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Development and Cross-Species Transferability of Novel Genomic-SSR Markers and Their Utility in Hybrid Identification and Trait Association Analysis in Chinese Cherry

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Abstract: Chinese cherry (*Cerasus pseudocerasus* (Lindl.) G.Don) is an economically important tetraploid fruiting cherry species native to China. Simple sequence repeats (SSRs)—due to their codominance, polymorphism, and stability—have been widely applied in genetic identification and trait-association analysis. In this study, using comparative genomics strategy and the data of one high-quality whole genome and seven preliminarily assembled genome sequences, we constructed a database containing 25,779 polymorphic SSR loci to efficiently develop novel markers. Sixty-four SSR loci covering eight linkage groups were selected to design primer pairs. Sixty (93.75%) primer pairs yielded specific bands and 32 (50.00%) exhibited moderate-to-high levels of informativeness (*PIC* ranging from 0.264 to 0.728) in 94 Chinese cherry accessions. A total of 38 primer pairs exhibited high transferability across 13 *Cerasus* taxa. The marker SAUCps203 was species-specific in *C. pseudocerasus* by checking with 114 accessions from *Cerasus* and 16 relatives, suggesting its potential application in accurate identification of Chinese cherry or its interspecific hybrid. Moreover, 1081 out of 1122 individuals from three cross F_1 populations of Chinese cherry were identified as true hybrid offspring by using only five SSR markers. Trait association analysis suggested that 20 SSR loci were significantly associated with soluble solids and fruit size, with explained phenotypic variance ranging from 9.02% to 26.35%. This study will provide a basis for SSR-based germplasm identification and further marker-assisted selection (MAS) of Chinese cherry.

Keywords: Chinese cherry; *Cerasus pseudocerasus*; genomic-SSR development; species-specific SSR marker; hybrid identification; trait association analysis

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1. Introduction

Chinese cherry (*Cerasus pseudocerasus*), belonging to the genus *Cerasus* of the family Rosaceae, is an important tetraploid fresh fruit crop ($2n = 4x = 32$) [1,2]. It originates in Southwest China and is widely distributed in the North and East China Plain, Qinling Mountains, Longmenshan Fault Zones, and Yun-Gui Plateau [3]. Its cultivation history dates back to 3000 years ago in China [4]. Chinese cherry is characterized by early bloom and maturity, and is full flavored, with rich nutritional ingredients and trace elements, wide adaptability, and intensive disease/pest resistance [5]. Therefore, the cherry industry is developing rapidly and has increasingly contributed to the rural vitalization of China.

During the past decade, we have carried out field investigation and assessment of cherry resources across its locality in China [5,6]. Some disadvantages—including small

fruit size, high acid content, and short shelf-life—have hindered the development of Chinese cherry industry to a certain extent [5–7]. Then we conducted intra- and inter-specific crossing by selecting excellent germplasm collections to breed new cultivars with early-matured, large, full-flavored fruit with a long shelf-life, and to accelerate genetic improvement of Chinese cherry. So far, we have obtained over 2000 individuals derived from five cross F_1 populations of Chinese cherry [8,9]. Among them, two landraces (NZH and HF) and one semi-wild germplasm (PJHH) with obvious character differences (Table S1) as parents have bred outstanding offspring. It is urgent to identify its authenticity to speed up further application.

Marker-assisted identification/selection (MAS) is an active way to get DNA markers associated with valuable traits that accelerate the breeding process by replacing the selection with a phenotype to the selection at the DNA or gene level, which has been widely employed in fruit trees [10,11]. SSRs, or microsatellites, are short tandem repeats of one to six nucleotides, which are widely distributed in coding and non-coding regions of the whole genome [12,13]. To date, SSRs have been an effective tool in DNA-based diagnostics—especially marker-assisted selection and genetic determination—due to their desirable genetic attributes with multi-allelic nature, codominant inheritance, and high reproducibility [14]. Over the past few decades, with the public release of genomes of Rosaceae crops (GDR, https://www.rosaceae.org, accessed on 17 February 2022) [15], large-scale SSR markers have been developed and applied for the marker assisted selection, genetic map construction, and trait-associated determination in the RosBREED project (https://www.rosbreed.org/, accessed on 17 February 2022) [16–19]. The availability of efficient and practical SSR markers is limited, and only 28 SSRs have been developed for the Chinese cherry [20,21].

Here, we developed novel genomic-SSR markers based on one high-quality whole genome and seven preliminarily assembled genome sequences, and evaluated their cross-species transferability and their utility in hybrid identification and trait association analysis by using 1236 samples of Chinese cherry and its relatives. Our objectives are: (i) to construct a genome-wide polymorphic SSR loci database for Chinese cherry; (ii) to develop novel SSR markers and evaluate their polymorphism and transferability within Chinese cherry and 17 relatives of *Cerasus*; (iii) to identify the authenticity of 1122 individuals from three cross F_1 populations; and (iv) to analyze three fruit quality traits associated SSR markers using phenotypic data of 65 Chinese cherry landraces.

2. Materials and Methods

2.1. Plant Materials

In this study, eight genomes (unpublished data) were sequenced with pair-end sequencing method on Illumina Hiseq2000 for SSR marker development: LuYg (the whole genome); and whole-genome resequencing of CQhzz, CBJ4, CBJ7, CPZB2, CXC1, CYX4, and WQX13 with an average depth of 5.53×. Among them, sequenced samples whose names are marked in green/red and bold were listed in Table S1. The seven landraces exhibited rich diversity in important economic characters based on field investigation and assessment of cherry resources across China [5,6,22]. A total of 1236 accessions were used for SSR marker evaluation and application, which contained 94 *C. pseudocerasus* accessions (92 landraces and 2 wild accessions), representing diverse genotypes, phenotypes, and geographical distributions [5,7,21]; 17 accessions from 13 Subg. *Cerasus* and Subg. *Microcerasus* taxa (Table S2); and 1122 F_1 individuals from HF × PJHH and reciprocal crosses of NZH and HF (Table S1). All of the F_1 seedlings were planted in the orchard of Sichuan Agricultural University (Chengdu campus), China.

2.2. SSR Identification, Primer Design, and Selection

Taking the LuYg as the reference genome, seven draft genomes were constructed using Chromosome software [23]. SSR motifs were screened using the MISA Perl script (http://pgrc.ipk-gatersleben.de/misa, accessed on 17 February 2022) with the following

parameters: repeat motifs of 2–6 bp and minimum repeat numbers of 6, 5, 5, 5, and 5 for di-, tri-, tetra-, penta-, and hexa-nucleotides, respectively. The sequences of SSR loci and their 600-bp flanking regions were extracted [24] and used to carry out homology comparison analyses in BlastN.

Sixty-four SSR loci evenly distributed across eight linkage groups were selected from the polymorphic SSR database for primer design. Primer pairs were designed in Software Primer 6.0 with the following parameters: (1) the size of the primer was between 18 and 27 bp; (2) the size of the amplicon was from 100 to 400 bp; (3) the melting temperature ranged from 50 to 60 °C; and (4) the GC content ranged from 40% to 65%.

2.3. PCR Amplification and Gel Electrophoresis

Total genomic DNA was extracted from silica gel-dried leaf tissues following the modified cetyltrimethyl ammonium bromide (CTAB) method [25]. PCR amplifications were performed in 20 µL reactions following Zhang et al. [21]. PCR products were detected through 8% polyacrylamide gels after electrophoresis.

2.4. Data Analysis

Data scoring of SSR fragments was performed using Quantity One software (Bio-Rad, Hercules, CA, USA). DNA marker (20 bp, TAKARA) was used to estimate the molecular size of the SSR fragments. The number of alleles (*Na*), effective number of alleles (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), and allele frequency were calculated with POPGENE 32 [26]. Polymorphism information content (*PIC*) was calculated using PowerMarker 3.25 [27]. NTSYS-pc2.11 software [28] was used to construct a neighbor-joining tree using an arithmetic average (UPGMA) algorithm based on Nei's genetic distance (Nei et al., 1983).

2.5. Hybrids Identification

Five new SSR markers were selected for hybrid identification (Table S3) and the results of all primers were combined to confirm hybrid authenticity. The amplified bands of the hybrids were classified into three types: (I) paternal-specific bands; (II) maternal-specific bands; and (III) parental-shared bands. F_1 seedlings with bands other than type I, II, or III were considered foreign origins. After excluding foreign origins, F_1 seedlings with paternal-specific bands were considered true hybrids and individuals with the absence of paternal-specific bands were defined as maternal hybrids.

2.6. Association Analysis

Association analysis was conducted in TASSEL 3.0 software [29] for total soluble solids (TSS), fruit longitudinal diameter (cm), and fruit transverse diameter (cm) of 65 Chinese cherry landraces (Table S2). Each specific band amplified by every SSR marker was analyzed as an independent locus. A general linear model (GLM) was employed, the Q matrix of population structure as a covariate was created using the model-based Bayesian clustering algorithm in STRUCTURE v2.3.4 [30]. Based on the ΔK statistic approach described by Evanno et al. [31], the best K was calculated and visualized using the program STRUCTURE HARVESTER [32]. Adopting more stringent restrictions, the significance of association analysis results was classified into four grades (p-value < 0.01, <0.005, <0.002, and <0.001). The physical positions of associated markers were mapped on the genomes of Chinese cherry (*Ppseudocerasus*_Genome), sweet cherry (PAV_r1.0) [33], and peach (*Prunus_persica*_v2.0) [34], respectively.

3. Results

3.1. Development of SSR Markers

A total of 49,756 microsatellites were identified across the whole genome sequence of LuYg. Based on the comparative genomic analyses (Figure 1a), 25,779 polymorphic SSR loci with various motif repeats were detected, accounting for 51.81% of total SSR loci in

the LuYg genome (Figure 1b). The repeats and positions of each differential SSRs in eight genomes were extracted consecutively, then the extracted information was reordered to form the differential SSR database. Among the eight linkage groups, the greatest number of differential SSRs (5762) was observed on LG1, and the least number (1409) was detected on LG7. Among polymorphic SSRs, 64 loci were randomly selected to design primers and were amplified in 11 Chinese cherry accessions. Sixty (93.75%) primer pairs produced clear and specific bands and 39 (60.94%) primer pairs were polymorphic (Table 1). The newly developed SSR markers were mapped to the genome of Chinese cherry sequenced on the Pacbio RS II platform (Figure 2).

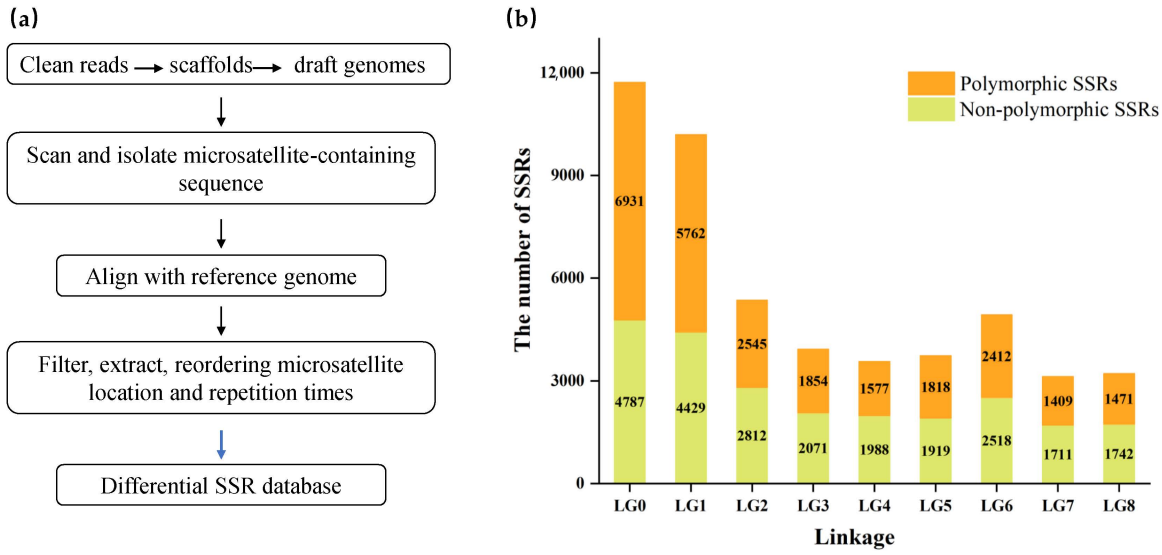

Figure 1. Construction of differential SSR database. (**a**) Flow chart of SSR marker development; (**b**) The number of non-polymorphic/polymorphic SSRs across the Chinese cherry genome in differential SSR database. LG0: the number of differential SSRs from the reads unmapped on LG1–LG8 were counted into LG0.

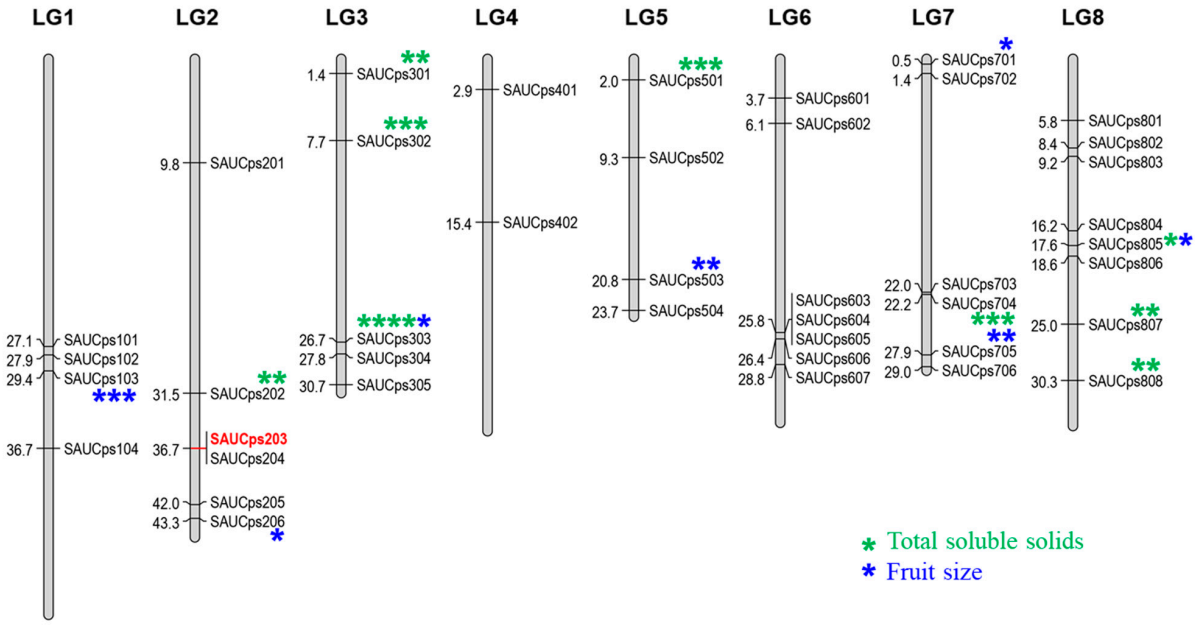

Figure 2. Graphical distribution of new SSR markers among linkage groups of Chinese cherry. SAUCps203 represents the SSR markers with species-specificity. (*: $p < 0.01$; **: $p < 0.005$; ***: $p < 0.002$; ****: $p < 0.001$).

Table 1. Thirty-nine developed SSR markers and their transferability across *Cerasus pseudocerasus* and relatives.

Marker	Linkage Group	Primer Sequence (5′-3′)	*Cerasus pseudocerasus*		Relatives (17 Accessions from 13 Species)
			Landrace (92 Accessions)	Wild (2 Accessions)	
SAUCps101	1	F: ATAATGGAGGTGACAGACGAGC R: GACCAAAAGAAAGCAACAAAATC	92	2	17
SAUCps305	3	F: ACAGAGCAGGCATCCGTATC R: CAGCAGCAACAACAACAACAG	92	2	17
SAUCps503	5	F: AGCCAACCAGCCACAACT R: CGCACCACGCCTTTCAAA	92	2	17
SAUCps602	6	F: TGAGGCCAGCCGAAGATGA R: TAGAGAGTGAAAGAGAGAAAGAGAC	92	2	17
SAUCps704	7	F: TTTTCTGGGAATCGGGATG R: GCAGTCGCTGTTTCAAACTCA	92	2	17
SAUCps705	7	F: CTATTGAATGAGGGTAAAGCG R: GAAGTAGAGGCCCGGATGA	92	2	17
SAUCps801	8	F: TCATTGGAGGAATCATATCGTGTTC R: AGTCTGTCGTGGCTGGAGA	92	2	17
SAUCps807	8	F: GAACTGCTGCGACTATGATTGA R: GCTCAACTGCCGTTTCTTCA	92	2	17
SAUCps601	6	F: AGGACAAAATCCAAGCATCA R: CCCACCACAATCTAAACACC	92	2	16 (hu)
SAUCps202	2	F: CCACTTGCTCTTCTATCTTCTT R: GCCCACCATCCACATCAA	92	2	15 (gl, ja)
SAUCps703	7	F: CCGAAGAACAGAAGAAGAAAGAAAG R: TGAGGATTGAGGAAGTGGGAAA	92	2	15 (gl, ja)
SAUCps502	5	F: CATAACATTCAGCCAACCTTT R: GACTTGAAGCCCTACACCAG	92	2	15 (gl, ja)
SAUCps204	2	F: GCACCGTATCTCATATCTTCC R: ACCCAGAGGCTTTCAATGT	92	2	15 (gl, ja)
SAUCps605	6	F: AACGGCATTGACTGGAGAT R: CTGTTGTTGATCGCCATAATTG	92	2	15 (ma, av×ps)
SAUCps402	4	F: CAAGTCTGTTAGAGGAGGAGGAG R: TGTGAGGATGAGGGCTGCTA	92	2	14 (gl, ja, to)
SAUCps504	5	F: AGATGGTCCTTCCGATTATTAT R: CTTGTTTTCTCTGTTATTGCGT	92	2	14 (cer, gl, ja)
SAUCps301	3	F: AACCCAACAAACGCTGATGTAT R: GCAAAGACCAAGGCTGACTC	92	2	12 (yed, hu, gl, ja, to)
SAUCps205	2	F: ACCAAAGAGGCAAACGATG R: TTTAGACCAATATGCAAGAAGAG	92	2	8 (av, vu, hu, gl, to)
SAUCps203	2	F: CGAACCTAAGACTACTGATCTA R: CTTGCTGCTTGTGGTAGAG	92	2	1 (av, av×ps, vu, cam, yed, ser, cer, ma, hu, gl, ja, to)
SAUCps302	3	F: GAGGGAAGGAGATGGGGACT R: GAAGGGTATGGCTTGGGACA	92	1	17
SAUCps401	4	F: CCTCTTCATCCGTCTTCTTCCT R: CAATCATCGTCACACTTCTTCCT	92	1	17
SAUCps701	7	F: GTGTCGATGTCATTGATGATGCTTA R: GTATGGAGAGGTGTCAGTGGAAG	92	1	17
SAUCps806	8	F: TTTGCCCACAACACCAGC R: GAAAGAAAGAAGACAGAGGAGG	92	1	16 (ja)
SAUCps803	8	F: AAATGACCGCACCCTAAAGCC R: GGAAAATCCCCACCAAAGACC	92	1	16 (cer)
SAUCps802	8	F: ACTTGCCTGTTTGTTCGT R: TGTCTTTGCCTTACCTTGA	92	1	1 (av, av×ps, vu, cam, yed, ser, cer, ma, hu, gl, ja, to)
SAUCps604	6	F: CCAAGTTCAGCCACAGAAG R: CATCCCGTTTCCGTTTCC	92	0	7 (av×ps, cam, yed, ser, cer, ma, hu, gl, ja, to)
SAUCps607	6	F: GCCTCTGCTTCTGCCTTCT R: GGGTTTGAACGGTCGGAAC	91	2	17
SAUCps501	5	F: AACCCTCCATTCCCATTTC R: CAATCAAAGACGCAACAAAGT	91	2	16 (gl)
SAUCps606	6	F: TTCCTCTTCCCACTCAATCACT R: CCTCCAACCACCGACACAT	91	2	16 (ma)

Table 1. *Cont.*

Marker	Linkage Group	Primer Sequence (5′-3′)	*Cerasus pseudocerasus*		Relatives (17 Accessions from 13 Species)
			Landrace (92 Accessions)	Wild (2 Accessions)	
SAUCps804	8	F: AGTCTAAGACCATCCGATCTACTTT R: CACCAGCAATCACTTCCTACAA	91	2	16 (ma)
SAUCps702	7	F: TTGCCTAACAAACAACTCTATCT R: TATCTCATCCATTTTTACCCTCC	91	2	13 (hu, gl, ja, to)
SAUCps808	8	F: CCTACTTTGTTCATTTCTTTCC R: ATCACGCACTACTAACTCCATT	91	2	8 (av, vu, ser, cer, ma, hu, gl, ja, to)
SAUCps206	2	F: TGTAATGACACTACTGCAACCA R: CCTTCCCAATGATGCTAATA	90	2	17
SAUCps104	1	F: GCCATTTCGTTTTATTCCTTTT R: TTTCTCCGTGCTCTCTCTGTTT	90	2	17
SAUCps805	8	F: TAAAGGGCGGTTCAAGTAGGA R: TGGGCATTCGTGTAGCAGAC	90	1	12 (yed, hu, gl, ja, to)
SAUCps103	1	F: ATCAGTCCAGCGAAGGG R: AACGCAATCTAGAAGAGGTATG	87	0	6 (av, av×ps, vu, cam, yed, ser, cer, ma, gl, ja, to)
SAUCps603	6	F: GATTAAACACTTGGATGCGATTGG R: GCCCATTGCGGATGCTCTA	81	1	13 (cam, gl, ja, to)
SAUCps304	3	F: AGATGTCCTCGTGTTGTAAGC R: CGTAGTTGGTCAGTTGGTCA	72	1	13 (hu, gl, ja, to)
SAUCps201	2	F: AATGGAAGAGGAAGGTATTTGAGTA R: CAGTTGTGGATTTAATCGAAGTTTT	64	2	17

Note: Abbreviations in parentheses in the 'Relatives' column represent accessions for which amplification failed. F: forward, R: reverse. Seventeen accessions of 13 taxa from relatives were *C. avium* (av, 4 accessions), *C. avium* × *C. pseudocerasus* (av × ps), *C. vulgaris* × *P./C. canescens* (vu×can), *C. vulgaris* (vu, 2 accessions), *C. campanulate* (cam), *C. yedoensis* (yed), *C. serrulate* (ser), *C. cerasoides* (cer), *C. mahaleb* (ma), *C. humilis* (hu), *C. glandulosa* (gl), *C. japonica* (ja), and *C. tomentosa* (to) (Table S2, accessions 95 to 111).

3.2. Characteristics of SSR Markers

3.2.1. Transferability in Chinese Cherry and Relatives

The transferability of 39 developed genomic SSR markers was evaluated in 94 Chinese cherry accessions (92 cultivated and 2 wild) and 13 *Cerasus* taxa (Table 1). Nineteen markers can be successfully amplified in all 94 Chinese cherry accessions, showing a 100% transferability rate. The remaining markers were applicable to 66–93 Chinese cherry accessions. Both SAUCps604 and 103 markers failed to amplify in two wild accessions.

All 39 SSR markers revealed cross-species transferability within *Cerasus*, and eight of them were applicable to all 13 taxa of *Cerasus*. These markers exhibited relatively high transferability ranging from 79.49% for *C. mahaleb* to 100.00% for *C. avium* × *C. pseudocerasus* among nine Subg. *Cerasus* taxa, while they showed lower transferability ranging from 53.85% for *C. glandulosa* to 74.36% for *C. humilis* among Subg. *Microcerasus* taxa. Therefore, the transferability of SSR markers was higher in the species more closely related to Chinese cherry.

3.2.2. Specificity in Chinese Cherry

Interestingly, we found a key SSR marker, SAUCps203, that was species-specific to Chinese cherry. All *C. pseudocerasus* accessions were successfully amplified, reproducing specific bands of approximately 260 bp (Figure 3). A specific band was also observed in the 'Landing 2′, an interspecific hybrid of *C. avium* × *C. pseudocerasus*. No bands were found in any other accessions of *Cerasus*, *Amygdalus*, and *Armeniaca* (Figure 3).

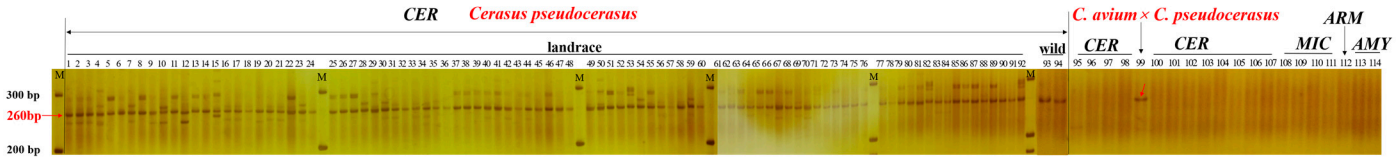

Figure 3. PCR profile of the SSR marker SAUCps203 with species-specificity in *Cerasus pseudocerasus*. Note: CER, Subgenus Cerasus; 1–94, *C. pseudocerasus*; 95–98, *C. avium*; 99, *C. avium* × *C. pseudocerasus*; 100, *C. vulgaris* × *P./C. canescens* and 101–102, *C. vulgaris*; 103–107, *C. campanulate*, *C. yedoensis*, *C. serrulate*, *C. cerasoides*, and *C. mahaleb*, respectively; MIC, Subgenus Microcerasus, including 108–111, *C. humilis*, *C. glandulosa*, *C. japonica*, and *C. tomentosa*, respectively; ARM, 112, Arm. mume; AMY: 113–114, Amy. persica and Amy. triloba, respectively (Table S2). The red arrow indicates the specific band.

3.2.3. Polymorphism in Chinese Cherry

A total of 143 allelic loci were detected among 94 Chinese cherry accessions using the 39 polymorphic markers, which meant that each marker corresponded to 3.6 allelic loci on average (ranging from 2 to 6), with an average major allele frequency (MAF) of 0.64 (ranging from 0.32 to 0.98), and mean polymorphic information content (*PIC*) of 0.415 (ranging from 0.039 to 0.728) (Table S4). Thirty-two newly developed SSR primer pairs exhibited moderate-to-high ($PIC \geq 0.25$) levels of informativeness (Table S4). According to the UPGMA cluster analysis, the similarity coefficient ranged from 0.42 to 0.99 (Figure S1). Two different clusters were observed in the cluster tree. Cluster I mainly consisted of the Chinese cherry accessions from the Sichuan Basin and Yunnan-Guizhou Plateau, and cluster II mainly included accessions from the North China Plain and East China Plain.

3.3. Application of SSR Markers

3.3.1. Hybrid Identification

Five SSR markers succeeded in identifying a total of 1122 individuals, 1081 of which were true hybrid offsprings, 20 may be foreign origins, and the remaining 21 needed further identification due to the absence of paternal-specific bands (Table 2 and Figure 4). True hybrid offspring dominated extremely, ranging from 91.72% to 97.12% with an average of 96.34% among three cross F_1 populations. A total of 204 and 103 true hybrids were identified by four SSR markers (SAUCps303, 102, 706, and 202) in reciprocal F_1 populations between NZH and HF respectively. Totally, 744 out of 766 individuals were true hybrids of HF×PJHH identified by three markers (SAUCps706, 202, and 304). In addition, SSR markers greatly differed from each other in efficiency, ranging from 25.84% for SAUCps102 to 82.00% for SAUCps706. Especially, SAUCps706 showed the highest efficiency and universality, being able to identify all 903 true hybrids and 17 foreign origins in three cross F_1 populations.

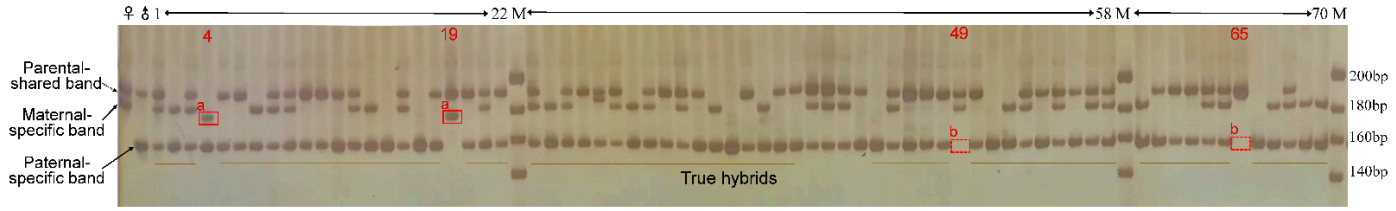

Figure 4. Banding patters based upon SSR marker SAUCps706 among F_1 individuals derived from the cross population of NZH×HF. ♀: female parent; ♂: male parent; M: DNA marker. 1–70 represented part of the F_1 individuals, no. 4 and no. 19 showed foreign origins, and no. 49 and no. 65 were maternal-type individuals. a: Non-parental shared, maternal-specific band, or paternal-specific band; b: the absence of paternal-specific band.

Table 2. Identification of true hybrids and foreign origins of 1122 F_1 individuals using five SSR markers.

Mating Design (♀× ♂)	Number of Progeny	Number		SAUCps303		SAUCps102		SAUCps706		SAUCps202		SAUCps304	
		T	F	T	F	T	F	T	F	T	F	T	F
NZH×HF	211	204 (96.68%)	7	158	5	84	4	203	6	180	3	NA	NA
HF×NZH	145	133 (91.72%)	10	109	6	0	4	65	10	85	3	NA	NA
HF×PJHH	766	744 (97.12%)	3	NA	NA	NA	NA	635	1	372	2	478	3
Total	1122	1081 (96.34%)	20	267	11	84	8	903	17	637	8	478	3
Efficiency		98.13% (1101/1122)		78.00%		25.84%		82.00%		57.49%		62.40%	

Note: Phenotypic characteristics of the cross parents are shown in Table S1. NZH: Nanzaohong; HF: Hongfei; PJHH: Pujianghonghua; NA: not available. T/F: the number of seedlings of true hybrids/foreign origins; Efficiency: the percentage of both true hybrids and foreign origins that can be identified by the SSR marker.

3.3.2. Trait-Marker Association Analysis

Sixty-five Chinese cherry landraces were divided into two groups with a distinct maximum of delta *K* (*K* = 2) by 39 SSR markers with 126 polymorphic loci (Figures S2 and S3). Based on the GLM, 10 loci TSS-associated were mapped on linkage groups 2, 3, 5, 7, and 8; and 10 loci associated for fruit size were mapped on six linkage groups (1–3, 5, 7, and 8) (Table 3). Adopting stringent restrictions, eight loci, four loci, and one locus were significantly associated with TSS at the $p = 0.005$, 0.002, 0.001 levels, respectively, with phenotypic variance explained (R^2) from 16.72% to 26.35%. Two and one loci were significantly associated with fruit size respectively at the $p = 0.005$ and $p = 0.002$ levels, with R^2 ranging from 12.60% to 17.94%. TSS-associated markers SAUCps302–255 and 302–234 (5.97 Mb), SAUCps303–330 (18.92 Mb) were located in the first QTL *qP-TSS3.1*^{m} (4.50–21.85 Mb) for TSS in sweet cherry genome [35], and SAUCps303–330 (24.59 Mb) were close to the peak association signals Glu.2013.3.1 (24.09 Mb) for glucose content in peach [36]. Based on the genome annotation of Chinese cherry, six potential candidate genes were searched in the space defined by ±10 kb on either side of the significantly associated markers (Table 3). Only one gene, *scaffold_7_1573*, possibly regulate TSS accumulation by responding to salicylic acid. For fruit size, three genes (*scaffold_8_1113*, *1115*, and *1116*) on LG8 may response to auxin, *scaffold_2_3446* regulate cell differentiation and *scaffold_3_2691* may be involved in pectin biosynthetic process.

Table 3. Three fruit traits associated with SSR loci and candidate genes.

Traits	Locus	R^2 (%)	Linkage Group	Position [1] (Mb)	Position [2] (Mb)	Position [3] (Mb)	*p*-Value	Candidate Genes (Ppseudocerasus_HiC_scaffold)	Functional Annotation
Total soluble solids	SAUCps202–308	13.68	2	31.5	12.74	19.22	0.005		
	SAUCps301–93	15.29	3	1.4	0.76	1.17	0.005		
	SAUCps302–255	16.72	3	7.7	5.97	6.24	0.002		
	SAUCps302–234	12.98	3	7.7	5.97	6.24	0.01		
	SAUCps303–330	26.35	3	27.8	18.92	24.59	0.001		
	SAUCps501–211	17.25	5	2.0	0.77	0.99	0.002		
	SAUCps704–248	17.18	7	22.2	12.60	15.73	0.002	scaffold_7_1573	Response to salicylic acid
	SAUCps805–206	15.49	8	17.6	-	-	0.01		
	SAUCps807–277	15.23	8	25.0	12.76	16.08	0.005		
	SAUCps808–238	13.91	8	30.3	18.50	20.49	0.005		
Longitudinal diameter	SAUCps103–204	14.91	1	36.7	27.45	32.94	0.01		
	SAUCps103–201	15.56	1	36.7	27.45	32.94	0.01		
	SAUCps103–198	19.74	1	36.7	27.45	32.94	0.002		
	SAUCps103–195	14.85	1	36.7	27.45	32.94	0.01		
	SAUCps303–324	14.61	3	27.8	18.92	24.59	0.01	scaffold_3_2691	Pectin biosynthetic process
	SAUCps503–218	12.61	5	20.8	13.53	15.20	0.005		
	SAUCps705–296	12.60	7	27.9	18.30	20.85	0.005		

Table 3. *Cont.*

Traits	Locus	R^2 (%)	Linkage Group	Position [1] (Mb)	Position [2] (Mb)	Position [3] (Mb)	*p*-Value	Candidate Genes (Ppseudocerasus_HiC_scaffold)	Functional Annotation
Transverse diameter	SAUCps206–171	12.32	2	43.3	12.74	28.87	0.01	scaffold_2_3446	Cell differentiation
	SAUCps701–353	9.02	7	0.5	0.35	3.60	0.01		
	SAUCps805–210	12.45	8	17.6	-	-	0.01	scaffold_8_1113, scaffold_8_1116	Response to auxin
								scaffold_8_1115	Positive effectors of cell expansion (modulate auxin transport)

Note: R^2: phenotypic variance explained by the QTL; Position [1]: physical positions on genome of Chinese cherry (Ppseudocerasus_Genome); Position [2]: physical positions on genome of sweet cherry (PAV_r1.0); Position [3]: physical positions on peach genome (Prunus_persica_v2.0); -: unmapped.

4. Discussion

4.1. Efficient Development of SSR Markers

In recent years, tremendous efforts dedicated to NGS-based sequencing have expedited the generation of SSR-enriched DNA libraries and ESTs (expressed sequence tags) [37]. In spite of accessibility to numerous SSR loci of diverse Rosaceae crops [33,38,39], significant efforts, time, and cost/resources are required [40]. The effective strategy used in this study is advantageous for screening informative SSR markers from large-scale markers by assessing their potential to detect fragment length polymorphism among different cultivated and wild Chinese cherry accessions. Similar strategies have been reported in rice [41], wheat [42], Nicotiana [43], and pear [44]. The efficiency of novel SSR markers was ascribed to greater potential of detecting amplification (93.75%) and polymorphism (59.38%), and application value compared with previous development of SSR makers relied either on genome sequencing (polymorphism 9.50%, 19/200) [21] or ESTs (polymorphism 5.00%, 8/160) [20] of single Chinese cherry accessions. Therefore, the database containing 25,779 polymorphic SSR loci will serve as a vital genomic resource for driving genomics-assisted breeding applications and genetic enhancement in Chinese cherry. Although this strategy is of high efficiency and high throughput, the SSR marker prediction of polymorphism is still difficult to make completely consistent with the PCR results. This issue may be caused by the phenomenon of "sliding" [45] or errors in sequencing data [46]. As sequencing quality improves, we believe that more crop sequencing data released for constructing polymorphic SSR databases will support more accurate predictions [15].

4.2. Efficient Application of Novel Markers

Species identification is essential for large-scale biodiversity monitoring and conservation [47]. Along with the morphological similarities that prevail, Chinese cherry interspecies and variety level make species identification more difficult [2]. The monomorphic fragment (260 bp) obtained with SAUCps203 was common and unique in all *C. pseudocerasus* lineages, which can be used for fast and accurate identification of *C. pseudocerasus*. This finding contributes a valuable tool for *C. pseudocerasus* lineage protection and discrimination and can also be an aid to taxonomic study.

Chinese cherry is a perennial woody plant that needs more than three years to show vegetative and reproductive characteristics. It is necessary to identify the authenticity of F_1 seedlings to shorten the period to breed true hybrids for future breeding programs. Here, over 1000 F_1 individuals were identified as true hybrids by using only five SSR markers, which exhibited the great potential for accurate identification of large populations due to its codominant inheritance.

Fruit size and TSS content impact consumer acceptance and are considered as major selection criteria in cherry breeding. As glucose is the major sugar in cherry impacting TSS content [48], we speculate that SAUCps303–330—close to glucose-associated peak signals in a previous study [36]—may serve as a potential functional marker for mining TSS-related genes. According to the genome annotation results of Chinese cherry, *scaffold_2_3446* at

5.96 kb downstream of SAUCps206–171 is putatively homologous to the *MSI4* gene which negatively regulates the cell cycle that may affect cell growth and division [49]. Additionally, *scaffold_8_1115* is predicted as a homologous gene of *SAUR20* [50], which was speculated to increase fruit size by accelerating cell expansion in the mesocarp during the second rapid expansion period of Chinese cherry. The above results might contribute to the body of knowledge on the genetic control of important fruit traits in Chinese cherry.

5. Conclusions

This study constructed a database containing 25,779 polymorphic SSR loci of Chinese cherry based on comparative genomic analyses. Among 64 new markers, 32 were moderately to highly polymorphic and 38 exhibited high transferability across 13 *Cerasus* taxa. SAUCps203, species-specific to *C. pseudocerasus*, was of great value for *C. pseudocerasus* lineage discrimination and protection. The 1081 true hybrids identified by five SSR markers are valuable genetic material for further study. Twenty SSR loci were estimated to be significantly associated with TSS content and fruit size, exhibiting potential application in discovery of candidate genes. This work presents a major advance of Chinese cherry in the confirmation and identification of SSR markers in genomics-assisted breeding applications and provides a reference for the efficient identification of QTLs underlying complex traits in cherry.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8030222/s1, Table S1: F_1 populations and their parents' phenotype characters; Table S2: List of information for each accession in this study; Table S3: Information of five SSR markers for hybrids identification; Table S4: Characteristics of 39 microsatellite loci across 94 Chinese cherry accessions; Figure S1: Phenograms by UPGMA cluster analysis of 94 Chinese cherry accessions; Figure S2: Population structure distribution of 65 Chinese cherry accessions; Figure S3: Curve diagram of K value.

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