Mol Biotechnol (2014) 56:1011–1020
DOI 10.1007/s12033-014-9780-y

RESEARCH

Use of SSR and Retrotransposon-Based Markers to Interpret the Population Structure of Native Grapevines from Southern Italy

Clizia Villano · Domenico Carputo · Luigi Frusciante · Xenia Santoro · Riccardo Aversano

Published online: 28 June 2014

Abstract Native grapevines are the quintessential elements of Southern Italy winemaking, and genomic characterization plays a role of primary importance for preservation and sustainable use of these unexploited genetic resources. Among the various molecular techniques available, SSR and retrotransposons-based markers result to be the most valuable for cultivars and biotypes distinctiveness. A total of 62 accessions including 38 local grape cultivars were analyzed with 30 SSR, four REMAP and one IRAP markers to assess their genetic diversity and obtain a complete genomic profiling. The use of VrZAG79, VrZAG112, VVS2, VVMD25 and VVMD5 combined with retrotransposon-based markers proved to be the most discriminating and polymorphic markers for the rapid and unambiguous identification of minority grapevines from Campania region, which is considered one of the most appreciated Italian districts for wine production. Results revealed 58 SSR marker-specific alleles, 22 genotype-specific SSR alleles, and four REMAP and IRAP private bands. Cases of synonymy and homonymy were discovered. In conclusion, we provided evidences that the integrating SSR and retrotransposon-based markers is an effective strategy to assess the genetic diversity of autochthonous grapes, allowing their easy identification.

Keywords *Vitis vinifera* · REMAP · IRAP · Campania · Fingerprinting · Private alleles · Molecular markers

Introduction

Cultivated grapevine, *Vitis vinifera* subsp. *vinifera* L. ($2n = 6x = 38$), is one of the major fruit crops worldwide, in terms of economic value and cultivated area. Literal, archeological, and paleo-botanical resources have essential tools to understand the spreading of viticulture in Europe and in particular in Italy and France, starting from the Caucasian area [1]. Nowadays, more than 450 varieties are registered in Italy [2], with some grapevines being cultivated only in Campania region (Southern Italy). These grapes are proudly used to make some of the world's finest wines, thanks not only to history, traditions, and excellent pedo-climatic conditions but also to a particularly heterogeneous collection of varieties, some of which have been growing here since the first half of the 19th century. This biodiversity includes registered varieties, ancient grapes, and autochthonous biotypes carefully preserved from genetic erosion. In this large panorama of genotypes, an efficient characterization system is important to avoid cases of synonymy (identical genotypes but different names) and homonymy (same names but different genotypes), to definite population structures, trace plant products, and protect breeders' rights. The oldest grape discrimination techniques, ampelography and ampelometry, are based on phenological traits analysis carried out during all vegetative cycle. In grape, these techniques can result elaborate and time consuming. It has been reported that morphological characterization is often inaccurate for the discrimination of closely related cultivars because of confounding environmental and developmental factors and

Electronic supplementary material The online version of this article (doi:10.1007/s12033-014-9780-y) contains supplementary material, which is available to authorized users.

C. Villano · D. Carputo · L. Frusciante · X. Santoro · R. Aversano (✉)
Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, 80055 Portici, Italy
e-mail: raversan@unina.it

control by epistatic and pleiotropic gene effects [3, 4]. In contrast, DNA-based molecular markers can overcome many of the limitations of phenotypic-based diversity analysis and provide a more direct measure of genetic diversity [5]. Among the most interesting markers, there are microsatellites (simple sequence repeats, SSR), popular for their reproducibility, co-dominant nature, polymorphism, hyper-variability, and high cross-species transferability [6]. They have been adopted as the standard markers for germplasm management in many plant species [7]. Also in grape, they have become markers of choice for compilation, standardization, and exchange of information concerning germplasm collections [6, 8–10]. In this contest, Laucou and collaborators [11], analyzing 4.370 grape accessions, defined a minimal set of nine SSR primers which proved to be discriminatory with a reduced probability of false identity. These primers have been already successfully employed by Bacilieri [12] to genotype several *V. vinifera* accessions, supporting archeological and historical data on grapevine domestication in Europe and Asia. Bergamini et al. [13], amplifying the same primers in over 2000 accessions of the Italian grapevine "Sangiovese", demonstrated the presence of an ancient variety ("Negrodolce", believed to have been lost) in its pedigree. More precise information can be provided combining SSR with other molecular markers [12]. In this regard, retrotransposon-based markers can be particularly interesting in that their use, combined with SSR, would allow the coverage of both inter- and intragenic DNA [11, 14, 15]. Retrotransposons are characterized by their capability to translocate and change their genomic location, whereby they generate transpositional polymorphism. They consist in elements, hundreds to thousands of nucleotides long. The long terminal repeats (LTRs) that bind a complete retrotransposon contain ends that are highly conserved in a given family of elements [16]. In grape, retrotransposons seem suitable additional markers also for their abundance in the genome [17], their presence in many copies [7], and their ability to track an insertion event and its subsequent vertical radiation through either pedigree or phylogeny analysis [18]. REMAP (REtrotransposon-Microsatellite Amplified Polymorphism) and IRAP (Inter-Retrotransposon Amplified Polymorphism) markers are cheap to establish and assay, easy to perform and reproduce [19]. In *V. vinifera* three retro-elements are known, *Gret1*, *Vine-1*, and *Tvv1* [20, 21]. Currently, only *Gret1* has been fully sequenced, and it is known to be associated with mutations causing most white-fruited *V. vinifera* genotypes due to its insertion into the promoter of *VvMybA1*, the transcription factor controlling the final step in anthocyanin biosynthesis during ripening [20, 22]. Pereira et al. [14] demonstrated that *Gret1* is useful as molecular marker, and it may play an important role in the expression of phenotypes that characterize a cultivar. REMAP has been used for genetic diversity assessment of various crops such as rice [23], wheat [24], and banana [25, 26]. IRAP has been employed in germplasm studies in barley [27], tobacco [28], and grape [29].

In light of the wealth of grape biodiversity and the availability of efficient marker systems, the main objective of the present study was to characterize through SSR and retrotransposon-based markers grape varieties grown in Campania. We have estimated several parameters of DNA diversity useful for clarifying genetic relationships and provided genomic molecular tools for germplasm protection and utilization.

Materials and Methods

Plant Material

Sixty-two grape cultivars were sampled from seven producing areas of Campania region. Details on the plant materials used are reported in Supplemental Table 1.

Microsatellite Analysis

Total genomic DNA was extracted from young leaves using the Qiagen Plant DNeasy Maxi Kit (Qiagen, Valencia, CA, USA), following the manufacturer's procedure. Microsatellite analysis was carried out with 20 nuclear and 10 chloroplast markers. They were chosen from six sources: eight (VVMD7, VVlb01, Vvlh54, Vvln16, VVIp60, VVIq52, VVMD25, and VVMD5) from Laucou et al. [11], eight (VrZAG series) from Sefc et al. [30], three (VVS series) from Thomas et al. [31], nine (CCMP series) from Weising et al. [32], VVIc05 from Merdinoglu et al. [33], and ccSSR5 from Chung et al. [34]. All SSRs characteristics are reported in Supplemental Table 2. PCR reactions were performed in a 20-μL volume containing 1× reaction buffer with 1.6 mM $MgCl_2$, 0.2 mM of each dNTP, 30 pM FAM-labeled forward SSR primer and reverse SSR primer, 1 unit of go*Taq* polymerase (Promega, Madison, WI, USA), and 30 ng of genomic DNA. Amplification consisted of 5 cycles at 94 °C for 45 s, Ta °C + 5 for 60 s, 72 °C for 30 s; 30 cycles at 94 °C for 45 s, Ta °C for 60 s, and 72 °C for 30 s with the annealing temperature reduced by 1 °C per cycle (touchdown PCR), then one elongation cycle at 72 °C for 20 min. Amplicons were separated with the ABI PRISM® 3130 DNA Analyzer system (Life Technologies, Carlsbad, California, USA). Size calibration was performed with the molecular weight ladder GenScan® 500 ROX™ Size Standard (Life Technologies, Carlsbad, California, USA). SSR alleles were detected and scored using Peak Scanner® software (Applied Biosystems, Foster

City, California, USA). The results validation was carried out with two biological replications, and PCR reactions were performed in triplicate. The alleles sizes were normalized using SSR data reported in the Vitis International Variety Catalog [35].

Retrotransposon-Based Genotyping

For REMAP and IRAP marker amplification, four primers were chosen: two were designed on the *Gret1* LTR regions, (5′-LTR: 5′-CGAGTTTGTGTAGATTACAC-3′, and 3′-LTR: 5′- GCATTTAGAAGGATTTAGCTT-3′) and two on microsatellite repeats (Microsat-GA [$(GA)_9$ C] and Microsat-CT [$(CT)_9$G]). We set up four REMAP markers combining LTR primers with microsatellite primers and one IRAP marker amplifying with both LTR primers as described in Pereira et al. [14]. PCRs were performed in a 20 μL reaction mixture containing 20 ng of genomic DNA as previously reported [36]. PCR products were separated in a 2 % agarose gel electrophoresis at 50 V for 30 min, 70 V for 30 min, and 100 V for 3 h. Bands were detected by GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, California, USA). For image acquisition and identification of band size, the Quantity One® 1-D Analysis Software (Bio-Rad Laboratories, Hercules, California, USA) was used. Results were confirmed with three technical replicates.

Data Analysis

The statistical software GenAlex 6.5 [37] was used for data analysis. In the REMAP and IRAP marker (dominant markers), the input file was created considering each band as one diallelic locus (1 means presence of band, 0 means absence of band); in the SSR analysis, each fluorescent peak obtained from capillary electrophoresis was treated as a unit character (allele) for its respective locus, as previously reported [38]. To estimate the degree of polymorphism, the number of alleles per locus for single-locus markers (SSR) and the number of bands per primer combination for multi-locus markers (REMAP and IRAP) were calculated. The statistical parameters useful for genetic diversity analysis were the expected heterozygosity ($He = 1 - \sum pi^2$, where pi is the frequency of the ith allele) [39], the fixation index ($F = 1/1 - Ho/He$) [40], the Shannon's Informative Index ($I = -1 * Sum\ (pi * Ln\ (pi))$ [41], the Power of Discrimination ($PD = 1 - \sum C$, where $C = pi * \{[(N*pi) - 1]/N - 1\}$), and the probability of identity ($PI = [1 - \sum pi^4 + \sum (2pipj)^2$, where pi and pj are the frequency of the ith and jth alleles, respectively) [42]. The expected heterozygosity is frequently used as synonym of the polymorphic information content [29]. For the chloroplast microsatellite data, the gene diversity equation was adapted to haploid data ($He = 1 - \sum pi^2$, where pi is the frequency of the ith haplotype) [43]. Because the chloroplast genome is uniparentally inherited and thus not recombining, it was treated as one locus, and the different haplotypes were treated as alleles. We also identified marker-specific and genotype-specific alleles, called private alleles. These are alleles found in a single genotype among the complete collection of genotypes studied.

A genetic distance matrix was calculated using the Dice coefficient [44]. Phylogenetic clustering trees were constructed by unweighted pair group method with arithmetic mean (UPGMA) [45] using MEGA 5 software [46]. Clusters robustness was tested by bootstrap resampling ($n = 1000$) with the software package WINBOOT [47]. To confirm the accuracy of clustering, we conducted a Bayesian analysis using the software STRUCTURE 2.3.1 [48]. For this analysis, we used the admixture model (because of the grape mating system and biology), correlating allele frequencies twenty times for each K (number of populations assumed), with a burn-in of 500.000 interactions followed by 750.000 interactions MCMC (Markov Chain Monte Carlo). Given that "Uva Fragola Nera" and "Uva Fragola Bianca" belong to the cultivar "Isabella", a hybrid between *V. labrusca* and an unidentified *V. vinifera* subsp. *vinifera* variety [49] , they were excluded from the STRUCTURE analysis since the inclusion of a limited number of samples from a population could bias stratification [48]. The most likely number of clusters was chosen using the ΔK method, as described in [50]. Pearson correlation between matrices of genetic and geographic distances, as well as between genetic distance matrices for SSR and retrotransposon-based markers, was calculated using the statistical tool XLSTAT 2013.2.05 (Addinsoft, Paris, France). Significance was evaluated by Mantel test [51].

Results

SSR Analysis

SSR used in this study allowed the differentiation of 62 grapevine accessions. We detected 183 alleles with size ranging from 62 bp (CCMP8) to 331 bp (VVIp60) (Supplemental Table 3) and an average of 12.35 alleles per *locus* for nuclear microsatellite (Table 1) and 3.6 for cytoplasm chloroplast microsatellite (Table 2). For nSSR, *He*, *F*, *I*, and *PD*, mean values were 0.794, 0.258, 1.582, and 0.692, respectively. *He* was significantly higher than 0.5 for 80 % of markers, and *F* value was negative for one marker, VrZAG21, and higher then 0.85 for six markers. F negative values indicate an excess of heterozygotes and

Table 1 Genetic parameters of 20 nuclear and cytoplasm microsatellites used to differentiate 62 grape varieties

Locus	Allele size range (bp)	Alleles per locus (no.)	Expected heterozygosity	Fixation index	Shannon's index	Power of discrimination
VrZAG79	104–180	12	0.700	0.332	1.534	0.715
VrZAG12	138–172	10	0.739	0.586	1.608	0.754
VrZAG21	178–214	14	0.776	−0.060	1.816	0.787
VrZAG29	102–116	7	0.491	0.573	1.048	0.493
VrZAG47	148–182	11	0.841	0.463	1.976	0.852
VrZAG62	185–213	10	0.838	0.173	**2.007**	0.852
VrZAG79	226–262	15	0.874	0.114	**2.306**	0.852
VrZAG112	220–262	17	0.882	0.177	**2.373**	**0.888**
VVIC05	142–169	14	0.823	0.334	**2.047**	**0.895**
VVMD27	173–203	10	0.846	0.161	1.980	0.836
VVS2	120–156	13	0.853	0.017	**2.187**	**0.864**
VVS4	154–176	9	0.671	0.158	1.345	0.686
VVS5	85–157	17	0.829	0.455	**2.191**	0.847
VVIb01	288–304	8	0.794	0.248	1.723	0.808
VVIh54	144–186	14	0.798	0.313	1.944	0.813
VVIn16	139–175	10	0.676	0.356	1.555	0.680
VVIp60	303–331	20	0.911	0.097	**2.633**	0.925
VVIq52	75–89	8	0.771	0.415	1.657	0.786
VVMD25	232–264	15	0.881	0.140	**2.335**	**0.895**
VVMD5	223–249	13	0.883	0.105	**2.279**	**0.897**
	Average	12.35	0.794	0.258	1.927	0.806

Numbers in bold indicate statistic significance

values above zero an excess of homozygotes. This suggests that many apparent homozygotes are likely to be heterozygotes with one amplified and one null allele, as suggested by Pelsy et al. [52]. The Shannon's index was greatly higher than two in nine loci (VrZAG62, VrZAG79, VrZAG112, VVIC05, VVS2, VVS5, Vvlp60, VVMD25, and VVMD5), with five of them sharing a high *PD* value (>0.86) (Table 2). For cpSSR, the gene diversity values ranged from 0.031 (CCMP7) to 0.716 (CCMP10), and the Shannon's Index was between 0.082 (CCMP7) and 1.391 (CCMP10). Allele frequencies (*Af*) were useful to distinguish common alleles ($Af > 0.5$) and specific alleles ($Af < 0.1$). Out of 47 marker-specific alleles identified, 19 were present in only one genotype, and were, therefore, named private alleles. VrZAG21 and VVIp60 revealed the highest number private alleles (4). Private alleles were detected in 14 grapes: 4 in "Catalanesca", 2 in "Mennavacca", and "Sommarello" and "Pellecchione" and 1 in "Coda di Volpe Bianca", "Ginestrello", "Rose'", "Malaga", "Coda di Volpe Nera", "Montepulciano", "Sanginella", "Abbuoto", "Piedirosso", "Aglianico del Taburno", "Serpentaro" e "Arilla Ischia" and "Pellecchione" (Table 3). Variety distribution in the UPGMA dendrogram was organized in five major clusters (Fig. 1). Two of them contained few grapevines: Cluster IV included two genotypes "Uva Fragola Bianca" and "Uva Fragola Nera", and Cluster V included only one genotype,

Table 2 Genetic parameters of ten cytoplasm microsatellites used to differentiate 62 grape varieties

Locus	Allele size range (bp)	Alleles per locus (no.)	Gene diversity	Shannon's index
CCMP1	123–127	4	0.432	0.811
CCMP2	207–208	4	0.091	0.191
CCMP3	100–106	2	0.471	0.917
CCMP4	117–127	4	0.470	0.887
CCMP5	99–102	4	0.675	1.186
CCMP6	106–109	4	0.482	0.876
CCMP7	142–144	2	0.031	0.082
CCMP8	62–86	3	0.282	0.610
CCMP10	104–112	5	0.716	1.391
ccSSR5	250–258	4	0.582	1.099
	Average	3.6	0.423	0.805

"Catalanesca". Thirty grapevines were included in Cluster I, seven in Cluster II, and twenty-two in Cluster III. Clustering robustness of some nodes was supported by bootstrap values higher than 70 %. Based on our molecular data (phylogenetic classification and microsatellite allele mismatches), it was possible to distinguish a case of synonymy (cultivars having more than one name) and homonymy (different cultivars mentioned under the same name) in the entire group of cultivars. For example In

Table 3 Locus specific alleles identified in 18 SSR loci

SSR locus	Allele (genotype)
VrZAG7	112, 152, 158, 170
VrZAG12	**140 (Mennavacca)**, 158
VrZAG21	178, **188 (Mennavacca), 196–204 (Catalanesca), 212 (Coda di Volpe nera)**
VrZAG47	148, 152, 164
VrZAG79	**226 (Pellecchione)**
VrZAG112	**220 (Ginestrello), 222 (Rose')**, 238
VVIC05	144, 150, 164
VVMD27	185
VVS2	138, **146 (Catalanesca)**
VVS4	154, 160, **163 (Malaga),** 172
VVS5	129, **133 (Coda di Volpe bianca),** 139, 153
VVIh54	144, 152, 156, **180 (Catalanesca)**
VVIn16	139, 147
VVIp60	**313 (Montepulciano), 314–328 (Sommarello), 329 (Sanginella)**
VVIq52	**87 (Abbuoto)**
VVMD25	232, 254
VVMD5	**243 (Piedirosso)**
ccmp8	**62 (Aglianico del Taburno)**

Private alleles and the corresponding genotypes are reported in bold

particular, "Procidana" showed a genetic constitution similar to "Coglionara". They are both grapevines mainly cultivated in the island of Ischia and based on our data they resulted synonymous. In must be noticed that synonymous cases are likely originated by mutation rather than sexual reproduction. Therefore, they share a common genetic origin but a different ampelographic classification. By contrast, the two "Barbera del Sannio" biotypes sampled in Castelvenere and Pietradefusi were molecularly homonymous to the one sampled in Ischia. The two "Livella" genotypes sampled in Ischia and Eboli resulted homonymous, while the two "Cacamosca" genotypes sampled in the same places were distinct cultivars.

The number of subpopulations was inferred by the model-based Bayesian clustering procedure. The Δk method is based on the rate of change in the log probability of data between successive K values and is a good estimator of the number of clusters (K) [497]. Δk values ranged from 0.191 (79 populations assumed) to 28.24 [3 populations assumed) (Supplemental Fig. 1) and suggested three ($K = 3$) as optimal number of population sampled. The three populations were divided in bar plots and labeled as A, B, and C (Fig. 2). Comparing Bayesian and phylogenetic analyses, we associated population A to Cluster II

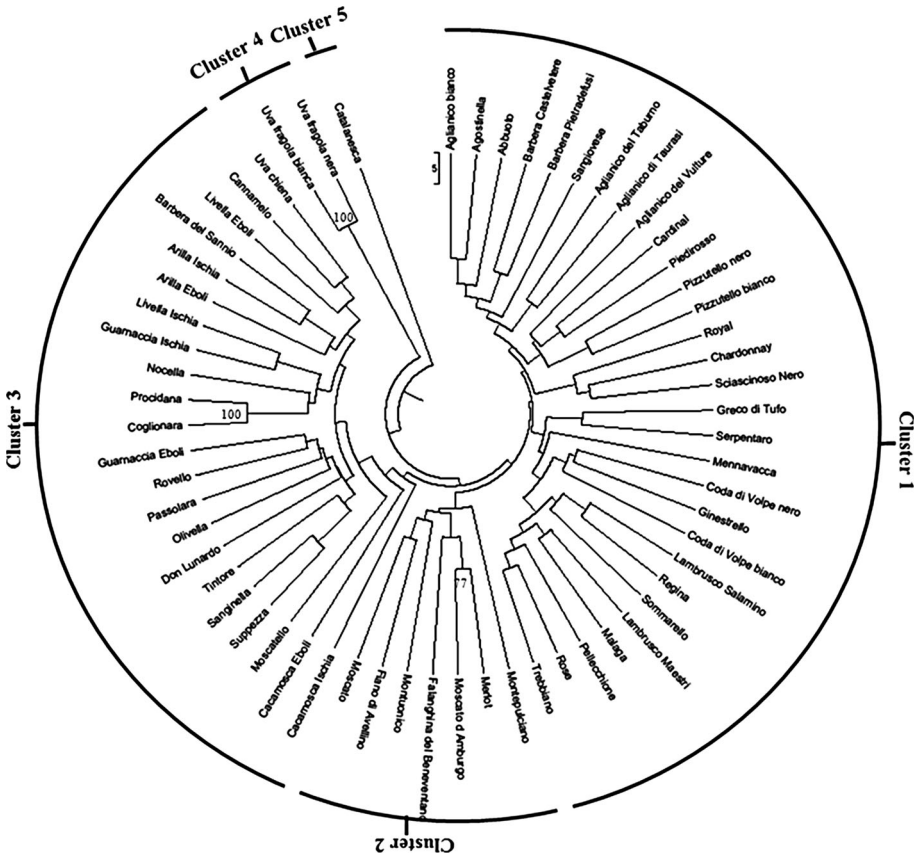

Fig. 1 Dendrograms of 62 grape genotypes obtained using UPGMA cluster analysis of SSR marker data. Bootstrap values higher than 70 % are indicated at nodes

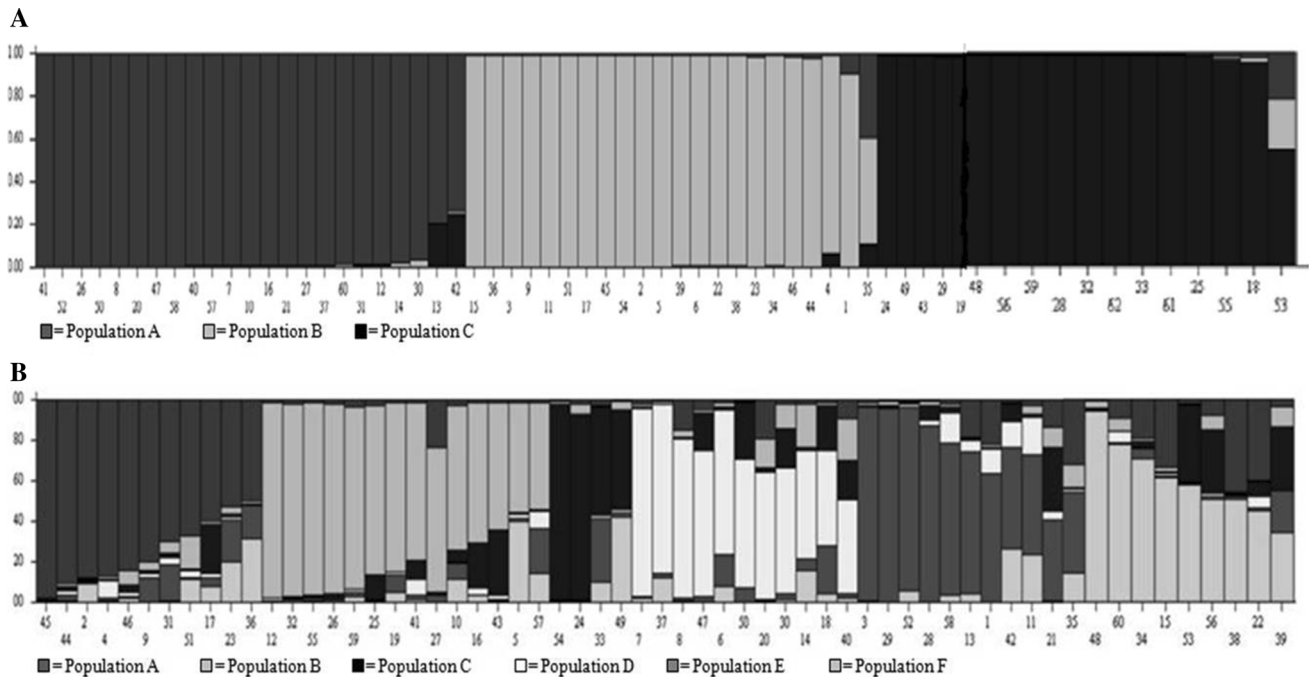

Fig. 2 Inferred population structure of the 62 grapevines genotypes using SSR (**a**), REMAP and IRAP (**b**) markers through the model-based program STRUCTURE. Each individual is represented by a *horizontal bar*, which is partitioned into *gray colored* segments that represent the individual estimated levels of the clusters

and a Cluster I's sub-cluster, population B to Cluster III, and population C to Cluster IV, V and a second Cluster I's sub-cluster. The bar plot graph (Fig. 2), based on STRUCTURE analysis, showed similar clusterization of UPGMA dendrogram.

Retrotransposon-Based Genotyping

The *Gret1* LTR element amplification revealed a unique multilocus pattern for each accession. This is due to the retrotransposons integration ability in either orientation, as head-to-head, head-to-tail, and tail-to-tail. Our analysis with retrotransposon-based markers was performed with 5 primer combinations, generating a total of 44 bands (Supplemental Table 4), of which 90 % was polymorphic (Tab. 4). Primer combinations amplifying the highest and lowest number of bands were 5′LTR/$(CT)_9$G (13 bands) and 3′LTR/$(CT)_9$G (4 bands), respectively. The PD value was higher than 0.3 in 3′LTR/$(CT)_9$G and in 5′LTR/3′LTR. These primer combinations shared also the highest *I* value (Table 4). Among REMAP patterns, three private bands were identified: two with the 5′LTR/$(GA)_9$C combination in "Moscatello" and "Sangiovese" and one with 3′LTR/$(GA)_9$C combination in "Pizzutello bianco" (Supplemental Table 4). The IRAP patterns revealed one private allele in "Cacamosca Ischia". All genotypes were clusterizing in eleven groups through UPGMA analysis (Fig. 3). Cluster IV was the largest one with 15 genotypes, while Clusters VI, VII, VIII, IX, X, and XI were the smallest, with an average of three genotypes each and a minimum of one genotype in Cluster XI. The estimated membership of each individual in each cluster was compared with the Bayesian analysis. Using the REMAP and IRAP dataset, the highest ΔK value was 17.14 in the sixth K (Supplemental Fig. 1). Six populations (A–F) were explained by six distinctive bar plot, each labeled by different gray gradations (Fig. 2). Six Bayesian populations assorted the same genotypes grouped in the UPGMA clusters: population B was comparable with the Cluster IV, C with Cluster VIII, D with Cluster IX and X, E with Cluster II and IV, and F with Cluster III and V.

Population B was comparable with the Cluster II, C with Cluster II, D with Cluster V, A with Cluster VIII, E with Cluster IX, and G with Cluster X.

Discussion

The genetic diversity of 62 grape accessions including 38 autochthonous grapevines from Campania was investigated using SSR and retrotransposon-based molecular markers. The gene pool of cultivated grapes has significant amounts of genetic variation and exhibits high differentiation, which needs to be characterized to increase knowledge on the available genetic resources. To provide further insights into the genetic structure and differentiation within and among grapevine samples, the analysis of the same genotypes with

Table 4 Results of REMAP and IRAP analyses used to differentiate 62 grape varieties

Marker	Primer combination	Fragments per marker (no)	Total fragments (no)	Polymorphism (%)	Expected heterozigosity	Shannon's index
REMAP						
	5′LTR/$(GA)_9$C	10	245	80.00	0.263	0.400
	5′LTR/$(CT)_9$G	13	191	100.00	0.211	0.352
	3′LTR/$(GA)_9$C	7	143	85.71	0.260	0.397
	3′LTR/$(CT)_9$G	4	89	100.00	0.427	0.617
IRAP						
	5′LTR/3′LTR	10	250	90.00	0.303	0.457
	Sum	44	918	–	–	–
	Average	8.8	183.6	91.14	0.293	0.445

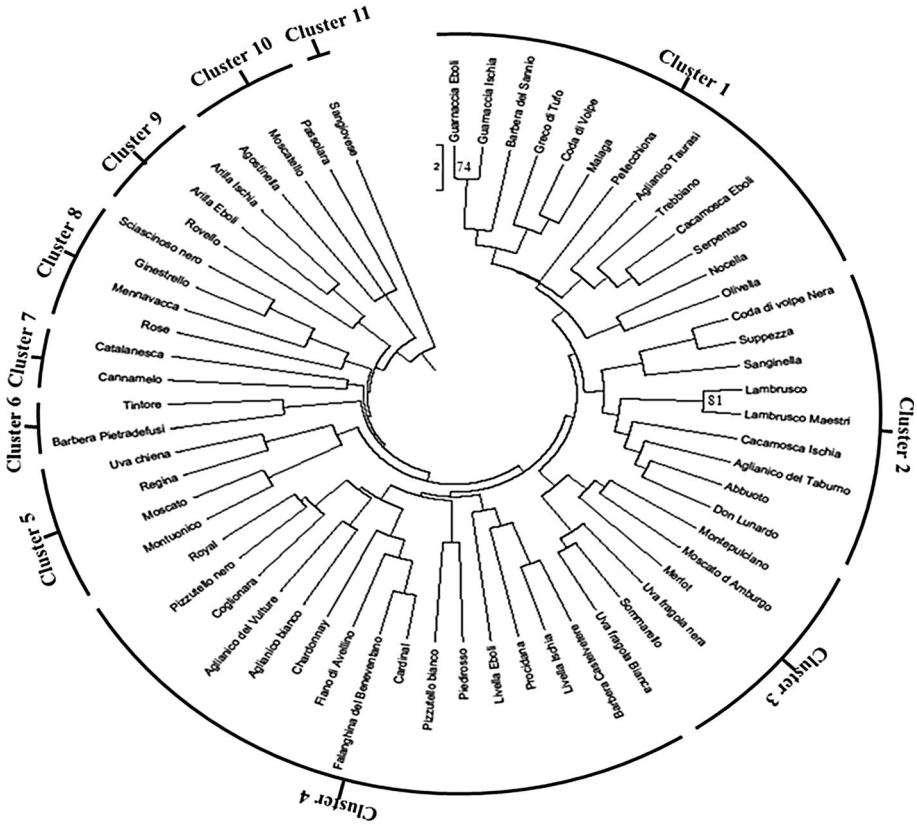

Fig. 3 Dendrograms of 62 grape genotypes obtained using UPGMA cluster analysis of IRAP and REMAP marker data. Bootstrap values higher than 70 % are indicated at nodes

different techniques is likely the best approach [12]. Therefore, in this study, we combined SSR with REMAP and IRAP markers. Based on their different nature, they can detect similar diversity patterns, but with differences in sensitivity. Marker sensitivity is intended as the capability of distinguishing inter and intra-specific variability. Through SSR analysis, it is possible to investigate inter-specific variability and identify genetically different varieties [11]. On the other hand, transposon-based markers can investigate intraspecific variability and identify different accessions within the same species [53]. Our results showed that five markers (VrZAG79, VrZAG112, VVS2, VVMD25, and VVMD5) should be recommended for the rapid and unambiguous identification of grapes from Campania region as they proved to be the most discriminating loci, with PD values ranging between 0.852 and 0.897 and Shannon's Index values from 2.187 to 2.373. This confirms findings by Costantini et al. [54], Zoghlami et al. [55], and Laucou et al. [11], who reported and used the same set of microsatellite in grape genotyping studies. With an average value of 12.35 alleles per locus, our microsatellite results are consistent with those of Santana

et al. [56], who found an average of 8.7 alleles per locus studying 65 grape accessions. It is interesting to note that out of 30 markers used, only 2 showed a FI value higher than 0.5 (Tables 1, 2) suggesting that 93.3 % of the microsatellite markers used are highly heterozygous. Our data were consistent with reports by Sant'Ana et al. [53], Lopes et al. [57], and Riahi et al. [58], who confirmed the high individual heterozygosity due to breeding programs applied to improve quality and productivity. Through statistical and phylogenetic analyses, synonymies, homonymies, and unique genotyping cases were identified. The three "Aglianico" biotypes ("Taburno", "Taurasi", and "Vulture") analyzed in this study confirmed the findings of Muccillo et al. [59], suggesting that "Aglianico Taurasi" and "Aglianico Taburno" biotypes are closer than the "Aglianico Vulture". One genotype, "Catalanesca", resulted unique and highly heterozygous and also produced the highest number of private alleles. "Uva Fragola Nera" and "Uva Fragola Bianca" clustered on their own in Cluster IV consistently with their genetic constitution. Indeed, they belong to the cultivar "Isabella", known to be not only a pure *V. vinifera* but also a hybrid between *V. labrusca* and an unidentified *V. vinifera* subsp. *vinifera* variety [49]. In SSR dendrogram, grouping all the native grapes of Ischia island clustered along with grapes sampled in the that area (Fig. 1); this might be explained by the presence of ancient genes correlated to the low rate of crossing occurring in the island of Ischia. Interestingly, cultivars producing white and red berries did not cluster separately. This can be explained by the epigenetic control of anthocyanin production in berry skin, not mapped by microsatellite [22].

Several studies reported the successful use of combined dominant and co-dominant markers for grapevine clone identification [16, 50, 58]. Microsatellites are usually the most frequently used genetic markers in intra-specific *Vitis* studies [11], while retrotransposon-based markers, classified as epigenetic markers, result the best for intra-species studies or rather to distinguish clones generated by vegetative propagation [49]. For this reason, the genetic diversity of our 62 grapevines was investigated also using four REMAP and one IRAP markers designed on the gypsy-type retroelement *Gret1*. The chosen methods have the characteristics of using fewer primers but providing sufficiently high polymorphisms to allow detection of intervarietal diversity and heterotic groups, as already demonstrated in apricot [60], bread wheat [61], and alfalfa [62]. We identified 44 different bands with five markers in a profile complexity ranging from 4 to 13 bands per marker. This is comparable with findings by Carcamo et al. [16], who detected a significant lower number of bands in 28 clones of grapevine "Tempranillo". This result could be related to a point mutation occurring in the primer sequence. Relatively few bands were obtained also in other grape studies [14, 29]. As far as we know, this is the first time that this technique has been used to produce complex multilocus profiles. Because of the nature of these markers, they could be successfully employed to build a cultivar identification diagram (CID). This is an open diagram successfully used in different plant species, as grapevine [63] and *Ginkgo* [64]. It can be very helpful for genetic resource conservation and utilization and plant variety protection [65]. The complete set of REMAP and IRAP markers was able to distinguish different biotypes of the same genotype as "Aglianico" or "Barbera del Sannio" series, but not the same genotype sampled in different areas like "Arilla Ischia" and "Arilla Eboli" or "Guarnaccia Ischia" and "Guarnaccia Eboli". This confirms the capability of these markers to discriminate the grape accessions but not structured sub-groups [52].

In conclusion, we have used and integrated two marker systems to detect genetic diversity and population structure in *V. vinifera* cultivars from a relatively small area. Using two appropriate techniques, we were able to classify this heterogeneous group based on the origin and spread through microsatellite as well as based on vegetative propagation through retrotransposon-based ones. Data suggested that a wide genetic variability is still present in grape germplasm cultivated in Campania. Homonymies and Synonymies were found, reinforcing the knowledge that molecular evaluations can provide further insights into genetic structure and differentiation of *Vitis* germplasm accumulated during centuries of cultivation and selection.

Acknowledgments We are thankful to Raffaele Garramone for helping in sampling and the public–private laboratory of genomics GenoPOM for the capillary electrophoresis analyses. We are grateful to the two anonymous reviewers for helpful suggestions and comments. Part of this research was carried out in the frame of the projects "Tecniche e Ricerche per la Riqualificazione dell'Originalità tramite Innovazioni nella Rintracciabilità di filiera nel Sannio beneventano (TERROIRS)" and "Salvaguardia della Biodiversità Vegetale in Campania (SALVE)", fundend by Benevento Chamber of Commerce and Region of Campania, respectively.

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