

Genetic Assessment of the Shrub *Syncepalum dulcificum* (SCHUMACH & THONN.) Daniell in Nigeria Using the Randomly Amplified Polymorphic DNA (RAPD)

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To cite this article:

Iloh Andrew Chibuzor, Orosun Bukola, Akinyele Olukemi Adejoke, Onyenekwe Paul Chidozie. Genetic Assessment of the Shrub *Syncepalum dulcificum* (SCHUMACH & THONN.) Daniell in Nigeria Using the Randomly Amplified Polymorphic DNA (RAPD). *International Journal of Genetics and Genomics*. Vol. 4, No. 6, 2016, pp. 45-50. doi: 10.11648/j.ijgg.20160406.11

Received: December 27, 2016; **Accepted:** January 7, 2017; **Published:** January 24, 2017

Abstract: In order to advocate for informed management decisions with regards to endangered species, we assessed genetic diversity and genetic structure in 40 individuals of six natural populations of the shrub *Syncepalum dulcificum* (SCHUMACH & THONN.) DANIELL growing in South Western Nigeria. Twelve (12) Random Amplified Polymorphic DNA (RAPD) primers were tested on total genomic DNA extracted from silica gel dried leaves. Bands were then scored for reproducibility and scoring error calculated. Several genetic diversity parameters were then tested using the POPGENE v1.32 software. Five (5) primers produced 227 reproducible and clear RAPD bands of which 47 were polymorphic (20.7%). The percentage of polymorphic loci (PPI) within populations ranged from 36% to 68%. Nei's gene diversity among population (Hs) was 0.03, while at species level (Ht) was 0.18. The coefficient of gene differentiation (Gst) among populations was estimated to be 0.83 with a gene flow rate (Nm) of 2.49 showing high genetic diversity within and among populations. The results however indicate a high similarity between the populations as well as close genetic relationship among them. We infer that *S. dulcificum* in Nigeria does not represent a genetically diverse population and this may be accounted for due the plant's its breeding system which is mainly autogamous. This study further suggests an *in-situ* form of conservation be set up as immediate rescue conservation procedure for the plant.

Keywords: DNA Markers, Genetic Diversity, RAPD, *Syncepalum dulcificum*

1. Introduction

When envisaging the effects of environmental effects on plant biodiversity, there is a need to consider understanding the diversity and population genetic structure of threatened biodiversity. It is therefore important to understand genetic variation within and between populations, as it could help in proper conservation planning and execution. Population genetics typically assess neutral genetic variation and provide estimates of genetic diversity and partitioning among a spatial hierarchy [1]. Studies in population genetics can also

estimate both contemporary and historic gene flow, and thereby aid in elucidating past intra-species relationships and distributions as it had been affected by climatic conditions [2]. Such genetic data, coupled with palaeo-ecological data can provide inferences to historical changes in plant biogeography and postglacial expansion/contraction [3], [4], [5]. Furthermore, low genetic diversity may reduce the potential of species or populations to survive in a changing environment [6]. Thus, it becomes imperative to take effective measures to protect these species against further loss especially in our changing world.

Determining the extant and pattern of genetic variation in

rare plants will promote more informed management decisions with regard to species maintenance, population augmentation, and potential translocation. Research may identify unique variants that warrant greater protection; conversely, research may identify populations sufficiently homogeneous (e.g., clonal) that reductions in population size will not impact viability. Finally, evaluating the risks associated with hybridization in rare plant populations will lead to a greater awareness of how to effectively manage these species in ecological zones that include widespread congeners [6].

Syncepalum dulcificum commonly known as miracle fruits/berry is a shrub of the family Sapotaceae [7]. The fruit is native to tropical West and Central Africa: Cameroon, Congo, Gabon, Zaire, Benin, Ghana and Nigeria. Opeke, [8] reported that it is commonly found growing in the wild in fringes of virgin forest as well as naturally in farms and secondary bushes. Achigan-Dako [9] describes the plant as a very promising species but unfortunately poorly documented resource. However, the species is well known for its taste modifying property and this has been traditionally exploited for centuries in Africa. For instance, in Benin republic, the fruit is eaten to aid consumption of tart or sour foods by the locals [9]. The utilization of the taste modifying property of the pulp and pigments in the food industry as well as its medicinal properties and use reported in the treatment of diabetes, malaria, hyperthermia; and enuresis whereas the almond is used to treat stomach ache, anaemia, and obesity [10].

Sadly, the knowledge on genetic improvement potential *S. Dulcificum* is still scanty; As the knowledge on the reproductive biology and mating system is still narrow [9]. Thus, the lack of knowledge of its distribution, genetic variation, will play a significant constraint for conservation programmes. In addition to this, short-lived or recalcitrant seeds often pose great challenges to the plants survival and existence. Hence, to fully understand these potential problems, estimates on the genetic diversity of populations are necessary to discern any problems and stimulate

measures, such as cultivation, agro-forestry practices, conservation efforts, and population restoration in the wild [11].

Studies on genetic diversity within populations have been simplified by the introduction of molecular analysis techniques, such as randomly amplified polymorphic DNA (RAPD) analysis [12], [13]. This fingerprinting technique can be used for determining genetic relationships of various species [14], [15], [16]. In addition, RAPD analyses are efficient, economical and tend to produce genetic markers suited to the assessment of population, race and species-specific genetic variation [17]. As molecular studies are fast becoming a tool in forest genetic diversity studies, we report the Randomly Amplified Polymorphic DNA (RAPD) assessment of the tree species: *Syncepalum dulcificum* in Nigeria. Our study hopes to quantify the genetic variation within and between populations (ii) to analyse the geographic distribution of diversity in the natural range, and (iii) to define a conservation strategy based on diversity patterns of the species.

2. Materials and Methods

2.1. Plant Material Collection

Fresh leaf tissues of *Syncepalum dulcificum* were collected from six locations (Ijebu-Ode, Forest Resources Management University of Ibadan botanical garden, Cocoa Research Institute of Nigeria (CRIN), National Centre for Genetic Resources and Biotechnology, Alonge village (Onigambari Forest Reserve) all in the South-western part of Nigeria. In total, 40 individuals representing six populations were investigated; with 2-4 individuals per population (Table 1). At least one herbarium specimen was prepared from each locality. Herbarium specimens are deposited in the University of Ibadan Herbarium. The coordinates for the field-collected material were obtained using a handheld GPS unit, and for all kinds of the geographical presentation, ArcView-ArcGIS v10.1 (ESRI, USA) was used.

Table 1. Sample collection, location and geo-references of *Syncepalum dulcificum* across South West Nigeria.

Location	Sample Name	Latitude	Longitude	Elevation (m)
Ijebu-Ode	A-E	003°56.826'N	06°50.972'E	111.9
	F	003°56.917'N	06°51.043'E	110.4
	G	003°56.931'N	06°51.057'E	109.4
	H	003°56.930'N	06°51.058'E	112.0
	I	003°56.939'N	06°51.066'E	110
	J	003°56.936'N	06°51.062'E	111.2
Forest Resources Management University of Ibadan Botanical garden	A	003°53.832'N	07°26.966'E	181
	B-H	003°53.779'N	07°27.464'E	205
	I-J	003°53.773'N	07°27.476'E	205
Forestry Research Institute of Nigeria (FRIN)	A-B	003°51.742'N	07°23.603'E	209
	C	003°51.757'N	07°23.608'E	209
	A	003°51.100'N	07°12.457'E	92
Cocoa Research Institute of Nigeria (CRIN) National Centre for Genetic Resources and Biotechnology	A-F	003°50.293'N	07°23.189'E	195
	G-J	003°50.296'N	07°23.188'E	195
	A-B	003°52.851'N	07°10.626'E	174
Alonge village (Onigambari Forest Reserve)	C-D	003°52.836'N	07°10.634'E	176

2.2. DNA Isolation

Total genomic DNA was extracted from silica gel dried leaf tissue. Extraction of total genomic DNA followed the CTAB procedure of Doyle and Doyle, [18] with the following modifications: 700 μ l of CTAB buffer were used for initial incubation, 500 μ l of isopropanol were used for DNA precipitation with two subsequent washing steps using 100 μ l of 70% ethanol each. Finally, DNA was dissolved in 200 μ l 1x TE including 2 μ l RNase (10mg·ml⁻¹).

2.3. Randomly Amplified Polymorphic DNA (RAPD) Analysis

Twelve (12) RAPD primers (Eurofins MWG Operon, Germany) were pre-screened in three replicates in order to select the best markers for further RAPD analysis of *S. dulcificum*. RAPD band reproducibility and scoring error were evaluated using the methods as described by Skroch and Nienhuis [19]. The final Polymerase chain reaction (PCR) protocol for the test samples was carried out in a volume of 28 μ l containing 20 μ l Dream master mix (Thermo Scientific, Lithuania) containing 10x buffer [10 Mm TrisHCl], 2 mM MgCl₂, 500 μ M deoxynucleotide triphosphates (dNTPs), 0.5 units of Taq DNA polymerase, 1 μ l bovine serum albumin (BSA) (Thermo Scientific, Lithuania), 2 μ l primer and 5 μ l of total genomic DNA was also added to the PCR cocktail. This screening was repeated three times to check reproducibility of the primer sets.

Modifying the methods of Qadir et al. [20], PCR reactions was performed on Peltier- based Thermal Cycler (LAB-KITS, Hong Kong.), with initial denaturation of 3 min at 94°C followed by 40 cycles of denaturation at 94°C for 1min, annealing at 40 °C for 1 min and extension at 72°C for 1 min, followed by a final extension for 10 min at 72°C.

The amplified products were separated in 1% agarose gel electrophoresis using 1X TBE buffer stained with GR Green and Visualized on gel documentation system (Flour Shot LAB-KITS, Hong Kong). The molecular size of the amplification products was estimated using 1kb DNA ladder (Biolabs, New England). The number of monomorphic bands, number of polymorphic bands and intensity of bands were recorded.

3. Data Analysis

To create a binary matrix, amplified fragments of 500-3000bp were scored visually as having present (1), or absent (0) peaks in the output traces. Only distinct peaks were scored as present, and the manual scoring procedure was repeated many times on separate occasions to reduce scoring errors. The similarity index was calculated from the data using the Nei similarity index coefficient [21]. Taking the Hardy-Weinberg (HW) principle to be at equilibrium, all genetic diversity parameters were calculated using the POPGENE v1.32 software [22].

4. Results

4.1. RAPD Marker Selection

RAPD was performed in triplicate using genomic DNA to test the reproducibility by checking the pattern of RAPD profiles of each sample. Five primers, OPA-02, OPD-01, OPJ-02, OPL-01 and OPJ-01 (Table 2) produced clear and reproducible polymorphic fragments in all samples, and all of the primers detected significant polymorphisms in the genomic DNA analysis.

Table 2. List of RAPD Primers and their sequences used for the reactions.

S/N	Primer	Sequences 5'-3'
1	OPA 02	TGCCGAGCTG
2	OPD 01	ACCGCGAAGG
3	OPJ 02	CCCGTTGGGA
4	OPL 01	GGCATGACCT
5	OPJ 01	CCCGGCATAA

4.2. Diversity Estimates

Nei's genetic variation statistics for all loci within population is represented in Table 3. Gene diversity (h) ranges from 0.12 to 0.18 Shannon's information index (I) also ranged between 0.19 to 0.28. In ranking, genetic diversity is rated as follows; 0.12, 0.15, 0.16 and 0.18. Percentage polymorphic loci (PPI) within populations ranged from 36 to 68. Nei's gene diversity among all populations (Hs) were 0.03 while at species level (Ht) it was 0.18.

Table 3. Nei's Generic Variation Statistics for All Loci per population of *S. dulcificum* across South West Nigeria.

S/N	Population	Sample Size	na*	ne*	h*	I*	No of PPI	% PPI
1	Alonge	4	1.40±0.50	1.25±0.33	0.15±0.19	0.22±0.28	10	40
2	CRIN	3	1.36±0.49	1.20±0.30	0.12±0.17	0.19±0.26	9	36
3	FRIN	3	1.48±0.51	1.31±0.38	0.18±0.20	0.27±0.30	12	48
4	IJEBUODE	10	1.60±0.50	1.25±0.32	0.15±0.17	0.25±0.25	15	60
5	NACGRAB	10	1.68±0.48	1.28±0.33	0.18±0.18	0.28±0.25	17	68
6	UI	10	1.52±0.51	1.25±0.30	0.16±0.18	0.24±0.26	13	52

*na: Observed number of alleles. * ne: Effective number of alleles. * h: gene diversity. * I: Shannon's Information index. PPI: Polymorphic loci.

4.3. Genetic Diversity Among Population

Nei's gene diversity among all population (Hs) 0.03 while at species level (Ht) 0.18 is represented in Table 4. The coefficient

of gene differentiation (G_{st}) among populations was also estimated as 0.83 with a gene flow (N_m) estimate of 2.49.

Table 4. Nei's Analysis of Gene Diversity among populations of *S. dulcificum* across South West Nigeria.

Sample size	Ht	Hs	G_{st}	N_m^*	No of polymorphic loci	% polymorphic loci
40	0.18	0.03	0.83	2.49	20	80

* N_m = estimate of gene flow from G_{st} E.g., $N_m = 0.5(1 - G_{st})/G_{st}$.

4.4. Genetic Divergence

Table 5 shows Nei's genetic distance (D) measured from Nei's unbiased measures of genetic identity and genetic distance. Results show that the highest genetic identity/distance was seen between FRIN and CRIN respectively. The lowest genetic identity/distance was seen between CRIN and Alonge.

Table 5. Nei's Original Measures of Genetic Identity (above diagonal) and Genetic distance (below diagonal) of *S. dulcificum* across South West Nigeria.

Pop ID	Alonge	CRIN	FRIN	IJEBUODE	NACGRAB	UI
Alonge	****	0.9907	0.9292	0.9695	0.9280	0.9588
CRIN	0.0094	****	0.9184	0.9725	0.9280	0.9534
FRIN	0.0734	0.0851	****	0.9571	0.9389	0.9652
IJEBUODE	0.0309	0.0279	0.0439	****	0.9730	0.9800
NACGRAB	0.0747	0.0748	0.0631	0.0274	****	0.9691
UI	0.0421	0.0478	0.0354	0.0202	0.0313	****

5. Discussion

Kelley, [23] reports that information about genetic variation within an endangered plant species, such as *S. dulcificum*, can assist in conservation efforts for that species and provide future breeders and researchers with additional data to support their work. Genetic variation study carried out on *S. dulcificum* is necessary and timely as the species has been reported to be endangered in the wild [24] while cultivation is somewhat problematic [25], [26]. Even though the plant is of high economic and medicinal interest [27], [28].

RAPD analysis has been successfully used for taxonomic and systematic classification, and phylogenetic or genetic diversity studies in plants [29]. The advantages of this technique as reported by Mahmood *et al.* [30] includes its speed or rapidity, simplicity as well as the requirement for only small amounts of DNA. Lack for any prior genetic information as well as consistency patterns irrespective of the plant source or age are some other advantages [30].

Although RAPD markers have few disadvantages, some of the problems regarding the reproducibility of RAPD data and scoring errors have been addressed [19]. As the standardization of DNA isolation techniques and PCR reaction conditions are keys in limiting errors, the use of intense staining, clearly resolvable bands and unvaried DNA samples have been shown to provide consistent results [31].

In the current RAPD study, all six populations were similar to each other based on the same band patterns that were obtained after RAPD amplification. These matching bands of similar patterns suggest either, that they originated from a common source, or that the technique was not able to detect variation. The RAPD technique is rarely able to discriminate between very closely related genotypes [32], and this could be the case in this study for *S. dulcificum*. A general

relationship between autogamous and level of heterozygosity can be attributed to low levels of genetic variation in autogamous (self- pollinated) plants where according to Hamrick [33], are cases of limited movement of genes through pollen, which then results to less differentiation among population.

Genetic diversity within population range between 0.12 - 0.18, such a close range in value suggests that *S. dulcificum* germplasm collection does not represent genetically diverse population. This study agrees with the findings of Achigan-Dako *et al.* [9] which states that one of the major contributory factors to the immediate degree of polymorphism seen in some plants might be due to some level of autogamous (selfing) breeding system

As reported by Barrett *et al.* [34], *S. dulcificum* practice high levels of selfing and thus might have smaller populations because selfing enables individuals to start colonies following dispersal, or to reproduce under conditions of low density. Predominant selfing has a profound effect on the genome, and autogamous species can provide important model systems for investigating the evolutionary consequences of restricted recombination [34].

Igic and Busch, [35] as well as Wright *et al.* [36] have also reported high rates of selfing to reduces heterozygosity levels, restrict recombination and multiple factors can contribute to reduced diversity in selfing populations, and distinguishing their relative contributions to overall reductions in diversity. Selfing lowers the effective population size up to two folds due to a reduction in the number of effective alleles in a population, this is very possible under neutrality [34]. Reduction in population could also be due to the life span of highly autogamous species which in most cases are short-lived and occupy unpredictable environments [34] hence making their populations more likely to be susceptible to environmental catastrophes leading to local extinctions. Such demographic factors associated

with small population size may, in part, contribute to higher extinction rates for *S. dulcificum* where low population was observed during collection. Also noticed was the level of gene flow estimate value (Nm) to be 2.49 was high and this according to Morjan and Rieseberg, [37] noted to be attributed to plants with some form of selfing breeding system. It's reported that plants species plant species with such breeding system have considerably lower migration rates than predominantly out-crossers.

6. Conclusion

The current work elucidates the usefulness of molecular markers in establishing distinct relationships within and among population where phenotypic expression using morphological methods alone may not be able to determine.

As *Synsepalum dulcificum* plant is been reported endangered, this study has been able to report the role which its autogamous breeding system may have played in the survival of the plant. Conscious effort needs to be put in place to study more on the reproductive biology and mating systems. There is also a need to improve plant survival via propagation i.e. domestication through controlled cross pollination which will help in exchange of gene between plus trees that might have been identified (improved breeding). Our study also recommends an *in-situ* form of conservation be set up as immediate rescue conservation procedure for the plant.

Acknowledgements

We are grateful to the Sheda Science and Technology Complex, Abuja Nigeria for providing the student fellowship to Orosun, Bukola in the Biodiversity and Climate Research Laboratory, Biotechnology Advanced Research Centre (BARC) under the West Africa Agricultural Research Programme (WAAPP) CARGS grant to Dr. Andrew Chibuzor ILOH. We are particularly thankful Miss Ogechi Nweke (Lab manger/Research Fellow) and Mr. Charles Osuji (Senior Lab Technologist) for the lab support.

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