Analysis of the relationship between ribosomal DNA ITS sequences and active components in *Rhodiola* plants

D.J. Zhang[1,2], W.T. Yuan[2], M.T. Li[2] and Y.H. Zhang[2]

[1]State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, Xining, China
[2]College of Eco-Environmental Engineering, Qinghai University, Xining, China

Corresponding author: D.J. Zhang
E-mail: djzhang@nwipb.ac.cn

Genet. Mol. Res. 15 (4): gmr15049337
Received September 21, 2016
Accepted September 21, 2016
Published December 23, 2016
DOI http://dx.doi.org/10.4238/gmr15049337

ABSTRACT. *Rhodiola* plants are a valuable resource in traditional Chinese medicine. The objective of this study was to evaluate the correlation between ribosomal DNA internal transcribed spacer (ITS) sequences and the three active components in *Rhodiola* plants. For this, we determined ITS sequence polymorphisms and the concentrations of active components salidroside, tyrosol, and gallic acid in different *Rhodiola* species from the Tibetan Plateau. In a total of 23 *Rhodiola* samples, 16 different haplotypes were defined based on their ITS sequences. Analysis of the active components in these same samples revealed that salidroside was not detected in species with haplotypes H_4, H_5, or H_{10}, tyrosol was not detected with haplotypes H_3, H_5, H_7, H_{10}, H_{14}, or H_{15}, and gallic acid was detected in with all haplotypes except H_{14} and H_{15}. In addition, the concentrations of salidroside, tyrosol and gallic acid varied between samples with different haplotypes as well as

those with the same haplotype, implying that no significant correlation exists between haplotype and salidroside, tyrosol or gallic acid concentrations. However, a statistically significant positive correlation was observed for among these three active components.

Key words: *Rhodiola*; Salidroside; Tyrosol; Gallic acid; Correlation; ITS sequence

INTRODUCTION

Plants of the genus *Rhodiola* L. are perennial herbs or subshrubs in the family Crassulaceae, with a height ranging from 10 to 30 cm. There are 96 *Rhodiola* species distributed in the world, of which 73 species are endemic to China. *Rhodiola* plants can tolerate various habitats; a few species grow in alpine meadows at an approximate altitude of 2000 m, while most are found in limestone, granite mountain terrain, glaciers, ridge meadows, or valley rocks at an approximate altitude of 3500 to 5000 m (Li et al., 2007). *Rhodiola* plants possess important pharmacological properties and serve as a valuable traditional Chinese medicine. For example, *Rhodiola* plants possess anti-anoxia, anti-fatigue, anti-aging, anti-depressant, anti-viral and anti-radiation properties, amongst others. Additionally, their use also can regulate the nervous system, and improve the function of the cardiovascular and immune system (Yuan et al., 2007). Internal transcribed spacer (ITS) regions ITS1 and ITS2 are present within the nuclear ribosomal DNA (nrDNA) region in plants, where they separate the 18S, 5.8S, and 26S genes. The nucleotide sequence of the nrDNA region varies substantially between different families and genera, although there is some conservation in length (Schmidt and Schilling, 2000). Because of this sequence variation, ITS sequences have been widely used for phylogenetic and phylogeographic studies in plants (Baldwin et al., 1995).

The well-known major active components in *Rhodiola* species are salidroside, tyrosol, and gallic acid (Yuan et al., 2007). These three active components are frequently used as marker compounds to evaluate the quality of *Rhodiola* material (Cui et al., 2008; Li et al., 2008). Several investigative methods, including high performance liquid chromatography (HPLC), have been used for qualitative and quantitative analysis of *Rhodiola* extracts and active components (Yuan et al., 2007). However, it is presently unclear if a correlation exists between *Rhodiola* ITS sequence variation and concentrations of active components. The objective of this study is to investigate nrDNA ITS region sequence polymorphisms alongside variation in active component concentrations in different *Rhodiola* species. This work provides a basis for further study of the molecular mechanisms involved in formation and accumulation of the main active components in *Rhodiola* plants, and provides a theoretical basis for the effective use and protection of *Rhodiola* resources.

MATERIAL AND METHODS

Materials

Twenty-three *Rhodiola* samples comprised of fresh leaves, roots, and whole plants were collected from different regions of the Tibetan Plateau. Leaf material was dried using silica gel before being transported to the laboratory. Root material was dried out of direct sunlight

and stored at room temperature until use. All specimens were identified by Mr. Yongchang Yang at the Northwest Plateau Institute of Biology, Chinese Academy of Sciences (Table 1).

Table 1. Samples used in this study.

Species	Locality	Voucher	GenBank accession No.
Rhodiola. papillocarpa S.H. Fu	Deqing, Yunnan	chen2012125	KR269896
R. yunnanensis S.H. Fu	Changdu, Xizang	chen2012446	KR269899
R. purpureoviridis S.H. Fu	Yulong, Yunnan	chen2012110	KR269897
R. cretinii S.H. Fu	Dege, Sichuan	chen2012481	KR269900
R. wallichiana S.H. Fu	Dingqing, Xizang	chen2012400	KR269898
R. wallichiana S.H. Fu	Maerkang, Sichuan	chen2012311	KR269904
R. wallichiana var. *Cholaensis* S.H. Fu	Daocheng, Sichuan	chen2012247	KR269902
R. brevipetiolata S.H. Fu	Changdu, Xizang	chen2012447	KR269903
R. subopposita S.H. Fu	Datong, Qinghai	ZDJ1205	KR269886
R. cabrida S.H. Fu	Sunan, Gansu	ZDJ1204	KR269885
R. subopposita S.H. Fu	Datong, Qinghai	ZDJ1206	KR269887
R. dumulosa S.H. Fu	Datong, Qinghai	ZDJ1201	KR269884
R. dumulosa S.H. Fu	Datong, Qinghai	ZDJ1207	KR269888
R. himalensis S.H. Fu	Daocheng, Sichuan	chen2012246	KR269905
R. concinna S.H. Fu	Deqing, Yunnan	chen2012127	KR269901
R. alsia S.H. Fu	Xinghai, Qinghai	chen2012332	KR269906
R. taohoensis S.H. Fu	Tongde, Qinghai	ZDJ1209	KR269889
R. taohoensis S.H. Fu	Tongde, Qinghai	ZDJ1210	KR269890
R. taohoensis S.H. Fu	Tongde, Qinghai	ZDJ1211	KR269891
R. taohoensis S.H. Fu	Tongde, Qinghai	ZDJ1212	KR269892
R. taohoensis S.H. Fu	Gonghe, Qinghai	ZDJ1213	KR269893
R. taohoensis S.H. Fu	Gonghe, Qinghai	ZDJ1214	KR269894
R. taohoensis S.H. Fu	Gonghe, Qinghai	ZDJ1215	KR269895
Sedum oaxacanum			EF632176
S. compactum			EF632175
S. alexanderi			EF632174
Hylotelephium erythrostictum			JQ954558

Methods

DNA extraction and PCR amplification of ITS regions

Total DNA was extracted from plant samples using the modified cetyltrimethylammonium bromide (CTAB) method (Zhang et al., 2008). The polymerase chain reaction (PCR) was performed using primers ITS1 (5'-AGAAGTCGTAACAAGGTTTCCGTAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990) in a 50 μL volume, containing 5.0 μL 10X PCR Buffer, 4 μL 2.5 mM dNTPs (including 1.5 μL 3 mM Mg^{2+}), 1 μL 5 pM of each primer, 0.25 μL (5 U) Taq DNA polymerase, 1.0 μL (15 to 20 ng) DNA template, and 37.75 μL double distilled water. The thermal cycler parameters were as follows: 94°C for 4 min, then 31 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 70 s, then a final extension at 72°C for 7 min. PCR amplicons were visualized on 1% TAE agarose gels before they were sent to Beijing Sunbiotech Co. (Beijing, China) for double-stranded DNA sequencing. Resulting ITS sequences were aligned with a *Rhodiola* species reference sequence obtained from the GenBank database (accession No. KF113720). Newly identified sequences were submitted to GenBank (accession Nos. KR269884 to KR269906). The ITS sequences of *Sedum oaxacanum*, *Sedum alexanderi*, *Sedum compactum*, and *Hylotelephium erythrostictum*, which belong to the Crassulaceae family alongside *Rhodiola*, were retrieved from GenBank (accession No. EF632176, EF632175, EF632174 and JQ954558, respectively) to use outgroups in the sequence alignment (Table 1).

Determination of active components

Concentrations of the three active components, salidroside, tyrosol, and gallic acid, were simultaneously measured by Agilent 1100 HPLC (Wang et al., 2006). To prepare samples for HPLC, 2 g dry root powder from the *Rhodiola* plants was mixed with 45 mL ethanol of different concentrations in a 50 mL volumetric flask. The samples were extracted by triplicate sonication at 40°C for 30 min, and then cooled to 20°C. Sonicated solutions were made up to 50 mL using ethanol at the concentration used for extraction and mixed well. Prior to HPLC analysis, 1 mL aliquots of the extracts were centrifuged at 8000 *g* for 5 min. Each sample was then measured three times by HPLC. The chromatographic conditions were as follows: column: Hypersil ODS (250 x 4.6 mm, 5 μm); mobile phase: acetonitrile-water (volume ratio of 1:9); flow rate: 1 mL/min; detection wavelength: 280 nm; and injection volume: 10 μL. Standard solutions were prepared by dissolving 0.4 mg salidroside, tyrosol, or gallic acid in 25% methanol. These were used as the stock solutions for the mixed controls. A standard curve was prepared using each of the solutions and 25% methanol as the diluent. Different concentrations of standard solutions were analyzed independently, and the measurements were repeated three times. The concentration of each active component was calculated according to the regression equation.

Data analysis

The ITS sequences were automatically aligned using CLUSTAL_X software (Thompson et al., 1997), followed by appropriate manual corrections to construct more rational alignments. The GC content was calculated using molecular evolutionary genetics analysis (MEGA) software (Kumar et al., 2004). Polymorphisms and haplotypes were analyzed using DNA sequence polymorphism analysis (DnaSP) (Rozas et al., 2003). Phylogenetic relationships between the ITS sequences were reconstructed by means of maximum parsimony (MP) using phylogenetic analysis using parsimony and other methods (PAUP*) version 4.0b10 (Swofford, 1993). Gaps were set as missing states. The MP analysis was performed using the following settings: tree bisection reconnection branch swapping, heuristic search, MULPARS, ACCTRAN, and 100 random addition sequence replicates. The phylogenetic tree was tested using the bootstrap method (1000 replicates).

Statistical analysis was performed using statistical product and service solutions (SPSS) version 13.0 to determine normal distribution of the three active components within the 23 samples. Correlation analysis between haplotypes and the three active components, and between the three active components alone, was also carried out.

RESULTS

ITS sequence analysis

ITS sequences obtained from the *Rhodiola* plants sampled in this study were aligned to a reference sequence. The length of ITS sequences ranged from 614 to 621 bp, and the average GC content was 54.2% (Table 2).

Table 2. Length and G+C content of the ITS sequences in *Rhodiola* samples.

No.	Species	Voucher	Length (bp)	Content of C (%)	Content of G (%)	Content of G+C (%)
1	*R. papillocarpa*	chen2012125	617	27.2	27.1	54.3
2	*R. wallichiana*	chen2012400	617	27.2	27.1	54.3
3	*R. brevipetiolata*	chen2012447	617	27.2	27.1	54.3
4	*R. wallichiana*	chen2012311	617	27.2	27.1	54.3
5	*R. wallichiana* var. *Cholaensis* S.H. Fu	chen2012247	614	26.2	27.2	53.4
6	*R. purpureoviridis*	chen2012110	614	26.2	27.2	53.4
7	*R. himalensis*	chen2012246	617	27.2	27.1	54.3
8	*R. cretinii*	chen2012481	615	27.3	27.6	54.9
9	*R. yunnanensis*	chen2012446	616	26.1	27.3	53.4
10	*R. concinna*	chen2012127	619	26.5	27.6	54.1
11	*R. alsia*	chen2012332	616	26.1	27.3	53.4
12	*R. dumulosa*	ZDJ1201	621	26.2	27.2	53.4
13	*R. cabrida*	ZDJ1204	616	26.6	27.4	54
14	*R. subopposita*	ZDJ1205	614	26.1	27	53.1
15	*R. subopposita*	ZDJ1206	616	26.3	26.9	53.2
16	*R. dumulosa*	ZDJ1207	614	27	27.9	54.9
17	*R. taohoensis*	ZDJ1209	615	27.3	27.6	54.9
18	*R. taohoensis*	ZDJ1210	617	27.2	27.1	54.3
19	*R. taohoensis*	ZDJ1211	614	26.9	27.9	54.8
20	*R. taohoensis*	ZDJ1212	617	27.4	27.7	55.1
21	*R. taohoensis*	ZDJ1213	614	27.2	27.2	54.4
22	*R. taohoensis*	ZDJ1214	614	27.4	27	54.4
23	*R. taohoensis*	ZDJ1215	614	27.2	28	55.2

We identified 75 variable sites across the sequenced region (Table 3).

Table 3. Polymorphisms within the ITS sequences of the *Rhodiola* samples

Species	Haplotype	Variable sites
		22568888111111111111111111111222222233444444444444445555555555555555555666
		16971568001111223466777999990013333790011111122499002222233444555778890 22
		68056828993701912368939403691716345678909846190123708369467564545 13
R. cabrida	H_1	GTT-TCCC-CCCGTCG-CG-TATCCGC-CGGA-CTCCGTTAT------TCGTCCTGCGCTTGGTTGC---TCC-C
R. alsia	H_2	GTC-TCCC-CCCGTCG-CG-GATCCGC-CGGA-TTCCGTTAT------TCGTCCTGCGCTTGGTTTA---TCC-C
R. concinna	H_3	GTC-TCCC-CCCGTCG-CG-GATCCGC-CGGA-CTCCGTTAT------TCGTCCTGTGCTTGGTCGC---TCC-C
R. dumulosa	H_4	GTC-TACC-CCCATCG-CG-GATTCGC-CCAA-CTCCGTTAT------CCGTCCTACGCTTTTTCGC---TCC-C
R. dumulosa	H_5	GTC-TACT-CCCATCG-CG-GATTCGC-CCAA-CTCCGTTAT------CCGTCCTACGCTTTGTCGC---TCC-C
R. subopposita	H_6	GTCGTACC-CCCGTTG-CG-GGTCTGC-CCAT-CTCCGCTAT------TCGTCCTACGCTCTGCCGC---TCT-C
R. himalensis	H_7	GTCGTACC-CCCGTTG-CG-GGTCCGC-CCAT-CTCCGCTAT------TCGCCTTGCACTTGGCCGCTC-TCT-C
R. taohoensis	H_8	GTC-ACCCTACCGTCA-CG-GATCCTTTCCTAACTCCGTTAT------TCGTCCTACGCCTGGTCGC---TCC-C
R. papillocarpa	H_9	GTC-TCCC-CTTGTCG-TA-GATCCGC-TTGA-CTCTATTAT------TCTTTTTACGCTTGGTCGC---TCC-C
R. purpureoviridis	H_{10}	GTC-TCCC-CTTGTCG-TA-GATCCGC-TTGA-CTCTATTAT------TCTTTTTACACTTGGTCGCTC-TCC-C
R. yunnanensis	H_{11}	GTC-TACC-CTTGTCG-TA-GATCCGC-TTTA-CTCTATAAT------TCTTTTTACGCTTGGTCGC---TCC-C
R. wallichiana	H_{12}	GCC-TCCC-CCTGTCGCCA-GACCCGC-TTGA-CTCCATTAT------TATTCTTGCAATTGGTCGC--ATGC-G
R. cretinii	H_{13}	GCC-TCAC-CCTGTCG-CA-GACCCGC-TTGA-CTCCATTATCATAATTATTCTTGCAATTGGTCGC--AAGC-G
R. Wallichiana var.	H_{14}	TCC-TCCC-CCTGTCG-TA-GATCCGC-TTGA-CTCCATTAT------TATTCTTGCAATTGGTCGC--ATGCTG
Cholaensis S.H. Fu	H_{15}	TCC-TCCC-CCTGTCG-TA-GATCCGC-TTGA-CACCATTAT------TATTCTTGCAATTGGTCGC--ATGCTG
R. wallichiana	H_{16}	GCC-TCCC-CCTGCCG-CAAGATCCGC-TTGA-CATCATTCA-----TTATTCTGGCGATTGGTCGCTCATGC-G
R. brevipetiolata		

ITS sequences of 23 samples were used to define 16 different haplotypes. Each of the different species possessed a different haplotype, with different samples of the same species,

except *R. wallichiana* and *R. dumulosa*, possessing the same haplotype (Table 4). These findings confirmed that a correlation exists between *Rhodiola* species and haplotype.

Table 4. Haplotypes and active components of *Rhodiola* samples examined in the study.

Voucher	Species	Haplotype	Active components		
			Salidroside	Tyrosol	Gallic Acid
chen2012125	*R. papillocarpa*	H_9	0.85	0.41	21.7
chen2012400	*R. wallichiana*	H_{12}	0.57	0.067	8.32
chen2012447	*R. brevipetiolata*	H_{16}	5.18	0.24	8.39
chen2012311	*R. wallichiana*	H_{15}	0.35	-	4.21
chen2012247	*R. wallichiana* var. *cholaensis* S. H. Fu	H_{14}	0.44	-	24.75
chen2012110	*R. purpureoviridis*	H_{10}	-	-	54.08
chen2012246	*R. himalensis*	H_7	0.17	-	57.87
chen2012481	*R. cretinii*	H_{13}	0.76	0.21	8.41
chen2012446	*R. yunnanensis*	H_{11}	2.67	0.3	14.22
chen2012127	*R. concinna*	H_3	0.39	-	10.35
chen2012332	*R. alsia*	H_2	4.79	0.79	24.54
ZDJ1201	*R. dumulosa*	H_4	-	-	0.088
ZDJ1204	*R. cabrida*	H_1	11.43	2.2	33.9
ZDJ1205	*R. subopposita*	H_6	12.95	3.23	12.24
ZDJ1206	*R. subopposita*u	H_6	41.41	0.86	14.16
ZDJ1207	*R. dumulosa*	H_5	-	-	6.91
ZDJ1209	*R. taohoensis*	H_8	-	-	5.26
ZDJ1210	*R. taohoensis*	H_8	-	-	5.43
ZDJ1211	*R. taohoensis*u	H_8	-	-	3.25
ZDJ1212	*R. taohoensis*	H_8	0.2	0.071	2.52
ZDJ1213	*R. taohoensis*	H_8	-	-	1.44
ZDJ1214	*R. taohoensis*	H_8	0.16	-	-
ZDJ1215	*R. taohoensis*	H_8	0.38	-	-

Phylogenetic analysis

Using *S. oaxacanum*, *S. alexanderi*, *S. compactum*, and *H. erythrostictum* as outgroups, 704 parsimonious trees were obtained by heuristic search. A representative MP tree is shown in Figure 1 (step length = 331, consistency index (CI) = 0.8731, retention index (RI) = 0.9077). The bootstrap value from 1000 replicates is shown for each branch. This phylogenetic analysis resulted in classification of the 27 analyzed sequences (including outgroups) into two groups (Figure 1). The first group consisted of outgroup species, including *H. erythrostictum*. *S. oaxacanum*, *S. alexanderi*, and *S. compactum*, and clustered into a single clade. The second group consisted of the 23 *Rhodiola* samples included in this study, and was divided into four subclades. *R. subopposita, R. himalensis*, and *R. dumulosa* clustered into the first subclade, whereas *R. cabrida, R. alsia*, and *R. concinna* clustered into a second subclade. *R. taohoensis* species sampled from different locations formed the third subclade, and the fourth subclade consisted of *R. papillocarpa*, *R. purpureoviridis*, *R. yunnanensis wallichiana*, *R. cretinii*, and *R. Brevipetiolata* species. All *Rhodiola* species in this study were clustered into a single clade, indicating that they form a closely related monophyletic group.

Quantitation of active components in Rhodiola samples

The concentrations of salidroside, tyrosol, and gallic acid in the roots of different *Rhodiola* species were measured by HPLC. Our results showed significant differences in the concentrations of the three active compounds between different *Rhodiola* species (Table 4).

The concentration of salidroside in *R. subopposita* was the highest, while concentrations in *R. purpureoviridis*, *R. dumulosa* (two samples), and *R. taohoensis* (four samples) were the lowest. The highest concentration of tyrosol was found in *R. subopposita*, while lowest concentrations were measured in *R. wallichiana*, *R. purpureoviridis*, *R. himalensis*, *R. papillocarpa*, *R. concinna*, *R. subopposita* (two samples), and *R. taohoensis* (five samples). The highest concentration of gallic acid was measured in *R. himalensis*, while lowest concentrations were measured in *R. taohoensis* (two samples).

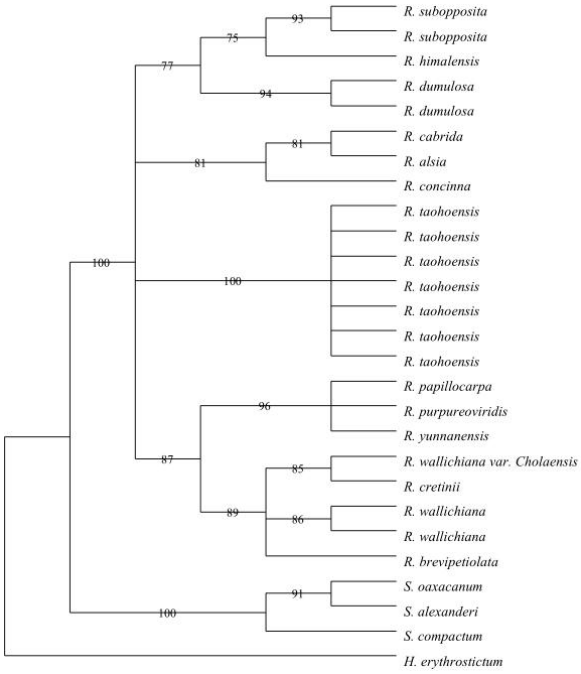

Figure 1. One of the 704 most parsimonious trees according to the ITS sequence data (step length = 331, CI = 0.8731, RI = 0.9077). The numbers on the branches represent the bootstrap support values of the most parsimonious tree.

Correlation analysis between haplotypes and active component concentrations

Among the 16 haplotypes defined by ITS sequence analysis, salidroside was not detected in samples with haplotypes H_4, H_5, or H_{10}. Tyrosol was not detected in H_3, H_5, H_7, H_{10}, H_{14}, or H_{15} haplotype samples, and gallic acid was not detected in samples with the H_{14} or H_{15} haplotypes (Table 4). Interestingly, chen2012400 and chen2012311, which were both *R. wallichiana* samples, and ZDJ1201 and ZDJ1207, which were both *R. dumulosa* samples, possessed different haplotypes and significantly different concentrations of salidroside, tyrosol, and gallic acid. In addition, two *R. subopposita* samples that possessed the H_6 haplotype and seven *R. taohoensis* samples that possessed the H_8 haplotype had significantly different salidroside, tyrosol, and gallic acid concentrations.

Normal distribution analysis showed that salidroside concentration detected in this study ranged from 0 to 2.67 mg/g, with a median of 0.38 mg/g. Tyrosol concentration ranged from 0 to 0.30 mg/g, with a median of 0 mg/g, and gallic acid concentration ranged from 3.25

to 21.70 mg/g, with a median of 8.39 mg/g (Table 5). Correlation analysis showed there was no significant correlation between haplotype and concentration of salidroside, tyrosol, and gallic acid ($P > 0.05$); however, there was a positive correlation between concentration of these three active components ($P < 0.05$) (Table 5).

Table 5. Concentrations and correlation analysis of active components in *Rhodiola* samples

Active components	Concentrations (mg/g)	Ratio of active components	rs	P value
Salidroside	0.38 (0-2.67)	Salidroside: tyrosol	0.874	<0.001
Tyrosol	0 (0-0.30)	Salidroside: gallic acid	0.488	0.018
Gallic acid	8.39 (3.25-21.70)	Tyrosol: gallic acid	0.443	0.034

DISCUSSION

The nrDNA ITS region has a fast rate of evolution, and displays a high degree of nucleotide variation but conservation in sequence length. These properties along with multiple copies of repeat sequences present in the nucleus make ITS sequences ideal for use in phylogenetic analyses. Many copies are highly similar or identical as a result of synchronous evolution of ITS sequences in most angiosperms. However, in recent years, in addition to molecular phylogenetic relationships between species and related genera, ITS sequences have been used to investigate intra-species variation in plants (Li et al., 2014). For example, analysis based on ITS sequence was successfully used in the identification of *Eucommia ulmoides* from different geographical origins in China, thus demonstrating its use as molecular marker (Ma et al., 2004). Differences in ITS sequences can be used to differentiate between species of medicinal plants, which provides significant guidance for the identification of genuine medicinal materials (Zhao et al., 2008).

Our results suggest that the paired variation of ITS sequences in *Rhodiola* species is relatively low despite their diverse morphology. This may be the result of rapid speciation observed in the areas used for sampling triggered by uplift of the Qinghai-Tibetan Plateau, and the extensive selection pressure that followed imposed by an alpine environment. Many plants contain biologically active components as secondary metabolites. The concentration and type of active components varies between plant species, which is likely to be the result of polymorphisms in the functional genes coding for the active components. However, the functional genes themselves are often too long to amplify and be used in sequence analysis techniques. Therefore, more suitable universal genes are used to study the relationship between gene polymorphisms and active components. Alternatively, a gene that is closely related to the variation in the active components can be identified through a study of the functional gene, to provide a basis for further investigation into the genetic mechanisms behind variation in active components (Liu, 2006). Many recent studies on the correlation between gene polymorphisms and active components have suggested that active components are associated with geographical distribution and haplotypes (Li et al., 2011; Wang et al., 2014; Zhang et al., 2015). However, few studies have focused on the correlation between species and active components.

In this study, our results showed that there was no correlation between haplotype and concentrations of salidroside, tyrosol, and gallic acid in *Rhodiola* species ($P > 0.05$). However, there was a positive correlation among concentrations of these three active components ($P < 0.05$).

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank Mr Yongchang Yang at the Northwest Institute of Plateau Biology, Chinese Academy of Sciences, for helping us in the identification of specimens. Research supported by the National Natural Science Foundation of China (#81160519, "Molecular mechanisms of formation and accumulation of salidroside"). The funders had no role in study design, in the collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the article for publication.

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