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Molecular characterization of amaranth landraces and assessment of interspecific relationships among *Amaranthus* spp. (L.) using RAPD markers

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Abstract

Amaranth landraces belonging to three species namely *Amaranthus tricolor*, *A. dubius* and *A. hypochondriacus* were studied for assessing genetic diversity and interspecific relationship using RAPD markers. Eighteen primers used for the study showed a very high level of polymorphism. Out of the total 376 brands detected, 89% were polymorphic. Genetic similarity indices ranged from 0.252 to 0.942 indicating a very high level of diversity within landrace collection which is mainly attributed to interspecific diversity. Three species distinctly formed three different groups on clustering. Clustering was influenced by morphological characters like leaf colour, but no geographical trends were clear. RAPD markers specific to each species were identified which can be utilized in future as SCARs. *A. dubius* and *A. hypochondriacus* were observed to be genetically closer than *A. tricolor*.

Key words: Amaranthus, RAPD markers, genetic diversity, interspecific relationships

Introduction

Amaranths are broad leaved plants well adapted to a range of humid environments. Grain amaranths are small seeded crops with dramatic history. The grain has high protein content with relatively high lysine levels. People around the world utilize either the grain or the leaves in a variety of recipies. Amaranths are also used medicinally and as protein supplement for children. Many alternative uses were also identified in countries like China, where plants are processed to protein, starch, oil and pigments for use in the food industry. In India, plants are mainly used as a protein rich leafy vegetable. Major species under cultivation in India include *Amaranthus tricolor*, *A. dubius* and *A. hypochondriacus*. Both wild and cultivated species are useful for genetic improvement. Amaranth has a broad genetic variability with diversity in the plant type (erect or prostrate), number of inflorescence (one to several), seed colour, earliness, protein content, plant height, seed and green matter yield, resistance to pest and disease and day length.

Characterizing germplasm collection facilitates effective use of genetic diversity in germplasm. DNA markers are most suitable for genetic diversity estimates their use as a means to assay diversity at the locus, chromosome and whole genome level is now well established.

Since the introduction of Random Amplified Polymorphic DNA (RAPD) markers in 1990 [1], their use in plant genetic analysis have increased in an exponential manner principally due to the ease of procedure. RAPDs are relatively quick, inexpensive and require no prior sequence information of target genome. Low amounts of DNA are sufficient, require no radioactive elements and detect a good number of polymorphisms. RAPD analysis have been used for genotype characterization, to assess genetic diversity, for genotype mapping, to identify markers linked to genes of interest and to assess inter or intra-populational genetic variability [2]. Recently, PCR based Simple Sequence Repeat (SSR) markers requiring small amount of DNA have also been developed [3] which have proved to be polymorphic but require nucleotide information for primer design and necessitate sophisticated electrophoretic systems for band resolution.

In the present study amaranth landraces collected from farmers were characterized using PCR based molecular markers - RAPD - in order to find out genetic diversity as well as to assess the genetic relationships among major cultivated amaranth species in India.

Material and methods

The plant material was collected from the germplasm maintained at Kerala Agricultural University under NATP project titled Sustainable Management of Plant Biodiversity of Amaranthus. Germplasm collection comprised 120 accessions collected from local farmers of Kerala. These include three major cultivated Amaranthus species namely, *A. tricolor*, *A. dubius* and *A. hypochondriacus*. Out of these landraces, fifteen

genotypes were selected after morphological classification based on leaf and inflorescence colour. The list of selected genotypes is given in Table 1. Selected *A. tricolor* accessions included red (5), green (3) and mixed (3) leaf types, *A. dubius* accessions included green (2) leaf types. *A. hypochondriacus* accessions consisted of grain (2) types.

PCR conditions were optimized with varying concentration of template DNA, Taq Polymers and $MgCl_2$ concentration. Primer survey was carried out and 45 primers from OPU, OPX, OPAK and OPA series (Operon technology inc, USA) were screened. Eighteen primers that gave reproducible and scorable bands were used for analysis (Table 2).

Table 1. List of amaranth genotypes selected for RAPD analysis

Sl. No.	Accession No.	Species name	Place of collection	Leaf colour	Inflorescence colour	Plant type
1.	563	*A. tricolor*	Thrissur	Red	Red	Red
2.	564	*A. tricolor*	Thrissur	Reddish green	Reddish green	Red
3.	510	*A. tricolor*	Kannur	Red	Red	Red
4.	538	*A. tricolor*	Alappuzha	Red	Red	Red
5.	550	*A. tricolor*	Thiruvananthapuram	Red	Red	Red
6.	501	*A. dubius*	Thrissur	Green	Green	Green
7.	577	*A. tricolor*	Malappuram	Reddish green	Red	Green
8.	581	*A. tricolor*	Ernakulam	Green	Green	Green
9.	584	*A. dubius*	Kozhikode	Green	Green	Green
10.	587	*A. tricolor*	Alappuzha	Green	Red	Green
11.	504	*A. tricolor*	Thrissur	Green	Green	Mixed
12.	542	*A. tricolor*	Thrissur	Red	Red	Mixed
13.	602	*A. tricolor*	Palakkad	Reddish green	Reddish green	Mixed
14.	539	*A. hypochondriacus*	Alappuzha	Red	Red	Grain
15.	568	*A. hypochondriacus*	Ernakulam	Green	Reddish green	Grain

No*.: Landraces collected and maintained at Agricultural University under NATP on sustainable management of plant biodiversity

DNA extraction: Total DNA was extracted by a method described for amaranthus by Dixit [4] which was a combination of procedures for plants by Dellaporta *et al.* [5] and for bacterial plasmids by Sambrook *et al.* [6]. Fresh leaves were rinsed, dried, weighed to 500 mg, ground in liquid nitrogen, transferred to eppendorf tubes with 1.5-2 ml extraction buffer (100 mM Tris HC1, pH 8.0, 50 mM EDTA, 500 mM NaCl and 10 mM β mercaptoethanol), and incubated at 65°C water bath for 30 minutes after mixing with 100 µl of 20% SDS. To this, 5M Potassium Acetate (500 µl) was added and kept on ice for 10 minutes. Supernatant was taken in new tube after centrifugation at room temperature. The supernatant was twice extracted with 1 ml Chloroform and pellets were retrieved after immediate centrifugation with chilled isopropanol at room temperature. Pellets were dissolved in 0.5 ml TE buffer after washing with 70% ethanol, gently mixed with 0.5 ml chilled 20% PEG in 1.2 M NaCl and kept on ice. After centrifuging at 4°C, pellets were washed in 70% ethanol, air dried and dissolved in 500 µl TE (10mM Tris HCl, 1mM EDTA) buffer. DNA concentrations were estimated by comparing with weighed standard DNA running along with samples on 1% agarose gel stained with ethidium bromide.

PCR Amplification: One sample from each of the four amaranth types was used for primer screening.

Table 2. Description of eighteen primers used for molecular characterization of amaranthus germplasm

Primer	Sequence	Band no.	Base pair (bp)	Polymorphism (%)
OPA 01	CAGGCCCTTC	19	200-2420	68.0
OPA 02	TGCCGAGCTG	21	160-2700	78.0
OPA 03	AGTCAGCCAC	23	220-2250	71.0
OPA 04	AATCGGGCTG	19	170-2150	84.0
OPA 05	AGGGGTCTTG	22	250-1740	90.9
OPA 07	GAAACGGGTG	25	260-3450	96.0
OPA 09	GGGTAACGCC	26	195-2700	92.3
OPA 10	GTGATCGCAG	18	210-2540	100.0
OPA 11	CAATCGCCGT	22	190-1575	90.9
OPA 12	TCGGCGATAG	20	180-2440	95.0
OPA 13	CAGCACCCAC	22	320-2725	12.1
OPA 15	TTCCGAACCC	12	450-2970	83.0
OPA 16	AGCCAGCGAA	20	300-2410	100.0
OPA 17	GACCGCTTGT	28	3645-4320	100.0
OPA 18	AGGTGACCGT	17	205-3230	94.0
OPA 19	CAAACGTCGG	26	310-3450	100.0
OPA 20	GTTGCGATCC	18	500-2350	88.8
OPAK 16	CTGCGTGCTC	20	295-2225	90.0

PCR was carried out in a thermal cycler (MJ Research). Each 25 µl reaction mixture contained 25 ng template DNA, IX reaction buffer (10 mM Tris HCl pH 8.3 and 50 mM KCl), 2.5 mM $MgCl_2$, IU Taq Polymerase, 100 µM of each dNTPs. Amplification cycle

was performed as an initial 3 cycles of denaturation of template DNA at 94°C for 1 minute, primer annealing at 40°C for 1 minute and primer extension at 72°C for 2 minutes followed by 40 cycles of 94°C for 30 seconds, 40°C for 30 seconds and 72°C for 1 minute. A final extension of 72°C for 10 minutes was performed followed by immediate cooling and storing at 4°C.

RAPD analysis: PCR products were separated on 1.5% agarose gel stained with ethidium bromide and run in IX TAB at constant 75V. Gels were visualized with UV transilluminator and photographed. All reactions were run in duplicate. All samples amplified well (Fig. 1) and selected 18 primers gave reproducible good bands on repeated trials.

Data scoring and statistical analysis: The band sizes were determined after comparing with Lambda DNA double digested with *EcoR*I and *HindD*III molecular marker, which was run along with amplified products. Each reproducible band was visually scored '1' for presence and '0' for absence. Binary data were used for statistical analysis. The data were analysed using NTSYS PC version 2.02i to generate Jaccard's similarity coefficient. The matrix was subjected to unweighted pair group method for arithmetic average analysis (UPGMA) to generate dendrogram using average linkage procedure.

Results and discussion

Any crop improvement programme depends on knowledge and understanding of genetic diversity available within the germplasm. Characterization and cataloguing of amaranth germplasm have been carried out using morpho-agronomic traits. Recently more versatile molecular marker techniques have replaced conventional techniques for characterizing genetic diversity. However, the use of biochemical and molecular markers in Indian Amaranth landraces is very limited or almost nil. RAPD markers have potential to measure variation with good coverage of entire genome. Careful standardization of each step overcomes the difficulties of RAPD technique reported earlier. We found that reliable data can be generated by strictly following standardised protocol, replication of amplification reaction and conservative criterion of band selection. Optimisation of various experimental steps, starting from DNA extraction protocol to the PCR amplification reaction and gel electrophoresis, was done in order to overcome the reported sensitivity problem of RAPD technique. Standardised DNA extraction protocol gave a very clear band of DNA with very good quantity and quality. Selected primers also gave clear reproducible bands on amplification. Each of the constituents in PCR amplification reaction was optimized after trying different concentration and the standardised protocol was strictly followed to get maximum reliability of the RAPD data.

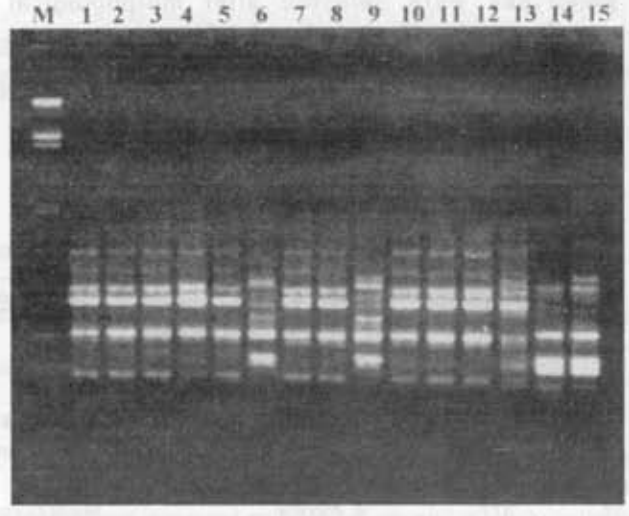

Fig. 1. RAPD profile of *Amaranthus* genotypes generated using random primer OPA 13: Lane 1-5, *A. tricolor*- red; Lane 6, *A. dubius*- green; Lane 7-8, *A. tricolor*- green; Lane 9, *A. dubius*- green; Lane 10, *A. tricolor*- green; Lane 11-13, *A. tricolor*- mixed: Lane 14-15, *A. hypochondriacus*- grain; Lane M, marker-Lambda DNA digested with ECoRI and Hind III.

RAPD profile analysis: Selected primers detected a very high level of polymorphism (Table 2). A total of 376 bands detected from 18 primers of OPAK 16 and OPA series (01, 02, 03, 04, 05, 07, 09, 10, 11, 12, 13, 15, 16, 17, 18, 19 and 20). The number of bands per primer ranged from 12 (OPA 15) to 28 (OPA 17) with an average of 20.8 scorable bands per primer. Out of the 376 bands selected, 335 were polymorphic (89%) revealing a high degree of polymorphism detected using RAPD markers. The molecular weights of amplification products ranged from 160 bp (OPA 02) to 4320 bp (OPA 17). Percentage of polymorphism detected with each primer was as high as 100% (OPA 10, 16, 17 and 19) to 68% detected by OPA 01.

The high level of polymorphism observed in the study agrees with the results of previous studies carried out in amaranth [7] and in other plant species like cowpea [8] and soybean [9], there by confirming the high diversity within the germplasm. Large number of bands obtained per primer indicated the efficiency of the selected primers to characterize the germplasm. Highest level of polymorphism evident with OP A 10, 16, 17 and 19 could be explained as the capability of RAPD primers to amplify the less conserved and highly repeated regions of the DNA. The high probability of amplified fragments, containing repeated sequence is also reported by Prasannalatha *et al.* [10]. Higher numbers of co-migrating bands monitor the reproducibility of amplification pattern, while polymorphic ones provided the key to genotype identification [11].

Rare and genotype specific bands were identified. A brief description of bands specific to each amaranth type is given in Table 3. The characteristic banding pattern of each species has helped to identify the markers that could distinguish clearly between three Amaranthus species (Fig. 1). Primers OP AS and OPA 17 had markers that were specific to each species. In OPA 17, 340 bp marker is present only in grain amaranth (*A. hypochondriacus*), 775 bp in *A. dubius*, while 650 and 945 bp markers were specific to *A. tricolor* accessions. The primer OPA5 also had distinct bands which could be turned into species - specific markers. In addition, certain markers characteristic to *A. tricolor*, *A. dubius* and *A. hypochondriacus* are also presented briefly in Table 3. Specific bands in primers like OPA 1, 5, 15, 16, 17, 19 and 20 can be converted to (Table 3) to sequence characterized amplified regions

Table 3. RAPD markers identified in the study for different *Amaranthus* species

Primer	Marker (bp)	Species
OPA 05	763	*A. tricolor*
	1025	*A. dubius*
	330	*A. hypochondriacus*
OPA 17	650	*A. tricolor*
	775	*A. dubius*
	340	*A. hypochondriacus*
OPA 01	220	*A. tricolor*
OPA 19	970	*A. tricolor*
OPA 12	338	*A. dubius*
OPA 15	450	*A. dubius*
OPA 20	500	*A. hypochondriacus*
OPA 16	873	*A. hypochondriacus*

(SCARs) which is useful for getting cultivar specific profiles. Genotype specific bands were also noticed in sesame [12]. Such studies are gaining importance in the changing international scenario.

Genetic Diversity Analysis: Genetic similarity matrix was calculated on the basis of Jaccard's algorithm for RAPD data (Table 4). The pair wise similarity values ranged between 0.256 and 0.942. Maximum similarity value was noticed between 1st and 2nd accessions, both being red *A. tricolor* (Trichur Red). Minimum similarity value of 0.256 was observed between 1st (Trichur Red - *A. tricolor*) and 6th (Trichur green - *A. dubius*) accessions. Wide range of similarity indicated high genetic diversity in amaranth collection. The genetic diversity was mainly due to interspecific dissimilarity. *A. hypochondriacus* showed only 25-30% genetic similarity with *A. tricolor*, while with *A. dubius* it showed an average similarity of 44%. *A. tricolor* and *A. dubius* are genetically 25-30% similar. Intraspecific dissimilarity is much less when compared to interspecific dissimilarity. Lowest similarity value (0.75) was observed between 4th (Allappey red) and 13th (Palghat mixed) accessions of *A. tricolor*, indicating that a diversity as high as 25% existed within various types of *A. tricolor* accessions collected from different geographical regions. A similarity value of 78% was observed within *A. dubius* accessions, between Trichur and Kozhikode green, where as both accessions of grain amaranth differed only 15% with respect to each other.

Wide range of similarity values suggested that the collected landraces of amaranth represents a genetically diverse population. Previous reports of molecular marker diversity on the amaranth landraces are lacking in India. Earlier studies were mainly based on morphological parameters. Morphological classification suggests only a moderate variation within the germplasm (Table 1). The level of allogamy (5-30%) found in amaranth also explains the variation. Genetic diversity observed in the germplasm using molecular markers indicates the high discrimination capacity of

Table 4. Genetic similarity matrix for *Amaranthus* genotypes generated using Jaccard's similarity coefficient

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1.000														
0.942	1.000													
0.913	0.910	1.000												
0.907	0.876	0.876	1.000											
0.892	0.935	0.886	0.877	1.000										
0.256	0.270	0.259	0.271	0.263	1.000									
0.887	0.886	0.868	0.851	0.899	0.257	1.000								
0.853	0.862	0.834	0.829	0.868	0.271	0.848	1.000							
0.273	0.288	0.276	0.277	0.287	0.778	0.280	0.262	1.000						
0.855	0.832	0.827	0.841	0.851	0.279	0.869	0.856	0.292	1.000					
0.864	0.859	0.845	0.828	0.862	0.268	0.864	0.869	0.293	0.899	1.000				
0.867	0.848	0.829	0.829	0.849	0.283	0.830	0.871	0.300	0.883	0.897	1.000			
0.773	0.793	0.757	0.750	0.788	0.300	0.791	0.779	0.313	0.799	0.811	0.812	1.000		
0.266	0.277	0.274	0.295	0.280	0.449	0.280	0.292	0.432	0.302	0.281	0.292	0.289	1.000	
0.278	0.289	0.286	0.301	0.290	0.441	0.290	0.307	0.438	0.312	0.291	0.307	0.307	0.857	1.000

RAPD markers. Individual genotypes were identified in the study and there were no duplicates. This is of major importance for solving problems concerning management of amaranth germplasm.

The clustering of RAPD based genetic similarity values using the UPGMA method are presented in dendrogram (Fig. 2). In the dendrogram, similarity coefficients between all possible pairs of genotypes ranged between 0.28 and 0.94. At 50% similarity all the accessions combined to form three major clusters, which clearly demarked the germplasm into three species. Cluster group I combined all the *A. tricolor* accessions (red, green and mixed types) into one, while clusters II and III comprised of *A. dubius* and *A. hypochondriacus* accessions respectively. At 40% similarity level, *A. dubius* and *A. hypochondriacus* accessions combined to form one cluster. At 80% similarity level all the accessions were divided into 5 major clusters. Cluster I combined all the accessions under *A. tricolor*, except 13 (Palakkad mixed), which alone formed cluster II. Both the accessions of *A. dubius* separated into two and formed clusters III and IV. The cluster V comprised of both accessions of *A. hypochondriacus*. At higher similarity levels, clusters sub group into three small clusters. All red leaf coloured *A. tricolor* accessions remained together, while green and mixed types were very close except Malappuram green (green leaf type *A. tricolor*), which clustered along with red leaf type accessions.

The diversity observed in germplasm is mainly attributed to the genetic dissimilarity between the three collected species. This is evident from unique banding pattern in RAPD profile exhibited by each species (Fig. 1). Within each species, accessions showed much more homogeneity with an average of 15-20% genetic dissimilarity between each other. Morphologically similar individuals were also genetically dissimilar and grouped into different clusters. *A. dubius* accessions and green type *A. tricolor* accessions were morphologically similar in leaf and inflorescence colour. 1st and 2nd accessions of *A. tricolor* (both red) collected from Trichur was found to be the most genetically similar genotypes on clustering, but were morphologically distant. RAPD profiles revealing low levels of intraspecific variation and high level of polymorphism is also supported by the observations of Ruas *et al.* [13] in six species of *Chenopodium*.

Even though clustering does not always strictly follow morphological classification, morphological characters like leaf colour seem to have high influence on clustering. Red leaf coloured accessions within *A. tricolor* formed sub group 1 while green and mixed type remained together in sub group 2 of cluster I. At 75% similarity level, Palakkad mixed accession (13th) with reddish green leaf and inflorescence colour remained separate as sub group 3. *A. tricolor* accession Malappuram green (7th) with reddish green leaves and red inflorescence was morphologically classified under green type and was observed to cluster along with red leaf coloured *A. tricolor* accessions in sub group 1. From the dendrogram, no geographical trends were clear among the population following UPGMA. Lack of agreement of RAPD data with morphological or geographical classification was also noticed earlier by Kaundun and Park [14]. Steiner *et al.* [15] noted that geographic distance among genotype collecting site was not associated with plant genetic distance but ecologic similarity was related to genetic similarity. Clustering of cultivars from same or nearby region was also reported by Shashidhara *et al.* [16].

India is one of the centers of domestication of these three major species of amaranthus under cultivation. These are difficult to characterize taxonomically due to similarity between large number of species, small difficult to see diagnostic features geographical distribution, which is the reason for many synonyms. From the RAPD study it is evident that interspecific dissimilarity is the main reason for genetic

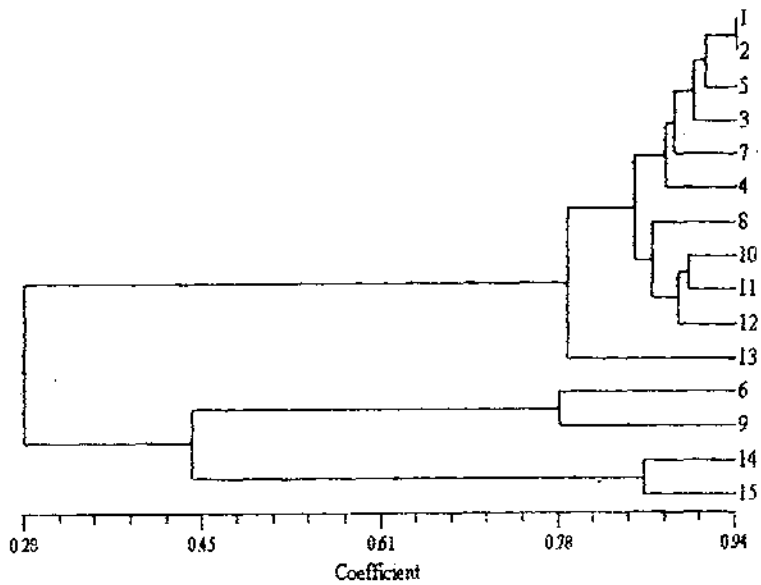

Fig. 2. Dendrogram developed from UPGMA cluster analysis showing genomic relationship between *Amaranthus* species: 1-5, *A. tricolor*- red; 6, *A. dubius* - green; 7-8, *A. tricolor* - green; 9, *A. dubius* - green; 10, *A. tricolor* - green, 11-13, *A. tricolor* - mixed; 14-15, *A. hypochondriacus* - grain

diversity in amaranth land races, since three species observed to vary upto 75% in the population. All types of *A. tricolor* accessions, including red, green and mixed type, had an average of 25-30% similarity to either *A. dubius* or grain amaranth accessions, while *A. dubius* and grain amaranth accessions were comparatively more similar (44%). The closeness of *A. dubius* and *A. hypochondriacus* clusters was observed earlier by Pal and Khoshoo [17]. *A. tricolor* is known to have a distant relationship with *A. hypochondriacus* (*A. hybridus* complex) as noticed by Xu and Sun [18]. High interspecific dissimilarity is also attributed to the differences in the basic chromosome level of each species. Chromosome number of *A. hypochondriacus* is normally n = 16 while in leafy amaranth it is n = 17 [19].

Our results indicate that RAPD marker is an excellent tool to assess genetic variability within amaranth germplasm collection. Intraspecific variability in amaranth is high enough to distinguish each genotype into unique one. Intraspecific dissimilarity is the main reason for genetic diversity in amaranth landraces. Chances of getting rare alleles are greater when large numbers of samples are studied from a population. It is also proposed that conservation efforts should target whole diverse population rather than selected individuals from diverse population.

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