EVALUATION OF GENETIC DIVERSITY IN PEARL MILLET [*Pennisetumglaucum* (L.) R. Br.] GENOTYPES FOR DROUGHT TOLERANCE USING RAPD TECHNIQUE

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DEPARTMENT OF BIOCHEMISTRY

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NOVEMBER, 2014.

DECLARATION

I hereby declare that this thesis entitled "**Evaluation of genetic diversity in pearl millet [*Pennisetum glaucum* (L.) R. Br.] genotypes for drought tolerance using RAPD technique"** is a record of my own research work under the supervision of Prof. H.M. Inuwa and Prof. A. J. Nok. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project has been presented elsewhere for the award of a degree in any other university.

Nweke, Ogechi

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Name of Student   Signature   Date

CERTIFICATION

This project titled "**Evaluation of genetic diversity in pearl millet [*Pennisetum glaucum* (L.) R. Br.] genotypes for drought tolerance using RAPD technique''** by Nweke, Ogechi meets the regulations governing the award of the degree of Masters in Science of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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Prof. H.M. Inuwa   Date
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Prof. A. J. Nok   Date
Member, Supervisory Committee

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Prof. I. A. Umar   Date
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Dean, School of Postgraduates Studies

DEDICATION

This research work is dedicated to God Almighty, my source of strength, my refuge and my fortress.

ACKNOWLEDGEMENT

ABSTRACT

Drought is one of the environmental factors that have adverse effects on farming in Nigeria, and is a global problem constraining crop production. This has necessitated research for the improvement of crops that are tolerant to drought. Pearl millet is an important staple food crop that adapts to growing in areas characterised by drought, low soil fertility and high temperature. Although, it can be grown in areas where other cereals crops would not survive it produces poor yields in areas with unpredictable rainfall. Hence, there is a need to exploit genetic diversity of this crop, to enhance its yield. Random amplified polymorphic DNA (RAPD) analysis was applied on some pearl millet genotypes, to assess the degree of polymorphisms and also investigate the genetic diversity. Ten (10) genotypes were evaluated, DNA was extracted and polymerase chain reaction for RAPD amplification was carried out using 10 different primers. Bands obtained after agarose gel electrophoresis was scored as '1' for presence of a band and '0' for absence of a band. The primers revealed scorable polymorphism and therefore recommended for further evaluation in pearl millet breeding programme. A total of 48 distinct fragments were produced. Of these, 44 (91.6%) were found to be polymorphic. P16 (TCGGCGGTTC), P18 (TGAGCCTCAC), P20 (CTGCGCTGGA), P27 (CCGTGCAGTA), P29 (GGCTAGGGGG) and P30 (TACGGTGCCG) primers produced the highest number of polymorphic bands, with 100% polymorphism, while P26 (ATCGGGTCCG) produced the least number of polymorphic bands (66.7.%). A 600bp fragment was amplified in varieties suspected to be drought tolerant and absent in others e.g. COMPOSITE, SOSAT C88, DMR-15, EX-BORNO and JKBH 778. Polymorphic PCR products (unique and shared) were compared and similarity coefficients were generated using Nei's analysis. The similarity coefficients were employed to construct a dendrogram showing genetic relationships using unweighted paired group method with arithmetic averages (UPGMA). The analysis indicates a highest similarity (73%) between ZANGO and PEO 5984 varieties while lowest similarity (3%) was observed between COMPOSITE and PEO 5684. These varieties with low similarity can be selected and crossed to obtain drought tolerant varieties in pearl millet improvement programmes. The use of RAPD technique was effective and helpful in determining the genetic relationship among the 10 pearl millet genotypes and identifying markers that may be associated with drought tolerance.

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ABBREVIATIONS

µg	Microgramme
ng	Nanogramme
µl	Microlitre
ml	Millilitre
µM	Micromolar
bp	Base pair
mg	Milligram
V	Volt
mM	Millimolar
T.E	Tris Ethylene Diamine Tetraacetic Acid
rpm	Revolution per minute
et al	et alia (and others)
RNase	Ribonuclease
EDTA	Ethylene Diamine Tetraacetic Acid
UV	Ultraviolet
CTAB	Cethyl Trimethyl Ammonium Bromide
SDS	Sodium Dodecyl Sulphate
DNA	Deoxyribonucleic Acid
SDW	Sterile Distilled Water
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
SSR	Simple Sequence Repeats
ISSR	Inter-Simple Sequence Repeats
SNP	Single Nucleotide Polymorphism

CHAPTER ONE

1.0 INTRODUCTION

1.1 Millets

Millets are a group of small - seeded species of cereal crops or grains belonging to the family Gramineae and widely grown around the world for food and fodder (Ojediran *et al.*, 2010). Millets are cultivated mostly in the dry, semi-arid to sub-humid drought-prone agro ecosystems (Obilana and Manyasa, 2002). The most widely cultivated species in order of worldwide production are: Pearl millet (*Pennisetum glaucum*) (known as Bajra in India, in *Nigeria:* Gero (Hausa), Dauro, Maiwa, Emeye (Yoruba), Foxtail millet (*Setaria italica*), Proso millet (*Panicum miliaceum*), Finger millet (*Eleusine coracana*) (Ojediran *et al.*, 2010). Nkama (1998) outlined the uses and traditional food preparations of pearl millet in Nigeria. The grain serve as food for the majority of people of Africa who utilize it in the form of a meal produced from flour called 'tuwo', refreshing drink 'kunu', dessert 'dan wake' and palp 'ogi', millet beer in Cameroon, millet flour called ' Bajari' in western India .As food, Pearl millet is nutritionally equivalent or superior to most cereals; containing high levels of methionine, cysteine, and other vital amino acids for human health (Obilana and Manyasa, 2002). They are also unique sources of pro-vitamin A (yellow pearl millets) and micronutrients (Zn, Fe and Cu) (FAO/ICRISAT, 1996). There is also growing interest in the crop because of the technological possibilities of its utilization in industrial applications for starch production (Ojediran *et al.*, 2010). Pearl millet is highly adapted to drought, representing an essential component of the food security and livelihood of many million poor farmers that inhabit drylands and semi-arid ecosystems throughout Sub-Saharan Africa (Gari, 2002). Small-scale farmers conserve and cultivate innumerable cultivars of Pearl millet, often adapted to local agro-ecological factors, livelihood needs, and cultural values (Gari, 2002).

Millets are extremely important in the African semi-arid tropics, produced in 18.50 million ha by 28 countries covering 30% of the continent. This is a significant 49% of the global millet area, with a production of 32 million tonnes by 2007 (FAO, 2009). Millet production is distributed differentially among a large number of African countries; Nigeria (7,700,000 tonnes), Niger (2,781,928 tonnes), Burkina Faso (1,104,010 tonnes), Mali (1,074,440 tonnes), Sudan (792,000 tonnes), Uganda (732,000), Chad (550,000) and Ethiopia (500,000) (FAO, 2009).

1.2 Drought Tolerance

Drought is a meteorological term and is commonly defined as a period without significant rainfall. Generally drought stress occurs when the available water in the soil is reduced and atmospheric conditions cause continuous loss of water by transpiration or evaporation (Jaleel *et al*., 2009). Drought stress is one of the major causes of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Wang *et al.,* 2003) Under this stress usually a water deficit in plant tissues develops, thus leading to a significant inhibition of photosynthesis (Cornic, 1994).

Drought tolerance is the ability to withstand water-deficit with low tissue water potential (Mitra, 2001). The responses of plants to tissue water-deficit determine their level of drought tolerance (Mitra, 2001). The ability to maintain the functionality of the photosynthetic machinery under water stress, therefore, is of major importance in drought tolerance. The plant reacts to water deficit with a rapid closure of stomata to avoid further loss of water through transpiration (Zlatev, 2009).

Different mechanisms may render a plant drought tolerant:

- The ability of a plant to escape periods of drought, especially during the most sensitive periods of its development.

- The ability of a plant to recover from a dry period by producing new leaves from buds that were able to survive the dry spell.

- The ability of a plant to endure or withstand a dry period by maintaining a favourable internal water balance under drought (Sorells *et al.,* 2000).

Drought stress tolerance is seen in almost all plants but its extent varies from species to species and even within species (Jaleel *et al*., 2009). It has been predicted that in the coming years rainfall patterns might shift due to an increase of the global temperature caused by burning of fossil fuels and the corresponding increase in atmospheric dioxides (Guido and Paul, 1994). Consequently, farming communities in the Northern Hemisphere could become increasingly dependent on drought tolerant varieties (Clarke *et al.,* 1992; Zavala-Garcia *et al.,* 1992). Selection for drought tolerance while maintaining maximum productivity under optimal conditions has been difficult (Sorells *et al*., 2000).

1.3 Characterization of Genetic Diversity

Characterization of diversity has long been based mainly on morphological traits (Somasundaram and Kalaiselvam, 2011). However, morphological variability is often restricted. Characters may not be obvious at all stages of the plant development and appearance may be affected by the environment (Somasundaram and Kalaiselvam, 2011). Nowadays, a variety of different genetic markers have been proposed to assess genetic variability as a complementary strategy to more traditional approaches in genetic resources management. Molecular tools provide valuable data on diversity through their ability to detect variation at the DNA level (Somasundaram and Kalaiselvam, 2011). For evaluation of

species diversity, it is essential that individuals can be classified accurately (Somasundaram and Kalaiselvam, 2011). A number of different techniques are available for identifying genetic differences between organisms. The choice of technique for any one specific use will depend upon the material being studied and the nature of the questions being addressed. Molecular techniques differ in the way they sample within the genome and in the type of data that they generate (Somasundaram and Kalaiselvam, 2011).

Random amplified polymorphic DNA (RAPDs) were the first PCR-based molecular markers to be employed in genetic variation analyses (Welsh and McClelland, 1990; Williams *et al.,* 1991). RAPD markers are generated through the random amplification of genomic DNA using short primers (decamers), separation of the fragments obtained on agarose gel in the presence of ethidium bromide and finally, visualization under ultraviolet light (Williams *et al.,* 1991). The use of short primers is necessary to increase the probability that, although the sequences are random, they are able to find homologous sequences suitable for annealing (Williams *et al.,* 1991). DNA polymorphisms are then produced by "rearrangements or deletions at or between oligonucleotide primer binding sites in the genome" (Mondini *et al*., 2009). This approach requires no prior knowledge of the genome analyzed and can be employed across species using universal primers. The major drawback of this method is that the profiling is dependent on reaction conditions which can vary between laboratories; even a difference of a degree in temperature is sufficient to produce different patterns (Bardakci, 2001). Additionally, as several discrete loci are amplified by each primer, profiles are not able to distinguish heterozygous from homozygous individuals (Bardakci, 2001).

1.4 Statement of the problem

I. It has been established that drought stress is a very important limiting factor at the initial phase of plant growth, affecting both elongation and expansion growth by preventing the plant crop from expressing their full genetic potential.

II. Drought has adverse effects on crop yield in majority of farmed regions in Nigeria and Africa (Shao *et al*., 2008). Pearl millet is one of the most important staple food and fodder crop in these regions (Poncet *et al.*, 1998).

III. Post-flowering drought stress (terminal drought) has been identified as one of the major environmental factors that reduces both the yield and yield stability of this crop.

IV. Improving the adaptation of pearl millet to terminal drought stress environments is, therefore, a major objective in pearl millet breeding programmes.

V. Breeding crop varieties for increased drought tolerance has traditionally been slow, but efficiency could be improved if attributes that help maintain yield under water-limited conditions are identified and used as selection criteria (Yadav *et al.*, 1999).

VI. Molecular marker technology provide opportunities to identify individual genetic factors and their functions in determining complex phenotypes such as drought tolerance (Prioul *et al*., 1997; Quarrie 1996), but also help breeders in selection through the use of linked markers.

VII. Hence, the development of drought tolerant lines has become increasingly important.

1.5 Justification of the Study

I. Pearl millet grain has relatively high nutritional values for a cereal. Its grain has higher protein and fat content than wheat or rice and its amino acid composition is more appropriate for human nutrition than that of wheat or polished rice (Stich *et al*., 2010).

II. Pearl millet does not receive the scientific and political support that a crop of such global importance and critical food security significance deserves (Gari, 2002). This neglect is largely due to its socio-ecological condition, as it is a crop of poor farmers in marginal agricultural areas (Gari, 2002).

III. In particular, pearl millet has a superior adaptation to drought and poor soils, a reliable harvest under such conditions, growing where no other crops succeed, requiring minimal inputs, and providing good nutritional sources (Gari, 2002). It constitutes a reliable basis for enhancing food security and developing crop diversification in the most challenging agro-ecological areas of Africa.

IV. The conservation, use, and availability of millet genetic diversity is increasingly important in view of the evolving needs and manifold challenges of small-scale farmers in arid and semi-arid lands throughout Sub-Saharan Africa.

V. Efficient and systematic exploitation of this diversity is the key to any crop improvement programme (Stitch *et al.,* 2010). For pearl millet, several studies have examined these issues. Busso *et al.,* (2000) determined the influence of farmer management on pearl millet landrace diversity in two villages in North-Eastern Nigeria. Bhattacharjee *et al.,* (2002) assessed the genetic diversity within and between ten Indian pearl millet landraces. The genetic diversity of 46 wild and 421cultivated genotypes of pearl millet from Niger was analyzed by Mariac *et*

al., (2006). Oumar *et al.,* (2008) examined the phylogeny and origin of pearl millet.

VI.   However, no molecular study examined the genetic diversity of pearl millet using RAPD in Nigeria.

Therefore, there is a need to evaluate the genetic diversity of the available drought tolerant genotypes. This information will help in breeding pearl millet for drought tolerance in semi – arid region of Northern Nigeria.

1.6 Objective of the Study

1.6.1 General Objective

To screen pearl millet (*Pennisetum glaucum* L. R. Br.) varieties for the presence of possible drought tolerance marker genes.

1.6.2 Specific Objectives

I.   To isolate genomic DNA from pearl millet (*Pennisetum glaucum* L. R. Br) varieties.

II.   To carry out RAPD amplification of the DNA isolated from the different millet genotypes.

III.   To show genetic diversity among the pearl millet genotypes using the RAPD patterns obtained.

IV.   To attempt to establish some specific DNA markers associated with drought tolerance in pearl millet using RAPD pattern.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Pearl Millet

2.1.1 Background

Pearl millet is an important food for millions of people inhabiting the semi-arid tropics (Badau, 2006). It is a major source of calories and vital component of food security in the semi arid areas in the developing world (Badau, 2006). Pearl millet (*Pennisetum glaucum* (L.) R. Br.) produces staple grain for about 90 million people living in the world's hottest, driest rain-fed crop/livestock production systems (Thudi *et al.,* 2010). It is widely grown as a multi-purpose cereal grain crop for feed, fodder, fuel and mulch over more than 30 million hectares (H) predominantly in the semi-arid tropics of sub-Saharan Africa and the Indian subcontinent (ICRISAT, 2012; Thudi *et al.,* 2010). Its grain protein content, concentration of essential amino acids and calorific content are all superior to those of maize (Davis *et al.*, 2003). Despite being a hardy crop for dry land areas, the grain production potential of pearl millet is constrained by several biotic (fungal, bacterial, viral and insect) and abiotic (high temperature, drought, and soil salinity, acidity, and infertility) stresses (Bidinger and Hash, 2004; Thudi *et al.,* 2010).

2.1.2 Origin and Geographical Distribution

Pearl millet (*Pennisetum glaucum*) is the most widely grown type of millet. Grown in Africa and the Indian subcontinent since prehistoric times (Manning *et al.,* 2010). It is generally accepted that pearl millet originated in Africa and was subsequently introduced into India (Fuller, 2003). Pearl millet is believed to have descended from a West African wild grass which was domesticated more than 40,000 years ago, then spread to East Africa, Southern

Africa and India (NRC, 1996). It reached tropical America in the 18th century and the United States in the 19th century (Brink and Belay, 2006). Pearl millet is commonly grown as a grain crop in the semi-arid regions of West Africa and the driest parts of East and southern Africa and the Indian subcontinent. It is also grown as a fodder crop in Brazil, the United States, South Africa, and Australia. However, it is believed that the crop may have originated in the Abyssinia region (now known as Ethiopia) (ICIPE, 2002). Millet has been a staple in these areas since early times, but was replaced by rice as the main staple in Southeast Asia and India (Oelke *et al.,* 1990; Brink and Belay, 2006). In China, record indicates that millet was grown as early as 4500 BCE, or possibly earlier, while other records indicate that several varieties arrived in China from Africa (Lu *et al.,* 2009). Still others report it was grown by the lake dwellers of Switzerland during the Stone Age (Railey, 2011). Pearl millet was introduced to the U.S. in 1875, but was not well accepted for human consumption (Oelke *et al.,* 1990).

Archaeobotany has placed pearl millet domestication on the margins of the Sahel more than 3000 years ago (Tostain 1998; D'Andrea *et al.,* 2001). Recent proposals situate this event in the third millennium BC in the far western Sahel, perhaps in Mauritania and/or northeast Mali (Fuller *et al.,* 2007). One of the emblematic sites of Central Nigeria, the Nok region, has produced a large amount of millet dated to 800-450 cal B C (Kahlheber *et al.,* 2009). Perhaps even more surprising is the fact that millet was also cultivated in areas much further in regions that are now rainforest (Höhn *et al.,* 2007).

2.1.3 Plant Taxonomy
Pennisetum glaucum (Pearl Millet)

Kingdom: Plantae - Plants

Subkingdom: Tracheobionta - Vascular plants

Superdivision: Spermatophyta - Seed plants

Division: Magnoliophyta - Flowering plants

Class: Liliopsida - Monocotyledons

Subclass: Commelinidae

Order: Cyperales

Family: Poaceae - Grass family

Genus: Pennisetum – Fountain grass

Species: *Pennisetum glaucum*

Synonyms for *Pennisetum glaucum*, include *Pennisetum typhoides, Pennisetum typhoideum, Pennisetum americanum, Setaria glauca, Setaria lutescens, Panicum americanum*, and *Panicum glaucum* (ICIPE, 2002), but *Pennisetum glaucum* is the current officially accepted name. Common names of pearl millet include; in English; bulrush millet, cattail millet, candle millet, in French: mil à chandelles or petit-mil. (Baker, 2003). In *Nigeria:* gero (Hausa), dauro, maiwa, emeye (Yoruba), India*:* bajra, bajri, cumbu, sajje (NRC, 1996).

2.1.4 Plant description

Pearl millet is an upright bunch grass that tillers from the base and has an extensive root system that provides drought tolerance (Newman *et al.,* 2010). Stems are 1/2–1 inch diameter. It is a leafy plant with leaf blades that are 8 - 40 inches long and 1/2 - 3 inches wide. The ligule, or junction of leaf blade to leaf sheath, is a fringe of hairs 0.08 - 0.1 inch long (Newman *et al.,* 2010). The sheath has very sparse hairs at the base of the collar and is often hairless. The inflorescence (flower) is a single raceme 4 - 20 inches long that resembles the flower of the aquatic plant known as cattail (Newman *et al.,* 2010). The flowers can be either cross-pollinated or self-pollinated. The female part (stigma) emerges before the male part is ready to shed its pollen. As a result, cross-pollination normally occurs. However,

where the timing overlaps, some self-pollination can occur. Grain begins developing as soon as fertilization occurs and is fully developed 20 - 30 days later. The whole process, from fertilization to ripening, takes only about 40 days (NRC, 1996). The seeds range in colour from white to brown, blue, or almost purple. Most are slate gray. They are generally tear shaped and smaller than those of wheat (as shown in Plate I). The average weight is about 8 mg. Some thresh free from glumes, while others require husking. The seeds are quick to germinate. If conditions are favourable, they sprout in about 5 days. Freshly harvested seed may not germinate immediately; however, a dormancy of several weeks after harvesting has been reported (NRC, 1996). Pearl millet is a diploid (2n = 14) (NRC, 1996). The genome size of pearl millet is about five times larger than that of rice (430 M bp), larger than that of sorghum (750 M bp) and almost equal to that of maize (2400 M bp) (Arumuganathan and Earle, 1991). Photograph of pearl millet plants growing in the field is shown in PlateII.

Plate I: Photograph of Pearl millet seed (NRC, 1996)

Plate II: Photograph of Pearl millet plants in the field (USDA, 2005).

2.1.5 Nutritional Benefits of Pearl Millet

Pearl millet grain contains 27% to 32% more protein, higher concentration of essential amino acids, twice the fat and higher gross energy than maize (Davis *et al.,* 2003). The energy density of pearl millet grain is relatively high, arising from its higher oil content relative to maize, wheat, or sorghum (Hill and Hanna, 1990). Collins *et al.,* (1997) noted commercial layers given feed containing pearl millet grain had lower omega-6 to omega-3 fatty acid ratio, endowing the eggs with a fatty acid profile more favourable to human health. The amino acid profile of pearl millet grain is better than that of normal sorghum or normal maize and is comparable to those of the other grains such as wheat, barley, and rice (Ejeta *et al.,* 1987) with a less disparate leucine/isoleucine ratio (Hoseney *et al.,* 1987; Rooney and McDonough, 1987). The lysine content of the protein reported in pearl millet grain ranges from 1.9 to 3.9 g per 100 g protein (Ejeta *et al.,* 1987). Generally, Pearl millet grain appears to be generally free of any major anti-nutritional factors, such as the condensed tannins which reduce protein availability (Gulia *et al.,* 2007).

2.1.6 Pest and Diseases of Pearl Millet

 Pearl millet is attacked by various diseases and pests in the field. The most important of the fungal diseases of pearl millet is downy mildew, caused by the fungus (*Sclerospora graminicola* (Sacc), which has been estimated to cause losses of $10 - 15\%$ in Nigeria (Aliyu *et al.,* 2008). Other diseases of pearl millet in Nigeria are Smut (*Moesziomyces pennicullariae* (Bref)Vankysyn). (*Tolysposporium pennissscullariae* (Bref)) and Ergot (*Claviceps fusiformis (Loveless)* (Thakur and King, 1988), Rust (*Puccinia substriata*) and Leaf spot (*Pyricularia grisea*). Some of the plant diseases will affect dry matter yield (Newman *et al.,* 2010). Pest such as stemborer (*Coniesta ignefusalis*), head miner (*Raghuva* sp), the head beetle (*Pacnoda* sp.) and grasshoppers attack pearl millet. *Quelea quelea* is the major vertebrate pest and

Striga a parasitic flowering plant/weed that affect pearl millet (Rai and Kumar, 1994, Aliyu *et al.,* 2008).

2.1.7 Uses of Pearl Millet

Pearl millet is the staple food for over 100 million people in tropical African countries and India (Andrews and Kumar, 2006). Decorticated and pounded into flour it is consumed as a stiff porridge in Africa, or as flat unleavened bread ('chapatti') in India. In Africa there are various other preparations such as couscous, rice-like products, snacks of blends with pulses, and fermented and non-fermented beverages (NRC, 1996, Andrews and Kumar, 2006). In West Africa, the main food dishes from pearl millet vary by country. The stiff or thick porridges (Tuwo or Tộ) are the most popular, commonly consumed in all the Sahelian countries across the region (Obilana, 2003). The steam cooked product 'Couscous' is more commonly consumed in the Francophone countries including Senegal, Mali, Guinea, Burkina Faso, Niger and Chad (Obilana, 2003). The thin porridge 'bouillie' is also popular in these countries. Three countries among others have unique foods from pearl millet specific to them. In Nigeria and Niger the thin porridge 'Fourra' is very popular while 'Soungouf; 'Sankhal' and 'Araw' are very popular in Senegal (Obilana, 2003). In several Indian preparations parched seeds are used. The stems are widely applied for fencing, thatching and building, as fuel and as a poor-quality fodder. Split stems are used for basketry. A dye for leather and wood is obtained from red- and purple-flowered types. In African traditional medicine, the grain has been applied to treat chest disorders, leprosy and poisonings, and the ground grain as an anthelmintic for children (Brink and Bellay, 2006). A root decoction is drunk to treat jaundice; the vapour of inflorescence extracts is inhaled for respiratory diseases in children. Outside Africa and India pearl millet is mostly grown as a green fodder crop for silage, hay making and grazing (Brink and Bellay, 2006). Following the discovery that pearl millet can

suppress root-lesion nematodes (*Pratylenchus penetrans*), it is increasingly being used as an alternative to soil fumigation in tobacco and potato cropping in Canada (Andrews and Kumar, 2006).

2.1.8 Pearl Millet Production

Pearl millet is ranks sixth in global cereal hectarage (Rai *et al.,* 1991). Figures for pearl millet production are approximate and some of the available data do not distinguish between the different types of millet (FAO, 2003). It is estimated that approximately 87% of global millet production is pearl millet (ICRISAT and FAO, 1996), this means that pearl millet accounts for about half of the world's millet crop (Taylor, 2004). According to FAOSTAT (2005), the global millet production in 2004 was about 28 million tons, annually grown on more than 29 million hectares in the semi-arid tropical regions of Asia, Africa and Latin America. India is the largest producer of this crop, both in terms of area (9.1 million hectares) and production (7.3 million tons), with an average productivity of 780 kg ha–1 during the last five years. Nigeria produces about 6.7 million tons on a total of 5.8 million hectares (USDA, 2005). Other major pearl millet producing countries are; Niger which accounts for 1.9 million tons, Mali 0.7 million tons , Burkina Faso 0.8 million tons, Senegal 0.6 million tons (Taylor, 2004). This compares with the production of about 27 million metric tonnes of maize, the closest cereal grain substitute in both production and commercial processing systems (Rohrbach, 2000).

2.1.9 Pearl Millet in Nigeria

Millet in Nigeria is predominantly produced in Bauchi, Borno, Gombe, Kano, Katsina, Sokoto and Yobe States (Aliyu *et al.,* 2008). In Nigeria, three types of pearl millet [*Pennisetum glaucum* (L.) R. Br.] are grown mainly for their grain. Gero millet is the major

type, while Dauro (transplanted) millet is restricted to the plateau and southern part of Kaduna states (Zarafi and Emechebe, 2005). Maiwa is grown by few farmers in Niger, Taraba, Kaduna and Adamawa states, because of its long growing season (Zarafi and Emechebe, 2005).

2.1.10 Drought Tolerance Mechanisms in Pearl Millet

In pearl millet, stomata play an important role in minimizing crop water use during pre-anthesis water deficit (Winkel *et al.,* 2001). However, controlling leaf water losses when water is non-limiting for plant development was also considered as a suitable adaptation strategy (Kholová *et al.,* 2008; 2010a, b).

2.2 Drought Response in Plants

Drought is an important environmental stress in agriculture adversely affecting plant growth and productivity especially in semi arid regions of the world (Cattivelli, 2007). Hence, the biochemistry of drought response in plants needs to be studied.

Firstly, drought can affect plants in differential stages of their growth; either early during plant establishment, in vegetative developmental stage (intermittent drought) or at the end of growing season in reproductive stage (terminal drought). Out of these, terminal drought is shown to contribute to the most severe yield losses as it affect spikelet establishment and reduces its fertility (Bernier *et al.,* 2007 and Kholova, 2010)

Secondly, plants have developed several strategies to deal with drought which has different intensities and effects on different levels of their phenological, morphological and anatomical structure as well as on the levels of various physiological and biochemical processes (Kholova, 2010).

Therefore, since diverse drought patterns exist and various plant adjustments to counteract the drought effects, it is very important to define which type of drought stress is targeted by a breeding programme (Kholova, 2010).

2.2.1 Physiological Adaptation to Drought in Plants

Plants have developed various ways of adapting to drought stress physiologically. Firstly, drought tolerant plants diminish their metabolic functions to cope with stress and resumes as soon as water potential increases (Bartels, 2005). Secondly, drought tolerant plants close their stomata during stress, this strategy is to limits the loss of water to air, it is an abscisic acid-mediated regulation. Stomata, highly specialized cells involved in gas exchange, can account for a high water loss through leaf transpiration (Blum 1996). This adaptation leads to accumulation of gases such as carbon dioxide, which diminish photosynthesis (Bohnert and Sheveleva, 1998). In addition, hygrotropism is another interesting adaptation strategy, in which roots detect a water gradient and redirect their growth towards it (Lamberts *et al.,* 2000).

Some other adaptations include: some drought tolerant plants consisting of spongy tissues acting as water reservoirs, rolling of leaves, floral abscission, growth impairment, reduction of foliar area to limit evaporation by the plants and floral induction (Passioura, 1996 and Lin *et al.,* 2007).

2.2.2 Biochemical Adaptations to Drought in Plants

Osmotic adjustment is one of the mechanisms that plants have evolved for adaptation to drought stress (Tabaeizadeh, 1998). This involves synthesis of osmoprotectants, osmolytes or compatible solutes, which are hydrophilic highly soluble molecules, able to produce a solvation surface, that capture water molecules to be later available during water limitation (Bartels and Sunkar, 2005). These molecules accumulate in plant cells in response to drought

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stress and are subsequently degraded after stress relief. They act as osmotic balancing agents (Tabaeizadeh, 1998). Examples of these osmolytes are **amino acid**s, glycine-betaine, sugars and sugar alcohols, which are non-toxic molecules at high concentrations, thus not interfering with cellular metabolism (Chen and Murata, 2002).

2.2.3 Molecular Adaptation to Drought in Plants

Studies have established that drought tolerance is a quantitative trait involving the participation of complex set of genes (Way *et al*., 2005; Montalvo-Hernandez *et al*., 2008). There are certain changes in expression patterns in plants which have been monitored when drought stress is perceived by a plant. Adaptive responses are observed as a consequence of such changes which includes, early flowering, growth inhibition, among others (Bray, 2002). This involves genes whose products involve early response like signal transduction, transcription and translation factors, late response genes such as water transport, osmotic balance, oxidative stress and damage repair (Shinozaki and Yamaguchi-Shinozaki, 2000). Some of the drought tolerance mechanisms are described as follows:

2.2.3.1 Drought sensing and signal transduction

Although, the sensor for **drought stress** is still unknown, it is generally accepted that the organ with such ability is the root. The plant regulator abscisic acid (ABA) is a key endogenous messenger mediating this stress response (Raghavendra *et al*., 2010). Diverse hypotheses suggest that a redox imbalance could trigger the response to drought; a second possibility is changes in cell wall-membrane integrity (Kacperska, 2004). In Arabidopsis, two histidine kinase receptors (ATHK), belonging to the two-component family have been described as induced in early **drought stress** (Urao *et al*., 1999). Additional protein kinases have been described, such as the Arabidopsis leucine rich protein RPK1 (Hong *et al*., 1997)

and SPK1 from Phaseolus vulgaris (Montalvo-Hernandez *et al*., 2008), the most abundant transcript under **drought stress.**

2.2.3.2 Induced Genes at Transcriptional Level

Plant **gene expression** is controlled at different levels and a significant number of drought-induced genes appear to be controlled at the transcriptional level. Massive transcriptome analyses using RNA microarrays have established that ABA-dependent and -independent **signal transduction** pathways operate in drought-stressed plants (Shinozaki and Yamaguchi-Shinozaki, 2000). Bioinformatics analyses have identified several Transcription Factors (TF) induced under **drought stress** (Bartels and Sunkar, 2005; Marcotte *et al*., 1989; Abe *et al*., 1997; Ashraf *et al.,* 2008; Ashraf, 2010).

2.2.3.3 Drought-Induced Proteins

Translational control is another mechanism regulating plant responses to abiotic stress (Xoconostle-Cazares *et al.*, 2010). Synthesized proteins have direct functions in membrane and protein protection (Xoconostle-Cazares *et al*., 2010). They involve the acquisition of water and ions, as well as their transportation and homeostasis maintenance in basal cell functions (Xoconostle-Cazares *et al.*, 2010). Late Embryogenesis Abundant protein (LEA) family belongs to this group and was described as highly accumulated in plant embryos (Dure *et al*., 1981; Galau *et al*., 1986). LEA proteins are expressed at basal levels and can be induced to high levels during osmotic and drought stress (Ingram and Bartels, 1996; Barrera-Figueroa *et al*., 2007). Aquaporin (AQP) family is another example of stress-protecting proteins, facilitating water uptake and allocation by forming cellular water pores. AQP are membrane spanning proteins and can be located in plasma membrane or (Johansson *et al*., 2000). Heat Shock Proteins (HSP), highly accumulated during stress and also known as molecular chaperones, are widely distributed in nature. They are involved in protein folding

and assembly, as well as in the removal and disposal of non-functional proteins (Wang *et al*., 2004). HSP are induced by drought and salinity (Alamillo *et al*., 1995; Campalans *et al*., 2001).

2.2.3.4 Oxidative Stress:

Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defences (Betteridge, 2000). One of the main effects of the dehydration in plants is the production of Reactive Oxygen Radicals (ROS) such as singlet oxygen, superoxide anion radicals, hydroxyl radicals and **hydrogen peroxide** (Smirnoff, 1998; Bartels, 2001). Since ROS are mainly produced in chloroplasts, the photosynthetic activity is compromised during stress; drought tolerance is unequivocally related to efficient antioxidant cellular processes (Kranner *et al*., 2002; Montero-Tavera *et al*., 2008). Factors participating in antioxidative activity are non-enzymatic and enzymatic (Bartels and Sunkar, 2005); among the former are vitamins C and E, glutathione, flavonoids, alkaloids, carotenoids, polyamines, etc. Enzymatic activities include catalase, super-oxide dismutase, peroxidase and metallothionein (Seki *et al*., 2001).

The study of the molecular, biochemical and physiological mechanisms the plants employ to respond to **drought stress** has provided scientific knowledge for plant breeding. Despite drought tolerance being a quantitative trait, key genes can significantly contribute to ameliorate the damage produced by water limitation (Xoconostle-Cazares *et al*., 2010).

2.3 Developing drought tolerant crops

A number of genetically-improved drought-tolerant crops have been developed by different approaches, such as conventional breeding, marker-assisted breeding and transgenic approach (Xoconostle-Cazares *et al*., 2010). For modern agriculture, a combination of the

aforementioned techniques will likely be needed to produce new varieties showing drought tolerance in the field (Mittler and Blumwald, 2010).

2.3.1 Conventional Breeding

Conventional improvement to obtain new individuals is based on their genetic variation and uses the selection to incorporate better characteristics into the progeny. For this purpose, two plants possessing desirable traits are selected and then crossed to exchange their genes, so that the offspring has new genetic arrangements. Individual plants are tested for the expression of the desirable characteristic and its maintenance in future plant generations (McCouch, 2004). In practice, drought tolerance is selected together with plant productivity; non-commercial varieties displaying drought tolerance are crossed with susceptible, higher-yield plants (McCouch, 2004; Hieng *et al*., 2004; Lizana *et al*., 2006, Xoconostle-Cazares *et al.,* 2010).

2.3.2 Marker - assisted Breeding

Marker assisted breeding is the application of molecular biotechnologies, specifically molecular markers, in combination with linkage maps and genomics to alter and improve plant traits on the basis of genotype assays (Ribaut *et al.,* 2010).Genetic improvement can be assisted by using recognizable tags linked to target genes; these are known as molecular markers, which are based on polymorphisms that occur naturally in the DNA sequence. Different methods are employed to detect linked markers such as Restriction Fragments length polymorphisms (RFLPs), Sequence Characteristic Amplified Regions (SCARs), the Random Amplified Polymorphic DNA (RAPDs), Simple Sequence Repeats (SSRs), Amplified Fragment Length Polymorphism (AFLPs), among others (Van Berloo *et al*., 2008). The genetic factors responsible for part of the phenotypic variation observed for a quantitative characteristic are called Quantitative Trait Loci (QTLs). The use of molecular

markers to assist the selection of new varieties has an enormous potential to accelerate this process (Ashraf and Akram, 2009; Ashraf, 2010).

2.3.3 Plant Breeding Through Genetic Engineering

The capacity to stably insert a wide collection of drought-related genes to plant genomes has opened amazing opportunities for crop improvement. Recombinant DNA technology as well as **plant regeneration** is now applicable to a significant number of crop species (Gosal *et al*., 2009). Key genes observed as induced under abiotic stress in other plants or microorganisms have been employed to generate transgenic plants (Xoconostle-Cazares *et al*., 2010)

2.4 Genetic Diversity

Genetic diversity is genetic variability among individuals of a variety, or population of a species (Brown, 1983). It results from the many genetic differences between individuals and may be manifest in differences in DNA sequence, in biochemical characteristics (e.g. in protein structure or isoenzyme properties), in physiological properties (e.g. abiotic stress resistance or growth rate) or in morphological characters such as flower colour or plant form (Nevo *et al.,* 1984; Brown, 1988). Four components of genetic diversity can be usefully distinguished; the number of different forms (alleles) ultimately found in different populations, their distribution, and the effect they have on performance and the overall distinctness between different populations (Brown, 1988; Hamrick *et al.,* 1992). The variation that underpins genetic diversity arises from mutation and recombination. Selection, genetic drift and gene flow act on the alleles present in different populations to cause variation in the diversity in them (Nevo *et al.,* 1984; Brown, 1988). The selection can be natural or it can be artificial, as is the case with much of the variation present in crop species (Brown, 1988; Hamrick *et al.,* 1992). Genetic diversity is the basis for survival and adaptation and makes it possible to continue and advance the adaptive processes on which

evolutionary success and, to some extent human survival, depends (Ramanatha Rao *et al.,* 2002). Survival and adaptation can be viewed in terms of time, space and fitness. Fitness includes adaptation, genetic stability and variability (Ramanatha Rao *et al.,* 2002). The process of extinction can be due to biotic or abiotic stresses, caused by factors such as competition, predation, parasitism and disease, or to isolation and habitat alteration due to slow geological and climatic change, natural catastrophes or human activities (Ramanatha Rao *et al.,* 2002). Given these threats, it is essential that the genetic diversity in plant genetic resources be properly understood and efficiently conserved and used (Ramanatha Rao *et al.,* 2002).

A number of methods are currently available for analysis of genetic diversity. These methods have relied on pedigree data (Bernardo, 1993), morphological data (Smith and Smith, 1992; Bar-Hen *et al.,* 1995), agronomic performance data, biochemical data obtained by analysis of isozymes (Hamrick and Godt, 1997) and storage proteins (Smith *et al.*, 1987) and DNA-based markers that allow faster and more reliable differentiation of genotypes and assessment of genetic diversity (Law *et al.,* 1998; Cooke and Reeves, 1998; Donini *et al.*, 2000; Koebner *et al.,* 2003; Mohammadi and Prasanna, 2003; Supriya, 2010).

2.4.1 Genetic Diversity in Pearl Millet

Pearl millet is a highly cross-pollinated species and genetic diversity in the species is distributed both within and among cultivars (Poncet *et al.*, 1998). Pearl millet exhibits a tremendous amount of polymorphism at both phenotypic and genotypic levels (Liu *et al.,* 1994). This is due to its highly out crossing breeding behaviour, its apparent origin from several independent domestication events (Poncet *et al.*, 1998) and the wide range of stressful environments in which it has traditionally been cultivated (Liu *et al.,* 1992). Analyzing pearl

millet genetic diversity, its origin and its dynamics is important for germplasm conservation and to increase knowledge useful for breeding programs (Supriya, 2010). The more complicated distribution of diversity in pearl millet, as well as the higher degree of marker polymorphism, makes genetic diversity studies in this crop more complicated than in the other crops (Supriya, 2010). Thus, the breeding behaviour of pearl millet, and the structure of genetic diversity within this species, has strong implications for the use of molecular markers in its diversity assessment (Supriya, 2010). In pearl millet, limited efforts have been made to study genetic diversity and various markers used included isozymes (Tostain *et al*., 1987; Tostain and Marchais, 1989; Tostain, 1992), RFLPs (Bhattacharjee *et al.,*2002), AFLPs (vom Brocke *et al*., 2003), and microsatellites (Budak *et al*., 2003; Mariac *et al.,* 2006; Chakauya and Tongoona, 2008; Kapila *et al.,* 2008). The availability of DNA-based markers has provided more powerful tools for the detailed assessment of genetic diversity in cultivated and wild plants (Melchinger *et al*., 1994). Development and utilization of PCR-based markers such as SSR, RAPD, AFLP, ISSR, EST, STS is a valuable asset for estimating genetic diversity, the identification of unique genotypes as potentially important new sources of alleles for enhancing important characteristics, analyzing the evolutionary and historical development of cultivars at the genomic level in pearl millet breeding programme (Budak *et al.,* 2003; Kapila *et al*., 2008) (Supriya, 2010).

2.5 Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) is one of the polymerase chain reaction (PCR) based DNA markers, defined as the amplification of genomic DNA with single primer of arbitrary nucleotide sequence" (Weising *et al.*, 1995). The standard RAPD technology utilises short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR (Welsh and McClelland, 1990). Amplification products are generally separated on agarose gels and

stained with ethidium bromide. Decamer primers are commercially available from various sources (e.g, Operon Technologies Inc., Alameda, California). Welsh and McClelland (1990) independently developed a similar methodology using primers about 15 nucleotides long and different amplification and electrophoretic conditions from RAPD and called it the arbitrarily primed polymerase chain reaction (AP-PCR) technique. PCR amplification with primers shorter than 10 nucleotides [DNA amplification fingerprinting (DAF)] has also been used producing more complex DNA fingerprinting profiles (Caetano-Annoles *et al.,* 1991). Although these approaches are different with respect to the length of the random primers, amplification conditions and visualisation methods, they all differ from the standard PCR condition (Erlich, 1989) in that only a single oligonucleotide of random sequence is employed and no prior knowledge of the genome subjected to analysis is required (Bardakci, 2001).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Apparatus

Electrophoresis unit (Sigma Aldrich SHU6), PCR Machine (Peltier based Thermal cycler Labkits PCR-MGL96+/Y, Gel documentation system (Alpha imager mini Alpha Innotech), UV Transilluminator (Clinx Science Instruments), UV Spectrophotometer (Eppendorf Biophotometer), Micropippettes (Ecopippette by CAPP and Rainin 84413) Centrifuge (Eppendorf 5417R, Eppendorf tubes (Eppendorf).

3.1.2. Reagents

Cetyl trimethylammonium bromide CTAB (Sigma Aldrich), Isopropanol(Sigma Aldrich), β-mercaptoethanol (BDH), Sodium acetate (BDH), Absolute ethanol (Sigma Aldrich), Chloroform isoamyl alchohol (BDH), RNase (Fermentas), Tris HCl (BDH), Polyvinylpyrrolidone (PVP) (BDH), Agarose gel (BDH), Primers (Inqaba Biotech). Deoxynucleotide triphosphates (dNTPs) (Thermo scientific), Taq DNA polymerase (Thermoscientific), Magnesium chloride (Thermo scientific), Sodium Dodecyl sulphate (SDS) (BDH), Potassium acetate (BDH), 100bp molecular weight marker (O'GeneRuler Fermentas).

3.2 Methods

3.2.1. Collection of Samples

A total of ten (10) genotypes of pearl millet were collected from Lake Chad Research Institute (LCRI) Maiduguri, Borno State. The samples included:

GWAGWA, SOSAT-C88, PEO 5684, JKBH 778, LCRI HOPE PROJECT 5532, PEO 5984, EX BORNO, ZANGO, DMR 15, COMPOSITE

The seeds were collected and grown for 10 days in transplanting bags in a screen house to obtain young fresh leaves for DNA extraction.

3.2.2. Genomic DNA Extraction

3.2.2.1 CTAB Method (Dellaporta *et al*., 1983)

200mg of young fresh leaves were harvested. The leaves were crushed in liquid nitrogen with mortar and pestle. 450 µl of the extraction buffer (CTAB 2%, 1.4M NaCl, 100mM Tris ph 8.0, 20mM EDTA PH 8.0, 1% PVP, 0.1% β-mercaptoethanol) PH 8.0 was added. It was incubated at 65°C in a water bath for 30 minutes, it was cooled for 5 - 10minutes at room temperature and centrifuged at 3,500 rpm for 20 minutes at room temperature. The supernatant was transferred into new clean tubes. Chloroform Isoamyl alchohol (24:1) 450 µl was added in the fumehood. It was mixed gently by inversion for 5 minutes. It was centrifuged for 5 minutes at room temperature. The supernatant (400 µl) was transferred into fresh tubes. 400 µl of ice cold isopropanol was added and mixed well gently by inversion for about 8 to 10 times. It was incubated at -20°C for 30 minutes. It was centrifuged at 3,500 rpm for 20 minutes at 4°C. The supernatant was decanted in the fume hood and air dry pellet. 200 µl and 3 µl of premixed low salt T.E buffer and RNase (10mg/ml) was added to each sample. It was incubated at 37°C in a waterbath for 30minutes. Chloroform isoamyl alchohol (24:1) (200 µl) was added in the fume hood. It was mixed gently by inverting the tubes 5 times. It was centrifuged at 3,500rpm at room temperature for 10 minutes. The supernatant (180 µl) was transferred to fresh eppendorf tubes. Absolute ethanol (500 µl) and 3M sodium acetate (30:1.5) was added. It was centrifuged at 3500 rpm for 15 minutes at 4°C. The supernatant was decanted and the pellet was washed with 200 µl of 70% ethanol. It was centrifuged for 5 minutes at 3500 rpm at 4°C, the wash step was repeated twice. The supernatant was decanted

and the pellet was air dried for 1 hour. The pellet was resuspended in 100 µl T. E. and stored at 4°C.

3.2.2.2 Modified SDS Method (Ihase *et al., 2014*)

Young fresh leaves from each pearl millet variety, weighing 0.2g were collected. The samples were crushed using mortar and pestle with liquid nitrogen. The ground leaf samples were collected into labelled eppendorf tubes and 800µl of the extraction buffer (100mM Tris HCl ph 8.0, 50mM EDTA, 500mM NaCl, 5% SDS) were added. It was mixed by inversion. It was centrifuged for 5 min at 12000 rpm at 4°C. The supernatant was transferred into fresh eppendorf tubes. 200 µl of 5M potassium acetate was added. It was mixed by inversion and an equal volume of Phenol-Chloroform-Isopropanol (25:24:1) was added and centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was transferred into fresh tubes without disturbing the interface and 800 µl of cold absolute ethanol was added, it was mixed well and incubated át -20°C for 20 min. It was then centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was decanted. Ethanol (70%) was added to wash the pellet, this step was repeated twice. The samples were air-dried for 30 min. 100 µl of low salt T. E. buffer was added. 2µl of RNase (10mg/ml) was added per tube. Samples were transferred to the water bath and incubated at 37°C for 1 hour. The samples were then prepared for agarose gel electrophoresis.

3.2.3. Estimation of Quality and Quantity of Isolated Genomic DNA

3.2.3.1 Agarose gel electrophoresis

Agarose gel (0.8 %) was prepared by weighing accurately 0.4g of agarose powder, which was added to 50 ml of 1X TBE (Tris boric EDTA) buffer, it was dissolved in a microwave oven for 2 min to obtain a clear solution. In the fume hood, 0.1% (a drop) of ethidium bromide (fluorescent dye) was added to the solution with vigorous shaking and allowed to cool for

40^0C. The gel was poured into the gel casting tray and was allowed to set for about 20 min. The prepared gel was placed in the electrophoresis tank and the buffer (1X TBE) was added to cover the gel. 3µl of the genomic DNA samples (mixed with a gel loading dye (6X Orange DNA loading dye) was loaded into the wells in the gel accordingly. The 100bp molecular weight marker (O'GeneRuler Fermentas) was also loaded in the first well on the agarose gel. Agarose gel electrophoresis was run for 30 min at 55volts. The gel was visualised using a gel documentation system and photographed.

3.2.3.2 Estimation of the quality and quantity of the extracted genomic DNA by UV Spectrophotometry

For spectrophotometric analysis, 2µl of DNA sample were diluted with 100µl with T.E. buffer and 100µl of the blank solution were prepared. The spectrophotometer readings was recorded, the blank sample was read in the machine first, the DNA samples diluted in T. E. buffer was recorded at 260nm/280 nm using Biophotometer (Eppendorf).

DNA concentration was calculated using absorbance values at 260 nm using the following formula.

Concentration of DNA (µl/ml) = <u>Absorbance at 260 nm x dilution factor x 50µg/ml</u>

1000

3.2.4 RAPD-PCR

A set of ten (10) primers obtained from Inqaba Biotech (South Africa) was used for RAPD-PCR. The primer designation and sequence are presented in Table 3.1.

Table 3.1: Primer Designation and Sequence

RAPD primer designation	Sequence
	5´-3´
P16	TCGGCGGTTC
P17	CTGCATCGTG
P18	TGAGCCTCAC
P19	TCGGCACGCA
P20	CTGCGCTGGA
P26	ATCGGGTCCG
P27	CCGTGCAGTA
P28	TAGCCGTGGC
P29	GGCTAGGGGG
P30	TACGTGCCCG

Adapted from Gorji *et al.,* (2010).

3.2.4.1 Preparation of stock solutions for primers

This was prepared according to manufacturer's instruction. Stock solutions of primers were prepared in a sterile T. E. buffer solution (10mM Tris HCl, pH 7.5 to 8.0, 1mM EDTA).

3.2.4.2 Preparation of T.E. Buffer

One times (1X) TE buffer solution consists of 10 mM Tris, adjusted to pH 7.5 with HCl and one (1) mM EDTA. 1 ml of Tris HCl was measured from an already prepared 1 M stock solution of Tris HCl and 0.2mls of EDTA was measured from 0.5M stock solution of EDTA. This solution was made up to 100mls with distilled water, sterilised and stored at room temperature.

3.2.4.3 Constitution of primers

Lypholised oligos were centrifuged to obtain pellets that might be displaced from the bottom of the tube during shipment. Primer stock solution was prepared (100 µM) with sterile T.E. buffer solution. Working solutions were diluted from the stock solution with sterile nuclease free water to prevent inhibition of enzymatic reactions and degradation of the nucleic acids by nucleases. From the stock solution, 25µl (15 µM concentration), was divided into smaller aliquots for long term storage to avoid frequent freeze thaw cycles and accidental contamination.

3.2.4.4 Preparation of working solutions for primers

Fifteen (15) µM concentration of primers for working solution was prepared from the stock solution. Microcentifuge tubes were labelled for working solutions appropriately. The tubes were disinfected with UV light in equipped laminar flow hood for 15 min. Eighty –five (85)ul of T.E. buffer were added to each tube. Twenty five (25) µl of the 100µM primer stock solution were added to each tube. It was vortexed for 30seconds at 3, 000 rpm. Tubes were

sealed with parafilm and placed in -20°C freezer. Ten (10) tubes to be used within the month were kept in -20°C, while the rest was stored in a -80°C freezer.

3.2.4.5 Procedure for RAPD-PCR

All reagents needed for RAPD-PCR were arranged in a freshly filled ice bucket. They were allowed to thaw completely before setting up the reaction. The RAPD-PCR Program/cycling conditions inputted into the PCR machine (Peltier Based Thermacycler Labkits PCR-MGL-96+/Y) are presented in Table 3.2

3.2.4.6 RAPD-PCR reaction mixture

PCR tubes were labelled accordingly with an ethanol resistant marker. A mastermix was prepared on ice in 5 eppendorf 1.5 ml tubes for the different primers (P16, P17, P18, P19, P20). The PCR reagents were pippetted into the tube in the following order; sterile water, 10X reaction buffer, dNTP mix, $MgCl_2$, Primer, and Taq DNA polymerase. It was mixed gently by pipetting up and down for at least 20 times. A 96 well plate was placed in the ice bucket as a holder for the 0.2ml thin walled PCR tubes. (Ten) 10ul of the DNA template was added into the PCR tubes. Fifteen (15) µl of the mastermix was gently added to the DNA template in the PCR tubes to make up the total volume of reaction to 25ul. The various components used for RAPD-PCR reaction are presented in Table 3.3.

Table 3.2: RAPD – PCR Cycling conditions

Steps	Cycles	Temperature	Time (min or secs)
Initial denaturation	1	94°C	4 min
Denaturation		94°C	1 min
Annealing	40	36°C	2 min
Extension		72°C	2 min
Final extension	1	72°C	15 min
Store/end		4°C	

Table 3.3: Master Mix for RAPD-PCR amplification

Reagents	1 sample (µl)	X 12 samples (µl)
Nuclease free water (SDW)	7.0	84.0
10X Reaction buffer	5.0	60.0
25Mm MgCL2	1.0	12.0
10Mm dNTPs	1.0	12.0
Taq DNA polymerase (5U/µl)	0.5	6.0
Primer	0.5	6.0
DNA template (50ng)	10.0	
Total	**25.0**	**180.0**

The tubes were placed in the PCR machine and covered. The RAPD-PCR program was run using these cycling conditions (Table 3.3). After the PCR programme was finished the tubes were removed from the thermocycler and stored at 4°C in the refrigerator. This procedure was repeated for primers P26, P27, P28, P29 and P30.

3.2.5 Agarose gel electrophoresis

One percent (1%) agarose gel was prepared by weighing accurately 0.5g of agarose powder, which was added to 50 mls of 1X TBE (Tris boric EDTA) buffer, it was dissolved in a microwave oven for 2 min to obtain a clear solution. In the fume hood, 0.1% (a drop) of Ethidium bromide (fluorescent dye) was added to the solution with vigorous shaking and allowed to cool for 40^0C. The gel was poured into the gel casting tray and was allowed to set for about 20 min. The prepared gel was placed in the electrophoresis tank and 1X TBE buffer was added to cover the gel. Five (5) µl of the RAPD - PCR product mixed with 2 µl of the gel loading dye (6X Orange DNA loading dye) was loaded into wells on the agarose gel. Seven (7) µl of the 100bp molecular weight marker (Fermentas) were also loaded in the first well. Agarose gel electrophoresis was run for 45 min at 55volts. It was then visualised with a gel documentation system and photographed.

3.2.6 Nei's Analysis

The RAPD profile was scored for the presence, (represented as 1) or absence (represented as 0) of bands of various molecular weight sizes in the form of binary matrix. Data was analyzed to obtain Nei's similarity coefficients among the isolates by using NTSYS-pc (Exeter Biological Software, Setauket, NY, Rohlf, 1993).

CHAPTER FOUR

4.0 RESULTS

4.1 Quality and quantity of extracted genomic DNA by UV spectrophotometer

Genomic DNA was extracted from ten varieties of pearl millet. The Optical density of DNA samples from the pearl millet varieties was measured by spectrophotometry (UV absorbance) at 260nm and 280nm, using Bio photometer (Eppendorf) as shown in Table 4.1. The samples exhibited an absorbance ratio (260nm/280nm) between 1.7 and 2.0 with a mean value of 1.9 (Figure 1). DNA concentrations ranged between 1500 to 1700 ng per extraction, which is sufficient for various downstream applications.

4.2 Quality and quantity of extracted genomic DNA by Agarose gel electrophoresis

The quality of the extracted DNA was also evaluated by agarose gel electrophoresis. The result of the extracted DNA run on a 0.8% agarose gel, stained with ethidium bromide and visualized with UV light is shown in Plate III and Plate IV. The bands on the electrophoresis were sharp and distinct.

Table 4.1: Quantity and Quality of Genomic DNA Extracted from Different Pearl millet

Sample No	Concentration (µg/µl)	Absorbance (260nm)	Absorbance (280nm)	Ratio (260/280nm
1	0.017	2.199	1.826	1.82
2	0.009	0.442	0.238	1.85
3	0.006	0.282	0.152	1.83
4	0.008	0.393	0.205	1.92
5	0.003	0.139	0.079	1.76
6	0.005	0.240	0.128	1.87
7	0.007	0.351	0.184	1.90
8	0.015	0.754	0.408	1.89
9	0.007	0.349	0.188	1.86
10	0.010	0.484	0.277	1.77

Plate III: Electrophoretogram of genomic DNA extracted from 10 pearl millet varieties (cultivars) using CTAB Method. M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532
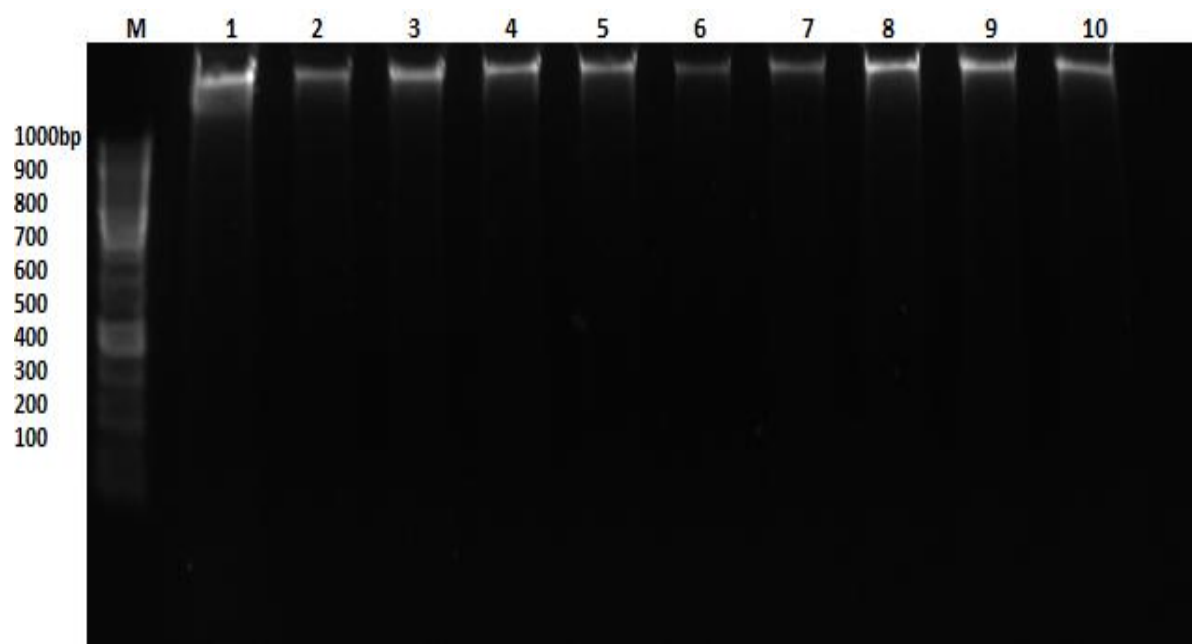
Plate IV: Electrophoretogram of genomic DNA extracted from 10 pearl millet varieties (cultivars) using modified SDS method M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532

4.3 RAPD-PCR Analysis

The ten primers amplified the DNA samples with clear differences in their banding pattern.

4.3.1 RAPD analysis using primer P16 (TCGGCGGTTC)

This primer amplified 8 bands with sizes ranging from 600-1000 bp (Plate V). Among such fragments, no common bands were identified in all the varieties. Two varieties DMR-15 and COMPOSITE produced 600bp fragment. Other varieties (PEO 5684, GWAGWA, ZANGO, PEO 5984, SOSAT C 88 and JKBH 778 all produced 1000bp fragments).

4.3.2 RAPD analysis using primer P17 (CTGCATCGTG)

Primer P17 produced a total number of 5 amplified fragments with sizes ranging from 400-1000 bp (Plate VI). RAPD analysis of P17 primer produced 4 fragments in PEO 5684, GWAGWA, ZANGO, PEO 5984 AND COMPOSITE ranging between 400bp -900bp. All the varieties produced a 1000bp fragment.

4.3.3 RAPD analysis using primer P18 (TGAGCCTCAC)

The results of RAPD marker analysis by using primer P18 for the ten pearl millet varieties are shown in Plate VII fragments of molecular sizes ranging from 500- 1000bp were produced in all varieties except DMR-15 and GWAGWA. A specific band was observed in SOSAT C88 with a molecular size of 700bp

Plate V: Electrophoretic banding patterns of RAPD with primer P16 (TCGGCGGTTC). M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532
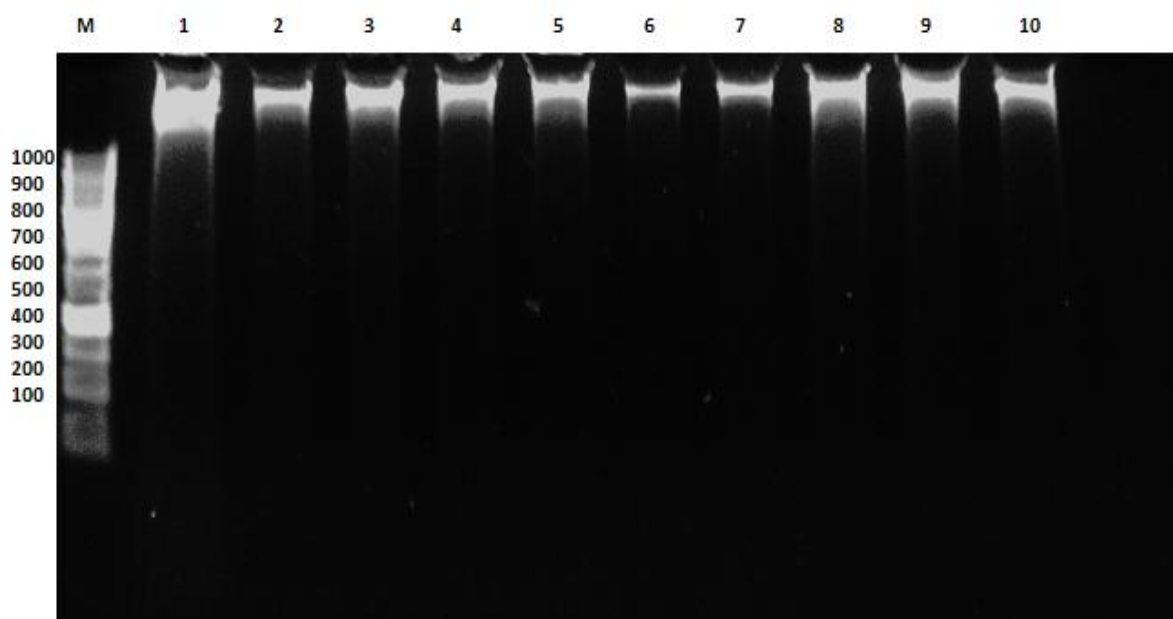
Plate VI: Electrophoretic banding patterns of RAPD with P17 (CTGCATCGTG). M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532
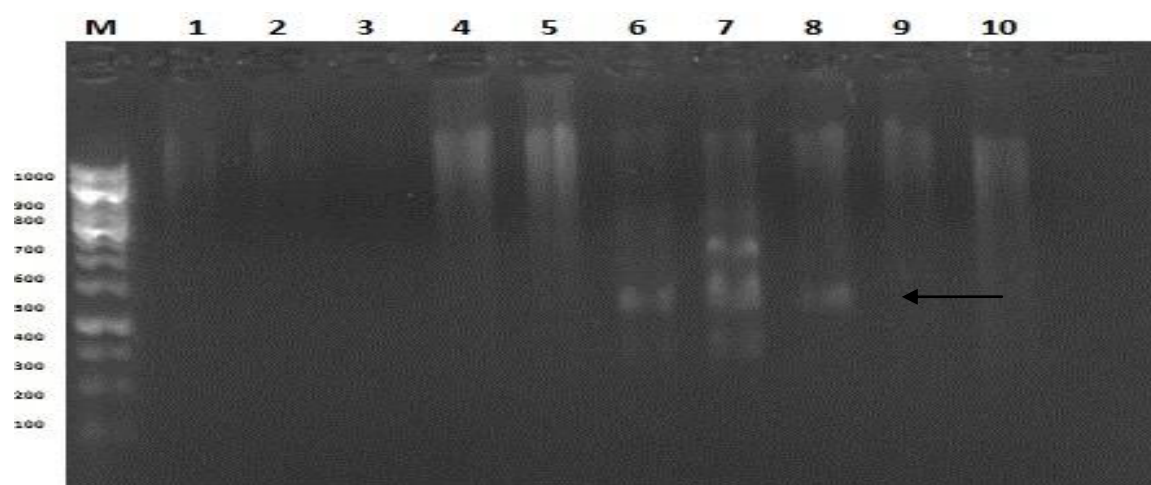
Plate VII: Electrophorectic banding Patterns of RAPD with P18 (TGAGCCTCAC). M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532

4.3.4 RAPD analysis using primer P19 (TCGGCACGCA):

This primer produced 7 amplified fragments in all varieties with molecular weights ranging from 200 to 1000 bp. A 1000bp fragment was produced in all the varieties (Plate VIII).

4.3.5 RAPD analysis using primer P20 (CTGCGCTGGA):

The analysis of P20 amplicons show a total of 4 fragments ranging from 300bp -1000bp distributed in the ten varieties of pearl millet (Plate IX).

4.3.6 RAPD analysis using primer P26 (ATCGGGTCCG):

The analysis using primer P26 produced a total number of 3 fragments ranging from 200 to1000 bp. Two specific bands were observed in DMR-15 variety at 400bp and 200bp (Plate X).
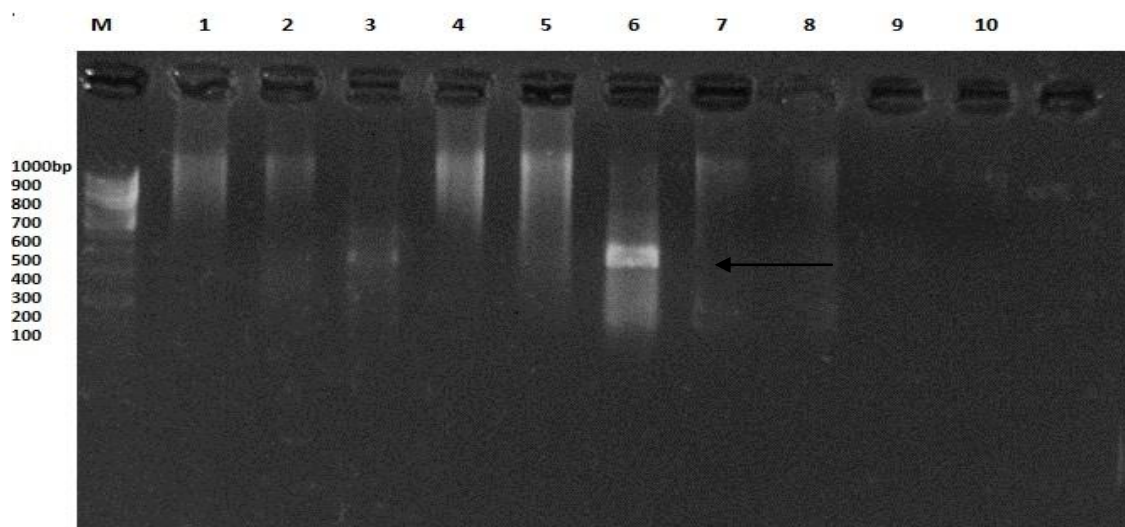
Plate VIII: Electrophoretic banding patterns of RAPD with P19 (TCGGCACGCA). M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532

Plate IX: Electrophoretic banding patterns of RAPD with P20 (CTGCGCTGGA). M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532
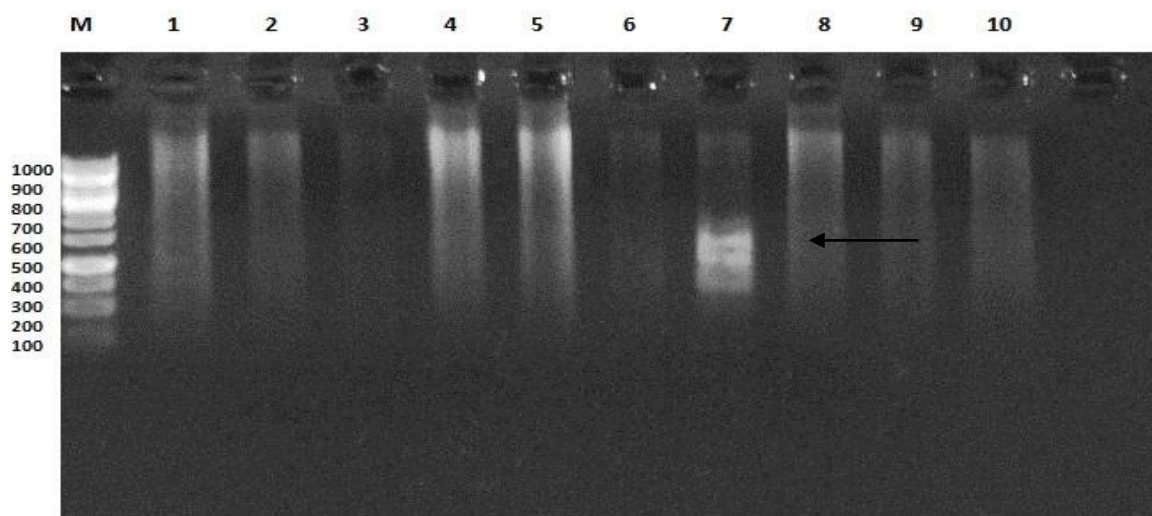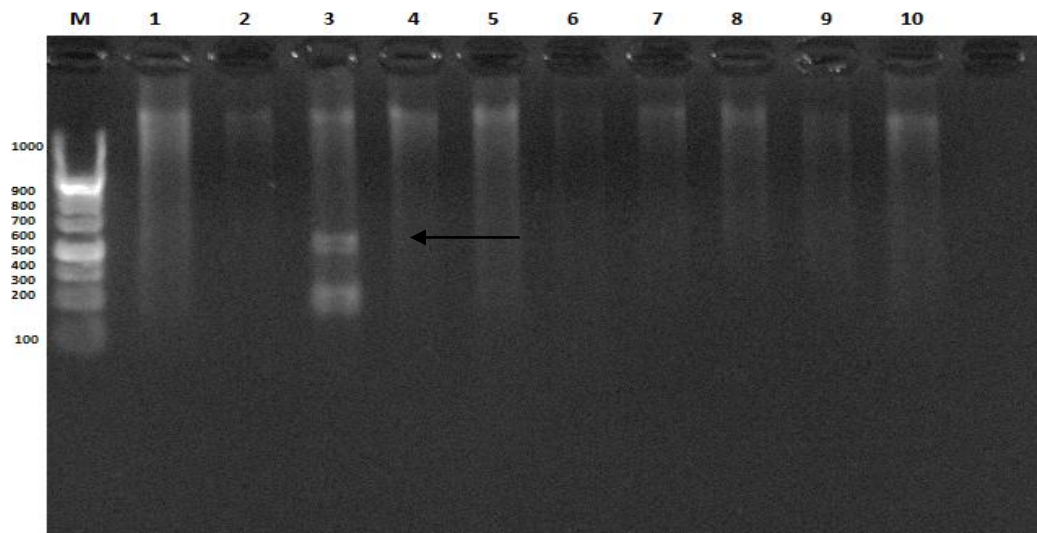
Plate X: Electrophoretic banding patterns of RAPD with P26 (ATCGGGTCCG). M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532

4.3.7 RAPD analysis using primer P27 (CCGTGCAGTA):

A total number of 6 fragments were produced by primer P17 with bands sizes ranging from 100bp – 1000bp (Plate XI). Some specific bands were observed in DMR 15 550bp and 700bp, PEO 5684 with bands sizes of 400bp and 100bp respectively.

4.3.8 RAPD analysis using primer P28 (TAGCCGTGGC):

The analysis using primer P28 obtained a total number of 5 fragments ranging from 200bp – 1000bp. A 1000bp fragment was produced in all the varieties, while GWAGWA variety had 2 specific bands with band sizes of 300bp and 200bp respectively (Plate XII).

4.3.9 RAPD analysis using primer P29 (TACGGTGCCG):

A total number of 5 fragments were observed with fragment sizes ranging from 500bp – 1000bp in all the varieties (Plate XIII).

4.3.10 RAPD analysis using primer P30 (TACGGTGCCG):

RAPD analysis using primer P30. A total number of five (5) fragments were amplified ranging from 300bp - 1000bp. Three specific bands 1000bp, 900bp and 300bp were observed in PEO 5684 (Plate XIV).
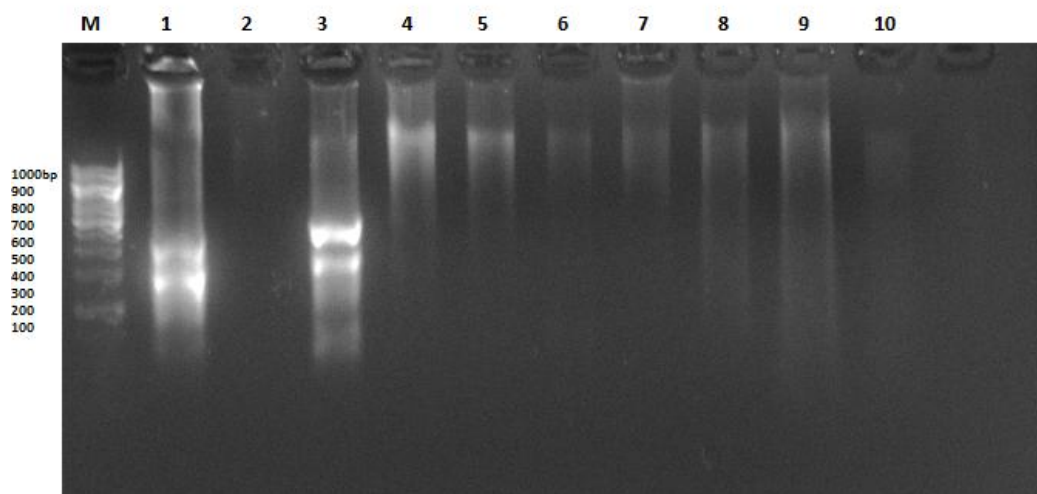
Plate XI: Electrophorectic banding patterns of RAPD with P27(CCGTGCAGTA) M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532
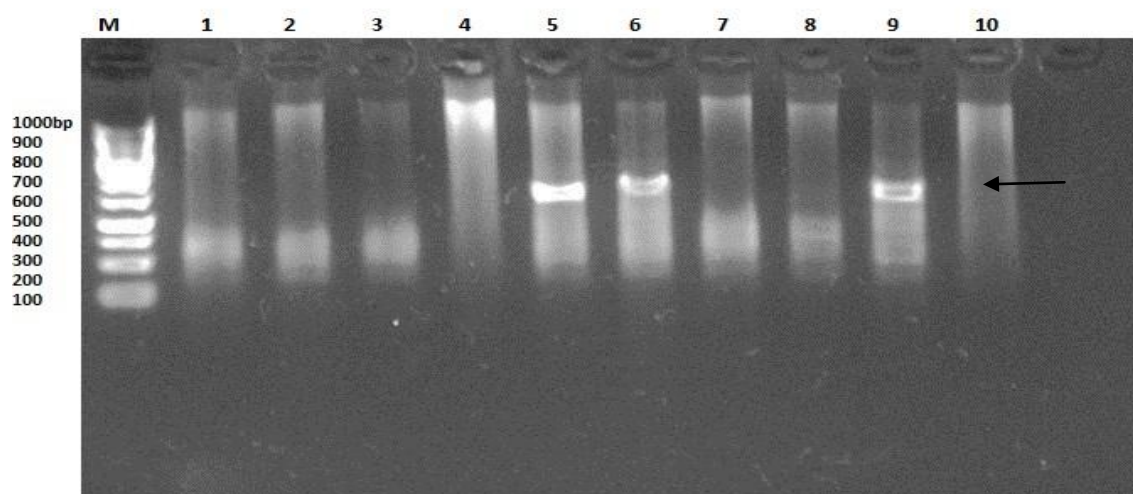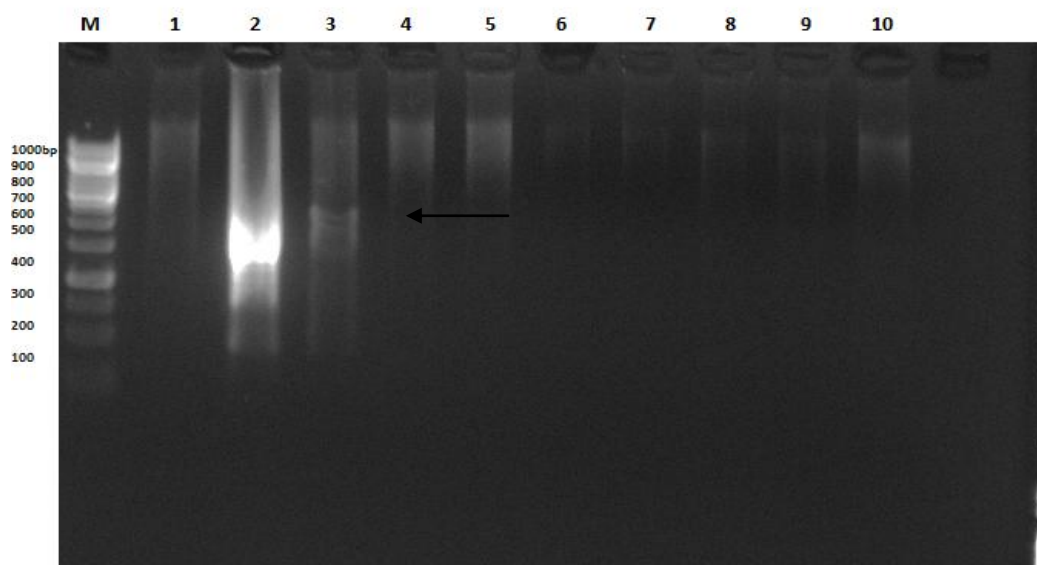
Plate XII: Electrophorectic banding patterns of RAPD with P28 (TAGCCGTGGC). M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532
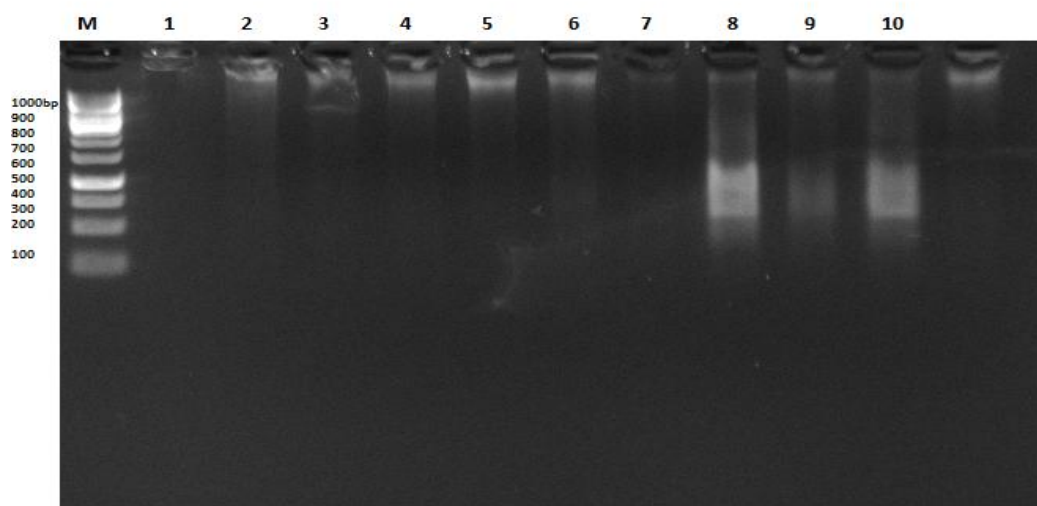
Plate XIII: Electrophorectic banding patterns of RAPD with P29 (GGCTAGGGGG). M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532
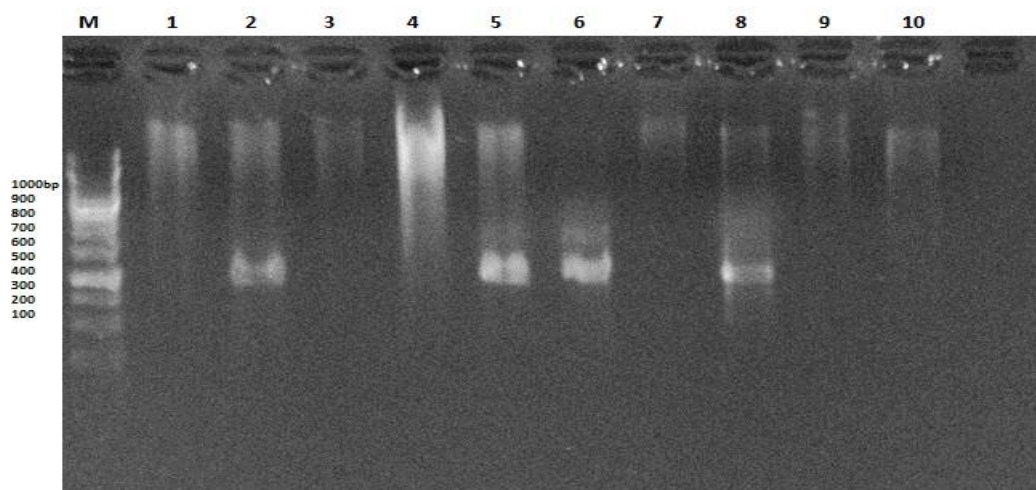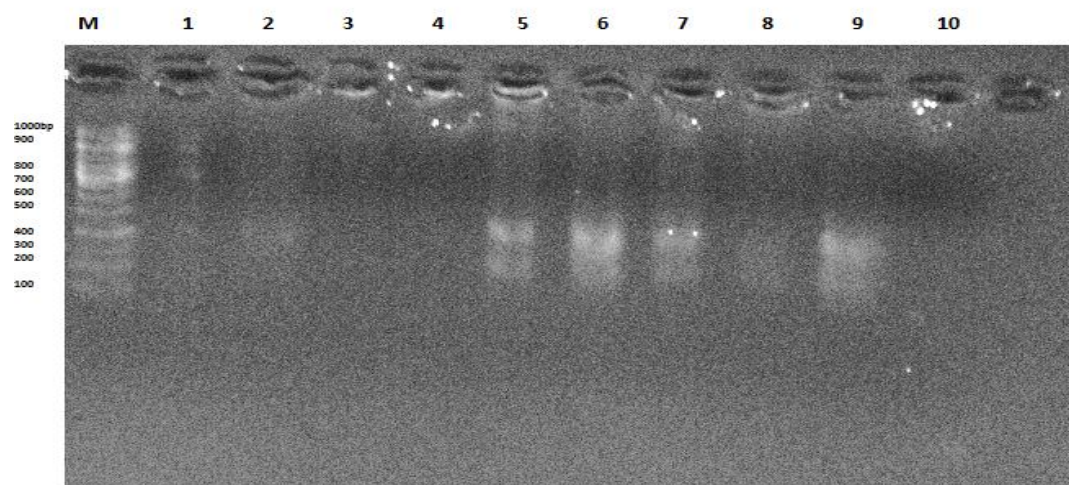
Plate XIV: Electrophorectic banding patterns of RAPD with P30 (TACGTGCCCG). M = Molecular weight marker 100bp. Lane 1 = PEO 5684, Lane 2 = GWAGWA, Lane 3 = DMR-15, Lane 4 = ZANGO, Lane 5= PEO 5984, Lane 6 = COMPOSITE, Lane 7 = SOSAT-C88, Lane 8 = JKBH -778, Lane 9 = EX-BORNO, Lane 10 = LCRI/HOPE 5532

A total of 184 bands were produced by the ten primers in all the ten varieties of pearl millet (Table 4.2). Primers P16 (TCGGCGGTTC), P17 (CTGCATCGTG), P18 (TGAGCCTCAC), P19 (TCGGCACGCA) and P28 (TAGCCGTGGC) amplified a fragment of 600bp size that was present in varieties suspected to be drought tolerant and absent in others. These include; COMPOSITE, SOSAT C88, DMR 15, EX BORNO and JKBH 778 (Table 4.2). Forty three (44) fragments were polymorphic. Percentage polymorphism was calculated (Table 4.3). P16 (TCGGCGGTTC), P18 (TGAGCCTCAC), P20 (CTGCGCTGGA), P27 (CCGTGCAGTA), P29 (GGCTAGGGGG) and P30 (TACGGTGCCG) produced the highest number of polymorphic bands, with 100% polymorphism; However, P26 (ATCGGGTCCG) produced the least percentage of polymorphic bands (66.70%) as shown in Table 4.3.

Table 4.2: Primer-specific Scoring of Bands in Ten (10) varieties of Pearl millet

varieties		PEO 5684	GWAGWA	DMR-15	ZANGO	PEO 5984	COMPOSITE	SOSAT-C88	JKBH-778	EX-BORNO	LCRI/ HOPE5532	Polymorphism
Primer	**MW**											
P16	1000bp	1	1	0	1	1	0	1	1	0	0	Polymorphic
	600bp	0	0	1	0	0	1	0	0	0	0	Polymorphic
P17	1000bp	1	1	1	1	1	1	1	1	1	1	Monomorphic
	900bp	1	1	0	1	1	0	0	0	0	0	Polymorphic
	600bp	1	0	0	0	0	1	0	0	0	0	Polymorphic
	500bp	0	0	0	0	0	1	0	0	0	0	Unique
	400bp	0	0	0	0	0	1	0	0	0	0	Unique
P18	1000bp	1	0	0	1	1	1	1	1	1	1	Polymorphic
	900bp	0	0	0	1	1	0	0	1	1	1	Polymorphic
	800bp	0	0	0	0	0	1	1	0	0	0	Polymorphic
	700bp	0	0	0	0	0	0	1	0	0	0	Unique
	600bp	0	0	0	0	0	1	1	1	0	0	Polymorphic
	500bp	0	0	0	0	0	1	1	0	0	0	Polymorphic
P19	1000bp	1	1	1	1	1	1	1	1	1	1	Monomorphic
	900bp	1	1	0	0	0	0	0	0	0	1	Polymorphic
	600bp	0	0	0	0	1	1	0	0	1	0	Polymorphic
	500bp	0	0	0	0	0	0	0	1	0	0	Unique
	400bp	1	1	1	0	1	1	1	1	0	0	Polymorphic
	300bp	1	1	1	0	1	1	1	1	0	0	Polymorphic
	200bp	1	1	1	0	1	1	1	1	1	0	Polymorphic
P20	1000bp	1	1	1	1	1	0	1	1	1	1	Polymorphic
	900bp	1	1	1	1	1	0	0	0	0	0	Polymorphic
	400bp	0	0	0	0	1	1	0	0	0	0	Polymorphic
	300bp	0	1	0	0	1	1	0	1	0	0	Polymorphic

Table 4.2: Primer-specific Scoring of Bands in Ten (10) varieties of Pearl millet

varieties		PEO 5684	GWAGWA	DMR-15	ZANGO	PEO 5984	COMPOSITE	SOSAT-C88	JKBH-778	EX-BORNO	LCRI/HOPE5532	Polymorphism
Primer	MW											
P26	1000bp	1	1	1	1	1	1	1	1	1	1	Monomorphic
	400bp	0	0	1	0	0	0	0	0	0	0	Unique
	200bp	0	0	1	0	0	0	0	0	0	0	Unique
P27	1000bp	0	0	1	1	1	1	1	1	1	0	Polymorphic
	900bp	0	0	0	1	1	0	0	0	0	0	Polymorphic
	400bp	0	0	1	0	0	0	0	0	0	0	Unique
	300bp	1	0	1	0	0	0	0	0	0	0	Polymorphic
	200bp	1	0	0	0	0	0	0	0	0	0	Unique
	100bp	0	0	1	0	0	0	0	0	0	0	Unique
P28	1000bp	1	1	1	1	1	1	1	1	1	1	Monomorphic
	900bp	1	0	1	1	1	0	0	0	0	0	Polymorphic
	600bp	0	1	1	0	0	0	0	0	0	0	Polymorphic
	300bp	0	1	0	0	0	0	0	0	0	0	Unique
	200bp	0	1	0	0	0	0	0	0	0	0	Unique
P29	1000bp	0	1	1	1	1	1	1	1	1	1	Polymorphic
	800bp	0	0	0	0	0	0	1	0	0	0	Unique
	700bp	0	0	0	0	0	0	1	1	1	0	Polymorphic
	600bp	0	0	0	0	0	0	1	1	1	0	Polymorphic
	500bp	0	0	0	0	0	0	1	1	1	0	Polymorphic
P30	1000bp	1	0	0	0	0	0	0	0	0	0	Unique
	900bp	1	0	0	0	0	0	0	0	0	0	Unique
	800bp	1	0	0	0	0	0	0	0	0	0	Unique
	400bp	1	1	0	0	1	1	1	1	1	0	Polymorphic
	300bp	0	0	0	0	1	1	1	1	1	0	Polymorphic
	TOTAL	21	18	19	14	22	22	22	21	16	9	

Table 4.3: Percentage (%) Polymorphisms Obtained by the Ten Primers Used

Primer	Range of fragment sizes in bp	Total No. of fragments	Polymorphic	Percentage polymorphism
P16	600-1000bp	2	2	100
P17	400-1000bp	5	4	80
P18	500-1000bp	6	6	100
P19	200-1000bp	7	6	85.7
P20	300-1000bp	4	4	100
P26	200-1000bp	3	2	66.7
P27	100-1000bp	6	6	100
P28	200-1000bp	5	4	80
P29	500-1000bp	5	5	100
P30	300-1000bp	5	5	100
TOTAL		48	44	91.6

4.4 Nei's Analysis

The relationships among the individual genetic populations studied using distance matrix method calculated based on Nei (1973) index of genetic similarity and distance. This was used to study the genetic distance between the varieties. The highest genetic similarity (73%) exists between ZANGO and PEO 5984 While the lowest similarity (3%) was observed between COMPOSITE and PEO 5684 (Table 4.4). Analysis of the values using Unweighted Pair Group Method with Arithmetic mean (UPGMA) as implemented in the UPGMA program of the NTSYS software package produced trees based on their genetic-relationship.

Table 4.4: Nei's Analysis of Genetic Distance and Genetic Similarity of the Pearl Millet Varieties (%).

	peo5684	Gwagwa	dmr15	zango	peo5984	composite	sosatc88	jkbh778	Exborno	Lcri5532
peo5684	0									
Gwagwa	37	0								
dmr15	16	35	0							
Zango	28	28	25	0						
peo5984	33	45	24	73	0					
Composite	3	14	11	4	37	0				
sosatc88	20	23	11	22	37	49	0			
jkbh778	24	35	14	34	58	45	70	0		
Exborno	19	19	16	42	55	40	58	71	0	
lcrihope5532	31	38	24	60	42	22	33	46	60	0

4.5 Phylogenetic Analysis

A Dendrogram was constructed using numerical taxonomic system (NTSYS) Exeter biological software to characterize *the sample* populations and presented in Figure 4.1. Phylogenetic analysis shows two major clusters (cluster A and B) at 40% genetic diversity with cluster B involving DMR15 variety clustered separately while the other varieties clustered at cluster A. Cluster A was sub-divided to another three distinct clusters (cluster I, II and III) with COMPOSITE (cluster III) clustered separately from the others. Cluster II consists of six samples showing relative genetic relationship, these include LCRI/HOPE - 5532 and EX-BORNO (60%) shows some genetic relationship with other samples in the sub-cluster. SOSAT-C88 also clustered with JKBH 778 (70%). The last sub-group from cluster II clustered ZANGO and PEO 5984 together (73%). The last sub-group from cluster A grouped PEO 5684 and GWAGWA (37%) as cluster I.
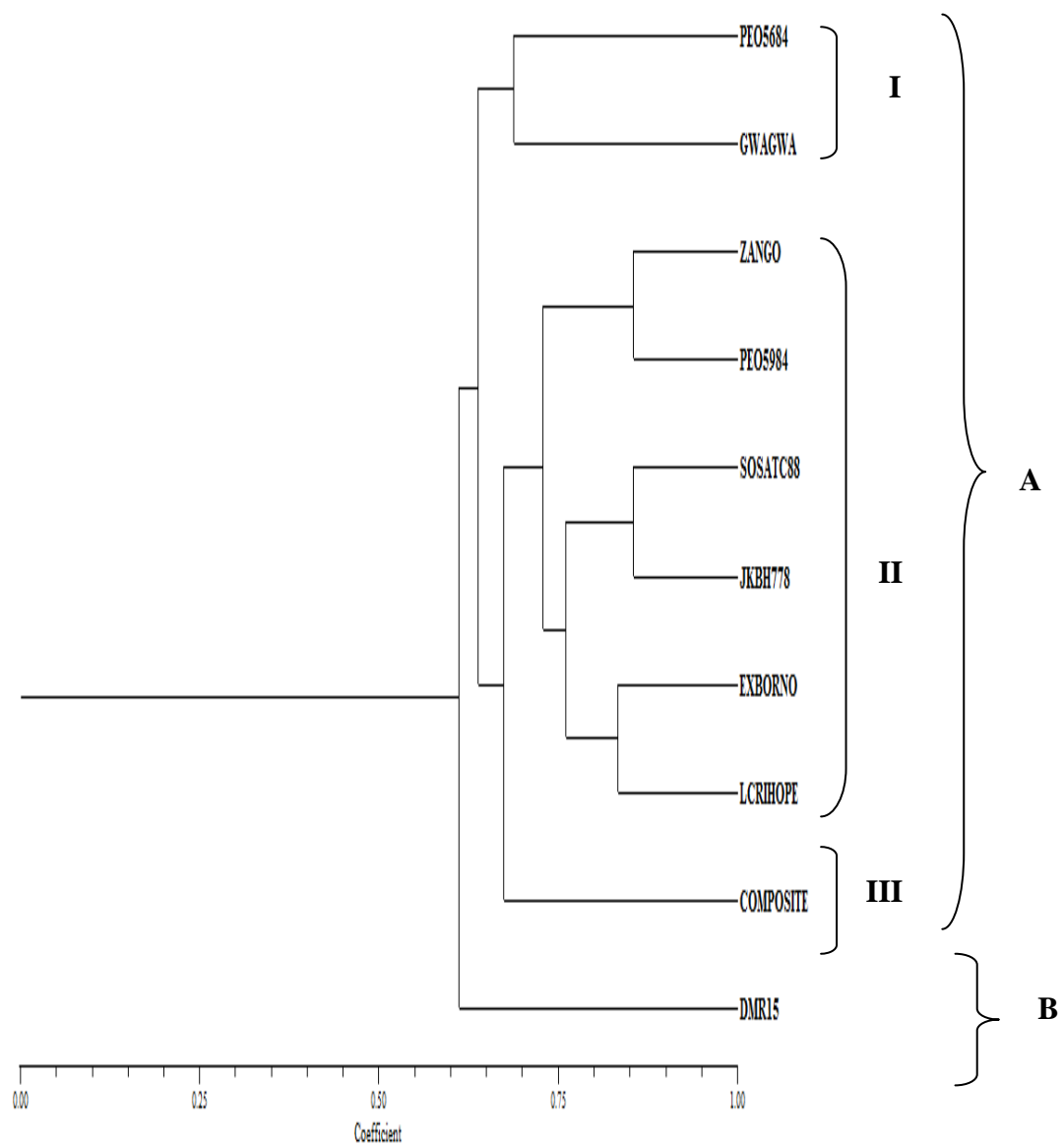
Figure 4.1: Dendrogram showing genetic relationship of the ten varieties of pearl millet

CHAPTER FIVE

5.0 DISCUSSION

DNA extraction from samples is the first step for all genetic diversity studies. Extraction of genomic DNA from Pearl millet using the SDS method with slight modifications was simple and easy. The modifications in this protocol involved shorter centrifugation time and a three time wash. This is because DNA extraction from some plants could be time consuming and laborious. This was not time consuming compared to the CTAB DNA extraction method. Protocols for the isolation of the DNA took 30 minutes. Young fresh leaf tissues were used in this method, because using younger leaves instead of older ones reduced nucleic acid contamination by plant metabolites that interfere with solubilisation of precipitated nucleic acids (Puchaa, 2004). A three-time wash combined with a short-run centrifugation was sufficient for DNA purification and removal of endogenous nucleases or other proteins, which agrees with the findings of Zidani *et al.,* (2005). The purity of genomic DNA extracted from pearl millet is dependent on the number of washes (Zidani *et al.,* 2005). Furthermore, high purity DNA is required for random amplified polymorphic DNA (RAPD) - PCR and other PCR based techniques (Khanuja *et al.,* 1999). The purity of DNA obtained in this study using this method varied between 1.7 and 1.9 which shows that the DNA is of high quality. Das *et al.,* (2009) also isolated DNA of high quality. The accepted range of 260nm/280nm ratios are commonly between 1.7 and 2.2, respectively for DNA purity. (Thermo Scientific, 2011).

High yield of DNA extracted is also required (Prasad and Padmalatha, 2006). The DNA yield using SDS method ranged from 1500ng to 1700ng per extraction showing that the DNA has a high yield. (Maniatis *et al.,* 1982, Gulia *et al.,* 2010). DNA isolated in this present study yielded strong and reliable amplification products showing its suitability for RAPD-PCR

using random decamer primers. The amplified fragments sizes ranged between 200 bp to 1000 bp. This proves that a high quality DNA is required for good amplification in RAPD-PCR. The efficiency and the speed show that this protocol is suitable for the extraction of DNA from leaf tissues of pearl millet for RAPD analysis. Thus, the DNA produced by this simple and modified protocol can be used in RAPD- PCR and may be used also for other PCR-based applications.

The RAPD-PCR analysis reveals ambiguous genetic polymorphisms among the ten varieties under study. The analysis of RAPD profiles indicates the existence of genetic variability between the varieties. RAPD PCR has proved to be a powerful tool for the identification of polymorphism in many crops including pearl millet. In the present study, RAPD show a high level of polymorphism and a high number of clearly amplified bands. A high level of polymorphism was in accordance with the report by Kale and Munjal (2005) and Jaya Prakash *et al*., (2006). Among the use of molecular markers, the most simple, fast and easy to perform assay is the use of RAPD markers to determine the existing genetic diversity and variation among and within the population. The first use of RAPD markers was reported by Williams *et al*., (1990) and Welsh and McClelland (1990). RAPD markers proved to be very informative and useful in monitoring the genetic diversity present in the ten pearl millet varieties. The use of RAPD has several advantages over other molecular marker techniques (Keil and Griffin, 1994). In contrast with other molecular techniques such as SNP, SSR, RFLP, DNA sequencing and allozymes, the technique of RAPD does not require previous knowledge of the genome in study. From this study, it is apparent that RAPD markers can be used with a great degree of confidence in pearl millet. The present study indicates that from the band patterns obtained with the use of the ten (10) primers, the different varieties of pearl millet can be identified.

The dendrogram obtained demonstrate clearly that genetic diversity exists among the ten varieties studied, In addition, the varieties are of distinct genotype and there may be no duplication among the ten pearl millet varieties. These findings of the present study agree with those of Govindaraj *et al.,* (2009) who reported genetic diversity analysis in some pearl millet accessions using molecular markers. A range of observations were made in the current analyses of genetic diversity of the ten pearl millet samples using their molecular traits. Overall, moderate level of dissimilarity was observed among the accessions. This indicates better possibilities for genetic improvement of the crop through selection and cross breeding (Fikreselassie, 2012). However, a very high level of similarity was revealed within genotypes that clustered together. The use of genotypes from different clustering groups for any breeding programme aiming to develop suitable varieties with specific characters as identified from the RAPD analysis is therefore strongly recommended and those that show high level of diversity are of great economic value as discovered in this study. They should be harnessed to improve accessions. Also, this study would avoid the use of materials with similar genetic background, as well as spending time, money and other resources on materials not having the best chance to produce the best result. The genetic diversity analysis among the varieties shows the genetic distance and similarity on the whole genome basis, which is the difference in their genetic makeup throughout the genome. If the genetic diversity in the germplasm is considerably less, measures should be taken to widen t he available gene pool and germplasm and analyzed time to time to reveal genetic diversity (Govindaraj *et al.,* 2009).

In this study, some RAPD markers that may be associated with drought tolerance were identified. Primers P16, P17, P18, P19 and P28 amplified a 600bp fragment that was present

in varieties COMPOSITE, SOSAT-C88, DMR-15, EX-BORNO and JKBH-778 which are suspected to be drought tolerant. These findings agree with the findings of Nazari and Pakniyat, (2008). They reported genetic diversity in wild and cultivated barley under drought stress using RAPD, The duo found markers associated with drought tolerance using RAPD markers. Gorji *et al.*, (2010) also reported on the use of RAPD markers in recognition of genotypes tolerant to drought in bread wheat. Yousef *et al.*, (2010) also used RAPD markers to identify new promising drought tolerant lines of rice under drought stress, they obtained the genetic diversity among 6 new rice lines and 4 cultivars with different responses to drought tolerance and established specific DNA markers associated with drought tolerance. Huseynova and Rustamova, (2010) reported on genetic diversity in 12 wheat genotypes. RAPDs associated with drought tolerance were used initially to search genetic diversity in wheat plants. They reported that a RAPD primer P6 (TCGGCGGTTC) produced respectively a 920-bp band present mainly in drought tolerant and semi-tolerant (absent in sensitive) genotypes. Thus, RAPD technology is a powerful tool in timely identifying markers related to drought tolerance in crop plants.

Results obtained from RAPD-PCR analysis are promising. It is the beginning of further research on marker-assisted selection. Selections based on genotypes identified from genetic diversity studies using RAPD will greatly increase breeding efficiency (Irada and Samira, 2010). It is hoped that the discovery of markers associated with drought tolerance with the aid of the genes involved, Sequence data can also be used to develop more robust PCR primers as diagnostics for drought tolerance. This study has shown that RAPD is quite efficient in diagnosing genetic diversity in pearl millet at DNA level. Hence, the wealth of information will help to select the markers which well distributed throughout the genome,

this can be exploited, not only to drive current advances in agriculture, but also to be used to get an insight into the genetic resources that can be employed in future hybridization programmes (Govindaraj *et al.,* 2009). RAPD is an effective tool for pearl millet germplasm management.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

In this study, the DNA from pearl millet *Pennisetum glaucum L. R. Br* was successfully isolated. The purity of the DNA ranged between 1.7- 1-9. RAPD analysis revealed ambiguous genetic polymorphisms among the ten varieties under study. Percentage polymorphism for all primers was 91.6%. Total fragments obtained were 48 of which 44 were polymorphic and 4 were monomorphic. Some DNA markers linked to drought tolerance were identified. Primers P16, P17, P18, P19 and P28 amplified a 600bp fragment that was present in varieties suspected to be drought tolerant. There is considerable genetic diversity among all the varieties. The highest genetic similarity (73%) exists between ZANGO and PEO 5984 varieties, while the lowest similarity (3%) exists between COMPOSITE and PEO 5684 varieties.

6.2 Conclusion

RAPD is quite efficient in evaluating the genetic diversity at the DNA level and can be used to distinguish between drought tolerant and sensitive pearl millet cultivars. The information obtained from this technique will help in selection of markers distributed throughout the genome. This technique also opens an excellent opportunity to develop drought tolerant pearl millet varieties and may be helpful in future pearl millet hybridization programmes aimed at improving drought tolerance.

6.3 Recommendation

This work is still open to further research and findings. Further work is therefore recommended as follows:

I. Pearl millet varieties that showed high level of diversity from this study should be used in pearl millet improvement programmes.

II. Markers obtained from this study should be further exploited for use in marker assisted selection for drought tolerance in pearl millet varieties in Nigeria.

III. RAPD analysis should be extended to additional pearl millet germplasm, especially those with unknown pedigree information. It would be possible to obtain information of their genetic relationship using this technique which could also help in designing breeding programmes.

IV. Molecular markers should be obtained from the pearl millet varieties in this study using other techniques like Simple Sequence Repeats (SSR) or microsatellites, inter-ISSR, SNPs etc. These methods can help generate greater specificity and tight associations in breeding for drought tolerance.

V. The 600bp fragment associated with drought tolerance should be isolated, sequenced, cloned and tested for drought tolerance in pearl millet or other species.

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APPENDIX I

RAPD-PCR optimisation

Stock solution	DNA Template (50ng)	Taq DNA polymerase (5U/µl)	X10 Reaction buffer	Primer (100µM)	dNTP mix (10mM each)	MgCl2 (20Mm)	ddH20	Total
Working solution		30U (6µl)	(75µl)	15µM (25µl)	6mM (15µl)	60mM		
Final concentration		1U/rxn	10%	0.5µM	0.2mM	2.0mM		
Final volume		2.0µl	2.5µl	0.83µl	0.5µl	2.5µl		25µl

Calculations for working solutions for 30 reactions

Taq DNA polymerase

5U in 1µl (in stock solution)
1U in 1µl (needed for the reaction)

1U = 1/5 = 0.2µl

Working solutions for 30 reactions
0.2µl × 30 = 6µl

X10 Reaction buffer

10% = 2.5µl
2.5 × 30 = 75µl

Primer

C1 = 15µM
C2 = 0.5µM
V1=
V2 =25µl

$$V1 = \frac{0.5uM \times 25µl}{15µM} = 0.83µl$$

0.83µ l × 30 = 24.9 (25µl)

dNTPs mix

C2 = 0.2
C1=10mM
V1=
V2 = 25µl

$$V1 = \frac{0.2 \times 25µl}{10mM} = 0.5µl$$
0.5µl × 30 = 15µl

MgCl2

C1 = 20mM
C2 = 2.0mM
V1=
V2= 25µl

$$V1= \frac{2.0mM \times 25µl}{20mM} = 2.5µl$$

2.5µl × 30 = 75µl
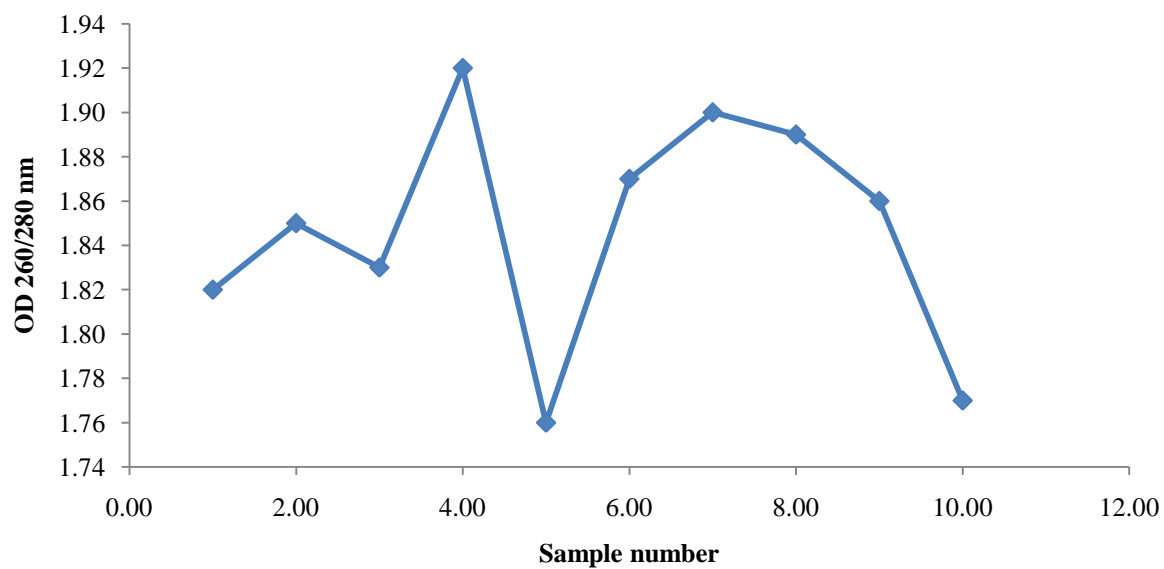
Optical density (OD) ratio of Genomic DNA samples extracted from the ten Pearl millet varieties