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Genetic Diversity on *Amaranthus hybridus* L., *Amaranthus viridis* L. and *Amaranthus spinosus* L. in parts of Rivers State, Nigeria

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ABSTRACT: The Amaranth plants are annuals or short-lived perennials with over 103 species of flowering plants in the family Amaranthaceae, distributed nearly worldwide. Several amaranth species are useful as food crops and are grown both for their leaves and for their edible seeds, which are a nutritious pseudocereal (nongrass seeds used like cereal grains). This paper reports the genetic diversity of three species of *Amaranthus* (*A. hybridus* L., *A. viridis* L. and *A. spinosus* L.) in Rivers state of Nigeria. Result obtained from this research showed high rate of diversity. DNA characterization and sequencing of the species were done through plastid Ribulose-1,5biophosphate Carboxylase large chain (*rbcL*) genetic marker to determine the rate of genetic variation among members of this genus in our study area. The sequence figures were firstly compared on Basic Local Alignment Sequence Tool for validation. Phylogenetic and molecular evolutional analysis was conducted using MEGA version 7. The dendogram of the molecular phylogeny generated from MEGA 7 software shows elevated rate of variation among studied species.

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Amaranthaceae (pigweed family) is an extensively recognized plant family comprising of annual or perennial herbs (Blunden *et al*., 1999). Species are primarily found worldwide especially in the tropic and sub–tropical regions. Several varieties are cultivated as ornamentals, vegetable or for grains (Flora of America, 2015), some are weeds (Brenner *et al*., 2000). *Amaranthus* species express high plasticity to environmental changes, and ensure their fitness by profuse seeds production. Amaranths species also show remarkable diversity linked to their extensive adaptability to diverse eco-geographic situations (Lee *et al*., 2008). Accurate genotype identification is therefore essential for examination of the genetic variability of local amaranths. For example in Indian a local *Amaranthus* species has been reported by Tui and Satyesh (2009) to develop several eco–types acclimatized to diverse ecological parameters, including cold, drought, and salinity because of their protracted cultivation history in different phytogeographic areas of Indo-Gangetic plains. Precise genotype and eco–type detection of economic crop is indispensable for germplasm preservation (Tui and Satyesh, 2009). Identification and conservation of germ–plasm are essential for upholding genetic variation. In order to choose ecotypes having high nutritional value in the place they are grown, there must be need to analyse or study their genetic material (Perez-Gonzalez, 2001). As reported in Štefúnová *et al*. (2014), *Amaranthus* L. is known to possess great inter- and intra–species disparity (Mosyakin and Robertson, 1996). Molecular apparatus have significant roles in the evaluation of phylogeny and species advancement which was utilized for provision of useful information for circulation and scope of genetic distinction within and amid species (Mondini *et al.,* 2009; Somasundaram and Kalaiselvam, 2011). Therefore, the objective of this paper is to provide an appropriate DNA sequence of the *rbcl* region of the selected species for unique identification using MEGA version 7 to construct a dendogram of the molecular phylogeny of the taxa to show elevated rate of variation among studied species.

MATERIALS AND METHODS

Collection and identification of plant materials: Matured plants of the three species from *Amaranthus* genus found in Rivers State; *Amaranthus spinosus, Amaranthus viridis and Amaranthus hybridus* were collected each from diverse ecological regions from three senatorial district of Rivers State namely; Obio/akpor local government area in Rivers East, Ahoada West local government area in Rivers West and Oyigbo local government area in Rivers South East senatorial district respectively. Just healthy and

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fresh parts were obtained. Three independent plants per eco geographical region were gathered from nine plants. The various conditions like land form, Altitude, Longitude, Latitude and Soil types from the sites were taken. Other pieces of information taken includes site of collection, collection number, date and name of collector. Identified pressed plant samples were deposited at the UPH Herbarium and also taken to the Forestry herbarium in Ibadan an internationally recognized herbarium for authentication and generation of herbarium number. Voucher numbers and ID numbers were assigned to accessions for supplementary study. The voucher samples were placed at the herbarium of the UPH Rivers State. Molecular analyses were done at the Centre for Biofuel Research in Rivers State and International institute for Tropical Agriculture (IITA) in Ibadan both in Nigeria while samples were taken to South Africa for sequencing.

Table 1: Collection sites of *Amaranthus* species from three eco-geographical regions of Rivers State Nigeria with their ecological conditions

s/no	Taxon	Senatorial district or Ecological region	Terrain	Altitude	Latitude	Longitude	Soil type	Process ID	Date collected
1	*A.Spinosus*	Rivers east	Upland	16.50 m	4°52'35"N	7°7'10"E	Sandy	112121	12/4/2018
2	*A.viridis*	Rivers east	Upland	13.12 m	4°52'36"N	7°7'11"E	Sandy	112122	12/4/2018
3	*A. hybridus*	Rivers east	Upland	12.10 m	4°53'22"N	6°55'44"E	Sandy loam	112116	12/4/2018
4	*A. Spinosus*	Rivers south east	Upland	11.07m	4°52'52"N	7°7'94"E	Sandy loam	112120	13/4/2018
5	*A.viridis*	Rivers south east	Upland	11.13m	4°53'22"N	7°8'45"E	Sandy loam	112117	13/4/2018
6	*A.hybridus*	Rivers south east	Upland	12.49m	4°53'81"N	7°8'03"E	Sandy Loam	112119	13/4/2018
7	*A. spinosus*	Rivers west	Coastal or Riverine	-7.62 m	4°59'29"N	6°27'52"E	Sandy loams	112118	15/4/2018
8	*A.viridis*	Rivers west	Coastal or Riverine	0.30 m	4°59'34"N	6°27'54"E	Sandy loams	112123	15/4/2018
9	*A.hybridus*	Rivers west	Coastal or Riverine	-17.68 m	4°59'33"N	6°27'55"E	Sandy loams	112115	15/4/2018

MATERIALS AND METHODS

DNA characterization method: DNA extraction: The plant DNA was obtained from fresh leaf samples. Fresh leaves of 0.5g weight each were utilized for the extraction. Zymo Quick DNA Plant/Seed Miniprep kit was employed for DNA extraction. The protocol of mentioned kit was strictly followed with little modifications.

PCR Amplification: Universal primers **1F, forward** (5′- ATGTCACCACAAACAGAAAC -3′) and **724R, reverse** (5′-TCGCATGTACCTGCAGTAGC-3′) (Lane, 1991) employed to amplify fragments of the ribosomal DNA (rDNA).

Table 2: Recipe for the Direct PCR Amplification method in the study

Component	Master Mix (µL)
10 x PCR buffer	2.5
50mM Mgcl2	1.5
5pMol forward primer	1.0
5pMol reverse primer	1.0
DMSO	1.0
2.5Mm DNTPs	2.0
Taq 5u/ul	0.15
100ng/µl DNA	2.0
H2O	13.85
Final Volume	25µL

Table 3: PCR cycling conditions for the gene regions amplified in this study

PCR STEPS		9 Cycle		35 Cycles				
Initial denaturation	Denaturation	Annealing temperature	Extension	Denaturation	Annealing temperature	Extension	Final extension	Hold temperature
94°C	94°C	65°C	72°C	94°C	55°C	72°C	72°C	10°c
5min	15sec	20sec	30sec	15sec	20sec	30sec	7min	∞

Qualification and Quantification of DNA and PCR Products: Both Agarose gel electrophoresis and Spectrophotometry methods were utilized for attainment of quality of DNA and PCR products before sequencing was done. The amplicon from the above reaction was submitted to gel electrophoresis in (1.5%) agarose gel using TBE 1X and the gel stained with Ethidium Bromide (13µL/50ml). The set up was allowed to run at 100volts for 40 minutes and viewed through UV illumination .The genomic DNA was photographed using a Gel Documentation System (Cleaver Scientific Ltd). Nucleic acids were further quantified and qualified by measuring their quantity and A260/A280 ratios using a Nanodrop lite

spectrophotometer (Thermo Scientific) (Spies, 2004; 2013; Awomukwu, 2015).

DNA Sequencing: After diluting the PCR products with dH_2O in 1:5, they were directly sequenced using the GeneAmp® PCR System 9700 Dual 384-Well thermal cycler. Regions that were amplified were sequenced in two ways with automated sequencer; the 310 Genetic Analyzer BigDye Terminator v1.1/3.1 Sequencing Kit, procedure was followed with slight adjustments. The constituent and quantity for sequencing PCR reactions were: 1 µl of 5x BigDye Sequencing Buffer, 0.5 µlBigDye® ready reaction mixes, 3 µl dH2O, 0.5 µL DMSO, 3 µl of 10 µM primer, and 2 µl PCR products were utilized. The Reactions for 384-Well Plates wereprepared to a total volume of 10µL per tubes (Spies, 2004; 2013; Awomukwu *et al*, 2015)**.** Ethanol/EDTA precipitation method was applied for clean-up.

Table 4: Recipe for the PCR sequencing method in the study

COMPONENTS	STANDARD MIX (µL)
Primer(separate tubes for F-primer and R-primer)	3.0
BigDye® ready reaction mixes	0.5
Distilled water	3.0
5x BigDye Sequencing Buffer	1.0
DMSO (Dimethyl sulf-oxide)	0.5
pGEM®-3Zf(+) double-stranded DNA Control Template	2.0
Total volume	10 µL

RESULTS AND DISCUSSION

DNA Characterization: The quality of the DNA samples of the *Amaranthus* species studied were verified by gel electrophoresis and bands were observed on 0.8 % agarose gel (plate 1a). Samples of extracted DNA samples were also quantified using spectrophotometry. The maximum absorbance ratio was 1.61 while the minimum was 1.41. Samples of each species were further selected for PCR and then sequencing.

PCR profile and sequencing: The DNA extracted were quantified and qualified by measuring the concentration and the A260/A280 Ratio with a Nanodrop Lite Spectrophotometer. The results show the DNA were suitable for PCR amplification and sequencing. The quantity of the DNA was measured in nanogram/microliter (ng//µl l) and the quality of the DNA was rated in the ratio of A260/A280. The DNA purity range from 1.4 to 1.6 while the Nucleic acid concentration range from 80.9 ng/µl to 209.5 ng/µl. The thick bands observed indicates the PCR amplified *rbcL* regions which range from500bp-580bp (Plate 1b) were suitable for sequencing. The sequences were aligned by ClustalW (Thompson *et al*., 1994).

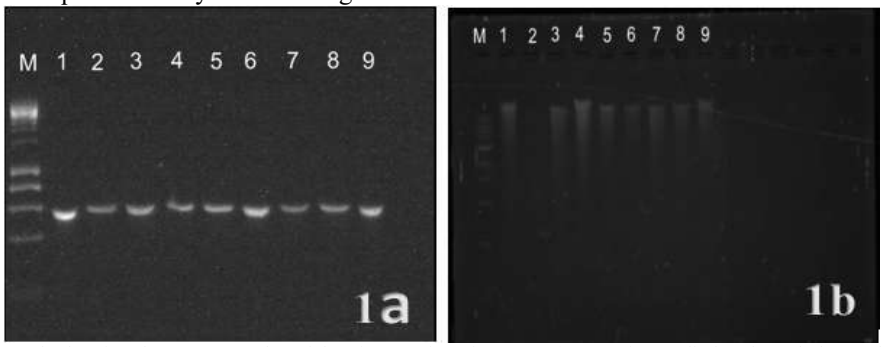

Plate 1a-1b: (1a) Bands showing the DNA samples of the nine species of *Amaranthus* species studied under gel electrophoresis (1b) Bands showing the amplified *rbcl* gene region of the nine species of *Amaranthus* studied under gel electrophoresis

Molecular Phylogenetic analysis and Dendogram: The *rbcL* region was used for the phylogenetic analysis in this study. Phylogenetic and molecular evolutional analysis were conducted using MEGA version 7 (Kumar, Stecher, and Tamura 2016). The difference in base composition bias per site is illustrated below (table 3.2) (Kumar and Gadagkar, 2001). The substitution patterns are homogeneous among lineages, the compositional distance will correlate with the number of differences between sequences. The analysis involved 9 nucleotide sequences. Codon positions included were first, second, third and Noncoding. All positions containing gaps and missing data were eliminated. There were (563) positions in the finishing dataset. The genetic resemblance coefficient concerning base composition in every Amaranthus had mean (0.051). The *Amaranthus spinosus* similarity ranged from (0.05-0.215), *Amaranthus viridis* from (0.012-0.34) and *Amaranthus hybridus* from (0.009-0.018), signifying genetic diversity variation of diverse populations.

Table 5: Estimates of base composition Bias difference between sequence

Taxa	Base composition Bias difference between sequences
A. spinosus Rivers east	0.0
A. hybridus Rivers east,	0.044
A. viridis Rivers south east,	0.02, 0.027
A. spinosus Rivers south east	0.05, 0.005, 0.023
A. hybridus south east,	0.03, 0.009, 0.007, 0.012
A. hybridus Rivers west	0.012, 0.016, 0.018, 0.016, 0.018
A. viridis Rivers west	0.034, 0.032, 0.012, 0.016, 0.023, 0.016
A. spinosus Rivers west	0.08, 0.229, 0.13, 0. 215, 0.187, 0.13, 0.13
A. viridis Rivers east,	0.004, 0.059, 0.016, 0.06, 0.034, 0.023,0.034, 0.066

The dendogram (fig1) generated from MEGA 7 software revealed the molecular diversity of the *Amaranthus* species being analysed. The evolution record was deduced through Neighbor-Joining protocol (Saitou and Nei, 1987). The most favorable tree having branch length (8.43375258) is shown. It is drawn to scale, branch-lengths similar in units to evolutionary distances ones applied for deduction of phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood (Tamura *et al*., 2004). The analysis included (9) nucleotide sequences. Codon positions were 1st, 2nd, 3rd and Non-coding. Every location with space and missing data were eliminated. There were total of (563) points in ultimate data-set. From the dendogram five groups or cluster were obtained. The first group was for *Amaranthus viridis* which shows highest similarity, where *A.viridis* East and South East were closer and a little distant *A.viridis* from Rivers west. The second group was for *A. hybridus,* where A. hybridus East and South East showed 100% similarity while *A.hybridus* Rivers West was so dissimilar that it formed the third cluster. The fourth group consisted of *A.spinosus* Rivers East and West while a more dissimilar *A. spinosus* Rivers South East branched earlier to form the fifth and final group. This disparity is in accord with the one generated from the anatomical and morphological researches.

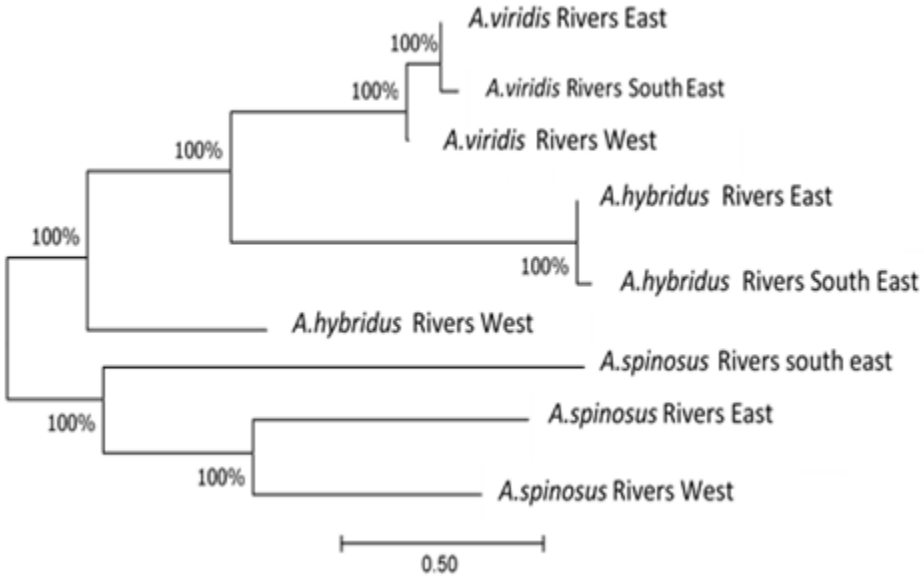

Fig 1: Dendogram of the molecular phylogeny of the nine *Amaranthus* species studied

Conclusion: The detection of *Amaranthus* species by application of sequences of the plastid gene Ribulose 1-5 Carboxylase/Oxygenase large subunits (*rbcL*) marker is marked as an encouraging tool for validation of plant species. DNA sequence of the *rbcl* region of every species studied supply data for unique identification of the taxa and also showed variations within related species from diverse eco geographical regions. The studies also demonstrated high rate of genetic variation existing in the *Amaranthus* species as illustrated by the Phylogenetic tree.

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