Analysis of SSR and AFLP Markers to Detect Genetic Diversity Among Selected Clones of Grapevine (*Vitis vinifera* L.) cv. Keshmeshi

H. Doulati Baneh*[1], S.A. Mohammadi[2], H. Mahmoudzadeh[1], F. de Mattia[3] and M. Labra[3]

(1) Department of Seed and Plant Breeding, Agricultural and Natural Resource Research Centre of West Azerbaijan, P.O. Box 365, Uremia, Iran
(2) Department of Agronomy & Plant Breeding, Faculty of Agriculture, University of Tabriz, Tabriz 51664, Iran
(3) Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, I-20126 Milan, Italy

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To assess the genetic differences between clones of grapevine (*Vitis vinifera* L.) cv. Keshmeshi, ten selected clones from a clonal selection programme were analysed by 23 Simple Sequence Repeats (SSR) and seven AFLP primer combinations. No intra-varietal differences between the clones could be detected by SSRs, whereas eight out of the 499 AFLP fragments generated by the seven primer combinations were polymorphic. The number of markers ranged from 44 (E34-M34) to 97 (E31-M32), with an average of 71.3 fragments per primer combination. Cluster analysis based on the AFLP data separated all the clones of Keshmeshi in two groups. The first group included nine white berry skin clones without any genetic differences, and the second group with only a red berry skin clone. AFLP could only distinguish the red berry clone of Keshmeshi from other white berry clones.

Keshmeshi is one of the most important grapevine (*Vitis vinifera* L.) cultivars grown in Iran, and used mainly for table grape and raisin production (Doulati Baneh *et al*., 2007). Long-time cultivation and vegetative propagation of this cultivar have led to the production of many Keshmeshi individuals with many different characters originating from somatic mutation and viral diseases. In various regions of Iran, Keshmeshi has different names, e.g. Bidaneh, Sefid bidaneh and Sultani (Doulati Baneh *et al*., 2007). Developing a successful grape production programme requires the propagation of virus-free, true-to-type cultivars and clones (Silvestroni *et al*., 1997). Over time, virus diseases, epigenetic effects and DNA mutations cause differences among cultivar accessions that in some cases have led to superior clones being identified and subsequently propagated by growers (Sensi *et al*., 1996). Clonal selection has become the most important way to improve the quality of grape cultivars. As a consequence, there is a need for reliable and precise methods of clonal characterisation for use by breeders and nurseries (Moreno *et al*., 1998). Clone identification has traditionally been based on ampelography and ampelometry, but their expression can be affected by developmental and environmental factors and may cause several false attributions (Imazio *et al*., 2002), while DNA-based methods are not influenced by environmental factors and a large number of potential polymorphic sequences or markers are available. DNA tools such as SSRs and AFLPs are largely used for the characterisation and differentiation of grapevine clones or closely related accessions (Imazio *et al*., 2002; Popescu *et al*., 2002; Kozjak *et al*., 2003; Karataş *et al*., 2007), but on the basis of cultivars, markers and primer combination, contrasting results about the usefulness of molecular markers to assess genetic differences among clones have been reported (Sensi *et al*., 1996; Cervera *et al*., 1998; Scott *et al*., 2000; Vignani *et al*., 2002). In the present research, the possibility of differentiating Keshmeshi grapevine clones derived from a clonal selection programme by analysing SSR and AFLP markers was investigated.

MATERIALS AND METHODS

Plant materials

In this study, ten clones of *Vitis vinifera* cv. Keshmeshi were taken from a clonal selection vineyard in the agricultural research centre of West Azerbaijan, Uremia, Iran (Table 1). The agronomical traits related to raisin production (inflorescence and bunch size, bunch weight, berry weight, size and shape, time and uniformity of ripening, berry colour, TSS and yield) were recorded in three consecutive years (2005 to 2007). The evaluation was performed on twelve individuals of each clone. To exclude the possible effects of viruses on morphological characters, all clones were evaluated for their sanitary status. ELISA tests confirmed that all the selected clones were free of grapevine fanleaf virus (GFLV), grapevine leafroll virus (GLRV) and arabis mosaic virus (Armv).

DNA extraction

DNA extraction was performed using 1 to 2 cm long young leaves harvested from rooted cuttings based on the CTAB method described by Labra *et al*. (2001), excluding the purification steps.

SSR analysis

Twenty-three SSR loci (Table 2) were used for clonal identification (Thomas & Scott, 1993; Bowers *et al*., 1999; Sefc *et al*., 1999). PCR was performed in a total volume of 10 µl containing 20 ng of DNA, 0.25 U *Taq* DNA polymerase, 10 µM of each primer, 200 µM of each dNTP and reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 nM Tris-HCl, pH 9.0 and 0.1% Triton X-100). The PCR cycles consisted of an initial denaturation for 7 min at 94°C, 35 cycles of denaturation (45 s at 94°C), annealing (30 s at 52°C) and extension (1 min at 72°C), and a final extension at 72°C for

*Corresponding author: e-mail: ah_Dolati@yahoo.com [Tel.: +98-441-2262222-6, Fax: +98-441-2262221]

7 min as described by Cresti (1997). The amplification products were separated by electrophoresis on 34 cm denaturing 6% polyacrylamide gel (run at 70 W, 60 min) and then silver stained according to the protocol of the Promega (Madison, USA) kit.

AFLP analysis

AFLP analysis (Vos *et al*., 1995) was performed as described in European Patent 0534858 (Keygene, Wageningen, The Netherlands). Genomic DNA (200 ng) was digested for 3 h with a unit of each of the restriction enzymes *Eco*RI and *Mse*I. The DNA fragments were ligated with *T4* DNA ligase to an *Eco*RI adapter (5 pmol) and an *Mse*I adapter (50 pmol) in a final volume of 50 µl at 37°C for 6 h. The resulting mixture was used as the template in a pre-amplification reaction containing DNA primers (E01 and M01, Table 2) complementary to the cores of the EcoRI and MseI adapters respectively. The pre-amplification mixture (50 µl) contained 20 µl digested/ligated DNA, 50 ng of the selected primers, 200 µM of each dNTP, 0.5 units Dynazyme II (Finnzymes, Keilaranta, Finland) and 5 µl Dynazyme (Finnzymes, Keilaranta, Finland) 10x buffer. After 2 min at 94°C, amplification was carried out for 20 cycles of denaturation (45 s at 94°C), annealing (30 s at 60°C) and extension (one min at 72°C). After a final extension step (7 min at 72°C), the pre-amplification products were diluted 1:50 (v/v) with water and used for selective amplification. This was carried out using one of the selective primers (E31, E32, E34 or E38; Table 2) complementary to the EcoRI adapter, and one of the primers (M31 M32 M34, M36, M38 or M39; Table 2) complementary to the MseI adapter. The EcoRI primer was end-labelled with γ33P-ATP (Amersham, Milan, Italy). The amplification mixture (20 µl final volume) contained 2.5 µl of the diluted pre-amplification mixture, 5 ng labelled EcoRI primer, 30 ng MseI primer, 200 µM of each dNTP, 0.5 units Dynazyme II (Finnzymes, Keilaranta, Finland) and 1 µl Dynazyme (Finnzymes, Keilaranta, Finland) buffer. After 2 min at 94°C, amplification took place for 36 cycles under the following conditions: denaturation for 30 s at 94°C; annealing for 30 s at 65°C for the first cycle, followed by lowering the temperature by 0.7°C for the next 12 cycles, then annealing at 56°C for the remaining 23 cycles, and extension for 60 s at 72°C. A total of 1.5 µl of the PCR-amplified mixture was added to an equal volume of loading buffer (80% (v/v) formamide, 1 mg ml-1 xylene cyanol FF, 1 mg ml-1 bromophenol blue, 10 mM EDTA, pH 8.0), denatured for 5 min at 92°C, loaded on a 43 cm 5% (w/v) denaturing polyacrylamide gel, and electrophoresed in TBE electrophoresis buffer for 3 h at 80 W. The gel was fixed in 10% (v/v) acetic acid and exposed to Kodak BioMax MR Film for 24 h. Polymorphic bands were scored by visual inspection of the resulting autoradiograms. AFLP analysis was performed in duplicate.

TABLE 1

Denomination and different agronomical traits of ten Keshmeshi clones.

Clone	Common name	Inflorescence number	Bunch number	Bunch length (cm)	Bunch width (cm)	Berry length (cm)	Berry width (cm)	TSS[1]	TA[2]	Mean bunch/shoot	Berry color
Kred	Keshmeshi Qermez	92**	86	26	15.3	1.4	1.3	22	1.2	1.8	Red
K1	Keshmeshi Sefid	85	80	29	12	1.3	1.2	20.5	0.98	1.8	Yellow
K3	Keshmeshi Sefid	96	96	29.5	11	1.5	1.4	21.5	0.96	2	Yellow
K4	Keshmeshi Sefid	90	81	29.5	12	1.7	1.4	21.5	0.81	1.6	Bright yellow
K9	Keshmeshi Sefid	100	90	25	14	1.5	1.3	22.5	0.97	1.7	Yellow
K12	Keshmeshi Sefid	80	70	28	12	1.4	1.2	24	0.76	1.9	Yellow
K25	Keshmeshi Sefid	127	121	25	13.4	1.3	1.1	21	1.05	1.7	Yellow
K35	Keshmeshi Sefid	80	70	28	14	1.4	1.2	23.2	0.95	1.8	Yellow
K59	Keshmeshi Sefid	136	115	25	14.3	1.3	1.1	21.4	1.01	1.9	Yellow
GRA1-1	Keshmeshi Sefid	80	73	26	13.5	1.7	1.3	22	0.95	1.85	Yellow
Standard error		0.36	0.15	0.12	0.13	0.16	0.02	0.02	0.1	0.2	

**The average values for each parameter have been reported

[1] Total soluble solid

[2] Total acidity of fruit juice

TABLE 2

Primer combinations used for AFLP analysis.

Name	DNA sequence
M01	5'-GATGAGTCCTGAGTAA-3'
E01	5'-GACTGCGTACCAATTC-3'
E31	5'-GACTGCGTACCAATTCAAA-3'
E32	5'-GACTGCGTACCAATTCAAC-3'
E34	5'-GACTGCGTACCAATTCAAT-3'
E38	5'-GACTGCGTACCAATTCACT-3'
M31	5'-GATGAGTCCTGAGTAAAAA-3'
M32	5'-GATGAGTCCTGAGTAAAAC-3'
M34	5'-GATGAGTCCTGAGTAAAAT-3'
M36	5'-GATGAGTCCTGAGTAAACA-3'
M38	5'-GATGAGTCCTGAGTAAACT-3'
M39	5'-GATGAGTCCTGAGTAAAGA-3'

TABLE 3

Allele length at 23 SSR loci developed from 10 clones of Keshmeshi.

Locus	Alleles	Locus	Alleles	Locus	Alleles
VVMD5	234:234	D12	158:184	VVS3	212:218
VVMD7	240:254	UCH29	211:300	VVS4	174:174
VVMD8	145:152	ISV2	143:143	VrZAG47	159:169
VVMD17	222:222	ISV3	133:139	VrZAG62	188:188
VVMD21	249:258	ISV4	197:197	VrZAG64	143:159
VVMD25	243:253	G7	106:118	VrZAG79	248:260
VVMD26	247:249	G10	149:149	VrZAG83	194:194
VVMD27	181:190	VVS2	145:151		

TABLE 4

AFLP primer combinations, total bands detected and number of polymorphic bands.

Primer combination	Total bands	Polymorphic bands
E32-M38	48	1
E34-M34	44	0
E38-M36	85	1
E34-M38	62	0
E31-M32	97	2
E32-M31	96	4
E34-M39	67	0

Statistical analysis

Allele sizes of the SSR bands were determined by internal size markers and by comparison with a standard set of microsatellite reference alleles (This *et al*., 2004). As far as AFLP is concerned, the amplified bands were scored as absent (0) or present (1) and the resulting data matrix was analysed using the GENSTAT V statistical program (Payne *et al*., 1993). Diversity levels were estimated on the basis of the percentage of polymorphic bands out of the total bands scored. Similarity-dissimilarity matrices were computed using the Jaccard coefficient. Dendrograms were constructed on the basis of UPGMA (unweighted pair-group method with arithmetical averages).

RESULTS AND DISCUSSION

SSRs

Ten clones of Keshmeshi grapevines with different quality and quantity traits (Table 1) were analysed at 23 SSR loci. Table 3 shows the allele size obtained for Keshmeshi at the analysed SSR loci. No polymorphism was found among the clones of the Keshmeshi cultivar and this technique could not differentiate the clones. These data suggest that the morphological and agronomical differences observed among these clones could not readily be explained on the basis of SSR regions. The same conclusion was reported by Silvestroni *et al*. (1997), who analysed Sangiovese and Fortana clones, and by Imazio *et al*. (2002), who applied SSR analysis to distinguish Traminer clones. However, some success in differentiating clones by SSR has been reported (Regner *et al*., 2000; Kozjak *et al*., 2003). In our research, clonal selection within the Keshmeshi cultivar was done on the basis of differences in quantitative traits, e.g. soluble solids, yield, berry weight and some qualitative traits, e.g. colour of the berry. These differences may result from slight changes in DNA sequences in the coding regions or epigenetic effects that are not detectable with SSR analysis.

AFLPs

Seven AFLP primer combinations generated 499 scorable fragments ranging from 44 (E34-M34) to 97 (E31-M32), with an average of 71.3 fragments per primer combination (Table 4). Eight out of the 499 AFLP fragments were polymorphic. Starting from this, cluster analysis (Figure 1) based on polymorphic AFLP markers separated the clones into two groups: 1) nine clones of Keshmeshi Sefid (yellow-skinned berries) without any polymorphism and 2) a Keshmeshi Qermez clone (red-skinned berries). The genetic similarity between the two groups was about 98.5, confirming their clonal origin.

The main difference between the Keshmeshi Qermez (Kred) and Keshmeshi Sefid clones is the colour of the berry skin, i.e. Keshmeshi Qermez is a red-skinned berry while Keshmeshi Sefid is a white-yellow berry. The berry colour is determined by the accumulation of anthocyanins, and a mutation appears to be responsible for the difference in berry colour. Recent research (Kobayashi *et al*., 2004; Hirochika *et al*., 2006) showed that *Myb*-related genes such as VvmybA1 regulate anthocyanin biosynthesis, and a retrotransposon-induced mutation in these genes is associated with the loss or synthesis of pigments in cultivars of *Vitis vinifera* L. Bud mutation from white-skinned Italia cultivars to red-skinned Ruby Okuyama caused by the deletion of a retrotransposon inserted in VvmybA1 has been reported

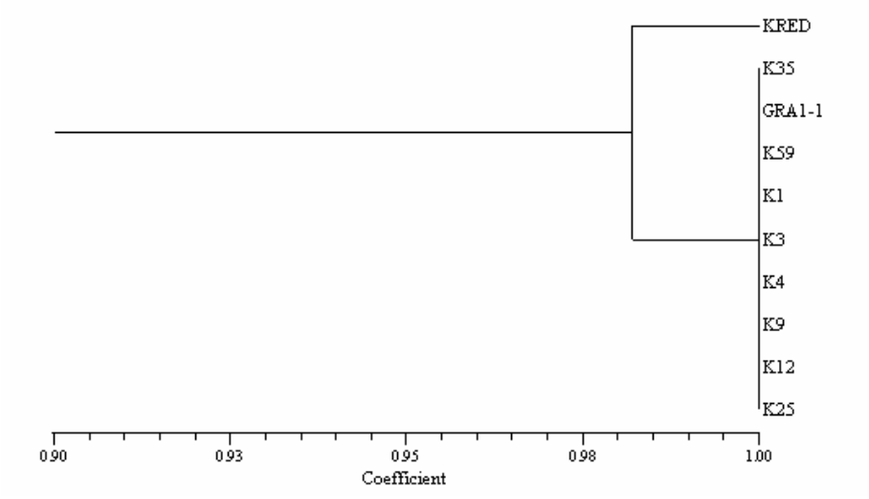

FIGURE 1

Dendrogram of genetic relationships among nine Keshmeshi Sefid clones and Keshmeshi Qermez (Kred) based on AFLP data.

(Kobayashi *et al*., 2005). Therefore, it could be hypothesised that a spontaneous retrotransposon-induced mutation in the *Myb* genes in a bud of the Keshmeshi Sefid cultivar caused the re-synthesis of pigments and, subsequently, Keshmeshi Qermez was derived. To clarify this difference between the two clones, the analysis should be extended by using retrotransposon markers.

Our results show that the nine Keshmeshi clones (group 1) are genetically uniform and that the differences (cluster, bunch and berry number, length, width, etc.) that exist among them are not readily explainable on the basis of AFLP analysis. This phenotypic variation could be explained by a differential expression of certain structural genes regulated by epigenetic changes, or by the occurrence of mutations. The existence of a correlation between changes in the methylation state of particular gene sequences and the expression of a mutant phenotype has been shown clearly by Imazio *et al*. (2002). Moreover, the mutation might be restricted to a very small region of the genome, or might involve a point mutation in a coding region that might be difficult to detect by AFLP markers. To explore a larger portion of the genome, more primers could be assayed (Fanizza *et al*., 2003).

Based on different number and primer combinations and different cultivars, there are contrasting results regarding the capability of AFLP to assess genetic differences among clones. Some authors (Cervera *et al*., 1998) failed to detect polymorphisms among clones, while others (Sensi *et al*., 1996; Scott *et al*., 2000) could differentiate clones by means of AFLP markers.

The sequencing of the grapevine genome was recently completed (Jaillon *et al*., 2007) and this opens the real possibility to improve DNA technology to analyse specific genes related to different morphological and agronomical traits. These new technologies will be useful in clarifying the genetic bases of clonal differences, and in excluding any homonymous or wrong attributions based only on observations of morphological characters.

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