

Optimization of DNA amplification fingerprinting parameters in coconut (*Cocos nucifera* L.)

† R. Manimekalai, K. Jeya Dev, Anuradha Upadhyay†, K. Devakumar, M. K. Rajesh
V.A. Parthasarathy*, V. Rajagopal and P. M. Kumaran

Central Plantation Crops Research Institute, Kasaragod, Kerala, India.

(Manuscript Received 27-11-2003; Revised : 6-3-2004; Accepted : 8-9-2004)

Abstract

DNA Amplification Fingerprinting (DAF) is a simple and powerful technique for genome analysis. It is being employed in various applications in a number of crops. For obtaining reproducible results from DAF technique, optimization of PCR parameters was done. 20 ng (2 ng/ μ l) template DNA, 15 μ M primer, 250 μ M each of dNTPs, 2 U (0.2 U/ μ l) Taq polymerase, 4 mM $MgCl_2$ and annealing temperature of 55 °C, with standard silver staining protocol gave good amplification with reproducibility. Subsequently these results will be used in tagging resistance genes for root (wilt) disease in coconut. The effect of GC content of primers was also discussed.

Key words:[†] Coconut, DNA amplification fingerprinting (DAF), optimization,

Introduction

DNA Amplification Fingerprinting (DAF) is an amplification based nucleic acid marker technique. DAF is distinguished from other amplification techniques by the high primer to template ratio, simplicity, reproducibility and high multiplex ratios. Independent of prior knowledge of DNA sequence, this requires experimental manipulation for reproducibility. Due to exponential nature of PCR amplification, small variations in the reaction components and thermal condition can greatly influence the final yield of the amplification products (Livak, 1996).

DAF has been used for a number of applications viz., analysing phylogenetic relationship (Cerny *et al.*, 1996; Prakash *et al.*, 1996; Nagaraju *et al.*, 2003), pedigree analysis, molecular characterization, genetic diversity analysis, genetic mapping (Caetano-Annolles *et al.*, 1999; He *et al.*, 1995), marker assisted selection (Prabhu and Gresshoff, 1994) in a variety of crops.

The DAF results depend on primer sequence, dNTPs, enzyme, Mg and primer concentration, annealing temperature etc. Present study attempts to optimize DAF parameters for coconut DNA to obtain reproducible results. We utilized DAF technique in coconut to check its suitability for detection of polymorphism and subsequent use in tagging root (wilt) resistance genes for selecting the mother palms for resistance breeding.

Materials and Methods

DNA from the cultivar West Coast Tall (WCT) was used for all the experiments. DNA was extracted as described by Upadhyay *et al.* (1999). For optimization, various levels of different reaction components were used. Two sets of DNA concentration (1, 3, 5 and 10, 20, 35 ng), two sets of primer concentration (5, 7, 10 and 7.5, 15, 20 μ M), dNTPs (200, 250, 500 μ M), Taq polymerase (1, 2 and 3 U), annealing temperature (45, 50 and 55 °C), $MgCl_2$ (3, 4, 5 μ M) with different combinations of PCR components were used.

* Indian Institute of Spices Research, Calicut, Kerala, India

† National Research Center for Grapes, Pune, India

*Corresponding author Email: partha_in@hotmail.com

DAF primers

A total of 16 decamer primers from University of British Columbia obtained through Dr. C. S. Prakash (Tuskegee University) were used (Table 1). The optimized conditions were tested with all the 16 primers.

Table 1. Primers, their sequence, GC/AT ratio and number of scorable bands

Primer	Sequence 5'-3'	GC/AT	No. of scorable bands
UBC2	CCTGGGCTTA	6:4	29
UBC8	CCTGGGGTA	7:3	31
UBC52	TTCCCGGAGC	7:3	27
UBC66	GAGGGCGTGA	7:3	30
UBC72	GGGCTCGTGG	8:2	34
UBC83	GGGCGCGTGG	8:2	18
UBC84	GGGCGCGAGT	8:2	25
UBC87	GGGGGGAAGC	8:2	26
UBC88	CGGGGGATGG	8:2	34
UBC100	ATCGGTCCG	7:3	14
UBC321	ATCTAGGGAC	5:5	18
UBC351	CTCCGGTGG	8:2	34
UBC356	GCGGCCCTCT	8:2	36
UBC701	CCCACAACCC	7:3	15
UBC729	CCCAACCCAC	7:3	28
UBC800	TCTCCCTCCT	6:4	33

PCR amplification

PCR amplification was carried out in 10 µl reaction volume in MJ Research DNA engine. A temperature profile of 94 °C for 5min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min and finally 72 °C for additional 8 min to complete the reaction was used. Amplification products were electrophoresed in 0.4 mm thick (16 cm X 18 cm) 5 % polyacrylamide-7 M urea denaturing gels. Wells were loaded with 5 µl of each amplification reaction products mixed with 5 µl of loading buffer (40% Urea, 3% Ficoll, 0.02% xylene cyanol FF, 0.02% Bromophenol blue in Tris- EDTA buffer, pH 8.3). Electrophoresis was carried out at 15W for 40 min. The gels were stained with silver nitrate using the technique of Bassam *et al.* (1991). Gels were preserved by drying at room temperature.

Fingerprint analysis

The interactions between DAF parameters were evaluated by using pooled level approach (Cobb and Clarkson, 1994). Number of scorable bands (N) was counted in each treatment. Pooled values (P) of N corresponding to particular treatment were used to calculate signal to noise ratio (S) for each reaction component.

$$S = -10 \log [1/n \sum_{i=1}^n S^2 / p_i^2]$$

where, n- number of levels

For each reaction component, the optimum level was deduced from observed maxima of S value.

Results and Discussion

DNA concentration

DNA concentration of 20 ng (2 ng/µl) was found to be giving distinct scorable bands. DNA of 1 ng, 3 ng, and 5 ng also gave amplification but the lower molecular weight bands were not scorable. 10 ng concentration gave amplification but reproducibility was not as high as in the case of 20 ng DNA.

DNA and primer concentration

Amplification was obtained with different primer concentrations (5, 7 and 10 µM) with different DNA concentrations (1, 3, and 5 ng). 1 ng template DNA didn't give good amplification with 5 µM primer, but gave amplification at 7 µM and 10 µM. Like wise 3 ng and 5 ng template DNA is not amplifiable at 5 µM primer concentration (Fig. 1a). Although amplification was obtained with lower template concentration, reproducible pattern was obtained with high template concentrations (> 1 ng/µl). Even though scorable amplification pattern was obtained with all three DNA concentrations (10, 20 and 35 ng) with different primer concentrations (7.5, 15 and 20 µM), 7.5 µM produced less intense of bands compared to 15 and 20 µM concentration. Consistent fingerprints were obtained with primer concentration of 15 µM and 20 ng template DNA with scorable range from 100 bp to 1500 bp (low and high molecular weight range) and S value was maximum at 15 µM primer and 20 ng DNA (Fig. 1b, Table 2)

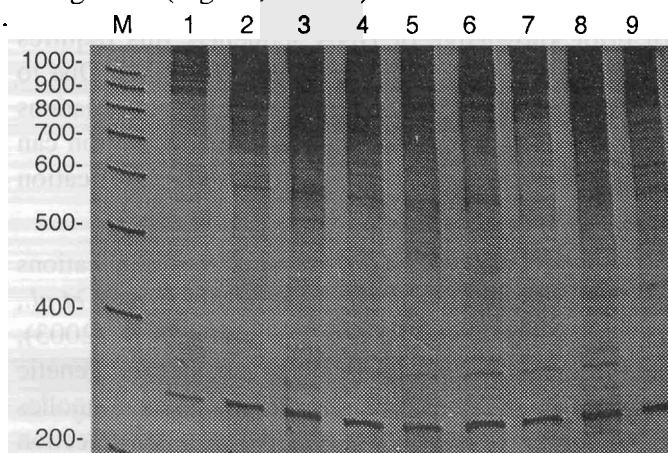


Fig. 1 a. PCR amplification of varying concentration of coconut DNA and primer. Total DNA from coconut cultivar WCT was amplified with Taq polymerase and primer (UBC66). Each electrophoretic lane was loaded with 5 µl of amplification reactions. Lanes 1-3: 1 ng DNA, Lanes 4-6: 3 ng DNA, Lanes 7-9: 5 ng DNA, each with 5, 7, and 10 µM primer respectively. M: Molecular weight markers in bp

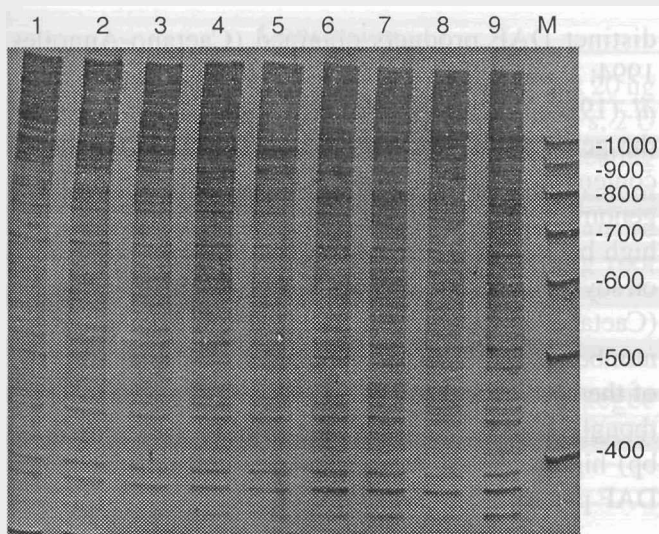


Fig. 1b. Lanes 1-3: 10 ng DNA, Lanes 4-6: 20 ng DNA, Lanes 6-9: 35 ng DNA with 7.5, 15 and 20 μ M primer respectively. M: Molecular weight markers in bp

Table 2. PCR parameter and S value

PCR components	S value
Primer	
7.5 μ M	27.4
15 μ M	29.5
20 μ M	25.2
DNA	
10 ng	27.1
20 ng	29.0
35 ng	25.9
dNTPs	
200 μ M	24.3
250 μ M	26.9
500 μ M	23.7
Enzyme	
1 U	25.2
2 U	28.2
3 U	26.8
Mg⁺⁺	
3 mM	27.8
4 mM	28.8
5 mM	27.9
Annealing temperature	
45 $^{\circ}$ C	27.3
50 $^{\circ}$ C	28.1
55 $^{\circ}$ C	28.2

DNA and Enzyme concentration

10, 20, and 35 ng DNA were tested with 1 U, 2 U and 3 U Taq DNA polymerase (15 μ M primer, 4 μ M MgCl₂, 250 μ M each dNTPs, 55 $^{\circ}$ C annealing temperature). All the three enzyme concentrations (1 U, 2 U and 3 U) gave amplification with all 3 DNA concentrations. 20 ng DNA with 2 U enzyme resulted in the most intense and sharp bands. 35 ng DNA produced

fused banding pattern with 2 U and 3 U enzyme. S value was maximum at 2 U of enzyme.

dNTPs concentration and annealing temperature

dNTPs concentration of 200 μ M (each dCTP, dGTP, dATP, dTTP) gave lesser intense bands therefore was not sufficient for amplifying DNA of 20ng with 15 μ M primer concentration. At 500 μ M concentration, amplification was obtained with some artifacts in high molecular weight range. dNTPs of 250 μ M, 20 ng DNA and 15 μ M primer gave good amplification pattern (Fig. 2). Although amplification was obtained with 3 concentrations of dNTPs both at 50 and 55 $^{\circ}$ C annealing temperature, band intensities varied at each level. At 50 $^{\circ}$ C annealing temperature, sharpness of bands was reduced with 500 μ M dNTPs, sharp intense bands were obtained with 250 μ M and faint banding pattern was observed with 200 μ M. At 55 $^{\circ}$ C annealing temperature scorable sharp bands were obtained with 500 μ M and 250 μ M, and bands were faint with 200 μ M. 250 μ M of each dNTPs found to be giving a more reproducible banding pattern compared to 500 μ M dNTPs. Annealing temperature of 50 $^{\circ}$ C resulted in non-specific amplification (Fig. 2). Low annealing temperature with high dNTPs and high primer concentration promote mispriming and accumulation of non-specific products (Caetano- Annolles, 1998). Hence 55 $^{\circ}$ C annealing temperature was selected for DAF analysis. Maximum S value was realized at 250 μ M dNTPs at 55 $^{\circ}$ C annealing temperature.

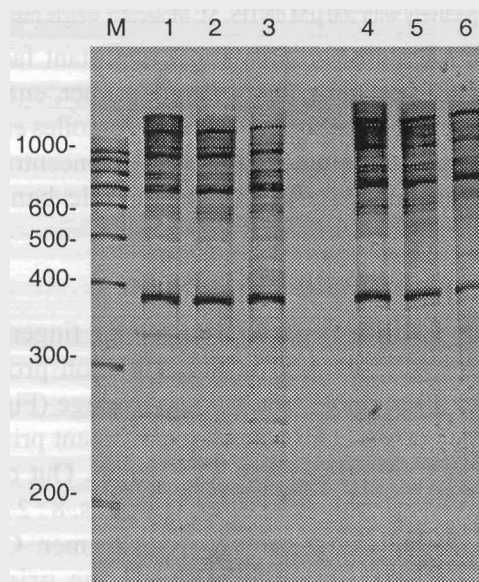


Fig. 2. PCR amplification of 20 ng DNA at varying dNTPs concentration with 50 $^{\circ}$ C and 55 $^{\circ}$ C annealing temperature. Lanes 1-3: 200 μ M, 250 μ M and 500 μ M dNTPs respectively with 55 $^{\circ}$ C annealing temperature. Lanes 4-6: 200 μ M, 250 μ M and 500 μ M dNTPs respectively with 50 $^{\circ}$ C annealing temperature. M: Molecular weight markers in bp

Mg⁺⁺ and dNTPs concentration

MgCl₂ concentration at 3 mM and 4 mM gave optimum intensity of bands with 250 μM dNTPs. 500 μM did not result in proper amplification. MgCl₂ at 5 mM produced non-specific bands that appeared diffused. S value maximized at 4 mM MgCl₂. 200 μM dNTPs produced background with all the three MgCl₂ concentration. Hence 4 μM, which gave optimum banding intensity and consistent profiles was selected for fingerprint analysis (Fig. 3)

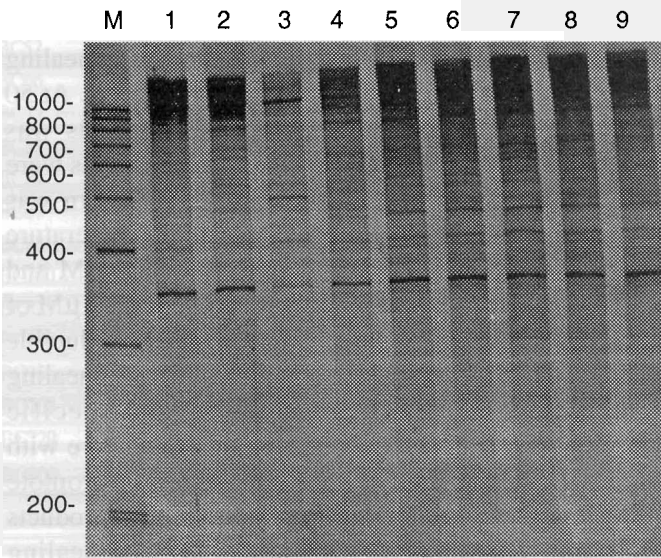


Fig. 3. PCR amplification of 20 ng DNA at varying MgCl₂ concentration with varying dNTPs concentration. Lanes 1-3: 5, 4 and 3 mM MgCl₂ respectively with 500 μM dNTPs. Lanes 4-6: 5, 4 and 3 mM MgCl₂ respectively with 250 μM dNTPs. Lanes 7-9: 5, 4 and 3 mM MgCl₂ respectively with 200 μM dNTPs. M: Molecular weight markers in bp

For DAF reproducibility most important factors suggested were annealing temperature, primer, enzyme and magnesium concentration (Caetano-Anolles *et al.*, 1999). Our results recommend that dNTP concentration also as important for getting reproducible banding pattern.

Fingerprint pattern with UBC primers

All the 16 primers gave characteristic fingerprint pattern with WCT DNA. The amplification profiles contained 25-30 scorable bands on an average (Fig. 4a and 4b; Table1). The DAF pattern for different primers were compared with different GC/AT ratio. Out of 16 primers, the GC/AT ratio is 7 : 3 for 7 primers, 8 : 2 for 6 primers, 6 : 4 and 5 : 5 each for one primer. Good amplification was obtained with all the primers irrespective of their GC content (Fig. 4b). This demonstrated that DAF pattern is independent of the primer GC content Earlier studies also suggested that the GC content of the primer had no correlation with either the amplification itself or with the total number of

distinct DAF products obtained (Caetano-Anolles, 1994; Prabhu and Gresshoff, 1994). In contrast, Sen *et al.* (1997) suggested the dependence of DAF pattern on the genome size of the species examined and the GC content of the primer used and reported that the larger genome size and high GC content of the primer result in high background smear DAF pattern in wheat. Studies on soybean, caucasian human and *Staphylococcus aureus* (Caetano-Anolles *et al.*, 1991) also indicated that the number of DAF products depended on the genome size of the species and the GC content of the primer. Even though the coconut has larger genome size (3.6 X 10⁹ bp) higher GC/AT ratio of primer did not give smear DAF pattern.

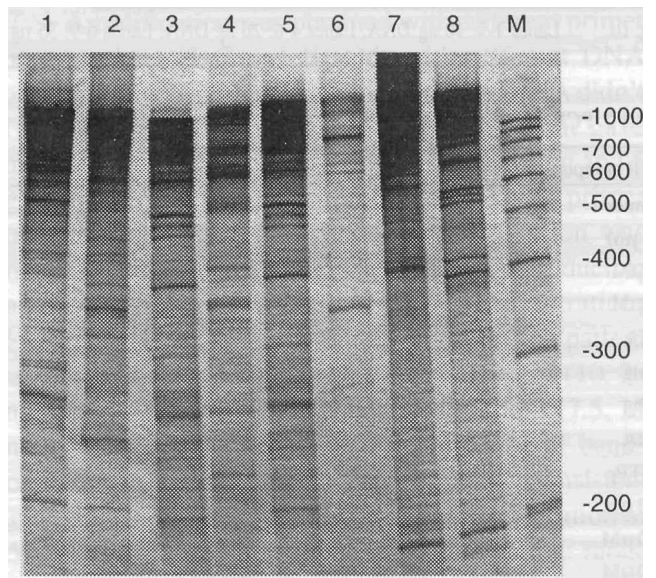


Fig. 4a. PCR amplification of 20 ng DNA with 16 UBC primers (15 μM primer, 2 U Taq DNA polymerase, 250 μM each dNTPs, 4 mM MgCl₂, 55 °C annealing temperature). Lanes 1-8: UBC2, UBC8, UBC52, UBC66, UBC72, UBC83, UBC84, UBC87 respectively. M: Molecular weight markers in bp.

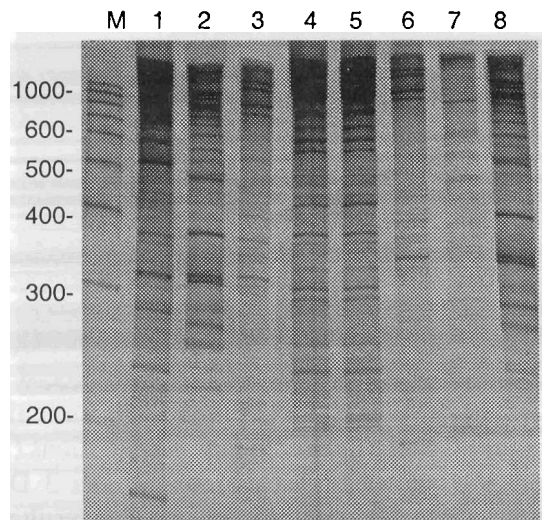


Fig. 4b. Lanes 1-8: UBC800, UBC729, UBC701, UBC356, UBC351, UBC321, UBC100, UBC88 respectively. M: Molecular weight markers in bp.

Conclusion

From the foregoing it can be concluded that 20 ng template DNA, 4 mM MgCl₂, 250 μM each dNTPs, 2 U Taq polymerase enzyme, 15 μM primer, 55 °C annealing temperature are essential for reproducible DAF pattern of coconut DNA. These PCR conditions can be successfully used for developing markers to tag root (wilt) resistance genes.

Acknowledgement

Authors thank Dr. C. S. Prakash (Tuskegee University) for providing the primer sequence and NATP for the financial assistance.

References

- Bassam, B.J., Caetano-Annolles, G. and Gresshoff, P.M. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* **196**: 80-83.
- Caetano-Annolles, G., Bassam, B.J. and Gresshoff, P.M. 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Biotechnology*. **9**:553-557.
- Caetano-Annolles, G. 1994. MAAP: A versatile and universal tool for genome analysis. *Plant Molec. Biol.* **25**: 1011-1026.
- Caetano-Annolles, G. 1998. DAF optimization using Taguchi methods and the effects of thermal cycling parameters on DNA amplification. *Biotechniques*. **25**:472-480.
- Caetano-Annolles, G., Schlarbaum, S.E. and Trigiano, R.N. 1999. DNA amplification fingerprinting and marker screening for pseudo-testcross mapping of flowering dogwood (*Cornus florida* L.). *Euphytica*. **106**: 209-222.
- Cerny, T.A., Caetano-Annolles, G., Triagiano, R.N. and Starman, T.W. 1996. Molecular phylogeny and DNA amplification fingerprinting of *Petunia taxa*. *Theor. Appl. Genet.* **92**: 1009-1016.
- Cobb, B. D. and Clarkson, J.M. 1994. A simple procedure for optimizing the polymerase chain reaction (PCR) using modified Taguchi methods. *Nucleic Acids Res.* **22**: 3801- 3805.
- He, G., Prakash, C.S. and Jarret, R.L. 1995. Analysis of genetic diversity in a sweet potato (*Ipomoea batatas*) germplasm collection using DNA amplification fingerprinting. *Genome*. **38**: 938-945.
- Livak, K. J. 1996. Quantitation of DNA/RNA using real time PCR detection. P-E *Applied Biosystems Technology Review*, Ref. no. 777902-001. 234 p.
- Nagaraju, V., He, G., Parthasarathy, V.A. and Prakash, C.S. 2003. Fingerprinting of coconut (*Cocos nucifera* L.) accessions using DNA markers. *J. Plant. Crops.* **31**: 8-13.
- Prabhu, R.R. and Gresshoff, P.M. 1994. Inheritance of polymorphic markers generated by DNA amplification fingerprinting and their use as genetic markers in soybean. *Plant Mol. Biol.* **26**:105-116.
- Prakash, C.S. and He, G. 1996. DNA marker based study of genetic relatedness in United States sweet-potato cultivars. *J. Amer. Soc. Hort. Sci.* **121**: 1059-1062.
- Sen, A, Balyan, H. S., Sharma, P. C., Ramesh, B, Alok kumar, Roy, J. K., Varshney, R. K., Gupta, P. K. 1997 DNA amplification fingerprinting (DAF) as a new source of molecular markers in bread wheat. *Wheat Information Service* **85**: 35-42.
- Upadhayay, A., Parthasarathy, V.A., Seema, G. and Anitha Karun 1999. An efficient method of DNA extraction from coconut leaves. *Agrotopica*. **11**:35-38

