Journal of Medicinal Plants Research Vol. 4(19), pp. 1948-1953, 4 October, 2010
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR10.364
ISSN 1996-0875 ©2010 Academic Journals

Full Length Research Paper

Molecular identification of original plants of *Spica prunellae* based on ITS sequence

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Accepted 15 September, 2010

The ITS sequences of the original plants of *Spica prunellae* and its confusable species including 16 individuals belonging to 6 species from 12 localities, were analyzed. The results showed that the length of ITS sequences was 584 bp after alignment and the overhanging terminals were cut. When alignment gaps were treated as missing data, there were 150 variable sites. The percent divergence between species was 10.9%, with a transitions/transversions ratio of 1.53. There were 11 variable and 9 informative sites among the ITS sequences of 4 species in *Prunella* Linn. There were specific sites at 5, 474 and 525 nt of the ITS sequence of *P. grandiflora*. There were 122 specific sites between genus *Ajuga* and genus *Prunella*. Both phylogenetic trees constructed by Maximum parsimony method and Neighbor-Joining method indicated that *Prunella vulgaris* Linn., *P. asiatica* Nakai. and *P. hispida* Benth. had a very close relationship. The character of ITS sequences can be used for providing the molecular markers for identifying Chinese *Prunella* (*S. prunellae*).

Key words: *Prunella vulgaris* Linn., *Prunella asiatica* Nakai, ITS sequence, molecular identification.

INTRODUCTION

According to Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2005), the dried fruit-spike of *Prunella vulgaris* L. (Fam. Labiatae) is used as the crude drug *Spica prunellae* (Common Selfheal Fruit-Spike) in practice. *P. vulgaris* is generally regarded as one of the original species of *S. prunellae* according to Modern Chinese Materia Medica (Xiao, 2002), which mainly distributes in Jiangsu, Anhui, Hubei and Henan provinces. Especially the species from Jiangsu with long spike and short spike-stalk has the best curative effect and is called 'Jing Common Selfheal'. Besides, fruit-spikes of *Prunella asiatica* Nakai and *Prunella hispida* Benth are regarded as other origins of *S. prunellae*.

Wang et al. (1996) found that the three species could be accurately distinguished according to their characters Delectis Florae Reipublicae Popularis Sinicae Agendae of bract, calyx and so on. But in practice it is still not easy to distinguish them. *Ajuga decumbens* Thunb. and *Ajuga multiflora* Bunge from the same family are also used as *S. prunellae* in some areas of China (Xiao, 2002; Academiae Sinicae Edita, 1977a).

Besides the introduced *Prunella grandiflora* (Linn.) Jacq., there are three other *Prunella* species including *P. vulgaris*, *P. asiatica*, and *P. hispida* in China according to *Flora Reipublicae Popularis Sinicae* (Delectis Florae Reipublicae Popularis Sinicae Agendae Academiae Sinicae Edita, 1977b) and *Flora of Chin*a (Wu and Raven, 1994), *Jiangsu Flora* (Jiangsu Institute of Botany, 1982) and *Anhui Flora* (Cooperation Group of the Anhui Flora, 1992) record that *P. vulgaris*, but not *P. asiatica* distributes in Jiangsu and Anhui, whereas *Flora Reipublicae Popularis Sinicae* records that *P. asiatica* instead of *P. vulgaris* distributes in Jiangsu and Anhui. This contradiction indicated that the confusion of distribution and taxonomy of *P. vulgaris* existed, then

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Table 1. List of samples.

Species	Locality	GenBank accession No.
Prunella vulgaris Linn.	Xinyu, Jiangxi	HQ228224
	Hunan	HQ228226
	Yingxiu, Sichuan	HQ228225
	Guiyang, Guizhou	HQ228227
P. asiatica Nakai.	Jurong, Jiangsu	HQ228220
	Panan, Zhejiang	HQ228221
	Wuhu, Anhui	HQ228222
	Xishui, Hubei	HQ228223
P. hispida Benth.	Kunming, Yunnan	HQ228228, HQ228229
P. grandiflora (Linn) Jacq.	Nanjing, Jiangsu	HQ228230, HQ228231
Ajuga decumbens Thunb.	Nanjing, Jiangsu	HQ228232, HQ228233
A. multiflora Bunge.	Jurong, Jiangsu	HQ228234, HQ228235

botanical origins of *S. prunellae* might be mixed and the original species of the genuine medicinal material Jing Common Selfheal were uncertain.

The internal transcriped spacer (ITS) of ribosomal DNA is becoming a useful molecular marker in identification of the origin of medicinal plants in addition to systematic development and classification researches due to its especial characteristics such as high repeatability, rapid mutation, stability under processing conditions (Lau et al., 2001; Zhao et al., 2001; Xu et al., 2006; Shen et al., 2001; Shen et al., 2005; Yu et al., 2003). In this paper, we reported to identify the original plants of *S. prunellae* and their confusable species based on ITS sequence.

MATERIALS AND METHODS

Samples of the 4 *Prunella* species including *P. vulgaris*, *P. asiatica*, *P. hispida* and *P. grandiflora* were collected from different habitats (Table 1); the species were wild except *P. grandiflora*. Voucher specimens were deposited in the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences and were identified based the morphological features according to *Flora of China*.

DNA isolation

Total DNA was extracted by the CTAB method described by Paterson et al. (1993). The extracted DNA was purified using E.Z.N.A.^{TM} Cycle Pure Kit (Beijing Bio-lab Materials Institute) and dissolved in double distilled water and stored at -20°C until use. Purified DNA was subjected to electrophoresis through a 0.8% agarose gel and the concentration was estimated by using an UV lamp.

Amplification and sequencing of the ITS regions

Primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAG G-3') and ITS4 (5'-TCC TCCGCTTATTGATATGC-3') were used for amplification. The optional parameters for 20 µL PCR reaction system contained 2.0 µL 10×PCR buffer, 2.0 µL 25 mmol·L^{-1}$MgCl_2$, 0.3 µL 10 mmol ·L^{-1} dNTP, 0.6 µL 10 µmol·L^{-1} of each primer, 1 U Taq DNA polymerase, and about 5 ng template DNA. PCR was carried out using the following conditions: initial denaturation at 94°C for 3 min; 35 cycles of denaturation (94°C for 45 s), annealing (58°C for 30 s), and extension (72°C for 1 min); and a final extension step at 72°C for 5 min. All amplicons were sequenced on one strand using the primer ITS5 by Shanghai BioAsia Biotechnology Co., Ltd.

Sequence analysis

Sequences were aligned with the program Clustal X (Thompson et al., 1997). Genetic distances were computed with MEGA3.1 (Kumar et al., 2001) using Kimura-two parameter model, and phylogenetic tree of NJ, MP was reconstructed by PAUP 4.0 (Swofford, 1999) with MP (Maximum parsimony) and NJ (Neighbor-Joining) methods respectively. The search algorithm was heuristic, and the reliability of each branch was estimated from bootstrap test (1000 replications). Alignment gaps were treated as missing data.

RESULTS

Sequence features of ITS regions

Because of the inconsistency about the boundary of ITS1, 5.8S and ITS2 regions existed between the *P. vulgaris* ITS (Accession No. AY506653) and *A. decumbens* ITS (Accession No. AF477768), the boundary of ITS1, 5.8S and ITS2 were redefined by the blasting the ITS sequences from *P. frutescens* (Accession No. AF477785), *S. splendens* (Accession No. AF477788), *P. vulgaris* (Accession No. AY506653) and *A. decumbens* (Accession No. AF477768). The result showed that the lengths of ITS1, 5.8S and ITS2 in *P. vulgaris* were 233, 163 and 229 bp, while 219, 163 and 223 bp in *A. decumbens*. 16 ITS sequences were aligned

Table 2. Percent divergence of ITS sequences from original plants of *S. prunellae* and their confusable species.

	P. vulgaris	*P. asiatica*	*P. hispida*	*P. grandiflora*	*A. decumbens*	*A. multiflora*
P. vulgaris	****	0.003	0.002	0.005	0.026	0.024
P. asiatica	0.007	****	0.004	0.004	0.026	0.024
P. hispida	0.006	0.007	****	0.005	0.026	0.025
P. grandiflora	0.015	0.008	0.015	****	0.026	0.024
A. decumbens	0.270	0.268	0.271	0.265	****	0.009
A. multiflora	0.249	0.244	0.253	0.244	0.044	****

Note: The numbers indicate Std. errors and percent divergences, respectively.

Table 3. Variable sites in the ITS sequence of four species in *Prunella*.

Species	Locality	Base site										
		5	**103**	**143**	**156**	**181**	**182**	**376**	**474**	**518**	**525**	**566**
P. vulgaris	Xinyu, Jiangxi	C	T	G	A	C	A	C	T	C	T	C
	Yingxiu, Sichuan	C	A	T	A	A	G	C	T	C	T	C
	Hunan	C	A	T	A	A	G	T	T	T	T	C
	Guiyang, Guizhou	C	A	T	A	A	G	T	T	T	T	C
P. asiatica	Jurong, Jiangsu	C	T	G	A	C	A	C	T	C	T	C
	Panan, Zhejiang	C	T	T	A	C	A	C	T	C	T	C
	Wuhu, Anhui	C	T	G	A	C	A	C	T	C	T	C
	Xishui, Hubei	C	T	G	A	C	A	C	T	C	T	C
P. hispida	Kunming, Yunnan	C	A	T	A	C	A	T	T	T	T	C
	Kunming, Yunnan	C	A	T	A	C	A	T	T	T	T	C
P. grandiflora	Nanjing, Jiangsu	G	T	G	T	C	A	C	A	C	C	C
	Nanjing, Jiangsu	G	T	G	A	C	A	C	A	C	C	A

with that of *P. vulgaris* or *A. decumbens* by Clustal X, and the overhanging terminals were deleted. Aligned sequence of 584 bp (including partial ITS1 and complete 5.8S and ITS2) was obtained. When alignment gaps were treated as missing data, 584 bp contained 150 variant sites and 149 informative sites. The percentage of sequence divergence between species (Table 2) was 10.9%, and the transitions/transversion ratio was 1.53. The mean nucleotide composition of all the sequences was 18.2% T, 32.8% C, 18.3% A, and 30.7% G; the percentage of G-C content was 63.5%.

Identification of species by ITS sequence analysis

The sequences were calculated with MEGA3.1 after pairwise arrangement, as shown in Table 3 and 4. *Prunella* species had 11 variant sites and 9 informative sites, and there were no variation at the coding region. *P. vulgaris*, *P. asiatica*, and *P. hispida* had 6 variant sites. When being compared with the three bases of 5(C), 474(C) and 525(T) in the other three species, *P. grandiflora* had three different bases: 5(G), 474(A) and 525(C). Therefore, it could be identified from the other 3 species. Further, it was easy to discriminate the counterfeits of *S. prunellae* - *A. decumbens* and *A. multiflora* based on the 122 informative sites present at genus *Ajuga* and genus *Prunella*.

There existed a certain degree of variation in ITS sequence among four species in *Prunella*. *P. vulgaris* from Jiangxi had the same ITS sequence as well as *P. asiatica* from Jiangsu, Anhui and Hubei had. The ITS sequences of *P. vulgaris* from Hunan and Guizhou were identical. The sequence of *P. vulgaris* from Sichuan was only different at 376 and 518 nt with populations from Hunan and Guizhou. The four populations of *P. asiatica* had same ITS sequences except 143 nt.

The sequences of two *P. hispida* individuals were the same, which had identical bases at 103 nt, 143 nt, 376 nt and 518 nt with *P. vulgaris* from Hunan and Guizhou, and the same bases at 181nt and 182nt with *P. asiatica*.

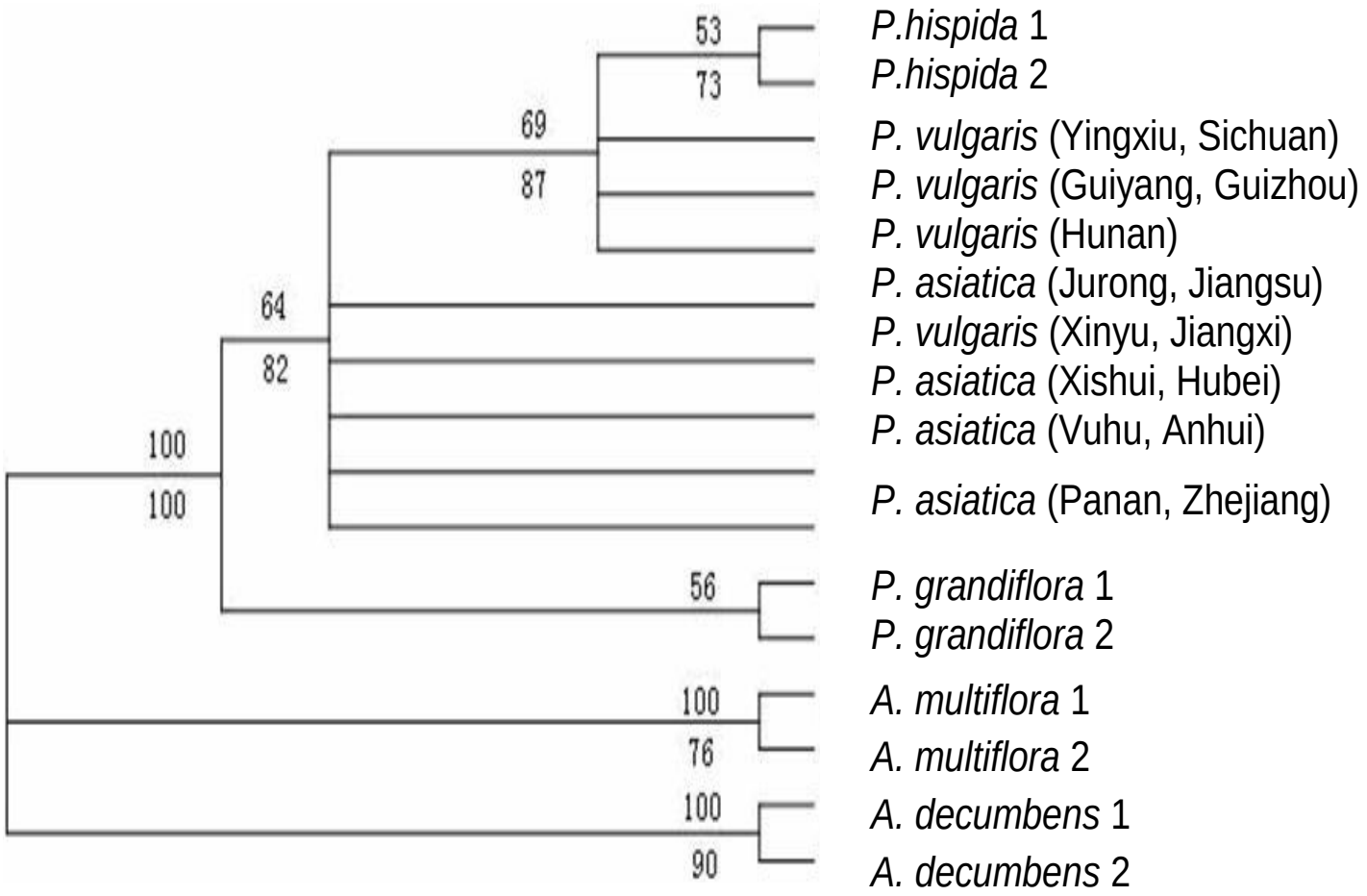

Figure 1. Phylogenetic treef *Spica prunellae* and their confusable species based on ITS sequences from original plants. Numbers above branch indicated bootstrap values (%) of Maximum parsimony method; numbers below branch indicated bootstrap values (%) of Neighbor-Joining method, respectively.

Though *P. hispida* could not be distinguished at a single site, a combination of six bases (103, 143, 181, 182, 376 and 518 nt) could be used as a standard to identify *P. hispida*.

In brief, ITS sequence could be used to identify *P. grandiflora*, *A. decumbens*, *A. multiflora* and *P. hispida*. *P. vulgaris* and *P. asiatica* could not be distinguished only based on ITS sequences.

Phylogenetic analysis of *Spica prunellae*

Phylogenetic trees were constructed by PAUP 4.0 with MP (Maximum parsimony) method and NJ (Neighbor-Joining) method respectively. The topology constructed by two methods was rather similar, only different in bootstrap values (Figure 1). It showed that *Prunella* species located in the same class, *A. multiflora* and *A. decumbens* assembled in another one. *P. grandiflora* had a rather distant phylogenetic relationship with the other 3 *Prunella* species. *P. hispida* was close to *P. vulgaris*. *P. vulgaris* and *P. asiatica* had an obscure relationship, as reflected by the relatively low bootstrap values of branches. In phylogenetic tree, *P. vulgaris* from Xinyu did not belong to the cluster of other *P. vulgaris* populations, but it had a closer relationship with *P. asiatica*, which indicated that it might be transition between the two species.

DISCUSSION

Molecular Identification of Spica prunellae

There were related reports about genuineness of Traditional Chinese Medicine, for example, *P. vulgari* is prescribed by *Chinese Pharmacopoeia* as the botanical origin of Spica prunellae from its closely related plant *P. grandiflora* and confusable species in genus *Ajuga*. Genuineness of traditional Chinese drugs refers to

genuineness of species, genuineness of producing area or genuineness of effective components. In this study, we focused on the genuineness of species. A lot of researches on genuineness of species have been done (Ge et al., 2007; Wen et al. 2007; Huang et al., 2007). The specific sites in ITS sequences could be used to identify *P. vulgaris*.

Jiangsu Flora (Jiangsu Institute of Botany, 1982) *a*nd *Anhui Flora* (Cooperation Group of the Anhui Flora, 1992) record that *P. vulgaris* grow in Jiangsu and Anhui, and that *P. asiatica* don't. Whereas *Flora Reipublicae Popularis Sinicae* records that *P. asiatica* instead of *P. vulgaris* grow in Jiangsu and Anhui. This contradiction indicated that there existed the confusion of distribution and taxonomy of *P. vulgaris*, so the botanical origins of Spica prunellae could be mixed and the original species of the genuine medicinal material Jing Common Selfheal is uncertain. As mentioned above, there are a few contradictions in the description of the morphological characters and distribution of *P. vulgaris* and *P. asiatica* in different literatures. Wang et al. (1994; 1996; 1999) found that *P. vulgaris*, *P. asiatica* and *P. hispida* had similar features of morphology, chemical composition and pharmacological effects, and that the cross distribution made it rather difficult to distinguish *P. vulgaris* from *P. asiatica*. One *P. vulgaris* population as well as *P. asiatica* had the same ITS sequence, and 4 specific sites of *P. hispida* were the same as those of *P. vulgaris*, with 2 specific sites of *P. hispida* identical with those of *P. asiatica*, which suggested that *P. asiatica*, *P. vulgaris* and *P. hispida* should be treated as one specie and *P. asiatica* should be regarded as the geographic subspecies of *P. vulgaris*.

Our results showed that *P. vulgaris*, the botanical origin of *S. prunellae* and local origins *P. asiatica* and *P. hispida* had a very close phylogenetic relationship, while taxonomy of three species should need more detailed evidences in future.

The origin of *S. prunellae* and genuineness of the species

S. prunellae is one of the genuine medicinal materials in Jiangsu. *P. vulgaris* is prescribed as the botanical origin of *S. prunellae* in Chinese Pharmacopoeia (2005). However, according to Flora Reipublicae Popularis Sinicae, not *P. vulgaris* but *P. asiatica* distributes in Jiangsu. Based on this study, it is suggested that the original plants of *S. prunellae* should be *P. vulgaris* and *P. asiatica*, and that the botanical origin of Jing Common Selfheal should be *P. asiatica*.

ACKNOWLEDGEMENTS

We thank Prof. Qin Minjian of China Pharmaceutical University and Chen Bingluan of Guizhou University for collecting some plant materials. We also thank Shi Derong for editing English. This work was supported by Natural Science Foundation of Jiangsu Province (BK2005206) and Commonweal Foundation of Jiangsu Province (BM2006104).

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