RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2014.40.113

ScienceAsia 40 (2014): **113–120**

Identification of native *Dendrobium* species in Thailand by PCR-RFLP of rDNA-ITS and chloroplast DNA

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Received 12 Mar 2013
Accepted 10 Mar 2014

ABSTRACT: The PCR-restriction fragment length polymorphism (PCR-RFLP) approach was successfully developed to identify 25 native *Dendrobium* species in Thailand. PCR-RFLP of the rDNA-ITS with six restriction enzymes and three chloroplast (cp) DNA regions with five primer-enzyme combinations produced 24 types of DNA patterns altogether. Twenty-three out of the 25 species determined in this study were found to belong to unique classes and were successfully differentiated. Two species, *D. crumenatum* and *D. formosum*, possessing the same DNA pattern, however, were identified after cutting the chloroplast DNA fragment amplified by psbC-trnS primer with *Mbo*I enzyme. An effective procedure for identifying each *Dendrobium* species was developed. PCR-RFLP of the rDNA-ITS with *Taq*I, which is the most informative enzyme, was used for the early detection of 16 *Dendrobium* species. To identify the remaining *Dendrobium* species, PCR-RFLP analysis was performed using one more primer-enzyme combination. Our study provides a rapid, simple, and reliable identification method for these *Dendrobium* species.

KEYWORDS: orchid, DNA markers, species verification, internal transcribed spacer

INTRODUCTION

Orchidaceae is one of the largest and most diverse families of flowering plants, making up to one-tenth of all flowering plant species in the world[1]. The genus *Dendrobium*, with 1184 species, is the second largest genus in Orchidaceae[2]. They are found in various geographical zones and enormously diverse in growth habits along tropical and subtropical Asia and Northern Australia. In Thailand, *Dendrobium* spp. is the largest orchid genus with more than 150 native species classified into 14 sections[3]. It is also one of the most popular orchids for commercial production with the drastic increase of demand for cut flower and pot plants over the years. About 8000 novel *Dendrobium* hybrids have been bred through interspecific hybridizations for different flower morphological characteristics[4]. In contrast, most native *Dendrobium* species are critically susceptible to deterioration and fragmentation of natural ecosystem because of mass collection from the wild for trade of both flower and pot plant. Hence native *Dendrobium* species are now considered endangered species (CITES). However, several native *Dendrobium* species are morphologically similar, thereby making their labelling through vegetative anatomy very difficult except during flowering period. Identification of *Dendrobium* species becomes necessary for sustainable use and conservation of the plant genetic resources. The development of a simple and reliable approach to identify these plant species is therefore needed.

The internal transcribed spacer (ITS) sequences of the nuclear ribosomal RNA gene (rDNA) are large segment consisting of 18S rDNA, 5.8S rDNA and 26S rDNA clustered together with internal transcribed spacers, ITS1 and ITS2, between genes. Notably, ITS sequences have been extensively used to determine genetic diversity and to classify several plants species because they are highly variable[5,6]. Besides the ITS sequences, organelle DNA, chloroplast DNA

(cpDNA) and mitochondrial DNA (mtDNA), are the other targets of genetic diversity and classification studies due to their maternal inheritance, smaller-sized molecule compared to genomic DNA, and low level of recombination, which make them easier and more reliable to examine the phylogenetic applications[7,8]. The rDNA-ITS or organelle DNAs has been widely used for the above-mentioned applications not only by sequences analysis but also by comparison of their PCR-amplified fragments. The PCR amplified products of rDNA-ITS or organelle DNA, however, often exhibits a monomorphic band that cannot differentiate among several species within the same genus. Further digestion of their amplicons with restriction enzymes (PCR-restriction fragment length polymorphism; PCR-RFLP) is therefore conducted for displaying the restriction site polymorphisms[9]. PCR-RFLP of the rDNA-ITS[10] and cpDNA[11,12] have been extensively used for genetic diversity studies in several plants because of its simplicity, reliability, and practicality.

In *Dendrobium*, both the sequences of rDNA-ITS[13,14] and cpDNA[15] and the PCR-RFLP of cpDNA (*rbcL* gene)[16] were used for species identification and determination of their genetic relationship. Moreover, inter-simple sequence repeat (ISSR) marker was also used for species identification and phylogenetic relationship of 31 *Dendrobium* spp. in China[17]. These findings collectively suggest that *Dendrobium* spp. was highly diversified with complex genetic background at the species-level. The molecular identification of native *Dendrobium* spp. in Thailand however has not yet been reported despite inclusion of several species. In this study, PCR-RFLP of the rDNA-ITS and cpDNA have been conducted for rapid and reliable species identification of 25 native *Dendrobium* spp. in Thailand. We also developed a specific PCR-RFLP procedure to identify these *Dendrobium* spp., which can be used for trade and conservation.

MATERIALS AND METHODS

Plant materials and DNA extraction

Twenty-five *Dendrobium* species were taken from the in vitro collection of the Biotechnology Laboratory, Chulabhorn Research Institute, Bangkok, Thailand. Each species was morphologically identified following Seidenfaden[3] before culturing. Total genomic DNA was extracted from the leaves as described previously[18], and kept at −80 °C until used. The quality and concentration of DNA were measured by 1% agarose gel electrophoresis and spectrophotometric analysis.

Table 1 Names and sequences of the primers used.

Primer	Sequence (5′-3′)
Nuclear rDNA[a]	
OrRNA1	ATTGAACCTTATCATTTAGAGG
OrRNA2	GTACTTGTTCGCTATCGGTC
Chloroplast DNA[b]	
trnS	GAGAGAGAGGGATTCGAACC
trnfM	CATAACCTTGAGGTCACGGG
psbC	GGTCGTGACCAAGAAACCAC
trnS	GGTTCGAATCCCTCTCTCTC
trnH	ACGGGAATTGAACCCGCGCA
rnK	CCGACTAGTTCCGGGTTCGA

[a] Source: this study. [b] Source: Ref. 7.

Development of orchid specific primers for amplification of the rDNA-ITS region

PCR primers (18d, 5′-CACACCGCCCGTCECTCCT ACCGATTG-3′ and 26S, 5′-AGACTCCTTGGTCCG TGTTTCAAGAC-3′) of rDNA sequences were designed based on conserved sequences between rice and several eukaryotes[19]. These primers were used to amplify the region from partial sequence of 18S rDNA, ITS1, 5.8S rDNA, ITS2, to partial sequence of 26S rDNA of two orchid samples, one native species (*Dendrobium virgineum*) and one cultivated variety (*Dendrobium* cv. Khaosanan). Standard PCR reaction was performed using 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM specific primers, 0.5 U of *Taq* DNA polymerase (Invitrogen), and 25 ng genomic DNA in a final reaction volume of 20 µl. PCR cycling conditions contained the initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, primer extension at 72 °C for 45 s, then postcycling extension at 72 °C for 10 min. The amplified products were examined by electrophoresis on 1% agarose gel. The DNA fragments were subsequently extracted from the ethidium bromide-stained gel and ligated with pGEM-T Easy Vector System I (Promega). The ligated products were transformed into *Escherichia coli* DH5α competent cells. The nucleotide sequence of the DNA fragments was determined by Macrogen Sequencing Service (Macrogen). The nucleotide sequence was compared against the National Centre for Biotechnology Information (NCBI) database using the programs BLASTX and BLASTN (www.ncbi.nlm.nih.gov/BLAST). All nucleotide sequences were deposited at DDBJ. Novel orchid-specific primers, OrRNA1 and OrRNA2, were subsequently designed based on the conserved sites of two orchids and other plants sequences (Table 1) to amplify the ITS region comprising ITS1, 5.8S rDNA and ITS2 in all *Dendrobium* species.

PCR-RFLP analysis

Nuclear ITS and cpDNA fragments were amplified and subsequently cut with several restriction enzymes to provide PCR-restriction fragment length polymorphism (PCR-RFLP). For nuclear ITS fragments, OrRNA1 and OrRNA2 primers were used to perform the PCR. Simultaneously, cpDNA fragments including trnS to trnfM, psbC to trnS, and trnH to trnK regions were obtained using the primer sets from Ref. 7 (Table 1). Standard PCR reaction was carried out using 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM specific primers, 0.5 U of *Taq* DNA polymerase (Invitrogen), and 100 ng genomic DNA in a final reaction volume of 100 µl. PCR cycling conditions contained the initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 55 °C for 60 s, primer extension at 72 °C for 60 s, then postcycling extension at 72 °C for 5 min. The amplicons from each sample were digested with several restriction enzymes following the manufacturer's instruction (New England Biolabs). The digested DNAs were fractionated by electrophoresis on 2% agarose gel and stained with ethidium bromide. The investigation of the DNA restriction patterns of all *Dendrobium* species were performed repeatedly at least twice.

Data analysis

PCR-RFLP bands were manually scored as 1 (for presence) and 0 (for absence) from the images of the gels. Both monomorphic and polymorphic bands were included in the data set to avoid bias estimation of genetic variation. The resulting binary data matrix was calculated using a simple matching coefficient[20] with FREETREE software[21]. The dendrogram was subsequently constructed using the similarity matrix with the unweighted pair-group method with arithmetic averages (UPGMA) with 1000 permutations of bootstrapping using FREETREE, and the tree was displayed using MEGA4[22].

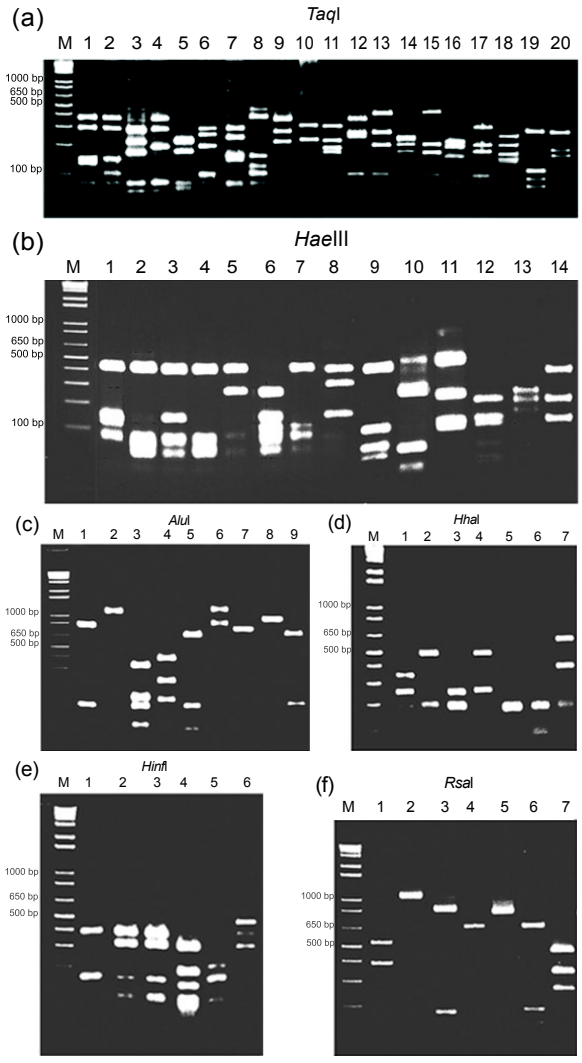

Fig. 1 PCR-RFLP patterns of rDNA-ITS of *Dendrobium* orchids after digestion with *Taq*I (a), *Hae*III (b), *Alu*I (c), *Hha*I (d), *Hin*fI (e) and *Rsa*I (f). The number above each lane are the type of DNA patterns found from each primer-enzyme combination. M is a 1 kb plus DNA ladder.

RESULTS AND DISCUSSION

To develop *Dendrobium* rDNA specific primers, approximately 1300 bp DNA fragments of rDNA-ITS were amplified from *D. virgineum* and *Dendrobium* cv. Khaosanan. Partial nucleotide sequences of the 1300 bp fragments (accession no. AB671740, AB671741 for *D. virgineum* and AB671742, AB671743 for *Dendrobium* cv. Khaosanan) were deposited in the database. The sequences of the two orchids were then aligned with rDNA-ITS region of several plant species, and the new specific primers, OrRNA1 - OrRNA2 (Table 1), were developed for ensuring the effective amplification of all *Dendrobium* species. Approximately 1100 bp of amplicons were observed in all 25 *Dendrobium* species. Six restriction enzymes having 4 bp or 6 bp with degenerated bases in the recognition sites for higher probability of restriction sites found on the amplicons, were subsequently used to produce the unique restriction pattern for each species. The DNA restriction pattern of 9, 14, 6, 7, 7, and 20 types were generated from the enzymes *Alu*I, *Hae*III, *Hin*fI, *Hha*I, *Rsa*I, and *Taq*I, respectively, (Fig. 1, and Table 2). When considered

Table 2 PCR-RFLP patterns of rDNA-ITS and chloroplast DNA of *Dendrobium* species.

Dendrobium sp.		Section[1]	PCR-RFLP patterns of sample generated by each primer-enzyme combination										
			*Alu*I[a]	*Hae*III[a]	*Hin*fI[a]	*Hha*I[a]	*Rsa*I[a]	*Taq*I[a]	*Hin*fI[b]	*Rsa*I[b]	*Taq*I[b]	*Hin*fI[c]	*Hin*fI[d]
D. hercoglossum	DHER	*Bre*	1	3	1	3	3	4*	1	1	1	2	1
D. palpebrae	DPAL	*Cal*	2	14	2	3	2	13	4	1	1	1	7
D. lindleyi	DLIN	*Cal*	2	9*	2	2	3	11*	4	1	1	2	6
D. signatum	DSIG	*Den*	1	1	1	1	1	1*	1	1	1	2	1
D. fridericksianum	DFRI	*Den*	1	1	1	1	1	2*	1	1	1	2	1
D. chrysanthum	DCHRY	*Den*	1	2*	2	2	2	3	2	1	1	2	2
D. lituiflorum	DLIT	*Den*	1	4	2	2	4	5*	2	1	1	1	3*
D. anosmum	DANO	*Den*	5	3	2	5	2	9	2	7	1	1	1
D. finlayanum	DFIN	*Den*	6*	8*	1	7*	3	10*	2	7	1	1	1
D. albosanguineum	DALB	*Den*	9*	3	2	6*	2	12*	2	7	1	1	6
D. parishii	DPAR	*Den*	5	3	2	5	2	9	4	6*	4*	2	1
D. primulinum	DPRI	*Den*	2	4	2	2	6*	14*	6*	1	1	1	1
D. unicum	DUNI	*Den*	8*	12*	2	2	2	13	1	1	1	2	5
D. capillipes	DCAP	*Den*	2	3	2	2	2	3	2	4*	3*	1	4
D. dixanthum	DDIX	*Den*	2	13*	6*	3	2	20*	4	1	2	1	7
D. ellipsophyllum	DELL	*Dis*	4*	7*	2	5	2	8*	2	7	1	1	1
D. cruentum	DCRUE	*For*	2	5	3*	3	2	6*	2	7	1	2	5
D. formosum	DFOR	*For*	3	6	2	4	2	7	3	2	2	1	4
D. infundibulum	DINF	*For*	2	10*	2	3	2	13	5*	5*	1	2	7
D. virgineum	DVIG	*For*	7*	11*	4*	2	5*	16*	8*	3*	1	2	4
D. christyanum	DCHRI	*For*	1	3	5*	3	3	18*	2	1	1	1	2
D. scabrilingue	DSCA	*For*	2	14	2	3	7*	19*	1	7	2	2	6
D. crumenatum	DCRUM	*Rho*	3	6	2	4	2	7	3	2	2	1	4
D. compactum	DCOM	*Sta*	2	5	2	2	2	15*	7*	1	1	1	1
D. acerosum	DACE	*Str*	2	3	1	3	4	17*	9*	1	1	2	1

[a] rDNA,[b] trnS-trnfM, [c] psbC-trnS, [d] trnH-trnK

[*] The number with asterisk represents a unique pattern which can be used to identify the respective species by only one primer enzyme combination.

[1] *Bre: Breviflores*; *Cal: Callista*; *Den: Dendrobium*; *Dis: Dictichophyllum*; *For: Formosae*; *Rho: Rhopalanthe*; *Sta: Stachyobium*; *Str: Strongyle*

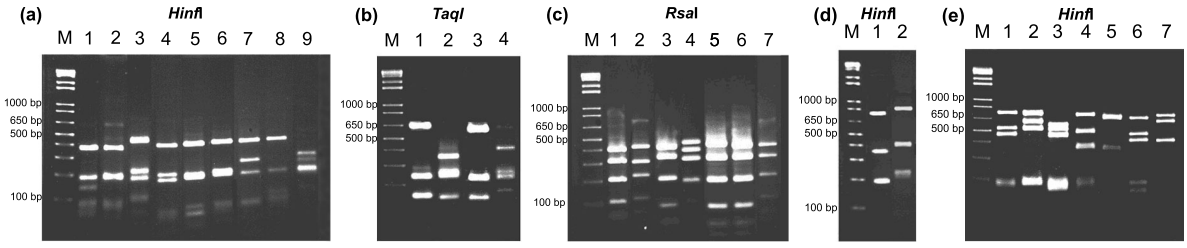

Fig. 2 PCR-RFLP patterns of cpDNA of *Dendrobium* orchids after digestion with restriction enzyme; (a) trnS-trnfM primer/*Hin*fI, (*b)* trnS-trnfM primer/*Taq*I, (c) trnS-trnfM primer/*Rsa*I, (d) psbC-trnS primer/*Hin*fI, and (e) trnH-trnK primer/*Hin*fI. The number above each lane are the type of DNA patterns found from each primer-enzyme combination. M is a 1 kb plus DNA ladder.

together, the combined restriction patterns of six enzymes could be classified into 23 classes. Almost all *Dendrobium* species used in this study showed the unique DNA pattern and could be differentiated from each other, except for the digested DNA patterns of *D. crumenatum* and *D. formosum*, as well as *D. anosmum* and *D. parishii*, which had the same pattern.

In case of cpDNA, three primer pairs including trnS to trnfM, psbC to trnS, and trnH to trnK regions were obtained[7] (Table 1). Five effective primer-enzyme sets of PCR-RFLP of cpDNA were performed to determine variation of the DNA pattern. Approximately 1100 bp of PCR products were found from all 25 *Dendrobium* species using trnS-trnfM primers. These amplicons were then cut with seven restriction enzymes, in which *Alu*I, *Hae*III, *Hha*I, and *Mbo*I produced one type, while *Hin*fI, *Taq*I, and *Rsa*I produced 9, 4, and 7 PCR-RFLP types, respectively, (Fig. 2a, 2b, 2c, Table 2). Additionally, psbC-trnS primers were used, and about 1500 bp PCR fragments from all species were subsequently digested with *Hae*III and *Hin*fI. Two definite polymorphic PCR-RFLP types, however, were found using *Hin*fI (Fig. 2d, Table 2). Finally, amplification with trnH-trnK primers gave about 2100 bp fragments which produced 7 definite

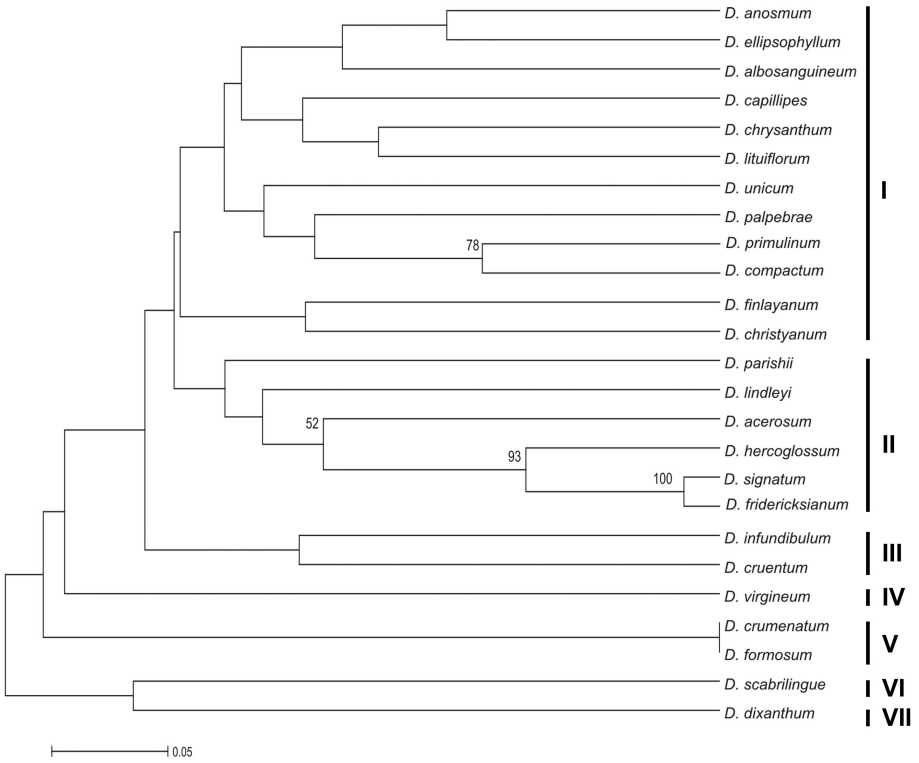

Fig. 3 Dendrogram of 25 Thai native *Dendrobium* species using UPGMA cluster analysis based on genetic similarities of DNA fingerprint from PCR-RFLP of the rDNA-ITS with six enzymes and five primer-enzyme combinations of cpDNA. Numbers (I–VII) indicates seven clusters in the dendrogram.

polymorphic PCR-RFLP types after digestion with *Hin*fI enzyme (Fig. 2e, Table 2). The PCR-RFLP patterns of the cpDNA with five primer-enzyme sets were further combined into 20 classes. Among these classes, only 17 unique classes of 17 *Dendrobium* species have been found. The discriminatory power of PCR-RFLP of chloroplast DNA within and among species in the genus *Abies* has also been reported, in which some species could not be differentiated[12]. Chloroplast DNA indicates common maternal inheritance, suggesting that the cpDNA of some species might have evolved from closely related maternal lineage. These results collectively suggest that analysis of cpDNA is less informative than analysis of nuclear rDNA-ITS in *Dendrobium* species. The efficacy of PCR-RFLP of rDNA-ITS has also been reported in mushroom[23] and *Withania somnifera*[10]. Notably, when the two data sets of rDNA-ITS and cpDNA were combined, 23 *Dendrobium* spp. could be identified by 24 classes of PCR-RFLP patterns. The DNA patterns of *D. anosmum* and *D. parishii*, which could not be discriminated by PCR-RFLP of rDNA-ITS, could be distinguished, whereas the *D. crumenatum* and *D. formosum* still had the same pattern, suggesting that the

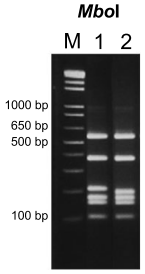

Fig. 4 PCR restriction patterns of *D. crumenatum* (1) and *D. formosum* (2) after amplification of cpDNA using psbC-trnS primer and cut with *Mbo*I. M is a 1 kb plus DNA ladder.

DNA regions of *D. crumenatum* and *D. formosum* used in this study are highly similar. These results were seen in the dendrogram where they were in the same clade (Fig. 3). However, if the amplified DNA fragments using psbC-trnS primer of *D. crumenatum* and *D. formosum* were cut by *Mbo*I, they could also be distinguishable (Fig. 4).

Identification of *Dendrobium* species by molecular techniques has been used in several studies including DNA marker-based and sequence-based ap-

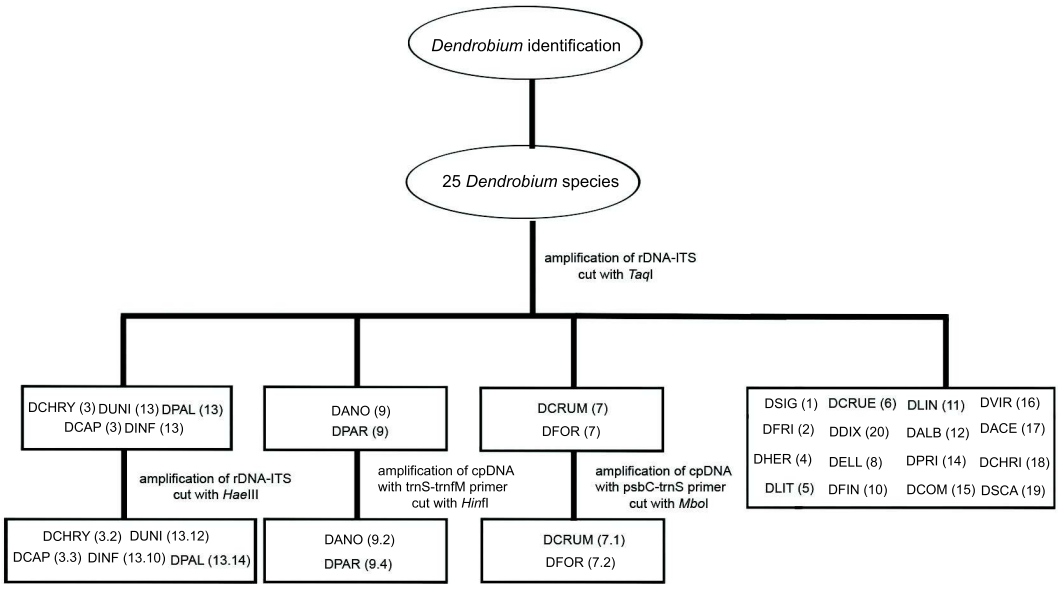

Fig. 5 Schematic representation of the effective procedure for identifying 25 native *Dendrobium* spp. found in Thailand. Firstly, amplification of rDNA-ITS were performed with the unknown *Dendrobium* spp., and then cut with *Taq*I, which can identify 16 *Dendrobium* spp. with a unique DNA pattern as shown by the number in parentheses (the same as the number with asterisk in Table 2). If the DNA pattern in the first step are type 3 or type 13, then cut the rDNA-ITS with *Hae*III which can further identify the other five species. If the DNA pattern of the first step is type 9, the amplification of cpDNA is then conducted with trnS- trnfM primer followed by cutting with *Hin*fI, which can identify two species. However, if the DNA pattern of the first step is type 7, the amplification of cpDNA is conducted with psbC-trnS primer followed by cutting with *Mbo*I, which can identify the two species. The species identified using 2 steps are shown by the number with two digits, where the first digit represents the DNA pattern of rDNA-ITS/*Taq*I and the second one is the DNA pattern of the second primer enzyme combination. The abbreviations of each species are described in Table 2.

proaches. The sequences of rDNA-ITS and the sequences of *matK* and *rbcL* genes of cpDNA [13,15] have been used to provide species identification of some *Dendrobium* plants. The sequence-based procedure could be the basis of a reliable protocol for species identification and could provide taxonomic information for clarifying evolutionary relevance of the taxa studied [24,25]. Nonetheless, this method is costly and time-consuming since it needs PCR amplification and DNA sequencing. The local orchid-research laboratory might not have all necessary equipment and materials. On the other hand, genetic diversity of 31 *Dendrobium* species from Yunnan region of China has been revealed through ISSR markers [17]. This method can provide species-specific ISSR markers in several species. The multiple bands from each marker exhibited in each species however made it difficult to determine those specific markers. In contrast, PCR-RFLP utilizes the digestion of amplified DNA fragments with a restriction endonuclease to display restriction site polymorphisms [9]. This approach could be performed simply through specific PCR products using a standard agarose gel electrophoresis. Species verification can therefore be done at the local research laboratory. PCR-RFLP approach has been used to investigate the phylogenetic relationship among 13 *Dendrobium* species using chloroplast *rbcL* gene [16].

Even though the genetic relationship of various *Dendrobium* spp. has been reported [14,15], the study of the phylogenetic relationship of several Thai native *Dendrobium* species has never been conducted. In this study, the dendrogram was reconstructed by the binary data matrix of PCR-RFLP from rDNA-ITS and cpDNA data sets (Fig. 3). These relationships were however not consistent with the taxonomic classification (section) and with our phylogenetic analysis based on DNA sequence (Srikulnath et al unpublished data), suggesting that the number of primer/enzyme combinations for PCR-RFLP analysis should be increased for more accurate investigation of the genetic relationship of each native *Dendrobium* spp.

In the present study, the effective procedure for identification of 25 native *Dendrobium* spp. in Thailand was clarified (Fig. 5). Firstly, all *Dendrobium*

spp. were amplified by PCR at rDNA-ITS region followed by cutting with *Taq*I as the first step. Sixteen *Dendrobium* species could be differentiated with a unique DNA pattern as shown by the number in parentheses (the same as the number with asterisk in Table 2) namely, *D. signatum* (1), *D. fridericksianum* (2), *D. hercoglossum* (4), *D. lituiflorum* (5), *D. cruentum* (6), *D. ellipsophyllum* (8), *D. finlayanum* (10), *D. lindleyi* (11), *D. albosanguineum* (12), *D. primulinum* (14), *D. compactum* (15), *D. virgineum* (16), *D. acerosum* (17), *D. christyanum* (18), *D. scabrilingue* (19), and *D. dixanthum* (20). For the species having the DNA pattern of either type 3 or type 13, the amplified fragments of rDNA-ITS were further cut with *Hae*III which could be used to identify the other five species, *D. chrysanthum* (3.2), *D. capillipes* (3.3), *D. infundibulum* (13.10), *D. unicum* (13.12), and *D. palpebrae* (13.14). However, if the DNA pattern of the first step was type 9, then cpDNA was amplified with trnS-trnfM primer followed by cutting with *Hin*fI, where *D. anosmum* (9.2) and *D. parishii* (9.4) were successfully distinguished. Finally, *D. crumenatum* and *D. formosum* which had the same DNA pattern as type 7 from the first step were then differentiated by amplifying the cpDNA with psbC-trnS primer and subsequent cutting with *Mbo*I.

Conclusively, our study provides a rapid, simple and reliable method to identify *Dendrobium* species, and the DNA patterns of all species in this study could be used as a reference for *Dendrobium* spp. identification and certification. This study also suggests that PCR-RFLP of rDNA-ITS and cpDNA could be an alternative method for species identification in some plants.

Acknowledgements: This work was financially supported by the National Research Council of Thailand (NRCT). We are very grateful to Amara Thongpan for valuable discussion and to Thiti Kanchanaketu for technical assistance in dendrogram construction. We acknowledge the laboratory of Biotechnology (Plant Tissue Culture section), Chulabhorn Research Institute, Laksi, Bangkok, Thailand, for providing all orchid samples.

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