CHARACTERIZATION OF SELECTED ZAMBIAN RICE (*ORYZA SATIVA*) ACCESSIONS USING SIMPLE SEQUENCE REPEAT MARKERS

By

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DECLARATION

I, **Munsaka Siankuku**, hereby declare that this dissertation represents my own work and that it has not previously been submitted for a degree, diploma or other qualification at this or another University.

Signature

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Date

APPROVAL

This dissertation of **Munsaka Siankuku** is approved as fulfilling part of the requirements for the award of the degree of Master of Science in Molecular Biology by the University of Zambia.

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DEDICATION

I dedicate this thesis to my Father and Mother, Mr. and Mrs. Siankuku. I thank God every day for giving me such loving and hard working parents. You are truly my inspiration; your support and continuous belief in me are what have kept me going.

ABSTRACT

The study was undertaken to assess the genetic diversity among some selected Zambian rice (*Oryza sativa* L.) accessions using Simple Sequence Repeat (SSR) molecular markers. There appears to be little or no information on the genetic or agronomic traits of the locally grown rice crop in Zambia. Information on the accessions that have been released as well as characteristics such as their responses to biotic factors such as fungal and bacterial pathogens or abiotic factors such as water stress is not available. A systematic rice seed system or breeding program appears to be non-existent. The aim of the study was to analyze the genetic diversity of selected rice accessions grown in various regions of Zambia. Thirty accessions were selected for genetic diversity analysis using ten simple sequence repeat molecular markers; RM5, RM168, RM7, RM13, RM225, RM452, RM211, RM205, RM413, and RM463. These were amplified by polymerase chain reaction (PCR) and analyzed by 1% agarose gel electrophoresis. Most of the primers showed distinct polymorphisms among the accessions studied, indicating the robust nature of microsatellites in revealing polymorphism. Great relatedness within and among accessions. Only RM 225 produced two to three bands in the accessions with the rest producing a single band was observed; with most of them appearing as single alleles, a single band averaging between 79-200 bp. Cluster analysis via a dendrogram revealed that most of the accessions were closely related and clustered into two major groups designated A and B. Only one accession (ZM8321) appeared to be distinct and distantly related to all the other 29 accessions. Cluster B contained the rest of the 29 accessions which were subdivided into 8 sub-clusters. Of the 29, accessions ZM8295 from Samfya District and ZM8313 from Kaputa District showed 100% similarity meaning that they are in fact the same accession. The results of the molecular characterization may provide a useful foundation for genetic improvement of rice as it creates baseline information to the rice germplasm to broaden the genetic base of cultivated rice in some parts of Zambia.

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LIST OF ABBREVIATIONS AND ACRONYMS

DNA: Deoxyribonucleic acid

PCR: Polymerase chain reaction

SSR: Simple sequence repeat

RPM: Revolutions per minute

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CHAPTER ONE

INTRODUCTION

1.0 Overview

This chapter establishes the background to the study and a statement of the problem. The aim of the study is stated as well as the research objectives and the questions. The significance of the study is also elaborated as well the hypothesis. The theoretical framework of the study is clearly stated so as to guide the researcher during the research process

1.1 Background

Rice is a cereal of the grass species *Oryza sativa* or *Oryza glaberrima*. It is a monocot, and is normally grown as an annual plant; in tropical areas, in some cases it has been known to survive as a perennial and can produce a ratoon crop for up to 30 years (IRRI, 2009). According to the international research institute (IRRI) report, (2009), rice plant can grow to about 1–1.8 m tall, and occasionally taller, depending on the accession and fertility of the soil. There are many accessions of rice, and culinary preferences tend to vary regionally. In some areas like the far east of Spain, there is a preference for softer and stickier accessions while other regions will prefer the aromatic accessions (Singh *et al*., 2003).

Rice is a grain cereal and is among the most widely consumed staple foods for a large part of the world's human population, especially in Asia. As an agricultural commodity, the crop is the third-highest in terms of worldwide production after maize and sugarcane, (FAOSTAT-2012). In terms of human consumption and caloric intake, it is the most important grain crop as it provides for about one fifth or more of the calories consumed world-wide by humans (Smith, 1998).

The cultivation of rice is well-suited to countries and regions with low cost of labor and high rainfall, as it requires intensive labor to cultivate, as well as a lot of water. However, rice can be grown practically anywhere, even on a steep hill or mountain areas with the use of water-controlling terrace systems.

Although its parent species are native to Asia and to certain parts of Africa, centuries of trade and exportation have made it quite common in a number of cultures world-wide. In Zambia, the crop is generally not considered a staple food crop for most people, but is mainly used as a supplementary food and is one of the major contributors to food security. According to the report on the state of plant genetic resources for food and agriculture (second report, 2008), rice in Zambia is grown in the Zambezi floods plains of Western Province, Chambeshi flats in the Northern Province, the Luangwa flood plains in Eastern Province, the lake basins of Mweru and Bangweulu in Luapula and sporadically on the Copperbelt in the dambo sites and along Kafue River and some seasonal streams. New germplasm introductions especially for upland rice have recently been planted. Some local accessions still used mostly by some small scale farmers in the rice producing areas are largely still based on the indica types (indica and aus) which are thought to have initially come from neighboring countries, particularly Angola and Tanzania. According to the Zambia national rice development strategy report (version 1, 2011), Zambia's main rice accessions are Supa, Malawi Faya, Kilombelo, Blue bonnet, Angola crystal, Sumba wanga, and Xiang Zhou 5. All these, however, usually mixed, leading to poor processing and making the resultant quality to be usually of a poor grade mixture. Consequently they do not fare favorably among consumers and institutional buyers, compared with the imported varieties. Consumer surveys (SNV 2009) have revealed that Zambians generally have a preference for the aromatic rice like 'Mongu', 'Nakonde' and 'Chama' hence the premium prices paid for local rice accessions. Most consumers tend to be quite price-sensitive and tend to show some brand loyalty. Apart from price, consumers mostly buy rice with regard to quality which includes color, size of the grains and free from grit, and other impurities. It has also been observed that most farmers recycle inferior seed that is usually a mixture of improved rice accessions, which would have been sourced from neighboring countries. This affects the final quality in that mixed grain may not mill well. Presently, there is no coordinated system for producing, multiplying and distributing rice seed as there is literally no regulation on rice germplasm quality which leads to the gradual dilution of pure accessions of rice (Zambia National Rice Development Strategy Report-Version 1, 2011).

Crops like maize have been characterized and varieties are known at genetic level but little or limited efforts aimed at variety selection take place in crops such as vegetables, rice, groundnuts, pigeon pea, cowpea, and tree and plantation crops (Zambia National Rice Development Strategy Report -Version 1, 2011).

For years, rice cultivars grown in Zambia have been known by their local/common names, mainly district or localities in which they are grown or place of origin e.g. Mongu, Nakonde, Chama etc. Few or no records of the genetic nature and background of such accessions are available. Molecular characterization could reveal their phylogeny and this information would be useful in utilizing the rice germplasm in genetic improvement of the existing rice accessions. Crop diversity is the biological base of all agriculture.

'Characterization' refers to the description of a character or quality of an individual or entity (Merriam-Webster, 1991). The word 'characterize' is synonymous to the word 'distinguish', that is, to mark as separate or different, or to separate into kinds, classes or categories. Therefore, characterization of genetic resources refers to the process of identifying or differentiating accessions. This identification may in broad terms refer to any difference in the appearance or make-up of an accession. In the agreed terminology of gene banks and germplasm management, the term 'characterization' refers to the description of characters that are usually highly heritable, easily seen by the eye and equally expressed in all environments (IPGRI/CIP, 2003). In genetic terms, characterization refers to the detection of variation as a result of differences in either DNA sequences or specific genes or modifying factors. Therefore, genetic characterization refers to the description of attributes that follow a Mendelian inheritance or rather attributes that involve specific DNA sequences (IPGRI/CIP, 2003).

Because of the nature of genetic characterization, it clearly contributes to an enhanced power for detecting diversity (including genotypes and genes) that exceeds that of traditional methods. Likewise, genetic characterization with molecular technologies offers greater power of detection than do phenotypic methods (e.g. isozymes). This is because molecular methods reveal differences in genotypes, that is, in the ultimate level of variation embodied by the DNA sequences of an individual and uninfluenced by environment. In contrast, differences revealed by phenotypic approaches are at the level of gene expression (proteins) (IPGRI/CIP, 2003).

This research was conducted to characterize selected rice accessions in Zambia at genetic level in order to create a foundation for genetic improvement. It was undertaken to provide an opportunity to maximize the use of conserved, traditional wild types of rice and modern rice accessions. Some genes can be of potential benefit in rice production and quality improvement. With the use of

marker assisted selection, characteristics such as drought and tolerance to soil acidity, disease resistance, good storability, grain type, sweetness, low nitrogen tolerance, nutrient content, and earliness can be genetically improved. Some traits are or may be negatively correlated with yield e.g. earliness and disease resistance. Therefore, appropriateness of developed or improved accessions as demanded by the breeders is important if utilization of local plant genetic resources for food and agriculture has to be a reality. Farmers with access to improved accessions and technologies are capable of producing more rice with better quality that can move beyond their village markets.

1.2 Statement of the Problem

Most of the locally grown rice accessions/cultivars are currently known by their local/common names. Few or no records of the genetic nature, purity and background of some of these accessions are available. Some of these accessions may have originated from other states/regions (Pratheepha, 2009).

The main challenge is that the genetic background of Zambian rice accessions has never been systematically characterized. There appears to be little or no information on the genetic or agronomic traits of the locally grown rice crop in Zambia. Information on any varieties that have been released as well as characteristics such as their responses to biotic factors such as fungal and bacterial pathogens or abiotic factors such as water stress is not available. A systematic rice seed system or breeding program appears to be non-existent. This could be because rice is not regarded as a staple food for the people of Zambia, even though it is one of the crops adding to food security in the country. The absence of information or records on rice genetic diversity, agronomic traits and a national seed system is one of the main constraints to the development of rice as a commercial crop.

1.3 Significance of the study

This study is of scientific significance in that:

It was set to characterize the genetic diversity of selected rice accessions in Zambia at molecular level in order to create a foundation for genetic improvement of the crop. The study was also conducted to contribute to the maximization of the use of conserved traditional wild relatives of rice and modern rice accessions.

Secondly, the study was designed to provide baseline information for rice germplasm in Zambia and to broaden the genetic base of cultivated rice. This was aimed at assisting breeders in adding value to certain characteristics of selected accessions in the country.

The study is also of significance inn that the results obtained could be of use to local gene bank curators in determining how genetically diverse and homogeneous the materials in their collections are.

1.4 Study Objectives

1.4.1 Aim of the Study

The aim of the study was to generate information on the genetic diversity of selected rice accessions from various parts of Zambia.

1.4.2 Specific Objectives of the Study

The specific objective of the study was to assess the genetic differences among selected rice accessions grown in Zambia

1.5 Study Hypothesis

The study tested the hypothesis that there is no genetic diversity in the rice accessions grown in Zambia.

1.6 Research Questions

The following research questions were framed to serve as a guide to the study:

i. What are some of the main rice accessions grown in Zambia?
ii. What are the major rice growing regions in Zambia?
iii. How genetically distinct are some of the accessions grown in Zambia?

Summary

This chapter gave a clear insight into the topic under research. The problem under investigation was stated while the objectives and significance of the study were clearly stated. The next chapter will look at literature. Literature will include global and Zambian information with regard to the research topic.

CHAPTER TWO

LITERATURE REVIEW

2.0. Overview

Having outlined the background of the study, and having stated the problem under investigation with the objectives of the study and their importance in the different literature, this chapter reviews studies undertaken by different scholars and any other authorized literature in related topics. The review process is presented under the following subheadings; (1) Adaptation and climatic requirements (2) Importance of rice in global agriculture (3) Molecular characterization of rice (4)Molecular markers (5)Attributes of Molecular characterization (6)Rice Nutrition

2.1 Adaptation and climatic requirements

The rice plant is native to tropical and subtropical southern Asia and southeastern Africa (Crawford and Shen, 1998). Because of the nature of the areas where it is predominantly grown, rice is regarded as a grass 'autogame', meaning a crop that is more easily grown in the tropics (Coclains-2003). Rice is usually grows well in hot and moist climate with temperatures of 16°C-27°C and rainfall ranging from 100cm to 200cm (Bose, 2013). It grows well on alluvial soil or on the fertile river basin but may also be grown in mixed soils or clayey loam soil (Bose, 2013).

2.2 Importance of rice in global agriculture.

Rice as one of the most important cereals of the world, it provides 21% of global human per capita energy and 15% of per capita protein (Maclean *et al.,* 2002). It is an important grain with regard to human nutrition and caloric intake, as it provides for about one fifth or more of the calories consumed by humans world-wide (Smith, 1998).The Asian cultivated rice (*Oryza sativa L.*) is one of the most important crops and major food source for about half of the global human population. The 57th session of the United Nations General Assembly noted that rice is the staple food of more than half the world's population, affirming the need to heighten the awareness of the role of rice in alleviating poverty and malnutrition. It also reemphasized the need for the world to focus

attention on the role that rice can play in providing food security and eradicating poverty and declared the year 2004 as the International Year of Rice (www.fao.org/ag/irc).

According to Rod Wing, director of the Arizona Genomics Institute at the University of Arizona and AXA-endowed scientist at the International Rice Research Institute who led the rice genome sequencing project, rice is a staple food for over half of the world's population. He has also noted that the crop will provide a major solution to the challenge of feeding the world's projected 9 billion population. It has been predicted that the world's population will increase to more than 9 billion people by the year 2050, and concerns have existed since the 1960s that food will become a luxury for the rich (IRRI.org-accessed in april-2015). The question has been how to scientifically optimize plants so that people in each region could also control their own food. With the complete sequencing of the rice genome, scientists and agriculturists can search for ways to cross Asian and African species to develop improved or new accessions of rice with the high-yield traits of Asian rice and the hardiness of African rice.

Another great attribute of rice as a crop is that it is very affordable throughout the world despite its great importance to much of the world's diet. Although the parent species of rice are native to Asia and to certain parts of Africa, centuries of trade and exportation have made it quite common in a number of cultures world-wide. In Zambia, the crop is generally not considered a staple food crop for most people. It is mainly used as a supplementary food and is one of the major contributors to food security in the country.
New germplasm introductions especially for upland rice have recently been made in the past years. (Zambia National Rice Development Strategy Report-Version 1, 2011).

Rice research that will develop new technologies for all farmers will play a key role in meeting the need for boosting food security and contributing to global efforts directed at poverty alleviation.

2.3 Molecular characterization of rice

Archaeological and Phylogeographical evidence suggests that rice was domesticated over thousands of years ago (about 10000 years ago) from its wild ancestor *O. rufipogon* in the region south of the Himalayan mountain range, likely in the present day Eastern and North East India, extending Eastward to Nepal, Myanmar and Thailand to Southern China (Khush 1997; Londo *et*

al., 2006). A recent study suggests that one of the two sub-species of Asian rice, *O. sativa spp* was domesticated in Southeast and South Asia while the other sub-species, *O. sativa ssp* japonica was domesticated in Southern China (Huang *et al*., 2012).

During domestication, accessions with desirable traits were selected, leaving most of the genetic diversity behind in the progenitors (Doebley *et al*., 2006). Zhu *et al*. (2007) estimated that the cultivated rice contains only close to 25% of the genetic diversity found in its wild progenitors depicting severe genetic erosion during domestication. Studies show that a considerable level of genetic diversity was lost during the agronomic improvement of commonly cultivated rice accessions and that indigenous crop accessions traditionally cultivated and maintained by farmers contain high level of genetic diversity and can serve as potential genetic resources for improving yield, resistance to pests and pathogens, and agronomic performance (Brush 1995; Hoisington *et al*. 1999; Mandel *et al*. 2011).

The rice genome has been well mapped and characterized. It is has an estimated genome size of 400-430Mb and appears to be the smallest of the major cereal crops genome (Arumuganathan amd Earle, 1991). According to Gale and Devos (1998), grass genomes, including those of rice, wheat, barley and sorghum, share a large degree of synteny, making rice an excellent model cereal.

2.4 Molecular markers

The use of molecular markers in routine research, trait association studies and plant breeding programmes is a highly developed field which has replaced the reliance on physical or morphological markers due to the limitations of the latter. Morphological markers including height, flower and seed colour are limited in their dependence on phenotypes whose manifestations only appear at maturity of crop plants. For instance, a crop plant which requires a minimum of 90 days to produce seed of a given color will mean that a researcher has to wait for that amount of time to analyze the results in a breeding programme. Additionally morphological markers tend to be affected by the genotype-environment interaction. For instance the height of a plant depends not only on its genotype but also on nutrients and water availability. If a plant is grown under water and nutritional stress, its development to full height may not be achievable thereby confounding the interpretation of results of a breeding programme. For these and other limitations, the use of physical markers has largely been replaced by molecular markers. Molecular markers are routinely used in many programmes including to determine genetic variability and assess germplasm purity

and homogeneity, verification of genotypes of interest and even estimation of relatedness between populations via estimation of genetic distances between breeding populations.

Molecular markers are DNA sequences. They tend to be neutral, can be analyzed at any developmental stage of a plant and some of them tend to be co-dominant. The analysis of molecular markers is done using DNA hybridization or amplification technologies. Examples of molecular markers analyzed by DNA hybridization are the classical Restriction Fragment Length Polymorphisms (RFLPs). These are analyzed by digesting genomic DNA with restriction enzymes in single digest reactions or in reaction combinations and separating the DNA fragments on agarose gel and transferring the DNA onto a membrane support by capillary action under strong salt conditions. The diversity of DNA fragment sizes is determined by locations of restriction sites in the genomic DNA and is analyzed using a radioactively- or non-radioactively-labeled nucleic acid probe. RFLPs are no longer routinely used in research due to need for large amounts of DNA as well as their tediousness.

Many of the molecular markers routinely in diversity analysis, marker-assisted selection and identification of candidate genes are based on nucleic acid amplification technologies. These include the classical Random Amplified Polymorphic DNAs (RAPDs), Simple Sequence Repeats, Cleaved Amplified Polymorphisms (CAPs) and Amplified Fragment Length Polymorphisms (AFLPs). Some of these depend on DNA amplification while others such as CAPs and AFLPs combine the use of restriction enzymes and DNA amplification and yet still others such as single nucleotide polymorphisms depend on nucleic acid sequencing. Molecular Marker based Genetic DNA markers can be defined as a DNA sequence or gene with a known position on a chromosome that can be used to identify individuals, species or inheritable traits and are predominantly used in molecular characterization and diversity studies due to their abundance and repeatability ((Duwick, 1984; McCouch *et al.*, 1997). Among different PCR based markers, the microsatellite markers based on simple sequence repeats (SSRs) are preferred over other molecular markers because of their merits in application such as high reproducibility, rapid analysis, low cost, easy scoring patterns and greater allelic diversity (Chen *et al*., 1997). These markers are distributed relatively throughout the genome and detectable due to high allelic diversity in cultivated accessions and distantly related species (McCouch *et al*., 1997). Singh *et al*. (2010) used a set of validated SSR molecular markers in rice parental lines and demonstrated their suitability for qualitative tract locus (QTL) mapping and finger printing studies in the crop. Therefore, genetic diversity analysis of the Zambian rice will help measure the extent of genotypic differences,

genetic relationships and assist in broadening the germplasm base of future aromatic rice breeding programs.

2.5 Attributes of Molecular characterization

Characterizing accessions is an activity that is normally regarded as the responsibility of the gene bank curator. It involves determining the expression of highly heritable characters, ranging from morphological features to seed proteins and possibly including molecular markers. Such traits enable easy and quick discrimination among phenotypes and allow simple grouping of the accessions, as well as a "check on the trueness-to-type of homogeneous samples, frequently according to criteria used by breeders and other germplasm users" (IPGRI Handbooks for Genebanks No. 6).

Information on the genetic make-up of accessions helps in decision making for conservation activities, and these range from collecting and managing through identifying genes to adding value to genetic resources. Molecular characterization by itself or in conjunction with other information (phenotypic traits or geo-referenced information) provides reliable information for assessing, among other factors, the amount of genetic diversity (Perera *et al*, 2000), the structure of diversity in samples or populations, rates of genetic divergence among populations and the distribution of diversity in populations found in different locations (Shim *et. al*., 2000).

Molecular characterization also helps in determining the breeding behavior of species, individual reproductive success and the existence of gene flow, that is, the movement of alleles within and between populations of the same or related species, and its consequences (Papa and Gepts, 2003). Molecular information improves or even allows the elucidation of phylogeny, and provides the basic knowledge for understanding taxonomy, domestication and evolution (Nwakanma, *et. al*., 2003). As a result, information from molecular markers or DNA sequences offers a good basis for better conservation approaches. Molecular information has also been used to determine the need for decreasing the size of germplasm collections.

A special and increasingly important role of genetic characterization is that of identifying useful genes in germplasm, that is, of maximizing conservation efforts (IPGRI Handbooks for Genebanks No. 6). Because the major justification for the existence of germplasm collections is use of the conserved accessions, it is important to identify those valuable genes that can help develop accessions that will be able to meet the challenges of current and future agriculture.

Assessment of genetic diversity and molecular characterization among rice accessions is very important for germplasm management, varietal identification and DNA fingerprinting. Plant genetic resource management consists of two phases: (i) germplasm conservation including acquisition of germplasm *in-situ* or *ex-situ*, preserving under controlled conditions, monitoring its viability, maintaining passport and other information and characterization of heritable morphological and molecular traits of germplasm; (ii) germplasm management including evaluation, utilization, genetic enhancement (Duvick 1990; Bretting and Widrlechner 1995).

Genetic diversity study can be regarded as a prerequisite step for selection of diverse parents for developing improved rice cultivars. Studies have revealed that the scope of crop improvement depends on the conserved use of genetic variability and diversity in plant breeding programs as well as the use of new biotechnological tools. According to Xu *et al*., (2000), molecular characterization can reveal the maximum genetic variation or genetic relatedness found in a population. Chakravarthi and Naravaneni (2006) reported on the usefulness of preservation and conservation of genetic resources since genetic diversity provides information to monitor germplasm and prediction of potential genetic gains. Information regarding genetic variability at molecular level is very important because it could be used to help identify and develop genetically unique germplasm that complement existing cultivars (Ni *et al*., 2002; Chakravarthi and Naravaneni, 2006). DNA based molecular markers have proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among the species of rice (Ragunathanchari *et al*., 1999, 2000; Shivapriya and Hittalmani, 2006).

With the increasing demand for food as a result of increasing populations as well as climatic changes, several biotechnological approaches have been adopted to increase quality and quantity of rice as well as its resistance to pests, diseases and environmental stresses; and genetic diversity studies provides the baseline information.

2.6 Rice Nutrition

Rice is mainly composed of carbohydrates, with little amounts of protein and literally has no fat, (Rice 101: Nutrition Facts and Health Effects). Different accessions of rice have different textures, tastes and nutritional value. Brown and wild rice contain the whole grain, this means both the germ and the bran of the grain are preserved. Many vitamins and minerals **(**Manganese, Selenium, Thiamin, Niacin. Magnesium, Copper) are concentrated in the bran and germ, which are

components of brown rice, but not white rice. Consequently, brown and wild rice are regarded as being healthier because they contain more nutrients and fiber. (Rice 101: Nutrition Facts and Health Effects). In contrast, white accessions of rice have the germ and bran of the grain polished away, which literally diminishes their nutritional profile and increases their glycemic load, or impact on blood sugar levels. White rice is about 90 percent carbohydrate, 8 percent protein and 2 percent fat, according to the book "Contemporary Nutrition: Functional Approach." White rice is a good source of magnesium, phosphorus, manganese, selenium, iron, folic acid, thiamine and niacin. It is low in fiber and its fat content is primarily omega-6 fatty acids, which are considered pro-inflammatory (Rice 101: Nutrition Facts and Health Effects; Coclain-2003).

Summary

This chapter presented the literature review. The major sections included: adaptation and climatic requirements, importance of rice in global agriculture, molecular characterization of rice, molecular markers, attributes of Molecular characterization and rice nutrition. The literature review was done in line with the themes derived from the objectives of the study. The knowledge gap was brought out from the reviewed literature. The observations from the reviewed literature were that a lot of baseline information has been generated for the rice plant in the past decades. Most of the studies were done on the varieties grown in the Asia-Pacific region. Nonetheless, little genetic information exists on the rice accessions grown in Zambia.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Overview

The previous chapter gave an insight of what different researchers found in their studies, their conclusions and their recommendations. In this chapter, the different methods, approaches and strategies used by the researcher to execute the study are explained. The methodology of the study is presented under the following headings: (1) experimental location (2) plant materials (3) genomic DNA extraction (4) molecular analysis by SSR markers (5) polymerase chain reaction (6) Gel electrophoresis (7) Information-analysis

3.1 Experimental location

The experiments were conducted at the Zambia Agricultural Research Institute (ZARI), Mount Makulu research station in Chilanga, Lusaka Province that lies on latitude -15.547745° and longitude 28.249392.

3.2 Plant materials and Chemicals

3.2.1 Plant materials

The study involved 30 rice accessions collected from the major rice growing regions in Zambia and curated at the National Plant Genetic Resource Centre (National Gene Bank) at ZARI in Chilanga. Table 3.1 shows the details and passport information of the accessions that were selected. 40 rice seeds of each accession were planted in a labeled plastic container filled with non-homogeneous sterile soil (5cm deep) collected from the University of Zambia agricultural fields and filled with water to a point where the water was 2cm above the soil. Seeds were let to grow in a wet and warm environment in the greenhouse under natural greenhouse light conditions and watered every day for up to 10 days with day temperatures ranging from 20°C-28°C. No inorganic fertilizers were used in the growing of the plants. Leaf samples of approximately 20mg were harvested from 10 day old seedlings and stored on ice until DNA was extracted. 10 plants were collected from each accession, giving a total of 300 plants for the 30 accessions used in the study.

Table 3.1: Passport information for each of the rice accessions

Serial no.	Accession no.	Local Name	Village	Latitude (south)	Longitude (east)	District	Province
1.	ZM 8316	Malawi Faya	Duncan	14°27.009'	30°49.228'	Nyimba	Eastern
2.	ZM 8317	Mpunga wa Makolo	Whyted	14°27.009'	30°49.228'	Nyimba	Eastern
3.	ZM 8318	Ndelema	Kapalakonje	11°10.800'	33°03.212'	Chama	Muchinga
4.	ZM 8321	Ndelema	Kapalakonje	11°10.800'	33°03.212'	Chama	Muchinga
5.	ZM 8328	Bakili	Mulopwe	11°11.639'	32°50.973'	Chama	Muchinga
6.	ZM 8338	Senga	Moonda	11°21.196'	32°59.531'	Chama	Muchinga
7.	ZM 8340	Sosha	Moonda	11°21.196'	32°59.531'	Chama	Muchinga
8.	ZM 8343	Chandege and Faya	Lwambu	11°21.847'	32°52.189'	Chama	Muchinga
9.	ZM 8346	Chandege -white and brown	Kazembe	11°06.773'	33°07.610'	Chama	Muchinga
10.	ZM 8361	Meya	Ng'anjo-Chiwato	11°02.34'	32°59.505'	Chama	Muchinga

11.	ZM 8267	Supa	Musonda	10°10.000’	31°59.000’	Mungwi	Northern
12.	ZM 8271	Chembe	Milishi	11°58.000’	28°44.000’	Chembe	Luapula
13.	ZM 8292	Chembe	Mutwale	11°53.000’	28°50.000’	Chembe	Luapula
14.	ZM 8295	Supa	Fibalala	11°39.000’	29°29.000’	Samfya	Luapula
15.	ZM 8298	Lusakasaka	Mwansabombwe FTC	9°46.000’	28°49.000’	Mwansabombwe	Luapula
16.	ZM 8299	Kalwena	Mwansabombwe FTC	9°46.000’	28°49.000’	Mwansabombwe	Luapula
17.	ZM 8303	Supa	Mwabu camp	8°33.000’	29°08.000’	Chienge	Luapula
18.	ZM 8308	Supa	Mwabu camp	8°30.000’	29°08.000’	Chienge	Luapula
19.	ZM 8311	Meli	Lambwe Chomba	8°24.000’	29°35.000’	Chienge	Luapula
20.	ZM 8313	Kaputa	kaputa camp	8°27.000’	29°39.000’	Kaputa	Northern
21.	MLK001	Supa/Zhazo	Liwena	13°35.289'	22°46.908'	Zambezi	North-Western
22.	MLK003	Supa	Liwena	13°34.370'	22°46.000'	Zambezi	North-Western
23.	MLK020	Kilombelo	Lukolwe Mission	13°10.179'	22°38.913'	Chavuma	North-Western
24.	MLK022	Malawi Faya	Lukolwe Mission	13°10.179'	22°38.913'	Chavuma	North-Western
25.	MLL026	Kajaketi	Samboko-Katongo	15°16.943'	23°08.292'	Mongu	Western

26.	MLK031	Aongola 7	Matondomange	15°34.870'	23°40.303'	Mongu	Western
27.	MLK055	Kajaketi	Kaeya Camp	16°06.180'	23°18.033'	Senanga	Western
28.	MLK059	Burma	Lyaangati Camp	15°48.020'	23°18.587'	Senanga	Western
29.	MLK062	Black rice	Lyaangati Camp	15°46.761'	23°18.085'	Senanga	Western
30.	MLK071	Malawi fire	Senanga Boma	16°06.580'	23°17.523'	Senanga	Western

3.3.1 Genomic DNA Extraction

All chemicals used for DNA extraction and nucleic acid amplification were procured from Himedia laboratories, Mumbai-India. Genomic DNA was isolated from young leaves of 10 day old plants following the mini modified DNA extraction method (Zheng *et al.*, 1995). Healthy rice leaf tissue samples (20mg) were collected and transferred to labeled sample plastic bags on ice. Tissue was cut and ground into a thick slurry using a polished glass motor and pestle and 500µl of DNA extraction buffer (100mM tris-HCl pH 8.0, 100mM EDTA, 100mM NaCl and 10% SDS) was added. Additional 400µl of the extraction buffer was added and the ground tissue was thoroughly mixed by pipetting. 500µl of the slurry was transferred to a 1.5ml eppendorf tube, and 500µl of Phenol+chloroform+isoamyl (25:24:1) was added. The contents were mixed well by inverting, and the tubes were spun for 5 minutes in micro-centrifuge at 5000 revolutions per minute (RPM). Aqueous supernatants were then transferred to new pre-labeled 1.5ml eppendorf tubes and the DNA was precipitated using 500µl ice cold isopropanol and 50µl 3M potassium acetate. After letting it to stand in the freezer (-30°C), the supernatant was spun for 5 minutes at 5000RPM and discarded to leave a pellet. 500µl 70% ethanol was then added to wash the pellet, spun for 5 minutes at 5000RPM and the supernatant was discarded to leave a pellet which was let to air-dry for 1hour. The DNA pellet was resuspended in 50µl of TE buffer (10mM tris-HCl pH 8.0, 1mM EDTA pH 8.0) and treated with 5µl of 10mg/ml RNaseA at 37°C for one hour. The quality of the DNA was assessed on 1% agarose gel while the DNA concentrations were measured using Nano drop spectrophotometry. DNAs was stored at –20°C until used.

3.3.2 Molecular analysis by SSR markers

Variation in the rice collections was assayed using simple sequence repeat molecular markers (SSR). Polymerase Chain Reaction (PCR) amplification of SSR markers was carried out using the 10 primer pairs listed in Table 3.2 randomly selected from 7 of the 12 chromosomes of rice. Each of the lyophilized primers was reconstituted in nuclease free water according to the manufacturer's recommendations to stock solutions of 100mM and were used to prepare working solution of 50ng/µl for each primer.

PCR reaction mixes were prepared in aliquots for 100 reactions by taking 100X the volume of a single reaction plus an additional single reaction to make up for pipetting errors.

Table 3.2: Rice SSR markers (primer pairs)

Locus name	Chromosome No.	Forward Primer	Reverse Primer	Size (bp)
RM-5	1	TGC ACC TTC TAG CTG CTC GA	GGG TTC TAG CCT AGC	113
RM225	6	TGC CCA TAT GGT CTG GAT G	CGGA AGG ACT AGG TGA AAG	140
RM-7	3	TTC GCC ATG AGG TCT CTC G	TT GTT GCT TTA CTA CCC TCC	180
RM-168	3	TGC TGC TTG CCT GCT TCC TTT	CGGCA CCT AAC TAA GCA AAG	116
RM-13	5	TCC AAC ATG GCA AGA GAG AG	GAC CTT AGC TTA CGG TGG	141
RM-452	2	CTGATCGAGAGCGTTAAGGG	GTCTT TGC ACC AAA CTA GGG	105
RM-463	12	TTCCCCTCCTTTTATGGTGC	GCGTC ACT GAC TCC TCT TGT	192
RM-413	5	GGCGATTCTTGGATGAAGAG	CTTCT GTT CTA ACC ACC CCT	79
RM-211	2	CCGATCTCATCAACCAACTG	GG AAA CTC TAG GAG CAC TTC	161
RM-11	7	TCTCCTCTTCCCCCGATC	GAT TCG GAG CGG GCG ATA	140

3.3.3 Polymerase chain reaction

A total of 3000 genomic DNA samples were amplified by the polymerase chain reaction. Template DNA extracted from 10 independent plants from each of the 30 accessions was amplified using the selected SSR primers. For accession ZM8338 for instance, ten samples of template DNA were amplified with all of the selected markers giving a total of 300 samples for that accession alone.

The PCR reaction mix was prepared by mix 2µl of template DNA, 2 µl 10x PCR buffer with $MgCl_2$ (25mM), 1 µl dNTPmix (10mM), 1µl each of forward and reverse primers (50ng), 0.2µl of *Taq* DNA polymerase and molecular grade nuclease free water was then added to make a final volume of 21µl. The PCR reaction was preceded by an initial template denaturation at 94ºC for 5 minutes followed by 35 cycles of 1 minute denaturation at 94ºC, 1 minute annealing at 55ºC, 1 minute extension at 72ºC and a final extension at 72ºC for 5 minutes.

3.3.4 Gel electrophoresis

The PCR amplification products were separated on 1% agarose gel slab at 100 volts for 1 hour in Tris Acetate EDTA (TAE) buffer. Ten PCR products for each accession were loaded in consecutive orders and identified by sequential numbers on the gel (see figure 4.1a and 4.1b). DNA fragments were revealed using the ethidium bromide staining procedure (Matin *et al*. 2012) while DNA fragment sizes were determined using 100 base pairs molecular weight markers. The gels were stained for 30-35 minutes and documented using BIO RAD gel documentation unit.

3.3.5 Analyses of fragment sizes and band intensity

The size of most intensely amplified fragments was determined by comparing the migration distance of amplified fragments relative to the 100 base pair fragment molecular weight marker used as a DNA ladder. Polymorphic information content (PIC) values were calculated for each SSR locus based on (Anderson *et al*., 1991). Polymorphic information content (PIC) provides an estimate of the discriminatory power of a locus or loci, by taking into account not only the number of alleles that are expressed, but also relative frequencies of those alleles, was estimated using the formula suggested by Botstein *et a*l., (1980).

The amplified bands were scored for each SSR primer pairs based on the presence or absence of bands, generating an information matrix of 1, 2 and 0 for each marker system. The matrices were

then analyzed using the Genstat statistical package 18th edition. Dendrograms displaying relationships among the 30 rice accessions were constructed using the Genstat statistical package.

Summary

This chapter looked at the methodology on how the research was carried out, the materials and how various techniques were used in the study. The materials and methods used in the study were explained, as well as the how the results were analyzed. This chapter established the methods that were used to successfully collect and analyze data. In the following chapter, the results from the study will be laid out.

CHAPTER FOUR

RESULTS

4.0 Overview

This chapter presents the findings on the characterization of selected Zambian rice accessions (*Oryza sativa*) using simple sequence repeat markers to determine the genetic diversity among the selected rice accessions. The chapter presents the sets of results from the determination of the quality and concentration of the DNA extracted to the polymerase chain reactions as well as gel electrophoresis. The information derived from the gel documentation unit after running the gel electrophoresis and some samples of the pictures are also presented in this chapter.

4.1 DNA quality concentrations

The quality of the extracted genomic DNA was determined by agarose gel electrophoresis while the concentrations of the DNA samples were determined by Nano-drop spectrophotometry. The concentrations ranged from 341.5ng/µl to 1089.69ng/µl. Purity information ratio of O.D. (A260/A280) for genomic DNA used in the extraction protocol optimization are shown in Table 4.1.

Table 4.1: Nano drop spectrophotometric concentration analysis of the extracted genomic rice DNA

Sample ID	**Nucleic Acid Conc.**	**Unit**	**A260**	**A280**	**260/280**	**260/230**	**Sample Type**	**Factor**
RM1	341.5	ng/µl	6.831	3.305	2.07	2.23	DNA	50
RM2	367.7	ng/µl	7.354	3.397	2.16	1.34	DNA	50
RM3	417.2	ng/µl	8.345	3.903	2.14	1.4	DNA	50
M1	1089.6	ng/µl	21.793	10.383	2.1	1.83	DNA	50
M2	485.2	ng/µl	9.703	4.711	2.06	1.57	DNA	50
M3	645.1	ng/µl	12.901	6.587	1.96	1.66	DNA	50

4.2 Marker analysis by spreadsheets

The presence or absence of amplification bands were tabulated in spreadsheets (see appendix IV). In the Table, 1 represented the presence of a band, 2 represented the presence of two bands and 0 represented the absence of a band. These where used to draw a dendrogram showing the level of relatedness or distinctness among the studied accessions.

4.3 PCR fragment analysis reveals distinct product band patterns

A total of 10 SSR markers (listed in table 3.1) were evaluated by gel electrophoresis for their polymorphism across 30 accessions of rice to determine the genetic diversity. The markers were found to be polymorphic among the rice accessions and generated reproducible and informative allelic profiles. Fragment profile as a measure of polymorphism was observed in DNA samples amplified using RM5 and RM 13 for instance. For RM5 a DNA fragment of 110 base pairs was observed in all accessions with examples of ZM84338, ZM8316, ZM8328 and ZM8321 shown in figure 4.1a. In the case of ZM8338 DNA extracted from seven independent plants amplified using RM5 showed variation with reference to PCR products. Three of these plants (36, 37 and 40) had amplification products whereas four (34, 35, 38 and 39) showed no amplification products. In the case of ZM8316 DNA from 10 independent plant samples was amplified to produce the expected fragment of 110 base pairs. In case of ZM8328, the RM5 marker was able to amplify all the ten samples except for two (51 and 57), Figure 4.1a.

DNA from 10 independent plants of accessions ZM8338, ZM8316, ZM8328 and ZM8321 were also amplified as the case was for the rest of the samples in the study. Figure 4.1b shows that there was amplification of template DNA from five of the samples (33, 34, 37, 38 and 39) while no amplification products were observed in three samples (35, 36 and 40). Molecular marker RM225 showed diversity in terms of DNA amplification profiles in fragment numbers and sizes as shown in figure 4.2. For instance nine DNA samples from ZM8338 amplified with RM225 showed amplification of three samples (43, 47 and 49) giving a fragment size of 140 base pair while the rest (42, 44, 45, 46, 48 and 50) showed no amplification products. In the case of ZM8316 only one sample (54) showed an amplification product with two fragments of 500 bp and 140 base pairs respectively. Similar results were obtained in the use of RM225 on 10 DNA samples of ZM8328 with the exception of sample 69, figure 4.2.

The level of polymorphism among the rice accessions was evaluated by calculating the number of alleles and PIC values for each of the 10SSR loci evaluated. A total of 300 DNA samples were analyzed, with 10 replicates from each accession across the 10 SSR markers. Several alleles were detected at the loci of all the microsatellite markers across the thirty rice accessions.

Among the 10 polymorphic markers, only RM 225 produced two to three alleles each, and the rest produced only one allele each. An accession was assigned a null allele for a microsatellite locus whenever an amplification product could not be detected for a particular genotype-marker combination. The amplicon size of all 30 genotypes for each marker alleles ranged between 79-200 bp.

In some cases high relatedness was observed in form of similar DNA size fragments especially in RM5 and RM13 which had approximately 110bp and 150bp respectively as shown in figure 4.1a and 4.1b. In all the reactions, an 110bp band was obtained except in lane 38 and 58 where no band was observed. DNA band intensity also differed with some reactions showing higher band intensity than others as the case was for lane 39 and 46 as shown in figure 4.1a.

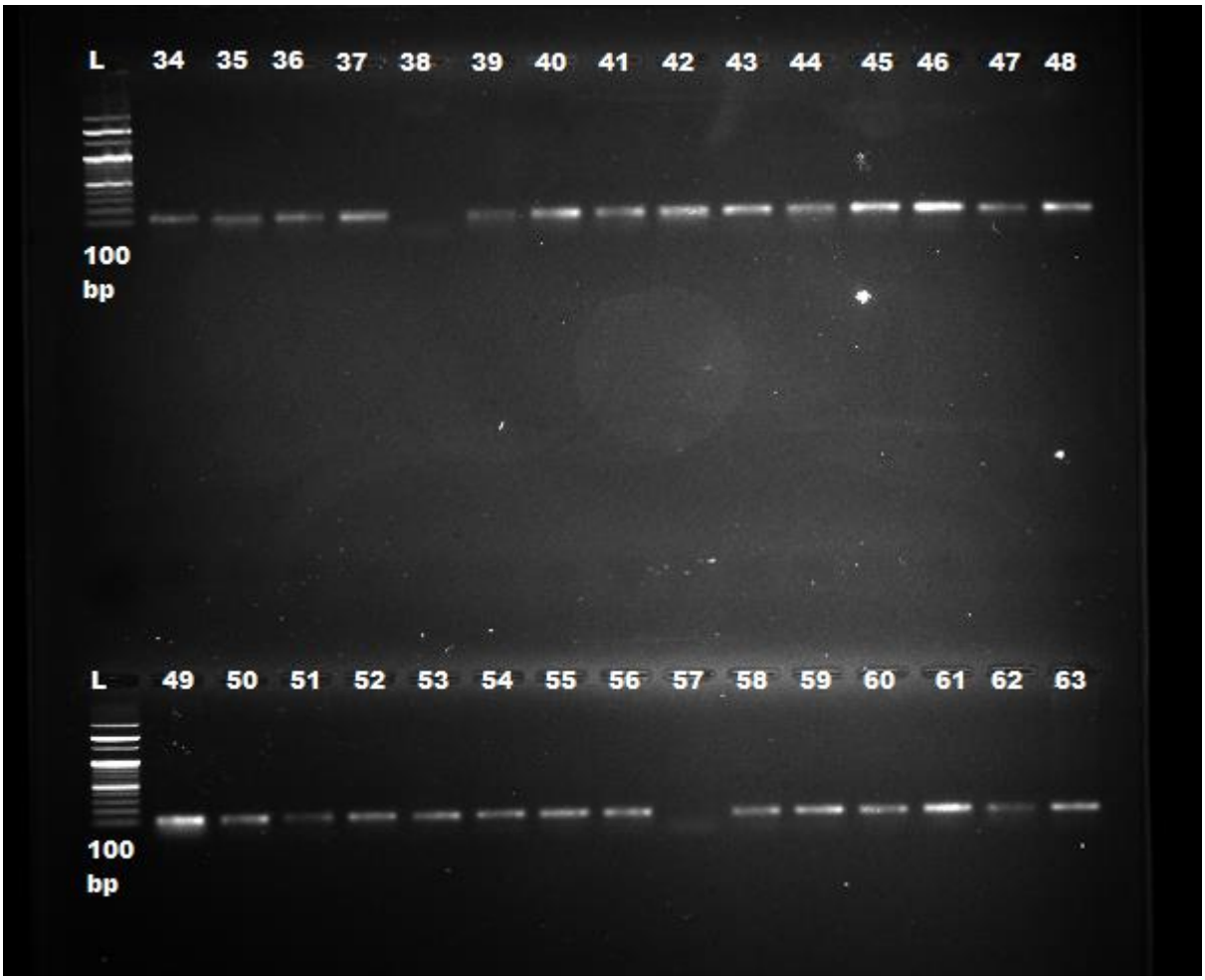

Figure 4.1a. DNA profile of four accessions with SSR marker RM 5 Legend: 34-40=ZM8338; 41-50=ZM8316; 51-60=ZM8328; 61-63=ZM8321 and L= 100bp Ladder marker (each accession had 10 replicates).

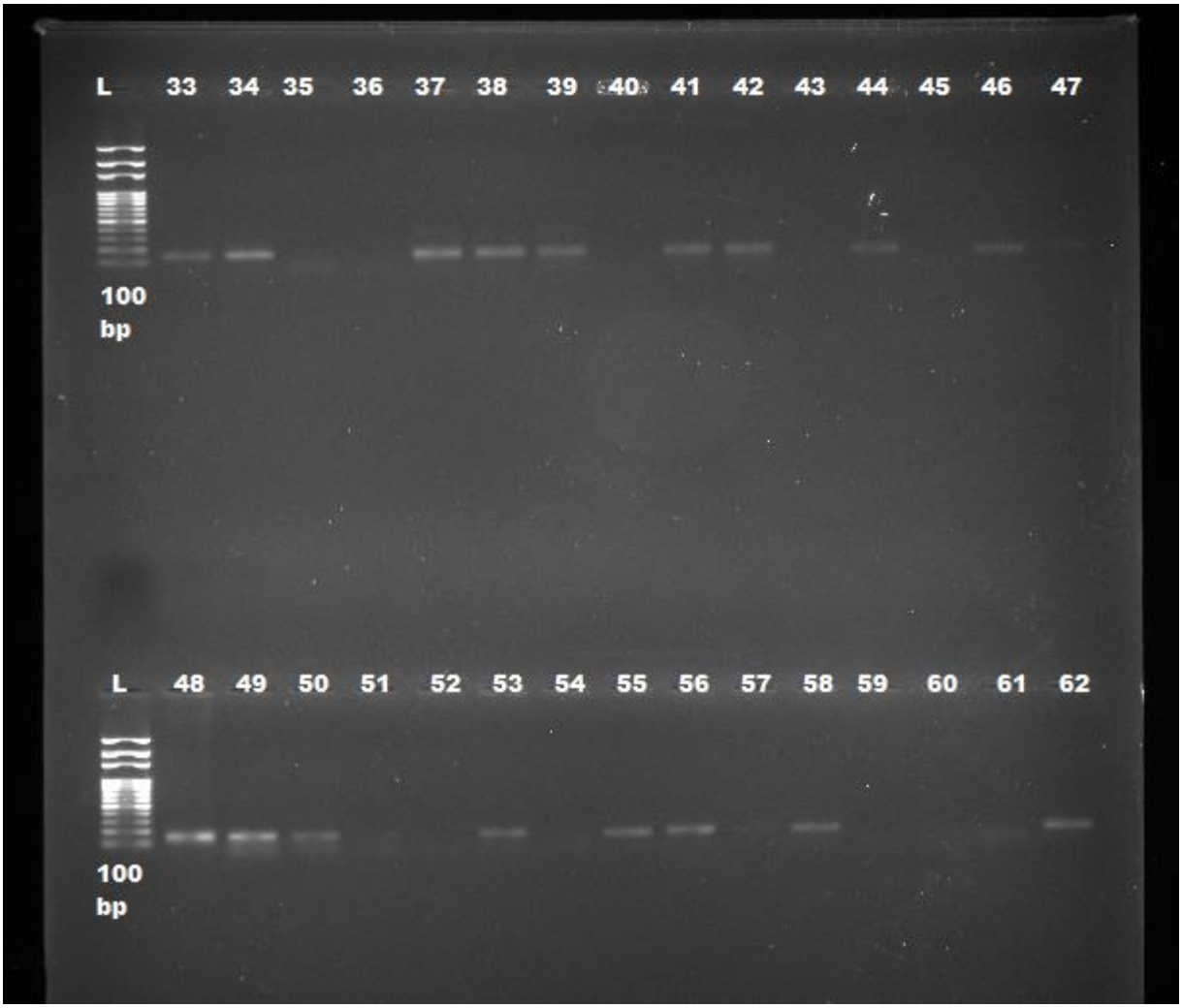

Figure 4.1b. DNA profile of four accessions with SSR marker RM 13.Legend: 33-40=ZM8338; 41-50=ZM8316; 51-60=ZM8328; 61-62=ZM8321 and L= 100bp Ladder marker (each accession had 10 replicates).

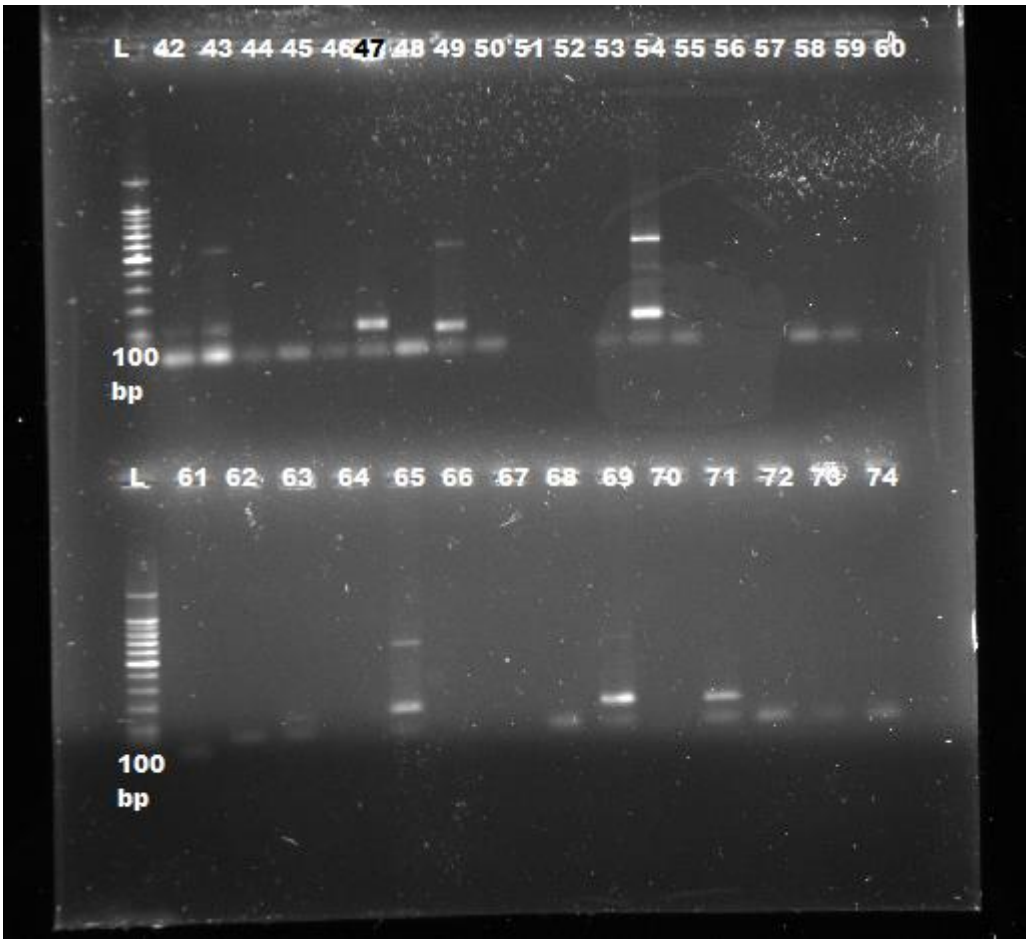

Figure 4.2. DNA profile of four accessions with SSR marker RM 225. Legend: 42-50=ZM8338; 51-60=ZM8316; 61-70=ZM8328; 71-74=ZM8321 and L= 100bp Ladder marker (each accession had 10 replicates).

4.4 Clustering of rice accessions

Cluster analysis was done to group the accessions by constructing a dendrogram illustrated in Figure 4.3 based on allelic information gathered from the genotype-marker interaction. The Genetic Similarity (GS) index ranged from a minimum of 80% to a maximum of 100%. The Genstat-dendrogram clustered the 30 genotypes into two major clusters (A and B) (Figure 4.3). Cluster A comprised of only one accession, ZM8321 commonly called Ndelema from Chama district in Muchinga province. Cluster B consisted of the 29 accessions which were subdivided into 10 sub-clusters that showed more genetic relatedness among the accessions with ZM8295

(Supa-Samfya district; Luapula Province) and ZM8313 (Kaputa-kaputa district, Northern Province) demonstratinng 100% relatedness.

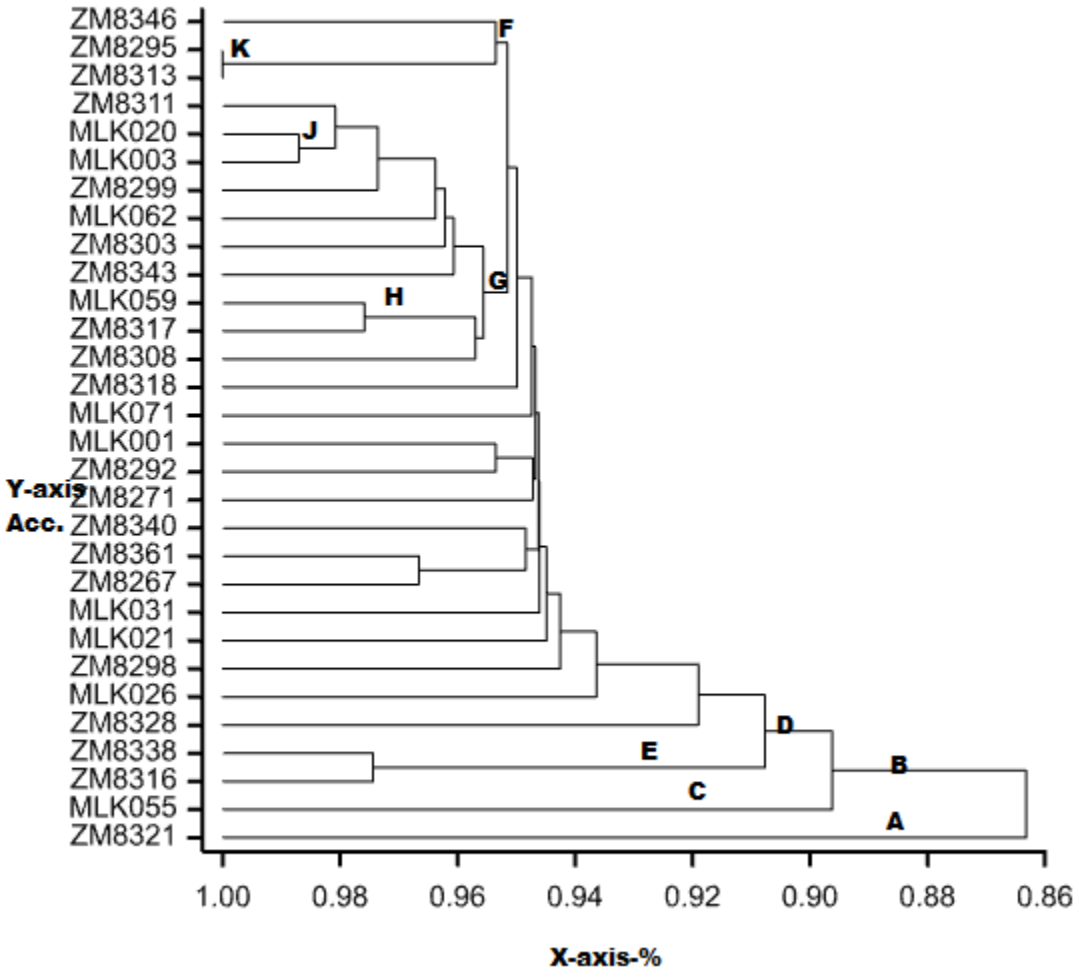

Figure 4.3. A Genstat clustering dendrogram showing the genetic relationships among 30 accessions on the alleles detected by 9 microsatellite markers. Legend: ZM8316= Malawi faya-Nyimba, Eastern; ZM8317- Mpunga wa Makolo-Nyimba, Eastern; ZM8318-Ndelema-Chama, Muchinga; ZM8321-Ndelema-Chama, Muchinga; ZM8328-Bakili-Chama, Muchinga; ZM8338-Senga-Chama, Muchinga; ZM8340-Sosha-Chama, Muchinga; ZM8343-Chandege and faya- Chama, Muchinga; ZM8346-Chandege-white and brown- Chama, Muchinga; ZM8361-Meya- Chama, Muchinga; ZM8267-Supa-Mungwi,Northern; ZM8271-Chembe-Chembe, Luapula; ZM8292-

Chembe-Chembe, Luapula; ZM8295-Supa-Samfya, Luapula; ZM8298-Lusakasaka-Mwansabombwe-Luapula; ZM8299-Kalwena- Mwansabombwe-Luapula; ZM8303-Supa-Chienge, Luapula; ZM8308-Supa-Chienge, Luapula; ZM8311-Meli-Chienge, Luapula; ZM8313-Kaputa-kaputa, Northern; MLK001-Supa/Zhazo-Zambezi, North-Western; MLK003-Supa- Zambezi, North-Western; MLK020-Kilombelo-Chavuma, North-Western; MLK022-Malawi Faya-Chavuma, North-Western; MLK026-Kajaketi-Mongu, Western; MLK031-Aongola 7-Mongu, Western; MLK055-Kajaketi-Senanga, Western; MLK059-Burma-Senanga, Western; MLK062-Black rice; MLK071-Malawi-Senanga, Western.

Summary

This chapter presented the findings of the study on the genetic diversity of the selected rice accessions from various growing regions in Zambia. The presentation of the findings was done under the themes: DNA quality concentrations, marker analysis by spreadsheets, PCR fragment analysis revealing distinct product band patterns and clustering of rice accession**s.** A dendrogram was generated that revealed the various levels of distinctness among the selected rice accessions. The next chapter will look at the discussion of results as presented in this chapter

CHAPTER FIVE

DISCUSSION

5.0 Overview

The previous chapter presented the research findings of the study in relation to the research questions. This chapter discusses the research findings on the genetic diversity of the selected rice accessions from some parts of Zambia. The discussion is presented in accordance with the research objective which was determine the genetic distinctiveness among selected accessions grown in Zambia.

5.1 Genetic distinctiveness among selected accessions grown in Zambia.

To contribute to national efforts towards development of rice as a crop in Zambia, the study endeavored to assess the genetic diversity of selected Zambian rice accessions using SSR makers. The overall objective of the study, therefore, was to characterize and analyze the genetic diversity of rice accessions from selected parts of Zambia as well as determine the extent of genetic diversity among the selected accessions. Success of a crop improvement program depends on the magnitude of genetic variability and the extent to which the desirable characters are heritable (Ravi *et al*., 2003). The assessment of genetic diversity has been demonstrated to be essential in establishing relationships among different cultivars (Sivaranjani *et al*., 2010, Kibria *et al*., 2009, Nagaraju *et al*., 2002). According to an FAO report (2002), genetic diversity has been utilized and preserved partially during the process of domestication and cultivation. It has also been estimated that not less than 15 percent of potential genetic diversity has been utilized in crop plants and a number of valuable allelic variations of traits of great economic importance are still unutilized. Some landraces and traditional accessions have been under cultivation by farmers since time immemorial, usually on the basis of practices inherited from their forefathers and easy availability of seeds. Molecular characterization could reveal their phylogeny and this information would be quite useful in utilizing the rice germplasm in genetic improvement of the existing rice accessions. Crop diversity is the biological base of all agriculture. Molecular Marker based Genetic Diversity Analysis (MMGDA) has the potential for assessing changes in genetic diversity over time and

space (Duwick, 1984). DNA markers are predominantly used in molecular characterization and diversity studies due to their abundance and repeatability (McCouch *et al.,* 1997).

Genetic characterization offers an enhanced power for detecting diversity (including genotypes and genes) that exceeds that of traditional methods. Likewise, genetic characterization with molecular technologies offers greater power of detection than do phenotypic methods (e.g. isozymes). This is because molecular methods reveal differences in genotypes, that is, in the ultimate level of variation embodied by the DNA sequences of an individual and uninfluenced by environment. In contrast, differences revealed by phenotypic approaches are at the level of gene expression (proteins) (IPGRI/CIP, 2003). This research aimed at characterizing selected rice accessions in Zambia at genetic level in order to create baseline information that could be useful in genetic improvement of the crop.

In the present study 30 rice accessions were collected rice and analyzed. The results of the study revealed a range of similarities and differences within and across accessions. There were great similarities among the accessions despite them being collected from different ecological zones of the country and being known by different local names in the various districts from which the accessions were collected.

The presence or absence of alleles were observed in independently replicated experiments. This suggested that the observed absence of amplification products were not polymerase chain reaction artefacts which could be interpreted as failed reactions. To the contrary it showed that the seed accessions from which the DNA was isolated in fact did not carry the targeted alleles. This also indicated that the observed presence or absence of PCR product bands revealed polymorphism between or even within what are currently considered to be pure accessions. In some PCR reactions, no bands were observed; this could mean that the reactions were not successful due to reaction parameters such as DNA concentration, quality or suboptimal magnesium ion concentration. The other possibility was that the PCR products were absent in some of the reactions due to the absence of the target DNA sequence in those rice accession, thereby contributing to the diversity of the accessions. The amplification reactions were repeated with similar results, ruling out reaction failure as the cause of the absence of band fragments.

Matin *et al*., (2012) proposed polymorphism information content (PIC) as an accurate indicator of allele diversity and frequency among accessions. The PIC values for the tested markers in this work ranged between 0.2 and 0.8. The SSR marker RM225 revealed the highest PIC value also

backed by results from agarose gel electrophoresis analysis of PCR products obtained with this particular marker, Figure 4.2. The information obtained in this work compared quite closely with those from previous estimates of microsatellites analyzed in related studies (Matin *et al*., 2012). The frequencies of null alleles were not included in the calculation of PIC values for each SSR locus.

Cluster analysis via a dendrogram revealed that most of the accessions were closely related and clustered into two major groups designated A and B. Only one accession (ZM8321) appeared to be distinct and distantly related to all the other 29 accessions. Cluster B contained the rest of the 29 accessions which were subdivided into 8 sub-clusters. Of the 29, accessions ZM8295 from Samfya District and ZM8313 from Kaputa District showed 100% similarity (cluster K) meaning that they are in fact the same accession despite the different names by which they are identified. Furthermore, despite the different names and accession numbers assigned to these two accessions, visual examinations revealed morphological similarities which confirm the genetic information obtained from the study (see appendix: I). These were closely related to ZM8346 (Chandege-white and brown- Chama, Muchinga) than the rest of the accessions. MLK020 and MLK003 showed relatedness at almost 99%, and are from the same agro-ecological zones but are known by different districts local names. ZM 8338 (Senga from Chama, Muchinga) and ZM 8316 (Malawi faya from Nyimba, Eastern) in cluster E showed 97.5% relatedness, which was a very high level of relatedness despite being known by different names in two closely located provinces as well as ecological zone in Zambia. Nonetheless, clusters F, G, H, J and K were closely related but quite distant from clusters A, B, C, D and E.

From the clustering, it was observed that a number of accessions were very similar to each other; for example, clusters E, H, J and K. this could mean these accessions paired together are the same despite being known by different local names and coming from distantly spaced growing regions. This information would help in decongesting the gene bank and also reduce the possibilities of breeders dealing with the same accessions while thinking they are distantly related. For all the sub clusters, few accessions where showed genetic differences with different local name, agro-ecological zone from which it was collected and outstanding morphological distinctiveness. Some few accessions showed the exact opposite. They were observed to be quiet distant in terms of relatedness with other accessions despite them being known by the same local name in different ecological zones e.g. Supa. This may mean that most of the accessions analyzed in this study were

heterogeneous even within those collected from the same agro-ecological zones and believed to be homogeneous and treated as being the same accession. For example, what may be referred to as Mongu rice may in fact be a mixture of accessions. The level of similarity observed at molecular was quite significant when compared to the observed variations. This could mean that a accession referred to by a district name is actually the same as another accession known by a different name of a district.

Therefore, it can be said that there is genetic distinctness among accessions selected in this study, but with a higher level of relatedness. It can also be seen that genotypes from far distinct cluster groups when crossed can give raise to a genetically wide range of offsprings, giving a diversity of genotypes to select from.

The study also revealed that although two variants could be present in the same cluster and share only about 75% similarity at the genetic level. By convention it is assumed that the two variants should have been the same and must be genetically similar, but our genetic diversity analysis contradicts this fact. Such kind of cultivar identity crisis is often witnessed throughout the world (Matin *et al*., 2012). Such discrepancies emphasize the importance of molecular characterization of rice germplasm prior to their deployment in varietal improvement. It is highly likely that there is more number of such cultivars whose genetic background is not yet unveiled and are used by farmers under unknown/false identities. The results revealed that all the primers showed distinct polymorphisms among the accessions studied, indicating the robust nature of microsatellites in revealing polymorphism. Therefore microsatellite marker based molecular fingerprinting could serve as a potential basis in the identification of genetically distant genotypes as well as in sorting of duplication for morphologically close accession.

Summary

This chapter presented the discussions of findings of the study on the genetic distinctiveness of selected rice accessions grown in some parts of Zambia. The discussion was done according to theme derived from the objectives of the study. Necessary arguments were put forward. The next chapter will present the conclusion of the study and recommendations.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.0 Overview

The previous chapter looked at the discussion of findings of the study. This chapter presents the conclusion of the study and the recommendations.

6.1 CONCLUSION

The present study revealed a range of genetic variation among the selected rice accessions. The results obtained showed that there was genetic distinctiveness among the selected rice accessions but with a higher degree of relatedness. It can also be concluded that the existing naming system of the accessions does not imply ultimate distinctiveness, neither does the growing region.

The results indicated that the SSR molecular markers are neutral and co-dominant and could be a powerful tool to assess the genetic variability of the cultivars. The information about the genetic diversity may be very useful for proper identification and selection of appropriate parents for breeding programs. Morphological and molecular characterization of such cultivars followed by their use in rice varietal improvement could lead to substantial gain.

6.2 RECOMMENDATIONS

Phenotypic information should be collected for the rice accessions to create information that will back up the molecular information obtained in this study.

Rice ecological zones should be identified for each accession to determine which accession gives better yield and traits when grown in a particular zone in order to enhance rice production.

Generating a systematic naming system for locally grown accessions should be generated should also be considered.

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WEB ACCESSED REFERENCE

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LIST OF APPENDICES

Appendix I: pictures of rice accessions-Figure 1A-30A

Figure 1A: ZM 8346

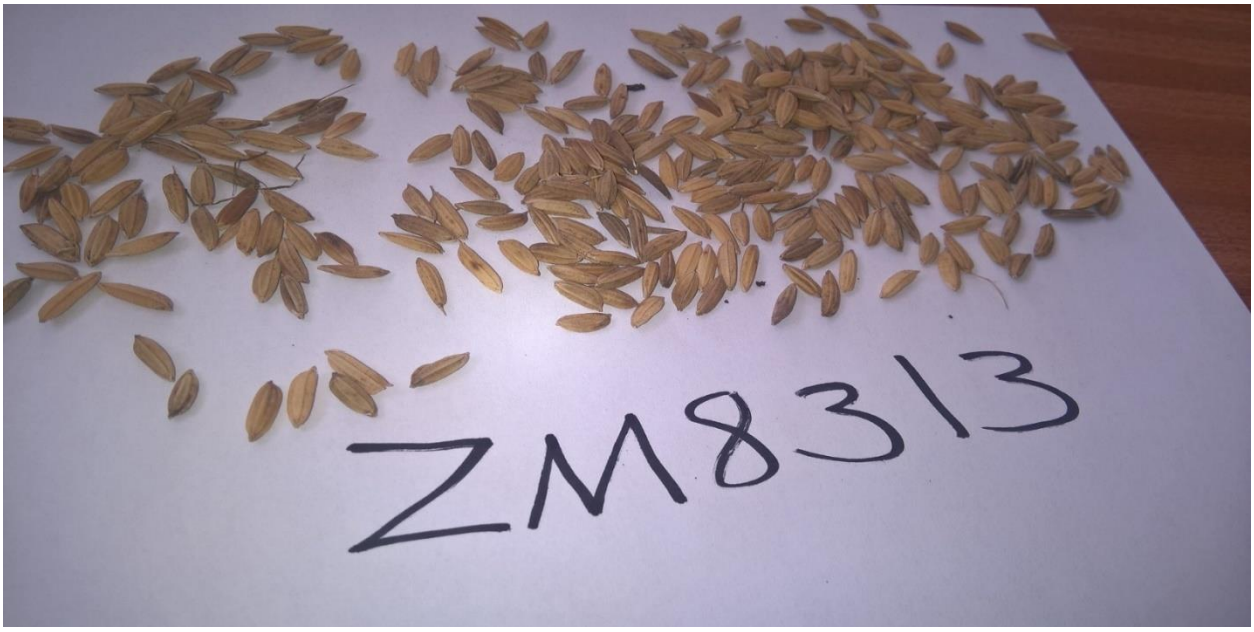


Figure 2A: ZM 8313

Figure 3A: ZM 8295

Figure 4A: ZM 8338

Figure 5A: ZM 8316

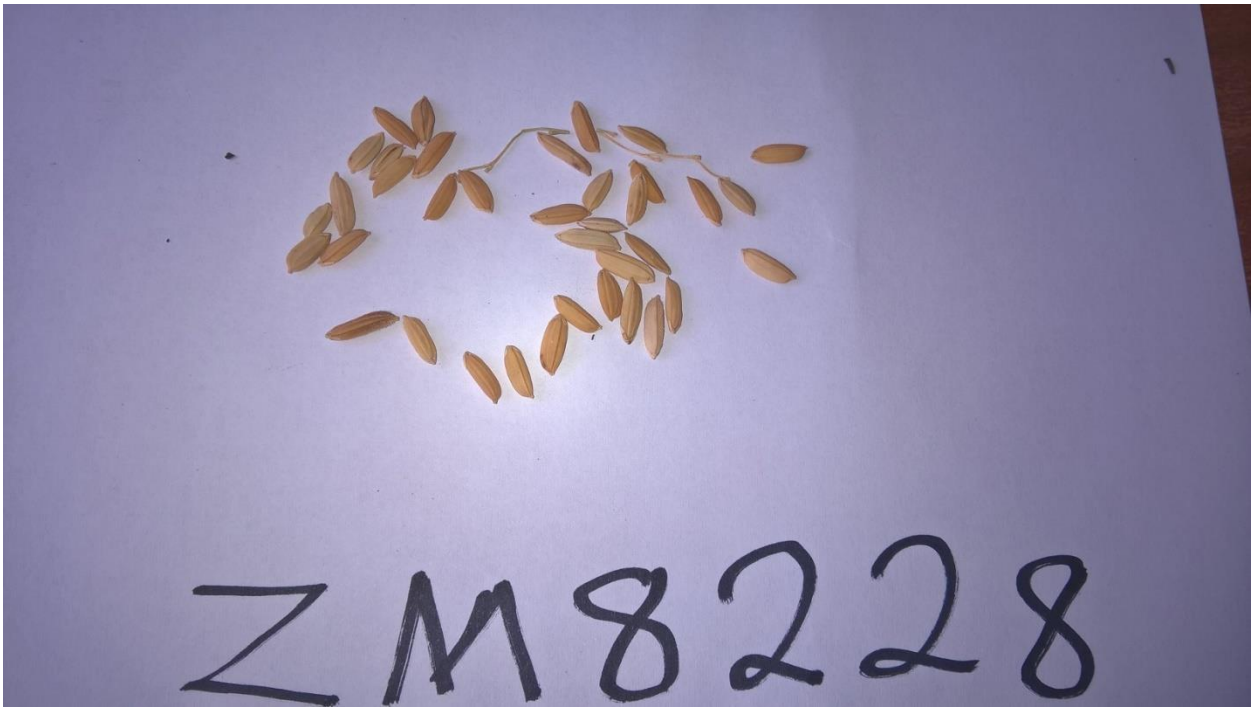


Figure 6A: ZM 8328

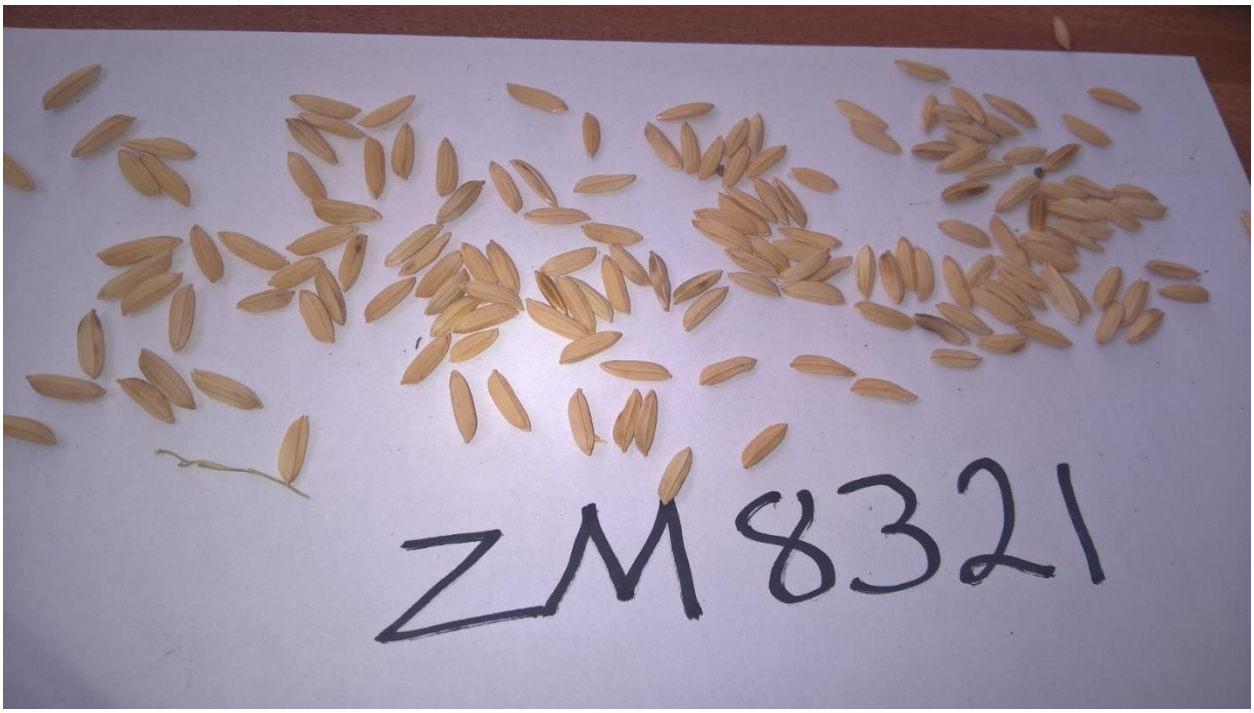


Figure 7A: ZM 8321

Figure 8A: MLK 026

Figure 9A: MLK 055

Figure 10A: ZM 8343

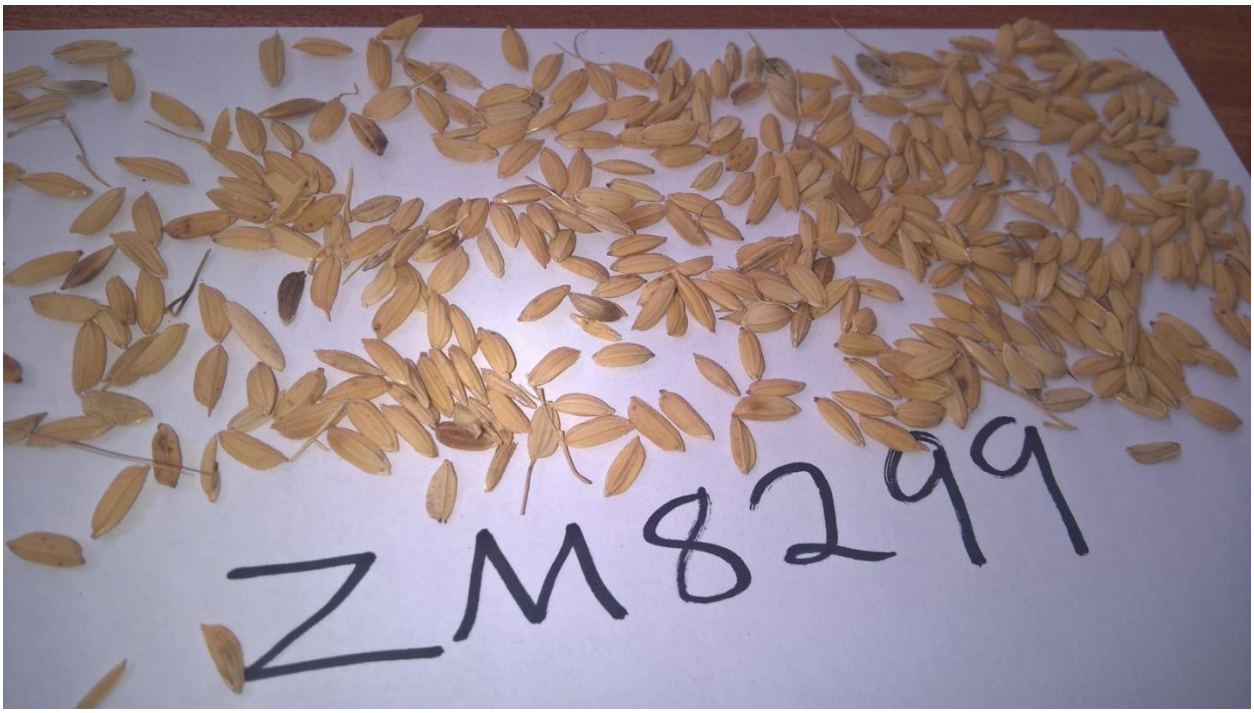


Figure 11A: ZM 8299

Figure 12A: MLK 021

Figure 13A: MLK 071

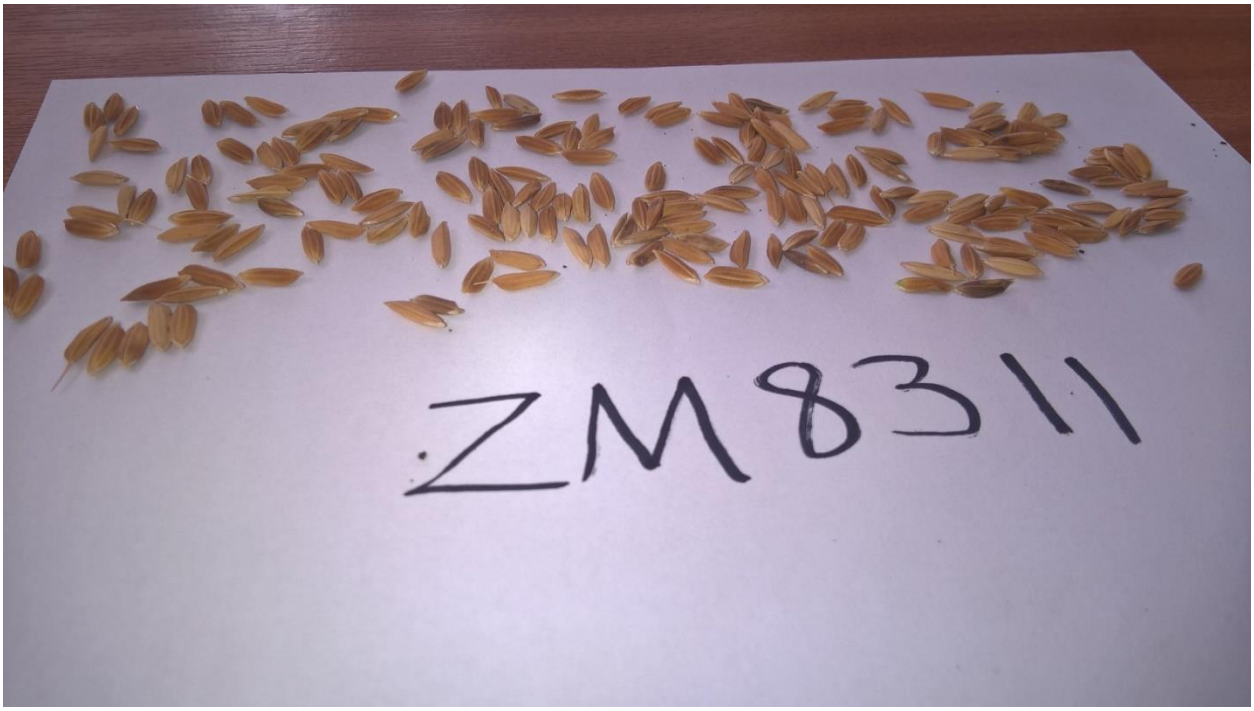


Figure 14A: ZM 8311

Figure 15A: ZM8361

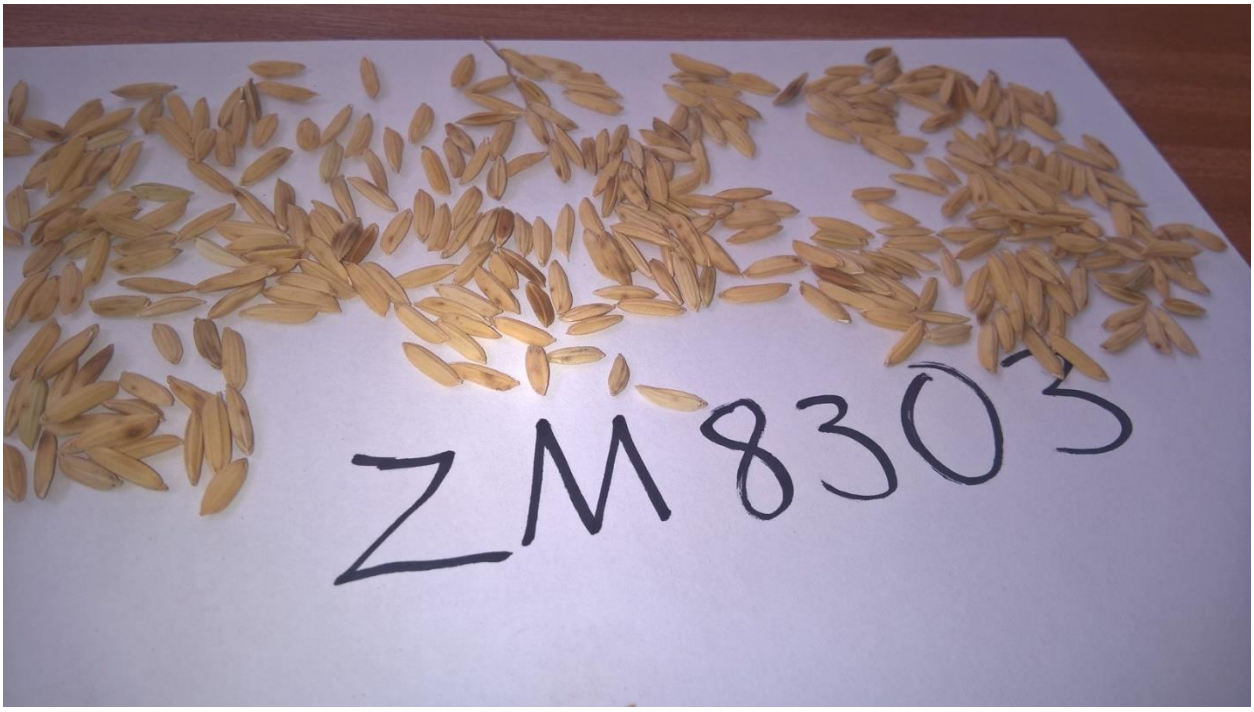


Figure 16A: ZM 8303

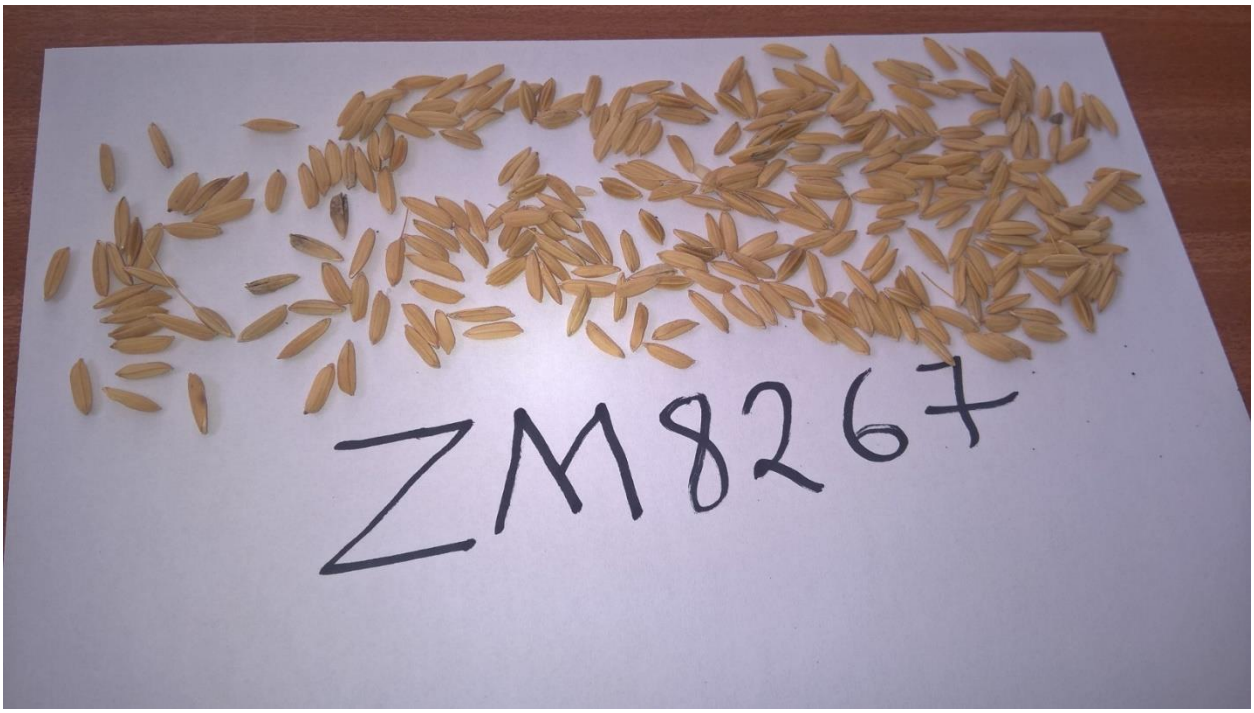


Figure 17A: ZM 8267

Figure 18A: ZM 8340

Figure 19A: ZM 8292

Figure 20A: MLK 001

Figure 21A: ZM 8318

Figure 22A: ZM 8271

Figure 23A: MLK 031

Figure 24A: ZM 8317

Figure 25A: MLK 059

Figure 26A: MLK 003

Figure 27A: ZM 8308

Figure 28A: MLK 062

Figure 29A: MLK 020

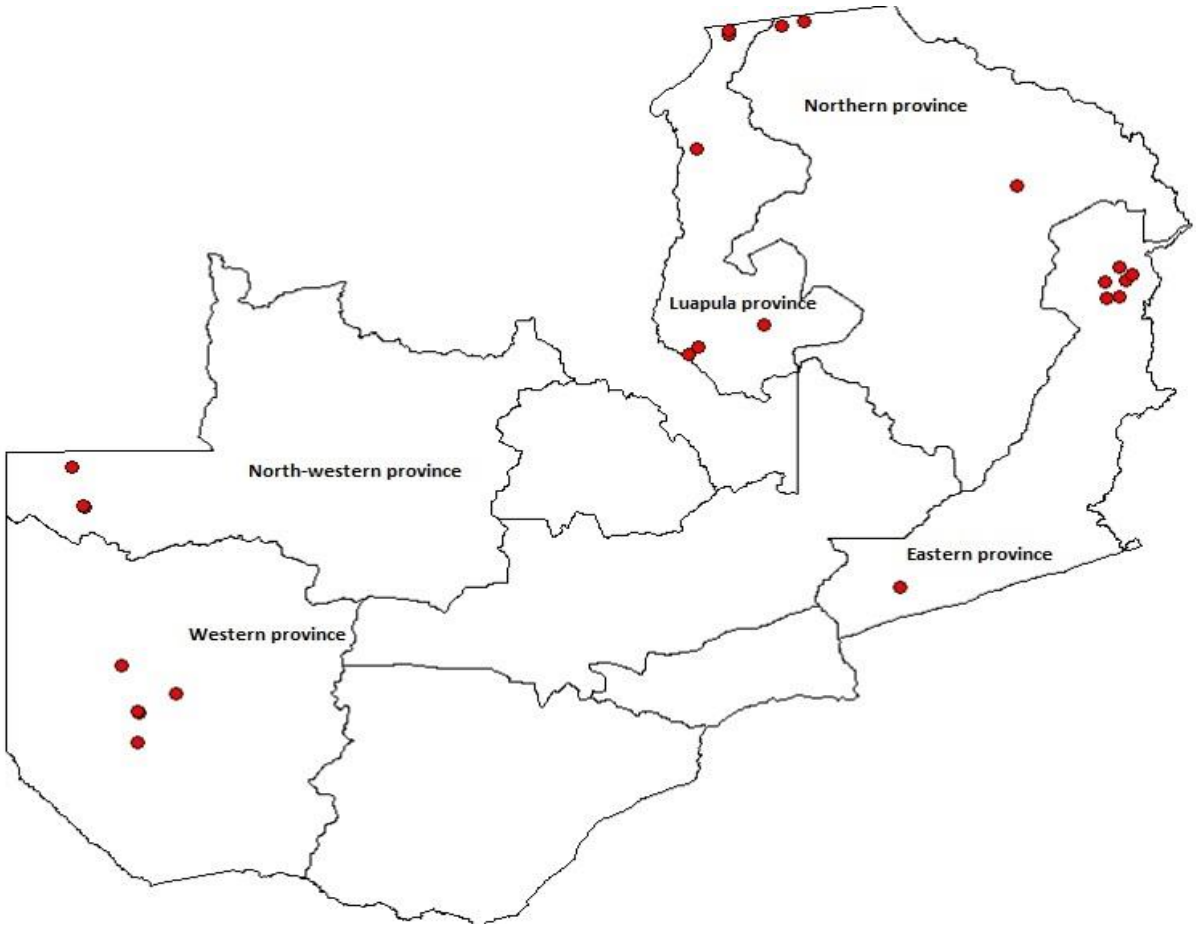
Figure 30A: ZM 8298

Appendix II: Raw passport information

Collector's no	Acc. No.	Local name	Village	Lat (s)	Lon (e)	Alt (m)	District	Province
MLK001	Not assigned	Supa/Zhazo	Liwena	13°35.289'	22°46.908'	1054	Zambezi	North-Western
MLK003	Not assigned	Supa	Liwena	13°34.370'	22°46.000'	1038	Zambezi	North-Western
MLK020	Not assigned	Kilombelo	Lukolwe Mission	13°10.179'	22°38.913'	1057	Chavuma	North-Western
MLK021	Not assigned	Malawi Faya	Lukolwe Mission	13°10.179'	22°38.913'	1057	Chavuma	North-Western
MLK026	Not assigned	Kajaketi	Samboko-Katongo	15°16.943'	23°08.292'	1040	Mongu	Western
MLK031	Not assigned	Aongola 7	Matondomange	15°34.870'	23°40.303'	1022	Mongu	Western
MLK055	Not assigned	Kajaketi	Kaeya Camp	16°06.180'	23°18.033'	1026	Senanga	Western
MLK059	Not assigned	Burma	Lyaangati Camp	15°48.020'	23°18.587'	1026	Senanga	Western
MLK062	Not assigned	Black rice	Lyaangati Camp	15°46.761'	23°18.085'	1020	Senanga	Western
MLK071	Not assigned	Malawi Faya	Senanga Boma	16°06.580'	23°17.523'	1005	Senanga	Western
	ZM 8267	supa	Musonda	10°10.000'	31°59.000'	1218	Mungwi	Northern
	ZM 8271	chembe	Milishi	11°58.000'	28°44.000'	1054	Chembe	Luapula
	ZM 8292	chembe	Mutwale	11°53.000'	28°50.000'	1150	Chembe	Luapula
	ZM 8295	supa	Fibalala	11°39.000'	29°29.000'	1182	Samfya	Luapula
	ZM 8298	Lusakasaka	Mwansabombwe FTC	9°46.000'	28°49.000'	1035	Mwansabombwe	Luapula
	ZM 8299	Kalwena	Mwansabombwe FTC	9°46.000'	28°49.000'	1035	Mwansabombwe	Luapula
	ZM 8303	supa	Mwabu camp	8°33.000'	29°08.000'	955	Chienge	Luapula

	ZM 8308	supa	Mwabu camp	8°30.000'	29°08.000'	966	Chienge	Luapula
	ZM 8311	meli	Lambwe Chomba	8°24.000'	29°35.000'	990	Chienge	Luapula
	ZM 8313	kaputa	kaputa camp	8°27.000'	29°39.000'	947	Kaputa	Northern
	ZM 8316	Malawi Faya	Duncan	14°27.009'	30°49.228'	952	Nyimba	Eastern
	ZM 8317	Mpunga wa Makolo	Whyted	14°27.009'	30°49.228'	901	Nyimba	Eastern
	ZM 8318	Ndelema	Kapalakonje	11°10.800'	33°03.212'	1009	Chama	Muchinga
	ZM 8321	Ndelema	Kapalakonje	11°10.800'	33°03.212'	1009	Chama	Muchinga
	ZM 8328	Bakili	Mulopwe	11°11.639'	32°50.973'	645	Chama	Muchinga
	ZM 8338	Senga	Moonda	11°21.196'	32°59.531'	702	Chama	Muchinga
	ZM 8340	Sosha	Moonda	11°21.196'	32°59.531'	702	Chama	Muchinga
	ZM 8343	Chandege and Faya	Lwambu	11°21.847'	32°52.189'	642	Chama	Muchinga
	ZM 8346	Chandege - white and brown	Kazembe	11°06.773'	33°07.610'	769	Chama	Muchinga
	ZM 8361	Meya	Ng'anjo-Chiwato	11°02.34'	32°59.505'	689	Chama	Muchinga

Appendix III: Map of Zambia showing major rice growing regions from which accessions used in the study where collected.



Appendix IV: Amplification score sheet

Accession	Plant	RM5	RM7	RM13	RM168	RM211	RM205	RM225	RM413	RM452	RM463
ZM8346	1	1	1	0	0	1	1	1	1	1	1
ZM8346	2	1	1	0	0	0	1	1	1	1	1
ZM8346	3	1	1	1	0	1	1	1	1	1	0
ZM8346	4	1	1	0	1	0	1	1	1	1	1
ZM8346	5	1	1	0	1	0	1	2	1	1	0
ZM8346	6	1	1	1	1	0	1	2	1	1	1
ZM8346	7	1	1	1	1	0	1	1	1	1	1
ZM8346	8	1	1	0	1	1	1	0	1	1	1
ZM8346	9	1	1	0	1	1	1	0	1	1	0
ZM8346	10	1	1	0	1	0	1	1	1	1	1
ZM8313	1	1	1	0	1	0	1	0	1	1	1
ZM8313	2	1	1	0	1	1	1	0	1	1	0
ZM8313	3	1	1	1	1	0	1	1	1	1	0
ZM8313	4	1	1	1	1	0	1	2	0	1	1
ZM8313	5	1	1	1	1	0	1	1	1	1	1
ZM8313	6	1	0	0	1	0	1	2	1	1	0
ZM8313	7	0	1	0	1	0	1	0	0	1	0
ZM8313	8	1	1	1	1	0	1	1	1	1	0
ZM8313	9	1	1	0	1	1	1	1	1	1	1
ZM8313	10	1	1	0	1	0	1	0	1	1	0
ZM8295	1	1	1	0	1	0	1	0	1	1	1
ZM8295	2	1	1	0	1	1	1	0	1	1	0
ZM8295	3	1	1	1	1	0	1	1	1	1	0
ZM8295	4	1	1	1	1	0	1	2	0	1	1
ZM8295	5	1	1	1	1	0	1	1	1	1	1
ZM8295	6	1	0	0	1	0	1	2	1	1	0
ZM8295	7	0	1	0	1	0	1	0	0	1	0
ZM8295	8	1	1	1	1	0	1	1	1	1	0
ZM8295	9	1	1	0	1	1	1	1	1	1	1
ZM8295	10	1	1	0	1	0	1	0	1	1	0
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ZM8338	2	1	1	1	0	0	1	1	1	1	0
ZM8338	3	1	1	1	1	1	1	1	1	1	1
ZM8338	4	1	1	1	0	0	1	1	1	1	0
ZM8338	5	1	1	1	1	1	1	0	1	1	1

ZM8338	6	1	1	0	0	0	1	1	1	1	1
ZM8338	7	1	0	0	0	1	1	2	1	1	1
ZM8338	8	0	1	1	0	0	1	0	1	1	0
ZM8338	9	1	1	1	0	0	1	1	1	1	0
ZM8338	10	1	1	1	0	0	1	1	0	1	1
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ZM8316	3	1	1	1	0	0	1	2	1	1	0
ZM8316	4	1	1	0	0	0	1	0	1	1	0
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ZM8316	6	1	1	0	0	0	1	1	1	1	1
ZM8316	7	1	1	1	0	1	1	1	1	1	0
ZM8316	8	1	0	1	0	1	1	0	1	1	0
ZM8316	9	1	1	1	1	1	1	2	1	1	1
ZM8316	10	1	1	1	1	1	1	0	1	1	0
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ZM8328	2	1	1	0	0	1	1	0	1	1	0
ZM8328	3	1	1	0	0	0	1	0	1	1	0
ZM8328	4	1	1	1	0	1	1	2	1	1	0
ZM8328	5	1	0	0	0	0	1	0	1	1	0
ZM8328	6	1	0	1	0	0	1	0	1	1	0
ZM8328	7	0	0	1	0	0	1	0	0	1	0
ZM8328	8	1	0	0	1	0	1	0	1	1	0
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ZM8328	10	1	1	0	1	0	1	0	1	1	0
ZM8321	2	1	1	0	1	1	1	1	0	0	1
ZM8321	3	1	1	1	1	0	1	1	0	0	1
ZM8321	4	0	1	1	0	0	1	0	0	1	1
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ZM8321	10	0	1	1	1	0	1	0	1	0	1
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MLK026	2	1	1	0	0	0	1	0	1	1	0
MLK026	3	1	1	0	1	0	1	0	1	1	0

MLK026	4	1	1	1	1	1	1	0	1	1	0
MLK026	5	1	1	1	0	0	1	0	0	1	0
MLK026	6	1	1	0	0	0	1	0	1	1	0
MLK026	7	1	1	1	0	0	1	0	0	1	0
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MLK026	9	1	1	0	1	0	1	1	1	1	0
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ZM8299	7	1	1	1	1	0	1	0	1	1	1
ZM8299	8	1	0	1	1	0	1	0	1	1	0
ZM8299	9	1	1	0	1	1	1	0	1	1	0
ZM8299	10	1	0	1	1	1	1	0	1	1	1

MLK021	1	1	1	0	1	0	1	1	0	1	1
MLK021	2	1	1	0	1	0	1	1	1	1	1
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MLK021	7	1	1	0	1	1	1	0	1	1	1
MLK021	8	1	0	0	1	1	1	1	1	0	1
MLK021	9	1	1	0	1	1	1	0	1	1	1
MLK021	10	1	1	0	1	1	1	1	1	1	0
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MLK071	2	1	1	0	1	0	1	1	1	0	0
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MLK071	6	1	1	1	1	1	1	0	1	1	0
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MLK071	8	1	1	1	1	0	1	0	1	0	1
MLK071	9	1	1	1	1	1	1	0	1	0	1
MLK071	10	1	1	0	1	0	1	1	1	1	1
ZM8311	1	1	0	0	1	1	1	1	1	1	0
ZM8311	2	1	1	0	1	0	1	1	1	1	1
ZM8311	3	1	1	0	1	0	1	1	1	1	1
ZM8311	4	1	0	1	1	0	1	0	1	0	0
ZM8311	5	1	1	0	1	0	1	0	1	1	1
ZM8311	6	1	1	0	1	0	1	1	1	1	0
ZM8311	7	1	1	0	1	1	1	0	1	1	0
ZM8311	8	1	1	1	1	1	1	0	1	1	0
ZM8311	9	1	1	1	1	0	1	0	1	1	0
ZM8311	10	1	0	0	1	1	1	0	0	1	1
ZM8361	1	1	0	0	0	0	1	1	0	1	1
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ZM8361	5	1	1	1	1	0	1	0	0	1	1
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ZM8361	8	1	1	0	1	0	1	0	1	1	1
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ZM8361	10	1	1	0	1	0	1	1	1	1	0
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ZM8303	2	1	1	0	1	0	1	0	1	1	0
ZM8303	3	1	1	1	1	0	1	0	0	1	0
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ZM8267	2	1	1	0	1	0	1	0	0	1	1
ZM8267	3	1	1	1	1	1	1	0	1	1	1
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ZM8267	6	1	1	0	1	0	1	1	0	1	1
ZM8267	7	1	1	1	1	0	1	1	0	1	0
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ZM8267	10	1	1	1	1	1	1	0	0	1	1
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ZM8340	3	1	1	0	1	0	1	1	0	0	0
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ZM8340	8	1	1	1	1	0	1	0	0	1	0
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ZM8292	1	1	1	0	1	0	1	1	1	1	0
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MLK001	1	1	1	1	1	1	1	1	1	1	1
MLK001	2	1	1	0	1	0	1	0	1	1	0
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MLK001	4	1	1	0	1	1	1	0	1	1	1
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MLK001	7	1	1	0	1	1	1	1	0	1	1
MLK001	8	1	0	0	1	1	1	1	0	1	0
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MLK001	10	1	0	0	1	1	1	1	0	1	1
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MLK031	1	1	0	1	1	0	1	0	0	1	0

MLK031	2	1	0	0	1	0	1	0	0	1	0
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MLK059	3	1	1	0	1	1	1	0	1	1	0
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MLK003	1	0	1	0	1	0	1	0	1	1	0
MLK003	2	1	1	1	1	0	1	1	1	1	0
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MLK003	7	1	1	1	1	1	1	0	1	1	0
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MLK003	9	1	1	0	1	0	1	0	1	1	0
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MLK062	1	1	1	0	1	1	1	1	1	1	0
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MLK062	10	1	1	0	1	0	1	0	1	1	0
MLK020	1	1	1	0	1	0	1	1	1	1	0
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MLK020	3	1	1	1	1	1	1	0	1	1	1
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ZM8298	1	0	0	0	1	0	1	1	1	1	0
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