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Analysis of sequence diversity through internal transcribed spacers and simple sequence repeats to identify *Dendrobium* species

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ABSTRACT. The Orchidaceae is one of the largest and most diverse families of flowering plants. The *Dendrobium* genus has high economic potential as ornamental plants and for medicinal purposes. In addition, the species of this genus are able to produce large crops. However, many *Dendrobium* varieties are very similar in outward appearance, making it difficult to distinguish one species from another. This study demonstrated that the 12 *Dendrobium* species used in this study may be divided into 2 groups by internal transcribed spacer (ITS) sequence analysis. Red and yellow flowers may also be used to separate these species into 2 main groups. In particular, the deciduous characteristic is associated with the ITS genetic diversity of the A group. Of 53 designed simple sequence repeat (SSR) primer pairs, 7 pairs were polymorphic for polymerase chain reaction products that were amplified from a

specific band. The results of this study demonstrate that these 7 SSR primer pairs may potentially be used to identify *Dendrobium* species and their progeny in future studies.

Key words: *Dendrobium*; Internal transcribed spacer; Molecular marker; Simple sequence repeat

INTRODUCTION

The Orchidaceae is one of the largest and most diverse families of flowering plants. There are almost 700 genera and around 20 000 species of orchids on Earth. Most Orchidaceae are rare and endangered, and have very high economic value. For instance, members of the *Dendrobium* genus are Orchidaceae with high economic potential as ornamental plants and for medicinal purposes. In addition, the species of this genus are able to produce large crops. *Dendrobium* varieties on the market are diverse and complex; however, it is difficult to distinguish one species from another, because of their very similar outward appearance before flower development, making identification problematic. To avoid mistakes in supply and demand in the *Dendrobium* market, it is important to use biological molecular markers as an identification method.

Molecular markers, such as simple sequence repeat (SSR), ISSRs, AFLP, and RAPDs, are efficient tools for the identification of cultivars, selection of parents for hybridization breeding, and conservation of genetic resources. Such molecular markers have already been used for the identification of *Morus* L. (Kalpana et al., 2012), *Prunus armeniaca* L. (Pedryc et al., 2009), *Boesenbergia* (Vanijajiva et al., 2005), *Fagopyrum tataricum* (Garima et al., 2012), and *Lycopersicon lycopersicum* L. (Meng et al., 2010) cultivars, among many other species. Molecular markers are used for the selection of parents for hybridization breeding, conservation of genetic resources, and analysis of genetic diversity, in addition to estimating the genetic diversity of different species of orchids (Huang et al., 2010). In particular, the internal transcribed spacer (ITS) technique has been frequently used to identify Orchidaceae using biological molecular markers. However, because of the diversity of the ITS technique, it is time-consuming and expensive to use. Therefore, to reduce the cost and improve the efficiency of Orchidaceae identification, more easily accessible and reliable molecular marker methods are needed to identify Orchidaceae. Recently, the SSR method has been developed to provide higher efficiency and lower cost compared to other biological molecular marker methods. This study used the original ITS sequences of 12 *Dendrobium* varieties to probe their phylogenetic relationship, and made use of SSR primers to identify these 12 *Dendrobiums*. Thus, we developed a technique to use SSR biological molecular markers to identify *Dendrobium* varieties. Singaporean (Yue et al., 2006) and Thai (Phuekvilai et al., 2009) researchers have also successfully used SSR markers as tools to identify Orchidaceae.

MATERIAL AND METHODS

Plant materials

Twelve plant samples were provided by Professor Chin of the National Ping Tong University of Science and Technology, Taiwan. These 12 *Dendrobium* samples may be distin-

guished into 2 main groups according to their red and yellow inflorescence. The characteristic traits of these samples are presented in Figure 1 and Table 1.

Figure 1. Pictures of the inflorescence of the 12 *Dendrobium* varieties studied. **A.** *Dendrobium nobile* Lindl; **B.** *Dendrobium moniliforme* (L.) Sw; **C.** *Dendrobium heterocarpum* Wall. ex. Lindl; **D.** *Dendrobium signatum* Rchb.f; **E.** *Dendrobium findleyanum* Parish & Rchb.f; **F.** *Dendrobium pendulum* Roxb; **G.** *Dendrobium linawianum* Rchb.f; **H.** *Dendrobium friedericksianum* Rchb.f; **I.** *Dendrobium unicum* Seidenf; **J.** *Dendrobium hercoglossum* Rchb.f; **K.** *Dendrobium wardianum* Warner; **L.** *Dendrobium nobile* var. *cooksonianum* Rchb.f.

Table 1. Species names, geographic distribution, color of the inflorescent, and ITS classification of 12 varieties.

Scientific name	Range	Color of the inflorescent	Deciduous	ITS classification
Dendrobium nobile Lindl.	Himalayas, India Bhutan, Nepal Myanmar, Thailand, China, Laos Vietnam	Red	Semideciduous	A
Dendrobium hercoglossum Rchb.f.	Malaysia, Thailand Laos, Vietnam Southern China	Red	Semideciduous	A
Dendrobium nobile var. *cooksonianum* Rchb.f.	Himalayas, India Bhutan, Nepal Myanmar, Thailand, China, Laos Vietnam	Red	Semideciduous	A
Dendrobium moniliforme (L.) Sw.	Himalayas, India Nepal, Bhutan Myanmar, China Taiwan, Korea Japan, Ryukyu	Red	Semideciduous	A
Dendrobium unicum Seidenf.	Thailand, Vietnam Laos	Orange	Deciduous	A
Dendrobium pendulum Roxb.	India, Myanmar Thailand, Laos China	Red	Deciduous	A
Dendrobium wardianum Warner	India, Myanmar China, Thailand	Red	Deciduous	A
Dendrobium heterocarpum Wall. ex Lindl.	India, Sri Lanka Nepal, Bhutan Myanmar, Thailand, Malaysia, China Sumatra, Java Borneo, Sulawesi	Yellow	Semideciduous	B
Dendrobium findleyanum Parish & Rchb.f.	Himalayas, Myanmar Thailand, Laos	Red	Semideciduous	B
Dendrobium signatum Rchb.f.	Myanmar, Thailand Laos, India	Yellow	Semideciduous	B
Dendrobium linawianum Rchb.f.	Taiwan, China	Red	Semideciduous	B
Dendrobium friedericksianum Rchb.f.	Thailand	Yellow	Semideciduous	B

DNA extraction

Stems or leaves were cut directly from the plant materials, frozen in liquid nitrogen, and stored at -80°C before analysis.

After extracting genomic DNA from samples using an Axygen plant DNA extraction kit, the extracted genomes were diluted to 5 ng/μL, and stored at -20°C.

Primer design

Fifty-three pairs of SSR primers were directly copied from the literature, including 7 pairs of primers developed from the *Vanda* orchid (Phuekvilai et al., 2009) and 2 pairs of primers developed from the *Mokara* orchid (Phuekvilai et al., 2009); 9 pairs of primers developed from the *Epidendrum* orchid (Pinheiro et al., 2009); 8 pairs of primers developed from the *Dendrobium* orchid (Boonsrangsom et al., 2008); 14 pairs of primers developed from the *Dendrobium* orchid (Yue et al., 2006); and 13 pairs of primers developed from the *Cymbidium* orchid (Huang et al., 2010).

A pair of ITS primers was designed based on the DNA sequence of a *Dendrobium* species with GenBank sequence accession No. EU477507.1. Consistent with the DNA sequence from 247 to 270 nucleotides, and the DNA sequence from 597 to 620 nucleotides, the forward and reverse primers were designed with a 24-nucleotide range.

PCR amplification

For the SSR, polymerase chain reaction (PCR) amplification was conducted in a 20-μL final volume containing 20 ng genome, 2 μL dimethylsulfoxide (DMSO), 2 μL PCR buffer, 400 μM dNTP, 1 μL of each primer (forward and reverse primers), 0.4 μL Taq DNA polymerase, and 9.2 μL water. The following PCR program was set to denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 40 s, Ta at 54°-59°C for 40 s, 72°C for 30 s, and a final extension at 72°C for 10 min, and was then held at 4°C.

For the ITS, PCR amplification was conducted in a 50-μL final volume containing 40 ng genome, 5 μL DMSO, 5 μL $MgSO_4$, 5 μL PFU buffer, 1000 μM dNTP, 1 μL of each primer (forward and reverse primers), 0.5 μL PFU DNA polymerase, and 23.5 μL water. The PCR program was set to denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 40 s, 55°C for 40 s, 72°C for 30 s, and a final extension at 72°C for 10 min, and was then held at 4°C.

ITS gene cloning

ITS PCR products purified on a gel were used with DNA ligase to incorporate the ITS fragment into a plasmid (p-True Blue) with an internal restriction enzyme (*Sma*I). Subsequently, heat shock was used to transform the vector into *Escherichia coli* (XL1-Blue), and the resultant ITS fragment was sequenced.

Analysis of phylogenetic relationships

ITS sequences of the plant materials were confirmed 3 times, and then the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 was used to analyze their phylogenetic relationship and construct a phylogenetic tree.

RESULTS

Phylogenetic relationships of the 12 *Dendrobium* samples

The 12 *Dendrobium* samples studied were differentiated into 2 main groups by ITS, which were designated as A and B. Group A contained *D. nobile* Lindl., *D. hercoglossum* Rchb.f., *D. nobile* var. *cooksonianum* Rchb.f., *D. moniliforme* (L.) Sw., *D. unicum* Seidenf., *D. pendulum* Roxb., and *D. wardianum* Warner, while Group B contained *D. heterocarpum* Wall. ex Lindl., *D. findleyanum* Parish & Rchb.f., *D. signatum* Rchb.f., *D. linawianum* Rchb.f., and *D. friedericksianum* Rchb.f. (as shown in Figure 2). We also found that these 12 *Dendrobium* varieties may be separated into 2 groups according to the color of their inflorescence; red (containing more anthocyanin) and yellow (containing more carotenoids). The red group contained *D. nobile* Lindl., *D. moniliforme* (L.) Sw., *D. findleyanum* Parish & Rchb.f., *D. pendulum* Roxb., *D. linawianum* Rchb.f., *D. hercoglossum* Rchb.f., *D. wardianum* Warner, and *D. var. cooksonianum* Rchb.f., while the yellow group contained *D. heterocarpum* Wall. ex Lindl., *D. signatum* Rchb.f., *D. friedericksianum* Rchb.f., and *D. unicum* Seidenf. As shown in Table 1 the comparisons of flower color groups and the ITS groups are quite similar, with the exception of the inflorescence *D. unicum* Seidenf., which is orange, and *D. findleyanum*

Parish & Rchb.f. and *D. linawianum* Rchb.f., which have the opposite color to the main color type within the respective ITS sequences. Furthermore, we also found that the deciduous *Dendrobium* cultivars also tend to reflect the ITS sequence in the A group.

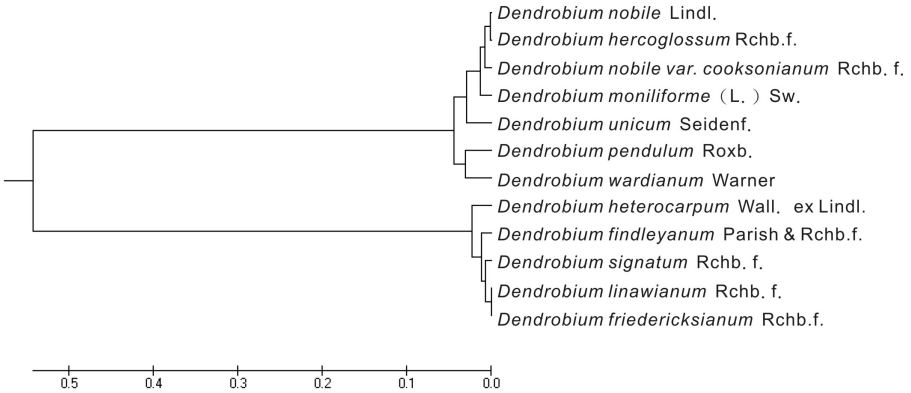

Figure 2. Dendrogram of genetic relationship among 12 *Dendrobium* varieties according to the ITS sequences using MEGA 4.0.

Analysis of SSR marker information

Among the 53 pairs of designed SSR primers, 7 pairs produced very specific bands for the 12 *Dendrobium* varieties used in this study. Specifically, 1 pair of the 9 primers was developed from *Epidendrum* orchids, 2 pairs of the 22 primers were developed from *Dendrobium* orchids, and 4 pairs of the 13 primers were developed from Chinese orchids. The selected primers and their sequences are shown in Table 2.

Table 2. Sequences and references for the 7 pairs of SSR primers that can identify the 12 *Dendrobium* varieties used in this study, selected from the 53 pairs designed for orchid classification.

Primer	Primer sequences		References
	Forward	Reverse	
B6-2	TAGGATGATGCACGGGAAA	GGGGGTTTTTATCATTGAGGA	Pinheiro et al., 2009
A5-3	GGAACGGAGAAGATTAAGACAACC	TGCCCTCACATGCCGTATT	Yue et al., 2006
A12-3	GTGACTCGAGCCTTGGAATACG	ACGCCGGTGAAAGAAGAAGAG	Yue et al., 2006
B5-3	GATTTATGTAGCCGACCCC	CCTGCTCCACTCACCTGTT	Huang et al., 2010
B6-3	AAAGTGACAGGGTAAGAGTGA	GGCGAAGATGTTGTTGAA	Huang et al., 2010
B9-3	AGCAACGATGGAGCAAGA	GCTGACCACGCTAACCTC	Huang et al., 2010
B11-3	GTCCCGAGCCTCACATAA	AAAGCAGTTCCATAAAGATTG	Huang et al., 2010

In the analysis of *D. nobile* Lindl. using the B6-2 primer, the major bands that were identified contained 330-, 240-, and 175-bp bands. As the 240-bp band was not identified from the analysis of the other cultivars, we regarded it as specific. Hence, it is possible to use this specific 240-bp band to distinguish *D. nobile* Lindl. from the other cultivars.

When using primer B11-3 for the analysis of *D. heterocarpum* Wall. ex Lindl., the major bands that were identified contained 350- and 270-bp bands. For *D. findleyanum* Parish

& Rchb.f., the major bands were 270, 180, and 170 bp. For *D. unicum* Seidenf., the major bands were 400, 210, and 180 bp. For *D. heterocarpum* Wall. ex Lindl., the major-specific band was 350 bp. For *D. findleyanum* Parish & Rchb.f., the major-specific band was 170 bp. For *D. unicum* Seidenf., the major-specific band was 210 bp. Therefore, this method could be used to identify the 12 *Dendrobium* species used in this study.

Among these 7 primer pairs, the B6-2 primer may be used to identify 7 of the 12 *Dendrobium* species used in this study, the B5-3 may be used to identify 5 species, primers A5-3 and B6-3 may be used to identify 4 species, the B11-3 may be used to identify 3 species, the B9-3 primer may be used to identify 2 species, and the A12-3 primer may be used to identify 1 species. However, of the 7 primer pairs, no single primer pair may be used to identify *D. pendulum* Roxb. The statistics are shown in Table 3 and **Figure S1**.

Table 3. Analysis of the polymorphic band patterns of the 12 varieties PCR using the 7 pairs of selected SSR primers.

ID	Primer name						
	B6-2	A5-3	A12-3	B5-3	B6-3	B9-3	B11-3
1	330 bp 240 bp SB 175 bp	790 bp 250 bp 240 bp SB 200 bp	400 bp 145 bp	800 bp 410 bp SB 305 bp	155 bp	270 bp 245 bp 175 bp 110 bp	ND
2	360 bp 330 bp 175 bp		400 bp 145 bp	590 bp SB 305 bp	155 bp	390 bp 270 bp 245 bp 110 bp	ND
3	295 bp SB 185 bp	825 bp 200 bp	145 bp	780 bp 710 bp SB	155 bp	245 bp 175 bp 110 bp	350 bp SB 270 bp
4	610 bp SB 330 bp 175 bp	200 bp 170 bp SB	400 bp 145 bp	ND	285 bp SB 155 bp	145 bp 200 bp SB	ND
5	810 bp 330 bp 175 bp	825 bp	400 bp	800 bp 305 bp 290 bp SB	155 bp	270 bp 245 bp 210 bp	270 bp 180 bp 170 bp SB
6	520 bp 185 bp	160 bp	400 bp	ND	155 bp	245 bp	180 bp
7	690 bp SB 360 bp 330 bp 175 bp	500 bp 250 bp	400 bp	780 bp 690 bp	230 bp SB 155 bp	270 bp 245 bp 175 bp 110 bp	ND
8	360 bp 330 bp 175 bp	950 bp SB 500 bp	400 bp 145 bp	690 bp 390 bp SB	155 bp	270 bp 245 bp 175 bp 110 bp	ND
9	ND	900 bp SB 590 bp 160 bp	305 bp SB 145 bp	ND	ND	390 bp 245 bp 110 bp	400 bp 210 bp SB 180 bp
10	630 bp SB 330 bp 215 bp SB	790 bp 160 bp	145 bp	500 bp	180 bp SB 155 bp	245 bp 210 bp 175 bp 110 bp	400 bp
11	520 bp 250 bp SB	590 bp 160 bp		500 bp	155 bp	185 bp SB 145 bp	ND
12	810 bp 175 bp	790 bp 200 bp	400 bp 145 bp	ND	300 bp SB 155 bp	270 bp 245 bp 175 bp	ND

SB = specific band; ND = non-decision (too complicated at the result of too many band patterns). 1 = *Dendrobium nobile* Lindl; 2 = *Dendrobium moniliforme* (L.) Sw; 3 = *Dendrobium heterocarpum* Wall. ex. Lindl; 4 = *Dendrobium signatum* Rchb.f; 5 = *Dendrobium findleyanum* Parish & Rchb.f; 6 = *Dendrobium pendulum* Roxb; 7 = *Dendrobium linawianum* Rchb.f; 8 = *Dendrobium friedericksianum* Rchb.f; 9 = *Dendrobium unicum* Seidenf; 10 = *Dendrobium hercoglossum* Rchb.f; 11 = *Dendrobium wardianum* Warner; 12 = *Dendrobium nobile* var. *cooksonianum* Rchb.f.

DISCUSSION

In this study, we found that 12 *Dendrobium* cultivars may be separated into 2 main groups based on the color of their inflorescence and their deciduous characteristics, with these groupings almost matching their ITS groupings. Moreover, we found that *D. pendulum* Roxb. and *D. wardianum* Warner had very similar shaped inflorescences, and were separated into a minor group according to their genetic relationship based on the ITS sequences. A report by Korean researchers indicated that the orchids used in their study could be differentiated into 3 groups according to leaf morphology, which matched their RAPD groupings (Chung et al., 2006). Another study, also published by Korean researchers, indicates that the phylogenetic tree derived from the RAPD analysis of *Cymbidiums* was similar to that derived from traditional classification (Choi et al., 2006). Thus, we believe that the genetic relationship based on the ITS sequence has a degree of credibility and accuracy, based on the findings of these two similar studies. Microsatellite markers may be used to differentiate populations of *Dendrobium* varieties (Cai et al., 2012). Thai researchers have shown that the polymorphic band patterns of some primer pairs may be used to identify Orchidaceae cultivars, and to demonstrate their genetic relationship (Phuekvilai et al., 2009). Singaporean researchers (Yue et al., 2006) have reported that SSR markers may be used for the identification of *Dendrobium* varieties. The 12 *Dendrobium* varieties used in this study are often used as parents for a range of commercial *Dendrobium* varieties. *D. nobile* Lindl. and *D. moniliforme* (L.) Sw. represent 2 very important genetic sources for the breeding of new *Dendrobium* cultivars. According to pedigree analyses, *D. nobile* Lindl. and *D. moniliforme* (L.) Sw. constitute 41.14 and 19.43%, respectively, of more than 500 *Dendrobium* cultivars (Yamamoto J, unpublished results). In this study, 3 of the 7 selected primers produced specific band patterns for *D. nobile* Lindl., and 1 primer of the 7 selected primers produced specific band patterns for *D. moniliforme* (L.) Sw. These 4 primers might represent useful tools for the identification of these 2 very important cultivars. The microsatellite markers found in this study revealed considerable genetic diversity in *Dendrobium*, which facilitated cultivar identification.

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Supplementary material

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