

Characterization of genetic biodiversity with *Vitis vinifera* L. Sangiovese and Colorino genotypes by AFLP and ISTR DNA marker technology

by

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S u m m a r y : A comparison between two recently developed, PCR-based DNA marker technologies (amplified fragment length polymorphism, AFLP; inverse sequence-tagged repeat analysis, ISTR) was carried out in a group of 19 *Vitis vinifera* L. accessions, including 13 putative Sangiovese-related grapevines and 6 "coloured" ecotypes whose fruits are of importance for conferring intense red colour to the wine. A large amount of polymorphic DNA fragments was revealed by both molecular techniques: 8 different AFLP and 5 ISTR primer combinations generated 264 and 249 polymorphic markers, respectively. Similarity relationships among the accessions were described by cluster analysis. The AFLP analysis revealed the existence of a uniform group for the Sangiovese (SG) ecotypes showing a high degree of genetic relatedness for the members of this cultivar. Among the coloured ecotypes (CLR), variability was more evident. Only the so called Colorino americano ecotype significantly diverged from both groups. ISTR analysis confirmed the genetic dissimilarity of Colorino americano and the existence of the SG and CLR groups, but in addition detected a higher proportion of polymorphism among the Sangiovese accessions compared to AFLP analysis. Sangiovese forte and Saragiolo apparently differed from the other SG-related grapevines in agreement with AFLP results. It is possible that the observed genetic dissimilarity between Sangiovese forte, Saragiolo and other SG-related types could be interpreted by the putative polyclonal origin of many grapevine cultivars, a concept which is generally accepted by the grapevine research community. Both AFLP and ISTR appear to represent innovative, efficient and sensitive molecular tools for investigating genetic diversity among *Vitis vinifera* ecotypes and for the eventual identification of clones.

Key words : AFLP markers, ISTR markers, phenotype, cultivar identification.

Introduction

Sangiovese and Colorino represent two Italian grapevine cultivars with a strong genetic variability due to their ancient origin. The high degree of variability within the ancient cultivars can be explained in terms of the polyclonal origin and of the different selection pressures applied in the various areas of their cultivation (CAMPOSTRINI *et al.* 1995). Sangiovese has always been of great economic importance in Italy, and even today it represents the main cultivated variety for Italian red wines. In Chianti wine, for example, Sangiovese is the main component (about 90 %) of the blending, while Colorino is one of the minor components, but very important for conferring the typical intense red colour to the wine.

Putative Sangiovese- and Colorino-related accessions along with many other grapevine cultivars and clones have been collected since 1987 from several areas of Tuscany and gathered in a germplasm collection of the Dipartimento di Ortoflorofruitticoltura, University of Florence, located in the Chianti area. Most of these ecotypes are grown in restricted areas and do not represent commercial clones cultivated nowadays in Italy. Genetic characterization is therefore of interest to register these ecotypes in the "Catalogo Nazionale delle Varietà di Viti" (Ministero Agricoltura e Foreste) that regulates propagation and marketing of grapevine in Italy. Furthermore a deeper knowledge of genetic traits will allow the utilization of these

ecotypes in breeding programs as a source of genetic variability as well as in clonal selection for the improvement of wine quality.

Grapevines have traditionally been identified on the basis of ampelography and ampelometry. However, for almost isogenic lines or when multiple synonyms have been traditionally used for a line, more traditional analytical approaches need to be complemented by molecular diagnostic tests in view of the limitation of ampelometry as recently discussed for several Italian cultivars (SILVESTRONI and INTRIERI 1995; SILVESTRONI *et al.* 1996).

PCR-based DNA marker technologies (microsatellite or simple sequence repeat polymorphism, SSRP; randomly amplified polymorphic DNA, RAPDs) have been developed for many cultivated crops including grapevine (BOWERS *et al.* 1993; COLLINS and SYMONS 1993; GOGORCENA *et al.* 1993; THOMAS and SCOTT 1993; CIPRIANI *et al.* 1994; BOTTA *et al.* 1995; MORENO *et al.* 1995; VIGNANI *et al.* 1995; XU *et al.* 1995) in addition to more laborious, slower methods like the detection of restriction fragment length polymorphism RFLP (BOURQUIN *et al.* 1993). More recently, the AFLP DNA marker technology which produces a large number of polymorphic bands was introduced (VOS *et al.* 1995) and used e.g. for fine mapping of cultivated crops including barley (BECKER *et al.* 1995). The AFLP method includes endonuclease digestion of genomic DNA, ligase-catalyzed adapter addition followed by a pre-amplification step before PCR amplification is performed in the pres-

ence of different primer combinations synthesized for the adapter region. A different approach for DNA fingerprinting is inverse sequence-tagged repeat (ISTR) analysis which is based on the selective PCR amplification of genomic DNAs in the presence of primers derived from *copia*-like repetitive elements. ISTR detects a similarly high amount of loci and percentage of polymorphic bands, but does not require any manipulation of DNA after isolation. This technology was originally developed for biodiversity studies in coconut (*Cocos nucifera* L.; ROHDE *et al.* 1995). Later on, ISTR analyses of various plant species (barley, potato, plum, pine trees, oil palm and others) and human as well as animal families with the identical primers developed from coconut sequences have demonstrated the general usefulness of this technology in the plant kingdom as well as for animal and human genome fingerprinting (ROHDE, in press). Here we present a comparative study between AFLP and ISTR polymorphism analysis on 19 *Vitis vinifera* types in an effort to assess the genetic uniformity and relatedness among Sangiovese and Colorino grapevines assembled in a germplasm collection.

Materials and methods

Plant material and DNA extraction: Plant material (Tab. 1) from the grapevine germplasm collection of the Dipartimento di Ortoflorofruitticoltura, University of Florence, was kindly provided by Prof. P. L. Pisani. The accessions were named after the collector's suggestions.

The SG group consisted of 12 putative Sangiovese-related ecotypes and the registered clone Sangiovese R 10 as a reference plant type. The CLR group consisted of 6 grapevines ecotypes characterized by the capability of conferring intense red colour to the wine. Among these 6 genotypes there were 4 putative Colorino-related types and 2 "coloured" accessions (Giacchè and Teinturier) of different origin.

Total DNA was extracted from young leaves by a modification of the method reported in MULCAHY *et al.* (1993). An additional Chroma-spin 1000 (CLONTECH Lab. Inc, CA) purification was performed to increase the quality of the DNA. DNA was quantified by visual comparison with lambda DNA on ethidium bromide-stained gels and stored at -20 °C in 10 mM Tris-HCl plus 1 mM EDTA, pH 8.0.

AFLP analysis:

A. Production and selection of fragments.

The AFLP procedure was performed by a modification of the method of Vos *et al.* (1995). Primary template DNA [1 µg] was partially digested with *EcoRI* [5U] and *MseI* [5U] for 2 h at 37 °C in a final volume of digestion of 50 µl containing 5 µl of 10 x restriction-ligation buffer [100 mM Tris-acetate, 100 mM MgOAc, 500 mM KOAc, 50 mM DTT, pH 7.5]. The digested DNA was ligated to biotinylated *EcoRI* [5 pmol] and non-biotinylated *MseI* [50 pmol] adapters with 1U T4 DNA ligase, 1.2 µl of 10 mM ATP and 1 µl of 10 x restriction-ligation buffer (see above).

Table 1

List of the grapevine genotypes used for AFLP and ISTR analyses

genotype	#
Colorino americano	1
Colorino del Valdarno	2
Colorino di Pisa	3
Colorino di Lucca	4
Giacchè	5
Teinturier	6
Sangiovese piccolo precoce	7
Sangiovese dell'Elba	8
Sangiovese R10	9
Morellino di Scansano	10
Sangiovese polveroso	11
Prugnolo gentile	12
Sangiovese polveroso Bonechi	13
Brunellone	14
Prugnolino medio	15
Prugnolino dolce	16
Morellino	17
Sangiovese forte	18
Saragiolo	19

The ligation reaction was performed for 3 h at 37 °C in a final volume of 60 µl. The sequence of the *EcoRI*-adapter was:

5'-biotin CTCGTAGACTGCGTACC
CTGACGCATGGTTAA-5'

and of the *MseI*-adapter:

5'-GACGATGAGTCCTGAG
TACTCAGGACTCAT-5'.

Streptavidine Dynabeads M-280 (DynaL, Oslo, Norway) were used to select biotinylated DNA fragments. The beads were washed once in 10 volumes STEX buffer [100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.1% (v/v) Triton X-100, pH 8.0]. Dynabeads were resuspended in 14 volumes of STEX and, per DNA sample, 10 µl of beads were used. After addition of the DNA to the beads, the mixture was brought to a final volume of 200 µl with sterile water and agitated gently for 30 min to ensure an efficient binding of biotinylated DNA. The beads were collected by means of a magnet (DynaL MPC) and washed 4 times with 1 x STEX. Each sample was stored at 4 °C in Tris-HCl buffer pH 8.0 plus 1 mM EDTA.

B. Selective PCR pre-amplification.

For selective pre-amplification of restriction fragments ligated to the *EcoRI* and *MseI* adapters, primers complementary to the core of each adapter containing one selective additional nucleotide base were used:

MseI+1 primer 5'-GATGAGTCCTGAGTAAA
EcoRI+1 primer 5'-GACTGCGTACCAATTCA

The 50 µl PCR mix consisted of 5 µl of DNA-adsorbed beads, 75 ng of each primer, 0.2 mM of each dNTP, 1.25 U Taq-Polymerase (GIBCO/BRL), 2.5 mM MgCl₂, 5 µl 10 x PCR-buffer.

The PCR amplification was carried out for 20 cycles at (92 °C, 60 s; 60 °C, 30 s; 72 °C, 60 s). To verify the efficiency of pre-amplification, 20-25 µl of the reaction mix were run on a 1 % agarose gel, electrophoresed and stained with 0.5 mg/ml ethidium bromide. The pre-amplification products were diluted 4 times in volume with sterile water.

C. Primer labeling with [γ -³³P]ATP and PCR amplification.

Amplification was carried out using selective primers, complementary to the *EcoRI* and *MseI* adapter regions, which contained 3 additional nucleotides at their 3' ends.

MseI+3 primers:

M32 5'-GATGAGTCCTGAGTAAAC

M33 5'-GATGAGTCCTGAGTAAAG

M38 5'-GATGAGTCCTGAGTAACT

M40 5'-GATGAGTCCTGAGTAAAGC

EcoRI+3 primers:

E37 5'-GACTGCGTACCAATTCAGC

E40 5'-GACTGCGTACCAATTCAGC

E41 5'-GACTGCGTACCAATTCAGC

One of the two primers (*EcoRI*+3 nucleotides primer) was labeled with [γ -³³P]ATP (Amersham).

The PCR mix for final volume of 20 µl consisted of 5 µl DNA as pre-amplified PCR products, 5 ng of labeled *EcoRI*+3 primer, 30 ng of unlabeled *MseI*+3 primer, 0.2 mM of each dNTPs, 0.4 U Taq-polymerase, 2 µl 10 x PCR buffer, 2.5 mM MgCl₂. The following PCR profile was used for the reaction: 1 cycle at (94 °C, 30 s; 65 °C, 30 s; 72 °C, 60 s), 24 cycles at (94 °C, 30 s; 56 °C, 30 s; 72 °C, 60 s). All PCR amplification steps were performed using a BIOMETRA TRIOblock thermocycler. A 1.5 µl aliquot of each reaction was added to an equal volume of sequencing loading buffer and after denaturation by heating to 93 °C, the samples were loaded on a 4.5 % sequencing polyacrylamide gel, and run in 1 x TBE electrophoresis buffer at 40 W. The gel was fixed, dried and exposed to X-ray films at room temperature for 24 h.

I S T R a n a l y s i s : ISTR analysis of grapevine DNAs was performed according to the protocol described earlier (ROHDE *et al.* 1995; ROHDE, in press) using the DNAs as isolated above without modifications and the following primer combinations: ISTR-F5/B2; ISTR-F6/B2; ISTR-F7/B2; ISTR-F6/B1; ISTR-F7/B1. The reaction was run in a final volume of 20 µl containing 25 ng of DNA, 1 x PCR buffer, 0.25 mM dNTPs, 2.5 mM MgCl, 50 ng of ³³P-labeled primers, and 1 U of Taq-polymerase. After the reaction, 1.5 µl aliquots were processed as described for the AFLP technique.

D a t a a n a l y s i s : AFLP and ISTR fingerprint profiles were evaluated by visual inspection of autoradiograms. Each profile was tested for consistency repeating the experiment two or three times. Only reproducible bands were used for statistical analysis. Band intensity variation was not considered as a criterion for polymorphism. Polymorphic DNA bands were scored as binary characters for their presence (1) or absence (0), and the resulting data were analysed using the NTSYS-pc program version 1.80 by F.J. ROHLF (Exeter, Software Setauket, USA, 1993).

Similarity-dissimilarity matrices were computed with Jaccard's coefficient for qualitative data according to the formula:

Jaccard's coefficient: $a/(n-d)$

n = total number of polymorphic bands

a = bands present in both compared ecotypes

d = bands absent in both compared ecotypes

Dendrograms were constructed by cluster analysis based upon UPGMA (unweighted pair-group method with arithmetical averages) algorithm.

The input data were also processed by Correspondence Analysis (BENZECRI 1973). By this method, the components are weighted according to their frequencies, it can be useful to stress the presence of a rare polymorphic band. The single band of the original data matrix (present or absent) was transformed with this method in a distance from a common centroid, giving new independent coordinates as in the Principal Component Analysis. On the base of these new coordinates the Euclidean distances matrix between the analysed accessions was computed.

Also for these matrices, dendrograms were constructed by cluster analysis based upon UPGMA. "Goodness" of each dendrogram was tested by using the MXCOMP program which allows direct comparison between the original similarity matrix and the cophenetic value matrix as suggested by ROHLF (1993). MXCOMP program was also used to check the correlation between the two molecular methods by comparing the similarity matrices obtained with Jaccard's coefficient and with Correspondence Analysis.

Results

AFLP analysis of 19 *Vitis vinifera* accessions revealed a total number of 264 polymorphic bands when analysed with the 8 different primer combinations. Fig. 1 shows one AFLP fingerprinting with primer combination E41/M38. For each genotype, almost all bands visible in the gels were consistent and reproducible. This study revealed that the number of total bands visible on gels varied in number from 39 to 73 with a mean of 57 total bands and a mean of 33 polymorphic bands for each primer combination. Of the AFLP bands observed, around 57.6 % were polymorphic (Tab. 2).

Grapevine genotypes were grouped by AFLP cluster analysis as shown in the dendrogram, calculated on the basis of Jaccard's coefficient, reported in Fig. 2 (a).

A very high $r = 0.98$, calculated by MXCOMP, revealed the "goodness" of this AFLP dendrogram. Among the considered grapevines, Colorino americano (identified by #1 in Tab. 1) diverged significantly from all other individuals. Minor divergence was observed in Teinturier (#6) and - for the Sangiovese-related types - in Saragiolo (#19), Prugnolino dolce (#15), Prugnolino medio (#16). Among the SG types, a high genetic correlation was observed for Sangiovese piccolo precoce (#7), Sangiovese dell'Elba (#8), Morellino di Scansano (#10), Sangiovese polveroso (#11), Prugnolo gentile (#12), and the registered clone Sangiovese Rauscedo 10 (#9).

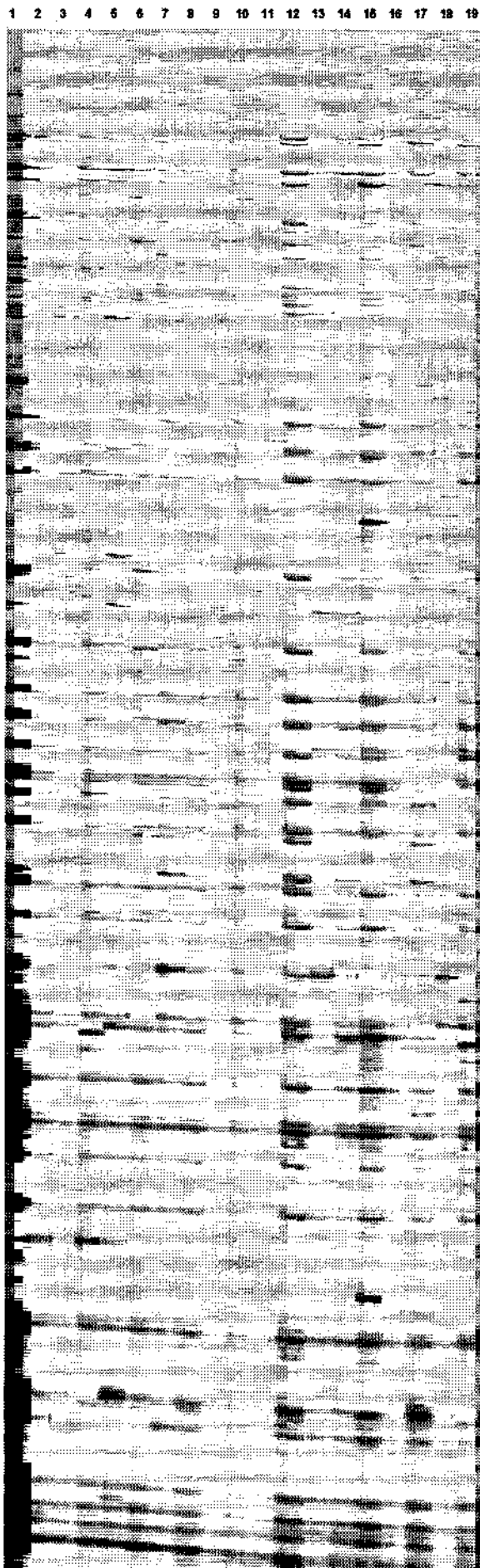


Fig. 1: AFLP fingerprinting of 19 accessions of *Vitis vinifera* (see Tab. 1) with the primer combination E41/M38.

Table 2

Comparison of polymorphisms detected by AFLP and ISTR analyses

primer pairs	total bands	total polymorphic bands	% polymorphic bands
AFLP			
E37/M32	39	30	76.9
E37/M38	41	13	31.7
E40/M32	67	38	56.7
E40/M33	70	44	62.8
E40/M38	52	23	44.2
E41/M33	73	42	57.5
E41/M38	61	34	55.7
E41/M40	55	40	72.7
ISTR			
F5/B2	34	26	76.4
F6/B2	109	65	59.6
F7/B2	124	103	83.0
F6/B1	57	42	73.6
F7/B1	26	13	50.0

ISTR fingerprinting (Fig. 3 shows one example with primer combination F5/B2) of the 19 accessions revealed 249 polymorphic markers for 5 primer combinations showing homogeneity with the results obtained by the AFLP technique: a comparison is therefore possible (Tab. 2). The number of total bands visible on each gel varied in number from 26 to 124 with a mean of 70 total bands and a mean of 50 polymorphic bands for each gel. The average percentage of polymorphisms is 71 % (Tab. 2).

According to ISTR data, grapevine accessions were clustered as shown by the dendrogram in Fig. 2 (b) with an $r = 0.97$.

This method confirmed the AFLP data and highlighted the diverging positions of Colorino americano (#1), Saragiolo (#19), and Teinturier (#6). In addition, however, the ISTR dendrogram provided variability within the Sangiovese group which was not detected by AFLP analysis. In fact polymorphic bands were detected among accessions Sangiovese piccolo precoce (#7), Sangiovese dell'Elba (#8), Morellino di Scansano (#10), Sangiovese polveroso (#11), Prugnolo gentile (#12) and Sangiovese Rauscedo 10 (#9). Furthermore Sangiovese forte (#18) diverged from SG-related types more than it was observed

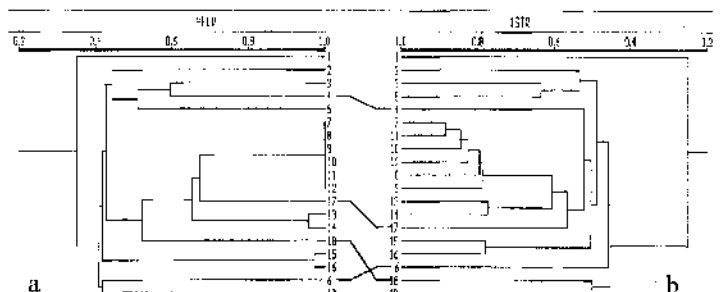


Fig. 2: Comparison of *Vitis vinifera* accessions based on genetic similarity matrices obtained with Jaccard's coefficient from AFLP (a) and ISTR (b) data.

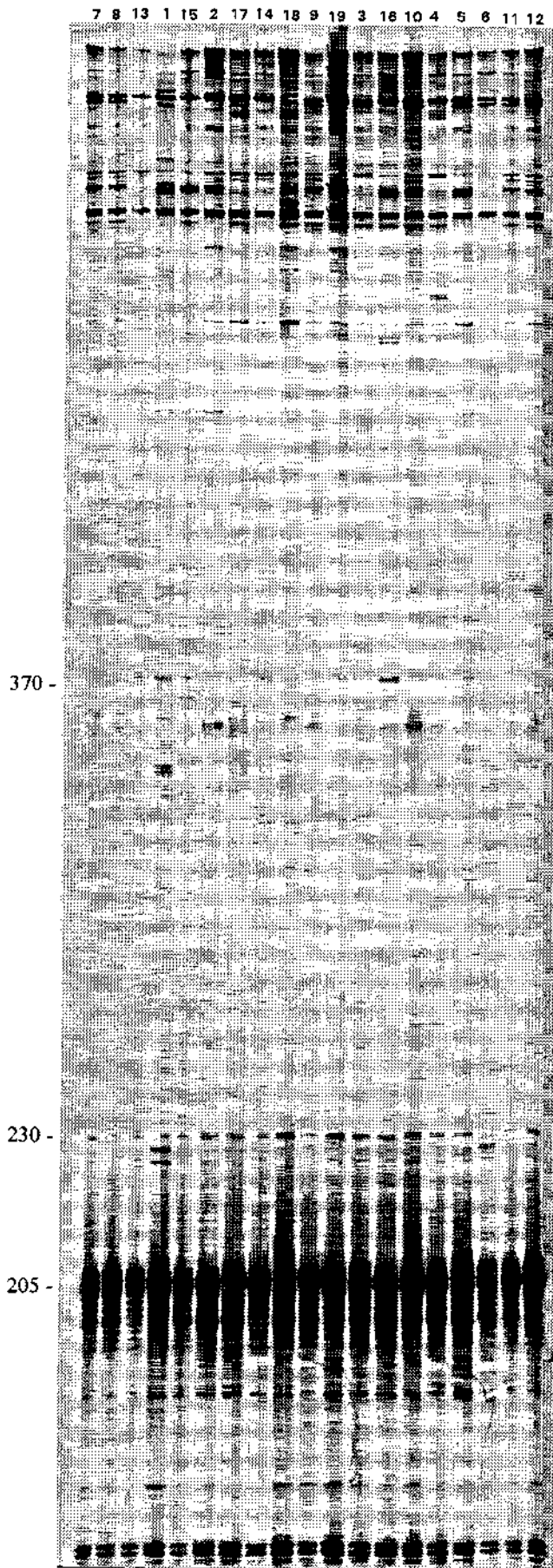


Fig. 3: ISTR fingerprinting of 19 accessions of *Vitis vinifera* (see Tab. 1) with the primer combination F5/B2. The indicated size markers (in nucleotides) were established from a sequence reaction run on the same gel.

with the AFLP dendrogram. Also Colorino di Lucca (#4) showed a slightly different relationship with the Colorino group.

AFLP and ISTR data were processed by Correspondence Analysis which generated other two dendrograms (Fig.4). The "goodness" is $r = 0.98$ for the AFLP dendrogram and $r = 0.96$ for the ISTR dendrogram. In both dendrograms, the shifting of Morellino (#17) and Sangiovese forte (#18) accessions towards more dissimilar positions is indicated, compared versus dendrograms calculated by Jaccard's coefficient (Fig.2). There is evidence that these two genotypes differ from the other SG-related types by some relevant polymorphic components.

A comparison between the AFLP and ISTR data sets was performed by the MXCOMP programme. A very high correlation was obtained comparing the matrices of Jaccard's coefficient ($r = 0.90$). This means that the two methods are favourably comparable. The matrices of Euclidean coefficient (correspondence analysis) were also compared ($r = 0.88$). Furthermore the slight difference between the two "r" values suggested that among the analysed accessions "private components" were of little relevance between AFLP and ISTR which showed a different ability in detecting rare components.

Discussion

Two newly developed, PCR-based DNA marker technologies were compared in their potential to reveal polymorphisms with selected genotypes of Sangiovese and "coloured" grapevines assembled in the germplasm collection of the University of Florence. Both technologies proved to represent powerful tools in the characterization of intraspecific variation among cultivars of *Vitis vinifera*. As with other PCR-based technologies like RAPD, small amounts of total DNA (ca. 20-50 ng) are sufficient as a template for the amplification of genetic loci by both AFLP and ISTR, and these DNA marker technologies are more sensitive than RFLP (BECKER *et al.* 1995) and generally more reproducible and reliable than RAPD (XU *et al.* 1995), especially for woody plants. With reference to microsatellite DNA amplification (SSRP) which allows allele polymorphism analysis at one specific locus, AFLP and ISTR provide a general overview on the genome structure based on multiple loci/single allele polymorphism analysis. AFLP and ISTR primers are universally applicable,

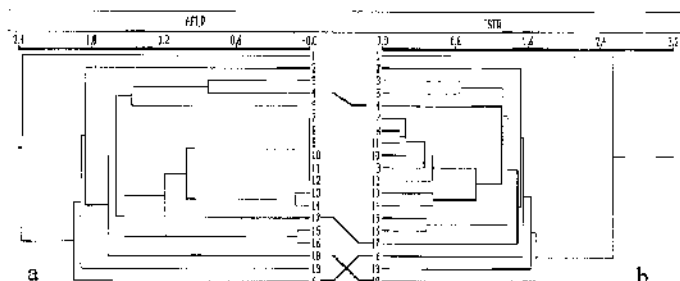


Fig. 4: Comparison of *Vitis vinifera* accessions based on genetic similarity matrices obtained with Correspondence Analysis from AFLP (a) and ISTR (b) data.

but ISTR offers an additional advantage in that isolated DNA is directly analyzed without the need of further manipulations (DNA restriction, adapter ligation, preamplification).

Both ISTR and AFLP generated comparable results as confirmed by visual inspection of the dendrograms and by the high correlation value obtained by comparison of the two data sets. These results clearly demonstrate that the "coloured" accessions are genetically distinct from Sangiovese and that Colorino americano differs from all the other genotypes. Similar conclusions were previously drawn from ampelometric studies (BANDINELLI *et al.* 1993), which by a general morphometric analysis of the same grapevines clearly distinguished between Sangiovese-related types and "coloured" grapevines. Even if, as a consequence of mutations, a certain degree of genetic variation could be expected within ancient cultivars, the high genetic divergence of Colorino americano, as evident from this present study both by AFLP and ISTR analysis with each primer combination, strongly argues that Colorino americano does not share the same origin as the other Colorino-related types. The high heterogeneity within the "coloured" group is also confirmed by the diverging position of Teinturier which shows a strong dissimilarity with the other members of the group.

Similarly for Sangiovese forte and Saragiolo, either the polyclonal origin theory or the exclusion of these accessions from the Sangiovese group, could be accepted.

Due to their specificity in separating cultivar genotypes, AFLP and ISTR together with other DNA marker techniques currently available for cultivated crops, will in the future complement the more traditional ampelometric analysis of grapevines. Detection of polymorphic bands among highly correlated Sangiovese accessions by ISTR analysis and preliminary approaches using AFLP marker technology on registered clones of Sangiovese showed encouraging results in terms of the possible application of these methods to the controversial and difficult aspect of clonal differentiation and/or identification. Further analyses using multiple primer combinations, will be necessary to assess the practical validity of the above mentioned techniques to clonal distinction in *Vitis vinifera*. In addition, the use of non-radioactive primers labeled with digoxigenin or fluorescent dyes will greatly improve the general applicability of the methods and open the possibility for automatic data analysis and consequently large scale processing.

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