Environmental and Experimental Biology (2012) 10: 125–132 *Original Paper*

Comparative genetic diversity analysis in Iranian local grapevine cultivars using ISSR and DAMD molecular markers

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Abstract

The conservation and characterisation of grapevine (*Vitis vinifera*) genetic resources in germplasm banks have been the basis of their use in breeding programmes that result in development of new cultivars. The genetic diversity of 21 grapevine accessions from the Kurdistan province (North-west of Iran) were investigated using inter-simple sequence repeat (ISSR) and directly amplified minisatellite DNA (DAMD) markers. Average polymorphism information content for ISSR and DAMD markers was 0.43 and 0.44, respectively, which revealed the equal resolving power in both marker types. The level of polymorphism generated by ISSR markers (64%) was relatively similar to DAMD (66%) markers. Genetic dissimilarity between pairs of genotypes ranged from 0.23 to 0.81 in ISSR and from 0.12 to 0.67 in DAMD marker analysis. Cluster analysis for ISSR and DAMD markers revealed that genotypes taken for the analysis can be divided in three and two distinct clusters. Genotype clustering showed acceptable congruence between ISSR, DAMD and morphological divergence between the studied genotypes. To our knowledge, this is the first detailed report of a comparison of performance among two targeted DNA region molecular markers (ISSR and DAMD) technique on a set of samples of grapevine. Overall, our results indicate that ISSR and DAMD fingerprinting could be used to detect polymorphism for genotypes of grapevine.

Key words: DAMD markers, genetic diversity, grapevine, ISSR markers, *Vitis vinifera*.
Abbreviations: DAMD, directly amplified minisatellite DNA; ISSR, inter-simple sequence repeat; PIC, polymorphism information content.

Introduction

Grapevine (*Vitis vinifera*) has been cultivated for about 5000 years and its vegetative propagation has favoured widespread diffusion of many cultivars around the world, generating numerous synonyms and homonyms. This sort of propagation, together with the sole use of a few cultivars allowed by the different Designations of Origin has led to a substantial decrease in grapevine diversity. In addition, the phylloxera attack suffered in the 19th century along with diseases like downy and powdery mildew have also contributed to this genetic erosion (This et al. 2006).

Due to its diversified climate Iran is suitable for grapevine growth and cultivation. Iranian grape germplasm is rich and complex and consists of a large number of grapevine cultivars and wild populations (Tafazzoli 1993). Unfortunately there is no any precise historical evidence about viticulture in Iran. However, McGovern (2003), suggested that human beings met wild grapes for the first time in the upland regions of eastern Turkey and in north-western Iran during the Palaeolithic era. The earliest evidence of grape cultivation dates back to the fourth millennium in the middle east (Zohary, Hopf 2000) and it has been proved that winemaking was already taking place in Iran during the second half of the sixth millennium B.C. (McGovern 2003).

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, which 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). Ampelography has long been the single method used for identifying grape cultivars (Boursiquot, This 1996) but, as this process is carried out on adult plants, a long period is required before the identification of accessions can be completed. Since many synonyms or homonyms exist for cultivars (This et al. 2006), passport data are not always sufficient to certify identities and errors arise. For

these reasons, as molecular marker technologies become available, they are being evaluated for their usefulness in grapevine cultivar identification and in assessing genetic diversity.

With respect to genetic diversity, the overriding question is how well do estimates based on molecular marker data correspond to actual levels of genetic diversity and/or to assessments of diversity based on morphology. In recent years, many new alternative and promising marker techniques have been developed in line with the rapid growth of genomic research (Gupta, Rustgi 2004). Minisatellite DNAs are tandemly repeated regions of genomes, many of which show high levels of length differences due to variation in the number of repeat units (Jeffreys et al. 1985). This technique was first reported by Heath et al. (1993) and termed as directed amplification of minisatellite-region DNA (DAMD). It is speculated that minisatellites and the DNA sequences flanking them are involved in inversions, which result in their distribution on both strands in opposite orientations. Despite the large number of grapevine germplasm collections held in Iran, most of them have not been characterised at either morphological or molecular levels. From a few morphological, AFLP and SSR marker-based studies in Iran, it has been reported that there exists high morphological and molecular diversity (Doulati Baneh et al. 2007; Ramezani et al. 2009; Hadadinejad et al. 2011). However, the results could hardly be inferred for Iranian germplasm accessions because of less representation in terms of geographical coverage of accessions.

Here, the use of ISSR and DAMD marker technique for studying genetic diversity was reported for the first time in Iranian grape genotypes. Objectives of the present study were as follows: (1) to determine the potential of ISSR and DAMD marker technique to generate polymorphic markers in grape; (2) to investigate whether DAMD markers could be effectively used in determining genetic relationships among grape genotypes compared to ISSR markers data.

Materials and methods

Plant material and morphological descriptor

Twenty one local *Vitis vinifera* L. varieties were sampled from the collection of Agricultural Research Station in Sanandaj (north-west of Iran). These varieties were collected from different regions of Kurdistan province (Table 1). Mean values were obtained from four plants based on seven morphoagronomic characteristics. The traits were chosen from the list of descriptors of the International Plant Genetic Resources Institute (1997), regarding importance

Table 1. Mean of different morphological traits evaluated in 21 local grapevine accessions used in this study. LA, leaf area; DM, days to maturity; BNL, bunch length; BRL, berry length; TBM, mass of ten berries; NBB, number of berries per bunch; BRW, berry width. *least significant differences at $P = 0.05$ probability level

No.	Cultivar	LA	DM	BNL	BRL	TBM	NBB	BRW
1	Nafti	15.4	136	8.6	9.0	6.3	144.3	3.1
2	Gave-Cham	14.6	152	12.5	13.1	12.7	146.3	1.0
3	Rasha	12.9	143	11.0	11.9	16.6	122.7	1.0
4	Vais-Gholi	19.9	127	16.2	18.2	34.1	44.0	2.8
5	Khoshnam	12.9	134	18.2	19.4	21.8	126.3	1.7
6	Maraie	11.9	129	6.6	7.2	6.3	44.0	1.1
7	Saih Par Rasheh	14.3	130	8.2	8.7	7.3	61.3	1.1
8	Gavan-Alahbab	17.0	135	13.8	15.6	56.4	56.3	2.2
9	Fakhri-Zodras	13.2	133	13.0	14.9	25.1	116.3	1.9
10	Keshmeshi	17.3	128	21.5	23.9	9.7	100.3	1.1
11	Askari	14.3	131	15.6	17.6	14.0	83.0	2.4
12	Gaznaie	14.7	138	13.0	14.5	28.3	69.7	1.5
13	BolMeskeh	13.1	134	10.7	11.5	22.7	79.3	1.8
14	Bozmarkot-Gaznaie	12.9	138	11.1	12.4	3.2	63.3	1.9
15	Sahebi	13.8	134	12.2	13.7	18.3	53.3	2.0
16	Fakhri	13.2	125	14.5	16.4	5.9	108.0	2.1
17	Sahani	16.1	125	16.6	18.3	7.6	45.7	1.6
18	Sepikeh	11.2	152	13.8	15.0	7.5	175.0	1.9
19	Perlit	12.6	120	20.4	22.8	3.3	281.7	1.1
20	Soraw	14.4	136	4.4	4.6	2.6	58.3	1.6
21	Siah-Zodras	16.6	131	11.4	18.7	21.3	20.0	2.4
	Mean	14.4	133.9	13.0	14.6	15.8	95.2	1.8
	LSD	1.1	4.5	3.8	4.8	5.2	11.6	0.7

in yield and fruit quality: leaf area, days to maturity, bunch length, berry length, mass of ten berries, number of berries per bunch, and berry width.

Genomic DNA isolation

DNA was extracted from 5 g of young leaves collected from five plants of each genotype using the CTAB method according to Lassner et al. (1989) with the modification described by Torres et al. (1993). The DNA final concentration was determined by agarose-gel electrophoresis using a known concentration of uncut λ DNA as a standard.

ISSR and DAMD marker analysis

For ISSR analysis, a set of 15 primers representing di, tri, tetra and pentamer repeats (UBC set # 9) was procured from the Biotechnology Laboratory, University of British Columbia, Canada. Following the optimisation of PCR conditions and pre-screening of the first 15 primers on a sample set, ten primers providing clear and informative amplicon profiles were selected to survey ISSR variation in the accessions listed in Table 2. DAMD markers (originally derived from the repeat elements of weed rice (Kang et al. 2002) were used in this study. Of 10 primers screened, seven with a GC content of 50 – 60% were selected to generate the DNA fingerprint profiles of all the genotypes (Table 2). PCR amplification was performed in 20 µL reaction containing 1X PCR buffer, 30 ng sample DNA, 2.5 µM primer, 200 µM of each dNTP, 1.5 – 2.5 mM $MgCl_2$ and 1.5 unit of Taq DNA polymerase (Cinnagene, Iran). All amplifications were carried out in a Eppendorf thermal cycler (Germany) as follows: 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at annealing temperature (T_a) (Table 1) and 2 min at 72 °C, and 8 min at 72 °C for final extension. Amplified products were electrophoresed in 1.2% agarose gels for 1 to 2 h at 100 V in 1X TBE and visualised by ethidium bromide staining.

Data analysis

Amplified bands obtained with all the molecular markers were scored visually for the presence (1) and absence (0) of bands for all the 21 genotypes. Nei's genetic distance (Nei 1973) among genotypes was determined and used for grouping of the genotypes by UNJ (Un-weighted Neighbor Joining) cluster method (Perrier et al. 2003). The fit of dendrogrammes obtained were checked by bootstrapping using 100 replications. NTSYS ver 2.02 (Rohlf 1998) and DARwin ver 5.0 (Perrier, Jacquemoud-Collet 2006) were used for clustering. The Mantel statistic was used to compare the dissimilarity matrices as well as the dendrograms produced by the ISSR and DAMD techniques through NTSYS software. Polymorphic information content (PIC) values were calculated for each ISSR and DAMD primers according to the formula: $PIC = 1 - \Sigma (P_{ij})^2$, where P_{ij} is the frequency of the i^{th} pattern revealed by the j^{th} primer summed across all patterns revealed by the primers (Botstein et al. 1980).

Results

DNA fingerprint database was produced using the two different PCR-based molecular markers (ISSR, DAMD) systems for 21 Vitis vinifera varieties belonged to different geographical locations of Kurdistan province (North-west of Iran). Our results indicated that primers obtained from the different regions of genomic DNA successfully amplified

Table 2. Primers used in ISSR and DAMD analyses. Ta, annealing temperature; GC,

Type	T_a (°C)	GC (%)	Sequence (5' to 3')	Marker
ISSR	50	47	AGAGAGAGAGAGAGAGT	UBC807
	52	47	TGTGTGTGTGTGTGTGA	UBC828
	54	50	GGATGGATGGATGGAT	UBC878
	44	52	CTCTCTCTCTCTCTCTG	UBC815
	52	52	CACACACAC ACACACAG	UBC818
	45	47	GAGAGAGAGAGAGAGAA	UBC812
	50	60	GGAGAGGAGAGGAGA	UBC880
	48	33	GAAGAAGAAGAAGAAGAA	UBC868
	48	47	CACACACACACACACAT	UBC816
	52	33	ATGATGATGATGATGATG	UBC864
DAMD-PCR	50	55	ATCCAAGGTCCGAGACAACC	URP1F
	50	60	GTGTGCGATCAGTTGCTGGG	URP2F
	50	65	GGCAAGCTGGTGGGAGGTAC	URP6R
	50	55	AGGACTCGATAACAGGCTCC	URP4R
	50	50	ATGTGTGCGATCAGTTGCTG	URP9F
	50	50	TACATCGCAAGTGACACAGG	URP13R
	50	55	AATGTGGGCAAGCTGGTGGT	URP17R

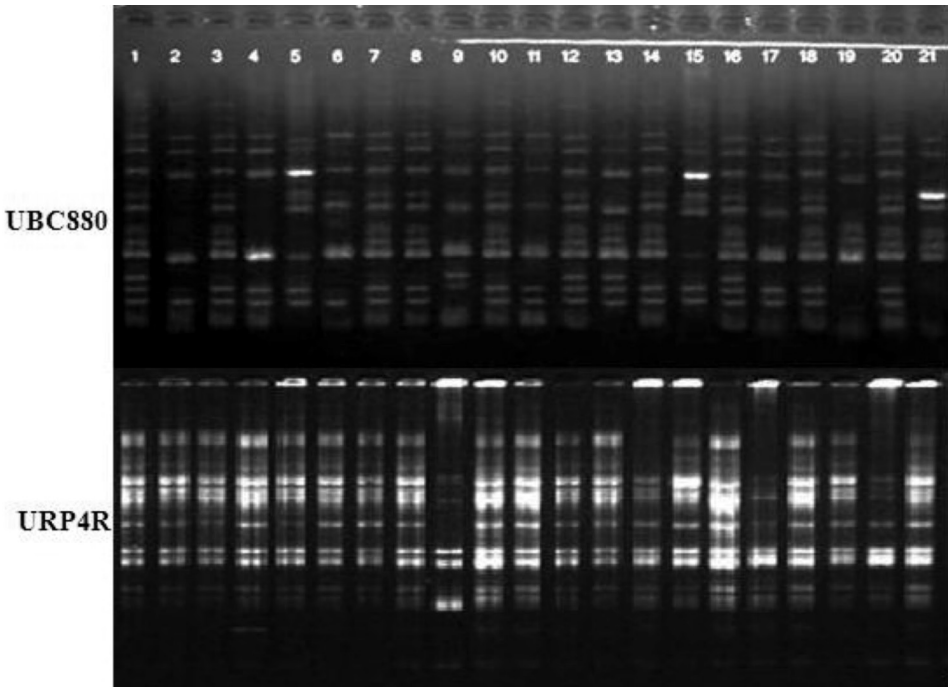

Fig. 1. Amplification profile obtained with UBC880 and URP4R primers detected in grapevine accessions.

accession template DNAs (Fig. 1). All of the two molecular markers used in this study were able to distinguish and identify each of 21 varieties. Salient features of fingerprint database obtained using different markers are given below.

A total of 106 bands were detected using ten ISSR markers, of which 69 were polymorphic (Table 3). ISSR polymorphic DNA bands varied between five (UBC816) and nine (UBC828 and UBC812), with an average of 6.9 per primer. Percent polymorphism ranged from 42 to 77% with an average polymorphism of 64.05% across all varieties. ISSR markers showed relatively high level of polymorphism in the examined germplasm, as the calculated PIC value for these markers was in the range of 0.27 to 0.49 with an average of 0.43 across the genotypes assayed (Table 3). A significant correlation ($r = 0.91$; $p < 0.01$) was observed between the total number of bands and

Table 3. Primers used in ISSR and DAMD analyses. Ta, annealing temperature; GC,

Type	Primer	No. of amplified bands	No. of polymorphic bands	Polymorphism (%)	PIC value
ISSR	UBC807	12	8	66	0.41
	UBC828	14	9	64	0.48
	UBC878	7	3	42	0.27
	UBC815	11	8	72	0.49
	UBC818	10	6	60	0.39
	UBC812	14	9	64	0.47
	UBC880	9	7	77	0.49
	UBC868	11	7	63	0.44
	UBC816	8	5	62.5	0.43
	UBC864	10	7	70	0.46
DAMD	URP1F	12	7	58	0.41
	URP2F	11	8	72	0.47
	URP6R	9	7	77	0.49
	URP4R	10	7	70	0.44
	URP9F	6	3	50	0.38
	URP13R	11	7	63	0.45
	URP17R	12	9	75	0.49

the number of polymorphic bands.

A polymorphic chain reaction (PCR) based approach involving the direct amplification of minisatellite region DNA (DMAD) with seven minisatellite core sequences as primers was used for diversity analysis of 21 grapevine varieties. The analysis indicated that the PCR profile and the optimised chemical concentrations resulted in reproducible and reliable DNA amplification (Fig. 1). In the grapevine varieties the number of amplified DAMD products varied from 6 to 12 fragments depending on the primers used. Total number of bands scored was 71 of which 48 were polymorphic (Table 3). PIC values ranged from 0.38 to 0.49, with an average value of 0.44 per locus. Based on the independent replications of DAMD, we observed that reproducible DNA markers were amplified and also noted that all DAMD primers used in this study produced RAPD-like results, but the numbers of bands were sharp and clear. The relatively high PCR stringencies in DAMD application effectively limited the PCR artifacts which commonly occur in RAPDs (Karaca et al. 2002; Ince et al. 2009).

Both molecular marker systems (ISSR and DAMD) were able to discriminate between 21 grape varieties. The level of polymorphism generated by ISSR markers (64%) was relatively similar to that of DAMD (66%) markers. Genetic dissimilarity between pairs estimated using binary data ranged from 0.23 to 0.81 in ISSR analysis and from 0.12 to 0.67 in DAMD markers analysis. Genetic relationships as determined by cluster analysis for ISSR, DAMD and pooled allelic data (ISSR + DAMD) are shown in Figs. 2, 3 and 4, respectively. Dendrogrammes obtained by ISSR (Fig. 2) and DAMD (Fig. 3) markers were relatively similar and most of the genotypes were placed in their respective groups, and also were matched with their morphological traits. A slightly significant positive correlation between ISSR and DAMD dendrogrammes observed. The values of the Mantel test showed positive correlation between the two marker types. The correlation coefficient was 0.44 between ISSR and DAMD, 0.39 between ISSR and pooled markers data (ISSR + DAMD) and 0.59 between DAMD and polled data (Table 4). The dendrogramme from DAMD data was most congruent with the general dendrogramme from pooled (ISSR + DAMD) data. Cluster analysis using ISSR and DAMD data grouped genotypes into three and two distinct clusters, respectively (Fig. 2 and 3). In both cluster analyses, genotypes number 1 and 13 (Nafti and Bolmeskeh) were not grouped with other genotypes and separated as a distinct branch. Genotypes belonging to cluster II in both ISSR and DAMD dendrogrammes were relatively the same. In the general dendrogramme (Fig. 4), genotypes grouped in four distinct clusters. Cluster I included some genotypes that are mostly known for their early maturity, high berry weight with red anthocyanin colouration. Cluster II and III consisted of genotypes that showed relatively lower bunch length, weight and, consequently, low fruit yield. Cluster IV contained four genotypes (Fig. 4). These genotypes have significantly higher number of berries per bunch with white anthocyanin colouration and significantly smaller berry size, in comparison to genotypes in other clusters.

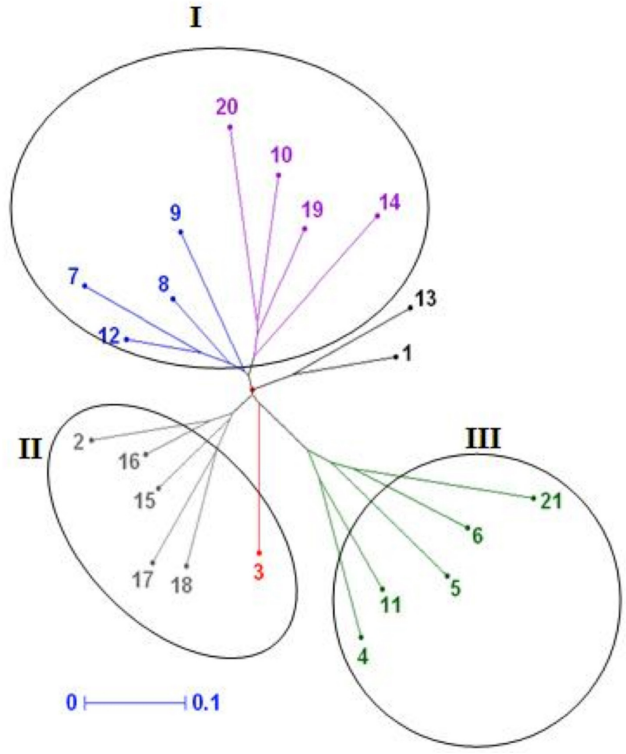

Fig. 2. Dendrogramme of the grapevine genotypes based on the dissimilarity matrix developed using ISSR markers.

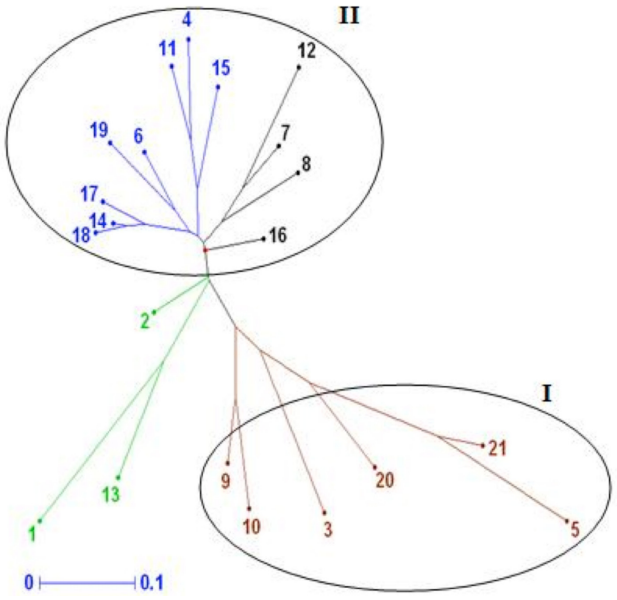

Fig. 3. Dendrogramme of the grapevine genotypes based on the dissimilarity matrix developed using DAMD markers.

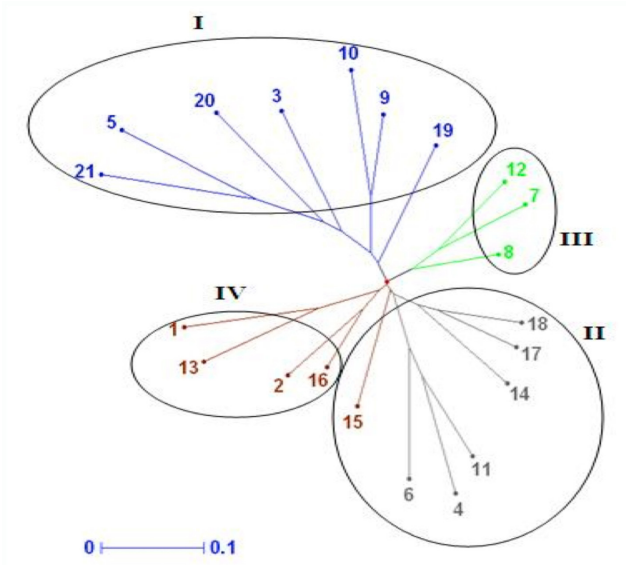

Fig. 4. Dendrogramme of the grapevine genotypes based on the dissimilarity matrix developed using ISSR and DAMD markers.

Discussion

The lack of knowledge regarding a substantial part of grapevine biodiversity in Iran led us to carry out surveys to find, catalogue and identify the genetic heritage of this species. Most of the Iranian grapevine growers are able to identify the most widespread cultivars in this region based on a few visible morphological traits, such as Keshmeshi, Fakhri and Gaznaie. However, when asked for the names of other different cultivars found, these were completely unknown to them.

In this study, we fingerprinted a set of 21 autochthonous grapes by means of ISSR and DAMD markers, in order to assess their genetic diversity. We demonstrated the usefulness and the reliability of these markers in the detection of DNA polymorphisms. Detection of genetic variation and determination of genetic relationships between individuals and populations are important considerations for the efficient conservation and utilisation of plant genetic resources. Data collected in the course of this study were used to compare the similarity values obtained with different marker systems. This was the first detailed report comparing the performance of two molecular marker types (ISSR and DAMD) based on tandemly repeated regions of genome on a set of samples of grapevine genotypes. Comparison studies on these molecular markers have been carried out for several plant species (Karaca, Ince 2008; Ince et al. 2009; Amirmoradi et al. 2012). We found relatively comparable genetic diversity within available grapevine accessions and confirmed previous analyses suggesting that grapevine is a very diverse species (Martinez et al. 2006; Ibanez et al. 2009). Our results are an addition to previous reports on clustering genotypes and varieties in different groups using different marker techniques. ISSR and DAMD generated a high number of polymorphic markers that could be used in diagnostic fingerprinting studies of grapevine. Based on the average percentage polymorphism and PIC, the efficiency of ISSR and DAMD markers for fingerprinting of grapevine genotypes is relatively the same. In general, these two techniques could be used in conjunction with each other (morphological descriptors) for diagnostic fingerprinting of grapevine. The selection of genotypes for this study was primarily based on some morphological traits. Therefore, we believe there is a need for molecular marker studies as a complementary tool to description of morphological traits in the field. This can reduce the amount of materials for study as well as the cost of experiments.

Table 4. Mantel test correlation coefficient among similarity matrices obtained using ISSR and DAMD markers

	ISSR	DAMD	ISSR + DAMD
ISSR	–		
DAMD	0.44	–	
ISSR + DAMD	0.39	0.59*	–

Despite the small number of ISSR and DAMD markers used in this study, the general congruence between ISSR and DAMD and their partial agreement with morphological divergence between studied genotypes suggest that either molecular marker method or combination of both is acceptable in expanded studies on grapevine germplasm. The slightly lower level of correlation between both molecular marker types ($r = 0.44$, $P < 0.05$) in the present study probably reflects that these markers are known to target different genomic fractions involving repeat and/or unique sequences, which may have differentially evolved or have been preserved during the course of natural or artificial selection. Many earlier reports showed discrepancy between dendrogrammes when two different molecular marker techniques were used (Sonia, Gopalakrishna 2007; Arif et al. 2009). Discordance between dendogrammes or trees obtained using different marker types can be explained by the genetically inert nature of markers when compared to functionally active, different regions of the genome targeted by different markering techniques, level of polymorphism detected and the number of loci and their coverage of the overall genome (Souframanien, Gopalakrishna 2004). Our results complement previous reports on clustering genotypes and varieties in different groups using different marker techniques. The genetic diversity pattern apparently differed between methods and classifying the germplasm yielded different results when different methods were applied.

It seems that since each of these methods demonstrates different aspects of diversity in different populations, simultaneous application of these methods can present researchers with a clearer view of diversity. However, the assessment of genetic variation in large samples of plant genetic resources requires a high costs, time and

amount of consumables. Thus, reliable, affordable and economical techniques should be preferred, at least in the first screening of genetic variability. Obviously, assessment of morphological traits takes several months and requires considerable costs; even in some cases, due to the effect of environment and mutual effects of genotype × environment in the emergence of these traits, it is necessary to repeat the experiments throughout several years and in different places. The current study confirmed the importance of molecular studies (cheap, fast and informative markers) beside the morphological data in detecting genetic variation among genotypes and in selecting diverse parents to carry out new crossing programmes successfully.

It was evident from the results that the dendrogramme based on molecular markers was not completely in accord with the morphological characteristics, as reported for other crops (Fernandez et al. 2002; Maric et al. 2004; Talebi et al. 2008). There are several possible explanations for such results: some of them are associated with the nature and structure of different molecular markers that are designed from various regions of genome. Another problem was the possibility of overestimating genetic similarity because fragments with the same size could have different origin (Talebi et al. 2012). Our results demonstrate that high genetic diversity exists between the investigated accessions. The magnitude and pattern of genetic variation detected in this study can be useful for more systematic germplasm management and utilisation in breeding programmes (Tanya et al. 2011). The exploitation of crosses between genetically distant parents (e.g., recombinant parents from local accessions and the introduced genotypes) and those from diverse local sources may produce higher heterosis, better genetic recombination and segregation in their progenies and result in varieties with a broad genetic base (Chahal, Gosal 2002). This study has implications for the management of genetic resources and their use in applied breeding programmes, particularly for the development of a core collection. Information about current genetic diversity permits the classification of our available germplasm into various/heterotic groups, which is particularly important in hybrid/cross-breeding programmes in grapevine.

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Received 20 October 2012; received in revised form 7 November 2012; accepted 12 November 2012