CHAPTER 6

Hybrids Derived from Crosses Between *Dendrobium* Cultivars and Fragrant Species: Identification by RAPD Technique

Molecular marker techniques can help resolved relationships among plant materials (Xiang *et al*., 2003). Randomly amplified polymorphic DNAs (RAPD) based on the polymerase chain reaction (PCR) has been used for cultivar identification and genetic relationship studies in *Cymbidium* (Obara-Okeyo and Kako, 1998), *Dendrobium* (Inthawong *et al*., 2006), *Phalaenopsis* (Been *et al*., 2002; Chen *et al*., 2001a, Taywiya *et al*., 2008), and *Vanda* (Lim *et al*., 1999). RAPD technique could be used to identify the markers linked to red floral trait, which is controlled by a single dominant gene, in *Phalaenopsis equestris*, from a cross between white floral parent and red floral parent, F_1 progenies and F_2 progenies. A total of 920 primers were used for screening of markers that were related to the red floral gene. It was found that OPQ 10 primer could provide a 380-bp DNA fragment (OPQ10-380) which was linked to the red floral gene (Chen *et al*., 2001b). The genetic relationships of *Phalaenopsis amboinensis*, *Phal. amabilis* and their hybrids were also identified by RAPD, using OPC 07 primer. It was found that the hybrids shared genetic combination of their parents (Chen *et al*., 2001a). Moreover, RAPD could indicate variation of somaclonal plants in *Phalaenopsis* True Lady "B79-19". About 1360 somaclonal plants were tested. No morphological difference was found in terms of leaf shape. However, after flowering there were some deformed flowers of certain clones. The RAPD data showed the difference of DNA patterns in somaclones that yielded deformed flowers. (Chen *et al*., 1998).

Three crosses between *Dendrobium* cultivars and fragrant species, *Den*. Emma white × *Den. parishii, Den*. Anna × *Den. parishii* and *Den*. Sonia 'Red' × *Den. parishii,* were made. In order to find the relationship among parental lines and progenies, RAPD technique was employed in this study. Firstly, suitable primers were tested among the parental lines. The ones that

provided distinguished bands were subsequently used in relationship study among the parental lines and progenies.

Materials and methods

Three *Dendrobium* cultivars, *Den.* Emma White, *Den.* Anna and *Den.* Sonia 'Red', four fragrant species, *Den. scabrilingue*, *Den. anosmum*, *Den. parishii* and *Den. peguanum*, and three sets of progenies derived from the crosses between, *Den.* Emma White and *Den. parishii* (Figure 11,E-H), *Den.* Anna and *Den. parishii* (Figure 11,I-L), and Den. Sonia 'Red' and *Den. parishii* (Figure 11,M-P), were evaluated using the RAPD technique.

Figure 11 Parental lines and progenies.

(A) *Den.* Emma White, (B) *Den.* Anna, (C) *Den.* Sonia 'Red' and (D) *Den. parishii.*.

(E-H) Progenies derived from the cross between *Den.* Emma White and *Den. parishii.*

(I-L) Progenies derived from the cross between *Den.* Anna and *Den. parishii.*

(M-P) Progenies derived from the cross between *Den.* Sonia 'Red' and *Den. parishii.*

The plants were individually grown in a three-inch pot using sphagnum moss as growing medium. Fertilizer of 21N-21P-21K was given to the plants once a week, and watering was done everyday. The studies were divided into two parts. The first part was on primer screening. One commercial cultivar, *Den.* Emma White, and four species, *Den. scabrilingue*, *Den. anosmum*, *Den. parishii* and *Den. peguanum* were used. The most suitable primers would be used in the second part. The relationships of parental lines and their progenies of *Den.* Emma White and *Den. parishii, Den.* Sonia 'Red' and *Den. parishii*, and *Den.* Anna and *Den. parishii* were evaluated.

Young leaves on new pseudobulb were cut and individually put in plastic bags, and then placed in an icebox and brought over to the laboratory, cleaned and rinsed with distilled water and 70% ethanol. About 0.1 g of leaf was employed for DNA extraction using the CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1987). Leaf tissue was ground in a mortar to give a fine powder form. After grinding, the powder was mixed with 1 ml 2X CTAB buffer and transferred into a 1.5 ml microcentrifuge tube. Next, 10 µl proteinase K (1 mg/µl) was added into the mixture and incubated at 60 ^{o}C for 30 min in water bath, gently shaked every 10 min. Then, 500 µl chloroform (24)-isoamyl (1) alcohol was added into this mixture, strongly shaked, and then put into the centrifuge at 10,000 rpm for 10 minutes. The liquid was transferred into a new tube and added with an equal amount of isopropanol, gently shaked and then incubated at 4 ^{o}C, overnight. After that, the mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded. Precipitate was washed with 500 µl of wash buffer (10 mM ammonium acetate, 75% ethanol) and centrifuged at 10,000 rpm for 5 min. Then, it was washed with 500 µl of 75% ethanol and centrifuged at 10,000 rpm for 5 min. This liquid was carefully discarded. The precipitate was air-dried. After that, it was resuspended by 100 µl of TE buffer (10 mM Tris-HCL, 0.5 mM EDTA), and 10 units of RNase A was added into this mixture and incubated at 37 ^{o}C for 30 min. This extracted DNA solution was diluted to 10 ng/µl using steriled deionized water. A total of 1µl of this dilution was used for PCR amplification. DNAs of parental lines, *Den.* Emma White and *Den. parishii* were screened by RAPD using 20 primers, OPF 01-20, in order to select for the suitable markers providing with polymorphic bands. PCR reactions were performed using a 20 µl mixture, containing 15.24 µl deionized water, 2 µl 10X PCR buffer, 0.8 µl 100 ng/µl primer, 0.6 µl 1.5 mM $MgCl_2$, 0.2 µl 100 µM dNTPs, 0.16 µl 0.8 U/µl *Taq* DNA polymerase and 1µl DNA template.

DNA amplification was done using programmed DNA thermocycle; started with two cycles of 94 ^{o}C denaturing for 1 min, 36 ^{o}C annealing for 10 sec and 72 ^{o}C extention for 70 sec; followed by thirty cycles of 60 sec at 90 ^{o}C, 45 sec at 42 ^{o}C and 70 sec at 72 ^{o}C; followed by one final extension cycle with 4 min at 72 ^{o}C and held at 4 ^{o}C. The amplification products were separated by electrophoresis in 1.8 % (w/v) agarose gel with 1X TBE buffer, stained by 0.5 μg/ml of ethidium bromide, photographed by Gene Snap program and evaluated by Gene Tool program, under the Gel Documentation. After the DNAs of the parental lines were screened, OPF primers, which showed clear and numerous polymorphic bands were selected for progenies screening.

Results and discussion

Primer screening for *Dendrobium* cultivars and four fragrant species

Den. Emma White and four fragrant species were screened using 20 primers, OPF 01-20 (Appendix, Figure 1). Four primers, OPF 01, OPF 04, OPF 07 and OPF 10, showed polymorphic DNA bands in the ranges of 248-1500, 206-1425, 445-1582 and 200-1452 bps, respectively, with high resolution and could be used to differentiate those five parents (Figure 12). OPF 01 primer yielded three DNA bands, 1500, 1300 and 821 bps only in *Den*. Emma white, but not the four fragrant species. On the other hand, there were two bands, which were found only in fragrant species: a 791-bp in *Den. parishii*, *Den. anosmum* and *Den. peguanum*, and a 271-bp in *Den. parishii*, *Den. anosmum* and *Den. scabrilingue*. Some DNA bands were found both in *Den*. Emma White and fragrant species when OPF 01 primer was used: a 1149-bp in *Den*. Emma White and *Den*. *peguanum*; and 1050 bps in *Den*. Emma White, *Den. parishii* and *Den. anosmum*; a 556-bp in *Den*. Emma white, *Den. parishii*, *Den. anosmum* and *Den. peguanum*; and a 404-bp in the four fragrant species. OPF 04 primer yielded five DNA bands, 1425, 1288, 714, 568 and 509 bps only in *Den*. Emma White, but not in the four fragrant species. On the other hand, there were one band, which was found only in fragrant species: a 1000-bp in *Den. parishii*, *Den. anosmum* and *Den. peguanum*, 832 and 607 bps in *Den. parishii*, *Den. anosmum, Den. scabrilingue* and *Den. peguanum*, and a 443-bp in *Den. parishii* and *Den. anosmum.* Some DNA bands were found both in *Den*. Emma White and fragrant species when OPF 04 primer was used: a 1123-bp in *Den*. Emma white, *Den. parishii*, *Den. anosmum* and *Den. peguanum*; and a 206-bp

in *Den. parishii* and *Den. anosmum.* OPF 07 primer yielded two DNA bands, 715 and 437 bps only in *Den*. Emma White, but not in the four fragrant species. On the other hand, there were six bands, which was found only in fragrant species: 1140, 1129, 937, 627, 538 and 445 bps in *Den. parishii* and a 1000-bp in *Den. peguanum*. Some DNA bands were found both in *Den*. Emma White and fragrant species when OPF 07 primer was used; a 1528-bp in *Den*. Emma White and *Den. anosmum,* a 1062-bp in *Den*. Emma White and *Den. scabrilingue*; a 874 bps in *Den*. Emma White, *Den. anosmum* and *Den. scabrilingue*; and a 796-bp in *Den*. Emma White, *Den. parishii* and *Den. anosmum.* OPF 10 primer yielded one DNA band, 891 bps in only *Den*. Emma White, but not in the four fragrant species. On the other hand, there were two bands, which was found only in fragrant species: 1452 and 600 bps in *Den. parishii* and *Den. anosmum*; a 1200-bp in *Den. parishii* and *Den. peguanum*; 750-bp in *Den. parishi*;*,* 700 and 650 bps in *Den. anosmum.* Some DNA bands were found both in *Den*. Emma White and fragrant species when OPF 10 primer was used; 548 and 400 bps in *Den*. Emma White, *Den. parishii* and *Den. anosmum* and *Den. scabrilingue*.

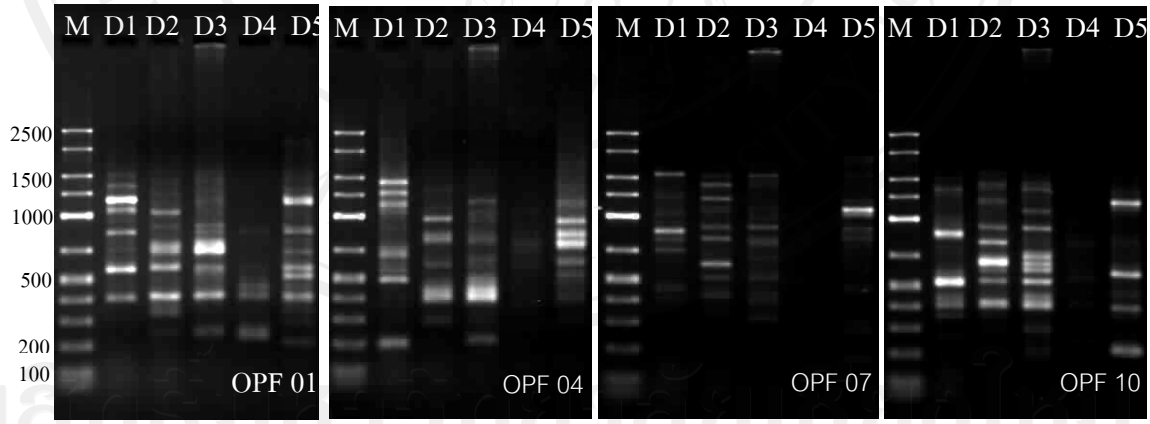

Figure 12 Polymorphic profiles obtained from *Den*. Emma White (D1), *Den. parishii* (D2), *Den. anosmum* (D3), *Den. scabrilingue* (D4) and *Den. peguanum* (D5) after amplification with four primers, OPF 01, OPF 04, OPF 07 and OPF 10.

Primer screening for the parental lines, *Dendrobium* Emma White and *Dendrobium parishii*

Parental lines, *Den.* Emma White and *Den. parishii*, were reproducibly tested with four primers, OPF 01, OPF 04, OPF 07 and OPF 10, for the best result. Banding patterns showed polymorphic DNA bands in the ranges of 400-1362, 206-1440, 400-1558 and 400-1300 bps,

respectively (Figure 13). OPF 01 primer yielded a 1362-bp DNA band specific to *Den*. Emma White and a 700 bps specific to *Den. parishii* while, there bands, 1050, 577 and 400 bps, were found in both parents. OPF 04 primer syielded six DNA bands, 1440, 1260, 1050, 700, 500 and 200 bps only in *Den*. Emma White and showed a 1000, 770 and 400 bps DNA bands in *Den. parishii*, but there was not common band presented in both parents. OPF 07 primer showed DNA bands, 1582, 850 and 700 bps only in a column of *Den*. Emma White and showed a 1440, 1200, 590 and 500 bps DNA bands in a column of *Den. parishii*, there was only one similar band, 770 bps, presented in both parents. OPF 10 primer showed a 850-bp DNA band in a column of *Den*. Emma White only and showed 1150, 900 and 680 bps DNA bands in a column of *Den. parishii*, there were three bands, 1300, 500 and 410 bps, presented in both parents. There was only one DNA band, 1000 bps, in the column of *Den. parishii* after reproducibly tested with OPF 04 primer that presented only in the fragrant species but not in the cultivar.

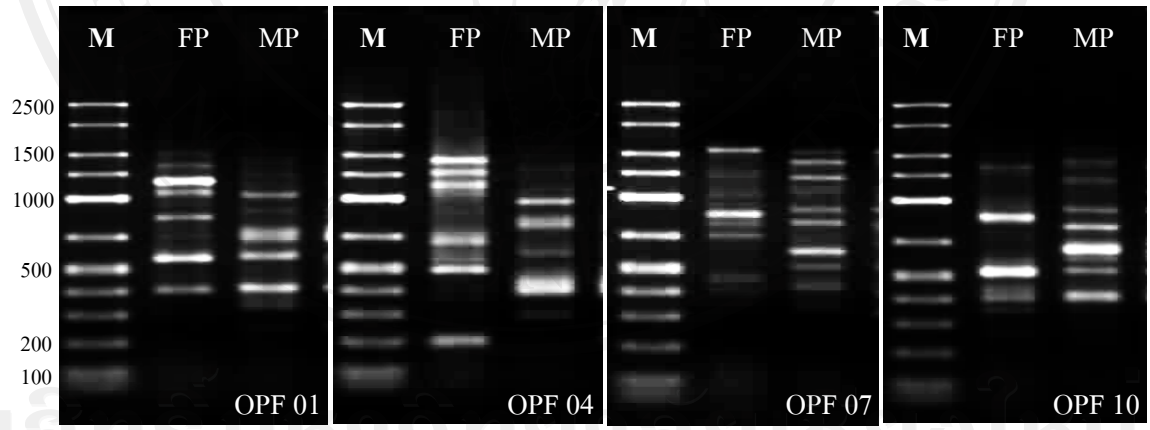

Figure 13 Polymorphic profiles obtained from parental lines, *Den*. Emma White as female parent (FP) and *Den. parishii* as pollen or male parent (MP) after amplification with four primers, OPF 01, OPF 04, OPF 07 and OPF 10.

Relationship of parental lines and their progenies

The four selected primers, OPF 01, OPF 04, OPF 07 and OPF 10, were used to analyze DNAs of the parental lines and their ten progenies. Poloymorphic bands specific to either parental lines were produced by the OPF 01 primer: 1149, 821 and 400 bps to *Den.* Emma White and

1250, 719, 556 and 270 bps to *Den. parishii* (Figure 14) their DNA patterns of 7 progenies showed monomorphic bands represented in both female and male parents, but 2 progenies, H3 showed 719-bp, and H6 showed 719 and 270 bps. OPF 04 primer yielded distinguishable polymorphic bands, seven of which were female parent, 1288, 1130, 714, 568, 509, 443 and 220 bps and five of which belong to male parent, 1130, 1000, 832, 607, and 443 pbs (Figure 15) whereas their 9 progenies, H2-H10, were showed clearly distinguishable DNA bands derived from the female and male parent, but a progeny, H1, showed only two common DNA bands found in both parent, OPF 07 primer showed six DNA bands of female parent, 1582, 1062, 847, 796, 715 and 473 bps and eight DNA bands of male parent, 1440, 1250, 1139, 937, 814, 627, 538 and 445 pbs, which were well defined with clearly distinguishable polymorphisms (Figure 16) whereas their 10 progenies, H1-H10, were showed clearly distinguishable DNA bands derived from the female and male parents. OPF 10 primers produced well defined polymorphic DNA bands of parental lines and their progenies with clearly distinguishable polymorphisms (Figure 17) whereas their 10 progenies, H1-H10, were showed clearly distinguishable DNA bands derived from the female and male parents.

The OPF 07 and OPF 10 primers produced well defined DNA bands which were polymorphic among parental lines and their progenies, thereby considered more suitable for identifying the relationship among parental lines and their progenies than OPF 01 and OPF 04. There were some bands of the parents not present in the progenies (Figure 14-17). Two bands, 874 and 473 bps, of the female parent and three bands, 1139, 627 and 445 bps, of the male parent were absent in progeny H1 (Figure 16). The high number of bands from parents absent in progenies of *Dendrobium* is probably due to the segregation of heterozygous chromosomes during meiosis. According to the report of Huang *et al*. (2000) stating that losing some DNA bands from parents caused by the segregation of heterozygous chromosome was also found in the genetic analysis of chrysanthemum hybrids, based on RAPD technique. Smith *et al*. (1996) described that chromosomal crossing-over during meiosis might have resulted in the loss of priming sites and thus markers of *Cyrtandra* species were presented in parents but not in progenies from interspecific crosses.

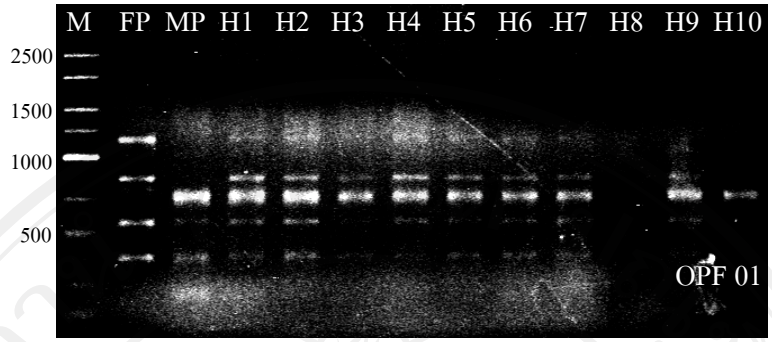

Figure 14 Polymorphic profiles obtained from *Den.* Emma White (FP), *Den. parishii* (MP) and their ten progenies (H1-H10) after amplification with OPF 01 primer.

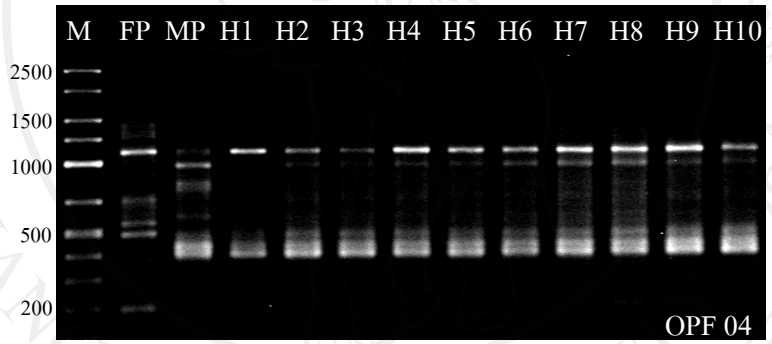

Figure 15 Polymorphic profiles obtained from *Den.* Emma White (FP), *Den. parishii* (MP) and their ten progenies (H1-H10) after amplification with OPF 04 primer.

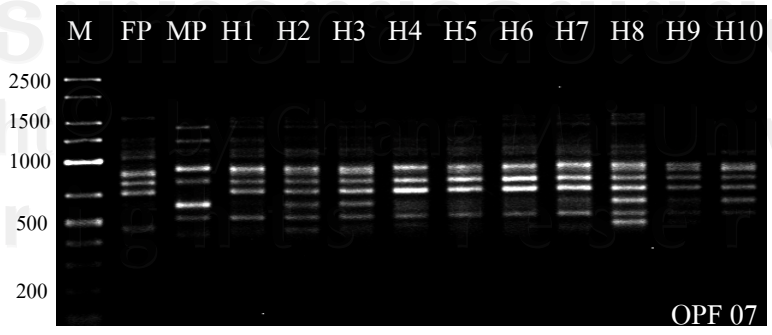

Figure 16 Polymorphic profiles obtained from *Den.* Emma White (FP), *Den. parishii* (MP) and their ten progenies (H1-H10) after amplification with OPF 07 primer.

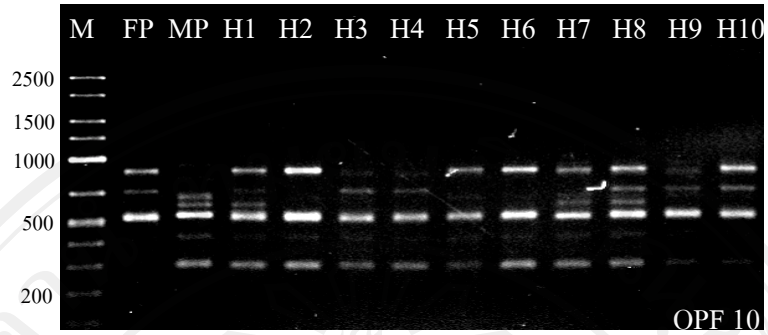

Figure 17 Polymorphic profiles obtained from *Den.* Emma White (FP), *Den. parishii* (MP) and their ten progenies (H1-H10) after amplification with OPF 10 primer.

Identification of scent markers by RAPD technique

Phenotypic distribution of progenies derived from the cross between *Dendrobium* Emma White and *Dendrobium parishii* was studied and described in chapter five. All of the progenies had fragrant flowers. As revealed by the primer screening study for *Dendrobium* cultivars, four fragrant species and parental lines, the 791-bp DNA band,, produced by OPF 01 primer, was consistently found in fragrant species, *Den. parishii*, *Den. anosmum* and *Den. peguanum* (Figure 12) and *Den. parishii* (Figure 14). This band, therefore , could be considered a scent marker. In order to confirm this result, two sets of progenies derived from crosses of *Den.* Anna × *Den. parishii* and *Den.* Sonia 'Red' × *Den. parishii* were evaluated using RAPD technique as previously done to observe the relationship between scent and non-scent of progenies and parents.

RAPD results from these two crosses, however, were not similar to what had been found in previous experiment from cross between *Den.* Emma White and *Den. parishii* in that the 791-bp band was not found. The further attempt was to identify polymorphic markers among the parents whose scent characters were different. Flowers of *Den.* Anna and *Den.* Sonia 'Red' were scentless whereas that of *Den. parishii* had distinctly strong fragrance. The RAPD results showed that the polymorphic bands that were specific to Den. parishii, were shared by both scent and scentless progenies (Figure 18-25).

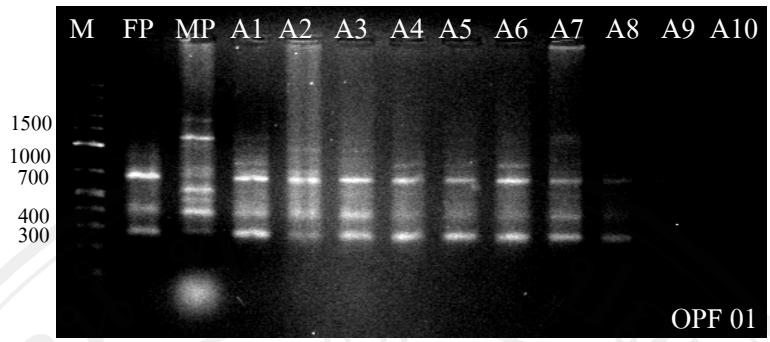

Figure 18 Polymorphic profiles obtained from *Den.* Anna (FP), *Den. parishii* (MP) and their five scent, A1-A5, and non-scent, A6-A10, progenies after amplification with OPF 01 primer.

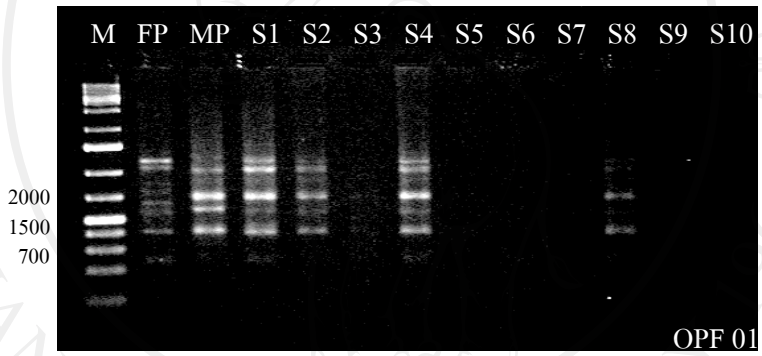

Figure 19 Polymorphic profiles obtained from *Den.* Sonia 'Red' (FP), *Den. parishii* (MP) and their five scent, S1-S5, and non-scent, S6-S10, progenies after amplification with OPF 01 primer.

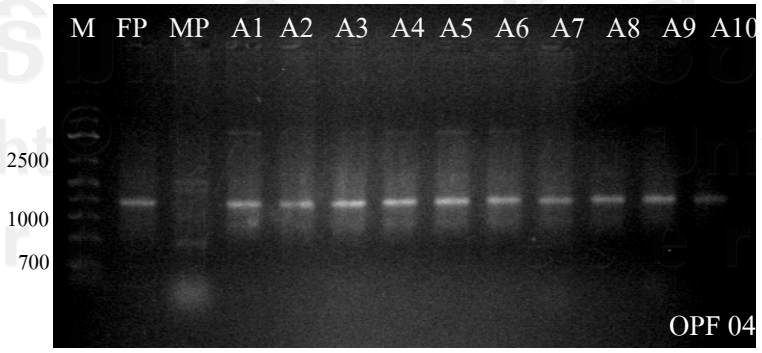

Figure 20 Polymorphic profiles obtained from *Den.* Anna (FP), *Den. parishii* (MP) and their five scent, A1-A5, and non-scent, A6-A10, progenies after amplification with OPF 04 primer.

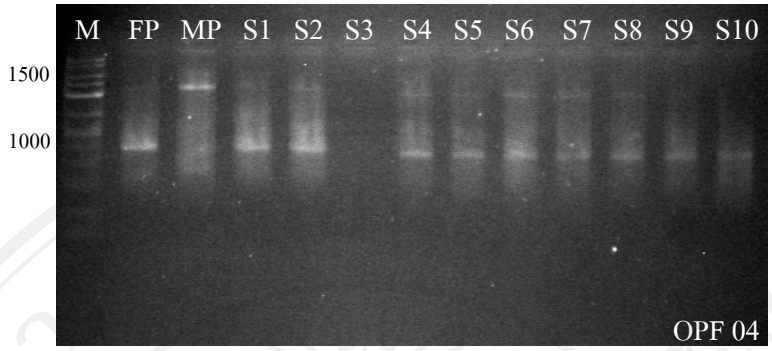

Figure 21 Polymorphic profiles obtained from *Den.* Sonia 'Red' (FP), *Den. parishii* (MP) and their five scent, S1-S5, and non-scent, S6-S10, progenies after amplification with OPF 04 primer.

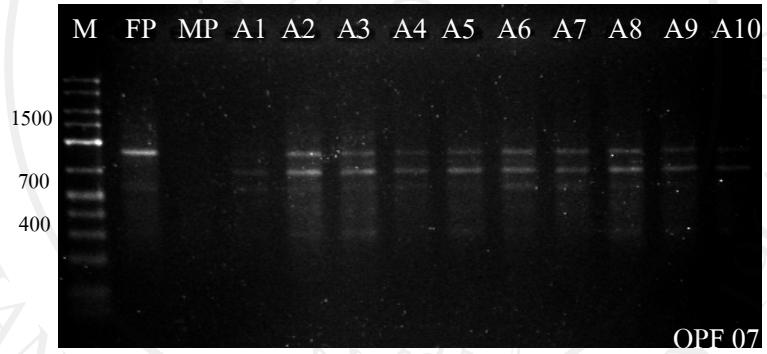

Figure 22 Polymorphic profiles obtained from *Den.* Anna (FP), *Den. parishii* (MP) and their five scent, A1-A5, and non-scent, A6-A10, progenies after amplification with OPF 07 primer.

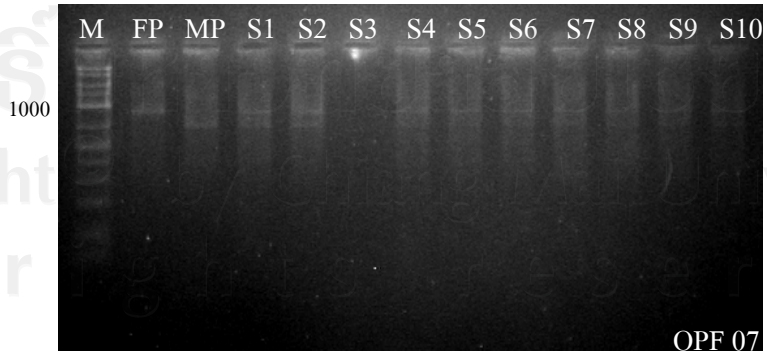

Figure 23 Polymorphic profiles obtained from *Den.* Sonia 'Red' (FP), *Den. parishii* (MP) and their five scent, S1-S5, and non-scent, S6-S10, progenies after amplification with OPF 07 primer.

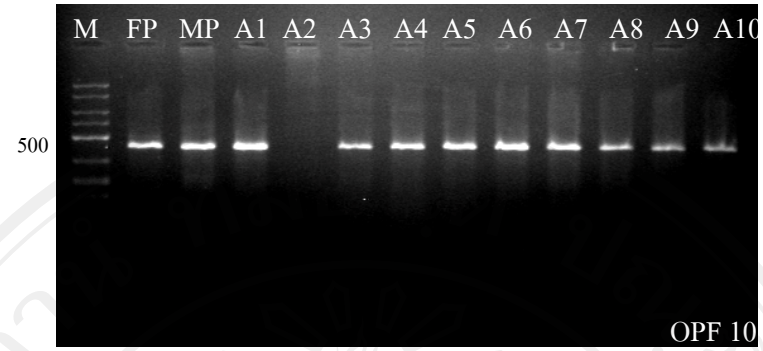

Figure 24 Polymorphic profiles obtained from *Den.* Anna (FP), *Den. parishii* (MP) and their five scent, A1-A5, and non-scent, A6-A10, progenies after amplification with OPF 07 primer.

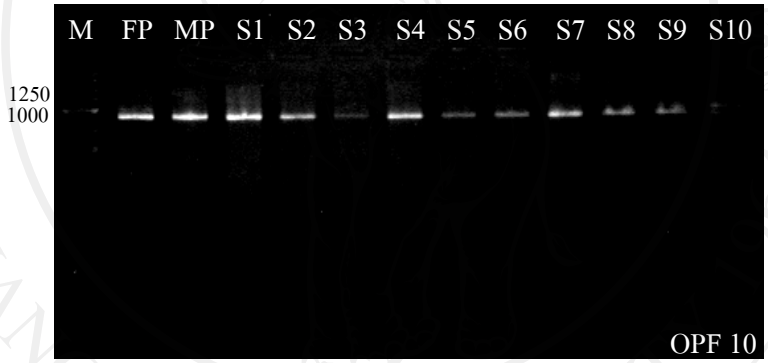

Figure 25 Polymorphic profiles obtained from *Den.* Sonia 'Red' (FP), *Den. parishii* (MP) and their five scent, S1-S5, and non-scent, S6-S10, progenies after amplification with OPF 10 primer.

The bands of *Den. parishii* were shared in both of scent and scentless progenies and could not separate scent and scentless progenies from each other by using RAPD technique. That might have been due to the primers which were not suitable and could not amplify DNA fragment in the location of genes that control scent character. In addition, the primers used in this study were slightly short, 10 mer, resulting in difficulties regarding annealing specificity and reproducibility. Flower scent might be a complex character, which is controlled by polygenes, and differ from other characters such as flower colors of which Chen *et al*. (2001b) stated that RAPD technique could be used to identify the markers linked to red floral trait, which was controlled by a single dominant gene, in *Phalaenopsis equestris*, from a cross between white floral parent and

red floral parent, F_1 progenies and F_2 progenies. In that study, a total of 920 primers were used for screening of markers that were related to the red floral gene. It was found that OPQ 10 primer could provide a 380-bp DNA fragment (OPQ10-380) which was linked to the red floral gene.

Even though OPF 10 could provide unique band, 791bps, for fragrant plant, it could not produce the same result when it was tested in other crosses. It indicated that there was a specific primer for certain cross. Since, fragrance is not a qualitative character, due to many different types of compounds in fragrance, thus, it should be controlled by polygenes. In the experiment where qualitative character such as color of *Phal. equestris* was tested, about 920 primers were used and only one primer could provide the marker linked to color gene. In this experiment, 20 primers were tested and one was found to present one band unique to fragrant plants. It might give some clue for future study that the possibility of finding a suitable marker of certain fragrant is quite promising. Thus, more primers are required in order to find a suitable marker as scent marker for further study.

Conclusions

Four primers, OPF 01, OPF 04, OPF 07 and OPF 10, could be used to identify relationship between parental lines and their progenies of cross Den. Emma White × *Den. parishii*. OPF 10 could amplify distinguishable band, 791 bps, in all fragrant plants. However when the same primer was employed in other crosses between non-fragrant and fragrant plants, Den. Anna × *Den. parishii* and *Den*. Sonia 'Red' × *Den. parishii*, the result was not reproducible. Primers used in the RAPD technique might be very specific for certain cross. Thus, more primers are in need for future study in order to obtain best result.