Assessment of the genetic diversity and genetic relationships of pomegranate (*Punica granatum* L.) in China using RAMP markers

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ABSTRACT

In this study, random amplified microsatellite polymorphism (RAMP) technique was used to examine the genetic relationships among 46 pomegranate genotypes, which were collected from 7 provinces in China. The results showed that a total of 127 bands were amplified by 14 pairs of high polymorphism primers, among which 113 (88.90%) were polymorphic bands, and that the effective number of alleles (N_e), Nei's gene diversity (H), and Shannon's information index (I) for pomegranate were 1.3345, 0.2126 and 0.2126, respectively. These results showed that there is the significant genetic diversity among pomegranate germplasm in China. When the genetic similarity coefficients (G_s) was 0.75, the 46 pomegranate cultivars were completely clustered into five groups by the unweighted pair group method with arithmetic mean (UPGMA). The cluster results indicated that the molecular classification was not consistent with the morphological/agronomical classification or geographic origin. The obtained genetic information assists toward the effective protection and sustainable utilization of pomegranate resources.

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1. Introduction

Pomegranate (*Punica granatum* L.) is a deciduous small shrub tree, which belongs to the Punicaceae plant family. This genus is composed of three species: *P. granatum, Punica nana*, and *Punica protopunica*, of which, *P. granatum* has $2n = 2x = 16$, 18 chromosomes, and has been cultivated from ancient times for its economic, ornamental and medicinal properties globally (Awamleh et al., 2009; Ebrahimi et al., 2010). The pomegranate is believed to have originated from Iran, and from there diversified to other regions such as Afghanistan, India, and other Mediterranean countries (Pirseyedi et al., 2010; Sarkhosh et al., 2006). It has been more than 2000 years since the pomegranate was introduced into China by Zhang Qian during the Western Han Dynasty. The long-term natural hybridization and gene mutation, as well as breeding strategies have led to various new complex varieties and types of pomegranates in China. A rough estimate of more than 230 pomegranate cultivars are found in 7 main production areas (Shandong, Shanxi, Anhui, Henan, Yunnan, Sichuan, and Xinjiang) in China (Yuan et al., 2007). At present, knowledge of pomegranate genetics is limited both in China and abroad due to a lack of research investment and proper tools. The presence of mixed varieties and other similar issues are serious causes of concern because of their effect on pomegranate production and domestic and international import/export. Development of highly reliable methods have become increasingly important to plant breeders.

DNA markers are independent from environmental interactions, unlimited in number and show a high level of polymorphism. Therefore, they are considered invaluable tools for determining genetic relationships, diversity, plant breeding, and genome mapping, as well as gene bank construction (Currò et al., 2010; Narzary et al., 2010). In recent years, RAPD (Sarkhosh et al., 2006; Noormohammadi et al., 2012), SRAP (Ranade et al., 2009; Soleimani et al., 2012), SSR (Hasnaoui et al., 2012; Soriano et al., 2011), ISSR (Zhao et al., 2011), AFLP (Ercisli et al., 2011; Maryam et al., 2010), and DAMD (Narzary et al., 2009) markers have been used to detect the genetic diversity/relationships of pomegranate germplasm by local and foreign researchers. Their results provide a molecular basis for the study of pomegranate germplasm resources. The reproducibility and frequency of polymorphic loci in the random amplified microsatellite polymorphism (RAMP) are vigorously enhanced by translocation of the 5′ anchor of repeating sequences to the 3′ end position and selective use of moderate arbitrary primers. RAMP is suitable for the genetic analysis of species whose genetic backgrounds are ambiguous (Min et al., 2008). RAMP function, as an effective genetic marker, has been widely used to analyze the genetic diversity and genetic relationships of different germplasms, such as wild barley (Davila et al., 1999), fig (Chatti et al., 2007), lactic acid bacteria (Chebeňová et al., 2010), and so on. However, there have been no reports on the use of RAMP to study the genetic diversity of pomegranates in China up to date. The objectives of the present study were to investigate the

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Table 1
Geographical origin, cultivar code, cultivar and main characteristics of Chinese the 46 pomegranate cultivars studied.

Geographical origin	Code	Cultivar[a]	Flower color	Flower form	Geographical origin	Code	Cultivar	Flower color	Flower form
	D1	Bingtangshiliu	Red	Monovalved	Yunnan	Y8	Yingpi	Red	Monovalved
	D2	Yihongyihao	Red	Monovalved		Y9	Huanghuashiliu	Yellow	Monovalved
	D3	Yiliu 88-1	Red	Monovalved		N1	Heyintongpi	Red	Monovalved
	D4	Mapicao	Red	Monovalved		N2	Tianhongmi	Red	Monovalved
Shandong	D5	Suoyidaqingpi	Red	Monovalved		N3	Heyinruanzi	Red	Monovalved
	D6	Lushiliu	Red	Monovalved	Henan	N4	Mudanhonghua	Red	Plena
	D7	Fenhongmudan	Pink	Plena		N5	Mudanhuanghua	Yellow	Plena
	D8	Taishanhong	Red	Monovalved		N6	Mudanbaihua	Red	Plena
	D9	Daqingpitian	Red	Monovalved		N7	Mudanhuashiliu	Red	Plena
	X1	Bairixue	White	Plena		N8	Suanhongpishiliu	Red	Monovalved
	X2	Moshiliu	Red	Monovalved		N9	Yueyuehong	Red	Monovalved
	X3	Zuimeiren	Red	Pavilion		H1	Dabenzi	Red	Monovalved
Shanxi	X4	Mozishiliu	Red	Monovalved		H2	Huohulu	Red	Monovalved
	X5	Jingpitian	Red	Monovalved		H3	Shuifenpi	Red	Monovalved
	X6	Tianhongtian	Red	Monovalved	Anhui	H4	Manaozi	Red	Monovalved
	X7	Yushiliu	Red	Monovalved		H5	Yushizi	Red	Monovalved
	Y1	Baihuashiliu	White	Monovalved		H6	Hongjumi	Red	Monovalved
	Y2	Hongpibaizi	Red	Monovalved		J1	Suanshiliu	Red	Monovalved
	Y3	Qingpibaizi	Red	Monovalved	Xinjiang	J2	Tianshiliu	Red	Monovalved
Yunnan	Y4	Lvpisuan	Red	Monovalved		J3	Hongpishiliu	Red	Monovalved
	Y5	Huopao	Red	Monovalved		C1	Shuijingshiliu	Red	Monovalved
	Y6	Nuoshiliu	Red	Monovalved	Sichuan	C2	Qingpiruanzi	Red	Monovalved
	Y7	Huahongpi	Red	Monovalved		C3	Huilihongpi	Red	Monovalved

[a] According to the names at the original growing places given to them.

genetic relationship of selected pomegranate cultivars in China as well as to estimate the genetic diversity using RAMP markers. The results obtained from this study can improve the conservation and management of pomegranate germplasm resources, and could be helpful in optimizing breeding programs.

2. Materials and methods

2.1. Plant materials

The 46 Chinese pomegranate cultivars used in this study (Table 1) were collected from the germplasm nursery Institute of Pomegranates the Mengzi County Yunnan Province, the germplasms nursery Institute of Pomegranates the Huili County Sichuan Province, and the germplasms nursery Institute of Pomegranates Shandong Province. Approximately 30–50 young leaves of each cultivar were sampled from around 3 to 5 adult trees that were apparently free of pests and diseases. All the samples were brought to the laboratory using ice, and washed with distilled water. The leaf samples were labeled, and stored at $-80\,^{\circ}$C until further use.

2.2. DNA extraction

Genomic DNA was extracted from 0.2 g of leaf tissue by the CTAB method of Yuan et al. (2007). The concentration and purity of DNA were assessed by 0.8% agarose gel electrophoresis and spectro-photometric analysis. The DNA extracts were diluted to a working concentration of 50 ng μl^{-1} and stored at $4\,^{\circ}$C.

2.3. Primer screening and RAMP-PCR amplification

Three pomegranate cultivars with different morphological traits were initially tested with a total of 100 primer combinations, and only those primers that produced polymorphic bands were selected. The selected primers were then used to amplify the genomic DNA of the 46 pomegranate cultivars. The annealing temperatures of the polymorphic primers which produced clear bands and low background were determined with a gradient PCR machine.

We used PCR conditions as described by Liu et al. (2007) with minor modifications. PCR amplification was performed with a 25 μl reaction volume, containing 1.5 mM of Mg^{2+}, 0.2 mM of each dNTP, 0.15 mM of each primer, 1 U of *Taq* polymerase, 25 ng of pomegranate DNA, and 2.5 μl of 10× buffer. The following thermal cycling protocol was used: an initial denaturation at $94\,^{\circ}$C for 4 min, followed by 45 cycles at $94\,^{\circ}$C for 45 s, 1 min at the respective optimal annealing temperature (Table 2), and $72\,^{\circ}$C for 2 min, and then a final extension at $72\,^{\circ}$C for 10 min.

2.4. RAMP data scoring and analysis

The RAMP-amplified products were separated by electrophoresis on 2% agarose gel (with 0.1‰ GV) for 1.5–2 h with 2 V cm^{-1}. The fragment patterns were photographed under UV light for further analysis. The 2 kb DNA marker (TaKaRa) was used as the molecular standard to confirm the presence of the DNA fragments.

The bands of DNA fragments on RAMP analysis were scored, using "1" to indicate the presence of a clear and repeatable band and "0" to indicate its absence. If the brightness of two bands had a difference of more than two times, they were recorded as different bands. A binary data matrix (1/0) was set up according to whether the bands existed or not. The PopGen32 software was used to compute for the percentage of polymorphic loci (P), the observed number of alleles (N_a), the effective number of alleles (N_e), the gene diversity (H), and the Shannon's information index (I). The Nei's genetic similarity coefficients (G_s) of the 46 pomegranate cultivars were calculated by NTSYS-pc (version 2.10) software. A genetic dendrogram was constructed for the 46 pomegranate cultivars based on the unweighted pair group method with arithmetic mean (UPGMA) using the SAHN clustering model, and its pattern robustness was tested using 1000 re-sampling permutations.

3. Results

3.1. Polymorphisms of amplification product

A total of 100 primer combinations were tested for RAMP amplification but only 14 pairs of primers (Table 2) produced unambiguous polymorphic bands and were used to amplify the DNA samples from 46 pomegranate cultivars. A total of