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Research Article

Use of DNA barcode *sensu lato* to identify traditional Tibetan medicinal plant *Gentianopsis paludosa* (Gentianaceae)

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Abstract *Gentianopsis paludosa* (Hook. f.) Ma (Gentianaceae) is an important species in Tibetan folk medicine commonly used to clear away the "heat evils" and toxic materials. A survey of market samples revealed that nine adulterant species, *Gentianopsis barbata*, *G. contorta, G. grandis, Halenia elliptica, Lomatogonium macranthum, L. rotatum, Swertia angustifolia, S. bifolia* and *S. erythrosticta,* are often marketed as *G. paludosa.* Methods to distinguish *G. paludosa* from its adulterants are limited by current morphological and chemical methods. DNA sequence analysis of the nuclear ribosomal DNA internal transcribed spacer region (ITS) was used in the differentiation of *G. paludosa* from the adulterant species. The data showed that the internal transcribed spacer regions differ significantly between *G. paludosa* and all nine adulterant species, so that they could be easily distinguished at the DNA level.

Key words Gentianaceae, *Gentianopsis paludosa*, identification, nrDNA ITS, Tibetan medicine.

Gentianopsis paludosa (Hook. f.) Ma (Gentianaceae), also known as "shi sheng bian lei", is an annually growing herb in bosk, meadow, and damp hillsides at an altitude of 2500–4500 m. The plant is distributed in China, India, Nepal, and Bhutan (Ho et al., 1988). In China, it grows in the northwest region and is called "ji he di" by local people (Yang, 1991). The whole herb of *G. paludosa* has long been used in traditional Tibetan folk medicine for benefiting the liver, clearing away the "heat evils" and toxic materials (Guo, 1987; Yang, 1991). Its main function in indigenous medicine is for the treatment of conjunctivitis, hypertension, haemorrhoids, hepatitis, nephritis, gastroenteritis, dyspepsia, fever, influenza, and diarrhoea (Guo, 1987). Phytochemical studies showed that *G. paludosa* contained xanthones, terpenoids, and flavonoids (Zhang et al., 1980; Wang et al., 2004, 2005). However, according to our investigation, there are at least nine adulterants on the market bearing the "shi sheng bian lei" commodity name: *Gentianopsis barbata* (Froel.) Ma, *Gentianopsis contorta* (Royle) Ma, *Gentianopsis grandis* (H. Smith) Ma, *Halenia elliptica* D. Don, *Lomatogonium macranthum* (Diels & Gilg) Fern., *Lomatogonium rotatum* (L.) Fries ex Nym., *Swertia angustifolia* Buch.-Ham ex D. Don, *Swertia bifolia* Batal., and *Swertia erythrosticta* Maxim. This widespread adulteration is causing serious confusion in the identification and variation in the quality of this medicinal material. Although these species differ in quality and effect, they share similar morphology and anatomy, for example, the inflorescence patterns and flower features (Ho et al., 1988). Identification of plant at the species level is traditionally achieved by careful examination of the specimen's macroscopic and microscopic morphology. This work usually needs to be carried out by a specially trained expert. Furthermore, many commercial products are sold either in dried forms or in processed material, rendering their authentication by morphological methods very difficult, if not impossible. Therefore, it is very difficult to distinguish *G. paludosa* from these adulterant species using morphological or histological characteristics.

DNA barcoding, taxon identification using a standardized DNA region, has received much attention recently. Based on different priorities given to the criteria used for designing the molecular markers, DNA barcoding has been derived from two approaches, DNA barcoding *sensu stricto* and DNA barcoding *sensu lato*. DNA barcoding *sensu stricto* corresponds to the identification of the species level using a standardized DNA fragment. The marker should be variable, standardized, and phylogenetically informative. This definition fit with the view of the Consortium for the Barcode of Life (CBOL, 2009). The definition of DNA barcoding *sensu lato* is much less restrictive. It corresponds to the identification of any taxonomic level using any DNA fragment. The marker should be extremely robust and short (Taberlet et al., 2007). DNA barcoding *sensu lato*, or

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Table 1 List of plant material used in this study

Taxon	Voucher	GenBank accession numbers
Gentianopsis barbata (Froel.) Ma	XCHY 2003108	AF346007
G. contorta (Royle) Ma	XCHY 2003123	AJ318539 AJ410318
G. grandis (H. Smith) Ma	XCHY 2001018	DQ317511 DQ317512
G. paludosa (Hook. f.) Ma	XCHY 2001019	Z48106 Z48131
Halenia elliptica D. Don	XCHY 2003005	AF346012
Lomatogonium macranthum (Diels & Gilg) Fern.	XCHY 2003045	AF346011
L. rotatum (L.) Fries ex Nym	XCHY 2003056	AF346010
Swertia angustifolia Buch.-Ham ex D. Don	XCHY 2001013	AJ318551 AJ410330
S. bifolia Batal.	Exp. Qingzang 705	DQ317490
S. erythrosticta Maxim	XCHY 2001003	AF255914

DNA-based taxon identification using diverse techniques, can be useful for scientists from special fields, such as traditional Chinese medicine, forensic science, and food industries.

In this study, the internal transcribed spacer (ITS) regions of *G. paludosa* and its nine adulterant species were sequenced and compared to explore the possibility of using them to differentiate these species.

1 Material and methods

1.1 Source of samples

Gentianopsis paludosa and nine adulterants were collected in Qinghai and Yunnan provinces of China. Table 1 lists all taxa included in this study, together with voucher information and GenBank accession numbers for ITS sequences. The plant specimens in this study were identified by the authors, and voucher specimens were deposited in the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

1.2 DNA extraction, polymerase chain reaction amplification and sequencing

DNAs were extracted using the CTAB method of Doyle & Doyle (Doyle & Doyle, 1987). For the whole ITS region amplification, the primers ITS4 and ITS5 were used (White et al., 1990). The polymerase chain reaction (PCR) reaction volumes (20 μL) contained 1.5 U of AmpliTaq DNA polymerase (TaKaRa Biotechnology, Dalian, China), and the amplification reactions were carried out in a GeneAmp 9600 thermal cycler (Perkin-Elmer 9600; Applied Biosystems, Foster City, CA, USA). The basic PCR programs were 3 min at 97°C, followed by 35 cycles of 45 s at 94°C, 30 s at 55°C, and 45 s at 72°C, with a final elongation of 7 min at 72°C. Double-stranded products were purified using a Watson (Shanghai, China) PCR Purification Kit. Sequencing reactions were carried out using an ABI PRISM Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems).The products of the sequencing reaction were electrophoretically separated on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Each sample was sequenced in both directions, and the sequencing was repeated three times.

1.3 Sequence analysis

The DNA sequences were compared and aligned using the programs MegAlign (DNAStar) (http://www.dnastar.com/t-sub-products-lasergene-megalign.aspx) and Mega 3.1 (http://www.megasoftware.net), and further verified by comparison with the sequences of other species in the genus *Gentianopsis* using a BLAST search at the National Center for Biotechnology Information website (http://www.nibi.nlm.nih.gov).

2 Results

The length of the ITS (ITS1 and ITS2) sequences in the 10 species ranged from 453 to 459 bp, giving an aligned matrix of 465 characters (Fig. 1). Of these, 330 sites were constant, 72 were variable, and 63 (19.10%) were parsimony informative. *Gentianopsis paludosa* had a 454-base sequence, and the percentage of G + C was 59.0% (Table 2). The pairwise distances between samples were estimated. The nucleotide sequence divergence between different populations of the same species was zero for *G. paludosa*, clearly showing that the ITS sequences of *G. paludosa* are homologous regardless of its geographical origin. *Gentianopsis paludosa* displayed sequence divergences at 73–58 positions from the six non-*Gentianopsis* species and 19–30 positions from the four *Gentianopsis* species. There is a much larger sequences divergence of 73 positions between *G. paludosa* and *Halenia elliptica*, and a small one of 19 positions between *G. paludosa* and *G. barbata*. Within the genus *Gentianopsis*, there is a small sequence divergence of 12 positions between *G. barbata* and *G. grandis*, and a much larger one of 30 positions between

Fig. 1. Aligned sequences of ribosomal DNA internal transcribed spacers from *Gentianopsis paludosa* and nine adulterant species in the region. Dashes are gaps required for alignment.

G. grandis and *G. contorta* (Table 3). The inter-species nucleotide sequence divergence between *G. paludosa* and the nine adulterant species therefore ranged from 19 to 73 positions, which is much larger than the intra-species variation of zero. On this basis the ITS (ITS1 and ITS2) region of *G. paludosa* could be adopted as a molecular marker for the accurate identification of this species from the adulterant species.

Table 2 Sequence characteristics of the internal transcribed spacer (ITS) regions of the studied species

Comparison	ITS
Length range (bp)	453–459
Aligned length (bp)	465
G+C content range (%)	57.77–61.66
G+C content mean (%)	59.89
Sequence divergence (%)	0.9–16.7
Number of variable sites (%)	72
Number of constant sites (%)	330
Number of informative sites (%)	63

3 Discussion

The World Wildlife Fund estimates that 80% of the world's population uses traditional medicines for healing and curing diseases (http://www.worldwildlife.org/what/globalmarkets/wildlifetrade/faqs-medicinalplant.html). The increased demand for botanical products is met by an expanding industry and accompanied by calls for assurance of quality, efficacy, and safety (Chen et al., 2010). Plants used as drugs, dietary supplements, and herbal medicines are identified at the species level. Unequivocal identification is a critical step at the beginning of an extensive process of quality assurance and is important for the characterization of genetic diversity, phylogeny, and phylogeography as well as the protection of endangered species (Sucher & Carles, 2008).

Using DNA-based methods, species identification has been achieved using DNA that was isolated from

Table 3 Pairwise distances between the studied species based on internal transcribed spacer regions

	1	2	3	4	5	6	7	8	9	10
1 *Gentianopsis paludosa*	—	0.069	0.044	0.046	0.135	0.133	0.133	0.156	0.151	0.167
2 *G. contorta*	30	—	0.053	0.060	0.128	0.131	0.126	0.158	0.154	0.163
3 *G. barbata*	19	23	—	0.028	0.106	0.103	0.110	0.126	0.122	0.131
4 *G. grandis*	20	26	12	—	0.117	0.115	0.115	0.142	0.138	0.151
5 *Swertia bifolia*	59	56	46	51	—	0.030	0.080	0.096	0.094	0.115
6 *S. erythrosticta*	58	57	45	50	13	—	0.078	0.096	0.094	0.117
7 *S. angustifolia*	58	55	48	50	35	34	—	0.106	0.106	0.131
8 *Lomatogonium macranthum*	68	69	55	62	42	42	46	—	0.009	0.122
9 *L. rotatum*	66	67	53	60	41	41	46	4	—	0.119
10 *Halenia elliptica*	73	71	57	66	50	51	57	53	52	—

Total character differences are below diagonal; mean character differences are above diagonal. —, Same sample has no character differences.

fresh and dried plant parts, plant extracts, processed herbal drugs, as well as finished products such as herbal teas, tablets, and capsules. In addition, DNA barcodes will be a useful and powerful tool for non-professional users such as customs officers, traditional drug producers and managers, and forensic specialists. DNA-based methods can be useful in quickly and efficiently pinpointing adulterated or misidentified raw materials, which can then be discarded without further need for time- and resource-consuming morphological, physical, and phytochemical examinations (Lau & Shaw, 2001; Ding et al., 2002).

The length of the ITS in *Gentianopsis paludosa* is short (456 bp), which advantages the amplification of degraded DNA. The inter-species nucleotide sequence divergence between *G. paludosa* and the nine adulterant species studied ranged from 19 to 73 positions, which is much larger than the intra-species variation of zero. The inter-specific divergence at ITS is higher than the intra-species divergence. Therefore, the ITS region could be potentially used to identify medicinal plant *G. paludosa* and its closely related species. This study agrees that with the exception of 5.8S, the ITS of nuclear ribosomal DNA and regions of the ITS could be potential barcodes (Chase et al., 2005; Kress et al., 2005).

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References

Chase MW, Salamin N, Wilkinson M, Dunwell JM, Kesanakurthi RP. 2005. Land plants and DNA barcodes: Short-term and long-term goals. Philosophical Transactions of the Royal Society B: Biological Sciences 360: 1889–1895.

Chen S-L, Yao H, Han J-P, Liu C, Song J-Y, Shi L-C, Zhu Y-J, Ma X-Y, Gao T, Pang X-H, Luo K, Li Y, Li X-W, Jia X-C, Lin Y-L, Leon C. 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS ONE 5: e8613.

CBOL Plant Working Group. 2009. A DNA barcode for land plants. Proceedings of the National Academy of Sciences USA 106: 12794–12797.

Ding X-Y, Xu L-S, Wang Z-T, Zhou K-Y, Xu H, Wang Y-Q. 2002. Authentication of stems of *Dendrobium officinale* by rDNA ITS region sequences. Planta Medica 68: 191–192.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11–15.

Guo B-Z. 1987. Medicinal Plants of Qinghai Province. Xining: Qinghai People's Publishing House. 458.

Ho T-N, Liu S-W, Wu Q-R. 1988. Gentianaceae. In: Flora Reipublicae Popularis Sinicae. Beijing: Science Press. 62: 290–410.

Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH. 2005. Use of DNA barcodes to identify flowering plants. Proceedings of the National Academy of Sciences USA 102: 8369–8374.

Sucher NJ, Carles MC. 2008. Genome-based approaches to the authentication of medicinal plants. Planta Medica 74: 603–623.

Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A, Vermat T, Corthier G, Brochmann C, Willerslev E. 2007. Power and limitations of the chloroplast *trn*L (UAA) intron for plant DNA barcoding. Nucleic Acids Research 35: e14

Lau T-W, Shaw P-C. 2001. Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of ribosomal DNA. Planta Medica 67: 456–460.

Wang H-D, Tan C-Y, Bai X-F, Du Y-G. 2004. Study on the chemical constituents from the herb of *Gentianopsis paludosa*. China Journal of Chinese Materia Medica 11: 1055–1056.

Wang H-D, Tan C-Y, Bai X-F, Du Y-G, Lin B-C. 2005. Studies on the chemical constituents from the herb of *Gentianopsis paludosa*. Chinese Pharmaceutical Journal 14: 1059–1061.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sninsky J, White T eds. PCR protocols: A guide to methods and application. San Diego: Academic Press. 315–322.

Yang Y-C. 1991. Tibetan medicines. Xingning: Qinghai People's Publishing House. 111.

Zhang B-C, Zhen R-D, Hu B-L. 1980. Study on the chemical constituents from *Gentianopsis paludosa*. Chinese Tradition Herb Drugs 11: 149–151.