The 4th
INDONESIAN
BIOTECHNOLOGY
CONFERENCE
(an International Forum for Biotechnology)

PROCEEDINGS

“Biotechnology for better food, health and environment”
Bogor, 5-7 August 2008

INDONESIAN BIOTECNOLOGI CONSORTIUM
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A New Method for Identifying the Progeny of Intergeneric Orchid Breeding Based on its Chloroplast $TRNL-F$ Sequences

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ABSTRACT

To determine the genotype of progeny in orchid breeding, a new method for selection of the progeny of intergeneric orchid breeding was carried out based on the chloroplast trn Leusine ($trnL$) gene and trn Phenylalanine ($trnF$) spacer region. Around 1,100-1,200 base pairs (bp) sequences of the specific region of the orchid chloroplast showed different structure in each genus of orchid as detected by Polymerase Chain Reaction using a pair of specific oligonucleotide primers. This technique is simple and useful for orchid breeders to easily and rapidly identify the success of the breeding program.

Keywords: $trnL-F$ region, molecular marker, intergeneric, orchid breeding

INTRODUCTION

Indonesia has a collection of more than 5,000 orchid species. That makes us as one of the most important target area for orchid exploitation (Irawati, 2002). At present, the Indonesian orchid flora is threatened of being decimated and numerous valuable components may even become extinct in the near future because of forest destruction and over-collecting for trade (Vermeulen, 2002). Production and commercialization of new lines of orchid hybrids has exploited the wild orchid flora. This would really be worth attempting, because it makes use of a resource unique to the country. In nature, orchid species rarely forms natural hybrids in zones of overlap. The integrity of the species is maintained by differences in flowering times, floral morphology, visual and olfactory cues, as well as by genetic incompatibility or inability of the hybrid to establish and reproduce. When one or more of the barriers or isolating mechanisms are removed, for example when orchids are pollinated by hand in the greenhouse, two different species will often produce viable hybrids quite readily. Artificial hybrids are
common between species in the same genus and between species of different genera in the same subtribe, but are more rarely between species in different subtribes (but within the same tribe). In fact, the genes from as many as six different genera are present in some hybrids, leading to the perception of orchids as the most promiscuous plants (Anonim, 2003). The earliest hybrids were intrageneric, that is, they were crosses between species in the same genus for example Calanthe Dominy (C. furcata X C. masuca) a cross made by John Dominy in 1856. Orchid hybridization is very important to increase its genetic variation. A new hybrid with genes from multiple genera has been created such as Pottinara (Brassavola X Laelia X Cattleya X Sophronitis). The Royal Horticultural Society registers all new orchid hybrids and published them regularly in major orchid periodicals and every five years in Sanders Complete list of Orchid Hybrids.

In this paper, trnL-F sequences of chloroplast DNA were used to produce a molecular marker for genotyping of hybrids between Phalaenopsis amabilis (L.) Blume and Dendrobium fimbriatum Hook.

MATERIALS AND METHODS

The plant materials and growth condition

P. amabilis (L.) Blume (Java form), Vanda tricolor Lindley and D. fimbriatum Hook were used as plant materials in this study (Fig. 1). The flowers of P. amabilis and D. fimbriatum were cross-pollinated and the plants were maintained in a glasshouse to produce fruits and fertile seeds. Seeds were sown on modified New Phalaenopsis (NP) medium (Islam et al., 1998) with 20 g/l sucrose as carbon source, 250 mg/l casein hydrolysate and 9 g/l agar instead of Gelrite. The medium contained 150 ml/l coconut water. The cultures were maintained under continuous white light at 25°C.

Fig. 1. Orchid plant used as materials. A. V. tricolor Lindley, B. P. amabilis (L.) Blume, C. D. fimbriatum Hook. Bars, 5 cm in A, 2 cm in B, and 1 cm in C

DNA isolation and purification

The leaves of seedlings were used for DNA extraction. DNA were isolated according to the instructions from the genomic DNA isolation kit from the manufacturer (Qiagen GmbH, Germany), without RNAse treatment.
Polymerase chain reaction and sequence analysis

Genomic DNA of orchids were used as templates for the amplification of trnL-F intergenic space of chloroplast DNA using primer C (5'-CGAAATCGGTAGACGCTACG) and primer F (5'-ATTGAACTGCTGACACGAG). The PCR program was 1 min at 94°C for initial denaturation, 35 cycles for 1 min at 94°C, for 1 min at 50°C and for 1 min 30 sec at 72°C. PCR products were purified using PCR purification column (Jet quick, GENOMED GmbH). The purified PCR products were sequenced using primer C or primer F, with Big Dye terminator (PE, Biosystem) with the following conditions: for 1 min at 96°C (initial denaturation), 25 cycles for 10 sec at 96°C, for 5 sec at 50°C and for 4 min at 60°C using 12.5 μl reactions following the dideoxynucleotide chain-termination method by using an ABI PRISM 377 DNA sequencer according to the manufacturer protocol (Perkin-Elmer, Foster City, CA). Alignment of the deduced amino acid sequences was performed with the GENETYX-MAC Version 13.0.4 and Sequencher version 4.8 (Gene codes corp., Ann Arbor, Michigan, USA).

Genotyping of orchid hybrids

The amplified DNA from each orchid, parental and sibling plants were independently digested with three kinds of restriction enzyme: EcoRI, BamHI and DraI and subjected to fingerprint analysis. The digested DNA were size-fractionated by electrophoresis in either vertical 6% polyacrylamide gel or 0.7% horizontal agarose gels, stained with ethidium bromide and visualized under UV-transilluminator. The genotype of each orchid was determined by the restriction fragment length polymorphism (RFLP) analysis of digested PCR product comparing the RFLP pattern of parental and the hybrids.

RESULTS AND DISCUSSION

Structure of the intergenic spacer region trnL-F in V. tricolor and P. amabilis

To analyze the structure of the intergenic spacer region trnL-F in V. tricolor and P. amabilis, the sequences of V. luzonica originated from the Phillipine which has been submitted in the Gene Bank were used as a reference by Goh et al. (2003) AY273699. We also compared the sequence of V. tricolor and P. amabilis to the sequence of Phragmipedium equadorensen published by Whitten et al. (2005) in the Gene Bank with the accession No. AY918864. Using a pair of universal primers C and F from Taberlet et al. (1991) we could amplify a fragment cp DNA of 1, 222 bp in length from V. tricolor and 1,196 bp in length from P. amabilis, that close to the length of that specific region of V. luzonica (1,145 bp) and P. ecuadorensen (1,001 bp). The sequence alignment of V. tricolor, P.
amabilis, *V. luzonica* and *P. ecuadorensis* showed that *V. tricolor* share 88% identity to *V. luzonica* and 86% identity to *P. ecuadorensis*. However, *P. amabilis* shared 86% identity to *V. luzonica* and 84% identity to *P. ecuadorensis*. Restriction map analysis showed that three kinds of endonuclease restriction enzymes, namely *EcoRI*, *BamHI*, and *DraI* can be used to provide the RFLP marker between these orchid genera (Fig. 4).

**Selection of orchid hybrids:**

*Dendrobium fimbriatum* X *Phalaenopsis amabilis*

Based on morphological analyses of parental orchids, *P. amabilis* and *D. fimbriatum*, and its hybrids, Suseno et al. (2005) found the different patterns of shoot developments and leaf shapes among the hybrid plants. They were grouped into four categories, namely Type 1, Type 2, Type 3 and Type 4 consisted of 46, 43, 2 and 2%, respectively and 7% outside of those 4 types that showed callus like structure, and 6% showed albino appearance (Fig. 2). Type 1 was characterized by small shoot with lanceolated leaves; Type 2 was characterized by large shoot with lanceolated leaves; Type 3 showed large shoots with ellipsoid leaves and Type 4 small shoots with ellipsoid leaves.

![Morphology of intergeneric hybrid orchid plants](image)

**Fig. 2.** Morphology of intergeneric hybrid orchid plants (*D. fimbriatum* X *Phalaenopsis amabilis*). A. *P. amabilis*, B. *D. fimbriatum*, C. Intergeneric D X P type 1-4, D. Callus like, E. Albino phenotype, F. Schematic figure of C. (Suseno et al., 2005)

We also did AFLP (Amplified Fragment Length of Polymorphism) analysis of the hybrids using the *DOH1* gene locus according to the *DOH1* cDNA sequences published by Yu et al. (2001). The results showed that all hybrid plants showed recombinant pattern in *DOH1* locus. Unfortunately, we found difficulties amplifying the *DOH1* locus because of the length of the large sizes of the fragments about 2,700 bp in *Phalaenopsis* and 2,600 bp in *Dendrobium*. A part of intergenic spacer *trnL*-F region, that is in a ranges of 1,100–1,200 bp in length was easier to be amplified, because it is a very specific region in the chloroplast DNA (Fig. 3) with no redundant sequences making it is efficient to be used as a molecular marker. We confirmed that the hybrids had both fragments from the parental plants (Fig. 4).
Fig. 3. Amplified DNA on the part of the intergenic trnL-F region. V, V. tricolor; P, P. amabilis; D, D. fimbriatum. I-1 to I-8 are intergeneric hybrids between D. fimbriatum and P. amabilis. The length of the PCR product is between 1,100-1,200 bp

Fig. 4. RFLP analysis of PCR products of intergeneric plants on the trnL-F region of chloroplast DNA. A. Dral digested PCR products, B. EcoRI digested PCR products: M. Lambda DNA/Phh; V. V. tricolor; P. P. amabilis; D. D. fimbriatum; I. intergeneric plants between D. fimbriatum and P. amabilis

The orchid genera used in this study, i.e. Vanda, Phalaenopsis and Dendrobium, showed RFLP patterns among them. The RFLP patterns among the 3 genera was very clear after the PCR product of trnL-F spacer fragment was digested with Dral. In vertical 6% acrylamide gel, V. tricolor showed 3 bands (770, 293 and 82 bp), P. amabilis was digested into two fragments (400 and 770 bp), while D. fimbriatum was cut into 2 fragments (770 and 300 bp). Intergeneric plants showed hybrid molecules belonging to both D. fimbriatum and P. amabilis was indicated by the presence of 3 bands (770, 400 and 300 bp). The results were confirmed by the RFLP pattern of trnL-F region digested with EcoRI restriction enzyme. P. amabilis showed 3 bands (639, 278 and 258 bp), while D. fimbriatum showed 3 fragments with a slightly different sizes (639, 278 and 235 bp). A 23 bp different of trnL-F spacer region between D. fimbriatum and P. amabilis can be used to distinguish these 2 orchid species, particularly if we separated the digested PCR product by vertical 6% acrylamide gel electrophoresis. These data suggested that for identifying the hybrid of
orchid, the trnL-F intergenic spacer region could be used as a good molecular marker and as an innovative method to confirm the phenotypic characters. The specification of the trnL-F structure was also useful for molecular phylogenetic analysis of Incarvillea (Bignoniaceae) (Chen et al., 2005) and comparing the genetic, geographic structure and history of specimen distribution of V. tricolor in Asia (Gardiner, 2005).

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