Armenian national grapevine collection: Conservation, characterization and prospects

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Abstract. The general strategy for grapevine genetic resources conservation in Armenia encompasses the collection of the still existing diversity and the use of protection techniques to minimize the losses over time. Being studied mainly by ampelography, the genetic diversity of Armenian grapevine needs to be re-investigated in accordance with modern requirements and international scales. The purpose of the presented research was the first large-scale molecular characterization of Armenian grape varieties by molecular methods using a set of 24 simple sequence repeat (SSR) markers encompassing the nine SSR markers recommended by the European project GrapeGen06. The obtained results indicate the uniqueness of the major part of the investigated varieties and reveal a substantial level of genetic variation within the Armenian grapevine. Based on the realized large-scale investigation a true-to-type inventory of Armenian grape germplasm will be realized and documented in the *Vitis* International Variety Catalogue and in the European *Vitis* database. The next step having strategic importance in terms of conservation of grape genetic resources in Armenia will be establishment of the first Armenian *Vitis* database with multi-crop passport description of all varieties preserved in grape collection.

1. Introduction

With long-standing history as unique grapevine diversity "hotspot" Armenia is a homeland for cultivated and wild grapes. The world's earliest known wine-making facility has been discovered during the excavation of the Areni-1 cave in 2007 dating back to 6200 years (the beginning of the IV Millennium BC), confirmed by archaechemical analyses [1]. Besides centuries of tradition in viticulture and winemaking Armenia possesses a high diversity of local indigenous and modern cultivars and wild grape populations. Armenian grape varieties evolved during thousands of years via human selection and their spectrum was further enriched by hybridization. Viticulture is a basic sector of Armenian agriculture and the production of brandy and wine is one of main branches of its export.

The first Armenian National Grapevine Collection, established at the Institute of Viticulture, Fruit-Growing and Wine-Making in 1950 (about 850 varieties, area 22 ha) was entirely eradicated after the USSR collapse in the early 1990ies [2]. In the following years three new ampelographic collections were formed, preserving 140 accessions, from which only 70 were native Armenian varieties. Due to various reasons, the conservation of Armenian grapevine germplasm in these three collections was also stopped. All these factors increased the risk of loosing endangered genetic diversity of Armenian grapevines. In this context, the establishment of the new grapevine collection is of crucial importance. To prevent genetic erosion increased efforts to collect and conserve old autochthonous, endangered and wild genotypes, as a reservoir of genotypes for future crop improvement are indispensable. Established in 2016, the Armenian National Grapevine Collection in Etchmiadzin, represents up to 80% of indigenous varieties. The rescue and preservation of grapevine genetic diversity in Armenia is particularly urgent for many reasons: i) the large number of traditional local varieties no longer cultivated, respectively existing as single vines il old lots, ii) the relevance of these resources for the development of cultivars iii) the occurrence of the grapevine's wild ancestor, *Vitis sylvestris* and iv) grape and wine production as a priority branch of economy and important source of income for the local population.

Political changes and economic decisions have endangered both the wild habitat of *Vitis sylvestris* and germplasm collections. A key example of such a loss, especially for Armenia, was the collapse of the Soviet Union and subsequently lack of fundling for the most important collections of grapevine and other perennial fruits. Nevertheless, Armenia possesses both,

significant wild grapevine populations and a rich panel of old autochthonous cultivars. However, intensive cultivation of a small number of commercial cultivars has resulted in an alarming reduction in genetic variability. Less known traditional cultivars are under-exploited. Many have only a local significance in the different wine-growing regions The only way to safeguard this heritage is via broad prospections in old vineyards by skilled people and its preservation in germplasm repositories followed by characterization, identification and evaluation of the agronomic features. The preservation of this rich diversity is very important for breeding of new cultivars and for future wine growing generations. The knowledge of genetic diversity and relationships among grapevine cultivars is important to recognize gene pools. The development of effective conservation and management strategies are needed.

The management of germplasm collections is a complex task that requires considerable technical, agronomic and scientific efforts. The major objectives are to maintain the accessions in good vegetative and productive conditions in order to ensure their long-term conservation, to ascertain trueness to type to provide reliable material for research, breeding, viticulture and exchange of germplasm. The first steps in the management of a collection are the careful documentation and characterization of each accession, which should be done by using the "Multi Crop Passport Descriptors" (MCPD) of Bioversity. MCPD-data provide basic information about the accession, including the accession name, the accession number, which is a unique code assigned by the curator of the collection, the berry colour, the provenance, donor, *etc*. The traditional method for the identification of grape cultivars is ampelography, which is mainly based on description and comparison of morphological characteristics of shoot tips, shoots, leaves and bunches. It is an accurate and reliable method, but it requires experienced staff. For a long time in Armenia ampelography was the only method used to identify cultivars. However, methods based on DNA analysis are now applied in the country. In particular the use of the nine microsatellite markers, recommended as the outcome of the European project GrapeGen06, proved to be particularly suitable for the comparison of allelic data with different international collections [3,4].

At present a comprehensive ampelographic and genetic investigation of the accessions conserved in Armenian National Grapevine Collection in Etchmiadzin is carried out. This is part of a bilateral project between the Institute of Molecular biology NAS RA and Julius Kühn-Institut (JKI), Institute for Grapevine Breeding Geilweilerhof. The objective is the discovery of breeding potential of Armenian grape germplasm by multidisciplinary characterization, aiming to conserve indigenous, rare and neglected grapevines and wild species, in order to increase knowledge about local genotypes and to preserve Armenian grape biodiversity.

The presented research is the first large-scale molecular characterization of Armenian grape germplasm using set of 24 simple sequence repeat (SSR) markers encompassing the nine SSR markers recommended by the European project GrapeGen06. After establishment of the Armenian National Grapevine Collection in 2016, thanks to the activity of researchers involved in the Armenian-German joint project and specialists from Vine and Wine Foundation of Armenia (VWFA), in total 293 accessions were introduced. They are due to abundant recent prospections carried out in the wine growing regions throughout the country. Thus the repository encompasses grape varieties grown since ancient times. Besides major wine and table varieties minor varieties, of local importance, grown especially in private and very old vineyards, as well as neglected local varieties, at risk of extinction were collected and conserved in the collection.

2. Material and methods

2.1. Plant material

In the scope of the here presented research 293 grapevine accessions preserved in the Armenian National Grape collection (Scientific Center of Agriculture, MA RA, Institute code: ARM006) were analyzed. Biological material includes mainly indigenous rare and neglected varieties, as well as interspecific and intraspecific hybrids and wild species. Two cultivars "Cabernet franc" and "Muscat a petits grains blancs" were used as references.

2.2. DNA extraction and SSR analysis

Genomic DNA was extracted from 100 mg of young leaf using peqGOLD Plant Mini Kit according to the manufacturer's protocol (peqLab, Germany). DNA concentration and quality were checked by spectrophotometric analysis and electrophoresis in 1% agarose gel. Microsatellite fingerprintings of grapevine varieties and wild accessions were performed on 24 microsatellite markers (nSSRs) well distributed across the nineteen grape chromosomes as previously described [5], two of the VMC series (VMC1b11, VMC4f3; *Vitis* Microsatellite Consortium [6], nine of the VVI series (VVIb01, VVIn16, VVIh54, VVIn73, VVIp31, VVIp60, VVIv37, VVIv67, VVIq52 [7], eight of the VVMD series (VVMD5, VVMD7, VVMD21, VVMD24, VVMD25, VVMD28, VVMD27, VVMD32 [8,9], VVS2 [10,11], VrZAG62, VrZAG79 [12], VrZAG67 and VrZAG83. Nine polymorphic microsatellite markers proposed by the GrapeGen06 (`http://www.montpellier.inra.fr/grapegen06`) project: VVMD5, VVMD7, VVMD25, VVMD27, VVMD 28, VVMD32, VVS2, VrZAG62 and VrZAG79 were used for comparison of genetic profiles with the SSR-marker database of the Julius Kühn-Institut (JKI), maintaining about 15.000 genetic profiles of the here cited markers [3]. Fifteen additional markers were analyzed for parent offspring analysis [13]. For fragment length determination by capillary electrophoresis on ABI 3130xl Genetic Analyzer (Applied Biosystems, Germany), all forward primers were 5° – labeled with a fluorescent dye (FAM, HEX, TAMRA or ROX). The combination of markers with different labels and diverse fragment lengths allows to perform the polymerase chain reaction (PCR) as multiplexes of up to 6 markers. The 2x KAPA2G Fast PCR Kit (Germany) was used to set up 5µl reaction mixtures containing master mix, 100pmol of each primer and 1 ng of template DNA. GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Germany) was used for the amplification starting with 3 min initial denaturation at 95 °C, followed by 30 cycles with denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s and extension at 72 °C

Table 1. Summary of identification results by comparison of genetic fingerprints.

Identification	**True variety names (*V*IVC)**		**Questionable genotypes**
JKI-SSR-marker-database	*Matching genetic profiles*	*Unknown genetic profiles, but matching previously analyzed Armenian varieties*	*Unknown genetic profiles*
No of accessions	151	69	73
No of prime names (VIVC) respectively of distinct genotypes	72	40	58
Genotypes in bibliographical references	65	29	
Varieties / sampling names; examples	Ararati Areni sev Dzhandzhal kara Garan dmak Kakhet Mskhali Voskeat	Aldara Artashati karmir Hakobi Vordi Khardji sev Shireyi Gevorg Tigrani Vanki	Babayan 5 Hayastani quyr 60-2/5, F, S Adelin sev

for 30 s. A final extension was performed at 72 °C for 7 min. 1 µl of the PCR product was used for fragment length determination and the results were processed with GeneMapper 4.0 software (Applied Biosystems, Germany).

Data analysis. The number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He) and polymorphism information content (PIC) were calculated for 291 Armenian grapevine varieties using Cervus software version 3.0.7 [14]) and GenAlEx softwares.

3. Results and discussion

In the scope of Armenian-German bilateral project the large-scale molecular characterization of 293 accessions conserved in the Armenian National Grapevine Collection was realized. The genetic diversity of grape varieties was analyzed by 24 simple sequence repeat markers encompassing the nine SSR markers recommended by the European project GrapeGen06.

The determination of the 293 accessions' identity requires a combination of molecular data and morphological features. Molecular analysis of Armenian grape varieties revealed the following three main cases: *synonyms:* different cultivar names, but identical fingerprints, *homonyms*: identical or very similar cultivar names, but different fingerprints, *questionables:* for some cases the variety, being true-to-type on the basis of ampelographic descriptors, turned out to be critical after comparison of their SSR profiles; or obvious differences between morphological descriptions in bibliography and the accessions features in the collection were detected.

The SSR profiles comparison based on *Vitis* International Variety Catalogue (*V*IVC) (`http://www.vivc.de/`) database assisted to determine accessions identities and provided in some cases unexpected information. Unique profiles, additional synonyms and homonyms also were identified. The summary of the identification results is presented in Table 1.

On the basis of the realized molecular analysis it turned out that 170 distinct genotypes are maintained in the collection and 123 accessions revealed to be duplicates. Regarding the identification of 220 (151 + 69) accessions, corresponding to 112 (72 + 40) distinct genotypes a matching profile existed in the *V*IVC database. The genetic profiles of the 151 accessions represent 72 distinct varieties. Some of them are listed in the second column of Table 1. These are well known Armenian grapevine varieties like Garan dmak, Mskhali, Voskeat, Ararati, Kakhet and unexpected ones like Dzhandzhal kara form Uzbekistan. The former importance of Areni sev is reflected by the 22 accessions discovered in various regions under different names. Some of them even were considered distinct cultivars mainly because of variation in cluster architecture.

In the scope of already realized projects (COST Action FA1003, DAAD) accessions from the previous collection were genotyped and thus further genetic profiles from Armenian varieties were available. Owing to these activities further 69 accessions could be assigned, corresponding to 40 cultivars. Some typical Armenian varieties are listed at the bottom of the third column in Table 1. During Soviet Union times Armenian grape breeders were actively creating new table and wine grape varieties. In the scope of the prospection some of them were discovered as well. Although no matching profile was found and descriptive references were lacking, their identity was considered as true to type if the parentage given by the breeders was corresponding.

Genetic identification is followed by ampelographic confirmation to ascertain the true identity of the variety and to ensure that no errors occurred during leaf sampling. Owing to the fact that no living references are available, morphological features of the varieties need to be compared with descriptions and photographs available in bibliography. Three toms of Armenian (published in 1947; 1962; 1981), three toms of Russian (published in 1946–1956; 1963–1970; 1984) ampelographies and the Caucasus and Northern Black Sea Region Ampelography (2012) are the most valuable sources for this purpose. In these books 65 varieties of the 72 matching genetic profiles and 29 from the 40 unknown genetic profiles are described, see Table 1. In 2018 the comparison of the accessions morphological features with descriptions and photos given in ampelographies started.

Table 2. Descriptive statistics and genetic diversity of 293 Armenian grape genotypes at 24 microsatellite loci.

Locus	Ra (*bp*)	Na	Ho	He	PIC
VVS2	123–155	15	0.808	0.879	0.866
VVMD5	226–266	12	0.849	0.848	0.829
VVMD7	233–265	15	0.785	0.827	0.803
VVMD25	237–269	14	0.789	0.797	0.764
VVMD27	176–198	11	0.800	0.768	0.729
VVMD28	218–282	23	0.778	0.820	0.805
VVMD32	240–292	18	0.785	0.775	0.755
VrZAG62	182–206	13	0.872	0.835	0.815
VrZAG79	237–261	12	0.858	0.848	0.829
VVIv67	338–391	26	0.587	0.710	0.694
VrZAG67	121–161	19	0.907	0.889	0.877
VrZAG83	180–201	7	0.628	0.643	0.575
VVIn16	147–157	6	0.605	0.652	0.605
VVIn73	258–272	7	0.509	0.468	0.436
VVIp60	306–332	14	0.552	0.697	0.648
VVMD24	200–220	13	0.781	0.766	0.734
VVMD21	239–267	12	0.691	0.748	0.706
VMC4f3	161–216	33	0.952	0.930	0.924
VVIb01	285–311	10	0.690	0.663	0.609
VVIh54	139–177	14	0.546	0.787	0.764
VVIq52	70–86	10	0.834	0.720	0.670
VVIv37	146–180	19	0.900	0.888	0.876
VMC1b11	167–205	16	0.845	0.797	0.771
VVIp31	163–195	17	0.966	0.891	0.879
Mean		*14.83*	*0.763*	*0.776*	0.748
Total		*356*			

Ra: range of allele size (bp), Na: number of alleles, Ho: observed heterozygosity, He: expected heterozygosity, PIC: Polymorphism information content.

The following example illustrates the procedure: Farmers transmitted three different designations for one and the same cultivar, because Sali, Karch mat and Tozot displayed the same genetic fingerprint. Each of them is described as a distinct cultivar. The three accessions were investigated in the field collection. They showed matching morphological features. After thorough examination it was concluded that it is most likely Tozot. Ampelographic determination of accessions/varieties identity in the collection will be continued in 2019.

Questionable genotypes exhibit unique genetic profiles. For 73 accessions no information could be given by the owners of the plants. For the time being, until the true name of the variety is found, it is planned equip the unknown sample with an appropriate designation and to gather the following descriptive material: i) appropriate name, ii) MCPD-data, iii) preliminary description, iv) herbarium, v) photos (shoot tip, leaves, bunches), vi) genetic profile.

The results obtained in the scope of the proposed project are proving the effectiveness of genotyping as a reliable and convenient tool for supporting ampelography in the correct identification of varieties maintained in the germplasm collection. By application of these approach similarities among accessions from Armenian collection and the varieties cultivated in other, mainly in post-Soviet Union countries were found and incorrect records of the accessions were discovered. Using molecular fingerprinting in our research we were able to document inconsistencies and inaccuracies in ampelographic descriptions, which is prerequisite for complete and precious description of all varieties preserved in germplasm collection.

Assessment of genetic diversity of Armenian grape germplasm by application of SSR markers: genetic diversity of the Armenian grape accessions was analyzed for nuclear microsatellites by estimating the range of allele size (*Ra,* bp), average number of observed alleles per locus (*Na*), observed heterozygosity (*Ho*), estimated heterozyosity (*He*) and polymorphism information content (*PIC*). Statistics about the discriminatory efficiency of the used 24 SSRs markers are presented in Table 2.

The high degree of observed genetic variability among the 293 accessions is proven by the high number of different alleles (356). The number of alleles per SSR locus ranged from 6 (VVIn16) to 33 (VMC4f3) and the mean allele number per locus was 14.83. For microsatellite markers efficiency were considered observed and expected heterozygosity (*Ho, He*) to evaluate the genetic variability among analysed grapevine. The observed and expected heterozygosity values were relatively high, with average at 0.763 and 0.776 accordingly. From the results obtained in the analyzed accessions the mean value of *Ho* was slightly lower than the *He*, which can indicate probable inbreeding, and in case of Armenian grape germplasm, fact of common origin and clonal propagation among the analyzed varieties. However as it is shown in Table 2, for the 14 loci from 24 (VVMD5, VVMD27, VVMD32 VRZAG62, VRZAG79, VrZAG67, VVIn73, VVMD24, VMC4f3, VVIb01, VVIq52, VVIv37, VMC1b11, VVIp31) analyzed the *Ho* was higher than *He,* ranged from 0.509 (VVIn73) to 0.966 (VVIp31) accordingly. High levels of heterozygosity are widely found among clonally propagated and outbreeding, perennial species including *V. vinifera* [15,16]. As an outbreeding species, grapevine possesses considerably heterozygous cultivars affected from severe inbreeding depression [17]. Obtained results demonstrated, that the expected heterozygosity (*He*) among analyzed varieties varied within a range between 0.4683 (VVIn73) and 0.930 (VMC4f3) indicating a high level of genetic diversity within the studied germplasm.

The value of polymorphism information content (*PIC*) estimates the usefulness of each microsatellite marker for reliable distinction. The calculated PIC values ranged from 0.436 (VVIn73) to 0.924 (VMC4f3) and classified the seven loci (VVIp31, VrZAG67, VVIv37, VVS2, VVMD5, VrZAG79 and VrZAG62) as highly informative markers. For cultivar distinction the value of estimated *PIC* is as indicator of SSRs effectiveness [18].

4. Conclusions

The comprehensive characterization of grape varieties implies ampelographic means in combination with molecular analysis. These tools ensure accurate identification.

In the scope of the present research the applied SSR markers proved to be beneficial for starting the sorting out of the new established Armenian grapevine collection at Etchmiadzin, e.g. adding synonyms, homonyms, clarification of questionable cases and identification of unique genotypes. Comparison of SSR fingerprints with genetic profiles in the *Vitis* International Variety Catalogue database revealed several unexpected identifications. The present study is a first step towards the genetic and ampelographic characterization of the Armenian grapevine germplasm. The obtained results indicate the uniqueness

of the major part of the investigated varieties and reveal a substantial level of genetic variation within the Armenian grapevine genepool. Based on the realized large scale investigation a true-to-type inventory of Armenian grape germplasm already started and will be documented in the *Vitis* International Variety Catalogue and in the European *Vitis* database. It should be underlined, that based on the results of the realized cooperative project between the Institute of Molecular Biology of NAS RA and the Institute of Grapevine Breeding, Geilweilerhof, by support of VWFA for the first time an Armenian *Vitis* database will be established. Data with entire ampelographic and molecular descriptions of plant material will be made available. Authenticity will have strategic importance in terms of conservation of grape genetic resources in Armenia and will be essential for improving the knowledge on Armenian grape germplasm.

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