CHARACTERIZATION OF DROUGHT TOLERANCE TRAITS IN SELECTED RICE (*Oriza sativa* L.) GENOTYPES GROWN IN SUDAN USING SIMPLE SEQUENCE REPEAT (SSR) MARKERS

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DICLARATION

I declare that this thesis is my original work and has not been submitted to any educational institution or University for the award of a certificate. Therefore, the materials quoted in this thesis, which are not mine, have been duly acknowledged.

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This thesis has been submitted for examination with our approval as University Supervisors.

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DEDICATION

This thesis is humbly dedicated to my beloved family: my mother, memories of my father, my wife, brothers and sisters.

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LIST OF ABBREVIATIONS AND ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of variance
ATP	Adenosine Tri-Phosphate
ARC	Agricultural Research Corporation
ARC	Africa Rice Center
BBRC	Biotechnology and Biosafety Research Center
DARwin	Dissimilarity Analysis and Representation software
DNA	Deoxyribonucleic Acid
IRRI	International Rice Research Institute
ISSR	Inter Simple Sequence Repeat
JICA	Japan International Cooperation Agency
KU	Kenyatta University
NERICA	New Rice for Africa
NADP	Nicotinamide Adenine Dinucleotide Phosphate
PCR	Polymerase Chain Reaction
PIC	Polymorphism Information Content
QTLs	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SAS	Statistical analysis software
SNPs	Single Nucleotide Polymorphisms
SSR	Simple Sequence Repeat
UNEP	United Nations Environment Programme
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USDA	United States Department of Agriculture
UV	Ultra Violet
WARDA	West Africa Rice Development Association

ABSTRACT

Rice (*Oryza sativa* L.) is one of the most important staple food crops providing the world's nutritional energy and 20% nutritional protein. It is cultivated under diverse ecological conditions ranging. Rice growth and productivity are unfavourably affected by various biotic and abiotic stresses; key among them being drought. Drought is the most important limiting factor for crop production in many regions of the world and is considered one of the major limiting factors of rice production and food insecurity in Sudan. Conventional breeding for drought tolerance is further slowed down by the complex nature of mechanisms underlying this stress although molecular markers offer a promising alternative approach. Exposure of plants to drought stress has been shown to lead to a significant effect in chlorophyll content and eventual reduction in photosynthesis. In this study, 23 rice genotypes grown in Sudan were examined for drought tolerance under greenhouse conditions. The focus was on tracking changes in chlorophyll content under drought stress and further screening of the plants for DNA polymorphisms using simple sequence repeat (SSR) markers for possible associations. The results showed that genotypes IR11A306, IRRI 154, Nerica 6, IR12N240, Nerica 4, Wakra and IRRI 150 exhibited high drought tolerance based on this assay. After 7 days of dehydration, IR11A306 recorded the highest increment of total chlorophyll followed by IRRI 154, Nerica 6 and IR12N240, while IR11A483 showed the highest reduction followed by Nerica 15, IR11N121 and IRRI 168. When plants were rehydrated and total chlorophyll measured, the highest increase and best recovery were observed in IR74371-70-1-1 followed by IRRI 168, IRRI 147, Nipponbare and Kosti 2. Genotype IR11A306 showed the least reduction in chlorophyll levels followed by Nerica 15, IRRI 150 and IRRI 122. Eighteen primers showed amplification of the SSR markers generating 569 alleles that ranged between 13 to 113 alleles per marker. These alleles further produced polymorphism information content (PIC) values of 0.51 to 0.99 per marker. The chlorophyll assay helped select genotypes that showed a steady recovery of chlorophyll content following drought stress while the markers studied could be useful for future molecular breeding for drought tolerance in rice.

CHAPTER ONE

1. INTRODUCTION

1.1. Background to the study

Rice (*Oryza sativa* L.) is one of the most important staple food crops providing 27% of the world's nutritional energy and 20% of overall nutritional protein (Bashir *et al*., 2007). It is cultivated under diverse ecological conditions ranging from irrigated to rainfed uplands, rainfed lowland, and deep water. Irrigated rice is cultivated on 55% of the world's rice production area and accounts for about 75% of total rice production. Furthermore, over 3 billion people in the world depend on rice for food (Awasthi and Lal, 2014). The growth and productivity of rice is adversely affected by various biotic and abiotic factors key among them being drought (Ndjiondjop *et al.*, 2010; Singh *et al*., 2012).

Drought is defined as a period without significant rainfall. Generally, drought stress occurs when available water in the soil is reduced and atmospheric conditions cause continuous loss of water by transpiration and/or evaporation (Jaleel *et al.*, 2009). Drought is the most important limiting factor for crop production in many regions of the world. It is a worldwide problem and has been shown to seriously influence grain production and quality (Ndjiondjop *et al.*, 2010). Particularly, drought conditions lead to a reduction in plant growth by affecting various physiological and biochemical processes (Farooq *et al.*, 2008). Furthermore, most cultivated rice varieties are susceptible to drought, therefore necessitating the need for continued improvement to overcome this problem (Uphoff *et al.*, 2015).

Sudan is one of the least-developed countries in Africa and one of the most vulnerable states to climate change and variability. This is manifested by most of its land which is arid and desert (Abbadi and Ahmed, 2006). A trend of decreasing annual rainfall (0.5%) over the past 60 years and increased rainfall variability were reported to be contributing to drought conditions in many parts of the country and problems such as these will certainly increase if the trend continues (Ahmed *et al*., 2012). Climate change affects agriculture and food production

systems in complex ways through effect on crop production. Drought is considered one of the major causes of food insecurity in Sudan (Mahgoub, 2014).

A few years ago was reported that agriculture in Sudan was the principal source of income and livelihood for between 60 percent and 80 percent of the population and the engine of growth for other economic sectors such as trade, industry and transport (Ndjiondjop *et al.*, 2010; Elgali *et al.*, 2010). However, occurrence of drought is considered one of the major causes of food insecurity in the country (Mahgoub, 2014). Being in an arid and desert ecological zone, Sudan was reported to be one of the most vulnerable countries to climate change owing to a high climatic variability and low development (Elasha *et al*., 2005).

Rice production has been practiced manly in the southern states of Sudan and if the estimated 300,000 hectares of rice growing area is properly utilized, it would suffice the local consumption demand to fill the gap for non-course food grain (Ahmed *et al.*, 2012). A few years ago, Mahgoub (2014) reported that no breakthrough with regards to increasing the cultivated area and improving new varieties has been achieved and this was mainly attributed to lack of understanding of the mechanisms underlying drought-related improvement in rice. Development of molecular markers and their use for genetic dissection of agronomically important traits have been identified as a powerful tool for studying complex plant traits such as drought tolerance (Sujib *et al*., 2011). Particularly, DNA-based molecular markers have been reliably used with availability of a large number of polymorphic markers enabling precise classification of the cultivars (Sohrabi *et al.*, 2013). Improvement of rice for drought tolerance using conventional breeding methods is slow due to geographical differences and the variations of seasons in drought timing and severity, the complex nature of drought tolerance traits and the difficulty in selection of combinations of traits (Courtois *et al*., 2003). Other factors that have slowed down this process include the low heritability, multiple gene control as well as genotype and environmental interactions (Cattivelli *et al*., 2008). The use of molecular markers to select accessions possessing genes and genomic regions that control target traits can fast-track the progress in breeding for drought tolerant

rice. This is because molecular markers are consistently transmitted from generation to generation and are not subject to environmental influences (Afiukwa *et al*., 2016). Studies have, however, utilized molecular markers to identify genotypes with traits directly related to drought tolerance (Afiukwa *et al*., 2016). These markers including Restriction Fragment Length Polymorphism (RFLP), Random Amplification of Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeats (ISSRs), Amplified Fragment Length Polymorphism (AFLP) and Single Nucleotide Polymorphisms (SNPs) have been available to assess the variability, diversity at molecular level and have been used to enhance traditional breeding programs to improve rice crop (Sonia, 2013). Of all these, however, SSRs are considered the markers of choice in many areas of genetic diversity studies in rice due to their efficiency, abundance in rice genome, high level of polymorphism and high but simple reproducible assays that are reliable (Singh *et al*., 2010). It has been demonstrated that exposure of plants to drought leads to a significant effect in chlorophyll contents as a result of the reduction in leaf growth (Chutia and Borah, 2012). Therefore, the main aim of this study was to understand the patterns of SSR markers linked to drought tolerance and their association with total chlorophyll content in rice under induced drought conditions. The results are discussed in the context of which rice genotypes best recover their ability to photosynthesise following drought conditions.

1.2. Problem statement

Rice is the third most consumed crop for the Sudanese people, yet its production is very low due to various challenges (Ahmed *et al*., 2012). They include shortage of rainfall and use of unimproved seed which lack adaptability to drought conditions (Courtois *et al*., 2003). Drought is a prolonged period of abnormally low rainfall, leading to a shortage of water (Jaleel *et al*., 2009). Drought is particularly frequent in the uplands and shallow rain-fed lowland fields in many parts sub-Saharan Africa encompassing areas where rice is grown. Drought scenarios are expected to worsen in future with predicted climate change also expected to lead to more complex interactions of drought with other abiotic and biotic stresses (Uphoff *et al.*, 2015). Sudan along with other countries in the

Sahel belt has suffered several long and devastating droughts in the past few decades (Mahgoub, 2014). The most severe drought conditions occurred in 1980-1984 and was accompanied by widespread displacement of people and localized famine (Ramadan *et al.*, 2015). These drought episodes in addition to erosion of natural resources as a result of climate change were among the root causes of social strife, conflict and considered one of the major causes of food insecurity in Sudan. There has been no breakthrough with regards to improving rice mainly due to lack of understanding of the mechanisms underlying drought-related improvement (Mahgoub, 2014). This study therefore was to determine the SSR markers linked to drought tolerance traits and their association with phonotypic traits in rice genotypes cultivated in Sudan. The study also determined the total chlorophyll content following drought stress by withholding water from the plants and later rewatering them.

1.3. Study justification

Rice is an important staple food in the world but the demand far outweighs the supply. Drought is a major limitation to rice production Sub-Saharan Africa thus the need for research on drought tolerance (Sujib *et al*., 2011). Rice production fields around the world are threatened by drought and this has necessitated development of lines that can tolerate drought. Progress in developing drought-tolerant rice genotypes has been slow, despite considerable efforts mainly because of limited understanding of the mechanisms affecting tolerance traits in rice (Uphoff *et al.*, 2015). Consequently, various national programs systematically incorporated drought tolerance as a breeding objective involving biotechnological approaches (Fukai *et al.*, 2009). Thefore, determining levels of total chlorophyll and related changes following induction of drought is vital for understanding which rice genotypes have the best recovery after stress hence have potential for improvement programs. On the other hand, molecular markers have potential to identify genotypes with traits directly related to drought tolerance. Of all existing ones, SSR markers are more popular in rice because they are highly informative, mostly monolocus, codominant, easily analyzed and cost-effective (Emanuelli *et al*., 2013). They have been used to detect high level of allelic diversity as well as

genetic variations among rice subspecies. Furthermore, these markers were found to be efficient in detecting genetic polymorphisms and discriminating among genotypes from germplasms of various sources including finer levels of variation among closely related breeding lines within the same variety (Garcia *et al*., 2007). Therefore, their use in the current study could be instrumental in allowing evaluation of genetic diversity of the rice genotypes cultivated in Sudan under drought conditions. In addition, associating SSR Markers and total chlorophyll content change under drought stress reveals possible relationship between the two parameters and further provide insight into the molecular mechanisms that control drought with regards to chlorophyll.

1.4. Hypotheses

i. Induction of drought stress has no effect on total chlorophyll content in selected rice genotypes grown in Sudan.

ii. The SSR markers related to drought show distinct patterns in the selected rice genotypes.

iii. The SSR Markers show an association with total chlorophyll content change under drought stress.

1.5. Objectives

1.5.1. General objective

To characterize selected rice genotypes grown in Sudan through Simple Sequence Repeats (SSRs) associated with drought tolerance traits and levels of chlorophyll contents under drought stress.

1.5.2. Specific objectives

i. To determine the effect of drought stress on total chlorophyll content in selected rice genotypes grown in Sudan.

ii. To determine the genetic patterns of selected rice genotypes grown in Sudan using SSRs markers.

iii. To determine the association between SSR Markers and total chlorophyll content change under drought stress.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Origin and distribution of rice

Debates on the origins of the domesticated rice are numerous. In 2011, genetic evidence showed that all forms of Asian rice, both *Indica* and *Japonica* sprang from a single domestication of the wild rice *Oryza rufipogon* that occurred 8,200–13,500 years ago in China (Molina *et al*., 2011). *Japonica* is one of the two major eco-geographical races of *O. sativa* (*O. sativa japonica*), the other being *Indica* (*O. sativa Indica*). These are a group of rice varieties from northern and eastern China grown extensively in some areas of the world (Garris *et al*., 2005). A study in 2012 showed, through a map of rice genome variation, that domestication of rice occurred in the Pearl River valley region of China from East Asia, then spread to South and Southeast Asia (Chutia and Borah, 2012). Before this research, the commonly accepted view, based on archaeological evidence, was that rice was first domesticated in the region of the Yangtze River valley in China (Vaughan *et al*., 2008).

The precise date of the first domestication is unknown although based on the molecular clock estimate; the date is estimated to be 8,200 to 13,500 years ago. This is consistent with known archaeological data on the subject (Khush, 1997). An older theory, based on one chloroplast and two nuclear gene regions, (Londo *et al*. 2006) had proposed that *O. sativa Indica* was domesticated in eastern India, Myanmar, and Thailand; and *Japonica* in southern China and Vietnam. Since the functional allele for nonshattering, the critical indicator of domestication in grains (as well as five other single-nucleotide polymorphisms) is identical in both *Indica* and *Japonica,* Ndjiondjop *et al.* (2010) determined that a single domestication event for *O. sativa* happened in the region of the Yangtze River valley.

2.2. Classification of rice

Rice is a monocotyledonous plant that belongs to the *Plantae* Kingdom, a member of *Poaceae* family and genus *Oryza. Oryza sativa*, commonly known as Asian rice, is the plant species most commonly referred to in English as rice (Bashir *et al.*, 2007). *Oryza sativa* is a grass with a genome consisting of 430Mb across 12 chromosomes. It was renowned for being easy to genetically modify and it was identified as a model organism for cereal biology (Ono *et al.*, 1988). *Oryza sativa* contains two major subspecies: the sticky, short-grained *Japonica* or *Sinica* variety, and the nonsticky and the long-grained *Indica* variety. *Japonica* varieties were usually cultivated in dry fields, in temperate East Asia, upland areas of Southeast Asia, and high elevations in South Asia, while *Indica* varieties were mainly lowland rice, grown mostly submerged, throughout tropical Asia (Khush, 1997). A third subspecies, which is broad-grained and thrives under tropical conditions, was identified based on morphology and initially called *Javanica*. This is, however, has been reported as Tropical Japonica (Kim *et al.*, 2012). Glaszmann (1987) used isozymes to sort *O. sativa* into six groups: *Japonica, Aromatic, Indica, Aus, Rayada,* and *Ashine* while Cubry *et al*. (2014) used simple sequence repeats to sort *O. sativa* into five groups: Temperate *Japonica,* Tropical *Japonica* and Aromatic comprising the *Japonica* varieties, while *Indica* and *Aus* comprising the *Indica* varieties.

2.3. Importance of rice

Rice has shaped the culture, diets and economies of thousands of millions of people (Garris *et al.,* 2005). Rice is an important staple food crop for more than 60 percent of the world's population (Londo *et al*., 2006). In 2014, more than 430 million metric tons of rice was consumed worldwide, according to the United States Department of Agriculture (Cubry *et al*. 2014). Ready to eat products such as popped and puffed rice, instant or rice flakes, canned rice and fermented products were produced. Rice straw has been used as cattle feed, for thatching roof and in cottage industry for preparation of hats, mats, ropes, straw board and as litter material (Lin et al., 2007). Furthermore, rice husks has been used as animal feed, for making paper, growth medium in horticulture and as a source of

fuel (Ndjiondjop *et al.* 2010). Rice bran, on the other hand, is used in cattle and poultry feed, defatted bran, which is rich in protein and can be used in the preparation of biscuits and as cattle feed (Chutia and Borah, 2012). Rice bran oil is also used in soap industries. Refined oil can be used as a cooling medium like cotton seed oil/corn oil (Khush, 1997).

2.4. Nutritional value of rice

Rice is a nutritive staple food which provides instant energy as its most important component is carbohydrate as starch (Vaughan *et al.*, 2008). However, it is poor in nitrogenous substances with an average composition of these substances estimated at only 8 percent while its fat content or lipids are negligible (Londo *et al.*, 2006). Rice flour is rich in starch and is used for making various food materials. It is also used in some instances by brewers to make alcoholic malt. The variability in composition and characteristics of rice is really broad and depends on variety and environmental conditions under which the crop is grown (Lin et al., 2007). In husked rice, protein content ranges in between 7 percent to 12 percent (Chutia and Borah, 2012). The use of nitrogen fertilizers during production increases the percentage content of some amino acids (Khush, 1997). The comparative nutritional value of cereals in Table 1 shows the differences in nutritional content of rice bran and raw rice. Brown rice is rich in some vitamins, especially B1 or thiamine (0.34 mg), B2 or riboflavin (0.05 mg), niacin or nicotinic acid (4.7 mg). In contrast, the white rice is poor in vitamins (0.09 mg of vitamin B1, vitamin B2 0.03 mg and 1.4 mg of niacin) and minerals. These are found mostly in the outer layers of the grain, which are removed by polishing process or "bleaching" whereas parboiled rice is rich in these vitamins as a result of their particular process (Molina *et al*., 2011).

Table 2.1: Nutritional value of rice and wheat per 100 grams

Cereals	Protein (gm)	Fat (gm)	CHO (gm)	Minerals (gm)	Calcium (gm)	Fiber (gm)	Energy (K cal)
Wheat whole	11.8	1.6	71.2	1.5	41	1.2	346
Wheat flour	12.1	1.7	69.4	2.7	48	1.9	341
Rice bran	13.5	16.2	48.4	6.6	67	4.3	393
Rice (raw)	6.8	0.5	78.2	0.6	10	0.2	345
Rice (par boiled)	8.5	0.6	77.4	0.9	10	0.2	349

Source: Vaughan *et al*. (2008)

2.5. Climatic requirements for rice

Rice needs a hot and humid climate. It is best suited to regions with high humidity, prolonged sunshine and an assured supply of water (Lin et al., 2007). The average temperature required throughout the life period of the crop ranges from 21 to 37 ̊C. Maximum temperature under which the crop can produce is 42 ̊C (Kim *et al.*, 2012). At different stages, the minimum temperature for sprouting is 10 ̊C but at the time of tillering, the crop requires a higher temperature than that needed for growth. The minimum temperature for flowering is 23 ̊C. Temperature requirement for blooming is in the range of 26.5 to 29.5 ̊C. The minimum temperature for grain formation is 21 ̊C and at the time of ripening the temperature should be between 20 to 25 ̊C. Photoperiodically, rice is a short day plant (Vaughan *et al.*, 2008). However, there are varieties which are non-sensitive to photoperiodic conditions (Lin *et al.*, 2007).

2.6. Rice production in Sudan

Rice is the third most consumed crop for the Sudanese people (Ahmed and Abdel, 2012). Rice production has been practiced manly in the southern states of Sudan and it has been grown since 1905 on an area of almost 5.5 thousand hectares and the total production is almost 8 thousand tones far below the requirement of the country's food requirements (Morgan *et al*., 2002). The same authors showed that, in Sudan the area under the crop increased from 2,100 ha in 1984 to 7,562 ha in 2013 with a corresponding increase in production from 2,000 ton to 25,000 ton. The estimated 300,000 hectares of rice growing area has not yet properly utilized (Ahmed and Abdel, 2012). Over the last few decades, consumption in Sudan has consistently increased and the demand for rice is steadily rising, as the country

annually imports 15 million dollars' worth of rice (CFI, 2013). This has led to the introduction of new varieties, such as NERICA upland rice (ARC, 2010). The Africa Rice Center (ARC) has developed 18 varieties of NERICA suited for upland cultivation and 60 varieties suited for lowland cultivation. Furthermore, the ARC (2014) argues that these qualities make NERICA varieties more competitive than their parent crops and other traditional rice crops. These qualities make NERICA varieties better suited for the upland environment of sub- Saharan Africa and therefore, there are numerous economic opportunities associated with producing NERICA rice in Sudan (Amin, 2015). In 2010, NERICA4 was introduced into the Gezira State through a joint partnership with Japan International Cooperation Agency (JICA) and the Sudanese government. Since then, encouraging productivity and high profit returns have led to the expansion of the project into five other states (Gedaref, Sennar, White Nile, River Nile and Northern State) and movement into large-scale production (JICA, 2014).

2.7. Effect of drought on plant morphological and physiological traits

Drought stress is a very important factor for plant growth as it affects both elongation and expansion growth (Yue *et al.*, 2005). Among the various crops cultivated across the world, rice is probably more susceptible to drought compared to the others (Molina *et al*., 2011). Water stress has been shown to reduce leaf area, cell size and intercellular volume (Kramer, 1969; Cubry *et al*. 2014). A variety that is more resistant to water flow from the stomata into the atmosphere is considered good for drought tolerance (Kim *et al*., 2012). The reduction in soil moisture may lead to lower water content in the leaves causing guard cells to lose turgor pressure and hence the size of stomatal pores reduce and/or causing stomatal closure. In addition, increased stomatal resistance may lead to reduced water transport in the leaves further causing a decrease in stomatal conductance (Singh, *et al*., 2012).

Reduction in stomatal conductance subsequently decreases transpiration by closing of the stomata and this results in prolonged plant survival by extending the period of availability of essential soil water reserves in the root zone (Gummuluru *et al*., 1989). Stomatal closure also helps to maintain high leaf water content

which leads to a reduction in photosynthetic activity (Hsiao, 1973). Higher photosynthetic rates could, in turn, favor a higher biomass and crop yields. The presence of cuticular wax is also important for water stress and is more in dryland-adopted as compared to irrigated rice (Srividhya *et al*., 2011). This phenomenon results in leaves that are thick and leathery which prevents water loss from the surface of the rice plant (Singh *et al.*, 2012). In general, drought stress leads to exhibit some morphological response in plants such as early maturity, early vigor and rapid growth. Physiological characters such as diffusive resistance of stomata, osmotic adjustment, leaf rolling, closing and opening of stomata, position of stomata, leaf water retention and leaf senescence. These observations were associated with drought tolerance (Londo *et al*., 2006). Increased seed size, early maturity and reduced plant height at the drought-prone location is of prime importance in increasing seed yield (Singh *et al*., 2012).

2.8. Effect of drought stress on rice crop

Drought is recognized as a major abiotic stress that limits rice productivity and adversely affects grain quality in rain-fed and upland ecosystems. Rice is most sensitive to drought stress during reproductive development at which time moderate water shortages can result in a significant reduction in grain yield (Afiukwa *et al*., 2016). The extent to which drought affects yield varies depending on the intensity and the time of occurrence of the stress within the crop growth cycle (Srividhya *et al*., 2011). The situation has become more serious with increasing global climate change (Yue *et al*., 2005). Plant responses to drought are well known and believed to be complex involving numerous physiological changes. For instance, leaf rolling and death of leaves are good examples of useful criteria in assessing levels of drought tolerance in a large scale screening (Zadoks *et al.*, 1974). The leaves of any crop plant frequently roll when the plant is suffering from water stress condition. Exposure to drought stress leads to a significant effect in Chlorophyll-a and Chlorophyll-b contents (Ranjbarfordoei *et al.*, 2000). When the leaf temperature was increased, the stomata become closed and transpiration rate decreased sharply with leaf rolling (Singh *et al*., 2012). Leaf rolling can be scored visually in rice either in the morning or mid-day. Delayed

rolling is used as an important selection criterion for drought tolerance in rice, which could be improved by incorporating the gene(s) into those lines/genotypes that perform better under irrigated but not well under water stress condition (Singh *et al*., 2012). A plant having the characteristics of delayed leaf rolling under water stress and faster recovery rates after removing the water stress in rice (Singh and Afria, 1988) was considered a good trait because flag leaf in rice crops plays an important role in grain filling and development (Gummuluru *et al*., 1989).

In a study conducted in drought stress, drought caused changes in root structure such as increased branching and density (Eghball and Maranville, 1993). Cultivars having deep and thick roots are reported to be good at tolerating drought stress and are further positively correlated with xylem vessel area, which is vital for the conductance of water from the soil to the upper parts of the plants to meet the evaporative demand (Singh *et al*., 2012). It was reported that large and vigorous root systems and the continued production of new root hairs were required for maximum response to nutrient supply and optimum environmental conditions and this was positively correlated with the dry matter accumulation within the shoot (Lowy and Willumsen, 1993).

It has been observed that drought affected plants generally exhibited small root system configuration and in many cases caused a reduction in the size of the root system which was directly proportional to the magnitude of water storage (Londo *et al*., 2006). According to Slayter and Codington, (1973), two types of effects of water deficit on root development were exposed. First, there was a reduction in the rate of meristematic activity and root elongation and this was directly associated with the level of internal water deficit. Secondly, the effect of suberization on the water and nutrients uptake is proportional of the root system as a whole. Under drought stress, production of root system is very important and was reported to have a good correlation with plant yield (Londo *et al.,* 2006).

2.9. Genetic diversity and its implication for crop improvement

Genetic diversity refers to the amount of genetic variability among individuals of a genotype or population of a species (Brown, 1983). During the assessments of genetic diversity, the number of alleles and their distribution as well as the effect on performance, and the overall distinctness between different populations can be determined (Rao and Hodgkin, 2002). Knowledge of genetic variation and relationships between genotypes/clones is critical to identify cultivars, understand the genetic variability for further improvement and provide evidence of evolutionary forces shaping the cultivar diversities as well as give appropriate conservation strategies (Thormann *et al*, 1994). Thus, knowledge of genetic diversity in plant genetic resources is essential for efficient utilization and conservation of germplasm.

Progress in developing drought-tolerant rice varieties has been slow despite considerable past efforts (Lafitte *et al,* 2004). Consequently, various national programs have systematically incorporated drought tolerance as a breeding objective (Fukai *et al,* 2009). However, research groups have revived interest in drought tolerance breeding and the use of new genomics tools to enhance crop productivity (Asins *et al*., 2010). Any development of drought-adapted rice varieties will affect rice production in sub-Saharan Africa, where rice consumption is growing faster than anywhere else in the world, and where government policies are moving the continent rapidly away from imports and toward self-sufficiency (Emanuelli *et al.,* 2013). Rice cultivars combining improved drought tolerance with responsiveness to favorable conditions were said to be among the most promising and deliverable technologies for alleviating poverty (Gupta and Varshney, 2000).

2.10. Genetic markers for diversity studies in crops

Molecular markers are identifiable DNA sequences, found at specific locations of the genome and transmitted by the standard laws of inheritance from one generation to the next (Varshney *et al.,* 2007). Molecular genetic techniques, both on their own and in combination with other biotechnological approaches, have a significant impact on plant genetic resource conservation and use (Cerqueira *et*

al., 2014). Initially, the molecular techniques were used largely for the analysis of specific genes for understanding gene action, gene mapping and the development of gene transfer technologies. The techniques have been applied to problems of direct relevance such as understanding the distribution and extent of genetic variation within and between species (Vikram *et al.*, 2011). Developments in molecular biology have opened the possibility of employing various types of molecular tools to identify and use genomic variation for the improvement of several organisms (Emanuelli *et al.*, 2013).

2.10.1. Restriction Fragment Length Polymorphism (RFLP)

The RFLP is the most widely used hybridization-based molecular marker. The RFLP markers were used for the first time in 1975 to identify DNA sequence polymorphisms for genetic mapping of a temperature-sensitive mutation of adeno-virus serotypes (Grodzicker *et al*., 1975). It was then used for human genome mapping (Botstein *et al*., 1980), and later adopted for plant genomes (Helentjaris *et al*., 1986). The technique is based on restriction enzymes that reveal a pattern difference between DNA fragment sizes in individual organisms. The major strength of RFLP markers is its high reproducibility, co-dominant inheritance, good transferability between laboratories, no sequence information required and relatively easy to score due to the large size difference between fragments (Meksem *et al*., 1995). However, there are several limitations for RFLP analysis since it requires the presence of high quantity and quality of DNA: it depends on the development of specific probe libraries for the species; the technique is not amenable for automation; the level of polymorphism is low and few loci are detected per assay; it is time consuming, laborious, and expensive (Ahmed *et al*., 2012). Therefore, for over ten years RFLP markers have been replaced by other suitable markers (Semagn *et al*., 2006).

2.10.2. Randomly Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) has been collectively termed multiple arbitrary amplicon profiling (Caetano-Anolles, 1994).This technique was the first to amplify DNA fragments from any species without prior sequence information (Semagn *et al*., 2006). The key innovation of RAPD is the use of a

single arbitrary oligonucleotide primer to amplify template DNA without prior knowledge of the target sequence (Heun and Helentjaris, 1993). The amplification of nucleic acids with arbitrary primers is mainly driven by the interaction between primer, template annealing sites and enzymes, and determined by complex kinetic and thermodynamic processes (Caetano-Anollés, 1997).

Several factors have been reported to influence the reproducibility of RAPD reactions: quality and quantity of template DNA, PCR buffer, concentration of magnesium chloride, primer to template ratio, annealing temperature, Taq DNA polymerase brand or source, and thermal cycler brand (Tsumura *et al.*, 1996). The concern about reproducibility of RAPD markers, however, could be overcome through choice of an appropriate DNA extraction protocol to remove any contaminants (Mishra *et al*., 2003), by optimizing the parameters used (Varshney *et al.,* 2007), by testing several oligonucleotide primers and scoring only the reproducible DNA fragments and by using appropriate DNA polymerase brand (Meksem *et al*., 1995).

Most RAPD fragments result from the amplification of one locus, and two kinds of polymorphism occur: the band may be present or absent, and the brightness (intensity) of the band may be different. Band intensity differences may result from copy number or relative sequence abundance (Devos and Gale, 1992) and may serve to distinguish homozygote dominant individuals from heterozygotes, as more bright bands are expected for the former. However, some authors (Thormann *et al*., 1994) found no correlation between copy number and band intensity. The fact that fainter bands are generally less robust in RAPD experiments (Heun and Helentjaris, 1993) suggest that varying degrees of primer mismatch may account for many band intensity differences.

2.10.3. Amplified Fragment Length Polymorphism (AFLP)

The AFLP technique combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA (Lynch and Walsh, 1998). The key feature of AFLP is its capacity for "genome representation": the simultaneous screening of representative DNA regions distributed randomly throughout the genome (Meksem *et al*., 1995). The AFLP markers can be generated for DNA of any organism without initial investment in primer/probe development and sequence analysis. Both good quality and partially degraded DNA can be used for digestion but the DNA should be free of restriction enzyme and PCR inhibitors (Blears *et al.*, 1998).

2.10.4. Inter Simple Sequence Repeat (ISSR)

The ISSR involves amplification of DNA segments present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite directions (Semagn *et al*., 2006). The ISSRs are semi-arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Each band corresponds to a DNA sequence bordered by two inverted microsatellites (Tsumura *et al.*, 1996). The ISSRs use longer primers (15–30 mers) as compared to RAPD primers (10 mers), which permit the subsequent use of high annealing temperature leading to higher stringency (Vikram *et al.*, 2011). The amplified products are usually 200–2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis. It does not require genome sequence information which leads to multilocus, highly polymorphous patterns and produces dominant markers (Mishra *et al*., 2003).

2.10.5. Single Nucleotide Polymorphism (SNP)

Public accessibility to the genome sequences of several organisms has enabled the study of sequence variations between individuals, cultivars, and subspecies. These studies revealed that single nucleotide polymorphisms (SNPs) are highly abundant and distributed throughout the genome in various species including plants (Semagn *et al*., 2006). The abundance of polymorphisms in plant genomes makes the SNP marker system an attractive tool for mapping, marker-assisted breeding and map-based cloning (Batley *et al*., 2003). The acronym, a SNP

marker is just a single base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position. Hence, allele discrimination can not be based on size differences on a gel (Gupta and Varshney., 2000). All methods for SNP genotyping combine two elements: first, the generation of an allele-specific product, and second the analysis thereof (Caetano-Anolles, 1994).

2.10.6. Simple Sequence Repeats (SSRs)

Microsatellites, Simple Sequence Repeats (SSRs), or Short Tandem Repeats (STRs), are repeating sequences of 1-6 base pairs of DNA. SSR markers are highly informative due to co-dominance, multiallelism, heritability, abundance and wide coverage of the genome. Another advantage is the conservation of flanking regions across generations, which allows repeated use of the technique (Emanuelli *et al*., 2013). Depending on length of the repeated sequence, the markers have been termed "satellites", "minisatellites", and/or "microsatellites". The strategy for using SSRs as genetic markers is that the repeat region may vary in length between genotypes but the DNA flanking the repeat is sufficiently conserved that the same primers will work in multiple genotypes (Afiukwa *et al.*, 2016). Thus, SSR polymorphism between two varieties is due to the differences in the length of the repeat between the two conserved sequences.

The SSRs are excellent markers because of their locus identity, multi-allelism, and PCR basis. Although SSR markers are developed for use in a single species, it is possible to extend known markers for use in related species (Emanuelli *et al.*, 2013). This is possible because the flanking regions are conserved and the number of duplications is variable. Therefore, once an SSR marker is available in a related species, attempting to transfer known markers can be advantageous for the individual who does not have original developed SSR markers. The availability of new microsatellite markers is important in effectively contributing to the genetic analysis of *Oriza Sativa* (L).

Genetic improvement of rice for drought tolerance through conventional breeding is slow due to the spatial and seasonal variations in drought timing and severity, the complex nature of drought tolerance itself and the difficulty in selecting for

combinations of traits which best suit combating drought-induced yield reductions (Emanuelli *et al.*, 2013). Among the factors accounting for the slow progress in developing drought-tolerant rice is the low heritability, multiple gene control, epistatic gene interaction, high incidence of genotype x environment interactions. which could seriously influence 'actual' yields (Gupta and Varshney., 2000). The use of molecular markers to select accessions possessing genes and genomic regions that control target traits can fast-track the progress in breeding for drought tolerant rice because molecular markers are transmitted faithfully from generation to generation and are not subject to environmental influences (Garcia *et al.*, 2007). For plant breeding applications, SSR markers have been proven and recommended as markers of choice (Gupta and Euphytica., 2000). The SSR markers have been extensively used to identify genetic variation among rice subspecies (Ni *et al*., 2002), evaluate genetic diversity among rice genotypes for drought tolerance and other abiotic stresses (Ramadan *et al*., 2015; Vanniarajan *et al*., 2012).Therefore, SSRs marker is the system of choice for genetic analysis in rice because of its abundance in the rice genome, high level of polymorphism and high but simple reproducible assays involved (Afiukwa *et al.*, 2016).

2.11. Quantitative Trait Loci (QTL)

Polygenic inheritance refers to inheritance of a phenotypic characteristic (trait) that is attributable to two or more genes and can be measured quantitatively. Multifactorial inheritance refers to polygenic inheritance that also includes interactions with the environment (Asins *et al.*, 2010). Unlike monogenic traits, polygenic ones do not follow patterns of Mendelian inheritance (discrete categories). Instead, their phenotypes typically vary along a continuous gradient depicted by a bell curve (Miles and Wayne, 2008). A Quantitative Trait Loci (QTL) is a section of DNA (the locus) that correlates with variation in a phenotype (the quantitative trait). The QTL typically is linked to, or contains, the genes that control that phenotype (Emanuelli *et al*., 2013). The QTL analysis has become the method of choice for genetic dissection of any trait in plant and animal species. The list of cloned QTLs has been expanded in the past few years. A cloning approach starts by searching for functional candidates, i.e. genes that

have been shown, or are suspected, to have a functional role in the phenotype of interest (Miles and Wayne, 2008). Then, the most promising candidates are selected from a large number of functional candidate genes, by testing their linkage to QTLs for the trait of interest, thereby identifying positional candidates, genes co-locating with QTLs (Asins *et al*., 2010). The QTLs are mapped by identifying which molecular markers correlate with an observed trait. This is often an early step in identifying and sequencing the actual genes that cause the trait variation. Quantitative traits are phenotypes (characteristics) that vary in degree and can be attributed to polygenic effects such as the product of two or more genes and their environment (Miles and Wayne, 2008). Thus, because of the low value of heritability of grain yield (GY) under stress and the lack of effective trait selection index related to drought tolerance, it is very important to find molecular marker associated with water stress tolerance in rice (Yue *et al*., 2006). Many SSR markers have been reported to be linked to drought tolerance traits or QTLs in rice such as yield under drought, maximum root length, relative spikelet fertility, basal root thickness and root dry weight (Kanbar and Shashidhar 2011).

CHAPTER THREE

3. MATERIAL AND METHODS

3.1. Plant material

Seeds of twenty three (23) *Oryza sativa* L. genotypes grown in Sudan with varying degree of tolerance to drought were obtained from Biotechnology and Biosafety Research Center (BBRC), Agricultural Research Corporation (ARC), Sudan and used. These included twelve upland drought tolerant genotypes (NERICA 4, NERICA 6, NERICA 14, NERICA 15, NERICA 16, NERICA 1, NERICA 7, Umgar¸ Kosti 1, Kosti 2 and Wakra), ten (IRRI 150, IR 11A306, IR 11A483, IRRI 147, IRRI 122, IR 12N240, IR 74371-70-1-1, IR11N121, IRRI 154 and IRRI 168) still under research in Sudan and two (IAC 165 and Nipponbare) from Kenyatta University (KU), Kenya. Genotype description is presented in Table 2.

3.2. Determination of chlorophyll content from rice

Analysis of total chlorophyll content was conducted on rice leaves grown in the greenhouse at the Plant Transformation Laboratory, Kenyatta University, Kenya. Five seeds were directly sown in plastic pots (size 10x10x5 cm) containing an equal volume of 500 gm garden soil placed in buckets containing water for under-watering. The pots arranged in a completely randomized block design with three replicates and the control comprised all the studied genotypes. The greenhouse conditions were 12 hours light/12 hour darkness photoperiod; 28ºC day and 24ºC night temperature and 60% of humidity. The pots were randomized and irrigated after every five days. At the 3-4 leaf stage (corresponding to around 3 weeks after sowing), the plants were irrigated (underwaterd) continually until 60 days followed by the first chlorophyll extraction according to Botstein *at al*. (1980). Five leaf discs were punched from rice plants using a paper punch and ground to a fine powder under liquid nitrogen. Total chlorophyll was then extracted using absolute acetone. Leaves were collected into sterile plastic tubes and ground into fine powder in the dark. Absolute acetone (2 ml) was added and the mixture was then centrifuged at 13,000 rpm for 5 min. The supernatant was then collected into

new Eppendorf tubes. The chlorophyll was quantified by measuring the absorbance using a spectrophotometer (Spectrometre UV- Visible- UV- 3100 PC-VWR) under 660nm wavelength. Three spectrophotometer readings were taken and an average calculated for each genotype. The plants were exposed to drought stress conditions by draining water from the buckets and placing back the plastic pots. The plants were maintained under this condition for seven days after which a second chlorophyll extraction was done. Plants were then put under the normal condition by rewatering the buckets and a 3rd chlorophyll extraction was done seven days later using the same procedure as described above.

Table 3.1: Origin, ecosystem and level of drought tolerance of rice genotypes used in the study.

Name	**Origin (Country, Group)**	**Ecosystem**	**level of drought tolerance**
NERICA 6	Sierra Leone	Upland	Drought tolerant
NERICA 16	Sierra Leone	Upland	Drought tolerant
NERICA 15	Sierra Leone	Upland	Drought tolerant
NERICA 14	Sierra Leone	Upland	Drought tolerant
NERICA 4	Sierra Leone	Upland	Drought tolerant
Wakra	Indonesia	Upland	Drought tolerant
Kosti 2	WARDA	Upland	Drought tolerant
Kosti 1	WARDA	Upland	Drought tolerant
IRRI 150	Philippines	lowland	---
IR11A306	IRSEA	lowland	---
IR11A483	IRSEA	lowland	---
IRRI 147	Philippines	lowland	---
IRRI 122	Philippines	lowland	---
NERICA 1	Sierra Leone	Upland	Drought tolerant
IR12N240	IRSEA	lowland	---
IR74371-70-1-1	India	lowland	Drought tolerant
IR11N121	IRSEA	lowland	---
IRRI 154	Philippines	lowland	---
IRRI 168	Philippines	lowland	---
Umgar	China	Upland	Drought tolerant
Nipponbare	Japan	Upland	---
IAC 165	Brazil	Upland	---
NERICA 7	Sierra Leone	Upland	Drought tolerant

Source: Ekeleme *et al*. (2009)

3.3. Genomic DNA extraction

Genomic DNA was isolated from fresh three week-old rice leaf samples grown in the greenhouse using a DNA extraction method described by Murray and Thompson, (1980) (Appendix 1). Quality of the extracted DNA was determined using agarose gel electrophoresis and the DNA later quantified using a nanodrop.

3.4. Screening rice genotypes using SSR markers

Nineteen (18) SSR markers previously reported to have an association with drought tolerance traits in rice (Afiukwa *et al*., 2016) were tested using PCR amplification (Appendix 2) to determine whether there were any polymorphisms among the rice accessions under this study. A list of primers and their description is shown in Table 3. PCR was done in 25µl reaction mixture comprising 1X PCR master mix with its buffer (New England Biolabs), 0.25µM of each primer (forward and reverse), 10ng/µl of template DNA and deionised water. The reactions were carried out in a thermocycler (Eppendorf Inc.) under the following conditions; an initial heating step of 95ºC for 3 min (denaturation) followed by 35 cycles of 94ºC for 30 seconds, annealing at 55ºC for 30 seconds and an extension period of 68ºC for 30 seconds. A final extension period of 68ºC for 5 min was also included. The amplified PCR products were resolved on a 2% agarose gel in Tris acetic EDTA (TAE) buffer after staining with SYBR green and running it at 100 volts for 1 hour alongside a 50bp DNA ladder. The gels were visualized under UV light (at 360nm) from a transilluminator and documented using a digital camera. The amplified bands were scored for each SSR marker, generating a binary data matrix of 1 (for presence) and 0 (for absence) for each primer. This information was then used to determine the number of alleles and the Polymorphism Information Content (PIC) by using the formula described by Botstein *et al.*, (1980) :

$$PIC_j = 1-\sum_{i=1}^{n} P_i^2-\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2 P_i^2 P_j^2$$

Where P_i and P_j are the frequencies of the i^{th} and j^{th} alleles of a given marker, respectively, n = number of different alleles

Table 3.2: List of SSR markers used in the current study

Primer name	Primer sequence	SSR motif	Chr. Num.	Size of bands	Source
RM38	F: ACGAGCTCTCGATCAGCCTA R: TCGGTCTCCATGTCCCAC	(GA)16	8	250	Srividhya *et al*., 2011
RM252	F: TTCGCTGACGTGATAGGTTG R: ATGACTTGATCCCGAGAACG	(CT)19	4	216	McCouch *et al*., 2002
RM170	F: TCGCGCTTCTTCCTCGTCGACG R: CCCGCTTGCAGAGGAAGCAGCC	(CCT)7	6	121	Yue *et al*., 2005
RM318	F: GTACGGAAAACATGGTAGGAAG R: TCGAGGGAAGGATCTGGTC	(GT)15	2	140	Srividhya *et al*., 2011
RM279	F:GCGGGAGAGGGATCTCCT R: GGCTAGGAGTTAACCTCGCG	(GA)16	2	174	Ordonez *et al*., 2010
RM7390	F: CTGGTTAACGTGAGAGCTCG R: GCAGATCAATTGGGGAGTAC	(GATA)8	9	140	McCouch *et al*., 2002
RM432	F: TTCTGTCTCACGCTGGATTG R: AGCTGCGTACGTGATGAATG	(CATC)9	7	187	Vikram *et al*., 2011
RM5367	F: AGTACCTCTCACTCGCCTGC R: TGTCAGCTGTGAGTGAAGTCG	(TC)13	12	185	McCouch *et al*., 2002
RM5423	F: ATCCCACTTGCAGACGTAGG R: ACAGCAGCAAGGTGCCTC	(TC)16	1	202	McCouch *et al*., 2002
RM5850	F: TTAGGTGTGTGAGCGTGGC R: ATACACAGATGACGCACACG	(ATA)27	6	181	McCouch *et al*., 2002
RM36	F: CAACTATGCACCATTGTCGC R: GTACTCCACAAGACCGTACC	(GA)23	3	192	Brondani *et al*., 2002
RM3558	F: ACGAGAGATCTTCTTTGCAG R: CCTCTATTTATGCCTCTACGC	(GA)12	4	161	McCouch *et al*., 2002
RM517	F: GGCTTACTGGCTTCGATTTG R: CGTCTCCTTTGGTTAGTGCC	(CT)15	3	266	Hong *et al*., 2005
RM6130	F: GGCAGAGAGAGCTGCATCTC R: GACGACGACGAACCCAAC	(CGC)8	4	116	McCouch *et al*., 2002
RM583	F: AGATCCATCCCTGTGGAGAG R: GCGAACTCGCGTTGTAATC	(CTT)20	1	192	Vikram *et al*., 2011
RM1141	F: TGCATTGCAGAGAGCTCTTG R: CAGGGCTTTGTAAGAGGTGC	(AG)12	1	100	McCouch *et al*., 2002
RM260	F: ACTCCACTATGACCCAGAG R: GAACAATCCCTTCTACGATCG	(CT)34	12	111	McCouch *et al*., 2002
RM525	F: GGCCCGTCCAAGAAATATTG R: CGGTGAGACAGAATCCTTACG	(AAG)12	2	131	McCouch *et al*., 2002

3.1. Data analysis

Complete randomized block was the design of choice for the experiment in the greenhouse. The analysis of variance (ANOVA) was performed to compare chlorophyll contents among genotypes under the imposed drought conditions using Statistical analysis software (SAS) version 9.2. A Tukey's HSD test at 95% confidence interval was used for mean separations and the data presented as means with their respective standard errors. Percentage change in total chlorophyll contents following dehydration and rehydration in rice genotypes was calculated using the equations:

$$\% \text{ chlorophyll change after dehydration} = \frac{\text{Total chlorophyll after dehydration- Total chlorophyll at normal watering} * 100\%}{\text{Total chlorophyll at normal watering}}$$

$$\% \text{ chlorophyll change after rehydration} = \frac{\text{Total chlorophyll after rehydration- Total chlorophyll at normal watering} * 100\%}{\text{Total chlorophyll at normal watering}}$$

The SSR marker amplification data (1 and 0) were used to determine population genetic structure and presented as a dendrogram generated by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in the Dissimilarity Analysis and Representation (DARwin) version 6 with 1000 bootstrap replicates. Marker-trait association analysis was done by physically comparing the pattern of the SSR markers clustered from the genotypes with the percentage change in the total chlorophyll content as a result of drought stress.

CHAPTER FOUR

4. RESULTS

4.1. Total chlorophyll content in normal condition at 2 months after planting

Under normal watering conditions (control), NERICA 7 showed the highest chlorophyll content followed by significantly lower levels in NERICA 15, Kosti 1, NERICA 1, NERICA 14, IAC 165, IRRIN121 and Wakra. Genotype IRRI 154 recorded the lowest chlorophyll content followed by Nipponbare, IR12N240, IR11A483, Kosti 2, IR74371-70-1-1 and IR11A306 (Figure 1).

4.2. Effect of drought on rice chlorophyll content after 7 days of dehydration

Withholding water for seven days resulted in a marked effect on total chlorophyll content in all rice genotypes in this study. The effect was first noted in the color change in the leaves with those under stress turning yellow compared to plants growing under normal conditions (Plate 1). Total chlorophyll content was significantly affected despite all genotypes responding differently to the drought stress treatment (Figure 1). After dehydration, IR11A306 recorded the highest increment in total chlorophyll followed by NERICA 4, Kosti 1, Wakra, NERICA 6, IR12N240, IAC 165 and IRRI 150. Genotype IR11A483 showed the highest reduction followed by Nipponbare, Kosti 2, IR74371-70-1-1, IR11N121, IRRI 168 , NERICA 7, NERICA 15 and NERICA 1.

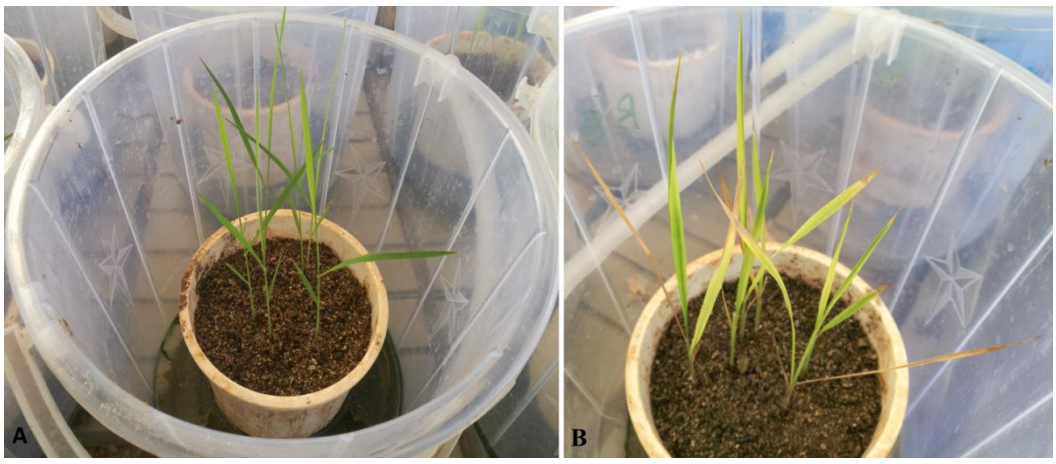

Plate 4.1: Plants of Kosti 1 genotype in the greenhouse before and after exposure to drought stress by withholding water for 7 days. A shows the control plants and B shows the plants at seven days drought stress (leaf color change).

4.3. Effect of drought on rice chlorophyll content after 7 days of rehydration

When plants were rehydrated and total chlorophyll content measured, genotypes recorded significantly different levels of chlorophyll levels (Figure 1). The highest increase and best recovery were observed in IAC 165 followed by Kosti 1, IR74371-70-1-1, IRRI 147, NERICA 6, Umgar and IRRI 168. Genotype NERICA 15 showed the least reduction in chlorophyll levels followed by IR11A483, IR11A306, IRRI 122, IRRI 150, IR12N 240 and IRRI 154.

4.4. Chlorophyll change after dehydration and after rewatering

All the genotypes responded differently to drought stress and recorded different values of change in total chlorophyll (Table 4). At the end of the dehydration period, genotype IR11A306 recorded the highest increase in total chlorophyll (187.12) followed by IRRI 154 (150.53), NERICA 6 (69.28), IR12N240 (57.33), NERICA 4 (16.79), Wakra (6.89) and IRRI 150 (2.77), while IR11A483 (-82.88) showed the highest reduction followed by NERICA 15 (-77.98), IR11N121 (-77.61), IRRI 168 (-73.87), NERICA 7 (-65.89), NERICA 1 (-61.71), NERICA 14 (-59.69) and Nipponbare (-59.32). After rehydration, the highest increase and best recovery were observed in IR74371-70-1-1 (257.54) followed by IRRI 168 (63.93), IRRI 147 (43.39), Nipponbare (14.18), Kosti 2 (7.89), IAC 165 (7.31) and Umgar (2.67). Genotype IR11A306 showed the least reduction in chlorophyll levels (-97.03) followed by NERICA 15 (-96.17), IRRI 150 (-92.17), IRRI 122 (-91.03), IR12N240 (-83.52), IRRI 154 (-83.36), NERICA 16 (-79.81), NERICA 4 (-78.029) and Wakra (-69.46). In general, Nerica 4 and Wakra performed best under both conditions (before and during dehydration) but their chlorophyll contents declined after rehydration. NERICA 6, IRRI 154 and IR11A306 showed a weak performance in normal conditions and after rehydration, but a good performance during the dehydration period (Table 4).

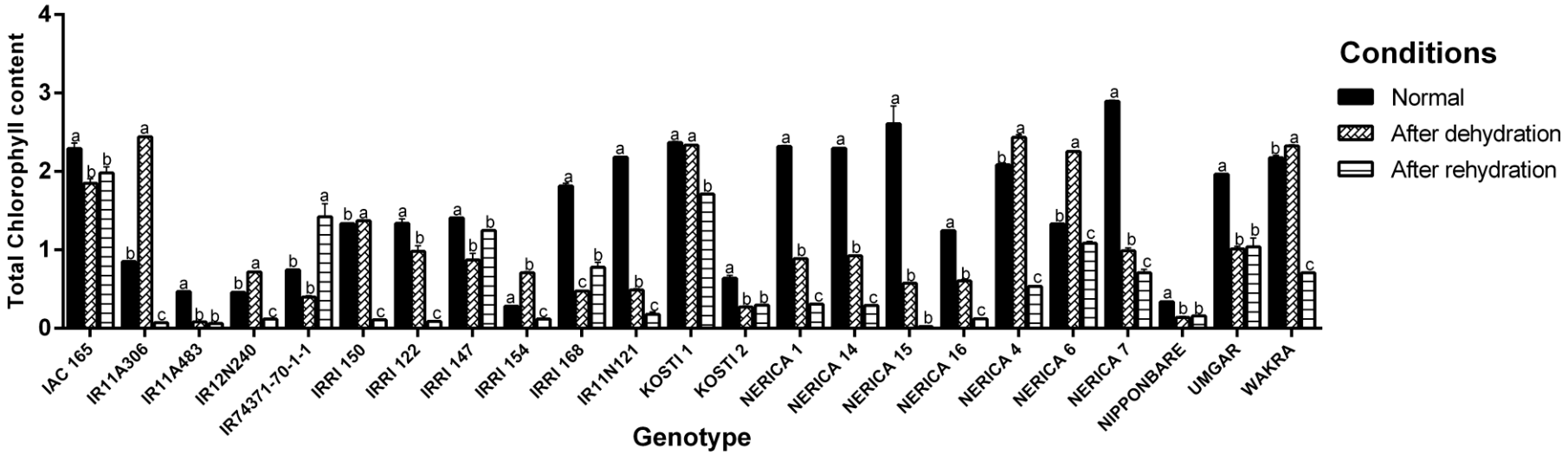

Figure 4.1: Total chlorophyll content in rice leaves measured using a spectrophotometer at 660nm wavelength under normal (before dehydration), after dehydration and after rehydration conditions (means of each genotype with the same letter are not significantly different).

Table 4.1: Percentage change in total chlorophyll content following dehydration and rehydration in rice genotypes

S/N	Genotype	% Chlorophyll change after 7 days dehydration	Rank	% Chlorophyll change after 7 days rehydration	Rank
1	IAC 165	-19.41	9	7.31	6
2	IR11A306	187.12	1	-97.03	23
3	IR11A483	-82.88	23	-22.98	8
4	IR12N240	57.33	4	-83.52	18
5	IR74371-70-1-1	-46.51	12	257.54	1
6	IRRI 150	2.77	7	-92.17	21
7	IRRI 122	-26.72	10	-91.03	20
8	IRRI 147	-38.22	11	43.39	3
9	IRRI 154	150.53	2	-83.36	18
10	IRRI 168	-73.87	20	63.93	2
11	IR11N121	-77.61	21	-63.56	12
12	KOSTI 1	-1.48	8	-26.64	9
13	KOSTI 2	-57.43	15	7.89	5
14	NERICA 1	-61.71	18	-65.39	13
15	NERICA 14	-59.69	17	-68.54	14
16	NERICA 15	-77.98	22	-96.17	22
17	NERICA 16	-51.33	14	-79.81	17
18	NERICA 4	16.79	5	-78.029	16
19	NERICA 6	69.28	3	-51.96	11
20	NERICA 7	-65.89	19	-28.31	10
21	NIPPONBARE	-59.32	16	14.18	4
22	UMGAR	-48.54	13	2.67	7
23	WAKRA	6.89	6	-69.46	15

4.5. Population structure of rice genotypes and SSR polymorphism

For determination of the levels of genetic diversity in the population under the current study, a total of 18 SSR markers successfully amplified (Plate 2, 3, 4 and 5).

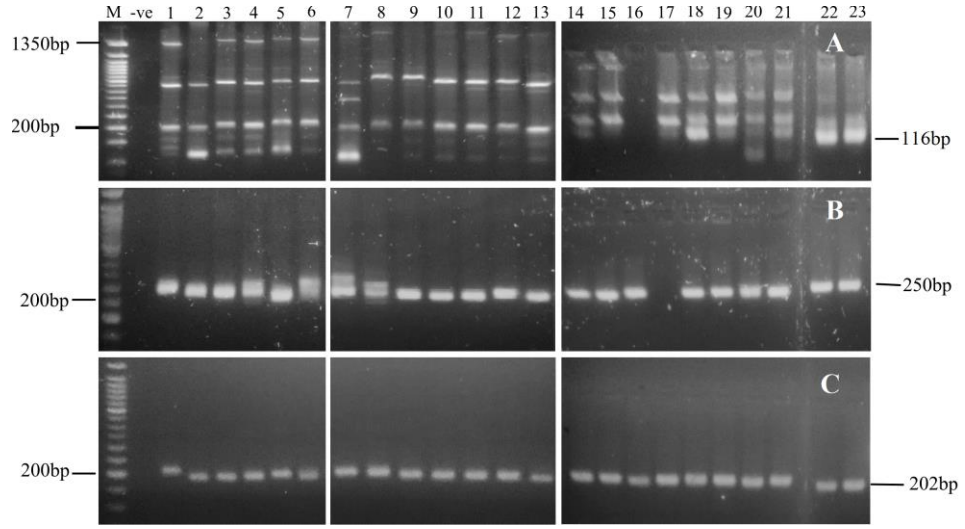

Plate 4.2: Representative gels of fragments amplified by PCR targeting SSR markers common to rice. M =50 bp ladder, -ve= negative control (A no template control omits the DNA template from the reaction), Numbers 1- 23 represent each of the rice genotypes, Primer identities are represented by A = RM6130, B = RM 38, C = RM5423.

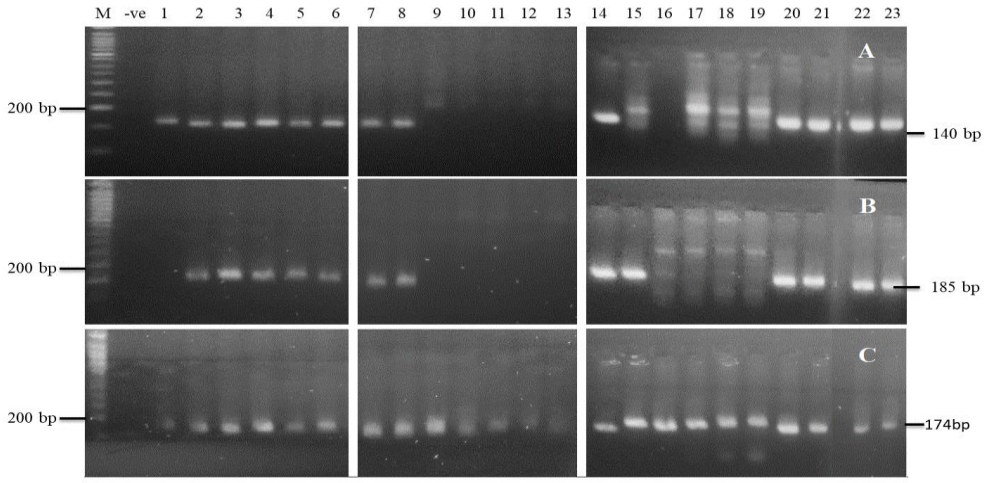

Plate 4.3: Representative gels of fragments, A =7390, B = RM5367, C = RM279.

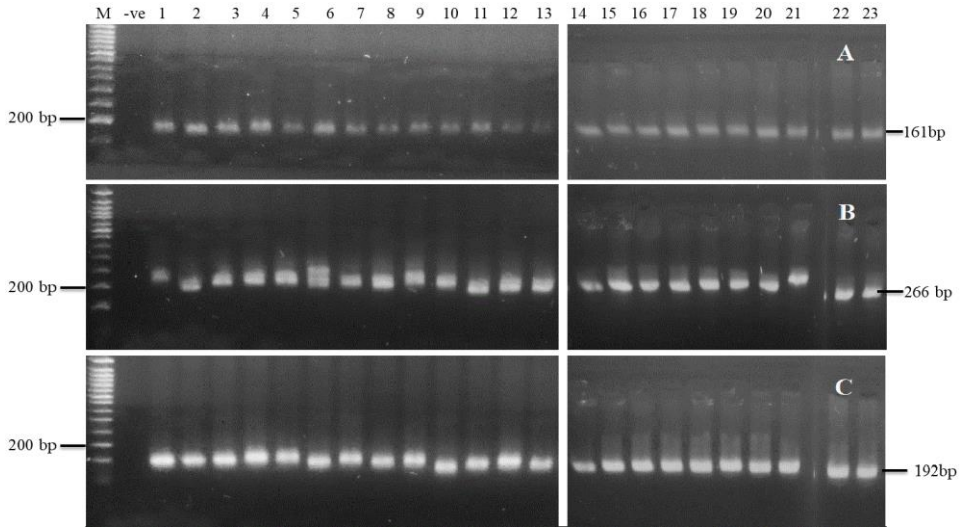

Plate 4.4: Representative gels of fragments, A = RM3558, B = RM517, C = RM583.

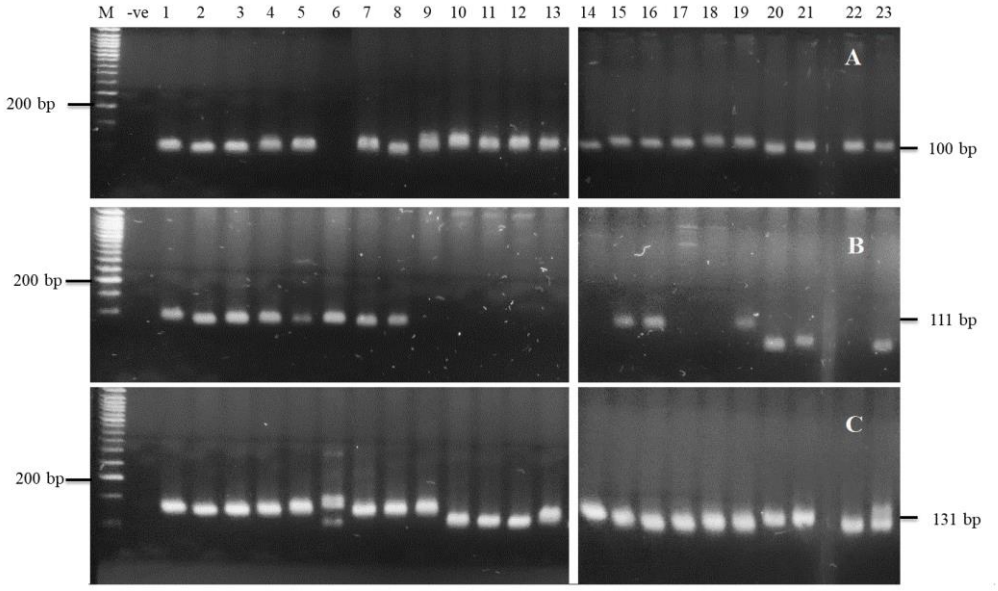

Plate 4.5: Representative gels of fragments, A = RM1141, B = RM260, C = RM525.

4.6. Allele frequency, Number of alleles and PIC

Information on allele frequency, allele number and PIC is summarized in Table 5. The 18 SSR primers resulted in a total of 569 alleles with 13 to 113 alleles per primer at an average of 31.7 and allele frequency ranged between 0.52 and 1. The PIC values ranged from 0.51 to 0.99 with a mean value of 0.88.

4.6. Genetic distance between the studied rice genotypes

Analysis of the polymorphism data (Table 6) showed low and moderate to high genetic distances that ranged from 0.00003 to 0.356. The highest similarity coefficient (0.356) was found between IR74371-70-1-1 and Nerica 6. Furthermore, NERICA 6 shared a high similarity percentage (0.352) between genotype IRRI 150, IR11A306, IR11A483 and IRRI 147. On the other hand, the lowest similarity percentage (0.00003) was observed between Umgar and Nipponbare. Although NERICA 16 and NERICA 7 share the same ecosystem, they recorded a relatively low similarity percentage (0.0001).

Table 4.2: Genetic diversity indices detected in rice genotypes by SSR markers

SSR primer	Allele frequency	Allele Number	PIC
RM38	0.96	27	0.95
RM252	1	27	0.99
RM170	1	33	0.99
RM318	1	37	0.99
RM279	0.74	28	0.73
RM7390	0.52	29	0.51
RM432	0.74	57	0.73
RM5367	1	23	0.99
RM5423	0.96	22	0.95
RM5850	0.57	13	0.56
RM36	1	26	0.99
RM3558	1	23	0.99
RM517	1	24	0.99
RM6130	0.96	113	0.95
RM583	1	23	0.99
RM1141	0.96	22	0.95
RM260	0.61	15	0.6
RM525	1	27	0.99
Mean	0.89	31.7	0.88

4.7. Cluster analysis

The neighbour-joining analysis grouped the 23 studied rice genotypes into 3 major clusters (Figure 2). Cluster 1 had 2 distinct sub-groups with all the members in this sub-group originating from the upland ecosystem. The genotypes that clustered in this group were Kosti 2, NERICA 14, Kosti 1, Wakra, NERICA 4, NERICA 15, NERICA 6, NERICA 7, NERICA 16, IAC 165 and Nerica 1, while sub-cluster 2 comprised Nipponbare, Umgar and IR12N240. The second cluster comprised lowland rice genotypes and these were further grouped into two sub-cluster; sub-cluster 1 that had IR11A306, IRRI 150, IR11A483, IRRI 147, IRRI 122 and IR74371-70-1-1 and sub-cluster 2 that had IRRI 154 and IR11N121. A lowland genotype IRRI 168 formed cluster 3 on its own.

4.8. Marker-trait association under drought stress analysis

This study further used the above analysis to manually (physically) compare the pattern in which the SSR markers grouped the genotypes and the change in total chlorophyll content in each genotype. It was observed that none of the markers clearly grouped the genotypes according to the patterns of change in total chlorophyll contents. Although the majority of cluster 1 are known as upland and drought tolerant (Figure 4), only NERICA 6, NERICA 4 and Wakra showed an increase in total chlorophyll content at the end of the dehydration period while the other genotypes showed a significant reduction. Genotypes in cluster 2 and cluster 3 were from undefined ecosystem. Here, IR11A306, IRRI 154 and IRRI 150 showed an increase in total chlorophyll content at the end of the dehydration period while the rest of the genotypes in both clusters showing a reduction in chlorophyll. On the other hand, genotype Nipponbare, Kosti 2, IAC 165 and Umgar in cluster 1 showed a recovery after rehydration while the other genotypes in the same cluster showed a decline in the their chlorophyll. As well, most of cluster 2 and 3 genotypes showed no recovery after rehydration except IR74371-70-1-1, IRRI 168 and IRRI 147 genotypes showed the best recovery.

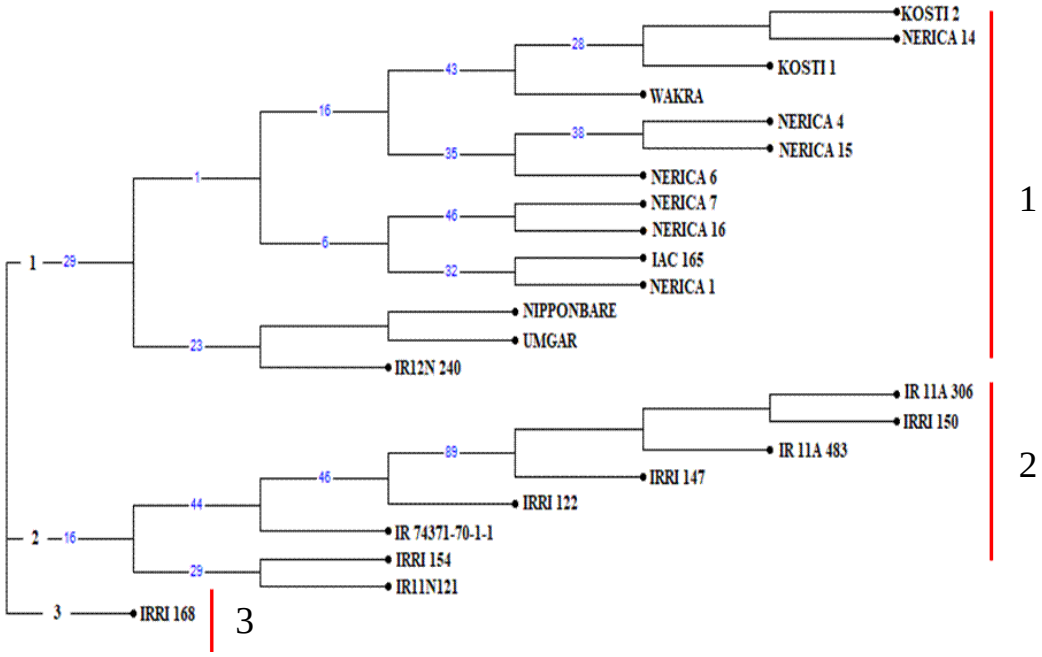

Figure 4.2: A dendrogram showing 23 rice genotypes generated from 18 SSR markers. Numbers 1, 2 and 3 represent separate clusters.

Table 4.3: Similarity coefficient among rice genotypes based on SSR markers

Units	NERICA 6	NERICA 16	NERICA 15	NERICA 14	NERICA 4	WAKRA	KOSTI 2	KOSTI 1	IRR 150	IR 11A 306	IR 11A 483	IRRI 147	IRRI 122	NERICA 1	IR 12N 240	IR 74371-70-1-1	IRRI N 121	IRRI 154	IRRI 168	UMGAR	NIPPONBARE	IAC 165
NERICA 16	0.2223																					
NERICA 15	0.125	0.1175																				
NERICA 14	0.1714	0.0839	0.0666																			
NERICA 4	0.125	0.1175	0.000036	0.0666																		
WAKRA	0.2275	0.14	0.1227	0.0588	0.1227																	
KOSTI 2	0.1714	0.0839	0.066	0.000036	0.0666	0.0588																
KOSTI 1	0.1714	0.0838	0.066	0.000054	0.0666	0.0588	5.4E-05															
IRRI 150	0.3525	0.2036	0.2476	0.214	0.2476	0.2701	0.214	0.214														
IR 11A 306	0.3525	0.2036	0.2476	0.214	0.2476	0.2701	0.214	0.214	3.6E-05													
IR 11A 483	0.3524	0.2036	0.2476	0.214	0.2476	0.2701	0.214	0.214	5.4E-05	0.000054												
IRRI 147	0.3524	0.2036	0.2476	0.214	0.2476	0.27	0.214	0.214	7.3E-05	0.000073	0.000054											
IRRI 122	0.3339	0.1851	0.2291	0.1955	0.2291	0.2516	0.1955	0.1955	0.0667	0.0667	0.0667	0.0666										
NERICA 1	0.244	0.0772	0.1392	0.1056	0.1392	0.1617	0.1056	0.1056	0.2254	0.2254	0.2253	0.2253	0.2068									
IR 12N 240	0.205	0.0561	0.1001	0.0665	0.1001	0.1226	0.0665	0.0665	0.1726	0.1726	0.1726	0.1726	0.154	0.0779								
IR 74371-70-1-1	0.3565	0.2077	0.2517	0.2181	0.2517	0.274	0.2181	0.2181	0.1655	0.1655	0.1655	0.1655	0.147	0.2294	0.1767							
IRRI N 121	0.3408	0.192	0.236	0.2024	0.236	0.2585	0.2024	0.2024	0.1965	0.1965	0.1964	0.1964	0.1779	0.2137	0.1609	0.2005						
IRRI 154	0.2839	0.1351	0.179	0.1455	0.179	0.2015	0.1455	0.1454	0.1395	0.1395	0.1395	0.1395	0.121	0.1568	0.104	0.1436	0.0625					
IRRI 168	0.2438	0.095	0.13905	0.1054	0.139	0.1615	0.1054	0.1054	0.1316	0.1316	0.1316	0.1316	0.1131	0.1167	0.064	0.1357	0.12	0.063				
UMGAR	0.205	0.0561	0.1001	0.0665	0.1001	0.1226	0.0665	0.0665	0.1726	0.1726	0.1726	0.1726	0.1541	0.0779	0.00005	0.1767	0.161	0.104	0.064			
NIPPONBARE	0.205	0.0561	0.1001	0.0665	0.1001	0.1226	0.0665	0.0665	0.1726	0.1726	0.1726	0.1726	0.1541	0.0779	0.00005	0.1767	0.161	0.104	0.064	3E-05		
IAC 165	0.2594	0.0926	0.1546	0.121	0.1546	0.1771	0.121	0.121	0.2408	0.2408	0.2408	0.2407	0.2222	0.0588	0.0933	0.2449	0.229	0.1722	0.1322	0.0933	0.0933	
NERICA 7	0.2223	0.000036	0.1175	0.0839	0.1175	0.14	0.0839	0.0838	0.2036	0.2036	0.2036	0.2036	0.1851	0.0772	0.0561	0.2077	0.192	0.1351	0.095	0.0561	0.0561	0.0926

CHAPTER FIVE

5. DISCUSSION

5.1. Effect of drought stress on total chlorophyll content

Drought stress affects morphological and physiological traits during plant growth and development. Particularly, drought stress leads to a reduction in leaf area, cell size and intercellular volume (Ndjiondjop *et al*., 2010). Furthermore, it has been demonstrated that exposure of plants to drought leads to a significant effect in chlorophyll contents as a result of the reduction in leaf growth (Chutia and Borah, 2012). In the current study, each rice genotype was examined under drought stress by monitoring the change in total chlorophyll content following seven days of withholding water and a further seven days of rewatering. The data showed a significant increase in levels of chlorophyll in some genotypes while others recorded marked chlorophyll decline as a result of dehydration at the end of the drought period. It has been reported that chlorophyll supports more efficient energy conversion into ATP (Adenosine Tri-Phosphate) and NADPH (Nicotinamide Adenine Dinucleotide Phosphate) which are then used as a source of energy to build carbohydrates from CO_2 (Peña *et al*., 1986**)**. Hence chlorophyll reduction in these genotypes might be an indication that photosynthesis might have already been inhibited when the color of leaves started to change and that was clearly observed.

Consequently, some of the genotypes showed rapid recovery of chlorophyll (based on the positive percentage increase of chlorophyll) after rehydration as a result of total chlorophyll content that were maintained at higher levels indicating chloroplast integrity. Similar results were reported by Peleg *et al* (2011). On the other hand, some genotypes showed no recovery (based on the negative percentage loss of chlorophyll) following drought as a consequence these plants failed to recover from drought stress and continued to lose more green leaf area. Overall, these observations indicated variations among these genotypes with respect to chlorophyll levels and how the individual lines respond to drought stress. It is important for a tolerant plant to maintain high levels of chlorophyll

under drought stress to ensure continued photosynthesis. In cereals, higher total chlorophyll content under stress conditions has been reported and this is an indicator of drought tolerance (Gummuluru *et al*., 1989).

5.2. Extent of SSR variability within rice population

The uses of molecular markers to select germplasm possessing genes and genomic regions that control target traits can fast-track the progress of breeding for drought tolerant rice. This is because molecular markers are transmitted faithfully from generation to generation and are not subject to environmental influences (Afiukwa *et al.*, 2016). The SSR markers are efficient and the system of choice for genetic analysis in rice because of their abundance in the rice genome, high level of polymorphism, reliable and high but simple reproducible assays (Singh *et al*., 2010). For these reasons, therefore, SSR markers have been used in the molecular characterization of rice as well as other crop species (Semagn *et al.*, 2006). In the current study 18 primers were used to detect variability in 23 rice genotypes. The 18 markers used in this study were successfully determined the genetic patterns of the selected rice genotypes. The data showed values that were comparatively higher than those of Aficukwa *et al.* (2016) who reported between 4 to 25 alleles per primer and PIC values of 0.76 to 0.95 using 16 SSR primers in 30 rice genotypes and higher to Ramadan *et al.,* (2015) used 46 primers in 7 rice genotypes who reported a total number of 127 alleles with a range of 2 to 6 alleles per marker PIC values of 0.21 to 0.79. This reflects the high discriminatory ability of the used markers and therefore affirms their use in genetic characterization studies (Singh *et al*., 2010). Moreover, according to Sajib *et al*., (2012) PIC value effectively demonstrates the power of SSR markers in measuring genetic variation among the genotypes.

5.3. Similarity and cluster analysis

Genetic similarity coefficients of pair-wise comparisons estimated on the basis of the polymorphic microsatellite loci ranged from 0.00003 to 0.356, indicating a wide range of genetic variation among the genotypes. These results gave an indication of the relationship between NERICA and other genotypes. The highest

similarity coefficient (0.356) was found between IR 74371-70-1-1 and NERICA 6 (0.913) that could be due to the ecosystem where NERICA and IR74371-70-1-1 are drought tolerant genotypes (Kaneda, 2007). The lowest similarity coefficient (0.00003) was observed between Umgar and Nipponbare. Also, NERICA 6 shared a high similarity coefficient (0.352) between genotype IRRI 150, IR11A 306, IR 11A483 and IRRI 147. These genotypes are known to be from different ecosystem and brought lately to Sudan and still under study. Based on this result, these genotypes could give a promising performance and can be produced widely in Sudan for their relationship with drought tolerant NERICA rice genotypes.

Tracking the population structure of rice genotypes under this study grouped them into 3 major clusters. The dendrogram analysis provided an evidence of the ecosystem of each genotype and the genetic relationship between them as cluster 1 (with two sub-clusters) comprised 93 % upland and drought tolerant genotypes and the rest grouped in cluster 2 and 3. The clustering was largely depending on drought tolerance according to absence or presence of the banding by SSR markers. The obtained results reflected the existence of considerable amount of molecular diversity among the tested genotypes and hence demonstrated of the feasibility of genetic improvement of drought tolerance using those genotypes in breeding program. The results also demonstrated the powerfulness of molecular analysis in assessing genetic diversity. The SSR markers have been widely used to characterize rice germplasm and evaluate genetic relationships among cultivars. Chandra *et al*. (2004) studied the extent of genetic diversity among 27 rice accessions from diverse hydrological habitats using 26 SSR markers; a dendrogram was constructed based on the similarity index. Clustering represented the genetic similarity among the accessions as well as their hydrological habitat adaptation. Allelic variations among 169 SSR loci have been used to evaluate genetic diversity among 234 accessions of rice and clearly detected five distinct groups, corresponding to Indica, aus, aromatic, temperate Japonica and tropical Japonica (Garris *et al*., 2005). Siwach *et al*. (2004) was able to differentiate 24 rice genotypes into two major groups, corresponding to Basmati and non-Basmati types, based on SSR analysis at 50 loci. Cluster analysis based on 32 SSR loci

clearly placed 35 Asian rice cultivars into two major groups, namely aromatic and non-aromatic coarse grain rice (Pervaiz *et al.*, 2009). Analysis based on SSR markers was able to divide Indian elite rice cultivars into clusters according to complex physiological characters namely early duration maturity, medium duration maturity and semi deepwater and deepwater rice (Davierwala *et al*., 2000).

5.4. Patterns of SSR polymorphism and their association with chlorophyll under drought stress

Rice is considered the most sensitive plant to drought stress during reproductive development at which time moderate water shortages can result in a significant reduction in grain yield (Afiukwa *et al*., 2016). Drought stress affects morphological and physiological traits during plant growth and development. Particularly, drought stress leads to a reduction in leaf area, cell size and intercellular volume (Ndjiondjop *et al*., 2010). Furthermore, it has been demonstrated that exposure of plants to drought leads to a significant effect in chlorophyll contents as a result of the reduction in leaf growth (Chutia and Borah, 2012). A plant having the characteristics of delayed leaf rolling under water stress and faster recovery rates after removing the water stress in rice was considered as a good trait because flag leaf in rice crops plays an important role in plant growth and development (Gummuluru et al., 1989). Production of root system under drought is very important and has good correlation with yield under drought stress (Londo *et al*., 2006). Furthermore, response to water stress in plants at the molecular level undoubtedly constitutes an area of major interest for a complete understanding of the process (Ndjiondjop *et al*., 2010).

Improvement of rice for drought tolerance using conventional breeding methods is slow due to the differences in geographical locations and variations of seasons in drought timing and severity, the selection of combinations difficulty of traits and the complex nature of drought tolerance (Courtois *et al*., 2003). Furthermore, other factors that underline the slow progress include low heritability, multiple gene control, genotype and environment interactions. All these were shown to substantially influence crop yields (Cattivelli *et al*., 2008).

NERICA is a new group of upland rice genotypes that perfectly adapt to the rain-fed upland ecology in sub-Saharan Africa where smaller holders farmers lack the means to irrigate (Kaneda, 2007) and were identified as tolerant to drought both at vegetative and reproductive stages (Ekeleme *et al*., 2009). Therefore, in this study NERICA genotypes were used as indicators of drought tolerance trait.

A comparison between the pattern in which the SSR markers clustered the genotypes and change in chlorophyll allowed determination of marker-trait associations for drought tolerance showed that none of the markers typically grouped the genotypes according to the change pattern of the total chlorophyll content. A majority of cluster 1 genotypes were from the upland ecosystem (drought tolerant genotypes) and showed an increase in the chlorophyll content at the end of dehydration as an expected result while the others showed a significant reduction but only four genotypes out of fourteen recovered and showed an increment in the total chlorophyll content after rehydration. Cluster 2 and 3 comprised lowland genotypes with regard to the ecosystem. But some of them showed an increase in the chlorophyll content at the end of the dehydration period and only three out of nine genotypes showed the best recovery. Therefore, it could be deduced from our results that the markers were able to group the genotypes based on their degree of drought tolerance reflecting the strength of the SSR markers in analysing and explaining the population genetic structure as earlier demonstrated by Garris *et al.* (2005). Notable, also, was the observation that although NERICA 1, NERICA 14, NERICA 15, NERICA 16 and NERICA 7 have been described as drought tolerant (Somado *et al.*, 2008), they did not show the expected performance under the current study conditions.

6. CONCLUSION AND RECOMENDATIONS

6.1. Conclusion

The study determined the effect of drought stress on total chlorophyll content in selected rice genotypes grown in Sudan and showed variation among the genotypes screened with respect to chlorophyll levels and how the individual lines respond to drought stress. For determination of the levels of genetic diversity in the population, the study determined the genetic patterns of the selected rice genotypes. A total of 18 SSR markers successfully amplified resulting in a total of 569 alleles with 13 to 113 alleles per primer with an average of 31.7. The PIC values ranged from 0.51 to 0.99 with a mean value of 0.88. Furthermore, the determined the association between SSR Markers and total chlorophyll content change under drought stress by comparing the pattern in which the SSR markers grouped the genotypes and the change in total chlorophyll content in each genotype and showed that none of the markers typically grouped the genotypes according to the patterns of change in total chlorophyll contents.

From the study, it could be concluded that the markers used in this study would be useful for more efficient selection of drought tolerant lines. The most significant application of these identified SSR markers for drought tolerance is to collect those favourable alleles into the elite rice genotypes through marker assisted breeding. In addition, the markers could be utilized in further studies for association mapping and marker assisted selection for drought tolerance in Sudanese rice genotypes. Therefore, these results could play a role in developing genotypes that tolerate drought stress by selecting the best parental lines for developing and improving drought-tolerant rice genotypes.

6.2. Recommendations

I. The information generated in this study would be used to facilitate rice breeding for guiding to choose divergent potential parental line selection for developing new genotypes that are tolerant to drought stress.

II. The SSR markers used in this study could be used for other rice genetic studies as they were able to reveal genetic diversity even among closely related genotypes.

III. From the result of this study, it is recommend that further studies for drought tolerance should be done for genotype IR74371-70-1-1, IRRI 168, IRRI 147, Nipponbare, IAC 165, IR11A306, IRRI 154, IR11A483, IR12N240 and IRRI 150 for their remarkable positive response to drought episode.

IV. Genotypes mentioned above could give promising new genotypes. Therefore, they are recommended to be produced widely in Sudan.

V. The other parameters (root length, plant height, leaf width, yield, etc.) apart from the determination of the effect of drought stress on chlorophyll content are recommended to be investigated for the rice genotypes used in this study at different stages of growth to determine drought tolerance.

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APPENDICES

Appendix I: Genomic DNA extraction procedure

1. Add 1% beta-mercaptoethanol to the CTAB-buffer.
2. Preheat the CTAB-buffer in the water bath to 65 ̊c.
3. Prepare 700 ul CTAB-buffer in Eppendorf tube.
4. Grind tissues into a fine powder. Transfer tissue-powder to the Eppendorf tube and mix very well and incubate in the water bath at 65 ̊c for 30 minutes.
5. Add 700 ul chloroform and mix very well, spin down at max speed for 2 minutes and transfer upper phase into a new Eppendorf tube.
6. Add 700 ul chloroform and mix well. Spin down at max speed for 2 minutes.
7. Transfer upper phase into a new Eppendorf tube. Add 466 ul of isopropanol and mix well. Store overnight at -20 ̊c for DNA precipitation.
8. Spin down at max speed (11000 rpm for 30 minutes). Keep the pellet, discard the supernatant. Add 1 ml of 70% ethanol and mix well. Spin down at 11000 rpm for 15 minutes.
9. Keep the pellet, discard the supernatant.
10. Dissolve pellet in 90 ul water in the water bath at 65 ̊c for 15 minutes.
11. Keep the pellet in the fridge for future use.

Appendix II: Polymerase Chain Reaction (PCR) and gel documentation procedure

The PCR mixture preparation:

1. Add 12.5 ul Taq mix buffer (0.25U/ul Taq DNA polymerase, 2X PCR buffer, 0.4mM dNTPs, 3.2 mM MgCl2, 0.02% bromophenol blue) to a new PCR tube.
2. Add 1.0 µl each of the forward and reverse primers.
3. Add 1.5 µl of DNA template and leave one tube without DNA template for negative control.
4. Top up the mixture to 25.0 µl with nuclease-free water.

The PCR profile:

94 c^o for 3 min followed by 35 cycles of 94 c^o for 30 sec, 55 c^o for 30 sec and 68 c^o for 30 sec with a final extension at 68 c^o for 5 min.

Gel running and documentation

1. Mix 3 ul loading dye and 2 ul cybr-green with 2 ul of 50 bp ladder.
2. Add 3 ul loading dye and 2 ul cybr-green to the PCR product in each PCR tube and mix well.
3. Pipette the ladders mixture into the first well and the second well for the negative control.
4. Pipette 20 ul of the mixture into the subsequent wells.
5. Run the gel at 100v for 1 hour.
6. Visualize under UV light and document using a digital camera.

Appendix III: ANOVA tables for chlorophyll content

1. Chlorophyll content under normal condition

Tukey's Studentized Range (HSD) Test for _st__

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	0.0083
Critical Value of Studentized Range	5.42914
Minimum Significant Difference	0.2856

Means with the same letter are not significantly different.

Tukey Grouping			Mean	N	Accesion_
	A		2.89500	3	NERICA 7
	B		2.60900	3	NERICA 15
	B				
C	B		2.37000	3	KOSTI 1
C					
C			2.32000	3	NERICA 1
C					
C			2.29500	3	NERICA 14
C					
C			2.29250	3	IAC 165
C					
C	D		2.18100	3	IRRI N 121
C	D				
C	D		2.17500	3	WAKRA
C	D				
C	D	E	2.08500	3	NERICA 4
	D	E			
	D	E	1.96450	3	UMGAR
		E			
		E	1.81400	3	IRRI 168
	F		1.40800	3	IRRI 147
	F				
	F		1.33800	3	IRRI 122
	F				
	F		1.33500	3	IRR 150
	F				
	F		1.33300	3	NERICA 6
	F				
	F		1.24600	3	NERICA 16
	G		0.85000	3	IR 11A 306
	G				
H	G		0.74400	3	IR 74371-70-1-1
H	G				
H	G	I	0.64000	3	KOSTI 2
H		I			
H	J	I	0.47000	3	IR 11A 483
	J	I			
	J	I	0.45700	3	IR 12N 240
	J				
	J		0.33800	3	NIPPONBARE
	J				
	J		0.28300	3	IRRI 154

2. Chlorophyll content after dehydration

The GLM Procedure

Tukey's Studentized Range (HSD) Test for _nd_

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	0.002836
Critical Value of Studentized Range	5.42914
Minimum Significant Difference	0.1669

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Accesion_
A	2.44050	3	IR 11A 306
A			
A	2.43500	3	NERICA 4
A			
B A	2.33500	3	KOSTI 1
B A			
B A	2.32500	3	WAKRA
B			
B	2.25650	3	NERICA 6
C	1.84750	3	IAC 165
D	1.37200	3	IRR 150
E	1.01100	3	UMGAR
E			
E	0.98750	3	NERICA 7
E			
E	0.98050	3	IRRI 122
E			
E	0.92500	3	NERICA 14
E			
E	0.88850	3	NERICA 1
E			
F E	0.87000	3	IRRI 147
F			
F G	0.71900	3	IR 12N 240
F G			
F G	0.70900	3	IRRI 154
G			
H G	0.60650	3	NERICA 16
H G			
H G	0.57450	3	NERICA 15
H			
H I	0.48850	3	IRRI N 121
H I			
H I	0.47400	3	IRRI 168
I			
J I	0.39800	3	IR 74371-70-1-1
J			
J K	0.27250	3	KOSTI 2
K			
L K	0.13750	3	NIPPONBARE
L			
L	0.08050	3	IR 11A 483

3. Chlorophyll content after rehydration

Tukey's Studentized Range (HSD) Test for _rd_

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	0.007062
Critical Value of Studentized Range	5.42914
Minimum Significant Difference	0.2634

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Accesion_
A	1.98250	3	IAC 165
B	1.71300	3	KOSTI 1
C	1.42300	3	IR 74371-70-1-1
C			
D C	1.24750	3	IRRI 147
D			
D	1.08400	3	NERICA 6
D			
D E	1.03800	3	UMGAR
E			
F E	0.77700	3	IRRI 168
F			
F	0.71000	3	WAKRA
F			
F	0.70800	3	NERICA 7
F			
F G	0.53500	3	NERICA 4
G			
H G	0.30750	3	NERICA 1
H G			
H G	0.29400	3	KOSTI 2
H G			
H G	0.29100	3	NERICA 14
H			
H I	0.17800	3	IRRI N 121
H I			
H I	0.15700	3	NIPPONBARE
H I			
H I	0.12250	3	NERICA 16
H I			
H I	0.11850	3	IR 12N 240
H I			
H I	0.11800	3	IRRI 154
H I			
H I	0.10750	3	IRR 150
H I			
H I	0.08800	3	IRRI 122
H I			
H I	0.07250	3	IR 11A 306
H I			
H I	0.06200	3	IR 11A 483
I			
I	0.02200	3	NERICA 15