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The use of AFLP and SSR molecular markers to decipher homonyms and synonyms in grapevine cultivars: the case of the varietal group known as "Schiave"

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Abstract ''Schiave'' collectively refers to grapevine cultivars presently grown on the Southern and Northern slopes of the Eastern Alps and bearing different names (Schiava, Trollinger, Rossara, Rossola, Geschlafene, Gansfüsser, Urban and others). Their common origin has been suggested by historic, linguistic and ampelographic considerations. This hypothesis has now been assayed by using more direct approaches based on AFLP and SSR analysis. The present paper shows: (1), that AFLP and SSR give comparable results when used to study genomic similarity among the Schiave grapevine cultivars, and (2) that ''Schiave'' is used to group grapevine cultivars that are genetically heterogeneous. In fact, a dendrogram constructed from an AFLP analysis of the 33 best-known Schiave (or correlated) cultivars, shows different, and in some cases relevant, degrees of genomic dissimilarity. The analysed cultivars cluster into at least five taxonomic groups with specific geographic distribution along the valleys of Valtellina, Bergamo and Brescia and those of South Tyrol and Swabia. It is concluded that the common definition ''Schiave'' refers to a similar cultivation practice in contiguous regions rather than to a common genetic background.

Keywords *Vitis vinifera* L. · AFLP · SSR · Schiave

Introduction

About 5000–8000 grapevine (*Vitis vinifera* L.) cultivars are presently classified (Alleweldt 1997). Diffusion from their site of origin, presumably in the Middle East Asia, and differentiation of cultivated grapevine in the last 2000 years, followed routes that essentially parallel the development of civilisation. The study of biodiversity within the existing cultivars is relevant for preserving germplasm, identifying cultivars and planning breeding programmes. Historic evidence, together with ampelographic data and with the analysis of variety homonyms and synonyms have frequently been used to infer the origin and relationships of domesticated grapevines. However, conclusions on these grounds are frequently questioned: synonyms and phylogeny need verification and identification, and mistakes need to be corrected.

The availability of new molecular tools to screen biodiversity among plant genomes (Karp et al. 1998) provides a more direct approach to address these questions. Some of these tools have already proven useful for the analysis of the grapevine DNA. Bourquin et al. (1993) and Bowers et al. (1996) verified the effectiveness of RFLP (restriction fragment length polymorphism) analysis. PCR (polymerase chain reaction)-based approaches have proven more versatile and less labour-consuming. RAPD (random amplified polymorphic DNA) analysis has been used for the study of genetic similarity among grape cultivars (Mulchay et al. 1995; Xu et al. 1996; Stavrakakis and Biniari 1998), its limitation being that it is not adequately reproducible in different laboratories (Botta et al. 1995; Jones et al. 1997). The AFLP (amplified fragmnt length polymorphism) approach was used (Sensi et al. 1996) and recommended (Cervera et al. 1998) for the characterisation of grapevine genetic resources since it allows simultaneous analysis of a large number of loci in a single assay and provides stable and reproducible patterns of band amplification. Microsatellite simple sequence repeat (SSR) analysis has also been used to the same purpose (Botta et al. 1995; Sefc et al. 1997). The latter approach, which amplifies hypervariabile regions containing tandemly arranged di-, tri- or tetranucleotide repetitions, is expected to detect cryptic levels of DNA variation among closely related genomes (Bowers et al. 1996; Powell et al. 1996). SSR analysis depends on

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the identification of simple sequence repeats and of their border sequences (Bowers et al. 1996, 1999).

The present paper addresses two questions: (1), do AFLP and SSR analyses of grapevine genomes give comparable results when used to study grapevine biodiversity, and (2) does the name ''Schiave'' group genetically related grapevine cultivars?

Grapevine cultivars collectively referred to as ''Schiave'' (or ''Vernatsch'' in Tyrolean) are presently grown in the Italian regions of Lombardia, Veneto and Friuli, and the South Tyrol in Austria and in Germany. The origin of the name ''Schiave'', together with local modifications has been widely debated. One hypothesis is that it groups grapevines of a common origin from the ancient state of Slavonia, in Eastern Europe (from which Sclava or Schiava originate). A second hypothesis is that the collective name has nothing to do with genetics, but rather refers to common agricultural practices, such as intensive pruning and training as small plants. In fact ''Schiave'' sounds like ''slaves'' in English, *i.e.* deprived of its natural habit of growth, as frequently used for grapevine in the Middle Ages, and grown in constraint. This agricultural training contrasts with traditional viticulture based on light pruning and large plant dimensions. Molecular data will show that the second hypothesis is correct. They also show that the AFLP and SSR tools are equally effective for studies on grapevine biodiversity.

Materials and methods

Biological material

The 33 grapevine (*V. vinifera* L.) cultivars were from different collections, as specified in Table 1.

DNA extraction

Young leaves were harvested from rooted cuttings and stored in NDS buffer (0.6 M EDTA, 10 mM Trizma base, 1% N-laurylsarcosine) for up to 30 days at 4°C. Genomic DNA was extracted following the QIAmp Tissue Protocol (Qiagen, Dassel, Germany). The recovered DNA was further purified using the Elu-Quik DNA Purification kit (Schleicher and Schuell, Dassel, Germany).

AFLP analysis

AFLP analysis (Vos et al. 1995) of the purified DNA preparations was performed as described in the European Patent 0534858 (Keygene, Belgium). In particular, DNA (1 µg) was digested for 16 h with *Mse*I (4-base cutter) and *Pst*I (6-base cutter). DNA fragments were ligated (with 1 unit of T4 DNA ligase for 3 h at 37°C) to biotinylated *PstI* (5 pmol) and non-biotinylated *Mse*I (50 pmol) adapters (Vos et al. 1995). Biotinylated fragments were bound to paramagnetic Streptavidine Dynabeads M-280 (Dynal, Oslo, Norway) and washed 3-times with STEX buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 1 mM EDTA and 0.1% Triton X-100). The beads were suspended in 200 µl of 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0).

Table 1 Reference cultivars used for the SSR and AFLP analysis, area of cultivation and site of germplasm collection

No.	Cultivar	Area of cultivation	Germplasm collection[a]
1	Blau Affenthaler	Würtenberg, Swabia	WE
2	Blau Trollinger	Würtenberg, Swabia	LB
3	Blau Gansfüsser	Würtenberg, Swabia	LB
4	Buchhölzer Vernatsch	Alto Adige, Italy	LB
5	Geschlafene	Alto Adige, Italy	LB
6	Weiβ Hennish	Austria and Germany	WE
7	Rot Heunisch	Austria and Germany	LB
8	Weiβ Heunish	Austria and Germany	LB
9	Muskat Trollinger	Würtenberg, Swabia	WE
10	Pavana	Valsugana, Trentino, Italy	ISMA
11	Pignola bergamasca	Bergamo, Italy	AP
12	Pignola Berolda	Valtellina, Italy	FF
13	Pignola Oltrepo'	Oltrepo' Pavese, Italy	CIVIFRUCE
14	Pignola friulana	Friuli, Italy	ISPERVIT
15	Rossara nera	Trentino, Italy	ISMA
16	Rossara bianca	Veneto, Italy	ISPERVIT
17	Rossera	Bergamo, Italy	CVVP
18	Rossola Motti	Valtellina, Italy	FF
19	Rossola Tona	Valtellina, Italy	FF
20	Rossolino nero	Valtellina, Italy	FF
21	Schiava '900	Lombardia, Italy	AP
22	Schiava bianca	Lombardia, Italy	CVVP
23	Schiava dolce	Lombardia, Italy	AP
24	Schiava grigia	Alto Adige-Trentino, Italy	LB
25	Schiava grossa	Alto Adige-Trentino, Italy	ISMA
26	Schiava lombarda	Lombardia, Italy	CVVP
27	Schiava nostrana	Lombardia, Italy	AP
28	Schiavetta	Veneto, Italy	ISPERVIT
29	Trollinger	Würtenberg, Swabia	LB
30	Urban	Würtenberg, Swabia	WE
31	Brugnola	Parma, Brescia, Italy	FF
32	Weiβer Hortling1	Alto Adige, Italy	LB
33	Weiβer Hortling2	Alto Adige, Italy	WE

[a] LB: Experimental Centre of Laimburg, Bolzan, Italy; AP: Experimental Centre of the Amministrazione Provinciale, Brescia, Italy; FF: Centro Sperimentazione Agraria, Fondazione Foianini, Sondrio, Italy; IASMA, Agricultural Research Institute, S. Michele all'Adige, Trento, Italy; WE: Staatliche Lehr- und Versuchsanstalt, Weinsberg, Germany; CIVIFRUCE, Regional Centre of Agriculture, Riccagioia, Pavia, Italy; ISPERVIT: Experimental Institute of Viticulture, Cornegliano Veneto, Italy; ICA: Istituto Coltivazioni Arboree, University of Milan, Italy; CVVP: Centro Vitivinicolo Provinciale, Brescia, Italy

PCR amplification

Was performed using four primer combinations, *Pst*I 1-*Mse*I 1, *Pst*I 2-*Mse*I 2, *Pst*I 3-*Mse*I 3, *Pst*I 2-*Mse*I 3 (Table 2). Amplification was in a 10-µl vol containing 10 ng of DNA, 50 ng of primer *Pst*I, 50 ng of primer *Mse*I, 200 mM of each dNTP, (dCTP was labelled with γ-^{33}-P), 1.5 mM $MgCl_2$, 1 µl of 10 × PCR buffer (Bioline, London, U.K.) and 0.5 units of Bio-X-Act polymerase (Bioline, London, UK). Amplification was with a PTC 100 thermal controller (MJ Research Inc., USA) with 20 cycles of denaturation (30 s at 94°C), annealing (30 s at 65°C, with a decrease of 0.7°C in each cycle) and elongation (1 min at 72°C) followed by 23 cycles of denaturation (30 s at 94°C), annealing (30 s at 56°C) and elongation (1 min at 72°C). The PCR was concluded by a final step of 30 min at 65°C.

SSR analysis

DNA was analysed at five microsatellite loci: VVS1, VVS2, VVS3, VVS4 andVVS5 (Thomas and Scott 1993). The PCR amplification mixture contained, in a total volume of 25 µl, 0.25 µM of the DNA primer specified for each microsatellite locus by Thomas and Scott (1993), 200 µM of each dNTP, 100 ng of purified grapevine DNA, 0.5 U of Dynazyme (Celbio, Italy) and Dynazyme buffer as specified by the supplier. Amplification was with a PTC 100 thermal controller (MJ Research Inc., USA) with 30 cycles of denaturation (60 s at 94°C), annealing (50 s at 52°C) and extension (1 min at 72°C). The reaction was concluded by a final step of 7 min at 72°C.

Analysis of the DNA amplification products

In the case of AFLP, 1.5 µl of the PCR-amplified mixture was added to an equal volume of loading buffer (80% formamide, 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue, 10 mM EDTA, pH 8.0), denatured for 5 min at 92°C, loaded onto a 4.5% sequencing polyacrylamide gel and electrophoresed in TBE (1 M Trizma base, 1 M boric acid, 20 mM EDTA, pH 8.0) electrophoresis buffer for 3 h at 50 W. The gel was fixed in 10% acetic acid and exposed to an X-ray film.

In the case of the SSR analysis, 10 µl of the PCR-amplified mixture was analysed by denaturating electrophoresis onto a 10% polyacrylamide gel in TBE buffer for 16 h at 100 mV. The gel was stained in 0.5 µg/ml of ethidium bromide and analysed in a Gel Doc 2000 (Biorad, USA). In both cases bands in the gels were found to be reproducible in separate analyses.

Data analysis

AFLP fingerprints were evaluated by visual inspection of autoradiograms. DNA bands were scored for their presence (1) or absence (0) and the resulting data matrices were analysed using the NT-SYS PC program (F.J. Rohlf, Exeter, Software Setauket, USA, 1993). Variation in band intensity was not considered as a criterion for polymorphism. Similarity dissimilarity matrices were computed with the Dice coefficient (Ds) for qualitative data. A dendrogram was constructed by cluster analysis based upon the UPGMA (unweighted pair-group method with arithmetical averages) algorithm. The input data were also processed by PCA (principal coordinate analysis).

SSR fingerprints were evaluated by visual inspection of stained gels. The similarity index between two individuals was calculated by measuring Ps, i.e. the proportion of shared alleles summed over loci / (2 × number of compared loci) (Bowcock et al. 1994). The resulting similarity matrix was used for the UPGMA analysis.

The ''goodness'' of each dendrogram was verified by using the MXCOMP program which allows direct comparison between the original similarity matrix and the co-phenetic value matrix (Rohlf 1993). The program produces the correlation coefficient *r*, whose values range from zero (no correlation) to 1 (maximum correlation). The MXCOMP program was also used to verify correlations between data produced with AFLP and microsatellite analysis.

Results

Ten grapevine cultivars belonging to the varietal group known as ''Schiave'' were selected among those cultivated in different regions on the Southern and Northern slopes of the Alps. These were used for DNA analysis to establish genomic similarity among cultivars. In order to verify the degree of correlation of results produced by different approaches, DNA was extracted from young leaves and AFLP and SSR analysis performed.

In the case of AFLP, four primer combinations were used. Electrophoretic analysis of the amplified products revealed a total of 276 bands, of which 128 (46%) were polymorphic. The similarity matrix, produced by measuring the proportion of shared bands between two individuals, resulted in the dendrograms of Fig. 1A. The calculated correlation coefficient (*r*) of 0.94 verified the

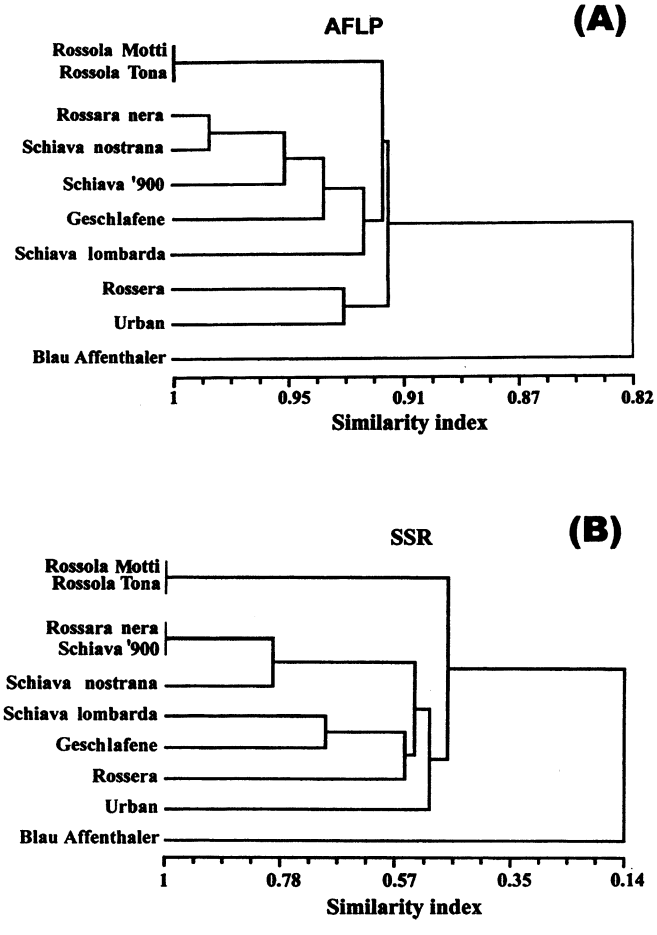

Fig. 1 Dendrograms, based on the Dice similarity index, showing the genetic relationship among ten grapevine cultivars collectively referred to as ''Schiave'', as determined by AFLP (**A**) and SSR (**B**) analysis. Grapevine cultivars were those listed in Table 1. Experimental details are described in Materials and methods

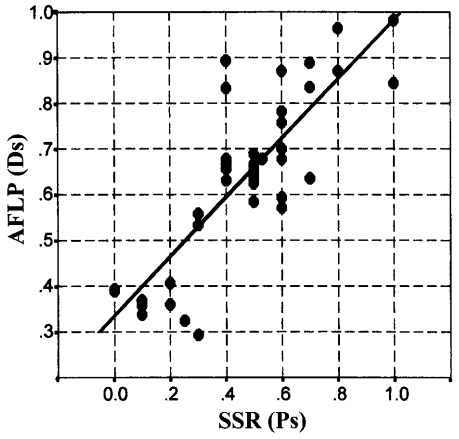

Fig. 2 Correlation between the similarity matrixes obtained in the AFLP (as measured with the Ds index) and SSR (as measured with the Ps index) analysis. Experimental details were those used for the dendrograms of Fig. 1. Data analysis was as specified in Materials and methods

Table 2 DNA primers for AFLP analysis

Name	DNA sequence	Variable extension
*Mse*1	5'-GATGAGTCCTGAGATAGACC-3'	ACC
*Mse*2	5'-GATGAGTCCTGAGATAGACA-3'	ACA
*Mse*3	5'-GATGAGTCCTGAGATAGGAA-3'	GAA
*Pst*1	5'-GACTGCGTACATGCAAA-3'	AAA
*Pst*2	5'-GACTGCGTACATGCAAC-3'	AAC
*Pst*3	5'-GACTGCGTACATGCACA-3'	ACA

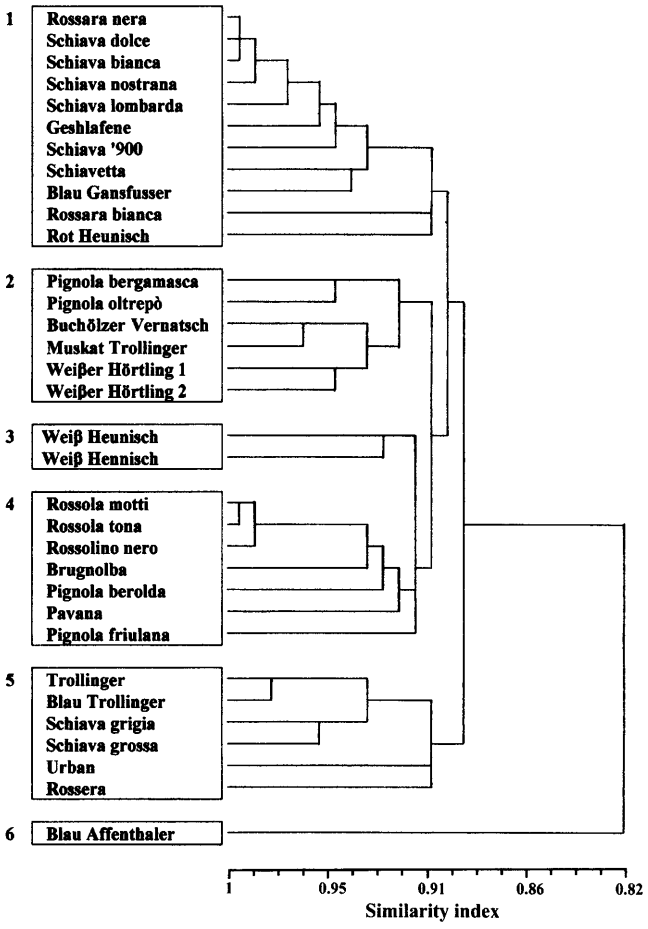

Fig. 3 Dendrogram, based on the Dice similarity index, showing the genetic relationship among 33 grapevine cultivars collectively referred to as ''Schiave'', as determined by AFLP analysis. Grapevine cultivars were those listed in Table 1. On the basis of genomic similarity, the dendrogram distinguishes six groups. Experimental details are described in Materials and methods

''goodness'' of the dendrogram. PCA for the first three coordinates confirmed the verified genomic relationships (data not shown).

In the case of SSRs, the five loci VVS1, VVS2, VVS3, VVS4 and VVS5 were examined. Amplified DNA fragments were analysed in polyacrylamide gel. The number of alleles and their size range for each microsatellite locus are shown in Table 3. Values of gene diversity were also calculated: all of them were high, ranging from 0.7115 to 0.7995, thus substantiating their usefulness as genomic markers. The highest rate of polymorphism (six different alleles) was produced at the VVS5 locus, in accordance with Thomas et al. (1994). The similarity matrix, produced by measuring the proportion of shared alleles between two individuals, produced the dendrogram of Fig. 1B. In this case, the correlation coefficient was $r = 0.93$, and the PCA confirmed the genomic relationships (data not shown).

The comparison between the AFLP and SSR similarity matrices was performed using the MXCOMP programme. The linear distribution of the compared values is shown in Fig. 2, where a correlation coefficient of 0.80 was calculated based on the results produced with the two analytical tools. The Mantel test demonstrated that the probability of these correlations being casual is less than 0.001. Therefore, although addressed to different genomic loci, AFLP and SSR analysis give corresponding estimates of genetic relationships among the tested cultivars.

Table 3 Number of alleles, range of allele size, and gene diversity of the five microsatellites loci used for SSR analysis

Locus	Range of allele size (bp)	Number of alleles	Gene diversity
VVS1	185–227	3	0.7115
VVS2	137–160	4	0.7445
VVS3	218–266	3	0.7450
VVS4	165–186	5	0.7855
VVS5	88–121	6	0.7995

The AFLP technique was then selected to define genetic relationships among the 33 grapevine cultivars collectively known as ''Schiave'' and thus to decipher homonyms and synonyms. AFLP analysis was performed with the four primer combinations *PstI* 1-*MseI* 1, *Pst*I 2-*Mse*I 2, *Pst*I 3-*Mse*I 3, *Pst*I 2-*Mse*I 3 (Table 2). Electro-

phoretic analysis of the amplified products revealed a total of 318 bands, of which 184 (57%) were polymorphic. Cluster analysis, based on the Dice's similarity index, produced the dendrogram of Fig. 3. The degree of genetic similarity was highest (100%) between the two accessions Rossola Motti and Rossola Tona, and lowest (80%) between Blau Affenthaler and the other accessions. The high correlation coefficient (r = 0.94), as calculated by MXCOMP, revealed the ''goodness'' of this AFLP dendrogram.

Discussion

The availability of different molecular tools to analyse biodiversity now provides the possibility to put an order into the large array of synonyms and homonyms within grapevine cultivars. AFLP analysis was strongly recommended by Crevera et al. (1998) as the method of choice for this purpose. The AFLP methodology offers the possibility to screen a higher number of anonymous loci than any other known tool: a single primer combination is frequently sufficient to ensure cultivar distinction. On the other hand, Sefc et al. (1997) showed that the analysis of microsatellites, which are hypervariable and are spread all over the genome of most eukaryotes, may be used to differentiate closely related genomes. Their consideration was that, due to the established high variability of the repeated nucleotides in microsatellite regions, each individual may hold a unique fingerprint. Furthermore, the inheritance of microsatellite alleles is co-dominant and is well suited to the study of family structure.

On this ground the question remained whether genomic similarities established with AFLPs or SSRs have a satisfactory degree of correspondence. In the present study we show that this is indeed the case, since the similarity matrixes obtained in the AFLP and SSR analysis shows a high correlation. Both AFLP and SSR data are suitable for the establishment of a database. This is important if we want to compare data obtained at different times and in different laboratories. In conclusion we propose that AFLP and SSR are equally effective to define genetic relationship among cultivars. The application of both tools may be recommended when the goal is the definition of the identity/diversity of different accessions.

In the present study, a dendrogram constructed upon an AFLP analysis of the 33 best known ''Schiave'' cultivars showed different, and in some cases relevant, degrees of genomic dissimilarity. The analysed accessions cluster in at least five different taxonomic groups and one outgroup (Fig. 3).

The first group includes accessions cultivated in Lombardia and Alto Adige confirming the proposal, based on historic data, of the circulation of grapevine cultivars within these adjacent regions. This group also includes: (1) Rossara nera, which traditionally belongs to the group of ''Schiave'' on the basis of amphelographic data and is genetically very similar to the ''Schiave'' cultivated in Lombardia; (2) Geschlafene, the German name for Rossara, which turns out to be genetically similar to the ''Schiave'' of Lombardia (it should be mentioned that in the Middle Ages the local name for ''Schiava'' in the region of Trentino was Sclaf, from which Geschlafene was derived); and (3) Rot Heunisch. It is interesting to note that this accession turns out to be considerably different from the Weiβ Heunish (which plots in the 3rd group), grown in the same regions. This is a case of false synonymy, where Heunish refers to the site of cultivation rather than to a common genetic background. Heunish cultivars, named Gouais in France, gave rise to some modern French cultivars, among which is Chardonnay (Meredith, personal communication).

The second group includes the two Pignola of Lombardia. Here again we have a case of false synonymy with Pignola Berolda and friulana (in the 4th group): thus, most-likely Pignola refers to the shape of the bunch, which resembles a pine cone. Bucholzer Vernatsch, which is considered a typical ''Schiava'', is genetically dissimilar from the ''Schiave'' of the 1st group but is similar to Muskat Trollinger, a cultivar used to make aromatic wines.

The third group, which includes Weiβ Heunisch and Weiβ Hennish, shows a case of false synonyms with rot Heunisch, which plots in the 1st group.

The fourth group includes a number of accessions cultivated in neighboring regions of Valtellina and Friuli. Rossola Motti, Rossola Tona and Rossolino nero show a very high genomic similarity and may be considered as variants of a common genotype. It may also be mentioned that the assonance between Rossola and Rossara (in the 1st and 5th group) does not correspond to genetic similarity.

The fifth group shows a strong correlation between Schiava grigia and Schiava grossa, cultivated in Trentino and Trollinger, and Blau Trollinger grown in Swabia. Considering the high genomic similarity, the latter two may be considered as variants of a common cultivar. Their close relationship with the ''Schiave'' grown in Trentino agrees with the consideration that the denomination Trollinger derives from Tirolinger (with this name grapevines cultivated in the nearby region of Tyrol were identified).

The 6th group includes a single accession, Blau Affenthaler, with a genomic constitution that is considerably distinct from all others.

It is concluded that the analysed cultivars, which represent the large majority of the presently cultivated ''Schiave'' grapevines, cannot be considered as members of a common taxonomic group. They are genetically distinguished and cluster in different taxonomic groups. The use of molecular markers allowed us to decipher true and false homonyms and synonyms. Especially instructive is the case of the assonance among Rossara, Rossola and Rossera that identifies accessions with large genomic dissimilarities. The proposal is that the term ''Schiave'' should refer to a similar cultivation practice in analogous mountain regions rather than to a common genetic background.

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