

Evaluation of genetic diversity in some *Amaranthus* spp. using morphological and Random Amplified Polymorphic DNA(RAPD) analysis

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Abstract

Amaranth, is an annual plant used as leafy vegetables, grains and ornamentals. It is a plant with a wide range of variability. Ten Amaranth genotypes were selected based on their morphological features and were evaluated over two seasons in 2016 at the Teaching and Research Farms, Federal University of Agriculture, Abeokuta, (FUNAAB), while the molecular analyses were carried out at the Laboratory of the Biotechnology Centre, FUNAAB. DNA extracted from young amaranth leaves were amplified using four Random Amplified Polymorphic DNA (RAPD) primers (OPA-02, OPA-10, OPB-11 and OPB-12). Data collected were subjected to statistical analyses. The Analysis of Variance revealed significant variation in the accessions with respect to height at flowering and number of branches only. Heritability estimates ranged from 60 – 94% for petiole length and number of branches, respectively. A significant and negative correlation was observed between number of branches and seed weight (-0.35). Molecular cluster analysis showed that all the accessions studied shared a similarity at a coefficient of 0.55 and two major clusters were formed at a coefficient of 0.60. For hybridization studies, accessions NGB-96 and NGB/09/09 which were from distant cluster could be used as parents to take advantage of the inherent variability. Other markers such as Simple Sequence Repeats (SSR) could be used to further reveal diversity in the accessions.**

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Introduction

Amaranthus is a cosmopolitan genus of herbs of the *Amaranthaceae* family. They possess small flowers that are arranged in dense clusters. Their stems and leaves are deeply pigmented (Costea and DeMason, 2001). *Amaranthus* species consist of grain and weedy types with the common grain types being *A. hypochondriacus*, *A. cruentus* and *A. caudatus* and the major weedy types include *A. viridis*, *A. spinosus*, *A. retroflexus* and *A. hybridus* (Oboh, 2007). Amaranths are very promising crops because of their content of protein, fat and active substances which make them highly palatable and nutritious. *Amaranthus* is a very good source of vitamins, fiber, dietary minerals and balanced

amino acids. The protein content of its seeds (15 to 18%) are greater than that of wheat (Dodok et al., 1996). In Nigeria, especially Yoruba community all species are referred to as "tete" even though they may add a second name to indicate a particular variety or species. In Nigeria however, little work has been done to assess the interspecies relationships among these species at the molecular level (Tony-Odigie et al., 2012) hence they are referred to as underutilized crops. *Amaranthus* are highly valued, so breeders use desirable traits for selection in order to improve the economic yield of the plant. However, conventional breeding techniques proved to be time consuming. Therefore, molecular techniques for detecting differences in the DNA of individual plants were used to examine

variability in cultivars. In addition, molecular characterization of germplasm aids plant breeders in selecting appropriate material for further genetic improvement of cultivars. The Polymerase Chain Reaction (PCR) based molecular markers have been used for analysis of genetic variations in natural populations. One such technique using arbitrary primers, namely Random Amplified Polymorphic DNA (RAPD) provides a convenient and rapid assessment of the differences in genetic composition of individuals. Others which may also be used includes Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) analysis and Single Nucleotide Polymorphism (SNP) technique which happens to be the most recent and advanced in molecular characterization. RAPD markers offer quick screening of genetic materials, construction of linkage maps in plants and animals and in bulk segregant analysis for identifying markers linked to target genes (Huang et al., 2003). Arif et al. (2010) stated that Sequence-based analyses sometimes fail to distinguish between species because of the significant similarity between their DNA

sequences in the amplified region. They also reported that RAPD primers are able to distinguish taxa below the species level because RAPD analyzes regions of the genome for genetic polymorphism and is applied widely, particularly in plant molecular biology for the detection of both coding and non-coding regions of the genome. *Amaranthus* species are among the underutilized crops and have future prospects for research and development with focus on leafy amaranths in Sub-Saharan Africa. The present study was carried out to determine the genetic relationships among selected *Amaranthus* species using Random Amplified polymorphic DNA (RAPD) markers and agromorphological characters.

Materials and Methods

Ten accessions of *Amaranthus* spp. obtained from National Center for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Oyo State, Nigeria were used in this study (Table 1). The experiments were carried out at the Teaching and Research Farms and the Biotechnology Centre Federal University of Agriculture, Abeokuta.

Table 1: Name and Sources of accessions used in the study

S/N	ACCESSION NO.	SOURCE
1.	NGB-96	NACGRAB, IBADAN
2.	NG/AA/03/11/101	NACGRAB, IBADAN
3.	NG/AA/MAY/09/027	NACGRAB, IBADAN
4.	NG/SA/DEC/07/042	NACGRAB, IBADAN
5.	NGB/01234	NACGRAB, IBADAN
6.	NG/TO/02/12/154	NACGRAB, IBADAN
7.	NGB/0126	NACGRAB, IBADAN
8.	NG/SA/DEC/09/041	NACGRAB, IBADAN
9.	NGB/01276	NACGRAB, IBADAN
10.	NGB/09/09	NACGRAB, IBADAN

Field Evaluations

Two field evaluations were carried out in May and August, 2016. The experiments were laid out in a Randomized Complete Block Design (RCBD) with three replicates and in single row plots. A block consisted of 10 rows and planting was done in 50cm rows and each accession was given one row. The intra row spacing was 20 cm, and inter row spacing of 50cm.

Data Collection

Data were collected on the following agronomic parameters: Days to emergence, Height at flowering, Plant height (cm), Inflorescence length (cm), Petiole length (cm), Number of branches, Stem girth (cm), Number of leaves, Leaf length (cm), Leaf width (cm), 1000- seed weight (g) and Seed weight per plant (g).

Statistical Analyses of Agronomic Data

Data were pooled across seasons and subjected to analysis of variance (ANOVA) using SAS (1999) statistical software. Duncan's Multiple Range Test at 5% probability levels was used to separate significant means. Heritability in the broad-sense was calculated for the various agronomic characters. Correlation coefficients were estimated to determine the inter-relationship between plant characters.

Table 2: Primers and the sequences used for PCR amplification

S/N	Primer Name	Sequence
1	OPB 02	TGATCCCTGG
2	OPB-10	CTGCTGGGAC
3	OPB-11	GTAGACCCGT
4	OPB-12	CCTTGACGCA

DNA Electrophoresis

Agarose gel electrophoresis was used to determine the quality and integrity of the DNA by size fractionation on 1.0% agarose gels. Agarose gel was prepared by dissolving and 1.0g agarose in 100 ml 0.5X TBE buffer solution. The gel was cooled down to 45°C and 10 µl of 5 mg/ml ethidium bromide was added and mixture poured into an electrophoresis chamber set with

MOLECULAR ANALYSIS

This was conducted at the Biotechnology Central Laboratory, FUNAAB. The plant samples were taken at two weeks from the field experiment for DNA extraction, PCR amplification and molecular analysis.

Genomic DNA extraction

The genomic DNA of the plant was isolated using CTAB method. Young Amaranth leaves (2g) were grinded in 600µl of extraction buffer and was incubated at 65°C for 20mins. The sample was then allowed to cool to room temperature, chloroform was then added and the tube was agitated gently to allow a homogenous mixture. Thereafter, the sample was spun at 14,000rpm for 15mins and equal volume of isopropanol was added to precipitate the DNA. The sample was refrigerated for one hour and later spun at 14,000 rpm for 10 mins and resulting pellet was washed with 70% ethanol and air dried for 30mins. Thereafter, re-suspension in 100µl of sterile distilled water was done. The quality of DNA was detected by agarose gel electrophoresis and the size of fragment obtained was 25kb for all the samples. The genomic DNA was used in PCR amplification using RAPD markers

the combs inserted. In order to determine the DNA integrity, 3µl of the DNA with 5µl sterile distilled water and 2 µl of 6X loading dye was mixed together and loaded in the wells created on the gel. The gel was 'ran' at 80V for 2 hours. Photographs of the gels were then taken under UV light.

Dilution of DNA for PCR

About 10 μ l of each DNA was taken into Eppendorf tube and 1000 μ l volume was made up by adding 990 μ l distilled water. The final concentration was made up to 20-50 ng/ μ l after dilution with 1xTBE buffer.

PCR reaction mix

The reaction mix was a total volume of 20 μ l consisting of 5 μ l of genomic DNA (70 ng/ μ l), 8.2 μ l of distilled water, 3.0 μ l of the primer, 10x buffer at 2.0 μ l, 1.6 μ l dNTPs and 0.2 μ l of Taq DNA polymerase. The thermo cycler profiles was an initial denaturation temperature for 3mins at 94 $^{\circ}$ C, followed by 45 cycles of denaturation temperature at 94 $^{\circ}$ C for 20 seconds, annealing at 37 $^{\circ}$ C for 40 seconds and primer extension at 72 $^{\circ}$ C for 40 seconds, followed by final extension temperature at 72 $^{\circ}$ C for 5 mins.

Statistical Analysis

Reproducible RAPD bands were scored manually in the binary mode with '1' indicating the presence, and '0' indicating the absence of a band, while the SSR profiles were scored with '1' indicating upper band and '0' for lower band. Data were analyzed using NTSYS-pc 2.02J (Rohlf, 2000). Dendrograms were then constructed using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair Group Method with Arithmetic Means (UPGMA) to infer genetic relationships and phylogeny.

Results

The analysis of variance (Table 3) revealed significant differences in the accessions with respect to plant height at 50% flowering and number of branches. Means of agronomic characters evaluated in ten accessions of amaranth are presented in Table 4. A wide range of variability was observed for height at flowering and number of branches/plant. However, days to emergence, petiole length, stem girth and leaf width showed no significant variability among the 10 accessions evaluated. Accession NGB/0126 had highest seed weight/plant (4.63 g). Plant height at flowering ranged from 28.21 cm for NG/AA/MAY/09/027 to 42.20 cm for NG/SA/DEC/07/042. NG/AA/MAY/09/027 had the longest

inflorescence of 38.54 cm, while accession NGB/01276 was the shortest (24.30 cm). The highest number of branches recorded was 21.80 for NG/SA/DEC/07/042 while NG/AA/03/11/101 had the least (7.80). Accession NG/AA/MAY/09/027 had the lowest seed weight/plant (1.09 g), whereas the highest was recorded for NGB/0126 (4.63 g). Variance components and heritability estimates of the characters are presented in Table 5. Plant height had the highest phenotypic variance (55796.98) and genotypic variance (42545.16). Higher PCV values compared to corresponding GCV values are indications of environmental influence in the variations observed among accessions used. Genetic coefficients of variation (GCV) and phenotypic coefficients of variation (PCV) were highest for plant height with values of 143.80 and 164.68, respectively and lowest for days to emergence (11.29 and 13.87, respectively). Heritability (broad-sense) was generally high for most of the characters estimated, while other characters showed moderate heritability.

Correlation coefficients between the pairs of the characters are shown in Table 6. Significant positive correlations were observed between; height at flowering and number of leaves (0.44**), inflorescence length and leaf length (0.34*) and number of branches and number of leaves (0.69**), while days to emergence and inflorescence length (-0.66**) and number of branches and seed yield (-0.35*) showed significant negative correlations. Dendrogram resulting from RAPD analysis showing similarity coefficients of ten Amaranth genotypes is presented in Fig. 1. All accessions shared a genetic similarity of 0.55. Two major clusters, 'A' and 'B' were observed with cluster A having four accessions, while B has six at a similarity coefficient of 0.60. At 0.82 coefficient, four accessions showed genetic similarity namely NG/SA/DEC/07/042, NGB/01234, NGB/0126 and NG/SA/DEC/09/041 and at 0.91 coefficient NGB-96 and NG/AA/03/11/101 were extremely similar. The two dimensional (2D) plots of the ten accessions of amaranth (Figure 2) showed the same trend with the dendrogram. It also revealed the distinction of accession NGB/09/09 from all others. Figure 3 shows a representative banding pattern generated by

using OPB-10 primer. The bright bands reveal by the primer shows the loci common to the accessions for any particular character.

Discussion

A wide variation was observed in the accessions with respect to number of branches and height at flowering which indicates that these characters could be used in discriminating the materials. Most of the agronomic parameters evaluated for Accessions NG/SA/DEC/07/042 shows it could be a useful material for hybridization studies. The high number of branches and leaves prior to flowering for accession NG/01276 indicates the potential of the plant to be used as a leafy vegetable whereas NGB/0126 with high seed weight indicates its potential for use as grain amaranths.

GCV together with heritability estimates would give a better picture of the extent of genetic advance that can be made through selection for estimating genetic gain under selection (Murtadha et al., 2000; Johnson et al., 1963). Generally, phenotypic variances were higher than genotypic variances indicating the variation observed among accessions studied was not only due to genotype but also due to the effect of the environment. High genotypic variance accompanied by corresponding low environmental variances observed for height at flowering, number of branches/plant, number of leaves and seed weight suggest that these traits are under genetic control rather than environmental influence. Hence, improvement of these traits could be made through selection. In selecting for high yield, the mode of inheritance for yield components must be understood. High heritability of number of branches indicated that the character is mainly governed by genetic influence, hence selection of accessions based on these characters will be rewarding. Moderate heritability estimates for petiole length, leaf width and stem girth shows the influence of the environment in the exhibition of these characters as such selection of these characters based on phenotypic appearance may not be reliable because of the influence of the environment.

Correlation is a measure of the intensity of the association between variables (Lukhele, 1981). Correlation by contrast, indicates whether two variables are interdependent or

vary together, hence it is a measure of closeness of association as reported by Ariyo (1987), Board et al., (1999) and Ojo (2003) in other crops. It is established that phenotypic character expression incorporates both genotypic and environmental effects. Therefore, the non-significant phenotypic correlation between two characters, relative to its significant genotypic counterpart, is indicative of appreciable environmental effects. Generally, genotypic relationships are of premium importance in plant breeding.

Significant positive correlation observed between height at flowering and number of leaves/plant, number of branches/plant and number of leaves/plant implies that taller plants at flowering will have more leaves produced per plant. The significant negative correlation observed between number of branches and seed weight implies that selecting plants with fewer branches will result to higher seed weight. This could be due to the 'source'-'sink' relationship where most of the assimilates will be used to compensate for vegetative growth at the expense of the seeds. When production for leafy vegetables is desired, selection for taller plants is key as this will translate to more number of leaves produced.

The dendrogram and 2D plots from the molecular study gives an idea about the relatedness and spatial distribution, respectively, of the accessions used in the study. It clearly showed the similarities between the accessions but accessions NGB-96 and NG/AA/03/11/101 could not be separated even at a highest similarity coefficient which suggest the closeness in the materials. In order to confirm this assertion, the number of markers could be increased or different markers could be used. Accessions NGB-96 and NG/AA/03/11/101 are more closely related to one another compared to other accessions and they share a genetic similarity of 91% using the RAPD-PCR molecular system with four different primers.

Conclusion

Significant variation existed in the accessions evaluated. The study also revealed that significant positive and negative correlation occurred for some of the agronomic characters. However, most of the traits are highly heritable and therefore successful breeding through

selection for most of the traits will be rewarding. The dendrogram and two dimensional (2D) plot showed that the accessions were differentiated from one another based on the polymorphic bands generated in the PCR. The ability to resolve genetic variation may be more directly related to the number of polymorphism detected by the marker techniques. The close relationship between NGB-96 and NG/AA/03/11/101 was also revealed by this study, hence for hybridization studies, members from distant cluster, for example accessions NGB-96 and NGB/09/09 could be used as parents to take advantage of the inherent variability.

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Table 3: Analysis of variance of plant characters evaluated in ten genotypes of *Amaranthus*

Source of variation	Degree of freedom	Days to emergence	Height at flowering (cm)	Plant height (cm)	Inflorescence length (cm)	Petiole length (cm)	Number of branches	Stem girth (cm)	Number of leaves	Leaf length (cm)	Leaf width (cm)	1000-seed weight (g)	Seed weight/plant (g)
Rep	4	28.42	21.38	42958.74	49.52	198.06	42.03	55.79	11240.87	10.88	72.12	0.15	0.41
Accession	9	76.42	88.89**	55796.98	118.34	200.48	96.76*	57.92	12365.53	6.43	95.40	0.17	5.59
Error	36	77.26	36.93	39755.45	72.97	239.87	18.47	59.14	6081.39	5.44	103.30	0.18	3.83

*, ** Significant at 5% and 1% probability levels,

Table 4: Mean Performance of the Ten Accessions of *Amaranthus* evaluated in this

Accessions	Days to emergence	Height at flowering (cm)	Plant height (cm)	Inflorescence length (cm)	Petiole length (cm)	Number of branches	Stem girth (cm)	Number of leaves	Leaf length (cm)	Leaf width (cm)	1000-seed weight (g)	Seed weight/plant (g)
NGB-96	61.80a	32.03bc	85.36b	35.22ab	7.46a	13.20cde	4.10a	138.00abc	12.24ab	4.76a	0.38ab	2.12ab
NG/AA/03/11/101	59.80a	31.64bc	87.10b	34.92ab	20.06a	7.80e	4.04a	64.20c	11.00b	5.48a	0.44ab	3.87ab
NG/AA/MAY/09/027	60.40a	28.21c	87.74b	38.54a	24.08a	18.00abc	15.10a	135.40abc	11.92ab	5.18a	0.85a	1.09b
NG/SA/DEC/07/042	67.00a	42.20a	88.16b	27.18ab	6.60a	21.80a	4.92a	208.00a	13.02ab	5.36a	0.29ab	2.05ab
NGB/01234	58.60a	31.97bc	85.22b	31.20ab	8.28a	17.80abc	4.76a	151.60abc	14.66a	5.40a	0.27ab	3.29ab
NG/TO/02/12/154	64.00a	30.31bc	80.42b	30.18ab	7.48a	17.60abc	4.64a	131.60abc	11.94ab	5.04a	0.34ab	2.25ab
NGB/0126	69.40a	32.22bc	197.72ab	26.78ab	7.18a	11.00de	3.64a	75.40bc	12.00ab	13.22a	0.28ab	4.63a
NG/SA/DEC/09/041	66.00a	33.45bc	392.40a	25.56b	7.86a	18.20abc	4.40a	181.40ab	13.70ab	17.42a	0.18b	3.28ab
NGB/01276	65.40a	38.79ab	79.96b	24.30b	7.42a	21.00ab	5.84a	216.00a	11.16b	5.28a	0.27ab	2.79ab
NGB/09/09	57.80a	36.51abc	250.26ab	34.94ab	6.06a	15.00bcd	4.22a	130.20abc	11.86ab	5.44a	0.28ab	1.85ab

Means followed by the same letter along the columns are significantly different ($P < 0.05$) using DMRT.

Table 5: Mean, variance components, genotypic and phenotypic coefficients of variation and broad sense heritability of the characters

Characters	Mean	Phenotypic Variance	Genotypic variance	Phenotypic coefficient of variation	Genotypic coefficient of variation	Broad Sense Heritability (%)
Days to emergence	63.02	76.42	50.67	13.87	11.29	66
Height at flowering (cm)	33.73	88.89	76.58	27.95	25.94	86
Plant height (cm)	143.43	55796.98	42545.16	164.68	143.80	76
Inflorescence length (cm)	30.88	118.34	94.01	35.23	31.40	79
Petiole length (cm)	10.25	200.48	120.52	138.17	107.13	60
Number of branches	16.14	96.76	90.60	60.95	58.97	94
Stem girth (cm)	5.57	57.92	38.21	136.73	111.05	66
Number of leaves	143.18	12365.53	10338.40	77.66	71.01	84
Leaf length (cm)	12.35	6.43	4.62	20.53	17.40	72
Leaf width (cm)	7.26	95.40	60.97	134.57	107.58	64
1000-seed weight (g)	0.36	0.17	0.11	116.52	94.31	66
Seed weight (g)	2.72	5.59	4.31	86.90	76.34	77

Table 6: Correlation coefficients between the pairs of the characters evaluated in the *Amaranthus* accessions

	Height at flowering (cm)	Plant height (cm)	Inflorescence length (cm)	Petiole length (cm)	Number of branches	Stem girth (cm)	Number of leaves	Leaf length (cm)	Leaf width (cm)	1000-seed weight (g)	Seed weight/plant (g)
Days to emergence	0.04	-0.05	-0.66**	0.06	-0.12	-0.14	-0.23	-0.15	0.16	-0.12	0.17
Height at flowering (cm)		-0.17	-0.18	-0.09	0.18	-0.02	0.44**	0.27	0.18	-0.11	0.04
Plant height (cm)			-0.03	-0.07	0.04	-0.06	0.07	-0.01	-0.06	-0.17	0.05
Inflorescence length (cm)				0.11	-0.07	0.22	0.09	0.34*	-0.03	0.13	-0.11
Petiole length (cm)					-0.13	-0.02	-0.09	0.06	-0.02	0.06	0.03
Number of branches						0.08	0.69**	0.22	0.08	-0.01	-0.35*
Stem girth (cm)							0.12	0.09	-0.02	-0.05	-0.05
Number of leaves								0.42**	0.04	0.01	-0.01
Leaf length (cm)									0.18	-0.21	-0.05
Leaf width (cm)										-0.11	-0.12
1000-seed weight (g)											0.05

** . Correlation is significant at the 0.01 level (2-tailed).* . Correlation is significant at the 0.05 level (2-tailed).

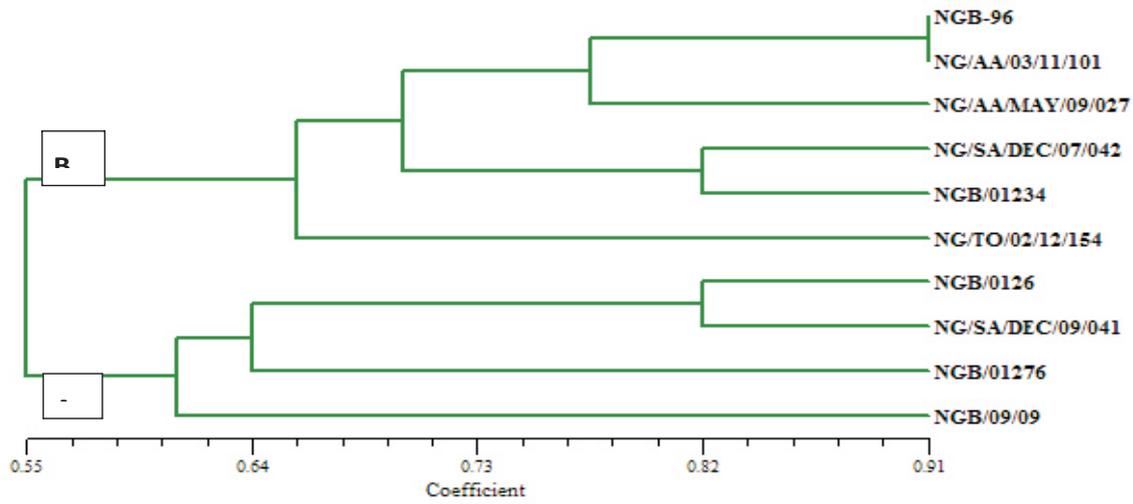


Figure 1: Dendrogram resulting from RAPD analysis showing similarity coefficients of ten *Amaranthus* genotypes used in this study

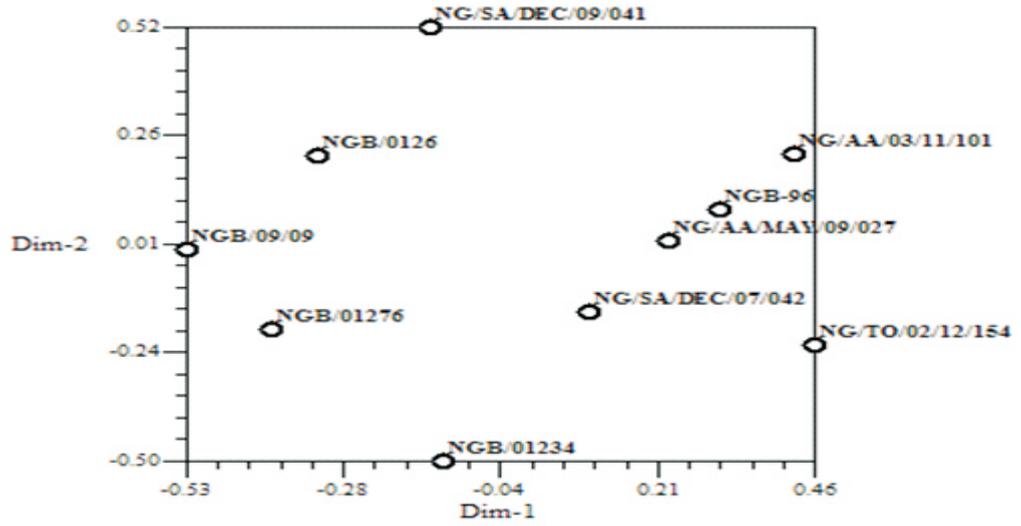
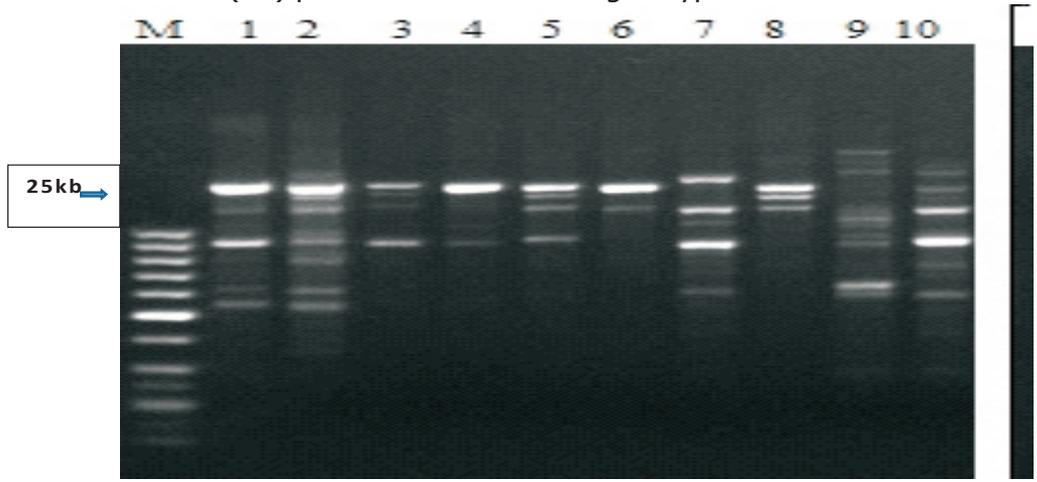


Figure 2: Two-dimension (2D) plot of ten *Amaranthus* genotypes based on RAPD markers



LEGENDS

M-Marker, 1-ngb-96, 2-ng/sa/dec/07/0423, 3-ngb/0126, 4-ngb/01234, 5-ngb/01276, 6-ngb/09/09, 7-ng/to/02/12/154, 8-ng/aa/may/09/027, 9-ng/aa/03/11/101, 10-ng/sa/dec/09/0412