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Genetic Diversity and Relationships within *Citrus Species* Based On Sequence-Related Amplified Polymorphism Markers (Sraps)

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Abstract

Sequence-related amplified polymorphism (SRAP) markers were used to detect molecular marker polymorphisms among five parents and four crosses of citrus and their relatives in Aurantioidea. Four SRAP primer combinations produced a total of 160 polymorphic fragments with an average of 40 per primer combination and the an-average polymorphism information content (PIC) of 0.86. The unweighted pair group method arithmetic average (UPGMA) analysis demonstrated that the accessions had a similarity range from 0.35 in the cross between Lemon and Clementine to 0.43 in the Grapefruit parent with a mean of 0.37. The dendrogram separated the parents and the resulted crosses of Citrus species into two main sub-clusters with a similarity value of 0.37. Only one member of the first sub-cluster which is Clementine or the parent of all the resulted crosses.In the second main sub-cluster, Only one member of the first sub-sub-cluster which is Grapefruit or the parent of one cross. The second sub-sub-cluster has consisted of one parent separated alone (Succari parent) and another sub-cluster. This sub-cluster is formed from the sub-sub-cluster including the parent Cleopatra mandarin and the resulting from cross Cleopatra mandarin x Clementine. The last sub-cluster has consisted of one group containing the parent Lemon and the resulted cross Lemon x Clementine. The other group consisted of two crosses; Grapefruit x Clementine and Succari x Clementine.

Keywords: molecular marker polymorphisms; cluster; parent; Lemon; Cleopatra mandarin; Clementine; Grapefruit; Succari

Introduction

The genus Citrus L. belongs to the subtribe Citrineae, the tribe Citreae within the subfamily Aurantioideae of the Rutaceae family (**Webber, 1967**). The Aurantioideae is one of seven subfamilies of Rutaceae which consists of two tribes and 33 genera. Each of the tribes Clauseneae and Citreae are composed of three subtribes. Clauseneae includes Micromelinae, Clauseninae and Merrillinae, and Citreae has Triphasiinae, Citrinae and Balsamocitrinae. The Citrinae is distinct from all the other subtribes in the subfamily by having pulp vesicles in the fruit. This subtribe contains three groups; primitive citrus fruit, near citrus fruit, and true citrus fruit trees. True citrus fruits have six genera: Clymenia, Eremocitrus, Micro-citrus, Poncirus, Fortunella and Citrus (**Swingle and Reece, 1967**).

Citrus taxonomy and phylogeny are very complicated, controversial and confusing, mainly due to sexual compatibility between Citrus and related genera, the high frequency of bud mutations and the long history of cultivation and wide dispersion (**Nicolosi *et al.*, 2000**). In addition, the level of difference concerning species status in Citrus is uncertain. Citrus taxonomy was based on mainly morphological and geographical data in the past and many classification systems have been formulated. Two of these systems suggested by Swingle (**Swingle and Reece, 1967)** and **Tanaka (1977)** have been the most widely accepted. The number of recognized species is the major difference between the two systems. Swingle recognized 16 species in the genus Citrus, whereas **Tanaka (1977)** recognized 162 species. **Scora (1975) and Barrett and Rhodes (1976)** suggested that there are only three 'basic' true species of Citrus within the subgenus Citrus as follows: citron (*C. medica* L.), mandarin (C. reticulata Blanco), and pummelo (*C. maxima* L. Osbeck). Later, Scora (1988) added C. halimi as another true species. Other cultivated species within Citrus were derived from hybridization between these true species or closely related genera followed, mainly, by natural mutations. Recently, this thesis has gained support from various

biochemical and molecular studies (**Federici *et al.*, 1998; Nicolosi *et al.*, 2000; Barkley *et al.*, 2006)**. Elucidating relationships, taxonomy, and diversity is important for developing breeding strategies, conserving biodiversity, and improving breeding efficiency.

Compared to morphological data, molecular markers provide abundant information, are highly efficient, and are insensitive to environmental factors. Many studies have utilized molecular markers to examine phylogenetic relationships among Citrus and its related genera, including isozymes (**Herrero *et al.*, 1996),** RFLP **(Federici *et al.*, 1998),** ISSR **(Gulsen and Roose, 2001a, b; Fang *et al.*, 1998),** RAPD **(Nicolosi *et al.*, 2000, Federici *et al.*, 1998),** cpDNA sequence **(Morton *et al.*, 2003),** SSR **(Barkley *et al.*, 2006) and** AFLP **(Pang *et al.*, 2007**). The most prominent finding from these studies was clonal variation within the major citrus groups such as lemon, sweet orange and grapefruit. However, accessions arising from spontaneous mutation are often difficult to distinguish (**Barkley *et al.*, 2006**). The most important advance was that molecular evidence supported the hybrid origin of many so-called species (i.e. sweet orange, grapefruit, and lemon) and identified their putative parental species (**Nicolosi *et al.*, 2000; Gulsen and Roose, 2001b; Pang *et al.*, 2007**). To date, molecular markers have significantly clarified the genome structure of the genus Citrus.

Sequence-related amplified polymorphism (SRAP) is a PCR- based marker system as described by **Li and Quiros (2001)**. The SRAPs is a simple and efficient marker system that can be adapted for a variety of purposes in different crops, including map construction, gene tagging, genomic and cDNA fingerprinting, and map-based cloning. It has several advantages over other systems. It is simple, has a reasonable throughput rate, discloses numerous co-dominant markers, targets open reading frames (ORFs), and allows easy isolation of bands for sequencing. Recently, they have been used to determine genetic relationships in Cucurbita pepo (**Ferriol *et al.*, 2003**), Cucurbita maxima (**Ferriol *et al.*, 2004**), peach and nectarine (**Ahmad *et al.*, 2004**), buffalograss (**Budak *et al.*, 2004; Gulsen *et al.*, 2005**), tomato (**Ruiz and Garcia- Martinez, 2005**), persimmon (**Guo and Luo, 2006**), okra (**Gulsen *et al.*, 2007**), and pea (**Esposito *et al.*, 2007**). Up to now, there is no report of measuring genetic diversity and relationships between Citrus and related genera by SRAP markers. The Aurantioideae are an important group of plants with many species of commercial importance. It is, therefore, important to understand the internal relationships among the different taxa of the subfamily for advancing breeding techniques and developing better conservation strategies. In this study, we investigated SRAP markers to better identify genetic diversity and relationships among five parents and their crosses of Citrus species.

Materials and Methods

Plant materials:

Nine genotypes of the genus Citrus were chosen for this study (Table 1). All genotypes were provided for DNA extractions. The materials were generated to represent the variability of the whole collection.

DNA extraction and SRAP analysis:

The total genomic DNA was extracted from young leaves by the CTAB method as described by **Doyle and Doyle (1990).** DNA concentration was measured with a NanoDrop, ND 100 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and 10 ng/mL DNA templates were made using TE (10 mM Tris– HCl, 0.1 mM EDTA, pH 8.0). All SRAP primer combinations were initially screened using a group of ten samples. (Table 2). The twenty-one primers that produced scorable polymorphic bands were used to amplify the rest of the accessions (Table 3). Each 15 mL reaction consisted of 1.33 mM of primers, 200 mM of each dNTP, 1.5 mL of 10× PCR Buffer (Biorun, Nantes, France), 2 mM of $MgCl_2$, 0.8 mg/mL Bovine serum albumin (Biological Industries, Beit Haemek, Israel) 5.8 mL ddH_2O, 1 unitof Taq polymerase (Biorun, Nantes, France) and 20 ng of template.

Table 1. Plant material used in this study as common and cultivar name.

Species name	Common or cultivar name
Citrus limon (L.) Burmf.	Lemon
Citrus reticulata Blanco	Cleopatra mandarin
Citrus paradisi Macf.	Grapefruit

Citrus sinensis (L.) Osbeck	Succari
Citrus Clementina L.	Clementine
Citrus species	Lemon x Clementine
Citrus species	Cleopatra mandarin x Clementine
Citrus species	Grapefruit X Clementine
Citrus species	Succari X Clementine

Table 2: The forward and reverse SRAP primer information for this study

Forward primers	Reverse primers
me1, 5′-TGAGTCCAAACCGGATA-3′,	em2, 5′-GACTGCGTACGAATTTGC-3′,
me2, 5′-TGAGTCCAAACCGGAGC-3′,	em3, 5′-GACTGCGTACGAATTGAC-3′,
me3, 5′-TGAGTCCAAACCGGAAT-3′,	em4, 5′-GACTGCGTACGAATTTGA-3′,
me4, 5′-TGAGTCCAAACCGGACC-3′,	em5, 5′-GACTGCGTACGAATTAAC-3′,

Data analysis:

DNA Thermal Cycler (Nyx Technik, San Diego, CA, USA) was used and cycling parameters included 2 min of denaturing at 94 8C, five cycles of three steps: 1 min of denaturing at 94 8C, 1 min of annealing at 35 8C temperature was increased to 50 8C, and for extension, one cycle 5 min at 72 8C. PCR products were separated on 2% agarose gel in 1× TBE buffer (89 mM Tris, 89 mM Boric Acid, 2 mM EDTA) at 115 V for 3.5 h, and photographed under UV light for further analysis. A 100 bp DNA ladder was used as the molecular standard to confirm the appropriate SRAP markers.

Data analysis:

Each band was scored as present (1) or absent (0) and data were analyzed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) software package (**Rohlf, 1993**). A similarity matrix was constructed based on Dice's coefficient (**Dice, 1945**) which considers only one to one matches between two taxa for similarity. The similarity matrix was used to construct a dendrogram using the unweighted pair group method arithmetic average (UPGMA) to determine genetic relationships among the germplasm studied. The representativeness of dendrograms was evaluated by estimating cophenetic correlation for the dendro- gram and comparing it with the similarity matrix, using Mantel's matrix correspondence test (**Mantel, 1967**). The result of this test is a cophenetic correlation coefficient, *r*, indicating how well the dendrogram represents similar data.

Polymorphism information content (PIC) values were calculated according to **Smith *et al.*, (1997)**, using the algorithm for allprimer combinations as follows:

where f^2 is the frequency of the *i*th allele. PIC provides an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles that are expressed but also the relative frequencies of those alleles. PIC values range from 0 (mono-morphic) to 1 (very highly discriminative, with many alleles in equal frequencies).

Results and discussion

SRAP amplification:

A total of 4 SRAP primer combinations were screened and atotal of 160 bands with high intensity were scored. The number of bands scored per primer combination ranged from 32 to 53, with a mean of 40. All fragments scored for each primer combination were polymorphic. Rare bands may be caused by mutations combined with selection pressure, gene flow, and drift. They are not desirable in associationstudies (**Pritchard *et al.*, 2000**), but desirable in cultivar identification; therefore, this population may not be appropriate for marker-trait association studies.

Table (3) revealed the total number of bands for each primer combination which ranged from 32 bands for the primer Em2R and me3F to 53 bands for the primer Em3 R and me4 F. The percentage of polymorphism ranged from 78 % for the primer Em2R and me3F to 96 % for the primer Em3 R and me4 F. the lowest unique bands were observed in Em2R and me3F (18) while the highest number was detected in Em3 R and me4 F primer (42). Data in Table (4) revealed the molecular weight of the four primer combinations. Table (5): indicate the number of present bands (1) and the absent bands for the four primer combinations in each genotype from parents and their crosses.

Table 3. SRAP primer combinations, numbers of polymorphic fragments resulted from this study.

Ser. No.	Primer name	Total number of bands	Polymorphic bands		Mono morphic bands	Poly morphism Percentage %	size range (bp)
			Non-unique bands	unique (bp) bands			
1	**Em2R& me3 F**	**32**	**7**	**18**	**7**	**78**	**88.7-2100**
2	**Em3 R & me4 F**	**53**	**9**	**42**	**2**	**96**	**119.8-2251.4**
3	**Em4 R & me2F**	**38**	**6**	**28**	**4**	**89**	**95.4-1321.7**
4	**Em5 R & me1 F**	**37**	**5**	**27**	**6**	**86**	**121.14-2292**
	Total	**160**	**27**	**115**	**19**	**88**	**-**

Table 4. Numbers and specific markers molecular weights for the nine genotypes using four SRAP primers**.**

primers	1	2	3	4	5	6	7	8	9	T
Em2R & me3 F	----	**(2)** 2099, 245 bp	**(1)** 308 bp	**(4)** 815, 322, 234,178 bp	**(2)** 301, 254 bp	**(3)** 1072, 798, 209 bp	**(3)** 925, 764, 260 bp	**(1)** 278	**(2)** 905, 181 bp	**18**
Em3 R & me4 F	**(2)** 511, 424 bp	**(4)** 1891, 636, 467, 281 bp	**(7)** 200, 767, 157, 625, 429, 281, 249 bp	**(5)** 959, 866, 584, 416, 149 bp	**(7)** 2251, 241, 1654, 942, 647, 476, 295 bp	**(3)** 1568, 744, 214 bp	**(3)** 893, 484, 311 bp	**(5)** 909, 659, 271, 228, 163 bp	**(6)** 1996, 1347, 850, 605, 222, 152 bp	**42**
Em4 R & me2F	**(2)** 205, 140 bp	**(2)** 865, 257 bp	**(4)** 878, 302, 229, 170 bp	**(4)** 887, 224, 202, 160 bp	**(5)** 1289, 844, 235, 166, 127 bp	**(4)** 1321, 932, 227, 162 bp	**(3)** 233, 179, 117 bp	**(3)** 309, 205, 136 bp	**(2)** 794, 297 bp	**29**
Em5 R & me1 F	**(5)** 2292, 1538, 687, 297, 189 bp	**(4)** 1746, 712, 290, 141 bp	**(3)** 694, 581, 246 bp	**(2)** 573, 254 bp	**(3)** 275, 216, 172 bp	**(3)** 1386, 227, 178 bp	**(2)** 1336, 187 bp	**(3)** 283, 205, 136 bp	**(2)** 1424, 252 bp	**27**
Total	**9**	**12**	**15**	**15**	**17**	**13**	**11**	**12**	**12**	**116**

Table 5. Indicate the number of positive bands (1) and the negative bands for the four primer combinations.

Primer	Genotype (1)		Genotype (2)	Genotype (3)	Genotype (4)	Genotype (5)	Genotype (6)	Genotype (7)	Genotype (8)	Genotype (9)
Em2R & me3 F	1	9	10	10	12	12	10	10	8	9
	0	23	22	22	20	20	22	22	24	23
Em3 R & me4 F	1	5	6	9	9	9	7	8	9	8
	0	48	47	44	44	44	46	45	44	45
Em4 R & me2F	1	8	6	9	10	10	11	9	8	7
	0	30	32	29	28	28	27	29	30	31
Em5 R & me1 F	1	12	11	11	9	9	10	9	9	8
	0	25	26	26	28	28	27	28	28	29
Total	**1**	**34**	**27**	**39**	**40**	**40**	**38**	**36**	**34**	**32**
	0	**126**	**127**	**121**	**120**	**120**	**122**	**124**	**126**	**128**

Phylogenetic analysis

Based on SRAP data, a similarity matrix was calculatedaccording to Dice's coefficient (**Dice, 1945**). A similarity dendrogram was constructed using UPGMA cluster analysis (Fig. 1). The genotypes studied had similarity values ranging from 0.31 to 0.43, indicating a high level of variation.

The dendrogram separated the members of the subtribe Citrine into two groups with a similarity value of 0.37. The dendrogram separated the parents and the resulted crosses of Citrus species into two main sub-clusters with a similarity value of 0.37. Only one member of the first sub-cluster which is Clementine or the parent of all the resulted crosses.In the second main sub-cluster, Only one member of the first sub-sub-cluster which is Grapefruit or the parent of one cross. The second sub-sub-cluster has consisted of one parent separated alone (Succari parent) and another sub-cluster. This sub-cluster is formed from the sub-sub-cluster including the parent Cleopatra mandarin and the resulting from cross Cleopatra mandarin x Clementine. The last sub-cluster has consisted of one group containing the parent Lemon and the resulted cross Lemon x Clementine. The other group consisted of two crosses; Grapefruit x Clementine and Succari x Clementine. The parental sweet orange tree was a hybrid of pummelo andmandarin (**Scora, 1975; Barrett and Rhodes, 1976**), which was latersupported by **Nicolosi *et al.*, (2000). Barkley *et al.*, (2006)** suggested that sweet orange has a majority of its genetic makeup from mandarin and only a small proportion from pummelo which was consistent with this study. **Federici *et al.*, (1998) and Nicolosi *et al.*, (2000)** found that *C. tachibana* and *C. amblycarpa* were clustered with mandarins based on RFLP, RAPD, SCAR, and cpDNA data, which was consistent with this study. Calamondin and 'Cleopatra' nested closely with the mandarins, with a similarity value between 0.70 and 0.73, respectively. Calamondin was reportedly a hybrid of kumquat and mandarin (**Barrett and Rhodes, 1976**). Calamondinand 'Cleopatra' were clustered within the mandarins (**Herrero *et al.*, 1996; Novelli *et al.*, 2000; Barkley *et al.*, 2006)**, which was also consistent with our SRAP-based results.

Table 6. Genetic similarity matrix for 9 genotypes of citrus species based on amplicons from 4 SRAP primer combinations.

	1	2	3	4	5	6	7	8	9
1	1								
2	0.41	1							
3	0.43	0.36	1						
4	0.41	0.35	0.34	1					
5	0.37	0.34	0.36	0.33	1				
6	0.42	0.36	0.36	0.34	0.31	1			
7	0.41	0.40	0.36	0.38	0.37	0.39	1		
8	0.41	0.40	0.36	0.38	0.34	0.36	0.37	1	
9	0.42	0.38	0.37	0.39	0.35	0.34	0.38	0.38	1

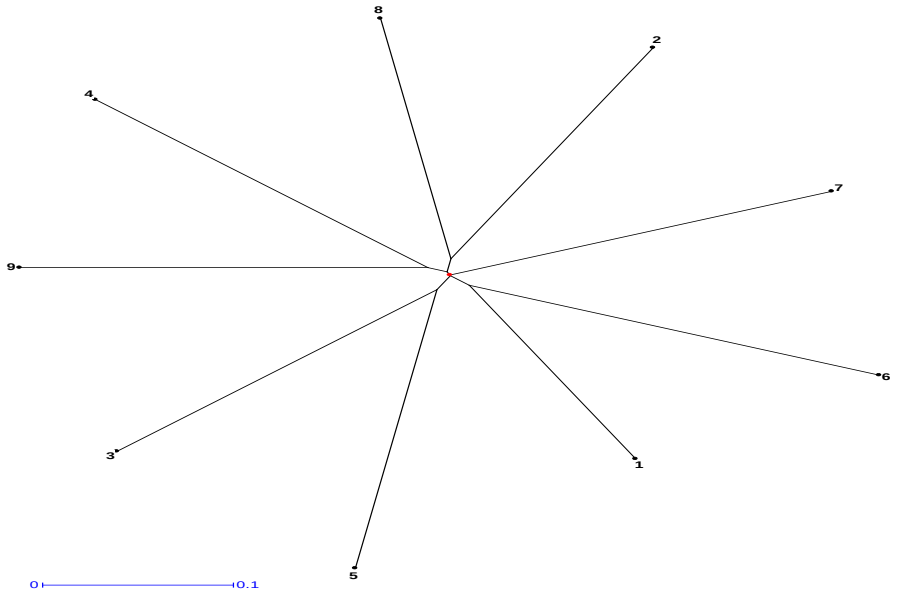

Fig. 1a. Neighbor-Joining tree based on Jaccard similarity coefficient showing the genetic relationship among 9 cultivated Citrus using SRAP markers.

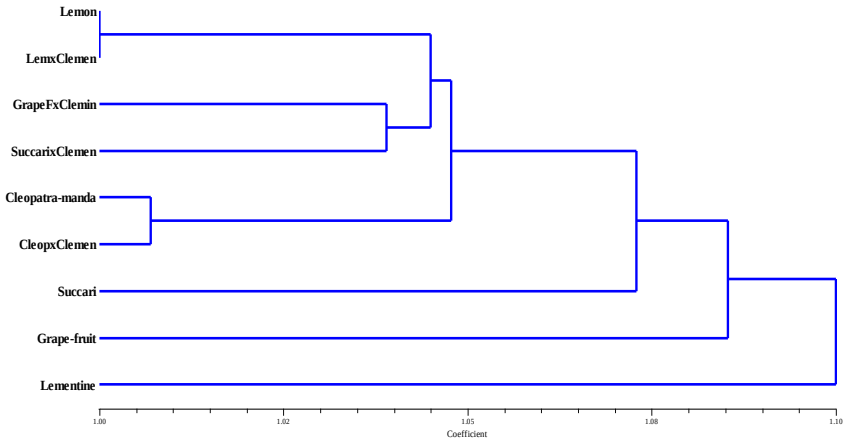

Fig. 1b. Neighbor-Joining tree based on Jaccard similarity coefficient showing the genetic relationship among 9 cultivated Citrus using SRAP markers.

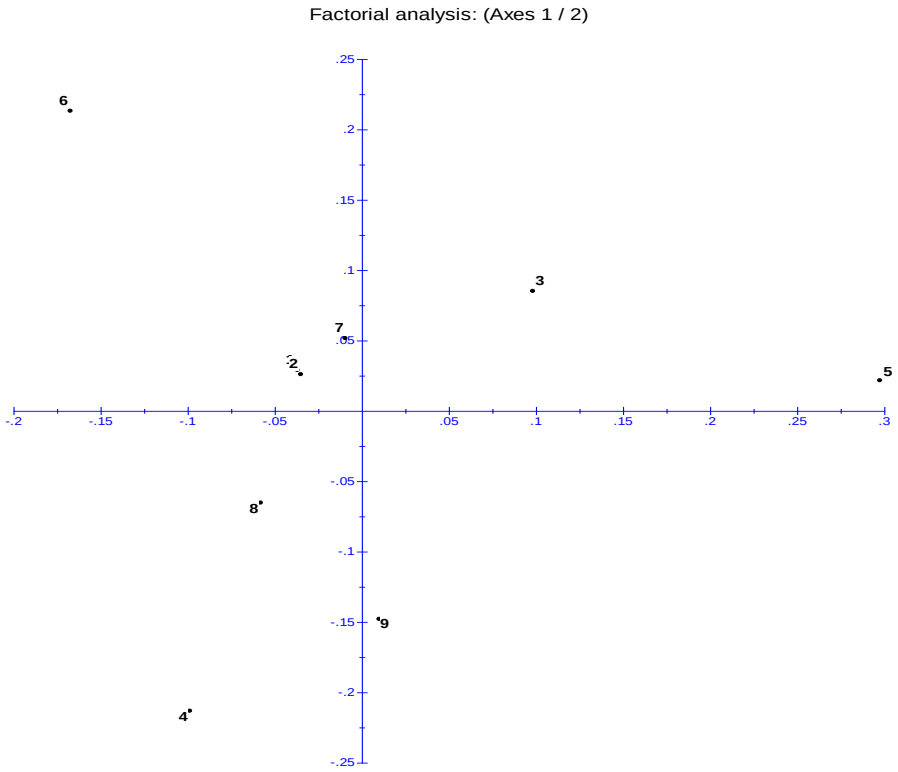

Fig. 2. Dispersion of 9 cultivated Citrus genotypes (*Citrus* species L.) in the two-dimensional plane of the principal coordinates analyses.

Barkley *et al.*, (2006) found that the mandarins were the most polymorphic among theancestral species. Monophyly in this group as detected with the UPGMA analyses indicates common ancestry among mandarins.

Genetic variation among common grapefruit cultivars was reported to be very low (**Fang and Roose, 1997; Corazza-Nunes *et al.*, 2002**). Grapefruit, highly polyembryonic, was reported as a hybrid of pummelo and sweet orange (**Barrett and Rhodes, 1976**; **Nicolosi *et al.*, 2000**), and allgrapefruit cultivars originated from the single parent through mutations (**Corazza-Nunes *et al.*, 2002**). Three pummelo accessions were clustered together. **Luro *et al.*, (2000)** also determined a high level of similarity values (>0.90) among pummelo accessions. Pummelo was proposed as one of the 'true basic species' in cultivated *Citrus* (**Barrett and Rhodes, 1976**), and, maybe, contributed to the genomes of the members of this subgroup.

Sour oranges, 'Rangpur', bergamot, 'Gou Tou Cheng', and *C. taiwanica* were clustered together. Two of the three sour orange accessions were closely related and the 'Australian' sour orange slightly differed from them with a similarity value of 0.85. Sour orange was reported as a hybrid of mandarin and pummelo in previous studies (**Barrett and Rhodes, 1976; Barkley *et al.*, 2006; Abkenar *et al.*, 2007**). The similarity value of *C. taiwanica* with sour orange was ~0.80. Similarly, sour orange and *C. taiwanica* were clustered in the same group based on ISSR data (**Fang *et al.*, 1998**). It was reported that *C. taiwanica* was probably a hybrid between *C. aurantium* and some other species of *Citrus* having long leaves (**Swingle and Reece, 1967**). 'Gou Tou Cheng' was found to be related to sour orange in this study. **Nicolosi *et al.*, (2000)** reported similar results. 'Rangpur' and bergamot were established in the same branch and closely related to sour orange. **Torres *et al.*, (1978)** reported that 'Rangpur' lime, despite its name, is quite different morphologically and genotypically from limes and was listed under *C. reticulata*. **Nicolosi *et al.*, (2000)** indicated that 'Rangpur' was a hybrid of citron and mandarin and clustered with the citrons. According to **Barkley *et al.*, (2006), Webber (1943)** believed that rangpurs were more similar to mandarins, but thought that they possibly were hybrids between limes and mandarins or possibly hybrids of limes and sour mandarins; therefore, the origin and parentage of the rangpurs have been unclear, but they have generally been classified with mandarins in most

previous studies. **Hodgson (1967)** suggested the origin of Bergamot was obscure but probably related to sour orange. This accession was identified as a hybrid of citron and sour orange (**Nicolosi *et al.*, 2000**) and clustered with sour orange (**Federici *et al.*, 1998**).

In general, conclusions of the SRAP analysis were highly correlated to those of previous studies of the subfamily Aur- antioideae which includes many genera. SRAP markers could be more advantageous over SSR markers due to the occasional loss of amplification sites of SSR primers in distant *Citrus* relatives and its relative simplicity. They may have potential in studies of diversity, linkage mapping, cultivar identification, and germplasm organization. Currently, we are using SRAP markers to identify relationshipsbetween a large number of Citrus collections and integrate SRAP markers intonew *Citrus* linkage maps.

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دراسة علي تعريف خمسة أنواع من الموالح علي أساس التشابهه و التباين في بعض القياسات الوراثية بإستخدام تقنية SRAP –PCR

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أجريت هذه الدراسة على خمسة آباء وأربع هجن مختلفة من الموالح لإلقاء الضوء على تباينات التراكيب الوراثية والبصمة الوراثية بينهما من خلال تقنية (SRAP) من خلال تضخيم بعض المواقع الوراثية لها حيث تميزت هذه التقنية بقدراتها العالية على التمييز بين الأصناف المختلفة تحت الدراسة. حيث أظهرت نتائج تحاليل البصمة الوراثية أن العدد الكلي للحزم المتضخمة وصل إلى 160 حزمة من خلال تحليل التباين المستخدم بينهم نسبة تباين وصلت إلى حوالي (0.86). كما أظهرت النتائج وجود تسابه بين الجريب فروت واليوسفي كلمنتين. وأختلفت الأصناف المختبرة في عدد الحزم المنفردة الموجبة من صنف إلى أخر داخل مجموعة الموالح المختبرة، أما بالنسبة لشجرة القرابة الوراثية فقد اظهرت النتائج المتحصل عليها إمكانية تمييز هذه المجموعة من الأصناف التابعة لجنس الموالح سواء كانت إباء أو هجن عن بعضها البعض بكفاءة عالية باستخدام تكنيك (SRAP) وبالتالي يمكن أن نوصي باستخدام هذا التكنيك للتمييز الوراثي بين الأنواع النباتية المختلفة.