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MICROSATELLITE MARKERS FOR GRAPEVINE: A STATE OF THE ART

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1. INTRODUCTION

Microsatellite markers have recently become the favourite type of DNA marker for identification of grapevine cultivars, and their properties enable a wide range of applications from cultivar identification based on various parts of the grapevine plant to pedigree reconstruction and genome mapping. Before such highly informative markers were made available, numerous methods were tested with the goal to provide a reliable method of genotype identification for the management of germplasm collections. Such a desired method, could then be potentially used as an accurate certification system for global in-

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ternational trade of grapevine and rootstock plant material.

The traditional methods for the identification and differentiation of cultivars, ampelography and ampelometry, are based on morphological differences between the varieties. However, several restrictions are imposed on these methods:

- As mainly full-grown leaves are taken into consideration for morphological identification, the methods can be applied only during the vegetative period to fully-grown plants. However, vine plant material is traded in the form of woody canes, which makes cultivar identification almost impossible. If problems occur, it is usually several years after a vineyard is planted before a mistake is noticed. In the case of rootstocks, the situation is even more difficult. Grafted rootstocks are never allowed to develop leaves in vineyards and this makes correct ampelographic identification impossible. The influence of the rootstock genotype on the growth of the grafted scion and on the quality of the harvested grapes is considerable. Therefore, the choice of suitable rootstock cultivars for different environmental conditions is a major economic factor in viticulture, and false classification of rootstock cultivars can involve economic disadvantages for the viticulturists.
- The phenotypes of plants are heavily influenced by environmental conditions as well as nutritional state and health. Different environments may cause variation in the morphological features considered in ampelography, and identification of infected, under-nourished or otherwise untypical plants is susceptible to mistakes.
- The total number of grapevine cultivars in ampelographic collections world-wide is estimated to be up to 15 000 and the number of cultivars in use is very large. Even if the plants are in excellent condition, it is extremely difficult to differentiate all varieties by morphological features.
- Application of ampelographic methods require skilled individuals, who however cannot have access and knowledge of the thousands of different cultivars in use worldwide, simply because no collection may contain exhaustively these genetic resources. Ampelography experts usually know grapevine cultivars in use in their region and are not familiar with those from other regions. Additionally, reproducibility and standardisation of observations between distant ampelographers has proved difficult to achieve.

For these reasons, alternative methods for cultivar identification, which better illustrate differences at the genotype level, are required. Diverse techniques for the molecular characterisation of organisms have been developed in the past twenty years (Karp *et al.*, 1998), and most of them have also been applied to cultivar differentiation in grapevines.

The usefulness of various enzyme systems for isoenzyme analysis of grapevine cultivars has been assessed by Schaefer (1971), Stavrakakis and Loukas (1983), Benin *et al.* (1988), Eiras-Dias *et al.* (1989), and Calo *et al.* (1989). However, the expression of enzymes may depend on the developmental stage of the plant or on environmental conditions. Therefore, only enzyme systems, which show no variation under different conditions, can be considered as isoenzyme markers. This limits the number of markers available, and consequently restricts the degree of polymorphism and differentiation achievable with isoenzyme analysis (Parfit and Arulsekar, 1989; Walter *et al.*, 1989). A practical

disadvantage is also the absolute requirement of fresh plant material at identical developmental stages.

The next step in the development of molecular markers was the analysis at the DNA level, since the DNA of a certain plant is identical in all cells of any tissue at any stage of development. DNA can be obtained from every kind of plant tissue available, e.g. wood, leaves or berries, and analyses can therefore be carried out at any time of the year. DNA characteristics are not influenced by environmental or sanitary conditions of the plants. Thus, DNA based analyses are free from various kinds of external limitations.

Restriction fragment length polymorphism (RFLP) analysis was successfully employed to detect cultivar specific DNA fingerprints for grapevine and rootstock varieties (Striem *et al.*, 1990, Bourquin *et al.*, 1991; 1992; 1993; 1995; Thomas *et al.*,1993; Guerra and Meredith, 1995). Compared with isoenzyme analysis, the RFLP method offers the advantages of robustness in various environments and higher levels of detectable polymorphism. However, complicated banding patterns may cause difficulties in the evaluation of results (Striem *et al.*, 1990). Further problems are the requirement of large amounts of high quality DNA, as well as the time-consuming and costly primary development of probes and time-consuming and costly analysis procedure.

On the contrary, thanks to the PCR (Polymerase Chain Reaction) technology, Random Amplified Polymorphic DNA (RAPD) analysis is a cheap, easy, fast method for the detection of genetic differences between organisms. Satisfying levels of polymorphism were detected in grapevine and rootstock cultivars using RAPD analysis (Collins and Symons 1993; Jean-Jaques *et al.*, 1993; Gogorcena *et al.*, 1993; Tschammer and Zyprian 1994; Moreno *et al.*, 1995; Xu *et al.*, 1996; Stavrakakis and Biniari, 1998; This *et al.*, 1997; Ye *et al.*, 1998). However, the major disadvantage of this method is the dependence of the results on strict experimental conditions. Different thermocyclers, Taq polymerases or DNA and primer concentrations (Büscher *et al.*, 1993) as well as experimenters can influence results. Stability of the results can be achieved by carefully sticking to standardized reaction conditions (This *et al.*, 1997), but on the whole, standardisation of the RAPD procedure and comparison of the results between laboratories is a difficult goal to achieve.

The conversion of polymorphic RAPD markers to SCAR (Sequence Characterized Amplified Region) markers with specific PCR primers allows the exploitation of RAPD polymorphisms with good reproducibility (Bauer and Zyprian, 1997). The wider development of SCARs from RAPD markers for the purpose of genotype identification was however slowed by the availability of microsatellite markers. RAPD markers converted to SCARs may however remain useful in genome mapping when they can be associated with a phenotype, as shown in the case of seedlessness (Lahogue *et al.*, 1998; This *et al.*, 2000).

Because none of the methodologies described above met the requirements of an ideal identification system, the search for more promising markers went on and microsatellite markers, primarily assessed in human and animal organisms, were also developed for grapevine.

2. WHAT ARE MICROSATELLITES ?

The existence of repeated simple sequence motifs (e.g. CACACA…) in plant nuclear DNA was demonstrated by Delseny *et al.* (1983). It was subsequently shown that simple sequence (also called microsatellite) repeats were ubiquitous in most organisms, including plant and organelle genomes (Lagercrantz *et al.*, 1993; Wang *et al.*, 1994), and that these sequences represented a major source of genetic variation (Tautz *et al.*, 1986) suitable for plant genetics (Morgante and Olivieri, 1993).

Among the three subclasses of repetitive DNA (satellite DNA, minisatellites and microsatellites; Tautz, 1993), microsatellites show the lowest degree of repetition. The typical microsatellite sequence consists of five to about one hundred tandem repeats of short, simple sequence motives composed of 1 to 6 nucleotides (e.g. (GA)n, (GATA)n; Figure 17.1). In eukaryotes, an estimated 10^4 to 10^5 microsatellite loci are scattered randomly throughout the genome. This abundance of microsatellite sequences in eukaryote genomes constitutes an almost unlimited source of polymorphic sites that may be exploited as genetic markers. Microsatellites are usually considered as selectively neutral, provided that they are not located inside or close to a coding sequence, where they may cause the disruption of gene function or be influenced by selection pressures on a gene in their vicinity.

Microsatellites were initially used as molecular probes for random fingerprinting of eukaryotic genomes by classical hybridisation methods, until the availability of the polymerase chain reaction (PCR) enabled targeting of specific loci.

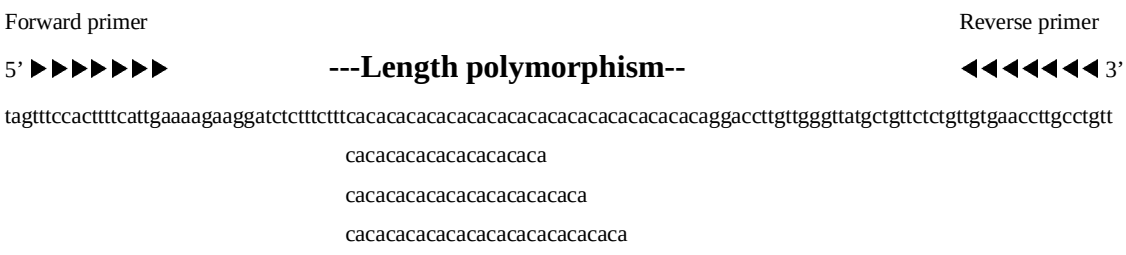
PCR amplified microsatellite markers have the advantages of being locus specific and highly polymorphic. Determination of allele sizes is achieved by high-resolution electrophoresis. The markers are co-dominant and thus allow the discrimination of homozygotes and heterozygotes. Microsatellite profiles are represented by the allele sizes detected at the analysed loci and given in base pairs.

The identification and establishment of microsatellite markers in an organism is a costly and time-consuming procedure involving the construction and screening of genomic libraries and the design and optimisation of PCR primers. Fortunately, microsatellite primers obtained for one species are very often usable in closely related species of the same genus and sometimes even through genera of the same family. This has been the case for most published microsatellite markers from *Vitis vinifera* and *Vitis riparia*, which amplified DNA from other *Vitis* species.

Finally, from a practical point of view, reproducibility and standardisation of microsatellite profiling is usually easy to achieve, which thus facilitates transfer and comparison of data between distant laboratories.

3. DEVELOPMENT OF MICROSATELLITE MARKERS IN *VITIS*

Thomas *et al.* (1993) first investigated the use of repetitive DNA for identifying grapevine cultivars. They showed that microsatellite sequences were abundant in grapevine and very



- Description
  - tandem repeats of short nucleotide motifs (1 to 6 bp)
  - a low degree of repetition (5-100 repeats)
  - random distribution in the genome
  - length polymorphism detected by PCR amplification and electrophoresis
- Advantages
  - locus-specific amplification
  - high polymorphism
  - distributed throughout the nuclear genome
  - frequent occurrence
  - a co-dominant inheritance

Figure 17.1. A dinucleotide microsatellite locus.

informative for identifying *V. vinifera* cultivars. Moreover primer sequences were conserved across other *Vitis* species and *Muscadinia* (Thomas and Scott, 1993). It was also demonstrated through pedigree analysis that the microsatellite alleles were inherited in a co-dominant Mendelian manner (Thomas and Scott, 1993) confirming their suitability for genetic mapping and investigation of genetic relatedness (Thomas *et al.*, 1994).

Other groups throughout the world became interested in grapevine microsatellite markers, which resulted in the development of additional markers (Bowers *et al.*, 1996; 1999b; Sefc *et al.*, 1999). All published primer sequences for grapevine SSR markers are available from the Greek *Vitis* database (Lefort and Roubelakis-Angelakis, 2000a; 2000b; http://www.biology.uoc.gr/gvd).

The approach used for microsatellite marker development in these studies was the construction of a genomic library from a grapevine or rootstock cultivar, screening of the library with microsatellite probes, sequencing of microsatellite containing clones, design of PCR primers from the sequences flanking the microsatellite, optimization of PCR conditions and characterization of the microsatellite polymorphism.

The major drawback of microsatellite markers is the initial time and resources required to develop the markers from genomic libraries. In total, about 40 microsatellite markers have been developed in three different laboratories using the above procedure (Thomas *et al.*, 1993; Bowers *et al.*, 1996; 1999b; Sefc *et al.*, 1999), with some shortcuts (Thomas and Scott, 1994), which also provided useful SSR markers. However, for the marker type to become of greatest use to the viticulture research community, an additional effort was required to develop a large number of microsatellite markers for mapping the *Vitis* genome and the *Vitis* Microsatellite Consortium (VMC) was formed in 1997. This international collaboration which includes the private company Agrogene (France) and 21 research laboratories worldwide recently met the target of developing 333 new *Vitis* markers from a microsatellite enriched genomic library. The consortium also produced at the same time 700 unique DNA sequences of the genome of *Vitis vinifera*.

4. EST DERIVED MICROSATELLITE MARKERS: A NEW STRATEGY

Many of the first studies on microsatellites in humans, animals and plants relied on public databases such as EMBL and Genbank as a source of microsatellite loci. This approach is still being used, and recent examples are microsatellites identified for potato (Milbourne *et al.*, 1998), sorghum (Brown *et al.*, 1996), barley (Becker and Heun, 1995), tomato (Smulders *et al.*, 1997), soybean (Akkaya *et al.*, 1992) and many other species (for a review, see Gupta *et al.*, 1996). Relatively new types of databases are those compiled from cDNA and EST (Expressed Sequence Tag) type data. Identification of microsatellites in ESTs is an approach that has been used in humans (Haddad *et al.*, 1997), and is becoming available in plants, as EST databases become more common. To date this specific approach has been reported successful for rice (Miyao *et al.*, 1996; Cho *et*

al., 2000) and grapes (Scott *et al.*, 2000b). Screening of EST databases is a quick and comparably cheap way to identify microsatellite loci of any repeat type and motif, and yields primers with highly conserved binding sites, thus enabling specific amplification over wider taxonomic ranges than do primers situated in non-coding regions of the genome. Since EST derived markers are associated with gene-rich regions of the genome, they may prove extremely useful for genome mapping. Pressure for sequence conservation in these regions may however reduce the level of polymorphism as compared with microsatellites that fully function as neutral markers.

Of course, the identification of microsatellite loci from EST databases relies on the prior existence of this type of sequence data. For grapevine, a large data set has been reported by Ablett *et al.* (1998), and has been screened for microsatellite markers by Scott *et al.* (2000b). Over 100 microsatellites were readily identified, and the further investigation of some of these loci showed enough polymorphism to allow the discrimination of several grapevine and rootstock cultivars, and transferability of the primers to species of the related genera *Cissus* and *Cayratia*.

5. IDENTIFICATION OF CULTIVARS OF *VITIS VINIFERA* AND ROOTSTOCKS FROM *VITIS* SPECIES

5.1 Source and quality of DNA used for PCR amplification

DNA suitable for PCR has been successfully isolated from wood (cambium tissue scrapings from beneath the bark of vine trunks, whole wooded canes), green canes, petioles, young leaves, floral tissue, pre-véraison and post-véraison berries and the rachis of grape bunches. The procedure described for leaf tissue (Thomas *et al.*, 1993) proved to be suitable for all the above tissue with only the berry tissue requiring an increase in the buffering capacity of buffer A. Bulky tissue such as canes and berries can be frozen in liquid nitrogen and while still frozen ground to a fine powder in an electric coffee grinder. Small scale DNA preparations from 10 - 200 mg of tissue from leaf and callus have also been successfully used for PCR (Franks *et al.*, 1998). Mature leaf tissue is the most difficult tissue for extracting good quality DNA. Canes and berries can be a source of very clean DNA and it is of interest to note that the original genomic library used for grapevine microsatellite isolation was made from DNA isolated from post-véraison berries of Sultana (Thomas and Scott, 1993). Other published methods (Lodhi *et al.*, 1994; Wolf, 1996; Lefort and Douglas, 1999) or the QIAGEN DNeasy Plant Mini Kit yield sufficient good quality DNA from as little as 100 mg fresh weight of plant material.

5.2. Analysis methods available and comparison between them

A number of different methods for analysing microsatellite markers have been assessed including the incorporation of 35S dATP during PCR and separation of the products on denaturing polyacrylamide gels (Thomas and Scott, 1993), 32P end-labelled primers in

the amplification reaction and separation on denaturing polyacrylamide gels, ethidium bromide staining of non-denaturing polyacrylamide gels (Scott *et al.*, 2000b), hybridisation of end-labelled (32P, biotin) microsatellite oligonucleotide probes to fragments transferred to nylon membranes after separation on denaturing polyacrylamide gels (Kijas *et al.*, 1995) and silver staining of denaturing polyacrylamide gels (Bowers *et al.*, 1996; 1999b; Bowers and Meredith, 1997). However, the most effective method has been the use of primers tagged with fluorochromes and fragment separation on denaturing polyacrylamide gels. The configurations used by a number of different groups include:

(1) ABI 373A or 377 DNA sequencers (PE Applied Biosystems) with sizing of the fragments using GeneScan or Genotyper (PE Applied Biosystems) (Thomas *et al.*, 1994; Botta *et al.*, 1995; Filippetti *et al.*, 1999).
(2) ABI Prism 310 Genetic Analyzer (PE Applied Biosystems) in combination with GeneScan Software (Grando and Frisinghelli, 1998; Grando *et al.*, 1999; 2000a; 2000b; 2000c).
(3) ALFexpress DNA sequencer (Pharmacia) in combination with the Fragment Manager software for allele sizing (Sefc *et al.*, 1997; 1998a; 1998b; 1998c; 1998d; 1999; 2000; Lopes *et al.*, 1999; Maletic *et al.*, 1999).
(4) Licor 4200 DNA sequencer (Licor) in combination with the GeneProfiler software (Scanalytics) (Lefort *et al.*, 2000a; 2000b; 2000c; Lefort and Roubelakis-Angelakis, 2000a; 2000b).

The advantages of the systems using fluorescent labelling are many and include the possibility of multiplexing loci in a single lane with an internal size standard and automated size calling of the alleles. Allele size calling between gels and different gel-based ABI sequencing machines has shown a high reproducibility, while differences in size calling are observed when results from different electrophoresis systems are compared. For example, an allele estimated to be 150 bp by silver staining may run at 148 bp in the Pharmacia system, while it may be called 153 by the ABI sequencer. These deviations, however, are constant within a certain locus, and can easily be determined by analysing a small set of genotypes in each of the systems. Later on, the allele sizes determined by different systems can be corrected for the observed deviation to one established standard system, and data from different sources become easily comparable again. Another possibility to facilitate the comparison of data is the presentation of microsatellite profiles by the actual number of repeat motifs in each allele.

5.3. Identification of grapevine cultivars and rootstocks by using nuclear SSRS

The initial grapevine microsatellite study by Thomas and Scott (1993) at CSIRO Plant Industry, Australia, reported the DNA identification of 26 *V. vinifera* cultivars and 6 additional *Vitis* species as well as *Muscadinia rotundifolia*. This was subsequently extended to more than 80 genotypes including rootstocks, wine grapes, table grapes and those used for raisin production (Thomas *et al.*, 1994). Currently a database of DNA

microsatellite profiles of close to 200 genotypes is held at CSIRO. These pioneering studies, showed the advantages of the method for addressing the crucial question of an exact identification of grapevine cultivars.

Interpretation of microsatellite bands was shown to be relatively easy, and the data could be stored conveniently in the form of allele sizes in base pairs. Grapevines are propagated vegetatively, so that, except for somatic mutations, the individual vines of a cultivar are genetically identical to each other. In principle, the microsatellite data obtained from one individual represent the microsatellite profile of that cultivar. The results of microsatellite analysis can be reproduced and compared in different laboratories or at different periods, provided standardisation is carefully performed (see above). Consistent microsatellite profiles were obtained for example using DNA of the same grapevine cultivars genotyped in different years (Botta *et al.*, 1995) and different laboratories (Sefc *et al.*, 1998; Grando and Frisinghelli, 1998; Lefort *et al.*, 2000a). SSR analysis offers great automation potential using automated sequencers and software for data collection.

The extraordinary potential of microsatellite markers for grapevine and rootstock cultivar discrimination has been demonstrated in several studies (Thomas and Scott, 1993; Thomas *et al.*, 1994; Cipriani *et al.*, 1994; Bowers *et al.*, 1996; Sefc *et al.*, 1998a; 1998b; 1998c; 1998d; 1999; Sanchez-Escribano *et al.*, 1999). Theoretically, five unlinked markers each with five equally frequent alleles could produce over 700 000 different genotypes (Bowers *et al.*, 1996). In reality, however, these ideal conditions are seldom fulfilled. In order to minimise the number of markers necessary for a reliable discrimination and identification of cultivars, the most informative loci have to be selected.

Simple allele counts are however apt to overestimate the value of a given marker, as deviations from the isofrequent allele distribution severely decrease the information content of that marker, even though the number of alleles may be high (Sefc *et al.*, 1999, Tessier *et al.*, 1999). Therefore, measures considering allele frequencies are better descriptors of locus variability. Two such measures are the *Probability of Identity (PI)* (Paetkau *et al.*, 1995; applied to grapevine genotyping by Sefc *et al.*, 1999, 2000), which is derived from allele frequencies, and the *Discrimination Power (D)* (Tessier *et al.*, 1999), based on banding pattern or genotype frequencies at a given locus. Both measures describe the probability, that two unrelated cultivars can be distinguished by a certain marker, allowing the distinction of two actually different cultivars. At hyperpolymorphic *Vitis* microsatellite loci, *pI* values can be as low as 0.05, which corresponds to a 5 % probability for genotype sharing among unrelated cultivars. Combining the data of five unlinked highly informative microsatellites, for example, the theoretical probability for non-distinguishable microsatellite profiles from different cultivars is below 10^{-5} (Sefc *et al.*, 1999). A higher discrimination power D implicates a lower probability of confusion of cultivars (1-D), and D values as high as 0.895 and 0.697 have been calculated for one highly and one moderately polymorphic microsatellite locus, respectively (Tessier *et al.*, 1999).

The information content of a given marker may differ between cultivar collections from different regions, as allele frequencies vary between grapevine gene pools. On the whole, however, a marker set containing the most informative markers as defined in one

cultivar collection will also yield a high level of discrimination power in other gene pools (Sefc *et al.*, 2000). Such a marker set has been recently recommended as a potential descriptor for grapevine cultivars (Cabello *et al.*, 1999).

Due to the high discriminative power of microsatellite analysis, the finding of identical genotypes in two different plants is strong evidence that these plants in fact belong to the same cultivar. Therefore, microsatellite analysis can be used to determine cultivar identity and to identify plant material of unknown varietal origin by comparing the genotype obtained from the sample with reference genotypes of cultivars stored in a database.

Some examples of practical applications of microsatellite based cultivar identification are outlined below.

In the course of a project, which aimed to reduce virus contamination of grapevine material by *in vitro* meristem culture, thermotherapy and subsequent propagation of the treated material for the production of certified planting material, cultivar identity of the *in vitro* plantlets prior to the propagation steps was carried out by microsatellite analysis. Two samples per clone were analysed at four microsatellite loci, and the resulting genotypes were compared to a reference database. This quality control step proved to be essential, as mistakes in cultivar assignment were detected (Sefc *et al.*, 1998d).

Similarly, evidence of false identities of rootstocks or cultivars in vineyards was found in Greece (Lefort and Roubelakis-Angelakis, unpublished). In a vineyard, rootstocks believed to be 1103 Paulsen and Ruggeri 140 appeared to be different from the reference plants of two ampelographic collections and remain unknown until now. Another example concerned 3 cultivars from the same vineyard where genotyping at nine SSR loci showed that if Liatiko was really Liatiko, a so-called Kotsifali proved to be an offspring of Liatiko and a so-called Kotsifaloliatiko was in fact a cultivar named Fegi.

Practical application of microsatellite genotyping of grapevine cultivars may also involve using DNA extracted from berries, raisins, wood or wine. In most studies dealing with the genetic characterisation and identification of vine cultivars, DNA has been extracted from leaves, which is the most convenient material for this purpose. However, it may be necessary in some cases to use other tissue as a DNA source, e.g. when harvested berries are to be examined or when the varietal composition of wine itself is put to the question. Wood tissue is also the only option for extracting DNA from grafted rootstocks, in which case cambium scrapings beneath the bark are taken (Bourquin *et al.*, 1992; Hong *et al.*, 1998). The procedure has also been successfully used for customers of the commercial grapevine DNA profiling service offered in Australia (Thomas, unpublished).

Wolf *et al.* (1999) also used wood from rootstock cuttings as a source of DNA suitable for RAPD PCR in order to identify 29 grapevine rootstock varieties. They assayed both cambium and wood and did not report any quantitative differences between both tissues in terms of DNA yield.

Cases of misidentification of Teleki 5C and SO4 such as that reported for the University of California collection (Walker and Boursiquot, 1992) can now be solved by DNA profiling (Thomas *et al.*, 1994). Recent studies have used microsatellite markers to identify 58 rootstocks (Hong *et al.*, 1998) and 110 accessions of 25 grape taxa (Lamboy and

Alpha 1998) with only five SSR markers. The high polymorphism of SSR markers through *Vitis* taxa is advantageous in reducing the number of markers needed for identification.

In a survey of table grapes for sale in various supermarkets and market places in Austria (Sefc *et al.*, 1998b), DNA was extracted from berries by grinding them in a mortar with liquid nitrogen after removal of the seed and proceeding according to the method established by Thomas *et al.* (1993). The genetic profiles resulting from microsatellite analysis of the berry samples were compared with a reference database. Only about two thirds of the samples were shown to be correctly labelled. Fruit offered as "Austrian table grapes" were shown to match the cultivar Blauer Portugieser and Hungarian grapes labelled as "Plattenseer" displayed the microsatellite profile of Chasselas. Discrepancies also included two white grapes described as "Muskat", which matched the microsatellite profile of Italia. One sample of blue grapes, denoted as "Cardinal", did not match the corresponding reference in the database and was identical to two other grape samples falsely presented as "Italia" and "Muskat", respectively. On comparison with the table grape database established by Sanchez-Escribano *et al.* (1999), these grapes matched the microsatellite profile of the cultivar "Michele Palieri". This inspection of the correctness of marketed table grape labels, though on a small scale, revealed a substantial degree of inaccuracy in information provided to the consumer.

5.4. Synonyms

Microsatellite analysis can further be applied to the confirmation and definition of synonyms, i. e. identical genotypes known under different names. The identification of duplicates is of particular importance in germplasm collections when maximum genetic variability should be maintained while keeping the number of specimens at minimum. For several cultivars, synonymy had been suspected or assumed based on ampelographic observations and could be confirmed by microsatellite analysis (see Cipriani *et al.*, 1994; Botta *et al.*, 1995; Bowers *et al.*, 1996; Sefc *et al.*, 1998a; Lopes *et al.*, 1999; Maletic *et al.*, 1999; Lefort *et al.*, 2000a).

For instance, microsatellite profiles of Italian wine grapes supported synonymy of the cultivars Refosco di Faedis and Refoscone (Cipriani *et al.*, 1994) as well as of Favorita, Pigato and Vermentino (Botta *et al.*, 1995). Among table grape cultivars, synonymy was confirmed for Keshmesh/Thompson Seedless and Dattier/Rhazaki (Bowers *et al.*, 1996). In a Portuguese cultivar collection, a number of assumptions of synonymous cultivars were verified: The cultivars within the following groups, Fernão Pires/Maria Gomes, Boal Cachudo/Boal da Madeira/Malvasia Fina, Síria/Crato Branco/Roupeiro, Periquita/Castelão Francês/João de Santarém/Trincadeira and Verdelho da Madeira/Verdelho dos Açores/Verdelho roxo, yielded identical microsatellite profiles. The Portuguese cultivar Moscatel de Setúbal was shown to be identical to the widely grown cultivar Muscat of Alexandria (Lopes *et al.*, 1999) and Moschato Alexandreias (Lefort *et al.*, 2000a). Interestingly not all synonymies found previously in an isoenzymic polymorphism study with a four enzymes system (Eiras-Dias and Bruno-Sousa, 2000) in

Portuguese grapevine were confirmed by SSR profiling, illustrating the increased power of microsatellite markers to differentiate between genotypes. In the South-Eastern part of Austria, a cultivar grown under the name of Morillon was considered to be a synonym of Chardonnay, which was supported by the identical microsatellite profiles of the two cultivars (Sefc *et al.*, 1998a). Among Croatian cultivars, Plavina and Brajdica are synonyms as confirmed by microsatellite analysis (Maletic *et al.*, 1999).

Apart from these cases, where microsatellite data confirmed previous ampelographic results, unexpected synonyms were discovered among cultivars from different collections. In a comparison of genotypes obtained from Croatian cultivars with those obtained from grapevines of neighbouring regions (Maletic *et al.*, 1999), synonyms were detected among Croatian cultivars from Istria and North Italian grapevines: Teran Bijeli was shown to be identical to Prosecco, and Muskat Ruza Porecki turned out to be a synonym of Rosenmuskateller. A grapevine cultivar grown in continental Croatia, Moslavac, was identical to the Hungarian cultivar Furmint. Ampelographic studies prompted by the results of genetic analysis further supported the microsatellite-based definition of these synonyms, as was the case for the synonymy between Moschato Mazas and Moschato Kerkyras in Greece (Lefort *et al.*, 2000a; 2000c).

In contrast, in one case putative synonymy was clearly rejected by microsatellite analysis. The Croatian cultivar named Hrvatica was supposed to be identical to the Italian cultivar Croatina, as both names mean "Croatian girl". However, it was shown that Hrvatica and Croatina differ at several loci and thus represent two distinct cultivars (Maletic *et al.*, 1999).

Grando *et al.* (2000a) evaluated the local biodiversity of ancient grapevine germplasm spread on the Trentino region (North Italy) at 7 microsatellite markers. The study was carried out on 36 recovered vines (relic varieties) and 12 local grapevine varieties which are still widely cultivated in Trentino, such as Lagrein, Lambrusco Foglia Frastagliata, Marzemino, Nosiola, Teroldego Schiava Grossa. Surprisingly eleven cases of synonymy were identified. These included identical identities among non-cultivated varieties (4 cases), among local varieties and non-cultivated vines (5 cases) and among non-cultivated varieties and international grapevine cultivars. The last two cases identified Vernaccia Nera with Merlot and Francesa Nera with Carmenère. Interestingly, other identical vine genotypes had received many different denominations in a geographically narrow wine producing area. The names are however often related, eg. Biancaccia and Biancazza, Vernaccia and Vernazzola or have similar references eg. Schiava Grigia (Schiava gray) and Cenerina (Cinereo, ashen). All the alleles found in this survey had already been described in other cultivars of *Vitis vinifera*, except for some alleles detected in Nera dei Baisi, which were characteristic for non-*vinifera Vitis* species.

As expected, the 12 local varieties still grown in Trentino contained little diversity at SSR loci. Moreover some of them revealed a considerable genetic similarity, ranging from 50 to 75% shared alleles, while all were still distinguishable.

Concerning rootstocks, 5A Teleki and 5BB Kober have been shown to have the same DNA profile indicating that the two different names were mistakenly given to the same rootstock in the past (Thomas *et al.*, 1994).

On the other hand it may happen that cultivars reported as synonyms are really not synonyms but very closely related plants as reported in the following study. It has been experimentally shown that a self-pollinated Sangiovese plant can produce seedlings, which are difficult to distinguish phenotypically from the mother plant but are genetically distinct based on DNA profiling (Filippetti *et al.*, 1999). Interestingly two of the 24 seedlings analysed had an identical genotype across 10 microsatellite loci but could be differentiated based on phenotype (Filippetti *et al.*, 1999), suggesting that either more SSR loci or AFLP markers would be necessary for finding genetic differences. As a result of the ancient history of grapevine cultivation in Europe, it might be possible that some grapevine cultivars may consist of more than one genotype in consequence of mixed plantings and the propagation of closely related plants which were phenotypically similar (Rives, 1961). This concept is known as the polyclonal origin of a cultivar.

5.5. Clonal lines and somatic mutants

While distinction of cultivars could be achieved to various degrees with all of the mentioned molecular markers systems, it has so far not been possible to detect polymorphisms among the different clones of a cultivar with any of these markers (Collins and Symons, 1993; Jean-Jacques *et al.*, 1993; Bourquin *et al.*, 1992; 1995; Thomas and Scott, 1993; Cipriani *et al.*, 1994; Bowers *et al.*, 1996; Loureiro *et al.*, 1998; Sefc *et al.*, 1998a; Ye *et al.*, 1998). When two different microsatellite genotypes were detected among clones of the cultivar Fontana, it was concluded that Fontana was of polyclonal origin and was in fact composed of two distinct cultivars. Yet, the two clonal groups proved to be closely related and may have been derived from a single parent and one of its seedlings (Silvestroni *et al.*, 1997).

In order to search for polymorphisms among clones, additional multi-locus techniques have been applied. While inter-simple sequence repeats (ISSR) PCR failed to detect variation among clonal accessions of Garnacha (Moreno *et al.*, 1998), the first positive results have been reported using AFLP (Amplified Fragment Length Polymorphism) in a study of clones and local selections from the Rioja region (Cervera *et al.*, 1998). Recent evidence of such a powerful resolution was shown by Scott *et al.* (2000a), who identified a somatic mutant of Flame Seedless by the AFLP technique.

5.6. Pedigree reconstruction

5.6.1 Methodology

Most of the grapevine cultivars in existence today are centuries old and are thought to have arisen by several processes: domestication of wild vines, either in the early vine domestication sites south of the Middle East or later in the vine growing regions of Europe, spontaneous crosses between wild vines and cultivars and crosses between two cultivars. Due to the importance of grapevine cultivars both as a crop plant and as a cultural heritage, it is extremely interesting to understand the genetic events, which led to today's cultivar range. The longstanding and lively interest in the history of grape culti-

vars is reflected by a variety of speculations concerning the origin of popular cultivars such as Cabernet Franc, Silvaner and Traminer.

Ancient wild vines involved in the original crosses cannot be identified any more as they no longer exist, but parents, which are themselves cultivars, may still be cultivated or kept in collections. Using the methods of molecular analysis, such parent cultivars and their offspring can be recognised, and pedigrees describing the genetic history of grapevine cultivars can be reconstructed.

While molecular marker types such as isoenzyme and RAPD techniques are of limited use for parentage studies (Ohmi *et al.*, 1993; Büscher *et al.*, 1994), microsatellites have proved to be the marker of choice for this purpose since they are transmitted in a codominant Mendelian manner. In a cross, each of the parents passes one allele per locus to the offspring (Table 17.1) and in consequence, each allele displayed by the offspring must also be present in at least one of the two parents. By examining the microsatellite allele composition of an individual and its two presumptive parents, it is possible to confirm or reject the proposed parentage. Microsatellite markers are used routinely in forensic investigations dealing with paternity disputes, and have recently found application in pedigree reconstruction in grapevines. In contrast to parentage analysis in humans, prior assumptions on possible relationships seldom exist among grapevines, and furthermore the chronological order of appearance of the grapevine cultivars remains widely unknown, providing no information on the generation sequence.

Table 17.1. Example for the codominant Mendelian inheritance of microsatellite alleles in the controlled cross St. Laurent x Blaufränkisch. At each locus, one allele per parent cultivar was transmitted to the offspring cultivar Zweigelt. Numbers represent the allele sizes in base pairs [data from Sefc *et al.* (1997)].

Locus	St. Laurent	Zweigelt	Blaufränkisch
ssrVrZAG 7	157:157	155:157	155:155
ssrVrZAG 15	175:177	165:175	165:165
ssrVrZAG 21	200:206	202:206	202:206
ssrVrZAG 25	225:236	225:236	225:225
ssrVrZAG 30	149:151	147:151	147:149
ssrVrZAG 47	163:167	157:163	157:172
ssrVrZAG 64	139:163	139:159	139:159
ssrVrZAG 67	126:152	126:139	139:149
ssrVrZAG 79	238:246	236:238	236:250

In order to achieve a satisfying level of confidence for the definition of pedigrees solely by microsatellite marker analysis, it is necessary to include data from a high number of unlinked loci. The search for parent cultivars and their offspring cultivar in a cultivar collection is carried out by comparing microsatellite alleles within all possible sets of three to identify pairs of cultivars that could have contributed the alleles of the third (offspring) cultivar. Applying a low number of around ten microsatellite markers to this purpose may result in the detection of cultivar groups where alleles at these loci are just by chance consistent with the parent-offspring scenario. The occurrence of false positives can be reduced with increasing the number of unlinked microsatellite markers.

Analysis of at least 25 markers is recommended for reliable pedigree studies in closely related organisms like grapevines. Using 11 highly informative SSR markers showed for instance numerous parentage possibilities in a group of 257 Greek and foreign cultivars, while the gradual inclusion of further markers reduced the number of possible genetic relationships. Pedigree reconstruction by nuclear markers can also be efficiently completed with the use of chloroplast microsatellite markers, providing information on the direction of the cross (see below chloroplast microsatellite markers).

5.6.2. Examples for the reconstruction of grapevine crosses

Among the first and most surprising results of parentage studies in grapevines was the discovery of the origin of the cultivar Cabernet Sauvignon (Bowers and Meredith 1997, Sefc *et al.*, 1997). Cabernet Sauvignon has been described as the world's most renowned grape variety for the production of fine red wine (Robinson, 1994) and has been grown in France at least since the 17th century. A close relationship between Cabernet Sauvignon and another French cultivar, Cabernet Franc, had been suspected from the morphological similarity of the two cultivars. Microsatellite profile comparisons among Central European grapevine cultivars confirmed Cabernet Franc as one parent of Cabernet Sauvignon. The identification of the white wine cultivar Sauvignon Blanc as the second parent of the famous red vine variety, however, was a surprise.

The further search for relatives among French grapevines led to another unexpected finding: 16 grapevine cultivars grown in north-eastern France turned out to be the progeny of a single pair of parents, Gouais Blanc and Pinot (Bowers *et al.*, 1999a). Among the progeny are prominent varieties as Chardonnay, Gamay Noir, Aligoté, Auxerrois and Melon.

While it seemed plausible that the highly valued Pinot should have given rise to a number of successful offspring cultivars, the contribution of Gouais Blanc as a parent of quality wine cultivars was entirely unexpected. The variety is believed to have been introduced to France from Eastern Europe and was widespread in the Middle Ages. However, it was so mediocre that, at various times and regions, it has been banned from vineyards and is no longer planted in France.

Cabernet Franc X Sauvignon Blanc

↓

Cabernet Sauvignon

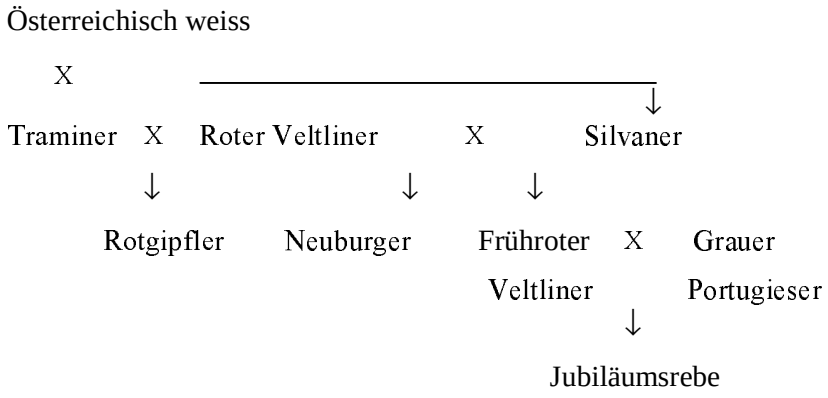
A comparison of the genetic profiles of cultivars from Central Europe resulted in the reconstruction of a four generation-pedigree illustrating the close relationship between the cultivars Österreichisch Weiß, Traminer, Roter Veltliner, Silvaner, Rotgipfler, Neuburger, Frühroter Veltliner, Grauer Portugieser and Jubiläumsrebe (Sefc *et al.*, 1998c).

Gouais Blanc X Pinot

↓

Aligoté
Aubin Vert
Auxerrois
Bachet Noir
Beaunoir
Chardonnay
Dameron
Franc Noir de la Haute Saône
Gamay Blanc Gloriod
Gamay Noir
Knipperlé
Melon
Peurion
Romorantin
Roublot
Sacy

Genetic profiles showed that Silvaner is most likely the offspring of a cross between Traminer and Österreichisch Weiß, and thus rejected the hypothesis that Silvaner was selected from a wild vine on the banks of the river Danube. Traminer was mentioned first in 1349 and is nowadays widely distributed, whereas Österreichisch Weiß is an ancient variety that has no economic importance today. Since the growing of Österreichisch Weiß was rather restricted to the Eastern part of Austria, the descendance of Silvaner also indicates its geographic origin from that region.



A second cross including the cultivar Traminer, Traminer x Roter Veltliner, led to the Austrian variety Rotgipfler. Roter Veltliner was also involved in two further crosses leading to the cultivars Neuburger and Frühroter Veltliner. Neuburger was previously supposed to descend from a natural cross between Pinot blanc and Silvaner. Microsatellite data, however, excluded the parentage of Pinot and suggested a cross between Silvaner and Roter Veltliner. Surprisingly, the same parents gave rise to another cultivar, Frühroter Veltliner, which is also known under the name Malvasier.

Breeding reports of Jubiläumsrebe, a white wine variety for the production of dessert wine selected in the 1920's, denoted the cultivar as a cross between the red wine cultivars Blauer Portugieser and Blaufränkisch. However, microsatellite analysis demonstrated that Jubiläumsrebe is actually an offspring of a cross between Grauer Portugieser and Frühroter Veltliner.

Malvasia Fina (Boal Cachudo, Boal da Madeira) X Síria (Roupeiro, Crato Branco)

↓

Boal Ratinho

A search for possible parent-offspring combinations among the microsatellite profiles of grapevines from a Portuguese collection revealed the origin of the cultivar Boal Ratinho, a white vine from the Carcavelos region near Lisbon, as the progeny of a cross between Malvasia Fina and Síria. Both the offspring and the parent cultivars have been grown in Portugal since ancient times. Various synonyms are used for the two parental cultivars, of which Boal Cachudo and Boal da Madeira for Malvasia Fina and Roupeiro and Crato Branco for Síria have been confirmed in the same microsatellite study (Lopes *et al.*, 1999).

In a survey of grapevines known as Petite Syrah in California, it was shown that most of the accessions grown under this name are identical to the cultivar Durif (Meredith *et al.*, 1999). Durif is morphologically very similar to the cultivar Peloursin, and is described as either a seedling or a selection of Peloursin produced in 1880 in France. Microsatellite studies at 25 loci confirmed the former possibility, and identified the cultivar Syrah as a likely parent of Durif.

Peloursin X Syrah

↓

Durif

6. GENETIC STUDIES OF THE EUROPEAN *VITIS VINIFERA* GERMPLASM

Viticulture has a longstanding tradition in Southern and Central Europe. As early as

2300 BC, the Neolithic populations of the Minoan and Cycladic cultures adopted viticultural practices from their Eastern neighbours (Marangou, 1991), who domesticated grapevine and elaborated viticulture and wine techniques as soon as 6000 BC (McGovern *et al.*, 1995; 1996). From 800 BC, Greeks and Phoenicians spread viticultural practices further west in the course of their colonisation of the Mediterranean basin. Likewise, it is widely supported by ancient texts that the Romans carried viticulture northwards to their colonies in the now French, German and Austrian regions. But it is not unlikely that a less elaborate viticulture could have existed in these regions before the Romans and the Greeks exported their know-how there. Wild vines were abundant in Southern and Central Europe at this time and may have served for the selection and domestication of locally cultivated grapevine varieties. Besides, the spread of viticultural knowledge may also have involved the introduction of cultivated varieties to new vine growing areas, and later on, exchange of grapevine cultivars between regions may have been substantial. Nowadays, regionally typical sets of grapevine cultivars are established in the vine growing regions of Europe, while at the same time, some particularly successful cultivars such as Cabernet Sauvignon, Chardonnay, Pinot Noir, Rheinriesling, Sauvignon Blanc and Syrah have been included in the programs of virtually all major wine producing areas.

However, it is hard to say how many of the cultivars typical for a certain region are indeed unique to that region, and not synonyms to varieties cultivated elsewhere, and to what extent the diverse local cultivar sets represent genetically distinct gene pools. A promising approach to answer these questions leads via the establishment and comparison of standardised genetic profiles derived from regional cultivar samples. Using microsatellite markers, identical profiles provide strong support to the identification of synonyms within and among regions. The genetic variability within a gene pool is assessed from the allelic constitution of cultivar samples and set in relation to the values derived from other samples. Furthermore, the genetic differentiation of local grapevine gene pools from other cultivars estimates the status of these gene pools as distinct genetic units.

A comparative study of grapevine cultivars typical for European vine growing regions ranging from Greece to Portugal (Sefc *et al.*, 2000) showed a uniformly high genetic variability in all of the investigated grapevine gene pools. The high number of heterozygous individuals within the gene pools exceeded the expected degree of heterozygosity based on a random combination of alleles. Prior to domestication, vine plants were dioecious and attained a high level of heterozygosity by outbreeding. As a side effect, deleterious recessive traits accumulated in the genome (Olmo, 1976), and a certain level of heterozygosity became a vital condition for the plants. Later on, the selection of successful plants in the course of domestication further favoured heterozygous plants, and the observed excess of heterozygosity is probably a consequence of both natural and human selection against homozygosity in grape plants.

The question, whether cultivars from the different regions constitute genetically distinct gene pools was approached by comparing the microsatellite allele frequencies derived from the diverse grapevine samples. Genetic differentiation was detected between

all samples, even in the case of grapevine cultivars from adjacent areas like Croatia and North Italy. According to these findings, the various regions of grapevine cultivation each harbour independent sources of genetic variation and therefore constitute valuable resources of genetic traits for grapevine breeders. The maintenance of local varieties should be a primary objective in order to prevent genetic erosion in grapevine.

7. CHLOROPLAST SSR MARKERS

Five universal microsatellites primers developed recently (Weising and Gardner, 1999) were tested in 77 grapevine cultivars from Greece and other geographic origins (Lefort *et al.*, 2000a; 2000b). All five SSR markers amplified and gave reproducible results. The 5 cp SSR loci yielded a total of 13 alleles whose combinations yielded 17 chloroplast haplotypes. Allele sizes at each locus were found in the range of previously reported sizes for 27 angiosperm species (Weising and Gardner, 1999). Four out of the 5 loci were polymorphic in the grapevines cultivars examined, and gene diversity was high with 17 haplotypes detected in 77 grapevine cultivars.

The maternal inheritance of the chloroplast genome, which is typical for angiosperm plants, is supported by the cp-microsatellite analysis of the documented cross between cvs. Bicane as the female and Muscat de Hambourg as the male parent, which gave rise to the cv. Italia (Ambrosi, 1999). The origin of the French cultivar Cabernet Sauvignon was established, with the use of nuclear SSRs, as being Cabernet franc and Sauvignon blanc (Bowers and Meredith, 1997). Genotyping with cp SSR enabled the precise direction of this supposedly spontaneous cross to be determined: since Cabernet Sauvignon and Sauvignon Blanc shared the same haplotype, Sauvignon blanc should then be the chloroplast donor, and, as such, the female partner in the cross.

Globally no clear genetic structure was made visible by cpSSRs profiling of 77 Greek grapevine cultivars. This can be seen as a consequence of extensive exchanges of cultivars between Greek regions.

8. USE OF SSR MARKERS FOR GENETIC MAPPING OF *VITIS VINIFERA* IN COMBINATION WITH OTHER MARKERS

The availability of new markers for grapevine led naturally to their use in mapping in addition to other markers already placed on the genome: isoenzymes, RFLPs, CAPS, AFLPs and RAPDs. The development of a genetic linkage map is the first step required for the detection of factors controlling the expression of economically important traits, which should hopefully make positional gene cloning and thus homologous recombination possible in the next future. Much of the effort in constructing such a map is directed toward identifying useful, polymorphic markers, which may be used in numerous other pedigrees and related taxa.

Microsatellite primers developed for *Vitis vinifera* proved to amplify homologous PCR products in many of the North American *Vitis* species (Thomas and Scott, 1993; Sefc *et al.*,

1998a) and in Asian *Vitis* species (Grando *et al.*, 1999). These findings have opened the possibility of using SSR markers for the combination of genetic maps from different *Vitis* species. Grando *et al.* (2000b, 2000c) constructed a genetic linkage map based on microsatellite and AFLP markers in an F1 population from a cross between *Vitis vinifera* cv. Moscato bianco x *Vitis riparia*, that segregates for downy mildew resistance. Individual linkage maps for each parent were generated following the double pseudo-testcross strategy and parental linkage groups were joined by the SSR loci and AFLPs segregating 3:1. Approximately 800 AFLPs and 40 microsatellites were assembled for both maps.The markers cover a total distance of 1600 cM and reflect the 19 linkage groups, which are expected for grapevine based on the number of 38 chromosomes in the diploid genome (Grando *et al.*, 2000b; 2000c).

Very recently, another team was able to place a gene controlling sex in grapevines on a genetic map (Dalbo *et al.*, 2000). The maps for the cultivars Horizon ('Seyval' x "Schuyler") and Illinois (*V. cinerea* B9 x *Vitis* rupestris B38), built with RAPDs, CAPS, AFLPs and SSRs, covered 1199 cM and ten homologous linkage groups were identified using 16 SSRs and 2 CAPS. A single locus controlling sex was mapped close to a SSR marker.

9. COMPUTER PROGRAMS FOR MICROSATELLITE DATA ANALYSIS

9.1. Introduction

The information gathered in the course of the establishment of microsatellite profiles from grapevine cultivars can be evaluated according to different perspectives:

a. Management of germplasm collections:
- Identification of cultivars,
- Identification of synonyms, and
- Reconstruction of pedigrees.

b. Evaluation of microsatellite markers:
- Level of polymorphism,
- Allele frequencies, and
- Frequency of null alleles.

c. Characterisation of grapevine gene pools:
- Genetic variability,
- Allelic and genotypic composition, and
- Differentiation among gene pools.

d. Cluster analysis:
- Establishment of similarity or distance measures, and
- Construction of phenograms.

Various computer programs support the analysis of microsatellite data. In the following, some of the programs offering applications for cultivar analysis will be briefly in-

troduced. The programs are available for free from the Internet. The respective user guides should be consulted for detailed information.

9.2. Identity 1.0

IDENTITY 1.0 (Wagner and Sefc, 1999; http://www.boku.ac.at/zag/identity.htm) was designed for the analysis of microsatellite data gathered in the course of a study of grapevine cultivars.

9.2.1. Management of germplasm collections

The program searches the microsatellite profiles for identical genotypes, suggesting these as synonymous cultivars, and for groups of three cultivars whose allelic composition is consistent with their being parents and offspring. Likelihood statistics offer support for the proposed parent-offspring groups and are calculated as described in Bowers and Meredith (1997).

9.2.2. Evaluation of microsatellite markers

The program provides locus by locus calculation of:

- Number of alleles,
- Allele frequencies with their standard deviations and upper 95% confidence limits,
- Expected (Nei, 1973) and observed heterozygosity values,
- Probability of identity (Paetkau *et al.*, 1995),
- Frequency of null alleles (Brookfield, 1996),
- Paternity exclusion probability (Weir, 1996), and as well as overall Probability of Identity and Paternity Exclusion Probability.

An interesting additive feature is a conversion tool for formatting data in the input format required by MICROSAT (see below).

9.3. Popgene

POPGENE (Yeh and Boyle, 1997; http://www.ualberta.ca/~fyeh/) is a comprehensive program for the analysis of haploid and diploid data from various marker types.

Applications relevant to the microsatellite analysis of grapevine cultivars:

9.3.1. Evaluation of microsatellite markers

The program offers the calculation of:

- Allele number,
- Effective allele number (Hartl and Clark, 1989),
- Allele frequency,
- Genotypic frequency,
- Percentage of polymorphic loci,

- Shannon's information index (Shannon, 1949), and
- Expected (Nei, 1973; 1978) and observed heterozygosity/homozygosity.

9.3.2. Characterisation of grapevine gene pools

- Hardy Weinberg Tests,
- Gene diversity (Nei, 1973),
- Fixation Index (Wright, 1978), and
- F-Statistic (Hartl and Clark, 1989; Weir and Cockenham, 1984).

9.3.3. Cluster analysis

- Genetic distances: Nei's (1972) genetic distance and Nei's (1978) unbiased genetic distance, and
- UPGMA dendrogram.

9.4. Other computer programs

9.4.1. Other programs for cluster analysis

MICROSAT (Minch, 1997; http://lotka.stanford.edu/microsat.html) is a computer program designed for the calculation of various genetic distance measures between individuals or groups of individuals from microsatellite data.

The distance matrices created by MICROSAT can be converted into dendrograms using the programs Neighbor (for UPGMA and Neighbor Joining algorithms), Kitsch or Fitch, which are included in the PHYLIP software package (Felsenstein, 1989, http://evolution.genetics.washington.edu/phylip.html). Dendrograms can be visualised using the programs Drawtree from the PHYLIP package or TREEVIEW (Page, 1996; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

9.4.2. Other programs for the characterisation of grapevine gene pools

GENEPOP (Raymond and Rousset, 1995; ftp://ftp.cefe.cnrs-mop.fr/genepop/) is a population genetics program providing tests for Hardy Weinberg equilibrium, linkage disequilibrium between loci and population differentiation. Similarly, the program FSTAT (Goudet, 1995; http://www.unil.ch/izea/softwares/fstat.html) estimates population-genetic characteristics like population differentiation, gene diversities and deviations from Hardy Weinberg equilibrium.

GENETIX (Belkhir *et al.*, 1996-1998) is a software providing a choice of tests commonly used in population genetics including the Mantel test and genetic distances.

10. GENETIC DATABASES OF SSR PROFILES

A database of grapevine genetic identity profiles at 6 nuclear microsatellite loci was reported in 1994 for more than 200 cultivars, with the initial aim to offer it to the public research community (Thomas *et al.*, 1994). Not long after the publication, commercial

interest in using the database as the basis of a commercial grapevine DNA identification service at the Australian Wine Research Institute (AWRI) resulted in restricted access (Scott *et al.*, 1996). The AWRI service has operated since 1996 and has received from customers a diverse range of samples including wood tissue, berry clusters, leaf tissue and canes for a multitude of purposes. Information contained in the database includes detailed information about the plant used as the source of the cultivar DNA profile with all plants sourced from national or state germplasm collections. A similar methodology inspired a second database service recently released under the appellation of "the Greek *Vitis* Database" (Lefort and Roubelakis-Angelakis, 2000a; 2000b). This kind of database is aimed not only to make widely available the genetic information about Greek cultivars but also and especially to facilitate germplasm management of Greek grapevine resources in Greece, disseminated in several collections. This database service is in fact a cluster of multimedia databases, offering information, genetic profiles at nine nuclear microsatellite loci for more than 300 cultivars, mainly of Greek origin, as well as genetic profiles obtained with 5 chloroplast microsatellite loci and an image database of 240 cultivars. Allele size calling has been brought in agreement with other laboratories according to the results published in a study of European grapevines (Sefc *et al.*, 2000) in order to make results useable and comparable between different regions of the world. Other database services developed on the same model will soon offer similar information about grapevine germplasm in Albania, Macedonia, Bulgaria and some other countries of the Balkans and Caucasus. As foreseen by Thomas *et al.* (1994), international collaboration should result in the near future in an international database of SSR profiles of cultivars from all collections worldwide, supporting the management of ampelographic collections as well as a microsatellite-based certification system.

11. ON THE WAY TO COMMERCIAL CERTIFICATION OF CULTIVARS

DNA profiling will become increasingly important for the registration and protection of new grapevine cultivars. New varieties can be protected by a patent or by Plant Breeders Rights (PBR). Patents can be broad and cover not only specific cultivars but plants in general while PBR is specific for plant cultivars only. The International Union for the Protection of New Varieties of Plants (UPOV) recommends crop specific phenotypic descriptors for PBR submissions. This is in the process of review as there is a current UPOV working group investigating the use of DNA profiling for cultivar identification and PBR protection. Four new Australian wine grape cultivars (Cienna, Vermilion, Rubienne and Tyrian) were recently granted protection under PBR and part of the PBR submission included DNA microsatellite profiles. Some customers of the commercial grapevine DNA identification service have used the service specifically for protecting intellectual property.

However, DNA profiling mainly has the potential to become a quality control tool for nurseries and wineries. The increasing strict regulations imposed on wines and the mar-

ket push for high quality wine may eventually lead to wineries establishing as part of their audit trail from vineyard to bottle the inclusion of DNA profiling to ensure that the cultivar name(s) on the label of the bottle is as accurate as possible. DNA profiles can be obtained from DNA extracted from free run juice after crushing (Thomas, unpublished). Under experimental conditions cultivar mixes down to 10% have been detected (Thomas, unpublished). However, isoenzyme polymorphisms, recently assessed in total grape must (Moreno-Arribas *et al.*, 1999), could still be locally competitive against microsatellites, when the heterogeneity of the cultivars in the vineyard is reduced. Some customers of the commercial grapevine DNA identification service have used it specifically for determining the cultivar in a vineyard and for the identification of grapes delivered to the winery. New regulations passed in the last 20 years in some regions such as the European Union strictly define the cultivars, which can be used for wine, raisins and table grapes in each member country. While, in the case of raisins and table grapes, it is more a matter of consumer information in order to ensure accurate labelling of products, in the case of wine, and especially wines of appellation, intervening commercial interests are so important that molecular certification of grafted cultivars (where respective identities of the rootstock or of the cultivar can be asserted with certitude) will be an important tool for enforcing these regulations and thus better protecting the viticulturists from mistakes, or fraudulent commercial behaviour, which can be of severe consequence. Such a technological audit trail could thus be applied to all sectors of the grapevine industry from the nursery and the vineyard, to the wineries and packaging and distribution of table grapes and raisins.

12. CONCLUSION AND PROSPECTS FOR THE FUTURE

Microsatellite markers have proved extremely useful for accurate cultivar identification, uncovering synonyms, determining genetic relatedness and for genetic mapping. Most likely the application of such DNA markers will spread and contribute to answer many questions in grapevine genetics. What is not certain however, is whether microsatellite markers will remain the preferred marker type of the future given the rapid pace of DNA marker technology. But it is obvious from this review that no other molecular marker or technology, including ampelography, has provided so much useful information in such a short period of time for grapevine genetics.

Other markers such as AFLPs (amplified fragment length polymorphisms; Sensi *et al.*, 1996; Cervera *et al.*, 1998; Goto-Yamamoto, 2000; Martinez-Zapater *et al.*, 2000) or ISSR (inter-simple sequence repeats; Moreno *et al.*, 1998) have already been characterised in grapevine and their potential is under scrutiny. They could quickly find application where microsatellites are not sensitive enough, that is to say predominantly in identifying closely related cultivars, supposedly isogenic lines or somatic mutants (Scott *et al.*, 2000a). Molecular identification of genotypes would certainly rely in the future.

It is also envisaged that the use of microsatellite markers for the identification of cultivars or rootstock material, during propagation in nurseries and at different key-points

of the trade system, as well as certification of grape juice before vinification will almost certainly soon be routinely used by the wine industry.

The research reported in this review also clearly demonstrates the advantages of international collaborations for grapevine molecular research. It is expected that international collaborations will continue to play a major role in the future as the more resources applied to a problem the faster the progress. This is especially true for genetic mapping studies, which aim to find markers closely linked to agronomically useful genes for marker-assisted breeding and for the isolation of these genes by map-based cloning strategies. This will certainly be the main fundamental application of microsatellites, once identification of genetic resources have been exhaustively accomplished and most of the information made available through a network of web-backed genetic databases specific to national germplasm collections. The next few years are expected to be exciting and rewarding as these international efforts continue to yield new and unexpected results.

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