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Identification of genetic diversity among mutant lemon and mandarin varieties using different molecular markers

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Abstract: Mandarin (*Citrus reticulate* Blanco) and lemon (*C.limon* L.) are among the most cultivated *Citrus* species in the world.Among different approaches, gamma radiation is widely used to induce mutations in citrus breeding studies. Gamma radiation causes DNA damage, randomly inducing several mutations in the genome. Molecular techniquesarewidely used to detect such induced mutations and genetic diversity in plants.In this study, simple sequence repeats (SSR), inter-simple sequence repeat (ISSR) and single-strand conformation polymorphism (SSCP) markers were used to detectthe induced mutations andgenetic diversityin someirradiated mandarin and lemon genotypes in comparison with standard citrus varieties. The irradiated genotypes used in this study derived from populationsthat had been originally obtained with gamma radiation with different gray doses applied on Yerli Mandarin and Antalya YerliYuvarlak lemon varieties. Out of 82 primers (22 SSCP, 30 ISSR and 30 SSR) tested, 55 primers (9 SSCP, 24 ISSR and 22 SSR primers) successfully amplified a total number of 363 amplicons.A single band with an approximate size of 500 nucleotides was determined using the primer ISSR 868 as differentiating between seedy parent and irradiated seedless mandarin genotypes. The polymorphism information content (PIC) values of SSCP, ISSR, and SSR markers ranged from 0.61–0.99, 0.31–0.96, and 0.33–0.96, respectively. Cluster analysis classified lemons and mandarins into 2 subgroups as mutant and commercial lemon genotypes. ISSR markers were found to be more effective to determine the genetic differences among the varieties in comparison to SSCP and SSR markers. The results clearly showed that SSCP, SSR and ISSR markers are important tools to distinguish mutant genotypes andconfirmed their usefulness for phylogenetic studies.

Keywords: Citrus, genetic differences, mutation breeding, seedlessness, thornlessness

1. Introduction

Citrus is one of the most important fruit crops with an increasing consumptionworldwide (Crowley, 2011). The most important species of citrus are sweet oranges, mandarins, lemons, and grapefruits (Mabberley, 2008). With the rise in consumer population, the demand for citrus fruit production has been increasing, and there has been a great deal of research aiming to develop new citrus varieties (Terolet al., 2008; Crowley, 2011). One of the main goals for the genetic improvement of fresh fruit in the world has been the development of varieties yielding fruits that are seedless, easy-peeling, flavorful and aromatic with a long ripening period (Raza et al., 2003). Also, the achievement of thornless trees is desirable because of their ease of handling and laborsaving advantages (Yoshida et al., 1999). Additionally, development of resistant varieties to biotic and abiotic stresses is very important for breeders.

Conventional breeding methods could be laborious, time-consuming, and expensive. Therefore, mutation breeding has been an alternative way of achieving new citrus cultivars. Mutation could be induced artificially with the help of various physical and chemical agents called mutagens (Lamo et al., 2017). Techniques such as radiation or chemical mutagens application are advantageous for increasing variability in crop species, since spontaneous mutations often occur with an extremely low frequency (Sutarto et al., 2009).

The first experiment on artificial mutation was carried out on citrus seeds using x-rays by Haskin and Moore in 1935 (Cameron and Frost, 1968). Afterwards, mutation

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based breeding approaches were used to improve some properties of fruit peel color (Chapot, 1975; Hensz, 1985; Usman et al., 2018), and to obtain seedless grapefruits (Usman et al., 2018), earlier grapefruit and orange varieties (Donini, 1982; Tang et al., 1994), highly fruitful and compact canopy oranges (Donini, 1982), seedless grapefruits (Hearn, 1984), seedless sweet oranges (Hearn, 1984; Huang et al., 2017), seedless or sparse seeded mandarins (Russo et al., 1981; Starrantino et al., 1988; Sutarto et al., 2009; Williams and Roose, 2010; Bermejo et al., 2011; Khalil et al., 2011; Bermejo et al., 2012; Goldenberg et al., 2014; Zhang et al., 2017), seedless and Mal Secco tolerant lemons (Gulsen et al., 2007), and seedless pummelos (Huang et al., 2003; Sutarto et al., 2009).

Detection of genetic differences, mutations, and phylogenetic relationship studies in citrus is important to ensure long-term success of citrus breeding program. Molecular markers have already been used for genetic characterization and improvement of many crop species. Recently, molecular and conventional breeding techniques are used together to obtain new varieties (Gentile and La Malfa, 2019).Molecular marker techniques are based on different principles, but they all can contribute to successfully identify the genome-wide variability (Polat, 2018). In this study, simple sequence repeats (SSR), inter-simple sequence repeat (ISSR) and single-strand conformation polymorphism (SSCP) molecular markers were used to identify the genetic differences in selected mutant citrus genotypes and in reference varieties.

SSCP technique is easy and efficient to detect any small alteration in a PCRamplified product (Yadav and Kale, 2009).SSCP is originally used for rapid mutation analysis and has advantages on mutation detection (Değirmenci and Kunter, 2008). A pair of SSCP primers was designed to amplify the coding region of gf-2.8 in 2salt–tolerant mutants and their parents (Wang et al., 2001). However, 22 candidate mutants of Thompson Seedless and KalecikKarası, selected on the basis of morphological observations with 46 control plants were analyzed with 15 SSCP markers. Unfortunately, polymorphic bands were rarely obtained from the SSCP analysis, and they were also not reproducible (DeğirmenciKarataş et al., 2010).

SSCP markers have been used for the determination of allelic diversity in genes responsible for Fe-chlorosis in citrus (Simsek et al., 2011;Aka Kacar et al., 2014). SSCP analyses were also conducted to detect small differences in sweet oranges (Mahmoudzadeh, 2013). However, this is the first study to use of SSCP molecular markers in lemon and mandarin mutation breeding.

SSR based molecular typing is one of the most widely used marker system in genetic relationship studies because SSR markers are transferable, highly polymorphic, useful, codominant, reproducible, effective, randomly and widelydistributed along the genome (Hussein et al., 2003; Biswas et al., 2011; Carrillo-Medrano et al., 2018). SSR markers have been the most commonly preferred markers in molecular biology for mapping (Chen et al., 2008; Gulsen et al., 2010) and genetic diversity analyses in citrus (Barkley et al., 2006; Barkley et al., 2009; Uzun et al., 2011; Kacar et al., 2013; Biswas et al., 2014; Polat, 2015; Carrillo-Medrano et al., 2018).ISSR based molecularcharacterization is carried out using a single marker (Capparelli et al., 2004) allowing a cost-effective mapping (Sankar and Moore, 2001), and it was successfully used to determine genetic diversity in citrus (Capparelli et al., 2004; Shahsavar et al., 2007; Uzun et al., 2010).

Recently, various SSR (Polat et al., 2015; Mallick et al., 2017) and ISSR (Chang Feng et al., 2008; Agisimanto et al., 2016) markers were used extensively to study phylogenetic relationships and differences among mutant citrus varieties. In the present study, genetic differences of some mandarin and lemon genotypes derived from the Yerli Mandarin and Antalya Yerli Yuvarlak lemon following gamma radiation as well as of some standard commercial citrus varieties were determined using ISSR, SSR, and SSCP markers.

2. Materials and methods

2.1. Plant materials

Nine stable mutant thornlessness lemons, oneparent [nonmutant Antalya Yerli Yuvarlak Limonlemon (L. Parent)], 34 stable mutantseedless mandarins,1 parent [nonmutant Yerli Mandarinmandarin (M. Parent)] obtained from a mutation breeding project (Turgutoğlu et al., 2014) were used. 3commercial lemon, 5 mandarin, 1 citron, 1tangelo and 1 tangor varieties were obtained from the Batı Akdeniz Agricultural Research Institute (BATEM) Citrus genetic gene pool, and were also included in the study as references.Stable mutant genotypes were chosen at the M_1V_3 stage and from different applications of ^{60}Co doses as shown in Table 1.

2.2. DNA isolation

Total genomic DNA was isolated from young leaves using the CTAB method as described by Doyle and Doyle (1990). The quality and concentration of the extracted DNAs were checked with a spectrophotometer (Thermo Scientific™ NanoDrop, V, Finland) and by 1% high resolution agarosegel electrophoresis.

2.3. SSCP analysis

Expressed sequence tags (ESTs) primers (Table 2) were developed by NCBI (National Center for Biotechnology Information) screening. Primer design was carried out using Primer 3 software (Rozen and Skaletsky, 2000), allowing the prevention of hairpin loops and dimers. PCR reactions for SSCP based molecular markers in citrus were performed according to the published protocol (Simsek et

Table 1. Plant materials, their cultivar or code name.

DNA no	Species	Variety or mutant	^{60}Co doses (Gray)	DNA no	Species	Variety or mutant	^{60}Co doses (Gray)
1	Lemon*	L. Parent	-	25	Mandarin	16-25	60
2	Lemon	1-1	60	26	Mandarin	16-33	60
3	Lemon	1-2	80	27	Mandarin	9-80	90
4	Lemon	1-3	80	28	Mandarin	12-75	120
5	Lemon	1-4	80	29	Mandarin	16-3	60
6	Lemon	1-5	80	30	Mandarin	8-100	100
7	Lemon	1-6	90	31	Mandarin	12-1	20
8	Lemon	1-7	90	32	Mandarin	16-16	60
9	Lemon	1-8	90	33	Mandarin	12-63	20
10	Lemon	1-9	90	34	Mandarin	8-89	100
11	Lemon	1-10	90	35	Mandarin	13-67	20
12	Mandarin**	M. Parent	-	36	Mandarin	1-3	100
13	Mandarin	12-60	120	37	Mandarin	1-5	100
14	Mandarin	12-13	20	38	Lemon	Kütdiken	-
15	Mandarin	12-3	20	39	Lemon	Interdonato	-
16	Mandarin	16-9	60	40	Lemon	Kaba limon	-
17	Mandarin	16-47	60	41	Citron	Etrog citron	-
18	Mandarin	11-50	120	42	Mandarin	Klemantin fina	-
19	Mandarin	11-74	120	43	Mandarin	Okitsu	-
20	Mandarin	12-66	120	44	Mandarin	Murcott	-
21	Mandarin	12-7	20	45	Tangelo	Orlando	-
22	Mandarin	13-49	20	46	Tangor	King	-
23	Mandarin	12-62	120	47	Mandarin	Nova	-
24	Mandarin	16-15	60	48	Mandarin	Robinson	-

*No mutant 'Antalya Yerli Yuvarlak Limon' lemon variety, **No mutant 'Yerli Mandarin' mandarin variety.

al., 2011; Aka Kaçar et al., 2014) with minor modifications. PCR amplification was conducted in 21 µL of reaction volume containing 1 µL each primer (0.3 µM), 8 µL of 2X master mix, 0.5 µL$MgCl_2$(2.5 mM), 0.05 µLTaq polymerase (0.6 U), and 50 ng of genomic DNA respectively. PCR conditions were conducted at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 1 min at 55°C, 72 °C for 1 min, and followed by a 6 min, final extension at 72 °C. The complete reactions were held at 4 °C by using Bio-Rad DNA-Engine Gradient Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). To obtain single-stranded DNA, 0.5 µl amplified the double-stranded PCR products in 9.8 ml Hi-Di Formamide and 0.2 ml LIZ-500 were separated by ABI 3130xl.

2.4. ISSR analysis

A total of 30 ISSR primers, previously evaluated by Fang and Roose (1997), Gulsen et al. (2010) and Chang Feng et al. (2008), were used for all genotypes as shown inTable 3. PCR reaction components and PCR amplification parameters were performed as described by Fang and Roose (1997) with some modifications. PCR amplification was carried out in 10 µl of reaction volume containing 0.3µM primer, 5 µL of 2X master mix, 2.5 mM $MgCl_2$, 0.6 U Taq polymerase and 50 ng of genomic DNA. PCR conditions were conducted at 94 °C for 2.5 min followed by 35 cycles of 94 °C for 45 s, 45 s at 48 °C, 72 °C for 1.5 min, and followed by a 10 min final extension at 72 °C. The completed reactions were held at 4 °C by using Bio-Rad DNA-Engine Gradient Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amplified PCR fragment was separated by 2% high resolution agarose gel electrophoresis in 1X TAE buffer (40 mMTrisAcetate, 1 mM EDTA, pH 8.0) for 3–4 h at 110 V using 1 kb and 100 bp DNA ladder (Vivantis). The fragment patterns were photographed

Table 2. List of SSCP primersand their bases sequences 5'-3'.

No	Forward	Reverse
1	GACCACTTTGATGGCTGTGA	GCAGAAGTCAGTAAACC
2	CATCACCAGCATATGGGACA	CAGAAGCGGCAAGAATGT
3	ATCGTGTTATCGCCATGGTT	CGTAACGTGCCAAAGTTTTT
4	CGTGTTGCCTGATTCATTTG	GCTTCAGGAATAACCCCAGA
5	AGTGAAATGCATTATCTGTTGCAG	TTATCAATATCTCTTGATTGCACA
6	CTGACCCCACTGAGATGGAT	CCCTTATTGCCATGATGCTT
7	CGTCGACTATGCCTGCTACA	GCAGGTCTCTTCACCGAGTC
8	CAAAATTCACGATCGCCTTT	AGGGCCAAACCTTTTCTCAT
9	TGGAATTGAAGGCAAAAAGG	TGCTCTTCTGGCAGTTCTCA
10	TGCTAGCTCCAAGGACAGGT	CGACCACAAGCTGATAGCAA
11	AGCAACTGGCTGGTCTTACAA	CCTCTTCGAATGGCTGAAAC
12	TTCTCTCTCAGGTTCCTTGTACTTT	GCTTTCTTTTCTATTTGTTTTCTGA
13	TGTTTCCCCTCTGCTTTCAC	ATTTGGCAAGGCCTCTCAG
14	AAGAAGAAATGGGGGAAACG	CTTGAGGCCATGGAGGATT
15	AATTCTAATTGGGGCCAACC	AATGCAAAATTCCGTGAACC
16	ACCGTTTGATCGAGTATGC	GCTGTCACTCCACCCGTAGT
17	AGCGTGCTCTCTCGTTAGAT	GAAGGATCATTGTCGAAACC
18	TCTGCAATTCACACCAAGTA	GGAAATCTAACGAGAGAGCA
19	CTGCAATTCACACCAAGTAT	CCAAGGAAATCTAACGAGAG
20	TTGCGTTCAAAGACTCGATG	CTGCGCCAAGGAAATCTAAC
21	TCAAAAATTCAAGGGTTCAC	GCCAAGGAAATATAACGAGA
22	AGCTCGAGAGGCTTTTGTTT	CGTGAACCCTTGAATTTTTG

under UV light (ENDURO GDS Gel Documentation System) in dye (EZ-ONE N472-KIT, Ambresco) for further analysis.

2.5. SSR analysis

A total of 30 SSR primers previously evaluated in Biotechnology Research And Application Laboratory of Çukurova University by Aka-Kacaret al. (unpublished) and Pestanana et al. (2011) were used (Table 4). PCR amplifications were conducted as described by Kacar et al. (2013) in 20 µL of reaction volume containing 1 µL each primer (0.3 µM), 1 µL M13 primer, 8 µL of 2X master mix, 0.5 µL $MgCl_2$ (2.5 mM), 0.2 µL Taq polymerase (0.6 U), 5 µL ddH_2O, and 50 ng of genomic DNA. PCR conditions were conducted at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min, and followed by a 6 min final extension at 72 °C by using a thermal cycler (Bio-Rad DNA-Engine Gradient Cycler, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The fragments were separated on 6% denaturing polyacrylamide gel that had been preheated for 25 min. Electrophoresis was conducted at 1500 V, 50 W, 35 mA, and 48 °C by a Li-Cor DNA Analyzer 4300. A 50-350 bp DNA ladder (MWG Biotech AG, Ebersberg, Germany) was run along side the amplified PCR products to determine DNA sizes.

For data analysis, SSCP, ISSR, and SSR profiles were scored for the presence (1) or absence (0) of fragments for each primer. Statistical analysis was carried out using the software PAST (Paleontological Statistics) (http://folk.uio.no/ohammer/past/). The UPGMA clusterand principal coordinate analysis (PCO) were constructed based on Dice's coefficient (Dice, 1945). Polymorphism rates (Pr) were calculated using following formula, Pr = (number of polymorphic bands/total number of bands in that assay unit) × 100. Polymorphism information content (PIC) values were calculated as described by Smith et al. (1997). Marker index (MI) values were calculated as given by Powell et al. (1996) and Smith et al. (1997).

3. Results

3.1. SSCP analysis

Following SSCP analysis, only 9 primers (of 22 available) revealed polymorphic DNA profiles. The total number of amplified amplicons showed 59 fragments, and the number of amplified DNA fragments by each primer

Table 3. Primer names and total number of amplicons, sizes (bp), polymorphic amplicons, PIC, MI and percentages of polymorphism (%) as revealed by ISSR markers among the 48 citrus genotypes.

No	Locus	Band sizes (bp)	Na	Pa	Pr (%)	PIC	MI
1	112	350-550-700-850-900-1000	6	3	50	0.52	26
2	731	250-300-450-550-700-850-1100	7	2	28	0.63	18
3	811	200-300-350-400-450-500-520-600-700-750-800-900-1000-1400-1600	15	13	86	0.69	59
4	812	150-230-300-350-450-520-700-900-1000-1400	10	2	20	0.95	19
5	813	350-500-600-680-710-900-1000-1100-1300	9	8	88	0.89	78
6	818	200-250-270-350-370-400-500-600-700	9	8	88	0.68	60
7	815	300-400-520-550-750-800-830-1000-1100-1300	10	10	100	0.69	69
8	823	250-350-400-450-500-650-800-1100-1500	9	4	44	0.89	39
9	845	250-350-480-600-650-900-1000-1100-1300	9	8	88	0.62	55
10	852	220-300-520-650-700-1000-1200-2000	8	7	87	0.44	38
11	876	180-220-350-400-600-700-1000-1300-1500	10	5	50	0.78	39
12	881	300-350-400-500-650-750-1750	7	4	57	0.74	42
13	Triaag3	800-790-600-450-300-100-190-1100-200	9	8	88	0.68	60
14	868	300-600-650-400-450-800-750-900-990	9	7	78	0.81	63
15	857	1800-1700-1600-600-500-250-850-550	8	6	75	0.56	42
16	873	2000-1500-1100-800-850-500-870-900-400	9	8	88	0.89	78
17	880	2100-1900-1400-790-700-300-1100-	7	6	86	0.44	38
18	856	1800-1700-1000-350-1900-600-400	7	2	29	0.86	25
19	834	1500-1400-850-700-600-500-220-420-900-320	10	9	90	0.76	68
20	803	250-260-600-800-450-310-300-190-290	9	8	89	0.31	31
21	807	290-310-690-800-1000-1200-1500-510	8	7	88	0.63	55
22	Diga_3C	350-650-410-800-550-200	6	3	50	0.73	37
23	$HVH(GA)_7$	1400-1100-1000-900-800-720-250-550-570-450	10	10	100	0.74	74
24	$HVH(TCC)_7$	1100-910-2400-1300-750	5	4	80	0.96	77
25	874	-					
26	875	-					
27	872	-					
28	898	-					
29	896	-					
30	895	-					
Total			206	152			
Mean			8.58	6.33	71.9	0.68	49.6

ranged from 4–9 fragments with an average of 5.44 alleles per locus (Table 5).The size of the detected alleles ranged from 125–415 bp as shown in Table 3. The PIC values for the 9 polymorphic primers ranged from 0.61 to 0.99, with a mean of 0.80. However, the MI values ranged from 19 to 88, with a mean of 63.71.

The SSCP-based dendrogram obtained from UPGMA cluster analysis of genetic distances and PCO are showed in Figures 1 and 2. Lemons and mandarins are generally divided into 2 main groups, A and B. The SSCP assay was efficient enough todistinguish genotypes as a separate taxonomic entity.

3.2. ISSR analysis

For the ISSR analysis, we used 30 ISSR primers but only 24 primers produced well-resolved band fragments. These 24 ISSR primers were then used for screening, and 206 bands were scored. The number of amplified DNA fragments by each primer ranged from 2–13 fragments with an average

Table 4. Primer names and total number of amplicons, sizes (bp), polymorphic amplicons, PIC, MI and percentages of polymorphism (%) as revealed by SSR markers among the 48 citrus genotypes.

No	Locus	Amplicon sizes (bp)	Na	Pa	Pr (%)	PIC	MI
1	83	225-230-220-210	4	4	100	0.73	73
2	84	170-180-190-200-165	5	5	100	0.75	75
3	506	175-170-165-180	4	4	100	0.75	75
4	59	260-270	2	2	100	0.36	36
5	54	170-180-175	3	2	66	0.55	36
6	171	175-160-145-150-155-165	6	6	100	0.91	91
7	D09	240-245-250-247-255-252-235	7	7	100	0.86	86
8	CP05	230-240-245-250-210	5	5	100	0.79	79
9	488	220-225-235-240-250-260	6	6	100	0.86	86
10	178	210-220-215-205-225-290	6	6	100	0.93	93
11	458	220-230-235-240-250-260	6	6	100	0.78	78
12	472	235-250-255-240	4	3	75	0.82	61
13	494	260-270-250-245	4	4	100	0.67	67
14	M121	200-190	2	1	50	0.96	48
15	C08	230-240-250-245	4	3	75	0.82	61
16	L10	310-290-300-320	4	4	100	0.77	77
17	173	215-200-225	3	3	100	0.67	67
18	473	210-220-205	3	3	100	0.75	75
19	105	200-180-185-220-210-225-230	7	7	100	0.83	83
20	191	180-175-177-185-190-170	6	6	100	0.85	85
21	AC01	190-150-170	3	3	100	0.84	84
22	495	230-240-210-250	4	4	100	0.86	86
23	39		-	-	-		
24	163		-	-	-		
25	140		-	-	-		
27	139		-	-	-		
28	166		-	-	-		
29	174		-	-	-		
30	176		-	-	-		
Total			98	94	-		
Mean			3.27	3.13	68.87	0.57	72.8

of 8.58 alleles per locus (Table 3).The size of the detected alleles ranged from 150 to 2100 bp as shown in Table 4. The PIC values for the 24 polymorphic primers ranged from 0.31 to 0.96, with a mean of 0.68. On the other hand, the MI values ranged from 18 to 78, with a mean of 49.6.

The ISSR-based dendrogram obtained from UPGMA cluster analysis of genetic distances and PCO are showed in Figure 1 and Figure 2. Lemons and mandarins are generally divided into 2 main groups as A and B. Similar to the results of SSCP, the ISSR assay was efficient enough todistinguish genotypes as a separate taxonomic entity. PCR amplifications of ISSR 868 is given Figure 3. A total of 24 ISSR primers were screened for linkage to the irradiated seedless trait in mandarin, but only 1 closely linked ISSR 868 marker with 500 bp was identified.

3.3. SSR analysis

Only 22 of the 30 SSR primers used produced well-resolved band fragments. These 22 SSR primers produced 98scorable bands, and the number of amplified DNA fragments by each primer ranged from 2 to 7 fragments

Table 5. Primer names and total number of amplicons, sizes (bp), polymorphic amplicons, PIC, MI and percentages of polymorphism (%) as revealed by SSCP markers among the 48 citrus genotypes.

No	Locus	Amplicon sizes (bp)	Na	Pa	Pr (%)	PIC	MI
1	5	192-200-205-298-303-305	6	6	100	0.61	61
2	8	163-167-205-214-405-410-415	7	4	57	0.71	40
3	7	202-210-301-310	4	4	100	0.73	73
4	9	350-343-345-355-359	5	1	20	0.99	19
5	13	186-214-215-196-199-201	6	3	50	0.84	42
6	4	125-128-140-166-215-220-176-183-190	9	9	100	0.82	82
7	16	243-253-266-351	4	4	100	0.80	80
8	18	210-232-289-297-287-290-299-305-308	9	9	100	0.88	88
9	21	139-191-200-236-240-315-326-268-288	9	9	100	0.88	88
Total			59	49	-	-	-
Mean			6.55	5.44	80.77	0.80	63,71

with an average of 3.27 alleles per locus. The size of the detected alleles ranged from 165 to 320 bp. The PIC values ranged from 0.33 to 0.96, with a mean of 0.57. On the other hand, the MI values ranged from 36 to 93, with a mean of 72.8.6 (Table 4).

The SSR-based dendrogram obtained from UPGMA cluster analysis of genetic distances and PCO showed in Figure4 and Figure 5. Similar to the results of SSCP and ISSR, the SSR assay was efficient enough to distinguish genotypes as a separate taxonomic entity.A similarity matrix and PCO based on SSCP, ISSR, and SSR data were given in Figures 4 and 5.

4. Discussion

To comprehend and interpret these structural differences correctly, one must clearly distinguish between the 2 levels of investigations in genetics: first, at the level of the phenotypes; and second, at the DNA level (Lönnig, 2005). However, there are several undesirable factors that are associated at the level of the phenotypic characterizationsuch ashigh dependency by environmental factors and the long time it takes to identify some traits such as pomological traits in fruit plants (Fang and Roose, 1997; Nicolosi et al., 2000). Furthermore, phenotypic characterization is hindered by long juvenility and harvest time, and it is labor intensive and requires large plots of land to grow large populations of plants (Stuber et al., 1999; Fu et al., 2011; Nadeem et al., 2018). In recent years, rapid development in molecular marker technology has partially solved some of the challenges such high frequency of bud mutation, a longhistory of cultivation, and wide cross-compatibility (Federici et al., 1998; Agisimanto et al., 2016). DNA markers have been widely used for determination of mutant genotypes in many plants (Uzun et al., 2003, Atak et al., 2004) including effective differentiation of mutant genotypes in citrus breeding (Deng et al., 1995; Feng et al., 2008; Polat et al., 2015; Agisimanto et al., 2016; Mallick et al., 2017).

Studies involving *Citrus* taxonomy and phylogenetic relationships are complex and quite difficult due to wide cross-compatibility among the species, nucellar embryony, apomixes, high frequency of bud mutation, long history of cultivation, and paucity of remaining wild citrus stands (Nicolosi et al., 2000; Moore, 2001; Biswas et al., 2011). DNA markers have also been extensively preferredfor cultivar identification in citrus (Biswas et al., 2014, Garcia-Lor et al., 2015). They represent some of the most powerful tools for the analysis of genomes and enable the association ofheritable traits with underlying genomic variation (Duran et al.,2009; Amar et al., 2011). It is important that the choice of molecular marker technique has to be a compromise between reliability, labor, and ease of analysis, statistical power, and confidence of revealing polymorphisms (Agarwal et al., 2008).

In this work, codominant SSR markers (Biswas et al., 2011), dominant ISSR markers (Lombardo et al., 2011), and SSCP markers that is sensitive and informative on mutation detection (DeğirmenciKarataş et al., 2010) were chosen for the determination of seedless mutant mandarins from nonmutant seedy parent mandarin and thornless lemons from nonmutant thorny parent lemon in citrus mutation breeding. In this study the genome was scanned at a high number of loci, as each of the 82 different primer combinations amplified an excess of 363 bands. Even with the large number of fragments generated to find markers linked to the gene(s) affecting theseedless trait in mandarin, only one polymorphism with 500 bp was found, mainly because the parent is almost genetically identical.

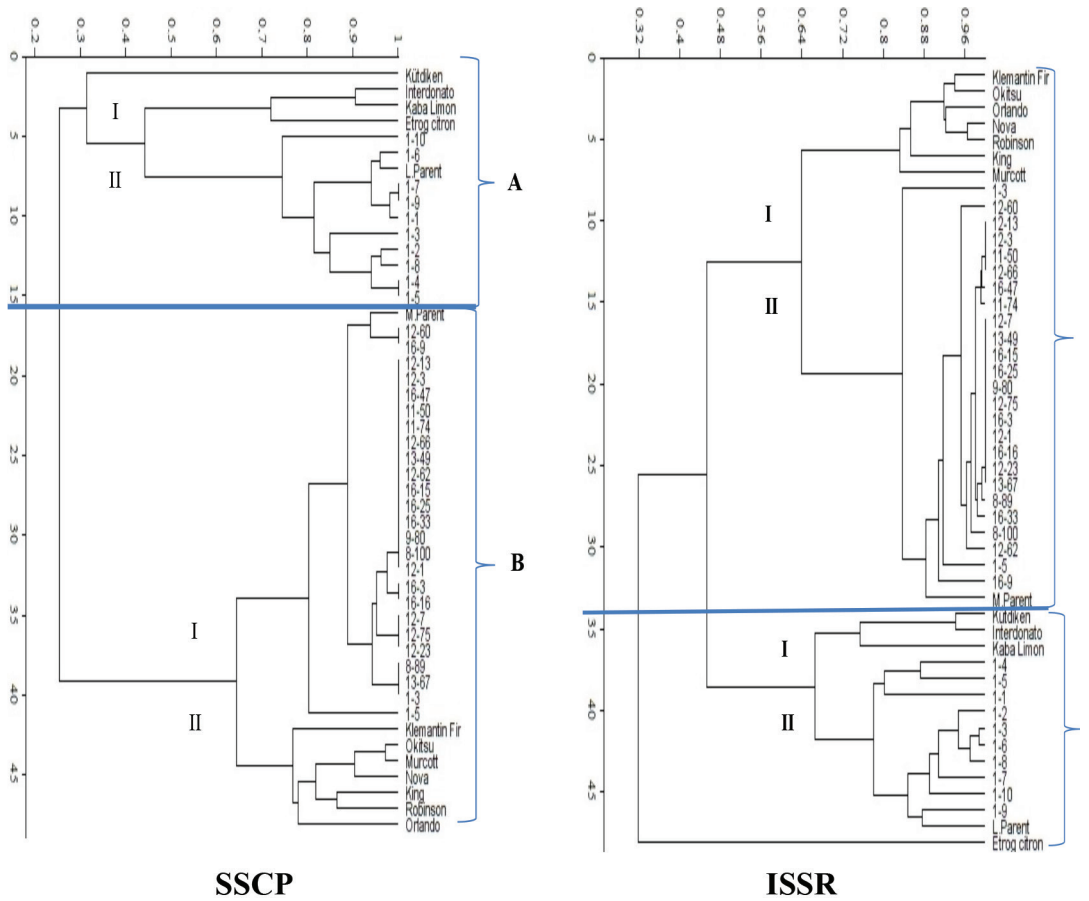

Figure 1. UPGMA dendrogram of SSCP and ISSR markers for 48 citrus genotypes.

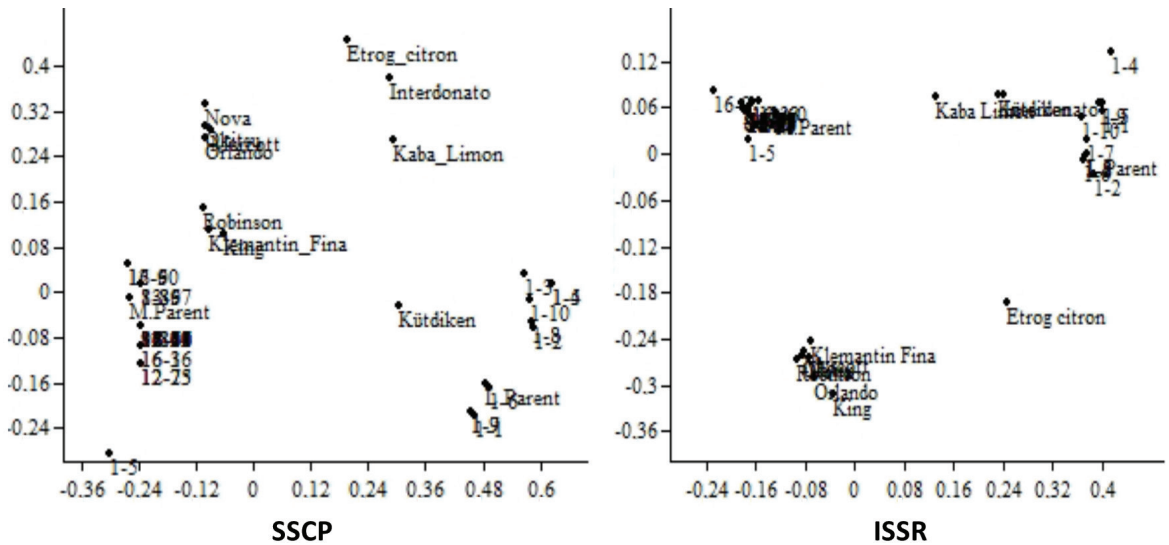

Figure 2. Principal coordinate analyses of SSCP and ISSR markers for 48 citrus genotypes.

ISSR 868 primer has the potential to accelerate breeding programs aimed at modifying unwanted sideeffects of the seedless mandarin mutation by marker-assisted selection.

However, there are no detailed reports available on the use of SSCP markers for the determination of genetic differences among citrus mutant genotypes. Herein, we

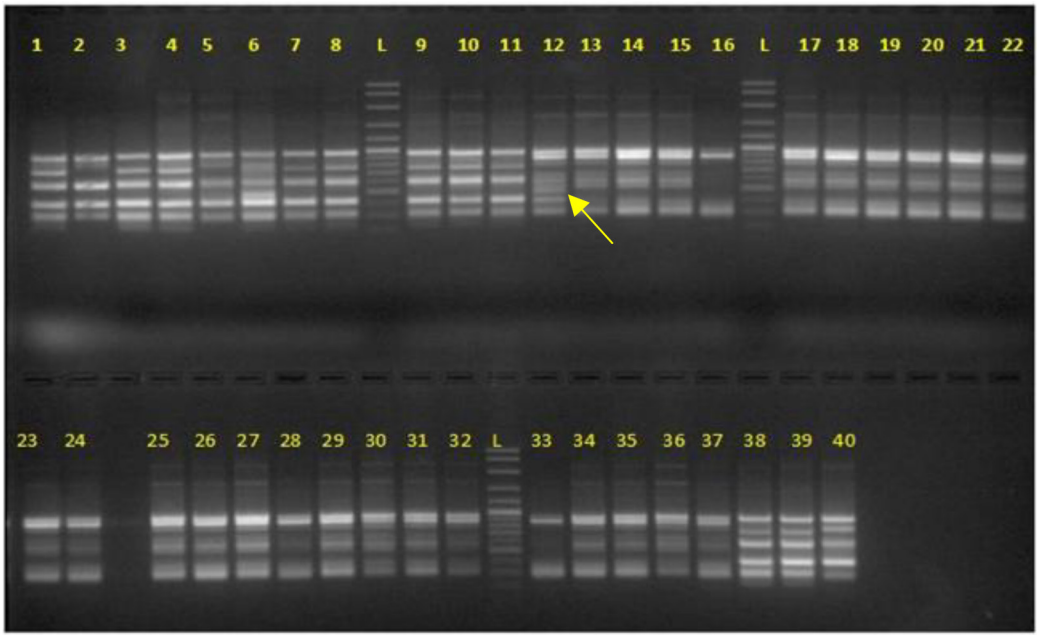

Figure 3. PCR amplifications of ISSR 868 primer. L: 100 bp DNA ladder, 1: L. parent, 2-11: Lemon mutant genotypes, 12: M. parent, 13-37: Mandarin mutant genotypes, 38-40: Commercial lemon varieties.

report the use of SSCP marker to detect polymorphism among mutant seedless Yerli Mandarin mandarin clones and mutant thornless Antalya YerliYuvarlak Limon lemon clones obtained by gamma irradiation and the parental materials as well as some commercial citrus varieties. Correlation coefficient matrices (r) for SSCP, ISSR and SSR markers were found to be high as they were 0.97, 0.95, and 0.98 respectively, suggesting that the cluster analysis strongly represents the similarity matrix. In other words, these results show that utilization of these markers can offer great benefits to our lemon and mandarin breeding program in several ways, such as identifying genotypes and studying the genetic similarity among genotypes. Aka-Kacar et al. (2005) suggested that correlation coefficient matrix is very good, good, weak or very weak if $r \geq 0.9$, $0.8 \leq r < 0.9$, $0.7 \leq r < 0.8$ or $r < 0.7$, respectively.

SSCP is one of most useful for the detection of point mutations (Orita et al., 1989; Konstantinos et al., 2008). Thus, a combined analysis offers increased sensitivity (Konstantinos et al., 2008). However, chemical mutagens are preferably used to induce point mutations, physical mutagens such as gamma raysinduce gross lesions, such as chromosomal abbreviation or rearrangements (Lamo et al., 2017). Therefore, the SSCP marker should be considered as a scanner method rather than identificationmethod (Fujita and Silver, 1994).Mutations in the *Gf-2.8* gene were identified by the analysis of the SSCP in wheat (Wang et al., 2001). Similarly, identification of the waxy gene (Sato and Nishio, 2003)and RuBisCO/LS gene mutations were carried out by SSCP markers in rice (Kajiwaraet al., 2005). On the contrary, although SSCP markers were used for the determination of early flowering mutant corn lines following chemical mutagenesis, it was not possible to identify any molecular markers associated with the observed variations in flowering time (Cristov et al., 2004). DeğirmenciKarataş et al. (2010)analyzed22 candidate mutants of ThompsonSeedless and KalecikKarası obtained via gamma ray using SSCP markers together with 46 control plants. Polymorphic bands were rarelyobtained in the SSCP analysis, and they were not reproducible. Our study to be pioneer for the utilization of SSCP markers for the characterization of mutant citrus genotypes, and it validated the use of ISSR and SSR markers.

ISSR markers are extensively used in establishing the genetic differences of mutant genotypes in many citrus species. For example, 15 of 100 ISSR primers were polymorphic between mutants and parental orange cultivars. Genetic diversities were determined with primers 812, 834, and 841 between seedless big fruit branch and the contrast branch, among Anshun No. 1 and Valencia and Jincheng oranges respectively. Combining the results of morphological and molecular analysis, it was certified that the branch of C. sinensis cv. and Anshun No. 1 exhibited genetic variation (Chang Feng et al., 2008). Besides that, DNA of plantlets from the 20, 40, and 60 Gy exposure were individually amplified and compared to the control for early detection of gamma ray mutagenesis using ISSR markers related to seedlessness mandarin, but no variant

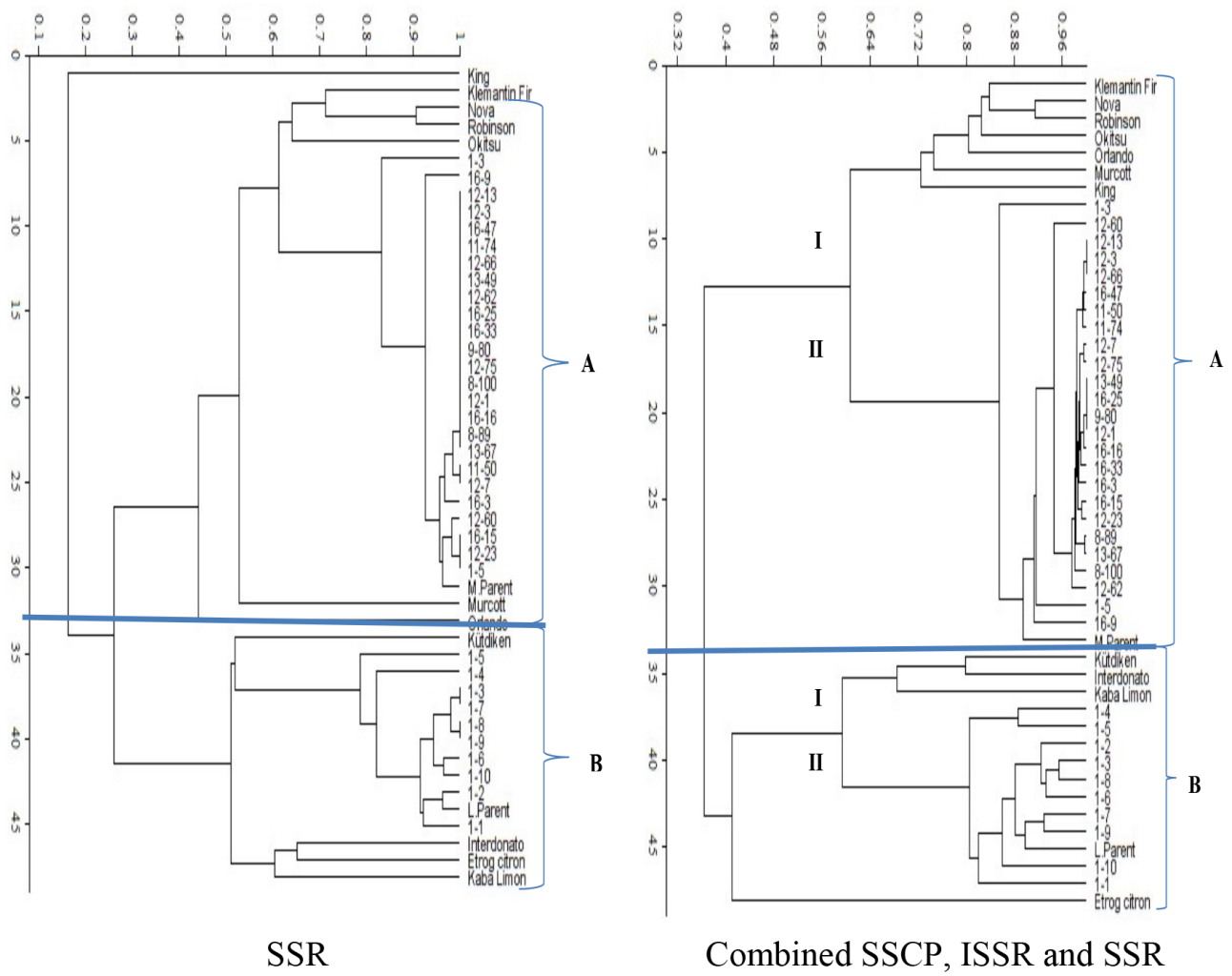

Figure 4. UPGMA dendrogram of SSR and combined SSCP, ISSR and SSR markers for 48 citrus genotypes.

was observed from the plantlets produced (Agisimanto et al., 2016).They could be because Agisimanto et al. (2016) used only three ISSR primers, namely, (GA)8YG, (TCC)5RY and HVH(CA)8 respectively. In our study, most of ISSR primers were successful in separating the mutant mandarins and mutant lemons.Similarly, ISSR markers have been used in various genetic diversity studies among lemons (Capparelli et al. 2004), mandarins (Scarano et al., 2002; Pal et al., 2013), and to distinguish highly related citrus cultivars, otherwise difficult to distinguish with other molecular marker methods (Shahsavar et al., 2007; Uzun et al., 2010; Pal et al., 2013).In our cluster analysis, lemon relatives were indicated as a separate group from mandarins. Etrog Citron was the accession that is the most distinct with approximately 0.30 from the othergenotypes.As expected, Etrog Citron in *C.medica* L. taxonomic classification is separated from both lemons and mandarins. Gülşen and Roose (2001) reported that all lemons, rough lemons, and sweet lemons, as well as some other suspected hybrids were clustered with citrons using ISSR markers. Most lemons had nearly identical genotypes, suggesting that they originated from a single clonal parent via a series of mutations.

Likewise, SSR markers are widely used for both to determine the genetic differences of mutant genotypes (Polat et al., 2015; Mallick et al., 2017) and to distinguish highly related citrus cultivars (Barkley et al., 2006; Uzun et al., 2011; Kacar et al., 2013) in many citrus species.

Citrus has complex taxonomic and phylogenetic structures (Nicolosi et al., 2000; Uzun et al., 2011; Garcia-Lor et al., 2015), and 2 important taxonomic systems made by Swingle et al. (1967) and Tanaka (1977) are usually accepted. Lemon [*Citrus limon*(L.) Burm. f.] was reported as a hybrid of citron and sour orange (*Citrus aurantium* L.) (Nicolosi et al., 2000; Gülşen and Roose, 2001). Most lemons have highly similar morphological characteristics and they are originated by mutation from a single parental lemon tree (Gülşen and Roose, 2001; Uzun

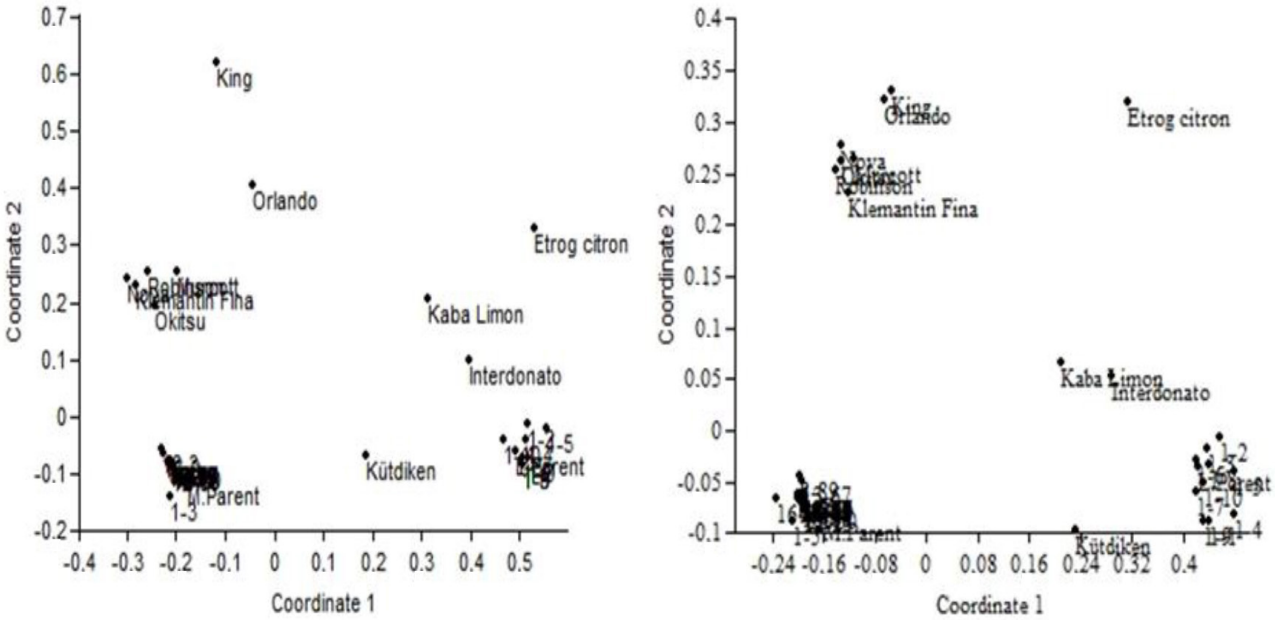

Figure 5. Principal coordinate analyses (PCO) of SSR and combined SSCP, ISSR, and SSR markers for 48 citrus genotypes.

et al., 2011). Interdonato lemon was reported as a hybrid between lemon and citron (Hodgson, 1967; Gulsen and Roose, 2000). Meanwhile, lemonsare distant from other *Citrus* spp., like that observed with SSR and SRAP data by Uzun et al. (2011).Unlike lemons, mandarin is considered true citrus species (Campos et al., 2005), and therefore, mandarins have low genetic polymorphism (Mallick et al., 2017). Used mandarins as material were distinct from the other mandarins with SSR and SRAP (Kacar et al. 2013).

In conclusion, despite some fragmented information available in the literaturereported by several scientists during the past year, our data confirmed that SSCP, SSR and ISSR methods are effective tools for the identification of closely related accessions and determination of the mutant thornless lemon and seedless mandarin genotypes. The combination of SSCP, SSR, and ISSR based marker methods also guarantee some advantages. The molecular markers seem to be convenient for the finely tuned determination of highly related plants, and also the results presented here can form the basis for the design of future citrus breeding projects. Besides, new developments in genomic research have given access to an enormous amount of sequence information as well as new insights into the function and interaction of genes and the evolution of functional domains, chromosomes and genomes. Future DNA marker techniques, such as the use of oligonucleotide arrays can be used to determine the genetic differences in citrus. On the other hand, topics such as genome editing can play a beneficial role in the efficient utilization of plants to citrus breeders.

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References

Agarwal M, Neeta S, Harish P (2008). Advances in molecular marker techniques and their applications in plant sciences. Plant Cell Reports 27: 617-631.

Agisimanto D, Noor NM, Ibrahim R, Mohamad A (2016). Gamma irradiation effect on embryogenic callus growth of *Citrus reticulata* cv. limaumadu. Sains Malaysiana 45: 329-337.

Aka-Kacar Y, Demirel A, Tuzcu O, Yesiloglu T, Ulas M et al. (2005). Preliminary results on fingerprinting lemon genotypes tolerant to mal secco (*Phomatracheiphila* Kanc. et Ghik) disease by RAPD markers. Biologia 60: 295-300.

Aka Kacar Y, Simsek O, Donmez D, Boncuk M, Yesiloglu T et al. (2014). Genetic relationships of some Citrus genotypes based on the candidate iron chlorosis genes. Turkish Journal of Agriculture and Forestry 38: 340-347.

Amara MH, Biswas MK, Zhang Z, Guoa WW (2011). Exploitation of SSR, SRAP and CAPS-SNP markers for genetic diversity of Citrus germplasm collection. Scientia Horticulturae 128: 220-227.

Atak Ç, Alikamanoğlu S, Açık L, Canbolat Y (2004). Induced of plastid mutations in soybean plant (*Glycine max* L. Merrill) with gamma radiation and determination with RAPD. Mutation Research 556: 35-44.

Barkley NA, Roose ML, Krueger RR, Federici CT (2006). Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). Theoretical and Applied Genetics 112: 1519-1531.

Barkley NA, Krueger RR, Federici CT, Roose ML (2009). What phylogeny and gene genealogy analyses reveal about homoplasy in citrus microsatellite alleles. Plant Systematics and Evolution 282: 71-86.

Bermejo A, Pardo J, Cano A (2011). Influence of gamma irradiation on seedless citrus production: pollen germination and fruit quality. Food and Nutrition Sciences 2: 169-180.

Bermejo A, Pardo J, Cano A (2012). Murcottseedless: influence of gamma irradiation on citrus production and fruit quality. Spanish Journal of Agricultural Research 3: 768-777.

Biswas MK, Chai L, Amar MH, Zhang X, Deng XX (2011). Comparative analysis of genetic diversity in Citrus germplasm collection using AFLP, SSAP, SAMPL and SSR markers. Scientia Horticulturae 129: 798-803.

Biswas MK, Xu Q, Mayer C, Deng X (2014). Genome wide characterization of short tandem repeat markers in sweet orange (*Citrus sinensis*). Plos One 9 (8): e104182. doi: 10.1371/journal.pone.0104182

Cameron JW, Frost NB (1968). Genetic, breeding and nucellar embryony. In: The Citrus Industry. (Eds.): Reuther W, Batchelor LD and Webber HJ. Berkeley, CA, USA: University California Press, pp. 325-379.

Campos ET, Espinosa MAG,Warburton ML, Varela AS, Monter AV (2005). Characterization of mandarin (*Citrus* spp.) using morphological and AFLP markers. Interiencia 30: 687-693.

Capparelli R, Viscardi M, Amoroso MG, Blaiotta G, Bianco M (2004). Bianco Inter-simple sequence repeat markers and flow cytometry for the characterization of closely related Citrus limon germplasms. Biotechnology Letters 26: 1295-1299.

Carrillo-Medrano SH, Gutierrez-Espinosa MA, Robles-González MM, Cruz-Izquierdo S (2018). Identification of Mexican lemon hybrids using molecular markers SSR. Revista Mexicana de Ciencias Agrícolas 9: 11-23.

Chang Feng C, Qiao H, Lin H, Qi Gao G, Ju Rong Q et al. (2008). The identification of *Citrus sinensis* cv. bud mutation by ISSR markers. Southwest China Journal of Agricultural Sciences 21: 1378-1380.

Chapot H (1975). The citrus plant. In: Citrus, CIBA-GEIGY Agrochemicals, Technical Monograph No 4. Basle, Switzerland: CIBA GEIGY Agrochemicals, pp. 6-13.

Chen C, Bowman KD, Choi YA, Dang PM, Rao MN et al. (2008). EST-SSR genetic maps for *Citrus sinensis* and *Poncirus trifoliata*. Tree Genetics and Genomes 4: 1-10.

Christov NK, Todorovska EG, Fasoula DA, Ioannides IM, Atanassov AI et al. (2004). Molecular characterization of chemical mutagenesis induced diversity in elite maize germplasm. Genetika 36: 47-60.

Crowley JR (2011). A molecular genetic approach to evaluate a novel seedless phenotype found in Tango, a new variety of mandarin developed from gamma-irradiated W. Murcott. PhD thesis, University of California Riverside, Riverside, CA, USA.

Değirmenci D, Kunter B (2008). SSCP (Single Strand Conformation Polymorphism) markörler aracılığıyla mutasyonların analizi. Mustafa Kemal Üniversitesi Ziraat Fakültesi Dergisi 13: 13-24.

DeğirmenciKarataş D, Kunter B, Coppola G, Velasco R (2010). Analysis of polymorphism based on SSCP markers in gamma-irradiated (Co^{60}) grape (*Vitis vinifera*) varieties. Genetics and Molecular Research 9: 2357-2363.

Deng ZN, Gentile A, Nicolosi E, Domina F, Vardi A et al. (1995). Identification of in-vivo and in-vitro lemon mutants by RAPD markers. Journal of Horticultural Sciences 70: 117-125.

Dice LR (1945). Measures of the amount of ecologic association between species. Ecology 26: 297-302.

Doyle JJ, Doyle JL(1990). Isolation of plant DNA fromfresh tissue. Focus 12: 13-15.

Donini B (1982). Mutagenesis applied to improve fruit trees: techniques, methods and evaluation or radiation induced mutations. In: Induced Mutations in Vegetatively Propagated Plants. Vienna, Austria: International Atomic Energy Agency (IAEA), pp. 29-36.

Duran C, Appleby N, Edwards D, Batley J (2009). Molecular genetic markers: discovery, applications, data storage and visualisation. Current Bioinformatics 4: 16-27.

Fang DQ, Roose ML (1997). Identification of closely related citrus cultivars with Inter simple sequence repeat markers. Theoretical and Applied Genetics 95: 408-417.

Federici CT, Fang DQ, Scora RW, Roose ML (1998). Phylogenetic relationships within the genus Citrus (Rutaceae) and related genera as revealed by RFLP and RAPD analysis. Theoretical and Applied Genetics 96: 812-822.

Fu J, Peng ZJ, Cai XD, Guo WW (2011). Regeneration and molecular characterization of interspecific somatic hybrids between Satsuma mandarin and two seedy sweet oranges for scion improvement. Plant Breeding 130 (2): 287-290. doi:10.1111/j.1439-0523

Fujita K, Silver J (1994). Single-strand conformational polymorphism. PCR methods and applications 4: 137-140.

Garcia-Lor A, Luro F, Ollitrault P, Navarro L (2015). Genetic diversity and population structure analysis of mandarin germplasm by nuclear, chloroplastic and mitochondrial markers. Tree Genetics and Genomes 11: 123.

Gentile A, LaMalfa S (2019). New Breeding Techniques for Sustainable Agriculture. In Innovations in Sustainable Agriculture. Cham, Switzerland: Springer International Publishing, pp. 411-437.

Goldenberg L, Yaniv Y, Porat R, Carmi N (2014). Effects of gamma-irradiation mutagenesis for induction of seedlessness, on the quality of mandarin fruit. Food and Nutrition Sciences 5: 943-952.

Gülsen O, Roose ML (2001). Lemons: Diversity and relationships with selected citrus genotypes as measured with nuclear genome markers. Journal of American Society for Horticultural Science 126: 309-317.

Gulsen O, Uzun A, Pala H, Canihos E, Kafa G (2007). Development of seedless and mal secco tolerant mutant lemons through budwood irradiation. Scientia Horticulturae112: 184-190.

Gulsen O, Uzun A, Canan I, Seday U, Canihos E (2010). A new citrus linkage map based on SRAP, SSR, ISSR, POGP, RGA and RAPD markers. Euphytica 173: 265-277.

Hearn CJ (1984). Development of seedless orange and grapefruit cultivars through seed irradiation. Journal of American Society for Horticultural Science 109: 270-273.

Hensz RA (1985). "Rio Red" a new grapefruit with a deep-red color. Journal Rio-Grande Valley Horticultural Society 38: 75-76.

Hodgson RW (1967). Horticultural varieties of citrus. In: Reuther W, Webber HJ, Batchelor LD (eds) The Citrus industry. Berkeley, CA, USA: University of California Press, pp. 431-591.

Huang JC, Xiao Y, Zhao CX, Chen ZS, Wang XY (2003). Induction of superior seedless mutation of *C. grandis* Osbeck cv. Sshatianyou by irradiation. Acta Agriculturae Nucleatae Sinica 17: 171-174.

Huang JH, Wen SX, Zhang YF, Zhong QZ, Yang L et al. (2017). Abnormal megagametogenesis results in seedlessness of a polyembryonic 'Meiguicheng' orange (*Citrus sinensis*) mutant created with gamma-rays. Scientia Horticulturae 217: 73-83.

Hussein EHA, Abd-alla SMM, Awad NA, Hussein MS (2003). Genetic analysis in some Citrus accessions using Microsatellites - and AFLP-based markers. Arabian Journal of Biotechnology 6: 203-222.

Kacar Y, Uzun A, Polat I, Yesiloglu T, Yılmaz B et al. (2013). Molecular characterization and genetic diversity analysis of mandarin genotypes by SSR and SRAP markers. Journal of Food, Agriculture and Environment 11: 516-521.

Khalil SA, Sattar A, Zamir R (2011). Development of sparse-seeded mutant kinnow (*Citrus reticulata* Blanco) through budwood irradiation. African Journal of Biotechnology 10: 65. doi: 10.5897/AJB10.1810

Kajiwara H, Kaneko T, Nishimura M, Ishizaka M (2005). Proteomic identification of RuBisCO/LS gene mutations in radiation mutant of rice by two-dimensional gel electrophorosesis, mass spectrometry, single strand conformation polymorphism and nucleotide sequencing. Rice Genetics Newsletter 20: 1-3.

Konstantinos KV, Panagiotis P, Antonios VT, Agelos P, Argiris NV (2008). PCR–SSCP: A method for the molecular analysis of genetic diseases. Molecular Biotechnology 38: 155-163.

Lamo K, Bhat DJ, Kour K, Solanki SPS (2017). Mutation studies in fruit crops: A Review. International Journal of Current Microbiology and Applied Sciences 6: 3620-3633.

Lombardo G, Schicchi R, Marino P, Palla F (2011). Genetic analysis of *Citrus aurantium* L. (Rutaceae) cultivars by ISSR molecular markers, Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology: Official Journal of the Societa Botanica Italiana. doi:10.1080/11263504.2011.557101

Lönnig WE (2005). Mutation breeding, evolution, and the law of recurrent variation. Recent Research Developments in Genetics Breeding 2: 45-70.

Mabberley DJ (2008). Mabberley's plant-book: a portable dictionary of plants. 3rd ed.. UK: Cambridge University Press.

Mahmoudzadeh H (2013). Bazı Portakal çeşitlerinde genetik farklılıklarını SSCP markırları ile belirlenmesi. Yüksek Lisans Tezi, Çukurova Üniversitesi, Adana, Turkey…

Mallick M, Bharadwaj C, Srivastav M, Sharma N, Awasthi OP (2017). Molecular characterization of Kinnow mandarin clones and mutants using cross genera SSR markers. Indian Journal of Biotechnology 16: 244-249.

Nadeema MA, Nawazb MA, Shahidc MQ, Dogan Y, Comertpay G et al. (2018). DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing. Biotechnology and Biotechnological Equipment 32: 261-285.

Nicolosi E, Deng ZN, Gentile A, La Malfa S, Continella G et al. (2000). Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theoretical and Applied Genetics 100: 1155-1166.

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single strand conformation polymorphisms. Proceeding National Academy Science 86: 2766-2770.

Pal D, Malik SK, Kumar S, Choudhary R, Sharma KC et al. (2013). Genetic variability and relationship studies of mandarin (*Citrus reticulata* Blanco) using morphological and molecular markers.Agricultural Research 2: 236-245.

Pestanana RKN, Amorim EP, Ferreira CF, Amorim VBO, Oliveira LS et al. (2011). Agronomic and molecular characterization of gamma ray induced banana (*Musa* sp.) mutants using a multivariate statistical algorithm. Euphytica 178: 151-158.

Polat I, Turgutoglu E, Kurt S (2015). Determination of genomic diversity within mutant lemon (*Citrus Limon* L.) and mandarin (*Citrus Reticulata*) using molecular markers. Pakistan Journal of Botany 47: 1095-1102.

Polat İ (2015). Genetic diversity analysis of Valencia and Navel group sweet orange cultivars by SSR markers. Derim 32: 47-62.

Polat İ (2018). Advanced innovative tools in lemon (*Citrus limon* L.) Breeding. Advances in Plant Breeding Strategies:Fruits. Chapter 12. Volume 3. Editors: Al-Khayri, Jameel M., Jain, S. Mohan, Johnson, Dennis V. (editors). Berlin, Germany: , Springer, pp. 437-463.

Powell W, Machray GC, Provan J (1996). Polymorphism revealed by simple sequence repeats. Trends in Plant Science 1: 215-222.

Raza H, Khan MM, Khan AA (2003). Review: seedlessness in Citrus. International Journal of Agriculture and Biology 5: 388-391.

Rozen S, Skaletsky HJ (2000). Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (editors). Bioinformatics Methods and Protocols: Methods in Molecular Biology. Totowa, NJ, USA: Humana Press, pp. 365-386.

Russo F, Donini B,Starrantino S (1981). Mutagenesis applied for citrus improvement. In: Proceedings International Society of Citriculture; Tokyo, Japan. pp. 68-70.

Sankar AA, Moore GA (2001). Evaluation of inter-simple sequence repeat analysis for mapping in Citrus and extension of the genetic linkage map. Theoretical and Applied Genetics 102: 206-214.

Sato Y, Nishio T (2003). Mutation detection in rice waxy mutants by PCR-RF-SSCP. Theoritical and Applied Genetics 107: 560-567.

Scarano MT, Abbate L, Ferrante S, Lucretti S, Tusa N (2002). ISSR-PCR technique: a useful method for characterizing new allotetraploid somatic hybrids of mandarin. Plant Cell Reports 20: 1162-1166.

Shahsavar AR, Izadpanah K, Tafazoli E, Sayed Tabatabaei BE (2007). Characterization of citrus germplasm including unknown variants by inter-simple sequence repeat (ISSR) markers. Scientia Horticulturae112: 310-314.

Smith JSC, Chin ECL, Shu H, Smith OS, Wall SJ, et al. (1997). An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparisons with data from RFLPs and pedigree. Theoretical and Applied Genetics 95: 163-173.

Starrantino A, Russo F, Donini B, Spina P (1988). Lemon mutations obtained by gamma irradiation of the nucellus cultured in vitro. In: 6th International Citrus Congress; Tel Aviv, Israel. pp. 231-235.

Stuber CW, Polacco M, Senior ML (1999). Synergy of empirical breeding, marker assisted selection and genomics to increase crop yield potential. Crop Science 39: 1571-1583.

Sutarto I, Agisimanto D, Supriyanto A (2009). Development of promisingseedless citrus mutants through gamma irradiation. Induced Plant Mutations in the Genomics Era. Rome, Italy: Food and Agriculture Organization of the United Nations, pp. 306-308.

Simsek O, Aka Kacar Y, Yesiloglu T, Olliutrault P (2011). Determination by SSCP markers of the allelic diversity of candidate genes for tolerance to iron chlorosis in citrus germplasm. Proc. 2nd IS on Citrus Biotechnology. Acta Horticulturae 892, ISHS.

Swingle WT, Reece PC (1967). The botany of citrus and its wild relatives. In: Reuther W, Webber HJ, Batchelor LD (editors). The Citrus industry. Berkeley, CA, USA: University of California Press, pp. 389-390.

Tanaka T (1977). Fundamental discussion of Citrus classification. Studia Citrologica 14: 1-6.

Tang XL, Wu ST, Peng CL, Li ZO (1994). Development of seedless citrus cultivars through gamma ray re-irradiation. In: Proceedings series; International Atomic Energy Agency; Vienna, Austria; Food and Agriculture Organization of the United Nations; Rome, Italy. p. 748.

Terol JF, Naranjo MA, Ollitrault P, Talon M (2008). Development of genomic resources for *Citrus clementina*: Characterization of three deep-coverage BAC libraries and analysis of 46,000 BAC end sequences. BMC Genomics 9: 12

Turgutoğlu E, Demir G, Kurt Ş, Ağsaran B, Polat İ (2014). Çekirdeksiz, dikensiz ve periyodisite göstermeyen mutant Yerli MandarinveAntalya YerliYuvarlak Limon tiplerinin belirlenmesi (Proje ara sonuç raporu). Araştırma Projeleri Faaliyet Raporları. Antalya, Turkey: Batı Akdeniz Tarımsal Araştırma Enstitüsü Müdürlüğü, pp. 34-48.

Usman M, Fatima B, Awais M, Shoukat D, Fatima A et al. (2018). Prospects of mutation breeding in grapefruit (*Citrus paradisi* Macf.). In: International Horticulture Conference ; Rawalpindi, Pakistan. p. 29.

Uzun B, Lee D, Donini P, Çağırgan I (2003). Identification of a molecular marker linked to the closed capsule mutant trait in sesame using AFLP. Plant Breeding 122: 95-97.

Uzun A, Gulsen O, Yesiloglu T, Aka-Kacar Y, Tuzcu O (2010). Distinguishing grapefruit and pummeloaccessions using ISSR markers. Czech Journal of Genetics Plant Breeding 46 : 170-177.

Uzun A, Yesiloglu T, Polat I, Aka Kacar Y, Gulsen O et al. (2011). Evaluation of genetic diversity in lemons and some their relatives based on SRAP and SSR markers. Plant Molecular Biology 29: 693-701.

Wang CT, Huang ZJ, He CF, Bi CL, Shen YZ (2001). Detection of the wheat salt-tolerant-mutant using PCR-SSCP combining with direct sequencing. Yi Chuan Xue Bao Journal 28; 852-855 (in Chinese).

Williams TE, Roose ML (2010). Tango-a new, very low-seeded, late-season irradiated selection of 'W. Murcott' mandarin from the University of California Riverside. Proceeding of the International Society of Citriculture 1: 202.

Yadav BR, Kale DS (2009). Single strand conformation polymorphism (SSCP) Analysis by Nondenaturing PAGE. Genetics & Genomics: Genotyping. http://www.protocol-online.org.

Yoshida T, Kawase K, Nesumi H (1999). Inheritance of thornlessness in Trifoliate Orange [*Poncirus trifoliata* (L.) Raf.]. Journal of Japanese Society for Horticultural Science 68: 1104-1110.

Zhang S, Shi Q, Albrecht U, Shatters Jr RG, Stange R et al. (2017). Comparative transcriptome analysis during early fruit development between three seedy citrus genotypes and their seedless mutants. Horticulture Research 4: 17041. doi: 10.1038/hortres.2017.41