GENETIC POLYMORPHISM ANALYSIS OF SELECTED MAIZE LINES BY PCR–RAPD MARKERS FOR PREDICTION OF COMBINATIVE POTENTIAL OF INBRED LINES FOR HYBRIDIZATION WITH HIGH LEVEL OF HETEROSE

ANALÝZA GENETICKÉHO POLYMORFIZMU VYBRANÝCH LÍNIÍ KUKURICE POMOCOU PCR-RAPD MARKÉROV ZA ÚČELOM PREDPOVEDANIA KOMBINAČNÉHO POTENCIÁLU INBREDNÝCH LÍNIÍ PRE KRÍŽENIA S VYSOKOU ÚROVŇOU HETERÓZY

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We explored the genetic diversity between tested maize inbred lines (*Zea mays L.*). Six inbred lines came from three basic populations: Iodent Reid (Idt), Iowa Stiff Stalk Synthetic (SSS), and Lancaster Sure Crop (Lsc). Three of them are representative lines for Idt and SSS populations and the other three inbred lines are crosses between Idt × Lsc, Idt × Lsc × SSS and Idt × SSS. The target of this research was to detect a genetic polymorphism by three PCR–RAPD markers and based on results select an acceptable inbred combination for a crossing program, which produces experimental hybrids with a high level of genetic gain. Lines were diversified by cluster analysis UPGMA to three groups by using three different PCR–RAPD primers. Each group reflects a classification of line to the source population according to the origin. Similarity index (Nei, Li 1979) demonstrated a high level of similarity between individuals of the same inbred lines A and X. Low levels of similarity were observed between Y9 and A (0.25) with the use of PP1; between C7, B8 and X4 (0.00) using PP2 and between Z6, X4, X5 and A (0.4) using PP3. The analysis of genetic polymorphism by PCR–RAPD markers has proved the stability of tested lines and shows diversity between lines.

Key words: genetic polymorphism, maize, inbred line, PCR–RAPD markers

The prediction of a combinative potential of inbred lines entering hybridisation is a fundamental step of efficient maize hybrid production. A combinative potential from genetic polymorphism and genetic diversity between inbred lines can be determined. Heterosis is a phenomenon of commercial maize breeding and the result of combinative potential.

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The maize genome is a source of tremendous phenotypic and molecular diversity. Whether measured by allozymes, microsatellites (or Simple Sequence Repeats – SSRs) or DNA sequences, maize has long been known to be genetically diverse. On the DNA sequence level, exotic and elite maize genotypes contain more diversity than humans [2].

Populations' knowledge and lines genetic diversity is the keystone in breeding and germplasm preservation. The classification of elite germplasm into heterotic groups and assignment of inbred lines to established heterotic groups are major decisions in any breeding program for hybrid maize [9].

Random amplified polymorphic DNA (RAPD) is a technique for amplifying anonymous stretches of DNA, using PCR (PCR–RAPD) with arbitrary primers [5].

In the past five decades, a large number of maize inbreds have been developed from a limited number of elite lines and elite line synthetics. This engenders the danger of a loss of genetic diversity and restricts the possibility of crosses between genetically divergent parents. Knowledge of the genetic relationship among breeding materials could help to avoid the great risk for an increasing uniformity in the elite germplasm and could ensure long-term selection gains [13].

Today in Slovakia, there is one breeding station (Zeainvent inc.) that deals with the development of inbred lines and maize breeding, selection, according to pedigree, knowledge of the relationship to the basic populations, and information about the combinative potential are the most frequently used genetic tools. Molecular genetics, specifically genetic polymorphism analysis of inbred lines by molecular markers, could be an interesting instrument to shorten the breeding time from the developing line to an excellent hybrid.

MATERIAL AND METHODS

Plant material and the origin of plant material

Genetic polymorphism analysis was carried out on six maize inbred lines which came from three basic populations Iodent Reid (Idt), Iowa Stiff Stalk Synthetic (SSS), Lancaster Sure Crop (Lsc). Two of them are representative lines for Idt and one inbred line is typical for the SSS population. The rest of the lines are crosses between Idt × Lsc, Idt × Lsc × SSS and Idt × SSS.

Iodent Reid (Idt) is a population developed from the Iodent Reid population, which was founded in 1909. The target of this breeding program was the selection for the best combinative potential to Lsc.

Iowa Stiff Stalk Synthetic (SSS) population comes from the Reid Yellow Dent population and was developed in four decades of systematical breeding and selection for the stiff stalk trait.

The *Lancaster Sure Crop (Lsc)* population comes from outcross dent cultivars. The goal of the breeding program was to select individuals characterised by a longer cob and a strong connection to stalk, to overcome harvest losses.

Plant material and DNA extraction

Biological material was used – private inbred material from Sempol Trnava, Slovakia and the publicly available line B84, originally from the USA. In total six maize inbred lines were analysed. Line A which comes from Iodent Reid population (Idt) was represented by one tested sample A in three repetitions (A1, A2, and A3). Lines X and Z come from the Iowa Stiff Stalk Synthetic (SSS). The first SSS sample was represented by inbred X in two repetitions (X4, X5) and the second SSS sample Z like one repetition (Z6). The other tested lines (C7, B8, Y9) originated from an experimental crossing between populations Iodent Reid, Iowa Stiff Stalk Synthetic and Lancaster Sure Crop. C7, B8, Y9 and were used only in one repetition (tab. 1).

Biological material was prepared in *in vitro* conditions on MS cultivation medium [15]. Seeds were cultivated at a constant temperature and constant light conditions. On the fifth day of cultivation the samples were taken.

DNA was isolated from the fresh leaf tissue of maize by the R o g e r s and B e n d i c h method [18]. The quantity and quality of isolated DNA was compared with known

T a b l e   1

Genetic origin of biological material

Synthetic population	Inbred line	Tested samples
Idt	A	A_1 A_2 A_3
SSS	X	X_4 X_5
	Z_6	Z_6
Idt × LSC	C_7	C_7
Idt × LSC × SSS	B_8	B_8
Idt × SSS	Y_9	Y_9

Idt – population Iodent Reid, SSS – population Iowa Stiff Stalk Synthetic, LSC – population Lancaster Sure Crop

DNA (Human genomic DNA, 245 ng $\times$ μl^{-1}) using 0.8 % (w/v) agarose (3:1, Amresco) gel. The final concentration was adjusted to 10 ng $\times$ μl^{-1}.

PCR–RAPD reaction and electrophoresis conditions:

We optimised each condition of PCR–RAPD reaction and we checked the stability of the conditions three times before the start of this project. By doing this, we ensured the reproducibility of results. We tried three primers to search the particular level of phylogenetic diversity. Each time we set up a reaction with the same conditions of PCR mix and with the same conditions of thermocycler. There was just one factor that changed: the type of primer.

In our case we started the reaction with six inbreds. Within inbred line A, tree samples were analysed in each reaction as repetition. Within inbred line X two samples were analysed in each reaction as repetition. Other inbreds were analysed in the form of one sample.

For the preparation of 25 μl PCR reaction, the master mix of 1 × PCR buffer (10 × PCR containing $MgCl_2$; Invitrogen™), 0.5 U Taq polymerase (Taq DNA Polymerase Recombinant 2U $\times$ ml^{-1}; Invitrogen™); 3 mmol $\times$ dm^{-3} $MgCl_2$ (50 mmol $\times$ dm^{-3} Magnesium Chloride; Invitrogen™); 0.24 mmol $\times$ dm^{-3} dNTP (100 mmol $\times$ dm^{-3} dNTP

of each; Promega™), Nuclease-Free Water and 40 ng $\times$ ml^{-1} of DNA were used.

Following PCR–RAPD primers PP1 5´ GGGA-GAGGGA 3´; PP2 5´ CCGCAGTCTG 3´; and PP3 5´ ACATCGCCCA 3´ like decamers where used. The time and temperature profile of the reaction was as follows: 2 minutes at 94 °C for the first denaturation followed by 44 PCR cycles: (1 minute at 94 °C for denaturation, 1 minute at 36 °C for primer annealing, 2 minutes at 72 °C for amplification); and 7 minutes at 72 °C final amplification.

For each reaction negative controls were prepared. Reactions proceeded in the thermocycler PTC – 150 minicycler (MJ Research). The PCR–RAPD products were subjected to horizontal electrophoresis at a constant voltage (60 V) and amperage (30 mA) during 5 hours on 2 % agarose gels, containing ethidium bromide (0.5 μg $\times$ dm^{-3}) and visualised under UV light.

The gels were recorded by the KODAK EDAS 290 documentary system under UV radiation. The amplified DNA fragments size was identified by comparison to DNA marker (250 bp; Invitrogen) and evaluated by the picture analytical system Kodak 1D.

Genetic distance analysis

The presence or absence of DNA fragments was determined in form of binary matrixes and consequently generated by MS Excel.

The N e i and L i [16] similarity index based on PCR–RAPD profiles were calculated in the Microsoft Excel Application according to the SI_{NL} = 2 x sum of the common bands A and B / (sum of the bands in the lane A + sum of the bands in the lane B). Then the genetic distance values were calculated according to the clusters in compliance with the averages of Euclidian distance for the objective position of PCR–RAPD in the statistical program SYNTAX. The dissimilarity of every single cluster is showed by the Euclidian Distances Averages of Clusters (EDAC).

RESULTS AND DISCUSSION

The origin of inbred lines was estimated by studding pedigrees and multi-annual monitoring

M A1 A2 A3 X4 X5 Z6 C7 B8 Y9 N.C.   M A1 A2 A3 X4 X5 Z6 C7 B8 Y9 N.C.

2000   2000

1750   1750

1500   1500

1250   1250

1000   1000

750   750

500   500

250   250

Fig. 1. PCR–RAPD profile of maize inbred lines using primer PP1
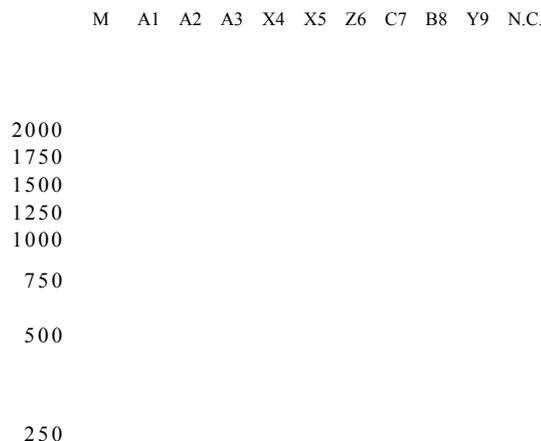N.C. – negative control; M – marker; A1, A2, A3, X4, X5, Z6, C7, B8, Y9 – inbred lines

Fig. 2. PCR–RAPD profile of maize inbred lines using primer PP2
N.C. – negative control; M – marker; A1, A2, A3, X4, X5, Z6, C7, B8, Y9 – inbred lines

during the selection of the breeding material.

From PCR–RAPD products (fig. 1, 2, 3) amplified by primers PP1, PP2 and PP3 the matrixes of present and absent DNA fragments were established, based on whatever the distance indexes has been determined.

Inbred lines A1, A2, and A3 (Idt) are samples of the one line A (genotype), and this might explain their high genetic similarity as well as their position in a dendrogram. Also lines X4 (B84) and X5 (B84) originate from one common line X (B84) of the SSS population. Line Z6 (SSS), is characterised by the highest level of genetic dissimilarity to line A (Idt), however Z6 is genetically the most similar to line X (B84). Line Y9 (Idt ×SSS) is genetically similar to lines C7 (Idt × LSC) and B8 (Idt × LSC × SSS). Line C7 (Idt × LSC) is genetically similar to line A (Idt), but genetically dissimilar to lines X4, X5 a Z6 (SSS). The origin of line B8 (Idt × LSC × SSS) was confirmed by its position between lines C7 (Idt × LSC) and Y9 (Idt × SSS).

Dendrogram (fig. 4) shows that lines A1, A2, A3 (Idt), C7 (Idt × LSC), B8 (Idt × LSC × SSS), Y9 (Idt × SSS) create one group marked as (I). The second group (II) consists of inbred lines: X4, X5, Z6 (SSS).

The first group consists of two sub-classes (Ia) and (Ib). Inbred lines A1, A2, A3 belong to (Ia) with majority rate Idt germplasm and C7, B8, Y9 belong to (Ib) as (Idt × LSC) and (Idt × LSC × SSS) hybrids. Inbred line with crossbreed source (Idt × LSC) is situated nearer to under-class (Ia), crossbreed (Idt × LSC × SSS) is in the middle of dendrogram and crossbreed (Idt × SSS) is situated nearer to inbred lines with SSS germplasm.

The knowledge of genetic similarity and genetic dissimilarity is meaningful for practical breeding. Based on genotype cluster analysis a hybridisation programme of selected maize inbred lines might be deduced. Because of the genetic dissimilarity of genotypes of the SSS and the Idt population, the mutual hybridisation might be considered.

Based on the genetic polymorphism analysis we recommend these models of hybridisation: A × Z6, A × X (B84) and C7 × Z6.

The relationship between genetic diversity and heterosis in maize is narrowly connected with maize hybrids construction [12].

Based on the comparison of standard breeding techniques and molecular biology techniques like PCR–RAPD markers, we can consider that PCR–RAPD markers are a useful technique for determining the genetic dissimilarity between inbred lines. C a r v a l h o et al.

M A1 A2 A3 X4 X5 Z6 C7 B8 Y9 N.C.

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2000
1750
1500
1250
1000

 750

 500


 250
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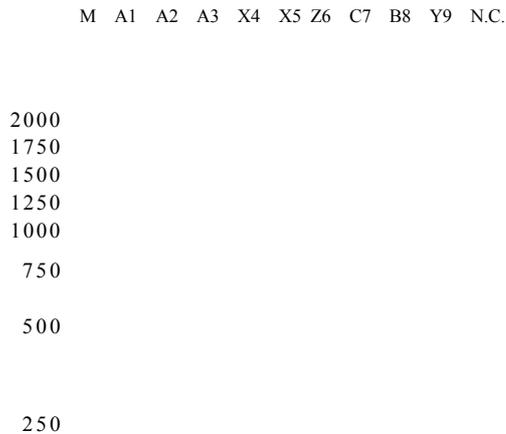
Fig. 3. PCR–RAPD profile of maize inbred lines using
         primer PP3
         N.C. – negative control; M – marker; A1, A2,
         A3, X4, X5, Z6, C7, B8, Y9 – inbred lines

Fig. 4. UPGMA dendrogram of the unweighted pair-
         group method with arithmetic averaging cluster
         analysis revealed by random amplified poly-
         morphic DNA marker technique
         Idt – Iodent Reid, SSS – Iowa Stiff Stalk Syn-
         thetic, Lsc – Lancaster Sure Crop, Crossed lines
         Idt × Lsc, Idt × Lsc × SSS and Idt × SSS.

[4] used PCR–RAPD markers to specify genetic distances between maize landraces. Study from L a n z a   et al. [11], confirmed the usefulness of PCR–RAPD markers as a help for the traditional breeding program, where thousands of crosses have to be done and F1 grain yield has to be evaluated in experimental designs. According to their results, PCR–RAPD-based genetic distance could be used to help in the choice of the crosses to be made among tropical maize lines derived from a broad genetic base population. In this way the number of evaluated single cross hybrids can be reduced. In the study by H a h n et al. [8] 51 maize inbreed lines were assayed, which were widely used for the production of commercial hybrids in Central and Northwestern Europe and had been previously analysed by PCR–RAPD.

PCR–RAPD is a simple and fast technique, but very sensitive for setting conditions of reaction. This is the reason for precise optimisation. Molecular markers like PCR–RAPD markers show us a particular view to polymorphism. Due to genetic polymorphism searching by more types of markers or more types of molecular techniques in one project, it is possible to obtain more exact results and less biased information.
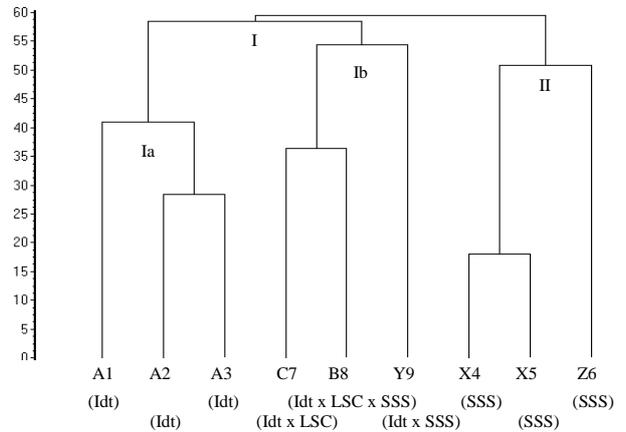
G a r c i a   et al. [6] studied the com-parison of PCR–RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical maize inbred lines. Comparative analysis was used in a project made by B u d a k et al. [3] based on a phylogenetic relationship using PCR–ISSRs, PCR–SSRs, PCR–RAPDs, and PCR–SRAPs.

The choice of used primers in our study was arbitrary. The number of used primers can be specified according to the requirements of exactness and the authenticity of results. G a r c i a et al. [6] used 32 primers for the polymorphism study of 18 inbred lines.

We applied three PCR–RAPD primers (PP1, PP2 and PP3). Based on electrophoretical separation of PCR products we were able to count bands and define a percentage of the monomorphic and polymorphic bands occurrence. During PCR–RAPD by PP1 primer, 41 bands were amplified in total, 25 % of them were monomorphic, and 75 % polymorphic ones. Analysis using PP2 primer detected 36 bands, 11 % of them were monomorphic, and 99 % polymorphic ones. By PP3 primer 69 bands were amplified in total, 31 % of them were monomorphic and 69 % polymorphic ones.

The dendrogram of genetic distance between selected maize inbred lines was made based on PCR–RAPD profiles. Inbred lines A (Idt) and Z6 (SSS) have the highest genetic dissimilarity,

according to the dendrogram. This fact supports the breeders practice and conception about genetic dissimilarity between inbred lines from Idt and from SSS.

There are more areas of research in molecular genetics where PCR–RAPD markers show genetic polymorphism. Y a n g et al. [20] used PCR–RAPD markers for genetic analysis to find plants which are resistant to stalk rot in maize. For this trait, gene *Rfg1* is responsible.

S u e n a g a et al. [19] created a new linkage map by PCR–RAPD, RFLP, PCR–ISSR markers based on intervarietal crosses of wheat. O k a m o t o et al. [17] worked with PCR–RAPD markers on genes identification by cDNA. New markers were developed by Z h a n g et al. [21] using PCR–RAPD for marker assisted selection.

Except for PCR–RAPD there is the PCR–ISSR method which is valuable molecular tool used for the same purpose as ours. B e ž o et al. [1] evaluated genetic distance into populations of potatoes, flax and barley. Based on the PCR–ISSR analysis they were able to be identified and classified as unknown genotype (without pedigree) into a pool of breeding material.

Integration pedigree analyses with DNA analyses enabled a more efficient estimation of combinational potential tested inbred lines without the secondary aspects of the environment. By that approach, testing of inbred lines is shortened and the information about genetic diversity can be directly used by the breeder for hybridisation.

Other new studies which have a focus on genetic diversity are based on transposition. L a i et al. [10] uncovered that those structural differences between maize lines are due to helitron transposition. The authors also established that the genes that have been captured and moved by helitrons appear to be predominantly pseudogenes.

M o r g a n t e et al. [14] found out that helitrons cause structural polymorphism. 20 % of gene fragments were not found in syntenous locations in two maize inbred lines due to helitron activity.

G u o et al. [7] studied allelic variation in the gene expression by using F1 (hybrid) individuals. In hybrids, the two alleles share a common genetic background, thus eliminating background effects and transacting factors as contributors to observed variation. Eleven of the fifteen genes exhibited allelic variation in expression. The authors propose that widespread differences in allele expression could contribute to heterosis.

CONCLUSION

The significant relationship between inbred lines has been found. Based on PCR–RAPD markers, the estimation of the lines origin has been confirmed.

Based on these results, is possible to state that the PCR–RAPD method is suitable for the practical determination of genetic polymorphism among maize inbred lines.

A low level of genetic dissimilarity was demonstrated between individuals of the same inbred lines A (A1, A2, A3) and X (X4, X5). The highest genetic dissimilarity was observed between Y9 and A (0.75) using PP1; between C7, B8 and X4 (1.00) using PP2 and between Z6, X4, X5 and A (0.6) using PP3.

The deficit of the PCR–RAPD method (problem with reproducibility) we overcame by a precise optimisation of reaction. We made three assays and each with a new primer. Because of the same reaction settings we could compare variability between assays.

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SÚHRN

Bola sledovaná genetická rozdielnosť medzi testovanými líniami kukurice siatej *(Zea mays L.)*. Šesť línií pochádzalo z troch základných populácií: Iodent Reid (Idt), Iowa Stiff Stalk Synthetic (SSS), Lancaster Sure Crop. Tri z nich sú charakteristické línie pre populácie Idt a SSS a ďalšie tri línie sú krížence medzi Idt × Lsc, Idt × Lsc × SSS and Idt × SSS. Cieľom tohto výskumu bolo určiť genetický polymorfizmus pomocou troch PCR–RAPD prajmerov a na základe výsledkov vybrať prijateľné kombinácie línií pre program kríženia, ktorý produkuje

experimentálne hybridy s vysokou hladinou genetického zisku. Línie boli rozdelené do troch skupín pomocou zhlukovej analýzy UPGMA, použitím troch rôznych PCR–RAPD prajmerov. Každá skupina odráža zatriedenie línií do zdrojovej populácie, alebo podobnosť ku danej populácii. Index podobnosti podľa Nei a Li poukázal na vysokú podobnosť medzi jedincami tej istej línie A a X. Nízka hladina podobnosti bola pozorovaná medzi líniami Y9 a A (0,25) s použitím prajmera PP1; medzi C7, B8 a X4 (0,00) s použitím prajmera PP2 a medzi Z6, X4, X5 a A (0,4) s použitím prajmera PP3. Analýzy genetického polymorfizmu pomocou PCR–RAPD markérov dokázali stabilitu testovaných línií a poukázali na variabilitu medzi líniami.