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Application of mitochondrial nad 1 intron 2 sequences to molecular identification of some species of *Dendrobium* Sw.

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Abstract: Objective Application of a new molecular marker to the identification of *Dendrobium* (Orchidaceae) species. **Methods** Complete sequences of the mitochondrial nad 1 intron 2 for nine species of *Dendrobium* Sw. were amplified and determined. **Results** Seventeen variable sites were found in the aligned 872 bp of nad 1 intron 2 sequences. Eight of the nine *Dendrobium* species except *D. loddigesii* could be identified by the nad 1 intron 2 sequences. **Conclusion** The mitochondrial nad 1 intron 2 sequences could be used as a new molecular marker for the identification of *Dendrobium* species.

Key words: *Dendrobium* Sw.; mitochondrial nad 1 intron 2; molecular marker

线粒体 nad 1 内含子 2 序列在石斛属植物分子鉴定中的应用

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摘 要: 目的 在兰科石斛属药用植物鉴定中应用新的分子标记。**方法** 扩增并测定 9 种石斛属植物线粒体中 NADH 脱氢酶亚基 1 编码基因(nad 1)内含子 2(intron 2)的全长序列。**结果** 比对后的 nad 1 intron 2 序列长 872 bp, 其中有 17 个变异位点, 可以鉴别除粉花石斛 *Dendrobium loddigesii* 以外的 8 种植物。**结论** 线粒体 nad 1 intron 2 序列可以作为一种新的分子标记用于石斛属植物的鉴定。

关键词: 石斛属; 线粒体 nad 1 intron 2; 分子标记

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The Chinese crude drug "Shihu" is derived from the dried or fresh stems of *Dendrobium* species (Orchidaceae). As a precious traditional Chinese medicine with great demand, substitutes or adulterants made up of "Shihu" are found in markets and used both clinically and in Chinese patent medicine factories. According to the *Chinese Pharmacopoeia* (2000 ed), only five species are listed as the botanical origin of qualified "Shihu", i. e. *D. loddigesii* Rolfe, *D. fimbriatum* Hook, *D. chrysanthum* Wall. ex Lindl, *D. officinale* Kimura et Migo, and *D. nobile* Lindl. The spiral or spring shaped commodity made up of the stems of *D. officinale* is known as "Tiepi Fengdou", while the commodities made up of the stems of the other four are known as "Huangcao". Both "Tiepi Fengdou" and "Huangcao" are subject to substitutes or adulterants[1], which had been authenticated based on ITS region of nuclear ribosomal genome, respectively[2,3], matK gene sequences of chloroplast genome[4], and RAPD technique[5].

Mitochondrial DNA (mtDNA) variation has been successfully used in the studies of phylogeography, population subdivision, and behavior of animal species, or human origin and migration. Yet mtDNA is rarely applied to phylogenetic or population genetic studies of plants. Unlike to animals, plant mtDNA shows low nucleotide substitutions rate and frequent structural rearrangements. However, there are a few cases in which

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plant mtDNA markers have been useful in resolving phylogenetic relationship and molecular genetic identification of closely related species, in detecting phylogeogra-phic patterns or in unraveling population genetic structure[6]. In most of these studies, the second intron of the mitochondrial NADH dehydrogenase subunit 1 (nad 1) gene has been shown to be useful in revealing intra-specific polymorphisms[7,8]. The nad 1 gene is fragmented into five single-copy coding segments that are scattered over at least 40 kb. The five nad 1 coding segments are designated nad 1a—nad 1e in wheat mitochondria[9]. Restriction mapping and Southern blot analysis have demonstrated that nad 1a is at minimum of 20 kb away from nad 1b. Moreover, at least two genes, namely atp 6 and rps 13, are located between them. The nad 1c/d and nad 1d/e segments are estimated to be separated by at least 7 and 12 kb, respectively. The nad 1b and nad 1c segments are separated by 1 422 bp in wheat mitochondria, which is the nearest distance. The nad 1 intron 2 is located exactly between nad 1b and nad 1c. Up to now, nad 1 intron 2 sequences have been applied to neither phylogenetic relationship studies nor identification of medicinal plants. Furthermore, no intron 2 sequences of *Dendrobium* species have been reported so far. The purpose of the present study is to detect sequence variation of nad 1 intron 2 in nine *Dendrobium* species which are used as the origin of "Huangcao" in markets.

1 Materials

All materials were collected from different places in China (Table 1). Due to the restriction in collecting wild *Dendrobium* species, each species was tested on two plants from only one collection, except *D. nobile*, *D. chrysanthum*, and *D. loddigesii* which were represented by two different collections. The voucher specimens were identified by Dr. XU Hong and Dr. DING Xiao-yu and deposited in the Herbarium of China Pharmaceutical University, Nanjing, China.

10×PCR Reaction buffer, 25 mmol/L $MgCl_2$, *Taq* DNA polymerase (Promega), dNTPs Mix (Sangon), primers (synthesized by Shanghai Boya Bioengineering), DNA Purification Kit (Shanghai Watson Bioengineering); PTC—200 Thermocycler (MJ Research), ABI Prism 310 Genetic Analyzer.

Table 1 Taxa included in this study and GenBank accession numbers of nad 1 intron 2 sequences

Names of species	Habitats	Specimen voucher	GenBank accession numbers of nad 1 intron 2 sequences
D. williamsonii	Yunnan	Yunnan99001	AY441760
D. aphyllum	Yunnan	Yunnan99002	AY441761
D. nobile	Lijiang, Yunnan	Yun-99055	AY441762
D. nobile	Xishuangbanna, Yunnan	Yunnan99003	
D. chrysanthum	Yunnan	Yunnan99004	AY441763
D. chrysanthum	Guangxi	Guangxi20001	AY484503
D. crepidatum	Yunnan	Yunnan99005	AY441764
D. loddigesii	Wenshan, Yunnan	Yunnan99006	AY441765
D. loddigesii	Hainan	Hainan99007	AY484504
D. thyrsiflorum	Xishuangbanna, Yunnan	Yunnan99008	AY441766
D. crystallinum	Xishuangbanna, Yunnan	Yunnan99009	AY441767
D. primulinum	Xishuangbanna, Yunnan	Yunnan99010	AY441768

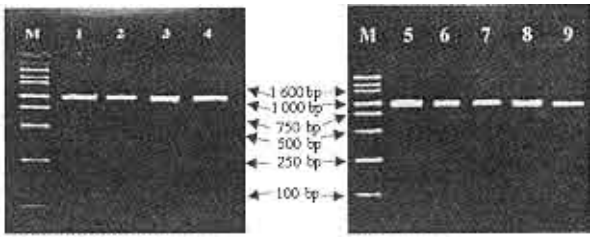
2 Methods

Total genomic DNA was extracted from fresh leaf and stem by a protocol modified from Rogers' method. Amplification of nad 1 intron 2 was carried out in 30 μL reaction volumes containing 3 μL 10 × PCR reaction buffer, 1. 8 μL 25 mmol/L $MgCl_2$, 2 μL 2 mmol/L dNTPs Mix, 1. 0 unit of *Taq* DNA polymerase, 1 μL 10 μmol/L forward and reverse primers and approximately 70—80 ng of total DNA. The primers used for amplification of nad 1 intron 2 were located in nad 1b and nad 1c respectively, which are P-1 forward primer (5′-GCA TTA CGA TCT GCA GCT CA-3′) and P-2 reverse primer (5′-GGA GCT CGA TTA GTT TCT GC-3′)[10]. The profile for the cycles of amplification was: an initial 4 min at 94 ℃ followed by 30 sec at 94 ℃ for denaturation, 45 sec at 54—57 ℃ for primer annealing and 1 min at 72 ℃ for primer extension, repeated for 30 cycles, and a final extension of 8 min at 72 ℃. PCR reaction was carried out by a PTC-200 Thermocycler. PCR products were purified by means of DNA Purification Kit. DNA products and the purified products were detected by ethidium bromide staining under UV after electrophoresis in 1. 0% agarose gel. According to the manufacturer's instruction, the purified PCR products were sequenced with the BigDye terminator mix on an ABI Prism 310 Genetic Analyzer. Forward and reverse primers were used to sequence all samples. The boundaries

of nad 1 intron 2 were determined by comparison with the corresponding sequence of *Triticum aestivum* L. (Gramineae) retrieved from the GenBank (Accession X57967). The sequences were aligned using Clustal X and analyzed using the program MEGA.

3 Results and discussion

The complete mitochondrial nad 1 intron 2 from nine *Dendrobium* species was amplified and sequenced. The sequences of nad 1 intron 2 range in size from 826 to 862 bp while the G+C content range from 56.0% to 56.7%. The result of agarose gel electrophoresis is shown in Fig. 1. Seventeen variable sites were found in the aligned 872bp sequences (Fig. 2). The divergence and numbers of substitutions between the sequences in pairwise comparisons are shown in Table 2. Although the nad 1 intron 2 is highly conserved (the inter-specific variation ranges from 0.726% to 0.234%), there are some variable sites which could be used as molecular characters to identify most of the species except *D. loddigesii*.



M-2000 DNA Marker 1-*D. williamsonii* 2-*D. aphyllum* 3-*D. nobile* 4-*D. chrysanthum* 5-*D. crepidatum* 6-*D. loddigesii* 7-*D. thyrsiflorum* 8-*D. crystallinum* 9-*D. primulinum*

Fig. 1 Agarose gel electrophoresis of PCR amplified products of mitochondrial nad 1 intron 2 sequences from nine species of *Dendrobium* Sw.

Table 2 Numbers of substitutions (lower triangle) and percent of sequence divergence (upper triangle) for mitochondrial nad 1 intron 2 of nine species in *Dendrobium* Sw. with gaps deleted in each pairwise comparison of sequences (Distance method: nucleotide number of differences and *p*-distance; pairwise deletion)

	D. williamsonii	*D. aphyllum*	*D. nobile*	*D. chrysanthum*	*D. crepidatum*	*D. loddigesii*	*D. thyrsiflorum*	*D. crystallinum*	*D. primulinum*
D. williamsonii	—	0.584	0.584	0.587	0.605	0.350	0.469	0.467	0.350
D. aphyllum	5	—	0.701	0.704	0.726	0.467	0.587	0.584	0.467
D. nobile	5	6	—	0.469	0.484	0.467	0.587	0.584	0.467
D. chrysanthum	5	6	4	—	0.363	0.469	0.590	0.587	0.469
D. crepidatum	5	6	4	3	—	0.242	0.608	0.605	0.484
D. loddigesii	3	4	4	4	2	—	0.352	0.350	0.234
D. thyrsiflorum	4	5	5	5	5	3	—	0.469	0.352
D. crystallinum	4	5	5	5	5	3	4	—	0.350
D. primulinum	3	4	4	4	4	2	3	3	—

	1122233346	6666777
	4645539941	2358368
	2790172818	9839669
D. williamsonii	TCGTAGAACC	AACGCCC
D. aphyllum	......G.T.	CG..A..
D. nobile	....C.G..T	.GT....
D. chrysanthum	.G..C.GC..	.G.....
D. crepidatum	C-..C.G...	.G...T.
D. loddigesii	......G...	.G...T.
D. thyrsiflorum	..TG..G...	.G.....
D. crystallinum	.....TG...	.G....T
D. primulinum	......G...	.G.C...

A dot indicates identity, a dash indicates a deletion, and asterisks indicate parsimony-informative sites.

Fig. 2 Variable sites of mitochondrial nad 1 intron 2 sequences from nine species of *Dendrobium* Sw. (Taxa names are shown on the left)

Authentic variable sites has not been found in the nad 1 intron 2 sequence of *D. loddigesii*. The point mutation G→A (position 392, 638) is recognized as the molecular marker for *D. williamsonii*. *D. aphyllum* has an authentic variable site at position 441 (T substituted for C) and two at position 629 (C substituted for A) and 736 (A substituted for C). The authentic variable site for the other six species are demonstrated in Table 3. Therefore,

Table 3 Molecular characters of mitochondrial nad 1 intron 2 sequences for identification of nine species of *Dendrobium* Sw. except *D. loddigesii*

	Position from 5′ end of nad 1 gene intron 2	Base of identified species	Base of other species
D. williamsonii	392	A	G
	638	A	G
D. aphyllum	441	T	C
	629	C	A
	736	A	C
D. nobile	618	T	C
	653	T	C
D. chrysanthum	167	G	C or-
	398	C	A
D. crepidatum	142	C	T
	167	—	C or G
D. thyrsiflorum	249	T	G
	250	G	T
D. crystallinum	337	T	G
	789	T	C
D. primulinum	689	C	G

such markers (SNPs) can be found in the mitochondrial nad 1 intron 2 sequences and used as a new molecular marker for the identification of *Dendrobium* species.

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利用浸苗法将野生天麻总 DNA 导入马铃薯的研究

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摘 要:目的 研究野生天麻总 DNA 对马铃薯的转化, 分析马铃薯转化植株中野生天麻的药用成分天麻素。**方法** 采用浸苗法将野生天麻总 DNA 导入马铃薯试管苗, 通过紫外扫描法、PCR 扩增筛选转化植株, 对转化植株进行 SDS-聚丙烯酰胺凝胶电泳(SDS—PAGE)蛋白分析, 通过 TLC 法检测天麻素。**结果** (1)在 200 株转化的马铃薯中有 21 株的紫外扫描图谱与正常对照组有显著差异, 且在 220 nm 有明显吸收峰。(2)5 株经 PCR 扩增出野生天麻抗真菌蛋白(GAFP)基因。(3)转基因马铃薯与正常马铃薯的蛋白表达有明显差异, 并且在转基因马铃薯中有一条与 GAFP 相同的条带。而正常马铃薯中无此条带。(4)通过薄层色谱法检测出 3 株转基因马铃薯表达野生天麻的有效药用成分天麻素。**结论** 采用浸苗法进行外源总 DNA 导入是可行的。

关键词: 野生天麻; 马铃薯; 浸苗法; 天麻素

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A genetic transformation study of introducing wild *Gastrodia elata* DNA into *Solanum tuberosum* by soaking seedling method

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Abstract: Objective Using the soaking seedling method to introduce wild *Gastrodia elata* DNA into potato plantlets and analyze gastrodin of transformed *Solanum tuberosum*. **Methods** After the tuber grown up, the solution of gastrodin was extracted from the potatoes which were transformed by wild *G. elata* DNA. The transformed plants were scaned by ultraviolet. PCR was used to analyze GAFP gene by SDS-PAGE. TLC was used to analyze the gastrodin of transformed *S. tuberosum*. **Results** (1)The 21

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