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# Genetic relationships among grapevine cultivars grown in Oltrepò Pavese (Italy)

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## Summary

**In order to evaluate the genetic distance among 47 grapevine accessions, including major and minor local cultivars grown in Oltrepò pavese (Pavia, Italy), DNA has been analysed with the AFLP approach (Vos *et al.* 1995). The electrophoretic analysis of the products amplified by 3 primer combinations showed high polymorphism.**

**Furthermore, considering that DNA markers for haploid, uniparentally inherited genomes, such as the chloroplast DNA, are important indicators of pedigree, CpSSR analysis was also performed and this identified cases of maternal common origin among the analysed cultivars.**

**The results show that these molecular tools allow univocal genotype identification and that the analysed germplasm has a wide genetic dissimilarity. Results are coherent with the postulation of a polycentric origin for the Oltrepò pavese cultivars and of a multiple varietal flow from different viticultural regions. The molecular information gathered in this research is essential for the establishment of an appropriate presentation programme of autochthonous varieties.**

K e y w o r d s : biodiversity, chloroplast SSR, AFLP, *Vitis vinifera* ssp *sativa.*

A b b r e v i a t i o n s : CpSSR = Chloroplast Simple Sequence Repeat; SSR = Nuclear Simple Sequence Repeat; AFLP = Amplified Fragment Length Polymorphism; JC = Jaccard's coefficient.

## Introduction

Italy is characterized by a rich, complex and diversified viticultural heritage. Oltrepò pavese, located on the Northern Apennines that rise South of the Po river, gives a relevant contribution to this variability since in this area about 40 grape varieties occur. Barbera and Croatina, the main coloured varieties, are the base for the production of local DOC red wines. Other major traditional varieties include Chardonnay, Riesling italico, Riesling renano, Moscato bianco, Cortese, Uva rara, Vespolina and Pinot. In 1884 more than 200 varieties were listed in Oltrepò pavese by the Pavia Ampelographic Provincial Committee. Due to the economic pressure for a mass market, nowadays, less than 20 of them are extensively grown, while some others are scattered or occasionally cultivated (Scienza *et al.* 1999).

To limit the current loss of intravarietal diversity, germplasm collections have been established. An essential pre-requisite to this purpose is cultivar genotyping which is also important for breeding programmes. Nowadays, the availability of different molecular tools to analyse grapevine genomes allows to produce data on genetic diversity (Karp *et al.* 1998), to put in order the large number of synonyms and homonyms and to estimate phylogenetic relationships among different cultivars (Thomas *et al.* 1994; Sensi *et al.* 1996; Labra *et al.* 1999; Labra *et al.* 2001 b).

In a previous report (Rossoni *et al*. 2001) genotyping of major and minor Oltrepò pavese grapevine cultivars was achieved by nuclear SSR analysis. This suggested wide genetic diversity and some parentage relations.

In order to evaluate the genomic distance among the analysed cultivars, their DNA has been analysed with the AFLP approach (Vos *et al.* 1995). Better than any other known molecular tool this approach offers the possibility to screen a higher number of anonymous genomic *loci* and frequently ensures cultivar distinction even with a single primer combination (Cervera *et al*. 1998).

Furthermore, considering this usefulness of DNA markers for haploid uniparentally inherited genomes to identify cases of maternal common origin among the cultivars CpSSR analysis was also performed.

The aim of this report is to study the genetic relationship of the varieties in Oltrepò pavese and its viticultural history.

## Material and Methods

P l a n t m a t e r i a l a n d D N A e x t r a c t i o n : The 47 grapevine cultivars (*Vitis vinifera* L.) listed in Tab. 1 were obtained from CI.VI.FRU.CE (Voghera, Pavia, Italy). They included all major cultivated varieties, as well as ancient varieties from old Oltrepò pavese vineyards and collections.

Young leaflets were collected from rooted cuttings, frozen in liquid nitrogen and ground to fine powder. Genomic DNA was extracted from this powder as described by Labra *et al.* (2001 b).

A F L P a n a l y s i s : AFLP analysis (Vos *et al.* 1995) was performed as described in the European Patent 0534858 (Keygene, Belgium). Genomic DNA was digested (3 h) with *Eco*RI (0.5 U) and *Mse*I (0.5 U), and ligated with EcoRI (5 pMol) and MseI adapters (50 pMol). Primer pairs used in the pre-amplification reaction were M01 and E01 (Labra *etal* . 2001 a). Amplification was carried out using 3 pairs of selective primers E32-M36, E32-M38, E33-M32, describing in the original protocol (European Patent 0534858, Keygene, Bel-

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Table 1

Grapevine accessions used for the CpSSR and AFLP analyses. Berry colour, status of cultivation, place of origin and haplotype detected by CpSSR are indicated for each accession

| No. | Cultivar | Berry color | Status | Place of origin | Haplotype S |
|---|---|---|---|---|---|
| 1 | Barbera | Black | cultivated | Piemont | 107/116 F |
| 2 | Barberone | Black | collection | Piemont | 107/116 F |
| 3 | Basgano di Moline Freisa | Black | collection | Lombardy | 106/115 B |
| 4 | Basgano di Oliva Gessi | Black | collection | Lombardy | 107/115 E |
| 5 | Colombaia bianca | White | collection | Lombardy | 107/115 E |
| 6 | Colombaia nera | Black | collection | Lombardy | 107/116 F |
| 7 | Cortese | White | cultivated | Piemont-Lombardy | 106/114 A |
| 8 | Croà acino rosso o Vermiglio | Black | collection | Lombardy | 106/115 B |
| 9 | Croà acino grande | Black | collection | Lombardy | 107/116 F |
| 10 | Croà acino piccolo | Black | collection | Lombardy | 107/116 F |
| 11 | Croatina | Black | cultivated | Lombardy | 107/116 F |
| 12 | Croatina bianca | White | cultivated | Lombardy | 107/115 E |
| 13 | Croatina internodo corto | Black | collection | Lombardy | 107/115 E |
| 14 | Malvasia bianca di Candia | White | cultivated | Lombardy | 107/115 E |
| 15 | Moradella | Black | cultivated | Lombardy | 107/115 E |
| 16 | Moradella di Montalto | Black | collection | Lombardy | 107/116 F |
| 17 | Moretto | Black | collection | Lombardy | 107/115 E |
| 18 | Moscato bianco | White | cultivated | Lombardy | 107/116 F |
| 19 | Moscato nero | Black | collection | Lombardy | 107/116 F |
| 20 | Moscato rosa antico | Pink | collection | Lombardy | 107/116 F |
| 21 | Nebbiolo | Black | collection | Piemont | 107/115 E |
| 22 | Negrara | Black | collection | Trentino-Lombardy | 107/116 F |
| 23 | Pignola | Black | collection | Lombardy | 107/116 F |
| 24 | Pinot bianco | White | cultivated | French | 106/114 A |
| 25 | Pinot grigio | Grey | cultivated | French | 106/114 A |
| 26 | Pinot nero | Black | cultivated | French | 106/114 A |
| 27 | Pollini bianca | White | collection | Lombardy | 107/115 E |
| 28 | Pollini tipo Barbera | Black | collection | Lombardy | 107/115 E |
| 29 | Pulitana | Black | collection | Lombardy | 106/114 A |
| 30 | Riesling italico | White | cultivated | French-Lombardy | 107/115 E |
| 31 | Riesling renano | White | cultivated | Germany | 107/115 E |
| 32 | Rossarone chiuso | Black | collection | Lombardy | 107/116 F |
| 33 | Rossarone gentile | Black | collection | Lombardy | 107/116 F |
| 34 | Rossarone grande | Black | collection | Lombardy | 107/115 E |
| 35 | Rossamia | Black | collection | Lombardy | 107/115 E |
| 36 | Tabernello | Black | collection | Lombardy | 107/116 F |
| 37 | Timoraccio | White | collection | Piemont | 107/115 E |
| 38 | Uva bianca dura invernale | White | collection | Lombardy | 106/114 A |
| 39 | Uva crova | Black | collection | Lombardy | 107/115 E |
| 40 | Uva della cascina | Black | collection | Lombardy | 107/115 E |
| 41 | Uva di Mornico | Black | collection | Lombardy | 107/115 E |
| 42 | Uva di Nigazzo | Black | collection | Lombardy | 107/116 F |
| 43 | Uva dura antica | Black | collection | Lombardy | 107/115 E |
| 44 | Uva rara | Black | cultivated | Lombardy | 107/115 E |
| 45 | Verdea comune | White | cultivated | Lombardy | 107/115 E |
| 46 | Vernassa | White | collection | Liguria-Lombardy | 107/116 F |
| 47 | Vespolina | Black | cultivated | Lombardy | 107/116 F |

gium). The EcoRI primers were labelled with $\gamma$-$^{33}$P-ATP (Amersham, Italy). Amplification reactions were performed as specified by Labra *et al.* (2001 a). Polymorphic bands were scored by visual inspection of the resulting autoradiograms.

CpSSR analysis: Five primers pairs for chloroplast microsatellite amplification, ccmp2, ccmp3, ccmp4, ccmp5, ccmp10 (Weising and Gardner 1999) designed for angiosperms have been used. The PCR reaction was performed by adding 15-20 ng of genomic DNA to a 10 µl PCR mixture

containing 0.2 µM of the reverse and forward primers specified for each micro-satellite *locus*, 0.2 mM of each dNTP, 0.4 U Dynazyme (Celbio, Italy) and Dynazyme buffer as specified by the supplier. The PCR amplification programme was: 4 min at 94 °C; 32 cycles of denaturation (1 min at 94 °C), annealing (1 min at 50 °C) and extension (1 min at 72 °C); then a final step for 30 min at 72 °C. Loading buffer 6x (2 µl) was added to the 10 µl mixture and 5 ìl of this were analyzed on 8 % acrylamide/bis-acrylamide non-denaturing gel and developed with the silver staining approach as described by Echt *et al.* (1999). Allele size was defined by visual inspection using a Gel Doc 2000 instrument (Biorad, USA). AFLP and CpSSR fingerprint was confirmed in duplicate.

Data analysis: In accordance with numeric taxonomy principles AFLP bands were scored as binary characters for absence (0) or presence (1) and the resulting data matrices analysed using the NTSYSpc 2.1 statistical programme. Levels of diversity were estimated as the percentage of polymorphic bands out of the total bands scored. The AFLP similarity-dissimilarity matrices were computed with the Jaccard's coefficient (Sneath and Sokal 1973). The final products of data processing were dendrograms constructed by cluster analysis based upon UPGMA (unweighed pair-group method with arithmetical averages).

In the case of CpSSR allele size was obtained by visual inspection. Combining two polymorphic CpSSR *loci* cultivar haplotyping was defined.

## Results

DNA of 47 grapevine cultivars, including major and minor traditional local cultivars from a local collection was extracted and analysed by AFLP using 3 primer combinations.

The electrophoretic analysis of the amplified products revealed a total of 156 bands, 90 of which (57.7 %) were polymorphic. These data were used to compute an UPGMA dendrogram defining genomic relationship among cultivars (Figure).

The Jaccard's similarity index among cultivars varied from 1.0 (full genetic similarity) to 0.564 (high genetic dissimilarity), indicating a large genetic variability among the analysed varieties. In all cases, except for Pinot nero, Pinot bianco and Pinot grigio, the analysis assured full cultivar distinction.

In the case of CpSSR analysis, 5 *loci* were analysed. Only 2 showed DNA polymorphism, with 2 different alleles in the case of ccmp3 (106 and 107 bp) and 3 alleles for ccmp10 (114, 115 and 116 bp). Six possible haplotypes were defined by the combination of allele size in the 2 polymorphic *loci*. Of these, 4 haplotypes (named A, B, E, F) were detected in the analysed grapevine populations. Haplotype E was the most common, being present in 21 samples (Tabs 1 and 2), while haplotype B was rarest; it was present only in 2 accessions (Basgano di Oliva Gessi and Croà acino rosso).

## Discussion and Conclusion

The present work addressed to the definition of genetic relationships among grapevines that are part of the Oltrepò

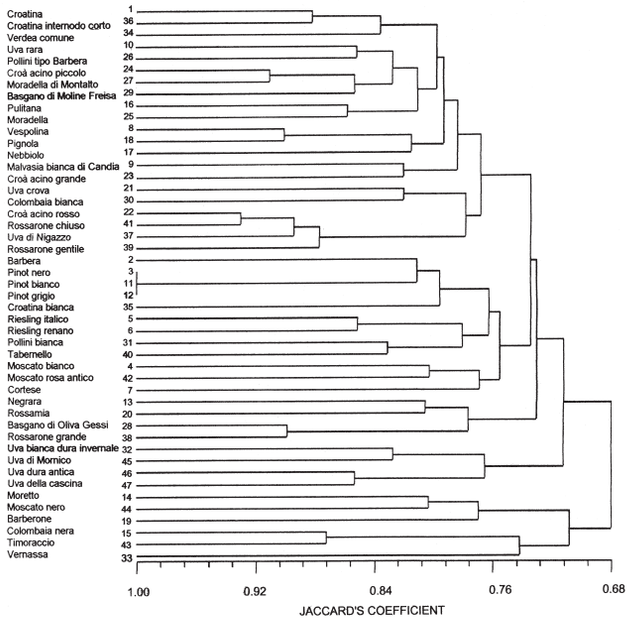


Figure: Dendrogram based on the Jaccard's similarity index, showing the genetic relationship among 47 Oltrepò pavese grapevine cultivars as determined by CpSSR and AFLP analyses.

Table 2

Possible haplotypes identified by the combination of the ccmp3 and ccmp10 chloroplast SSR *loci*

| Haplotype | CpSSR *locus* | |
|---|---|---|
| | Ccmp3 | Ccmp10 |
| A | 106 | 114 |
| B | 106 | 115 |
| C | 106 | 116 |
| D | 107 | 114 |
| E | 107 | 115 |
| F | 107 | 116 |

pavese heritage. The dendrogram obtained from the AFLP analysis (Figure) showed high genetic variability and revealed clear cases of synonymy and homonymy. In the case of 3 putative synonymous varieties, *e.g.* Rossarone gentile, Rossarone chiuso and Rossarone grande, results confirmed the close relationship between the first two but evidenced high genetic diversity in the case of Rossarone grande. This was confirmed by CpSSR analysis that grouped the first two in haplotype F and the latter in haplotype E. These conclusions are also supported by previous data obtained by nuclear SSR analysis (Rossoni *et al.* 2001). The analysis of this case confirms that chloroplast and nuclear SSR are appropriate markers to investigate genetic relationships among closely related accessions (Bowers *et al.* 1997).

High genetic dissimilarity was recorded by AFLP analysis in the case of the other 3 synonymous accessions, Croà acino piccolo, Croà acino rosso and Croà acino grande. This confirms previous suggestions on their independent genealogy (Rossoni *et al.* 2001).

Possible common ancestors are suggested in the case of Croatina and Croatina internodo corto which have close positions in the dendrogram. On the other hand, these two cultivars showed different haplotypes in the CpSSR analysis thus suggesting a different maternal origin.

The 3 Pinot accessions were shown to be identical, on the bases of AFLP and CpSSR analysis as well as on previously reported nuclear SSR data (Rossoni *et al.* 2001). Bellin *et al.* (2000) found few polymorphic AFLP bands within Pinot clones but concluded that these were not reproducible. Thus, new tools are needed for clonal characterization and clonal discrimination remains an open issue.

Uva bianca dura invernale, Uva di Mornico, Uva della Cascina and Uva dura antica, known as the oldest varieties cultivated in Oltrepò pavese, were also analysed. From the high genetic dissimilarity, evidenced by AFLP and CpSSR analyses, it was concluded that these old varieties had different origin and no common maternal progenitor.

In conclusion, in spite of the confirmed synonyms and the verified genetic similarities, the high genetic diversity of grapevines of Oltrepò pavese suggests a polycentric origin of today's grapevine assortment. The Oltrepò pavese region has been an important commercial cross since Roman times. Thus, it is most likely that cultivars with different morphological and genetical traits were introduced by various colonizing populations turning this region into a relevant centre of grapevine diversity. High diversity is still preserved in the region and the molecular tools used in this study now offer a concrete basis for its protection and for *insitu* and *ex situ* preservation programmes.

A comparison of results produced by AFLP and SSR suggests that the AFLP approach, by investigating a large number of anonymous *loci* offers the most appropriate tool to estimate genetic distance among varieties. In the case of synonymous varieties (*e.g.* Rossarone grande and Rossarone chiuso) or hypothetical parental relationship, the use of SSR markers is preferable, since they explore highly polymorphic regions and they are co-dominant markers, while AFLP are dominant.

We conclude that only the application of both analytical approaches will produce satisfactory data for the screening of intravarietal diversity and for analysing synonymies, homonymies, false attributions and pedigrees. In the case of hypothetical parental relationships, chloroplast SSR analysis has the capacity to elucidate maternal origin.

The computational analysis data of this and of a previous report (Rossoni *et al*. 2001) is saved on a CD-Rom.

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## References

Bellin, D.; Velasco, R.; Grando, M. S.; 2000: Intravarietal DNA polymorphisms in grapevine (*Vitis vinifera* L.). Acta Hortic. **546**, 343-349.

Bowers, J. E.; Meredith, C. P.; 1997: The parentage of a classic wine grape, Cabernet Sauvignon. Nat. Genet. **16**, 84-87.

Cervera, M. T.; Cabezas, J. A.; Sancha, J. C.; Martinez de Toda, F.; Martinez-Zapater, J. M.; 1998: Application of AFLPs to the characterisation of grapevine *Vitis vinifera* L. genetic resources. A case of study with accessions from Rioja (Spain). Theor. Appl. Genet. **97**, 51-59.

Echt, C. S.; May-Marquardt, P.; Hseih, M.; Zahorchak, R.; 1996: Characterization of microsatellite markers in eastern white pine. Genome **39**, 1102-1108.

Karp, A.; Isaac, P.; Ingram, D.; (Eds.); 1998: Molecular Tools for Screening Biodiversity. Chapman & Hall, London, UK.

Labra, M.; Carreno-Sanchez, E.; Bardini, M.: Basso, B.; Sala, F.; Scienza, A.; 2001 a: Extraction and purification of DNA from grapevine leaves. Vitis **40**, 101-102.

Labra, M.; Failla, O.; Fossati, T.; Castiglione, S.; Scienza, A.; Sala, F.; 1999: Phylogenetic analysis of grapevine cv. Ansonica growing in the island of Giglio, Italy, by AFLP and SSR markers.Vitis **38**, 161-167.

Labra, M.; Winfield, M.; Ghiani, A.; Grassi, F.; Sala, F.; Scienza, A.; Failla, O.; 2001 b: Genetic studies on Trebbiano and morphologically related varieties by SSR and AFLP markers. Vitis **40**, 187-190.

Rossoni, M.; Fasoli, V.; Labra, M.; Spinardi, A.; Failla, O.; Scienza, A.; Sala, F.; 2001: Exploration of elite grapevine germplasm of Oltrepò pavese (northern Italy) using genetic, chemotaxonomic and morphological markers. Adv. Hort. Sci. **15,** 72-78.

Scienza, A.; Valenti, L.; Molina, M.; (Eds.); 1999: Vitigni antichi della Lombardia. Ampelografia del germoplasma locale. Provincia di Pavia.

Sefc, K. M.; Lopes, M. S.; Lefort, F.; Botta, R.; Roubelakis-Angelakis, K. A.; Ibanez, I.; Pejiè, H. W.; Wagner, J.; Glossl, H.; Steinkellner, H.; 2000: Microsatellite variability in grapevine cultivars from different European regions and evaluation of assignment testing to assess the geographic origin of cultivars. Theor. Appl. Genet. **100**, 489-505.

Sensi, E.; Vignani, R.; Rohde, W.; Biricolti, S.; 1996: Characterisation of genetic biodiversity with *Vitis vinifera* L. Sangiovese and Colorino genotypes by AFLP and ISTR DNA marker technology. Vitis **35**, 183-188.

Sneath, P. H. A.; Sokal, R. R.; 1973: Numerical Taxonomy. The Principles and Practice of Numerical Classification. W H. Freeman, San Francisco, USA.

Thomas, M. R.; Scott, N. S.; 1993: Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequenced-tagged sites (STSs). Theor. Appl. Genet. **86**, 985-990.

Thomas, M. R.; Cain, P.; Scott, N. S.; 1994: DNA typing of grapevine: A universal methodology and database for describing cultivars and evaluating genetic relatedness. Plant. Mol. Biol. **25**, 939-949.

Vos, P.; Hogers, R.; Bleeker, M.; Reijans, M.; Van de Lee, T.; Hornes, M.; Frijters, A.; Pot, J.; Peleman, J.; Kuiper, M.; Zabeau, M.; 1995: AFLP: a new technique for DNA fingerprinting. Nucl. Acids Res. **23**, 4407-4414.

Weising, K.; Gardner, R.; 1999: A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. Genome **42**, 9-19.

Zabeau, M., Vos, P.; 1992 : European patent application. Publication n° 0543858 A1.

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