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Genetic diversity in *Vernonia amygdalina* Delile accessions revealed by random amplified polymorphic DNAs (RAPDs)

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Abstract

The bitter leaf plant (*Vernonia amygdalina* Delile) is an indigenous shrub tree species highly cultivated in West and Central Africa for its nutritional and medicinal values. We used 10 random amplified polymorphic DNA (RAPD) markers to assay 30 accessions of *V. amygdalina* Delile ecotypes (bitter and less bitter leaves, respectively) collected from the southern states of Nigeria (Cross River, Edo, and Oyo). The results obtained showed that RAPD markers were highly polymorphic (98.0%) and generated a total of 29 PCR bands – ranging from two for OPH-03 to 10 for OPB-01 primers. The polymorphic information content was highest for the OPB-01 primer (0.768). Moreover, gene diversity (H_e = 0.800) was high, and cluster analysis delineated the accessions into seven groups, which indicated that a significant genetic diversity was present among the accessions studied. The geographical distinctness observed among the accessions indicated a possible effect of plant isolation by distance and a restricted gene flow. The results obtained in this study showed a genetic variability that could be exploited for varietal delineation and used to improve this indigenous species in Nigeria. This is the first report on the molecular study of genetic diversity in *V. amygdalina.*

Key words: *Vernonia amygdalina*, genetic diversity, RAPD – random amplified polymorphic DNA

Introduction

The bitter leaf plant, *Vernonia amygdalina* Delile, is a member of the genus Vernonia that comprises approximately 1 000 species of forbs and shrubs belonging to the family Asteraceae – the largest of the tribe Vernonieae. It grows mainly in South America and Africa (Izevbigie et al., 2003). *V. amydalina* is highly cultivated in West and Central Africa and can be consumed in various forms. The leaf, stem, and root extracts of the plant are used for various medicinal, herbal, and nutritional purposes (Ijeh and Ejike, 2011).

V. amygdalina is most commonly used in traditional medicine, where leaf decoctions are used to treat fever, malaria, diarrhea, dysentery, hepatitis, and cough, as a laxative, and as a fertility inducer (Ucheck, 2004). The plant has acquired special relevance recently, because it has been shown to possess potent anti-tumorigenic properties in clinical studies (Izevbigie et al., 2004). Pharmacological studies in animals have shown that the leaf extract has both hypoglycemic and hypolipidemic properties and, thus, could potentially be useful in the management of diabetes mellitus (Akah and Okafor, 1992; Ebong et al., 2008). Nutritionally, in the tropics, *V. amygdalina* is used mainly to make soups as well as an appetizer and a febrifuge (Ijeh et al., 1996; Iwu et al., 1996).

Generally called bitter leaf, some variations have been observed in the level of bitterness, ranging from very bitter to less bitter, with the "bitter" type possessing a deep green coloration and a deep bitter taste and the "less bitter" type possessing a fairly light green coloration with little or no bitter taste. Despite the usefulness of various parts of the plant as a remedy against several diseases, to date, there is no information available in the literature with regard to the genetic variation among accessions or different types of *V. amygdalina*. This study was, therefore, conducted with the objective

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Table 1. Thirty *V. amygdalina* accessions used for the study with a description of the ecotype and collection sites in Nigeria

	Sample name	Ecotype	State	Local government area	Latitude [°N]	Longitude [°E]	Alt. [m]	Region
1	VA-1E	bitter	Edo	Oredo	06°44.567	006°07.263	41	south south
2	VA-2E	bitter	Edo	Oredo	06°44.811	006°07.601	55	south south
3	VA-3E	bitter	Edo	Oredo	06°44.507	006°07.201	47	south south
4	VH-4E	less bitter	Edo	Oredo	06°44.470	006°07.201	78	south south
5	VH-5E	less bitter	Edo	Esan west	06°36.340	006°14.070	307	south south
6	VA-6E	bitter	Edo	Esan west	06°36.217	006°14.103	310	south south
7	VA-7E	bitter	Edo	Esan west	06°36.487	006°14.312	318	south south
8	VA-8E	bitter	Edo	Esan west	06°36.587	006°14.261	308	south south
9	VA-9E	bitter	Edo	Esan west	06°44.780	006°07.061	372	south south
10	VH-10E	less bitter	Edo	Esan central	06°44.234	006°13.136	405	south south
11	VA-11E	bitter	Edo	Esan central	06°45.235	006°05.328	373	south south
12	VA-12E	bitter	Edo	Esan central	06°44.701	006°07.103	376	south south
13	VA-13I	bitter	Oyo	Akinyele	06°44.116	005°12.091	48	south west
14	VA-14I	bitter	Oyo	Akinyele	06°44.317	005°12.110	42	south west
15	VH-15I	less bitter	Oyo	Akinyele	06°44.367	005°12.101	40	south west
16	VA-16I	bitter	Oyo	Akinyele	06°44.461	005°12.163	80	south west
17	VH-17I	less bitter	Oyo	Akinyele	06°44.613	005°07.204	87	south west
18	VH-18I	less bitter	Oyo	Akinyele	06°44.473	005°07.211	81	south west
19	VH-19C	less bitter	Cross river	Biase	05°37.940	008°01.062	28	south south
20	VA-20C	bitter	Cross river	Biase	05°37.980	008°01.026	16	south south
21	VA-21C	bitter	Cross river	Biase	05°37.859	008°00.965	23	south south
22	VH-22C	less bitter	Cross river	Biase	05°38.060	008°01.096	21	south south
23	VA-23C	bitter	Cross river	Biase	05°36.915	008°02.798	39	south south
24	VA-24C	bitter	Cross river	Akamkpa	05°24.105	008°13.358	102	south south
25	VA-25C	bitter	Cross river	Akamkpa	05°24.101	008°13.334	98	south south
26	VH-26C	less bitter	Cross river	Akamkpa	05°22.061	008°16.373	89	south south
27	VA-27C	bitter	Cross river	Calabar south	04°57.517	008°20.598	52	south south
28	VA-28C	bitter	Cross river	Calabar south	04°57.598	008°20.413	48	south south
29	VH-29C	less bitter	Cross river	Calabar south	04°57.505	008°20.593	63	south south
30	VA-30C	bitter	Cross river	Calabar south	04°57.403	008°20.512	42	south south

of determining the genetic variation and molecular diversity in *V. amygdalina* types available in Nigeria by using random amplified polymorphic DNA (RAPD) markers.

Materials and methods

Sample collection

Leaf samples of *V. amygdalina* used for this study were collected from three states in the southern part of Nigeria – namely, Cross River (n = 12), Edo (n = 12), and Oyo (n = 6; Table 1). A total of 20 bitter and 10 less bitter types of *V. amygdalina* samples were randomly collected from different locations within a total of eight Local Government Areas (LGA), with due consideration for a representation of the two main ecotypes (bitter and less bitter types) as identified by the locals during collection. The samples were preserved in a fresh state in her-

Table 2. Sequences of Operon RAPD markers used in the study

Primer name	Sequence (5′ to 3′)
OPB-01	GTTTCGCTCC
OPB-14	TCCACTCTAA
OPH-03	AGACGTCCAC
OPH-04	GGAAGTCGCC
OPT-15	GGATGCCACT

metic containers using dry ice before being taken to the laboratory for analyses.

DNA extraction

Young leaves weighing between 100 and 200 mg were ground into a powder using liquid nitrogen, and DNA extraction was done in accordance with a modified Dellaporta protocol (Dellaporta et al., 1983). The extracted DNA samples were quantified using 1.5% agarose gel electrophoresis and spectrophotometry.

Polymerase Chain Reaction (PCR) amplifications with RAPD markers

PCR amplification consisted of 4 µl 50 ng/µl DNA, 2.5 µl 10 × buffer (Bioline, Madison, USA), 1.5 µl 50 mM $MgCl_2$ (Bioline, Madison, USA), 2 µl 2.5 mM dNTPs (Bioline, USA), 0.2 µl 500 U DNA polymerase (Bioline, Madison, USA), and 1 µl 10 pm each RAPD primer (Table 2), and 1 µl DMSO. PCR samples were adjusted to a total volume of 25 µl by adding 12.8 µl DEPC-treated water (Invitrogen Corporation, USA). The PCR cycling profile used for the reaction comprised an initial DNA denaturation step at 94°C for 5 min, 44 cycles of 94°C for 30 s; annealing temperature at 49°C for 1 min, and final extension at 72°C for 1 min, and, finally, an 8-min extension at 72°C was maintained.

Data analysis of RAPD profile

The data matrix of RAPD profiles (Fig. 1) for fragments of similar size from each individual were scored as either the presence (1) or absence (0) of band. Data obtained from scoring the RAPD bands were used for the genetic similarity matrix using Jaccard's similarity coefficient (Jaccard, 1908). The polymorphic information content (PIC) of the markers used was calculated using the method propounded by Botstein and coworkers (1980). The phylogenetic relationship was determined by a cluster analysis using an unweighted pair–group method with arithmetic averages (UPGMA) with the NTSYS-pc software version 5.0.157.

Results

Gene diversity and polymorphism

A total number of 29 bands were detected from the five decamer primers used on the 30 accessions that were assayed (Table 3). The number of amplified bands (Fig. 1) ranged from two for OPH-3 to 10 for OPB-01 primers. The allelic frequency was highest (0.60) for OPB-14 and OPH-14 and lowest for OPH-03 and OPB-01. Gene diversity (H_e) was significantly high among the samples studied, as revealed by OPB-01 (H_e = 0.800), followed by OPH-04 (H_e= 0.720). OPH-03, on the other hand, showed the smallest diversity among the studied samples (H_e = 0.300). The polymorphism information content followed the same trend (Table 3); it was highest in OPB-01 (0.768) and lowest in OPH-03 (0.268). A dendrogram prepared on the basis of results from a cluster analysis (Fig. 2) showed that the accessions studied were grouped into seven main clusters. Cluster 1 consisted of six accessions of bitter and less bitter *V. amygdalina* types, obtained mainly from the southern parts of Edo State in Oriedo LGA. Cluster 2, on the other hand, consisted of accessions of bitter and less bitter *V. amygdalina* plants from the coastal southern part of the Cross River State in Calabar South LGA. Accessions obtained from Biase and Akamkpa LGAs in the humid forest vegetation of Cross River State made up Cluster 3. Cluster 4 consisted of accessions from the three main states and was the most diverse among all seven clusters. Cluster 5 consisted of accessions obtained from Akinyele LGA, Ibadan in Oyo State. Clusters 6 and 7 appeared as out-groups with distinct grouping away from other clusters. All four accessions grouped in these three clusters were obtained from the northerly and higher altitude regions of Edo State in Esan Central and Esan West LGAs.

Discussion

In this study, RAPD analyses provided an insight into the genetic diversity, genetic structure, and distribution of two ecotypes of *V. amygdalina* ("Bitter" and "Less bitter") obtained from three southern states of Nigeria (Cross River, Edo, and Oyo). Previously, random

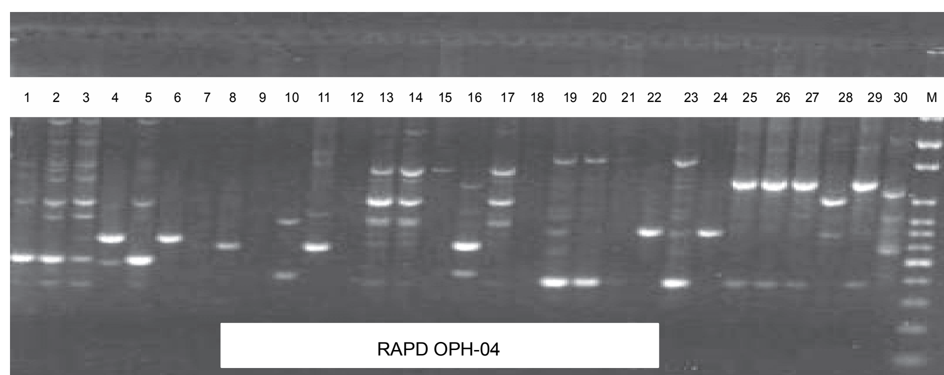

Fig. 1. Amplification patterns from *V. amygdalina* accessions (1–30) using the RAPD OPH-04 primer. *M* is the marker of molecular weight (100-bp DNA ladder and PUC/18 *Sau 3AI-PUC 18/Taq1* digest)

Table 3. List of band frequencies generated through amplification

Marker	Band frequency	Sample size	Number of bands	Gene diversity	PIC
OPB-14	0.600	30	3	0.480	0.4992
OPB-01	0.200	30	10	0.800	0.7680
OPH-14	0.600	30	5	0.560	0.3648
OPH-04	0.400	30	9	0.720	0.6720
OPH-3	0.200	30	2	0.300	0.2680

PIC – polymorphism information content

amplified polymorphic DNA (RAPD) markers were used to determine the extent of the genetic diversity in different plant species, including sweet cherry (Lisek et al., 2005), citrus (Baig et al., 2009), capsicum (Rabelo da Costa et al., 2006), pinus (Monteleone et al., 2006), and even in Leishmania parasites (Mkada-Driss et al., 2014), and provided useful information for the exploitation of available genetic variability. The large number of alleles and the high gene diversity (He = 0.800) observed in this study (Table 3) proved that significant genetic variability occurs among the *V. amygdalina* species grown in Nigeria.

Five of the polymorphic RAPDs decamer primers used in this study (Table 2) were found useful for the delineation of accessions collected from different parts of the country. These observations are similar to those reported by Lisek and coworkers (2005) wherein, by using six primers, it was possible to distinguish all cultivars of the sweet cherry studied. Similarly, Hsiang and coworkers (2000) have successfully used seven RAPD primers to distinguish among juniper and cedar cultivars. All markers used in this study were highly polymorphic (98.0% polymorphic bands), similarly to the report of Shafie and coworkers (2009) who detected higher polymorphic bands (95.60%) in RAPD markers than in inter-simple sequence repeat (66.67%) markers in *Artemisia capillaris.*

The seven clusters created during the cluster analysis (Fig. 2) showed that plants within the same location were generally distinct from those from other locations, even within the same state. Samples within the same local government areas were generally less diverse, with the exception of a few cases, as was found for materials from Edo State. It is intriguing to find that, within the same state of Cross River, *Vernonia* accessions obtained from Biase and Akamkpa LGAs were classified in Cluster 3 and were distinct from the tested accessions from Calabar South that were classified under Cluster 2. A similar trend, and in fact a greater genetic diversity, was observed among *Vernonia* accessions collected from Edo State. All accessions obtained from Oriedo LGA were clustered in Cluster 1 and were distinct from those

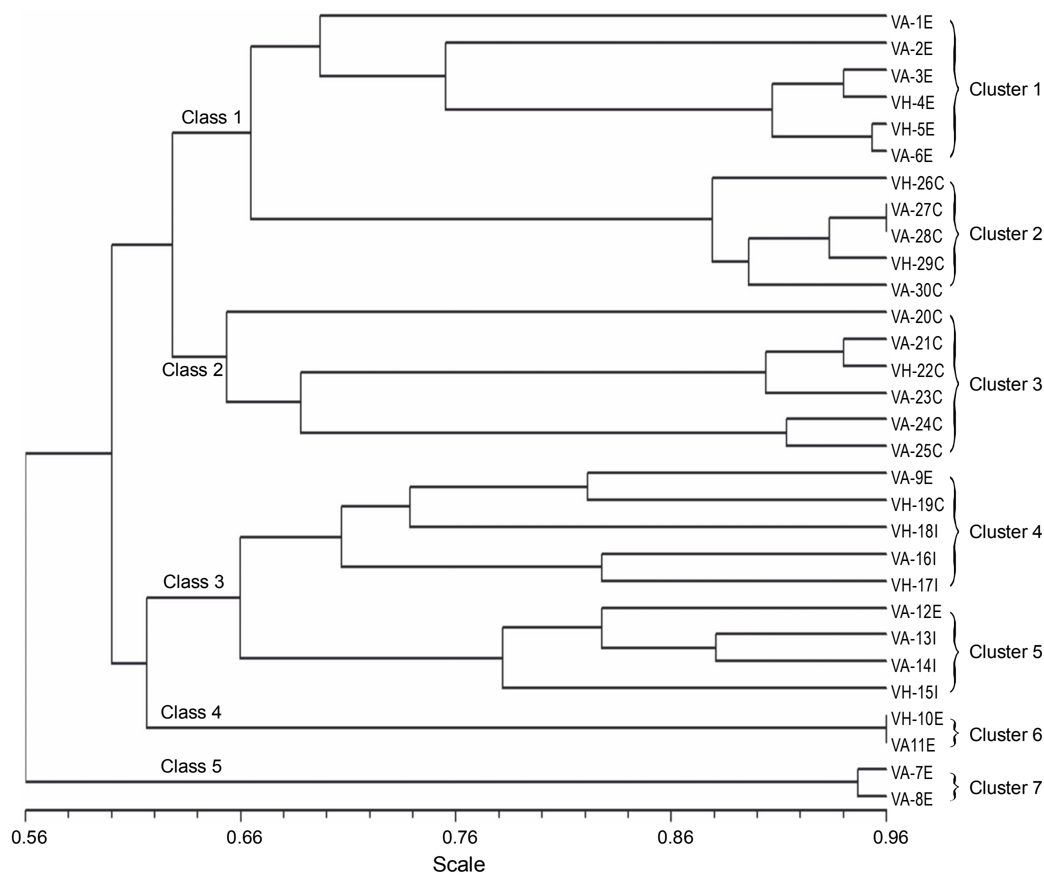

Fig. 2. Dendrogram showing the relationships between *V. amygdalina* types (bitter denoted as VA) and (less bitter denoted as VH)

collected from Esan Central and Esan West LGAs grouped in clusters 6 and 7. The geographical differentiation observed among *Vernonia* accessions in this study indicates that plant isolation by distance (IBD) – which may be due to the Founder effect as a result of a genetic drift – as well as a local adaptation could possibly explain the diversity observed in the investigated species. The Founder effect is the loss of genetic variation due to the establishment of a new population by a very small number of individuals from the original population; the gene and genotype frequencies of a new population may not represent these aspects of the original population (Provine, 2004). This observation is not surprising as *Vernonia* spp. is more often propagated vegetatively through the exchange of stem cuttings than via traditional methods. On the other hand, seeds are usually dispersed by wind over a restricted distance within each location. This implies that the genetic diversity could be narrow within the same geographical area, as only a few individuals could explain the diversity observed.

Some accessions, however, from different geographical locations sharing similar genetic profiles were observed and grouped into Cluster 4. Moreover, it was observed that there was no differentiation between bitter and less bitter ecotypes among accessions from the same location. This indicates that, although these plants may have different phytochemical constitution, the RAPDs decamer primers used in this study did not show any significant differentiation among accessions on the basis of the phytochemical constitution. This could be due to the dominant nature of RAPD markers and a random restriction of the DNA fragments, which do not differentiate between homozygous and heterozygotes markers, thereby masking the possible heterozygous nature of this trait.

Conclusions

This study – the first report of molecular characterization among *Vernonia amygdalina* accessions – showed that a significant genetic diversity is present within this

species. The geographical differentiation observed between the plants even within the same state showed the presence of an enormous amount of genetic variability that can be exploited for the genetic improvement of this highly valued medicinal and nutritious traditional plant. We, therefore, suggest that the genetic diversity observed among *Vernonia* accessions studied was attributable to plant isolation by distance – secondary to geographical delimitation rather than phytochemical variation. Furthermore, we showed that the use of the RAPD marker system is an effective tool to evaluate genetic diversity and phylogenetic differences among *V. amygdalina* accessions. These results, therefore, provide a basis for further evaluation and exploitation of the available genetic diversity by using other marker systems such as the co-dominant microsatellites (SSRs), single-nucleotide polymorphisms (SNPs), and a sequence analysis to unravel greater genetic information and locate important mutations that could have been responsible for some of the observed phenotypic differences such as the intensities of bitterness.

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