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DETECTION OF THE GENETIC VARIABILITY OF AMARANTHUS BY RAPD AND ISSR MARKERS

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Abstract

RAPD and ISSR markers were used to analyse intra and inter-specific variability of 16 *A. caudatus,* 18 *A. cruentus* and 21 *A. hypochondriacus* accessions. The potential of both approaches was evaluated using three random and three microsatellite primers amplifying in total of 1126 (RAPD), respectively 1013 (ISSR) scorable fragments. Similarity values among accessions of three *Amaranthus* species ranged from 0.00 to 1.00 in both types of markers. Based on the intra-specific variability the polymorphism percentage varied from 70 to 100% (RAPD) or from 90 to 100% (ISSR) respectively. Resolving power (Rp) of ISSR primers have been higher (5.28 in average) in comparison to RAPD primers (4.84 in average). Percentage of distinguished accessions by ISSR and RAPD primers ranged from 29 to 89% (based on primer's type) and from 19 to 72%, respectively. Cluster analysis based on RAPD and ISSR data has shown the individual species separation except of three accessions. Two of *A. caudatus* genotypes originated from India clustered with *A. hypochondriacus* accessions and one of *A. hypochondriacus* genotype originated from Nepal clustered with *A. cruentus* accessions. This study has demonstrated, that a single primer marker systems as RAPD and ISSR are able to generate a sufficient level of informative characters for intra and inter-specific analysis of *Amaranthus* genus.

Key words: *Amaranthus caudats*, *A. cruentus*, *A. hypochondriacus*, Markers.

Introduction

Amaranthus unique nutritional properties make it a valuable food resource. It has higher amount of protein with balanced essential amino acids contents (Tucker, 1986). This species also exhibit a remendous amount of morphological diversity and a wider adaptability to different eco-geographical situations (Lee *et al*., 2008). The study of genetic diversity among populations of different phytogeographic regions is important, as survival, perpetuation, and continuance of a species to meet the demands of changing environments largely depend on the extent of variability available in its gene pool (Ray & Roy, 2009).

As summarized in Štefúnová *et al*. (2014), the genus *Amaranthus* L. is reported as to possesing high inter- and intraspecies variability (Mosyakin & Robertson, 1996). Molecular tools have important roles in studies of phylogeny and species evolution, and have been applied to provide a valuable data of the distribution and extent of genetic variation within and between species (Mondini *et al*, 2009; Somasundaram & Kalaiselvam, 2011).

The independence of DNA markers on environmental conditions, their high heritability, relatively inexpensive way of testing, predetermines their use over phenotypic markers. In comparison to the morphological markers are DNA markers more suitable for detection of intra- and interspecies variability (Gilbert *et al*., 1999; Duran *et al*., 2009).

According to the isozyme and RAPD data (Chan & Sun, 1997), grain amaranths are more closely related to each other and to *Amaranthus hybridus* than to *Amaranthus powellii* or *Amaranthus quitensis*.

At the interspecific level, the grain amaranth species are less divergent from each other than their wild progenitors (Sun *et al*., 1999). Genetic relationship amongst amaranth species determined by RAPDs is consistent with their cytogenetic and evolutionary relationships (Ranade *et al*., 1997). Gupta & Gudu (1991) studied hybrid development from interspecific crosses between species (*Amaranthus hypochondriacus*, *Amaranthus caudatus* and *Amaranthus cruentus*) to find out genetic relationships between them. Based on hybrid development, it was suggested that *Amaranthus hypochondriacus* and *Amaranthus caudatus* were genetically closer than the other two combinations of species studied. *Amaranthus cruentus* seemed to be genetically closer to *Amaranthus hypochondriacus* than it was to *Amaranthus caudatus*. Popa *et al*. (2010) studied the genetic diversity and phylogenetic relationships among six species of *Amaranthus* from different geographic regions using the RAPD markers. Molecular analysis showed relatively low intra- and inter-species polymorphism. However, by using some specific RAPD primers have been amplified some amplicons that, after further analysis, could be regarded as molecular markers.

Molecular marker analyses have contributed to the understanding of origin and evolution of cultivated amaranths and wild species (Trucco & Tranel, 2011). Several techniques, such as internal transcribed spacer (ITS) of rDNA, amplified fragment length polymorphism (AFLP), and double-primer fluorescent intersimple sequence repeat (ISSR) were employed to examine the taxonomic status and phylogenetic relationships of grain amaranths and their wild relatives. Both, AFLP and double-primer fluorescent ISSR have a great potential for generating a large number of informative characters for phylogenetic analysis of closely related species (Xu & Sun, 2001). Both of the techniques, AFLP and ISSR were also utilized for amaranth mutant lines evaluation by Labajová *et al*. (2013) and Žiarovská *et al*. (2013).

Materials and Methods

Template DNA preparation: The seeds of 16 *A. caudatus* L, 18 *A. cruentus* L. and 21 *A. hypochondriacus* L. genotypes (Table 1) were obtained from North Central Regional PI Station (NC 7), Iowa State University, Ames. The amaranth seedlings were cultivated under *In vitro* conditions on Murashige & Skoog (1962) medium. DNA from fresh young leaves was isolated according to Rogers and Bendich *et al*. (1994) optimized protocol. Each genotype was represented by ten individuals.

PCR condition and temperature profile for RAPD and ISSR: According to the optimization, PCR conditions were assigned 20 mmol.dm^{-3} Tris-HCl, pH 8,0, 50 mmol.dm^{-3} KCl, 1 U Taq polymerase, 3 mmol.dm^{-3} $MgCl_2$, 0.1 mmol.dm^{-3} deoxy ribonucleotides, 0.4 µmol.dm^{-3} RAPD primer, 0.2 µmol.dm^{-3} ISSR primer and 20 ng DNA in 25µl total reaction volume. Following RAPD and ISSR primers have been used: LA-01 (CCTGGGTGGA), LA-03 (CCTGGGCCTC), LA-08 (CCGGCCTTCC); $(CA)_6$ AG, $(CT)_8$AC and $(GA)_6$CC. The PCR cycling conditions were as follows: 94°C for 2 minutes, then followed by 45 cycles at 94°C for 1 minute, 36°C (RAPD primers) and 50 °C (ISSR primers) for 1 minute, 72°C for 2 minutes with a final 7 min extension at 72°C and then cool down to 4°C. Amplification was performed in a MyCycler (Biorad). Each reaction was repeated three times. The PCR products were separated by electrophoresis on 2% agarose gel (3: 1, Amresco) containing 0.5 µg.ml-1 ethidium bromide in a 1 × TBE buffer and then photographed under UV light using GeneSnap (Syngene).

Similarity analysis and clustering: The amplified DNA fragments were scored for each cultivar as 1 (band present) and 0 (band absent) using the GeneSnap (Syngene) software. Genetic similarity was calculated on the basis of Nei and Li (1979) coefficient. The similarity matrix thus generated was used for cluster analysis by unweighted pair-group method with arithmetic average (UPGMA) with statistic program SYNTAX. Resolving power (Rp) of a primer was calculated as Ib. Band informativness (Ib) was represented into a 0–1 scale by the formula: Ib=1-(2 × 0.5-p), where p is the proportion of the 20 samples containing the band (Prevost & Wilkinson, 1999; Chakrabarti *et al*., 2001). Genotypes distinctiveness coefficient was calculated as the proportion of distinguished genotype of the total number of analyzed genotypes.

Results and Discussion

Intra-specific genetic analysis of three amaranth species by RAPD markers: Decamer primers used for analysis of three species of amaranth differed in GC base content. The LA01 primer comprises of 70% and the remaining two primers (LA03 and LA08) comprises of 80% GC bases content. Certain correlation can be observed between GC base content and the polymorphism percentage of analyzed amaranth species (Tables 2, 3 and 4). By decamers with 70% or 80% of GC base content have been recorded the polymorphism at an average of 70% or 90% respectively. As a species-specific could be recognized the primer LA03. With respect to analyzed species, the percentage of polymorphism differed (73% *A. caudatus*, 85% *A. cruentus* and 100% *A. hypochondriacus*). Such a trend in the other two primers has not been observed.

By RAPD analysis of 16 genotypes of *A. caudatus* a total of 341 scorable DNA fragments were amplified using three random primers. Among the accessions of *A. caudatus* the similarity index values varied from 0.14 to 1.00 with a mean value of 0.73. A primer having resolving power of 6.88 (coefficient of genotypes distinctiveness 0.62) was sufficient to distinguish 10 out of 16 accessions of *A. caudatus*. By the analysis of 18 genotypes of *A. cruentus* a total of 375 scorable DNA fragments were amplified using three random primers. Within the accessions of *A. cruentus* the values ranged from 0.33 to 1.00 with a mean value of 0.75. A primer having resolving power of 5.11 distinguished 13 (coefficient of genotypes distinctiveness 0.72) from a total of 18 genotypes of *A. cruentus*. Analysis of 21 genotypes of *A. hypochondriacus* a total of 410 scorable DNA fragments were fragments using three random primers. For *A. hypochondriacus* accessions the similarity index values varied form 0.00 to 1.00 with a mean value 0.68. A primer having resolving power of 4.00 distinguished 13 (coefficient of genotypes distinctiveness 0.62) out of 21 accessions of *A. hypochondriacus*. Generally we can state that regardless of the amaranth species and primer's type, the values of the similarity index have indicated a significant intra-specific variability. The polymorphism levels has reached in average of 80% for 16 accessions of *A. caudatus*, 18 accessions of *A. cruentus* and 87% (in average) for 21 genotypes of *A. hypochondriacus.* The higher percentage of intraspecific polymorphism in *A. hypochondriacus* iindicates its greater genetic variation than the other amaranth species studied.

Inter-specific genetic analysis of three amaranth species by RAPD markers: Cluster analyses performed with the RAPD data matrix generated by three used primers could group genotypes at the inter-specific level into four main clusters (Fig. 1) with the number of genotypes 14, 19, 18 and 4. The first cluster was composed of 14 genotypes of *A. caudatus*. The remaining two accessions with codes 5 and 6 (PI 480816, PI 480854) were included in the second cluster consisting of genotypes of *A. hypochondriacus*. Similar character of separation was observed even within intra-specific analysis of *A. caudatus* by ISSR markers. By the primer LA01 were indicated genotypes separated with the similarity index value of 1.0 (most similar). The second cluster contained 17 genotypes of *A. hypochondriacus*. The third cluster contains all 18 analyzed genotypes of *A. cruentus* and fourth cluster, allocated to the cluster of *A. cruentus* genotypes, consisted of the remaining four genotypes of *A. hypochondriacus*. Most of the genotypes have been allocated into main cluster based on amaranth species.

Table 1. Species and accessions of *Amaranthus* used in this study.

Code	Species	Accession	Origin	Note
1	*Amaranthus caudatus*	Ames 12751	Nepal	Unknown origin
2		PI 490440	Peru	Unknown origin
3		PI 490604	Bolivia	Unknown origin
4		PI 490642	Peru	Unknown origin
5		PI 480816	India	Unknown origin
6		PI 480854	India	Unknown origin
7		PI 511693	Peru	Cultivated material
8		PI 511711	Ecuador	Cultivated material
9		PI 568147	Bolivia, Tarija	Cultivated material
10		PI 175039	India	Unknown origin
11		PI 553073	USA, New Yersey	Cultivar
12		PI 166045	India	Unknown origin
13		PI 632249	USA, Iowa	Unknown origin
14		Ames 5600	India	Unknown origin
15		Ames 5685	USA, Pennsylvania	Unknown origin
16		NSL 109789	Italy	Unknown origin
17	*Amaranthus cruentus*	Ames 1959	Ghana	Cultivar
18		Ames 5129	Nigeria	Unknown origin
19		Ames 21948	Papua New Guinea	Unknown origin
20		PI 566896	USA, Arizona	Landrace
21		PI 511719	Guatemala	Cultivated material
22		PI 511876	Mexico	Landrace
23		PI 527567	Burundi	Landrace
24		PI 604558	Mexico	Landrace
25		PI 612169	China	Cultivated material
26		Ames 5638	Mexico, Puebla	Unknown origin
27		Ames 5648	Mexico, Sonova	Unknown origin
28		PI 477913	Mexico	Cultivar
29		Ames 5493	Mexico, Morelos	Unknown origin
30		Ames 5369	Kongo	Cultivar
31		Ames 5310	Mexico, Sonova	Landrace
32		Ames 25121	Nigeria, Oyo	Wild
33		Ames 2215	Mexico, Sonova	Landrace
34		PI 566897	India, Kerala	Cultivated material
35	*Amaranthus hypochondriacus*	Ames 2064	Nepal	Breeding material
36		Ames 2086	Nepal	Unknown origin
37		Ames 2061	Nepal	Unknown origin
38		Ames 21046	India	Cultivar
39		PI 481464	Nepal	Unknown origin
40		PI 538794	Russia	Cultivated material
41		PI 542595	China	Cultivated material
42		PI 568130	USA, Iowa	Breeding material
43		PI 511731	Mexico	Cultivated material
44		Ames 12744	Nepal	Unknown origin
45		Ames 1972	Nigeria	Breeding material
46		PI 274279	India, Himachal Pradesh	Unknown origin
47		PI 337611	Uganda	Landrace
48		PI 477915	India	Breeding material
49		PI 477916	Mexico	Cultivar
50		PI 477917	Mexico	Cultivar
51		Ames 2178	Nepal	Breeding material
52		Ames 5132	Mexico, Chihuahua	Landrace
53		Ames 5209	Mexico, Mexiko	Landrace
54		Ames 5321	Mexico, Chihuahua	Landrace
55		Ames 5467	Mexico, Oaxaca	Unknown origin

Table 2. Characteristics of intra-specific variability of analysed accessions of *Amaranthus caudatus* (L.) by RAPD.

Characteristics	Type of primer		
	LA01	LA03	LA08
Total number of amplified DNA fragments	157	114	70
Average of amplified fragments per accession*	10	7	4
Number of levels of DNA fragments distribution	17	11	11
Number of monomorphic levels	4	3	1
Percentage of polymorphism*	77	73	91
Resolving Power of a primer Rp	6.88	3.25	4.50
Coefficient of genotypes distinctiveness (Number of distinguished accessions)	0.62 (10)	0.56 (9)	0.19 (3)
Range of similarity index (SI_{NL}) among accessions (average value)	0.48–1.00 (0.74)	0.14–1.00 (0.81)	0.20–1.00 (0.63)
Size range of amplified fragments in bp.	500–2000	350–2500	350–2000

* Rounded to whole numbers, SI_{NL} – Similarity Index (0 –the less similar, 1 – the most similar)

Table 3. Characteristics of intra-specific variability of analysed accessions of *Amaranthus cruentus* (L.) by RAPD.

Characteristics	Type of primer		
	LA01	LA03	LA08
Total number of amplified DNA fragments	142	101	132
Average of amplified fragments per accession*	8	6	7
Number of levels of DNA fragments distribution	17	13	17
Number of monomorphic levels	5	2	2
Percentage of polymorphism*	71	85	88
Resolving Power of a primer Rp	3.11	5.22	5.11
Coefficient of genotypes distinctiveness (Number of distinguished accessions)	0.61 (11)	0.72 (13)	0.72 (13)
Range of similarity index (SI_{NL}) among accessions (average value)	0.67–1.00 (0.84)	0.33–1.00 (0.67)	0.46–1.00 (0.74)
Size range of amplified fragments in bp.	400–2000	350–1500	400–2000

* Rounded to whole numbers, SI_{NL} – Similarity Index (0 –the less similar, 1 – the most similar)

Table 4. Characteristics of intra-specific variability of analysed accessions of *Amaranthus hypochondriacus* (L.) by RAPD.

Characteristics	Type of primer		
	LA01	LA03	LA08
Total number of amplified DNA fragments	142	119	149
Average of amplified fragments per accession*	7	6	7
Number of levels of DNA fragments distribution	10	12	12
Number of monomorphic levels	3	0	1
Percentage of polymorphism*	70	100	92
Resolving Power of a primer Rp	4.00	5.24	6.29
Coefficient of genotypes distinctiveness (Number of distinguished accessions)	0.62 (13)	0.43 (9)	0.52 (11)
Range of similarity index (SI_{NL}) among accessions (average value)	0.40–1.00 (0.77)	0.00–1.00 (0.64)	0.15–1.00 (0.63)
Size range of amplified fragments in bp.	600–2000	350–1500	400–2000

* Rounded to whole numbers, SI_{NL} – Similarity Index (0 –the less similar, 1 – the most similar)

The specificity of four genotypes of *A. hypochondriacus*, has placed them beyond the cluster of genotypes of *A. cruentus*. Genotypes with numbers 35 (Ames 2064), 49 (PI 477916), 52 (Ames 5132) and 54 (Ames 5321) originated from different regions of Mexico (49, 52 and 54) or from Nepal (35), respectively. Most of the allocated genotypes represent landraces (52 and 54), the breeding material (35) and cultivar (49). Either in terms of genetic background, or in terms of origin, it is not possible to clearly identify the reasons for exclusion, these four genotypes of *A. hypochondriacus* into a separate cluster. In terms of inter-species assessment the clusters have been organized in the following order: *A. caudatus – A. hypochondriacus – A. cruentus.*

Intra-specific genetic analysis of three amaranth species by ISSR markers: The main characteristics of intra-specific variability are represented in Tables 5, 6 and 7. In terms of polymorphism we can assume that the frequency of repeated CA, CT and GA dimers of microsatellite DNA has been within analyzed species of amaranth relatively balanced.

By the ISSR analysis of 16 genotypes of *A. caudatus* a total of 384 scorable DNA fragments were amplified using three microsatellites primers. The values of the similarity index among accessions ranged from 0.17 to 1.00. A primer having resolving power of 7.38 distinguished 13 (coefficient of genotypes distinctiveness 0.81) from a total of 16 genotypes of this variety. ISSR analyses of 18 genotypes of *A. cruentus* a total of 333 scorable DNA fragments were amplified using three microsatellites primers. The values of the similarity index among accessions ranged from 0.00 to 1.00. Primer with a resolving power 10.33 distinguished 16 (coefficient of genotypes distinctiveness 0.89) from a total of 18 genotypes.

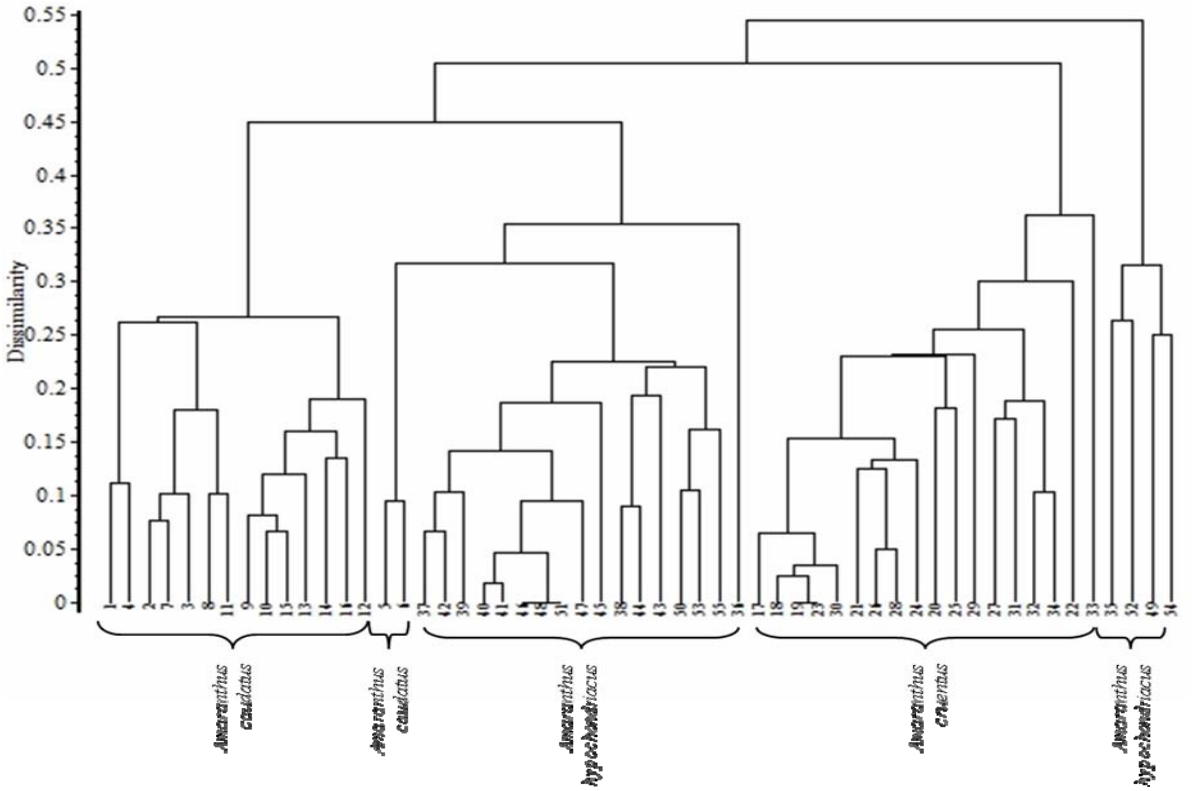

Fig. 1. Dendrogram of *Amaranthus* L. accessions (codes as in Table 1) constructed with UPGMA method on the basis of RAPD analysis.

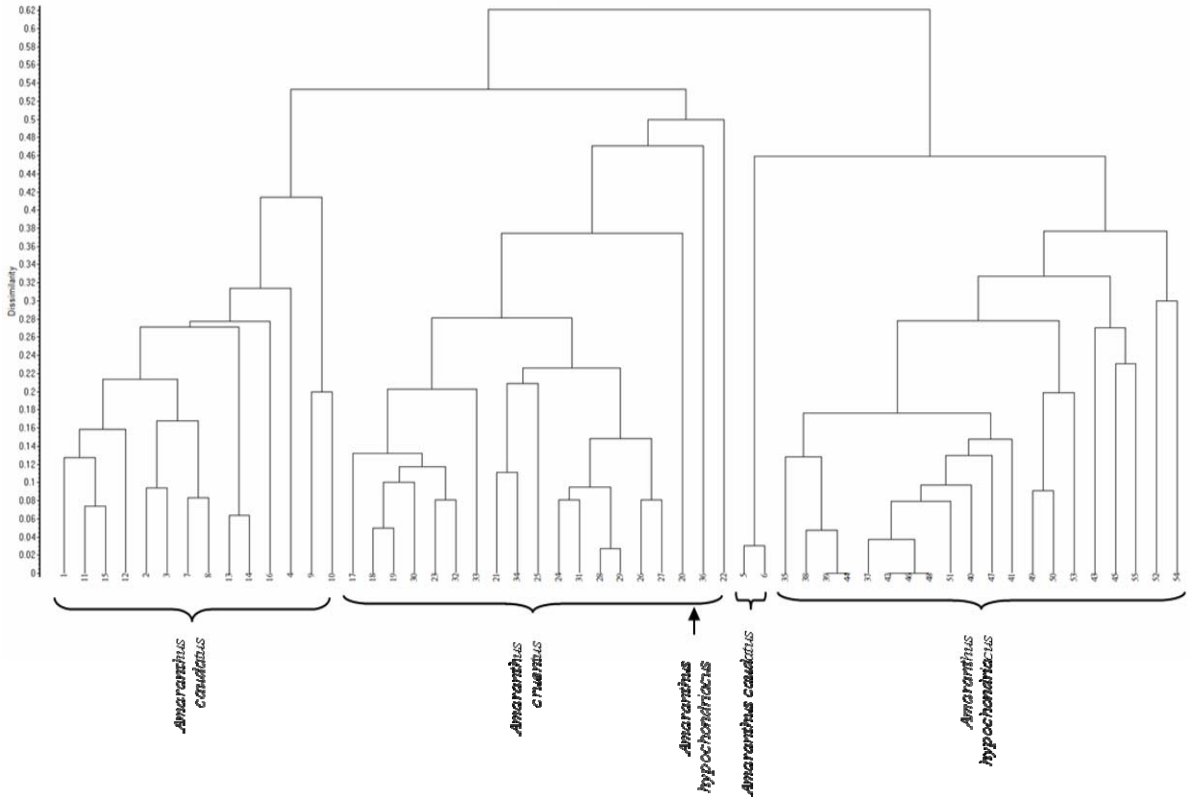

Fig. 2. Dendrogram of *Amaranthus* L. accessions (codes as in Table 1) constructed with UPGMA method on the basis of ISSR analysis.

Table 5. Characteristics of intra-specific variability of analysed accessions of *Amaranthus caudatus* (L.) by ISSR.

Characteristics	Type of primer		
	$(CA)_6AG$	$(CT)_8AC$	$(GA)_6CC$
Total number of amplified DNA fragments	149	93	142
Average of amplified fragments per accession*	9	6	9
Number of levels of DNA fragments distribution	17	14	18
Number of monomorphic levels	2	1	3
Percentage of polymorphism*	88	93	83
Resolving Power of a primer Rp	7.38	5.88	6.50
Coefficient of genotypes distinctiveness (Number of distinguished accessions)	0.81 (13)	0.38 (6)	0.50 (8)
Range of similarity index (SI_{NL}) among accessions (average value)	0.33–1.00 (0.70)	0.17–1.00 (0.61)	0.44–1.00 (0.71)
Size range of amplified fragments in bp.	400–2000	600–2800	400–1500

* Rounded to whole numbers, SI_{NL} – Similarity Index (0 –the less similar, 1 – the most similar)

Table 6. Characteristics of intra-specific variability of analysed accessions of *Amaranthus cruentus* (L.) by ISSR.

Characteristics	Type of primer		
	$(CA)_6AG$	$(CT)_8AC$	$(GA)_6CC$
Total number of amplified DNA fragments	65	97	171
Average of amplified fragments per accession*	4	5	10
Number of levels of DNA fragments distribution	11	13	21
Number of monomorphic levels	0	2	0
Percentage of polymorphism*	100	85	100
Resolving Power of a primer Rp	3.44	3.00	10.33
Coefficient of genotypes distinctiveness (Number of distinguished accessions)	0.56 (10)	0.39 (7)	0.89 (16)
Range of similarity index (SI_{NL}) among accessions (average value)	0.00–1.00 (0.62)	0.31–1.00 (0.79)	0.15–1.00 (0.60)
Size range of amplified fragments in bp.	500–2400	400–2000	500–2000

* Rounded to whole numbers, SI_{NL} – Similarity Index (0 –the less similar, 1 – the most similar)

Table 7. Characteristics of intra-specific variability of analysed accessions of *Amaranthus hypochondriacus* (L.) by ISSR.

Characteristics	Type of primer		
	$(CA)_6AG$	$(CT)_8AC$	$(GA)_6CC$
Total number of amplified DNA fragments	68	103	125
Average of amplified fragments per accession*	3	5	6
Number of levels of DNA fragments distribution	12	10	18
Number of monomorphic levels	1	1	1
Percentage of polymorphism*	92	90	94
Resolving Power of a primer Rp	2.67	2.48	5.81
Coefficient of genotypes distinctiveness (Number of distinguished accessions)	0.57 (12)	0.29 (6)	0.38 (8)
Range of similarity index (SI_{NL}) among accessions (average value)	0.18–1.00 (0.70)	0.44–1.00 (0.79)	0.27–1.00 (0.64)
Size range of amplified fragments in bp.	400–1600	350–2000	500–1600

* Rounded to whole numbers, SI_{NL} – Similarity Index (0 –the less similar, 1 – the most similar)

By the ISSR analysis of 21 genotypes of *A. hypochondriacus* a total of 296 scorable DNA fragments were amplified. The values of the similarity index among accessions ranged from 0.18 to 1.00. A primer having resolving power of 2.67 distinguished 12 (coefficient of genotypes distinctiveness 0.57) from a total of 21 genotypes.

Inter-specific genetic analysis of three amaranth species by ISSR markers: For the evaluation of the three cultivated species of the amaranth genus by ISSR markers were generated three main clusters (Fig. 2) with the number of genotypes 14, 19 and 22. Within the first cluster have been located 14 accessions of *A. caudatus.* 18 accessions of *A. cruentus* have been allocated into second cluster together with one accession (Ames 2086, code 36) of *A. hypochondriacus*. The following, third cluster consisted of 20 genotypes of *A. hypochondriacus* and two genotypes of *A. caudatus* (PI 480816 and PI 480854). The allocation specificity of above mentioned one accession of *A. hypochondriacus* and two accessions of *A. caudatus* have been confirmed by both DNA markers.

Only two accessions of *A. hypochondriacus* PI 274279 and PI 477915 (codes 46 and 48) have been identified by both types of markers as identical (Figs. 1 and 2). Whereas the clustering based on RAPD markers has shown that *A. caudatus* is closely related to *A. hypochondriacus*, the ISSR-based UPGMA analysis has clearly identified the *A. caudatus* as closely related to *A. cruentus.*

Amaranth became rediscovered and is intensively studied from different point of view from antioxidant activity through celiac diseases diet and biofuel applications up to the molecular based polymorphism analyses. It has excellent nutritional value and ample capacity for growth under drought, heat, and soil nutrient deficiency (Jimenez, 2013). *Amaranthus* is a cosmopolitan

genus comprising large number of species with many morphotypes cultivars/accessions having diversified morphological features. The species, morphotypes within *Amaranthus* are very closely related requiring microclassification even revision in present taxonomic status (Das, 2012). Molecular markers including the random amplified polymorphic DNA (RAPD) marker have been employed to study the genetic diversity and phylogenetic relationships between *Amaranthus* species (Lymanskaya, 2012; Džunková *et al*., 2011; Solano & Porfirio, 2010; Ray & Roy, 2009; Lee *et al*., 2008).

Our results, in terms of the applicability and the effectively of marker system, correspond to ones reported by other authors (Ray & Roy, 2009; Balwant *et al*., 2013). RAPD primers used for genetic diversity and relationships analyses among 6 *Amaranthus* species from 8 phytogeographic regions yielded a total of 262 amplicons, ranging from ~250 to ~3000 bp in size with an average of 13.1 amplicons per primer, of which 254 amplicons (96.94%) were polymorphic. The genetic similarity coefficient among all the *Amaranthus* species ranged from 0.16 to 0.97 with a mean similarity coefficient of 0.56, indicating that variation existed in the genetic diversity of different populations. Decamer primers sufficiently produced clear and reproducible RAPD profiles (Ray & Roy, 2009). Regardless of the amaranth species and primer's type, the values of the similarity index among the accessions ranged from 0.00 to 1.00 indicating a significant intra-specific variability. Genetic diversity in a set of 31 *Amaranthus* accessions ranged from 0.58 to 0.98 (Balwant *et al*., 2013).

A primer having resolving power of 4.00 distinguished 13 (coefficient of genotypes distinctiveness 0.62) out of 21 accessions of *A. hypochondriacus*. Resolving power of a primer has been found to correlate strongly with genotype diagnosis and so has potential for a number of applications (Prevost & Wilkinson, 1999). Solano and Porfirio (2010) detected within the amaranth population low genetic variability, with polymorphism levels of 27.66% for *A. caudatus* race 'Sudamericana' (12 accessions) and 65.96% in *A. hypochondriacus* race 'Azteca' (38 accessions). Also, the similarity index values reaching 0.15 confirmed a low diversity within populations. In our study the polymorphism levels has reached 80% for 16 accessions of *A. caudatus*, 18 accessions of *A. cruentus* and 87% for 21 genotypes of *A. hypochondriacus* and similarity index values varied from 0.00 up to 1.00 depending on amaranth species and used primer. According to Ray and Roy (2009) the percentage of RAPD polymorphism at the intraspecific level, were found to be 22.5%, 18.3% and 23.3% in the grain types *A. hypochondriacus*, *A. caudatus* and *A. cruentus*, respectively. In our study we also confirmed the highest percentage of polymorphism in *A. hypochondriacus*. The same authors state that the levels of RAPD polymorphism are lower in grain amaranths in comparison to leafy types, due to their relatively long cultivation history or selection pressure in domestication. In spite of the apparent phenotypic similarity, RAPD markers were able to detect sufficient polymorphisms to distinguish the same amaranth plant species collected from different phytogeographic regions. All the populations of a species have clustered together. Moreover, the RAPD-based UPGMA analysis clearly separated leafy types from grain types into 2 separate clusters (Ray & Roy, 2009).

The amaranths are notorious for their ability to hybridize. Accidental interspecific hybrids in great variety have been reported. Most of the accidental hybrids have been found in Europe, where species native to many different parts of the world have come into contact as weeds or been brought together in botanical gardens (Sauer, 1950, 1967). Most forms of *A. caudatus* show no trace of hybridization with weeds or other domesticates. Even in Asia, where *A. caudatus* and *A. hypochondriacus* share an enormous range as grain crop, only a few sterile hybrids are known (Sauer, 1967). Within the cluster of *A. hypochondriacus*, the genotype from Nepal Ames 2086 (code 36) has been allocated separately (Fig. 1). Based on ISSR markers, was the same genotype assigned to accessions of *A. cruentus* (Fig. 2). This might be due to the occurrence of the atypical plant of possible *A. hypochondriacus* as a result of the crossing with *A. cruentus* which has been introduced into India (Sauer, 1967). Some of the accessions of *A. hypochondriacus,* landraces (52 and 54) have been separated into individual cluster. One possible explanation can be made based on observation of Sauer (1967) who states that in southern Mexico, particularly Oaxaca, local populations of *A. hypochondriacus* show some resemblances to *A. cruentus* which is also planted there as a grain crop. Similar atypical plants appear in the *A. hypochondriacus* grain crop of Madras, India, where *A. cruentus* has also been introduced. Considering the above, may the genetic background of *A. hypochondriacus* explains the highest number of polymorphism observed in comparison with other species.

There is always a controversy on the relationship between genetic divergence and geographical origin. There is no doubt that when morphological variations cause confusion or misidentification, RAPD analysis can aid the correct identification of species in amaranth genetic resources (Ray & Roy, 2009).

In terms of inter-species assessment the clusters have been organized in the following order: *A. caudatus – A. hypochondriacus – A. cruentus.* Our results correspond to results by Solano and Porfirio (2010). Analysis of 141 RAPD fragments generated from 16 primers revealed that *A. hypochondriacus* and *A. caudatus* are genetically most similar to each other compared to *A. cruentus*. In Amaranthus there is a higher genetic diversity within species and races than among species and races. Ray & Roy (2009) and Transue *et al*. (1994) also has concluded that based on RAPD markers is *A. caudatus* closely related to *A. hypochondriacus*. However, on the basis of restriction-site variation of PCR-amplified chloroplast and nuclear DNA, *A. caudatus* and *A. cruentus* are found to be more closely related to each other (Lanoue *et al*., 1996). The varying results obtained from different studies may be caused by differences in the marker systems employed, in the number of species/accessions sampled, and in the methods of data analysis.

ISSR appears to produce reliable and highly polymorphic band profiles. The key additional feature of ISSR lies in its technical simplicity and speed. ISSR analysis is quick, reproducible and generates sufficient

polymorphisms to have potential for large-scale DNA fingerprinting purposes (Prevost & Wilkinson, 1999). The UPGMA tree generated based on the AFLP and ISSR data sets are the most consistent in topology and the most coherent with the current morphology-based intra- and interspecific classifications (Xu & Sun, 2001). AFLP as suitable markers techniques applied for amaranth germplasm identification was used by several authors (Costea *et al*., 2006; Wassom *et al*., 2005).

The work of Lee *et al*. (2008) demonstrating wide potential applicability of microsatellite markers for the study of intra- and inter-specific genetic diversity as well as evolutionary relationships among cultivated and wild amaranths, which may be of considerable value for the conservation and use of amaranth genetic resources. In our study ISSR markers generated higher values of polymorphism in observed species in comparison to RAPD markers. The resolving power (Rp) of ISSR primers has been higher in comparison to RAPD primers. Also the percentage of distinguished accessions by ISSR primers reached higher values. Džunková *et al*. (2011) in order to simplify the identification of amaranth accessions in gene banks or seed laboratories, a comprehensive method based on band position and relative band intensity data from the glutelin patterns of the chip microfluidic electrophoresis was developed.

By both types of marker systems (RAPD and ISSR) have been almost all the studied accessions found genetically diverse which may be useful in breeding for improving the genotypes of amaranth as well as for the identification of the appropriate genotypes suitable for certain environmental conditions. Similar results provide Balwant *et al*. (2013) where all the 31 *Amaranthus* accessions were found genetically diverse. In phylogenetic tree based on ISSR, Xu and Sun (2001) identified that nearly all intraspecific accessions of amaranth can be placed in their corresponding species clusters, indicating that these taxa are well-separated species.

Conclusions

Three amaranth species were analyzed to determine genetic diversity among and within them, to obtain the corresponding genetic fingerprints, and to carry out comparisons for differentiating and distinguishing between the genetic variants. Random amplified polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers were used to distinguish and characterize 55 genotypes of three *Amaranthus* species. RAPD primer having resolving power above 7.38 was sufficient to distinguish 13 out of 16 genotypes of *A. caudatus*. 16 out of 18 genotypes of *A. cruentus* have been distinguished by the primer having resolving power above 10.33 and primer resolving power higher than 4.00 was sufficient to distinguish 13 out of 21 genotypes of *A. hypochondriacus.* Resolving power of ISSR primers seemed to be species dependent. Each of three ISSR primers used generated large numbers of polymorphisms, ranging from 83 to 100%. This percentage was higher in comparison to RAPD markers. Microsatellite primer $(CA)_6AG$ was able to distinguish 13 out of 16 genotypes of *A. caudatus* and 12 out of 21 genotypes of *A. hypochondriacus*. Primer $(GA)_6CC$ has distinguished 16 out of 18 genotypes of *A. cruentus.* The average value of RAPD primers resolving power was 4.84 and ISSR primers 5.28. Percentage of distinguished genotypes by RAPD markers ranged from 19 to 72% and by ISSR primers from 29 to 89%.

The present work has shown that both DNA markers systems generate sufficient polymorphisms to have potential for large-scale DNA fingerprinting purposes followed by intra and inter-specific analysis. As different origins and breeding stages of the tested species were involved, the ability of intra and inter-species grouping of RAPD and ISSR markers was proved in this study.

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