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Development of Polymorphic SSR Markers in Chinese Water Chestnut Based on RAD-seq

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Abstract Chinese water chestnut (*Eleocharis dulcis*) is an important characteristic vegetable that grows in shallow waters. In order to develop simple sequence repeat (SSR) molecular markers for genetics research on *Eleocharis dulcis*, simplified genome sequencing by restriction-site associated DNA sequencing (RAD-seq), SSR search and primer design were performed. A total of 5039 SSR loci were detected, from which 4 137 pairs of primer were designed. Among them, the proportion of tri-nucleotides motifs was the highest (1 894), accounting for 45.89% of the total loci; followed by di-nucleotide with 1 406, accounting for 34.07%.. A total of 100 pairs of randomly selected primers were verified. 93% of the primers were successfully amplified. To assess the polymorphism of these primers, two varieties of Chinese water chestnuts were used to amplify the SSR marker using 83 pairs of primers. The 83 pairs of primers generated a total of 232 fragments. A total of 128 fragments were polymorphic. 60 pairs of primers out of 83 pairs generated at least one polymorphic band, and the ratio of polymorphic primers was 72.28%. These SSR markers developed from RAD tags were proved to be effective, which will provide a useful tool for genetic diversity research and molecular breeding in Chinese water chestnuts.

Keywords RAD sequencing; SSR; Chinese water chestnut; Molecular marker

Chinese water chestnuts (*Eleocharis dulcis*) are commonly known as ‘Mati’ in Chinese, because they are shaped somewhat like a horse's feet. It is a perennial shallow-water herb, widely distributed in Southeast Asia, America, Europe and Oceania, etc. (Li et al., 2006, Changjiang Vegetables, (8): 39-43). China is one of the places of origin of Chinese water chestnuts. Guangxi is the province with the largest planting area of Chinese water chestnuts in China, with the planting area of 20 000 hm^2 in 2016, accounting for about 44.98% of the total area of the country (Zhao et al., 2018, Economic Management Press, pp.41). Chinese water chestnut bulbs are a popular food with high nutritional and medicinal components, unique taste, low fat content and no cholesterol (Hummel and Kiviat, 2004; Zhan et al., 2014).

The variety diversity of Chinese water chestnuts is high. The wild varieties usually produce small corms, only 1 cm in diameter, whereas the cultivated varieties have stronger stems and larger, sweeter corms, purplish red to brown, up to 4 cm in diameter. At present, the research of Chinese water chestnut mainly focuses on variety breeding (Chen et al., 2011a; 2011b; Lai et al., 2018), tissue culture (Pan et al., 2011; Gao et al., 2016), cultivation techniques (Li, 2017, Northern Horticulture, (3): 66-67; Xu et al., 2019, Chinese Vegetables, (1): 100-102), physiological and biochemical characteristics (Hussain et al., 2019; Zhang et al., 2019), cloning and expression of related genes (Cheng et al., 2016; Song et al., 2019; Song et al., 2020) and so on. In the study of genetic resources, Jiang et al. (2012; 2015) analyzed the genetic diversity of 24 Chinese water chestnut varieties by using RAPD and ISSR techniques, and classified the Chinese water chestnut resources into 5 groups. Zhang et al. (2016, Journal of Yangzhou University (Agriculture and Life Sciences), 37(4): 119-122) optimized the ISSR response system and applied it to the genetic diversity analysis of 35 Chinese water chestnut germplasm resources, and divided the Chinese water chestnut resources into two categories. In the first category, there is only one kind of Chinese water

chestnut in Fuzhou, Jiangxi province, and the rest are classified into the second category. Liu et al. (2015) used transcriptome sequencing to develop simple sequence repeats (SSR) markers for transcription sequences of Chinese water chestnut and obtained 2 570 SSR loci.

Simple repeat sequences (SSR) are short motif arrays ranging in length from 1 to 10 base pairs and are widely distributed in eukaryotic genomes (Gemayel et al., 2012). SSR markers have been widely used in plant genotyping and marker-assisted breeding due to their characteristics of high information, co-dominance, simultaneous detection of multiple alleles, good repeatability, replication and transfer between related species, etc. (Collard and Mackill, 2007).

The development of next generation sequencing (NGS) techniques provides a more convenient means of studying species without reference genomes (Zalapa et al., 2012). Restriction-site associated DNA sequencing (RAD-seq), a type of simplified genome sequencing, is highly utilized in the development of SSR markers. This method was first proposed by Miller et al. (2007). Firstly, restriction endonuclides were used to obtain a large number of polymorphic fragments, which effectively simplified the complexity of the genome. After double-ended sequencing, simplified genome sequence data was obtained, from which genome-based microsatellite loci could be developed (Barchi et al., 2011).

The aim of this study was to detect and analyze SSR sequence in the genome of Chinese water chestnut by RAD-seq and screen effective primers for the development of SSR markers in the genome of Chinese water chestnut. The results of this study will provide a tool and theoretical basis for assessing the genetic diversity and phylogeny of Chinese water chestnut using SSR markers.

1 Results and Analysis

1.1 A simplified genome sequence that is sequenced and assembled

RAD-seq obtained a total of 39.4 G of raw data. After data validity filtering, the amount of Clean Base (bp) data obtained was 39.2 G, and the data efficiency was 99.78%. The percentages of bases with Phred values greater than 20 and 30 accounted for 96.75% and 91.43% of the total bases, respectively, and the GC content was 33.87%. The number of Clean Reads obtained was 130 963 222 and 77 437 310 after weight removal. The repeat rate of Clean reads was 40.87%, and the number of reads with EcoRI restriction sites was 75 883448. Cd-hit was used to cluster reads containing EcoRI cleavage sites (Li and Godzik, 2006), and reads similar to RAD-tag were grouped together as a cluster. A total of 24 848 206 cluster tag numbers were obtained. The classes with fewer than 10 clustered reads were filtered, and 450 203 clusters were obtained, which contained 43 491 152 available reads. Each type of Contig after selection was locally assembled, and the Contig less than 100 bp were removed. A total of 393 324 Contig were obtained, and the average length of each Contig was 263 bp (Table 1). In this study, the data validity of RAD sequencing was relatively high, and the number of sequences with enzyme restriction sites was large. After clustering and assembly, high-quality Contig was obtained, which provided reliable data for SSR analysis and primer design.

1.2 4 127 pairs of Chinese water chestnut SSR primers were developed

SSR analysis of the assembled Contig obtained 5 039 double-ended SSR loci containing 100 bp sequences, among which 4 127 loci were available and primers were successfully designed (Appendix 1). Among the available SSR loci, repetition types ranged from two base to six base, among which Trinucleotide was the highest with 1 894, accounting for 45.89% of the total number, and its average length was 13.92 bp. Dinucleotide was followed by 1 406, accounting for 34.07% of the total, with an average length of 16.33 bp (Table 2). The number of SSR repeats ranged from 4 to 30, and the number of SSR repeats with 4 times was the largest, which was 1 885, accounting for 45.67% of the total number (Figure 1). In the repeating units, the number of AT and TA was the largest, both were 269, accounting for 6.52% of the total, and the average length was 17.37 bp and 16.52 bp, respectively. TAT has the largest number of Trinucleotide, accounting for 4.12% of the total number of 170, with an average length of 14.24 bp. AAAT has the largest number of Dinucleotide, with a total of 49, accounting for 1.19% of the total, and an average length of 17.71 bp (Table 3). In this study, SSR obtained by RAD sequencing and primers designed

according to sequencing data were abundant, and SSR types were distributed evenly, indicating that sequencing data could cover the genome more evenly.

Table 1 Summary statistics of the RAD-seq

Data types	Data size
Raw base (bp)	39 377 398 500
Clean base (bp)	39 288 966 600
Effective rate (%)	99.78
Q20 (%)	96.75
Q30 (%)	91.43
GC content (%)	33.87
Clean reads	130 963 222
Removed duplication reads	77 437 310
Clean duplication rate (%)	40.87
Digestion reads	75 883 448
Digestion ratio (%)	97.99
Cluster tag number	24 848 206
Cut tag number	450 203
Cut pair reads	43 491 152
Total contig base (bp)	103 788 703
Total contig number	393 324
Average contig length (bp)	263
N50 length (bp)	111 338

Table 2 Statistics of 4127 SSR loci

SSR motif	Counts	%	Average motif length (bp)	Number of repeats								
				3	4	5	6	7	8	9	10	>10
Dinucleotide	1406	34.07	16.33	-	-	-	567	258	175	104	75	227
Trinucleotide	1894	45.89	13.92	-	1321	313	120	47	37	21	10	25
Tetranucleotide	516	12.50	18.19	-	333	121	40	13	4	3	2	-
Pentanucleotide	190	4.60	22.95	-	137	37	7	2	2	1	-	4
Hexanucleotide	121	2.93	25.74	-	94	20	6	1	-	-	-	-

Note: % indicates the ratio of this SSR type to the total number

1.3 The developed SSR primers of Chinese water chestnut showed high effectiveness

In order to verify the effectiveness of SSR primers developed based on RAD-seq sequence analysis, 100 pairs of different types of SSR primers were randomly selected for verification (Appendix 2). After PCR and electrophoresis detection, the success rate of 100 pairs of primers was 93% (Figure 2). In order to further evaluate the polymorphism of SSR primers of Chinese water chestnut, two varieties with obvious differences in phenotype were selected, and 83 pairs of primers were selected from the successfully amplified primers for polymorphism verification. The results showed that 83 pairs of primers amplified 232 bands, of which 128 were polymorphic, and the average number of bands amplified by each pair of primers was 4.26. The average number of polymorphic bands was 2.3, with an average polymorphism percentage of 45.76%, and 60 pairs of polymorphic primers with a ratio of 72.28% (Figure 3; Appendix 3). The SSR primers of Chinese water chestnut reported in this study had a high success rate of amplification, and the primers with polymorphism reached 70%, indicating good polymorphism.

2 Discussion

Molecular markers play a very important role in the application of marker assisted breeding to breed new varieties (Collard and Mackill, 2007). SSR markers are abundant in plant genomes, featuring co-dominance and heritable characteristics. The detection method is easy to operate, with high repeatability and reliable results. Therefore,

SSR markers are widely used in genetic diversity analysis, fingerprint construction and genetic linkage studies (Gupta and Varshney, 2000). In this study, we developed a set of SSR markers from the simplified genomic data of Chinese water chestnut, which has high application value in the follow-up study of Chinese water chestnut.

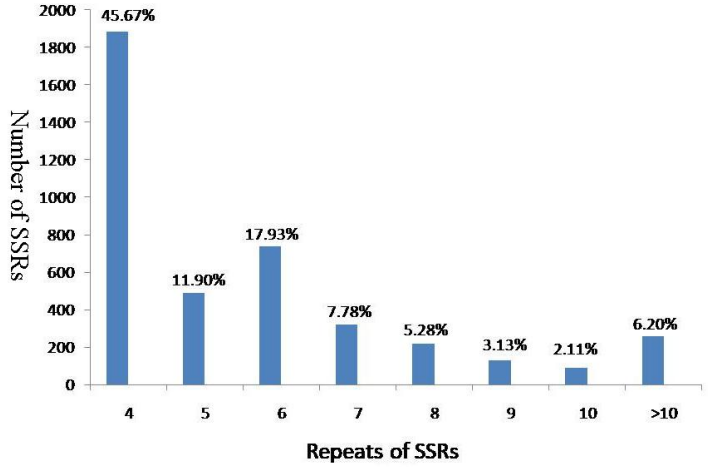

Figure 1 Frequency distribution of SSR repeats

Table 3 Frequencies and repeat numbers for the 20 most present SSR motifs

SSR motif	Counts	Average length (bp)	% of the total
AT/TA	269	17.37	6.52
TA/AT	269	16.52	6.52
TC/GA	211	15.80	5.11
CT/AG	206	16.81	4.99
TAT/ATA	170	14.24	4.12
AAT/ATT	165	14.16	4.00
ATA/TAT	159	14.85	3.85
AG/CT	145	15.94	3.51
ATT/AAT	141	13.62	3.42
GA/TC	139	15.91	3.37
TTA/TAA	137	14.08	3.32
TTG/CAA	110	15.03	2.67
TAA/TTA	87	13.97	2.11
TG/CA	74	15.46	1.79
AAG/CTT	66	12.82	1.60
TTC/GAA	59	13.07	1.43
TCT/AGA	53	13.87	1.28
GAA/TTC	52	13.96	1.26
ACA/TGT	51	15.24	1.24
AAAT/ATTT	49	17.71	1.19

As an important aquatic vegetable, there are few studies on the genetic diversity of Chinese water chestnut, and only a few reports on the use of RAPD and ISSR to analyze Chinese water chestnut germplasm resources and the development of SSR based on transcriptional data (Jiang et al., 2012; 2015; Liu et al., 2015; Zhang et al., 2016, Journal of Yangzhou University (Agricultural and Life Sciences Edition), 37(4): 119-122). There have been no studies to develop molecular markers based on the whole genome sequence. Based on the direct correlation between protein coding sequences and genes, the developed SSR markers may be related to functional genes and phenotypes (Wang et al., 2015). However, SSR (Genomic-SSR) developed based on genome has higher polymorphic information content (PIC) and lower genetic similarity than EST-SSR developed based on gene coding sequence. Therefore, genomic SSR has advantages in DNA fingerprinting, breeding selection, identification of variety specific markers, and genetic diversity analysis (Parthiban et al., 2018). With the

development of next-generation sequencing (NGS) technologies, RAD-seq based technologies provide a low-cost tool for the development of thousands of genetic markers in species including non-model organisms (Andrews et al., 2016). The aim of this study was to use the RAD-seq technique to simplify the genome sequencing of Chinese water chestnut, and to obtain a set of genome-based molecular markers, which could provide a basis for the study of genetic diversity of Chinese water chestnut cultivars in later stage.

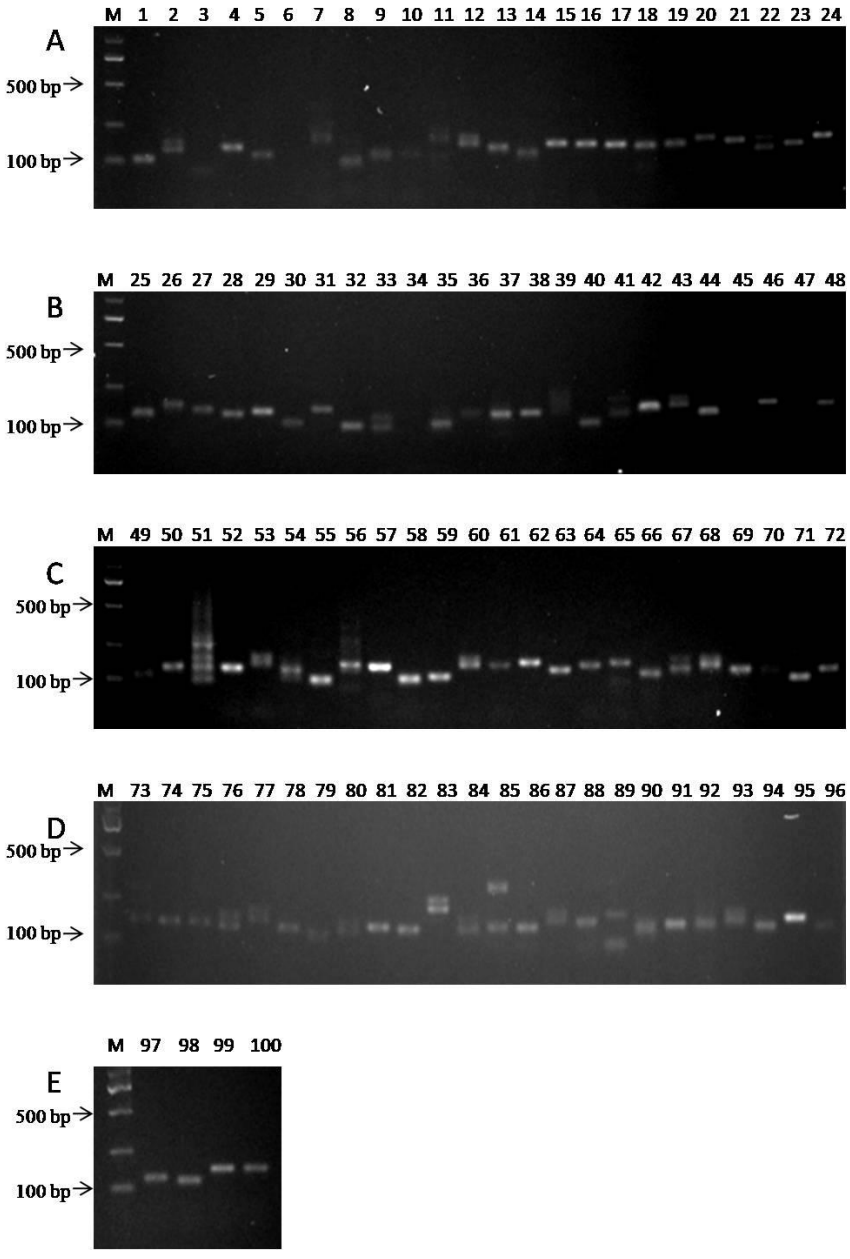

Figure 2 The fast screen of the selected 100 pairs of primer

Note: M: DL2000 DNA marker; The stripes indicated by the arrows represent the number of bases in the fragment

In this study, a total of 393 324 Contig were obtained, and 5 039 SSR loci were obtained through analysis, among which 4 127 SSR loci could be used for primer design. Compared with ISSR and RAPD (Jiang et al., 2012; 2015; Liu et al., 2015; Zhang et al., 2016, Journal of Yangzhou University (Agricultural and Life Sciences Edition), 37(4): 119-122), two molecular marker methods using random primers for screening, the number of SSR markers obtained by screening based on RAD-seq technology is greatly improved, and there is no need for complex preliminary experimental screening (Baird et al., 2009). In previous reports, in SSR analysis of Chinese water chestnut by transcript sequence (EST), 2 379 Contigs, 2 570 SSR loci were obtained, and only 1 606 loci could be

used for SSR primer design (Liu et al., 2015). The SSR loci and designable primers obtained in this study were higher than the SSR loci developed in transcripts. Studies have shown that in plants, non-coding regions contain more SSR than coding regions (Morgante et al., 2002; Fujimori et al., 2003), which may be the reason for the greater number of SSR obtained by RAD-seq. In this study, the development of SSR markers based on RAD-seq resulted in more markers, which has certain positive significance for supplementing the molecular marker library of Chinese water chestnut.

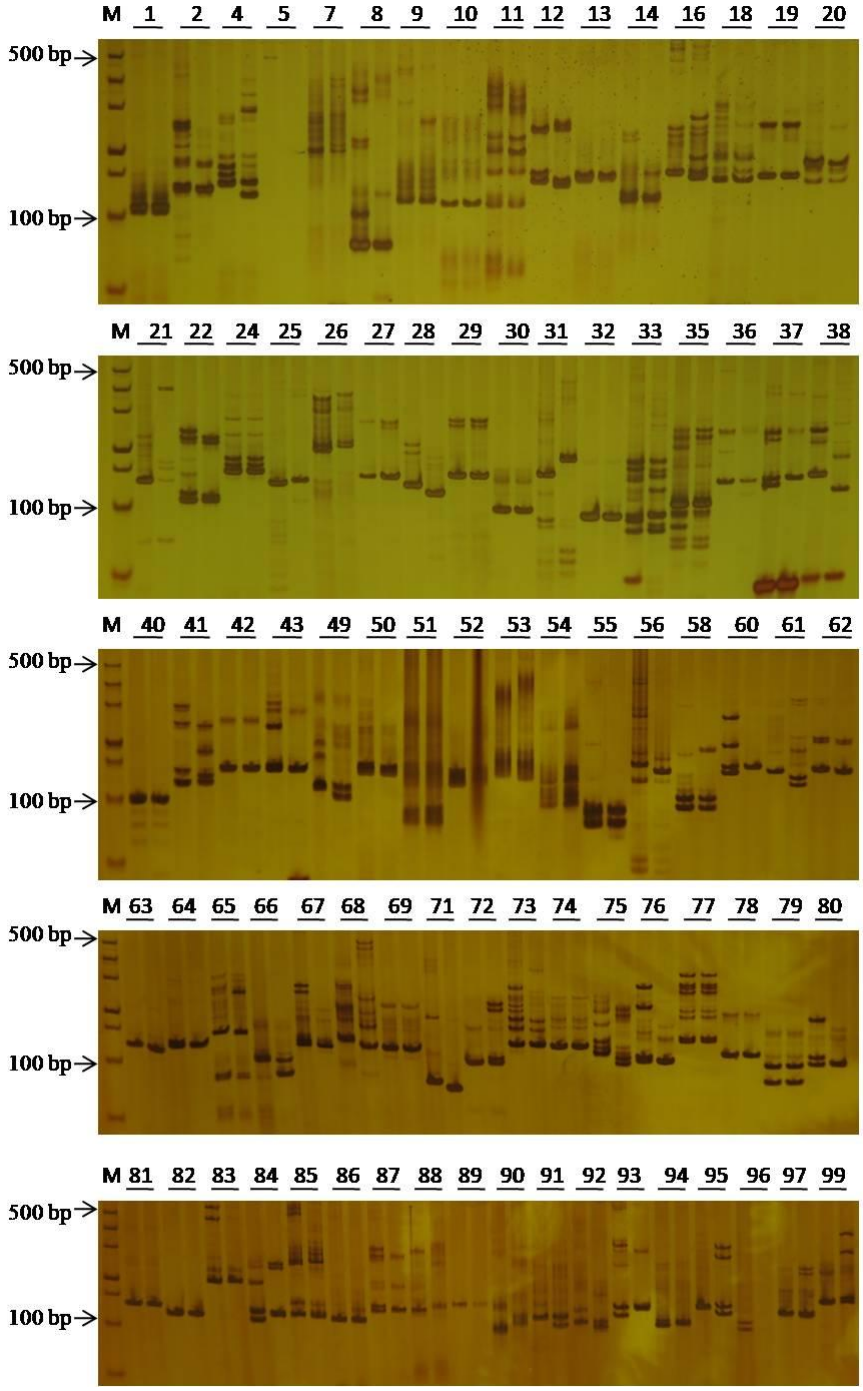

Figure 3 Polymorphic analysis of 83 pairs of SSR primers

Note: M: DL2000 DNA marker; For each pair of primers, the left was Guilin Chinese water chestnut and the right is wild Chinese water chestnut

Among transcript-based SSR markers, Dinucleotide was the highest in abundance (Liu et al., 2015). At the genomic level, SSR in most plants has the highest Trinucleotide ratio, for example, *Arabidopsis thaliana* has a Trinucleotide ratio of 58% and Dinucleotide ratio of 21.9% (da Maia et al., 2009). In this study, among the SSR markers developed based on RAD-seq, the highest repetition type was Trinucleotide, with a ratio of 45.89% and a number of 1 894. Compared with Dinucleotide SSR, Trinucleotide SSR is easier to conduct polymorphism analysis in PCR detection, because Trinucleotide SSR has fewer false strips (Song et al., 2002). Therefore, SSR markers developed in this study have higher success rate and polymorphism in practice. In this study, the SSR with the highest repetition units were AT and TA, with both ratios of 6.52%. Among the SSR markers developed based on transcript, GA had the highest repetition units, and Dinucleotide had the highest abundance (Liu et al., 2015). It is not completely consistent with the method based on RAD-seq in this study, indicating that SSR in CDS region and SSR in the whole genome have certain differences in repeating units. However, in other plants, such as two crops of Solanum family, tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*), the highest SSR locus of repetitive unit is AT. The ratios were 8.55% and 8.04%, respectively (da Maia et al., 2009), which were consistent with Chinese water chestnut.

In order to verify the effectiveness of SSR markers, 100 SSR loci of different types were randomly selected for verification. The amplification success rate was 93%, and the product size was basically consistent with the expected product size, indicating that the SSR markers identified in this study were highly effective. Eighty-three pairs of primers were selected from the successfully amplified primers to analyze the polymorphism of the two Chinese water chestnut varieties with significant differences. Sixty pairs of primers had polymorphism loci, the ratio of which was 72.28%, indicating that the SSR marker developed by this method had high polymorphism.

In this study, the number of SSR markers of Chinese water chestnut was higher, and their availability and polymorphism were higher, which provided a basis for the study of genetic diversity of Chinese water chestnut germplasm resources in the later period.

3 Materials and Methods

3.1 Sample collection and DNA extraction

The tested Chinese water chestnut materials were local variety 'Guilin Mati' and wild Chinese water chestnut. Leaf stem liquid nitrogen was collected for quick freezing and stored at -80 °C for later use. DNA was extracted using the Biospin Plant Genome DNA Extraction kit (Bioer Technology, #BSC13S1). Refer to the instruction manual for specific operation. The resulting DNA was tested for concentration and integrity using 1% agarose gel electrophoresis. The purity of each DNA was tested using NanoDrop and the exact concentration of DNA was tested using Qubit.

3.2 RAD library construction and sequencing

Enzyme digestion: 600 ng DNA was added into 50 μL enzyme digestion reaction system containing 20 U EcoRI, digested at 37 °C for 60 min, and then terminated at 65 °C for 20 min.

Plus P1 connector: P1 primer (100 nmol/L) 2.5 μL, ATP (10 mmol/L) 1 μL, 10×NEB Buffer 1 μL, T4 DNA ligase 1 μL, ddH_2O 5 μL were mixed evenly, incubated at room temperature for 20 min, and then at 65 °C. The connection reaction was terminated at 20 min.

Interrupt: Use Bioruptor to randomly cut the product into segments averaging 500 bp. DNA fragments ranging from 300 bp to 800 bp were separated by 1.5% agarose gel electrophoresis. The MinElute gel extraction kit was used for gelling recovery.

Terminal repair: Flat termination of the reclaimed DNA fragment with end blunting enzyme and A-tail addition of the flat terminating fragment using 15 U Klenow enzyme at 37 °C, 10 min.

Add P2 connector: After the fragment was repurified, use 1 μL P2 connector (10 μmol/L), and connect as per P1 connector.

PCR amplification of enriched fragments: Qubit fluorometer was used for quantification and then PCR reaction was performed. The PCR reaction system was as follows: DNA 20 ng, Phusion Master Mix 20 μL, primers P1 and P2 5 μL (10 μmol/L) each, and ddH_2O supplement 100 μL. The PCR reaction procedure was as follows: 98 °C 30 s, 60 °C 15 s, 72 °C 15 s amplified 18 cycles. After agarose gel electrophoresis, 300~700 bp fragments were cut for gel recovery. After the obtained DNA content was adjusted to 3 ng/μL, the machine could be sequenced.

Computer sequencing: Illumina HiSeq was used for RAD double-ended sequencing.

3.3 SSR analysis and primer design

SSR search software was used to analyze the DNA sequence after RAD-seq assembly and detect simple repeats in the sequence. Search the motif of the two, three, four, five and six nucleotide sequences analyzed, and filter the SSR that is too close. The final SSR confirmation criteria are as follows: the minimum length of repeat unit is 2, the maximum length of SSR repeat unit is 6, the minimum length of SSR sequence is 12, the length of upstream and downstream sequence of SSR is 100 bp, and the minimum distance between two SSR is 12 bp. After SSR location in the genome was confirmed, Primer Premier 3.0 software was used to design primers from SSR flanking sequences in batch mode. The range of target amplification products was set as 125~250 bp, the optimal annealing temperature was 63 °C, the optimal length of primers was 24 bp, and the maximum annealing temperature difference of each pair of primers was 1.

3.4 Validation of primer effectiveness and polymorphism

In order to verify the effectiveness of SSR primers developed based on RAD-seq sequence analysis, 100 pairs of different types of SSR primers were randomly selected for verification. The PCR reaction system is as follows: 2×TSINGKE Master Mix (Tsingke Biotechnology, TSE002) 10 μL, forward primers and reverse primers 1 μL each, genomic DNA 10 ng, ddH_2O complement 20 μL. The PCR reaction procedure was as follows: predenaturation at 94 °C for 5 min, 35 cycles (94 °C 30 s, 60 °C 30 s, 72 °C 90 s), 72 °C for 5 min. PCR products were tested by 1% agarose gel electrophoresis to determine the effectiveness of primers. In order to further evaluate the polymorphism of SSR primers, two Chinese water chestnut varieties with obvious differences in phenotype were selected, and 83 pairs of primers were selected to verify their polymorphism. The PCR reaction system and procedure are as above. The PCR products were electrophoresis with 18% polyacrylamide gel (PAGE), 120 V for 2 h, then washed twice with ddH_2O, the gel was placed in 0.1% $AgNO_3$ solution for shaking and staining for 10 min, and washed twice with ddH_2O rapidly. The washed gel was immersed in 0.4% formaldehyde solution (containing 1.2% NaOH) for development, and a large amount of ddH2O was added in time to terminate development according to development conditions. According to the statistics of the isolated PCR fragments, the location with clear bands was denoted as a site, and the same site with different bands in two varieties was denoted as a polymorphic site.

Authors' contributions

HFL was mainly responsible for experimental operation, data analysis and the writing of the first draft of the paper. QZY, JHP, LLL, CQ and HSY participated in the experimental design and result analysis. DWQ was the proposer and leader of the project, responsible for guiding the experiment and paper writing and revision. All authors read and approved the final manuscript.

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