J Plant Biotechnol (2016) 43:174-180
DOI:http://dx.doi.org/10.5010/JPB.2016.43.2.174

ISSN 1229-2818 (Print)
ISSN 2384-1397 (Online)

Research Article

Genetic diversity analysis of *Glycyrrhiza uralensis* using 8 novel polymorphic microsatellite markers

Yurry Um · Mei-Lan Jin · Yi Lee · Mok Hur · Seon Woo Cha · Chan Sik Jung · Seong Min Kim · Jeong-Hoon Lee

Received: 30 May 2016 / Revised: 7 June 2016 / Accepted: 16 June 2016

Abstract Licorice plant (*Glycyrrhiza* spp.) is an important herb, but the major portion of the national demand is imported to Korea because the domestic production base is vulnerable. We performed basic molecular breeding research for domestic cultivation and production. All publicly available *G. uralensis* EST sequences, which totaled 56,089, were assembled into 4,821 unigenes and examined for microsatellites. Eight polymorphic microsatellite loci were identified and 16 *G. uralensis* and 6 *G. glabra* accessions, which were collected from different locations, were genotyped using the microsatellites. Genetic diversity within the accessions was estimated by construction of a dendrogram. The dendrogram was clustered into two groups. The results showed that there is a correlative genetic relationship between species. The microsatellite markers were found to be useful for diversity analysis as they are able to successfully distinguish the *Glycyrrhiza* accessions.

Keywords *Glycyrrhiza*, Microsatellites, Genetic diversity

Introduction

Our experimental focus for this study was to analyze the genetic relationship between the collections of licorice plant (*Glycyrrhiza* spp.), which is one of the most ancient medicinal herbs and has been used as a Chinese herbal medicine to treat infectious diseases for over 3,000 years (Zhang and Ye 2009). Moreover, the use of licorice dates back thousands of years, and it is widely used as a crude drug, a natural sweetener for foods, and a flavoring agent for American tobacco (Sudo et al. 2009). Licorice is made from the dried roots and rhizomes (stolons) of *Glycyrrhiza uralensis*, *G. glabra*, and *G. inflata*. The plants are leguminous and native to Northern Asia, the Middle East, and Southern Europe. There are approximately 30 *Glycyrrhiza* species in the world (Asl and Hosseinzadeh, 2008). Toward improving natural resource conservation and breeding, recent research projects using several molecular marker types were focused on licorice plant's genetic diversity and population analysis (Erayman et al. 2014; Liu et al. 2015).

Divergence of wild populations has been analyzed using several molecular markers. Molecular genetic diversity studies for *Glycyrrhiza* species were performed with random amplified polymorphic DNA (RAPD) (Khan et al. 2009), inter-simple sequence repeat (ISSR) (Yao et al. 2008), and simple sequence repeat (SSR) (Erayman et al. 2014; Liu et al. 2015). Yamazaki et al. (1994) adopted RAPD and restriction fragment length polymorphism (RFLP) analyses to analyze genetic relationships of *Glycyrrhiza* species and found that *G. glabra* L. and *G. uralensis* Fisch are different species. Among the molecular markers, microsatellites or SSRs have been proved to be highly informative DNA markers due to their high degree of polymorphism and co-dominant mode of inheritance and genome-wide distribution. SSR markers are widely used for various genetic analyses such as genome mapping, cultivar identification, marker-assisted selection, genetic diversity analysis, phylogenetic relationship analysis, and population and evolutionary studies (Wang et al. 2009; Kalia et al. 2011). EST-based microsatellite markers are gene-targeted markers with known or putative functions; we can select the alleles if they are related to a targeted trait (Sorrells and Wilson 1997). In addition, EST-based microsatellite markers could be used in different species because of their highly conserved nature (Varshney et al. 2005ab). With

Y. Um · M. L. Jin · M. Hur · S. W. Cha · C. S. Jung · J. H. Lee (✉)
Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, Rural Development Administration, Eumseong 27709, Korea
e-mail: artemisia@korea.kr

Y. Lee
Department of Industrial Plant Science and Technology, Chungbuk National University, Cheongju 28644, Korea

S. M. Kim
Department of Plant Resources, College of Industrial Science, Kongju National University, Yesan 32439, Korea

advances in sequencing technology, a huge amount of EST data has been produced for various species, including *G. uralensis*. These data are available in databases and can be economically and easily mined for EST-based microsatellite markers. In this work, publicly available *G. uralensis* EST sequences were assembled for the development of microsatellites. Identified microsatellites were used to test the genetic relationship and transferability in *Glycyrrhiza* species.

Materials and Methods

Plant materials

A total of 22 *Glycyrrhiza* accessions, including two *Glycyrrhiza* species, used in this study were collected from Canada, Mongolia, Uzbekistan, China, and Korea (Table 1). The accessions include *G. glabra* and *G. uralensis*. Plant materials were collected from 1-year-old fresh, healthy plants grown in a field in Eumseong, Korea. *Glycyrrhiza* leaf tissues were harvested from each genetic resource. The harvested leaf tissues were frozen in liquid nitrogen for genomic DNA isolation. Genomic DNA was isolated using DNeasy Plant mini kit (QIAGEN, Tokyo, Japan) according to the manufacturer's specifications. All DNA samples were preserved at -80°C until analyses were performed.

Data processing and assembly

All available *G. uralensis* sequences (56,089 ESTs; Table 2) were downloaded from NCBI (www.ncbi.nlm.nih.gov/) in August 2014. SeqClean (https://sourceforge.net/projects/seqclean/files/seqclean-x86_64.tgz/download) was used to remove potential impurities such as vectors, adapters, linkers, etc. The parameters of SeqClean were set so that the program would not remove poly(A)/(T) tails, as having poly(A)/(T) tails in the sequence could be helpful (Morin et al. 2008). The data analysis was performed using MISA script (http://pgrc.ipk-gatersleben.de/misa/) software (Chevreux et al. 2004) to search for SSR occurrences per contig. The default settings for the parameters were used with a few changes as follows: uniform read distribution (off), masking poly(A)/(T) tails (on), no quality file requirement (on), quality clipping (on), extra gap penalty (on), and non-IUPAC consensus pre seq type was forced. Ontological analysis of the EST assembly was conducted using Blast2Go (Conesa et al. 2005).

Table 1 *Glycyrrhiza* spp. accessions used in this study

No.	Sample name	Species	Location
1	1A	*G. uralensis*	KOR
2	1B	*G. uralensis*	〃
3	1C	*G. uralensis*	〃
4	2A	*G. glabra*	CAN
5	2B	*G. glabra*	〃
6	2D	*G. glabra*	〃
7	2E	*G. glabra*	〃
8	3A	*G. uralensis*	MNG
9	3C	*G. uralensis*	〃
10	3E	*G. uralensis*	〃
11	4C	*G. uralensis*	MNG
12	5A	*G. uralensis*	MNG
13	5B	*G. uralensis*	〃
14	5C	*G. uralensis*	〃
15	5D	*G. uralensis*	〃
16	5E	*G. uralensis*	〃
17	6A	*G. glabra*	UZB
18	6B	*G. glabra*	〃
19	7A	*G. uralensis*	CHN
20	7B	*G. uralensis*	〃
21	7C	*G. uralensis*	〃
22	7E	*G. uralensis*	〃

KOR, Korea; CAN, Canada; MNG, Mongolia; UZB, Uzbekistan; CHN, China

PCR amplification and genotyping

A total of 4,821 unigenes obtained from the assembly were subjected to SSRIT to identify SSRs (Temnykh et al. 2001) and to design primers using Primer3 (Koressaar and Remm 2007; Untergrasser et al. 2012). The default settings of the program were used with some adjustments: product size 250 ~ 500 bp, optimum 300 bp; primer size 18 ~ 25 nt, optimum 22 nt; primer Tm 55 ~ 60°C, optimum 57°C; primer GC content 40 ~ 60%, optimum 50%. Single PCRs were carried out for the 22 accessions with 8 EST-based microsatellite primers. The PCR mix contained 2 pmol and FAM-labelled primers and 2 pmol of the forward primer in a 25 ml reaction volume with 2.5 ml PCR buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 50 ~ 100 ng template DNA, and 1 U Taq DNA polymerase. Conditions for all PCR amplifications were as follows: 94°C for 5 min, then 30 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 45 s, final extension at 72°C for 10 min. PCR products were separated in a 2.0% agarose gel to visualize PCR amplification. PCR primer sets used in these experiments are listed in Table 2. Forward primers were labeled with a virtual dye 6-FAM, NED, VIC or PET (Applied Biosystems). After PCR amplification, 0.2 ml of PCR products was mixed with 9.8 ml Hi-Di formamide (Applied Biosystems) and 0.2 ml of GeneScanTM 500 LIZ® size standard (Applied Biosystems, Foster City, CA, USA). The mixture was denatured at 95°C for 5 min and placed on ice. The amplified fragments were separated by capillary electrophoresis on the ABI 3730 DNA analyzer (Applied Biosystems) using a 50-cm capillary with a DS-33 install standard as a matrix. We analyzed the amplicon size using the GeneMapper software (version 4.0; Applied Biosystems).

Data analysis for genetic diversity

For each accession, fragments amplified with the EST-based microsatellite markers were scored as present (1) or absent (0). Genetic parameters including major allele frequency (MAF), number of alleles (NA), genetic diversity (GD), expected heterozygosity (HE), and polymorphic information content (PIC) were measured by calculating the shared allele frequencies using the PowerMarker software (version 3.25) (Liu and Muse 2005). The data were then used to compute the PIC value for each polymorphic marker fragment according to the formula: $PIC_i = 2f_i\ (1\text{-}f_i)$ where f_i is the frequency of band presence (Roldán-Ruiz et al. 2000). The correlation of geographic and genetic distances from principal coordinate analysis (PCoA) was performed using NTSYS software v. 2.2 (Rohlf 2000; Rohlf 2004). A dendrogram was made using unweighted neighbour-joining method. Bootstrap analysis was performed with 1,000 replications. Mantel's test was used to determine the correlation of the dissimilarity matrix and the dendrogram.

Results and Discussion

Assembly and annotation

A total of 56,089 EST sequence reads from a publicly available *Glycyrrhiza* EST database (NCBI) were cleaned using SeqClean software (Table 2). After cleaning, 55,492 ESTs were assembled with CLC Genomics Workbench (CLC bio, Aarbus, Denmark). As a result, 55,492 reads were assembled into 12,061 singletons and 2,867 contigs. The singleton and contigs represented 4,821 unigenes ranging from 81 to 3,786 nucleotides in length, with an average length of 656 nt. Microsatellite search module (MISA) was used to identify the microsatellite loci from the 4,821 unigenes. In total, 3,053 SSRs distributed in 4,821 unigenes (63.3%) were found-roughly, one SSR for each 8.7 kb of the marker evaluation and polymorphism detection,

Abundance and distribution of EST-based microsatellites

Hexa-nucleotide repeats ranked in highest abundance, accounting for 63.78% of the SSRs. Hepta-, di-, and tri-

Table 2 Statistics on EST sequences in *Glycyrrhiza uralensis*

Contents	Numbers
Total number of sequences downloaded	56,089
Total size of downloaded sequences (bp)	26,955,389
Number of cleaned EST sequences	55,942
Total number of sequences examined	4,821
Total number of identified SSRs	5,536
Number of SSRs containing sequences	3,053
Number of sequences containing more than one EST-SSR	1,484

Table 3 Frequencies of microsatellite repeat by type in the *Glycyrrhiza* EST database

Repeat types	Number of SSRs	Proportion of all SSRs (%)
Dinucleotide	458	8.27
Trinucleotide	386	6.97
Tetranucleotide	71	1.28
Pentanucleotide	159	2.87
Hexanucleotide	3,531	63.78
Hepta-nucleotide	596	10.77
Octa-nucleotide	159	2.87
Nona-nucleotide	156	2.82
Deca-nucleotide	20	0.36
Total	5,536	100.00

Table 4 The most abundant microsatellite motifs in the EST database. Only motifs accounting for 5% or more of each repeat type (i.e. di-, tri-, tetra-nucleotide, etc.) are included

SSR type	SSR motif	Number of SSR motifs	Percentage of SSR motif
Di-nucleotide	AG/CT	1,421	44.71
	GA/TC	903	28.41
	AC/GT	355	11.17
	CA/TG	241	7.58
	AT/AT	169	5.32
Tri-nucleotide	GAA/TTC	300	10.37
	AGA/TCT	294	10.16
	ACA/TGT	226	7.81
	AAG/CTT	224	7.74
	AAC/GTT	178	6.15
	CAA/TTG	168	5.81
	CAC/GTG	147	5.08
Tetra-nucleotide	CAAG/CTTG	61	11.89
	AAAT/ATTT	47	9.16
	AAAC/GTTT	28	5.46
	TATG/CATA	27	5.26
Penta-nucleotide	CATAC/GTATG	201	17.96

nucleotide repeats were next in abundance, accounting for 10.77, 8.27, and 6.97% of the SSRs. Penta-, octa-, nona-, and tetra-nucleotide repeats were rarer (2.87, 2.87, 2.82, and 1.28 %, respectively), and deca-nucleotides together accounted for less than 1% of the identified SSRs. Among di-nucleotide repeats, (AG/CT)n accounted for 44.71% of di-nucleotide repeats (Table 3). Among tri-nucleotide repeats, (GAA/TTC)n and (AGA/TCT)n were the most abundant repeat motifs and represented 10.37 and 10.16% of tri-nucleotides, respectively. Among the tetra-nucleotide repeats, (CAAG/CTTG)n was most abundant, with each accounting for 11.89% of the tetra-nucleotides. Only one motif (CATAC/GTATG)n accounted for 17.96% of hepta-nucleotides (Table 4).

Marker evaluation and polymorphism detection

A total of 125 primer pairs were designed from di- to tetra-nucleotide EST-SSRs. The EST-SSR primers were selected based on high G/C content, melting temperature of 55 ~ 60°C. These primers were tested on a subset of *G. uralensis* accessions (sample No.1 line). Most of the primers (95 primer pairs, 76%) produced amplicons; however, 30 primer pairs (24%) failed to amplify the loci. Finally, eight EST-based microsatellite markers with clear amplification fragments on agarose gel were selected and tested for polymorphism in 2 *Glycyrrhiza* species. Detailed information and accession genetic parameters regarding these eight novel polymorphic *Glycyrrhiza* EST-based

Table 5 Primers used for *Glycyrrhiza* EST-based microsatellite markers

Marker	Accession No.	SSR Modif.	SSR specific primer (5'→3')		Product size (bp)
			Forward	Reverse	
GlySSR1	FS239000	(tatg)5	GACTGGAATCTCAAACGCAATA	AAAATCAAAGCGTGACCAGATA	330-352
GlySSR2	FS239076	(ttctc)6···(aag)7	TTCTTTGACTCACTCACCCCTA	AGTGATTCACGAGTTCATCGTC	260-291
GlySSR3	FS239091	(gaat)6	CGGTATAGCGTGGTAGTCTGAG	TTTTTGAAGCTGTTGAATGGTT	287-341
GlySSR4	FS239392	(catggg)9	CTGTTTTCCCCTGTTTTCTTCT	TCTGTTGCTCCTCTGTTTGAGT	260-290
GlySSR5	FS239689	(ag)18	TTGCGAGTGAGAGGAAGTTAAG	CCAGAGTACACACGATTTTGGT	382-407
GlySSR7	FS240731	(ag)18	CCAAGGAATTAGAACTGCGACT	AGTGCCATGAGAGAAGTTGAAA	270-311
GlySSR10	FS238963	(aac)6...(tgg)6	ATGGCAGGTATCATTCACAAGAT	TGTCCTTGATCTTCTCCACAAAC	332-344
GlySSR11	FS238990	(gga)7	GTAATGCCGTTGGAGGATGAC	AGGGCAGAATCTAAGTGCAGAA	352-375

Table 6 Characteristics of 8 polymorphic microsatellite loci from *Glycyrrhiza* ssp

Marker	MAF	N_A	GD	H_E	PIC
GlySSR1	0.62	4	0.56	0.32	0.52
GlySSR2	0.28	6	0.79	0.48	0.76
GlySSR3	0.88	3	0.22	0.08	0.20
GlySSR4	0.42	5	0.71	0.44	0.66
GlySSR5	0.32	7	0.79	0.44	0.76
GlySSR7	0.50	6	0.67	0.32	0.63
GlySSR10	0.64	5	0.55	0.28	0.52
GlySSR11	0.70	4	0.47	0.40	0.44
Mean	0.55	5	0.60	0.35	0.56

MAF, major allele frequency; N_A, number of alleles; GD, genetic diversity; H_E, expected heterozygosity; PIC, polymorphism information content

microsatellite primers are shown in Table 5. According to gene annotation with BLASTx, the eight polymorphic *Glycyrrhiza* EST-based microsatellite containing genes were homologous to the genes involved in amino acid synthesis. EST-based microsatellite variations in these genes could show their potential role in differentiation or evolution (Varshney et al. 2005), which can be helpful for diversity studies, cross-species transferability, and breeding in larch species (Zhang et al. 2015).

Major allele frequency (MAF) varied from 0.28 for GlySSR2 to 0.88 for GlySSR3 with average MAF of 0.55. A total of 40 alleles, ranging from 3 for GlySSR3 to 7 for GlySSR5, were observed among 22 *Glycyrrhiza* accessions, with an average of 5.0 alleles per locus. The average values of genetic diversity (GD) were 0.60, ranging from 0.22 for GlySSR3 to 0.79 for GlySSR2 and GlySSR5, respectively. The average expected heterozygosity (HE) was 0.35, ranging from 0.08 for GlySSR3 to 0.48 for GlySSR2. PIC values were calculated for each polymorphic marker using a method that gives a maximum value of 0.50 (Roldan-Ruiz et al. 2000). For all accessions, average PIC values ranged from 0.20 for GlySSR3 to 0.76 for GlySSR2 and GlySSR5, with an average PIC of 0.56.

Assessment of genetic diversity in *Glycyrrhiza* with EST-based microsatellite markers

A total of 40 polymorphic fragments from 8 EST based microsatellite markers were used to assess genetic diversity in the *Glycyrrhiza* species accessions and *Astragalus membranaceus* as an out group resource. The data were used to compute a genetic similarity matrix using the PowerMarker and unweighted neighbour-joining algorithm with NTSYS. The resulting dendrogram was very highly correlated with the distance matrix as determined. Finally, the *Glycyrrhiza* species accessions were divided into two groups (Fig. 1). Clade Ⅰ contained 16 accessions, all of which were land races collected from different *G. uralensis*-growing locations in Mongolia, China, and South Korea. This cluster had an average genetic distance value (coefficient) of 0.88, with minimum and maximum coefficient of 0.76 and 1.00, respectively. Clade Ⅱ contained two clusters encompassing 6 accessions, which showed clearly the clas-

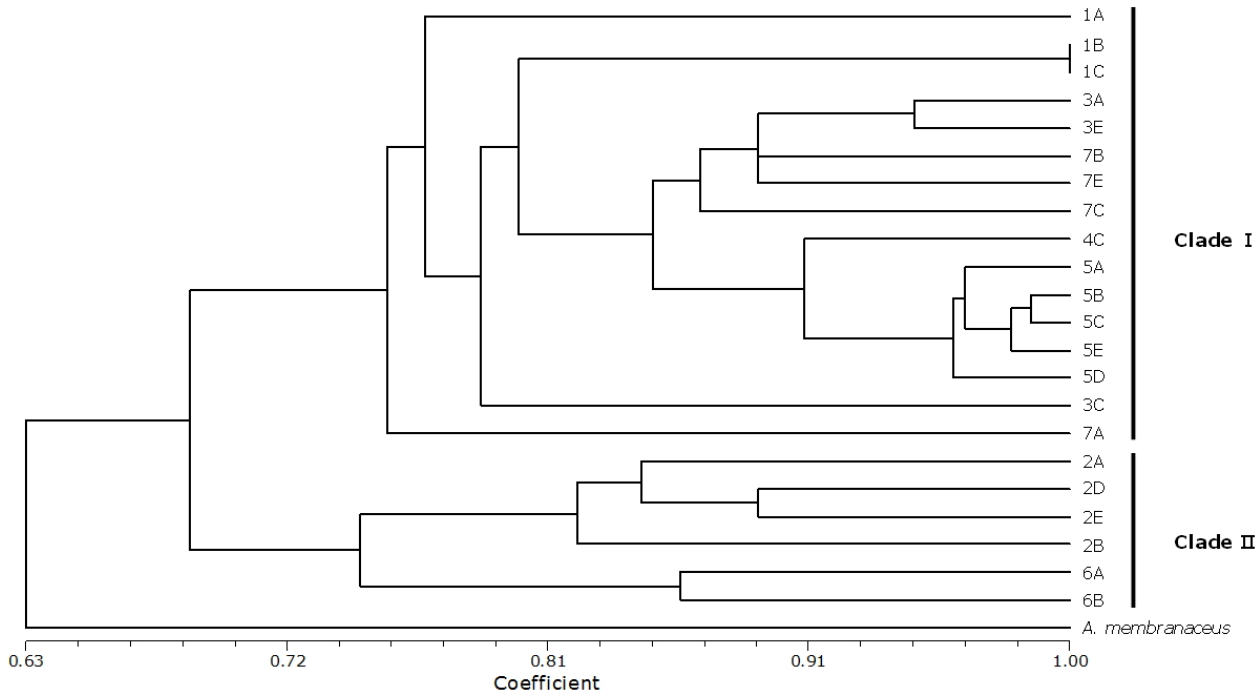

Fig. 1 Dendrogram of the *Glycyrrhiza* accessions based on the Dice coefficient as calculated from the EST-based microsatellite marker data. A total of 22 *Glycyrrhiza* accessions were used for grouping with *Astragalus membranaceus* as the out group. Bootstrap values (1,000 replications) greater than 50% are indicated on branches

sification. These were land races collected from different *G. glabra*-growing locations in Canada and Uzbekistan.

Eight microsatellite markers would be useful for studies of the genetic diversity, population structure, and evolutionary relationships among *Glycyrrhiza* species. The amplification was further examined in two specie-*G. uralensis* and *G. glabra*-which are also indigenous and important medicinal plants in South Korea.

The development of functional markers such as EST-based microsatellite is becoming a valuable study for plants, especially in marker-assisted breeding one. However, the number of EST-based microsatellite markers for *Glycyrrhiza* species is very few because the public resource is limited. In this research, a set of EST-based microsatellites, derived from *G. uralensis* EST sequences available from the NCBI database, was newly developed. A total of eight polymorphic EST-based microsatellite markers were finally selected and tested across *G. uralensis* and *G. glabra*. These novel EST-based microsatellite markers would be helpful for future research on genetic diversity, population structure, linkage map construction, or QTL mapping in *Glycyrrhiza* species. At this time, the number of EST-based microsatellite markers is limited, but this situation will be substantially changed by the application of next-generation sequencing. At present, large-scale transcript sequences for *G. uralensis* (Liu et al. 2015) have been deposited in the NCBI database and the information can be easily employed to develop functional genomic-based molecular markers at the genome scale in future studies.

Acknowledgements

This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01028801)" Rural Development Administration, Republic of Korea.

References

Asl MM, Husseyinzadeh H (2008) Review of Pharmacological Effects of *Glycyrrhiza* sp. and its Bioactive Compounds. Phytother Res 22:709-724

Chevreux B, Pfisterer T, Drescher B (2004) Using the miraEST Assembler for Reliable and Automated mRNA Transcript Assembly and SNP Detection in Sequenced ESTs. Genome Res 14:1147-1159

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M (2005) Blast2GO: a Universal Tool for Annotation, Visualization and Analysis in Functional Genomics Research. Bioinformatics 21:3674-3676

Erayman M, Ilhan E, GÜZEL Y, Eren AH (2014) Transferability

of SSR Markers from Distantly Related Legumes to *Glycyrrhiza* species. Turk J Agric For 38:32-38

Kalia RK, Rai MK, Kalia S, Singh R, Dhawan AK (2011) Microsatellite markers: An Overview If the Recent Progress in Plants. Euphytica 177:309-334

Khan S, Mirja KJ, Tayyab M, Abdin MZ (2009) RAPD Profile for Authentication of Medicinal Plant *Glycirrhiza glabra* L. Medicinal Aromatic Plant Sci Biotech 3:8-51

Koressaar T, Remm M (2007) Enhancements and Modifi Cations of Primer Design Program Primer3. Bioinformatics (Oxford, England) 23:1289-1291

Liu KJ, Muse SV (2005) PowerMarker: An Integrated Analysis Environment for Genetic Marker Analysis. Bioinformatics 21:2128-2129

Liu Y, Zhang P, Song M, Hou J, Qing M, Wang W, Liu C (2015) Transcriptome Analysis and Development of SSR Molecular Markers in *Glycyrrhiza uralensis* Fisch. PloS one 10:e0143017

Morin RD, Bainbridge M, Fejes A, Hirst M, Krzywinski M, Pugh TJ, McConald H, Varhol R, Jones SJM, Marra MA (2008) Profiling the HeLa S3 Transcriptome using Randomly Primed cDNA and Massively Parallel Short-read Sequencing. Biotechniques 45:81

Rohlf FJ (2000) Phylogenetic Models and Reticulations. J Classif 17:185-189

Rohlf FJ (2004) NTSYS-pc Ver. 2.11 T. Exter Software, New York

Roldán-Ruiz I, Dendauw J, Van Bockstaele E, Depicker A, De Loose M (2000) AFLP Markers Reveal High Polymorphic Rates in Ryegrasses (*Lolium* spp.). Mol Breeding 6:125-134

Sorrells ME, Wilson WA (1997) Direct Classification and Selection of Superior Alleles for Crop Improvement. Crop Sci 37:691-697

Sudo H, Seki H, Sakurai N, Suzuki H, Shibata D, Toyoda A, Totoki Y, Sakaki Y, Iida O, Shibata T, Kojoma M, Muranaka T, Saito K (2009) Expressed Sequence Tags from Rhizomes of *Glycyrrhiza uralensis*. Plant Biotechnol 26:105-107

Temnykh S, DeClerck G, Lukashova A, Lipovich L, Cartinhour S, McCouch SR (2001) Computational and Experimental Analysis of Microsatellites in Rice (*Oryza sativa* L.): Frequency, Length Variation, Transposon Associations, and Genetic Marker Potential. Genome Res 11:1441-1452

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3-New Capabilities and Interfaces. Nucleic Acids Res 40:e115-e115

Varshney RK, Graner A, Sorrells ME (2005a) Genic Microsatellite Markers: Features and Applications. Trends Biotechnol 23: 48-55

Varshney RK, Sigmund R, Borner A, Korzun V, Stein N, Sorrells ME, Langridge P, Graner A (2005b) Interspecific Transferability and Comparative Mapping of Barley EST-SSR Markers in Wheatrye and Rice. Plant Sci 168:195-202

Wang ML, Barkely NA, Jenkins TM (2009) Microsatellite Markers in Plants and Insects. Part I: Applications of Biotechnology. Genes Genom Gen 3:54-67

Yamazaki M, Sato A, Shimomura K, Saito K, Murakoshi I (1994) Genetic Relationships among *Glycyrrhiza* Plants Determined by RAPD and RFLP Analyses. Biol Pharm Bull 17:1529-1531

Yao H, Zhao Y, Chen DF, Chen JK, Zhao TS (2008) ISSR Primer Screening and Preliminary Evaluation of Genetic Diversity in Wild Populations of *Glycyrrhiza uralensis*. Biol Plantarum 52:117-120

Zhang G, Sun Z, Zhou D, Xiong M, Wang X, Yang J, Wei Z (2015) Development and Characterization of Novel EST-SSRs from *Larix gmelinii* and Their Cross-Species Transferability. Molecules 20:12469-12480

Zhang Q, Ye M (2009) Chemical Analysis of the Chinese Herbal Medicine Gan-Cao (licorice). J Chromatogr A 1216:1954−1569