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# Marker assisted genetic diversity among *Amaranthus* Species through RAPD

**Varun Kumar Pandey, Dr. BL Jatt and Dr. Ratnaker Pandey**

**Abstract**
Amaranths have high nutritional value due to higher amount of protein (14-18%) with balanced essential amino acid and presence of phenolic content. It has high photosynthetic efficiency, low input requirements and high yield potential for grain vegetable and fodder production, and capacity to grow in diverse conditions such as water deficiency, salt stress, and soil mineral nutrient deficiency. Genetic diversity and relationships among 6 Amaranthus species were studied from different phyto geographic regions and analyzed using a random amplified polymorphic DNA (RAPD) marker. After screening of 20 RAPD (decamer) primer of operon family, 451amplicon produced ranging from 290bp to 3000bp in size, of which 408 were polymorphic while rest are monomorphic. The genetic similarity coefficient among all the Amaranthus species ranged from 0.10 to 1.0 with a mean similarity coefficient of 0.67, observed from arithmetic dendrogram, populations of the same species clustered together. The majority of polymorphism (96%) among Amaranthus Species demonstrates that variation occurs in the different populations of Amaranthus Sp.

**Keywords:** Genetic Diversity, Amaranthus, Phyto geographic region, RAPD

## Introduction
*Amaranthus*, collectively known as amaranth, is a cosmopolitan genus of annual or shortlived perennial plants. Some amaranth species are cultivated as leaf vegetables, pseudocereals, and ornamental plants. Most of the *Amaranthus* species are summer annual weeds and are commonly referred to as pigweed. Catkin-like cymes of densely packed flowers grow in summer or autumn. Approximately 60 species are recognized, with inflorescences and foliage ranging from purple, through red and green to gold. Members of this genus share many characteristics and uses with members of the closely related genus *Celosia*.
The grain amaranths (*A. hypochondriacus*, *A. cruentus*, and *A. caudatus*) belong to the Genus *Amaranthus* L., which includes 60-70 species (Sauer 1976). The three grain amaranths are classified along with their putative progenitor species (*A. hybridus* L., *A. quitensis* H.B.K., *and A. powellii*S. Wats.) in what is termed the *A. hybridus*complex and are thought to be paleoallotetraploids (2*n*=4*x*=32), although chromosome counts of both 32 and 34 have been reported for *A. cruentus* (Pal *et al*. 1982, Greizerstein and Poggio 1994, 1995). While the grain amaranths have been cultivated for centuries in the Americas, they have been underutilized since the Spanish Conquest when they were replaced by Old World crops and their cultivation suppressed due to their deeply rooted use in indigenous religious practices (Sauer 1976, 1993; Iturbide and Gispert 1994). In the last few decades, the grain amaranths have begun to reclaim some of their importance, largely due to the recognition of the nutritional value of their seed for human consumption (Bressani *et al*. 1992; Tucker 1986). As summarized in Štefúnová *et al*. (2014) [18], the genus Amaranthus L.is reported as to possessing high inter- and interspecies variability (Mosyakin & Robertson, 1996) [13]. 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) [12].
Molecular markers present numerous advantages over conventional phenotype based

alternatives. The choice and selection of an adequate marker system depends upon the type of study to be undertaken and whether it will fulfil at least a few of the mentioned criteria: (a) highly polymorphic between two organisms, inherited codominantly, (b) evenly distributed throughout the genome and easily visualized, (c) occurs frequently in the genomes, (d) stable over generations, (e) simple, quick and inexpensive, (f) small amounts of DNA samples required, and (g) no prior information about the sample's genome (Agarwal *et al*., 2008; Hatzopoulos *et al*., 2002) [1]. Because of their high polymorphism level and discerning power, molecular markers have been used as a powerful tool for olive gene pools' characterization. Molecular markers have played a crucial role to distinguish, characterize, and to elucidate olive germplasm origin and diversity. Different molecular markers have been applied for olive genetic diversity assessment, such as the dominant random amplified polymorphic DNA (RAPD) (Belaj *et al*., 2003; Cordeiro *et al*., 2008; Gemas *et al*., 2004; Gomes *et al*., 2009; Martins-Lopes *et al*., 2007, 2009; Trujillo *et al*., 1995) [5, 6, 9, 14]

A new DNA polymorphism assay was first described in 1990 by Williams *et al*. (1990) [22] and Welsh & McClelland (1990) [21]. The random amplified polymorphic DNA (RAPD) marker is based on the amplification by PCR of random DNA segments, using single primers of arbitrary nucleotide sequence. The amplified DNA fragments, referred to as RAPD markers, were shown to be highly useful in the construction of genetic maps. With RAPD method the resulted polymorphisms are detected by electrophoresis as different DNA fragments. The different DNA fragments are generated once the primers used usually anneal with multiple sites in different regions of the genome, producing multiple amplified products that often contain repetitive DNA sequences (Paran & Michelmore, 1993) [15].

Molecular marker analyses have contributed to the understanding of origin and evolution of cultivated amaranths and wild species. Amaranth species were analysed 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. Markers were used to distinguish and characterize genotypes of Amaranthus species. This study has demonstrated, that RAPD markers were used to analyse intra and inter specific variability and are also able to generate a sufficient level of informative characters for intra and inter-specific analysis of Amaranthus genus.

Amaranthus remains a species with tremendous potential and although considerable work has been done to exploit the biological activity and medicinal applications of this plant, countless possibilities for investigation still remain in relatively newer areas of its function. Despite increasing attention to amaranths, the germplasm of amaranths has not been well characterized. However, there is an increased interest for a meaningful understanding of the genome of the amaranths and the extent of its genetic diversity. By both types of marker systems (RAPD and SSR) 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.

## Materials & Method

### Plant Materials

Plants of 7 Amaranthus species were collected from 3 different phytogeography regions for experimental purpose (Table 1).

**Table 1:**

| | |
|---|---|
| *A. cruentus* | A.1 |
| Amaranthus *spinus* | A.2 |
| Amaranthus *tricolour* | A.3 |
| A. *caudatus* | A.4 |
| Amaranthus gangeticus | A.5 |
| *A. hypocondriacus* | A.6 |
| *Amaranthus sp.* | A.7 |

### Plant DNA extraction

Total genomic DNA from all the Amaranthus sp. was extracted by the protocol as described by Dellaporta *et al.* (1983) using 1% Cetyl Trimethyl Ammonium Bromide (C-TAB) Method. In Which seeds were grounded, to fine powder using liquid nitrogen, 100 g of PVP (Polyvinyl Pyrolidone) was added and transferred to 15 ml extraction buffer containing 2% w/v C-TAB, 1.4 M NaCl, 200 mM EDTA, 0.1% □-mercaptoethanol,100 mM Tris pH 8.0, preheated to 65 $^0$C and incubated at 65 $^0$C for 1 hrs. With occasional shaking. The homogenate was cooled to room temp and extracted with 15 ml of chloroform: isoamyl alcohol (24:1). Centrifuge at 12,000 rpm (C-24 Remi, 12 x 15 ml Angle Head), 23 $^0$C, for 15 min. The clear aqueous phase was separated. To this, 5 ml of 5 M NaCl and 10 ml isopropanol were added and stored at 4 $^0$C overnight. This was again centrifuged at 12,000 rpm (C-24 Remi, 12 x 15 ml Angle Head), 23 $^0$C for 15 min, and the supernatant decanted and retained the pellets. The pellet was air dried. Pellet was dissolved in 500 µl. of TE and left for 10 minutes. Solution was transferred to 1.5 ml of eppendorf tube. Remaining traces of DNA were dissolved and adding 500µl of TE in centrifuge tube, to get total 1 ml of DNA. In order to remove RNA impurities 5 □l (100 □g/ml), RNase per ml of DNA was added and incubated for 1 hr. at 37 $^0$C. 1 ml of DNA is divided in to two eppendorf tube each tube contains 500 □l of DNA. 250 □l phenol and 250 □l chloroform was added and mixed gently and then centrifuged it at 14,000 rpm. (C-24 Remi, 12 x 15 ml Angle Head) for 15 minutes; at room temperature. Supernatant was isolated and the equal volume of chloroform was added mixed gently and centrifuged at 8,000 rpm (C-24 Remi, 12 x 15 ml Angle Head) at room temperature. Supernatant was isolated and 1/10 vol. of sodium acetate (3 M) and 2.5 vol. of chilled absolute alcohol was added mixed gently and kept it for 1 hr. or overnight at 20 $^0$C. It was further centrifuged at 8,000 rpm (C-24 Remi, 12 x 15 ml Angle Head) at 4$^0$C for 20 minutes; supernatant was decanted and retained the pellet. Pellet was washed twice with 70% alcohol and air dried the pellet. The pellet was dissolved in TE (200l).

### RAPD Marker Analysis with Designed primer

DNA was isolated and then diluted to the concentration of DNA were dissolved in 100 □l TE buffer to give a working dilution of DNA, equivalent to 1 mg/□l. This was further diluted to working dilution of DNA equivalent to 50 ng/□l to 100 ng/□l, in order to check the detection limit of our PCR assay. Highlighting products was performed on agarose gel (1.5%), staining with ethidium bromide and visualization under UV light using Gel Doc UVP LLC system. For the selection of primer corresponding to genetic diversity in Amaranthus *sp*, the PCR was carried out with total DNA from

seeds using specific degenerate primers.RAPD analyses were performing by using different types of decamer primers of Operon kits (Operon Technologies, Alameda, CA) followed by gel electrophoresis.

**Primers used**

An initial analysis of Amaranthus seeds was conducted with 35 arbitraryprimers (Operon Technologies, Alameda, CA, USA). Out of these random decamer primers, only 20screened for experimental purpose, as detectable distinct bands and were used for the study. DNA from the 7 varieties of amaranth was amplified in PCR using 20 operon family primers. Table 2.

**Table 2:**

| Sl.No | Primer | Sequence | Sl.No | Primer | Sequence |
|---|---|---|---|---|---|
| 1 | OPA-20 | GTTGCGATCC | 11 | OPBA-10 | GGACGTTGAG |
| 2 | OPT-04 | CACAGAGGGA | 12 | OPBA-13 | AGGGCGAATG |
| 3 | OPC-15 | GACGGATCAG | 13 | OPBA-20 | GAGCGCTACC |
| 4 | OPA-04 | AATCGGGCTG | 14 | OPBB-01 | ACACTGGCTG |
| 5 | OPF-13 | GGCTGCAGAA | 15 | OPBB-06 | CTGAAGCTGG |
| 6 | OPBA-01 | TTCCCCACCC | 16 | OPBB-09 | AGGCCGGTCA |
| 7 | OPBA-02 | TGCTCGGCTC | 17 | OPBB-11 | TGCGGGTTCC |
| 8 | OPBA-06 | GGACGACCGT | 18 | OPBB-15 | AAGTGCCCTG |
| 9 | OPBA-07 | GGGTCGCATC | 19 | OPBB-17 | ACACCGTGCC |
| 10 | OPBA-08 | CCACAGCCGA | 20 | OPBC-01 | CCTTCGGCTC |

The PCR reaction contained 50 ng of genomic DNA, 1.5 mM of assay buffer, 2.5 mM dnTps each of deoxyadenosine-triphosphate (dATP), deoxythymidine-51-triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and deoxycytidine triphosphate (dCTP), 2.5µM of primer, 1.5 mM MgCl2 and 0.5u/µl units of Taq DNA polymerase. Amplifications were performed in a Agilent techno sure cycler 8800(programmed for an initial denaturation at 94 $^0$C for 5 min, followed by 44 cycles of denaturation at 94 $^0$C for 30 min, annealing at 37 $^0$C for 45 min, 1 min extension at 72 $^0$C and a final extension of 5 min at 72 $^0$C, and then at 4 $^0$C until storage. Amplified PCR products were electrophoresed on a 1.5% agarose gel in 10X Tris/Borate/EDTA (TBE) buffer at 100 volts for 2 h. The ethidium bromide-stained gels were documented using a gel documentation system (UVP Trans illuminator). The reproducibility of the amplification products was checked twice for each polymorphic primer.

**Table 3:**

| Temperature ($^0$C) | Duration (min.) | Activity | Cycles |
|---|---|---|---|
| 94 | 5 | Denaturation | 1 |
| 94 | 30 | Denaturation | 42 |
| 37 | 45 | Annealing | |
| 72 | 1 | Extension | |
| 72 | 5 | Extension | 1 |
| 4 | | Storage | |

**Molecular Data Analysis**

Dendrogram was constructed using genetic distance calculation with the formulas of Sequential, hierarchical, and nested clustering (SHAN) was done using NTSYSpc version 2.1,

Rohlf. FJ (2000) [17]. Binary matrix was constructed based on the presence ("1") or absence ("0") of RAPD bands and clustering was done with UPGMA cluster method (Unweighted Pair Group Mathematical Average).

**Results**

Decamer primers used foranalysis of seven species of amaranth for genetic diversity. Samples were subjected to RAPD analysis, for this 20 primers used: The primer OPA-20And OPF13comprises of 73% to 76% and while the rest of the primers comprises above of 90% GC bases content. Certain correlation can be observed between GC base content and the polymorphism percentage of analyzed amaranth species and have been recorded the polymorphism an average of 70% or above 90% respectively. As a species specific could be recognized the primer LA03. With respect to analyzed species, the percentage of polymorphism differed (73.68% *Amarnthus. sp*, 85% *A. caudatus* and 100% *A. hypochondriacus*).

**Table 4:**

| Sl No | Primer | Sequence | # amplified bands | # Polymorphic bands | # Monomorphic bands | % of Polymorphism |
|---|---|---|---|---|---|---|
| 1 | OPA-20 | GTTGCGATCC | 19 | 14 | 5 | 73.68 |
| 2 | OPT-04 | CACAGAGGGA | 18 | 17 | 1 | 94.44 |
| 3 | OPC-15 | GACGGATCAG | 24 | 23 | 1 | 95.83 |
| 4 | OPA-04 | AATCGGGCTG | 22 | 21 | 1 | 95.45 |
| 5 | OPF-13 | GGCTGCAGAA | 17 | 13 | 4 | 76.47 |
| 6 | OPBA-01 | TTCCCCACCC | 24 | 21 | 3 | 87.50 |
| 7 | OPBA-02 | TGCTCGGCTC | 21 | 19 | 2 | 90.48 |
| 8 | OPBA-06 | GGACGACCGT | 25 | 22 | 3 | 88.00 |
| 9 | OPBA-07 | GGGTCGCATC | 26 | 25 | 1 | 96.15 |
| 10 | OPBA-08 | CCACAGCCGA | 21 | 17 | 4 | 80.95 |
| 11 | OPBA-10 | GGACGTTGAG | 18 | 18 | 0 | 100.00 |
| 12 | OPBA-13 | AGGGCGAATG | 25 | 22 | 3 | 88.00 |
| 13 | OPBA-20 | GAGCGCTACC | 20 | 17 | 3 | 85.00 |
| 14 | OPBB-01 | ACACTGGCTG | 21 | 20 | 1 | 95.24 |
| 15 | OPBB-06 | CTGAAGCTGG | 24 | 21 | 3 | 87.50 |
| 16 | OPBB-09 | AGGCCGGTCA | 23 | 20 | 3 | 86.96 |
| 17 | OPBB-11 | TGCGGGTTCC | 23 | 21 | 2 | 91.30 |
| 18 | OPBB-15 | AAGTGCCCTG | 29 | 27 | 2 | 93.10 |
| 19 | OPBB-17 | ACACCGTGCC | 26 | 26 | 0 | 100.00 |
| 20 | OPBC-01 | CCTTCGGCTC | 25 | 24 | 1 | 96.00 |
| | | Total | 451 | 408 | 43 | 90.47 |

By RAPD analysis of 7 variety of *Amaranthus* a total of 451scorable DNA fragments were amplified using 20 random primers. Among the varieties of *Amaranthus* the similarity index values varied from 0.23 to1.00 with a mean value of 0.63. A primer having resolving power of 5 (coefficient of genotypes distinctiveness 0.62) was sufficient to distinguish the different varieties of Amaranth. The primer OPA-20, OPF-13and OPBA-08Relatively low polymorphism was detected. 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 73.68% *Amarnthus. sp*, 85% *A. caudatus* and 100% *A. hypochondriacus.* The higher percentage of intraspecific polymorphism in *A. hypochondriacus* indicates its greater genetic variation than the other amaranth species studied. Cluster analyses performed with the RAPD data matrix generated by 20 used primers could group genotypes at the inter-specific level into four main clusters (Fig. 1) with the number of genotypes 12, 14, 17 and 8.

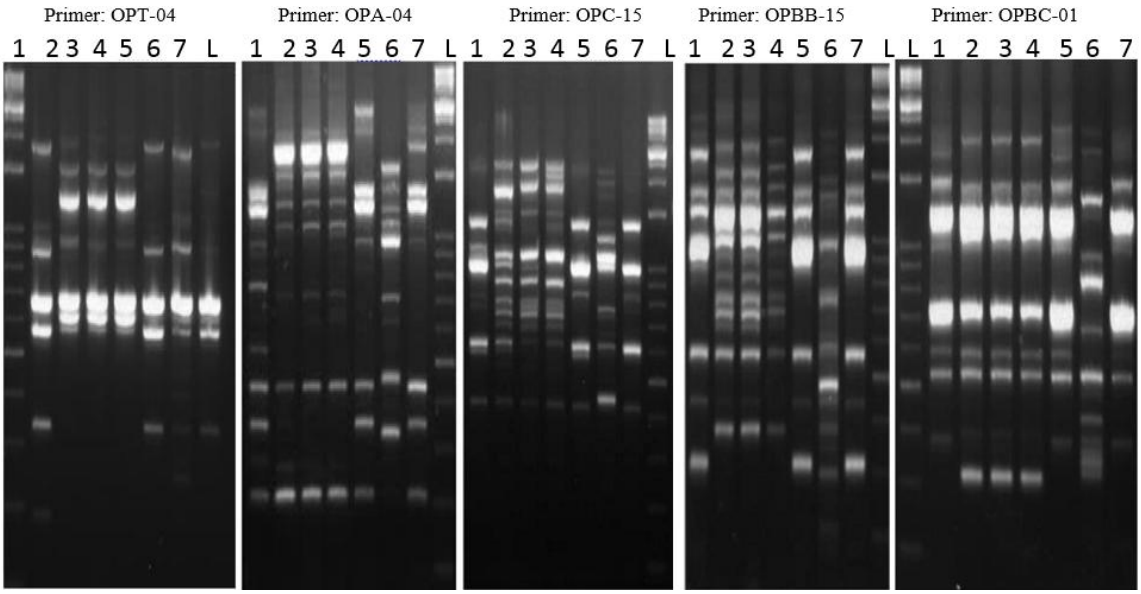


**Fig 1:** Electrophoretic profile of amplification products obtained with primers

Analyzing the results obtained, we found that A2, A3 and A4 showed an identical profile (or very similar) of amplification products, even if the intensity of the bands varied between the varieties of Amaranth. A similar aspect was also highlighted at A6 variant with control. On the results with OPBA07, OPBA08 and OPBA13 primer, THREE groups of samples could be associated: first included A2, A3 and A4, variants are grouped in the second one. Similar results were obtained with the primer OPBB09: one group includes A2, A3, A4 and A6.

Differences in electrophoresis pattern of PCR products were also detected with the primers OPA 04 and OPC 15 (Fig.). It may be noted that by using OPA-04 primer are two groups of electrophoresis profiles: the first group is composed of the following A5 and A7, the second group includes A3 and A4 with control, and A6 variant shows a slight contrast to the other.

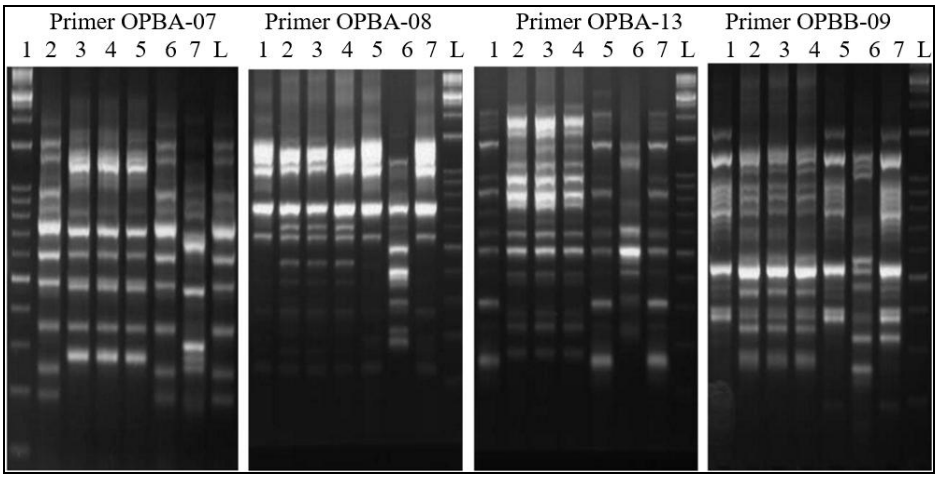


**Fig 2:**

When using OPBB 15 primer, clustering variants were similar, although in this case, the electrophoresis profile of the A1, A5 and A7 variant was very close to that of variants A2, A3 and A4. Significant differences Occurred when OPBC primer was used (Fig.1): A2, A3 and A4 variants had identical profiles, as is the A4 variant and control. Regarding A1 variant, it deviates less in profile than A5, while in the A 6 are higher.

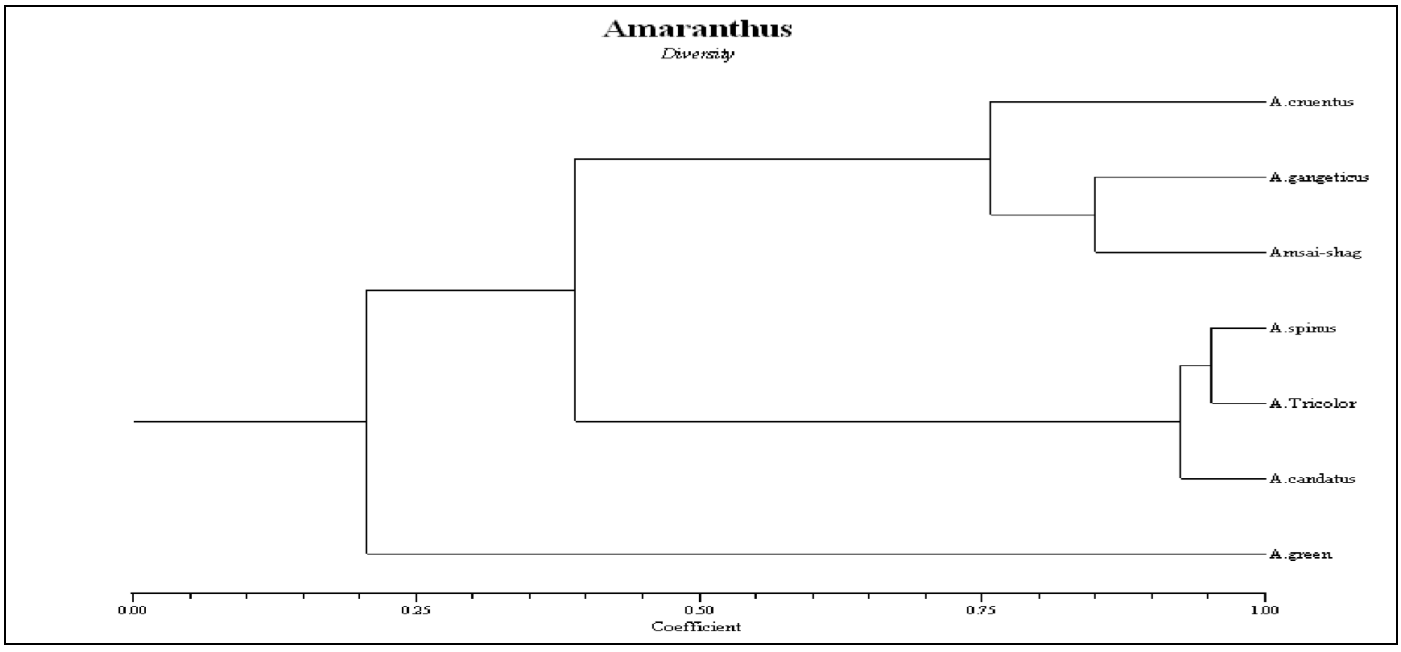


**Fig 3:** Clustering based on the seed of 7 varieties of *Amaranthus* according to NTSYSpc version 2.1 index

Amplicons in the size range from ~290 to ~3000 bp were scored for an estimation of genetic Similarities among the Amaranthus species. A total of 451 amplified bands were generated, of which 43 were monomorphic (10.48%), while 408 were polymorphic, resulting in 91% polymorphism.

The assessment data can be seen that A1 and A6 variant are identical, so they belong to *A. Hypocondriacus* species. In the other variants, A2, A3 and A4, the genetic distance is between 0.3 and 0.5, and can be considered part of the same species.

The UPGMA dendrogram revealed two major clusters and 6 sub-clusters. The dendrogram reflects that in spite of the apparent phenotypic similarities between some species, RAPD markers were able to detect sufficient polymorphisms to also distinguish them. Percentages of RAPD polymorphism were found to be 44.78%, 49.66%, 49.88%, and 43.01% respectively.

## Discussion

Amaranth became rediscovered and is intensively studied through the molecular based polymorphism analysis. *Amaranthus* is a cosmopolitan genus comprising large number of species with many morphotypes cultivars having diversified morphological features. The species, morphotypes within *Amaranthus* are very closely related requiring micro classification even revision in present taxonomic status (Das, 2012) [7]. 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) [10, 19, 20, 11].

At present experimental finding, RAPD primers used for genetic diversity and relationships analysis among 7 *Amaranthus* species from 3 phytogeographic regions yielded a total of 451 amplicons, ranging from ~290 to ~3000 bp in size with an average of 12 amplicons per primer, of which 408 amplicons (96.94%) were polymorphic while the 43 amplicon were found as monomorphic in finding. The genetic similarity coefficient among all the *Amaranthus* species ranged from 0.20 to 1.00 with a variation existed in the genetic diversity of different populations. Decamer primers sufficiently produced clear and reproducible RAPD profiles (Ray & Roy, 2009) [20]. So the amaranth species and primer's type, the values of the similarity index among the species 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). The dendogram finding show that the A. spinus and A. tricolour are most similar to each other likewise the A. gangeticus and A. sp. A. cruentus and A. caudatus show a big difference cluster as shown in fig substantialy different from all other varieties of Amaranth species The line joins ton the A2, A3 and A5, A7 that means the two groups are more similar to each other other than A6. The results revealed an intraspecific polymorphism in *A. hypocondriacus*, was detected among the other studied species. However, the low level of polymorphism in A 7 species may reflect the high level of inbreeding in these *Amaranthus* species. Therefore the primers which we have used they were successful in amplifying the bands from the genus *Amaranthus* [Bardini, M., Lee, D, 2004] [4]. Analysis with the RAPD markers we used to clarify the phylogenetic relationships among the studied species revealed a relatively high level of polymorphism However, our current study showed that applicable molecular Method have useful in the characterization at biological level Moreover, RAPD analysis using specific primers revealed some amplicons that, after confirmation, could be regarded as molecular markers for the species tested. This report will help us to find strategies for marker-assisted identification of amaranth genotypes in order to conservation of amaranth genetic resources for different inbreeding approaches.

## Conclusion

Result finding show the more polymorphism implies that the phenotypic character all seven variety of Amaranth were more diverse and more versatile in ecological nature which helps the future inbreeding research. Seven amaranth species were analyzed to determine genetic diversity within them, to obtain the corresponding genetic variations, and to carry out comparisons for differentiating and distinguishing between the genetic variants. Random amplified polymorphic DNA (RAPD) were used to distinguish and characterize the genotypes of seven varieties of *Amaranthus* species. Each of 20 decamer primers used for generating the large numbers of polymorphisms, ranging from 87 to 100%. The present work has shown that both DNA markers systems generate sufficient polymorphisms for DNA fingerprinting as well as for intraspecific analysis for different origin and breeding purpose.

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