อาร์เอพีดี-พีซีอาร์ที่เหมาะสมในการจำแนกสายพันธุ์งา
Optimization of RAPD-PCR for Identification of Oilseed Sesame

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บทคัดย่อ

อาร์เอพีดีเป็นเทคนิคที่ใช้ตรวจสอบความแตกต่างในระดับดีเอ็นเอที่ง่าย ไม่ซับซ้อนและเชื่อถือได้ ในการเพิ่มปริมาณดีเอ็นเอในปฏิกิริยาอาร์เอพีดี-พีซีอาร์นั้นพบว่ามีหลายปัจจัยที่มีผลต่อการเพิ่มปริมาณดีเอ็นเอ ซึ่งทำให้การวิเคราะห์ผลคลาดเคลื่อนไป วัตถุประสงค์ของการศึกษานี้เพื่อหา RAPD protocol ที่เหมาะสมสำหรับใช้ในการจำแนกสายพันธุ์งา โดยดำเนินการศึกษาหาระดับความเข้มข้นที่เหมาะสมของดีเอ็นเอต้นแบบ แมกนีเซี่ยมคลอไรด์ และ Taq DNA polymerase ที่ใช้ในการเพิ่มปริมาณดีเอ็นเอของงา จากการศึกษาพบว่าการเพิ่มปริมาณดีเอ็นเอที่เหมาะสมของสายพันธุ์งาในปฏิกิริยาอาร์เอพีดี-พีซีอาร์ในปริมาตร 20 ไมโครลิตรนั้นได้จากระดับความเข้มข้นของดีเอ็นเอต้นแบบ 40 นาโนกรัม แมกนีเซี่ยมคลอไรด์ 3.5 มิลลิโมลาร์ และ Taq DNA polymerase 1.5 ยูนิต จากนั้นดำเนินการคัดเลือกไพร์เมอร์อาร์เอพีดีที่ใช้ในปฏิกิริยาพีซีอาร์ที่สามารถให้แถบดีเอ็นเอที่ชัดเจน จากการคัดเลือกไพร์เมอร์ทั้งหมด 29 ไพร์เมอร์ พบว่า 18 ไพร์เมอร์ให้แถบดีเอ็นเอชัดเจนและสามารถใช้เป็นเครื่องหมายโมเลกุลดีเอ็นเอในการจำแนกพันธุ์งาได้ อาร์เอพีดี-พีซีอาร์เป็นเทคนิคที่มีประสิทธิภาพอีกเทคนิคหนึ่งที่ใช้ในการจำแนกความแตกต่างของสายพันธุ์และสามารถนำไปใช้ในการศึกษาความสัมพันธ์ทางพันธุกรรมของเชื้อพันธุ์งาได้

คำสำคัญ: การจำแนกพันธุ์พืช การปรับปฏิกิริยาพีซีอาร์ที่เหมาะสม งา อาร์เอพีดี-พีซีอาร์

Abstract

Random Amplified Polymorphic DNA (RAPD) technique is a simple and reliable method to detect DNA polymorphism. Several factors can affect the amplification profiles, thereby causing false bands and non-reproducibility of assay. In this study, we aimed to optimize RAPD protocol for identification of sesame varieties. Several concentrations of template DNA, $MgCl_2$, and *Taq* DNA polymerase were performed in four sesame varieties. Reproducible amplification patterns were obtained using 40 ng of template DNA, 3.5 mM of $MgCl_2$, and 1.5 U of *Taq* DNA polymerase in 20 μl of PCR reaction. The optimized RAPD-PCR reaction was then used for RAPD primer survey. Twenty-nine 10-base oligonucleotide primers were tested to determine which could produce scorable RAPD markers. Out of 29 primers

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screened, 18 revealed clear patterns of DNA amplifications and can be used as DNA markers to distinguish the sesame varieties. These selected primers will be useful for further genetic diversity study of sesame germplasm. The RAPD-PCR was proved to be a valuable tool for cultivar identification and genetic diversity study of sesame germplasm.

Keywords: cultivar identification, PCR optimization, RAPD-PCR, sesame

Introduction

The random amplified polymorphic DNA technique offers a powerful tool to detect DNA polymorphisms. The method is based on polymerase chain reaction (PCR) in which a single arbitrary primer able to anneal and prime at multiple locations through the genome which can produce a spectrum of amplified products that are characteristics of the template DNA (Welsh and McClelland, 1990; William et. al., 1990). The relative low cost of the technique and requirement of small amount of DNA template provide advantages in the utilization of RAPD in cultivar identification.

Sesame (Sesamum indicum L.), is one of the most ancient crops known to man (Bedigian and Harlan, 1986). It was a highly prized oilseed due to its resistance to drought, the ease to extract oil from seeds and the high stability of the oil (Langham and Wiemers, 2002). Sesame is grown primarily for its nutritious seed that is rich in unsaturated fatty acids (oleic and linoleic acid), protein and calcium as well as vitamin E, and small quantities of vitamin A, B1 and B2. Furthermore, its oils contain antioxidant called sesaminol, sesamin, sesamolin and sesamol which involve in a high degree of resistance against oxidative rancidity besides γ tocopherol (vitamin E) which is found in other plants (Ashri, 1989; Ubonratchathani Field Crop Research Center, 1998). Sesame seeds are widely used in various culinary preparations which its oil is mainly used in cooking and salad, and for making margarine. The oil is also mainly used in cosmetic preparations, pharmaceutical products, paints, soaps, and insecticides (Ashri, 1989, 1998). The meal left after oil extraction (sesame cake) contains 35-50% protein which makes a rich feed for poultry and livestock.

Methods for identification of sesame cultivars are primarily based on analysis of morphological characters. These, however, are unreliable indicators of plant genotype, and are influenced by both genetic and environmental factors. RAPD is an alternative method in detection of polymorphism in sesame. Being a fast and simple method, RAPD can be quickly and efficiently applied to identify useful polymorphisms (Waugh and Powell, 1992). RAPD markers are dominant markers and some loss of information may occur in comparison to the dominant markers such as RFLP and SSR markers. However, these can be compensated with the advantage of RAPD markers nature which not only requires no previous knowledge of DNA sequences but also requires small amount of DNA template (Welsh and McClelland, 1990; William et. al., 1990).

In this study, we described the optimization of RAPD-PCR technique through examining the

critical parameters, concentrations of DNA templates, MgCl2 and Taq DNA polymerase, as required to obtain reproducible DNA amplification for characterization of sesame varieties. Screening of RAPD primers suitable for sesame identification was performed using optimized RAPD-PCR.

Materials and Methods

Plant materials

The cultivars of sesame analyzed in this study are listed in Table 1. Sesame seeds were obtained from Ubonratchathani Field Crop Research Center and grown in greenhouse at Office of Field Experimentation and Central Laboratory, Faculty of Agriculture, Ubon Rajathanee University.

Table 2 RAPD primers with arbitrary sequences used in optimization of RAPD-PCR

Primer name	Sequence 5' to 3'
1. OPA 01	5' CAG GCC CTT C 3'
2. OPA 02	5' TGC CGA GCT G 3'
3. OPA 03	5' AGT CAG CCA C 3'
4. OPA 04	5' AAT CGG GCT G 3'
5. OPA 09	5' GGG TAA CGC C 3'
6. OPA 11	5' CAA TCG CCG T 3'
7. OPA 13	5' GTG CAC CCA C 3'
8. OPA 18	5' AGG TGA CCG T 3'
9. OPA 19	5' CAA ACG TCG G 3'
10. OPA 20	5' GTT GCG ATC C 3'
11. OPB 11	5' GTA GAC CCG T 3'
12. OPD 12	5' CAC CGT ATC C 3'
13. OPE 02	5' GGT GCG GGA A 3'
14. OPK 07	5' AGC GAG CAA G 3'
15. OPK 08	5' GAA CAC TGG G 3'
16. OPK 20	5' GTG TCG CGA G 3'
17. OPM 06	5' CTG GGC AAC T 3'
18. OPM 07	5' AGG CGG GAA C 3'
19. OPM 13	5' GGT GGT CAA G 3'
20. OPM 17	5' TCA GTC CGG G 3'
21. OPM 18	5' TCA GTC CGG G 3'
22. OPM 20	5' AGG TCT TGG G 3'
23. OPU 05	5' TTG GCG GCC T 3'
24. OPU 11	5' AGA CCC AGA G 3'
25. OPU 16	5' CTG CGC TGG A 3'
26. OPU 18	5' GAG GTC CAC A 3'
27. OPU 19	5' GTC AGT GCG G 3'
28. K 2	5' ACC TCG CCA C 3'
29. K 4	5' TGC TGG TTC C 3'

Total DNA Extraction

Total genomic DNA was extracted from 3 week-old sesame plants using the protocol as described by Lodhi et.al. (1994). The additional phenol-chloroform extraction and ethanol precipitation of DNA was included in the procedure to ensure the purity of DNA. Briefly, one to two grams of leaf tissue were ground in liquid nitrogen and incubated with 2% CTAB (cetyltrimetylammonium bromide) extraction buffer for 1 hour at 65 °C. The DNA was extracted once with chloroform-octanol (24:1). The aqueous phase was mixed with a half volume of 5 M NaCl, precipitate with two volume of cold 95% ethanol. DNA pellet was washed with cold 76% ethanol twice and dissolved in TE buffer (10mM Tris HCl and 0.1 mM EDTA, pH 8.0). The dissolved DNA samples were treated with RNase A (10 mg/ml) at 37°C for 1 hour. The DNA solution was further purified by extracted with phenol/chloroform/ isoamyl mixture (25:24:1) to remove protein contaminants, then precipitated with 100 % ethanol. The DNA is pellet after the precipitation step, washed with cold 70% ethanol to remove salt and small organic molecules, and re-suspended in TE buffer.

RAPD-PCR optimization

The primer OPA 04 was used to optimize the RAPD technique (Kit A, Operon Biotechnologies, Germany). The amplification was performed in a final volume of 20 µl containing 1X PCR buffer (Promega.), 200 µM of each dNTPs (Promega.), and 0.6 µM of primer with different concentrations of DNA templates (40 and 60 ng), $MgCl_2$ (2.5, 3, 3.5 mM) and *Taq* DNA polymerase (1 and 1.5 units) (Fermentus) in order to determine the optimal concentration for PCR reaction. The reaction were carried out in a thermal cycler (Perkin Elmer 9700) according to following amplification profile of initial denaturation at 94°C for five min, followed by 35 cycles of one min at 94°C, 1 min at 36 °C and two min of 72 °C. The reaction was further extended at 72°C for 10 min.

Amplification products were separated in 1.3 % agarose gel stained with ethidium bromide in Tris-Borate-EDTA (TBE) buffer for 2 hours at 80 Volts. Subsequently, gels were visualized on UV light in Gel documentation (Wealtec). DNA fragment size were estimated by comparison with standard marker, 1 kb DNA ladder (Promega).

RAPD analysis and primer selection

The optimized RAPD-PCR reaction was used for RAPD primer survey. Four sesame varieties, namely KU18, WL9, BL5 and BL6 were randomly selected for primer screening. The survey was carried out using 29 random 10-mer primers (Operon Biotechnologie, Germany) (Table 2). The primers that gave reproducible and scorable amplifications were selected and will be used for RAPD fingerprint of 14 sesame varieties.

Table 2 RAPD primers with arbitrary sequences used in optimization of RAPD-PCR

Primer name	Sequence 5' to 3'
1. OPA 01	5' CAG GCC CTT C 3'
2. OPA 02	5' TGC CGA GCT G 3'
3. OPA 03	5' AGT CAG CCA C 3'
4. OPA 04	5' AAT CGG GCT G 3'
5. OPA 09	5' GGG TAA CGC C 3'
6. OPA 11	5' CAA TCG CCG T 3'
7. OPA 13	5' GTG CAC CCA C 3'
8. OPA 18	5' AGG TGA CCG T 3'
9. OPA 19	5' CAA ACG TCG G 3'
10. OPA 20	5' GTT GCG ATC C 3'
11. OPB 11	5' GTA GAC CCG T 3'
12. OPD 12	5' CAC CGT ATC C 3'
13. OPE 02	5' GGT GCG GGA A 3'
14. OPK 07	5' AGC GAG CAA G 3'
15. OPK 08	5' GAA CAC TGG G 3'
16. OPK 20	5' GTG TCG CGA G 3'
17. OPM 06	5' CTG GGC AAC T 3'
18. OPM 07	5' AGG CGG GAA C 3'
19. OPM 13	5' GGT GGT CAA G 3'
20. OPM 17	5' TCA GTC CGG G 3'
21. OPM 18	5' TCA GTC CGG G 3'
22. OPM 20	5' AGG TCT TGG G 3'
23. OPU 05	5' TTG GCG GCC T 3'
24. OPU 11	5' AGA CCC AGA G 3'
25. OPU 16	5' CTG CGC TGG A 3'
26. OPU 18	5' GAG GTC CAC A 3'
27. OPU 19	5' GTC AGT GCG G 3'
28. K 2	5' ACC TCG CCA C 3'
29. K 4	5' TGC TGG TTC C 3'

Results and Discussions

Optimization of RAPD-PCR reaction

It is well known that problems related to standardization of amplification conditions with final reagent concentration in PCR cocktails have been observed. The laboratory practices showed that variable such as final reagent concentrations and template DNA, both quality and quantity, also influence the attainment of reproducible results. As essentially any primer can amplify very small amount of DNA from any organism, however, DNA purity is extremely important in order to obtain clear and discriminate patterns.

Our preliminary results indicated that DNA extraction from sesame has been difficult due to the presence of contaminant such as polyphenol and polysaccharides. These compounds have also been reported to cause difficulty in DNA purification in other plant species; and inhibit enzyme action. Polysaccharides are visually in DNA extracted by their viscous, glue like texture and make the DNA unmanageable in pipetting and unamplifiable in the PCR reaction by inhibiting Taq DNA polymerase (Fang et.al. 1992). Therefore, we have modified and employed the DNA extraction method for sesame which involved a modified CTAB extraction (modified from Doyle and Doyle, 1990), employing high salt concentrations to remove polysaccharides, the use of polyvinylpyrrolidone (PVP) to remove polyphenols (Lodhi et.al. 1994), and extended RNase treatment and a phenol-chloroform extraction. We have obtained clean DNA from sesame leaves using the modified DNA extraction procedure mention above and these DNA samples were ready for RAPD optimization.

The three parameters including template DNA, $MgCl_2$ and Tag DNA polymerase were studied to ensure that sesame RAPD profiles were selected in an informative and reproducible manner. The optimized RAPD-PCR technique was performed using several concentrations of template DNA (40 and 60 ng), $MgCl_2$ (2.5, 3, 3.5 mM) and *Tag* DNA polymerase (1 and 1.5 units), while maintaining constant concentrations of each dNTPs (200 mM), primer (0.6 μM) and PCR buffer (1X), using the same amplification profile in the thermocycler. The concentration of those parameters was selected based on clear and scorable DNA bands produced. The optimum reaction mixture which gave best fingerprint pattern was obtained using 40 ng of DNA template, 3 mM of $MgCl_2$ and 1.5 U of *Tag* DNA polymerase (Figure. 1). A higher or lower concentration resulted in either sub-optimal or complete lack of PCR amplifications.

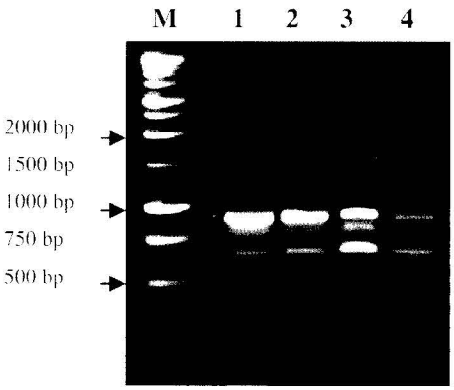

Figure 1 Agarose gel eletrophoresis of DNA fragments obtained by the optimized RAPD-PCR of four sesame varieties using primer OPA 04. (M = 1 Kb ladder DNA marker, 1 = KU 18, 2 = WL 9, 3 = BL 5 and 4 = BL 6)

The quality and quantity of DNA template are factors that also affect RAPD reproducibility (Fraga et.al., 2005). For the majority of organism, the amount of DNA template between 10 and 100 ng are sufficient to obtained complex and reproducible RAPD patterns. High amount of DNA usually inhibit amplification due to competition of the primers for the template DNA (Micheli et.al., 1994). Consequently, several quantities of DNA template should be tested for organisms that have not fully investigated, not only to ensure a large number of bands, but also in order to verify the optimized conditions for PCR.

Since the $MgCl_2$ is co-factor of the *Taq* polymerase enzyme, it influences the DNA amplification process. Lower quantities than the necessary ones lead to the amplification failure or to deficient amplification, to low experiments reproducibility. High concentrations of magnesium ion result in reaction specificity (Persing, 1993). In our experiment the 3.5 mm $MgCl_2$ was suitable to amplify sesame DNA.

Another factor that affects reproducibility of RAPD pattern is the quantity of Taq DNA polymerase. The intensity of bands increases correspondingly with increasing *Taq* DNA polymerase up to 2 U. After this concentration, the amplification profile is not affected by the enzyme concentration. Assay with 2.5 U showed that increased enzyme concentrations lead to decrease specificity. Thus, demonstrating another factor that affects the reproducibility and fidelity of RAPD pattern (Fraga, et.al. 2005).

Primer selection and survey

To determine the suitability of the RAPD technique in identifying DNA polymorphisms among sesame cultivars, twenty nine 10-base oligonucleotide primers from Operon Kits (Table 2) were initially screened against four sesame varieties, namely, KU18, WL9, BL5 and BL6. The initial screening under the optimized RAPD-PCR reaction was essential to select RAPD primers yielding distinctive DNA patterns which could be used as RAPD markers for sesame fingerprints. Of the 29 decamer primers screened, 24 revealed amplification products while 5 primers did not amplified detectable products (OPA09, OPE02, OPM17, OPM18 and OPU11) (Figure. 2). Among the 24 primers, the amplifications obtained using 6 primers (OPA17, OPA19, OPB11, OPD12, OPM07, and OPU16) were shown ambiguous RAPD patterns, therefore these primers were discarded from analysis. Following this initial screening the 18 primers were chosen to further identify and detect genetic variation of sesame germplasm (Table 3).

Preliminary RAPD fingerprint of sesame

We have shown preliminary RAPD fingerprint of 14 sesame varieties using the three selected primers, K2, OPM13 and OPA06 (Figure. 3). These primers gave distinctive and satisfactory amplification patterns. Generally, the prominent amplified DNA products were readily reproducible. Therefore, the 18 selected primers from this study will be useful for further genetic diversity study in Thai oilseed sesame germplasm.

Due to its fastest and simplest, RAPD technique has been widely used extensively for a variety of purposes which include cultivar identification (Virk et.al., 1995, Galderisi et.al., 1999), estimation of genetic diversity (Cao and Oard, 1997, Wang et.al., 1999). In sesame, RAPD has been employed to determine genetic diversity in Indian and exotic germplasm (Bhat et.al., 1999) and in Turkish sesame populations (Ercan et.al., 2004).

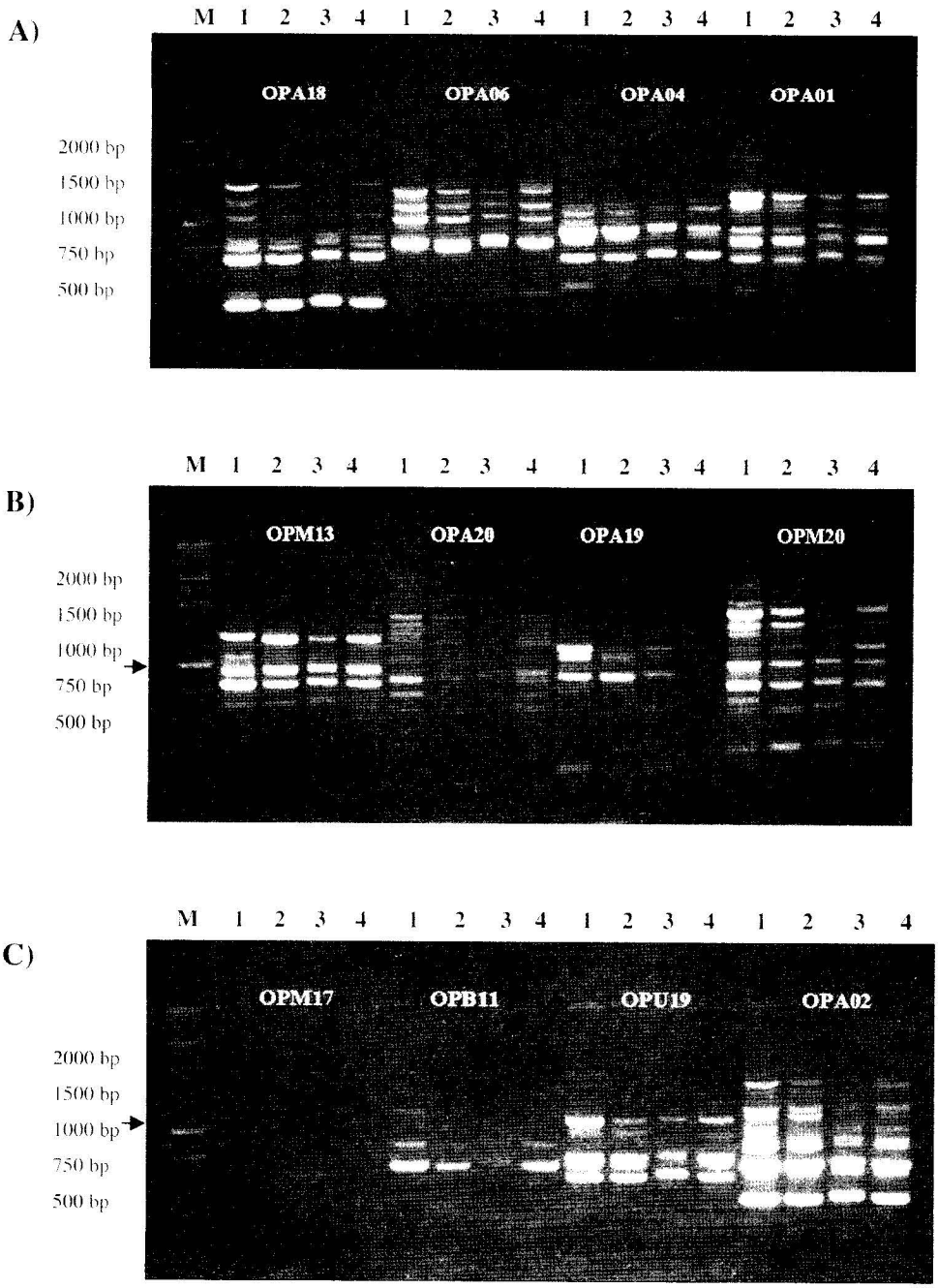

Figure 2 Agarose gel electrophoresis of DNA fragments obtained from RAPD primer screening of 4 sesame varieties (M = 1Kb ladder DNA marker, 1 = KU18, 2= WL 9, 3 = BL 5, 4 = BL 6). Panel A) Primer OPA18, OPA06, OPA04 and OPA02 Panel B). Primer OPM13, OPA20, OPA19 and OPM20

Panel C) Primer OPM17, OPB11, OPU19 and OPA02.

Table 3 The 18 selected primers along with their sequence, % GC content and number of DNA amplification products obtained from sesame screening.

Primer name	Sequence 5' to 3'	% G+C content	Number of DNA bands
1. OPA 01	5' CAG GCC CTT C 3'	70	7
2. OPA 02	5' TGC CGA GCT G 3'	70	8
3. OPA 03	5' AGT CAG CCA C 3'	60	5
4. OPA 04	5' AAT CGG GCT G 3'	60	7
5. OPA 06	5' GGT CCC TGA C 3'	70	7
6. OPA 13	5' GTG CAC CCA C 3'	70	8
7. OPA 18	5' AGG TGA CCG T 3'	60	7
8. OPA 20	5' GTT GCG ATC C 3'	60	10
9. OPK 07	5' AGC GAG CAA G 3'	60	9
10. OPK 08	5' GAA CAC TGG G 3'	60	7
11. OPK 20	5' GTG TCG CGA G 3'	70	8
12. OPM 13	5' GGT GGT CAA G 3'	60	7
13. OPM 20	5' AGG TCT TGG G 3'	60	9
14. OPU 05	5' TTG GCG GCC T 3'	70	10
15. OPU 18	5' GAG GTC CAC A 3'	60	6
16. OPU 19	5' GTC AGT GCG G 3'	70	5
17. K 2	5' ACC TCG CCA C 3'	70	9
18. K 4	5' TGC TGG TTC C 3'	60	4

Conclusion

We have presented a procedure to identify sesame varieties using RAPD analysis. RAPD fingerprint strategy should be applicable to sesame genotyping due to its rapidness, simplicity, low cost and potential to generate polymorphisms. Our results suggest that there is a considerable possibility for increasing the efficiency of the RAPD-PCR reaction if precise standardization protocol is determined. RAPD technique can be used successfully not only for cultivar identification but also for evaluation of genetic diversity of sesame germplasm.

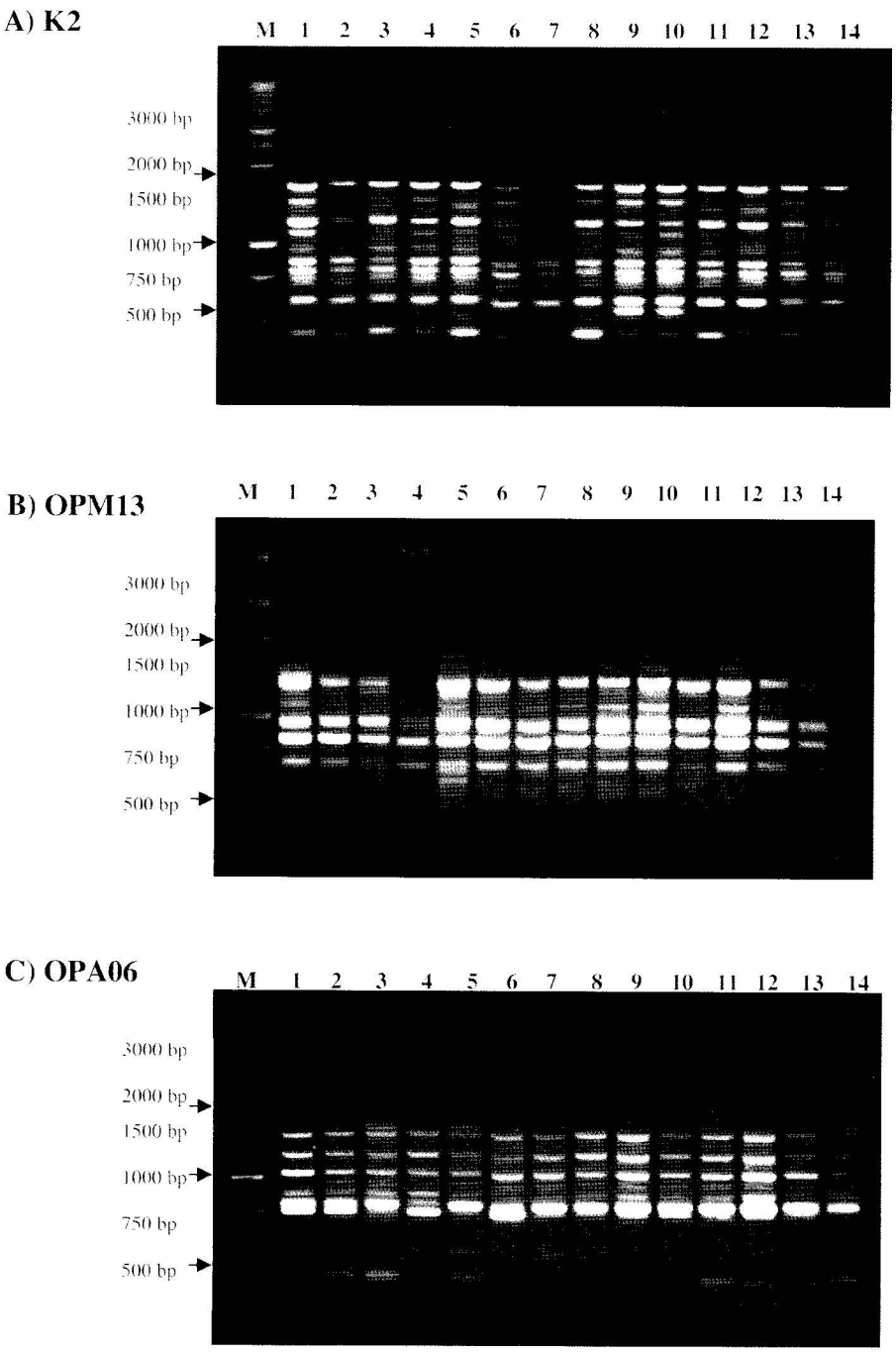

Figure 3 RAPD fingerprint of 14 sesame varieties using the selected primers K2 (A), OPM13 (B), and OPA06 (C). M = 1 Kb ladder DNA, 1 = WL 9, 2 = BL 5, 3 = BL 6, 4 = BL 1, 5 = Ubonratchathani 1, 6 = Ubonratchathani2, 7 = Ubonratchathani3, 8 = KKU1, 9 = Nakhonsawan, 10 = Phitsanulok, 11 = Bureerum, 12 = Chaibadan, 13 = WL7, 14 = Red sesame

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