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GENETIC VARIATION WITHIN THE AQUATIC PLANT SPECIES *CRYPTOCORYNE* ×*PURPUREA* IN TASIK BERA, MALAYSIA AS REVEALED BY RAPD ANALYSIS

Ahmad Sofiman Othman*, Yu Choo How, Chin Choke Lin and Mashhor Mansor.

School of Biological Sciences, Universiti Sains Malaysia, 11800 Pulau Pinang, Malaysia

Abstrak: Variasi genetik dua populasi semulajadi tumbuhan akuatik yang jarang ditemui Cryptocoryne ×purpurea telah dikaji dengan menggunakan penanda DNA polimorfik amplikasi rawak (RAPD). Empat pencentus rawak menjana sejumlah 57 jalur dan 49 (86%) daripadanya adalah polimorfik menggambarkan variasi genetik yang tinggi untuk spesies ini. Rajah pohon berdasarkan kaedah Neighbor-Joining (NJ) dijana menggunakan data kesamaan genetik membezakan sampel-sampel ke dalam dua kelompok berbeza setiap satunya untuk populasi tunggal. Graf PCO dua-dimensi yang dijana menggunakan data kesamaan genetik juga serasi dengan topologi rajah pohon NJ. Analisis varians molekul (AMOVA) menunjukkan bahawa sebahagian besar variasi genetik (67%) berada antara populasi sementara hanya 33% daripada variasi genetik berada di dalam populasi. Aras genetik antara individu yang rendah terhasil daripada mutasi somatik memandangkan spesies hybrid ini menunjukkan kesterilan debunga yang tinggi. Namun, peristiwa hibridisasi yang berbeza diikuti pemencilan populasi dan pengumpulan mutasi somatik yang tak bersandaran mungkin telah menyebabkan variasi genetik antara populasi yang tinggi.

Abstract: Genetic variation of two natural populations of the rare aquatic plant species Cryptocoryne ×purpurea were investigated using random amplified polymorphic DNA (RAPD) markers. Four random primers generated a total of 57 bands and 49 (86%) of them were polymorphic indicating considerable genetic variation at the species level. A Neighbor-Joining (NJ) tree constructed using genetic similarities data separated the samples into two distinct groups each representing a single population. Two-dimensional PCO graph generated from genetic similarity data was also in agreement with the NJ tree topology. Analysis of molecular variance (AMOVA) showed that a large proportion of the genetic variation (67%) resided between populations while only 33% of the genetic variation resided within population. The low level of genetic diversity among individuals was likely generated from somatic mutation since this hybrid species exhibited high pollen sterility. However, separate hybridization events followed by population isolation and subsequent independent accumulation of somatic mutations could have resulted in the high genetic variation between populations.

Keywords: Cryptocoryne ×purpurea, RAPD, Genetic Variation

INTRODUCTION

Cryptocoryne ×*purpurea* Ridley is a perennial aquatic plant species and has a restricted distribution in Peninsular Malaysia. The plant was first collected from Kota Tinggi, Johore by Ridley in 1892. In 1985, Niels Jacobsen, a Danish

*Corresponding author: sofiman @usm.my

botanist found this plant in Tasik Bera (Bera Lake), Pahang namely in Pos Iskandar (formerly known as Fort Iskandar). Currently the species can be found in two locations within Tasik Bera i.e., Kelantong Swamp and Pos Iskandar.

It was noted that the pollen of *C.* ×*purpurea* is completely sterile (Jacobsen 1977). One possible explanation could be that the plant is of hybrid origin with most likely parents being *C. cordata* and *C. griffithii*. This assumption was based on the fact that the *C.* ×*purpurea* possesses characteristics from the two species namely the broad collar zone of limb of the spathe (*C. cordata*) and the purple and rough limb (*C. griffithii*). The pollen sterility explains why fruits are unknown (Jacobsen 1977).

The main taxonomic character for delimitation of *Cryptocoryne* species is the variations of the shape and colour of the limb of the spathe. Cordate leaves characterize *C.* ×*purpurea* with purple marking underneath. The spathe is a long tube and the limb has a rough purplish surface, and a broad, yellow red collar zone. There is some variation in the colouration and also in the surface structure of the limb of the spathe in plants from different localities (as well as in some of the plants in cultivation of unknown origin), an indication that the hybrid has arisen several times independently from different parental populations.

RAPD analysis employs single short arbitrary primers able to generate genome-specific, multiple amplification products (Welsh & Mc Clelland 1990; Williams *et al.* 1990). RAPD markers are essentially unlimited in number and usually very polymorphic (Shrestha *et al.* 2002). Most variation among individuals for RAPD probably arise from base-pair substitutions, insertion or deletions that modify the primer site or from the genomic sequence that separate the primer site to a distance that will not permit amplification (Williams *et al.* 1990). RAPD has been successfully used in the determination of population genetic study in rare/endangered plants (eg. Chen *et al*. 2006; Fu *et al*. 2003; Martin *et al*. 2003) and plants with economic values (eg. Shrestha *et al.* 2002).

The objective of the present study was to estimate and partition the genetic variation within and among the two populations of *C.* ×*purpurea* using RAPD.

MATERIALS AND METHOD

Plant Material

Individuals of *C.* ×*purpurea* were collected from two localities within Tasik Bera, Pahang which are roughly 20 km apart. Nineteen individuals from Kelantong Swamp and twenty individuals from Pos Iskandar were used for DNA isolation and subsequent RAPD analysis (Table 1). Leaves were cleaned and dried with silica gel prior to DNA extraction.

Table 1: The list and labels of plants used in the study

Sample name	Location	Sample name	Location
1-1	Kelantong Swamp	2-1	Pos Iskandar
1-2	Kelantong Swamp	2-2	Pos Iskandar
1-3	Kelantong Swamp	2-3	Pos Iskandar
1-4	Kelantong Swamp	2-4	Pos Iskandar
1-6	Kelantong Swamp	2-5	Pos Iskandar
1-7	Kelantong Swamp	2-6	Pos Iskandar
1-8	Kelantong Swamp	2-7	Pos Iskandar
1-9	Kelantong Swamp	2-8	Pos Iskandar
1-10	Kelantong Swamp	2-9	Pos Iskandar
1-11	Kelantong Swamp	2-10	Pos Iskandar
1-12	Kelantong Swamp	2-11	Pos Iskandar
1-13	Kelantong Swamp	2-12	Pos Iskandar
1-14	Kelantong Swamp	2-13	Pos Iskandar
1-15	Kelantong Swamp	2-14	Pos Iskandar
1-16	Kelantong Swamp	2-15	Pos Iskandar
1-17	Kelantong Swamp	2-16	Pos Iskandar
1-18	Kelantong Swamp	2-17	Pos Iskandar
1-19	Kelantong Swamp	2-18	Pos Iskandar
1-20	Kelantong Swamp	2-19	Pos Iskandar
		2-20	Pos Iskandar

DNA Extraction

DNA was isolated from dried samples using the hexadecyltrimethylammonium bromide (CTAB) method (Doyle & Doyle 1987). DNA concentration was estimated using Eppendorf Biophotometer. Optical density of the DNA at OD_{260} and OD_{280} was measured and the quality of DNA was calculated using the formula: OD_{260} X 50 μl/ml.

Amplification Conditions

RAPD analysis was performed with the initial screening of sixty primers (OPERON Technologies Inc.) namely, OPA 1-20, OPH 1-20 and OPE 1-20. These primers were screened on four randomly selected individuals i.e. two from each locations. Of these primers, four viz. OPA 7, OPE 14, OPH 4 and OPH 5 were chosen based on clarity and reproducibility of bands generated. RAPD reactions were carried out in a volume of 25 μl containing 2.5 μl 10X *Taq* DNA polymerase buffer, 2.0 mM $MgC1_2$, 2.5 μl 2.0 mM dNTP, 1.0 μl pmol primer, 0.1 U *Taq* DNA polymerase (Promega, USA) and 1.0 μl template DNA.

Amplification of genomic DNA was made on a MJ Research PTC-200 Peltier Thermal Cycler and commenced with 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, annealing at 34°C for 1 min and extension at 72 °C for 2 min and a final extension at 72°C for 6 min. PCR products were fractionated by 1.5% agarose gel electrophoresis and stained with ethidium bromide and visualized and photographed under ultraviolet light.

Data analysis

RAPD bands were scored as present (1) or absent (0) for each plant in a binary matrix. Genetic similarity among the samples was calculated according to Jaccard's similarity coefficients as implemented in RAPDistance (Armstrong *et al*. 1995). Jaccard's similarity coefficients are defined as a/(a+b+c) where a is the number of positive matches, b and c refer to the number of bands in both samples 1 and 2. These genetic similarity values are used for clustering analysis using the Neighbor-Joining method as implemented in the computer program PHYLIP (Felsenstein 1993). The resulting Neighbor-Joining dendogram was visualized using TreeView (Page 1996).

Principal Coordinate Analysis (PCO) based on the similarity matrix was performed using MVSP 3.1 (Multi-Variate Statistical Package; Kovach Computing Services 1985-2003). The relationship among plant individuals were described in two dimensional graph.

AMOVA (Excoffier 1992) was used to estimate variance components and to test the significance of partitioning of RAPD variation within and among population.

Table 3: Analysis of molecular variance (AMOVA) within and among populations of *C.* ×*purpurea* using RAPDs

Source of Variation	df	SSD	MSD	Estimated Variance	Total (%)	*P*
Among population	1	1775.307	175.307	8.777	67	-
Within population	37	157.871	4.265	4.265	33	0.001*

* *P* values are the probability of obtaining a more extreme component by chance alone.
Notes:
df – degrees of freedom
SSD – sum of squared deviatios
MSD – mean squared deviation

RESULTS

DNA extraction

Good quality DNA was obtained using CTAB DNA extraction method by Doyle and Doyle (1987). The DNA then was treated with RNase before PCR amplification.

Genetic polymorphism and RAPD patterns

Four random primers, namely, OPA 7, OPE 14, OPH 4 and OPH 5 generated a total of 57 scorable bands ranging from 330 bp to 2600 bp. Products with molecular weight less than 330 bp cannot be consistently amplified thus they were not included in the analysis. Out of these 57 bands, 49 of them were polymorphic and 8 were monomorphic (Table 2). The number of bands ranged from 11 to 17 per primer.

Table 2: Details of the four random primers used and results obtained

Primer	Sequence	Amplified bands	Polymorphic bands
OPA - 07	GAAACGGGTG	17	15
OPE - 14	TGCGGCTGAG	13	13
OPH - 04	GGAAGTCGCC	17	16
OPH - 05	AGTCGTCCCC	11	5
	Total	57	49

Genetic relatedness

The use of Jaccard's similarity to calculate genetic relatedness among all samples results in different similarity values between samples. The smallest similarity value was 0.25 between samples 2-14 and 1-9 while the biggest similarity values separated all samples into two main groups; group 1 and group 2 as shown in the NJ tree (Figure 1). The groupings correspond to individuals from the two different locations, that is, group 1 are those individuals from Kelantong Swamp and group 2 comprises of individuals from Pos Iskandar. The relationship among individuals and populations can also be summarized by principal coordinate analysis (Figure 2). About 59.8% of the total variation was described by the first two axes. This analysis revealed the same clustering pattern as the NJ dendogram which resolved into two distinct clusters. Analysis of molecular variance AMOVA revealed that 67% of the total genetic variance occurred was due to among populations variance and 33% occurred among individuals within populations.

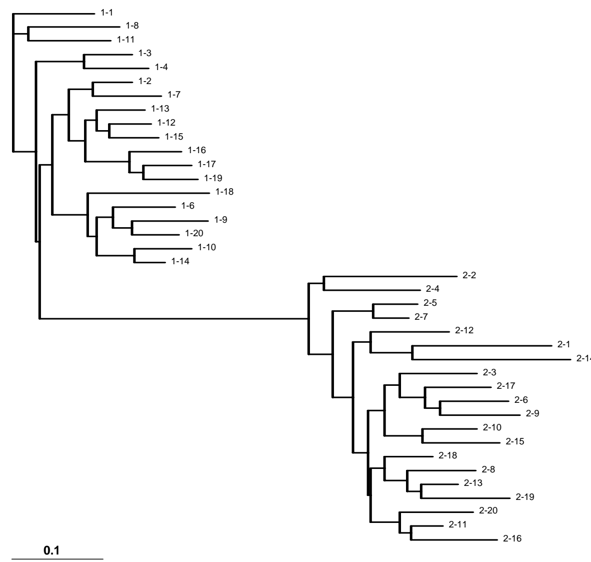

Figure 1: A Neighbor-Joining tree illustrating the genetic relationships among 39 individuals of *C.* ×*purpurea* from two populations. The scale above represents genetic distance.

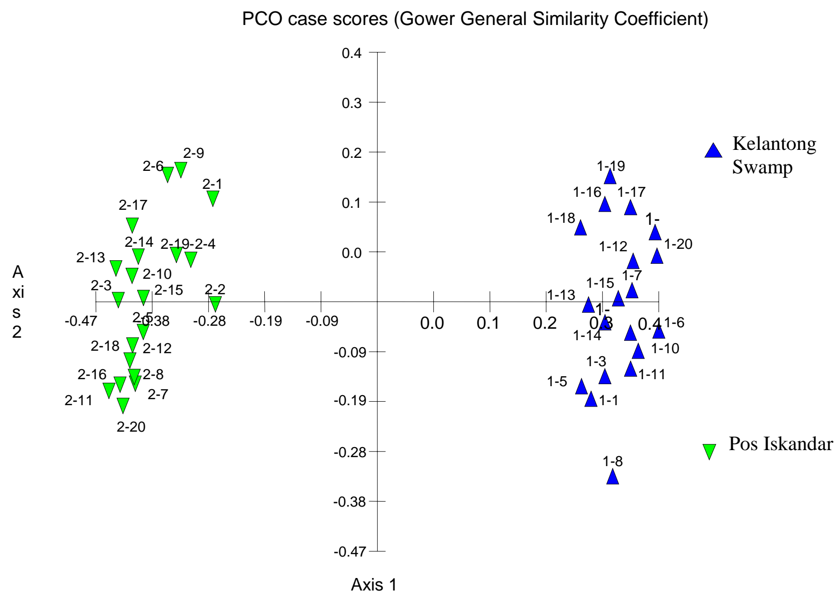

Figure 2: Principal coordinate analysis of RAPD data of *C.* ×*purpurea* clearly separated samples from Kelantong swamp populations from those of Pos Iskandar

DISCUSSION

This study provides the first characterization of molecular genetic diversity among populations of *C.* ×*purpurea,* namely, from Tasik Bera. It also highlights the usefulness of RAPD markers as an efficient and inexpensive tool for conservation based studies, namely, for endangered or plants of restricted distribution.

The low genetic variation among individuals of *C.* ×*purpurea* within a population is evident from the fact that out of the 60 primers initially screened, only four primers produced 49 polymorphic markers. This also corresponds to the finding that genetic variation in aquatic angiosperms is generally low (Laushman 1993) and this might be due to a cocktail of reasons such as the uniform nature of the aquatic habitat, high incidence of asexual reproduction and long distance dispersal of shoots or seeds.

It is assumed that genetic diversity was lower for clonal plants than for non-clonal plants (Harper 1977). However, sufficient growing body of data indicated that populations of clonal plants could maintain substantial amounts of genetic variation (Ellstrand & Roose 1987; Eckert & Barett 1993; Widen *et al*. 1994). In our case, as pointed by Jacobsen (1986) the whole population of *C.* ×*purpurea* in Tasik Bera is assumed to be hybrids that have low pollen fertility. Therefore, this species cannot reproduce sexually and propagate effectively through production of runners and dominant buds. We visited the two locations in Tasik Bera twice during our studies both during the month of June or August. We observed flowers on both occasions but never found any seeds.

The only possible explanation for the clonal diversity found in *C.* ×*purpurea* populations is due to somatic mutations. Clonal plants can be very long-lived and therefore, somatic mutations may eventually lead to some mutation (Persson & Gustavson 2001). In Tasik Bera, *C.* ×*purpurea* grow in shaded, slow running stream away from direct sunlight. The distance between the two locations is quite far (approximately 20km apart) and very much isolated. In between these two localities lies a large body of water (swampy lake) that is directly exposed to sunlight. These two populations might have originated from a single hybridization event and were separated and isolated and accumulates somatic mutations. Alternatively, they can also be a result of two or several hybridization events with different genetic make up and later being isolated they can accumulate somatic mutations. The high level of among populations genetic diversity obtained in this study seems to suggest the latter event might be more probable.

The information gained from investigating the genetic variation of populations of rare or endangered plant species is important in developing a sound conservation program for these plants. Results from the NJ tree and the PCO clearly show that the two populations are quite distinct. This further supports the view that the two populations might be a result of two separate hybridization events. Although *C.* ×*purpurea* represents an evolutionary dead-end, the two populations are genetically quite different. Therefore, a good conservation strategy for the species would be to protect both localities/habitats from any disturbance that could threaten the survival of the species in the wild.

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