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ORIGINAL PAPER

Genetic variation in nine *Shorea* species (Dipterocarpaceae) in Indonesia revealed by AFLPs

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Abstract *Shorea* is the largest and most important genus of the Dipterocarpaceae. The genetic diversity and structure of nine *Shorea* species from two different locations, namely Nanjak Makmur in Sumatra and Sumalindo in Borneo, were evaluated using amplified fragment length polymorphism (AFLP) markers. A total of 274 trees were investigated at 85 polymorphic AFLP loci. Levels of genetic diversity of these species ranged from $\overline{H_e}$=0.100 for *S. acuminata* to $\overline{H_e}$=0.165 for *S. blumutensis*. The population of rare species *S. blumutensis* possessed the highest genetic diversity suggesting that geographically restricted species can have levels of genetic variation comparable to closely related widespread common congeners. Analyses of molecular variance revealed that the genetic variation was mainly found among species in both locations (57.7% in Sumatra; 56.3% in Borneo). The unweighted pairgroup method using arithmetic averages dendrogram of all samples revealed an almost complete separation of species. Thus, AFLP markers proved appropriate for phylogenetic studies of *Shorea* species. Specific markers have been detected showing high-frequency differences among species and between regions within species. Sequence information of these markers can be used to develop specific polymerase chain reaction markers for wood identification. The possibility of interspecific hybridization was discussed.

Keywords AFLP · Conservation of genetic resources · Dipterocarpaceae · Genetic diversity · Genetic structure · *Shorea* · Tropical Southeast Asia

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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 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 of genetic resources.

The development of DNA-based molecular marker techniques has advanced the studies of genetic diversity considerably over the last decade, which allows fingerprinting of plant genomes and reveals direct and reliable polymorphisms 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 (a large 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 polymorphisms (Garcia et al. 2004; Xiao et al. 2006; Bouajila et al. 2007; Masum Akond et al. 2008). This marker technique has often been applied to study of genetic variation in forest tree species (Gailing and von Wuehlisch 2004; Castillo-Càrdenas et al. 2005; Lara-Gomez et al. 2005; Tang et al. 2008). The application of AFLPs as simple, universal markers for 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. 2006a, b) and any other dipterocarps (Luu 2005).

The objectives of the present study are: (1) to evaluate genetic variation within and differentiation among nine *Shorea* species from two different locations, namely Nanjak Makmur on Sumatra and Sumalindo on Borneo using AFLP markers; (2) to test if AFLPs are suitable markers to distinguish among species, between regions within species, and to dissect phylogenetic relationships among species within the genus *Shorea*; and (3) to test whether widespread common dipterocarp species possess more genetic variation within populations than rare species or species with scattered distribution. Furthermore, we want to detect AFLP markers with pronounced frequency differences among species, and between regions within species, which contribute to 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 locations (Nanjak Makmur in Sumatra and Sumalindo in Borneo) in Indonesia. The six populations from Nanjak Makmur Sumatra are located at an elevation of approximately 100 m, 10°22′ S and 101°40′ E, including the two populations Spar_NS and Slep_NS previously studied using AFLPs (Cao et al. 2006a). The five populations from Sumalindo Borneo are located at 200 m, 00°55′–00°56′ N and 115°18′–116°36′ E. Information on the taxonomic status of the nine *Shorea* species and species distribution on islands Sumatra and Borneo, location, and sample size of populations is shown in Table 1 and Fig. 1. According to Ashton (1982) and Newman et al. (1996a, b), distributions of *Shorea* species studied are somewhat different from each other. *S. parvifolia* is the most common dipterocarp species and distributed in Sumatra and Borneo, followed by *S. leprosula*, *S. palembanica* and *S. johorensis*. *S. leprosula* is very common in Sumatra, but much less common in northwestern and eastern Borneo. *S. palembanica* is common on river

Table 1 Taxonomic status, species distribution, location, and sample size of 11 sampled populations of nine *Shorea* species in Indonesia

Shorea species	Section	Subsection	Species distribution	Sample location	Population abbreviation	Sample size
1. *S. parvifolia*	*Mutica*	*Mutica*	S, B, commonest	Nanjak	Spar_NS	26
2. *S. acuminata*	*Mutica*	*Mutica*	S, common	Makmur in	Sacu_NS	32
3. *S. dasyphylla*	*Mutica*	*Mutica*	S, B, scattered	Sumatra	Sdas_NS	20
4. *S. blumutensis*	*Richetioides*	*Richetioides*	(NE) S, rare		Sblu_NS	21
5. *S. leprosula*	*Mutica*	*Mutica*	S, B, common		Slep_NS	16
6. *S. macroptera*	*Mutica*	*Auriculatae*	(E) S, B, common		Smac_NS	26
7. *S. parvifolia*	*Mutica*	*Mutica*	S, B, commonest	Sumalindo	Spar_SLB	31
8. *S. leprosula*	*Mutica*	*Mutica*	S, B, common	in Borneo	Slep_SLB	26
9. *S. palembanica*	*Brachypterae*	*Brachypterae*	S, B, common		Spal_SLB	25
10. *S. platyclados*	*Brachypterae*	*Brachypterae*	S, B, common		Splat_SLB	27
11. *S. johorensis*	*Brachypterae*	*Brachypterae*	(E) S, B, common		Sjoh_SLB	24
Total						274

Taxonomic status (section and subsection) of *Shorea* species is according to Ashton (1982). Information of species distribution on islands Sumatra and Borneo was obtained from Ashton (1982) and Newman et al. (1996a, b). *S. blumutensis* is geographically restricted to lowland dipterocarp forests in northeastern Sumatra. *S. dasyphylla* is widespread throughout Sumatra and Borneo, but has a scattered distribution.
S Sumatra, *B* Borneo, *(NE)* northeastern, *(E)* eastern

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

banks and freshwater swamps and rarely occurs on low moist hillsides in Sumatra and Borneo. *S. johorensis* is found in eastern Sumatra and commonly in eastern Borneo. *S. acuminata* has a narrow distribution, because it is commonly found only in Sumatra. Over a wide altitudinal range, *S. dasyphylla* is quite widespread throughout Sumatra and Borneo, but has a scattered distribution compared to other *Shorea* species. *S. blumutensis* is confined only to northeastern Sumatra, with rare occurrences in lowland dipterocarp forests. *Shorea macroptera* has four subspecies with different geographical distributions in Sumatra (ssp. *macroptera*) and Borneo (the other three subspecies), only ssp. *macroptera* is subject to the AFLP study. *S. platyclados* is widespread in mountainous districts usually between 700 and 1,300 m, and not as common as *S. parvifolia*, *S. leprosula*, and *S. palembanica*.

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. Populations have a minimum sample size of 20 trees (except for *S. leprosula* in Nanjak Makmur, $n=16$). The estimated density for the nine species is 0.15 to 3 trees per hectare. Species identification was done on the basis of leaf morphological 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 silica gel-dried leaf tissue following the DNeasy 96 Plant Kit protocol of the manufacturer (Qiagen, Hilden, Germany). Five-microliter 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 polymerase chain reaction (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 labeled 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. Binary character matrices were compiled for further analysis. The exact test for linkage disequilibrium was conducted for all marker pairs in each species using the program ARLEQUIN version 3.01 (Excoffier et al. 2006).

POPGENE version 1.31 software (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), 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 quantifying the degree of AFLP polymorphism within populations ($I=-\Sigma p_i \log_2 p_i$, where p_i is the frequency of the presence or absence of a AFLP band; Lewontin 1972), 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 unweighted pairgroup method using arithmetic averages (UPGMA) dendrogram was constructed using the program NTSYS-pc version 2.0 (Rohlf 1998) based on Nei's (1978) genetic distances. Hierarchical analyses of molecular variance (AMOVA; Excoffier et al. 1992) based on the pairwise squared Euclidean distances among molecular phenotypes (presence or absence of bands) were conducted using ARLEQUIN version 3.01 (Excoffier et al. 2006) to further quantify the amount of genetic variation residing at two levels (i.e., among and within populations; among species and within species on each island). The same program was used to generate the matrix of pairwise F_{st} values, which indicate the genetic differentiation between populations, and are analogous to G_{st} if a locus consists of two alleles as applicable in dominant marker analyses (e.g., RAPD; Nybom and Bartish 2000). The significance levels for AMOVA were evaluated using a permutation approach (1,023 replications).

Another UPGMA clustering indicating the patterns of variation within and among populations was performed based on similarity values using the software NTSYS-pc version 2.0 (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

Test for linkage disequilibrium

The linkage disequilibrium for all marker pairs was tested in each species. The percentage of loci in significant linkage disequilibrium ($p<0.01$) ranged from 3.6% for *S. macroptera* to 14.3% for *S. platyclados*, suggesting independence of most AFLP markers.

Genetic diversity

A total of 85 unequivocally recognizable polymorphic AFLP fragments were scored, ranging in size from 76 to 486 bp. Table 2 shows that the genetic diversity varied among species. At the single species level, the rare species *S. blumutensis* and the scattered species *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 variation and the extent of distribution of a species.

Genetic structure

The results of AMOVAs (Table 3) showed highly significant ($p<0.001$) genetic differentiation among populations

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

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

PPL percentage of phenotypically polymorphic loci, n_e effective number of alleles per locus, H_e Nei's (1978) 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	*df*	SSD	MSD	Variance components	Total (%)	*p* value
Among populations	10	2,027.812	202.781	7.925	56.9	<0.001
Within populations	263	1,580.769	6.011	6.011	43.1	
Nanjak Makmur Sumatra						
Among species	5	1,025.881	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	

df 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

(mostly among species). The main portion of the total variation resided among species. AMOVAs at two hierarchical levels indicated remarkable values of among-species variation in Nanjak Makmur Sumatra (57.7%) and in Sumalindo Borneo (56.3%). Likewise, genetic differentiation among species (G_{st}) calculated using the POPGENE software (Yeh et al. 1999) was high in Nanjak Makmur Sumatra (G_{st}=0.52) and in Sumalindo Borneo (G_{st}=0.46). The pairwise F_{st} values (Table 4) generated from AMOVA indicated that all populations were significantly differentiated ($p<0.05$) with a range from 0.297 (Sdas_NS/Spar_SLB) to 0.711 (Sblu_NS/Slep_SLB). The F_{st} values between *S. dasyphylla* and *S. parvifolia* showed the lowest level (F_{st}=0.297 for Sdas_NS/Spar_SLB and F_{st}=0.312 for Sdas_NS/Spar_NS) followed by 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), and the smallest (0.067) between the two populations of *S. leprosula* from the two different regions (Slep_NS/Slep_SLB). Additionally, 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).

An UPGMA dendrogram (Fig. 2) displayed 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 one population of *S. parvifolia* (Spar_SLB) and *S. dasyphylla* (Sdas_NS) formed one cluster as sister to the other population of *S. parvifolia* (Spar_NS). The clustering of these *Shorea* species was in accordance with the division of wood varieties. Nevertheless, the dendrogram only partially reflected the taxonomic subdivision among sections. *S. blumutensis* (Sblu_NS, sect. *Richetioides*, Yellow Meranti) was divergent from all the other species of the wood variety Red Meranti. Within the Red Meranti group, *S. acuminata* (Sacu_NS, sect. *Mutica* subsect. *Mutica*) branched first followed by the two

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

Population 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

+: $p<0.05$

Table 5 Nei's (1978) unbiased measures of genetic distance for nine *Shorea* species

Population ID	1	2	3	4	5	6	7	8	9	10	11
1. Spar_NS											
2. Sacu_NS	0.207										
3. Sdas_NS	0.094	0.195									
4. Sblu_NS	0.316	0.277	0.252								
5. Slep_NS	0.228	0.263	0.199	0.389							
6. Smac_NS	0.191	0.235	0.146	0.256	0.257						
7. Spar_SLB	0.087	0.222	0.077	0.295	0.209	0.210					
8. Slep_SLB	0.217	0.259	0.203	0.371	0.067	0.218	0.198				
9. Spal_SLB	0.171	0.243	0.146	0.237	0.189	0.149	0.165	0.185			
10. Splat_SLB	0.130	0.256	0.151	0.228	0.235	0.162	0.170	0.197	0.150		
11. Sjoh_SLB	0.181	0.226	0.169	0.262	0.183	0.124	0.200	0.163	0.149	0.123	

populations of *S. leprosula* (Slep_SLB, Slep_NS, sect. *Mutica* subsect. *Mutica*) and a larger cluster. The latter cluster consisted of two sister groups, one containing *S. parvifolia* and *S. dasyphylla* (Spar_NS, Spar_SLB and Sdas_NS, sect. *Mutica* subsect. *Mutica*), and the other containing *S. palembanica*, *S. platyclados*, *S. johorensis* (Spal_SLB, Splat_SLB, and Sjoh_SLB, sect. *Brachypterae* subsect. *Brachypterae*), and *S. macroptera* (Smac_NS, sect. *Mutica* subsect. *Auriculatae*).

Genetic relationships 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, b). 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, b), for which the genetic assignment was not consistent with the morphological diagnosis. 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). Generally, the topology of Fig. 3 is similar to that of Fig. 2. All the individuals were clearly divided into two wood varieties, Red Meranti and Yellow Meranti. Within Red Meranti, the separation of sections (*Brachypterae* and *Mutica*) was not clear. *S. macroptera* from section *Mutica* (subsection *Auriculatae*) grouped together with species of section *Brachypterae*. Different from Fig. 2, *S. parvifolia* populations from Sumatra and Borneo grouped together in one cluster which is sister to *S. dasyphylla*. 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.

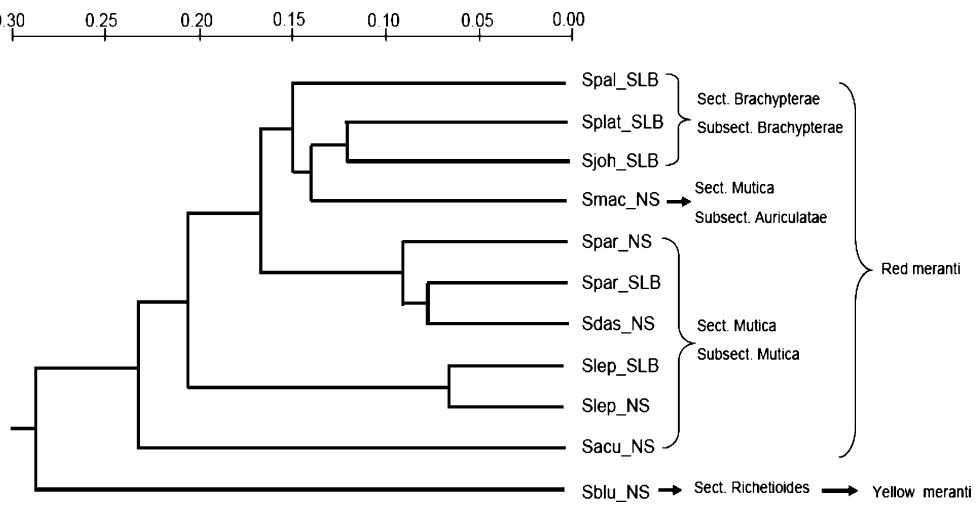
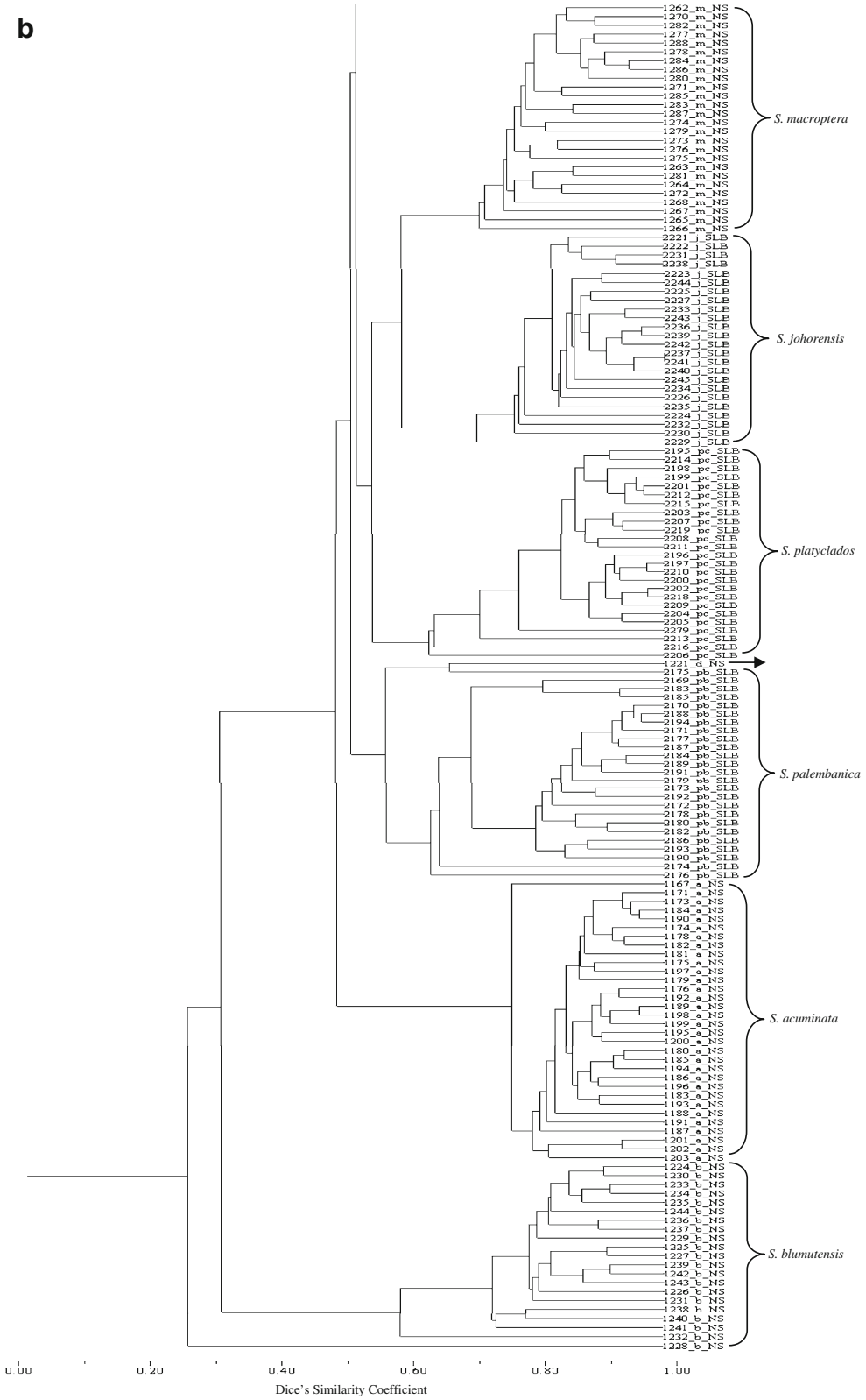


Fig. 2 UPGMA dendrogram representing the genetic distances among populations of *Shorea* species based on Nei's (1978) genetic distance. The taxonomic status (section and subsection) of *Shorea* species is according to Ashton (1982). Timber grouping is according to Symington (1943)

Fig. 3 **a** UPGMA dendrogram based on the Dice (1945) index. *NS* Nanjak Makmur Sumatra, *SLB* Sumalindo Borneo; individuals with arrows are not associated with the morphological diagnosis. **b** UPGMA dendrogram based on the Dice (1945) index. *NS* Nanjak Makmur Sumatra, *SLB* Sumalindo Borneo

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). Markers 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 Fig. 3b). Marker 61 (f) shows a high frequency (1.0) in *S. macroptera* and low frequencies (<0.1) in most

Fig. 3 (continued)



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. This is an indication that fragments of the same size may be not homologous. Markers 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. Markers 39 (i) and 73 (j) differentiate between Nanjak Makmur Sumatra and Sumalindo Borneo for *S. parvifolia*. In Sumalindo Borneo, marker 39 (i) has a high frequency (>0.8) in *S. platyclados* and a 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

Nei's gene diversity (H_e) and Shannon's information index (that does not rely on the estimation of allele frequencies) showed identical tendencies (Table 2). Since most studies applied H_e as diversity estimate (Cao et al. 2006a), the present study focused on this measure to compare genetic diversity among species. We are aware that the relatively small number of 85 fragments may result in biases in the estimation of genetic diversity (Szmidt et al. 1996; Isabel et al. 1999). Estimates of genetic diversity are dependent on the frequency of null homozygotes and the fixation index F of populations. Multilocus estimates of gene diversity from a higher number of markers are more robust to variations in the fixation index F of populations (Kremer et al. 2005). Since *Shorea* species are predominantly outcrossing (Ashton 1969; Chan 1981; Sakai et al. 1999; Lee et al. 2000), the assumption of Hardy–Weinberg equilibrium can be justified. The absence of linkage disequilibrium for most marker pairs in the present study and in earlier study on *S. leprosula* and *S. parvifolia* using the same AFLP primer combination suggests that genome-wide variation patterns can be assessed even by the comparatively limited number of fragments (Cao et al. 2006a).

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. 2000; 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).

Compared to other tree species (including dipterocarps) studied using AFLPs (Castillo-Càrdenas et al. 2005; Lara-Gomez et al. 2005; Luu 2005; Tang et al. 2008), the nine *Shorea* species investigated here showed moderate levels of genetic diversity (H_e) ranging from 0.100 (*S. acuminata*) to 0.165 (*S. blumutensis*). The differences of genetic diversity estimates among species are small. These levels of genetic diversity can be attributed to the population evolutionary history of *Shorea* species like bottleneck effects and genetic drift at the last glacial maximum when tropical rain forests were restricted to comparatively small refugia on Borneo and Sumatra (Gathorne-Hardy et al. 2002). However, no evidence for strong bottleneck effects was found for any of the investigated species.

Comprehensive studies on more than 141 plant species revealed the mating system as the most important factor to shape genetic diversity and its distribution at nuclear markers (Hamrick and Godt 1996; Duminil et al. 2007). The results also 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 (Ashton 1969; Bawa 1992).

In Nanjak Makmur Sumatra, *S. leprosula* contained a slightly higher level of genetic diversity (H_e=0.134) than *S. parvifolia* (H_e=0.110), which confirmed the tendency revealed in a previous study using AFLPs (Cao et al. 2006a). Nevertheless *S. leprosula* showed a slightly lower level of genetic diversity (0.115) than *S. parvifolia* (0.122) in Sumalindo Borneo. In general, endemic tree species and species with a narrow geographic distribution harbor less genetic variation than widespread species (Hamrick et al. 1992). In the present study, however, the rare species *S. blumutensis* exhibited genetic diversity (H_e=0.165) higher than widespread species including scattered species *S. dasyphylla* and other common species. Previous studies also have shown that some rare and geographically restricted species have levels of genetic variation comparable to closely related widespread common congeners (Young and Brown 1996; Gitzendanner and Soltis 2000). These results confirmed that not all types of rarity have the same genetic implications. However, our results are based on only one rare species and need to be confirmed through studying more rare species and more populations in each species in the future.

Genetic structure

Figures 2 and 3 showed a clear separation of populations from different islands for both species *S. leprosula* and *S. parvifolia*. The pairwise F_{st} values (0.336 for *S. parvifolia*, 0.404 for *S. leprosula*; Table 4) and the genetic distances (0.087 for *S. parvifolia*, 0.067 for *S. leprosula*; Table 5) indicated strong differentiation between populations within both species. Comparably high genetic differentiation was observed among six populations of *S. parvifolia* and seven populations of *S. leprosula* from both Borneo and Sumatra in a previous study (Cao et al. 2006a). The ecological and life history traits of the genus *Shorea*, in particular limited gene flow via pollen and seed dispersal, contributed to the

considerable differentiation between populations. *Shorea* species are pollinated mainly by small insects, such as beetles and thrips, which can migrate actively only over a limited distance (Appanah and Chan 1981). Pollination predominantly within a distance of 100 m between pollen and seed parent was observed for *S. acuminata* (Naito et al. 2008). Pollination distances for *S. leprosula* were slightly longer if the density of adults was low, but mainly within 200 m (Fukue et al. 2007). Seeds of *Shorea* species are dispersed by wind or gravity. Although seed dispersal distances can be up to 500 m or even further, more than half of the mature seeds land within 50 m of the parent tree under forest conditions (Chan 1980; Takeuchi et al. 2004), and seed dispersal is limited even for species with comparatively light seeds and long-winged fruits (Fukue et al. 2007).

The AMOVA analysis of the present study indicated highly significant ($p<0.001$) genetic differentiation among species. AFLP diversity distributed among species both in Nanjak Makmur Sumatra (57.7%) and in Sumalindo Borneo (56.3%) is higher than within-species diversity (42.3%, 43.7%, respectively; 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 genetic differentiation among species is attributed to the species' reproductive isolation from each other. However, for most species studied here, the observed levels of genetic variation are based on single populations. Hence, the significant genetic differentiation among species revealed here needs to be confirmed in further studies based on more populations for each species.

Except for three samples 2220_pc_SLB, 2217_pc_SLB, and 1221_d_NS, which are indicated with arrows in Fig. 3, the UPGMA clustering (Figs. 2 and 3) showed a very good separation of species and populations without an obvious relation to the sampling locations.

Diagnostic markers

Some 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 specific information on homology of the same-sized AFLP fragments shared among different species and, by means of sequence comparisons to databases, on putative function of the fragments, if any. It might be possible to develop simple diagnostic markers from strongly differentiating AFLP bands. These markers may be useful as tools to unambiguously identify the species status of wood samples, and to distinguish the region of the origin of wood for widespread species (Finkeldey et al. 2007).

Fig. 4 Frequency distribution of AFLP markers in *Shorea* species. *Spar*, *S. 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*; *NS* Nanjak Makmur Sumatra, *SLB* Sumalindo Borneo

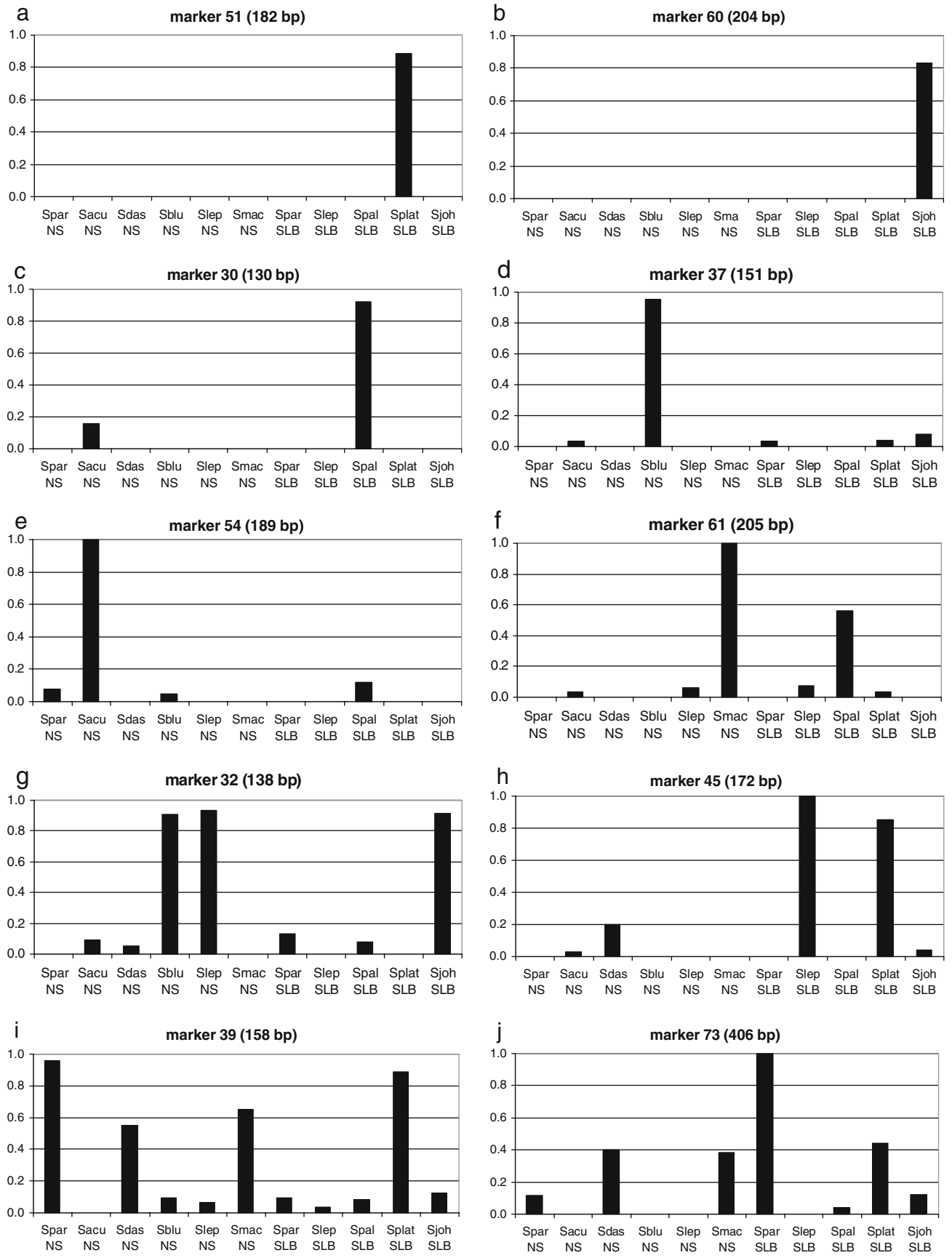
Equal-sized fragments may be non-homologous in different species. This is likely the case for marker 32 that distinguishes between regions for S. *leprosula*, but it is also present with high frequencies in *S. blumutensis* and *S. johorensis* from different regions, respectively.

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 not very likely. *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; Ishiyama et al. 2003). 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 cannot be excluded. A combination of species-specific markers is instrumental to detect recent hybridization events between sympatrically occurring species such as *S. parvifolia*, *S. leprosula*, and *S. acuminata*.

Usefulness of the 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 cannot be distinguished (Mueller and Wolfenbarger 1999). Hence, size homoplasy effects possibly limit the usefulness of this marker type for 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 analyzed per experiment (Pejic et al. 1998). These advantages can counteract the size homoplasy effects.


a
marker 51 (182 bp)
b
marker 60 (204 bp)
c
marker 30 (130 bp)
d
marker 37 (151 bp)
e
marker 54 (189 bp)
f
marker 61 (205 bp)
g
marker 32 (138 bp)
h
marker 45 (172 bp)
i
marker 39 (158 bp)
j
marker 73 (406 bp)
1.0
0.8
0.6
0.4
0.2
0.0
Spar NS
Sacu NS
Sdas NS
Sblu NS
Slep NS
Sma NS
Smac NS
Spar SLB
Slep SLB
Spal SLB
Splat SLB
Sjoh SLB

Therefore, genome-wide AFLP datasets can provide high power in testing specific phylogenetic relationships in particular for closely related taxa within the same genus (Rokas et al. 2003). In closely related species, fragments of the same size are more likely to be orthologous than in distantly related species. Even though it is impossible to determine an ancestral (versus derived) character state for AFLP markers, taxonomic relationships revealed by AFLP markers are in wide accordance with the phylogenetic trees derived from sequence variation in chloroplast regions within Dipterocarpaceae (Cao et al. 2006b; Gamage et al. 2006; Indrioko et al. 2006).

Most of the phylogenetic studies based on AFLPs used only a limited number of samples per species. With our experimental design, i.e., nine species with the sample size ranging from 16 to 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. *S. blumutensis* (section *Richetioides*, Yellow Meranti) is isolated far from all the other species of wood variety Red Meranti. In a phylogenetic study based on the *PgiC* gene (Kamiya et al. 2005), section *Richetioides* (Yellow Meranti) was likewise basal to a clade comprising *Shorea* species of wood varieties Red Meranti and Balau as sister groups. In cpDNA studies (Tsumura et al. 1996; Kajita et al. 1998; Kamiya et al. 1998; Dayanandan et al. 1999), there was also a clear separation of wood varieties, but their relative position in the phylogenetic trees was not resolved. Additionally, phylogenetic studies based on cpDNA (Indrioko et al. 2006) and AFLPs (Cao et al. 2006b) differentiated genera and tribes within Dipterocarpaceae, but sectional subdivision within genus *Shorea* and separation of wood varieties were not reflected. AFLP fragments that strongly differentiated among species in this study will be further sequenced in order to verify the homology of the same-sized fragments.

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