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Polymorphism of Some Native Sistan Grapes Assessed by Long and Short Primers for RAPD Markers

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Abstract: Grapevines have Bronze ages archive in Sistan area of Iran. In order to study the genetic variation and taxonomic relationships between 6 cultivars of the Sistan grapevines (*Vitis vinifera* L.) at molecular level, random amplified polymorphic DNA (RAPD) markers were used. The data were subjected to statistical analyses and genetic resemblance was calculated using Dice similarity index. The grapevines related to the different geographic areas of Sistan were assessed by 50 short (10 mer) and long (15-21 mer) primers. Out of 50 primers which were tested, 21 primers gave reproducible results. Selected primers created 497 bands. Resulting profiles showed that the produced bands varied in size from 300 to 3500 base pairs. The numbers of reliable polymorphic fragments for short and long primers were 86 and 334 bands, respectively. In multiplication reaction the items in the size area of 564 to 1904 base pair resulted for short primers and 564 to 4277 base pair for long primers. From the bands calculated a matrix that was analyzed by the unweighted pair group method on arithmetic averages to draw a dendrogram. The population was classified in 4 main groups in which Red Yaghooti and White Yaghooti had the maximum and Red Yaghooti and Laal had the minimum similarity coefficients. In our study, by comparing the results gained from technique long and short primers in RAPD, the potential value of long primers for the production of polymorphism in grapes was identified.

Key words: Genetic similarity, *Vitis vinifera*, RAPD, primers

INTRODUCTION

Many grapevines varieties have reduced around the world duo to marked tendency to use only desirable cultivars with high quality and quantity. In Iran the first high price of early ripening grapevines in the market are from Zabol, but after 8 years drought many cultivated grapevines are towards extinction and few of vineyards have remained at the least variation. Furthermore, raisins of seeded and seedless grapevines recently were found by Iranian and Italian archeologists in Zabol Shahre Sokhta (about 5000 years old ancient site with historical graveyards and architectural style). The long history of grapevine has determined a complex picture in which many biotypes or even cultivars are misidentified or called by different names in different areas. The distinction of clones or nearly related cultivars by morphological characters is often difficult (Shiran *et al*., 2007).

Grapevines are propagated by cuttings and the resulting clones of a given population are genetically identical to each other and to mother plants (Mullins *et al*., 2000). Knowledge of genetic diversity among adapted cultivars or elite breeding materials has a significant impact on the improvement of plants. It can be obtained from pedigree analysis, morphological traits (Mullins *et al*., 2000) or using molecular markers (Adiguzel *et al*., 2006).

Traditional methods for the recognition of the characters of grapevine were based on observation of morphological traits (Ohmi *et al*., 1993) and these properties are also affected by the environmental conditions and growth factors and it results in the decrease in efficiency of these markers. Molecular markers provide the best estimate of genetic diversity since they are independent of the confounding effects of environmental factors. Isoenzymes have been used in the last two decades with the same aim (Dolezalova *et al*., 2003). It was not suitable for predicting F1 yield and heterosis (Yu *et al*., 2005).

The random amplified polymorphic DNA (RAPD), rather than protein can be used to detect DNA

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polymorphism without predetermined genetic data. Each product is derived from a region of the genome that contains two short segments in inverted orientations, on opposite strands that are complimentary to the primer and sufficiently close together for the amplification to work. The RAPD has been used for many crop plants in resent years, such as pepper (Kumar *et al*., 2007), tomato (Rajput *et al*., 2006), *Pinus patula* trees (Malabadi *et al*., 2006), Date palm (Javouhey *et al*., 2000; Eshraghi *et al*., 2006), wheat cultivars (Shehata, 2004) and mints (Momeni, 2006). Many authors have also reported of identification in grapevines by the use of RAPD markers (Collins and Symons, 1993; Moreno *et al*., 1995; Grando *et al*., 1996; Stavrakakis *et al*., 1997; Fanizza *et al*., 1999; Ulanovsky *et al*., 2002).

The aim of present report was to offer a reliable method for the identification, recognition and examination of the phylogenetic relationships among 6 grapevines cultivars in Zabol. The application of DNA markers for genotype analysis of grapevines are very widespread and may contribute to unravel the Iranian grapevine germplasm diversity.

MATERIALS AND METHODS

Plant material: After 8 years drought in the reign of Sistan (Iran), 6 grape cultivars namly Fakhri, Laal, Sangak, Red Yaghooti, Roche Safid (Whight Yaghooti) and Cheshm Gavi which all of them belong to *Vitis vinifera* were hardly collected from the demolished vineyards of Sistan which located in the east of Iran. The grapes cuttings were established in the Zabol university green house and then maintained in *in vitro* culture in the BioCenter of Zabol University. The media (4.2 g L^{-1}) of Murashige and Skoog (1962) obtained from Emperial Laboratories, 20 g L^{-1} sucrose, 0.1 mg g L^{-1} IAA for rooting and 1.5 mg g L^{-1} BAP were used to produce enough shoots and leaves *in vitro*. The leaves materials from jars and plants in pots were utilized for DNA isolation during the study.

DNA extraction: In the presence of liquid nitrogen 0.5 g of young leaves was grinded and their genomic DNA was extracted by protocol of Lodhi *et al*. (1994). The PVP (50 mg polyvinyle pirrolidone) was added to the buffer to eliminate polyphenols (Newbury and Possingham, 1997) and 5 M NaCl used for eliminating the excess of polysaccharides (Do and Adams, 1991). After that, DNA was solubilized in TE buffer (Tris 10 M, EDTA 1 mM and pH 8.0) and stored at -20°C. DNA concentration was assessed by a biophotometer (Eppendorf AG. Hamburg) and gel electrophoresis. The sample with optimal A_{260}/A_{280} values ranging from 1.8-2.0 was used for the RAPDs amplification and the DNA concentration of 10 ng was adjusted to be used in the laboratory.

RAPD amplification: Genomic DNAs were amplified using 50 different RAPD primers (Cinnagen, Iran) after optimization of amplification reaction. Polymerase Chain Reaction (PCR) was performed using a Gradient Eppendorf Termal Cycler based of what reported by Lodhi *et al*. (1997). Each PCR reaction was carried out more than three times to be observed the repeatability of amplification patterns. Out of fifty primers which were tested, seven (10 mer) and fourteen (15-21 mer) gave reproducible results (Table 1). The reaction mixture with

Table 1: Primers and number of polymorphic bands obtained from six cultivars

No.	Primer sequence	No. of polymorphic bands	Annealing temperature	Polymorphism (%)	Primer code
1	5/-CGGCCCACGT-3/	17	35	70	B-302
2	5/-CGGAGAGCGA-3/	12	35	70	B3-18
3	5/-CTGGGGCCGT-3/	14	35	70	B-341
4	5/-GCGTGACCCG-3/	10	35	71	B-345
5	5/-GGAGCCCCCT-3/	12	35	66	B-349
6	5/-CCCACTACTG-3/	10	35	66	B-372
7	5/-CGCCCGCAGT-3/	11	35	68	B-389
8	5/-ATACCAAACGACGAG-3/	17	42	70	P-36
9	5/-TGCGCAACGTTGTTG-3/	14	44	70	P-35
10	5/-GTAAAACGCGGCCAGT-3/	15	48	68	CO-7
11	5/-GGGTAACGCCACGTAG-3/	18	49	78	OPO-8
12	5/-AATCAAAATCACCGGTG-3/	21	43	87	CO-9
13	5/-GTAAAACGACGGCCAGT-3/	23	52	88	P-33
14	5/-CCTGTAGTGGGTTAGGTCGT-3/	30	60	100	CO-4
15	5/-CGCCGCTCCCGATTGGCCTTT-3/	35	59	100	CO-8
16	5/-ACAGGGAACGCGGATCTCTA-3/	27	60	100	CO-1
17	5/-AGCCTCGATTATCTGCGACA-3/	28	58	100	CO-10
18	5/-AAATGCGGCAATGATACGCC-3/	31	58	100	CA-2
19	5/-ACGTTTTCCCATTGCCAA-3/	21	48	87	CA-3
20	5/-CTAAGCTGCTTTTGTTTGAGC-3/	32	58	100	GY-169
21	5/-GTTCAGGGCTGTTTATAG-3/	22	52	88	GY-107

total volume of 25 L containing of 7×PCR buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl, Cinnagene), 0.1% triton×100, 2 mM $MgCl_2$, 200 μM dNTPs, 2U of *Taq* DNA polymerase (Cinnagene), 0.4 μM primer and 10 ng genomic DNA was used. The Termal Cycler with the following profile was programmed (I) 40 cycles of 30 sec at 94°C, 1 min at 36°C for short primers and 44-60°C for long primers and 2 min ramp until 72°C. (ii) final step of 72°C for 8 min and then cooled to 4°C. The PCR products were separated by electrophoresis on 1.4% agarose gel in 0.5×TAE buffer (0.045 M Tris, 0.001 M EDTA, glacial acetic acid 57.1 mL) and stained with ethidium bromide. EcoR1/HindIII digested lambda DNA was used as molecular size marker. The gels were visualized by Gel Documentation system (Vilber Lourmat-France) and image of gels were printed.

Data analysis: Reproducible, polymorphic bands of the both replicated RAPD-PCR were scored as (1) for presence and (0) for absence in each cultivar. The generated data matrixes were subjected to statistical analyses using NTSYS-pc software (Rohlf, 1998). Genetic similarities were calculated using Dice similarity index as in Nei and Li (1979). Dendrograms showing genetic relationships of 6 cultivars and lines were constructed using the unweighted pair-group method on arithmetic averages algorithm (UPGMA). For each dendrogram the cophenetic correlation coefficient between the matrix of genetic similarities and the matrix of cophenetic values were computed and tested the significancy of them using Mantel matrix correspondence test (Mantel, 1967).

RESULTS

The plants that were established in the jars gave many micro cutting by using modified Murashige and Skoog (1962) medium containing 2.0 mg L^{-1} BAP (6-Benzyl amino porin) and for rooting IAA (indole-3-acetic acid) was used according to the procedure outlined above. In order to have fresh leaves, rooted grapevines in *in vitro* were potted and kept in growth room at 25°C.

Higher yields of DNA from 6 different grapevine leaves were obtained by using the modified CTAB procedure (lodhi *et al.*, 1994) rather than Dellaporta *et al.* (1983). Partially expanded leaves resulted in good yields instead of very young leaf tissues in the jars. From 0.5 g fresh leaves the DNA yielded 0.5×10^{-6} ng ranged with A_{260}/A_{280} between 1.8 and 2.0.

The evaluation of the 50 tested primers, 21 primers resulted optimal amplified DNA (Table 1) and the others had bands with poor resolution and were difficult to analyze. Total generated bands were 497 for Fakhri, Laal, Sangak, Red Yaghooti, Roche Safid (White Yaghooti) and Cheshm Gavi. The DNA polymorphisms among the

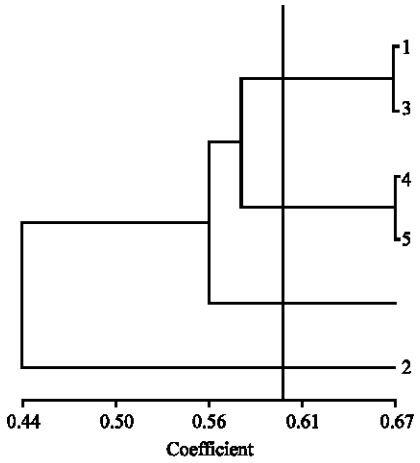

Fig. 1: Dendrogram illustrating of 6 grapevine cultivars by UPGMA cluster analysis based on RAPD data. 1-F; 2-L; 3-S; 4-Re; 5-Ro; 6-Ch

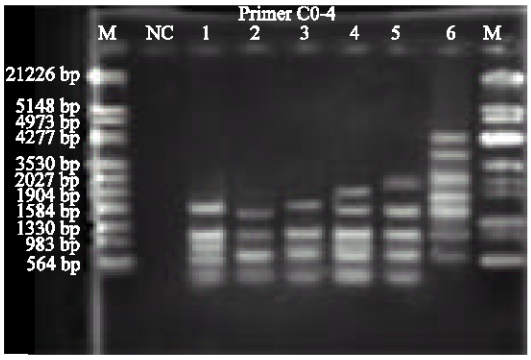

Fig. 2: RAPD profiles on grapevine DNA generated with the primer C0-4 (20-mer). 1-F; 2-S; 3-Re; 4-Ro; 5-Ch; 6-L

grapevine cultivars showed 420 bands of which similarity indices ranged from 0.4112 to 0.6718. All primers produced at least one variable locus in all cultivars.

The 6 cultivars were identified by means of the 21 primers (long and short) that were selected their ability to generate unique polymorphic amplification fragments. The mean similarity indices for all paired comparisons (Table 2) was 0.5347 and ranged from 0.4112 (Laal and Red Yaghooti) to 0.6718 (Red Yaghooti and Roche Safid).

Similar results were obtained with unweighted pair group method with arithmetic mean (UPGMA). When six cultivars were analyzed with UPGMA, they were clustered into four groups (Fig. 1). The branch on numbers 1-3 and 4-5 illustrated on dendrograms point up the highest similarity. However, at these dendrograms, four major clusters were formed at 40% different genetic distances.

A typical gel displaying the amplification products using long primer is shown in Fig. 2. Long primers

Table 2: Matrix of similarity coefficient among the 6 grapevine cultivars as estimated by dices coefficient

Cultivars	Fakhri	Red Sangak	Roche Yaghooti	Cheshm Safid	Gavi	Laal
F	100.00					
S	45.50	100.0				
Re	67.10	46.4	100.0			
Ro	53.70	41.1	60.3	100.0		
Ch	62.41	42.1	55.9	67.2	100.0	
L	55.26	46.4	53.2	55.8	58.7	100.0

F-Fakhri, S-Sangak, Re-Red Yaghooti, Ro-Roche Safid, Ch-Cheshm Gavi, L -Laal

generated more DNA fragments, a wider range of DNA fragment sizes (typically from 564 to 4277 bp, compared with 564 to 1904 bp obtained with 10 mer primers) and greater number of polymorphic fragments, than short ones.

DISCUSSION

A prerequisite for grapevine improvement in tissue culture is the availability of highly efficient methods for plant propagation *in vitro*. Although, the grapevine was among the first plant to be cultured *in vitro* by Morel in 1944 (Mullins *et al*., 2000), our study showed the most promising approach to the problems of drought and maintaining the grapevine germplasm in Zabol.

Classical ampelography (Galet, 1979) and other morphometric methods (Swanepoel and Devilliers, 1987) are useful for cultivar identification but are not enough reliable to attribute for grapevine identification at the clonal level. Furthermore, the same cultivar may have different names and varied cultivars may have the same name. The RAPD analysis in this study showed the aptitude of RAPD markers for differentiation of some native grapevines. This is the first report on the use of molecular markers for evaluating genetic relationships of Sistanian native grapevines.

Optimal template DNA and primers concentration were 10 ng and 0.4 μM, respectively. In this study more leaves from *in vitro* were used to obtain proper weight of DNA because they were more succulent in compare with the *in vivo* leaves. However purred DNA from different leaves yielded the same patterns for RAPD. Contaminated DNA and varied concentration of primers and buffers also showed unexpected results. As indicated in several studies, DNA quality and quantity (Williams *et al*., 1990), choice of DNA polymerase (Schierwater and Ender, 1993), $MgCl_2$ concentration (Park and Kohel, 1994, Williams *et al*., 1990), primer concentration (Williams *et al*., 1990), ethidium bromide vs. silver detection of products (Caetano and Anolles *et al*., 1992) and presence of RNA (Yoon and Glawe, 1993) have been reported to affect the RAPD outcome.

In this study the long primer (CO-8) produced more DNA fragments and more polymorphic bands which emerged by primers CO-1, CO-4, CO-8, CO-10, CA-2 and GY-169 with 80%, but short primer B-345 had less bands (14 bands). Rafalski *et al*. (1991) suggested 10 mer primers in RAPD analysis and Ye *et al*. (1996) observed more polymorphic bands yielded by long primers on grapevine which were consistent with study on Poplar (Castiglione *et al*., 1993). It is not exactly clear why more polymorphic bands were produced by long primers. It should be pointed out, that the long primers had lower G+C content (Ye *et al*., 1996) from 29-61% compared to 10 mer primers from 45-61% which were used by Welsh and McClelland (1991) and 45-60% used by Castiglione *et al*. (1993). They were able to generate more fragments from 10 mer primers. In this study lower G+C content (41-66%) belonged to long primers and short primers consist of 60-80% G+C content. The annealing temperature for long primers was higher than short primers. It is not clear that whether the produced RAPD-PCR bands by long primers were due to higher temperature, purred DNA, length of primer, lower G+C or a combination of them. More experiment should be carried out to determine the effects of each mentioned above. The possibility of extra bases at 5 end anneal to the templates to encourage the bases to start a new template-primer complex has been considered (Ye *et al*., 1996). Furthermore, long primers may have more repetitive DNA region for targeting which may not be easy accessible with 10 mer primers.

In our experiment 15 primers in some loci produced monomorphic bands in which primer P-36 had the longest chain with 4277 bp and primers CA-2, CY-107 and B-302 produced monomorphic bands with 564 bp.

Analysis of these RAPD data for band's similarity indices showed differentiation in all cultivars. To find genetic variation in 6 cultivars, the software of SPSS, PopGen 32 (data are not shown) and NTSYS were used. In these assessments, the six genotypes were clustered in four groups. The smallest distance value was obtained between Yaghooti and Roche Safid (White Yaghooti) which seems can be closely regrouped. Flowering, bery set and ripening of the both cultivars are not the same as Fakhry and Sangak which are clustered together and ripen 40 days later. Some cultivars present different intermediate level of similarity and are grouped with other ones. Breeding programs for traditional cultivars is based on intra-specific hybridization and given poor fruit quality on inter-specific hybrid. The genotypes of *Vitis vinifera*, have been recommended for both seeded and seedless varieties in breeding programs (Mullins *et al*., 2000). Consequently, it is suggested that from two regrouped varieties (Yaghooti and Roche Safid) and (Fakhry and

Sangak) one of each may be attribute to the breeding program (mass selection projects) and Laal with Cheshm Gavi can be pronounced as two different groups.

The use of UPGMA illustrated differentiation among six cultivars which this method has been already confirmed by Uolanosky *et al*. (2002) on grape and other plants such as Date palm (Eshraghi *et al*., 2006). The dendogram (Fig. 1) had the ability to divide the cultivars into 2 different groups of early (Laal) and late (Cheshme Gavi) ripening grapes.

In conclusion, the use of RAPD as a technique for genomic identification and clustering for grapevine cultivars could be encouraged. It is also necessary to enlarge the number of cultivars and use different molecular markers to estimate precise genetic diversity on grapevine cultivars. Furthermore, comparison of works among different laboratories should be carried out to standard methodology for evaluating the ancient grapevines in Sistan.

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