Molecular Diversity Analysis of Some Ile-Ife Moss Species Expressed by Random Amplified Polymorphic DNA

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Abstract- Molecular studies using Random Amplified Polymorphic DNA profiling was carried out on some moss species from Ile-Ife, Nigeria in order to determine their genetic variation and species relationships. The result obtained revealed that the chloroplast DNA isolated using the SDS method produced higher yields though lesser quality, while those isolated using the Kit method produced lesser yield but higher quality. All the seven primers employed in this study showed RAPD fragments with varying bands ranging from 200 Bp to 3000 Bp. Out of a total of 55 individual fragments obtained from the seven primers used, 27 bands were polymorphic while 28 were species specific (unique). Monomorphic bands were absent. The values of the pair-wise comparison of the genetic distance (GDMR) and the percentage genetic variation of the moss species studied showed that they were genetically distinct from each other. The UPGMA phenogram of the RAPD data separated the moss species studied into seven main groups at 0.6 level of similarity. From the findings made, it was concluded that the moss species studied were quite distinct and distant genetically therefore there was unlikelihood of gene flow across them.

I. INTRODUCTION

Molecular tools have been very rudimental to the understanding of plant evolution and relationships at all levels of taxonomy within the past two decades (Stech and Quandt, 2010). Random Amplification of Polymorphic DNA (RAPD) profiling has been one of the most commonly used molecular techniques and it is a type of Polymerase Chain Reaction (PCR) analysis whereby DNA segments are amplified at random (Kumar and Gurusubramanian, 2011).

RAPD has been useful in the study of genetic diversity/polymorphism, germplasm characterization, genetic structure of populations, detection of somaclonal variation, cultivar identification, hybrid purity, genome mapping, developing genetic markers linked to a trait in question, population and evolutionary genetics, plant and animal breeding, animal-plant-microbe interactions, pesticide/herbicide resistance, animal behavior study, and forensic studies (Stenoien and Sastad, 1999; Kumar and Gurusubramanian, 2011; Alam *et al.*, 2012). It has also been used by many researchers in studying the population structure and genetic diversity of moss species in many parts of the world (Stenoien and Sastad, 1999; Clarke *et al*., 2009; Alam *et al*., 2012).

Mosses constitute a group in the division bryophyte (Buck and Goffinet, 2000; Adebiyi *et al*., 2012; Oyesiku, 2012). They are non-vascular plants that occur over a wide range of habitats such as rocks, soil, logs, tree trunks and concrete walls. They play significant ecological role in the ecosystem (Saxena and Harrinder, 2004) as they play very important role in soil formation, plant succession, as well as regulate the soil moisture (Renzaglia and Vaughn, 2000; Alam *et al*., 2012; Aline *et al*., 2012, Oyesiku, 2012).

In Nigeria, studies had focused on description and provision of taxonomic keys of some mosses (Egunyomi, 1984; Akande, 1992), provision of checklist of bryophyte species from defined habitats (Egunyomi, 1979, 1980, 1981, 1984; Makinde and Odu, 1993), phytochemical properties, antimicrobial and insecticidal activities of mosses (Ande *et al*., 2010; Adebiyi *et al*., 2012; Adebiyi *et al*., 2013; Femi-Adepoju *et al*., 2014; Tedela *et al*., 2014), their effectiveness as bio-indicators in the monitoring of environmental pollution (Batagarawa and Lawal, 2010; Ekpo *et al*., 2012; Ite *et al*., 2014) and assessment of genetic diversity within accessions (Bolaji *et al.*, 2018). Bolaji and Faluyi (2017) had also reported the chromosome numbers of some Nigerian moss species.

There is an acute dearth of information on the species relationships of the bryophyte species from Nigeria. This study seeks to take advantage of the molecular tool (Random Amplified Polymorphic DNA profiling), in elucidating the taxonomic status and genetic relationship of the bryophyte species in Nigeria.

II. MATERIALS AND METHODS

Molecular studies using Random Amplified Polymorphic DNA profiling was carried out to determine the genetic variation among seven moss species following the method of Alam *et al.* (2012). The moss species selected for this study were *Hyophila crenulata* C, Mull. Ex Dus, *Calymperes afzelii* Sw., *Bryum coronatum* Schwaegr, *Thuidium gratum* (P. Beauv) Jaeg., *Archidium ohioense* Schmp ex. C. Mull, *Barbula lambarenensis* P. Vard. and *Stereophyllum nitense* Mitt. Samples were collected from various locations in Ile-Ife, Nigeria (Table 1), during the raining seasons (between April and November) while the plants were in full bloom and in their optimal conditions. Samples

collected were identified at the herbarium of the University of Ibadan, Nigeria.

Extraction of Genomic DNA and Determination of DNA Quantity and Purity

The total genomic DNA was isolated from fresh leaf samples of the moss species using SDS method (Dellaporta *et al*., 1983) and also QIAamp DNA Mini Kit (250) cat no. 51306 according to the manufacturer's protocol. All DNA preparations were tested for quality (purity) and quantity (yield), using Nanadrop 2000 spectrophotometer. A DNA preparation was considered to be good if it had A 260 nm/ A 280 nm ratio as approximately 1.8. Sterile water was used as blank.

Table 1: Materials Used in the Study

Moss species	Collector/Source/Location	Habit	Habitat Description
Hyophila crenulata	Bolaji; Ile-Ife, 7º31.45′N; 4º31.46′E, Nigeria 7º31.48′N; 4º31.46′E, Nigeria	Acrocarpous	On concrete walls under tree shades
Calymperes afzelii	Bolaji; Ile-Ife, 7º31.5′N; 4º31.33′E, Nigeria	Acrocarpous	On tree bark around the base of tree (*Terminalia catapa, Funtumia elastic, Albizia zygia* and *Cola millenii*)
Bryum coronatum	Bolaji; Ile-Ife, 7º31.5′N; 4º31.34′E, Nigeria	Acrocarpous	On concrete slabs along a pathway under shade
Thuidium gratum	Bolaji; Ile-Ife, 7º31.25′N; 4º31.31′E, Nigeria 7º31.45′N; 4º31.48′E, Nigeria 7º31.25′N; 4º31.31′E, Nigeria 7º31.46′N; 4º31.48′E, Nigeria	Pleurocarpous	On tree bark around the base of tree (*Milicia excelsia, Dalbergi spp, Steculia tragacantha, Funtumia elastica* and *Celtic zenkeri*)
Archidium ohioense	Bolaji; Ile-Ife, 7º31.47′N; 4º31.41′E, Nigeria	Acrocarpous	On rock surface at the top of an open hill
Barbula lambarenensis	Bolaji; Ile-Ife, 7º31.46′N; 4º31.48′E, Nigeria	Acrocarpous	On concrete walls
Stereophyllum intense	Bolaji; Ile-Ife, 7º31.26′N; 4º31.31′E, Nigeria 7º31.9′N; 4º31.39′E, Nigeria 7º31.46′N;4º31.48′E, Nigeria	Pleurocarpous	On tree bark around the base of a tree (*Acacia* spp., *Funtumia elastic, Dalbergia spp* and *Lagerstomia indica*)

Checking the DNA Quality and Integrity by Agarose Gel Electrophoresis

1 % agarose gel was prepared by adding 1 g agarose to 100 ml of 0.5X TBE buffer in a conical flask. The mixture was heated on a microwave, swirling the conical flask, until the agarose dissolves completely. The mixture was left to cool down to 55 – 60 °C. 5 µl ethidium bromide was added and mixed well. The solution was poured into the gel tray and allowed to solidify for about 30 minutes at room temperature. Agarose gel electrophoresis of the isolated genomic DNA was carried out by loading 3 µl of the DNA and 3 µl of the loading dye into the gel wells and running at 80 V for about 40 min. The DNA bands were visualized on the UV trans-illuminator. The profile was photographed using Kodak Gel Documentation system (Model EDAS 290).

RAPD-PCR Reactions

A total of 7 random decamer primers were employed in the experiments for genome screening. The polymerase chain reaction (PCR) reactions were performed in a total reaction volume of 10µl containing 7µl PCR master mix and 3 µl DNA template. The PCR master mix contains Taq DNA polymerase (0.1 µl), 50mM $MgCl_2$ (0.4 µl), 10× PCR buffer (1 µl), DMSO (0.8 µl), 2.5Mm dNTPs (0.5 µl) and primer (1 µl) made up to 7 µl with 2.9 µl nuclease-free water (Table 2).

DNA amplification was carried out in a thermocycler with the following thermal profile: 5 min at 94 ºC (initial denaturation) followed by 40 cycles of 30 sec at 94 ºC (denaturation), 30 seconds at the primer annealing temperature and 1 min at 72 ºC (primer elongation), a final step of 7 min at 72 ºC (final extension) and held at 10 ºC (Table 3). After completion of the cycle, PCR products were stored at –20 ºC until required for electrophoresis. Amplification products were separated on 1.5 % (w/v) agarose gels with 0.5× TBE buffer. Electrophoresis was run at 100 V for 90 min. 10 µl of 100bp was run simultaneously, loading on gel as a molecular standard. The gel was visualized, photographed and analyzed. The ladder used was 1 kb Plus DNA ladder from Thermo Scientific.

RAPD-data Analysis

Statistical analysis for band was carried out by scoring for presence (1) or absence (0) of bands, identification of

monomorphic and polymorphic bands, determination of number of alleles amplified and calculation of percentage polymorphism. Percent Polymorphism % = No. of polymorphic Bands / Total no. of Bands. The molecular sizes of the fragments (Bp range) of each gel were estimated on the basis of the corresponding marker lane. These data were then used to calculate the percentage polymorphism so as to study the genetic diversity among the seven moss samples.

Determination of Genetic Variation and Genetic distance

The genetic variation across the species was calculated using the pair – wise difference value (PD %) calculated as PD = $(F_{ab}/ (F_a + F_b)) \times 100$ where F_{ab} is the number of fragments that differed between two individuals 'a' and 'b', while F_a and F_b is the number of fragments scored in DNA profiles of each individual respectively (Gilbert *et al.*, 1991). The genetic distance (GD_{MR}) was calculated as $GD_{MR} = [(N_{10} + N_{01})/2N]^{0.5}$ where GD_{MR} is the Modified Rogers' Distance; N_{10} is the number of bands (or alleles) present only in individual i; N_{01} is the number of bands present only in individual j; and N is the total number of bands (Mohammadi and Prasanna, 2003). To examine the genetic relationship among species, a phenogram was generated from the binary data using the Unweighted Pair Group Method with Arithmetic (UPGMA) mean cluster analysis of PAST version 1.49 (Hammer *et al.*, 2001).

Table 2: PCR Mixture Used in the Study

10× PCR buffer	1.0µl
50 Mm $MgCl_2$	0.4 µl
5 pMol forward primer	0.5 µl
5 pMol reverse primer	0.5 µl
DMSO	0.8 µl
2.5 Mm DNTPs	0.8 µl
Taq 5u/ul	0.1
100 ng/ µl DNA	3.0
Nuclease-free water	2.9 µl

Table 3: PCR Program Used in the Study

Initial denaturation	40 Cycles			**Final Extension Holding Temperature**	
	Denaturation	**Annealing Temperature**	**Extension**		
94 ºC	94 ºC	37 ºC	72 ºC	72 ºC	10 ºC
	30 sec	30 sec	1 min	7 min	∞

III. RESULTS AND DISCUSSION

The genomic DNA isolated from both the SDS and Kit methods showed clear bands on 1 % agarose gel (Figure 1). However, the chloroplast DNA isolated using the SDS method produced higher yields though lesser quality (Table 4); while those isolated using the Kit method produced lesser yield but higher quality (Table 5). This observation was also noted by Schlink and Reski (2002) who made similar observation while trying to isolate high-quality DNA from *Physcomitrella patens*. This suggests that where quality is more paramount than quantity, the selection of the kit method should be preferred over the tradition SDS method when isolating genomic DNA from the moss species studied.

Also the DNA isolated using the SDS method failed to amplify, while those of the kit method amplified during the RAPD-PCR reactions. This could be due to the presence of compounds or phytochemicals that could inhibit PCR reactions in the DNA isolated by the SDS method.

All seven primers employed in this study produced RAPD fragments with varying bands. The bands ranged in molecular sizes from approximately 200 Bp to 3000 Bp (Table 6). The number of fragments amplified by each primer varied. A total of 55 individual fragments were obtained from the seven primers with 27 being polymorphic and 28 being species specific (unique). No monomorphic band was observed. Primer OPB – 07 (850 Bp – 3000 Bp) produced the highest number of fragments thus engendering the highest percentage polymorphism (67%). Primer OPT – 06 produced the lowest number of fragments with a total of five bands. OPB-04 gave the least polymorphism with 25% polymorphism (Table 7).

According to Welsh *et al.*, 1991, the number and size of fragments generated strictly depend on the nucleotide sequence of the primers that act on the template DNA generating genome-specific fingerprints from the amplified random DNA fragments.

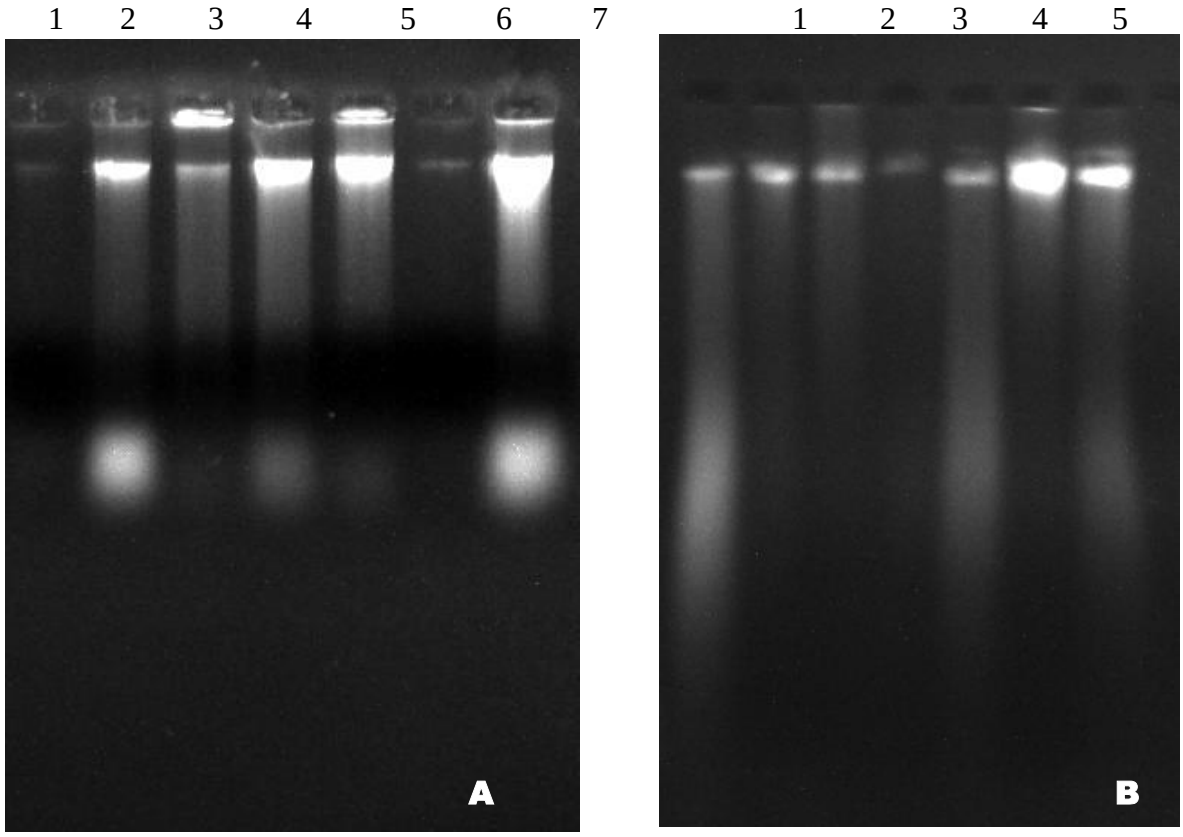

Figure 1: Gel Representation of DNA Extracted from the Moss Species Studied
A: Gel representation of DNA extracted using the SDS method; B: Gel representation of DNA extracted using the Kit method
(Lanes 1 – 7: *Stereophyllum nitense*, *Thuidium gratum*, *Hyophila crenulata*, *Calymperes afzelii*, *Archidium ohioense*, *Bryum coronatum*, *Barbula lambarenensis*)

Table 4: Concentration and Purity of DNA Samples Extracted by SDS Method

Sample	Nucleic Acid	Unit	260/280
Hyophila crenulata	190.5	ng/µl	1.58
Calymperes afzelii	289.2	ng/µl	1.82
Bryum coronatum	353.6	ng/µl	1.73
Thuidium gratum	391.1	ng/µl	1.86
Archidium ohioense	210.5	ng/µl	1.47
Barbula lambarenensis	260.4	ng/µl	1.25
Stereophyllum nitense	553.1	ng/µl	1.57

Table 5: Concentration and Purity of DNA Samples Extracted by Kit Method

Sample	Nucleic Acid	Unit	260/280
Hyophila crenulata	44.1	ng/µl	1.53
Calymperes afzelii	39.1	ng/µl	1.56
Bryum coronatum	89.9	ng/µl	1.66
Thuidium gratum	64.4	ng/µl	1.78
Archidium ohioense	127.3	ng/µl	1.75
Barbula lambarenensis	87.4	ng/µl	1.82
Stereophyllum nitense	225.4	ng/µl	1.69

Table 6: Arbitrary RAPD Primers Used in the Study and Fragment Size Ranges

Primer	Sequence	Fragment size(range in Bp)
OPB – 07	GGTGACGCAG	850 – 3000
OPT – 06	CAAGGGCAGA	850 – 2000
OPH – 02	TCGGACGTGA	500 – 2000
OPB – 11	GTAGACCCGT	650 – 3000
OPH – 07	CTGCATCGTG	650 – 1650
OPT – 20	GACCAATGCC	200 – 1650
OPB – 04	GGACTGGAGT	650 – 1650

The values of the pair-wise comparison of the genetic distance (GD_{MR}) between the moss species studied ranged from 0.5 – 0.71(Table 8). The least of the genetic distance occurred between *Calymperes afzelii* and *Thuidium gratum* with a genetic distant value of 0.5. This was followed by *Calymperes afzelii* and *Hyophila crenulata* with genetic distance of 0.62. *Barbula lambarenensis* and *Hyophila crenulata*, *Bryum coronatum* and *Hyophila crenulata*, *Calymperes afzelii* and *Barbula lambarenensis* share a genetic distance of 0.66. All other pairs shared a genetic distance of 0.71. This indicates that the species were highly genetically distant from each other.

Similarly, the pair-wise comparison of the genetic variation (PD) between the species showed that most of the species varied from each other by a genetic variation value of 100%. The least of this value occurred between *Calymperes afzelii* and *Thuidium gratum* (Table 8). This also indicates that the moss species studied are genetically distinct from each other. Reports by Bolaji and Faluyi (2017), on the morphology, anatomy and chromosome number and behavior of these species further corroborate these findings that these species are quite distinct not only morphologically but also genetically.

The absence of monomorphic fragments indicates that the species were quite dissimilar genetically.This fact is further supported by the pair-wise comparison of their percentage genetic variation and genetic distance. This is further corroborated by the taxonomic groupings of these species into distinct families and orders by Goffinet, 2012.

According to Ramanadevi and Thangaraj (2014), high genetic variation implies unlikely possibility of gene flow. This suggests that there is unlikelihood of gene flow across the seven moss species studied.

Table 7: Total Bands Scored by the Primers and their Percentage Polymorphism

S/N	Primer	Total No. of bands	No. of Polymorphic Bands	No. of Monomorphic Bands	No. of Unique Bands	Polymorphism (%)
1	OPB-07	12	8	-	4	67
2	OPT-06	5	2	-	3	40
3	OPH-02	9	5	-	4	56
4	OPB-11	6	2	-	4	33
5	OPH-07	8	4	-	4	50
6	OPT-20	7	4	-	3	57
7	OPB-04	8	2	-	6	25
	TOTAL	55	27	-	28	49

Table 8: Pair-wise Comparison of Genetic Variation (%) of Species Studied (Below Diagonal) and Genetic Distance (GD_{MR}) of Moss Species Studied (Above Diagonal)

Species	*Stereophyllum intense*	*Thuidium gratum*	*Hyophila crenulata*	*Calympers afzelii*	*Archidium ohioense*	*Bryum coronatum*	*Barbula lambarenensis*
Stereophyllum nitense	****	0.71	0.71	0.71	0.71	0.71	0.71
Thuidium gratum	100	****	0.62	0.50	0.71	0.71	0.67
Hyophila crenulata	100	74	****	0.63	0.71	0.66	0.66
Calympers afzelii	100	50	79	****	0.71	0.71	0.66
Archidium ohioense	100	100	100	100	****	0.71	0.71
Bryum coronatum	100	100	88	100	100	****	0.71
Barbula lambarenensis	100	91	88	87	100	100	****

The UPGMA phenogram of the RAPD data separated the moss species studied into seven main groups at 0.6 level of similarity. The seven groups were *Bryum coronatum*, *Barbula lambarenensis*, *Archidium ohioense*, *Stereophyllum nitense*, *Hyophila crenulata*, *Thuidium gratum* and *Calymperes afzelii* (Figure 2). The groupings agree with their taxonomic groupings into separate families by Schofield (1985).

The ability to understand the genetic relationships within species at the molecular level has greatly increased through the application of Random Amplified DNA (RAPD) technique (Sabir *et al*., 2012). Reports have shown that it is a quick and cost effective molecular technique (Clark and Lanigan, 1993) that has been used by several researchers in the study of population differetiation and in the study of genetic relationships (Liu *et al.*, 1998; Callejas and Ochando, 2002; Ali *et al*., 2004).

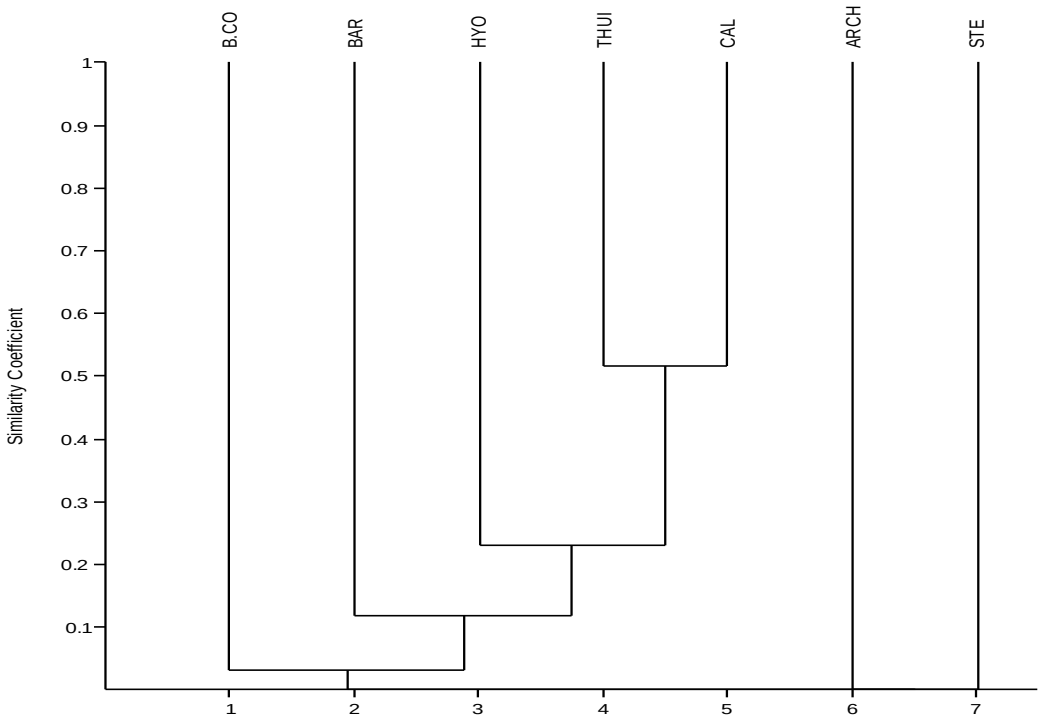

Figure 2: UPGMA Phenodrogram Showing Genetic Relatedness of the Moss Species Studied based on RAPD Data

Acronyms: Hyo represents *Hyophila crenulata*; Cal represents *Calymperes afzelii*; B.coro represents *Bryum coronatum*; Thui represents *Thuidium gratum*; Arch represents *Archidium ohioense*; Barb represents *Barbula lambarenensis*; Stereo represents *Stereophyllum nitense.*

IV. CONCLUSION AND RECOMMENDATION

The molecular studies revealed that the moss species studied were not only distant morphologically but also genetically. The UPGMA phenogram of the RAPD data separated the moss species studied into groupings that were consistent with those of Schofield (1985).

Much is still left to be done in the morphological, morphometric, cytological, and molecular characterization of bryophytes in Nigeria, taking into consideration the real and potential genetic resources available in their vast populations. Efforts should be made to characterize more of these species and also determine their reproductive biology as this could be an important step in determining the strategy for their conservation.

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