Development of Highly Polymorphic Expressed Sequence Tags-Simple Sequence Repeat Markers and Their Application in Analysis of Genetic Diversity of Chinese Bayberry (*Morella rubra*)

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Abstract. Chinese bayberry (*Morella rubra*) is an economically important subtropical evergreen fruit crop native to China and other Asian countries. For facilitating cultivar discrimination and genetic diversity analysis, a total of 38 high-quality and highly polymorphic expressed sequence tags-simple sequence repeat (EST-SSR) markers, with little or no polymerase chain reaction (PCR) stutter bands, including 21 screened from those obtained previously and 17 newly developed markers, were developed. The average number of alleles (N_a) per locus was 5.6, and polymorphism information content varied from 0.34 to 0.86, with a mean value of 0.57. With these markers, all 42 Chinese bayberry accessions analyzed were successfully discriminated and the phylogenetic relationship between accessions was revealed. The accessions can be separated into two groups with six subgroups. The grouping of four main cultivars in three subgroups and 12 white-fruited accessions, each with little or no anthocyanin accumulation in ripe fruit, into five subgroups suggested the preservation of broad diversity among cultivated populations. These EST-SSR markers and the findings obtained in this study can assist the discrimination of cultivars and lines and contribute to genetic and breeding studies in Chinese bayberry.

Chinese bayberry (*Morella rubra*, former name *Myrica rubra*), the only species in *Morella* commercially cultivated for fresh fruit, is a subtropical evergreen tree native to China and other Asian countries (Chen et al., 2004; Jia et al., 2014; Zhang et al., 2009a). Chinese bayberry is planted as an important fruit crop in China and commercial production of this fruit is mainly restricted to southern China, with a history of over 2000

years, but also in Japan and recently Australia. The fruit ripens in early summer, an off season for the fresh fruit market in the northern hemisphere, with a characteristic flavor welcomed by consumers. The fruit is also rich in bioactive compounds like anthocyanins, which have multiple benefits for human health, such as scavenging of reactive oxygen species and prevention of cancers (Liu et al., 2013; Sun et al., 2013; Zhang et al., 2005, 2015a).

There are as many as 305 cultivars or lines reported for Chinese bayberry (Zhang and Miao 1999), but the diversity and phylogenetic relationship are far from being well studied compared with many other fruit species (Zhang et al., 2009a). In industry, the availability of a molecular identity for each elite cultivar is frequently required. For example, some accessions are quite close to each other and cannot be distinguished morphologically. This is quite common especially for white-fruited cultivars, also named Bai or Shuijing in Chinese, with ripe fruit accumulating little or no anthocyanins.

The genetic diversity of Chinese bayberry germplasm has been analyzed with different molecular markers, initially using random amplified polymorphic DNA (Lin et al., 1999; Xie et al., 2008), inter simple sequence repeat (Pan et al., 2008; Qian et al., 2006; Qiu et al., 2002; Xie et al., 2008), and amplified fragment length polymorphism (AFLP) (Zheng et al., 2006; Zhang et al., 2009a) as well as simple sequence repeat (SSR) (Jia et al., 2015; Jiao et al., 2012; Terakawa et al., 2006; Xie et al., 2011a, 2011b; Zhang et al., 2015b; Zhang et al., 2012; Zhang et al., 2009b) recently. Occasionally, some other molecular markers such as sequence-related amplified polymorphism (Lin et al., 2013), inter primer binding site, and start codon targeted polymorphism (Chen and Liu, 2014) are used. Compared with other molecular markers, SSRs have certain advantages such as high abundance, high reproducibility, codominant inheritance, and their multiallelic nature and have been widely applied in parentage analysis, hybrid identification, cultivar discrimination, and genetic diversity studies in various fruits (Heidi and Andrew, 2007). However, despite the availability of SSR markers from previous studies, the number of high-quality and highly polymorphic markers, with only weak or no PCR stutter bands, is still limited. PCR stutter bands are common for some SSR markers, especially those with a high number of dinucleotide repeats, which results from enzyme slippage during SSR-PCR amplification, severely affecting the size discrimination of PCR products (Guichoux et al., 2011; Hite et al., 1996).

With advances in sequencing technology, data from RNA-Seq can offer a multitude of SSR loci. Previously, we developed 109 EST-SSR markers based on 41,239 unigene sequences generated from the transcriptome data of Chinese bayberry (Feng et al., 2012; Zhang et al., 2012). In this study, 21 high-quality and highly polymorphic EST-SSR markers were screened out from these previously obtained 109 markers and 17 new ones were developed. With these markers, 42 Chinese bayberry accessions were discriminated and their diversity and phylogenetic relationships were analyzed.

Materials and Methods

Plant materials. Forty-two Chinese bayberry (*Morella rubra* Sieb. et Zucc., former name *Myrica rubra*) accessions and one *M. nana* Cheval. accession, as the outgroup,

Table 1. List of 42 Chinese bayberry accessions included in this study.

No.	Accession	Note	Origin
1	Anhuibaimei	White	Anhui
2	Baxiandao	Black	Fujian
3	Dongkui	Red	Fujian
4	Dayezhong	Black	Fujian
5	Fugongyihao	Black	Fujian
6	Fujianbaimei	White	Fujian
7	Ranbingsanhao	Red	Fujian
8	Ruansianhaibian	Black	Fujian
9	Taipingyangwuhao	Red	Fujian
10	Wangzianhai	Black	Fujian
11	Yingsianhaibian	Black	Fujian
12	Wusu	Black	Guangdong
13	Dayexidi	Black	Jiangsu
14	Dongshanbaimei	White	Jiangsu
15	Dongshanwumei	Black	Jiangsu
16	Gousetou	Red	Jiangsu
17	Guo-1	Red	Jiangsu
18	Jietou	Red	Jiangsu
19	Langdangzi	Black	Jiangsu
20	Lazhua	Black	Jiangsu
21	Shijiazhong	Red	Jiangsu
22	Taohong	Black	Jiangsu
23	Wang-1	Black	Jiangsu
24	Xishanbaimei	White	Jiangsu
25	Xishanwumei	Black	Jiangsu
26	Zaoshu	Red	Jiangsu
27	Zitiao	Black	Jiangsu
28	Cixibaimei	Pink	Cixi, Zhejiang
29	Biqi	Black	Cixi, Zhejiang
30	Shuangqiaobaimei	White	Linhai, Zhejiang
31	Songshanshuimei	Black	Linhai, Zhejiang
32	Yongquanzaoshumei	Red	Linhai, Zhejiang
33	Zaodamei	Black	Linhai, Zhejiang
34	Dingao	Black	Wenzhou, Zhejiang
35	Yuyaobaimei1	White	Yuyao, Zhejiang
36	Yuyaobaimei2	White	Yuyao, Zhejiang
37	Yuyaobaimei3	White	Yuyao, Zhejiang
38	Yuyaobaimei4	White	Yuyao, Zhejiang
39	Yuyaobaimei5	White	Yuyao, Zhejiang
40	Yuyaobaimei6	White	Yuyao, Zhejiang
41	Lizhizhong	Black	Yuyao, Zhejiang
42	Wandao	Black	Zhoushan, Zhejiang
43	<i>Morella nana</i>	Red	Yunnan

were included in this study. Detailed information about the accessions is presented in Table 1. Young leaves were collected and subsequently frozen in liquid nitrogen and then stored at -80°C until use.

DNA extraction. Total genomic DNA was extracted from leaf tissue using an improved

cetyltrimethylammonium bromide protocol as described by Zhang et al. (2009a).

Development of new EST-SSR markers. MicroSatellite identification tool (MISA) (<http://pgsc.ipk-gatersleben.de/misa/misa.html>) was used to identify microsatellite repeats in Chinese bayberry RNA-Seq data (Feng et al., 2012). Only di or trinucleotide SSR loci with 16 base pairs (bp) as minimum length were considered. The SSR primers were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOD=BlastHomeAd) with following coefficients: primer size from 18 to 22 nucleotides and PCR product size 150–300 bp.

SSR-PCR amplification. For gene cloning to confirm the authenticity of PCR amplification, conventional SSR-PCR was conducted with 25 μL of reaction mixtures containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 ng genomic DNA, 5 pmol of each primer, 1.5 mM MgCl_2 , 0.2 mM of each deoxynucleotide triphosphate, and 0.5 units of Taq DNA polymerase (TaKaRa, Dalian, China) using an Eppendorf Mastercycler (Eppendorf Scientific, Inc., Westbury, NY) with an initial denaturation at 94°C for 5 min followed by 30 cycles of 30 s at 94°C , 30 s at 58°C , 30 s at 72°C , and a final extension of 7 min at 72°C . For ease of PCR product size analysis, fluorescent labeled primers were applied with strategies described by Schuelke (2000). For this purpose, the PCR reaction mixtures contained 2 pmol forward primer with M13 tail (M13 universal sequence, TGTAACGACGGC-CAGT) at the 5'-end, 8 pmol of downstream primer, 6 pmol of universal fluorescence dye-labeled primer, either 6-carboxyfluorescein (6-FAM), or 5-hexachloro-fluorescein (HEX), with modified amplification conditions: an initial denaturation at 94°C for 5 min followed by 20 cycles of 30 s at 94°C , 30 s at 60°C , 30 s at 72°C , another 12 cycles of 30 s at 94°C , 30 s at 55°C , 30 s at 72°C , and a final extension of 15 min at 72°C . The primers were synthesized by Shanghai HuaGene Biotech Co., (Shanghai, China) and the primer information is listed in Table 2.

SSR-PCR product size identification and sequence authentication. Size identification of fluorescence dye-labeled SSR-PCR products was completed by TsingKe Biological Technology Co., Ltd. (Beijing, China) using capillary electrophoresis in an ABI PRISM 3130 DNA Analyzer (Applied Biosystems, Foster City, CA). The authenticity of SSR-PCR amplification was confirmed through conventional gene cloning and sequencing.

Data analysis. SSR data were scored as several alleles per locus distinguished by their sizes. The genetic statistics, including the N_a , observed heterozygosity (H_o), expected heterozygosity (H_e), and polymorphic information content (PIC) were calculated with the POWERMARKER software version 3.25 (Liu and Muse 2005). A dendrogram was made by using MEGA 5.0 software (Tamura et al., 2011). The data and the dendrogram were processed based on Nei's genetic distance coefficient (Nei, 1973).

Results and Discussion

EST-SSR marker development. On the basis of RNA-Seq data from Chinese bayberry, we previously developed 109 EST-SSR markers (Zhang et al., 2012). In this study, these markers were reevaluated using the criterion of producing only weak or no PCR stutter bands while also being highly polymorphic. First, 40 EST-SSR markers with PIC over 0.4 were picked and the corresponding primers were synthesized. Among these, 21 markers were successfully selected, with authentic (confirmed by cloning and sequencing) and high-quality (with no or only weak PCR stutter bands) SSR-PCR amplification following both conventional program and fluorescence dye-labeling procedures. Serious stuttering problem was encountered for 19 EST-SSR markers, with 14 having dinucleotide motifs and the remaining five having compound ones.

Another 17 EST-SSR markers were newly developed in this study. First, 62 EST-SSR markers were mined from RNA-Seq data, with 41 containing trinucleotide motifs and the remaining 21 with dinucleotide ones. After analysis through SSR-PCR amplification, gene cloning and sequence authentication, as well as size discrimination to exclude those markers with serious PCR stutter bands or being nonpolymorphic for eight selected cultivars (Biqi, Dongkui, Shuijing, Wandao, Dingao, Dayexidi, Wusu, and Zaodamei), 17 were found to be of both high-quality and highly polymorphic, with 15 of them being trinucleotide SSRs and the other two dinucleotide ones.

The polymorphism of these 38 EST-SSR markers was evaluated with 42 Chinese bayberry accessions and one accession of the outgroup *M. nana*. A total of 211 alleles were found, with the N_a at a specific polymorphic locus ranged from 2 (MrEST-SSR-64) to 17 (MrEST-SSR-52) and the mean value 5.6, which is higher than the value reported previously (Jia et al., 2014; Xie et al., 2011a; Zhang et al., 2012). The value of H_e varied from 0.33 to 0.88 with the mean of 0.59. The H_o ranged from 0.26 to 0.87, with an averaged value of 0.61. The value of PIC ranged from 0.34 to 0.86, with the mean 0.57, which was also in the range of values reported previously (Jia et al., 2014; Jiao et al., 2012; Xie et al., 2011; Zhang et al., 2012). The detailed genetic diversity statistics for each of the EST-SSR markers used in this study are listed in Table 2.

Genetic relationships among 42 Chinese bayberry accessions. According to the unweighted pair group method using arithmetic mean clustering result, the 42 Chinese bayberry accessions can be separated into two groups with six subgroups (Fig. 1). This is similar to the clustering of two groups with seven subgroups we previously obtained by using AFLP (Zhang et al., 2009a) although the Chinese bayberry accessions involved in these two studies are quite different.

The accessions involved in this study mainly focus on commercially important

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Table 2. Characterization of the 38 microsatellite loci for Chinese bayberry, including primer sequences, repeat motifs, number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphism information content (PIC), and GenBank accession numbers.

Primer	Primer sequence(5'-3')	Repeat motif	N_a	H_o	H_e	PIC	GenBank
MrEST-SSR-8	F:AAACAACAAAGATTCTCCGAAC R:GGACGCTGGAGACTGAGACA	(TC) ₈	6	0.69	0.40	0.64	JX105249
MrEST-SSR-13	F:GTACTCTAGGGAGGAGGCATAA R:AAGTTGGAGTGGGAGGTTCAG	(AG) ₈	5	0.67	0.51	0.62	JX105254
MrEST-SSR-21	F:TCTCCTTCGCTATCTCCCTGT R:TGCGTTGAAATTTAGAATCTGT	(TC) ₉	6	0.69	0.63	0.64	JX105262
MrEST-SSR-25	F:AAATAAGGGGTGTGTAATGACG R:GCTGAAATAGCCATTGGGAGAT	(TC) ₁₀	6	0.74	0.58	0.70	JX105266
MrEST-SSR-27	F:CCCCATCTTTTGTCTCTTTCT R:GTGATTCCGCCCATTTCTGTTAT	(TC) ₁₁	6	0.58	0.65	0.54	JX105268
MrEST-SSR-33	F:GCGGCTACAGGTGCCAGAGAC R:GAACAAGTGGAAATGCAAGGT	(AG) ₁₂	10	0.77	0.74	0.75	JX105274
MrEST-SSR-52	F:CACATTTAGCAGCGACAGAC R:CACCAGGGAGATAGAAGCAG	(GA) ₁₆	17	0.87	0.88	0.86	JX105293
MrEST-SSR-55	F:CCGGAGAACAGAAGGCACATA R:GAGGGAGAGACGAAGAAGATT	(GA) ₁₆	10	0.79	0.77	0.77	JX105296
MrEST-SSR-59	F:TGGATGGTTAATTCGATCGTT R:ATTTAGCTGGTTTATGTGGGG	(GAA) ₇	2	0.45	0.47	0.35	JX105300
MrEST-SSR-63	F:TGTCTGTGGGTGGAGGTTA R:TTCCACCAGATGATACGATGC	(AAG) ₁₀	5	0.57	0.51	0.51	JX105304
MrEST-SSR-64	F:TTTCATTGAAGCCCACCTCC R:AAGTAATGGACGCTCCCTCT	(CAC) ₈	2	0.26	0.41	0.34	JX105305
MrEST-SSR-65	F:GTTTCAGAGTCCGAATCATCCT R:CGCCACCACATCCTTCAAATA	(GAA) ₈	4	0.60	0.63	0.54	JX105306
MrEST-SSR-66	F:TAGGGTTGGTGATTGTGCGTG R:TCTTGGGGCTGACTTTGATGC	(AGA) ₈	4	0.37	0.39	0.35	JX105307
MrEST-SSR-68	F:GAAAAATCAACGGGGAGAAGG R:TATTGGACACAACGAAGCAGC	(AAG) ₈	3	0.52	0.44	0.44	JX105309
MrEST-SSR-69	F:CCATCCAAAACAAAACCCAA R:CTCACCGTCTATCCTGCCAAA	(CAC) ₈	4	0.63	0.53	0.56	JX105310
MrEST-SSR-70	F:GCCTTTTGTTCCTTTTCTA R:TCTTCCATTACGGTTCCTAGC	(GAC) ₉	7	0.70	0.70	0.64	JX105311
MrEST-SSR-84	F:CAATCCGCATCGGACGCCTACC R:TTACAGACATTTCCACAAACAAC	(AT) ₆ (GT) ₈	8	0.76	0.65	0.73	JX105325
MrEST-SSR-92	F:TATGGAGTTAAGGAAGAGGGGG R:GATGTTTACATGGAAGACGTG	(TC) ₉ TG(TC) ₁₀	6	0.72	0.77	0.68	JX105333
MrEST-SSR-98	F:CCAATCCACCTCTTAAAGCC R:TGGTAAGTATCAACCCAAGC	(CGT) ₈ N ₁₃ (CTT) ₄	5	0.57	0.52	0.49	JX105339
MrEST-SSR-102	F:GAAACGGTATCGGTCGGAGTCT R:GAGGTGGAGGTGGTGGTTGTAG	(CCT) ₇ CCG(CCT) ₄	4	0.54	0.50	0.45	JX105343
MrEST-SSR-105	F:AGACGATGAGAGGTCTGGGGCC R:AAACTGCTGTGGTAATGGGAA	(CAG) ₇ N ₉ (CAA) ₆	6	0.71	0.56	0.65	JX105346
MrEST-SSR-110	F:AGAGACCTTGTATTGAGTTTCG R:TCGTCTCCTTCCATAACCAC	(AAG) ₅	4	0.64	0.47	0.57	KU253590
MrEST-SSR-111	F:GGGAAGAAGAAAAACAAAAGAAG R:GGAGACCAGGACGAGAACCAGAAG	(AAG) ₅	6	0.76	0.70	0.72	KU253591
MrEST-SSR-112	F:AACAGCGACAACACCATTTCC R:CCTTACTAGTATTGCCATTGCTG	(TTC) ₈	7	0.69	0.67	0.64	KU253592
MrEST-SSR-113	F:GTTTCAGAGAGCTCGGTCGTC R:GTATCTCCCGCTCCGACTTG	(GA) ₁₀	6	0.71	0.69	0.66	KU253593
MrEST-SSR-114	F:TTGTAACGTAACCCCTCCCTC R:GATCTCTGCCGCCGTTTGG	(AG) ₇	5	0.71	0.63	0.66	KU253594
MrEST-SSR-115	F:GTCTCGCTTCTCACTCTGC R:GGAAAGCTTTGGTGAGGGA	(AAG) ₁₀	5	0.40	0.49	0.37	KU253595
MrEST-SSR-116	F:ACCCTGTTTCTCTGCTTTC R:GGCCTCCACCCAATTTCCC	(CTT) ₈	4	0.67	0.60	0.60	KU253596
MrEST-SSR-117	F:ACGAGGTCTAAAATCCTGCACT R:CCAACCCCTGTCTCTGAACC	(GAA) ₈	4	0.57	0.58	0.48	KU253597
MrEST-SSR-118	F:CCATGGAGATCAACAATGGTGT R:CCGAGAGCTTGCGAGTTGAT	(GAG) ₁₀	5	0.68	0.70	0.61	KU253598
MrEST-SSR-119	F:TGTCCTCTAGCTCTTCCGCA R:GGCACTGATCAAGGTAAAGAAGC	(TTC) ₈	4	0.62	0.65	0.56	KU253599
MrEST-SSR-120	F:CTCAGATCCTTCCACGTTCCC R:CACTGGATCAGGACACCCTC	(AAG) ₈	4	0.47	0.46	0.42	KU253600
MrEST-SSR-121	F:AGATGCAGTCTTGACGCCTT R:GGGTCTGTCTTCCATTACGGT	(CGA) ₈	6	0.68	0.70	0.62	KU253601
MrEST-SSR-122	F:GAGAAGCTACCGGATCTCGC R:CACTGACAAAAACGCACCCA	(GGA) ₇	7	0.55	0.53	0.48	KU253602
MrEST-SSR-123	F:TGACTGAATTGGGTGATGCTC R:ATCTGGGCCTCCTTCAAACC	(GAA) ₉	5	0.52	0.44	0.48	KU253603

(Continued on next page)

Table 2. (Continued) Characterization of the 38 microsatellite loci for Chinese bayberry, including primer sequences, repeat motifs, number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphism information content (PIC), and GenBank accession numbers.

Primer	Primer sequence(5'-3')	Repeat motif	N_a	H_o	H_e	PIC	GenBank
MrEST-SSR-124	F:GTCCAAGACCTGTCCTAGCG R:AATCTCACATGCTCCTCGCA	(CTT) ₈	5	0.34	0.42	0.31	KU253604
MrEST-SSR-125	F:GCCATCACAACTGTCAAATCCT R:TGTCTGTGGGTTGGAGGTTA	(CTT) ₁₀	5	0.57	0.49	0.52	KU253605
MrEST-SSR-126	F:GGGAAATCTTTCTGACCGCA R:GAGATGACACTACTGATTCTGGA	(GAA) ₈	3	0.46	0.67	0.57	KU253606

Fig. 1. Dendrogram for 42 Chinese bayberry accessions derived from unweighted pair group method using arithmetic mean cluster analysis based on 38 highly polymorphic EST-SSR markers. The symbols before the accession codes indicates the location from where the accession originated, with ▼, ■, ★, ●, §, and ▲ standing for Anhui, Fujian, Guangdong, Jiangsu, Yunnan, and Zhejiang, respectively. The accessions labeled with ◇ indicated white-fruited accessions, with ripe fruit containing little or no anthocyanins.

cultivars or elite lines, especially those collected from three traditional production provinces, Zhejiang, Fujian, and Jiangsu (for map, refer to Fig. 1 of Zhang et al., 2009). Previously, the accessions from Zhejiang and Jiangsu have been relatively more intensively studied but systematic research on Fujian accessions has lagged behind (Lin et al., 2013).

Subgroup A included six white-fruited accessions collected from a mountain in Yuyao, Zhejiang Province. They are close to each other, indicating they originate from the same gene pool, possibly through natural seed distribution, but they also show differences, precluding the possibility of synonyms, i.e., the same cultivar with different names.

Subgroup B contained only two accessions, ‘Wusu’ from Guangdong and ‘Baxiandao’ from Fujian.

Six accessions, five from Zhejiang and ‘Langdangzi’ from Jiangsu, were included in subgroup C. Two out of four of the main cultivars with largest production, Biqi and Wandao are genetically close to each other, which are consistent with the report by Jiao et al. (2012) and Qian et al. (2006). Biqi and Wandao are close geographically, originating from Cixi, Zhejiang and Zhoushan, Zhejiang, which are less than 100 km apart, and are similar morphologically, with average fruit weight around 11 g and black in color for both cultivars.

It is interesting that Dingao from Zhejiang, one of the four main commercial cultivars,

clustered together with 13 accessions from Jiangsu in subgroup D. Previously, ‘Dingao’ was reported to be clustered with ‘JS2011-16’, which is derived from Suzhou, Jiangsu Province (Jiao et al., 2012). The relationship between ‘Dingao’ and accessions from Jiangsu needs to be further investigated.

Subgroup E included 10 accessions, eight of them were from Fujian. ‘Taohong’ from Jiangsu and ‘Anhuibaimei’ from Anhui were also in this group.

Subgroup F contained five accessions, including Yongquanzaoshuimei, Songshanshuimei, and Shuangqiaobaimei, three main cultivars in Linhai, Zhejiang. These three cultivars were clustered tightly together with Dongkui, which originated from Huangyan, Zhejiang located within 50 km from Linhai. A Fujian accession, ‘Taipingyangwuhao’, also appeared in this subgroup.

As expected, the outgroup *M. nana* has a high genetic distance to *M. rubra* accessions.

Four main cultivars, according to annual production, Dongkui, Biqi, Dingao, and Wandao were included in this study. It was found that they were distributed in three subgroups (subgroup C, D, and F), suggesting the preservation of broad diversity. Further extension of elite cultivars from subgroups B and E, where most cultivars from Fujian clustered, could strengthen this diversity.

Twelve white-fruited accessions, with no or little anthocyanin accumulation in ripe fruit, appeared in five subgroups, suggesting the preservation of broad diversity in white-fruited accessions as well. Obviously, the clustering of accessions is not correlated with color, which was also observed in our previous study by Zhang et al. (2009) using AFLP markers.

Also, similar to that observed previously (Zhang et al., 2009), accessions from the same geographical region were often, but not always, more closely related than those from different regions. The clustering of cultivars from different regions suggested the existence of extensive gene flows among accessions.

In conclusion, a total of 38 high-quality and highly polymorphic EST-SSR markers were developed and used to analyze the genetic diversity of 42 Chinese bayberry accessions. All accessions were distinguished and no synonyms were found. The accessions clustered in two groups with six subgroups. Broad diversity was observed in the four main producing cultivars, and among 12 white-fruited accessions. These EST-SSR markers and the findings obtained in this study can assist the discrimination of cultivars or lines as well as contribute to genetic or breeding studies in Chinese bayberry.

Literature Cited

- Chen, F.Y. and J.H. Liu. 2014. Germplasm genetic diversity of *Myrica rubra* in Zhejiang province studied using inter-primer binding site and start codon-targeted polymorphism markers. *Sci. Hort.* 170:169–175.
- Chen, K.S., C.J. Xu, B. Zhang, and I.B. Ferguson. 2004. Red bayberry: Botany and horticulture. *Hort Rev.* 30:83–114.
- Feng, C., M. Chen, C.J. Xu, L. Bai, X.R. Yin, X. Li, A.C. Allan, I.B. Ferguson, and K.S. Chen. 2012. Transcriptomic analysis of Chinese bayberry (*Myrica rubra*) fruit development and ripening using RNA-Seq. *BMC Genomics* 13:19.
- Guichoux, E., L. Lagache, S. Wagner, P. Chaumeil, P. Léger, O. Lepais, C. Lepoittevin, T. Malausa, E. Revardel, F. Salin, and R.J. Petit. 2011. Current trends in microsatellite genotyping. *Mol. Ecol. Resour.* 11:591–611.
- Heidi, M.M. and C.C. Andrew. 2007. Almost forgotten or latest practice? AFLP applications, analyses and advances. *Trends Plant Sci.* 12: 106–117.
- Hite, J.M., K.A. Eckert, and K.C. Cheng. 1996. Factors affecting fidelity of DNA synthesis during PCR amplification of d(C-A)n.d(G-T)n microsatellite repeats. *Nucleic Acids Res.* 24:2429–2434.
- Jia, H.M., Y. Jiao, G.Y. Wang, Y.H. Li, H.J. Jia, H.X. Wu, C.Y. Chai, X. Dong, Y. Guo, L. Zhang, Q.K. Gao, W. Chen, L.J. Song, E. Weg, and Z.S. Gao. 2015. Genetic diversity of male and female Chinese bayberry (*Myrica rubra*) populations and identification of sex-associated markers. *BMC Genomics* 16:394.
- Jia, H.M., Y.T. Shen, Y. Jiao, G.Y. Wang, X. Dong, H.J. Jia, F. Du, S.M. Liang, C.C. Zhou, W.H. Mao, and Z.S. Gao. 2014. Development of 107 SSR markers from whole genome shotgun sequences of Chinese bayberry (*Myrica rubra*) and their application in seedling identification. *J. Zhejiang Univ. Sci. B* 15:997–1005.
- Jiao, Y., H.M. Jia, X.W. Li, M.L. Chai, H.J. Jia, Z. Chen, G.Y. Wang, C.Y. Chai, E. Weg, and Z.S. Gao. 2012. Development of simple sequence repeat (SSR) markers from a genome survey of Chinese bayberry (*Myrica rubra*). *BMC Genomics* 13:201.
- Lin, B.N., L.J. Xu, and C.L. Jia. 1999. Studies on identification and classification of genomic DNA in *Myrica* by RAPD analysis (in Chinese with English abstract). *Acta Hort. Sin.* 26:221–226.
- Lin, Q.H., Q.Z. Zhong, and Z.H. Zhang. 2013. Genetic diversity analysis of 18 Chinese bayberry germplasm resources with SRAP (in Chinese with English abstract). *Chinese J. Trop. Crops.* 34:1667–1671.
- Liu, X.F., C. Feng, M.M. Zhang, X.R. Yin, C.J. Xu, and K.S. Chen. 2013. The *MrWD40-1* gene of Chinese bayberry (*Myrica rubra*) interacts with MYB and bHLH to enhance anthocyanin accumulation. *Plant Mol. Biol. Rpt.* 31:1474–1484.
- Liu, K. and S.V. Muse. 2005. PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics* 21:2128–2129.
- Nei, M. 1973. Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci. USA* 70:3321–3323.
- Pan, H., X.H. He, Y.W. Li, Y.Z. Guo, and G.X. Huang. 2008. Genetic diversity of wild *Myrica* resources in Guangxi analyzed by inter-simple sequence repeats (ISSRs) (in Chinese with English abstract). *J. Fruit Sci.* 25:353–357.
- Qian, J.L., W.S. Yu, H.K. Wang, X.M. Lou, and Z. Zhang. 2006. Analysis on ISSR marker of primary cultivars of bayberry in Jiangsu and Zhejiang (in Chinese with English abstract). *J. Plant Resour. Environ.* 15:17–20.
- Qiu, Y.X., C.X. Fu, and H.H. Kong. 2002. Inter-simple sequence repeat (ISSR) analysis of different cultivars in *Myrica rubra* (in Chinese with English abstract). *J. Agr. Biotech. Sin.* 10:343–346.
- Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nat. Biotechnol.* 18:233–234.
- Sun, C.D., H.Z. Huang, C.J. Xu, X. Li, and K.S. Chen. 2013. Biological activities of extracts from Chinese bayberry (*Myrica rubra* Sieb. et Zucc.): a review. *Plant Foods Hum. Nutr.* 68:97–106.
- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28:2731–2739.
- Terakawa, M., S. Kikuchi, S. Kanetani, K. Matsui, T. Yumoto, and H. Yoshimaru. 2006. Characterization of 13 polymorphic microsatellite loci for an evergreen tree, *Myrica rubra*. *Mol. Ecol. Notes* 6:709–711.
- Xie, X.B., Y.Y. Qiu, L.P. Ke, X.L. Zheng, G.T. Wu, J.Q. Chen, X.J. Qi, and S. Ahn. 2011b. Microsatellite primers in red bayberry (*Myrica rubra*). *Amer. J. Bot.* 98 e93–e95.
- Xie, X.B., Y.Y. Qiu, X.J. Qi, X.L. Zheng, L.J. Qiu, and Y.J. Zhang. 2008. Analysis of genetic relationship between male and female plants in *Myrica rubra* by RAPD and ISSR (in Chinese with English abstract). *J. Fruit Sci.* 25:198–202.
- Xie, R.J., J. Zhou, G.Y. Wang, S.M. Zhang, L. Chen, and Z.S. Gao. 2011a. Cultivar identification and genetic diversity of Chinese bayberry (*Myrica rubra*) accessions based on fluorescent SSR markers. *Plant Mol. Biol. Rpt.* 29:554–562.
- Zhang, W.S., K.S. Chen, B. Zhang, C.D. Sun, C. Cai, C.H. Zhou, W.P. Xu, W.Q. Zhang, and I.B. Ferguson. 2005. Postharvest responses of Chinese bayberry fruit. *Postharvest Biol. Technol.* 37:241–251.
- Zhang, S.Y., C. Feng, C.J. Xu, C.Q. Zhu, and K.S. Chen. 2015b. Polymorphisms in different EST-SSR types derived from the Chinese bayberry (*Myrica rubra*, Myricaceae) transcriptome. *Genet. Mol. Res.* 14:6037–6041.
- Zhang, S.M., Z.S. Gao, C.J. Xu, and K.S. Chen. 2009a. Genetic diversity of Chinese bayberry (*Myrica rubra* Sieb. & Zucc.) accessions revealed by amplified fragment length polymorphism. *HortScience* 44:487–491.
- Zhang, X.N., H.Z. Huang, Q.L. Zhang, F.J. Fan, C.J. Xu, C.D. Sun, X. Li, and K.S. Chen. 2015a. Phytochemical characterization of Chinese bayberry (*Myrica rubra* Sieb. et Zucc.) of 17 cultivars and their antioxidant properties. *Intl. J. Mol. Sci.* 16:12467–12481.
- Zhang, S.Y., X. Li, C. Feng, C.Q. Zhu, D. Grierson, C.J. Xu, and K. S. Chen. 2012. Development and characterization of 109 polymorphic EST-SSRs derived from the Chinese bayberry (*Myrica rubra*, Myricaceae) transcriptome. *Amer. J. Bot.* 99:e501–e507.
- Zhang, Y.J. and S.L. Miao. 1999. Resources and utilization of Chinese bayberry varieties (in Chinese with English abstract). *South China Fruit.* 28:24–25.
- Zhang, S.M., C.J. Xu, Z.S. Gao, K.S. Chen, and G.Y. Wang. 2009b. Development and characterization of microsatellite markers for Chinese bayberry (*Myrica rubra* Sieb. & Zucc.). *Conserv. Genet.* 10:1605–1607.
- Zheng, J.T., W.S. Zhang, L. Bao, X.X. Wang, Y.W. Zhu, Q.S. Fu, C.J. Xu, and K.S. Chen. 2006. AFLP identification and characterization of Yongxuan 56, a new line of Biqi Chinese bayberry (in Chinese with English abstract). *J. Fruit Sci.* 23:397–400.