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DNA finger printing analysis of fourteen species of orchids using ISSR technique

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Abstract

The DNA fingerprint can be used for identification the orchid cultivars and also used in orchid breeding in the future. The DNA fingerprint and genetic diversity among 12 *Dendrobium*, 1 *Ascocentrum*, and 1 *Rhynchostylis* orchid cultivars were studied using Inter Simple Sequence Repeat (ISSR) marker technique. The results showed that eighth PCR products which were derived from ten oligonucleotide primers amplification (PP1, PP2, PP3, PP4, PP5, PP6, PP7, PP8, PP9 and PP10). There were three primers; PP3, PP5 and PP7 could generate different bands among fourteen orchid cultivars and contained band size from 186-2159 base pair that amplified from the good quality orchid DNAs in the PCR. Hence these ISSR technique markers not only can be used for identification orchid cultivars but also for genetic database useful for future breeding program.

Keywords: DNA fingerprint, *Dendrobium* orchid, Inter simple sequence repeat technique

1. Introduction

The genus *Dendrobium* (*Dendrobium*: *Orchidaceae*) is one of the most important genera in the orchid family *Orchidaceae* with 1190 species as listed by the Royal Botanic Gardens, Kew, UK (1). *Dendrobium* plants are distinctive ecological diversification distributing in tropical and subtropical Asia and North-Australia. *Dendrobium* plants are also among the most popular orchids for commercial production of cut flower and pot plants (2). Since 18th century, more than 8000 novel *Dendrobium* hybrids and cultivars have been bred in horticulture through inter-specific hybridization for novel flower morphological characteristics (3). Most wild *Dendrobium* speices only survive in certain habitats and are susceptible to the deterioration and fragmentation of natural ecosystems. Characterization of the genetic diversity and examination of the genetic relationship among *Dendrobium* species are important for the sustainable conservation and increased use of plant genetic resources. Traditionally, comparative vegetative anatomy and plant systematic were two common strategies to assess the relationships among the taxa *Dendrobium* (4). Problems associated with variability and plant growth conditions are easy to cause confusion in the species identification. The extensive development of molecular techniques for genetic analysis in the past decade has led to the increase of the knowledge of orchid genetic diversity. Molecular techniques, in particular the use of molecular markers, have been used to monitor DNA sequence variation in

and among orchid species and cultivars (5). Inter simple sequence repeats (ISSRs) involves polymerase chain reaction (PCR) to amplify DNA fragments between two simple sequence repeats (SSRs) with inverse orientations using primers with a single SSR motifs anchored at the 3'- or 5'-end by a few nucleotides (6). ISSR markers are present in both nuclear and organelles genomes, and usually are highly polymorphic in plant populations, providing a genotyping system with features of consistency, reliability and co-dominancy (7). Inter simple sequence repeated (ISSR) have become a good DNA molecular marker for research on populations of the same species. The main advantages of ISSR are no need for DNA sequence information prior to amplification, low cost, simple operation, high stability, and abundance of genomic information. Because of these reasons, ISSR are being used for population authentication and population molecular ecology studies (8).

In the present study we collected 12 *Dendrobium* species from Khon Kaen University and Rajamangala University of Technology Isan, Kalasin Campus for research objective to test the ISSR technique for molecular diagnosis of *Dendrobium* species.

2. Materials and Methods

2.1 Plant materials

12 *Dendrobium* species, *Ascosentrum miniatum* and *Rhynchostylis giyantean* were collected in Khon Kaen University (KKU) and Rajamangala University of Technology Isan, Kalasin Campus (KSC/RMUTI) (Table 1). Fresh foliage samples were harvested from two or three individuals of each species and kept at -20 ^{o}C for genomic DNA extraction.

2.2 Genomic DNA extraction

Genomic DNA was extracted from leave samples using a cetyltrimethyl ammonium bromide (CTAB) method as described previously with minor modification (9). Briefly, 0.2g of fresh plant tissues were grounded in liquid nitrogen into a fine powder, and transferred into an Eppendorf tube with 700 ml of 2X CTAB [2% CTAB, 1.4 M NaCl, 100 mM Tris base, 20 mM EDTA, 1% PVP] and 2-mercaptoethanol 2 l mixing well and incubation at 60 ^{o}C for 1 hr. Then the chloroform:isoamyl alcohol (24:1) 700 ml was added and mixed thoroughly. The sample was collected by centrifuging at 10,000 rpm for 10 min. Genomic DNA was precipitated by adding 700 ml iso-propanal. After washing with 75% ethanol, the dried genomic DNA pellet was resuspended in 100 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA). The concentration of genomic DNA samples was determined by UV spectrophotometer and necessary dilutions were done, followed by verification with 0.8% agarose gel electrophoresis. The DNA samples were stored at -20 ^{o}C until further analysis.

2.3 Quantification of isolated DNA

Spectrophotometer method was used to quantify the purity of DNA sample. 10 ml of DNA dissolved in 1X TAE buffer (also used as blank) volume made up to 1 ml. Absorbance was taken at 260 and 280 nm.

Table 1. Orchid species used in this study.

Species name	Code	Sources
D. unicum	Du	KSC/RMUTI
D. lindleyi	Dl	KSC/RMUTI
D. primulinum	Dp	KSC/RMUTI
D. finlayanum	Df	KSC/RMUTI
D. anosmum	Da	KSC/RMUTI
D. friederichsianum	Dfr	KSC/RMUTI
D. lituiflorum	Dli	KSC/RMUTI
D. crepidatum	Dc	KKU
D. parishii	Dpa	KSC/RMUTI
D. hercoglossum	Dh	KKU
D. signatum	Ds	KKU
D. thyrsiflorum	Dt	KSC/RMUTI
R. giyantean	Rg	KSC/RMUTI
A. miniatum	Am	KSC/RMUTI

2.4 ISSR analysis

ISSR primers were selected (Table 2) for ISSR amplification using all samples. PCR amplication reactions of extracted DNA sample was carried out with ISSR primer in Biometra® TGRADIENT thermo cycler. PCR reaction was performed using 25 ml of PCR reaction mixture solution containing 5 ml of 10X PCR buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.1% gelatin), 1.5 ml of $MgCl_2$ (25 mM), 2.5 ml of primer (5mM), 2.5 ml of dNTP (25 mM), 50 ng of genomic DNA, and 1 unit of Taq DNA polymerase. Touch-down PCR was performed with conditions: 94 ^{o}C for 5 min followed by 30 cycles of 94 ^{o}C for 60s, 44-56 ^{o}C for 45s, and 72 ^{o}C for 90s, and a final extension at 72 ^{o}C for 10 min. For ISSR markers profiling, PCR products were subjected to electrophoresis on 1.5% agarose gels, followed by staining with ethidium bromide. The electrophoretic patterns of the PCR products were recorded digitally using Gel-Doc 2000 image analysis system (IN GENIUS, SYNGENE BIO IMAGING).

Table 2. Primer oligonucleotide sequence.

Primer	Sequence (5'——3')	Tm (^{o}C)	Ann.T. (^{o}C)
PP1	(AG)8CT	53.7	48.7
PP2	(AC)8CA	53.7	48.7
PP3	(AG)8AA	51.4	46.4
PP4	(TG)8GG	56	51
PP5	(AG)8CC	56	51
PP6	(AC)8GA	53.7	48.7
PP7	(CTC)6	60.5	55.5
PP8	(AC)8AG	53.7	48.7
PP9	(AC)8GA	53.7	48.7
PP10	(ACTG)2 (ATCG)2	49.2	44.2

3. Results

3.1 Genomic DNA isolation

Spectrophotometer method was used to quantify the purity of DNA samples. According to results *D. friederichsianum* was in pure form and contained maximum concentration (Table 3).

Table 3. Different samples and their absorbance ratio and quantification value.

Sample	Ratio A260/A280	Quantification value
Du	1.71	662 mg/ml
Dl	1.86	103 mg/ml
Dp	1.67	366 mg/ml
Df	1.85	629 mg/ml
Da	1.77	396 mg/ml
Dfr	1.88	715 mg/ml
Dli	1.76	570 mg/ml
Dc	1.94	559 mg/ml
Dpa	2.21	116 mg/ml
Dh	1.71	261 mg/ml
Ds	1.86	210 mg/ml
Dt	1.68	684 mg/ml
Am	1.70	376 mg/ml
Rg	1.75	438 mg/ml

3.2 Results of ISSR

A total of 10 primers was used in ISSR, 8 primers (PP1, PP2, PP3, PP5, PP6, PP7, PP8 and PP9) were able to reproducible bands in all orchid species. Total primers produced 460 bands ISSR fragments (complete data not shown) and both monomorphic and polymorphic bands were observed. Using primer PP7 the monomorphic bands in Du, Dl, Dp, Df, Da, Dli, Dpa and Dh with length 400-500 bp suggesting that this primer would be used as differential markers (Figure 1, 2, and 3).

4. Discussion and Conclusion

In view of the large and increasing number of *Dendrobium* hybrids and cultivars and the importance of wild exotic germoplasm for breeding programs, it is necessary to evaluate genetic diversity and phylogenetic relationship of the genus *Dendrobium*. Phylogenetic knowledge could provide useful information for the selection of closely related species for the introgression of horticultural traits in *Dendrobium* plants. Because only a

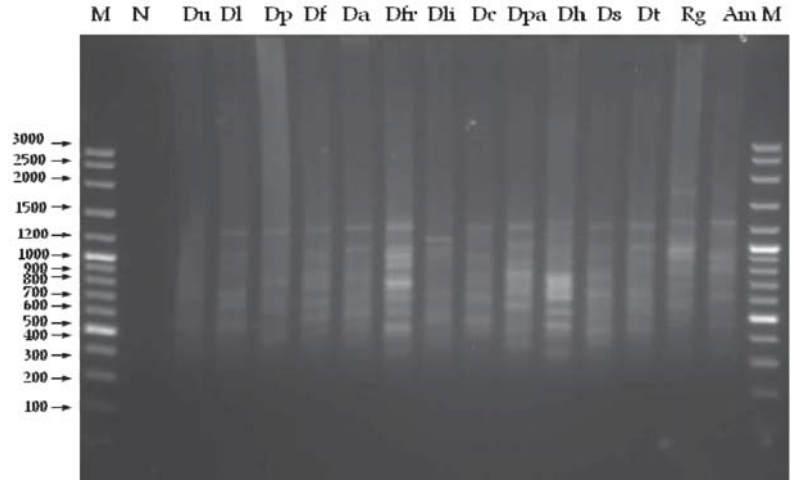

Figure 1. Agarose gel photographs of the PCR products amplified by ISSR PP3 primer.

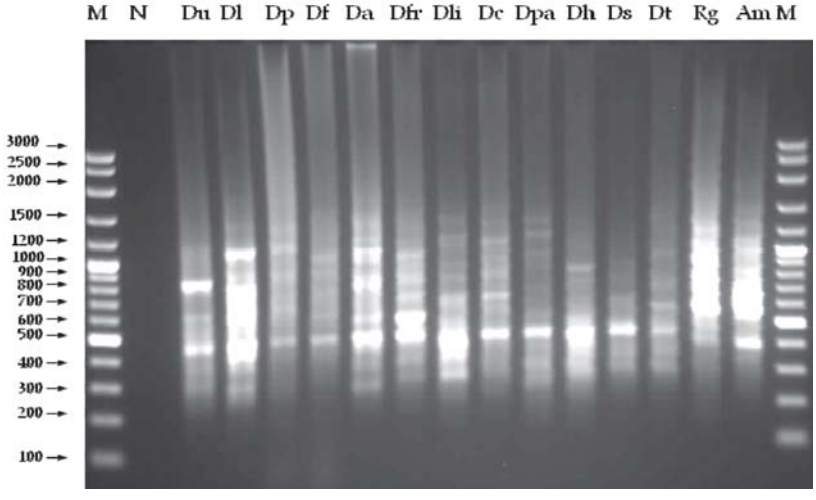

Figure 2. Agarose gel photographs of the PCR products amplified by ISSR PP5 primer.

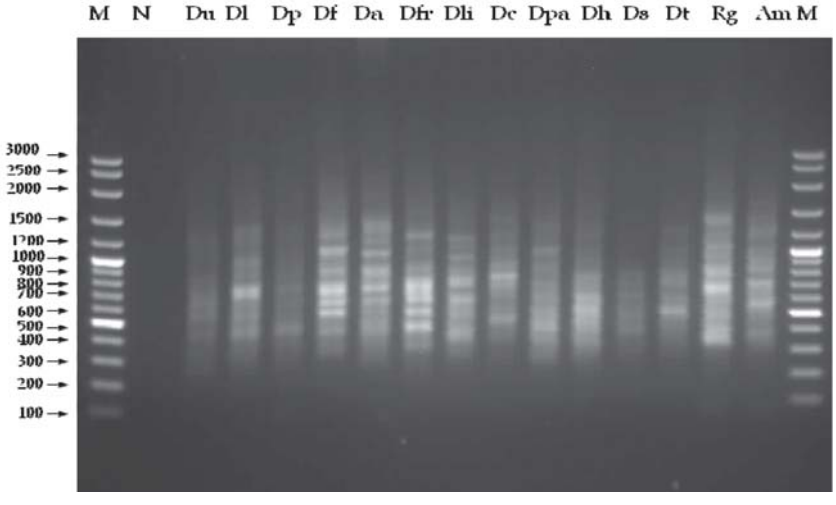

Figure 3. Agarose gel photographs of the PCR products amplified by ISSR PP7 primer.

few morphological parameters were used as key characters in traditional plant systematic, their variations possibly caused confusion in classification even with conflicts among taxonomists (2). The extensive development of molecular techniques for genetic analysis in the past decade has led to the increase of the knowledge of orchid genetic diversity. Molecular techniques, in particular the use of molecular markers, have been used to monitor DNA sequence variation in and among orchid species and cultivars (5). Variation of the ribulose-bisphosphate carboxylase gene (rbcL) and restriction enzyme sites of chloroplast DNA were used for phylogenetic analysis among 13 taxa in subtribe *Dendrobiinae* (11). The *Dendrobium* internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA (nrDNA) were reported and used to determine genetic relationship among 17 species in Hong Kong (12), 12 species in Taiwan (13), and the section Dendrocoryne in Australia (10). Molecular evidences from these studies suggested that the genus *Dendrobium* was polymorphic with complex genetic background at the species level. These molecular studies bring useful information on the determinism of genetic variation and the organization of genetic diversity within *Dendrobium* plants. Sze *et al* (14) constructed a DNA microarray for high throughput identification of the plant resource of commercial FDSH [Fengdu Shihu (*Dendrobium officinale*)]. The 5S ribosomal DNA intergenic spacer region in *Dendrobium capillipes*, *Dendrobium hercoglossum*, *Dendrobium jenkinsii*, *Dendrobium moniliforme*, *Dendrobium nobile*, *Dendrobium officinale*, *Dendrobium williamsonii*, and *Dendrobium wilsonii* was amplified by a single primer pair and sequenced. The sequences displayed polymorphism. In order to identify *Dendrobium loddigesii*, Qian *et al* (15) prepared six pairs of diagnostic amplification refractory mutation system primers based on nuclear ribosomal DNA ITS sequences of *Dendrobium loddigesii* and eleven adulterants. Liu *et* al (16) studied the rDNA ITS sequences of 17 species of Herba Dendrobii in order to identify Herba Dendrobii and adulterant species at the molecular level. Wang *et al* (17) demonstrated that ISSR technique was very effective in determining genetic diversity of 31 *Dendropbium* at species level. Yang *et al* (18) employed ISSR molecular marker technology to identify species and habitats of medical *Dendrobium* plants. Takamiya *et al* (19) constructed an ITS1-5.8S-ITS2 sequence database of close to 200 *Dendrobium* species. They identified 21 plant sources of Dendrobii Herba based on sequences of the ITS regions of nuclear ribosomal DNA. Pathak and Jaroli (20) indicated that RAPD technology is an effective method to differentiate 8 *Dendrobium* species. The RAPD and ISSR technique applied for investigation was very highly sensitive and reproducible. Hou *et al* (21) provided a basic guideline in which the *Dendrobium officinale* individuals with rare alleles deserve to be protected with first priority, and those individuals with the most common alleles should also be concerned. The 15 trinucleotide microsatellites developed in them study can be used as an important tool for further assessing. Feng *et al* (22) constructed some mapping populations of *Dendrobium* species, and proved that these populations were feasible for genetic linkage map construction. Concerning the universal use of genetic linkage maps, codominant markers, including SSRs or SNPs should be the first choice for future mapping effort, to maximize the marker coverage and distribution in *Dendrobium* genome. Related knowledge about more plant species is prerequisite for efficiently managing and exploiting *Dendrobium* genetic resources. We report here the use of ISSR genotyping technique in measuring genetic variation and determination of genetic relationships among 12 *Dendrobium* species in North-eastern-region of Thailand. Despite the horticultural and medicinal significance, the available information of phylogenetic relationships and genetic diversity of the genus *Dendrobium* is still very limited (10). We found that ISSR polymorphisms were highly reproducible and

informative for species identification in *Dendrobium* plants. In the present study, we analyzed 10 ISSR primers (Table 2) and species-diagnostic ISSR markers were characterized for *D. unicum*, *D. lindleyi*, *D. primulinum*, *D. finlayanum*, *D. anosmum*, *D. friederichsianum*, *D. lituiflorum*, *D. crepidatum*, *D. parishii*, *D. hercoglossum*, *D. signatum* and *D. thyrsiflorum* (Table 1). Although, the $(CA)_8$ primers were reported to be used to fingerprint populations of *Dendrobium officinale* (8), they tested 76 ISSR primers and selected 10 ISSR primers which produced clear and reproducible bands, but in this research the $(AC)_8$ primers (PP8; $(AC)_8$AG and PP9; $(AC)_8$GA) were not included in this study. Genetic diversity was revealed to be extremely high in *D. unicum*, *D. lindleyi*, *D. primulinum*, *D. finlayanum*, *D. anosmum*, *D. friederichsianum*, *D. lituiflorum*, *D. crepidatum*, *D. parishii*, *D. hercoglossum*, *D. signatum* and *D. thyrsiflorum* by ISSR data analysis. Results of ISSR data indicated that genetic variation existed within theses *Dendrobium* populations. Consistently, the analysis using ISSR showed evident differences in genetic composition between each germplasm collection and its wild population, which was probably attributable to the fact that initially the *Dendrobium* individuals with distinct genotypes in each wild population were unevenly selected for establishing the germplasm collection. However, unconscious selection in wild populations also led to genetic changes in evidence. In conclusion, our study demonstrated that ISSR technique was effective in determining genetic diversity of the genus *Dendrobium* at species level. Cluster analysis based on ISSR profiles identified *Dendrobium* species and provided a molecular diagnosis tool for the authentication of valuable medicine plant species. The ISSR markers reported in the present study will facilitate the understanding of inter-species gene flow, genetic structure of species, genetic diversity and evolutionary relationship in the genus *Dendrobium*.

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