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TECHNICAL NOTE

Development and characterization of SSR markers for pomegranate (*Punica granatum* L.) using an enriched library

N. Hasnaoui · A. Buonamici · F. Sebastiani · M. Mars · M. Trifi · G. G. Vendramin

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Abstract In the present work, we report the development of 11 microstallite markers (SSR) for *Punica granatum*. Evaluated on a set of 27 pomegranate accessions sampled in Tunisia, they displayed 25 alleles, with number of alleles per locus ranging between 1 and 4, and an observed heterozygosity from 0.037 and 0.592. This set of SSR markers can be very useful for studies dealing with genetic diversity assessment of germplasm, with cultivars/varieties fingerprinting and pedigree analysis of this economically important fruit species.

Keywords Pomegranate · *Punica granatum* L. · SSR markers · Microsatellites

To the Memory of Professor Mohamed Marrakchi, who passed away in April 2008.

N. Hasnaoui (✉) · M. Trifi
Laboratory of Molecular Genetics, Immunology & Biotechnology, Faculty of Sciences of Tunis, 2092 El-Manar, Tunis, Tunisia
e-mail: nejibhasnaoui@yahoo.fr

A. Buonamici · G. G. Vendramin
CNR-Instituto di Genetica Vegetale, 50019 Sesto Fiorentino, Florence, Italy

F. Sebastiani
Dipartimento di Biotecnologie Agrarie, Genexpress, Università di Firenze, 50019 Sesto Fiorentino, Florence, Italy

N. Hasnaoui · M. Mars
U.R. Agro-Biodiversity, Higher Agronomic Institute, 4042 Chott Mariem, Sousse, Tunisia

The pomegranate (*Punica granatum* L.), one of the oldest known edible fruits, is originating from Persia and surrounding areas and from there it has been spread in many other regions. *P. granatum* is the predominant member of two species comprising the Punicaceae family. The second species, *P. protopunica*, is endemic of the Island of Socotra (Yemen; Mercure 2007). In its origin centre (Iran) and surrounding regions (Afghanistan, Pakistan, India, Oman) pomegranate is encountered as wild types as well as cultivated varieties/accessions (Al-Said et al. 2009; Mousavinejad et al. 2009; Narzary et al. 2009), but in Mediterranean basin, to nowadays only cultivated varieties were reported (Martinez et al. 2006; Jbir et al. 2008; Durgac et al. 2008). Its fruit has a thick skin and an enlarged calyx surmounted by its persistent sepals. Like all cultivated plants, pomegranate is liable to variation, and several of its forms have been considered distinct species. Around the world, many hundreds of cultivated as well as wild types of pomegranate exist.

Microsatellites were developed using an enriched library following the protocol described by Edwards et al. (1996). In brief, total genomic DNA was digested with *Rsa*I. The digested fragments (200–800 bp) were ligated to a *Mlu*I adaptor (consisting of a 21-mer oligonucleotide (5′-CTCTTGCTTACGCGTGGACTA-3′). Ligated DNA was PCR amplified using the oligo-adaptor as primer. PCR products were used for the enrichment step through hybridization with Hybond N+ filters (GE Healthcare) bounding oligonucleotide repeats (GA, GT, AT, GC, CAA, ATT, GCC). PCR products were cloned into the pGEM-T easy vector (Promega) and transformed using the JM109 High Efficiency Competent Cells (Promega, Madison Wis.).

A set of 62 positive clones was randomly chosen and sequenced in one direction. Sequencing was performed using the DYEnamic ET Terminator Sequencing Kit (GE Healthcare) and the reactions were run in a capillary automatic

Table 1 Characteristics of *Punica granatum* microsatellites markers

Locus name	Primer sequence 5′–3′ (F: forward; R: reverse)	Repeat motif	Allele size range (bp)	*A*	H_O	H_E	Deviation from HW equilibrium	Null allele frequency	EMBL accession no.
Pom004	F-TCCTTTTTACCCAATTTTCA	$(CT)_{10}$	149	1	0	–	–	0	FM994084
	R-TGCACATCTTTTGCTGTAAG								
Pom006	F-TACTAGGTGGAACCGAACTT	$(GA)_4$ ACT $(GA)_{26}$	145–171	4	0.222	0.209	NS	0	FM994088
	R-CCTTGACAACCTCATCTCAT								
Pom010	F-CCTCATTGCTGATGAATCTT	$(AG)_{19}$	231–233	2	0.259	0.283	NS	0.015	FM994089
	R-ACTCGAGAAGCTCTGTGAAG								
Pom013	F-CACACCCTTCATCAAAAGAT	$(CT)_{19}$	165–169	3	0.111	0.108	NS	0	FM994090
	R-GGACTAACAACCAGCCATAG								
Pom014	F-CGCATTTGGTTGTAGAAGAC	$(GA)_{27}$	197–207	3	0.111	0.108	NS	0	FM994091
	R-AGGAGCGTCTGTTTTAATCTT								
Pom021	F-GACTGGAAGAAGCAGAGACT	$(AC)_{28}$	201–205	3	0.481	0.544	NS	0.034	FM994092
	R-GAAAAGGAAGTAGCAGAGCA								
Pom024	F-GGAGATTTGAATTGGGAAGT	$(AG)_{27}$	230–242	3	0.592	0.500	NS	0	FM994093
	R-GTGGACTAACTCAAGCAAGG								
Pom039	F-TAGTTGAATAGGCCACATCC	$(CA)_{12}$	143	1	0	–	–	0	FM994085
	R-CTATACAGTCCGAGGACCAC								
Pom046	F-CTTCCTCCTACCGAACTATG	$(GA)_{21}$	241–245	2	0.037	0.037	NS	0	FM994098
	R-CCCACTTTGACACTTCTACC								
Pom055	F-GAGACAATTGGGATCAGAAA	$(GCC)_5GCACTCA(TC)_8$	245–257	2	0.037	0.037	NS	0	FM994096
	R-AGTCGACGAACTGTGAAATC								
Pom056	F-CTCGCCATACTACTGAAAGG	$(CT)_{10}$	214	1	0	–	–	0	FM994086
	R-ATTGGGTGAGATATGTTTGG								

A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity ($N = 27$); NS, not significant

sequencer (MegaBACE 1000, GE Healthcare). Among the 62 sequenced clones, 54 (87%) showed microsatellite motifs. After performing a reverse sequencing step, 36 sequenced clones were discarded because of non desired properties like extremely long or too short repeat stretches or SSR too close to the cloning site. Specific primers complementary to microsatellites flanking regions were designed using the PRIMER 3 software (Rozen and Skaletsky 2000). The SSR primers were tested for their ability to produce a readable pattern using genomic DNA of three Tunisian pomegranate cultivars. The PCR reactions were performed in a final volume of 15 µl containing 10 ng of DNA, 1 × GoTaq reaction Buffer (Promega), 2.5 mM of $MgCl_2$, 0.2 µM of each primer, 200 µM of each dNTP and 1 U Go*Taq* polymerase (Promega). The amplifications were carried out in a thermocycler (T1, Biometra) using the following temperature profile for the preselected 18 primer pairs: an initial step of 3 min at 95°C followed by 10 touchdown cycles of 30 s at 94°C, 40 s at 65°C (−1°C per cycle), 30 s at 72°C and 25 cycles of 30 s at 94°C, 30 s at 55°C, 40 s at 72°C with final extension time of 8 min at 72°C. PCR products were resolved on 2% agarose gel. After these steps, 11 SSR primer pairs could finally selected because of the high quality single-banded amplification products of the expected size. The selected primers were labeled with fluorochromes (FAM, HEX, and TAMRA) to be used in a capillary automatic sequencer (MegaBACE 1000, GE Healthcare) for genotyping 27 *P. granatum* cultivars originating from different regions of pomegranate cultivation in Tunisia. Alleles were sized using the size standard MegaBACE ET400-R (GE Healthcare) and the software Fragment Profiler version 1.2 (GE Healthcare). Arlequin 3.1 (Excoffier et al. 2005) was used to calculate the number of alleles (A), to estimate the observed (H_O) and expected heterozygosity (H_E) and to test the Hardy–Weinberg equilibrium.

The 11 SSR loci developed provided 25 alleles, with an allele's number per locus ranging between 1 and 4. Among the developed SSR loci, three (Pom004, Pom039 and Pom056) revealed only one allele within the analyzed set of 27 accessions. The remaining 8 loci were polymorphic. This reflects a quite narrow genetic background of the *P. granatum* local resources, but it is likely that more alleles could be detected by genotyping pomegranate cultivars/varieties from other geographic origin and/or wild populations. The observed and expected heterozygosity ranged from 0.037 to 0.592 and 0.036 to 0.491, respectively. The low level of heterozygosity might be the result of the asexual propagation of pomegranate cultivars, since vegetative propagation is the unique mode used for its cultivation, and/or by the limited sample size analyzed. Besides, no significant deviation from the Hardy–Weinberg equilibrium was observed. No evidence of significant occurrence of null alleles could be detected at any locus (always below 5%, Table 1), calculated using Identity software (Wagner and Sefc 1999). No significant linkage disequilibrium among the analyzed loci using sequential ($P = 0.05$) Bonferroni correction was observed, estimated using F-STAT version 2.9.3. (Goudet 2001).

To the best of our knowledge, this is the first report on SSR markers for *P. granatum*, a category of useful genetic marker to assess genetic diversity and helpful for conservation studies in this important fruit species.

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