Plant Breeding: Clonality – A Concept for Stability and Variability During Vegetative Propagation

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1 Introduction

Clonality means asexual reproduction or propagation without meiosis. This results in offspring that are identical to the ancient progenitor except when mutations occur. It seems that clonality is a dynamic concept. Genetic variation is added by numerous mechanisms to an asexual living strategy to enhance variation and provide an open system for adaptation and selection. For a cultivated woody perennial crop genetic variation has additional meanings since clonality is used to sustain identical genotypes and select for new clones of grapevine cultivars through clonal selection.

Grapevine (*Vitis* ssp. L.) is used as a role model for investigating clonality in a woody perennial since it is a well-investigated cultivated plant that is propagated vegetatively. Grapevine cultivars (*Vitis vinifera* L.) are composed of clones showing homogeneous ampelographic characteristics, and distinguished by minor differences. Asexually derived grapevine lineages afford one of the best systems to study clonality in plants. Estimates of intra-varietal genetic diversity have increased as detection systems with enhanced resolution become available. The most powerful applied marker system for analyzing clonality in grapevine is AFLP-PCR (amplified fragment length polymorphism-polymerase chain reaction). Less resolution has been obtained with RAPD-PCR (random amplified polymorphic DNA) or ISSR-PCR (inter simple sequence repeat). Microsatellites have recently been used to detect clonal variability. The sources of genetic variation in grapevine clones are manifold. Somatic mutations, somaclonal variation, retrotransposition, chimerism and epigenetic changes have all been recently confirmed with molecular marker techniques.

Most perennial plants combine sexual reproduction with some form of clonal propagation. The balance between these forms of reproduction affects propagule size, establishment dynamics and the transmission of genetic variation. This balance is thought to greatly influence population demography, genetic diversity, the accumulation of mutations, metapopulation dynamics and the evolutionary potential of populations (Dorken and

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Eckert 2001). Clonal growth (clonality) is known as a mechanism of foraging for resources, competition and compensation of environmental heterogeneity, but also as a low-risk strategy for maintaining local populations and the fittest genotypes within a population (Auge and Brandl 1997). In general, long-lived woody perennials are highly heterozygous and inbreeding results in a decline in vigor and fertility, as in the case with naturally cross-pollinated crops. Vegetative reproduction occurs both naturally in habitats of plant communities and artificially through vegetative propagation of commercially desired plant clones. Clonality is a term that is often vaguely applied in association with asexual reproduction. Here, this term is used to describe the asexual reproduction of an individual resulting in a set of clones. A clone is defined as an individual that descended from a single common ancestor by mitosis. A group of clones originating from the same ancestor (monozygotic) is defined to be genetically identical except for the effect of mutations.

Clonality in woody perennials occurs by means of stolon formation, layering, root suckering, vegetative sprouting, formation of rhizomes and axis splitting. The relative importance of sexual versus clonal recruitment may vary widely among plant species because clonal reproduction allows populations to persist and expand in habitats where, for one reason or another, sexual reproduction cannot occur. The production of asexually derived offspring in woody perennials occurs in many ways. There are species in which new individuals may arise without disturbance; in others, however, asexual reproduction is in direct response to injury or imbalance (Berg and Hamrick 1994). Woody perennials are economically used as vegetatively propagated plants and sold as 'clones' with identical phenotypes. Such cultivated clones consist of single desired genotypes that are multiplicated and spread worldwide as identical individuals with superior combinations of genes. Vegetatively propagated plant material can be used to control growth phases, to shorten time to flowering or conversely to rejuvenate plantings. Furthermore, two superior genotypes may be combined in one plant through grafting techniques.

Clonality has been studied under a variety of perspectives involving morphology, physiology, ecology and evolution of natural asexually reproducing plant populations. The work on clonality of the last decades is relatively limited in the study of woody perennials (e.g. Rajora 1999; Schenk 1999; Chung and Epperson 2000) and has been brought together in several reviews (Eckert 1999). Most approaches to study clonality have focused on plant populations in their natural habitat (de Kroon and van Groenendael 1997) to examine ecological effects and the natural environment for selective forces on the populations. There is increasing interest in analyzing the clonality of cultivated clones to assist in clonal selection processes of

superior genotypes. Moreover the need to identify and discriminate among clones has been intensified by legal defenses to breeders' rights. A new area of research focuses on transgenic clones with investigations of performance and identification.

Grapevine (*Vitis vinifera* ssp. L.) is an excellent crop plant for clonality study because of its economic importance and reliance on vegetative propagation. Today's cultivars resulted from the selection of advanced genotypes of ancient origin mostly generated by intentional and spontaneous crosses centuries ago. Each ancient cultivar expresses distinct phenotypes, resulting in sets of morphologically different clones. These clones have spread worldwide adjusting to different environments and cultivation techniques. The causes discussed for clonal variation are virus infection, polyclonality and mutations. For grapevine clones the concept of individuality is straightforward and relies on propagation records and morphological features. Since grapevine is a high-value crop, significant viticultural research in describing and analyzing the phenotypes has been performed since the 19th century. More recently, tissue culture, transformation and molecular genetic techniques have been used in genomic and biotechnological approaches to improving grapevines.

This chapter does not intend to provide a comprehensive literature review on grapevine genetic studies, but to present examples of publications that underline the discussion of clonality.

2 Clonal Variation Assessment

Degrees of clonality in *Vitis* have been assessed through phenotypic and genotypic parameters with differing intentions. Molecular markers in combination with phenotypic traits are used to identify clones. Molecular markers can further be used to quantify neutral genetic variation within and among 'populations' of clones. Several causes have been proposed to account for the variation that occurs among cultivated clones. One is that differing clones are not true clones, but rather resemble a population of very closely related individuals, perhaps siblings, of similar morphology, a concept termed polyclonality. Another explanation considers the interference of pathogens such as viruses on phenotypes with identical genotypes. Both concepts are widely used with respect to woody perennial clones; however, neither proves to measure clonality in its strict sense. The third explanation considers accumulation of the phenotype-altering mutations as the primary factor resulting in true clones (clones identical by descent).

2.1 Polyclonality

Traditionally, phenotypic variation within cultivars has been accepted. Polyclonality can account for the existence of such variation. According to Rives (1961) more than one seedling, all marked with morphological uniformity, gave rise to many grapevine cultivars. Polyclonality in woody perennial cultivars was proposed long ago, but only recently proven by molecular marker techniques. The advent of simple sequence repeat (SSR) techniques permits polyclones to be clearly detected (Vignani et al. 1996; Silvestroni et al. 1997; Filippetti et al. 1999).

2.2 Pathogen-Infected Clones

Virus diseases contribute to increasing the phenotypic variability within grapevine cultivars. Grapevines, in common with other woody perennials, are subject to infection by virus and virus-like pathogens. In addition, there are several other debilitating diseases caused by viroids, phyto- or mycoplasms. In the case of grapevine, plants with recognizable symptoms of virus disease are normally rejected as a source of budwood or cuttings. One problem in viticulture, as in other fruit crops, is that seemingly healthy plants can be symptomless carriers of disease-causing viruses. The extent to which phenotypic variation within a population of clones is due to the genotype or to the presence of viruses has been subject to experimentation with differing results. Studies show that differences among clones exist regardless of their phytosanitary status. Other studies suggest that the overall performance of grapevine clones is improved after sanitation of the plant material (Mannini 2000). According to current knowledge, genomic changes in DNA have not been observed, and thus pathogen-infected clones with altered phenotypes are not true clones.

2.3 Mutation

Clonal variation can be induced through mutations to induce stable genetic changes. These genetic changes are visualized by observed changes in morphology or by applying molecular genetic markers. There is abundant evidence for the occurrence of somatic mutations in plants (e.g. Klekowski and Godrey 1989) and molecular genetic variation has been found among naturally occurring clones in several plant species. Somatic variation might be expected to be most common at loci with high mutation rates, e.g. as reported in some cases for microsatellite DNA sequences (Schlotterer et al.

1998; Udupa and Baum 2001). There is little information about mutation rates during somatic development of plants (Gill et al. 1995).

2.3.1 Phenotype

Grapevine development is controlled by two main factors over the growing season: the pre-growth conditions of the vine, including its size, characteristics of its buds and the amount of stored reserves; and the environmental conditions that regulate and modify shoot and fruit development. Furthermore, how vineyards are managed has a large impact on the phenotypic performance of clones (e.g. Clingeleffer 1988; Cirami et al. 1993). Clones of a cultivar often differ substantially in viticultural performance and in their ability to produce quality wines. Clonal descriptions usually focus on viticultural traits (e.g. yield components: cluster number, cluster weight, numbers of berries/cluster, berry weight, berry size), while traits pertaining to wine quality are very difficult to evaluate. The literature concerning the selection of clones based on viticultural traits is vast. A summary of the German clonal selection program covers the strategies and their success (Schöffling and Stellmach 1993).

Clonal selection focuses on the phenotype and viticultural performance. The selection procedure takes between 20 and 30 years of repeated studies and phytosanitary restoration before a clone will be 'certified'. Differences among the phenotypic plasticity vary among cultivars; examples for diverse *V. vinifera* cultivars are Pinot, Traminer and Nebbiolo, whereas Zinfandel is a good example of very limited genetic diversity. The plasticity of Pinot results in a set of color types (red, grey, white berries), differences in cluster architecture (loose, tight cluster), berry size and growth habit, while Zinfandel, with a very limited genetic base at its point of origin (Maletic et al. 2003), has not developed significant clonal variation even though it has existed for centuries. The phenotypic identification of grapevine clones is an essential means of grapevine improvement due to the reliance on traditional varieties for wine making. Clonality studies, however, require reproducible morphological measurements. Furthermore, because of environmental and pathogen effects on clonal expression, it can be difficult to define true genetic clonal differences.

2.3.2 Genotype

The identification of individuals is challenging in perennial clonal organisms. Grape clones are often poorly defined, and tracking and confirming

their identity with worldwide distribution and hundreds or thousands of mothervines is extremely difficult. Molecular markers are now routinely implemented in studies of clonality. There are a range of molecular markers and several reviews discuss the application of different marker classes, their pros and cons in respect of the studied organisms (e.g. Lavi et al. 1994). Most markers applied in clonality studies are PCR-based and generate multi-locus fingerprints. Microsatellites have a unique standing in grapevine genetic research. More than 400 SSR loci have been developed for *Vitis* and are applied for identification and mapping purposes. A brief description of the marker systems follows:

AFLP (amplified fragment length polymorphism): AFLP involves the restriction of DNA with two different endonucleases, followed by a ligation with appropriate adaptors and amplification of DNA fragments in two steps. Various primer combinations with differing selection ends are employed to generate multilocus dominant markers.

SSR (simple sequence repeats): Often referred to as microsatellites, SSR consist of short stretches of tandemly repeated motifs, 2–4 bp in length. Once flanking primer sites are identified, they can be amplified using PCR to generate a locus and scored by size (corresponding to alleles). SSR are inherited in a codominant fashion, allowing an assessment of within-population structure.

RAPD (random amplified polymorphic DNA): Short random primers are employed in PCR reaction to amplify random DNA segments. The presence of a band indicates successful amplification; absence indicates no amplification due to, e.g., mutation in the primer recognition site. RADP in diploid organisms behave in a dominant/recessive fashion.

ISSR (inter simple sequence repeat): Non-anchored ISSR markers are arbitrary, multiloci, PCR-based markers that amplify intermicrosatellite sequences at multiple loci throughout the genome. The marker is PCR-based and dominant.

RFLP (restriction fragment length polymorphism): RFLP analyses involve the restriction of DNA and the production of DNA patterns based on variations occurring in the length of DNA fragments generated by a specific endonuclease. Marker systems applying RFLP may be combined with PCR or hybridization techniques and may vary in the classes of DNA employed.

Some authors have proposed combining different marker systems to enhance resolution of the methods. The combination of markers is relevant in clonality studies for identifying true clones that arise from the accumulation of mutations. In these cases, analysis with allele-specific markers is followed by screening for intra-variety diversity with multilocus dominant markers.

3 Studies Analyzing Clonal Variation in Grape

Many clonal variation studies in grapevine have been performed in recent years in an effort to discriminate among clones and provide techniques for reliable identification. The experimental design and data interpretation

vary significantly depending on the study's aims. In the following sections the experimental conditions are summarized.

3.1 Experimental Design and Plant Material

The plant material studied varies among certified variety clones, accessions of particular varieties, which are morphologically similar or dissimilar, color types of clones, sports and their motherplants. The numbers of clones analyzed vary greatly among studies and within a variety. The phytosanitary status of the samples ranged from clones taken from commercial vineyards (virus status unknown), from academic grapevine collections, from tissue culture regenerated plant material, to certified virus-free material (via thermotherapy or shoot tip culture). To prevent the inclusion of different but morphologically similar varieties, some studies test the plant material for polyclonal origin with sets of SSR markers to provide a solid basis to genetically analyze clonality.

3.2 Data Measurement and Interpretation

Analysis of data from these clonal studies varied from the identification of discriminating markers to performing statistical analysis. Computing similarity–dissimilarity or genetic distances matrices with differing coefficients was frequently done together with either constructing dendrograms or principal component analysis. Frequently cophenetic values were calculated or the mantel test performed to test for linkage of genotypic and phenotypic data. The statistical treatment depends on the marker system used. Defining clonal variation via SSR markers is simple because of differing, missing or additional allele sizes, whereas multilocus marker systems need further statistical analysis. Cervera et al. (1998) employed genetic similarity values as an approximate value to characterize clonal genotypes. The value of = 0.97 (corresponding to more than 97% similarity in markers) is utilized as a standard. However, a similarity value will depend on the marker system used, the statistical coefficient applied and the number and diversity of samples/clones studied.

3.3 Clonal Variation in Grapevine Clones?

Clonal variation can be repeatedly detected among clones of grapevine cultivars. The degree of genetic variation depends mainly on the molecular

marker chosen and the scope and range of plant samples employed. According to the studies performed, AFLP-PCR provides the best resolution of genetic variation deriving from somatic mutations. Table 1 shows published work employing AFLP markers. All of these studies, except one, detected genetic variation among clones of differing grapevine cultivars. The scale of diversity found seems to depend on the number of markers produced and the clonal plant material studied. Cultivated, registered clones seem to exhibit more intra-variety variability than clones derived from mutated shoots from the same vine. The reproducibility of AFLP markers has been challenged (e.g. Goto-Yamamoto 2000; Merdinoglu et al. 2000), but recent studies report no such problems, possibly due to careful scoring procedures and optimization of conditions (e.g. Imazio et al. 2002; Fanizza et al. 2003; Forneck et al. 2003a).

RAPD-PCR was implemented in earlier studies to search for genetic differences among clones. Five studies found clone-specific RAPD markers, whereas three could not detect differences among clones (Table 2). Other studies investigated genetic variation among somaclones (multiple clones arising from the same mothervine through in vitro passages). In general, the genetic variation detected is low and determined by the nature of the marker system.

SSR markers are well suited to grapevine, although few SSR studies have been conducted to search for differences among clones. However, clones, biotypes (clones that show phenotypic divergence) or sports are often studied together while investigating variety identification, parentage and diversity. Early SSR studies did not discover differences in alleles among clones and general opinion confirmed SSR marker to be unsuitable for clonal detection. This was thought because of their codominant inheritance and stability. SSR marker were successfully adopted for establishing pedigree analyses of grapevine cultivars (e.g. Bowers and Meredith 1997) and used for cultivar identification. SSR markers have been employed to study polyclonality of grapevine cultivar accessions (e.g. Filippetti et al. 1999; Kozjak et al. 2003). Given the increasing use of SSR markers in genetic analyses of long-lived plants, it is important to characterize their stability at the inter-variety level. Studies have confirmed genetic variation in SSR sequences and specified variation through missing alleles (null alleles), the addition of one or several alleles (chimerism) and the development of new alleles with differing sizes (Table 3). Recent studies analyzing the chimeric state of grapevine clones have been directed using SSR markers (e.g. Franks et al. 2002). The results of these studies suggest that chimerism occurs frequently in grapevine clones.

ISSR markers were applied by several groups to enhance the resolution and reproducibility of the RAPD technology. Limited levels of genetic

Table 1. Studies implementing AFLP markers to differentiate among clones

Variation	Cultivar and (number of clones or sports)	AFLP technology	Specific comments	Source
Clonal variation	Sangiovese (6), Colorino (4), among others	8 *Mse*I/*Eco*RI combinations with 458 bands	Intra-specific variation	Sensi et al. (1996)
Clonal variation	Pinot gris (1), P. noir (1)	4 *Mse*I/*Eco*RI combinations	Clone-specific patterns	Goto-Yamamoto (2000)
Clonal variation	Napoleon (9), Flame Seedless (5), Italia (5), among others	4 *Mse*I/*Eco*RI combinations with 440 bands	Ten intra-varietal polymorphic bands for F. Seedless and Napoleon	Cervera et al. (2000)
Clonal variation	Traminer (3), Savignin (5), P. blanc (2), P. gris (2), Chasselas (2), Auxerrois (2), Silvaner (2)	8 *Mse*I/*Eco*RI combinations with 197 bands	Clone-specific patterns for all clones	Merdinoglu et al. (2000)
Clonal variation	Flame Seedless (2)	*Mse*I/*Eco*RI combinations with 3,000 bands	Two differentiating bands	Scott et al. (2001)
Clonal variation	Albarino (28 accessions)	2 *Mse*I/*Eco*RI combinations with 199 bands	Distinction among closely related varieties and evidence of intra-varietal variation	Cervera et al. (2001)
Clonal variation	Tempranillo (31 accessions)	2 *Mse*I/*Eco*RI combinations with 206 bands	One to two polymorphic bands among clone groups	Cervera et al. (2002)
Clonal variation	Traminer (24), among others	3 *Mse*I/*Eco*RI combinations with 153 bands	Clones show an average similarity of 97.1%. Eight clones could not be separated	Imazio et al. (2002)
Clonal variation	P. noir (20), P. blanc (10), P. gris (6) among others	10 *Mse*I/*Eco*RI combinations with 422 bands	Clone-specific patterns. Three clones could not be separated	Forneck et al. (2003b)
No variation	Italia (3) (+ 1 sport)	49 *Mse*I/*Eco*RI combinations with 3.880 bands		Fanizza et al. (2003)

Table 2. Studies implementing RAPD marker to differentiate among clones. Studies analyzing somaclonality are not included

Variation	Cultivar and (number of clones or sports)	Specific comments	Source
Clonal variation	Sultania (2)	Genetic differences found in bands produced from 17 of 110 primers, between this pair with a bandsharing ratio of 96.1% (compared to unrelated cultivars with 78.5%)	Striem et al. (1994)
Clonal variation	Pinot, Chardonnay (no. not specified)	Genetic variation among Pinot noir and Pinot gris observed using 118 markers. 'Frequency of polymorphism was not higher between the color types than within one color type'	Regner et al. (2000b)
Clonal variation	Riesling (10)	Genetic variation was found among 9 of 10 clones tested using a 'clone'-specific marker. All clones could be differentiated using primer combinations	Regner et al. (2000a)
Clonal variation	Traminer (3), Savignin (5), Pinot blanc (2), P. gris (2), Chasselas (2), Auxerrois (2), Silvaner (2)	Genetic differences among all clones tested in banding patterns produced from 138 primers	Merdinoglu et al. (2000)
Clonal variation	Traminer (12)	Genetic differences found in bands produced from 20 of 26 primers	Regner and Kaserer (2002)
Clonal variation/ no variation	Chardonnay (3), Pinot noir (+4 sports), Niagara (+3 sports), Concord (+1 sport)	No differences found among 'known' clones of P. noir and Chardonnay using 53 primers. Low genetic variation found among Niagara and Concord	Ye et al. (1998)
Clonal variation/ no variation	Aubin vert (3), Räuschling (2), Aubin blanc (2), Chardonnay (3), Aligoté (3), Pinot (5), St. Laurent (2)	Genetic variation found in banding patterns produced from 20 primers. Clones of Pinot, Aubin vert and Chardonnay could not be differentiated	Tschammer and Zyprian (1994)

Table 3. Studies implementing SSR markers to differentiate among clones. Cultivar expressing clonal variation appears in *italics*

Variation	Variety and (number of clones studied)	SSR loci analyzed	Origin of mutation according to author(s)	Source
Variation	Fortana (5)	VVS1, VVS2, VVS4, VVMD3, VVMD6	Polyclonality	Silvestroni et al. (1997)
Clonal variation	Gewürztraminer (3), Savignin (5), Pinot blanc (2), *P. gris* (2), Chasselas (2), Auxerrois (2), Silvaner (2)	VVS2	Allelic mutation	Merdinoglu et al. (2000)
Clonal variation	Traminer (12)	VRG1, VRG2, VRG3, VRG4, VRG7, VRG9, VRG10, VRG11, VRG15	Allelic mutations (null alleles, additional alleles)	Regner and Kaserer (2002)
Variation	Nebbiolo (15)	VVMD7, VVS5	Polyclonality	Botta et al. (2000)
Clonal variation	Riesling (10)	VRG1, VRG2, VRG3	Allelic mutations (null alleles, additional alleles)	Regner et al. (2000a)
Clonal variation	Pinot noir (25), Chardonnay (22)	15 VMC loci	Chimerism, allelic mutations	Riaz et al. (2002)
Clonal variation	Primitivo (4), Pinot noir (3), P. blanc (3), P. gris (2), P. meunier (4)	VVS1, VVS2, VVS19, VVS29, VVS5, VVS16, VVMD7	Chimerism, allelic mutation	Franks et al. (2002)
Clonal variation	Pinot (145)	VVS2, VVS5, VVMD25, VVMD30, VVMD32, VVMD7, VMC3b12, VMC3c9, VMC8g6, VrZAG25, VrZAG79, VMC5g7	Chimerism, allelic mutation	Hocquigny et al. (2003)
Variation	Refosk (55 accessions)	29 loci	Polyclonality	Kozjak et al. (2003)
Clonal variation	Muscat d'Alsace (5), Grefo di Tufo (6), Primitivo (5), Corvina (3)	VVMD32, VVS2, VVMD7, ISV8, VMC6e4	Chimerism, allelic mutations	Crespan (2004)

Table 3. *Continued*

Variation	Variety and (number of clones studied)	SSR loci analyzed	Origin of mutation according to author(s)	Source
No variation	Pinot noir (3), P. gris (3), P. blanc (5), Cabernet franc (3), C. sauvignon (3), Chardonnay (5), Refosk (4), Ribolla (7)	VVS1, VVS2, VVS3, VVS4, VVS5		Cipriani et al. (1994)
No variation	Sangiovese (12)	VVS2, VVS4, VVS29, VVMD5, VVMD6, VVMD7, VVMD8		Vignani et al. (1996)
No variation	Albarino (18 accessions)	VVS1, VVS2, VVS29, VVMD5, VVMD6, VVMD7		Loureiro et al. (1998)
No variation	Portugieser (2), Silvaner (2), Gutedel (2), Riesling (2), Pinot blanc (1), P. gris (1), P. blanc (1)	VVS1, VVS2, VVS3, VVS4, VVS29, VVMD5, VVMD7, VVMD28, VVMD32, VVMD36		Sefc et al. (1998)
No variation	Pinot clones (number not specified)	34 loci		Regner et al. (2000b)

variation can be revealed by using these techniques. Regner et al. (2000a) found clone-specific ISSR markers among a set of ten *V. vinifera* cv. Riesling clones. Such polymorphic markers were also found by Specht (2002) analyzing Pinot blanc, P. gris, Auxerrois and Chardonnay clones.

Few studies have been published using RFLP-PCR to examine clonal variation and no differences were detected. Gogorcena et al. (1993) tested nine Pinot noir clones together with P. blanc and P. gris, finding no differences. Likewise Bourquin et al. (1995) did not detect differences among rootstock clones.

Marker systems to trace mutations associated with transposition or retrotransposition events in the genome are of great use in clonality studies. Several strategies have been developed and two different strategies were adopted in grapevine to analyze clonal variation. Inverse sequence-tagged repeat (ISTR) analysis (Rohde 1996) makes use of the ubiquitous presence of reverse transcriptase sequences and can be extended into a generally applicable, multiple locus strategy for grapevine. Sensi et al. (1996) found intra-varietal genetic differences among putative clones of *V. vinifera* cv. Sangiovese using ISTR markers. Another technique, sequence-specific amplification polymorphism (S-SAP), uses a combination of AFLP and sequence-specific PCR (based on long terminal repeats). This technique is a powerful method to detect the insertional polymorphism of retrotransposons (Waugh et al. 1997). The discriminating power of S-SAP has been studied by Pelsy et al. (2002, 2003) who found resulting S-SAP markers in good agreement with SSR markers obtained from the same set of samples. S-SAP markers can be employed for clonal studies of *V. vinifera* cv. Pinot, and have shown the potential to discriminate among clones (Forneck and Wedig, in prep.).

3.4 Pitfalls

Experimental errors limiting clonality measures may hamper clonality studies. Potential drawbacks can result due to sampling errors. Misnaming or mixing of grapevine clones has been reported in grapevine research. Although molecular genetic techniques are now routinely used, methodological pitfalls may result in misinterpretation. However such pitfalls may occur, they need to be considered in the data interpretation. A problem with AFLP analysis is the appearance of artifact amplicons. These bands may be caused by reduced specificity of restriction enzymes (Goto-Yamamoto 2000), or when buffer conditions are inadequate. Furthermore, banding patterns can be affected by the DNA extraction method (Konradi et al. 2002). Another critical point is that genetic data generated by AFLP and

RAPD markers may use foreign DNA (e.g. contaminating microbes) as template-producing 'false positives'. RAPD-PCR was employed by several groups to discriminate among clones. However, this technique is not always reproducible and has been dismissed by most authors (e.g. Büscher et al. 1993; Regner et al. 2000b). SSR markers are advantageous since they are species-specific and display typical allele sizes for a particular locus. A quality assessment of different detection systems showed that differences in SSR allele size estimates do occur and can affect the utility of SSR markers. Furthermore, visualization of alleles depends on the resolution of the technique chosen. This may be critical for the correct identification of null or chimeric alleles.

3.5 Somaclones and Protoclones

Another grouping among clones are the soma- and protoclones, which refer to plantlets regenerated through in vitro techniques. The processes underlying somaclonal variation are believed to require multiple genetic and/or epigenetic events that affect patterns of expression or result in gene mutation. For further information a comprehensive review of somaclonal variation has been brought together by various authors and edited by Roubelakis-Angelakis (2001). The first indications of somaclonal variation of regenerated or tissue-culture-propagated plants were gained through phenotypic observations of altered morphology from the donor plant. Genetic variation has been identified using RAPD markers in protoclones of *V. vinifera* cv. Seyval blanc (Schneider et al. 1996) and by applying AFLP markers to characterize somaclones of anther-derived grapevines (Popescu et al. 2002). The latter group also observed changes in methylation patterns in *V. vinifera* cv. Mission. No somaclonal variation of ploidy level was found by Kuksova et al. (1997) in plants regenerated from leaf explants through somatic embryogenesis. Spontaneous somaclonal variation in regenerated Seyval blanc protoclones was confirmed by Reustle and Matt (2000). These authors detected cytogenetic variation (tetraploid regenerates) and clone-specific RAPD-patterns.

3.6 Chimerism

Chimeric grapevines have been observed in the past and used in clonal selection programs. A chimera coexists of at least two different genotypes. By convention a periclinal chimera is a plant with a two-layered tunica above a corpus showing one or more genetically different entire shoot

apical cell layers. The underlying mutations can enter these meristems in two ways. Cells can mutate in the initial shoot meristem, or a mutated somatic cell may be incorporated into an adventitious meristem. In both ways the meristem develops into a shoot with the mutant phenotype (sport). The literature on chimerism in woody perennials and grapes is sparse. Descriptions of chimeric grapevine plants were reported in the middle of the 19th century describing red- and white-colored Pinot clusters occurring on one vine. First experimental evidence was provided through cytological studies by Thompson and Olmo (1963), who showed chimeras coexisting with different ploidy levels. Molecular analysis has added proof with recent studies revealing genetic diversity and chimeric state of some P. meunier clones (Franks et al. 2002), P. noir and P. gris clones (Hocquigny et al. 2003), and P. noir and Chardonnay clones (Riaz et al. 2002) with SSR markers.

4 Epigenetic Effects on Clonal Variation

Epigenetic effects cover the study of the mechanisms by which genes bring about their phenotypic effects. Several age-related processes have been described that affect the different stages of growth. In the case of woody perennials the epigenetic 'imprint' on the DNA may persist for several years of culture. There are at least two distinct classes of epigenetic information that can be inherited by chromosomes and thus transmitted via vegetative propagation. One class is DNA methylation, in which a nucleic acid base is modified by a DNA methyltransferase. The other class of epigenetic information involves changes in chromatin proteins (van Steensel and Henikoff 2003). These findings motivated the development of technologies to reveal epigenetic patterns that can be used to elucidate genetic variation. One of the most popular strategies is to use methyl-sensitive restriction endonucleases to map and quantitatively assay the relative abundance of methylated C-residues (e.g. MSAP, methylation sensitive amplified polymorphism). Very few studies have been conducted to reveal methylation patterns among grapevine clones. Imazio et al. (2002) found changes in methylation patterns among clones of *V. vinifera* cv. Traminer. The authors suggest that the phenotypic differences observed are due to synergetic effects of genomic and epigenetic variation. Another study was performed to analyze changes in methylation patterns as potential causes of the recalcitrance of regeneration of *Vitis* ssp. (Harding et al. 1996). This group also found that methylation patterns change during tissue culture passages of *V. vinifera* cv. Sultania.

5 Concluding Remarks

Clonality is a dynamic concept. Genetic variation is sustained by mutation, which provides the basis for the clonal selection of superior clones in woody perennial crops. The old nomenclature that exists for defining clones (offspring produced asexually without recombination events) persists in the light of recent DNA-based studies. The terminology on clonality is expanding and new definitions have been constructed describing clones in all taxa (Loxdale and Lushai 2003). A term describing clones in woody perennial crops could be 'clones are the assemblage of biotypes deriving from a single zygote through somatic mutations. Clones may expose genetic variation. Chimeric clones bearing mutations in a divided genome are thus clones as are polyploid clones.'

Genetic variation is added by numerous mechanisms to enhance variation in asexually reproducing organisms, and allow for adaptation and selection processes. For a cultivated woody perennial crop genetic variation has additional meanings since clonality is used to maintain identical genotypes and allow for the selection of new clones of grapevine cultivars. Research on clonality and grapevine clones will progress. New innovative techniques will facilitate closer examinations of the mechanisms of the genome using microchip and microarray techniques. Expanding research in grapevine clones by incorporating advances from other genomics efforts and techniques will have a large impact on grape clonality research. The position and timing of the mutations will be studied to manipulate variation-inducing events. Furthermore, quantification of such variation will be of great interest.

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