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Collection and genetic characterization of *Vitis vinifera* ' Žilavka' by microsatellites and AFLP markers

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ABSTRACT

'Žilavka' has been grown in Bosnia and Herzegovina since the XIVth century and is exploited for wine production. Although not sufficiently studied, this grapevine cultivar has high economic potential for the country. Five survey missions resulted in the collection of eighty 'Žilavka' accessions that varied in terms of names and characteristics. Because of the unknown origin of the cultivar, these accessions were analyzed with microsatellites markers in order to obtain a standard 'Žilavka' genotype. AFLP markers were used to investigate the genetic basis of variability within the cultivar. 'Žilavka' grapevines were screened on 14 microsatellite loci, thus revealing 4 different genotypes arising from mutations observed at 10 polymorphic loci. AFLP analysis of 52 'Žilavka' accessions revealed 35 different genotypes, with an average polymorphism of 57 %. Cluster analysis showed no grouping of different Žilavka accessions according to their names, characteristics or collection locations. The standard 'Žilavka' genotype was further compared to 211 cultivars from Slovenia (49), Austria/Germany (20), France (13), Portugal (27), Croatia (19), Greece (32), Spain (21) and Italy (30) in order to assess their genetic relationships. In pairwise comparisons, the highest genetic similarity was found with Slovenian cultivars 'Glera' and 'Briška Glera' (64 %) and the highest genetic dissimilarity (100 %) with two Italian cultivars, 'Nebbiolo Lampia' and 'Vespolina'. Inventory, collection and genetic characterization of 'Žilavka' accessions are important steps towards cultivar standardization, identification of parental cultivars and investigation of cultivar origin, required for its sustainable use.

Key words: grapevine, microsatellite, standard genotype, variability, AFLP

IZVLEČEK

ZBIRANJE VINSKE TRTE SORTE ŽILAVKA (*Vitis vinifera* L.) IN GENETSKA KARAKTERIZACIJA Z MIKROSATELITNIMI IN AFLP MARKERJ

'Žilavka' je pomembna vinska sorta z območja Bosne in Hercegovine kjer jo gojijo že od XIV stoletja. V preteklosti ni bila dovolj proučevana vendar ima velik ekonomski potencial za omenjeno območje. Akcesije žilavke (80), ki imajo različna imena in se razlikujejo po nekaterih osnovnih karakteristikah so bile nabrane na različnih področjih Bosne in Hercegovine. Za pridobitev standardnega genotipa žilavke smo uporabili mikrosatelitne markerje in z analizo 14 lokusov odkrili mutacije na 10 lokusih in določili 4 različne genotipe. Vse akcesije (52) katerim smo na osnovi mikrosatelitov določili standardni genotip smo nadalje analizirali z AFLP markerji in tako določili 35 različnih genotipov. Povprečni polimorfizem pri AFLP analizi je bil 57 %. S klastrsko analizo nismo odkrili skupin povezanih z različnimi karakteristikami, poimenovanjem ali izvorom akcesij žilavke. Standardni genotip žilavke smo nadalje primerjali z genotipi 211 kultivarjev iz Slovenije (49), Avstrije/Nemčije (20), Francije (13), Portugalske (27), Hrvaške (19), Grčije (32), Španije (21) in Italije (30), da bi ugotovili kakšna je sorodnost z njimi. V analizi primerjave parov smo ugotovili, da je žilavka najbolj sorodna (64 %) s slovenskima kultivarjema 'Glera' in 'Briška Glera' ter najbolj genetsko oddaljena od italijanskih kultivarjev 'Nebbiolo Lampia' in 'Vespolina'. Zbiranje, vrednotenje in genetska karakterizacija akcesij žilavke je pomembna za standardizacijo kultivarjev, analizo starševstva in izvora sorte.

Ključne besede: vinska trta, mikrosateliti, standardni genotip, variabilnost, AFLP

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1 INTRODUCTION

Vitis vinifera 'Žilavka' is a white grapevine cultivar and is used traditionally for wine production. In the region of Herzegovina (Bosnia and Herzegovina), this cultivar has been grown for more than 600 years, the Bosnian King Tvrtko having been mentioned as drinking Žilavka wine in the 14th century (Vuksanović and Kovačina 1984). In the 19th century, due to the grape quality and, particularly, to its resistance to bunch rot (*Botrytis cinerea* Pers.), the Austro-Hungarians exploited this cultivar by the production of a special dessert wine of the *Malaga* type (Mijatović 1988). Apart from its historic importance and long tradition, Žilavka wine has high economic potential for Bosnia and Herzegovina, since it is exported and is popular in neighboring countries. It is also grown in adjacent regions of Croatia, Macedonia, Montenegro and Serbia. Vuksanović and Kovačina (1984), Mijatović (1988), Tarailo (1991) and Cindrić *et al*. (2000) have provided an ampelographic description of this cultivar, which is characterized by high morphological heterogeneity of various traits: the shape, size and compactness of the cluster, the color, shape and size of the berry, vegetative potential, grape and wine quality, and resistance to bunch rot (*Botrytis cinerea* Pers.). In 1978 and 1991, clonal selection was performed at various locations and different types of 'Žilavka' were mentioned. Tarailo (1991), who described the clonal selection of 'Žilavka', also reported four different 'Žilavka' types and claimed that they are different genotypes. 'Žilavka' heterogeneity might be a result of ecological/growth factors or genetic mutations. Many authors designate 'Žilavka' as an autochthonous cultivar to Herzegovina (Cindrić *et al.* 2000; Milosavljević 1998; Burić 1985; Vuksanović and Kovačina 1984; Licul and Premužić 1979; Tarailo *et al.* 1978; Avramov 1974), where it can be found under various synonymic names: 'Žilavka Mostarska' (Cindrić *et al.* 2000; Milosavljević 1998; Mijatović 1988), 'Žilavka Hercegovačka' (Cindrić *et al.* 2000; Avramov 1974), 'Žilavka Bijela' (Mijatović 1988; Avramov 1974), 'Žilavka Žutka' and 'Žilavka Zelenka' (Aničić *op cit*. Mijatović 1988). In our study, two different marker systems, microsatellites (SSR – short sequence repeats) and AFLP (amplified fragment length polymorphism), were applied for genetic analysis, thus providing a complementary tool to ampelographic descriptions. Microsatellites are among the most frequently used DNA markers for cultivar identification, revealing synonyms and homonyms and geographical origin, studying genetic relationships within large groups of cultivars and for clonal variability characterization. AFLP has been found to be an efficient and sensitive method for establishing genetic intra-varietal variability. A combination of SSR and AFLP molecular markers is used to obtain a more accurate and complete framework for genomic identity of different accessions within the cultivar (Vignani *et al.* 2002; Labra *et al.* 2001).

2 MATERIALS AND METHODS

2.1 Plant material and DNA extraction

Survey expeditions were organized to the region of Herzegovina as the traditional centre of 'Žilavka' cultivation. 'Žilavka' was collected from 5 different locations, which were inventoried according to the information received from local people. Four locations belong to the Mostar area (43º20'58"N; 17º48'45"E) and one is from the Trebinje area (42º42'40"N; 18º20'44"E). Three of them are

old vineyards established during the seventies, one is a collection vineyard and one is a modern vineyard. Fresh leaves were collected from each of 80 chosen accessions. Plant material of accessions with different characteristics and names ('Starinska Žilavka', 'Žilavka', 'Stara Žilavka', 'Žuta Žilavka', 'Zelena Žilavka') were included in the study (data available upon request).

Total genomic DNA was extracted from fresh leaf tissue by CTAB (cetyltrimethylammonium bromide) extraction buffer (Kump and Javornik 1996), re-suspended in TE buffer (1 M Tris-HCl, 0,5 M EDTA, pH 8.0) and stored at 4 ºC.

2.2 Microsatellite analysis

Eighty 'Žilavka' accessions were analyzed at 14 microsatellite (SSR) loci: VVS2 (Thomas and Scott 1993), VVMD5 and VVMD6 (Bowers *et al.* 1996), VVMD24 24, VVMD25 25 and VVMD27 27 (Bowers *et al.* 1999), VrZAG21, VrZAG47, VrZAG62, VrZAG64, VrZAG67 and VrZAG79 (Sefc *et al.* 1999), and Uch11 and Uch29 (Lefort *et al.* 2001). The PCR reaction in a total volume of 10 µl contained 20 ng of extracted DNA, 10 × PCR buffer (Fermentas), 0.2 mM dNTP's (Fermentas), 2 mM $MgCl_2$ (Fermentas), 0.5 µM of each primer and 0.5 U of *Taq* polymerase (Fermentas). One of each of the primer pairs was labeled with fluorescent Cy-5 dye. Amplification of SSR loci was done in a Whatman Biometra T-Gradient thermocycler with the following steps: hot start for 5 minutes at 95 ºC; 26-40 cycles of: denaturation at 94 ºC for 30-45 seconds, annealing at 50-56 ºC for 30-45 seconds and an extension step at 72 ºC for 90 seconds. Amplification of loci Uch11 and Uch29 was done by a tailing protocol using three different primers: 0.2 µM of each of unlabelled Uch primers and 0.075 µM of 18 bp M13 tail sequence attached to the forward primer for subsequent fluorescent labeling (5' TGTAAAACGACGGCCAGT '3). An optimized touchdown protocol was used for PCR of these two loci: initial denaturation of 94 ºC for 5 minutes: 5 cycles of 94 ºC for 45 seconds, 60 ºC for 30 seconds with a decrease of -1 ºC per cycle and 72 ºC for 1.5 minutes; followed by 25 cycles at the annealing temperature of 55 ºC. PCR reactions were completed at 72 ºC for 8 minutes (incubation). Amplified SSR fragments were denatured for 4 minutes at 95 ºC and separated on 6 % polyacrylamide gel electrophoresis containing 7 M urea and detected by an ALFexpress DNA automated sequencer (GE Healthcare). The allele sizes were analyzed with AlleleLocator version 1.03 software (Amersham Pharmacia Biotech, 1998). Alleles were precisely sized against ALFexpress sizer 50 - 500 bp (GE Healthcare) and by internal DNA standards of various sizes amplified from plasmid.

2.3 AFLP Analysis

Fifty-two 'Žilavka' accessions were analyzed by 6 different AFLP primer combinations: *Mse*I-CAA/*Pst*I-ACA, *Mse*I-CAA/*Pst*I-AAC, *Mse*I-CAT/*Pst*I-ACA, *Mse*I-AG/*Pst*I-AGA, *Mse*I-CT/*Pst*I-ACA and *Mse*I-AG/*Pst*I-ACA. Isolated DNA (250 ng) was digested by 2.5 U of *Pst*I and 2.5 U of *Mse*I restriction endonucleases (New England Biolabs) in a reaction volume of 40 µl at 37 ^{o}C for 3 h. After digestion, 5 pmol of *Pst* and 50 pmol of *Mse* double stranded adaptors were ligated (1 Weiss U of T4 DNA ligase) to the sticky ends of the genomic fragments in a final volume of 50 µl at 37 ^{o}C for 3 h. Adaptors were obtained by mixing equimolar amounts of primers *Pst*RI linker1 and *Pst*RI linker2 for *Pst* adapters and *Mse*I linker1 and *Mse*I linker2 for *Mse* adapters. The pre-amplification procedure was done in a total reaction volume of 50 µl containing: 5 µl of ligation reaction serving as template, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP's, 1.25 U *Taq* polymerase, 50 ng of both *Pst*I and *Mse*I primers with one selective nucleotide (*Pst*I+A and *Mse*I+C). Reactions were amplified in a Whatman Biometra T-Gradient thermocycler, repeating 20 cycles of: 94 ºC for 30 seconds, 56 ºC for 60 seconds and

72 ºC for 105 seconds. The PCR products from the pre-amplification step were diluted 10-fold and 5 µl of the dilution was used for selective amplification. Selective amplification was done by 15 ng of both *Pst*I and *Mse*I primers with two or three selective nucleotides at the 3' end and *Pst*I primers were Cy5 labeled at their 5' end to allow automated laser fluorescence analysis. Selective reactions were performed in a 10 µl PCR reaction mix with the same reaction components as for the pre-amplification step, except that 0.3 U of *Taq* polymerase was used. The reaction was amplified with an initial touch down protocol: 94 ºC for 30 seconds, 65 ºC for 30 seconds and 72 ºC for 60 seconds with a touchdown of - 0.7 ºC of annealing temperature per cycle, followed by 23 cycles at 94 ^{o}C 30 s, 56 ^{o}C 30 s and 72 ^{o}C 1 minutes, and the samples were immediately transferred onto ice. The amplification reaction was mixed with an equal volume (10 µl) of formamide loading buffer (5 mg dextrane blue in 1 ml of formamide), denaturated by heating to 94 ^{o}C for 4 minutes. Five µl of sample was loaded onto 5% polyacrilamide denaturating gel (5% acrylamide-bisacrylamide 19:1, 1×TBE, 7 M urea) and electrophoresed on an ALFexpress DNA automated sequencer (GE Healthcare). An external size standard ranging from 50 to 500 bp was run together with samples to allow further sizing of the fragments. AFLP bands ranging from 50 to 500 base pairs were analyzed using AlleleLocator version 1.03 software, (Amersham Pharmacia Biotech, 1998). In the first instance, all 80 accessions were included in AFLP testing but only 52 produced scorable fingerprints and were included in the AFLP analysis.

2.4 Data analysis

Microsatellite and AFLP amplified bands across all analyzed loci were scored by state as present and given numerical value 1 or absent 0, making a presence-absence matrix of bands. This matrix served as a basis for clustering and assessing overall similarity among the analyzed accessions. A similarity index was calculated from band sharing data of each pair of accessions using the Jaccard (1908) coefficient of similarity. A dendrogram was constructed from the matrix of pairwise distances using an unweighted pair group method (UPGMA) for clustering in the NTSYS-PC software package, version 1.80.

The obtained microsatellite profile of 'Žilavka' was further compared to 211 already genotyped cultivars in Europe in order to assess their genetic relationships.

For AFLP analysis, the percentage of polymorphism was calculated for each primer pair combination from the total number of amplified bands and the number of polymorphic bands.

3 RESULTS AND DISCUSSION

3.1 Microsatellite analysis

Eighty 'Žilavka' accessions were screened on 14 microsatellite loci. These analyses resulted in 4 different genotypes arising from mutations observed at 10 polymorphic loci (Tab. 1). Seventy-seven accessions had the same allelic profile across all 14 loci and could be considered as a standard genotype for 'Žilavka'. Three accessions (9/2, 10/2 and 11/2), which were thought to be 'Žilavka', resulted in polymorphism at 5 to 6 loci (Tab. 1) and could not be considered to be 'Žilavka'.

Table 1: Standard 'Žilavka' genotype profiled at 14 microsatellite loci with three distinguished genotypes and 'Chardonnay' as reference cultivar.

Genotype	'Žilavka'	9/2	10/2	11/2	'Chardonnay'
VVS2	132:152	n.a.	132:152	132:152	137:152
VVMD5	226:238	226:238	226:238	226:238	232:236
VVMD6	208:208	208:208	190[a]:208	208:208	198:208
VVMD24	208:208	208:208	208:208	208:208	208:216
VVMD25	240:242	250[a]:250[a]	n.a.	240:242	240:256
VVMD27	179:194	179:194	181[a]:194	179:194	n.a.
VrZAG21	201:203	201:206[b]	n.a.	201:206[b]	200:206
VrZAG47	158:173	158:173	158:173	158:173	160:168
VrZAG62	189:189	189:189	189:189	189:205[a]	188:196
VrZAG64	144:144	160[b]:164[b]	144:144	160[b]:164[b]	160:164
VrZAG67	151:161	141[b]:151	141[b]:141[b]	141[b]:151	140:153
VrZAG79	249:249	237[b]:249	242[a]:258[a]	237[b]:249	244:246
Uch11	235:235	235:235	235:235	235:249[a]	248:263
Uch29	208:295	208:295	295:306[a]	208:295	289:297

[a]*new alleles different from standard* 'Žilavka' *genotype,*
[b]*alleles shared between distinguished* 'Žilavka' *genotypes, n.a. no amplification*

However, detailed morphological description of these accessions should be performed for clear definition. Grapevine cultivars are vegetatively propagated and individuals of one cultivar are expected to be genetically identical to each other, but some cultivars may comprise several different genotypes due to a polyclonal origin (Kozjak *et al*. 2003) or somatic mutations can be accumulated over years of clonal propagation.

Comparison of standard 'Žilavka' from our analyses with 'Žilavka' from Croatia, genotyped by Maletić *et al*. (1999) resulted in the same allelic profiles at all 7 comparable loci, which confirmed the trueness to type of the 'Žilavka' genotype.

The standard 'Žilavka' genotype was further compared to 211 cultivars, from Slovenia (49), Austria/Germany (20), France (13), Portugal (27), Croatia (19), Greece (32), Spain (21) and Italy (30) in order to assess their genetic relationships. Comparison was enabled by using one reference cultivar, 'Chardonnay', for allele size standardization. The proportion of shared alleles was used as the basis for distance measurement among analyzed varieties. In pairwise comparisons of 'Žilavka' with cultivars from the various European regions, the highest genetic similarity was found with a Slovenian cultivar 'Glera' (64 %) and the highest genetic dissimilarity (100 %) with two Italian cultivars, 'Nebbiolo Lampia' and 'Vespolina'. A phenogram of all 211 genotypes was constructed (data available on request) in order to illustrate the genetic relationships between 'Žilavka' and other European cultivars. 'Žilavka' clustered together with Slovenian old cultivars 'Glera', 'Briška Glera', 'Vitovska', 'Zunek', 'Duranja' and 'Popetre'.

3.2 AFLP analysis

Fifty-two accessions of standard 'Žilavka' genotype analyzed at 14 microsatellite loci were included in further AFLP analysis. Six primer pair combinations generated 163 scorable bands, of which 87 (57%) were polymorphic, expressing intra-varietal variability (Tab. 2).

Table 2: Characteristics of analyzed AFLP loci expressed by number of amplified bands, polymorphic bands and % of polymorphism.

Primer pair combination	Number of amplified bands	Number of polymorphic bands	Polymorphism (%)
*Mse*I-CAA / *Pst*I-ACA	15	13	87
*Mse*I-CAA / *Pst*I-AAC	20	18	90
*Mse*I-CAT / *Pst*I-ACA	13	5	38
*Mse*I-AG / *Pst*I-AGA	44	27	61
*Mse*I-CT / *Pst*I-ACA	36	11	31
*Mse*I-AG / *Pst*I-ACA	35	13	37
Total	**163**	**87**	**57 (Average)**

The degree of polymorphism was higher than reported previously for some cultivars, e.g., 'Carmenere' (2.3%) (Moncada and Hinrichsen 2007), 'Cabernet Sauvignon' (5.9%) (Moncada *et al*., 2005) or 'Traminer' (34%) (Imazio *et al.* 2002). The high level of detected polymorphism might be explained by the accumulation of somatic mutations over years of cultivation. 'Žilavka' was spontaneously propagated for many years without formal selection of clones, which might also have resulted in higher heterogeneity within the cultivar.

Based on AFLP data, the 52 'Žilavka' accessions revealed 35 genotypes, which are clustered on the dendrogram (Fig. 1).

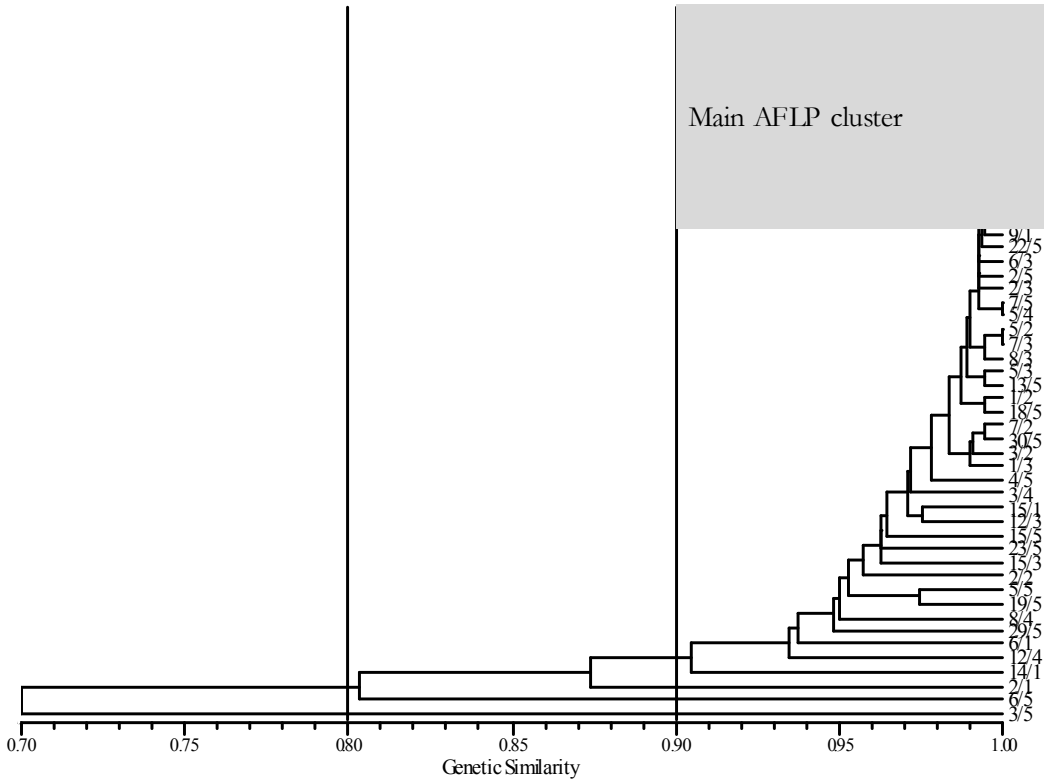

Figure 1: Dendrogram showing genetic similarity among 'Žilavka' accessions generated from AFLP data

The clustering of accessions did not correlate with their names, characteristics or sampling locations. The main AFLP cluster consists of 16 non-differentiated accessions of the

standard 'Žilavka' genotype, which had previously been differentiated by their different names, characteristics or sampling locations (data available upon request), which means that AFLP analysis revealed no type specific marker.

Three out of 52 accessions had an AFLP genetic similarity lower than 0.90 (33/5, 6/5 and 2/1). On the basis of some reports (e.g., Cervera *et al.* 2000, 1998), accessions showing similarities > 0.90 can be considered to belong to the same cultivar, while the cultivars that are different show similarities between 0.65 - 0.90). These three accessions all belong to the standard 'Žilavka' genotype according to the SSR analysis, but further detailed morphological analyses should be done before reaching any final conclusions.

4 DISCUSSION

Identification and distinction of 'Žilavka' accessions was possible on the basis of two marker systems. Microsatellite markers enabled determination of a standard 'Žilavka' genotype and, together with AFLP markers, revealed intra-varietal polymorphism. The two molecular markers, SSR and AFLP, explore different parts of the genome and have a different structure in terms of primer sequences and amplified motifs, so they are both valuable for obtaining reliable results on accession variability.

Molecular analysis has shown that 'Žilavka' is not a highly homogenous cultivar (high polymorphism is observed), which can be explained by non-formal clonal selection of Žilavka in Bosnia and Herzegovina and also by the long cultivation period, resulting in an accumulation of mutations. Microsatellite genotyping enabled us to exclude the accessions that are different from the true-to-type 'Žilavka', since it is known that different cultivars show at least four allelic differences, while clones show fewer differences but can also be distinguished in some cases by SSR analysis (Laucou *et al*. 2011). In our study, 80 different accessions of 'Žilavka' were sampled from 5 locations (data available on request) but molecular analysis revealed no clustering of 'Žilavka' accessions according to the observed characteristics, names or locations. Detailed research on ampelographic data needs to be performed to explain the high AFLP polymorphism obtained for three accessions (33/5, 6/5 and 2/1) and to confirm their different origin. The remaining accessions analysed with AFLP markers expressed lower AFLP polymorphism (< 0.90) explaining their intra-varietal variability and true-to-type identity, which was also confirmed by SSR genotyping.

On the basis of the results obtained by molecular analysis, we can clearly distinguish accessions genotypically different from the true-to-type 'Žilavka', which is a step toward cultivar identification, standardization and collection.

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