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Phylogenetic assay and pollen structure of few species of the genus *amaranthus* L.

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Abstract

In Amaranthaceae, morphology based identification methods are usually time consuming and may sometimes lead to misidentification and always may not provide good resolution at the species levels. The phenotypic variability of the taxa may lead to misidentifications and creation of new false identity. DNA sequencing has been used to explain evolutionary relationships for more than 20 years in molecular systematics. The aims of DNA barcoding include identification of known specimens/species and new discovery of unknown plant species for enhancing taxonomy for the good of the science and betterment of society. The study basically emphasised on palynological studies and molecular profiling of Amaranthus species using universal markers rbcL and matK. The pollen morphology of the species of Amaranthus shows significant differences in polar length and equatorial diameter whereas the aperture was pantoporate with evenly distributed microspines. The phylogenetic assay showed bootstrap value of 96 and 98 for matK while 79 and 98 for rbcL dendrograms. All barcodes yield quality sequences.

Keywords: DNA Barcoding, *rbc*L, *mat*K, Palynology.

Introduction

The flowering plant family Amaranthaceae which corresponds to the classical family Amaranthaceae Juss. 69 genera and 772 species as well as the twice larger related family Chenopodiaceae have been subject to repeated taxonomical revisions from the time they were first described (1789 and 1799) respectively to the present[1-3]. Recently it has been proposed to combine them into one large family Amaranthaceae as a result of molecular analysis[4]. The morphology of pollen grains, is an important source of information for plant systematics. In certain cases, palynological data have been crucial for taxonomic conclusions[5]. But for closely related species of the genus *Amaranthus* L., such data can lead to confusions regarding the classification though pollen grains morphological studies shows very slight variation. Traditionally, most plant identifications are based on morphological characters, but such identification is not always reliable and efficient[6].

DNA barcoding basically relies on short and standardized gene regions for the identification of plant species. The agricultural and horticultural applications of barcoding such as for marketplace regulation and copyright protection remain poorly explored. This study examines the use of effectiveness/quality of the standard plant barcode markers (*matK* and *rbc*L) for the identification of plant species that are medicinal and are of interest for pharmaceutical industry both in private and public nurseries and to authenticate the medicinal plants. A wide variety and different range of molecular techniques including the random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism in plant (AFLP), restriction fragment length polymorphism of the selected taxa (RFLP), microsatellite and single nucleotide polymorphism (SNP) have been proposed to identify plant species/specimen and cultivars[7-11]. DNA barcoding method has emerged as a relatively new/novel and perhaps more universal tools with which to analyze diversity of both plants and animals and to fix specimens to their respective species even in the absence/ unavailability of key morphological diagnostic features[12,13]. Although there are still some reserves against the performance of DNA barcoding as compared, for example, to morphology, an early study, through a thorough comparison of DNA barcoding and morphology-based species identification recorded a number of limitations to the morphology particularly when it comes to cryptic species[2]. As a taxonomic tool this technique has widely acceptable and also has been successfully used in large scale biodiversity projects where regional flora and fauna are documented including regulated and threatened taxa[14-17]. Although a number of plant loci including, *trnH-psbA*, *rpoc1*, *rpoB*, *trnL*, *rbcL* and *matK* were initially proposed as potential plant barcodes based on assessments of similarity with genebank and recoverability, sequence derived quality and levels of plant species discrimination, the Consortium for the Barcode of Life recommended the 2-loci combination of *rbc*L +*matK* as the most standard plant barcode for analysis[18-23].

The DNA barcode data generated in the present study will serve in the future in commercial agricultural and medicinal plant industries for the purpose of control of counterfeited product, and could also serve in ecological studies of local flora as demonstrated elsewhere[24-26]. In addition, the evolutionary processes such as hybridization especially interspecific hybridisation and different polyploidy are common in plants, so such species boundaries are difficult to define[27,28]. Thus, screening for single or multiple regions using appropriate primers are necessary for DNA barcoding studies in the nuclear and plastid genomes in plants that has been an important point of research. Since *matK* and *rbc*L sequences from Amaranthaceae were the most abundant in GeneBank, they were chosen for the study. Similarly authentication is a critical aspect of research in selection of plants for study, so an attempt was done to authenticate the *Amaranthus* L. using the palynological and DNA barcodes data inorder to make a unique identity among the plants.

Materials and methods

The fresh leaves of of *Amaranthus spinosus L., Amaranthus caudatus* L., *Amaranthus tricolor* L., *Amaranthus dubius* Mart., and *Amaranthus viridis* L. were used for isolating genomic DNA. Pollen grains were collected from the anther of 15 flowers of each species and fixed in glacial acetic acid for acetolysis as per the technique[29]. The sculpturing pattern, values of P(pollen axis ratio) and E(equatorial diameter) and aperture number were viewed and data were measured using the Scanning Electron Microscope (SEM) JSM-6390 LA coated with gold, examined and photographed using JSM-6390 LA. All the experiments were evaluated statistically with SPSS version 20.0, the results were represented in mean±SEM (standard error of mean). One way analysis of varience (ANOVA) followed by DMRT to find out any significant difference in pollen characters among five *Amaranthus* sps. resulting from analytical experiments carried out. P value less than 0.05 were adopted as statistically different.

The DNA was isolation by using branded Sigma kit GenElute Plant Genomic DNA Mini-preparation Kit. GeneAmp PCR System 9700, Applied Biosystems, a PCR thermal cycler for PCR amplification, using the primers of *rbc*L and *matK*. The primer details were given in table 1 and PCR amplification data for conditions provided are given in Table-2.

Table-1: The universal primers *rbc*L and *matK* and their sequences.

Target gene	Primer	Direction	Sequence strand (5'→ 3')
matK	390 f	forward	CGATCTATTCATTCAA TATTTC
	1326r	reverse	TCTAGCACACGAAAGT CGAAGT
*rbc*L	rbcLa_f	Forward	ATGTCACCACAAACA GAGACTAAAGC
	rbcL724 _rev	Reverse	GTAAAATCAAGTCCAC CRCG

Table-2: PCR amplification profile.

matK			*rbcL*		
98 °C	-30 sec		98 °C	-30 sec	
98 °C	- 5 sec	40 cycles	98 °C	- 5 sec	40 cycles
50 °C	-10 sec		60 °C	-10 sec	
72 °C	-15 sec		72 °C	-15 sec	
72 °C	- 60 sec		72 °C	- 60 sec	
4 °C	- ∞		4 °C	-∞	

Sequencing reaction was done in a PCR thermal cycler named GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit of Applied Biosystems, USA model. The sequence quality was checked Applied Biosystems Sequence Scanner Software v1. Using Geneious Pro v5.6 Sequence alignment and required editing of the obtained sequences were carried out [30]. The DNA sequences of *Amaranthus* spp. under study were subjected to BLAST analysis for better identification at the species level. Sequences obtained were aligned and compared using Multiple Sequence Alignment software program of BioEdit Sequence Alignment Editor , CLUSTAL W Multiple Alignment[31,32]. DNA barcodes namely *rbc*L and *mat*K for constructing neighbouring tree model using MEGA 7.0, and a tree was constructed using a combination of rbcL and matK[33].

Results and discussion

The SEM photomicrographs of radial, longitudinal axis, pore aperture, aperture number and distance are shown in Figure-1. The pore structure is similar to the Type II of that is the pores possess microspines, granulate surface and are evenly spread, with numerous pores[34]. The present palynological result support existing data which have indicated that *Amaranthus* is pantoporate in terms of pore number. Most of the species examined in Amaranthaceae have pantoporate with *Amaranthus* type of pollen[35]. The aperture of the pollen examined showed great variation in *Amaranthus tricolor* L. , were their size and number is reduced as reported that there is a *Amaranthus* type of porate aperture in the family Amaranthaceae[36]. The pollen grains of *Amaranthus* species are spheriodal to oblate spheroidal, pantoporate and pantotreme, with this a key is prepared inorder to make a comparison and identification among the species.

Shape- Spheroidal
Diameter 22.12-22.21μm
Aperture distance 3.23 -3.66 μm .... *Amaranthus viridis* L.
Shape- Oblate-spheroidal
Diameter 21.80-21.84μm
Aperture distance 1.00 -1.22 μm .... *Amarantus spinosus* L.
Diameter 18.00-19.86 μm
Aperture distance 2.05-2.57 μm .... *Amarantus tricolor* L.

Shape- Prolate-spheroidal
Diameter 20.32-21.34µm
Aperture distance 3.02-3.14 µm ....*Amarantus dubius* Mart.
Diameter 20.46 -20.83 µm
Aperture distance 2.12-2.21 µm .... *Amarantus caudatus* L.

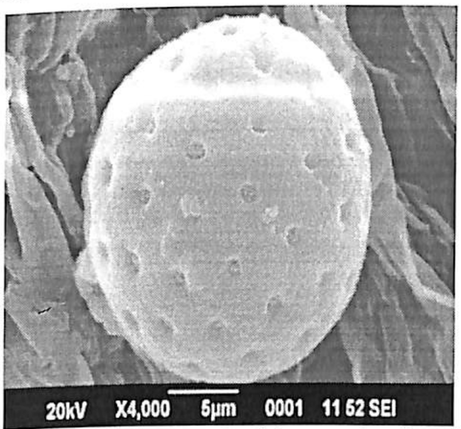

Figure-1: SEM photograph of *Amaranthus* L. pollen.

This study demonstrated differences in pollen characteristics among the *Amaranthus* species. However, pollen morphology/palynological data will have limited use in species identification because of similarities across the species. To increase the usefulness of pollen morphology in species identification, additional analysis of naturally occuring population as well as sampling from different geographical regions would be needed to account for the study[37]. The statistical analysis of pollen characters using SPSS version 20.0 by Duncan's Multiple Range test ($\alpha/p = 0.05$) showed that there is a significant difference between the species of *Amaranthus* L. as because the $p < 0.05$.Thus it infers that the palynological characters are significant among five selected species. Similar attempt was made to pollen morphological differences in *Amaranthus* L. species and the hybrids formed by interspecific hyhridisation where the mean value with same letter are not significant as reported[37].

From the DNA barcoding studies using the universal primers *rbc*L and *mat*K, the study revealed the correct differentiation among *Amaranthus* species. It has been proved experimentally that DNA markers can act as a powerful/authentic tool for identification of cultivars and species for phylogenetic evaluation[38,39].

The accession numbers of the DNA sequences submitted and size of the sequences are given in the Table-5. The tree constructed by neighbour joining tree feature using MEGA 7 is given in the Figure-2.

Table-4: Pollen characters among 5 *Amaranthus* species

Species	*Amarantus viridis* L.	*Amarantus spinosus* L.	*Amarantus dubius* Mart.	*Amarantus caudatus* L.	*Amaranthus tricolor L.*
Pore distance	$1.254^{d}\pm0.006$	$1.19^{d}\pm0.022$	$1.172^{e}\pm0.19$	$1.226^{d}\pm0.01$	$1.146^{e}\pm0.02$
Aperture distance	$3.448^{c}\pm0.081$	$2.924^{c}\pm0.146$	$2.672^{d}\pm0.07$	$2.73^{c}\pm0.06$	$2.534^{d}\pm0.07$
Diameter(l)	$20.872^{b}\pm0.85$	$20.634^{b}\pm0.766$	$19.91^{c}\pm0.48$	$20.006^{b}\pm0.49$	$19.652^{c}\pm0.45$
Diameter(e)	$21.086^{b}\pm0.321$	$21.376^{b}\pm0.257$	$21.308^{b}\pm0.32$	$21.386^{b}\pm0.36$	$21.364^{b}\pm0.37$
Aperture number	$24.2^{a}\pm0.74$	$25.8^{a}\pm0.374$	$28^{a}\pm0.44$	$30^{a}\pm0.89$	$16.8^{a}\pm0.58$

Each value that is expressed in as mean±Std. Error done in triplicates. Data analysed by SPSS version 20.0 by Duncan's Multiple Range test ($\alpha/p=0.05$). Mean values followed by the different Superscript in the columns are significantly different among the pollen characters.

Table-5: Accession number of sequence submitted in the GenBank.

Plant samples	Place of Collection	rbcL Accession No.	matK Accession No.
A.viridis	Edakochi	KJ773261	KJ772535
A.spinosus	Thevara	EF590496	EF590394
A.dubius	Thrissur	KX090210	KX090202
A.caudatus	Boat jetty	KX090209	KC747133
A. tricolor	Kodugallur	JF940812	JF953165