Journal of Applied Botany and Food Quality 85, 224 - 228 (2012)

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Genetic characterization and relatedness among autochthonous grapevine cultivars from Northeast Turkey by Simple Sequence Repeats (SSR)

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(Received October 10, 2012)

Summary

25 autochthonous grapevine cultivars from Northeast Anatolia in Turkey together with two well-known standard cultivars, Cabernet Sauvignon and Merlot were fingerprinted using six pairs of SSR primers to assess their genetic diversity and relatedness. All six SSR primers produced successful amplifications and revealed DNA polymorphisms that were subsequently used to assess genetic relatedness of the cultivars. A total of 52 alleles were detected with a mean value of 8.67 alleles per locus indicating allele richness. The average expected heterozygosity (He) and observed heterozygosity (Ho) were 0.759 and 0.809, respectively. Considering the number of alleles generated, the highest number was observed in VVS2 loci (14 alleles/locus), while the lowest in VrZAG83 loci (5 alleles/locus). The Unweighted Pair-Group Method with Arithmetic mean (UPGMA) dendrogram constructed based on the SSR data yielded two main clusters. First cluster included only cv. Kibris and the second cluster included rest of the cultivars including Cabernet Sauvignon and Merlot. The results showed that SSR markers have proved to be an efficient tool for fingerprinting grapevine cultivars and conducting genetic diversity studies in grapevine.

Introduction

Turkey, located at the junction of two main plant gene centers, has a very old and rich grapevine germplasm including over 1500 cultivars. Archaeological excavations also confirm that grapevine cultivation is a very old tradition in Anatolia dating back to 4000 BC (Selli et al., 2007). Each grape-growing region in Turkey has particular local grapevine cultivars which differ from each other in color, taste, shape, bunch density etc. There is also a wide variation in terms of synonymous cultivars in each region and correct identification of these cultivars is of great importance in cultivar standardization and determination of total cultivar number (Ergul et al., 2006).

Northeast Anatolia region in Turkey has older and special grapevine cultivation. There were a lot of churches in this region and grape berylliums of different shapes can be seen on the walls of churches. McGovern (2008) stated that the region, together with other regions of the South Caucasus and Anatolia, is the cradle of domestication and primary viticulture in the Old World. In this region, Yusufeli district is the main traditional viticulture area including over 30 very old and local grapevine cultivars. This area is also very rich in terms of wild grapes (*Vitis vinifera* ssp. *sylvestris*). In Christian period, these cultivars were used for wine-making. However, at present, wine production is not practiced because of the conservative life style of the people in this region. Hence, the cultivars previously used for wine-making are currently used in juice production. Northeast Anatolia region differs greatly from the other regions in Turkey in terms of climatic characteristics and thus it is expected that the grape germplasm of this region would have economically important adaptive traits that can potentially be incorporated into grape breeding programs in future.

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During the last decade, the genomic resources that are available to the grapevine research community have increased enormously in parallel to a renewed interest in grapevine (*Vitis vinifera* L.) germplasm resources and analysis of genetic diversity in grapes. It is well recognized that genetic variation is invaluable for crop improvement and understanding gene function, and this fact applies to grapevine as well (This et al., 2006).

Knowledge of the genetic diversity and relationships among the grapevine cultivars is important for recognizing gene pools, identifying pitfalls in germplasm collections, and developing effective conservation and management strategies (Ergul et al., 2006). The grapevine is highly heterozygous and propagation by cuttings or layers maintains their heterozygosity. Therefore, morphological classifications provide rough guidelines, while molecular evaluations provide further insight into the genetic structure and differentiation within and among taxa which is useful for geneticists, plant breeders, and gene bank managers (Papanna et al., 2009)

Lots of studies have investigated the genetic variation within grapevine by using different molecular marker systems such as Random Amplification of Polymorphic DNA (RAPD) (Benjak et al., 2005), Amplified Fragment Length Polymorphism (AFLP) (Doulati Baneh et al., 2007), Inter Simple Sequence Repeats (ISSR) (Argade et al., 2009), Single Nucleotide Polymorphism (SNP) (Pindo et al., 2008) and SSR (De Mattia et al., 2009; Leao et al., 2009). Among these marker systems, SSRs have been widely used by researchers to determine the genetic diversity within grapevine cultivars. Six so-called "core set" SSR markers (i.e., VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, and VrZAG79) are recommended for the direct comparison of results from different laboratories (This et al., 2004).

In Northeast part of Turkey, there are several specific grape germplasms and Coruh Valley is one of the most important grape germplasm centers of this region. There are a number of autochthonous grapevine cultivars which were used many centuries ago. Therefore, it can be valuable to characterize this germplasm both morphologically and genetically. In this study, an attempt to assess the level of genetic diversity and genetic relationship among 25 autochthonous grapevine cultivars from Northeast Anatolia in Turkey has been made. It is expected that the information presented here would be useful for selection and more efficient utilization of this germplasm in grape breeding programs in future.

Material and methods

Plant material

Leaf samples of 25 autochthonous grapevine cultivars used in this study were collected from Yusufeli district in Northeast Anatolia region in Turkey. A total of 25 grapevine cultivars together with two reference cultivars, Cabernet Sauvignon and Merlot were included in SSR analysis. Some important ampelographic traits of these cultivars provided by the International Union for the Protection of New Varieties of Plants (UPOV) are presented in Tab. 1.

Tab. 1: Basic descriptive characteristics of 25 grapevine cultivars used in this study.

Cultivar	Utility	Berry Color	Berry shape
Alvan	Juice	Dark red-violet	Round
Artvin	Juice	Green-yellow	Ovate
Beyaz Istanbul	Table	Green-yellow	Elliptic
Beyaz turfanda	Table	Green-Yellow	Ovate
Ciklap	Juice	Green-Yellow	Round
Erik	Table	Yellow	Ovate
Gelin parmagi	Juice	Green-Yellow	Narrow elliptic
Gines	Table	Dark red-violet	Round
Goh	Juice	Blue-black	Round
Hatkul	Juice	Blue-black	Round
Kara turfanda	Juice	Blue-black	Round
Karul	Table	Green-Yellow	Narrow elliptic
Keci memesi	Juice	Dark red-violet	Narrow elliptic
Kibris	Table	Dark red-violet	Ovate
Kirmizi Istanbul	Table	Rose	Ovate
Kiskinbur	Juice	Rose	Round
Kokulu	Juice	Blue-black	Round
Kutuk	Juice	Green-Yellow	Ovate
Mandagozu	Juice	Blue-black	Elliptic
Mezarlik	Juice	Yellow	Round
Nanebur	Juice	Dark red-violet	Elliptic
Razaki	Table	Dark red-violet	Ovate
Sulu Kurta	Juice	Dark red-violet	Round
Tokat	Table	Green-yellow	Round
Yag	Juice	Dark red-violet	Round

DNA extraction

Genomic DNA was extracted from young leaf tissue using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) according to the instructions provided by the manufacturer. Subsequently, a RNAse treatment was performed on the eluted DNA samples. Purity and concentration of the DNA were checked both on 1% (w/v) agarose gels and by NanoDrop® ND-1000 Spectrophotometer.

SSR analysis

Six SSR markers (i.e., VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, and VrZAG79) were used in **Polymerase Chain Reaction** (PCR) studies. PCR was conducted in a volume of 10 µL and contained 15 ng genomic DNA, 5 pmol of each primer, 0.5 mM dNTP, 0.5 unit GoTaq DNA polymerase (Promega), 1.5 mM $MgCl_2$ and 2 µL 5X buffer. The forward primers were labeled with WellRED fluorescent dyes D2 (black), D3 (green) and D4 (blue) (Proligo, Paris, France). Reactions without DNA were included as negative controls. PCR amplification was performed by using the Biometra® PCR System. The amplification conditions consisted of an initial denaturation step of 3 mins at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52-56°C and 2 mins at 72°C with a final extension at 72°C for 10 mins. The PCR products were first separated on a 3% (w/v) agarose gel run at 80 V for 2 hrs. The gel was then stained with ethidium bromide at a concentration of 10 mg/mL. A DNA ladder (100 bp) (Promega) was used for the approximate quantification of the bands. The amplification products were visualized under UV light, and their sizes were estimated relative to the DNA ladder. For further determination of polymorphisms, the PCR products were run on CEQTM 8800 XL Capillary Genetic Analysis System (Beckman Coulter, Fullerton, CA). The analyses were repeated at least twice to ensure reproducibility of the results. Allele sizes were determined for each SSR locus using the Beckman CEQTM Fragment Analysis software. In each run, Cabernet Sauvignon and Merlot cultivars were included as reference cultivars.

Genetic analysis

The genetic analysis program "IDENTITY" 1.0 (Wagner and Sefc, 1999) was used according to Paetkau et al. (1995) for the calculation of number of alleles, allele frequency, expected and observed heterozygosity, estimated frequency of null alleles, and probability of identity per locus. Genetic dissimilarity was determined by the program "MICROSAT " (version 1.5) (Minch et al., 1995) using proportion of shared alleles, which was calculated by using "ps (option 1 - (ps))", as described by Bowcock et al. (1994). The results were then converted to a similarity matrix, and a dendrogram was constructed with the UPGMA method (Sneath and Sokal, 1973) using the software NTSYS-pc (Numerical Taxonomy and Multiware Analysis System,version 2.0) (Rohlf, 1988).

Results and discussion

The study presents SSR analysis results of a total of 27 grapevine cultivars comprising 25 autochthonous grapevine cultivars sampled from Northeast Anatolia and two reference cultivars Cabernet Sauvignon and Merlot, to determine genetic diversity and relatedness among them.

As shown in Tab. 2, a total of 52 alleles ranging from 5 to 14 alleles per locus with a mean value of 8.67 alleles per locus were detected. Polymorphic bands were obtained with all primers. VVS2 loci were the most polymorphic among the six loci, with the highest effective number of alleles (14 alleles), followed by the loci of VrZAG79 (11 alleles) and VVMD27 (9 alleles). The VrZAG83 loci generated the lowest number of alleles (5 alleles) (Tab. 2).

The mean He and Ho were determined as 0.759 and 0.809 over six loci, respectively. Among the six loci, the Ho values were the highest (0.889) in VVS2 loci indicating high genetic diversity while the lowest (0.593) in VrZAG83 loci (Tab. 2).

Among the six loci, only VVMD7 generated more than two alleles (tri-allelic loci) in two varieties (Karul and Nanebur) (Tab. 3).

In present study, the frequency of null alleles (r) in VVS2 and

Tab. 2: Number of alleles, allele range (bp), expected heterozygosity, observed heterozygosity, and probability of identity values of grapevine cultivars.

Loci	N	He	Ho	PI	r
VVMD7	6	0.756	0.852	0.186	-0.0547
VVMD27	9	0.748	0.852	0.134	-0.0592
VrZAG79	11	0.831	0.852	0.088	-0.0112
VVMD24	7	0.763	0.815	0.150	-0.0296
VVS2	14	0.903	0.889	0.032	0.0072
VrZAG83	5	0.612	0.593	0.330	0.0119
Total	52	4.613	4.853		
Average	8.67	0.759	0.809		

N: number of alleles; Ho: observed heterozygosity; He: expected heterozygosity; PI: probability; r: null allele frequencies

Tab. 3: Allele sizes, in base pairs, of six microsatellites loci from 27 grapevine cultivars.

Cultivar	VVMD7	VVMD27	VrZAG79	VVMD24	VVS2	VrZAG83
Artvin	238:246	181:185	248:256	207:211	139:143	185:191
Alvan	238:246	185:189	244:246	207:207	145:149	191:191
Beyaz Istanbul	236:246	185:185	244:252	207:211	135:151	185:197
Beyaz turfanda	238:246	185:191	244:246	207:215	135:141	185:191
Ciklap	238:246	179:185	240:246	207:215	139:151	191:191
Erik	236:244	179:181	248:256	207:207	139:149	185:185
Gelin parmagi	238:246	181:185	246:250	217:217	125:149	191:191
Gines	236:242	183:185	246:246	207:215	133:145	191:191
Goh	238:246	181:185	248:256	207:215	143:143	191:191
Hatkul	238:246	179:185	244:244	205:217	123:143	191:191
Kara turfanda	236:244	179:185	246:250	205:217	135:149	185:191
Karul	230:238:246	179:185	238:246	201:207	135:143	187:191
Keci memesi	240:246	185:189	242:246	205:217	141:145	185:191
Kibris	238:238	195:195	246:248	205:211	159:159	185:191
Kirmizi Istanbul	236:244	181:185	248:256	207:211	137:143	185:191
Kiskinbur	238:246	185:185	248:250	205:215	125:143	185:191
Kokulu	238:246	179:183	234:244	205:207	123:151	185:187
Kutuk	238:246	175:179	244:248	205:207	133:137	185:185
Mandagozu	236:246	185:185	238:248	207:211	131:133	191:197
Mezarlik	236:244	183:185	244:248	207:215	133:133	181:185
Nanebur	238:242:246	179:185	240:248	207:209	139:143	185:191
Razaki	236:244	181:185	248:256	207:211	139:143	185:191
Sulu Kurta	246:246	181:185	246:250	217:217	123:149	191:191
Tokat	244:244	177:185	244:246	205:207	137:143	185:191
Yag	236:244	181:185	246:250	217:217	125:149	191:191
Cabernet Sauvig.	236:236	175:189	246:246	207:217	139:151	197:197
Merlot	236:244	189:191	258:258	207:211	139:151	191:197

VrZAG83 loci was positive, but these low values suggest the absence of null alleles (Tab. 2).

The most informative loci, with regards to the probability of identity (PI), were VVS2 and VrZAG79 which generated 14 alleles (PI: 0.032) and 11 alleles (PI: 0.088), respectively, whereas the least informative locus was VrZAG83 which generated 5 alleles (PI: 0.330) (Tab. 2).

Allele sizes (bp) of 27 cultivars from six SSR loci are shown in Tab. 3. The most frequent alleles were 246 (30.38%) in VVMD7, 185 (44.44%) in VVMD27, 246 (27.78%) in VrZAG79, 207 (38.89%) in VVMD24, 143 (18.52%) in VVS2 and (53.70%) in VrZAG83, respectively. The number of microsatellite different genotypes ranged from 8 (VrZAG83) to 21 (VVS2) with an average of 13.1 and a total of 79.

The genetic similarity ranged from 0.17 to 0.92 among cultivars and the cultivars Kutuk and Yag; Kokulu and Yag as well as Kibris and Cabernet Sauvignon were found to be more distant at 0.17 similarity ratio (Fig. 1). According to genetic similarity ratio, cultivars Kirmizi Istanbul and Razaki were the closest (0.92) (Fig. 1). The average similarity ratio was 0.53 indicating high genetic diversity among cultivars.

To elucidate the genetic relationship among grapevine cultivars, a dendrogram generated from the UPGMA cluster analysis over six SSR loci classified 27 cultivars into two main groups depicted in Fig. 1. A single cultivar (cv. Kibris) was clustered separately from the remaining cultivars. The second main cluster included 24 autochthonous cultivars and the two reference cultivars. This cluster was further divided into two subgroups. The first subgroup comprised the two reference cultivars (Cabernet Sauvignon and Merlot) and two autochthonous cultivars (cvs. Mandagozu and Beyaz Istanbul). In this subgroup reference cultivars clustered separately from the other two cultivars. The second subgroup comprised the remaining 22 autochthonous cultivars.

In this study we report for the first time the use of SSR markers for assessing genetic relatedness among 25 autochthonous grapevine cultivars from Northeastern Turkey. The results obtained in the present study show that microsatellites can be effectively used for fingerprinting purposes in grapevine. In fact, all tested microsatellite primer pairs produced various levels of amplifications. The mean value of 8.67 alleles per locus obtained with the microsatellites that produced polymorphic amplification patterns is consistent with the reported data of 4-16 alleles per locus in *V. vinifera* germplasms assessed with microsatellites in different countries (Bowers et al., 1996; Fatahi et al., 2003; Martin et al., 2003; Martinez et al., 2006; Tangolar et al., 2009).

The high levels of within-group variation and the simple genetic structure observed in the dendrogram probably suggest a complex history of development of grapevine cultivars in Northeast Anatolia. Introduction and spread of wild and semi-domesticated grapes, especially from its native Near Eastern range, domestication of indigenous wild grape, natural hybridization between indigenous and introduced vines, and human selection may have contributed to this high variation. The cv. Kibris was clustered separately on dendrogram. If we look at Tab. 3, we can see that this cultivar has unique alleles in at least five loci. This cultivar was brought to Coruh

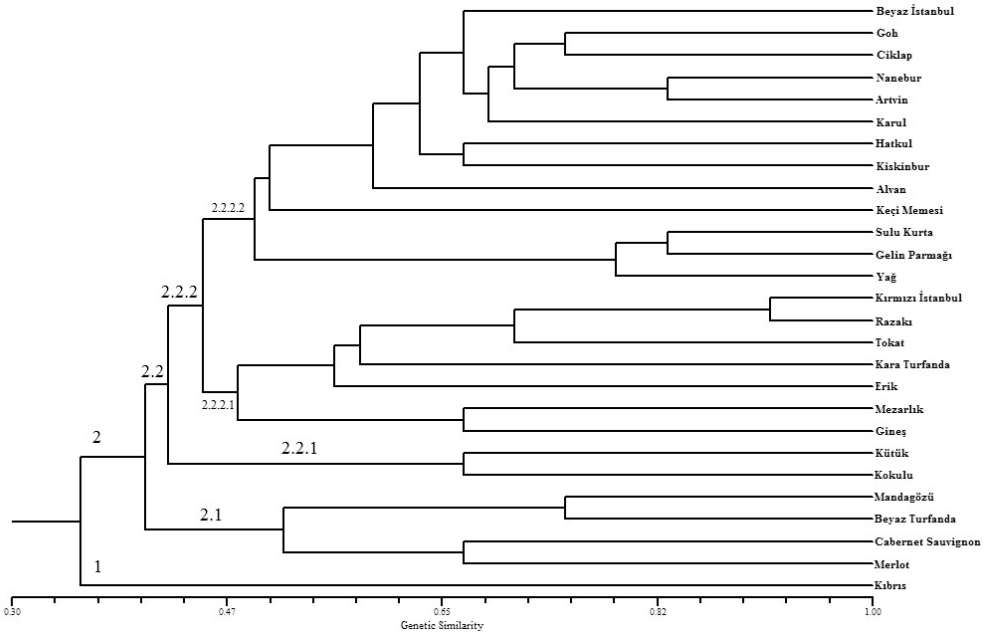

Fig. 1: Dendrogram showing the relationships of 27 grapevine cultivars based on UPGMA cluster analysis of six SSR loci.

Valley from Cyprus Island and due to the protective position of the island, the genetic structure of the cultivar may have been protected.

Based on the number of alleles generated and probability of identity values, the most informative loci were VVS2 (14 alleles per locus, PI:0.032) and VrZAG79 (11 alleles per locus, PI: 0.088), whereas the least informative locus was VrZAG83 generated (5 alleles per locus, PI: 0.330). Previously, among the six SSR primers, the highest number of alleles was obtained from VVS2 primer (Fatahi et al., 2003; Nuñez et al., 2004; Sellı et al., 2007; Tangolar et al., 2009) while the lowest number of alleles was detected in VrZAG83 primer (Sefc et al., 2000; Snoussi et al., 2004; Tangolar et al., 2009).

Microsatellites have been becoming the marker of choice for fingerprinting and genetic diversity studies in a wide range of living organisms. The approach described in this paper shows that microsatellite analysis is a powerful tool for characterization of grapevine cultivars as well. Taken together with their co-dominant nature and reproducibility, SSR markers are very useful for the analysis of genetic diversity, genomic mapping and marker-assisted selection in many plant species compared to many other marker systems. In this study, the mean Ho level was slightly higher than that of He (80.9% for Ho and 75.9% for He). Comparable observed heterozygosity levels were reported between 74.3-85.5% in different grape-cultivated areas in the world (Bowers et al., 1996; Sefc et al., 2000; Fatahi et al., 2003; Martinez et al., 2006; Tangolar et al., 2009).

Such high levels of heterozygosity are commonly observed among clonally propagated, outbreeding, perennial species since they are favoured during selection and are known to confer greater adaptability, vigour and productivity on clonal varieties (Aradhya et al., 1998; Sefc et al., 2000). Grapes, being an outbreeding species, have highly heterozygous cultivars, carry a heavy genetic load and suffer from severe inbreeding depression (Olmo, 1976).

Our findings also indicated that there was a high genetic distance value among cultivars ranging from 0.17 to 0.92. Considering the environmental conditions of the region, it is expected that the grapevine germplasm in the region would have economically important adaptive traits that can potentially be incorporated into grapevine breeding programs. Hence, it is expected that the results of this study will assist current grapevine breeding efforts in Turkey as well as maintain the genetic integrity of the genetic resources.

In summary, the gene pool of cultivated grapes surveyed in Northeast Anatolia has significant amounts of genetic variation. In regard to germplasm management, our results show that the germplasm collection is highly variable and most variation is common to all genetic groups identified.

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