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Research Article
DNA Fingerprinting of *in vitro* Micropropagated Pomegranate Genotypes

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Abstract

Background and Objective: Pomegranate is grown for its rich flavour in numerous tropical and subtropical areas, like Egypt and the Kingdom of Saudi Arabia (KSA). Assessing the genetic background of the pomegranate is the key to its expansion through the Middle East, where tissue culture reproduction strategies could be used to solve environmental and economic problems. This study aimed at studying the genetic stability of 2 pomegranate genotypes *in vitro* micro-propagated in the Kingdom of Saudi Arabia by using the random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) and inter simple sequence repeats (ISSR) tools. **Materials and Methods:** The two above mentioned molecular tools were used to evaluate the DNA fingerprints of Taify and Yemeni pomegranate genotypes 12 weeks post *in vitro* propagation in Taif, Kingdom of Saudi Arabia compared to the mother plant. Shoot tip explants of 4-5 cm long were grown on Murashige and Skoog (MS) medium supplemented by 1.0 mg L^{-1} NAA, 2.00 mg L^{-1} IBA and 2 g L^{-1} activated carbon for 4 weeks for rooting. On 12 weeks DNA extracts were prepared from the acquired plantlets obtained and used as templates for each of RAPD-PCR and ISSR tools. **Results:** The RAPD-PCR and ISSR assays generated a total of 79-94 and 57-72 DNA fragments, respectively. In case of RAPD-PCR 80 and 90% of the primers used and developed monomorphic fragments of the Yemeni and Taify genotypes, respectively, particularly OPG08 primer for Taify genotype and OPA04 and OPD07 primers for the Yemeni genotype. Regarding ISSR, no DNA polymorphic for the micropropagated clones were recorded compared to the mother plant. **Conclusion:** The ISSR assay's findings indicated the genetic homogeneity between the *in vitro* micropropagated clones of both pomegranate genotypes and the mother plants.

Key words: Pomegranate, micropropagation, molecular tools, genetic homogeneity, fingerprinting, random amplified polymorphic DNA, polymerase chain reaction

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Since the ancient times and throughout various countries including the Middle East, the pomegranate (*Punica granatum* L.) has been cultivated and that leads to the presence of numerous unique region-specific genotypes, where the largest pomegranate growing country in the world is India[1]. The medicinal applications of the pomegranate attributed to all parts of the tree including fruit, leaves, flowers and roots[2]. Pomegranates have exceptionally high antioxidant capacity due to the high concentration and chemical composition of phenolic compounds[3]. These phenolic compounds have confirmed activities such as antimicrobial, anticancer and anti-arteriosclerotic behaviours[4].

The Punicaceae family consists of one genus, *Punica* and 2 species: *P. granatum* and *P. protopunica*[5]. Pomegranate has several essential medical uses and is of economic importance to the countries where it grows. Substantially increasing the production of pomegranate will have a positive impact on medical treatment and will raise the pomegranate's economic value. Plant tissue culturing in general and micropropagation in particular are practical approaches for the broad-ranging propagation of plants[6]. In order to effectively produce fruit varieties with enhanced desirable qualities, genotyping studies of pomegranate varieties from various regions are required[7]. Plant tissue culturing is a biotechnology strategy that has the capacity to generate plants in large quantities, although it requires substantial time and cost. Somatic variations, though, are a problem in the micropropagation of several plant species.

However, the downside of the micropropagation procedure is the somaclonal variations between all the subclones derived from older parent plants. Moreover, plantlets extracted from *in vitro* cultures may have somaclonal variations that are often inherited[8]. Researchers have documented several procedures for the regeneration of pomegranate (*P. granatum* L.)[9] and it has been shown that most of the popular protocols for organogenesis uses seedlings derived from plant materials[10]. Thus, genetic studies are necessary to improve the *in vitro* propagation of pomegranate cultivated in Taif province, KSA[11].

An efficient system for the *in vitro* propagation of 2 Iranian pomegranate cultivars (Malas Saveh and Yousef Khani) has been developed through the use of shoot-tip and nodal explants[12]. Random amplified polymorphic DNA (RAPD) is one of the simplest methods used to assess genetic variation in plants[13]. Another, more efficient technique for detecting genetic variations is the use of an inter-simple sequence repeats (ISSR) assay[14,15]. Additionally, *in silico* polymerase chain reaction (PCR) analysis is a technique where the sequence of PCR primers and the published whole genome of a plant species are used to identify the physical location of the chromosomal regions targeted by primers[16]. This analysis can be used to infer PCR primers' genome coverage and nearby or adjoining genes can be targeted through a marker assay[16].

Genotyping studies of pomegranate in different regions are necessary for both the appropriate selection, cultivation and advertising of desirable attributes of fruits[7]. On the hand, plant tissue culture is a biotechnology technique has the ability to produce plants with very large amount in considerable time and cost. However, the somatic variations can be considered as an issue in the micropropagation of some plants[11,12]. Using shoot tip and nodal explants *in vitro* propagated an efficient system for *Punica granatum* L. of 2 Iranian pomegranate genotypes (Malas Saveh and Yousef Khani)[12]. One of the simple methods used for studying the genetic variation is the random amplified polymorphic DNA[13,15].

Another technique with more efficiency for detection of genetic variation is inter simple sequence repeats[16]. Possible genetic changes in the genome of the micro-propagated plantlets among the subsequently sub-culturing could be done, therefore, this study aimed to determine the DNA fingerprinting of plantlets of two pomegranate genotypes (Taify and Yemeni) 12 weeks post *in vitro* micropropagated, were investigated by both of RAPD-PCR and ISSR.

MATERIALS AND METHODS

It is worth noting that the experimental part of this study was carried out at the Scientific Research Center at Taif University, KSA during the period of 2018-2019.

Plant material: Two healthy pomegranate (*P. granatum* L.) genotypes (Taify and Yemeni) were collected from the Taif region, KSA. All the juvenile branches as well as the scale leaves were excluded. The shoot-tip explants were washed with running water to eliminate dust, superficially disinfected with alcohol (70%) for 1 min. Then incubated in 0.1% mercuric chloride (Hg_2Cl_2)[17] for 5 min and then rinsed with sterilised distilled water.

Micropropagation: The shoot-tip explants of the two pomegranate genotypes were cultivated in a shoot-induction medium for 4 weeks. By subculturing the propagated shoots with the same Murashige and Skoog (MS) medium[18], the shoot proliferation of the various subcultures

(10 subcultures, five weeks for each) and elongation periods (four weeks) were calculated. For the rooting phase, shoots with a length of 4-5 cm was cultivated in the MS medium. The plantlets were grown in pots containing sterile soil and kept for adaptation in a managed greenhouse. All cultures were incubated at 26±2°C, with a 16 h/8 h light/dark cycle and under cool white fluorescent light at a 3000 lux light intensity.

Isolation of DNA: Genomic DNA was extracted from young, micropropagated pomegranate leaves by approach described in Bousquet *et al.*[19]. The validity of the collected DNA was tested by electrophoresis of the gel and maintained at -20°C.

RAPD and ISSR: For determining the genetic stability of micropropagated pomegranate plantlets, ten arbitrary 10-base RAPD-PCR primers (OPA04, OPA06, OPB05, OPB07, OPC08, OPC09, OPD07, OPG08, OPK05 and OPO02) were used according to the protocol by Williams *et al.*[20]. RAPD reactions were conducted in triplets in 25 µL reaction volume and DNA concentration of 100 ng μL^{-1}. The PCR program was 94°C for 4 min (1 cycle), 35 cycles where each cycle was: 94°C for 45 sec, annealing at 35 °C for 1 min and elongation at 72°C for 1 min. The final elongation step was extended for 10 min and then kept at 4°C till electrophoresis analysis.

For the ISSR analysis, ten ISSR primers [$(AG)_8YC$, $(AG)_8YG$, $(AC)_8YT$, $(AC)_8YG$, $(GT)_8YG$, $CGC(GATA)_4$, $GAC(GATA)_4$, $(AGAC)_4GC$, $(GATA)_4GC$, $(GACA)_4AT$] were used for determining the genetic stability of micropropagated pomegranate plantlets following the method of Chandrika *et al.*[15]. ISSR reactions were conducted in triplets in 25 µL reaction volume using the same DNA template concentration. PCR conditions were: 1 cycle at 94°C for 5 min, 35 cycles each cycle was as follow: denaturation at 94°C for 1 min, annealing temperature at 44°C for 1 min and elongation at 72°C for 1 min and 1 cycle at 72°C for 10 min.

Electrophoresis: In 1.5% agarose gel supplemented with (0.5 µg mL^{-1}) ethidium bromide in 1×TBE buffer the amplified PCR products of RAPD and ISSR analysis were separated. PCR products were visualized on UV transilluminator and photographed using a gel documentation system[21].

PCR analyses: Both of RAPD-PCR and ISSR analyses, was done at the Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt. For analyses and interpretation, the amplified bands were scored as (0) absence and (1) for presence. Unstable (non-reproducible) and weak bands were omitted from the analysis. The genetic identity index was used to describe the genetic similarity between micropropagated plantlets and to hierarchically identify clones throughout UPGMA model using the NTSYS software[22]. The index of genetic identities was used to determine genetic similarity among the micropropagated plantlets and to hierarchically classify the clones according to UPGMA model, using the software NTSYS[22].

RESULTS

In this study two pomegranate genotypes (Taify and Yemeni) were micropropagated by the shoot-tip explant process (Fig. 1) up to 52 weeks. The DNA fingerprints of 20 micropropagated plantlets, 10 for each genotype were determined by using RAPD-PCR and ISSR molecular tools. Results of RAPD-PCR analysis showed presence of some differences between Taify and Yemeni pomegranates, as shown in Table 1 and Fig. 2.

A total numbers of 94 and 79 fragments were generated from the DNA templates of the micropropagated Taify and Yemeni plantlets, respectively. The molecular weights of the DNA fragments of RAPD analysis were ranged from 234.1-1875.5 bp for Taify genotype and from 346.4-4372.1 bp

Table 1: Characters of DNA amplified fragments generated via RAPD-PCR analysis of 10 *in vitro* micropropagated clones of Taify and Yemeni pomegranate genotypes

	Types of DNA amplified fragments									
	Taify					Yemeni				
RAPD-PCR primers	TADF	M_W range	MF	PF	UF	TADF	M_W range	MF	PF	UF
OPA04	06	234.1-805.6	06	00	00	14	748.3-4372.1	04	01	09
OPA06	06	597.5-1392.1	06	00	00	07	346.4-2498.6	07	00	00
OPB05	07	663.8-1514.3	07	00	00	06	437.9-2649.3	06	00	00
OPB07	09	405.9-1002.1	09	00	00	08	402.9-2091.9	08	00	00
OPC08	09	419.9-0927.4	09	00	00	09	437.7-1723.3	09	00	00
OPC09	11	388.3-1796.5	11	00	00	11	561.3-1922.4	11	00	00
OPD07	10	358.7-1883.3	10	00	00	09	521.7-0714.5	07	00	02
OPG08	17	347.1-1925.2	11	03	03	06	476.9-1958.2	06	00	00
OPK05	10	498.8-1681.4	10	00	00	05	747.5-1859.2	05	00	00
OPO02	09	482.6-1875.7	09	00	00	04	606.5-1449.7	04	00	00
Total	94	234.1-1875.7	88	03	03	79	346.4-4372.1	67	01	11

TADF: Total amplified DNA fragments, M_W: Molecular weight, MF: No. of monomorphic, PF: No. of polymorphic, UF: No. of unique fragments

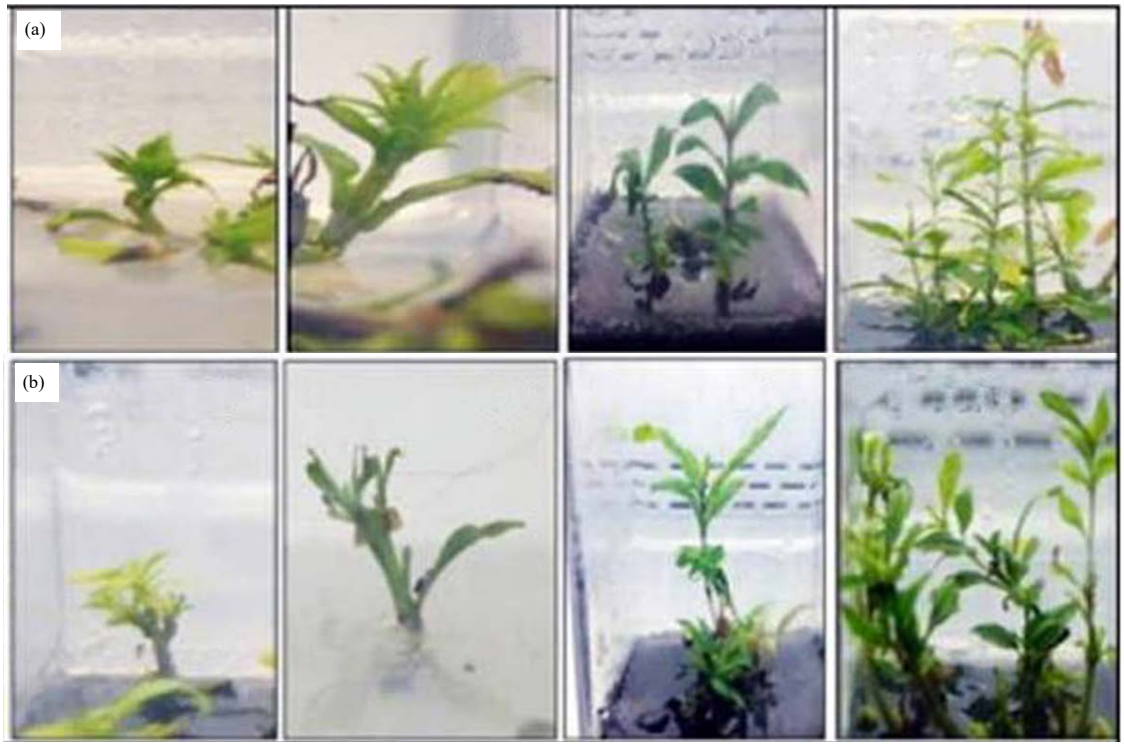

Fig. 1(a-b): Micropropagation steps of two pomegranate genotypes (a) Taify and (b) Yemeni

Table 2: Characters of DNA polymorphisms generated via ISSR-PCR analysis of 10 *in vitro* micropropagated clones of Taify and Yemeni pomegranate genotypes

	Types of DNA amplified fragments									
	Taify					Yemeni				
ISSR primers	TADF	M_W range	MF	PF	UF	TADF	M_W range	MF	PF	UF
ISSR-P01	05	521.9-1081.2	05	00	00	05	384.0-1426.9	05	00	00
ISSR-P02	05	374.7-0988.3	05	00	00	07	340.9-1691.6	07	00	00
ISSR-P03	05	328.9-0794.9	05	00	00	06	462.7-1606.5	06	00	00
ISSR-P04	06	413.1-0983.1	06	00	00	08	317.8-1866.0	08	00	00
ISSR-P05	05	349.8-1024.4	05	00	00	09	453.7-2016.2	09	00	00
ISSR-P06	06	579.8-1505.2	06	00	00	10	553.1-2255.9	10	00	00
ISSR-P07	04	717.5-1029.3	04	00	00	07	370.4-2848.9	07	00	00
ISSR-P08	05	523.4-1029.2	05	00	00	07	381.9-2494.7	07	00	00
ISSR-P09	05	708.5-1177.7	05	00	00	07	465.9-2268.5	07	00	00
ISSR-P10	11	496.4-1533.3	11	00	00	06	260.9-1500.7	06	00	00
Total	57	328.9-1533.3	57	00	00	72	260.9-2848.9	72	00	00

TADF: Total amplified DNA fragments, M_W: Molecular weight, MF: No. of monomorphic, PF: No. of polymorphic, UF: No. of unique fragments

for the Yemeni variety (Table 1). At the level of fragments type of RAPD-PCR, for Taify genotype, 88, 3 and 3 monomorphic, polymorphic and unique fragments, respectively, were recorded. Regarding the Yemeni genotype, 67, 1 and 11 monomorphic, polymorphic and unique fragments, respectively, were recorded (Table 1).

The RAPD findings showed that 80 and 90% the used primers produced monomorphic bands using the templates of Yemeni and Taify genotypes, respectively. This was obvious from the fragments generated by using OPG08 primer for Taify genotype and OPA04 and OPD07 primers for the Yemeni genotype. This was approved whereas OPG08 primer generated 11, 3 and 3 monomorphic, polymorphic and unique fragments. The OPA04 primer produced 4, 1 and 9 monomorphic, polymorphic and unique fragments. OPD07 produced 7 and 2 monomorphic and unique fragments (Table 1 and Fig. 2). The average frequency of each RAPD-PCR primer was 9.4 and 7.9, for Taify and Yemeni pomegranate genotypes, respectively.

ISSR data illustrated by Table 2 and Fig. 3 showed the genetic stability of both the Taify and Yemeni genotypes as no polymorphism was found compared to mother plants using

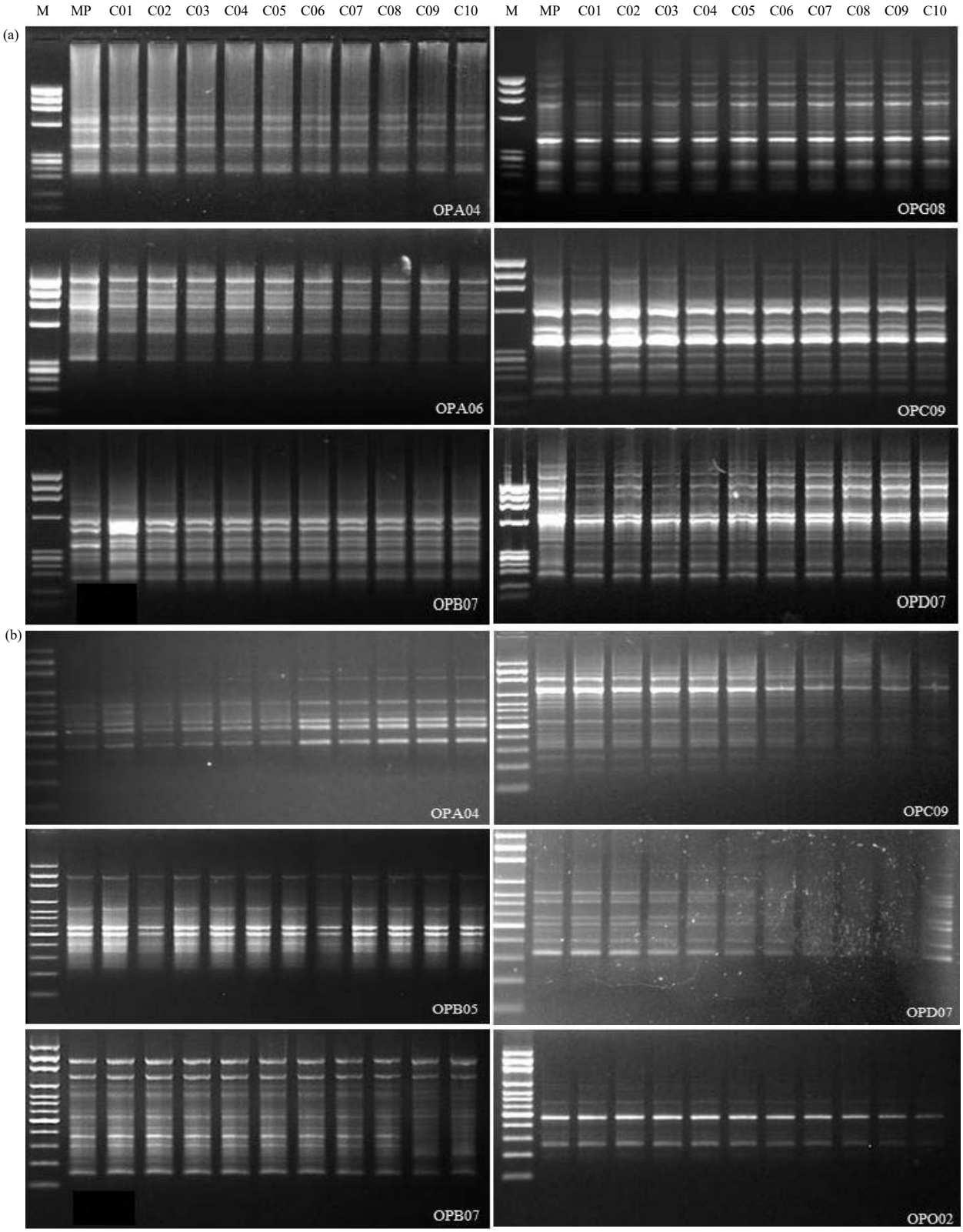

Fig. 2(a-b): Agarose gel electrophoresis of DNA polymorphisms generated by 12 RAPD-PCR primers belonging to 6 Operon groups (A, B, C, D, G and O) using the DNA templates of 10 *in vitro* micropropagated clones (C01-C10) of (a) Taify and (b) Yemeni pomegranate genotypes

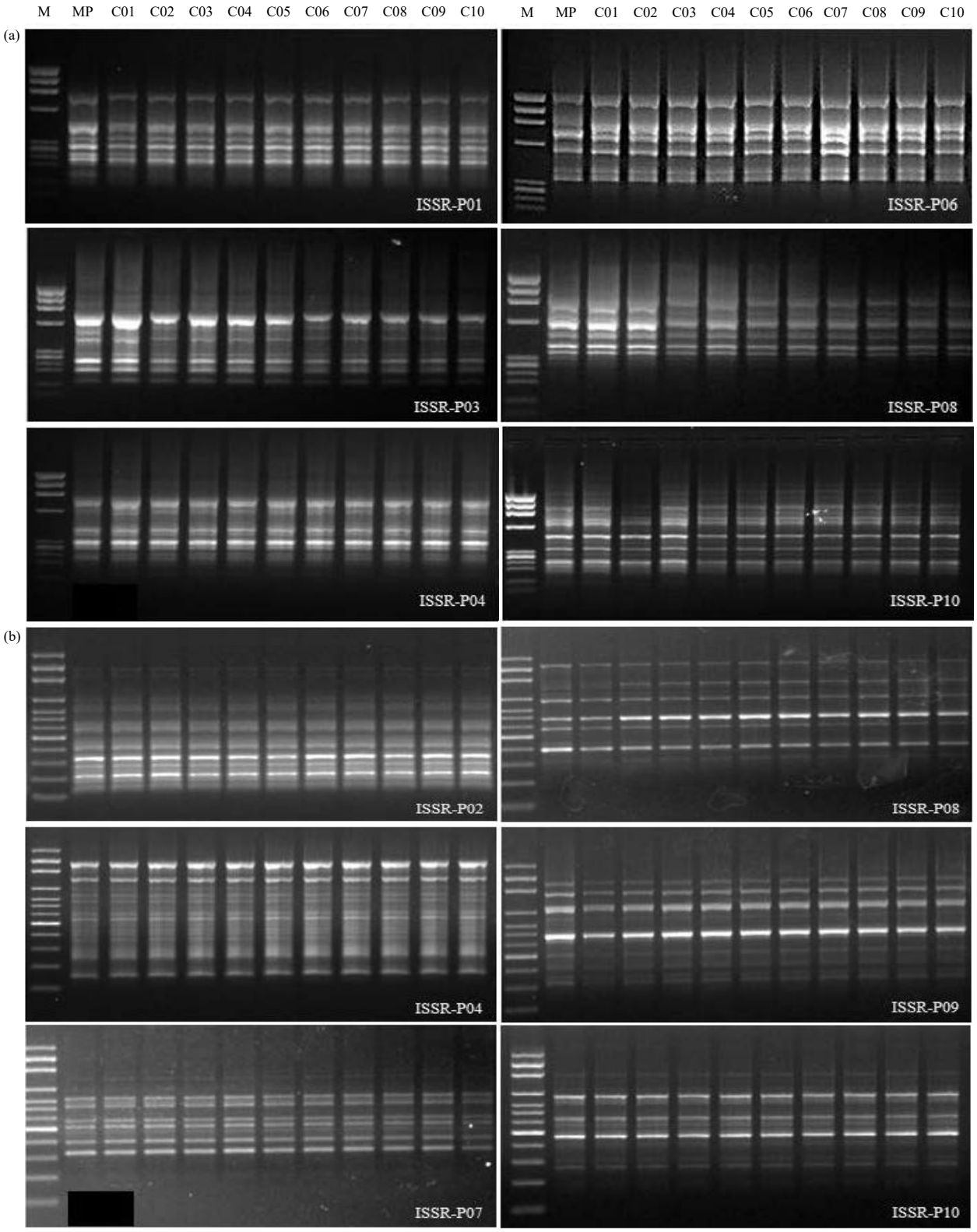

Fig. 3(a-b): Agarose gels electrophoresis of (1.5%) stained with ethidium bromide shows the DNA polymorphisms of DNA extracts of 10 *in vitro* micro-propagated clones (C01-C10) of (a) Taify and (b) Yemeni pomegranate genotypes using 12 ISSR-PCR primers

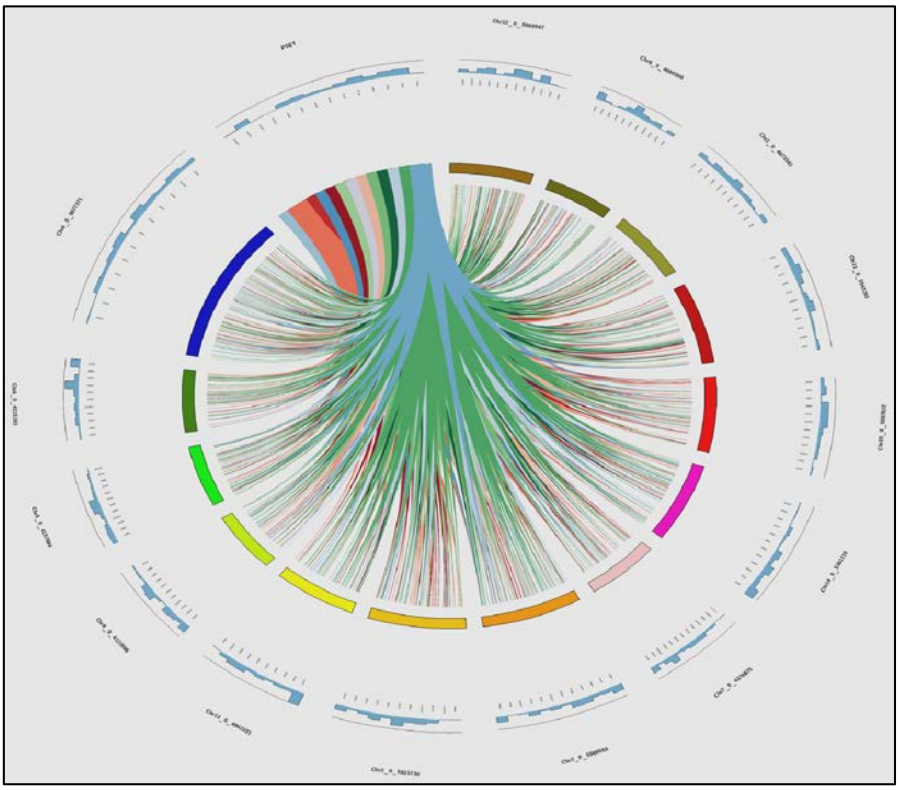

Fig. 4: Pomegranate genome, where the colored links depict targeted genomic regions using the RAPD and ISSR primers as produced through *in silico* PCR analysis using KASPspoon software

ISSR primers. Results showed that ISSR produced 57 fragments with the Taify genotype ranging from 328.9-1533.3 bp. While the Yemeni genotype produced 72 fragments in sizes ranging from 260.9-2848.9 bp (Table 2).

About 656,115 bp (0.9%) of the pomegranate genome have been covered using the primer set used in this study, with the $(AG)_8$YG primer covering the longest genomic region (0.1%) of the total genome.

All PCR primers of this study relying on the published genome of pomegranate (Fig. 4). The molecular analysis revealed that 14 primers generated hits in the pomegranate genome, while other primers did not revealed any hits.

DISCUSSION

Researchers have documented several procedures for the regeneration of pomegranate (*P. granatum* L.)[9]. Chauhan and Kanwar[10] reported that most of the popular protocols for organogenesis uses seedlings derived from plant materials. Thus, genetic studies are necessary to improve the *in vitro* propagation and preservation of pomegranate cultivated and grown in Taif Province, KSA[11].

An effective micropropagation protocol was developed by El-Dessoky *et al.*[11] for the two pomegranate genotypes (Taify and Yemeni) in the KSA. This protocol was successfully applied for micropropagation of the same two pomegranate genotypes by the shoot-tip explant process up to 52 weeks.

RAPD and ISSR were recorded to be easy tools among several molecular markers, which make them efficient tools when screening for DNA polymorphism[11,13]. However, the use of RAPD markers can result in questionable reproducibility[15,23,24]. In this study the same molecular tools, i.e., RAPD and ISSR were applied to determine the DNA fingerprint of the two pomegranate genotypes (Taify and Yemeni) in the KSA that were micropropagated *in vitro* in four subcultures. Results showed that the number of total amplified fragments generated via RAPD-PCR (94 and 57 for Taify and 79 and 72 for Yemeni) was more than that produced among ISSR. Also, no unique DNA fragments were generated with ISSR compared to 14 fragments distributed 11 and 3 for Taify and Yemeni genotypes, respectively. RAPD was successfully exploited in the assessment of the genetic stability among many micropropagated plantlets[11,24].

The evaluation of the genomic stability of micropropagated plantlets through ISSR was recorded for several plants[14,25]. Dessoky *et al.*[11] evaluated the genetic stability of these micropropagated plants by using the same two molecular tools. The lack of polymorphic fragments in the ISSR analysis of the two pomegranate genotypes under investigation were verified the genetic uniformity between the *in vitro* micropropagated clones of the Taify and the Yemeni pomegranate genotypes compared to the mother plants. It is worth mentioning that the micropropagated Iranian pomegranate genotypes (Malas Saveh and Yousef Khani) have shown differences in growth and vegetative morphological characteristics compared to the parent plants[12]. Similar results have been conducted using SCoT and RAPD assays in banana, olives and grapes[24,26,27]. The hits in the pomegranate genome of the two genotypes of this study could be due to the genome sequence gap of next-generation sequencing. One can recommend that further studies should be done at the level of physiological and horticultural characters of the micropropagated plants, which could be reflect the possible changes in the genome of the micropropagated plants.

CONCLUSION

Two molecular tools were used to determine the DNA fingerprinting of two pomegranate genotypes (Taify and Yemeni) micropropagated via 12 subcultures. The ISSR assay's findings indicate genetic homogeneity between the *in vitro* micropropagated pomegranate and the mother plants. On the other direction, three RAPD-PCR primers of groups A, D and G proved the presence of genetic changes in the genome of the micropropagated plants compared to the mother plant.

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