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A MICROSATELLITE-BASED SYSTEM FOR THE IDENTIFICATION AND LEGAL PROTECTION OF GRAPEVINE VARIETIES

Paper prepared by experts from Spain

A MICROSATELLITE-BASED SYSTEM FOR THE IDENTIFICATION AND LEGAL PROTECTION OF GRAPEVINE VARIETIES

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Summary

More than 6,000 plants have been analyzed to evaluate a system based on 9 microsatellites that could be useful for most of the issues related to the genotyping of grapevine varieties: variety identification; DUS testing; and identification of Essentially Derived Varieties (EDVs). The 9 microsatellite markers selected are: VVS2, VVMD5, VVMD27, VVMD28, ssrVrZAG29, ssrVrZAG62, ssrVrZAG67, ssrVrZAG83 and ssrVrZAG112. The selection of these markers was based on different criteria: availability (public), map position (genetically independent), polymorphism (high), allele size range (that allows multiplexing), and 'quality' (ease of amplification, absence of known null alleles, absence of alleles differing only in 1 bp). A genotyping system was optimized, including the design of a multiplex PCR with the 9 markers, and capillary electrophoresis and fluorescence analysis in an automatic sequencer. More than 1,300 accessions (2,600 plants) of *Vitis vinifera* L. from the collection of grapevine varieties at the "El Encín" state have been analyzed with this system. Until now, the conclusion reached for all those accessions studied that presented the same genotype at the 9 microsatellite loci is that they arise from the same embryo. Once the genotype table has been built up, any plant belonging to any of these varieties can be easily and unequivocally identified, with the exception of the sports and EDVs, which require a morphological description. Regarding the technical exams for legal protection of new varieties (DUS test), the lower number of different alleles between different varieties, and the higher number of different alleles within a variety (mutations) have been used to determine a minimum distance that would allow the establishing of Distinctness. Uniformity and Stability have been evaluated by studying about 4,000 plants of 19 different varieties.

Introduction

Grapevine is one of the oldest cultures in the world. Their plants are woody, and asexually multiplied through cuttings. There are a lot of varieties in the world (between 5,000 and 10,000) and many of them have been cultured for several centuries. Most are local varieties, and there are numerous synonyms (one variety having different names) and homonyms (different varieties having the same name) within and between countries.

There are two clearly distinguishable markets for grapevine: wine and table. The first one is much more important economically, and stable, regarding the varieties used, because in many cases the wine producers of a given place need to use certain varieties to get a "quality" label. So, breeding efforts are mainly focused on clone selection. The table market is much more dynamic and most of the varieties being cultivated now are different from those cultured 30 years ago. Crossbreeding is much more active for table grapes, and new interesting varieties are quickly spread to other producer countries. As a consequence, the issue of

variety legal protection (similar to a patent or to intellectual property) in grapevines affects mainly these table varieties.

In the grapevine species, a new variety can come from a sexual cross, where an embryo is produced, or from an established variety through a somatic mutation and asexual propagation. In this case, the new variety is called a 'sport' (which may be an Essentially Derived Variety, EDV, within a legal scope). This, in addition to the already mentioned existence of synonyms and homonyms, has made of the identification of grapevine varieties a difficult issue over time. Competent organizations, particularly the *Organisation internationale de la vigne et du vin* (OIV) and UPOV, have been working separately to improve the system, which is based mainly on morphological characteristics. At the thirty-seventh session of the Technical Working Party for Fruit Crops (TWF, UPOV), held in Salvador, Brazil, from August 21 to 25, 2006, it was agreed that a meeting for harmonization of the UPOV Test Guidelines and the OIV Descriptor concerning Grapevine would be agreed. That meeting was scheduled to be held on November 7 and 8 in Germany. OIV will present 6 descriptors that correspond to 6 microsatellites studied during the GENRES project (This et al. 2004). This reflects the current situation today: many grapevine institutes use microsatellite markers to identify their varieties, in addition to, or instead of, the morphological descriptions. Their incorporation into the official system for the legal protection of varieties must be seriously considered in the near future, but it is first necessary to have a thorough study to evaluate each microsatellite individually.

The purpose of this paper is to show the evaluation of 9 microsatellites through the study of a very large number of plants, and how the conclusions reached allow the establishment of rules that could be useful for different issues related to grapevine varieties: from the identification of a variety to the legal DUS test, and the identification of sports and putative Essentially Derived Varieties.

System of 9 microsatellites

Microsatellite markers were first described in grapevine in 1993 (Thomas and Scott 1993) and have been broadly used all over the world to characterize many different varieties. Many groups have developed their own markers, but the main international attempt to harmonize a microsatellite-based system for the identification of grapevine varieties was taken within the frame of the GENRES project, in Europe, and involved 10 different laboratories, including ours (This et al. 2004). Unfortunately, at the beginning of that project there was no information on map position of the microsatellites, and among the 6 markers chosen, two pairs were genetically linked. For that reason, and because they are not able to discriminate all the different varieties (not sports) tested (Martín et al. 2003), this set of 6 microsatellite is not an optimal selection. We selected a new set, based on the previously used microsatellites, and started by raising the number of markers up to 9. The criteria taken into account to make the new selection were: Availability (public), map position (genetically independent), polymorphism (high), size of alleles (that allow multiplexing), and 'quality' (ease of amplification, absence of known null alleles, absence of alleles differing only in 1 bp).

Finally, the 9 microsatellite markers chosen were: VVS2 (Thomas and Scott 1993), VVMD5, VVMD27, VVMD28 (Bowers et al. 1996; Bowers et al. 1999), ssrVrZAG29, ssrVrZAG62, ssrVrZAG67, ssrVrZAG83 and ssrVrZAG112 (Sefc et al. 1999). A system was optimized, including the design of a multiplex PCR with the 9 markers, and capillary

electrophoresis and fluorescence analysis in an automatic sequencer. Simplex PCRs were done when necessary.

About 6,000 plants have been studied using this system, mainly within two different projects, one to characterize the collection of grapevine varieties of "El Encín", in Madrid, and other to study the uniformity and stability of these markers using 19 varieties.

Study of a grapevine collection

The set of 9 markers is being used to characterize more than 1,300 accessions (2 plants per accession) of *Vitis vinifera* L. from the collection of grapevine varieties of "El Encín", Madrid, including the key Spanish wine varieties (Ibáñez et al. 2003). Until now, almost 1,000 accessions have been completed with all the 9 microsatellites, and many have also been morphologically studied.

This set of accessions was compared pair-wise and a high number of full matches was found. All those accessions that presented the same genotype for the 9 microsatellite loci were further studied to determine if they were morphologically identical and/or well-known synonymies. In cases where that was true, one representative accession was kept within the set and the others were discarded. Those that could not be discarded in this way were analyzed with 20 microsatellites (16 different to the 9 previously used). In all these cases, the accessions that fully matched using the 9 microsatellites also matched with the 16 additional microsatellites. Given that it is extremely improbable to get the same genotype in 25 microsatellites by random, we concluded that they, in fact, arose from the same original embryo.

The next step was to compare full-matches-but-1-allele. Only one case was detected: Chasselas blanc and Chasselas Gros Coulard differed only in one allele in microsatellite ssrVrZAG83 (possible mutation to a null allele). The full match with the additional 16 microsatellites confirmed that the later was a sport of the former. Other similar cases have been described in the literature: Black Corinth (Ibáñez et al. 2000), Pinot (Regner et al. 2000), White Riesling (Regner et al. 2000), Greco di Tufo, Muscat d'Alsace (Crespan 2004).

The third step was to compare full-matches-but-2-alleles. Only two cases were detected: Alphonso Lavallée with Princeps, and Pizzutello Moscato Biondo with Galletta rosa. In both cases they differed in two alleles of two different microsatellites. The amplification of 16 additional microsatellites revealed that these varieties were really distinct.

Finally 468 accessions were considered as different genotypes, including the four cited in the previous paragraph.

On the other hand, although we have not studied it so systematically, we have never found a case where two varieties considered different using microsatellites showed identity through morphology markers.

Study of Uniformity and Stability

Microsatellites are highly polymorphic sequences, because of their higher mutation rate. This causes concern about their use for certain applications, especially those related with legal issues. For that reason, a considerable effort is being made in IMIDRA to evaluate the uniformity and stability of this set of microsatellites. The aim of a project conducted between

2002 and 2005 was to study with the 9 microsatellites about 4,000 plants of 19 grapevine varieties: Cardinal, Crimson seedless, Flame seedless, Italia, Napoleon, Ohanes, Red Globe, Sugraone, Thompson seedless (table), Airen, Cabernet Sauvignon, Chardonnay, Garnacha, Merlot, Monastrell, Muscat d'Alexandria, Palomino Fino, Sauvignon Blanc and Tempranillo (wine). The varieties were carefully selected, considering different uses of the grapes (wine and table), color of the skin of the berry (recommended by the UPOV for grouping grapevine varieties), the presence or absence of seeds (very important trait in new varieties), temporal origin (ancient and more recent) and geographical origin (from different continents).

To evaluate uniformity, we studied the set of 9 microsatellites in 50 plants of 3 different plantations of each of the 19 varieties (2,850 plants). The plant material used in all the cases was young leaves, and came mainly from Spain, but some also came from South Africa (Cabernet sauvignon, Chardonnay, Sugraone, and Thompson seedless), and Chile (Cabernet sauvignon).

Because grapevine is a woody plant, a controlled study of stability, through multiplying plants of all the varieties, would be difficult and unpractical, and would allow studying only one, or a very limited number of cycles of multiplication. For this reason, and considering that the same varieties are multiplied in different places, producing plants that are certainly in very different cycles of multiplication, we decided to study stability looking at plants of plantations from different origins, and when possible, of different age.

To evaluate stability, we studied 9 microsatellites in 5 plants of 10 different plantations (including the 3 studied for uniformity) of each of the 19 varieties (950 plants). The plant material used in all the cases was young leaves, and came mainly from Spain, but was also sent from Chile, France, Germany, Hungary, South Africa, and USA.

In fact, the uniformity and stability are being tested in both studies: if a change in one plant was found in a sample of 5 or of 50 plants, it could affect the conclusion on uniformity, while if we found a change in all the plants of a sample of 5 or of 50 plants it could affect the conclusion on stability.

We have not evaluated the plants morphologically, in such a way as to establish any relationship between microsatellite and morphological uniformity or stability.

Summarizing, for each allele of each microsatellite of each variety we have studied about 185 individual values. The analyses were carried out in an automatic sequencer, using software (GeneScan®) that calculates the sizes of the amplified DNA fragments using an internal standard and a lineal regression. For that, there is an experimental variation for the values obtained for a given allele.

In the study, we have not detected differences that could not be explained as technical variations, with the exception of several putative chimeras. This phenomena has been detected in Merlot (VVMD27), and Cardinal (VVMD5), and had been previously described in grapevine (Franks et al. 2002; Crespan 2004; Bertsch et al. 2005).

UPOV establishes in the Guidelines for the conduct of tests for distinctness, uniformity and stability in grapevine (*Vitis* L.) (document TG/50/8): ‘For the assessment of uniformity […] In the case of a sample size of 10 plants, the maximum number of off-types allowed would be 1’. Taking this into consideration, the conclusion of the study, at least for these 19 varieties, is that the 9 microsatellites are very uniform and stable.

Although we have not evaluated the plants morphologically, the results show that the use of this system of microsatellite analysis would not require any additional effort by the breeder to keep uniformity and stability in their new varieties.

Identification of varieties

The studies done using the system of 9 microsatellites allow the establishment of some general rules for their use as an identification tool for grapevine varieties. In summary, if we analyze these 9 microsatellites from two different plants, we will find three alternative types of results:

A. Full match: we will conclude beyond doubt that the two plants are of the same variety, or clones of the same variety, or synonyms, or one is a sport or EDV of the other. The final decision will rely on other characteristics (e.g. morphological, agronomical, etc.), but the advantage compared to the present situation is clear: only two or a few varieties have to be compared with the morphological descriptors to reach a conclusion.
B. Two or more different alleles: the plants belong to different varieties.
C. One different allele: more microsatellites should be studied. If there is a full match in the new microsatellites, the conclusion should be like the first case. Otherwise, it would be the second case.

Legal Identification of varieties

Several times the system has been used for the identification of varieties for existing legal implications. These included the defense of a protected variety for putative infringements of plant breeders' rights, the variety identification of plants sold by a nursery, the determination if a plant belonged to a list of authorized varieties or to the list of commercial varieties.

The general procedure we established is the analysis of the sample with the set of 9 microsatellites, and comparison with a dedicated database. When a match was found between the sample and a given variety, a forensic procedure for establishing the probability of such matches was applied. A reference collection was chosen, and matching probabilities of grape microsatellite genotypes were calculated under the assumption of independent breeding programs. Even eliminating some microsatellites from the analysis, the matching probabilities were so low that the forensic procedure using microsatellites seemed to provide a sound basis for legal identification, including assessing infringements of plant breeders' rights, even with a less valuable set of microsatellites (Ibáñez and Eeuwijk 2003). As an example, using the 9 microsatellite described here, and a conservative, small reference collection, the likelihood ratio for a random match with a given protected variety is 1 in 156,000,000.

Distinctness, Uniformity, and Stability

A variety may be considered to be clearly distinguishable if the difference in characteristics is: (a) consistent, and (b) clear. Regarding the microsatellite system developed, the consistency should not be a problem at all: examining 2-3 plants, it can be seen if the difference is consistent or a technical artifact. 'The clear distinctness, in general known as minimum distance, that should exist between two plant varieties so that they are considered distinct according to the UPOV Convention is a difficult question' (ISF Position Papers

2003). The key point when analyzing distinction is to establish the minimum distance, and for that, we have to determine:

(a) the lowest number of different alleles between different varieties
(b) the highest number of different alleles within a variety.

If there is a clear border between those two numbers, we will be able to establish an acceptable minimum distance, using a fixed criterion (Roberts 2004).

From the reported study of a large grapevine collection, both questions can be answered. With respect to the point a), using a large collection of almost 500 genotypes, two cases were found where the number of different alleles was 2. On the other hand, within a variety, and considering the 9 microsatellites used here and 1,000 accessions, the higher number of different alleles was 1.

In conclusion, considering the two extreme values obtained (2 and 1 alleles), a *minimum distance of 2 alleles* should be considered as an acceptable fixed criterion to establish distinction. This is supported by Crespan, who estimated a general mutation rate per plant and microsatellite in grapevine of 8×10^{-5} (Crespan 2004). So, it would be very improbable to find in the same plant two independent mutations producing two allele differences. From a practical point of view, finding 1 or 2 alleles of difference between two grapevine samples is very rare, and is worthy to use additional microsatellites to confirm or reject the distinction.

Regarding the microsatellite uniformity and stability, the results showed that the 9 used here are very uniform and stable, making it unnecessary for the breeder to make any additional effort to keep uniformity and stability in the process of developing new varieties. In case chimeras were found, the microsatellite should be discarded for that variety in question, and replaced by another microsatellite, if the remain were not enough (less than 3 different alleles).

Conclusions

About 6,000 plants have been analyzed for different purposes, with a system of 9 microsatellites: VVS2, VVMD5, VVMD27, VVMD28, ssrVrZAG29, ssrVrZAG62, ssrVrZAG67, ssrVrZAG83 and ssrVrZAG112. The system has proved to be suitable for variety identification and legal protection (DUS test), and the following main rules can be indicated: for identification, a complete match means identity (or sport); for Legal Protection: minimum distance of 2 alleles and no additional effort for breeders (uniform and stable).

For the near future, the question remaining would be to include the set of microsatellites as characterization descriptors by the competent organizations. The system is being used at the present time to characterize the whole reference collection of the Spanish Plant Variety Office.

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