Vitis **44** (1), 35–38 (2005)

Characterisation of the grapevine cultivar Picolit by means of morphological descriptors and molecular markers

L. Zulini, E. Fabro and E. Peterlunger

Dipartimento di Scienze Agrarie ed Ambientali, Università di Udine, Italia

Summary

The phenotypic and genotypic variability of cv. Picolit, an ancient, female-flower cultivar from north-eastern Italy was investigated by means of ampelographic and ampelometric descriptors and by molecular markers, such as microsatellites and AFLPs. Thirty nine samples were collected from old plants (30-100 years old), which showed some differences in morphology and growth. In two samples (P6 and P7) morphological differences were found. These samples showed a different allelic profile at 18 out of the 21 SSRs analysed and were therefore considered not to belong to the cv. Picolit. Of the remaining samples, 35 gave the same allelic pattern at all SSRs and they were therefore considered 'true-to-type' Picolit, whereas two of them (P4 and P8) showed several variations, including extra alleles. One of the possible causes of such differences is chimerism. The AFLP analysis, from which samples P6 and P7 were excluded, enabled screening of a larger portion of the genome and confirmed the differences of the P4 and P8 samples from the remaining ones. P4 and P8 were different from the majority of samples at 13 and 37 AFLP *loci* respectively. A few further polymorphic bands were recorded in the remaining samples, but they were disregarded since they were not always reproducible. This research confirmed the appreciable somatic stability of SSR markers even in long-lived, vegetatively propagated plants, and the occasional occurrence of solid mutations and chimerisms.

K e y w o r d s : *Vitis vinifera*, Simple Sequence Repeat, AFLP, fingerprinting, chimerism, clonal selection.

Introduction

In the last 20 years molecular markers have provided a powerful tool for cultivar identification, and among these markers microsatellites (Simple Sequence Repeats, SSRs) and AFLP (Amplified Fragment Length Polymorphism) proved to be very useful. Microsatellites, in particular, are very often used as molecular markers for cultivar identification, pedigree analysis, population studies, and genome mapping. They are 1-6 base long core sequences tandemly repeated several times. Their hypervariability is due to the frequent change of the number of repeats of the core sequence at a given locus. Microsatellite repeats are largely interspersed in plant genomes and they were estimated to occur every 1.2 kb in *Arabidopsis* (Morgante *et al.* 2002). The SSR analysis gives reliable and repeatable results. Owing to all these features, microsatellites have been largely used in grape fingerprinting and genetic analysis (Thomas and Scott 1993; Cipriani *et al.* 1994; Bowers *et al.* 1996; Maletic *et al.* 1999; Sefc *et al.* 2000; Crespan and Milani 2001; Pellerone *et al.* 2001; Rossetto *et al.* 2002; Zulini *et al.* 2002; Hvarleva *et al.* 2004). The high heterozygosity of the grapevine genome (69-88 % according to Thomas and Scott 1993) contributes to widen the possible combinations of alleles at any SSR locus and this increases the discriminating power of these markers.

AFLP (Vos *et al.* 1995) is a molecular marker produced by selective amplification of restricted DNA fragments. A typical feature of this kind of marker is the high number of bands (~ loci), up to 50-100, that can be simultaneously analysed on each sample. This allows screening of a large part of the genome and therefore enhances the possibility to identify spot mutations that may distinguish clonal variations within a given cultivar (Cervera *et al.* 2000, Scott *et al.* 2000, Cervera *et al.* 2001, Bellin *et al.* 2001).

Both, morphological descriptors and molecular markers are important tools for the identification and characterisation of grapevine germplasm with the aim to study and preserve the genetic variability existing in viticulture; thus rare and endangered cultivars may be identified and re-evaluated to obtain typical products which may characterise a particular territory on the wine market.

The aim of this research was to characterise the phenotypic and genetic variability of Picolit, an autochthonous grapevine cultivar grown in Friuli (north-eastern Italy), by means of ampelographic descriptors, SSR and AFLP markers. Picolit has hermaphrodite flowers with reflex stamina producing sterile pollen, low berry set and low productivity. Sweet dessert wines are obtained from Picolit usually after wilting of berries. The determination of its genetic variability is the first step to improve its production and to valorise this cultivar.

Material and Methods

Thirty-nine Picolit grapevine plants were identified in commercial vineyards, with a preference for long-lived individuals (30-100 years old). The second criterion of choice was the coverage of phenotypic variability observed during several visits to old vineyards in the area of the Picolit AOC "Colli Orientali del Friuli" (Friuli eastern hills).

Correspondence to: Dr. L. Zulini, Istituto Agrario di San Michele all'Adige, Unità Operativa Viticoltura, Via E. Mach 1, 38010 San Michele all'Adige, Italy. Fax: +39-0461-650-956. E-mail: luca.zulini@iasma.it

From each plant (biotype) 10 adult leaves were sampled and dried for ampelographic and ampelometric observations. Twelve descriptors were used, of which 6 were ampelographic and 6 ampelometric descriptors, excerpted from the OIV list as revised by the Genres 081 European Union project (Genres 081, 1997).

At bud burst shoot tips were sampled and from these DNA was extracted using the DOYLE and DOYLE method (1990), as modified by CIPRIANI and MORGANTE (1993).

Twenty-one microsatellite loci were analysed. They were chosen among the most polymorphic, *i.e.* with the greatest discrimination power: VVS2 (THOMAS and SCOTT 1993), VVMD5, VVMD7, VVMD27 (BOWERS *et al.* 1996), VrZAG47, VrZAG62, VrZAG79 (SEFC *et al.* 1999), VMC4A1, VMC4C6, VMC4D2, VMC4D3, VMC4D4, VMC4F3, VMC4G6, VMC4H2, VMC4H5, VMC4H6 (DI GASPERO *et al.* 2000), UDV10, UDV15, UDV23, UDV24 (DI GASPERO pers. comm.). PCR amplification and fragment analysis were carried out as described by ZULINI *et al.* (2002).

The AFLP analysis was carried out using 23 *Eco*RI/*Mse*I primer combinations and the AFLP® Analysis System I (GIBCOBRL®, Life Technologies). DNA (500 ng·sample^{-1}) was digested using 2.5 U of *Mse*I and *Eco*RI restriction enzymes during 4 h at 37 °C, ligated to *Eco*RI and *Mse*I adapters in 25 µl of a mix containing 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM MgAc, 50 mM Kac, 1 mM DTT, 50 mM KCl, 50 % glycerol (v/v), 4.6 pmol of *Eco*RI adapter, 46 pmol of *Mse*I adapter, 2 U of T4 DNA ligase. The ligation was incubated for 18 h at 10 °C. Restricted-ligated DNA was diluted 1:10 by adding TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and pre-amplified using primers complementary to the *Eco*RI e *Mse*I adapters with an additional selective base. PCR for pre-amplification was performed in a final volume of 50.4 µl in 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM of each dNTP, 47 ng of each primer (*Mse*I + C and *Eco*RI + A), 2 U of *Taq* DNA polymerase (Amersham Pharmacia biotech) and 5 µl of diluted DNA. PCR reaction was carried out in a PCR PTC-100TM (MJ Research, Inc.) using the following thermal profile: (94 °C for 30 s, 56 °C for 60 s, 72 °C for 60 s) for 28 cycles. Pre-amplificated DNA fragments were diluted 1:25 with TE buffer and used as template DNA for the selective amplification. In this amplification *Eco*RI and *Mse*I primers were used, with the same sequences of pre-amplification primers, but with three selective bases at the 3' end. *Eco*RI primers were labelled with γ^{33}P-ATP at the 5' end. PCR was performed in 20 µl of a mixture containing 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.9 mM of each dNTP, 30.15 ng of *Mse*I primer, 13.9 ng of labelled *Eco*RI primer, 1 U of *Taq* DNA polymerase (Amersham Pharmacia biotech) and 5 ml of pre-amplificated DNA. Selective amplification was carried out in a Perkin Elmer® 9700, with the following thermal profile: (94 °C for 30 s, 65 °C (-0.7 °C for cycle) for 30 s, 72 °C for 60 s) for 12 cycles; (94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s) for 23 cycles. In the AFLP analyses were used 23 primer combination: *Eco*RI + AAC/*Mse*I (+ CAA, + CAC, + CAG, + CTA); *Eco*RI + AAG/*Mse*I (+ CAA, + CAC, + CAG, + CTA, CTC); *Eco*RI + ACA/*Mse*I (+ CTG, + CTT); *Eco*RI + ACT/*Mse*I (+ CAT, + CTC, + CTG, + CTT); *Eco*RI + ACC/*Mse*I (+ CTG, + CTT); *Eco*RI + ACG/*Mse*I (+ CAC, + CAG); *Eco*RI + AGC/*Mse*I (+ CTA, + CTC); *Eco*RI + AGG/*Mse*I (+ CAT, + CTA). Twenty microliters of the amplified products were mixed with 15 µl of loading dye, heated at 95 °C for 5 min, and 3 µl loaded in each lane of a 6 % polyacrylamide sequencing gel and visualized after exposing the gels using Amersham Hyperfilm^{TM} MP film. Only easily scorable bands, which were clearly visible and/or consistently amplified in repeated gels, were considered for the analysis; bands were defined as polymorphic bands (present in only some samples) and monomorphic bands (present in all the samples).

Results and Discussion

By ampelographic and ampelometric analyses two different phenotypes of cv. Picolit were identified. The first phenotype was observed in 37 samples, it was therefore considered the 'true-to-type' Picolit. The second phenotype was rather different in several traits, and was observed in the samples P6 and P7 (Fig. 1, Tab. 1). Characters such as petiole sinus shape and opening are under strong genotype control, they are not strongly influenced by environment, *i.e.* their differences indicate a different genotype.

Fig. 1: Leaf lamina of 'true-to-type' Picolit and samples P6, P7 (see also Tab. 1).

Table 1

OIV descriptors showing morphological differences between Picolit and P6, P7 leaves

Biotype	Descriptor / level of expression		
	OIV 67[a]	OIV 79[b]	OIV 80[c]
Picolit	2	1	1
P6, P7	4	5	3

[a] shape of blade of the mature leaf: 2 = wedge-shaped, 4 = circular.
[b] degree of petiole sinus opening: 1= open, 5 = overlapping.
[c] shape of base of petiole sinus: 1= U-shaped, 3= V-shaped.

The microsatellite analysis confirmed that samples P6 and P7 were not Picolit, as they showed a different allelic profile at 18 out of the 21 SSR loci examined (Fig. 2). The microsatellite analysis of the remaining 37 Picolit biotypes

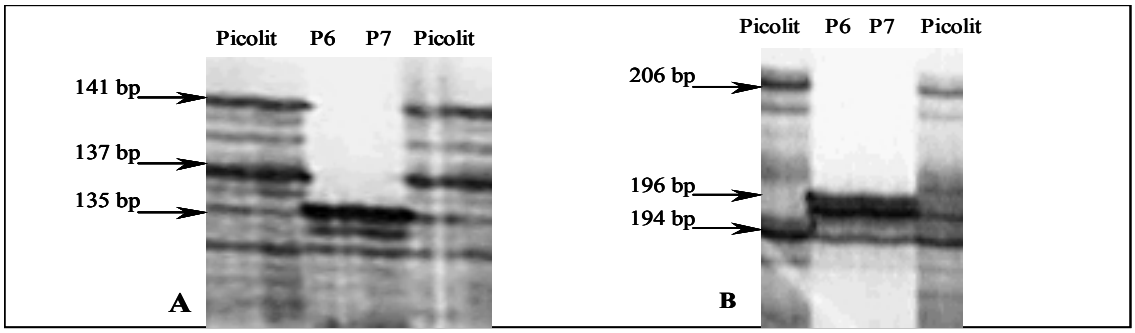

Fig. 2: Autoradiogram of a polyacrylamide gel separation of the γ^{33}P-labelled PCR-amplified microsatellite loci VVS2 (**A**) and VrZAG62 (**B**). The samples P6 and P7 show different alleles as compared with the 'true-to-type' Picolit (bp = base pairs).

resulted in 35 samples with the same allelic pattern at all the examined SSR loci: the profile of these samples may therefore be considered typical for this cultivar. The remaining two samples, P4 and P8, gave patterns different from the typical one at several loci.

The P4 sample showed an extra allele at the locus VMC4D3, and was heterozygous at the locus VMC4G6, at which the 'true-to-type' genotypes were homozygous (Tab. 2). The P8 sample showed the presence of a second allele at three loci (VVMD7, VMC4A1, VMC4H6) which were homozygous in the 'true-to-type' genotypes; P8 gave extra alleles in as much as 4 loci (Tab. 2). The microsatellite analyses were reproducible and repeated with different DNA samples for both clones, P4 and P8. While sample P4 could be considered a Picolit clone, slightly different from the most common genotype, P8 is more distant from the 'true-to-type' Picolit.

Table 2

Variability found in 37 clones of Picolit analysed at 21 SSR loci

SSR *locus*	most clones	P4	P8
VVS2	137-141	=[a]	=
VVMD5	232-238	=	n.a.[b]
VVMD7	247	=	239-247
VVMD27	181-185	=	=
VrZAG47	159-163	=	=
VrZAG62	194-206	=	=
VrZAG79	239-259	=	239-251-259
VMC4A1	278	=	274-278
VMC4C6	159-168	=	
VMC4D2	96-110	=	
VMC4D3	115-117	101-115-117	101-115-117
VMC4D4	158-164	=	=
VMC4F3	180-184	=	=
VMC4H2	77-79	=	65-77-79
VMC4H5	122-124	=	=
VMC4G6	125	125-127	125-127-135-141
VMC4H6	164	=	160-164
UDV010	158-174	=	=
UDV015	168-178	=	=
UDV023	184-200	=	=
UDV024	191-199	=	=

[a] = same pattern as most clones.
[b] n.a. = no amplification.

The presence of extra alleles, *i.e.* alleles exceeding the expected two, is rather frequent in grape. As much as 6 % of the 371 SSRs isolated and screened in a panel of a few cultivars by the VMC (Vitis Microsatellite Consortium) apparently yielded 3-4 bands. The presence of extra alleles could be due either to the occasional amplification of a second locus or to the presence of chimerism. The first cause, although likely, is just a speculation and is supported by the high chromosome number of the genus *Vitis* (2n = 2x = 38). Conversely, the occurrence of chimerism in grape has been recently demonstrated by Riaz *et al.* (2002), who found that a third allele, if occurring, was present in leaves but not in roots or woody tissue. At the same time, Franks *et al.* (2002) were able to isolate and grow separately the L1 and L2 cell layers of Pinot Meunier, a grape cultivar consistently showing three alleles at two different SSR loci. The fingerprinting of the two tissues demonstrated that L1- and L2- derived tissues carried only two alleles each, one of which in common, the other brought by either one or the other L layer.

As to the profile of the P8 clone, we do not know the cause(s) that generated such a profile.

The AFLP analyses, that allowed the exploration of a portion of the grapevine genome wider than the SSR analysis basically confirmed the results already discussed. Beside the samples P6 and P7, which were not included in the AFLP analysis, samples P4 and P8 were different from the majority of samples at 13 and 37 loci, respectively. A few further polymorphisms were recorded in other samples, but, since they were not always reproducible, they have been disregarded. In the analysis of clones of cv. Italia lack of reproducibility of AFLP single polymorphic bands was found by Fanizza *et al.* (2003), too.

Microsatellite analysis coupled with the morphological description proved to be an effective method for identification of cultivars and to distinguish between genotypes raised from sexual reproduction.

Clonal variations are still hard to find by analysis of only a few SSR loci, but extension of the molecular analysis to a larger part of the genome increases the chance to find mutated clones. This has been demonstrated by Riaz *et al.* (2002), who found differences within Pinot noir and

Chardonnay clones by screening 100 SSR loci. The AFLP approach, although less reliable because of the inconstancy of some 'ghost' bands, can also help to identify clonal variations, as has been demonstrated recently (CERVERA *et al.* 2000, SCOTT *et al.* 2000, BELLIN *et al.* 2001).

Cv. Picolit proved to be quite homogeneous, in spite of the different sites of sample origin. Moreover, it showed a remarkable somatic stability since several plants showing the same SSR and AFLP patterns, were up to 100 years old.

Acknowledgment

Research was funded by the Consortium 'Colli Orientali del Friuli' by a contribution of the European Union within the frame of the 5B project. Authors also thank Dr. G. Di Gaspero for kindly providing unpublished primers of the UDV microsatellite series. The revision and discussion of the manuscript by Prof. R. TESTOLIN, University of Udine, is greatly acknowledged.

References

BELLIN, D.; VELASCO, R.; GRANDO, M. S.; 2001: Intravarietal DNA polymorphisms in grapevine (*Vitis vinifera* L.). Acta Hortic. **546**, 343-349.

BOWERS, J. E.; DANGL, G. S.; VIGNANI, R.; MEREDITH, C. P.; 1996: Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). Genome **39**, 628-633.

CERVERA, M. T.; CABEZAS, J. A.; SÁNCHEZ-ESCRIBANO, E.; CENIS, J. L.; MARTINEZ-ZAPATER, J. M.; 2000: Characterization of genetic variation within table grape varieties (*Vitis vinifera* L.) based on AFLP markers. Vitis **39**, 109-114.

CERVERA, M. T.; RODIGRUEZ, I.; CABEZAS, J. A.; CHAVEZ, J.; MARTINEZ-ZAPATER, J. M.; CABELLO, F.; 2001: Morphological and molecular characterization of grapevine accessions known as Albillo. Am. J. Enol. Vitic. **52**, 127-135.

CRESPAN, M.; MILANI, N.; 2001: The Muscats: a molecular analysis of synonyms, homonyms and genetic relationship within a large family of grapevine cultivars. Vitis **40**, 23-30.

CIPRIANI, G.; FRAZZA, G.; PETERLUNGER, E.; TESTOLIN, R.; 1994: Grapevine fingerprinting using microsatellite repeats. Vitis **33**, 211-215.

CIPRIANI, G.; MORGANTE, M.; 1993: Evidence of chloroplast DNA variation in the genus Actinidia revealed by restriction analysis of PCR-amplified fragments. J. Genet. Breed. **47**, 319-326.

DI GASPERO, G.; PETERLUNGER, E.; TESTOLIN, R.; EDWARDS, K. J.; CIPRIANI, G.; 2000: Conservation of microsatellite loci within the genus *Vitis*. Theor. Appl. Genet. **101**, 301-308.

DOYLE, J .J.; DOYLE, J. L.; 1990: Isolation of plant DNA from fresh tissue. Focus **12**, 13-15.

FANIZZA, G.; CHAABANE, R.; RICCIARDI, L.; RESTA, P.; 2003: Analysis of a spontaneous mutant and selected clones of cv. Italia (*Vitis vinifera*) by AFLP markers. Vitis **42**, 27-30.

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.

Genres081 EU Project, 1997: Primary Descriptor List for Grapevine Cultivars and Species (*Vitis vinifera* L.). Institut für Rebenzüchtung Geilweilerhof, Siebeldingen, Germany.

HVARLEVA, T.; RUSANOV, K.; LEFORT, F.; TSVETKOV, I.; ATANASSOV, A.; ATANASSOV, I.; 2004: Genotyping of Bulgarian *Vitis vinifera* L. cultivars by microsatellites analysis. Vitis **43**, 27-34.

MALETIC, E.; SEFC, M. K.; STEINKELLNER, H.; KONTIC, J. K.; PEJIC, I; 1999: Genetic characterization of Croatian grapevine cultivars and detection of synonymous cultivars in neighbouring regions. Vitis **38**, 79-83.

MORGANTE, M.; HANAFEY, M.; POWELL, W; 2002: Microsatellites are preferentially associated with non repetitive DNA in plant genomes. Nat. Genet. **30**, 194-200.

PELLERONE, F. I.; EDWARDS, K. J.; THOMAS, M. R.; 2001: Grapevine microsatellite repeats: isolation, characterisation and use for genotyping of grape germplasm from Southern Italy. Vitis **40**, 179-186.

RIAZ, S.; GARRISON, K. E.; DANGL, G. S.; BOURSIQUOT, J. M.; MEREDITH, C. P.; 2002: Genetic divergence and chimerism within ancient asexually propagated winegrape cultivars. J. Am. Soc. Hortic. Sci. **127**, 508-514.

ROSSETTO, M.; MCNALLY, J.; HENRY, R. J.; 2002: Evaluating the potential of SSR flanking regions for examining taxonomic relationship in the Vitacee. Theor. Appl. Genet. **104**, 61-66.

SCOTT, K. D.; ABLETT, E. M.; LEE, L. S.; HENRY, R. J.; 2000: AFLP markers distinguishing an early mutant of Flame seedless grape. Euphytica **113**, 245-249.

SEFC, K. M.; REGNER, F.; TURETSCHEK, E.; GLÖSSL, 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.; LOPES, M. S.; LEFORT, F.; BOTTA, R.; ROUBELAKIS-ANGELAKIS, K. A.; IBANEZ, J.; PEJIC, I.; WAGNER, H. W.; GLÖSSL, J.; 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**, 498-505.

THOMAS, M. R.; SCOTT, N. S.; 1993: Microsatellite repeats in grapevine reveal DNA polymorphism when analyzed as sequence-tagged sites (STSs). Theor. Appl. Genet. **86**, 985-990.

VOS, P.; HOGERS, R.; BLEEKER, M.; REIJANS, M.; 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.

ZULINI, L.; RUSSO, M.; PETERLUNGER, E.; 2002: Genotyping wine and table grapevine cultivars from Apulia (Southern Italy) using microsatellite markers. Vitis **41**, 183-187.

Received September 20, 2004