MORPHOLOGICAL, PHYSIOLOGICAL AND MOLECULAR SCREENING OF AERIAL YAM (*D.bulbifera L.*) AND TEST FOR SODIUM CHLORIDE TOLERANCE

BY

KALU, SUCCESS ENI
GBT/Ph.D./15/005

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DECLARATION

I, KALU, SUCCESS ENI with Registration Number, Reg No: GBT/Ph.D./15/005 hereby declare that this thesis titled "Morphological, Physiological and Molecular Screening of Aerial Yam (*Dioscorea bulbifera* L.) and Test for Sodium Chloride Tolerance" is the product of my research effort under the supervision of Prof. I. A. Ekpo and Dr. (Mrs.) A. N. Osuagwu and has not been presented elsewhere for the award of a degree or certificate. All sources have been duly acknowledged.

Signature: .....

Date: 24/10/2019

CERTIFICATION

This is to certify that this thesis titled "Morphological, Physiological and Molecular Screening of Aerial Yam (*Dioscorea bulbifera* L.) and test for Sodium Chloride Tolerance" carried out by Kalu, Success Eni with Registration number GBT/Ph.D./15/005 has been examined and found worthy of the award of the degree of Doctor of Philosophy (Ph.D.) in Genetics and Biotechnology (Environmental Biotechnology).

1. EXTERNAL EXAMINER
   Name: Prof. C. O. Ibegbulem — Signature: .....
   Status: Professor — Date: 24/10/19

2. CHIEF SUPERVISOR
   Name:Prof. Imo A. Ekpo — Signature: .....
   Status: Professor — Date: 24-10-2019

   SUPERVISOR
3. Name: Dr. (Mrs) A. N. Osuagwu — Signature: .....
   Status: Senior Lecturer — Date: 24/10/2019

4. HEAD OF DEPARTMENT
   Name: Udensi, O. Ugorji (Ph.D) — Signature: .....
   Status: Senior Lecturer — Date: 24/10/2019

5. GRADUATE SCHOOL REPRESENTATIVE
   Name: Dr. Oluwatosin O. O. Kennedy — Signature: .....
   Status: Associate Professor — Date: 24-10-19

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ABSTRACT

Aerial yam (*Dioscorea bulbifera* L.) is an indigenous edible energy giving crop used also as herbal remedy for some common ailments like diabetes, dysentery, cholera and conjunctivitis. It is one of the lesser cultivated and utilized plants in the Niger-Delta region of Nigeria and threatened to extinction. It is fast growing and has the ability to adapt to different soil types and habitat conditions and therefore seen as having the potential of tolerating stress conditions thus capable of providing food security and wellbeing in this era of climate change. This study therefore sought to determine the tolerance of *D. bulbifera* to table salt stress condition through morphological, physiological and molecular studies. Bulbils of *D.bulbifera* were sourced from local farmers in Abia State, Akwa Ibom State and Cross River State of the Niger- Delta region of Nigeria, and grown in a green house. Randomized Complete Block Design (RCBD) with three replications was used for the study. At four weeks after sprouting, the plants were treated with 250ml of different concentrations of sodium chloride (0, 100, 200 and 300mM) twice a week for six weeks. Morphological data of leaf colour, number of leaves/plant, root length, leaf length, petiole length, leaf area, vine length and number of bulbils were measured weekly during the treatment period. Chlorophyll-a and chlorophyll- b concentrations, peroxidase and glucose-6-phosphate dehydrogenase activities were analyzed after the completion of the treatment. Diversity study was carried out using micro- satellite molecular markers. Results revealed that sodium chloride reduced the vine length, number of leaves, number of bulbils, chlorophyll-a and chlorophyll-b concentrations of the plant, and caused increase in the root length, peroxidase and glucose-6- phosphate dehydrogenase activities. Although sodium chloride reduced the growth of the *D.bulbifera*, all the lines were able to tolerate the salt concentrations $\leq$ 200mM ($P<0.05$) as they were able to grow to maturity and produce

bulbils. Significant differences in growth and yield were found between the lines ($P<0.05$). Bulbils with round shape tended to show more tolerance to the salt as revealed from the morphological studies. The diversity study revealed genetic differences between the lines. In conclusion, *D. bulbifera* is able to tolerate table salt stress and can thrive in saline soils of concentration ≤200mM, *D. bulbifera* with round shape are more tolerant to the stress condition than the spindle shaped, and finally, diversity in *D. bulbifera* in Niger Delta region of Nigeria is low. (Word count: 387)

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LIST OF ABBREVIATIONS, GLOSSARIES AND SYMBOLS

^0C	Degree celsius
cm	Centimetre
CO_2	Carbon(iv)oxide
CTAB	CetylTrimethyl ammonium bromide
D. bulbifera	*Dioscorea bulbifera*
DNA	Deoxyribose nucleic acid
dNTP	deoxyribonucleotide triphosphate
EC	Electrical conductivity
EDTA	Ethylene diaminetetraacetic acid
g	Gram
H_2O	Water
ISSG	Invasive species specialist group
Kg	Kilogram
Ltd	Limited
$MgCl_2$	Magnesium chloride
$mgg^{-1}FW$	milli gram per gram fresh weight
mL	MilliLitre
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
ng/µl	Nanogram per micro litre
O_2	Oxygen
PCR	Polymerase chain reaction
PIC	Polymorphic information content

PVF	Polyvinyl fluoride
RCBD	Randomized complete block design
ROS	Reactive Oxygen Species
SSR	Simple sequence repeats
TE	Tris-EDTA
UV	Ultra violet

CHAPTER ONE

INTRODUCTION

1.1 Background information

Environmental deterioration caused by human activities such as deforestation, irrigation practices and excessive use of agrochemicals, etc. has resulted to biotic and abiotic stresses in plants (Shao and Chu, 2005). Abiotic stresses contribute to low productivity of crops (Shanker and Venkateswarly, 2011). Acquaah (2007) has estimated 70% of the step- down in yield of crop plants to abiotic stresses. Salinity as an abiotic stress has contributed to low productivity of plants worldwide (Vinocur and Altman, 2005; Shrivastara and Kumar, 2015). It has been proven via research that salt stress has severe effect on plants (Sivritepe and Eris, 1999; Song *et al.,* 2006). These effects include dehydration and generation of ionic imbalance.

Water, a solvent of life is essential for growth and survival of plants. Without water, it becomes difficult for plants to manufacture their food through the process of photosynthesis. Water also contributes to temperature regulation in plants. Without water, carbohydrates, proteins, nucleic acids and enzymes lose their physical and chemical properties which may influence their activities (Nkang and Mofunanya, 2016). Dehydration causes stresses on plants which may influence their biochemical activities and bring about low productivity. High salinity causes dehydration in plants and obviously prompts their low yield.

Dehydration in plants may cause the stomata to close. Stomata are tiny pores found in the leaves of plants which function in gaseous exchange. They regulate the amount of gaseous substances e.g. carbon (iv) oxide (CO_2), oxygen (O_2) and water vapour (H_2O) that enter and leave the plant cells. The closure of the stomata reduces the amount of carbon (iv) oxide that enter the leaves thereby inhibiting carbon

fixation and exposing the chloroplasts to excessive excitation energy which might increase the generation of reactive oxygen species (ROS) (Parvaiz and Satyawati, 2008). ROS have the capability to react with cellular components resulting in significant damage to cellular membranes. However, some enzymes such as peroxidase, catalase, etc., are able to break down these ROS into simpler substances that are less harmful to the plants thus controlling the levels of these ROS. In plant cell, these enzymes are considered as defensive team that protect the cells from oxidative damage (Mittler, 2002) and following salt stress condition, these enzymes tend to increase in amount (Mittova *et al.*, 2003).

Aerial yam (*Dioscorea bulbifera*) is in the family *Dioscoreaceae*. It is an indigenous edible crop cultivated for its cheap source of nutrient which is of economic importance to the rural dwellers of the Niger-Delta region of Nigeria. Not only do they depend on it as a supplement to other sources of energy giving food, it is also used as a herbal remedy for some common ailments like diabetes, dysentery, conjunctivitis and cholera, (ISSG, 2012). It is fast growing and can adapt to different soil types, weather and habitat conditions and therefore, having the potential of tolerating salt stress and providing food security in era of famine occasioned by climate change.

D. bulbifera is also one of the less cultivated and utilized crops, as such it is faced with extinction threat in this part of the world. In view of the foregoing, it becomes needful to call for more publicity and attention to the plant to enhance its conservation and improvement.

Simple sequence repeats (SSR) is a good molecular marker for diversity studies. It is highly polymorphic, co-dominant, abundant and evenly distributed in the genome and easy to assay, it reveals variation among plant species (Chakraborti *et al.*,

2011) and so capable of giving good results for diversity studies of the local landraces of *D. bulbifera*. This research work was set out to investigate the tolerance of *D.bulbifera* to sodium chloride stress through morphological, physiological and molecular studies using SSR markers.

1.2 Rationale of the study

The issue of climate change cannot be over emphasized as it is global and predicted to become worse in future. It is expected to increase the intensity of abiotic stresses on plants (Shrivastara and Kumar, 2015) leading to more reduction in yield of crops. Human activities to meet man's needs have resulted to the addition of salts to the top soil that eventually reduce productivity of plants. Inadequate fertilizer application, bush burning, and indiscriminate use of detergents have added much salt to the top soil which are washed down the soil by rain and flood to the root zones of plants. High soil salinity among others is a limiting factor for plant growth and productivity worldwide and its cumulative effect is of great concern to both agricultural sector and environmentalists.

Despite the effect of salinity on plants, some species of plants such as tomato (Srinieng and Karnchanatat, 2015) are reported to have the capacity of tolerating the stress conditions and grow to maturity. Although *Dioscorea bulbifera* is reported to grow rapidly and adapt to different soil types, weather and habitat conditions, Morisawa (1999) attributed its inability to thrive in coastal areas to be as a result of salt intolerance. There is need to investigate this claim, as other factors may directly or indirectly be responsible for this observation.

The choice of *D. bulbifera* for this research is based on the following facts: its indigenous state, its aggressive fast growing and high yielding characteristics, its uses,

its underutilization and threat of extinction. These characteristics make it a good candidate for this research. Hereupon, an understanding of its response to salt stress viz-a- viz its genetic diversity will provide the needed baseline data for efficient conservation and improvement of the species.

1.3 Objectives of the study

This study is designed to evaluate the specific effect of various levels of NaCl salinity on the morphology and physiology of *D. bulbifera* and the genetic diversities among the cultivars used for the study. The specific objectives are to:

1. Determine the cultivars of *D. bulbifera* that show tolerance to salinity through morphological studies.
2. Quantify some specific enzymes in the cultivars which are associated with salt tolerance. These are:
   (i.) Glucose- 6- phosphate dehydrogenase activity
   (ii) Peroxidase activity.
3. Determine the relationship between enzyme activity in *D. bulbifera* leaves and saline concentrations.
4. Determine the chlorophyll content of the cultivars in relation to saline concentrations.
5. Assess genetic variation among the cultivars of *D. bulbifera* using simple sequence repeats (SSR) markers.

CHAPTER TWO

LITERATURE REVIEW

2.1 Characteristics of *D. bulbifera*

Dioscorea bulbifera (aerial yam, air yam or air potato) is a crop plant belonging to the family *Dioscoreaceae*. Although considered as an aggressive weed (Croxton *et al.*, 2011) and included in the Global Compendium of weeds as an invasive plant (Randall, 2012), it is an important cultivated species of high economic value in tropical countries (Tortoe *et al.*, 2012). It is said to have originated from Asia and Africa (Lebot, 2009).

D. bulbifera is dioecious (that is, its male and female organs are borne on different individuals), herbaceous and monocotyledonous. Langeland *et al.* (2008) reported that *D. bulbifera* undergoes both sexual and asexual reproduction, and that is a reason for its rapid and wide spread growth habit. Whereas, the best soil for its growth is loamy soil rich in inorganic material (Wilkin, 2001), it cannot inhabit coastal areas and this has been attributed to its salt intolerance (Morisawa, 1999).

D. bulbifera is one of the most important species within its genus (Lebot, 2009). Most of its varieties of are cultivated for food and so serve as important food crop (Hammer, 1998). It also has a high therapeutic potential and has been used traditionally for the treatment of diarrhea, dysentery, conjunctivitis, fatigue and depression (Ghosh *et al.*, 2012). It is also used in the production of synthetic steroidal hormones for the manufacture of birth control pills (ISSG, 2012). It is classified as shown below:

Domain Eukaryota

Kingdom Plantae

Phylum	Spermatophyta
Sub phylum	Angiospermae
Class	Monocotyledonae
Order	Dioscoreales
Family	Dioscoreaceae
Genus	Dioscorea
Species	*Dioscorea bulbifera*

(Retrieved from https://en.m.wikipedia.org>wiki>Diosc..., June, 2018).

2.2 Chemical composition and anti-nutritional factor of *D. bulbifera*

Dioscorea bulbifera is a good source of carbohydrate, proteins, fats and mineral nutrients (Hussain Bhat *et al.*, 2019). It contains a quite number of micro nutrients such as iron, copper, zinc, boron, iodine, manganese and molybdenum (Taponjou *et al.*, 2013). The tuber is composed of 4.5% crude protein, 1.9% crude lipid, 87.5% nitrogen-free extract, 0.86% potassium, 0.14% magnesium and 0.12% phosphorus (Temple and Sen, 1993). The analysis of the bulbils flour indicated the presence of 6.22% dw moisture, 2.36% dw fat, 8.12% dw protein, 3.44% dw ash, 0.91% dw cellulose, 79.86% dw carbohydrate, 373.16 kcal/100g energy, 486mg/100g oxalic acid and 365.4mg/100g citric acid (Achy *et al.*, 2016).

Although *D. bulbifera* contain essential nutrients for good health, it may pose a health problem due to the presence of anti-nutritional factor. As such, proper processing is require before consumption (Bhandari and Kawabata, 2004). The wild species of *D. bulbifera* contain anti-nutritional factor such as, free phenols, tannins, hydrogen cyanide, total oxalate, amylase (Arinathan et al., 2009; Shajeela *et al.*, 2011) and phytate (Achy et al., 2016)

2.3 Salinity: Meaning, types and causes

Salinity can be defined as a measure of salt content (e.g. sodium chloride) in soil or water. From the agricultural point of view, it is occurrence of salt in amount more than what is necessary for plants (Yadav *et al.*, 2011). It is a serious environmental problem in the world (Jouyban, 2012). Soil is said to be saline if the electrical conductivity (EC) of the saturation extract in the root zone is above $4dSm^{-1}$ (approximately 40mM sodium chloride at 25^0C) and most plants are affected at this value of EC and even at lower value of EC (Jamil *et al.*, 2011).

Salinity may be grouped into primary and secondary salinity. Primary salinity is prevalent in the arid and semi-arid regions and occurs when salt accumulates in the soil or water of an area over a long period of time as a result of natural processes like weathering of rocks and wind and rain deposition (Jouyban, 2012). Secondary salinity involves the addition of salts from human activities like land clearing. The latter may take the form of "dry land salinity" or "irrigation- induced salinity". Dry land salinity occurs as a result of vegetation imbalance, that is, when deep rooted plant that has been removed by man is replaced with shallow- rooted plant that uses less water. This makes room for more water to seep from the soil to the ground water thereby raising the water table and concentrating the salts at the surface of the soil after evaporation. Irrigation- induced salinity occurs when excess water is applied to crops. This excess water moves to the groundwater thus raising the water table and salts to the surface (Arzani, 2008).

Salinity is one of the major sources of abiotic stresses and a major constrain to crop production and quality worldwide (Kumar *et al.*, 2013; Shahbaz and Ashraf, 2013). It has affected an estimated 45 million hectares of irrigated land and is expected to increase due to climate change (Munns and Tester, 2008). Soil salinity

has been much enhanced also as a result of agricultural practices (Zhu, 2001). Deforestation and clearing of agricultural lands have concentrated salts through capillary action in the root zone (Rengasmy, 2010).

2.4 Effect of salinity on plants morphology/ growth

Saline soils limit plants growth and yield (Jouyban, 2012; Paul, 2012) and no toxic substance restricts plant growth more than salt on world scale (Bernstein *et al.*, 1995; Xiong and Zhu, 2002). Not only does it severely limit plant growth, it also affects plants yield by way of tampering with the process of photosynthesis through reduction in chlorophyll content among others (Netondo *et al.*, 2004; Srinieng *et al.*, 2015). It has however been estimated that about 50% of present cultivated land would be lost by the year 2050 if salt stress persists (Wang *et al.*, 2003).

One of the essential elements for proper plant growth is water and it is absorbed by plant through the roots. Absorption of water by plant roots is controlled by the concentration of salts in the soil water as well as the water present in the plant. High soil salinity may cause inability of the plant to absorb enough water from the soil. This is because high salinity causes flow of water from the plants roots back into the soil resulting to inability of the plant to absorb enough water from the soil. The roots of different plants contain different amounts of salts that allow a flow of water from the soil into the plant roots. So, as the amount of salt in the soil increases, the water in the roots are drawn to the soil and the plants are unable to absorb enough water for their growth process thus resulting in stunted growth and poor yields of crops. In fact, the primary effect of excess salinity is that it reduces the amount of water uptake by the plant roots and in this way, causes poor growth.

Increased salinity causes undue concentration and absorption of ions, particularly chloride, which may turn out to be toxic to the plants with the tendency to terminate the assimilation of other major nutrients such as nitrogen, phosphorus, calcium, potassium and magnesium thereby leading to death of the plant (Abd EL-Azim and Ahmed, 2009; Jouyban, 2012; Srinieng *et al.*, 2015). Effect of salinity on growth of plant can be studied by examining its effect on the morphology of the plant. There is a consensus among researchers that salinity has negative impact on the morphology of plants. Higher concentrations of sodium chloride causes decrease in plant height of *Vicia faba* L. (Abdul Qados, 2011). Gónmez-Bellot *et al.* (2013) reported decrease in total root length of *Euonymus japonica* treated with salt. Ramezani *et al.* (2011) have also reported the same decrease in *Echium amoenum*. A general decrease in fresh or dry weight of plants by salinity has also been observed. Sodium chloride was observed to cause loss in fresh and dry weight of leaves and roots in canola plants (Bybordi *et al.*, 2010), root fresh weight of maize (*Zea mays* L.); an effect which increases with higher level of salinity (Usman *et al.*, 2012). Reduction in dry weight of *Solanum melongena* L. has also been reported by Chartzoulakis and Loupassaki (1997). Salinity reduces total leaf area of plants (Munns and Termaat, 1986). Romero *et al.* (1998) gave credence to this when they reported decrease in the leaf area of *Citrus* under salt stress condition. Jampeetong and Brix (2009) also reported decreased in leaf area and root length with salinity in *Salvinia natans*. Hence it is not surprising that sodium chloride decreased total leaf area and of course height of *Capsicum annuum* L. (Chartzoulakis and Klapaki, 2000). Furthermore, leaf area of *Solanum melongena* L. is reduced when sodium chloride concentration is greater than 10 mM (Chartzoulakis and Loupassaki, 1997).

Salinity causes reduction in chlorophyll concentration or contents of plants (Stepien and Johnson, 2009). Sodium chloride decreases chlorophyll content in *Prunus cerasus* (sweet cherry), (Erturk *et al.*, 2007). Findings by Chen *et al.* (2003) also revealed that there is reduction in concentration of chlorophyll at sodium chloride concentrations higher than 100mM in *Chrysanthemum indicum*. Just as Di Martino *et al.* (2003) recorded decrease in chlorophyll-a and b in *Spinacia oleraceae* (Spinach) exposed to salinity stress, Abdul Qados (2011) reported same decrease in *Vicia faba* L. and Srinieng *et al.* (2015) in tomato. Jampeetong and Brix (2009) reported reduction in chlorophyll contents of *Salvinia natans* treated with sodium chloride. Contrasting the reduction in chlorophyll concentration by salinity, Acosta- Motos *et al.* (2015) reported increase in chlorophyll levels in *Eugenia* treated with sodium chloride.

2.5 Effect of salinity on enzyme activity

Enzymes are biocatalysts. They regulate the rate at which chemical reactions occur in living organisms without themselves being altered at the end of the process. Without enzymes, most of the reactions in living organisms will not occur at a perceptible rate; enzymes are found to function in every aspect of cell metabolism and they are fundamental for any organism to survive. The antioxidant defense system in the plant cell is also enzymatic (Peng *et al.*, 2011).

Enzymes can be grouped based on their specific activities into two common groups: catabolic enzymes and anabolic enzymes. The catabolic enzymes function in the "breakdown" of substances in living organisms while anabolic enzymes functions in "build up" of substances in living organisms. Peroxidase, an enzyme commonly found in plants, animals and microorganisms has been suggested to function in the

breakdown of toxic hydrogen peroxide releasing oxygen gas and water (Petersen and Anderson, 2005).

Peroxidase uses several phenolic substrates for the breakdown of hydrogen peroxide and so is considered a general indicator for oxidative stress (Hiraya *et al.*, 2001). Other functions of peroxidase include cell wall lignification and degradation, defense responses to insects, pathogens and physical wounding. Researchers have found that increase in total peroxidase activities are often found during infection of higher plants by pathogen with highest increases in resistant plants (Novacky and Hampton 1968). Sodium chloride causes increased peroxidase activity in plants (Bybordi *et al.*, 2010; Weisany *et al.*, 2012).

Glucose-6 phosphate dehydrogenase is an enzyme that functions in the pentose phosphate cycle. The pentose phosphate cycle is considered as a major source of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and pentose needed for nucleic acid biosynthesis. The pentose phosphate pathway is central to plant metabolism (Kruger and Von Scheawen, 2003). Glucose 6- phosphate dehydrogenase (G6PDH) catalyses the first step of the oxidative pentose phosphate path way. It converts glucose 6-phosphate to 6 phospho gluconate and generates a molecule of NADPH for anabolic metabolism like the synthesis of fatty acids and amino acids. In plant tissues, different forms of G6PDH are seen. It is found in the cytosol, chloroplasts (Herbert *et al.*, 1979), peroxisomes (Knight *et al.*, 2001) and in the plastids of heterotrophic plant tissues (Journet and Douce, 1985). G6PDH deficient cells are highly sensitive to oxidative stress compared with cells showing endogenous G6PDH.

Water deficit in plants as a result of salt stress leads to the formation of ROS such as superoxide, hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet

oxygen which are highly reactive and may cause cellular damage through oxidation of lipids, proteins and nucleic acids (Apel and Hirt, 2004; Kumar *et al.*, 2013). This is the reason most plants cannot tolerate high salt concentrations of the soil and so cannot be grown on a salt affected land as reported by Glenn and Brown (1999). However, some plants have the ability to grow under salinity due to the presence of various mechanisms in them for salt tolerance (Flowers and Yeo, 1995). One possible defense mechanism involved in the tolerance response in plants is the presence of plant ROS network consisting of anti-oxidants and anti-oxidant enzymes for maintaining the levels of ROS. In plant cells, anti-oxidant enzymes such as peroxidase protect cells from oxidative damage (Mittler, 2002). The protection of cells by anti-oxidative enzymes is considered the main mechanism for resistance to oxidative damage (Manish *et al.*, 2011).

The activities of the anti- oxidative enzymes such as peroxidase etc. increase under salt stress (Mittova *et al.*, 2003, 2004; Panuccio *et al.*, 2014). Gulsen (2004) reported tolerant response in buffalo grasses to be associated with higher levels of peroxidase activity. In support of increase in anti-oxidant enzymes activities under salt stress, Weisang *et al.* (2012) recorded significant increase in catalase and peroxidase activities in *Glycine max* under salt stress conditions. Also in support, Gharsallah *et al.* (2016) reported that ascorbate peroxidase and catalase increase in both leaf and root tissue during all stages of the stress treatment within the San Miguel tolerant tomato plant.

2.6 Genetic diversity and simple sequence repeats

Plants and animals contain genes which are their basic building blocks. Diversity is the differences that occur among organisms of the same species or of

different species. The diversity in the genes (genetic diversity) enables the organism to adapt to changes in the environment; this is because plant diversity is the basis for the development of plant characters useful for human needs by breeders (Tanto and Demissie, 1996). Population genetic theory predicts that absence in genetic diversity will result in species inability to adapt to the changing selection pressure (Young and Merriam, 1992). It is therefore not surprising that the conservation of plant genetic diversity today is of great interest due to environmental imbalance resulting from human activities.

Genetic diversity which enables plant species to adapt and survive changes in the environment (NRC, 1991) can be detected using molecular markers and as such, development and use of molecular makers for plant diversity studies has been reported for a wide variety of plants. Different types of techniques are employed in estimation of genetic diversity: Random Amplified Polymorphic DNA (RAPD), DNA Amplification Fingerprinting (DAF), Simple Sequence Repeats (SSRs) and many others. Simple sequence repeats (SSRs) also known as micro- satellites are "stretches of DNA containing tandem repeating di, tri or tetra nucleotide units ubiquitously distributed throughout the eukaryotic genomes; they are thought to be the major source of genetic variation in quantitative traits" (Mahalakshmi *et al.*, 2002). The SSR has provided a high level of information for a variety of plant species and this has led to the initiation of SSR discovery programs for most agronomically important crops (Panaud *et al.*, 1996; Milbourne *et al.*, 1998).

Yan *et al.* (2014) developed fourteen micro satellite markers for *D. bulbifera* which showed high levels of polymorphism. These markers include: DBSSR1 to BDSSR14. Similarly, Silva et al. (2016) in their study developed an enriched genomic library for *Dioscorea bulbifera* and designed seven SSR primers— Db2, Db3, Db4,

Db5, Db6, Db7 and Db8— six of which they reported polymorphic. They used these six and added four polymorphic loci developed for other *Dioscorea* species thus resulting to ten polymorphic microsatellite markers in evaluating 42 air yam (*D. bulbifera*) accessions in Brazil. From the ten polymorphic primers, thirty-three alleles (bands) were found with two to four alleles per locus. An average of 3.3 alleles per locus and 100% polymorphism was recorded with the average polymorphic information content of 0.595.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Glassware's and equipment

Glasswares and equipment used for this study were: spectrophotometers (Genova nano 24V DC, 50/60 Hz 50VA, Bibly Scientific Ltd, Uk and Perkin Elmer Cetus, Lambda 3B), Eppendorf centrifuge (5424R), UV Transilluminator (Cambridge, UK, camera, Canon EOS70D, Japan), freezer (U40HEFG), grinder (MM400), automated thermal cycler (Thermo Fisher Scientific Oy, Finland), Eppendorf thermo mixer (F1.5), incubator, microwave oven, weighing balance, forceps, Eppendorf tubes, sample tubes, micro pipettes, magnet and ruler.

3.1.2 Chemicals and reagents

Chemicals and reagents used for this study which include: sodium chloride, acetone, guaiacol, hydrogen peroxide, phosphate buffer, glucose-6 phosphate, beta Nicotinamide Adenine Dinucleotide Phosphate, chloroform, propanol, ethanol, and magnesium chloride were purchased from a chemical store. Primers and other chemicals for molecular studies were purchased from Inqaba Biotec West Africa Ltd.

3.1.3 Plant materials

Bulbils of *D. bulbifera* with various shapes (Plate 1) were obtained from local farmers in Abia State, Akwa Ibom State and Cross River State and established in Biological Sciences Research Farm to have the various lines (Table 1) for the experiment.

PLATE 1: *Dioscorea bulbifera* bulbils of various shapes

(a) Elongated shape
(b) Round shape
(c) Angular shape

TABLE 1

Source of *Dioscorea bulbifera* bulbils for morphological and molecular studies and characteristics

Lines	Location of collection	Shape of the bulbil
Dboh1.	Ohafia	Elongated
Dboh2.	Ohafia	Elongated
Dboh3.	Ohafia	Elongated
Dboh4.	Ohafia	Elongated
Dboh5.	Ohafia	Elongated
Dbak6.	Akpabuyo	Elongated
Dbak7.	Akpabuyo	Elongated
Dbak8.	Akpabuyo	Round
Dbak9.	Akpabuyo	Angular
Dbak10.	Akpabuyo	Elongated
Dbor11.	Oron	Elongated
Dbor12.	Oron	Elongated
Dbor13.	Oron	Elongated
Dbor14.	Oron	Elongated
Dbor15.	Oron	Round
Dbok16.	Okobo	Round
Dbok17.	Okobo	Round
Dbok18.	Okobo	Elongated
Dbok19.	Okobo	Round
Dbok20.	Okobo	Elongated
Dboh21.	Ohafia	Elongated
Dboh22.	Ohafia	Round
Dbok23.	Okobo	Round
Dbok24.	Okobo	Elongated
Dbor25.	Oron	Elongated
Dbor26.	Oron	Elongated
Dbak27.	Akpabuyo	Elongated
Dbak28.	Akpabuyo	Round
Dbok29.	Okobo	Round
Dbor30.	Oron	Elongated
Dbak31.	Akpabuyo	Elongated
Dboh32.	Ohafia	Round
Dbak33.	Akpabuyo	Elongated
Dbok34.	Okobo	Elongated
Dbok35.	Okobo	Elongated
Dbor36.	Oron	Elongated
Dbor37.	Oron	Round
Dbak38.	Akpabuyo	Elongated
Dboh39.	Ohafia	Elongated
Dboh40	Ohafia	Elongated

3.1.4 Soil sample for the study and experimental location

Top soil (0 -20cm depth) was randomly collected from three locations at the Biological Science Experimental Farm, University of Calabar, and bulked to form a composite sample. Experiment was conducted in the Greenhouse at the Department of Genetics and Biotechnology, University of Calabar, Calabar.

3.2 Methods

3.2.1 Experimental design

Randomized Complete Block Design (RCBD) with three (3) replicates was used for the study. Treatment was the various concentrations of NaCl used and block was the various lines of *D. bulbifera*.

3.2.2 Experimental procedures

Known weight (5kg) of composite samples of soil was put into labeled polyethylene bags with drainage holes at the base. The bulbils of *D. bulbifera* were planted in poly bags, three bulbils per bag and watered with 250ml of distilled water daily until sprouting was established. After sprouting, the plants were reduced to one per bag. At four weeks after sprouting, the plants were treated with 250ml of the various concentrations of sodium chloride solution (0, 100, 200 and 300mM) twice a week for four weeks.

Morphological data on vine length, number of leaves per plant, leaf area, leaf length, petiole length were measured and recorded every week from the day of treatment application to two weeks after treatment application. Root length, number of bulbils per plant, as well as weight of bulbils per plant were obtained at maturity. Biochemical indices such as chlorophyll concentration of the leaves, Glucose 6-phosphate dehydrogenase and Peroxidase activities were measured

spectrophotometrically from the leaf extracts of the plant samples two weeks after the last treatment application.

3.2.3 Determination of peroxidase activity

The peroxidase activity was determined spectrophotometrically. The steps used in the peroxidase assay included: sample collection, extract preparation and test/ peroxidase activity determination.

Sample collection: The leaf samples of *D. bulbifera* were aseptically taken from the plants using forceps and inserted into labelled specimen tubes, covered with its cover slip and then taken to the laboratory.

Extract preparation: The leaf samples collected above were weighed and 0.5g of each of the leaf samples was frozen at -80^0C and ground using a mortar and pistol and transferred into 2ml eppendorf tubes. Then, 0.8ml of the extraction buffer (phosphate buffer containing polyvinyl fluoride, PVF) was added, vortexed and centrifuged at 15000rpm for 4min. The supernatant was collected using micropipette and kept on ice at 4^0C

Test/peroxidase activity determination: To determine the peroxidase activity, 0.6ml of the extract was pipetted into a cuvette and 0.1ml guaiacol, 2ml of buffer solution, and 0.1ml hydrogen peroxide were added; hydrogen peroxide being the last to be added.

The spectrophotometer was turned on, allowed to boot and the wavelength set at 436 nm. The cuvette containing the reaction mixture was placed in the spectrophotometer and absorbance values read and recorded after 1min, 2min, 3min, 4min and 5min. Peroxidase specific activity was then calculated using the formula:

Enzyme specific activity units g^{-1} f wt = $[500/\Delta t] \times [1/1000] \times [TV/UV] \times [1/F\ wt]$

Where Δt = change in time/minute

TV = total volume of the extract (ml)

UV = volume used (ml)

F wt = weight of the fresh leaf tissue (g)

3.2.4 Determination of Glucose-6 Phosphate dehydrogenase (G6PDH) activity

The activity of glucose-6-phosphate dehydrogenase was determined spectrophotometrically. The steps used for the assay included samples collection, Extracts preparation and glucose 6 phosphate dehydrogenase activity determination.

Samples collection: The leaves of the plants were harvested at mid-day into specimen tubes, covered and taken to the laboratory.

Extract preparation: The plant samples were frozen and ground using mortar and pestle, hydrated with an extraction buffer (0.8ml mixed phosphate buffer, pH 7.0 and incubated at 65^0C. The homogenate was centrifuged at 15000rpm for 4 minutes. The supernatant was collected into an Eppendorf tube and stored on ice.

G6PDH activity determination: G6PDH activity was then measured in a spectrophotometer at wavelength 340nm. The reaction mixture that was pipetted into a cuvette included: 0.6ml of the extract, 2.0 ml of purified water, 2.0 ml of buffer, 0.1 ml of Glucose 6 phosphate, 0.1 ml of beta-Nicotinamide Adenine Dinucleotide phosphate (NADP). The reaction mixture was equilibrated to 25^0C. The reduction of $NADP^+$ was measured every 1 min at 340nm absorbance.

Enzyme activity in units/ml was calculated from the formula:

Units/ml enzyme = Δ A340 nm/minute/ 6.22V

Where:

V = volume in millilitres of enzyme used.

3.2.5 Determination of chlorophyll content

The chlorophyll contents of the leaf were determined using acetone incubation method. The steps used also included samples collection, extracts preparation and chlorophyll content determination.

Samples collection: The leaf samples of the plant were collected into specimen tubes, covered and taken to the laboratory.

Preparation of the extracts: To prepare the extracts, 2g of each of the leaf tissues samples of *D. bulbifera* was placed in a 2.0ml Eppendorf tubes containing Zirconia beads and frozen at -80^0C using freezer. The leaf tissues were crushed using grinder and 20ml of 80% acetone added to the crushed tissue and incubated at 25^0C for 10 minutes using Eppendorf thermo mixer. Subsequently, the mixture was centrifuged at 15000rpm for 4 minutes and the supernatant pipetted into a cuvette.

Determination of chlorophyll content: The supernatant in the cuvette was placed in a spectrophotometer and absorbance values read and recorded at wavelengths of 643nm and 663nm. The chlorophyll content was calculated from the formula as described by Strickland and Parsons (1972) as follows:

Chl a = $(11.6\ A_{663} - 1.3\ A_{643})\ VX^{-1}$

Chl b = $(19.1\ A_{643} - 4.7\ A_{663})\ VX^{-1}$

Where chl a and chl b contents are in mg g-1 FW

A663 and A643 are absorbance at 663 and 643nm

V = volume (ml) of 80% acetone used

X = Fresh weight of sample used

3.2.6 DNA extraction

Genomic DNA was extracted from fresh leaf apex of young leaves using cetyl trimethyl ammonium bromide (CTAB) method as described by Mignouna *et al.*

(1998). Young fresh leaf samples were collected and each placed in 2.0ml Eppendorf tubes. Zirconia bead was added to each of the tubes and frozen in a -80^{0}C freezer. The frozen samples were then crushed using a grinder after which the beads were removed with the use of a magnet. Then, 500µl of CTAB buffer was added to each tube mixed using a vortex mixer and incubated for one hour at 65^{0}C at 300rpm using an Eppendorf thermo mixer. Afterwards, the samples were centrifuged at 15000rpm for 20 minutes using an Eppendorf centrifuge. The supernatants were pipetted into a new 2.0 ml Eppendorf tubes and 500µl of chloroform were added to each tube. The mixtures in each tube were mixed by inversion and centrifuged. The supernatants were collected into new tubes and chloroform added to each again, centrifuged at 15,000rpm for 20 minutes and supernatants collected. Then, 500µl iso propanol was added, centrifuged at 15,000rpm for 20 minutes and decanted. Again, 500µl of 70% ethanol was added, centrifuged, decanted and dried. TE buffer (50µl, pH7.5) was added, incubated for 5minutes and the DNA stored in freezer until needed for further analysis.

3.2.7 DNA qualification and quantification

To ascertain the quality of the extracted DNA, agarose gel electrophoresis was employed whereas it was quantified using a spectrophotometer at 260nm. DNA with high concentrations (concentrations above 25.0 ng/μL) were diluted in TE buffer to obtain DNA concentration of 25.0 ng/μL

3.2.8 Polymerase chain reaction (PCR)

A total of ten SSR primer pairs were used in the study (Table 2). The primers were diluted in nuclease free water as stated by the manufacturing company to have a 100µm stock solution which was vortex, incubated briefly and centrifuged for 1

minute. A working solution was prepared from the stock solution by pipetting 20µl into a new Eppendorf tube and 180µl of nuclease free water added. The PCR cocktail was prepared. The cocktail included 2.0µl of 10X tag buffer, 2.5µl of $MgCl_2$, 1µl dNTPs, 0.4µl of forward primer and reverse primer, 1µl of 25ng/ µl template DNA, 2.0 µl tag polymerase and making the total volume to 20µl with nuclease free water. The reaction was conducted in a 96-well microtitre plate using an automated thermal cycler. The PCR programme consisted of initial denaturation at 95^0C for 15 minutes, followed by 35 cycles of denaturation at 95^0C for 30s, annealing at 45- 55^0C for 30s and extension at 72^0C for 1 min, with a final extension step at 72^0C for 5 min.

3.2.9 PCR products analysis

The PCR products were analyzed using agarose gel electrophoresis. The 1.5% agarose gel solution used was prepared by dissolving 3g of agarose in 200ml of TAE buffer, homogenated by heating in a microwave oven for 4 minutes and allowed to cool briefly for a minute. Then, 14µl Ethydium bromide was added to the gel solution, mixed properly and poured into the electrophoretic tray with the comb inserted and then allowed to solidify. At solidification, the comb was removed and the tray was placed in the electrophoretic tank fully immersed by the TAE buffer. Then, 1µl loading dye was added to the PCR products which were transferred to the wells. PCR was run at 80v for one hour twenty minutes after which the gel was viewed in an UV Transilluminator and the picture taken with a camera.

TABLE 2

Characteristics of 10 SSRs primers used for the diversity studies of the *D. bulbifera*

S/N	Name	Forward and reverse sequence	Annealing Temp. (^{0}C)
1.	Db2	F: CACGACCTCCTGGAAGACAACT	56.7
		R: ATATAGCACGGGAGGCACAAAC	54.84
2.	Db5	F: TGTCTATTATATTGCTCTTTCT	45.52
		R: CGTTTCTAATTTCTGGGTAT	45.63
3.	DBSSR4	F: ACACACACACACAGAGAGAGAG	54.84
		R: GAAAAGGAGAAGCCGAAT	45.77
4.	Db3	F: TTTTACCCAGGATTTAGAAGAA	47.38
		R: GGACTGGAGCCACAAGATT	51.09
5.	Db6	F: AAGCCGGTATCATTCAACAAAA	49.25
		R: CCCTCGCCAACATCAAGTAA	51.78
6.	Db7	F: CCGCAAGGCTCAAAAAGTTAGG	54.84
		R: TCGTGGATGAAGATGGGTGGAC	56.7
7.	Db8	F: TCCCAAGAAATCCAGAATA	44.62
		R: ATGCATGCCAAAACAAATA	42.46
8.	DBSSR2	F: ACACACACACACAGAGAGAGAG	54.84
		R: AACGCATCCCACCACTTC	50.32
9.	DBSSR3	F: ACACACACACACAGAGAGAGAG	54.84
		R: CACGATGGAGGAACACTT	48.04
10.	DBSSR5	F: ACACACACACACAGAGAGAGAG	54.84
		R: TTGATTGAAAAGGGAGGCT	46.77

3.2.10 SSR fragment analysis

The fragment sizes in base pairs for each genotype across SSR markers were converted to binary data, where alleles were transformed into presence (1) or absence (0) of an SSR band. The cluster pattern of the genetic diversity was constructed using Darwin 5.0 software. Polymorphic Information Content (PIC) was estimated using Alerquin software.

3.3 Measurement of morphological parameters

3.3.1. Vine length

The vine length was measured using a meter rule. A thread was used to take the length of the plant from the bottom to the tip of the stem which the measurement was then taken with the use of a meter rule.

3.3.2. Number of leaves per plant

The number of leaves from each plant was gotten by counting the leaves one after the other.

3.3.3. Root length

The root length was measured with a meter rule. The measurement started from the base to the tip of the root.

3.3.4. Number of bulbils per plant

The number of bulbils per plant was obtained by counting the bulbils from each plant.

3.3.5. Weight of bulbils per plant

The weight of the bulbils was determined with the use of an electronic weighing balance. All of the bulbils per plant were placed on the weighing balance and readings recorded.

3.3.6 Leaf length

The leaf length was measured using a meter rule. The rule was placed between the stalk and the apex of each leaf and measurement taken through the mid rib.

3.3.7. Petiole length

The petiole length was measured with the use of a meter rule. The meter rule was placed at the base of the plant running down the petiole to the node.

3.3.8. Leaf area

The leaf area was obtained by graphing the leaves on a paper. The squares covered by the leaves were counted and recorded as the area of the leaves in cm^2.

3.3.9. Leaf colour

The leaf colour was determined by comparing the colour of the leaf with a colour chart.

3.4 Data analysis

Data were analyzed using analysis of variance (ANOVA) and significant differences were determined using least significant difference (LSD).

CHAPTER FOUR

RESULTS

4.1 Results

4.1.1 Peroxidase activity

Peroxidase activity increases with increasing concentration of sodium chloride as indicated in Table 3. The mean value for peroxidase activity for line 2 was recorded as 0.007±0.00, 0.019±0.00, 0.028±0.00 and 0.035±0.00 at 0.00mM, 100mM, 200mM and 300mM, respectively, which were significantly different from each other. For line 7, the mean values recorded were 0.006±0.00, 0.019±0.00, 0.027±0.00 and 0.032±0.00 at 0.00mM, 100mM, 200mM and 300mM respectively. Line 10 recorded 0.012±0.00, 0.023±0.00, 0.030±0.00 and 0.036±0.00 at 0.00mM, 100mM, 200mM and 300mM, respectively.

4.1.2 Glucose-6-phosphate dehydrogenase activity

The mean and standard error values of glucose-6-phosphate dehydrogenase activity in leaves of *D. bulbifera* treated with the different concentrations of the salt is shown in Table 4. Sodium chloride causes increase in glucose-6-phosphate dehydrogenase activity in *D. bulbifera*. The enzyme activity increases as the concentration of the sodium chloride increases. For line 2, the values were 0.008±0.087 at 0.00mM, 0.024±0.083 at 100mM, 0.043±0.078 at 200mM and 0.055±0.075 at 300mM concentrations of the salt. For line 7, the values were 0.005±0.088, 0.027±0.82, 0.048±0.076 and 0.062±0.073 at 0.00mM, 100mM, 200mM and 300mM concentrations of the salt, respectively. For line 10, the values were 0.004±0.087 at 0.00mM, 0.023±0.083 at 100mM, 0.041±0.078 at 200mM and 0.053±0.075 at 300mM. For line 15, the values were 1.506±0.171 at 0.00mM, 1.528±0.179 at 100mM, 1.542±0.185 at 200mM and 1.555±0.190 at 300mM.

TABLE 3

Peroxidase activity in leaves of *D. bulbifera* treated with different concentrations of sodium chloride

LINES	TREAMENTS				
	0.00mM	100mM	200mM	300mM	LSD
2.	0.01 ± 0.00^{d}	0.02 ± 0.00^{c}	0.03 ± 0.00^{b}	0.04 ± 0.00^{a}	0.002
7.	0.01 ± 0.00^{d}	0.02 ± 0.00^{c}	0.03 ± 0.00^{b}	0.03 ± 0.00^{a}	
10.	0.01 ± 0.00^{d}	0.02 ± 0.00^{c}	0.03 ± 0.00^{b}	0.04 ± 0.00^{a}	
15.	0.01 ± 0.00^{d}	0.02 ± 0.00^{c}	0.03 ± 0.00^{b}	0.03 ± 0.00^{a}	
18.	0.01 ± 0.00^{d}	0.01 ± 0.00^{c}	0.02 ± 0.00^{b}	0.03 ± 0.00^{a}	

Values are mean ± S.E of peroxidase activity determinations.

Means with different superscript letter on the same row indicates significant difference ($p<0.05$).

TABLE 4

Glucose-6-phosphate dehydrogenase activity in the leaves of *D. bulbifera* treated with different concentrations of sodium chloride

LINES	TREATMENTS				
	0.00mM	100mM	200mM	300mM	LSD
2.	0.01 ± 0.09^{d}	0.02 ± 0.08^{c}	0.04 ± 0.08^{b}	0.06 ± 0.08^{a}	0.003
7.	0.01 ± 0.09^{d}	0.03 ± 0.08^{c}	0.05 ± 0.08^{b}	0.06 ± 0.07^{a}	
10.	0.01 ± 0.09^{d}	0.02 ± 0.08^{c}	0.04 ± 0.08^{b}	0.05 ± 0.08^{a}	
15.	1.51 ± 0.17^{d}	1.53 ± 0.18^{c}	1.54 ± 0.19^{b}	1.56 ± 0.19^{a}	
18.	1.50 ± 0.17^{d}	1.52 ± 0.18^{c}	1.54 ± 0.18^{b}	1.55 ± 0.19^{a}	

Values are mean ± S.E of glucose-6 phosphate dehydrogenase activity determinations.

Means with different superscript letter on the same row indicates significant difference ($p<0.05$).

Line 18 recorded 1.504±0.170 at 0.00mM, 1.524±0.178 at 100mM, 1.539±0.184 at 200mM and1.553±0.190 at 300mM concentrations of the salt. The increases were significant at p-level of 0.05.

4.1.3 Chlorophyll-a

Table 5 gives the means of chlorophyll-a concentration of the leaves of the *Dioscorea bulbifera* treated with the different concentrations of the sodium chloride. The table showed reduction in the chlorophyll-a content of the plant by the salt. Generally, the 0.00mM recorded higher values while the 300mM recorded lowest values, although lines 3, 9, 11, 12 recorded higher values at100mM which were not significantly different from the 0.00mM. Line 1 recorded a value of 139.7±0.00, 139.9±0.00, 136.2±0.00 and 134.6±0.00 at 0.00mM, 100mM, 200mM and 300mM, respectively. Line 2 recorded 144.3±0.00 at 0.00mM, 144.0±0.00 at 100mM, 139.6±0.00 at 200mM and 137.4±0.00 at 300mM. Line 3 recorded 143.4 at 0.00mM, 143.7±0.00 at 100mM, 138.2±0.00 at 200mM and 137.8±0.00 at 300mM.

4.1.4 Chlorophyll b

The effect of sodium chloride on chlorophyll-b content of *D. bulbifera* is shown in Table 6. The Table showed that sodium chloride reduced the chlorophyll-b content of the plant. The reduction increased with increasing concentrations of the sodium chloride. For instance, from the Table 11, line 19 recorded 232.8±0.00 at 0.00mM, 232.7±0.00 at 100mM, 223.8±0.00 at 200mM and 102.6±0.00 at 300mM. Line 20 recorded a mean of 232.5±0.00 at 0.00mM, 232.2±0.00 at 100mM, 224.4±0.00 at 200mM and 104.9±0.00 at 300mM concentrations of the sodium chloride.

TABLE 5

Chlorophyll-a content (mgg^{-1} FW) of *D. bulbifera* treated with different concentrations of sodium chloride

LINES	TREATMENTS				
	0.00mM	100mM	200mM	300mM	LSD
1.	139.70±70.48^{a}	139.90±68.90^{a}	136.20±101.02^{b}	134.60±116.81^{c}	0.51
2.	144.30±38.69^{a}	144.00±40.47^{a}	139.60±71.28^{b}	137.40±89.93^{c}	
3.	143.40±44.16^{a}	143.70±42.30^{a}	138.20±82.90^{b}	137.80±86.38^{c}	
4.	144.20±39.28^{a}	144.00±40.47^{a}	139.40±72.89^{b}	138.00±84.63^{c}	
5.	146.20±28.31^{a}	146.00±29.33^{a}	142.60±49.33^{b}	139.70±70.48^{c}	
6.	146.80±25.37^{a}	146.40±27.32^{a}	143.30±44.79^{b}	139.20±74.51^{c}	
7.	146.50±26.82^{a}	146.20±28.31^{a}	142.70±48.67^{b}	139.90±68.90^{c}	
8.	147.10±23.96^{a}	146.40±27.32^{b}	144.30±38.69^{c}	140.80±62.02^{d}	
9.	152.10±6.40^{a}	152.30±5.93^{a}	147.90±20.40^{b}	143.90±41.07^{c}	
10.	151.90±6.89^{a}	151.50±7.92^{a}	147.90±20.40^{b}	143.40±44.16^{c}	
11.	152.40±5.71^{a}	152.50±5.48^{a}	148.50±17.92^{b}	144.30±38.69^{c}	
12.	152.70±5.05^{a}	152.80±4.84^{a}	148.30±18.73^{b}	144.20±39.28^{c}	
13.	155.80±0.61^{a}	155.70±0.69^{a}	152.90±4.63^{b}	148.00±19.98^{c}	
14.	157.10±0.03^{a}	157.30±0.01^{a}	154.10±2.51^{b}	149.40±14.50^{c}	
15.	154.90±1.45^{a}	154.40±2.08^{b}	152.80±4.84^{c}	147.80±20.83^{d}	
16.	155.20±1.13^{a}	155.60±0.77^{a}	153.40±3.67^{b}	149.00±15.97^{c}	
17.	204.10±486.85^{a}	204.40±493.13^{a}	199.60±397.45^{b}	192.40±273.27^{c}	
18.	204.20±488.94^{a}	204.00±484.77^{a}	199.00±386.22^{b}	193.70±293.97^{c}	
19.	204.40±493.13^{a}	204.10±486.85^{a}	201.10±426.25^{b}	194.50±307.09^{c}	
20.	204.20±488.94^{a}	204.10±486.85^{a}	199.40±393.69^{b}	194.00±298.86^{c}	

Values are mean ± S.E of chlorophyll-a determinations.

Means with the same superscript letter on the same row are not significantly different ($p<0.05$)

TABLE 6

Chlorophyll-b content ($mgg^{-1}FW$) of *D. bulbifera* treated with different concentrations of sodium chloride

LINES	TREATMENTS				
	0.00mM	100mM	200mM	300mM	LSD
1.	192.10±0.00^a	191.30±0.00^a	140.80±0.00^b	34.70±0.00^c	5.4
2.	189.80±0.00^a	189.20±0.00^a	140.70±0.00^b	34.10±0.00^c	
3.	196.00±0.00^a	195.40±0.00^a	143.60±0.00^b	32.90±0.00^c	
4.	187.70±0.00^a	187.10±0.00^a	140.20±0.00^b	31.20±0.00^c	
5.	204.40±0.00^a	203.60±0.00^a	166.70±0.00^b	73.40±0.00^c	
6.	206.90±0.00^a	206.70±0.00^a	168.20±0.00^b	75.20±0.00^c	
7.	207.60±0.00^a	207.60±0.00^a	166.90±0.00^b	74.60±0.00^c	
8.	206.20±0.00^a	206.40±0.00^a	168.40±0.00^b	74.90±0.00^c	
9.	213.70±0.00^a	212.90±0.00^a	179.60±0.00^b	79.80±0.00^c	
10.	212.90±0.00^a	219.90±0.00^a	182.30±0.00^b	82.20±0.00^c	
11.	217.30±0.00^a	216.10±0.00^a	179.90±0.00^b	87.10±0.00^c	
12.	214.20±0.00^a	214.00±0.00^a	176.80±0.00^b	85.70±0.00^c	
13.	219.20±0.00^a	218.80±0.00^a	211.00±0.00^b	99.80±0.00^c	
14.	214.80±0.00^a	213.90±0.00^a	209.80±0.00^b	99.20±0.00^c	
15.	212.40±0.00^a	212.40±0.00^a	210.10±0.00^b	98.60±0.00^c	
16.	216.20±0.00^a	215.80±0.00^a	210.70±0.00^b	99.20±0.00^c	
17.	230.90±0.00^a	230.20±0.00^a	223.30±0.00^b	102.20±0.00^c	
18.	232.40±0.00^a	231.90±0.00^a	223.80±0.00^b	103.70±0.00^c	
19.	232.80±0.00^a	232.70±0.00^a	223.80±0.00^b	102.60±0.00^c	
20.	232.50±0.00^a	232.20±0.00^a	224.40±0.00^b	104.90±0.00^c	

Values are mean ± S.E of chlorophyll-b determinations.

Means with the same superscript letter on the same row are not significantly different ($p<0.05$)

4.1.5 Genetic diversity characteristics of the SSR markers

The summary of the genetic diversity characteristics generated from the 10 SSR markers used for this study is shown in Table 7. Marker Db7 had the highest major allele frequency of 0.51 while marker Db6 had the lowest frequency of the major allele of 0.22. The average frequency of the major allele was recorded as 0.37. Generally, the allele frequency of all the markers was below 0.95, indicating that they were all polymorphic in character. DBSSR5 recorded the highest number of allele detected (10 alleles). This was followed by Db2 and Db5 which recorded 9 alleles each. Db3 and DBSSR4 recorded 8 alleles each while Db8, DBSSR3, DBSSR2 and Db7 recorded 7, 6, 5 and 3 alleles, respectively.

Gene diversity was high, ranging from 0.60 in Db7 to 0.93 in Db6 with a mean of 0.76. The discriminatory power of each SSR marker was assessed by calculating the Polymorphic Information Content (PIC) values. The PIC value ranged between 0.33 in Db7 and 0.87 in DBSSR5 with a mean value of 0.7645. Apart from the Db7, the PIC of all the markers was above 0.50 indicating they were polymorphic.

Plate 2 showed SSR marker (Db2) profile generated for the *D. bulbifera* lines used in the study. Generally, the pattern of movement of the DNA bands in the gel distinguishes one line from the other.

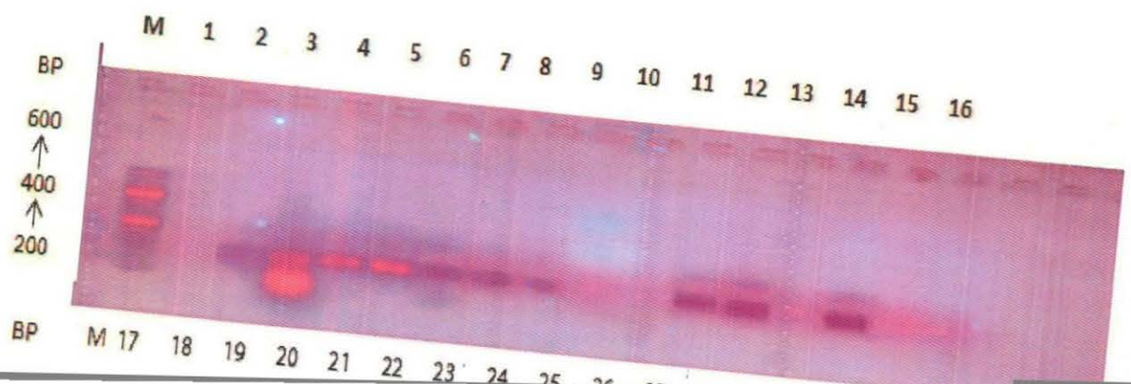
4.1.6 Principal component analysis/ Analysis of genetic variance

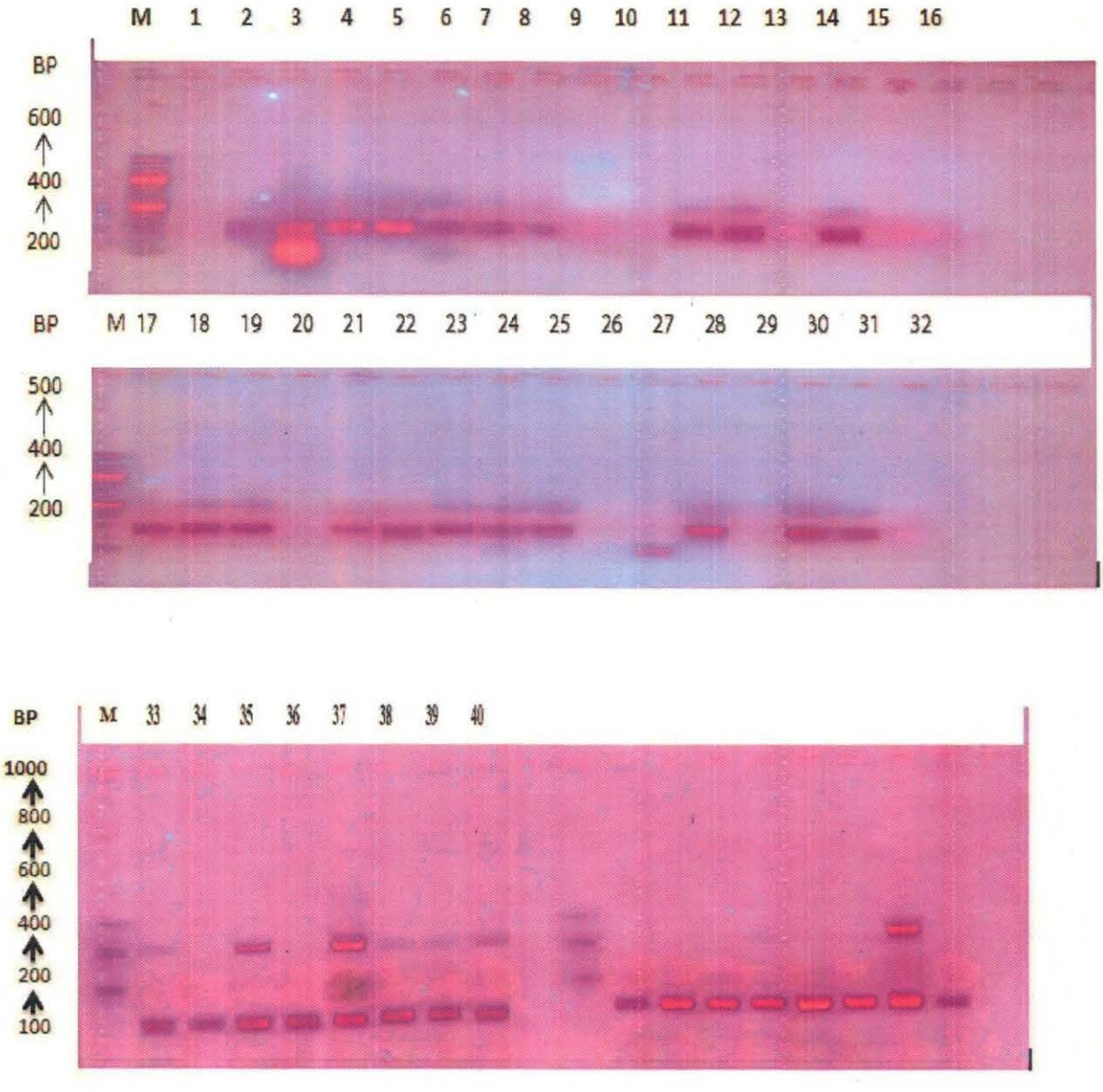
The principal components, eigen values and genetic variation among the forty samples of the *D. bulbifera* is recorded in Table 8. The Principal Component Analysis (PCA) revealed five components which contributed to high genetic variation (67.48%) among the lines of the *D. bulbifera* used for the study. This means that 67.48% of the variations in molecular data are attributable to variations in the DNA molecules of the different

TABLE 7

Genetic diversity attributes of the ten SSR markers used to analyze forty lines of *D. bulbifera*

S/N	Marker	Major. Allele. Frequency	Allele No	Gene Diversity	PIC
1	Db2	0.44	9.00	0.68	0.78
2	Db3	0.31	8.00	0.82	0.87
3	Db5	0.33	9.00	0.85	0.85
4	Db6	0.22	6.00	0.93	0.76
5	Db7	0.51	3.00	0.60	0.33
6	Db8	0.42	7.00	0.63	0.79
7	DBSSR5	0.34	10.00	0.81	0.88
8	DBSSR2	0.37	5.00	0.80	0.79
9	DBSSR3	0.41	6.00	0.66	0.74
10	DBSSR4	0.35	8.00	0.83	0.87
	Mean	**0.37**	**7.10**	**0.76**	**0.76**





Legend

M ladder

BP basepairs

1 -40 lines of *D. bulbifera* used for the study

PLATE 2: SSR primer (Db2) profile generate for *Dioscorea bulbifera*

TABLE 8

Eigen values for the principal components analysis of the 40 lines of the *Dioscorea bulbifera*

Principal components	Eigen values	% variation	Cumulative variation
1	0.658	28.63	28.63
2	0.345	15.00	43.63
3	0.260	11.32	54.95
4	0.158	6.89	61.84
5	0.129	5.64	67.48

lines. Principal component1 showed an eigen value of 0.658 and contributed 28.63% to the total genetic variation observed in the lines of *Dioscorea bulbifera*. The second principal component had an eigen value of 0.345 and contributed 15% to the total variation observed in the population. Principal component 3 had an eigen value of 0.260 and 11.32% contribution to the genetic variation. Principal component 4 and 5 had an eigen value of 0.158 and 0.129 and contributed to 6.89% and 5.64%, respectively, to the total genetic variation observed among the lines of *D. bulbifera*.

4.1.7 Dendrogram

The dendrogram (FIG. 1) demonstrates the relationship among the lines of *D. bulbifera*. It groups the lines into two main clusters from the average distant point denoted by a red dot at the middle, with the first cluster having 39 members and the other containing only one member. The dendrogram further divided the cluster 1 into five sub-clusters which indicates the existence of genetic diversity among the lines used. Lines Dbok16, Dbor15, Dbok23 and Dbak8 belong to the same cluster and also have distant relationship with other lines (Fig. 1).

4.1.8 Analysis of molecular variance (AMOVA)

The analysis of molecular variance (AMOVA) as shown in Table 9 reveals a higher genetic variation (76.45%) among the population than within population variation (23.55%) inferred from molecular characteristics of the *D. bulbifera* used for the study. This is to say that 76.45% of the genetic variation in the molecular data was attributable to variations in DNA molecules of the different samples while 23.55% of the variation was attributable to the interaction between the environment and DNA molecules of *D.*

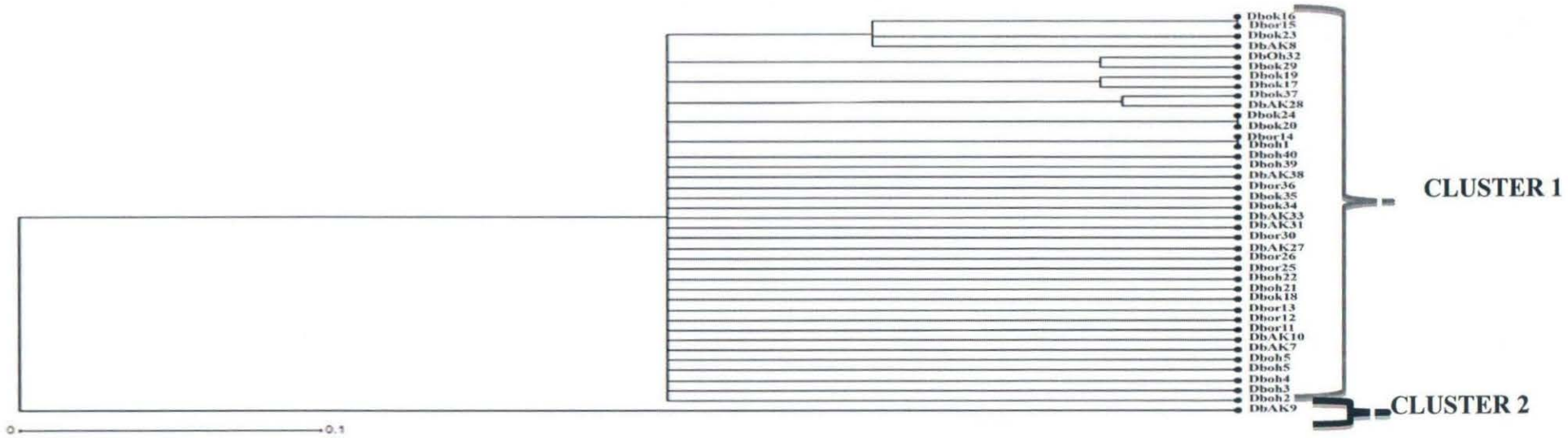


FIG. 1: Dendrogram demonstrating the relationship among the forty lines of *D. bulbifera* based on DNA

Key:

Dbok *D. bulbifera* collected from Okobo, Akwa Ibom State

Dbor *D. bulbifera* collected from Oron, Akwa Ibom State

DbAK *D. bulbifera* collected from Akpabuyo, Cross River State

Dboh *D. bulbifera* collected from Ohafia, Abia State

TABLE 9

AMOVA table for SSR analysis of forty samples of *D. bulbifera*

SOV	DF	TSS	MSS	Estimated Variance	% mol. Variance	p-value	Phqt
Among pop.	**12**	192.3	16.025	1.234	76.45	0.010	0.587
Within pop.	27	203.89	6.82	8.775	23.55		
Total pop.	39	295.11	7.755	10.005	100		

Key: Phqt = estimate of the population genetic differentiation based on permutation

bulbifera. The fixation index from the table is 0.587 which gives a significant variation ($p<0.05$)

4.1.9 Vine length

The effect of various concentrations of sodium chloride on the vine length of *D. bulbifera* are shown in Table 10 which gives the means and standard errors of vine length of *D. bulbifera* treated with various concentrations of sodium chloride, Sodium chloride causes reduction in vine length of the plant. This is seen in lines 1, 2, 4 etc. For line 1, a value of 180.25±0.00 was recorded for 0.00mM of the salt, 176.0±0.00 for 100mM, 163.25±0.00 for 200mM and 151.0±0.00 for 300mM. Line 2 has its values as 183.0±0.00, 174.0±0.00, 167.0±0.00 and 157.0±0.00 for 0.00mM, 100mM, 200mM and 300mM concentrations of the salt, respectively. Line 4 recorded 184.5±0.00 for 0.00mM, 182.75±0.00 for 100mM, 167.75±0.00 for 200mM and 159.0±0.00 for 300mM. Nevertheless, some lines recorded a higher value at 100mM concentration of the salt. For instance, line 9 has a value of 254.0±0.00 for 0.00mM, 256.0±0.00 for 100mM, 232.0±0.00 for 200mM and 218.0±0.00 for 300mM, although the value 256.0±0.00 for 100mM was not significantly different from 254.0±0.00 of 0.00mM. But for lines 3 and 17, the higher values at 100mM were significantly different from the other values. Line 3 has a mean value of 188.25±0.00 for 100mM, 192.00±0.00 for 100mM, 169.25±0.00 for 200mM and 157.25±0.00 for 300mM. For all the lines, 300mM recorded the lowest values. Significant differences ($p< 0.05$were recorded among the lines.

4.1.10 Number of leaves/plant

The result of the effect of sodium chloride on the number of leaves of *D. bulbifera* is shown in Table 11. The Table gives the mean values of the number of leaves of *D.*

TABLE 10

Vine length (cm) of *D. bulbifera* treated with different concentrations of sodium chloride

	TREATMENTS				
LINES	0.00mM	100mM	200mM	300mM	LSD
1.	180.25±765.87^{a}	176.00±881.17^{b}	163.25±1275.56^{c}	151.00±1723.00^{d}	2.1
2.	183.00±695.57^{a}	174.00±938.22^{b}	167.00±1152.01^{c}	157.00±1495.46^{d}	
3.	188.25±570.75^{b}	192.00±489.15^{a}	169.25±1080.90^{c}	157.25±1486.33^{d}	
4.	184.50±658.65^{a}	182.75±701.82^{a}	167.75±1128.06^{b}	159.00±1423.19^{c}	
5.	185.75±628.65^{a}	182.25±714.40^{b}	169.00±1088.69^{c}	157.75±1468.15^{d}	
6.	192.50±478.74^{a}	186.75±605.15^{b}	173.00±967.42^{c}	162.00±1318.14^{d}	
7.	191.75±494.39^{a}	190.00±531.89^{a}	169.75±1065.41^{b}	159.75±1396.55^{c}	
8.	194.25±443.21^{a}	192.00±489.15^{b}	176.25±874.16^{c}	165.00±1217.12^{d}	
9.	254.00±51.96^{a}	256.00±66.49^{a}	232.00±10.22^{b}	220.25±76.65^{c}	
10.	240.50±0.68^{a}	238.75±0.00^{a}	228.25±24.71^{b}	220.00±78.73^{c}	
11.	252.25±40.71^{a}	254.00±51.96^{a}	234.75±3.60^{b}	218.00±96.42^{c}	
12.	243.25±4.51^{a}	245.00±8.71^{a}	229.00±21.31^{b}	215.00±126.29^{c}	
13.	275.00±293.81^{a}	273.00±262.28^{a}	252.00±39.22^{b}	243.00±4.02^{c}	
14.	297.00±758.81^{a}	298.25±791.74^{a}	261.00±110.65^{b}	253.00±45.36^{c}	
15.	293.50±670.35^{a}	291.00±610.52^{b}	260.00±100.93^{c}	251.75±37.75^{d}	
16.	336.00±2115.35^{a}	338.00±2203.26^{a}	312.00±1200.02^{b}	303.00±923.22^{c}	
17.	354.00±2970.97^{b}	357.00±3127.67^{a}	338.00±2203.26^{c}	326.00±1702.64^{d}	
18.	344.00±2477.73^{a}	341.00±2338.48^{b}	318.00±1404.69^{c}	310.00±1135.38^{d}	
19.	318.00±1404.69^{a}	315.00±1300.34^{b}	306.00±1011.46^{c}	294.00±682.65^{d}	
20.	329.00±1821.76^{a}	326.00±1702.64^{b}	310.00±1135.38^{c}	296.00±732.98^{d}	

Values are mean ± S.E of vine length determinations.

Means with the same superscript letter on the same row are not significantly different ($p<0.05$)

TABLE 11

Number of leaves of *D. bulbifera* treated with different concentrations of sodium chloride

LINES	TREATMENTS				
	0.00mM	100mM	200mM	300mM	LSD
1.	25.00±5.07^{a}	24.00±7.42^{b}	22.00±13.47^{c}	19.00±25.90^{d}	0.5
2.	25.00±5.07^{a}	24.00±7.42^{b}	21.00±17.16^{c}	19.00±25.90^{d}	
3.	25.00±5.07^{b}	27.00±1.70^{a}	21.00±17.16^{c}	18.00±30.94^{d}	
4.	24.00±7.42^{a}	24.00±7.42^{a}	20.00±21.31^{b}	17.00±36.42^{d}	
5.	25.00±5.07^{a}	23.00±10.22^{b}	20.00±21.31^{c}	18.00±30.94^{d}	
6.	25.00±5.07^{a}	24.00±7.42^{b}	20.00±21.31^{c}	17.00±36.42^{d}	
7.	32.00±1.12^{a}	32.00±1.12^{a}	28.00±0.69^{b}	24.00±7.42^{c}	
8.	31.00±0.34^{a}	30.00±0.01^{b}	28.00±0.69^{c}	23.00±10.22^{d}	
9.	30.00±0.01^{b}	31.00±0.34^{a}	27.00±1.70^{c}	24.00±7.42^{d}	
10.	31.00±0.34^{a}	31.00±0.34^{a}	28.00±0.69^{b}	25.00±5.07^{c}	
11.	32.00±1.12^{a}	32.00±1.12^{a}	29.00±0.13^{b}	25.00±5.07^{c}	
12.	31.00±0.34^{a}	30.00±0.01^{b}	26.00±3.16^{c}	24.00±7.42^{d}	
13.	36.00±8.71^{a}	36.00±8.71^{a}	30.00±0.01^{b}	27.00±1.70^{c}	
14.	39.00±19.10^{a}	39.00±19.10^{a}	32.00±1.12^{b}	29.00±0.13^{c}	
15.	38.00±15.19^{a}	37.00±11.73^{b}	32.00±1.12^{c}	29.00±0.13^{d}	
16.	40.00±23.46^{b}	41.00±28.26^{a}	37.00±11.73^{c}	33.00±2.35^{d}	
17.	45.00±51.96^{b}	46.00±59.00^{a}	40.00±23.46^{c}	38.00±15.19^{d}	
18.	42.00±33.52^{a}	42.00±33.52^{a}	37.00±11.73^{b}	35.00±6.14^{c}	
19.	39.00±19.10^{a}	38.00±15.19^{b}	33.00±2.35^{c}	31.00±0.34^{d}	
20.	40.00±23.46^{a}	39.00±19.10^{b}	35.00±6.14^{c}	35.00±6.14^{c}	

Values are mean ± S.E of vine length determinations.

Means with the same superscript letter on the same row are not significantly different ($p<0.05$)

bulbifera treated with the different concentrations of sodium chloride. This indicates that sodium chloride reduced the number of leaves of *D. bulbifera*. The reduction which was more pronounced with higher concentrations of the salt was observed in lines 1, 2, 5, 12, 19, etc. For line 1, 0.00mM recorded the highest number of leaves with mean value of 25.00±0.00, followed by 100mM which recorded a value of 24.00±0.00. 200mM recorded a mean value of 22.00±0.00 while 300mM recorded 19.00±0.00 which was the lowest. Nevertheless, lower concentrations of the salt (≤ 100mM) may stimulate growth through increase in the number of leaves as observed in lines 3, 16 and 19. For line 3, 0.00mM recorded a value of 25.00 ±0.00, 100mM recorded 27.00 ±0.00, 200mM recorded 21.00±0.00 and 300mM recorded 18.00±0.00. For line 16, 0.00mM recorded 40.00±0.00, 100mM recorded 41.00±0.00, 200mM recorded 37.00±0.00 and 300mM recorded 33.00±0.00.

4.1.11 Root length

The means and standard error values of the root length of *D. bulbifera* treated with various concentrations of sodium chloride are recorded in Table 12. The result shows significant differences between the treated plants and the control plants as regard the root length. This indicates that sodium chloride increases the root length of the plant. 300mM concentrations of the salt had the highest root length value while the controls had the lowest values in all the lines. For line 1, the mean and standard error values of the root length for 0.00mM was 13.00± 1.28, 100mM had 13.20± 1.13, 200mM had 15.00± 0.21 and 300mM had17.00± 0.04. For line 2, 0.00mM recorded a value of 13.40± 0.99, 100mM recorded 13.50± 0.93, 200mM recorded 14.50± 0.39 and300mM recorded 15.90± 0.02 values. Significant differences were also observed among the lines.

4.1.12 Number of bulbils

The mean and standard error values of the number of bulbils of *D. bulbifera* treated with the different concentrations of the salt is shown in Table13. Sodium chloride significantly reduces the number of bulbils in *D. Bulbifera* as indicated in the table. The number of bulbils decreased with increasing concentrations of the sodium chloride. For line 1, the values were 2.00±0.06, 2.00± 0.06, 2.00±0.06, and 0.00±0.84 at 0.00mM, 100mM, 200mM and 300mM, respectively. For line 2, the values were 2.00± 0.06 at 0.00mM, 3.00±0.01 at 100mM, 2.00±0.06 at 200mM and 0.00±0.84 at 300mM. For line 3, the values were 3.00±0.01, 3.00±0.01, 2.00±0.06 and 0.00±0.84 at 0.00mM, 100mM, 200mM and 300mM, respectively. For line 17, the table recorded 6.00±1.19 at 0.00mM, 5.00±0.57 at 100mM, 3.00±0.01 at 200mM and 1.00±0.34mM.

4.1.13 Leaf length

The mean values of the leaf length of *D. bulbifera* treated with various concentrations of sodium chloride are shown in Table 14. The Table reveals no significant difference in leaf length between the treated plants and the controls, but significant differences existed between the lines. For line 1, 0.00mM recorded a mean value of 8.35±0.00, 100mM recorded a value of 8.35±0.00, 200mM recorded 8.4±0.00 and 300mM also recorded 8.4±0.00. For line 2, 0.00mM recorded a value of 8.00±0.00, 100mM recorded 8.0±0.00, 200mM recorded 8.0±0.00 and 300mM also recorded 8.0±0.00. For line 9, 0.00mM recorded 8.75±0.00, 100mM recorded 8.25±0.00, 200mM recorded 8.25±0.00 and 300mM recorded 8.25±0.00.

4.1.14 Petiole length

The means and standard error values of petiole lengths of *D. bulbifera* treated with various concentrations of sodium chloride are recorded in Table 15.

TABLE 13

Number of bulbils of *D. bulbifera* treated with different concentrations of sodium chloride

LINES	TREATMENTS				
	0.00mM	100mM	200mM	300mM	LSD
1.	2.00±0.06^{a}	2.00±0.06^{a}	2.00±0.06^{a}	0.00±0.84^{b}	0.34
2.	2.00±0.0b^{a}	3.00±0.01^{a}	2.00±0.06^{b}	0.00±0.84^{c}	
3.	3.00±0.01^{a}	3.00±0.01^{a}	2.00±0.06^{b}	0.00±0.84^{c}	
4.	3.00±0.01^{a}	3.00±0.01^{a}	1.00±0.34^{b}	0.00±0.84^{c}	
5.	4.00±0.18^{a}	4.00±0.18^{a}	4.00±0.18^{a}	1.00±0.34^{b}	
6.	3.00±0.01^{a}	3.00±0.01^{a}	2.00±0.06^{b}	0.00±0.84^{c}	
7.	4.00±0.18^{a}	3.00±0.01^{b}	3.00±0.01^{b}	2.00±0.06^{c}	
8.	3.00±0.01^{b}	4.00±0.18^{a}	2.00±0.06^{c}	1.00±0.34^{d}	
9.	4.00±0.18^{a}	4.00±0.18^{a}	3.00±0.01^{b}	0.00±0.84^{c}	
10.	3.00±0.01^{a}	3.00±0.01^{a}	3.00±0.01^{a}	0.00±0.84^{b}	
11.	4.00±0.18^{a}	3.00±0.01^{b}	3.00±0.01^{b}	0.00±0.84^{c}	
12.	3.00±0.01^{b}	4.00±0.18^{a}	2.00±0.06^{c}	1.00±0.34^{d}	
13.	3.00±0.01^{a}	3.00±0.01^{a}	2.00±0.06^{b}	0.00±0.84^{c}	
14.	5.00±0.57^{a}	5.00±0.57^{a}	3.00±0.01^{b}	1.00±0.34^{c}	
15.	4.00±0.18^{a}	4.00±0.18^{a}	3.00±0.01^{b}	0.00±0.84^{c}	
16.	4.00±0.18^{a}	4.00±0.18^{a}	4.00±0.18^{a}	0.00±0.84^{b}	
17.	6.00±1.19^{a}	5.00±0.57^{b}	3.00±0.01^{c}	1.00±0.34^{d}	
18.	8.00±3.10^{a}	7.00±2.03^{b}	5.00±0.57^{c}	2.00±0.06^{d}	
19.	6.00±1.19^{a}	6.00±1.19^{a}	3.00±0.01^{b}	2.00±0.06^{c}	
20.	4.00±0.18^{a}	3.00±0.01^{b}	2.00±0.06^{c}	0.00±0.84^{d}	

Values are mean ± S.E of number of bulbils determinations.

Means with the same superscript letter on the same row are not significantly different ($p<0.05$)

TABLE 14

Leaf length of *D. bulbifera* treated with different concentrations of sodium chloride

LINES	TREATMENTS				
	0.00mM	100mM	200mM	300mM	LSD
1.	8.35±0.00	8.35±0.00	8.40±0.00	8.40±0.00	NS
2.	8.00±0.02	8.00±0.02	8.00±8.02	8.00±0.02	
3.	8.15±0.01	8.15±0.01	8.13±0.01	8.13±0.01	
4.	7.95±0.03	7.93±0.03	7.93±0.03	7.93±0.03	
5.	8.08±0.01	8.03±0.02	8.03±0.02	8.00±0.02	
6.	8.18±0.00	8.15±0.01	8.15±0.01	8.13±0.01	
7.	8.23±0.00	8.18±0.00	8.18±0.00	8.15±0.01	
8.	8.28±0.00	8.23±0.00	8.25±0.00	8.23±0.00	
9.	8.75±0.05	8.25±0.00	8.25±0.00	8.25±0.00	
10.	8.00±0.02	8.00±0.02	8.00±0.02	8.00±0.02	
11.	8.00±0.02	8.00±0.02	8.00±0.02	8.75±0.05	
12.	8,95±0.09	8.90±0.08	8.90±0.08	8.75±0.05	
13.	8.58±0.02	8.53±0.01	8.50±0.01	8.50±0.01	
14.	8.40±0.00	8.40±0.00	8.40±0.00	8.40±0.00	
15.	8.50±0.01	8.50±0.01	8.00±0.02	8.00±0.02	
16.	8.75±0.05	8.75±0.05	8.50±0.01	8.50±0.01	
17.	8.98±0.10	8.95±0.09	8.93±0.09	8.93±0.09	
18.	8.08±0.01	8.05±0.01	8.05±0.01	8.03±0.02	
19.	8.75±0.05	8.50±0.01	8.50±0.01	8.25±0.00	
20.	8.25±0.00	8.25±0.00	8.25±0.00	8.25±0.00	

Values are mean ± S.E of leaf length determinations.

The values are not significantly different from each other.

The Table reveals no significant differences between the treated plants and the controls but significant differences existed between the lines. For line 1, the mean and standard error values for 0.00mM was 5.725±0.02, 5.65±0.03 for 100mM, 5.625±0.03 for 200mM and 5.6±0.03 for 300mM. For line 2, 0.00mM concentration of the salt recorded a value of 5.65±0.03, 100mM recorded 5.625±0.03, 200mM recorded 5.525±0.03 and 300mM recorded 5.6±0.03. The highest mean value for petiole length was found in line 9 with a value of 8.6±0.68 while the lowest value was 4.5±0.30 found in lines 6, 7, etc.

4.1.15 Leaf area

The means and standard error values of the leaf area of *D. bulbifera* treated with sodium chloride salt are shown in Table 16. No significant difference was found in the leaf area between the treated plants but between the lines existed significant differences. For line 1, the value at 0.00mM was 67.5±0.00, at 100mM was 66.75±0.07, at 200mM, the value was 66.75±0.07 and at 300mM, the value was 67.0±0.03. For line 2, the values were 66.75±0.07 at 0.00mM, 67.0±0.03 at 100mM, 66.75±0.07 at 200mM and 66.5±0.12. For line 4, the values were 67.0±0.03, 66.75±0.07, 66.75±0.07 and 66.75±0.03, respectively. For the line 5, the value at 0.00mM was 61.25±4.42, at 100mM, the value was 60.75±5.15, at 200mM, the value was 60.75±5.15 and at 300mM, the value was also 60.75±5.15. For line 10, the value was 71.25±1.54 for all.

4.1.16 Leaf colour

The effect of sodium chloride on the leaf colour of *D. bulbifera* is shown in plate 3. The control plants of all the lines as well as the plants treated with 100mM concentration of the salt were deep green in color while the plants treated with 200 and 300mM concentrations of the salt had their leaves yellowing in colour. The yellowing in colour was more pronounced at 300mM concentration of the salt.

TABLE 15

Petiole length of *D. bulbifera* treated with different concentrations of sodium chloride

LINES	TREATMENTS				
	0.00mM	100mM	200mM	300mM	LSD
1.	5.73±0.02	5.65±0.03	5.63±0.03	5.60±0.03	NS
2.	5.65±0.03	5.63±0.03	5.53±0.04	5.63±0.03	
3.	5.48±0.05	5.58±0.03	5.58±0.03	5.53±0.04	
4.	5.68±0.02	5.55±0.04	5.58±0.03	5.55±0.04	
5.	4.55±0.28	4.60±0.26	4.55±0.28	4.60±0.26	
6.	4.50±0.30	4.58±0.27	4.50±0.30	4.60±0.26	
7.	4.50±0.30	4.70±0.23	4.48±0.31	4.55±0.28	
8.	4.55±0.28	4.55±0.28	4.55±0.28	4.60±0.26	
9.	8.60±0.68	8.60±0.68	8.60±0.68	8.60±0.68	
10.	8.55±0.65	8.55±0.65	8.60±0.68	8.60±0.68	
11.	8.55±0.65	8.45±0.60	8.58±0.67	8.53±0.64	
12.	8.40±0.58	8.60±0.68	8.60±0.68	8.60±0.68	
13.	7.38±0.17	7.40±0.18	7.35±0.17	7.40±0.18	
14.	7.40±0.18	7.40±0.18	7.40±0.18	7.40±0.18	
15.	7.40±0.18	7.35±0.17	7.40±0.18	7.35±0.17	
16.	7.35±0.17	7.40±0.18	7.40±0.18	7.38±0.17	
17.	4.55±0.28	4.60±0.26	4.55±0.28	4.60±0.26	
18.	4.50±0.30	4.58±0.27	4.50±0.30	4.55±0.28	
19.	4.50±0.30	4.68±0.24	4.50±0.30	4.55±0.28	
20.	4.55±0.28	4.55±0.28	4.40±0.30	4.60±0.26	

Values are mean ± S.E of petiole length determinations.

The values are not significantly different from each other.

TABLE 16

Leaf area of *D. bulbifera* treated with different concentrations of Sodium Chloride

LINES	TREATMENTS				
	0.00mM	100mM	200mM	300mM	LSD
1.	67.50±0.00	66.75±0.07	66.75±0.07	67.00±0.03	NS
2.	66.75±0.07	67.00±0.03	66.75±0.07	66.50±0.12	
3.	67.25±0.01	67.50±0.00	67.25±0.01	67.25±0.01	
4.	67.00±0.03	66.75±0.07	66.75±0.07	66.75±0.07	
5.	61.00±4.77	60.75±5.15	60.75±5.15	60.75±5.15	
6.	61.25±4.42	61.25±4.42	61.25±4.42	60.75±5.15	
7.	60.75±5.15	61.00±4.77	60.75±5.15	60.75±5.15	
8.	61.00±4.77	61.00±4.77	60.75±5.15	60.75±5.15	
9.	70.50±0.98	70.75±1.16	70.50±0.98	70.50±0.98	
10.	71.25±1.54	71.25±1.54	71.25±1.54	71.25±1.54	
11.	70.75±1.16	71.50±1.76	70.50±0.98	70.75±1.16	
12.	72.00±2.23	72.00±2.23	71.50±1.76	71.25±1.54	
13.	69.00±0.24	69.00±0.24	68.75±0.17	68.75±0.17	
14.	69.00±0.24	68.75±0.17	68.50±0.10	68.50±0.10	
15.	69.25±0.33	69.00±0.24	69.00±0.24	68.75±0.17	
16.	69.00±0.24	68.75±0.17	68.75±0.17	68.75±0.17	
17.	69.75±0.55	69.75±0.55	69.75±0.55	69.75±0.55	
18.	69.75±0.55	70.00±0.68	69.75±0.55	69.75±0.55	
19.	70.25±0.82	70.25±0.82	69.75±0.55	69.75±0.55	
20.	69.75±0.55	70.25±0.82	69.75±0.55	69.75±0.55	

Values are mean ± S.E of leaf area determinations.

The values are not significantly different from each other.

a b

PLATE 3: Leaf colour of *D. bulbifera* treated with 0.00mM and 100mM concentrations of sodium chloride.

Key:

a Leaf colour treated with 100mM of NaCl

b Leaf colour treated with 200mM of NaCl

a b

PLATE 3: Leaf colour of *D. bulbifera* treated with 0.00mM and 100mM concentrations of sodium chloride.

Key:

a Leaf colour treated with 100mM of NaCl

b Leaf colour treated with 200mM of NaCl

c d

PLATE 4: Leaf color of *D. bulbifera* treated with 200mM and 300mM concentrations of sodium chloride.

Key:

c leaf colour of *D. bulbifera* treated with 200mM of NaCl

d Leaf colour of *D. bulbifera* treated with 300mM of NaCl

CHAPTER FIVE

DISCUSSION, SUMMARY, CONCLUSION AND RECOMMENDATION FOR FURTHER STUDIES

5.1 Discussion

Sodium chloride was observed to cause increase in peroxidase and glucose-6 phosphate dehydrogenase activities in *D. bulbifera*. Increase in these enzymes activities suggests formation of excess hydrogen peroxide by the salt and eventually oxidative stress. Therefore, as a defense response to the oxidative stress, the peroxidase and glucose-6 phosphate dehydrogenase activities were increased. This agrees with authors (Mittova *et al.*, 2003; Panuccio *et al.*, 2014) who reported increase in anti oxidative enzymes under salt stress.

A reduction in chlorophyll a and b concentrations in the treated plants especially at 300mM concentration of the salt was recorded. This implied that salinity caused reduction in the chlorophyll a and b contents of the plant. Chlorophyll is an important material for photosynthesis. Reduction in chlorophyll content leads to reduction in the rate of photosynthesis. This phenomenon accounts for the reduction in the growth of the treated plant.

Sodium chloride reduced the growth of *Dioscorea bulbifera*. Specifically, it decreases the vine length, number of leaves and number of bulbils of the *D. bulbifera*. The decrease increases with increasing concentration of sodium chloride and was more significant at 300mM concentration of the sodium chloride. At 100mM, the height and number of leaves of *D. bulbifera* of some of the lines was at higher value, although not significantly different from that of the controls. From this, it can be deduced that higher concentration of sodium chloride (>100mM) caused reduction in growth of *D. bulbifera* and lower concentration of sodium chloride (≤100mM) tend to

Significant differences in growth were recorded among the lines. Lines 13 – 20 showed more growth than the other lines. Lines 1 – 5 which showed lower growth had spindle or elongated bulbils shape. Bulbils with spindle or elongated shaped could be said to be less tolerant to the salt treatment than the round or ovoid shaped.

For estimation of the efficiency and discriminating power of the primer, major allele frequency, allele number, gene diversity and polymorphic information content (PIC) were the important feature to note. The result of these features is shown in table 7. The polymorphic information content is used to evaluate the discriminatory power of the primer (marker) within the population (Junjian *et al.*, 2002) and it is calculated from allele number and frequency (Norman *et al.*, 2012). Polymorphic information content values are positive and range from 0 to 1. A marker is said to be informative if the polymorphic information content is greater than or equals 0.50 (Tessier*et al.*, 1999). The result of the study as recorded in table 7 showed that each of the primer recorded a value greater than 0.50 for the polymorphic information content except for the marker Db7. An average value of 0.77 was also observed for gene diversity. Therefore, the SSR primers used for the study were informative and efficient in discriminating the species. This however confirmed the work of Silva *et al.* (2016) which recorded that these primers produce bands which were polymorphic except for the Db7.

For effective use and conservation of the germplasm of *Dioscorea bulbifera*, genetic diversity study of the plant is inevitable. The principal component analysis of the molecular data shows the existence of genetic differences in the germplasm of *Dioscorea bulbifera* collected from the different locations of the Niger – Delta region of Nigeria. This analysis revealed 67.48% variation of the total variation in the species to be attributed to difference in the DNA molecules. This of course is in

support of Sanou (1993) who noted several setbacks in using only morpho-agronomic characters for diversity studies. This occurrence of genetic differences among the species of *Dioscorea bulbifera* informs the possibility of improvement of the plant through selection (Obidiegwu *et al.*, 2009).

The dendrogram generated for the different lines of the *D. bulbifera* used for this study showed two clusters. Cluster 1 contained a total of 39 lines of the *D. bulbifera* which is sub divided into 5 clusters, while cluster 2 contained only 1. This clustering pattern shows that the first sub cluster down the phylogenetic tree contained many of the lines with close genetic similarities. Selection and vegetative propagation may have likely contributed to this high genetic similarity among the lines of the plant used for the study. Also, in each of the sub-cluster, it is observed that lines collected from different locality had close genetic relationship with each other. This showed that geographical location within this region of Nigeria may not account for the existence of variation among the species but the interaction of the gene and environmental factors which include man's activities.

Lines Dbok16, Dbor15, Dbok23 and Dbak8 found at the top of the phylogenetic tree which appears to have the highest yield and more tolerant to the salt stress based on the morphological studies had round shaped bulbils. It could therefore be said that bulbils shape can be used as a criteria for choosing high yielding and more salt tolerant species of *D. bulbifera* and that round shaped bulbils of *D. bulbifera* are more yielding and tolerant to salt stress than the elongated.

5.2 Summary

The purpose of this research was to evaluate the tolerance of *D. bulbifera* to salt (sodium chloride) stress condition and to determine its genetic diversity. This was

justifiable by the need to improve plants productivity (especially indigenous plants) despite climate change.

Human activities like irrigation practice, use of organic chemicals, etc., to make ends meet have resulted to abiotic stresses in plants. Salt stress as an abiotic stress has contributed to low productivity of plants. However, some plants— rice, potatoes, maize, etc.— have been reported to tolerate this stress condition, and so are able to grow to maturity and produce.

Aerial yam (*D. bulbifera*) is an indigenous edible crop cultivated for its cheap source of energy. It is less utilized but has been reported to be very helpful to man's wellbeing. It is used traditionally for the treatment of some common ailments like diarrhea, cholera, diabetes and dysentery. It is fast growing and has the ability to tolerate and adapt to different soil types, weather and habitat conditions.

The plants used were sourced from Abia, Akwa Ibom and Cross River States of the Niger-Delta region of Nigeria and planted in a greenhouse at the Department of Genetics and Biotechnology, University of Calabar, Calabar. The plants were treated with 250ml of different concentrations of sodium chloride (0, 100, 200 and 300mM) weekly for four weeks during which data were collected. The plants were then allowed to maturity.

Analysis of variance (ANOVA) was used to analyze data. The result on the plants morphology showed that although the growth of *Dioscorea bulbifera* was reduced by salt when compared with the control, the plant was able to tolerate the stress condition through increased peroxidase and glucose-6 phosphate dehydrogenase activities and so were able to grow to maturity and produce. The result also revealed significant morphological differences among the different lines of the plants used for the study.

The molecular studies of the plant, using simple sequence repeats markers revealed genetic diversity among the lines of the plants. The plants with round or ovoid shaped bulbils were more tolerant to the salt and appeared at the top of the phylogenetic tree. This pointed to the possibility of improvement of the plant which is very essential to human wellbeing.

5.3 Conclusion

Dioscorea bulbifera has the ability to grow fast, tolerate salt stress condition (≤200mM of NaCl) and could be listed as a moderate salt tolerant plant. It has the potential of providing food security in this era of climate change. Its diversity and utilization is low in Niger Delta region of the Nigeria. There is therefore a call for improvement and proper utilization of the plant to prevent its extinction.

5.4 Contribution to knowledge

1. *D. bulbifera* can thrive in saline soils of concentrations ≤200mM
2. Round shaped bulbils are more tolerant to saline soils of ≤200mM than the elongated.
3. The diversity of *D, bulbifera* in Niger- Delta of Nigeria is low

5.5 Recommendation for further studies

It is therefore recommended that:

1. Further studies on *Dioscorea bulbifera* include wild species as more diversity could be established.
2. Traits like high bulbil yield, high number of bulbil per plant, high bulbil weight, tolerance to salt, etc. be established and linked with the SSR markers for efficient study.

REFERENCES

Abd EL-Azim, W. M & Ahmed, S. (2009). Effect of salinity and cutting date on growth and chemical constituents of *Achilea fragratissima* Forssk under Ras Sudr conditions. *Research Journal of Agriculture and Biological Science*, 5(6), 1121-1129.

Abdul Qados, A. M. S. (2011). Effect of salt stress on plant growth and metabolism of bean plant *Vicia faba* L. *Journal of the Saudi Society of Agricultural Sciences*, 10(1), 7-15.

Achy, J. Y., Koffi, P. K., Ekissi, G. S., Konan, H. K & Kouame, P. (2016). Assessment of physico-chemical properties and anti-nutritional factors of flour from yam (*Dioscorea bulbifera*) bulbils in Southeast Cote, D' Ivoire. *International Journal of Advanced Research*, 4(12), 871- 881.

Acosta-Motos, J. R., Diaz-Vivancos, P., Alvarez, S., Fernandez-Garcia, N., Sanchez-Blanco, M. J & Hernandez, J. A. (2015). NaCl- induced physiological and biochemical adaptive mechanism in the ornamental *Mytus cummunis* L. plants. *Journal of Plant Physiology*, 183, 41- 51.

Acquaah, G. (2007). *Principles of plant genetics and breeding*. Oxford, United Kingdom. Blackwell.

Andreu, P., Arbeloa, A., Lorente, P & Marin, J. A. (2011). Early Detection of salt Stress Tolerance of Prunnus Rootstocks by Excised Root culture. *Horticultural Science*, 40, 80 – 85.

Apel, K & Hirt, H. (2004). Reactive Oxygen Species: Metabolism, oxidative stress and signal transduction. *Annual Review of Plant Biology*, 55, 373 – 399.

Arinathan, V.,Mohan, V. R & Maruthupandian, A. (2009). Nutritional and anti-nutritional attributes of some under-utilized tubers. *Tropical and subtropical Agroecosystems*, 10, 273- 278.

Arzani, A. (2008). Improving salinity tolerance in crop plants: A biotechnological view. *In Vitro Cellular & Developmental Biology – Animal*, 44, 373- 383.

Bernstein, N., Silk, W. K & lauchli, A. (1995). Growth and development of Sorghum leaves under conditions of NaCl stress: possible role of some mineral elements in growth inhibition. *Planta*, 196, 699 – 705.

Bhandari, M. R & Kawabata, J. (2004). Assessment of anti-nutritional factors and bioavailability of calcium and zinc in wild yam (*Dioscorea* spp.) tubers in Nepal. *Food Chemistry*, 85(2), 281- 287.

Bybordi, A., Tabatabaei, S. J & Ahmedov, A. (2010). Effect of salinity on the growth and peroxidase and IAA oxidase activities in canola. *Journal of food, Agriculture and Environment*, 8(1), 109-112

Glenn, E.P & Brown, J.J. (1999). Salt tolerance and crop potential of halophytes. *Critical Review in Plant Science*, 18, 227- 255

Gónmez-Bellot, M.J., Álvarez, S., Bañón, S., Ortuño, M.F & Sánchez-Blanco, M.J. (2013).Physiological mechanisms involved in the recovery of euonymus and laurustinussubjected to saline waters. *Agricultural Water Manage*ment, 128, 131–139.

Gulsen, O. (2004). Buffalo grasses: Their organelle DNA, Chinch bug resistance variation and peroxidase enzyme response to Chinch bug injury. University of Nebraska-Lincoln, Lincoln, Pp 133.

Hammer, R. L. (1998). Diagnosis: Dioscorea. *Wild Land Weeds*, 2(1), 8- 10.

Herbert, M., Burkhard, C. & Schnarrenberger, C. (1979). A survey for isoenzymes of glucose – phosphate isomerase, phosphoglucomutase, glucose 6- phosphate dehydrogenase and 6 phosphogluconate dehydrogenase in C_3^-, C_4^- and CAM-plants and green algae. *Planta*, 145, 95 – 104.

Hiraya, S., Sasaki, K., Ito, H., Chashi, & Matsui, H. (2001). A large family of class III plant peroxidases. *Plant Cell Physiology*, 42, 462-468.

Hussain Bhat, M., Fayaz, M., Kumar, A & Jain, A. K. (2019). Phytochemical screening and nutritional analyses of some edible parts of *Dioscorea bulbifera* L. *Chemical Science Transactions*, 8(1), 20- 27.

Invasive Species Specialist Group (2012). Global invasive species database (GISD). Auckland, New Zealand, University of Auckland, Retrieved from http://www.issg.org/database, June 19, 2016.

Jackson, M. L. (1973). *Soil chemical analysis*. New Delhi: Pretice Hall of India, Private Limited.

Jamil, A., Riaz, S., Ashraf, M & Foolad, M. R. (2011). Gene expression profiling of plants under salt stress. *Critical Review of Plant Science*, 30(5), 435- 458.

Jampeetong, A. & Brix, H. (2009). Effects of NaCl salinity on growth, morphology, photosynthesis and proline accumulation of *Salvinia natans*. *Aquatic Botany*, 91(3), 181- 186.

Jayeola, A. A. & Oyebola, T. O. (2013). Morpho-molecular studies in the natural populations of *Dioscorea bulbifera* in Nigeria. *Journal of Genetics and Molecular Biology*, 14, 1061 - 1128.

Journet, E. P & Douce, R. (1985). Enzymatic capacities of purified cauli flower bud plastids for lip synthesis and carbohydrate metabolism. *Plant Physiology*, 79, 458 – 467.

Jouyban, Z. (2012). The effect of salt stress on plant growth. *Technical Journal of Engineering and Applied Sciences*, 2(1), 7 – 10.

Junjian, N., Colowit, P. M., & Mackill, D. (2002). Evaluation of genetic diversity in rice subspecies by microsatellite markers. *Crop Science,* 42, 601-607.

Khan, A. A., Roa, S. A & McNeilly, T. (2003). Assessment of salinity tolerance based upon seedling root growth response functions in maize (*Zea mays* L.). Euphytica, 131, 81- 89.

Knight, J. S., Emes, M. J & Debnam, P. M. (2001). Isolation and characterization of a full length genomic clone encoding a plastidic glucose 6-Phosphate dehydrogenase from *Nicotiana tabacum* L. *Planta*, 212, 499 – 507.

Kruger, N. J & Von Schaewen, A. (2003). The oxidative pentose phosphate pathway: Structure and organization. *Current Opinion in Plant Biology*, 6, 236 -246.

Kumar, K., Kumar, M., Kim, S. R., Ryu, H & Cho, Y.G. (2013). Insights into genomics of salt stress response in rice. *Rice Journal*, 6, 27.

Langeland, K. A., Cherry, H. M., McCormick, C. M & Craddock Burks, K. A (2008). Identification and Biology of Non-native Plants in Florida's Natural Areas. Gainesville, Florida, USA: University of Florida IFAS Extension.

Lebot, V. (2009). Tropical root and tuber crops: cassava, sweet potato, yams and aroids. In: Pests and diseases. (Lebot V, eds). CAB International, Wallingford, 253 – 264.

Mahalakshmi, V., Aparna, P., Ramadevi, S & Rodomiro, O. (2002). Genomic sequence derieved simple sequence repeats markers: A case study with Medicago specie. *Electronic Journal of Biotechnology*, 5(3), 233 – 242.

Mignouna, H. D., Ellis, N. T. H., Knox, M. R., Asiedu, R. & Quat, N. N. (1998). Analysis of genetic diversity in Guinea yams *(Dioscorea* spp.) using AFLP finger printing. *Tropical Agriculture (Trindad),* 75, 2.

Milbourne, D., Meyer, R. C., Collins, A. J., Ramsay, L. D & Gebhardt, C. (1998). Isolation, characterization and mapping of simple sequence repeat loci in potatoe. *Molecular General Genetics*, 259, 233 – 245.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends Plant Science*, 7, 405 – 410.

Mittova, V., Guy, M., Tal, M & Volokita, M. (2004). Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. *Journal of Experimental Botany*, 399, 1105–1113.

Mittova, V., Tal, M., Volokita, M & Guy, M. (2003). Up-regulation of the leaf mitochondrial and peroxisomal antioxidative systems in response to salt-

induced oxidative stress in the wildsalt-tolerant tomato species *Lycopersicon pennellii. Plant Cell and Environ*ment, 26, 845–856.

Morisawa, T. L (1999). Weed notes: *Dioscorea bulbifera, D. alata, D. sansibarensis*. California, USA: The Nature Conservancy, Wildland Invasive Species Program. http://tncweeds.ucdavis.edu.

Munns, R. & Termaat, A. (1986). Whole plant response to salinity. *Australian Journal of Plant Physiology*, 13, 143- 160.

Munns, R. & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, 59, 651 – 681.

Netondo, G. W., Onyango, J. C & Beck, E. (2004). Sorghum and salinity: II. Gas exchange and chlorophyll fluorescence of sorghum under salt stress. *Crop Science*, 44, 806 -811.

Nkang, A. & Mofunanya, A .A. J. (2016). *General plant physiology* eds. Calabar, Cross River state. e2oMedia Limited.

Norman, P. E., Tongiona, P & Shanahan, P. E. (2011). Diversity of the morphological traits of yam (*Dioscorea* spp) genotypes from Sierra Leone. *Journal of Applied Bioscience,* 45, 3045-3058.

Norman, P. E., Tongiona, P & Shanahan, P. E. (2012). Diversity I chromosome number and raphide morphology of yam (*Dioscorea* spp) genotypes from Sierra Leone. *African Journal of Plant Science,* 6(4), 157-162.

Novacky A & Hampton, R. E. (1968). Peroxidase isozymes in virus infected plants. *Phytopathology*, 58, 301-305.

Obidiegwu, J. E., Asiedu, R., Ene-Obong, E. E., Muoneke, C. O & Kolesnikova-Allen, M. (2009). Genetic characterization of some water yam (*Dioscorea alata* L.) accessions in West Africa with some simple sequence repeats. *Journal of Food, Agriculture an Environment,* 7, 634-638.

Panaud, O., Chen, X. L & McCough, S. R. (1996). Development of microsatellite markers and characterization of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa* L.). *Molecular General Genetics*, 252, 597 – 607.

Panuccio, M. R., Jacobsen, S. E., Akhtar, S.S & Muscolo, A. (2014). Effect of saline water on seed germination and early seedling growth of the halophyte quinoa. *AoB Plants*, 6.

Parvaiz, A. & Satyawati, S. (2008). Salt stress and phytobiochemical responses of plants – a review. *Plant Soil and Environment*, 54, 89 – 99.

Paul, D. (2012). Osmotic stress adaptations in rhizobacteria. *Journal of Basic Microbiology*, 52, 1- 10.

Peng, J., Haitao, S., Jingling, C., Hong, Z., Yingying, Z & Yonghua, Z. (2011). Effect of 1-methyl cyclopropene in chilling injury and quality of peach fruit during cold storage. *Journal of Food* Science, 76, 485- 491.

Petersen, C. E & Anderson, B. J. (2005). *Investigations in the Biology* 1151. Ilenois USA. Laboratory Stipes Publishing L. L. C. Champaign,

Ramezani, E., Sepanlou, M. G & Naghdi, B. H. (2011). The effect of salinity on the growth, morphology and physiology of *Echium omoenum* Fisch. & Mey. *African Journal of Biotechnology*, 10(44), 8765 – 8773.

Randall, R. P (2012). *A Global Compendium of Weeds*. Perth, Australia: Department of Agriculture and Food, Western Australia, Pp 1124.

Rayment, G. E & Higginson, F. R. (1992). *Australian Laboratory Handbook of soil and water Chemical Methods*. Melbourne, Inkata Press.

Rengasmy, P. (2010). Soil processes affecting crop production in salt affected soils. *Functional Plant Biology*, 37, 613 – 620.

Romero-Aranda, R., Moya, J. L., Tadeo, F.R., Legaz, F & Primo-Millo, E. (1998). Physiological and anatomical disturbances induced by chloride salts in sensitive and tolerant citrus: beneficial and detrimental effects of cations. *Plant Cell and Environment*, 21, 1243-253.

Sanou, J. (1993). Evaluation de la variabilite au seind'une collection de fonio (*Digitaria exilis* Stapf). Structuration, Potentialites agronomiques. Memoird'ingenieur, option agronomic. Universite Ouagadougou, Burkina Faso.

Shahbaz, M. & Ashraf, M. (2013). Improving salinity tolerance in cereals. *Critical Review of Plant Science*, 32, 237- 249.

Shajeela, P. S., Mohan, V. R., Louis Jesudas, L & Soris, P. T. (2011). Nutritional and anti-nutritional evaluation of wild yam (*Dioscorea* spp.). *Tropical and subtropical Agroecosystems*, 14, 723- 730

Shanker, A. K & Venkateswarly, B. (2011). Abiotic stress in plants – mechanisms and adaptations. In Tech Publisher, Rijeka, Croatia, Pp 428.

Shao, H. & Chu, L. (2005). Plant molecular biology in China: opportunities and challenges. *Plant Molecular Biology Reporter*, 23(4), 345 – 358.

Shrivastara, P. & Kumar, R. (2015). Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi Journal of Biological Science, 22(2), 123- 131.

Silva, D. M., Siqueira, M. V. B. M., Carrasco, N. F., Montello, W. C., Nascimento, W. F. & Veasey, E. A. (2016). Genetic diversity among air yam (*Dioscorea*

bulbifera) varieties based on single sequence repeat markers. *Genetics and Molecular Research,* 15(2), gmr – 15027929.

Sivritepe, N. & Eris, A. (1999). Determination of salt tolerance in some grapevine cultivars (*Vitis vinifera* L.) under in vitro conditions. *Turkish Journal of Biology*, 23(4), 473 – 485.

Song, J., Ding, X., Feng, G & Zhang, F. (2006). Nutritional and osmotic roles of nitrate in a euhalophyte and a xerophyte in saline conditions. *New Phytologist*, 171(2), 357 – 366.

Srinieng, K., Saisavoey, T & Karnchanatat, A. (2015). Effect of salinity stress on antioxidative enzyme activities in tomato cultured in vito. Parkistan Journal of Botany, 47(1), 1-10.

Stepien, P. & Johnson, G.N. (2009). Contrasting responses of photosynthesis to salt stress in the glycophyte Arabidopsis and the halophyte Thellungiella: Role of plastidterminal oxidase as an alternative electron sink. *Plant Physiology*, 149, 1154- 1165.

Strickland, J. D. H & Parsons, T. R. (1972). *A Practical Handbook of Seawater Analysis.* Bulletin of the Fisheries Research Board of Canada. Ohawa, 167.

Tapondjou, L. A., Jenelt-Siens, K., Bottger, S. & Melzig, M. F. (2013). Steroidal saponnins from the flowers of *Dioscorea bulbifera. Var. Sativa Phytochemistry*, 95, 341- 350

Tessier, C., David, J., This, P., Boirsiquot, J. M & Charrier, A (1999). Optimization of the choice of molecular markers for varietal identification in *Vitis vinifera* L. *Theorical and Applied Genetics*, 98, 171 – 177.

Temple, V. J & Sen, K. K. (1993). Composition of aerial yam tubers (*Dioscorea bulbifera*). *Bioscience Research Communications*, 5(1), 5- 8.

Tortoe, C., Johnson P.N.T., Abbey L., Baidoo E., Anang D., Acquaach, S.G & Saka, E. (2012). Sensory properties of pretreated blast-chilled (*Dioscorea rotundata*) as a convenience food product. *African Journal of Food Science* and *Technology,* 3, 59–65.

Usman, M., Hag, A.U., Ahsan, T., Amjad, S., Riasat, Z & Umar, M. (2012). Effect of NaCl on morphological attributes of maize (*Zea mays* L.). *Botany Research International*, 5(3), 57- 60.

Vinocur, B. & Altman, A. (2005). Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Current Opinion in Biotechnology*, 16(2), 123 – 132.

Wang, W. X., Vinocur, B., & Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*, 218, 1–14.

Weisany, W., Sohrabi, Y., Heidari, G., Siosemardeh, A & Ghassemi-Golezani, K. (2012). Changes in antioxidant enzymes activity and plant performances by salinity stress and zinc application in soybean (*Glycine max* L.). *Plant Omics Journal*, 5(2), 60- 67.

Wilkin, P. (2001). Dioscoreaceae of South- Central Africa. *Kew Bulletin,* 56, 361-404.

Xiong, L & Zhu, J.K. (2002). Molecular and genetic aspects of plant responses to osmotic stress. Plant *Cell and Environment*, 25, 131- 159.

Yadav, S., Irfan, M., Ahmad, A & Hayat, S. (2011). Causes of salinity and plant manifestations to salt stress: A review. *Journal of Environmental Biology*, 32, 667 - 685.

Yan, D.D., Li, Y., Sun, X. Q., Ivo, J. L.,Hang, Y. Y., Li, M. M. (2014). Isolation and characterization of polymorphic micro satellite loci from aerial yam (*Dioscorea bulbifera* L.). *Genetics and Molecular Research*, 13 (1), 1514-1517.

Zhang, M., Fang, Y., Ji, Y., Jiang, Z & Wang, L. (2013). Effect of salt stress on ion content, antioxidant enzymes and protein profile in different tissues of *Broussonetiapapyrifera. South African Journal of Botany*, 85, 1 – 9.

Zheng, J., Zhao, L., Shen, B., Jiang, L & Zhu, A. (2016). Effects of salinity on activity and expression of enzymes involved in ionic, osmotic, and antioxidant responses in *Eurya emarginata. Acta Physiologiae Plantarum*, 38, 70.

Zhu, J.K. (2001). Plant salt tolerance. *Trends Plant Science*, 6, 66- 71.

APPENDIX 1

The ANOVA table of number of leaves of *Dioscorea bulbifera* treated with sodium chloride

SOV	Df	SS	MS	Fcal	Ftab(0.05)
Total	79	4056.49			
Treatment	3	734.54	244.8	317.9	2.77
Block	19	3278.24	172.5	224.0	4.05
Error	57	43.71	0.77		

LSD = 0.5

The ANOVA table for root length of D. *bulbifera* treated with sodium chloride

SOV	Df	SS	MS	Fcal	Ftab
Total	79	610.32	-	-	-
Treatment	3	256.96	85.65	81.6	2.77
Block	19	293.30	15.44	14.7	4.05
Error	57	60.06	1.05		

LSD = 0.54

The ANOVA table of leaf length of *D. bulbifera* treated with sodium chloride

SOV	Df	SS	MS	Fcal	Ftab
Total	79	12.55	-	-	-
Treatment	3	0.1020236	0.03	2.0	2.77 NS
Block	19	5.9880859	0.315	18.5	4.05
Error	57	0.9865701	0.017		

LSD 0.3

The ANOVA table of petiole length of *D. bulbifera* treated with sodium chloride

SOV	Df	SS	MS	Fcal	Ftab	
Total	79	203.6	-	-	-	
Treatment	3	0.015	0.05	1.67	2.77	NS
Block	19	203.4175	10.71	3570	4.05	
Error	57	0.1675	0.003			

LSD 0.029

The ANOVA table for leaf area of *D. bulbifera* treated with sodium chloride

SOV	Df	SS	MS	Fcal	Ftab	
Total	79	1489.3	-	-	-	
Treatment	3	1.09	0.36	0.041	2.77	NS
Block	19	993.6	52.29	6.02	4.05	
Error	57	494.6	8.68			

LSD 1.56

The ANOVA table of vine length of *D. bulbifera* treated with sodium chloride

SOV	Df	SS	MS	Fcal	Ftab
Total	79	304987.75	-	-	-
Treatment	3	13465.88	4488.63	284.99	2.77
Block	19	290624.31	15296.02	971.18	4.05
Error	57	897.55	15.75		

LSD 2.1

The ANOVA table of number of bulbils of *D. bulbifera* treated with sodium chloride

SOV	Df	SS	MS	Fcal	Ftab
Total	79	239.4875	-	-	-
Treatment	3	145.3375	48.45	117.88	2.77
Block	19	70.7375	3.72	0.0091	4.05 NS
Error	57	23.4125	0.411		

LSD 0.34

The ANOVA table of Chlorophyll a in *D. bulbifera* treated with sodium chloride

SOV	Df	SS	MS	Fcal	Ftab
Total	79	39486.0589	-	-	-
Treatment	3	814.14	271.38	288.70	2.77
Block	19	38618.52	2032.55	2162.29	4.05
Error	57	53.40	0.94		

LSD 0.51

The ANOVA table of chlorophyll-b in *D. bulbifera* treated with sodium chloride

SOV	Df	SS	MS	Fcal	Ftab
Total	79	278232.91	-	-	-
Treatment	3	240720.26	80240.09	770.58	2.77
Block	19	31577.51	1661.97	15.96	4.05
Error	57	5935.15	104.13		

LSD 5.40

ANOVA TABLE of Glucose 6 dehydrogenase activity in *D. bulbifera* treated with sodium chloride

SOV	Df	SS	MS	Fcal	Ftab
Total	19	10.7874452	-	-	-
Treatment	3	0.007127	0.0023756	740.4	3.49
Block	4	10.7802797	2.6950699	840030.5	3.26
Error	12	0.0000385	0.0000032		

LSD 0.003

The ANOVA TABLE of peroxidase activity in *D. bulbifera* treated with sodium chloride

SOV	Df	SS	MS	Fcal	Ftab
Total	19	0.0020452	-	-	-
Treatment	3	0.0018892	0.0006297333	547.6	3.49
Block	4	0.0001422	0.00003555	30.9	3.26
Error	12	0.0000138	0.00000115		

LSD 0.002

APPENDIX 2

DNA profile scoring

SAMPLE	Db2	Db5	DBSSR4	Db3	Db6	Db7	Db8	DBSSR2	DBSSR3	DBSSR5
Line 1	0	1	0	0	0	1	1	1	1	0
Line 2	1	1	1	0	0	1	1	1	1	1
Line 3	1	1	0	1	1	1	1	1	1	1
line 4	1	1	1	0	0	1	0	0	0	0
Line 5	1	1	0	0	0	1	1	1	1	0
Line 6	1	1	1	0	0	1	1	1	0	0
Line 7	1	1	0	0	0	1	1	1	1	1
Line 8	0	1	0	0	0	0	1	1	0	0
Line 9	0	0	0	0	0	0	0	0	0	1
Line 10	1	0	1	0	0	0	1	1	0	0
Line 11	1	1	1	0	0	1	1	1	1	0
Line 12	0	1	1	0	1	1	1	1	0	1
Line 13	1	0	1	0	0	0	0	0	0	1
Line 14	0	1	0	0	0	1	1	1	1	0
Line 15	0	0	0	0	0	0	1	1	0	0
Line 16	0	0	0	0	0	0	1	1	0	0
Line 17	0	0	1	0	1	1	0	0	1	1
Line 18	0	0	0	0	1	1	0	0	0	1
Line 19	0	0	1	1	1	1	0	0	1	1
Line 20	1	1	0	0	0	0	1	1	1	0
Line 21	1	1	0	0	0	0	1	1	1	1
Line 22	1	1	1	1	1	1	1	1	1	1
Line 23	0	0	0	0	0	0	1	1	0	1
Line 24	1	1	0	0	0	0	1	1	1	0
Line 25	1	0	0	1	1	1	1	1	1	0
Line 26	1	1	1	0	0	0	1	1	1	1
Line 27	1	1	1	0	0	0	1	1	0	0
Line 28	1	0	1	0	1	1	1	1	0	1
Line 29	0	0	1	0	0	1	1	1	0	1
Line 30	1	0	1	0	0	1	1	1	1	0
Line 31	1	1	0	0	0	0	1	1	0	1
Line 32	0	0	1	0	0	1	1	1	1	1
Line 33	1	0	0	0	0	0	1	1	1	1
Line 34	1	0	1	0	0	0	1	1	0	1
Line 35	1	1	0	0	1	1	1	1	1	1
Line 36	1	0	1	1	0	0	1	1	1	1
Line 37	1	0	1	0	0	1	1	1	0	1
Line 38	1	0	0	0	1	0	0	0	0	1
Line 39	1	0	0	1	1	0	1	1	0	1
Line 40	1	1	1	1	1	1	1	1	0	1

Dissimilarity matrix showing genetic distances among 40 lines of *Dioscorea bulbifera*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1	0																																							
2	0.36	0																																						
3	0.47	0.53	0																																					
4	0.14	0.25	0.44	0																																				
5	0.52	0.47	0.15	0.45	0																																			
6	0.54	0.39	0.16	0.47	0	60	0																																	
7	0.75	0.57	0.76	0.66	0.60	0.54	0																																	
8	0.19	0.33	0.50	0.19	0.45	0.47	0.66	0																																
9	0.11	0.30	0.50	0.14	0.54	0.52	0.69	0.27	0																															
10	0.19	0.39	0.33	.019	0.52	0.50	0.72	0.22	0.22	0																														
11	0.14	0.33	0.36	0.16	0.49	0.47	0.75	0.25	0.19	0.16	0																													
12	0.90	0.60	0.91	0.78	0.72	0.54	0.39	0.81	0.81	0.90	0.90	0																												
13	0.23	0.37	0.34	0.20	0.49	0.44	0.68	0.23	0.23	0.09	0.15	0.89	0																											
14	0.10	0.80	0.99	0.98	0.74	0.62	0.52	0.11	0.10	0.11	0.17	0.26	0.12	0																										
15	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0																									
16	0.52	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0																								
17	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0																							
18	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0																						
19	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0																					
20	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0																				
21	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0																			
22	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0																		
23	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0																	
24	0.22	0.30	0.40	0.14	0.51	0.34	0.50	0.36	0.19	0.21	0.32	0.40	0.38	0.62	0.32	0.30	0.67	0.17	0.50	0.24	0.60	0.23	0.16	0																
25	0.16	0.20	0.45	0.24	0.40	0.45	0.66	0.14	0.19	0.28	0.24	0.78	0.14	0.94	0.20	0.23	0.40	0.34	0.61	0.46	0.44	0.16	0.28	0.33	0															
26	0.34	0.33	0.47	0.14	0.50	0.41	0.43	0.26	0.19	0.22	0.22	0.54	0.21	0.86	0.12	0.60	0.54	0.10	0.54	0.31	0.23	0.34	0.19	0.22	0.22	0														
27	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.45	0.65	0.32	0.75	0.65	0													
28	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.45	0.65	0.32	0.75	0.11	0.32	0.65	0												
29	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.67	0.87	0.97	0											
30	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.34	0.15	0.98	0.73	0.76	0.65	0										
31	0.52	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.88	0.86	0									
32	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.14	0.50	0.44	0.60	0.16	0.45	0.65	0.32	0.75	0.65	0.96	0								
33	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.18	0.14	0.50	0.44	0.60	0.16	0.45	0.65	0.32	0.75	0.65	0							
34	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.14	0.50	0.44	0.60	0.16	0.45	0.65	0.32	0.75	0.65	0						
35	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.14	0.50	0.44	0.60	0.16	0.45	0.65	0.32	0.75	0.65	0.76	0.45	0.89	0.56	0					
36	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0				
37	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.87	0.67	0,98	0.34	0.			
38	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0/78	0.88	0.82	0.43	0		
39	0.22	0.30	0.40	0.14	0.51	0.34	0.50	0.36	0.19	0.21	0.32	0.40	0.38	0.62	0.32	0.30	0.67	0.17	0.50	0.24	0.60	0.23	0.16	0.45	0.76	0.34	0.18	0.92	0.22	0.30	0.47	0.14	0.50	0.44	0.60	0.16	0.83	0.96	0	
40	0.16	0.20	0.45	0.24	0.40	0.45	0.66	0.14	0.19	0.28	0.24	0.78	0.14	0.94	0.20	0.23	0.40	0.34	0.61	0.46	0.44	0.16	0.28	0.33	0.32	0.55	0.60	0.16	0.19	0.22	0.22	0.78	0.18	0.92	0.22	0.30	0.47	0.78	0.94	0

Warning:
At least one negative eigenvalue.
(Smallest eigenvalue: -0.00998533728599652)
Non Euclidean dissimilarity

Axis	**Eigenvalue**	**Inertia%**
Axis	Eigenvalue	Inertia%
1	0.06581	28.63
2	0.03448	15
3	0.02603	11.32
4	0.01583	6.89
5	0.01296	5.64

Unit coordinates on factorial axes

WARNING: negative eigenvalue => cosinus² may be negative or greater than one!

1st column = coordinate
2nd column = cosinus² x 1000

	Axis 1		Axis 2		Axis 3		Axis 4		Axis 5
	Coord.	Cos²	Coord.	Cos²	Coord.	Cos²	Coord.	Cos²	
Coord.	Cos²								
1	0.2607	609	-0.1864	311	0.2639	624	0.0190	3	-0.0926
77									
2	-0.0078		-0.1047		-0.0348		0.0282		-0.0560
3	-0.0525	89	-0.0952	292	0.1077	374	-0.1885		0.0066
1									
4	-0.1187	59	-0.4393	808	-0.2851	340	0.0781	26	-0.2369
235									
5	0.1945	626	-0.1661	456	0.0272	12	-0.0896	133	-0.0406
27									
6	0.1515	468	-0.1529	477	-0.1077	236	0.1031	217	-0.0455
42									
7	0.0257	72	-0.0483	256	0.0466	238	-0.0764	640	-0.0980
8	0.4479		0.1062	57	0.0743	28	-0.0041	0	-0.2117
225									
9	-0.5398	525	0.4832	420	0.0926	15	0.0470	4	-0.2465
109									
10	0.2042	406	0.0684	45	-0.2811	769	0.1280	159	0.1564
238									
11	0.1345	465	-0.2197		-0.0617	98	0.0474	58	0.0100
3									
12	-0.0725	147	-0.0511	73	0.1124	353	0.1056	311	-0.0887
220									
13	-0.4303	680	0.1050	40	-0.4564	765	0.1293	61	0.0015
0									
14	0.2607	609	-0.1864	311	0.2639	624	0.0190	3	-0.0926
77									
15	0.4225	700	0.2831	314	0.1376	74	0.0982	38	0.1016
40									
16	0.4225	700	0.2831	314	0.1376	74	0.0982	38	0.1016
40									
17	-0.4540	870	-0.2300	223	0.1355	78	0.0914	35	0.0568
14									
18	-0.5774	879	-0.0101	0	0.3470	317	0.0137	0	-0.0916
22									
19	-0.4496	798	-0.2349	218	0.1587	99	0.0214	2	0.1713
116									
20	0.2968	927	-0.0378	15	-0.0953	96	-0.1850	360	-0.0232
6									
21	0.0847	232	0.0730	173	-0.0361	42	-0.1420	654	-0.0899
262									

22	-0.0774	338	-0.1400		0.0166	16	-0.0731	302	0.0421
100									
23	0.0625	30	0.4317		0.1565	189	0.0714	39	-0.0100
1									
24	0.2968	927	-0.0378	15	-0.0953	96	-0.1850	360	-0.0232
6									
25	0.0318	12	-0.1518	268	0.1401	228	-0.1847	397	0.2445
695									
26	0.0387	124	-0.0081	5	-0.1165		-0.0086	6	-0.0465
179									
27	0.2429	727	-0.0244	7	-0.2597	831	0.0478	28	-0.0438
24									
28	-0.1332	993	-0.0035	1	-0.0185	19	0.0630	222	0.0947
502									
29	-0.0547	56	0.0594	67	0.0925	162	0.3092		0.0072
1									
30	0.1010	221	-0.1752	665	-0.0523	59	0.1016	224	0.1594
551									
31	0.1016	239	0.1843	784	-0.1109	284	-0.1077	268	-0.1887
822									
32	-0.0337	31	-0.0290	23	0.1339	492	0.1924		0.0580
92									
33	0.0417	41	0.1763	741	-0.0109	3	-0.1165	324	0.0837
167									
34	-0.0141	5	0.1794	810	-0.1829	842	0.1007	255	0.0497
62									
35	-0.0421	157	-0.0761	513	0.0958	812	-0.1523		-0.0489
212									
36	-0.0225	12	0.0427	42	-0.0939	205	-0.0174	7	0.1867
810									
37	-0.0620	303	0.0264	55	-0.0668	352	0.1343		0.0284
64									
38	-0.5069	783	0.1858	105	-0.1741	92	-0.3084	290	0.0201
1									
39	-0.0846	82	0.2072	495	0.0159	3	-0.1831	386	0.1803
375									
40	-0.0899	325	-0.0865	301	-0.0165	11	-0.0254	26	0.0145
8									

Fit criterion...

Tree file: SUCCESS KALU BINARY TEXT DOC.arb
Dissimilarity file: SUCCESS KALU BINARY TEXT DOC.dis

Fit criterion for tree: SUCCESS KALU BINARY TEXT DOC.arb and dissimilarity: SUCCESS KALU BINARY TEXT DOC.dis
Edge length sum: 1.6191
Mean error: 0.0044
Mean absolute error: 0.0487
Maximum absolute error: 0.241
Mean square error: 0.0052

Cophenetic r: 0.9544

Alleles for the locus 0:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 1:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	A

Alleles for the locus 2:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	A

Alleles for the locus 3:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	A

Alleles for the locus 4:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	A

Alleles for the locus 5:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	A

Alleles for the locus 6:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	S

Alleles for the locus 7:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	A

Alleles for the locus 8:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	A

Alleles for the locus 9:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	A

Alleles for the locus 10:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	A

Alleles for the locus 11:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	A

Alleles for the locus 12:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	T

Alleles for the locus 13:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	A

Alleles for the locus 14:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	M

Alleles for the locus 15:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	T

Alleles for the locus 16:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	T

Alleles for the locus 17:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	C

Alleles for the locus 18:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	C

Alleles for the locus 19:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	T

Alleles for the locus 20:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	C

Alleles for the locus 21:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 22:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 23:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 24:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	T

Alleles for the locus 25:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	C

Alleles for the locus 26:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 27:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 28:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 29:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 30:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	Y

Alleles for the locus 31:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 32:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 33:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 34:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 35:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 36:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 37:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 38:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 39:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Alleles for the locus 40:

No.	Freq.	s.d.	Allele:
1	0.98551	0.01449	-
2	0.01449	0.01449	G

Expected heterozygosity

	Locus#	Mean	s.d.	Tot. Het.
1	0.02899	0.02899	0.00000	0.02899
2	0.02899	0.02899	0.00000	0.02899
3	0.02899	0.02899	0.00000	0.02899
4	0.02899	0.02899	0.00000	0.02899
5	0.02899	0.02899	0.00000	0.02899
6	0.02899	0.02899	0.00000	0.02899
7	0.02899	0.02899	0.00000	0.02899
8	0.02899	0.02899	0.00000	0.02899
9	0.02899	0.02899	0.00000	0.02899
10	0.02899	0.02899	0.00000	0.02899
11	0.02899	0.02899	0.00000	0.02899
12	0.02899	0.02899	0.00000	0.02899
13	0.02899	0.02899	0.00000	0.02899
14	0.02899	0.02899	0.00000	0.02899
15	0.02899	0.02899	0.00000	0.02899
16	0.02899	0.02899	0.00000	0.02899
17	0.02899	0.02899	0.00000	0.02899
18	0.02899	0.02899	0.00000	0.02899
19	0.02899	0.02899	0.00000	0.02899
20	0.02899	0.02899	0.00000	0.02899
21	0.02899	0.02899	0.00000	0.02899
22	0.02899	0.02899	0.00000	0.02899
23	0.02899	0.02899	0.00000	0.02899
24	0.02899	0.02899	0.00000	0.02899
25	0.02899	0.02899	0.00000	0.02899
26	0.02899	0.02899	0.00000	0.02899
27	0.02899	0.02899	0.00000	0.02899
28	0.02899	0.02899	0.00000	0.02899
29	0.02899	0.02899	0.00000	0.02899
30	0.02899	0.02899	0.00000	0.02899
31	0.02899	0.02899	0.00000	0.02899
32	0.02899	0.02899	0.00000	0.02899
33	0.02899	0.02899	0.00000	0.02899
34	0.02899	0.02899	0.00000	0.02899
35	0.02899	0.02899	0.00000	0.02899
36	0.02899	0.02899	0.00000	0.02899
37	0.02899	0.02899	0.00000	0.02899
38	0.02899	0.02899	0.00000	0.02899
39	0.02899	0.02899	0.00000	0.02899
40	0.02899	0.02899	0.00000	0.02899