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MORPHOLOGICAL AND MOLECULAR STUDIES OF THREE SPECIES OF *Boerhavia* L. FROM ILE-IFE, NIGERIA

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ABSTRACT

A wide range of plasticity and polymorphism have been documented in the genus *Boerhavia*. More so, the possible presence of natural hybrids among the species of the genus has made species identification more difficult. This study employed morphological characters, pollen parameters as well as Random Amplified Polymorphic DNA (RAPD) to identify and elucidate the phylogenetic relationship that exists among the three species of *Boerhavia* distributed in Ile-Ife, Nigeria. Qualitative and quantitative morphological characters were observed and measured respectively. The acetolysis of the pollen grains was carried out according to Erdtman method. Fresh and young leaves of the *Boerhavia* species studied were collected for genomic DNA extraction using modified Dellarpota procedure. The quality and concentration of DNA was assessed by gel electrophoresis on 2% agarose with known concentrations of undigested lambda DNA. Subsequently, the DNA quantification was done according to standard measurement. The Principal Components Analysis of the morphological traits and the pollen grain studies indicated that the three *Boerhavia* species studied have a very high level of relationship. However, the Single Linkage Cluster Analysis of the morphological data and the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of RAPD data revealed that *B. erecta* is distantly related to *B. coccinea* and *B. diffusa*. Despite the fact that the three *Boerhavia* species studied possess high level of similarity, they can still be distinguished from each other. In conclusion, *B. coccinea* and *B. diffusa* are more closely related to each other than *B. erecta*.

Key words: RAPD marker, Pollen grains, Phylogenetic relationship, Polymorphism

INTRODUCTION

The genus *Boerhavia* popularly known as spiderlings belongs to the 4 0'Clock family (Nyctaginaceae). It consists of approximately 50 species (Levin *et al*., 2001; Struwig and Siebert, 2013; Debasmita *et al*., 2015). The members of the genus flower and fruit all year round and are widely distributed throughout the tropical and subtropical regions of the world. The distribution is also extended to the warm and temperate regions (Struwig and Siebert, 2013; Debasmita *et al*., 2015). The genus was divided into two groups based on the nature of their inflorescence (Fosberg, 1978). Four species of the genus were reported in West Africa and they are all present in Nigeria (Hutchinson and Dalziel, 1952).

Three out of these species: *B. coccinea* Mill., *B. diffusa* L. and *B. erecta* L. are found to be commonly grown in southern part of Nigeria (Hutchinson and Dalziel, 1952; Ekeke and Agogbua, 2019). *Boerhavia* is characterized by elongated stems with prominent nodes (Chen and Wu, 2007). Spheroidal, pantoporate and spinulose pollen grains have been reported in *Boerhavia* from different regions (Nowicke and Skvarla, 1979, Struwig *et al.,* 2013; Pramanick *et al.,* 2015, Maw, 2019). Chromosome number of 2n=52 was documented for *B. coccinea* and *B. erecta* while 2n=26, 52, 54 and 116 were recorded for *B. diffusa* (Bogle, 1974; Virendra and Subramaniam, 1986; Spellenberg, 2000). The epidermal cells observed in *Boerhavia* species in Nigeria are either polygonal in shape with straight anticlinal walls or irregular with curved or undulating walls (Fadeyi *et al*., 1989). Ajao *et al.* (2017) documented C_3 photosynthetic metabolism pathway in *B. diffusa* while C_4 pathway was observed in *B. erecta, B. coccinea* and *B. repens* L. in Nigeria.

Woodson (1961) opined that *B. caribaea* Jacq. and *B. coccinea* from the New World and *B. diffusa* of the Old World are the same. He only recognized *B. diffusa* and *B. erecta,* thereby placing *B. diffusa* and *B. coccinea* in the same taxa. Moreover, a wide range of plasticity and polymorphism have been identified

to exist among the species of *Boerhavia* and therefore, they were treated as complex groups. In addition, the possible presence of natural hybrids between species of *B. diffusa* complex and *B. repens* complex has made it difficult to distinguish between these species (Meikle and Hewson, 1984; Whitehouse, 1996). Ekeke and Agoghua (2019) stated that there was no clear intraspecific variation among the three *Boerhavia* species they studied based on morphological characters and trichome types.

Molecular markers have been increasingly employed in studies of genetic diversity in order to elucidate the phylogenetic relationship among different species (Miah *et al*., 2013; Patwardhan *et al.,* 2014). Patwardhan *et al.,* (2014) suggested that molecular markers which are relatively recent have gained popularity in the phylogenetic studies and can be used in conjunction with the classical phylogenetic approach- a morphology-based method, since they are not totally free of errors. Random amplified polymorphic DNA (RAPD) is a commonly used polymerase chain reaction (PCR) based assay. In addition, it is a quick and efficient technique in producing species-specific finger prints (Stepniak *et al*., 2002). Moreover, RAPD markers have been shown to be useful in studying the genetic diversity of *Boerhavia* populations (Shukla *et al*., 2003).

Species of *Boerhavia* including *B. erecta* and *B. coccinea* have been reported to have many medicinal uses. *Boerhavia diffusa* was documented as a promising source of drug that will be useful in rejuvenating new cells in the body. In addition, *B. diffusa* was reported to possess many bioactive phytochemicals which make it a potential candidate for curing various ailments in humans and animals (Patil *et al*., 2016; Patil and Bhalsing, 2016; Kumar *et al.,* 2018). Considering the medicinal value as well as plasticity and polymorphism of the species of this genus, it very crucial to carry out a detailed study on the available *Boerhavia* species in Ile-Ife. This study therefore, employed morphological traits (vegetative and reproductive), pollen grain attributes and RAPD marker to identify and elucidate the phylogenetic relationship that exists among the three *Boerhavia* species distributed in Ile-Ife, Nigeria.

MATERIALS AND METHODS

Species Collection and Planting

The seedlings of three available *Boerhavia* species in Ile-Ife: *B. diffusa, B. erecta* and *B. coccinea* were collected from different locations in Ile-Ife (7° 29' 57.247"N and 4°33'35.241"E; 7°30'05.171"N and 4°33'25.571"E; 7°29'12.07"N and 4°29'35.42"E respectively). The plants were identified at IFE herbarium, Obafemi Awolowo University, Ile-Ife, Nigeria. The collected seedlings were planted and raised to maturity. Matured seeds were harvested, planted and raised for two generations to ensure that they were breeding true. Five plants from each species were used for the study.

Morphological Study

Qualitative and quantitative morphological characters were observed and measured respectively. The qualitative character observed include: habit, leaf pigmentation, leaf base, leaf apex, leaf margin, leaf arrangement, stem type, stem pigment, seed colour, fruit colour, leaf pubescence and the flower colour. The quantitative characters measured include corolla breadth, corolla length, fruit length, leaf area and plant height.

Pollen Grain Study

The acetolysis of the pollen grains from the three species studied was carried out according to Erdtman (1960) method. Fresh pollen grains collected in 70% ethanol were crushed with a glass rod. The suspension was then sieved through a fine mesh into clean tubes, centrifuged at 1000 x g for 5 minutes and the alcohol was decanted. Five (5) cm^3 of glacial acetic acid was added to the residue, mixed, centrifuged again and the supernatant was decanted. Six (6) cm^3 of freshly prepared acetolysis mixture was added to the residue in the test tube, heated in water bath to boiling point and stirred intermittently with glass rod. The mixture was centrifuged and waste acetolysis mixture was decanted. Glacial acetic acid (10 cm^3) was added to the residue, stirred, centrifuged and washed with several changes of distilled water. The acetolysed pollen was stored in about 2 cm^3 dilute glycerine. The pollen grains were mounted in glycerine covered with clean coverslip sealed with melted wax. The pollens were viewed and measured using attached ocular micrometer under the light microscope.

Photomicrographs of the pollen grains were taken at x 1000 magnification.

Pollen size was calculated as:

$$Polar\ axis \times Equitorial\ diameter$$

$$^{P}/_{E}\ ratio = Polar\ axis \div Equatorial\ diameter$$

Molecular Study

The molecular study was conducted at the Biotechnology laboratory, National Horticultural Research Institute (NIHORT), Ibadan, Nigeria. Fresh and young leaves of the three *Boerhavia* species studied were collected for genomic DNA extraction using modified Dellaporta procedure (Dellaporta *et al.* 1983). The quality and concentration of DNA was assessed by gel electrophoresis using 2% agarose with known concentrations of undigested lambda DNA (Sigma, St. Louis, MO, USA). Quantification of DNA was done using absorbance measurement in a spectrophotometer (Beckman Coulter DU530) at 260 nm.

RAPD Amplification

A total of 21 RAPD primer pairs were used in this study following modified method of Saiki (Saiki *et al.* 1988). Polymerase chain reaction (PCR) assay for the RAPD reactions was conducted in an Eppendorf Mastercycler (Nexus Thermal Cycler, USA Scientific, Inc.). The composition of PCR mix of RAPD marker is presented in table 1. The DNA concentration of each sample was diluted to 50 ng/µl before use. RAPD amplification was conducted following the thermal profile: denaturation at 94 °C for 2 mins and 30 cycles of denaturation at 94 °C for 15 s, annealing at 65 °C for 20 s, extension of 72 °C for 30 s while the final extension was at 72 °C for 5 mins. Each cycle reduced the annealing temperature by 1 °C with the final 30 cycles running at 55 °C annealing temperature. A final elongation cycle of 72 °C for 5 mins completed the reaction. PCR products (3 µl) were loaded on 2% agarose gel in 1 X TBE buffer alongside 50 base pair (bp) standard size ladder. Gel banding pattern on gel stained with ethidium bromide was viewed in a Bio rad UV mini dark room.

Molecular Data Analysis

Each genotype for the RAPD primers was scored visually on the basis of their presence (1) or absence (0) separately for each species. The sizes of fragment (molecular weight in base pair) were estimated using the DNA hyper-ladder 2 (Bioline) as base pair ladder which was run along with the amplified products. The scores obtained using all 21 polymorphic primers for RAPD were then used for constructing matrix. The statistical software NTSYS-PC (Rohlf, 2005) and POWER MARKER software were used to construct a UPGMA dendrogram using hierarchical clustering. Using NTSYS software, a dissimilarity matrix was calculated utilizing Jaccard (1908) coefficient. Cluster analysis based on the dissimilarity matrix, was performed with UPGMA (Sneath and Sokal, 1973) of the NTSYS–PC version 2.2 (Rohlf, 2005).

Statistical Analysis

The quantitative data recorded were subjected to one way ANOVA and the means were separated by Duncan's multiple range test. All the morphological data were further subjected to Single Linkage Cluster Analysis and Principal Components Analysis (PCA).

Table 1: PCR Mix of RAPD Markers used in Amplification of *Boerhavia* DNA

Reaction Component	Volume Used (μl)
$MgCl_2$	0.4
10x Buffer	1.0
DNTPs	0.8
DMSO	0.8
Primer	1.0
Sterile H_2O	2.9
Taq polymerase	0.1
Template	3.0
TOTAL	10

Abbreviations: DMSO (dimethyl sulfoxide); DNTPs (Deoxynucleoside triphosphates); Taq (*Thermus aquaticus*)

RESULTS

Morphological Study

The leaves of all the three *Boerhavia* species are fleshy and sparsely pubescent on the abaxial surface. The abaxial surfaces of the three species are greenish though much paler in *B. erecta*. The leaves are ex-stipulate. In *B. diffusa,* marginal hairs are present which is diagnostic for this species. The petiole is greenish; with purple pigmentation in *B. erecta*. The flower is bisexual with two stamens. Each stamen is made up of a filament with a double-lobed anther. The flower is cup-shaped and the fruit is sticky, one-seeded with five ribs in all the species studied. The stems in all the species are sparsely pubescent (Tables 2 and 3). The Principal Components Analysis showed that all the three species of *Boerhavia* studied are closely related (Figure 1). However, the Single Linkage Analysis showed that *B. coccinea* and *B. diffusa* are more related while *B. erecta* was separated out (Figure 2).

Pollen Grain Study

Monad spinulated spheroidal pollen grains were observed in all the three *Boerhavia* species studied. The pollen size in *B. erecta* was significantly smaller than what was documented in *B. coccinea* and *B. diffusa* (Figure 3 and Table 4). The exine thickness was significantly different among all the species studied. The nexine thickness and spine length in *B. erecta* were significantly wider and longer respectively than what was recorded in the other two species. However, the pore size was significantly smaller in *B. erecta* when compared with *B. coccinea* and *B. diffusa*. In *B. erecta*, the spinules appeared shining. Two layers of pores were observed; the areas containing these pores were depression-like. Polar axis-equatorial ratio (P/E ratio) is 1 in *B. erecta*. The P/E ratio is slightly greater than 1 in *B. coccinea* while slightly less than1 in *B. diffusa*. The inner pores in *B. diffusa* appeared to have some thickening (annuli) around them.

Table 2: Qualitative Characters of Three *Boerhavia* Species Studied

Plant Species	*B. diffusa*	*B. coccinea*	*B. erecta*
Habitat	Lawns, roadsides	Lawns, ruderal	Dump site, ruderal
Habit	Wide spreading prostrate to decumbent herbs	Wide spreading prostrate to decumbent herbs	Decumbent to erect herbs
Leaf shape	Ovate-orbicular	Ovate-orbicular	Deltoid
Leaf Margin	Entire	Entire	Entire
Leaf type	Simple	Simple	Simple
Leaf Apex	Acute, rounded	Acute, rounded	Acute
Leaf base	Cordate	Cordate	Truncate
Leaf Indumentum	Marginal hairs present	Glabrous	Glabrous
Leaf arrangement	Opposite	Opposite	Opposite
Leaf Colour	Green	Green	Green
Leaf pigmentation	None	None	Purplish-red along margin
Inflorescence	Axillary/terminal	Axillary/terminal	Axillary/terminal
Flower	It is bisexual and actinormorphic. 2-12 clustered up to the wide flower head.	It is bisexual and actinormporphic. 2-12 clustered up to the wide flower head.	It is bisexual and actinormorphic. 3-9 clustered up to the wide flower head.
Anther colour	Purple	Yellow.	Lemon
Filament colour	Light Pink	Light Pink	White
Stigma colour	Purple	Yellow	White
Stigma type	Capitate	Capitate	Capitate
Style colour	Light pink	Yellow	White
Corolla colour	Purple or purplish red	Pink	White with red pigmentation in some species.
Fruit type	Anthocarp	Anthocarp	Anthocarp
Fruit Indumentum	Pubescent	Pubescent	Glabrous
Fruit shape	Club-like	Club-like	Obconical
Longevity	Perennial	Perennial	Annual

Table 3: Quantitative Characters of Three *Boerhavia* Species Studied

Plant species	*B. diffusa*	*B. coccinea*	*B. erecta*
Corolla length (mm)	*4.15±0.02^{a}	4.11±0.03^{a}	3.28±0.04^{b}
Corolla breadth (mm)	3.23±0.04^{a}	4.22±0.02^{b}	3.17±0.02^{a}
Plant height (cm)	73.35±4.61^{a}	73.95±4.18^{a}	87.16±12.78^{a}
Fruit length (mm)	4.23±0.04^{a}	4.16±0.03^{ab}	4.10±0.03^{bc}
Leaf Area (cm^2)	19.43 ± 1.11^{a}	19.78 ± 0.99^{a}	17.53 ± 1.16^{a}

* Means ± standard error, $^{a, b, c}$: Means within each row with different superscript are significantly different ($P<0.05$)

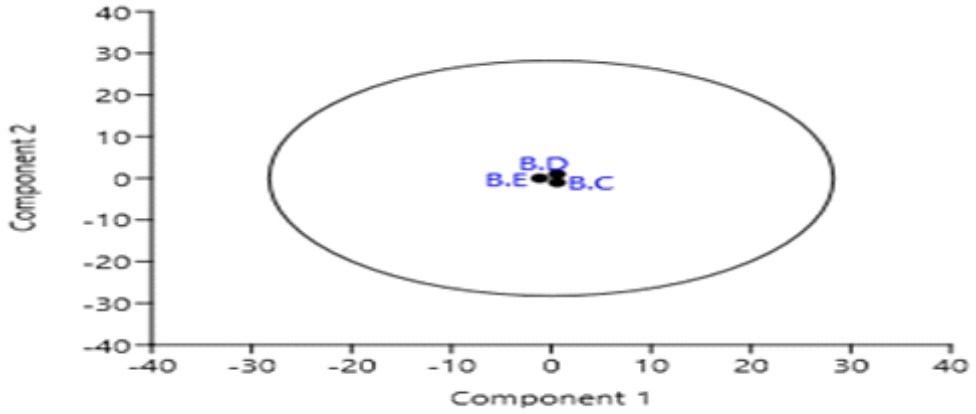

Figure 1: Principal Components Analysis of All Quantitative and Qualitative Characters Measured in *Boerhavia* Species Studied
Abbreviations: B.E- *B. erecta*, B.D- *B. diffusa*, B.C- *B. coccinea.*

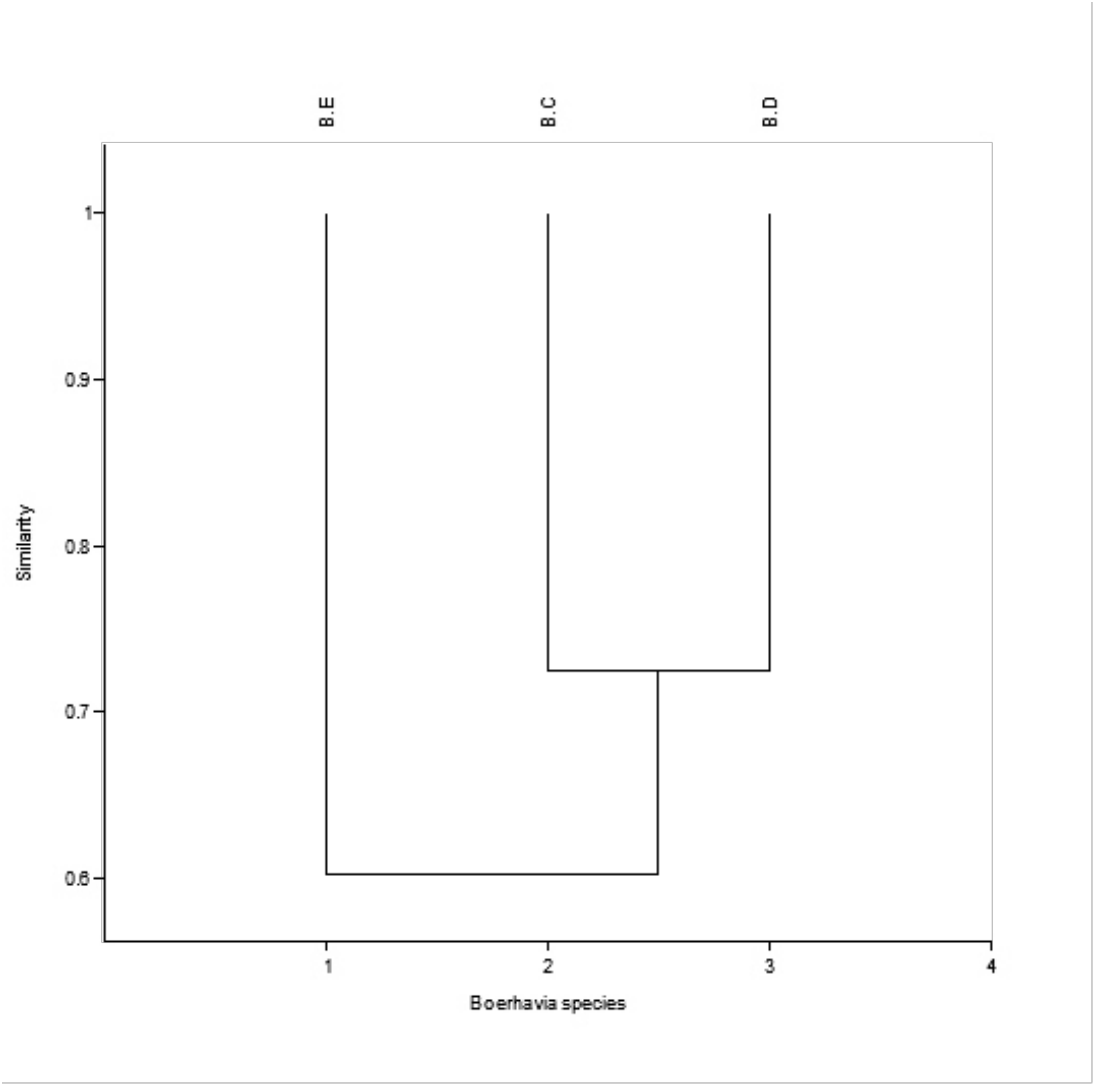

Figure 2: Single Linkage Cluster of All Quantitative and Qualitative Characters of *Boerhavia* Species Studied
Abbreviations: B.E – *B. erecta*, B.D – *B. diffusa*, B.C – *B. coccinea.*

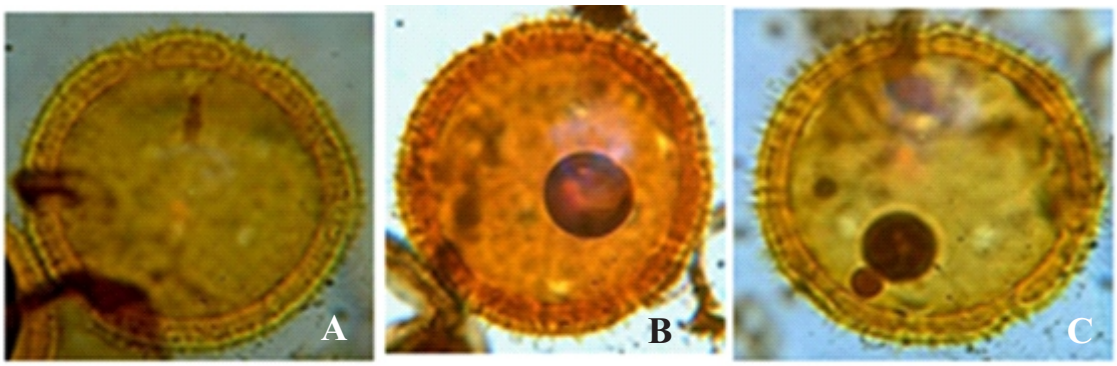

Figure 3: Pollen Morphology in *Boerhavia* Species Studied

A- *Boerhavia. coccinea* B- *Boerhavia. diffusa* C- *Boerhavia. erecta*

RAPD Analysis

The extracted DNA obtained from the three *Boerhavia* species studied showed sharp and clear bands (Figure 4). Electrophoretic data were scored manually, and each band in the RAPD profile was treated as an independent character (locus) with two states (alleles), presence (+) or absence (-). The Polymorphism Information Content (PIC) values recorded for the three species studied were well above zero (Table 5). On the basis of the analysis of RAPD profiles amplified by the arbitrary primers, a phylogenetic tree was constructed and it revealed the relationship among the three species studied (Figures 5 and 6). The phylogenetic tree separated *B. erecta* from the rest.

Table 4: Pollen Parameters of Three *Boerhavia* Species Studied

Species	Polar axis Aperture (µm)	Equatorial Diameter (µm)	Pollen size (µm)	Exine Thickness (µm)	Exine pattern	Nexine Thickness (µm)	Spine (µm)	Pores (µm)
B. erecta	*62.9± 0.31^{a}	62.9±0.30^{b}	3956.5 ±28.1^{b}	10.2±0.04^{c}	Spinulate	5.1±0.06^{b}	1.9± 0.07^{b}	3.1± 0.07^{b}
B. coccinea	64.8± 0.31^{b}	63.2±0.38^{b}	4096.5 ±30.2^{a}	7.6 ±0.02^{a}	Spinulate	4.4±0.10^{a}	1.5± 0.03^{a}	4.7± 0.09^{a}
B. diffusa	62.4± 0.18^{a}	64.9±0.20^{a}	4046.9 ±16.2^{a}	9.2±0.12^{b}	Spinulate	4.2±0.04^{a}	1.6± 0.05^{a}	4.8± 0.09^{a}

* Means ± standard error; Means within each column with different superscript are significantly different (P<0.05).

Pollen description: Polar axis (total length); Equatorial diameter (total width); Pore- spherical opening in pollen; Exine- the outermost hard part of the pollen containing patterns.

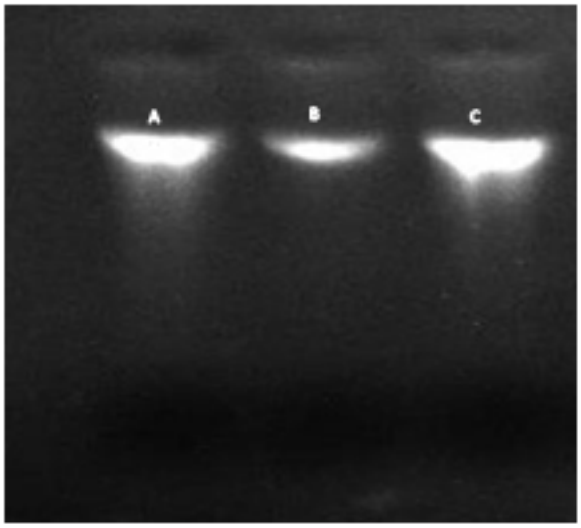

Figure 4: Genomic DNA of the Three Species Visualized on Agarose Gel Electrophoresis.
A. *Boerhavia coccinea* B. *Boerhavia diffusa* C. *Boerhavia erecta*

Table 5: Number of Alleles, Gene Diversity and Polymorphism Information Content (PIC) of RAPD Markers

Marker	Major Allele Frequency	Sample Size	*No. of obs.	Allele No	Availability	Gene Diversity	**PIC
OPT20	0.3333	3.0000	3.0000	8.0000	1.0000	0.6667	0.5926
0PT08	0.6667	3.0000	3.0000	2.0000	1.0000	0.4444	0.3457
OPT08	0.3333	3.0000	3.0000	7.0000	1.0000	0.6667	0.5926
OPT09	0.3333	3.0000	3.0000	5.0000	1.0000	0.6667	0.5926
OPT16	0.3333	3.0000	3.0000	8.0000	1.0000	0.6667	0.5926
OPB08	0.6667	3.0000	3.0000	4.0000	1.0000	0.4444	0.3457
0PB08	0.3333	3.0000	3.0000	5.0000	1.0000	0.6667	0.5926
OPT01	0.6667	3.0000	3.0000	5.0000	1.0000	0.4444	0.3457
OPT05	1.0000	3.0000	3.0000	2.0000	1.0000	0.0000	0.0000
0PT05	0.3333	3.0000	3.0000	7.0000	1.0000	0.6667	0.5926
OPB10	0.3333	3.0000	3.0000	5.0000	1.0000	0.6667	0.5926
OPT06	0.6667	3.0000	3.0000	4.0000	1.0000	0.4444	0.3457
OPH08	0.6667	3.0000	3.0000	5.0000	1.0000	0.4444	0.3457
OPT12	0.3333	3.0000	3.0000	5.0000	1.0000	0.6667	0.5926
OPH07	0.3333	3.0000	3.0000	5.0000	1.0000	0.6667	0.5926
OPB05	0.3333	3.0000	3.0000	7.0000	1.0000	0.6667	0.5926
OPB04	0.3333	3.0000	3.0000	6.0000	1.0000	0.6667	0.5926
OPH05	0.3333	3.0000	3.0000	8.0000	1.0000	0.6667	0.5926
OPT07	0.3333	3.0000	3.0000	8.0000	1.0000	0.6667	0.5926
OPB13	0.3333	3.0000	3.0000	8.0000	1.0000	0.6667	0.5926
OPB20	0.3333	3.0000	3.0000	7.0000	1.0000	0.6667	0.5926
Mean	0.4444	3.0000	3.0000	5.8000	1.0000	0.5820	0.5056

Abbrevations: * Number of Samples Observed, ** Polymorphism Information Content

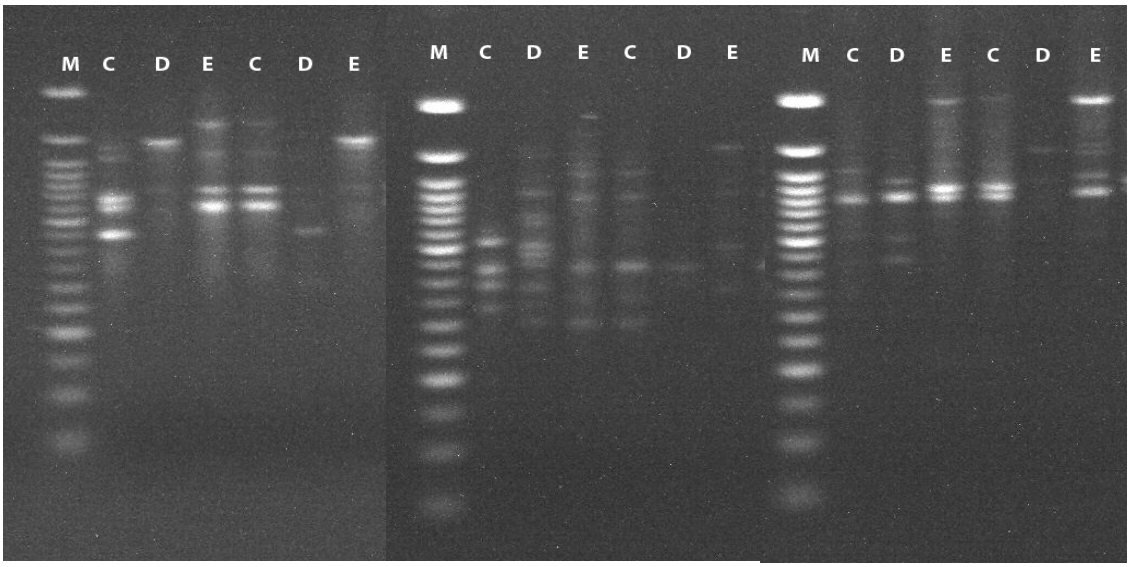

Figure 5: RAPD Profile of *Boerhavia* Species from Some of the Random Primers. M- 100 bp DNA ladder, C- *B. coccinea*, D – *B. diffusa*, E- *B. erecta*

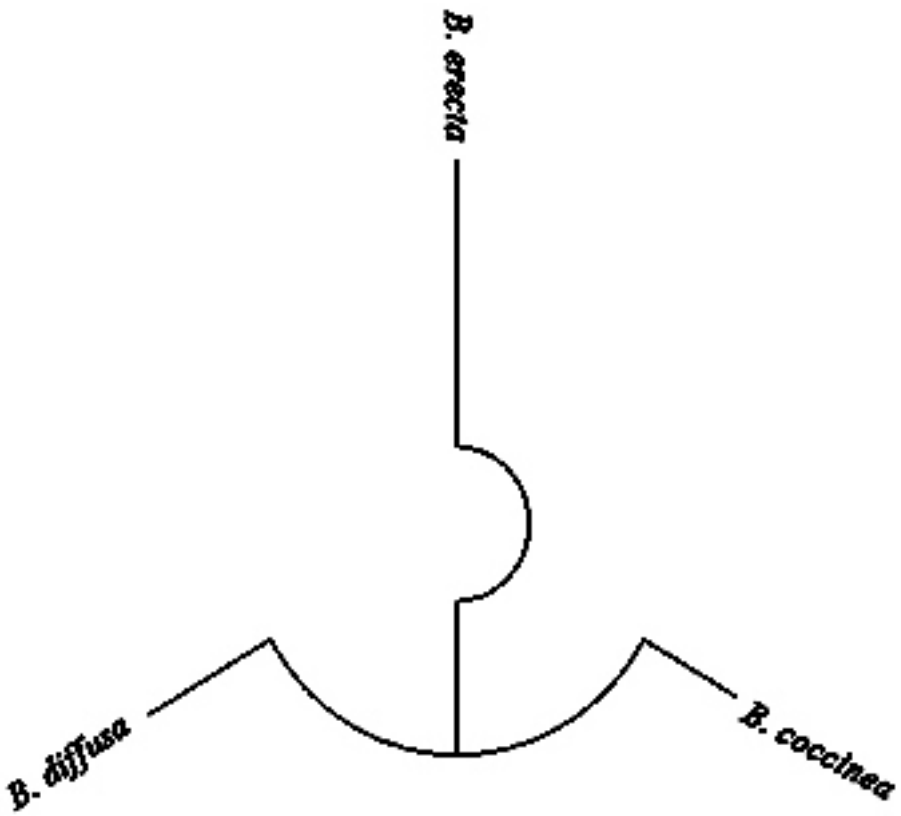

Figure 6: Dendrogram of the Three Species of *Boerhavia* Studied Based on UPGMA Cluster Analysis of RAPD Data

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DISCUSSION

The habits observed in the three *Boerhavia* species studied were similar to what was documented by Chen and Wu (2007). The leaves of *B. diffusa* are pubescent at the margins unlike *B. erecta* and *B. coccinea* that are glabrous. This feature is diagnostic for *B. diffusa* even when inflorescences are not present. In addition, the features noted on leaf margins, petioles and fruits of *B. erecta* were in line with observations of Chen and Wu (2007). However, pigmentation was absent in *B. coccinea*

and *B. diffusa* according to the observations from this present study. The flowers of *B. erecta* studied are white. Chou *et al.* (2004) reported that *B. erecta* found in Taiwan can have either white or pink flowers. Each flower of the three species studied has the same number of stigma and stamens; one capitate stigma and two stamens. However, varying number of stamens has been recorded for these species in literatures; 2-4 stamens were recorded for *B. erecta* and 1-3 for *B. coccinea* (Spellenberg, 2004; Chen and Wu, 2007). The deltoid leaf shape as well as truncate leaf base is diagnostic for *B. erecta*. More so, its longevity, sizes of the leaf and corolla, filament colour and the shape of the fruit separated it from the other two species which have light pink filament and pubescent club-like shaped fruits. Additionally, *B. coccinea* and *B. diffusa* shared similar characters like perennial habit, ovate-orbicular leaf shape and cordate leaf base which grouped them together.

From the pollen grains study, the quantitative characters were shown to overlap. The only character that separated the three *Boerhavia* species studied from each other was the exine thickness with the least recorded in *B. coccinea*. This indicates that pollen characteristics are not enough to distinguish the three species studied from each other. This observation is similar to those reported in previous studies that pollen morphological attributes in the genus *Boerhavia* have limited value taxonomically, due to the fact that they are homogenous and have overlapping characters (Perveen and Qiaser, 2001; Struwig *et al.*, 2013; Maw, 2019).

The result showed that the extraction protocol used in this study was effective for genomic DNA extraction in *Boerhavia* species. The PIC values recorded in this present study were well above zero. This pointed out that there are allelic variations in the DNA of the three *Boerhavia* species studied; the PIC values would have been 1.0 if the alleles are totally different from each other. Filho *et al.* (2010) and Parthiban *et al.* (2018) reported that such discriminating power and high polymorphism primers can be used effectively in constructing genetic linkage maps. The dissimilarity among sequences indicates genetic divergence as a result of molecular evolution over a long period of time (Patwardhan *et al.,* 2014). This pointed out that the three species studied shared some level of similarities. The phylogenetic tree showed that the three species studied had a common origin; indicating that the three *Boerhavia* species had a common ancestor. However, the phylogenetic tree constructed in this study, revealed that *B. erecta* was distantly related to *B. coccinea* and *B. diffusa*.

Both the morphological and molecular studies showed the existence of the same phylogenetic relationship among the three *Boerhavia* species studied. A combination of the two methods therefore strengthens the result for the determination of phylogenetic relationships among organisms to a great extent (Patwardhan *et al.,* 2014). This study revealed that *B. coccinea* and *B. diffusa* shared more similar characters than *B. erecta* making them more closely related. The features shared may be under the control of similar genes that are present in both *B. coccinea* and *B. diffusa*. However, *B. erecta* has quite a number of characters that are unique to it which are likely to be under the influence of different alleles. This might have been the reason why it is distantly related to the other two species. The fact that they all have the same origin showed that they shared many similar genes controlling the expression of similar phenotypes that were observed among the three species.

Despite the fact that the three *Boerhavia* species studied possess a high level of similarity, they can still be distinguished from each other. The study concluded that *B. coccinea* and *B. diffusa* are more closely related to each other than *B. erecta*.

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