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Analysis on SSR Loci in Transcriptome and Development of EST-SSR Molecular Markers in *Lonicera macranthoides*

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Abstract Huizhanmao-rendong (*Lonicera macranthoides*), as a species of original plant of wild honeysuckle flower (*Flos Lonicerae*), the traditional Chinese medicine, is widely distributed in South China and features high medicinal and economic value. In this study, the SSR loci of 74 057 unigenes in the transcriptome of *L. macranthoides* were analyzed by MISA. Among them, 15 587 unigenes contained 20 161 SSR loci, and the frequency of SSR was 21.05%. The types of SSR sites in the transcriptome of *L. macranthoides* were distributed from single nucleotide to six nucleotide repeats. The main repeats were dinucleotide (9 704) and trinucleotide (5 847), accounting for 48.13 % and 29.00 % of the total SSRs, respectively. The main repeat units were AG/CT (6 086), AT/AT (3 071) and A/T (2 826), accounting for 30.79%, 15.23%, and 14.02% of the total SSRs, respectively. There were 4 897 and 3 531 repeats (the numbers of repeats were the most) in 6 and 5 SSR loci, accounting for 24.29 % and 17.51 % of the total SSR number, respectively. By using primer 6.0 software, 17 611 pairs of SSR primers were designed, and 30 pairs of primers were randomly selected for amplification verification. Among them, 14 pairs of amplified bands showed polymorphism, and then the genetic diversity of 6 varieties of *L. macranthoides* was analyzed by using these 14 pairs of primers. The results will provide a scientific basis for species identification, genetic diversity analysis, and molecular marker-assisted breeding of *L. macranthoides*.

Keywords *Lonicera macranthoides*; SSR; Mlecular marker; Transcriptome

Huizhanmao-rendong (*Lonicera macranthoides*) belongs to *Lonicera* genus in the family of Caprifoliaceae, which was recorded in the *Chinese Pharmacopoeia* (2015 edition) as a original plant of wild honeysuckle flower (*Flos Lonicerae*), and has high medicinal and economic value. For thousands of years, the buds or first-blooming flowers of *Lonicera lonicera* named “Wild honeysuckle flower” have been used as traditional Chinese medicine to treat inflammation, fever and headache (Chu et al., 2011; Zhang et al., 2016). *Lonicera lonicera* is widely distributed in southern China, and a large number of studies have been carried out on its chemical composition, biological activity and molecular mechanism of chlorogenic acid biosynthesis (Chen et al., 2015; Hu et al., 2016; Chen et al., 2017). However, there are relatively few studies on SSR molecular markers of *Lonicera lonicera*. In related reports on molecular markers of *Lonicera japonica*, potential ISSR and SRAP markers were found for *Lonicera japonica* (Wang et al., 2009; Chen et al., 2011) and analyzed the SSRs, SCAR and RAPD markers used for genetic identification in *L. japonica* (Jiang et al., 2012; Fu et al., 2013; Cheng et al., 2016). Although previous studies have provided a large number of available molecular genetic markers for *Lonicera* SPP., little is known about SSR markers for *Lonicera macranthoides* SPP.

Simple sequence repeats (SSRs) are short tandem repeats of single, two, three, four, five and six nucleotide motifs randomly distributed in eukaryotic and prokaryotic genomes (Powell et al., 1996). In the field of plant breeding, SSR markers have been widely used to evaluate genetic diversity, phylogeny, genetic linkage mapping, genotype identification, variety protection, quantitative trait loci analysis and molecular marker-assisted selection (MAS), etc. (Wang et al., 2002). It is a powerful tool for studying plant population genetics, molecular ecology and MAS breeding because of its good reproducibility, polymorphism and codominance.

At present, *Lonicera macranthoides*, as the main original plant of wild honeysuckle flower, the bulk medicinal materials, has a large market demand and various varieties. There were differences in chemical composition of *Lonicera macranthoides* from different regions and varieties. The varieties on the market are confused, and it is difficult to distinguish, resulting in uneven quality of medicinal materials. Despite the small number of molecular markers available, resources for molecular markers in *Lonicera macranthoides* are still scarce. EST-SSR polymorphisms have many loci, which can reflect rich genetic variation information, and are commonly used to identify Chinese medicinal herbs. At present, the morphological identification methods such as variety identification, character identification and microscopic identification are difficult to meet the rapid and accurate needs of people. At the same time, the identification results are easy to enrol subjective color, and the reliability is poor. Although the physical and chemical identification of the active components of traditional Chinese medicine can distinguish the authenticity of traditional Chinese medicine, the identification effect of doping phenomenon is not good. In order to facilitate the rapid and accurate identification and protection of *Lonicera macranthoides* and other base plant species of shān yín huā, and carry out related genetic research, it is urgent to develop SSR molecular markers. In this study, we obtained a large number of high-quality transcriptome data from *Lonicera macranthoides* based on the Illumina HiSeq 2000 platform and developed a large number of transcriptom-based SSRs. At the same time, we analyzed the distribution characteristics of SSR loci in the transcriptome of *Lonicera macranthoides*, and verified 14 SSR markers for genetic diversity analysis of *Lonicera macranthoides*.

1 Results and Analysis

1.1 The number of SSR in the transcriptome of *Lonicera macranthoides*

A total of 74 057 Unigenes were obtained by transcriptome sequencing of *Lonicera macranthoides*. Among them, 15 587 Unigenes contained SSR loci, 3 481 Unigenes contained more than one SSR loci, and a total of 20 161 SSR loci were found (1 784 SSR loci presented in compound formation). The frequency of SSR loci was 27.22 %, and the occurrence frequency was 21.05% (Table 1).

Table 1 Statistical of SSR loci in transcriptome sequence of *Lonicera macranthoides*

Item	Statistic
Number of sequence	74 057
Total length of sequence (bp)	69 256 202
Number of SSR loci	20 161
Number of sequence containing SSR loci	15 587
Number of sequence containing more than 1 SSR loci	3 481
Number of SSRs present in compound formation	1 784
Frequency of SSR loci (%)	27.22
Occurrence frequency of SSR loci (%)	21.05

1.2 SSR distribution characteristics of *Lonicera macranthoides* transcriptome

There were 2 897 mononucleotide SSR sites and 9 704 dinucleotide SSR sites in the transcriptome of *Lonicera macranthoides*. There were 5 847, 516, 616 and 581 SSRS for trinucleotide, quadnucleotide, pentanucleotide and hexanucleotide, respectively. There were 295 SSRS with different motif sequence types in the *Lonicera macranthoides* transcriptome (Table 2), and there were 2, 4, 10, 28, 74 and 177 types of mononucleotide, dinucleotide, trinucleotide, quadnucleotide, pentanucleotide and hexanucleotide repeats, respectively. In terms of distribution frequency, the dominant repeat motif type was dinucleotide AG/CT, which accounted for 30.19 % of the total SSR, followed by AT/AT and A/T, which accounted for 15.23 % and 14.02 %, respectively. Among the trinucleotide motifs, AAG/CTT was the most abundant, accounting for 8.48% of the total SSR. However, the distribution frequency of quadnucleotide, pentanucleotide and hexanucleotide motif types was low.

Table 2 Distribution of SSR repeat type in *Lonicera macranthoides*

Repeat type	Total motif amount	Percentage (%)	Number of motif type	Motif (amount)	Percentage (%)
Mononucleotide	2 897	14.37	2	A/T (2826)	14.02
	-	-	-	C/G (71)	0.35
Dinucleotide	9 704	48.13	4	AG/CT (6086)	30.19
	-	-	-	AT/AT (3071)	15.23
	-	-	-	AC/GT (538)	2.67
	-	-	-	CG/CG (9)	0.04
Trinucleotide	5847	29.00	10	AAG/CTT (1709)	8.48
	-	-	-	ATC/ATG (869)	4.31
	-	-	-	ACC/GGT (705)	3.50
	-	-	-	Other (2 564)	
Quadnucleotide	516	2.56	28	AAAG/CTTT (154)	0.76
	-	-	-	ACAT/ATGT (70)	0.35
	-	-	-	AGAT/ATCT (49)	0.24
	-	-	-	Other (243)	
Pentanucleotide	616	3.06	74	AAAAG/CTTTT (86)	0.43
	-	-	-	AAAAT/ATTTT (57)	0.28
	-	-	-	AAGAG/CTCTT (50)	0.25
	-	-	-	Other (423)	
Hexanucleotide	581	2.88	177	AACCCT/AGGGTT (13)	0.06
	-	-	-	ACGGCG/CCGTCG	0.06
	-	-	-	Other (555)	

By analyzing the distribution of SSR locus repeats in the transcriptome of *Lonicera macranthoides* (Figure 1), we found that the maximum number of repetitions of 6, with 4,897, accounting for 24.29% of the total number of SSR, followed by 5, 7,8, and 9. There were 3 531 (17.51%), 3 246 (16.10%), 1 798 (8.92%) and 1 412 (7.00%), respectively. 56.11% of the SSR loci with repeats less than 12 were dinucleotide repeats, and 98.71% of the SSR loci with repeats greater than or equal to 12 were single nucleotide repeats. The SSR loci of mononucleotide, dinucleotide, trinucleotide, quadnucleotide, pentanucleotide and hexanucleotide repeat types had the highest number of repeats with 12, 6, 5, 5, 4 and 4, respectively.

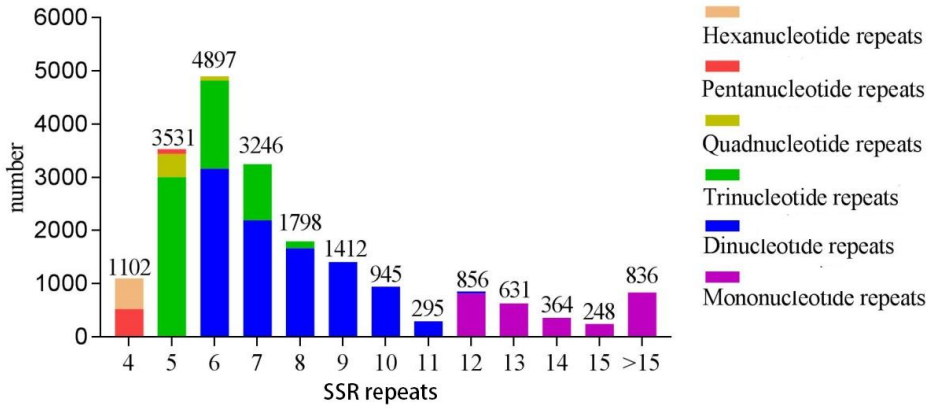

Figure 1 Distribution of SSR repeats in *Lonicera macranthoides* transcriptome

1.3 Evaluation of SSR availability of *Lonicera macranthoides* transcriptome

The polymorphism of SSR markers is an important basis to judge their availability, and the length of SSR markers is an important factor to affect the polymorphism. Studies have shown that SSR length is greater than or equal to 20 bp with high polymorphism, 12~19 bp with medium polymorphism, and below 12 bp with very low polymorphism (Temnykh et al., 2001). In this study, there were 5 336 SSRs with high polymorphism, accounting

for 26.47% of the total number of SSRs. There were 13 041 SSRs with medium diversity, accounting for 64.68% of the total SSRs. In conclusion, a total of 91.15% of SSR had moderate or above level polymorphism. In general, SSR with low motifs is more likely to produce polymorphism than SSR with high motifs (Li et al., 2017). In this study, there were 2 379 SSRS with three low-level repeat motifs (single nucleotide, dinucleotide and trinucleotide) in SSRs that greater than or equal to 20 bp, accounting for 44.58 % of the total number of SSRs. These results indicated that the SSR derived from the transcriptome of *Lonicera macranthoides* had high polymorphism, which had high application value in the study of molecular markers of *Lonicera macranthoides* and *Lonicera* L..

1.4 Development and validation of EST-SSR marker in *Lonicera macranthoides*

In order to develop SSR molecular markers, Primer Premier 6.0 was used to design primers with the upstream and downstream sequences not less than 150 bp, and five pairs of primers were designed for each sequence. After SSRFinder was used to verify and remove the unqualified primers, a total of 17 611 pairs of SSR loci specific primers were designed for 3,523 SSR sequences, accounting for 17.47% of the total SSR of *Lonicera macranthoides*.

Thirty pairs of primers with different SSR loci were randomly selected and used to amplify the DNA of six different *Lonicera macranthoides* varieties, among which 14 pairs of primers showed polymorphism. POPGENE software was used to analyze the genetic diversity, and four indicators including number of alleles (Na), number of effective alleles (Ne), expected heterozygosity (He) and observed heterozygosity (Ho) were analyzed (Table 3). Each polymorphic locus had two to four alleles, with Ho index ranging from 0.00 to 0.65 and He index ranging from 0.06 to 0.66.

2 Discussion

SSR marker is an ideal molecular marker, which mainly has the following advantages compared with other molecular markers (You, 2015) : (1) It is widely distributed, uniform and abundant in the whole genome; (2) High polymorphism ratio and high polymorphism among individuals of the same species; (3) SSR loci are multiallelic genes, which can provide rich genetic information; (4) With codominance, homozygotes and heterozygotes can be distinguished; (5) Good stability and repeatability. According to the development mode, SSR markers can be further divided into genome SSR markers and expressed sequence tag SSR markers (EST-SSR). The development of EST-SSR based on transcriptome sequencing is time-saving, cheap and cost-effective. Compared with genomic SSR markers, EST-SSR markers mainly have the following advantages (Parthiban et al., 2018; Xiang et al., 2018): (1) For species without reference genome sequence, the development cost is low and efficient; (2) EST-SSR is derived from a relatively conserved transcriptional region, which has higher generality and conservation than genomic SSR markers, and can be used in similar species. (3) Genes with specific functions are closely related and more easily associated with phenotypic traits.

Lonicera macranthoides, as a basal plant of the traditional Chinese medicine, wild honeysuckle flower, is the main species in the central South and southwest China. With the increase of market demand, introduction area and new producing area, the use of commercial medicinal materials of wild honeysuckle flower is chaotic. The first is that there are four species of wild honeysuckle flowers (*Lonicera macranthoides*, *Lonicera hypoglauca* Miq., *Lonicera confusa* (Sweet) DC., and *Lonicera fulvotomentosa* Hsu et S. C. Cheng). In addition, there is a high similarity in morphology and chemical composition between *Lonicera macranthoides* species and varieties, which is often mixed without screening in the market, resulting in uneven quality of medicinal materials (Li et al., 2020). Second, due to the active and extensive introduction and cultivation of *Lonicera macranthoides*, there are huge differences in ecological environment among different producing areas, and there may be great differences in chemical composition between *Lonicera macranthoides* from different producing areas of the same variety (Jiang et al., 2012). Therefore, *Lonicera macranthoides*, as a common base plant of the bulk medicinal material, is in urgent need of a set of scientific, rapid and convenient germplasm identification method. EST-SSR polymorphisms have many loci, which can reflect rich genetic variation information, and are commonly used as the identification of Chinese medicinal herbs.

Table 3 Characteristics of 14 polymorphic genic SSR markers in *Lonicera macranthoides*

Locus	Repeatmotif	Size (bp)	Forward primer (5' to 3')	Reverse primer (5' to 3')	Na	Ne	Ho	He
SSR01	AT (2*9)	152	CTCTTCGATGTTGTTCATCCAGT	AAGATGAAGATGAAGGAGGAGGA	4	2.2963	0.3871	0.5738
SSR03	GCC (3*7)	132	CGTTGTTACTGAATTCTCGCTTT	GGGTTTTCGGAGATTACTGATTT	3	1.8375	0.4516	0.4632
SSR05	ATGA (4*5)	134	AGATAAACCAGAGGGGATAGCTG	CAGAAACAGCAGTTTTGGCTAAT	2	1.1373	0.0000	0.1227
SSR09	CTG (3*5)	151	TCTCAGTAAAGACCTTTGCCTTG	AGTACCACCAGGAACACCTGTAA	2	1.3709	0.3226	0.2750
SSR10	GGT (3*7)	159	CCAGGGTTTGATAATTGTGATGA	CTCTGCATATACGGTGATGATTG	3	1.9572	0.5806	0.4971
SSR11	AG (2*6)	154	GATGATTGGATTGAGGGGATATT	AACACGTAAATGAATATGGTCCG	4	1.5924	0.2581	0.3781
SSR13	ATCTC (5*4)	127	CGAAACCATCTCTTTGCCTTAC	TGAGATTTCTCTTCCAACACCAT	3	2.8348	0.6452	0.6579
SSR16	AT (2*7)	159	TAAAAGGGCTTACACTTGTTCCC	AATGACTTCAACTTTATGCCGC	3	1.2603	0.0968	0.2099
SSR18	TA (2*7)	120	GCTTTCTTGGTATCTTGAAGGTG	ACAAACACGCAAGTTTGCAATAG	2	1.1373	0.0000	0.1227
SSR19	TA (2*11)	154	GCCATTGATTCAGTTGATTATGA	ACCATCCATCTCTTCCTTTCACT	3	1.8375	0.4516	0.4632
SSR27	ACC (3*5)	148	CTCCGCCTACCAACACCAATA	AGAGTTCTTGTACGAAATCCCG	2	1.1014	0.0323	0.0936
SSR28	TC (2*7)	103	ACTTTAAGTTTCACCCGCATCTT	GATCATGTAATGGTTGGAGGCTA	2	1.0666	0.0645	0.0635
SSR29	ATA (3*5)	136	ACATGATACCCTCCGTACAAAAC	CCCATAGAGAGAATTGAAAGAGAGA	2	1.0666	0.0645	0.0635
SSR30	TGA (3*5)	156	AACAAGTCAGGAAGTTGCTCAAG	AACCACTAGTTCAACCTTACAGGG	2	1.8445	0.0000	0.4654

EST-SSR search was conducted on 74 057 Unigene sequences of *Lonicera macranthoides*. A total of 20 161 SSR loci were found, the frequency of SSR locus was 27.22 %, and the frequency of occurrence was 21.05%. The SSR frequency and frequency of occurrence was slightly lower than 32.51% and 24.64% of *Lonicera caerulea* L.var.edulis Regel. (Zhang et al., 2016). Among EST-SSR distribution characteristics of each species, dinucleotide or trinucleotide was the most frequent repeat type, such as *Lonicera caerulea* L.var.edulis Regel. (Zhang et al., 2016), tea tree (Jin et al., 2006), pepper (Wu et al., 2015), dùzhòng (Huang et al., 2013), Dangshen (Liu et al., 2018) and prickly pear (Yan et al., 2015) etc.. In this study, dinucleotide repeats were the most common in the SSR repeats of *Lonicera macranthoides*, among which AG/CT had the highest frequency, which was consistent with the results of most studies on *Lonicera caerulea* L.var.edulis Regel.. In addition, AAG/CTT was the highest trinucleotide repeat frequency in *Lonicera macranthoides* and *Lonicera caerulea* L.var.edulis Regel.. It is also consistent with the results that AAG/CTT is the main repeat type of trinucleotide in dicotyledonous plants. It is also worth mentioning that AG/CT was the most common dinucleotide repeat motif in *Lonicera macranthoides*, followed by AT/AT and A/T, which accounted for 30.19 %, 15.23 % and 14.02 % of the total SSR, respectively. The single nucleotide repeat A/T was the most common in *Lonicera caerulea* L.var.edulis Regel., followed by AG/CT and AT/AT, which accounted for 33.95 %, 27.21 % and 13.40 % of the total SSR, respectively. Finally, in this study, 30 pairs of EST-SSR primers were designed to amplify the target fragment, and then 6 DNA samples of *Lonicera macranthoides* were used to verify the SSR locus polymorphism, and 14 EST-SSR markers with high polymorphism were successfully developed. However, the actual availability of these 14 EST-SSR markers and whether they have universality and transferability within the *Lonicera* genus need to be further studied by collecting more *Lonicera macranthoides* materials.

3 Materials and Methods

3.1 Transcriptome data of *Lonicera macranthoides*

In this study, the transcriptome sequencing sample of *Lonicera macranthoides* 'Jincuilei' was collected from Xiaoshajiang Town, Longhui County, Shaoyang City, Hunan Province. Total RNA of flower organs was extracted and sent to BGI Technology Co., LTD., Shenzhen. And then transcriptome sequencing was performed based on the HiSeq 2000 high-throughput sequencing platform. Finally, 74 057 Unigenes were obtained.

3.2 SSR screening and primers design of *Lonicera macranthoides*

SSR loci in *Lonicera macranthoides* transcriptome were searched by SSR software MicroSAtellite (MISA). The length of the pre and post sequences on Unigene of all SSR repeat units was screened, and only the SSR whose pre and post sequences were not less than 150 bp were retained and used to design primers. The software Primer 6.0 was used to design primers based on the pre and post sequences of SSR repeat units. Each SSR generated five primers, and then the primers were screened: First, the primers cannot exist SSR; The second is to remove those primers that are compared to different unigenes and screen those primers that are uniquely matched. Thirdly, SSRfinder is used to verify SSR, and product sequence is used to find SSR, check whether the results are the same as MISA results, and screen out the same SSR products.

3.3 DNA extraction and screening of polymorphism primers

Leaf DNA of different *Lonicera macranthoides* varieties was extracted with CTAB Plant genomic DNA Rapid Extraction Kit (Beijing Adley Biotechnology Co., LTD.). PCR amplification reactions were performed on an ABI Veriti 96 PCR instrument. The PCR reaction system of 25 μL was as follows: 12.5 μL 2 × Taq Master Mix (Vazyme), 1 μL of 10 μM forward and reverse primers, 1.5 μL genomic DNA, and 4 μL ddH_2O. The PCR reaction procedure was as follows: predenaturation at 95℃ for 3 min; The optimal Tm annealing time was 15 s at 95℃ and 15 s at 72℃ for 30 cycles. Extend at 72℃ for 5 min. Genetic diversity was analyzed by POPGENE1.32 software.

Authors' contributions

LSS and QZQ were the experimental designers and executors of the study, who completed the data analysis and wrote the first draft of the paper. ZHJ, LYX and CN participated in the experimental design and analysis of experimental results. WXM and LXM were the architects and principals of the project, supervising the experimental design, analyzing data , writing and revising paper. All

authors read and approved the final manuscript.

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