IDENTIFICATION OF GRAPEVINE CULTIVARS USING MICROSATTELITE MARKERS

IDENTIFICAREA CULTIVARELOR DE VIȚĂ DE VIE CU AJUTORUL MARKERILOR MOLECULARI

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Abstract. *Molecular markers provide an alternative means of distinguishing cultivars and could, also, be used for investigating taxonomically relationships between species, as well as for genome mapping. Here we describe how this method is applied to distinguish between Vitis vinifera cultivars using as selection criteria the allele polymorphism for the UCH 40 locus. The applied procedure comprising DNA extraction, PCR amplification, electrophoresis and sizing of PCR products is extensively described.*

Rezumat. *Markerii moleculari sunt larg folositit astazi in vederea identificarii unor cultivare, clasificarii taxonomice a unor specii cat si alcatuirii de harti genetice. In aceasta lucrare este prezentat modul in care pot fi identificate unele cultivare de vita de vie folosind drept criteriu de selectie polimorfismul alelelor la locusul UCH 40. Pentru a analiza secventele de ADN repetitiv (microsatelitii) la un anumit locus sunt necesare extractia ADN-ului, amplificares fragmentelor respective de ADN (PCR), electroforeza si analiza fragmentelor amplificate. Aceste etape de lucru cat si rezultatele obtinute sunt descrise in detaliu in prezenta lucrare.*

INTRODUCTION

The increasing international trade of grapevine and rootstock plant material as well as of wine necessitates a reliable identification of genotypes. Molecular markers provide an alternative means of distinguishing grapevine cultivars that is independent of the phenotypic characters used in ampelography and could, also, be used for investigating taxonomically relationships between *Vitis* species, as well as for genome mapping at *Vitis vinifera*. They can be easily scored and used for cultivar identification, parentage determination, population genetics, physical and genetic mapping. Among these markers, microsatellites or simple sequence repeats (SSRs) are recommended for grapevine genotyping.

Microsatellite are highly mutable loci which may be present at many sites in an eukariote genome, consisting of stretches of tandemly repeated nucleotide motifs which can be as short as 4, 3, 2 and even 1 nucleotide. The sequence knowledge of these regions is used for designing specific amplifying primers, which then define a sequence – tagged microsatellite site (STMS)(1). There are several important advantages of STMSs:

-they are (usually) a single locus, which because of the high mutation rate is often multiallelic;
-they are codominant markers and can be detected by a PCR assay, using a pair of flanking unique oligonucleotides as primers and so, allele frequencies can be determined directly;
-they show extensive polymorphism due to site – specific length variation as a consequence of the occurrence of different number of repeat units;
-they are very robust tools that can be exchanged between laboratories and their data are highly informative (1).

The usefulness of these markers has been assessed in samples of grapevine varieties cultivated in different regions of Europe, Australia, California etc. (2). However, due to the predominance of certain alleles or the occurrence of null alleles in some populations, the information content of a given marker may vary between the cultivars from these regions, but the consistency of the results obtained in many laboratories supports the establishment of a common electronic database for identification of grapevine cultivars (1-2). In this study set of grapevine cultivars was genotyped at the UCH 40 locus in order to identify them.

MATERIAL AND METHODS

DNA extraction

Plant material used for nucleic acid extraction was obtained from a collection of *Vitis vinifera* cultivars. Leaf samples (100 mg) from individual plants were frozen in liquid nitrogen, ground to a fine powder and resuspended in 1-ml DNA extraction solution and 10 ml 2-mercaptoethanol. After 15 min incubation in 65^{o}C (water bath), 0,5 ml volume of chloroform / isoamyl alcohol (24: 1) was added and mixed (no by vortex) and the phases were separated by centrifugation at 14000 rpm for 1 min. 0,8 ml of supernatant was collected and an equal volume of isopropanol was added to precipitate the DNA. DNA precipitation was improved by ice incubation for 15 min. After 1min centrifugation at 14000 rpm, the supernatant was removed and 1 ml ethanol 70 % was added to the pellet. Ethanol was removed after centrifugation at 14000 rpm for 1 min and the pellet was resuspended in 100 µl TE.

Determining DNA concentration

DNA concentration was estimated in each sample, spectrophotometrically, by measuring A_{260}. 5 µl of DNA of each sample was added to 995 µl of distillated water and the optical density of the diluted DNA was measured at 260 nm and DNA concentration was calculated with the following formulas:

DNA concentration (µg / µl) = (A_{260}) x OD of 1.0 x 200 / 1000 µl

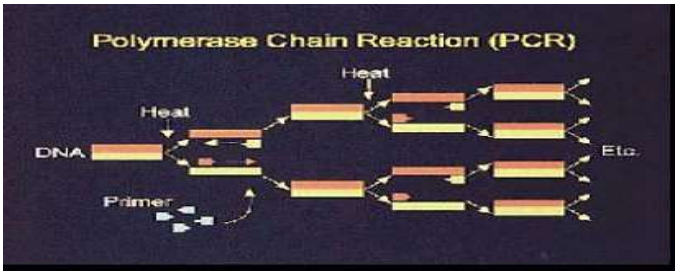

Fig. 1 *Polymerase chain reaction for the amplification of miscrosatellite locus*

PCR amplification of microsatellite locus

1 µl (50 ng) of DNA was added to 19 µl of the PCR reaction mix containing 0,5µl Taq polymerase, 2µl PCR 1xbuffer, 0,6µl $MgCl_2$, 1µl dNTPs, 0,87 µl Forward primer, 0,26 µl Reverse primer and deionized water to reach the final volume of 19 µl. The following thermal cycling protocol was applied for the UCH40 locus amplification: 9^0C for 10 min,62^0C for 30 s,95^0C for 30 s,72^oC for 15 s,29 cycles of 30 s at 62^0C,72^0C for 5 min and immediately terminated at 4^0C (**Fig. 1**).

Electrophoresis and sizing of PCR products

Before loading on sequencing gel, PCR products were checked by electrophoresis through agarose gel (2%, TBE 5x containing 54 g Tris base, 27,5 g boric acid, 20 ml 0,5 M EDTA, pH 8,0) and ethidium bromide staining to verify quality and specificity of amplification. DNA samples were prepared for standard sequencing gel as followed: denaturation for 2 min at 75*C and, immediately, chilling on ice.

Polyacrylamide gel (3, 75 %) was prepared from 6 ml TBE buffer (5x), 12,6 g urea and 4,5 ml of 50% acrylamide solution. The volume was adjusted to 30 ml using deionized water. In this solution, mixed and filtered well, 200 µl of 10% APS and 20 µl TEMED were added and the gel was poured on the plate and let to polymerize for 1 ½ hrs. After polymerization, the gel was mount onto the sequencing apparatus, in TBE buffer tank and prerun for 10 min before loading. DNA samples were loaded and the gel was run for 5 hrs in a LI-COR DNA Sequencer. The Data Collection program was used to control the electrophoresis parameters.The GENEIMAGER software sized microsatellite allele length.

RESULTS AND DISCUSSIONS

The DNA typing procedure using STMS markers detected Mendelian inherited co-dominant alleles at a single locus in the grapevine genome (10). Allele polymorphism is observed as different lengths of the microsatellite when separated on polyacrylamide gels (**Fig. 2**).

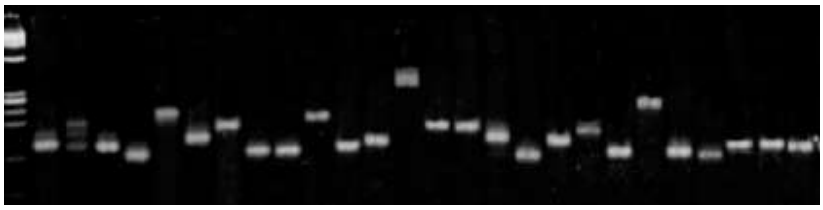

Fig. 2 - *Allele polymorphism for the UCH40 locus in grapevine cultivars*

Typically, PCR amplification yields two bands corresponding to one allele from the maternal ancestor and two bands corresponding to the other paternal ancestor, if the individual is heterozygous at the analyzed locus, one band if it is homozygous. Some rootstocks had no allele for the analyzed locus and were given a null – null genotype. A null allele could occur when DNA representing the locus is present but its amplification by PCR is prevented by DNA mutations (point mutations DNA, deletions or insertions) at or between the priming sites or the DNA representing the locus is not present in the cultivar genome. The microsatellite profile at UCH 40 locus was polymorphic. The

cultivars shown different genotypes and a high level of heterozygosity could be observed (**Fig. 2**).

The highly heterozygosity observed at this locus (**Fig. 2**) is, probably, a consequence of both natural and human selection against homozigosity in grape plants. Prior to domestication, vine plants were dioecious and outbreeding and therefore attained a high level of homozygosity. As a side effect, deleterious recessive traits accumulated in the genome and, in consequence, a certain level of heterozygosity has become a vital condition for the plants. The selection for highly heterozygous plants was intensified in the course of domestication and cultivation of grapevines, when the genotypes were selected, according to their agronomic performance (1-2).

For the analyzed locus, the microsatellite profile of each cultivar is unique and this can be used, in combination with data provided by other loci analysis, for an accurate identification of grapevine cultivars. Microsatellite, as molecular marker, prove to be a useful tool for genome mapping, population and phylogenic studies, parentage analysis, individual identification, cancer diagnostic etc. The methods for microsatellite detection are easy, quickly and feasible for these studies (3).

CONCLUSIONS

1. The microsatellite profile at UCH 40 locus in grape cultivars is polymorphic.
2. The highly heterozygosity observed at UCH 40 locus is, probably, a consequence of both natural and human selection against homozigosity in grape plants.
3. The microsatellite profile of each cultivar at UCH 40 locus is unique and this can be used, in combination with data provided by other loci analysis, for an accurate identification of grapevine cultivars.

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