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Highly variable *Vitis* microsatellite loci for the identification of Pinot Noir clones

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Summary

Nineteen new microsatellite loci of *Vitis* were elaborated by following the procedure of tagging an SSR-enriched library. Primers for these VRG markers were used for genotyping grapevines. Only the markers VRG 1, VRG 2, VRG 4, VRG 7, VRG 9, VRG 10, VRG 15 and VRG 16 show heterozygous alleles and Mendelian segregation. Other VRG loci such as VRG 5, VRG 6, VRG 11, VRG 12, VRG 13 and VRG 17 produce a multiallelic profile and some of them show distorted segregation. Variability of the VRG loci is rather high as compared to other grapevine SSR markers. Stable VRG markers such as VRG 16 can be useful for the identification of cultivars. Highly variable VRG microsatellites could be successfully applied to trace polymorphism within the variety Pinot Noir. Clones of Pinot Noir could be differentiated using these markers. By applying the PhyQuest program, a dendrogram showing the genetic divergence within Pinot Noir clones was constructed.

Key words: *Vitis*, clonal selection, genotyping, Pinot Noir.

Introduction

Pinot Noir is one of the major traditional grapevine cultivars worldwide with a long historical background. About 40,000 ha are cultivated mainly in temperate zones. Due to the large family and several closely related cultivars, the genetic relationship among the Pinots was not clear (AMBROSI *et al.* 1994). Application of SSR (Simple Sequence Repeats) markers revealed the relationship of the Pinots and helped to clarify their origin (REGNER *et al.* 2000 a). The high variability within cv. Pinot Noir has also generated other perspectives in regard to the genetic relationship of Pinot-related cultivars (FORNECK *et al.* 2002). It was supposed that Pinot Meunier is not an individual cultivar but a chimeric vine (FRANKS *et al.* 2002). Chimeric types of Pinot Gris are supposed to be the origin of all Pinots (HOCQUINGY *et al.* 2004). Even Pinot Noir, Pinot Gris and Pinot Blanc belong to the same cultivar, their main difference being berry color.

Microsatellite markers became widely accepted for genotyping grapevines with the aim to identify cultivars (THOMAS *et al.* 1993, TESSIER *et al.* 1999, SEFC *et al.* 2001). Due to their high degree of polymorphism, their codomi-

nant inheritance and their reproducibility, SSR markers are preferred to other DNA based markers (VOSMAN *et al.* 2001). In fact, internationally accepted scoring systems based on these markers are already introduced to improve germplasm management (THIS *et al.* 2004).

Most stable SSR markers are located in noncoding areas due to their two nucleotide repeats. SSR markers, which are derived from EST (Expressed Sequence Tag) or cDNA databases, could be aligned with higher relevance to the phenotype of the vine. The sequence of most SSRs seems to be highly conserved as usual in noncoding areas. Therefore utility of SSRs is primarily given for genotyping cultivars of grapevine and not for detecting spots (BORREGO *et al.* 2002).

Since grapevines are cultivated since ancient times, several traditional varieties like Pinot show a high degree of variability (BASSERMANN-JORDAN 1975). The level of variability depends on the cycles of propagation, the spread to different locations and the intensity of cultivation by growers (SCHÖFFLING and STELLMACH 1993). Since the 19th century clonal selection has been used to improve agricultural performance of several grapevine cultivars (AMBROSI *et al.* 1994). In clonal selection the natural variation of a grapevine cultivar is used for specific viticultural tasks enabling breeders to offer different phenotypes within the term of a single cultivar. For this reason, clones nowadays do not represent a huge spectrum of variability within a traditional variety but reflect the result of selection criteria by viticulturists. For breeding purpose it would be advantageous to identify propagation material of individual clones and it would also be helpful to estimate the heterozygosity of the involved material and to compare new genotypes with clones already established. A further interest is to gain an indication of genetic differences of these clones and to demonstrate the variability of individual Pinot Noir clones.

The first results from differentiating clones by genetic markers (BELLIN *et al.* 2001, REGNER *et al.* 2001, RIAZ *et al.* 2002) show that this topic is not limited by tools but by the amount of loci applied. If many markers are used the probability to find differing alleles increases. RAPD (Random Amplified Polymorphic DNA), Inter SSR, AFLP (Amplified Fragment Length Polymorphism) and even SSR markers could be applied to find polymorphism in White Riesling (REGNER *et al.* 2000 b), Traminer (IMAZIO *et al.* 2002; REGNER *et al.* 2002) and other cultivars (HOCQUINGY *et al.* 2004). For identification of clones only sequence-characterized markers will be reproducible and stable in analysis.

Material and Methods

Construction of a library and elucidation of the SSRs. Genomic DNA of *Vitis riparia* was purified from leaves of *in vitro*-grown plants. Isolation was performed following the procedure published by THOMAS *et al.* (1993). DNA restriction fragments with sizes of 200-400 bp were introduced into the multicloning site of a pUC 18 vector. Positively transformed clones of the *E. coli* XL1 blue strain were screened first for insertions by testing β -galactosidase production and afterwards were tested by hybridisation against a (GA)_n probe. The sequences were developed in the frame of the VMC (Vitis Microsatellite Consortium = Collaboration Agreement for the Development of Grape Microsatellite Markers 1998).

Segregation analysis and genotyping. For the segregation analysis of the developed VRG (Vitis Riparia Götzhof) markers, 68 offsprings derived from a cross of Welschriesling \times Sirius were used. They are all kept at the Department for Grapevine Breeding at the Federal College and Institute for Viticulture and Horticulture in Klosterneuburg (HBLA u. BA Klosterneuburg). In order to prove their utility in genotyping, these markers were also analyzed in 45 traditional cultivars well established in the HBLA u. BA Klosterneuburg (Tab. 1).

DNA was extracted from young leaves of field-grown plants following the protocol of THOMAS *et al.* (1993) modified by REGNER *et al.* (1998). Amplification was performed in 20 μ l of the buffer solution, which consisted of 16 mM (NH₄)₂SO₄, 67 mM Tris-HCl, pH 8.8, 1.5 mM MgCl₂, 0.01 % Tween 20, 0.1 mM of each dNTP (GenXpress, Maria Wörth, Austria), 0.2 μ M primer (labelled with the fluorescent dyes 6FAM, TET and HEX), 1 Unit Biotherm Taq DNA polymerase (GenXpress, Maria Wörth, Austria), and 50 ng of genomic DNA of grapevine. Labelling with different fluorescent coloring agents facilitated the application in multiplex PCR assays. The amplification of the SSR loci was performed in an Omnigene (Hybaid, Teddington, Great Britain) thermocycler processed for 36 cycles. The general PCR protocol applied was 2 min denaturation at 94 °C and 35 cycles with an annealing phase of 30 s and temperatures between 45 °C and 55 °C, 1 min synthesis phase at 72 °C and denaturation for 15 s at 92 °C. The annealing temperature for each locus was set according to the T_m of forward and reverse primer -10 °C temperature. A final extension of the fragments was performed at 72 °C for 5 min.

Yield of DNA fragments was estimated by running an aliquot of the sample on a 2 % agarose gel stained with ethidium bromide. The samples were denaturated by heating up with formamide and loaded together with a size standard (Genescan 350 Tamra, Appl. Biosystems, Warrington, Great Britain) on 6 % polyacrylamid gel. Detection of the SSR fragments was carried out by an automated sequencer (ABI 373, Perkin-Elmer, Vienna). Analyses were done twice in the case of polymorphism with a different plant sample. Characterization of Pinot Noir clones. Thirteen Pinot Noir clones were used in this study. They are all kept at the HBLA u. BA in Klosterneuburg. Their specific

Table 1

Fortyfive traditional grapevine cultivars used for the characterisation of VRG SSR loci

| | |
|------------------------|--------------------|
| Silvaner | Portugieser Blau |
| Chasselas | Muskat Ottonel |
| Traminer | Perle von Csaba |
| St. Laurent | Sauvignon Blanc |
| Cabernet Franc | Semillon |
| Veltliner Frührot | Rotgipfler |
| Chardonnay | Burgunder |
| Neuburger | Roesler |
| Wildbacher Blau | Rathay |
| Blauburger | Jubiläumsrebe |
| Bianca | Goldburger |
| Müller-Thurgau | Muskateller |
| Grenache | Heunisch |
| Schwarzriesling | Aligote |
| Steinschiller | Orangetraube |
| Merlot | Veltliner Grün |
| Lambrusco | Cabernet Sauvignon |
| Veltliner Rot | Zierfandler |
| Blaufränkisch | Riesling |
| Bouvier | Sultanina |
| Königin der Weingärten | Sangiovese |
| Scheibkern | Seifert |
| Vernatsch | |

Table 2

Pinot Noir clones used in this study. Selection criteria and specific origin

| No. | Pinot Noir clones | Selection criteria | Origin |
|-----|-------------------|------------------------|--------------------|
| 1 | Fr 52-86 | Typical Pinot | Freiburg (D) |
| 2 | Fr 52-57 | Intensive colour | Freiburg (D) |
| 3 | Gm A | Low yield | Geisenheim (D) |
| 4 | Kl 5/43 | High wine quality | Klosterneuburg (A) |
| 5 | Mariafeld | Loose cluster | Wädenswill (CH) |
| 6 | W2/10 | High yield | Wädenswill (CH) |
| 7 | Kl 3/45 | Wines of high typicity | Klosterneuburg (A) |
| 8 | Fr 52-80 | Typical Pinot | Freiburg (D) |
| 9 | Re 16/9 | Small bunches | Retz (A) |
| 10 | Poly VI | High yield | Unknown |
| 11 | Oberlin | Low yield | Pully (F) |
| 12 | Re 14/13 | High wine quality | Retz (A) |
| 13 | W 10/5164 | High yield | Wädenswill (CH) |

origin and their selection criteria are shown in Tab. 2. The grapevines were evaluated according to the O.I.V descriptors (O.I.V. 1983) and were analyzed with 35 SSR markers commonly used in identification of grapevine cultivars. These included VVS markers developed by THOMAS and

SCOTT (1993), VVMD markers by BOWERS *et al.* (1996, 1999) and VRZAG markers by SEFC *et al.* (1999). With the aim to find polymorphism within the cultivar, all newly developed SSR (VRG) markers were also applied. The protocol for DNA extraction, amplification and detection of the SSR fragments was the same as described above.

Statistical analysis: The calculation of the SSR based heterozygosity index was performed by using the software PhyQuest (TIEFENBRUNNER *et al.* 2002). A distance matrix could be calculated to obtain a dendrogram where the genetic similarity was observed.

Results and Discussion

Nineteen VRG SSR clones were sequenced and primers were designed for the amplification of these loci (Tab. 3). Their segregation pattern was analyzed in 68 seedlings derived from a cross of Welschriesling x Sirius as shown in Tab. 4. Mendelian segregation of heterozygous alleles could be detected in VRG 1, VRG 2, VRG 4, VRG 7, VRG 9, VRG 10, VRG 15 and VRG 16. VRG 15 shows distorted behavior in the small scale segregation analysis. The loci VRG 5, VRG 6, VRG 11, VRG 12, VRG 13 and

Table 3

Primer Sequences, sizes in base pairs (bp) and melting temperature (Tm) for the amplification of 19 VRG SSR loci and their repeat type

| Name (VMC code) | Sequence | Size (bp) | Tm (°C) | repeats |
|----------------------|---------------------------|-----------|---------|------------|
| VRG1F | AAggTTTccTgccggcgATAAcc | 23 | 72 | (TC)18x |
| VRG1R | ccATTggTAAATAAAgTccc | 20 | 56 | |
| VRG2F | cTccTccgcTcAcTcAccATTTTc | 24 | 69 | (TC)16x |
| VRG2R | cATgccTgcAggTcgAcTcTAgAgg | 25 | 71 | |
| VRG3F | ggTAcccggggATcTAcTAc | 20 | 59 | (GA)34x |
| VRG3R | AcAgcAAAacgcAgccc | 17 | 63 | |
| VRG4F | gTTcATcTgTcTAAAccTAggg | 22 | 56 | (GA)17x |
| VRG4R | AcAAgcTgcTTAcTccgcTccgg | 23 | 71 | |
| VRG5F | gATcccTTcATgcTATcccATggc | 24 | 70 | (TC)19x |
| VRG5R | AgcTTgcATgccTgcAggTcgAcTc | 25 | 75 | |
| VRG6F(VMCNG3a7F) | AgcccggAATcATAgcTcAc | 20 | 64 | (GA)39x |
| VRG6R(VMCNG3a7R) | ggcTgTcgggTAAccTcc | 18 | 63 | |
| VRG7F(VMCNG3c8F) | gcgATgAcgTccAATccAc | 19 | 65 | (GA)35x |
| VRG7R(VMCNG3c8R) | cccATTcTATcgcTcccAgT | 20 | 63 | |
| VRG8F(VMCNG3f8F) | AAgggATgATggTgTgcAAc | 20 | 63 | (GA)46x |
| VRG8R(VMCNG3f8R) | cTcAgcTcTcTcTcTAGgcTcTTAc | 25 | 61 | |
| VRG9F(VMCNG3h8F) | AAgATAATgggAAggAgAAcActg | 24 | 62 | (TC)10+5x |
| VRG9R(VMCNG3h8R) | ATgATggATTccccTTAgAc | 22 | 64 | |
| VRG10F(VMCNG3a10F) | gTgccTTgTcTTcAccTTAgcc | 22 | 64 | (TC)26x |
| VRG10R(VMCNG3a10R) | TTTgTgAgAAgTggAggTcggA | 22 | 64 | |
| VRG11F(VMCNG3c10F) | cTgcAcTTgATgATcAcAAAgA | 22 | 61 | (GA)34+13x |
| VRG11R(VMCNG3c10R) | gTgAgATcgAcTTgcgATAgAg | 22 | 61 | |
| VRG12F(VMCNG3e10F) | TcAggAgTTAgccATcAgggTc | 22 | 64 | (GA)40x |
| VRG12R(VMCNG3e10R) | ggccTAcATAggcAgggAAgT | 21 | 64 | |
| VRG13F(VMCNG3g10F) | TTTgggTggATTccTTTTTA | 20 | 59 | (GA)35+3x |
| VRG13R(VMCNG3g10R) | TcTgcTTTcTcTcTgTcTTg | 22 | 61 | |
| VRG14F(VMCNG3a11F) | AccAgcggcAATAATgTcAgT | 21 | 64 | (TC)13x |
| VRG14R(VMCNG3a11R) | gAccTcgTggTcgAccgA | 18 | 66 | |
| VRG15F(VMCNG3b11F) | cAggccgAgTTTcAaccggT | 19 | 66 | (GA)34x |
| VRG15R(VMCNG3b11R) | AggATcccgcggc | 14 | 66 | |
| VRG16F(VMCNG3f11F) | AATTcTAcAAccATgAgTccc | 22 | 61 | (GA)18 x |
| VRG16R(VMCNG3f11R) | TTATcTcAccAAAgcTcTTccA | 22 | 66 | |
| VRG17F(VMCNG3a12.1F) | AATgcAAgTggcAAgcAATgAc | 22 | 66 | (TC)5+2x |
| VRG17R(VMCNG3a12.1R) | cTgcTgcTTTTcATgTcggTTT | 22 | 66 | |
| VRG18F(VMCNG3b12F) | ccTggTTcAcAATTTcggcT | 20 | 64 | (GA)46x |
| VRG18R(VMCNG3b12R) | TgTcAgTgAccgcAcTaccg | 20 | 65 | |
| VRG19F(VMCNG3f12F) | TTAgcccgTgAATAAgAAgTg | 22 | 62 | (TC)29x |
| VRG19R(VMCNG3f12R) | ggccTTAcTAGTAAccgTgTgc | 22 | 62 | |

Table 4

Segregation pattern of VRG markers analyzed in 68 offspring derived from a cross Welschriesling × Sirius. Distribution of parental alleles and missing alleles in the seedlings was calculated ($p > 0.05$, degree of freedom = 1). H (primary allele). A (secondary allele)

| SSR locus | Observed H | Observed A | Missing values | Total | Chi-square value of distribution |
|-----------|------------|------------|----------------|-------|----------------------------------|
| VRG 1 | 34 | 28 | 6 | 68 | 0.58 |
| VRG 2 | 25 | 43 | 0 | 68 | 4.76 |
| VRG 3 | 53 | 10 | 5 | 68 | 29.34 |
| VRG 4 | 28 | 38 | 2 | 68 | 1.51 |
| VRG 6 | 27 | 41 | 0 | 68 | 2.88 |
| VRG 7 | 34 | 34 | 0 | 68 | 0.00 |
| VRG 9 | 39 | 26 | 3 | 68 | 2.60 |
| VRG 10 | 28 | 39 | 1 | 68 | 1.81 |
| VRG 11 | 32 | 35 | 1 | 68 | 0.13 |
| VRG 15 | 47 | 19 | 2 | 68 | 11.87 |
| VRG 16 | 34 | 34 | 0 | 68 | 0.00 |

VRG 17 produce multiallelic profiles and VRG 5 and VRG 12 show distorted segregation. At VRG 3, VRG 13, VRG 14, VRG 19 no stable inheritance in the population was observed. Although VRG 3 and VRG 13 could be used for genotyping in Pinot, only few of the descendants bear the same alleles as their parents at these two loci. VRG 8 and VRG18 did not result in stable amplification of the micro-satellite DNA (Tab. 5).

Analysing these markers in 45 traditional cultivars (Tab. 1) their potential in genotyping grapevines could be estimated. VRG 9 was the most useful marker for the identification of grapevines due to its polymorphism, distribution of alleles (Tab. 5), incidence in all used grapevines and stable inheritance of the locus.

Even though morphological and agronomic differences were found and used as selection criteria for the 13 Pinot Noir clones studied, both O.I.V. descriptors (data not shown) and the analysis with VVS, VVMD and VRZAG markers (Tab. 6) confirmed the “trueness to type” of these clones. These published markers, often used in identification of grapevine cultivars, did not indicate any polymorphism. They show stable alleles within the Pinot clones and seem to be useless for detecting intravarietal genetic variation. They were selected out of a pool of markers due to their experimental stability.

Nevertheless, some of the developed VRG markers, resulted in different allelic profiles (Tab. 7). The polymorphism of differing alleles could be reproduced in a second

Table 5

Alleles of 8 VRG markers: Size in base pairs (AS), frequencies in traditional cultivars (Tab. 1) (AF), expected (H_e) and observed (H_o) heterozygosity in 45 grapevines

| VGR 1 | | VGR 2 | | VGR 3 | | VGR 4 | | VGR 9 | | VGR 10 | | VGR 15 | | VGR 16 | |
|-------|------|-------|------|-------|------|-------|------|-------|------|--------|------|--------|------|--------|------|
| AS | AF | AS | AF | AS | AF | AS | AF | AS | AF | AS | AF | AS | AF | AS | AF |
| 198 | 0.1 | 108 | 0.08 | 196 | 0.03 | 107 | 0.24 | 224 | 0.2 | 91 | 0.02 | 136 | 0.4 | 246 | 0.5 |
| 221 | 0.02 | 155 | 0.2 | 197 | 0.3 | 150 | 0.5 | 225 | 0.25 | 93 | 0.2 | 140 | 0.08 | 250 | 0.02 |
| 224 | 0.4 | 157 | 0.08 | 198 | 0.1 | 193 | 0.2 | 226 | 0.01 | 95 | 0.3 | 142 | 0.08 | 254 | 0.02 |
| 226 | 0.18 | 158 | 0.02 | 212 | 0.02 | 195 | 0.06 | 232 | 0.02 | 97 | 0.03 | 148 | 0.02 | 260 | 0.1 |
| 228 | 0.2 | 159 | 0.4 | 214 | 0.1 | 198 | 0.02 | 236 | 0.06 | 101 | 0.1 | 150 | 0.2 | 261 | 0.1 |
| 230 | 0.1 | 162 | 0.2 | 240 | 0.44 | | | 237 | 0.02 | 103 | 0.06 | 164 | 0.12 | 263 | 0.26 |
| | 167 | 0.02 | | | | | 240 | 0.07 | 107 | 0.06 | 186 | 0.1 | | | |
| | | | | | | | 241 | 0.05 | 109 | 0.04 | | | | | |
| | | | | | | | 242 | 0.01 | | | | | | | |
| | | | | | | | 246 | 0.11 | | | | | | | |
| | | | | | | | 247 | 0.08 | | | | | | | |
| | | | | | | | 248 | 0.11 | | | | | | | |
| H_e | 0.74 | | 0.74 | | 0.69 | | 0.64 | | 0.85 | | 0.84 | | 0.73 | | 0.66 |
| H_o | 0.68 | | 0.75 | | 0.65 | | 0.51 | | 0.83 | | 0.77 | | 0.52 | | 0.58 |

Table 6

Allelic profiles (sizes in base pairs) at 35 stable SSR loci (VVS, VVMD, VRZAG) analyzed in 13 Pinot Noir clones. Due to the experience of mapping different populations we could determine VVMD6 as a homozygous locus while VRZAG 12 shows a null allele

| Locus | Pinot Noir clones (1-13) | |
|-----------|--------------------------|-----|
| VVS 1 | 183 | 190 |
| VVS 2 | 137 | 151 |
| VVS 3 | 214 | 220 |
| VVS 4 | 168 | 173 |
| VVS 29 | 171 | 179 |
| VVMD 5 | 228 | 238 |
| VVMD 6 | 199 | 199 |
| VVMD 7 | 239 | 243 |
| VVMD 8 | 140 | 142 |
| VVMD17 | 212 | 220 |
| VVMD 21 | 249 | 249 |
| VVMD 24 | 216 | 218 |
| VVMD 25 | 243 | 253 |
| VVMD 26 | 251 | 257 |
| VVMD 27 | 185 | 189 |
| VVMD 28 | 221 | 239 |
| VVMD 31 | 216 | 216 |
| VVMD 32 | 241 | 273 |
| VVMD 34 | 254 | 264 |
| VVMD 36 | 254 | 254 |
| VRZAG 7 | 157 | 157 |
| VRZAG 12 | 153 | |
| VRZAG 15 | 167 | 179 |
| VRZAG 21 | 202 | 208 |
| VRZAG 25 | 227 | 238 |
| VRZAG 29 | 114 | 118 |
| VRZAG 30 | 151 | 151 |
| VRZAG 62 | 189 | 195 |
| VRZAG 64 | 140 | 164 |
| VRZAG 67 | 128 | 154 |
| VRZAG 79 | 240 | 246 |
| VRZAG 82 | 251 | 271 |
| VRZAG 83 | 190 | 202 |
| VRZAG 93 | 188 | 188 |
| VRZAG 112 | 242 | 244 |

trial and the results were confirmed with a second sample of each clone. The genetic differences are not surprising, however, the possibility to find some deviations at SSRs is rare (HOCQUINGY *et al.* 2004). Mutations located in the range of an SSR marker characterized by the sequence enabled us to use them for clonal identification. Besides the VRG marker data also some other SSR polymorphism within the Pinot clones could be detected (data not shown). It seems that the SSR variability within a cultivar depends on the age and frequency of propagation of the cultivar as well as on the location in the genome. In mapping studies of Welschriesling x Sirius higher variable regions could

be illuminated. The occurrence of null alleles or the loss of heterozygosity as seen in clone Fr 52-57 at locus VRG 2 (Table 7) is one main source for polymorphic SSR loci. The appearance of a third or fourth allele due to chimerism is another kind of variability (HOCQUINGY *et al.* 2004). Mutations at the annealing site can easily inhibit the amplification of the allele and cause null alleles. A null allele was accepted if the repetition resulted in the same marker size without changing the PCR protocol for easier annealing conditions. Usually homozygous alleles would be recognized by higher amounts of fragment DNA. The formation of new alleles at VRG 2 could be observed in Fr 52-86 and Kl 5/43. At several other VRG loci new allele sizes can be observed due to changes in the DNA sequence. If the size is out of the frame of the locus, a larger rearrangement in the genome could have taken place. A third allele with the expected length for that locus could have its origin in periclinal chimera (FRANKS *et al.* 2002).

All these deviations make an identification system of clones within the variety feasible. Each genotype differs from all others at least by combining several SSR loci. As clonal material is derived by vegetative propagation these changes happen during cell division.

Polymorphism was used to calculate a proximity matrix of the clones. The clustering analysis based on these data (Figure) showed that clone Re 16/9 represents the most individual genotype, followed by W 2/10, Kl 3/45, Fr 52-80. Re 16/9 was collected from an ancient vineyard with various cultivars near Retz (Lower Austria). Clones from Freiburg were situated closely together in the dendrogram, maybe due to their similar selection area. As the groups contain either local individuals, loose cluster types or dark color types, the morphological range of Pinot Noir could be confirmed as a very broad one. Nowadays it can be supposed that the same clonal individuals have been dispersed all over Europe.

Genetic analysis as a tool to verify differences between clones is a useful method for breeding. Most grape growers prefer to cultivate clonal material of a traditional cultivar. In France and other countries with controlled production systems (appellation d'origine contrôlée) wines must be produced from specific clones to be accepted for the common A.O.C. labelling. Therefore identification of clonal material is highly appreciated for clonal breeding. While RAPD, AFLP, InterSSR and MSAP (Methylation-Sensitive Amplified Polymorphism) (IMAZIO *et al.* 2002) markers produce higher levels of polymorphism, SSR markers are more appreciated due to their stability, reproducibility and known position in the genome (ADAM-BLONDON *et al.* 2004). For clonal identification it will be very helpful to gain SSR markers like the VRGs, which are tagging higher variable regions in the genome.

The chance to identify an individual clone raises the question of any clonal protection. Comparing the costs of transgenic plants and traditionally bred clones, it is not logical that genetically modified (GVO) grapevines should be patented whereas clones do not get a proper protection. It would be very helpful if private growers would offer their clonal material to others. But they will not do so without the prospect of any benefit. Therefore interesting genetic

Table 7

Allele sizes (base pairs) at the VRG SSR loci analyzed in 13 Pinot Noir clones. Boldface numbers are deviating from the main profile of Pinot Noir. Unique alleles are favored for the identification of a specific clone. Corresponding clone numbers are listed in Tab. 2

| Clone | VRG 1 | VRG 2 | VRG 3 | VRG 4 | VRG 7 | VRG 9 | VRG 10 | VRG 11 | VRG 13 | VRG 15 | VRG 16 | | | | | | | | | | | | | |
|-------|------------|------------|-------|------------|------------|------------|------------|------------|--------|--------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|-----|------------|-----|-----|
| 1 | 221 | 226 | 155 | 158 | 240 | 198 | 164 | 194 | 226 | 248 | 119 | 110 | 128 | 151 | 199 | 248 | 262 | | | | | | | |
| 2 | 198 | 226 | 155 | 240 | 198 | 164 | 194 | 226 | 248 | 119 | 94 | 105 | 114 | 128 | 174 | 95 | 139 | 151 | 248 | 262 | | | | |
| 3 | 221 | 226 | 155 | 162 | 240 | 297 | 339 | 164 | 194 | 226 | 248 | 119 | 110 | 128 | 95 | 139 | 151 | 199 | 248 | 262 | | | | |
| 4 | 221 | 226 | 155 | 158 | 198 | 194 | 194 | 226 | 248 | 119 | 94 | 114 | 128 | 95 | 151 | 95 | 151 | 199 | 248 | 262 | | | | |
| 5 | 221 | 226 | 155 | 162 | 198 | 164 | 194 | 226 | 248 | 119 | 105 | 105 | 128 | 95 | 139 | 151 | 199 | 248 | 262 | | | | | |
| 6 | 221 | 226 | 155 | 162 | 197 | 214 | 240 | 150 | 194 | 226 | 248 | 119 | 99 | 105 | 95 | 139 | 151 | 199 | 248 | 262 | | | | |
| 7 | 221 | 226 | 155 | 162 | 240 | 198 | 164 | 194 | 226 | 248 | 119 | 99 | 110 | 128 | 95 | 139 | 151 | 199 | 248 | 262 | | | | |
| 8 | 221 | 226 | 155 | 162 | 198 | 194 | 194 | 226 | 248 | 119 | 94 | 107 | 198 | 164 | 194 | 226 | 248 | 119 | 95 | 139 | 151 | 199 | 248 | 262 |
| 9 | 221 | 226 | 155 | 162 | 198 | 107 | 198 | 164 | 194 | 226 | 248 | 119 | 110 | 114 | 128 | 95 | 139 | 151 | 199 | 248 | 262 | | | |
| 10 | 198 | 224 | 155 | 162 | 240 | 198 | 164 | 194 | 226 | 248 | 119 | 105 | 128 | 151 | 114 | 128 | 151 | 199 | 248 | 262 | | | | |
| 11 | 221 | 226 | 155 | 162 | 240 | 198 | 194 | 226 | 248 | 119 | 105 | 114 | 128 | 151 | 114 | 128 | 151 | 199 | 248 | 262 | | | | |
| 12 | 221 | 226 | 155 | 162 | 240 | 198 | 194 | 226 | 248 | 119 | 131 | 94 | 105 | 100 | 114 | 128 | 151 | 199 | 248 | 262 | | | | |
| 13 | 221 | 226 | 155 | 162 | 240 | 198 | 194 | 226 | 248 | 119 | 131 | 105 | 110 | 128 | 110 | 128 | 151 | 199 | 248 | 262 | | | | |

material is kept away from the wine community due to the lack of any financial benefit for clonal selection.

Hence, if the genetic analysis reveals that the genetic basis of an individual clone differs from all other registered clones, the prerequisites of novelty are fulfilled. Additionally, for any kind of protection, uniformity and stability must be shown. In the past these criteria could not be evaluated for clones. Despite morphological differences, differentiation of clones is neglected by the International Union for Protection of Varieties (UPOV). Nowadays UPOV does not accept genetic differences as the only criterium for showing distinctness. One possible way to protect an

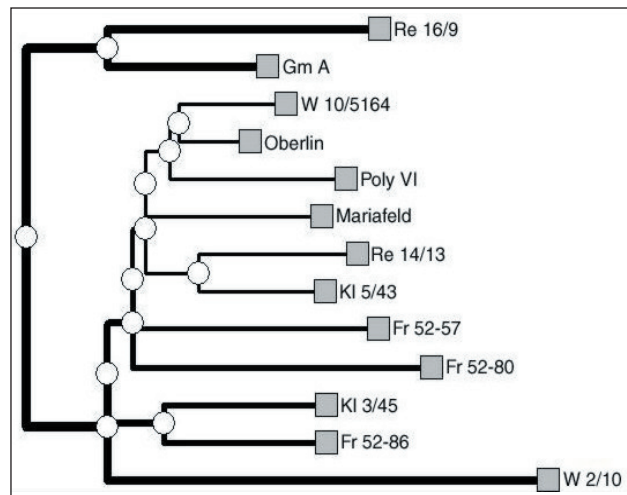


Figure: Dendrogram generated using the proximity matrix from the VRG SSR data of 13 Pinot Noir clones. The distance was calculated following Genelocus with hierarchical weighing of states.

individual clone could be the use of plants with defined unique sequences. Usually transgenic plants are accepted for patenting due to their additional transferred sequence. If a clone can be differentiated from all the others by determining a specific sequence there is no reason to refuse this protection approach.

Hopefully, concerning the proceedings in genetic mapping and definition of specific clones, we will reach the point that mutations can easily be linked to the corresponding DNA. The DUS (distinctness, uniformity, stability) test as criterion for variety protection will be applicable even to clonal material by using highly variable SSR markers. Moreover this genetic information about a clone allows to avoid duplicates in clonal collections and enables control of clonal identity.

What are the possible sources of the variation and their spread in grapevine? Repetitive sequences like SSRs are more frequently mutated than others. The slippage of one or more additional repeats is supposed (RAFALSKI *et al.* 1996). Even the enlargement of SSR loci during evolution has been postulated (ORTI *et al.* 1997). Nevertheless, the flanking regions of the SSR insert could also be changed. The annealing process of the primers at the conserved sites depends on the coincidence of the sequence. Mismatches could avoid amplification. If the mutated alleles and the original ones are coexisting in a vine, more than two alleles would be observed. It is supposed that in this case a chimeric grapevine would be detected. During longer propagation periods chimeric vines also may be separated into two different genotypes. This would explain the finding of different allelic profiles within samples of a single variety. The only risk for this procedure is that new mutations occur due to *in vitro* manipulations or spontaneous events.

The variability of Pinots (Gris, Blanc, Noir and Meunier) led to the assumption that Pinot Gris is the origin of the Pinots (HOCQUINGY *et al.* 2004). Due to the limited use of SSR loci and the non-representative sample for the whole development of the Pinots, it can be supposed that

research on the variability of Pinots should be intensified. There is a lack of connecting the genetic profile with the corresponding clones; therefore no comparison is possible. Future work on variability within a cultivar is requested to present data as a guideline for identification. Clonal profiles shown in this report are easily reproducible.

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References

- ADAM-BLONDON, A. F.; ROUX, C.; CLAUD, D.; BUTTERLIN, G.; MERDINOGLU, D.; THIS, P.; 2004: Mapping 245 SSR markers on the *Vitis vinifera* genome: A tool for grape genetics. *Theor. Appl. Genet* **109**, 1017-1027.
- AMBROSI, H.; DETTWEILER, E.; RÜHL, E.; SCHMID, J.; SCHUMANN, F.; 1994: Farbatlas Rebsorten. 300 Sorten und ihre Weine. Ulmer Stuttgart.
- BASSERMANN-JORDAN F.; 1975: Geschichte des Weinbaus. Pfälzische Verlagsanstalt. Neustadt/Weinstraße.
- BELLIN, B.; VELASCO, R.; GRANDO, S. M.; 2001: Intravarietal DNA polymorphisms in grapevine (*Vitis vinifera* L.). *Acta Hort.* **546**, 343-349.
- BORRERO, J.; DE ANDRÉS, M. T.; GÓMEZ, J. L.; IBÁÑEZ, J.; 2002: Genetic study of Malvasia and Torrontes groups through molecular markers. *Am. J. Enol. Vitic.* **53**, 125-130.
- BOWERS, J. E.; DANGL, G. S.; MEREDITH, C. P.; 1999: Development and characterization of additional microsatellite DNA markers for grape. *Am. J. Enol. Vitic.* **50**, 243-246.
- BOWERS, J. E.; DANGL, G. S.; VIGNANI, R.; MEREDITH, C. P.; 1996: Isolation and characterisation of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). *Genome* **39**, 628-633.
- FORNECK, A.; KONRADI, J.; BLAICH, R.; 2002: A genetic variation analysis of *Vitis vinifera* cv. Pinot Noir. *Acta Hort.* **603**, 167-170.
- FRANKS, T.; BOTTA, R.; THOMAS, M. R.; 2002: Chimerism in grapevines: Implications for cultivar identity, ancestry and genetic improvement. *Theor. Appl. Genet.* **104**, 192-199.
- HOCQUIGNY, S.; PELS, F.; DUMAS, V.; KINDT, S.; HELOIR, M. C.; MERDINOGLU, D.; 2004: Diversification within grapevine cultivars goes through chimeric states. *Genome* **47**, 579-589.
- IMAZIO, S.; LABRA, M.; GRASSI, F.; WINFIELD, M.; BARDINI, M.; SCIENZA, A.; 2002: Molecular tools for clone identification: The case of the grapevine cultivar Traminer. *Plant Breed.* **121**, 531-535.
- OFFICE INTERNATIONAL DE LA VIGNE ET DU VIN (O.I.V.) 1983: Le code des caractères descriptifs des variétés et espèces de *Vitis*. Ed. Dedon, Paris.
- ORTI, G.; PEARSE, D. E.; AVISE, J. C.; 1997: Phylogenetic assessment of length variation at a microsatellite locus. *Proc. Natl. Acad. Sci. USA* **94**, 10745-10749.
- RAFALSKI, J. A.; VOGEL, J. M.; MORGANTE, M.; POWELL, W.; ANDRE, C.; TINGEY, S. V.; 1996: Generating and using DNA markers in plants. In: B. BIRREN, E. LAI (Eds): *Non Mammalian Genomic Analysis*, 75-134. Academic Press, New York.
- REGNER, F.; KASERER, H.; 2002: Investigations into the genetic variability of Traminer clones. *Mitt. Klosterneuburg* **52**, 177-186.
- REGNER, F.; STADLBAUER, A.; EISENHELD, C.; 1998: Heunisch x Fränkisch ein wichtiger Genpool europäischer Rebsorten. *Vitic. Enol. Sci.* **53**, 114-118.
- REGNER, F.; STADLBAUER, A.; EISENHELD, C.; 2001: Molecular markers for genotyping grapevine and for identifying clones of traditional varieties. *Acta Hort.* **546**, 331-342.
- REGNER, F.; STADLBAUER, A.; EISENHELD, C.; KASERER, H.; 2000 a: Genetic relationship among Pinots and related cultivars. *Am. J. Enol. Vitic.* **51**, 7-14.
- REGNER, F.; WIEDECK, E.; STADLBAUER, A.; 2000 b: A differentiation and identification of White Riesling clones by genetic markers. *Vitis* **39**, 103-107.
- RIAZ, S.; GARRISON, K. E.; DANGL, G. S.; BOURSQUOT, J. M.; MEREDITH, C. P.; 2002: Genetic divergence and chimerism within ancient asexually propagated winegrape cultivars. *J. Am. Soc. Hortic. Science* **127**, 508-514.
- SCHÖFFLING, H.; STELLMACH, G.; 1993: Klon-Züchtung bei Weinreben in Deutschland. Waldkirchner Verlag, Waldkirchen.
- SEFC, K. M.; REGNER, F.; TURETSCHKE, E.; GLOESSL, J.; STEINKELLNER, H.; 1999: Identification of microsatellite sequences in *Vitis riparia* and their applicability for genotyping of different *Vitis* species. *Genome* **42**, 367-373.
- SEFC, K. M.; LEFORT, F.; GRANDO, M. S.; SCOTT, K.; STEINKELLNER, H.; THOMAS, M. R.; 2001: Microsatellite markers for grapevine: A state of the art. In: K. A. ROUBELAKIS-ANGELAKIS (Ed.): *Molecular Biology and Biotechnology of Grapevine*, 433-463. Kluwer Publ., Amsterdam.
- TESSIER, C.; DAVID, P.; THIS, P.; BOURSQUOT, J. M.; CHARRIER, A.; 1999: Optimization of the choice of molecular markers for varietal identification in *Vitis vinifera* L. *Theor. Appl. Gen.* **98**, 171-177.
- THIS, P.; JUNG, A.; BOCCACCI, P.; BORRERO, J.; BOTTA, R.; COSTANTINI, L.; CRESPIAN, M.; DANGL, G. S.; EISENHELD, C.; FERREIRA-MONTEIRO, F.; GRANDO, S.; IBÁÑEZ, J.; LACOMBE, T.; LAUCOU, V.; MAGALHAES, R.; MEREDITH, C. P.; MILAN, N.; PETERLUNGER, E.; REGNER, F.; ZULINI, L.; MAUL, E.; 2004: Development of a standard set of microsatellite reference alleles for identification of grape cultivars. *Theor. Appl. Gen.* **109**, 448-458.
- THOMAS, M. R.; MATSUMOTO, S.; CAIN, P.; SCOTT, N.; 1993: Repetitive DNA of grapevine: Classes present and sequences suitable for cultivar identification. *Theor. Appl. Gen.* **86**, 173-180.
- THOMAS, M. R.; SCOTT, N. S.; 1993: Microsatellite repeats in grapevine reveal DNA polymorphism when analysed as sequence-tagged sites (STSs). *Theor. Appl. Gen.* **86**, 985-990.
- TIEFENBRUNNER, A.; TIEFENBRUNNER, M.; TIEFENBRUNNER, W.; WAHRA, A.; 2002: A software tool as an aid to the identification of species of *Longidorus Micoletzky (Nematoda: Dorylaimoidea)*. *Nematology* **4**, 845-852.
- VOSMAN, B.; COOKE, R. J.; GANAL, M.; PEETERS, R.; ISAAC, P.; BREDEMEIJER, G.; 2001: Standardization and application of microsatellite markers for variety identification in tomato and wheat. *Acta Hort.* **546**, 307-316.

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