ISSN: 0974 - 0376

The Ecoscan : Special issue, Vol. VII: 253-258: 2015
AN INTERNATIONAL QUARTERLY JOURNAL OF ENVIRONMENTAL SCIENCES
www.theecoscan.in

MOLECULAR STUDIES OF POMEGRANATE (*PUNICA GRANATUM* L.) GENOTYPES USING RAPD MARKERS

Komal Kathuria *et al.,*

KEYWORDS

RAPD Profiles
Cultivars Specific Band Combination
Identification and Characterization

Proceedings of National Conference on Harmony with Nature in Context of Bioresources and Environmental Health
(HORMONY - 2015)
November 23 - 25, 2015, Aurangabad,
organized by
Department of Zoology,
Dr. Babasaheb Ambedkar Marathwada University
Aurangabad (Maharashtra) 431 004
in association with
National Environmentalists Association, India
www.neaindia.org

The Ecoscan
AN INTERNATIONAL QUARTERLY JOURNAL OF ENVIRONMENTAL SCIENCES

KOMAL KATHURIA*, R. BHARGAVA, P. K. YADAV AND KARUN GURJAR
Department of Horticulture, College of Agriculture,
Swami Keshwanand Rajasthan Agricultural University,Bikaner-334006
e-mail: kworld1412@gmail.com

ABSTRACT

An experiment was conducted to develop RAPD profiles for 10 pomegranate (*Punica grantum* L.) genotypes using 14 random decamer primers of OPF series at Plant Physiology laboratory of Central Institute for Arid Horticulture, (C.I.A.H), Bikaner during year 2014-2015. The total genomic DNA from leaf tissues of each genotype was isolated using DNeasy Plant Mini Kit (QIAGEN) and subjected to PCR using set of primers and template DNA. A total of 114 bands were obtained, different sets of primers showed different RAPD patterns with respect to the genotypes, the generated bands varied 3 to 11 in numbers, and the size of base pairs of genotypes ranged between 300-6000 bps. Primers OPF 3 and OPF 6 maximum number of bands *i.e.* 11 bands; however primer OPF 12 produced minimum bands *i.e.* only 3 bands. RAPD profiles developed using primers OPF-1, OPF-4, OPF-5, OPF-6, OPF-8, OPF-9, OPF-10 showed the typical/cultivars specific band combinations which can be useful for identification of cultivars. Primers OPF 11 and OPF 12 showed low value for the varietal identification. RAPD patterns could serve as an ideal tool for identification and characterization of the cultivars as well as evaluation of genetic diversity among various genotypes of the crop.

***Corresponding author**

INTRODUCTION

Pomegranate (*Punica granatum* L.) commonly known as *Anar* belongs to family Punicaceae is one of oldest and prominent edible fruit crop extensively cultivated in tropical, subtropical and arid regions of the world.It is believed to be originated in Central Asia especially the Transcaucasia-Caspian region in Iran, from where it spread to the rest of the world (Harlan, 1992; Smith, 1976; Levin, 1994 and Verma *et al.*, 2010). It was cultivated in ancient Egypt and early in Greece, Italy and Iraq. Later, it spread to Asian countries like Turkmenistan, Afghanistan, Iran, India, China, North Africa and Mediterranean Europe (Melgarejo and Martinez, 1992) and to some extent in the drier parts of South East Asia, Malaya, the East Indies and Africa, USA, China, Japan and Russia (La Rue, 1980 and Mars, 1994). The genus *Punica* consists of two species, *Punica granatum* L. and *P. protopunica* Balf. However, *P.nana* L. is an ornamental dwarf pomegranate and has been considered as a distinct species by some authors (Melgarejo and Martinez, 1992). Due to hardy nature, tolerance to salinity, drought and extreme climatic conditions, suitability under arid regions, fragile agro-ecosystem, high yield potential, good keeping quality and demand in market supports this fruit to occupy a major share of horticultural produce. Light Pink to blood red arils as edible, deserts, ice-creams, custards, juices, syrups, as culinary purposes are popular, also possess medicinal and nutritional value. Powdered dried arils often used as condiment, an acidulate preparations of curries, others products includes products from industrial, pharmaceutical and tanning industries.

Genetically, crop is heterozygous, wide genetic diversity has been developed in nature and this diversity in majority of cultivars has been stabilized, since it propagates vegetatively. The classification of these cultivars rests mostly on floral and fruit characters, these parameters becomes insufficient to clearly distinguish the cultivars. DNA based markers have gained reputation as taxanomic markers. Random amplified polymorphic DNA (RAPD) technique was first introduced by Williams *et al.* (1990). (RAPD) markers can be used for detection of DNA polymorphism without the requirement for predetermined genetic data. Each product is obtained from a region of the genome consisting two short fragments in inverted directions, on opposite complementary strands, that are complementary to primer and adequately close together for amplification (Williams *et al.*, 1990). RAPD markers have been employed to understand and unravel the diversity and systematics of pomegranate by different workers from time to time in Iranian and Turkish accessions (Talebi Bedaf *et al.*, 2003; Sarkhosh *et al.*, 2006; Ercisli *et al.*, 2007; Jambhale *et al.*, 2007; Yang *et al.*, 2007; Zamani *et al.*, 2007; Sheidai *et al.*, 2008; Sarkhosh *et al.*, 2009; Ercisli *et al.*, 2011; Zhang *et al.*, 2012). In India, limited studies have been done in this aspect of the pomegranate. The PCR based technique offers numerous advantages like wider applicability, accuracy and speed of estimation, less costly and could be an ideal tool for the cultivar identification. Hence, in this study attempt has been made to characterize and study various pomegranate genotypes and to develop RAPD patterns for the identification of cultivars.

MATERIALS AND METHODS

Collection of plant material and isolation of genomic DNA

The experimental material for the investigation consisted of young leaf samples of 10 pomegranate (*Punica granatum* L.) genotypes (Jalore Seedless, Ganesh ,G-137, Khog, Mridula, Bassein Seedless, Bhagwa, Phule Arakta , GKVK-1, Dholka) collected from Pomegranate Germplasm Repository ,C.I.A.H., Bikaner. The genomic DNA was isolated from pomegranate leaf sample using DNeasy Plant Mini Kit (QIAGEN, Ltd, Crawley, UK). All the chemicals and biochemicals used in analysis were of fine analytical grade and obtained from standard manufacturer *viz.*,Himedia, Qiagen, Sd fine-chem Ltd, Sigma-Aldrich, Merck, *etc*. For the experiment, double distilled water was used. The glasswares and disposable plastic wares were of standard make obtained from Borosil, Axygene and Eppendorf. All reagents, solutions, Ependroff tubes, microcentrifuge tubes, PCR tubes and tips were sterilized at 15 psi, 121ºC for 20 minutes in autoclave before use. Fourteen primers of OPF series (OPERON TECHNOLOGIES Inc. Alameda, California) were used for analysis (Table 2).

Preparation of Reaction Mixture and programming of PCR

The reaction mixture for RAPD analysis consisted of PCR master mix (2x) (12.5 μl), sterilized distilled water (7.0 μL), template DNA (4.5 μL)**,** primer(1.0 μL)**.** The programming of PCR follows three steps;step 1 consist of single segment for initial denaturation of DNA at 94ºC for 5 minutes, step 2 includes three segments; wherein in segment 1 denaturation of DNA at 94ºC for 0.5 minutes, in segment 2 of step 2 at 36ºC for 1 minutes there is primer annealing followed by segment 3 at 72ºC for 2.5 minutes (extension of annealed primer). Laststep 3 of the cycle consisted of process in which there is final extension of DNA72ºC for 7 minutes. There are total 35 cycles in amplification before the DNA gets amplified (Table 1). Genomic DNA was electrophorated on 0.8 per cent agarose gel. Agarose gel was prepared by dissolving 0.8 g of agarose in 100 mL 1 X TAE buffer and heated in a microwave oven. 10 μL of Ethidium bromide (5 mg/mL) was added to the above solution TAE buffer was added to it after cooling agarose to room temperature. Gel solution was then poured into the gel casting plate inserted with appropriate comb. After setting of gel, the plate was placed in electrophoresis chamber and submerged using 0.5 X TAE buffer and comb was removed gently. 8 μL genomic DNA samples were mixed with 2 μL of 6 X gel loading dye and were loaded in the wells. Electrophoresis was carried out at constant voltage (70 V for 45 minutes). Agarose gel were photographed by Syngene Gel Documentation system and stored as JPEG files.

RESULTS AND DISCUSSION

The total genomic DNA from leaf tissues of each genotype was isolated using DNeasy Plant Mini Kit (QIAGEN, Ltd, Crawley, UK). The integrity of the isolated DNA was verified by visualization of DNA on Agarose gel (0.8 per cent) with uncut ë DNA as standard. A single sharp band was observed for isolated DNA in all 10 cultivars corresponding in size to lDNA. Purity of the DNA *i.e.*,DNA bereft of proteins, polyphenols, RNA*etc* showed that the quality of the DNA extracted was excellent as shown in Figure 1.Random amplification of polymorphic DNA was done by using 14 primers of OPF series obtained from OPERON TECHNOLOGIES (Inc. Alameda, California). Clear and distinct bands amplified by RAPD primers were scored for the presence (1) and absence (0) for the corresponding band among the cultivars.A total of 114 bands were obtained, different sets of primers showed different RAPD patterns with respect to the genotypes, the generated bands varied 3 to 11 in numbers, and the size of base pairs of genotypes ranged between 300-6000 bps. Primers OPF 3 and OPF 6 maximum number of bands i.e. 11 bands; however primer OPF 12 produced minimum bands i.e. only 3 bands as shown in Table 3. RAPD profiles developed using primers OPF-1, OPF-4, OPF-5, OPF-6, OPF-8, OPF-9, OPF-10 showed the typical/cultivars specific band combinations which can be useful for identification of cultivars. RAPD patterns in 10 pomegranate genotypes using the primer OPF 6 is as shown Figure 2. Molecular studies on 25 pomegranate cultivars using RAPD markers were carried out earlier by Yang *et al.* (2007), 12 RAPD primers yielded 110 polymorphic bands. Ecrisli *et al.* (2007) used 76 primers to generate RAPD profiles,15 out of 76 primers produced good and reproducible polymorphic bands, out of 88 fragments bands generated 13 fragments were monomorphic and 75 were polymorphic.

Scoring of Bands and Development of RAPD profiles for varietal identification

RAPD primers were scored for the presence (1) and absence (0) for the corresponding band among the cultivars.Cultivars Specific Bands in RAPD profiles using different set of primers can be used for the identification of cultivars.RAPD profile developed using OPF 1 primer reveals total 7 bands on the

Table 1: PCR amplification programme for RAPD

Sl no	Conditions	Temperature	Duration
1	Initial Denaturation	94ºC	5 min
2	Denaturation	94ºC	0.5 min
3	Annealing	36ºC	1 min
4	Extention	72ºC	2.5 min
5	Final Extension	72ºC	7 min
6	Final hold	4ºC	Forever
7	Total cycles	35	-

Table 2: List of RAPD primers used, sequence of primers and their melting temperature

Sl no	Primer name	Sequence [5′ 3′]	Tm
1	0PF-1	ACGGATCCTG	32
2	0PF-3	CCTGATCACC	32
3	0PF-4	GGTGATCAGG	32
4	0PF-5	GGTGATCAGG	32
5	0PF-6	GGGAATTCGG	32
6	0PF-7	CCGATATCCC	32
7	0PF-8	GGGATATCGG	32
8	OPF-9	CCAAGCTTCC	32
9	OPF-10	GGAAGCTTGG	32
10	OPF-11	TTGGTACCCC	32
11	OPF-12	ACGGTACCAG	32
12	OPF-13	GGCTGCAGAA	32
13	OPF-14	TGCTGCAGGT	32
14	OPF-15	CCAGTACTCC	32

Table 3: List of RAPD primers, primer's sequence, total no of bands obtained and size of amplicons

S No.	Primer Name	Primer Sequence 5'- 3'	Total No Bands Obtained	Size of amplicons(bps)
1	OPF-1	ACGGATCCTG	7	550-2500
2	OPF-3	CCTGATCACC	11	550-6000
3	OPF-4	GGTGATCAGG	8	600-1800
4	OPF-5	CCGAATTCCC	9	300-2000
5	OPF-6	GGGAATTCGG	11	400-3000
6	OPF-7	CCGATATCCC	10	800-3500
7	OPF-8	GGGATATCGG	10	350-2200
8	OPF-9	CCAAGCTTCC	10	1100-5000
9	OPF-10	GGAAGCTTGG	9	400-1900
10	OPF-11	TTGGTACCCC	6	400-1900
11	OPF-12	ACGGTACCAG	3	700-1300
12	OPF-13	GGCTGCAGAA	7	600-3000
13	OPF-14	TGCTGCAGGT	4	300-2300
14	OPF-15	CCAGTACTCC	9	300-2500

Table 4: Typical band combinations for varietal identification

Cultivars	Typical band combination
Jalore Seedless	OPF 5 (bp 1400)+ OPF 7 (bp 3500)+ OPF 10 (bp 1800)
Ganesh	OPF 4 (bp 1800)+ OPF 10 (bp 1800)
G-137	OPF 4 (bp 1800)+ OPF 9 (bp 1100)
Khog	OPF 1 (bp 600)+OPF 4 (bp1800))+ OPF 5 (bp 600)
Mridula	OPF 4 (bp 1800)+ OPF 6 (bp 3000)+ OPF 7(bp 3500)+OPF 9 (bp 1100)
Bassein Seedless	OPF 1 (bp 1000)+ OPF 5 (bp 1400)+ OPF 9 (bp 1100)
PhuleArakta	OPF 5 (bp 600) + OPF 8 (bp 2000)
GKVK-1	OPF 5 (bp 600) + OPF 7(bp 3500)+ OPF 10 (bp 1800)+ OPF 15 (bp 900)
Dholka	OPF 5 (bp 600)+OPF 6(bp 3000) + OPF 7 (bp 3500)

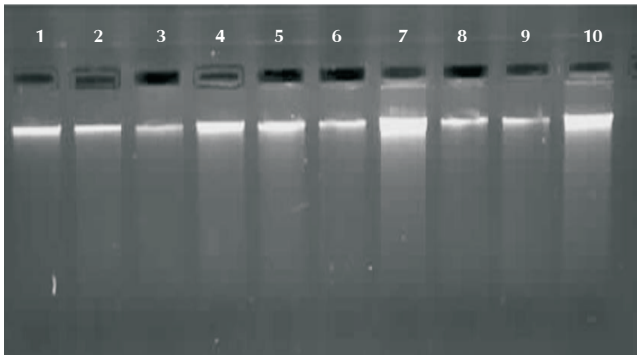

Figure 1: DNA of ten genotypes of Pomegranate

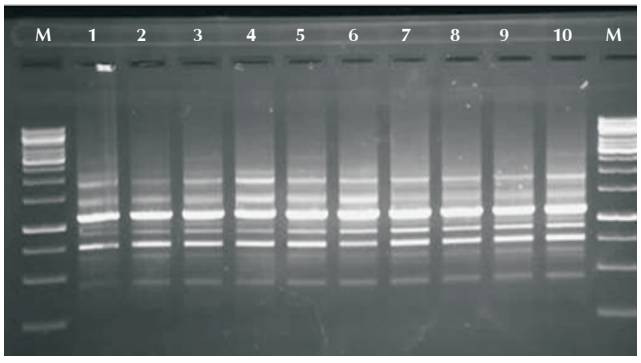

Figure 2: RAPD patterns of bands obtained in ten genotypes of pomegranate using primer OPF 6. Lane M = DNA size marker (1KB) *(1 = Jalore Seedless, 2 = Ganesh, 3 = G-137,4 = Khog, 5 = Mridula, 6 = Bassein Seedless, 7 = Bhagwa , 8 = Phule Arakta, 9 = GKVK-1, 10 = Dholka)

composite profile which ranged from 550bp-2500bp, band corresponding bp 1000 showed variability, since, this band was recorded only in genotype Khog and Bassein Seedless, hence, this band can be used to these two cultivars. A total of 8 bands on the composite profile which ranged from 600bp-1800bp were obtained using primer OPF 4.The band corresponding bp 1800 present in four cultivars *viz.*, Ganesh, G-137, Khog and Mridula. The band corresponding bp 600 was present in four cultivars (Khog, PhuleArakta, GKVK-1 and Dholka). Hence, such bands or band combinations can be used to identify cultivars.RAPD profile using OPF 5 primer reveals that a total of 9 bands which ranged from 300bp-2000bps. The bands corresponding to bps 300, 650, 800, 1200 and 1600 were present in all the genotypes. The bands corresponding bps 600, 1400, 1800 and 2000 showed variability. The band corresponding bp 600 was present in four cultivars (Khog, PhuleArakta, GKVK-1 and Dholka). Hence, this band was considered as a typical band for these cultivars and can be used identify these four cultivars from the rest of cultivars. The band corresponding bp 1400 was present in Jalore Seedless, Khog and Bassein Seedless.RAPD profile developed by primer OPF 6 reveals 11 bands on the composite profile which ranged from 400bp-3000bp. The band corresponding bp 3000 was only present in cultivars Mridula and Dholka. Hence, this band can be considered as typical band and can be used to identify these two cultivars. 10 bands on the composite profile which ranged from 350bp-2200bp were obtained in RAPD profile using OPF 8 primer. The band corresponding bp 2000 was present only in Phule Arakta. Hence, this band was considered as typical band to identify cultivar Phule Arakta. RAPD profile using OPF 9 primer reveals total of 10 bands appeared on the composite profile which ranged from 1100bp-5000bp.The band corresponding to bp 1100 was present in cultivars G-137, Khog, Mridula and Bassein Seedless, can be used for varietal identification. OPF

10 primer reveals total 9 bands on the composite profile which ranged from 400bp-1900bp.The band corresponding to bp 650 was present only in Khog. Hence, this band was considered as the typical band for the identification of cultivar 'Khog' from the rest of cultivars. The band corresponding to bp 1800 was present only in Jalore Seedless, Ganesh, G-137, Khog and GKVK-1 cultivars.

Based on the RAPD profiles generated, it was recorded that bands produced from primers OPF 11, OPF 12, were present in all taxa under study. Hence, these primers have low value for varietal identification. However, the other primers in combination can help in varietal identification (Table 4.)

RAPD markers have been employed to understand various aspects such as assessment of genetic diversity, genome mapping and systematics of by different workers from time to time. A low level of polymorphism was observed among 28 Iranian pomegranate genotypes, due to clonal or vegetative propagation of the cultivars tested (Talebi Bedaf *et al.*,2003). Toor *et al.* (2005) carried out analysis of 20 accessions of date palm using RAPD markers. Forty-seven decamer primers of OPA, OPB and OPC series were used, 34 decamer primers were selected for final RAPD analysis and screened on the basis of easily scorable and repeatable amplification products over two replications. A total of 191 amplicons were obtained with 34 primers with an average of 5.61 bands per primer. Out of these 191 bands, 159 (83.24%) were found to be polymorphic. Most of the primers produced fragments below 1.5 kb range, though a few amplicons crossed 3 kb range. Dehesdtani *et al.* (2007) evaluated the genetic diversity of *Citrus spp.* using RAPD marker. Four of 21 random primers produced reproducible polymorphic bands. A total of 864 fragments of size 150 to 2100 b.p. were obtained, 70.13 per cent were found to be polymorphic. Singh *et al.* (2007) studied genetic relationship among 50 *ber*genotypes representing species Z. *mauritiana,* Z. *nummularia*and Z. *spina-christi.* Out of 120 primers initially tested, 46 were highly reproducible and generated 368 bands with 86.2 per cent polymorphism (316 polymorphic bands). The number of amplified product per primer ranged from 2 (OPF-9) to 17 (OPO-3) with an average of eight bands per primer. Sheidai*et al.* (2008) studied the genetic variations in 11 pomegranate genotypes using 15 RAPD markers. Thirteen primers were found polymorphic producing a total of 173 bands out of which 73 bands were common in all cultivars, while six bands were specific, which could be used in the cultivar discrimination. Primers OPB12 and OPA13 produced the highest number of polymorphic bands (12 bands out of 16 = 0.75% and 11 bands out of 25 = 0.44%), while primers OPR15 and OPA15 produced the least number of polymorphic bands (2 out of 12 = 0.16%). Molecular screening in the near isogenic lines in rice cultivars for bacterial leaf blight resistance in rice was carried out by Ankita A. Patel *et al.* 2015 showed total 261 bands with average 3 alleles were amplified per primers, amplicon size range between 113- 400 bps. .This PCR based technique offers numerous advantages like wider applicability, accuracy and speed of estimation, less costly and could be an ideal tool for the cultivar identification.Thus, the foregoing research is a recourse to the modern methods wherein RAPD profiles could serve as an ideal tool for cultivar identification, characterization and in appraising systematics of pomegranate crop.

REFERENCES

Ankita, A., Patel, Jadega., Gunvanth, C., Patel Heli, J., Prajapati Vijay, I., Zala Harshvardhan, N., Shah, K., Kumar, P. and Zaman, M. 2015. Molecular Analysis of Bacterial Leaf Blight resistance XA 5 gene in near Isogeneic lines in Rice (*Orzyasativa* L.). *The Ecoscan.* **9(1):** 635-641.

Dehesdtani, S. K., Kazemitabar and Rahimian, H. 2007. Assessment of genetic diversity of navel sweet orange (*Citrus sinensis*,Navel) cultivars grown in Mazandaran Province using RAPD markers. *Asian J. Plant Sci.* **6:** 1119-1124.

Ercisli, S., Agar, G., Orhan, E., Yildirim, N. and Hizarci, Y. 2007. Interspecific variability of RAPD and fatty acid composition of some pomegranate cultivars (*Punicagranatum* L.) growing in Southern Anatolia Region in Turkey. *Biochem. Syst. Ecol.* **35(11):** 764-769.

Ercisli, S., Gadze, J., Agar, G., Yildirim, N. and Hizarci, Y. 2011. Genetic relationships among wild pomegranate (*Punicagranatum L.*) genotypes from Coruh Valley in Turkey. *Genet. Mol. Res.* **10(1):** 459-464.

Harlan, J. R. 1992. Crops and Man, 2nd Edn.*American Society of Agronomy and Crop Science Society of America*, Madison, WI, p. 295.

Jambhale, V. M., Patil, S. C., Pawar, S. V., Jadhav, A. S., Patil, H. E. and Dahake, K. D. 2007. Molecular characterization of pomegranate cultivars with RAPD markers. *Asian J. Hort.* **2(1):** 176-179.

Levin, G. M. 1994. Pomegranate (*Punicagranatum*L.) plant genetic resources in Turk-menistan.*Plant Genet. Res.Newsletter.* **97:** 31-36.

Melgarejo, M. P. and Martinez, V. R. 1992. El Granado. *Ediciones Mundi-Prensa*, Madrid, S.A., p. 163.

Sarkhosh, A., Zamani, Z., Fatahi, R. and Ebadi, A. 2007. RAPD markers reveal polymorphism some Iranian pomegranate (*Punicagranatum L.*) genotypes. *Sci. Hortic.* **111(1):** 24-29.

Sarkhosh, A., Zamani, Z., Fatahi, R. and Ranjbar, H. 2009. Evaluation of genetic diversity among Iranian soft-seed pomegranate accessions by fruit characteristics and RAPD markers. *Sci. Hortic.* **121:** 313-319.

Sheidai, M., Noormohammadi, Z., Saneghi, A. and Shahreiyari, Z. H. 2008. RAPD analysis of eleven Iranian pomegranate (*Punicagranatum* L.) cultivars. *Acta Biol. Szege.* **51(1):** 61-64.

Singh, D., Sharma, P., Singh, A.K., Singh, R. and Singh, N. K. 2007. Molecular profiling and genetic relationship among ber (*Ziziphus sp.*) genotypes using RAPD markers. *The Indian J. Genet. Plant Breed.* **67(2):** 121-127.

Smith, P. M. 1976. Minor crops. In: Simmonds, N.W. (Ed.), *Evolution of Crop Plants*, Longman, London, pp. 301-324.

Talebi, B. M., Shareifi, B. and Bahar, M. 2003. Analysis of genetic diversity in pomegranates cultivars of Iran using random amplified polymorphic DNA (RAPD) markers. In: Proceedings of *3rd National Congress on Biotechnology*, volume 2, Mashad, Iran. **2:** 343-345.

Toor, A. K., Sharma, R., Singh, G. and Mohapatra, T. 2005. Genetic variation in date palm (*Phoenix dactylifera* L.) cultivars assessed using RAPD markers. *Indian J. Genet. Plant Breed.* **65(4):** 335-336.

Verma, N., Mohanty, A. and Lal, A. 2010. Pomegranate genetic resources and germplasm conservation: a review. In: Chandra, R (Ed.), Pomegranate. *Fruit, Veg. Cereal Sci. Biotech.* **2(4):** 120-125.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Res.* **18(15):** 6531-6535.

Yang, R. P., Long, W. H., Zhang, H., Xu, B. and Li, W. X. 2007.

RAPD analysis of 25 *Punicagranatum*germplasm resources collected in Yunnan province. *J. Fruit Sci.* **2:** 226-229.

Zamani, Z., Sarkhosh, A., Fatahi, R. and Ebadi, A. 2007. Genetic relationships among pomegranate genotypes studied by fruit characteristics and RAPD markers. *J. Hortic. Sci. Biotechnol.* **82(1):** 11-18.

Zhang, Y. P., Tan, H. H., Cao, S. Y., Wang, X. C., Yang, G. and Fang, J. G. 2012. A novel strategy for identification of 47 pomegranate (*Punicagranatum* L.) cultivars using RAPD markers. *Genet. Mol. Res.* **11:** 3032-3041.