Short Communication

Genotyping natural hybrids of *Cryptocoryne purpurea* in Borneo and Peninsular Malaysia

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ABSTRACT. A preliminary study was undertaken on the genotype of *Cryptocoryne purpurea* and the application of fingerprinting to study the genetic relatedness of population from four different localities, namely Kota Tinggi (Johor), Tasik Bera (Pahang), Lundu (Sarawak) and Sungai Lawak (Kalimantan). A total of 20 reproducible bands were generated and unique bands were found in all localities that demonstrated some degree of variations among the samples. Dendrograms and genetic similarity indices generated using NYSYSpc ver. 2.2 clearly divided *C. purpurea* hybrids into two clusters based on geographical distribution.

Keywords: Cryptocoryne, M13, RAPD-PCR, genotyping, geographical variations.

INTRODUCTION

Cryptocoryne (Araceae), a genus of riverine plants, is locally known as *Keladi Paya* (Peninsular Malaysia), *Kiambang Batu* (Malays-Sarawak), *Kelatai* (Iban), and *Tropong Ajer* (Banjarmasin, Kalimantan). They are popularly used as aquarium plants due to their interesting features including leaves and spathes of many different attractive colours and shapes (Mansor, 1991).

Presently, there are approximately 50 species of *Cryptocoryne* found in South East Asia with 15 species occurring in Malaysia (Jacobsen, 1991). *Cryptocoryne* has a high degree of endemism and is frequently observed in co-existence with two or more species. Co-existence of these *Cryptocoryne* species may lead to interbreeding and produce new hybrids within the same area. An example of this is for *C. purpurea*, that has been reported to be endemic to Tasik Bera in Pahang, Malaysia (Jacobsen, 1985). However, Jacobsen (2002), revealed the presence of a hybrid, *C. purpurea* nothovar. *borneoensis*, in Sampit, Kalimantan

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Tengah, Indonesia. He suspected that the hybrid is possibly between *C. griffithii* and *C. cordata* var. *zonata*. It was initially referred to as *C. griffithii* based on morphological characters (Jacobsen, 1991) but subsequently discovered to be *C. purpurea* (Jacobsen, 2002). The capability of *C. purpurea* to hybridise naturally may create more complexity in terms of taxonomic studies and classification. Confusion in identification occurred such as in the mis-identification of *C. purpurea* in Pahang and also when a hybrid of *C. purpurea* was discovered in Kalimantan Tengah (Ipor *et al*., 2005). Ipor *et al.* (2005), discovered a new species in Sungai Stungkor, Sarawak, and predicted that it is also a hybrid of *C. purpurea*. Therefore, this study was undertaken to investigate the genetic relationship between hybrids of *C. purpurea* located in Peninsular Malaysia and the proposed hybrid found in Sarawak (Ipor *et al*., 2005).

MATERIALS AND METHODS

Plant materials

The plant samples of *C. purpurea* were collected at four localities, namely Tasik Bera (Pahang), Sungai Stungkor, Lundu (Sarawak) and Kota Tinggi (Johor) in Malaysia, and Sungai Lawak, Sampit in Kalimantan Tengah, Indonesia (Figure 1). Plant materials from Sarawak and Pahang were fresh samples, while dried samples from Johor and Kalimantan were kindly provided by Professor Dr Niels Jacobsen from The Royal Veterinary and Agricultural University, Denmark, and Mr Takashige Idei from Japan. The fresh samples were surface sterilised with 70% ethanol and rinsed with distilled water before DNA extraction. There were 15 accessions used in the study (Table 1).

DNA extraction and purification

DNA extractions were carried out using the modified CTAB method described by Doyle and Doyle (1987). Approximately, 600 µl CTAB extraction buffer with 5 µl β-mercaptoethanol were preheated to 60°C in a water bath for about 15 minutes. The sample (0.2 g) was first grounded in liquid nitrogen using a mortar and pestle. Then, preheated CTAB was added to the ground tissue, incubated at 60°C for about 2 hours with mixing every 15 minutes. After that 600 µl of CIA (24:1 chloroform:isoamyl

Figure 1. Map of Peninsular Malaysia and Borneo indicating sampling sites. The sampling sites are Tasik Bera in Pahang, Kota Tinggi in Johor, Sungai Stungkor in Sarawak and Sungai Lawak, Sampit in Kalimantan Tengah, Indonesia.

Table 1. Sample inventory and the origin of *Cryptocoryne purpurea* from Peninsular Malaysia and Borneo.

Samples	Origin
J1	Johor
J2	Johor
J3	Johor
K1	Kalimantan
K2	Kalimantan
K3	Kalimantan
L1	Sg Stungkor
L2	Sg Stungkor
L3	Sg Stungkor
L4	Sg Stungkor
P1	Tasek Bera
P2	Tasek Bera
P3	Tasek Bera
P4	Tasek Bera
P5	Tasek Bera

alcohol) was added into the tube and mixed before centrifugation at 13,000 rpm for 15 minutes. The upper aqueous layer was transferred to a new 1.5 ml centrifuge tube and mixed again with 600 µl of CIA. The mixture was centrifuged for about 5 minutes at 13,000 rpm. Finally, the upper layer was transferred to a new tube containing 600 µl of cold isopropanol and stored overnight at -20°C. The DNA was recovered by centrifugation for 13,000 rpm for 15 minutes and the DNA pellet was washed with 800 µl wash buffer and centrifuged for 15 minutes. Supernatant was discarded and allowed DNA pellet to dry, 50 µl TE buffer was added and DNA was stored at 4°C until use. An additional purification step was required using Wizard® Genomic DNA Purification Kit (Promega) for samples isolated from dried materials.

PCR Amplification and Analysis

DNA fingerprinting was carried out using M13 universal primer (5'-TTATGAAACGACGGCCAGT- 3'). The amplifications were carried out using MyCycler (BioRad). A 25 µl total volume of PCR mixture was prepared comprising of 1X PCR buffer (Vivantis), 2 mM of $MgCl_2$ (Vivantis), 0.2 mM of dNTPs (Vivantis), 10 pmol of M13 primer, 1 unit *Taq* polymerase (Vivantis) and 20 ng of DNA templates. The PCR cycle employed was as follows: 2 minutes for initial denaturation at 94°C and 35 cycles of 1 minute for denaturation at 94°C, 1 minute of annealing at 48°C, 2 minutes of extension at 72°C and a final extension step of 72°C for 10 minutes. The amplicons were analysed on 2% agarose gel.

Data Analysis

PCR amplicons were documented and scored using Kodak Gel Documentation System (BioRad). The DNA bands were scored as discrete variables using "0" to show absence and "1" to show presence of band. The data was analysed using genetic data analysis software, Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) version 2.2. The data was quantified by similarity index, Jij = Cij / (ni + nj – Cij), where Jij= the number of individuals i and j, ni= the number of bands in individual i, nj= the number of bands in

individual j. Dendrogram was constructed by the unweighted paired group method of arithmetic average (UPGMA) based on Jaccard's similarity coefficient as described by Sneath and Sokal (1973).

RESULTS

The DNA was isolated successfully using both fresh and dried samples. The DNA was of high quality and this was shown in subsequent PCR amplification. A PCR profile was established for all individual and reproducibility of the profile was confirmed by repeating the amplification for five times except for sample K1 from Kalimantan. Figure 2 shows the PCR profile derived using the M13 universal primer. Different banding patterns were observed and the total of loci was 20 bands. Banding sizes ranging from 300 bp to 1200 bp were used in the data analysis. For the purpose of scoring, only 14 samples were used because sample K1 was found to produce inconsistent results. Each band obtained was scored as "0" for absent or "1" for presence of band for each sample. Banding patterns were analysed to create phylogenetic tree using NTSYSpc ver 2.2 (Figure 3) and the data was converted into a binary (Table 2).

DISCUSSIONS

Although the indigenous *Cryptocoryne* species grows well in natural wetlands, demand in the aquarium industry and also habitat destruction, rapid exploitation and deforestation due to human activities have decreased the species' population number. Thus, it is crucial that the species be protected.

Polymerase chain reactions have been increasingly used and have been useful in determining the relationship between species or varieties and also individuals such as somaclones (Bhattacharya & Ranade, 2001; Fu *et al*., 2002; Giménez *et al*., 2005). For this work, this technique was employed to determine the relationship between populations

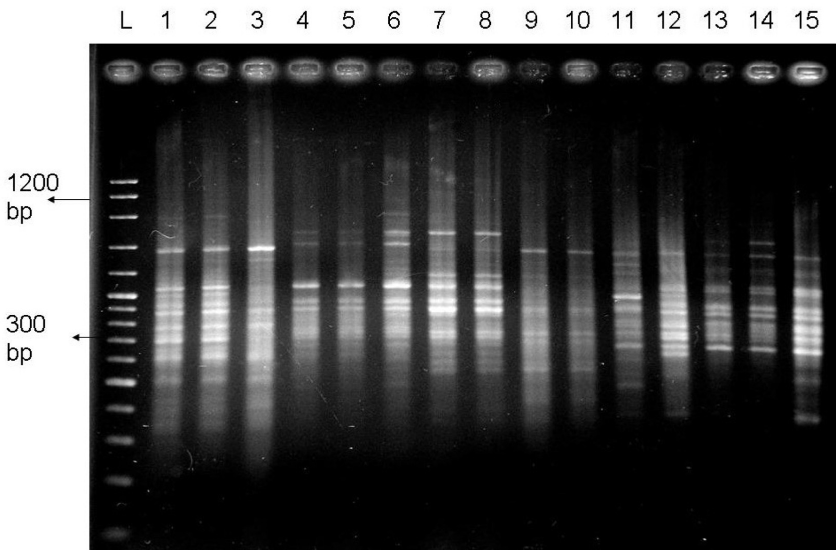

Figure 2. A 2% agarose gel image of PCR profile visualized under UV. Lanes 1-3 are samples from Kota Tinggi (Johor), Lanes 4-6 are samples from Sungai Lawak, Sampit (Kalimantan Tengah), Lanes 7-10 are samples from Sungai Stungkor (Sarawak), Lanes 11-15 are samples from Tasik Bera (Pahang). L is 100 bp Ladder Plus (Vivantis).

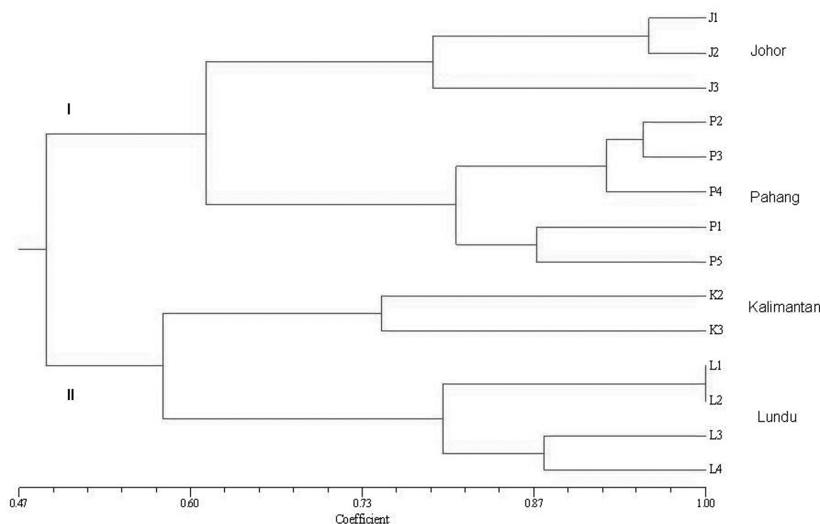

Figure 3. Dendrogram showing cluster analysis of genetic similarities among 14 samples of *Cryptocoryne purpurea* using M13 universal primer. Samples are separated into two clusters according to geographical distribution with samples from Sungai Stungkor being genetically closer to samples from Kalimantan.

	J1	J2	J3	K2	K3	L1	L2	L3	L4	P1	P2	P3	P4	P5
J1	1.00													
J2	0.957	1.00												
J3	0.762	0.818	1.00											
K2	0.333	0.316	0.353	1.00										
K3	0.400	0.476	0.316	0.750	1.00									
L1	0.522	0.583	0.455	0.632	0.762	1.00								
L2	0.522	0.583	0.455	0.632	0.762	1.00	1.00							
L3	0.500	0.476	0.421	0.500	0.556	0.857	0.857	1.00						
L4	0.444	0.421	0.471	0.429	0.375	0.737	0.737	0.875	1.00					
P1	0.783	0.750	0.636	0.421	0.476	0.417	0.417	0.381	0.421	1.00				
P2	0.636	0.609	0.571	0.667	0.500	0.522	0.522	0.500	0.444	0.783	1.00			
P3	0.571	0.545	0.600	0.706	0.526	0.545	0.545	0.526	0.471	0.727	0.952	1.00		
P4	0.600	0.571	0.526	0.625	0.556	0.571	0.571	0.556	0.500	0.762	0.900	0.947	1.00	
P5	0.636	0.609	0.571	0.556	0.600	0.522	0.522	0.500	0.444	0.870	0.818	0.857	0.900	1.00

Table 2. Genetic similarity matrix for 14 samples of *Cryptocoryne purpurea*.

of *Cryptocoryne* in Peninsular Malaysia and Borneo that have been separated geographically for millions of years. The results were scored and a dendrogram was constructed based on the similarity matrix that divided the *C. purpurea* into two clades. Apart from that, the work also showed that variations were found between the samples. The dendrogram showed that samples located within the same area are grouped in the same clade indicating a close relationship between individuals. For clade 1, the group consists of the species collected from Peninsular Malaysia *viz*. Johor and Pahang. For clade II, the group consists of the species collected from Borneo *viz*. Kalimantan Tengah and Sarawak. The values from genetic similarity matrix showed clear differences between individuals based on localities. For example, the genetic similarity of samples taken from Johor (31.6%) and Kalimantan Tengah (47.6%). For samples originating from Johor and Sarawak, the genetic similarity is

between 42.1% and 58.3%. Meanwhile, a high value of genetic similarity was also detected for samples derived from sites that are within the same geographical location such as those from Peninsular Malaysia, i.e. Johor (54.5%) and Pahang (78.3%), as compared to those in Borneo, i.e. Sarawak (37.5%) and Kalimantan Tengah (76.2%). The present study indicates that the closer the distance of the plants, the genetic content of the population is more similar. Bahulikar *et al*. (2004) reported that ISSR and AFLP analyses of *Nicotiana attenuata* in Utah and Arizona contain low genetic differentiation among sites and higher genetic differentiation within populations.

Although a clear separation of individuals based on geographical location was shown by the analysis, variations between individuals within the same location were also observed. This can be seen from the genetic similarity matrix of samples from Johor where genetic similarity index is between 0.762 to 0.957. The genetic similarities between individuals in Pahang were between 0.727 to 0.952. According to Avise (1994), nearly all related species showed at least some degree of genetic differentiation among geographical areas.

CONCLUSION

M13 Universal Primer was useful in determining the genetic relatedness of *C. purpurea* from different locations in Peninsular Malaysia and Borneo. The DNA fingerprints of *C. purpurea* hybrids showed higher degree of genetic similarity between close locations and also variation within a population. The study showed that the samples from Johor and Pahang are closely related compared to samples located in Kalimantan and Sarawak.

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