumatra and Kalimantan (Borneo), is the region of the highest species diversity of the Dipterocarpaceae. The taxonomic subdivision of the family Dipterocarpaceae and its phylogeny remain in dispute (Maury 1979; Kostermans 1985; Takhtajan 1997). Attempts have been made to clarify the phylogeny of Dipterocarpaceae based on PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism) markers and nucleotide sequences of chloroplast DNA genes (Tsumura et al. 1996; Kajita et al. 1998; Morton et al. 1999; Dayanandan et al. 1999), RAPD (Random

Amplified Polymorphic DNA) markers (Rath et al. 1998), and more recently, partial sequences of nuclear genes (Kamiya et al. 2005). The objectives of the present study are to assess genetic differentiation among Indonesian dipterocarp trees of 54 species in nine genera based on AFLP (Amplified Fragment Length Polymorphism; Vos et al. 1995) markers, and to compare our results with those of a phylogenetic study based on PCR-RFLPs of five specific chloroplast regions using the same material (Indrioko 2005; Indrioko et al., submitted). Compared with phylogenetic studies based on PCR-RFLPs, gene sequences and RAPDs, AFLP assays can detect large numbers of polymorphic loci distributed throughout the genome without previous knowledge of sequence information (Powell et al. 1996), and AFLP markers are highly reproducible (Jones et al. 1997). Thus the AFLP marker technique will provide additional information on genome-wide differentiation patterns between species. This technique has been successfully used to analyse differentiation at the species or genus level (Sharma et al. 1996; Koopman et al. 2001; Brouat et al. 2004; Banfer et al. 2004; Gailing and Wuehlisch 2004). We expect to obtain novel insights into the evolution of dipterocarps in Southeast-Asia by the combination of different molecular marker techniques with traditional taxonomic assessments in order to provide more accurate information on phylogenetic relationships and novel tools to identify specific groups with diagnostic markers for the conservation of dipterocarps.

Leaf samples of 81 trees from 54 species belonging to nine genera and two tribes (Dipterocarpeae and Shoreae) were collected from natural forests on Sumatra and Kalimantan (Borneo), the arboretum Haurbentes (Java) and Bogor Botanical Garden (Java) in Indonesia (Table 1).

Total genomic DNA was extracted from small piece (about 2 cm^2) of silicagel-dried leaf tissue following the Dneasy 96 Plant Kit protocol of the manufacturer (Qiagen, Hilden, Germany). AFLP reactions were performed as described by Vos et al. (1995) with minor modifications. The genomic DNA was digested with the two restriction enzymes *Eco*RI/*Mse*I. The corresponding double-stranded (ds) *Eco*RI-adapter and *Mse*I-adapter were ligated to the ends of the restriction fragments to generate template DNA for PCR amplification. Fragments were amplified with two primer pairs complementary to the adapters and restriction site sequences with one to three additional “selective” nucleotides at their 3’- ends. The *Eco*RI + 1 primer (E01) and *Mse*I + 1 primer (M03) with selective nucleotides A and G, respectively, were used for a preselective amplification. The *Eco*RI + 3 primer (E35) with ACA as selective nucleotides was combined with *Mse*I + 3 primer (M63) with GAA as selective

nucleotides for the selective amplification. Primer E35 was labelled with fluorescent dye 6-FAM. All PCR reactions were performed in the Peltier Thermal Cycler (PTC-200 version 4.0, MJ Research). PCR-amplification products were diluted in proportion of 1:20. Then, 2 µl diluted product of each probe was mixed with 12 µl Hi-Di^{TM} formamide and the internal size standard GeneScan 500 ROX (2 µl for 96 samples) from Applied Biosystems, and run on an automatic sequencer (ABI PRISM 3100 Genetic Analyser, Applied Biosystems). The size of the AFLP fragments was assessed with the software packages GeneScan 3.7 and Genotyper 3.7 (Applied Biosystems).

A total of 125 polymorphic markers of the AFLP bands within a readable range from 50-500 bps was manually selected and scored for presence (1) and absence (0) of a band at a particular position. Results were converted into a binary character matrix for further analyses. An UPGMA (Unweighted Pair Group Method using Arithmetic Averages) dendrogram was generated with the software package PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford 1998) based on the mean number of pairwise character differences.

The results of the present study are shown in Fig. 1. Variation was observed both among and within species, if more than one plant of a particular species was investigated. However, plants belonging to the same species were more similar to each other than to any other individuals, confirming the suitability of the selected method to assess species differentiation. The observed differentiation among species generally supported the taxonomic classification of the Dipterocarpaceae by Ashton (1982). All the investigated dipterocarps divided into two major groups, one corresponding to tribe Dipterocarpeae with an haploid chromosome number of $x = 11$ (upper cluster, containing genera *Dipterocarpus, Anisoptera, Cotylelobium*, *Upuna* and *Vatica*), the other corresponding to tribe Shoreae with $x = 7$ (lower cluster, containing genera *Hopea*, *Parashorea*, *Shorea* and *Dryobalanops*) (Ashton 1982). Only *Shorea fallax* did not fall into these main clusters. Robustness of groups is supported by bootstrap consensus values (Felsenstein 1985) larger than 50% indicated at branch nodes. In tribe Dipterocarpeae, all genera were clearly resolved. Members of *Anisoptera* formed a strongly supported monophyletic group with a bootstrap value of 92 sister to genus *Dipterocarpus. Cotylelobium* and *Upuna* were closely related with a bootstrap value of 67, and have affinity to the *Vatica* cluster. *V. bantamensis* (two trees, section *Sunaptea*) is sister to species of *Vatica* section *Vatica* (*V. venulosa, V. bella, V. granulata, V. rassak* and *V. pauciflora*). Within tribe Shoreae except *S. fallax*, *Dryobalanops* with bootstrap value of 87

and *S. virescens* with bootstrap value of 100 formed a monophyletic group basal to the remaining branches. Most species of *Hopea* and *Shorea* were clearly separated. The species of *Hopea* and *Parashorea* formed a cluster sister to the species-rich group of *Shorea*. Diagnostic markers (consistency index CI = 1), which allow to unambiguously identify genera, species groups or species, are found and marked with small vertical bars on the corresponding branches.

The Asian dipterocarps have been classified into two tribes based on morphology, wood-anatomy and cytology (Ashton 1982; Gottwald and Parameswaran 1966). Tribe Dipterocarpeae with the genera *Dipterocarpus, Anisoptera*, *Cotylelobium*, *Upuna* and *Vatica* is characterized by valvate fruit-sepals, scattered resin canals and a basic chromosome number x = 11. Tribe Shoreae includes the genera *Hopea, Parashorea,* and *Shorea*, which have imbricate fruit sepals, resin canals in tangential bands and a basic chromosome number x = 7 in common. Genus *Dryobalanops* combines caryological and morphological characters of tribe Dipterocarpeae and Shoreae (subvalvate fruit-sepals, scattered resin canals and x = 7). Phylogenetic relationship among these dipterocarp trees using PCR-RFLP markers of cpDNA (Indrioko 2005; Indrioko et al., submitted) generally agreed with the traditional classification (Ashton 1982; Gottwald and Parameswaran 1966). In the cpDNA trees, tribe Dipterocarpeae and tribe Shoreae (without genus *Dryobalanops*) formed two monophyletic groups. Dependent on outgroup selection, *Dryobalanops* clustered either with tribe Dipterocarpeae or with tribe Shoreae and occupied a basal position in the cpDNA phylogeny (Indrioko 2005; Indrioko et al., submitted). The resultant UPGMA tree topology of the present study based on AFLPs resembles the relationship inferred from traditional taxonomic assessment (Ashton 1982; Gottwald and Parameswaran 1966) and the phylogeny based on PCR-RFLPs of cpDNA (Indrioko 2005; Indrioko et al., submitted).

Like morphological and caryological characteristics (Ashton 1982; Gottwald and Parameswaran 1966) and PCR-RFLP markers of cpDNA (Indrioko 2005; Indrioko et al., submitted), AFLP markers also did not support an unambiguous allocation of genus *Dryobalanops* to tribe Shoreae. In the present study, genus *Dryobalanops* grouped together with *S. virescens* basal to the major group of tribe Shoreae. Furthermore, it shares the chromosome number x = 7 with tribe Shoreae, but wood anatomical characters with tribe Dipterocarpeae. Fruit-sepal aestivation is intermediary (subvalvate type) between tribe Dipterocarpeae (valvate) and tribe Shoreae (imbricate) (Maury-Lechon and Curtet 1998). Thus, the basal position of *Dryobalanops* to either tribe Shoreae or tribe Dipterocarpeae in AFLP and cpDNA trees supported the morphological and carylogical characters.

In the present study, genus *Parashorea* clustered together with *Hopea.* In the cpDNA analysis (Indrioko 2005; Indriko et al., submitted), *Parashorea* clustered with *Shorea*. Molecular data from Tsumura et al. (1996), Kajita et al. (1998) and Kamiya et al. (2005) also indicated that *Parashorea* is a very close relative of *Shorea*. This incongruence may suggest interspecific hybridization or ancestral polymorphisms.

S. virescens formed a group with *Dryobalanops.* Both are basal to major members of tribe Shoreae in this study. Based on cpDNA variation (Indrioko 2005; Indrioko et al., submitted), *S. virescens*, *S. materialis* and *S. fallax* formed a separate clade basal to the other *Shorea* species and genus *Parashorea*. Thus, a separation of *S. fallax* and *S. virescens* from the other *Shorea* species was observed at cpDNA and at AFLP markers. In the present study, the basal position of *S. fallax* and *S. virescens* to the other members of tribe Shoreae (*Hopea*, *Parashorea* and *Shorea*) may be related to high within-species variation as shown for *S. fallax* in a phylogenetic tree derived from the nuclear gene *PgiC* (Kamiya et al. 2005). In the latter study, the diagnostic haplotypes of *S. fallax* fell into five different subclades of clade "Red Meranti", suggesting the possibility of introgressive hybridization. However, shared ancestral polymorphisms or balancing selection may also have led to ambiguous results in phylogenetic analyses (Kamiya et al. 2005).

Different sections within the species-rich genera *Hopea* and *Shorea* have been proposed by Ashton (1982). These sections were poorly separated by variation at AFLPs in this study and by the variation observed at cpDNA (Indrioko 2005; Indrioko et al., submitted). However, Kamiya et al. (2005) were able to separate different timber groups corresponding to particular sections within *Shorea* based on partial sequences of the *PgiC* gene.

In contrast to the phylogenetic tree obtained from cpDNA analysis, in the present AFLP study, moderate to high bootstrap support could only be obtained for a large cluster within *Dipterocarpus* (78), for the genus *Anisoptera* (92), for the cluster of *Cotylelobium lanceolatum* and *Upuna borneensis* (67) and for the genus *Dryobalanops* (87) in addition to clear separation at the species level.

AFLP fragments from different descent can be the same in size. Due to the difficulty in identifying homologous alleles (Mueller and Wolfenbarger 1999), interpretation of these non-homologous fragments as homologous markers is not avoidable and causes homoplasy in phylogenetic trees. Hence, low bootstrap support at the genus level and also dubious grouping of *Parashorea* may be related to a high level of size homoplasy revealed by AFLP markers.

Additionally, the number of detected diagnostic AFLP bands in the present study is limited, and supported only a few clusters. However, the major results of cpDNA analyses are

supported by AFLP differentiation patterns: the intermediate position of *Dryobalanops*, the basal position S. *fallax* and *S. virescens*, the subdivision in tribe Dipterocarpeae and Shoreae and a quite well resolution of genera with some exceptions.

AFLP fragments that are diagnostic for specific groups of taxa or that show strong frequency differences between taxa will be characterised more closely. These markers can be used as a tool for the certification of wood from different origins to reduce illegal logging activities.

In summary, our results supported the usefulness of AFLP markers to distinguish species and to differentiate genera and tribes within the subfamily Dipterocarpoideae. At this level, genetic variation observed at AFLPs is largely consistent with molecular phylogenies and the taxonomic subdivision of the subfamily. However, size homoplasy effects and considerable variation at AFLPs within species (Cao et al., in prep.) are regarded as main reasons for a poor differentiation of species and sections within genera.

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Table 1. Samples used in this study. Classification is based on Ashton (1982).

Tribe, genus and species	No. of samples	Sampling location	Origin
Tribe: DIPTEROCARPEAE (*x* = 11)			
Dipterocarpus			
• *D. grandiflorus* BLCO	2	Arboretum, Haurbentes	Borneo
• *D. oblongifolius* BL.	1	Botanical garden, Bogor	Borneo
• *D. retusus* BL.	1	Botanical garden, Bogor	Java
• *D. rigidus* RIDL.	1	Botanical garden, Bogor	Sumatra
• *D. tempehes* SLOOT.	1	Arboretum, Haurbentes	Borneo
Anisoptera			
section Anisoptera			
• *A. costata* KORTH.	2	Arboretum, Haurbentes	Borneo
• *A. marginata* KORTH.	1	Botanical garden, Bogor	Borneo
• *A. reticulata* ASHTON	1	Natural forest, Malinau	Borneo
Cotylelobium			
• *C. lanceolatum* CRAIB	1	Botanical garden, Bogor	Borneo
Upuna			
• *U. borneensis* SYM.	1	Botanical garden, Bogor	Borneo
Vatica			
Section *Sunaptea*			
• *V. bantamensis* (HASSK.) B. & H. *ex* MIQ.	2	Botanical garden, Bogor	Java
Section *Vatica*			
• *V. bella* SLOOT.	1	Botanical garden, Bogor	Sumatra
• *V. granulata* SLOOT.	1	Botanical garden, Bogor	Sulawesi
• *V. pauciflora* (KORTH.) BL.	2	Botanical garden, Bogor	Sumatra
• *V. rassak* (KORTH.) BL.	1	Botanical garden, Bogor	Maluku
• *V. venulosa* BL.	1	Botanical garden, Bogor	Sumatra
Tribe: SHOREAE (*x* = 7)			
Dryobalanops			
• *D. aromatica* GAERTN. F.	1	Botanical garden, Bogor	Sumatra
• *D. lanceolata* BURCK	2	Botanical garden, Bogor	Borneo
Parashorea			
• *P. globosa* SYM.	1	Botanical garden, Bogor	Sumatra
• *P. lucida* (MIQ.) KURZ	1	Botanical garden, Bogor	Sumatra
Hopea			
Section *Dryobalanoides*			
• *H. dryobalanoides* MIQ.	2	Arboretum, Haurbentes	Sumatra
• *H. griffithii* KURZ	1	Botanical garden, Bogor	Borneo
• *H. mengerawan* MIQ.	2	Arboretum, Haurbentes	Sumatra
• *H. nigra* BURCK	1	Botanical garden, Bogor	Borneo
Section *Hopea*			
• *H. bancana* (BOERL.) SLOOT.	2	Arboretum, Haurbentes	Sumatra
• *H. celebica* BURCK	1	Botanical garden, Bogor	Sulawesi

Tribe, genus and species	No. of samples	Sampling location	Origin
• *H. odorata* ROXB.	2	Arboretum, Haurbentes	Sumatra
• *H. sangal* KORTH.	2	Arboretum, Haurbentes	Sumatra
Shorea			
Section *Mutica*			
• *S. acuminata* DYER	3	Natural forest, Tebo	Sumatra
• *S. leprosula* MIQ.	2	Arboretum, Haurbentes	Borneo
• *S. parvifolia* DYER	2	Arboretum, Haurbentes	Sumatra
• *S. macroptera* DYER	1	Botanical garden, Bogor	Sumatra
Section Brachypterae			
• *S. andulensis* ASHTON	1	Natural forest, Muara Teweh	Borneo
• *S. balangeran* (KORTH.) BURCK	1	Botanical garden, Bogor	Sumatra
• *S. platyclados* SLOOT. *ex* ENDERT	2	Arboretum, Haurbentes	Sumatra
• *S. scaberrima* BURCK	1	Botanical garden, Bogor	Borneo
• *S. johorensis* FOXW.	2	Arboretum, Haurbentes	Borneo
• *S. palembanica* MIQ.	2	Arboretum, Haurbentes	Borneo
• *S. selanica* BL.	2	Arboretum, Haurbentes	Moluccas
• *S. fallax* MEIJER	1	Botanical garden, Bogor	Borneo
Section Anthoshorea			
• *S. javanica* K. & V.	1	Botanical garden, Bogor	Java
• *S. montigena* SLOOT.	1	Botanical garden, Bogor	Moluccas
• *S. virescens* PARIJS	2	Arboretum, Haurbentes	Borneo
Section Pachycarpae			
• *S. mecistopteryx* RIDL.	3	Arboretum, Haurbentes	Borneo
• *S. macrophylla* (DE VRIESE) ASHTON	2	Arboretum, Haurbentes	Borneo
• *S. splendida* (DE VRIESE) ASHTON	2	Arboretum, Haurbentes	Borneo
Section Ovalis			
• *S. ovalis* (KORTH.) BL.	2	Arboretum, Haurbentes	Sumatra
Section Richetioides			
• *S. blumutensis* FOXW.	2	Natural forest, Tebo	Sumatra
• *S. xanthophylla* SYM.	2	Natural forest, Tebo	Sumatra
• *S. acuminatissima* SYM.	1	Natural forest, Ketapang	Borneo
• *S. faguetiana* HEIM	1	Botanical garden, Bogor	Borneo
Section Shorea			
• *S. seminis* (DE VRIESE) SLOOT.	1	Arboretum, Haurbentes	Borneo
• *S. guiso* (BLCO) BL.	2	Arboretum, Haurbentes	Borneo
• *S. materialis* RIDL.	1	Botanical garden, Bogor	Sumatra
Sum	81		

Legend: Latitude and longitude of each sampling location

1. Arboretum, Haurbentes, Jasinga, West Java: 6°10' - 6°35' S and 106°20'-106°30' E
2. Botanic garden, Bogor, West Java: 6°36' - 6°37' S and 106°32'-106°33' E
3. Natural forest, Tebo, Jambi, Sumatra: 1°00' - 1°45' S and 102°00'-102°45' E
4. Natural forest, Malinau, East Borneo: 2°45' - 3°21' N and 115°48'-116°34' E
5. Natural forest, Muara Teweh, Central Borneo: 0°00' - 0°20' S and 114°30'-115°10' E
6. Natural forest, Ketapang, West Borneo: 1°00' - 1°15' S and 110°45'-111°00' E

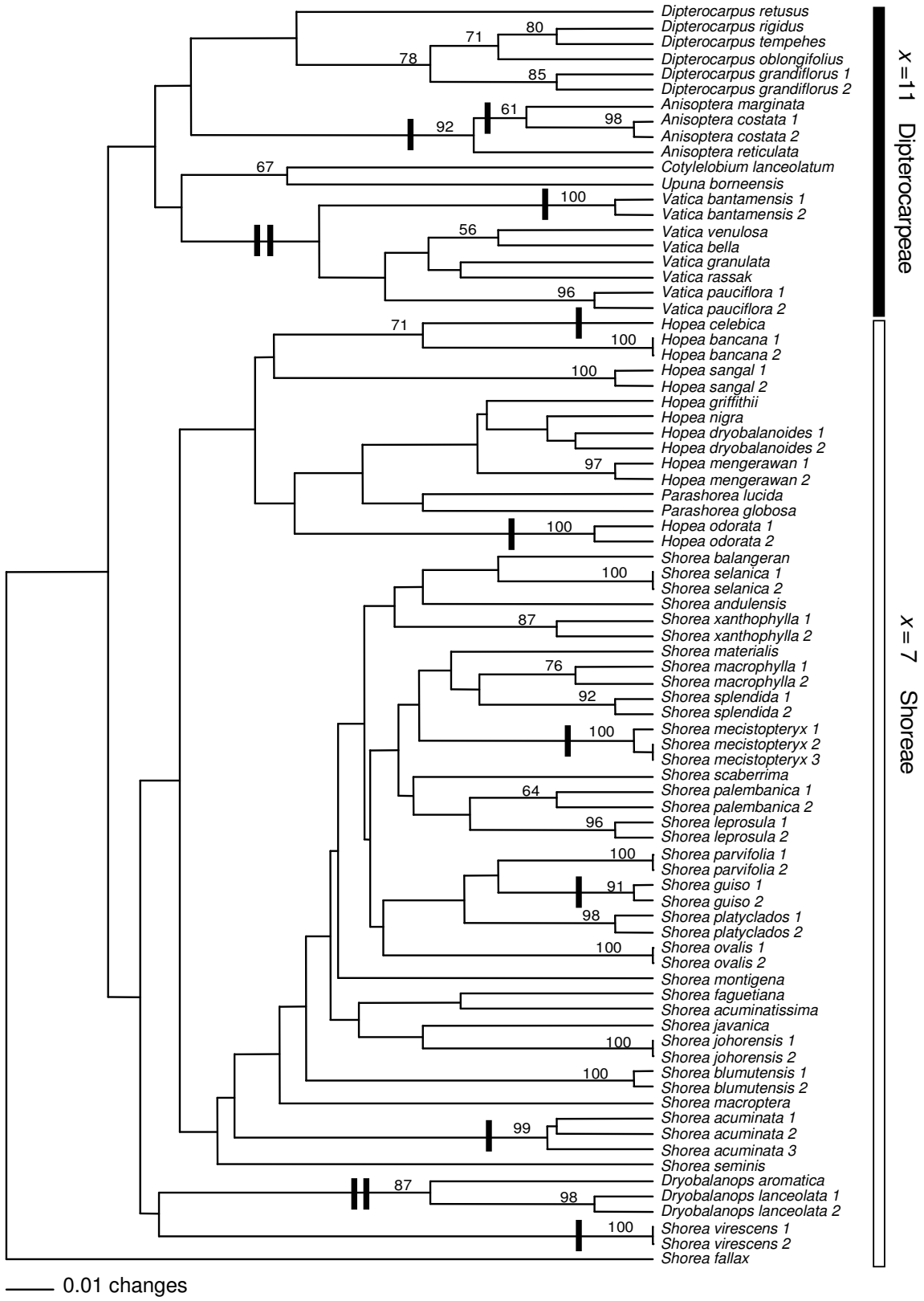

Fig. 1. UPGMA dendrogram generated with program PAUP version 4.0 (Swofford 1998), using 81 samples of 54 species in nine genera based on 125 AFLP fragments. The upper cluster corresponds to tribe Dipterocarpeae with an haploid chromosome number $x = 11$; the lower cluster to tribe Shoreae with $x = 7$. Numbers at branch nodes indicate UPGMA bootstrap support (1000 replicates). Small vertical bars on branches show clusters or species that are supported by diagnostic AFLP bands (consistency index CI=1).

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Part 2

Genetic diversity within and among populations of *Shorea leprosula* Miq. and *S. parvifolia* Dyer (Dipterocarpaceae) in Indonesia detected by AFLPs

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Abstract: Genetic diversity within and among populations of *Shorea leprosula* and *S. parvifolia* from Indonesia was investigated using AFLP markers. The results indicated that *S. leprosula* is genetically more variable than *S. parvifolia*. At the population level, a higher level of genetic diversity was revealed for *S. leprosula* with a percentage of polymorphic loci (PPL_p) of 53.32% and an expected heterozygosity (H_{ep}) of 0.16 in comparison with *S. parvifolia* showing PPL_p of 51.79% and H_{ep} of 0.14. At the species level, *S. leprosula* showed PPL_s of 92.86% and H_{es} of 0.21, while *S. parvifolia* showed PPL_s of 85.71% and H_{es} of 0.21. Genetic differentiation (G_{st}) indicated that 25% and 31% of total genetic diversity in *S. leprosula* and *S. parvifolia*, respectively, were attributed to the differences among populations. AMOVA analysis at two hierarchical levels exhibited that most genetic variation resided within populations with a proportion of 70.2% for *S. leprosula* and 66.2% for *S. parvifolia*. The AMOVA at three hierarchical levels based on the complete data set revealed that the genetic difference between the two species was remarkably higher with a proportion of 44.1% than differences within and among populations (38.1% and 17.8%, respectively). The genetic differentiation between islands was significant for *S. leprosula* but not for *S. parvifolia*. The observed genetic diversity agreed with the life history traits of *Shorea* species. Highly differentiating individual AFLP markers were found for each species, which will serve as diagnostic markers for the identification of wood of different species, from different islands and regions.

Keywords: AFLP, conservation, genetic diversity, *Shorea leprosula, S. parvifola,* tropical rainforests

Introduction

Shorea leprosula Miq. and *S. parvifolia* Dyer are the most common and widespread emergents of lowland dipterocarp forests in Indonesia, particularly on Sumatra and Borneo (Kalimantan). They are very important timber trees and the main sources of light red meranti (Ashton 1982; Newman et al. 1996). Recently, due to the exploitation for their valuable timber, both species are heavily affected by deforestation, forest fragmentation, and nonsustainable forest management including selective logging. Since the stability and the evolutionary potential of a species depend on its genetic variation, it is important to obtain knowledge of the amount of genetic diversity in order to provide information for the development of strategies for the conservation and sustainable utilization of a species.

The development of high-resolution DNA-based genetic markers facilitates assessment of genetic diversity in plant species. In contrast to pedigree data, morphological, physiological, cytological and many biochemical markers, DNA-based molecular markers can reveal variation among accessions directly at the DNA level, and are not affected by environmental factors. The most commonly used DNA markers for the fingerprinting of plant genomes and genetic diversity studies include restriction fragment length polymorphisms (RFLPs) (Botstein et al. 1980), random amplified polymorphic DNA (RAPDs) (Welsh and McClelland 1990), microsatellites or simple sequence repeats (SSRs) (Morgante and Olivieri 1993) and amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995). Since AFLP markers are distributed across the whole genome, they have a high multiplex ratio, meaning a large number of different genetic loci that may be simultaneously analysed per experiment (Pejic et al. 1998). Additionally, due to other advantages such as high reproducibility (Jones et al. 1997), a high degree of resolution for discriminating closely related germplasms (Gagne et al. 2000), no sequence information prior to the generation of AFLP fingerprints and high transferability across species, the AFLP marker technique is a very effective, fast and reliable tool to reveal restriction fragment polymorphisms in contrast to other markers (Vos et al. 1995). Many studies have applied this technique not only to DNA fingerprinting (Vos et al. 1995) and genetic diversity studies (Russell et al. 1997; Paul et al. 1997; Kremer et al. 2005), but also to genome mapping (Becker et al. 1995), phylogenetic studies (Aggarwal et al. 1999; Zhang et al. 2001) and parentage analysis (Lima et al. 2002).

To date, several studies have been conducted on the genetic variation of *Shorea* species using RAPD markers (Harada et al. 1994), allozyme markers (Lee et al. 2000a), SSR markers (Nagamitsu et al. 2001; Obayashi et al. 2002; Lee et al. 2004; Ng et al. 2004) and DNA sequences (Ishiyama et al. 2003). However, the application of the AFLP technique to *Shorea*

and other dipterocarp trees has rarely been reported (Luu 2005). The purpose of the present study is to estimate the genetic variation within and among populations of *S. leprosula* and the closely related species *S. parvifolia* using the AFLP technique. We want to test the following hypotheses: (1) there is high variation within and between species; (2) AFLPs are applicable to differentiate the two species; (3) individual AFLP markers can show high differentiation among geographic regions for one species, and thus are useful for wood certification purposes; (4) hybridization between species is a rare event.

Material and methods

Leaves of adult trees and saplings were collected from 12 large natural populations on Sumatra and Borneo and one plantation on Java, which are located in seven regions for *S. leprosula* (AS, NS, PS, TS, SB, TB and HA) and six regions for *S. parvifolia* (AS, NS, PS, TS, SB, BB). Information on the geographic distribution and sample size of the sampled populations for the two species is shown in Fig. 1 and Table 1. The collection site in each population had an area of 100-300 ha**.** A minimum distance of 30 m was kept between sample trees. The estimated density for *S. leprosula* and *S. parvifolia* is 0.15 ~ 3 trees per hectare. Species identification was done on the basis of leaf morpholgical characters (e.g., leaf length, petiole length, leaf width**,** distance from petiole to the widest part of the leaf, number of venation, number of lobes, dometiana length, leaf shape).

DNA extraction

Total genomic DNA was extracted from a small slice (approx. 2 cm^2) of dried leaf tissue following the Dneasy 96 Plant Kit protocol of the manufacturer (Qiagen, Hilden, Germany). Then, five µl DNA was run electrophoretically on a 0.8 % agarose gel at 100 V in TAE buffer, visualized by staining with ethidium bromide, and photographed in ultraviolet light to check DNA quantity and quality. Extracted DNA was stored at -20 °C.

AFLP analyses

AFLP analyses were carried out according to the protocol of Vos et al. (1995) with minor modifications. The total genomic DNA of each sample was digested simultaneously with the two restriction enzymes *Eco*RI and *Mse*I. The double-stranded (ds) *Eco*RI and *Mse*I adaptors were ligated to the ends of the restriction fragments to generate template DNA for PCR amplification, which consists of two successive steps. The preselective amplification

amplified the restricted DNA fragments with the primer combination E01/M03, each having one selective nucleotide A and G, respectively. These products were used for the selective amplification with the primer combination E35/M63, each possessing the three selective nucleotides ACA and GAA, respectively. Primer E35 was labelled with fluorescent dye 6-FAM. All PCR reactions were conducted in a Peltier Thermal Cycler (PTC-200 version 4.0, MJ Research). The amplified restriction products were resolved electrophoretically on an ABI Genetic Analyser 3100 together with the internal size standard GeneScan 500 ROX (fluorescent dye ROX) from Applied Biosystems. The size of the AFLP fragments was determined with the software packages GeneScan 3.7 and Genotyper 3.7 (Applied Biosystems).

Data analyses

Each band in AFLP fingerprint pattern was considered as a separate putative locus. Only the loci with clearly amplified bands were manually selected and scored for presence (1) and absence (0) of a band. Then, the binary matrices of AFLP phenotypes were assembled for different analyses.

Genetic parameters were computed using the POPGENE version 1.31 software (Yeh et al. 1999) under the assumption of Hardy-Weinberg equilibrium, including the percentage of polymorphic loci (*PPL*; Nei 1973), the number of alleles per locus (n_a), effective number of alleles per locus (n_e; Hartl and Clark 1989), gene diversity (h = expected heterozygosity H_e; Nei 1973), Shannon's information index introduced in population genetics by Lewontin (1972) indicating the degree of AFLP polymorphism within populations using the formula: $I = -\Sigma p_i \log_2 p_i$, where p_i is the frequency of the presence or absence of a given AFLP phenotype (band), total genetic diversity (H_t), genetic diversity within populations (H_s), genetic diversity among populations (D_{st}), the relative magnitude of genetic differentiation among populations ($G_{st} = D_{st} / H_t$; Nei 1987) and gene flow (N_m, the numbers of migrants per generation) estimated from G_{st} according to McDermott and McDonald (1993), where $N_m = 0.5(1-G_{st}) / G_{st}$. Allelic frequencies were calculated here based on the square root of the frequency of the null (recessive) allele. Nei's (1978) unbiased measures of genetic identity and genetic distance were calculated for all pairwise combinations of populations studied here. An UPGMA (Unweighted Pair Group Method using Arithmetic Average) dendrogram, as suggested by Sneath and Sokal (1973), was constructed using the program NTSYS-pc version 2.0 (Rohlf 1998) based on Nei's genetic distances (1978). An analysis of molecular variance (AMOVA) based on the pairwise squared Euclidean distances among molecular phenotypes was carried

out applying ARLEQUIN version 2.0 (Schneider et al. 2000) to partition the genetic diversity at two or three hierachical levels as described by Excoffier et al. (1992). Pairwise F_{st} values obtained from AMOVA were used to measure the genetic differentiation between populations, which are analogous to G_{st} if a locus consists of two alleles as applicable in dominant marker analysis (e.g., RAPD; Nybom and Bartish 2000). The significance levels for AMOVA were obtained after 1023 random permutations. To test the correlation between genetic distances and geographic distances of natural populations, Mantel tests (Mantel 1967) were conducted separately for both species without plantation *S. lepr*_HA using programs JeEep.com-Coordinate Translation (http://jeeep.com/details/coord/) and GenALEx version 5 (Peakall and Smouse 2001). The correlation coefficient r and the their corresponding *p* value were calculated with 999 random permutations.

Additionally, another UPGMA clustering indicating the patterns of variation within and among populations was performed with the program NTSYS-pc version 2.0 (Rohlf 1998) based on similarity values calculated from all possible pairwise combinations of individuals using the Dice index (Dice 1945), which emphasizes shared traits among individuals (AFLP bands in this study) and ignores the absence of shared traits. The cophenetic correlation coefficient was calculated using the Mantel z statistic in order to test the goodness of fit of the UPGMA cluster analysis to the distance data (Mantel 1967; Rohlf 1998). A value of $r > 0.9$ is considered a very good fit.

Results

Genetic diversity

A total of 56 clear, repeatable bands was scored, ranging in size from 63 bp to 483 bp. Table 2 shows that the genetic diversity varied among populations and between the species *S. leprosula* and *S. parvifolia*. In general, populations of *S. leprosula* showed a higher genetic variation with the mean values of $PPL_p = 53.32\%$, $n_{ap} = 1.533$, $n_{ep} = 1.274$, $H_{ep} = 0.161$ and $I_p = 0.245$ relative to populations of *S. parvifolia* with the mean values of $PPL_p = 51.79\%$, $n_{ap} = 1.518$, $n_{ep} = 1.227$, $H_{ep} = 0.138$ and $I_p = 0.215$. At the species level, *S. leprosula* harbored also higher values of genetic diversity with $PPL_s = 92.86\%$, $n_{as} = 1.929$, $n_{es} = 1.347$, $H_{es} = 0.211$ and $I_s = 0.330$ than *S. parvifolia* with $PPL_s = 85.71\%$, $n_{as} = 1.857$, $n_{es} = 1.336$, $H_{es} = 0.205$ and $I_s = 0.319$. At the regional level, Asialog Sumatra (AS) had the highest diversity values of all parameters both for *S. leprosula* ($PPL = 66.07\%$, $n_a = 1.661$, $n_e = 1.355$, $H_e = 0.208$, $I = 0.316$) and for *S. parvifolia* ($PPL = 76.79\%$, $n_a = 1.768$, $n_e = 1.371$, $H_e = 0.222$, $I = 0.337$), whereas Tering Borneo (TB) and TNBT Sumatra (TS) had the lowest diversity values in *S. leprosula*

(n_e =1.184, H_e = 0.115, I = 0.182) and *S. parvifolia* (n_e = 1.159, H_e = 0.097, I = 0.152), respectively. In *S. leprosula*, population *S. lepr*_HA did not show the highest diversity values in spite of its greatest sample size. Comparing the populations from the same regions, *S. leprosula* exhibited a higher level of genetic diversity than *S. parvifolia* regardless of sample size except for population *S. lepr*_AS with only nine samples.

Genetic structure

The results of AMOVA are shown in Table 3. The AMOVA with three hierarchical levels based on the complete data set revealed that 44.1% of the total variation was due to the difference between the two species and higher than the proportion of within-population variation (38.1%) and among-population variation within species (17.8%). AMOVAs at two or three hierarchical levels performed separately for *S. leprosula* and *S. parvifolia* indicated that the main portion of the total variation resided within populations. At two hierarchical levels, the within-population variation in *S. leprosula* (70.2%) was higher than in *S. parvifolia* (66.2%), and the among-population variation in *S. leprosula* (29.8%) was lower than in *S. parvifolia* (33.8%). At three hierarchical levels based on the geographical regions (islands), a significant proportion of the total variation in *S. leprosula* (30.2%) was attributable to the difference between islands, while variation between islands was not significant in *S. parvifolia* (10.2%, p = 0.073). The pairwise F_{st} values (Table 4) generated from AMOVA indicated that most of the populations were significantly differentiated ($p < 0.05$) with F_{st} values ranging from 0.051 to 0.722, except for population pair *S. lepr*_PS / *S. lepr*_TS showing F_{st} value of 0.026 ($p > 0.05$). This low differentiation value suggests gene exchange between the two populations on Sumatra. Similar to the results of AMOVA at two hierarchical levels, the genetic differentiation among populations (G_{st}) and gene flow (N_m) calculated by POPGENE software showed lower G_{st} = 0.25 and higher N_m = 1.54 in *S. leprosula* and conversely higher G_{st} = 0.31 and lower N_m = 1.09 in *S. parvifolia*. G_{st} values for individual AFLP markers ranged from 0.014 to 0.963 in *S. leprosula* and from 0.010 to 0.806 in *S. parvifolia*.

Genetic distances and cluster analyses

Table 5 shows the pairwise Nei's (1978) genetic distances between populations. The largest distance (0.386) was found between populations of the two species (*S. lepr*_SB / *S. parv*_BB), the smallest (0.014) between populations of *S. leprosula* (*S. lepr*_HA / *S. lepr*_TS). The distances ranged from 0.014 (*S. lepr*_HA / *S. lepr*_TS) to 0.141 (*S. lepr*_TB / *S. lepr*_NS) in

S. leprosula and from 0.022 (*S. parv*_PS / *S. parv*_NS) to 0.176 (*S. parv*_BB / *S. parv*_NS) in *S. parvifolia*.

An UPGMA dendrogram (Fig. 2) displayed genetic relationships among all the populations investigated based on the matrix of Nei's (1978) genetic distances among populations (Table 5). A total of 13 populations was divided into two major groups, the first one corresponding to *S. leprosula*, the second one corresponding to *S. parvifolia*. In *S. leprosula*-group, populations *S. lepr*_HA and *S. lepr*_TS were genetically the most similar. Two populations from Borneo (*S. lepr*_TB and *S. lepr*_SB) differed clearly from the other populations, and showed a close relationship to each other. The planted population *S. lepr*_HA from Java clustered with the populations from Sumatra, which allows us to assume its natural habitat in Sumatra. *S. lepr*_AS was separated from the other populations of the Sumatra-group. In *S. parvifolia*-group, populations *S. parv*_PS and *S. parv*_NS were genetically the most similar. Two populations from Borneo (*S. parv*_SB and *S. parv*_BB) formed a cluster along with an additional population from Sumatra (*S. parv*_TS), and were separated from the remaining populations from Sumatra. In this group, the population clustering was not strictly in accordance with their geographic origin on Borneo and Sumatra. This is consistent with the results of the AMOVA analysis. According to Mantel tests, the correlations between genetic distances and geographic distances were not significant both in *S. leprosula* without plantation *S. lepr*_HA ($r = 0.504$, $p = 0.068$) and in *S. parvifolia* ($r = 0.193$, $p = 0.240$), but the cluster analysis (Fig. 2) based on the genetic distances among populations reflected geographic proximity except for populations *S. lepr*_HA and *S. parv*_TS.

Genetic relationships among all the individuals of *S. leprosula* and *S. parvifolia* investigated were examined further by another UPGMA cluster analysis based on the Dice (1945) similarity index (Fig. 3a, Fig. 3b). All of 268 individuals were separated into two major clusters, namely an upper cluster (Fig. 3a) containing all of the individuals of *S. leprosula,* and a lower cluster (Fig. 3b) containing all of the individuals of *S. parvifolia*. In general, most individuals from the same region were grouped in the same cluster or close clusters.

In the upper *S. leprosula*-cluster (Fig. 3a), two major groups can be identified. One group contains most individuals from Sumatra island, which belong to populations *S. lepr*_PS, *S. lepr*_TS, *S. lepr*_NS, *S. lepr*_HA, *S. lepr*_AS, and one individual (1413_l_SB) from Borneo. The other group contains most individuals from Borneo island, which belong to populations *S.*

*lepr*_TB and *S. lepr*_SB. Three individuals (606_l_AS, 766_l_PS and 851_l_TS) were isolated from the two major groups.

In the lower *S. parvifolia*-cluster (Fig. 3b), two major subclusters can be identified, one containing all the individuals of populations *S. parv*_BB and *S. parv*_TS, most individuals of population *S. parv*_SB, 17 individuals of population *S. parv*_AS and two individuals from *S. parv*_PS, the other containing all the individuals of population *S. parv*_NS, most individuals of population *S. parv*_PS, 15 individuals of population *S. parv*_AS, and one individual (1384_p_SB) from Borneo. Individual 584_p_AS was separated from the two major subclusters. Individuals of population *S. parv*_AS, which showed the highest genetic diversity (Table 2) in *S. parvifolia*, were scattered throughout the whole lower cluster. In contrast, all samples of population *S. parv*_BB, which showed a low genetic diversity, grouped together in one cluster. Also all but one sample of population *S. parv*_SB grouped together in one cluster.

Generally, the topology of Fig. 3 is similar to that of Fig. 2. The resolution among the individuals from different populations in *S. parvifolia* (Fig. 3b) was more clear than that in *S. leprosula* (Fig. 3a), reflecting that genetic differences among populations in *S. parvifolia* (G_{st} = 0.31) were higher than that in *S. leprosula* (G_{st} = 0.25). The cophenetic correlation indicated a very good fit of the cluster analysis to the data of genetic distances (r = 0.91).

Distribution of AFLP markers in both species

The genetic variation found in this study was based on the differences of AFLP band patterns of the individuals. Fig. 4 shows the differences in the frequencies of all 56 AFLP markers between *S. leprosula* and *S. parvifolia* (top part), which were sorted descendantly, and the frequencies of corresponding markers in *S. leprosula* and *S. parvifolia* (middle and lower part), respectively. The great differences (> 0.6) in frequencies of AFLP markers between the two species indicate high frequencies in one species and very low frequencies or absence in the other. Seven AFLP markers are present in *S. leprosula* and absent in *S. parvifolia*, four markers present in *S. parvifolia* but absent in *S. leprosula*. There are also markers with high frequencies (> 0.6) present both in *S. leprosula* and in *S. parvifolia.*

Furthermore, some amplified AFLP fragments showed remarkable frequency differences not only between species *S. leprosula* and *S. parvifolia*, but also between the islands Borneo and Sumatra and among populations within species. Fig. 5 shows that marker 27 (a) has high frequencies (> 0.8) in *S. leprosula*, but it is absent in *S. parvifolia*; marker 28 (b) is present in *S. parvifolia* with high frequencies (> 0.9), but with very low frequencies (< 0.25) or absent in

S. leprosula; markers 11 (c) and 52 (d) differentiate between the islands Borneo and Sumatra for *S. leprosula*; marker 10 (e) is present in only one population (AS) with high frequency (0.89), but absent in other populations of *S. leprosula*, and present in most populations of *S. parvifolia* with the highest frequency in one population (AS, 0.76); marker 18 (f) is characteristic only for population BB of *S. parvifolia*.

Discussion

Genetic diversity within populations

Like most tropical trees, *S. leprosula* and *S. parvifolia* are predominantly outcrossing (Ashton 1969; Chan 1981; Sakai et al. 1999; Lee et al. 2000b). Outcrossing species with a greater potential for gene movement are typically characterized by high amounts of species-level and within-population diversity (Loveless and Hamrick 1984; Hamrick and Godt 1989). Information on the high genetic diversity within species and populations for *S. leprosula* and *S. parvifolia* is available from previous studies using random amplified polymorphic DNA (RAPD), isozyme and microsatellite markers (Harada et al. 1994; Lee et al. 2000a; Nagamitsu et al. 2001; Lee et al. 2004; Ng et al. 2004). The high level of genetic diversity at both the population and species levels observed for *S. leprosula* based on isozymes (Lee et al. 2000a) is among the highest genetic diversity recorded for tropical rain forest trees (Hamrick and Loveless 1986; Loveless 1992).

For AFLP markers, less comparable data are available in the literature. Estimates of genetic diversity for *S. leprosula* and *S. parvifolia* are moderate as compared to other tree species studied using AFLPs (Table 6). *S. leprosula* contains higher levels of genetic diversity than *S. parvifolia* according to a previous study using microsatellites (Lee et al. 2004). This result is supported in the present study (Table 2), particularly by comparison of the populations of *S. leprosula* and *S. parvifolia* from the same regions (NS, PS, TS, and SB). The slightly lower diversity of population *S. lepr*_AS as compared to population *S. parv*_AS may be due to the small sample size of *S. lepr*_AS.

S. leprosula and *S. parvifolia* are both diploid species (2n = 14; Jong and Lethbridge 1967; Kaur et al. 1978), belong to the same genus *Shorea* section *Mutica* (Ashton 1982), have similar ecological and life history traits (i.e., regionally distributed, long-lived, high fecundity, predominantly outcrossing, animal pollinated, and late successional), which can lead to a high genetic diversity within populations of both species. However, the higher level of genetic diversity in *S. leprosula* as compared with *S. parvifolia* may be due to the species'

evolutionary history, in which the ancestors of *S. leprosula* had acquired very high genetic diversity during speciation (Lee et al. 2000a). This also suggests that *S. leprosula* is more robust to survive under changing natural conditions.

The planted population *S. lepr*_HA showed a lower level of genetic diversity than population *S. lepr*_AS despite its largest sample size, but a higher level than remaining populations of *S. leprosula* from the regions NS, PS, TS, SB and TB. Hence, a "founder effect" resulting from artificial selection of limited number of samples from natural population has not been observed.

Genetic structure

Tropical trees tend to possess most of their genetic diversity within populations (Hamrick and Loveless 1989). Similarly, the analysis of molecular variance (AMOVA) revealed that most of the AFLP diversity resided within populations both for *S. leprosula* (70.2%) and for *S. parvifolia* (66.2%) in this study. The genetic diversity partitioned among populations was also highly significant ($p < 0.001$, Table 3). The overall degree of population differentiation was higher both in *S. leprosula* ($G_{st} = 0.25$) and in *S. parvifolia* ($G_{st} = 0.31$) compared with some other plant species studied at AFLP loci (Table 6). Such results seem to be in contradiction with expectations of low diversity among populations for long-lived and outcrossing species (Loveless and Hamrick 1984; Hamrick and Godt 1989). The ecological and life history traits, i.e. gene flow via limited pollen and seed dispersal, are the important determinants for these results. *Shorea* species are pollinated mainly through small and low-energy insects, beetles (*Chrysomelidae* and *Curculionidae*, Coleoptera) and thrips (*Thrips* and *Megalurothrips*, Thysanoptera), which have extraordinary short generation times (~8 d), and can migrate only short distance (Appanah and Chan 1981; Sakai et al. 1999). Due to gravity, seed dispersal seldom exceeds 50 m radius from the mother trees (Chan 1980). Only populations *S. lepr*_PS and *S. lepr*_TS, which are located in close vicinity (Table 1, Fig. 1) on Sumatra, revealed very low and non-significant genetic differentiation ($F_{st} = 0.026$, $p > 0.05$, Table 4), suggesting a higher degree of gene flow between these populations. *S. parvifolia* holds an overall higher degree of differentiation between populations than *S. leprosula*, indicating lower gene flow in *S. parvifolia* ($N_m = 1.09$) than in *S. leprosula* ($N_m = 1.54$). A barrier to gene flow is reflected by the significant differentiation (30.2%, $p < 0.05$) between the Borneo and Sumatra islands for *S. leprosula*. Conversely, the differentiation between islands is not significant for *S. parvifolia* ($p = 0.07$). In the UPGMA dendrogram (Fig. 3b), all of samples of *S. parv*_TS, most samples of *S. parv*_AS and two samples of *S. parv*_PS from Sumatra grouped together

with *S. parv*_BB and *S. parv*_SB from Borneo. Populations on Sumatra and Borneo are likely to be reproductively isolated from each other, but the islands were connected by a large land area in the recent geological past due to lower sea levels during the last glaciation (Voris 2000). Thus, the non-significant differentiation among islands observed for *S. parvifolia* may reflect the connection of populations on both islands during their evolutionary past.

In contrast to the high level of population differentiation detected in this study, a low level of population differentiation (G_{st} = 0.085) was obtained in a previous study for *S. leprosula* in Peninsular Malaysia based on allozymes (Lee et al. 2000a). The low G_{st} value can be attributed to the limited number of detected loci. In addition, some information on DNA sequences are not expressed at the protein level, reducing the degree of detectable genetic variation. In the present study based on AFLP markers, genetic variation is detected simultaneously at a large number of loci (56 loci) across the entire genome in a single assay. Therefore, the AFLP technique is more effective in measuring population differentiation. Accordantly, the study of *Dipterocarpus* cf. *condorensis* Pierre (Luu 2005) has revealed higher genetic differentiation among populations based on AFLPs (G_{st} = 0.111) as compared to isozymes (G_{st} = 0.039).

The relatively high levels of genetic diversity within populations, high genetic differentiation between populations (regions) and the occurrence of unique and frequent AFLP markers in populations and on islands underline that efforts aiming at *in situ* and *ex situ* conservation should be undertaken. If logging activities are not controlled, there will still be the risk of less gene flow, increase in inbreeding and genetic differentiation and finally gene erosion by genetic drift as reported in previous studies (Murawski et al. 1994; Obayashi et al. 2002).

Diagnostic markers

Possibly due to genetic selection, drift and random allele fixation, some alleles have become private to one species, one region or one island or show pronounced frequency differences between groups (Fig. 5). This is reflected in exceptionally high G_{st} values at individual loci for the differentiation within species (maximum G_{st} = 0.96 in *S. leprosula,* G_{st} = 0.81 in *S. parvifolia*), which are otherwise characteristic for highly differentiating chloroplast markers. *S. leprosula* and *S. parvifolia* are characterised by highly differentiating AFLP markers. Sequencing of these highly differentiating AFLP markers will provide information on their position in genome. Such typical markers will be used as diagnostic markers to identify the

wood of different species, different regions and islands, and contribute to the strategies of forest conservation.

Hybridization

S. leprosula and *S. parvifolia* are closely related species, distributed sympatrically (Fig. 1), share a common pollinator, and their flowering time is overlapping (Chan and Appanah 1980), which indicate the possibility of hybridization between *S. leprosula* and *S. parvifolia*. Moreover, interspecific hybrids among *Shorea* species have been already reported in former studies (Chan and Appanah 1980; Chan 1981; Ashton 1982; Harada et al. 1994; Ishiyama et al. 2003). Nevertheless, UPGMA cluster analyses based on AFLP markers (Fig. 2, Fig. 3a and Fig. 3b) showed a clear distinction between *S. leprosula* and *S. parvifolia* in the present study. Additionally, each species harboured unique AFLP markers (7 in *S. leprosula* and 4 in *S. parvifolia*) and many markers (including some unique markers) with pronounced frequency differences between species (Fig. 4). Similar but less pronounced frequency differences were found in an allopatric pair of taxa (*Fagus sylvatica* and *F. orientalis;* Gailing and von Wuehlisch 2004). Also based on chloroplast markers and leaf morphological characteristics (used for species identification), *S. leprosula* and *S. parvifolia* can be clearly distinguished. However, there is not a single morphological characteristic specific for either species.

All pairwise F_{st} values for interspecific population pairs were highly significant. However, the population pair *S. lepr*_AS and *S. parv*_AS showed the lowest genetic differentiation (F_{st} = 0.446, Table 4) among these values. Marker 10 (167 bp, Fig. 5) revealed a striking pattern with a very high frequency (0.89) in only one population (Asialog) for *S. leprosula* and the highest frequency in the same region (Asialog) for *S. parvifolia* (0.76). This observation may be an indication that gene flow occurs to some extent between species in this region. Therefore, a rare event of hybridization between the two species can be speculated. However, the high frequency of the same fragment in region Asialog for both species can also be exlpained as a shared ancestral variation. A more intensive study of phenoytpic and genetic variation in this region will help to quantify the extent of hybridization between species.

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Table 1 Geographic location and sample size of *Shorea leprosula* and *S. parvifolia* populations in this study

Shorea species	Location	Island	Population abbreviation	Sample size	Latitude	Longitude	Altitude (m)
S. leprosula	Haurbentes	Java	*S. lepr*_HA	34	6° 54'– 7° 54' S	106° 41' – 107° 42' E	250
	Tering	Borneo	*S. lepr*_TB	23	00°– 00° 10' N	115° 22' – 116° 38' E	200
	Asialog	Sumatra	*S. lepr*_AS	9	2° 02' – 2° 22' S	103° 15' – 103° 33' E	100
	Pasir Mayang	Sumatra	*S. lepr*_PS	11	00° 8' – 03° 9' S	101° 19' – 103° 20' E	100
	TNBT	Sumatra	*S. lepr*_TS	22	01° 5' – 02° 6' S	102° 13' – 103° 14' E	200
	Nanjak Makmur	Sumatra	*S. lepr*_NS	16	10° 22' S	101° 40' E	100
	Sari Bumi Kusuma	Borneo	*S. lepr*_SB	18	01° 59' – 00° 36' S	111° 18' – 114° 42' E	400
S. parvifolia	Batu Ampar	Borneo	*S. parv_BB*	16	00° 45' – 00° 50' N	116° 48' – 117° 00' E	50
	Asialog	Sumatra	*S. parv_AS*	33	2° 02' – 2° 22' S	103° 15' – 103° 33' E	100
	Pasir Mayang	Sumatra	*S. parv_PS*	23	00° 8' – 03° 9' S	101° 19' – 103° 20' E	100
	TNBT	Sumatra	*S. parv_TS*	14	01° 5' – 02° 6' S	102° 13' – 103° 14' E	200
	Nanjak Makmur	Sumatra	*S. parv_NS*	28	10° 22' S	101° 40' E	100
	Sari Bumi Kusuma	Borneo	*S. parv_SB*	21	01° 59' – 00° 36' S	111° 18' – 114° 42' E	400
Total				268			

Table 2 Genetic diversity within populations of *Shorea leprosula* and *S. parvifolia*

Population	Sample size	Polymorphic loci	*PPL*	n_a	n_e	H_e	*I*
1. *S. lepr*_HA	34	34	60.71%	1.607	1.318	0.186	0.280
2. *S. lepr*_TB	23	26	46.43%	1.464	1.184	0.115	0.182
3. *S. lepr*_AS	9	37	66.07%	1.661	1.355	0.208	0.316
4. *S. lepr*_PS	11	29	51.79%	1.518	1.280	0.163	0.248
5. *S. lepr*_TS	22	28	50.00%	1.500	1.281	0.160	0.238
6. *S. lepr*_NS	16	28	50.00%	1.500	1.250	0.151	0.232
7. *S. lepr*_SB	18	27	48.21%	1.482	1.249	0.145	0.221
Mean	19	30	53.32%	1.533	1.274	0.161	0.245
Total	133	52	92.86%	1.929	1.347	0.211	0.330
SD				0.260	0.352	0.184	0.251
8. *S. parv*_BB	16	19	33.93%	1.339	1.201	0.115	0.171
9. *S. parv*_AS	33	43	76.79%	1.768	1.371	0.222	0.337
10. *S. parv*_PS	23	33	58.93%	1.589	1.228	0.143	0.228
11. *S. parv*_TS	14	21	37.50%	1.375	1.159	0.097	0.152
12. *S. parv*_NS	28	28	50.00%	1.500	1.189	0.119	0.188
13. *S. parv*_SB	21	30	53.57%	1.536	1.211	0.135	0.214
Mean	22.5	29	51.79%	1.518	1.227	0.138	0.215
Total	135	48	85.71%	1.857	1.336	0.205	0.319
SD				0.353	0.353	0.185	0.256

PPL: percentage of phenotypically polymorphic loci; n_a: observed number of alleles per locus; n_e: effective number of alleles per locus; H_e: Nei's (1973) gene diversity; *I*: Shannon's information index [Lewontin (1972)]; SD: standard deviation of total values

Table 3 Summary of analysis of molecular variance (AMOVA) for AFLP phenotypes

Source of variation	d.f.	SSD	MSD	Variance components	Total (%)	*p* value
Between species (LP)	1	763.658	763.658	5.294	44.1	< 0.001
Among populations within species	11	522.992	47.545	2.135	17.8	< 0.001
Within populations	255	1166.679	4.575	4.575	38.1	
S. leprosula						
Among populations	6	242.087	40.348	1.937	29.8	< 0.001
Within populations	126	576.191	4.573	4.573	70.2	
Between islands (SB)	1	151.409	151.409	2.302	30.2	< 0.05
Among populations within islands	5	90.678	18.136	0.759	9.9	< 0.001
Within populations	126	576.191	4.573	4.573	59.9	
S. parvifolia						
Among populations	5	280.905	56.181	2.333	33.8	< 0.001
Within populations	129	590.487	4.577	4.577	66.2	
Between islands(SB)	1	85.858	85.858	0.745	10.2	0.073
Among populations within islands	4	195.047	48.762	1.971	27.0	< 0.001
Within populations	129	590.487	4.577	4.577	62.8	

L: *Shorea leprosula*; P: *S. parvifolia*; S: Sumatra; B: Borneo; d.f.: degree of freedom; SSD: sum of squared deviation; MSD: mean squared deviation; *p* value: the probability of obtaining a more extreme component estimate by chance alone

Table 4 Pairwise F_{st} and significant *p* values

Pop. ID	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *S. lepr*_HJ	0.000	+	+	+	+	+	+	+	+	+	+	+	+
2. *S. lepr*_TB	0.426	0.000	+	+	+	+	+	+	+	+	+	+	+
3. *S. lepr*_AS	0.201	0.451	0.000	+	+	+	+	+	+	+	+	+	+
4. *S. lepr*_PS	0.131	0.433	0.140	0.000	-	+	+	+	+	+	+	+	+
5. *S. lepr*_TS	0.105	0.430	0.183	0.026	0.000	+	+	+	+	+	+	+	+
6. *S. lepr*_NS	0.122	0.471	0.169	0.069	0.051	0.000	+	+	+	+	+	+	+
7. *S. lepr*_SB	0.392	0.214	0.352	0.373	0.394	0.426	0.000	+	+	+	+	+	+
8. *S. parv*_BB	0.655	0.717	0.637	0.645	0.664	0.689	0.722	0.000	+	+	+	+	+
9. *S. parv*_AS	0.555	0.576	0.446	0.475	0.525	0.532	0.573	0.267	0.000	+	+	+	+
10. *S. parv*_PS	0.612	0.662	0.576	0.573	0.607	0.623	0.661	0.494	0.156	0.000	+	+	+
11. *S. parv*_TS	0.616	0.697	0.595	0.602	0.625	0.653	0.695	0.292	0.251	0.478	0.000	+	+
12. *S. parv*_NS	0.644	0.683	0.615	0.619	0.642	0.659	0.687	0.559	0.223	0.127	0.559	0.000	+
13. *S. parv*_SB	0.598	0.666	0.554	0.561	0.600	0.628	0.643	0.388	0.255	0.402	0.357	0.437	0.000

Significance level = 0.05

Table 5 Nei's (1978) unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) for *Shorea leprosula* and *S. parvifolia*

Pop. ID	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *S. lepr*_HA	_	0.883	0.947	0.969	0.986	0.976	0.901	0.726	0.798	0.773	0.773	0.733	0.788
2. *S. lepr*_TB	0.125	_	0.878	0.887	0.888	0.868	0.969	0.691	0.766	0.741	0.725	0.714	0.752
3. *S. lepr*_AS	0.055	0.130	_	0.966	0.959	0.951	0.921	0.723	0.825	0.788	0.769	0.756	0.800
4. *S. lepr*_PS	0.031	0.120	0.035	_	0.986	0.969	0.908	0.759	0.836	0.815	0.801	0.769	0.825
5. *S. lepr*_TS	0.014	0.119	0.042	0.015	_	0.985	0.912	0.747	0.826	0.799	0.799	0.760	0.808
6. *S. lepr*_NS	0.024	0.141	0.051	0.032	0.016	_	0.901	0.694	0.780	0.754	0.748	0.717	0.757
7. *S. lepr*_SB	0.104	0.031	0.083	0.096	0.092	0.104	_	0.680	0.771	0.747	0.727	0.720	0.763
8. *S. parv*_BB	0.320	0.370	0.324	0.276	0.292	0.365	0.386	_	0.919	0.869	0.958	0.839	0.927
9. *S. parv*_AS	0.226	0.266	0.192	0.179	0.191	0.248	0.260	0.084	_	0.972	0.920	0.954	0.945
10. *S. parv*_PS	0.258	0.300	0.239	0.205	0.225	0.282	0.291	0.140	0.029	_	0.875	0.979	0.913
11. *S. parv*_TS	0.258	0.322	0.262	0.222	0.225	0.290	0.319	0.043	0.083	0.134	_	0.844	0.926
12. *S. parv*_NS	0.311	0.337	0.280	0.263	0.275	0.333	0.329	0.176	0.047	0.022	0.170	_	0.909
13. *S. parv*_SB	0.238	0.285	0.223	0.192	0.213	0.278	0.270	0.076	0.057	0.091	0.078	0.095	_

Table 6 Genetic diversity and differentiation for *Shorea leprosula* and *S. parvifolia* in comparison with other tree species and perennial herbaceous plant at AFLP loci

Species	Origin	Climatic zone	Pollinator	No. of population	No. of primer pair	No. of loci	*PPL* (%)	H_e	G_{st} (F_{st})	Sources
Shorea leprosula	Indonesia	tropical	insect	7	1	56	53.32	0.161	0.25	present study
Shorea parvifolia	Indonesia	tropical	insect	6	1	56	51.79	0.138	0.31	present study
Dipterocarpus cf. *condorensis*	Vietnam	tropical	insect	5	1	72	71.2	0.215	0.111	Luu (2005)
Acer skutchii	Mexican	subtropical	insect, wind	3	1	161	66.5	0.15	0.075	Lara-Gomez et al. (2005)
Pinus monticola	North America	temperate	wind	4	3	163	31.3	0.123	0.094	Kim et al. (2003)
Hibiscus tiliaceus	South China	tropical and subtropical	insect	8	8	566	88.5	0.198	0.152	Tang et al. (2003)
Malus sylvestris (wild)	Europe	temperate	insect	4	3	139	76.6	0.225	0.05	Coart et al. (2003)
Pelliciera rhizophorae	Colombian Pacific Coast	neotropical	bird	6	4	225	42.4	0.117	0.265	Castillo-Cárdenas et al. (2005)
Acanthopanax sessiliflorus	East Asia	temperate	wind	10	8	70	50.4	0.187	0.069	Huh et al. (2005)
Trollius europaeus (perenn.herb. plant)	Europe	temperate	insect	18	3	117	50	0.194	0.39	Despres et al. (2002)

PPL: percentage of polymorphic loci within populations; H_e: expecetd heterozygosity within populations; G_{st} (F_{st}): proportion of total genetic diversity partitioned among populations

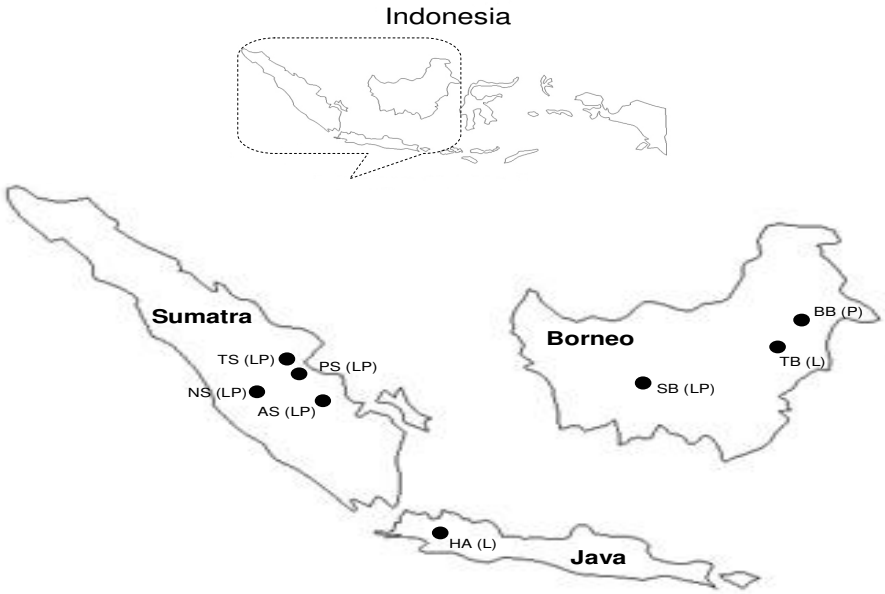

Fig. 1 Geographic distribution of 13 populations of *Shorea leprosula* and *S. parvifolia* in Indonesia
L: *S. leprosula*; P: *S. parvifolia*; HA: Haurbentes Java; AS: Asialog Sumatra; NS: Nanjak Makmur Sumatra; PS: Pasir Mayang Sumatra; TS: TNBT Sumatra; BB: Batu Ampar Borneo; SB: Sari Bumi Kusuma Borneo; TB: Tering Borneo

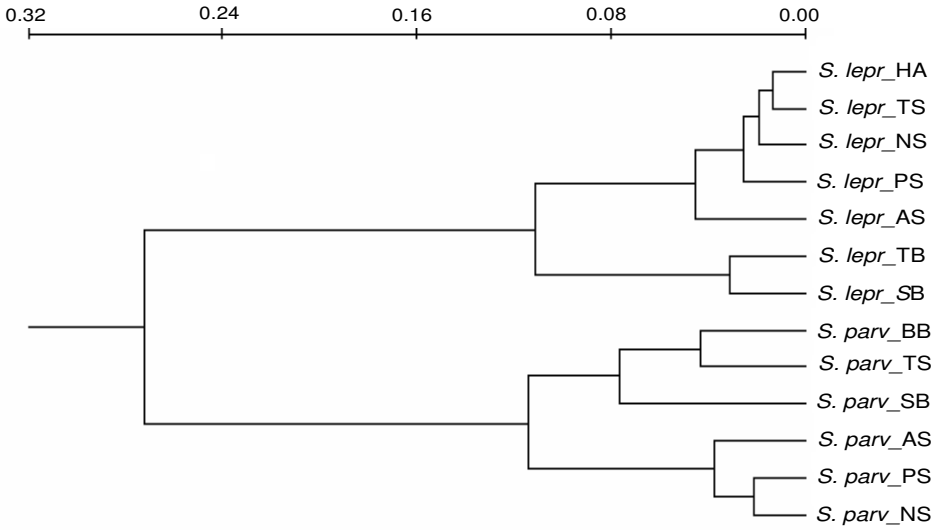

Fig. 2 UPGMA dendrogram representing the genetic distances among populations of *Shorea leprosula* and *S. parvifolia* based on Nei's (1978) genetic distance

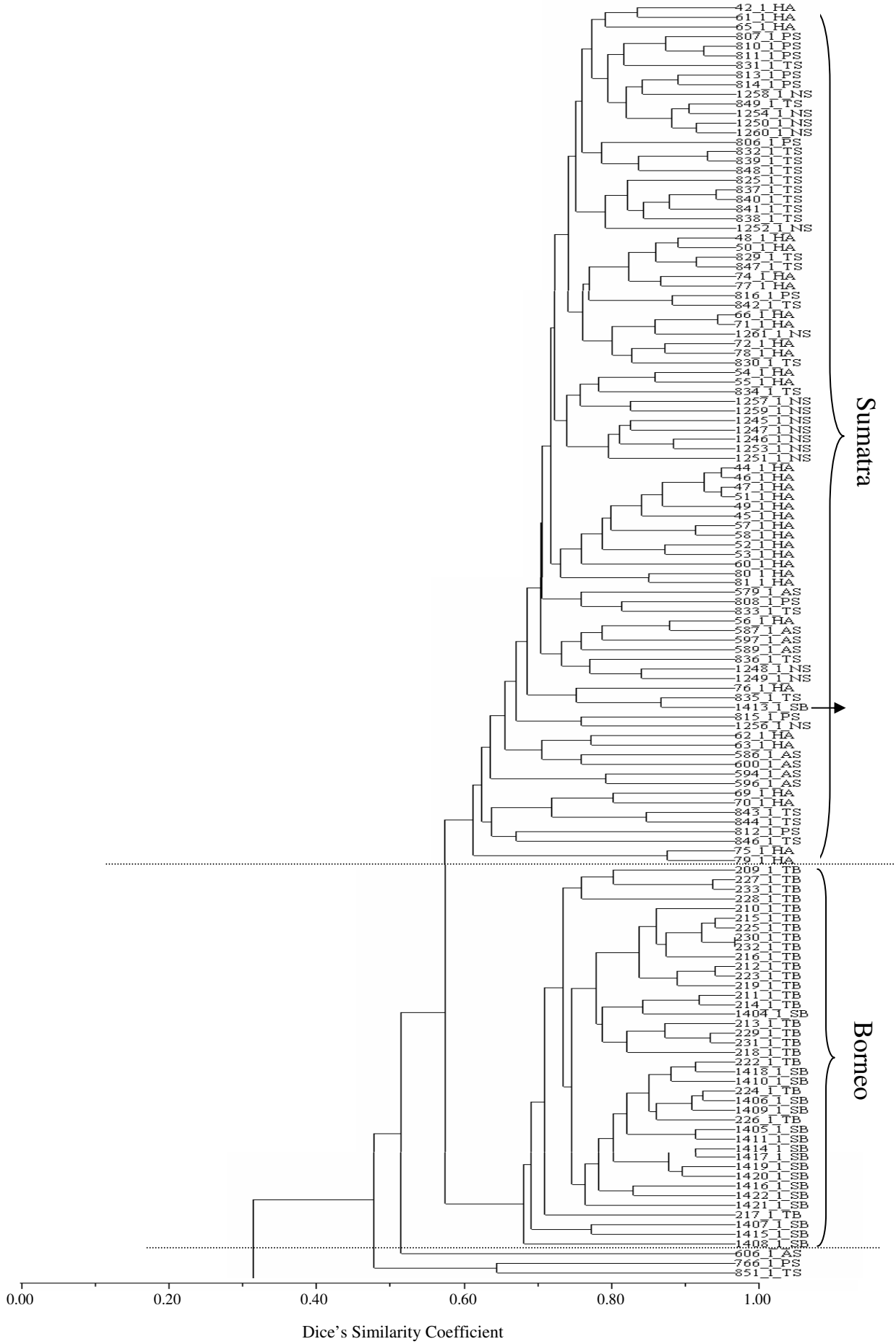

Fig. 3a Upper cluster of UPGMA dendrogram based on the Dice (1945) index

Fig. 3b Lower cluster of UPGMA dendrogram based on the Dice (1945) index

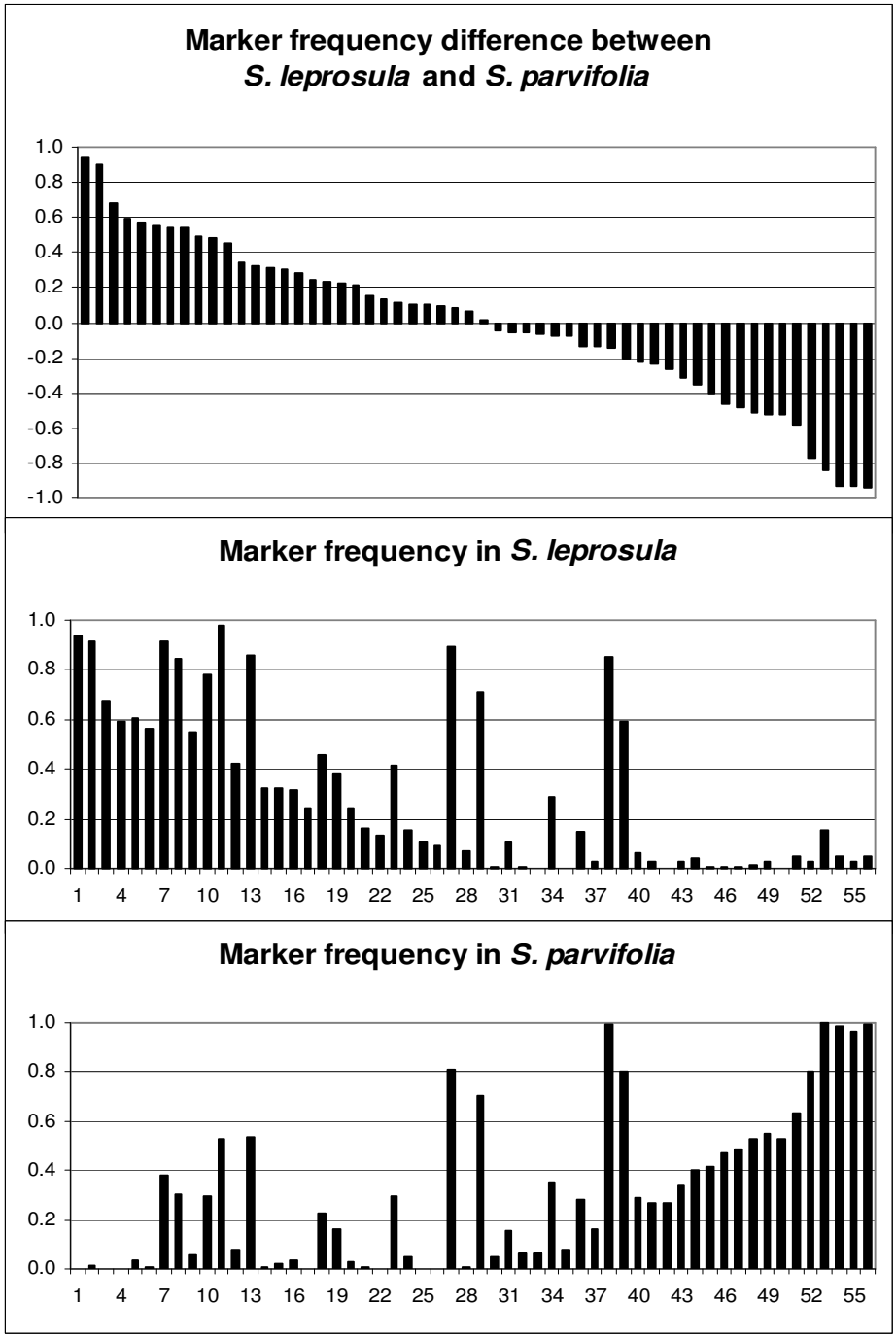

Fig. 4 Differences in the frequencies of AFLP markers (bands) for *S. leprosula* and *S. parvifolia*

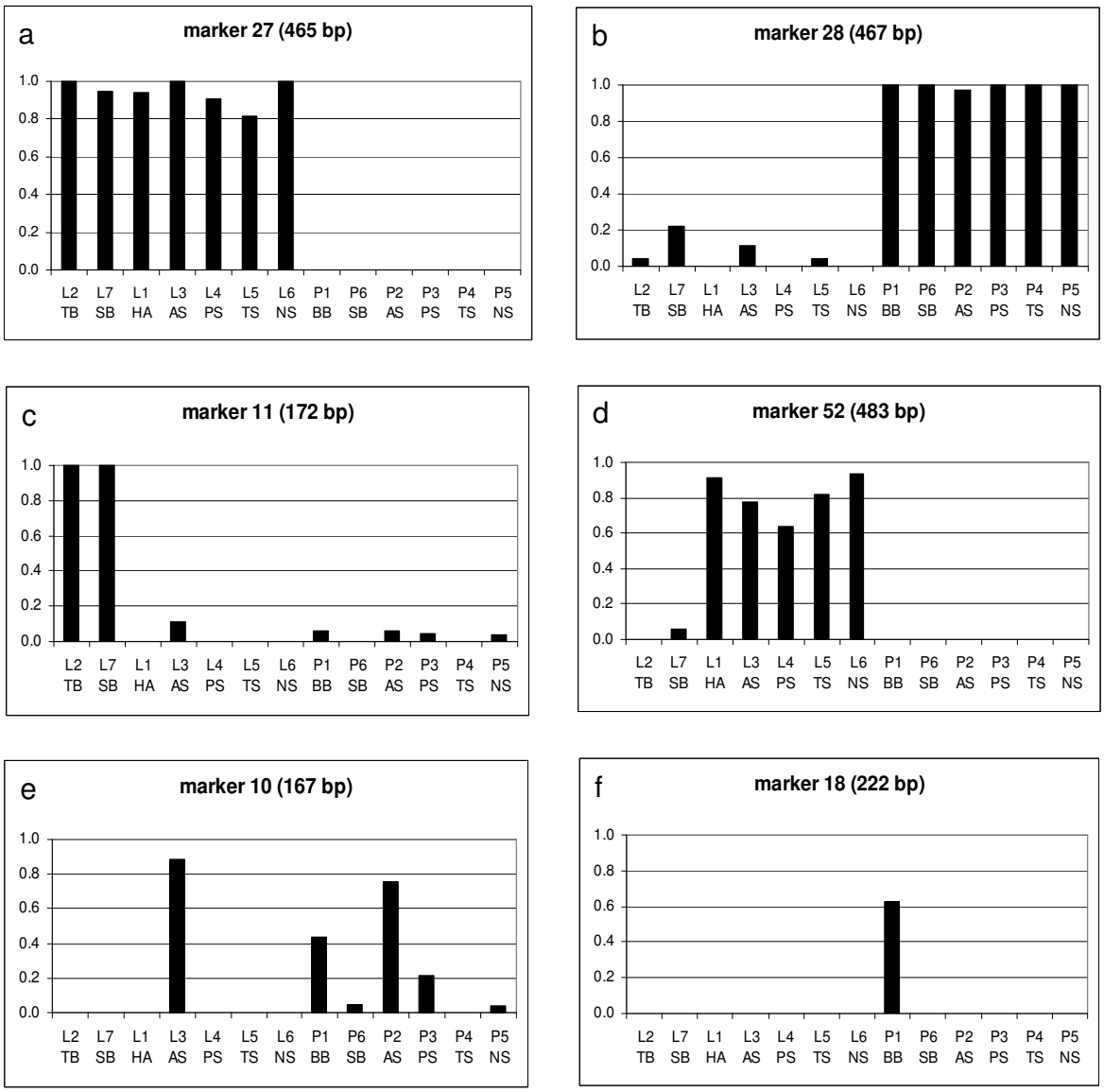

Fig. 5 Frequency distribution of diagnostic markers for *S. leprosula* (L) and *S. parvifolia* (P) HA: Haurbentes Java; AS: Asialog Sumatra; NS: Nanjak Makmur Sumatra; PS: Pasir Mayang Sumatra; TS: TNBT Sumatra; BB: Batu Ampar Borneo; SB: Sari Bumi Kusuma Borneo; TB: Tering Borneo

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Part 3

Genetic variation in nine *Shorea* species in Indonesia revealed by AFLPs

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Abstract: *Shorea* is the largest and most important genus of the Dipterocarpaceae. The genetic variation and structure of nine *Shorea* species from two different locations, namely Nanjak Makmur on Sumatra and Sumalindo on Kalimantan (Borneo) were evaluated using AFLP markers. A total of 274 trees were investigated at 85 polymorphic AFLP loci, including 141 individuals of six species from Nanjak Makmur Sumatra and 133 individuals of five species from Sumalindo Borneo. The results showed similar levels of mean genetic variation for species from Nanjak Makmur Sumatra and from Sumalindo Borneo ($\overline{H_e}$ = 0.138 for Sumatra; $\overline{H_e}$ = 0.129 for Borneo). *S. blumutensis* and *S. dasyphylla* from Nanjak Makmur Sumatra possessed the highest genetic diversity with H_e of 0.165 and H_e of 0.164, respectively. *S. acuminata* from Nanjak Makmur Sumatra harboured the lowest genetic diversity with H_e of 0.100. The hypothesis that widespread species (e.g. *S. leprosula* and *S. parvifolia*) show a higher level of genetic variation than rarespread species (*S. blumutensis*, *S. dasyphylla*) is rejected. AMOVA analysis revealed that the genetic variation was mainly found among species both in Nanjak Makmur Sumatra (57.7%) and in Sumalindo Borneo (56.3%). Surprisingly, the UPGMA dendrogram of all samples revealed an almost complete separation of clusters according to species affiliation. Thus, AFLP markers proved appropriate to dissection of phylogenetical relationships among *Shorea* species. Species-specific markers with high frequencies (> 80%) have been detected in two species (*S. platyclados* and *S. johorensis*). Several other markers showed high frequency differences among species, and between regions within species (for *S. leprosula* and *S. parvifolia* that are represented in both regions). The homology of equal-sized AFLP fagments has to be confirmed by sequencing. Sequence information can be used to develop specific PCR markers for wood identification purposes. The possibility of interspecific hybridization is discussed.

Keywords: AFLP, conservation, Dipterocarpaceae, genetic diversity, genetic structure, *Shorea*, tropical Southeast-Asia

Introduction

Shorea Roxb. is the dominant emergent tree genus in tropical Asia (Ashton 1982). It is the largest genus of the Dipterocarpaceae, the most important tree family in tropical Southeast-Asia both from an ecological and an economic perspective. It encompasses about 200 species, of which 163 are distributed in Malesia, mostly in Indonesia, in particular on Sumatra and Borneo (Kalimantan). The timber is highly valued and used for construction (shipbuilding, bridges, piers), decking and outdoor furniture (Ashton 1982). The distribution area and the abundance of many *Shorea* species in Indonesia are shrinking due to the changes of land use systems from forests to other uses including agriculture and cultivation of oil palms, and due to the exploitation of the valuable timber. The genetic consequences of forest decline on dipterocarps are largely unknown. Patterns of genetic variation within and among species and have rarely been studied in primary and secondary dipterocarp forests in Indonesia, although an understanding of the spatial distribution of genetic diversity is crucial for the development of strategies for an efficient conservation and sustainable use.

The development of DNA-based molecular marker techniques has advanced the studies of genetic diversity considerably over the last decade, which allow fingerprinting of plant genomes and reveal direct and reliable polymorphism at the DNA level (e.g., De Verno and Mosseler 1997; Rogers 2002). We chose amplified fragment length polymorphisms (AFLPs; Vos et al. 1995) for this study. Due to the high multiplex ratio (number of polymorphic loci generated in a single experiment; Rafalski et al. 1996) and reproducibility (Jones et al. 1997), AFLP is an efficient marker technique for fingerprinting and assessing genetic polymorphism (Russell et al. 1997; Pejic et al. 1998; Farooq and Azam 2002; Garcia et al. 2004). This marker technique has often been applied to study of genetic variation in forest trees species (e.g., Tang et al. 2003; Gailing and von Wuehlisch 2004; Castillo-Càrdenas et al. 2005; Lara-Gomez et al. 2005). The application of AFLPs as simple, universal markers to the assessment of genetic variation of tropical forest trees has recently been emphasized by Kremer et al. (2005), but only few reports based on the AFLP marker technique are available for *Shorea* (Cao et al. submitted a; Cao et al. submitted b) and any other dipterocarps (Luu 2005).

The objective of the present study is to evaluate genetic variation within and differentiation among nine *Shorea* species from two different locations, namely Nanjak Makmur on Sumatra and Sumalindo on Borneo (Kalimantan) using AFLP markers. The following hypotheses are addressed: (1) widespread, common dipterocarp species possess more genetic variation within populations than rare species; (2) the average levels of genetic variation within populations are similar at the investigated sites; (3) AFLPs are suitable

markers to distinguish among species, between regions within species, and to dissect phylogentic relationships among species within the genus *Shorea*. Furthermore we want to detect AFLP markers with pronounced frequency differences among species, and between regions within species, in order to contribute to the development of molecular genetic tools for the identification of wood from different species and origins.

Materials and Methods

Plant material

The leaves of adult trees and saplings were collected from 11 natural populations of nine *Shorea* species distributed in two different regions (Nanjak Makmur on Sumatra and Sumalindo on Borneo) in Indonesia. Information concerning geographic distribution and sample size of the sampled populations is shown in Table 1 and Fig. 1. The collection site for each species within regions had an area of 100-300 ha**.** A minimum distance of 30 m was kept between sample trees to avoid excessive sampling of related plants. All species have a minimum sample size of 20 trees (16 trees in case of *S. leprosula* in Nanjak Makmur). The estimated density for the nine species is 0.15 to 3 trees per hectare. Species identification was done on the basis of leaf morpholgical characters such as leaf length, petiole length, leaf width**,** distance from petiole to the widest part of the leaf, number of venation, number of lobes, dometiana length, and leaf shape.

DNA extraction

Total genomic DNA was extracted from a small slice (ca. 2 cm^2) of silicagel-dried leaf tissue following the DNeasy 96 Plant Kit protocol of the manufacturer (Qiagen, Hilden, Germany). Five µl DNA was separated electrophoretically on a 0.8 % agarose gel at 100 V in TAE buffer, visualized by staining with ethidium bromide, and photographed in ultraviolet light to check DNA quantity and quality. Extracted DNA was stored at -20 °C.

AFLP assays

The AFLP fingerprinting technique of Vos et al. (1995) was employed with slight modifications. Total genomic DNA of each sample was digested with the two restriction endonucleases *Eco*RI and *Mse*I. The *Eco*RI-adaptor and the *Mse*I-adaptor were ligated to the ends of the restriction fragments. The restriction-ligation reactions were performed overnight (14-16 h) at room temperature to generate template DNA for PCR amplification. The preselective amplifications were conducted using primer pair E01/M03 with selective

nucleotides A and G, respectively. The selective amplifications were achieved using primer pair E35/ M63 having selective nucleotides ACA and GAA, respectively. Primer E35 was labelled with fluorescent dye 6-FAM. All PCR reactions were conducted in the Peltier Thermal Cycler (PTC-200 version 4.0, MJ Research). The amplified restriction products were separated electrophoretically on the ABI Genetic Analyser 3100 together with the internal size standard GeneScan 500 ROX (fluorescent dye ROX) from Applied Biosystems. The size of the AFLP fragments was detected with the software packages GeneScan 3.7 and Genotyper 3.7 (Applied Biosystems).

Data analysis of AFLP markers

Each AFLP band was assumed to correspond to a dominant allele at a single locus. Only unambiguous bands of total AFLP fingerprint patterns were manually selected and scored as present (1) or absent (0) in each sample. The binary character matrices were compiled for further analysis.

POPGENE ver. 1.31 computer program (Yeh et al. 1999) was used to calculate the parameters of genetic variation under the assumption of Hardy-Weinberg equilibrium, including the percentage of polymorphic loci (*PPL*, Nei 1973), the number of alleles per locus (n_a), effective number of alleles per locus (n_e, Hartl and Clark 1989), gene diversity (h = expected heterozygosity H_e, Nei 1973), Shannon's information index for phenotypic diversity (Lewontin 1972) quantifying the degree of AFLP polymorphism within populations: $I = -\Sigma pi \log_2 pi$, where p_i is the frequency of the presence or absence of a AFLP band, total genetic diversity (H_t), genetic diversity within populations (H_s), genetic diversity among populations (D_{st}), and the relative magnitude of genetic differentiation among populations ($G_{st} = D_{st} / H_t$, Nei 1987). Allelic frequencies were calculated based on the square root of the frequency of the null (recessive) allele. Nei's (1978) unbiased measures of genetic identity and genetic distance were calculated for all pairwise combinations of populations. An UPGMA (unweighted pairgroup method using arithmetic average) dendrogram was constructed using the program NTSYS-pc ver. 2.0 (Rohlf 1998) based on Nei's genetic distances (1978). Hierarchical analyses of molecular variance (AMOVA, Excoffier et al. 1992) based on the pairwise squared Euclidean distances among molecular phenotypes were conducted using the program ARLEQUIN (Schneider et al. 2000) to further quantify the amount of genetic variation residing at two levels. The same program was used to generate the matrix of pairwise F_{st} values indicating the genetic differentiation between populations. The

significance levels for AMOVA were evaluated using a permutation approach (1023 replications).

Another UPGMA clustering indicating the patterns of variation within and among populations was performed based on similarity values using the NTSYS-pc software (Rohlf 1998). The estimates of genetic similarity were calculated from all possible pairwise combinations of individuals according to Dice index (1945): $S_{ij} = 2a/(2a+b+c)$, where S_{ij} is the similarity between two individuals i and j, a is the number of bands present in both i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. The goodness of fit of the clustering to the genetic distance matrix, on which it was based, was calculated using the Mantel z statistic (Mantel 1967; Rohlf 1998).

Results

Genetic diversity

A total of 85 unequivocally recognisable polymorphic AFLP fragments were scored, ranging in size from 76 bp to 486 bp. Table 2 shows that the genetic diversity varied among species. At the single species level, the rare species *Shorea blumutensis* and *S. dasyphylla* possessed the highest level of diversity with the values of n_e = 1.266, H_e = 0.165, I = 0.257 and n_e = 1.273, H_e = 0.164, I = 0.251, respectively. The relatively widespread species *S. acuminata* harbored the lowest diversity values of n_e = 1.159, H_e = 0.100, I = 0.162. There was no positive association between the level of genetic vatiation and the extent of distribution of a species. At the regional level, Nanjak Makmur Sumatra exhibited a slightly higher level of diversity with the mean values of $\overline{n_e}$ = 1.226, $\overline{H_e}$ = 0.138 and $\overline{I}$ = 0.214 relative to Sumalindo Borneo with the mean values of $\overline{n_e}$ = 1.211, $\overline{H_e}$ = 0.129 and $\overline{I}$ = 0.204. The differences in the mean diversity estimates were statistically not significant (t-Test).

Genetic structure

The results of AMOVAs (Table 3) showed highly significant ($p < 0.001$) genetic differentiation among populations (mostly among species). The main portion of the total variation resided among species. AMOVAs at two hierarchical levels performed separately for Nanjak Makmur Sumatra and Sumalindo Borneo indicated that the among-species variation in Nanjak Makmur Sumatra (57.7%) was slightly higher than in Sumalindo Borneo (56.3%). Similarly, the genetic differentiation among species (G_{st}) calculated using

POPGENE software showed a slightly higher G_{st} = 0.52 in Nanjak Makmur Sumatra than in Sumalindo Borneo (G_{st} = 0.46). The pairwise F_{st} values (Table 4) generated from AMOVA indicated that all the populations were significantly differentiated ($p < 0.05$) with F_{st} values ranging from 0.297 (Sdas_NS / Spar_SLB) to 0.711 (Sblu_NS / Slep_SLB). The F_{st} values between *S. dasyphylla* and *S. parvifolia* show the lowest level (F_{st} = 0.297 for Sdas_NS / Spar_SLB and F_{st} = 0.312 for Sdas_NS / Spar_NS) followed by a F_{st} values of 0.336 for the population pair of *S. parvifolia* (Spar_NS / Spar_SLB) and 0.404 for the *S. leprosula* pair (Slep_NS and Slep_SLB).

Genetic distances and cluster analyses

Table 5 shows Nei's (1978) pairwise genetic distances between populations. The largest distance (0.389) was found between *S. blumutensis* and *S. leprosula* in Nanjak Makmur Sumatra (Sblu_NS / Slep_NS), the smallest (0.067) between the two populations of *S. leprosula* from the two different regions (Slep_NS / Slep_SLB). Additionally, the small genetic distances were also found between the two populations of *S. parvifolia* (0.087 for Spar_NS / Spar_SLB), and between the species *S. dasyphylla* and *S. parvifolia* (0.094 for Sdas_NS / Spar_NS, 0.077 for Sdas_NS / Spar_SLB), reflecting the relatively high genetic similarities.

An UPGMA dendrogram (Fig. 2) displays the genetic relationships among all the 11 populations of nine species investigated based on the matrix of Nei's (1978) genetic distances (Table 5). In most cases, the species were clearly separated from each other, but *S. parvifolia* (Spar_NS and Spar_SLB) and *S. dasyphylla* (Sdas_NS) formed one group. The four species *S. macroptera*, *S. platyclados*, *S. johorensis*, and *S. palembanica* (Smac_NS, Splat_SLB, Sjoh_SLB and Spal_SLB) clustered together, close to the *S. parvifolia* / *S. dasyphylla* cluster. The two populations of *S. leprosula* (Slep_SLB and Slep_NS) formed one single cluster. *S. acuminata* and *S. blumutensis* (Sacu_NS and Sblu_NS) were isolated from the other species.

Genetic relationship among all the individuals of 11 populations of the nine species were revealed further by another UPGMA analysis based on the Dice (1945) similarity index (Fig. 3a, Fig. 3b). Individuals of the same population and species were grouped together in the same cluster except for three individuals (*S. platyclados*: 2220_pc_SLB and 2217_pc_SLB; *S. dasyphylla*: 1221_d_NS; see arrows in Fig 3a and 3b). Generally, the topology of Fig. 3 is similar to that of Fig. 2. Nine species can be clearly identified in nine clusters. Individuals of *S. parvifolia*, *S. dasyphylla* and *S. leprosula* were grouped in one large cluster (Fig. 3a). *S. macroptera*, *S. johorensis* and *S. platyclados* formed another large cluster. *S. palembanica*, *S.*

acuminata and *S. blumutensis* clustered to the base of the tree in separate clusters. Samples of *S. blumutensis* were separated far from the individuals of the other species (Fig. 3b). The cophenetic correlation showed a very good fit of the cluster analysis to the matrix of genetic distances (r = 0.88).

Distribution of AFLP markers

The genetic diversity revealed at AFLPs is attributed to the differences in distribution patterns of markers in individuals. Some AFLP markers showed remarkable frequency differences not only among species, but also between regions within the species *S. leprosula* and *S. parvifolia* (Fig. 4). Marker 51 (a) and 60 (b) are private to *S. platyclados* and *S. johorensis*, respectively, with high frequencies (> 0.8). Marker 30 (c), 37 (d) and 54 (e) have high frequencies (> 0.9) in *S. palembanica*, *S. blumutensis* and *S. acuminata*, respectively, and low frequencies (< 0.2) in the other species (see also Figure 3b). Marker 61 (f) shows a high frequency (1.0) in *S. macroptera* and low frequencies (< 0.1) in most other species. In Sumalindo Borneo, marker 32 (g) shows a high frequency (> 0.9) only in *S. johorensis* and low frequencies (< 0.2) in the other species. The same marker 32 (g) is present with high frequencies (> 0.9) in *S. blumutensis* and *S. leprosula* in Nanjak Makmur Sumatra. Marker 32 (g) and marker 45 (h) are present in Nanjak Makmur Sumatra and Sumalindo Borneo, respectively, with high frequencies (> 0.9) for *S. leprosula* and absent in the other region. Marker 39 (i) and 73 (j) differentiate between Nanjak Makmur Sumatra and Sumalindo Borneo for *S. parvifolia*. In Sumalindo Borneo, marker 39 (i) has high frequency (> 0.8) in *S. platyclados* and low frequency (< 0.2) in the other species. The homology of equal-sized fragments in different species has to be tested (see Discussion).

Discussion

Genetic diversity within species

The breeding system in plants is one factor that affects gene flow, and hence influences the genetic variation within and among populations (Loveless and Hamrick 1984). Outcrossing is predominant in many tropical tree species (Nason and Hamrick 1996). *Shorea* species are predominantly outcrossing and strongly self-incompatible (Chan 1981, Sakai et al. 1999, Lee et al. 2000b, Nagamitsu et al. 2001, Obayashi et al. 2002). Outcrossing plants in general exhibit higher levels of genetic diversity within populations than selfing plants (Hamrick and Godt 1996), but lower differentiation.

Compared to other tree species (including dipterocarps) studied using AFLPs (Tang et al. 2003, Castillo-Càrdenas et al. 2005, Lara-Gomez et al. 2005, Luu 2005), the nine *Shorea* species investigated here showed moderate levels of genetic diversity (H_e) ranging from 0.100 to 0.165. In Nanjak Makmur Sumatra, *S. leprosula* contains a higher level of genetic diversity (H_e = 0.13) than *S. parvifolia* (H_e = 0.11) in spite of its smaller sample size, supporting the results of previous studies using microsatelites (Lee et al. 2004) and AFLPs (Cao et al. submitted b). Nevertheless, *S. leprosula* showed a slightly lower level of genetic diversity (0.115) than *S. parvifolia* (0.122) in Sumalindo Borneo. In Nanjak Makmur Sumatra, the rare species (*S. blumutensis*, *S. dasyphylla*) exhibited higher levels of genetic variation than widespread species (*S. leprosula* and *S. parvifolia*). Endemic tree species and species with a narrow geographic distribution harbour on average less genetic variation than widespread species (Hamrick et al. 1992). Our results do not follow this general trend and contradict our expectation (hypothesis 1; see above). They point towards surprisingly high levels of genetic variation in uncommon dipterocarps.

Isozyme studies suggested that forest trees are on the average among the most variable organisms (Hamrick and Godt 1996). However, large differences among tree species exist with regard to the (average) genetic variation within populations. The expected heterozygosity (H_e) at isozyme gene loci of 16 uncommon tree species growing on Barro Colorado island ranged from 0.026 to 0.254 (mean: 0.142) (Hamrick and Murawski 1991). The range of H_e for 17 Australian eucalypts (*Eucalyptus* spp.) was from 0.068 to 0.278 (mean: 0.174) (Moran 1992). Even higher differences are reported for pines (*Pinus* spp.) (Ledig 1986). Compared to these studies, the differences of genetic diversity estimates among species reported here are low: the expected heterozygosity of the most variable population (*S. blumutensis*; H_e = 0.165) exceeded the least variable population (*S. acuminata*; H_e = 0.100) by only 65%; even smaller differences were observed for the other diversity estimates, which all indicated moderate genetic variation within the studied populations. The exact reasons for the rather low variance of diversity estimates are unknown, but it is unlikely that genetic drift had a strong impact on genetic structures in the recent past of some populations, but not in others. Thus, the result confirmed previous findings that dipterocarps, like most other tropical trees, are able to avoid very low effective population sizes even if they occur in low density (e.g. Ashton 1969; Bawa 1992).

Genetic structure

Fig. 2 and Fig. 3 show a clear separation of populations within both species *S. leprosula* and *S. parvifolia*. The pairwise F_{st} values (Table 4) and the genetic distances (Table 5) indicate strong differentiation between populations within both species. The ecological and life history traits of the genus *Shorea*, in particular limited gene flow via pollen and seed dispersal over large distances, contribute to the considerable differentiation between populations. *Shorea* species are pollinated mainly by small insects, such as thrips and small beetles (Appanah and Chan 1981; Momose et al. 1998), and produce single-seeded fruits; more than half of the mature seeds land within 50 m of the parent tree (Chan 1980). AMOVA analysis of a former study based on AFLPs (using the same selective primer pair) showed similar high population differentiation of 29.8 % among seven populations of *S. leprosula* and 33.8% among six populations of *S. parvifolia* (Cao et al. submitted b). Thus, the suitability of AFLP markers revealed in previous works (Kim et al. 2003; Luu 2005; Cao et al. submitted b) to detect strong and significant differences among populations within species is confirmed by the present study.

The AMOVA analysis of the present study indicated highly significant ($p < 0.001$) genetic differentiation among species. Most of the AFLP diversity resided among species both in Nanjak Makmur Sumatra (57.7%) and in Sumalindo Borneo (56.3%) (Table 3). Similarly, the overall degree of species differentiation computed using POPGENE was high in both regions ($G_{st} = 0.52$ in Nanjak Makmur Sumatra, $G_{st} = 0.46$ in Sumalindo Borneo). The high level of genetic differentiation among species in both locations may be due to effective reproductive isolation of the species and a rather long separated evolutionary history of populations.

The UPGMA clustering (Fig. 2, Fig. 3) showed a very good separation of species and populations without an obvious relation to the sampling locations. For example, comparatively low genetic differentiation was observed between populations Sdas_NS and Spar_SLB, which belong to different species located on different islands.

Diagnostic markers

Due to the different effects of natural selection and genetic drift on different species, private and frequent markers were found for some species and populations (regions) within species in this study. These markers showed very high G_{st} values indicating their suitability to differentiate among species, and between regions within species. Sequencing of these highly differentiating AFLP markers will provide information on the homology of AFLP fragments

sharing the same fragment size in different species and, by means of sequence comparisons to data bases, on the putative function of fragments, if any. It might be possible to develop simple diagnostic markers from strongly differentiating AFLP bands. These tools may be useful to unambiguously identify the species status of wood samples, and to distinguish the region of origin of wood for widespread species (Finkeldey et al. in press).

Possibility of hybridization

In the UPGMA dendrogram (Fig. 3), two samples of *S. platyclados* from Sumalindo Borneo (2220_pc_SLB and 2217_pc_SLB) clustered together with individuals of *S. parvifolia* from the same region. One sample of *S. dasyphylla* from Nanjak Makmur Sumatra (1221_d_NS) was grouped together with individuals of *S. palembanica* from Sumalindo Borneo. Morphologically, leaves of these samples are distinct and easy to identify. Thus, misidentification of these samples is impossible. *S. platyclados* is distributed sympatrically with *S. parvifolia* (Fig. 1). The habitats of *S. dasyphylla* and *S. palembanica* exist both on Sumatra and Borneo (Ashton 1982). Moreover, *Shorea* species flower at the same time (Appanah and Chan 1981). Although hybridization among Dipterocarpaceae species is rare, the rare event of interspecific gene exchange in *Shorea* is possible. In fact, interspecific hybrids among *Shorea* species have already been reported in former studies (Chan and Appanah 1980; Chan 1981; Ashton 1982; Harada et al. 1994). The three samples 2220_pc_SLB, 2217_pc_SLB and 1221_d_NS may be of hybrid origin. However, ancestral shared polymorphisms in these species can not be excluded.

Usefulness of AFLP technique in phylogenetic studies

AFLPs are anonymous and dominant markers. No sequence information prior to the generation of AFLP fingerprints is required, and homologous and non-homologous fragments can not be distinguished (Mueller and Wolfenbarger 1999). Hence, size homoplasy effects can be caused in phylogenetic studies. However, AFLP markers are distributed across the whole genome, and they have a high multiplex ratio, meaning a large number of different genetic loci that may be simultaneously analysed per experiment (Pejic et al. 1998). These advantages can counteract the size homoplasy effects. Therefore, genome-wide AFLP data sets may provide high power in testing specific phylogenetic relationships in particular for closely related taxa within the same genus (Rokas et al. 2003).

Most of the phylogenetic studies based on AFLPs used only limited number of samples per species. With our experimental design, i.e. nine species with the sample size ranging from

16- 32, we have revealed not only pronounced within-species variation (Table 3), but also strong genetic differentiation among species indicating a strong phylogenetic signal of AFLP markers. Additionally, the UPGMA dendrogram (Fig. 3) showed a clear resolution of almost all the samples into nine species-clusters based on high frequency differences of AFLP markers among species.

In conclusion, the present study proved the reliability of AFLP markers to differentiate among species, and confirmed that AFLPs are suitable molecular markers to detect phylogenetic relationships among species as revealed in previous study (Cao et al. submitted).

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Table 1 Geographic location and sample size of 11 populations of nine species in Indonesia

Shorea species	Location	Island	Distribution	Population abbreviation	Sample size	Latitude	Longitude	Altitude (m)
S. parvifolia	Nanjak Makmur	Sumatra	common	Spar_NS	26	10°22' S	101°40' E	100
S. acuminata			common	Sacu_NS	32			
S. dasyphylla			scattered	Sdas_NS	20			
S. blumutensis			rare	Sblu_NS	21			
S. leprosula			common	Slep_NS	16			
S. macroptera			common	Smac_NS	26			
S. parvifolia	Sumalindo	Borneo	common	Spar_SLB	31	00°55'-	115°18'-	200
S. leprosula			common	Slep_SLB	26	00°56' N	116°36' E	
S. palembanica			common	Spal_SLB	25			
S. platyclados			common	Splat_SLB	27			
S. johorensis			common	Sjoh_SLB	24			
Total					274			

Information of species distribution on islands was obtained from Newman et al. (1996a; 1996b).

Table 2 Genetic diversity within populations of nine *Shorea* species in Indonesia

Population	Distribution	Sample size	Polymorphic loci	*PPL*	n_a	n_e	H_e	*I*
1. Spar_NS	common	26	38	44.71%	1.447	1.176	0.110	0.174
2. Sacu_NS	common	32	42	49.41%	1.494	1.159	0.100	0.162
3. Sdas_NS	scattered	20	47	55.29%	1.553	1.273	0.164	0.251
4. Sblu_NS	rare	21	53	62.35%	1.624	1.266	0.165	0.257
5. Slep_NS	common	16	36	42.35%	1.424	1.224	0.134	0.204
6. Smac_NS	common	26	45	52.94%	1.529	1.259	0.155	0.238
Mean		24	44	51.18%	1.512	1.226	0.138	0.214
7. Spar_SLB	common	31	44	51.76%	1.518	1.199	0.122	0.193
8. Slep_SLB	common	26	39	45.88%	1.459	1.192	0.115	0.178
9. Spal_SLB	common	25	52	61.18%	1.612	1.245	0.149	0.232
10. Splat_SLB	common	27	56	65.88%	1.659	1.235	0.144	0.230
11. Sjoh_SLB	common	24	47	55.29%	1.553	1.183	0.115	0.186
Mean		27	48	56.00%	1.560	1.211	0.129	0.204

Information of species distribution on islands was obtained from Newman et al. (1996a; 1996b).
PPL: percentage of phenotypically polymorphic loci; n_a: observed number of alleles per locus; n_e: effective number of alleles per locus; H_e: Nei's (1973) gene diversity; *I*: Shannon's information index [Lewontin (1972)]

Table 3 Summary of the analysis of molecular variance (AMOVA) for AFLP phenotypes

Source of variation	d.f.	SSD	MSD	Variance components	Total (%)	*p* value
Among populations	10	2027.812	202.781	7.925	56.9	< 0.001
Within populations	263	1580.769	6.011	6.011	43.1	
Nanjak Makmur Sumatra						
among species	5	1025.881	205.176	8.547	57.7	< 0.001
within species	135	844.473	6.255	6.255	42.3	
Sumalindo Borneo						
among species	4	808.186	202.047	7.395	56.3	< 0.001
within species	128	736.295	5.752	5.752	43.7	

d.f.: degree of freedom; SSD: sum of squared deviation; MSD: mean squared deviation; *p* value: the probability of obtaining a more extreme component estimate by chance alone

Table 4 Pairwise F_{st} and significant *p* values based on AFLP phenotypes

Pop. ID	1	2	3	4	5	6	7	8	9	10	11
1. Spar_NS	0.000	+	+	+	+	+	+	+	+	+	+
2. Sacu_NS	0.584	0.000	+	+	+	+	+	+	+	+	+
3. Sdas_NS	0.312	0.537	0.000	+	+	+	+	+	+	+	+
4. Sblu_NS	0.675	0.657	0.593	0.000	+	+	+	+	+	+	+
5. Slep_NS	0.598	0.643	0.508	0.690	0.000	+	+	+	+	+	+
6. Smac_NS	0.489	0.602	0.412	0.601	0.587	0.000	+	+	+	+	+
7. Spar_SLB	0.336	0.595	0.297	0.670	0.559	0.514	0.000	+	+	+	+
8. Slep_SLB	0.612	0.660	0.547	0.711	0.404	0.576	0.565	0.000	+	+	+
9. Spal_SLB	0.537	0.622	0.477	0.618	0.561	0.492	0.546	0.599	0.000	+	+
10. Splat_SLB	0.504	0.665	0.476	0.622	0.636	0.497	0.542	0.617	0.534	0.000	+
11. Sjoh_SLB	0.547	0.628	0.488	0.636	0.585	0.479	0.575	0.600	0.530	0.502	0.000

Significance level = 0.05

Table 5 Nei's (1978) unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) for nine *Shorea* species

	1	2	3	4	5	6	7	8	9	10	11
1. Spar_NS	****	0.813	0.910	0.729	0.796	0.826	0.917	0.805	0.843	0.878	0.835
2. Sacu_NS	0.207	****	0.823	0.758	0.769	0.790	0.801	0.772	0.785	0.774	0.798
3. Sdas_NS	0.094	0.195	****	0.777	0.820	0.865	0.926	0.816	0.864	0.860	0.845
4. Sblu_NS	0.316	0.277	0.252	****	0.678	0.774	0.744	0.690	0.789	0.796	0.769
5. Slep_NS	0.228	0.263	0.199	0.389	****	0.774	0.811	0.935	0.828	0.790	0.833
6. Smac_NS	0.191	0.235	0.146	0.256	0.257	****	0.811	0.804	0.862	0.850	0.884
7. Spar_SLB	0.087	0.222	0.077	0.295	0.209	0.210	****	0.820	0.848	0.844	0.819
8. Slep_SLB	0.217	0.259	0.203	0.371	0.067	0.218	0.198	****	0.831	0.822	0.850
9. Spal_SLB	0.171	0.243	0.146	0.237	0.189	0.149	0.165	0.185	****	0.860	0.862
10. Splat_SLB	0.130	0.256	0.151	0.228	0.235	0.162	0.170	0.197	0.150	****	0.885
11. Sjoh_SLB	0.181	0.226	0.169	0.262	0.183	0.124	0.200	0.163	0.149	0.123	****

Fig. 1 Geographic distribution of 11 populations of nine *Shorea* species studied
1: *S. parvifolia*; 2: *S. acuminata*; 3: *S. dasyphylla*; 4: *S. blumutensis*; 5: *S. leprosula*; 6: *S. macroptera*; 7: *S. parvifolia*; 8: *S. leprosula*; 9: *S. palembanica*; 10: *S. platyclados*; 11: *S. johorensis*; NS: Nanjak Makmur Sumatra; SLB: Sumalindo Borneo

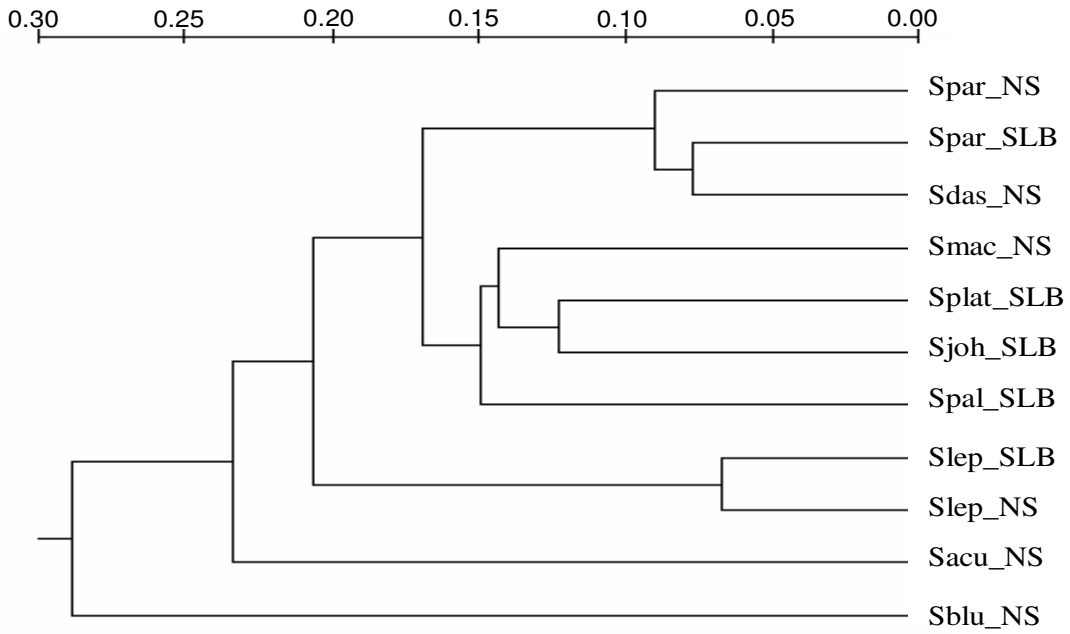

Fig. 2 UPGMA dendrogram representing the genetic distances among populations of *Shorea* species based on Nei's (1978) genetic distance

Fig. 3a UPGMA dendrogram based on the Dice (1945) index
NS: Nanjak Makmur Sumatra; SLB: Sumalindo Borneo

Fig. 3b UPGMA dendrogram based on the Dice (1945) index
NS: Nanjak Makmur Sumatra; SLB: Sumalindo Borneo

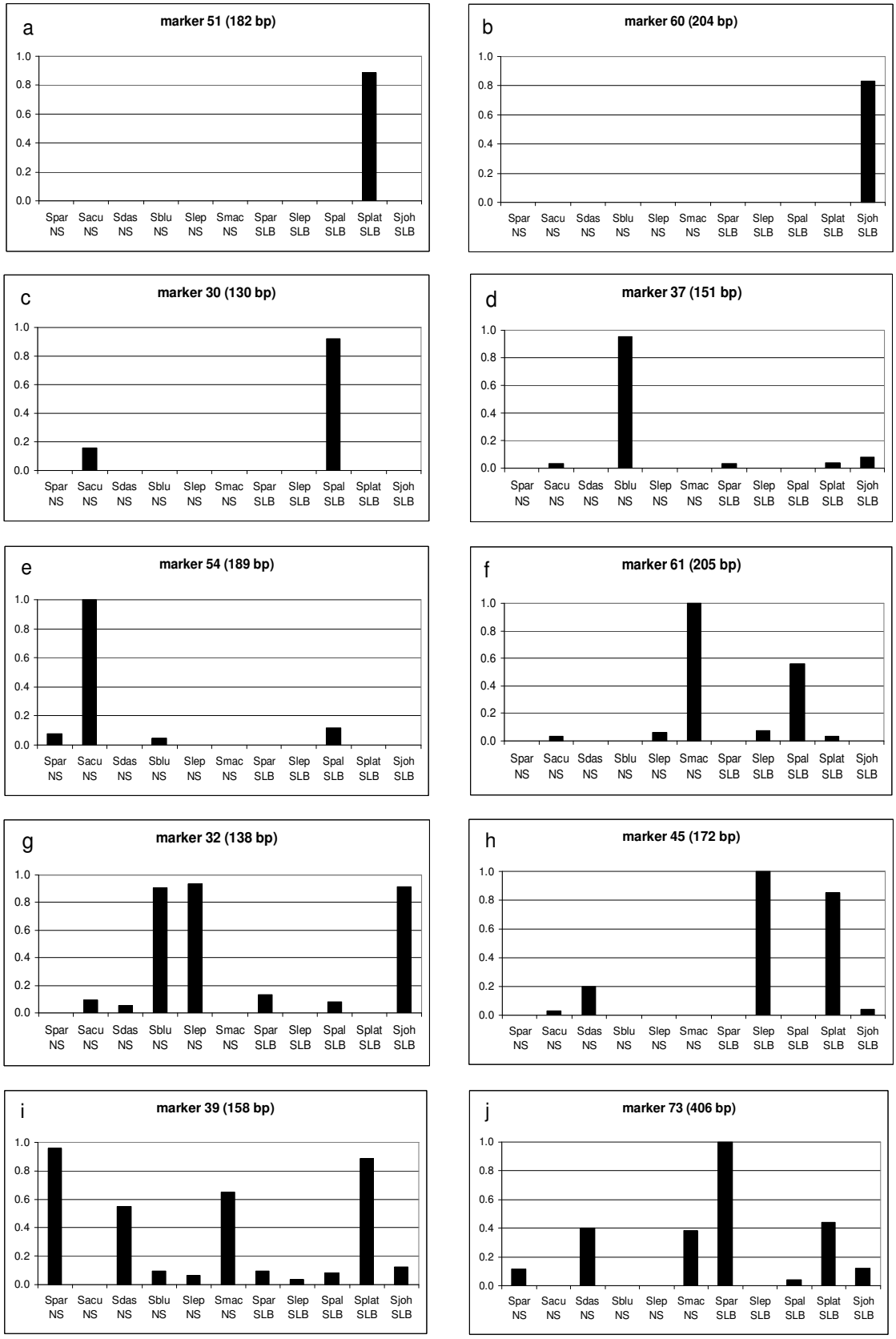

Fig. 4 Distribution of AFLP markers in *Shorea* species
NS: Nanjak Makmur Sumatra; SLB: Sumalindo Borneo

Spar: *Shorea parvifolia;*	Sacu: *S. acuminata;*	Sdas: *S. dasyphylla;*
Sblu: *S. blumutensis;*	Slep: *S. leprosula;*	Smac: *S. macroptera;*
Spal: *S. palembanica*	Splat: *S. platyclados*	Sjoh: *S. johorensis*

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Appendix 1: List of publication

1. Cao C-P, Gailing O, Siregar I, Indrioko S, Finkeldey R (2004) Comparison of phylogenies based on AFLP and PCR-RFLP. Botanikertagung in Braunschweig S. 446

Abstract:

Southeast-Asia is the diversity center of the Dipterocarpaceae, a pantropical tree family with more than 500 species in 17 genera. Asian forests dominated by dipterocarps are hot-spots of global biodiversity, and the timber industry of many Southeast-Asian countries critically depends on wood of dipterocarps. Indonesia, particularly Sumatra and Kalimantan, is the region of the highest species diversity of the Dipterocarpaceae. The taxonomic subdivision of the Dipterocarpaceae family and its phylogeny remain under dispute. Only few attempts have been made to clarify species' relationships based on molecular markers. We assessed genetic differentiation among dipterocarp trees belonging to 58 species at AFLP (Amplified Fragment Length Polymorphism) loci and compared our results to a study using variation of cpDNA. We expect to obtain novel insights into the evolution of dipterocarps in Southeast-Asia by the combination of different molecular techniques and traditional taxonomic assessments. AFLPs will also be used to explore patterns of genetic variation within and among populations of selected species.

2. Indrioko S, Cao C-P, Luu HT, Siregar I, Siregar U, Leinemann L, Gailing O, Finkeldey R (2005) Studien zur genetischen Variation südostasiatischer Dipterocarpaceen als Grundlage für ihre Erhaltung. In: Korn H, Feit U (eds) Treffpunkt Biologische Vielfalt V. Bundesamt für Naturschutz, Bonn, 235-239

3. Cao C-P, Finkeldey R, Gailing O (2005) Geographic differentiation within *Shorea leprosula* and *Shorea parvifolia* (Dipterocarpaceae) as revealed by amplified fragment length polymorphism (AFLP) markers. XVII International Botanical Congress. Wien. S. 462-463

Abstract:

Shorea leprosula and *Shorea parvifolia* are widely distributed in Indonesia and constitute economically and ecologically important species. We analysed six populations of *Shorea parvifolia* and seven populations of *Shorea leprosula* from a total of six locations in Sumatra and Borneo and one plantation at 56 well scorable AFLP markers. Cluster Analysis (NTSYS pc2) revealed a clear differentiation between species. A high differentiation among

geographic origins could be observed for *Shorea parvifolia* (G_{st} = 0.31) and *Shorea leprosula* (G_{st} = 025). For both species, AFLP markers with very strong differentiation between geographic regions could be found. For *Shorea leprosula*, we detected one "diagnostic" marker that differentiated between origins from Sumatra and Borneo with a frequency difference of 96%. For two additional populations of both species, specific "private" AFLP markers could be detected. We want to characterize these markers more closely for wood certification purposes.

4. Cao C-P, Gailing O, Siregar I, Indrioko S, Finkeldey R (submitted) Genetic variation at AFLPs for the Dipterocarpaceae and its relation to molecular phylogenies and taxonomic subdivisions

Abstract: (see part 1)

5. Cao C-P, Finkeldey R, Siregar I, Siregar U, Gailing O (submitted) Genetic diversity within and among populations of *Shorea leprosula* Miq. and *S. parvifolia* Dyer (Dipterocarpaceae) in Indonesia detected by AFLPs

Abstract: (see part 2)

6. Cao C-P, Gailing O, Siregar I, Siregar U, Finkeldey R (submitted) Genetic variation in nine *Shorea* species in Indonesia revealed by AFLPs

Abstract: (see part 3)

Appendix 2: Protocol of AFLP

1. - Restriction - Ligation - Mix RLM (for 10 probes):

T4 DNA Ligase buffer 10 ×	2.0 µl
0.5M NaCl	2.0 µl
BSA (1 mg/ml)	1.0 µl
*Mse*I (10 u/µl)	0.8 µl
*Eco*RI (10 u/µl)	4.0 µl
T4 DNA Ligase (4 u/µl)	1.9 µl
HPLC H_2O	8.3 µl
	20.0 µl

- **Restriction - Ligation - Reaction RLR (for 10 probes):**

T4 DNA Ligase buffer 10 ×	10.0 µl
0.5M NaCl	10.0 µl
BSA (1 mg/ml)	5.0 µl
*Mse*I Adaptor pair	6.0 µl
*Eco*RI Adaptor pair	6.0 µl
HPLC H_2O	23.0 µl
	60.0 µl

- **for each probe:**

6 µl RLR + 4 µl genomic DNA + 2 µl RLM

mix stays overnight (14 – 16 h at 22 °C room temperature)

2. Preselective amplification:

HPLC H_2O	13.5 µl
PCR-buffer (10×)	2.5 µl
dNTPs (10 mM)	0.38 µl
Primer M03	0.25 µl
Primer E01	0.25 µl
Taq-polymerase	0.1 µl
DNA (Restriction-Ligation-Reaction product)	8.0 µl
	25 µl

PCR protocol (aflp-preamp):

72 °C 2 min

[94 °C 10 sec, 56 °C 30 sec, 72 °C 2 min]$_{20}$

60 °C 30 min

4 °C forever

3. Selective amplification:

HPLC H_2O	9.25 µl
PCR-buffer (10×)	1.67 µl
dNTPs (10 mM)	0.25 µl
*Mse*I primer (M63)	0.25 µl
*Eco*RI primer (E35)	0.25 µl
Taq-polymerase	0.0675 µl
DNA (aflp-preamp product)	4.0 µl
	≅ 15 µl

PCR protocol (aflp-selec):

94 °C 2 min
[94 °C 10 sec, 65 °C 30 sec, 72 °C 2 min]$_1$
[94 °C 10 sec, 64 °C 30 sec, 72 °C 2 min]$_1$
[94 °C 10 sec, 63 °C 30 sec, 72 °C 2 min]$_1$
[94 °C 10 sec, 62 °C 30 sec, 72 °C 2 min]$_1$
[94 °C 10 sec, 61 °C 30 sec, 72 °C 2 min]$_1$
[94 °C 10 sec, 60 °C 30 sec, 72 °C 2 min]$_1$
[94 °C 10 sec, 59 °C 30 sec, 72 °C 2 min]$_1$
[94 °C 10 sec, 58 °C 30 sec, 72 °C 2 min]$_1$
[94 °C 10 sec, 57 °C 30 sec, 72 °C 2 min]$_1$
[94 °C 10 sec, 56 °C 30 sec, 72 °C 2 min]$_{24}$
60 °C 30 min
4 °C forever

Appendix 3: Sequences of adaptors and primers used

*Eco*RI adapter		5'-CTCGTAGACTGCGTACC-3' 3'-CATCTGACGCATGGTTAA-5'
MseI adapter		5-'GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
*Eco*RI + 0 primer	E00	5'-GACTGCGTACCAATTC-3'
*Eco*RI + 1 primer	E01	5'-GACTGCGTACCAATTC**A**-3'
*Eco*RI + 3 primer	E35	5'-GACTGCGTACCAATTC**ACA**-3'
*Mse*I + 0 primer	M00	5'-GATGAGTCCTGAGTAA-3'
*Mse*I + 1 primer	M03	5'-GATGAGTCCTGAGTAA**G**-3'
*Mse*I + 3 primer	M63	5'-GATGAGTCCTGAGTAA**GAA**-3'

Appendix 4: PCR-RFLP analysis

1. PCR amplification

The tested universal oligonucleotide primers have been used for the amplification of selected cpDNA gene regions of *Shorea* species. The sequences of these primers are described in the following table:

Sequences of primer pairs used in PCR-RFLP of cpDNA

Gene / IS	Gene product/position	Forward and reverse primer sequence (5'-3')	Note(*)
trnL-F	tRNA-Leu (UAA)- tRNA-Phe (GAA)	CGA AAT CGG TAG ACG CTA CG ATT TGA ACT GGT GAC ACG AG	A
rbcL	RuBisCo large subunit	TGT CAC CAA AAA CAG AGA CT TTC CAT ACT TCA CAA GCA GC	B

(*): A = Taberlet et al. (1991); B = Tsumura et al. (1996)

Temperature profile of PCR steps

Step 1: initial denaturation for 15 minutes at 95°C

Step 2: 35 cycles of:

denaturation for 1 minute at 94 °C

annealing for 1 minute at 56 °C (for trnL-F) or 50 °C (for the other primers)

extension for 2 minutes at 72 °C

Step 3: final extension for 10 minutes at 72 °C

Reaction mix (15 µl) of PCR reagents

Template DNA (5-10 ng)	2.0 µl
Forward primer (5 pmol/µl)	1.8 µl
Reverse primer (5 pmol/µl)	1.8 µl
Distilled water	1.9 µl
HotStar Taq® Master Mix Kit (Qiagen, Hilden)	7.5 µl

The extracted DNA was diluted to 1-10% prior to PCR. Amplification products were analysed electrophoretically in order to check if the PCR was successful.

2. Restriction of PCR product using enzymes (PCR-RFLP)

Cp DNA fragments amplified with selected universal primers were then digested with restriction endonucleases, i.e. *Alu*I and *Hinf*I (Roche, Mannheim) as described in table below. These enzymes recognize specific target sequence of four-base sites (four-cutter).

Restriction endonuclease for digesting the amplification product

Name	Sequence (5'-3')	Incubation Temp. (℃)
*Alu*I	AG↓CT	37
*Hinf*I	G↓ANTC	37

The restriction solution was incubated for at least three hours and at most one night. This solution was prepared as follows:

Restriction solution (11.5 µl)

Restriction endonuclease	1.0 µl (1 unit)
Enzyme specific buffer	1.0 µl
Amplification product	5.0 µl
Distilled water	4.5 µl

The PCR-RFLP products were separated electrophoretically after digestion in order to compare the DNA band patterns of the samples investigated with those of clearly identified samples and to determine the species name of the samples investigated.

Appendix 5: Chloroplast simple sequence repeat (cpSSR) analysis

The universal primers *ccmp*6 (consensus chloroplast micrisatellite primers) (Weising and Gardner 1999) were used for cpSSRs analysis.

Sequence of primer pairs used for cpSSRs analysis

cpSSR Locus	Location	Forward and reverse primer sequence (5' - 3')
ccmp 6	ORF77 - ORF 82 intergenic spacer	CGA TGC ATA TGT AGA AAG CC CAT TAC GTG CGA CTA TCT CC

Temperature profile of PCR steps

Step 1: initial denaturation for 15 minutes at 95°C

Step 2: 35 cycles of:

denaturation for 1 minute at 94 °C

annealing for 1 minute at 50 °C

extension for 1 minutes at 72 °C

Step 3: final extension for 10 minutes at 72 °C

The reaction mix of PCR reagents is the same as for PCR-RFLP.

Genotyping of PCR product

Amplification products were separated by capillar electrophoresis on an automated sequencer ABI Prism 3100® Genetic Analyzer (Applied Biosystems) with polymer 3100 POP-4™ (Applied Biosystems). The length of electrophoresis products (in base pairs = bps) was measured with the help of th internal size standard GS 500 ROX™ (Applied Biosystems). Individual alleles were analysed using GeneScan© version 3.7 (Applied Biosystems) and genotyped using Genotyper© version 3.7 NT (Applied Biosystems). The reagents for genotyping were described as follows:

Reagent mix for genotyping (for 96 probes)

GS 500 ROX™ (Applied Biosystems)	2 µl
HiDi Formamide (Applied Biosystems)	1.152 ml

The reagent mix was distributed equally for 96 sample tubes, and then 2 µl of amplificatiom product of each sample was added to the tubes.

Appendix 6: Species, size in bp and presence and absence of diagnostic markers (from part 1)

Species	Size in bp, presence (1) and absence (0) of diagnostic markers												
	238	319	188	213	426	304	246	338	332	364	363	144	361
Anisoptera reticulata	**1**	0	0	0	0	0	0	0	0	0	0	0	0
Anisoptera marginata	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0
Anisoptera costata 1	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0
Anisoptera costata 2	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0
Vatica bantamensis 1	0	0	**1**	**1**	**1**	0	0	0	0	0	0	0	0
Vatica bantamensis 2	0	0	**1**	**1**	**1**	0	0	0	0	0	0	0	0
Vatica venulosa	0	0	**1**	**1**	0	0	0	0	0	0	0	0	0
Vatica bella	0	0	**1**	**1**	0	0	0	0	0	0	0	0	0
Vatica granulata	0	0	**1**	**1**	0	0	0	0	0	0	0	0	0
Vatica rassak	0	0	**1**	**?**	0	0	0	0	0	0	0	0	0
Vatica pauciflora 1	0	0	**1**	**1**	0	0	0	0	0	0	0	0	0
Vatica pauciflora 2	0	0	**1**	**1**	0	0	0	0	0	0	0	0	0
Hopea celebica	0	0	0	0	0	**1**	0	0	0	0	0	0	0
Hopea odorata 1	0	0	0	0	0	0	**1**	0	0	0	0	0	0
Hopea odorata 2	0	0	0	0	0	0	**1**	0	0	0	0	0	0
Shorea mecistopteryx 1	0	0	0	0	0	0	0	**1**	0	0	0	0	0
Shorea mecistopteryx 2	0	0	0	0	0	0	0	**1**	0	0	0	0	0
Shorea mecistopteryx 3	0	0	0	0	0	0	0	**1**	0	0	0	0	0
Shorea guiso 1	0	0	0	0	0	0	0	0	**1**	0	0	0	0
Shorea guiso 2	0	0	0	0	0	0	0	0	**?**	0	0	0	0
Shorea acuminata 1	0	0	0	0	0	0	0	0	0	**1**	0	0	0
Shorea acuminata 2	0	0	0	0	0	0	0	0	0	**1**	0	0	0
Shorea acuminata 3	0	0	0	0	0	0	0	0	0	**1**	0	0	0
Shorea virescens 1	0	0	0	0	0	0	0	0	0	0	**1**	0	0
Shorea virescens 2	0	0	0	0	0	0	0	0	0	0	**1**	0	0
Dryobalanops aromatica	0	0	0	0	0	0	0	0	0	0	0	**1**	**1**
Dryobalanops lanceolata 1	0	0	0	0	0	0	0	0	0	0	0	**1**	**1**
Dryobalanops lanceolata 2	0	0	0	0	0	0	0	0	0	0	0	**1**	**1**

Legend: the question mark (?) means that band was ambiguous.

Appendix 7: Frequency distribution of AFLP markers in *Shorea leprosula* and *S. parvifolia* and genetic Differentiation (G_{st}) among populations at each locus generated with POPGENE ver 1.31 (Yeh et al. 1999) (from part 2)

		1	2	3	4	5	6	7	8	9	10
Pop. ID	Sample size	63	64	108	130	138	141	143	144	150	167
*S. lepr*_HA	34	0.088	0.000	0.353	0.000	0.912	0.000	0.088	0.265	0.206	0.000
*S. lepr*_TB	23	0.000	0.130	0.000	0.000	0.957	0.000	0.304	0.174	0.000	0.000
*S. lepr*_AS	9	0.222	0.222	0.000	0.111	0.778	0.111	0.222	0.778	0.000	0.889
*S. lepr*_PS	11	0.000	0.364	0.000	0.000	0.818	0.000	0.000	0.091	0.000	0.000
*S. lepr*_TS	22	0.000	0.182	0.000	0.000	0.864	0.000	0.000	0.273	0.000	0.000
*S. lepr*_NS	16	0.000	0.188	0.000	0.000	1.000	0.000	0.313	0.688	0.125	0.000
*S. lepr*_SB	18	0.111	0.278	0.000	0.000	1.000	0.000	0.278	0.000	0.000	0.000
	G_{st}	0.058	0.037	0.173	0.049	0.169	0.049	0.058	0.221	0.069	0.632
*S. parv*_BB	16	0.125	1.000	0.000	0.000	0.000	0.750	0.000	0.188	0.000	0.438
*S. parv*_AS	33	0.606	1.000	0.000	0.000	0.030	0.545	0.030	0.485	0.030	0.758
*S. parv*_PS	23	0.652	1.000	0.000	0.000	0.000	0.217	0.000	0.217	0.000	0.217
*S. parv*_TS	14	0.071	1.000	0.000	0.000	0.000	0.857	0.000	0.214	0.000	0.000
*S. parv*_NS	28	1.000	1.000	0.000	0.107	0.000	0.107	0.000	0.571	0.000	0.036
*S. parv*_SB	21	0.952	1.000	0.000	0.190	0.048	0.667	0.000	0.238	0.000	0.048
	G_{st}	0.499	0.000	0.000	0.060	0.014	0.181	0.013	0.065	0.013	0.251
Between-LP	G_{st}	0.206	0.848	0.024	0.009	0.539	0.153	0.041	0.003	0.013	0.047

		11	12	13	14	15	16	17	18	19	20
Pop. ID	Sample size	172	176	181	193	199	201	221	222	228	242
*S. lepr*_HA	34	0.000	0.382	0.235	0.618	0.000	0.000	0.000	0.000	0.647	0.059
*S. lepr*_TB	23	1.000	0.348	0.348	0.261	0.000	0.000	0.043	0.000	0.957	0.000
*S. lepr*_AS	9	0.111	0.000	0.556	0.889	0.000	0.000	0.111	0.000	0.889	0.111
*S. lepr*_PS	11	0.000	0.182	0.636	0.636	0.000	0.000	0.000	0.000	0.727	0.182
*S. lepr*_TS	22	0.000	0.000	0.500	0.455	0.045	0.000	0.000	0.000	0.682	0.000
*S. lepr*_NS	16	0.000	0.125	0.625	0.813	0.000	0.000	0.000	0.000	0.313	0.125
*S. lepr*_SB	18	1.000	0.389	0.667	0.889	0.000	0.000	0.111	0.000	0.833	0.000
	G_{st}	0.963	0.080	0.050	0.143	0.020	0.000	0.033	0.000	0.139	0.037
*S. parv*_BB	16	0.063	0.000	0.000	0.000	0.500	0.375	0.000	0.625	1.000	1.000
*S. parv*_AS	33	0.061	0.030	0.273	0.061	0.030	0.061	0.152	0.000	0.606	0.970
*S. parv*_PS	23	0.043	0.000	0.130	0.000	0.000	0.043	0.348	0.000	0.261	0.957
*S. parv*_TS	14	0.000	0.143	0.357	0.143	0.000	0.000	0.143	0.000	1.000	1.000
*S. parv*_NS	28	0.036	0.000	0.286	0.000	0.000	0.000	0.107	0.000	0.679	1.000
*S. parv*_SB	21	0.000	0.048	0.238	0.048	0.000	0.000	0.190	0.000	0.952	1.000
	G_{st}	0.010	0.037	0.041	0.034	0.240	0.135	0.044	0.345	0.450	0.138
Between-LP	G_{st}	0.069	0.049	0.035	0.201	0.012	0.017	0.026	0.019	0.000	0.732

		21	22	23	24	25	26	27	28	29	30
Pop. ID	Sample size	372	406	409	411	412	415	465	467	469	108.6
*S. lepr*_HA	34	0.000	0.000	0.000	1.000	0.000	0.853	0.941	0.000	1.000	0.059
*S. lepr*_TB	23	0.043	0.000	0.783	0.435	0.000	1.000	1.000	0.043	1.000	0.174
*S. lepr*_AS	9	0.333	0.000	0.000	0.889	0.000	0.889	1.000	0.111	0.889	0.111
*S. lepr*_PS	11	0.000	0.000	0.000	0.636	0.091	0.818	0.909	0.000	1.000	0.182
*S. lepr*_TS	22	0.000	0.000	0.000	1.000	0.000	0.682	0.818	0.045	1.000	0.045
*S. lepr*_NS	16	0.000	0.000	0.063	1.000	0.000	0.875	1.000	0.000	1.000	0.250
*S. lepr*_SB	18	0.000	0.000	0.111	0.833	0.000	0.833	0.944	0.222	0.889	0.000
	G_{st}	0.164	0.000	0.412	0.394	0.040	0.112	0.236	0.057	0.263	0.034
*S. parv*_BB	16	1.000	1.000	0.000	0.688	0.000	1.000	0.000	1.000	1.000	0.000
*S. parv*_AS	33	0.909	0.545	0.061	0.303	0.455	0.970	0.000	0.970	0.545	0.485
*S. parv*_PS	23	1.000	0.174	0.087	0.130	0.826	1.000	0.000	1.000	0.130	0.087
*S. parv*_TS	14	1.000	0.786	0.000	1.000	0.000	1.000	0.000	1.000	1.000	0.000
*S. parv*_NS	28	1.000	0.107	0.071	0.000	0.964	1.000	0.000	1.000	0.000	0.107
*S. parv*_SB	21	0.905	0.905	0.048	0.143	0.238	1.000	0.000	1.000	0.952	0.000
	G_{st}	0.226	0.448	0.013	0.583	0.441	0.149	0.000	0.149	0.806	0.169
Between-LP	G_{st}	0.648	0.187	0.015	0.202	0.160	0.126	0.626	0.789	0.292	0.003

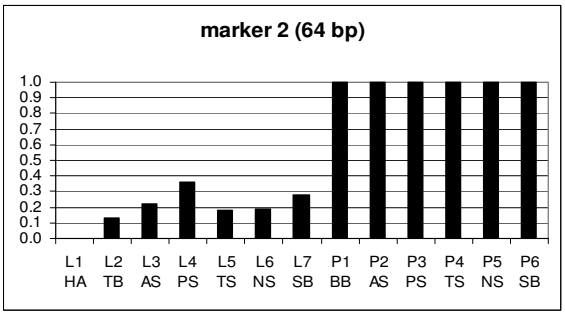
		31	32	33	34	35	36	37	38	39	40
Pop. ID	Sample size	109.6	126.4	134.7	137.7	146.3	156.6	157.5	160.3	161.1	166.4
*S. lepr*_HA	34	0.471	0.618	0.441	0.059	0.647	0.029	0.000	0.794	0.441	0.529
*S. lepr*_TB	23	0.087	0.478	0.130	0.000	0.696	0.000	0.000	1.000	0.043	0.000
*S. lepr*_AS	9	0.667	0.444	0.333	0.000	1.000	0.333	0.111	0.556	0.778	0.000
*S. lepr*_PS	11	0.364	0.273	0.273	0.091	0.909	0.000	0.000	0.364	0.364	0.000
*S. lepr*_TS	22	0.318	0.455	0.091	0.045	0.818	0.000	0.000	0.364	0.227	0.000
S. lepr_NS	16	0.188	0.375	0.375	0.000	0.875	0.000	0.063	0.375	0.250	0.000
*S. lepr*_SB	18	0.278	0.000	0.000	0.000	0.833	0.000	0.111	0.944	0.833	0.000
	G_{st}	0.084	0.072	0.066	0.022	0.140	0.147	0.031	0.346	0.248	0.282
*S. parv*_BB	16	0.000	0.313	0.000	0.938	0.375	0.000	0.688	0.000	0.000	0.000
*S. parv*_AS	33	0.000	0.303	0.000	0.273	0.515	0.727	0.515	0.000	0.030	0.000
*S. parv*_PS	23	0.000	0.304	0.000	0.174	0.043	0.174	0.870	0.000	0.000	0.000
*S. parv*_TS	14	0.071	0.357	0.000	0.429	0.000	0.571	0.071	0.000	0.000	0.000
*S. parv*_NS	28	0.000	0.464	0.000	0.000	0.429	0.000	0.893	0.000	0.036	0.000
*S. parv*_SB	21	0.000	0.000	0.000	0.571	0.190	0.000	0.000	0.000	0.952	0.000
	G_{st}	0.031	0.048	0.000	0.307	0.103	0.279	0.307	0.000	0.711	0.000
Between-LP	G_{st}	0.092	0.008	0.069	0.082	0.153	0.057	0.172	0.275	0.034	0.036

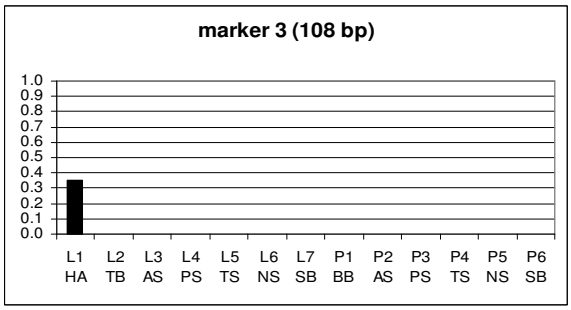
		41	42	43	44	45	46	47	48	49	50
Pop. ID	Sample size	170.8	172.4	182.3	196.6	199.3	223.8	321.5	326.5	417.6	418.5
*S. lepr*_HA	34	1.000	0.941	0.588	0.000	0.912	0.824	0.000	0.412	0.471	0.000
*S. lepr*_TB	23	0.913	0.000	0.739	0.000	0.870	0.087	0.000	0.000	0.000	1.000
*S. lepr*_AS	9	0.667	0.778	0.444	0.111	1.000	0.222	0.111	0.000	0.556	0.111
*S. lepr*_PS	11	0.818	1.000	0.091	0.000	1.000	0.727	0.000	0.000	0.818	0.091
*S. lepr*_TS	22	0.909	0.773	0.591	0.000	0.955	0.818	0.000	0.000	0.682	0.045
*S. lepr*_NS	16	0.875	0.750	0.438	0.125	0.938	1.000	0.000	0.000	0.625	0.000
*S. lepr*_SB	18	0.833	0.000	0.611	0.056	0.833	0.056	0.000	0.000	0.056	0.944
	G_{st}	0.120	0.466	0.079	0.036	0.133	0.459	0.049	0.207	0.185	0.782
*S. parv*_BB	16	0.938	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000
*S. parv*_AS	33	0.939	0.758	0.000	0.879	0.455	0.000	0.485	0.000	0.091	0.030
*S. parv*_PS	23	0.826	0.826	0.000	0.826	0.913	0.043	0.217	0.000	0.000	0.043
*S. parv*_TS	14	0.357	1.000	0.000	0.714	0.143	0.000	1.000	0.000	0.000	0.000
*S. parv*_NS	28	0.786	0.536	0.071	0.607	0.286	0.000	0.036	0.000	0.071	0.000
*S. parv*_SB	21	0.810	0.905	0.286	0.810	0.238	0.000	0.190	0.000	0.238	0.048
	G_{st}	0.139	0.289	0.104	0.163	0.310	0.018	0.715	0.000	0.061	0.011
Between-LP	G_{st}	0.014	0.036	0.151	0.355	0.253	0.198	0.129	0.028	0.085	0.081

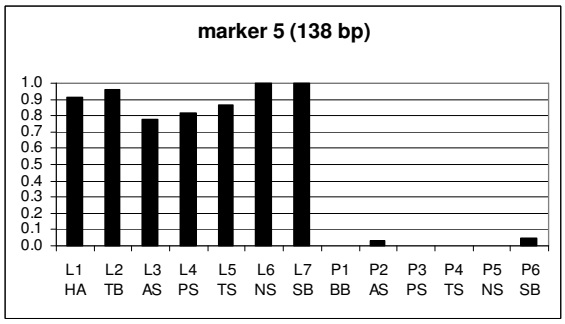
		51	52	53	54	55	56
Pop. ID	Sample size	468.3	482.5	59.8	60.5	73.8	82.4
*S. lepr*_HA	34	0.000	0.912	0.029	0.706	0.029	0.029
*S. lepr*_TB	23	0.000	0.000	0.043	1.000	0.435	0.174
*S. lepr*_AS	9	0.000	0.778	0.000	0.778	0.000	0.000
*S. lepr*_PS	11	0.000	0.636	0.000	0.909	0.182	0.091
*S. lepr*_TS	22	0.000	0.818	0.000	0.818	0.091	0.000
*S. lepr*_NS	16	0.000	0.938	0.000	0.875	0.063	0.000
*S. lepr*_SB	18	0.000	0.056	0.000	1.000	0.222	0.000
	G_{st}	0.000	0.324	0.014	0.196	0.086	0.049
*S. parv*_BB	16	0.063	0.000	0.063	0.688	0.688	0.750
*S. parv*_AS	33	0.333	0.000	0.636	0.576	0.333	0.485
*S. parv*_PS	23	0.391	0.000	0.913	0.565	0.130	0.130
*S. parv*_TS	14	0.071	0.000	0.071	0.857	0.143	0.857
*S. parv*_NS	28	0.500	0.000	0.964	0.321	0.286	0.107
*S. parv*_SB	21	0.000	0.000	0.000	0.381	0.143	0.381
	G_{st}	0.116	0.000	0.498	0.095	0.124	0.215
Between-LP	G_{st}	0.079	0.222	0.172	0.094	0.014	0.094

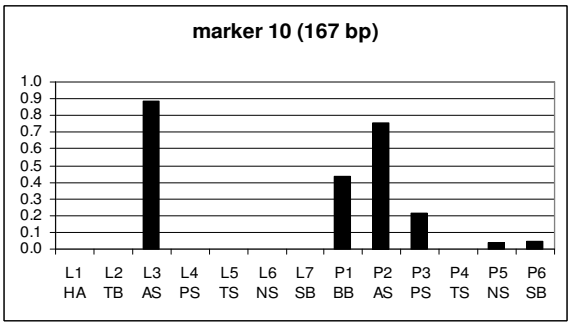
L-*S. leprosula*; P- *S. parvifolia*

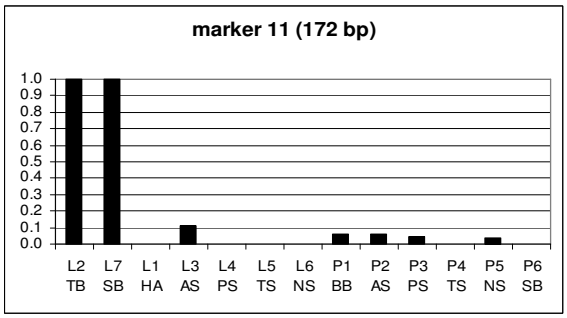
Appendix 8: AFLP marker distribution in populations of *Shorea leprosula* and *S. parvifolia* (from part 2)

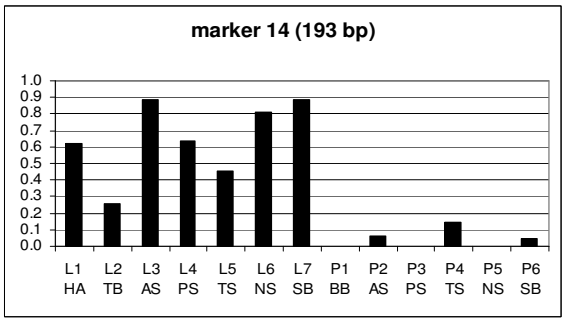


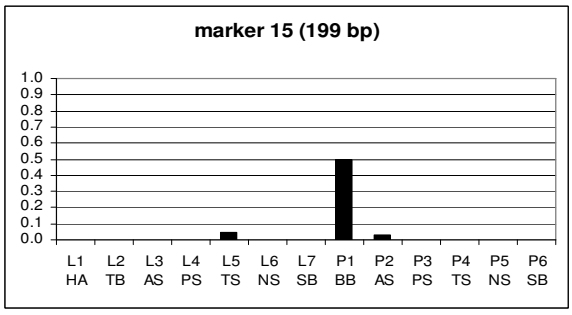


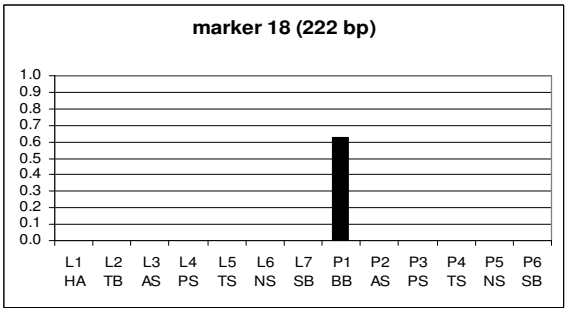


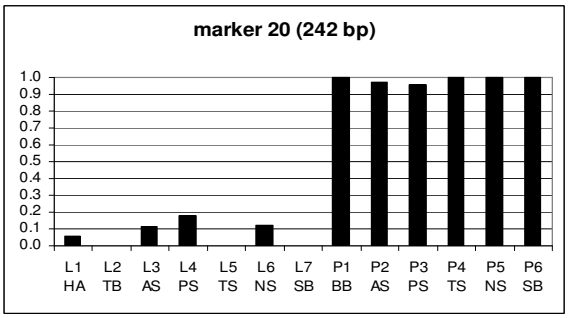


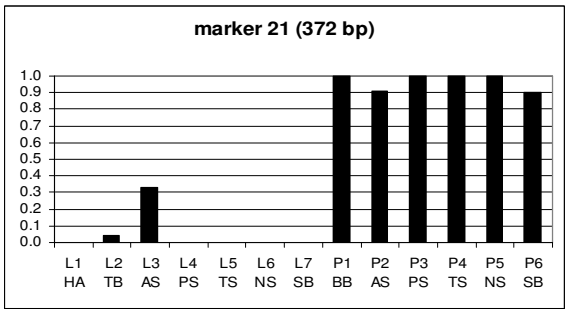


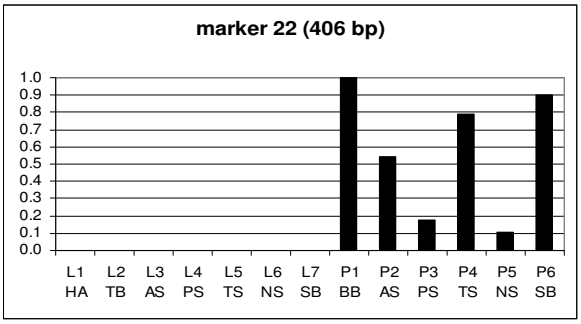


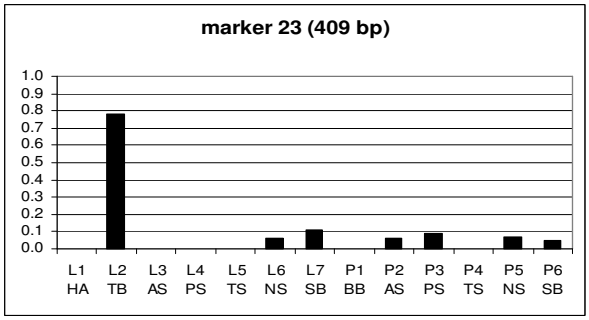


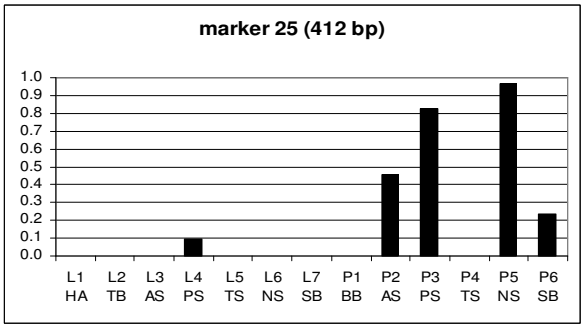


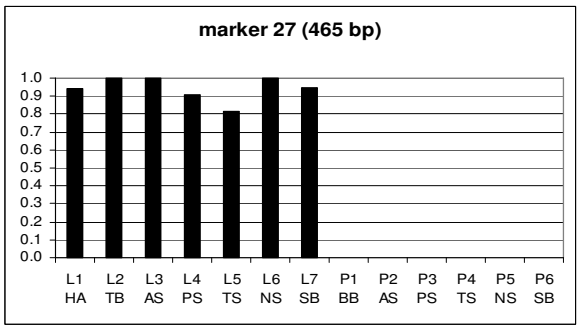


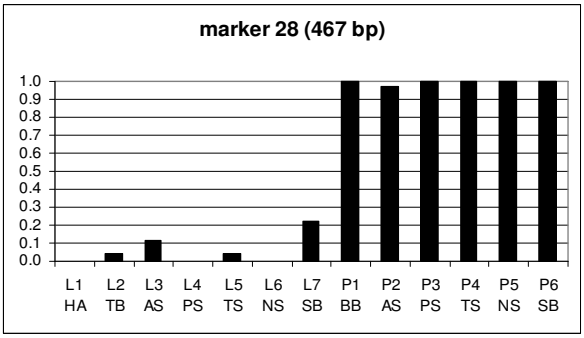


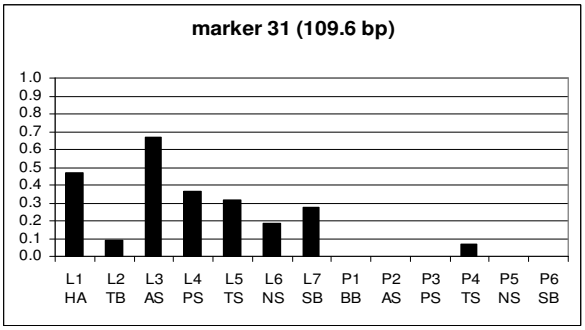

marker 22 (406 bp)
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
L1 HA L2 TB L3 AS L4 PS L5 TS L6 NS L7 SB P1 BB P2 AS P3 PS P4 TS P5 NS P6 SB

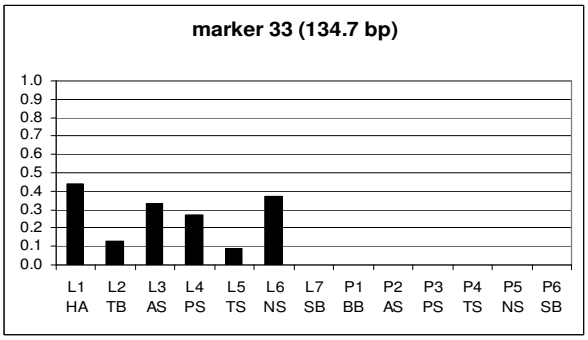

marker 23 (409 bp)
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
L1 HA L2 TB L3 AS L4 PS L5 TS L6 NS L7 SB P1 BB P2 AS P3 PS P4 TS P5 NS P6 SB

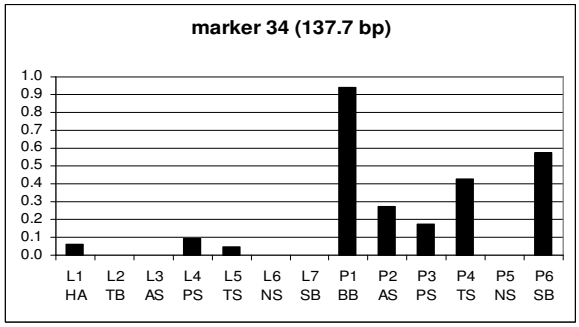

marker 25 (412 bp)
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
L1 HA L2 TB L3 AS L4 PS L5 TS L6 NS L7 SB P1 BB P2 AS P3 PS P4 TS P5 NS P6 SB

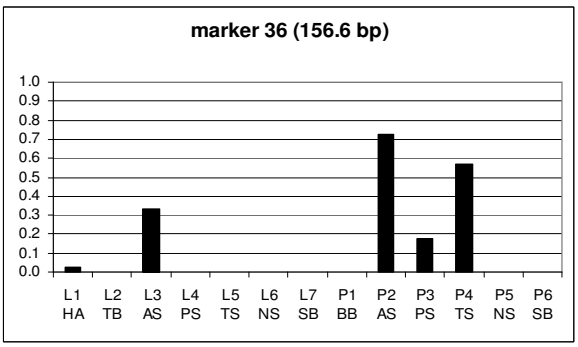

marker 27 (465 bp)
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
L1 HA L2 TB L3 AS L4 PS L5 TS L6 NS L7 SB P1 BB P2 AS P3 PS P4 TS P5 NS P6 SB

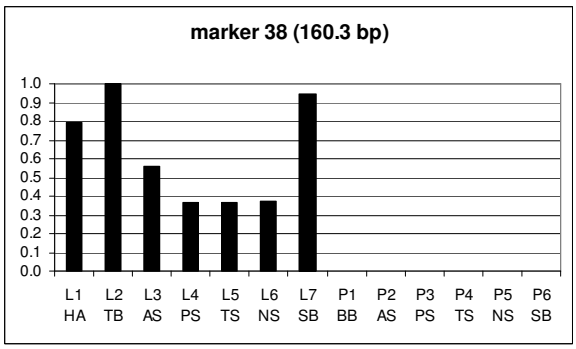

marker 28 (467 bp)
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
L1 HA L2 TB L3 AS L4 PS L5 TS L6 NS L7 SB P1 BB P2 AS P3 PS P4 TS P5 NS P6 SB

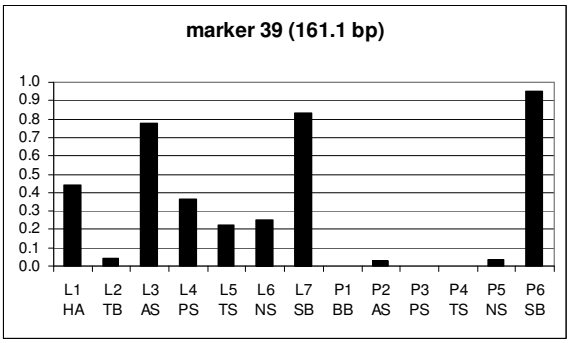

marker 31 (109.6 bp)
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
L1 HA L2 TB L3 AS L4 PS L5 TS L6 NS L7 SB P1 BB P2 AS P3 PS P4 TS P5 NS P6 SB

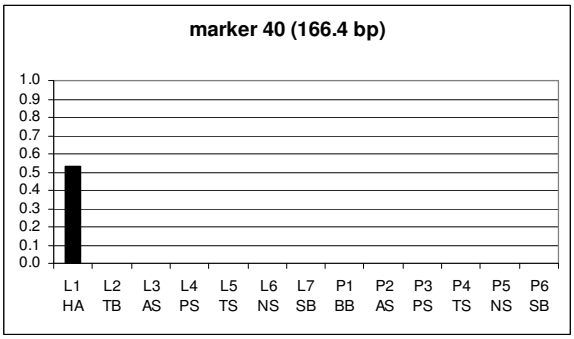

marker 33 (134.7 bp)
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
L1 HA L2 TB L3 AS L4 PS L5 TS L6 NS L7 SB P1 BB P2 AS P3 PS P4 TS P5 NS P6 SB

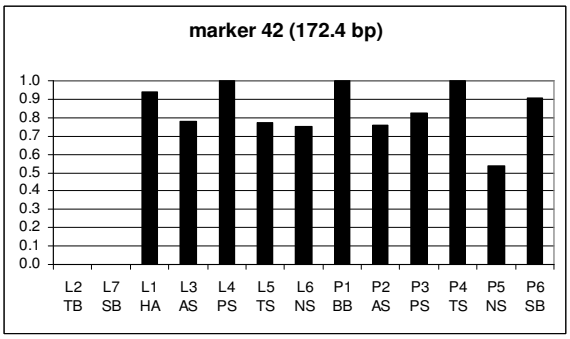

marker 34 (137.7 bp)
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
L1 HA L2 TB L3 AS L4 PS L5 TS L6 NS L7 SB P1 BB P2 AS P3 PS P4 TS P5 NS P6 SB

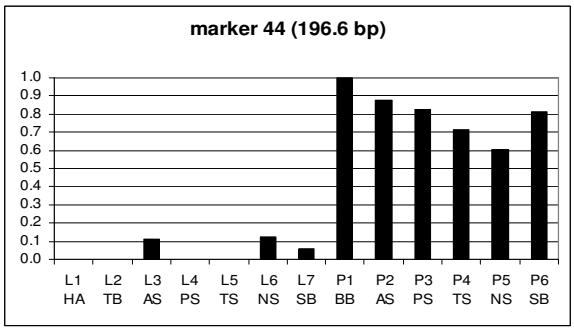

marker 36 (156.6 bp)
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
L1 HA L2 TB L3 AS L4 PS L5 TS L6 NS L7 SB P1 BB P2 AS P3 PS P4 TS P5 NS P6 SB

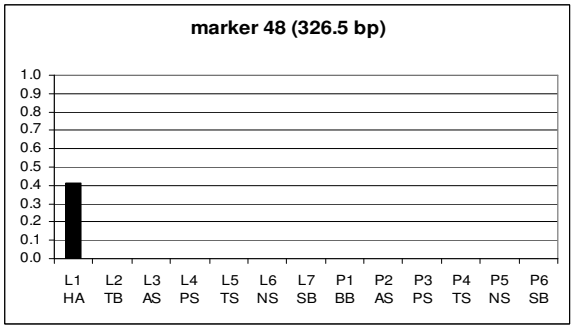

marker 38 (160.3 bp)
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
L1 HA L2 TB L3 AS L4 PS L5 TS L6 NS L7 SB P1 BB P2 AS P3 PS P4 TS P5 NS P6 SB

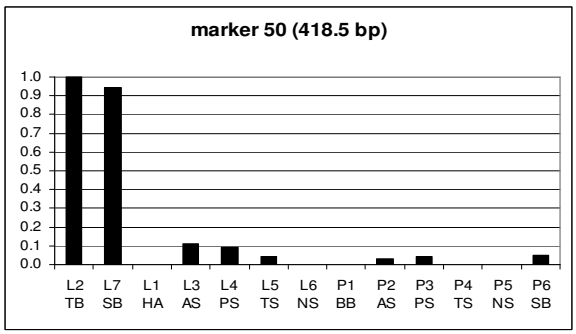


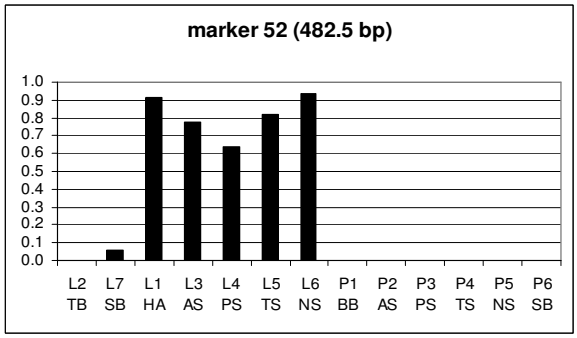












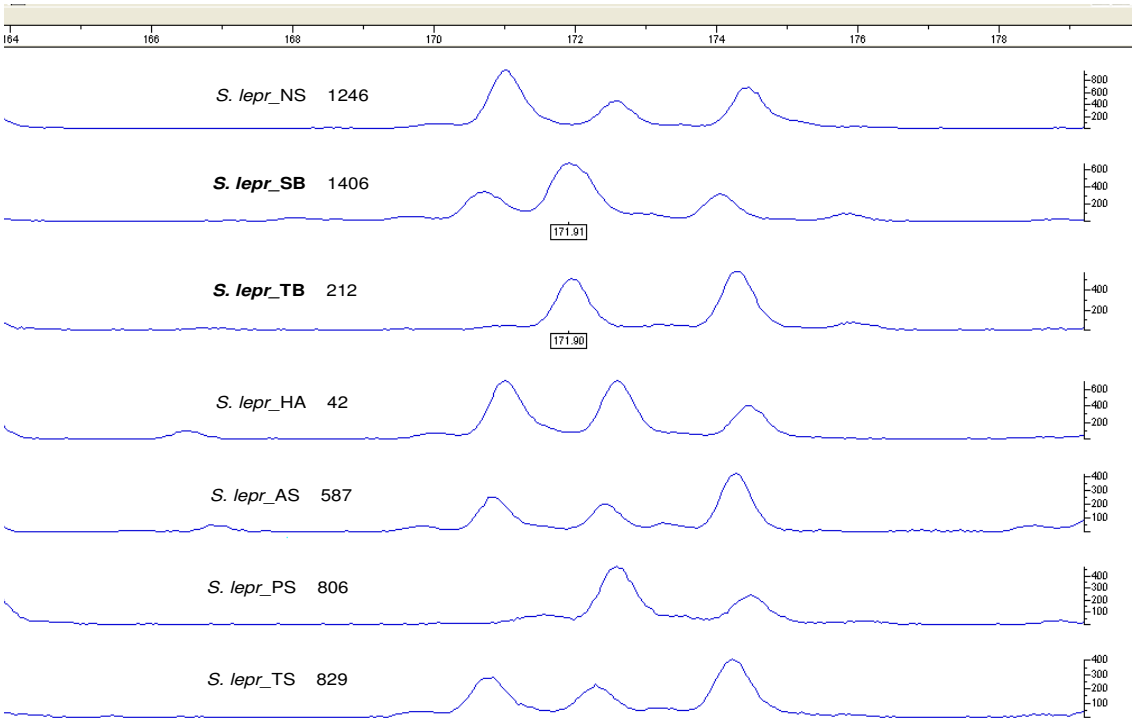
Legend:

L- *S. leprosula*
HA- Haurbentes Java;
NS- Nanjak Makmur Sumatra;
TS- TNBT Sumatra;
SB- Sari Bumi Kusuma Borneo;

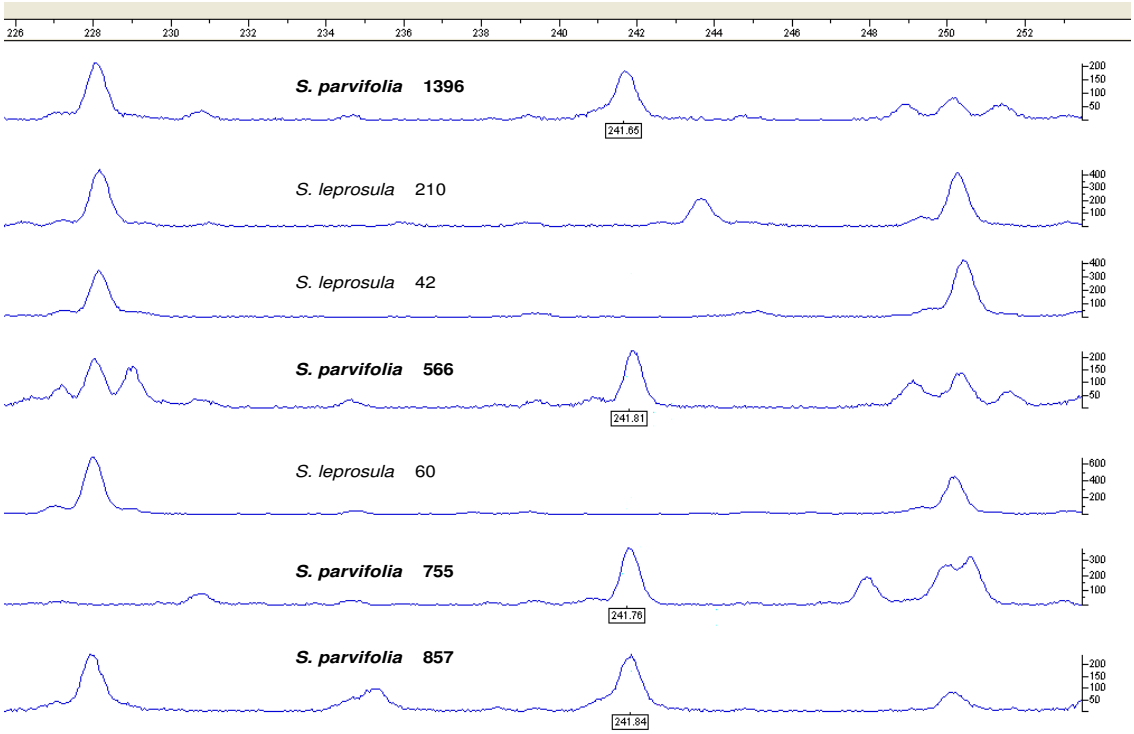
P- *S. parvifolia*
AS- Asialog Sumatra;
PS- Pasir Mayang Sumatra;
BB- Batu Ampar Borneo;
TB- Tering Borneo

Appendix 9: AFLP diagnostic markers (primer E35M63, from part 2)

(1). Marker 11 (172 bp) diagnostic to populations *S. lepr*_SB and *S. lepr*_TB of *Shorea leprosula*



(2). Marker 20 (242 bp) diagnostic to *S. parvifolia*



Legend: HA- Haurbentes Java; AS- Asialog Sumatra; NS- Nanjak Makmur Sumatra; PS- Pasir Mayang Sumatra; TS- TNBT Sumatra; SB- Sari Bumi Kusuma Borneo; TB- Tering Borneo

Appendix 10: Genetic differentiation between *Shorea leprosula* and *S. parvifolia* at each locus obtained using POPGENE ver 1.31 (Yeh et al. 1999) (from part 2)

	Locus	Sample size	H_t	H_s	G_{st}	N_m
1	63	268	0.334	0.266	0.206	1.930
2	64	268	0.497	0.076	0.848	0.090
3	108	268	0.045	0.044	0.024	20.655
4	130	268	0.030	0.029	0.009	57.919
5	138	268	0.461	0.212	0.539	0.427
6	141	268	0.240	0.203	0.153	2.764
7	143	265	0.088	0.084	0.041	11.729
8	144	267	0.291	0.290	0.003	149.652
9	150	268	0.037	0.037	0.013	39.150
10	167	268	0.170	0.162	0.047	10.160
11	172	268	0.173	0.161	0.069	6.789
12	176	268	0.133	0.127	0.049	9.814
13	181	268	0.309	0.299	0.035	13.977
14	193	268	0.316	0.253	0.201	1.993
15	199	268	0.037	0.037	0.012	40.187
16	201	268	0.033	0.033	0.017	28.491
17	221	268	0.095	0.093	0.026	18.962
18	222	268	0.037	0.036	0.019	25.490
19	228	267	0.497	0.497	0.000	2000.000
20	242	267	0.496	0.133	0.732	0.184
21	372	267	0.484	0.170	0.648	0.272
22	406	267	0.265	0.216	0.187	2.181
23	409	268	0.103	0.101	0.015	32.175
24	411	268	0.473	0.378	0.202	1.977
25	412	268	0.247	0.208	0.160	2.623
26	415	268	0.362	0.316	0.126	3.472
27	465	267	0.474	0.177	0.626	0.299
28	467	266	0.498	0.105	0.789	0.134
29	469	266	0.486	0.344	0.292	1.214
30	108.6	268	0.126	0.126	0.003	172.774
31	109.6	268	0.165	0.150	0.092	4.960
32	126.4	268	0.317	0.315	0.008	58.922
33	134.7	268	0.120	0.112	0.069	6.778
34	137.7	268	0.183	0.168	0.082	5.607
35	146.3	268	0.453	0.384	0.153	2.777
36	156.6	268	0.146	0.138	0.057	8.354
37	157.5	268	0.284	0.235	0.172	2.407
38	160.3	268	0.338	0.245	0.275	1.318
39	161.1	267	0.256	0.248	0.034	14.307
40	166.4	268	0.068	0.065	0.036	13.260
41	170.8	268	0.472	0.465	0.014	35.557
42	172.4	268	0.496	0.478	0.036	13.255
43	182.3	268	0.294	0.250	0.151	2.807
44	196.6	267	0.407	0.262	0.355	0.908
45	199.3	268	0.497	0.372	0.253	1.479
46	223.8	268	0.284	0.228	0.198	2.020
47	321.5	267	0.212	0.185	0.129	3.392
48	326.5	268	0.053	0.051	0.028	17.486
49	417.6	268	0.239	0.218	0.085	5.383
50	418.5	268	0.171	0.157	0.081	5.682
51	468.3	266	0.135	0.125	0.079	5.850

	Locus	Sample size	H_t	H_s	G_{st}	N_m
52	482.5	268	0.297	0.231	0.222	1.756
53	59.8	268	0.268	0.222	0.172	2.403
54	60.5	268	0.498	0.452	0.094	4.849
55	73.8	268	0.204	0.201	0.014	36.659
56	82.4	268	0.217	0.197	0.094	4.797
	Mean	268	0.266	0.203	0.238	1.602
	SD		0.025	0.014		

Estimates of genetic diversity and gene flow of *Shorea leprosula* and *S. parvifolia* generated with POPGENE ver 1.31 (Yeh et al. 1999) (from part 2)

	Sample size	H_t	H_s	G_{st}	N_m
S. leprosula	133	0.214	0.161	0.246	1.535
SD		0.034	0.021		
S. parvifolia	135	0.202	0.138	0.314	1.091
SD		0.034	0.016		
S. lepr.-S. parv.	268	0.266	0.203	0.238	1.602
SD		0.025	0.014		

H_t, total genetic diversity; H_s, genetic diversity within populations; G_{st}, proportion of total genetic diversity partitioned among populations; N_m, gene flow from $N_m = 0.5(1 - G_{st})/G_{st}$; SD, standard deviation

Appendix 11: Genetic diversity and differentiation for *Shorea leprosula* at each locus obtained using POPGENE ver 1.31 (Yeh et al. 1999) (from part 2)

	Locus	Sample size	H_t	H_s	G_{st}	N_m
1	63	133	0.061	0.058	0.058	8.170
2	64	133	0.187	0.180	0.037	13.024
3	108	133	0.054	0.045	0.173	2.399
4	130	133	0.016	0.015	0.049	9.616
5	138	133	0.378	0.314	0.169	2.465
6	141	133	0.016	0.015	0.049	9.616
7	143	130	0.170	0.160	0.058	8.112
8	144	133	0.320	0.249	0.221	1.759
9	150	133	0.048	0.045	0.069	6.713
10	167	133	0.172	0.064	0.632	0.292
11	172	133	0.415	0.015	0.963	0.019
12	176	133	0.199	0.183	0.080	5.745
13	181	133	0.426	0.404	0.050	9.445
14	193	133	0.493	0.423	0.143	3.008
15	199	133	0.007	0.006	0.020	24.788
16	201	133	0.000	0.000	****	****
17	221	133	0.038	0.037	0.033	14.804
18	222	133	0.000	0.000	****	****
19	228	133	0.500	0.431	0.139	3.104
20	242	132	0.070	0.068	0.037	12.982
21	372	132	0.064	0.053	0.164	2.549
22	406	133	0.000	0.000	****	****
23	409	133	0.162	0.095	0.412	0.713
24	411	133	0.420	0.254	0.394	0.769
25	412	133	0.013	0.013	0.040	11.951
26	415	133	0.457	0.406	0.112	3.966
27	465	132	0.239	0.183	0.236	1.622
28	467	131	0.063	0.059	0.057	8.218
29	469	133	0.172	0.127	0.263	1.400
30	108.6	133	0.116	0.112	0.034	14.392
31	109.6	133	0.314	0.288	0.084	5.455
32	126.4	133	0.342	0.317	0.072	6.443
33	134.7	133	0.226	0.211	0.066	7.054
34	137.7	133	0.028	0.027	0.022	22.026
35	146.3	133	0.470	0.404	0.140	3.081
36	156.6	133	0.055	0.047	0.147	2.908
37	157.5	133	0.041	0.040	0.031	15.402
38	160.3	133	0.498	0.325	0.346	0.944
39	161.1	132	0.404	0.304	0.248	1.514
40	166.4	133	0.086	0.062	0.282	1.274
41	170.8	133	0.447	0.394	0.120	3.669
42	172.4	133	0.499	0.266	0.466	0.574
43	182.3	133	0.424	0.391	0.079	5.825
44	196.6	132	0.043	0.042	0.036	13.415
45	199.3	133	0.342	0.296	0.133	3.258
46	223.8	133	0.481	0.261	0.459	0.590
47	321.5	133	0.016	0.015	0.049	9.616
48	326.5	133	0.064	0.051	0.207	1.920
49	417.6	133	0.412	0.336	0.185	2.209
50	418.5	133	0.394	0.086	0.782	0.139
51	468.3	133	0.000	0.000	****	****
52	482.5	133	0.489	0.331	0.324	1.043

	Locus	Sample size	H_t	H_s	G_{st}	N_m
53	59.8	133	0.011	0.010	0.014	35.440
54	60.5	133	0.420	0.338	0.196	2.058
55	73.8	133	0.146	0.133	0.086	5.296
56	82.4	133	0.043	0.041	0.049	9.625
	Mean	133	0.214	0.161	0.246	1.535
	SD		0.034	0.021		

H_t, total genetic diversity; H_s, genetic diversity within populations; G_{st}, proportion of total genetic diversity partitioned among populations; N_m, gene flow from $N_m = 0.5(1 - G_{st})/G_{st}$; SD, standard deviation

Appendix 12: Genetic diversity and differentiation for *Shorea parvifolia* at each locus obtained using POPGENE ver 1.31 (Yeh et al. 1999) (from part 2)

	Locus	Sample size	H_t	H_s	G_{st}	N_m
1	63	135	0.494	0.247	0.499	0.501
2	64	135	0.000	0.000	****	****
3	108	135	0.000	0.000	****	****
4	130	135	0.050	0.047	0.060	7.847
5	138	135	0.013	0.013	0.014	34.695
6	141	135	0.449	0.368	0.181	2.262
7	143	135	0.005	0.005	0.013	38.698
8	144	134	0.298	0.279	0.065	7.197
9	150	135	0.005	0.005	0.013	38.698
10	167	135	0.259	0.194	0.251	1.490
11	172	135	0.034	0.033	0.010	49.327
12	176	135	0.037	0.036	0.037	12.865
13	181	135	0.205	0.196	0.041	11.703
14	193	135	0.042	0.041	0.034	14.340
15	199	135	0.097	0.074	0.240	1.582
16	201	135	0.084	0.072	0.135	3.205
17	221	135	0.153	0.146	0.044	10.956
18	222	135	0.121	0.079	0.345	0.948
19	228	134	0.469	0.258	0.450	0.611
20	242	135	0.119	0.103	0.138	3.127
21	372	135	0.183	0.141	0.226	1.708
22	406	134	0.495	0.274	0.448	0.617
23	409	135	0.044	0.044	0.013	37.215
24	411	135	0.413	0.172	0.583	0.357
25	412	135	0.418	0.234	0.441	0.634
26	415	135	0.056	0.048	0.149	2.847
27	465	135	0.000	0.000	****	****
28	467	135	0.056	0.048	0.149	2.847
29	469	133	0.491	0.095	0.806	0.120
30	108.6	135	0.119	0.099	0.169	2.460
31	109.6	135	0.012	0.012	0.031	15.894
32	126.4	135	0.271	0.258	0.048	9.935
33	134.7	135	0.000	0.000	****	****
34	137.7	135	0.388	0.269	0.307	1.131
35	146.3	135	0.250	0.224	0.103	4.355
36	156.6	135	0.258	0.186	0.279	1.289
37	157.5	135	0.454	0.315	0.307	1.127
38	160.3	135	0.000	0.000	****	****
39	161.1	135	0.235	0.068	0.711	0.203
40	166.4	135	0.000	0.000	****	****
41	170.8	135	0.492	0.423	0.139	3.089
42	172.4	135	0.433	0.308	0.289	1.233
43	182.3	135	0.062	0.055	0.104	4.320
44	196.6	135	0.478	0.400	0.163	2.566
45	199.3	135	0.344	0.237	0.310	1.113
46	223.8	135	0.007	0.007	0.018	26.697
47	321.5	134	0.488	0.139	0.715	0.199
48	326.5	135	0.000	0.000	****	****
49	417.6	135	0.068	0.064	0.061	7.741
50	418.5	135	0.020	0.020	0.011	44.828
51	468.3	133	0.228	0.202	0.116	3.813
52	482.5	135	0.000	0.000	****	****

	Locus	Sample size	H_t	H_s	G_{st}	N_m
53	59.8	135	0.442	0.222	0.498	0.504
54	60.5	135	0.459	0.415	0.095	4.757
55	73.8	135	0.277	0.243	0.124	3.545
56	82.4	135	0.412	0.324	0.215	1.831
	Mean	135	0.202	0.138	0.314	1.091
	SD		0.034	0.016		

H_t, total genetic diversity; H_s, genetic diversity within populations; G_{st}, proportion of total genetic diversity partitioned among populations; N_m, gene flow from $N_m = 0.5(1 - G_{st})/G_{st}$; SD, standard deviation

Appendix 13: Frequency distribution of AFLP markers in *Shorea* species and genetic Differentiation (G_{st}) among populations at each locus generated with POPGENE ver 1.31 (Yeh et al. 1999) (from part 3)

		1	2	3	4	5	6	7	8	9	10
Pop. ID	Sample size	76	77.3	78	79	81	82	84.5	90	91	92
Spar_NS	26	0.731	1.000	0.000	0.385	1.000	0.000	0.000	1.000	0.538	0.154
Sacu_NS	32	1.000	1.000	0.344	0.375	1.000	0.375	0.813	1.000	0.844	0.156
Sdas_NS	20	0.250	0.950	0.000	0.900	0.650	0.000	1.000	1.000	0.850	0.250
Sblu_NS	21	0.476	0.143	0.952	0.238	0.905	0.190	0.286	0.095	0.905	0.905
Slep_NS	16	0.750	0.875	0.188	0.000	1.000	0.000	0.563	1.000	1.000	0.000
Smac_NS	26	0.000	1.000	0.000	1.000	0.846	0.000	0.115	0.615	1.000	0.885
	G_{st}	0.433	0.582	0.530	0.528	0.316	0.129	0.521	0.757	0.273	0.412
Spar_SLB	31	0.290	0.516	0.000	0.032	0.645	0.032	0.000	1.000	0.258	0.000
Slep_SLB	26	0.154	0.769	0.154	0.038	1.000	0.000	0.000	0.923	1.000	0.077
Spal_SLB	25	0.400	0.920	0.000	0.320	0.880	0.280	0.400	0.040	0.840	0.080
Splat_SLB	27	0.667	0.185	0.000	0.111	1.000	0.000	0.000	1.000	0.926	0.000
Sjoh_SLB	24	0.583	0.667	0.000	0.708	0.875	0.000	0.083	0.292	1.000	0.000
	G_{st}	0.084	0.178	0.065	0.225	0.275	0.109	0.151	0.710	0.476	0.024
Total	G_{st}	0.025	0.117	0.070	0.065	0.003	0.002	0.131	0.028	0.000	0.125

		11	12	13	14	15	16	17	18	19	20
Pop. ID	Sample size	95	96	98	99	100	105.3	106.3	108.6	110	110.7
Spar_NS	26	0.077	1.000	0.000	0.077	0.115	0.038	0.308	0.115	0.000	0.462
Sacu_NS	32	0.219	1.000	0.031	1.000	0.250	1.000	1.000	0.000	0.063	0.000
Sdas_NS	20	0.300	1.000	0.100	0.300	0.050	0.150	0.000	0.100	0.000	0.150
Sblu_NS	21	0.143	0.952	0.667	0.857	0.905	0.571	0.286	0.381	0.905	0.571
Slep_NS	16	1.000	0.000	0.438	0.188	0.375	0.000	0.250	0.250	0.500	0.188
Smac_NS	26	0.077	1.000	0.308	0.154	1.000	0.000	0.000	0.346	0.077	0.346
	G_{st}	0.648	0.824	0.175	0.571	0.580	0.711	0.650	0.062	0.438	0.101
Spar_SLB	31	0.194	0.613	0.000	0.000	0.065	0.000	0.097	0.000	0.065	0.097
Slep_SLB	26	1.000	0.077	0.077	0.077	0.231	0.000	0.115	0.000	0.000	0.154
Spal_SLB	25	0.000	0.080	0.760	0.080	1.000	0.000	0.240	0.000	0.120	0.800
Splat_SLB	27	0.000	0.926	0.074	0.074	0.000	0.037	0.000	0.074	0.000	0.444
Sjoh_SLB	24	0.083	0.958	0.792	0.917	0.000	0.042	0.125	0.000	0.125	0.042
	G_{st}	0.850	0.438	0.346	0.540	0.843	0.012	0.030	0.030	0.026	0.253
Total	G_{st}	0.000	0.168	0.009	0.037	0.018	0.115	0.061	0.043	0.052	0.001

		21	22	23	24	25	26	27	28	29	30
Pop. ID	Sample size	112	120	124	125	125.5	126.5	127	129	130	130.4
Spar_NS	26	1.000	0.846	0.077	0.000	0.962	0.192	0.000	0.000	0.115	0.000
Sacu_NS	32	0.063	1.000	0.094	0.000	1.000	0.219	0.000	0.063	0.031	0.156
Sdas_NS	20	1.000	1.000	0.000	0.000	0.600	0.050	0.650	0.000	0.000	0.000
Sblu_NS	21	0.143	0.381	0.952	0.000	0.048	0.000	0.571	0.952	0.000	0.000
Slep_NS	16	1.000	1.000	0.063	0.000	0.938	0.250	0.000	0.000	0.000	0.000
Smac_NS	26	1.000	1.000	0.038	0.000	0.769	0.077	0.000	0.192	0.731	0.000
	G_{st}	0.923	0.571	0.610	****	0.419	0.039	0.290	0.623	0.364	0.069
Spar_SLB	31	1.000	1.000	0.000	0.000	0.806	0.161	0.032	0.000	0.000	0.000
Slep_SLB	26	1.000	1.000	0.038	0.000	1.000	0.038	0.038	0.308	0.000	0.000
Spal_SLB	25	1.000	1.000	0.040	0.360	0.640	0.040	0.040	0.000	0.000	0.920
Splat_SLB	27	1.000	0.111	0.481	0.667	0.037	0.000	0.074	0.000	0.000	0.000
Sjoh_SLB	24	1.000	1.000	0.083	0.083	0.792	0.083	0.083	0.042	0.000	0.000
	G_{st}	****	0.930	0.163	0.229	0.398	0.026	0.004	0.118	****	0.670
Total	G_{st}	0.173	0.000	0.016	0.077	0.005	0.006	0.032	0.036	0.044	0.062

		31	32	33	34	35	36	37	38	39	40
Pop. ID	Sample size	130.8	137.8	139.8	141	144	148	151	152.3	157.5	160.3
Spar_NS	26	0.000	0.000	0.000	0.077	0.308	0.038	0.000	0.462	0.962	0.000
Sacu_NS	32	0.000	0.094	0.000	0.000	0.000	0.000	0.031	0.969	0.000	0.000
Sdas_NS	20	0.000	0.050	0.300	0.000	0.300	0.050	0.000	0.050	0.550	0.100
Sblu_NS	21	0.000	0.905	0.333	0.000	0.000	0.048	0.952	0.095	0.095	0.810
Slep_NS	16	1.000	0.938	0.000	0.000	0.500	0.250	0.000	1.000	0.063	0.000
Smac_NS	26	0.000	0.000	0.000	0.000	0.115	0.654	0.000	1.000	0.654	0.000
	G_{st}	1.000	0.632	0.123	0.033	0.110	0.229	0.731	0.725	0.411	0.466
Spar_SLB	31	0.000	0.129	0.000	0.516	0.000	0.161	0.032	0.645	0.097	0.129
Slep_SLB	26	1.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.038	0.538
Spal_SLB	25	0.040	0.080	0.000	0.000	0.080	0.000	0.000	0.920	0.080	0.000
Splat_SLB	27	0.074	0.000	0.074	0.000	0.148	0.000	0.037	0.963	0.889	0.074
Sjoh_SLB	24	1.000	0.917	0.000	0.083	0.083	0.000	0.083	1.000	0.125	0.125
	G_{st}	0.954	0.552	0.030	0.218	0.028	0.069	0.016	0.288	0.445	0.147
Total	G_{st}	0.074	0.015	0.019	0.029	0.023	0.031	0.047	0.072	0.015	0.000

		41	42	43	44	45	46	47	48	49	50
Pop. ID	Sample size	161	162.3	163.3	170.7	172	172.5	174.3	177	179.8	181
Spar_NS	26	0.038	1.000	1.000	0.769	0.000	0.500	0.308	0.000	0.462	0.308
Sacu_NS	32	0.000	1.000	0.875	0.906	0.031	0.719	0.188	0.000	0.531	0.156
Sdas_NS	20	0.800	0.650	1.000	0.650	0.200	0.900	0.300	0.000	0.800	0.350
Sblu_NS	21	0.143	0.048	0.000	0.143	0.000	0.048	0.190	0.143	0.095	0.381
Slep_NS	16	0.188	1.000	1.000	0.875	0.000	0.750	0.938	0.000	1.000	0.875
Smac_NS	26	0.423	0.962	0.308	0.615	0.000	0.000	0.115	0.000	1.000	0.231
	G_{st}	0.264	0.661	0.735	0.169	0.075	0.285	0.328	0.063	0.528	0.195
Spar_SLB	31	0.548	0.710	1.000	0.903	0.000	0.935	0.290	0.000	0.194	0.129
Slep_SLB	26	0.192	1.000	1.000	0.769	1.000	0.000	0.885	0.000	1.000	0.731
Spal_SLB	25	0.400	1.000	0.960	0.440	0.000	1.000	0.040	0.160	0.960	0.040
Splat_SLB	27	0.852	1.000	0.074	0.111	0.852	0.074	0.000	0.037	0.778	0.000
Sjoh_SLB	24	0.375	1.000	0.542	0.000	0.042	0.208	0.042	0.875	1.000	0.167
	G_{st}	0.147	0.483	0.643	0.332	0.766	0.725	0.441	0.492	0.535	0.278
Total	G_{st}	0.025	0.052	0.000	0.024	0.174	0.003	0.004	0.068	0.025	0.018

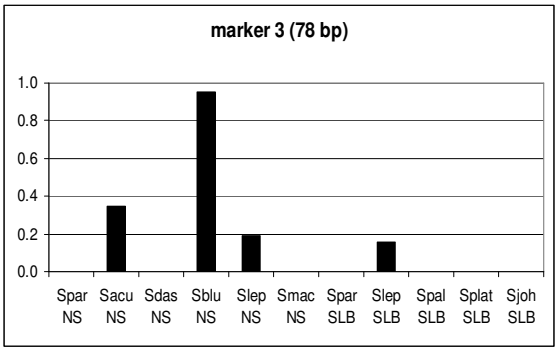
		51	52	53	54	55	56	57	58	59	60
Pop. ID	Sample size	181.8	182.3	188.4	189	191	193	195.5	196.5	199.4	203.8
Spar_NS	26	0.000	0.038	0.000	0.077	1.000	0.000	0.000	0.654	0.346	0.000
Sacu_NS	32	0.000	0.031	0.000	1.000	0.000	0.000	0.000	0.000	0.031	0.000
Sdas_NS	20	0.000	0.000	0.000	0.000	1.000	0.000	0.050	1.000	0.300	0.000
Sblu_NS	21	0.000	0.048	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000
Slep_NS	16	0.000	0.438	0.000	0.000	1.000	0.813	0.000	0.063	0.938	0.000
Smac_NS	26	0.000	0.269	0.000	0.000	0.962	0.000	0.077	0.500	0.000	0.000
	G_{st}	****	0.120	****	0.930	0.887	0.522	0.023	0.611	0.457	****
Spar_SLB	31	0.000	0.097	0.000	0.000	1.000	0.000	0.000	0.968	0.097	0.000
Slep_SLB	26	0.000	0.885	0.000	0.000	1.000	0.538	0.000	0.077	0.615	0.000
Spal_SLB	25	0.000	0.000	0.600	0.120	1.000	0.000	0.000	0.160	0.520	0.000
Splat_SLB	27	0.889	0.074	0.000	0.000	0.926	0.111	0.630	0.074	0.185	0.000
Sjoh_SLB	24	0.000	0.042	0.000	0.000	1.000	0.000	0.000	0.000	0.208	0.833
	G_{st}	0.615	0.495	0.317	0.050	0.230	0.222	0.340	0.622	0.114	0.537
Total	G_{st}	0.077	0.015	0.042	0.073	0.138	0.001	0.029	0.012	0.000	0.068

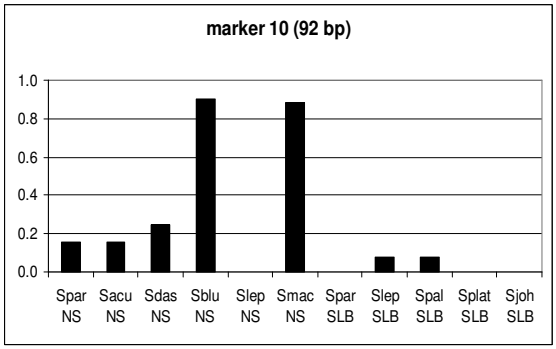
		61	62	63	64	65	66	67	68	69	70
Pop. ID	Sample size	204.5	228	241	242	250	350	351	371.8	380	382
Spar_NS	26	0.000	0.654	0.000	1.000	1.000	0.000	1.000	1.000	0.000	0.000
Sacu_NS	32	0.031	1.000	0.031	0.094	0.031	0.250	0.031	0.031	0.000	0.000
Sdas_NS	20	0.000	0.700	0.000	0.800	0.650	0.200	0.850	0.450	0.000	0.000
Sblu_NS	21	0.000	0.000	0.190	0.952	0.000	1.000	0.000	0.000	0.714	0.619
Slep_NS	16	0.063	0.438	0.000	0.188	1.000	0.313	0.750	0.000	0.000	0.000
Smac_NS	26	1.000	0.962	0.692	0.154	0.462	0.654	0.692	0.615	0.115	0.308
	G_{st}	0.947	0.441	0.307	0.574	0.695	0.532	0.505	0.631	0.364	0.248
Spar_SLB	31	0.000	1.000	0.000	1.000	0.677	0.194	0.806	0.742	0.032	0.000
Slep_SLB	26	0.077	0.923	0.000	0.000	1.000	0.654	0.269	0.000	0.000	0.000
Spal_SLB	25	0.560	0.040	0.040	0.680	0.960	0.880	0.040	0.000	0.000	0.040
Splat_SLB	27	0.037	0.370	0.037	0.741	1.000	0.222	0.889	0.296	0.222	0.037
Sjoh_SLB	24	0.000	1.000	0.042	0.250	1.000	0.250	0.917	0.000	0.000	0.000
	G_{st}	0.231	0.683	0.008	0.495	0.376	0.236	0.322	0.322	0.081	0.012
Total	G_{st}	0.020	0.010	0.031	0.000	0.168	0.000	0.000	0.032	0.016	0.035

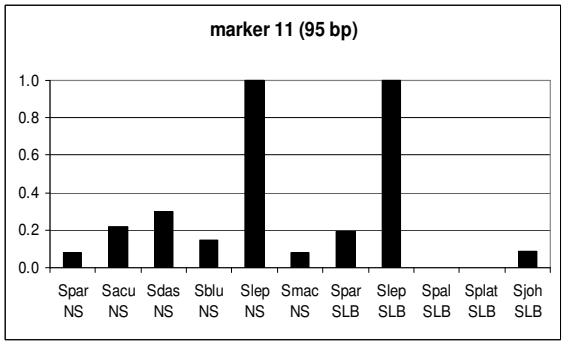
		71	72	73	74	75	76	77	78	79	80
Pop. ID	Sample size	390	391	406	409	411	412	414.7	417.5	418.5	465.3
Spar_NS	26	0.385	0.038	0.115	0.038	0.000	0.962	1.000	0.000	0.000	0.000
Sacu_NS	32	1.000	0.000	0.000	0.469	0.031	0.781	0.906	0.000	0.000	0.313
Sdas_NS	20	0.000	0.050	0.400	0.350	0.200	0.300	0.550	0.000	0.000	0.000
Sblu_NS	21	0.000	0.048	0.000	0.762	0.048	0.000	0.857	0.000	0.000	0.048
Slep_NS	16	0.000	0.000	0.000	0.063	1.000	0.000	0.875	0.625	0.000	1.000
Smac_NS	26	0.000	0.000	0.385	1.000	0.077	0.038	0.615	0.346	0.077	0.000
	G_{st}	0.826	0.012	0.129	0.513	0.820	0.501	0.198	0.250	0.033	0.827
Spar_SLB	31	0.065	0.000	1.000	0.000	0.452	0.194	1.000	0.097	0.000	0.065
Slep_SLB	26	0.000	0.000	0.000	0.346	0.808	0.000	0.885	0.077	0.962	1.000
Spal_SLB	25	0.000	0.800	0.040	0.120	0.000	0.000	0.960	0.000	0.000	0.160
Splat_SLB	27	0.037	0.000	0.444	0.926	0.074	0.000	1.000	0.000	0.111	0.074
Sjoh_SLB	24	0.042	0.000	0.125	1.000	0.000	0.000	1.000	0.000	0.042	0.125
	G_{st}	0.011	0.537	0.725	0.657	0.333	0.083	0.292	0.028	0.680	0.772
Total	G_{st}	0.085	0.050	0.061	0.004	0.001	0.108	0.136	0.027	0.093	0.003

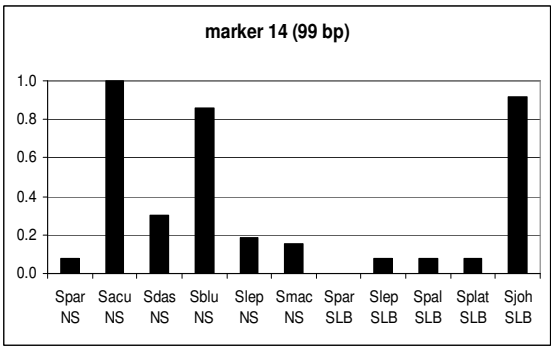
		81	82	83	84	85
Pop. ID	Sample size	466.8	467.8	469.3	482.4	486.4
Spar_NS	26	0.962	0.538	0.000	0.000	0.000
Sacu_NS	32	0.094	0.000	0.875	0.000	0.000
Sdas_NS	20	0.450	0.000	0.450	0.000	0.000
Sblu_NS	21	0.000	0.000	0.000	0.095	0.000
Slep_NS	16	0.000	0.000	1.000	0.875	0.000
Smac_NS	26	0.000	0.923	0.615	0.000	0.000
	G_{st}	0.763	0.584	0.531	0.553	****
Spar_SLB	31	1.000	0.097	0.903	0.000	0.000
Slep_SLB	26	0.423	0.038	0.846	0.038	0.000
Spal_SLB	25	0.000	0.560	0.040	0.000	0.720
Splat_SLB	27	0.074	0.778	0.074	0.074	0.815
Sjoh_SLB	24	0.125	0.958	0.000	0.000	0.042
	G_{st}	0.716	0.386	0.484	0.020	0.431
Total	G_{st}	0.003	0.035	0.015	0.042	0.142

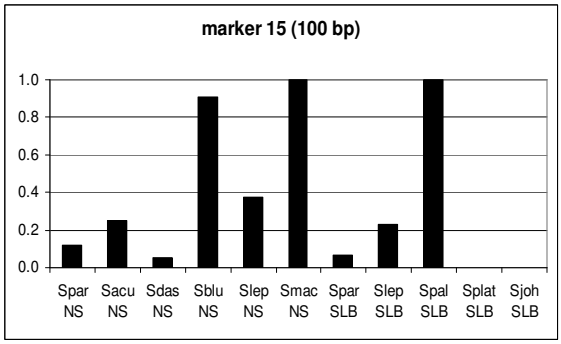
Appendix 14: AFLP marker distribution in *Shorea* species (from part 3)

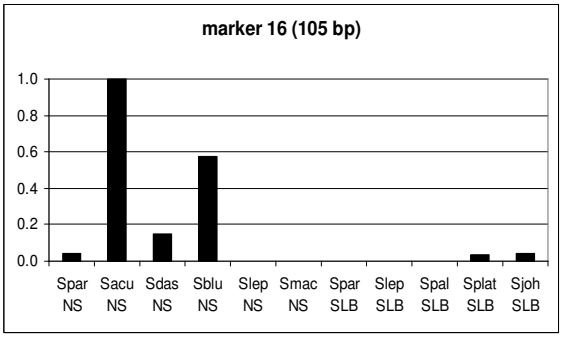


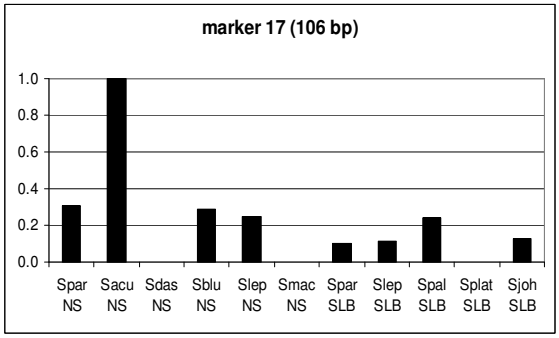


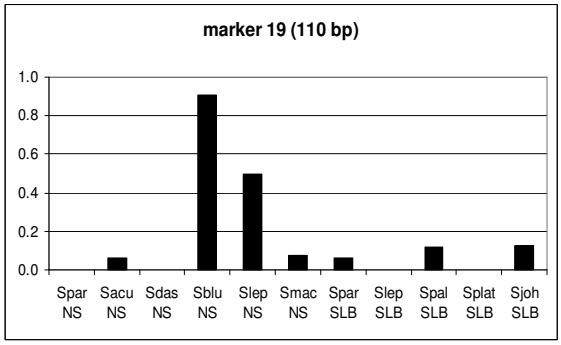


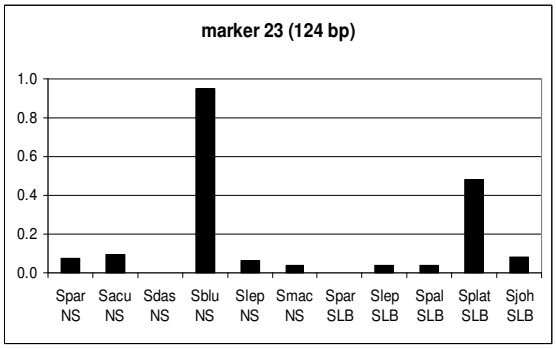


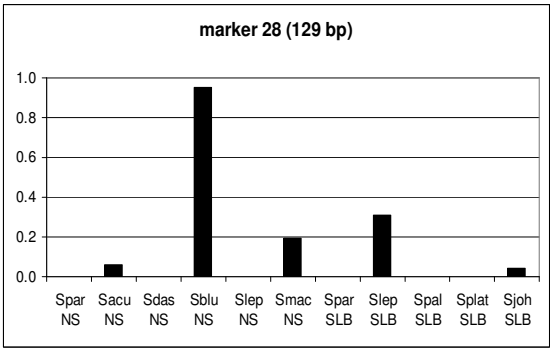


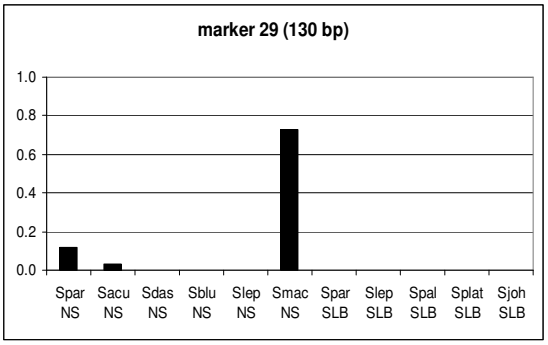








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0.6
0.4
0.2
0.0
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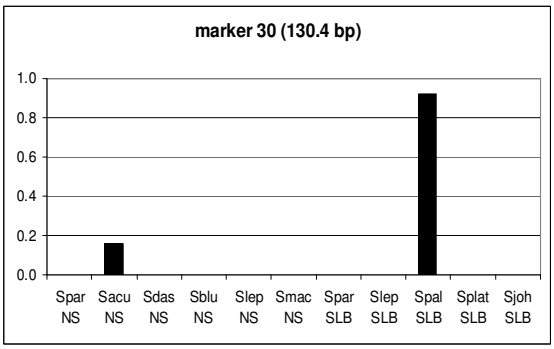
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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0.4
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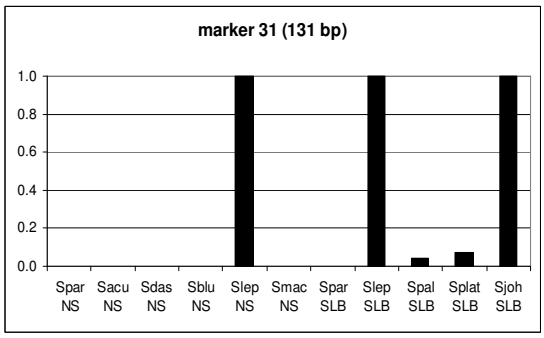
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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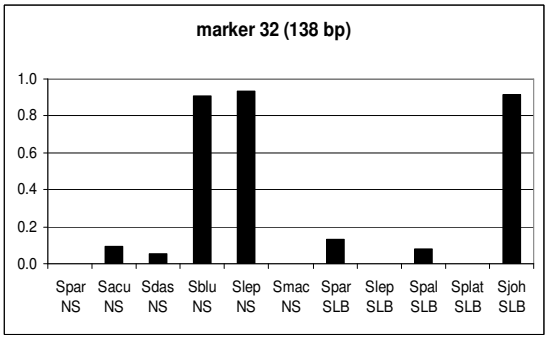
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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Sacu NS
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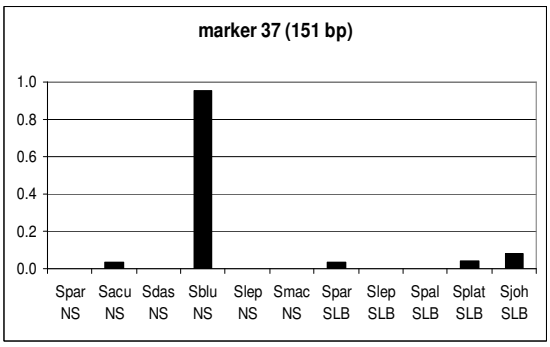
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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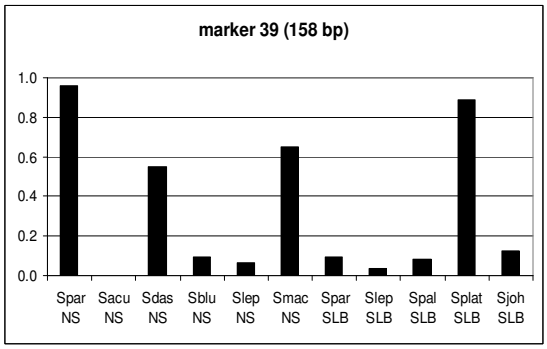
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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Sacu NS
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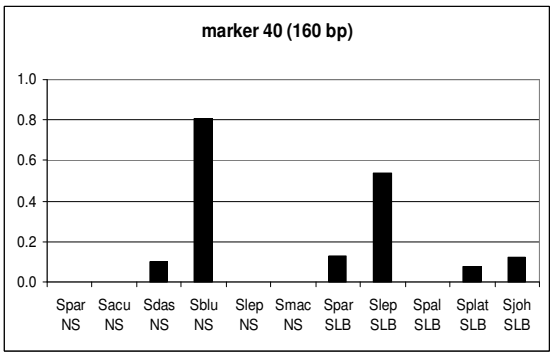
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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Smac NS
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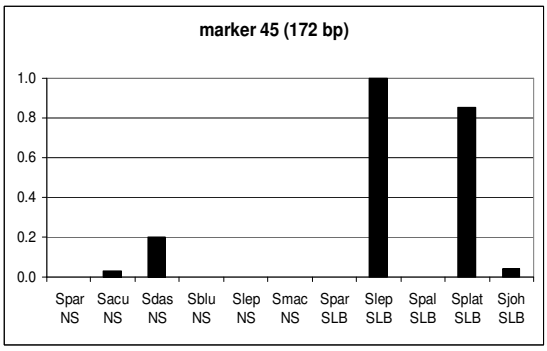
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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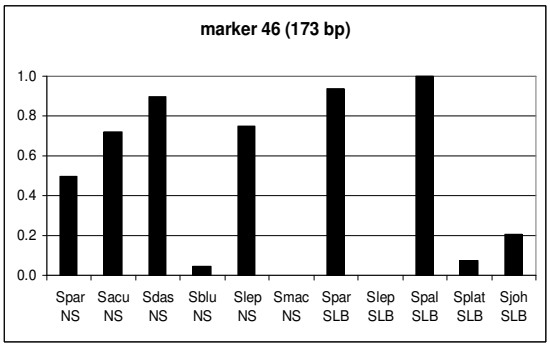
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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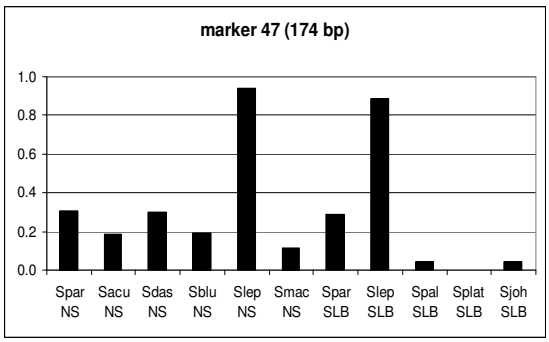
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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Sblu NS
Slep NS
Smac NS
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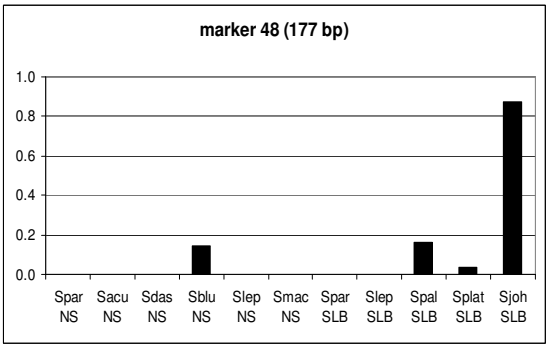
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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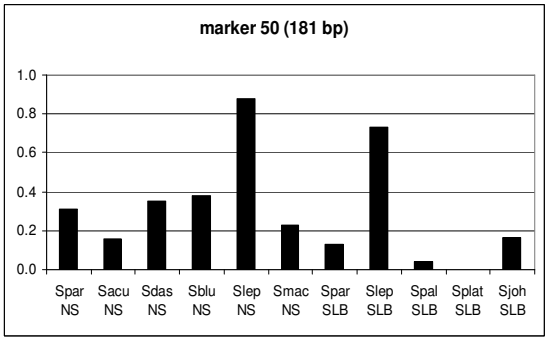
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
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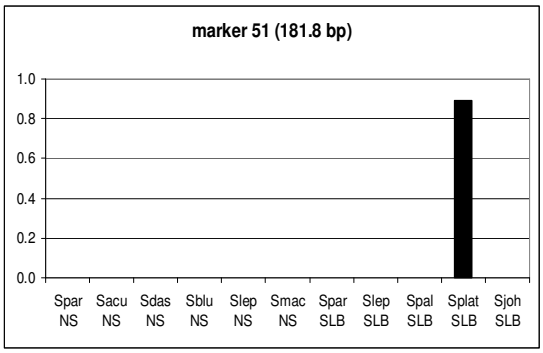
Splat SLB
Sjoh SLB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Slep NS
Smac NS
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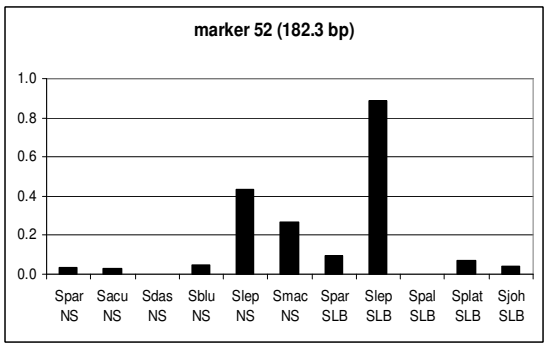
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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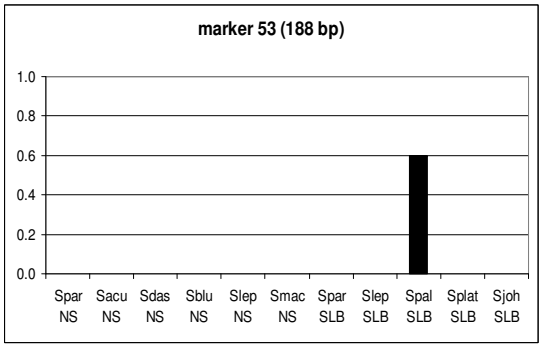
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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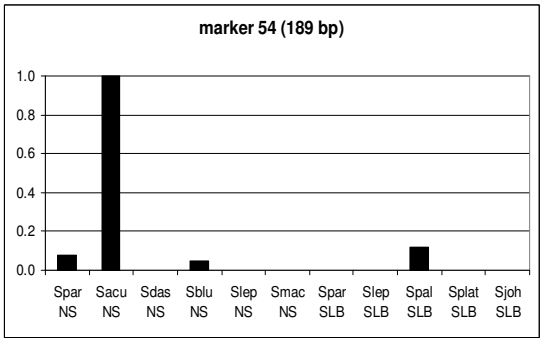
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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Sacu NS
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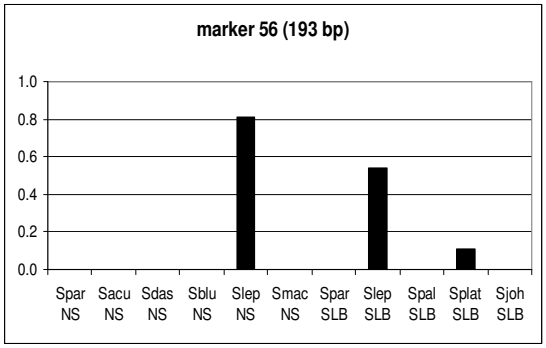
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB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Smac NS
Spar SLB
Slep SLB
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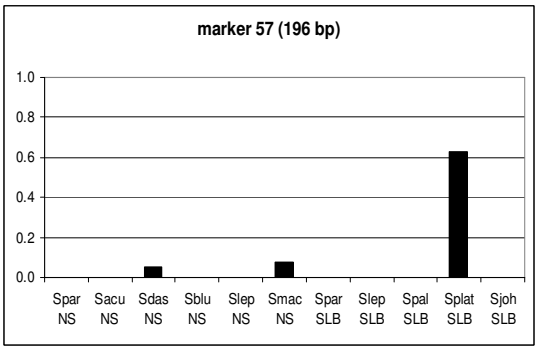
Splat SLB
Sjoh SLB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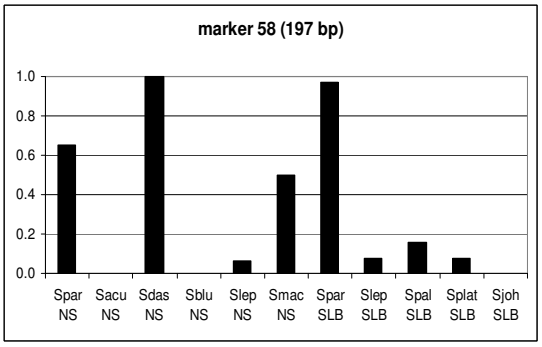
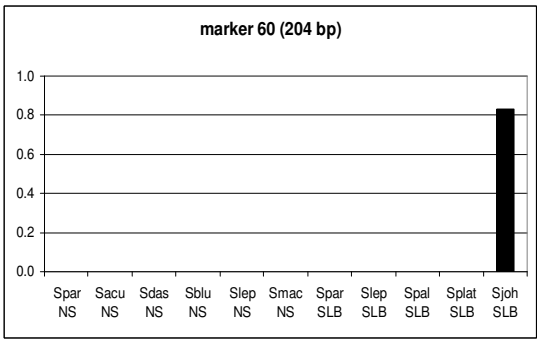
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 58 (197 bp)
1.0
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 60 (204 bp)
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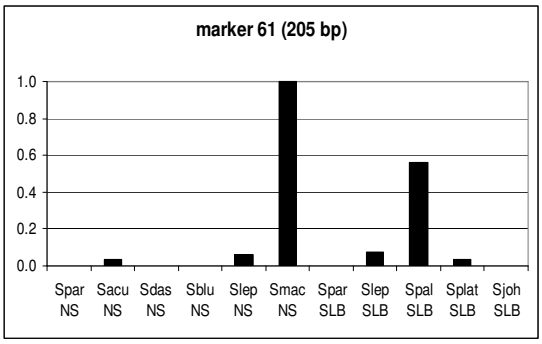
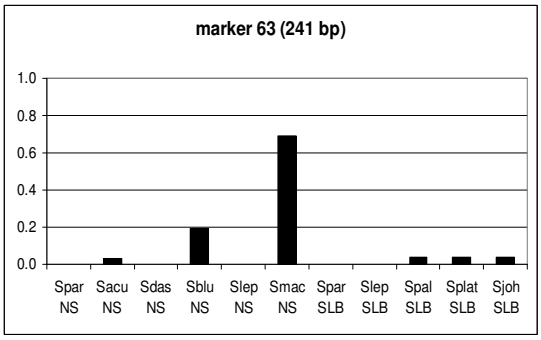
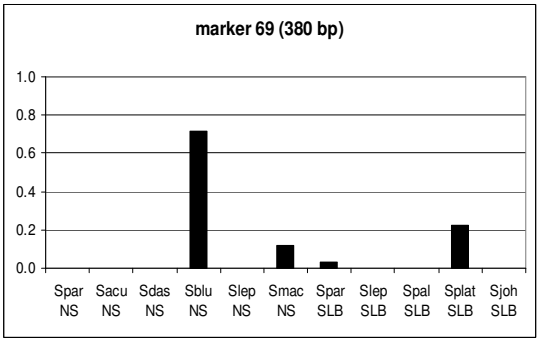
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 61 (205 bp)
1.0
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 63 (241 bp)
1.0
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 69 (380 bp)
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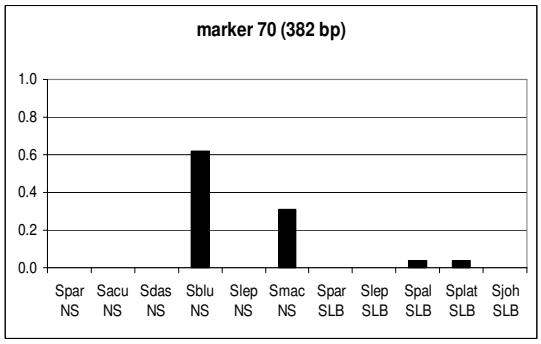
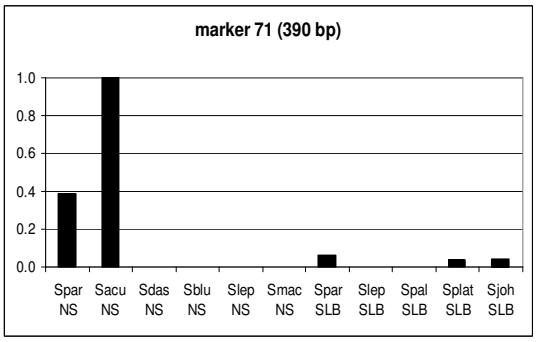
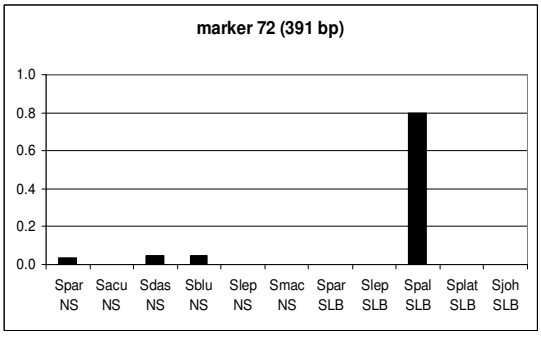
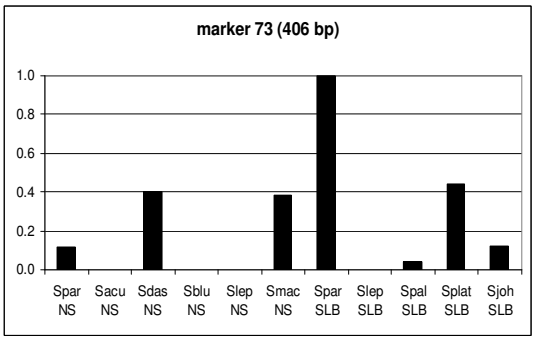
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 70 (382 bp)
1.0
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 71 (390 bp)
1.0
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 72 (391 bp)
1.0
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 73 (406 bp)
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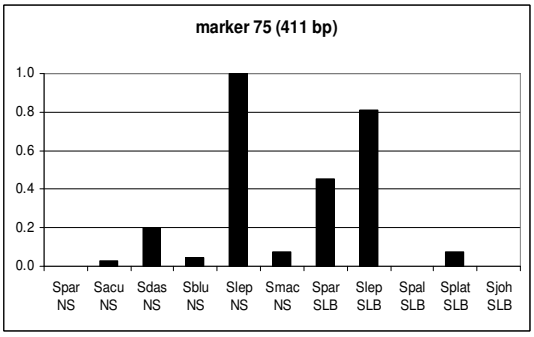
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 75 (411 bp)
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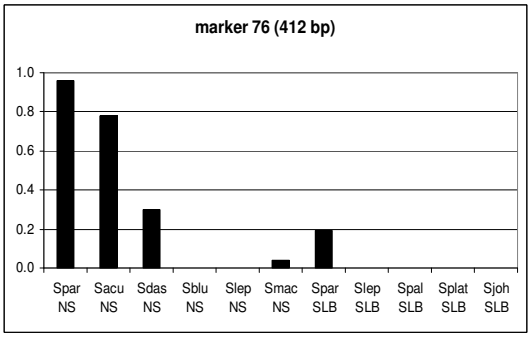
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 76 (412 bp)
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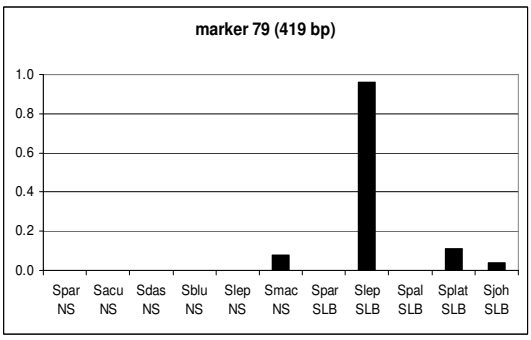
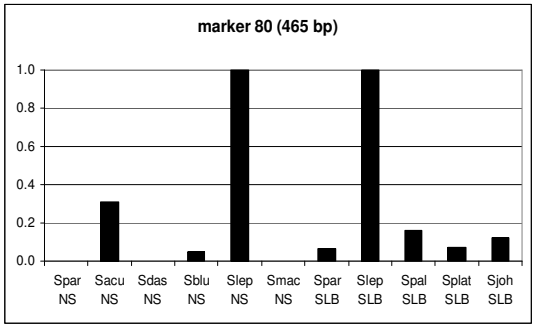
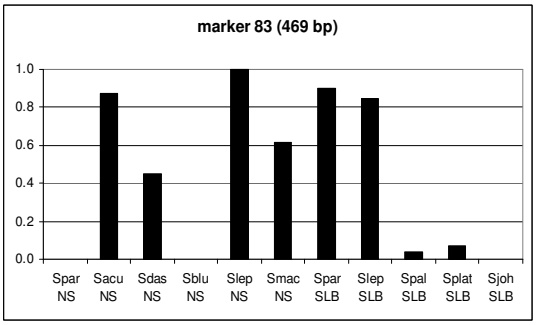
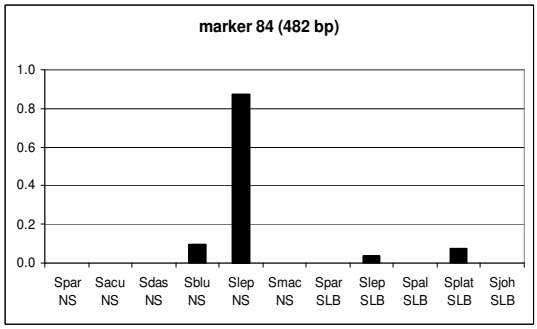
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB


marker 79 (419 bp)
1.0
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB







NS: Nanjak Makmur Sumatra

SLB: Sumalindo Borneo

Spar: *Shorea parvifolia;*

Sacu: *S. acuminata;*

Sdas: *S. dasyphylla;*

Sblu: *S. blumutensis;*

Slep: *S. leprosula;*

Smac: *S. macroptera;*

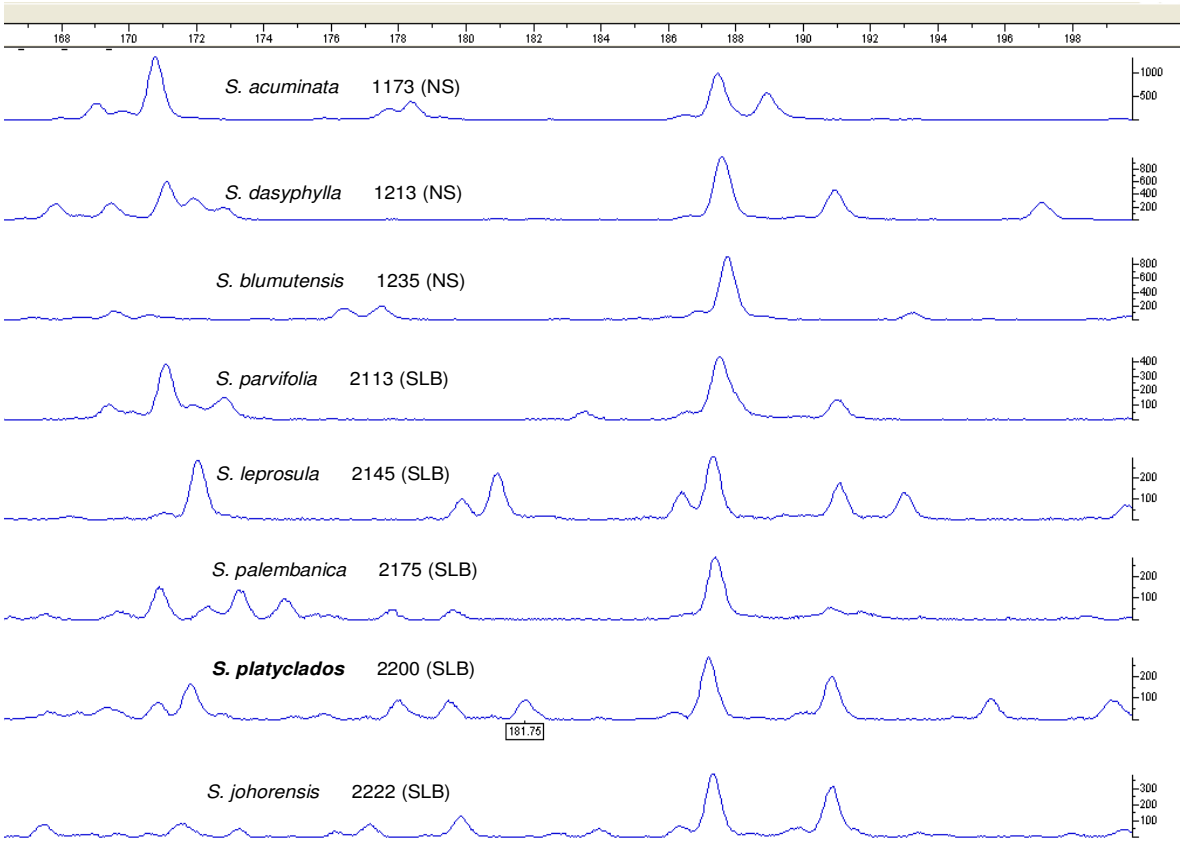
Spal: *S. palembanica*

Splat: *S. platyclados*

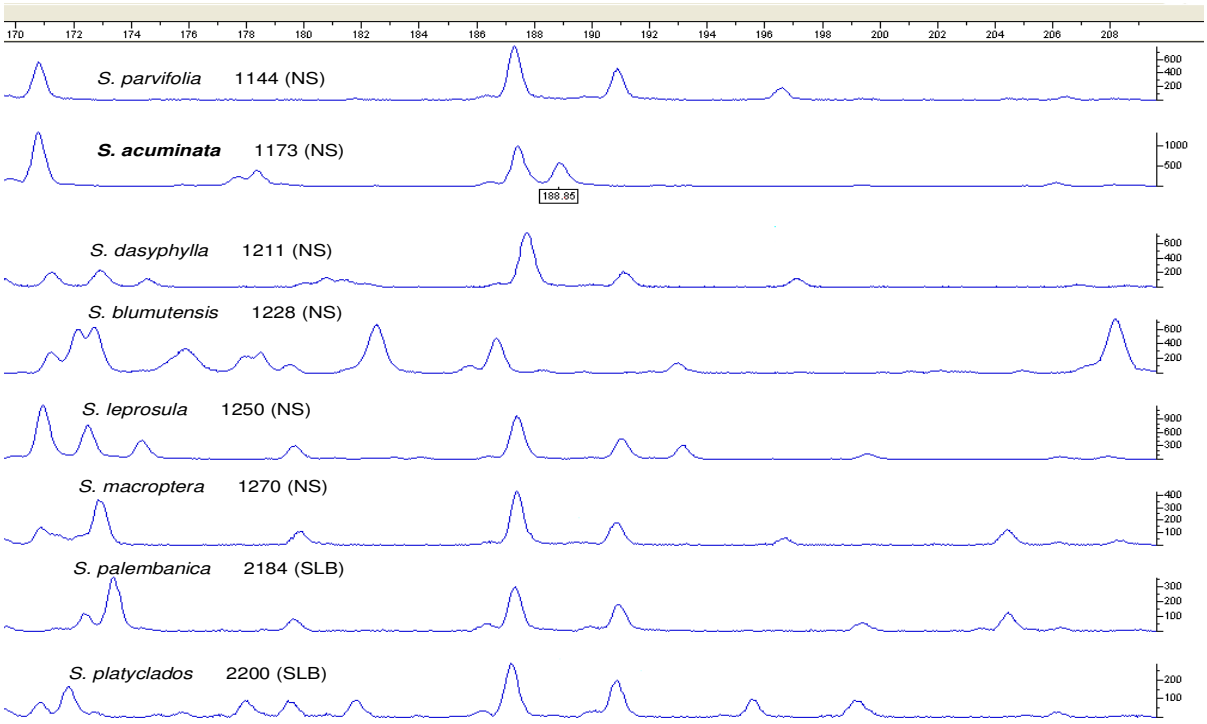
Sjoh: *S. johorensis*

Appendix 15: AFLP diagnostic markers (primer E35M63, from part 3)

(1). Marker 51 (182 bp) diagnostic to *Shorea platyclados* in Sumalindo Borneo (SLB)



(2). Marker 54 (189 bp) diagnostic to *S. acuminata* in Nanjak Makmur Sumatra (NS)



Appendix 16: Genetic diversity and differentiation for *Shorea* species in Nanjak Makur Sumatra at each locus obtained using POPGENE ver 1.31 (Yeh et al. 1999) (from part 3)

	Locus	Sample size	H_t	H_s	G_{st}	N_m
1	76	141	0.479	0.272	0.433	0.655
2	77.3	141	0.376	0.157	0.582	0.359
3	78	141	0.293	0.138	0.530	0.443
4	79	141	0.468	0.221	0.528	0.447
5	81	141	0.338	0.231	0.316	1.081
6	82	141	0.098	0.085	0.129	3.372
7	84.5	141	0.457	0.219	0.521	0.459
8	90	141	0.387	0.094	0.757	0.161
9	91	141	0.416	0.303	0.273	1.333
10	92	141	0.398	0.234	0.412	0.713
11	95	141	0.363	0.128	0.648	0.271
12	96	141	0.324	0.057	0.824	0.107
13	98	141	0.257	0.212	0.175	2.355
14	99	141	0.445	0.191	0.571	0.375
15	100	141	0.457	0.192	0.580	0.362
16	105.3	141	0.365	0.106	0.711	0.204
17	106.3	141	0.368	0.129	0.650	0.270
18	108.6	141	0.193	0.181	0.062	7.558
19	110	141	0.290	0.163	0.438	0.642
20	110.7	141	0.273	0.246	0.101	4.459
21	112	141	0.432	0.033	0.923	0.042
22	120	141	0.316	0.135	0.571	0.375
23	124	141	0.260	0.101	0.610	0.320
24	125	141	0.000	0.000	****	****
25	125.5	141	0.488	0.284	0.419	0.694
26	126.5	141	0.129	0.124	0.039	12.370
27	127	141	0.220	0.156	0.290	1.222
28	129	141	0.258	0.098	0.623	0.303
29	130	141	0.168	0.107	0.364	0.874
30	130.4	141	0.027	0.025	0.069	6.767
31	130.8	141	0.278	0.000	1.000	0.000
32	137.8	140	0.391	0.144	0.632	0.291
33	139.8	141	0.109	0.096	0.123	3.554
34	141	141	0.013	0.013	0.033	14.694
35	144	141	0.202	0.180	0.110	4.067
36	148	141	0.184	0.142	0.229	1.687
37	151	141	0.231	0.062	0.731	0.184
38	152.3	141	0.499	0.137	0.725	0.190
39	157.5	141	0.395	0.233	0.411	0.716
40	160.3	141	0.184	0.098	0.466	0.573
41	161	141	0.275	0.202	0.264	1.396
42	162.3	141	0.415	0.141	0.661	0.257
43	163.3	141	0.463	0.123	0.735	0.180
44	170.7	141	0.496	0.412	0.169	2.466
45	172	141	0.040	0.037	0.075	6.148
46	172.5	141	0.441	0.315	0.285	1.253
47	174.3	141	0.347	0.233	0.328	1.023
48	177	141	0.024	0.023	0.063	7.488
49	179.8	141	0.498	0.235	0.528	0.446
50	181	141	0.362	0.292	0.195	2.063
51	181.8	141	0.000	0.000	****	****
52	182.3	141	0.140	0.123	0.120	3.670

	Locus	Sample size	H_t	H_s	G_{st}	N_m
53	188.4	141	0.000	0.000	****	****
54	189	141	0.292	0.020	0.930	0.038
55	191	141	0.464	0.053	0.887	0.064
56	193	141	0.171	0.082	0.522	0.458
57	195.5	141	0.021	0.021	0.023	20.990
58	196.5	141	0.411	0.160	0.611	0.318
59	199.4	141	0.304	0.165	0.457	0.593
60	203.8	141	0.000	0.000	****	****
61	204.5	141	0.288	0.015	0.947	0.028
62	228	140	0.500	0.280	0.441	0.635
63	241	141	0.170	0.118	0.307	1.130
64	242	141	0.489	0.209	0.574	0.372
65	250	141	0.495	0.151	0.695	0.219
66	350	141	0.423	0.198	0.532	0.441
67	351	140	0.493	0.244	0.505	0.490
68	371.8	141	0.399	0.148	0.631	0.293
69	380	141	0.160	0.102	0.364	0.874
70	382	141	0.167	0.125	0.248	1.514
71	390	141	0.323	0.056	0.826	0.106
72	391	141	0.023	0.022	0.012	41.546
73	406	141	0.153	0.133	0.129	3.383
74	409	141	0.448	0.218	0.513	0.474
75	411	141	0.317	0.057	0.820	0.110
76	412	140	0.380	0.190	0.501	0.498
77	414.7	140	0.473	0.380	0.198	2.032
78	417.5	141	0.174	0.131	0.250	1.497
79	418.5	141	0.013	0.013	0.033	14.694
80	465.3	141	0.319	0.055	0.827	0.104
81	466.8	137	0.344	0.082	0.763	0.156
82	467.8	139	0.307	0.128	0.584	0.356
83	469.3	139	0.474	0.223	0.531	0.442
84	482.4	141	0.205	0.092	0.553	0.405
85	486.4	141	0.000	0.000	****	****
	Mean	141	0.289	0.138	0.522	0.458
	SD		0.024	0.009		

H_t, total genetic diversity; H_s, genetic diversity within populations; G_{st}, proportion of total genetic diversity partitioned among populations; N_m, gene flow from $N_m = 0.5(1 - G_{st})/G_{st}$; SD, standard deviation

Appendix 17: Genetic diversity and differentiation for *Shorea* species in Sumalindo Borneo at each locus obtained using POPGENE ver 1.31 (Yeh et al. 1999) (from part 3)

	Locus	Sample size	H_t	H_s	G_{st}	N_m
1	76	133	0.373	0.342	0.084	5.424
2	77.3	133	0.485	0.398	0.178	2.312
3	78	133	0.032	0.030	0.065	7.175
4	79	133	0.249	0.193	0.225	1.719
5	81	133	0.384	0.278	0.275	1.317
6	82	133	0.065	0.058	0.109	4.110
7	84.5	133	0.101	0.086	0.151	2.815
8	90	133	0.487	0.141	0.710	0.205
9	91	133	0.425	0.223	0.476	0.551
10	92	133	0.032	0.031	0.024	19.970
11	95	133	0.353	0.053	0.850	0.088
12	96	133	0.479	0.269	0.438	0.642
13	98	133	0.350	0.229	0.346	0.944
14	99	133	0.277	0.127	0.540	0.427
15	100	133	0.355	0.056	0.843	0.093
16	105.3	133	0.016	0.016	0.012	40.854
17	106.3	133	0.114	0.110	0.030	16.420
18	108.6	133	0.015	0.015	0.030	15.932
19	110	133	0.062	0.060	0.026	18.752
20	110.7	133	0.310	0.231	0.253	1.475
21	112	133	0.000	0.000	****	****
22	120	133	0.306	0.022	0.930	0.038
23	124	133	0.134	0.113	0.163	2.569
24	125	133	0.231	0.178	0.229	1.686
25	125.5	133	0.500	0.301	0.398	0.757
26	126.5	133	0.064	0.063	0.026	18.885
27	127	133	0.053	0.053	0.004	114.365
28	129	133	0.073	0.064	0.118	3.728
29	130	133	0.000	0.000	****	****
30	130.4	133	0.246	0.081	0.670	0.247
31	130.8	133	0.484	0.022	0.954	0.024
32	137.8	133	0.274	0.123	0.552	0.406
33	139.8	133	0.015	0.015	0.030	15.932
34	141	133	0.129	0.101	0.218	1.794
35	144	133	0.062	0.060	0.028	17.700
36	148	133	0.033	0.031	0.069	6.799
37	151	133	0.031	0.030	0.016	30.673
38	152.3	133	0.337	0.240	0.288	1.235
39	157.5	133	0.280	0.155	0.445	0.623
40	160.3	133	0.177	0.151	0.147	2.904
41	161	133	0.417	0.355	0.147	2.899
42	162.3	133	0.192	0.099	0.483	0.535
43	163.3	133	0.465	0.166	0.643	0.278
44	170.7	133	0.423	0.283	0.332	1.007
45	172	133	0.440	0.103	0.766	0.153
46	172.5	133	0.471	0.130	0.725	0.190
47	174.3	133	0.285	0.159	0.441	0.633
48	177	133	0.255	0.129	0.492	0.516
49	179.8	133	0.431	0.200	0.535	0.435
50	181	133	0.228	0.165	0.278	1.301
51	181.8	133	0.231	0.089	0.615	0.313
52	182.3	133	0.260	0.131	0.495	0.510

	Locus	Sample size	H_t	H_s	G_{st}	N_m
53	188.4	133	0.136	0.093	0.317	1.076
54	189	133	0.025	0.023	0.050	9.469
55	191	133	0.103	0.079	0.230	1.671
56	193	133	0.140	0.109	0.222	1.753
57	195.5	133	0.144	0.095	0.340	0.972
58	196.5	133	0.315	0.119	0.622	0.304
59	199.4	132	0.310	0.275	0.114	3.890
60	203.8	133	0.209	0.097	0.537	0.431
61	204.5	133	0.145	0.112	0.231	1.661
62	228	133	0.484	0.154	0.683	0.233
63	241	133	0.024	0.024	0.008	60.933
64	242	133	0.484	0.245	0.495	0.510
65	250	133	0.260	0.162	0.376	0.829
66	350	132	0.410	0.314	0.236	1.618
67	351	131	0.488	0.331	0.322	1.051
68	371.8	133	0.227	0.154	0.322	1.054
69	380	133	0.052	0.048	0.081	5.668
70	382	133	0.015	0.015	0.012	41.908
71	390	133	0.029	0.028	0.011	43.317
72	391	132	0.209	0.097	0.537	0.431
73	406	133	0.392	0.108	0.725	0.190
74	409	133	0.479	0.164	0.657	0.262
75	411	133	0.285	0.190	0.333	1.003
76	412	133	0.040	0.037	0.083	5.504
77	414.7	132	0.127	0.090	0.292	1.215
78	417.5	133	0.035	0.034	0.028	17.515
79	418.5	133	0.291	0.093	0.680	0.235
80	465.3	131	0.370	0.085	0.772	0.148
81	466.8	133	0.393	0.112	0.716	0.199
82	467.8	131	0.459	0.282	0.386	0.795
83	469.3	132	0.395	0.204	0.484	0.533
84	482.4	133	0.023	0.022	0.020	24.104
85	486.4	130	0.357	0.203	0.431	0.661
	Mean	133	0.239	0.129	0.462	0.583
	SD		0.027	0.009		

H_t, total genetic diversity; H_s, genetic diversity within populations; G_{st}, proportion of total genetic diversity partitioned among populations; N_m, gene flow from $N_m = 0.5(1 - G_{st})/G_{st}$; SD, standard deviation

Appendix 18: Genetic diversity and differentiation for *Shorea* species in Nanjak Makur Sumatra and Sumalindo Borneo at each locus obtained using POPGENE ver 1.31 (Yeh et al. 1999) (from part 3)

	Locus	Sample size	H_t	H_s	G_{st}	N_m
1	76	274	0.442	0.431	0.025	19.189
2	77.3	274	0.482	0.425	0.117	3.768
3	78	274	0.187	0.174	0.070	6.665
4	79	274	0.394	0.368	0.065	7.204
5	81	274	0.360	0.359	0.003	189.316
6	82	274	0.083	0.083	0.002	255.948
7	84.5	274	0.340	0.295	0.131	3.316
8	90	274	0.445	0.432	0.028	17.492
9	91	274	0.420	0.420	0.000	2000.000
10	92	274	0.265	0.232	0.125	3.494
11	95	274	0.359	0.359	0.000	2000.000
12	96	274	0.474	0.394	0.168	2.475
13	98	274	0.302	0.299	0.009	53.838
14	99	274	0.382	0.368	0.037	13.151
15	100	274	0.418	0.411	0.018	27.778
16	105.3	274	0.233	0.206	0.115	3.850
17	106.3	274	0.269	0.252	0.061	7.640
18	108.6	274	0.117	0.112	0.043	11.152
19	110	274	0.196	0.186	0.052	9.050
20	110.7	274	0.290	0.290	0.001	362.857
21	112	274	0.285	0.236	0.173	2.385
22	120	274	0.311	0.311	0.000	2000.000
23	124	274	0.206	0.203	0.016	31.197
24	125	274	0.114	0.105	0.077	5.973
25	125.5	274	0.496	0.493	0.005	93.209
26	126.5	274	0.100	0.100	0.006	77.324
27	127	274	0.149	0.144	0.032	14.993
28	129	274	0.181	0.174	0.036	13.346
29	130	274	0.096	0.092	0.044	10.763
30	130.4	274	0.135	0.126	0.062	7.552
31	130.8	274	0.401	0.372	0.074	6.248
32	137.8	273	0.343	0.338	0.015	32.206
33	139.8	274	0.068	0.066	0.019	26.439
34	141	274	0.068	0.066	0.029	16.792
35	144	274	0.142	0.138	0.023	20.826
36	148	274	0.119	0.115	0.031	15.881
37	151	274	0.146	0.140	0.047	10.211
38	152.3	274	0.458	0.425	0.072	6.410
39	157.5	274	0.348	0.343	0.015	32.788
40	160.3	274	0.181	0.181	0.000	2000.000
41	161	274	0.348	0.339	0.025	19.745
42	162.3	274	0.331	0.314	0.052	9.129
43	163.3	274	0.464	0.464	0.000	2000.000
44	170.7	274	0.474	0.463	0.024	20.665
45	172	274	0.269	0.222	0.174	2.372
46	172.5	274	0.456	0.455	0.003	180.479
47	174.3	274	0.320	0.319	0.004	121.388
48	177	274	0.138	0.129	0.068	6.897
49	179.8	274	0.480	0.468	0.025	19.472
50	181	274	0.307	0.301	0.018	26.729
51	181.8	274	0.114	0.105	0.077	5.958

	Locus	Sample size	H_t	H_s	G_{st}	N_m
52	182.3	274	0.198	0.195	0.015	32.250
53	188.4	274	0.065	0.062	0.042	11.554
54	189	274	0.184	0.170	0.073	6.316
55	191	274	0.348	0.300	0.138	3.115
56	193	274	0.157	0.157	0.001	441.221
57	195.5	274	0.080	0.077	0.029	17.074
58	196.5	274	0.372	0.368	0.012	42.673
59	199.4	273	0.307	0.307	0.000	2000.000
60	203.8	274	0.102	0.095	0.068	6.829
61	204.5	274	0.228	0.223	0.020	24.602
62	228	273	0.498	0.493	0.010	49.872
63	241	274	0.107	0.103	0.031	15.655
64	242	274	0.487	0.487	0.000	2000.000
65	250	274	0.467	0.388	0.168	2.470
66	350	273	0.417	0.417	0.000	1800.381
67	351	271	0.491	0.491	0.000	1906.629
68	371.8	274	0.332	0.321	0.032	15.391
69	380	274	0.113	0.111	0.016	30.419
70	382	274	0.102	0.098	0.035	13.996
71	390	274	0.207	0.189	0.085	5.394
72	391	273	0.113	0.107	0.050	9.466
73	406	274	0.279	0.262	0.061	7.753
74	409	274	0.463	0.462	0.004	137.374
75	411	274	0.303	0.302	0.001	461.160
76	412	273	0.253	0.225	0.108	4.136
77	414.7	272	0.365	0.316	0.136	3.178
78	417.5	274	0.114	0.111	0.027	18.053
79	418.5	274	0.154	0.139	0.093	4.862
80	465.3	272	0.343	0.342	0.003	162.462
81	466.8	270	0.367	0.366	0.003	159.324
82	467.8	270	0.390	0.376	0.035	13.638
83	469.3	271	0.445	0.438	0.015	33.004
84	482.4	274	0.127	0.122	0.042	11.281
85	486.4	271	0.189	0.162	0.142	3.026
	Mean	274	0.278	0.266	0.044	10.864
	SD		0.019	0.018		

H_t, total genetic diversity; H_s, genetic diversity within populations; G_{st}, proportion of total genetic diversity partitioned among populations; N_m, gene flow from $N_m = 0.5(1 - G_{st})/G_{st}$, see McDermott and McDonald, Ann. Rev. Phytopathol. 31:353-373 (1993); SD, standard deviation

Summary

The Dipterocarpaceae are a dominant, species-rich tree family comprising more than 500 species in 17 genera. From an ecological and economic perspective, it is the most important tree family in Asian tropical rain forests, which are a centre of global biological diversity. Indonesia, in particular Sumatra and Kalimantan (Borneo), is the country with the highest species diversity of Dipterocarpaceae. *Shorea* is the largest and most important genus within the family. It encompasses about 200 species, of which 163 species are distributed in the Malayan archipelago, mainly on Sumatra and Kalimantan (Borneo) in Indonesia. Dipterocarps are heavily affected by deforestation, forest fragmentation and nonsustainable forest management. Many dipterocarp forests are exploited for the valuable timber. Information on the phylogeny of Indonesian dipterocarps is scarce. Genetic variation patterns within dipterocarp species based on DNA markers have been rarely reported.

The advent of restriction enzymes and the polymerase chain reaction (PCR) has allowed the assessment of genetic variation directly at the DNA level. A range of powerful and rapidly developing techniques are now available. AFLP (Amplified Fragment Length Polymorphisms) is a DNA fingerprinting technique that is based on the selective PCR amplification of restriction fragments. The AFLP marker technique is a very effective, fast and reliable tool to reveal restriction fragment polymorphisms. Main advantages are the large number of loci uncovered in a single assay, the high reproducibility, and the universal applicability of AFLPs since no sequence information prior to the generation of AFLP fingerprints. Many studies have applied this technique to DNA fingerprinting for animals and plants and genetic diversity studies. Applications also include QTL (quantitative trait locus) mapping, phylogenetic studies and investigations of the reproduction system by means of parentage analysis.

This dissertation is composed of three parts using the AFLP marker technique to characterize genetic variation of Indonesian dipterocarps:

(1) genetic variation of Dipterocarpaceae and its relation to molecular phylogenies and taxonomic subdivisions (part1);

(2) genetic diversity within and among populations of two common dipterocarps, *Shorea leprosula* Miq. and *S. parvifolia* Dyer, in Indonesia (part 2);

(3) genetic variation in nine *Shorea* species in Indonesia (part 3).

Part 1: A total of 81 samples of dipterocarp trees (Dipterocarpaceae; subfamily Dipterocarpoideae) from Indonesia belonging to 54 species in all nine genera native to the country were investigated at 125 AFLP loci in order to assess genetic differentiation among species. The resultant UPGMA tree clearly separated all investigated dipterocarps into two major groups corresponding to tribe Dipterocarpeae with base chromosome number of $x = 11$ containing genera *Dipterocarpus*, *Anisoptera*, *Cotylelobium, Upuna*, and *Vatica*, and to tribe Shoreae with $x = 7$ containing genera *Hopea*, *Parashorea*, *Shorea,* and *Dryobalanops*. Genus *Dryobalanops* grouped together with *Shorea virescens* basal to other members of tribe Shoreae. Evidences from chloroplast DNA, caryological and morphological characters also suggested an intermediate position of this genus between tribe Dipterocarpeae and tribe Shoreae. The results of this study using the AFLP marker technique are in accordance with the topology of molecular phylogenetic trees derived from PCR-RFLP and sequence analysis of chloroplast DNA and with the classification based on caryological and morphological characters (with a few exceptions) and generally support the traditional taxonomic assessments. This suggests that the widely accepted taxonomic subdivisions of the subfamily Dipterocarpoideae are reflected in genome-wide differentiation patterns. The possibility of interspecific hybridization is discussed.

Part 2: AFLPs were used to assess the genetic diversity and differentiation in the most common and widespread emergent dipterocarp tree species - *Shorea leprosula* Miq. and *Shorea parvifolia* Dyer - from Indonesia. A total of 268 individuals from natural populations in Sumatra and Borneo and from a plantation on Java were analysed at 56 AFLP loci. Samples included 133 individuals from seven populations of *S. leprosula* and 135 individuals from six populations of *S. parvifolia*. *S. leprosula* is genetically more variable than *S. parvifolia*. At the population level, a higher level of genetic diversity was revealed for *S. leprosula* with a percentage of polymorphic loci (PPL_p) of 53.3% and an expected heterozygosity (H_{ep}) of 16% in comparison with *S. parvifolia* showing PPL_p of 51.8% and H_{ep} of 14%. At the species level, *S. leprosula* showed a percentage of polymorphic loci (PPL_s) of 92.9% and an expected heterozygosity (H_{es}) of 0.21, while *S. parvifolia* showed PPL_s of 85.7% and H_{es} of 0.21. Genetic differentiation (G_{st}) calculated on the basis of estimated allele frequencies indicated that 25% and 31% of total genetic diversity in *S. leprosula* and *S. parvifolia*, respectively, were attributed to the differences among populations. Likewise, AMOVA analysis at two hierarchical levels exhibited that most genetic variation resided within populations with a proportion of 70.2% for *S. leprosula* and 66.2% for *S. parvifolia*.

The AMOVA at three hierarchical levels based on the complete data set revealed that the genetic difference between the two species was remarkably higher with a proportion of 44.1% than differences within and among populations (38.1% and 17.8%, respectively). The genetic differentiation between islands was significant for *S. leprosula* but not for *S. parvifolia*. The observed genetic diversity within populations and genetic differentiation among populations agreed with the life history traits of *Shorea* species (i.e., regionally distributed, long-lived, high fecundity, predominantly outcrossing, gene flow via limited pollen and seed dispersal, and late successional). Some private markers with high frequencies were found, which can serve as diagnostic markers for the identification of wood of different species from different islands and regions.

Part 3: Genetic variation and genetic structure of nine *Shorea* species from two different locations, namely Nanjak Makmur on Sumatra and Sumalindo on Kalimantan (Borneo), was evaluated using AFLP markers. A total of 274 trees were investigated at 85 polymorphic AFLP loci, including 141 individuals of six species from Nanjak Makmur Sumatra and 133 individuals of five species from Sumalindo Borneo. The results indicated that the six species from Nanjak Makmur Sumatra and the five species from Sumalindo Borneo showed similar levels of genetic variation ($\overline{H_e}$ = 0.138 on Sumatra; $\overline{H_e}$ = 0.129 on Borneo). *S. blumutensis* from Nanjak Makmur Sumatra showed the highest genetic diversity with H_e of 0.165. *S. acuminata* from Nanjak Makmur Sumatra harboured the lowest genetic diversity with H_e of 0.100. The hypothesis that uncommon species (*S. blumutensis* and *S. dasyphylla*) show a lower level of genetic variation than widespread species (e.g., *S. leprosula* and *S. parvifolia*) is rejected. AMOVA analysis revealed that the genetic variation was mainly found among species, both in Nanjak Makmur Sumatra (57.7%) and in Sumalindo Borneo (56.3%). The UPGMA dendrogram of all samples revealed an almost complete separation of clusters according to species affiliation. Thus, AFLP markers proved suitable to dissect phylogenetic relationships among *Shorea* species confirming the validity of results of the first part. Species-specific markers with high frequencies (> 80%) have been detected in two species (*S. platyclados* and *S. johorensis*). Several other markers showed high frequency differences among species, and between regions within species (for *S. leprosula* and *S. parvifolia* that are represented in both regions). The homology of equal-sized AFLP fagments has to be confirmed by sequencing. Sequence information can be used to develop specific PCR markers for the identification of the origin of wood.

In conclusion, AFLPs proved to be suitable tools to analyse phylogenetic relationships among species of the Dipterocarpaceae and to investigate patterns of genetic diversity within and among populations. The taxonomic classification of dipterocarps is largely congruent with genome-wide variation detected at AFLPs. Similar levels of genetic variation were identified within populations of rare and widespread species. Considerable differentiation among populations was detected for two common *Shorea* species (*S. leprosula* and *S. parvifolia*).

Results can be applied within the context of the development of strategies for the conservation of genetic resources of dipterocarps, and as a basis for development of molecular tools to identify the origin of dipterocarp wood. The taxonomic status of species is a useful basis for conservation programmes since it is related to genome-wide differentiation patterns. Results support the importance to conserve as many species as possible, since even rare species can harbour considerable levels of genetic variation. Conservation of genetic resources requires the preservation of large populations *in situ* or *ex situ* even for rare species; a few trees maintained in a botanical garden or arboretum are of limited use. Strong differentiation among species and, at least for particular AFLP fragments, among islands or regions points towards the existence of DNA variation patterns useful to identify the origin of plant material. It might be possible to convert these “informative” AFLP fragments into molecular markers useful to control the origin of wood. These tools will be helpful within the context of forest certification since they will provide non-manipulable methods to observe the chain-of-custody from natural forests to finished wood products in consumer countries.

Zusammenfassung

Über 500 Arten aus 17 Gattungen gehören zur Familie der Dipterocarpaceen (*Dipterocarpaceae*). Es ist die aus ökonomischer und ökologischer Sicht bei weitem wichtigste Familie in den Tropenwäldern Asiens, die eines der weltweit wichtigsten Zentren biologischer Vielfalt darstellen. Indonesien, insbesondere Sumatra und Kalimantan (Borneo), ist das Land mit der höchsten Artendiversität der Dipterocarpaceen. *Shorea* ist die größte und wichtigste Gattung der Familie mit etwa 200 Arten, von denen 163 in malaiischen Archipel überwiegend auf Sumatra und Kalimantan auftreten. Viele Dipterocarpaceen sind stark von Entwaldung, Fragmentierung und gegen die Nachhaltigkeit verstoßende Waldbewirtschaftung betroffen. Ihr wertvolles Holz führt vielfach zur Übernutzung von Dipterocarpaceen-Wäldern. Nur wenig ist über die Phylogenie und Evolution indonesischer Dipterocarpaceen bekannt. Genetische Variationsmuster innerhalb von einzelnen Arten sind in Indonesien fast völlig unerforscht.

Die Untersuchung von Variation der DNS wurde durch die Verfügbarkeit von Restriktionsenzymen und durch die Möglichkeit, DNS-Bereiche mittels der Polymerase-Kettenreaktion zu vervielfältigen, stark vereinfacht. Viele Methoden zur molekularen Charakterisierung von genetischer Variation stehen heute zur Verfügung. Die AFLP-Methode (Amplifizierte Fragmentlängen Polymorphismus) ist eine Technik, mit der sich durch die selektive Amplifizierung von Restriktionsfragmenten ein „genetischer Fingerabdruck“ erstellen lässt. Die Methode erlaubt eine sehr effektive, schnelle und zuverlässige Erkennung von Variation bei Restriktionsfragmenten. Hauptvorteile sind eine große Anzahl von Markern, die in nur einer Reaktion untersucht werden können, eine in der Regel gute Wiederholbarkeit und die Möglichkeit, Variation der DNS beobachten zu können, ohne dass zuvor Informationen über bestimmte Sequenzen der DNS vorliegen müssen. Die Methode wurde häufig bei Tieren und Pflanzen als „genetischer Fingerabdruck“ und zur Untersuchung genetischer Variation genutzt. Sie wurde auch zur Erstellung von genetischen Karten, für die Charakterisierung des Reproduktionssystems mittels Elternschaftsanalysen und für phylogenetische Untersuchungen eingesetzt.

Diese Dissertation besteht aus drei Hauptteilen, die auf der Nutzung der AFLP Technik zur Untersuchung genetischer Variation indonesischer Dipterocarpaceen basieren.

(1) Genetische Variation von AFLPs bei indonesischen Dipterocarpaceen und ihre Beziehung zu molekularen Phylogenien und zur taxonomischen Untergliederung der Familie;

(2) Genetische Diversität innerhalb von und zwischen Populationen von zwei häufigen Dipterocarpaceen Indonesiens, *Shorea leprosula* Miq. und *S. parvifolia* Dyer;
(3) Genetische Variation innerhalb von Populationen bei neun *Shorea*-Arten Indonesiens.

Teil 1: Einundachtzig Individuen, die 54 verschiedenen Arten aller neun in Indonesien heimischen Gattungen der Dipterocarpaceen (Unterfamilie Dipterocarpoideae) repräsentieren, wurden an 125 AFLP-Genorten untersucht, um die genetische Differenzierung zwischen den Arten zu erkennen. Das Dendrogramm auf der Grundlage der genetischen Ähnlichkeiten trennt alle untersuchten Individuen in zwei Gruppen. Eine Gruppe enthält die den Dipterocarpeae zugehörigen Arten mit einer Chromosomenanzahl von $x = 11$ (Gattungen: *Dipterocarpus*, *Anisoptera*, *Cotylelobium, Upuna* und *Vatica*), die andere Gruppe umfasst die Shoreae-Arten mit der Chromosomenanzahl $x = 7$ (Gattungen: *Hopea*, *Parashorea*, *Shorea* und *Dryobalanops*). Die Gattung *Dryobalanops* gruppiert gemeinsam mit *Shorea virescens* basal zu den anderen Shoreae. Untersuchungen der DNS aus Chloroplasten sowie karyologische und morphologische Studien legen eine intermediäre Position dieser Gattung zwischen den Dipterocarpae und den Shoreae nahe. Die an AFLPs beobachtete genetische Variation stimmt mit der Topologie phylogenetischer Bäume auf der Basis von PCR-RFLPs und Sequenzanalysen von Chloroplasten-DNS (cpDNS) grundsätzlich gut überein und stützt die gängige taxonomische Untergliederung der Dipterocarpoideae. Die genomweite genetische Differenzierung stimmt damit grundsätzlich mit der Taxonomie von Dipterocarpaceen Indonesiens überein. Die Möglichkeit von Hybridisierung zwischen Arten wird vor diesem Hintergrund diskutiert.

Teil 2: AFLPs wurden zur Charakterisierung genetischer Variation innerhalb von und zwischen Populationen der häufigsten Dipterocarpaceen Indonesiens, *Shorea leprosula* Miq. und *Shorea parvifolia* Dyer, genutzt. Insgesamt wurden 268 Bäume von natürlichen Populationen Sumatras und Borneos sowie einer Plantage auf Java an 56 AFLP-Genorten untersucht. Sieben Populationen mit 133 untersuchten Individuen der Art *S. leprosula* und sechs Populationen mit 135 untersuchten Individuen der Art *S. parvifolia* wurden betrachtet. *S. leprosula* zeigt etwas höhere genetische Variation als *S. parvifolia*. Für *S. leprosula* beträgt der durchschnittliche Anteil polymorpher Genorte innerhalb von Populationen (PPL_p) 53,3 % und die durchschnittliche erwartete Heterozygotie (H_{ep}) 16 %. Die korrespondierenden Werte für *S. parvifolia* betragen PPL_p = 51,8 % und H_{ep} = 14 %. Auf der Ebene der beiden Arten wurde über alle Populationen der jeweiligen Art hinweg ein Anteil polymorpher Genorte von 92,9 % für *S. leprosula* und von 85,7 % für *S. parvifolia* geschätzt. Die erwartete

Heterozygotie ist auf dieser Ebene mit 21 % für beide Arten ähnlich. Auf der Basis der geschätzten Allelhäufigkeiten wurde ein Anteil von G_{st} = 25 % (*S. leprosula*) beziehungsweise G_{st} = 31 % (*S. parvifolia*) an der Gesamtvariation innerhalb von Arten ermittelt, der durch Differenzierung zwischen Populationen zustande kommt. Eine Analyse molekularer Varianz (AMOVA) ergab, dass sich 70,2 % der Variation innerhalb von *S. leprosula* Populationen und 66,2 % innerhalb von *S. parvifolia* Populationen finden. Wurde die AMOVA für den gesamten Datensatz durchgeführt, so zeigte sich, dass 44,1 % der Gesamtvariation zwischen den Arten vorliegt. Weitere 17,8 % finden sich zwischen den Populationen innerhalb einer Art und die restlichen 38.1 % innerhalb der Populationen. Die Differenzierung zwischen Populationen der unterschiedlichen Inseln ist für *S. leprosula*, nicht aber für *S. parvifolia,* signifikant. Die beobachtete genetische Variation entspricht den biologischen Charakteristika der beiden untersuchten Arten als regional verbreitete, langlebige, überwiegend fremdbefruchtende Arten von Klimaxwäldern mit hoher Fertilität und begrenzter Verbreitung genetischer Information durch Pollen und Samen. Einige Marker wurden gefunden, die ausschließlich bei einer Art beziehungsweise auf bestimmten Inseln oder in bestimmten Populationen auftraten. Diese Marker eignen sich möglicherweise zur Etablierung von Methoden, um den Ursprung von Holz von einer fraglichen Art oder aus einer fraglichen Region überprüfen zu können.

Teil 3: Die genetische Variation von neun *Shorea*-Arten aus zwei verschiedenen Gebieten wurde untersucht. Insgesamt wurden 274 Bäume untersucht, nämlich 141 Bäume, die sechs Arten angehören, aus dem Gebiet „Nanjak Makmur“ auf Sumatra sowie 133 Bäume, die fünf Arten angehören, aus dem Gebiet „Sumalindo“ auf Kalimantan. Zwei Arten (*S. parvifolia* und *S. leprosula*; siehe Teil 2) wurden in beiden Gebieten in die Analysen einbezogen. Die durchschnittliche Höhe genetischer Variation innerhalb von Populationen war in den beiden Gebieten ähnlich ($\overline{H_e}$ = 13,8 % für „Nanjak Makmur“ und $\overline{H_e}$ = 12,9 % für „Sumalindo“). Die höchste Variation wurde bei *S. blumutensis* beobachtet (H_e = 16,5 %), die geringste bei *S. acuminata* (H_e = 10.0 %). Die Erwartung, dass die genetische Variation seltener Arten (*S. blumutensis* und *S. dasyphylla*) geringer ist als die Variation häufiger Arten (insbesondere *S. leprosula* und *S. parvifolia*), wurde nicht bestätigt. Eine AMOVA ergab, dass sich in beiden Regionen der größte Anteil an der beobachteten Variation zwischen den Arten findet (57,7 % in „Nanjak Makmur“ und 56,3 % in „Sumalindo“). Ein Dendrogramm illustriert eine nahezu vollständige Separierung der untersuchten Individuen in Gruppen, die den jeweiligen Arten entsprechen. Dies unterstützt die Eignung von AFLPs zur Analyse phylogenetischer Beziehungen zwischen *Shorea*-Arten und damit die phylogenetische Relevanz der im ersten

Teil dargestellten Untersuchung. Artspezifische Marker, die innerhalb einer Art mit einer Häufigkeit von mindestens 80% beobachtet wurden, wurden bei den beiden Arten *S. platyclados* and *S. johorensis* beobachtet. Einige Marker zeigten große Variation zwischen den Arten und für die in beiden Regionen untersuchten Arten *S. leprosula* und *S. parvifolia* auch zwischen den Regionen. Die Homologie von AFLP-Fragmenten gleicher Größe bei verschiedenen Arten kann durch Sequenzierung überprüft werden. Sequenzinformation kann dann genutzt werden, um spezifische DNS-Marker für die Identifikation des Ursprungs von Holz zu entwickeln.

Zusammenfassend ist festzustellen, dass AFLPs geeignet sind, um phylogenetische Beziehungen zwischen Arten der Dipterocarpaceen zu analysieren und um genetische Differenzierungsmuster innerhalb von und zwischen Populationen zu erkennen. Die gängige taxonomische Klassifikation der Dipterocarpaceen Indonesiens stimmt gut mit genomweiten Differenzierungsmustern überein. Bei häufigen und seltenen Arten wurde ähnlich hohe genetische Variation beobachtet. Bei den häufigen Arten *Shorea leprosula* und *S. parvifolia* wurde hohe Differenzierung zwischen Populationen beobachtet.

Die Ergebnisse können genutzt werden, um Strategien zur Erhaltung der genetischen Ressourcen von Dipterocarpaceen zu entwickeln und um molekulare Marker zur Erkennung der Holzherkunft zu etablieren. Die taxonomische Untergliederung der Arten ist aufgrund des hier erstmals nachgewiesenen Bezugs zu genetischen Variationsmustern eine sinnvolle Basis für die Erhaltung genetischer Ressourcen. Es sollte die genetische Variation von so vielen Arten wie möglich erhalten werden, weil sogar seltene Arten über hohe Diversität verfügen. Die Konservierung genetischer Ressourcen setzt die Erhaltung großer Populationen *in situ* oder *ex situ* sogar bei seltenen Arten voraus. Wenige in botanischen Gärten oder Arboreten erhaltene Bäume einzelner Arten sind von allenfalls geringem Wert.

Hohe Differenzierung zwischen Arten und, zumindest für ausgewählte AFLP-Fragmente, auch zwischen Inseln oder Regionen belegt die Existenz von Variationsmustern der DNS, die die Erkennung der Herkunft von Holz der Dipterocarpaceen durch molekulargenetische Untersuchungen aussichtsreich erscheinen lässt. Es dürfte möglich sein, diese „informativen“ AFLP-Fragmente zu Markern zu konvertieren, die die Kontrolle der Holzherkunft ermöglichen. Diese Merkmale werden für die Zertifizierung von Wäldern, Forstbetrieben oder Holzprodukten nützlich sein. Ihre Beobachtung stellt eine nicht manipulierbare Methode dar, die es erlaubt, die Verarbeitungs- und Vermarktungskette bei Dipterocarpaceen weltweit vom Tropenwald bis zum Konsumenten zu verfolgen.

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