GENETIC VARIATION AND GENETIC STRUCTURE OF TWO CLOSELY RELATED DIPTEROCARP SPECIES, *DRYOBALANOPS AROMATICA* C.F.GAERTN. AND *D. BECCARII* DYER

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ABSTRACT

Large-scale genetic structure revealed in tree populations in SE Asia, as well as in many temperate forests, has been shaped by climatic fluctuation in the late Pleistocene, most importantly by that in the last glacial period. In a comparative study of the phylogeographic patterns of two closely related dipterocarp species, *Dryobalanops aromatica* C.F.Gaertn. and *D. beccarii* Dyer, we investigated how changes in land area associated with changes in climate affected large-scale genetic structure. We examined the genetic variation of *D. aromatica*, collected from nine populations throughout the Sundaic region, and of *D. beccarii*, collected from 16 populations mainly in Borneo, using seven polymorphic microsatellite markers. The two species were clearly distinguishable in the STRUCTURE analysis, although hybridisation probably occurred in sympatric populations and also in several other populations. The *D. aromatica* populations were divided into two main groups by the STRUCTURE analysis: Malay–Sumatra and Borneo. Mixing of the Sumatra and Borneo clusters occurred on the Malay Peninsula, supporting the hypothesis that tropical rainforests expanded over a dried Sunda Shelf during the last glacial period. The two main genetic clusters might have been formed by repeated cycles of fluctuation in land area. The *D. beccarii* populations in Borneo were divided into four geographically distinct groups: western Sarawak, central inland Sarawak, central coastal Sarawak and Sabah. The population on the Malay Peninsula (Gunung Panti) was an admixture of the four Bornean clusters. This suggests that this population is a relic of the recent range expansion of *D. beccarii* during the last glacial period.

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INTRODUCTION

The Sundaic region is a part of western Malesia comprising Peninsular Malaysia, Sumatra, Borneo, Java and their surrounding islands. Approximately 15,000 plant species are endemic to the Sundaic region – about 5% of the global total of plant species (Myers *et al.*, 2000). This region with its immense and unique biodiversity in parallel with ongoing rapid degradation of forests has been identified as one of the hottest hotspots in the world (Myers *et al.*, 2000; Conservation International, 2010). Since the beginning of the 20th century, especially in the second half, deforestation has been accelerating as a result of increasing human activities such as intensive logging, conversion of forest areas to large-scale plantations and expansion of agricultural land (e.g. FAO, 2010; Langner *et al.*, 2007; Hansen *et al.*, 2010). The overall annual deforestation level in this region remained above 1% from 2000 to 2010 (Miettinen *et al.*, 2011). The importance of conservation of rainforests in tropical Asia coupled with sustainable development of rural communities has been broadly discussed (Sodhi *et al.*, 2004; Ghazoul & Sheil, 2010; Corlett, 2014).

In the last two decades, various kinds of genetic markers have been developed and adapted for studying plant genetic diversity and population genetic structure in tropical forests, as well as in temperate forests. Genetic diversity and genetic structure in plant species are both influenced by many factors, but at different levels. Some of them are intrinsic to the life history of a species (breeding system, modes of seed and pollen dispersal, life form, gregariousness), whereas others are historical processes (large-scale distribution range shifts) associated with changes in climate, especially during the ice ages and human impact (habitat fragmentation by cutting, density change as a result of selective logging) (Hamrick *et al.*, 1992; Heuertz *et al.*, 2004). Fine-scale spatial genetic structure can often be generated in the first case, as has been reported in tropical rainforest trees (Takeuchi *et al.*, 2004; Harata *et al.*, 2011; Kettle *et al.*, 2011; Ismail *et al.*, 2012; Kettle *et al.*, 2012; de Morais *et al.*, 2015). However, climatic fluctuations during the Quaternary strongly affected their large-scale genetic structure (Comes & Kadereit, 1998; Hewitt, 2000; Ishiyama *et al.*, 2008; Iwanaga *et al.*, 2012; Kamiya *et al.*, 2012; Ohtani *et al.*, 2013). Drastic environmental changes have been caused by human activity; however, the effect on the genetic diversity and structure of tree populations may not occur concurrently, because of the long life cycle of trees (Finger *et al.*, 2012). However, some change in breeding systems, caused by selective logging and the resulting inbreeding depression, have already been reported in Asian tropical rainforest trees (Obayashi *et al.*, 2002; Naito *et al.*, 2005; Fukue *et al.*, 2007; Naito *et al.*, 2008; Ismail *et al.*, 2012).

The lowland evergreen forests of the SE Asian tropics are characterised by an abundance of dipterocarp trees (Dipterocarpaceae). Over 400 species in 11 genera are recognised in the area centring on the Sundaic region (Ashton, 1982). *Dryobalanops* C.F.Gaertn. is one of the smallest genera of Dipterocarpaceae, and contains only seven known species: *D. aromatica* C.F.Gaertn., *D. oblongifolia* Dyer, *D. beccarii* Dyer, *D.*

fusca Slooten, *D. keithii* Symington, *D. lanceolata* Burck and *D. rappa* Becc. All seven species occur in Borneo, and the genus is centred in the north-western part of this island, where four of the species (*D. fusca*, *D. keithii*, *D. lanceolata* and *D. rappa*) are endemic (Ashton, 1982). *D. aromatica*, *D. beccarii*, *D. lanceolata* and *D. oblongifolia*, which are locally known as kapur (Ashton, 1982; Symington, 2004), grow tall (more than 70 m) and have been an important source of timber. They are essentially outbreeding, and are pollinated by insects. The seeds are dispersed by gravity or by gyration, as they have winged fruits (Appanah & Turnbull, 1998). They used to be common (Newman *et al.*, 1998; Chua *et al.*, 2010), but their habitat is currently greatly reduced, and four of the seven species are listed as threatened in the 2017 IUCN Red List: *D. fusca* and *D. keithii*, both Critically Endangered, and *D. beccarii* and *D. lanceolata*, both Endangered (IUCN, 2017).

In this study, we examined the genetic structure of *D. aromatica*, which occurs throughout Peninsular Malaysia, Sumatra and Borneo. We also examined *D. beccarii*, which has a broad distribution in Borneo from latitude 2° N northwards, and grows especially on inland slopes, but whose distribution is limited outside Borneo. Only one isolated population is known outside of Borneo, in Panti Forest Reserve in Johor, Malaysia (Ashton, 1982). These two species are genetically closely related (Dwiyanti *et al.*, 2015). The two species are recognised as morphologically and ecologically distinct, but identification is often difficult in the field, especially in the absence of flowers or fruits (Symington, 2004; Chua *et al.*, 2010). We examined the genetic variation and genetic structure of these species using microsatellite markers. Microsatellites are essentially neutral, and changes in allele frequency are based on purely stochastic processes (Li, 1997). These markers therefore have great potential to detect genetic structure that is shaped by stochastic processes related to historical changes in climate. Comparative analysis of genetic diversity and structure will provide deeper insight into the phylogeographic orientation of the present distribution of the species. This can also be useful for zoning potential natural vegetation projected on the scattered and fragmented habitats (Tüxen, 1956). This will provide spatial extent and configuration of forests for determining conservation and management units, to define the first approximation of areas to which the species is locally adapted (Manel *et al.*, 2003).

MATERIALS AND METHODS

Plant materials

Samples of leaf material were collected from nine populations of *D. aromatica* and 16 populations of *D. beccarii* (Table 1, Fig. 1). Samples were collected from across the current distribution ranges of these species in Peninsular Malaysia, in Sumatra and in Borneo. In total, 219 *D. aromatica* individuals and 235 *D. beccarii* individuals were sampled. Plant materials were dried in the field using silica gel and kept at −30° C until they were processed for DNA extraction. In the laboratory, samples were ground to a

D. aromatica				
Code	**Population**	**Sample size**	**Location**	**Coordinates**
A1	Singkil	23	Sumatra	2.3589N, 97.8722E
A2	Barus	6	Sumatra	2.0692N, 98.3578E
A3	Mursala	51	Sumatra	1.6731N, 98.4967E
A4	Kancing	13	Peninsular Malaysia	3.3042N, 101.6114E
A5	Gunung Panti	23	Peninsular Malaysia	1.8278N, 103.8669E
A6	Lingga	20	Lingga Isl.	1.5086N, 104.6372E
A7	Similajau	32	Sarawak	3.4503N, 113.2808E
A8	Lambir	28	Sarawak	4.2131N, 114.03E
A9	Limbang	27	Sarawak	4.7544N, 114.9908E
D. beccarii				
Code	**Population**	**Sample size**	**Location**	**Coordinates**
B1	Gunung Panti	3	Peninsular Malaysia	1.8278N, 103.8669E
B2	Gunung Gading	28	Sarawak	1.6902N, 109.8458E
B3	Kubah N.P.	15	Sarawak	1.6131N, 110.19694E
B4	Kuching	7	Sarawak	1.6781N, 110.4147E
B5	Bako N.P.	28	Sarawak	1.7253N, 110.4664E
B6	Bukit Lingang	6	Sarawak	1.5308N, 111.7794E
B7	Lubok Antu	5	Sarawak	1.3006N, 111.8467E
B8	Batang Ai N.P.	35	Sarawak	1.2219N, 111.9464E
B9	Bukit Tangii	32	Sarawak	2.6080N, 111.95E
B10	Mukah	15	Sarawak	2.4678N, 112.6169E
B11	Nyabau	10	Sarawak	3.23361N, 113.1E
B12	Similajau N.P.	13	Sarawak	3.4503N, 113.2808E
B13	Bukit Tiban	8	Sarawak	3.4581N, 113.4978E
B14	Sungai Asap	10	Sarawak	3.0378N, 113.93389E
B15	Bakun Dam	6	Sarawak	2.7564N, 114.0631E
B16	Deramakot	14	Sabah	5.3533N, 117.4086E

Populations are in the order that they occur from west to east along each line of latitude. N.P. National Park.

Table 1 Sampling locations of *D. aromatica* and *D. beccarii*.

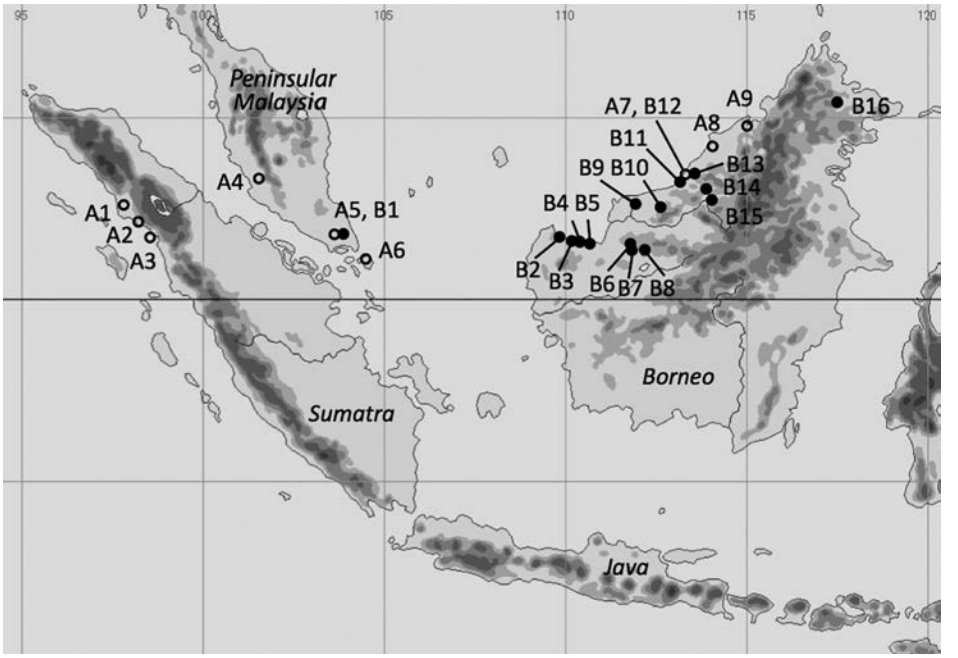

Fig. 1 Map of the sampling locations of nine populations of *D. aromatica* (open circles) and 16 populations of *D. beccarii* (closed circles).

fine powder using a TissueLyser II (QIAGEN Japan, Tokyo). Total genomic DNA was extracted using the modified CTAB method (Doyle & Doyle, 1990).

Microsatellite genotyping

The genetic variation of eight microsatellite loci was examined for both species. Of these, seven (Dra187, Dra428, Dra426, Dra519, Dra266, Dra471 and Dra569) had been developed for *D. aromatica* (Nanami *et al.*, 2007) and one (Sle384) for *Shorea leprosula* (Lee *et al.*, 2004). The forward primer for each marker was labelled with 6-FAM, VIC, NED or PET phosphoramidite (Applied Biosystems Japan, Tokyo). A Type-it Microsatellite PCR kit (QIAGEN Japan, Tokyo) was used to amplify the microsatellite loci. Multiplex PCR amplification was performed in a volume of 5 µl, containing 1×Type-it Multiplex PCR Master mix, 0.2 µM of forward and reverse primers, and approximately 40 ng of genomic DNA. A 2720 Thermal Cycler (Applied Biosystems Japan) was used under the following conditions: initial denaturation at 95° C for 5min, then 31 cycles of denaturation at 95° C for 30s, annealing for 90s and extension at 72° C for 30s, followed by a final incubation at 60° C for 30min. The annealing temperature was 52–57° C. Fragment sizes were determined using an ABI PRISM 310 Genetic Analyzer and visualised using the GeneMapper 3.0 software (Applied Biosystems Japan).

Microsatellite data analysis

The existence of null alleles was examined using Micro-checker (Oosterhout *et al.*, 2004). The linkage disequilibrium between pairs of loci was examined with FSTAT 2.9.3.2 (Goudet, 2001), based on 100 permutations for each test. Basic statistics, including the mean number of alleles per locus (N_a), effective number of alleles (N_e) for each population of each species, observed heterozygosity (H_o), expected heterozygosity (H_e) and Wright's inbreeding coefficient (F), were calculated using GenAlEx version 6.41 (Peakall & Smouse, 2006). Allele richness (A_r) was calculated using FSTAT 2.9.3.2 (Goudet, 2001). The F-statistics F_{IS}, F_{ST} and F_{IT} (Weir & Cockerham, 1984) were calculated to determine the level of population differentiation, using FSTAT. Standard errors and confidence intervals were estimated using the jack knife and bootstrap methods, respectively, implemented in FSTAT. Selective neutrality for the loci was examined by means of an F_{ST} outlier method (Beaumont & Nichols, 1996) using the LOSITAN software (Antao *et al.*, 2008). We performed 50,000 simulations with a stepwise mutation model. Candidate loci which may have been under positive selection or balancing selection were identified as outliers based on 99% confidence intervals. Because the sample size of some populations was very small, we used populations with a sample size of more than ten for all the above estimates.

A Bayesian model-based clustering analysis implemented in STRUCTURE version 2.3.4 (Pritchard *et al.*, 2000) was used to estimate the number of genetically homogeneous groups of individuals or clusters (K) and determine the genetic structure of the sampled populations. A Markov chain Monte Carlo (MCMC) simulation of 4×10^5 steps, preceded by a burn-in of 2×10^5 steps, was performed using admixture and allele size-correlated models, with the LocPrior option. The program was run with ten replications each for the range K=1–10 for the combined *D. aromatica* and *D. beccarii* dataset, and also for the two datasets separately. In order to evaluate the likelihood of K, we uploaded the STRUCTURE-generated results to the online program STRUCTURE HARVESTER (Earl & von Holdt, 2012) and obtained plots of the mean likelihood value (Ln PrX/K) and ΔK for successive values of K. We then determined the optimum values of K, following Evanno *et al.* (2005). For certain values of K the replicated results were aligned using CLUMPP version 1.1.2 (Jakobsson & Rosenberg, 2007) and visualised using DISTRUCT (Rosenberg, 2004). The existence of population bottlenecks was assessed using BOTTLENECK version 1.2.02 (Cornuet & Luikart, 1998), by testing for positive or negative deviations in observed heterozygosity from the expected value under mutation-drift balance. We used a two-phase model (TPM) and tested it using the Wilcoxon signed-rank test because of the small number of loci (Luikart *et al.*, 1998). Geographic barriers to genetic exchange were visualised using the software BARRIER version 2.2 (Manni *et al.*, 2004). The software implements Monmonier's maximum difference algorithms and identifies genetic barriers on a geographic map, based on the genetic distance matrix and coordination data (Monmonier, 1973). The number of barriers is set arbitrarily. The reliability of

the barriers was evaluated by bootstrapping, based on 100 permutations of the dataset generated by the Microsatellite Analyzer (MSA) software (Dieringer & Schlötterer, 2003). The distance matrix was constructed based on Nei's (1978) pairwise standardised genetic distance.

RESULTS

Null alleles and linkage disequilibrium

The existence of null alleles was examined for the eight microsatellite loci. Null alleles were detected in 14 out of 25 populations (three in *D. aromatica* and 11 in *D. beccarii*) at the locus Dra471. In addition, they were detected in four, five, three, one and four populations at the loci Dra187, Dra428, Dra519, Dra266 and Sle384, respectively. We excluded Dra471 from the following analysis. Linkage disequilibrium was examined separately for *D. aromatica* and *D. beccarii.* Seven out of 183 tests on *D. aromatica* and two out of 169 tests on *D. beccarii* were significant at the 5% level after applying the Bonferroni correction. Furthermore, none of the remaining seven loci (after the exclusion of Dra471) were identified as outliers by LOSITAN. We concluded that these loci were neutral and sufficiently independent for the application of Bayesian and other methods to the population demography analysis.

Genetic variation

A total of 132 alleles were detected at the seven loci across the combined *D. beccarii* and *D. aromatica* dataset. The estimated population genetic parameters are listed in Table 2. The 95% confidence interval (CI) for H_e was 0.603–0.611 for *D. aromatica* and 0.321–0.487 for *D. beccarii*. The 95% CI for A_r was 5.32–8.91 for *D. aromatica* and 3.30–6.71 for *D. beccarii*. H_e was significantly larger in *D. aromatica* than in *D. beccarii*. A_r was larger in *D. aromatica* than *D. beccarii*, but not significantly. The coefficient of genetic differentiation, F_{ST}, was significantly greater than zero in both species (at the 99% CI level). It was larger in *D. beccarii* than in *D. aromatica* (at the 95% CI level). The overall inbreeding coefficient, F_{IS}, was not significantly different from zero at the 99% CI level in either species (Table 3). A significant heterozygosity deficit was detected by the BOTTLENECK analysis in the A8 (Lambir) and A9 (Limbang) populations of *D. aromatica*, and in the B5 (Bako) and B9 (Bukit Tangii) populations of *D. beccarii*. This suggests a recent population expansion in these populations. None of the populations showed any signs of a recent bottleneck.

Genetic structure

The STRUCTURE analysis was performed first on the combined *D. aromatica* and *D. beccarii* dataset. The most likely number of genetic clusters was $K = 2$, since a single

D. aromatica									Wilcoxon test‡		
Population		***N***	N_a	N_e	A_r	H_o	H_e	***F***	**a**	**b**	**c**
Singkil	Mean	23	7.00	3.39	5.28	0.646	0.657	0.026	0.718	0.531	1.000
	SE†		1.36	0.56	0.77	0.085	0.052	0.087			
Mursala	Mean	51	7.71	3.71	5.24	0.538	0.635	0.192	0.718	0.344	0.688
	SE		1.48	0.70	0.79	0.104	0.099	0.090			
Kancing	Mean	23	4.57	2.82	4.01	0.609	0.585	–0.083	0.406	0.656	0.812
	SE		0.69	0.52	0.54	0.034	0.057	0.096			
Gunung Panti	Mean	20	5.71	3.43	4.79	0.500	0.655	0.204	0.945	0.148	0.297
	SE		0.84	0.62	0.64	0.062	0.058	0.114			
Lingga	Mean	13	6.43	3.33	5.86	0.637	0.648	0.014	0.054	0.961	0.109
	SE		1.04	0.52	0.91	0.055	0.057	0.025			
Similajau	Mean	32	5.57	2.61	4.28	0.580	0.565	0.031	0.344	0.711	0.688
	SE		0.57	0.37	0.39	0.094	0.064	0.107			
Lambir	Mean	28	7.43	3.10	5.24	0.551	0.542	–0.041	0.0039	1.000	0.0078
	SE		1.46	0.67	0.87	0.109	0.110	0.050			
Limbang	Mean	27	7.86	3.47	5.58	0.519	0.566	0.149	0.027	0.981	0.055
	SE		1.57	0.956	0.96	0.102	0.100	0.090			
Total	Mean	27.1	6.54	3.223	7.11**	0.572	0.607	0.061	–	–	–
	SE	1.41	0.42	0.22	0.76	0.029	0.026	0.032			
D. beccarii									Wilcoxon test‡		
Population		***N***	N_a	N_e	A_r	H_o	H_e	***F***	**a**	**b**	**c**
Gunung Gading	Mean	28	2.57	1.57	2.13	0.301	0.273	–0.006	0.594	0.500	1.000
	SE†		0.53	0.24	0.37	0.141	0.097	0.205			
Kubah	Mean	15	2.71	1.74	2.46	0.381	0.357	–0.048	0.578	0.500	1.000
	SE		0.36	0.25	0.29	0.114	0.086	0.155			
Kubah	Mean	15	2.71	1.74	2.46	0.381	0.357	–0.048	0.578	0.500	1.000
	SE		0.36	0.25	0.29	0.114	0.086	0.155			
Bako	Mean	28	3.43	1.68	2.83	0.265	0.301	0.120	0.016	0.992	0.031
	SE		0.78	0.30	0.54	0.099	0.100	0.115			
Batang Ai	Mean	35	3.71	1.92	2.70	0.441	0.365	–0.168	0.281	0.781	0.562
	SE		0.78	0.38	0.48	0.133	0.105	0.059			
Bukit Tangii	Mean	32	4.86	2.20	3.74	0.509	0.475	–0.069	0.039	0.976	0.078
	SE		1.01	0.30	0.55	0.098	0.091	0.034			
Mukah Hill	Mean	15	4.14	2.36	3.62	0.400	0.429	0.038	0.148	0.945	0.297
	SE		0.86	0.74	0.71	0.088	0.088	0.076			
Nyabau	Mean	10	4.86	2.79	4.86	0.543	0.529	–0.013	0.055	0.960	0.109
	SE		0.738	0.58	0.68	0.115	0.097	0.095			
Similajau	Mean	13	5.14	3.56	4.94	0.648	0.644	–0.027	0.766	0.289	0.578
	SE		0.91	0.82	0.77	0.065	0.065	0.068			
Sungai Asap	Mean	10	2.71	1.98	2.71	0.329	0.356	0.025	0.578	0.500	1.000
	SE		0.61	0.52	0.56	0.084	0.099	0.079			
Deramakot	Mean	14	2.86	1.60	2.62	0.245	0.311	0.160	0.055	0.961	0.109
	SE		0.40	0.23	0.32	0.078	0.080	0.121			
Total	Mean	20.0	3.70	2.14	5.00*	0.406	0.404	0.004	-	-	-
	SE	1.09	0.24	0.16	0.56	0.034	0.030	0.033			

N sample size, N_a actual number of alleles, N_e effective number of alleles, A_r allele richness calculated based on ten samples, H_o observed heterozygosity, H_e expected heterozygosity, *F* inbreeding coefficient. † Standard error. ‡ The results of Wilcoxon tests for the BOTTLENECK analysis. Probabilities for testing (a) heterozygosity deficit, one-tailed test; (b) heterozygosity excess, one-tailed test; and (c) heterozygosity excess or deficit, two-tailed test.

Table 2 Summary of population genetics parameters estimated for *D. aromatica* and *D. beccarii*.

	F_{IT}	F_{ST}	F_{IS}
D. aromatica			
Mean	0.257 ± 0.054	0.189 ± 0.021	0.083 ± 0.050
95% CI	(0.139–0.361)	(0.150–0.227)	(0.004–0.185)
99% CI	(0.151–0.392)	(0.136–0.237)	(–0.004–0.221)
D. beccarii			
Mean	0.302 ± 0.035	0.308 ± 0.046	–0.070 ± 0.052
95% CI	(0.247–0.383)	(0.232–0.396)	(–0.070–0.052)
99% CI	(0.231–0.413)	(0.208–0.423)	(–0.089–0.069)

F-statistics (Weir & Cockerham, 1984) were calculated using FSTAT version 2.9.3.2. Standard errors of the means were calculated using the jackknife method. Confidence intervals (CIs) were calculated by the bootstrap method.

Table 3 Summary of *F*-statistics for *D. aromatica* and *D. beccarii*.

Δ*K* peak appeared at *K* = 2 (Fig. 2a). The bar plot for *K* = 2 is shown in Fig. 3a. Two clusters clearly differentiate the two species; however, these two clusters were mixed in the A7, B11, B12 and B16 populations (and to a lesser extent in A3 and B15). The B12 (*D. beccarii*) and A7 (*D. aromatica*) populations are sympatric in Similajau, Sarawak. In addition, *D. aromatica* has been recorded in the Deramakot area, where population B16 was sampled (Chua *et al.*, 2010). Intensive hybridisation between these two species is suspected in these populations. The STRUCTURE analysis of the *D. aromatica* dataset alone revealed three peaks of Δ*K* at *K* = 2, 4 and 6, but showed that the most likely number of genetic clusters was *K* = 2 (Fig. 2b). The bar plot for *D. aromatica* at *K* = 2 (Fig. 3b) showed that the populations were separated into two groups: Malay-Sumatra and Borneo. We noted, however, that the Bornean cluster was mixed to some extent with populations A5, in Peninsular Malaysia, and A6, on the Lingga Islands. At *K* = 4, the Sumatran populations were separated from the Malay populations, and the central Sarawak population (A7) was separated from the eastern Sarawak populations (A8 and A9). At *K* = 6, the populations in Sumatra and in Peninsular Malaysia were each further divided into two groups.

The STRUCTURE analysis of the *D. beccarii* dataset alone revealed two prominent peaks of Δ*K*, at *K* = 3 and 6 (Fig. 2c). At *K* = 3 (Fig. 3c), the populations in Borneo were divided into three regional groups based on the dominant clusters: one in western Sarawak (B2, B3, B4, B5, B6 and B7), one in central to eastern Sarawak (B8, B9, B10, B13, B14 and B15) and one in central coastal Sarawak (B11 and B12) and Sabah (B16). Interestingly, the population in Peninsular Malaysia (B1) is a mixture of all three of these clusters. At *K* = 6, the central Sarawak group was differentiated into two groups: B8 and the others (B9, B10, B13, B14 and B15). The central coastal Sarawak populations (B11 and B12) were differentiated from the Sabah population (B16), and the western Sarawak

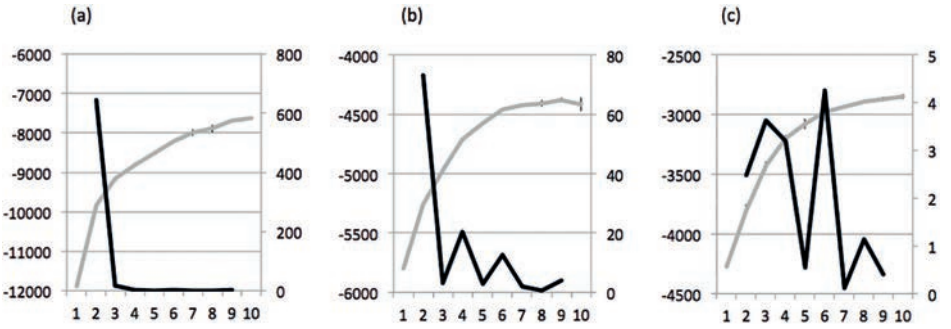

Fig. 2 Plots of the mean LnP(*K*) (grey lines) and the Δ*K* (black lines) obtained from the STRUCTURE analyses of (a) the combined *D. aromatica* and *D. beccarii* dataset, (b) *D. aromatica* and (c) *D. beccarii*. Standard deviations of the mean LnP(*K*) values are indicated with vertical bars.

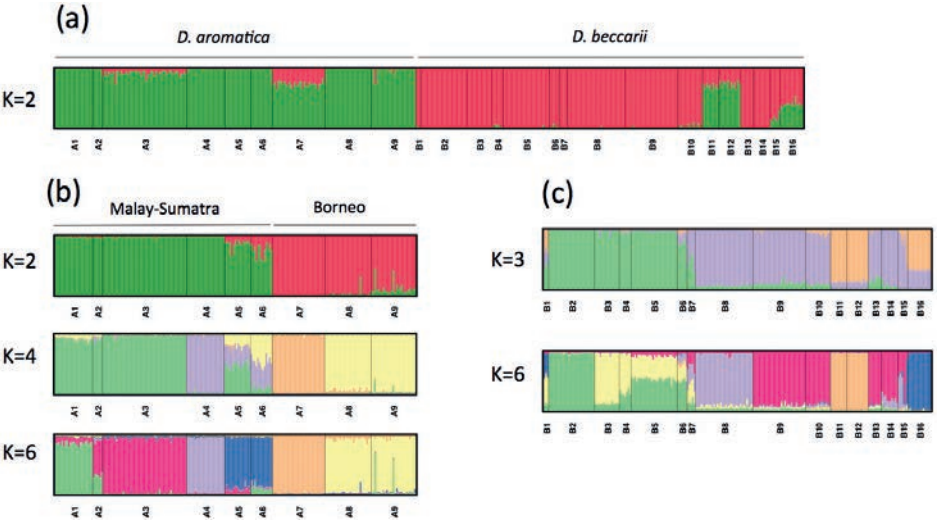

Fig. 3 Profile of membership coefficients for each individual (bar plot) of *D. aromatica* and *D. beccarii*, obtained from the STRUCTURE analysis. (a) Estimated genetic structure for $K = 2$ for the combined dataset from nine populations of *D. aromatica* and 16 populations of *D. beccarii*. (b) Bar plots for the nine populations of *D. aromatica* for $K = 2$, 4 and 6. (c) Bar plots for the 16 populations of *D. aromatica* for $K = 3$ and 6.

populations were differentiated into two groups: B2 and the others (B3, B4, B5, B6 and B7), which were mixtures of two or three clusters.

In order to identify the boundaries or barriers where the genetic differentiation occurred and determine the strength of these barriers, we performed BARRIER analyses separately for *D. aromatica* and *D. beccarii*. Setting the number of barriers at four, the strongest barrier (100% bootstrap probability) appeared between the Malay–Sumatra and Borneo groups in *D. aromatica* (Fig. 4a). There was a slightly weaker barrier

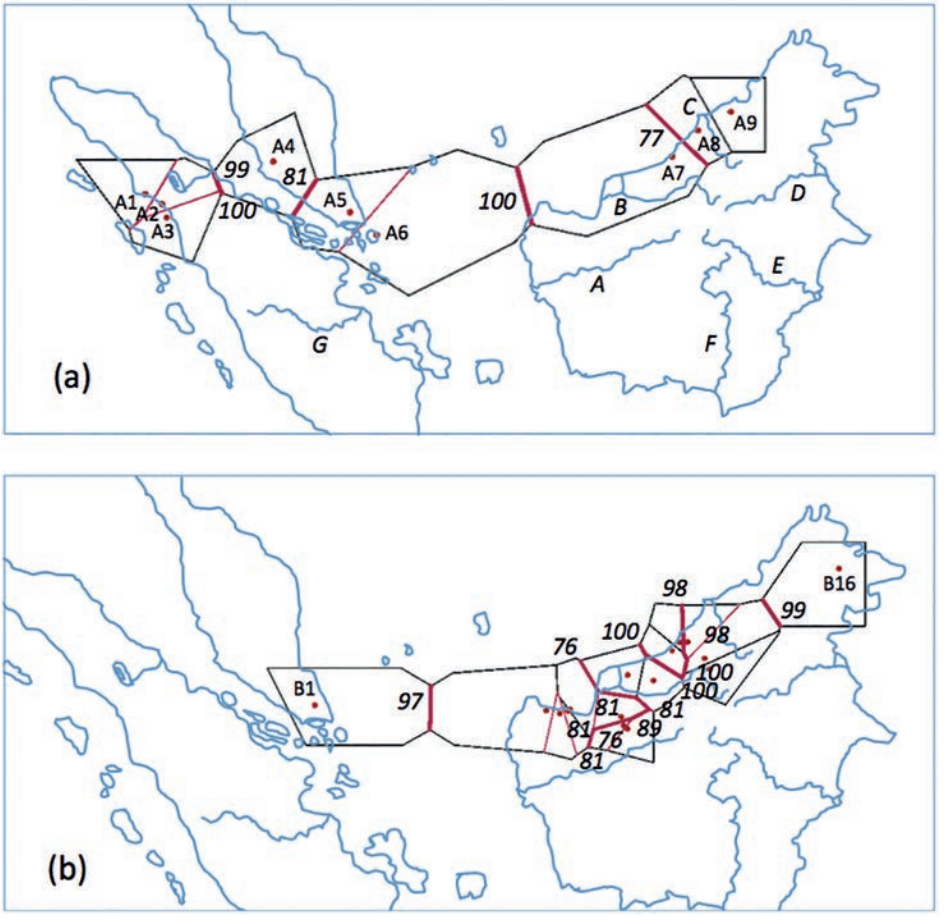

Fig. 4 Genetic barriers estimated by the BARRIER analysis, projected on a geographic map, for (a) *D. aromatica* and (b) *D. beccarii.* Red lines indicate barriers, with bootstrap probabilities (%) next to them. The polygonal neighbourhood for each population is indicated by the Voronoï tessellation (black lines). In both figures solid red dots indicate sampling locations. The large rivers noted *A*, *B*, *C*, *D*, *E*, *F* and *G* are the Kapuas, Rajang, Baram, Kayan, Mahakam, Barito and Batang Hari Rivers, respectively. Population codes are omitted for *D. beccarii*, except for B1 and B16, but can be found in Fig. 1.

between Sumatra and Peninsular Malaysia (99–100%). The other barriers occurred between A4 and A5 in Peninsular Malaysia (81%) and between A7 and A8 in Borneo (77%). We also set the number of barriers at four for *D. beccarii* (Fig. 4b). The strongest barriers (98–100%) isolated the central coastal Sarawak group (B11 and B12) from the others. There were slightly weaker barriers between B1 and the others (97%) and between B16 and the others (99%). The western Sarawak group (B2, B3, B4 and B6) was also separated from the central Sarawak group (B7, B8, B9, B10, B13, B14 and B15) by the barriers crossing the area (76–89%).

DISCUSSION

The smaller H_e and larger F_{ST} obtained for *D. beccarii* suggest that the populations of this species are more intensively isolated, with more limited gene flow among them, than those of *D. aromatica*. The overall inbreeding coefficient, F_{IS}, was not significantly different from zero in either species, indicating that mating in both species is essentially random. The STRUCTURE analysis of the combined *D. aromatica* and *D. beccarii* dataset revealed one single prominent peak of ΔK at K=2 (Fig. 2a). For K=2, the two species were clearly separated into different clusters. This may indicate that the microsatellite markers we used are effective for discriminating between these two species. However, in several of the populations, considerable mixing of the two clusters was observed. The strong similarity of the bar plot pattern of B11 and B12, in coastal central Sarawak, to that of the sympatric A7 population of *D. aromatica* (Similajau) is confirmed by the similarly high proportions of the *D. aromatica* cluster observed in all three populations: 0.7593 ± 0.0079 (S.E.), 0.7110 ± 0.0095 and 0.7551 ± 0.0099 for A7, B11 and B12, respectively. However, the samples of the two species were collected at different times, and it is likely that we collected material from the same group of trees as different species. The mixing of the two clusters in these three populations raises the possibility that these trees are hybrids of *D. aromatica* and *D. beccarii*. The larger contribution of the *D. aromatica* cluster in these populations, together with the habitat in the lowland coastal area they inhabit, both favour *D. aromatica* and suggest that introgression is proceeding towards this species. Mixing of the two clusters was also observed in the A3, B15 and B16 populations, suggesting that hybridisation is occurring. Occasional hybridisation has also been reported among three closely related *Shorea* species – *Shorea parvifolia* Dyer, *S. leprosula* Miq. and *S. curtisii* Dyer ex King – in Peninsular Malaysia (Ishiyama *et al.*, 2008) and in Singapore (Kamiya *et al.*, 2011), and these species are in the same section, *Mutica*. A case of hybridisation was also observed in the genus *Dryobalanops*, between *D. aromatica* and *D. lanceolata*, in the sympatric habitat of Lambir Hills National Park. The putative hybrids grow in the middle of the habitats occupied by *D. aromatica* and *D. lanceolata* and showed intermediate leaf morphology (Itoh *et al.*, unpublished data). Hybridisation may occur where two closely related species come into contact during range expansion (Rieseberg *et al.*, 2007; Ishiyama *et al.*, 2008). A recent study has shown that introgression is likely to take place preferentially from the resident species towards the invading species (Currat *et al.*, 2008). This suggests that the hybridisation is the result of invasion by *D. beccarii* into *D. aromatica* territory in the central coastal Sarawak populations. However, population size change was not detected in any of the A7, B11 or B12 populations by the BOTTLENECK analysis (Table 2). The very low variation in the proportion of the clusters represented in these populations indicated by the STRUCTURE analysis suggests that the hybridisation was an old event, and populations are not under a wave of invasion. The lack of pure *D. aromatica* or *D. beccarii* individuals in these populations also supports this speculation. In another pair of sympatric populations in Gunung Panti

(A5 and B1), the two species are clearly differentiated, and no signs of hybridisation were observed.

The STRUCTURE analysis of *D. aromatica* alone showed that the populations were divided into two groups: Malay–Sumatra and Borneo (Fig. 3b). A strong barrier separated them (Fig. 4a). The proposed "savanna corridor" (Heaney, 1991), a large belt of grassland thought to have existed in the middle of the dry Sunda Shelf, could be an environmental barrier that prevents gene flow. The mixing of the Sumatra and Borneo clusters in the Peninsular Malaysian populations (Fig. 3b) suggests a past admixture of the clusters and that the savanna corridor was open at some point, allowing some gene flow. The separation of Borneo from the eastern edge of the Sunda Shelf around 8,000 years ago by rising seawater (Voris, 2000) could finally have blocked all gene flow. The secondary barrier that was indicated in the Malacca Strait probably corresponds to the opening of that strait about 4,000 to 3,000 years ago (Voris, 2000). The formation of the clusters, however, can be traced back to the earlier similar changes in land area in the Pleistocene. Similar groupings were also observed in *Shorea leprosula* revealed by cpDNA sequence variations (Ohtani *et al.*, 2013) and in *S. parvifolia* revealed by nuclear DNA sequence variations (Iwanaga *et al.*, 2012). The estimated splitting time is 2.6 to 0.7 MYA and 0.28 to 0.09 MYA for *S. leprosula* (Ohtani *et al.*, 2013) and *S. parvifolia* (Iwanaga *et al.*, 2012), respectively, setting the time of clustering long before the last glacial period. Our result supports the idea that present tropical rain forests in the Sundaic region are in a refugial state after experiencing expansion and overlap in the last glacial age (Cannon *et al.*, 2009; Raes *et al.*, 2014).

The STRUCTURE and BARRIER analyses suggested that individuals of *D. beccarii* were separated into four geographically distinct groups in Borneo: western Sarawak, central inland Sarawak, central coastal Sarawak and Sabah. There were strong barriers between Peninsular Malaysia and Borneo, and between Sabah and Sarawak. Another barrier separated the central coastal from the central inland Sarawak populations. A deep genetic split between Sabah and Sarawak has been observed in other species, such as the legume genus *Spatholobus* Hassk. (Ridder-Numan, 1998), the stone oak *Lithocarpus* Blume (Cannon & Manos, 2003) and *Macaranga* Thouars species (Bänfer *et al.*, 2006), as well as in fauna such as orang-utans (Warren *et al.*, 2001), rodents (Gorong *et al.*, 2004) and some birds, for example forktails (Moyle *et al.*, 2005). Because of the limited distance of gene flow both via insect-mediated pollen dispersal and gravity or gyration dispersal of seeds (Harata *et al.*, 2011; Kettle *et al.*, 2011; de Morais *et al.*, 2015), high mountain ridges and large rivers could be considered as effective barriers. The Crocker range, lying between B16 and the others, has probably acted as a barrier. Although the genetic structure in Sarawak is not as pronounced as the differentiation between the populations in Sabah and Sarawak, there is nevertheless some differentiation. Several large rivers in Sarawak, such as the Rajang River in the centre of the region, restrict distribution ranges and may have functioned as barriers against gene flow.

The clustering suggested by the STRUCTURE analysis is especially interesting in that the isolated population of *D. beccarii* in Peninsular Malaysia (B1) was a mixture of

the four Bornean clusters. Peninsular Malaysia is separated from Borneo by a distance of about 500 km. Over such a distance gene flow is unlikely; it is more plausible that the mixture occurred when *D. beccarii* expanded into Peninsular Malaysia during the last glacial period. We therefore suggest that B1 is a relic of the most recent range expansion of *D. beccarii*, during the last glacial period.

In this study we have found that historical events, most importantly those occurring after the last glacial period, have imprinted their effects deeply on the genetic structure of tropical rain forest trees. The differential pattern in genetic variation and genetic structure of these two closely related *Dryobalanops* species suggests that they may have been influenced mainly by two factors. One is the longer span of historical change traced back to the speciation of the genus *Dryobalanops* in the Pliocene. Today the distribution of *Dryobalanops* species is somewhat scattered and restricted, and absent altogether from Java; however, abundant fossils of *Dryobalanops* have been recorded in a broad range of Sundaland including Java and even extending to Burma (Slooten, 1932; Mandang & Kagemori, 2004). The current species distribution is therefore often considered as remnants, which in former times expanded in a much larger area (Slooten, 1932). The second is the difference caused by species-specific intrinsic factors such as pollen and seed dispersal abilities, light acquisition capacity, nutrient utilisation capacity and drought tolerance. Comparative studies of the fine genetic structure of these two species may be needed for further understanding of the generation of the large-scale genetic structure in *Dryobalanops*.

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