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MOLECULAR DISCRIMINATION AMONG DIVERSE GRAPEVINE GENOTYPES BY RAPD MARKERS IN RELATION TO DROUGHT STRESS

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ABSTRACT

Four grapevine genotypes (*Vitis vinifera* L.), comprised two landraces and two released varieties were tested in this investigation. Five RAPD informative primers utilized in the initial screening due to their ability to produce polymorphic and unambiguous markers between the grapevine accessions. The selected primers were OPB1, OPO3, OPO5, OPO7, and OPO9. These primers produced a total of 75 DNA fragments, of which 68 fragments (90.7%) were reproducible polymorphic amplified fragments, while 7 DNA fragments were conserved among the 4 genotypes tested. All the accessions studied showed a unique banding patterns for the 5 primers used. The results indicated that 13 negative and 38 positive markers were detected. The marker fragments size ranged between 360 to 1620 bps for the negative markers and from 290 to 2810 bps for the positive markers. The most effective primer was OPO3, which discriminated all studied genotypes. The degree of the genetic similarity between all accessions tested ranged from 27.8% to 77.1%. The dendrogram tree resulting from the UPGMA suggests the existence of grapevine genotypes groups with higher similarities. The markers used in the present investigation proved to be quite powerful in detecting high polymorphism and RAPD-PCR is a reliable method for discrimination and genetic analysis of grapevine germplasm.

Barrani landrace considered as drought tolerant grapevine genotype against Thompson seedless variety which gave the lowest values of internodes length, leaf area and total chlorophyll content

under low available water added treatment (highest water deficit stress).The primer OPO3 was more informative than all other primers used as it discriminated these two contrasting genotypes by six and three positive and negative bands, respectively.

Key words: Grapevine, *Vitis vinifera*, RAPD-PCR, Dendrogram and Drought stress.

INTRODUCTION

The grape is unique, not only because of its status as a major global horticultural crop but also for its ancient historical connections with human culture. Currently, *Vitis vinifera* is among the most important plant species being cultivated on area of about 7.9 million ha with more than 10000 grape cultivars worldwide and an annual production of approximately 58 million tons **(FAO, 2004)**. It considered the second major fruit crop in Egypt and it is the fourth crop of a great potentiality for export to world markets. Total grapes area in Egypt reached 165.786 feddan producing about 1.432 million ton according to the annual year book of statistics of ARE, 2008. Accurate grapevine identification is necessary because of the global problem which has arisen as a result of the long history of cultivation and distribution of vegetative cutting from new cultivars that were wrongly identified and renamed. The spread across cultural boundaries has also increased the problem due to different countries or regions adopting different names for the same cultivar. Traditional methods of discrimination and identification of grape varieties have relied on morphological characters whose expression is affected by developmental and environmental factors. Knowledge about germplasm diversity and genetic relationships among breeding materials (landraces, traditional varieties, new recombinants...etc.) are highly valuable tools in plant improvement strategies. A number of methods have relied mainly on the availability of genetic markers; there are three types of genetic markers, morphological, biochemical and molecular markers. Marker based selection is the area where it could have the greatest impact in plant development. Molecular markers have proved to be a valuable, rapid and fundamental tool for genetic studies and genotypic characterization. The random amplified polymorphic DNA (RAPD) technique **(Williams *et al.*, 1990 and Welsh and Mc Clelland,1990)** has several distinct advantages: The

cost per reaction is low, only a small amount of plant material is required for DNA extraction (A few nanograms of DNA from the organism under study is enough) non–radioactive detection and no-need for expensive equipments in addition to the method does not require any prior knowledge of the genome sequence **(Karatash and Sabit Agaoglu, 2008).** The RAPD technique has been successfully used for genetic studies in grapevines **(Martinez *et al.*, 2003, Benjak *et al.*, 2005 and Kocsis *et al.*, 2005)** which increased the understanding of relatedness of cultivars and facilitated research in vitis genetics **(Reisch, 2000).**

The aim of this study was to identify and discriminate grapevine genotypes which dominate in newly reclaimed areas under desert conditions of Egypt and to determine their genetic similarities based on RAPD–PCR analysis.

MATERIAL AND METHODS

Plant Material:

The present study was carried out during three successive years lasted 2011 at Maryout Experimental Station of Desert Research Center by growing cuttings of four diverse grapevine genotypes in 25 cm diameter plastic bags. After three months, three of them (the released variety, Thompson seedless and the two landraces, Barrani and Saint Catherine) were treated by four levels of water deficit (control, 75%, 50% and 25% of available water level). Each treatment contained five replicates arranged in split plot design where, genotypes allocated main plots and the levels of irrigation water in sub-plots. The recorded data statistically analyzed according **Snedecor and Cochran (1989)**. Molecular analysis was practiced by using five RAPD selected primers to differentiate among four diverse genotypes tested.

Molecular Analysis:

Young leaves were collected from 5 trees for each cultivar and immediately frozen in liquid nitrogen and stored at -20°C. DNA was extracted from the leaf samples following the protocol for minipreps by using CTAB **(Dellaportta *et al.*, 1983)**. For RAPD analysis, the method described by **Williams *et al.* (1990)** was used to optimize the RAPD conditions.

Five 10-mer primers were used to generate the RAPD markers. All primer codes and sequences are presented in Table (1). The PCR reaction conditions were optimized and reaction mixtures (50 µl total volume) consisted of 10X PCR buffer, $MgCl_2$ (50 mM), dNTPs (2 mM), primer (5 µl), template DNA (10 ng/µl), Taq DNA polymerase (5 units). DNA amplification was carried out for 45 cycles in Perkin Elemar G. thermal-cycler. Amplified products were size-separated by electrophoresis in 1.5% agarose gel and visualized by ultraviolet illumination after staining with ethidium bromide.

Table (1): Code of the operon primers tested and their nucleotide sequences.

Code	Sequence
OP-O3	5/CTGTTGCTAC 3/
OP-O5	5/ CCCAGTCACT3/
OP-O7	5/ CAG CAC TGAC3/
OP-O9	5/ TCCCACGCAA 3/
OP-B 1	5/ GTT TCG CTC C3/

Data Analysis:

RAPD assays were repeated twice for each primer and only clear bands were scored, with particular attention to sharp bands. Faint ones were ignored. The data of the primers products were used to estimate genetic similarity (GS) between different pairs of genotypes tested by calculated Dice coefficient according **Sneath and Sokal , 1973**. GS = 2Nij / (Ni+Nj), where Nij is the number of bands present in both genotypes i and j; Ni is the number of bands present in genotype i and Nj is the number of bands present in genotype j. Based on the similarity matrix, a dendrogram showing the genetic relationships between genotypes, was constructed using the NTSYS-pc version 2.21b (published 22 June 2009).www.exetersoftware.com software package. Genetic relationship among the genotypes was estimated with the dendrogram constructed using DICE computer package to estimate the pairwise differences matrix and plot the phonogram among genotypes. Unique bands detected in a particular genotype but not in others were used as positive DNA markers. The absence of a common band for a given genotype was referred to negative specific marker.

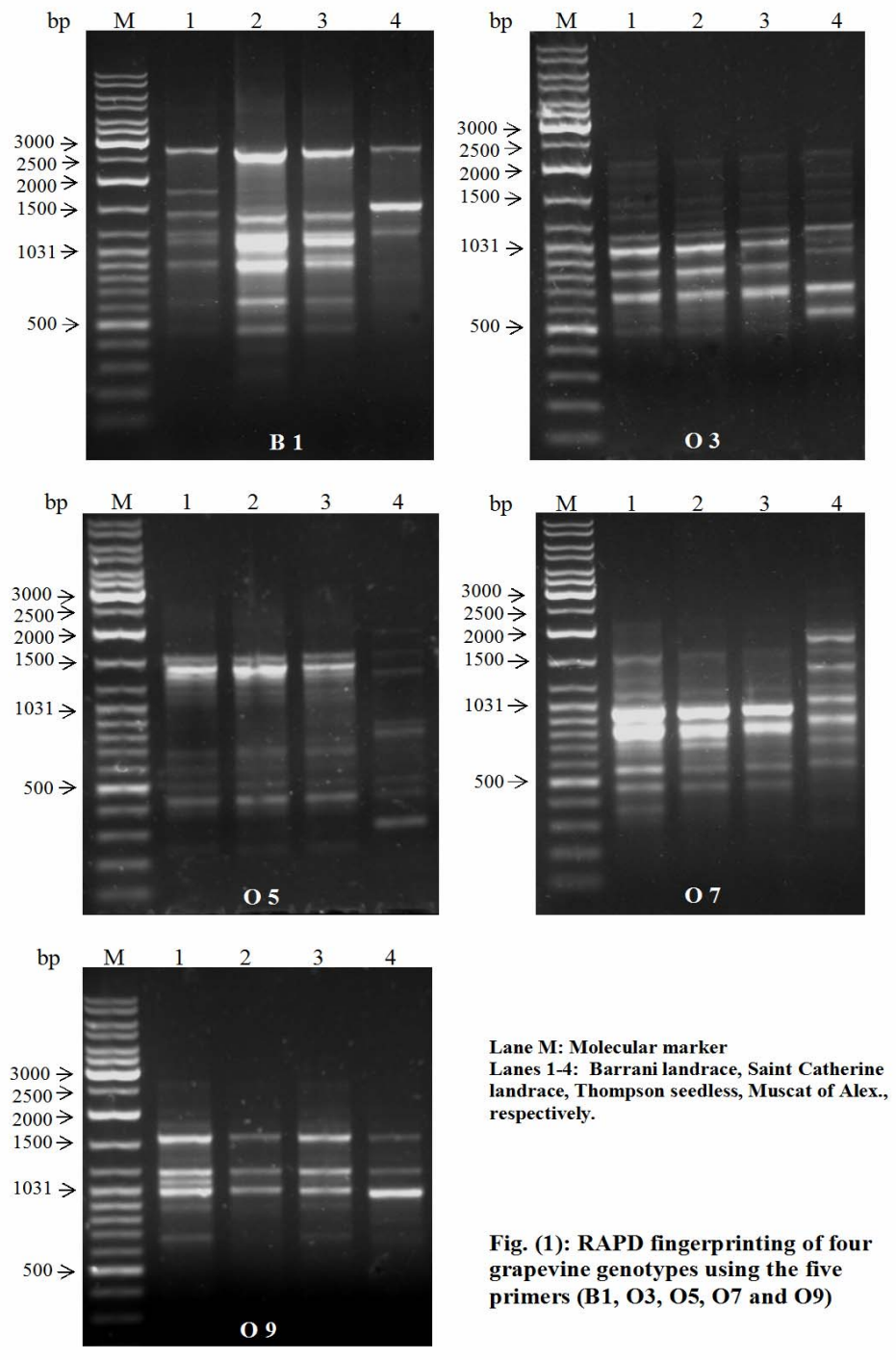
RESULTS AND DISCUSSION

(1) Degree of Polymorphism:

In order to investigate the genetic diversity of the four grapevine genotypes, five informative RAPD primers were selected due to their ability to produce polymorphic and unambiguous markers between them (Fig. 1). The primers revealed total number of 75 amplified fragments (Table, 2). Out of them, 68 were determined as polymorphic amplified fragments size ranged from 260 to 2810 bps and the number of bands per primer varied from 9 (OPO9) to 18 (OPO3). The five primers discriminated all of the studied genotypes and each of such genetic materials showed a unique banding pattern. The most effective primer was OPO3, which produced 18 bands. This primer gave ratio of polymorphic bands (94.4%) which was the highest ratio of polymorphism while the lowest ratio was 75 % (OPO9) as shown in Table (3). The 5 primers used in the present study produced high degree of polymorphism. These primers could be recognized to be appropriate primers for studies related to genetic diversity of grapevine genotypes. The suitability of RAPD technique for genetic diversity studies and grapevine germplasm evaluation has been reported by several authors **(Kocsis *et al.*, 2005; Bodea *et al.*, 2009 and Maia *et al.*, 2009).**

(2) Specific DNA Markers:

The presence or absence of unique DNA Fragments with different sizes in a particular genotype could be used as positive or negative specific DNA markers for such genotype and might be helpful in genotype identification and discrimination. In the present investigation 38 positive and 13 negative markers were detected by the five tested primers (Table, 2). The marker fragments size ranged from 290 to 2810 bps for the positive markers and from 260 to 1620 bps for the negative marker in genotype No.1 (Barrani landrace) and 9 positive markers (2 from OPB1 at 2750 and 1780bp), (2from OPO3 at 2100 and 1710 bp), (3 from OPO7 at 2180, 1540 and 365 bp) and (3 from OPO9 at 1830, 1580 and 1110 bp) were detected. It is note worthy that no negative markers were observed in the same genotype among the five RAPD primers results.



Lane M: Molecular marker
Lanes 1-4: Barrani landrace, Saint Catherine landrace, Thompson seedless, Muscat of Alex., respectively.

Fig. (1): RAPD fingerprinting of four grapevine genotypes using the five primers (B1, O3, O5, O7 and O9)

Table (2): RAPD polymorphism in the four grapevine genotypes using the five primers.

Amplicon	Primer code	bp	1	2	3	4	MM *
AF1	B1	2810	0	0	0	1	PM
AF2		2750	1	0	0	0	PM
AF3		2700	0	0	1	0	PM
AF4		2600	0	1	0	0	PM
AF5		1780	1	0	0	0	PM
AF6		1540	0	0	0	1	PM
AF7		1430	1	0	1	0	
AF8		1370	0	1	0	0	PM
AF9		1225	1	0	0	1	
AF10		1160	0	0	1	0	PM
AF11		1120	1	1	0	0	
AF12		920	1	1	1	0	
AF13		630	1	1	1	1	
AF14		480	1	1	1	0	NM
AF15		370	0	1	0	0	PM
AF16		290	0	1	0	0	PM
AF17	O3	2310	0	0	1	1	
AF18		2170	0	1	0	0	PM
AF19		2100	1	0	0	0	PM
AF20		1950	0	0	0	1	PM
AF21		1710	1	0	0	0	PM
AF22		1650	0	0	1	1	
AF23		1550	0	1	0	0	PM
AF24		1460	1	0	0	1	
AF25		1345	1	1	0	0	
AF26		1235	0	1	0	1	
AF27		1190	0	0	1	0	PM
AF28		1125	1	1	0	0	
AF29		1040	1	1	1	1	
AF30		850	1	1	1	0	NM
AF31		730	0	0	0	1	PM
AF32		680	1	1	1	0	NM
AF33		600	0	0	0	1	PM
AF34		500	1	1	0	0	

MM*: Molecular marker, PM: Positive marker and NM: Negative marker

Table (2) Cont.

Amplicon	Primer code	bp	1	2	3	4	MM*
AF35	O5	2130	0	0	0	1	PM
AF36		1740	0	0	0	1	PM
AF37		1625	0	0	1	0	PM
AF38		1585	1	1	0	0	
AF39		1435	1	1	0	1	NM
AF40		1390	0	1	0	0	PM
AF41		1190	1	1	1	0	NM
AF42		1090	1	1	0	0	
AF43		940	0	0	0	1	PM
AF44		860	0	0	0	1	PM
AF45		720	1	1	1	0	NM
AF46		610	1	1	1	0	NM
AF47		535	1	1	1	1	
AF48		460	1	1	1	1	
AF49		350	0	0	0	1	PM
AF50		260	1	1	1	0	NM
AF51	O7	2180	1	0	0	0	PM
AF52		1900	0	0	0	1	PM
AF53		1690	0	0	1	0	PM
AF54		1600	0	1	0	0	PM
AF55		1540	1	0	0	0	PM
AF56		1450	0	0	0	1	PM
AF57		1225	0	0	1	1	
AF58		1155	1	1	0	0	
AF59		1100	0	0	0	1	PM
AF60		1000	1	1	1	0	NM
AF61		910	0	0	0	1	PM
AF62		830	1	1	1	0	NM
AF63		740	0	1	0	1	
AF64		580	1	1	1	1	
AF65		475	1	1	1	0	NM
AF66		365	1	0	0	0	PM
AF67	O9	1830	1	0	0	0	PM
AF68		1620	0	1	1	1	NM
AF69		1580	1	0	0	0	PM
AF70		1210	1	1	1	1	
AF71		1110	1	0	0	0	PM
AF72		1025	1	1	1	1	
AF73		900	1	1	1	0	NM
AF74		820	0	0	0	1	PM
AF75		680	1	0	1	0	

MM*: Molecular marker, PM: Positive marker and NM: Negative marker

Table (3): Amplification results of the five RAPD primers for the four grapevine genotypes tested.

Primer code	TAF	PB	P%	Genotype								TSM
				1		2		3		4		
				AF	SM	AF	SM	AF	SM	AF	SM	
B1	16	15	93.8	8	2	8	4	6	2	4	4	12
O3	18	17	94.4	9	2	9	2	6	1	8	5	10
O5	16	14	87.5	9	0	10	1	7	1	8	5	7
O7	16	15	93.8	8	3	7	1	6	1	7	7	12
O9	9	7	75	7	3	4	0	5	0	4	2	5
Total	75	68	-	41	10	38	8	30	5	31	23	46

TAF= Total number of amplified fragments, PB = Polymorphic bands, P%= polymorphism percentage, AF= Amplified fragments / genotype, SM= Genotype- specific marker including either the presence or absence of a given band, TSM= Total number of specific markers

(3) *Genetic Similarity:*

According to the similarity matrix of the 4 grapevine genotypes (Table, 4), the highest similarity (0.771) was found between genotypes No.1 and No. 2 (Saint Catherine landrace) while the lowest similarity (0.290) was between genotypes No.2 and No.4 (Muscat of Alex.). The primers used in the present investigation proved to be quite powerful in detecting high polymorphism as well as distinguishing the tested grapevine genotypes. Figure (2) revealed the dendrogram tree of the four grapevine diverse accessions resulting from the UPGMA of values presented in Table (4). Cluster analysis suggests the existence of groups with higher similarities. The two genotypes No.1 and No.2 were clustered together in the main group, while the most distant relationship was scored between genotype No. 4 and all other genotypes tested. In the present investigation, the high level of polymorphism detected using RAPD analysis and the determination of DNA markers, suggested that RAPD approach showed considerable potential for grapevine genotypes identification, discrimination and explaining the interrelationships among genetic materials tested. These findings are more or less in line with those previously reported by **Singh *et al.* (2005)** and **Afiah *et al.* (2008).**

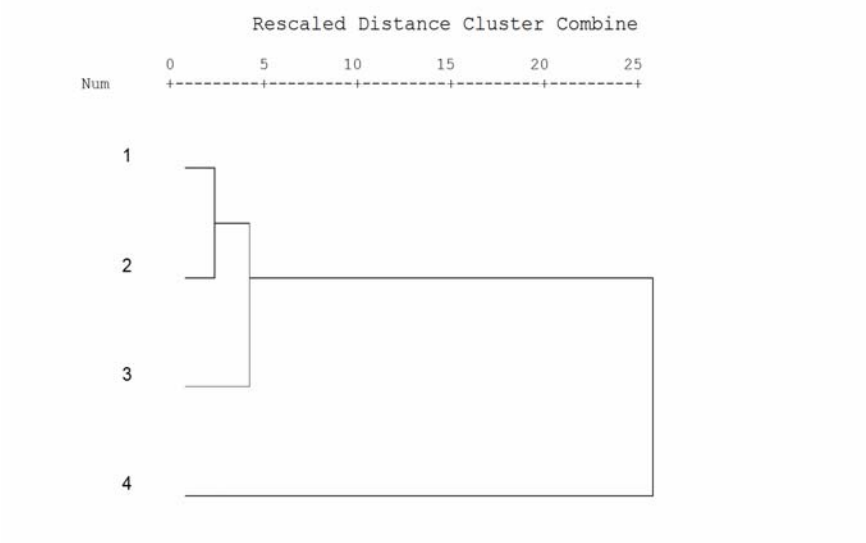
One concern is that much of the genetic variation for improving abiotic stress tolerance has been lost during domestication, selection and modern breeding, leaving pleiotropic effects of the selected genes for development and adaptation. Such genes are critical in matching cultivars to their target agronomic environment, and since there is little leverage in

changing these, other sources of variation may be required. In grapevine and many other crops, greater variation to abiotic stresses exists in primitive landraces and newly bred lines gene pools. These markers can be successfully used for identification of the best genotypes associated with stress tolerance gene(s). Similar conclusion was obtained in different grapevine genotypes by **Kim *et al.* (2002), Aras *et al.* (2005), Solouki *et al.* (2007), Salayeva *et al.* (2010), Butiuc- Keul *et al.* (2010)** and **El-Sayed *et al*. (2011).**

Table (4): Similarity matrices among four genotypes of grapevine based on RAPD analysis.

Genotypes	1	2	3
2	0.771		
3	0.592	0.588	
4	0.278	0.290	0.361

1-4:Barrani, Saint Catherine Landraces, Thompson seedless, and Muscat of Alex , respectively



1-4: Barrani landrace, Saint Catherine landrace, Thompson seedless, Muscat of Alex , respectively.

Fig. (2). The genetic distances among four grapevine genotypes using five RAPD primers.

Barrani landrace considered as drought tolerant grapevine genotype against Thompson seedless variety which gave the lowest values of internodes length, leaf area and total chlorophyll content in the second

growing season (Table, 5) under low available water added treatment (highest water deficit). The primer OPO3 was more informative than all other primers used as it discriminated the highly tolerant genotype (Barrani landrace) against the moderate one (Thompson seedless) by six and three positive and negative bands, respectively. The primer OPB1discriminated Barrani landrace by four positive bands of amplicon fragments (AF2, 5, 9 and 11) and two negative bands (AF3 and 10) for the sensitive variety Thompson seedless. **Striem *et al.* (1996)** identified twelve RAPD markers that could be associated to the seedless character. Meanwhile, OPO5 verified the contrasting genotypes by three positive bands (AF38, 39 and 42) and unique negative band (AF37). The primer OPO7 discriminated the same genotypes by three and two positive and negative oligonucleotide fragments, respectively. Also, OPO9 gave three positive (AF 67, 69and 71) and unique negative (AF68) molecular markers for the best potentiality of Barrani grapevine landrace as shown in Table (2). These findings are in line with the earlier reports of **Ren *et al.* (2000), Renger *et al*. (2005), El-Halfawy *et al*. (2006), Lima *et al.* (2006)** and **Afiah *et al*. (2008).**

Table (5): Response of the three grape genotypes to irrigation water deficit in 2010 growing season.

Genotypes	Treatments	Leaf area (cm^2)	Internodes length	Relative water content %	Total chlorophyll content
Barrani Landrace	25%	9.82	3.22	73.08	1.12
	50%	15.64	3.68	75.72	1.86
	75%	24.92	3.94	81.68	2.14
	100%	23.46	5.60	83.04	2.04
	mean	18.45	4.11	78.38	1.79
Saint Catherine Landrace	25%	12.08	2.70	73.92	1.48
	50%	16.66	3.58	74.76	1.15
	75%	23.86	3.86	81.71	2.00
	100%	21.40	5.26	84.94	1.97
	mean	18.50	3.85	78.83	1.90
Thompthone Seedless Variety	25%	9.34	2.68	73.72	1.30
	50%	15.22	3.74	80.66	2.01
	75%	23.82	3.36	81.76	1.79
	100%	25.58	4.80	87.52	1.96
	mean	18.46	3.64	80.91	1.77
Treatments grand mean	25%	10.41	2.87	73.57	1.30
	50%	15.84	3.67	77.05	1.30
	75%	24.20	3.72	81.71	1.98
	100%	23.48	5.72	85.17	1.99
LSD 0.05	Genotypes (G.)	N.S.	0.30	1.13	N.S.
	Treatments (T.)	1.88	0.42	1.47	0.27
	G. X T.	2.88	N.S.	0.45	N.S.

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المفاضلة الجزيئية بين تراكيب وراثية متباينة من العنب بواسطة تحليل RAPD-PCR وعلاقة ذلك بتحمل الجفاف

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[٤] قسم الاصول الوراثية النباتية - مركز بحوث الصحراء - المطرية - القاهرة

فى هذه الدراسة تم اختبار اربعة تراكيب وراثية من محصول العنب (صنفان وسلالتان وليدتا البيئة Landraces) باستخدام خمسة من البادئات العشوائية المختارة RAPD-PCR والتى تعطى نسبا عالية للتعدد المظهرى Polymorphism تساعد فى التفريق بين مدخلات العنب الوراثية Grapevine accessions.

- باستخدام تفاعل البلمرة المتسلسل PCR اعطت البادئات العشوائية الخمسة المستخدمة ٧٥ حزمة تمثل قطعا صغيرة من الحامض النووى DNA (تتكون كل منها من عشرة تتابعات جزيئية) كان المتباين منها ٦٨ حزمة بنسبة ٩٠،٧ % تعدد

مظهرى. كما تميزت كل من التراكيب الوراثية تحت الدراسة بحزمة وحيدة unique او اكثر لكل من البادئات العشوائية المستخدمة فى ما عدا السلالة وليدة بيئة برانى عند استخدام البادىء OPO5 وكذلك الصنف طومسون والسلالة وليدة بيئة سانت كاترين عند استخدام البادىء OPO9. كما اوضحت النتائج تسجيل ٣٨ كاشف جزيئى موجب (تراوح الحجم الجزيئى لها ما بين ٢٩٠ - ٢٨١٠ bp) بالاضافة الى ١٣ كاشف جزيئى سالب (تراوح الحجم الجزيئى لها ما بين ٣٦٠ - ٢٦٢٠ bp).

- يعتبر البادىء العشوائى OPO3 هو الاعلى كفاءة حيث امكن بواسطته تمييز كافة التراكيب الوراثية المختبرة بنسبة تباين ٩٤,٤% كما كانت نسبة التماثل فى اعلى درجاتها (٧٧،١%) بين التراكيب الوراثية الاول والثانى (السلالة وليدة بيئة برانى والسلالة وليدة بيئة سانت كاترين) كما كانت اقل ما يمكن(٢٧،٨%) بين الاول والرابع (السلالة وليدة بيئة برانى والصنف مسكات اسكندرية). وقد ترجمت شجرة القرابة ذلك حيث كان التركيب الوراثى الاول والثانى فى المجموعة الاكثر قرابة يليها الثالث (الصنف طومسون عديم البذور) فى درجة قرابته لكليهما فى حين كان التركيب الوراثى الرابع هو الاكثر تباعدا عن كافة تراكيب العنب الوراثية المختبرة.

- تؤكد هذه النتائج امكانية توصيف المدخلات الوراثية للعنب بواسطة تفاعل البلمرة المتسلسل لتحليل بادئات التضخيم العشوائى RAPD-PCR analysis كما اوضحت الدراسة وجود تلازم بين ارتفاع درجة تحمل السلالة وليدة بيئة سيدى برانى (الارضية والمناخية) لنقص مياه الرى مقارنة بالصنف الحساس Thompson seedless وستة من الكاشفات الجزيئية الموجبة بالاضافة الى ثلاثة من الكاشفات الجزيئية السالبة عند تحليل نتائج البادئ العشوائى الامثل OPO3.