

# High Levels of Genetic Diversity within One Population of *Rheum tanguticum* on the Qinghai-Tibet Plateau have Implications for Germplasm Conservation

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**Abstract:** *Rheum tanguticum*, a source plant for the traditional Chinese medicine Dahuang (rhubarb), is an endangered species. In this study, we first assessed genetic diversity using 102 internal transcribed spacer (ITS) sequences cloned from one population of *R. tanguticum* on the Qinghai-Tibet Plateau. In total, 1773 polymorphic sites were identified, including 707 sites in the ITS1 region, 773 sites in the ITS2 region, and 293 sites on the 5.8S gene. Overall, transitions were the most common type of mutation and represented 77% of all base substitutions found within the ITS regions of *R. tanguticum*. We found that the average intra-specific genetic distance was  $0.1260 \pm 0.0755$  and ranged from 0 to 0.3618. Furthermore, a neighbor-joining phylogenetic tree constructed using these sequences strongly supported the conclusion that abundant intra-specific variation exists within the ITS regions of *R. tanguticum*. Interestingly, we found that eighty-seven clones of *R. tanguticum* had high similarity to the ITS sequence of *R. rhabarbarum* and ten clones had high similarity to the ITS sequence of *R. officinale*; however, five clones of *R. tanguticum* had similarity to *Fagopyrum esculentum*. Therefore, we observed a significant amount of ITS sequence variation at both the intra-genomic and intra-specific levels in *R. tanguticum*. Based on these results, we suggest that *in situ* conservation is preferable for *R. tanguticum* protection.

**Keywords:** Genetic diversity, intra-specific variation, *Rheum tanguticum*, internal transcribed spacer, endangered species conservation.

## INTRODUCTION

*Rheum tanguticum* Maxim. Ex Balf. (Polygonaceae) is one of the three main sources of Dahuang (rhubarb), a well-known traditional Chinese medicine (TCM). Its phenolic compounds and polysaccharides have shown some promising pharmacological properties [1, 2]. The dried rhizomes and roots of *R. tanguticum* have been widely used as laxatives and anti-inflammatory agents in China [3], while the standardized rhubarb extracts, such as emodin, have become available on the international market [4, 5]. *R. tanguticum*, also known as Tangut rheumor chicken-feet rheum, is native to the Qinghai-Tibet Plateau at altitudes ranging from 2300 to 4200 m [6, 7]. In recent years, the wild resources of *R. tanguticum* were seriously threatened by over-exploitation and an extensive loss of habitat; thus, this species has been included on the important conservation of wild plants list for the Qinghai province [8]. Some measures have been taken to reduce the difference between the commercial demand and the wild resource depletion, such as cultivation in Guoluo [9]. However, reductions to wild *R. tanguticum* populations are a major concern for development of superior

pharmaceutical crop cultivars of the Tangut rheum. Analysis of genetic variations in the endangered species at the population level is critically important for conservation strategies because sufficient genetic variation can increase the species chance of survival [10]. Therefore, the analysis of genetic variation within and between populations becomes important not only for the conservation of the endangered species [11-15] but also for pharmaceutical crop development.

The internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA) has been used extensively in studies on genetic diversity, phylogenetics, and evolution at the species or genera levels due to its fast rate of evolution and great variation [16-18]. The ITS region is composed of three parts: ITS1, ITS2, and the highly conserved 5.8S rDNA gene, which is located between ITS1 and ITS2 [19]. Compared to organellar DNA, the ITS of nrDNA, which is inherited from both parents, can provide much more information [20]. Furthermore, this region is simple to amplify using PCR and a set of universal primers, which are available for a wide range of organisms, and it can be easily sequenced due to its relatively small size [21]. Importantly, high levels of variation are usually observed within ITS regions and are ideal for revealing genetic diversity and population differentiation in many species [22-24]. However, to date, there have been no reports of ITS sequence variation at the intra-specific level in *R. tanguticum*. In previous reports, genetic diversity of *R. tanguticum* was revealed based on ISSR and SSR analysis

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[8, 25]. These studies demonstrated most of genetic variation within populations of *R. tanguticum* rather than between.

The goals of the present study were to identify and characterize the distribution of sequence variation based on the ITS sequences within one population of *R. tanguticum*, analyze potential reasons for sequence variations, and propose conservation strategies for this species.

## MATERIALS AND METHODOLOGY

### Plant Materials

Twenty-nine specimens of *R. tanguticum* were collected from the Baihe pasture of Zoige in the Sichuan Province of China (latitude: 33°18'35"N to 33°21'32"N; longitude: 102°27'12"E to 102°28'9"E; altitude: 3436-3459 m) (see the Appendix Table S1). A typical habitat for *R. tanguticum* is shown in (Fig. 1).



Fig. (1). The habitat of *Rheum tanguticum*.

### Genomic DNA Preparation and PCR Amplification

Genomic DNA was extracted from 20 mg of silica gel-dried leaves using the Plant Genomic DNA Kit (Tiangen Biotech Co., China) after grinding for 1 min at 30 revolutions/s with a DNA Extraction Grinder (MM400; Retsch, Haan, Germany). Polymerase chain reaction (PCR) amplifications of the ITS region were carried out using a Peltier Thermal Cycler ABI9700 (ABI lab, Inc., USA) and the following pair of universal primers: ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC -3') [26]. PCR amplification was performed using 20-50 ng of genomic DNA as template and a total volume of 25  $\mu$ L, which also contained the following: 1 $\times$ PCR buffer (without MgCl<sub>2</sub>), 2.0 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 0.1  $\mu$ M each ITS primer (synthesized by Sangon Co., China), and 1.0 U Taq DNA Polymerase (Bicolor BioScience & Technology Co., China). PCR reaction conditions consisted of an initial denaturation step for 5 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C and extension for 1.5 min at 72°C (+3s/cycle), with a final extension step at 72°C for 7 min [27]. The PCR products were separated on 1.0% agarose gels in 0.5 $\times$ TBE buffer for visualization. Direct sequencing of the PCR products from *R. tanguticum* was unsuccessful,

indicating the presence of polymorphisms within individual specimens.

### Cloning and Sequencing of the ITS Region

The PCR products were purified using the TIAN quick Midi Purification Kit (Tiangen Biotech Co., China). The recovered products were ligated into the pMD19-T vector (Takara Biotech Co., China) following the addition of dATP residues to the 3' ends of the PCR products using the DNA A-Tailing Kit (Takara Biotech Co., China). The cloned products were then transformed into *E. coli* DH5 $\alpha$  cells using standard recombinant DNA techniques.

*E. coli* colonies were cultured in LB culture medium containing ampicillin, and following selection with colony PCR, five positive clones from each sample were sequenced using an ABI3730XL sequencer (Majorbio Co., Shanghai, China). In total, we obtained 105 clones from 29 samples (excluding fungal sequences). Three of the clones (including KF514611, KF514546 and KF514613) showed significant differences when compared with the other sequences (~90bp deletions at the start of the ITS region) and were not analyzed in this paper (see the sequence alignment in Appendix Fig. S1, S2, and S3).

### Sequence Alignment and Date Analysis

The sequences of the ITS regions of *R. tanguticum* were aligned using Codoncode Aligner3.7.1 (CodonCode Co., USA). Genetic variations were analyzed with a Kimura 2-parameter (K2P) distance matrix, which was constructed using the MEGA5.0 software program [28]. The neighbor-joining (NJ) phylogenetic trees were built in the MEGA5.0 software program using the 102 sequenced clones from *R. tanguticum* as well as four other GenBank sequences from other species: *Rheum officinale* (JN187108), *Rheum nobile* (GQ206264), *Rheum rhabarbarum* (JQ288756), and *Fagopyrum esculentum* (EF653685). The 5.8S gene and the ITS1 region were obtained through sequence alignment with *R. rhabarbarum* and *F. esculentum*, and the ITS2 region was obtained using hidden Markov model (HMM)-based annotation methods [29].

## RESULTS

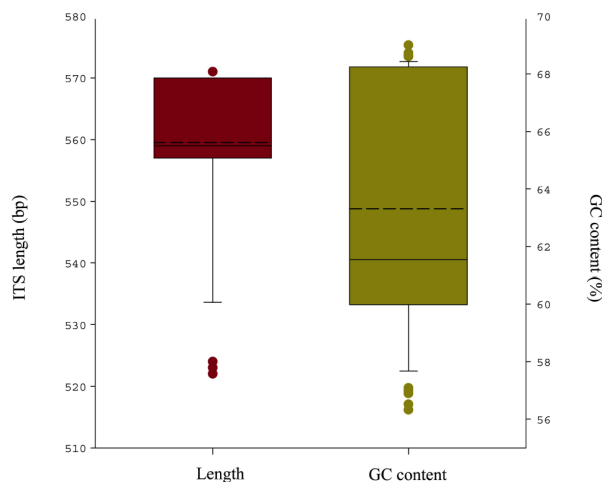
### Sequence Length and GC Content

The average and distribution of the ITS sequence lengths, as well as GC contents, are shown in (Fig. 2). The ITS sequence length ranged from 522-571 bp in the 102 clones from *R. tanguticum*. The average length of the ITS sequences was 559 bp, and the median length was similar to the average. The GC content of the ITS sequences from *R. tanguticum* ranged from 56- 69%, with an average value of 63% and a median of 62%.

### Sequence Variation and Polymorphic Distributions of the ITS Region within Individual Genomes

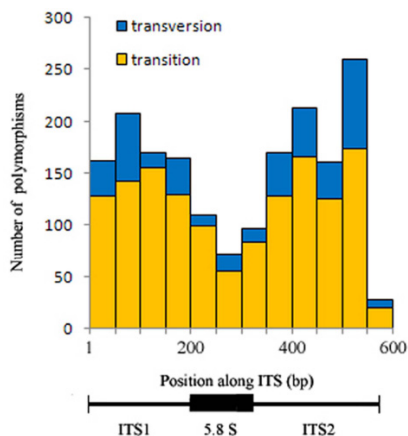
We did not analyze insertions and deletions (indels) in the present study, as they are often spread across multiple sites, particularly in long homopolymeric tracts, and are difficult to identify in an accurate and reliable manner. Therefore, we limited ourselves to the detection and comparative

analysis of base substitution-only type polymorphisms (e.g., transitions and transversions).



**Fig. (2).** ITS sequence length and GC content in 102 clones of *Rheum tanguticum*. Box plots show the interquartile range (IQR) of the data, which is defined as the difference between the 75th and 25th percentiles. The solid and dotted lines in the box represent the median and average values, respectively, for length and GC content.

Despite the exclusion of indels from our study, we identified a remarkably high level of intra-individual variation in *R. tanguticum*, ranging from 0 (in the sample RTM02) to 168 polymorphisms (in the sample RTM09). In total, 1773 polymorphic sites were identified: 707 sites in the ITS1 region, 773 sites in the ITS2 region, and 293 sites on the 5.8S exon (Fig. 3). Overall, the ITS2 regions had nearly one fold more polymorphisms than the ITS1 region. Within the ITS1 region, the polymorphisms were evenly distributed over the 200 bp, whereas polymorphisms in ITS2 were primarily found in the 350-550 bp regions. Transversions were detected at the highest frequency in the 500-550 bp region of ITS2. Furthermore, we found transitions to be the most common type of mutation, representing 77% (1366 of 1773 sites) of all base substitutions in the ITS regions of *R. tanguticum*.



**Fig. (3).** The distribution of polymorphisms (base substitution-only) in the *Rheum tanguticum* rDNA array, as derived from ITS sequencing data and shown in 50-bp bins.

Sample RTM10 of *R. tanguticum* is a typical representative of the samples (see the Appendix Table S1), and a multiple sequence alignment of five clones of this sample is shown in Fig. (4). There were 105 variable sites between KF514583 and the three clones KF514579, KF514580, and KF514581. Only one variable site was observed between KF514583 and KF514582 and a small amount of variation was identified among the other three clones.

#### Analysis of ITS Sequence Variations by Genetic Distance

We divided the *R. tanguticum* mutations into two groups, containing intra-specific and intra-genomic variations, which were then analyzed using K2P genetic distances. In particular, we looked at the distribution of divergences within the classes at a resolution 0.02 distance units (Fig. 5). Our results showed that the average intra-specific distance was  $0.1260 \pm 0.0755$  and that *R. tanguticum* had a relatively high level of sequence divergence, ranging from 0 to 0.3618. In addition, K2P intra-genomic distances ranged from 0 to 0.3338 within individual *R. tanguticum* specimens (see the Appendix Table S1). These results demonstrate a high level of variation within the ITS sequences of *R. tanguticum*.

#### Analysis of Phylogenetic Relationships Based on ITS Sequences Using NJ Trees

We constructed a NJ tree based on the 102 ITS sequences of *R. tanguticum* as well as the four GenBank sequences from *R. officinale*, *R. nobile*, *R. rhubarbarum* and *F. esculentum* (Polygonaceae) (Fig. 6). The tree was divided into three groups: (1) ten clones from six samples of *R. tanguticum* (green region) that had high similarity to the ITS sequence of *R. officinale*, (2) a second group containing *R. nobile*, and (3) the remaining eighty-seven clones (including the blue, yellow, and red regions, as well as KF514638) that had high similarity to the ITS sequence of *R. rhubarbarum*. Overall, the NJ tree supported the hypothesis that the ITS sequences from *R. tanguticum* were polyphyletic. Unexpectedly, five clones of *R. tanguticum* (purple region) clustered most closely with *F. esculentum*.

## DISCUSSION

The present study revealed a high level of intra-specific genetic polymorphisms in the ITS regions of *R. tanguticum*, which was consistent with the molecular evidence provided by ISSR and SSR analysis in previous reports [8, 25, 30]. The high levels of genetic diversity within the population of *R. tanguticum* may be due to several factors. First, mating systems are thought to be one of the most important factors determining genetic diversity in *R. tanguticum*. In the wild, rheum plants are self-incompatible [31], and it has been proven that self-incompatibility is crucial for the maintenance of high levels of genetic variability within many species [32]. Second, the physiological structure of *R. tanguticum* may be another important factor. Indeed, *R. tanguticum* possesses panicles containing numerous small flowers that can produce large amounts of trigonous achenes, which are small, light, and show a high germination rate between plants [33]. Therefore, for developmental reasons, this species may have more opportunity to accumulate genetic

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KF514579 TGTCGAAACC TGCACAAGCA GACAGACCCG CAAACCCGTC TCTAACCCGC CGTCGGGGGG ATCGGGCTCT CTTTGAGCC 80bp
KF514580 ..... .G..... .AC.....
KF514581 ... A. .... .G..... .AC.....
KF514582 ..... .GT...A. ....AT. .G..T..A. .... .C.A.....G .....
KF514583 ..... .GT...A. ....AT. .G..T..A. .... .C.A.....G .....

KF514579 CCCTCCCCG GCGTGCGCCA ACCAAACCCG GACGCGGATT ATGCCAAGGA CTATGAACAA GAGCGTGTC CATGCACCTC 160bp
KF514580 ...- .T.... .A... ..GT.T.... GC..... .C.... .G...A
KF514581 .....T.... .....G.....
KF514582 ...C.TA..A .T..... .G...A... GC..... .CA..T .G..TG...T
KF514583 ...C.TA..A .T..... .G...A... GC..... .CA..T .G..TG...T

KF514579 GCGCGCGTG CGACATTGCA TCGTTTCTAC TTAACAGAAC GACTCTCGAC AATGGATATC TTGGCTCTCG CATCGATGAA 240bp
KF514580 ..T...A... ..C..G .....T .....G...C.....C.....T.....
KF514581 .....G.....
KF514582 A...T... ..G.C..G .A..... .AG...A.....C.....
KF514583 A...T... ..G.C..G .A..... .AG...A.....C.....

KF514579 GAACGTAGCG AAATGCGATA CTTGGTGTGA ATTGCAGAAT CCCATGAACC ATCGAGTCTT TGAACGCAAG TTGTGCCCGA 320bp
KF514580 .....T.....T.....C.....T.....CA.....
KF514581 .....
KF514582 ...T.....A..A... ..C.....
KF514583 ...T.....A..A... ..C.....

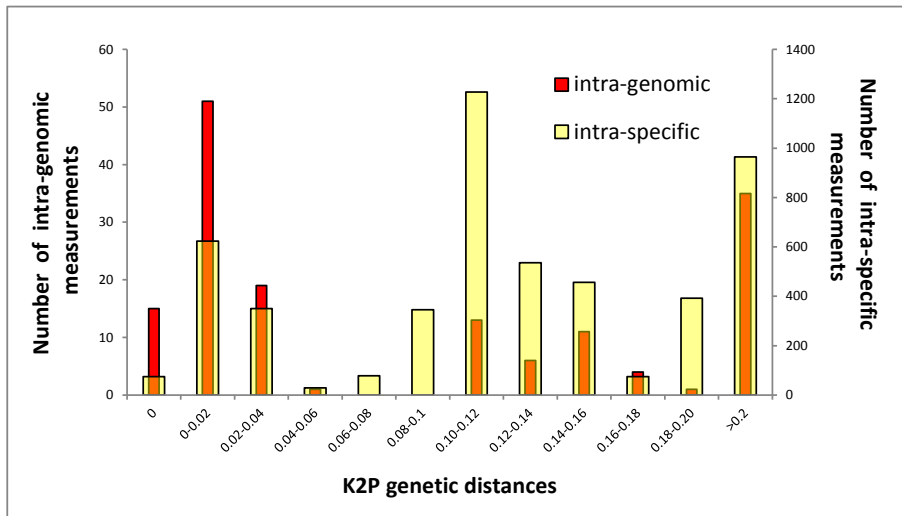
KF514579 AGCCTTCTGG CCGAGGGCAC GTCTGTCTGG GCGTCACGCA CCTTATCGCA CATGCCCCCT CCGAGGGGCC GAGGCAGAGA 400bp
KF514580 .....GC....C..CC......G...A--.G...G..A
KF514581 .....G.....
KF514582 .....A.....A......A......GCG...C..CC......T.G......G.....AG
KF514583 .....A.....A......A......GCG...C..CC......T.G......G.....AG

KF514579 CTGGCCTCCC ATGCGTCTCG GTGCACGACC GGCCTAAACG CAGGCCCCGC GGCCACGAGA AGCCGCAACG ATGTGTGGTG 480bp
KF514580 ..A....AT G...C... ..G.T .....A....G......T.T..G...A.....
KF514581 .....T.....
KF514582 T.....T.G...C...A .A..T... ..A...T. ....AT ...G.....ATG.....
KF514583 T.....T.G...C...A .A..T... ..A...T. ....AT ...G.....ATG.....

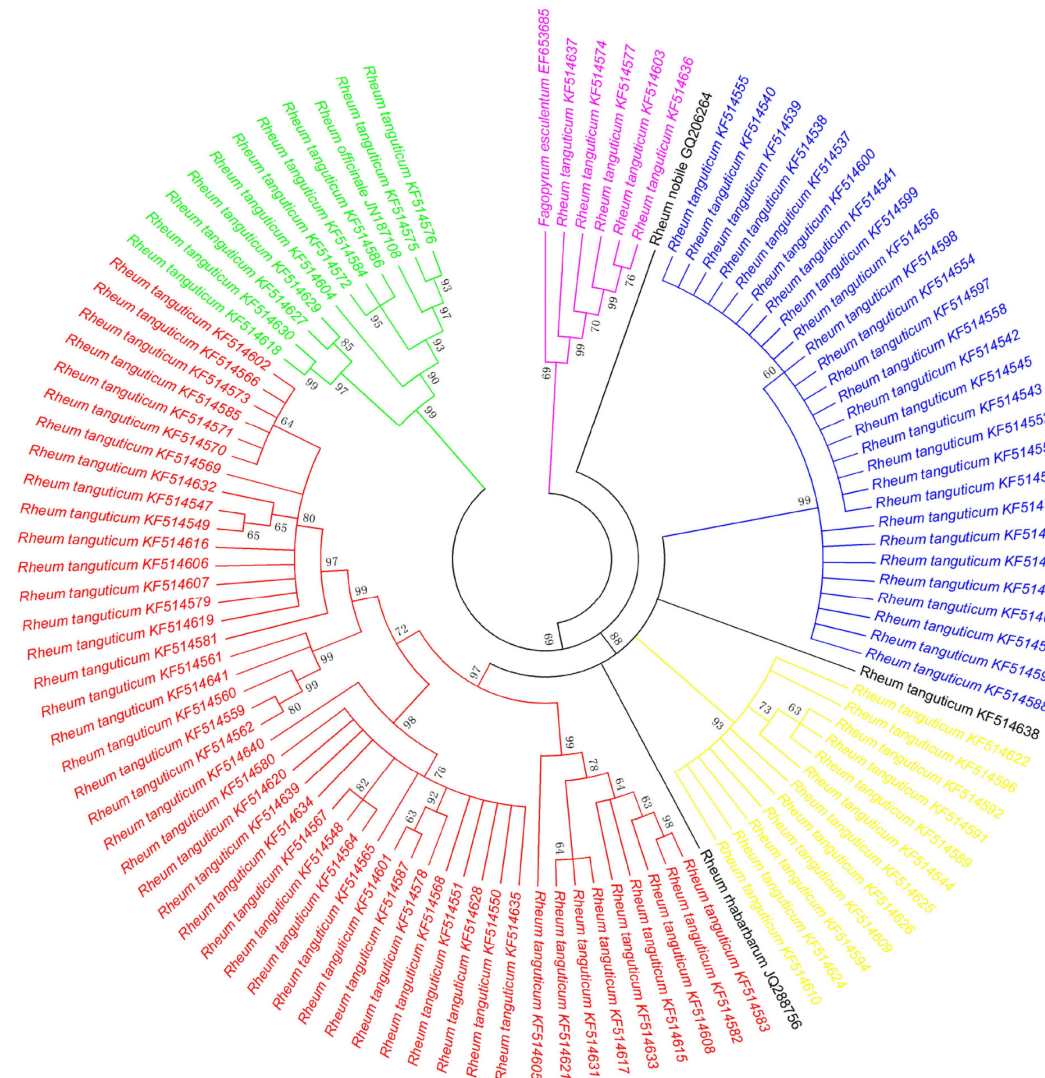
KF514579 TACCAGCGGC CTCGTCCCGT GAAGCATCGC ATCGTGTCTC GCGCGGACCC TGGGCGCCAA AGGGCCTCGA CCACCGTTG 559bp
KF514580 ...T.....A.G....T..G.....C.....C.....
KF514581 .....C...T.....C.....
KF514582 .....A.A...T..G....G.....C...T.....C.....
KF514583 .....A.A...T..G....G....G.....C...T.....C.....

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**Fig. (4).** The complete alignment matrix of the ITS sequence from sample RTM10 of *Rheum tanguticum*.



**Fig. (5).** The relative distributions of intra-genomic divergence (red) between clones within individual *Rheum tanguticum* samples and intra-specific variation (yellow) for the ITS sequences. x axis, K2P genetic distance; y axis, frequency.



**Fig. (6).** Circle Neighbor-Joining trees based on the ITS sequences of *Rheum tanguticum* and several closely related species. Bootstrap scores (1000 replicates) are shown ( $\geq 60\%$ ) for each branch.

mutation compared to other species [34]. Finally, the environment may drive *R. tanguticum* to accumulate high levels of genetic diversity. *R. tanguticum* is distributed primarily throughout the eastern region of the Qinghai-Tibet Plateau [7], which is highly susceptible to climate change. For example, Quaternary climatic oscillations during glacial and interglacial stages in this region may have driven periods of retreat and expansion in this species.

In addition, we captured some genetic information from *R. tanguticum* that had not been revealed previously when we studied genetic diversity between clones. From analyses of the phylogenetic tree, ten clones of *R. tanguticum* had high similarity to ITS sequence of *R. officinale*, which is supported by morphological classification [35]. However, eighty-seven clones had high similarity to ITS sequence of *R. rhabarbarum*, which was consistent with palynological research [36, 37]. Pollen morphology demonstrated that *R. tanguticum* and *R. rhabarbarum* were both microechinate-foveolate, while *R. nobile* and *R. officinale* were different. Unexpectedly, five clones of *R. tanguticum* were similar to *F. esculentum*. These two species belong to different genera of the Polygonaceae, which may indicate that the ITS of nrDNA inherited from parents can reveal horizontal gene-transfer and hybridization events in the species evolutionary history. The phenomena of identical variants across genera in nature were also found in the nuclear genome of *Eleutherococcus giraldii* and *Panax ginseng* of Araliaceae [38] and in the nuclear genome of *Dendropanax hainanensis* and *Merillinopanax membranifolius* of Araliaceae [39].

We suggest that the preferable choice for protection of *R. tanguticum* is *in situ* conservation because of its high intra-specific genetic variation and its living environment. Genetic diversity is important to consider when formulating conservation strategies for species because it is critical to the capability of a species to adapt to environmental changes [40]. In other words, we must select appropriate protection methods to maintain as much genetic diversity as possible. For example, due to low intra-specific variation and fewer wild resources of *P. ginseng*, tissue culture, somatic embryogenesis, and cultivation were applied [41-43]. However, high genetic variation and highly structured geographic patterns were found in *Lamiophlomis rotata* (Lamiaceae), so *in situ* method was used to preserve it in as many populations as possible [44]. In addition, we also consider the physiological characteristics and the living conditions of species when formulating conservation strategies. The protection of *Eucalyptus impensa* in Western Australia is a classic example. After *in situ* conservation, this species was still threatened due to frequent fire and disease. Additionally, the seeds of this species were difficult to obtain, thus tissue culture and micropropagation methods, such as *in vitro* methods, were used in the propagation of *E. impensa* and had success [45]. Nevertheless, over-exploitation has severely threatened the availability of *R. tanguticum* in nature. Hence, based on the high genetic diversity of *R. tanguticum*, the first step will be to prohibit excavation and establish nature reserves for remaining populations. The second step will be to conduct artificial cultivation to breed excellent germplasms of *R. tanguticum* and solve the difference between conservation of *R. tanguticum* and increasing market requirements.

## CONCLUSIONS

In summary, the present study revealed a high level of genetic diversity in the ITS regions of *R. tanguticum*. In addition, we identified genetic information from *R. tanguticum* that has not been revealed previously. Our results showed that ten clones of *R. tanguticum* had high similarity to the ITS sequence of *R. officinale*, eighty-seven clones had high similarity to the ITS sequence of *R. rhabarbarum*, and five clones of *R. tanguticum* were similar to *F. esculentum*. Based on these results, *in situ* conservation is the preferable choice for protection of *R. tanguticum*.

## CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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## ABBREVIATIONS

ITS	= Internal transcribed spacer of nuclear ribosomal DNA
ITS1	= Internal transcribed spacer 1 of nuclear ribosomal DNA
ITS2	= Internal transcribed spacer 2 of nuclear ribosomal DNA
nrDNA	= Nuclear ribosomal DNA
TCM	= Traditional Chinese medicine
PCR	= Polymerase chain reaction
NJ tree	= Neighbor-joining phylogenetic tree
K2P	= Kimura 2-parameter distance
MEGA5.0	= Molecular Evolutionary Genetics Analysis version 5.0
HMM	= Hidden Markov model
Indels	= Insertions and deletions

## REFERENCES

- [1] Liu, L.; Yuan, S.F.; Long, Y.; Guo, Z.J.; Sun, Y.; Li, Y.H.; Niu, Y.B.; Li, C.; Mei, Q.B. Immunomodulation of *Rheum tanguticum* polysaccharide (RTP) on the immunosuppressive effects of dexamethasone (DEX) on the treatment of colitis in rats induced by 2,4,6-trinitrobenzene sulfonic acid. *Int. Immunopharmacol.*, **2009**, *9* (13-14), 1568-1577.
- [2] Krenn, L.; Presser, A.; Pradhan, R.; Bahr, B.; Paper, D.H.; Mayer, K.K.; Kopp, B. Sulfemodin 8-O-β-D-Glucoside, a new sulfated anthochinone glycoside, and antioxidant phenolic compounds from *Rheum emodi*. *J. Nat. Prod.*, **2003**, *66* (8), 1107-1109.

- [3] Chinese Pharmacopoeia Commission. *Pharmacopoeia of the People's Republic of China*; China Medical Science and Technology Press: Beijing **2010**.
- [4] Huang, Q.; Shen, H.M.; Shui, G.H.; Wenk, M.R.; Nam Ong, C. Emodin Inhibits Tumor Cell Adhesion through Disruption of the Membrane Lipid Raft-Associated Integrin Signaling Pathway. *Cancer Res.*, **2006**, *66* (11), 5807-5815.
- [5] Srinivas, G.; Anto, R.J.; Srinivas, P.; Vidhyalakshmi, S.; Senan, V.P.; Karunakaran, D. Emodin induces apoptosis of human cervical cancer cells through poly (ADP-ribose) polymerase cleavage and activation of caspase-9. *Eur. J. Pharmacol.*, **2003**, *473* (2-3), 117-125.
- [6] Liu, S.W. *Flora of Qinghai*. Qinghai People Press: Xining, **1997**, pp. 155-156.
- [7] Bao, B.J.; Grabovskaya-Borodina, A.E. *Flora of China*. Li, A.R.; Bao, B.J., Ed.; Science Press: Beijing, Missouri Botanical Garden: St. Louis, **2003**, *5*, pp. 341-350.
- [8] Hu, Y.P.; Wang, L.; Xie, X.L.; Yang, J.; Li, Y.; Zhang, H.J. Genetic diversity of wild populations of *Rheum tanguticum* endemic to China as revealed by ISSR analysis. *Biochem. Syst. Ecol.*, **2010**, *38* (3), 264-274.
- [9] Jiao, X.L.; Zhang, F.L. Cultivation methods of *Rheum tanguticum* in Guoluo. *China Sci. Technol. Inform.*, **2006**, *5*, 116.
- [10] Cote, C.T. Genetic variation in rare and common plants. *Annu. Rev. Ecol. Syst.*, **2003**, *34*, 213-237.
- [11] Huang, Y.; Zhang, C.Q.; Li, D.Z. Low genetic diversity and high genetic differentiation in the critically endangered *Omphalogramma souliei* (Primulaceae): implications for its conservation. *J. Syst. Evol.*, **2009**, *47* (2), 103-109.
- [12] Torres, E.; Iriando, J.M.; Escudero, A.; Pérez, C. Analysis of within-population spatial genetic structure in *Antirrhinum microphyllum* (Scrophulariaceae). *Am. J. Bot.*, **2003**, *90*(12), 1688-1695.
- [13] de Jesus, O.N.; Silva, Sde.O.; Amorim, E.P.; Ferreira, C.F.; de Campos, J.M.; Silva, Gde.G.; Figueira, A. Genetic diversity and population structure of Musa accessions in *ex situ* conservation. *BMC Plant Biol.*, **2013**, *13*, 41.
- [14] Tero, N.; Aspi, J.; Siikamäki, P.; Jäkäläniemi, A.; Tuomi, J. Genetic structure and gene flow in a metapopulation of an endangered plant species, *Silene tatarica*. *Mol. Ecol.*, **2003**, *12* (8), 2073-2085.
- [15] Ding, G.; Zhang, D.Z.; Ding, X.Y.; Zhou, Q.; Zhang, W.C.; Li, X.X. Genetic variation and conservation of the endangered Chinese endemic herb *Dendrobium officinale* based on SRAP analysis. *Plant Syst. Evol.*, **2008**, *276*, 149-156.
- [16] Chinese Plant BOL Group. Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. *Proc. Nat. Acad. Sci. USA*, **2011**, *108* (49), 19641-19646.
- [17] Hřibová, E.; Čížková, J.; Christelová, P.; Taudien, S.; de Langhe, E.; Doležel, J. The ITS1-5.8S-ITS2 sequence region in the Musaceae: structure, diversity and use in molecular phylogeny. *PLoS One*, **2011**, *6* (3), e17863.
- [18] Nieto Feliner, G.; Rosselló, J.A. Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants. *Mol. Phylogenet. Evol.*, **2007**, *44* (2), 911-919.
- [19] 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.; Leon, C. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS One*, **2010**, *5* (1), e8613.
- [20] Chase, M.W.; Fay, M.F. Barcoding of plants and fungi. *Science*, **2009**, *325*, 682-683.
- [21] Poczai, P.; Hyvärinen, J. Nuclear ribosomal spacer regions in plant phylogenetics: problems and prospects. *Mol. Biol. Rep.*, **2010**, *37* (4), 1897-1912.
- [22] Yamaji, H.; Fukuda, T.; Yokoyama, J.; Pakd, J.H.; Zhou, C.Z.; Yang, C.S.; Kondo, K.; Morota, T.; Takeda, S.; Sasaki, H.; Maki, M. Reticulate evolution and phylogeography in *Asarum* sect. *Asi-sarum* (Aristolochiaceae) documented in internal transcribed spacer sequences (ITS) of nuclear ribosomal DNA. *Mol. Phylogenet. Evol.*, **2007**, *44* (2), 863-884.
- [23] Matsumura, S.; Yokoyama, J.; Fukuda, T.; Maki, M. Intraspecific differentiation of *Limonium wrightii* (Plumbaginaceae) on north-western Pacific Islands: Rate heterogeneity in nuclear rDNA and its distance-independent geographic structure. *Mol. Phylogenet. Evol.*, **2009**, *53* (3), 1032-1036.
- [24] Sass, C.; Little, D.P.; Stevenson, D.W.; Specht, C.D. DNA Barcoding in the Cycadales: Testing the Potential of Proposed Barcoding Markers for Species Identification of Cycads. *PLoS One*, **2007**, *2* (11), e1154.
- [25] Chen, F.J.; Wang, A.L.; Chen, K.M.; Wan, D.S.; Liu, J.Q. Genetic diversity and population structure of the endangered and medically important *Rheum tanguticum* (Polygonaceae) revealed by SSR markers. *Biochem. Syst. Ecol.*, **2009**, *37* (5), 613-621.
- [26] White, T.J.; Bruns, T.; Lee, S.; Taylor, J. *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*. In: Innis, M.A.; Gelfand, D.H.; Sninsky, J.J.; White, T.J., Ed.; Academic: San Diego, **1990**; pp. 315-322.
- [27] Kress, W.J.; Wurdack, K.J.; Zimmer, E.A.; Weigt, L.A.; Janzen, D.H. Use of DNA barcodes to identify flowering plants. *Proc. Natl. Acad. Sci. USA*, **2005**, *102* (23), 8369-8374.
- [28] Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.*, **2011**, *28* (10), 2731-2739.
- [29] Keller, A.; Schleicher, T.; Schultz, J.; Müller, T.; Dandekar, T.; Wolf, M. 5.8S-28S rRNA interaction and HMM-based ITS2 annotation. *Gene*, **2009**, *430* (1-2), 50-57.
- [30] Wang, X.M.; Yang, R.; Feng, S.F.; Hou, X.Q.; Zhang, Y.Q.; Li, Y.; Ren, Y. Genetic Variation in *Rheum palmatum* and *Rheum tanguticum* (Polygonaceae), Two Medicinally and Endemic Species in China Using ISSR Markers. *PLoS One*, **2012**, *7* (12), e51667.
- [31] Komatsu, K.; Nagayama, Y.; Tanaka, K.; Ling, Y.; Basnet, P.; Meselhy, M.R. Development of a high performance liquid chromatographic method for systematic quantitative analysis of chemical constituents in rhubarb. *Chem. Pharm. Bull.*, **2006a**, *54* (7), 941-947.
- [32] Borba, E.L.; Semir, J.; Shepherd, G.J. Self-incompatibility, inbreeding depression and crossing potential in five Brazilian *Pleurothallis* (Orchidaceae) species. *Ann. Bot.*, **2001**, *88*, 89-99.
- [33] Xiao, S.P.; Chen, M.; Huang, L.Q.; Gao, F. Primary study on shapes of fruits and germination characters of seeds of Radix et Rhizoma Rhei. *China J. Chin. Mater. Med.*, **2007**, *32* (3), 195-199.
- [34] Nybom, H. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Mol. Ecol.*, **2004**, *13* (5), 1143-1155.
- [35] Li, A.R. *Flora Republicae Popularis Sinicae*; Science Press: Beijing, **1998**.
- [36] Wang, A.L.; Yang, M.H.; Liu, J.Q. Molecular phylogeny, recent radiation and evolution of gross morphology of the rhubarb genus *Rheum* (Polygonaceae) inferred from chloroplast DNA *trnL-F* sequences. *Ann. Bot.*, **2005**, *96*, 489-498.
- [37] Yang, M.H.; Zhang, D.M.; Zheng, J.H.; Liu, J.Q. Pollen morphology and its systematic and ecological significance in *Rheum* (the Rhubarb genus, Polygonaceae) from China. *Nord. J. Bot.*, **2001**, *21*, 411-418.
- [38] Song, J.Y.; Shi, L.C.; Li, D.Z.; Sun, Y.Z.; Niu, Y.Y.; Chen, Z.D.; Luo, H.M.; Pang, X.H.; Sun, Z.Y.; Liu, C.; Lv, A.; Deng, Y.P.; Larson-Rabin, Z.; Wilkinson, M.; Chen, S.L. Extensive pyrosequencing reveals frequent intra-genomic variations of internal transcribed spacer regions of nuclear ribosomal DNA. *PLoS One*, **2012**, *7* (8), e43971.
- [39] Mitchell, A.; Wen, J. Phylogeny of *Brassaiopsis* (Araliaceae) in Asia based on nuclear ITS and 5S-NTS DNA sequences. *Syst. Bot.*, **2005**, *30*, 872-886.
- [40] Jump, A.S.; Marchant, R.; Peñuelas, J. Environmental change and the option value of genetic diversity. *Trends Plant Sci.*, **2009**, *14* (1), 51-58.
- [41] Kevers, C.; Gal, N.L.; Monteiro, M.; Dommes, J.; Gaspar, T. Somatic embryogenesis of *Panax ginseng* in liquid cultures: a role for polyamines and their metabolic pathways. *J. Plant Growth Regul.*, **2000**, *31*, 209-214.
- [42] Kim, Y.J.; Kim, M.K.; Shim, J.S.; Pulla, R.K.; Yang, D.C. Somatic embryogenesis of two new *Panax ginseng* cultivars Yun-Poong and Chun-Poong. *Russian J. Plant Physiol.*, **2010**, *57*, 283-289.
- [43] Chen, X.C.; Liao, B.S.; Song, J.Y.; Pang, X.H.; Han, J.P.; Chen, S.L. A fast SNP identification and analysis of intraspecific varia-

- tion in the medicinal *Panax* species based on DNA barcoding. *Gene*, **2013**, *530*, 39-43.
- [44] Liu, J.; Wang, L.; Geng, Y.; Wang, Q.; Luo, L.; Zhong, Y. Genetic diversity and population structure of *Lamiophlomis rotata* (Lamiaceae), an endemic species of Qinghai-Tibet Plateau. *Genetica*, **2006**, *128* (1-3), 385-94.
- [45] Bunn, E. Development of *in vitro* methods for *ex situ* conservation of *Eucalyptus impensa*, an endangered mallee from southwest Western Australia. *Plant Cell Tissue Organ Cult.*, **2005**, *83*, 97-102.

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