Plant Gene and Trait 2020, Vol.11, No.2, 1-8
http://genbreedpublisher.com/index.php/pgt

Research Article Open Access

Development and Application of SSR Makers with Transcriptional Sequencing Data of Female and Male Flowers in Chestnut

Wenming Qiu Yuhai Xu Xiujuan He Zhu Tong Cui Xiao Na Zhang Zhonghai Sun

Institute of Fruit and Tea, Hubei Academy of Agricultural Sciences, Fruit and Tea Subcenter of Hubei Innovation Center of Agricultural Science and Technology, Wuhan, 430064

Corresponding author email: hbfruit@126.com

Plant Gene and Trait, 2020, Vol.11, No.2 doi: 10.5376/pgt.2020.11.0002

Received: 27 Apr., 2020

Accepted: 28 Apr., 2020

Published: 28 Apr., 2020

Preferred citation for this article:

Qiu W.M., Xu Y.H., He X.J., Tong Z., Xiao C., Zhang N., and Sun Z.H., 2020, Development and application of SSR makers with transcriptional sequencing data of female and male flowers in chestnut, Plant Gene and Trait, 11(2): 1-8 (doi: 10.5376/pgt.2020.11.0002)

Abstract In present study, nineteen simple sequence repeat (SSR) loci were isolated and characterized from male and female flower-specific expression genes, which derived from a transcriptional sequencing database of female and male flowers in chestnut. Among these pairs of SSR primers, five pairs showed good polymorphism and productive stability by PCR analysis. Seventeen chestnut cultivars were used to perform SSR-PCR experiments and the PCR products were determined with capillary electrophoresis (CE) method. The results showed that seventeen cultivars were divided into three groups. The first group included six cultivars such as ‘Chaoduanzhi 1’, ‘Chaoduanzhi 2’, ‘Yimeng Duanzhi’ and ‘Yanhong’, which belongs to northern chestnut varieties. The second group contained ten cultivars, which were mainly southern chestnut varieties composing of some local varieties of Hubei province. The last group contained only one cultivar ‘Jinliwang’, which was separately different relationship from other cultivars in the first and second groups. Taken together, these results indicated that the SSR markers exhibited good repeatability and high polymorphism. Our study provided novel insights into analyzing genetic diversity and developing specific molecular marker assisted with male and female flower of chestnut.

Keywords Chestnut; Transcriptional sequencing; SSR; Capillary electrophoresis

The main commercially cultivated species of Castanea in the world are Chinese chestnut (*Castanea mollissima*), Japanese chestnut (*C. crenta*), American chestnut (*C. dentata*) and European chestnut (*C. sativa*). China is the origin center of chestnut plants, and has the largest yield of edible chestnut in the world. According to FAO statistics in 2018, the total edible chestnut output in the world was 2.354×109 kg, of which Chinese chestnut output was 1.96×109 kg, accounting for 83.5% of the world's output (http://www.fao.org/faostat/zh/# data/QC). Chestnut has unique flavor and rich nutrition. It is a healthy food with low fat and sulfur, rich in starch, crude protein and minerals. Many countries and regions have the traditional habit of consuming chestnut, and the demand of chestnut at home and abroad is large. China has plenty of chestnut germplasm resources and also many local varieties. And the formation and distribution of Chinese chestnut are obviously regional, which can be briefly divided into two types: northern type and southern type. With the development of chestnut industry, the ability to explore and create new germplasm of chestnut is increasing, and the introduction and selection of local chestnut are frequent. Thus it is difficult to identify varieties by using the appearance and morphological characteristics, actually in practice there are problems such as mixed varieties, homonymous foreign matters, heteronymy and homonymy (Cheng et al., 2012). Therefore, it is necessary to study the diversity of chestnut resources and identify varieties, which is benefit for the selection and comprehensive development of new varieties of chestnut.

Simple sequence repeat (SSR) molecular markers exist widely in the genome, with low cost, good stability, high polymorphism, co-dominant inheritance and other advantages (Hokanson et al., 1998). Researchers have developed many SSR markers by constructing gene library or using Fagaceae genome database (http://www.fagaceae.org) and public EST database (Ai et al., 2007; Xiang et al., 2010; Santos et al., 2015). SSR markers were widely used to study the genetic diversity of Chinese chestnut, European chestnut, Japanese

chestnut and American chestnut (Marinoni et al., 2003; Tian et al., 2009; Jiang et al., 2015; Liu et al., 2016). However, the SSR markers were mainly from European chestnut, and there lacks SSR markers from Chinese chestnut, especially the SSR markers related to target genes. In theoretical research and breeding practice, molecular markers related to target traits or target genes are more valuable and precious.

With the reduction of high-throughput sequencing cost, combining transcriptome sequencing with bioinformatics analysis could quickly identify SSR loci and efficiently develop SSR markers, which had been successfully applied in many plants (Yan et al., 2015; Rao et al., 2016; Zhao et al., 2019). In this study, based on RNA-sequence database of male and female flowers of Chinese chestnut, SSR loci were identified in the genes specifically expressed in male or female flowers. Then these SSR loci were screened and verified by PCR and capillary electrophoresis, and SSR markers with good repeatability and polymorphism were developed. This research could provide more high-quality molecular markers, and facilitate development of specific molecular markers linked with male and female flowers of chestnut, which had important meanings for the study of diversity of chestnut resources and the analysis of differentiation and development of male and female flowers of chestnut.

1 Results and Analysis

1.1 DNA quality analysis

The DNA was checked by spectrophotometer, with OD260/OD280 was in the range of 1.8~2.0, OD260/OD230 was about 2.0; then the DNA were detected by electrophoresis on 1% agarose gel (Figure 1). It showed that the main DNA bands were bright and clear, no dispersion bands which meant there was no proteins or RNAs in the DNA sample. These results indicated that the DNA was good and could be used for SSR-PCR amplification in the next step.

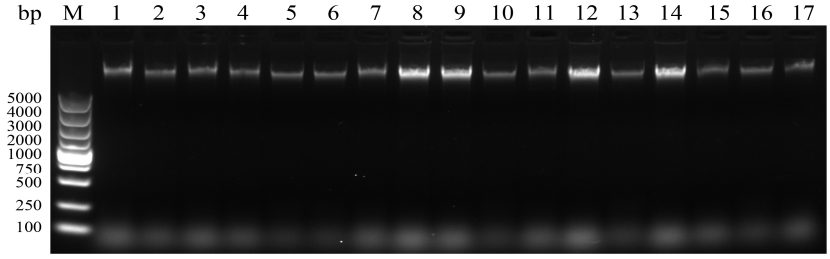

Figure 1 The results of DNA by agarose gel electrophoresis
Note: M: DL5000 DNA Marker; lane 1~17: 17 DNA samples

1.2 SSR primer screening

To evaluate and screen the 19 SSR primers (Table 1)， PCR was conducted using 8 chestnut DNA samples. The results showed nine primers could get clear bands, among them five primers had polymorphic bands, and S3, S10, S15 showed high polymorphism (Figure 2). Each pair of primers could detect 2-7 gene loci within the 600 bp fragment range, which indicated that these primers could be used for subsequent genetic analysis.

1.3 Capillary electrophoresis analysis

The PCR products of 3 pairs of polymorphic primers (S3, S10, S15) in 17 samples were detected by PCR QIAxcel automatic capillary electrophoresis detection platform (PCR-QIAxcel). QIAxcel ScreenGel 1.4 software was used to analyze the amplified fragment size from 15 bp to 600 bp. The results showed 27 polymorphic alleles were detected in the 17 samples, each pair of primers could detect 9 alleles on average. The capillary electrophoresis pattern of primer S15 was shown in Figure 3. Among them, the abscissa was the length of the fragment and the ordinate was the fluorescence intensity (RFU). The higher of the RFU presented the higher concentration of the PCR product. It was shown that the peak value of amplified signal was the same as that on agarose gel, and the size of the band was basically the same as expected, indicating that the primers had good stability and reproducibility. According to the electrophoretic analysis, primer S15 detected 7 polymorphic loci in the 17 chestnut samples (such as 125 bp, 187 bp, 241 bp, 317 bp, 388 bp, 463 bp, 508 bp;

with an error±3 bp). In addition, there were no significant specific peaks, which demonstrated that the SSR primers had high repeatability and polymorphism under current PCR reaction conditions.

Table 1 SSR primers related specific genes between female & male flowers of chestnut

Primer No.		Primer sequence (5'-3')	Repeat type	Optimal annealing Temperature (°C)
S1	F	GATTGCTGGCGATCGTTATT	Perfect	51.2
	R	ACTTGTGAACCCAAAGCACC	$(TA)_9$	
S2	F	TTGATTCCCTGAGGATGGAG	Perfect	51.2
	R	GGTTGTTGCAAAGCCATTCT	$(TGA)_5$	
S3	F	GTGGGAACAAGAAGAAGCCA	Perfect	49.2
	R	AAGTCATTTTGCATGGAGCA	$(AGA)_5$	
S4	F	GAACACGATCAAGAAGTTCCG	Perfect	52.5
	R	TCTCTCTGTGGCTGTTGTGG	$(AGA)_5$	
S5	F	GGCAATGAGCTTGAGCTTAGA	Perfect	52.5
	R	TTGATTACATCAGTACATCTCCTTCC	$(AT)_8$	
S6	F	CTCATGCATGCAAGCACTTT	Perfect	51.2
	R	TGCATTGTGTTACCTTCCCA	$(TTA)_5$	
S7	F	TGACAGAAATGGCGGATAAA	Perfect	49.2
	R	CACAGGTGGCTCTCAAGTCA	$(AAG)_5$	
S8	F	CCCAATGAACAAATTCTGGG	Perfect	51.2
	R	GCTGTGGCATCATCATCATC	$(GAT)_6$	
S9	F	CGAATGCGAGATTGGATGTA	Perfect	51.2
	R	CGTTGATCAATTTACGTGCC	$(ATG)_5$	
S10	F	CCAAGGAGGTTTGCAAGGTA	Perfect	52.5
	R	GGGTGTAGATGGTGGTGGTC	$(AGCC)_6$	
S11	F	CAAAACCTCCACAATTCTCACC	Perfect	52.5
	R	CTCAACCCCACCCCTTTATT	$(TCT)_5$	
S12	F	TTGATGAGTTCAGGCTGGTG	Perfect	52.5
	R	CACTCAACTCACCCCCTCAC	$(GA)_7$	
S13	F	CACGGAGTACGTGTGTTGCT	Perfect	54.3
	R	GAGGAGTTCGAGCTCACCAT	$(CGG)_5$	
S14	F	AGTGGAGATGGTGCTCAAGG	Perfect	52.5
	R	GCTGCTGAGAATTGCTTTCC	$(GGC)_5$	
S15	F	GATGATGAGAGCTTGGCCTC	Perfect	52.5
	R	AAGAAGAAGATGCAGACCGC	$(CCG)_5$	
S16	F	CCACCAATCAGTTCCTCCAG	Perfect	52.5
	R	GCAACATCACCGTCAATGAG	$(GCC)_6$	
S17	F	CGTCAGGTTGAAGTCGAACA	Perfect	52.5
	R	CACTCCTTGCACTAGCCCAT	$(TGC)_5$	
S18	F	ACAACAGCCACCAACTCCTC	Perfect	54.3
	R	CGTTCAGCTCCTCGTAGTCC	$(CGG)_7$	
S19	F	TAGACTGTAGGATGGCCACG	Perfect	54.3
	R	GACCGTTCATGGTCTTCCAG	$(CTC)_5$	

1.4 Genetic analysis of 17 chestnut varieties

According to the results of capillary electrophoresis, the phylogenetic tree of 17 varieties was constructed (Figure 4). The results showed that the genetic diversity coefficients of 17 varieties were ranged from 0.48 to 0.93, and these varieties could be divided into three groups with the coefficient of 0.74 as threshold. Among them, the first group consisted of six varieties such as ‘Chaoduanzhi 1’, ‘Chaoduanzhi 2’, ‘Yimeng Duan- zhi’ and ‘Yanhong’, which were mainly northern Chinese chestnut varieties. The second group contained 10 varieties, which were mainly local varieties in Hubei Province, among them ‘Yaozili’ and ‘Xuanhuahong’ represented Hubei Dawu variety, while ‘Jinyou 1’, ‘Jinyou 2’ and ‘Luotian Hongli’ were mainly from Luotian,

Hubei Province. The third group contained only one variety ‘Jinliwang’, which was far away from the other two groups.

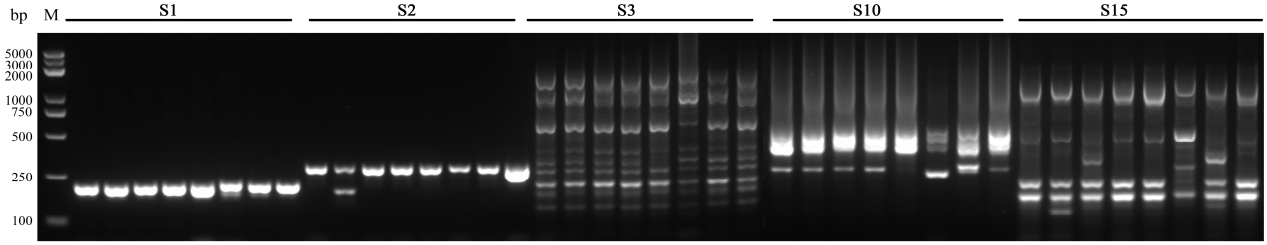

Figure 2 Profile of different primers amplification in 8 samples of *Castanea mollissima*

Note: M: DL5000 DNA Marker; S1, S2, S3, S10 and S15 were the primers’name; The 8 lanes for each primer pair showed the DNA samples of Chaoduanzhi 2, Luotian Hongli, Yaozili, Hongguang Youli, Jinyou 2, Jinliwang, Yanhong and Jiuyuehan respectively from left to right

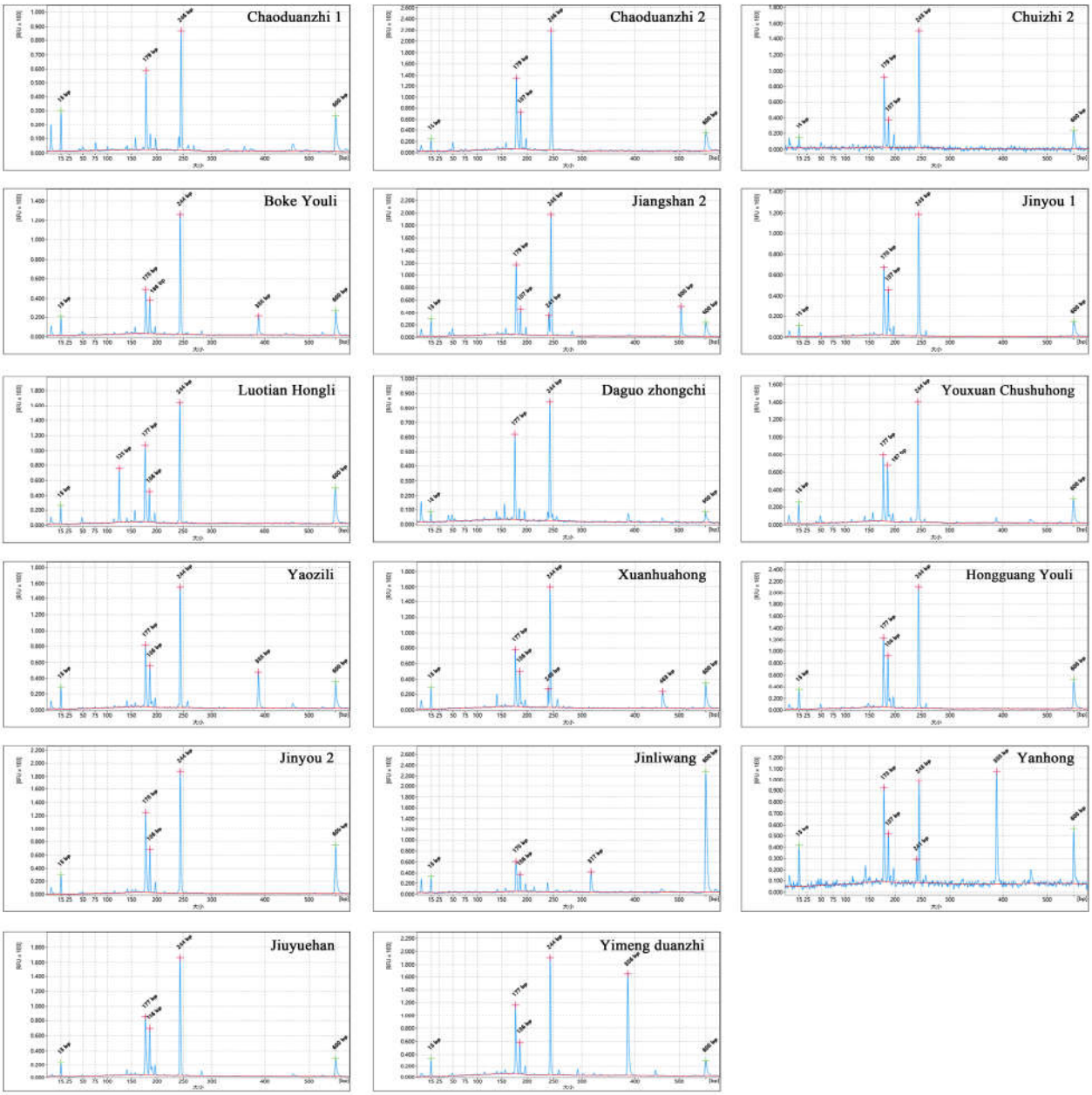

Figure 3 Profile of primer S15 amplification by PCR-QIAxcel in 17 samples of *Castanea mollissima*

The abscissa was the length of the fragment and the ordinate was the fluorescence intensity (RFU), the labels of typical peaks meant the fragment size

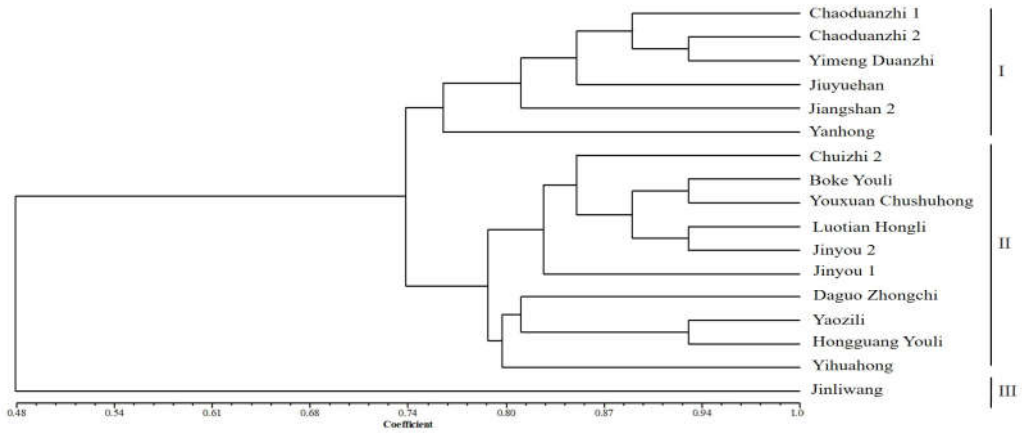

Figure 4 Phylogenetic tree of 17 chestnut varieties based on SSR analysis

2 Discussion

Chestnut is the largest cultivated species in the genus Castanea. It is widely distributed in China and has been cultivated for a long time. Previous studies on agronomic traits or molecular markers of chestnut suggested that both natural and local populations had high genetic polymorphisms (Wang et al., 2006; Jiang et al., 2014). And previous researchers used DNA markers such as RAPD, AFLP, ISSR and SSR to analyze genetic variation or identify varieties of Chinese chestnut (Ai et al., 2006; Wang et al., 2007; Cheng et al., 2012; He et al, 2014). SSR marker was the most widely used as its high repeatability, stability and reliability. As the capillary electrophoresis was easy, efficient, accurate and sensitive method to detect the PCR results, combining SSR-PCR with capillary electrophoresis has been successfully used for chestnut SSR genetic analysis (Liu et al., 2016).

The SSR markers developed in this study were from Chinese chestnut. To test these markers, randomly 17 Chinese chestnut variety were analyzed. And the phylogenetic tree of these varieties were consistent with their distribution, origination and variety characteristics reported previously, which objectively reflected the genetic relationship of these varieties. For example, the first group mainly contained Northern Chinese chestnut varieties, among which three short branch varieties were from Shandong Province, and they were closely related and clustered together; while 'Jinliwang'was derived from Japanese chestnut, although it had been domesticated for a long time in China, it still retained obvious characteristics of Japanese chestnut, such as its leaves are lanceolate (Xu et al., 2010), so it had a distinct clustering relationship with other 16 Chinese chestnut varieties. These results indicated that the SSR primers developed in this study had good polymorphism and reliability, and could be used for genetic analysis of germplasms from genus Castanea including chestnut.

In addition, it was worth noting that the SSR markers developed herein were based on the genes specifically expressed in male and female flowers of Chinese chestnut compared with previous studies. Theoretically, these markers were closely linked with the genes involved in the differentiation and development of male and female flowers of Chinese chestnut, and these SSR markers could be regarded as traits-related markers. Actually, in practice the yield and quality of chestnut were seriously affected by the shortage of female flowers and overload of male flowers. However, the research on the genetic and molecular level of the differentiation and development of male and female flowers in chestnut was very lacking (Qiu et al., 2016). So based on our study herein, the next step was to analyze and verify the SSR markers in the male and female flowers, and to screen SSR markers closely linked to the specific genes of male and female flowers of chestnut. Expected SSR markers could be applied in the molecular marker assisted breeding program, and benefit for the breeding of 'multiple female and less male'varieties of chestnut in the future.

3 Materials and Methods

3.1 Materials and sampling

The 17 chestnut varieties (Table 2) used in this study were planted in Chestnut Resource Nursery of Institute of

fruit and Tea, Hubei Academy of Agricultural Sciences. The trees were grafted for about 10 years, with seedling chestnut (*C. mollissima*) as rootstock. And for each variety, at least 3 healthy trees were used for sampling. The leaf samples of peripheral, inner, upper and lower parts of the tree were randomly collected and stored in ice box temporarily. After being brought back to the laboratory, the leaves were washed with clear water, and then frozen rapidly with liquid nitrogen and stored at -80℃ for use.

Table 2 Name and source of chestnut cultivars

No.	Name of cultivar	Source	No.	Name of cultivar	Source
1	Chaoduanzhi 1	Taian, Shandong	10	Yaozili	Dawu, Hubei
2	Chaoduanzhi 2	Taian, Shandong	11	Xuanhuahong	Dawu, Hubei
3	Chuizhi 2	Linyi, Shandong	12	Hongguang Youli	Luotian, Hubei
4	Boke Youli	Luotian, Hubei	13	Jinyou 2	Wuhan, Hubei
5	Jiangshan 2	Dawu, Hubei	14	Jinliwang	Wuhan, Hubei
6	Jinyou 1	Wuhan, Hubei	15	Yanhong	Changping, Beijing
7	Luotian Hongli	Luotian, Hubei	16	Jiuyuehan	Luotian, Hubei
8	Daguo Zhongchi	Luotian, Hubei	17	Yimeng Duanzhi	Junan, Shandong
9	Youxuan Chushuhong	Yixing, Jiangsu			

3.2 DNA extraction and quality evaluation

The genomic DNA of leaf samples were extracted by CTAB method (Doyle and Doyle, 1987), and each sample was repeated three times. DNA was dissolved in double distilled water and detected with NanoDrop 1000 ultra-micro spectrophotometer (Thermo Fisher Scientifific Inc., Wilmington, DE, USA) to check the DNA concentration and absorbance value. The integrity and quality of DNA were analyzed using electrophoresis on 1% agarose gel. The qualified DNA was diluted to about 50 ng/μl with double distilled water and stored at -20℃ for use.

3.3 Screening of SSR loci related to male and female flowers specifically expressed genes

Based on the RNA-sequencing database of female and male flowers of chestnut generated previously in our lab, differential expressed genes between male and female flowers were identified according to the criteria of false discovery rate<0.01 and fold changes≥2 (Qiu et al., 2016). In this study, rpkm (reads per kilobase per million mapped reads) value was used to screen male and female flower specifically expressed genes (SEGs), for example, rpkm=0 in male flowers, rpkm>0 in female flowers, the gene was selected as candidate gene of female flower specific expression. With this method, a total of 239 genes were identified, including 59 female flower SEGs and 180 male flower SEGs. According to the unique sequences of these SEGs, microsatellite (MISA) software was used to identify and locate simple sequence repeats (SSR) sites (Thiel et al., 2003), and primer 3 software was used to design SSR primers (Table 1). The primer DNA was synthesized by Tianyihuiyuan Biotechnology Co., Ltd. (Wuhan) and purified by PAGE method.

3.4 SSR primers amplification and screening

Among the 17 chestnut DNA samples, 8 samples were randomly selected ('Chaoduanzhi 2', 'Luotian Hongli', 'Yaozili', 'Hongguang Youli', 'Jinyou 2', 'Jinliwang', 'Yanhong' and 'Jiuyuehan') and 19 pairs of SSR primers (Table 1) were used for PCR analysis. PCR was performed using 25 μL reaction mix containing 200 ng template DNA, 2×es-Taq master mix (premixed es-Taq polymerase, 3 mmol/L $MgCl_2$, 400 μmol/L dNTPs) (Kangweishiji, Beijing) 12.5 μL, 1 μL forward primer (10 μm) and 1 μL reverse primer (10 μm). Then the PCR reaction mix were incubated at 94℃ for 3 min, 94℃ for 30 s, annealing for 30 s, 72℃ for 1 min, 35 cycles, and 72℃ for 5 min. The PCR products and DL5000 (DNA marker) were analyzed using electrophoresis on 2.5% agarose gel containing Gelred solution (Biotium). After electrophoresis under 130 V for 50 min, the pictures were taken by gel imaging system (Bio-Rad).

3.5 Capillary electrophoresis analysis

Furthermore, SSR primers with high polymorphism were selected to amplify the 17 samples of chestnut DNA. The PCR program was the same as above, with only the annealing Temperature was different (Table 1). The PCR products were analyzed by automatic capillary electrophoresis Qiaxcel Advanced (Qiagen, Germany).

Qiaxcel® DNA high resolution kit OM800, QX alignment marker (15/600 bp) and QX DNA Size Marker (25~500 bp) were purchased from Qiagen, and the results were analyzed with Qiaxcel ScreenGel software (Qiagen, Germany). According to the PCR-QIAxcel amplification results, the capillary electrophoresis data were transformed into'1,0'matrix. The genetic similarity coefficient of 17 samples was calculated by NTSYS-pc 2.10 software. And the phylogenetic tree was constructed by UPGMA clustering method with default parameters.

Authors' contributions

Wenming Qiu designed and conducted the experiments, completed data analysis and wrote the manuscript. Xiujuan He and Zhu Tong involved in sampling. Na Zhang involved in SSR-PCR electrophoresis and data collection. Yuhai Xu and Cui Xiao revised the manuscript. Zhonghai Sun was the principal investigator of this research, involved in experimental design, data analysis, writing and modification of the manuscript. All authors read and approve the final manuscript.

Acknowledgments

This research is supported by the project of Hubei Agricultural Science and Technology Innovation Center (2019-620-000-001-23), the supporting project of Hubei Academy of Agricultural Sciences (2019fcxjh03) and the young top talents program of Hubei Academy of Agricultural Sciences (q2018028).

Reference

Ai C.X., Yu X.M., Liu Q.Z., and Zhang L.S., 2006, Extraction and RAPD and SSR analysis of genomic DNA in *Castanea* plants, Xibei Zhiwu Xuebao (Acta Botanica Boreali-Occidentalia Sinica), 26(3): 624-627

Ai C.X., Yu X.M., Zhang L.S., and Liu Q.Z., 2007, Development and characterization of SSR markers in Chinese patial chestnut (*Castanea mollissima*) cultivars, Nongye Shengwu Jishu Xuebao (Journal of Agricultural Biotechnology), 15 (2): 283-289

Cheng S.Y., Yuan H.H., Cheng H., Li L.L., Wang Y., Xu F., and Jiang D.Z., 2012, A preliminary identification on the genetic resources of *Luotian Chestnut* by ISSR markers, Hubei Nongye Kexue (Hubei Agricultural Sciences), 51(14): 3096-3100

Doyle J.J., and Doyle J.L., 1987, A rapid DNA isolation procedure for small quantities of fresh leaf tissue, Phytochemical Bulletin, 19 (1): 11-15

Hokanson S.C., Szewc-McFadden A.K., Lamboy W.F., and McFerson J.R., 1998, Microsatellite (SSR) markers reveal genetic identities, genetic diversity and relationships in a *Malus* × *domestica* borkh. core subset collection, Theor. Appl. Genet., 97: 671-683
https://doi.org/10.1007/s001220050943

He X.J., Qiu W.M., Xu Y.H., 2014, Analysis of Genetic Diversity of Chestnut Germplasm Resources in Hubei Province by Leaf Morphologic Characteristics and RAPD Markers, Nanfang Guoshu (South china fruits), 43(2):17-21

Jiang X.B., Gong B.C., Liu Q.Z., Chen X., Wu K.Y., Deng Q.E., and Tang D., 2014, Phenotypic diversity of important agronomic traits of local cultivars of Chinese Chestnut, Yuanyi Xuebao (Acta Horticulturae Sinica), 41(4): 641-652

Jiang X.B., Tang D., Gong B.C., and Lai J.S., 2015, Genetic diversity and association analysis of local cultivars of Chinese Chestnut based on SSR markers, Yuanyi Xubao (Acta Horticulturae Sinica), 42(12): 2478-2488

Liu G.B., Cao J., Wang J.B., Lan Y.P., 2016, Genetic diversity analysis of ancient Chestnut trees based on fluorescent SSR markers, Forest Research, 29 (6): 940-945

Marinoni D., Akkak A., Bounous G., Edwards K.J., and Botta R., 2003, Development and characterization of microsatellite markers in *Castanea sativa* (Mill.), Molecular Breeding, 11: 127-136
https://doi.org/10.1023/A:1022456013692

Qiu W.M., Xu Y.H., He H.J., 2016, Identification and expression analysis of *MADS*-box genes related to female and male flowers development in Chestnut, Yuanyi Xuebao (Acta Horticulturae Sinica), 43 (8): 1593-1604

Rao L.B., Yang H.B., Guo H.Y., Duan H.P., Chen Y.T., Development of SSR molecular markers based on transcriptome sequences of *Alnus*, Linye Kexue Yanjiu (Forest Research), 29 (6): 875-882

Santos C., Zhebentyayeva T., Serrazina S., Nelson C.D., and Costa R., 2015, Development and characterization of EST-SSR markers for mapping reaction to Phytophthora cinnamomi in *Castanea* spp. Scientia Horticulturae, 194: 181-187
https://doi.org/10.1016/j.scienta.2015.07.043

Thiel T., Michalek W., Varshney R., and Graner A., 2003, Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.), Theor. Appl. Genet., 106: 411-422
https://doi.org/10.1007/s00122-002-1031-0
PMid:12589540

Tian H., Kang M., Li L., Yao X.H., Huang H.W., 2009, Genetic diversity in natural populations of *Castanea mollissima* inferred from nuclear SSR markers, Shengwu Duoyangxing (Biodiversity Science), 17(3): 296-302
https://doi.org/10.3724/SP.J.1003.2009.09043

Wang T.K., Bai S.H., Dong C.H., Hu Y.J., Qi Y.S., Zhang J.Z., 2007, Genetic diversity of Chestnut germplasm in Yanshan region revealed by AFLP, Molecular Fenzi Zhiwu Yuzhong (Plant Breedin), 5(1): 121-127

Wang Y., Kang M., and Huang H.W., 2006, Subpopulation genetic structure in a panmictic population as revealed by molecular markers: A case study of *Castanea Sequinii* using SSR markers, Zhiwu Shengtai Xuebao (Journal of Plant Ecology), 30(1): 147-156 https://doi.org/10.17521/cjpe.2006.0021

Xiang W., Ning Z.L., Kang M., and Huang H.W., 2010, Characteristic and generality of EST-SSRs of *Castanea mollissima*, Redai Yaredai Zhiwu Xuebao (Journal of Tropical and Subtropical Botany), 18(6): 665-669

Xu Y.H., Jiang Y.C., Wang Z.J., Fang B., Wang Q.F., Zhang L.T., Su X.G., and Wang C.H., 2010, Breeding of a new processing Chinese chestnut cultivar Jinliwang, Guoshu Xuebao (Journal of Fruit Science), 27(1): 156-157

Yan X.Q., Lu M., and An H.M., 2015, Analysis on SSR information in transcriptome and development of molecular markers in *Rosa roxburghii*, Yuanyi Xuebao (Horticulturae Sinica), 42(2): 341-349

Zhao T., Chang S.X., Leng Q.Y., Xu S.S., Yin J.M., and Niu J.H., 2019, Development of SSR molecular markers based on transcriptome sequencing of *Bougainvillea*, Fenzi Zhiwu Yuzhong (Molecular Plant Breeding), 17 (13): 4331-4341