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Full Length Article

Authentication of Chinese Herbal Medicines *Dendrobium* Species and Phylogenetic Study Based on nrDNA ITS Sequence

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Abstract

Dendrobium plants are one of the most valuable traditional Chinese medicinal herbs. Because of excessive exploitation and habitat degradation, *Dendrobium* species are endangered. Moreover, during no flowering stages, it is very difficult and even impossible to distinguish *Dendrobium* species based on morphological characteristics. Therefore, exploiting other DNA barcodes as complementary molecular marker for distinguishing *Dendrobium* spp. are necessary. Hence, developing an uncomplicated and exact method for authenticating *Dendrobium* spp. is imperative. In this study, nuclear ribosomal DNA internal transcribed spacer (ITS) sequences of 320 samples from 73 *Dendrobium* spp. were analyzed. The average intra- and inter-specific genetic divergences were 0.007 and 0.220, respectively. The TaxonGap method indicated that between-species variation of about 80.9% of the species were higher than within-species variation. The neighbor-joining tree showed that 73 *Dendrobium* spp. were divided into four major clades. Phylogenetic relationships between *Dendrobium* spp. supported traditional morphological methods and previously published molecular data. This study indicated that ITS region was not only suitable for species identification, but also for phylogenetic analysis of *Dendrobium.* However, it was also found that ITS could not readily resolve all species determination problems in *Dendrobium*. © 2018 Friends Science Publishers

Keywords: *Dendrobium*; ITS; DNA barcode; Molecular authentication; Phylogenetic study

Introduction

Dendrobium, which is one of the largest genera of Orchidaceae, includes more than 1500 species and is mostly distributed in New Zealand, eastern and northern region of Australia, and subtropical and tropical Asia (Takamiya *et al*., 2011; Feng *et al*., 2015a; Xu *et al*., 2015). *Dendrobium* has important economic significance, and some species are very famous ornamental plants (Wang *et al*., 2009). Moreover, the fresh or dried stems of *Dendrobium* species have the efficacy of maintaining gastric tonicity, enhancing production of body fluid, nourishing Yin, and antipyresis (Chinese Pharmacopoeia Committee, 2015). About 40 *Dendrobium* plants were used as traditional Chinese herbs, and four kinds of *Dendrobium* (*D. nobile*, *D. chrysotoxum*, *D. fimbriatum*, *D. officinale*) are recorded in Chinese Pharmacopoeia (2015). Due to over-exploitation, degradation and loss of habitat, many *Dendrobium* plants are under serious threat of extinction and are contained in Appendices I and II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

The unequivocal authentication of *Dendrobium* plants at species level is crucial to protect genetic resources, and assure quality, efficacy and security in clinical use (Puihaybut *et al.*, 2005; Wang *et al.*, 2009). Morphological characters have traditionally been used to authenticate *Dendrobium* species. However, this method is often impacted and limited by phenotypic plasticity (Ding *et al.*, 2009; Feng *et al*., 2015a; Lau *et al*., 2015). Moreover, morphological features of many *Dendrobium* plants are very similar during the lack of flowering periods. Therefore, it is very difficult and even impossible to distinguish *Dendrobium* species using morphological taxonomy. Hence, developing an uncomplicated and exact method for authenticating *Dendrobium* species is urgent.

In the past decade, the rapid development of molecular biotechnology has allowed Chinese medical herbs to be identified. It has been proved that molecular markers, particularly DNA barcoding, were extremely powerful tool to differentiate closely related species. DNA barcode is an innovative technology to authenticate organisms based on a short DNA fragment. Due to several advantages, such as

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generality, simplification, high copy number, interspecific variability and intraspecific uniformity, the nuclear ribosomal DNA internal transcribed spacer (ITS) region has been recommended as an ideal DNA barcode candidate to authenticate Chinese herbal medicine (Selvaraj *et al.*, 2012).

Many researches have demonstrated that the identification of medicinal plants by using ITS regions was effective and accurate (Shiba *et al.*, 2006; Kim *et al.*, 2007; Lian *et al.*, 2008; Feng *et al.*, 2010). The objectives of this study were a) to establish a fast, simple and reliable method for distinguishing *Dendrobium* species and b) to investigate their phylogenetic relationship.

Materials and Methods

Material Collection and DNA Extraction

In total 320 specimens from 73 *Dendrobium* species were analyzed. Among them, the fresh leaf materials of 26 *Dendrobium* samples were collected from *Dendrobium* forest tree germplasm repository of Longling County Research Institute of *Dendrobium*, which located in Longling county, Baoshan city, Yunnan province, China (Table 1). All other sample data were from GenBank (Table S1). The gathered samples were stored in 4℃ with color-changing silica gel for subsequent DNA extraction. All specimens were coded (Table 1) and reserved in the herbarium of Chengdu University of Traditional Chinese Medicine. Tiangen plant genomic DNA kit was used to extract genomic DNA. Extracted DNA was stored at -20°C until required.

PCR Amplification and DNA Sequencing

The ITS sequences of 26 species of *Dendrobium* were amplified by the forward primer 5'-CGTAACAAGGTTTCCGTAGGTGAAC-3' and reverse primer 5'-TTATTGATATGCTTAAACTCAGCGGG-3' (Geng *et al.*, 2015). The 25 μL PCR amplification system included 12.5 μL 2 × Taq master mix buffer (Tiangen, China), 15 ng DNA per reaction volume as well as 1 pmol of each primer. PCR program was performed with 94°C denaturation temperature for 4 min, followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, 1 min at 72°C, and a final extension for 10 min at 72°C. After electrophoresis analysis, PCR products were sequenced bi-directionally by Invitrogen™ Life Technology.

Data Analysis

The forward and reverse sequences of ITS were spliced together with Contig Express program (Lu and Moriyama, 2004). ITS, ITS1, 5.8S and ITS2 boundaries were confirmed by contrast with ITS region of *Dendrobium* retrieved from NCBI. The 26 ITS sequences obtained in this study were deposited in NCBI database. GenBank accession numbers were shown in Table 1.

Table 1: *Dendrobium* species, ITS length, GC content and accession numbers

Species	Voucher	Length (bp)			GC content (%)			GenBank No.
		ITS	ITS1	ITS2	ITS	ITS1	ITS2	
D. bellatulum	DB1	640	230	247	54.4	53.0	52.6	KY499211
D. primulinum	DP2	640	233	244	51.3	45.5	52.9	KY499212
D. stuposum	DS4	640	233	244	54.1	53.2	51.6	KY499214
D. brymerianum	DB5	640	233	244	52.7	51.1	50.4	KY499215
D. cariniferum	DC6	636	230	243	53.6	52.2	52.3	KY499216
D. falconeri	DF7	638	230	245	52.0	48.3	51.4	KY499217
D. wardianum	DW8	637	228	246	55.3	52.6	55.7	KY499218
D. porphyrochilum	DP9	640	231	246	47.5	44.2	45.1	KY499219
D. aurantiacum var. *denneanum*	DA10	640	233	244	52.5	50.2	50.8	KY499220
D. aphyllum	DA11	639	232	244	51.3	45.7	52.5	KY499221
D. chrysotoxum	DC13	641	233	245	55.2	52.8	55.1	KY499223
D. nobile	DN14	636	231	242	52.5	49.4	52.9	KY499224
D. crystallinum	DC15	641	232	246	55.9	54.7	54.9	KY499225
D. fimbriatum	DF16	639	233	243	55.4	56.7	52.7	KY499226
D. thyrsiflorum	DT18	641	231	247	52.6	49.4	52.6	KY499228
D. moschatum	DM19	640	233	244	50.0	48.9	46.3	KY499229
D. strongylanthum	DS20	640	231	246	47.2	43.3	44.7	KY499230
D. henryi	DH21	636	231	242	52.2	50.2	50.4	KY499231
D. officinale	DO22	636	231	242	52.4	50.6	50.4	KY499232
D. jenkinsii	DJ23	643	234	246	50.4	46.6	48.8	KY499233
D. moniliforme	DM24	637	232	242	53.8	52.2	52.5	KY499234
D. pendulum	DP25	637	228	246	53.1	49.6	53.3	KY499235
D. devonianum	DD26	641	234	244	49.8	45.3	48.8	KY499236
D. chrysanthum	DC27	637	233	241	53.1	51.5	51.5	KY928056
D. longicornu	DL28	640	230	247	54.2	53.0	52.6	KY928057
D. williamsoni	DW29	636	230	243	53.5	51.7	52.3	KY928058

The multiple alignment of ITS sequences of 320 samples from 73 *Dendrobium* species was performed by Clustal X 1.8 (Thompson *et al.*, 1997) and genetic distances were calculated using MEGA 7 (Kumar *et al.*, 2016). The number of polymorphic sites was estimated by DnaSP 5.10 (Librado and Rozas, 2009). The intra-specific genetic variation was assessed through analyzing average intra-specific distance, coalescent depth and theta. The inter-specific genetic divergence was determined through calculating average inter-specific distance, the minimum inter-specific distance and theta prime. The distributions of inter- vs. intraspecific variability were compared by DNA barcode gaps (Meyer and Paulay, 2005). The ability of discrimination species was determined by TaxonGap v2.4.1 (Slabbinck *et al.*, 2008). The phylogenetic trees were constructed by MEGA 7 software using the neighbor-joining algorithm based on the K2P-distance matrices from with 1000 bootstrap replicates. *Aphyllorchis montana* (GenBank accession FJ454867) was used as outgroup for rooting the trees.

Results

ITS Sequence Characteristics

The length of 26 ITS sequences obtained in this study varied from 636 to 643 bp. Since the sequence of the 5.8S region is relatively conservative, it will not be analyzed. The lengths of ITS1 and ITS2 were in the range of 228–234 bp and 241–247 bp, respectively (Table 1).

Table S1: GenBank accession number of different *Dendrobium* species retrieved from NCBI in this study

Species name	GenBank No.	Species name	GenBank No.
D. bellatulum	KJ210419、KJ210420、KP159302、KT778752、KX600501	*D. salaccense*	JN388577、KJ210494、KJ210493
D. primulinum	KT778755、KX522641、HM054747、HM054750、HM054751	*D. sinense*	KF143511、KJ210497、JN388578、KJ210498
D. gratiosissimum	DQ058790、FJ384737、JN388590、KF143464、KF143465	*D. somai*	AF521616、EU840692、HM590380
D. aurantiacum	KJ210416、KJ210417、KJ210418	*D. sulcatum*	KF143517、AB593670、EU477510
D. chrysanthum	AF362047、KT778753、AB873182、HM054594、JN388584	*D. terminale*	DQ058801、KX522647
D. longicornu	AB847661、KF143485、KF143484、KJ210467、KJ210468	*D. tosaense*	KC331000、EU003113、KC330999、KC331001、KC331002
D. williamsonii	HQ114225、KJ210509、KJ210508、KJ672700、KX522632	*D. trigonopus*	KJ210505、DQ058793、FJ384741、HQ114228、KF143521
D. acinaciforme	HQ114253、KJ210407、KJ210408	*D. wilsonii*	KC205190、KR075037
D. aduncum	GU339110、KC346887、KX600499、JN388580	*D. xichouense*	KJ672648、KC568304、KF143527、KJ210514
D. capillipes	KX522648、AF362035、JN388582	*D. stuposum*	KF143516、JN388599、GU339104、KJ672695、HQ114237
D. catenatum	KJ881387、KJ881388、KJ881389、KJ881390	*D. brymerianum*	AB593511、KF143432、KJ210422、KJ210423、HQ114233
D. chameleon	AB593527、AF521607、AY239960、HM590385	*D. cariniferum*	AB847645、AF362027、KF143435、KF143436、KJ672654
D. christyanum	AB972351、KJ210425、GU339106、KF143441、KJ210426	*D. falconeri*	AB593560、FJ384734、KF143458、KJ210447、HQ114239
D. compactum	AB847650、KF143445	*D. wardianum*	JN388600、KT778762、DQ058789、HQ114231
D. crumenatum	AB972336、JN388587、AB593537、HM054625、HM590370	*D. porphyrochilum*	KF143500、KJ210489、KX600505
D. densiflorum	AF362029、DQ058786	*D. aurantiacum* var. *denneanum*	AF362040、KF143448、AF362043、FJ384731
D. dixanthum	AB593552、AB972354、DQ058788、GU339103、KF143454	*D. aphyllum*	KF143430、HQ114247、KJ210413、KJ210414、KJ944622
D. ellipsophyllum	AB593554、AB972338、AF362033、KF143455	*D. infundibulum*	KF143477、KJ210459
D. equitans	AF521609、EU840701、HM590388、KJ672668	*D. chrysotoxum*	AB593533、AB873185、HQ114221、KT778756、KX522645
D. exile	AF362024、KF143456、KF143457、KJ210444、KJ210445	*D. nobile*	JF713113、JF713114、JF713115、JF713116、KJ210484
D. findlayanum	AB593563、KF143462、AB972340、JN388589	*D. crystallinum*	AB593538、GU339116、HQ114243、KJ944633、KT778764
D. flexicaule	FJ384743、AF355570	*D. fimbriatum*	AB873184、HQ114229、JN388588、KF143461、KJ672672
D. gibsonii	AB593568、GU339105、KX522636	*D. thyrsiflorum*	HM054758、HQ114227、KF143519、KJ210503、KX600503
D. hancockii	AF362025、AF362038、DQ058787、JN388591、KP159297	*D. moschatum*	AB593616、HM054713、HM054714、KT778750、KX522635
D. harveyanum	KF143468、KC568299、AB593576、KJ210452、JN388594	*D. strongylanthum*	DQ058797、KJ210499、KJ210500、KJ210501、KJ210502
D. heterocarpum	GU339101、AB593582、JN388592、JN388593、KX600513	*D. henryi*	AB593579、EF629323、KF143470、KJ210455、KJ210456
D. hookerianum	KJ210458、AB593584、KF143475、KX509992	*D. officinale*	FJ384723、HQ114245、JF803236、KC205184、KC205185
D. leptocladum	AF521612、AB593598、EU840697、HM590373	*D. jenkinsii*	KJ210460、DQ058785、KC568297、KF143479、KX600506
D. linawianum	KP159299、AB593599、EU003115、EU003117	*D. moniliforme*	KP159303、GU339111、HQ114246
D. lindleyi	DQ058784、GU339114、JN388568	*D. pendulum*	KF143498、KF143496、AB593633、DQ058791、HQ114234
D. lituiflorum	KJ944624、KR075041	*D. hercoglossum*	KC346889、KP159300、JN388576、AB972333
D. lohohense	AB593605、AF363024、JN388574、KF143482	*D. loddigesii*	KP159301、AB873183、KT778746、JN388569
D. minutiflorum	KF143488、KF143487	*D. huoshanense*	KC568300、KU556796
D. miyakei	AF521614、HM590386、KJ210473	*D. crepidatum*	KJ210431、KJ210432、AB593534、KJ210433
D. monticola	DQ058799、DQ058798	*D. transparens*	KF143520、AB593679、KX600508、KT778761
D. parciflorum	KF143466、HQ114252、HQ700437、JN388575、KJ210450	*D. devonianum*	KP743545、HQ114244、KC346888、KJ210441, KJ210442
D. parishii	AB593630、KJ944629、AB972344、EU121417		

The content of GC varied from 47.2% to 55.9% for the ITS, 43.3% to 56.7% for the ITS1, and 44.7% to 55.7% for the ITS2 (Table 1). The mean GC content of ITS, ITS1 and ITS2 was 52.5, 50.1 and 51.3%, respectively. The number of polymorphic sites within ITS, ITS1 and ITS2 were 330 (50.3% of the total ITS region), 153 (62.7% of the total ITS1 region) and 150 (60.2% of total ITS2 region), respectively.

Intra- and Inter-specific Genetic Divergence

A perfect molecular marker for authenticating species should have smaller variations within species and larger variation between species. So the inter- and intraspecific divergences of ITS were assessed. The intraspecific variations of *Dendrobium* plants in this study ranged from a minimum 0.000 (*D. officinale*, *D. moniliforme*, *D. salaccense, D. bellatulum, D. primulinum, D. trigonopus, D. infundibulum, D. crystallinum, D. christyanum, D. jenkinsii, D. loddigesii*, *D. nobile, D. falconeri, D. sinense, D. gibsonii, D. catenatum, D. lituiflorum, D. tosaense, D. monticola*) to a maximum of 0.050 (*D. terminale*), with an average of 0.007. The interspecific divergences ranged from a minimum of 0.000 (*D. bellatulum* vs. *D. christyanum*) to a maximum of 0.436 (*D. monticola* vs. *D. somai*), with an average of 0.220.

Intra- and interspecific variations of *Dendrobium* plants were characterized by six metrics (average intra-specific distance, coalescent depth, theta, average inter-specific distance, the minimum inter-specific distance and theta prime) (Meier *et al.*, 2008). The results indicated that intraspecific variations of ITS sequences were relatively smaller and inter-specific divergences were very obvious (Table 2). Therefore, ITS region, which has more significant variations between species than within species, is a valid DNA marker to authenticate *Dendrobium* species.

Table 2: Analyses of inter-specific divergence and intra-specific variation of the ITS sequences in 320 samples of 73 *Dendrobium* species

Measurement	Kimura 2-Parameter (K2P) Value
Theta	0.0068±0.0100
Coalescent depth	0.0109±0.0142
All intraspecific distance	0.0049±0.0092
Theta prime	0.1796±0.0344
Minimum interspecific distance	0.0725±0.0535
All inter-specific distance	0.1795±0.0571

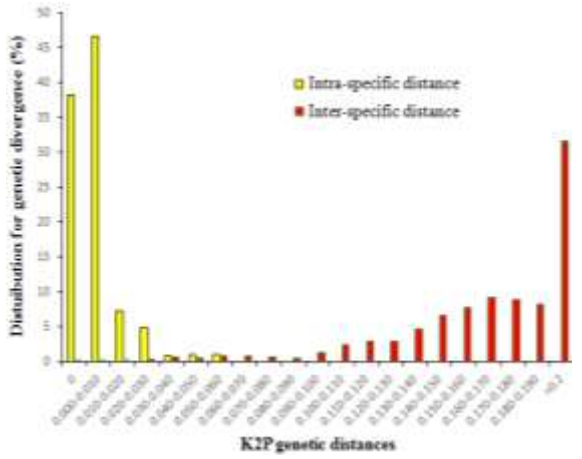

Fig. 1: Relative distribution of inter-specific divergence between congeneric *Dendrobium* species and intra-specific variation in the ITS region using K2P model

Evaluation of Barcode Gap

The distribution of genetic distances showed that there was a slight overlap in intar/interspecific divergences (Fig. 1). The interspecific divergence that equaled to 0 was only 0.09% and the proportion <0.060 was only 3.30%. Moreover, most of the *Dendrobium* species were found to have a distinct ITS sequence. The result suggested that ITS had clear barcoding gaps and was suitable to identify *Dendrobium* plants.

Species Authentication Capability of ITS

The species identities of these sequences obtained in this study were ascertained by TaxonGap method. The result of TaxonGap method showed that the ITS regions of 61 species had adequate specificity to be distinguished from their neighbors (Fig. 2). The result indicated that between-species divergence of about 80.9% of the species were bigger than within species divergence. Therefore, it is perorated that ITS region could be used as a DNA marker to discriminate *Dendrobium* plants. However, it has exceptions: 16.4% of the species (light grey bar) had similar sequences with their genetically close species for *D. aurantiacum* and *D. aurantiacum* var. *denneanum*, *D. bellatulum* and *D. christyanum*, *D. catenatum* and *D. moniliforme*, *D. cariniferum* and *D. williamsonii*, *D. hercoglossum* and *D. linawianum*, *D. strongylanthum* and *D. monticola* (Fig. 2).

Phylogenetic Analysis

Phylogenetic tree of 73 *Dendrobium* species was divided into four clades (Fig. 3). Clade 1 was more intricate, with 43 species from six sections (sect.): sect. *Dendrobium* (36 species), *Pedilonum* (*D. xichouense*), *Breviflores* (*D. aduncum* and *D. hercoglossum*), *Grastidium* (*D. leptocladum*), *Chrysotoxae* (*D. chrysotoxum*), *Stuposa* (*D. stuposum*). There was no report about to which section *D. catenatum* belongs, but it was clustered with sect. *Dendrobium*. Clade 2 was the most intricate, with 27 species from eight sections: sect. *Chrysotoxae* (*D. thyrsiflorum*, *D. lindleyi*, *D. densiflorum*, *D. sulcatum*, *D. jenkinsii*), *Formosae* (*D. bellatulum*, *D. trigonopus*, *D. cariniferum*, *D. longicornu*, *D. sinense*, *D. infundibulum*, *D. christyanum*, *D. williamsonii*), *Dendrobium* (*D. capillipes*), *Stachyobium* (*D. strongylanthum*, *D. compactum*, *D. minutiflorum*, *D. porphyrochilum*, *D. monticola*), *Pedilonum* (*D. chameleon*, *D. miyakei*), *Crumenata* (*D. exile*, *D. crumenatum*, *D. equitans*), *Aporum* (*D. terminale*, *D. acinaciforme*), *Strongyle* (*D. parciflorum*). Clade 3 was the simplest, only contained one species *D. ellipsophyllum*, which belonged to sect. *Distichophyllum*. Clade 4 was also simple, only included two species (*D. salaccense* and *D. somai*) that both are from sect. *Grastidium*.

Discussion

It is very important to develop a rapid and accurate identification method for protecting *Dendrobium* resources and assuring its clinical medication safety. In the study, we used ITS region to authenticate *Dendrobium* species with a large sample size. The results showed that ITS region of *Dendrobium* had enough variation, and it possessed a high capability of successfully distinguishing different species from *Dendrobium*. For instance, *D. densiflorum* and *D. thyrsiflorum*, which both belong to Sect. *Chrysotoxae*, have very similar morphological characteristics (Tsi *et al.*, 1999), but ITS region could accurately differentiate them. In addition, *D. hercoglossum* and *D. aduncum* from sect. *Breviflores*, which were also very difficult to distinguish based on morphological characteristics (Tsi *et al.*, 1999), could be successfully differentiate using ITS sequences. Nevertheless, it should be noticed that ITS could not readily resolve all species determination problems in *Dendrobium*. For instance, ITS sequences of *D. bellatulum* and *D. christyanum* were identical. Moreover, the genetic divergences of ITS sequences of *D. cariniferum* and *D. williamsonii*, *D. strongylanthum* and *D. monticola*, *D. catenatum* and *D. moniliforme*, and *D. hercoglossum* and *D. linawianum* were so small that they failed to be

Fig. 2: The heterogeneity and separability for individual taxa of ITS based on 73 *Dendrobium* species by TaxonGap

Fig. 3: Phylogenetic tree based on ITS sequences of 73 *Dendrobium* species. *Aphyllorchis montana* was designated as outgroup for rooting tree. Numbers above the branches indicate bootstrap values

discriminated by ITS. Furthermore, increasing species or sample set will probably increase the number of *Dendrobium* that cannot be distinguished by ITS. Thus, it is necessary to exploit other barcode loci as supplementary molecular marker to distinguish these species.

Due to their vegetative similarity, abundant species and lapped morphologic variation within some species (Xu *et al.*, 2015), *Dendrobium* taxonomy is considered as the most complicated challenges in Orchidaceae (Feng *et al.*, 2015b). As the findings of other molecular researches, this study also found that infrageneric taxa of *Dendrobium* were not monophyletic (Xiang *et al.*, 2013; Feng *et al.*, 2015a, b). Phylogenetic tree indicated that sect. *Dendrobium* was paraphyletic. *Dendrobium chrysotoxum* from Sect. *Chrysotoxae*, and sects. *Stuposa* and *Breviflores* clustered together with sect. *Dendrobium*, which strongly endorsed the opinion that they should be contained in sect. *Dendrobium* (Feng *et al.*, 2015b). Futhermore, *D. catenatum* was also grouped together with sect. *Dendrobium.* Therefore, we think *D. catenatum* should also be included in sect. *Dendrobium.* The neighbor-joining (NJ) tree also showed that these species of sect. *Chrysotoxae* formed two clades (1, 2), which indicated that sect. *Chrysotoxae* was probably polyphyletic, and this result was consistent with precious reports (Feng *et al.*, 2015b). It was

reported that sect. *Grastidium* was monophyletic (Xiang *et al*., 2013). However, NJ tree of this study showed that except for *D. salaccense* and *D. somai* from sect. *Grastidium* (clade 4), *D. leptocladum* was clustered together with sect. *Dendrobium*. The result suggested that sect. *Grastidium* might not be monophyletic.

Conclusion

In this study, ITS sequences of 320 samples from 73 *Dendrobium* species were analyzed. The application of DNA barcoding is very beneficial to safe use and protection of *Dendrobium* plants. In crux, ITS region was not only suitable for species identification but also for phylogenetic analysis of *Dendrobium* spp.

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