RESEARCH NOTE

Mining microsatellite markers from public expressed sequence tag sequences for genetic diversity analysis in pomegranate

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Introduction

A set of microsatellites (SSRs) markers were developed for pomegranate (*Punica granatum* L.) through bioinformatic mining of GenBank public database (http://www.ncbi.nlm.gov/genbank/). In total, 787 pomegranate expressed sequence tag (EST) sequences were downloaded from GenBank and assembled with CAP 3, producing 415 unigenes that contained 46 contigs and 369 singletons. Eighty bi-type to penta-type SSRs were obtained from these unigenes, among which 59 EST-SSR primer pairs were successfully designed. Twenty-eight primer pairs were selected as representative samples for evaluating usability and 18 (64.3%) were usable markers. Fifteen of the 18 markers revealed polymorphism among 42 pomegranate accessions, detecting 2–5 alleles (mean = 2.80). The observed heterozygosity (H_o) and polymorphic information content (PIC) values ranged from 0.119 to 0.619 (mean = 0.381) and from 0.091 to 0.656 (mean = 0.402), respectively. Genetic diversity of the accession set was elucidated using the newly-developed markers, revealing a distinctive genetic background of the accessions from east–central China. The EST-SSR markers developed in this study will provide more SSR markers available for genetic studies in pomegranate.

The genus *Punica* consists of two species, *P. granatum* and *P. protopunica*, of which *P. granatum*, pomegranate, is a predominant species and has been widely cultivated in various areas for thousands of years, while *P. protopunica* is only endemic to the Island of Socotra (Stover and Mercure 2007). Pomegranate is usually cultivated for its edible fruits and/or for decorative purposes. In the last few years, it has been found that pomegranate fruit acts as antioxidant, antidiabetic and hypolipidemic, and shows antibacterial, antiinflammatory, antiviral, and anticarcinogenic activities (Viuda-Martos *et al.* 2010). Therefore, recent years have seen an increased demand for pomegranate fruits on the part of consumers, researchers and food, and medical industry.

The pomegranate is native to Persia and has been cultivated and naturalized over the entire Mediterranean region since ancient times (Meerts *et al.* 2009). In West Han Dynasty, it was introduced to China by Zhang Qian, who was sent to the West regions as an envoy (Sun *et al.* 1983). Since then, a lot of variations have been occured naturally and by artificial selection in China, among which some have been used as elite genotypes (landraces) for cultivation in certain regions. A better understanding of genetic variation of pomegranate contributes to cultivar fingerprinting, germplasm conservation, and breeding of new cultivars. To date, several kinds of DNA marker systems have been employed for pomegranate diversity analysis, such as random amplified polymorphic DNA (RAPD) (Sarkhosh *et al.* 2006; Zamani *et al.* 2007), amplified fragment length polymorphism (AFLP) (Yuan *et al.* 2007; Jbir *et al.* 2008), and sequence-related amplified polymorphism (SRAP) (Ranade *et al.* 2009). These studies provide information for genetic studies of pomegranate. Microsatellites or simple sequence repeats (SSRs), generally considered as one of the most powerful Mendelian markers, have been widely used in germplasm identification, population genetics, and evolutionary studies in a variety of plant species. However, the number of available SSR markers in pomegranate is limited; only ~150 SSR primer sequences are published (Koohi-Dehkordi *et al.* 2007; Currò *et al.* 2010; Hasnaoui *et al.* 2010, 2012; Pirseyedi *et al.* 2010). These SSR markers are much less than what is demanded in genetic studies in pomegranate. In the present study, bioinformatic tools were used to explore new microsatellite loci from public EST database and to assess their potential for genetic diversity analysis in pomegranate.

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Keywords. expressed sequence tags; genetic diversity; microsatellites; polymorphism; pomegranate.

Materials and methods

Plant material and DNA extraction

To evaluate the usability of EST-SSR markers and to screen polymorphic loci, 42 pomegranate accessions were used in the present study (table 1). Eighteen were widely cultivated cultivars from China and abroad, and 24 landraces were from Shandong, Shaanxi, and Henan provinces in China with a limited cultivation area. All the materials were supplied by Department of Plant Genetics and Breeding, Henan Agricultural University, China and subjected to DNA extraction using a CTAB method described by Doyle and Doyle (1990).

Pomegranate EST data retrieval, assembly and SSR primer development

A total of 787 pomegranate ESTs were downloaded from GenBank on March 10, 2011 and then assembled using CAP 3 with the criteria set at 93% identity and 40-bp overlap (Huang and Madan 1999). This assembly generated 415 unigenes containing 46 contigs and 369 singletons. In general, a contig is a set of overlapping DNA segments derived from a single genetic source while a single DNA segment that dose not cluster with other segments is termed a singleton; the contigs plus singletons emerging from assembly process are collectively referred to as unigenes. Mining for SSR loci in the unigenes was conducted using the web tool, SSRIT (http://www.gramene.org/db/markers/ssrtool), with the criteria as follows: five repeats for dinucleotide and trinucleotide repeats, and four repeats for tetranucleotide and pentanucleotide repeats. Primer Premier program (Premier Biosoft International, Palo Alto, CA) was used to design primers flanking the putative SSRs. The input parameters for the program were: a length of 17–23 bp, a GC content of 40–60%, and an estimated amplicon size of 100–300 bp.

PCR amplification and gel electrophoresis analysis

PCR amplification was performed on a PTC-200 thermal cycler (MJ Research, Watertown, USA). The 15 μL reaction volume contained 1× PCR buffer, ~50 ng of sample DNA, 0.4 μM of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 0.5 unit of *Taq* DNA polymerase (Sangon, Shanghai, China). The reaction program was set as follows: 5 min at 94°C, followed by 34 cycles of 40 s at 94°C, 40 s at optimal annealing temperature (T_a) (determined by touch-down PCR with temperature set between 50°C and 60°C) and 1 min at 72°C, and then 8 min at 72°C for final extension. Amplified products were electrophoresed on 8% denaturing polyacrylamide gels (19 : 1, acrylamide : bis; 8 M urea) and the gels were silver-stained according to the protocol in the technical manual of silver sequence DNA staining reagents (Promega, Madison, USA). The size of amplicons for each microsatellite was estimated by reference to a DNA ladder (pUC19 DNA/*Msp*I marker, Sangon, Shanghai, China).

Assessment of EST-SSR polymorphism and cluster analysis

For SSR-PCR, amplified bands were visually scored as presence (1) or absence (0) in the form of binary matrix. The H_o

Table 1. A list of a set of pomegranate accessions containing 18 commercial cultivars (C) and 24 landraces (L).

Number	Accession name	C/L	District of collection	Number	Accession name	C/L	District of collection
1	Sanbai	C	G	22	LR4	L	SX
2	Linxuan 1	C	SX	23	LR5	L	SX
3	Linxuan 14	C	SX	24	LR6	L	SD
4	Qingpiruanzi	C	SX	25	LR7	L	SD
5	Dahongtian	C	SX	26	LR8	L	SD
6	Dahongsuan	C	SX	27	LR9	L	H
7	Yudazi	C	H	28	LR10	L	H
8	Nanyanghong	C	H	29	LR11	L	H
9	Heyinshiliu	C	H	30	LR12	L	H
10	Yushiliu	C	H	31	LR13	L	H
11	Kaifenglijihong	C	H	32	LR14	L	H
12	Hongjumi	C	SD	33	LR15	L	H
13	Baipisuan	C	SD	34	LR16	L	H
14	Taishanhong	C	SD	35	LR17	L	H
15	Xinjiangdazi	C	X	36	LR18	L	H
16	Valenciana	C	SP	37	LR19	L	H
17	Hicaznar	C	TK	38	LR20	L	H
18	Tunisiruanzi	C	TI	39	LR21	L	H
19	LR1	L	SD	40	LR22	L	H
20	LR2	L	SD	41	LR23	L	H
21	LR3	L	SD	42	LR24	L	H

Single-letter abbreviation for the district of collection of the accessions: G, Gansu (China); H, Henan (China); SX, Shaanxi (China); SD, Shandong (China); X, Xinjiang (China); SP, Spain, TK, Turkey; TI, Tunis.

and PIC were calculated for each SSR marker using PowerMarker v3.25 software (Liu and Muse 2005). Jaccard's coefficient (Jaccard 1908) was calculated based on the binary matrix using NTSYS-pc 2.10e software (Rohlf 2000). Cluster analysis was performed via construction of a dendrogram using the unweighted pair group method using arithmetic averages (UPGMA) and the SAHN module of NTSYS-pc 2.10e.

Results and discussion

Pomegranate EST-SSR distribution, frequency, and other features

Eighty SSR loci were identified from 415 unigenes. Sixty-six unigenes (15.9%) contained SSRs with repeat motifs ranging from dinucleotide to pentanucleotide, and the mean distance of the SSRs in the unigenes was ~11.4 kb. Of the EST-SSRs, the most common three repeat motifs were AG (32, 40.0%), AT (14, 17.5%), and AC (7, 6.25%); each of the other motifs were less than 5% in frequency. Although the number of ESTs used here is limited, the types and frequencies of the predominant repeat motifs are in line with previous reports (Kumpatla and Mukhopadhyay 2005). The abundance of AG motif in ESTs seems to be a common feature in the dicot species. The SSR-containing unigenes were BLAST-searched against GenBank, of which, 12 (18.2%) were known gene products, 8 (12.1%) were hypothetical proteins, and the remaining 56 (69.7%) were unknown genes.

Primer design was performed using 66 SSR-containing unigenes, of which 53 were allowed to design 59 primer pairs and the remaining 13 unigenes failed to design primer mainly due to short distance (less than 20 bp) flanking the SSRs.

Table 2. Characteristic of 18 pomegranate EST-SSR loci. Shown for each primer pair are the forward (F) and reverse (R) primer sequences, repeat motif, annealing temperature (T_a), expected product size and GenBank accession number, respectively.

Locus	Repeat motif	Primer sequence (5′–3′)	T_a(°C)	Expected product size (bp)	GenBank accession
EPS01	$(GGA)_7$	F: TCTATTCCACATAGAAAGAGGGG R: ATGATGTCTATGCAATTGGCTG	57	121	JG771258
EPS03	$(AT)_{12}$	F: CGCTGGTCACACTACTTACTCG R: TTGTAGTGGAAGACACAGCAGC	55	263	JG771964
EPS04	$(CT)_{10}$	F: AAAGGGGAAAAAGACGAAGAAG R: CCCTGTCCTTAAGTCTGAGTGG	55	257	JG771772
EPS05	$(CTT)_{11}$	F: TTGTTGGGTATTCCTCTTCTC R: ACATCATACACCTTGCCCTC	55	197	JG771683
EPS06	$(TC)_{13}$	F: AAATCGCATCCCTCCGTCT R: CTGTTCGCCAGGGTAAAGA″	56	147	JG771472
EPS07	$(AGA)_6$	F: CATGGGAGAACAGACAACTCAA R: AGCCAGTCTGTTAACTATCGCC	55	252	JG771464
EPS08	$(AG)_{18}$	F: TTCCCGAGAAAGTTGCATATCT R: TAGTCCGTGAGGATTTTGTCCT	56	204	JG771444
EPS09	$(AG)_{15}$	F: TAATCCCATTCCAAACAAGTCC R: ATATTGACGGAGGCTTCACTGT	57	199	JG771396
EPS10	$(GAT)_6$	F: TAGCACAGGGGAAATCTGAAAT R: GGAAGAGTTTGGTTCAGGATTG	56	130	JG771286
EPS11	$(AT)_9$	F: AACTTCTGGTGTCTCTTCCACC R: GTGTGGAGTTGAAGATCGATGA	58	147	JG771255
EPS12	$(CT)_9$	F: TCTCTCTCCCTCATTTCCTCTG R: GTCTCTACCATCTCAGCAGCCT	55	199	JG771218
EPS13	$(TA)_9$	F: GCTAGCGAATGAAATGTCTT R: GAGTATAGCAGTAGGGGAGATG	52	178	JG771230
EPS14	$(GTTG)_6$	F: GGGACACGCTGGAGTACAAT R: GACCCCTCTCTCTCCTGCTT	58	267	JG771277
EPS16	$(TGG)_8$	F: AGGCTTCATGACCCATCATC R: AGGAAGAGTTCGGGTTCGAT	57	191	JG771376
EPS17	$(TCC)_5$	F: TGTGGGTGTGGGAACATAATAA R: TCAACAGGACAAAGGATGAAGA	58	275	JG771421
EPS19	$(CT)_9$	F: TGGGGATTATCGTTGTCTTCA R: TCCAAGCTGAACTCGTTCCT	58	236	JG771896
EPS22	$(TA)_8$	F: ATATTTGCCTCAATGGCGTC R: AAACAGCTCCTCTTCCCCAT	57	300	JG771743
EPS24	$(GT)_7$	F: CAAACGCCTTCATGAAACTACA R: GACAAAAATTTCCAGCTCCATC	58	104	JG771352

Table 3. The results of primer screening for 42 pomegranate accessions.

Locus name	Cultivars (18 accessions)			Landraces (24 accessions)			Total (42 accessions)		
	N	H_o	PIC	*N*	H_o	PIC	*N*	H_o	PIC
EPS01	2	0.333	0.346	3	0.417	0.531	3	0.381	0.461
EPS03	1	0.000	0.000	2	0.250	0.278	2	0.143	0.172
EPS04	2	0.222	0.278	2	0.333	0.330	2	0.286	0.308
EPS05	2	0.167	0.198	2	0.291	0.219	2	0.238	0.210
EPS06	2	0.222	0.278	3	0.458	0.500	4	0.357	0.432
EPS07	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
EPS08	3	0.500	0.494	4	0.708	0.698	5	0.619	0.656
EPS09	3	0.389	0.438	3	0.542	0.531	4	0.476	0.509
EPS10	3	0.278	0.549	2	0.417	0.375	3	0.357	0.461
EPS11	1	0.000	0.000	2	0.208	0.153	2	0.119	0.091
EPS12	2	0.333	0.401	3	0.375	0.448	3	0.357	0.431
EPS13	2	0.556	0.346	2	0.541	0.278	2	0.548	0.301
EPS14	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
EPS16	2	0.611	0.494	2	0.500	0.413	2	0.548	0.459
EPS17	2	0.278	0.370	3	0.625	0.656	3	0.476	0.559
EPS19	3	0.389	0.537	3	0.583	0.622	3	0.500	0.594
EPS22	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
EPS24	2	0.222	0.278	2	0.375	0.444	2	0.310	0.387
Mean	1.94	0.250	0.278	2.28	0.368	0.360	2.5	0.318	0.335

N, number of alleles; H_o, observed heterozygosity; PIC, polymorphic information content values are given for each locus. The locus names are same as given in table 2.

High success rate of primer construction (80.3%), which was higher than those reported by Varshney *et al.* (2002) in cereal species, was obtained here, probably resulting from high-quality sequences (unigenes) used for primer construction.

EST-SSR assays and their informativeness

Twenty-eight primer pairs representing a variety of motifs (four dinucleotide, six trinucleotide, two tetranucleotide, and one pentanucleotide repeats) were randomly selected for genotyping pomegranate accessions. Of the 28 primer pairs, 21 pairs amplified distinct bands among the 42 pomegranate accessions. The remaining 7 pairs had no PCR product. Eighteen of the 21 primer pairs amplified fragments around their expected band sizes, whereas the remaining three generated fragments 150-200 bp larger than expected. In a separate experiment, we sequenced these three amplified fragments and found that three introns not too long (157 bp, 166 bp, and 186 bp in length) existed in the regions amplified by the three primer pairs (detailed data not shown). Information of the three primer pairs is not included in our results because it is difficult to detect SSR variation by using conventional electrophoresis techniques. The 18 well-developed EST-SSR primer pairs are described in table 2.

Among the 42 pomegranate accessions, 15 EST-SSR loci revealed allelic variation and three loci (ESP07, ESP14 and ESP22) were monomorphic (table 3). The 15 polymorphic markers detected 2–5 alleles with a total of 42 alleles and an average of 2.80 alleles per marker. A typical EST-SSR profile obtained using primer pair ESP08 is shown in figure 1. To assess the usefulness of the newly-developed markers, H_o and PIC were calculated for each polymor-

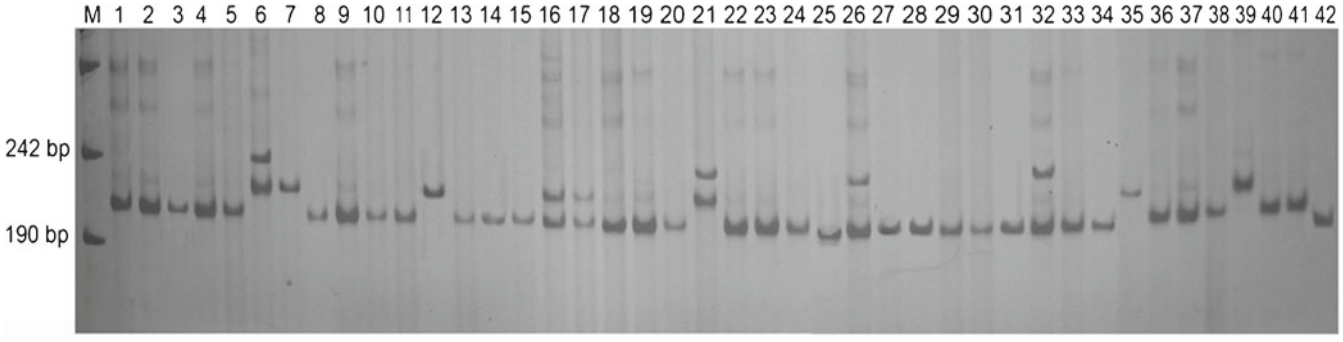

Figure 1. EST-SSR polymorphism revealed by primer pair EPS08 among the 42 pomegranate accessions. Lane M is molecular weight marker (pUC19 DNA/*Msp*I marker). Lanes 1–42 represent the 42 pomegranate accessions; the lane number is the same as the accession number of table 1.

phic EST-SSR marker. The H_o values of these markers ranged from 0.119 (ESP11) to 0.619 (ESP08) with a mean of 0.381. The PIC had a similarity to the H_o values, and varied from 0.091 (ESP11) to 0.656 (ESP08) with a mean of 0.402. Generally, SSR loci from transcribed regions had a low level of polymorphism compared to genomic SSRs (Kumpatla and Mukhopadhyay 2005). However, the allelic number and allelic diversity detected by the EST-SSR markers in this study were nearly the same as those reported for genomic SSRs (Currò *et al.* 2010; Hasnaoui *et al.* 2010, 2012; Pirseyedi *et al.* 2010), where a number of pomegranate cultivars from several countries were tested. Most probably, this is due to the application of many pomegranate landraces with abundant genetic variation in our study.

The 18 EST-SSR markers produced 1–3 alleles (mean = 1.94), H_o values of 0.00–0.611 (mean=0.250), and PIC values of 0.00–0.549 (mean=0.278) in pomegranate cultivars, while in pomegranate landraces, 1–4 alleles (mean=2.28), H_o values of 0.00–0.708 (mean=0.368), and PIC values of 0.00−0.698 (mean=0.360) were observed. Two SSR loci (ESP03 and ESP11) were monomorphic in pomegranate cultivars but polymorphic in pomegranate landraces. Most markers revealed higher H_o or PIC values in pomegranate landraces than in pomegranate cultivars. The average values of the three indices (N, H_o, and PIC) were also higher in landraces than in cultivars (table 3). Obviously, much more genetic variation exists in Chinese pomegranate landraces, which can be explored for breeding purpose. However, three SSR loci i.e., ESP10, ESP13, and ESP16, were more informative in cultivars than in landraces. Probably, the three loci experienced an artificial selection for agronomic characters in pomegranate.

Diversity and genetic relationship analysis

The genotype data of all the 42 alleles were used to calculate pairwise similarity among the 42 accessions. The similarity coefficient values ranged from 0.706 to 0.975 with a mean of 0.842. The pomegranate landraces had a lower mean similarity coefficient (0.794) than that of pomegranate cultivars (0.879), which also showed an abundant genetic variability within the landraces. It was noted that the similarity coefficient values calculated from EST-SSR data were much higher than those obtained from RAPD (Sarkhosh *et al.* 2006; Zamani *et al.* 2007), AFLP (Jbir *et al.* 2008), and SRAP (Ranade *et al.* 2009). This is probably due to the differences between the different samples or marker systems tested. Genetic diversity among the 42 pomegranate accessions was further analysed by construction of a dendrogram (figure 2). All the accessions were clustered into two major groups (I and II) at a similarity level of 0.722. On the whole, this division was in line with geographical origins of the accessions. All non-Chinese accessions (Valenciana, Hicaznar, and Tunisiruanzi) and a Chinese landrace LR13 were clustered in group I; except for LR13, group II netted all Chinese accessions. Obviously, Chinese and non-Chinese

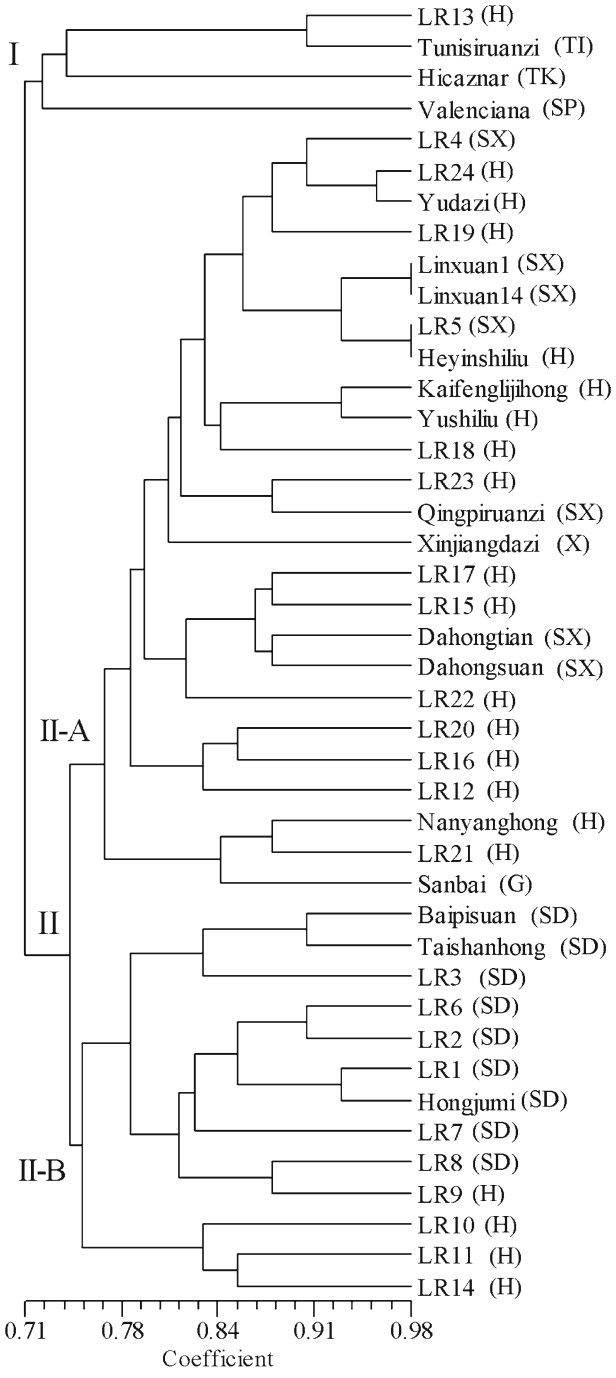

Figure 2. A dendrogram of genetic relationship among 42 pomegranate accessions based on Jaccard's similarity coefficients from the EST-SSR data. Geographic distributions of the accessions are provided in parenthesis.

accessions were separated, suggesting different genetic backgrounds between them. As an exception, LR13 was clustered closely with Tunisiruanzi, a widely cultivated cultivar that was introduced in China in 1980s, indicating that it could have a lineage of Tunisiruanzi (probably be a seedling tree of Tunisiruanzi). Group II could be divided into two subgroups (II-A and II-B) at a similarity coefficient level of

0.758. Subgroup II-A contained the accessions with multiorigins, i.e., the accessions from Gansu, Shaanxi, and Xinjiang, and most accessions from Henan. However, subgroup II-B was mainly comprised of the accessions from east–central China, i.e., all the accessions from Shandong and four landraces (LR9, LR10, LR11, and LR14) from the northeast regions of Henan. Compared to the accessions from other regions, the accessions from east–central China (including Shandong province and the northeast regions of Henan province) had a distinctive genetic background. Our results were concordant with the report of Yuan *et al*. (2007), where pomegranate populations from Henan, Xinjiang and Shaanxi were closest while the populations from Shandong and Anhui were closest in Nei's genetic distance. From these, the newly-developed EST-SSR markers could be applied in diversity study in pomegranate.

It is concluded that we for the first time developed and characterized 18 EST-SSR markers for pomegranate in response to a limited number of SSR markers currently available. Most of the markers were polymorphic and could detect more than one allele in a set of 42 pomegranate accessions containing cultivars and landraces. Also, the genetic diversity among the accessions was well resolved using these markers. The newly-developed markers together with other published ones can provide a supply for genetic studies in pomegranate.

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