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The use of RAPD marker for verification of *Dendrobium* hybrid, *D. santana* x *D. friedericksianum* orchid

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Abstract *Dendrobium* is one of the most important orchids due to its beautiful flower. The use of random amplified polymorphic DNA (RAPD) marker for verification of *Dendrobium* hybrid orchid between *D. santana* and *D. friedericksianum* was investigated. Twelve 10-mer primers of RAPD were used for the above purpose. The results showed that 6 primers which were OPA03, OPB07, OPB18, OPJ04, OPR11 and OPT06 could amplify and give a clear profiles of DNA from all samples. The primer $OPA03_{240,\ 310}$, $OPB07_{380,\ 420,\ 850}$, $OPJ04_{550,\ 980}$, $OPT06_{310,\ 450,\ 620}$, $OPR11_{180,\ 500,\ 1,040}$, $OPB18_{300,\ 410,\ 560,\ 1,010}$ could be used as markers for verification of hybrid between *D. santana and D. friedericksianum*.

Keywords: Orchid, *Dendrobium* hybrid, RAPD, primer

Introduction

Orchidaceae is one of the world largest families of flowering plants of angiosperms. Among various orchids, *Dendrobium* is the third largest genus in the family Orchidaceae comprising of about 1,184 species worldwide (Leitch *et al.*, 2009). *Dendrobium* is outstanding in many ways as it diverse shapes, forms and colors. The *Dendrobium* species are broadly categorized into horticultural, agricultural, medicinal or dual-purpose species depending upon their utility.

D. santana is a hybrid between *D. moniliforme* (dwarf Nobile) and *D. friedericksianum* (Thai wild orchid) which can produce flowers all year round and even can bloom since small size of seedling. The mature orchids give numerous odorless flowers of thick yellow petals. The hybrid of *D. santana* and *D. friedericksianum* should be heat tolerant orchid that is very easier to grow and produces flower all season of growing. Thus, it is suitable for growing as economic orchid. The hybrids can be a prototype for a study of *in vitro* flowering and produced as souvenirs for increasing values of agricultural products. Characteristics of flower of *D. santana* and *D. friedericksianum* are shown in Figure 1.

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Dendrobium is one of the most admirable orchids for all growers, especially in Thailand, because *Dendrobium* has very attractive all year blooming with colourful flowers and various textures on petals. According to high demands of *Dendrobium* from both domestic and international growers, new hybrids and mutant species having outstanding characteristics of flowers are very important for fulfilling market demands. The registrations of new species and hybrids are essential for preventing arrogation or plagiarism. The registration methods need accurate and exact verification by using DNA markers or DNA fingerprinting.

Figure 1. Flower characteristics of *D. Santana* (A and B); *D. friedericksianum* (C and D)

RAPD markers have been identified for revealing high levels of polymorphism in many medicinal plants due to their low investment and requirement of short period of time. Though it has some certain limitations, this technique can amplify multiple DNA positions at one time without any knowledge of base order of targeted DNA. However, several primers have to be examined for amplifying whether they can produce the profiles of DNA or not. The DNA profiles are the outcome of process that primers are attached with 2 nearby DNA of different opposite facing fragments (5'--> 3'). RAPD is very

convenient and fast technique using just little content of DNA for verification with ethidium bromide. RAPD was reported in using for verification of relation and variation of *Dendrobium* (Ferreira *et al.,* 2006; Khosravi *et al.,* 2009; Z ha *et al.,* 2009; Xue *et al.,* 2010; Antony *et al.,* 2012) and other orchids such as *Phalaenopsis* (Goh *et al.,* 2005; Niknejad *et al.,* 2009), *Vanda* (Lim *et al.,* 1999; Kishor and Sharma, 2009) and *Cattaleya* (Pinheiro *et al.,* 2012).

Hybrid verifications using RAPD markers have been reported in some orchids such as *Dendrobium* (Inthawong *et al.,* 2006). Parentage relation of 5 pairs *Dendrobium* hybrids were verified by RAPD with OPF03 and OPF14 primers. Feng *et al.* (2013) verified heredity relationship of Dendrobium hybrids between *D. nobile* and *D. moniliforme* (diploid with 2n = 38) by RAPD and found that 286 primers created 500 DNA fragments of 150 base pairs. Among those 136 fragments matching with *D. nobile* and 122 fragments matching with *D. moniliforme.* RAPD was also reported to apply for verification of other hybrids of orchids, for example, *Renanthera imschootiana* and *Vanda coerulea* (Kishor and Sharma, 2009). Among those primers OPA1 could specify heredity relationship of hybrid having exact monomorphic band with parental orchid at position of 837 base pairs. The exact monomorphic bands with mother orchid were at 1,611, 998 and 541 base pairs, and the hybrid created polymorphic band at 1,941, 794 and 618 base pairs.

Therefore, the objective of this study was to use RAPD markers for verification genetic relationship of *Dendrobium* hybrid between *D. santana* and *D. friedericksianum*.

Materials and methods

RAPD (Random amplified polymorphic DNA analysis) for verification of hybrid

DNA extraction

DNA was extracted from young leaves of *D. santana*, *D. friedericksianum*, and hybrid between *D. santana* and *D. friedericksianum* using hexadecyltrimetyammonium bromide (CTAB) according to the method described by Doyle and Doyle (1990) with some modification. The samples of 1 g fresh weight of young leaves were put into cool grinder and then CTAB buffer was added with 1% PVP-40, 1.4 mM NaCl, 20 mM Na_2EDTA (pH 8.0), 2% CTAB, and 750 µl of 2% mercaptoethanol. The samples were grinded, poured into 2 ml microcentrifuge tubes and incubated at 60℃ for 60 minutes. The sample tubes were shaken every 10 minutes for 60 minutes, added with 700 µl of chloroform and centrifuge at 12,000 rpm for 15 minutes. The supernatant was transferred to new centrifuge tubes, and added with 650 µl

isopropanol solutions. Tubes were shaken gently and leaved for 5 minutes for precipitation until DNA fragments could be seen. The solution was poured off, rinsed the DNA sediment with 70% cool ethanol for 2 times, dried the sediment at room temperature, dissolved the sediment with 50 µl TE buffer (10 µl Tris-HCl, pH 7.0, and 1 µl Na_2EDTA) at room temperature, then stored the DNA solution at 4℃.

The extracted DNA was qualified by agarose gel electrophoresis method on 0.7% agarose gel dissolved with 1x TE buffer, mixed 2 µl extracted DNA with 2 µl loading dye, dropped on agarose gel with standard DNA, examined through 100 volts electromotive force with TBE buffer (Tris Base Boric acid), 0.5 µl Na_2EDTA (pH 8.0) for 20 minutes. Then, compare concentrations of the extracted DNA with standard DNA (λDNA), dyed DNA with ethidium bromide, investigated results under 260 nm ultraviolet through Gel documentation and adjusted DNA concentration with TE buffer to 2,300-2,800 µg/l for polymerase chain reaction (PCR).

DNA proliferation by PCR technique

DNA contents were increased by PCR technique with 10 nucleotide of 12 RAPD primers, which were OPA03, OPAB01, OPAB09, OPAB14, OPB04, OPB07, OPB08, OPB18, OPJ04, OPN15, OPR11 and OPT06. According to Sirinya (2008), PCR components of 25 µl were composed with 1 µl of 60 ng prototype DNA, 2.5 µl of 10x *Taq* buffer, 1.5 µl of 0.2 µM primers, 2.0 µl of 250 µM dNTPs (dCTP, dGTP, dTTP and dATP), 0.2 µl of 5.0 unit *Taq* polymerase, and 17.8 µl of dH_2O. DNA was synthesized by DNA Thermal Cycler at 94℃ denaturation for 2 minutes (1 round), following 94℃ denaturation for 30 seconds, 37℃ annealing for 1 minute, 72℃ extension for 2 minutes (2 rounds), 94℃ denaturation for 30 seconds, 35℃ annealing for 1 minute, 72℃ extension for 2 minutes (2 rounds), 93℃ denaturation for 30 seconds, 35℃ annealing for 1 minute, 72℃ extension for 2 minutes (41 rounds), 72℃ extension for 5 minutes (1 round) to generate completed primers extension and kept at 4℃. The result of PCR was examined by dropping 12 µl of DNA solution on 1.5% agarose gel and electrophoresed in TBE buffer through 100 volts electromotive force for 1 hour and 50 minutes, followed by staining with 0.5 µg/µl ethidium bromide for 10 minutes, rinsed with distilled water for 10 minutes, investigated DNA fragments under 260 nm ultraviolet, took photograph with gel documentation, selected primers that can increase DNA from each sample, analyzed data by counting appeared content of DNA fragments, position of appearance and disappearance of fragments on DNA fingerprint of parentage and hybrids of each primer.

Results

RAPD analysis for hybridity verification

A RAPD analysis was performed by using 12 primers of 10 nucleotides to randomly match between DNA fragments of *D. santana*, *D. friedericksianum* and hybrid between *D. santana* and *D. friedericksianum.* There were total of 334 DNA fragments consisting of 42 polymorphic bands and 133 monomorphic bands that were matched with *D. friedericksianum.* Other 159 monomorphic bands were matched with *D. santana* (Table 1).

Table 1. Primer types of RAPD for hybrid verification between *D. santana* and *D. friedericksianum* and amplified DNAs from those primers

Primers	Sequences (5' – 3')	Total Bands	Monomorphic Bands		Polymorphic Bands	Length of DNA (bp) fragments	Clear profiles
			D. friedericksianum	***D. santana***			
OPA03	AGT CAG CCA C	54	24	29	1	250-1,100	++++
OPAB01	CCG TCG GTA G	15	6	8	1	200-1,100	+++
OPAB09	GGG CGA CTA C	17	5	11	1	200-1,100	+++
OPAB14	AAG TGC GAC C	17	8	8	1	200-1,200	++
OPB04	GGA CTG GAG T	19	6	3	10	200-1,517	+++
OPB07	GCT GAC GCA G	37	22	12	3	200-1,300	++++
OPB08	GTC CAC ACG G	14	5	7	2	300-1,517	++
OPB18	GGG AAT TCG G	27	9	12	6	200-1,200	++++
OPJ04	CCG AAC ACG G	30	12	16	2	300-1,000	++++
OPN15	CAG CGA CTG T	25	8	12	5	200- 1,517	+
OPR11	GTA GCC GTC T	47	16	25	6	200- 1,517	++++
OPT06	CAA GGG CAG A	32	12	16	4	300-1,200	++++
Total		334	133	159	42	**200-1,517**	

++++ All samples were increased with clear fragments. +++ increased with moderately clear fragments. ++ increased with unclear fragments. + unclear and not amplify.

According to the RAPD analysis for hybrid verification by using 12 primers, there were 6 primers, OPA03, OPB07, OPB18, OPJ04, OPR11, and OPT06 that can increase DNA with clear fragments of all samples (Figure 2-6) for verification genetic of *D. santana*, *D. friedericksianum* and hybrid between

the two species. The hybridity showed DNA patterns derived from both parents at size of 240 and 310 base pairs (Figure 2), 380, 420 and 850 base pairs (Figure 3), 550 and 980 base pairs (Figure 4), 310, 450 and 620 base pairs (Figure 5), 180, 500 and 1,040 base pairs (Figure 6) and 300, 410, 560 and 1,010 base pairs (Figure 7) respectively. The primer OPA03 at specific base pair of 240 and 310 ($OPA03_{240, 310}$), the primer OPB07 at specific base pair of 380, 420 and 850 ($OPB07_{380, 420, 850}$), the primer OPJ04 at specific base pair of 550 and 980 ($OPJ04_{550, 980}$), the primer OPT06 at specific base pair of 310, 450 and 620 ($OPT06_{310, 450, 620}$), the primer OPR11 at specific base pair of 180, 500 and 1,040 ($OPR11_{180, 500, 1,040}$), and the primer OPB18 at specific base pair of 300, 410, 560 and 1,010 ($OPB18_{300, 410, 560, 1,010}$), could be used as markers for verification of hybrid.

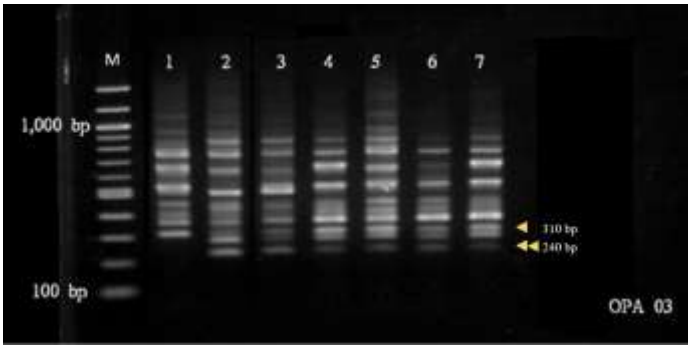

Figure 2. DNA patterns of *D. friedericksianum* (lane 1) *D. santana* (lane 2) and hybridity of *D. santana* and *D. friedericksianum* (lane 3-7) analyzed with RAPD of OPA03 primer M = 100 base pairs ladder DNA; ◄ = indicate bands specific to *D. friedericksianum;* ◄◄= indicate bands specific to *D. santana*

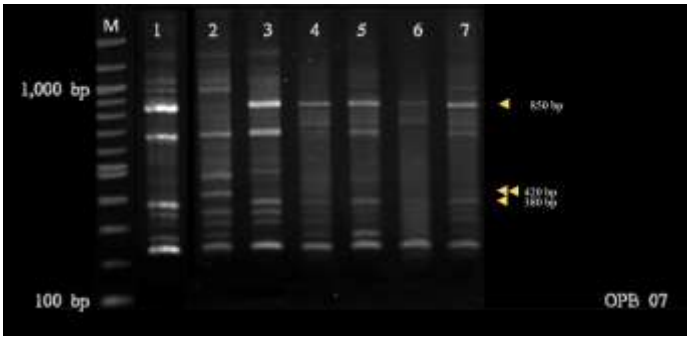

Figure 3. DNA patterns of *D. friedericksianum* (lane 1) *D. santana* (lane 2) and hybridity of *D. Santana* and *D. friedericksianum* (lane 3-7) analyzed with RAPD of OPB07 primer, M = standard DNA of 100 base pair ◄ = indicate bands specific to *D. friedericksianum;* ◄◄ = indicate bands specific to *D. santana*

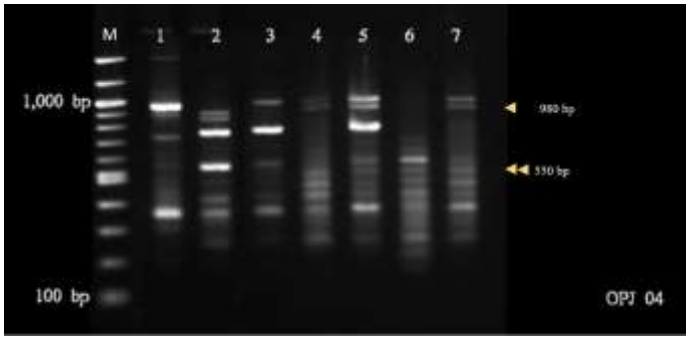

Figure 4. DNA patterns of *D. friedericksianum* (lane 1) *D. santana* (lane 2) and hybridity of *D. santana* and *D. friedericksianum* (lane 3-7) analyzed with RAPD of of OPJ04 primer, M = standard DNA of 100 base pair ◄ = indicate bands specific to *D. friedericksianum* ◄◄ = indicate bands specific to *D. santana*

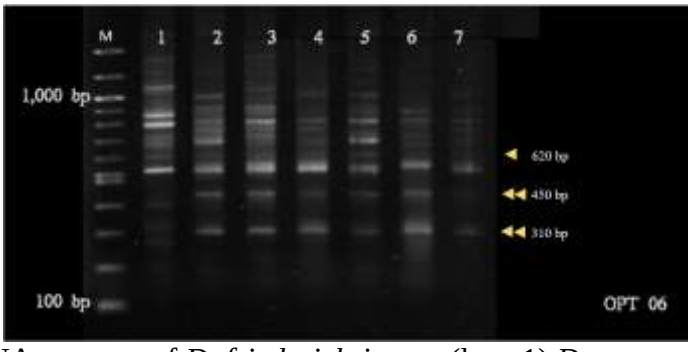

Figure 5. DNA patterns of *D. friedericksianum* (lane 1) *D. santana* (lane 2) and hybridity of *D. santana* and *D. friedericksianum* (lane 3-7) analyzed with RAPD of OPT06 primer, M = standard DNA of 100 base pair, ◄ = indicate bands specific to *D. friedericksianum*, ◄◄ = indicate bands specific to *D. santana*

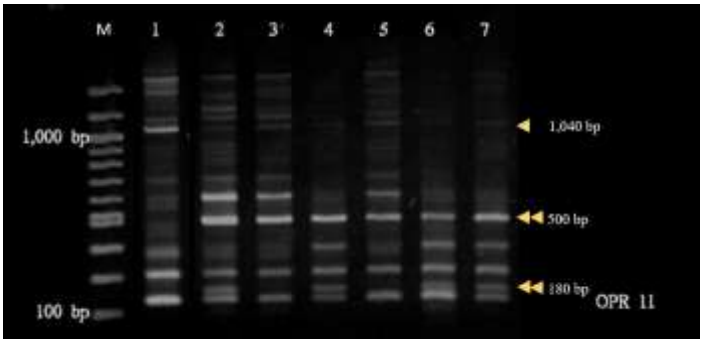

Figure 6. DNA patterns of *D. friedericksianum* (lane 1) *D. santana* (lane 2) and hybridity of *D. santana* and *D. friedericksianum* (lane 3-7) analyzed with RAPD of OPR11 primer, M = standard DNA of 100 base pairs, ◄ = indicate bands specific to *D. friedericksianum* , ◄◄ = indicate bands specific to *D. santana*

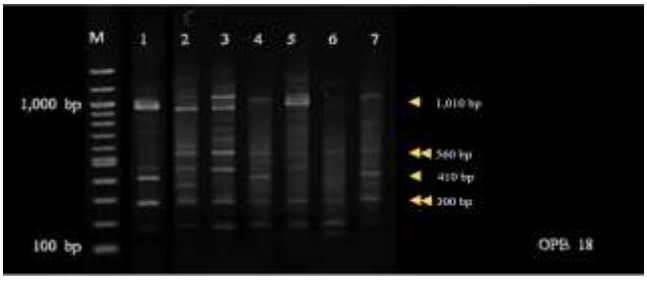

Figure 7. DNA patterns of *D. friedericksianum* (lane 1) *D. santana* (lane 2) and hybridity of *D. santana* and *D. friedericksianum* (lane 3-7) analyzed with RAPD of OPB18 primer, M = standard DNA of 100 base pair, ◄ = indicate bands specific to *D. friedericksianum*, ◄◄ = indicate bands specific to *D. santana*

Discussion

According to the hybridity verification of *D. santana* and *D. friedericksianum* with RAPD, 6 primers of OPA03, OPB07, OPB18, OPJ04, OPR11, and OPT06 gave the clear results. The DNA contents could be increased from all samples and showed clear DNA fragments for verifying hybrid between *D. santana* and *D. friedericksianum*. There were 3 types of the DNA fragments of the hybrid, which were DNA fragments that was monomorphic to *D. santana*, *D. friedericksianum*, and DNA fragments that were different from parents. The fragments could be derived from hybridization caused by DNA intervention or DNA disappearance from the original positions that were matched by primers, so the DNA fragments were changed. The primer $OPA03_{240,\ 310}$, $OPB07_{380,\ 420,\ 850}$, $OPJ04_{550,\ 980}$, $OPT06_{310,\ 450,\ 620}$, $OPR11_{180,\ 500,\ 1,040}$, $OPB18_{300,\ 410,\ 560,\ 1,010}$ could be used as markers for verification of hybrid.

Chattopadhyay *et al*. (2012) assessed the genetic variability among five species of *Dendrobium* i.e. *D. bellatulum, D. densiflorum, D. fimbriatum, D. nobile* and *D. apphyllum* by using RAPD marker and found that RD-03, RD-04, RD-05, RD-11 and RD-14 could be used to distinguish the difference among those species. The size of amplified DNA fragments produced by these primers varied between 250 to 800 bp. Inthawong *et al.* (2006) verified parentage relationship including with hybrid of 5 species of *Dendrobium* by RAPD technique, and found that the hybrid had genetic characteristics different from parents. The profiles of hybrid analyzed by RAPD with the appropriate primers showed clear RAPD fragments such as primer $OPF03_{736,\ 1014}$ that was the DNA marker of D017 x D022 hybrid, primer $OPF03_{1219,\ 1239}$ that was the DNA marker of D037 x D022 hybrid, primer $OPF14_{831,\ 1182}$ that was the DNA marker of D030 x D031 hybrid, and primer $OPF0_{273,\ 476,\ 490,\ 564}$ that was the DNA marker of D037 x D034 hybrid. In some publications, many breeders have

employed RAPD technique for determination of the hybridity of interspecific hybrids in several orchids, such as *Cattaleya* (Benner *et al.*, 1995) and *Phalaenopsis* (Jheng *et al*., 2012).

Meanwhile, Parab *et al.* (2008) used RAPD as genetic marker to characterize the population of *Aerides maculosum*. Among the total primers tested for molecular analysis of *A. maculosum* 100% polymorphic pattern was obtained with 7 RAPD primers (OPA 3, OPA 8, OPA 13, OPD 2, OPD5, OPD 7 and OPD 8). Total 13 RAPD primers produced a clear and reproducible banding pattern. The 13 RAPD primers produced 101 fragments with an average of 7.76 bands per primer. Out of which 94 bands with the mean of 7.23 per primer were polymorphic for all the populations. The percentage of polymorphism across all the samples varied from 50 to 100% (average 93.09 %). The polymorphism was higher with OPA 3, OPA 8, OPA 13, OPD 2, OPD 5, OPD 7 and OPD 8, while lowest results were obtained in OPA 14 primer. While Poobathy *et al*. (2013) analyzed genetic variation of *Dendrobium.* Sonia-28 by RAPD after preparing artificial seeds that were collected under low temperature. The result showed that there were variation in DNA patterns among regenerated plants from storing artificial seeds under low temperature. Kishor *et al.*, (2008) reported that RAPD markers could be used for early determination of hybridity and easy identification of the reciprocal crosses of *Aerides vandarum* and *Vanda stangeana* at the protocorm stage. In summary, RAPD technique provides a useful tool for breeding application, which is a simple and fast procedure, requires a low quantity of DNA, and effective for the characterization of the hybrids without resorting to complex process.

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