ISSN: 2347-4688, Vol. 11, No.(2) 2023, pg. 603-614

Current Agriculture Research Journal

www.agriculturejournal.org

Evaluation of the Genetic Stability of *Amaranthus viridis* L. Species in Selected Regions of Western Ghats using Random Amplified Polymorphic DNA (RAPD)

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Abstract

Random Amplified Polymorphic DNA is cheap and fast molecular technique to identify the genetic resemblances or variations in DNA in various plants. The proposed research work was intended to compare the genetic diversification of *Amaranthus viridis* species in selected regions of Western Ghats in Tamilnadu and Kerala by RAPD analysis.The biometric morphological data among the five A. *viridis* accessions were compared. Their genomic DNA was isolated and finger prints were obtained using three RAPD markers. The percentage polymorphism, polymorphism information content, effective multiplex ration and resolving power were calculated. UPGMA dendrogram was constructed and their genetic relatedness was compared using Jaccard coefficient. Upon finger printing, 66 bands were counted for the three RAPD primers used,among which 46 bands were polymorphic band numbers from 8 to 21. Percentage of polymorphic bands ranged between 44.44% and 80.77%. EMRvalues for 66 polymorphic loci ranged between 3.56 and 17.01. The UPGMA dendrogram comparing the genomic profiles of the 5 A. *viridis* accessions using Jaccard coefficient revealed their genetic distances where the maximum Jaccard's coefficient value observed was 0.96 between AVKATN and AVNEK. Among the 5 selections the peak similarity index (0.9565) was witnessed between AVKATN and AVNEK. The cuurent study revealed that AVKATN and AVNEK had the highest their genetic distances, whereas AVKATN and AVNEK were genetically similar among the 5 A. *viridis* accessions. Several polymorphic bands which ranged up to 80.77% have proved their genetic variations.

Article History

Received: 04 March 2023

Accepted: 17 July 2023

Keywords

Amaranthus Viridis;
Genetic Stability;
Random Amplified Polymorphic DNA;
RAPD;
Western Ghats.

Introduction

Random Amplified Polymorphic DNA, a molecular method now available has been successfully applied to notice the genetic likenesses or variations in DNA in several plants.[1,2] The supremacy of RAPD is that it is a speedy method, facile to execute and

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Doi: https://dx.doi.org/10.12944/CARJ.11.2.22

relativelyinexpensive. It is proximately applied to the scrutiny of many organisms since universal primer sets are utilized without any necessity for earlier information about sequences.[3,4] Applications of RAPD involve DNA sequence polymorphisms detection, mapping diverse populations,[5,6] markers isolation associated to different traits or particular targeted intervals[7,8] as well as implementation in parentage analysis and identification of varieties.[9,10]

RAPD also allows evolutionary researchers to find the genetic characters of various species that areclosely related. The invention of the RAPD methodhad a key influence on the investigation of the genomesin eukaryotes and backed to the development of several DNA markers. RAPDs are predominantly beneficial to analyse the gene structure of populations as they disclose polymorphisms in non-coding areas of the genetic material.[11]

The typical RAPD technology uses short synthetic oligonucleotides generally10 bases lengthy of arbitrary sequences as primers with low annealing temperatures to amplify nanogram quantities of total genomic DNA using PCR.[12] RAPD markers distinctive to organisms from one species in a genus are species-specific.[13] Identification of hybrid populations is possible by choosing population-specific markers.[14]

The dissimilarity coefficients between species could be analysed and clustering can be carried out using unweighted pair group method and arithmetic average. The UPGMA dendrogram is constructed with dissimilarity matrix to reveal the degree of fit with the cophenetic correlation values. Dendograms can clutch the accessions of species into groups based on their eco-geographical regions that provide beneficial insights into their phylogenetic relationships.[15] Phylogenetic relationships can also be evaluated by Principal Component Analysis (PCoA) which explains the percentage of cumulative variance among species. The 2DPCoA analysis demonstrates grouping of the diverse accessions signifyingthe clustering of accessions.[15]

The current study was designed to compare the biometric morphological data among the five *Amaranthus viridis* L.accessions from selected regions of Western Ghats in Tamilnadu and Kerala, to isolate genomic DNA and finger print the five A. *viridis* accessions using three RAPD markers, to calculate the percentage polymorphism, effective multiplex ration, polymorphism information content, marker index and resolving power after data scoring, to construct UPGMA dendrogram using Jaccard's coefficient and compare the genomic profiles of A. *viridis* accessions, to find the similarity matrix on the basis of RAPD data among 5 A. *viridis* accessions and to perform 3D Principal Component Analysis (PCoA) for genetic relatedness among the five A. *viridis* accessions.

Materials and methods

Collection of plants

The five accessions of *Amaranthus viridis* L. (SH1431, SH1432, SH1433, SH1434, SH1435) used in the current study were collected from selected regions of Western Ghats in Tamilnadu (ChunkanKadai and Kaliakkavilai) and Kerala(Neyyatintakra, Maruthoor, M.C. Road) (Figure 1) during the month of December, 2018.

The accessions were authenticated and deposited in the Sundaranar Herbarium, Manonmaniam Sundaranar University, Tirunelveli, Tamilnadu, India. The accessions were mapped representing their geographical distribution in Western Ghats of Tamilnadu, India using GoogleMyMaps(Figure 2).

The details of the latitude and longitude of the accessions collection sites and the names of the places along with the districts and state names from where the accessions where collected is represented in Table1.

Biometric Observations

For the analysis of morphology, 3 plants from every accession were analysed and the data were recorded during collection for three morphometric traits as per the procedure followed by,[16] height of the plants, length of leaf blade and width of leaf blade. The type of soil at the collection site was observed during collection and its pH (1g of soil dissolved in 100mL distilled water) was also measured. The local name of the accessions were enquired to the people in the collection site and noted.

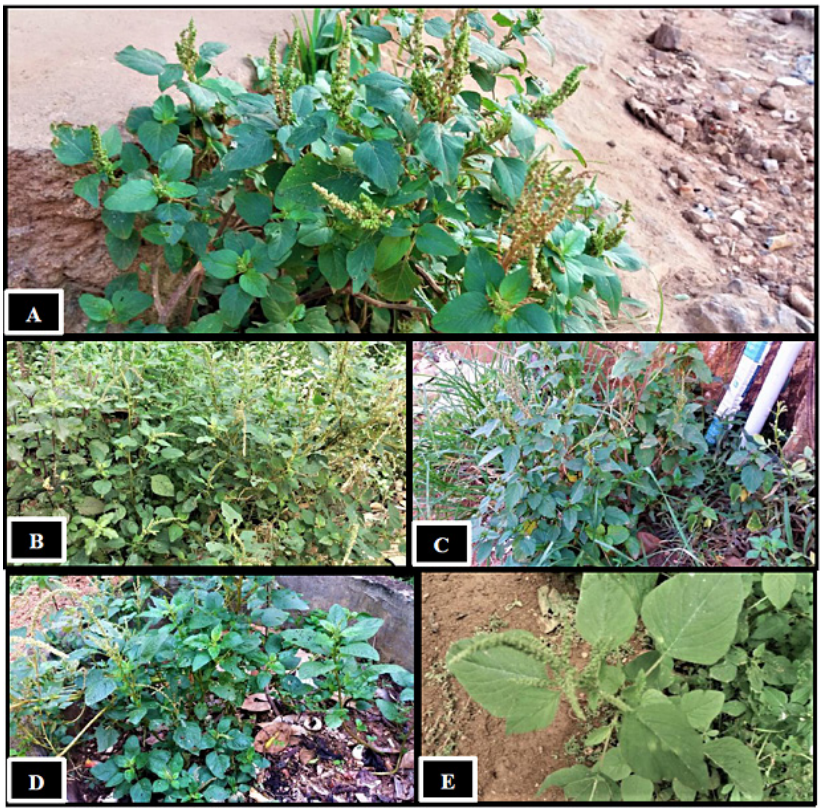

Fig. 1: *Amaranthus viridis* accessions collected from A: ChunkanKadai, B: Kaliakkavilai, C: Neyyatintakra, D: Maruthoor and E: M.C. Road.

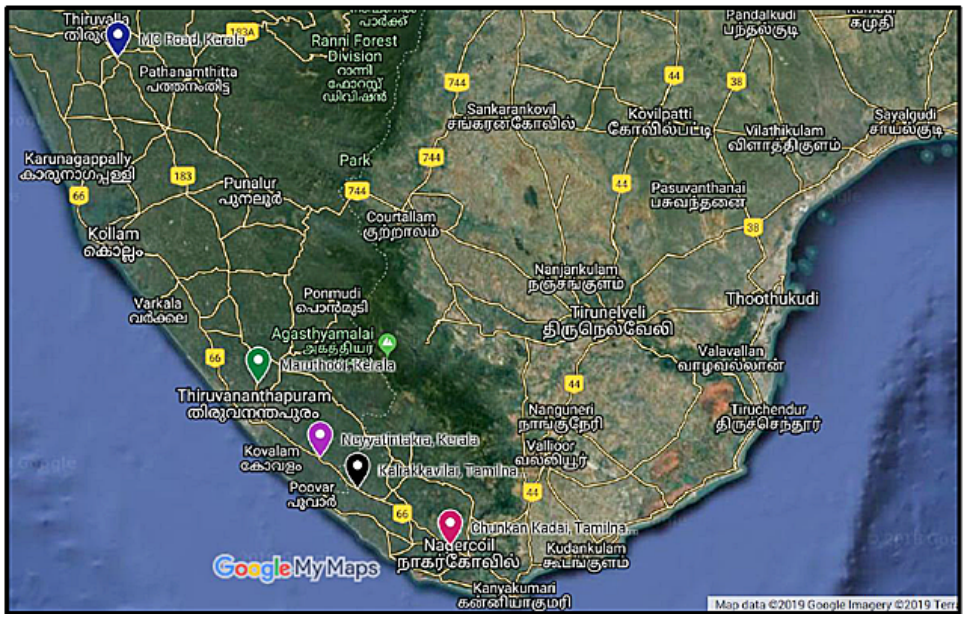

Fig. 2: A distribution map showing locations (selected regions of Western Ghats in Tamilnadu and Kerala) of collection of 5 *Amaranthus viridis* accessions.

Table 1. Accession number, Collection location and geographic coordinates of *Amaranthus viridis* accessions used in the current study.

	Acc. No.	Code	State	District	Place	Latitude	Longitude	Soil type	pH of soil
	SH 1431	AVCHTN	Tamil Nadu	Kanya kumari	Chunkan Kadai	8.1993°N	77.3870°E	red loamy	6.5
	SH 1432	AVKATN	Tamil Nadu	Kanya kumari	Kaliakka vilai	8.3318°N	77.1744°E	loamy clay	7.5
	SH 1433	AVNEK	Kerala	Thiruva nantha-puram	Neyyatin takra	8.4016°N	77.0871°E	sandy loam	6.5
	SH 1434	AVTHK	Kerala	Thiruva nantha-puram	Maruthoor	8.5724°N	76.9451°E	brown loam	7
	SH 1435	AVKOK	Kerala	Kollam	MC Road	8.7695°N	76.8828°E	laterite soil	7

Finger printing of *Amaranthus viridis* L. accessions using RAPD markers

Genomic DNA Isolation

The genomic DNA from leaf samples was isolated using CTAB method.[17]

Quantification of DNA and Purity Assessment

The yield of DNA per gram of fresh leaves was found using a ultraviolet–visible spectrophotometer at 260nm and 280nm. The purity of DNA was determined by calculating the ratio of absorbance at 260nm-280nm. The purity and concentration of DNA was also checked by analysing the isolated DNA samples on 1% agarose gel.

Primers Used

Three primers were utilized for RAPD study of the five accessions of A. *viridis* included Primer1: 5'-CCCAGCAACTGATCGCACAC-3', Primer2: 5'-AGGACTCGATAACAGAC-3' and Primer3: 5'-ATGTAAGCTCCTGGGGATTCAC-3'(Operon Technologies, Inc., USA).

PCR Amplification

Each reaction mixture (25µl) for PCR amplification consisted Template (50ngDNA)-1µL; Primer(100ng/µl)-2.0µl; 10XAssay Buffer-2.5µl; dNTPs(10mM)-1.0µl; TaqPol(3U/µl)-0.5µl; Water-18.0µl. The Thermocycler was automated for aprimary denaturation process of five minutes at 94°C, then forty cycles of one minute at 94°C, annealing for forty five seconds at 37°C, extension process was at 72°C for two minutes and last extension at 72°C for five minutes. The hold temperature was at 4°C. Amplified DNA was loaded onto 1% agarose gel and tinged with ethidium bromide.

Data Scoring

Clear-cut polymorphic bands clearly resolved, were scored apparently for their occurrence or nonappearance with each primer. The scores were obtained in the form of a matrix with '1' and '0', which specify the presence and absence of bands. Calculation of percentage polymorphism, polymorphism information content (PIC), effective multiplex ration (EMR), resolving power (RP) and marker index (MI)

Percentage Polymorphism

The percentage polymorphism among different accessions with a primer was estimated by the formula[18]

Percentage polymorphism=(No.of Polymorphic band/Total no. of bands) × 100

Effective Multiplex Ratio (EMR)

EMR was calculated by the formula[18]
EMR=np(np/n)
np=No. of polymorphic bands
n=Total No. of bands

Polymorphism Information Content (PIC)
The PIC value for each primer was calculated as proposed by[19]

PIC = 1–[f2 +(1–f)2]

Where f=No. of bands present
No. of bands absent + No. of bands present

Marker Index (MI)
MI was found using the formula[20]
MI= PIC×EMR

Primer Resolving Power (Rp)
Rp was estimatedby the[21] formula:
Rp = ΣIbi
whereIbi =1–[2|0.5–p|]

Where, 'p' is the fraction of accessions with the ith band and Ibi is the informativeness of the ith band.

Construction UPGMA Dendrogram using Jaccard's Coefficient
The genetic relativeness was measured by computing the Jaccard index for pairwise comparisons on the basis of proportions of shared bands produced by the 3 primers. Similarity matrix, similarity coefficients, cluster analysis and dendrogramwas created using NTSYSpc.2.017,UPGMA and SHAN programs.

Dendrogram Drawing and Clustering Analysis
For dendrogram drawing, in "Clustering" tab, input .NTS file. The name and path of output file in the field "Output file" was specified. The desired method for clustering from the "Clustering method" part was selected as UPGMA. The find option under the field 'in case of ties' part was clicked. 'Tie tolerance' and 'beta' fields were unchanged. 'Compute' toolbar was clicked. 'Report listing' window was closed once the procedure was successful. "Pot tree" toolbar was clicked. A dendogram was displayed.

Genetic Similarity
The similarity index between species was found using the protocol of.[22] Genetic similarity between individuals X and Y was calculated using the formula:
SXY = 2NXY / (NX + NY)
Where, SXY = rate of band sharing
NX= No. of bands in individual 1
NY = No. of bands in individual 2
NXY = total number of bands shared by both 1 and 2 individual

Principal Component Analysis
In NTSYSpc, Similarity left menu was selected and Qualitative Data and J (J = Jaccard's coefficient) was chosen for Coefficient. Output file was saved as type .NTS, Positive (= 1.00000) and Negative (=0.00000) codes were set as default.

Statistical Analysis
Variability of the data was represented as the mean ± standard error (SE), where n=3. Data were examined using the one way ANOVA(analysis of variance) to identify significant differences between means. Duncan's test was used to compare the means than differed significantly using at $p<0.05$ probability level with SPSS software.

Table 2: Local names and morphological data of *Amaranthus viridis* accessions and their soil types

A. viridis accession	Local name	Height of Plant(cm) (mean+SE)	Length ofleaf blade (cm) (mean+SE)	Width of leaf blade (cm) (mean+SE)
AVCHTN	Kuppaikeerai	65.33+0.88b	5.93+0.20a	4.2+0.12a
AVKATN	Kuppaikeerai	54.67+0.88a	6.27+0.22ab	5.4+0.31a
AVNEK	Kuppacheera	82+1.15c	8+0.06c	5.33+0.74a
AVTHK	Kuppacheera	86.33+0.88d	8.23+0.23c	5.5+0.68a
AVKOK	Kuppacheera	54+0.58a	6.67+0.15b	5.27+0.14a

Values trailed by the same letter (a, b, c, d) are not significantly different ($p < 0.05$) by Duncan's multiple range test.

Results

Biometric Observations

The five accessions of *Amaranthus viridis* L. (AVCHTN, AVKATN, AVNEK, AVTHK, AVKOK) tested for their variation in the height of plant, length of leaf blade, width of leaf blade, the soil type in which they were cultivated and difference in pH of the soil is represented in Table 2.

Plant Height

The plant heights of the 5 accessions used in the current study ranged from 54+0.58cm (AVKOK) to 86.33+0.88cm (AVTHK).

Leaf Blade Length

The largest leaf blade length (8.23+0.23cm) was observed in AVTHK and the smallest leaf blade length (5.93+0.20cm) was observed in AVCHTN.

Leaf Blade Width

The leaf blade width was maximum (5.5+0.68cm) in the accession AVTHK and the accession AVCHTN had minimum (4.2+0.12cm) blade width.

Soil Type

Different accessions of A.*viridis* was found to grow in different types of soil types red, brown laterite, while majority of them among the accessions were reported to exist in loamy soil type.

pH of Soil

The pH of the soil where the accessions collected where mostly found to occur close to the neutral pH from the range 6.5-7.5.

Genomic DNA Isolation, Quantification and Quality Assessment

Samples of DNA were measured usingUV-VIS spectroscopyat 260nm.The 260/280 ratio ranged from 1.6 to 1.72 which were less than 1.8 for all the accessions reflecting high purity DNA. The whole DNA isolated from the garden-fresh *Amaranthus viridis* accessions leaves were quantified by means of agarose gel electrophoresis (1%) and resulted in, high molecular weight, good quality DNA.

Finger Printing of *Amaranthus Viridis* L. Accessions using RAPD Markers

The three RAPD markers used in the current research to detect the genetic relationship generated amplification products (Figure 3). Polymorphic bands percentage was from 44.44% to 80.77%. The primer with maximum number of polymorphic bands was Primer3 (21alleles). '1' and '0',specifies if bands were present and absent (Table 3).

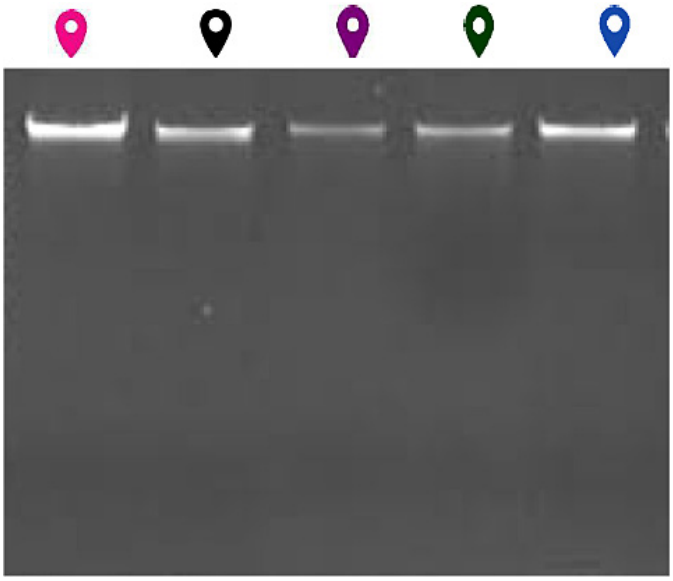

Fig. 3: RAPD profiles of Amaranthus viridis accessions from selected regions of Western Ghats in Tamilnadu and Kerala generated by 3 primers. Numbers 1-5 on the lanes represent AVCHTN, AVKATN, AVNEK, AVTHK and AVKOK accessions respectively.

Table 3: Data scoring of RAPD gel using three primers in the five *A. viridis* accessions

Primer1						
Base pairs	**Acc1**	**Acc2**	**Acc3**	**Acc4**	**Acc5**	**Mono/polymorphic**
4000	1	0	0	0	0	Poly/unique
2500	1	1	1	1	1	mono
2000	1	0	0	0	0	Poly/unique
1750	1	1	1	1	1	mono
1500	1	1	1	0	1	poly
1300	1	0	0	0	0	Poly/unique
1150	0	0	0	0	1	Poly/unique
Primer2						
Base pairs	**Acc1**	**Acc2**	**Acc3**	**Acc4**	**Acc5**	**Mono/polymorphic**
750	1	0	0	0	0	Poly/unique
650	1	0	0	0	0	Poly/unique
500	1	1	1	1	1	mono
400	0	0	0	0	1	Poly/unique
375	1	0	0	0	0	Poly/unique
300	0	0	0	1	1	Poly
275	1	1	1	0	0	poly
175	0	1	1	1	1	poly
<100	0	1	1	1	1	poly
Primer3						
1750	0	0	0	1	1	poly
1500	1	1	1	0	0	poly
1250	1	0	0	1	1	poly
800	1	1	1	1	1	mono
700	1	0	0	1	1	poly
650	1	1	1	0	0	poly
550	1	1	1	0	0	poly
200	1	0	1	1	1	poly

The three RAPD primers produced 66 bands totallyamongthat 46 bands were polymorphic with bands numbers between 8 and 21, with an average of 15.33 bands per primer (Table 4).

Calculation of percentage polymorphism, polymorphism information content (PIC), effective multiplex ration (EMR), marker index (MI) and resolving power (RP)

Table 4: Details of primers,polymorphism information content (PIC), effective multiplex ration (EMR), percentage polymorphism, marker index (MI) and resolving power (RP) in *Amaranthus viridis* accessions.

Primer	Sequence (5'-3')	GC content (%)	Mono-morphic bands	Poly-morphic bands	Percentage Polymor phism (%)	EMR	PIC	MI	RP
Primer1	5'-CCCAGCAA CTGATCGCAC AC-3'	60	10	8	44.44	3.56	0.5	1.78	6.66
Primer2	5'-AGGACTCG ATAACAGAC-3'	47.06	5	17	77.27	13.14	0.5	6.57	7.72
Primer3	5'-ATGTAAGC TCCTGGGGA TTCAC-3'	50	5	21	80.77	17.01	0.46	7.82	9.86
	Average	52.35	6.67	15.33	67.49	11.24	0.49	5.39	8.08

Effective Multiplex Ratio

EMR for 66 bands was from 3.56 to 17.01 with a mean of 11.24 per primer. The peak value was observed with the primer3 and the lowest value for the primer.[1]

Polymorphism Information Content

The PIC scores for polymorphic locifluctuated from 0.46 to 0.5, with a mean value of 0.49 per primer. The maximum PIC score was found for the primers1 and 2 and the lowest for the primer.[3]

Marker Index

The marker index value oscillated from 1.78 to 7.82 with a mean valueof 5.39 perprimer grouping. The peak score (7.82) was found for the primer3and the lowest for the primer1.

Resolving Power

The RP valueswerefrom 6.66 to 9.86 with a mean of 8.08 per primer blend. The maximum value (9.86) was observed for the primer3 and the lowest for the primer1. The details of primers, polymorphism information content (PIC), resolving power (RP),percentage polymorphism, effective multiplex ration (EMR) andmarker index (MI) in A.*viridis* accessions is listed in Table 4.

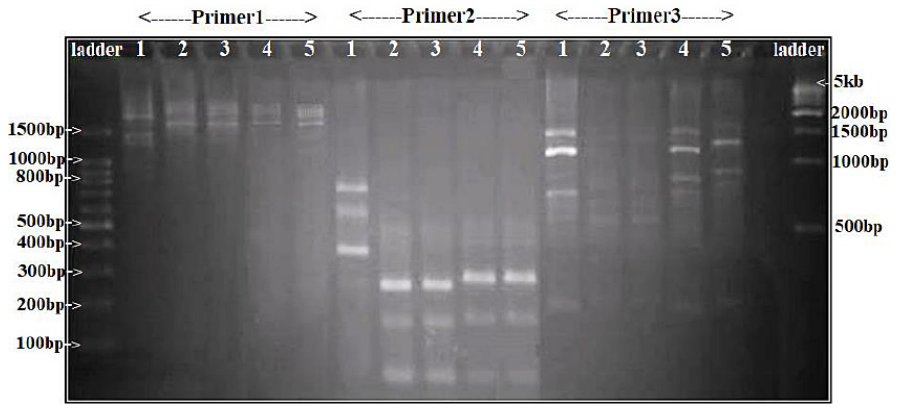

Fig. 4: UPGMA dendrogram comparing the genomic profiles of different Amaranthus viridis accessions collected from selected regions of Western Ghats in Tamilnadu and Kerala using Jaccard coefficient.

Construction UPGMA Dendrogram using Jaccard's Coefficient

The UPGMA dendrogram comparing the genomic profiles of the 5 *Amaranthus viridis* accessions in selected regions of Western Ghats in Tamilnadu and Kerala using Jaccard's coefficient is shown in Figure 4.

A UPGMA dendrogram showed peak Jaccard's coefficient value of 0.96 between AVKATN and AVNEK, indicating the highest genetic similarity and least value(0.58) was noticed between AVCHTN with the rest. A value of 0.87 was found between AVTHK and AVKOK. Clusters analysis exposed the establishment of one major cluster (1) and two sub clusters (1B and 1A). Cluster 1A includes AVKATN and AVNEK while cluster 1B includes AVTHK and AVKOK.

Genetic Similarity

Following the method of Nei& Li (1979) the similarity index values calculatedpair-wise among the 5 accessions are shown inTable 5. The similarity coefficients lied between 0.4828 and0.9565. Among the 5 varieties, the maximum similarity index (0.9565) was foundamong AVKATN and AVNEK, least value (0.4828) was observed among AVCHTN and AVTHK.

Table 5: Genetic similarity matrix constructed on RAPD analysis of 5 *Amaranthus viridis* populations in selected regions of Western Ghats using Nei and Li's formula.

	AVCHTN	AVKATN	AVNEK	AVTHK	AVKOK
AVCHTN	1				
AVKATN	0.6207	1			
AVNEK	0.6667	0.9565	1		
AVTHK	0.4828	0.5455	0.6087	1	
AVKOK	0.5	0.56	0.6154	0.88	1

Principal Component Analysis

For the ease of interpretation, the genetic similarity values between pairs of genotypes in RAPD were subjected to principal component analysis (PCoA) to obtain 3D graphical representations between the 5 genotypes. Three-dimensional representation using Principal Component Analysis (PCoA) for genetic relatedness among 5 *Amaranthus viridis* accessions were clubbed into three individualsets(Figure 5). In these combinations, all accessions from tamilnadu were allotted to group I. Group II involved of only AVTHK and Group III consisted of only AVKOK.

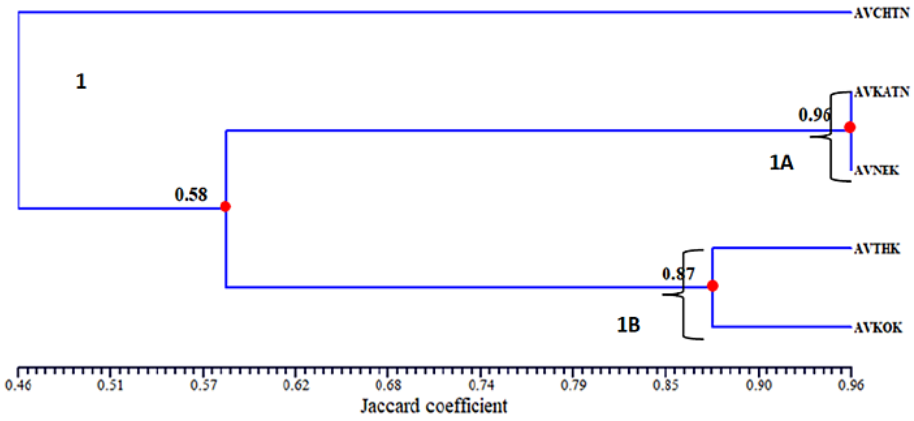

Fig. 5: Three-dimensional representation using Principal Component Analysis (PCoA) for genetic relatedness among 5 *Amaranthus viridis* accessions in selected regions of Western Ghats in Tamilnadu and Kerala.

Discussion

In the current study, the five accessions of *Amaranthus viridis* L. (AVCHTN, AVKATN, AVNEK, AVTHK, AVKOK) collected from selected regions of Western Ghats in Tamilnadu (ChunkanKadai and Kaliakkavilai) and Kerala (Neyyatintakra, Maruthoor, M.C. Road) varied in their plant height (54+0.58cm to 86.33+0.88cm), leaf blade length (5.93+0.20cm to 8.23+0.23cm), leaf blade width(4.2+0.12cm to 5.5+0.68cm), the soil type in which they were cultivated and difference in pH of the soil (6.5-7.5). [23]observed that A. *viridis* in Africa consisted of petiole that is upto 10cm long.

In this study, we have also analysed the finger printing of five *Amaranthus viridis* L. accessions using RAPD markers to evaluate their genetic diversity. Also, investigation by[24]]using random amplified polymorphic DNA (RAPD) marker indicated the genetic diversity and relationships among 6 Amaranthus species from 8 phytogeographic regions of the Indo-Gangetic plains. RAPD primers yielded a total of 262 amplicons, of which 254 amplicons(96.94%) were polymorphic. Description of grain Amaranth germplasm using RAPD analysis in south west Nigeria has been described previously by.[25] In a recent study,[26] have performed marker assisted genetic diversity studies.

In the current study, the percentage polymorphism, polymorphism information content (PIC),effective multiplex ration (EMR), resolving power (RP) and marker index (MI) calculated whereEMR for 66 polymorphic loci ranged between 3.56 and 17.01, PIC values ranged from 0.46 and 0.5, the MI oscillated from 1.78 to 7.82 and the RP range was between 6.66 to 9.86. A similar study on nuclear polymorphism was done by[13] in *Colletotrichum gloeosorioides* isolates of mango. According to a study by,[27] the PIC index existed from 0.19-0.81 Effective Multiplex Ratio (EMR) of the primers ranged from 0.5-7.0 with a mean value of 2.47.

In the current research, the UPGMA dendrogram comparing the genomic profiles of the 5 *Amaranthus viridis* accessions in selected regions of Western Ghats in Tamilnadu and Kerala where the peak value observed was 0.96 between AVKATN and AVNEK, indicating the highest genetic similarity. Cluster 1A included AVKATN and AVNEK while cluster 1B included AVTHK and AVKOK. Previously[28] have assessed the molecular diversity in Amaranthus spp.[29] had reported genetic relationship among 8 Amaranthus palmeri inhabitants.

Our further experiments revealed the Jaccard Similarity values achieved comparison of every pair wise among the 5 varieties of A.*viridis* where the similarity coefficients analysed using 3 RAPD markers extendedbetween0.4828 and0.9565. Amongst the 5 selections the peak similarity index (0.9565) was witnessed between AVKATN and AVNEK and the least similarity index (0.4828) was detected between AVCHTN and AVTHK. It has been earlier established by[24] that disparityoccurred in the genomics of *Amaranthus* populations in the Indo-Gangetic grasslands.[25] characterized grain Amaranth germplasm based on their physical, dietary, as well as RAPD analysis.

In this study, we have also analyzed the genetic similarity values for RAPD between pairs of genotypes by subjecting to principal component analysis (PCoA) to obtain 3D graphical representations between the 5 genotypes for ease of interpretation.[30] has already described the PCoA of physical characteristics in[36] variations to differentiate the diversity by GBS.

Conclusion

Upon finger printing of five *Amaranthus viridis* L. accessions in selected regions of Western Ghats in Tamilnadu and Kerala, using three RAPD markers to evaluate their genetic diversity, several polymorphic bands which ranged up to 80.77% has proved their genetic variations. Also, the UPGMA dendrogram comparing the genomic profiles of the five *A. viridis* accessions using Jaccard coefficient has revealed AVKATN and AVNEK had the highest their genetic distances, whereas AVKATN and AVNEK were genetically similar among the 5 varieties. An enhanced understanding of genetic diversity among plant species and its distribution is vital for its protection and potential use. Other molecular diversity analysis studies may use RFLPs, AFLPs or SSRs.

Acknowledgements

We are grateful to Dr. P. Ravichandran, Head of the Department of Plant Sciences in Manonmaniam Sundaranar University, Tamilnadu, India for

authentication of the accessions. I am also thankful to the staff members of Biotechnology Department, Malankara Catholic College for theirencouragement throughout this work. We are alsothankful to Rev. Fr. Jose Bright, Correspondent/Secretary and Dr. J. Thampi Thanka Kumaran, Principal, Malankara Catholic College, Mariagiri for their constant encouragement and support.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Conflict of Interest

The authors have no conflict of interest.

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