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SSR Molecular Markers Development Based on Whole Genome Sequences in *Glycyrrhiza uralensis* Fisch.

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Abstract It is different in terms of active ingredients from various sources and origins on *Glycyrrhiza* Linn.. The development of specific molecular markers is of great significance for identification of germplasm resources. In the study, simple sequence repeat (SSR) motifs were analyzed based on the whole genome data in *Glycyrrhiza uralensis* Fisch.. SSRs were identified with MISA tool in all scaffold sequences. Suitable SSRs were used to design primers by using Primer3-2.4.0 software and 100 pairs of SSR primers were randomly utilized for the validity of molecular markers. The results showed 193 207 SSRs were detected from the whole genome sequence of *G. uralensis* distributed in 9 250 scaffolds. Of these, mono-nucleotide repeat SSRs was the main type, accounting for 60.73%, followed by dinucleotide (26.11%) and trinucleotide repeat (10.95%). Of the 284 repeat motifs, A/T (58.28%), AG/CT (10.48%), AT/AT (10.48%), AC/GT (5.12%) and AAT/ATT (3.57%) were the main repeat base. In addition, SSR primers for 140294 SSRs were designed using Primer3-2.4.0 software. We eventually developed 701 302 pairs of SSR markers in *G. uralensis*. Subsequently, 100 pairs of SSR primers were randomly selected for the further verifying. PCR results indicated that 63 of them (63%) had amplified the target bands, and 12 pairs of primers could generate polymorphic bands in seven samples. A large number of SSR markers developed will be used for identification of *Glycyrrhiza* germplasm resources and future molecular marker assisted breeding.

Keywords *Glycyrrhiza uralensis* Fisch.; Genome; Repetitive sequence; SSR marker; Development

Glycyrrhiza uralensis Fisch. is a perennial herb or subshrub of *Glycyrrhiza* in Leguminosae. Its roots and rhizomes have the effects of moistening lung and relieving cough, tonifying spleen and supporting Qi, clearing away heat and relieving toxin, harmonizing drugs and so on, which is one of the traditional Chinese medicines (Chinese Pharmacopoeia Commission, 2015). In addition to its medicinal value, *Glycyrrhiza uralensis* Fisch. also has the functions of wind prevention, sand fixation, and water and soil conservation. In recent years, with the application of *Glycyrrhiza uralensis* Fisch. in agriculture, food, cosmetics, industry and other fields, its demand has also increased accordingly. Due to over excavation, the distribution area of wild *Glycyrrhiza uralensis* Fisch. has been decreasing year by year, and even facing the depletion of wild resources, which has led to the continuous increase of the planting area of cultivated *Glycyrrhiza uralensis* Fisch.. Large-area planting bases for cultivated *Glycyrrhiza uralensis* Fisch. have been established in Inner Mongolia, Xinjiang, Jilin and Shanxi respectively (Li, 1993). Studies have shown that even the medicinal ingredients of *Glycyrrhiza uralensis* Fisch. with the same origin are different (Ruo et al., 2018). In order to better identify species and distinguish germplasm resources, it is particularly important to develop molecular markers associated with specific traits of *Glycyrrhiza uralensis* Fisch..

Molecular markers have been widely used in genetic identification and genetic background analysis of medicinal plants. The development of molecular markers of *Glycyrrhiza uralensis* Fisch. mainly involves random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) (Liu et al., 2015), inter-simple sequence repeat (ISSR) (Li et al., 2010), start codon targeted polymorphism (SCoT), expression sequence tag - simple sequence repeat (EST-SSR) (Li et al., 2015) and simple sequence repeat (SSR) markers (Liu et al., 2015). Among them, SSR markers are DNA fragments composed of 1~6 bp nucleotides, which are widely distributed in the genomes of eukaryotes. An SSR sequence appears at an average of about 10 kb nucleotides, and there are

highly conserved sequences at both ends. Due to the different SSR sequences and repetition times of different species, SSR markers show a high degree of conservatism and repeatability in different species, so that different species can be accurately identified. At present, most of the published SSR markers of *Glycyrrhiza uralensis* Fisch. were developed based on the sequencing results of *Glycyrrhiza uralensis* Fisch. transcriptome. But there were such limited molecular markers of *Glycyrrhiza uralensis* Fisch. that could not meet the needs of identification of germplasm resources and genetic diversity analysis (Liu et al., 2015). In this study, based on the whole genome sequences in *Glycyrrhiza uralensis* Fisch., the composition and frequency of SSRs in *Glycyrrhiza uralensis* Fisch. genome were statistically analyzed by bioinformatics methods, and a large number of SSR markers were designed. Seven *Glycyrrhiza uralensis* Fisch. samples were used to verify the designed markers. The developed polymorphic markers can be used for the identification of germplasm resources, genetic diversity analysis and molecular marker assisted breeding, which could lay a foundation for artificially breeding excellent *Glycyrrhiza uralensis* Fisch. varieties.

1 Results and Analysis

1.1 Distribution of SSRs in *Glycyrrhiza uralensis* Fisch. genome

Using MISA to search for repeat loci based on 12 528 scaffolds in the whole genome of *Glycyrrhiza uralensis* Fisch., it was found that 9 250 scaffolds contained SSR loci, the frequency was 73.52%, and the number of SSRs was 193 207, including 32 112 complex microsatellites. There were 510 SSR loci in each Mb sequence of the genome, and on average, each scaffold contained 179 SSR loci. 1~6 nucleotide positions were all present, and the number of mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, and hexanucleotide repeat units were 117 343, 50 445, 21 159, 2 889, 844 and 527, which respectively accounted for 60.73%, 26.11%, 10.95%, 1.50%, 0.44% and 0.27% of the total number of repeat units (Table 1; Figure 1A).

Table 1 Number and distribution of SSR loci in *Glycyrrhiza uralensis* Fisch. genome

Repeat types	Repeat times									Total	Percentage (%)
	5	6	7	8	9	10	11	12	>12		
Mononucleotide						64 634	26 849	12 780	13 080	117 343	60.73
Dinucleotide		15 663	10 074	7 569	5 660	3 594	2 558	1 706	3 621	50 445	26.11
Trinucleotide	9 779	4 595	2 532	1 440	748	520	338	242	965	21 159	10.95
Tetranucleotide	1 623	638	288	134	47	50	32	23	54	2 889	1.50
Pentanucleotide	632	141	44	11	4	5	2		5	844	0.44
Hexanucleotide	352	107	39	14	2	10	2		1	527	0.27
Total	12 386	21 144	12 977	9 168	6 461	68 813	29 781	14 751	17 726	193 207	100

1.2 Repeat type and frequency of SSR in *Glycyrrhiza uralensis* Fisch. genome

Through the analysis of the repeat loci, it was found that among the mononucleotide repeat units, the frequency of A/T repeat sequences was 112 592, accounting for 95.95%, and the frequency of G/C type was 4 751, accounting for 4.05% (Figure 1B). There were 4 types of dinucleotide repeat units. Among them, AG/CT and AT/AT repeat sequences have a high frequency, which were 20 251 and 20 247 respectively, accounting for 80.28% of dinucleotide repeat units, followed by AC/GT, and the least was CG/CG type, accounting for only 0.12% (Figure 1C). There were 10 types of trinucleotide repeat units, of which AAT/ATT has the highest frequency, accounting for 32.58%. The frequencies of AAC/GTT, AAG/CTT and ACC/GGT repeat units accounted for 13.72%, 17.56% and 18.56% respectively. And the frequencies of the other six types were lower, all lower than 10% (Figure 1D). There were 30 types of tetranucleotide repeat units, of which AAAT/ATTT has a high frequency, accounting for 32.36%. The frequencies of AAAG/CTTT, AATT/AATT, ACAT/ATGT and AGAT/ATCT accounted for 10.56%, 10.76%, 10.52% and 10.07% respectively. The frequencies of the other 25 types were lower than 10% (Figure 1E). There are 78 types of pentanucleotide repeat units, of which AATAT/ATATT has the highest frequency, accounting for 11.61%, and the frequency of the other types was lower than 10% (Figure 1F). There are 157 types of hexanucleotide repeat units, and the frequency was generally low. Among them, the frequencies of AGAGGG/CCCTCT, AAGAGG/CCTCTT and AAAAAT/ATTTTT were relatively higher, accounting for 3.42%, 3.61% and 2.66% respectively (Figure 1G).

Figure 1 Type and frequency distribution of SSRs

Note: A: Distribution frequency of 6 types of SSR; B: Repeat types and frequency of mononucleotide; C: Repeat types and frequency of dinucleotide; D: Repeat types and frequency of trinucleotide; E: Repeat types and frequency of tetranucleotide; F: Repeat types and frequency of pentanucleotide; G: Repeat types and frequency of hexanucleotide

1.3 Design and verification of SSR primers

140 294 repeat loci were selected from all SSRs for primer development, 5 pairs of primers could be designed for most loci, and a total of 701 302 pairs of SSR markers were designed (Table 2). 100 pairs of primers designed for scaffolds sequences were randomly selected for marker validity detection. 63 pairs of primers amplified the target bands in seven wild or cultivated *Glycyrrhiza uralensis* Fisch. from Yanchi County in Xinjiang, Longxi County in Gansu, Shizuishan City in Ningxia, Datong City, Yuci City and Yangqu County in Shanxi, indicating that the effectiveness of primer design was 63%. Among them, 12 pairs of primers could generate polymorphic bands in seven samples, which could be used to identify *Glycyrrhiza uralensis* Fisch. from different habitats (Figure 2).

Table 2 Information of 12 primer pairs

Primer No.	Primer sequence (5'-3')	Repeat unit	Annealing temperature (°C)	Expected range (bp)
Gcs1	F:TCCTGTTTGACCGAACCAACTT R:TGCACGCAGAGAAGGGAATGAA	(TTAA)5	61	183
Gcs2	F:TCCTGTTTGACCGAACCAACTT R:TGCACGCAGAGAAGGGAATGAA	(A)11	61	238
Gcs3	F:GGGAGTGAAGGAGAAGCGAAGA R:GGGTCGAGAGGAGAGGGAGAGA	(TGTGGC)5	63	209
Gcs4	F:AGGTGTTGGGCATGCGTTTCAG R:GCAGAAGTAGCCAGCTTGATTT	(TTA)9	59	216
Gcs5	F:CACAACGCAACGCAACCCAAC R:GGCCTTGGGAGAAGAAGAAGTA	(AACAA)5	60	250
Gcs6	F:CGTTCTGGTTTTGGTCCTATGG R:CCTATGGTCAACTGGTGCTTAA	(AT)11	58	248
Gcs7	F:AGAGGAGTGTGGGAAGGTCTAC R:GCTAGTTTGGGACGTACTTTAA	(TCTT)5	56	247
Gcs8	F:GCAGTAGTGTGTCTTGTGTGAG R:GGGGCTTCAATTCAGGGTTTCC	(GGCAGA)5	61	216
Gcs9	F:GGGTTTCGATCGTCACAAGTTCA R:TCTTCTCTCTCGCGCCCAATTC	(AG)7(TG)9	62	153
Gcs10	F:CCCGGGTGACAAGGCTTTT R:TGGAAATGTTAAGGTCACCGAC	(GAAACA)5	57	243
Gcs11	F:TGGCTAAAAGGGACACAAAAGA R:TTTTCGTAGGCAGAGAGAATGG	(G)10n(G)10	58	198
Gcs12	F:TCCCCATCCTCATCACCATCGT R:AAGATTGCCCTCCTCCTCTGA	(GTC)5(TCA)7	62	156

Figure 2 PCR of the primers Gcs6 in 7 *Glycyrrhiza uralensis* Fisch.

Note: M: D2000 DNA Marker; 1~7: Represent the number of *Glycyrrhiza uralensis* Fisch. material (Table 1)

2 Discussion

In recent years, with the continuous development of sequencing technology, more and more plant genome sequences such as *Salvia miltiorrhiza*, *Dendrobium catenatum*, *Chrysanthemum*, *Mentha longifolia* and *Glycyrrhiza uralensis* Fisch. have been published (Song et al., 2018). These data laid the foundation for the development of molecular markers based on sequencing technology, such as SSR (Song et al., 2019), EST-SSR, SNP (Yang et al., 2019), Indel (Liu et al., 2019) and KASP, and their application to identification of germplasm

resources. In this study, 193 207 SSR loci were screened out using MISA software based on the whole genome sequences in *Glycyrrhiza uralensis* Fisch.. The genome has an average of 482 SSR loci per Mb sequence. The study found that using MISA software to screen SSR loci based on the whole genome, 117 511 SSR loci were screened out from 239 Mb genome sequences of *Apocynum venetum*, with an average of 492 SSR loci Mb⁻¹. 488 592 SSR loci were screened out from 1.18 Gb genome sequences of *Eucommia ulmoides*, with an average of 404 SSR loci Mb⁻¹ (Wu et al., 2015). The *Corchorus capsularis* genome was about 338 Mb, with an average of 467 SSR loci Mb⁻¹ (Yang et al., 2019). The genome size of tetraploid *Arachis hypogaea* Linn., which belongs to legume like *Glycyrrhiza uralensis* Fisch., was about 2.7 Gb, with an average of 245 SSR Mb⁻¹ (Wang et al., 2019). The *Glycyrrhiza uralensis* Fisch. genome was about 400 Mb, and the distribution frequency of SSR loci in the whole genome was similar to these species, basically in line with the tendency that genome size was negatively correlated with the number or distribution frequency of SSR (Morgante et al., 2002).

It was found that in most species genomes, the distribution frequency of genome SSRs will gradually decrease with the increase of the number of repeat units. Dinucleotide and trinucleotide repeats were the main repeat types, and the difference lies in the difference of repeat units (Sonnet et al., 2000). In this study, 1~6 nucleotide loci existed in the whole genome SSR loci of *Glycyrrhiza uralensis* Fisch., with the largest number of mononucleotide repeat units, accounting for 60.73%, followed by dinucleotide and trinucleotide repeat units, accounting for 26.11% and 10.95% respectively, which was consistent with the results that the proportion of mononucleotide repeat units in the genome of *Salvia splendens* Ker-Gawler (41.6%) (Wang et al., 2018), *Eucommia ulmoides* (54.34%) (Wu et al., 2015) and *Punica granatum* L. (51.95%) (Hong et al., 2019) was the highest. And it was similar to the species that the proportion of dinucleotide repeat units was the highest, such as *Fagopyrum tataricum* (69.72%) (Fang et al., 2019), *Rhododendron simsii* Planch. (94.58%) (Gong et al., 2018), *Scutellaria baicalensis* Georgi (68.32%) (Qi et al., 2015), *Gastrodia elata* Bl. (78.88%) (Zhou et al., 2017). There was a tendency that the distribution frequency of genome SSR loci decreased with the increase of length of repeat units. It was generally believed that the species which has a large number of mononucleotide and dinucleotide repeat units in the genome has a high level of evolution (Toth et al., 2000). The mononucleotide and dinucleotide in the genome of *Glycyrrhiza uralensis* Fisch. accounted for 86.84% of all SSR loci, indicating that *Glycyrrhiza uralensis* Fisch. may have a late origin and a high level of evolution. In this study, the SSR of *Glycyrrhiza uralensis* Fisch. contained 284 types of abundant repeat units. The number of repeat sequences composed of bases with same motif but different lengths was different. The dominant units were A/T (58.28%), AG/CT (10.48%), AT/AT (10.48%), AC/GT (5.12%) and AAT/ATT (3.57%), which was similar to the base preference of *Gastrodia elata* Bl. (Zhou et al., 2017), *Punica granatum* L. (Hong et al., 2019) and *Eucommia ulmoides* (Wu et al., 2015), and A/T was the main repeat unit.

There were also many studies on molecular markers of *Glycyrrhiza uralensis* Fisch., including RAPD markers (Wu et al., 2003), 26 pairs of markers of other legume species (For et al., 2014), 81 pairs of SRAP primers (Ai et al., 2017) and universal primers to amplify ITS and *psbA-trnH* sequences to achieve the purpose of molecular identification of different primordial *Glycyrrhiza uralensis* Fisch. (Yang et al., 2017), but generally, the number of markers was very limited. With the development of sequencing technology, both single primer SCoT markers and EST-SSR markers developed by expressing sequence tags (Song et al., 2017; Xin, 2019) have the problem of insufficient marker coverage. In terms of transcriptome data development markers, 10 378 SSRs and 7 032 SSRs were screened from the transcriptome data of *Glycyrrhiza inflata* Batalin and *Glycyrrhiza glabra* L., respectively, and a total of 110 pairs of SSR primers were designed (Liu et al., 2015; Xin, 2019). Obviously, the traditional methods and molecular markers based on sequencing data have been far from meeting the requirements of identification of genetic diversity and genetic relationship of *Glycyrrhiza uralensis* Fisch.. The number of SSR markers identified in this study reached 701 302 pairs, and most of them could amplify target bands in *Glycyrrhiza uralensis* Fisch. from different sources, indicating that these markers had great application potential and could significantly improve the efficiency of identification, development and molecular marker assisted selection of *Glycyrrhiza uralensis* Fisch. germplasm resources.

3 Materials and Methods

3.1 Experimental materials

The leaves of wild *Glycyrrhiza uralensis* Fisch. used in the experiment were collected from Shanxi, Ningxia and other places respectively, and dried and preserved with silica gel. While the cultivated licorice was the hydroponic seedlings. Our group collected seeds from the origin and cultured them in hydroponics. The materials included 3 cultivated *Glycyrrhiza uralensis* Fisch. and 4 wild *Glycyrrhiza uralensis* Fisch., which were identified by Professor Zhang Shuosheng from Shanxi University of Chinese Medicine (Table 3). Genome DNA was extracted by improved CTAB method (Zhang et al., 2014).

Table 3 Tested samples from different sources

Code	Location	Growth mode	Phenotype
GC1	Datong City, Shanxi	Wild	Red
GC2	Yuci City, Shanxi	Wild	Red
GC3	Shizuishan City, Ningxia	Wild	Red
GC4	Yanchi County, Xinjiang	Cultivation	Red
GC5	Longxi County, Gansu	Cultivation	Yellow
GC6	Datong City, Shanxi	Cultivation	Red
GC7	Yangqu County, Shanxi	Cultivation	Red

3.2 Extraction of SSR from licorice genome sequence

We used MISA (<https://webblast.ipk-gatersleben.de/misa/>) software to search for repeat loci in the published *Glycyrrhiza uralensis* Fisch. genome (Mochida et al., 2016). The search criteria were as follows: nucleotide (base pair, bp) 1 bp, 2 bp, 3 bp, 4 bp, 5 bp, 6 bp, the repeat times were not less than 10 times, 6 times, 5 times, 5 times, 5 times, 5 times successively, and the composite SSR (the distance between 2 SSRs) was less than 100 bp.

3.3 Design of SSR primers

Primer 3-2.4.0 software was used to extract 300 bp sequence containing SSR repeat locus flanking as template sequence for marker development. The design criteria of primers were as follows: the GC content was 30%~70%, the product size was 100~250 bp, the T_m value was between 55°C~65°C, the difference of T_m value between upstream and downstream primers was no more than 5°C, the primer length was 18~22 bp, and the GC content of primers was 30%~70%.

3.4 Validation of *Glycyrrhiza uralensis* Fisch. SSR primers

The PCR reaction system was 10 µL: 1×*Taq* DNA polymerase buffer, 2.5 mmol/L MgCl₂, 1 U DNA polymerase, 100 µmol/L dNTPs, 0.2 µmol/L upstream primers, 0.2 µmol/L downstream primers, 50 ng template DNA, and add distilled water to 10 µL. The PCR reaction conditions were as follows: pre-denaturation at 95°C for 4 min, denaturation at 94°C for 1 min, renaturation at 55°C~60°C for 45 s, extension at 72°C for 1 min, and for 35 cycles, and final extension at 72°C for 5 min. The amplified products were mixed with 6× sample solution, and subjected to non-denaturing polyacrylamide gel electrophoresis for 1 h. After the electrophoresis, silver staining was performed.

Authors' contributions

ZHX was the main writer and reviser of the manuscript; ZSS and ZHX were in charge of the project, mainly responsible for project conception and experimental design; DCH mainly arranged and analyzed the data of the manuscript; WYL and LR gave specific guidance to the experiment; ZD and YMR completed the experiments. All authors read and approved the final manuscript.

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