

Genetic diversity in Amaranth (*Amaranthus* spp.) based on phenotypic and RAPD markers

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Abstract

Genetic similarities, which can be detected with phenotypic and molecular markers, are important for effective use of available germplasm. It provides information on genetic diversity for reliable gene recombination. Thirty accessions of amaranth were assessed for variation with 15 phenotypic characters and 10 random amplified polymorphic DNA (RAPD) primers. Significant ($p < 0.05$) phenotypic variation was observed among the accession at the vegetative phase and most of the accessions had leaves with green pigmentation. Sink factor such as leaf, stem and root weights, and dry matter, were homogenous traits which contributed significantly to diversity among the accessions. The RAPD primers produced 115 bands with 88 (77%) polymorphic loci. The DNA classification between some of the accessions was similar to the phenotypic diversity. Information on phenotypic diversity and traits contributing to the diversity can be used deductively with genetic diversity to guide effective hybrid program.

Keyword: Accession, genetic divergence, Mahanalobis, oligonucleotide, polymorphism, UPGMA,

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Introduction

Genetic diversity is an important component in any breeding program and the extent of genetic divergence will determine the limits of selection for improvement. It is indispensable in developing new crop varieties with desirable characters. Therefore, assessment of genetic diversity in available germplasm is important as prerequisite for crop improvement. Diversity is usually determined based on morphological characters which include quantitative and qualitative traits (Govindaraj, 2015). However, this approach is not adequate due to small number of phenotypic markers that are usually influenced by environmental factors. However, complementing the morphological information with genetic diversity at the level of DNA would be more reliable. Molecular markers are usually consistent and more accurate because they are unlimited in number and are

not affected by environmental factors or developmental stage of the plant (Winter and Kahl, 1995).

Random Amplified polymorphic DNA (RAPD) markers were considered in this study. It uses arbitrary primers that provide a large number of multi-locus markers and has been considered efficient for study of genetic diversity in plants (Chan and Sun, 1997; Mandal and Das, 2002; Fracaro et al., 2005; De Masi et al., 2006; Popa et al., 2010). RAPD is less costly and convenient to generate data faster with less labour compared to other molecular makers.

Amaranths are broad-leafed plants, economic and important vegetable in West Africa (Grubben and Denton, 2004). The genus *Amaranthus* consist of about 70 species distributed throughout the world. *A. cruentus*, *A. dubius* and *A. tricolor* are among the common species cultivated as vegetable in Africa

(Grubben and Denton, 2004; Maundu et al., 2009). The tender leaves and stem are consumed cooked. It is a nutritious food with high content of protein, vitamins, minerals (Alegbejo, 2013; Shukla et al., 2010). Amaranth is generally management resource-efficient and warm season crop with ability to adapt to heat and drought (Katiyar et al., 2000; Robert et al., 2008).

Despite the nutritional quality and agricultural potential of amaranths, limited attention has been placed on its genetic improvement. This study was carried out to classify thirty accessions of amaranth into distinct groups based on phenotypic and RAPD markers, and to determine characters that are selection criteria to genetic diversity.

Materials and Methods

Plant materials and field evaluation

A total of thirty accessions of amaranth collected from Germplasm Units of National Centre for Genetic Resources (NACGRAB), Nigeria, National Horticultural Research Institute (NIHORT), Nigeria and Asian Vegetable Research and Development Centre (AVRDC), Tanzania (Table 1) were used in the study. The accessions were evaluated during the late growing seasons of 2010 and 2011 at the Teaching and Research Farm of the Federal University of Agriculture, Abeokuta, Nigeria. Abeokuta is located between Lat 7°10'N and 7°58'N, Long 3°20'E and 4°37'E, and Alt 159 masl.

Randomized complete block design with three replicates was used. The accessions were grown in three-row plots at a spacing of 0.30 x 0.30 m within and between rows. Seedlings were raised in the nursery with top soils and transplanted to the field at 3 weeks after planting. A plant per hill was maintained at 2 weeks after transplanting to give a total of 15 plants per plot. Inter-plot spacing was 0.50 m. At 5 weeks after transplanting, ten plants were randomly selected per plot and measured for

plant height (cm), stem width (cm) at 0.20 m from soil level, number of leaves; length and width of 3rd leaf from top of the plant (cm), petiole length (cm), leaf area (cm²), fresh weights of leaf (including petiole), stem (g), root (g) and whole plant, and dry matter (g) - 20 g fresh leaf oven dried at 50°C for 12 hrs. The following characters were estimated leaf/stem ratio = $\frac{\text{leaf length (cm)}}{\text{stem width (cm)}}$; Specific leaf area = $\frac{\text{leaf area (cm}^2\text{)}}{\text{leaf dry matter (cm}^2\text{/g)}}$; Biomass weight as total fresh weight of leaf and stem (g); Harvest index = $\frac{\text{Biomass weight (g)}}{\text{Whole plant weight (g)}}$. Also, the genetic materials were observed for some qualitative traits (Table 1).

Data collected were subjected to analysis of variance across the years (genotype and year were considered as fixed factors). Significant effects were separated with Duncan's multiple range test. Mean values of the genotype were subjected to the procedures of principal component, factor and single linkage cluster analyses using statistical analysis system (SAS) ver 9.1.1 (SAS Institute, 2000). Also, FASTCLUS and canonical analysis were performed on the standardized data (mean = 0; standard deviation = 1) to classify the accessions into distinct groups

RAPD analysis

Leaves of 2 week-old seedlings of ten individuals per accessions were collected for the random amplified polymorphic DNA (RAPD) assays. Genomic DNA was extracted from the fresh leaves with cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle, 1991). A total of 22 oligonucleotide primers with arbitrary sequence were collected from the Biotech Centre Laboratory, International Institute of Tropical Agriculture (IITA), Nigeria and assayed on five randomly selected accessions. A final 10 primers (Table 5) which gave high polymorphism and distinct bands were used for the RAPD analysis. The PCR amplification was performed in 25 µl reaction volume with 5.0 µl of 25 ng genomic DNA, 1.6 µl of 25 mM MgCl₂, 2.0 µl of 25 nM

Table 1. Qualitative trait and source of thirty amaranth accessions

Accession	Source	Leaf pigmentation	Leaf shape	Leaf margin	Leaf size
NG08	NACGRAB	Green	Elliptical	Entire	Large size
NG11	NACGRAB	Green	Lanceolate	Entire	Large size
NG104	NACGRAB	Green	Elliptical	Entire	Small size
NG16	NACGRAB	Green with pink stripe	Elliptical	Entire	Large size
NG27	NACGRAB	Green	Lanceolate	Entire	Large size
NG123	NACGRAB	Green with pink stripe	Elliptical	Entire	Medium size
NG132	NACGRAB	Green	Lanceolate	Entire	Large size
NG141	NACGRAB	Green	Lanceolate	Entire	Large size
NG145	NACGRAB	Green	Elliptical	Entire	Large size
NG174	NACGRAB	Pink with green strips	Elliptical	Entire	Large size
NG184	NACGRAB	Pink	Elliptical	Entire	Large size
NG621	NACGRAB	Green	Elliptical	Entire	Large size
NH86	NIHORT	Green	Lanceolate	Undulate	Large size
NH117	NIHORT	Pink	Elliptical	Entire	Medium size
NH120	NIHORT	Pink	Lanceolate	Crenate	Large size
NH151	NIHORT	Pink	Elliptical	Entire	Large size
NH161	NIHORT	Green	Elliptical	Undulate	Small size
NH169	NIHORT	Pink	Elliptical	Undulate	Large size
NH3194	NIHORT	Green	Rhombic	Undulate	Large size
AM06	AVRDC	Pink with green strips	Elliptical	Entire	Large size
AM11	AVRDC	Pink	Elliptical	Entire	Medium size
AM14	AVRDC	Green	Elliptical	Entire	Medium size
AM18	AVRDC	Pink	Lanceolate	Entire	Medium size
AM19	AVRDC	Green	Lanceolate	Entire	Medium size
AM20	AVRDC	Pink with green strips	Elliptical	Entire	Large size
AM31	AVRDC	Pink	Elliptical	Entire	Large size
AM32	AVRDC	Pink with green strips	Elliptical	Entire	Medium size
AM42	AVRDC	Green	Lanceolate	Entire	Medium size
ACNL	AVRDC	Pink with green strips	Elliptical	Entire	Medium size
UG40	AVRDC	Pink	Elliptical	Entire	Medium size

National Centre For Genetic Resources and Biotechnology (NACGRAB), Nigeria
 National Horticultural Research Institute (NIHORT), Nigeria
 Asian Vegetable Research and Development Centre (AVRDC), Tanzania

dNTPs, 0.5 µl of 1.0 unit Taq DNA polymerase, 1.0 µl of a random primer, 2.5 µl of 10x buffer (200 mM Tris-HCl, 500 mM KCl, pH 8.0) and 12.4 µl distilled deionized water. The PCR amplification was performed with the following cycle: 1 cycle at 94°C for 5 min, then 45 cycles at 94°C for 1 min, 35°C for 4 min and 72°C for 2 min. The reaction was terminated with a 4-min DNA extension step at 72°C (Williams et al., 1990). The amplified products were electrophoresed in 1.2 % agarose gels with a 200 bp DNA ladder at 120 V for 1.5 h and stained with ethidium bromide. The RAPD profiles were visualized and photographed under ultraviolet light imaging system (Biotechnology Centre, IITA, Nigeria).

Distinct RAPD fragments with the same molecular weight were considered as identical bands, and scored for the presence (1) or absence (0) across the accessions for each primer. The scores were entered into a binary rectangular matrix and Jaccard's coefficient

(Jaccard, 1908) was used to estimate genetic similarity matrix using SIMQUAL program of NTSYS-pc version 2.10 (Rohlf, 1998). The similarities matrix was used to construct a dendrogram using unweighted pair group method based on arithmetic average (UPGMA). Total number of loci, number of polymorphic loci, and allele frequency were determined using POPGEN Version 1.32 software program (Yeh et al., 1997). Polymorphic information content (PIC) was estimated as $2fi(1 - fi)$ where fi is the frequency of the amplified allele of marker i (Roldán-Ruiz et al., 2000)

Results

The analysis of variance revealed significant variation among the accessions for the characters evaluated (Table 2). The accessions effect varied with year for stem width, petiole length and leaf length. Most of the accessions had leaf and stem with green pigmentation.

Table 2. Mean squares of characters evaluated in amaranth across two years

Source of variation	Block (year) (df = 4)	Year (df = 1)	Accession (df = 29)	Accession x Year (df = 29)	Error (df = 116)
Plant height (cm)	28.97	54.90	176.32**	37.85	48.61
Stem width (cm)	44.02**	398.93**	7.66**	6.71**	2.52
Number of leaves	294.13**	357.13**	22.41	13.15	19.57
Petiole length (cm)	10.49**	2.53	18.33**	5.52**	2.80
Leaf length (cm)	6.83	150.73**	57.77**	16.87**	8.39
Leaf width (cm)	5.93**	20.99**	6.63**	1.66	1.63
Leaf/stem	0.01	0.02	0.19**	0.03	0.03
Leaf area	869.77	8569.48**	1878.11**	576.39	402.60
Specific leaf area (cm ² /g)	18.78	269.25**	80.69	107.47	70.30
Fresh leaf weight (g)	6.35	2.05	64.73**	10.01	6.67
Fresh stem weight (g)	25.34	43.39	477.83**	21.92	30.04
Root weight (g)	2.23	0.93	36.79**	3.05	2.47
Whole plant weight (g)	121.68	0.63	1215.07**	81.53	134.33
Harvest index	0.01	0.02*	0.04*	0.01	0.01
Dry matter (g)	1.42	0.40	41.58**	4.94	3.44

Whole plant, fresh leaf, stem and root weights and petiole length with high vector score (≥ 0.30) accounted more for the variation among the accessions along the principal component axis (PA) 1 (Table 3). PCA1 represented 51% of the cumulative variation of the first three PCAs and PCA2 with 32% variation was significant for specific leaf area, leaf area and leaf length. Also, considering factor scores above 0.50 to ensure that most characters were loaded once across the factor axes (Table 3), sink factor (fresh leaf, stem, root and whole plant weights, and dry matter) were loaded under factor axis (FA) 1 and photosynthetic-related factor (specific leaf area, leaf area and leaf length) under FA2. Whole plant weight with communality of 0.94 accounted more for the variation across the axes.

The amaranth accessions were classified into seven (7) groups (Table 4). High performance for the sink factors was associated with amaranths in Group 4, and this potential accounted for high dry matter yield of the group. Photosynthetic-related traits were associated

with accessions in Group 1. Group 3 had high number of leaves. Distance between the groups was described using the mahalanobis D² distance of the canonical procedure (Figure 1). Groups 4 and 5 were at a significant distance from each other and from the other groups. Distance between groups 2 and 6; 1 and 7; 6 and 7 were not significant.

The ten oligonucleotide primers generated a total number of 115 bands, with 88 (77%) as the number of polymorphic loci (Table 5). The fragments ranged from 150 to 5500 bp in length. Average PIC value was 0.44 and PIC ranged from 0.31 to 0.50. The levels of polymorphism revealed by each primer among the accessions ranged from 60% (OPU-13) to 90% (OPH-17). The UPGMA dendrogram generated from RAPD primers revealed five main clusters at 0.56 similarity coefficient with many polymorphisms within the clusters (Figure 2). Although, the genetic dendrogram distinguished some of the accessions contrary to the phenotypic diversity,

Table 3. Vector scores of characters, variance and eigen value of the first three principal component and factor axes

Character	Principal component axis			Factor axis			Communality
	1	2	3	1	2	3	
Plant height (cm)	0.19	0.12	0.12	0.40	0.21	0.17	0.32
Stem width (cm)	0.07	0.26	-0.47	0.14	0.44	-0.65	0.68
Number of leaves	-0.01	-0.21	0.32	-0.02	-0.36	0.45	0.41
Petiole length (cm)	0.35	0.07	0.12	0.71	0.11	0.17	0.70
Leaf length (cm)	0.18	0.36	0.47	0.36	0.61	0.65	0.93
Leaf width (cm)	0.13	0.23	-0.41	0.27	0.39	-0.57	0.77
Leaf : stem	0.00	-0.05	0.23	0.00	-0.09	0.33	0.93
Leaf area (cm ²)	0.23	0.43	0.30	0.48	0.72	0.41	0.92
Specific leaf area (cm ² /g)	-0.05	0.54	0.07	-0.11	0.90	0.10	0.85
Fresh leaf weight (g)	0.40	-0.10	-0.01	0.82	-0.17	-0.02	0.90
Fresh stem weight (g)	0.38	0.01	-0.24	0.78	0.02	-0.34	0.94
Root weight (g)	0.39	-0.17	0.02	0.80	-0.29	0.03	0.90
Whole plant weight (g)	0.45	-0.05	-0.16	0.91	-0.08	-0.22	0.94
Harvest index	-0.04	0.21	-0.13	-0.08	0.35	-0.18	0.90
Dry matter (g)	0.28	-0.35	0.10	0.58	-0.58	0.14	0.76
Eigen value	4.22	2.81	1.92				
Variation (%)	0.28	0.19	0.13				
Cumulative (%)	0.28	0.47	0.60				

Table 4. Phenotype and character classification of thirty accessions of amaranth

Cluster	Principal component axis						
	1	2	3	4	5	6	7
	AM19, NG145, NH169, NH086	AM06, AM18, AM20, NG011, NG174, NG184, NG27, NH120, NH151, NH161, NH3194	AM11, NH117, UG40	AM31, NG08	NG016, NG621	AM42, NG014, NG123, NG132	ACNL, AM14, AM32, NG141
Plant height (cm)	23.92 (8.29)	21.97 (6.70)	23.85 (9.73)	24.16 (8.33)	23.96 (7.15)	16.21 (4.61)	28.95 (9.41)
Stem girth (cm)	5.47 (2.10)	3.98 (2.66)	2.81 (2.29)	5.15 (2.72)	5.87 (2.81)	4.05 (1.96)	4.60 (2.90)
Number of leaves	6.98 (5.50)	8.04 (4.72)	9.64 (6.25)	6.45 (5.62)	7.43 (5.08)	3.90 (2.81)	5.52 (5.10)
Petiole length (cm)	9.99 (1.79)	8.54 (1.81)	11.08 (0.75)	11.06 (2.72)	9.19 (2.86)	10.26 (1.23)	11.45 (3.21)
Leaf length (cm)	15.48 (4.14)	9.09 (2.28)	12.87 (7.73)	11.32 (3.87)	9.22 (1.52)	8.32 (0.52)	11.12 (3.86)
Leaf width (cm)	8.60 (1.47)	7.92 (1.29)	7.87 (1.60)	8.84 (1.62)	9.37 (1.92)	10.01 (2.45)	8.41 (1.09)
Leaf area (cm ²)	85.33 (26.18)	45.95 (11.37)	67.46 (51.3)	66.54 (31.53)	54.73 (11.39)	52.99 (10.98)	59.55 (20.38)
Specific leaf area (cm ² /g)	19.18 (9.08)	8.56 (7.10)	10.52 (15.41)	6.00 (2.64)	15.62 (6.59)	12.67 (4.13)	8.90 (4.47)
Fresh leaf weight (g)	7.05 (3.35)	5.51 (2.93)	11.04 (3.23)	12.78 (5.55)	8.43 (2.35)	7.88 (1.40)	9.30 (3.52)
Fresh stem weight (g)	13.02 (5.00)	9.87 (3.10)	15.51 (4.48)	31.37 (12.00)	22.80 (17.72)	11.91 (4.26)	12.97 (5.26)
Leaf:Stem	0.56 (0.17)	0.55 (0.22)	0.72 (0.11)	0.45 (0.28)	0.55 (0.28)	0.71 (0.19)	0.75 (0.20)
Root weight (g)	3.06 (1.57)	2.76 (1.00)	7.49 (2.83)	8.25 (3.77)	3.92 (2.42)	3.07 (1.32)	5.12 (2.44)
Whole plant weight (g)	23.53 (8.32)	19.06 (5.78)	34.25 (9.42)	59.11 (29.78)	35.86 (19.37)	23.27 (5)	27.83 (8.02)
Harvest index	0.84 (0.09)	0.80 (0.08)	0.78 (0.06)	0.80 (0.14)	0.84 (0.11)	0.84 (0.08)	0.79 (0.09)
Dry matter (g)	5.29 (2.22)	6.86 (2.74)	8.62 (3.05)	11.18 (2.54)	4.05 (1.84)	4.38 (1.01)	7.51 (2.49)

Standard deviation in parenthesis

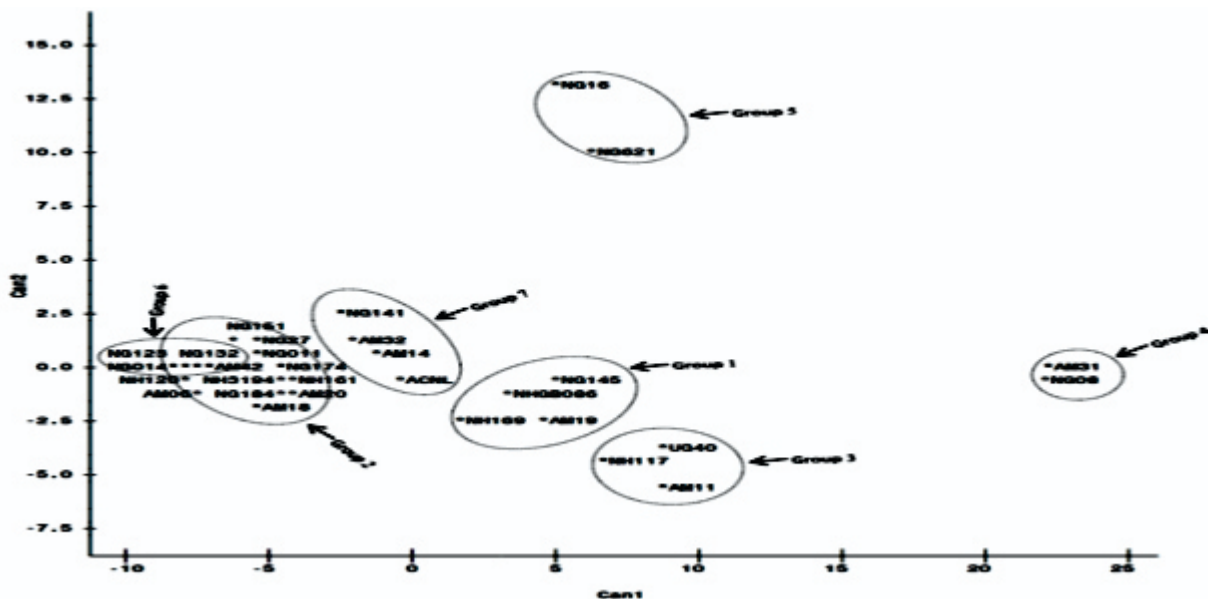


Figure 1. Bi-plot of canonical axes 1 and 2 describing genetic distance between the groups within thirty amaranth accessions

Table 5. Size of band, total number of loci, number of polymorphic loci and polymorphic information content of 10 RAPD primers for 30 accessions of amaranth

Primer		Band size (bp)	Total number of loci	Number of Polymorphic loci	Polymorphic information content
OPB – 17	AGGGAACGAG	200 – 1500	16	12	0.48
OPH – 12	ACGCGCATGT	300 – 2500	17	15	0.49
OPH – 17	CACTCTCCTC	200 – 2500	20	18	0.50
OPI – 06	AAGGCGGCAG	150 – 3000	11	8	0.43
OPI – 09	TGGAGAGCAG	250 – 4500	8	6	0.46
OPI – 19	AATGCGGGAG	200 – 5500	10	7	0.43
OPT – 08	AACGGCGACA	200 – 3500	7	4	0.31
OPU – 13	GGCTGGTTCC	250 – 4000	5	3	0.37
OPU – 14	TGGGTCCTC	220 – 3700	9	6	0.43
OPV – 19	GGGTGTGCAG	250 – 3000	12	9	0.47

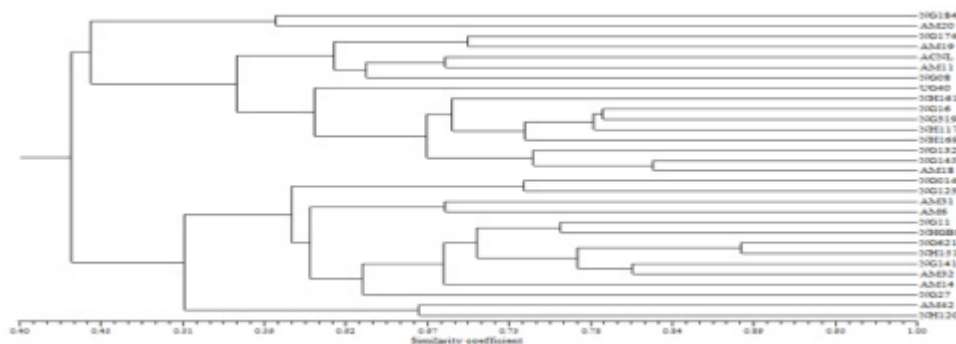


Figure 2. Genetic diversity of thirty accessions of amaranth based on RAPD markers some information were relatively similar. NG621 was 88% genetically similar to NH151; and AM18 was related to NG145 at 0.83 similarity coefficient. NG184 and AM20 were at least 56% divergent from the other accessions

Discussion

Amaranth is a common and cheap source of vegetable cultivated in Africa, and it is important for feeding and nutrition of the growing population. Therefore, research efforts to improve and develop new varieties are imperative to provide essential nutrients to consumers whose diets rely heavily on the crop. Part of the effort involves selection of parental materials based on existence of genetic variation.

Among the amaranth in the study, at least, two accessions were different for the characters evaluated. At the early stage of growth of the amaranths, it was possible to identify varying genotypes and select potential parents with high value for the characters. Hence, at the vegetative phase, there is potential for genetic improvement of amaranths which are usually consumed when tender. Studies on vegetable amaranths reported presence of wide range of diversity in both agronomic and qualitative traits (Varalakshmi, 2004; Bola, 2007). Most of the accessions were of green leaf pigmentation and large leaf size which are farmers and consumers preferred traits. Also, differential performance of the accession across years indicated the opportunity to discover and develop superior genotype in varying environmental conditions. Olaniyi (2006) attributed genotype x environment to differences in ecological distribution and genetic differences among the genotypes.

It is not enough to understand genetic variation on individual character but knowledge

on genetic divergence, considering several traits simultaneously, is central and valuable in plant breeding. The germplasm can be classified into similar and manageable groups for hybridization processes. Assessment of genetic diversity is mostly and easily accessible by phenotypic variation. Although, often influenced by environmental factors, morphological markers allow for assessment of diversity in the presence of varying environmental conditions. Also, if the traits are heritable phenotypic diversity can reflect the genotypic diversity (Smitha and Krishnakumary, 2011). Advent of molecular markers has also contributed to speedy and reliable information on genetic diversity. It has gain relevance to determine the quality of phenotypic classification relative to genetic diversity (Popa et al., 2010). The morphological divergence and similarity between some of the amaranth accessions reflected the DNA classification. Beyond the grouping pattern, morphological data revealed trait or group of traits that contributed considerably to the diversity. Sink attributes such as leaf, stem and root weights, and dry matter were homogeneous traits and are important as primary selection criteria for vegetative improvement in amaranth. In conclusion, information from the phenotypic diversity can be used deductively with the molecular markers to guide effective hybrid program.

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