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DETECTION OF GENETIC STRUCTURE AMONG RAMIN (*Gonystylus bancanus* (Miq.) Kurz) POPULATIONS IN PENINSULAR MALAYSIA USING A RAPID DNA FINGERPRINTING TECHNIQUE

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ABSTRACT

Here, we demonstrate the power of DNA fingerprints, generated by Direct Amplification of Length Polymorphism (DALP) analysis to understand the genetic structure and variation of five natural populations of ramin (*Gonystylus bancanus*) in Peninsular Malaysia. Six primer sets generated 309 distinct fragments ranging from 100-1200 bp among 156 individuals. These loci were highly polymorphic (83%) and no two individuals shared the exact same fingerprint. Most of the genetic variation was found within populations (only 13% among population variance) although cluster analysis indicated that most individuals could be correctly assigned to their original population. Genetic similarity among populations was not correlated with geographic distance, possibly due to environmental differences among the sampled peat forests. The DNA fingerprinting approach presented here would be highly effective for tracking individual logs from forest to market and detecting illegal smuggling. These DNA fingerprints could also be applied for correct deployment of seedlings in enrichment planting schemes to avoid breakdown of co-adapted complexes for survival and growth in the extreme peat swamp environment.

Key words: Genetic structure, genetic variation, genetic relatedness, DALP analysis, *Gonystylus bancanus* (ramin), conservation, sustainable management

INTRODUCTION

While equatorial tropical rainforests are home to the majority of Earth's biodiversity, we know relatively little about the geographic patterns of genetic diversity in these forests. A recent study (Vamosi *et al.*, 2008) highlights the extinction risk for many of the plants living in these forests, further highlighting the urgency for rapid generation of knowledge about endangered species. Additionally, the harvests of economically valuable natural resources from tropical rainforests rely primarily on 'wild' populations, which are often undergoing their first round of extraction. The response and resiliency of these 'wild' populations after extraction are dependent on their life history strategies and level of heterogeneity (Wickneswari *et al.*, 1999, Wickneswari & Boyle 2000). Limited studies on the impact of commercial logging on breeding system and genetic diversity of tropical rain-forest species have been carried out (Kitamura *et al.*, 1994, Lee *et al.*, 2002a,b, Wickneswari *et al.*, 2004). Hence, the sustainable management and regulation of these 'wild' products can be extremely difficult. Here, we examine the use of a universal molecular marker (DALP – Desmarais *et al.*, 1998) for rapidly generating informative DNA fingerprints in an endangered species of tropical timber and its spatial genetic variation. We also discuss the possible use of our results for the regulation of its international trade.

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Gonystylus bancanus (commonly called 'ramin') is a popular, creamy yellow light hardwood. Largely restricted to the lowland peat swamp and mixed swamp forest of Indonesia and Malaysia, ramin is the most vulnerable timber species in Southeast Asia. Remaining populations are becoming increasingly fragmented and scattered due to deforestation (Lim *et al.*, 2004). Due to over exploitation, all species in the genus were upgraded in 2004 by CITES from Appendix III to II. Ramin also has been classified as a species with high risk of extinction by the International Union for Conservation of Nature and Natural Resources (IUCN) (Lim *et al.*, 2004). According to the Malaysian Timber Industry Board (MTIB), the export of ramin decreased from 44,139 cubic meters in 2003 to about 12,899 cubic meters in 2005, with only 2,694 cubic meters remaining in 2007. This decline indicates the probable rapid decline of its easily accessible natural stocks. *Gonystylus* is distributed throughout Southeast Asia, including the Nicobar, Solomon and Fiji Islands (Airy Shaw 1953). The centre of *Gonystylus* diversity is found on the island of Borneo. Most of the species are found in hill forests and are generally rare (e.g. *G. othmanii*) (Tawan 1999). Few are economically valuable.

In Malaysia, the peat swamp forest extends for 1.54 million ha out of which 0.30 million ha are in Peninsular Malaysia, 1.2 million ha in Sarawak and 0.12 million ha in Sabah. This represents about 7.9% of the total forested area in Malaysia (FRIM-UNDP/ GEF 2004). The floristic composition of peat swamp forests is often substantially different than other lowland habitats (Cannon & Leighton 2004) and its conversion and degradation can lead to the release of significant amounts of carbon into the atmosphere (Page *et al.*, 2002) and substantial change in hydrological patterns (Wösten *et al.*, 2008). Peatlands are generally quite vulnerable to disturbance and become easily degraded. In Peninsular Malaysia, ramin can be found mainly in the central and the southern part of the peninsula where it occurs in low-lying plains behind the coast. The main source of this vulnerable timber is the Peat Swamp Forest in South East Pahang (FRIM-UNDP/ GEF PSF 2004). Five natural populations were selected for this study, including both the eastern and western parts of the peninsula and the northern and southern extent of its range.

Direct Amplification of Length Polymorphism (DALP) combines the advantages of high-resolution fingerprint technique with the additional possibility to sequence each new marker. The DALP method involves the use of random PCR primers to generate multi-band genome DNA patterns and enable sequencing of defined polymorphic bands for the study of distinct loci (Desmarais *et al.*, 1998) and does not require any prior knowledge of the study organism. If successful, this technique could be used to quickly generate DNA fingerprinting tools for a wide range of tropical species. For CITES species like ramin, these fingerprinting tools could be used for the dual purposes of taxonomic identification and the determination of the geographic origin of individual logs (Deguilloux *et al.*, 2003). These tools could prevent illegal smuggling.

Our objectives were to determine the level of genetic variation among populations and individuals detectable using the DALP technique and the potential for distinguishing individuals and their geographic origin.

MATERIALS AND METHODS

Plants materials, DNA extraction and DALP fingerprinting

Five natural populations of ramin from four forest reserves (i.e. Belara F.R., Pekan F.R., Nenasi F.R. and Air Hitam F.R.) were chosen from three states in Peninsular Malaysia. The populations from Pekan F.R consist of two different populations, Pekan 1 and Pekan 2 (Figure 1). Pekan 1 is located to the east of Pekan F.R. compared to Pekan 2. Samples of ramin from Pekan 1 were sampled at conservation forest area (compartment 46) and virgin forest (compartment 101). The distance between the compartments is about 4 km. However Pekan 2 (compartment 139) is located very close to an oil palm plantation and an aborigine settlement. The distance between Pekan 1 and Pekan 2 is about 21 to 46 km. The number of samples collected from each population of ramin is summarized in Table 1. Whole genomic DNA was extracted from the inner bark of ramin by using modified method of cetyl trimethyl ammonium bromide (CTAB) (Doyle & Doyle 1987). After that the extracted DNA were purified with the DNeasy Plant Mini Kit (Qiagen, German) under the protocol DNA Preparation from step one until step twelve. The concentration and quality of DNA were determined by using agarose gel (0.8%) electrophoresis in 1 x TBE buffer.

A total of 156 ramin samples from five populations in Peninsular Malaysia were investigated using Direct Amplification of Length Polymorphism (DALP) analysis. For this purpose a set of oligonucleotides (hereafter called 'selective') was designed, all sharing the same 5' core sequence of the universal M13 sequencing primer. The selective primers were DALP221, DALP231, DALP232, DALP233, DALP234, DALP235, DALP241 and DALP242. The selective primers were combined with one of the common M13 reverse primers which is the 'reverse' primer, DALPR. Table 2 indicates the sequences of the selective primers and DALPR. In combination with the reverse primer,

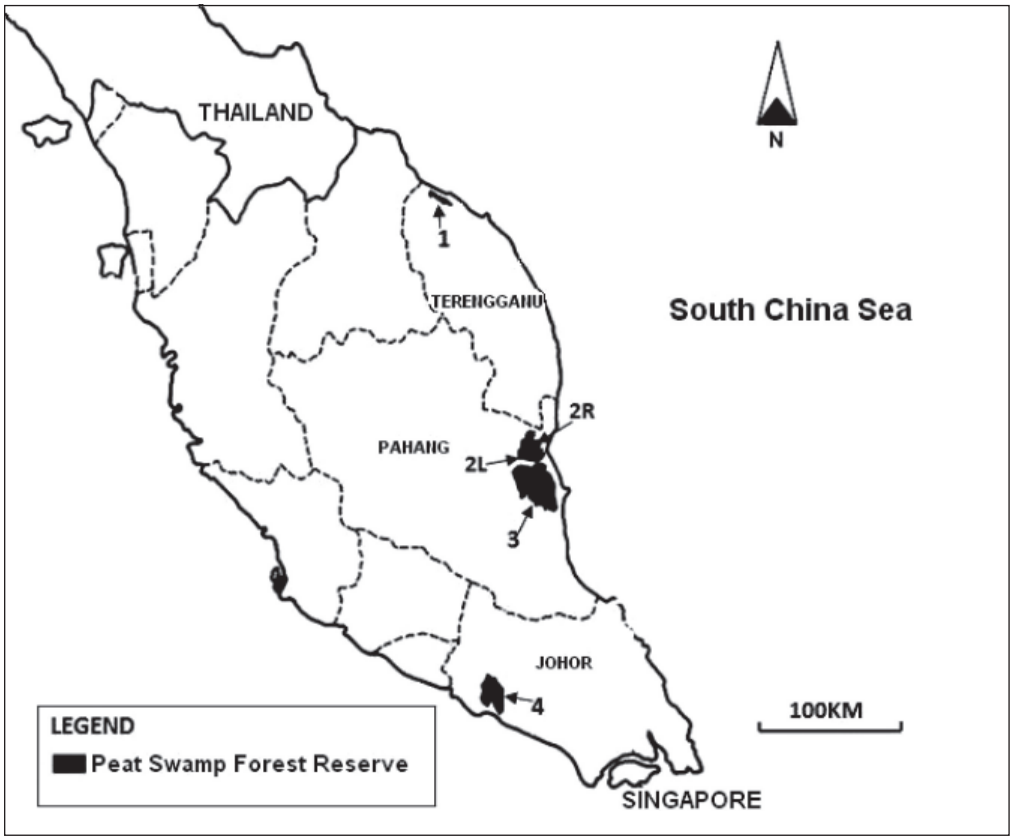

Fig. 1. Locations of the five populations of *G. bancanus* from four forest reserves in Peninsular Malaysia. 1. Belara F.R., 2. Pekan F.R. (2R = Pekan 1, 2L = Pekan 2), 3. Nenasi F.R., 4. Air Hitam Utara F.R.

Table 1. Five populations of *G. bancanus* in Peninsular Malaysia

States	Forest	Population	No. of samples
Pahang	Pekan FR	Pekan 1 (R)	38
	Pekan FR	Pekan 2 (L)	24
	Nenasi FR	Nenasi (N)	32
Johor	Air Hitam Utara FR	Air Hitam (A)	30
Terengganu	Belara Forest	Belara (B)	32

Table 2. The sequences of the DALP selective primers and DALPR

Primer	Sequences (5' to 3')
DALP221	GTTTTCCCAGTCACGACGC
DALP231	GTTTTCCCAGTCACGACAGC
DALP232	GTTTTCCCAGTCACGACGAC
DALP233	GTTTTCCCAGTCACGACACG
DALP234	GTTTTCCCAGTCACGACCAG
DALP235	GTTTTCCCAGTCACGACCAC
DALP241	GTTTTCCCAGTCACGACTCAG
DALP242	GTTTTCCCAGTCACGACCTAG
DALPR	TTTCACACAGGAAACAGCTATGAC

all the selective primers produced specific multi-banded patterns where inter-individual length variations can be detected. The PCR reactions were carried out in a final volume of 20 µl containing 2 µl DNA, 5 pmole each primer, 0.5 U *Taq* DNA polymerase, 2 mM $MgCl_2$, 0.2 mM dNTPs and 1 x reaction buffer. The PCR program consisted of pre-denaturation at 94ºC for 4 min, followed by 40 cycles of 94ºC for 30 s, 48 to 53ºC for 45 s, and 72ºC for 90 s, then a final extension at 72ºC for 5 min. Amplified DNA fragments were separated according to their length in denaturing polyacrylamide gel with 7% urea (10 x TBE [108g Tris base, 55g boric acid, 0.5 M EDTA (pH 8.0)], 30% polyacrylamide solution, ddH_2O) for 4 hours at 250 Volt. The PCR reactions were repeated three times for each individual.

Data analysis

The banding pattern for each individual was scored as a matrix of presence (1) or absence (0) for each DALP marker. Only reproducible banding patterns were scored, i.e. consistent results for two of the three replicate amplifications. Band sizes and alignments were determined using software, GelCompar Version 4.1 (Applied Maths Kortrijk, Belgium). The total proportion of polymorphic bands was estimated as

the number of loci at which the most common allele had a relative frequency of less than 0.95.

The genetic variation was quantified using Shannon Diversity Index, $H_0 = - \Sigma p_i \log_2 p_i$, where p_i is the frequency of phenotype i (King & Schaal 1989). H_0 can be calculated and compared for different populations. If $H_{pop} = 1/n\ (\Sigma H_0)$ is the average diversity over the n different populations, and $H_{sp} = - \Sigma p \log_2 p$ is the diversity calculated from the phenotypic frequencies p in all the populations considered together, then the proportion of diversity present within populations, H_{pop}/H_{sp}, can be compared with that between populations, $(H_{sp} - H_{pop})/H_{sp}$. Genetic distances (GD) between all pairs of individuals were calculated as $GD = 1 - S_{xy}$ according to the method developed by Nei and Li (1979)

$$S_{xy} = 2n_{xy} / (n_x + n_y)$$

where S_{xy} = the number of bands shared by individual x and individual y, n_x = the number of bands in individual x and n_y = the number of bands in individual y. Genetic distance among individuals and the corresponding Neighbor Joining tree were generated using PAUP 4.0 Beta Version (Swofford 1993).

RESULTS

Out of 8 selective primers used, 6 successfully yielded good amplification and consistent banding patterns, namely DALP221, DALP231, DALP232, DALP233, DALP234 and DALP235. Amplification of these minisatellite markers successfully detected 309 fragments with sizes ranging from 100 to 1200 bp where 83% (256 fragments) were reproducible. The six primers varied considerably in the number of observed polymorphic loci (Table 3). DALP235 exhibited the highest percentage (94.1%) of the polymorphic loci while DALP233 the lowest percentage (73.5%). The comparison between the five populations showed that the Nenasi population was the most polymorphic (86.8%) and the Belara population was the least polymorphic (76.1%).

Each DALP primer generated multi-locus patterns for the 156 individuals assayed. For primer, bands with the same migration distance were considered to be homologous (Desmarais *et al.,* 1998). Genetic variation within populations varied considerably amongst the different primers (Table 4). Overall the Nenasi population exhibited relatively the highest levels of variability within population (13.173) whereas the Belara population

Table 3. The numbers and percentages of polymorphic loci detected with six DALP primers for five populations of *G. bancanus*

Primers	No. of polymorphic loci for each primer (*)	Percentage of polymorphic loci	Number of polymorphic loci (*)				
			Pekan 1	Pekan 2	Nenasi	A. Hitam	Belara
DALP221	37 (50)	74	27 (39)	18 (23)	29 (37)	32 (41)	32 (41)
DALP231	53 (58)	91.4	48 (57)	47 (57)	50 (56)	43 (54)	42 (54)
DALP232	54 (70)	77.1	48 (67)	43 (61)	57 (66)	40 (59)	38 (59)
DALP233	36 (49)	73.5	28 (38)	35 (40)	38 (47)	26 (34)	25 (36)
DALP234	28 (31)	90.3	26 (30)	30 (31)	30 (30)	28 (31)	24 (31)
DALP235	48 (51)	94.1	48 (51)	42 (49)	45 (51)	48 (51)	46 (51)
Total	256 (309)	–	–	–	–	–	–
Average percentage of polymorphic loci	–	82.8	79.8	82.4	86.8	80.4	76.1

* The total number of loci.

Table 4. Estimates of Shannon diversity index (H_0) for five populations of *G. bancanus*

Primers	Populations				
	Pekan 1	Pekan 2	Nenasi	A. Hitam	Belara
DALP221	8.654	5.744	8.528	9.118	8.949
DALP231	16.056	15.163	16.094	13.517	13.497
DALP232	15.863	15.135	17.69	13.642	14.401
DALP233	9.52	10.632	12.748	8.658	9.122
DALP234	8.444	8.561	9.43	7.88	6.932
DALP234	15.254	13.27	14.55	15.511	15.354
Average	12.299	11.418	13.173	11.388	11.376

exhibited the lowest levels of variability within population (11.376). However, the mean H_o among populations was not significant but H_o for each primer was significant at $p \leq 0.05$.

Shannon Diversity Index of phenotypic frequencies were used to partition the diversity. The proportion of within population genetic diversity (H_{pop}/H_{sp}), as estimated by the Shannon Diversity Index of phenotypic frequencies was considerably greater than among population diversity ($H_{sp} - H_{pop}/H_{sp}$) (Table 5). On average, the genetic differentiation among populations was only 13%, indicating that most variation occured within populations. DALP232 detected the most (15.346) and DALP221 detected the least variability within population. DALP221 detected the highest genetic variation among populations (0.404) whereas DALP231 detected the least genetic variation among populations (0.049).

Eight main clusters (A, B, C, D, E, F, G and H) were observed in the Neighbor Joining tree (Fig. 2). Two clusters were comprised of a single population: Cluster B (Pekan 1, 11 inds.) and Cluster G (Nenasi, 20 inds.). Clusters C and D were primarily comprised

Table 5. Partitioning of genetic diversity within and between populations of *G. bancanus* for six DALP primers

Primers	H_{pop}	H_{sp}	H_{pop}/H_{sp}	$(H_{sp} - H_{pop})/H_{sp}$
DALP221	8.199	13.750	0.596	0.404
DALP231	14.865	16.443	0.904	0.096
DALP232	15.346	16.152	0.950	0.050
DALP233	10.136	11.990	0.845	0.155
DALP234	8.249	8.701	0.948	0.052
DALP235	14.788	15.543	0.951	0.049
Average	11.931	13.763	0.866	0.134

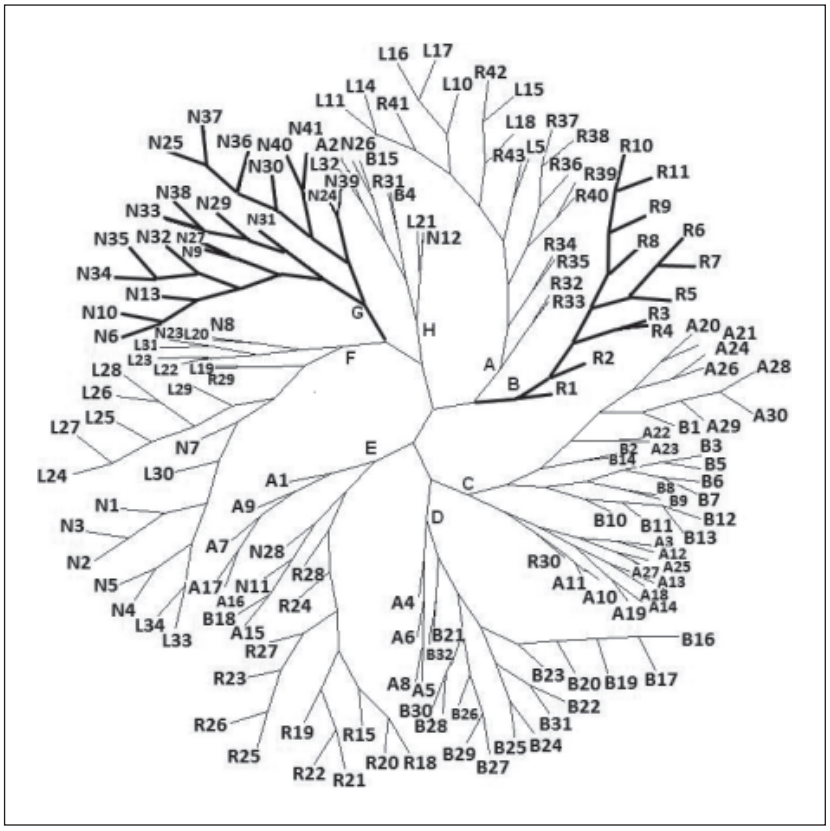

Fig. 2. Unrooted tree based on Neighbor Joining clustering for the 156 individuals of *G. bancanus* from five populations in Peninsular Malaysia. Individual labels are preceded by population identifiers in the following manner: Pekan 1 (R), Pekan 2 (L), Nenasi (N), Belara (B) and Air Hitam (A).

of individuals from only two populations (Belara, 29 inds. and Air Hitam, 23 inds., with one individual from Pekan1) and the populations were generally well-segregated. The remaining clusters were comprised of mixtures of individuals from different populations, although individuals from the same population were generally distinct. Only Cluster F (Pekan 2, 14 inds.; Nenasi, 8 inds.; and Pekan 1, 1 ind.) was closely related to Cluster G. Cluster E contained a combination of individuals from four populations (Pekan 1, 12 inds.; Air Hitam, 6 inds.; Nenasi, 2 inds.; and Belara, 1 ind.) and Cluster H contained individuals from five populations (Nenasi, 2 inds.; Pekan 2, 2 inds.; Belara, 2 inds.; Pekan 1, 1 ind.; and Air Hitam, 1 ind.).

DISCUSSION

In this investigaton, the DALP technique effectively generated detailed DNA fingerprints without any preliminary development of genetic resources specific to ramin. A large number of reproducible fragments were generated by a small set of DALP primers and the majority of these fragments were polymorphic and the populations were largely cohesive and identifiable. In a comparison among different marker systems, Roy *et al.* (2008) demonstrated that results from a DALP fragment analysis were congruent with microsatellite and DNA sequence data. The data indicates high gene flow and no evidence for host-specificity. Similar to our study, the DALP fragment analysis was used for the first time on a fungal model and produced easy and fast polymorphic patterns of dominant markers. DALPs provided useful additional tools to distinguish genetic structure and gene flow at various levels of relatedness among individuals (Desmarais *et al.,* 1998; Langar *et al.,* 2003; Wang *et al.,* 2003).

The low level of differentation among populations observed for our study subject which is an endangered tropical timber species agrees with results from genetic studies of other tropical trees. The genetic variation within and among nine populations of *Calycophyllum spruceanum* was analyzed and most variation resided within populations (91%), although variation among populations was highly significant ($P < 0.001$) (Russell *et al.,* 1999). Cao *et al.* (2006) revealed that most genetic variation resided within populations; 70.2% for *Shorea leprosula* and 66.2% for *S. parvifolia*. Hamrick (1990) had reported that most genetic variation for woody perennial plants is within populations. For the breeders, the high genetic variability present in the populations is important in improvement programmes. For conservation of genetic diversity, i.e establishment of germplasm banks, the results indicate that a fairly large sample is necessary to represent the variability in a species (Gauer & Cavalli-Molina 2000).

The cluster analysis indicated that many individuals could be assigned to their original populations, for example clusters B and G, each comprised of a single population. The cluster analysis also revealed that Pekan 1, Pekan 2 and Nenasi were genetically similar to the Belara and Air Hitam populations, which may be due to their close geographic proximity in the Malaysian state of Pahang. Nevertheless, the three populations occurred in different clusters because the ecological background of each population was different. Pekan 1 population and Nenasi population are located in virgin forest and relatively undisturbed compared to Pekan 2 population.

The DNA fingerprinting approach determined that genetic similarity among populations was not always correlated with geographic distance, possibly due to environmental differences among the sampled peat forests. Although Air Hitam and Belara are geographically distant, both populations were closely related (Cluster C). This unique condition could be explained by clinal variation due to ecological or edaphic similarities between Belara population (Terengganu) and Air Hitam (Johor) in comparison to the Pahang populations. As suspected by Anderson (1961), water logging at the periphery of a swamp and periodic drought in the center of a swamp were major factors determining local ecological conditions and the distribution of complex organic compounds and concentrations of major and trace minerals may vary among and within peat swamps. Both the Belara and Air Hitam populations are water logged only in the monsoon season while the Pahang populations are water logged throughout the year. This implies that the Belara and Air Hitam populations may have undergone adaptation to a periodic dry season as reflected by their genetic similarities and the resultant divergence from the Pahang populations.

Alternatively, individuals in the Belara and Air Hitam populations may have originated from different ancestral areas than the Pahang populations. The Air Hitam population, because of its location on the western coast of the peninsula, may have originated from Sumatra, whereas the Belara population, on the northern of peninsula, may have originated from the part of southern Thailand. Furthermore, Thailand and Sumatra may have at one time joined into one continent that had broken apart in the process known as continental drift. Therefore, Air Hitam and Belara populations are closely related whereas the Pahang populations, on the eastern side

of the central mountain range, may have originated from a different ancestral population. However, the information available is not enough to explain and make any conclusion on these populations. This would require further study involving sampling of all populations of ramin in Southeast Asia to provide better insight of differences observed in Peninsular Malaysia. As reported by Lim *et al.* (2004), the *Gonystylus* genus is distributed throughout Southeast Asia, including the Nicobar, Solomon and Fiji Islands whilst Stibig *et al.* (2002) illustrated the range of the genus *Gonystylus* in Sumatra, the Malay Peninsula (including Peninsular Malaysia and parts of southern Thailand) and Borneo.

We can conclude from this study that Pekan 1, Pekan 2 and Nenasi be treated as an intact and continuous population with Belara, Pekan 1, Pekan 2, Nenasi and Air Hitam as a greater but not continuous population of ramin as reflected by their positions in the dendrogram based on genetic similarities (Figure 2).

CONCLUSION

Here, we demonstrated that the DALP fingerprinting technique is an effective and cheap way of obtaining highly detailed and powerful genetic markers within a single species of endangered tropical tree. Most individuals possessed a unique DNA fingerprint and the geographic origin of many of these individuals could be reliably identified using simple statistical analyses. Given the many challenges and lack of bioinformatic resources for forest geneticists working in Southeast Asia on 'wild' but economically important species, our study illustrates that a large number of meaningful markers can be obtained without a great deal of expensive preliminary genetic work. These DALP fingerprints also provide the advantage of many more informative bands, which can be directly sequenced, providing even further insight and opportunity into the genetic investigation of the target species. Besides as an alternative for tracking the geographic origin of species, the sets of primers can also be used as a tool to determine the most suitable seedlings for enrichment planting studies which is essential for devising optimum management strategies for their sustainable utilization and conservation.

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