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Population Genetic Diversity in The Genus Dryobalanops Gaertn. f. (Dipterocarpaceae) Based on Nuclear Microsatellite Markers

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Abstract

The genus *Dryobalanops* Gaertn. f. is a small but important genus that contains seven species and is found in the Southeast Asian tropical rain forests. Seven polymorphic nuclear microsatellite markers were analyzed in 46 natural populations of six extant *Dryobalanops* species (N = 700 individuals) covering the distribution range in Western Malesia. The mean gene diversity at the species level ranged from 0.392 in *D. rappa* to 0.635 in *D. aromatica*. The F_{ST} value ranged from 0.156 in *D. keithii* to 0.283 in *D. beccarii* and all F_{ST} values were highly significantly greater than zero. These results suggest that gene flow between populations has been limited and intensive genetic drift has occurred in all of the species. Plants with narrowly distributed species such as *D. keithii* and *D. rappa*, which are endemic to Borneo, tend to have lower levels of genetic diversity compared with widespread species such as *D. aromatica*. STRUCTURE analysis revealed that at least two of the six species could be effectively discriminated each other. The results of this study will be helpful for building effective conservation schemes as well as for providing basic data for future provenance trials of Dryobalanops species.

Keywords: *Dryobalanops spp.; genetic diversity; genetic structure; microsatellite.*

1. Introduction

The genus *Dryobalanops* (Dipterocarpaceae), locally known as "Kapur", is a prominent genus with few species (a total of seven species). Three species, i.e. *D. aromatica* Gaertn. f., *D. beccarii* Dyer and *D. oblongifolia* Dyer have a broad distribution range over Borneo, Sumatra and the Malay Peninsula. *D. aromatica* occurs naturally in Sumatra (Angkola, Sibolga, Kelasan, Upper Singkil, Bengkalis, Siak and Mursala Island), the Malay Peninsula (Johor, Pahang, Selangor and Terengganu), Lingga Archipelago (Lingga and Singkep Island) and Borneo (Sabah, Sawarak and Brunei). Two subspecies are recognized for *D. oblongifolia*, i.e., subsp. *oblongifolia* Dyer and subsp. *occidentalis* P.S. Ashton, with the former occurring in Borneo (Sarawak, West Kalimantan, Central Kalimantan and East Kalimantan) and the latter in Sumatra (Bengkalis, Riau, Jambi and Palembang) and Malay Peninsula (Johor, Kelantan, Pahang, Perak, Selangor, and Terengganu). *D. beccarii* occurs in the Malay Peninsula (Johor) and Borneo (Sabah and Sarawak) [1,2]. The other four species, i.e. *D. lanceolata* Burck, *D. rappa* Becc., *D. keithii* Symington and *D. fusca* Slooten are endemic to Borneo. The timber of *Dryobalanops*, which differs in density and strength between species, is an important, moderately heavy and durable construction timber. This genus has excellent traits that allow rapid growth in plantations or through silvicultural manipulations [3]. The species also yields particular commodities categorized as non-wood forest products, such as oil and camphor crystals, which are obtained from the inner portion of the stems. Camphor especially obtained from *D. aromatica* attracted early Arab traders, and was worth more than its weight in gold [4].

Similar to other dipterocarps, the distribution area where *Dryobalanops* species once abundant has been shrinking since the last half century due to over-exploitation and changes in land use systems to agriculture and oil palm cultivation [5]. Increased use of forest resources and a shrinking forestland base threaten the sustainability of forest genetic resources and highlight the

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importance of conservation and sustainable management of these resources [6]. In fact, the lowland dipterocarp forests where these species often predominate, are ecologically important for nursing the rich biological diversity and for controlling the atmosphere-water conditions at a global level. Protecting these rain forests is one of the biggest environmental issues in this century. Obtaining information on population genetic parameters such as genetic diversity and structure of these tree species is a fundamental requirement for devising conservation schemes and future provenance trials.

Genetic diversity is essential to the long-term survival and evolution of tree species. It is the foundation of sustainability because it provides raw material for adaptation, evolution, and survival of species and individuals, especially under changed environmental and disease conditions [7]. Loss of diversity may cause a risk of extinction because of a lack of adaptive ability to a changing environment [8]. Genetic diversity is also the basis for tree improvement through selective breeding. Therefore, maintaining genetic diversity and conserving forest genetic resources are critical to forest sustainability, ecosystem stability and species' continued adaptation and survival [6]. In recent years, a variety of DNA-based techniques have been employed to study genetic diversity and structure in tree populations (e.g., Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) and Cleaved Amplified Polymorphic Sequences (CAPS)). Among the classes of molecular markers, microsatellites, or simple sequence repeats (SSRs), are considered to be ideal markers for genetic studies because they combine several suitable features for evaluating genetic variation such as co-dominance, multiallelism and high polymorphism. This allows precise discrimination, even among closely related individuals, and the possibility of efficient analysis by a rapid and simple polymerase chain reaction (PCR) assay [9, 10, 11]. Thus, the aims of our study are 1) to estimate the amount of genetic variation, 2) to examine the genetic structure and 3) to evaluate the discrimination abilities of microsatellite markers for the six *Dryobalanops* species.

2. Material and methods

2.1. Plant materials

A total of 700 individual leaf samples were collected from six species including two subspecies in the genus *Dryobalanops* (*D. aromatica*, *D. beccarii*, *D. lanceolata*, *D. rappa*, *D. keithii*, *D. oblongifolia* subsp. *oblongifolia* and *D. oblongifolia* subsp. *occidentalis*) across 46 natural populations in the Western Malesian region (Table 1). Samples of *D. fusca* were also collected, but not used for this study because of its polyploid nature. Plant materials were dried in the field with silica gel, stored in a freezer at –80°C and subsequently used for DNA extraction. Silica gel-dried leaves were ground to a fine powder using a Tissue Lyser II (QIAGEN Japan, Tokyo). Total genomic DNA was extracted from each sampled leaf using the modified CTAB method [12].

2.2. Microsatellite genotyping

Seven nuclear microsatellite loci (Dra187, Dra428, Dra426, Dra519, Dra266, Dra471 and Dra569), developed for *D. aromatica* [13], were used in this study. The forward primer of each marker was labeled with 6-FAM, VIC, NED, or PET phosphoramidite (Applied Bio systems Japan, Tokyo). A Type-it Microsatellite PCR kit (QIAGEN Japan, Tokyo) was used to amplify 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 about 40 ng of genomic DNA. An Applied Bio systems 2720 thermal cycler was used for the PCR amplification under the following conditions: initial denaturation at 95°C for 5 min, then 31 cycles of denaturation at 95°C for 30 s, annealing for 1 min 30 s and extension at 72°C for 30 s, followed by a final incubation at 60°C for 30 min. The annealing temperatures depended on the primer pairs and species used. Details of the markers, including the annealing temperatures used, are shown in Table 2. Fragment sizes were determined using an ABI PRISM^{TM} 310 Genetic Analyzer and visualized using Gene Mapper 3.0 software (Applied Bio systems Japan, Tokyo).

2.3. Data analysis

Basic statistics of genetic diversity, including number of alleles per locus (N_a), observed heterozygosity (H_O), expected heterozygosity (H_E) and fixation index (F), were calculated using Gen AlEx software version 6.41 [14]. F_{ST} was calculated to determine the level of population differentiation using ARLEQUIN Version 3.5.1.2 [15]. An analysis of molecular variance (AMOVA) was also carried out using ARLEQUIN version 3.5.1.2 to apportion the variance among species [15]. Genetic distances between populations were estimated using the Nei genetic distance, D [16], as implemented in Gen Alex version 6.41 [14]. The Bayesian model-based clustering method implemented in STRUCTURE software version 2.3.4 [17] using admixture model with LOCPRIOR option and allele frequency correlated model was further used to estimate the number of genetically homogeneous populations (K) and determine the genetic structure of the sampled populations. A burn-in of 20,000 steps followed by 100,000 steps of MCMC (Markov chain Monte Carlo) simulation with 8 replications each, for a range of K-values from 1 to 10 was performed on the entire data set. To determine the appropriate number of clusters (K), the statistic ΔK [18] was calculated based on the rate of change in the log probability of data between successive K-values using STRUCTURE HARVESTER [19].

3. Results and discussion

3.1. Cross-species amplification

All seven microsatellite primer pairs developed for *D. aromatica* were successfully amplified across all six *Dryobalanops* species. In a total of 700 individuals analyzed, 145 alleles were detected. The seven loci yielded only 16 alleles in *D. oblongifolia* subsp. *occidentalis*, followed by *D. keithii* with 23 alleles and *D. rappa* with 33 alleles. The other four species, *D. oblongifolia* subsp. *oblongifolia, D. beccarii, D. lanceolata* and *D. aromatica* had 55, 67, 68 and 110 alleles, respectively. The number of allele per locus ranged from 16 (Dra426) to 45 (Dra266). Microsatellites have several advantages for use in population studies, including their high mutation rate and distribution throughout the genome [20].

Table 1 Populations and geographic locations of six *Dryobalanops* species studied

Species	Population code	*N*	Location	Latitude	Longitude
D. aromatica	Daro1	6	Barus (Central Tapanuli, North Sumatra, Sumatra, Indonesia)	2° 04'09" N	98° 21' 28" E
	Daro2	51	Mursala Island (Central Tapanuli, North Sumatra, Sumatra, Indonesia)	1° 40' 23" N	98° 29' 48" E
	Daro3	13	Lingga Island (Lingga Archipelago, Riau Archipelago, Sumatra, Indonesia)	0° 09' 31" S	104° 38' 14" E
	Daro4	23	Kanching Forest Reserve (Selangor, Malay Peninsula, Malaysia)	3° 18' 15" N	101° 36' 41" E
	Daro5	20	Gunung Panti Forest Reserve (Johor, Malay Peninsula, Malaysia)	1° 49' 40" N	103° 52' 01" E
	Daro6	32	Similajau National Park (Bintulu, Sarawak, Borneo, Malaysia)	3° 27' 01" N	113° 16' 51" E
	Daro7	28	Lambir Hills National Park (Miri, Sarawak, Borneo, Malaysia)	4° 12' 47" N	114° 01' 48" E
	Daro8	27	Limbang (Limbang, Sarawak, Borneo, Malaysia)	4° 45' 16" N	114° 59' 27" E
D. beccarii	Dbec1	3	Gunung Panti Forest Reserve (Johor, Malay Peninsula, Malaysia)	1° 49' 40" N	103° 52' 01" E
	Dbec2	28	Gunung Gading National Park (Lundu, Sarawak, Borneo, Malaysia)	1° 41' 25" N	109° 50' 45" E
	Dbec3	15	Kubah National Park (Kuching, Sarawak, Borneo, Malaysia)	1° 36' 47" N	110° 11' 49" E
	Dbec4	7	Kuching (Kuching, Sarawak, Borneo, Malaysia)	1° 40' 41" N	110° 24' 53" E
	Dbec5	28	Bako National Park (Kuching, Sarawak, Borneo, Malaysia)	1° 43' 31" N	110° 27' 59" E
	Dbec6	6	Bukit Lingang (Sri Aman, Sarawak, Borneo, Malaysia)	1° 31' 51" N	111° 46' 46" E
	Dbec7	5	Lubok Antu (Sri Aman, Sarawak, Borneo, Malaysia)	1° 18' 02" N	111° 50' 48" E
	Dbec8	35	Batang Ai National Park (Sri Aman, Sarawak, Borneo, Malaysia)	1° 13' 19" N	111° 56' 47" E
	Dbec9	32	Bukit Tangii (Sibu, Sarawak, Borneo, Malaysia)	2° 36' 29" N	111° 57' 00" E
	Dbec10	15	Mukah Hill Protected Forest (Mukah, Sarawak, Borneo, Malaysia)	2° 28' 04" N	112° 37' 01" E
	Dbec11	10	Nyabau Forest Reserve (Bintulu, Sarawak, Borneo, Malaysia)	3° 14' 01" N	113° 06' 00" E
	Dbec12	13	Similajau National Park (Bintulu, Sarawak, Borneo, Malaysia)	3° 27' 01" N	113° 16' 51" E
	Dbec13	8	Bukit Tiban (Miri, Sarawak, Borneo, Malaysia)	3° 27' 29" N	113° 29' 52" E
	Dbec14	10	Sungai Asap (Kapit, Sarawak, Borneo, Malaysia)	3° 02' 16" N	113° 56' 02" E
	Dbec15	6	Bakun Dam (Bintulu, Sarawak, Borneo, Malaysia)	2° 45' 23" N	114° 03' 47" E
	Dbec16	14	Deramakot (Sandakan, Sabah, Borneo, Malaysia)	5° 21' 12" N	117° 24' 31" E
D. lanceolata	Dlan1	39	Chankol (Sri Aman, Sarawak, Borneo, Malaysia)	1° 14' 58" N	111° 31' 50" E
	Dlan2	4	Sungai Apah (Kapit, Sarawak, Borneo, Malaysia)	1° 46' 10" N	113° 03' 47" E
	Dlan3	15	Niah National Park (Miri, Serawak, Borneo, Malaysia)	3° 47' 53" N	113° 47' 12" E
	Dlan4	24	Sungai Asap (Kapit, Sarawak, Borneo, Malaysia)	3° 02' 16" N	113° 56' 02" E
	Dlan5	30	Lambir Hills National Park (Miri, Sarawak, Borneo, Malaysia)	4° 12' 47" N	114° 01' 48" E
	Dlan6	7	Bakun Dam (Bintulu, Sarawak, Borneo, Malaysia)	2° 45' 23" N	114° 03' 47" E
	Dlan7	4	Murum (Kapit, Sarawak, Borneo, Malaysia)	2° 38' 48" N	114° 21' 57" E
	Dlan8	8	Balikpapan (Balikpapan, East Kalimantan, Borneo, Indonesia)	1° 07' 16" S	116° 51' 04" E
	Dlan9	17	Deramakot (Sandakan, Sabah, Borneo, Malaysia)	5° 21' 12" N	117° 24' 31" E
	Dlan10	10	Sepilok (Sandakan, Sabah, Borneo, Malaysia)	5° 51' 54" N	117° 56' 56" E
D. rappa	Drap1	16	Sematan (Lundu, Sarawak, Borneo, Malaysia)	1° 48' 55" N	109° 45' 57" E
	Drap2	4	Chankol (Sri Aman, Sarawak, Borneo, Malaysia)	1° 14' 58" N	111° 31' 50" E
	Drap3	9	Bukit Lima Forest Park (Sibu, Serawak, Borneo, Malaysia)	2° 17' 00" N	111° 51' 59" E
	Drap4	4	Brunei Darussalam (Borneo, Brunei Darussalam)	4° 15' 50" N	114° 34' 36" E
D. keithii	Dkei1	14	Deramakot (Sandakan, Sabah, Borneo, Malaysia)	5° 21' 12" N	117° 24' 31" E
	Dkei2	18	Sepilok (Sandakan, Sabah, Borneo, Malaysia)	5° 51' 54" N	117° 56' 56" E
D. oblongifolia	Dobob1	5	Kubah National Park (Kuching, Sarawak, Borneo, Malaysia)	1° 36' 47" N	110° 11' 49" E
subsp. *oblongifolia*	Dobob2	4	Kuching (Kuching, Sarawak, Borneo, Malaysia)	1° 40' 41" N	110° 24' 53" E
	Dobob3	8	Bukit Gahar (Serian, Sarawak, Borneo, Malaysia)	1° 08' 29" N	110° 33' 23" E

	Dobob4	11	Mukah Hill Protected Forest (Mukah, Sarawak, Borneo, Malaysia)	2° 28' 04" N	112° 37' 01" E
	Dobob5	12	Nyabau Forest Reserve (Bintulu, Sarawak, Borneo, Malaysia)	3° 14' 01" N	113° 06' 00" E
D. oblongifolia subsp. *occidentalis*	Doboc1	2	Gunung Panti Forest Reserve (Johor, Malay Peninsula, Malaysia)	1° 49' 40" N	103° 52' 01" E

N number of sample

Table 2 Primer information for 7 microsatellite loci used in this study

Locus and dye	Primer sequence (5'-3')	Annealing Temperature (°C)					
		D. aromatica	*D. beccarii*	*D. lanceolata*	*D. rappa*	*D. keithii*	*D. oblongifolia*
Dra187 (FAM)	F: TCTCTCTTATCCAACTCTCTCA R: AGGGAACTAAAGCAGACATCAC	57	57	50	54	52	54
Dra428 (VIC)	F: CTATTGATGCCCTTATAGCTTT R: ACGAGCCTCTCTACTCTATAAT	57	57	50	54	52	54
Dra426 (NED)	F: CCAACGCTGCTCAAAGTTCGTG R: GCTGGCTGGCATAATATAATCC	57	57	50	54	52	54
Dra519 (PET)	F: TCAAGCCAGAAGAGATAGAGAC R: ATATTCCTTTCATATTATTGGG	57	57	50	54	52	54
Dra266 (FAM)	F: AGACTTAATAATGGAGGACGAG R: CCACAATTAGCCACCATCTTAC	54	54	55	48	49	53
Dra471 (VIC)	F: TCTCAGTCTCACAATCTATCCA R: TTTCTGTGTCATTTTAGCAACC	54	54	55	48	49	53
Dra569 (NED)	F: GTAAAACCAATACACGTACATA R: ATGGAAGTCATTTCATCTATTT	54	54	55	48	49	53

At the same time, however, foreign microsatellite loci may yield conflicting results; the amplification of microsatellites could fail or null alleles could occur when they are applied on closely or more distantly related different species [21, 22]. In this study, all seven microsatellite loci exhibited a large number of alleles per locus, suggesting that these loci will be of potential use in studying genetic diversity, population structure and investigating the reproductive strategy of these *Dryobalanops* species.

3.2. Genetic diversity

The genetic diversity parameters are shown in Table 3. Among the studied populations, the lowest mean number of alleles per locus was found in *D. oblongifolia* subsp. *occidentalis* (N_a = 2.29), followed by *D. rappa* (N_a =2.96). The highest allelic diversity was found in populations of *D. aromatica* (N_a = 6.63). The low level of mean number of alleles in *D. oblongifolia* subsp. *occidentalis* could be caused by the small sample size [23].

At the population level, observed heterozygosity (H_O) ranged from 0.238 (Dbec1) to 0.681 (Daro3). The highest expected heterozygosity was found in Daro5 (H_E = 0.729) and the lowest was found in Drap2 (H_E= 0.277). This result is consistent with genetic diversity at the species level, which showed that the mean observed heterozygosity (H_O) ranged from 0.378 in *D. beccarii* to 0.578 in *D. aromatica* and the mean expected heterozygosity was lowest in *D. rappa* (H_E= 0.392) and highest in *D. aromatica* (H_E= 0.635) (Table 3). The estimates of genetic diversity for the six *Dryobalanops* species in this study were comparable to those of other dipterocarp species studied using microsatellite, such as *Shorea leprosula* [24], which occurs in the Malay Peninsula, Sumatra and Borneo and *Shorea javanica* [25], which occurs in Sumatra. However, this study also showed that the levels of genetic diversity in *Dryobalanops* species were lower than *Shorea lumutensis* [26], which occurs in the Malay Peninsula. The mating system is the most important factor that shapes genetic diversity and its distribution at nuclear markers [27, 28]. The present study also confirmed that dipterocarps, like most other tropical trees, are able to avoid very low effective population sizes even if they occur at low density [29, 30].

The extent of a geographic range has been shown to be strongly associated with the level of genetic variation maintained within populations [31]. Plant species with widespread distributions such as *D. aromatica*, tend to harbor higher levels of genetic variation than species with more restricted ranges, such as *D. rappa*, which is endemic to Borneo [20, 32]. Rare and endemic species with small population sizes tend to have reduced genetic diversity due to bottlenecks, genetic drift and inbreeding [33, 34, 35]. *D. aromatica* is widely distributed and abundant in the Malay Peninsula, Sumatra and Borneo, whereas *D. rappa* only occurs in Borneo (Sabah, Sarawak and Brunei). Therefore, a high outcrossing rate, widespread distribution, and large population sizes may be the factors responsible for maintaining higher levels of genetic variation in *D. aromatica.*

3.3 Genetic structure and species relationship

Genetic differentiation within species was analyzed using the F_{ST} index (Table 4) for all seven loci and populations.

The largest F_{ST} value was observed in *D. beccarii* (0.283) and the smallest was in *D. keithii* (0.156) (Table 4). However, all F_{ST} values were significantly larger than zero at very high level. This shows that gene flow among populations has been limited and intensive genetic drift has proceeded in all of the species as a result. The levels of gene flow among populations via pollen and seed primarily determine genetic differentiation among populations [36]. Species with discontinuous, isolated populations can be expected to show increased levels of genetic differentiation.

Table 3 Genetic diversity values in six *Dryobalanops* species

Species	Population	Population code	*N*	N_a	N_e	H_O	H_E	*F*
D. aromatica	Barus	Daro1	6	4.00	2.75	0.548	0.569	0.117
	Mursala	Daro2	51	8.57	4.04	0.630	0.725	0.127
	Lingga	Daro3	13	7.14	3.71	0.681	0.689	0.007
	Kancing	Daro4	23	4.86	2.87	0.540	0.598	0.070
	Gunung Panti	Daro5	20	6.86	4.27	0.521	0.729	0.289
	Similajau	Daro6	32	5.29	2.62	0.616	0.571	-0.084
	Lambir	Daro7	28	7.86	3.38	0.587	0.623	0.032
	Limbang	Daro8	27	8.43	3.52	0.503	0.575	0.192
	Mean			**6.63**	**3.39**	**0.578**	**0.635**	**0.094**
D. beccarii	Gunung Panti	Dbec1	3	2.00	1.66	0.238	0.341	0.333
	Gunung Gading	Dbec2	28	3.29	1.75	0.357	0.353	0.045
	Kubah	Dbec3	15	3.00	1.84	0.457	0.415	-0.085
	Kuching	Dbec4	7	3.14	2.39	0.408	0.484	0.086
	Bako	Dbec5	28	4.14	2.06	0.291	0.392	0.232
	Bukit Lingang	Dbec6	6	2.71	1.62	0.286	0.323	0.084
	Lubok Antu	Dbec7	5	2.57	1.65	0.343	0.346	-0.042
	Batang Ai	Dbec8	35	4.29	2.13	0.453	0.447	-0.031
	Bukit Tangii	Dbec9	32	4.86	2.22	0.420	0.479	0.108
	Mukah Hill	Dbec10	15	4.57	2.39	0.381	0.436	0.080
	Nyabau	Dbec11	10	5.00	3.12	0.600	0.605	0.021
	Similajau	Dbec12	13	5.14	3.65	0.659	0.673	0.017
	Bukit Tiban	Dbec13	8	2.86	1.80	0.304	0.360	0.109
	Sungai Asap	Dbec14	10	3.14	2.16	0.329	0.415	0.153
	Bakun Dam	Dbec15	6	2.86	2.16	0.262	0.442	0.355
	Deramakot	Dbec16	14	3.29	1.90	0.265	0.369	0.150
	Mean			**3.55**	**2.16**	**0.378**	**0.430**	**0.098**
D. lanceolata	Chankol	Dlan1	39	6.29	3.64	0.462	0.688	0.324
	Sungai Apah	Dlan2	4	3.43	2.53	0.500	0.513	0.033
	Niah	Dlan3	15	4.43	2.58	0.543	0.493	-0.104
	Sungai Asap	Dlan4	24	4.00	2.26	0.464	0.460	-0.013
	Lambir	Dlan5	30	4.14	2.25	0.443	0.456	0.007
	Bakun Dam	Dlan6	7	2.43	1.80	0.429	0.375	-0.138
	Murum	Dlan7	4	2.14	1.93	0.286	0.362	0.123
	Balikpapan	Dlan8	8	2.86	2.14	0.286	0.379	0.200
	Deramakot	Dlan9	17	4.57	2.55	0.597	0.538	-0.122
	Sepilok	Dlan10	10	3.71	2.08	0.486	0.446	-0.077
	Mean			**3.80**	**2.38**	**0.449**	**0.471**	**0.017**
D. rappa	Sematan	Drap1	4	3.43	2.61	0.509	0.496	0.040
	Chankol	Drap2	4	2.43	1.94	0.286	0.277	-0.071
	Bukit Lima	Drap3	9	3.43	2.62	0.508	0.474	-0.104
	Brunei	Drap4	16	2.57	1.95	0.393	0.321	-0.208
	Mean			**2.96**	**2.28**	**0.424**	**0.392**	**-0.076**
D. keithii	Deramakot	Dkei1	14	3.28	2.19	0.459	0.469	0.087
	Sepilok	Dkei2	18	2.71	1.95	0.444	0.371	-0.021
	Mean			**3.00**	**2.07**	**0.452**	**0.420**	**0.033**
D. oblongifolia subsp. *oblongifolia*	Kubah	Dobob1	5	3.86	2.89	0.514	0.614	0.200
	Kuching	Dobob2	4	3.29	2.60	0.536	0.576	0.058

	Bukit Gahar	Dobob3	8	4.71	2.97	0.500	0.616	0.234
	Mukah Hill	Dobob4	11	4.43	2.93	0.481	0.582	0.168
	Nyabau	Dobob5	12	3.71	2.26	0.452	0.525	0.118
	Mean			**4.00**	**2.73**	**0.497**	**0.583**	**0.155**
D. oblongifolia subsp. *occidentalis*	Gunung Panti	Doboc1	2	**2.29**	**2.09**	**0.500**	**0.464**	**-0.022**

N Number of samples, N_a mean number of alleles, N_e mean number of effective alleles, H_O mean observed heterozygosity, H_E Nei's mean expected heterozygosity and *F* inbreeding coefficient

Generally, the presence of genetic structure has likely been influenced by geographic history. During the Pleistocene in SE Asia, sea levels fluctuated repeatedly, decreasing as low as 120 m below present level and occasionally rising above present levels [37]. At the last glacial maximum (LGM), about 20,000 years ago, the continental shelf dried and formed a large landmass called Sunda land [38]. Tree populations are thought to have had large population sizes and might be associated with greater levels of genetic variation. After the LGM, the sea level rose because of earth warming and Sunda land was subsequently separated into large islands, i.e., Sumatra, Borneo, Java and the Malay Peninsula. Tree populations might have been further isolated by the formation of geological structures such as mountains, rivers, basins and volcanoes. Such geographical structures might have served as phylogeographic barriers and limited gene flow via seed and pollen, and caused genetic differentiation as a result.

An AMOVA of the entire microsatellite data set (Table 5) revealed that 54.83% of the total molecular variance was observed within individulas, 18.92% was attributed to differences among species and 18.87% was attributed to differences among populations within species. The remainder (7.38%) was among individuals within populations. All components of molecular variance were highly significant (P < 0.0001). STRUCTURE analysis with prior information about geographical origin of populations showed that the highest likelihood value (Ln Pr*X*/*K*) occurred at *K* = 3, indicating that the investigated six species of *Dryobalanops* could be split into three genetically distinct groups. When *K* = 3, *D. aromatica*, *D. beccarii* and other four species in a group could be separated (cluster I, cluster II and cluster III, respectively in Fig. 1a). Subsequently, applying STRUCTURE analysis for the four species (excluding *D. aromatica* and *D. beccarii*), the Δ*K* values showed a clear single peak at *K* = 3. This showed that *D. rappa* and *D. keithii* in the same cluster (cluster III' in Fig. 1b) as well as *D. oblingifolia* subsp. *oblongifolia* and *D. oblongifolia* subsp. *occidentalis* in one cluster (cluster I' in Fig. 1b). Cluster I' also appeared in *D. lanceolata*. Cluster II' was found only in *D. lanceolata* (Fig. 1b). This pattern suggests that the microsatellite analyses performed in this study were successful in revealing significant genetic heterogeneity between the species and could assist to discriminate *Dryobalanops* species but not completely. In our previous study [39] we could show by using chloroplast DNA (cpDNA) variation that *D. keithii, D. lanceolata, D. rappa*, *D. oblongifolia* and *D. fusca* had species specific polymorphisms and could be distinguished each other, but *D. aromatica and D. becarrii* could not be distinguished because they shared common haplotypes. This indicates that combining the cp DNA polymorphism with present microsatallite result we could effectively discriminate these all six species at DNA level.

Table 4 Genetic differentiation among populations in six *Dryobalanops* species

Species	No. Population	F_{ST}	*P* value
D. aromatica	8	0.194	<0.0001
D. beccarii	16	0.283	<0.0001
D. lanceolata	10	0.246	<0.0001
D. rappa	4	0.159	<0.0001
D. keithii	2	0.156	<0.0001
D. oblongifolia subsp. *oblongifolia*	5	0.194	<0.0001

Table 5 Summary of the analysis of molecular variance (AMOVA) in six *Dryobalanops* species

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among species	6	720.38	0.58	18.92***
Among populations within species	39	754.17	0.57	18.87***
Among individuals within populations	654	1384.88	0.22	7.38***
Within individuals	700	1168.00	1.67	54.83***
Total		4027.43	3.04	

df degree of freedom; *** *P* < 0.0001

4. Conclusions

This study revealed that all *Dryobalanops* species are highly genetically structured, which may be a consequence of limited gene flow and intensive genetic drift in the populations. These characteristics of genetic structure have significant implications for conservation strategies. Firstly, *in situ* conservation strategies should be adopted to protect and restore all existing populations of each *Dryobalanops* species. Considering the significant genetic differentiation among populations in each species, as many of the populations as possible should be preserved and the unit must be large enough to ensure that total variation is maintained. Anthropogenic destruction should be prevented to allow these species to propagate and increase in size through natural regeneration. Secondly, when *ex situ* conservation is being carried out, the sample size of populations should be large enough to ensure the genetic diversity within each population. In order to increase the gene flow and genetic diversity especially for rare endemic species, germplasm resources should be established with seeds from multiple sources.

The pattern of genetic structure revealed in this study also will be useful for silvicultural purpose in these highly potential species. To establish a provenance trial series, seeds could be collected from the areas of the differentiated genetic background, which can be indicated by mapping the structured population groups on geographic map. Differential growth performance and other physiological characteristics could be attributed to differential genetic backgrounds.

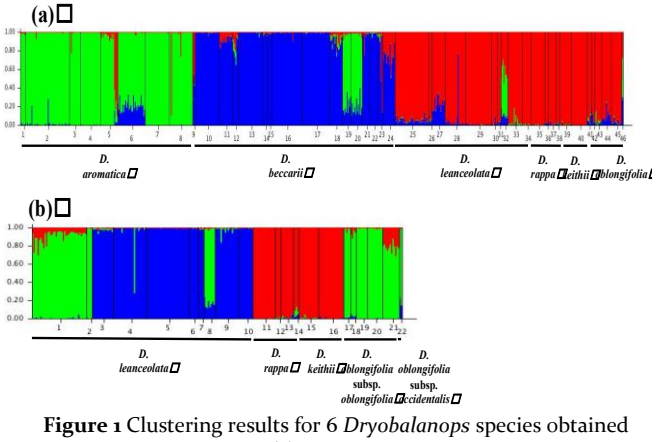

Figure 1 Clustering results for 6 *Dryobalanops* species obtained from STRUCTURE analysis. (a) Estimated genetic structure for K = 3 with 46 populations of 6 *Dryobalanops* species. The cluster in green is designated cluster I, cluster in blue is designated cluster II and the cluster in red is designated cluster III. (b) Estimated genetic structure for K = 3 for 4 *Dryobalanops* species with 22 populations. The cluster in green is designated cluster I', cluster in blue is designated cluster II', and cluster in red is designated cluster III'

Glossary

Gene flow: the movement of genes between previously separate populations, which is mediated by seed migration or pollen dispersal in plants.

Genetic differentiation: the accumulation of differences in allelic frequencies between completely or partially isolated populations due to evolutionary forces such as selection or genetic drift. The extent of genetic differentiation is measured by Wright's fixation index, F_{ST}.

Genetic diversity: the richness of genetic variation in a population or species, which is commonly evaluated by average heterozygosity or number of alleles at certain loci.

Genetic structure: the state of a population being differentiated into several subpopulations with significant value of F_{ST}.

Genetic variation: any genomic or DNA variation in a population or species as a result of mutations. The situation of a locus containing high level of genetic variation is called polymorphism.

Microsatellite: sequences of DNA made up of tandemly repeated motifs, from one to six bases in length, which are arranged head-to-tail generally without interruption.

PCR (polymerase chain reaction): a technique for amplifying DNA sequences in vitro by separating the DNA into two strands and incubating it with oligonucleotide primers and DNA polymerase.

Primer: a short oligonucleotide complementary to target DNA and acts as the leader for DNA extension.

Random genetic drift: random fluctuations in the numbers of gene variants in a population. Genetic drift takes place when the occurrence of variant forms of a gene, called alleles, increases and decreases by chance over time.

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