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CHARACTERIZATION METHODS AND FINGERPRINTING OF AGRONOMICALY IMPORTANT CROP SPECIES

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Continued usage of morphological data to describe agronomically important genotypes indicates that those data retain popularity as descriptors of plant species. However, the need is arising for their more detailed description by laboratory-based biochemical and molecular methods. The standardization of those techniques has been achieved by ISTA and UPOV through DUS testing that is the basis of the system of protection of Plant Breeders Rights. Not only by morphological characterization but also by combining morphological, biochemical and molecular aspects in identification and description of agronomically important genotypes, it is possible to reveal their unique genetic profiles e.g. fingerprints.

In this review we summerised the techniques that proved to be successfuly applied in biochemical and molecular characterisation of agronomicaly important genotypes. Obtaining their unique genetic

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profiles due to application of those methods, it is now possible to precisely characterize them with most certainty and reproducibility.

Key words: ISTA, UPOV, DUS, PBR, SDS-PAGE, RAPD, AFLP, SSR

INTRODUCTION

Seed producers and end-users need to know the identity of their plant species and cultivars. Plant breeders must, therefore, describe the cultivated species and know their origin as well as explain and monitor in detail the lines, cultivars and hybrids, because they are the end-product of a major investment of money, time and breeding work. In order for a newly developed genotype to be placed on national and international lists of newly developed varieties, it must be first fully defined by various kinds of descriptions and tests. One of those is the Distinctness, Uniformity and Stability (DUS) test, which is the basis of the system for the protection of plant breeders rights (PBRs).

This test, which has thus far been based mostly on morphological genotype characterization, requires that the newly developed genotype be different from the existing ones but also that it exhibit uniformity and stability with respect to traits it has been bred for. The breeding activities of describing plant species, varieties and cultivars based on traits exhibiting genetic variability can be used to advance successful germplasm storage and monitor their genetic diversity. In the last decade, the development of biochemistry, genetics and molecular biology has opened a new chapter in the field of describing agronomically important genotypes and providing their much more detailed characterization, not only in terms of how distant their germplasms are from those of the existing ones but also in the sense of monitoring the uniformity and stability of their characteristics relative to each other.

This chapter is known as fingerprinting or profiling, i.e. defining the identity card of an agronomically important genotype at the molecular level.

The subject of this paper was a review of methods used for describing the characteristics of a genotype, the different levels of identification and the possibility of them complementing each other to obtain genotype fingerprints or genetic identity of sorts.

Morphological data

Characters used to determine a genotype's identity card are morphological data, protein-based data and data obtained at the molecular level (DNA data).

In our country, phenotypic genotype evaluation based on morphological characteristics has been the prevalent method of genotype assessment, because it provides unique genotype identification. However, it is necessary to consider the practicality and validity of using only morphological data for genotype identification. The new standards go beyond the mere identification of morphological characteristics per se and require the determination of genetic distances among genotypes, which cannot be achieved by using morphological data

only (SMITH and SMITH, 1992). Firstly, the larger part of morphological variability cannot be measured consistently because of genotype x environmental interactions (G x E) and the effects of a large number of quantitative genes. Secondly, many obvious morphological variabilities must be eliminated because of the fact that most genotypes look similar. Thirdly, genetic control is not known for most morphological characteristics, although most genotypes are known to be visibly similar phenotypically (SMITH and SMITH, 1989; SMITH *et al.*, 1991). It is therefore impossible to determine how complete an example of morphological description the genome is or to what extent similar phenotypes reflect similar genotypes.

Also, an increased number of cultivars that are very close genetically further complicates the already ardous and extremely time-consuming morphological identification. This problem is particularly pronounced with species having a limited level of genetic diversity or when selection is directed towards similarity in morphological characteristics. This occurs in wheat when nontraditional crosses are used that negate previous correlations between morphology and grain quality (LOOKHART and BIETZ, 1990).

Based on the above, it can be concluded that morphological characteristics cannot always be interpreted so that they provide the correct assessment of genetic differences. Morphological data only cannot meet the criteria set by DUS tests.

Protein data

Because of the desire to more clearly define and more reliably describe the existing and newly developed genotypes and to bring their categorization into accord with European standards, laboratories around the world are working increasingly on the application of biochemical (proteins) and molecular (DNA-based) methods.

Today, protein characters are used routinely and are widely utilized as sources of reliable data in the taxonomy and genetics (TANKSLEY and ORTON, 1983) of many agricultural and horticultural species (LEE *et al.*, 1996).

The successful exploitation of proteins in genotype identification is based on the fact that they are direct products of gene transcription and translation. Therefore, they can be regarded as markers of structural genes that code them. The proximity of the processes between protein synthesis and primary genetic information (DNA) also largely reduces or even eliminates any environmental interaction in protein composition. Thus, analyses of protein composition have in fact become analyses of gene expression, while methods for protein composition comparison enable the measuring of genetic variability among individuals and populations (COOKE, 1994). For protein identification, it is necessary to use proteins that exist in multiplied molecular (i.e., polymorphic) form and relatively large amounts, so that they can be extracted easily. For these reasons, seed proteins are extremely useful for identification purposes and are used widely. They include albumins (water-soluble proteins, mostly enzymes), globulins (salt-soluble reserve proteins typical of legume seeds), prolamins (alcohol-soluble reserve proteins typical of cereal seeds), and glutelins (detergent-soluble structural or enzymatic proteins).

The choice of organ to be used for extracting protein components is also an important factor contributing to the success of identification, as confirmed by LEE *et al.* (1996). According to these authors, seeds are a suitable source of tissue, because they are in the final stage of development, and so are seedlings, because they are a rich source of enzymatically active proteins, which, in turn, are suitable for the incorporation of radioactive components.

Chromatography (HPLC), electrophoretic separation (polyacrylamide gels, or PAGE, starch gels) of negatively charged molecules, and the serological method of measuring response degree of the antigen-antibody reaction can all be used for obtaining protein profiles and successful genotype identification.

MORGAN (1989) found that HPLC can separate seed proteins of all cereals and that the resulting protein profiles can be used to distinguish among genotypes. Several different reverse phase HPLC systems and methods have been developed and used to obtain quantitatively present or absent peaks of genotypes' protein profiles. The amount of protein detected on a certain chromatographic peak can quickly provide very reliable information not only on the quality of proteins present in the genotype but also on their quantity. This method is extremely discriminating, reliable, fast and and automated and can be used for genotype characterization. The drawback is that it requires expensive investments to purchase adequate equipment.

Use of the electrophoretic method in genotype identification depends on what plant species the genotype belongs to and what reproductive method it uses. Thus, there are two main methods of application:

1. Direct (multi-locus) application, where proteins are analyzed that are polymorphic and genetically coded by multiple loci. A good example are reserve proteins found in cereal seeds. They are coded by multigene loci and products of single loci may contain several electrophoretically separated bands. The criterion for distinguishing among genotypes is the existence or absence of a particular protein band (or set of bands) that appears on a defined position or positions on the gel. This type of application is used with self-pollinated plant species such as wheat, barley, pea, etc. ISTA (International Seed Testing Association) has tested and defined standard reference methods for electrophoretic identification of wheat (COOKE, 1988), barley (WEISS *et al.*, 1991) and pea and included in the ISTA rules, which are used across the world to identify the desired genotypes of those plant species. This method is also used with great success with plant species that reproduce vegetatively or asexually (GILLILAND, 1989).
2. Indirect (single-locus) application, which involves the testing of proteins that, although polymorphic, originate from a single locus (isozymes or allozymes). Distance among genotypes is demonstrated either as the occurrence of different isozymic phenotypes (banding pattern) in self-pollinated species and those that reproduce vegetatively or as a difference in the frequency of occurrence of isozymic phenotypes in open-pollinated plant species.

Special attention should be paid to open-pollinated species that show expression of different phenotypic characteristics. Open-pollinated individuals

may also be genetically distant and may contain different combinations of homozygous and heterozygous genes, including those that code for reserve seed proteins or isozymes. Because of the specific genetic structure, there are two ways of their electrophoretic identification:

1. Analysis of seed mixture extract (bulk metod) to encompass the genotype's protein profile
2. Use of an indirect method for assessing individual plants, determining variability levels within genotypes. Because of the difficulties in reading the gels of complex reserve protein profiles, it is much simpler to analyze individual isozymic loci.

The SDS-PAGE method of protein profile sample mixture is included in ISTA international rules as a technique for distinguishing among and identifying commercial genotypes of different plant species.

This method was used for genetic characterization of maize inbred lines and hybrids at the Biotechnology laboratory of the Maize Research Institute in Belgrade, as well as, for first screening of genetic purity of hybrid seed. All analyzed genotypes have unique protein pattern and unique code (combination of numbers and leters) have been assigned to them.

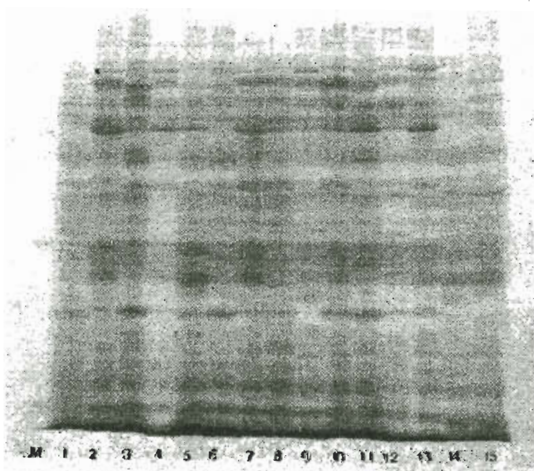

Fig. 1. - Maize genotypes identification (1-15) by the SDS-PAGE method

Different protein electrophoresis methods have indisputable importance in plant species and genotype identification. According to SMITH *et al.* (1992), 50 plant species were found in total in which electrophoresis methods had been successfully used for identification purposes. Although proteins are a product of primary DNA transcription, environmental conditions may still affect the levels of qualitative and quantitative seed proteins (HIGGINS, 1984).

Protein may also interact with other components, especially those found in the seed. Such ineractions with components found in the root, leaves or tubers may reduce the repeatability of protein profiles. This means that the organ to be used as a protein source must be carefully selected. Another important factor affecting protein profile stability and reliability is the method of data analysis, both

in quantitative and qualitative terms. Some authors think that preliminary quantitative studies must be carried out to test the validity of the two-dimensional protein profile. Genetic components of variability have been studied in this way in barley and maize inbred lines (HIGGINBOTHAM and SMITH, 1989). BURBIDGE *et al.* (1986), however, argue that in wheat it is much more advisable to take into account the qualitative aspects of wheat protein profiles, because it has been determined that in that case environmental effects are minimized or nonexistant.

Studies demonstrating the stability of protein profiles fall into two categories: 1. tests of profile stability in light of changed environmental conditions; 2. demonstration of genetic profile control. GORG *et al.* (1988) think that two-dimensional electrophoresis is the best method of protein separation, because it provides both the qualitative and quantitative protein profile data. This method is used widely and is the most reliable one for determining of protein profile stability in genotypes grown under different environmemntal conditions.

GOODMAN and STUBER (1980) and their team worked for many years on determining the genetic control of protein profiles. Developing stable isozyme systems, they concluded that their profiles were comparable and repeatable in many different crop species as well as that they did not depend on environmental conditions. TANKSLAY and ORTON (1983) argue that in many crop species it is possible to genetically characterize izoenzyme variants for many enzyme systems.

According to ZLOKOLICA *et al.* (2000), one of the most polymorphic genetic markers is the MDH isoenzyme system (Fig. 2), which provides a highly reliable picture of genetic variability within a population as well as divergence among alfalfa lines. Figure 3 shows genetic identification of sunflower lines using the PHI/PGD isoenzyme system.

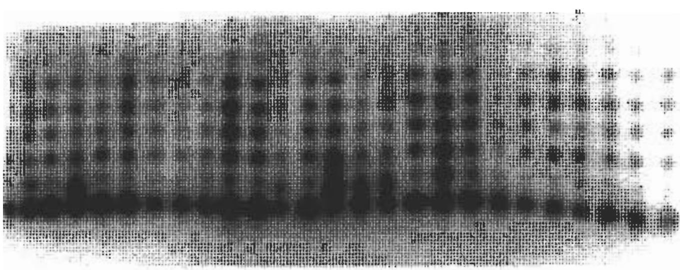

Fig. 2. - Determination of genetic uniformity of alfalfa by biochemical markers (MDH isoenzyme system)

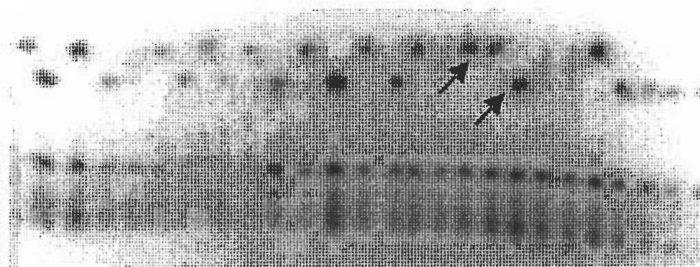

Fig. 3. - Determination of sunflower genetic variability by biochemical markers (PHI/PGD isoenzyme system), arrows indicate genetic variability within sunflower lines

Based on these observations, the method for determining cultivar purity as well as genotype description by protein profile development have found their place in European descriptors as the obligatory and standard method for genotype identification and description.

It can be concluded that protein profiles are suitable for providing crop identity cards, as they are stable genotype descriptors.

DNA data

Most proteins used to obtain electrophoretic or chromatographic profiles are mostly expressions of genes that are unevenly distributed in the genome. This prevents these proteins from providing a sample that would cover the entire genome. According to MARSHALL and BROWN (1975), more than 75% of nucleotide substitutions have no effect on the characteristics of the resulting proteins and will therefore remain undetected by protein analysis techniques. According to STUBER *et al.* (1988), genome coverage by biochemical markers is 30% at the most.

LEE *et al.* (1996) concluded that biochemical characterization methods cover only part of a plant species genome, and in some cases, such as with oilseed rape, there is even a lack of protein polymorphism, which limits the success of genotypic identification.

The complete coverage of a genome can be achieved only by the use of molecular variability indicators (DNA polymorphism), i.e. molecular markers. The use of the technologies at the molecular level expands their possible use in genotype identification and DUS testing. In the last decade, molecular techniques have been rapidly developing and there is now a whole range of different methods that, if applied properly, can be used as an absolute indicator of distance, stability and similarity among different genotypes and their genetic constitutions (ZLOKOLICA *et al.*, 1999).

The most commonly used methods for DNA profiling and genotype characterization by determining their distance and uniformity are the RFLP, PCR-based techniques (RAPD, AFLP and SSR). They are used selectively depending on the crop species and genetic constitution of the genotype.

In the 1970s, when endonucleases, or restriction enzymes, were isolated and purified, it became possible to cut up high molecular total genome DNA (10^9 order of magnitude) into shorter fragments. The short DNA sequences (fragments), cut up in a specific way by a certain type of enzyme, vary in number and length. This variability is called Restriction Fragment Length Polymorphisms (RFLPs). In the simplest case, this method can be viewed as variability in the size of restriction DNA fragments that can be detected by electrophoretic separation on agarose gel. Depending on the type of restriction enzyme, a large number of DNA fragments are obtained after cutting (one cut per 4,000 nucletide bases). In wheat, for example, around 4,000,000 fragments can be produced. Such a large number of fragments produce an unclear, smeared picture during electrophoretic separation on the gel and provide no information due to the high density of the fragments. Because of this, DNA probes had to be invented (a small portion of cloned DNA homologous with the fragments of interest and capable of hybridizing them). The selected fragments of interest are transferred from the agarose gel onto a nylon or nitrocellulose membrane filter in a procedure named Southern blotting. The filter with the radioactively labeled probe is exposed to conditions that promote

hybridization and subsequently photographed using X-rays. This way the distribution of fragments that identify a particular genotype is obtained. LEE *et al.* (1996) concluded that the RFLP method has significant potential for genotype discrimination and that, especially with oilseed rape, it is highly discriminative, reliable, relatively fast, suitable for sample comparation, and capable of being carried out at any time of year. They also stressed that this method is advantageous over most methods of morphological characterization presently used in DUS tests.

The disadvantages of this method when used for identification purposes, namely its duration, increased costs, technical difficulties and poor availability of the specific probes, can be overcome by the development of other methods that would eliminate some of these shortcomings. The development of the PCR (Polymerase Chain Reaction) method made it possible to surmount these difficulties.

PCR - Based methods

The polymerase chain reaction is a rapid procedure for *in vitro* enzymatic multiplication of specific DNA segments. Uses for this method are practically unlimited and still on the rise. The method is characterized by speed, simplicity, safety (no radioactive probes are used as in the case of RFLP), repeatability and accuracy. It is therefore used in direct cloning of DNA or cDNK, *in vitro* mutagenesis and DNA engineering, analysis for presence of undesirable agents, prenatal diagnosis of genetic diseases, analysis of variability of allelic sequences, etc. Another use of this method that is of importance and relevance to this paper is its application in genetic fingerprinting, i.e. molecular genotype identification.

There are two ways in which the PCR method can be used. One requires the knowledge of specific sequences of the DNA portion of interest, while the other makes use of randomly created sequences, prior knowledge of which is not required.

Many PCR-based methods have been developed that include either one or the other of the two ways of utilizing the PCR technique. We will discuss only several of them (RAPD, AFLP and SSR), as they are the most commonly used ones in genotype identification.

The RAPD method is based on the detection of polymorphism of multiplied random sequences that are distributed throughout the genome. The first studies (WELSH and MC CLELLAND, 1990; WILLIAMS *et al.*, 1990) with soybean and rice showed that genotypes could be clearly distinguished using this method. Using the RAPD method as opposed to biochemical identification methods, LEE *et al.* (1996) found the discrimination level among genotypes to be over 95%. This method was a potential solution for the identification and distinguishment of oilseed rape genotypes, since biochemical indicators could detect no polymorphism with this plant species. After comparing the RFLP and RAPD methods, the same authors concluded that both methods were higly discriminative, suitable for work, relatively fast, and equally capable of sample comparison and that they should hence be given very serious consideration as potential descriptors in DUS tests for oilseed rape.

Genetic characterization of 20 soybean genotypes from the Maize Research collection was done by set of 27 RAPD primers (NIKOLIĆ *et al.*, 2005).Total, 86

RAPD fragment of different molecular weight were obtained out of which 37.2% were polymorphic (Fig. 4).The reproducibility of RAPD essay was tested in two rounds of amplification with all soybean genotypes. Results confirmed that problem related with reproducibility of RAPD can be resolved by rigorous attention to details.

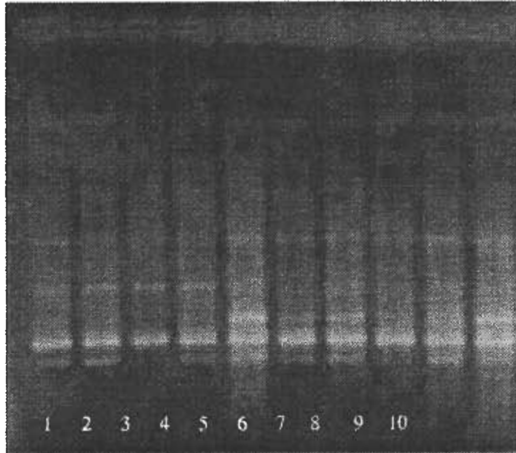

Fig. 4. - RAPD analysis of 10 soybean genotypes by primer GEN 2-80-7

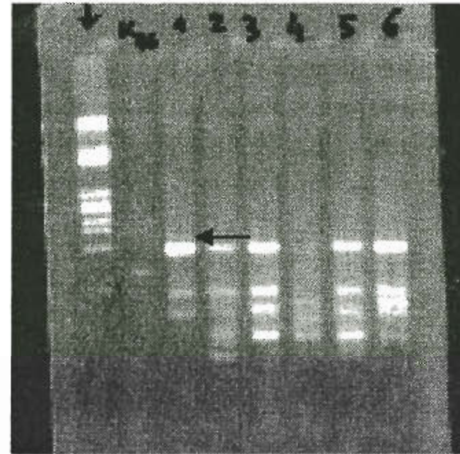

Fig. 5. - RAPD analysis of 6 maize genotypes. M- marker (Ladder DNA), arrow indicate polymorphic bands among genotypes

DRINIĆ *et al.* (2004) determined genetic diversity of maize inbred lines of different orgin on the basis of RAPD markers and examined usefulness of RAPD markers for assigning inbred lines to heterotic groups (Fig. 5).The results agree with another reports that stated that the level of polymorphism for RAPD markers is high in maize. Cluster analysis based on genetic distance calculated from RAPD data showed clear grouping of inbred lines into main heterotic groups.

MC GREGOR *et al.* (2000) and NOLI *et al.* (personal communication) report that the RAPD method can also be successfully used to identify potato genotypes and determine genetic distances among them.The AFLP method is based on restriction fragment length polymorphism. AFLP is a PCR-based and dominant method just like RAPD, but it is technically more advanced in being able to distinguish between homozygous and heterozygous genotypes (VOS and KUIPER, 1998). It is used for genome mapping in different plant species, DNA fingerprinting, and analysis of genetic relations and diversity among genotypes.

This technique is more reliable than the RAPD method, since, according to MC GREGOR *et al.* (2000), it has greater reproducibility (99.6%) than RAPD (83.4%), and it has proven to be the most effective method for potato DNA fingerprinting, as it managed to distinguish among 39 cultivars of this crop species using one primer combination.

According to VOS *et al.* (1995), the AFLP method exhibited a high level of reliability and showed a significant degree of polymorphism among wheat genotypes. According to LAW *et al.* (1998), the level of discrimination obtained by AFLP is far greater than that offered by morphological characters.

This method was used for maize genotype identification at the laboratory for molecular markers of the Small Grains Department of the Institute of Field and Vegetable Crops in Novi Sad and the polymorphic profiles of maize genotypes were obtained using 10 primer combinations (Fig. 6.).

The findings of the authors mentioned above, are supported by our own when using the AFLP method. We have found this method to be repeatable and to produce dense DNA profiles with a high percentage of polymorphism (max. percentage obtained: 81.5%). During statistical data processing by the NTSYS pc. program, genetic distance among the maize genotypes was determined too (Jaccard's similarity coefficient), confirming that this method can be used in maize genotype identification as well (GALOVIĆ, 2002). Although more reliable and precise and capable of producing a larger number of polymorphic fragments, this technique also requires more man-hours.

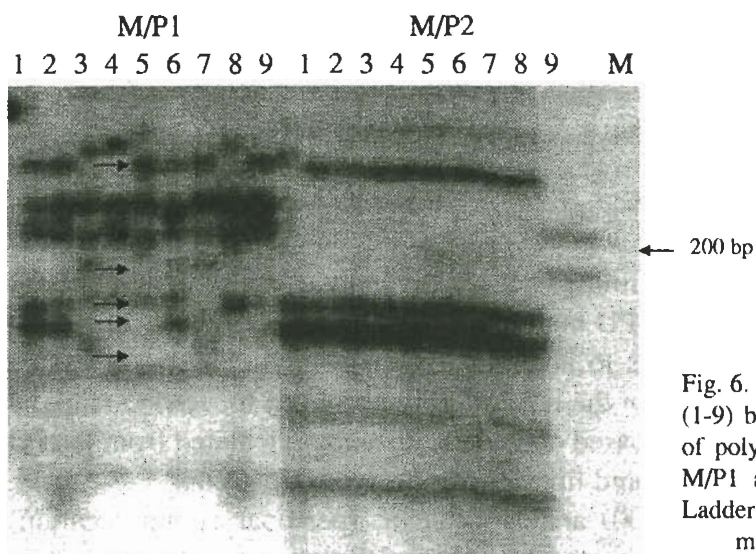

Fig. 6. - Maize genotype identification (1-9) by the AFLP method (fragment of polyacrylamide gel) using primers M/P1 and M/P2 , M- marker (Low Ladder DNA), arrows indicate polymorphism among genotypes

Another efficient PCR-based method for genotype identification is the use of so-called microsatellites (Simple Sequence Repeats - SSRs). In this case, fragment polymorphism is based on the total length of the sequence, which is determined by a large number of repeatable units. This method is codominant and hence able to distinguish between heterozygotes for different fragments in diploid genomes. Individual loci corresponding to specific pairs of primers (oligonucleotide sequences) are therefore codominant and may be multiallelic. According to JONES *et al.* (1997), the PCR product obtained as a result of this method is highly reproducible, and although this marker system is species-specific, costly to create and requires prior knowledge of the sequence, once it has been developed, it pays off financially. Microsatellite systems for genotype identification have been developed for many agronomically important crop species, such as wheat, soybean, barley, potato and others. It's reproducibility and specificity have been proven by RODER *et al.*, 1998 where the authors found out that microsatellites shown a much higher level of polymorphism and informativness in hexaploid wheat than any other

marker system. It has been established (MC GREGOR *et al.*, 2000) that this system can produce polymorphism frequency of 100%. This marker system can also be used as a simple and reliable marker system for the verification of the integrity and genetic stability of wheat genebank accessions (BORNER *et al.*, 2000).

According to KOBILJSKI and GALOVIĆ (2002), the presence of the Rht 8 and Ppd 1 genes in the wheat genome can be determined with a great deal of certainty using the microsatellite WMC 261, which makes it possible to assess wheat genotypes for stem height and earlier maturity. Using the microsatellite method with 10 specific SSR primers (GALOVIĆ, 2005), polymorphism was found among 25 wheat genotypes, which proved the possibility of describing them by this method on molecular level (Fig. 7.).

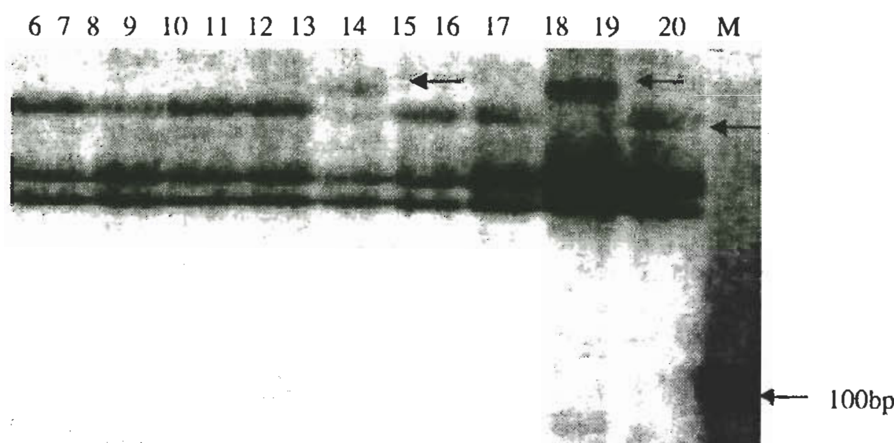

Fig. 7. - Wheat genotype identification (6-20) by the SSR method (fragment of polyacrylamide gel) using specific primer GWM165 (M-Low Ladder DNA), arrows indicate polymorphism among genotypes

SSR markers have been also used to evaluate genetic diversity among 12 maize inbred lines and to predict heterosis in their crosses (DRINIĆ *et al.*, 2002). Fifty eight out of 60 alleles, detected in maize inbred lines by the analysis of a random set of 21 SSR loci, were polymorphic. Data shows that inbreds most closely related by their pedigree are also closely related on the basis of marker information. The correlation coeficient between heterosis for grain yield and genetic distances based on SSR markers are positive and mainly significant, while their magnitude is not large enough to be beneficiary in prediction of heterosis.

CONCLUSION

Modern technologies have made genotype identification of agronomically important crop species much more accurate and rapid and independent of environmental conditions. Genetic similarities or differences revealed by genetic profiles obtained by these methods provide an identity card of sorts for each genotype tested.

The identification methods must be chosen in accordance with the genotype's genetic base, because only then the genetic profiles will be highly reliable.

Use of DNA markers as diagnostic tools in genotype identification along with DUS tests is possible and a thing of the future, but it is only when these methods are used in conjuction with morphological characterization that a comprehensive picture of an agronomically important genotype can be obtained.

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METODE KARAKTERIZACIJE I LIČNA KARTA AGRONOMSKI ZNAČAJNIH BILJNIH VRSTA

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Izvod

Stalna upotreba morfoloških podataka u opisu agronomski važnih genotipova ukazuje na činjenicu da su ovi podaci zadržali popularnost deskriptora biljnih vrsta. Međutim javlja se potreba njihovog detaljnijeg opisa, laboratorijski baziranim, biohemijskim i molekularnim metodama. Standardizacijom ovih novih tehnika u svetu, zbog njihovih brojnih mogućnosti u oblasti identifikacije genotipova, gde se ustanovljavaju standardi za njihovu zaštitu, bave se međunarodne organizacije ISTA (International Seed Testing Association) i UPOV (Union pur la Protection des Obtenious Vegetales). Rezultat ovih nastojanja da se standardizuju tehnike za identifikaciju genotipova su DUS (Distinctness, Uniformity, Stability) testovi koji predstavljaju osnovu za deskripciju biljnih vrsta i genotipova u svrhu sistema zaštite intelektualne svojine biljnih oplemenjivača (Protection of Plant Breeders Rights - PBR). Ovakva svojevrsna standardizacija novih metoda polako postaje i naša svakodnevnica. Kombinacijom morfoloških, biohemijskih i molekularnih podataka može se postići sveobuhvatna karakterizacija agronomski značajnih biljnih vrsta i genotipova. Primenom novih tehnika moguće je otkriti njihov jedinstveni genetički profil i dobiti ličnu kartu (fingerprinting) svakog agronomski važnog genotipa.

U ovom radu prikazan je pregled tehnika koje su se pokazale uspešne pri primeni biohemijske i molekularne karakterizacije agronomski važnih genotipova. Otkrivanjem njihovog jedinstvenog genetičkog profila, zahvaljujući primeni ovih metoda, sada je moguća njihova precizna karakterizacija sa visokim nivoom reproducibilnosti i pouzdanosti.

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