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REGULAR ARTICLE

THE POTENTIAL OF ISSR MARKERS IN AMARANTH GAMMA-RADIANCE MUTANTS GENOTYPYING

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ABSTRACT

Amaranth is one of the re-discovered crops. Amaranth DNA polymorphism changes caused by γ-radiation in selected amaranth lines - *Amaranthus cruenthus* L. were observed using genotypes Ficha and Hybrid K-433 (the result of interspecific hybridization of species *A. hypochondriacus* × *A. hybridus*). Mutant lines are M8 generation positively selected plants for weight of thousand seeds. To determine the polymorphism between individual samples ISSR technique was used. Primer Ama1 detected interspecific and primer Ama2 revealed intra – and interspecific polymorphisms, too. Primer Ama1 distinguished only the cultivar Ficha and hybrid K-433 accessions. Primer Ama2 created more polymorphic DNA profiles and has the ability to distinguish not only Ficha cultivar and hybrid K-433, but mutant accessions among themselves. A total of 11-13 band levels for the primer Ama1 and 14-16 for the primer Ama2 were obtained.

Keywords: Amaranth, ISSR, inter- and intra specific variability.

INTRODUCTION

Genus *Amaranths* L. includes 60, according other authors 87 species and has status of healthy and re-discovered plant. **(Jeroboam *et al.*, 1997; Monica *et al.*, 2003)**. Great interest is oriented on amaranth like pseudocereal, with nutritional value comparable or better like cereals (protein 12-19%, lysine 5-7%), easily digestible starch, presence of cholesterol lowering fractions in seed oil, high carotene content in leaves (2 000 to 11 000 iu/ 100 gms), edible organic dyes, absence of gluten and absence of antinutritional factors like prolamins and gliadin. Thanks to these properties amaranth products could be widely used not only in diet for affected by celiac disease and phenylketonuria, but also like a source of exclusive BIO foods **(Matušová, 2008; Jamriška, 2006; Habánová & Habán, 2003; Mohinder, 1999)**. Amaranth includes wide group of freely growing, weed and also exploited plants, which originate from free hybridization to systematic breeding **(Kulakow & Jain, 1990)**. The goals of general amaranth breeding programs define these problems: high rate of heterozygosity, low heritability for some important traits and disease resistance especially in the case of amaranth (*A. caudatus,* L.). Breeding goals on the nutrition level include proteins, fats, starch, vitamins and organic dyes content and composition **(Gajdošová *et al.*, 2002)**. In last decade properties and possible usage of amaranth grain evoke interest among scientists. There was a lot of work done on the field of genetic diversity and evolutionary relationship study based on isozymes, allozymes and RAPD markers **(Chan & Sun, 1997; Hauptli & Jain, 1984; Pratt & Clark, 2001)**. In addition to hybridization method radiation mutagenesis is employed like effective breeding tool. FAO/IAEA database listed more than 2300 radiation-modified plants including amaranth.

Mutation technology was employed as a tool to create genetic variation in *Amarantus tricolor* in order to select lines with improved drought tolerance. The results of γ-radiance treatment were manifested in 1 mutant genotype with better grow vigor under the stress conditions and 2 mutant lines which retained more water in leaves under drought conditions compared to the wild type. Next research of mutant lines showed increased content of protein concentration. Also mutant lines were compared with wild type based on RAPD markers. From 19 arbitrary primers used, only two primer sets showed polymorphisms. The differences observed during the RAPD analyses of the two mutants as compared to the wild type, could be indicative of specific genomic areas possibly involved in drought tolerance **(Kgang, 2008)**.

Treatment by γ-radiation was used also for enhancing quality and quantity of amaranth grain of two selected genotypes: *Amaranthus cruentus* genotype 'Ficha' and hybrid K-433.

Thanks to positive selection which was performed from 2nd to 8th mutant generation several putative mutant lines of *A. cruentus* and hybrid K-433 were selected characterized by highly significantly increased weight of thousand seeds (in comparison with control) with an obvious tendency to stabilization of this trait. At those selected plants genetically fixed WTS and secondary yield increase can be expected **(Gajdošová, 2008)**.

Hricová *et al.* (2011) realized biochemical research of mutant lines *Amaranthus cruentus* genotype 'Ficha' and hybrid K-433. They states that nutritional value of selected mutant lines in comparison with untreated controls remain unchanged. The highest result for nutritional value was observed in mutant line C82/1.

Optimization of simple enzyme polymorphism analyses for genotype Ficha and mutant line of K-433 hybrid was described in **Múdry *et al.* (2011)** research. The best result was obtained in the case of phosphoglucomutase, where both genotypes were distinguished.

There is possibility to study the genome of mutant amaranth plants by molecular markers and by flow cytometry to distinguish γ-radiation induced changes on the molecular level. Molecular markers are used to gain DNA fingerprint (unique profile) of particular genotype, which characterize concrete genotype and allows studying genetic similarity and dissimilarity among tested genotypes.

The aim of the paper was to analyze unique DNA profile of 11 genotypes based on ISSR molecular markers and characterize differences among the DNA fingerprints.

MATERIAL AND METHODS

Mutant lines of amaranth (7 days plants) and control plants were used in analysis. The characteristic of mutant lines and their control samples is listed in the Tab 1. Individuals are characterized by statistically significant increase of weight of thousand seeds **(Gajdošová & Libiaková, 2002; Gajdošová *et al.*, 2005)**.

Table 1 Characteristic of mutant lines

Mutant lines	Origin of mutant lines
C 15/1, C 26/2, C 26/3,C 27/5, C 82/1, C 236/1, Control A	cultivar Ficha (*Amaranthus cruentus*)
D 54/1, D 279/1, D 282/1, Control B	hybrid K-433, result from interspecific hybridization of species (*A. hypochondriacus* x *A. hybridus*)

Genomic DNA was isolated using the isolation kit Invisorb® Spin Plant Mini Kit (Invitek). The obtained DNA was quantified by fluorometer (Qubit TM). PCR-ISSR technique was used for monitoring DNA polymorphism of mutation affected samples.

ISSR reactions were performed in a buffer solution 1 × PCR containing 100 mmol × dm^{-3} Tris-HCl (pH 8.8), 500 mmol × dm^{-3} KCl a 1.5 mmol × dm^{-3} $MgCl_2$ (Applichem), together with 0.08 mmol × dm^{-3} d NTP (Invitrogen™) 750 nmol × dm^{-3} Ama1 primer or 1000 nmol × dm^{-3} Ama2 primer (Table 2) (Microsynth), 1 U Taq polymerase (Applichem) and 50ηg of template DNA. PCR water was added into 15 µl final volume of reaction.

Primers used in ISSR reactions are listed in the Tab 2. Time and temperature profile of ISSR reaction is in the Tab 3.

Table 2 Characterization of applied primers

Name of primer	Sequences of primer	Characterization of primer
Ama1	$(GACA)_4$	unanchored, trimer
Ama2	$(GATA)_2\,(GACA)_2$	unanchored, tetramer

Table 3 Time and temperature profile of PCR-ISSR reactions

Steps of ISSR reaction	Time profile	Temperature profile	Number of cycle
Initial denaturation	3 minute	95 °C	-
Denaturation	15 second	95 °C	Depending on the primers*
Annealing	40 second	49.5 °C	
Extension	2 minute	72 °C	
Final extension	7 minute	72 °C	-
Cooling	10 minute	4 °C	-

*Primer Ama1: 29 cycles and primer Ama2: 32 cycles.

Amplified fragments were electrophoretically separated in agarose gel (Applichem) together with 1 × TBE and Gel Red 10 000 × (Biotium). To determine the profile changes of the multiplicated fragment the control plants were used. Electrophoresis was conducted at a voltage of 60V at 3h. Electrophoreograms were processed with documentation system G:Box in GeneSnap program - Product version: 7.09 (Syngene) and GeneTools - Product version: 4.01 (Syngene). ISSR profiles were evaluated for the presence and absence of amplification products using parameters listed in the Tab 4.

Table 4 Parameters for ISSR analysis evaluation

Integration parameters		
Method	Rolling disk - radius 30	
Peak detection	width	7
	heigh	3
	peak volume	min 1%
filter	type	Savitsky - Golay
	width	3

Efficiency of primers was evaluated on the basis of their amplification abilities. On the basis of separation of amplification products on agarose gel, the amount of fragments of each primer was determined.

On the basis of DNA fragments separated according to the size by electrophoresis, the matrix of presence and positions of DNA fragments was prepared. From matrix there were calculated similarity indices (SI, Similarity Index) - Jaccard's (1908) coefficient (GDJ) by the relationship: GDJ =1- [N11 / (N11 + N10 + N01)], where N11 is the number of bands–alleles present in both individuals; N10 is the number of bands–alleles present only in the individual i; N01 is the number of bands – alleles present only in the individual j. GDJ takes into consideration only matches between bands–alleles that are present and ignores pairs in which a band–allele is absent in both individuals.

Unweighted pair group method with arithmetic means UPGMA cluster analysis based on genetic similarity by the Jaccard similarity coefficient was measured.

RESULTS AND DISCUSSION

Results

The evaluation of characterizing and distinguishing primer properties and abilities was done. Amaranth accessions were analyzed using microsatellite markers of which both produced reproducible polymorphic banding patterns. A total of 11-13 band levels for the primer Ama1 and 14-16 for the primer Ama2 were obtained.

Primer Ama1 distinguishes only the cultivar Ficha and hybrid K-433 accessions without the ability to reveal the polymorphism among their mutant lines (figure 1), but the unique fragment in the figure 2 was synthetized for mutant accession for both, cultivar and hybrid genotypes, too.

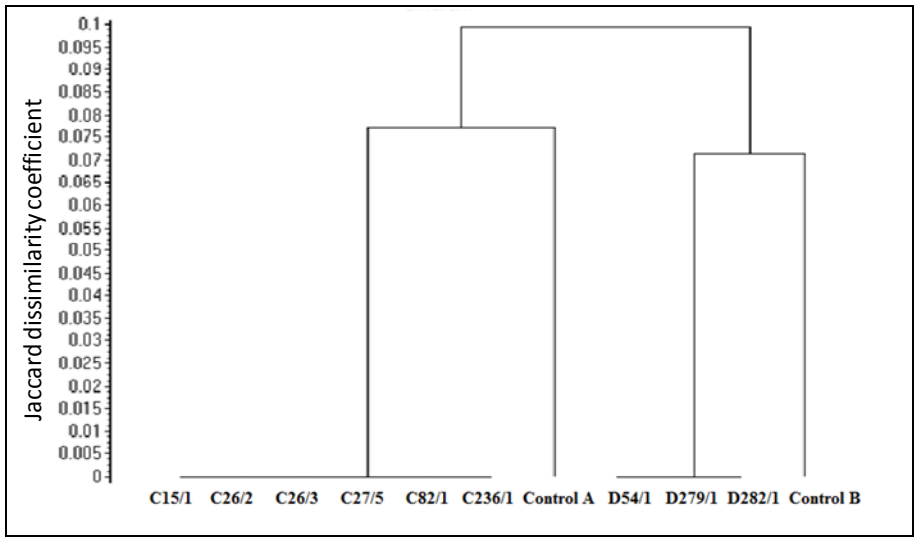

Figure 1 Dendrogram of Ficha accessions for Ama2 primer

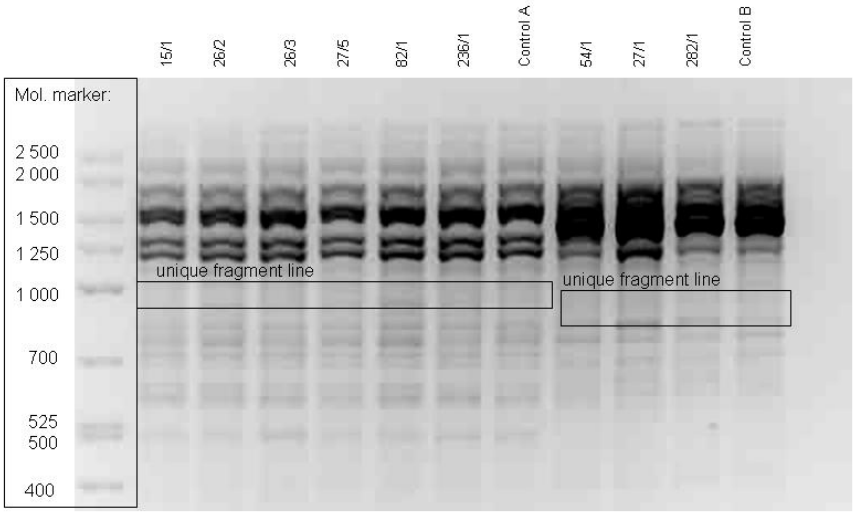

Figure 2 Ficha cultivar and hybrid K-433 - profile of Ama1

Primer Ama2 DNA fragments profile was more polymorphic and the primer has the ability to distinguish not only Ficha cultivar and hybrid K-433, but mutant accessions among themselves, too (figure 3).

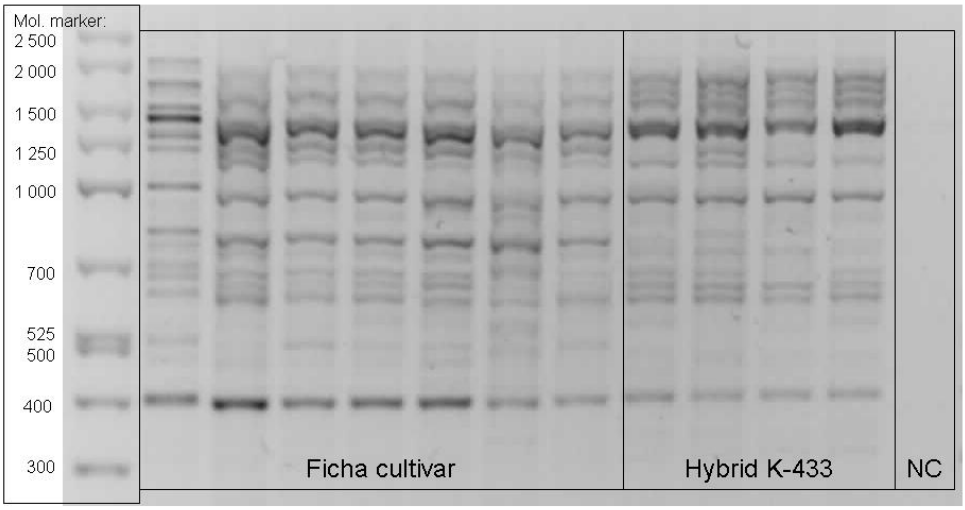

Figure 3 Electrophoreogram of tested Amarant accessions using the primer Ama2

The Jaccard simmilarity coefficient (1978) for the used marker system ranged in the spectrum of 0,526 - 1. Absolute certainty that two samples are genetically identical can only be obtained by comparison of their complete genomes. However, samples do not necessarily have to be completely identical in order to be considered redundant. For example, two samples collected from the same outcrossing population will have a very small probability to be completely identical, yet they will share a similar genetic backround. Therefore, we can consider redundancy by quantifying the genetic diversity between samples based on the screening of germplasm for a large number of polymorphic markers. Statistical tools may then be used to evaluate whether samples display sufficient genetic variation in order to consider them distinct. Variability of genetic coefficient values (Tab 5) and dendrogram clusters corresponds to the analysed intergenic space of Ficha mutant lines compared to Control A as well as mutant hybrid K-433 genotypes compared to Control B (Tab 6).

Table 5 Jaccard similarity coefficient for Ficha accessions based on Ama2 ISSR results

genotype	**C 15/1**	**C 26/2**	**C 26/3**	**C 27/5**	**C 82/1**	**C 236/1**	**Control A**
C 15/1	1						
C 26/2	0,579	1					
C 26/3	0,684	0,647	1				
C 27/5	0,650	0,611	0,722	1			
C 82/1	0,550	0,588	0,611	0,579	1		
C 236/1	0,526	0,563	0,588	0,556	0,529	1	
Control A	0,675	0,733	0,867	0,813	0,688	0,667	1

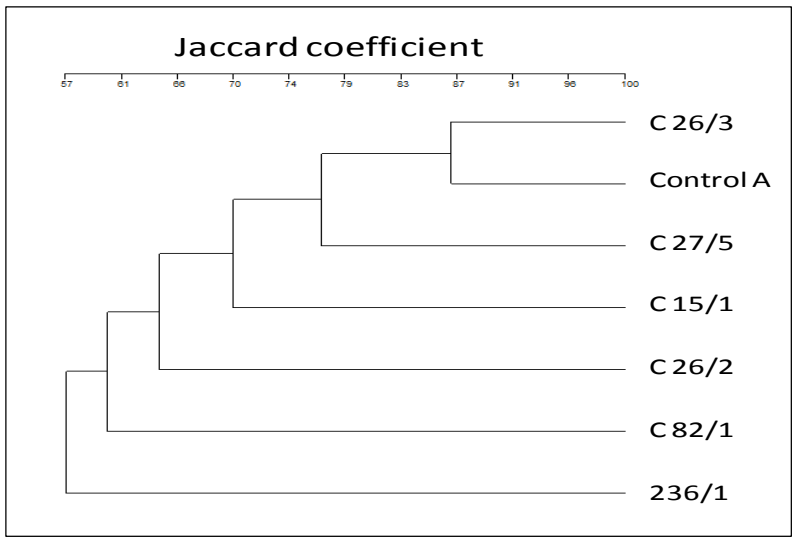

Figure 4 Dendrogram of Ficha accessions for Ama2 primer

Intergenic space polymorphism evaluated by Ama2 ISSR primer shows in the Ficha accessions dendrogram variability (figure 4) of tested mutans plants corresponding to the fact that they all are the offsprings and the only difference is the treatment of gamma-rays and the control genotype without this treatment. The Jaccard similarity coefficient was in all cases higher than 0,5 what shows thew high level of the same intergenic fragments in their intergenic space. As the most similar to the Control A was the C 26/3.

Table 6 Jaccard similarity coefficient for hybrid K-433 accessions based on Ama2 ISSR results

genotype	**D54/1**	**D279/1**	**D282/1**	**ControlB**
D54/1	1			
D279/1	0,867	1		
D282/1	0,929	0,813	1	
ControlB	0,867	0,765	0,933	1

Using Ama2 primer for hybrid K-433 accessions the results corresponds to those for Ficha. The Jaccard similarity coefficient is in the range of 0,765 - 1 and DNA fragment profile was polymorphic in only 4 band levels (figure 5).

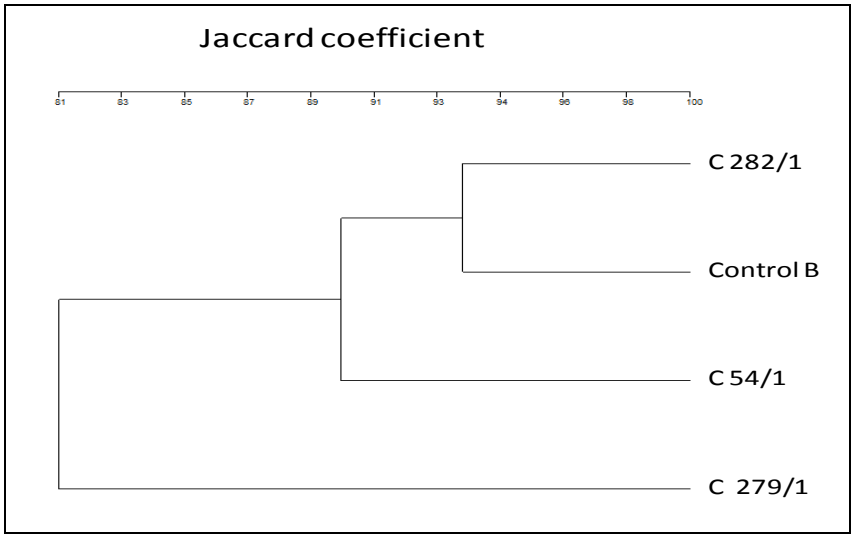

Figure 5 Dendrogram of hybrid K-433 accessions for Ama2 primer

Dendrogram based of Ama2 primer PCR results shows very high intergenic ISSR profile similarity of C 282/1 and Control B genotypes.

Discussion

Mutagenesis is widely used method in plant breeding. Breeders use mutagenesis to improve characters and properties of plants and to increase genetic polymorphism within plant genomes. Mutagenesis experiments were realized by **Morita *et al.* (2009); Agrawal *et al.* (2001)** with rice, **Ramesh *et al.* (2001); Zayats (2001)** with barley, **Das *et al.* (2001)** with potato, **Kgang (2008)** with amaranth **Gajdošová *et al.* (2002, 2005).**

Plant genome reaction on γ-radiation was similar in the case of amaranth, poppy and fenugreek. Expression of mutagenesis was incremenent of seed yield. Furthermore, in the case of poppy seed and fenugreek the content of morphine and diosgenin, were increased, too. However, there was not found any considerable change in biochemical seed composition due to γ-radiation in amaranth. **(Floria & Ichim, 2006a; Floria & Ichim, 2006b; Gajdošová *et al.*, 2005, 2008).**

We observed amaranth DNA polymorphism changes caused by γ-radiation in selected amaranth lines.

Different molecular methods are used to distinguish DNA polymorphisms of inter- and intra- species variability between plants. These techniques lead to population mapping. Moreover, mutation breeding produces population of genotypes, which need to be sorted according genetic diversity. Methods like RAPD, AFLP, ISSR, SSR, SNP, MFLP and

isozyme are being used for searching DNA polymorphism in mutant population and lead to accurate localization of genes and QTLs for the target traits **(Shu, 2008).**

Ray, Roy (2009) determined genetic diversity and relationships among *Amaranthus* species by RAPD and SCAR markers. RAPD primers yielded a total of 262 amplicons, of which 96.94% were polymorphic. A mean similarity coefficient among all the *Amaranthus* species was 0.56. This coefficient indicates variation, which exists among different populations. SCAR markers are useful for germplasm identification for conservation and defying amaranth ecotype. **Ranade *et al.* (1997)** evaluated inter- and intra-species variability present in the genus *Amaranthus* at the molecular level by RAPD molecular markers.

To determine the polymorphism between individual samples, we used PCR-ISSR technique as a suitable tool for the determination of the variability between related subjects, like authors, eg. **Souframanien (2002)**; **Joshi *et al.* (2004)**; **Joshi *et al*. (2004)** used PCR-ISSR technique to distinguish parental lines and hybrids in rice, bajra, sorghum and sunflower hybrids. Distinguishing ability of ISSR primers used in our project was similar to mentioned experience. First primer, Ama1, reveals inter-species DNA polymorphism between cultivar Ficha and hybrid K-433 without the ability to find DNA polymorphism among their mutant lines. DNA profiles were more polymorphic with Ama2 primer. Ama2 primer has the ability to observe inter-specific and intra-specific polymorphism, too. Consequently, Ama2 primer distinguished cultivar Ficha from hybrid K-433 and also there was found variability among mutants. In comparison, the examination of genetic diversity and relationships between cultivated and wild *Amaranthus* species using isozyme and RAPD markers high levels of genetic diversity were found between species, but genetic uniformity was observed among selected genotypes **Chan & Sun (1997)**.

The distinguishing ability of Ama1 and Ama2 primer depend on the properties of individual primer like **Ranade et al. (1997)** observed. They searched effectiveness of several RAPD markers. Out of the 65 primers tested, 40 primers produced polymorphic patterns. However, only 24 primers of these gave consistent RAPD profiles with all the genotypes tested; 16 primers resulted in inconsistent profiles. The remaining 25 primers entirely failed to give any amplification in more than half of the genotypes.

Kgang (2008) used mutation to create genetic variation in *Amarantus tricolor* with improved drought tolerance. Mutant lines were compared with wild type based on RAPD markers. From 19 primers used, only two primers showed polymorphisms between the amaranth wild type and the two mutant lines.

Joshi-Sacha & Gopalakrishna (2007) detected the polymorphism between a radiation induced *Sesbania rostrata* mutant and the parental genotype using three different marker systems. Of the 200 RAPD primers used, only 3% produced a polymorphism between the mutant and the parental genotype, whereas 12.5% of the AFLP primers and 15.7% of the ISSR primers produced polymorphisms. Similarly, ISSR method was used by **Nolan *et al.* (2010)** to find genetic diversity of *A. pumilus* populations. Genetic variation was detected among and within *A. pumilus* populations, according to the author´s statement found genetic variability was low. Due to its desirable characteristics in plant breeding trials, genetic variation within *A. pumilus* was also compared to variation of grain varieties *A. hypochondriacus*, L. and *A. cruentus*, L. From the results follows, that genetic diversity within *A. pumilus* was lower than either grain species. The observed genetic variability provides information about population and its background. Accordingly, similarity of mutant population in comparison to untreated control, from which mutant population comes from, is high. Jaccard similarity coefficient reveals 74% average similarity between mutant population and untreated control of Ficha cultivar and 86% average similarity between K-433 mutant population and untreated control. The same collection of amaranth mutant lines was tested for possible change in biochemical composition by **Hricová *et al.* (2011)**. Authors states that nutritional value of selected mutant lines in comparison with untreated controls remain unchanged. The highest result for nutritional value was observed in mutant line C82/1, which was 17 % higher than control average and 22 % higher than mutant lines average. Accordingly, γ-radiance has no big influence on studied intergenic and intragenic space of DNA. Further research should be done to find, which mechanism cause such visible change like is the change of weight of thousand seeds.

On the basis of knowledge obtained by analysis of polymorphisms of amaranth mutant lines and knowledge others authors, we can assume, that there is important to find out primers with suitable properties for examination of the particular level of DNA polymorphism, which need to be tested among accessions of mutant lines of amaranth.

The project of **Mallory *et al.* (2008)** determined and characterized a set of highly informative, reproducible microsatellite markers for the amaranths grains. A total of 179 microsatellites were polymorphic across accessions from the three grain amaranths (*A. hypochandriacus* L., *A. cruentus* L., and *A. caudatus* L.). From the thirty-seven used primers only 21% of them were polymorphic between the parents of a segregating population. The transferability of these markers to *A. hybridus*, *A. powellii*, S. Wats., and *A. retroflexus*,

L. is reported and suggests that these markers may be useful in studying other species within the genus *Amaranthus*, including several economically important weeds.

CONCLUSION

Comparing the ability of primers Ama1 and Ama2 to characterize and distinguish the cultivar or hybrid genotypes and mutant or control plants we can assume the potential of effective amaranth genotypes determination by ISSR. Amaranth accessions were analysed using microsatellite markers of which both produced reproducible polymorphic banding patterns and were able to distinguish both, the species specifity and mutant lines specifity, too. The calculated Jaccard similarity coefficient was in all cases higher than 0.5 what shows the high level of the same intergenic fragments in their intergenic space as the species-specific fingerprinting pattern.

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