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Development and characterization of SSR markers in Chinese jujube (*Ziziphus jujuba* Mill.) and its related species

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ABSTRACT

In order to study the extensively genetic diversities of more than 700 cultivars of Chinese jujube, it is necessary to utilize various informative DNA markers. SSR markers are highly polymorphic, co-dominant, locus-specific markers widely used in genetic studies, but less used in Chinese jujube because of no specific primers available. In this study, we used the approach of selectively amplified microsatellite (SAM) to develop SSR markers for Chinese jujube and its related species. Three cultivars (Dongzao, Dalilongzao and Jinsixiaozao) were selected to perform the approach of SAM with CT repeats. There were totally 25 primers obtained, of which we selected 16 primers available to detect the polymorphism in populations of 24 Chinese jujube cultivars, two wild jujube varieties and two Indian jujube cultivars. Based on these primers, genetic relationships of the 28 samples were constructed in a dendrogram according to the UPGMA cluster analysis. The samples were clustered into three main groups, including Chinese jujube, wild jujube and Indian jujube as expected. The 16 sequence-specific SSR primers could efficiently distinguish all the 24 cultivars of Chinese jujube, except for two cultivars, Jinsixiaozao and its 'stoneless' mutant, Wuhexiaozao. As a result, SAM was a very efficient method in targeted developing sequence-specific SSR primers in Chinese jujube. Furthermore, SAMs could also be used as high polymorphic molecular markers independently. The further study would focus on developing other oligonucleotide repeat types and applying more SSRs available in the genetic research of Chinese jujube.

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1. Introduction

Chinese jujube, belonging to the genus of Rhamnaceae family, is a specific species native to China with thousand-year history of cultivation, and famous for its nutrient-enriched fruits. According to China Fruit's Monograph – Chinese Jujube Volume, there are more than 700 cultivars of Chinese jujube in China (Qu and Wang, 1991). These different cultivars and varieties contain large variation in genetic traits, such as fruit shape, flavor, color, botany traits and propagation ability. Methods such as morphology, isoenzymes, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and sequence-related amplified polymorphism (SRAP) markers were previously used to study on the origination, taxology and genetic diversity of Chinese jujube (Liu, 1999; Peng et al., 2000; Lu et al., 2005; Li et al., 2010). But there are some deficiencies in these methods. The methods of morphology and isoenzyme are inefficient to distinguish cultivars, and the results are often liable to be influenced by the environment. Molecular markers such as RAPD, AFLP and SRAP are more powerful to distinguish cultivars, but these tools often fail to reach clear conclusions in the identification of duplicates or mislabeled genotypes and less amplified regions (Zhang et al., 2009; Li and Quiros, 2001). So the study in population genetics of Chinese jujube should be carried out with more feasible methods.

Microsatellites or simple sequence repeats (SSRs) are tandem repeats of short DNA sequences extensively distributed throughout the whole genome in both coding and non-coding regions. The distinguishing features of SSR loci are characterized with high information content, co-dominant inheritance pattern, reproducibility and locus specificity (Kashi et al., 1997; Röder et al., 1998a,b). Though this technology is efficient, the SSR primers are genus/species-specific, so the application of SSR markers should be developed with specific primers in different species. Because of this reason, there were still no any reports about SSR markers used in Chinese jujube.

In the previous study, based on the isolating and sequencing clones containing putative SSR regions, together with designing and testing flanking primers, genomic SSRs were developed (Elaine et al., 1992; Luchezar et al., 1993; Jon et al., 1993; Huang et al., 1999; Collevatti et al., 1999), but their development is typically costly, time consuming, and labor intensive. Several techniques that generate multi-locus SSR fingerprints are able to utilize SSRs as molecular markers without the expense of single locus isolation. The approach of SAM (selectively amplified microsatellite)

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Table 1
Cultivars used in the development and polymorphic analysis of SSR primers.

Code	Cultivar/variety	Latin name
1	Dongzao	*Ziziphus jujuba* Mill. 'Dongzao'
2	Gagazao	*Ziziphus jujuba* Mill. 'Gagazao'
3	Hamazao	*Ziziphus jujuba* Mill. 'Hamazao'
4	Zaocuiwang	*Ziziphus jujuba* Mill. 'Zaocuiwang'
5	Jiuyueqing	*Ziziphus jujuba* Mill. 'Jiuyueqing'
6	Xiangfenmuzao	*Ziziphus jujuba* Mill. 'Xiangfenmuzao'
7	Zaoqiangtangzao	*Ziziphus jujuba* Mill. 'Zaoqiangtangzao'
8	Chuanganxiaozao	*Ziziphus jujuba* Mill. 'Chuanganxiaozao'
9	Heyangjidanzao	*Ziziphus jujuba* Mill. 'Heyangjidanzao'
10	Cangxianshazao	*Ziziphus jujuba* Mill. 'Cangxianshazao'
11	Hetaowen	*Ziziphus jujuba* Mill. 'Hetaowen'
12	Xinzhengmayazao	*Ziziphus jujuba* Mill. 'Xinzhengmayazao'
13	Dalilongzao	*Ziziphus jujuba* Mill. 'Dalilongzao'
14	Tailihong	*Ziziphus jujuba* Mill. 'Tailihong'
15	Chahuzao	*Ziziphus jujuba* Mill. 'Chahuzao'
16	Lajiaozao	*Ziziphus jujuba* Mill. 'Lajiaozao'
17	Mantouzao	*Ziziphus jujuba* Mill. 'Mantouzao'
18	Fengmiguan	*Ziziphus jujuba* Mill. 'Fengmiguan'
19	Qingxianjianzao	*Ziziphus jujuba* Mill. 'Qingxianjianzao'
20	Wuhexiaozao	*Ziziphus jujuba* Mill. 'Wuhexiaozao'
21	Jinsixiaozao	*Ziziphus jujuba* Mill. 'Jinsixiaozao'
22	Longzao	*Ziziphus jujuba* Mill. 'Longzao'
23	Hupingzao	*Ziziphus jujuba* Mill. 'Hupingzao'
24	Linyilizao	*Ziziphus jujuba* Mill. 'Linyilizao'
25	Gaolang No. 1	*Ziziphus mauritiana* Lam. 'Gaolang No. 1'
26	Cuimi	*Ziziphus mauritiana* Lam. 'Cuimi'
27	Wild jujube 1	*Ziziphus spinosa* Hu. 'variety 1'
28	Wild jujube 2	*Ziziphus spinosa* Hu. 'variety 2'

is a new method to develop SSR markers created by Hayden and Sharp (2001) that combines SAMPL (selective amplification of microsatellite polymorphism loci) technique with 5′-anchored PCR (Witsenboer et al., 1997; Fisher et al., 1996). This approach provides a source of SAMs which can be converted to locus-specific SSR markers with the design and synthesis of a single primer specific to the conserved region flanking the repeated sequences. With the advantages of high polymorphism of alleles, high recovery of useful SSR and cost-efficiency, SAM technique was used to develop SSR markers for genetic study, such as in bread wheat, litchi, and sweet cherry (Gupta and Varshney, 2000; Li, 2003; Ai et al., 2007). Moreover, SAM technique can also be used as polymorphic molecular markers independently for population and genetic mapping studies. In this study, SAM technique was used to targetedly develop the sequence-specific SSR primers for Chinese jujube, and the primers were investigated among different Chinese jujube cultivars to provide an effective detecting method for the genetic analysis in the population of Chinese jujube and its related species.

2. Materials and methods

2.1. Plant materials and DNA isolation

Three cultivars of Chinese jujube (Dongzao, Dalilongzao and Jinsixiaozao) were selected to develop the sequence-specific SSR primers with the approach of SAM. A total of 24 familiar cultivars of Chinese jujube (*Ziziphus jujuba* Mill.), two wild jujube varieties (*Ziziphus spinosa* Hu.) and two Indian jujube cultivars (*Ziziphus mauritiana* Lam.) shown in Table 1 were selected to analyze the applicability and polymorphism of the SSR primers. 24 cultivars of Chinese jujube were collected in the germplasm orchard of Cangxian county, Hebei province of China. Two wild jujube varieties (1 and 2) different from each other in the shape of the branches and leaves were collected in the arboretum of Chinese Academy of Forestry and two Indian jujube cultivars (Gaolang No. 1 and Cuimi) were collected in Nankou farm of Beijing. All samples were healthy young leaves and were collected in June 2008. The plant materials were treated with liquid nitrogen and stored at −80 °C until being used. Genomic DNA was extracted using cetyltrimethyl ammonium bromide (CTAB) method and detected on 0.8% agarose gel by electrophoresis.

2.2. Preparation of template DNA

500 ng of genomic DNA isolated from Dongzao, Dalilongzao and Jinsixiaozao was digested for 3 h at 37 °C with 10 U of *Mse*I and 20 U of *Pst*I in 50 μl of 1× NEB (New England Biotechnologies) buffer II and 100 ng/μl BSA. The following steps were performed according to the method described by Hayden and Sharp (2001): 5 and 50 pmol *Pst*I and *Mse*I adapters were added, respectively, and the mixture was heated at 45 °C for 5 min to melt any cohesive adapter termini that had annealed. A solution containing 1× NEB buffer II, 100 ng/μl BSA, 6 mM dATP and 1 U T4 ligase (NEB) was added to give a total volume of 60 μl and incubated at room temperature for 16 h. The adapters were prepared by incubating equimolar amounts of two synthetic oligomers in annealing buffer (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM EDTA) at 65 °C for 10 min, followed by slow cooling to room temperature over 2 h.

Amplification of the *Pst*I–*Mse*I restriction fragments was performed in a 20 μl reaction mixture containing 0.2 mM dNTP, 1× PCR buffer (TaKaRa Biotechnology Dalian Co., Ltd.), 5 pmol each *Mse*I and *Pst*I suppressor primer, 2 μl digested/ligated template DNA and 1 U *Ex Taq* polymerase (TaKaRa Biotechnology Dalian Co., Ltd.). PCR amplification was performed for 20 cycles (60 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C) in a 96-well thermocycler (ABI 9700, US). The reaction mixture was then diluted 1:20 with TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). Preamplification was performed in a 20 μl reaction mixture containing 0.2 mM dNTP, 1× PCR buffer, 5 pmol each *Mse*I and *Pst*I adapter primer with selective nucleotides (one for *Mse*I adapter primer and two *Pst*I adapter primer), 2 μl of the diluted pool of amplified *Pst*I–*Mse*I fragments and 1 U *Ex Taq* polymerase. PCR was performed as described above and the reaction products were diluted 1:20 with TE.

SAM amplification was performed in a similar 20 μl reaction mix, with 5 pmol *Mse*I adapter primer + NN, 20 pmol SSR primer and 2 μl of diluted preamplification products as template DNA. The 5′-anchored SSR primer was designed to faithfully anchor to the 5′-end of microsatellite repeats, and consisted of a mixture of two synthetic oligomers. PCR was performed for 37 cycles with the profile 60 s at 94 °C, 60 s annealing (see below) and 30 s at 72 °C. The annealing temperature for the first cycle was 65 °C, reducing by 1 °C/cycle for the next seven cycles. The next five cycles were with annealing at 59 °C. Following the twelfth cycle, the denaturation and annealing steps were reduced to 30 s and the remaining 25 cycles were continued using annealing at 57 °C. Sequences of the adapters, suppressors and 5′-anchored SSR primers (Beijing AuGCT Biotechnology Co., Ltd.) in the SAM experiment are shown in Table 2.

After the three steps of amplification, samples were mixed with a half volume of gel loading buffer (98% formamide, 10 mM EDTA, 0.25% xylene cyanol as tracking dye), heated for 10 min at 94 °C, chilled quickly on ice and run on 6.0% denaturing polyacrylamide gels under standard conditions. The gels were visualized with silver staining.

2.3. Sequencing of SAMs and designing of primers

Selected SAM-amplification products with sharp and legible bands of the three cultivars were excised from dried polyacrylamide gels, re-amplified and sequenced after cloning into vector pGM-T (TIANGEN Biotech Beijing Co., Ltd.). The plasmids were sequenced (Beijing AuGCT Biotechnology Co., Ltd.) and the sequence-specific primers were designed with Oligo software (Version 6).

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