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Full Length Research Paper

Identification of the medicinal plant *Dipsacus asperoides* from three other species in genus *Dipsacus* (Dipsaceae) by internal transcribed spacer of ribosomal deoxyribonucleic acid (rDNA ITS)

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The internal transcribed spacer of ribosomal deoxyribonucleic acid (rDNA ITS including ITS1, 5.8S, and ITS2) of six populations of *Dipsacus asperoides* and two adulterants (*Dipsacus japonicus* and *Dipsacus chinensis*), were sequenced by polymerase chain reaction (PCR) products sequencing method and a phylogenetic tree was constructed by neighbor-joining method with Mega 3.1 software. The sequences of rDNA ITS region ranged from 596 to 602 bp. In comparison to the sequences among intraspecies of *D. asperoides*, variation of the rDNA ITS was significant among *D. asperoides* and its adulterants. Together with the reported rDNA ITS regions of *Dipsacus mitis* in GenBank (Accession No. AY236187), total of 122 variable sites and 29 informative sites were present among these interspecies. Notably, 14 of the total 29 informative sites were species-characteristic traits. Such a differentiation in the ITS sequence, could provide molecular markers to distinguish *D. asperoides* from the adulterants at the DNA level and to complement conventional morphological methods. And the phylogenetic analysis was conducted to obtain the information on variation and evolution of the four species. Based on sequence divergence and phylogenetic pattern, the results illustrated that *D. asperoides* was more closely related to *D. mitis* than to *D. chinensis* and to *D. japonicus*.

Key words: *Dipsacus*, *Dipsacus asperoides*, phylogenetic analysis, internal transcribed spacer of ribosomal deoxyribonucleic acid (rDNA ITS).

INTRODUCTION

Dipsacus asperoides distributed chiefly in Yunnan, Hubei, Shanxi, Sichuan and Guizhou Provinces of China, is one of the most medicinally important species in genus *Dipsacus* L. (family Dipsacaceae), whose dried roots, known as Dipsaci Radix or “Xu Duan”, have been used as a source of medicinal herb in traditional Chinese medicines. Dipsaci Radix is used as circulation promoter, haemostatic, miscarriage preventer and for the treatment of ache and weakness of the loins and knees, seminal emission, metrorrhagia, fetal distress, rheumatic

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Dipsacus L. consists of nine species and one variety in (Chinese Pharmacopoeia Commission, 2010). The genus arthralgia, carbuncles and sores, traumatic injuries China (Flora of China Editorial Board, 1986). Although, each of the species can be identified, based on morphological characters, there are several morpholo-gical synapomorphies: setose or pubescent shoots and leaves, terete tap root, a capitulum inflorescence, radial symmetry flowers, bractlets without limbs, and a bicarpellate ovary. As terete form of the tap root as a plastic character, it is difficult to discriminate the medicinal roots from the non-medicinal in a pile of medicinal materials. According to the Pharmacopoeia of People’s Republic of China, tap roots of *D. asperoides* are exclusively used as medicines (Chinese Pharmacopoeia Commission, 2010). Recently, due to an increased demand for Dipsaci Radix, some adulterants from

Dipsacus japonicus Miq. and *Dipsacus chinensis* Bat., with similar morphology in dried roots, were mixed into Dipsaci Radix. Because Dipsaci Radix is quite expensive and frequently adulterated, a reliable method for authentication of putative specimens and the species is needed to protect consumers and to ensure the safe and effective treatment. The similarity among the species may represent an evolutionary convergence; however, no rigorous or explicit phylogenetic analysis has been conducted for the genus *Dipsacus*.

Sequences of the internal transcribed spacer of ribosomal deoxyribonucleic acid (rDNA ITS) have been useful for molecular authentication and phylogenetic construction at the intrageneric and intrafamilial level (Baum et al., 1994; Douzery et al., 1999; Wang et al., 1999; Ding et al., 2002; Le Roy et al., 2002; Crockett et al., 2004). In this study, we employed ITS sequences of the nuclear ribosomal repeat to help authenticate *D. asperoides* from adulterants on molecular level and to complement conventional morphological methods. Specific objectives were to: (1) identify the difference of rDNA sequences between *D. asperoides* and its adulterants in main habitants of China to provide molecular markers against adulterants, and (2) resolve phylogenetic relationship of these species in *Dipsacus*.

MATERIALS AND METHODS

Plant materials

A total of three species of the genus *Dipsacus* was collected in nature from June to August, 2006 (Table 1). Except *D. japonicus*, which was represented by only one collection, *D. asperoides* and *D. chinensis* were test on six and two populations from different geographical area in China, respectively. Fresh leaves from the collected species were stored in ziplock bags with silica gel. Voucher specimens of the collected species were identified by Dr. Wang Zaigui, and reserved in the Herbarium at College of Life Sciences, Anhui Agricultural University, China.

Deoxyribonucleic acid (DNA) extraction

The leaves dried by silica gel were used for DNA exaction. Total DNA was extracted following the cetyltrimethylammonium bromide (CTAB) method of Frederick et al. (1998). Each sample (about 0.2 g) was ground in liquid nitrogen, then, the powder was suspended in extraction buffer (pH 8.0), containing 2% CTAB, 0.2% 2-mercaptoethanol, 1.4 mol/L NaCl, 20 mmol/L ethylene diamine tetraacetic acid (EDTA) and 100 mmol/L Tris-HCl; and was incubated at 65°C for 1 h, followed by extraction with chloroform/isoamyl alcohol. Thereafter, DNA was precipitated with cold isopropanol.

Polymerase chain reaction

DNA amplifications were performed in 100 µl reactions containing approximately 100 ng genomic DNA, 0.15 mM of each deoxynucleotide triphosphate (dNTP), 10 pM of each primer (ITS3, 5'-CGT AAC AAG GTT TCC GTA GGT GAA C-3' and ITS5, 5'-TTA TTG ATA TGC TTA AAC TCA GCG GG-3', designed according to Fushimi et al. (1996), and 2 units of Taq polymerase (Promega). PCR profile included pre-denaturation for 4 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and finally, 10 min at 72°C. The PCR products were purified with DNA Clean-Up Purification Kit (Promega). Sequencing reactions were performed using the purified PCR products by Institute of Crop, Chinese Academy of Agricultural Sciences. All DNA sequences were deposited at GenBank (Table 1 for Accession No.).

Sequence analysis

The DNA sequences obtained were aligned and consensus sequences were constructed using Clustal X software. The boundaries between the coding and spacer regions were determined by comparing with the sequence of *Dipsacus mitis* (GenBank Accession No. AY236187) in *Dipsacus*. The consensus sequences were exported to Mega 3.1 software. Sequence divergence values were computed by the Kimura Two-Parameter method using the Compute Pairwise program of Mega 3.1. A neighbor-joining tree was constructed from the sequence divergence using the Bootstrap test of phylogeny program of Mega 3.1.

RESULTS

Characteristics of internal transcribed spacer (ITS) sequences of Dipsacus

ITS sequences of four *Dipsacus* species were shown to be significantly different from one another. The total length of ITS1, 5.8S and ITS2 regions of four species in *Dipsacus* ranged from 596 to 602 bases, with an ITS1 of 220 to 227 bases, a 5.8S of 164 bases, and an ITS2 of 209 to 212 bases (Table 2). Of the aligned positions from ITS1, 5.8S and ITS2 regions of the ten samples representing four of the *Dipsacus* species, 122 sites were variable, 29 of which were phylogenetically informative. As most mutations were base substitutions, only nine deletions, each consisting of one nucleotide, were inferred in the aligned sequences with five in ITS1 and four in ITS2. The distribution of informative sites in ITS1 and ITS2 was 14 and 15, respectively (Tables 3 and 4). Notably, 14 of these informative positions, including sites 65, 80, 86, 123,136, 149, 154, 201, 204, 210, 409, 447, 456 and 583 (Tables 3 and 4, Figure 1), were species-characteristic traits and could be used to differentiate *D. asperoides* from three other species in genus *Dipsacus*. On the other hand, when six intraspecific samples of *D. asperoides* were aligned, there presented 10 variable sites, but no informative sites, among which there were only 1 to 2 variable sites in two samples of *D. asperoides*, collected at nearby the same area, that is, ASP2 and ASP3, and ASP4 and ASP5.

Phylogenetic analyses

As shown in Table 5, sequence divergences among four

Table 1. Accessions of *Dipsacus* sampled for the ITS studies.

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Species	Abbreviation	Voucher	Geographical origin	GenBank accession No.
D. asperoides	ASP1	D06014	Dali, Yunnan, China	EU925563
D. asperoides	ASP2	D06004	Lijiang, Yunnan, China	EU925564
D. asperoides	ASP3	D06005	Lijiang, Yunnan, China	EU925565
D. asperoides	ASP4	D06025	Shangri-la, Yunnan, China	EU925566
D. asperoides	ASP5	D06029	Shangri-la, Yunnan, China	EU925567
D. asperoides	ASP6	D06036	Yichang, Hubei, China	EU925568
D. chinensis	CHI1	D06001	Lijiang, Yunnan, China	EU925569
D. chinensis	CHI2	D06027	Shangri-la, Yunnan, China	EU925570
D. japonicus	JAP	D06083	Yanqing, Beijing, China	EU925571
*D. mitis**	MIT			AY236187

*, The ITS sequence of *D. mitis* was screened from GenBank.

Table 2. Sequence sizes and percent G+C content of ITS regions from *Dipsacus*.

Taxa	ITS1 (bp)	5.8S (bp)	ITS2 (bp)	Total ITS (bp)	G+C (%)
ASP1	222	164	212	598	63.2
ASP2	220	164	212	596	62.9
ASP3	220	164	212	596	63.1
ASP4	223	164	212	599	63.1
ASP5	224	164	212	600	63.2
ASP6	222	164	212	598	62.6
CHI1	224	164	211	599	63.7
CHI2	225	164	212	601	63.9
JAP	226	164	212	602	64.4
MIT	227	164	209	600	63.5

Note: Species abbreviations are given in Table 1.

Table 3. Parsimony-informative sites in ITS1 regions from 10 samples of *Dipsacus*.

Sites	46	47	48	65	80	86	123	136	148	149	154	201	204	210
ASP1	G	A	A	A	A	A	T	C	G	A	A	A	A	T
ASP2	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ASP3	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ASP4	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ASP5	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ASP6	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CHI1	A	T	C	G	C	G	C	A	T	G	G	G	T	C
CHI2	A	T	C	G	C	G	C	A	T	G	G	G	T	C
JAP	*	*	*	G	C	G	C	A	*	G	G	G	T	C
MIT	A	T	C	G	C	G	C	A	T	G	G	G	T	C

Note: Species abbreviations are given in Table 1. Asterisk (*) indicates the same base composition as SP1 sequence.

of the *Dipsacus* species were estimated to range from 0.0438 to 0.1686 and averaging 0.0962. The highest divergence was between *D. japonicus* and *D. chinensis*. The lowest values were between *D. mitis* and *D. asperoides*. *D. japonicus* was highly divergent from the rest of the species within *Dipsacus*, with divergence values ranging from 0.1353 to 0.1686, with an average of 0.1540. Apart from *D. japonicus* and *D. mitis*, which were

Table 4. Parsimony-informative sites in ITS2 regions from 10 samples of *Dipsacus*.

Sites	390	395	409	421	446	447	456	461	504	507	532	540	552	583	589
ASP1	G	G	C	G	T	A	T	C	T	T	T	C	A	T	G
ASP2	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*
ASP3	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*
ASP4	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*
ASP5	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*
ASP6	*	T	*	T	*	*	*	*	*	*	*	*	G	*	T
CHI1	C	C	T	*	G	G	C	T	G	G	C	T	G	C	T
CHI2	C	C	A	*	G	G	C	T	G	G	C	A	G	C	T
JAP	T	*	A	C	C	G	C	G	A	G	A	*	G	C	T
MIT	*	*	A	T	*	G	C	*	*	*	*	A	G	C	*

Note: Species abbreviations are given in Table 1. Asterisk (*) indicates the same base composition as ASP1 sequence.

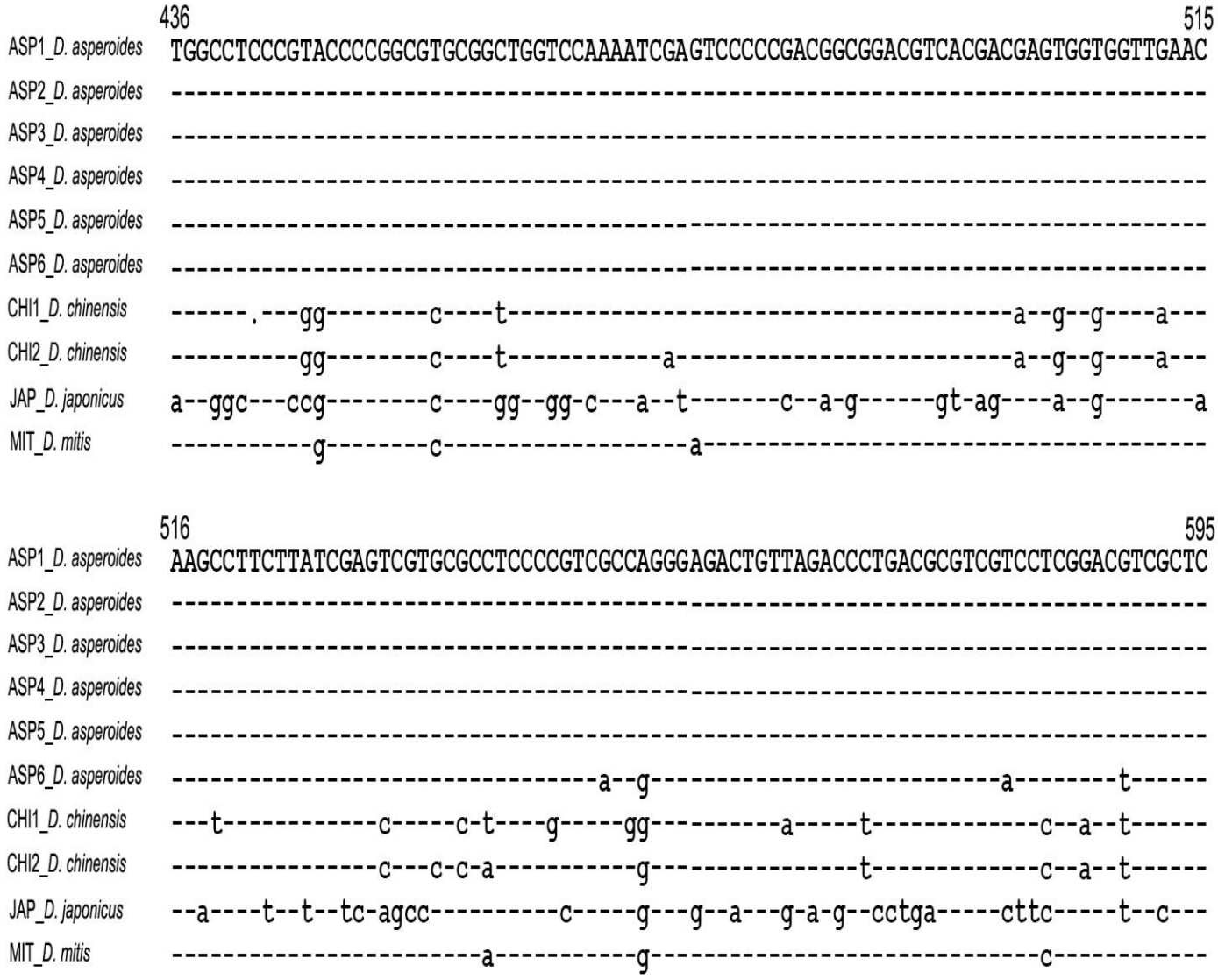

Figure 1. Alignment of partial DNA sequences of ITS2 regions. Numbers above sequences indicated ITS of *D. asperoides* (ASP1) from position 436 to 595. Base substitutions at sites 447, 456 and 583, presented in lower case, could provide molecular markers to differentiate *D. chinensis*, *D. japonicus* and *D. mitis* from *D. asperoides*.

Table 5. Kimura Two-Parameter sequence divergences of ITS regions from *Dipsacus*.

	ASP1	ASP2	ASP3	ASP4	ASP5	ASP6	CHI1	CHI2	JAP	MIT
ASP1	—									
ASP2	0.0102	—								
ASP3	0.0085	0.0017	—							
ASP4	0.0034	0.0102	0.0085	—						
ASP5	0.0034	0.0102	0.0085	0.0000	—					
ASP6	0.0120	0.0154	0.0171	0.0120	0.0120	—				
CHI1	0.0941	0.0979	0.0999	0.0940	0.0940	0.0941	—			
CHI2	0.0807	0.0844	0.0864	0.0806	0.0806	0.0807	0.0224	—		
JAP	0.1519	0.1603	0.1581	0.1498	0.1498	0.1540	0.1686	0.1580	—	
MIT	0.0474	0.0528	0.0510	0.0438	0.0438	0.0528	0.0674	0.0509	0.1353	—

Note: Species abbreviations are given in Table 1.

represented by only one sample, it appeared to be less divergent from the six populations of *D. asperoides*, with divergence values ranging from 0.0000 to 0.0171%, averaging 0.0089. The sequence divergence is also low in two populations of *D. chinensis*, with divergence value of 0.0224.

The neighbor-joining analysis produced a tree (Figure 2), by which the relationship among the four *Dipsacus* species became resolved. The Bootstrap analysis showed support (Bootstrap value more than 60%) for several clades (Figure 2). All six populations of *D. asperoides* were clearly clustered into one group. One population of *D. asperoides* from Hubei was sister to those from Yunnan, which was supported by Bootstrap value of 100%. In the neighbor-joining tree (Figure 2), *D. mitis* was suggested to form a sister clade to the group of *D. asperoides*, while *D. japonicus* was basal within the tree.

DISCUSSION

The ITS region has been widely applied in authentication analysis of Chinese Traditional Medicines (Fushimi et al., 1996; Wang et al., 1999; Ding et al., 2002). The ITS sequences of the four species in *Dipsacus* were shown to be significantly different from one another by an average of 0.0962. The difference by base substitutions and insertion-deletion in the ITS data set is big enough to differentiate the medicinal *D. asperoides* from the non-medicinal species, which match the classification based on morphological characters. Therefore, the ITS region of the *Dipsacus* species may be adopted as a molecular marker for accurate identification of these species. Compared with those in ITS2 with 4 of the total 29, there are more informative sites in ITS1 of the four species with 10 of the total 29 (Tables 3 and 4), which were species-characteristic traits and could provide an important molecular marker for differentiation of *D. asperoides* from the three other species in genus *Dipsacus*. Alternatively, it should be likely that the specific primers corresponding to conserved regions of 5.8S and 18S genes and flank to ITS1 could be used to amplify the region for subsequent analysis. Moreover, our results indicate that the interspecific sequence divergences among the four *Dipsacus* species, which range from 0.0438 to 0.1686, are much more significant than the intraspecific variation among six populations of *D. asperoides* or two of *D. chinensis*. Together with the neighbor-joining tree, the sequence divergences further suggest that the intraspecies of *D. asperoides*, which have close sister relationship, always distribute in near geographical area.

Scientific studies have shown that ITS regions can be used to infer phylogeny among closely related taxa and to identify species or strains (Baum et al., 1994; Douzery et al., 1999; Le Roy et al., 2002; Crockett et al., 2004;). Wendel et al. (1995) cautioned about the potential misleading phylogenetic reconstruction from rDNA sequences due to the bi-directional interlocus concerted evolution after hybridization and polyploidization (for example, allopolyploidy). All trees constructed with algorithms that assume dichotomous evolution will be potentially misleading in groups that have experienced reticulate evolution. At present, there are no data available concerning the types of polyploid (allo- versus auto-) in *Dipsacus*. We, therefore, plan to test our ITS phylogeny with classification derived from morphological characters.

The neighbor-joining tree showed that *D. mitis* was sister to the clades of *D. asperoides* samples. Morphologically, it aligns well with *D. asperoides* with the following synapomorphies: tri- to quinquepartite or pinnately parted leaves, capitulum less than 4 cm in diameter, and white or cream flowers (Flora of China Editorial Board, 1986). These results might indicate that *D. mitis* is closely related to *D. asperoides* on phylogenetic development.

Phylogenetic position of *D. chinensis* was positioned between the clade of *D. japonicus* and the clade consisting of *D. mitis* and *D. asperoides*. The average ITS

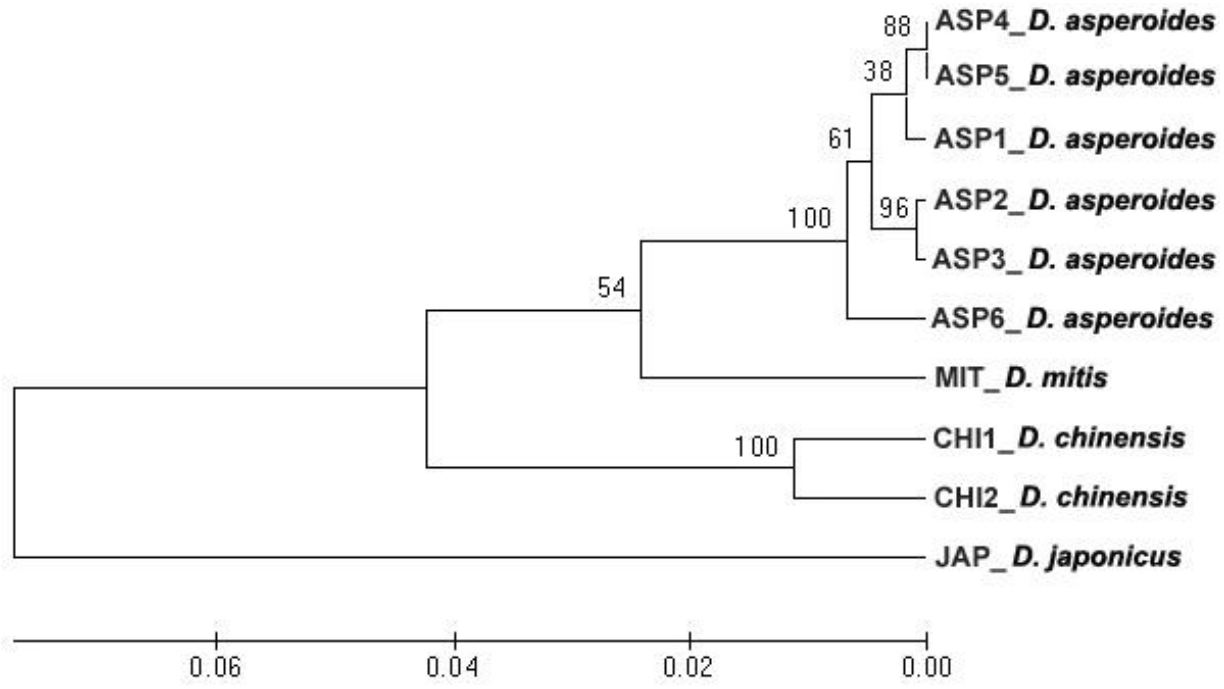

Figure 2. The neighbor-joining tree of *Dipsacus* constructed from the entire ITS sequences. Numbers above lines are the bootstrap values in 1000 replicates.

sequence divergence between *D. chinensis* and *D. japonicus* was greater than that between *D. chinensis* and the other species of the genus. Interestingly, *D. chinensis* is morphologically quite distinct from both *D. japonicus* and *D. asperoides*. Morphologically, *D. chinensis* is characterized by the presence of pinnately divided leaves, and capitulum more than 4 cm in diameter (Flora of China Editorial Board, 1986). By morphological characters, the exact phylogenetic position of *D. chinensis* has not been resolved. With the neighbor-joining tree, it can be inferred that *D. chinensis* is closely related to the *D. mitis* and *D. asperoides* clade. On the other hand, morphologically with lignified root and purplish red flowers (Flora of China Editorial Board, 1986), *D. japonicus* is cladistically basal species within the ITS clades of the genus, indicating that it is very distinct from the rest of species of the genus *Dipsacus* in terms of its ITS sequence as well as morphology.

The low ITS sequence divergence and high level of morphological similarities between *D. mitis* and *D. asperoides* suggest that they may have diverged recently. In contrast, a high level of ITS sequence divergence was observed between *D. japonicus* and *D. asperoides*, which suggested a relatively early divergence.

In conclusion, the present results illustrate that the sequence of ITS region can group the medicinal species (*D. asperoides*), the non-medicinal species (*D. mitis*, *D. chinensis* and *D. japonicus*) into four distinct clades in good agreement with the traditional classification. Analysis of the concerned sequences has provided defined molecular markers for authentication and for assessing the phylogeny of these *Dipsacus* species.

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