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Plastid *trn*L intron polymorphisms among *Phalaenopsis* species used for identifying the plastid genome type of *Phalaenopsis* hybrids

Chi-Chu Tsai [a,b,*], Yu-Chung Chiang [c], Yu-Shium Lin [a], Wen-Lin Liu [a], Chang-Hung Chou [d,**]

[a] *Kaohsiung District Agricultural Research and Extension Station, Pingtung 900, Taiwan*
[b] *National Pingtung University of Science and Technology, Pingtung 912, Taiwan*
[c] *National Sun Yat-sen University, Kaohsiung 804, Taiwan*
[d] *Research Center for Biodiversity, China Medical University, Taichung 404, Taiwan*

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ABSTRACT

The *trn*L intron sequences of plastid DNA for over 95% of the living native species of *Phalaenopsis* were determined in this study, and nearly all *Phalaenopsis* species were found to bear unique *trn*L intron sequences resulting from mutations, insertions/deletions, or both. These *trn*L intron sequences have been deposited into GenBank database for further identifying the plastid genome type of *Phalaenopsis* hybrids. Molecular evidence has demonstrated that maternal inheritance of the plastid genome occurs during interspecific hybridization of *Phalaenopsis* species. Therefore, the plastid genome type of *Phalaenopsis* hybrids can be determined by comparing the *trn*L intron sequences of the hybrids to GenBank database. The plastid genome type of the hybrids that is revealed through this analysis can be used to re-evaluate their genealogies because plastid DNA is maternally inherited. We examined *trn*L intron sequences from three *Phalaenopsis* hybrids including *P.* Yungho Gelb Canary, *P.* Timonthy Christopher, and *P.* Rainbow Chip to re-evaluate their genealogies from the recording of the Sander's List of Orchid Hybrids. No heterogeneous *trn*L intron sequences were found for any of the *Phalaenopsis* hybrids examined. After sequence comparing to GenBank database, the plastid genome types of the hybrids are determined. The conflict of genealogy and the plastid genome type in two hybrids *P.* Timonthy Christopher and *P.* Rainbow Chip can be found. This conflict results from their female parent *P.* Cassandra with wrong registration in Sander's List of Orchid Hybrids at Royal Horticultural Society (RHS).

1. Introduction

Moth orchids (*Phalaenopsis* spp.) are some of the most beautiful and popular plants. They consist of approximately 66 native species worldwide, 56 of which are extant (Christenson, 2001). Based on the classification of Christenson (2001), the *Phalaenopsis* genus is divided into five subgenera, namely *Proboscidioides*, *Aphyllae*, *Parishianae*, *Polychilos*, and *Phalaenopsis*, which were determined mainly by plant size and floral morphology (including callus, lip structure, pollinium number, etc.). The subgenus *Polychilos* was further subdivided into four sections, including *Polychilos*, *Fuscatae*, *Amboinenses*, and *Zebrinae*. In addition, subgenus *Phalaenopsis* was also subdivided into four sections, namely, *Phalaenopsis*, *Deliciosae*, *Esmeralda*, and *Stauroglottis*. Species of *Phalaenopsis* are found throughout tropical Asia and the larger islands of the Pacific Ocean. All *Phalaenopsis* species, excluding the natural tetraploid species *Phalaenopsis buyssoniana* Rchb.f., have 38 ($2n = 38$) chromosomes (Tanaka and Kamemoto, 1984; Christenson, 2001). Recently, the plastid genome of *Phalaenopsis aphrodite* have been completely sequenced (Chang et al., 2006), and molecular phylogenies of *Phalaenopsis* species also have been conducted based on the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA) and plastid DNA (Tsai et al., 2006a, 2009, 2010a,b). In addition, molecular data was applied to determine the inheritance of the natural hybrid, *Phalaenopsis x intermedia*, showing *P. aphrodite* was the maternal parent and *Phalaenopsis equestris* was the paternal parent (Tsai et al., 2006b).

Most plastid genomes are multicopy circular molecules (120–160 kbp) that retain highly conserved structures among vascular plants, mosses, and algae (Palmer, 1985). The majority of angiosperm species undergo uniparental maternal plastid genome inheritance (Kuroiwa, 1991; Mogensen, 1996), and recombination of genes between plastids is rare (Chiu and Sears, 1985). The degeneration time of pollen plastid progeny has been suggested to be the interval of time between pollination and fertilization (Chiu and Sears, 1993). Electron microscopy suggested that the plastids were excluded from the early generative cell during the

* Corresponding author at: Kaohsiung District Agricultural Research and Extension Station, Pingtung 900, Taiwan. Fax: +886 8 7746735.
** Corresponding author.
E-mail addresses: tsaicc@mail.kdais.gov.tw (C.-C. Tsai), choumasa@mail.cmu.edu.tw (C.-H. Chou).

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first pollen mitosis in *Syringa oblata* (Liu et al., 2004). In fact, the most common mechanism for maternal plastid inheritance is the exclusion of plastids during the first pollen mitosis via unequal plastid distribution (Lycopersicon type) or during generative or sperm cell development via plastid degeneration (Solanum type) (Hageman and Schroder, 1989; Mogensen, 1996). In addition, the maternal inheritance of plastid DNA for both interspecific hybrids and intergeneric hybrids between *Phalaenopsis* and *Doritis* has been determined based on specific DNA markers (Chang et al., 2000).

Universal primers for the *trn*L intron and *trn*L-*trn*F spacer were developed by Taberlet et al. (1991) and have been used successfully to identify DNA sequences that are useful for phylogenetic markers at the intrageneric level, such as within *Miscanthus*, *Saccharum* (Poaceae; Hodkinson et al., 2002), *Moraea* (Iridaceae; Goldblatt et al., 2002), and *Allium* (Liliaceae; Van Raamsdonk et al., 2003). Furthermore, because organellar genomes are often uniparentally inherited, plastid and mitochondrial DNA polymorphisms have become molecular markers for investigating sex-biased dispersal and the directionality of introgression (Wills et al., 2005).

In this study, the plastid *trn*L intron sequence was determined for 54 native *Phalaenopsis* species. The inheritance of the plastid genome of three interspecific hybridizations of *Phalaenopsis* species was determined based on inspection of the *trn*L intron sequence. In addition, the native *trn*L sequences were used to identify the plastid genome type of various *Phalaenopsis* hybrids.

2. Materials and methods

2.1. Plant materials

In this study, 54 native *Phalaenopsis* species, and three *Phalaenopsis* hybrids including *P.* Yungho Gelb Canary, *P.* Timonthy Christopher, *P.* Rainbow Chip were examined (Table 1). In all cases, fresh leaves were taken from living plants grown in greenhouses at the Kaohsiung District Agricultural Research and Extension Station (KDARES) in Pingtung, Taiwan.

2.2. DNA extraction, PCR amplification, and electrophoresis

Total DNA of samples studied was extracted using a cetyltrimethylammonium bromide (CTAB) method that has been previously described (Doyle and Doyle, 1987), and approximate DNA yields were determined using a spectrophotometer (Hitachi U-2001, Tokyo, Japan). Primer sets were then used to amplify the *trn*L intron region of the chloroplast DNA (cpDNA) of all of the *Phalaenopsis* plants described in Taberlet et al. (1991), using polymerase chain reaction (PCR) conditions that have been previously described (Tsai, 2003). PCR products were separated by agarose gel electrophoresis [0.8% (w/v)] in 1× TBE buffer, stained with 0.5 $\mu g\,ml^{-1}$ ethidium bromide and photographed under UV light.

2.3. DNA recovery and sequencing

PCR products were separated on 0.8% agarose gel, and the DNA was subjected to purify and quantify prior to sequencing. PCR products were sequenced on an ABI 3700 sequencer (Applied Biosystems Inc., Foster City, CA, USA) using the dideoxy chain termination method. Sequencing was performed using the Big Dye Terminator labeling mix following the manufacturer's instructions.

2.4. BLAST searching

The *trn*L intron sequences from the 54 native *Phalaenopsis* species were deposited into GenBank, whereby these sequences were made publically available through various NCBI databases. To determine which *trn*L intron sequence was present in each *Phalaenopsis* hybrid, an optimized sequence comparison algorithm was used to search NCBI databases to identify optimal local alignments to a query sequence based on the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Inspection of the BLAST results identified the native *Phalaenopsis trn*L intron sequence that represented the corresponding plastid genome type for each of the *Phalaenopsis* hybrids.

3. Results and discussion

3.1. Plastid trnL intron polymorphisms in the genus Phalaenopsis

PCR-amplified DNA sequencing was used to determine the *trnL* intron genotypes of 54 *Phalaenopsis* species, representing over 95% of the living species diversity within this genus, and these sequences were submitted to GenBank (accession numbers: AY265742–48, AY265750–61, AY265763–87, AY265793, AY265795–800, DQ194981–82, DQ195040). The variation in length for the *trn*L intron sequences of the *Phalaenopsis* species ranged from 627 bp in *Phalaenopsis pulcherrima* to 721 bp in *Phalaenopsis mannii*. Nearly all of the *Phalaenopsis* species had a unique *trn*L intron sequence resulting from mutations, insertions/deletions (indels), or both. Within the subgenus *Phalaenopsis*, 117 indels and 28 polymorphic sites were identified by multiple sequence alignment of the *trn*L intron sequences of 12 species of this subgenus. Each species of the subgenus *Phalaenopsis* encoded a unique *trn*L intron sequence with the exception of *Phalaenopsis schilleriana* and *Phalaenopsis philippinensis*, which had identical *trn*L intron sequences. These two species belong to the section *Phalaenopsis* (Fig. 1). *P. philippinensis* had been treated as *Phalaenopsis x leucorrhoda*, a natural hybrid between *P. aphrodite* and *P. schilleriana*, until this was reassessed by Tharp et al. (1987). An artificial hybridization between *P. aphrodite* (♀) and *P. schilleriana* (♂) was conducted by Dr. Robert J. Griesebach to determine the morphology between the hybrids and *P. x leucorrhoda* (see Fowlie, 1991). This result did not support the previous observation that *P. x leucorrhoda* was a natural hybrid of *P. aphrodite* (♀) and *P. schilleriana* (♂). However, from the comparison of *trn*L intron between *P. schilleriana* and *P. philippinensis*, it revealed that *P. philippinensis* may be a recent natural hybrid between *P. schilleriana* as the maternal parent and *P. aphrodite* as the paternal parent.

Within the subgenus *Polychilos*, 290 indels and 42 polymorphic sites were found among the sequences in the multiple sequence alignment of the *trn*L intron sequences of 34 species of this subgenus. One variable length polymorphism was found within the *trn*L intron sequence of the various species of the subgenus *Polychilos*, and this variation occurred within a known hot spot region. This hot spot is highly enriched with A and T nucleotides and contains AAT/ATT/AT repeat sequences. The A+T rich nature of the hot spot region of the plastid DNA is well known and has also been reported elsewhere (Ogihara et al., 1991, 1992). The A+T content within the hot spot region ranges from 83.0% to 100.0%, which is higher than that observed for the entire *trn*L intron ranging from 71.8% to 76.2%. Moreover, variable length polymorphisms that occur within hot spot regions of plastid DNA have been described in several reports (Tassopulu and Kung, 1984; Ogihara and Tsunewaki, 1988; Ogihara et al., 1991; Guo and Terachi, 2005). Two mechanisms, slipped-strand mispairing and molecular recombination, are thought to account for indels in the noncoding regions of the plastid genome during evolution (Kelchner, 2000). Each species of the subgenus *Polychilos* had a unique *trn*L intron sequence with the exception of *Phalaenopsis fuscata* and *Phalaenopsis kunstleri*, which had the same sequence. These two species belong to the section *Fuscatae* (Fig. 2). Actually, these two species were confused for each other