
RAPD (random amplified polymorphic DNA) marker as a tool for hybrid oil palm verification from half mature zygotic embryo culture

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Hybrid verifications within the F1 population (Tenera) between Pisifera (72, 110, 172, 206, 558 and 777) and Dura (366 and 865) were detected by randomly amplified polymorphic DNA (RAPD) analysis. Firstly, DNA from half mature zygotic embryo of six combinations of hybrid were isolated and detected by RAPD using 7 arbitrary 10-mer primers. From all random primers tested they could amplify parental DNA. Secondly, half mature zygotic embryo consisted of coleoptiles region of those combinations was cultured on Murashige and Skoog (MS) medium plus various kinds and concentrations of auxins. The result revealed that OPT06 primers provided clearly DNA pattern and could be used for hybrid verification of the crosses 865 (D) × 206 (P). The highest frequency of callus formation (46.60%) was obtained on the medium supplemented with 2.50 mg/l 3,6-dichloro-o-anisic acid (dicamba) containing MS medium, significant difference to other kinds and concentrations of auxins.

Key words: hybrid verification, half mature zygotic embryo, callus, RAPD

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a diploid monocotyledon belonging to the family Arecaceae. It is a valuable economically important source of vegetable oil, the most traded vegetable oil in the international market, and increasingly used in the food industry (Corley, and Tinker, 2003). For Thailand, Thai government aims to increase palm oil production in order to serve the biodiesel industry, due to a sharp rise in global oil prices, which necessitates the finding of alternative energy sources. Due to a continuous rise in demand for biodiesel, it was estimated that domestic consumption of biodiesel will grow to 31.3 billion liters per year in the next six years. By the

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year 2012, the areas need to cultivate oil palms approximately required 9.1 million rai (Watcharas, 2008). Indeed, the large amount of oil production in the oil palm fruit is unique biological characteristic of this palm species (Hartley, 1977). All commercial palms are F1 hybrids between selections with small or without kernels (pisifera) and large thick kernels (dura), but the hybrids (tenera) show very high variation in oil yield with the best plants yielding 40% more than average. As oil palm is normally propagated by seeds, hence, a high variation in the field and available sustainable genetic trait production in traditional breeding is limited, the low palm oil yield is obtained. Nowadays, plant micropropagation is applied for plant breeding in order to overcome some limitations, and clonal propagation oil palm through tissue culture is common (Aberlenc-Bertossi *et al.*, 1999; Rajesh *et al.*, 2003). However, lacking of good hybridity of oil palms is the major problem of oil palms cultivation in Thailand. Therefore, it is necessary to verify the hybridity at early stage before commercial plantation. The test of hybridity is to assure the uniformity and stability of the field performance and yield. At the present, molecular markers have been used in many agricultural areas, especially plant breeding. The uses of biochemical markers such as isozyme analysis are an established method that can be used for determination of hybridity. However, this method is limited due to many factors affecting protein expression including development of plant tissue and the environment. Low number of markers restricted polymorphism may also affect the utility of these markers (Walter *et al.*, 1989). RAPD (Randomly amplified polymorphic DNA) is a valuable tool for identifying genetic variation because it is economic, quick and simple (Williams *et al.*, 1990). It permits identification of DNA polymorphisms and can be used to amplify particular fragments of genomic DNA (Bielawski *et al.*, 1996). DNA fragment profiles have been employed to analyze the genetic relationships of plant species (Ayana *et al.*, 2002). RAPD analysis is based on the presence or absence of polymorphisms in individuals or groups of individuals (Tingey and Tufo, 1993). In *Elaeis*, RAPD markers have been employed for the analysis of genetic variation among African germplasm accessions (Shah *et al.*, 1994) and successfully used for the detection of somaclonal variants among regenerant populations (Rival *et al.*, 1998). In this research work, half seed of six combinations of hybrid was verified their hybridity by the use of RAPD. The left of half seed was brought to induction of callus for micropropagation. Hybrid cross was screened for commercial propagation for high oil yield producing clone.

Materials and methods

Plant materials

Mature fruits of oil palm from six hybrids, cross #77 [366 (D) × 172 (P)], cross #58 [366 (D) × 72 (P)], cross #118 [366 (D) × 206 (P)], cross #119 [865 (D) × 206 (P)], cross #130 [865 (D) × 110 (P)] and cross #137 [366 (D) × 777 (P)] were kindly provided by Assoc. Prof. Dr. Theera Eksomtramage (Agricultural Research Station, Klong Hoy Kong, Hat Yai, Songkhla, Thailand). All seeds were extracted from the fruits, cracked by hammer and trimmed by pruning scissors to remove the excess kernel. Mature Zygotic embryos were embedded by kernel in cube of 3×3×3 cm³ which sterilized in 70% alcohol for two minutes and followed by 20% (w/v) sodium hypochlorite together with two to three drops of Tween-20 for further 20 minutes. The cubes were then thoroughly washed in sterile water for three times. The embryos were excised from the cubes and cultured on media.

RAPD analysis

Mature zygotic embryos (MZE) were cultured on Murashige and Skoog (MS) supplemented with either 40 mg/l NAA or 2, 4-D or dicamba at the concentration of 2.5 and 5.0 mg/l for 4 weeks. Elongated MZEs with fully developed haustorium was cut into half. First half of MZE (HMZE) consisted of mainly haustoria were collected and isolated DNA followed the method of Te-chato (2000). RAPD analysis of genomic DNA was carried out using 7 decamer random dinucleotide primers (OPB08, OPR11, OPT06, OPT19, OPAB01, OPAB09 and OPAB14) obtained from Operon Tech. (California, USA) (Table 1). The RAPD analysis was performed according to the methodology of Saichon (2004). Each amplification mixture of 25 µl contained 2.5 mM MgCl₂, 10x *Taq* buffer, 100 µM of each dNTP, 0.3 mM of primer, 1.5 units of *Taq* polymerase and 20 ng of template DNA. The thermal profile for RAPD-PCR was started from 39 cycles of 95°C for 1 min 37°C for 1 min 72°C for 2 min, followed by 1 cycle of 95°C for 1 min 37°C for 1 min and finally 72°C for 10 min. PCR products were then electrophoresed in 1.5% (w/v) agarose gels in 0.5X TBE buffer at 100 V. The gels were stained with ethidium bromide for 15 minutes and viewed under ultraviolet light with gel documentation. Reproducibility of the amplification patterns was verified by using different DNA preparations from the parents.

Effect of various kinds and concentration of auxins on callus formation

Second HMZE consisted of apical shoots were inoculated in culture tubes containing 10–15 ml of modified MS medium supplemented with either 40 mg/l NAA or 2, 4-D or dicamba at the concentration of 2.5 and 5.0 mg/l for callus induction. All media were solidified with 0.75% agar. The pH of all culture media was adjusted to 5.7 with 0.1 N KOH before adding agar and autoclaving at 1.05 kg/cm², 121°C for 15 minutes. The cultures were placed under light conditions (3,000 lux illumination for 16 h photoperiod) at 25±2°C and subcultured every 4 weeks on the same medium component for 3 months.

Data recording

For experimental design and statistical analysis, completely randomized design (CRD) with 4 replicates (each replicate consist of 10 embryos) was performed. The percentage of cultures that produced callus, type of callus and number of the embryos per tube were recorded after 1 month for 3 months of culture by counting under a stereo microscope (Nikon, SMZU). Data were analyzed using analysis of variance (ANOVA).

Results and discussion

RAPD analysis

All primers could be amplified and provided polymorphic patterns among parents. The number of bands for each primer varied from 8 to 17 with an average of 13.86 fragments per primer. The size of the amplified products ranged from 100 to 1517 bp. A total of 97 RAPD fragments were scored from the seven random primers (Table 1). The results revealed that there was only one primer, OPT06 provided clearly DNA pattern (Fig. 1). OPT06 primer had the greatest capacity for discriminating polymorphic fragments in half-embryo cultured and gave the highest percentage of polymorphic at 87.50, followed by OPT19 (86.67) and OPB08 (65.54), respectively (Table 1). Cross #119 obtained from the parent 865 (Dura) × 206 (Pisifera) was a good model for verification of hybrid. Results of DNA pattern among cross #119 were found specific fragment could be used to distinguish between dura, pisifera and hybrids. All hybrids showed the DNA patterns between the two parents and more additive bands according to RAPD analysis (Fig. 3,4). These results were similar to those reported by Carvalho *et al.* (2004) in chestnut. They indicated that RAPD marker was successfully used for detecting the material propagated

in vitro and the donor plants of chestnut. Hybrid of oil palms of genotype #119 gave the percentage of hybrids at 23.81%. Moreover, some bands were amplified from the F1 hybrid but not from parents. The reason could be due to the complex heredity background of each plant (Chowdhury *et al.*, 1993; Cordeiro *et al.*, 2000; Chen *et al.*, 2004) or heterosis. Our results showed that RAPD markers are effective tools for indicating six crosses of hybridization in genomic DNA of parents and for distinguishing among individual hybrids of oil palm.

Table 1. Primers used in RAPD analysis of genetic purity in the parents plant of *Elaeis guineensis* Jacq. and number of scoreable bands for each primer.

Primer	Sequence fragments	Amplified fragments	Monomorphic fragments	Polymorphic fragments	Polymorphic (%)	Band mol. Weight (bp)
OPB08	GTCCACACGG	13	5	8	65.54	150-1480
OPT06	CAAGGGCAGA	8	1	7	87.50	290-1100
OPT19	GTCCGTATGG	15	2	13	86.67	100-2100
OPR11	GTAGCCGTCT	16	7	9	56.25	200-1517
OPAB01	CCGTCGGTAG	13	9	4	30.77	300-1500
OPAB09	GGGCGACTAC	17	6	11	64.71	210-1500
OPAB14	AAGTGCGACC	12	9	3	25.00	210-1517
Total band scored		97	40	57	58.76	

Effect of various kinds and concentrations of auxins on type of callus formation

HMZE consisted of shoot tip of all genotypes swell at 10-14 days of culture and started to form callus at 4-5 weeks of culture from HMZE. After 6 weeks of subculture onto various kinds and concentrations of auxins, almost auxins promoted callus formation from HMZE of cross #119. Four types of calluses could be distinguished; friable, compact, nodular and root-like calluses. The friable calluses were yellow, translucent and succulent. The compact calluses were muddy white and compact. The nodular calluses were yellow or pale yellow and compact. The root-like calluses were elongate in shape, white color and soft texture. Kinds and concentrations of auxins used in media had a significant effect on type of calluses. Characteristics of the callus obtained in NAA, 2, 4-D and dicamba containing the medium were quite different. Dicamba provided a yellow compact callus (so called nodular callus) whereas 2, 4-D gave both a white friable callus and a white elongative soft callus and NAA can not provided callus formation (Fig. 5). The highest percentage of friable, compact and nodular calluses was obtained from 2.50 mg/l dicamba after 3 months of culture (Table 2). Maria and Heidi (2002) also reported that dicamba was effective for callus induction from culturing of wheat (*Triticum aestivum* L.). Similar result was also found in immature

embryo culture of winter (Carman *et al.*, 1988) and spring wheat cultivars (Hunsinger and Schauz, 1987.). Dicamba is promising auxins which has been reported to be an effective on promoting direct and indirect embryogenic callus induction from cultured mature zygotic embryo and young of leaf oil palm (Te-chato, 1998a). Time consuming for callus induction in culture medium supplemented with dicamba was shorter earlier than 2, 4-D and NAA. Similar result was obtained from culturing young leaf of the same plant (Te-chato, 1998b; Te-chato *et al.*, 2003). In addition, 2, 4-D containing medium was reported to induce nodular structure from epidermal cells of mature zygotic embryo while dicamba induced from both the epidermis and vascular tissues (Thawaro and Te-chato, 2007.).

Conclusion

The result of our experiment indicated that RAPD can be used for identification of hybridity. The application of OPT06 as a primer could amplify hybrid DNA and can be used for testing hybridity of the cross #119 obtained from 865 (D) × 206 (P). For culturing of MZE or HMZE MS medium supplemented with 2.50 mg/l dicamba gave the highest friable, compact, nodular callus formation at 34.65%, 15.84% and 46.60%, respectively, which significant difference to others auxins.

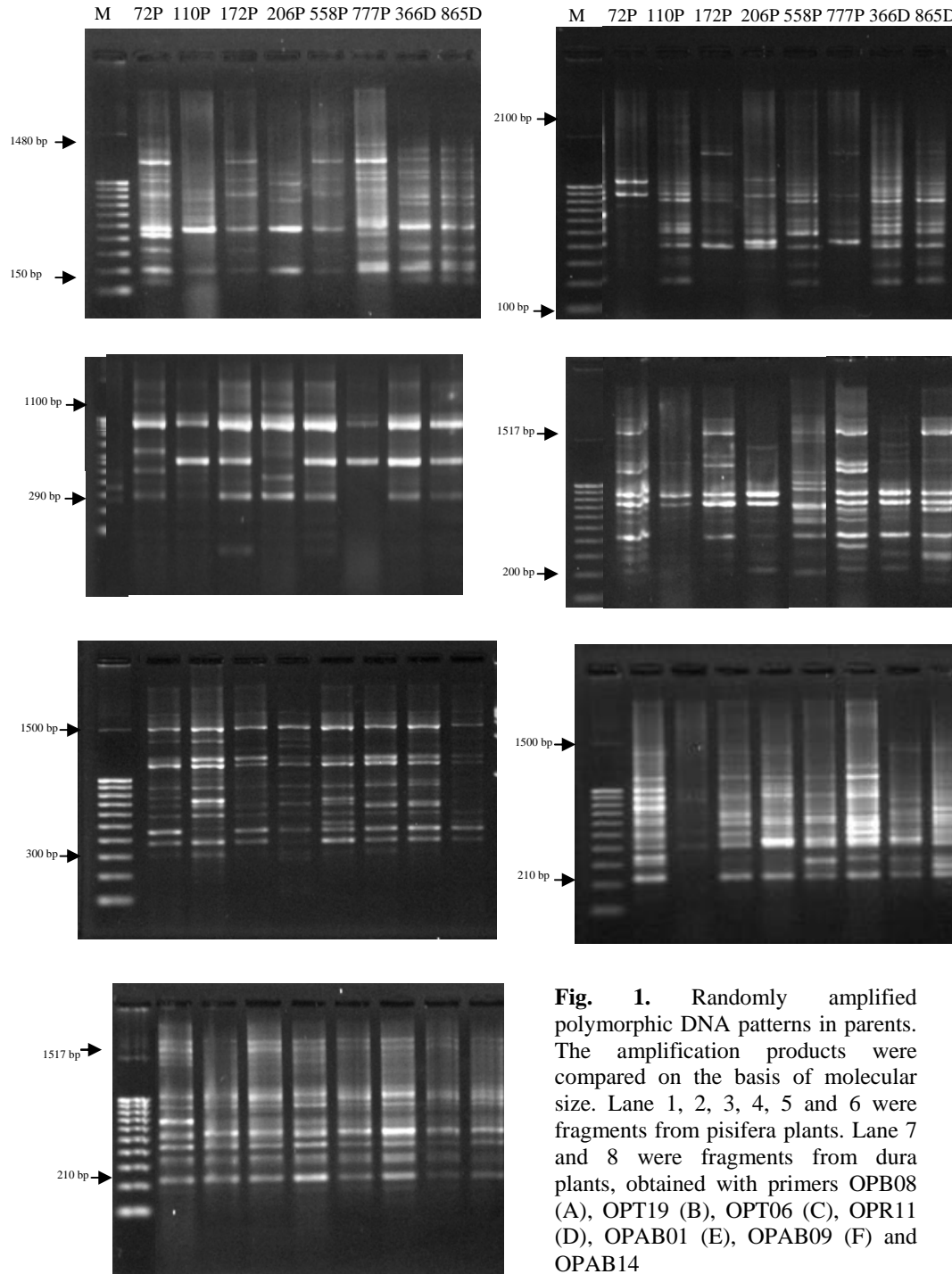


Fig. 1. Randomly amplified polymorphic DNA patterns in parents. The amplification products were compared on the basis of molecular size. Lane 1, 2, 3, 4, 5 and 6 were fragments from pisifera plants. Lane 7 and 8 were fragments from dura plants, obtained with primers OPB08 (A), OPT19 (B), OPT06 (C), OPR11 (D), OPAB01 (E), OPAB09 (F) and OPAB14

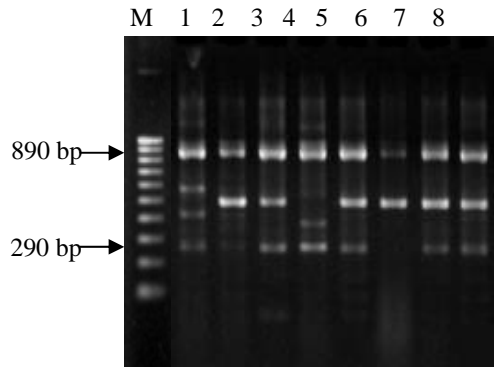


Fig. 2. Randomly amplified polymorphic DNA patterns in parents. The amplification products were compared on the basis of molecular size. Lane 1, 2, 3, 4, 5 and 6 were fragments from pisifera plants. Lane 7 and 8 were fragments from dura plants, obtained with primers OPT06.

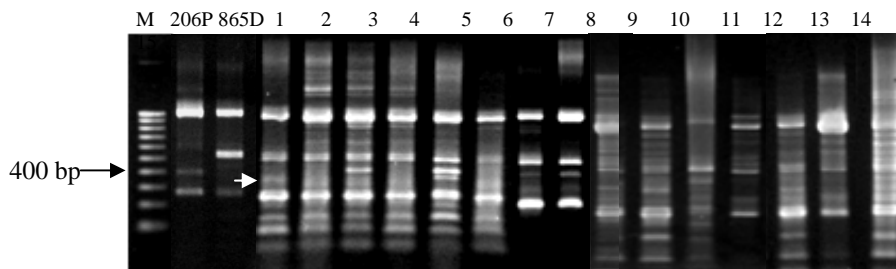


Fig. 3. RAPD patterns in hybrids and parents of cross #119 [865 (D) × 206 (P)]. The amplification products were compared on the basis of molecular size. Lane P and D were fragments from parents. Lane 1-15 were fragments from hybrids, obtained with primers OPT06.

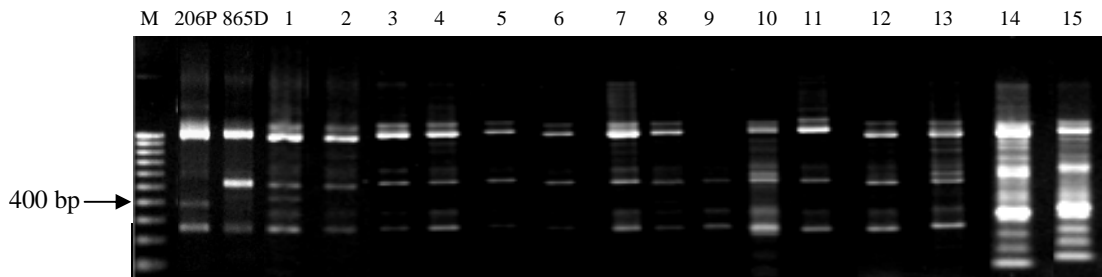


Fig. 4. RAPD patterns in hybrids and parents of cross #119 [865 (D) × 206 (P)]. The amplification products were compared on the basis of molecular size. Lane P and D were fragments from parents. Lane 1-15 were fragments from hybrids, obtained with primers OPT06.

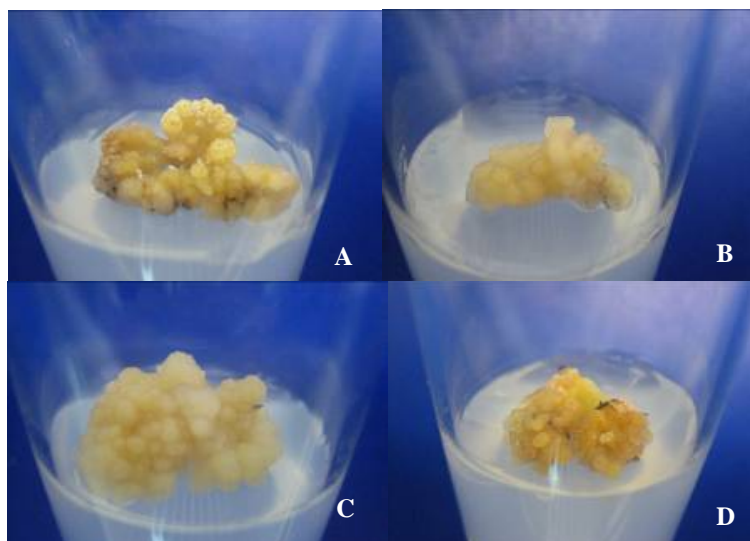


Fig. 5. Callus formation from culturing mature zygotic embryo of cross #119 [865 (D) × 206 (P)] cultured on solidified MS medium supplemented with various auxins for 3 months. (A) 2.5 mg/l 2,4-D, (B) 5.0 mg/l 2,4-D, (C) 2.5 mg/l dicamba and (D) 5.0 mg/l dicamba (bar = 2.50 mm).

Table 2. Effect of various kinds concentrations of auxin on type of callus induction from mature zygotic embryos of mature zygotic embryo of D×P # 119 after culture for 3 months.

Auxin	Concentrations (mg/l)	Type of callus formation (%)			
		Friable callus	Compact callus	Nodular callus	Root like callus
NAA	40	0c	0c	0c	0b
2,4-D	2.5	29.09b	8.18b	40.19b	11.82a
	5	35.29a	11.76a	39.22b	13.73a
Dicamba	2.5	34.65a	15.84a	46.60a	6.93b
	5	29.13b	11.65a	42.57ab	12.62a
F-test		*	*	*	*
C. V. (%)		45.07	32.22	35.34	39.17

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