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ORIGINAL RESEARCH ARTICLE

Protection of viticultural biodiversity: genetic and phenotypic characterisation of grapevine varieties from the northwest coastal area of Tuscany (Italy)

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ABSTRACT

To explore the grapevine biodiversity of some minor viticultural districts located in the northwest coastal area of Tuscany, a total of 99 grapevine samples were collected from field borders or no longer cultivated areas and analysed at the molecular level using 12 microsatellite markers (SSR). The results revealed the presence of 58 unique genotypes among which 42 already known varieties (36 belonging to the *Vitis vinifera* species and 6 *Vitis* interspecific crosses) and 16 hitherto unreported genetic profiles. About 50 % of the accessions (52 out of 99) were vegetatively propagated and planted in a vineyard collection to protect them from extinction and to record their phenotypic characteristics. This research has highlighted the potential for using several grapevine cultivars that were probably once widespread and can henceforth be cultivated again to help revive the eco-wine tourism of the Tuscan heroic vineyards and add prestige to the local wine panorama.

KEYWORDS: *Vitis vinifera*, grapevine germplasm, genetic identification, SSR markers, ampelography, anthocyanins, biodiversity, viticulture

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INTRODUCTION

Italy is among the most established and top-quality wine-producing countries in Europe, with viticulture having increasingly become one of the leading businesses of the agri-food economy. Furthermore, Italy has one of the widest ampelographic platforms, constituting a wealth of grapevine varieties that can be considered an invaluable source of plant biodiversity (Bacilieri *et al.*, 2013; De Lorenzis *et al.*, 2019).

In the last 150 years, the Italian total vineyard surface area has undergone a sharp reduction, especially in some agricultural districts, decreasing from over one million hectares in the 1970s to approximately 700,000 hectares today (Boselli *et al.*, 2019; OIV - International Organisation of Vine and Wine., 2022). There are several concurrent causes of this drastic abandonment of vine cultivation (Torquati *et al.*, 2015): i) industrialisation and urbanisation (above all, in certain regions such as Lombardy, one of the most productive of northern Italy) (Raimondi *et al*., 2015), ii) rural depopulation (a chronic outmigration of mostly young adults looking for job opportunities elsewhere), iii) a significant increase in the pressure of vine pests of American origin, predominantly phylloxera, downy mildew and powdery mildew (Fontaine *et al.*, 2021; Gadoury *et al*., 2012; Granett *et al*., 2001). Moreover, many traditional autochthonous cultivars have been replaced by the so-called "international varieties" (i.e., Cabernet Sauvignon, Merlot, Sauvignon Blanc, Chardonnay), comprising a few genotypes that determine most of the worldwide grapevine diversity (Wolkovich *et al*., 2018), especially due to their high adaptability to different environments (Otto *et al*., 2022).

This sudden evolution of the grapevine landscape has caused genetic erosion, both in cultivated and wild grapevines (Gisbert *et al*., 2018). Thus, several varieties once commonly grown and cited in the most popular ampelography treatises are no longer available or are in danger of disappearing. For these reasons, many research groups working in the regions traditionally suited to viticulture are engaged in the protection of ancient autochthonous, less known or forgotten vines (Ghrissi *et al*., 2022; Goryslavets *et al*., 2015; Gristina *et al*., 2017; Maraš *et al*., 2014; Margaryan *et al*., 2021; Moravcova *et al*., 2006; Sargolzaei *et al*., 2021; Schneider *et al*., 2014; Zombardo *et al*., 2021).

The wine-growing area involved in this study is located in Tuscany (central Italy), one of the most renowned regions in the world from an agricultural, touristic and landscape point of view. In particular, Candia dei Colli Apuani, Garfagnana, and Lunigiana are three wide valleys in the northwest coastal area, on the border of Levante Ligure (east Liguria), lying between the Apennines and the Apuan Alps and overlooking the Tyrrhenian Sea. Historically, these territories maintained broad administrative independence and have been relevant for maritime trade with foreign countries or land trade with the rest of Italy (Piccardi and Pranzini, 2018). As already mentioned, the vineyards also in these places are disappearing. The main reason is land abandonment with a subsequent advanced average age of farmers, exacerbated by environmental constraints (i.e., harsh sites, significant elevations, steep slopes, hydrogeological hazards) and the high pressure of fungal diseases linked to abundant rainfall (over 1200 mm/year) (García-Ruiz and Lana-Renault, 2011; Jackson, 2008; Storchi *et al*., 2018). In this area, so-called "extreme viticulture" is still carried out, consisting of terraced vineyards confined to isolated areas with limited accessibility and thus associated with the impossibility of mechanised agronomic practices and prohibitive management costs (Strub and Loose, 2021). Consequently, there are no major commercial wineries but only family-run production units, 90 % of which have a fragmented cultivated area, often smaller than 5 hectares (Storchi *et al*., 2018).

The growing concern about land abandonment and the low profitability of vineyard production has resulted in actions being carried out aiming to recover old vine varieties. Native grapevine germplasm renewal could prove fundamental in not only avoiding genetic erosion but also achieving differentiated productions and better adapting plant material to the local climatic conditions. Considering the viticultural vocation of this important transit area, it is likely that a rich biodiversity can be found. The heterogeneity of the grapevine germplasm in remote districts is, in fact, an indicator of the movement of people and goods, and it is assumed that the ampelographic heritage has been enriched by grape varieties imported from elsewhere over the centuries.

The research work described here aimed at rescuing individual vines discovered along field borders or in areas that are no longer cultivated and are located in different municipalities of the Candia dei Colli Apuani, Garfagnana, Lunigiana and Levante Ligure territories. Genotyping of 99 recovered vines was carried out using a set of 12 SSR markers (Simple Sequence Repeats), an internationally recognised and widely used method for grapevine genetic identification (Pelsy, 2010). The rarest and most interesting genotypes were propagated and gathered in a grapevine germplasm collection to allow ex-situ conservation and phenotypic characterisation.

MATERIALS AND METHODS

1. Plant materials

The plant materials (young leaves or small shoot portions) of 99 relic vines were sampled from 15 different Italian municipalities belonging to the territories of Candia dei Colli Apuani (2, Massa Carrara province), Garfagnana (3, Lucca province), Lunigiana (8, Massa Carrara province) and Levante Ligure (2, Genoa province), encompassing an area of approximately 1050 km^2 (Figure 1).

The vine specimens (all non-grafted) were identified in reforested wine-growing areas or field borders by local farmers who carried out visual inspections. After the plant material collection, each sample was named after the original location, the contact person or the putative varietal name referred to (Table 1). The sampling took place progressively, starting from the early 2000s and based on reports relating to

viticultural material found within the wide area of interest. Confirmatory genetic analyses were carried out on all samples in 2021 and 2022.

2. SSR Genotyping

Genotyping was performed using 12 SSR markers: nine as the minimum standard set for grapevine identification (VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, VrZAG79) (Maul *et al*., 2012), plus VMC6E1 (ISV2), VMC6G1 (ISV4) (Crespan, 2003) and VMCNG4b9 (Welter *et al*., 2007). PCR reactions were performed using forward primers labelled with fluorescent dyes (6-FAM, PET, VIC, or NED); two multiplex panels of fluorescent-labelled microsatellite loci were used. Simultaneous PCR amplifications were conducted in a final volume of 12.5 µl containing 1× PCR reaction buffer, 10 ng of genomic DNA, 0.2 mM of each dNTPs, 2 mM $MgCl_2$, 1.5 U Taq DNA Polymerase (Thermo Fischer Scientific, Waltham, MA). Depending on the locus, primer concentrations ranged from 0.11 to 0.48 µM (see Additional file 1). Reactions were performed on a GeneAmp PCR System 9700 using the following profile: a hot start of 95 °C for 5 min, 30 amplification cycles of 45 sec at 95 °C, 1 min at 55 °C, 30 sec at 72 °C, and a final extension step of 30 min at 72 °C. 0.5 µL PCR product for each sample was mixed with 9.35 µL of formamide and 0.15 µL of GeneScan™

TABLE 1. List of the 99 grapevine samples grouped by their genotype. Original locations (Municipality; Province: MS = Massa Carrara; LU = Lucca, GE = Genoa), berry colour, true-to-type prime name/Unknown, SSR profile ID number, and *Vitis* International Variety Catalogue (VIVC) code (if available) are reported. The sample names in bold correspond to the 52 grapevine accessions currently present in the experimental vineyard. The asterisks (*) indicate 47 accessions with already adult vines (over 3 years old) that were described based on the OIV primary descriptor priority list. Nyd = berry colour not yet determined.

Part 1/3

Sample Name	Original location: Municipality (Province)	Berry color	True-to-Type Prime Name /Unknown	SSR-profile ID	VIVC code
	Known grapevine varieties – *Vitis vinifera*				
1-Vite pergolona	Careggine (LU)	white	Afus Ali	1	122
2-Gorfigliano Chiesa vecchia 1	Minucciano (LU)	white	Agostenga	2	107
3-Gorfigliano Chiesa vecchia 2	Minucciano (LU)				
4-Gorfigliano Chiesa vecchia 3	Minucciano (LU)				
5-Gorfigliano Chiesa vecchia 4	Minucciano (LU)				
6-Gorfigliano Chiesa vecchia 5	Minucciano (LU)				
7-Bianchetta 1*	Massa (MS)	white	Albarola	3	238
8-Bianchetta 2*	Massa (MS)				
9-Albarola*	Massa (MS)				
10-Montelama	Zeri (MS)				
11-Aleatico	Careggine (LU)	black	Aleatico	4	259
12-Piccioli Nera	Fosdinovo (MS)	black	Ancellotta	5	447
13-Massaretta 1*	Carrara (MS)	black	Barsaglina	6	1013
14-Barsaglina del Nonno*	Massa (MS)				
15-Massaretta 2*	Carrara (MS)				
16-Barsaglina 1*	Massa (MS)				
17-Barsaglina 2*	Massa (MS)				
18-Colombana 1*	Massa (MS)	black	Bonamico	7	1540
19-Colombana 2*	Massa (MS)				
20-Buonamico*	Massa (MS)				
21-Groppello 2*	Aulla (MS)				
22-Merla 2	Fosdinovo (MS)	black	Cabernet Sauvignon	8	1929
23-Bianca 11 Poli	Careggine (LU)	black	Caloria	9	2010
24-Pollera Corlaga*	Pontremoli (MS)				
25-Merla 1	Fosdinovo (MS)	black	Canaiolo Nero	10	2037

Part 2/3

Sample Name	Original location: Municipality (Province)	Berry color	True-to-Type Prime Name /Unknown	SSR-profile ID	VIVC code
	Known grapevine varieties – *Vitis vinifera*				
26-Biancolina	Massa (MS)	white	Chasselas Blanc	11	2473
27-Schiava Nera	Massa (MS)	black	Ciliegiolo	12	2660
28-Sillico 4	Pieve Fosciana (LU)				
29-Nera 1 Poli	Careggine (LU)				
30-Vite colore grosso	Careggine (LU)				
31-Zeri	Zeri (MS)				
32-Bracciola Tendola*	Pontremoli (MS)	black	Della Borra	13	3511
33-Bracciola Coloretti*	Fivizzano (MS)				
34-Zeri Ternesa 3	Zeri (MS)	black	Dolcetto	14	3626
35-Durella 1*	Pontremoli (MS)	white	Durella Gentile	15	24871
36-Durella 2*	Aulla (MS)				
37-Sillico 2	Pieve Fosciana (LU)	black	Farinella	16	26367
38-Pulichese Nero	Massa (MS)	black	Gallizzone	17	16968
39-Vignali 4	Zeri (MS)	white	Luglienga Bianca	18	6982
40-Pollera*	Aulla (MS)	white	Malvasia Bianca di Candia	19	23555
41-Pollera Bianca*	Aulla (MS)				
42-Groppello*	Fivizzano (MS)	black	Marinello	20	26721
43-Antona Nera Polini*	Massa (MS)	black	Montepulciano	21	7949
44-Bosa 4	Careggine (LU)	black	Muscat Hamburg	22	8226
45-Occhio di Pernice	Careggine (LU)				
46-Morone 2*	Pontremoli (MS)	black	Parmesana	23	25342
47-Morone 3*	Pontremoli (MS)				
48-Podenzana 3	Podenzana (MS)	dark red violet	Pollera Nera	24	9585
49-Pollera B1*	Fivizzano (MS)				
50-Podenzana 2	Podenzana (MS)				
51-Livornese*	Massa (MS)	white	Rollo	25	10171
52-Sillico 1	Pieve Fosciana (LU)	black	Sangiovese	26	10680
53-Passerina Nera	Careggine (LU)				
54-Vite Schiava	Careggine (LU)	black	Schiava Grossa	27	10823
55-Rossara 1*	Aulla (MS)	dark red violet	Schiava Lombarda	28	10825
56-Vignali 2	Zeri (MS)	black	Sciaccarello	29	10837
57-Vignali 3	Zeri (MS)				
58-Vigna di Posara	Fivizzano (MS)	black	Tintoria Lloyd	30	24446
59-Pizzamosca*	Pontremoli (MS)	white	Tocai Friulano	31	12543
60-Bracciola Bianca Antona*	Massa (MS)	white	Trebbiano Toscano	32	12628
61-Bianca 1 Poli	Careggine (LU)				
62-Morone 1*	Pontremoli (MS)	black	Uva Crova	33	24563
63-Sillico 5	Pieve Fosciana (LU)				
64-Sillico 6	Pieve Fosciana (LU)				
65-Verduschia Antona	Massa (MS)	white	Verdea	34	12944
66-Verdella 2	Aulla (MS)	white	Verdicchio Bianco	35	12963
67-Verdella	Aulla (MS)				
68-Vermentino B12*	Massa (MS)	white	Vermentino	36	12989
69-Vermentino Bianchi*	Massa (MS)				
70-Vermentino 17*	Massa (MS)				
Vermentino VCR1*	Reference				
71-VL4*	Massa (MS)				
72-Pulichese Bianco	Massa (MS)				

Part 3/3

Sample Name	Original location: Municipality (Province)	Berry color	True-to-Type Prime Name /Unknown	SSR-profile ID	VIVC code
	Known grapevine varieties – *Vitis vinifera*				
73-VP2*	Massa (MS)	black	Vermentino Nero	37	12992
74-VP3*	Massa (MS)				
	Known grapevine varieties – Vitis interspecific crosses				
75-Rutili	Massa (MS)	black	Baco Noir	38	870
76-Piervitali	Fivizzano (MS)	white	Muscat de St. Christol	39	8209
77-Giacché*	Massa (MS)	black	Jacquez	40	5627
78-Sillico 3	Pieve Fosciana (LU)	black	Rosette	41	10209
79-Zeri Vignali 1	Zeri (MS)	white	Villard Blanc	42	13081
	Unknown Grapevine Varieties				
80-Agata	Lavagna (GE)	rose	Unknown 01	43	-
81-Baldini Bianca 2*	Mulazzo (MS)	white	Unknown 02	44	-
82-Bianca Mazzoni	Fivizzano (MS)	white	Unknown 03	45	-
83-Bosa 3	Careggine (LU)	black	Unknown 04	46	-
84-Collezione Bosa	Careggine (LU)				
85-Castel del Piano 1	Licciana Nardi (MS)	nyd	Unknown 05	47	-
86-Vite Mammolo	Careggine (LU)	black	Unknown 06	48	-
87-Marchese Grigio*	Pontremoli (MS)	grey	Unknown 07	49	-
88-Merlarola*	Pontremoli (MS)	black	Unknown 08	50	-
89-Marchese Nera	Pontremoli (MS)				
90-Mesco Vite di Sestri	Sestri Levante (GE)	white	Unknown 09	51	-
91-Monferrato*	Aulla (MS)	black	Unknown 10	52	-
92-Rossara 2*	Massa (MS)				
93-Nera 4*	Massa (MS)	black	Unknown 11	53	-
94-VL5*	Massa (MS)				
95-Pergola Bosa	Careggine (LU)	black	Unknown 12	54	-
96-Podenzana 1	Podenzana (MS)	black	Unknown 13	55	-
97-Schiava Bianca*	Mulazzo (MS)	white	Unknown 14	56	-
98-Tané*	Fivizzano (MS)	rose	Unknown 15	57	-
99-Uva Rosa*	Fivizzano (MS)	dark red/violet	Unknown 16	58	-

500 LIZ Size Standard (Life Tech, Carlsbad, CA, USA). Afterwards, the capillary electrophoresis was run using an ABI 3130xl Genetic Analyzer (Life Tech, CA, USA). Allele calling was performed employing GeneMapper version 5.0 (Thermo Fisher Scientific, Waltham, MA, USA) and using a homemade bin-set built with some reference varieties. Allele sizes were recorded in bp (using the *V*IVC allele sizing), and genotypes showing a single peak at a given locus were considered homozygous. The resulting SSR profiles were compared with those in the CREA Viticulture and Enology molecular database (a constantly updated repository that to date contains 4982 unique *Vitis* spp. microsatellite profiles), in the *Vitis* International Variety Catalogue (https://www.vivc.de/), and in the literature to ensure unambiguous varietal identification.

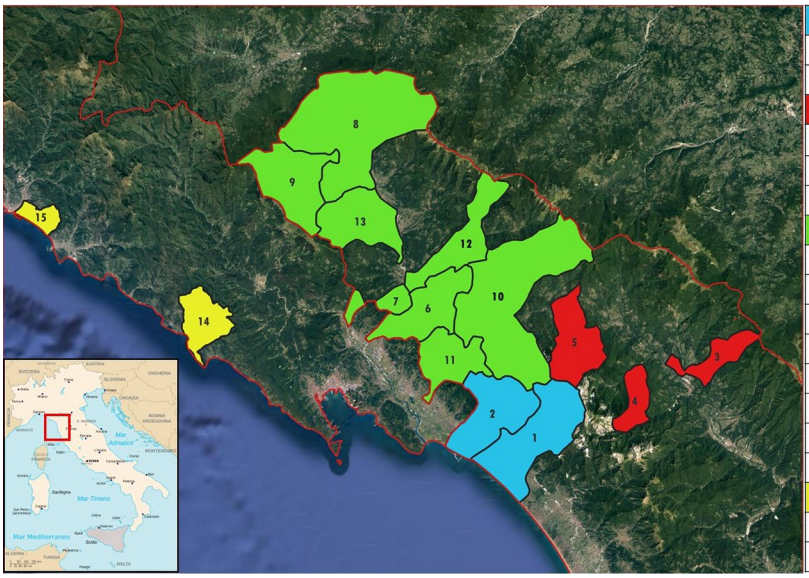
3. Statistics on SSR Markers

Statistics on the SSR markers data were computed using Cervus 3.0.7 software (freely available at http://www.fieldgenetics.com/pages/home.jsp) and GenAlEx 6.51b2 software (Peakall and Smouse, 2012). Recognised hybrids (6 samples) were excluded from the computation. Cervus was also used to infer putative trios (parent-parent-offspring related genotypes). The search for parent-offspring relationships, however, was limited to the 58 genetic profiles found in this work.

4. Genetic Similarity and Ancestry Evaluation

Genetic distances were computed with GenAlEx 6.51b2 software (Peakall and Smouse, 2012). Recognised hybrids were excluded from computation. PCoA was also performed using GenAlEx software. Genetic distances were used to run MEGA X - Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al*., 2018) software, version 10.0.5 for a NJ (Neighbor-Joining) phylogenetic tree elaboration (Saitou and Nei, 1987).

Ancestry values and number of subpopulations were calculated on 52 genotypes, excluding hybrids, using STRUCTURE 2.3.1 software (Pritchard *et al*., 2000).



Candia dei Colli Apuani	
1	Massa (MS)
2	Carrara (MS)
Garfagnana	
3	Pieve Fosciana (LU)
4	Careggine (LU)
5	Minucciano (LU)
Lunigiana	
6	Aulla (MS)
7	Podenzana (MS)
8	Pontremoli (MS)
9	Zeri (MS)
10	Fivizzano (MS)
11	Fosdinovo (MS)
12	Licciana Nardi (MS)
13	Mulazzo (MS)
Levante Ligure	
14	Sestri Levante (GE)
15	Lavagna (GE)

FIGURE 1. Geographical locations and list of the 15 Italian municipalities from which the 99 grapevine samples included in the study were taken.

The four areas considered are: Candia dei Colli Apuani (light blue), Garfagnana (red), Lunigiana (green) and Levante Ligure (yellow). In brackets the abbreviation of the provinces of Tuscany (MS = Massa Carrara; LU = Lucca) and Liguria (GE = Genoa).

Ten independent runs were performed for K values from 1 to 8, with a burn-in period of 50,000, followed by 50,000 MCMC replications. The best K was calculated using the ΔK parameter (Evanno *et al.*, 2005). The chosen membership coefficient threshold for membership to a given cluster was Q = 0.80. Data reported in Additional file 2 were obtained after an alignment of the 10 independent runs calculated from K = 2 to K = 8 using CLUMPP software (Jakobsson and Rosenberg, 2007), version 1.1.2 (freely available at https://rosenberglab.stanford.edu/clumpp).

5. Grapevine Germplasm Collection

From the year 2005 onwards, an experimental vineyard with the purpose of local viticultural germplasm collection was set up at "Podere Scurtarola" farm in the municipality of Massa (Tuscany, Italy; 44°04'80" N, 10°10'98" E, 200 m a.s.l.), covering an area of approximately 0.5 hectares. In this environment, the average annual rainfall is about 1200 mm (data from Consorzio LaMMa, available at https://www.lamma.rete.toscana.it/), and a mean Huglin index value of 2010 °C (Santos *et al.*, 2012).

The vineyard is terraced and situated on a steep slope (35°, corresponding to a gradient of more than 70 %). The soil has a sandy clay loam texture (63 % sand, 25 % clay, 12 % silt), and the inter-row has permanent spontaneous grass cover to reduce erosion and the risk of landslides.

A preliminary virus screening was carried out on additional plant material from the sampled vines, according to the directives of the Italian clonal selection protocol for grapevine (Grapevine fanleaf virus – GFLV; Grapevine leaf roll associated virus 1, 2, and 3 - GLRaV-1,-2, 3; Grapevine virus A - GVA, Grapevine virus B - GVB; Grapevine fleck virus - GFkV; Arabis mosaic virus - ArMV). Portions of woody branches taken from the mother plants of the formerly screened virus-free samples were propagated by grafting on 1103 Paulsen rootstock. The root cuttings (at least 20 specimens each) were planted in the vineyard with an east-west orientation and a 0.8 x 2.30 m row spacing. Vermentino (VCR1 clone) was added as a reference variety, being the main grape cultivar in the area of interest. The vines were trained on an upward vertical shoot-positioned trellis, with spur cordon pruning and an average of 10 buds *per* vine. Over the years, homogeneous agronomic conditions were maintained on the vineyard and organic pest management was carried out.

6. Ampelographic Descriptions

The ampelographic description of some of the main vine organs was carried out on the 47 accessions of already adult vines (over 3 years old) grown in the vineyard collection; these are reported in bold and an asterisk in Table 1. The ampelographic characteristics were recorded according to the recommended OIV - International Organization of Vine and Wine methodology (descriptor list for grape varieties and *Vitis* species, available at https://www.oiv.int/public/medias/2274/code-2e-edition-finale.pdf), both for the unknown and the already known genotypes. A set of 14 standardised OIV primary descriptors (priority list) was used: young shoot (OIV 001, 004), shoot (OIV 016), young leaf (OIV 051), mature leaf (OIV 067, 068, 070, 076, 079, 081-2, 084, 087) and berry (OIV 223, 225). Moreover, the OIV 151 descriptor for flower sexual organs was added.

7. Anthocyanin Content and Pigment Profiles of Berry Skins

The anthocyanin content and pigment profile of berry skins for seven coloured-berry unknown genotypes were determined following the method described by Gomez-Alonso (Gómez-Alonso *et al.*, 2007) with some modifications.

Instead of separating the skin from the pulp, 100 g aliquots of entire berries were blended at maximum speed for 45 s using a 6805 model Osterizer blender (Sunbeam, FL, USA). The pigments were then extracted from 5 g of the resulting blend by adding 5 mL of a 3 % formic acid solution (CH_2O_2) to methanol (CH_3OH) (HPLC grade, Carlo Erba, Italy). After 45 min on an orbital shaker, the samples were centrifuged at 4000 rpm for 5 min, and the supernatant was filtered through a 0.45 µm RC syringe filter (Cole-Parmer Instrument Co. Europe, United Kingdom) and collected in 2.5 mL glass vials with screw caps. HPLC analyses were performed by injecting 10 µL of the sample into an HPLC system (Agilent Technologies, CA, USA) composed of an autosampler (1260 series), solvent degasser (1100 series), quaternary pump (1100 series), thermostatted column compartment (1100 series) and diode array detector (DAD, 1200 series). HPLC was driven by a Personal Computer running Agilent ChemStation for LC 3D System software (Agilent, California, USA). Anthocyanins were separated using a Luna® Omega 5 µm Polar C18 100 Å 250 x 4.6 mm column (Phenomenex, CA, USA) preceded by a guard column packed with the same material, maintained at 10° C for better separation of the pigments. Three different mobile phases were employed: mobile phase A comprised a 50 mM ammonium dihydrogen phosphate solution ($NH_4H_2PO_4$) in water (H_2O) with pH adjusted to 2.60 with 85 % phosphoric acid (H_3PO_4) (analysis grade, PanReac, Spain); mobile phase B comprised 20 % v/v of the mobile phase A in acetonitrile (C_2H_3N) (Carlo Erba, Italy); and mobile phase C comprised a 200 mM phosphoric acid solution (H_3PO_4) in water (H_2O) at pH = 1.5. The mobile phase gradient is described in (Gómez-Alonso *et al*., 2007); the chromatograms were collected at 520 nm, and the pigments were identified using peak retention times suggested by the authors. Pure standards of malvidin-3-O-glucoside chloride ($C_{29}H_{35}O_{17}Cl$ – Extrasynthèse, France) were injected to establish a calibration curve for the quantitative results.

RESULTS

1. Genetic Identification of Grapevine Samples

The molecular analyses performed on the collected plant materials allowed us to identify 79 vines, while 20 remained anonymous (Table 1): 73 samples corresponded to 36 already known *Vitis vinifera* varieties, 6 samples to 6 *Vitis* interspecific crosses, and 20 samples to 16 hitherto unreported genotypes. The 58 different SSR profiles obtained are shown in Table 2. Only Unknown 04, Unknown 08, Unknown 10 and Unknown 11 were found twice. The remaining unknown genotypes could be actual cultivars in high danger of extinction or plants obtained from single seeds. Unknown 03 is likely a hybrid (never genotyped as a rootstock), because it displays some alleles that are absent in *V. vinifera*, namely allele 237 at VVMD7, allele 133 at VMC6E1 (ISV2) locus and allele 179 at VrZAG62 (Crespan *et al*., 2009). This observation was also validated through a comparison with the up-to-date CREA-VE molecular database.

2. Statistics on SSR data and trio relationships

Diversity parameters were computed for the 12 detected SSR markers on 52 genotypes (hybrids were discarded). The results are reported in Table 3. The number of alleles found *per* locus among the samples ranged from 6 for VVMD5 and VMC6G1 (ISV4) loci to 10 for the VVMD28, VrZAG79, VMC6E1 (ISV2) and VMCNG4b9 loci, with a mean number of 8.67. The number of effective alleles is 4.896, and Shannon's index is 1.774. The mean observed heterozygosity is higher than expected (0.838 *vs* 0.789), as supported by the fixation index, negative for all loci and suggesting no inbreeding. The PIC values ranged from 0.667 for the VVMD25 locus to 0.806 for VMC6E1 (ISV2), with an average of 0.762. Overall, the combined non-exclusion probability (identity) value was very low: 5.59E-09. The probability of null alleles was negative for all loci.

Two trio combinations were found, in which the putative offspring share at least one allele *per* locus with its possible parents: Gallizzone may originate from a spontaneous cross between Farinella and Sciaccarello and Unknown 10 from Durella Gentile and Pollera Nera (Table 4). Given the low number of molecular markers used for this kind of comparison, this preliminary information needs to be reinforced with additional molecular data.

3. Genetic Similarity and Ancestry evaluation

The genetic distances computed with GenAlEx software are represented by the Principal Coordinate Analysis displayed in Figure 2. The first axis explains 9.76 % of the variation, while the second explains 7.97 % (for a total of 17.73 %). Unknown genotypes are evenly distributed in three of the plain quadrants.

The NJ tree inferred with the genetic distances (12 SSR markers from 52 genotypes) is shown in Figure 3. Even when representing the data in this way, the unknown genotypes are scattered throughout almost every branch of the tree. Interestingly, the Unknown 12 genotype can be seen to be very different from all the others and can be considered an out-group together with the Cabernet Sauvignon and Ancellotta cultivars.

When considering ancestry evaluation, the search for the best number of subpopulations (K) resulted in the most significant value being found for K = 2 (Figure 4). Seventeen genotypes stand out from the rest of the population, being or having been among the grapevine varieties typical of Liguria and Tuscany, like Albarola, Caloria, Ciliegiolo, Della Borra, Durella Gentile, Farinella, Gallizzone, Pollera Nera and Sciaccarello, plus eight of the unknown genotypes (Additional file 2). Twenty-three genotypes were grouped in the second cluster and encompassed very different varieties, from the ancient table grape Afus Ali (belonging to *proles orientalis*) to the wine grape Cabernet Sauvignon (belonging to *proles occidentalis*) (Negrul, 1946); this second group also comprised five unknown genotypes, while twelve genotypes were admixed. Observing the groups of genotypes with Q > 0.80 for increasing K values, a nucleus of four genotypes

TABLE 2. List of the 58 unique SSR profiles obtained at 12 SSR loci. Allele lengths are expressed in base pairs. Allele lengths for VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62 and VrZAG79 are provided using the VIVC allele sizing.

Part 1/2

SSR-profile ID	True-to-type Prime Name/ Unknown	SSR profile																							
Known grapevine varieties: *Vitis vinifera*		VVS2		VVMD5		VVMD7		VVMD25		VVMD27		VVMD28		VVMD32		VrZAG62		VrZAG79		VMC6E1 (ISV2)		VMC6G1 (ISV4)		VMCNG4b9	
1	Afus Ali	133	135	228	234	239	249	249	255	186	186	234	258	258	272	186	188	243	251	143	167	177	191	150	158
2	Agostenga	133	155	230	240	233	247	239	249	186	190	234	244	252	272	194	196	239	251	141	151	169	169	150	158
3	Albarola	133	155	234	238	243	263	239	241	180	182	234	244	252	262	188	194	249	259	151	165	177	177	162	168
4	Aleatico	133	135	228	230	239	249	249	255	180	195	236	246	264	272	186	196	249	255	143	143	169	169	150	158
5	Ancellotta	133	155	234	234	239	263	241	255	186	190	234	244	240	272	194	194	245	247	151	165	169	177	158	162
6	Barsaglina	133	155	248	248	239	257	241	241	182	195	234	236	256	262	194	196	249	259	141	151	177	197	150	158
7	Bonamico	133	135	228	230	253	263	241	255	184	186	234	236	252	272	200	202	239	245	151	169	169	169	150	162
8	Cabernet Sauvignon	139	151	234	242	239	239	239	249	176	190	234	236	240	240	188	194	247	247	141	165	169	191	168	176
9	Caloria	133	151	228	234	239	249	241	263	190	195	236	236	262	272	194	200	245	259	161	169	169	177	158	168
10	Canaiolo Nero	133	135	230	242	233	239	241	255	186	190	258	260	252	272	188	204	251	259	157	169	169	177	150	158
11	Chasselas Blanc	133	143	230	238	239	247	241	255	186	190	218	268	240	240	194	204	251	259	141	165	169	177	158	162
12	Ciliegiolo	133	133	228	238	247	263	241	241	180	184	234	246	252	252	194	204	245	259	141	143	177	177	158	158
13	Della Borra	133	133	228	234	239	249	239	241	184	190	236	258	258	272	194	202	245	251	165	169	187	187	158	162
14	Dolcetto	139	143	236	248	247	255	239	239	180	195	228	234	262	272	204	204	249	251	141	143	177	187	162	166
15	Durella Gentile	133	155	228	230	243	263	239	241	180	195	236	244	252	256	188	194	249	259	151	165	169	177	162	168
16	Farinella	133	151	234	234	247	249	239	241	180	182	234	236	272	272	202	204	251	259	151	165	169	187	158	162
17	Gallizzone	133	151	228	234	239	247	239	241	182	190	234	258	252	272	194	204	245	259	151	169	177	187	158	158
18	Luglienga Bianca	145	155	230	238	247	247	241	249	186	186	234	246	252	262	192	194	239	251	141	169	169	177	158	158
19	Malvasia Bianca di Candia	133	143	228	240	249	263	241	255	186	195	246	248	258	258	200	202	239	251	141	169	177	187	150	176
20	Marinello	133	139	234	234	239	249	239	243	186	190	258	268	254	272	186	200	239	245	141	151	169	187	158	162
21	Montepulciano	133	145	228	230	249	249	239	241	190	195	234	244	258	272	190	200	251	251	141	169	187	191	168	176
22	Muscat of Hamburg	135	149	234	240	247	249	249	255	180	186	236	244	272	272	186	192	239	255	141	161	169	187	158	158
23	Parmesana	133	143	238	242	243	247	241	255	186	186	246	260	252	272	188	192	243	251	169	169	169	169	150	158
24	Pollera Nera	133	151	230	230	239	249	241	263	190	195	236	258	252	262	194	200	245	259	165	169	169	177	158	168
25	Rollo	133	133	228	234	239	249	241	241	184	195	236	258	250	252	194	200	245	251	141	161	187	197	158	178
26	Sangiovese	133	133	228	238	239	263	241	241	180	186	234	244	252	256	194	196	243	259	143	165	177	197	158	168
27	Schiava Grossa	135	155	238	240	247	247	241	255	182	186	236	244	252	272	192	194	239	259	161	165	177	187	158	178
28	Schiava Lombarda	133	143	228	236	239	253	239	255	180	186	244	246	250	272	194	196	237	251	143	161	197	197	138	166
29	Sciaccarello	133	133	228	230	239	247	239	241	184	190	236	258	252	272	194	204	245	245	161	169	177	187	158	158

SSR-profile ID	True-to-type Prime Name/ Unknown	SSR profile																					
Known grapevine varieties: *Vitis vinifera*		VVS2		VVMD5		VVMD7		VVMD25		VVMD27		VVMD28		VVMD32		VrZAG62		VrZAG79		VMC6E1 (ISV2)		VMC6G1 (ISV4)		VMCNG4b9	
30	Tintoria Lloyd	133	151	238	240	239	247	249	255	190	195	228	234	252	272	194	196	245	251	151	165	169	191	158	158
31	Tocai Friulano	133	151	230	240	239	257	241	249	186	195	234	248	240	256	188	194	251	251	141	151	169	177	164	166
32	Trebbiano Toscano	133	143	228	234	249	253	241	255	180	184	244	248	250	272	194	200	245	251	141	161	177	187	162	176
33	Uva Crova	135	145	228	238	247	247	241	255	186	186	234	258	240	252	192	196	243	251	151	161	169	177	150	176
34	Verdea	133	133	236	242	247	247	239	255	180	190	236	260	250	272	194	204	245	249	141	169	187	187	158	158
35	Verdicchio Bianco	133	155	230	242	239	247	241	241	180	186	236	258	252	256	196	196	249	257	165	165	169	197	164	166
36	Vermentino	133	151	236	240	249	249	241	249	180	182	236	244	250	256	200	204	249	259	157	165	169	197	164	172
37	Vermentino Nero	135	143	228	242	239	253	255	255	186	190	234	260	252	262	188	192	239	251	165	169	169	177	158	158
Known grapevine varieties: *Vitis* interspecific crosses		VVS2		VVMD5		VVMD7		VVMD25		VVMD27		VVMD28		VVMD32		VrZAG62		VrZAG79		VMC6E1 (ISV2)		VMC6G1 (ISV4)		VMCNG4b9	
38	Baco Noir	133	145	236	268	239	265	239	239	182	208	244	258	272	272	196	200	243	255	133	143	183	197	152	158
39	Muscat de St. Christol	133	143	228	238	237	247	241	255	180	190	234	234	256	272	179	204	255	261	143	143	169	197	158	158
40	Jacquez	139	143	230	246	237	239	255	257	180	190	230	236	252	252	186	198	249	251	141	151	175	187	154	172
41	Rosette	133	147	238	246	247	251	241	243	182	187	248	258	262	262	194	194	243	261	151	151	177	181	160	168
42	Villard Blanc	133	143	234	238	237	251	241	255	182	190	234	236	240	256	179	194	255	261	133	143	187	197	150	158
Unknown grapevine varieties: *Vitis vinifera* and *Vitis* interspecific crosses*		VVS2		VVMD5		VVMD7		VVMD25		VVMD27		VVMD28		VVMD32		VrZAG62		VrZAG79		VMC6E1 (ISV2)		VMC6G1 (ISV4)		VMCNG4b9	
43	Unknown 01	133	141	228	240	243	249	241	249	180	182	234	244	250	256	188	204	249	251	165	165	169	191	172	176
44	Unknown 02	133	133	228	234	239	249	241	241	180	190	236	236	240	262	194	202	249	259	161	165	187	187	158	162
45	Unknown 03*	135	143	238	240	237*	251	255	255	182	190	234	258	240	256	179*	194	255	261	133*	151	177	177	150	158
46	Unknown 04	133	133	228	242	239	253	241	241	190	195	236	248	258	272	188	200	251	259	141	165	169	177	176	176
47	Unknown 05	133	143	228	230	239	247	241	255	180	184	258	268	252	262	194	194	245	259	161	165	169	177	158	162
48	Unknown 06	133	145	228	240	247	263	239	239	190	190	236	258	258	272	202	204	243	245	161	165	177	187	158	158
49	Unknown 07	139	143	228	234	239	247	239	255	186	190	258	268	272	272	186	204	245	251	137	141	169	177	158	166
50	Unknown 08	133	151	228	230	233	247	239	241	182	184	236	236	252	256	196	204	245	259	165	169	169	187	158	166
51	Unknown 09	133	133	228	234	247	263	239	241	180	195	234	258	252	262	192	194	249	251	143	151	169	177	158	168
52	Unknown 10	133	155	228	230	239	263	241	241	190	195	236	258	252	262	194	194	245	259	165	169	169	177	158	168
53	Unknown 11	143	143	228	228	239	247	239	255	180	190	258	258	250	272	186	194	249	251	165	169	191	197	138	162
54	Unknown 12	151	151	234	234	263	263	255	255	190	192	236	236	256	256	194	194	247	251	159	159	169	169	158	178
55	Unknown 13	133	133	228	230	247	247	241	255	184	195	234	236	252	272	196	204	245	251	165	169	177	177	158	168
56	Unknown 14	133	151	228	230	247	263	239	263	182	184	236	236	252	256	194	204	245	259	169	169	169	187	158	168
57	Unknown 15	143	145	234	236	247	253	241	255	186	190	236	244	250	262	194	194	245	251	161	165	177	197	158	166
58	Unknown 16	133	151	228	234	253	257	239	241	182	182	234	236	252	256	192	194	259	259	165	165	185	187	158	168

* Vitis interspecific crosses: typical non-vinifera alleles.

TABLE 3. Diversity parameters calculated for the 12 SSR markers from 52 genotypes (hybrids were excluded from computation) using GenAlEx and Cervus softwares. N = No. of genotypes analysed, Na = No. of different alleles, Ne = No. of effective allele, I = Shannon's Information Index, Ho = observed heterozygosity, He = expected heterozygosity, F = Fixation Index, PIC = mean polymorphic information content, NE-I = non-exclusion probability (identity), F(Null) = probability of null alleles.

Locus	N	Na	Ne	I	Ho	He	F	PIC	NE-I	F(null)
VVS2	52	9	3.487	1.628	0.769	0.713	-0.079	0.689	0.106	-0.0502
VVMD5	52	8	5.370	1.846	0.865	0.814	-0.063	0.790	0.058	-0.0385
VVMD7	52	9	5.121	1.818	0.827	0.805	-0.028	0.778	0.065	-0.0154
VVMD25	52	6	3.482	1.403	0.769	0.713	-0.079	0.667	0.128	-0.0395
VVMD27	52	8	5.622	1.812	0.885	0.822	-0.076	0.798	0.056	-0.0369
VVMD28	52	10	5.430	1.907	0.885	0.816	-0.084	0.793	0.057	-0.0397
VVMD32	52	9	5.452	1.867	0.846	0.817	-0.036	0.793	0.057	-0.025
VrZAG62	52	9	5.381	1.914	0.865	0.814	-0.063	0.795	0.054	-0.0323
VrZAG79	52	10	5.507	1.888	0.904	0.818	-0.104	0.795	0.057	-0.0533
VMC6E1 (ISV2)	52	10	5.815	1.914	0.865	0.828	-0.045	0.806	0.051	-0.0248
VMC6G1 (ISV4)	52	6	3.852	1.473	0.769	0.740	-0.039	0.697	0.111	-0.0244
VMCNG4b9	52	10	4.228	1.819	0.808	0.763	-0.058	0.743	0.076	-0.0287
Mean		8.67	4.896	1.774	0.838	0.789	-0.063	0.762		
Combined non-exclusion probability (identity)									5.59E-09	

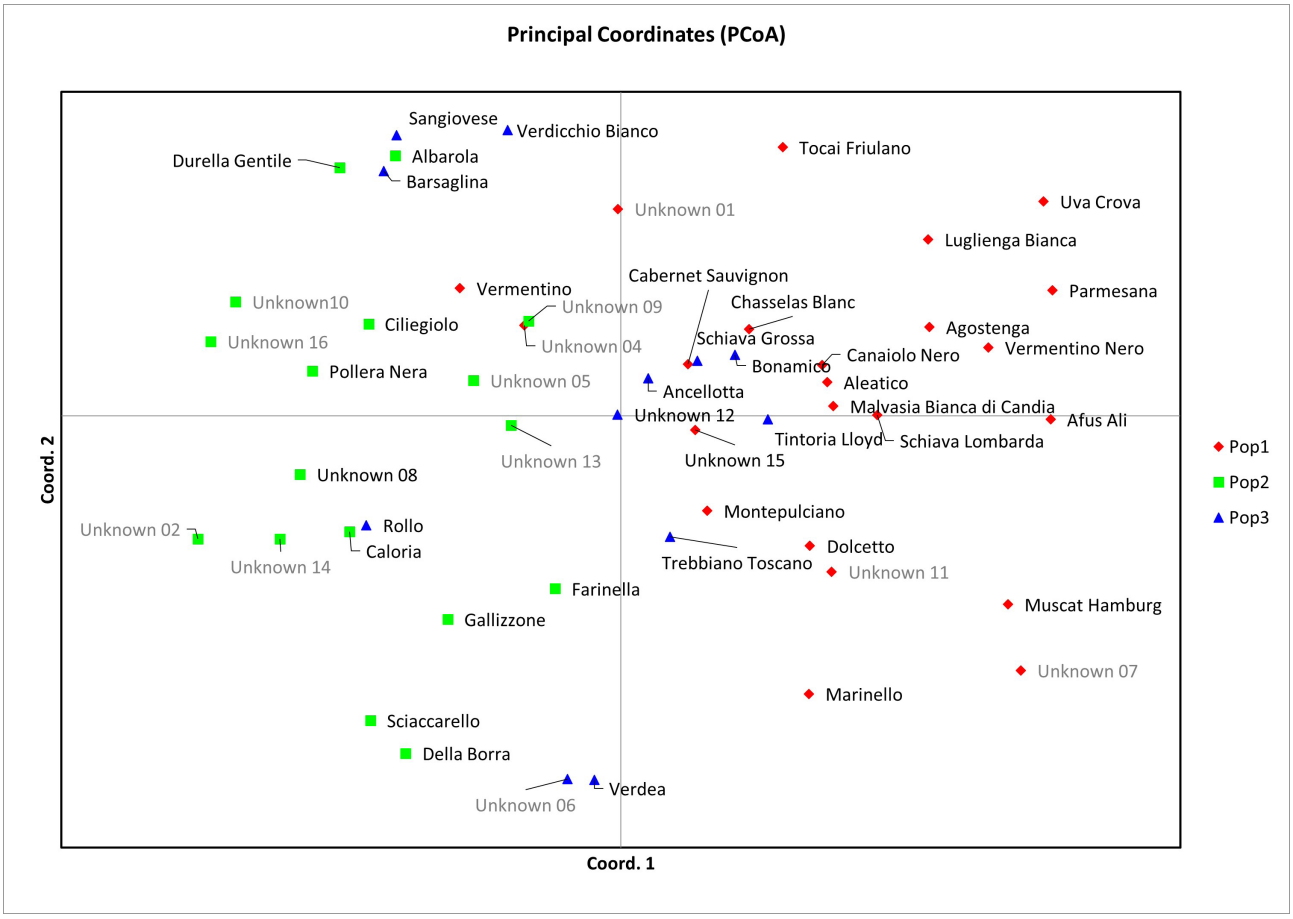


FIGURE 2. PCoA results obtained by comparing the genetic distances of 52 genotypes.

The first axis explains 9.76 % of the variation and the second 7.97 %. Pop1 and Pop2 refer to the two groups obtained for K = 2 using STRUCTURE analysis with Q > 0.8, Pop3 refers to the admixed genotypes.

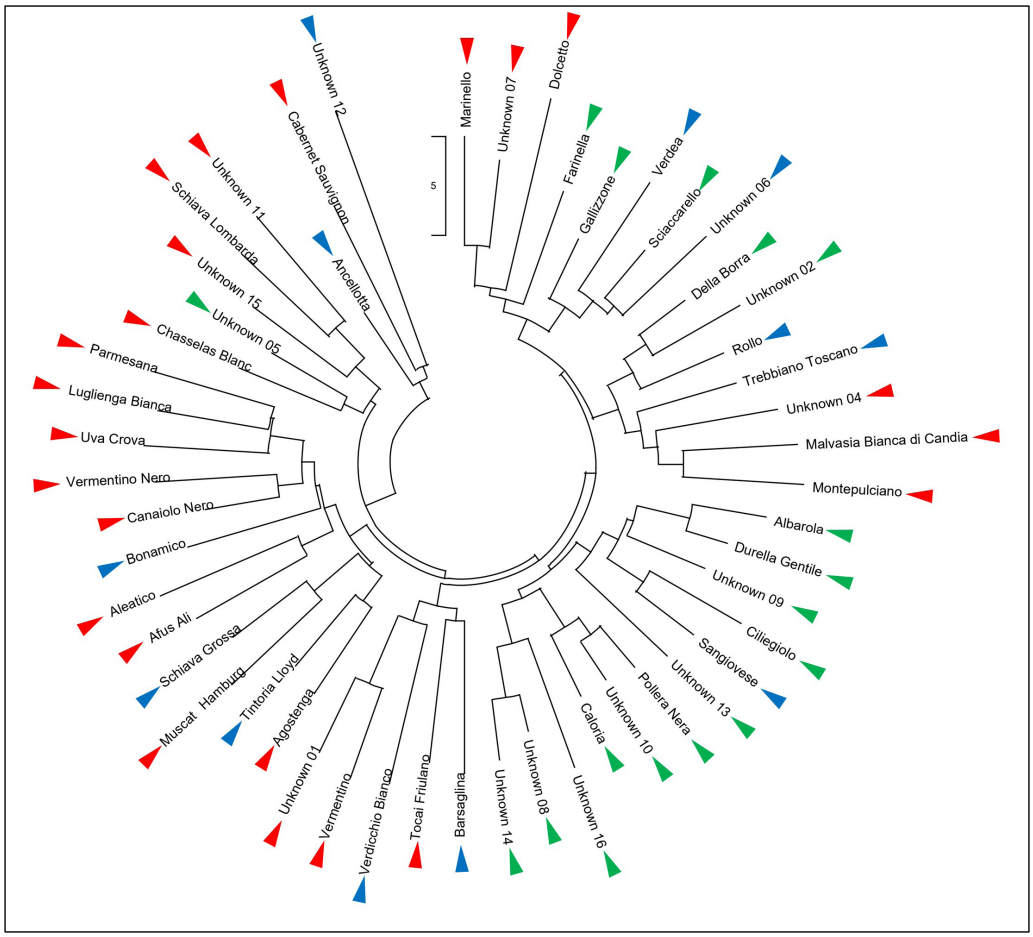


FIGURE 3. NJ tree of 52 genotypes with 12 SSR markers.

The optimal tree was inferred using the Neighbor-Joining method and MEGA X software (Kumar et al., 2018; Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the genetic distances used to infer the tree. The genetic distances were computed using GenAlEx software. The different coloured triangles refer to the results obtained from the STRUCTURE analysis for K = 2: red = Pop1, green = Pop2, blue = Pop3, admixed genotypes (Q < 0.8).

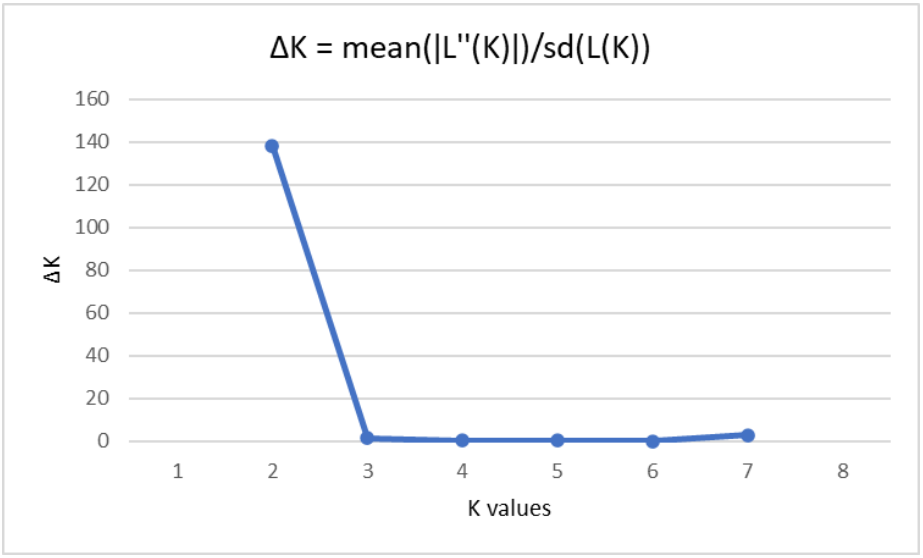


FIGURE 4. Estimate of the number of clusters obtained with STRUCTURE for K values ranging from 1 to 8 using 12 SSR.

Plot of ΔK values *versus* number of groups (K).

TABLE 4. Putative trios detected using Cervus sotfware.

Offspring	Candidate parent 1	Candidate parent 2	Trio LOD score
Gallizzone	Farinella	Sciaccarello	1.43
Unknown 10	Durella Gentile	Pollera Nera	1.24

TABLE 5. Ampelographic descriptions of 10 grapevine accessions belonging to 8 different unknown genotypes present in the vineyard germplasm collection at "Podere Scurtarola" farm, Massa (MS, Tuscany). The set of 14 standardised OIV primary descriptors plus OIV 151, grouped according to each target vine organ (young shoot: OIV 001, 004; shoot: OIV 016; young leaf: OIV 051; mature leaf: OIV 067, 068, 070, 076, 079, 081-2, 084, 087; berry: OIV 223, 225; flower: OIV 151), are shown for each SSR profile.

Sample name			81-Baldini Bianca 2	87-Marchese Grigio	88-Merlarola	91-Monferrato 92-Rossara 2	93-Nera 4 94-VL5	97-Schiava Bianca	98-Tané	99-Uva Rosa
SSR profile ID			44 Unknown 02	49 Unknown 07	50 Unknown 08	52 Unknown 10	53 Unknown 11	56 Unknown 14	57 Unknown 15	58 Unknown 16
Primary Descriptor	OIV Code	Characteristic	Description (Note)	Description (Note)	Description (Note)	Description (Note)	Description (Note)	Description (Note)	Description (Note)	Description (Note)
Young shoot	1	Opening of the shoot tip	Fully open (5)	Fully open (5)	Fully open (5)	Fully open (5)	Fully open (5)	Fully open (5)	Fully open (5)	Fully open (5)
	4	Density of prostrate hairs on the shoot tip	Medium (5)	Low (3)	Low (3)	High (7)	Medium (5)	Medium (5)	Low (3)	Medium/High (5-7)
Shoot	16	Number of consecutive tendrils	2 or less (1)	2 or less (1)	2 or less (1)	2 or less (1)	2 or less (1)	2 or less (1)	2 or less (1)	2 or less (1)
Young leaf	51	Colour of upper side of blade (4th leaf)	Green (1)	Green (1)	Yellow (2)	Green (1)	Bronze (3)	Yellow (2)	Bronze (3)	Bronze/ Copper reddish (3-4)
Mature leaf	67	Shape of blade	Wedge-shaped (2)	Circular (4)	Pentagonal (3)	Pentagonal (3)	Pentagonal (3)	Circular (4)	Pentagonal (3)	Circular (4)
	68	Number of lobes	Five (3)	Five (3)	Three (2)	Five (3)	Five (3)	Three (2)	Five (3)	Five/Seven (3-4)
	70	Area of anthocyanin colouration of main veins on upper side of blade	Only at the petiolar point (2)	Absent (1)	Absent (1)	Absent (1)	Five (3)	Absent (1)	Absent (1)	Up to the 1st bifurcation (3)
	76	Shape of teeth	Both sides convex (3)	Both sides convex (3)	Both sides convex (3)	Both sides straight (2)	Both sides straight (2)	Both sides straight (2)	Both sides convex (3)	Mixture between both sides straight and both sides convex (5)
	79	Degree of opening/ overlapping of petiole sinus	Very wide open/Open (1-3)	Open (3)	Closed (5)	Closed (5)	Closed (5)	Closed (5)	Open (3)	Overlapping (7)
	081-2	Petiole sinus base limited by vein	Not limited (1)	Not limited (1)	Not limited (1)	Not limited (1)	Not limited (1)	Not limited (1)	Not limited (1)	Not limited (1)
	84	Density of prostrate hairs between main veins on lower side of blade	Low (3)	Low (3)	Medium (5)	High (7)	None or very low (1)	Low (3)	None or very low (1)	High (7)
	87	Density of erect hairs on main veins on lower side of blade	Low (3)	Low (3)	None or very low (1)	None or very low (1)	None or very low/Low (1-3)	Low (3)	None or very low (1)	None or very low (1)
Berry	223	Shape	Globose (2)	Globose (2)	Globose (2)	Globose (2)	Globose (2)	Globose (2)	Globose (2)	Globose (2)
	225	Colour of skin	Green yellow (1)	Grey (4)	Blue black (6)	Dark red violet (5)	Blue black (6)	Green yellow (1)	Pink (2)	Dark red violet (5)
Flower	151	Sexual organs	Fully developed stamens and fully developed gynoecium (3)	Fully developed stamens and fully developed gynoecium (3)	Fully developed stamens and fully developed gynoecium (3)	Fully developed stamens and fully developed gynoecium (3)	Fully developed stamens and fully developed gynoecium (3)	Fully developed stamens and fully developed gynoecium (3)	Fully developed stamens and fully developed gynoecium (3)	Fully developed stamens and fully developed gynoecium (3)

remained fixed up to K = 8; these were Caloria, Pollera Nera, Sciaccarello and Unknown 14.

Overall, a confluence between the PCoA (Figure 2) and STRUCTURE results was noted. Interestingly, the group of 17 genotypes found with STRUCTURE analysis for K = 2 occupies the left side of the PCoA plain, even if not in an exclusive way. Moreover, several unknown genotypes enlarge the plain of genetic distances much more than renowned varieties to be different from each other, like Afus Ali and Cabernet Sauvignon; two additional unknown genotypes are positioned on the left-hand side, opposite Afus Ali and Cabernet Sauvignon.

4. Ampelographic Descriptions within the Grapevine Vineyard Collection

As a result of the profitable activity of local grapevine germplasm recovery implemented over the years, the vineyard collection has come to host more than half of the samples discovered (52 out of 99) following vegetative multiplication and planting. Currently, the vineyard contains 39 known and 12 unknown grapevine accessions, plus one *Vitis* interspecific cross. Among these, 47 accessions have over-3-year-old vines: 36 known grapevine varieties belonging to 18 different genotypes, 1 *Vitis* interspecific cross (Jacquez), and 10 belonging to 8 unknown genotypes, including a putative hybrid, Unknown 03 (Table 1). The ampelographic characteristics of adult vines were assessed and are shown in Table 5, limited to the 8 unknown genotypes. As for the 19 known genotypes, we verified that the morphology of the main organs correctly corresponded to the ampelographic descriptions already present in the official databases (data not shown, available on request).

5. Phenotypic description of the grapes for unknown genotypes: anthocyanin contents and pigment profiles

Seven unknown genotypes with coloured berries were characterised at the phenotypic level by analysing the quantity and quality of the skin anthocyanins (see Table 6).

The anthocyanin content was highly variable and reflected the visual characteristics detected on the bunch (Table 5, OIV descriptor 225). The genotypes with grey and pink berries (Unknown 07 and Unknown 15) exhibited only small amounts of anthocyanins, whereas quantities 20 times higher were found in Unknown 10 and Unknown 11.

Malvidin-3-O-glucoside was the most prevalent anthocyanin in Unknown 11 and Unknown 16, accounting for 45 % and 50 % respectively of total anthocyanins. Furthermore, these two genotypes shared a similar profile; in both cases, trisubstituted anthocyanins made up approximately 50 % of the total, and the proportion of acylated anthocyanins was around 36 %, with malvidin-3-O-p-coumaroylglucoside representing more than 20 % of total anthocyanins. Unknown 07 also had a higher percentage of trisubstituted anthocyanins, but with a more homogeneous distribution among the different pigments. The most represented were

TABLE 6. Anthocyanin content and pigment profiles of berry skins.

Sample	Unknown 07	Unknown 08	Unknown 10	Unknown 11	Unknown 13	Unknown 15	Unknown 16
Anthocyanins (mg/Kg)	80	1083	1928	2077	914	93	327
Delphinidin-3-O-glucoside (%)	24.5	3.5	3.3	7.0	6.8	3.6	4.1
Cyanidin-3-O-glucoside (%)	20.3	42.8	7.4	0.8	9.8	68.9	0.5
Petunidin-3-O-glucoside (%)	9.0	3.2	3.1	6.2	5.4	2.9	4.7
Peonidin-3-O-glucoside (%)	10.6	36.3	60.4	4.8	36.5	4.9	4.4
Malvidin-3-O-glucoside (%)	21.5	6.7	22.2	45.0	31.1	11.5	50.0
Delphinidin-3-O-acetylglucoside (%)	0.0	0.4	0.2	0.3	0.3	0.0	0.2
Cyanidin-3-O-acetylglucoside (%)	0.0	1.2	0.2	0.1	0.3	0.5	0.1
Petunidin-3-O-acetylglucoside (%)	0.0	0.2	0.0	0.3	0.5	0.0	0.3
Peonidin-3-O-acetylglucoside (%)	0.0	1.3	0.2	0.3	1.3	0.4	0.4
Malvidin-3-O-acetylglucoside (%)	3.2	0.5	0.2	3.8	1.8	1.8	9.6
Delphinidin-3-O-p-coumaroylglucoside (%)	4.0	0.2	0.1	1.9	0.3	0.5	0.1
Malvidin-3-O-caffeoylglucoside (%)	0.0	0.3	0.2	1.0	0.5	0.7	1.7
Cyanidin-3-O-p-coumaroylglucoside (%)	1.6	1.4	0.0	0.5	0.5	0.0	0.1
Petunidin-3-O-p-coumaroylglucoside (%)	0.0	0.2	0.1	2.2	0.2	0.4	1.4
Peonidin-3-O-p-coumaroylglucoside (%)	1.5	1.4	1.8	2.0	2.0	0.9	1.8
Malvidin-3-O-p-coumaroylglucoside (%)	3.8	0.4	0.6	23.8	2.7	3.0	20.6
Trisubstituted anthocyanins[1] (%)	55.0	13.4	28.6	58.2	43.3	18.0	58.8
Acylated anthocyanins[2] (%)	14.1	7.5	3.6	36.2	10.4	8.2	36.3

[1] Sum of Delphinidin-3-O-glucoside, Petunidin-3-O-glucoside and Malvidin-3-O-glucoside.

[2] Sum of acetylglucoside, caffeoylglucoside and p-coumaroylglucoside anthocyanins.

delphinidin-3-O-glucoside, cyanidin-3-O-glucoside and malvidin-3-O-glucoside, each with values above 20 %. Approximately 14 % of acylated anthocyanins were almost entirely represented by malvidin-3-O-acetylglucoside, delphinidin-3-O-p-coumaroylglucoside and malvidin-3-O-p-coumaroylglucoside. Unknown 08, 10 and 15 were characterised by a low percentage of trisubstituted anthocyanins. In Unknown 15, cyanidin-3-O-glucoside represented almost 70 % of the pigments, and, except for malvidin-3-O-glucoside (11.50 %), no other anthocyanin reached 5 %. Peonidin-3-O-glucoside represented approximately 60 % of the anthocyanins of Unknown 10, followed by malvidin-3-O-glucoside (22 %) and cyanidin-3-O-glucoside (7.4 %), while acylates were the lowest among all the samples (3.6 %). Unknown 08 was the genotype with the lowest percentage of trisubstituted anthocyanins, owing to the high percentages of cyanidin-3-O-glucoside and peonidin-3-O-acetylglucoside, which together accounted for almost 80 % of all anthocyanins. Finally, the most abundant anthocyanins in Unknown 13 were malvidin-3-O-glucoside and peonidin-3-O-glucoside, which, with similar percentages, constituted approximately 70 % of the profile. The percentages of trisubstituted and disubstituted anthocyanins were similar, while acylated anthocyanins represented about 10 % of the total pigments.

DISCUSSION

1. Research background, safeguard of viticultural germplasm reservoirs and biodiversity genetics

Italian viticulture is characterised by a surprising richness of grapevine varieties, the legacy of centuries of natural selection and human cultivation, considering that the winegrowing tradition in this country dates back to the Protohistoric Age (Crespan *et al*., 2023; Mannini, 2004). Rescuing forgotten grapevines and characterising retrieved unknown genotypes can contribute to providing a valuable source of viticultural biodiversity in the face of climate change (Van Leeuwen and Destrac-Irvine, 2017), as well as putative missing links in kinship studies of the noblest vines (Raimondi *et al*., 2020) and a cultural and productive heritage for their place of origin (Gutiérrez-Gamboa *et al*., 2020; Mannini, 2004). Beyond studies that have already been performed on grapevine germplasm collections and that give a general idea of existing biodiversity (Cipriani *et al*., 2010; Cretazzo *et al*., 2022; Emanuelli *et al*., 2013; Laucou *et al*., 2011; Maraš *et al*., 2014), there are several recent or ongoing studies in Italy based on SSR genotyping related to the *in situ* discovery of niche varieties that have survived in circumscribed remote environments (Fanelli *et al*., 2021; Pastore *et al*., 2020; Zombardo *et al*., 2021).

The coastal area of Tuscany (less famous than other wine districts in this region, such as Chianti and Montalcino), where this research project was implemented, has a strong tradition in viticulture that has undergone a sharp decline due to both environmental and anthropogenic factors (Schultz and Jones, 2010; Storchi *et al*., 2018). The study carried out in old, abandoned vineyard plots scattered across the Candia dei Colli Apuani, Garfagnana, Lunigiana, and Levante Ligure territories (Figure 1) allowed us to correctly identify the 99 grapevine samples collected over the years, obtaining an approximate idea of the past varietal composition. In detail, we highlighted some cases of synonymy between official and local names, found mismatches with the putative varietal name reported for some samples and identified 16 hitherto unreported genotypes (Table 1).

Interesting information emerged from the in-depth analysis of the obtained molecular results. Primarily, the number of alleles *per* locus revealed a vast biodiversity within the recovered pool of samples (Table 3). Even the identified trio combinations provide intriguing insights. When considering the possible origin of Gallizzone as a spontaneous cross between Farinella and Sciaccarello (Table 4), it should be noted that the latter variety was deemed one of the main parent varieties within the assortment of Italian grapevine germplasm, especially regarding central Italy (Crespan *et al*., 2023). Therefore, this preliminary indication is well supported by available information, at least on one of the putative parent candidates.

An ancestry evaluation was carried out using available data. Considering that the Italian grapevine germplasm was found to be admixed, until a clear distinction emerged from the different evolutionary history between Northern and Southern Italian grapevines (Mercati *et al*., 2021), and that the search for subgroups is usually applied to hundreds of genotypes (Bacilieri *et al*., 2013; Cipriani *et al*., 2010; Cretazzo *et al*., 2022), we nevertheless obtained interesting results from our limited population. The split of our pool of samples into two sub-populations allowed us to highlight one first group of 17 genotypes, of which eight (Caloria, Della Borra, Gallizzone, Pollera Nera, Sciaccarello, and three unknown genotypes) remained constantly joined, even for increasingly clustered groups (up to 7), until a complete population destructured for K = 8. It is worth noting that Della Borra, Pollera Nera, and Caloria have been recognised as progenies of the barely mentioned Sciaccarello (D'Onofrio *et al*., 2021). Given the restricted geographical area of sample collection, we can assume these genotypes to be the main grapevine varieties characterising the study area.

2. Contextualised information about known genotypes

Of the known grapevine varieties retrieved in the sample pool, the most represented genotypes, with at least five individuals each, were Vermentino (as expected), Agostenga, Barsaglina and Ciliegiolo. Vermentino is the most cultivated variety in this geographical area, and the white easy-to-drink wines produced from its grapes on the Tuscan coast are highly appreciated. For this reason, it was included in the vineyard collection as a reference variety. Agostenga is an early-ripening, white-berried variety grown today exclusively in some high mountain areas of Aosta Valley (where it is known as Prié Blanc or Blanc de Morgex). Recently, a synonymy with the Spanish cultivar Legiruela was confirmed, suggesting that it was moved to very

geographically distant areas several centuries ago (Schneider *et al*., 2010). All five samples were taken from an area of mid-elevation in the province of Lucca (Minucciano, about 700 a.s.l.). In Italy, Agostenga has been identified among relic vines in Garfagnana in previous studies (D'Onofrio et al., 2016), as well as in Southern Umbria (Zombardo *et al*., 2021), suggesting that in Central Italy, while no longer in existence, there was a minimal presence of this cultivar in the past. This sporadic presence may be due to the introduction of Agostenga as a grape for fresh consumption, as suggested by the reference to the month of August in its name.

Barsaglina is a minor, black-berried variety from Massa Carrara, hence the official synonym Massaretta (Robinson *et al*., 2013). This variety is cultivated in an area of less than 20 hectares located between Tuscany and Liguria, according to the last Italian census (2010); however, there are still concrete traces of a past diffusion of this variety in the territories considered.

Ciliegiolo (Italian for "small cherry", referring to the cherry aroma of its grapes), already cited as being one of the varieties grown around Florence in the 1600s (Soderini, 1590) is nowadays quite widespread in many Italian regions. It is now known that Ciliegiolo descends from Sangiovese and Muscat Rouge de Madère (or Moscato Violetto) (Cipriani et al., 2010; D'Onofrio et al., 2021).

Two more represented grapevine varieties in the pool of collected samples are Albarola and Bonamico. The first is a white-berried, late-ripening variety typical of "Cinque Terre" (Torello Marinoni *et al*., 2009). The second is a high-yield, late-ripening black variety spread all over Tuscany in the past, but now just hanging on in old vineyards in the Pisa, Pistoia and Lucca provinces (Robinson *et al*., 2013). Three out of the four assigned sample names were incorrect, as both Colombana and Groppello are different varieties, genetically distant from Bonamico.

Three samples collected in the municipality of Pontremoli (MS) were called "Morone" (Table 1); however, our DNA analyses revealed Morone 2 and Morone 3 to be Parmesana, and Morone 1 (with 2 other samples from Garfagnana) to belong to the Uva Crova genotype. Parmesana is a minor vine of the Emilia Romagna region (central Italy), which can be found under the name of Brugnera or Covretto in the area of Reggio Emilia (Pastore *et al*., 2020; Raimondi *et al*., 2015), Cavazza in Modena and Pallona/Giuncanesa in the bordering Garfagnana (D'Onofrio *et al*., 2016), all regions under the aegis of the Este Duchy in the past. Uva Crova is a vine retrieved in Candia dei Colli Apuani, historically found in Garfagnana under the name of Carraresa (D'Onofrio *et al*., 2016; Roncaglia, 1850) and in the Reggio Emilia province as Covra (Pastore et al., 2020). Despite the two different genetic profiles, these locally named Morone samples are all included in the "Crova, Covra, Covretto, Croa, and Croatina" family, given that the grapes have some morphological characteristics in common (Rossoni *et al*., 2001).

Three samples, all found in Lunigiana, belonged to the Pollera Nera genotype, one of the most representative cultivars of this area, where it is historically present (Acerbi, 1825). A recent kinship analysis revealed Pollera Nera to descend from the parent Sciaccarello, which was retrieved in this work along with some of its proven offspring: Caloria (which is also known under the generic synonym of Pollera, but has a different genetic profile from Pollera Nera), Della Borra (also locally known as Bracciola Nera, but genetically distinct from the "official" Bracciola Nera accession 1639 on *V*IVC), and Rollo (official synonym of Livornese Bianca in Tuscany, or Bruciapagliaio in Liguria) (Crespan *et al*., 2021; D'Onofrio *et al*., 2021; Di Vecchi Staraz *et al*., 2007; Torello Marinoni *et al*., 2009).

Two samples corresponding to Muscat of Hamburg were also found. This fine black table grape variety (progeny of Schiava Grossa x Muscat of Alexandria) (Crespan, 2003) is historically cultivated in Tuscany (mainly on the Lucca hills), where it is still used as a wine grape. This interesting example highlights how varieties of exogenous origin introduced to the local assortment become part of the production of niche wines that distinguish and enhance a small wine-growing region.

Vermentino Nero is a minor traditional vine of the province of Massa Carrara (Roncaglia, 1850) which is nearly extinct. It is nowadays grown within a few hundred hectares limited to this area and in Lunigiana, where it is also known as Merla. Vermentino Nero has a low degree of kinship with the much better-known white Vermentino; however, recent results indicate Vermentino Nero has a close genetic link with Canaiolo Nero, assuming that these two varieties share one still unknown parent (Raimondi *et al*., 2020).

The finding of six different *Vitis* interspecific crosses (five proven; 1 putative: Unknown 03) indicates that there was a sizeable cultivation of direct-producer hybrids in the past, even in central Italy (Zombardo *et al*., 2021). During the last century, hybrid vines were employed for stemming fungal diseases of foreign origin, but their use was gradually abandoned and even banned in the European Union in 1979 (Kapusta *et al*., 2018).

3. Unknown genotypes: genetic and phenotypic characteristics

The proportion of unknown grapevine varieties sourced within the pool of samples was quite significant (20 out of 99), which is more than a fifth of the total amount. This demonstrates that grapevines that do not conincide with any genotype included in the official databases had some relevance in the early 20th century. Some samples had given names that turned out to be incorrect, not corresponding with the putative variety (e.g., Schiava Bianca and Vite Mammolo). And for some others valid matches were found by comparing the ampelographic descriptions with the literature of the past (e.g., Tané, and Rossara/Monferrato) (Roncaglia, 1850).

In addition to the ampelographic description of the main vine organs (using the set of 14 standardised OIV primary descriptors), we chose to examine the anthocyanin profile of berry skins in our preliminary phenotypic evaluation of unknown genotypes. This parameter represents a relatively

stable quality trait that is an expression of some variety-dependent genes (Massonnet *et al*., 2017) and can be used for chemotaxonomic classifications of red-berried *Vitis Vinifera* cultivars (Mattivi *et al*., 2006). In red wine grapes, higher percentages of trisubstituted anthocyanins (delphinidin-3-O-glucoside, petunidin-3-O-glucoside, and malvidin-3-O-glucoside), as well as a high proportion of acylated form of anthocyanins (acetyl-glucosides, caffeoyl-glucosides, and p-coumaroyl-glucosides) are desirable, since they enhance colour stability and confer a better aging capacity to wines (Cheynier *et al*., 2006; Wang *et al*., 2023; Zhao *et al*., 2017).

The results (Table 6) indicate that the most valuable genotype for red winemaking is Unknown 11, due to its high percentage of malvidin-3-O-glucoside and acylated pigments. Unknown 16 has an equally interesting anthocyanin profile, despite its low anthocyanin content (mg/kg). Such composition can be crucial in conferring good stability to the colour matrix, enabling the production of wines with a vibrant pink hue.

CONCLUSIONS

The results of this research have shed some light on the composition of the autochthonous grapevine germplasm of Candia dei Colli Apuani, Garfagnana, Lunigiana and, to a lesser extent, Levante Ligure. It was possible to identify and characterise numerous known and unknown genotypes and to create a valuable vineyard collection that includes most of them. The exploitation of the precious grapevine reservoir provided by these wine-growing regions has ensured the protection of emblematic cultivars thought to be extinct or to have almost disappeared. Our work has laid down solid foundations for the potential use of certain grapevine varieties that were probably once preferred and can henceforth be cultivated again to help revive the eco-wine tourism of the Tuscan heroic vineyards.

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