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Comparison of different methods for SNP detection in grapevine

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Summary

Single nucleotide polymorphisms (SNPs) are the most abundant of all markers, both in animal and plant genomes. In crops and tree species considerable investment has been recently made on this genomic technology. While large-scale characterisation of SNPs by high-throughput techniques is possible, such high-throughput platforms are not available to all plant breeding laboratories. This report compares alternative multi-purpose and affordable methods for SNP assay in grapevine (*Vitis* spp.). In particular, the efficiency, sensitivity and reliability of single-strand conformation polymorphism (SSCP) on both non-denaturant gels and fluorescence-based capillary electrophoresis are compared with minisequencing (single nucleotide extension reaction). The results indicate that when multiplexing in combination with minisequencing is a mid-throughput, reliable and flexible technique for the detection of SNPs and can therefore be used effectively to improve marker assisted breeding in grapevine.

K e y w o r d s : grapevine, SNPs, SSCP, capillary electrophoresis, minisequencing, multiplex PCR.

Introduction

In plants, molecular diversity was first studied based on the existence of mutational events revealed by PCR-based genetic markers. Currently, detection of single nucleotide polymorphisms (SNPs) permits a more accurate approach to the analysis of sequence differences between alleles (Rafalski 2002). In fact, precise surveys of DNA diversity at the nucleotide level provide a snapshot of evolution at its most basic level. Nucleotide diversity reflects the combined history of selection, migration, recombination, and mating systems experienced by a species. Additionally, nucleotide diversity is one source of phenotypic variation (Buckler and Thornsberry 2002), and SNPs can be used as simple co-dominant genetic markers for high-resolution genetic mapping of traits, as well as for association studies based on candidate genes or on a whole genome scan (Rafalski 2002).

In medical science SNP markers are already used for genetic mapping of complex traits, pharmacogenomics and medical diagnostics (Kruglyak 1997, McCarthy and Hilfiker 2000, Suh and Vijg 2005). SNPs have been characterized in crop plant genomes such as maize (*Zea mays* L.; Ching *et al.* 2002), sugarbeet (*Beta vulgaris* L.; Schneider *et al.* 2001), barley (*Hordeum vulgare* L.; Kanazin *et al.* 2002), soybean (*Glycine max* L. Merrill; Zhu *et al.* 2003), wheat (*Triticum aestivum* L.; Bryan *et al.* 1999) and rice (*Oryza sativa, Oryza rufipogon*; Nasu *et al.* 2002).

A number of methods for SNP discovery and genotyping are available, although all are not equally useful and it is unclear which are the most suitable and most efficient (Gupta *et al.* 2001). Methods such as resequencing (Sanger *et al.* 1977), denaturing gradient gel electrophoresis (DGGE; Myers *et al.* 1986), single strand conformational polymorphism analysis (SSCP; Orita *et al.* 1989), minisequencing (Syvanen *et al.* 1990), heteroduplex analysis (HA; White *et al.* 1992), derived/cleaved amplified polymorphic sequences (dCAPs/CAPs; Konieczny and Ausubel 1993), dHPLC WAVE (Oefner and Underhill 1995), pyrosequencing (Ronaghi *et al.* 1998), TaqMan assay (Lee *et al.* 1999), targeting induced local lesions in genomes (TILLING; McCallum *et al.* 2000), and temperature gradient capillary electrophoresis (TGCE; Hsia *et al.* 2005) have all been used with success. Significant efforts towards large-scale characterisation of SNPs have been attempted with high-throughput techniques, such as DNA chips and microarrays (Gunderson *et al.* 2005) and the SNPlex™ genotyping system (Applied Biosystems; De la Vega *et al.* 2005). However, these platforms are expensive and not flexible since in order to be economically efficient consider only a fixed pool of genetic loci. Moreover, they are not practical for small to medium size laboratories and thus alternative techniques must be employed.

In this paper, affordable, moderately high-throughput, and multi-purpose methods for SNP assay (SSCP on both non-denaturant gel electrophoresis and fluorescence-based capillary electrophoresis, and minisequencing) are compared in grapevine where only a limited number of SNP-based studies have been completed (Owens 2003, Salmaso *et al.* 2004, Troggio *et al.* 2007).

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 : DNA was isolated from young leaves following the procedure by Doyle and Doyle (1990). Four cultivars of *Vitis vinifera* L. ('Moscato bianco', 'Teroldego rotaliano', 'Riesling italico', and 'Pinot Noir'), the hybrid 'Merzling' (the complex genotype 'Freiburg 993-60' derived from

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multiple crosses also involving wild species such as *V. rupestris* and *V. lincecumii*), and the accession Wr 63 of *Vitis riparia* Mchx. were considered. The six genotypes listed above, referred to with the abbreviations M, T, Ri, P, F and R, respectively, are the parents of different mapping populations: M x R (Grando *et al.* 2003), 'Syrah' x P (Troggio *et al.* 2007), and F x T (Salmaso *et al.* submitted). Six individuals from each F_1 population were also included in the analyses.

EST amplification: Twelve EST markers were chosen among well-characterized ESTs available at http://research.iasma.it/genomics. Of these, eleven are located on dense functional genetic linkage maps developed in grapevine (Vezzulli *et al.* 2006, Troggio *et al.* 2007, Salmaso *et al.* submitted) (Tab. 1). Genomic DNA from the six cultivars was amplified by PCR using the following conditions: 20 ng of DNA template, 1 x PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM of each primer, 1 Unit HotStarTaq DNA polymerase (Qiagen), and water to a final volume of 25 µl. PCR reactions were performed using a 15 min initial denaturation/activation step, followed by 30 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 2 min, with a final extension step of 10 min at 72 °C. PCR products were assessed by electrophoresis in 1.5 % agarose gels and visualized by ethidium bromide staining.

Polymorphism detection methods: Sequence diversity in the six grapevines was studied in the 12 ESTs by direct sequence analysis. PCR products were sequenced using ABI Prism® 3100 Genetic Analyzer (Applied Biosystems). Sequencing reactions (10 µl final volume) were prepared with 10-50 ng PCR product, 4 µl of ABI Prism® BigDye terminator sequencing ready reaction kit, and 5 pmol of the forward primer. Sequencing reactions were carried out using a 1 min initial denaturation step at 96 °C, followed by 35 cycles at 96 °C for 10 s, 55 °C for 5 s, and 60 °C for 4 min. DNA sequences were aligned with Pregap4/Gap4 software from Staden Package (Staden *et al.* 2000) and used to survey parental alleles for polymorphic sites. Haplotype inference was done by Clark's algorithm (Clark 1990).

The power to reliably detect the given SNPs within the 12 EST sequences was analysed by comparing different approaches:

SSCP on non-denaturant gel electrophoresis: This method was performed as described by Martins-Lopes *et al.* (2001) with modifications. An acrylamide gel solution sufficient for two gels was prepared as follows: 7.5 ml of a mutation detection enhancement (MDE, specific for heteroduplex and SSCP analysis, Biospa) gel solution, 3 ml of 50 % glycerol, 1.8 ml of 10 x TBE were dissolved in 17.7 ml of milliQ water, polymerised by the addition of 150 µl of 10 % ammonium persulfate (APS) and 18.8 µl of tetramethyllenediamine (TEMED, Amersham Biosciences). The gel (0.4-mm thick and 20-cm long) was bonded to one glass plate by treatment with 0.5 % of γ-methacryloxypropyl-trimethoxysilane (Sigma) and 0.3 % of glacial acetic acid dissolved in 100 % ethanol. The gel plate was covered with repel-silane ES (Amersham Biosciences). Nine µl of loading buffer (0.25 % bromophenol blue and 95 % Hi-Di formamide) were added to 5 µl of PCR product; after denaturation at 95 °C for 3 min, 6 µl out of the resulting sample were loaded on the gel, which was then run for 16 h at 135 V in 0.6 x TBE running buffer. Visualisation was carried out with silver staining as described in Bassam *et al.* (1991);

SSCP on fluorescence-based capillary electrophoresis: The PCR reaction was performed with forward or reverse primers labelled with HEX fluorescent dye. Gene Scan Polymer (GSP) was used as a sieving matrix. The run polymer was prepared according to the following conditions: 5 % GSP, 10 % glycerol, 1 x TBE and milliQ water and filtered through Millex®-G 0.22 µm pore size filter (Millipore). The loading buffer was prepared at a final concentration of 1 x TBE with 10 % glycerol and milliQ water. The loading solution consisted of 1 µl of fluorescent PCR-fragment (dilution ranges between 1:50 and 1:150), 0.4 µl purified Genescan®-500 ROX Size Standard (Applied Biosystems), 0.5 µl of 0.3 M sodium hydroxide (NaOH) and 11.25 µl of Hi-Di formamide. Electrophoresis was performed using 36-cm capillaries on an ABI Prism® 3100 Genetic Analyzer. PCR products were first denatured and then injected at 1 kV for 22 s and separated at 15 kV for 25 min. The run temperature was set at 30 °C. The data were visualized as coloured peaks in chromatograms analysed with Genescan software (Applied Biosystems).

Minisequencing on an automated sequencer capillary system: Primer extension reaction was carried out in four steps. a) Minisequencing primer design: For each locus under investigation primers flanking the SNP mutations, revealed from sequencing, were designed with the computer program GeneRunner v3.04 (Hastings Software, Hudson, NY) and a primer matching the following conditions was chosen. Specific parameters were considered as follows: primer length between 18 and 26 bases, melting temperature between 55 and 60 °C, GC content > 40 %, lack of hairpin loops and presence of dimers. Primer direction was either 5' → 3' end, if viable, using the mutation upstream sequence, or 3' → 5' end using the mutation downstream reverse complementary sequence. Primer multiplexes were created adding at a polyT chain at the 5'-end, providing for a difference of at least 6 bases between primers (Tab. 1). b) Template preparation: Multiplex PCR reactions were performed using the following conditions: 20 ng genomic DNA, 2 x PCR reaction buffer (Qiagen), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.16 µM each primer, 2 Units HotStarTaq DNA polymerase (Qiagen), and milliQ water to a final volume of 25 µl. These conditions were optimized based on the protocol of Henegariu *et al.* (1997). In order to remove unincorporated dNTPs and primers during the amplification reaction, 1.5 µl of exonuclease-phosphatase (ExoSAPIT, Amersham) was added to each 5 µl of multiplex PCR product and incubated at 37°C for 45 min followed by 75 °C for 15 min. c) Minisequencing reaction: The minisequencing reaction was performed using the SNaPshot™ Multiplex Kit protocol (Applied Biosystems) with some modifications. The purified PCR product (5.5 µl)

Table 1

PCR and minisequencing primer sequences (5'→3' direction) for each locus (EST) analysed

Locus	Linkage Group*	GenBank ID	Putative Function	PCR Primer Forward	PCR Primer Reverse	PCR product size	Minisequencing Primer**
IN0780	6 (S x P)	CF609950	putative casein kinase 1 [Oryza sativa]	CGTGTGCTATGGACGAACTG	GCAGCATCTAAGGCTGGAGA	235	GCAAGATTCTAATGCCTAAACAGA
IN0860	5 (S x P-IN)	CF610029	calmodulin mutant SYNCAM15 [synthetic construct]	GCGTCGGTTCGATCTCATT	CCTTGTTCCTCGGACTTCTC	301	$(T)_{11}$ACTCCACGAACCCATCCTT
IN0417	2 (S x P-IN)	CF609610	unkown [Arabidopsis thaliana]	GATGCCAGAAGGAGAACTGC	CTGGCACTGCACACCTCTT	218	$(T)_{16}$GGAGGGAAAAGGTGTTCATT
IN0523	13 (S x P-IN)	CF609712	phospholipase C [Vigna radiata]	CCTGAACTGGCTCTTCTTCG	CATAGGACAGTTGATGGCTGC	291	$(T)_{21}$CTTCTAATGCGCTTTGAATTC
IN0945	7 (F x T)	CF610106	galactomannan galactosyltransferase [Lotus japonicus]	GCGACAACATAACCAGCAAG	GAGTGACGGTGGAGGAATCT	323	AAGACCCTTCATTACACACTTCAC
IN0551	6 (S x P)	CF609738	unkown [Arabidopsis thaliana]	ACACCGACGCAACTTCTTCT	GGCTCCATTGGAAGTGGTAA	410	$(T)_{12}$ACGTGCGTTAACGGCATC
IN0681	11 (S x P)	CF609854	G protein-coupled receptor -related [Arabidopsis thaliana]	TTGTCATGGCATCAGCGG	TGATAATCATAACCGTGGTCTCC	311	$(T)_{24}$AGGCATTGGAGGAATGACTTAT
IN0251	1 (F x T)	CF609461	response regulator 9 [Zea mays]	GCGGATCTGAGCTGTTATGG	ACATGATACAAGCACCGTCG	253	$(T)_{20}$GGTAAATTCGGTGTTCAGATCT
IN0129	2 (S x P-IN)	CF609347	probable glycerate dehydrogenase [Arabidopsis thaliana]	TCCAACTCATCGACTCCTCC	GCATTGGTGACAGTGATGCC	195	CTAGGTCAATATGGTCTACGCC
IN0320	15 (IN)	CF609526	No hits found	GAGACGCTTAACTGAGTGTGG	TTGCTCCTTCAACTTATCAGC	263	$(T)_3$CCAGTCCATAAAACCTATAATCT CTTG
IN0135	Unmapped	CF609353	GHMYB9 - transcription factor [Gossypium hirsutum]	AGCCGCAGTTAAGGAAGAGG	CCAATACTGCAACTGCACTCC	243	$(T)_{17}$GAATCAGCCCACCATACCA
IN0886	5 (F x T)	CF610050	unkown [Arabidopsis thaliana]	TTAGGCACTGCCAATGTCAC	CCGAGGAAGGTGAATGATTG	373	$(T)_{21}$GTTACACAACCACCCTGAAGA

* 'Syrah' x 'Pinot Noir' (S x P) (Troggio *et al.* 2007) and 'Merzling' x 'Teroldego rotaliano' (F x T) (Salmaso *et al.* submitted) crosses and the integrated map (IN) built on the S x P, 'Syrah' x 'Grenache', and 'Riesling italico' x 'Cabernet Sauvignon' progeny (Vezzulli *et al.* 2006).

** PolyT chains were added at 5'-end of minisequencing primers to create three primer multiplexes.

was mixed with 2 µl of SNaPshot Multiplex ready reaction Mix (Applied Biosystems), 1 µl of SNaPshot primer mix (containing 0.8 µM for each minisequencing primer), and water to a final volume of 10 µl. Minisequencing reactions were performed by an initial incubation at 96 °C for 1 min, followed by 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. d) Electrophoresis on ABI Prism® 3100 Genetic Analyzer: After primer extension reaction, 0.5 µl of minisequencing reaction product was mixed with 9.4 µl Hi-Di formamide and 0.08 µl Genescan®-120LIZ Size Standard (Applied Biosystems) and denaturated at 95 °C for 2 min. Products were analysed on an ABI Prism® 3100 Genetic Analyzer using POP-4 polymer and a 36-cm capillary array. Peak signals were analysed with GeneScan Analysis software (Applied Biosystems). For the minisequencing technique a distinct colour was assigned to each ddNTP as follows: green/A, black/C, blue/G, red/T, whereas sequencing colours were assigned as follows: green/A, black/G, blue/C, red/T. The minisequencing reaction can produce one (homozygote) or two (heterozygote) peaks depending on the genotype at each locus.

Results

Sequencing, SSCP analyses and minisequencing results are reported in Tab. 2 for the 12 loci (ESTs) studied in the six grapevines. The products of SSCP on non-denaturant gel electrophoresis and SSCP fluorescence-based capillary electrophoresis are shown in Figs 1 and 2 for two of the 12 loci analysed. When SSCP revealed a polymorphism, this was not always fully informative. For instance, for the marker IN0886, three genotypes involving three different alleles could be detected on SSCP gel electrophoresis (arrows in Fig. 1 a). An additional allele was clearly detected by SSCP fluorescence-based capillary electrophoresis (Fig. 2 a, arrow). By acrylamide gel electrophoresis, the M, F and P genotypes were homozygous for the same allele (Fig. 1 a), common to one of the heterozygous T and Ri genotypes, while on capillary electrophoresis genotype F showed an extra allele. The finding of the extra allele was supported by the segregation noted in the progeny of the cross F x T (Fig. 2 a). The same was observed for markers loci IN0780 and IN0681 (not shown), where SSCP on capillary electrophoresis was more sensitive than SSCP on acrylamide gels. For the remaining nine loci (IN0129 is shown in Fig. 1 b for SSCP on gel, and Fig. 2 b for SSCP on capillary electrophoresis) no difference was observed when using the two techniques. Individually sequencing of the six different genotypes at the 12 loci considered confirmed the alleles identified by SSCP on fluorescence-based capillary electrophoresis (Tab. 2) except for IN0129 where an extra allele was detected for the Ri genotype. For IN0251, secondary peaks in the sequence reduced the accuracy of SNP detection.

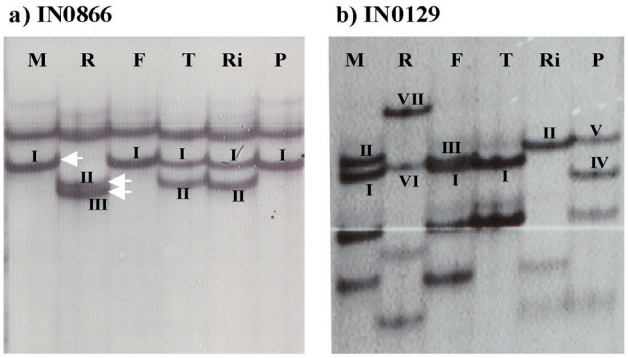

Fig. 1: SSCP of non-denaturant gel electrophoresis profiles genotypes for loci IN0886 and IN0129 of *Vitis vinifera* 'Moscato bianco' (M), *V. riparia* (R), the hybrid 'Merzling' (F), *V. vinifera* 'Teroldego rotaliano' (T), *V. vinifera* 'Riesling italico' (Ri), *V. vinifera* 'Pinot Noir' (P).

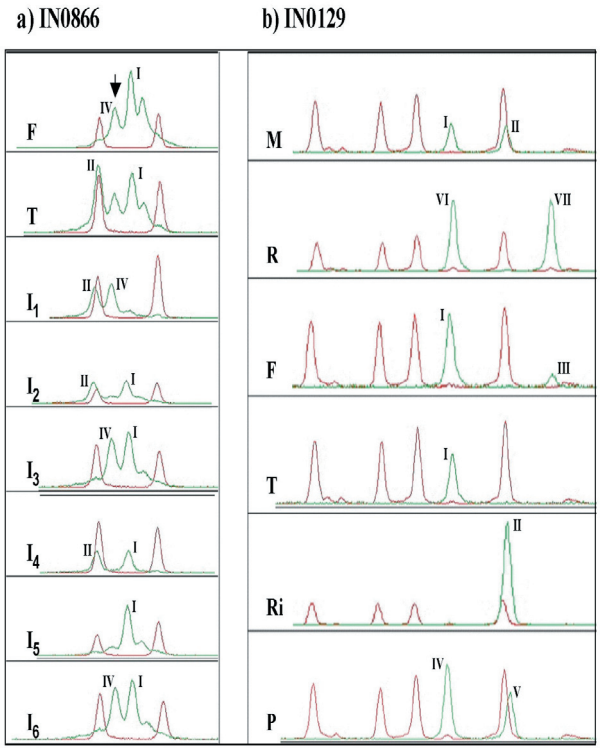

Fig. 2: **a**) SSCP fluorescence-based capillary electrophoresis profiles for locus IN0886 of the hybrid 'Merzling' (F), *Vitis vinifera* 'Teroldego rotaliano' (T), and six individuals of F x T; **b**) SSCP fluorescence-based capillary electrophoresis profiles for the locus IN0129 of *V. vinifera* 'Moscato bianco' (M), *V. riparia* (R), the hybrid 'Merzling' (F), *V. vinifera* 'Teroldego rotaliano' (T), *V. vinifera* 'Riesling italico' (Ri), *V. vinifera* 'Pinot Noir' (P). As only one primer was labelled, each allele gave a single peak (in green) corresponding to one of the two strands, whereas on the silver stained gel both strands could be detected. Red peaks represent GeneScan™-500ROX™ Size Standard (Applied Biosystems).

Results of minisequencing are shown in Fig. 3 for a multiplex of the IN0129, IN0320, IN0135, IN0886 markers for the six genotypes M, R, F, T, Ri, P. For IN0320, the genotypes at the SNP position detected with minisequencing analysis did not correspond to those expected from sequencing: three genotypes were heterozygous in spite of their apparent homozygosity established by sequencing (arrows in Tab. 2). The minisequencing multiplex was tested by skipping the final purification step from unincorporated [F]ddNTPs and loading the minisequencing products directly on an automated sequencer. As shown in Fig. 3, no interference between peaks of interest and [F]ddNTPs peaks was noted.

Table 2

Sequencing, SSCP analyses and minisequencing results for the 12 loci (ESTs) studied in *Vitis vinifera* 'Moscato bianco' (M), *V. riparia* (R), the hybrid 'Merzling' (F), *V. vinifera* 'Teroldego rotaliano' (T), *V. vinifera* 'Riesling italico' (Ri), *V. vinifera* 'Pinot Noir' (P)

Locus	Genotype	Sequencing Nucleotide position §	Haplotype	SSCP-gel Haplotype	SSCP-capil. Haplotype	Miniseq. Nucl. Pos.
		94 132 **171** 189 232 303 316 353				**171**
	M	-- --C-- -- --T-- -- --**C**-- -- --C-- -- --A-- -- --A-- -- --T-- -- --C-- --	1	I, I	I, I	C
		-- --C-- -- --T-- -- --**C**-- -- --C-- -- --A-- -- --A-- -- --T-- -- --C-- --	1			C
	R	-- --A-- -- --C-- -- --**C**-- -- --C-- -- --A-- -- --A-- -- --G-- -- --C-- --	2	II, III	II, III	C
		-- --A-- -- --C-- -- --**T**-- -- --C-- -- --C-- -- --T-- -- --G-- -- --T-- --	3			T
	F	-- --C-- -- --T-- -- --**C**-- -- --C-- -- --A-- -- --A-- -- --T-- -- --C-- --	1	I, I**	I, IV	C
IN0866		-- --A-- -- --C-- -- --**C**-- -- --T-- -- --A-- -- --A-- -- --G-- -- --C-- --	4			C
	T	-- --C-- -- --T-- -- --**C**-- -- --C-- -- --A-- -- --A-- -- --T-- -- --C-- --	1	I, II	I, II	C
		-- --A-- -- --C-- -- --**C**-- -- --C-- -- --A-- -- --A-- -- --G-- -- --C-- --	2			C
	Ri	-- --C-- -- --T-- -- --**C**-- -- --C-- -- --A-- -- --A-- -- --T-- -- --C-- --	1	I, II	I, II	C
		-- --A-- -- --C-- -- --**C**-- -- --C-- -- --A-- -- --A-- -- --G-- -- --C-- --	2			C
	P	-- --C-- -- --T-- -- --**C**-- -- --C-- -- --A-- -- --A-- -- --T-- -- --C-- --	1	I, I	I, I	C
		-- --C-- -- --T-- -- --**C**-- -- --C-- -- --A-- -- --A-- -- --T-- -- --C-- --	1			C
		40 48 51 65 67 100 101 **140** 175				**140***
	M	-- --A-- -- --C-- -- --G-- -- --G-- -- --C-- -- --C-- -- --C-- -- --**C**-- -- --G-- --	1	I, II	I, II	G
		-- --A-- -- --C-- -- --C-- -- --G-- -- --T-- -- --A-- -- --A-- -- --**C**-- -- --G-- --	2			G
	R	-- --C-- -- --T-- -- --C-- -- --G-- -- --C-- -- --C-- -- --C-- -- --**C**-- -- --G-- --	6	VI, VII	VI, VII	G
		-- --C-- -- --T-- -- --C-- -- --T-- -- --C-- -- --C-- -- --C-- -- --**T**-- -- --G-- --	7			A
	F	-- --A-- -- --C-- -- --G-- -- --G-- -- --C-- -- --C-- -- --C-- -- --**C**-- -- --G-- --	1	I, III	I, III	G
IN0129		-- --A-- -- --C-- -- --C-- -- --G-- -- --C-- -- --C-- -- --C-- -- --**C**-- -- --G-- --	3			G
	T	-- --A-- -- --C-- -- --G-- -- --G-- -- --C-- -- --C-- -- --C-- -- --**C**-- -- --G-- --	1	I, I	I, I	G
		-- --A-- -- --C-- -- --G-- -- --G-- -- --C-- -- --C-- -- --C-- -- --**C**-- -- --G-- --	1			G
	Ri	-- --A-- -- --C-- -- --C-- -- --G-- -- --T-- -- --A-- -- --A-- -- --**C**-- -- --G-- --	2	II, III**	II, II**	G
		-- --A-- -- --C-- -- --C-- -- --G-- -- --T-- -- --C-- -- --C-- -- --**C**-- -- --G-- --	8			G
	P	-- --A-- -- --C-- -- --G-- -- --G-- -- --T-- -- --C-- -- --C-- -- --**C**-- -- --G-- --	4	IV, V	IV, V	G
		-- --A-- -- --C-- -- --G-- -- --G-- -- --C-- -- --C-- -- --C-- -- --**C**-- -- --T-- --	5			G
		139 180				**139***
	M	-- --**T**-- -- --C-- --	1	I, II	I, II	A
		-- --**T**-- -- --G-- --	2			A
	R	-- --**T**-- -- --C-- --	1	I, III	I, III	A
		-- --**C**-- -- --C-- --	3			G
	F	-- --**T**-- -- --C-- --	1	I, I	I, I	A
IN0135		-- --**T**-- -- --C-- --	1			A
	T	-- --**T**-- -- --C-- --	1	I, I	I, I	A
		-- --**T**-- -- --C-- --	1			A
	Ri	-- --**T**-- -- --C-- --	1	I, I	I, I	A
		-- --**T**-- -- --C-- --	1			A
	P	-- --**T**-- -- --C-- --	1	I, I	I, I	A
		-- --**T**-- -- --C-- --	1			A
		59 145				**59***
	M	-- --**A**-- -- --C-- --	1	I, I	I, I	T**
		-- --**A**-- -- --C-- --	1			G**←
	R	-- --**C**-- -- --T-- --	2	II, II	II, II	G
		-- --C-- -- --T-- --	2			G
	F	-- --**A**-- -- --C-- --	1	I, II	I, II	T
IN0320		-- --**C**-- -- --T-- --	2			G
	T	-- --**A**-- -- --C-- --	1	I, I	I, I	T**
		-- --**A**-- -- --C-- --	1			G**←
	Ri	-- --**A**-- -- --C-- --	1	I, I	I, I	T
		-- --**A**-- -- --C-- --	1			T
	P	-- --**A**-- -- --C-- --	1	I, I	I, I	T**
		-- --**A**-- -- --C-- --	1			G**←
		42				**42**
	M	-- --**G**-- --	1	I, I	I, I	G
		-- --**G**-- --	1			G
IN0251	R	-- --**G**-- --	1**	I, II	I, II	G
		-- --**G**-- --	1**			G
	F	-- --**G**-- --	1**	I, IV	I, IV	G
		-- --**G**-- --	1**			G

Tab. 2, continued

Locus	Genotype	Sequencing Nucleotide position §	Haplotype	SSCP-gel Haplotype	SSCP-capil. Haplotype	Miniseq. Nucl. Pos.
		42				**42**
	T	-- --**G**-- --	1**	I, I	I, I	G
		-- --**A**-- --	2**			A
IN0251	Ri	-- --**G**-- --	1**	I, I	I, I	G
		-- --**A**-- --	2**			A
	P	-- --**G**-- --	1	I, I	I, I	G
		-- --**G**-- --	1			G
		20 65 **133**				**133***
	M	-- --A-- -- --G-- -- --**A**-- --	1	I, I**	I, III	T
		-- --A-- -- --T-- -- --**A**-- --	3			T
	R	-- --G-- -- --T-- -- --**G**-- --	2	II, II**	II, III	C
		-- --A-- -- --T-- -- --**A**-- --	3			T
	F	-- --A-- -- --G-- -- --**A**-- --	1	I, II	I, II	T
IN0780		-- --G-- -- --T-- -- --**G**-- --	2			C
	T	-- --A-- -- --G-- -- --**A**-- --	1	I, I	I, I	T
		-- --A-- -- --G-- -- --**A**-- --	1			T
	Ri	-- --A-- -- --G-- -- --**A**-- --	1	I, I	I, I	T
		-- --A-- -- --G-- -- --**A**-- --	1			T
	P	-- --A-- -- --G-- -- --**A**-- --	1	I, I	I, I	T
		-- --A-- -- --G-- -- --**A**-- --	1			T
		57 58 67 81 108 195 204 207 **231**				231*
	M	-- --A-- -- --A-- -- --A-- -- --T-- -- --C-- -- --G-- -- --G-- -- --C-- -- --**T**-- --	1	I, III	I, III	A
		-- --A-- -- --C-- -- --A-- -- --T-- -- --T-- -- --C-- -- --G-- -- --C-- -- --**T**-- --	3			A
	R	-- --C-- -- --C-- -- --A-- -- --T-- -- --C-- -- --G-- -- --A-- -- --G-- -- --**C**-- --	5	V, VI	V, VI	G
		-- --A-- -- --C-- -- --A-- -- --C-- -- --C-- -- --G-- -- --A-- -- --G-- -- --**C**-- --	6			G
	F	-- --A-- -- --C-- -- --A-- -- --T-- -- --C-- -- --G-- -- --G-- -- --C-- -- --**T**-- --	1	I, IV	I, IV	A
IN0860		-- --A-- -- --C-- -- --G-- -- --C-- -- --C-- -- --G-- -- --G-- -- --C-- -- --**T**-- --	4			A
	T	-- --A-- -- --A-- -- --A-- -- --T-- -- --C-- -- --G-- -- --G-- -- --C-- -- --**T**-- --	1	I, I	I, I	A
		-- --A-- -- --A-- -- --A-- -- --T-- -- --C-- -- --G-- -- --G-- -- --C-- -- --**T**-- --	1			A
	Ri	-- --A-- -- --C-- -- --A-- -- --T-- -- --C-- -- --G-- -- --G-- -- --C-- -- --**T**-- --	2	II, III	II, III	A
		-- --A-- -- --C-- -- --A-- -- --T-- -- --T-- -- --C-- -- --G-- -- --C-- -- --**T**-- --	3			A
	P	-- --A-- -- --A-- -- --A-- -- --T-- -- --C-- -- --G-- -- --G-- -- --C-- -- --**T**-- --	1	I, II	I, II	A
		-- --A-- -- --C-- -- --A-- -- --T-- -- --C-- -- --G-- -- --G-- -- --C-- -- --**T**-- --	2			A
		61 89 166 **235** 305				**235**
	M	-- --C-- -- --G-- -- --G-- -- --**T**-- -- --G-- --	1	I, I	I, I	T
		-- --C-- -- --G-- -- --G-- -- --**T**-- -- --G-- --	1			T
	R	-- --C-- -- --G-- -- --G-- -- --**T**-- -- --G-- --	1	I, IV	I, IV	T
		-- --T-- -- --G-- -- --G-- -- --**C**-- -- --C-- --	4			C
	F	-- --C-- -- --G-- -- --G-- -- --**T**-- -- --G-- --	1	I, III	I, III	T
IN0417		-- --C-- -- --G-- -- --T-- -- --**T**-- -- --C-- --	3			T
	T	-- --C-- -- --G-- -- --G-- -- --**T**-- -- --G-- --	1	I, II	I, II	T
		-- --C-- -- --T-- -- --T-- -- --**T**-- -- --G-- --	2			T
	Ri	-- --C-- -- --G-- -- --G-- -- --**T**-- -- --G-- --	1	I, II	I, II	T
		-- --C-- -- --T-- -- --T-- -- --**T**-- -- --G-- --	2			T
	P	-- --C-- -- --G-- -- --G-- -- --**T**-- -- --G-- --	1	I, II	I, II	T
		-- --C-- -- --T-- -- --T-- -- --**T**-- -- --G-- --	2			T
		176 182 188 199 215 269 302				**176**
	M	-- --**G**-- -- --T-- -- --C-- -- --A-- -- --G-- -- --C-- -- --A-- --	1	I, I	I, I	G
		-- --**G**-- -- --T-- -- --C-- -- --A-- -- --G-- -- --C-- -- --A-- --	1			G
	R	-- --**T**-- -- --T-- -- --C-- -- --A-- -- --G-- -- --T-- -- --A-- --	5	V, VI	V, VI	T
		-- --**T**-- -- --T-- -- --C-- -- --G-- -- --T-- -- --T-- -- --A-- --	6			T
	F	-- --**G**-- -- --T-- -- --C-- -- --A-- -- --G-- -- --T-- -- --A-- --	2	II, III	II, III	G
IN0523		-- --**T**-- -- --C-- -- --G-- -- --A-- -- --G-- -- --T-- -- --A-- --	3			T
	T	-- --**G**-- -- --T-- -- --C-- -- --A-- -- --G-- -- --T-- -- --A-- --	2	II, IV	II, IV	G
		-- --**G**-- -- --T-- -- --C-- -- --A-- -- --G-- -- --T-- -- --G-- --	4			G
	Ri	-- --**G**-- -- --T-- -- --C-- -- --A-- -- --G-- -- --T-- -- --A-- --	2	II, IV	II, IV	G
		-- --**G**-- -- --T-- -- --C-- -- --A-- -- --G-- -- --T-- -- --G-- --	4			G
	P	-- --**G**-- -- --T-- -- --C-- -- --A-- -- --G-- -- --C-- -- --A-- --	1	I, II	I, II	G
		-- --**G**-- -- --T-- -- --C-- -- --A-- -- --G-- -- --T-- -- --A-- --	2			G

Tab 2, continued

Locus	Genotype	Sequencing Nucleotide position §	Haplotype	SSCP-gel Haplotype	SSCP-capil. Haplotype	Miniseq. Nucl. Pos.
		232 **283** 343				**283**
	M	-- --C-- -- --**T**-- -- --C-- --	1	I, I	I, I	T
		-- --C-- -- --**T**-- -- --C-- --	1			T
	R	-- --C-- -- --**T**-- -- --C-- --	1	I, II	I, II	T
		-- --T-- -- --**T**-- -- --T-- --	2			T
	F	-- --C-- -- --**T**-- -- --C-- --	1	I, III	I, III	T
IN0551		-- --C-- -- --**T**-- -- --T-- --	3			T
	T	-- --C-- -- --**T**-- -- --C-- --	1	I, IV	I, IV	T
		-- --C-- -- --**A**-- -- --C-- --	4			A
	Ri	-- --C-- -- --**T**-- -- --C-- --	1	I, I	I, I	T
		-- --C-- -- --**T**-- -- --C-- --	1			T
	P	-- --C-- -- --**T**-- -- --C-- --	1	I, I	I, I	T
		-- --C-- -- --**T**-- -- --C-- --	1			T
		53 61 **210**				**210**
	M	-- --A-- -- --G-- -- --**C**-- --	1	I, I	I, I	C
		-- --A-- -- --G-- -- --**C**-- --	1			C
	R	-- --A-- -- --G-- -- --**C**-- --	1	I, II	I, II	C
		-- --A-- -- --G-- -- --**T**-- --	2			T
	F	-- --G-- -- --G-- -- --**C**-- --	3	I, III**	III, IV	C
IN0681		-- --A-- -- --A-- -- --**C**-- --	4			C
	T	-- --A-- -- --G-- -- --**C**-- --	1	I, I**	I, III	C
		-- --G-- -- --G-- -- --**C**-- --	3			C
	Ri	-- --A-- -- --G-- -- --**C**-- --	1	I, I	I, I	C
		-- --A-- -- --G-- -- --**C**-- --	1			C
	P	-- --A-- -- --G-- -- --**C**-- --	1	I, I	I, I	C
		-- --A-- -- --G-- -- --**C**-- --	1			C
		39 40 81 83 98 124 **167** 185 284 290				**167**
	M	-- --G-- -- --T-- -- --A-- -- --C-- -- --G-- -- --A-- -- --**T**-- -- --A-- -- --C-- ----C--	1	I, II	I, II	T
		-- --G-- -- --T-- -- --G-- -- --C-- -- --G-- -- --A-- -- --**C**-- -- --A-- -- --C-- ----C--	2			C
	R	-- --G-- -- --T-- -- --A-- -- --C-- -- --G-- -- --A-- -- --**C**-- -- --A-- -- --C-- ----C--	3	III, V	III, V	C
		-- --G-- -- --A-- -- --A-- -- --C-- -- --G-- -- --G-- -- --**C**-- -- --A-- -- --C-- ----T--	5			C
	F	-- --G-- -- --T-- -- --A-- -- --C-- -- --G-- -- --A-- -- --**C**-- -- --A-- -- --C-- ----C--	3	III, IV	III, IV	C
IN0945		-- --A-- -- --T-- -- --A-- -- --C-- -- --G-- -- --A-- -- --**C**-- -- --T-- -- --T-- ----C--	4			C
	T	-- --G-- -- --T-- -- --A-- -- --C-- -- --G-- -- --A-- -- --**T**-- -- --A-- -- --C-- ----C--	1	I, I	I, I	T
		-- --G-- -- --T-- -- --A-- -- --C-- -- --G-- -- --A-- -- --**T**-- -- --A-- -- --C-- ----C--	1			T
	Ri	-- --G-- -- --T-- -- --A-- -- --C-- -- --G-- -- --A-- -- --**C**-- -- --A-- -- --C-- ----C--	3	III, VI	III, VI	C
		-- --G-- -- --T-- -- --A-- -- --T-- -- --G-- -- --A-- -- --**C**-- -- --T-- -- --T-- ----C--	6			C
	P	-- --G-- -- --T-- -- --A-- -- --C-- -- --G-- -- --A-- -- --**C**-- -- --A-- -- --C-- ----C--	3	III, VII	III, VII	C
		-- --G-- -- --T-- -- --A-- -- --C-- -- --A-- -- --A-- -- --**C**-- -- --T-- -- --T-- ----C--	7			C

In bold: the SNPs characterized with minisequencing.

* 3' → 5' primer direction for minisequencing.

** results not consistent with the different SNP genotyping methods compared in this paper.

§ nucleotide position refers to the actual consensus reads.

Discussion

In this study, SSCP on both non-denaturant gel electrophoresis and fluorescence-based capillary electrophoresis, and minisequencing - affordable, moderately high-throughput, and multi-purpose methods for SNP assay - are compared in grapevine where only a limited number of SNP-based studies have been completed (Owens 2003; Salmaso *et al.* 2004, Troggio *et al.* 2007).

Capillary electrophoresis is a good alternative to acrylamide gel electrophoresis to survey for molecular markers and analyse differential gene expression. The method offers several advantages: automated sample loading, multi-capillary injection, faster separation, better reproducibility and increased sensitivity (Kimberly *et al.* 1997, Wenz *et al.* 1998). As in our case, it has recently been shown that this method is also valid for SSCP analysis (Baba *et al.* 2003). The temperature control provided by capillary electrophoresis was crucial to ensure consistent results, since single-stranded DNA assumes different secondary structures at different temperatures. Thus the high sensitivity of this method is also demonstrated, as it detected additional genotypes for 25 % of the loci analysed compared to SSCP on acrylamide gels.

Individual sequencing of the six different genotypes at the 12 loci considered confirmed the alleles identified by SSCP on fluorescence-based capillary electrophoresis (Tab. 2) except for one case. Moreover, for one EST the

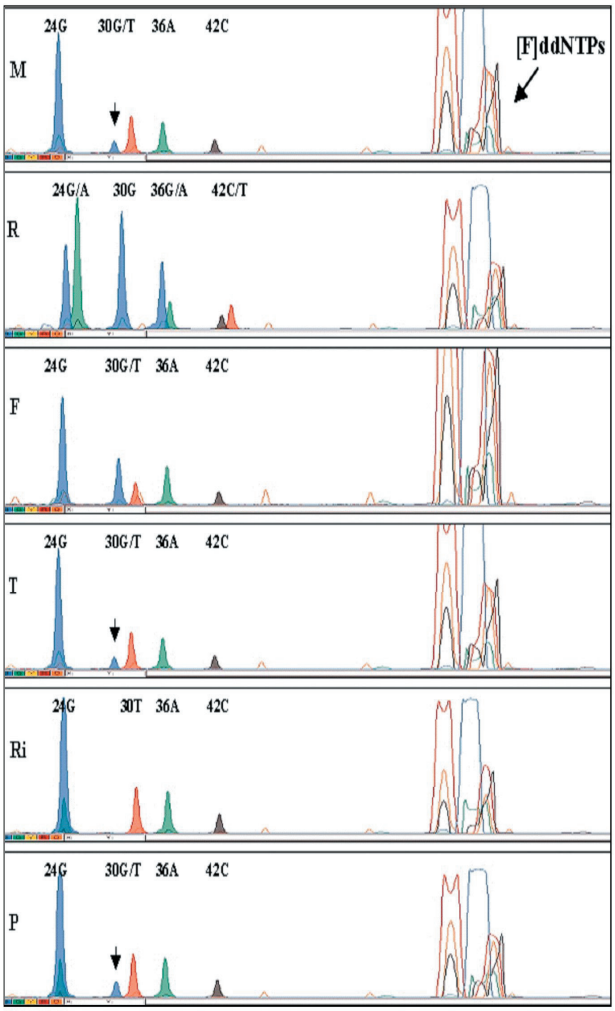

Fig. 3: Electropherograms of a SNaPshot products of loci IN0129 (primer length 24 bases), IN0320 (primer length 30 bases), IN0135 (primer length 36 bases), and IN0886 (primer length 42 bases) in *Vitis vinifera* 'Moscato bianco' (M), *V. riparia* (R), the hybrid 'Merzling' (F), *V. vinifera* 'Teroldego rotaliano' (T), *V. vinifera* 'Riesling italico' (Ri), *V. vinifera* 'Pinot Noir' (P). Relative sizes of SNaPshot products are determined by sizing against GeneScanTM-120LIZTM Size Standard (Orange peaks, Applied Biosystems). A distinct colour was assigned to each ddNTP as follows: green/A, black/C, blue/G, red/T.

presence of secondary peaks in the sequence reduced the accuracy of SNP detection and thus it was not possible to clarify the heterozygous allelic variations. Direct sequencing of PCR-amplified genomic fragments from diploid samples, in fact, resulted in mixed sequencing templates. Secondary peaks noted in sequence profiles may thus represent one of the two reads downstream of a heterozygous In/del. However, they are difficult to distinguish from sequencing artefacts in the region.

In the literature, only validation data for SSCP gel electrophoresis have been reported. Hayashi (1992) shows that at least 90 % of all point mutations are detectable by SSCP when the fragment size is approximately 200 nucleotides and 80 % when fragments are less than 400 nucleotides. Salmaso *et al.* (2004) report a 65 % SSCP detection efficiency in fragments with an average of 460 nucleotides, compared to 67 % detection efficiency in fragments with an average of 300 bp (present study). We conclude that the results with SSCP fluorescence-based capillary electrophoresis we produced are consistent with sequencing data and can be considered an efficient, accurate and reliable alternative to SSCP (Tab. 3). However, SSCP analysis has the relevant drawback that it does not allow multiplexing, at least at the PCR level (Scholl *et al.* 2001, Bertin *et al.* 2005).

A multiplex approach is a core enabling technology for high-throughput SNP genotyping. The procedure has been efficiently applied in this study with minisequencing. A multiplex approach, based on PCR amplification, PCR product purification and primer extension reaction of multiple primer combinations in a single tube reaction format was implemented for the same 12 markers (three different multiplexes) analysed separately. In one case, the genotypes at the SNP position detected with minisequencing analysis did not correspond to those expected from sequencing: three genotypes were heterozygous in spite of their apparent homozygosity established by sequencing (arrows in Tab. 2). Preferential amplification of one allele in PCR could explain this result. Less efficient priming of one allele versus another can occur due to mismatches between the PCR primer and the allelic template (Walsh *et al.* 1992). As the minisequencing primer tags a different site compared to the PCR primer, the weak allele during PCR amplification is revealed in the minisequencing reaction (low amplitude peaks, arrows in Fig. 3). Nevertheless, a multiplex approach has recently been demonstrated to be efficient at up to seven loci during the construction of high-density grapevine maps (Vezzulli *et al.* 2006, Troggio *et al.* 2007), which include up to 503 SNP-based markers. SNP-based marker multiplexes have been transferred among different mapping populations with an average efficiency rate of 65 % (Vezzulli *et al.* 2006). To help design multiplex PCR assays, a web-enabled system has recently been developed (MuPlex, Rachlin *et al.* 2005). With its capacity to investigate different mutation sites simultaneously, even if they are located in different regions of the same locus, the multiplex minisequencing system provides high throughput for SNP validation, as well as enough power for medium-throughput linkage analysis and association studies. The turnaround time of the minisequencing analysis using an ABI Prism® 3100 Genetic Analyzer (16 capillary array) is about 30 min per sample, including capillary filling, sample loading and separation. Thus, 5376 data points per day can be generated (48 runs/24 h x 16 capillaries x seven-plex reactions). When compared to the 45 min/sample turnaround time of the SSCP analysis using the same instruments for a total of 512 data points per day (32 runs/24 h x 16 capillaries), the throughput of the multiplex minisequencing analysis increases more than 10-fold. However, when different mutation sites for the same locus must be characterized to define a specific haplotype, the throughput difference between SSCP analyses and minisequencing is somewhat reduced. Although high-throughput technologies for SNP genotyping such DNA chips and microarrays exist, the advantage of both minisequencing and SSCP fluorescence-based capillary electrophoresis is evident: (i) the principal instrumentations are widely accessible across or within a laboratory; (ii) these

Table 3

Features of SNP genotyping methods

Methods	Most significant advantage	Disadvantage
SSCP-gel	Low-cost genotyping Inexpensive labelling method No expensive equipment required	Not suitable for high throughput Limited genotype discrimination
SSCP-capillary	Automated electrophoresis Accurate genotyping Reproducibility Rapid separation	Difficult to multiplex Expensive primer labelling
Minisequencing	Accurate genotyping Simplicity of assay Multiplexing capacity Easy data interpretation Mid-throughput	One SNP per reaction High cost Post-PCR purification Prior sequence information necessary

two simple and versatile techniques represent valid alternatives for genotyping since the same laboratory equipment is required; (iii) and throughput is sufficiently high for routine analysis in a medium size project dedicated to marker assisted selection.

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