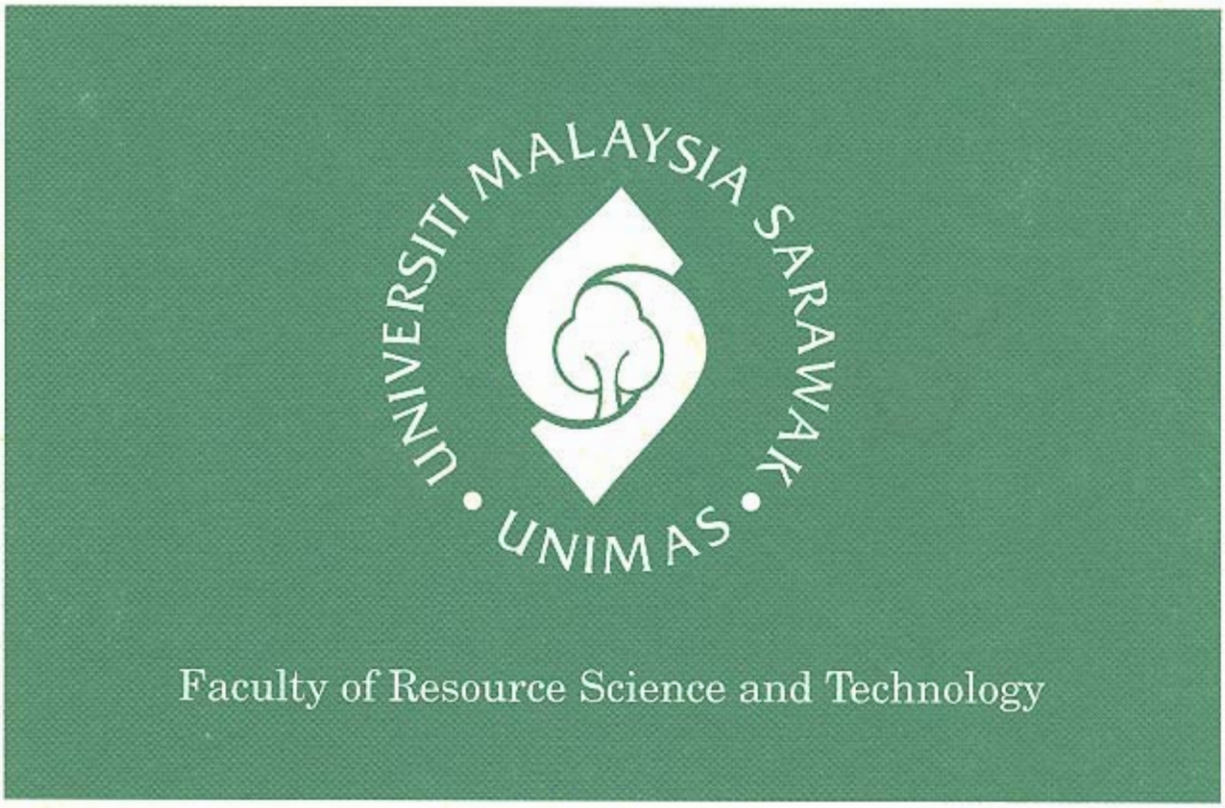

DNA FINGERPRINTING OF *CRYPTOCORYNE PALLIDINERVIA* ENGLER ACCESSIONS USING POLYMERASE CHAIN REACTION WITH M13 UNIVERSAL PRIMER

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This project is submitted in partial fulfilment of
the requirements for the degree of Bachelor of Science with Honours
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Faculty of Resource Science and Technology
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ABSTRACT

DNA fingerprinting of *Cryptocoryne pallidinervia* Engler accessions was described by Polymerase Chain Reaction (PCR). Genomic DNA of 18 accessions were amplified with the M13 universal primer (5'-TTATGAAACGACGGCCAGT-3'). A total of 18 distinctive PCR patterns were obtained which composed of 3 to 20 bands with the size ranging from 500bp to 3kb. The PCR profile was further analyzed to establish genetic relationship between the accessions through the construction of dendrogram. Cluster analysis of genetic relatedness tended to group *C. pallidinervia* accessions into four different major clusters. All big leaves accessions were grouped together whereas the small leaves accessions can be found in all clusters. Besides that, Bintulu accessions were highly different among the others since they grouped together in different clusters. Based on these results, it suggests that the PCR method with M13 universal primer is a rapid and reliable method to study genetic relatedness of *C. pallidinervia* accessions from different locations.

Keywords : DNA fingerprinting, M13 universal primer, Polymerase Chain Reaction, *Cryptocoryne pallidinervia.*

ABSTRAK

Pengecapjarian DNA bagi Cryptocoryne pallidinervia Engler telah dihuraikan oleh Polymerase Chain Reaction (PCR). DNA genomik bagi 18 aksesi telah diamplifikasi dengan menggunakan M13 universal primer (5'-TTATGAAACGACGGCCAGT-3'). Sebanyak 18 corak penjaluran PCR yang jelas diperolehi dan terdiri daripada 3 hingga 20 jalur dengan saiz DNA dari julat 500bp hingga 3kb. Profil PCR terus dianalisis untuk mencari hubungan genetik antara aksesi melalui pembentukan dendrogram. Analisis kelompok bagi hubungan genetik telah mengumpulkan C. pallidinervia kepada empat kelompok utama yang berbeza. Semua aksesi daun bersaiz besar telah dikumpulkan bersama manakala aksesi daun bersaiz kecil boleh dijumpai dalam semua kelompok Selain itu, aksesi Bintulu menunjukkan perbezaan yang paling tinggi berbanding aksesi lain kerana ia dikumpulkan dalam kelompok yang berbeza. Berdasarkan keputusan ini, didapati bahawa kaedah PCR dengan menggunakan M13 universal primer merupakan satu kaedah yang cepat dan berkesan untuk mengkaji hubungan genetik C. pallidinervia dari lokasi yang berbeza.

Kata kunci : Pengecapjarian DNA, M13 universal primer, Polymerase Chain Reaction, Cryptocoryne pallidinervia.

1.0 Introduction

Cryptocoryne has lately been given some attention because of its potential value as aquarium plants (Rataj & Horeman, 1977). It is a common fresh water aquatic plant. According to Mansor (1991), most of the species of *Cryptocoryne* are highly exploited for the aquarium industry and apparently fetch high prices in the international aquarium market. Fourteen species of *Cryptocoryne* had been recognised from Borneo and one of them are *Cryptocoryne pallidinervia* Engler (Figure 1.1). It is a plant of lowland forests where it grows in slow running rivers and streams and seasonally in undated forest pools under extremely acid conditions which are pH4 or so. The occurance of this species is endemic to Borneo which is in the peat swamp forests of Sarawak and West Kalimantan. To date, in Sarawak, it can be found in Kampung Keranji in Lundu, Lingga and Sungai Batu, Kampung Teriso in Sri Aman, Sungai Ayang, Dalat in Mukah and Kemena Waterfall in Bintulu (Figure 1.2). *C. pallidinervia* is characterized by the cordate, more or less bullate leaves. The spathe has a long tube and the limb is red with protuberances. The spadix has the male and female flowers situated adjacent to each other. It has green leaves and ovate with a cordate base. It also has a long brownish tube and a long kettle which is black purple inside. Its collar zone is yellowish with small red spots that become smaller towards the throat.

Figure 1.1 (a) *Cryptocoryne pallidinervia* in Kampung Keranji, Lundu; (b) *Cryptocoryne pallidinervia* in Lingga, Sri Aman; (c) *Cryptocoryne pallidinervia* in Sungai Batu, Kampung Teriso, Sri Aman; (d) *Cryptocoryne pallidinervia* in Sungai Ayang, Dalat, Mukah (photo taken by Dr. Isa B. Ipor)

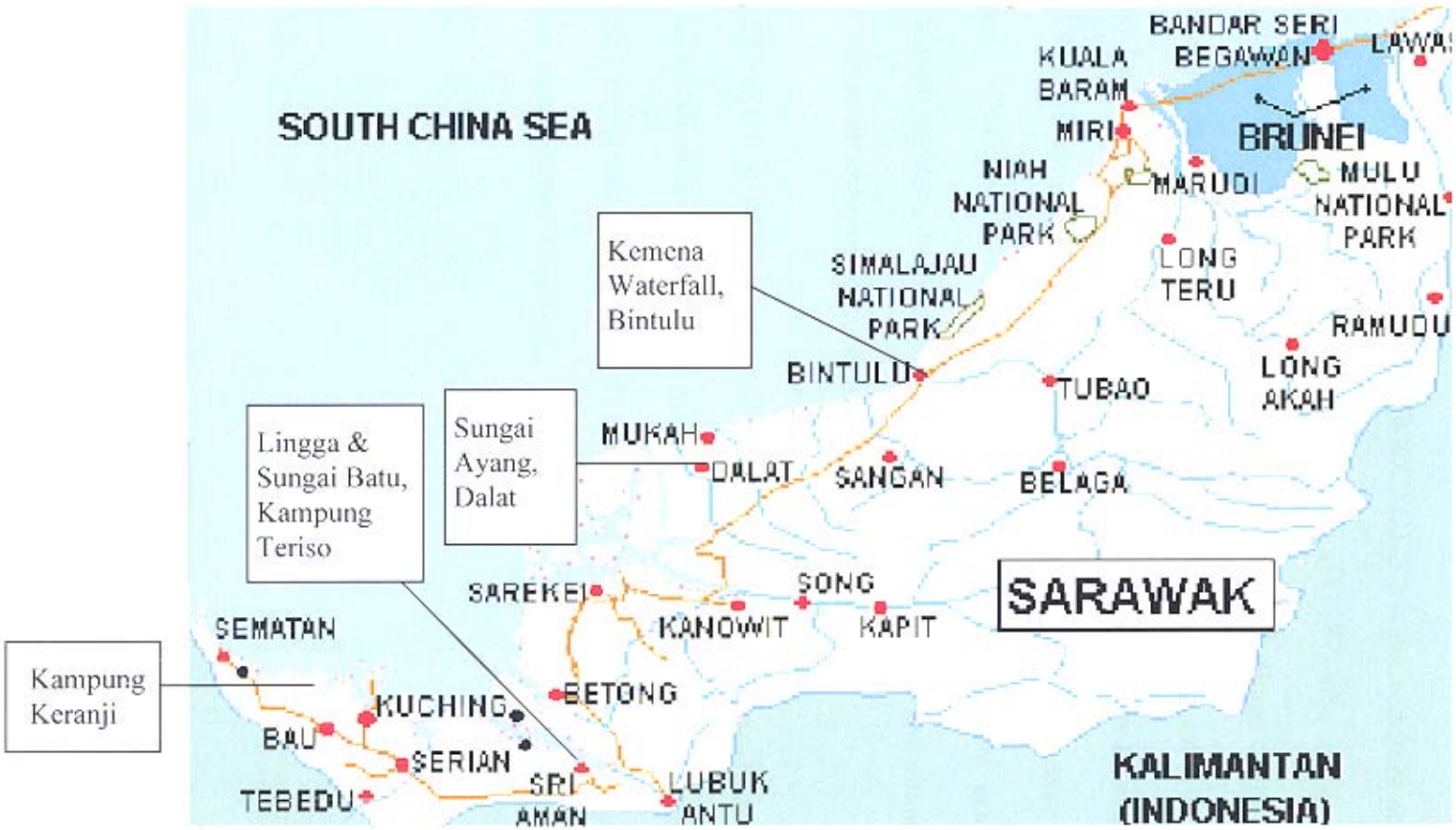

Figure 1.2 Distributions of *C. pallidinervia* in Sarawak

The use of M13 universal primer has been widely recognized as one of the marker system in DNA fingerprinting of plants (Welsh *et al*, 1991; Chong *et al*, 1995). M13 universal primer, the dominant marker, (5'-TTATGAAACGACGGCCAGT-3') is derived from protein III gene of M13 bacteriophage. This had been proven by Vassart *et al* (1987) who had discovered that the tandem repeat sequence from the protein III gene of M13 bacteriophage could effectively show DNA fingerprints in humans and some other mammals. Nybom and Schaal (1990) had explained that M13 probes had been proved useful in generating DNA fingerprints in a number of plants. Welsh *et al* (1991) had generated DNA fingerprints in maize using radioactive PCR with M13 universal primer. Chong *et al* (1995) reported that the M13 universal primer and non – radioactive PCR with ethidium bromide staining could verify species and clonal identity of 15 *Salix* promising clones for biomass production in Ontario, Canada.

The main objective of this study is to determine the genetic relatedness between accessions of *C. palidinervia* from five different locations in Sarawak, which are Kampung Keranji in Lundu, Lingga and Sungai Batu, Kampung Teriso in Sri Aman, Sungai Ayang, Dalat in Mukah and Kemena Waterfall in Bintulu.

2.0 Materials and methods

2.1 Plant materials

Eighteen accessions of *C. pallidinervia* from five different locations were studied, i.e. Kampung Keranji in Lundu, Lingga and Sungai Batu, Kampung Teriso in Sri Aman, Sungai Ayang, Dalat in Mukah and Kemena Waterfall in Bintulu. The details of the accessions are given in Table 2.1.

Table 2.1 Details of *C. pallidinervia* accessions

Place of collection	Leaves	Habitat	Abbreviation	Accession
Kampung Keranji, Lundu	Small	Flat land, dry river, rubber plantation	KRJ	a) KRJ2 b) KRJ3 c) KRJ5
Lingga, Sri Aman	Big	Logged over peat swamp forest	LNG	a) LNG2 b) LNG3 c) LNG4 d) LNG5
Sungai Batu, Kampung Teriso, Sri Aman	Big	Peat swamp riverine forest	TRS	a) TRS3 b) TRS4 c) TRS5
Sungai Ayang, Dalat, Mukah	Small	Sago farm, intertidal	DLT	a) DLT2 b) DLT3 c) DLT4 d) DLT5 e) DLT6
Kemena Waterfall, Bintulu	Small	Secondary forest	BTL	a) BTL1 b) BTL3 c) BTL4

2.2 *Plant DNA extraction*

Total genomic DNA was isolated from fresh leaves of *C. pallidinervia* using a modified CTAB method (Doyle & Doyle, 1987). A small piece of leaf tissue from *C. pallidinervia* was sampled by closing the lid of a 1.5 ml Eppendorf tube over it. Then, the tube, which contained the leaf tissue, needs to be immersed in liquid nitrogen for a few second until it is frozen. Before the leaf tissue macerate with a plastic grinder, it needs to be thawed at room temperature. 100 µl of CTAB extraction buffer (100 mM Tris – HCl pH 8.0, 20 mM EDTA, Na2, 14 M NaCl, 2 % (w / v) hexadecyltrimethylammonium bromide, 0.2 % (v / v) 2 – mercaptoethanol) was added into the tube and continue to grind until slurry was formed. Then, further 900 µl of extraction buffer was added and the lid of the tube was closed and it needs to be mixed gently. The tube had been incubated for 30 minutes in water bath at 65 °C. After 30 minutes, the tube was removed from the waterbath and was allowed to cool to ambient. Then, 400 µl of wet chloroform (chloroform : isoamylalcohol 24 : 1) was added and mixed gently to a single phase. After that, it was centrifuged in a benchtop microcentrifuge at 13 000 rpm for 5 minutes. The upper layer was removed and transferred to a clean tube. 600 µl of ice – cold propan – 2 – ol was added and mixed gently to precipitate the nucleic acid. Then it was centrifuged at 13,000 rpm for 2 minutes to pellet the DNA. After centrifugation, the supernatant was poured away and 1 ml of wash buffer (76 % ethanol, 10 mM ammonium acetate) was added and stored at –20 °C for 30 minutes. Then, it was centrifuged at 13,000 rpm for 2 minutes and the supernatant was poured away and dried the pellet by inverting the tube for 5 minutes. Lastly, the DNA pellet was dissolved in 50 µl TE (10 mM Tris – HCl pH 7.6, 1 mM EDTA) and it was stored at 4 °C until required.

2.3 Genomic DNA purification

Genomic DNA purification was done by using Wizard® Genomic DNA Purification Kit (PROMEGA). Firstly, 3 µl of Rnase Solution was added to the genomic DNA to purify the DNA. After it was inverted, it was incubated in waterbath at 37 °C for 30 minutes. After 30 minutes of incubation, the sample was cooled to room temperature for 5 minutes before proceeding. Then, 200 µl of Protein Precipitation Solution was added and inverted for 1 minute. The supernatant containing DNA was transferred into a clean tube after centrifuged it at 13,000 rpm for 10 minutes. Then, 600 µl of room temperature isopropanol was added to precipitate the nucleic acid. The mixture was centrifuged at 13,000 rpm for 5 minutes. After the supernatant was decanted, 600 µl of room temperature 70 % ethanol was added to wash the DNA. Then, it was centrifuged at 13,000 rpm for 5 minutes. The ethanol was then aspirated and the pellet was air – dried for 30 minutes. Lastly, 50 µl of DNA Rehydration Solution was added to dissolve the pellet. The DNA was then incubated overnight at 4 °C. The DNA concentration was estimated using 0.8% agarose gel electrophoresis together with λ Hind III marker of known concentrations.

2.4 PCR amplification

PCR amplification reaction was performed in volumes of 25 µl containing 1X PCR buffer (200 mM Tris – HCl pH 8.4, 500 mM KCl), 3 mM $MgCl_2$, 200 µM each of dNTPs which consist of dATP, dCTP, dTTP and dGTP, 0.2 µM M13 universal primer (5'-TTATGAAACGACGGCCAGT-3') (Welsh et al. 1991 & Chong et al. 1995), 1.0 U of Taq DNA polymerase and 15 ng of *C. pallidinervia* genomic DNA. The amplification was carried out in a

GeneAmp PCR System Model 2400 (Perkin - Elmer Co. Ltd.) thermocycler, programmed for 35 cycles of 1 minute of denaturing at 94 °C, 1 minute of annealing at 48 °C and 2 minutes of extension at 72 °C after an initial 2 minutes denaturing at 94 °C. The program was then followed by a final step at 72 °C for 10 minutes. After the final cycle, the samples were held at 4 °C until the tubes were removed from the thermocycler. The PCR products (10 μL) were separated on a 1.2 % agarose gel for two hours and 30 minutes at 80V in 1X TBE buffer. The gel was stained in GelStar staining solution for 1 hour and 30 minutes. Then, the gel was visualized under UV light. Polaroid 667 film was used to document the gel.

2.5 Data analysis

Photograph from GelStar - stained agarose gel was used to score the data for PCR analysis. The PCR bands were named after the primer and a hyphenated number corresponding to the order of their migrations. Starting from the slowest to the fastest migrating fragment, the PCR amplified bands were designated as M13 - 01, M13 - 02, M13 - 03 and so on. The presence of a band was scored as 1 and absence was scored as 0, based on the several criteria which are each locus was assumed as independent or non – allelic, there was no bias in scoring monomorphic fragments versus polymorphic fragments and only fragments in the range of 500bp to 3kb were considered in order to increase the data reliability. The data then, was analyzed using Ntsys – PC software. The data was quantified by the similarity index, $J_{ij} = C_{ij} / (ni + n_j - C_{ij})$ (Jaccard, 1908), where J_{ij} is the number of bands common to individuals i and j, n_i is the number of bands in individual i and n_j is the number of bands in individual j. A dendrogram was generated using the

Unweighted Pair – Group Method with Arithmetical Averages (UPGMA) as described by Sneath and Sokal (1973).

3.0 Result and discussion

3.1 *Plant DNA extraction*

Total genomic DNA extracted from *C. pallidinervia* leaves are shown in Figure 3.1. The modified CTAB miniprep DNA extraction method (Doyle & Doyle, 1987) together with Wizard® Genomic DNA Purification Kit (Promega Corporation, USA) method were successfully extracted from the genomic DNA of 18 *C. pallidinervia* accessions collected from five different locations in Sarawak. The quality of DNA was determined by gel electrophoresis method. A distinct band with less smearing was observed from each accession and these genomic DNA were used as template in PCR amplification to generate the DNA profile.

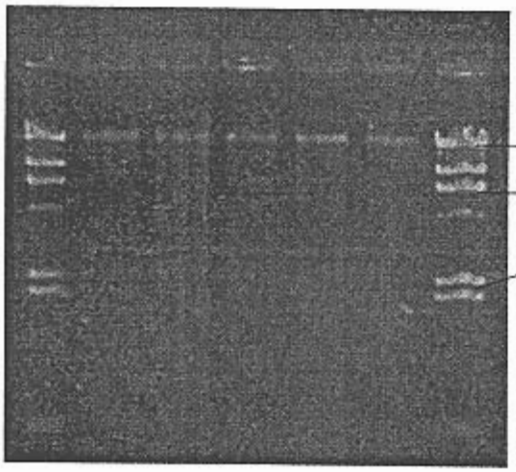

Figure 3.1 Agarose (0.8%) gel electrophoresis of genomic DNA for *C. pallidinervia* accessions collected from Bintulu. M is the λ Hind III marker (Promega Corporation, USA)

3.2 PCR amplification

Based on the PCR results, M13 universal primer was successfully amplified all *C. pallidinervia* accessions by producing multiple bands as shown in Figure 3.2. Repeating PCR for three times

proved the reproducibility of the fingerprinting obtained. Negative controls were performed for PCR and the result showed that no contamination or artificial product occurred during the PCR reaction.

A total of 18 *C. pallidinervia* accessions from Keranji, Lingga, Teriso, Dalat and Bintulu were analyzed by using PCR method. Based on the result, it showed a high degree of polymorphism among the accessions. 18 distinctive DNA profiles were produced which composed of 3 to 20 bands with the molecular weight ranging from 500bp to 3kb. Although most of the *C. pallidinervia* accessions showed a high degree of polymorphisms, but two accessions, DLT4 and DLT6 produced identical banding profiles.

Based on the data scoring table (Table 3.1), there were several accessions that obtained unique band. As an example, locus M13 – 07 was specific to accession TRS4, locus M13 – 14 was specific to accession TRS5, locus M13 - 21 was specific to accession LNG4, locus M13 – 24 was specific to accession DLT3 and locus M13 – 27 was specific to accession BTL4. Three bands (locus M13 – 25, locus M13 – 29 and locus M13 – 30) were found in all accessions except accession BTL1. Different presence or absence behaviors of the remaining bands were useful in differentiating the samples within accessions (Chong *et al*, 1995). For example, accessions KRJ2 and KRJ5 differed from other accessions at locus M13 – 33 as well as accessions TRS4 and DLT2, which differed at locus M13 – 06.

Table 3.1 Diagram of the fingerprinting profile to identify the *C. pallidinervia* accessions using M13 universal primer

LOCUS	KRJ 2	KRJ 3	KRJ 5	LNG 2	LNG 3	LNG 4	LNG 5	TRS 3	TRS 4	TRS 5	DLT 2	DLT 3	DLT 4	DLT 5	DLT 6	BTL 1	BTL 3	BTL 4
M13-01				—	—	—	—				—	—						
M13-02	—	—										—	—	—	—			
M13-03						—			—			—	—	—	—		—	
M13-04	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	
M13-05	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
M13-06									—		—							
M13-07									—									
M13-08						—	—	—	—	—	—	—	—	—	—			
M13-09				—			—											
M13-10	—	—	—	—		—	—	—	—	—	—	—	—	—	—			—
M13-11	—		—	—		—												—
M13-12		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
M13-13										—				—		—	—	—
M13-14										—								
M13-15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
M13-16	—	—	—	—	—	—	—		—	—	—	—	—	—	—			
M13-17					—	—			—					—				
M13-18	—		—						—				—	—	—			
M13-19		—	—	—	—	—	—	—	—	—	—		—	—	—			
M13-20	—										—							
M13-21						—												
M13-22	—		—	—													—	
M13-23																		—
M13-24												—						
M13-25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	—
M13-26	—	—	—	—			—	—	—	—	—	—		—				
M13-27																		—
M13-28		—	—						—	—						—		
M13-29	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	—
M13-30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	—
M13-31	—	—	—	—	—	—			—									
M13-32		—		—	—	—	—	—	—	—	—	—	—	—	—			
M13-33	—		—															

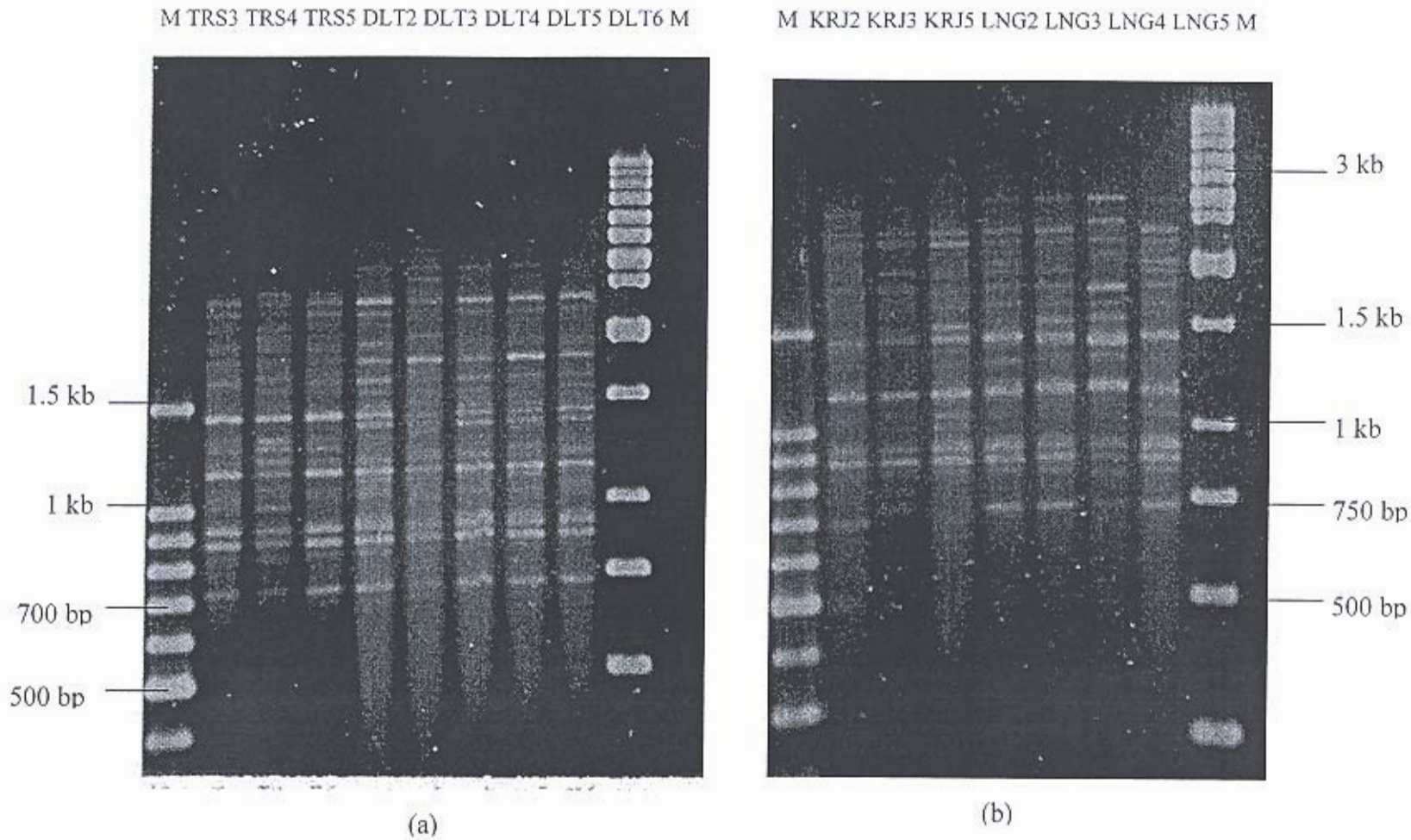

Figure 3.2 PCR profiles of some of the *C. pallidinervia* samples using M13 universal primer. Lane 1 is 100bp marker and Lane 10 is 1kb marker

3.3 Genetic relatedness of C. pallidinervia accessions

Genetic differences among 18 *C. pallidinervia* accessions were analyzed by using Jaccard similarity coefficient. The genetic relationship between the accessions is shown in Figure 3.3. This dendrogram was constructed based on the similarity matrix that had been generated by M13 universal primer (Table 3.3). The constructed dendrogram had divided *C. pallidinervia* accessions into four major groups of cluster (Table 3.2).

Table 3.2 Major groups of *C. pallidinervia* accessions based on the similarity matrix generated by M13 universal primer

Cluster	Accession
1	KRJ2
	KRJ5
	LNG2
2	KRJ3
	TRS5
	LNG5
	DLT2
	TRS3
	DLT3
	DLT4
	DLT6
	DLT5
	TRS4
	LNG3
	LNG4
3	BTL3
	BTL4
4	BTL1

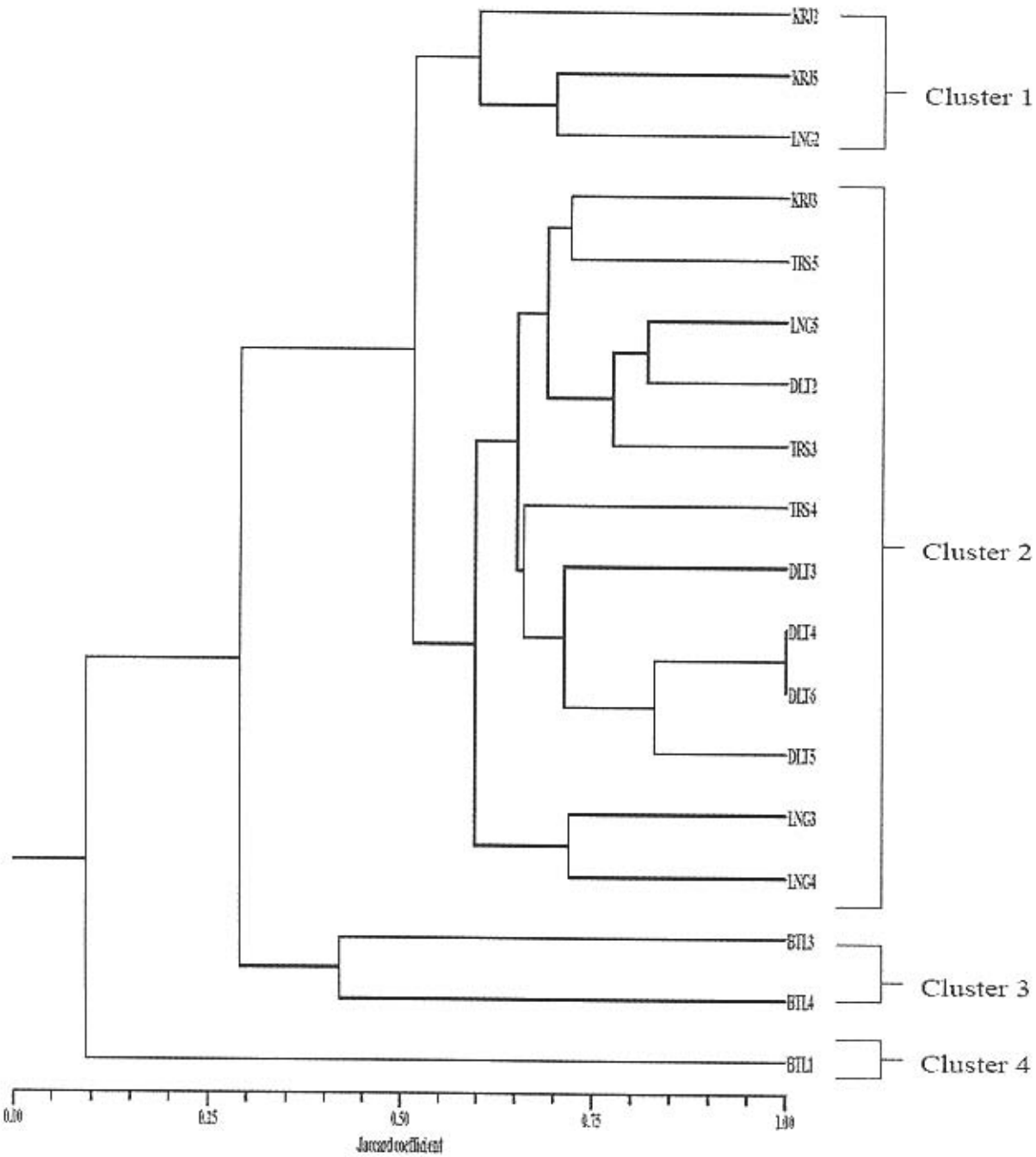

Figure 3.3 Dendrogram representing the genetically relatedness among the *C. pallidinervia* accessions

Table 3.3 Similarity matrix generated by M13 universal primer

	KRJ2	KRJ3	KRJ5	LNG2	LNG3	LNG4	LNG5	TRS3	TRS4	TRS5	DLT2	DLT3	DLT4	DLT5	DLT6	BTL1	BTL3	BTL4
KRJ2	1.00																	
KRJ3	0.50	1.00																
KRJ5	0.68	0.68	1.00															
LNG2	0.52	0.68	0.70	1.00														
LNG3	0.33	0.65	0.50	0.67	1.00													
LNG4	0.38	0.57	0.52	0.67	0.72	1.00												
LNG5	0.36	0.67	0.52	0.78	0.65	0.65	1.00											
TRS3	0.42	0.69	0.53	0.61	0.56	0.58	0.80	1.00										
TRS4	0.40	0.67	0.61	0.54	0.57	0.65	0.59	0.60	1.00									
TRS5	0.35	0.72	0.57	0.57	0.53	0.55	0.72	0.75	0.64	1.00								
DLT2	0.41	0.63	0.50	0.65	0.61	0.62	0.82	0.75	0.64	0.68	1.00							
DLT3	0.41	0.63	0.43	0.57	0.53	0.62	0.72	0.65	0.57	0.60	0.68	1.00						
DLT4	0.43	0.67	0.52	0.52	0.56	0.65	0.67	0.69	0.67	0.63	0.63	0.72	1.00					
DLT5	0.43	0.65	0.52	0.52	0.55	0.64	0.65	0.67	0.73	0.70	0.62	0.70	0.83	1.00				
DLT6	0.43	0.67	0.52	0.52	0.56	0.65	0.67	0.69	0.67	0.63	0.63	0.72	1.00	0.83	1.00			
BTL1	0.00	0.13	0.11	0.05	0.07	0.05	0.06	0.07	0.10	0.19	0.06	0.06	0.06	0.11	0.06	1.00		
BTL3	0.28	0.28	0.32	0.32	0.31	0.30	0.28	0.33	0.27	0.33	0.26	0.33	0.35	0.37	0.35	0.22	1.00	
BTL4	0.26	0.26	0.30	0.30	0.22	0.29	0.26	0.31	0.20	0.32	0.25	0.25	0.26	0.29	0.26	0.20	0.42	1.00

KRJ : Kampung Keranji, Lundu
LNG : Lingga, Sri Aman
TRS : Sungai Batu, Kampung Teriso, Sri Aman
DLT : Sungai Ayang, Dalat, Mukah
BTL : Kemena Waterfall, Bintulu

Cluster 1 contained three accessions from Kampung Keranji and Lingga with average value of J = 0.82. The greatest value was observed between accession KRJ5 and LNG2 with J = 0.70. The smallest value of J = 0.52 within this cluster was observed between accession KRJ2 and LNG2. The genetic variation of accessions from this cluster was greatly different even from the same location.

Cluster 2 was the biggest cluster composed of 12 accessions derived from Kampung Keranji, Lingga, Kampung Teriso and Dalat. The average value among the entire accessions was J = 0.76. The greatest value of J = 0.83 within this cluster was observed between accession DLT4 and DLT5 as well as accession DLT5 and DLT6. While the smallest value of J = 0.07 within this cluster was observed between accession DLT5 and LNG3. In this cluster, accession DLT4 and DLT6 had identical banding profiles. Since differences in the PCR banding profiles were high between and within accession in this study, so one possible explanation is that accession DLT4 and DLT6 were derived from the same source. Chong *et al* (1995) showed that banding profiles generated by M13 universal primer did not only verified the *Salix* species identity, but also differentiated among 14 of the 15 *Salix* clones. Two clones of *S. eriocephala*, ERIO21 and ERIO23 had identical banding profiles and therefore, suggested that these two clones were two ramets of a clone.

Based on the dendrogram, the Bintulu accessions were grouped together in Cluster 3 that comprised of two accessions, i.e. accession BTL3 and BTL4. While Cluster 4 is composed of only accession BTL1. The geographical distance between Bintulu and Dalat, Teriso, Lingga and Keranji may influence the Bintulu accessions. The distance between Bintulu and other locations was bigger compared to others (Table 3.4). For instance, the distance between Bintulu and Keranji is 400 Km and they were not closely related based on the dendrogram. In fact, their

genetic similarity value was 0.22. Therefore, it suggests that *C. pallidinervia* accessions from Bintulu can be considered as the subvarieties of *C. pallidinervia*. Besides the geographical distance factor, the differences between Bintulu accessions and other accessions may also be due to habitat. The habitat for all of the accessions is peat swampy land, lowland area and along the slow running rivers, except Bintulu accessions, which is in secondary forest (Table 2.1). Hence, the different geographic or environmental origins of these samples also have significant effects on the clustering of the DNA profiles obtained from PCR (Lai, 2003).

Table 3.4 Geographical distance between five different locations of *C. pallidinervia* accessions in Sarawak

	KERANJI	**LINGGA**	**TERISO**	**DALAT**	**BINTULU**
KERANJI		160 Km	142 Km	272 Km	400 Km
LINGGA			26 Km	177 Km	290 Km
TRISO				172 Km	285 Km
DALAT					130 Km
BINTULU					

From the dendrogram, it was observed that accessions from different locations tended to group together in a cluster, as shown in Cluster 2. Therefore, it suggests that the gene flow has occurred within the *C. pallidinervia* accessions. The gene flow occurs due to the pollination, which is one of the mating systems besides vegetative reproduction. The pollen is possible to be transferred from one location to another through insects especially between adjacent locations such as Lingga and Kampung Teriso. However, further research need to be done because there is lack of evidence regarding the pollen fertility in *C. pallidinervia*.

Leaves characters show the main differences among the accessions. All big leaves accessions were grouped together in Cluster 2, whereas small leaves accessions can be found in all of the clusters. Upadhyay *et al* (2002) reported in their studies on molecular analysis of phylogenetic relationships among coconut accessions that, when subjected to cluster analysis, all Dwarf accessions were grouped together while Tall accessions formed three groups.

4.0 Conclusion

The objective of this study is to determine the genetic relatedness between accessions of *C. palidinervia* in Sarawak. Towards the end, the results were highly satisfactory. Banding profiles generated with M13 universal primer did not only verified the species identity, but also differentiated 18 *C. pallidinervia* accessions. The observed PCR banding profiles with this primer were sufficient in differentiating the *C. pallidinervia* accessions. Therefore, the M13 universal primer was indeed valuable.

Polymerase Chain Reaction (PCR) is a rapid, high discriminative power of molecular typing method and it has the advantages of being less labor – intensive, easier and simple methods as well as minute amounts of genomic DNA are needed. The use of Jaccard similarity coefficient had determined the genetic relationship among the diversity of the accessions. Cluster analysis had divided the samples tested into four different major clusters.

As a conclusion, the result obtained showed that *C. pallidinervia* from different locations were genetically related. This is because accessions from different locations can group together in a cluster. However, Bintulu accessions were highly different among the others since they grouped together in different cluster. These preliminary results highlighted the need of further study in order to confirm the taxonomic identity of those accessions from Bintulu.

5.0 Acknowledgement

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