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Using of Inter Microsatellite Polymorphism to evaluate gamma-irradiated *Amaranth* mutants

J. Žiarovská*, K. Ražná and M. Labajová

Department of Genetics and Plant Breeding, Slovak University of Agriculture, Tr. A. Hlinku 2, 94976, Nitra, Slovak Republic

Abstract

Molecular markers ISSR were used to analyse γ-irradiated mutants of amaranth Ficha cultivar and K-433 hybrid; tested six microsatellite - primed PCR markers. First analysis of gamma-irradiated amaranth mutant lines was done using the ISSR. In the study, primers that distinguish the mutant lines from control plant were reported as well as primer with the differentiation ability for all analysed mutant lines. The very specific changes in the mutant lines´ non-coding regions based on inter microsatellite length polymorphism were analysed. Mutant lines of the Ficha cultivar (C15, C26, C27, C82, C236) shared a genetic dissimilarity of 0.16 and their ISSR profiles were more similar to the Ficha than those of K-433 hybrid mutant lines. The K-433 mutant lines (D54, D279, D282) shared genetic dissimilarity of 0.22 but are more distinct to their control plant as a whole, as those of the Ficha mutant lines. Different ISSR fingerprints patters of the mutant lines when compared to the Ficha cultivar and K-433 hybrid ISSR specific profiles may be part of a consequence of the complex response of the intergenic space of mutant lines to the gamma-radiance.

Key words: Amaranth, Ficha, ISSR, K-433, Mutant lines

Introduction

The genus *Amaranthus* is originated in the Americas, consists of 60–70 species and including 3 cultivated grain species (*A. caudatus*, *A. cruentus*, and *A. hypochondriacus*). Three ancient cereal *Amaranthus* species, *A. caudatus*, *A. cruentus* and *A. hypochondriacus* are nowadays widely studied and cultivated worldwide because of their exceptional nutritional value of both seeds and leaves (Brenner et al., 2000). Molecular genetic cluster analysis could help in defining the genetic similarities among *Amaranthus* species as well as among the lines. Previous molecular genetic analyses of *Amaranthus* species have been designed to determine ancestors of domestic species by using single marker system (Sun et al., 1999; Wetzel et al., 1999a). Using amplified fragment length polymorphism (AFLP) markers, Vos et al. (1995) reported an unweighted pair group method with arithmetic mean analysis (UPGMA) of *Amaranthus* accessions representing agronomically important weedy *Amaranthus* species from a wide geographical area to determine genetic similarity and potential for interspecies hybridization.

Different molecular markers including random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and amplified fragment length polymorphism (ALFP) have been used for more accurate study of genetic diversity and phylogenetic relationships between *Amaranthus* species (Xu and Sun 2001; Štefúnová and Bežo, 2002, 2003; Wassom and Tranel 2005; Lee et al., 2008; Ray et al., 2008; Popa et al., 2010).

ISSR is a source of genetic markers that was previously used for amaranth germplasm assessment (Xu and Sun 2001; Ray and Roy, 2007) and have higher reproducibility than RAPDs (Meyer et al., 1993; Fang and Roose, 1997). The conventional ISSR involves PCR amplification of DNA with a single primer composed of simple sequence repeats (SSRs; or microsatellites). Microsatellites are abundant throughout the eukaryotic genome, evolve rapidly and are widely used in plant germplasm evaluation for well characterized species as well as for underutilized ones (Balážová et al., 2007; Nováková et al., 2010;

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*Corresponding Author

J. Žiarovská
Department of Genetic and Plant Breeding, Slovak University of Agriculture, Tr. A. Hlinku 2, 94976, Nitra, Slovak Republic

Email: jana.ziarovska@uniag.sk

Svobodová et al., 2011). Microsatellite-anchored primer amplifies the region between two closely spaced, oppositely oriented SSRs, and the amplified products are separated by agarose gel electrophoresis. The resultant PCR fragments usually range from 0.3 to 2 kb in size and are often limited in number. Further improvement of the efficiency of this marker system can be achieved by double-primer fluorescent ISSR (Huang and Sun, 2000). Two SSR primers labeled with fluorescein Cy5 are added in each PCR amplification in this approach. Such modified ISSR generates a large number of DNA fragments with only a few pairs of primers and the technique became comparable to the efficiency of AFLP (Huang and Sun, 2000).

In the present study, ISSR technique was used for fingerprints characterization of the amaranth mutant lines. Mutation breeding is still one of the conventional breeding methods for plants. It is relevant with various fields like, morphology, cytogenetics, biotechnology, and molecular biology. Mutation breeding has become increasingly popular in recent times as an effective tool for crop improvement (Acharya et al., 2006) and an efficient means supplementing existing germplasm for cultivar improvement in breeding programs. FAO/IAEA database listed more than 2300 radiation-modified plants including amaranth. Mutation technology was employed as a tool to create genetic variation in *Amaranhtus tricolor* in order to select lines with improved drought tolerance. The results of γ-radiance treatment were manifested in 1 mutant genotype with better growth vigor under the stress conditions and 2 mutant lines which retained more water in leaves under drought conditions compared to the wild type. Another research of mutant lines reported by Kgang (2008) showed increased content of protein concentration. 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). For plant species, γ-radiation is mainly used for breeding or research purposes (Majer and Hidek, 2012; Rebodero and Lidon, 2012). 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. Positive selection was performed from 2nd to 8th mutant generation of several putative mutant lines of *A. cruentus* and hybrid K-433 and characterized which significantly increased weight of thousand seeds (Gajdošová et al., 2008).

Mutant lines and molecular approach selected by Gajdošová et al. (2008) were used in this study. The aim of the study was to perform the ISSR for amaranth mutant lines and their control genotypes. ISSR profiles were compared with the aim to investigate the consistency of the selected markers across individual mutant lines and to analyse changes in the ISSR polymorphism of mutant lines.

Materials and Methods

Selected seeds of *Amaranthus* mutant plants positively selected for the weight of the thousands seeds were used for analyses. Mutant lines together with their control genotypes were obtained from Institute of Plant Genetics and Biotechnology - Slovak Academy of Sciences in Nitra, Slovakia (Table 1, Figure 1). Mutant lines with the statistically confirmed increase of weight of thousand seeds were taken as accessions for ISSR analyses only. The same set of the mutant lines was used in wide biochemical analyses performed by Hricová et al. (2011) and Múdry et al. (2011).

DNA was extracted from leaf tissue of randomly selected six individual plants of each mutant line or control genotype based on the method of Rogers and Bendich (1994). The purity of DNA is one of the major factors affecting the success of genomic or genetic technologies studies. Nucleic acid isolation from polyphenol rich plants fail to produce good quality DNA or RNA as polyphenols adhere and interfere with DNA during isolation (Anuradha et al., 2013). For quantity setting of the extracted DNA, Nanodrop Nanophotometer™ was used. Determination of DNA quality was done by agarose gel electrophoresis on 1.5% agarose gel in 1xTBE buffer coloured by GelRed™.

Table 1. Species and accessions of *Amaranthus* studied.

Species	Accession	Type
Amaranthus cruentus	Ficha cultivar	Control A
	C 15	Mutant lines
	C 26	
	C 27	
	C 82	
	C 236	
A. hypochondriacus x *A. hybridus*	K-433 hybrid	Control B
	D 54	Mutant lines
	D 279	
	D 282	

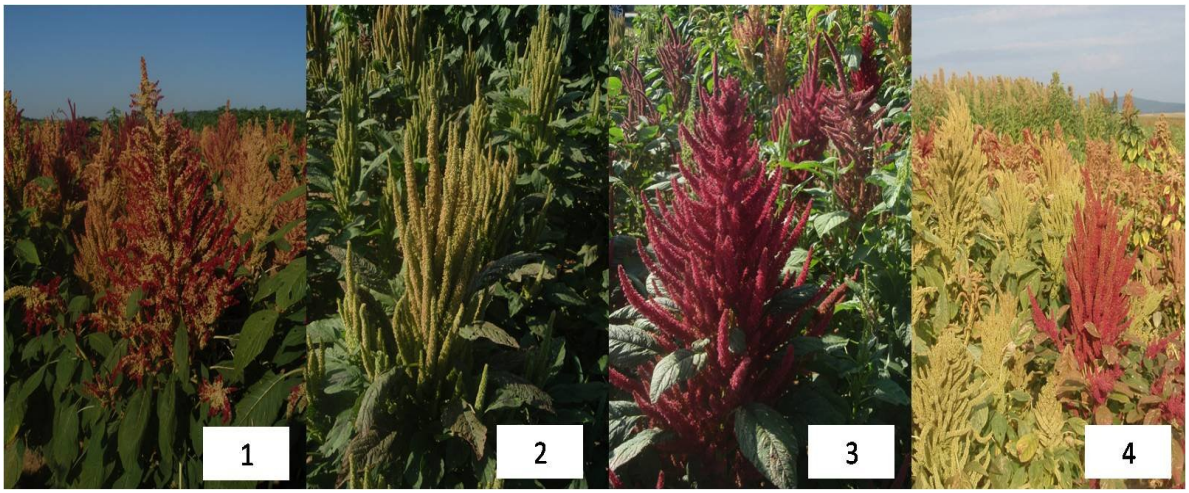

Figure 1. Analyzed specie of amaranth as in field conditions.
1 - Ficha cultivar - ControlA; 2 - C26 mutant line; 3 - K-433 hybrid - Control B; 4 - D282 mutant line.

An analysis of six randomly selected individual plants of each population was conducted with the ten selected ISSR primers. The used ISSR primers were as follows: (GTG)3GC, (CT)10T, (AG)10G, (GATA)2(GACA)2, (CTG)3GC, (GACA)4.

ISSR reactions were done in a volume of 15 µl containing 1.5 mmol × dm^{-3} $MgCl_2$ (Applichem), together with 0.08 mmol × dm^{-3} d NTP (InvitrogenTM) 600-800 nmol × dm^{-3} primer, 1 U Taq polymerase (Applichem) and 50ηg of template DNA.

The repeatability of ISSR banding patterns were examined, and factors affecting the number of band generated per primer were tested, such as DNA concentrations, $MgCl_2$ and primer concentrations.

Amplifications were carried out in a Bio-Rad C1000™ Thermocycler with the following program: an initial denaturation step at 95°C for 4 min followed by 45 cycles at 95°C for 60 s, annealing at 52°C for 60 s, and extension at 72°C for 2 min and a final cycle at 72°C for 10 min. The amplified products were separated using the Experion™ Automated Electrophoresis Station following the manufacturer´s instructions. The band of equal molecular weight and mobility generated by the same ISSR primer were considered to be individual loci. Only consistently reproducible, well resolved fragments were scored.

Amplified products were surveyed for polymorphism and scored in a binary mode (1 as presence of fragment, 0 as absence), assuming that each band represents a single locus. Each experiment was repeated three times. Non-reproducible bands were very rare and were excluded from the analyses along with weak bands.

Jaccard´s (1908) genetic dissimilarity index was used to calculate genetic similarity, employing the software SYN-TAX 2000 HIERCLUS (Podani, 2001). The same software was used for calculating of the cophenetic correlation coefficients for every used primer. Cluster analysis was conducted with UPGMA method in classifying the binary data derived to generate dendrogram to assess the relationships among the mutant lines and their control genotypes.

Results and Discussion

In this study, ISSR markers were applied to distinguish mutant lines of two species of the amarath - *Amaranthus cruentus* and *A. hypochondriacus* x *A. hybridus*. Molecular markers approach was successfully used to understand intra- and inter- specific genetic diversity and/or evolutionary relationships in *Amaranths* (Lanoue et al., 1996; Chan and Sun, 1997; Sun et al., 1999; Xu et al., 2001; Lee et al., 2008, Tony-Odigie et al., 2012; Mladenovic et al., 2012). Lee et al. (2008) reported the potential of microsatellite based markers for the *A. hypochondriacus* assessment; when they successfully amplified in all the tested species 12 loci and demonstrated the applicability of these markers for the study not only of intra-, but for inter-specific genetic diversity of amaranth, too. The same authors have summarized 12 repeat motifs in the amaranth genome with the number of alleles they provided as in the range from 5 to 12 alleles. The CA rich regions were reported, as well as GTG repeats.

Table 2. Individual ISSR primers polymorphism and their average Jaccard similarity indices.

Primer	No. of ISSR markers		Percentage polymorphism of ISSR markers	Cophenetic correlation coefficient	Average Jaccard similarity for Ficha mutant lines	Average Jaccard similarity for K-433 mutant lines
	total	polymorphic				
(GTG)3GC	18	10	56	0.91	0.86	0.74
(CT)10T	18	4	22	1	0.88	0.88
(AG)10G	16	10	63	0.93	0.81	0.91
(GATA)2 (GACA)2	14	3	21	0.83	0.92	0.72
(CTG)3GC	12	12	100	0.96	0.53	0.75
(GACA)4	18	18	100	0.99	0.75	0.75

Both of the above mentioned repeat motifs were used in the primers that were applied in this study. At the individual used primers level, ISSR polymorphism was measured as the proportion of polymorphic loci to the total number of loci scored in all accessions (Table 2). Primers with low percentage of polymorphism distinguish the mutant lines of both, Ficha and K-433 from control genotypes. Primers with higher percentage of polymorphism gave no clear and different ISSR fingerprints pattern for mutant and for control lines. The differentiation ability of the primers for distinguishing Ficha and K-433 based mutant lines were reported previously (Labajová et al., 2011).

In the study of the 20 primers tested, an initial screening resulted in selection of 10 microsatellite primed PCR primers that produced clear and reproducible ISSR profiles in Ficha and K-433. ISSR assays of each primer were performed three times, with only reproducible, amplified fragments being scored. Initially screening of all the used primers showed an identical profile of amplification products in the case of 14 primers.

To yield more accurate and reliable results, profiles for all 6 polymorphic primers were used to generate a UPGMA dendrogram shown in Figure 1. The UPGMA analysis separated mutant lines and their control genotypes into two main clusters.

ISSR is another source of genetic markers that have higher reproducibility than RAPDs (Meyer et al., 1993; Fang and Roose, 1997). Along with the RAPD technique, both of the still preferred method for fast initiating identification of genotypes as they are relatively inexpensive, utilizes arbitrary primers, and randomly samples a potentially large number of loci in a less complex pattern than other PCR based markers (Milella et al., 2005, 2011). Transue et al. (1994) and Chan and Sun (1997) used RAPD markers for the study of evolutionary relationships among grain amaranths and their wild relatives. Popa et al. (2010) studied genetic diversity and phylogenetic relationship among six species of *Amaranthus* by RAPD markers and showed that there is slightly intra and inter species polymorphism. Comparisons between RAPD and ISSR techniques and their effectiveness can be made, first because only a few studies about ISSR as a type of random markers in amaranth exist and second, RAPD products often contain repetitive DNA sequences (Paran and Michelmore, 1993). Both of the techniques are used for minor and underutilized species equally (Milella et al., 2005, 2011; Labajová et al., 2011; Svobodová et al., 2011; Al-Khalifah, 2012).

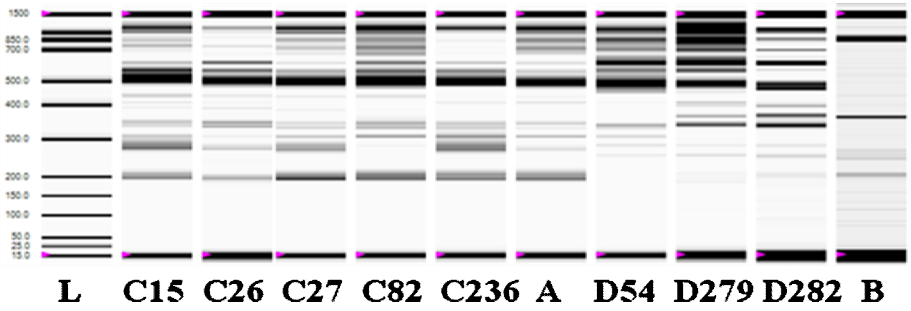

Figure 2. Electrophoreogram of tested Amaranth accessions using the primer $(GATA)_2(GACA)_2$. L - Marker; C -Ficha accession A - Control for Ficha; D - K-433 hybrid accessions: B - Control for K-433.

Table 3. Jaccard dissimilarity indices of analysed mutant lines and their control genotypes.

	C15	C26	C27	C82	C236	Ficha	D54	D279	D282	K-4333
C15	0.000									
C26	0.140	0.000								
C27	0.140	0.073	0.000							
C82	0.141	0.165	0.165	0.000						
C236	0.145	0.169	0.146	0.193	0.000					
Ficha	0.134	0.136	0.136	0.138	0.117	0.000				
D54	0.356	0.378	0.396	0.364	0.391	0.384	0.000			
D279	0.322	0.344	0.363	0.310	0.375	0.349	0.097	0.000		
D282	0.356	0.378	0.396	0.345	0.409	0.384	0.086	0.043	0.000	
K-433	0.422	0.427	0.462	0.414	0.424	0.435	0.222	0.205	0.222	0.000

Ray and Roy (2007) have used both of the techniques - RAPD and ISSR - for the phylogenetic relationships analysis between Amaranthaceae, where RAPD analysis have higher level of polymorphism among leafy amaranths and ISSR shows higher level of polymorphism in grain amaranths. Authors confirmed an effective discriminating power for both of the random primer systems. For the purposes of present study, ISSR markers were chosen rather than RAPD ones, because of the mutation potential of microsatellites in plants (Gao and Xu, 2008).

As ISSR fingerprints results show, all the mutant lines of the Ficha genotype (C15, C26, C27, C82, C236) shared a genetic dissimilarity of 0,16 and their ISSR profiles were more similar to the Ficha than those of K-433 mutant lines. The K-433 mutant lines (D54, D279, D282) shared genetic dissimilarity of 0.22 but as the dendrogram shows, were more distinct to their control genotype as a whole, as those of the Ficha mutant lines. The two major clusters were observed at a dissimilarity coefficient of a 0.38, which means that the used primers had generated two relatively different sets of fingerprints, one for the Ficha and its mutant lines and one for the K-433 and its mutant lines.

Amaranthus cruentus mutant lines were grouped in the first major cluster and the average Jaccard dissimilarity coefficient for all of the Ficha mutant lines was 0.11 (Table 3). Similarly, the second cluster is compounded only from K-433 mutant lines with the average dissimilarity coefficient of 0.07.

As the Table 3 shows, no genetic uniformity was observed among the Ficha mutant lines, nor among the K-433 mutant lines, but low average Jaccard dissimilarity coefficient indicate that their ISSR profiles were similar, but not the same completely.

As the dendrogram (Figure 3), mutant line C82 shared the specific positions to the Ficha control genotype - was the most distinct to the Ficha. The result corresponds to the results of the biochemical analyses of the same mutant lines as in this study, where C82 line is statistically different as the rest of the analysed Ficha lines (Hricová et al., 2011, personal communication; Múdry et al., 2011).

Our data indicates the use of ISSR molecular marker systems in amaranth gamma-rays mutant lines as an advanced technique, because of the possible linkage of changes of the non-coding regions to the specific changes of the genome after the gamma-rays treatment. And, it is significant as already positive selection for the thousand seeds weight was reported from the same by Gajdošová et al. (2005).

Molecular marker analyses have contributed to the elucidation of origin and evolution of cultivated amaranths, and allied wild species. Hauptli and Jain (1984) were among the first to use molecular markers to address evolutionary relationships among the grain amaranths. They observed that with the exception of the *A. caudatus – A. quitensis* pair, grain amaranths are more closely related to each other than either is to their putative wild progenitor. This work was based on isozyme polymorphisms and several authors have since expanded molecular diversity studies in the genus.

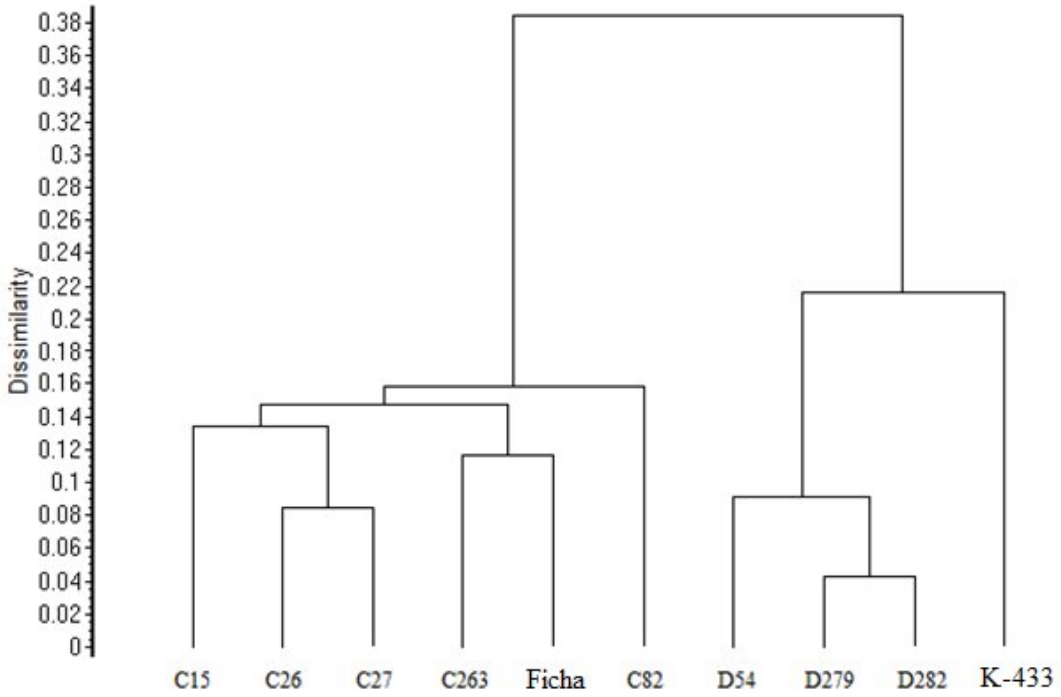

Figure 3. Dendrogram of Ficha and K-433 mutant lines as analysed by ISSR.

In a study including both isozyme and random amplified polymorphic DNA (RAPD) markers, Chan and Sun (1997) reported molecular phylogenies of cultivated and wild amaranths. Authors evaluated 23 different species, including the three cultivated for grain as well as accessions of all species in the *A. hybridus* aggregate. Studies based on restriction site variations in nuclear and cytoplasmic DNA reported that *A. caudatus* and *A. cruentus* are more closely related to each other and to their supposed progenitors than to *A. hypochondriacus* (Lanoue et al., 1996). Isozyme and RAPD markers were used by other authors and findings tends to agree with the different evolutionary hypothesis presented herein (Ranade et al., 1997; Zheleznov et al., 1997).

Xu and Sun (2001) reported that both, AFLP and double-primer ISSR detected high levels of polymorphism among species, averaging 96.7 and 99.5%, respectively. Within species, however, AFLP polymorphism was much lower than double-primer ISSR polymorphism. All these results confirms the reliability of the molecular marker based methods for distinguishing closely related material, such as lines.

Conclusions

Gamma-irradiated amaranth mutants were analysed with ISSR markers. The results showed specific changes in non-coding regions of mutants, based on inter microsatellite length polymorphism. Different ISSR fingerprints patterns of the mutant lines when compared to the Ficha and K-433 ISSR specific profiles may be part of a consequence of the complex response of the intergenic space of mutant lines to the gamma-radiance.

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