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# Biodiversity of grapevines (*Vitis vinifera* L.) grown in the Province of Verona

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## Summary

**PCR-based DNA microsatellite analysis has been applied to define the genetic relationships among 7 most representative grapevine cultivars grown in the province of Verona, 5 ancient grapevine and two varieties grown in different regions of Italy. For each variety three different clones or accessions were investigated to assess genotypical uniformity; in 5 cases we found out intravarietal dissimilarity. SSR data were used to create a distance matrix and then a polylogenetic tree. Results show a polygenetic relationship among some cultivated (Corvina, Rondinella, Molinara, Trebbiano di Soave-Verdicchio) and ancient (Dindarella-Pelara, Oseleta, Rossetta di montagna) varieties all grown in the Valpolicella hills, suggesting the possibility that their evolution occurred in the same area and with few common anchestors. Two situations of synonyms that had already described between Trebbiano di Soave and Verdicchio, and between Dindarella and Pelara, were confirmed by a molecular method as SSR analysis. Amplification of Trebbiano di Soave/Verdicchio *locus* VVMD36 yielded a fragment of 500 bp, this allele provides a fast and reliable tool to differentiate among Trebbiano grapevines.**

K e y w o r d s : Biodiversity, SSR, Verona.

## Introduction

Microsatellites or Simple Sequence Repeats (SSRs) are regions of the eukaryotic genome characterised by repetition (from 5 to about 100) of simple sequence motifs composed of 1 to 6 nucleotides. The abundance of microsatellite sequences in the eukaryote genomes represents an almost unlimited source of polymorphic sites that may be exploited as genetic markers. The number of repetitions can be highly variable, therefore SSRs are effective to study the phylogenetic relationship among species and varieties. SSRs are widely used for varietal identification due to their variability and co-dominant heredity (Morgante and Olivieri, 1993).

For a long time, the Verona area is famous for its viticulture and nowadays both, white and red wines receive good rating all over the world. Viticulture of Verona is based on a number of local grapevine varieties of unknown origin. A collection of ancient grapevines has recently been established and clonally propagated by the 'Centro per la Sperimentazione in Vitivinicoltura, Provincia di Verona' providing suitable material to investigate the relationships between ancient and actually grown grapevine varieties (Tosi and Bletzo 2000). Clones or accession from this collection were subjected to SSR analysis to produce a molecular characterisation of grapevines grown in the province of Verona.

## Material and Methods

Leaf samples of 13 grapevines cultivated in the province of Verona were harvested from the collection of the 'Centro per la Sperimentazione in Vitivinicoltura, Provincia di Verona', preserving the analyzed clones of Corvina, Garganega, Molinara, Corvinone, Rondinella, and sustaining a collection to preserve Durella, Trebbiano toscano, Trebbiano di Soave and Verdicchio and the ancient varieties Dindarella, Pelara, Oseleta, Cabrusina, Rossetta di montagna. Enantio accessions were collected from private wineries (Table). DNA extraction was performed according to a micromethod for DNA purification (Lefort and Douglas 1999) developed for hardwood species.

Samples were analysed by 10 SSR *loci*: VVS2 (Thomas and Scott 1993), VVMD5 and VVMD7 (Bowers *et al*. 1996), VVMD14, VVMD25, VVMD27, VVMD28, VVMD31 and VVMD36, (Bowers *et al*. 1999) and VrZAG62 (Sefc *et al*. 1999).

The PCR mixture contained 50 ng of genomic DNA, 0.5 U Taq DNA polymerase (Biotools), 1.5 mM $MgCl_2$, 200 µM dNTPs, 1 µM forward primer labelled with fluorophores (TET, HEX, FAM), 1 µM reverse primer. PCR amplification was performed using Robocycler gradient (Stratagene) programmed with: 2 min at 94 °C, followed by 40 cycles of 20 s at 94 °C, 30 s at the appropriate annealing temperature (as reported in literature cited above) and 30 s at 72 °C, plus a final elongation step at 72 °C for 3 min. PCR products were controlled on 1 % agarose gel.

At least three PCR reactions labelled with different fluorophores were grouped by mixing 1 µl of each reaction and loaded on to ABI Prism 310 Genetic Analyser. Length of SSR alleles were evaluated using the GeneScan software.

## Results and Discussion

Three clones or accessions for each grapevine variety were analysed by 10 SSR markers chosen among the most polymorphic ones. The Table reports SSR alleles for each

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T a b l e

Amplification band of 42 clones analysed with 10 SSR primers. Data are grouped for the three clones/accessions of each variety (row) and for amplification SSR data (column). Length of SSR amplification band is reported in bp. Differences among clones/accessions of the same variety are given in bold. * = approximate size estimated on agarose gel (see Figure)

| Clone or accession | VVS2 | ZAG64 | VVMD14 | VVMD25 | VVMD27 | VVMD28 | VVMD31 | VVMD36 | VVMD5 | VVMD7 |
|---|---|---|---|---|---|---|---|---|---|---|
| corvina ISVCV7 | 149:153 | 136:143 | 235:225 | 242:264 | 178:188 | 258:258 | 209:213 | 249:249 | 237:229 | 235:235 |
| corvina ISVCV13 | 149:153 | 136:143 | 235:225 | 242:264 | 178:188 | 258:258 | 209:213 | 249:249 | 237:229 | 235:235 |
| corvina ISVCV48 | 149:153 | 136:143 | 235:**229** | 242:264 | 178:188 | 258:258 | 209:213 | 249:249 | 237:229 | 235:235 |
| garganega ISVCV11 | 130:140 | 136:138 | 229:225 | 242:256 | 178:192 | 236:248 | 207:209 | 261:249 | 223:229 | 245:**247** |
| garganega ISVCV24 | 130:140 | 136:138 | 229:225 | 242:256 | 178:192 | 236:248 | 207:209 | 261:249 | 223:229 | 245:**247** |
| garganega ISVCV84 | 130:140 | 136:138 | 229:225 | 242:256 | 178:192 | 236:248 | 207:209 | 261:249 | 223:229 | 245:**249** |
| molinara ISVCV3 | 132:153 | 160:164 | 229:227 | 240:258 | 178:178 | 236:258 | 209:209 | 265:249 | 235:225 | 235:249 |
| molinara ISVCV87 | 132:153 | 160:164 | 229:227 | 240:258 | 178:178 | 236:258 | 209:209 | 265:249 | 235:225 | 235:249 |
| molinara ISVCV100 | 132:153 | 160:164 | 229:227 | 240:258 | 178:178 | 236:258 | 209:209 | 265:249 | 235:225 | 235:249 |
| corvinone ISVCV2 | 132:149 | 178:178 | 235:227 | 256:264 | 178:184 | 234:256 | 207:207 | 249:249 | 237:229 | 235:229 |
| corvinone ISVCV3 | 132:149 | 178:178 | 235:227 | 256:264 | 178:184 | 234:256 | 207:207 | 249:249 | 237:229 | 235:229 |
| corvinone ISVCV7 | 132:149 | 178:178 | 235:227 | 256:264 | 178:184 | 234:256 | 207:207 | 249:249 | 237:229 | 235:229 |
| rondinella ISVCV23 | 140:149 | 143:160 | 229:225 | 242:256 | 178:188 | 246:258 | 209:213 | 259:249 | 223:223 | 235:235 |
| rondinella ISVCV73 | 140:149 | 143:160 | 229:225 | 242:256 | 178:188 | 246:258 | 209:213 | 259:249 | 223:223 | 235:235 |
| rondinella ISVCV76 | 140:149 | 143:160 | 229:225 | 242:256 | 178:188 | 246:258 | 209:213 | 259:249 | 223:223 | 235:235 |
| trebbianoS acc.1 | 130:153 | 143:164 | 229:227 | 242:242 | 178:184 | 236:258 | 207:209 | 500:500* | 225:237 | 235:243 |
| trebbianoS acc.2 | 130:153 | 143:164 | 229:227 | 242:242 | 178:184 | 236:258 | 207:209 | 500:500* | 225:237 | 235:243 |
| trebbianoS acc.3 | 130:153 | 143:164 | 229:227 | 242:242 | 178:184 | 236:258 | 207:209 | 500:500* | 225:237 | 235:243 |
| verdicchio acc.1 | 130:153 | 143:164 | 229:227 | 242:242 | 178:184 | 236:258 | 207:209 | 500:500* | 237:225 | 235:243 |
| verdicchio acc.2 | 130:153 | 143:164 | 229:227 | 242:242 | 178:184 | 236:258 | 207:209 | 500:500* | 237:225 | 235:243 |
| verdicchio acc.3 | 130:153 | 143:164 | 229:227 | 242:242 | 178:184 | 236:258 | 207:209 | 500:500* | 237:225 | 235:243 |
| dindarella acc.1 | 140:153 | 136:160 | 235:229 | 250:264 | 178:188 | 246:258 | 209:209 | 259:249 | 229:223 | 235:245 |
| dindarella acc.2 | 140:153 | 136:160 | 235:229 | 250:264 | 178:188 | 246:258 | 209:209 | 259:249 | 229:223 | 235:245 |
| dindarella acc.3 | 140:153 | 136:160 | 235:229 | 250:264 | 178:188 | 246:258 | 209:209 | 259:249 | 229:223 | 235:245 |
| pelara acc.1 | 140:153 | 136:160 | 235:229 | 250:264 | 178:188 | 246:258 | 209:209 | 259:249 | 229:223 | 235:245 |
| pelara acc.2 | 140:153 | 136:160 | 235:229 | 250:264 | 178:188 | 246:258 | 209:209 | 259:249 | 229:223 | 235:245 |
| pelara acc.3 | 140:153 | 136:160 | 235:229 | 250:264 | 178:188 | 246:258 | 209:209 | 249:249 | 229:223 | 235:245 |
| oseleta acc.1 | 149:149 | 136:143 | 229:229 | 264:268 | 178:190 | 228:258 | 209:213 | 292:249 | 229:229 | 235:253 |
| oseleta acc.2 | 149:149 | 136:143 | 229:229 | 264:268 | 178:190 | 228:258 | 209:213 | **249**:249 | 229:229 | 235:253 |
| oseleta acc.3 | 149:149 | 136:143 | 229:229 | 264:268 | 178:190 | 228:258 | 209:213 | 292:249 | 229:229 | 235:253 |
| enantio acc.1 | 132:149 | 140:164 | 225:217 | 256:256 | 184:190 | 228:255 | 213:213 | 249:249 | 225:225 | 243:259 |
| enantio acc.2 | 132:149 | 140:164 | 225:217 | 256:256 | 184:190 | 228:255 | 213:213 | 249:249 | 225:225 | 243:259 |
| enantio acc.3 | **153**:149 | 140:164 | 225:217 | 256:256 | 184:190 | 228:255 | 213:213 | 249:249 | 225:225 | 243:259 |
| durella ISVCVI4 | 130:130 | 158:158 | 229:209 | 250:256 | 178:184 | 244:244 | 213:209 | 247:247 | 223:233 | 243:243 |
| durella ISVCVI6 | 130:130 | 158:158 | 229:209 | 250:256 | 178:184 | 244:244 | 213:209 | 247:247 | 223:233 | 243:243 |
| durella ISVCVI8 | 130:130 | 158:158 | 229:209 | 250:256 | 178:184 | 244:244 | 213:209 | 247:247 | 223:233 | 243:243 |
| cabrusina acc.1 | 143:149 | 138:138 | 229:217 | 242:250 | 184:188 | 234:254 | 209:213 | 249:249 | 229:243 | 243:249 |
| cabrusina acc.2 | 143:149 | 138:138 | 229:217 | 242:250 | 184:188 | 234:254 | 209:213 | 249:249 | 229:**229** | 243:249 |
| cabrusina acc.3 | 143:149 | 138:138 | 229:217 | 242:250 | 184:188 | 234:254 | 209:213 | 249:249 | 229:243 | 243:249 |
| rossetta acc.1 | 140:153 | 136:160 | 229:229 | 256:256 | 180:184 | 236:256 | 209:209 | 265:249 | 225:229 | 235:249 |
| rossetta acc.2 | 140:153 | 136:160 | 229:229 | 256:256 | 180:184 | 236:256 | 209:209 | 265:249 | 225:229 | 235:249 |
| rossetta acc.3 | 140:153 | 136:160 | 229:229 | 256:256 | 180:184 | 236:256 | 209:209 | 265:249 | 225:229 | 235:249 |

accession. P.I. (probability of identity, SEFC *et al*. 1999), considering the whole set of SSRs analysed, was $7.94 \cdot 10^{-9}$, therefore varieties with the same alleles should be considered synonyms. All markers but one are highly polymorphic, VVMD31 had only three alleles detected.

Intravarietal variability: In 5 grapevines a limited intravarietal polymorphism was detected. The clone ISV-CV 84 of Garganega showed a VVMD7 amplification band with two additional base pairs with respect to the allele carried by clones ISV-CV 11 and ISV-CV 24. The clone ISV-CV 48 of Corvina (the most popular Corvina clone due to its peculiar characteristics) showed a VVMD14 amplification band 4 bp longer than the same *locus* in other Corvina clones tested.

From the ancient varieties Oseleta and Cabrusina, two accessions out of three had an heterozygous amplification at the VVMD36 and VVMD5 *loci*, respectively, while the same *loci* were homozygous in the third accession examined. The disappearance of one allele may suggest that the *loci* have reached homozygosity, similar polymorphisms have already been described by REGNER *et al.* (2000). The VVS2 amplification product of the accession no. 3 of Enantio was 21 bp longer than the corresponding allele of the other two accessions. Differences in clones or accessions suggest that some grapevines grown in the Province of Verona should be considered populations of related plants, rather than uniform varieties. The high frequency of intravarietal polymorphism could also be ascribed to the relative recent rescue and cataloguing of many varieties.

Intervarietal variability and phylogenetic relationship: Preliminary phylogenetic analysis of the data points out the existence of two clusters of similarity among the analysed clones. The first cluster consists of Corvina, Oseleta, Rondinella, Garganega, Dindarella and Pelara, the second of Rossetta di montagna, Molinara, Trebbiano di Soave-Verdicchio, while other grapevines (Durella, Enantio, Cabrusina and Corvinone) do not show any clear relationship neither among themselves, nor with the varieties described above.

In the past, Corvinone was considered highly related to Corvina. A previous isoenzyme analysis differentiated Corvinone from Corvina supporting the registration of two independent grapevine cultivars (CANCELLIER and ANGELINI 1993). Our analysis not only confirms that Corvina and Corvinone are two distinct cultivars, but also points out that Corvinone is not related to any other cultivar of Verona.

Homonyms and synonyms: SSR analysis evinced two examples of synonyms. The first concerned Dindarella and Pelara. Pelara is traditionally differentiated from Dindarella because of its small number of berries per cluster; nevertheless our SSR analysis proved the absolute identity of these varieties. The phenotypic difference may be due to a specific mutation or to different health conditions. Trebbiano di Soave and Verdicchio were also found synonymous after SSR analysis, as reported in the National Grapevine Register.

Many grapevines in Italy are named Trebbiano such as Trebbiano toscano, Trebbiano romagnolo, Trebbiano di Modena, Trebbiano di Spagna, Trebbiano spoletino, Trebbiano di Lugana, Trebbiano di Soave. In the Province of Verona, Trebbiano di Soave is grown at the far east, while Trebbiano di Lugana is cultivated in the extreme west, in the area of Lugana. In spite of small differences among accessions grown in the different areas, mainly due to variation of soil and microclimate, the two varieties are considered to be identical. Notably, our data revealed that Trebbiano di Soave (and its synonym Verdicchio) exhibited a highly reproducible allele at the VVMD36 *locus* with the unique size of about 500 bp, as revealed in a 2 % agarose gel (Figure). Amplification of the genome of several Trebbiano cultivars (Trebbiano di Soave, Trebbiano di Lugana and three clones of Trebbiano toscano) showed that only Trebbiano di Soave and its synonyms (Trebbiano di Lugana and Verdicchio) have the 500 bp fragment at the VVMD36 *locus*. Instead, the three clones of Trebbiano toscano yielded a band of about 250 bp, suggesting that Trebbiano toscano is not closely related to Trebbiano di Soave (Figure). The 500 bp allele at the VVMD36 *locus* represents therefore a fast and reliable marker for the rapid identification of Trebbiano di Soave and synonyms, since it can be scored unambiguously on agarose gel.

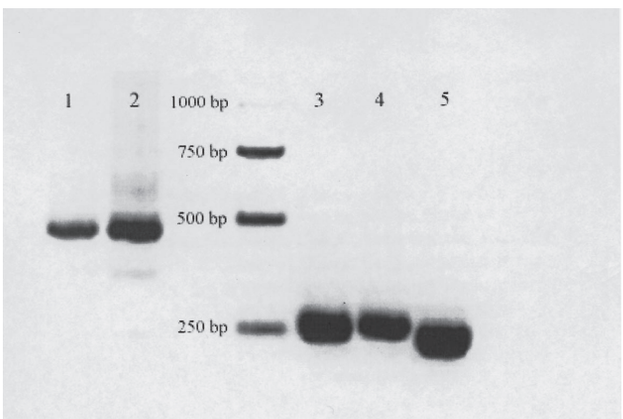


Figure: Gel electrophoresis in a 2 % gel of the VVDM36 *locus* amplified from the genome of Trebbiano di Lugana (lane 1), Trebbiano di Soave (lane 2) and three clones of Trebbiano toscano (lane 4: clone AP-TR 2, lane 5: clone Rauscedo 4, lane 6: clone Santa Lucia 30). Molecular markers in lane 3. Trebbiano di Lugana and Trebbiano di Soave show the same VVDM36 *locus* with a fragment of about 500 bp. The clone Santa Lucia 30 of Trebbiano toscano in lane 6 shows an amplification band smaller than the corresponding *locus* of clones AP-TR 2 and Rauscedo 4; this was confirmed on acrylamide gel (not shown).

Ampelographic analysis considers Garganega closely related to Grecanico dorato, a grapevine cultivated in Sicily. Comparison of our data with those of Garganega and Grecanico dorato reported in the Grape Molecular Collection of the Istituto Agrario di San Michele all'Adige (http://relay.ismaa.it:12164/genetica/gmc.html), confirmed that these cultivars are highly related and most likely represent the same grapevine.

## Conclusions

Several grapevines grown in the Province of Verona retained common genetic traits, suggesting that their evolu-

tion occurred in the same area and from few common ancestors. In addition, this report reveals the presence of several ancient varieties closely related to the most traditional grapevines of Verona, disclosing new genetic resources to further develop locally adapted high quality grapevines.

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