Journal of Agriculture, Environmental Resources and Management
ISSN2245-1800(paper) ISSN 2245-2943(online)
5(5)650-1220; Jan.2023; pp844-856

www.saerem.com

The Role of Mophormetric techniques and Dna sequencing in solving taxonomical problems: a case study of the Amaranths.

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Abstract.

The Amaranth flowering plants are of Amaranthaceae family. The taxonomy of Amaranthus is complicated and has principally been considered among systematics as a "problematic" genus. The close morphological similarities of Amaranthus especially in Nigeria which often times leads to confusion in species identification and collection. The related morphological likeness of Amaranthus in Rivers state and Nigeria as a whole often leads to misunderstanding in species classification and detection. This study on the Role of Mophormetric techniques and Dna sequencing in solving taxonomical problems was conducted using three species of the genus Amaranthus namely; A. hybridus L., A.viridis L. and A. spinosus L. from the three senatorial district of Rivers state. DNA characterization and sequencing of the species were done through plastid Ribulose-1,5biophosphate Carboxylase large chain (rbcL) genetic marker. 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. For the Mophormetric techniques, 52 quantitative and qualitative characters were achieved from morphological and anatomical characters and applied for construction of a dendogram using the Paleontological statistics (PAST) software. Results from both Phylogenetic tree and the dendogram clearly shows identified Amaranths species aligning on the tree, based on their level of similarity and differences. It is recommended that Ribulose biphosphate carboxylase large subunit (rbcL) be used in collaboration with the plastid marker Maturase K (MatK) and the nuclear ribosomal internal transcribed spacer (ITS) genetic marker.for more effective results. For the mophormetric techniques, it is recommended that more taxonomic lines of evidence be utilized to generate more characters to arrive at good taxonomic conclusion.

Key Words: Dna Sequencing, Morphometrics, Dendogram, Phylogenetic tree

Introduction

Resolving taxonomic problems between closely related species is notoriously difficult. Such species can generally only be diagnosed based on few characters that often have a host of problems. The taxonomy of Amaranthus is complicated and has principally been considered among systematics as a "problematic" genus (Costea and DeMason, 2001). The close morphological similarities of Amaranthus especially in Nigeria which often times leads to confusion in species identification and collection, diverse Amaranthus species may be difficult to differentiate. Amaranths species also show remarkable diversity linked to their extensive adaptability to diverse eco-geographic situations (Lee, Hong, Dixit, Chung, Ma, Lee and Park, 2008). Some Amaranthus species from one region can be morphologically distinct from same species found in other regions (Ozimede, Obute and Nyanayo, 2019a).

Taxonomic techniques have hugely developed over the last decades, and the birth of the internet and Molecular systematics has also affected all aspects of taxonomy. Nobody can deny that the technological progress has contributed positively to science (Guerra-Garcia, Espinosa and Garcia-Gomez, 2008).

Morphometrics represent the quantitative analysis of biological form that has been widely used in a lot of discipline including systematics (Henderson, 2006). Morphometrics, known as numerical taxonomy, is the application of various mathematical procedures to encode characters. The practice of numerical taxonomy embraces numbers of fundamental assumptions and philosophical attitudes towards taxonomic work (Kolawole,

Abdulrahaman, Mahboob and Oladele, 2016). It has the ability to integrate data from a variety of sources such as anatomy, cytology, ecology, genetics, geography, physiology, palynology, chemistry etc. (Soladoye, Onakoya, Chukwuma and Sonibare, 2010). Number of researchers have demonstrated by their work the importance of numerical taxonomic methods in plant classification and delimitation. For instance (Kolawole, Abdulrahaman, Mahboob and Oladele, 2016) adopted numerical methods to conduct a comparative study, explaining and establishing the taxonomic relationship between some species of Euphorbiaceae using anatomical and morphological characters.

Tautz, Arctander, Minelli, Thomas and Vogler (2003) and Hebert, Cywinska, Ball and deWaard, (2003) made radical proposals that have attracted considerable attention. Both assign larger roles to the use of DNA sequences in taxonomy. Given the importance of taxonomy it is not surprising that these proposals have received much attention. Hebert *et al*. (2003) proposed 'DNA barcoding', which is now pursued by the Consortium for the Barcode of Life (CBOL). In a barcoded world, specimen identification will be based on a partial DNA sequence for COI. Investigators will identify an unidentified specimen by first extracting its DNA, then amplifying and sequencing COI before comparing the sequence from the query with COI sequences for all known species. Although more modest in language, Tautz *et al*. (2003) proposed an even more central role for DNA sequences in taxonomy when they envisioned a 'DNA taxonomy' using DNA sequences as a scaffold; i.e., not only specimen identification but also the determination of species boundaries and hence species descriptions would be based on DNA sequences.

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. 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.

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	$4^0$52`35"N	$7^0$7`10"E	Sandy	112121	12/4/2018
2	*A.viridis*	Rivers east	Upland	13.12 m	$4^0$52`36"N	$7^0$7`11"E	Sandy	112122	12/4/2018
3	*A. hybridus*	Rivers east	Upland	12.10 m	$4^0$53`22"N	$6^0$55`44"E	Sandy loam	112116	12/4/2018
4	*A. Spinosus*	Rivers south east	Upland	11.07m	$4^0$52`52"N	$7^0$7`94"E	Sandy loam	112120	13/4/2018
5	*A.viridis*	Rivers south east	Upland	11.13m	$4^0$53`22"N	$7^0$8`45"E	Sandy loam	112117	13/4/2018

6	*A.hybridus*	Rivers south east	Upland	12.49m	$4^0$53`81"N	$7^0$8`03"E	Sandy Loam	112119	13/4/2018
7	*A. spinosus*	Rivers west	Coastal or Riverine	-7.62 m	$4^0$59`29"N	$6^0$27`52"E	Sandy loams	112118	15/4/2018
8	*A.viridis*	Rivers west	Coastal or Riverine	0.30 m	$4^0$59`34"N	$6^0$27`54"E	Sandy loams	112123	15/4/2018
9	*A.hybridus*	Rivers west	Coastal or Riverine	-17.68 m	$4^0$59`33"N	$6^0$27`55"E	Sandy loams	112115	15/4/2018

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.

Codification of taxonomic characters

Binary and multi state characters to be used in morphometric analysis from the studied operational taxonomic units (OTUs) were converted into numbers in an excel spreadsheet, used as a rough worksheet, before been transferred into the Paleontological statistic (PAST) worksheet for the construction of a dendogram.

Numerical analysis of morphological and anatomical characters

Taxonomic characters obtained from the morphological and anatomical investigations of (3) species of the *Amaranthus* genus studied from three senatorial district of Rivers State amounting to nine species in total were grouped by cluster analysis using the single linkage method based on similarity matrix of Euclidean distances of quantitative and qualitative characters. This statistical analysis was done via Paleontological statistics (PAST) software.

Results

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 sequences were aligned by ClustalW (Thompson *et al*., 1994). The results of the

extracted sequence from the rbcl region of studied plants are

presented in (Fig 1a- 1i)

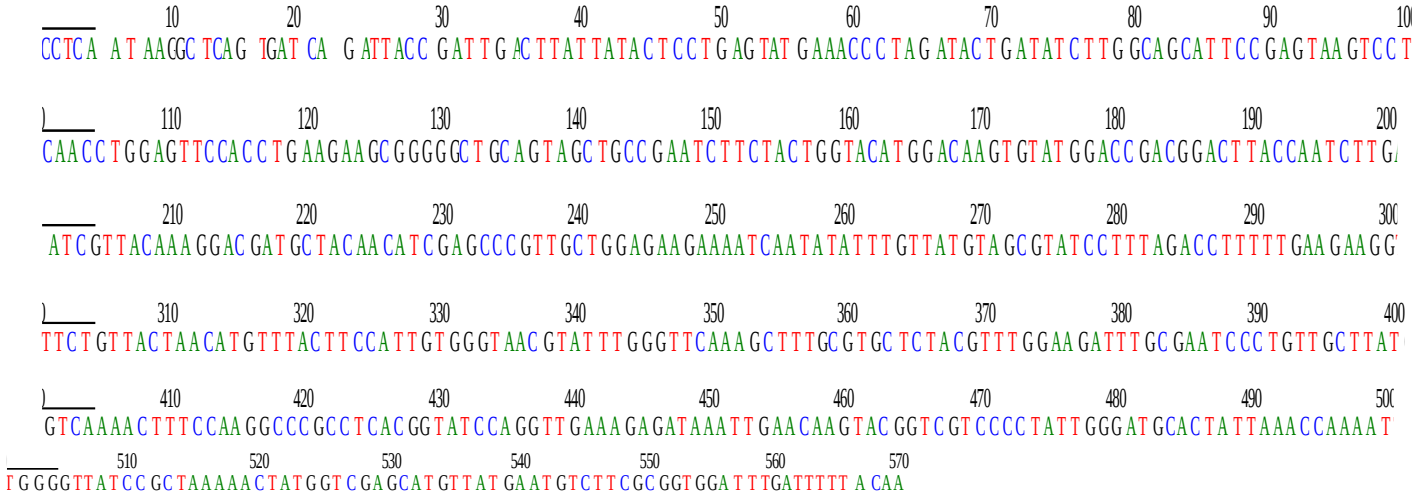


Fig 1a: Rivers east *Amaranthus viridis* DNA sequence extracted from the *rbcL* region

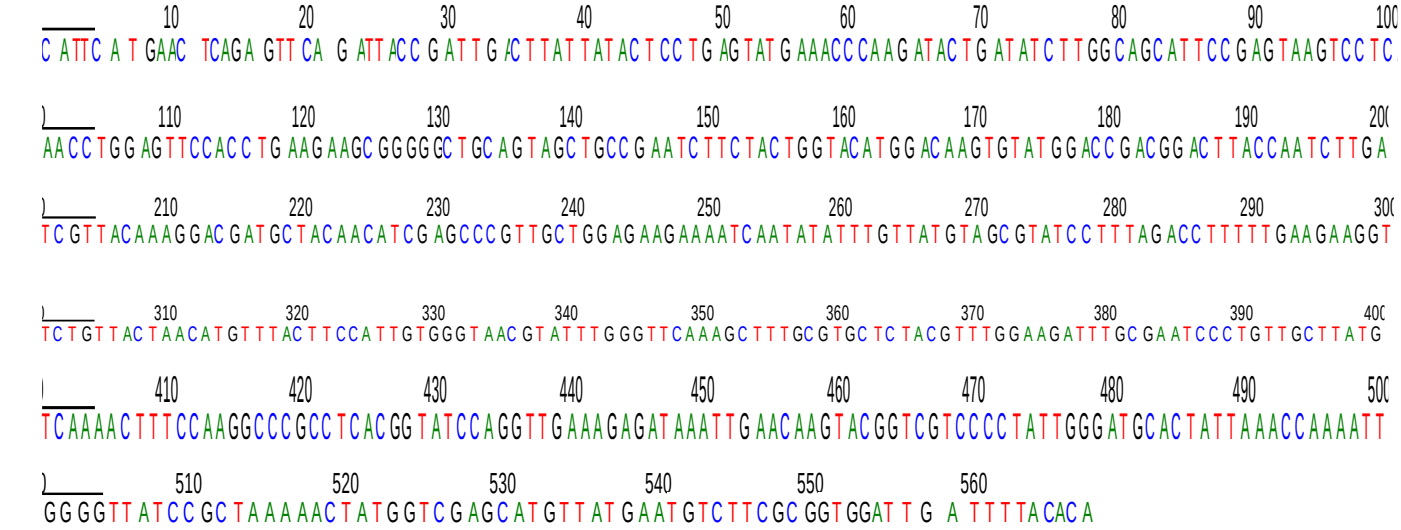


Fig. 1b: Rivers east *Amaranthus spinosus* DNA sequence extracted from the *rbcL* region

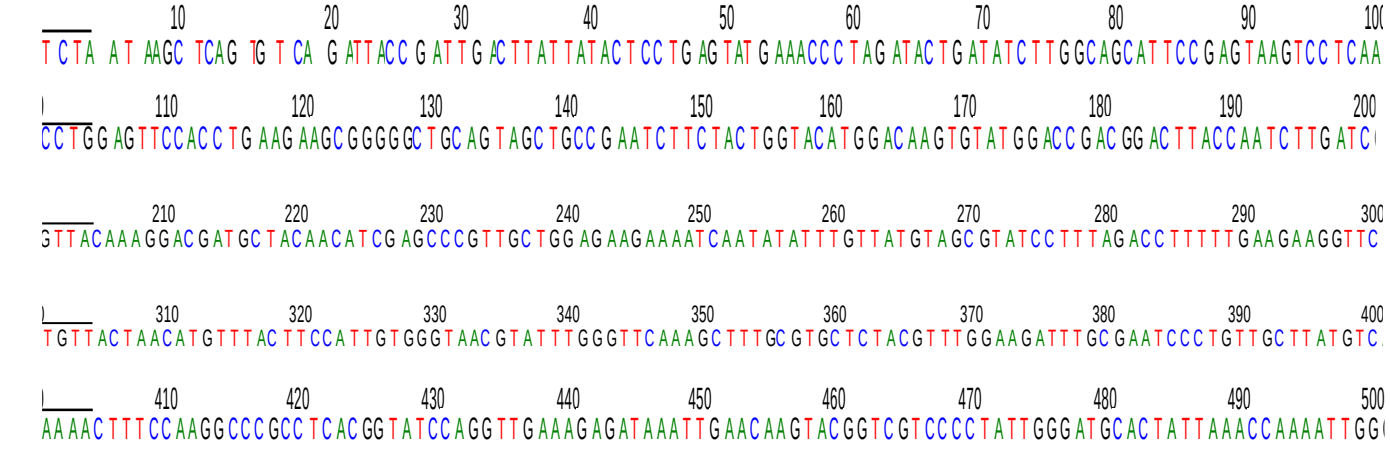


Fig 1c: Rivers east *Amaranthus hybridus* consensus DNA sequence extracted from the *rbcL* region

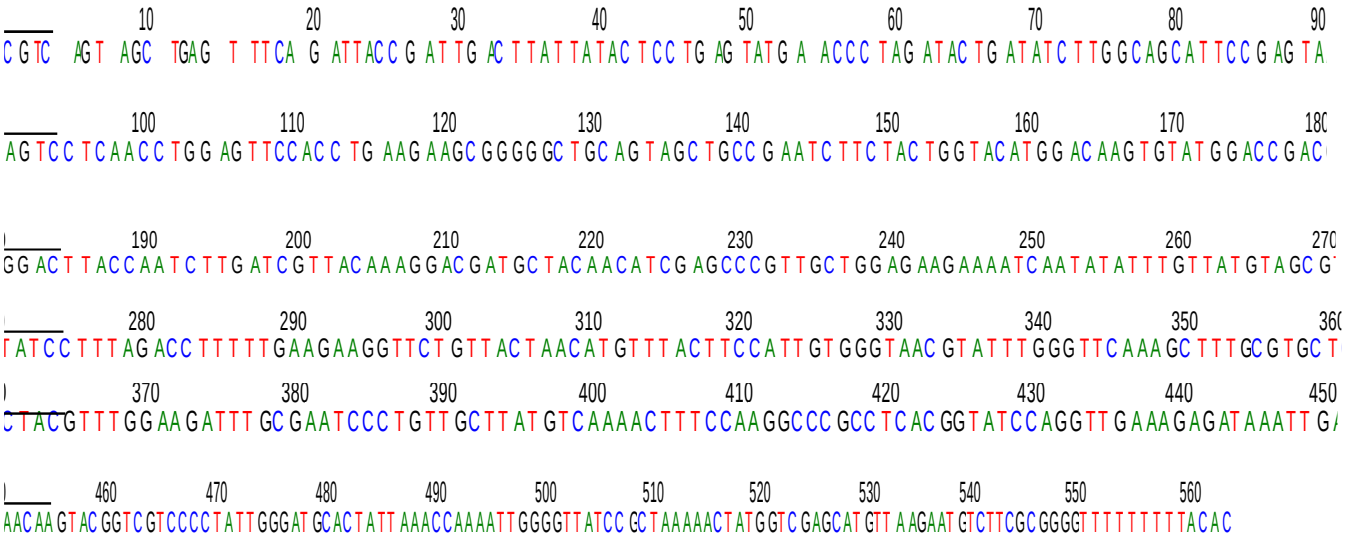


Fig 1d: Rivers south east *Amaranthus viridis* consensus DNA sequence extracted from the *rbcL* region

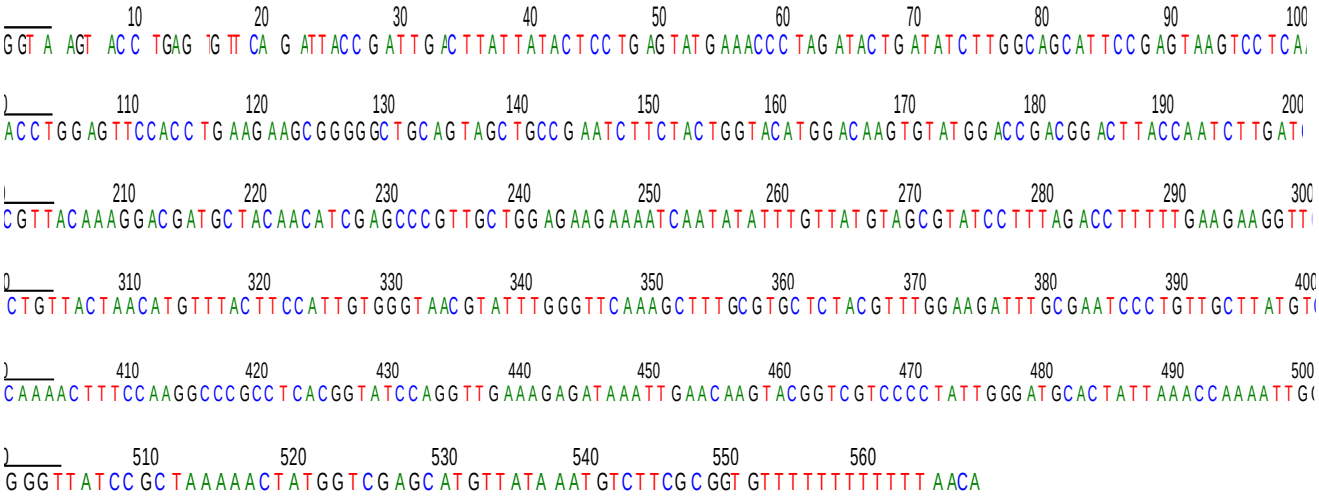
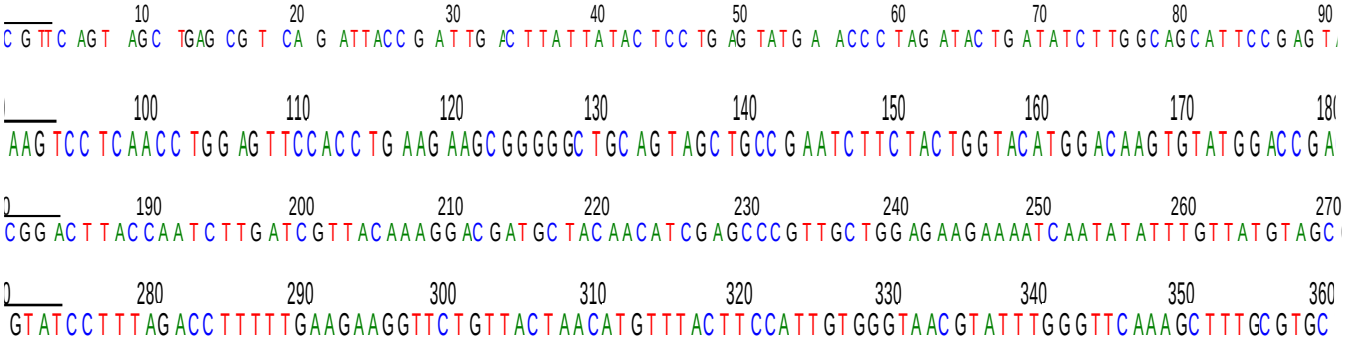


Fig 1e: Rivers south east *Amaranthus spinosus* consensus DNA sequence extracted from the *rbcL* region



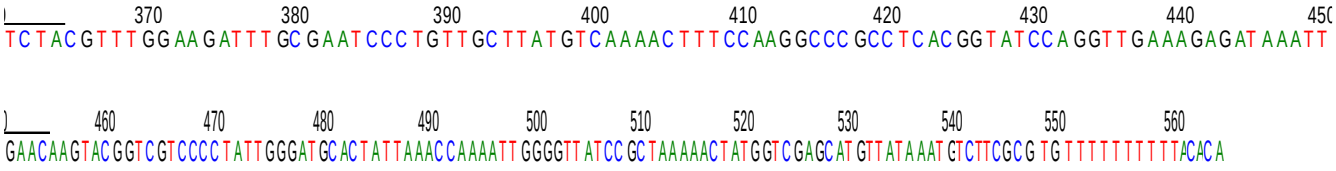


Fig 1f: Rivers south east *Amaranthus hybridus* consensus DNA sequence extracted from the *rbcL* region

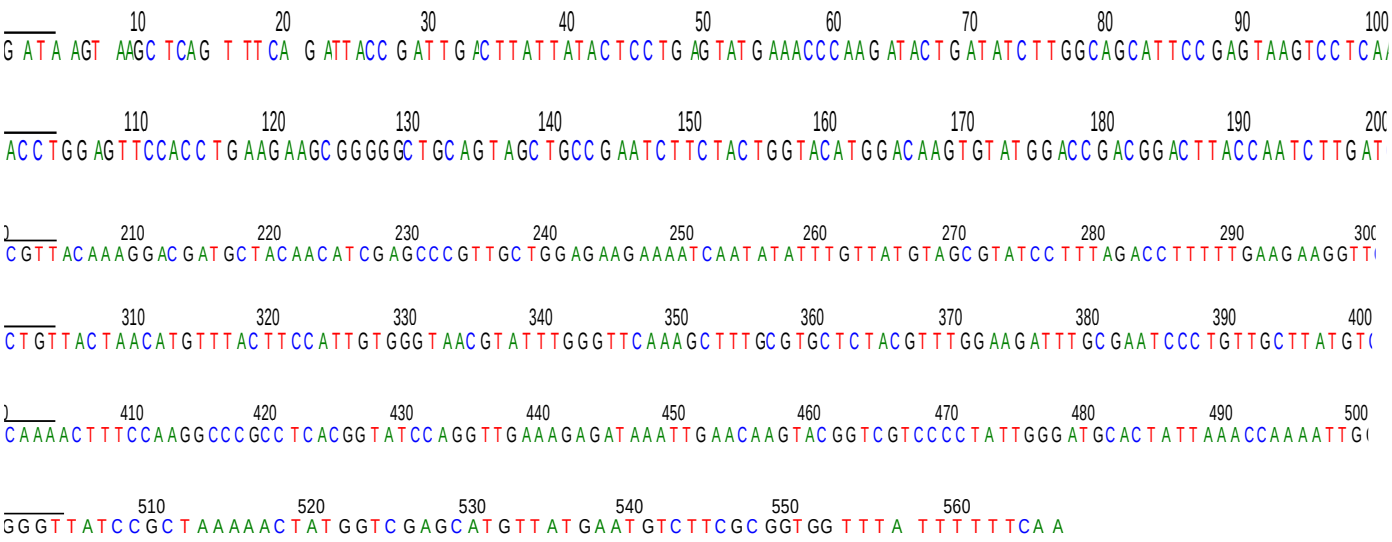


Fig 1g: Rivers south west *Amaranthus hybridus* consensus DNA sequence extracted from the *rbcL* region

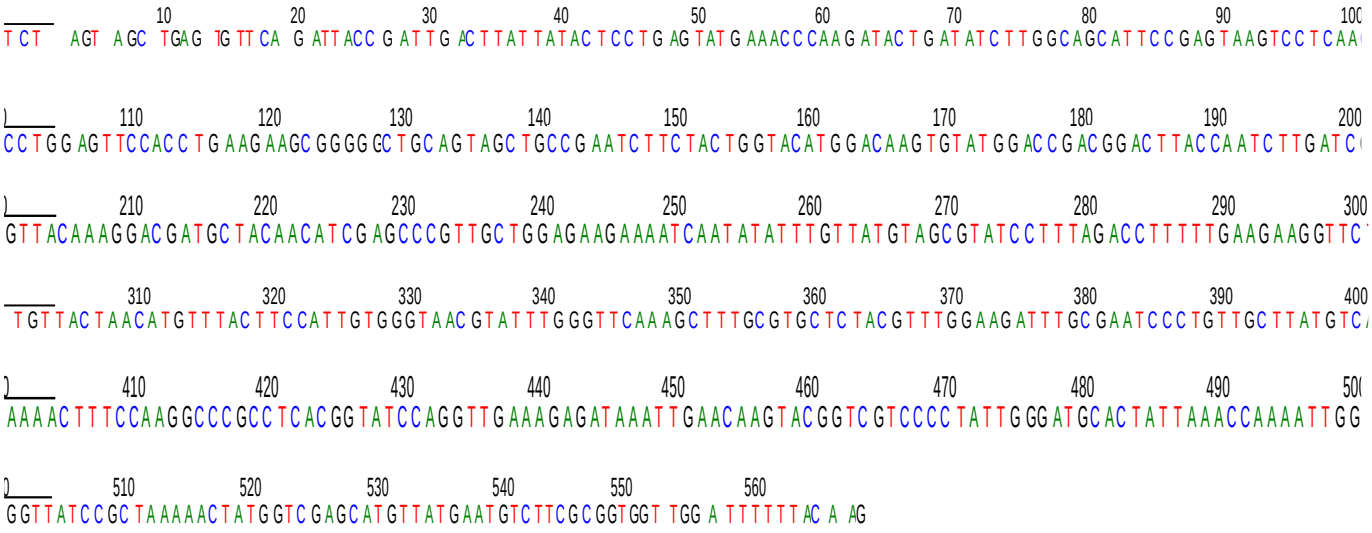
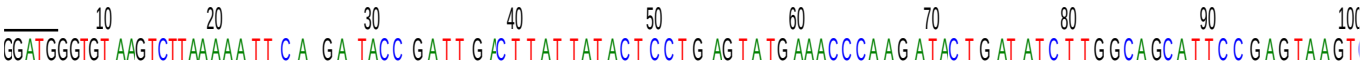


Fig 1h: Rivers south west *Amaranthus viridis* consensus DNA sequence extracted from the *rbcL* region



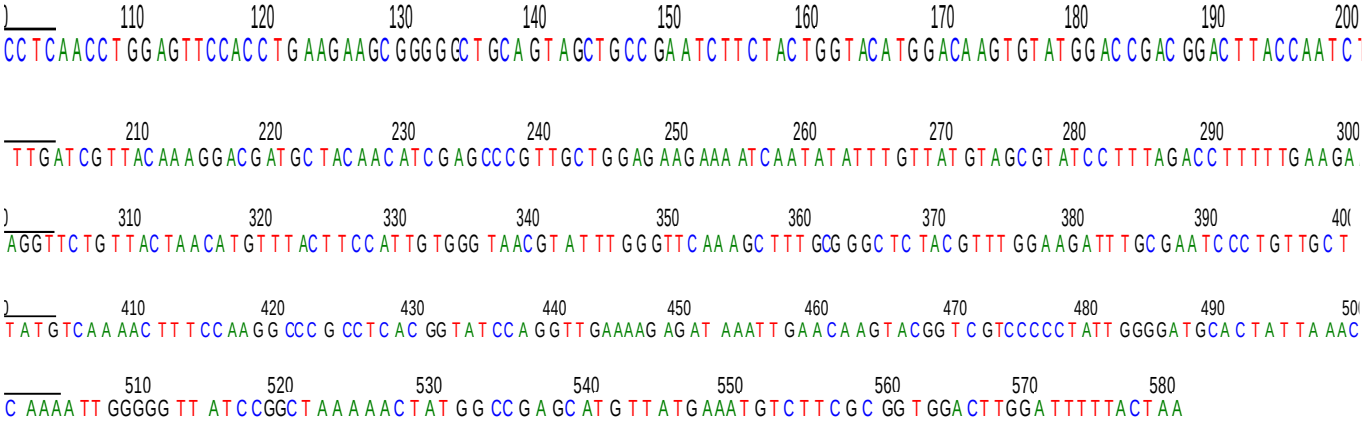


Fig 1i: Rivers south west *Amaranthus spinosus* consensus DNA sequence extracted from the *rbcL* region

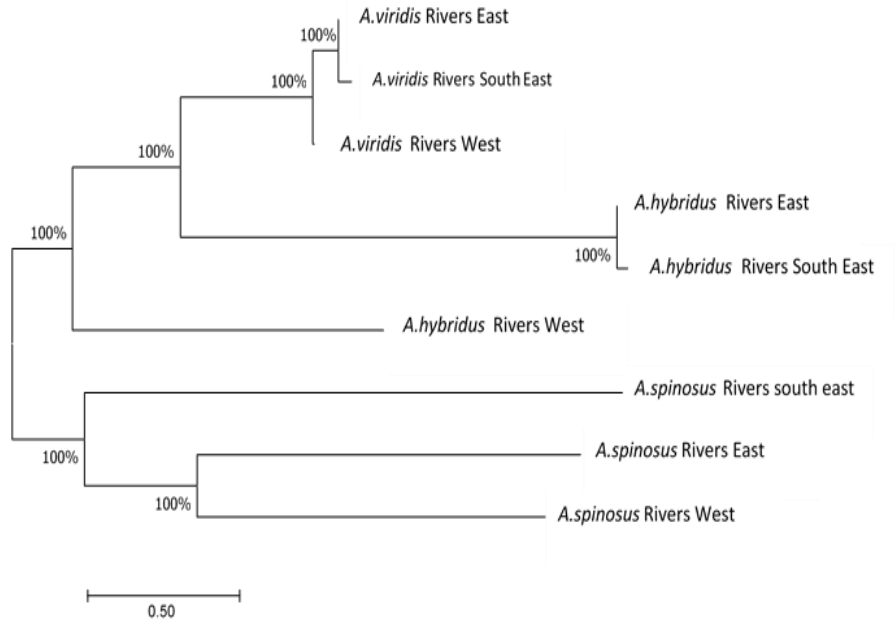


Fig 4.4: Dendogram of the molecular phylogeny of the nine Amaranthus species studied.

KEYS

OZEMADE RBCL 1	*A.spinosus* Rivers west
OZEMADE RBCL 2	*A.spinosus* Rivers east
OZEMADE RBCL 3	*A.viridis* Rivers east
OZEMADE RBCL 4	*A.hybridus* Rivers west
OZEMADE RBCL 5	*A.hybridus* Rivers east
OZEMADE RBCL 6	*A.viridis* Rivers west
OZEMADE RBCL 7	*A.hybridus* Rivers south east
OZEMADE RBCL 8	*A.viridis* Rivers south east
OZEMADE RBCL 9	*A.spinosus* Rivers south east

Table 2: 52 Morphological and Anatomical characters of the *Amaranthus* species used in multivariate analysis with their method of scoring

S/NO.	CHARACTERS	STATE	CODE
		Ovate	1
		Obovate	2
1	Leaf shape	Lanceolate	3

		Oblong	4
		Elliptical	5
		Acute	1
2	Leaf apex	Accuminate	2
		Obtuse	3
		Cuneate	1
3	Leaf base	Broadly cuneate	2
		Acute	3
		Rounded	4
		Entire	1
4	Leaf margin	Serrated	2
		Dentate	3
		Undulate	4
		Alternate	1
5	Phylotaxy	Opposite	2
		Whorled	3
		Reticulate	1
6	Venation	Palmate	2
		Cross-venulate	3
		Green	1
7	Stem colour	Reddish pink	2
		Yellow	3
		Wild	1
8	Habitat	Cultivated	2
		Herb	1
9	Habit	Shrub	2
		Tree	3
		4.1- 5.0 cm	1
		5.1- 6.0 cm	2
10	Petiole length	6.1-7.0 cm	3
		7.1 – 8.0 cm	4
		80-119 cm	1
11	Plant height	120-149 cm	2
		150-179 cm	3
		Uniseriate	1
12	Nature of epidermis	Biseriate	2
		Multiseriate	3
13	Nature of intercellular spaces	Small	1
		Big	2
		Parenchyma	1
14	Nature of Cortical tissue in midrib	Collenchyma	2
		Parenchyma and collenchyma	3
		Parenchyma and sclerenchyma	4
		3-4 layers thick	1
15	Nature of parenchyma cells in the cortex of Midrib	3-5 layers thick	2
		4-5 layers thick	3
		2-3 layers thick	1
16	Nature of xylem cells within midrib	2-4 layers thick	2
		2-6 layers thick	3
		3-4 layers thick	4
	Nature of collenchyma cells in	1-2 layers thick	1
17	Midrib	1-3 layers thick	2
		2-3 layers thick	3
18	Sand crystals in Midrib	Absent	0
		Present	1
		Parenchyma	1
19	Pith tissue in midrib	Collenchyma	2
		Sclerenchyma	3

20	Single phloem strand in midrib	Absent	0
		Present	1
		Crescent	1
21	Midrib shape in transverse section	Arc	2
		Rounded	3
		Crescent	1
22	Vascular bundles arrangement in midrib	Circular	2
		Arc	3
		4	1
		5	2
23	Number of vascular bundle in Midrib	6	3
		9	4
		Crescent	1
24	Shape of vascular bundles in midrib	Ring	2
		Ovate	3
25	Bundle shealth in Midrib	Absent	0
		Present	1
		Absent	0
26	Trichomes in midrib	Present	1
27	Protuberance in midrib	Absent	0
		Present	1
		Uniseriate	1
28	Nature of Petiole epidermis	Biseriate	2
		Multiseriate	3
29	Trichomes in petiole	Absent	0
		Present	1
		Parenchyma	1
30	Nature of Cortical tissue in petiole	Collenchyma	2
		Parenchyma and collenchyma	3
		Parenchyma and sclerenchyma	4
31	Nature of parenchyma cells in Petiole cortical tissues	3-4 layers thick	1
		4-5 layers thick	2
		4-6 layers thick	3
32			
	Nature of collenchyma cells in Petiole cortical tissues	2-3 layers thick	1
		3-4 layers thick	2
		3-5 layers thick	3
		4-5 layers thick	4
33	Sand crystals in petiole	Absent	0
		Present	1
		Parenchyma	1
34	Pith tissue in petiole	Collenchyma	2
		Sclerenchyma	3
		Crescent	1
35	Petiole shape in transverse section	Rounded	2
		Cordate	3
		V-shaped	4
36	Vascular bundles arrangement in petiole	Crescent	1
		Circular	2
		Arc	3
		7	1
37	Number of vascular bundle in petiole	9	2
		12	3
		19	4
		Crescent	1
38	Shape of vascular bundles in petiole	Ring	2
		Ovate	3

39	Protuberances in petiole	Absent	0
		Present	1
40	Shape of stem in transverse section	Rounded	1
		Irregular Ovate	2
41	Nature of stem Cortical tissues	Parenchyma	1
		Collenchyma	2
		Parenchyma and collenchyma	3
		Parenchyma and sclerenchyma	4
42	Sand crystals in stem cortex	Absent	0
		Present	1
43	Protuberance in Stem	Absent	0
		Present	1
44	Nature of stem Pith tissues	Parenchyma	1
		Collenchyma	2
		Sclerenchyma	3
45	Stem epidermal circumference	Angular	1
		Rounded	2
46	Nature of stem epidermis	Uniseriate	1
		Biseriate	2
		Multiseriate	3
47	Anomalous cambial ring around the vascular cylinder	Absent	0
		Present	1
48	Conjugative tissue in vascular cylinder	Thin parenchyma	1
		Lignified parenchyma	2
49	Nature of stem cortical parenchyma cells	2-4 layers	1
		3-4 layers	2
		3-5 layers	3
50	Nature of stem cortical collenchyma cells	3-4 layers	1
		3-5 layers	2
		4-5 layers	3
		5-6 layers	4
51	Trichomes in stem	Absent	0
		Present	1
52	Intercellular spaces in stem cell	Small	1
		Big	2

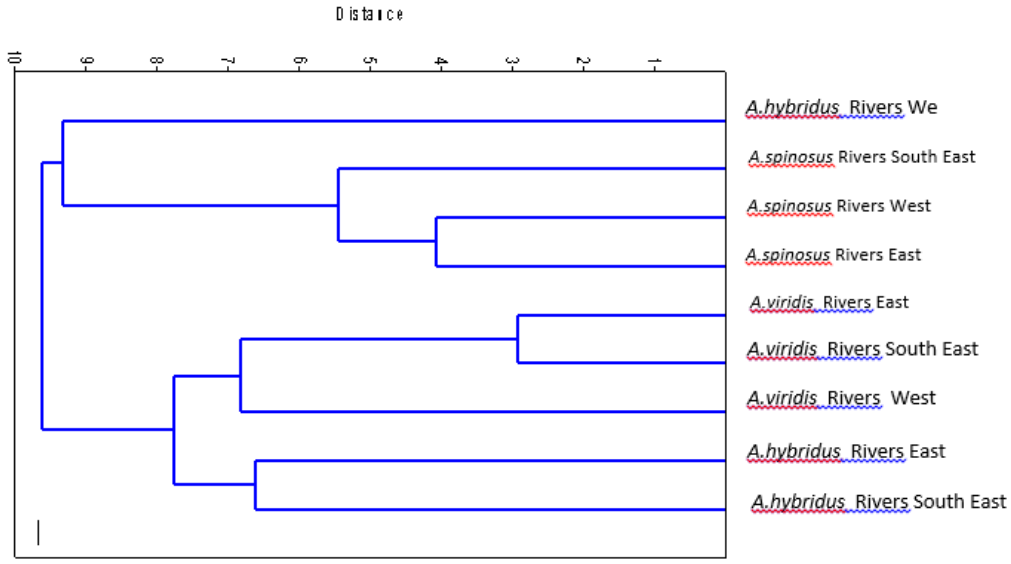


Fig 4.1: Dendogram of the nine *Amaranthus* species produced by cluster analysis using Past software.

Discussion: This finding on the role of numerical taxonomy in solving taxonomy problems is in agreement with that of other researchers who demonstrated how numerical techniques can be used to analyze data for biosystematics and taxonomic studies (Onyeweaku, Nyananyo, and Ozimede, 2020 and Ozimede, Obute and Nyanayo, 2019a). Numerical taxonomy also known as Morphometrics add a quantitative element to species identification, allowing more severe comparisons within a genus. In the numerical analysis of the three amaranths using 52 morphological and anatomical characters, the results revealed that variations in both morphology and anatomy characters are important, confirming their usefulness for species identification purposes. Same trends had been observed by previous studies on Ficus species (Sonibare, Jayeola and Egunyomi, 2004), Acalypha in South-Western Nigeria (Soladoye, Sonibare and Rosanwo, 2008), Senna species in South-western Nigeria (Soladoye, Onakoya, Chukwuma and Sonibare, 2010).

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 as illustrated by the Phylogenetic tree and is in conformity with the report of (Ozimede, Obute and Nyananyo, (2019b).

Recommendations: The Ribulose biphosphate carboxylase large subunit (*rbcL*) is a good genetic marker region for distinguishing the *Amaranthus* genus. But it will be more effective if is used in collaboration with the plastid marker Maturase K (*MatK*) and the nuclear ribosomal internal transcribed spacer (*ITS*) genetic marker. Presently in Nigeria, requirement exists for taxonomist to apply molecular detection for each indigenous plant for establishment of standard genetic library. It would alleviate the bewilderment by indistinct morphological identification.

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