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DNA-BASED METHODS USED FOR VARIETAL PURITY DETECTION IN WHEAT CULTIVARS

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Abstract

Wheat is a cereal that plays an important role in agriculture, for feed and human food. It is well known that the wheat (Triticum aestivum L.) has a large and complex genome (2n = 6x = 42 chromosomes) that makes difficult the genetic researches. Assessing genetic purity and varietal identification are important topics in wheat seed quality control. Several approaches that can be used to exploit new methods for genetic purity assessment and varietal identification of wheat are currently available in various international laboratories. Techniques based on morphological identification involve intense effort, making it sometimes difficult to verify crops varieties. Using molecular markers in order to determine the purity and variety identification of different seed types seems to be a better approach. Nowadays in the world there are numerous wheat varieties and cultivars with different properties. The molecular markers are not influenced by environment conditions and this makes them play an important role not only for seed purity evaluation but although for research purposes. Taking all that into consideration we can say that DNA-based methods using PCR technique are always useful tools to determine authenticity and purity not only for wheat varieties but for other crops. A better understanding of the factors controlling purity and varietal identification, as well as the effective utilization of new and developing genetics and genomics technologies, have great potential to improve the genetic purity assessment.

Key words*: genetic purity, molecular markers, varietal identification, wheat.*

WHY PUTTING A HIGH VALUE ON AGRICULTURE TODAY

It is well known that cereals are one of the most important sources of food for human consumption. From the total quantity of cereals produced annually, most of them are intended for food consumption, followed by their use for animal feed, and the rest are processed for industrial use or used as seeds. According to Food and Agriculture Organization (FAO), the 21st century faces multiple challenges in terms of ensuring the need for food, feed and fiber in the conditions of population growth worldwide (FAO, 2010). Thus, there will be an increase of the world population with 34 percent, being expected that by 2050 population will reach approximately 9.7 billion people (FAO, 2009; 2017).

Wheat production ranks third after corn and rice production. In terms of nutritional intake, wheat holds a second place, after rice, given the increased use of corn as animal feed. Nowadays, most of wheat production is used for food consumption, followed by use for animal feed and for industrial use, including biofuels (FAO, 2009; 2018).

In Romania, according to Ministry of Agriculture and Rural Development (MADR, 2018), the area cultivated with wheat registered an increase from 1,975.0 thousand ha in 2007 to 2,109.0 thousand ha in 2018, the average wheat production also recording growth: from 1,541 Kg/ha, as obtained in 2007 and reaching 4,803 Kg/ha in 2018. However, when it comes to wheat production and also other cereals production, can always be badly affected by the influence of climate changes in recent years.

This should be taken into account when it comes to the evolution of cultivated areas and the crops production in Romania.

BASIC REMARKS ABOUT WHEAT GENOME AND COMMON WHEAT EVOLUTION

Common wheat (*Triticum aestivum* L.) known as bread wheat is one of the most cultivated types of wheat. Although the wheat genome has always been considered impossible to sequence due to the large number of repetitive sequences (> 80%) and its size of 17 GB, being five times larger than the human genome (Paux et al., 2008), there are currently numerous studies on wheat genomics.

Triticum aestivum L. has high variability and is a self-pollinating plant species and the most important part about its genome is that common wheat is allohexaploid (2n = 6x = 42 chromosomes, AABBDD) (Novoselskaya, 2015) having six sets of chromosomes, each two sets from three different species (IWGSC, 2014). Thereby, bread wheat contains three subgenomes (ABD) derived from *Triticum urartu* (genome A), *Aegilops speltoides* (genome B) and *Aegilops tauschii* (genome D) (Jizeng et al., 2013; IWGSC, 2014) which sometimes makes its genetic study difficult and last but not least, a challenge to characterize this important genome (Erayman et al., 2004).

Another cultivated wheat species is *Triticum durum* which is tetraploid (2n = 4x = 28 chromosomes) and has two genomes (AABB). Species related to common wheat are rarely cultivated for commercially aim.

Hexaploid and tetraploid wheat species evolved through two important evolutionary processes and according to Baenziger (2016) the progenitor is not known but it seems that it was originally a diploid species. Accordingly, a first evolutionary process consisted of a divergent evolution, when this species evolved into many other diploid species, including *Triticum urartu*, *Triticum tauschii*, barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.). Second evolutionary step consisted of a convergent evolution that was achieved by natural hybridization and spontaneous doubling of the chromosome and made it possible the formation of polyploidy species. One such example is that of *Triticum urartu* (genome A donor) which, following hybridization with an unknown *Triticum* species (genome B donor), and formed *Triticum dicoccoides*, the progenitor of *Triticum durum* (AABB). A second hybridization process that occurred between *Triticum dicoccoides* and *Triticum tauschii* (genome D donor) resulted in the formation of the common wheat, whose genomic constitution is the one mentioned above: AABBDD (Baenziger, 2016).

GENETIC MARKERS CLASSIFICATION AND IMPORTANCE

Genetic markers are allele or DNA sequences that have a known position on the chromosome that contain a specific gene for phenotypic character. They are based on the polymorphism of the gene in terms of phenotypic expression. Genetic markers can be divided into two groups: traditional markers and molecular markers or DNA markers (Raza et al., 2019).

Traditional markers: this group includes three categories of markers: morphological markers, cytological markers and biochemical markers.

Morphological marker are markers that control morphological characters, and can be used to analyze different properties, such as seed shape or flower color, germination mode and other important agronomic parameters.

Advantages of these markers are that their use does not require any biochemical and molecular specific systems, being safe to apply. Amongst disadvantages we can mention that they are in a limited number being affected by different plant germination phases and many other natural elements.

Breeders use successfully these markers in plant breeding program (Raza et al., 2019).

Cytological markers: in terms of cytology we can say that structural features of chromosomes can be obtained by chromosome karyotype and bands, thus cytological markers consist of the combined differences that exist between the structure, size, configuration, sequence and location of the chromosomes, differences that denote the variations in the mode of dispersion of euchromatin and heterochromatin (Jiang, 2013). Cytological analysis can be a useful tool in characterization of wheat species (Kwiatek et al., 2019; Daiyan et al., 2019).

Biochemical markers or allozymes (isozyme) are markers that control biochemical properties. Allozymes analysis has been used for a long time for different purposes such as to mark genetic variability, taxonomy or to study crops genetics and although for plant breeding. DNA mutation involves a replacement of an amino acid and as a consequence modified charges in the electric net of the protein, although the molecule conformation can change.

The advantage of these markers lies in the simplicity of the process, proving themselves to be useful markers for breeders and for genetic studies of plant species.

As a disadvantage we can state the low level of polymorphism and the fact that allozymes are phenotypic markers which implies that they may be affected by external conditions (Kumar et al., 2009).

Molecular markers or DNA markers where extremely used for the determination of genetic diversity in major crops listing wheat (Prasad et al., 2000), rice (Nivedita et al., 2016), maize (Awaludin et al., 2013) and common bean (Debrah et al., 2018).

These markers consist of a small piece of nucleotide sequence and can be analyzed with the help of polymorphisms existing among the nucleotide sequence of various members. Considering that molecular markers are DNA sequence which encodes a particular character or gene of an organism, may be powerful instruments for evaluation of the transmission of hereditary characters, although, speaking of wheat, there is less influence in the transmission of hereditary characters between cultivars, compared to other self-pollinating plants (Raza et al., 2019).

Genetic markers are now frequently used by breeders in breeding programs including here marker assisted selection of cultivars with important agricultural traits, for crops varietal identification or assessment of genetic purity (Zorica, 2010).

MOLECULAR MARKERS AND DNA-BASED TECHNIQUES

The use of molecular markers (DNA-based markers) techniques can offer numerous advantages if we compare with conventional phenotypic approach.

Molecular markers are stable and can be detected in all tissues, they do not depend of plant growth, development and are not influenced by the environment.

Among the criteria to successfully use molecular markers techniques are the following: they have to be polymorphic, equal distributed throughout the plant genome, to highlight genetic differences, to generate reliable markers, be quick, easy to use and not very expensive, the amounts DNA samples needed to be small, to not require prior information about the plant genome or organism, be specific and to have specificity and reproducibility (Agarwal et al., 2008).

Taken into account that no method is perfect, in choosing the applied method it can count the population level at which the studies are performed, as well as the polymorphism provided by the marker used.

Depending on the technique used to identify them, DNA based molecular markers may be divided into two categories: non PCR based and PCR based markers (Agarwal et al., 2008; Kumal et al., 2009).

Non PCR based markers: this first type of markers includes hybridization-based markers such as Restriction Fragment Length Polymorphism (RFLP).

Polymerase chain reaction based markers (PCR based markers)*:* this second category includes among others the Random Amplified Polymorphic DNA markers (RAPD), Amplified Fragment Length Polymorphism markers (AFLP), Microsatellite or Simple Sequence Repeats markers (SSRs), Single Nucleotide Polymorphism markers (SNP), Randomly Amplified Microsatellite Polymorphisms (RAMP) markers, Inter Simple Sequence Repeats (ISSRs), Sequence Related Amplified Polymorphism (SRAP), Sequence Characterized Amplified Region (SCAR), Sequence Tagged Sites (STS), Cleaved Amplified Polymorphic Sequence (CAPS) etc. (Debrah et al., 2018; Raza et al., 2019). In Table 1 are listed some feature comparison of the most used molecular markers.

When talking about PCR-based markers we cannot put aside the development of the PCR technique by Cary Mullis in 1983, which had an important effect on the future development

of molecular biology techniques (Mullis et al., 1986).

In other words we can state that many purposes can be applied for this multitude of DNA markers such as genetic purity evaluation, gene identification, varietal identification, marker assisted selection, quantitative trait loci (QTLs) mapping, genotypic selection, etc. (Bernardo, 2008; Raza et al., 2019; Nadeem et al., 2018).

Table 1. Feature comparison of the most used molecular markers

Characteristics	RFLP	RAPD	AFLP	ISSR	SSR	SNP
Codominant/ Dominant	Codominant	Dominant	Dominant	Dominant	Codominant	Codominant
Reproducibility	High	High	Intermediate	Medium-High	High	High
Polymorphism level	Medium	Very high	High	High	High	High
Makers availability	Low	High	Medium	Medium	Medium	High
DNA quality	High	High	High	Low	Low	High
DNA quantity	High	Medium	Low	Low	Low	Low
Genome abundance	High	Very high	Very high	Medium	Medium	Very high
Analysis costs	High	Low	Moderate	High	High	High
Sequence information	No	No	No	No	Yes	Yes
PCR based	No	Yes	Yes	Yes	Yes	Yes
Visualization	Radioactive	Agarose gel	Agarose gel	Agarose gel	Agarose gel	Automated sequencers

(Kumal et al., 2009; Nadeem et al., 2018).

NON PCR BASED MARKERS TECHNIQUES

Among the first molecular markers technique can be listed methods that used Restriction Fragment Length Polymorphism markers (RFLPs). These markers are based on hybridization. The technique consists in extraction of high quality DNA which is an important step of this method. Another step is the use of restriction enzymes that cut DNA extracted at specific recognition sites resulting in many fragments of different lengths. The fragments are isolated by agarose or polyacrylamide gel electrophoresis (PAGE) (Raza et al., 2019).

Because of their complexity RFLP markers are not quite suitable for breeding (Zhao et al., 2019), they although show low polymorphism which is a major problem for using RFLP markers in wheat, in which case early studies showed that microsatellites markers are more suitable for evaluation of wheat cultivars (Röder et al., 1995). Compared to RFLP markers, SSR markers are more genome specific avoiding confusion created sometimes as a result of the difficulty in results interpretation, because RFLP probes can hybridize more than one positions in wheat genome (Song et al., 2005). Despite all inconvenience related to the use of RFLP markers, it should be mentioned that they were first successfully used in 1975 for genetic mapping of an adenovirus then for human genome mapping and later on they were choose as markers for plant genomes (Semagn et al., 2006).

PCR BASED MARKERS TECHNIQUES

Randomly Amplified Polymorphic DNA (RAPD) methods involves amplification of genomic DNA by PCR technique, with a single set of primers with an arbitrary nucleotide sequence. The resulting PCR products are isolated by gel electrophoresis. For the success of the technique a condition is that the selected marker should have the content in guanine-cytosine (GC) of at least 40% (Raza et al., 2019).

RAPD markers technique was successfully used for assessment of genetic diversity in wheat (Tamimi & Janabi, 2019) but also for other plants such common bean (Szilagyi et al., 2011), *Amaranthus* species (Popa et al., 2010),

proving that it has strong potential in wheat breeding (Eid, 2019).

Amplified Fragment Length Polymorphism (AFLP) technique merges the Restriction Fragment Length Polymorphism (RFLP) method and PCR technique (Raza et al., 2019). This method turned out to be very sensitive in detecting markers for genetic diversity studies of common wheat cultivars (Arabi et al., 2019; Sadeqi et al., 2019) or among durum wheat cultivars (Roncallo et al., 2019).

Simple Sequence Repeats markers (SSRs) are valuable tools for studying the genetic material of plants.

SSRs markers or microsatellites markers are sequences of one to six nucleotides repeated in tandem. Microsatellites are to be found in the genome but they also exist in other places such mitochondria and we talk about mitochondrial microsatellites and chloroplast when we talk about chloroplast microsatellites (cpSSRs) (Nadeem et al., 2018).

Because of their high polymorphism level they can be easily identified by PCR technique (Raza et al., 2019).

One of their particularities is that being co-dominant markers a comparative analysis of a DNA locus allows us the verification of the similarities between species being able to establishing varietal purity, as well as the phylogenetic relationships between plants (Lakhneko et al., 2016). With their help we could be able to differentiate between the homozygous and heterozygous genotypes (Xin et al., 2005).

SSR markers have been used although to authenticate and identify the genetic purity of various other crops such as maize (Wang et al., 2003), rice (Li et al., 1999; Xin et al., 2005), barley (Owen et al., 2019) etc.

According to various research studies these markers are suitable for uniformity and seed purity assessment of 90% of wheat varieties (Wang et al., 2015), as well as for fingerprinting or varietal identification (Sharma & Singh, 2015; Varshney et al., 2005).

As many studies shows they are used in most areas of crops genetics being as it is stated before high informative, locus specific with co-dominant inheritance.

SSR markers can discriminate between genotypes, being able not only to detect polymorphism but although to estimate genetic diversity in common wheat (Plaschke et al., 1995; Stachel et al., 2000; Prasad et al., 2000) or between durum wheat genotypes (Eujayl et al., 2002; Marzario et al., 2018) being able to differentiate wild species of wheat with A, D and C genomes or to asses genetic variation within these species (Salehi et al., 2018). They are by far the most used markers.

SSRs markers are found to be more informative than other markers in common wheat (Song et al., 2005) and they can be successfully used in another areas such as marker assisted selection (MAS) (Ciucă et al., 2018) with high implications in breeding programs (Stachel et al. 2000; Giura et al., 2019).

Another approach for microsatellite markers is so called sequence-tagged microsatellite site (STMS) when microsatellites markers that contains genomic fragment are cloned and sequenced for primers construction used in PCR amplification. The method involves intense effort, sequence information is needed as long as the characterization and cloning of the probe and mutations cannot be detected outside the target sites (Kumar et al., 2009).

Randomly Amplified Microsatellite Polymorphisms (RAMP) markers techniques, uses SSR primers for genomic DNA amplification that are radiolabeled containing a '5' anchor and '3' repeats. The resulting products are evaluated by agarose gel electrophoresis (Raza et al., 2019).

The technology does not require high costs and RAMP markers have a high level of polymorphism and high genome distribution (Nadeem et al., 2018).

They can be successfully applied and have good potential for studies on various plants molecular characterization (Davila et al., 1999; Guasmi et al., 2012; Salazar et al., 2014).

Single Nucleotide Polymorphism (SNP) markers technique is based on the fact that in many organisms it can occurs variations in the genome sequence in a single nucleotide position (Kumar et al., 2009).

SNPs may be alterations of C/T or G/A or reversions of C/A, A/T, C/G, or T/G depending on nucleotide exchange, in other words addition or deletion of one base (Raza et al., 2019).

The methods using SNPs markers has also gained popularity even though this markers are

only a bi-allelic type (Kumar et al., 2009), mostly because they are present in large numbers in plants and animals. Their frequency in plants may be between one SNPs at every 100-300 base pairs (Nadeem et al., 2018).
Using the SNPs technique it turned out to be useful for varietal identification in crops where polymorphisms is hard to find (Kumar et al., 2009) but although for genomic assisted breeding (Yong et al., 2017) in wheat varieties and cultivars (Vendramin et al., 2019; Körmöczi et al., 2019).

Inter Simple Sequence Repeats (ISSR)
The method uses microsatellites as primers in a PCR reaction with a single primer for many loci in order to amplify usually inter simple sequence repeats of different sizes. The primer for technique can follow the SSR motifs of two up to five nucleotides at microsatellite loci making possible the appearance of several amplification products (Ammiraju et al., 2001).
One of the main advantages of using ISSRs markers is that it does not require the sequence data to obtain the primer and the quantities for template DNA are low, and last but not least they have a random distribution throughout the genome (Kumar et al., 2009).
ISSRs can be used to find markers associated with genes that control important traits thus being important for the selection of varieties with the desired traits (Ammiraju et al., 2001).
This technique was used for varietal identification of different rice genotypes (Dharmaraj et al., 2018), proven to be useful in gene mapping studies (Kumar et al., 2009), and in analysis of wheat genetic diversity (Etminan et al., 2016).
A disadvantage for using ISSRs marker just as for RAPD markers is the reproducibility issues that may occur (Kumar et al., 2009).
Sequence Characterized Amplified Regions (SCARs) markers are DNA fragments that are amplified using PCR technique with specific primers of fifteen to thirty nucleotides sequences cloned from RAPD fragments related to a trait of interest (Kumar et al., 2009).
The SCARs markers are locus specific, easy to use and have highly reproducibility, being used in marker assisted selection and gene mapping studies (Dar et al., 2019).
A disadvantage may be the need of sequence data for the PCR primers construction (Kumar et al., 2009).
Start Codon Targeted (SCoT) markers are markers that are related to short conserved region in plant genes surrounding the translation start codon (ATG).
These markers have been used to evaluate genetic diversity, varietal identification of cultivars, and for quantitative trait loci (QTL) mapping, DNA fingerprinting of different species, including wheat, rice, pea, sugar cane and grapes (Dar et al., 2019; Etminan et al., 2019).
Cleaved Amplified Polymorphic Sequences (CAPS) is a technique that combine RFLP technique with PCR, being able to refer to these markers as PCR- Restriction Fragment Length Polymorphism (PCR-RFLP) markers.
The method consist in amplification of DNA target by PCR using specific primers of twenty-twenty five base pairs then digested with restriction enzymes and products are visualized in agarose or acrylamide gel (Nadeem et al., 2018).
The method requires low quantities of DNA target, has high reproducibility and these markers are codominant but compared to the RFLP technique the polymorphisms is more difficult to find (Kumar et al., 2009).
CAPS marker technique is useful in gene mapping studies or used for marker assisted selection in wheat (Wang et al., 2017; Zhu et al., 2018).

DIFFERENT TRENDS FOR SEED VARIETAL PURITY EVALUATION

Genetic purity testing and varietal identification are important topics in seed quality control. Techniques based on morphological identification involve intense effort, including both material and substantial human resources, making it sometimes difficult to assess the purity and variety of different types of seeds. In this context the use of molecular biology techniques can play an important role both in the identification of varieties much faster and in determining the genetic purity of the seeds in different countries.

Nowadays, the authenticity and purity identification of seeds regularly involve field tests where the purpose is the establishment of Distinctiveness (D), Uniformity (U) and Stability (S) of the variety (DUS test), test that can raise researchable issues (Chakrabarty & Choudhury, 2019). The DUS test takes a long time to perform being sensitive to environmental conditions.

In the 1990s there was a significant development of molecular biology techniques, which made it possible to identify the authenticity and varietal purity in the laboratory, by using molecular markers (Smith & Register, 1998).

The interest of the specialists for such topic is emphasized by many early studies so methods such as detection of polymorphism of prolamin proteins by vertical polyacrylamide gel electrophoresis are used as in order to determine the varietal purity in wheat seeds and triticale since 1997, 1998 (Vyhnánek & Bednár, 2003).

The methods based on PCR technology offer new opportunities for the analysis of varietal purity. The technology uses a small amount of DNA that sometime does not require high purification, and the results can be obtained much faster.

Due to the rapid advancement in biomolecular techniques, the use of molecular markers to test distinctiveness, uniformity and stability comes not only as a necessity but also an addition when their use may be able to replace in near future the morphological observations. This topic is of great interest within the International Union for the Protection of New Varieties of Plants (UPOV) discussions (Debrah et al., 2018).

The use of molecular markers has advantages in terms of method specificity. Their importance in this mater comes from the fact that compared with other biochemical markers such as isoenzymes, they are not dependent on the conditions of the external environment, being independent of the ontogenetic stage of the plant (Vyhnánek & Bednár, 2003) with high implications in plant breeding (Nadeem et al., 2018; Sarika et al., 2017).

Applying these technologies for varietal identification can greatly simplify the seed quality control process, increasing the objectivity and efficiency and at the same time decreasing the material effort in terms of the space necessary for the cultivation as well as the testing time in the field.

Regarding the protection of varieties, seed producers had initiatives on establishment of intellectual property rights in field plants since the end of the 19th century, which led eventually to the adoption of specific legislation regarding the protection of plant varieties in the United States of America but also in some European countries (Correa et al., 2015).

RULES AND REGULATIONS IN FORCE ON VARIETAL PURITY ASSESSMENT

Determining varietal purity by using molecular biology techniques plays an important role also for seed producers in establishing varietal identification of seeds, seeds certification or in case of litigation.

In accordance with the Romanian legislation if there is any suspicion regarding the seeds varietal purity, further analyses can be made. Thereby verification of the seeds varietal purity can be done by methods such as: protein electrophoresis, DNA-polymerase reaction (PCR), Magnetic Resonance Imaging (MRI), ELISA- immunoassay tests and/or other laboratory methods accepted by the institutions and bodies in the European Union and/or internationally (MADR, 2011).

When we talk about seed certification it is well known that the legislative framework is established by normative acts of the Ministry of Agriculture and Rural Development (MADR) in accordance with the requirements of the European Economic Community thereby the varietal purity of the varieties being regulated by law.

According to the law, seeds have sufficient identity and varietal purity or for seeds from a consanguineous line, sufficient identity and purity in terms of their characteristics.

The legislation also mentions the minimum purity of a variety which is examined mainly through field inspections (MADR, 2010).

The minimum purity of a cultivar is different depending on the seed biological category of which belongs as follows: for species such as *Avena nuda*, *Avena sativa*, *Avena strigosa*,

Hordeum vulgare, *Oryza sativa*, *Triticum aestivum*, *Triticum durum*, *Triticum spelta*, we must have a varietal purity of 99.9% for base category seeds, 99.7% for seeds certified C1, 99.0% C2 certified seed, and for maize lines a minimum varietal purity of 98% (MADR, 2010).

According to the Organisation for Economic Cooperation and Development (OECD) rules, the varietal purity of a hybrid obtained in culture can be verified either in post-control or by additional methods. In order to determine the varietal purity or for varietal identification molecular biology techniques such as DNA or RNA methods can be used. Techniques as RFLP, AFLP, PCR, or the use of molecular markers (SSR or SNP) are very useful when a character is not visible to the naked eye (OECD, 2012).

From all considerations regarding genetic markers listed above one can state the idea that the methods that involve the use of molecular markers are very effective in confirming the purity and authenticity of a plant (Singh et al., 2016).

Currently, one of the techniques used in laboratories to determine the varietal purity, according to The State Institute for Testing and Registration of Varieties (ISTIS) methodology is protein electrophoresis (ISTIS, 2010). The method of determination is in accordance with International Seed Testing Association (ISTA) rules (ISTA, 2019).

In order that the chosen method to be effective in assessing the varietal purity it is important that the tested variety to be accompanied by a reference material. However, for testing purposes, the applicant is not obliged to provide this reference material so, just the varietal purity of the tested sample is being evaluated, which can sometimes be a minus, not taking into account the reference material.

The analysis of the electrophoretic spectra generated by storage proteins allows us to detect varietal impurity for many wheat cultivars (Hassan et al., 2019; Metakovsky et al., 2019; Beom et al., 2018) and may have also implication for plant breeding. Problems may also arise as a result of the fact that the method based on the electrophoresis of storage alcohol-soluble proteins may have its shortcomings so for example in oats, identical spectra makes sometime difficult the use of electrophoresis method for laboratory variety control (Lyubimova & Eremin, 2019).

If we refer to the DUS test to confirm the uniformity we can apply any polymorphic genetic markers, for example, gliadin composition, may be successfully used in hexaploid wheat (Metakovsky et al., 2019).

Among others tests that can be applied for varietal identification we can also list chemical tests. These tests are simple and their application does not require substantial material resources and tests such as standard and modified phenol tests, potassium hydroxide tests and sodium hydroxide tests, growth response of seeds treated with GA_3 could be helpful in assessment of wheat varietal purity (Salem et al., 2019; Raut et al., 2019).

FUTURE ASPECTS, TRENDS AND IMPORTANCE OF USING MOLECULAR BIOLOGY TECHNIQUES

Lately, we have witnessed to a continuous development of molecular biology techniques and starting from RFLP markers to SSRs and SNPs markers we can say that today there are lots of marker associated methods that come in support of researchers, but also of seed producers or breeders. The evaluation of genetic purity of a variety is important both for the registration of the variety and also for its protection. As mentioned above, the current testing system evaluates a number of morphological characters which becomes an impediment in the event of evaluating hundreds of samples. In this context the use of molecular markers becomes of great help.

Among the most used molecular markers we list the SSRs markers that, as mentioned above, have a high degree of polymorphism, high distribution throughout the plant genome and are not affected by external conditions.

A new variety characteristics can be defined by molecular markers patterns and be compared with others patterns from anywhere in the world, to establish its uniqueness (Wang et al., 2015).

The final purpose of the sharp development of the technology we can say that is to create, analyse and not least to manipulate the genetic variation and to develop improved cultivars

(Moose & Mumm, 2008) a marker assisted breeding with high implication in economy (Eathington et al., 2007).

In the last period as a result of the climatic changes and in the conditions of world growth population special attention was paid to the selection of improved cultivars assisted by molecular markers.

For wheat the interest was in obtaining new lines with resistance to various diseases or pests (Simpfendorfer et al., 2013; Goutam et al., 2015), useful in breeding programs to obtain cultivars that are resistant to more than one disease (Miedaner et al., 2019; Ciucă et al. 2015, 2018; Cristina et al., 2015; Miedaner & Korzun, 2012) and/or to counteract the climatic changes the selection of valuable cultivars (Giura et al., 2019; Ciucă et al., 2009; Vlasenko et al., 2019).

As the population increases, it becomes just as important to obtain varieties that have improved content in various substances (i.e. protein content) (Prasad et al., 2003; Cristina et al., 2016).

The marker assisted selection (MAS) has been successfully used for many other crops such rice (Jena & Mackill, 2008; Qing et al., 2019), maize (Prasanna et al., 2010; Awaludin et al., 2013; Raj et al., 2020), barley (Miedaner & Korzun, 2012; Jain et al., 2019; Varshney et al., 2007), sorghum (Afolayan et al., 2019; Kage et al., 2015), the technique was used not only for cereals but also for fruits (Al-Khayri et al., 2018).

As mentioned before the DUS test involves at this point the assessment of morphological characters which are highlighted by field inspections according to the International Union for the Protection of New Varieties of Plants (UPOV) rules. The use of molecular technologies as single way to establish varietal identification in crops is still under discussion as is the use of molecular markers for varietal identification in crops such wheat (UPOV, 2011) or maize (UPOV, 2014).

Since cereals trade is of particular importance for a country's economy, it becomes imperative to protect the breeders rights and the application of molecular markers it becomes important in terms of rapid detection of wheat cultivars and not only (Chun et al., 2018).

Others new and efficient genotyping techniques such as next generation sequencing (NGS) (Adlak et al., 2019), genotyping by sequencing (GBS), chip based NGS, or techniques such as kompetitive allele specific PCR (KASP) (Nadeem et al., 2018; Rasheed et al., 2019) and allele-specific PCR, designate molecular markers as suitable markers for genotyping, making it possible to speed up the process of molecular marker assisted selection techniques in wheat breeding (Raza et al., 2019; Mwadzingeni et al., 2016).

Because the genome complexity in wheat is very high, advances in both bioinformatics and sequencing makes NGS applications in wheat very feasible (Berkman et al., 2012; Bernardo et al., 2020).

CONCLUSIONS

Molecular markers are of particular importance for the development of modern techniques of molecular biology with special implications for breeding program, in germplasm management and improvement of wheat cultivars but also for varietal identification, being able in certain situations to resolve legal dispute.

Thus far, when it comes to wheat and its genome complexity, the need to discover new molecular markers and associated methods is still a goal itself and a challenge for researchers.

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