Microsatellite isolation and characterization in pomegranate (*Punica granatum* L.)

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Abstract
Development of microsatellite markers has been an increasing trend in crop genetic studies because of their applicability in breeding programs. Here we report the development of inter simple sequence repeat (SSRs) in pomegranate (*Punica granatum* L.) using an enrichment method that makes use of magnetic beads. Enriched genomic libraries with AG and ATG microsatellite motifs were constructed, and 60 positive clones were detected by a colony PCR technique, of which 43 clones showed high-quality sequences. Out of these, 32 (74.4%) contained microsatellite sequences and 25 primer pairs were designed, of which 11 (44%) revealed polymorphisms, 12 (48%) showed monomorphic patterns and 2 (8%) generated poor amplification on a set of 20 pomegranate genotypes. Eleven microsatellite primers (two of them amplified two loci) were selected to assess polymorphism in the set of genotypes. There were 44 alleles amplified over 13 loci, with an average of 3.38 alleles per locus. The mean polymorphism information content (PIC) value was 0.433 over 13 loci, which shows that the majority of the microsatellite loci are highly informative. Cluster analysis was able to separate genotypes based on their geographical distribution and type (i.e., wild or domestic). This study shows the isolation efficiency of the magnetic beads technique, the abundance of microsatellites in pomegranate, and their potential application in pomegranate genome mapping and genotyping.
Keywords: Pomegranate; Microsatellite; Genetic diversity; SSR-enrichment libraries

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INTRODUCTION

Pomegranate (*Punica granatum* L.) is one of the oldest fruits known to man. This genus is composed of three species: *P. protopunica*, *P. nana* and *P. granatum* (2n = 16), of which *P. granatum* is mainly cultivated for fruit production (Moriguchi *et al*., 1987). The pomegranate tree has a wide geographical distribution that spans from Iran to the Himalayas in northern India, and has been cultivated since ancient times throughout the Mediterranean regions of Asia, Africa and Europe (Behzadi Shahrbabaki, 1998).

Iran is the largest producer of this fruit, generating considerable variation in genotypes. In a collection in the Yazd province in central Iran, there are 770 genotypes, including 740 that are cultivated, as well as additional wild and ornamental types. Nonetheless, genomic information on this crop is very limited owing to the lack of research investment and proper tools. It is therefore necessary to develop and utilize tools to evaluate genetic diversity among pomegranate genotypes to promote conservation and sustainable utilization of this crop (Behzadi Shahrbabaki, 1998). Pomegranate genotypes have been evaluated with respect to many morphological traits and morphometric criteria, but very little work has been done on molecular characterization of the pomegranate genome. Molecular markers have proved to be a powerful tool for assessing genetic variation, and phylogenetic and genetic relationships, as well as for studying relatedness among cultivars of many species.

Microsatellite markers (SSRs) have proven to be very useful for cultivar identification, pedigree analysis, evaluation of genetic distance among organisms

(Priolli *et al*., 2002) and genetic diversity studies in a wide range of plants due to their high polymorphism, abundance and co-dominant inheritance (Gupta *et al*., 1996). In addition, they are widely used in genetic and phylogenetic studies (Oliveira *et al*., 1997).

The major drawback to the use of microsatellites has been that they need to be isolated *de novo* from species that are being examined for the first time; such isolation can be time-consuming. This drawback has recently been overcome by the introduction of library enrichment and automatic sequencing (Zane *et al*., 2002). Molecular studies of the pomegranate have been restricted to examinations of randomly-amplified polymorphic DNA (RAPD) by (Dorgac *et al*., 2008; Sarkhosh *et al*., 2006; Talebi Bedaf *et al*., 2003) inter simple sequence repeats (ISSR) by Talebi Bedaf *et al*. 2005 (Talebi Bedaf *et al*., 2005) and amplified fragment length polymorphism (AFLP) by (Jbir *et al*., 2008; Rahimi *et al*., 2003). Koohi-Dehkordi *et al*. (2007) published the first report describing the isolation of microsatellites in pomegranate (Koohi-Dehkordi *et al*., 2007). They isolated 15 microsatellites, but only five of them were appropriate for PCR amplification and genotyping. These five microsatellites have been successfully applied in the assessment of the genetic diversity of cultivated and wild pomegranate (Koohi-Dehkordi *et al*., 2007). The development of a large number of pomegranate microsatellite markers could significantly facilitate the construction of pomegranate molecular maps, which are needed for efficient marker-assisted selection to improve pomegranate breeding.

In recent years, several strategies for microsatellite isolation have been developed; a review by Zane *et al*. (2002) describes some of the methods for obtaining microsatellite repeats (Zane *et al*., 2002). Screening genomic libraries by hybridizing with microsatellite probes and sequencing the hybridized positive clones is a traditional but laborious and costly approach (Billotte *et al*., 2001). In contrast, screening microsatellite-enriched, small-insert libraries can significantly reduce time and cost (Billotte *et al*., 2001). Enrichment can be done by hybridizing microsatellite-containing fragments with biotin-labeled probes that are captured by magnetic beads coated with streptavidin or fixed on a nitrate filter (Edwards *et al*., 1996). The eluted portion that remains after removing non-hybridized DNA is highly enriched for microsatellites (Butcher *et al*., 2000). Amongst the isolation methods, the enrichment method appears to be highly efficient and widely applicable (Zane *et al*., 2002; Hamilton *et al*., 1999).

The objectives of this study were: (1) to isolate and identify microsatellites from the pomegranate genome,

Table 1. Pomegranate genotypes used for polymorphism screening.

No.	Species/Type	Genotype	Origin
1	*P. granatum* / wild	Vahshi narak marvdasht	Marvdasht, Fars, Iran
2	*P. granatum* / wild	Shirin sisangan	Sisangan, Mazandaran, Iran
3	*P. granatum* / wild	Dare loshan	Gilan, Iran
4	*P. granatum* / wild	Jangalhaye asalem	Asalem, Gilan, Iran
5	*P. granatum* / wild	Vahshi jangali roodbar	Roodbar, Gilan, Iran
6	*P. granatum* / wild	Jangal daland	Golestan, Iran
7	*P. granatum* / wild	Bargmoordi	Chaharmahal bakhtiari, Iran
8	*P. granatum* / domestic	Post siahe abrand abad	Abrand abad, Yazd, Iran
9	*P. granatum* / domestic	Rabab neyriz	Neyriz, Yazd, Iran
10	*P. granatum* / domestic	Shirin post siah yazd	Yazd, Iran
11	*P. granatum* / domestic	Malase dane siahe yazd	Yazd, Yazd, Iran
12	*P. granatum* / domestic	Malase saveh	Saveh, Markazi, Iran
13	*P. granatum* / domestic	Alak saveh	Saveh, Markazi, Iran
14	*P. granatum* / domestic	Ghojagh varamin	Varamin, Tehran, Iran
15	*P. granatum* / domestic	Shirin taghlid kan	Tehran, Iran
16	*P. granatum* / domestic	Behaste ravare kerman	Ravar, Kerman, Iran
17	*P. granatum* / domestic	Yek kilooie malase sistan	Sistan va baloochestan, Iran
18	*P. granatum* / domestic	Shishe kabe ferdos	Khorasan, Iran
19	*P. granatum* / ornamental	Golnare zinati saveh	Saveh, Markazi, Iran
20	*P. granatum* / ornamental	Golnar fars	Fars, Iran

and (2) to detect polymorphisms using novel microsatellites in a collection of pomegranate genotypes of diverse origin.

MATERIALS AND METHODS

Plant materials and DNA isolation: Twenty pomegranate genotypes were selected on the basis of morphological traits (e.g. fruit color, seed color and taste) and also geographical regions including 11 commercial, seven wild and two ornamentals (Table 1). Young leaf samples from these genotypes were collected from the pomegranate collection of the Agricultural Research Center of Yazd, Iran. The DNA of cultivar Bargmoordi was used to develop the microsatellite-enriched libraries. Total genomic DNA was extracted from fresh pomegranate leaves according to the CTAB method described by Murray and Thompson (1980).

Enriched library construction: Microsatellites were isolated following the protocol of Hamilton *et al.* (Hamilton *et al*., 1999). Briefly, 15 µg of genomic DNA was digested in a mix of four restriction enzymes *(Nhe*I, *Alu*I, *Rsa*I and *Hae*III) that yielded fragments between 200-1000 bp long. The restricted fragments were purified using the QiAquick PCR purification kit (QIAGEN Co.).

Single-stranded overhangs of the obtained DNA fragments were removed using mung bean nuclease; genomic DNA ends were then dephosphorylated by calf intestinal alkaline phosphatase (0.01 U/pmol DNA) and ligated to phosphorylated SNX linkers (Forward 5′ CTAAGGCCTTGCTAGCAGAAGC3′, Reverse 5′ p-GCTTCTGCTAGCAAGGCCTTAGAAAA3′) to provide a PCR priming site. *Xmn*I was also included in the ligation procedure. The linker-ligated fragments were amplified by PCR using SNX-forward as the primer. These fragments were heat denatured and then hydrolyzed to biotinylated $(AG)_{15}$ at 65°C and $(ATG)_{10}$ at 62°C for about 17 hours in a hybridization oven. Fifteen microgram of streptavidin-coated magnetic beads was washed three times with 300 µl of 0.5X Saline Sodium Citrate (SSC) for 5 min per wash and suspended in 100 µl of 0.5X SSC. The hybridization solution was added and incubated on a heat block at 43°C for one hour and agitated for 5 min. The solution was then placed in an oven at a suitable temperature (68°C for AG and 62°C for ATG motifs) for hybridization. The solution was washed twice with 200 µl of 2X SSC, twice with 200 µl of 1X SSC and four times with 200 µl of 0.5X SSC for 5 min per wash in order to remove non-targeted DNA from the oligo-bead complex. Beads were separated from the solution between washes using a MagneSphere Magnetic Separation Stand (Promega Co.). Microsatellite-enriched DNA was eluted by adding 30 µl of preheated sterile water at 98°C, incubating at 98°C for 15 min and then separating the solute from the beads. The recovered single-stranded DNA was amplified using the SNX forward linker as a primer. The resulting double-stranded DNA products were digested with *Nhe*I to produce cloning ends. The repeat-enriched DNA was ligated into an *Xba*I-digested dephosphorylated pBluescript SK (+/-) vector, transformed into competent *Escherchia coli* (MC1061) cells, and plated on LB medium containing 10 mg/ml of ampicilin, X-gal (20 mg/ml) and IPTG (23 mg/ml). After 17 hours, the transformed microsatellite clones (white clones) were plated on new plates.

Sequencing and primer design: The colony-PCR technique was used to choose the positive clones. Sixty of those with 500-800 bp inserts were selected and sequenced (Macrogen Co., Korea). Sequences were analyzed by Chromas software ver. 2.13. Primers were designed for sequences that contained microsatellite repeats with the program Primer3 (http://www.fokker.wi.mit.edu/primer3/input.htm). The designed primers were synthesized commercially by MWG AG Biotech, Germany (Table 2). Twenty-five primer pairs were tested and optimized in 20 pomegranate cultivars.

PCR amplification and electrophoresis: The efficiency of designed primers was assessed among 20 pomegranate germplasms containing 11 domestic, 7 wild and 2 ornamental genotypes. PCR amplification was performed in a 15 µl volume containing 40 ng of pomegranate DNA, 1X reaction buffer, 0.1 mM of each dNTP, 0.5 µM each of forward and reverse primer, 1.3 mM $MgCl_2$ and 0.2 U *Taq* polymerase (Cinagen Co. Iran). All reactions were performed using an Eppendorf Thermal Cycler (AG22331). The following thermal cycling protocol was used: 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, 45 s at optimal annealing temperature (Table 2), which was determined by testing in the range of ±5°C from the theoretical annealing temperature, and 72°C for 1 min. Then a final extension reaction was allowed to proceed at 72°C for 7 min. Amplification PCR products were separated using 6% denaturing polyacrylamide gel

Table 2. Microsatellite primer sequences, repeat types, allele size ranges and number of alleles in 20 pomegranate genotypes.

Primer name	GeneBank accession No.	Motif repeat	Primer sequence 5´- 3´	Primer sequence 5´- 3´	Product size range	No. of alleles	H_O	H_E	PIC values	p-value	Tm (˚C)
pg1	FJ491733	$(AC)_{16}$	GGT CTG ACT GGA CCG TTG C	GA GAA CGA AGA TCC CGG TTT	222	1	---	---	---	---	55
pg2	FJ491733	$(TCCGT)_2TTCC(TTC)_4$	CGG GAT CTT CGT TCT CGA T	GGA ATC CGT GAG CTG AGA GT	151	2	---	---	---	---	59
pg3	FJ491734	$TGGC(TGG)_3$	CGA AGA ACG GCT AAT CAA CG	GAT CCA CAA CCA CGT CCA AC	121	1	---	---	---	---	61
pg4	FJ491735	$(TC)_{12}TT(TC)_{20}$	CTG ATG TAA TGG CTG AGC AAA	GCA CTT GAA CAA AGA GAA TGC	220-245	5	1	0.74	0.696	0.0000	56
pg5	FJ491736	$(AG)_4$ AC$(AG)_4$ AC $(AG)_4$	GCC ACC TCT GCA ATT CTC TC	GCA AAG GTT AGG CTC CGA AT	247-252	2	0	0.332	0.277	0.0000	58
pg6	FJ491737	$(AC)_{15}$	GGT TGC TCA TCC CTT GAC TC	GCG TCT GTC AGT GTC TTA GGC	180-210	5	1	0.607	0.530	0.0000	61
pg7	FJ491738	$(AG)_{20}$	CTT CCA TTC CCC TAG CAA CC	CTC CCC CGA ACT TAT CCT TC	195	- - -	---	---	---	---	---
pg8	FJ491739	$(AG)_{20}$	CAC CAT AGA CTT AAA CGA GCA CAA	GAA GCT CCA TTG CCT CGT C3	115-150	5	1	0.742	0.699	0.0000	59
pg9	FJ491740	$(AG)_{23}$	CGG TGG AAT TGG CAT AAG AC	CTG AAC TGG AAG TCG CAC AC	154	2	---	---	---	---	61
pg10(a)	FJ491741	$(AG)_9GG(AG)_{14}$	TGC TAG ACA GAA CTG GGA GAA C	AGA GAG TGG GGT TTC CAT TG	224-245	4	1	0.627	0.554	0.0000	60
pg10(b)[a]	FJ491741	$(AG)_9GG(AG)_{14}$	TGC TAG ACA GAA CTG GGA GAA C	AGA GAG TGG GGT TTC CAT TG	370-385	4	0.1	0.18	0.164	0.0469	60
pg11	FJ491742	$(ATAG)_6$	CTC CCT TCG GTT CTT GGT CT	ACG ATG GAG TGC TTG TGG AT	172	2	---	---	---	---	60
pg12	FJ491743	$(TC)_{17}$	CTC TCA AAG CCC TGA ACC TG	GAG CTA AGG TAA TGG AGC AGA AG	157	2	---	---	---	---	61
pg13	FJ491744	$(AG)_{32}$	AGG CTG AGA GTC TCC CCA AC	TTG AGA TCG AAC GAA TCG AC	193	1	---	---	---	---	55
pg14	FJ491745	$(AG)_{32}$	GCA CAT TTC TTC CAC CTT CC	GGT TAC AAT GCA CAG AGT CCA C	227-245	4	1	0.653	0.6	0.0028	61
pg15	FJ491745	$(CT)_7$	CGA GAC CCT TCA GTT CTT CG	CAG GCT CGT CCT GAA TAT CG	189	2	---	---	---	---	60
pg16	FJ491746	$(AT)_{11}$	CTG CAT ATC CCA AGT CAA GGT G	GGC TTG ATT CGA TGG GCT AT	242	- - -	---	---	---	---	---
pg17[a]	FJ491747	$(TCA)_{14}$	CAT CAG ACT ACG ATG GCA CT	GCA TAA TAG CCT TCA ATT TAC A	140-145	2	0.25	0.219	0.195	0.5229	55
pg18(a)	FJ491748	$(TCA)_{14}$	TCT AAG GGC AGA ATG GCA CT	TGG CAC TAG ATC CGT AAA TCT C	186-200	3	0.25	0.366	0.326	0.0001	59
pg18(b)[a]	FJ491748	$(TCA)_{14}$	TCT AAG GGC AGA ATG GCA CT	TGG CAC TAG ATC CGT AAA TCT C	335-347	3	0.3	0.320	0.269	0.7799	59
pg19	FJ491749	$(TCA)_{15}$	GCA TAG AAT GAC CTG AGT CGT G	TGA CAG CCC CTC TTG ATT TC	139	1	---	---	---	---	58

Continued on next page

pg20	FJ491750	$(AG)_{12}$	AGT CCA CCC TTT ACC ACA TCC	CTC CCA TAC AAC GGA CCA AT	119	2	---	---	---	---	58
pg21	FJ491751	$(AG)_{7}$	CAA GAC AGA AGC ACC ATC CA	TCT CCC AAA TCA GAC CAA CC	210-225	4	1	0.660	0.595	0.0010	54
pg22	FJ491752	$(ACAT)_3$ $(AT)_3$ $(AG)_{22}$ $(AT)_3$	CCC CGC ACT TAG AAT CTA TTA	TCC AGT TCC AAT CGA CAG AC	230-240	3	1	0.524	0.41	0.0002	55
pg23	FJ491753	$(TC)_{16}$	ACC ACT CCC ACC ATT ATT GC	GGA GGG AAG AGA CGA GCA TT	227-235	3	0.2	0.340	0.314	0.0000	56
pg24	FJ491754	$(TCA)_{8}$	GAA AGA GTG TGT GAG AGA ATC TGC	GGA CGG ATG CAA GAA ATG G	91	1	---	---	---	---	61
pg25	FJ491755	$(CT)_{13}$	ATC AAG AAC TCC CCC GAA CT	CCT TCT TCC ATT CCC CTA GC	193	1	---	---	---	---	64
Average in polymorphic loci			---	---	---	3.38	0.623	0.485	0.433	---	---

[a] Loci not deviating from the Hardy-Weinberg equilibrium (HWE).

electrophoresis in 1X TBE buffer, at a constant current of 30 mA. A 50-bp-plus DNA ladder (Fermentas Co.) was used as a molecular size standard and stained with silver nitrate (Sambrook *et al*., 2001).

Data analysis and genetic relationships: The number of alleles, expected heterozygosity (H_E), observed heterozygosity (*Ho*), genetic similarity and genetic distance as estimated by Nei's coefficient between pairs and dendrograms, based upon the unweighted pair group method with arithmetical averages (UPGMA), were analyzed using Powermarker (ver 3.25) and Mega3 software. Hardy-Weinberg equilibrium (HWE) and polymorphism information content (PIC) value for each primer was calculated with powermarker software.

RESULTS

Two enriched genomic libraries with dinucleotide AG and trinucleotide ATG microsatellite motifs were constructed. Among the observed clones, 60 positive clones were selected with the colony-PCR technique and sequenced. The sequences of 17 clones were insufficiently clear; however, among the 43 clones with high-quality sequences, SSR motifs were observed in 32 clones (74.4%). Among these 32, four clones were redundant (9.3%) and five sequenced clones were not suitable for primer design because of short or missing flanking regions (11.6%). The remaining 23 suitable clones for primer design showed 25 motifs (two clones each had two separate motifs).

Three classes of microsatellite-repeat arrays were revealed in these clones, including perfect (89%), imperfect (15%) and compound (6%) repeats.

Twenty-five SSR primer pairs were designed and their efficiency was assessed among 20 pomegranate genotypes. All primers amplified microsatellite loci in the selected genotypes. Two primer pairs (pg7 and pg16) amplified in all samples, but these produced extra bands that complicated band discrimination; therefore, they were excluded from the rest of the experiments, while 23 pairs amplified interpretable PCR products. Among these latter 23, 12 primer pairs turned out to be monomorphic (48%) and 11 primer pairs showed a polymorphism pattern in selected genotypes (44%), while two (pg10 and pg18) amplified two loci in the examined genotypes.

According to the results, a total of 44 alleles were mapped to 13 loci. Primer sequences, the number of

alleles, allele size variation, heterozygosity and PIC values at 13 polymorphic microsatellite loci observed among 20 pomegranate genotypes is given in Table 2. The observed number of alleles per locus ranged from two to five with an average of 3.38 alleles per locus, the observed heterozygosity ranged from 0.00 to 1.00 with an average of 0.623, and the PIC values were estimated to range from 0.164 to 0.699 with an average of 0.433.Tests for Hardy-Weinberg equilibrium (HWE) of the polymorphic loci revealed that most loci, except pg10b, pg17 and pg18b, were significantly deviated from HWE ($P < 0.01$) (Table 2). Significant deviation from HWE is probably due to self-pollination [low proportion of 13%, cross-pollination (Jalikop *et al*., 1990)] and also limitation in tested samples.

DNA polymorphism among 20 pomegranate genotypes was detected by 11 microsatellite markers and allowed for genetic distance estimates and clustering of 15 genotypes into three groups (Fig. 1), with five genotypes (Vahshi Jangali Roodbar, Bargmoordi, Vahshi Narake Marvdasht, Yek Kilooie Malase Sistan and Golnare Fars) considered as an individual cluster. Fifteen genotypes were classified into three different groups including group I (Dare Loushan, Jangalhaye Asalem, Jangale Daland and Shirine Sisanghan), group II (Shishe Kabe Ferdous, Ghojaghe Varamin, Shirine Taghlide kan, Golnare Zinatie Saveh, Alake Saveh and Malase Saveh) and group III (Rababe Neiriz, Bihaste Ravare Kerman, Poost Siahe Abrand Abad, Malase Dane Siahe Yazd and Shirine Poost Siahe Yazdi). In this dendrogram, wild and domestic genotypes were divided into different clusters and clustering of the genotypes was performed according to their geographical origin. In most cases, the clusters were not correlated with the morphological traits.

DISCUSSION

Efforts have been made to identify pomegranate cultivars and estimate genetic distances using DNA markers, mainly RAPD, AFLP, ISSR and SSRs. Although these markers have been successfully used for cultivar identification, we sought to increase the efficiency of SSR isolation from the pomegranate genome by employing SSR enrichment. In so doing, we evaluated the effectiveness of two differently-prepared, SSR-enriched libraries. By testing various combinations of 4- and 6-bp restriction enzymes, those four restriction enzymes that yielded DNA fragments between 200-

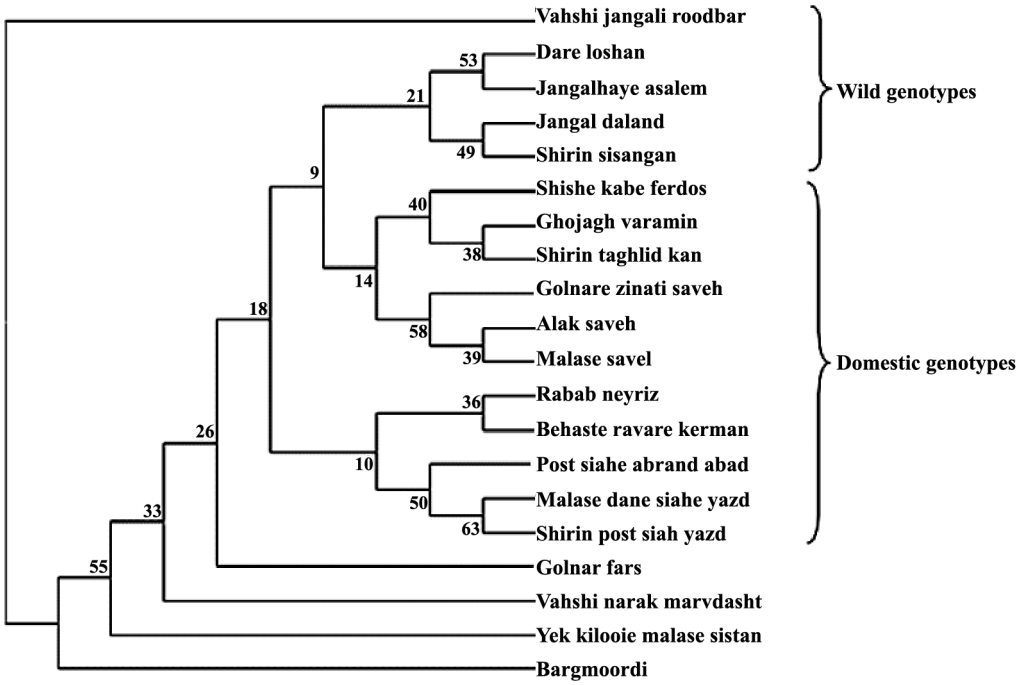

Figure 1. Dendrogram resulting from microsatellites based on genetic distance analysis of 20 pomegranate genotypes by Power marker ver. 3.25 and Mega3 software, estimated by Nei's coefficient in pair wise comparisons. Cluster analysis was performed using the UPGMA algorithm.

1000 bp long (*Nhe*I, *Alu*I, *Rsa*I and *Hae*III) were selected as beneficial fragments for enrichment and cloning. Hamilton *et al*. (1999) explained that the choice of restriction enzymes to size-fractionate the genome affects the number and repeat type of potential SSR markers.

A total of 32 unique clones containing microsatellite repeats were identified among 43 clearly-sequenced pomegranate genomic clones. An average enrichment of 74.4% was achieved, which suggests the enrichment procedure was successful for the two developed libraries, yielding a higher efficiency of enrichment than methods reported by other researchers, such as enrichment with a nylon membrane and magnetic beads (Zhao *et al*., 2005; Stajner *et al*., 2004; Ahmad *et al*., 2003; Hamilton *et al*., 1999; Rossetto *et al*., 1998).

The results of this study identify 9.3% redundant clones and 11.6% clones with insufficient flanking sequences, which is lower than the averages assessed in other plants. Squirrell *et al*. (2003) reported 14.5% redundant and 39.9% clones without enough flanking regions in *Brassica* sp. (Squirrell *et al*., 2003). Another study by Stajner *et al*. (2004) showed that in one enriched library for a GA motif, 29% of clones had an insufficient flanking region (Stajner *et al*., 2004). They suggested that this might be due to the application of restriction sites containing the microsatellite-repeat motifs, as was the case with the AG motif in the two restriction sites *Alu*I and *Nhe*I.

There were a low percentage of clones lacking microsatellite repeats (25%) in comparison with the average percentage observed in enriched libraries (36%) (Squirrell *et al*., 2003), indicating that the efficiency of the enrichment step was higher in this study and that the screening of the clones before sequencing was performed well. Therefore, it seems that the colony-PCR technique is a suitable method for screening clones before sequencing.

In this study, 32 out of 43 sequenced clones contained microsatellite repeats, (70% efficiency). This efficiency is comparable to that of other plants studied with an enriched efficiency between 50-90% (Butcher *et al*., 2000). Also Hamilton *et al*., (1999) explained that the efficiency of this method for hybridization-positive clones is approximately 20-95% depending on the enrichment repeat (Hamilton *et al*., 1999).

In similar research by Koohi-Dehkordi *et al*. (2007) for the isolation of pomegranate microsatellites, the efficiency of the enrichment method *via* the nylon membrane was reported as 51% in the AG library and 47% in the GT library (Koohi-Dehkordi *et al*., 2007), so it is clear that the efficiency of the method used in this study is higher. These results demonstrate that the magnetic bead enrichment method is a more efficient technique than other enrichment methods for identification of microsatellite motifs.

The mean number of alleles per locus was 3.38 and the observed heterozygosity ranged from 0.00 to 1.00 with an average of 0.623, suggesting a high degree of variation in isolated loci. The average PIC value was 0.433; these markers can thus be classified as ‘informative‘.

All of the designed primers amplified the expected size, and 11 of them revealed a polymorphism in 20 pomegranate genotypes (44%). This is comparable to *Brassica* sp. (43%) and other plants (49%) (Squirrell *et al*., 2003), and is a higher percentage than that obtained in the previous efforts of Koohi-Dehkordi *et al*. (2007), where only 5 of the 15 designed primers showed polymorphism in 29 pomegranate cultivars (30%) (Koohi-Dehkordi *et al*., 2007).

For two polymorphic primer pairs, double-band patterns were observed, which Ahmad *et al*. (2003) previously explained (Ahmad *et al*., 2003) and suggested that such complex banding patterns were due to additional locus. Multiple loci may be produced by duplication, which can only be confirmed by segregation analysis and mapping of the locus on the chromosome or by sequencing (Ahmad *et al*., 2003).

In this work, wild and domestic pomegranate genotypes were divided into different clusters according to their geographical origins, as was reported for the apricot (Sanchez-Perez *et al*., 2005). In most cases, the clusters were not in agreement with the morphological traits (fruit color, seed color and taste); this result was also observed in previous studies of pomegranate diversity with RAPD, ISSR and AFLP markers (Sarkhosh *et al*., 2006; Talebi Bedaf *et al*., 2003, 2005; Rahimi *et al*., 2003). It is known that some mutations and genetic changes that are easily recognizable phenotypically may not be detectable by application of molecular markers (Talebi Bedaf *et al*., 2003). The fragments of genome amplified with SSR primers may not be parts of codons for morphological characteristics.

Compared to previous works, the results from this experiment showed higher polymorphism among pomegranate genotypes. In summary, this study achieved the primary isolation and characterization of pomegranate microsatellites. The polymorphic microsatellites presented here function as efficient

genetic markers, and will assist in pomegranate genotype identification and assessment of genetic diversity.

Acknowledgements

This work has been financed by Isfahan University of Technology, Iran, whose support is highly appreciated. We thank the Yazd Agricultural Research Center, Iran for providing the plant materials.

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