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RFLP and inheritance patterns of chloroplast DNA in intergeneric hybrids of *Phalaenopsis* and *Doritis*

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Abstract. The mode of inheritance of chloroplasts was analyzed using restriction fragment length polymorphism (RFLP) in both interspecific hybrids of *Phalaenopsis* and intergeneric hybrids of *Phalaenopsis* and *Doritis*. Chloroplast DNA digested with *Dra* I followed by hybridization with an *rbc*L probe revealed that *Phalaenopsis amabilis*, *Phalaenopsis aphrodite*, and *Phalaenopsis stuartiana*, which belong to the taxonomic section PHALAENOPSIS, have the same size 2.0-kb fragment. Both *Phalaenopsis mannii* and *Phalaenopsis amboinensis* have a 2.3-kb fragment, while *Doritis pulcherrima* has a 3.5-kb fragment. In both interspecific and intergeneric hybrids, maternal inheritance of the chloroplast genome was detected. The hybrids of both reciprocal crosses (A x B and B x A) are registered with the same hybrid names in Sander's List of Orchid Hybrids at the Royal Horticultural Society, despite harboring chloroplast DNA from different parents. These results suggest that the chloroplast DNA can be used as a marker for identification of parentship and for phylogenetic studies of taxonomy.

Keywords: cpDNA inheritance pattern; *Doritis*; *Phalaenopsis*; RFLP.

Introduction

Phalaenopsis spp., and *Doritis* spp. belong to Tribes *Vandeae* and *Orchidaceae*, respectively. About 45 wild species of *Phalaenopsis* are distributed in Asia in a region 23° north and south of the equator (Sweet, 1980). A large collection of various *Phalaenopsis* spp. is maintained at the Taiwan Sugar Research Institute (TSRI) including 34 wild species and 1,239 superior hybrids (Chen and Wang, 1996). The botanic and horticultural characteristics of these plants have been analyzed for breeding purposes.

Unlike nuclear genes, the inheritance pattern of organelle genes varies greatly among different organisms (Birky, 1995). So far, the inheritance of cytoplasmic organelles in *Orchidaceae* has been studied only cytogenetically using DNA fluorochrome 4', 6'-diamidino-2-phenyl indole (DAPI) in conjunction with epifluorescence microscopy (Corriveau and Coleman, 1988). However, no molecular analyses have been carried out in *Phalaenopsis*. Here we present that the maternal inheritance pattern of chloroplast DNA (cpDNA) was detected in hybrids of *Phalaenopsis* and *Doritis* by using RFLPs visualized with a *rbc*L probe.

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Materials and Methods

Plant Materials

Intraspecific and interspecific crosses of *Phalaenopsis* and intergeneric crosses between *Phalaenopsis* and *Doritis* were performed and maintained in the greenhouse of TSRI (Table 1). Six wild species of *Phalaenopsis* and one wild species of *Doritis* were used in these experiments, including *P. amabilis* (L.) Blume, *P. amboinensis* J. J. Smith, *P. aphrodite* Rchb. F., *P. equestris* (Schauer) Rchb. f., *P. mannii* Rchb. f., *P. stuartiana* Rchb. f., and *D. pulcherrima* Lindl. Among them, *P. amabilis* (L.) Blume, *P. aphrodite* Rchb. F., and *P. stuartiana* Rchb. F. belong to the same taxonomic section PHALAENOPSIS (Fu et al., 1997). One intraspecific hybrid between *P.*

Table 1. Plant materials used in crosses.

Intraspecific crosses
P. equestris 'W9-55' x *P. equestris* 'W9-57'
P. equestris 'W9-57' x *P. equestris* 'W9-55'
Interspecific crosses
P. amabilis 'W1-2' x *P. amboinensis* 'W2-2'
P. amboinensis 'W2-2' x *P. amabilis* 'W1-2'
P. mannii 'W25-1' x *P. stuartiana* 'W40-5'
P. stuartiana 'W40-5' x *P. mannii* 'W25-1'
Intergeneric crosses
P. equestris 'W9' x *D. pulcherrima* 'W46-26'
D. pulcherrima 'W46-26' x *P. equestris* 'W9'

equestris (Schauer) Rchb. f. W9-55 and W9-57, two interspecific crosses between *P. amboinensis* J. J. Smith and *P. amabilis* (L.) Blume, and between *P. mannii* Rchb. f. and *P. stuartiana* Rchb. f., and an intergeneric cross between *P. equestris* (Schauer) Rchb. f. and *D. Pulcherrima* Lindl. were analyzed. The F1 progenies of the crosses and the reciprocal crosses were also analyzed.

Extraction of DNA

cpDNA extractions were performed following the protocol of Lichtenstein and Draper (1985). Plants were moved to a dark room for at least 3 days to reduce polysaccharide content (Baum and Bailey, 1989). Thirty gram of leaf tissues were cut to pieces and homogenized in chloroplast extraction buffer (50 mM Tris, 25 mM EDTA, 0.3 M mannitol, 1% polyvinylpyrrolidone) using a polytron (Kinematica AG, PT3000) at 10,000 rpm for 30 sec, and then at 8,000 rpm for 1 min. After filtration by cheesecloth, the filtrate was centrifuged at 3,500 rpm (Kokusan, H-251, type B rotor) for 10 min at 4°C. The pellet was resuspended in chloroplast suspension buffer (50 mM Tris, 25 mM EDTA, 0.3 M mannitol) and layered onto the top of a 30 to 52% sucrose gradient, and centrifuged at 28,000 rpm (Beckman L-8M, 55.2 Ti rotor) for 30 min at 4°C. The interface containing chloroplasts was collected and incubated with chloroplast suspension buffer supplemented with 2 M NaCl for 15 min and then centrifuged at 4,500 rpm (Kokusan, H-251, type B rotor) for 15 min at 4°C. This incubation and centrifugation were repeated without adding NaCl. The pellet was then resuspended with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and digested with proteinase K and SDS for 1 h at 37°C, followed by phenol/chloroform extraction and then ethanol precipitation.

For the extraction of genomic DNA, 1-2 g leaf tissues were cut to pieces in the extraction buffer (100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 2% CTAB and 1% polyvinylpyrrolidone) preheated to 65°C. Cell lysates were then incubated at 65°C with 1% of 2-mercaptoethanol for 45 min. Genomic DNAs were then extracted with phenol/chloroform and then ethanol precipitated. The concentration of nucleic acid was determined by TKO 100 Mini-Fluorometer.

Probe Preparation and PCR Reaction

The *rbc*L probe was labeled with digoxigenin (DIG) in a capillary polymerase chain reaction machine (Air Thermocycler, Idaho). For PCR, 6 ng of cpDNA was used as the template DNA, along with 10 mM mixed DIG-dNTP, 0.5 mM of two primers, 0.5 unit of *Taq* DNA polymerase (DIG DNA Labeling Kit, Boehringer Mannheim). The primer sequences were derived from *Oryza sativa* cpDNA, and provided by Dr. H. Dai (Institute of Botany, Academia Sinica, Taiwan, ROC). cpDNA amplified with these primers is a 540-bp conserved fragment of the *rbc*L gene from nucleotide (nt) 301 to nt 840. The sequences of the 30-mer primers are: primer 1 (RBCL-301U)- TTGGA CTGAT GGACT TACCA GTCTT GATCG; primer 2 (RBCL-840L)- TCTTC GCATG TACCT GCAGT CGCAT TCAAG.

RFLP Method and Southern Hybridization

The cpDNA (2 μg), genomic DNA samples (8 μg), and amplified *rbc*L fragments (2 μg) were digested with 20 units of restriction endonuclease according to the manufacturer's instructions (Boehringer Mannheim). Fourteen restriction enzymes were used for detection of the polymorphism including *Ava*I, *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*II, *Kpn*I, *Msp*I, *Pst*I, *Xba*I, and *Xho*I. Restricted fragments were then separated in a 0.8% agarose gel. After separation, agarose gels were depurinated with 0.25 N HCl for 5 min, denatured in 1.5 M NaCl, 0.5 M NaOH for 30 min, neutralized in 3 M NaCl, 0.5 M Tris, and then blotted onto nylon membrane (Boehringer Mannheim).

Prehybridization and hybridization reactions following the recommendation of the manufacturer (DIG Luminescent Detection Kit, Boehringer Mannheim) were performed in a hybridization oven (Hybaid). Prehybridization was carried out in 5 X SSC, 1 X blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS at 68°C for 1 h. For hybridization, 5-25 ng of denatured DIG-labeled *rbc*L probe was added for a 68°C overnight incubation. Nylon membranes were then washed with 2 X SSC, 0.01% SDS at room temperature for 5 min twice, and then in 0.1 X SSC, 0.1% SDS at 68°C for 15 min twice.

For detection, the washed nylon membrane was incubated with 0.15 M NaCl, 0.1 M maleic acid (pH 7.5) for 2 min, and then with 1 X blocking reagent for 30 min. Antibody against DIG conjugated with alkaline phosphatase (1:5000 diluted) was then allowed to interact with membrane for 30 min with light rocking (DIG Luminescent Detection Kit, Boehringer Mannheim). The

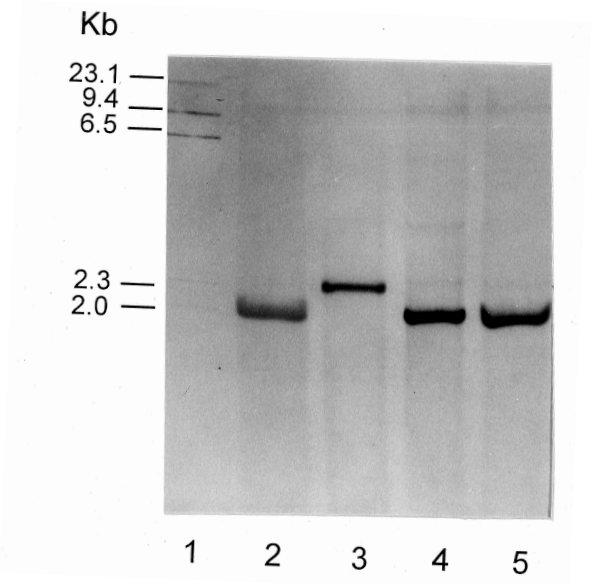

Figure 1. Polymorphisms are detected among different wild species. Total genomic DNAs of four wild type *Phalaenopsis* were digested with restriction enzyme *Dra*I, transferred onto nylon membrane, and probed with a *rbc*L fragment. Lane 1, DNA marker; lane 2, *P. amabilis* 'W1-2'; lane 3, *P. amboinensis* 'W2-2'; lane 4, *P. amabilis* 'W1-8'; lane 5, *P. aphordite* 'W3-16'.

unbound antibody was washed with 0.1 M NaCl, 0.1 M Tris, pH 9.5, and detected with the alkaline phosphatase substrate NTB/BCIP (20 µl/ml) overnight.

Results

RFLPs of Parental cpDNA

Several RFLP experiments were carried out to test the polymorphism in the intraspecific, interspecific, and intergeneric crosses and in the F1 progenies of both crosses and reciprocal crosses. Restriction enzyme digestion of cpDNA, PCR amplification of the *rbc*L fragment, and restriction digestion of the amplified *rbc*L fragments failed to resolve any polymorphism among them. Finally, when the amplified 540-bp *rbc*L fragment was used as a probe in Southern blot analysis, a polymorphism was detected between both *P. amabilis* and *P. amboinensis*, which belong to different taxonomic sections, PHALAENOPSIS and AMBOINENSIS, respectively. Total genomic DNA was digested with *Dra* I followed by hybridization with the 540-bp *rbc*L probe. A 2.0-kb fragment was detected in *P. amabilis*, while a 2.3-kb fragment was detected in *P. amboinensis* (Figure 1, lanes 2 and 3). However, no polymorphisms existed when clones W1-2 and W1-8 of *P. amabilis* were compared (Figure 1, lanes 2 and 4), nor between *P. amabilis* and *P. aphrodite* which belong to the same taxonomic section PHALAENOPSIS (Figure 1, lanes 4 and 5). No polymorphism existed when clones W9-55 and W9-57 of *P. equestris*, section STAUROGLOTTIS and their intraspecific hybrids were analyzed .

cpDNA Inheritance in Interspecific Hybrids of Phalaenopsis

In the analysis of the interspecific cross between *P. amboinensis* (parent A) and *P. amabilis* (parent B), results showed that parent A gave rise to a 2.3-kb fragment and parent B gave rise to a 2.0-kb fragment. In the cross A x B, all five individual F1 progenies showed a maternal inheritance pattern of the cpDNA by the presence of a 2.3-kb fragment. In contrast, all five F1 progenies of the reciprocal cross B x A, showed a 2.0-kb fragment derived from their maternal parent (Figure 2A).

In another interspecific cross, *P. mannii* (parent A) and *P. stuartiana* (parent B), a 2.3-kb fragment was obtained for *P. mannii*, section POLYCHILOS and a 2.0-kb fragment for *P. Stuartiana*, section PHALAENOPSIS. Both progenies of cross A x B and reciprocal cross B x A showed a maternal inheritance pattern of their cpDNA (Figure 2B).

cpDNA Inheritance in Intergeneric Hybrids of Phalaenopsis and Doritis

In analyzing the intergeneric cross between *P. equestris* (parent A) and *D. pulcherrima* (parent B), parent A gave rise to a 2.0-kb fragment, while parent B resulted in a 3.5-kb fragment. In all the five F1 progenies of cross A x B, a 2.0-kb fragment derived from parent A was detected; in the reciprocal cross B x A, all five F1 progenies showed a 3.5-kb fragment derived from parent B (Figure 3). These results suggest that a maternal inheritance pattern of chloroplast DNA is present in the intergeneric hybrids between *Phalaenopsis* and *Doritis*.

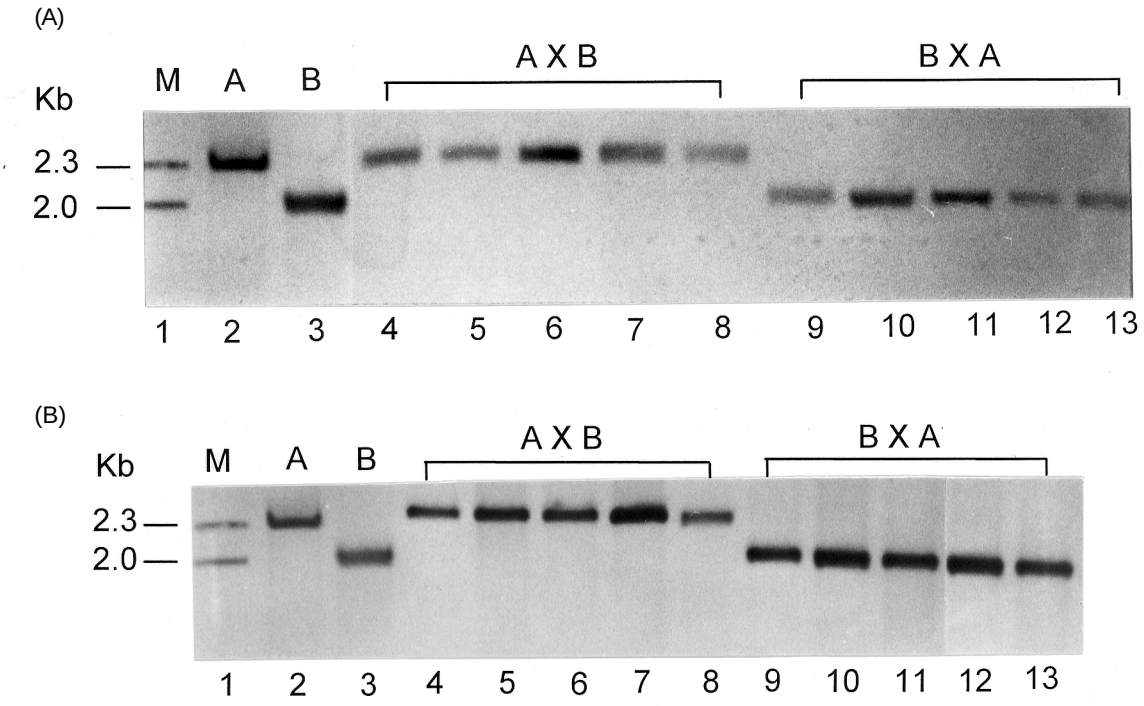

Figure 2. Maternal inheritance pattern is observed in interspecific crosses. (A) Interspecific cross between *P. amboinensis* (parent A) and *P. amabilis* (parent B); (B) Interspecific cross between *P. mannii* (parent A) and *P. stuartiana* (parent B). Lane 1, DNA marker; lane 2, parent A; lane 3, parent B; lanes 4-8, progenies of cross A x B; lanes 9-13, progenies of reciprocal cross B x A.

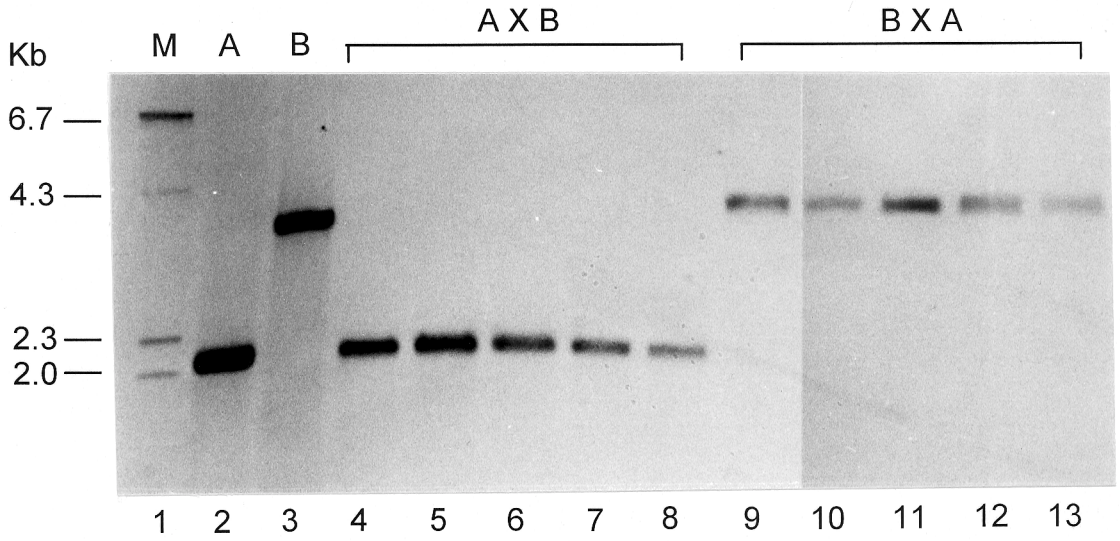

Figure 3. RFLP analysis of intergeneric hybrids of *P. equestris* (parent A) and *D. pulcherrima* (parent B). Lane 1, DNA marker; lane 2, parent A; lane 3, parent B; lanes 4-8, progenies of cross A x B; lanes 9-13, progenies of reciprocal cross B x A.

Discussion

It has been reported that the *rbc*L probe shows polymorphsim in bananas (Gawel and Jarret, 1991), *Nicotiana* (Kung et al., 1982), *Oryza* species (Kanno and Hirai, 1992), and cocoa (Laurent et al., 1993). Polymorphic patterns were detected in both interspecific reciprocal hybrids of *Phalaenopsis* and intergeneric reciprocal hybrids between *Phalaenopsis* and *Doritis* using RFLP and hybridized with the *rbc*L probe.

Since *Phalaenopsis* is monoecious, reciprocal crosses are available for the study of the inheritance patterns of cpDNA. Besides, there is no reproduction block withinin *Phalaenopsis* or between *Phalaenopsis* and *Doroitis*. Both interspecific and intergeneric crosses are accessible. In this study, wild species of *Phalaenopsis* were used rather than cultivars, which have gone through many generations of crossing. Their complicated genetic background could confuse the analysis and make interpretation difficult. The generation time of *Phalaenopsis* is two to three years, and the long duration from seedling to first blooming makes it hard to design an experiment that includes all the crosses needed to complete the assay of inheritance patterns of *Phalaenopsis* cpDNA. Besides, only those with both parental strains and F1 progenies maintained can be used for this study. Usually, hundreds to thousands of F1 progenies are produced by each individual pair of parental plants; however, only tens to hundreds become mature and bloom. Natural selection may have excluded other types of inheritance patterns. In addition, the conclusion that cpDNA is inherited maternally is based on 30 progenies, 5 from each of 6 crosses. It may be possible to detect small numbers of biparental or paternal progeny if hundreds of F1 progenies are analyzed. On the other hand, the lack of paternal inheritance pattern was not due to the sensitivity of the approach used in this report. We found that both the 2.0- and 2.3-kb fragments can be detected toward the one-hundred fold dilution in a tenfold dilution series, suggesting that a one-in-a-hundred chance of a paternal inheritance pattern can be detected if it is there. Hence,it is indeed the maternal inheritance pattern detected, rather than the lack of chance to detect other inheritance patterns such as paternal or beparental patterns.

In the Royal Horticultural Society (RHS) Sander's List of Orchid Hybrids, the hybrids from both cross A x B and reciprocal cross B x A of *Phalaenopsis* have the same hybrid name (Greatwood et al., 1993). From our analysis, the RFLP patterns of cpDNA in both cross A x B and reciprocal cross B x A were obviously different. It is possible that the cytogenetic difference between cross A x B and reciprocal cross B x A may be due to unique features of the hybrids of either cross. Thus the RFLPs of cpDNA using the *rbc*L probe may be useful in tracing the genetic background.

In the phylogenetic studies of taxonomy and evolution among wild species of *Phalaenopsis* by random amplified polymorphic DNA (RAPD) markers, it has been shown that *P. amabilis*, *P. aphrodite, P. equestris*, and *P. stuartiana* have the same genetic background (Fu et al., 1997). Interestingly, our results showed that these four species of *Phalaenopsis* have the same size 2.0-kb fragment in the RFLP analysis. Thus, the observation that *P. equestris*, section STAUROGLOTTIS is taxonomically close to *P. amabilis*, section PHALAENOPSIS was confirmed by both RAPD (Fu et al., 1997) and RFLP-Southern hybridization analyses.

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蝴蝶蘭和朵麗蘭種間及屬間雜種葉綠體 DNA 遺傳模式之探討

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本研究利用核酸片段長度多形性及*rbc*L探針之南方式轉印法，探討蝴蝶蘭種間、蝴蝶蘭及朵麗蘭屬間雜交後代之葉綠體遺傳模式。分析結果顯示：屬於 PHALAENOPSIS 分類區間的蝴蝶蘭產生一個 2.0 kb 長度的核酸片段，而屬於另一個分類區間的蝴蝶蘭則產生一個 2.3 kb 長度的核酸片段。朵麗蘭在此分析結果則顯示一個 3.5 kb 長度的核酸片段。在所有的種間及屬間雜交後代，都顯示葉綠體 DNA 為母系遺傳。此外，正、反交的後代（A x B 及 B x A），在英國皇家學會 Sander's 蘭花雜交種名錄皆登記為同一種，然而分析他們的葉綠體 DNA，則發現他們的葉綠體 DNA 各遺傳自不同的母系。因此，利用核酸片段長度多形性及*rbc*L探針之南方式轉印法，可應用於鑑定雜交種的母系親代，並且有助於演化分類上的研究。

關鍵詞：葉綠體遺傳模式；朵麗蘭；蝴蝶蘭；核酸限制片段長度多型性。