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Development of simple sequence repeat (SSR) markers from a genome survey of Chinese bayberry (*Myrica rubra*)

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Abstract

Background: Chinese bayberry (*Myrica rubra* Sieb. and Zucc.) is a subtropical evergreen tree originating in China. It has been cultivated in southern China for several thousand years, and annual production has reached 1.1 million tons. The taste and high level of health promoting characters identified in the fruit in recent years has stimulated its extension in China and introduction to Australia. A limited number of co-dominant markers have been developed and applied in genetic diversity and identity studies. Here we report, for the first time, a survey of whole genome shotgun data to develop a large number of simple sequence repeat (SSR) markers to analyse the genetic diversity of the common cultivated Chinese bayberry and the relationship with three other *Myrica* species.

Results: The whole genome shotgun survey of Chinese bayberry produced 9.01Gb of sequence data, about 26x coverage of the estimated genome size of 323 Mb. The genome sequences were highly heterozygous, but with little duplication. From the initial assembled scaffold covering 255 Mb sequence data, 28,602 SSRs (≥5 repeats) were identified. Dinucleotide was the most common repeat motif with a frequency of 84.73%, followed by 13.78% trinucleotide, 1.34% tetranucleotide, 0.12% pentanucleotide and 0.04% hexanucleotide. From 600 primer pairs, 186 polymorphic SSRs were developed. Of these, 158 were used to screen 29 Chinese bayberry accessions and three other *Myrica* species: 91.14%, 89.87% and 46.84% SSRs could be used in *Myrica adenophora*, *Myrica nana* and *Myrica cerifera,* respectively. The UPGMA dendrogram tree showed that cultivated *Myrica rubra* is closely related to *Myrica adenophora* and *Myrica nana*, originating in southwest China, and very distantly related to *Myrica cerifera*, originating in America. These markers can be used in the construction of a linkage map and for genetic diversity studies in *Myrica* species.

Conclusion: *Myrica rubra* has a small genome of about 323 Mb with a high level of heterozygosity. A large number of SSRs were identified, and 158 polymorphic SSR markers developed, 91% of which can be transferred to other *Myrica* species.

Background

Chinese bayberry is an important commercial horticultural crop. It has been cultivated for more than 7,000 years in southern China, but is little known elsewhere. The production area is currently 340,000 ha, with an annual production of 1.1 million tons. The plant is diploid (2n = 16), generally dioecious, with the female plants cultivated for fruit [1], growing well on poor soils due to the association of nitrogen-fixing bacteria with the root system. It is rich in anthocyanins exhibiting a wide range of pharmacological properties, such as anti-inflammatory, antitumor and antioxidative effects [2].

There are four species within the genus *Myrica* in China, namely *Myrica rubra* Sieb. & Zucc., *M. esculenta* Buch.-Ham., *M. nana* Cheval., and *M. adenophora* Hance. *M. rubra* is widely distributed, with many local cultivars in the Zhejiang, Jiangsu, Fujian and Guangdong provinces and a few from Guizhou, Yunnan and Hunan provinces. The best known cultivars are Biqi and

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Dongkui, both from the Zhejiang province. Although there are abundant germplasm resources, studies on genetics and breeding of the species are still in their infancy. Molecular marker technology is a popular tool for breeding and genetic research, and with the construction of a genomic library, 13 polymorphic microsatellite loci have been developed in *M. rubra* [3] and 11 from an expressed sequence tag library [4]. Recently, 12 primer pairs have been temporarily developed by ISSR-suppression PCR [5] with GSG $(GT)_6$ as the primer for enriching microsatellite sequences. Reports on the genetic diversity in Chinese bayberry using SSR markers have also recently been published [6,7], but the number of markers for Chinese bayberry is limited.

The reproducibility, multiallelicism, co-dominance, relative abundance and good genome coverage of SSR markers have made them one of the most useful tools for genetic diversity and linkage mapping. Genomic SSRs and EST-SSRs, considered complementary to plant genome mapping, have been reported in many fruit crops, such as walnut [8], cherry [9], apricot [10] and coconut [11]. EST-SSRs are useful for genetic analysis, but their relatively low polymorphism and the high possibility of no gene-rich regions in the genome are limitations to their use. In contrast, genomic SSRs are highly polymorphic and tend to be widely distributed throughout the genome, resulting in better map coverage [12].

With genetic maps serving as the basis for future positional gene cloning, making map-based cloning and marker-assisted selection possible, the development of more SSRs is essential. As sequencing technologies advance, whole-genome shotgun (WGS) sequences are becoming increasingly available. These DNA sequences are valuable resources for SSR development in many plant species, such as rice [13] and papaya [14]. In addition, they can be used to evaluate the frequency and distribution of different types of SSRs in the genome, and even help to estimate genome size and characters such as heterozygosis and repeats.

As a way of reducing the cost of genotyping research, Schuelke [15] proposed a method for fluorescent dye labelling of PCR fragments with a sequence-specific forward primer: the universal fluorescent-labelled M13(-21) primer, at the 5′ end, acts as the forward primer in a 'one-tube' reaction. As this method allows for high-throughput genetic analyses, with a high number of microsatellite markers widely used, we considered the possibility of using this approach for multiplex PCR, to improve the efficiency and save costs.

In this study, we mined and validated 158 SSR markers and describe their application for understanding the genetic relationship among 29 Chinese bayberry accessions and other *Myrica* species. These markers are useful for genotyping and genetic diversity analysis and linkage mapping of *Myrica rubra* and other *Myrica* species.

Results

Genome survey using whole genome shotgun data in Chinese bayberry

WGS generated 273,161 (>100 bp) high quality sequence reads from two DNA libraries (250 bp and 500 bp) of the androphyte individual 'C2010-55'. We used 9.01 G raw data for K-mer analysis and heterozygous simulation. For the 17-mer frequency distribution (Figure 1), the peak of the depth distribution was about 22. The estimated genome size was 323 Mb, using the formula: genome size = k-mer count/peak of the kmer distribution. The minor peak at 1/2 altitude of the main peak indicates the high level of heterozygosity in this genome (Figure 1). A total of 739,969 contigs were assembled with a total sequence length of 255.7 Mb. The length of N50 was 295 bp in our assembly, and the longest contig and scaffold 7,593 and 127,008 bp, respectively.

Frequency distribution of different types of SSR markers

A total of 17,172 out of 273,161 scaffolds (6%) retrieved from the genome survey sequence contained 28,602 SSRs (Table 1), of which 5,401 contained more than one SSR, and 1,444 SSRs were present in compound format. Among the derived SSR repeats, the dinucleotide was the most abundant repeat, accounting for 84.72% of total SSRs, followed by tri- (13.78%), tetra- (1.34%), penta- (0.12%), and hexa- (0.04%) nucleotides (Table 1). There was a large proportion of both dinucleotides and trinucleotides while the rest amounted to less 2%. The average frequency of occurrence was about 10.47% (Table 1).

The SSR frequency of each motif is presented in Additional file 1. The SSR motif consists of 69 types. Among the repeat motifs of the dinucleotide, the AG/CT repeat was the most common, representing 53.72%, followed by 39.20% AT repeats (Figure 2), and the predominant motifs of trinucleotide (AAG/CTT and AAT/ATT) repeats accounted for 37.15% and 32.56%, respectively (Figure 3).

Polymorphism of SSR markers

We first designed and synthesised 600 SSR primer pairs from those scaffolds more than 2Kb long. The majority of SSR loci were dinucleotide repeats (597, 99.5%), and the remainder trinucleotide. Initially, the effectiveness of these primer pairs was detected in two cultivars (Biqi and Dongkui) and *M. cerifera* through denaturing PAGE (Polyacrylamide gel electrophoresis), and 581 (96.8%) of these were amplified successfully in Biqi and Dongkui, and 400 (66.7%) in *M. cerifera*. The SSR loci (186, 31%) were identified as heterozygous loci either in Biqi or in Dongkui.

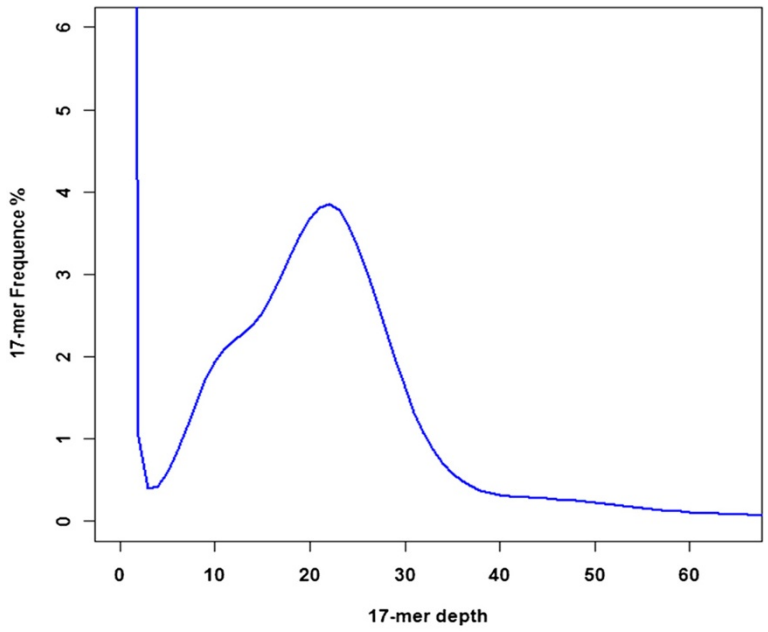

Figure 1 The distribution of 17-mer depth analysis based on whole genome shotgun data in Chinese bayberry.

Subsequently, they were used to screen 32 accessions, and detected an average of 8.25 alleles and from 3 to 15 alleles per locus (Table 2).

The PIC at each locus ranged from 0.256 to 0.883 with an average of 0.67 loci. The PCR product size ranged from 110 to 274 bp. All the primers produced amplicons in agreement with the expected sizes. Most of the SSR primers (139 primer pairs) showed significant deviation from HW equilibrium ($P < 0.05$). Partial correlation analysis showed that significant positive correlations existed between the repeat unit length and PIC ($P < 0.01$, $r = 0.2747$). This showed that these SSRs had high rates of transferability for *M. adenophora* (91.14%) and *M. nana* (89.87%) and low rates for *M. cerifera* (46.84%), indicating that these markers are suitable for genetic diversity analyses in other *Myrica* species.

One of the objectives of this study was to develop potential suitable SSR markers for genetic mapping using Biqi and Dongkui as crossing parents. We selected 99 heterozygous loci in Biqi and 105 in Dongkui (Table 3): 135 primer pairs can be used together in linkage mapping of the planned F_1 populations between Biqi and Dongkui.

Table 1 Occurrence of SSRs in the Genome Survey of Chinese bayberry

Type	Number	Proportion in all SSRs (%)	Frequency (%)
Dinucleotide	24,233	84.72%	8.87%
Trinucleotide	3,941	13.78%	1.44%
Tetranucleotide	383	1.34%	0.14%
Pentanucleotide	35	0.12%	0.013%
Hexanucleotide	10	0.04%	0.004%
Total	28,602	100%	10.47%

Genetic relationship analysis

The 32 accessions were divided into three groups (A, B and C, Figure 4), based on Nei's genetic distance coefficient [16] using UPGMA cluster analysis. The similarity among all the accessions varied from 0.54 to 0.84. At the species level, the UPGMA dendrogram produced clusters separating *M. nana* and *M. cerifera* into two distinct groups. The genetic

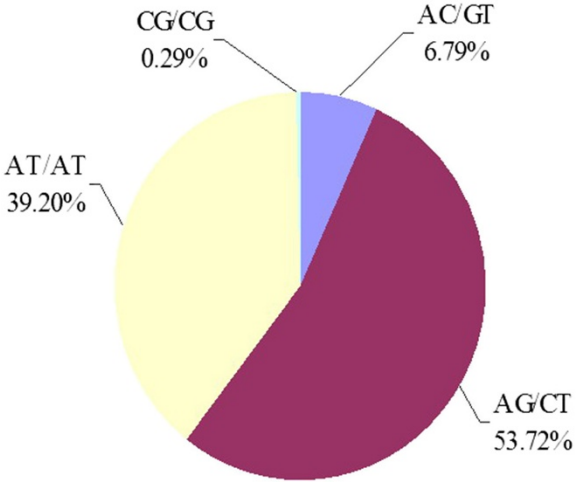

Figure 2 Percentage of different motifs in dinucleotide repeats in Chinese bayberry genome.

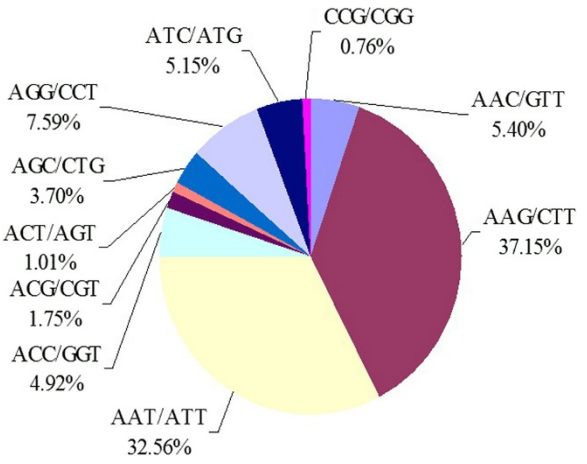

Figure 3 Percentage of different motifs in trinucleotide repeats in Chinese bayberry genome.

similarity between *M. cerifera* and *M. rubra* was 0.54, lower than the 0.74 previously reported by Xie [6].

The main cluster 'A' included the subgroups A-1 and A-2. Subgroup A-1 includes 16 accessions, mainly from the cities of Ningbo (12) and Hangzhou (3), where the popular and dominant cultivar is Biqi. This demonstrates that these natural elite seedling selections are truly distinct from the local cultivars. For their genetic relationships (Figure 4), the rare monoecious individual (C2010-4) is closely related to Biqi, while the accessions 'Shuijing' and 'Y2010-72' (both white fruit type) are clearly separated in the cluster, with low genetic distance.

Subgroup A-2 includes 14 accessions, with four from Wenzhou, two from Taizhou, and one each from the cities of Hangzhou and Ningbo, and the Hunan, Guangxi, Guizhou and Jiangsu provinces. This group includes the popular cultivar Dongkui. The four accessions from Wenzhou distributed in this cluster have genetic similarity. The accession 'Tongzimei' from the Hunan province is on an independent branch, showing that it is genetically distinct. 'Xiaolejiangchonghei' and '*M. adenophora*' grouped together, and are possibly in the same population. Six androphyte accessions, distributed in group A, are close to cultivars of the same geographic origin.

The accessions '*Myrica nana*' from Yunnan and '*Myrica cerifera*' from the USA were independently classified as the 'B' and 'C' group, indicating a distant relationship with cultivated *Myrica rubra*.

Discussion

Our major aims were to find a large set of SSR markers for *Myrica rubra* and understand the genetic diversity and relationship among representative cultivars, androphyate and related species. This research paves the way for constructing an SSR-based linkage map in *Myrica*.

The genome characteristics of genus *Myrica*

Shotgun sequencing is suitable for simple genomes, with no or few repeat sequences, such as Chinese bayberry. For such genomes, the genome can largely be assembled simply by merging together reads containing overlapping sequence [17]. We report the genome survey of Chinese bayberry using whole genome shotgun sequencing. The 17-nucleotide depth distribution suggests a genome size of 323 Mb, larger than peach (220 Mb, http://www.rosaceae.org/peach/genome), but close to our estimate of 250 Mb from flow cytometry using rice as the reference (date not shown). Although the highly homozygous material was selected on a limited number of SSR loci assays, the actual heterozygous rate, as revealed by 185 new SSR markers, was very high (63%). To overcome the key issue of heterozygosity and allow us to generate a high-quality genome sequence, we can use a unique homozygous form such as monoploid, derived using tissue culture or from nature and worth further study.

Marker development for under-utilised fruit crops

SSRs have been widely used for high-throughput genotyping and map construction as they have the advantage of high abundance, random distribution within the genome, high polymorphism information content and stable co-dominance [18-20]. They can be developed from either genomic or expressed sequence tag (EST) libraries. Although EST-SSRs are useful for genetic analysis, their disadvantages of relatively low polymorphism and high concentration in gene-rich regions of the genome may limit their usage, especially for the construction of linkage maps [21]. In this study, a total of 600 SSR primer pairs were designed from 28,602 SSR sites, and 581 (96.8%) primer pairs were effective. Due to the self-complementary nature to form dimers, AT/TA is not usually used to develop markers [12]. Our findings are in agreement with that published for peach, where the dinucleotide repeat motifs were also found to be the most common, and $(CT)_n$ as the most common repeat unit [22].

In the present study, the mean value of PIC was higher than the previously reported 0.62 [7], but the consistent relationship was observed between SSR polymorphism and repeat unit length. There are some reports of a positive relationship between degree of polymorphism and repeat unit length [23,24]. However, those polymorphic SSRs that are homozygous in both parents cannot be mapped in F_1 populations, although they are useful for mapping in F_2 or backcross populations [25], while heterozygous SSRs can be used for mapping in F_1 populations (Table 2). The estimated number of SSRs that can be mapped in the F_1 populations between Biqi and Dongkui was about 85%.

Table 2 Characteristics of 158 SSR markers in this study

Locus	GenBank Accession	Repeat motif	Primer sequence (5'-3')	Size range (bp)[a]	*Na*	*Ho*	*He*	PIC	P_{HW}
ZJU001[ab]	JQ318696	(GA)10	F:<NED > <Tail-1 > CCTCTCCACCCATGAGAAAC R:CAAATCATTCCCTGCTTTCC	160-188	7	0.1667	0.4271	0.4002	0.0000
ZJU002[ac]	JQ318697	(TC)13	F:<NED > <Tail-1 > TCAAAGAGACGTTGTGGCAG R:TCCGCTCACAGACAGAGAGA	219-229	4	0.2083	0.5257	0.4572	0.0005
ZJU003[ab]	JQ318698	(AG)11	F:<NED > <Tail-1 > GTCACCTTGCTCTTCTTGGC R:TCCTTGTACTTGTTCTGCTGGA	203-217	8	0.7407	0.8344	0.7949	0.0003
ZJU004[ac]	JQ318699	(GA)10	F:<NED > <Tail-1 > AACAGAACCATCGTCAAGGC R:GGTACAGTCGCTCCGGTTTA	204-210	4	0.3571	0.7325	0.6704	0.0003
ZJU005[ab]	JQ318700	(AG)14	F:<NED > <Tail-1 > CTTTGGACATGGCAACACAC R:TCCACTTTGACAGATTCCCA	200-228	11	0.3000	0.8679	0.8291	0.0000
ZJU006[ab]	JQ318701	(GA)10	F:<NED > <Tail-1 > CTCGCCCTCTCTCTCTACCA R:AGTTTATCCACCCGTGTCGT	193-205	5	0.2593	0.3305	0.3089	0.0000
ZJU007[ab]	JQ318702	(AG)13	F:<NED > <Tail-1 > TGATCCATTGGAACCATGTG R:TCAGTTGATGGTGCAGAAGC	193-209	8	0.5625	0.6617	0.6302	0.1868
ZJU008[ab]	JQ318703	(CT)10	F:<NED > <Tail-1 > GGAGAAATGAACGGTGGAGA R:TCCAAAGCTAATACCCACGC	191-215	10	0.7931	0.7973	0.7563	0.0002
ZJU009[ab]	JQ318704	(CT)10	F:<NED > <Tail-1 > AATTGTCGCAAGTAGTCGCC R:ATATCAACCCATGGGAGCAA	207-221	5	0.0741	0.3599	0.3371	0.0000
ZJU010[ab]	JQ318705	(CT)11	F:<NED > <Tail-1 > TGCAACATCGAAATTGGAAA R:ATGCCGGCAAGTCTTAGTGT	181-205	9	0.9032	0.8012	0.7614	0.0000
ZJU011[a]	JQ318706	(GA)10	F:<NED > <Tail-1 > GGAGGCTCGTCAGTCATCTC R:TTAGCGTCCCTTCTCTCTCG	200-216	9	0.2692	0.7926	0.7554	0.0000
ZJU012[ab]	JQ318707	(CT)12	F:<NED > <Tail-1 > CTTCACTCACCGCCTTTCTC R:AATGGCCTCCACATCTCAAG	184-218	13	0.5000	0.8571	0.8251	0.0000
ZJU013[ab]	JQ318708	(CT)10	F:<NED > <Tail-1 > ACTTGTCATTCCCACGTTCC R:CACTCCATCTCAACCACCCT	211-221	6	0.4444	0.5199	0.4515	0.0094
ZJU014[ab]	JQ318709	(AG)15	F:<NED > <Tail-1 > TGGAATGTCGATCTGAAACAA R:ACCAGCTTATACGACGGTGG	186-212	13	0.6875	0.9033	0.8791	0.0251
ZJU015[ab]	JQ318710	(GA)11	F:<NED > <Tail-1 > TTGGTGTGGTGGTAATGGTG R:AAATAATGCAAGCAGGTGGG	199-221	6	0.6154	0.6614	0.5902	0.0585
ZJU016[ab]	JQ318711	(TC)10	F:<NED > <Tail-1 > CCGTTGACTATTGCCCAGTT R:GGCAATTTCCAAATCGCTAA	196-216	11	0.6333	0.8469	0.8130	0.0179
ZJU017[ab]	JQ318712	(CT)13	F:<NED > <Tail-1 > ACTGAAGAACCAAACGTGGG R:GGTGTGTTTCTCTGTGTGCG	180-200	6	0.6250	0.7093	0.6518	0.0003
ZJU018[ab]	JQ318713	(CT)15	F:<NED > <Tail-1 > ACGAAATTTGACCAATCGCT R:AGGGTTTCTTCTGGTTCGGT	196-216	7	0.1429	0.7189	0.6667	0.0000
ZJU019[ab]	JQ318714	(GA)12	F:<NED > <Tail-1 > TTTCATAACCCGTTGGCTTC R:AAGGTGGAAACGTGTCAAGG	209-219	6	0.2800	0.6865	0.6317	0.0000
ZJU020[b]	JQ318715	(AG)10	F:<NED > <Tail-1 > CACAGGACATGTGATGGAGG R:CCATCCTGAGCTTTGTCGAT	201-213	7	0.5172	0.7453	0.6983	0.0000
ZJU021[a]	JQ318716	(TG)10	F:<NED > <Tail-1 > TCGCCAGCTTCCTAATGTCT R:GAGCGCATGTTGTTGCTAAA	190-212	8	0.7778	0.7428	0.7025	0.0663

Table 2 Characteristics of 158 SSR markers in this study *(Continued)*

ZJU022[ab]	JQ318717	(GA)10	F:<NED > <Tail-1 > AAGCTTAAGCAAGCGTCGAG R:TGCGAAGGGAAATTTCAGAC	188-208	9	0.6923	0.8575	0.8227	0.0109
ZJU023[ac]	JQ318718	(AG)15	F:<NED > <Tail-1 > GTGTTTGGGCAGCACCTATT R:AAAGAGTACAACAACGCGGG	200-226	14	0.6667	0.8840	0.8544	0.0251
ZJU024[ab]	JQ318719	(TC)10	F:<NED > <Tail-1 > CCGCATGTTTGATTGATGTC R:GCGTTGAGCGGAGAGATTAC	180-196	6	0.6000	0.7345	0.6716	0.1624
ZJU025[ab]	JQ318720	(TC)10	F:<NED > <Tail-1 > TTTGAGCGATAGTACGGAGG R:ATATGCTACGTTGGTTGCCC	216-234	8	0.2667	0.7537	0.7044	0.0000
ZJU026[ab]	JQ318721	(TC)10	F:<NED > <Tail-1 > CCAGACAGGTTAGCCACCAT R:GCCTCTGGATCTCGATTACG	200-220	10	0.4545	0.8573	0.8199	0.0000
ZJU027	JQ318722	(TTC)8	F:<NED > <Tail-1 > GTTGCAATTTGCCTCCATTT R:GGTGCCTATACTGCCAGCTC	203-227	6	0.3125	0.6250	0.5321	0.0003
ZJU028[ab]	JQ318723	(AG)10	F:<NED > <Tail-1 > CAACCATCCAAACCAAATCC R:TCTACCAATCGTGGCTAGGG	164-170	4	0.1724	0.2789	0.2566	0.0000
ZJU029[ab]	JQ318724	(AG)10	F:<NED > <Tail-1 > TCTTCCGGGATGTCTACAGG R:CAACAGCAATCGCAAAGAAA	189-205	6	0.5312	0.6925	0.6296	0.0480
ZJU030[ab]	JQ318725	(CA)13	F:<NED > <Tail-1 > AAGTGAGCTCTCCCTCCCTC R:CACCGAAATACTTGCCGTTT	193-205	7	0.4286	0.7208	0.6676	0.0000
ZJU031[ab]	JQ318726	(GA)16	F:<NED > <Tail-1 > GCACAGGAACACCAGGATCT R:CCAAGCCCTAATTCCCTTTC	179-195	8	0.8387	0.7948	0.7492	0.0000
ZJU032[ab]	JQ318727	(TC)11	F:<NED > <Tail-1 > ATTCCCACGTTCGTTCAGAC R:GATGCCTAACTCCGAATCCA	204-226	8	0.6786	0.6442	0.5852	0.0220
ZJU033[ab]	JQ318728	(TC)10	F:<NED > <Tail-1 > GCACAAGTTGCTGACATGCT R:AGTTGCATTCAACCCACACA	195-207	6	0.0690	0.6655	0.5897	0.0000
ZJU034[ab]	JQ318729	(CT)10	F:<NED > <Tail-1 > ATGGGAATGTGGAGAACGAG R:GCTTTGCTTCTTTGCTTTGG	191-209	8	0.4138	0.7762	0.7250	0.0000
ZJU035[ab]	JQ318730	(GA)14	F:<NED > <Tail-1 > TTGGATCCTGGTTACCTTCG R:AAACTGCATGCATGGTTCCT	201-217	8	0.1290	0.7425	0.6900	0.0000
ZJU036[ab]	JQ318731	(GA)10	F:<NED > <Tail-1 > CTGCCACTCTTACTGGCCTC R:ATGTGCCCAATCTTGACTCC	186-214	8	0.3333	0.5895	0.5516	0.0000
ZJU037[ab]	JQ318732	(TC)10	F:<NED > <Tail-1 > GTGATTTCCCTCCCATTGAC R:ACGAAGCGGGAAGTAGGATT	208-228	9	0.8125	0.7867	0.7429	0.0135
ZJU038[b]	JQ318733	(AG)10	F:<NED > <Tail-1 > CTTATGGCCCGTTTGTAACC R:AACGATTGCTTTAAGCGGAA	194-200	4	0.2273	0.5106	0.4646	0.0007
ZJU039[a]	JQ318734	(CT)10	F:<NED > <Tail-1 > AAACGAAAGTGGGCGTATTG R:CACCAGTGCGTCCTATGAGA	219-229	6	0.3077	0.6161	0.5745	0.0004
ZJU040	JQ318735	(TC)16	F:<NED > <Tail-1 > AAACTCCGTGCTGGAATCAA R:GCAGACAAGCCTTCCTGTTC	198-220	10	0.3182	0.8192	0.7798	0.0000
ZJU041[ab]	JQ318736	(TC)11	F:<PET > <Tail-2 > TGATCACCTTTCAGTTGGCA R:CACATTGGCAGAGTCCTGAA	226-244	5	0.2258	0.3199	0.3031	0.0000
ZJU042[ab]	JQ318737	(TC)10	F:<PET > <Tail-2 > AGGATTTCTCCAGAGGGACG R:GGTTCCGCATCAAACTACAAA	220-242	5	0.3571	0.5331	0.4880	0.0000
ZJU043[b]	JQ318738	(CT)10	F:<PET > <Tail-2 > AAACCGAGCTCTCCTAAGCC R:CTCGCAATTTCTCGGGATAC	225-245	4	0.5714	0.6383	0.5667	0.2655

Table 2 Characteristics of 158 SSR markers in this study *(Continued)*

ZJU044[ab]	JQ318739	(GA)12	F:<PET > <Tail-2 > GATGGTGGCTTGTCTTGGTT R:AAGTGGGACGTCAATTCCTG	235-255	8	0.2500	0.5091	0.4853	0.0000
ZJU045[ab]	JQ318740	(CT)10	F:<PET > <Tail-2 > GAGAGAGGGAGAGAGGCCAT R:GGAAGATTCATGGGAGAGGG	228-258	13	0.6129	0.8821	0.8544	0.0007
ZJU046[ab]	JQ318741	(AG)10	F:<PET > <Tail-2 > TTGCTGTAAGCATCGCAATC R:AAGCTCCGGTAACACACACC	226-242	7	0.3871	0.6256	0.5824	0.0000
ZJU047[ab]	JQ318742	(GA)13	F:<PET > <Tail-2 > TTCGATCATTCATGAGGCTG R:TTAATTGCATCCCGGATTTC	247-259	7	0.7097	0.7615	0.7074	0.0019
ZJU048[ab]	JQ318743	(CT)14	F:<PET > <Tail-2 > AGCGGACCGAGTTGTAGAGA R:CCAACCCTACAAAGCGAGAG	230-254	12	0.2903	0.8493	0.8166	0.0000
ZJU049[ab]	JQ318744	(GAA)8	F:<PET > <Tail-2 > GTGTCTGCAGCAACTTCCAC R:GTCGGAACCGAAGATGGTTA	234-267	10	0.8125	0.7262	0.6797	0.0000
ZJU050[ab]	JQ318745	(AG)11	F:<PET > <Tail-2 > GTCACAGCCTGGATAGCTCC R:GTCTCTCCTGGATGAGCTGC	233-245	7	0.3000	0.7288	0.6916	0.0000
ZJU051[ab]	JQ318746	(AG)12	F:<PET > <Tail-2 > AGAGAAAGACCGGGACCAAT R:GAGAAATAAAGCCGAGCGTG	229-233	3	0.4333	0.4198	0.3594	0.0012
ZJU052[ab]	JQ318747	(AG)16	F:<PET > <Tail-2 > CCCGAGCTGAACGAAATAGA R:GGATCAAAGCGTTGTCGTTT	230-248	9	0.4348	0.8628	0.8261	0.0000
ZJU053[ab]	JQ318748	(AG)10	F:<PET > <Tail-2 > AAATCCGAAACACCTCTCCC R:ATGTGGAGACTTCCACTGGG	222-240	8	0.5000	0.5655	0.5211	0.0001
ZJU054[ab]	JQ318749	(CT)13	F:<PET > <Tail-2 > TTGATTTGCTTTGTGCATTTG R:CAAACTACCGTGCCCAACAT	232-250	9	0.3000	0.8667	0.8268	0.0003
ZJU055[ab]	JQ318750	(CT)10	F:<PET > <Tail-2 > TTATGGGTTTCATTGGGCAG R:TCACCAGGCTACTGCATGTC	238-254	6	0.2500	0.7006	0.6357	0.0000
ZJU056[ab]	JQ318751	(GA)13	F:<PET > <Tail-2 > GACAAAGTGGGTGCCATTCT R:TGCATGCTTCCTTTCTTCCT	230-246	7	0.5714	0.7643	0.7122	0.0068
ZJU057[ab]	JQ318752	(CT)10	F:<PET > <Tail-2 > TCATGTGGAGATTGAAGCCA R:CGTCCCGAATGAAGATTTGT	230-244	6	0.1579	0.6814	0.6283	0.0000
ZJU058[ab]	JQ318753	(GT)10	F:<PET > <Tail-2 > TCCGGAGCTTTCAATCTCAT R:GCCTACGAACTCAGGTTCCA	252-274	11	0.7500	0.8274	0.7900	0.8036
ZJU059[b]	JQ318754	(TC)14	F:<PET > <Tail-2 > TGTTTGTTTCTTGCTATTTCCATC R:GACAGTTCCCACCAGCATTT	217-235	7	0.5200	0.7935	0.7505	0.0016
ZJU060[ab]	JQ318755	(GT)8(GA)9	F:<PET > <Tail-2 > TGGCCAGGAACTTTGTATCC R:GAAAGATTGGGAATGCTGGA	223-243	7	0.6562	0.8110	0.7691	0.0000
ZJU061[ab]	JQ318756	(TC)11	F:<PET > <Tail-2 > TTTGGAGGAAGCAAACAAGC R:TCCTGCGCCAACAATCTAAT	204-232	11	0.2812	0.7922	0.7506	0.0000
ZJU062	JQ318757	(TC)10	F:<PET > <Tail-2 > GTCGAGAGAACAAAGCGACC R:GTCCAATGCCGCACTAACTT	240-252	7	0.2400	0.3282	0.3135	0.0004
ZJU063[ab]	JQ318758	(TC)12	F:<PET > <Tail-2 > ACTCAGCAGGACCACCAACT R:TTAGACGGAAATTGGGCTTG	232-250	10	0.7000	0.8593	0.8270	0.1320
ZJU064[b]	JQ318759	(GA)10	F:<PET > <Tail-2 > ACCATGAAGCTGACCTGGAG R:TTTCGTGGTCCCACCTACTC	226-244	6	0.4348	0.7256	0.6666	0.0001
ZJU065[ac]	JQ318760	(CA)13	F:<PET > <Tail-2 > TCCAGAATATCATCTCTTGCCA R: ATATTCCTAACGTGTGCGGG	214-236	9	0.6333	0.7706	0.7219	0.0001

Table 2 Characteristics of 158 SSR markers in this study *(Continued)*

ZJU066[ab]	JQ318761	(GA)10	F:<PET > <Tail-2 > CTTTCCCTTGTCGCTTTCAG R:GGTCGCAGATCAGGTCAAGT	221-235	8	0.2593	0.6450	0.6075	0.0000
ZJU067[ab]	JQ318762	(CT)10	F:<PET > <Tail-2 > CAGACAGCGAGGAGACAACA R:GGTCTTTCGAACTTTGCTCG	217-263	11	0.6923	0.8273	0.7861	0.0070
ZJU068[ab]	JQ318763	(CT)10	F:<PET > <Tail-2 > GAAGCTAAACGCCAGAAACG R:ACTCCTCACACGAATGGGTC	227-239	6	0.2917	0.7535	0.6913	0.0000
ZJU069[bc]	JQ318764	(GA)10	F:<PET > <Tail-2 > TGCCATAATCCTGAGAGCCT R:TGTTCTGTAATGGCGTGGAA	224-258	8	0.2609	0.5594	0.5235	0.0004
ZJU070[ab]	JQ318765	(CT)11	F:<PET > <Tail-2 > GTGCTCGAGATGTCCTCCAT R:ACAATCCCATCGCATACAGG	221-247	7	0.5200	0.7861	0.7364	0.0000
ZJU071[ab]	JQ318766	(GA)10	F:<PET > <Tail-2 > CTAAGGTTGGTCCCTGTCCA R:CTTGTGTGGTGATGGTTTGG	228-234	3	0.3704	0.6157	0.5305	0.0110
ZJU072[ab]	JQ318767	(AG)10	F:<PET > <Tail-2 > AGTCAGCGTGGGAATGTACC R:TTTCAGAACAAGTTCGTCGC	223-237	7	0.5625	0.7604	0.7117	0.0000
ZJU073[a]	JQ318768	(AG)12	F:<PET > <Tail-2 > TACGCCAAGATCCAAAGACC R:TCTCGAGTTGAGTTTGGGCT	222-242	7	0.2105	0.7568	0.7087	0.0000
ZJU074[ab]	JQ318769	(CT)15	F:<PET > <Tail-2 > TGCAGAGGAACTGGTGACTG R:GAGAAGGCTCAGTGGGTGAG	215-239	10	0.5517	0.8234	0.7831	0.0007
ZJU075[b]	JQ318770	(CT)17	F:<PET > <Tail-2 > AATAAACACACAGGGCGAGG R:ATCGGGCAGACCAGAATATG	239-255	9	0.0769	0.8650	0.8307	0.0000
ZJU076[ab]	JQ318771	(AG)9	F:<FAM > <Tail-3 > ATGGTTACCGACGTCCTCTG R:AGTGCAGAGTGCGAGATCAA	131-169	11	0.8438	0.8353	0.8034	0.0000
ZJU077[ab]	JQ318772	(AC)9	F:<FAM > <Tail-3 > TTTGGAATTCAACAACATTTAGAC R:TGCAGCCTTGCTCCTCTTAT	137-153	8	0.2000	0.6590	0.6079	0.0000
ZJU078[ab]	JQ318773	(TC)10	F:<FAM > <Tail-3 > ACACCACGGTTCTTCGATTC R:GTAACGAGGCTCTTGCTTGC	130-146	6	0.5500	0.7513	0.6881	0.1339
ZJU079[ab]	JQ318774	(TC)13	F:<FAM > <Tail-3 > AAGGCTAGACCGCAATCTGA R:GGGCAACAGTTTACTTCCCA	116-134	9	0.8438	0.8596	0.8291	0.0008
ZJU080[ab]	JQ318775	(CT)9	F:<FAM > <Tail-3 > CTTGACGAAATGCAGACGAA R:TCCGGATCAGGGTCAAATAG	124-134	5	0.2903	0.3411	0.3172	0.0103
ZJU081[ab]	JQ318776	(GA)8	F:<FAM > <Tail-3 > TGCTCTTGCAGAGAGTCGAG R:TCATAATACCCTTGGCAAACA	137-157	6	0.5517	0.5820	0.5379	0.0003
ZJU082[ab]	JQ318777	(CT)10	F:<FAM > <Tail-3 > TATATCGAATCCCAAAGGCG R:AAGATATTGGTCCGGCTCCT	129-141	5	0.3438	0.4043	0.3792	0.0169
ZJU083[ab]	JQ318778	(AG)10	F:<FAM > <Tail-3 > TAGCCTTGGAGATTTAGGGC R:TTGAAATTTCGCAGCCTCTT	133-157	11	0.8667	0.8960	0.8692	0.0000
ZJU084[ab]	JQ318779	(AG)9	F:<FAM > <Tail-3 > TTTCGATTGGTGGTCTGTGA R:TTATTAACTTCACTTTGTTTATTCGG	124-138	6	0.1379	0.5197	0.4766	0.0000
ZJU085[ab]	JQ318780	(AG)9	F:<FAM > <Tail-3 > GCTTTAACCGAGTGATGGGA R:TAAAGGAGCGCTGGAAAGAA	150-184	8	0.6875	0.5992	0.5383	0.6352
ZJU086[ab]	JQ318781	(TC)10	F:<FAM > <Tail-3 > TCCTCTCTTTCACACTTCCGA R:GGTCGATCATTTCTCTCCCA	118-152	13	0.9062	0.8720	0.8445	0.0005
ZJU087[ab]	JQ318782	(GA)9	F:<FAM > <Tail-3 > CGAGTGTAGCTAGGAACGGC R:AATTGGACCTGCAAATCTCG	135-149	8	0.4688	0.7748	0.7273	0.0204

Table 2 Characteristics of 158 SSR markers in this study *(Continued)*

ZJU088[ab]	JQ318783	(CT)9	F:<FAM > <Tail-3 > GAGCTCCGAACTTCTTCCCT R:CTTCTCCACAGGACTCTGCC	126-150	13	0.9677	0.8773	0.8490	0.0053
ZJU089[ab]	JQ318784	(GA)8	F:<FAM > <Tail-3 > CGTTAGGATTCGGGAACAGA R:CAGGGCTAATGTGGACCAGT	138-152	7	0.8065	0.7382	0.6778	0.0000
ZJU090[ab]	JQ318785	(AG)9	F:<FAM > <Tail-3 > GGAAATCTCCGAATGTGATCC R:TGGTGGATGAACCACTCAAA	118-134	8	0.2903	0.6642	0.6089	0.0000
ZJU091[bc]	JQ318786	(TC)15	F:<FAM > <Tail-3 > AAAGAGCACACAGCCCTAGC R:GGCAGTGTCGCAGTGATAGA	124-146	10	0.4615	0.8695	0.8358	0.0012
ZJU092[ab]	JQ318787	(TG)10	F:<FAM > <Tail-3 > CTCTTGCCGACCTCATTGTT R:CGGGACTCGCATAAATCACT	127-151	11	0.6875	0.8264	0.7916	0.0041
ZJU093[ab]	JQ318788	(GA)10	F:<FAM > <Tail-3 > ATGCCATGTTGCATGAGTGT R:TATCCCGTAAGCAATCAGGG	130-156	12	0.9355	0.8662	0.8367	0.3689
ZJU094[ab]	JQ318789	(CT)10	F:<FAM > <Tail-3 > ATCACGGGTTCTGCTGTTCT R:CAGAAGAAGCCATTTCTGCC	124-150	10	0.9062	0.8646	0.8332	0.0000
ZJU095[ab]	JQ318790	(AG)9	F:<FAM > <Tail-3 > TACCCACCGTACCAAAGGTC R:GAATGAACCCAGGCGATAGA	114-130	7	0.4839	0.7070	0.6420	0.0004
ZJU096[ab]	JQ318791	(CT)10	F:<FAM > <Tail-3 > CATACTGCAATGCATCTCCC R:TCAATTTGTGTGCCCTTACG	126-154	13	0.8000	0.8757	0.8479	0.0310
ZJU097[ab]	JQ318792	(AG)10	F:<FAM > <Tail-3 > AATTGTTAGGGAGGGCTCGT R:TGCGTTGTGGAGACCATTTA	118-134	8	0.8438	0.7778	0.7297	0.0009
ZJU098[ab]	JQ318793	(CT)9	F:<FAM > <Tail-3 > GACGCTCCATCTCTGGTCTC R:CCCAAACCGCACTAGAGAAA	145-167	10	0.9355	0.8831	0.8549	0.0483
ZJU099[ab]	JQ318794	(GA)10	F:<FAM > <Tail-3 > TTGTTGCACTTGTGGGTGAT R:AACTACAAACAGCCCAACCG	130-150	9	0.7742	0.7763	0.7299	0.0000
ZJU100[ab]	JQ318795	(TC)9	F:<FAM > <Tail-3 > ACTTGTCCGGATTCCACAAC R:TCAAGGCACACAATAATGCAA	128-154	5	0.8333	0.6316	0.5629	0.2930
ZJU101[ab]	JQ318796	(AG)9	F:<FAM > <Tail-3 > TGATTGAGCTGCCAACAAAG R:TTTAACATTTGGCACCGACA	134-154	7	0.6667	0.7062	0.6527	0.8110
ZJU102[ab]	JQ318797	(GA)10	F:<FAM > <Tail-3 > GAACCACGAACTTCAACCGT R:AACCACCAAACTTAGCTTCCA	118-132	8	0.4231	0.5890	0.5441	0.0111
ZJU103[ab]	JQ318798	(AG)9	F:<FAM > <Tail-3 > TGAGGAGGGAGTTGAGTTGG R:GCGTCTTCCTCCTCCTTCTT	121-139	10	0.7097	0.7731	0.7359	0.0003
ZJU104[ab]	JQ318799	(TA)9	F:<FAM > <Tail-3 > ACGTGGCAGTTGAGTTGTTG R:TCAGATCTCCGTTGGAGCTT	114-128	6	0.3704	0.6296	0.5702	0.1383
ZJU105[ab]	JQ318800	(GA)11	F:<FAM > <Tail-3 > TGAGAAACGCAGCAAGAGAA R:CATCTCTCCCAAGCATCCTC	135-157	11	0.5806	0.8165	0.7801	0.0000
ZJU106[ab]	JQ318801	(GA)8	F:<FAM > <Tail-3 > GCAGTCGGATAGAGAGACGG R:TGTTGATCAAACACACCGAGA	134-146	7	0.3636	0.7717	0.7203	0.0000
ZJU107[ab]	JQ318802	(TC)10	F:<FAM > <Tail-3 > TGGTGTCACGATTCACTGGT R:CTGCATGTAATGGCAGTTCAA	114-130	8	0.4375	0.5322	0.5012	0.3632
ZJU108[b]	JQ318803	(CT)9	F:<FAM > <Tail-3 > TTGGTAGTGCACTGCAGGAG R:CGAGGGTCGAGTTCAGAGAG	132-160	13	0.3929	0.8253	0.7909	0.0000
ZJU109[ab]	JQ318804	(TC)10	F:<FAM > <Tail-3 > TCCGCTCTCCTCTCTGTCTC R:GTGAGTTGTGCTGCTGCAAT	138-164	11	0.8000	0.8441	0.8082	0.0003

Table 2 Characteristics of 158 SSR markers in this study *(Continued)*

ZJU110[ab]	JQ318805	(AG)9	F:<FAM > <Tail-3 > TTGCACGGTGGTAGCTGTAG R:ACTGTGGTCCGTCGAACTCT	143-159	5	0.7667	0.6486	0.5844	0.0000
ZJU111[ab]	JQ318806	(TC)8	F:<FAM > <Tail-3 > TTTCTAATGTTGTTCGCCCA R:TCATTCTCCTTGCAGATCCC	122-136	5	0.9000	0.5701	0.4652	0.0000
ZJU112[ac]	JQ318807	(GA)8	F:<FAM > <Tail-3 > GGAGAGTGAGAGATCGCAGC R:GGCAACACCCTCAGTATCGT	133-147	8	0.4839	0.6557	0.6212	0.0009
ZJU113[ab]	JQ318808	(AG)9	F:<FAM > <Tail-3 > AAACGCACCAGAGAAAGACG R:TCCATCTCTGGTCTCCATCC	138-154	6	0.6667	0.6588	0.5987	0.0130
ZJU114[a]	JQ318809	(GA)10	F:<FAM > <Tail-3 > CTAGAGCGCTCCACGATACC R:AGAACGCTTGGAGAATCGAA	132-160	12	0.8214	0.8740	0.8448	0.0388
ZJU115[ab]	JQ318810	(AG)14	F:<FAM > <Tail-3 > GGTCTGAGGCCTTCACTCTG R:GAGACCCAATAACCCATCCA	126-156	14	0.9677	0.9022	0.8775	0.0068
ZJU116[ab]	JQ318811	(AG)15	F:<FAM > <Tail-3 > CTTTCTCCGTCTGCTCCATC R:GTCCAAACTTGGAGCCCATA	110-136	13	0.6875	0.8199	0.7846	0.0001
ZJU117[ab]	JQ318812	(AAG)9	F:<FAM > <Tail-3 > TCTCAGATCCCTCCACGTTC R:CCACTGGATCAGGACAACCT	118-133	6	0.4688	0.6944	0.6426	0.0000
ZJU118[ab]	JQ318813	(CT)9	F:<FAM > <Tail-3 > CAAGCCACGTGCATACCTATT R:CAGCTGGCTTCTAACTGCAA	120-144	11	0.8750	0.8502	0.8171	0.0001
ZJU119[a]	JQ318814	(AG)11	F:<FAM > <Tail-3 > CTTTCGACTTCAGAGGCAGC R:TCCCTCTCAAACTTTGCCAC	136-152	9	0.4828	0.8348	0.7975	0.0000
ZJU120[ab]	JQ318815	(GA)8	F:<HEX > <Tail-4 > TTGGTTTCGTTTGCAAGTCA R:GTCATCCATCCAATCCATCC	164-180	6	0.9355	0.7012	0.6354	0.0073
ZJU121[a]	JQ318816	(CT)11	F:<HEX > <Tail-4 > AATCACCGAAGAAATCCACG R:ATTGCCCTCCCTTCTGTTCT	164-186	11	0.8621	0.8705	0.8426	0.0000
ZJU122[ab]	JQ318817	(TC)8	F:<HEX > <Tail-4 > TGACGGAAGGATACTGGCTC R:CCATCAGACATGGCTTTCCT	164-180	7	0.7742	0.7509	0.7012	0.0000
ZJU123[ab]	JQ318818	(CT)8	F:<HEX > <Tail-4 > TGAATTATTCGGTTCCCTGG R:TGCTTCAGTTCCAAACGAAA	172-176	3	0.4667	0.6367	0.5499	0.2152
ZJU124[ab]	JQ318819	(CT)10	F:<HEX > <Tail-4 > GTGGCAGCCTCTCTATCGTC R:ATGACGTACTGCCCTTGCTT	161-187	12	0.9355	0.8778	0.8498	0.0001
ZJU125[ab]	JQ318820	(TC)8	F:<HEX > <Tail-4 > TAAGGGCAGTCAGACCAACC R:CTGCAGCCTACAATGATCCA	164-186	4	0.2188	0.3884	0.3453	0.0000
ZJU126[ab]	JQ318821	(GA)10	F:<HEX > <Tail-4 > CCAATGTGGACAGGTGTGAG R:GGCAGTAGTCGCTTCCCATA	173-193	11	0.9677	0.8535	0.8228	0.0000
ZJU127	JQ318822	(GC)10	F:<HEX > <Tail-4 > AGGATCCTTGTCACCACCAG R:AATTCTTCCTTCCCAGCCTC	165-189	11	0.9259	0.8288	0.7900	0.0079
ZJU128[ab]	JQ318823	(AG)14	F:<HEX > <Tail-4 > CCCAATTGACACAAATTCCC R:TTGGCATAGCATTGTTCGTC	145-161	5	0.4194	0.5019	0.4496	0.1981
ZJU129[ab]	JQ318824	(CT)10	F:<HEX > <Tail-4 > GAGGTGCAATTACGTGGCTT R:TCAAGCATCAGCTGCTCAGT	161-189	10	0.7500	0.8031	0.7611	0.0234
ZJU130[ab]	JQ318825	(GA)8	F:<HEX > <Tail-4 > GATTGCATGCACCAAATCAC R:GAATGTCCACGACGTGAATG	160-176	5	0.3478	0.4599	0.4131	0.2852
ZJU131[ab]	JQ318826	(CT)14	F:<HEX > <Tail-4 > TTGAGAATCACAAACGCCTG R:GGTGGGTGAAATGCCTAGAA	153-187	13	0.8710	0.8990	0.8735	0.0009

Table 2 Characteristics of 158 SSR markers in this study *(Continued)*

ZJU132[ab]	JQ318827	(CT)11	F:<HEX > <Tail-4 > AGGCACCTTTCTTTCCTCTC R:CAAGGAAGGAGGTGACGAAG	164-178	5	0.6452	0.6568	0.5834	0.6586
ZJU133[ab]	JQ318828	(TC)11	F:<HEX > <Tail-4 > GCCCTGCAGTCTTTGTCAAT R:CAGCTTGCAGTGTTCATTCA	171-195	8	0.8710	0.7731	0.7267	0.0000
ZJU134[ab]	JQ318829	(GA)11	F:<HEX > <Tail-4 > AGTGCCCAAGCATGACTTCT R:AATCAGTTGTCCGAGGATGG	172-190	8	0.9688	0.7907	0.7507	0.0004
ZJU135[ab]	JQ318830	(AG)10	F:<HEX > <Tail-4 > AATTTACGGCTGTCCGTGAG R:CCTTGGGCTTCATGAACATT	173-191	10	0.9688	0.7966	0.7557	0.0000
ZJU136[ab]	JQ318831	(GA)10	F:<HEX > <Tail-4 > TCCCACAGATCTCTAGCCGT R:CGCTCAGTTCTTAATTTCTTACTGTC	173-201	13	0.7742	0.8953	0.8692	0.0004
ZJU137[ab]	JQ318832	(TC)8	F:<HEX > <Tail-4 > TGGATCTTGCTGCAGTTGTC R:AGCTAGCACTGGCCTAACCA	140-168	12	0.1875	0.6930	0.6612	0.0000
ZJU138[ab]	JQ318833	(CT)10	F:<HEX > <Tail-4 > GCACAGTTGAGTTATGGGCA R:CTCTTTCAAATCCACGCACA	152-170	8	0.3333	0.7746	0.7261	0.0001
ZJU139[ab]	JQ318834	(GA)12	F:<HEX > <Tail-4 > CCGAGCTTCGTTAGGACTTG R:CCAACAATACCCGAACCATC	138-164	6	0.3667	0.4418	0.4043	0.0000
ZJU140[b]	JQ318835	(CT)14	F:<HEX > <Tail-4 > TGTGCTCATCTTGGATCCTG R:ACATCAGCTTGCATCCCTCT	172-198	9	0.6538	0.6139	0.5474	0.0000
ZJU141[ab]	JQ318836	(CT)13	F:<HEX > <Tail-4 > CACAATCAGCTGCAGAATCAA R:AATGGCCGCTTGCAATATAA	175-201	11	0.6774	0.7996	0.7600	0.0002
ZJU142[ab]	JQ318837	(TC)13	F:<HEX > <Tail-4 > CATTCACCTCCTTTCGCAAT R:ATCCAACGGCTCAAAGAATG	166-184	9	0.6774	0.6912	0.6498	0.0231
ZJU143[ab]	JQ318838	(CT)12	F:<HEX > <Tail-4 > GTAGAGTAGATGCGCCTCGG R:ACGTACGAGCCATACACACG	181-197	7	0.6923	0.7044	0.6397	0.0000
ZJU144[ab]	JQ318839	(AG)12	F:<HEX > <Tail-4 > GCCACTCTTCCCTCAACGTA R:CAGGTCAGTCCTGATGGGAT	148-164	7	0.5161	0.6864	0.6252	0.0430
ZJU145[ab]	JQ318840	(CT)10	F:<HEX > <Tail-4 > TGTGGCTGTGTTCCTCCATA R:CAATGTTGGGTGCTTTGTTG	155-175	7	0.6875	0.7351	0.6912	0.0000
ZJU146[ab]	JQ318841	(AG)10	F:<HEX > <Tail-4 > TGGAAACTTTGTCGTGTGGA R:TTATATCGGGCAGCCAGAAC	154-168	6	0.2258	0.6663	0.6187	0.0000
ZJU147[ab]	JQ318842	(AG)10	F:<HEX > <Tail-4 > TTAGGAACCAAACTGGACGG R:TCAAATGCCGTGCTATTGAG	173-195	10	0.8333	0.7169	0.6811	0.0005
ZJU148[ab]	JQ318843	(AG)18	F:<HEX > <Tail-4 > AAGAGCAGGAACCGAACCTT R:ACCGAAAGACGAAGAAACGA	160-190	15	0.9375	0.9067	0.8829	0.4973
ZJU149[ab]	JQ318844	(TC)8	F:<HEX > <Tail-4 > AGCCCTCCATGTGTGCTTAT R:AGGGAGAGAGTGGTTCTGCC	139-163	11	0.8333	0.8718	0.8417	0.0022
ZJU150[ab]	JQ318845	(AG)10	F:<HEX > <Tail-4 > ACTTAACTGAGAGGCTGCGG R:GTGGAAACCGAACGTCCTAA	163-201	10	0.9000	0.8469	0.8123	0.0053
ZJU151[ab]	JQ318846	(CA)9	F:<HEX > <Tail-4 > GAATTGGAAATCCCTAGCCC R:CATTTGCGCATGTCTCCTTA	156-170	6	0.3750	0.5511	0.5188	0.0001
ZJU152[ab]	JQ318847	(AG)11	F:<HEX > <Tail-4 > AAACGAAGTCGTTCAATGCC R:CTTGATTTGGGCCTTCGATA	163-181	7	0.9355	0.7578	0.7040	0.0161
ZJU153[ab]	JQ318848	(AG)10	F:<HEX > <Tail-4 > CCAGCTCCGAATTAGCAAAC R:GTGGCGGTTTATCTCATCGT	173-191	6	1.0000	0.6667	0.5927	0.0000

Table 2 Characteristics of 158 SSR markers in this study *(Continued)*

ZJU154[ab]	JQ318849	(AG)11	F:<HEX > <Tail-4 > TTGTCAATTGCCCTTCCTTC R:TTCCTCCCTTTCCCACTTCT	156-184	10	0.9333	0.6847	0.6184	0.0000
ZJU155[ab]	JQ318850	(TC)9	F:<HEX > <Tail-4 > GAGAGCAATCAGTGAAGCCC R:GGGAGACGGATGTCGATTTA	160-188	8	0.8438	0.6731	0.6037	0.0000
ZJU156[ab]	JQ318851	(TA)8	F:<HEX > <Tail-4 > ATACGTCGAAAGATCCACCG R:TTCTGGAATCCTTCCCATTG	166-184	7	0.5484	0.6626	0.6063	0.0000
ZJU157[ab]	JQ318852	(AG)9	F:<HEX > <Tail-4 > CACTCACAACCAAAGCCAGA R:GTGCATAATCACAGGCATGA	154-186	13	0.9677	0.9064	0.8823	0.0171
ZJU158[ab]	JQ318853	(AT)10	F:<HEX > <Tail-4 > CCAGATGATCACGCAGCTTA R:CGTCCTCCAATACGTTCCTC	156-174	9	0.6452	0.8292	0.7917	0.0000
Mean					8.25	0.5636	0.7178	0.6730	

Note: [a b c] These SSRs are transferable for *M. adenophora*, *M. nana* and *M. cerifera*, respectively. SSR markers are listed according to ascending order in different fluorescent dyes. Shown for each primer pair are the repeat motif, primer sequences, size range (bp), number of alleles detected (*Na*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), polymorphism information content (PIC) and Chi-square test for Hardy-Weinberg equilibrium (P_{HW}). The annealing temperature was 60 °C; a, including length of tail sequences (18 bp total). P_{HW} over 0.05 are underlined.

Recently, based on mass sequence data of Chinese bayberry obtained by RNA-Seq, 41,239 UniGenes were identified and approximately 80% of the UniGenes (32,805) were annotated, which provides an excellent platform for future EST-SSR development and functional genomic research [26].

High efficient test methods

Normally, a universal M13 primer is labelled with one of a number of fluorescent dyes. The tailed primer provides a complementary sequence to the fluorescent labelled M13 primer, leading to the amplification of fluorescent PCR products, and then the PCR products of different sizes and/or labelled with different fluorescent dyes are mixed and tested [27]. In this research, a multiplex PCR strategy was designed using the universal M13-tailed primer and three additional tail primers, designed arbitrarily, in presumed four-plex amplifications in single PCR, for a major reduction in cost and time. However, as only a few primer combinations were successful, most resulting in weak bands, we did the PCR individually and mixed the PCR products. Further optimisation of multiplex PCR is needed to evaluate its general applicability.

Table 3 Distribution of the segregation types expected for the mapping population (Biqi × Dongkui)

Segregation type	Alleles	Number	Mapping in F_1
aa × aa	1	12	No
aa × bb	2	11	No
aa × ab	2	24	Yes
ab × aa	2	18	Yes
ab × ab	2	8	Yes
aa × bc	3	12	Yes
ab × cc	3	12	Yes
ab × ac	3	41	Yes
ab × cd	4	20	Yes
Total		135	

Evolution of *Myrica* species

In this study, both cultivated species and wild species were analysed and their genetic diversity could easily be differentiated. *M. nana* and *M. cerifera* were clearly genetically distant to *M. rubra*. *M. nana*, also known as the dwarf or Yunnan arbutus, is indigenous to the Yunnan and Guizhou provinces, and has a plant height of < 2 m. The juvenile period of fruit tree can be shortened for breeding purposes. Studies on embryo culture *in vitro* of the F_1 seeds of crosses between *M. rubra* and *M. nana*, [28], has shown good cross compatibility between *M. rubra* and *M. nana*, resulting in 70.5% normal seeds with intact embryo. *M. adenophora* and *M. nana* grow as wild trees, with the fruit of *M. adenophora* only suitable for medical purposes and not edible.

Our findings on the genetic similarity between *M. adenophora* and *M. rubra*, which are considered a progenitor-derivative species pair, are consistent with a previously published figure of 0.897 [29]. An earlier study observed little change in allelic diversity along the chronosequence and no evidence for heterosis, although there was a moderate change in genotypic diversity [30]. The markers developed in this study can be very useful in future population structure analysis.

Conclusions

In summary, the genome size of *Myrica* genus is small, about 320 Mb. A large set of SSRs were developed from a genome survey of *Myrica rubra*. The results suggest that they have high rates of transferability, making them suitable for use in other *Myrica* species.

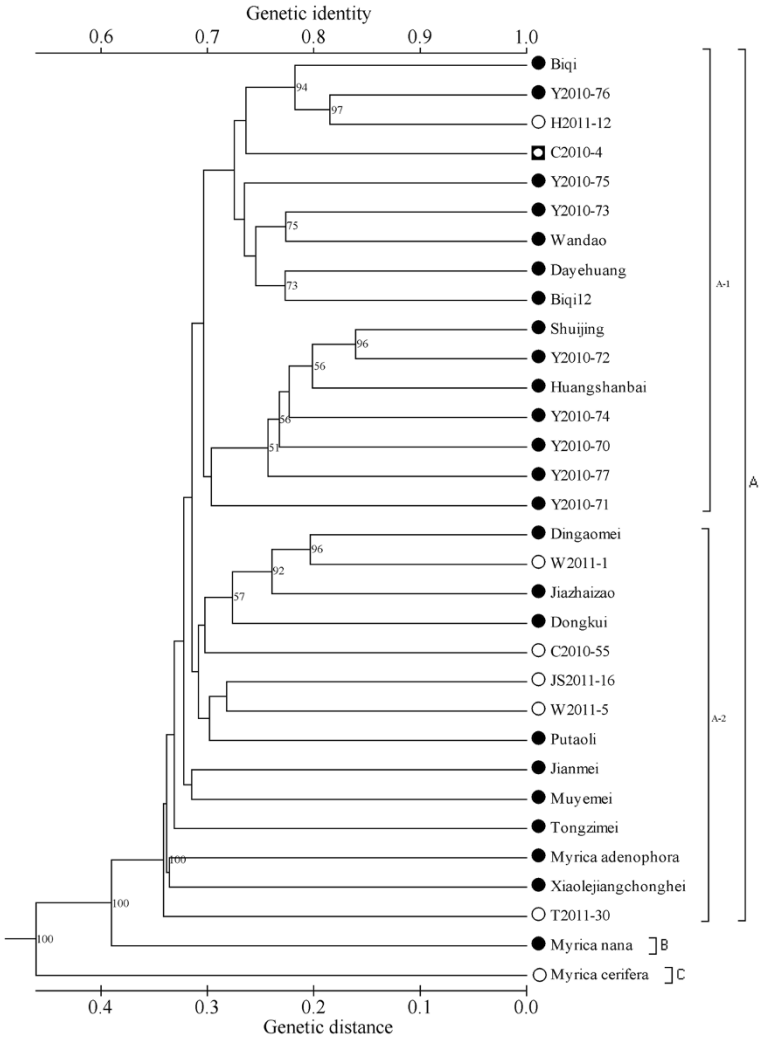

Figure 4 Dendrogram for 32 Chinese bayberry accessions derived from UPGMA cluster analysis based on 158 SSR markers. The symbols before the accession codes indicate the sex: ○, androphyte plant, ● , common cultivars, and ◘, monoecious plant. The numbers are bootstrap values based on 1000 iterations. Only bootstrap values larger than 50 are indicated.

Materials and methods

Plant materials and genome survey

We selected an androphyte 'C2010-55' for the genome survey because it was the most homozygous (10 out of 14 SSRs) individual among 230 accessions. Two DNA libraries of 250 and 500 bp insert size were constructed and sequenced by Illumina Hi-Seq 2000.

Twenty-nine accessions of the cultivated species (*M. rubra*) and 3 related species (*M. adenophora*, *M. nana*, *M. cerifera*), collected from different provinces in China (Table 4), were used to evaluate the suitability of the SSRs for genetic distance analysis. Young leaves were collected and frozen in liquid nitrogen prior to genomic DNA extraction using CTAB methods [4]. DNA concentrations were measured spectrophotometrically at 260 nm, and the extracts electrophoresed on 1% agarose to confirm the quality. The purified DNAs were standardised at 40 ng/μl and stored at -40°C.

SSR identification and primer design

We used MISA scripting language (http://pgrc.ipk-gatersleben.de/misa/misa.html) to identify microsatellite repeats in our sequence database. The SSR loci containing perfect repeat units of 2-6 nucleotides only were considered. The minimum SSR length criteria were defined as six reiterations for dinucleotide, and five reiterations for other repeat units. Mononucleotide repeats and complex SSR types were excluded from the study.

The SSR primers were designed using BatchPrimer3 interface modules (http://pgrc.ipk-gatersleben.de/misa/

primer3.html). We selected 600 primers that met the following parameters: 110–230 final product length, primer size from 18 to 22 bp with an optimum size of 20 bp, and the annealing temperature was set at 60°C. The repeat units over eight were used.

Tail-1(M13 universal sequence-TGTAAAACGACGGCCAGT), Tail-2(CGAGTCAGTATAGGGCAC), Tail-3 (ATCACGAAGCAGATGCAA) and Tail-4(GAGCATCTCGTACCAGTC) were added to the 5' end of each 150 forward primers of pairs respectively. Tail-2, Tail-3 and Tail-4 were designed so that the primer size was 18 bp and the annealing temperature was 53°C. Primers were synthesised by Invitrogen Trading (Shanghai) Co., Ltd. We primarily tested two cultivars (Biqi and Dongkui) and *M. cerifera* for 600 SSR loci by PAGE (polyacrylamide denaturing gel) to confirm their suitability. Tail-1, Tail-2, Tail-3 and Tail-4 labelled with one of the following dyes: NED, PET, FAM, and HEX, respectively.

Polymerase chain reaction and gel electrophoresis

Each 20 μl reaction mixture contained 10 × PCR buffer (plus Mg2+), 0.2 mM of each dNTP, 5 pmol of each reverse, 4 pmol of the tail primer, 1 pmol of the forward primer, 0.5 units of rTaq polymerase (TaKaRa,

Table 4 The 32 bayberry accessions included in this study

No.	Accession	Fruit/Flower colour[a]	Fruit maturity date	Region
1	Biqi	black	Late June	Cixi, Ningbo, Zhejiang
2	Dongkui	red	Early July	Taizhou, Zhejiang
3	Dayehuang	red	Mid-June	Hangzhou, Zhejiang
4	Dingaomei	black	Mid to late June	Wenzhou, Zhejiang
5	Huangshanbai	white	Early July	Hangzhou, Zhejiang
6	Jiazhaizao	black	Mid-June	Wenzhou, Zhejiang
7	Jianmei	red	Late June	Cixi, Ningbo, Zhejiang
8	Muyemei	black	Late June	Jinhua, Zhejiang
9	Putaoli	black	Mid June	Hangzhou, Zhejiang
10	Shuijing	white	Late June/Early July	Yuyao, Ningbo, Zhejiang
11	Tongzimei	black	Mid-June	Hunan
12	Wandao	black	Early July	Zhoushan, Zhejiang
13	Xiaolejiangchonghei	black	May	Guizhou
14	Biqi12	black	Late June	Yuyao, Ningbo, Zhejiang
15	Y2010-70	red	Late June/Early July	Yuyao, Ningbo, Zhejiang
16	Y2010-71	black	Mid to late June	Yuyao, Ningbo, Zhejiang
17	Y2010-72	white	Late June/Early July	Yuyao, Ningbo, Zhejiang
18	Y2010-73	red	Late June	Yuyao, Ningbo, Zhejiang
19	Y2010-74	red	Late June/Early July	Yuyao, Ningbo, Zhejiang
20	Y2010-75	black	Late June	Yuyao, Ningbo, Zhejiang
21	Y2010-76	white	Late June/Early July	Yuyao, Ningbo, Zhejiang
22	Y2010-77	red	Late June/Early July	Yuyao, Ningbo, Zhejiang
23	C2010-4	red	Late June	Cixi, Ningbo, Zhejiang
24	*C2010-55	red	-	Cixi, Ningbo, Zhejiang
25	*W2011-1	yellow- red	-	Wenzhou, Zhejiang
26	*W2011-5	red	-	Wenzhou, Zhejiang
27	*H2011-12	yellow-green	-	Hangzhou, Zhejiang
28	*JS2011-16	red	-	Suzhou, Jiangsu
29	*T2011-30	red	-	Taizhou, Zhejiang
30	*Myrica adenophora*	red	February to May	Guilin, Guangxi
31	*Myrica nana*	red	June to July	Yunnan
32	**Myrica cerifera*	yellow-green	-	Cixi, Ningbo, Zhejiang

Note: fruit colour for cultivar and flower colour for androphyte. * selected androphytes.

China) and 40 ng genomic DNA template. Each primer pair had an interval of 20 bp according to the expected size of amplicons.

DNA amplification was in an Eppendorf Mastercycler (Germany) programmed at 94°C for 5 min for initial denaturation, then 32 cycles at 94°C (30 s)/58°C (30 s)/72°C (30 s), followed by 8 cycles of 94°C (30 s)/53°C (30 s)/72°C (30 s). The final extension step was 10 min at 72°C. Each PCR product was run on 1% agarose gel at 110 V for a quality check.

Subsequently, PCR products were electrophoresed on 8% denaturing PAGE, according to Myers et al. [31], at 60 W in a Sequi-Gen GT Nucleic Acid electrophoresis cell (BioRad) for 4 h, depending on the fragment sizes to be separated, and visualised by silver staining [32]. Genotypes showing one and two bands were scored as homozygous and heterozygous, respectively, and the results recorded and photographed.

Multiplex PCR was designed and tested with products of different sizes and labelled with different fluorescent dyes. Each 20 μl reaction mixture contained 10 × PCR buffer (plus Mg^{2+}), 0.8 mM of each dNTP, 1 unit of rTaq polymerase, 40 ng genomic DNA template and a total of four primer pairs with 5 pmol of each reverse primer, 4 pmol of each tail primer, and 1 pmol of each forward primer. The PCR products were diluted, mixed with the internal size standard LIZ500 (Applied Biosystems) and loaded on an ABI 3130 Genetic Analyzer. Alleles were scored using GeneMapper version 4.0 software (Applied Biosystems, Foster City, CA, USA).

Data analysis

The raw genome sequence data was first filtered to obtain high quality reads, then assembled using SOAP (http://soap.genomics.org.cn/soapdenovo.html) denovo software to contig, scaffold and fill in gaps. In addition, we used SSPACE software to build the scaffold. K-mer analysis was to help estimate the genome size and characters, such as heterozygosis and repeats.

The number of alleles (A), observed heterozygosity (Ho) and expected heterozygosity (He) were calculated using POPGENE version 1.32 (http://www.ualberta.ca/~fyeh/popgene_download.html). Chi-square test for Hardy-Weinberg equilibrium allele frequencies and polymorphism information content (PIC) was calculated using PowerMarker version 3.25 [33] (http://statgen.ncsu.edu/powermarker/downloads.htm). Microsoft office Excel 2007 was used to analyse the correlation. Genetic similarity among all the accessions was calculated according to Dice's coefficients using Nei's coefficient index [16] with the Free Tree 0.9.1.50[34] (http://www.natur.cuni.cz/~flegr/programs/freetree.htm) software, and the dendrogram constructed using the unweighted pair-group method with arithmetic averaging (UPGMA) option. The confidence of branch support was then evaluated by bootstrap analysis with 1,000 replicates. The dendrogram was printed using MEGA version 5.05 software [35] (http://www.megasoftware.net/mega.php).

Additional material

Additional file 1: Occurrence of different SSRs in the genome survey of Chinese bayberry.

Authors' contributions

ZSG, HJJ, EVW and MLC designed the experiments. YJ, CYC, GYW collected plant materials. YJ, HMJ and XWL performed the SSR experiments and analysed the data. The whole genome shotgun and sequencing assembly was performed by ZC. YJ, ZSG and EVW drafted this manuscript.

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