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Full Length Research Paper

A perspective on geoherbalism: An example of scutellariae radix biogeography based on three chloroplast DNA regions

Bin Li[1,2], Shouzhuo Li[3] and Shiliang Zhou[1]*

[1]State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China.
[2]Research Institute of Forestry, Chinese Academy of Forestry, Beijing 100091, China.
[3]Chengde Medical College, Chengde 067000, Hebei, China.

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The term 'geoherbalism' is extensively used for both academic and commercial purposes. To examine the recent application of this term to a frequently used herbal medicine, 231 samples of genuine scutellariae radix, *Scutellaria baicalensis*, were collected from 13 populations, covering almost the entire distribution range. In addition, we sampled its two succedanea, that is, 39 samples from two populations of *Scutellaria rehderiana* and 45 samples from two populations of *Scutellaria viscidula* and a control (*Scutellaria regeliana*). The genetic differences among the samples in the three rapidly evolving chloroplast DNA regions (matK, psbK-psbI and trnL-trnF) were analyzed. All three of the medicinal species showed very low genetic diversity when compared to the control, possibly because of the long harvesting history. Based on the markers used, *S. baicalensis* was distinguishable from *S. rehderiana* but not from *S. viscidula*. The geoherbalic populations in Rehe were indistinguishable from the other populations. We conclude that the concept of geoherbalism should be restricted to its historical usage in referring to the genuine medicinal species rather than the geographical regions within the genuine species as proposed by some advocates.

Key words: Chloroplast DNA, genetic diversity, geoherbalism, scutellariae radix, *Scutellaria*.

INTRODUCTION

Historically, because of a lack of botanical knowledge, materials from morphologically similar but taxonomically distinct species were often regarded as the same medicine. Consequently, different species in a genus, occasionally in a family or very rarely in different families, might be misused for the same or similar diseases. Because of occasional failures, herbalists were driven to distinguish the adulterants from the effective materials and the succedanea from the genuine medicines. The concept of 'geoherbalism' was proposed to refer to the effectiveness of a medicine on certain diseases, thus, a geoherbalic medicine is that medicine proven to be the most effective of any other similar medicine.

*Corresponding author. E-mail: slzhou@ibcas.ac.cn. Tel: +86-10-62836503. Fax: +86-10-62590843.

Unfortunately, the concept has recently been used misleadingly to refer to medicine production within a restricted geographic area. In this report, using the example of scutellariae radix, we demonstrate that the concept of geoherbalic medicine is best applied in a sense that is equivalent to the genuine medicine.

Scutellariae radix or Huang-qin in Chinese is a key ingredient of many traditional Chinese prescriptions to treat liver problems, such as hepatitis, cirrhosis, jaundice and hepatoma and other diseases, such as leukemia, hyperlipemia, arteriosclerosis, and inflammation (Susan et al., 2004; Watanabe et al., 2002). Phytochemical analysis revealed that scutellariae radix contains large amounts of bioactive flavonoids, such as baicalein, baicalin and wogonin which exhibit inhibitory effects against cancer and human immunodeficiency virus (HIV-1) (Chang et al., 2002; Parajuli et al., 2009). The demand for scutellariae radix is growing annually in Chinese,

Japanese, Korean, European and American herbal medicine markets. Indeed, the consumption of scutellariae radix increased to 21 million kg in 2007 from 8 million kg in 1983 (Cui et al., 2009).

The genuine scutellariae radix consists of the roots of *Scutellaria baicalensis* growing in Northern China. There are six succedanea: *Scutellaria rehderiana* Diels and *Scutellaria viscidula* Bunge in Northern China and *Scutellaria amoena* C. H. Wright, *Scutellaria omeiensis* C. Y. Wu, *Scutellaria likiangensis* Diels and *Scutellaria tenax* W. W. Smith in South-western China. The three species in Northern China (that is, *S. baicalensis*, *S. rehderiana* and *S. viscidula*) appear similar to individuals who are outside the herbalist specialty and the roots are used indiscriminately (Cui et al., 2009).

Each species occupies a distinct territory and the genuine medicines were traditionally produced in very restricted areas. The concept of genuineness is driven by practitioners of Chinese medicine who argue that only the medicine produced in certain traditional areas should be regarded as 'genuine medicine'. Historically, the best accepted scutellariae radix was 'Rehe scutellariae radix', a product that originated in Rehe (now in some areas of the Provinces of Hebei, Inner Mongolia and Liaoning, China) and is, thus, considered to be the genuine scutellariae radix. Rehe scutellariae radix is derived from *S. baicalensis* and is believed to be of high medicinal quality (Foster, 1993).

Based on geoherbalism, the term 'geoherbalics' in this study is refer to a discipline aimed at identifying genuine medicines or the origins of genuine medicines. Genetic variation among populations is thought to be the genetic basis of geoherbalism (Yuan et al., 2009; Huang et al., 2004) and genetic analysis are the most frequently used methods in geoherbalics. Among the many molecular markers available, nucleotide sequences are the preferred markers that have been successfully used. The specific aims of this study were to answer: (1) how much genetic variation exists among species in *Scutellaria*; (2) does the genuine scutellariae radix significantly differ genetically from other succedaneous scutellariae radix; and (3) is Rehe scutellariae radix distinguishable from other populations, and, if so, how is it distinguishable?

MATERIALS AND METHODS

Plant materials

S. baicalensis occurs in nine Northern and North-Eastern Provinces of China, which are Gansu, Shaanxi, Shanxi, Inner Mongolia, Hebei, Shandong, Liaoning, Jilin and Heilongjiang (Figure 1). A total of 315 samples from 17 sites of the three medicinal species, *S. baicalensis*, *S. rehderiana* and *S. viscidula* and 24 samples from one population of the out group *Scutellaria regeliana* were collected (Table 1). Among these samples, 229 samples from 11 populations, including four Rehe populations (B2, B5, B6 and B11) and seven non-Rehe populations (B1, B4, B7, B8, B10, B12 and B13) were genuine scutellariae radix (*S. baicalensis*) and 46 samples from two populations of *S. rehderiana* and 47 samples from two populations of *S. viscidula* were succedaneum scutellariae radix. One population of *S. regeliana* was used as control. The localities of all of the sampled populations are shown in Figure 1. The fresh leaves were quickly desiccated by heating them on an electric blanket for a few hours.

DNA extraction, gene marker selection, amplification and sequencing

The total DNA of all of the desiccated leaf samples were extracted and purified using the DP305 Plant DNA Extraction Kit [Tiangen Biotech (Beijing) Co., Beijing, China], following the manufacturer's instructions.

A total of eight DNA regions (atpB-rbcL, atpH-atpF, ITS, matK, psbK-psbI, rbcL, trnH-psbA and trnL-trnF) were analyzed for their variability among the populations of *S. baicalensis* using one sample per population. Three chloroplast fragments (matK, psbK-psbI and trnL-trnF) were then chosen as the genetic markers for further use.

Polymerase chain reaction (PCR) amplification of these markers was performed in a 20 µl volume using a DNA engine DYAD thermal cycler (Bio-Rad, Hercules, CA, USA), with the following protocol: 94°C for 4 min; 40 cycles of 30 s at 94°C; 30 s at 52°C; 2 min at 72°C; and a final extension of 10 min at 72°C. The PCR products were purified using PEG 8000. The sequencing reaction protocol comprised 30 cycles of 15 s at 96°C; 15 s at 52°C; 4 min at 60°C and 30 min at 60°C. The sequencing PCR products were purified using NaAc, and both strands were sequenced using an ABI 3730xl DNA Analyzer using ABI BigDye v3.0 (Applied Biosystems, Foster City, CA, USA).

Sequence alignment and data analysis

The DNA sequences were assembled using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA), and the sequence alignments were adjusted manually using Se-Al 2.0 (Rambaut, 1996). Informative gaps were coded as presence (1) or absence (0) in relation to the out-group.

The sequence diversity, as represented by the number of variable sites (*s*), number of haplotypes (*h*), haplotype diversity (*hd*), and nucleotide diversity (π), was computed using DnaSP 5.0 (Librado and Rozas, 2009). The molecular variance components were partitioned into: among species, among populations within species and within populations for all of the samples, and among and within populations of *S. baicalensis* using AMOVA incorporated in Arlequin (Excoffier and Lischer, 2010). The haplotype frequency-based population differentiation (G_{ST}) and both haplotype similarity and haplotype frequency-based population differentiation (N_{ST}) were calculated using Arlequin. The population relationships were built using Network 4.5.1.6 (http://fluxus-engineering. com) based on the haplotypes.

RESULTS

Sequence variability of three chloroplast DNA markers

The aligned partial lengths of all 339 of the sequences were 875 bp, with two indels for matK, 428 bp, with four indels for psbK-psbI, and 825 bp, with three indels for trnL-trnF (Table 2). The indel for matK reflected the absence of a TTCAAAAAG repeat in *S. rehderiana*. The major indel for psbK-psbI resulted from a deletion in *S.*

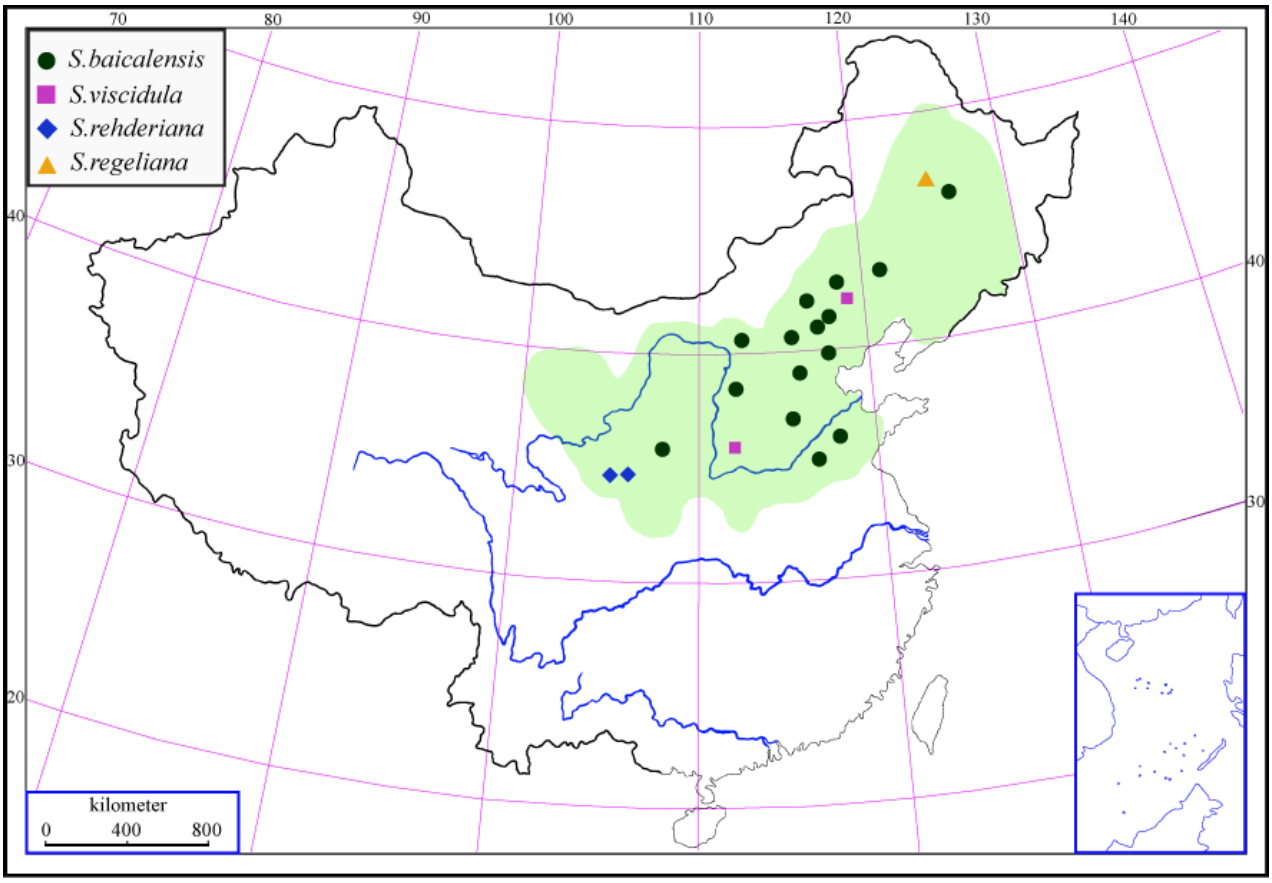

Figure 1. Distribution of sampled populations of *S. baicalensis*, *S. rehderiana*, and *S. viscidula* in China. *S. baicalensis* is indicated by circles, *S. rehderiana* by diamonds, *S. viscidula* by squares, and *S. regeliana* by a triangle.

regeliana and *S. rehderiana*. A major indel for trnL-trnF was a CTGAAAAC repeat. The number of variable sites ranged from 13 to 24 when all of the samples were analyzed and only three of four are within *S. baicalensis*. There were 23 haplotypes in total and 12 haplotypes within *S. baicalensis*. The nucleotide diversity was 0.00322 in total and 0.00033 within *S. baicalensis.*

Genetic variation

The genetic diversity of *S. baicalensis*, *S. rehderiana*, *S. viscidula*, and *S. regeliana* at both the species and population levels is summarized in Table 1. No variation was detected in *S. viscidula*, and *S. regeliana* exhibited the highest diversity, even though only one population was analyzed. *S. baicalensis* harbored very limited genetic diversity. At the population level, the population of *S. regeliana* exhibited the highest genetic diversity, and no variation was observed in the five populations. Within *S. baicalensis*, B02 (Hebei-Chengde), B10 (Inner Mongolia–Huhehaote) and B12 (Shandong–Linqu) exhibited relatively high levels of genetic diversity. However, the levels of genetic diversity seemed to be population dependent. The B12 and B13 populations were located in the same town, but their genetic diversity differed significantly (Table 1).

Differences in nucleotide sequences were found largely among the different species, accounting for 92.22% of the total molecular variation (Table 3). Approximately, 2.75% of the variation was from among the populations, and only 5.03% of the total variation was restricted to within the populations. To ascertain whether Rehe scutellariae radix is significantly different genetically from other populations, all of the populations within *S. baicalensis* were divided into two groups, including the Rehe and non-Rehe groups. However, within *S. baicalensis*, the component of molecular variation accounted for only 3.26% among the groups, 53.72% among the populations, and 43.02% within the populations (Table 3).

Population relationships and identification of species and populations

The genetic divergence between all of the species was significant, with the exception of between *S. baicalensis* and *S. viscidula* (N_{ST} = 0. 044, G_{ST} = 0.090) (Table 4).

Table 1. Species, population localities (province–city or county–town or village or mountain), sample size (*n*), voucher specimen, and genetic diversity at both population and species levels. *s*: number of segregating sites; *h*: number of haplotypes; *hd*: haplotype diversity; *π*: average number of nucleotide differences.

Taxon	Population locality	Population code	Voucher (2009)	*n*	*s*	*h*	*hd*	*π*
Scutellaria baicalensis Georgi				231	10	9	0.358	0.00022
	Hebei–Changli–a hill beside the city, 70 m	B01	S. L. Zhou et al. 090801-2	15	0	1	0	0
	Hebei–Chengde–Shang Bancheng, 310 m, cultivated	B02	S. Z. Li 090808	23	3	4	0.731	0.00046
	Hebei–Pingquan, 504 m, a white-flowered mutant	B03	Y. Zhong 090807-1	1	-	-	-	-
	Hebei–Shexian–Longhuxiang, Jiaozhicun, 685 m	B04	S. L. Zhou et al. 090804-1	22	2	3	0.394	0.00020
	Hebei–Weichang–Mountain beside the city, 900 m	B05	S. L. Zhou et al. 090730-1	22	1	2	0.368	0.00017
	Hebei–Weichang–Saihanba Forest farm, 1714 m	B06	S. L. Zhou et al. 090731-4	19	2	3	0.433	0.00022
	Hebei–Yuxian–Xiao Wutaishan, 1064 m	B07	Y. Zhong 090807-1	24	0	1	0	0
	Hebei–Yuxian–Xiao Wutaishan, 1580 m	B08	Y. Zhong 090808-1	21	2	3	0.452	0.00023
	Heilongjiang–Harbin–Botanical garden, 128 m, cultivated	B09	S. L. Zhou 090826	1	-	-	-	-
	Inner Mongolia–Huhehaote–Daqingshan, 1190 m	B10	Y. Zhong 090810-1	7	3	2	0.286	0.00041
	Inner Mongolia–Wongniute–the second Army Horse Breeding Farm	B11	S. L. Zhou et al. 090731-3	24	1	2	0.159	0.00008
	Shandong–Linqu–Wujing town, Rujiazhuang, 241 m, cultivated	B12	S. L. Zhou et al. 090802-1	20	5	5	0.695	0.00052
	Shandong–Linqu–Wujing town, Shangyinshicun, 593 m	B13	S. L. Zhou et al. 090802-2	24	1	2	0.431	0.00020
S. rehderiana Diels.				39	8	6	0.399	0.00044
	Gansu–Yuzhong–Xinglongshan, 2115 m	R1	S. L. Zhou 090915A	24	8	5	0.529	0.00054
	Gansu–Yuzhong–Xinglongshan, 2700 m	R2	S. L. Zhou 090915B	15	4	2	0.133	0.00026
S. viscidula Bunge				45	0	1	0	0
	Inner Mongolia–Chifeng–Balaqixiang, 400 m	V1	Y. Zhong 090813-1	22	0	1	0	0
	Shanxi–Shuozhou–Pinglu, Hutoushan, 1200 m	V2	Y. Zhong 090809-	23	0	1	0	0
S. regeliana Nakai				-	-	-	-	-
	Heilongjiang–Harbin–Sun Island, 114 m	Outgroup	S. L. Zhou 090822	24	8	6	0.594	0.00122

Within *S. baicalensis*, population B01 showed significantly larger in N_{ST} and G_{ST} values than those of the other populations (Table 5), which indicated that only population B01 was readily identifiable from the other populations.

The network (Figure 2) based on the combined

Table 2. Variability of the three chloroplast DNA regions among all assayed samples and within *S. baicalensis*. *L*: aligned length; *i*: number of informative indels; *s*: number of variable sites; *h*: number of haplotypes; *hd*: haplotype diversity; *π*: nucleotide diversity.

Region	*L*	All four species					Within *S. baicalensis*				
		i	*s*	*h*	*hd*	*π*	*i*	*s*	*h*	*hd*	*π*
matK	877	2	14	7	0.421	0.00223	0	3	4	0.183	0.00021
psbK-psbI	432	4	13	9	0.497	0.00481	0	3	4	0.396	0.00076
trnL-trnF	828	3	24	9	0.412	0.00348	1	4	4	0.160	0.00023
Total	2137	9	51	23	0.639	0.00322	1	10	12	0.535	0.00033

Table 3. Percentages of molecular variation components partitioned into among species, among populations within species, and within populations.

Source of variation	All four species (%)	Within *S. baicalensis* (%)
Among species	92.22	–
Among populations within species	2.75	–
Within populations	5.03	–
Among groups* within *S. baicalensis*	–	3.26
Among populations within groups	–	53.72
Within populations	–	43.02

*All 11 populations within *S. baicalensis* were divided into two groups. The Rehe group included population B2, B5, B6, B11 and the other group included population B1, B4, B7, B8, B10, B12, B13.

Table 4. Genetic divergence between the *Scutellaria* species. Below diagonal: haplotype frequency-based population differentiation (G_{ST}); above diagonal: both haplotype similarity and haplotype frequency-based population differentiation (N_{ST}).

Species	*S. baicalensis*	*S. rehderiana*	*S. viscidula*	*S. regeliana*
S. baicalensis	–	0.869	0.090	0.960
S. rehderiana	0.210	–	0.929	0.954
S. viscidula	0.044	0.682	–	0.968
S. regeliana	0.129	0.334	0.607	–

data set of all three of the chloroplast DNA fragments demonstrated that the medicinal species were significantly different from the non-medicinal out-group, *S. regeliana*.

Among the medicinal species, *S. rehderiana* was distinguishable from the other two species, even though they are closely related. *S. baicalensis* and *S. viscidula* were undistinguishable by the markers used because they shared haplotype 1. Twelve haplotypes were present within *S. baicalensis*, and most of the populations shared haplotypes 1 and 2. Population B01 contained the unique haplotype 5, which is a useful marker for identification.

Geographically, the haplotypes present in the northern populations (B06 and B10) were ancestral to the other haplotypes, indicating that the populations radiated from the north.

DISCUSSION

Historical shift of medicinal scutellariae radix and geoherbalism

Recorded in Shennong's Handbook of Materia Medica nearly 2000 years ago, scutellariae radix is one of the classical herbal medicines frequently included in many prescriptions. Currently, only *S. baicalensis* is considered to be genuine scutellariae radix. However, scutellariae radix was obtained from both Hubei and Shandong Provinces in the earliest record in a supplementary to Shennong's Handbook of Materia Medica for Herbalists(ca. 220 AD). Because of lack of plant taxonomic knowledge and difficulties with the identification of the radices of different species, all of the species with well-developed taproots had been

Table 5. Genetic differentiation between populations of *S. baicalensis*. Below diagonal: haplotype frequency-based population differentiation (G_{ST}); above diagonal: both haplotype similarity and haplotype frequency-based population differentiation (N_{ST}).

Population	B01	B02	B04	B05	B06	B07	B08	B10	B11	B12	B13
B01		0.700	0.829	0.850	0.819	1	0.815	0.700	0.926	0.706	0.833
B02	0.386		0.081	0.045	0.154	0.208	0.039	0.042	0.124	0.249	0.035
B04	0.603	0.076		0	0.080	0.076	0	0	0	0.272	0.032
B05	0.623	0.066	0		0.117	0.190	0	0	0.033	0.278	0
B06	0.596	0.086	0.012	0.022		0.133	0.110	0	0.089	0.282	0.154
B07	1	0.253	0.074	0.109	0.108		0.167	0.000	0.043	0.359	0.261
B08	0.567	0.045	0	0	0.021	0.123		0	0.035	0.264	0
B10	0.796	0.093	0.010	0.023	0.019	0.014	0.028		0	0.203	0
B11	0.802	0.163	0.007	0.017	0.041	0.022	0.032	0		0.312	0.095
B12	0.426	0.086	0.142	0.151	0.134	0.322	0.126	0.129	0.234		0.279
B13	0.562	0.044	0.000	0	0.034	0.150	0	0.042	0.050	0.136	

regarded as scutellariae radix, and some of them were used as succedanea until very recently (Wu and Li, 1977). From the records in medicinal books, such as Variorum of Shennong's Handbook of Materia Medica (ca. 500 AD) and subsequent books, it is clear that scutellariae radix and its succedanea were produced in the northern part of China, from Jiangsu to Shanxi and Shaanxi Provinces. Three species (*S. baicalensis*, *S. rehderiana* and *S. viscidula*) were generally used indiscriminately. To distinguish the genuine medicine from the succedanea, the term geoherbalism was used to refer to the genuine species. Although, this term has been used for five centuries, the recent application of the concept was only to the medicines produced in a certain locality; this is not the original sense of this term, which actually pertained to the genuine medicine versus the succedanea of different species. Thus, the use of the scutellariae radix term shifted from one species to another over time. The earliest scutellariae radix was unknown *Scutellaria* specie and then became *S. baicalensis*, *S. rehderiana* and *S. viscidula*. However, the geoherbalic medicine in the modern sense can only be *S. baicalensis*.

Both genuine and succedaneous scutellariae radix show low genetic diversity

Compared to the non-medicinal congener *S. regeliana*, the genetic variation or diversity of all three of the medicinal species is low. For the reason that no sequence-based genetic diversity evaluation exists, our estimations remain to be verified. Because of the relatively low evolution rates of chloroplast genes compared to those of nuclear genes, our results are low estimates. Furthermore, the significance of our estimates rests not on the absolute parameter values but on the relative levels of genetic diversity compared to the out-group. The low genetic diversity of the medicinal species is remarkable. Although, it is impossible to attribute the low genetic diversity solely to historical harvesting, such activities must have caused some loss of genetic diversity. In fact, both genuine and succedaneous scutellariae radix, show low genetic variation. Therefore, the geographical relationships are complicated among the populations, especially among the cultivated populations (Yuan et al., 2010). The frequent exchange of seeds for cultivation and escape from cultivation into the wild provide further reasons that the concept of geoherbalism should not be restricted to the population level but should apply to the species level.

Genuine scutellariae radix is partially distinguishable from some of its succedanea using chloroplast DNA markers

Scutellaria is a large genus consisting of 360 to 425 species (Paton, 1990), and the identification of Scutellariae species is taxon and marker dependent. Hosokawa et al. (2005) selected *rpl16* and *rpl16 to rpl14* to discriminate six species that are morphologically difficult to distinguish. The matK region, which was used in the present study, is considered to be one of the most variable chloroplast DNA markers and is recommended for use as a barcode for plants (CBOL Plant Working Group, 2009). The variability of psbK-psbI in Panax, ranked second among these eight chloroplast DNA regions tested (Zuo et al., 2010). The trnL-trnF region is one of the most widely used chloroplast DNA regions for phylogenetic reconstruction in plants. Each of these markers is capable of discriminating *S. baicalensis*, *S. rehderiana* and *S. regeliana,* but none of them can separate *S. baicalensis* from its sympatric species, *S. viscidula*. Morphologically, *S. viscidula* is easily distinguishable from *S. baicalensis* by its smaller size,

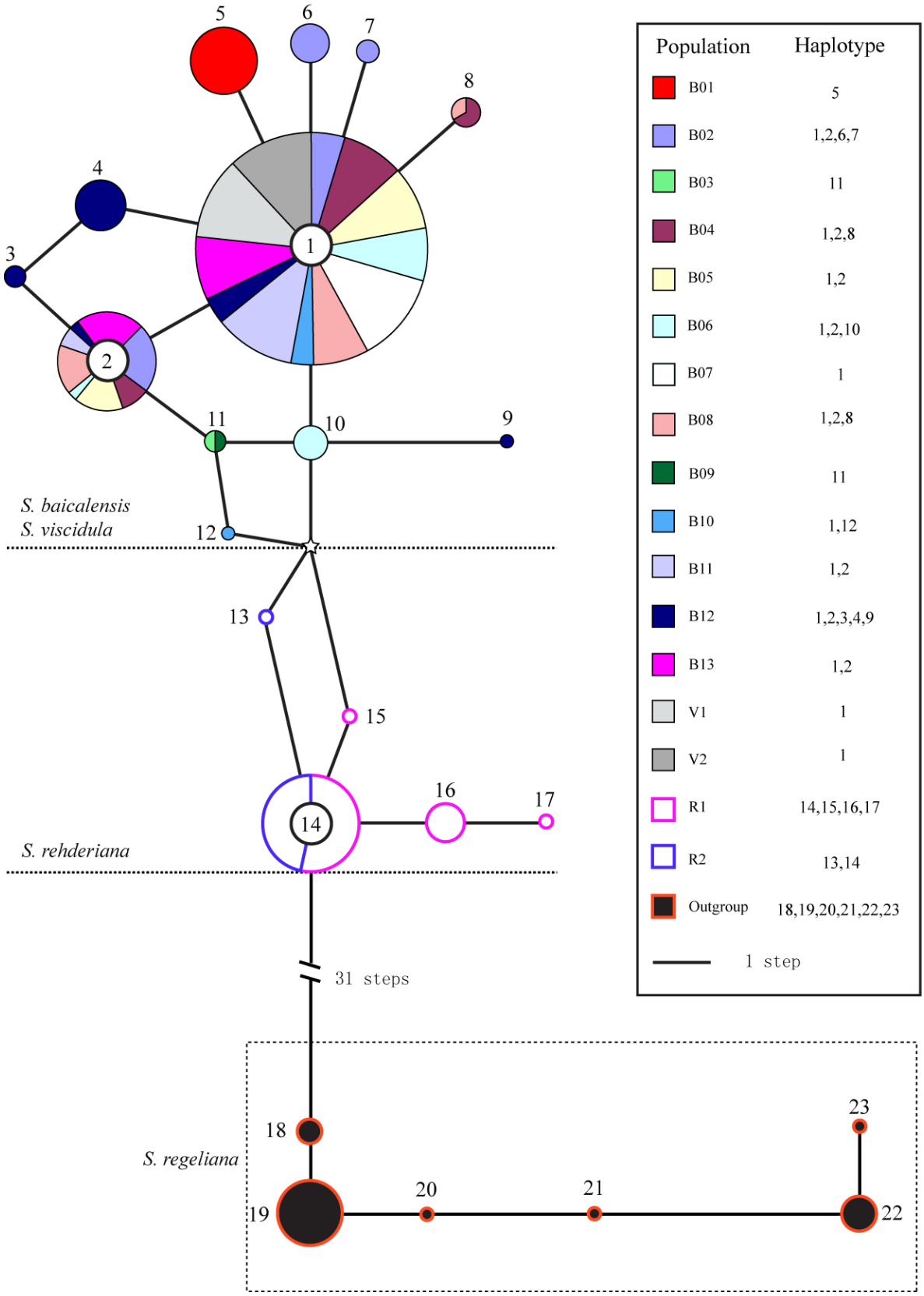

Figure 2. Network based on a combined data set consisting of nucleotide sequences for three chloroplast DNA fragments (matK, psbK-psbI and trnL-trnF) showing the genetic relationships of all haplotypes of *S. baicalensis*, *S. rehderiana*, *S. viscidula* and *S. regeliana*. Individual populations are indicated by different colors.

hairy stems and yellow flowers. We failed to sequence the *trnH-psbA* intergenic spacer because of the presence of multiple copies of the same length. Additional work is needed to analyze the nuclear genetic differences and to explain why the two species share the same chloroplast haplotype.

Geoherbalic Rehe scutellariae radix is indistinguishable from its counterparts

Although the DNA regions we used in this study are among the most variable markers used to distinguish species, they are not variable enough to discriminate between populations. The scutellariae radix that occurs in some areas of Hebei, Inner Mongolia and Liaoning Provinces is called Rehe scutellariae radix (B02, B05, B06 and B11) and is believed to be the geoherbalic scutellariae radix in a narrow sense. The markers used in this study were unable to distinguish these populations from the other populations. Two explanations of this result are possible: the markers used lack sufficient variability, and other markers, such as microsatellites, might be more informative or the Rehe scutellariae radix is truly indistinguishable from other populations. Situated in the center of the overall distribution of scutellariae radix, it is unlikely that Rehe scutellariae radix would possess a strong genetic basis that distinguishes it from adjacent populations. Rehe scutellariae radix gained its fame possibly not because of its superior quality compared to its counterparts but because genuine scutellariae radix was produced only in the Rehe area for a long period of time.

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