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Genetic studies on Trebbiano and morphologically related varieties by SSR and AFLP markers

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Summary

Several grapevine varieties are named Trebbiano and have similar phenotypical characteristics (*e.g.* whitish berries, late ripening, vigorous growth). Other vines share many of the morphological characteristics of Trebbiano but have different names. In order to clarify the relationships between some of these varieties, a study was carried out with 7 Trebbiano varieties and 17 cultivars that are morphologically similar to Trebbiano. AFLP and SSR analyses were performed to define genetic similarity among the Trebbiano cultivars as well as between the Trebbiano cultivars and related varieties. Results show a large degree of variability between most of the cultivars and suggest that they do not share a common ancestor. Genetic analysis revealed that cv. Verdicchio is identical with Trebbiano di Soave which is very similar to Trebbiano di Lugana.

K e y w o r d s : *Vitis vinifera* L., SSR, AFLP, Trebbiano.

A b b r e v i a t i o n s : SSR = Simple Sequence Repeat, AFLP = Amplified Fragment Length Polymorphism.

Introduction

Traditional methods of grape cultivar identification relied on morphological characters whose expression can be affected by developmental and environmental factors, while limited polymorphism has hampered the use of biochemical markers such as isozymes (Samaan and Wallace 1981; Parfitt 1989). In contrast, DNA-based methods are not influenced by environmental factors, and a large number of potential polymorphic sequences or markers is available. Using molecular tools such as AFLPs (Vos *et al.* 1995) and SSRs (Karp *et al*. 1998) it is possible to characterize and compare genotypes independently from phenotype. By identifying polymorphic sequences in genomic DNA, these tools allow to draw phylogenetic (Heun *et al*. 1997) and taxonomic (William and Clair 1993) conclusions and permit cultivar identification (Mulkahy *et al*. 1995; Xu and Bakalinsky 1996).

The availability of a range of molecular tools to analyse biodiversity provides the possibility to bring some order into the confusing array of synonyms and homonyms of grapevine cultivars; Cervera *et al*. (1998) strongly recommended AFLP analysis as the method of choice for this purpose. The AFLP approach allows to screen a larger number of anonymous loci than any other tool. On the other hand, several authors have shown that the analysis of microsatellites may be used to differentiate between closely related cultivars (Bowers and Meredith 1997; Lopes *et al*. 1999; Sefc *et al*. 2000). They considered that, due to the high variability of microsatellite regions, each individual should have a unique fingerprint. Furthermore, the inheritance of microsatellite alleles is co-dominant and is very suitable to study family structures. The combination of AFLP and SSR approaches is recommended if the goal is to define clonal (Pinot, Sangiovese) (Regner *et al.* 2000) or varietal (Moscati, Malvasia, Schiave) groups (Stavrakakis and Biniari 1998; Crespan and Milani 2001).

There are many grapevine varieties called Trebbiano; often an amendment is added to indicate the town (*e.g.* Trebbiano di Nizza), region (*e.g.*, Trebbiano Toscano) or country (*e.g.* Trebbiano di Spagna) in which the vine is grown, or to reflect some common morphological characteristic (Molon, 1906). In this paper, the genetic relationships between various varieties of Trebbiano have been investigated using the analyses of AFLPs and SSRs to determine whether they are genetically related.

Material and Methods

P l a n t m a t e r i a l : The cultivars (*Vitis vinifera* L.) used in this study are listed in Tab. 1. It comprises 7 Trebbiano vines, 17 cultivars selected on the basis of synonymy or sharing morphology with Trebbiano or on the basis of historical reports indicating some relationship.

D N A e x t r a c t i o n : Young leaves (length 1-2 cm) were harvested from rooted cuttings. They were frozen in liquid nitrogen and ground to a fine powder. Genomic DNA was extracted as described by Labra *et al.* 2001.

S S R a n a l y s i s : DNA was analysed at the following 8 microsatellite *loci*: VVS2, VVS3, VVS4 (Thomas and Scott 1993), VVMD5, VVMD6, VVMD7 (Bowers *et al*. 1996), VVMD27, VVMD28 (Bowers *et al.* 1999).

The analysis was performed by adding 15 ng of genomic DNA to a 20 µl PCR mixture containing 10 ng of the

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Table 1

Cultivars used for SSR and AFLP analyses, area of cultivation and site of germplasm collection

Cultivar	Area of cultivation	Germplasm collection
Biachetta trevigiana	Veneto	CI.VI.FRU.CE
Bianchetta dei castelli di Monfumo	Lombardy	CI.VI.FRU.CE
Clairette	France	CI.VI.FRU.CE
Grechetto	Umbria	CI.VI.FRU.CE
Erbaluce	Piedmont	CI.VI.FRU.CE
Falanghina	Campania	CI.VI.FRU.CE
Grecanico	Sicily	CI.VI.FRU.CE
Greco Bianco	Campania	CI.VI.FRU.CE
Greco Nero	Southern Italy	CI.VI.FRU.CE
Montonico	Central Italy	CI.VI.FRU.CE
Ortugo	Emilia Romagna	CI.VI.FRU.CE
Pagadebito	Emilia Romagna	CI.VI.FRU.CE
Petit Manseng	France	CI.VI.FRU.CE
Rossola	Ligury	CI.VI.FRU.CE
Trebbiano d'Abruzzo	Abruzzo	CVVP
Trebbiano di Lugana	Lombardy	CVVP
Trebbiano di Soave	Veneto	CVVP
Trebbiano di Spagna	Abruzzo	CVVP
Trebbiano Romagnolo	Emilia Romagna	CVVP
Trebbiano Spoletino	Umbria	CVVP
Trebbiano Toscano	Tuscany	CVVP
Verdejo	Spain	IASMA
Verdesse	France	IASMA
Verdicchio	Marche	CI.VI.FRU.CE

IASMA, Agricultural Research Institute, S. Michele all'Adige, Trento, Italy.
CIVIFRUCE, Regional Centre of Agriculture, Riccagioia, Pavia, Italy.
CVVP: Centro Vitivinicolo Provinciale, Brescia, Italy.

DNA primers specified for each microsatellite locus, 200 µM of each of the 4 dNTPs, 0.5 U Dynazyme (Celbio, Italy) and Dynazyme buffer as specified by the supplier. The forward primers were end-labelled with α^{33}P ATP (Amersham, Italy). PCR amplification was performed with a programmable thermal controller (PTC 100, MJ Research Inc., USA) using the following profile: 7 min at 94 °C; 35 cycles of denaturation (45 s at 94 °C), annealing (30 s at 52 °C) and extension (1 min at 72 °C); a final step for 7 min at 72 °C.

AFLP analysis: AFLP was performed as described by LABRA *et al*. (1999), except that genomic DNA (200 ng) was digested (3 h) with *Eco*RI (0.5 U) and *Mse*I (0.5 U) and ligated with EcoRI adapter (5 pmol) and MseI adapter (50 pmol). Primer pairs used in the pre-amplification reaction were M01 and E01, while the 4 pairs of primer (E31 - M32, E32 - M36, and E33 - M38) of Tab. 2 were used for the amplification reaction. Results were confirmed by repeating the analysis for a small number of samples.

Analysis of the DNA amplification products: In the case of the SSR analysis, 10 µl of the PCR-amplified mixture were added to 2 µl of loading buffer (80 % formamide, 1 mg ml^{-1} xylene cyanol FF, 1 mg ml^{-1} bromophenol blue, 10 M EDTA, pH 8.0) and a total of 3 µl was analysed by electrophoresis on a 4.5 % sequencing polyacrylamide gel and electrophoresed in TBE electrophoresis buffer (50 mM boric acid, 1 mM EDTA, pH 8.0) for 3 h at 80 W. The gel was fixed in 10 % acetic acid and exposed to an X-ray film. Visual inspection of the resulting autoradiograms allowed scoring of microsatellite bands. Allele sizes were determined using a Gel Doc 2000 (Biorad, USA).

In the case of AFLP, 1.5 µl of the PCR-amplified mixture were added to an equal volume of loading buffer (80 %

Table 2

DNA primers for AFLP analysis.

Name	DNA sequence	Variable extension
M01	5'-GATGAGTCCTGAGTAA-3'	A
E01	5'-GACTGCGTACCAATTC-3'	A
M32	5'-GATGAGTCCTGAGTAA-3'	AAC
M36	5'-GATGAGTCCTGAGTAA-3'	ACC
M38	5'-GATGAGTCCTGAGTAA-3'	ACT
E32	5'-GACTGCGTACCAATTC-3'	AAC
E33	5'-GACTGCGTACCAATTC-3'	AAG

formamide, 1 mg ml^{-1} xylene cyanol FF, 1 mg ml^{-1} bromophenol blue, 10 M EDTA, pH 8.0), denatured for 5 min at 92 °C and analysed by electrophoresis using a PAGE gel as described above.

Statistical analysis: Each microsatellite allele or AFLP band was scored as a binary character for its absence (0) or presence (1). In the case of microsatellite analysis, presence was scored as (1) independently of the heterozygous or homozygous state. The resulting data were analysed using the software programme Genstat 5. Similarity-dissimilarity matrices were computed using the Jaccard's coefficient (JC) (Sneath and Sokal 1973)

$$JC = a/(n-d)$$

where a is the band present in both compared genotypes, n the total number of polymorphic bands and d the band absent in both compared genotypes. The final products of the SSRs were subjected to a cluster analysis using UPGMA (unweighted pair-group method with arithmetical averages) and a dendrogram was drawn (Fig. 1). AFLPs were subjected to PCO analysis and the first two co-ordinates were plotted (Fig. 2).

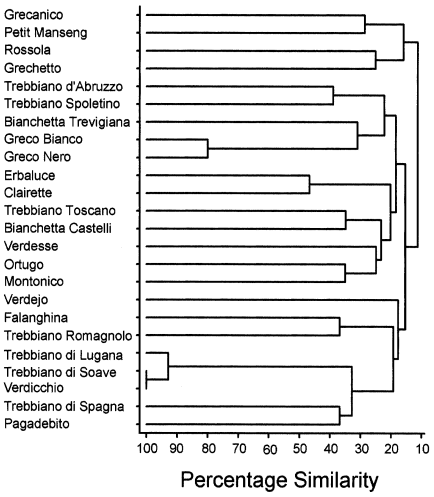

Fig. 1: Dendrogram of band sharing similarity based on the matrix of similarity derived from the SSR data matrix.

Results

The microsatellite analysis, conducted with all 24 samples, revealed extensive gene diversity both between the Trebbiano cultivars and between the cultivars of Trebbiano and related varieties (Fig. 1). Only three samples had a high proportion of bands sharing similarity: Trebbiano di Lugana, Trebbiano di Soave and Verdicchio. In fact, the last two samples were 100 % identical. Trebbiano di Lugana shared 93 % of bands with these two cultivars.

The analyses of AFLPs, carried out only on the 7 Trebbiano cultivars and Verdicchio, also revealed a large amount of variability between the recognised Trebbiano cultivars. The PCO plot of the first two co-ordinates, which accounts for 70 % of all the variability in the matrix of similarity, clearly separates cvs Trebbiano di Soave, Trebbiano di Lugana and Verdicchio from the other 5. A dendrogram of the results shows that Trebbiano di Soave and Trebbiano di

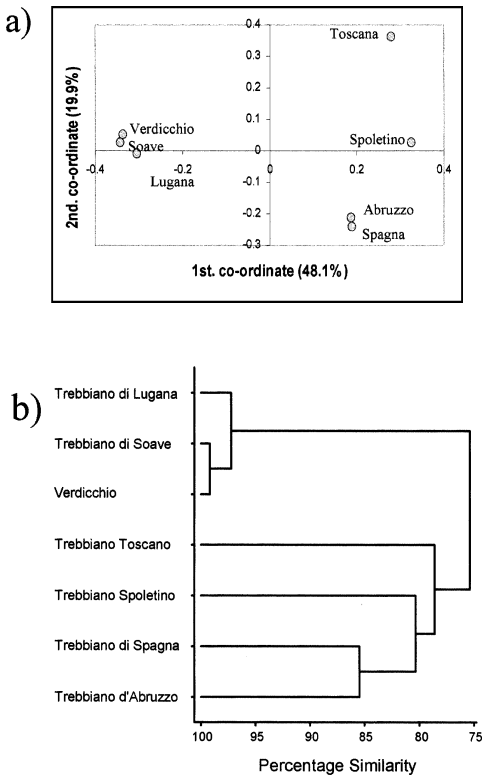

Fig. 2: **a**) Principal coordinate analysis of the AFLP data obtained from the 6 cultivars of Trebbiano plus the sample of Verdicchio; **b**) Dendrogram representing the same samples to show band sharing similarity.

Lugana were almost identical, sharing 97 % of bands. Interestingly, cv. Verdicchio is almost identical with Trebbiano di Soave (99 % band sharing). No other two cultivars shared more than 85 % of bands.

Discussion

Information on the origin and relationship among plant cultivars is of great interest both for germplasm preservation and for cultivar improvement by breeding and biotechnology. For grapevine, this information might be obtained by combining ampelometric and chemotaxonomic (protein polymorphism, anthocyanin profile) approaches with historical and cultural data. Historical, linguistic and morphological approaches have been used to study the relationship of cvs of Trebbiano. The first citation of the name Trebbiano goes back to Plinius who in his *Naturalis Historia* mentions *vinum trebulanum* as an ancient vine of Campania in southern Italy. Bacci (1959) suggested that the term Trebbiano is derived from the name of a town in the area of Luni in ancient Etruria (Tuscany, Italy). Others believe that it originates from the name of towns in which the grape was grown; e.g. Trebbiano Nizza (Pavia, Italy), Trebbiano Sarzana (La Spezia, Italy) or Trebbiano di Brescia (Brescia, Italy). It has also been suggested that the name is derived from the Ligurian Appenines river Trebbia.

The high degree of variability among the cultivars of Trebbiano used in this study indicates that they are of vari-

ous origin. In fact, of all the cultivars included in the study, only Trebbiano di Soave, Trebbiano di Lugana and Verdicchio were genetically similar. Indeed, cv. Verdicchio was shown to be identical to Trebbiano di Soave, and is obviously a synonym.

The names Greco bianco, Greco nero, Grecanico and Grechetto are derived from the adjective "Greek". Our results, however, do not show a close genetic similarity between these cultivars and Trebbiano. Therefore, the name Greco should not be considered a synonym of Trebbiano on the basis of a genetic relationship, but, more probably, reflects common oenological characteristics: *e.g.*, alcoholic, sweet and aromatic wines from overripe grapes.

An interesting genetic relation was detected between Erbaluce and Clairette that shared 50 % of SSR alleles. The names of these two varieties have a common semantic origin (Clairette from *clarus* - clear, bright, and Erbaluce from *albus* and *lux* - daylight and light). They also share several morphological traits and give rise to similar wines. However, they are not genetically similar to the Trebbiano group.

It can be concluded that the cultivars named Trebbiano probably do not have a common ancestor. However, they do share many morphological characteristics, *e.g.* whitish berries, large bunches, late ripening, vigorous growth (MOLON 1906; BACCI 1959). Therefore, in agreement with the hypothesis of HOHNERLEIN-BUCHINGER (1996) the name Trebbiano is not derived from the *Trebulanum*, a particular place of origin, but from the Frankonian term "Draibio" that means vigorous shoot. The introduction of these vigorous and productive varieties can be considered to be a consequence of the agrarian policy of Charlemagne, who promoted a rapid renewal of medieval Italian viticulture after the crisis of the Roman empire.

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