Genetic study of 46 Greek grape cultivars by random amplified polymorphic DNA markers (RAPD- PCR).

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Abstract: Forty eight random decamer primers of arbitrary nucleotide were used to amplify genomic DNA through the polymerase chain reaction (RAPD- PCR) in order to identify and discriminate 46 greek grapevine cultivars (*Vitis vinifera* L.) grown in Greece and to determine the genetic similarities among these cultivars. More than 700 (89,7% of total electrophoretic bands) reproducible polymorphic fragments were generated by this method. Forty four of the cultivars studied showed a unique banding pattern for more than 7 of the primers used. The most effective primer was OPE-04, since it discriminated all studied cultivars.
Herefrom, the degree of genetic similarity was calculated and the dendrogram of the 46 Greek cultivars was constructed. The results indicate that there is genetic variation among grape cultivars studied with the values of the genetic similarity ranging from 0,644 to 0,963. The results show that RAPD-PCR is a reliable and very useful method for the identification and genetic analysis of grape cultivars.

Introduction

The discrimination and identification of grape varieties are very difficult due to their large number. It has been estimated that there are more than 8000 grape cultivars, under 24.000 different names (Viala and Vermorel 1909). In Greece more than 700 grapevine cultivars (*Vitis vinifera* L.) are grown and for the discrimination and classification of Greek cultivars have been used classic ampelographic (Krimbas 1943, Davidis 1967, Vlachos 1986) and biochemical (Stavrakakis 1982) methods.
The aim of this study was to identify and to discriminate 46 Greek grape cultivars and to determine their genetic similarities based on RAPD-PCR analysis. This method, based on random amplified polymorphic DNA obtained by polymerase chain reaction analysis allows the direct comparisons of the genetic material of grape cultivars. This DNA marker has been used to discriminate cultivars of several plants (Carlson et al. 1991, Stiles et al. 1993) including grapevine (Bourquin et al. 1995, Grando et al. 1995, Moreno et al. 1995, Stavrakakis at al 1997; Stavrakakis and Biniari 1998). In this work 48 decamer primers of arbitrary nucleotide sequence were used to amplify genomic DNA through the polymerase chain reaction (RAPD - PCR) in order to identify and discriminate grape cultivars grown in different areas in Greece.

Material and Methods

Grapevine material

Forty-six grapevine cultivars grown in Greece were chosen for identification (Tab. 1). Leaf material of these cultivars was obtained from the grape germplasm collection of NAGREF in Athens, Agriculture University of Athens such as different grapevine areas in Greece.

DNA extraction

Grapevine DNA was extracted from young and fully expanded leaves according to Thomas et al. (1993) with minor modifications. 1 g of leaves from individual vines were frozen in liquid

nitrogen and ground to a fine powder, thawed and resuspended in 12,5 ml of buffer A [0.25 M NaCl, 0.2 M TRIS-Cl (pH 8.0), 50 mM EDTA, 0.1 v/v 2-mercaptoethanol, 2.5% w/v polyvinyl-pyrrolidone (MW 40.000)]. A crude nuclei pellet was obtained by centrifugation at 7.000 rpm for 10 min at 4 ^{o}C. The pellet was resuspended in 2,5 ml of extraction buffer B [0.5 M NaCl, 0.2 M TRIS-Cl (pH 8.0), 50 mM EDTA, 1% v/v 2-mercaptoethanol, 2.5% w/v polyvinyl-pyrrolidone, 3% sarkosyl, 20% ethanol] and incubated at 37^{o}C for 45 min. An equal volume of chloroform/isoamyl alcohol (24:1) was then mixed in and the phases were separated by centrifugation at 14.000 rpm for 15 min. The aqueous layer was collected and 0.54 volume of frozen isopropanol (-20 ^{o}C) was added to precipitate the DNA. The DNA was fished and resuspended in 300 µl TE (10 mM Tris - HCl, pH 7.4, 1 mM EDTA) containing 15 µg.ml^{-1} RNase A and incubated for 15 min at 37 ^{o}C. Protein was removed by the addition of a half volume of 7.5 M ammonium acetate, followed by centrifugation and the DNA in the supernatant was precipitated with a 0.25 of cold isopropanol; ca. 120 µg DNA per g FW was obtained.

Amplification conditions

For RAPD analysis the protocol reported by Williams et al. (1990) was followed with minor modifications. Amplification reactions were performed in volumes of 25 µl containing 60 ng of genomic DNA, 10 mM TRIS-Cl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 200 M each of dATP, dGTP, dCTP, dTTP, 50 ng primer and 1 unit of Taq DNA polymerase (Qiagen). Forty-eight random decamer oligonucleotides were used (Tab. 2) as primers for the amplification of RAPD sequences.

Amplification was performed in a Perkin Elmer DNA Thermal Cycler 9600. After 5 min at 94^{o}C, 34 cycles of PCR were performed, (1 min at the 94^{o}C, 1 min at the 44^{o}C, 2 min at the 72^{O}C) followed by a 10 min at 72^{o}C for extension.

Gel electrophoresis

Aliquots of the RAPD products were analyzed in 2% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate and 1mM EDTA, pH 8). After staining in ethidium bromide (1µg.ml^{-1}) the gels were photographed on a Gel Doc 1000 (Biorad).

All of the reactions were repeated at least twice with independently isolated genomic DNA as templates. The degree of genetic similarity (I) detected electrophoretically between each pair of cultivar studied was calculated by using the SM coefficient and using the NTSYS-pc package 1.8 developed by Rohlf (Exeter Software, New York, USA), generating the appropriate dendrogram (Fig.2).

Table 2: Synthetic nucleotides were used as primers for RAPDs analysis and number of bands.

Primer code	Nucleotide sequence 5´→ 3´	Total number of fragments amplified	Primer code	Nucleotide sequence 5´→ 3´	Total number of fragments amplified
1224	CAGGCCCTTC	16	**OPM-03**	GGGGGATGAG	16
1225	AGGTGACCGT	19	**OPM-04**	GGCGGTTGTC	16
1226	CGCAGGATGG	17	**OPM-05**	GGGAACGTGT	13
1227	GTGTGCCCCA	18	**OPM-06**	CTGGGCAACT	22
OPF- 01	ACGGATCCTG	17	**OPM-07**	CCGTGACTCA	19
OPF-02	GAGGATCCCT	17	**OPM-08**	TCTGTTCCCC	18
OPF-03	CCTGATCACC	14	**OPM-09**	GTCTTGCGGA	14
OPF-04	GGTGATCAGG	20	**OPM-10**	TCTGGCGCAC	12
OPF-05	CCGAATTCCC	21	**OPM-11**	GTCCACTGTG	11
OPF-06	GGGAATTCGG	8	**OPM-12**	GGGACGTTGG	18

OPF-07	CCGATATCCC	14	**OPM-13**	GGTGGTCAAG	19
OPF-08	GGGATATCGG	11	**OPM-14**	AGGGTCGTTC	17
OPF-09	CCAAGCTTCC	16	**OPM-15**	GACCTACCAC	19
OPF-10	GGAAGCTTGG	13	**OPM-16**	GTAACCAGCC	11
OPF-11	TTGGTACCCC	13	**OPM-17**	TCAGTCCGGG	10
OPF-12	ACGGTACCAG	5	**OPM-18**	CACCATCCGT	20
OPF-13	GGCTGCAGAA	15	**OPM-19**	CCTTCAGGCA	10
OPF-14	TGCTGCAGGT	13	**OPM-20**	AGGTCTTGGG	18
OPF-15	CCAGTACTCC	2	**OPE-01**	CCCAAGGTCC	14
OPF-16	GGAGTACTGG	10	**OPE-04**	GTGACATGCC	18
OPF-17	AACCCGGGAA	12	**OPE-08**	TCACCACGGT	9
OPF-20	GGTCTAGAGG	16	**OPE-12**	TTATCGCCCC	15
OPM-01	GTTGGTGGCT	22	**OPE-17**	CTACTGCCGT	14
OPM-02	ACAACGCCTC	17	**OPQ-01**	GGGACGATGG	12

Table 1: Studied cultivars and sampling areas.

Cultivar code	Cultivar	Sampling area	Cultivar code	Cultivar	Sampling area
1.	**Agiorgitiko**	(a), (b)	24.	**Mandilaria**	(a), (d)
2.	**Aidani Aspro**	(a), (d)	25.	**Mavrodafni**	(a), (b)
3.	**Aidani Mavro**	(a)	26.	**Mavrotragano**	(a)
4.	**Athiri**	(a), (d),(f)	27.	**Mavro Messenikola**	(a)
5.	**Assyrtiko**	(a), (d)	28.	**Monemvassia**	(a), (d)
6.	**Vapsa**	(a)	29.	**Moschato Samou**	(a), (d)
7.	**Verzami**	(a)	30.	**Moschofilero**	(a), (b)
8.	**Vilana**	(a), (c)	31.	**Negoska**	(a)
9.	**Vidiano**	(a), (c)	32.	**Debina**	(a)
10.	**Glykerithra**	(a)	33.	**Xynomavro**	(a), (e)
11.	**Goustolidi**	(a)	34.	**Platani**	(a), (d)
12.	**Dafni**	(a), (c)	35.	**Plyto**	(a), (c)
13.	**Thrapsathiri**	(a),(c)	36.	**Potamissio**	(a), (d)
14.	**Katsano**	(a), (d)	37.	**Razaki Archanon**	(a), (c)
15.	**Kolliniatiko**	(a)	38.	**Refosco**	(a)
16.	**Korinthiaki**	(a), (b)	39.	**Roditis**	(a), (f)
17.	**Kotsifali**	(a), (c),(f)	40.	**Robola Aspri**	(a)
18.	**Kritiko**	(a)	41.	**Romeiko**	(a), (c)
19.	**Lagorthi**	(a), (c)	42.	**Savvatiano**	(a), (f)
20.	**Ladikino**	(a), (c)	43.	**Sideritis**	(a)
21.	**Limnio**	(a), (d)	44.	**Soultanina**	(a), (c)
22.	**Liatiko**	(a), (c)	45.	**Stavroto**	(a)
23.	**Malagouzia**	(a)	46.	**Fraoula Kokkini**	(a)

(a): Institute of Vine, NAGREF, Athens, (b): Vineyards of Peloponnesus, (c): Vineyards of Crete, (d): Vineyards of Aegean' islands, (e): Vineyards of Macedonia, (f): Agriculture University of Athens, Athens.

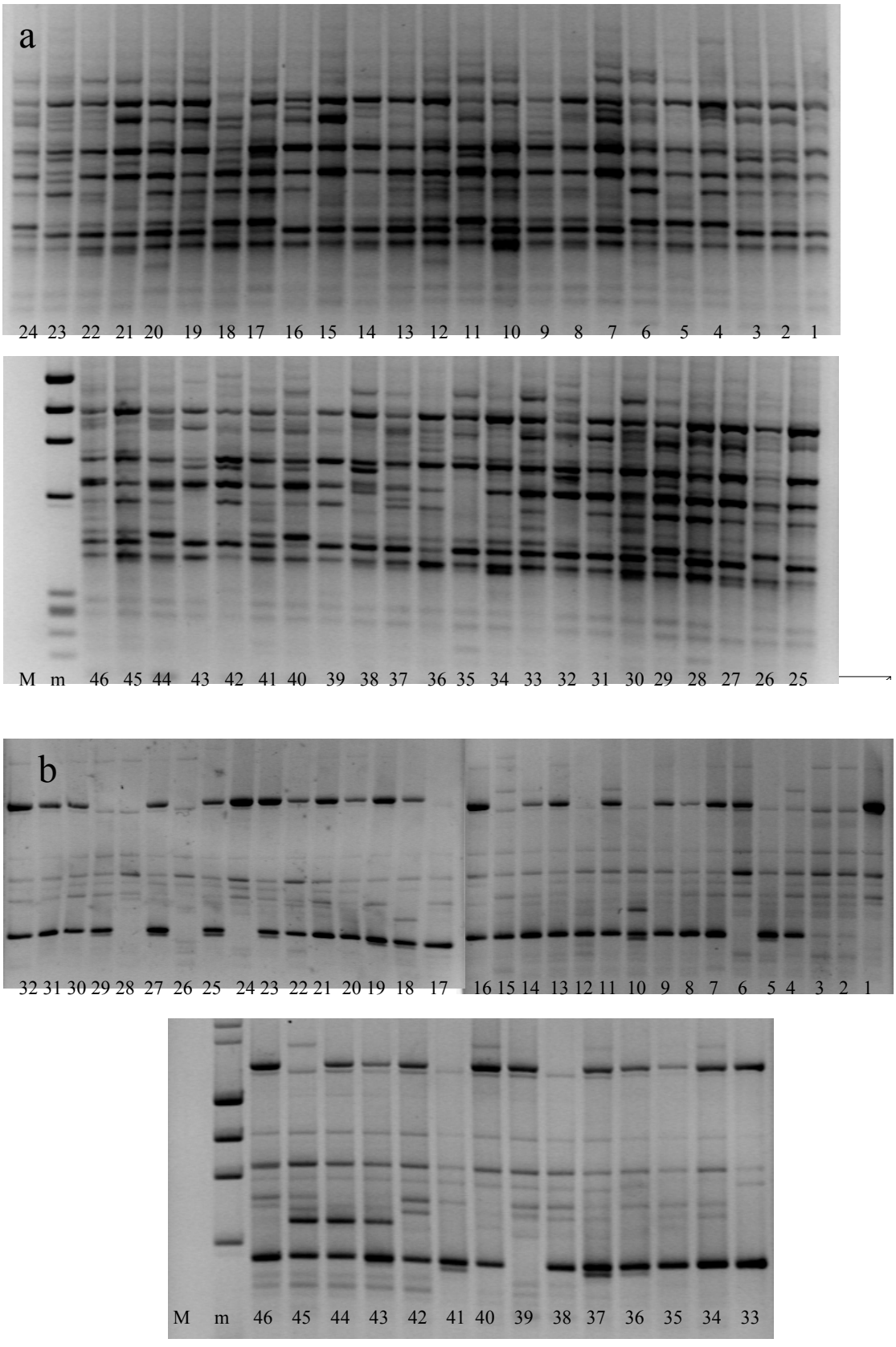

Figure 1: RAPD patterns obtained using primers OPM-01 (a) and OPF-03 (b) for all studied cultivars.

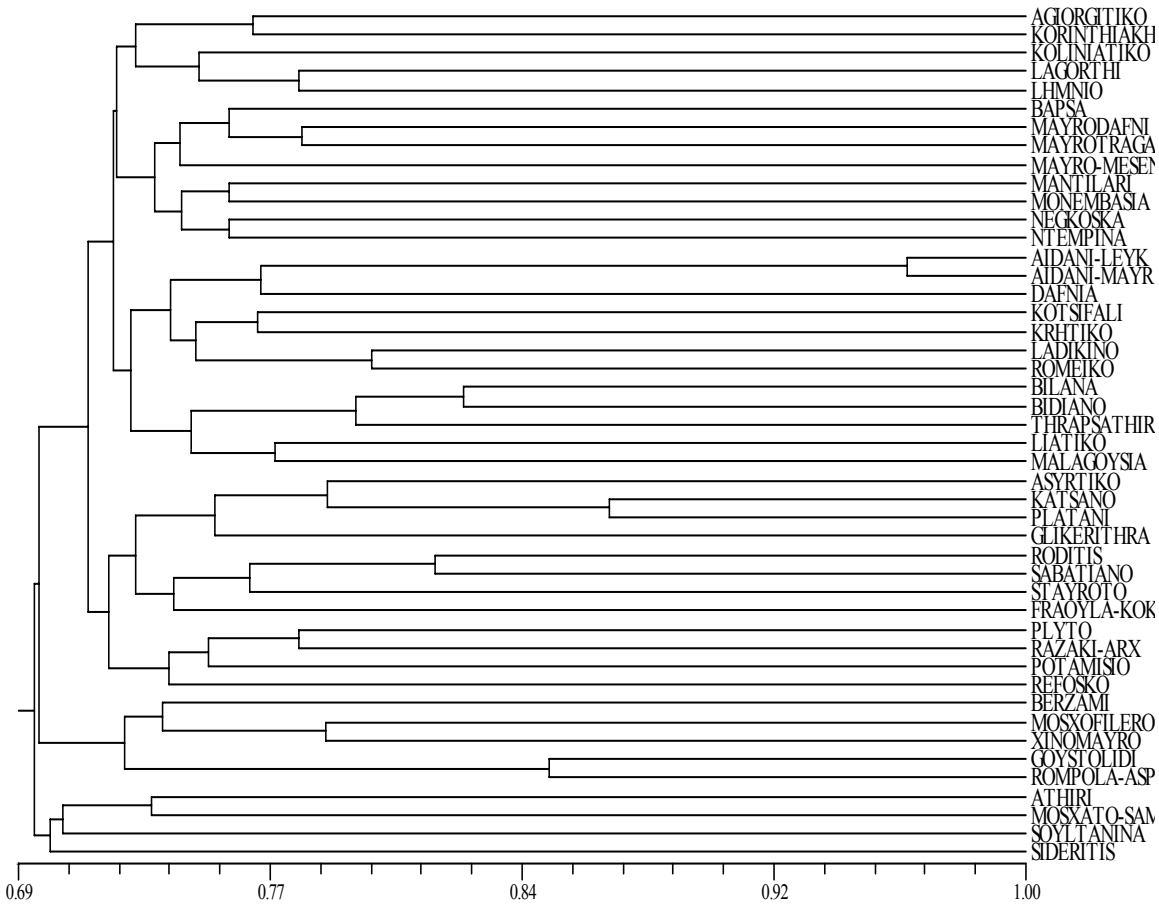

Figure 2: Dendrogram based on 710 RAPDs amplification products showing the relationship among studied cultivars.

Results and Discussion

Forty eight random decamer primers were used to amplify genomic DNA from 46 greek grapevine cultivars. Each primer provided at least two polymorphic bands. 710 reproducible fragments (89,7% of total electrophoretic bands) were generated by this method. The total number of amplified fragments is shown in Tab. 2. Among the 48 primers used, the primers OPM-01, OPE-04, OPM-18, 1225, OPM-13, OPF-04 and OPF-05 have more polymorphic DNA fragments and discriminated all or 44 of the studied cultivars. Forty four of the cultivars studied showed a unique banding pattern for more than 7 of the primers used. The most effective primer was OPE-04 with which all studied cultivars were discriminated. Examples of RAPD patterns amplified with primers OPM-01 and OPF-03 are shown in Fig. 1.

In general, there was genetic variation among grape cultivars studied with the values of the genetic similarity ranging from 0,644 to 0,963. Higher genetic similarity was found in the pairs of cultivars Aidani aspro- Aidani mavro (I= 0,963), Katsano- Platani (I= 0,872), Vilana-Vidiano (I= 0,827), Goustolidi-Robola Aspri (I= 0,853) and Thrapsathiri - Vidiano (I= 0,818). The very high degree of genetic similarity (0.963) between the cultivars Aidani aspro and Aidani mavro indicates that these cultivars originated from a common stock. Maybe due to mutation, the berries of cultivar Aidani mavro have a black reddish color while in Aidani aspro there are white. This is not surprising since mutation of berry' s color is rather common event in Greek grapevines (Krimbas 1943). This result is in agreement with previous studies (Krimbas 1943, Davidis 1967).

The pairs of group cv Vilana- Vidiano (I= 0,827) and Thrapsathiri- Vidiano (I= 0,818) cultivated in Crete showed relatively high genetic similarity indicating that they are related

cultivars. The same holds for the pairs of grape cultivars Katsano and Platani (I= 0,872) which are cultivated in Cyclades islands and cultivars Goustolidi - Robola Aspri (I= 0,853) which are cultivated in Ionian islands. On the other hand it's a surprise the comparatively low degree of genetic similarity between Athiri and Thrapsathiri (I=0,713) which are considered closely related cultivars. This can be explained by the geographic origin of studied samples.
The results show that there is genetic variation among studied greek cultivars and that RAPD-PCR is a reliable and very useful method for the identification and genetic analysis of grape cultivars.

References

1. BOURQUIN J.C, OTTEN L and WALTER B (1995): PCR- RFLP analysis of *Vitis*, *Ampelopsis* and *Parthenocissus* and its application to the identification of rootstocks. Vitis 34 (2): 103-108.
2. CARLSON J.E, TULSIERAM L.K, GLAUBITZ J.C, LUK V.W.K, KAUFFELDT C and RUTLEDGE R (1991): Segregation of random amplified DNA markers in F1 progeny of conifers. Theor. Appl.Genet. 83: 194-200.
3. DAVIDIS, X.O. (1967): An Introduction to Ampelography. Privetely printed, Athens (in Greek).
4. GRANDO M.S, DE MICHELI L, BIASETTO L and SCIENZA A (1995): RAPD markers in wild and cultivated *Vitis vinifera*. Vitis 34: 37-39.
5. KRIMBAS, V. (1943): Greek Ampelography. Vol. I. Ministry of Agriculture, Athens (in Greek).
6. MORENO S, GOGORCENA Y and ORTIZ J.M (1995): The use of RAPD markers for identification of cultivated grapevine (*Vitis vinifera* L.) Scientia Horticulturae 62: 237-243.
7. ROHLF F.J. (1993): NTSYS-pc. Numerical taxonomy and multivariate analysis system, Version 1.80. Exeter Software, Setauket, New York.
8. STAVRAKAKIS,M.N.(1982): Identification of grape cultivars (*Vitis vinifera* L.) by pollen isozyme polymorphisms. Ph.D Thesis. Agric. Univ. of Athens. Athens.
9. STAVRAKAKIS M.N and BINIARI K (1998): Genetic study of grape cultivars belonging to the muscat family by random amplified polymorphic DNA markers. Vitis 37 (3),119-122.
10. STAVRAKAKIS M.N, BINIARI K and HATZOPOYLOS P (1997): Identification and discrimination of eight Greek grape cultivars (*Vitis vinifera* L.) by random amplified polymorphic DNA markers. Vitis 36,175-178.
11. STILES J.I, LEMME C, SONDUR S, MORSHIDI M.B and MANSHARDT R (1993): Using randomly amplified polymorphic DNA for evaluating genetic relationships among papaya cultivars. Theor. Appl.Genet.85: 697-701.
12. THOMAS M.R, MATSUMOTO S, CAIN P and SCOTT N.S (1993): Repetitive DNA of grapevine: classes present and sequences suitable for cultivar identification. Theor. Appl.Genet. 86: 173-180.
13. VIALA P and VERMOREL V (1909): Traité Général d'Ampélographie. Maison éd Paris, 7 volumes.
14. VLACHOS, M. (1986): Ampelography. Thessaloniki (in Greek).
15. WILLIAMS J.G.K, KUBELIK A.R, LIVAK K.J, RAFALSKI J.A and TINGEY S.V (1990): DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18(22): 6531-6535.