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Genetic Variation among Clones and Sports of 'Pinot noir' (*Vitis vinifera* L.)

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Summary

The genetic variation among sports and clones of *Vitis vinifera* cv. 'Pinot' cultivars ('Pinot noir', 'Pinot gris', and 'Pinot blanc') was analysed utilizing 375 AFLP-PCR markers. Thirty two 'Pinot' genotypes of differing origin, age and phenotype were analysed to assay genetic variation among 'Pinot' clones and to look after a clonal "genetic structure" present within the selections. Results showed that the degree of genetic similarity within the majority of clones was high (99 %), though a minor group of 'Pinot noir' clones and one 'Pinot gris' clone exhibited higher variation up to 5 % . The genetic variation as indicated in molecular diversity indices were highest in the 'Pinot noir' group (0,0362±0,0192), decreased in the 'Pinot gris' group (0,0230±0,0145) and was lowest in the 'Pinot blanc' group (0,0038±0,0031). No colour-type clustering of 'Pinot' clones or a berry colour linked marker could be assessed. Unique markers serving as pre-requisite to individually fingerprint clones were found in all 'Pinot' cultivars.

Key words. AFLP – clonal genotype – clonal variation – grapevine

Introduction

The grapevine cultivars classified as the 'Pinot'-group *Vitis vinifera* cv. 'Pinot noir', 'Pinot gris', and 'Pinot blanc' are known for producing high quality wines throughout viticulture regions worldwide. 'Pinot' ssp. belong to the oldest *Vitis vinifera* varieties documented, though their distinct geographical origin remains unknown. As such they display genetic diversity and numerous phenotypic variants, mainly within the cultivar 'Pinot noir'. Selections made from these variants are clones, which have acquired traits as a result of the accumulation of somatic mutations. Grapevine cultivars are propagated vegetatively to sustain these genotypic traits, resulting in sets of clones for each grapevine cultivar offering scope for improved vineyard performance through productivity, maturity, fruit cluster characteristics and vine growth habit differences (Konrad et al. 2003). The identification and preservation of clonal variation is of importance to sustain genetic diversity which plays a key role in modern viticulture.

Clonal variation (variation within grapevine cultivars) is traditionally discussed to be attributed to polyclonal origin, mutation and effects of pathogens (Riaz et al. 2002; Hocquigny et al. 2004; Forneck 2005). By definition applied in this paper, a clone is a population of individuals which are the descendants by vegetative propagation of a single parent plant and vary genetically solely through mutations. The genetic component of phenotypic variation among sets of clones within a cultivar is of great interest for grape breeding whereas identification of clones pertains issues of patenting and legal rights. Cultivar identification in grapevines is traditionally based on morphological and physiological plant characters using ampelographic descriptors. However, such morphological markers do not permit the differentiation among clones, which are identified largely on the basis of their documented propagation history. To allow true genetic identification of clones, molecular marker systems need to be used. First reports on the detection of clonal variation in grapevines were given by Silvestroni et al. (1997) using microsatellites. SSR-markers have also been used for the detection of chimeric variants among clones (Riaz et al. 2002; Franks et al. 2002; Hocquigny et al. 2004). Because of the possibility to screen large numbers of anonymous loci AFLP-marker were proposed for the identification of closely related genotypes such as somatic mutants and clones (Scott et al. 1999; Imazio et al. 2002). Clonal variation based on AFLP-banding patterns were detected in grapevine (Ye et al. 1998; Cervera et al. 2000, 2002; Moncada et al. 2005), whereas other marker systems (e.g. RAPD-PCR, ISSR-PCR) proved to be less efficient (Forneck 2005).

The study presented aims to reveal information on the genetic variation within clonal genotypes of the 'Pinot' group and addresses three questions:
(1)is there clonal variation among 'Pinot' clones?
(2)Can a "clonal genetic structure" be defined?
(3)Can unique markers for individual clones be found?

Material and Methods

Plant material

The plant material was obtained from collections at Universität Hohenheim (GER), Staatliches Weinbauinstitut

Freiburg (GER), Forschungsanstalt Geisenheim (GER), Staatliche Lehr- und Versuchsanstalt Weinsberg (GER) and from grape breeders (Table 1). Samples of 'P. noir', 'P. gris' and 'P. blanc' clones were collected along with 'Domina' ('Bl. Portugieser' x 'P. noir'), 'W. Riesling', 'Chardonnay' and 'Regent' [('G. Silvaner' x 'Müller Thurgau') x 'Chambourcin'], which were employed as methodological controls and statistical outgroup. The 'Pinot' clones encompassed both registered German grapevine clones with well-documented propagation histories and new clonal selections with younger records. All clones varied in age, propagation history and reported geographic origin. Two replicates originating from the same mother plant of each clone were greenhouse cultivated.

DNA extraction and AFLP-PCR analysis

DNA was extracted from young leaves using a modified protocol of Thomas et al. (1993) by adding two additional purification steps employing Chloroform / Isoamylalcohol (19:1). AFLP - analysis was performed according to Vos et al. (1995) with minor modifications. In the preamplification step *Eco*RI and *Mse*I primers without selective nucleotides were utilized; for the selective amplification eight primer combinations were applied: ATG/AGT, ATC/ATG, AGG/CAG, AGG/ATG, AGG/AGT, AGG/ATC, ATC/CAG, ATC/ATG (*Eco*RI/*Mse*I + selective nucleotides). PCR protocols followed the protocols described in Forneck et al. (2000). The amplification products were separated at 1600V on a 6 % polyacrylamide gel and silver stained as described in Bassam and Caetano-Annoles (1995).

Statistical analysis

AFLP markers were scored for presence or absence and expressed in binary. Markers with unusual variability usually consisted of weak bands and were eliminated from the dataset. All polymorphic markers were replicated ensuring reproducibility. Statistical analysis was performed using the NTSYS-PC (vers. 1.8) (Exeter Software, Setauket, N.Y.) for genetic similarity calculations applying the Simple Matching coefficient for cluster analysis applying the UPGMA algorithm. The ARLEQUIN (version 2.0) (Schneider et al. 2000) was employed to calculate the molecular diversity indices according to Tajima (1983).

Results

Clonal variation among 'Pinot' clones

Eight Primer combinations generated 375 markers in the range of 100–500 bp. All primer combinations producing polymorphic AFLP markers were duplicated ensuring reproducibility. In total 72 (19,2 %) polymorphic markers were detected. Twenty six of the 33 'Pinot' clones studied showed at least 99 % genomic similarity (Fig. 1). One group exhibiting higher genetic variation (up to 5 % dissimilarity of the randomised fingerprinted genome) was a group of 'Pinot noir' clones including We M847, We M848, We M242, We M819, one Swiss 'Pinot noir' clone (CH 9/18), a recent 'Pinot noir' selection FR1602 and the 'Pinot gris' clone FR 49-207.

Clonal "genetic structure" within the 'Pinot' clones

The assemblage of AFLP markers produced a most common, unique genotype for 'Pinot' within the range of clones analysed in this study. The genotype "9" is shared by seven 'Pinot' clones either colour. Another triple clone genotype is "7" displaying the genotypes of three 'Pinot noir' clones and a double clone genotype is "25/26" standing for two 'Pinot blanc' clones (Table 3) indicating the close relationship within the 'Pinot' group.

No colour-type grouping of 'Pinot' clones could be observed during the course of our study. However, the genetic variation within groups of clones was highest in of 'Pinot noir' followed by 'Pinot gris' and 'Pinot blanc' (Table 2). Despite the fact that the comparison of genetic variation among colour-type 'Pinots' is hampered because of imbalance in numbers of genotypes analysed, the decreasing variation could be confirmed by molecular diversity measures (Table 3), although these values do not differ significantly.

Table 1. 'Pinot' clones utilized in this study.

No.	Cultivar	Origin[a]	Clone	No.	Cultivar	Origin[a]	Clone	No.	Cultivar	Origin[a]	Clone
1	'P. noir'	1	We M 847	12	'P. noir'	3	FR 54-102	23	'P. blanc'	3	FR 70
2	'P. noir'	1	We M 848	13	'P. noir'	3	FR 10	24	'P. blanc'	3	FR 74
3	'P. noir'	1	We M 242	14	'P. noir'	3	FR 11	25	'P. blanc'	3	FR 72
4	'P. noir'	1	We M 819	15	'P. noir'	3	FR1602	26	'P. blanc'	3	D 55
5	'P. noir'	2	1-86 Gm	16	'P. noir'	4	F 105 S	27	'P. blanc'	3	D 57
6	'P. noir'	2	1-36-4 Gm	17	'P. noir'	5	INRA 777	28	'P. gris'	3	FR 49-207
7	'P. noir'	2	2-6 Gm	18	'P. noir'	6	9/18	29	'P. gris'	3	D 42
8	'P. noir'	2	20-13 Gm	19	'P. n. precoce'	1	n.i.	30	'P. gris'	3	D 43
9	'P. noir'	2	20 Gm	20	'P. blanc'	2	1-46 Gm	31	'P. gris'	2	2-26 Gm
10	'P. noir'	3	FR 12 L	21	'P. blanc'	2	2-53 Gm	32	'P. gris'	2	2-15-4Gm
11	'P. noir'	3	FR 52/86	22	'P. noir'	3	FR 54-102	33	'P. gris'	7	H 1

P.: Pinot; n.: noir; n.i.: not identified

[a]1: LVWA Weinsberg, GER; 2: FA Geisenheim, GER; 3: WI Freiburg, GER; 4: Frank, Kenzingen-Nordweil, GER; 5: ENTAV, F, 6: R.A.C. Changins, CH; 7: Hauser-Bühler, Vogtsburg-Bickensohl, GER.

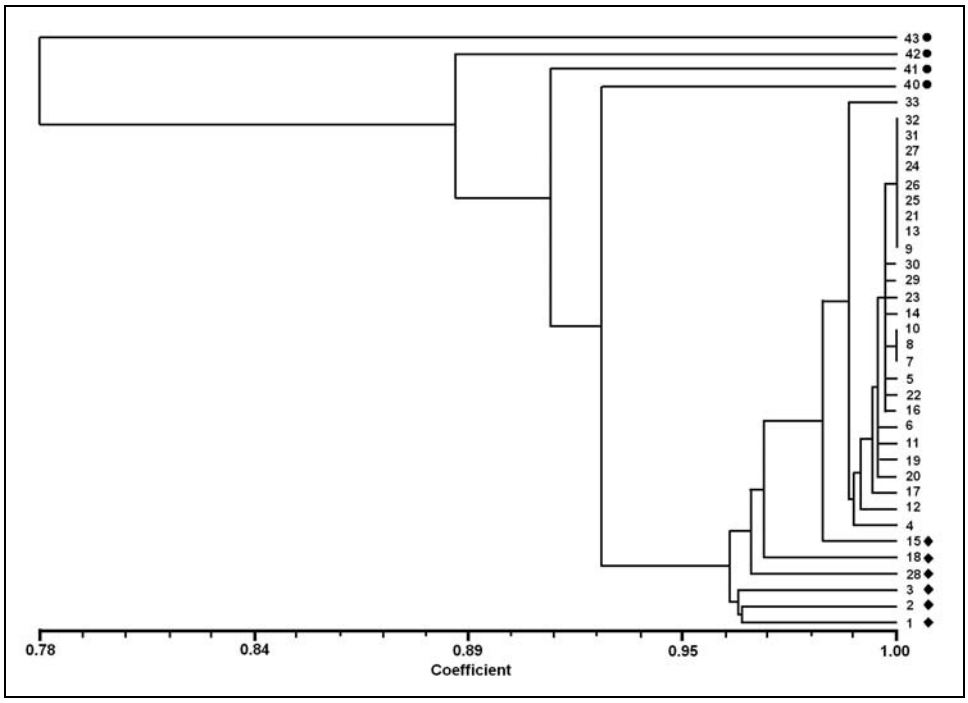


Fig. 1. Dendrogram generated of 33 'Pinot' clones (labels correspond to Tab. 1) based on Simple Matching Coefficient subjected to UPGMA Clustering procedure. In addition: 'Regent' (43), 'Domina' (42), 'Riesling' (41) and 'Chardonnay' (40) as outgroup marked with "●". The 'Pinot' clones, forming a group exhibiting genetic variation higher 1 % are individually marked with "◆".

Table 2. Molecular diversity indices (Tajima 1983) of grouped 'Pinot' clones according to colour-type.

'Pinot' cultivar (N)	Average gene diversity over loci
'Pinot noir' (19)	0.036±0.019 a[a]
'Pinot gris' (8)	0.023±0.014 a
'Pinot blanc' (6)	0.004±0.003 a
'Pinot' clones total (33)	0.026±0.015

[a]indices followed by the same letter are not significantly different (α=0.05).

Unique markers for individual clonal fingerprinting

The search for unique markers to individually fingerprint clones requires a marker in adequate seize, abundance and reproducibility. This screening intended to evaluate the chance of finding clone-specific markers, derived through mutation which is dependant on the number and diversity of the samples under study. Within the range of 33 'Pinot' clones studied solely five markers specific to one particular clone (1,13 %) could be detected. Within the 'Pinot noir' group (19 clones) it was possible to identify eight (2,13 %), within the 'Pinot gris' group (8 clones) eleven (2,93 %) and within the 'Pinot blanc' (6 clones) group two (0,53 %) unique markers.

Discussion

Among the grapevines (*V. vinifera* ssp. L.) 'Pinot noir' offers an extensive pool of different phenotypes identified and selected through clonal selection. This might be due to elevated mutation rates in gene regulating sequences including transposable elements (eg Böhm and Zyprian 1998; Pelsy and Merdinoglu 2002; Kobayashi et al. 2004) possibly interacting with environmental cues. The range of 'Pinot' clones tested included multiple selections made from various geographic origins in France, which are of considerable age and selections made from these (sub-clones) by breeders in France, Switzerland and various breeding stations in Germany. Grapevine strictly asexually propagated and long-lived is prone to the accumulation and proliferation of mutations. There is little research on the degree, mechanisms and environmental interaction of such genomic change in higher plants other than empirical studies. And it is recently that science found interest in using long-lived woody perennial clones (and sports) for model studies (eg Scott et al. 2000). Clonal selection is an economically and successfully applied breeding strategy in grapevine and knowledge on the durability, proliferation and accumulation of mutant traits is important. The chances of mutated lines to survive depend on whether they are located in somatic, germinal or multipotent tissues, furthermore the motility of mutant lines, the mutation rate, the plant's lifespan and the number of cells constituting the propagule (Grosberg and Strathmann 1998). Selection occurs through influences of bottlenecks, which in the case of grapevine are mainly directed by breeding.

Clonal variation among the 'Pinot' clones

V. vinifera cv. 'Pinot noir', 'Pinot gris' and 'Pinot blanc' originate from a single hybridisation event (Bowers et al. 1993) but derived from mutations exhibiting colour-type phenotypes (sports). The occurrence of colour-type sports in 'Pinot noir' happens frequently, mainly on older vines after stress influence such as severe winter frost. Field observations show a higher degree of noir – gris and to a lesser extent gris – blanc mutations; noir – blanc mutations are very

Table 3. Genotype Frequencies of 'Pinot' Clones analysed.

Common genotype	Genotype frequency	'P. noir' (19)	'P. blanc' (8)	'P. gris' (6)
"9"	7	20 Gm Fr 10	3-31 Gm Fr 74 D57	2-26 Gm 2-15-34 Gm
"7"	3	FR 12L, 20-13 Gm 2-6 Gm		
	1	We M847		
	1	We M898		
	1	We M842		
	1	We M819		
	1	1-86 Gm		
	1	1-36-4 Gm		
	1	FR 52/86		
	1	FR 54/102		
	1	FR 11		
	1	FR 1602		
	1	F 105S		
	1	INRA 777		
	1	CH 9/18		
	1	'P. noir précoce'		
	1		3-31 Gm	
	1		FR 70	
"25/26"	2		FR 72 D55	
	1			FR 49-207
	1			D42
	1			D43
	1			H1

rarely observed. Thus it seems unlikely to define a common mutation event for the cultivar 'Pinot blanc' and 'Pinot gris' which rather display an assemblage of independently arisen sports of various 'Pinot noir' mother plants.

The 'Pinot' clones analysed in this study showed varying degrees of genetic variation ranging from 95–100 % similarity, confirming the results of previous work (eg MONCADA et al. 2005). The extent of clonal variation in *Vitis* ssp. seems to be dependant to the cultivar. 'Cabernet Sauvignon' (MONCADA et al. 2005), 'Pinot noir' (FORNECK et al. 2003; REGNER et al. 2006), 'Sangiovese' (SENSI et al. 1996) and 'Traminer' (MERDINOGLU et al. 2000; IMAZIO et al. 2002) are cultivars with evident clonal variation. In contrast other cultivars such as 'Carmenere' (MONCADA et al. 2005) 'Riesling' or 'Zinfandel' display less genetic variation. The reasons underlying this phenomen may be manifold. Chimerism plays an important role in the formation of clonal phenotypes (skin colour; leaf pubescence) and has been detected in major grapevine cultivars as 'Pinot noir', 'Chardonnay' and 'Pinot meunier' (RIAZ et al. 2002; FRANKS et al. 2002; HOCQUIGNY et al. 2004). Another driving force for clonal variation are transposition events within long-lived perennial clones as detected by retrotransposon-based markers (e.g. PELSY et al. 2003). Age of the organism is discussed to be a factor for the accumulation of mutations. In the case of cultivated grapevine, through the hybridisation event may be of considerable age, rejuvenation of genotypes occurs regularly and hamper these factors.

The clonal variation detected in this study exhibits a structure. Clones that are genetically similar, corresponding to one most common genotype (defined by stochastic processes) and those clustering in genetic distance. In the case of the 'Pinot noir' genotypes studied a genetically more variable group of clones, originally sampled in Switzerland representing of loose-berried phenotype, which have a common selection history (time of selection and breeding station). These clones have been subsequently under clonal selection and preserved the existing clonal variation. In addition two other clones ('P. gris' (FR 49-207) and 'P. noir' (9/18) showed clonal variation in average of 3,7 % which is considerably lower than the genetic variation detected among cultivars (Fig. 1).

Dominant, random AFLP-markers failed to determine a genetic structuring among 'Pinot' colour-types. This would have been a fortunate though unlikely incident which has been overcome by current research presenting evidence that the deletion of a transcription factor involved in the anthocyanin biosynthesis caused the mutation from 'Pinot noir' to 'Pinot blanc' (YAKUSHIJI et al. 2006).

Clonal fingerprinting and identification

Differentiation of grapevine cultivars meets the demands of growers and breeders. However, legislation is not able to protect grapevine clones within existing legal frames as new varieties. According to UPOV (International Union for the Protection of new Varieties of plants) rules uniformity and stability are prerequisites for variety protection, but clones do not fulfil these criteria. Plant Patents (Plant Patent Act) are used to protect plant material that can be vegetatively propagated but this excludes the patenting of grapevine clones, because law requires that a patented plant can be described as unique by objective criteria that go beyond the ability of current technology to differentiate clones. Presently trademarks are being used to protect grapevine clonal selections. Protection of legal rights on clones will become increasingly important and should be encompassed using molecular markers such as sets of SSR-markers and other DNA profiling tools (RIAZ et al. 2002; THIS et al. 2004). The AFLP polymorphisms identified may serve as basis for the development of marker system to differentiate among sets of clones. These AFLP markers need to be converted into SCAR markers and a combination of adequate, clones-specific SCAR marker combinations applied in multiplex PCR-technology, may likely have the potential and resolution for clone identification within limited and know sets of samples. The clonal fingerprint, to evidently identify a clone may be difficult to achieve without yet knowing the extent and underlying mechanisms of the genetic variation in clones.

Conclusion

The 'Pinot' cultivars are thought to belong to the oldest varieties in Europe and are propagated vegetatively for

centuries allowing the accumulation of somatic mutations giving rise to grapevine clones. Numerous clones of a cultivar, representing identical genotypes apart from mutations, acting as "pseudo" near isogenic lines with defined morphological traits, offer perspective to perform genomic diversity studies and the search for markers linked to traits. Certainly, AFLP fingerprinting has potential to assist in quality control in nurseries and wineries to comply with regulations imposed on wines and their accurate labeling (Sefc et al. 2001). Furthermore it should play a role in the efficient management of germplasm collections and in the study of biodiversity of grapevines.

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