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ASSESSMENT OF GENETIC SIMILARITY AND PURITY DEGREE AMONG SEVERAL ROMANIAN MAIZE INBRED LINES USING SSR MARKERS

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Abstract

Maize (Zea mays ssp. mays) is today one of the most important cereal crops used not only for human consumption but although for feed or for industrial purposes, without the genetic evolution and the active intervention of breeders in the plant constant improvement, maize would not have today significance.
In this study, seeds from thirteen maize inbred lines (LC1-LC13) were analysed using eight SSRs markers recommended for seed varietal purity assessment. The seeds classes for maize inbred lines used in this study were pre-basic and basic seeds. Three of the maize inbred lines chosen for testing from both pre-basic and basic categories were analysed in order to verify that the varietal purity is preserved. High genetic similarity was between inbred line LC1 and LC2. SSR marker phi015 was the most polymorphic marker followed by umc1545, umc1448 and umc1117. The SSRs markers that showed low polymorphism were umc1061 and phi109275. The aim of this study was to select the most informative SSRs markers which fit to prove the varietal purity and to assess genetic diversity for maize seeds.

Key words: *SSR markers, maize, varietal purity, genetic diversity.*

INTRODUCTION

The history of maize (*Zea mays* ssp. *mays)* is thought to have begun 9,000 years ago, when the inhabitants of southern Mexico domesticated the plant called teosinte. Due to genetic evolution and human intervention in plant improvement maize is nowadays one of the main crops in the world, along with wheat and rice (Balter, 2007).
In Romania, maize is one of the main cereal crops. Statistical data on cultivated areas with cereals and their production from 2010 to 2019 showed an annual increase in cultivated areas but also in crops production. In 2020, according to data provided by INS (2021), in Romania, the area cultivated with cereals decreased by 2.4%, compared to 2019. Due to climatic changes especially the severe drought the production decreased by 37.6% with low yields for the vast majority of crops. Maize represents in Romania 55.8% of the total cereal production, wheat 35.2%, and barley 6.2%. The area cultivated with maize was the largest and the maize yields ranked second in the European Union (INS, 2021).
In recent years it has been observed in maize an extensive progress in breeding programs which led to a large number of new hybrids and different types of varieties selections (Bocianowski et al., 2021; Bițică, 2016).
Molecular markers and especially simple sequence repeats (SSRs) markers have wide applicability such as plant genetic diversity analysis, studies of germplasm conservation of maize genotypes (Vivodík et al., 2018), varietal purity or varietal identification (Chaudhary et al., 2018). In crop improvement strategies, SSRs markers are important tool for breeders in marker assisted selection (MAS) (Șuteu et al., 2014; Ahmad et al., 2017).
As a result of climate change and the growth of the world's population, it is becoming a general interest in selection and creating new maize hybrids showing valuable characters such as

drought or pests resistance (Sun et al, 2021; Li et al., 2016; Gaikpa et al., 2021), improved content in essential nutrients (Zawadi et al., 2021; Prasanna et al., 2020).
SSRs markers are preferred in research studies because they are codominant and have a high degree of polymorphism and reproducibility thus easy to identify by PCR technique (Raza et al., 2019; Vasile et al., 2020).
Selection of the most informative SSRs markers suitable for varietal purity assessment and genetic diversity of maize inbred lines chosen is the main purpose of this study.

MATERIALS AND METHODS

Plant material consisted of seeds from thirteen Romanian maize inbred lines (LC1-LC13) obtained from Central Laboratory for Quality of Seeds and Planting Material (LCCSMS), Romania. The seeds classes for these thirteen maize inbred lines used in this study were pre-basic second generation and basic seeds. In order to verify that the varietal purity is preserved for three of the maize inbred lines seeds from both pre-basic and basic categories were analysed.

DNA extraction

The DNA extraction was performed using the DNA extraction kit NucleoSpin Plant II (Macherey-Nagel) which contains two lysis buffers PL1 with CTAB and PL2 buffer with SDS. The DNA extraction protocol was adapted in order to meet the desired purity and concentration requirements for extracted DNA. The buffer chosen for DNA extraction in the present study was PL1 buffer based on CTAB. In order to highlight the fact that the choice of the extraction method is the correct one, an experimental plan was made which consisted in DNA extraction from dry maize seeds from all maize inbred lines and embryo and plant material resulting from maize seed germination for three of the maize inbred lines. Thus, after homogenizing the seeds samples chosen for testing a number of 3-5 seeds from each of the thirteen maize inbred lines were ground and about 40 mg of ground powder was transferred to a sterile microcentrifuge tube and PL1 buffer was added. The mixture was vortexed thoroughly and RNase A solution was added. The amount of buffer was also adjusted, thus increasing the amount recommended by the manufacturer for a better homogenization of the mixture. Incubation time at 65°C was increased from 10 to 30 mins. After centrifugation, the lysates were cleared by filtration using kit column and mixed with PC binding buffer. The mixture was loaded on a silica membrane column and the contaminants were removed by washing the column three times with kit wash buffers. The genomic DNA was eluted with kit elution buffer containing 5 mM Tris/HCl, pH 8.5 and frozen at -20°C for longer storage.
DNA extraction from maize inbred line LC9, LC10 and LC11 was also made of 1-4 ground embryos. The seeds were placed in distilled water at room temperature for 2 hours after which the embryos were grounded.
In order to perform DNA extraction from germinated seeds 3-5 seeds from maize inbred lines LC9, LC10 and LC11 were covered with filter paper soaked in water. Germination was performed at a temperature of 26-28°C and the plant material for DNA extraction was taken after 48 hours and the germinated material was ground. In both cases, the DNA extraction followed the same steps mentioned above.
The concentration and quality of the extracted genomic DNA was assessed by spectrophotometry using the Biochrom Biowave DNA UV-Vis spectrophotometer. DNA amplification was verified by performing a PCR endogenous assay using primers to detect maize *hmg* (high mobility group) reference gene (Bonfini et al., 2012).

SSRs markers

Eight SSRs markers were chosen for this study in order to assess varietal purity and genetic diversity for the thirteen Romanian maize inbred lines. These SSRs markers are recommended as suitable for verification of maize varieties (ISTA, 2021). The SSRs markers, PCR primers sequence and approximate allele size range obtained for SSRs markers used in this study is presented in Table 1 (ISTA, 2021; Woodhouse et al., 2021).

Table 1. SSRs markers and PCR primers sequence

SSRs marker	Forward	Reverse	Approximate allele size range (bp)
umc1545	GAAAACTGCATCAACAACAAGCTG	ATTGGTTGGTTCTTGCTTCCATTA	70-96
umc1448	ATCCTCTCATCTTTAGGTCCACCG	CATATACAGTCTCTTCTGGCTGCTCA	160–190
umc1117	AATTCTAGTCCTGGGTCGGAACTC	CGTGGCCGTGGAGTCTACTACT	146–170
umc1061	AGCAGGAGTACCCATGAAAGTCC	TATCACAGCACGAAGCGATAGATG	100–120
phi109275	CGGTTCATGCTAGCTCTGC	GTTGTGGCTGTGGTGGTG	150-160
phi102228	ATTCCGACGCAATCAACA	TTCATCTCCTCCAGGAGCCTT	140-170
phi083	CAAACATCAGCCAGAGACAAGGAC	ATTCATCGACGCGTCACAGTCTACT	143-166
phi015	GCAACGTACCGTACCTTTCCGA	ACGCTGCATTCAATTACCGGGAAG	89-121

(ISTA, 2021; Woodhouse et al., 2021)

PCR conditions

In order to be able to choose the best PCR reaction conditions, optimizations of the PCR reaction were made. Thus, the final primer concentration was varied from 1 µM to 0.6 µM in the final mix also the final PCR reaction volume was adjusted from 20 µL to 15 µL for SSR marker umc1545. For all SSRs markers a temperature profile was created in order to choose the optimal annealing temperature. PCR products amplification was done in BIO- RAD T100^{TM} Thermal Cycler system. The annealing temperatures chosen for testing were 61°C, 60°C, 59°C, 57°C and 56°C.

PCR reaction components and final concentration chosen for all SSRs markers was 1x Green GoTaq® Flexi Buffer, upstream and downstream primers 0.6 µM, 0.2 mM PCR Nucleotide Mix 10mM, 1.5 mM $MgCl_2$ solution 25mM, 0.5U GoTaq G_2 Hot Start DNA Polymerase (Promega). Additional reagents: nuclease-free water up to 15µL final volume and about 60-90 ng/µL template DNA.

The thermal cycling profile for PCR products amplification with SSRs markers were: initial denaturation 5 min at 95°C, denaturation 30 s at 95°C, annealing 30 s at 59°C for SSR marker umc1545 and 60°C for the remaining selected SSR markers, extension 30 s at 72°C, 35 cycles, final extension 5 min at 72°C.

To verify DNA amplification a different PCR reaction mix was performed containing PCR mix composed of 1x Fast Start PCR Master (Roche) a ready-to-use hot start PCR mix, 0.3 µM upstream and downstream primers final concentration, 5 µL template DNA and nuclease-free water up to 50µL final volume. The thermal cycling profile was initial denaturation 4 min at 95°C, denaturation 30 s at 95°C, annealing 30 s at 60°C, extension 1 min at 72°C, 37 cycles, final extension 7 min at 72°C. Fragments separation and highlighting of the resulting PCR amplification products was performed by agarose gel electrophoresis. The agarose gel concentration was between 2.4 and 2.8 % agarose (Agarose I™, VWR Life Science) in 1X TAE (TAE Buffer, 10X, Molecular Biology Grade/ Promega).

For nucleic acid visualization in agarose gel Red Safe™ Nucleic Acid Staining Solution (Intron) and ECO Safe Nucleic Acid Staining Solution was used. The power supply was provided by Consort EV243, the migration being performed at a voltage between 64-67 V. The migration time was between 1h 45 min and 2 hours. PCR products were visualized in UV light using Vilber Lourmat E-BOX VX2 imaging system.

RESULTS AND DISCUSSIONS

An important first step when using molecular biology methods is that the chosen DNA extraction method to result in a genomic DNA which meets the methods desired concentration and purity requirements. As mentioned in other studies the DNA requirements when using SSR markers are small amount of DNA. The genomic DNA does not require high purity ratio (Raza et al., 2019; Vasile et al., 2020).

As stated before the chosen buffer for DNA extraction in the present study was CTAB based lysis buffer and variations were only related to DNA extraction from dry maize seeds, embryos and plant material resulting from maize seed germination. These variations were applied to

only three of the maize inbred lines tested (LC9, LC10 and LC11).

Following the analysis of the spectrophotometric data resulting from the extracted DNA evaluation, it was found that the lowest values of concentration were obtained after DNA extraction from embryos, an average yield of 15 ng/µL. Given the fact that the concentration values are heterogeneous and there are insufficient data to exclude this type of approach an optimization of the extraction method from embryos is needed.

The highest concentration values were obtained after extraction from plant material resulting from seed germination, namely an average yield of 94 ng/µL. This type of approach was not considered appropriate for the present study because it requires a longer time for DNA extraction.

DNA extraction from dry maize seeds has proven to be the fastest and most effective method. The DNA yield for all thirteen maize inbred lines used in this study was between 28-57 ng/µL. Even if the DNA concentration is not very high compared to the DNA extraction after seeds germination it proved to be sufficient for PCR amplification.

Regarding the purity ratio for all measurements, it could be seen that the ratio was 1.8 for A_{260}/ A_{280} ratio and 1.94 for A_{260}/ A_{230} ratio.

Verification of the extracted DNA was performed in this study by amplifying the extracted DNA with specific primers for *Zea mays* reference gene (*hmg*).

All the resulting PCR products showed amplification regardless the extraction method. Figure 1 shows the 79bp amplification products for maize *hmg* (high mobility group) reference gene, for the PCR products obtained by amplifying the extracted DNA from dry maize seeds, embryos and plant material resulting from maize seed germination.

Although the data analysis related to DNA concentration showed that there are very heterogeneous values between the three extraction methods, no significant differences were observed for PCR products obtained by amplifying the extracted DNA with *Zea mays* reference gene (*hmg*).

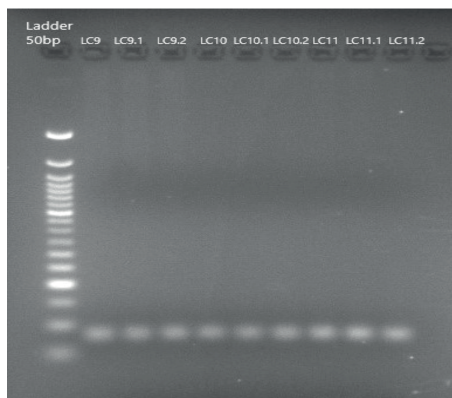

Figure 1. Agarose gel electrophoresis of PCR product obtained by amplifying the extracted DNA with *Zea mays* reference gene (*hmg*)
Legend: LC9-LC11 - PCR products obtained by amplifying the extracted DNA from dry seeds; LC9.1-LC11.1 - PCR products obtained by amplifying the extracted DNA from embryos; LC9.2-LC11.2 - PCR products obtained by amplifying the extracted DNA from seeds germination

Another important step when using methods based on SSRs markers is to optimize the working method in order to choose the right PCR conditions.

Choosing the most informative markers is also a priority for the success of a study (Raza et al., 2019; Vasile et al., 2020).

The eight SSRs markers chosen for this study are recommended as suitable for verification of maize varieties (ISTA, 2021).

In order to choose the best PCR conditions, a temperature gradient was created for all SSR markers.

No significant differences were observed in highlighting the amplification products at the chosen annealing temperatures. Figure 2 shows the results of PCR amplification products from two maize inbred line LC10 and LC11 with the umc1061 SSR marker at annealing temperatures of 61°C, 60°C, 59°C, 57°C and 56°C.

The final annealing temperature chosen for the umc1545 SSR marker was 59 °C and for the other SSRs markers 60°C.

Variations in primers concentration and final volume did not significantly influence product amplification, thus it was decided to reduce the concentration of primers and the final reaction volume for umc1545 SSR marker.

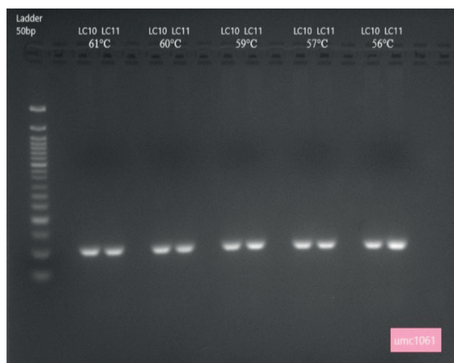

Figure 2. Agarose gel electrophoresis of PCR product obtained with umc1061 SSR marker at different annealing temperatures

In order to increase the specificity of the PCR reaction and to eliminate the risk of producing non-specific amplification products, it was decided to use a hot start enzyme in the PCR reaction.

Under the chosen conditions for testing all SSR markers presented good amplification products.

Another goal in this study was to characterize using SSRs markers the chosen thirteen maize inbred lines which will serve eventually as the seed parents to various maize hybrids.

The approximate allele size range (bp) obtained for all SSRs markers used in this study is presented in Table 1. The estimation of the values for the alleles obtained after PCR products electrophoresis running was made using the image analysis E-CAPT software.

After analysing the images, it was observed that all the selected markers showed some degree of polymorphism on the chosen maize inbred lines. Thus for SSRs markers umc1545, umc1448 and umc1117 four alleles were observed, for SSRs markers umc1061 and phi109275 two alleles were estimated, for SSRs markers phi102228 and phi083 three alleles were estimated and for SSR marker phi015 were estimated five allele. The highest number of estimated alleles was after using the SSR marker phi015. In Figure 3 we can observe the agarose gel electrophoresis of PCR product obtained with SSR marker phi015 for all thirteen maize inbred lines and the five alleles estimated (89, 112, 121,116, 97 bp) after image analysis with Vilber Lourmat, E-CAPT software.

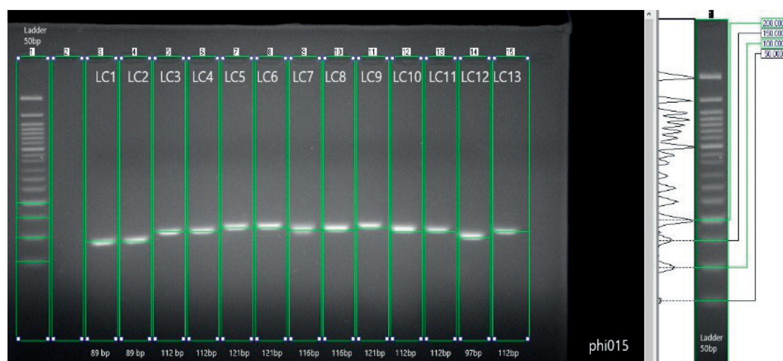

Figure 3. Agarose gel electrophoresis of PCR product obtained with phi015 SSR marker and approximate allele size range (bp) estimated after image analysis

As expected, the PCR products evaluation for maize inbred lines LC1 and LC2 (pre-basic second generation seeds and basic seeds) revealed high similarity with all SSRs markers used in this study. For maize inbred lines LC3, LC13 (pre-basic second generation seeds and basic seeds) and LC4, LC10 (basic and pre-basic second generation seeds) the PCR products evaluation of the eight SSRs markers, revealed a degree of similarity between 25-37% which may suggests some kind of contamination or an improvement of the initial inbred line desired by the breeder. After analysing the PCR products, high similarity was observed for inbred lines LC3 and LC13 with SSRs markers phi015, phi102228 and umc1061 and for LC4 and LC10 high similarity was observed with SSRs markers phi015 and phi102228.

For a better accuracy of determining the genetic purity of the seeds, it is recommended to analyse a larger number of seeds (Jhansi et al., 2015; ISTA, 2021).

Although maintaining genetic purity is an important factor in breeding process, genetic purity deterioration can occur due to various causes such as variations in plant adaptation to different environmental conditions or the influence of certain diseases, natural crossing but also due to precarious mechanical handling of seeds (Sendekie, 2020). It should be noted that the evaluation of the PCR products was performed in agarose gel and this method has its limitations (Sserumaga et al., 2014).

In order to assess genetic diversity for all thirteen maize inbred lines used in this study a dendrogram showing the relationship of maize inbred lines based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis was performed using Dice coefficient for comparison among sets of variables. For statistical data analysis a binary matrix was made, thus the amplified polymorphic bands were marked as present with "1" and absent with "0" and the dendrogram was generated using an online dendrogram construction utility (Garcia - Vallvé & Puigbo, 2009) retrieved from http: // genomes. urv. cat / UPGMA/. In Figure 4 is presented the relationship of maize inbred lines based on UPGMA cluster analysis.

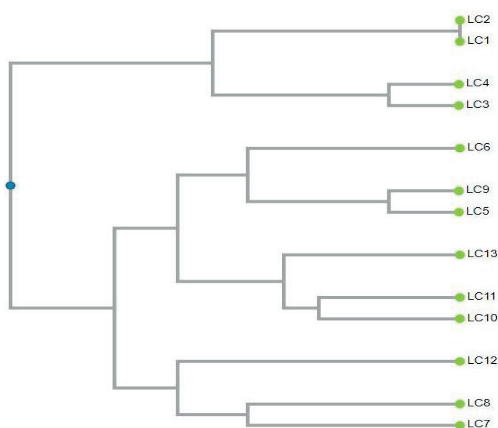

Figure 4. Dendrogram showing the relationship of maize inbred lines based on UPGMA cluster analysis

The dendrogram showed three clusters: cluster I consisting of LC7, LC8 and LC12, cluster II consisting from two sub-clusters (LC10, LC11, LC13 in sub-cluster II-1 and sub-cluster II-2 with LC5, LC9 and LC6) and cluster III consisting of LC1, LC2, LC3 and LC4.

Between inbred lines LC1 and LC2 can be seen 100 % similarity. High similarity it was also observed between maize inbred lines LC3 and LC4 and between inbred lines LC5 and LC9. Approximately 80% similarity was observed between maize inbred lines from LC1 to LC4 and between maize inbred lines LC5 to LC13. Evaluation of genetic relationships between different inbred lines can therefore be estimated using SSRs markers, being able to differentiate among closely related maize inbred lines (Sserumaga et al., 2014).

To better highlight the obtained allele it is possible to take into account to optimize the PCR reaction conditions as well as the use of much more SSRs markers.

Improvements can also be made in the way of highlighting the resulting products, namely the use of either a high resolution agarose instead of routine use agarose or highlighting the PCR products in polyacrylamide gel (Shiri, 2011; Adu et al., 2019; Zhang et al., 2021). Another effective way to analyse the data obtained after using the SSRs markers is capillary electrophoresis (Sserumaga et al., 2014; Tsonev et al., 2015; Bocianowski et al., 2021) which can reduce the possible risks related to results misinterpretation. However, it must be taken into account that using this method increases the final cost of the analysis.

The high genetic diversity of maize offers important opportunities for breeders in plant selection process where inbred lines are important in the development of hybrid varieties with high disease resistance, improved nutritional principles, higher yield or drought tolerance (Madobe et al., 2021; Oluwaranti et al., 2018; Zhang et al., 2021). That's why SSRs markers are important tools in assessing the varietal purity of maize inbred lines and the resulting hybrids in breeding process but also in the variety protection (Jhansi et al., 2015; Wani et al., 2017).

The availability of SSRs markers has played an important role in the development of the agricultural field, so for maize it was possible to observe an improvement in breeding programs as a result of the development of techniques that use molecular markers (Ahmad et al., 2017). The methods that use SSR markers have an advantage in terms of simplicity, efficiency, accessibility and reproducibility of the method,

so SSR markers are preferred in studies on testing the varietal purity and genetic diversity in maize and other crops (Sudharani, 2014; Jhansi et al., 2015; Wani et al., 2017; Adu et al., 2019).
In seeds quality control it is important to maintain or to confirm the genetic purity of an inbred line. Thus using methods based on SSRs markers or other techniques such as competitive allele specific PCR (KASP) or next generation sequencing (NGS) technology are very useful when phenotypic approach methods are far too laborious (Semagn et al., 2014; Raza et al., 2019; Vasile et al., 2020; Chen et al., 2021). The main problem is still the final cost of the implemented method and methods based on using SSRs markers are much more affordable than other modern technologies.

CONCLUSIONS

The chosen DNA extraction method was suitable and the extracted DNA met the desired criteria for the chosen PCR amplification method.
All SSRs markers chosen in this study gave good PCR amplification products under the tested reaction conditions and remained unaffected by variation made from initial conditions.
All the selected markers showed some degree of polymorphism on the chosen maize inbred lines. The highest number of estimated alleles was after using the SSR marker phi015 (five products were estimated) followed by markers umc1545, umc1448 and umc1117 (four alleles were estimated), markers phi102228 and phi083 (three alleles were estimated) and markers umc1061 and phi109275 (two alleles were estimated). Thus, the most polymorphic SSR marker of the eight SSRs markers used in this study was phi015 and the SSRs markers that showed low polymorphism were umc1061 and phi109275.
High genetic similarity was observed between lines LC1 and LC2 proving that that the varietal purity is preserved among seeds categories. Regarding varietal purity assessment among seeds categories for a better evaluation of the results much more seeds need to be analysed.
The SSRs markers turned out to be very useful tool for varietal purity and genetic diversity assessment being able to differentiate among closely related maize inbred lines and place the maize inbred lines into groupings based of genetic similarity.
Assessment of genetic diversity and varietal purity among different maize inbred lines can play an important role in hybrid maize breeding process.
This study can be a starting point for evaluating other maize inbred lines and selecting lines with desired traits in hybrid maize breeding program.

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