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BRIEF COMMUNICATION

Phylogenetic relationship of China *Dendrobium* species based on the sequence of the internal transcribed spacer of ribosomal DNA

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Abstract

The genetic relationship of 36 *Dendrobium* species in China was determined based on sequence analysis of the internal transcribed spacer (ITS) region of ribosomal DNA. Aligned sequences of the complete ITS region obtained from the 36 *Dendrobium* species and 2 outgroup species (*Epigeneium amplum* and *Epigeneium nakaharaei*) by using PCR amplification and direct DNA sequencing. The nrDNA ITS1 of *Dendrobium* was 225 - 234 bp and ITS2 was 239 - 248 bp. Phylogenetic tree was constructed, and seven main clusters were generated among the 36 *Dendrobium* species. From the results, *D. moulmeinense* was not grouped in the classification of *Dendrobium* and *E. amplum* and *E. nakaharaei* were shown to be divergent from *Dendrobium* species. The phylogenetic relationships revealed by ITS DNA analysis partially supported previously published morphological data.

Additional key words: *Epigeneium* species, *Orchidaceae*, PCR, phylogenetic tree.

The genus *Dendrobium* was established by Olaf Swartz in 1799 and is the second genus largest of *Orchidaceae* family plants (Tsi 1980). There are more than 1400 *Dendrobium* species distributed all over the world (Lavarack *et al*. 2000). Seventy-six species of *Dendrobium* are distributed in China which has been subdivided into twelve sections. *Dendrobium* species comprise some very important ornamental plants in the world and some *Dendrobium* species have long been used in traditional Chinese medicine (Lavarack *et al*. 2000). 1992 China Red list of threatened plants recorded the status of several *Dendrobium* species as endangered. Although the circumscription of genus *Dendrobium* has been clearly defined, relationships within the group remain unresolved because of morphological diversification and reproductive features (Tsi 1980), and very few *Dendrobium* species have been DNA-fingerprinted to date.

Different regions of nuclear ribosomal DNA (rDNA) can be used to examine lineages with different levels of divergence (Christopher *et al*. 2005). The internal transcribed spacer (ITS) region of 18S-26S rDNA has proven to be a useful source of characters for phylogenetic studies in many angiosperm families (Baldwin *et al*. 1995). Several studies have shown that the ITS regions can be used to infer phylogeny among closely related taxa and to identify species or strains (Lau *et al*. 2001, Zeng *et al*. 2008). In this study, direct DNA sequencing of PCR-amplified products was used to identify the ITS sequences of 36 *Dendrobium* species. Based on the ITS sequence obtained from each *Dendrobium* species, the genetic relationships and classifications among these 36 *Dendrobium* species could be determined, and molecular markers can be usefully exploited to identify and evaluate various *Dendrobium* species in the future.

The plants were collected from the nature and cultivated in laboratory. The fresh leaf materials were collected from 36 species of *Dendrobium* and two outgroup species from *Epigeneium*, *E. amplum* and *E. nakaharaei*. DNA was extracted using cetyldimethyl ethylammonium bromide (CTAB) according to Doyle and Doyle (1990). The DNA concentration was determined using a spectrophotometer and concentration 5 μg cm^{-3} for use in amplification reactions. In order to amplify the internal transcribed spacer of rDNA of *Dendrobium* plants, an appropriate primers pair identified as P1 (5'- ATTGAA TGGTCCGGTGAAGTGTTCG-3') and P2 (5'-AATTCC CCGGTTCGCTCGCCGTTAC-3') was used. (Douzery *et al*. 1999). The primers used to amplify the ITS of *E. amplum* and *E. nakaharaei* are the same as those in the

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Abbreviations: CTAB - cetyldimethyl ethylammonium bromide; GC - guanine and cytosine; ITS - internal transcribed spacer; PCR - polymerase chain reaction.

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Clements *et al*. (2002). Amplification reactions were performed in 0.025 cm^3 mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, with 0.2 mM each dNTP, 12.5 pM of each primer, 2.5 U of Taq DNA polymerase (*TaKaRa*, Dalian, China) and 20 ng genomic DNA. Amplification reactions were performed in a *PTC-200* DNA thermal cycler (*M.J. Research*, Watertown, MA, USA). The PCR profile consisted of an initial 5 min premelt at 94 °C and 35 cycles of 1 min at 94 °C, 1 min at 52 °C, and 2 min at 72 °C, followed by a final 10 min extension at 72 °C. Amplified products were electrophoresed in a 1.0 % (m/v) agarose gels with 1× TBE buffer, stained with ethidium bromide, and photographed using *UVP Autochemi* system (*UVP Inc*, Upland, CA, USA). The PCR products were purified using *QIAquick* gel extraction kit (*Qiagen*, Shanghai, China). The ITS sequences were sequenced directly using an *ABI377* automated sequencer (*Applied Biosystems*, Foster City, CA, USA). Sequencing primers were the same as those used for PCR. In order to confirm the sequences, each sample was sequenced two times.

The boundaries of the ITS regions were determined by comparison with the sequences of *Orchidaceae* (Douzery 1999). The ITS sequences were aligned using *Clustal X* (Thomson *et al*. 1997). Gaps were treated as missing data. Transition/transversion (ns/nv) ratio was determined using the program *MEGA version 3.1* (Kumar *et al*. 2004). Maximum parsimony analyses and phylogenetic tree were performed using *PAUP 4.0* (Swofford 1998) with all changes weighted equally, using *HEURISTIC* searches with *TBR* branch swapping and 100 random addition sequences. Multiple most parsimonious trees were summarized as strict consensus tree. Bootstrap analysis (BS) was conducted using 1000 replicates. Sequence divergences between

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

Species	Length			GC content [%]			GenBank No.
	ITS	ITS1	ITS2	ITS	ITS1	ITS2	
D. jenkinsii	644	233	248	50.93	47.64	49.60	EF629321
D. moulmeinense	633	231	241	53.23	54.55	51.87	EF629322
D. henryi	636	231	242	52.20	49.78	50.41	EF629323
D. hainanense	636	228	245	53.62	48.25	54.69	EF629324
D. christyantum	639	229	247	54.46	53.28	52.63	EF629325
D. moschatum	639	232	244	49.61	47.84	46.31	EF629326
D. flexicaule	635	230	242	52.13	50.43	50.00	AF355570
D. aphyllum	638	231	244	51.57	46.32	52.46	AF355573
D. wardianum	636	228	248	54.30	52.63	52.42	AF420245
D. primulinum	639	234	243	51.49	46.15	53.09	AF362913
D. brymerianum	640	233	244	52.81	51.50	50.41	AF362036
D. devonianum	641	234	244	49.30	44.02	48.77	AF311779
D. cariniferum	636	230	243	53.93	52.61	52.26	AF362027
D. denneanum	640	233	244	52.50	50.64	50.00	AF362040
D. chrysotoxum	641	233	245	55.23	52.79	55.10	AF362023
D. huoshanense	637	232	242	54.32	52.59	53.72	AF355569
D. acinaciform	640	233	244	51.72	47.21	52.05	AF362034
D. nobile	636	231	242	53.62	50.65	53.31	AF372039
D. crystaclinum	639	232	244	54.77	52.16	54.92	AF363023
D. fimbriatum	635	230	242	52.44	50.00	51.24	AF362041
D. lohohense	635	230	242	52.91	51.74	50.41	AF363024
D. crepidatum	631	225	243	51.35	48.00	50.21	AF355574
D. loddigesii	640	233	244	50.62	43.78	52.05	AF311778
D. densiflorum	641	231	247	52.73	49.35	53.04	AF362029
D. thyrsiflorum	641	231	247	52.73	49.35	53.04	AF362032
D. chrysanthum	636	234	239	53.46	51.71	53.14	AF355572
D. officinale	635	231	241	52.44	51.08	50.21	AF311776
D. moniliforme	635	232	240	53.86	52.59	52.08	AF311777
D. hancockil	640	232	245	55.00	51.29	55.92	AF362025
D. aurantiacum	640	233	243	55.40	56.65	52.67	AF362044
D. pendulum	639	231	245	53.52	48.92	55.10	AF362912
D. hercoglossum	635	233	239	54.17	51.07	54.39	AF363685
D. gratiosissimum	636	229	243	54.72	51.97	55.14	AF311780
D. salaccens	640	233	244	52.03	49.79	49.59	AF362026
D. ellipsophyllum	640	230	247	57.34	54.78	59.11	AF362033
D. capillipe	638	228	247	50.94	47.81	49.80	AF362035
E. amplum	650	232	249	58.77	57.76	60.64	AY240010
E. nakaharaei	650	232	249	55.85	53.02	57.43	AY240012

species for total nucleotides were calculated using the *DNADIST* program of *PHYLIP version 3.573*.

Each PCR product of the 36 *Dendrobium* species was a single fragment (approximately 650 bp in length) appeared. Their GenBank accession numbers was shown in Table 1. The nucleotide sequences were further determined, and we found that they were comprised of a partial sequence of the 26S rRNA gene, the ITS region, 5.8S rRNA gene and a partial sequence of the 18S rRNA gene. The boundaries of the internal transcribed spacers (ITS1, ITS2) and nuclear rDNA coding regions in the 36 species were determined by comparison to several published sequences obtained from a range of angiosperms (Douzery 1999). The length of the ITS region in *Dendrobium* species varied from 631 to 644 bp. ITS region length of *E. amplum* and *E. nakaharaei* was 650 bp. ITS regions from *Dendrobium* species varied from 225 to 234 bp for the ITS1 region, 163 bp for 5.8S rRNA gene, and 239 to 248 bp for the ITS2 region. The content of guanine and cytosine (GC) varied from 49.30 to 57.34 % for the ITS region, 47.38 to 56.65 % for the ITS1, and 46.31to 59.11 % for the ITS2 region (Table 1). The average GC content of ITS, ITS1 and ITS2 was 52.98, 50.30 and 52.25 %, respectively.

Polymorphic sites within ITS1, 5.8S rRNA gene and ITS2 between 36 taxa were 141 bp (60.98 % of the total

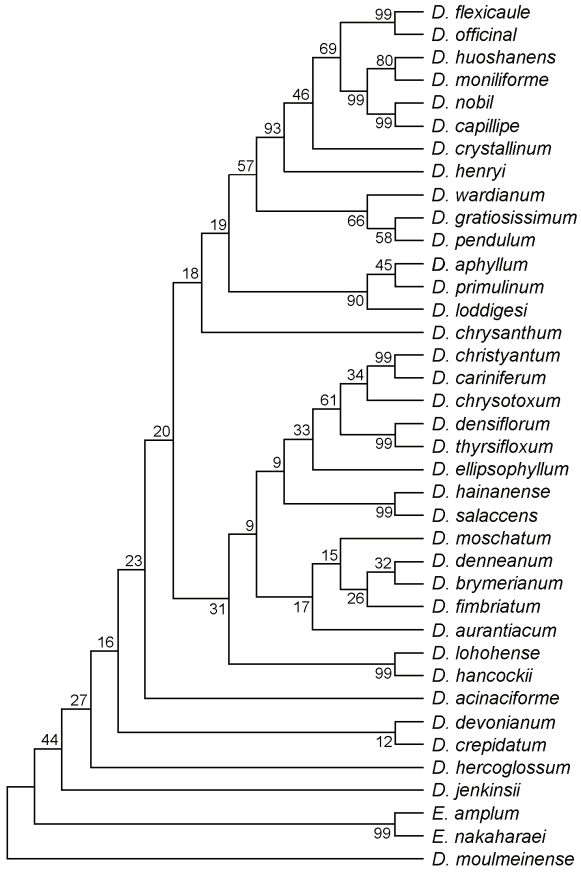

Fig. 1. Strict consensus trees derived from the parsimony analysis of ITS (length = 1 448 steps, CI = 0.46, RI = 0.58). Bootstrap percentages are shown above or below each branch

ITS1 region), 23 bp (14.11 % of total 5.8S rDNA) and 139 bp (57.03 % of the total ITS2 region). The transition/ transversion of ITS region was 1.3, but the transition/ transversion of ITS1, 5.8s and ITS2 were 1.1, 4.0 and 1.2, respectively. It was proved that the 5.8S rDNA regions are conserved than ITS. The sequence divergences of ITS region were estimated to be 0.2 - 36.3 % using the Kimura two parameter models. The highest divergence was between *D. jenkinsii* and *D. moulmeinense*. Within the 36 *Dendrobium* individuals of this study, sequence divergence in ITS regions was significantly greater than in 5.8S rDNA regions. The rather high sequence divergences among the genera of *Dendrobium* seem to be consistent with the previous report (Xu *et al*. 2001).

The maximum parsimony analysis, treating gaps as missing data, 1 445 MPT was found with a length of 1448 steps, consistency index (CI) = 0.46 (excluding uninformative characters), and retention index (RI) = 0.58. The strict consensus tree with bootstrap percentages (BP) is presented in Fig. 1. The present findings showed close genetic variation among the 36 *Dendrobium* species. Seven clusters representing species-specific grouping were obtained (Fig. 1). Cluster I, comprised of 15 *Dendrobium* species of section *Dendrobium*. Cluster II included 7 species of section *Dendrobium*, *D. salaccens* of section *Grastidium* and *D. hainanense* of section *Strongyle*, *D. christyantum* and *D. cariniferum* of the section *Formosae*, *D. thyrsiflorum*, *D. densiflorum* and *D. chrysotoxum* of the section *Chrysotoxae*, *D. ellipsophyllum* of the section *Distichophyllum* closely grouped together with 99 % BP. *D. acinaciform* of section *Aporum* was grouped in cluster III. *D. devonianum* and *D. crepidatum* of section Dendrobium was grouped in cluster IV. *D. hercoglossum* of section *Breviflores* was grouped in cluster V. *D. jenkinsii* of section *Chrysotoxae* was grouped in cluster VI. Based on the dendrogram, two outgroups, *E. amplum* and *E. nakaharaei*, were shown to be divergent from *Dendrobium* species. *D. moulmeinense* is a little known species from Yunnan province of China and isn't grouped in the classification of *Dendrobium* by Tsi *et al*. (1999). Therefore, our results do not support *D. moulmeinense* being placed in the section *Dendrobium*. Based on the classification of *Dendrobium* proposed by Tsi *et al*. (1999), the 35 *Dendrobium* species in this study should be grouped into eight sections, namely *Dendrobium*, *Grastidium*, *Chrysotoxae*, *Distichophyllum*, *Breviflores*, *Formosae*, *Aporum* and *Strongyle*. The phylogenetic tree of *Dendrobium* species deduced from the ITS sequence, however, does not completely match the classification based on morphological characters (Tsi *et al*. 1999). Our findings are consistent with the findings of Tsai *et al*. (2004). *E. nakaharaei* should be placed in the genus *Epigeneium* and not being placed in the genus *Dendrobium*.

ITS region are specific and the ITS sequences of ribosomal DNA has been used for the analysis of diversity (Christopher *et al*. 2005), identification of varieties/ species (Lau *et al*. 2001), phylogenetic relationship (Clements *et al*. 2002) and conservation and management

of genetic resources (Adams *et al*. 2006). More data, especially more sources of evidence are required which can be formalized for *Dendrobium* (Van den Berg *et al*. 2005, Ould *et al*. 2007, Dikshit *et al*. 2007). Our further work will focus on sampling more species throughout their range worldwide; comparing the results of the molecular phylogenetic analyses based on more sequence data with available morphological evidence; and employing sequences of more conserved gene.

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