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BRIEF COMMUNICATION

Primer design and optimization for RAPD analysis of *Nepenthes*

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Abstract

Primers with higher G+C content produced better random amplified polymorphic DNA (RAPD) profiles in *Nepenthes*. The occurrence of clustered G's and C's in the center of the primer seemed also to influence the banding patterns. It was also observed that for certain polymerases, the use of different buffers other than that recommended by the manufacturer provided a better amplification profile for *Nepenthes*.

Additional key words: GC content, PCR buffer, RAPD markers.

The Random Amplified Polymorphic DNA (RAPD) method of DNA amplification (Williams *et al.* 1990) has been widely used as a means of generating genetic markers in many organisms. These markers can represent polymorphic sites between different taxa and is thus useful in population studies, phylogenetics and genetic linkage mapping. Despite its vast potential, many research groups have encountered problems in reproducing RAPD fingerprint patterns. Optimisation of the RAPD method is thus imperative and should improve reproducibility of RAPD experiments. As the RAPD method is a modification of the polymerase chain reaction (PCR), parameters that can be optimised for RAPD are similar to that of PCR. Here we describe 2 of those parameters, primer design and buffer-polymerase compatibility.

Primer design affects the success of RAPD. Although in essence, the RAPD method uses arbitrary primer sequences, many of these primers must be screened in order to select primers that provide useful amplification information. Studies have shown that the use of high G+C % primers in RAPD generally works well in many organisms ranging from cassava to fungi (Kubelik *et al.* 1995, Wong *et al.* 1997). We tested 20 random primers with 60 - 70 % G+C content on genomic DNA extracted from leaves of several *Nepenthes* plants. Based on this sequence, a few primers were designed. These were categorized into primers that should yield useful amplification and primers that should have no or few amplification products.

Several studies have also shown that the composition/brand of reaction buffer used, has a marked effect on RAPD product quality and quantity (Levi *et al.* 1993, Johansson *et al.* 1995, Hilton *et al.* 1996). We also attempted to find the best combination of buffer and polymerase for RAPD analyses of *Nepenthes*.

Leaf samples and voucher specimens of *Nepenthes ampullaria* Jack, *Nepenthes gracilis* Korth. and *Nepenthes rafflesiana* Jack (*Nepenthaceae*) were collected from Johor, Malaysia. The leaves (0.5 g) were ground to a fine powder in liquid nitrogen in a mortar using a pestle. The powder was resuspended in 1 cm^3 DNA extraction buffer containing 1.875 % hexadecyltrimethylammonium bromide (CTAB), 37.5 mM Tris HCl (pH 8), 7.5 mM EDTA (pH 8), 1.3 M NaCl, 29 mM 2-mercaptoethanol, 20 mg cm^{-3} polyvinylpyrrolidone (PVP)-10, and 2 % sodium dodecyl sulphate (SDS). The contents were subsequently heated at 65 °C for 30 min. This was followed by addition of 1 cm^3 of chloroform and centrifugation at 10 000 *g*. A second extraction was carried out on the supernatant by addition of 0.2 cm^3 extraction buffer containing 10 % CTAB and 0.7 M NaCl together with 1 cm^3 chloroform. DNA was ethanol precipitated and resuspended in 0.2 cm^3 sterile distilled water. This was treated with 0.001 cm^3 RNase (10 mg cm^{-3})

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and incubated for 30 min at 37 °C. The DNA was extracted with phenol:chloroform (1:1) and precipitated with 0.02 cm^3 3 M sodium acetate and 0.5 cm^3 100 % ethanol. The DNA was washed in 70 % ethanol and resuspended in 0.05 cm^3 TE. The RAPD analysis was carried out in a 0.025 cm^3 reaction volume containing 25 ng template, 0.4 mM dNTP (*New England Biolabs,* USA), 4 mM $MgCl_2$, 1 mM primer, 1.25 U Taq polymerase and 1 × PCR reaction buffer ($MgCl_2$ free). Samples were cycled in a *PTC 100 Peltier Thermocycler* (*MJ Research Inc.*, Watertown, USA) using the following thermal profiles: 95 °C, 1 min; 32 °C, 1 min; 72 °C, 2 min for 45 cycles followed by a final extention at 72 °C for 10 min. Amplification products were resolved in 2 % agarose gels in 1 × TAE buffer and visualized using ethidium bromide staining (Sambrook *et al.* 1989). Thirty different primers were used. OPU primers (Kit U) were purchased from *Operon Technologies* (Alameda, USA). C and D series primers were from *Genset Singapore Biotech Pte Ltd.* (Singapore) and Taq polymerases from *Promega* (Madison, USA) was used. Twenty Operon Kit U arbitrary primers were screened. These primers have G+C content of 60 - 70 %. Out of the 20 primers, only 9 produced a substantial amount of amplification fragments for most of the templates (Table 1). The other primers either produced no or few amplication products. Of the 9 primers, 7 have a 70 % G+C content. These primers have a common G+C-rich centre. Based on this G+C content, primers C1 - C5 were designed. D1 - D5 primers were designed using the reverse as a rule (A's and T's in place of G's and C's). These primers are based on the OPU primers which worked badly during the screening (Table 1).

Table 1. Primers used.

Source of primers	Names	Sequence: (5' - 3')	G+C [%]
Operon series	OPU 1	ACGGACGTCA	60
	OPU 5	TTGGCGGCCT	70
	OPU 10	ACCTCGGCAC	70
	OPU 14	TGGGTCCCTC	70
	OPU 15	ACGGGCCAGT	70
	OPU 16	CTGCGCTGGA	70
	OPU 18	GAGGTCCACA	60
	OPU 19	GTCAGTGCGG	70
	OPU 20	ACAGCCCCCA	70
C series	C1	ACGGGCGCCA	80
	C2	ACGGGCCCCA	80
	C3	ACGGCCGCCA	80
	C4	ACGGCCCCCA	80
	C5	ACGCGCGCGA	80
D series	D1	CAATAATAAC	20
	D2	CATACCCATC	50
	D3	CCTTTTCCTC	50
	D4	CCTTCCTTCC	60
	D5	CTCTCTCTCT	50

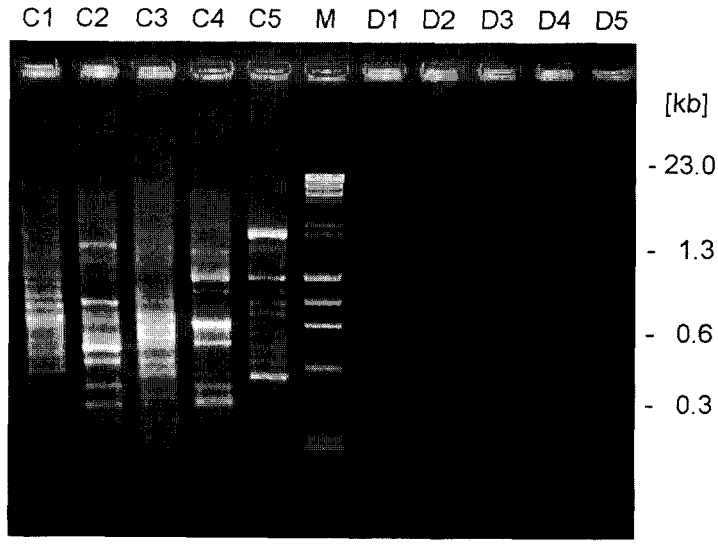

Fig. 1. Amplification profiles of *Nepenthes rafflesiana* with primers C1 - C5 and D1 - D5. *Lane* M - λ *Hind* III and ϕx-174 *Hae* III DNA markers.

Our results show that true to the general pattern observed from the screening, all the C series primers produced relatively more amplification fragments for all templates used. D series primers produced no amplification fragments for most of the templates (Fig. 1). This result conforms to our sequence hypothesis that strings of Gs and Cs in primers yield better amplification.

We also noted that the use of the recommended manufacturer's reaction buffer was not optimum for the RAPD method for *Nepenthes*. It was found that the buffer provided by the Promega Taq polymerase (100 mM Tris HCl, 500 mM KCl, 1 % Triton) was less efficient for the

RAPD method of *Nepenthes* when compared with a buffer containing 200 mM Tris HCl, 500 mM KCl with no Triton added.

In conclusion, primers with 70 - 80 % G+C content with clustered G's and C's in the centre produced more informative banding profiles for *Nepenthes*. It can be speculated that the *Nepenthes* genome consists of many regions of multiple G's or C's, thus resulting in increased amplification when such complementary primers are used. The success of RAPD analysis of *Nepenthes* is also dependent upon the buffer-polymerase compatibility. Manufacturer's recommended buffer-polymerase combination may not work optimally for all RAPD experiments.

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