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Genetic diversity in African nutmeg (*Monodora myristica*) accessions from South Eastern Nigeria

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Twenty-one accessions of African nutmeg (*Monodora myristica* Gaertn. Dunal), an endangered spice plant, were collected from the South-East and South-South regions of Nigeria and analyzed for genetic diversity using random amplified polymorphic DNA (RAPD) markers. Twenty-one (21) decamer primers were tested out of which 10 that gave reproducible band patterns were selected for the study. A total of 77 bands were generated, ranging from 3 for OPB17 to 13 for OPT07, and were all polymorphic. The mean polymorphic information content (*PIC*) and genetic diversity (H_e) were 0.673 and 0.697, respectively, indicating high genetic variation among the accessions. Cluster analysis delineated the accessions into four major groups. The maximum similarity index (0.88) based on Dice coefficient was recorded between AGL-01 and CRS-01 while the least (0.13) was between UGA-02 and EKW 01. The derived data was thus able to determine the extent of molecular variation underlying RAPD size polymorphism. Results obtained from this study proved that RAPD could be successfully used as a molecular tool for diversity study in *M. myristica*. The distributive pattern of genetic variation of *M. myristica* accessions provides important baseline data for conservation and improvement strategies for this species.

Key words: African nutmeg, random amplified polymorphic DNA (RAPD), genetic variation, polymorphic information content (PIC), similarity index, *Monodora myristica.*

INTRODUCTION

Monodora myristica (Gaertn.) Dunal also known as Calabash nutmeg, Jamaican nutmeg or African nutmeg, is a useful but neglected tropical tree of the family Annonaceae. It is perennial and found mainly in the evergreen and deciduous forests of tropical African countries, (GRIN, 1985; Iwu, 1993). The plant can grow up to 35 m in height and 2 m in girth with conspicuous, attractive and scented flowers. The large subspherical fruits contain brown, oval, aromatic seeds (Figure 1) which when ground are used as a spice or condiment in African cuisines, providing a flavour resembling that of nutmeg, *Myristica fragrans* (Celtnet Recipes, 2011). In addition, the bark, seed and leaves are used in treating various ailments in African traditional medicine, (Erukainure et al., 2012).

Genetic diversity plays an important role in the survival and adaptability of a species (Frankham, 2005) and is

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Figure 1. Parts of *Monodora myristica* plant. A = Trunk, B = Leaf, C = Flower, D = Broken pod with seeds, E = Intact pods.

essential for its conservation and management (Razvi et al., 2013). When genetic diversity is low, the species is susceptible to diseases or natural hazards and the entire crop species could easily be wiped out.

Molecular tools have been found to be more useful and accurate in the study of inter-species and intra-species genetic diversity in several plants. Randomly amplified polymorphic DNA (RAPD) markers being technically easy to use and fast, have been more commonly used and successfully too, for determination of inter- and intra-species genetic diversity in plants. Some of these include *Zingiber officinale (*Ashrat et al., 2014), *Andrographis paniculata* (Gosh et al., 2014), Phaseolus *vulgaris (*Razvi et al., 2013), *Ocimum* spp. (Sairkar et al., 2012) *Chrysanthemum* (Martin et al., 2002), *Annona crassiflora* (Cota et al., 2011), *Prosopis* (Goswami and Ranade, 1999), date palm (Corniquel and Mercier, 1994), *Papaya* (Stiles et al., 1993), poplars (Bradshaw et al., 1994) and amaranths (Ranade et al., 1997). However, to date, no such attempt has been reported for *M. myristica.*

M. myristica is listed presently under Kew's difficult seeds due to its inability to grow easily outside its natural habitat (Burkill, 1985; Baskin and Baskin, 1998). The plant is largely harvested from the wild and greatly affected by wild fires, urbanization, reckless and uncontrolled felling of trees for timber and firewood without replanting. As part of our conservation efforts to rescue this important species from extinction, there is need, therefore, to determine the extent of genetic diversity to guide the selection of a core collection in a local gene bank. This information could also be used as a guide for further documentation of available genetic and phenotypic variations towards initiating a breeding program for this underutilized crop species. The present study was carried out with this in mind, and should provide the much needed baseline data or information for further studies.

Table 1. Accession codes and location information for 21 accessions of *M. myristica.*

Name	State	LGA	Latitude	longitude
AKS 02	Akwa Ibom	Ikono	05°09.119'	007°48.483'
CRS 01	Cross River	Calabar municipal	04°57.440'	008°19.775
AGL02	Anambra	Anaocha	6°5'33"	7°2'59"
AGL08	Anambra	Anaocha	6°5'30"	7°2'54"
AGL03	Anambra	Anaocha	6°5'14"	7°2'44"
EKW01	Anambra	Aguata	6°0'56"	7°5'10"
AGL09	Anambra	Anaocha	6°5'21"	7°2'45"
UMN04	Anambra	Aguata	6°2'4"	7°3'1"
AGL07	Anambra	Anaocha	6°5'28"	7°2'54"
UMN01	Anambra	Aguata	6°2'8"	7°3'3"
AGL01	Anambra	Anaocha	n/a	n/a
UGA02	Anambra	Aguata	5°56'37"	7°4'53"
AGL10	Anambra	Anaocha	6°5'28"	7°2'54"
UGA01	Anambra	Aguata	5°56'34"	7°4'50"
UMN02	Anambra	Aguata	6°2'6"	7°2'59"
UMN03	Anambra	Aguata	6°2'2"	7°2'53"
AGL06	Anambra	Anaocha	6°5'28"	7°2'54"
AGL05	Anambra	Anaocha	6°5'28"	7°2'50"
AKS01	Akwa ibom	Ikono	05°09.115'	007°48.478'
AGL04	Anambra	Anaocha	6°5'22"	7°2'55"
AGL11	Anambra	Anaocha	6°5'28"	7°2'53

n/a: not obtained

Table 2. RAPD Primer sequences used for the study.

Primer	Sequence (5' to 3')
OPT14	TCACCTCCTG
OPT07	GGCAGGCTGT
OPH06	ACGCATCGCA
OPT16	GGTGAACGCT
OPT20	GACCAATGCC
OPT13	GAGGAGCATC
OPB17	AGGGAACGAG
OPT04	CACAGAGGGA
OPB12	CCTTGACGCA
OPB11	GTAGACCCGT

MATERIALS AND METHODS

Sample collection

Young, healthy, leaf samples were collected from 21 accessions of *M. myristica* from different locations in South East and South South Nigeria. The accession codes and collection sites are given in Table 1. The collections were preserved in silica gel during transportation to the laboratory.

DNA extraction, PCR amplification and RAPD analysis

All laboratory experiments were carried out at the International Institute of Tropical Agriculture, Ibadan, Nigeria. Total genomic DNA was extracted from 6 g of young leaves per accession using the modified cetyl trimethyl ammonium bromide (CTAB) method, as adopted by Razvi et al. (2013). After extraction, the yield of DNA was measured using a Nanodrop (ND-1000) UV Spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples on 0.8% agarose gel. Polymerase chain reactions (PCR) were carried out in a Peltier thermal cycler (BioRAD DNA engine) using the primers listed in Table 2. The PCR amplifications were performed as follows: 49 cycles of 94°C for 20 s (denaturation), 38°C for 40 s (annealing) and 72°C for 1 min (elongation) followed by a final extension step of 7 min at 72°C. Amplicons were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide and bands were visualised and photographed under a UV trans-illuminator (GDS-8000, Bio-imaging system).

For RAPD analysis, 21 10-mer oligonucleotide primers (Operon Technologies Inc., CA. USA) were tested and ten were selected and used for characterization of genotypes based on their ability to produce reproducible bands. The selected RAPD primers along with their base sequences are presented in Table 2. The amplified bands as seen in the gel pictures were scored. For each primer, the amplified bands were scored as present (1) or absent (0). Summary statistics for the genetic data based on the following parameters: genetic diversity as described by Weir (1996) and polymorphic information content as described by Botstein et al. (1980), were calculated using the Powermarker software version 3.25 (Liu and Muse, 2005).

The similarity indices were determined using the Dice's method (dij= b+c/2a+(b+c) where dij is dissimilarity between units i and j, xi, xj: variable values for units i and j a: number of variables where xi = presence and xj = presence, b: number of variables where xi = presence and xj = absence, c: number of variables where xi = absence

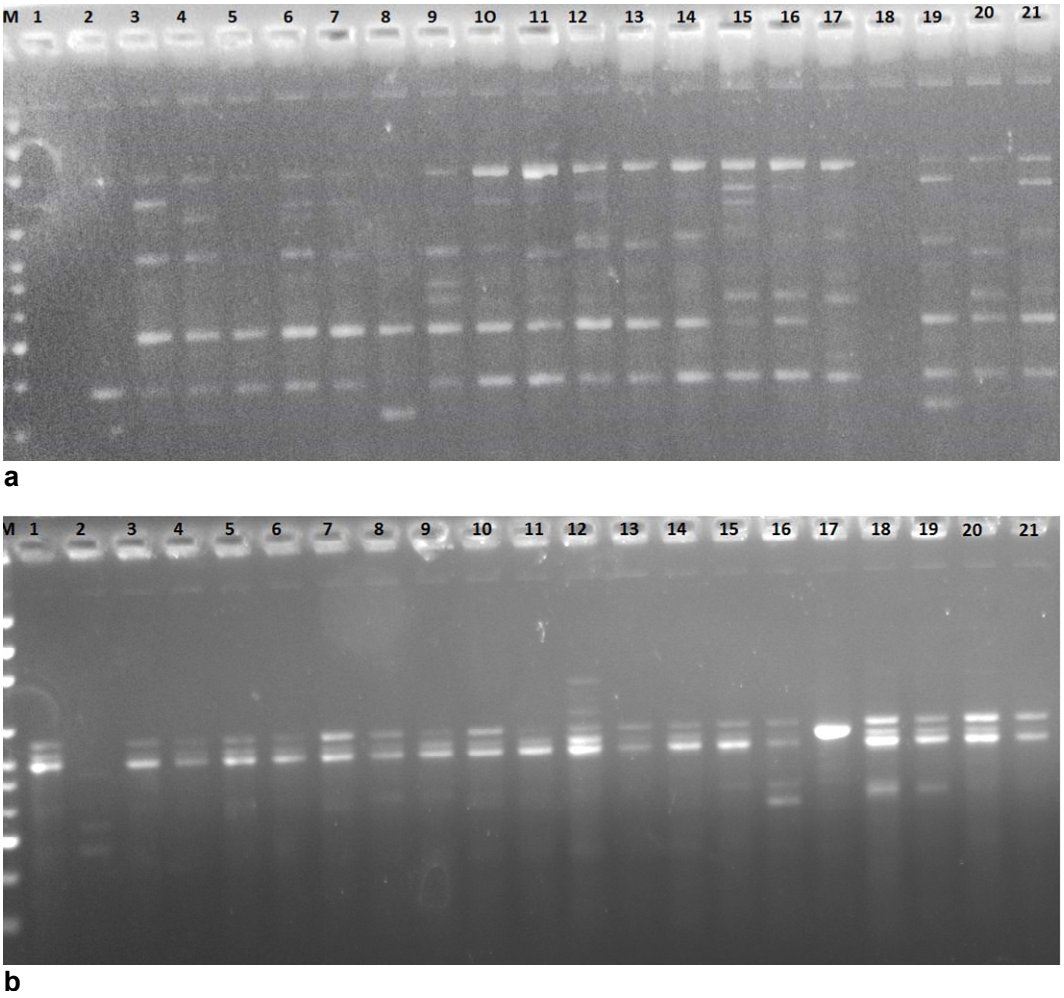

Figure 2. Typical RAPD profiles of 21 accessions of *M. myristica* amplified with a) OPT16 and b)OPH06. M, marker DNA; 1, AKS02; 2, CRS01; 3, AGL02; 4, AGL08; 5, AGL03; 6, EKW01; 7, AGL09; 8, UMN04; 9,AG07; 10, UMN01; 11, AG01; 12, UGA02; 13, AGL10; 14, UGA01; 15, UMN02; 16, UMN03; 17, AGL06; 18, AGL05; 19, AKS01; 20, AGL04; 21, AGL11.

Table 3. Summary statistics for genetic information based on 10 RAPD markers on 21 accessions of *M. myristica*.

Marker	Number of Bands	Gene Diversity	PIC
OPT14	7	0.698	0.683
OPT07	13	0.880	0.869
OPT16	10	0.939	0.935
OPH06	11	0.813	0.791
OPT20	3	0.608	0.562
OPT13	10	0.540	0.525
OPB17	3	0.558	0.481
OPT04	7	0.766	0.746
OPB12	7	0.640	0.617
OPB11	6	0.535	0.517
Mean	7.7	0.698	0.673

PIC, Polymorphic information content.

and xj = presence with the software Darwin 5.0 (Perrier and Jacquemoud-Collet, 2006). Hierarchical cluster analysis using the unweighted pair group method with arithmetical means (UPGMA) was carried out using the Darwin 5.0 software.

RESULTS

Different bands were observed for most accessions, indicating a high level of polymorphism among the species. Typical RAPD profiles for selected primers are shown in Figure 2. The primer OPT07 had the highest number of bands (13) while OPB17 and OPT20 had the lowest with 3 bands each (Table 3). The average similarity index based on Dice's similarity matrix ranged from 0.13 to 0.88 (Table 4) and the genetic differences obtained based on the 10 RAPD markers ranged from 0.540 to 0.938 with an average index of 0.698 (Table 3). The dendogram further delineated the accessions into four clusters with several

Table 4. Similarity indices based on the Dice's method among 21 accessions of *Monodora myristica* in South-eastern Nigeria.

	AKS-2	CRS1	AGL2	AGL8	AGL3	EKW1	AGL9	UMN4	AGL7	UMN1	AGL1	UGA2	AGL10	UGA1	UMN2	UMN3	AGL6	AGL5	AKS1	AGL4
CRS1	0.75																			
AGL2	0.39	0.81																		
AGL8	0.26	0.75	0.31																	
AGL3	0.28	0.73	0.45	0.17																
EKW1	0.35	0.78	0.19	0.21	0.32															
AGL9	0.39	0.80	0.36	0.28	0.29	0.22														
UMN4	0.25	0.76	0.37	0.25	0.26	0.33	0.37													
AGL7	0.41	0.73	0.39	0.37	0.39	0.32	0.39	0.43												
UMN1	0.39	0.80	0.31	0.43	0.44	0.34	0.44	0.45	0.28											
AGL1	0.60	0.88	0.40	0.62	0.62	0.38	0.56	0.64	0.44	0.32										
UGA2	0.42	0.76	0.25	0.33	0.43	0.13	0.30	0.40	0.38	0.39	0.40									
AGL10	0.46	0.68	0.33	0.35	0.37	0.24	0.26	0.49	0.28	0.38	0.46	0.28								
UGA1	0.44	0.64	0.44	0.38	0.35	0.27	0.30	0.46	0.35	0.47	0.54	0.31	0.21							
UMN2	0.44	0.53	0.62	0.44	0.35	0.51	0.41	0.47	0.43	0.48	0.57	0.52	0.43	0.46						
UMN3	0.52	0.61	0.58	0.57	0.50	0.53	0.50	0.55	0.45	0.47	0.40	0.54	0.41	0.53	0.30					
AGL6	0.54	0.86	0.74	0.60	0.64	0.65	0.64	0.68	0.63	0.62	0.72	0.64	0.59	0.67	0.64	0.74				
AGL5	0.41	0.74	0.49	0.45	0.38	0.47	0.38	0.39	0.47	0.35	0.38	0.48	0.49	0.47	0.48	0.38	0.66			
AKS1	0.42	0.81	0.39	0.37	0.39	0.40	0.28	0.35	0.53	0.46	0.55	0.46	0.46	0.49	0.44	0.52	0.60	0.36		
AGL4	0.41	0.68	0.50	0.35	0.31	0.38	0.37	0.44	0.36	0.42	0.42	0.36	0.28	0.37	0.37	0.46	0.59	0.40	0.46	
AGL11	0.33	0.70	0.41	0.27	0.29	0.32	0.23	0.43	0.43	0.45	0.59	0.35	0.19	0.35	0.35	0.41	0.53	0.38	0.33	0.25

sub-clusters, indicating genetic variations among the accessions. Fourteen accessions, namely, AGL11, AGL10, AGL09, UGA01, AGL04, UGA-02, EKW-01, AGL-02, UMN04, AKS02, AGL03, AGL08, AKS01 and AGL05 made up cluster 1 with many sub clusters, while 5 accessions, namely, UMN01, AGL07, AGL01, UMN-03 and UMN-02 fell into cluster 2; cluster 3 had accession AGL-06 only while cluster 4 had CRS 01 which was the most distant accession (Figure 3).

DISCUSSION

The present study was an effort to document available genetic variation in African nutmeg populapopulations in the South East and South South zones of Nigeria. The study is particularly critical as the spice is endangered and difficult to propagate.

The number of fragments observed in this study using RAPD markers was satisfactory for interpretation and conversion into molecular data for the populations studied. According to Ferreira and Grattapaglia (1995), RAPD markers are sensitive and may generate different quantities of amplified fragments depending on the quality and quantity of the DNA used, as well as the amplification conditions.

The mean genetic diversity of 0.698 obtained in the present study was quite high compared to values from other species using similar techniques. An average of 0.31 was reported for *Anonna crassiflora* (Cota et al., 2011). The high values obtained in our study are quite encouraging and conform to that expected for tree species which generally present greater genetic variation within populations (Porth and El-Kassaby, 2014). The genetic similarity indices usually range from zero to one, with values closer to one indicating greater genetic diversity. In the present study it ranged from 0.13 (between UGA-02 and EKW-01) to 0.88 (between AGL-01 and CRS-01), indicating high intra-specific variation in this species. The closest similarity was obtained between accessions UGA-02 and EKW-01 from Aguata Local Government area of Anambra state. However, the both correlation

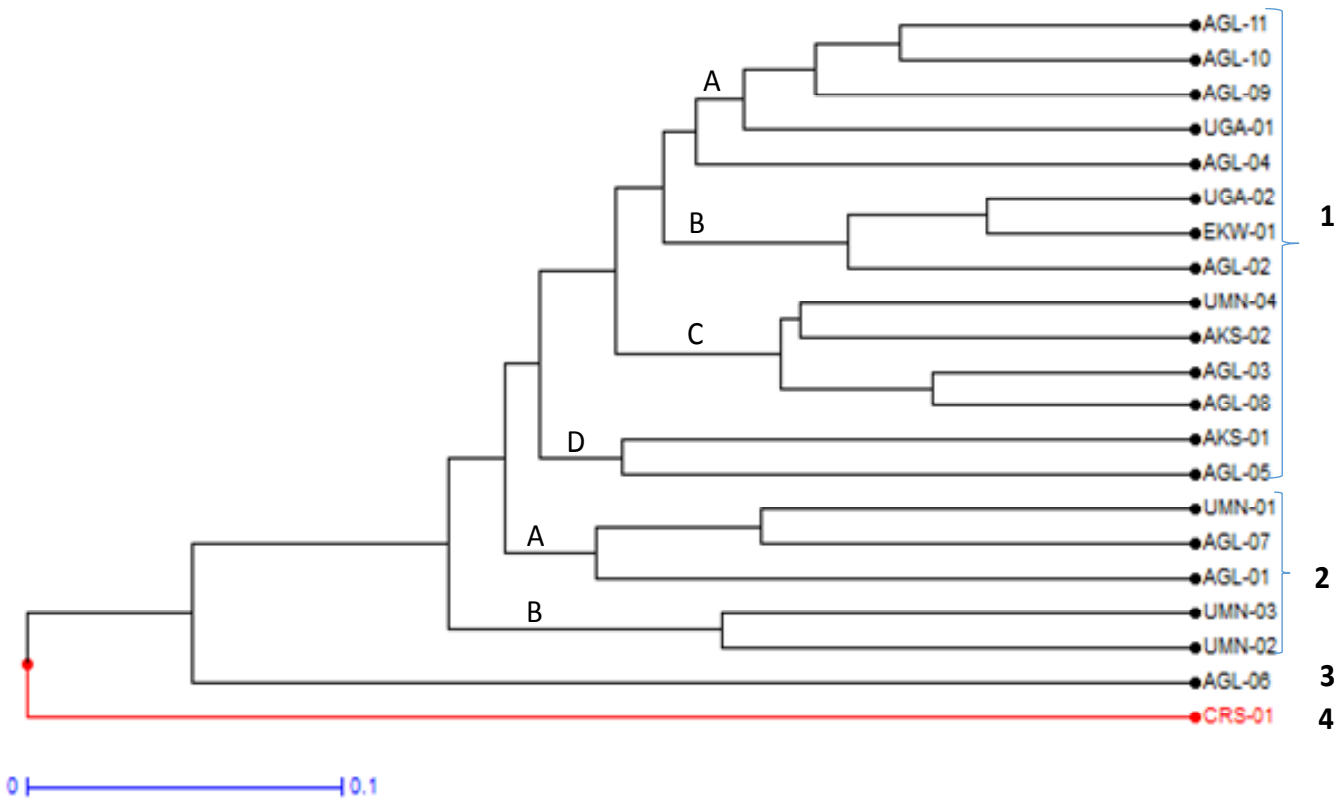

Figure 3. Dendogram based on UPGMA for genetic data in the 21 accessions of *M. myristica.*

between genetic and geographical distances of the accessions did not generally indicate much tendency towards isolation by distance as further illustrated in the clustering pattern, except for CRS-01 (the only accession from Cross River State) which was quite distinct from all the rest. CRS-01 accession was obtained from a coastal region which has an average monthly rainfall of 305.708 mm and total annual rainfall volume of 3668.5mm, which is quite high when compared with the volume of rainfall in other locations. This accession appears to have evolved special adaptations over the years to cope with the higher annual rainfall experienced in this zone, even though such features could not be clearly ascertained from this study. The distribution of the 20 accessions in four different clusters indicates that even though samples were selected from different geographical areas, evolutionary forces such as genetic drift and natural selection pressure remain the major causes of diversity among the accessions. They produce divergent phylogenetic branching which can be recognized because the molecular sequences on which they are based share a common ancestor.

The RAPD-PCR technique has not been used previously in diversity studies on *M. myristica* but has been successfully used for diversity studies in various other species including *Changium symrymioedes* (Fu et al., 2003), *Eremanthus erythropappus* (Freitas et al., 2008), *Ocimum* spp. (Saikar et al., 2012), *Hybiscus* spp. *(*Prasad, 2014) and *Annona crassiflora* (Cota et al., 2011) among others.

The RAPD pattern observed in this study was able to distinguish the minute differences among the accessions. Molecular markers, unlike morpho-agronomic traits, are not influenced by environmental conditions and, therefore, are more reliable tools not only to characterize genotypes, but also to measure genetic relationship more precisely (Razvi et al., 2013).

The present investigation has shown clearly that *M. myristica* is rich in diversity despite reported losses due to urbanization, unsustainable harvests and indiscriminate felling of trees for firewood and timber. Apart from maintaining food and health security, a rich genetic diversity is a basic resource for improvement programs. It also helps the species to withstand different biotic and abiotic stresses under changing environmental conditions (Porth and El-Kassaby, 2014).

The identification and development of primers that can generate reproducible bands in this species is useful for further studies on improvement, taxonomy and conservation. Information provided in this study with the use of RAPD-PCR has shown it clearly to be quite efficient for genetic variability studies in *M. myristica* and probably other related species and biological specimens. The scope can, however, be increased by using more informative and co-dominant markers in further genetic con-

servation efforts to safeguard this highly valued butundomesticated spice tree species from extinction.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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