QUALITY ANALYSIS AND MOLECULAR DIVERSITY OF CASSAVA (*Manihot esculenta* Crantz) GERMPLASM IN KENYA

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented, wholly or in part, for an award of degree in any other university.

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Recommendation

We wish to confirm that this thesis was carried out under our supervision and has our approval to be presented for examination as per the Egerton University regulations.

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DEDICATION

This work is dedicated to the memory of my dear late parents Patrick Ng'ang'a and Beatrice Wambui who set an example of love, discipline and hard work that has stayed with me to this day.

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ABSTRACT

Cassava (*Manihot esculenta* Cranzt) is an important food security crop for resource poor rural communities particularly in Africa. Little is however known about variability of critical root nutritional, biochemical quality traits and molecular diversity of Kenyan cassava germplasm. This led to a study whose objective was to determine the nutritional quality traits of different genotypes of cassava cultivated in the central Rift Valley region of Kenya and to identify genetic constitution of cassava accessions from different regions of Kenya using molecular tools. Roots from fourteen accessions comprising Kenyan local landraces and improved clones were screened for their nutritional traits including the contents of cyanogenic glycosides, protein and the micro nutrients iron and zinc while another 69 accessions were tested for molecular diversity using Simple Sequence Repeats (SSRs). Trait stability and the effects of the environment on the expression of the nutritional traits were evaluated using various genotype (G) x environment (E) interaction study models. There were significant ($p<0.05$) differences in all the nutritional traits in the three test sites of Baringo, Kericho and Nakuru in Kenya. Contents of cyanogenic glucosides in both roots and leaves, total root proteins, root iron and zinc ranged from 31.8 ppm to 90.8 ppm; 20.8 ppm to 154.4 ppm; 1.15 % to 3.47 %; 17.81 ppm to 59.69 ppm and 39.39 ppm to 118 ppm, respectively. The sites were also significantly ($p<0.05$) different from each other with the highest cyanogenic content in leaves and roots expressed at the Nakuru site. Regression coefficients (b_i) obtained ranged from -2.21 to 2.29 for all traits combined while sensitivity to environmental change (SE^2_i) revealed that cassava genotypes differed in their level of sensitivity. The root cyanide trait had the highest mean SE^2_i which indicated that it was the least stable quality trait in the cassava germplasm. The observed values for protein and mineral contents suggest the potential for improving the nutritive value of local cassava germplasm. Seven pairs of micro satellite (SSR) primers previously developed from cassava were used to detect polymorphic 21 alleles in a sample of 69 accessions. The cluster analysis of similarity matrix obtained at 68 % with SSR data showed that the 69 accessions were grouped into five marker based groups. This study proved that SSRs could be used to identify cassava accessions as well as in the assessment of level of genetic relatedness among accessions.

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LIST OF ABBREVIATIONS

1. AFLP - Amplified Fragment Length Polymorphism
2. CIAT - Centro Internacional de Agricultura Tropical
3. EDTA - Ethylenediaminetetra-acetic Acid
4. FAO - Food and Agriculture Organization
5. IITA - International Institute of Tropical Agriculture
6. PCR - Polymerase Chain Reaction
7. RAPD - Random Amplified Polymorphic DNA
8. RFLP - Restriction Fragment Length Polymorphism
9. SDW - Sterile Distilled Water
10. SSA - Sub Saharan Africa
11. SSR - Simple Sequence Repeat
12. WHO - World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Cassava (*Manihot esculenta* Crantz) is a widely grown root crop in several tropical countries of Africa, Latin America and Asia between latitudes 30^{o} N and 30^{o} S, a belt that coincides with most of the less developed countries of the world (Phillips, 1974). For a long time, this crop has been ranked among the four major food crops in developing countries, after rice, wheat and maize (Cock, 1985). In some countries, cassava provides over 50% of the average caloric intake of the population. Most of the world production of cassava is used for human consumption, animal feed and the starch industry. In Africa, over 90% of cassava produced is consumed as human food with only 6% devoted to livestock feed (Ntawuruhunga, 2005). Cassava starch can perform the same functions as maize, rice and wheat starch. In India and Malaysia, good quality cassava starch is produced for specific local applications as well as for the export market. Several medium to large scale cassava processing units are therefore operational mainly for production of cassava native starch and glucose syrups (Ntawuruhunga, 2005). Cassava is primarily a carbohydrate source containing little protein and is often considered an inferior food. However, in much of the world where severe malnutrition and starvation exists, the populations suffer not only from protein deficiency but also from carbohydrate deficiency indicating that their carbohydrate supplies are not adequate (CIAT, 2001).

Cassava's adaptability to relatively marginal soils and erratic rainfall conditions, its high productivity per unit of land and labor, the certainty of a yield even under the worst conditions and the possibility of maintaining a continuous supply year round, makes this crop a basic component of the farming system in most areas of Sub-Saharan Africa (Nweke and Enette, 1999). The crop has a potential to contribute significantly to food security and poverty alleviation due to its suitability and adaptability to traditional farming systems and socio-economic circumstances of the farming communities (Kinyua, 2005). Other advantages include flexibility in harvesting (year-round availability) and planting periods, and long

period of in-ground storage after maturity. Famine therefore is rare in areas where cassava is grown, since it provides a stable base to the food production system and has the potential for bridging the food gap (Iglesias *et al*., 1997; Nweke and Enette, 1999 and Ekwele *et al*., 2001).

In Kenya, cassava is largely grown for household food security and in some areas for sale in fresh or processed form. Total output of cassava is estimated to be 864,000 tons at an average productivity of 9 tons per hectare (Kariuki *et al*., 2002). Production takes place mainly in the low altitude areas of the coast province, mid-altitude zones in Central and Eastern provinces and in Nyanza and Western provinces (Njeru and Munga, 2003). The area under cassava in western, central and the coastal regions is estimated at 49,000, 19,000 and 34,000 ha respectively, representing nearly all the country's total area under this crop. Western and central areas have higher yields averaging about 9.0 t/ha compared to the coast with only 4.0 t/ha (Mohamed, 2002).

Although Cassava is an important starchy tuberous root crop grown in the tropics, its potential toxicity due to cyanide is of great public health concern. The toxicity is due to the presence of cyanogenic glucosides, mainly linamarin (Essers, 1994). Due to this, consumption of cassava has sometimes led to chronic ailments such as Konzo and sometimes death (Essers, 1994).

Micronutrient malnutrition is a burgeoning public health problem in developing countries particularly in Africa. This type of malnutrition results primarily from use of diets deficient in essential vitamins and minerals like iron and zinc. Known as “hidden hunger,” micronutrient malnutrition can exist even when poor people have enough to eat, but lack the resources to buy fruits, vegetables, meat, and other foods rich in vitamins and minerals, (FAO, 2003). Diets poor in micronutrients cause illness, blindness, premature death, reduced productivity and impaired mental development, particularly among women and children in developing countries (UNICEF, 2004). Micronutrient deficiencies are estimated to cost Sub-Saharan African economies more than U.S. $2.3 billion in lost productivity (UNICEF, 2004).

Cassava has a low nutritional value, although high in carbohydrates when compared to most cereals but is low in its protein and micro nutrient content. The crop can however be improved through biofortification to increase its protein and micronutrient content (Harvest plus, 2002). This is an obvious first step in building a food systems approach to reducing malnutrition. This approach addresses the root causes of micronutrient malnutrition, targets the poorest people, involves built-in delivery mechanisms, and is scientifically feasible and cost-effective (Harvest plus, 2002). As part of an integrated food systems approach, it represents the best means for enabling rural households to improve family health and nutrition in sustainable ways (Harvest plus, 2002). One approach towards the development, use and conservation of biofortified foods involves the screening of the existing plant genetic resources for germplasm with the nutrient resources and quality so desired (Harvest plus, 2002).

Although genetic resources have traditionally been evaluated on the basis of morphological and agronomic traits, these do not necessarily reflect food value and inherent genetic relationship among germplasm. Indeed, most morphological and physiological descriptors are greatly influenced by the environment and show continuous variation and high plasticity, with most of them only scorable at maturity. The use of reliable and standardized genetic descriptors is therefore critical in enhancing the efficiency of identification and use of high value plant germplasm to ameliorate hunger and malnutrition (Wachira, 2002). Accurate characterization and evaluation of accessions within the cassava germplasm resources in Kenya as well as assessment of the level of genetic variation in the resource is important in devising optimum management strategies for sustainable utilization and conservation of the resource.

DNA fingerprinting for cultivar or varietal identification has become an important tool for genetic identification in plant breeding and germplasm management. When planning DNA fingerprinting, one of the most important decisions is the marker system and technique to be used (McGregor *et al*., 2000). The molecular marker analysis will provide a powerful tool for characterizing cassava germplasm in Kenya (Fregene *et al.,* 2003) and coupled with

biochemical typing for quality traits, groups of accessions can be identified for immediate farm use as well as use in biofortifying future cultivars through breeding.

1.2 Statement of the Problem

In spite of the growing popularity of cassava as a source of dietary calories, it is one of about the 3,000 economically important plant species that contain cyanogenic glucosides (Vetter, 2000) and therefore potentially toxic to consumers (Roseling, 1988). Long term consumption of cassava containing high levels of cyanogenic glucosides has also been associated with tropical ataxic neuropathy, spatic paraparesis and in areas with low iodine intake, development of hypothyroidism, goiter and cretinism (Bradbury and Holloway, 1988). Cyanide is very toxic to humans and there are reports of death due to cyanide poisoning emanating from consumption of fresh cassava roots (Akentonwa *et al*., 1994). Incidences of human death due to cyanide poisoning have been reported especially during periods of food shortage. The underlying reason for exposure to cyanide during these periods is the ineffective processing that is used to quickly avail food for the hungry (Akentonwa *et al*., 1994). Proper processing of cassava roots, including fermenting, drying, and grinding into flour has been found to reduce toxicity. Exposure to cyanide has been crudely estimated to be 15-50 mg/day in endemic areas. Farmers also frequently import varieties from one area to another without the knowledge that the cyanide biochemical trait is polygenic and hence is greatly influenced by the environment and can easily shoot up when cassava is grown under new and different environments TRIP, (1993).

Malnutrition is a rampant problem in Sub-Saharan Africa (SSA). It ranks first among the top ten preventable health risks. The nutrient deficiencies of greatest global health concerns include protein, iron and zinc. Interventions to alleviate these deficiencies rely on supplementation and food fortification programmes which are not sustainable and hardly reach all the affected persons. Sustainable solutions to malnutrition can be developed through linking agriculture, nutrition and health. Enriching cassava with micronutrients through breeding (Dixon, 2005), would go a long way towards preventing protein, iron and zinc deficiencies in SSA including Kenya where cassava is a widely consumed staple.

Studies to evaluate the food quality, nutritional and genetic diversity of the popular cassava germplasm have not previously been carried out in Kenya. A sustainable agricultural system requires that components of diversity be used in a way and at a rate that will not lead to along term decline of diversity, thus maintaining its potential to meet the needs and aspirations of present and future generations. Genetic diversity is however threatened by the introduction and adoption of modern high yielding varieties (Wachira, 2002). A dramatic increase in the use of small number of highly selected accessions has led to loss of valuable genetic resources. The proportion of genetic diversity accessed by the popular varieties has often not been determined yet it is critical to the sustainable use of cassava genetic resources in Kenya. Since cassava is predominantly vegetatively propagated, over reliance on a few varieties which may also share a common ancestry may minimize the on-farm diversity and thus increase the risks posed by such co-evolving biotic factors as pests and diseases to cassava farming.

Impact projections suggest that cassava demand in the next 9 years is expected to increase considerably in cassava growing countries, and therefore, special efforts should be made to improve root yield and address the problems with cassava consumption such as cyanogenesis and mineral compositions (Pinstrup-Andersen *et al*., 1999) as well as the sustainable use of this vital genetic resource.

1.3 Justification

Cassava is a major food security crop but which has a problem of cyanide poisoning and low nutrition. Apparently, no controlled studies have been carried out to determine the levels of cyanide and nutritional quality traits for different genotypes and environments in potential cassava producing areas of the central Rift Valley where cassava is grown and consumed though with high incidences of cyanide poisoning and low nutrition.

As a starting point in breeding for higher micronutrient contents in cassava, popular varieties should be screened for useful variability of quality traits and later be used as parents for the development of breeding populations. It is expected that through genetic recombination, micronutrient contents can be enhanced to levels that are significant for

human nutrition. Because of evidence of adequate variability in the protein, iron and zinc content of roots and leaves, work should be done on these traits. The aim will be to identify and select from the varieties of superior agronomic performance, germplasm with the highest protein, iron and zinc contents which can be recommended for exploitation and further use in the development of an advanced generation of biofortified cassava varieties.

Most researchers and policy makers have driven farmers in cultivating a few popular cassava varieties leading to the fixation of a few plants of a particular genetic basis and potentially, loss of some valuable genetic resources. The level of genetic variation within the popular cassava germplasm in Kenya is yet to be determined. Cassava genetic resources in Kenya are currently conserved in *ex-situ* field gene banks in Kakamega, Katumani and Mtwapa which are general cassava growing areas. The proportion of diversity accessed from these resources by the popular cultivars is yet to be determined. It is prudent for farmers to practice sustainable agriculture by enhancing their on-farm diversity to buffer their enterprises from co-evolving disease and pest factors.

Genetic diversity can most efficiently be quantified using molecular markers. The Simple Sequence Repeat markers (SSR) are one such marker system that has been used for many genetic applications, including the assessment of genetic variability in germplasm collections and pedigree reconstruction (Fregene *et al.,* 2003). It is envisaged that this study will help to bring to in the breeding program germplasm with important quality traits that were left out previously in the quest to develop fortified cassava varieties of the future whilst ensuring a broad genetic base for such future varieties.

1.4 Objectives

1.4.1 General objective

To determine the nutritional quality traits of different genotypes of cassava cultivated in the central Rift Valley region of Kenya and to identify genetic constitution of sixty nine cassava accessions from different regions of Kenya using molecular tools.

1.4.2 Specific objectives

1. To determine the effect of genotype and environments on cyanide levels and their stability in fourteen popular cassava accessions cultivated at three sites (Nakuru, Kericho and Baringo) of central Rift Valley in Kenya.
2. To evaluate the levels of protein, zinc and iron and their stability on fourteen cassava accessions grown in three sites (Nakuru, Kericho and Baringo) of central Rift Valley in Kenya.
3. To estimate the level of genetic relatedness in 69 cassava accessions sourced from the national *ex-situ* field gene banks based on simple sequence repeat polymorphisms.

1.5 Null Hypotheses

1. There is no effect of genotype and environment on cyanide levels in cassava accesssions grown in central Rift Valley.
2. Protein, iron and zinc levels are high and do not vary in cassava roots of the fourteen test cassava accessions grown in central Rift Valley.
3. There is no genetic diversity within the 69 cassava accessions.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cassava Crop

Cassava (*Manihot esculenta* Crantz) belongs to the family Euphorbiaceae. Of the 98 species that belong to the genus *Manihot*, cassava is the only species that is widely cultivated for food production (Nassar, 2005). It is a perennial shrub grown throughout the tropical and subtropical regions of the world. It originated and was domesticated in the Neotropics. The crop is widely grown between the latitudes 30^0 N and 30^0 S, a belt that coincides with most of the developing countries of the world (Phillips, 1974).The crop was introduced into Africa in the 16^{th} century, where it is now cultivated across an extensive area, known as the "cassava belt". In Kenya, cassava was introduced between the 16^{th} and 19^{th} century by the European explorers. It was introduced along with other crops such as beans, maize and sweet potatoes. It adapted well to the environment and by the start of the 20^{th} century, it was widely grown in the country (Suttie, 1970).

Globally, cassava is the fourth largest source of carbohydrates for human consumption after rice, wheat and maize. The cassava root consists of 30-40% dry matter and it contains mostly carbohydrates. It is also rich in vitamin C, carotene, calcium, and potassium, though poor in protein (Hendershot, 1972). Cassava ranks fourth of the food crop in developing countries after rice, maize and wheat (Cock, 1985). The leaves are relatively rich in protein and can be consumed as vegetables (Adegbola, 1977; Nassar, 2005). The crop is particularly used as a reserve food when staple main crops fail as it is stored in the ground for several seasons. Cassava is planted in about 16 million hectares, with about 50% being in Africa, 30% in Asia and 20% in Latin America. Annual global production is estimated at 189.09 million tons (MT). Africa produces about 101.91 MT annually while Nigeria, the largest producer in the world produces 33.37 MT (FAO, 2002). Cassava is an essential part of the diet of more than half a billion people around the globe (Opare-Obisaw, 2004).

Cassava is grown virtually throughout Kenya. However, the Western, Coastal and semi-arid Eastern) regions of Kenya have the highest production in that order (Kariuki *et al*.,

2002). Traditional cassava utilization in Kenya is limited to roasting and boiling of fresh roots for consumption in all the growing areas (Khaemba, 1983). In Nyanza and Western provinces of Kenya, roots are also peeled, chopped into small pieces, dried and milled into flour for Ugali. This is normally in combination with a cereal (maize or sorghum). In the Coast province cassava leaves are used as vegetable (Khaemba, 1983) while in Machakos and Kitui, cassava roots are used as snack.

The bulk of cassava produced in the country is used for human consumption and surpluses are processed into starch or used for animal feed. However, the present production is adequate for both the demands of starch production and as a food source (Khagram, 1983). The market for fresh cassava as food is more lucrative than for starch extraction but the market for fresh cassava is limited (Karisa, 1983). Except for cassava crisps there is no commercial processing of cassava for human consumption. This area could be explored to make it more acceptable to a larger section of the population thereby increasing the demand for cassava products and the income of the farmer.

Cassava's role varies greatly in different parts of the world; in Sub Saharan Africa and elsewhere, the crop has been important for preventing famine during times of drought and civil unrest. In Asia and Latin America, the roots also provide raw materials for small and large scale processing into livestock feed and starch (CIAT, 2001). Cassava has a comparatively high biological efficiency of food (energy production) because of its rapid and prolonged growth as well as the fact that it produces 2.2 times more calories per hectare than maize at a lower resource cost (Hahn *et al*., 1979).

2.2 Cyanide in Cassava

Many edible plants contain cyanogenic glucosides, whose concentrations can vary widely as a result of genetic and environmental factors, location, season, and soil types (JECFA, 1993). All cassava tissues, with the exception of seeds, contain the cyanogenic glucosides linamarin (>90% total cyanogens) and lotaustralin (<10% total cyanogens). Cyanogenesis is initiated in cassava when the plant tissue is damaged. Rupture of the vacuole releases linamarin, which is hydrolyzed by linamarase, a cell wall-associated β-glucosidase

(McMahon *et al*., 1995). Cassava tubers vary widely in their cyanogenic glucoside content, although most varieties contain 15–400 mg cyanide/kg fresh weight. Occasionally, varieties of cassava tubers contain 1300–2000 mg cyanide/kg fresh weight, and cassava leaves contain 1000–2000 mg cyanogenic glucosides/kg on a dry matter basis (Padjmaja, 1995).

Movement of a genotype from one location to another could alter its cyanogenic potential because of differences in climate and soil characteristics (Grace, 1977;TRIP, 1993). Bruijn (1971) noted that different genotypes of cassava do not react the same way with the changing environmental conditions with regard to hydrogen cyanide content. In cassava, there is a parallel development of the leaves and the storage root that competes simultaneously for the current production of assimilates (Ekanayake, 1993). Even after the roots start to fill, the cassava crop continues to produce leaves for photosynthesis. It has been reported that low levels of cyanogenic potential coincide with the active root bulking phases (Bokanga *et al.*, 1994).

Exposure of developing cassava roots to drought does not always lead to low cyanogenic potential values (Bokanga *et al.*, 1994). Some caution should therefore be exercised in using the level of cyanogenic glucosides as a distinguishing characteristic for cassava cultivars because of the plasticity of this trait. The cyanide yielding capacity of cassava roots is not only dependent on the genetic character of the genotype grown, but also on several environmental and growth factors (Rosling, 1988). This results in considerable variation of toxicity between the same varieties grown under different conditions. Despite this plasticity, characterization of cyanide yielding capacity of several genotypes in one experiment is useful to match genotypes to environment with respect to this quality trait. According to Cooke and De la Cruz (1982), cassava plant toxicity does not however change with plant age.

Most cassava cultivars are incorrectly called non-cyanogenic because the cyanoglycoside content is less than 100 mg kg^{-1} fresh roots; but there are cyanogenic cultivars, also called bitter cassavas, which may contain cyanoglycosides of up to 500 mg kg^{-1} fresh roots (Wheatley *et al*., 1993). To date, there is no acyanogenic cassava cultivar

reported, and several studies on cassava linamarin synthesis suggest that the cyanoglucosides accumulated in roots are synthesized in shoots and then transported to roots where they are stored. However, McMahon *et al*., (1995) demonstrated that secondary roots were capable of synthesizing linamarin at rates equivalent to leaves.

In cassava, the production of cyanide or cyanogenesis is the result of the hydrolysis of linamarin by linamarase to form an acetone cyanydrin, which is either spontaneously or enzymatically transformed by α-hydroxynitrile lyase to release hydrogen cyanide (HCN). Cassava linamarase and hydroxynitrile lyase have been purified and characterized, and their cDNAs have been isolated (Cooke *et al*., 1978; Eksittikul and Chulavatnatol, 1988; McMahon *et al*., 1995; White et al., 1998). Even though linamarin and linamarase are present in most of the plant tissues, no HCN is detected under physiological conditions, suggesting that the enzymes and their substrate exist in two different compartments (McMahon *et al*., 1995).

Cultivar-dependent differences in the cyanogenic components of cassava have been very controversial. Extensive research has been done to understand the physiological basis to account for these differences. Several factors, intrinsic and extrinsic, including the genotype and the environmental conditions such as dryness and soil composition have been assigned for the differences observed (Bokanga, 1994).

2.2.1 Effects of cyanide on human health

Because of the presence of cyanoglucosides, cassava is potentially toxic to human populations that subsist on cassava-based diets and low ingestion of protein. Almost all of the tissues of cassava contain large amounts of cyanogenic glucosides, such as linamarin and lotaustralin. Linamarin accounts for 95% of the total cyanoglucosides, therefore, most of the research on Cassava cyanoglucosides has been focused on the biochemistry and metabolism of these compounds (Padjmaja, 1995). Considerable medical evidence exists to associate consumption of high cyanide cassava with toxic effects in humans (Lundquist, 1992) and when consumers associate ill health with cassava toxicity, the immediate reaction is to discard or substitute cassava with other crops and this problem has led to cassava being rejected in some areas.

A number of animal and human cyanide and cyanogenic glucoside toxicological studies have cited evidence of poor diet, low protein, and vitamin B_{12} and folic acid deficiencies as factors that may exacerbate the observed toxicities. Cyanide poisoning from high-cyanogenic cassava is typically associated with insufficient consumption of Cystein and Methionine in the diet. Reduced sulfur-containing compounds are substrates for the detoxification of cyanide catalyzed by the enzymes rhodanese and/or β-cyanoalanine synthase (Castric *et al*., 1972; Kakes, 1990; Nambisan, 1993). The dose–effect curve of the acute effects in humans is steep. Whereas slight effects occur at exposure to hydrogen cyanide levels of 20–40 mg/m^3, 50–60 mg/m^3 can be tolerated without immediate or late effects for 20 min to 1 h, 120–150 mg/m^3 is dangerous to life and may lead to death after 0.5–1 h, 150 mg/m^3 is likely to be fatal within 30 min, 200 mg/m^3 is likely to be fatal after 10 min, and 300 mg/m^3 is immediately fatal. It should however be emphasized that this represents crude average exposure estimates, based on various studies (DECOS, 2002).

As a respiratory poison, free cyanide (hydrogen cyanide or the cyanide ion) has high acute toxicity due to its primary toxic effect of inhibiting cytochrome oxidase (by binding haem iron), the terminal enzyme of the mitochondrial electron transport chain (Isom and Way, 1974). Tissue utilization of oxygen is impaired and with time, a state of histotoxic anoxia occurs (oxidative metabolism is brought to complete cessation). Cyanide can also inhibit approximately 40 enzymes, including a number of other important metalloenzymes containing, for the most part, iron, copper, or molybdenum (e.g., alkaline phosphatase, carbonic anhydrase, catalase, peroxidase, ascorbic acid oxidase, xanthine oxidase, and succinic dehydrogenase); these reactions may also contribute to cyanide toxicity (Rieders, 1971; Ardelt *et al*., 1989; US EPA, 1990; ATSDR, 1997).

The effects of acute cyanide exposure are dominated by central nervous system and cardiovascular disturbances. Typical signs of acute cyanide poisoning include tachypnoea, headache, vertigo, lack of motor coordination, weak pulse, cardiac arrhythmias, vomiting, stupor, convulsions, and coma (Ballantyne, 1983; Way, 1984; Johnson and Mellors, 1988). Tropical ataxic neuropathy, an upper motor neuron disease characterized by irreversible

paraparesis (Ernesto *et al*., 2002), was described in Nigeria and dietary cassava was proposed to be the causative factor.

An epidemic of spastic paraparesis occurred in a drought-stricken cassava staple area of Mozambique in 1981–1982 (Tylleskär *et al*., 1991). Altogether, 1102 cases were identified. The highest recorded village prevalence rate found by active case detection was 29 per 1000 inhabitants; 65% of the cases were under 15 years of age (Tylleskär *et al*., 1991). In contrast to tropical ataxic neuropathy, the onset of mantakassa was acute. General symptoms around the time of onset included fever, pain (especially in the legs), paraesthesiae, headache, dizziness, and vomiting; many patients also complained of weakness in the arms and difficulty in speaking and in seeing. Outbreaks of konzo have been reported in the Democratic Republic of the Congo (formerly Zaire) since 1938. The outbreaks have occurred during droughts and dry seasons. Again, the affected populations have relied almost exclusively on bitter cassava roots as the staple food (Tylleskär *et al*., 1991).

Iodine deficiency and goitre, hypothyroidism, and cretinism are endemic in many areas of Africa. Several surveys in the endemic areas have demonstrated that there is also a strong correlation between cassava consumption and the thyroid effects (Delange and Ermans, 1970; Delange; Ermans, 1971; Ermans *et al.*, 1972 and Delange, 1994). Based on a geographical link between the prevalence of diabetes and cassava consumption (McGlashan, 1967), dietary exposure to cyanides has been linked to the malnutrition-related diabetes mellitus, also known as the "type-J" or "type-Z" diabetes (Hugh-Jones, 1955 and Abayomi, 1994). Approximately 80% of absorbed cyanide is metabolized to thiocyanate in the liver by the mitochondrial sulfur transferase enzyme rhodanese and other sulfur transferases. Thiocyanate is excreted in the urine. Minor pathways for cyanide detoxification involve reaction with cystine to produce aminothiazoline- and iminothiazolidinecarboxylic acids and combination with hydroxycobalamin (vitamin B_{12a}) to form cyanocobalamin (vitamin B_{12}); with these end-products also being excreted in the urine.

2.3 Nutritional Value of Cassava

Cassava is a starchy staple food whose roots are very rich in carbohydrates, a major source of energy. In fact, the cassava plant is the highest producer of carbohydrates among crop plants with perhaps the exception of sugarcane. It has been reported that cassava can produce 2.5×10^5 calories/ha/day compared to 1.76×10^5 for rice, 1.1×10^5 for wheat, 2.0×10^5 for maize, and 1.14×10^5 for sorghum (Coursey and Haynes, 1970; Nestel, 1973; Phillips, 1974; Nartey, 1977; Nassar, 1986). The chemical composition of cassava varies in different parts of the plant, and according to variety, location, age, method of analysis, and environmental conditions.

Although cassava roots are rich in calories, they are grossly deficient in proteins, fat, and some of the minerals and vitamins. Consequently, it is of lower nutritional value than are cereals, legumes, and even some other root and tuber crops such as yams (Hendershot, 1972; Nassar, 1986). The cassava root contains carbohydrates, 64 to 72 per cent of which are made up of starch, mainly in the form of amylose and amylopectin. About 17 per cent sucrose is found in sweet varieties, and small quantities of fructose and dextrose have been reported (Hendershot, 1972; Nassar, 1986). The lipid content of cassava is only 0.5 per cent.

Cassava is poor in proteins (1 to 2 per cent), and the amino acid profile of the cassava root is very low in some essential amino acids, particularly lysine, methionine and tryptophan. The peel of cassava roots contains slightly more protein than is found in the flesh. Therefore, peeling results in loss of part of the valuable protein component of the root. However, fermentation of the roots results in protein enrichment by a factor of some 6 to 8 (Hendershot, 1972; Nassar, 1986).

Cassava leaves are richer in proteins than the roots. Although the leaves contain far less methionine than the roots, the levels of all other essential amino acids exceed the FAO's recommended reference protein intake. For this reason, cassava-leaf protein is claimed to be superior to soybean protein. Supplementation of cassava products such as leaf-meal with methionine or any other of the nutrients it lacks serves to improve its biological value

significantly and has been widely practiced in industry for the processing of food for human consumption and animal feeds (Adegbola, 1977).

Cassava root is reasonably rich in calcium and vitamin C, but the thiamine, riboflavin, and niacin contents are not as high. Large proportions of these nutrients have been reported to be lost during processing (Bradbury *et al.,* 1988*).* All of this should be taken into account in cassava-processing in order to retain as much as possible of these nutrients.

2.3.1 Micronutrients deficiency in cassava

Iron deficiency is the most common micronutrient deficiency in the world and a main cause of anemia, a condition in which the blood contains low levels of red blood cells (WHO 2006). Two billion people worldwide are estimated to suffer from anemia. Approximately 50 percent of all anemias can be attributed to iron deficiency. Iron deficiency causes fatigue, reduces work capacity, and weakens the immune system. Severe anemia also heightens the risk of women dying during childbirth and impairs children's' physical growth, mental development, and learning capability (WHO 2006). Sixty percent of pre-school children in Kenya are estimated to have iron deficiency anemia. In Ethiopia, Mali, and Mozambique, the rate exceeds 75 percent (UNICEF 2004).

In Kenya, approximately 30,000 people are estimated to be at risk of inadequate zinc intake (FAO, 2004). The clearest indicator of zinc deficiency is stunting in children (FAO, 2003). The human body relies on zinc to heal wounds, grow and repair body tissue, properly clot blood, and ensure sound fetal development. Severely malnourished children or those with persistent diarrhea or respiratory problems may be zinc deficient (GAIN, 2006).

Iron and zinc are important micronutrients for proper functioning of the human body (GAIN, 2006). Many families in Sub Saharan Africa (SSA) consume cassava as a staple, especially the poor who can seldom afford supplementary foods that are rich in essential nutrients (Dixon, 2005). Very often in major cassava producing areas, boiled or roast cassava takes the place of bread to go with in the morning or evening tea/coffee. Unfortunately, the tannins in both tea and coffee decrease iron availability and this worsens the situation for a

diet that is already low in iron (Dixon, 2005). As a consequence of predominance of cassava in their diets, the poor suffer from micronutrient malnutrition. The most vulnerable groups are the women, adolescents and children who also constitute the larger portion of the population in SSA. These age and gender groups have higher requirements for micronutrients because they are more active and/or still growing (Dixon, 2005).

Since cassava roots are grossly deficient in proteins, fat and some of the minerals and vitamins (Hendershot, 1972), continued consumption can lead to malnutrition especially to the poor children and women of SSA who entirely consume cassava as a staple.

2.4 Biofortification

Biofortification is a revolutionary process that nutritionally enhances staple crop varieties with higher levels of vitamins and minerals. This process holds great potential to improve the health of the poor in developing countries, particularly in rural areas. As an additional tool in the fight to improve the nutritional status of the poor, biofortification complements existing nutrition interventions, such as mineral supplements, commercial food fortification, and dietary diversity (CGIAR, 2001).

There are principally three ways to fight micronutrient malnutrition; supplementation, fortification, and food-based approaches (Mc Clafferty, 2003). Biofortified crops which include varieties bred for increased mineral and vitamin content provide higher levels of micronutrients in the foods that the poorest people grow and consume daily. It can complement the other two approaches by providing a sustainable and low-cost means of reducing the number of persons requiring treatment through supplementation and commercial fortification. Once the fixed investments are made in developing micronutrient-rich staple food crops at central research locations, recurrent costs are maintained low, and benefits can be made available to all developing countries around the world (Mc Clafferty, 2003).

A major advantage of improving the micronutrient content of staple crops is that the resulting varieties stand a high chance of being adopted by farmers in target regions. These crops are already produced and consumed by the majority of poor households in the

developing world. Because changes in mineral content need not alter the appearance, taste, texture, or cooking quality of improved staples, they should be fully acceptable to consumers and markets. Particularly where scientists can combine high micronutrient content with high yield, farmer adoption and the market success of such nutritionally improved varieties are guaranteed (Mc Clafferty, 2002).

The biofortification approach involves developing breeding methodologies, breeding nutritional quality traits into current crop varieties, and adapting these varieties to diverse environments. No large, recurrent investments are required after nutritious varieties have been initially disseminated, and the costs do not increase with the number of people treated. To achieve the full benefits of the plant breeding approach for poor people in developing countries, it is estimated that an investment of up to US$1.4 million per year per major staple crop over 8-10 years will be required (Mc Clafferty, 2003). Despite this lofty cost, the expected returns are great and every small effort should be made towards achieving the goal of developing nutritionally enriched varieties. Varieties of six nutritionally enriched staple crops—beans, cassava, maize, rice, sweet potato, and wheat—would cover roughly 90 percent of the population at risk from micronutrient malnutrition in the developing world (Mc Clafferty, 2003).

One of the most advanced biofortification projects are Golden Rice, which is rice with vitamin A. In development are also other crops like mustard, canola, sweet potato, millet and cassava that are biofortified with vitamin A, as well as rice and common legumes with zinc and iron (AFIC, 2004).The International Food Policy Research Institute recently launched an ambitious programme to encourage plant breeders working with staple crops to breed for varieties with more vitamins and minerals. The targeted crops are wheat, maize, cassava and beans and the targeted micronutrients are iron, zinc, vitamin A and for cassava, iodine (AFIC, 2004).

2.5 Stability of Quality Traits

Even as efforts are made to identify and develop healthy and nutritious biofortified cassava cultivars, the quality traits of such cultivars must be stable across environments. Local

landraces of cassava have been cultivated widely in traditional cassava areas in Kenya. These landraces have however hardly been tested outside their traditional environments for biochemical quality traits. Similarly, they have not been compared for stability of their biochemical quality traits relative to newly introduced and improved accessions. The stability of cassava quality traits over a wide range of environments would be generally regarded desirable as the resulting products would be uniform in quality under different growing enviroments. Stability measures used to characterize genotype x environment (G x E) interactions among cultivars at various stages of germplasm development employ different models that measure response to environmental changes and the consistence of that response (stability) (Lin *et al*., 1986). The most widely used approach has been based on linear regression of cultivar traits on an environmental index (b_i) derived from the average performance of all cultivars in a site (Finlay and Wilkinson, 1963; Eberhart and Russell, 1966; Freeman, 1973; Chakroun *et al*., 1990: Wachira *et al.*, 2001). The conventional analysis of variance has also been widely used to detect G x E interactions (Casler and Hovin, 1984). The variance of a genotype across environments, which is also a measure of sensitivity to environmental changes (SE_i^2), has also been used as a stability measure (Wachira *et al.*, 2001).

Studies have shown that genotypic variation exists for biochemical quality traits content among cassava genotypes. A positive relationship was found between iron and zinc concentration, suggesting that selecting for high iron concentration in cassava roots will not have a negative effect on zinc or vice versa. Therefore, it can be concluded that a potential exists for developing cassava clones high in positive biochemical traits (Dixon, 2005).

2.6 Genetic Diversity of Cassava Germplasm

Diversity is an invaluable genetic resource that has been threatened by the introduction and adoption of modern, high yielding cultivars (Wachira, 2002). Cassava probably originated in wild *Manihot esculenta* populations growing along the southern rim of the Amazon Basin in Brazil. The savannas of Colombia, Venezuela, Guatemala and southern Mexico had earlier been proposed as likely places of origin due to the large number of landraces present there

(Rogers 1965). However the *Manihot* species found in these locations are only distantly related to present day cassava (Fregene *et al*., 1994; Schaal *et al*., 1994; Roa *et al*., 1997).

Given the "out of Brazil" theory for the spread of cassava to the rest of the world, many founder events should have occurred, with the concomitant effect of reduced diversity and increased genetic differentiation. Furthermore, the primary method of propagation i.e vegetative or asexual propagation – would be expected to lead to further reduction in genetic diversity over time because of the accumulation of systemic pathogens and the spread of a few, vigorous, well-adapted landraces with a capacity to produce many stakes for planting. Nonetheless, levels of molecular marker diversity observed for cassava landraces from Africa and several Neotropical countries are comparable with those from Brazil (Beeching *et al*., 1993; Fregene *et al*., 2000).

However, the traditional farming system of slash and burn, followed by 3 to 15 years of fallow, as practiced by small farmers, by far the largest producers of cassava in the Neotropics and Africa, and the allogamous nature of cassava produce a large pool of volunteer seedlings. Natural and human selection acts on these to produce new varieties that maintain a high level of genetic diversity (Doyle, 2001).

An elevated level of inter- and intra-varietals diversity is also known to exist in farm fields; for example, the diversity found in a single field of a Makushi Amerindian community in southern Guyana was shown to equal that of the core-of-core collection of 38 accessions representing the world's cassava collection held at the International Center for Tropical Agriculture (CIAT), Cali, Colombia (Elias, 2000 a).

Variation in allele frequency at many unlinked loci is the preferred way to assess genetic diversity and differentiation, and to estimate the strengths of the various forces shaping them. A study of unlinked loci from cassava landraces across the three continents would shed more light on the dynamics of genetic diversity and differentiation, the latter that may represent heterotic pools. High levels of genetic differentiation, potentially representing

heterotic pools, have been described for maize (Shull, 1952; Tomes, 1998) and robusta coffee (Leroy *et al*, 1993).

In Kenya, identification and collection of cassava germplasm has predominantly relied on vegetative characteristics. The collections are maintained in the field in a continuous vegetative phase by cyclic pruning or periodic renewal of the entire collection every 2-4 years. In traditional farming systems, the concept of a variety can encompass very diverse genetic entities. Traditional naming and classification systems are often based on traits that are perceived subjectively and therefore in so doing it is not uncommon to find confusion between varieties or use of different names for the same cultivar (Elias *et al.*, 2001). Collections of cassava held on farms or the major ex-situ gene banks are however not based on optimized collection strategies and genetic redundancy may be expected. A germplasm collection should ideally be well characterized and represent the maximum variation of the taxon with a reasonable number of accessions. It should also include wild relatives, landraces, cultivars (obsolete and current) and genetic stocks (Wachira, 2002).

The first step towards the efficient use, protection and conservation of cassava germplasm must therefore be the development and use of reliable and standardized genetic descriptors which not only distinguish individuals and varieties but also reflect inherent variation and genetic relationships among collection holdings. Such descriptors are important in that they can be used for identification of germplasm for commercial use and for establishing genetic relationships (Wachira, 2002).

Molecular descriptors particularly Simple sequence repeat (SSR) markers are attractive to study because they are abundant in plant and animal genomes, they have high levels of polymorphism, and are adaptable to automation (Donini *et al*., 1998). In cassava, SSR markers have been used to search for duplicates in the Centro Internacional de Agricultura Tropical (CIAT) core collection (Chavarriaga-Aguirre *et al*., 1999) and to analyze variation in natural populations of putative progenitors (Olsen and Schaal, 2001). The proposed study of the organization of genetic diversity is expected to benefit cassava

germplasm conservation and enhancement efforts in Kenya, and contribute to the elucidation of forces that shape genetic differentiation in this asexually propagated allogamous crop.

2.6.1 Molecular markers

The creation of novel improved crop varieties is increasingly relying on markers. Molecular genetic markers, based on DNA sequence polymorphism, are now widely used to complement phenotypic and protein-based markers. Over the past 20 years, DNA-based markers have been established in many agricultural crops. Molecular markers linked to desirable traits have been used to accelerate plant breeding (Ribaut and Hoisington, 1998), for example by replacing phenotypic assays with single-marker assays when possible and cost-effective. Many traits of interest to plant breeders, however, are complex and polygenic. Therefore the creation of an adapted elite variety will increasingly involve the deliberate combination of various genomic regions from many different individuals (Peleman and van der Voort, 2003). Comprehensive knowledge of genetic diversity in the cultivated and wild germplasm – the source of novel genomic regions, alleles and traits (Xiao *et al.*, 1998; Li *et al*., 2003) is very important. Applying molecular markers in this context requires moving from single marker assays to genome-wide marker profiles; genomic fingerprints covering genetic diversity at hundreds of loci. For genetic diversity analysis, a reliable measure of the differences and the relatedness between individuals will require whole-genome profiling (Xiao *et al.*, 1998; Li *et al*., 2003).

A range of techniques can be used to detect polymorphism at the nucleic acids i.e DNA level. The array of techniques falls into three broad categories with respect to strategy; (i)Non- Polymerase Chain Reaction (PCR) based approache such as Restriction Fragment Length Polymorphism (RFLP); (ii) PCR arbitrary priming e.g Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeat (ISSR); (iii) Targeted PCR e.g Sequence Tag Sites (STS), Cleaved Amplified Polymorphism (CAPs) Simple Sequence Repeats (SSR) and Single Strand Conformation Polymorphism (SSCP) (Wachira, 2002). Non-PCR based approaches are labor intensive and have low throughput and are therefore not widely used. New polymorphic assay

procedures based on the PCR (Mullis and Faloona, 1989) have been the choice markers of many crops.

2.6.1.1 Restriction Fragment Length Polymorphism

Restriction Fragment Length Polymorphism (RFLP) is the variation (s) in the length of DNA fragments produced by a specific restriction endonuclease from genomic DNAs of two or more individuals of a species (Kahl, 2001). The technology was first developed in the 1980s for use in human genetic applications and was later applied to plants. By digesting total DNA with specific restriction enzymes, an unlimited number of RFLPs can be generated. RFLPs are relatively small in size and are co-dominant in nature. If two individuals differ by as little as a single nucleotide in the restriction site, the restriction enzyme will cut the DNA of one but not the other. Restriction fragments of different lengths are generated. All RFLP markers are analyzed using a common technique. However, the analysis requires a relatively complex technique that is time consuming and expensive. The hybridization results can be visualized by auto radiography if the probes are radioactively labelled), or using chemiluminescence's (if non-radioactive, enzyme-linked methods are used for probe labeling and detection). Any of the visualization techniques will give the same results. The visualization techniques used will depend on the laboratory conditions. The RFLP technique is expensive and may require the use of hazardous radioactive probes that are not available to many resource-poor research programs in developing countries, and must be physically transferred from site to site under strict safety protocols. Nonetheless, RFLP has successfully been used in genetic diversity of coconut trees in Cote d'Ivoire (Lebrun *et al.*, 1999) and cassava (Angel *et al.*, 1992).

2.6.1.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a molecular biology technique for enzymatically replicating DNA without using a living organism, such as *E. coli* or yeast. Like amplification using living organisms, the technique allows a small amount of DNA to be amplified exponentially. Since PCR is an *in vitro* technique, it can be performed without restrictions on the form of DNA and it can be extensively modified to perform a wide array of genetic

manipulations (Mullis and Faloona, 1989). The technique is commonly used in medical and biological research laboratories for a variety of tasks, such as the detection of hereditary diseases, the identification of genetic fingerprints, the diagnosis of infectious diseases, the cloning of genes, paternity testing, and DNA computing.

PCR is used to amplify specific regions of a DNA strand. This can be a single gene, just a part of a gene, or a non-coding sequence. A PCR process usually amplifies only short DNA fragments, usually up to 10 kb. Certain methods can copy fragments up to 47 kb in size, which is still much less than the chromosomal DNA of a eukaryotic cell - for example, a human cell which contains about three billion base pairs. PCR, as currently practiced, requires several basic components. These components are; DNA template, which contains the region of the DNA fragment to be amplified; two primers, which determine the beginning and end of the region to be amplified; a DNA polymerase, which copies the region to be amplified; deoxynucleosides-triphosphate (dNTPs), from which the DNA Polymerase builds the new DNA and buffer, which provides a suitable chemical environment for the DNA Polymerase

The PCR process consists of an initiation step where the mixture is heated at temperatures greater than 90°C to ensure that the DNA strands as well as the primers have melted. The DNA polymerase can be present at the initiation step, or it can be added after. Annealing step follows by heating at 36-68°C depending on the specific PCR technique and its thermal cycling profile. The primers always jiggle around, caused by the Brownian motion. Short bondings are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and start copying the template. Once there are a few bases built in, the melting temperature (T_m) of the double-stranded region between the template and the primer is greater than the annealing or extension temperature. Lastly, there is elongation by heating at 72°C. This is the ideal working temperature for the DNA polymerase. The primers, having been extended for a few bases, already have a stronger hydrogen bond to the template than the forces breaking these attractions. Primers that are on positions with no exact match melt away from the template (because of the higher temperature) and are not extended.

The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template). The above steps are repeated 25 times or more to increase the copy number of amplified PCR products though with good primers and fresh polymerase, 15 to 20 cycles may be sufficient. The mixture is then held at 4°C.

The PCR products can be visualized on the basis of their size using gel electrophoresis. Gel electrophoresis is a procedure that consists of injecting DNA into a gel matrix and then applying an electric current to the gel. As a result, the smaller DNA strands migrate faster than the larger strands through the gel matrix toward the positive terminal. The size of the PCR product can be determined by comparing it with a DNA ladder, which contains DNA fragments of known molecular weight, also within the gel (Rabinow, 1996; Mullis, 1998; Sambrook and Russell, 2001).There are several types of PCR based marker systems including RAPD, AFLP and SSR.

2.6.1.2.1 Random Amplified Polymorphic DNA

Random Amplified Polymorphic DNA marker (RAPD) are any DNA segments that are amplified using short oligodeoxynucleotide primers of arbitrary nucleotide sequence (amplifiers) (Kahl, 2001). RAPDs are produced by PCR using genomic DNA and arbitrary primers (Welsh and McClelland, 1990; Devos and Gale, 1992).The enzyme DNA Taq polymerase is used to amplify DNA segments between closely spaced sequences (<2 kb) and complimentary to the short random oligomers (typically 10-mers). RAPD polymorphism results from changes in the primer-binding site in the DNA sequence. The PRC products can be separated by gel electrophoresis. The RAPD markers are dominant (rely on dominant traits) and therefore not informative enough. This marker system may also have low transferability and repeatability between laboratories. RAPDs have been used in cassava diversity studies in Ghana (Asante and Offei, 2003) and on Kenyan tea (Wachira *et al.*, 1995).

2.6.1.2.2 Amplified Fragment Length Polymorphism

Amplified Fragment Length Polymorphism (AFLP) refers to any difference between corresponding DNA fragments from two organisms that are detected by the amplified restriction length polymorphism technique. The AFLP technique combines components of RFLP analysis with the PCR technology (Kahl, 2001). Total genomic DNA is digested with a pair of restriction enzymes, normally a frequent and a rare cutter. Adaptors of known sequence are then ligated to the DNA fragment at the 3´ and 5´ ends. Primers complementary to the adaptors are used to amplify the restriction fragments. The PCR-amplified fragments can then be separated by gel electrophoresis and banding patterns visualized low. A range of enzymes and primers are available to manipulate the complexity of AFLP fingerprints to suit application. Care is needed in selection of primers with selective bases. The AFLP markers are also dorminant and cannot distinguish between homozygotes and heterozygotes. They have been used in inheritance and genetic diversity studies of *Pinus sylvestris* L. (Lerceteau and Szmidt, 1999) and cassava (Elias *et al*., 2000 b).

2.6.1.2.3 Simple Sequence Repeats

Simple Sequence Repeats (SSR) also known as Micro satellites are any one of a series of very short (2-10 bp), middle repetitive, tandemly arranged, highly variable (hyper variable) DNA sequences dispersed throughout fungal, plant, animal and human genomes (Kahl, 2001; Tautz and Rentz, 1984; Tautz, 1989). The di-, tri- or tetra-nucleotide repeats are arranged in tandem arrays consisting of 5-50 copies, such as $(AT)_{29}$, $(CAC)_{16}$, or $(GACA)_{32}$. The SSRs are abundant in plants, occurring on average every 6-7 kb (Cardle *et al*., 2000). These repeat motifs are flanked by conserved nucleotide sequences from which forward and reverse primers can be designed to PCR-amplify the DNA section containing the SSR. SSR alleles, amplified products of variable length, can be separated by gel electrophoresis and visualized by silver staining, auto-radiography (if primers are radioactively labeled) or via automation (if primers are fluorescently labelled). SSR markers are co-dominant and can therefore distinguish homozygotes and heterozygotes making them ideal for a genetic diversity study. SSR analysis is amenable to automation and multiplexing and allows genotyping to be

performed on large numbers of lines, and multiple loci to be analyzed simultaneously. SSRs can be identified by searching among DNA databases (e.g. EMBL and Gene bank), or alternatively small insert (200-600 bp) genomic DNA libraries can be produced and enriched for particular repeats (Powell *et al*., 1996). From the sequence data, primer pairs (of about 20 bp each) can be designed (software programmes are available for this). SSR markers have been used to determine genetic diversity in cassava around the world (Elias *et al*., 2001; Fregene *et al.,* 2003 and Moyib *et al.,* 2007) and in cattle (Guo *et al.,* 2005)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant Materials

Fourteen cassava accessions which included local landraces and some improved lines from the International Institute of Tropical Agriculture (IITA) and International Centre for Tropical Agriculture (CIAT) were used in this biochemical study (Table 1a). The accessions were planted at three sites; Kericho (Soin), Nakuru (Subukia), and Baringo (Marigat) districts of central Rift Valley in Kenya (Table 2). The cassava germplasm were selected for the study because of their high yields and disease resistance. The germplasm are also variably popular in Western and Eastern Kenya where they are predominantly grown. Another sixty nine (69) accessions were randomly sampled (Table 1b) from the national *ex-situ* gene banks at Kakamega and Katumani. Within them, few samples from advanced IITA lines and CIAT were sampled to act as checks. All these germplasm were subjected to molecular diversity studies using SSR markers.

Table 1a: Cassava Germplasm (Accessions) for biochemical characterization.

	Accessions		**Accessions**
1	Migyera	8	MM96/3868
2	SS4	9	MM96/1871
3	Mucericeri	10	MH95/0183
4	KME-1	11	Serere
5	MM96/4466	12	990067
6	MM96/7151	13	990072
7	MM96/4884	14	KME-61

Table 1b: Sixty nine accessions of cassava sampled from the national genebanks of KARI-Katumani and KARI-Kakamega.

Accession Number	Accession	Origin	Accession Number	Accession	Origin	Accession Number	Accession	Origin
1	Kiringis	landrace	24	Matuja-2	Landrace	47	Mucericeri-1	landrace
2	Obarodak	landrace	25	Nyadai	Landrace	48	990072-A	IITA
3	Kaleso	landrace	26	Tamisi	Landrace	49	990056	IITA
4	Kibandameno	landrace	27	Sabina	Landrace	50	990014	IITA
5	Gachaga	landrace	28	Bwanatereka	Landrace	51	Kitwa	landrace
6	Marakwet	landrace	29	Nabwire	Landrace	52	Migyera-2*	IITA
7	Waite	landrace	30	Opondo	Landrace	53	SS4*	IITA
8	Nyamambakaya	landrace	31	Serere-1	CIAT	54	Mucericeri-2*	landrace
9	CK-8	landrace	32	Bumba	Landrace	55	KME-1*	landrace
10	CK-9	landrace	33	Opongi	Landrace	56	MM96/4466-B*	IITA
11	Sabulenya	landrace	34	Iuderudu	Landrace	57	MM96/7151*	IITA
12	Nyakatanegi-1	landrace	35	MH95/0183-A	IITA	58	MM96/4884	IITA
13	Adhiambolela	landrace	36	MM96/1871-A	IITA	59	MM96/3868*	IITA
14	Kamisi	landrace	37	MM96/4466-A	IITA	60	MM96/1871-B*	IITA
15	Otugo	landrace	38	MM96/7688	IITA	61	MH95/0183-B*	IITA
16	Nyakatanegi-2	landrace	39	Migyera-1	IITA	62	Serere*	CIAT
17	Nyakatanegi-3	landrace	40	Kapchelelyo	Landrace	63	990005	IITA
18	Agriculture	IITA	41	Kapchetuya	Landrace	64	990072*	IITA
19	Matuja-1	landrace	42	KME-1	Landrace	65	MM96/5280	IITA
20	Mercury	landrace	43	Ex-Mariakani	Landrace	66	Arror 1	Wild type
21	Mageuna	landrace	44	960249	IITA	67	Arror 2	Wild type
22	Number 8	landrace	45	990183	IITA	68	Arror 3	Wild type
23	Sifros	landrace	46	Mue	Landrace	69	Arror 4	Wild type

** Accessions that were used in the biochemical study*

Table 2: Edaphic and Climatic descriptors of trial sites

Trial site	Altitude m-asl	Soil type	Annual Rainfall (mm)	Mean maximum Temperature (^{0}C)	Mean minimum Temperature (^{0}C)
Baringo (Marigat)	1066	Sandy	590	32.5	16.8
Kericho (Soin)	1315	Nitisols	1356	24.5	14.6
Nakuru (Subukia)	1676	Andosols	800	27.2	13.8

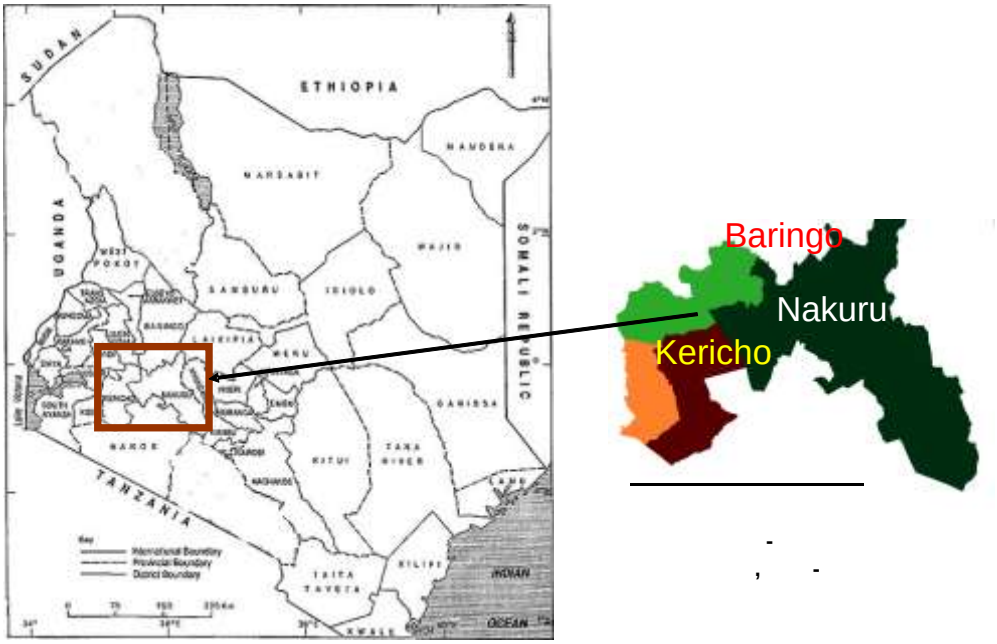

Figure 1: Map of Kenya showing cetral Rift Valley of Kenya where the study was done

3.2 Evaluation of Cassava Varieties

The accessions were planted in a randomized complete block design (RCBD) with three replications. The plot sizes were 3M x 4M while the plant spacing was 1M x 1M giving twelve plants per plot. Cassava cuttings measuring 15 cm long from each variety were planted in five rows. No fertilizers or herbicides were applied during the course of the experiment. Whereas the trials at Nakuru and Kericho sites were rain fed, the trial at Baringo was under

irrigation at least once per week. Destructive sampling of the plants was done on the 12th month after planting.

Harvest consisted of randomly uprooting 1 plant within a plot depending with the biggest root sizes and the corresponding youngest fully open leaves. Cyanide test was done in the field immediately after harvest on the leaves and roots. Root samples for biochemical analysis to determine the contents of protein, iron and zinc in the KARI Njoro quality laboratory were collected immediately whereas disease free leaves (about six) were randomly harvested from plots and subjected to molecular analysis in the KARI Njoro and Egerton University biotechnology laboratories.

3.3 Test for Cyanide Levels in Cassava

A modified picrate testing procedure as described by NARAPR, (2004) was used to determine cyanide levels in the test cassava germplasm in the field as follows; Two cm leaf disc was corked from young but fully expanded leaf. Using forceps, the leaf disc was placed into a glass vial (25mm outer diameter and 84mm high).Two to three drops of toluene were pipetted onto the leaf disc to release hydrogen cyanide from it. Filter papers (0.7cm by 1.0cm) were dipped into sodium picrate solution which was prepared by mixing 25g anhydrous sodium carbonate and 5g moist picrate acid in 1 litre of distilled water. The filter paper was suspended from the cap of the vial without touching the leaf disc. The filter paper gradually changed from light yellow to deep red, the shade depending on the amount of hydrogen cyanide released from the leaf disc. The set up was left to stand for four hours and color intensity noted.

For the cassava roots, one gram of the sample was placed inside the test tube. Five drops of toluene were added into the tube. A filter paper saturated with alkaline picrate was suspended above the sample by holding it with a plastic cap or rubber cork. Contact between the strip and the sample inside the tube was avoided and left at room temperature for twenty four hours and color intensity noted.

Both the leaf and root samples were rated on color using a scale of 1-9 depending on the basis of intensity of red color (Figure 2). The score structure ranged from scale of 1-4 representing 0 – 50 ppm, scale of 5-6 representing 50-100 ppm and scale of 7-9 representing over 100 ppm. This enabled the cassava genotypes to be ranked as low, medium and high in cyanide content respectively.

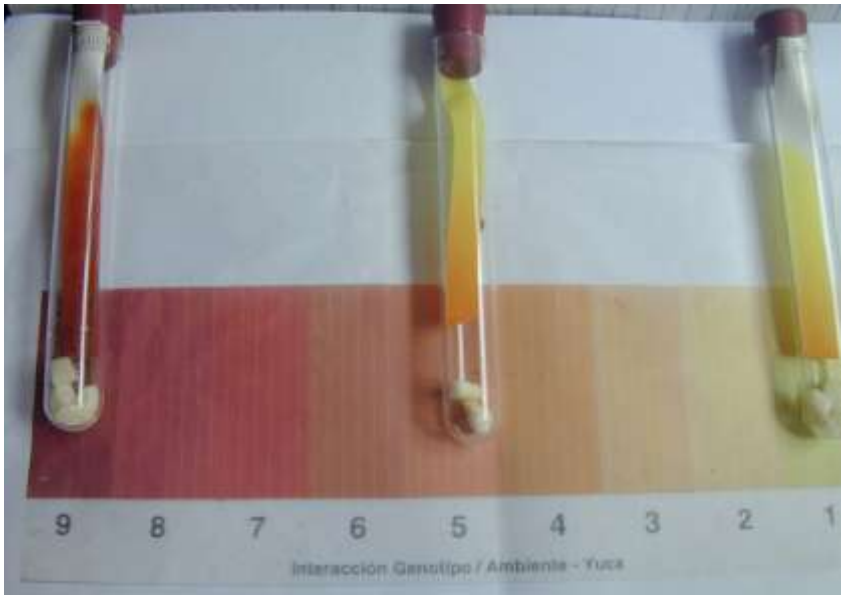

Figure 2: Color chart for cyanide analysis of cassava accessions using modified picrate testing procedure.

3.4 Biochemical Assays

3.4.1 Protein test

Protein analysis was done on root test samples four weeks after sampling following the Association of American Cereal Chemists (AACC, 1983). This was determined by Kjeldahl method. Protein content was the protein mass expressed as a percentage of the total sample. One gram catalyst (made up of 1000g Potassium sulphate, 5g Selenium and 25g Copper sulphate mixed together thoroughly) was weighed and put in numbered digestion tubes. One gram of sample was put in a digestion tube and 7.5 mls concentrated Sulphuric acid (Nitrogen free) added to it. This was digested in a digester (Tecator, Sweden) for 30 minutes at 398^{o}C or until the mixture cleared. It was then removed from the digestion block and left to cool for 20-30 minutes. After cooling, 25mls of distilled water was added to the

mixture then followed by addition of 25 mls NaOH which was added slowly to avoid the vigorous reaction of the acid and base. Distillation followed after addition of a base, into a conical flask with 0.1N boric acid for 4 minutes which contained bromophenol blue dye. Blue Color in boric acid changed to green upon receiving nitrogen in form of ammonia. This was then back titrated using 0.1N hydrochloric acid which changed the green color of the mixture in conical flask to blue. The titre volume was recorded and was used in the following formula for calculation of average percent protein;

$$\text{Average Protein \%} = \frac{(T-B) \times N \times 14.007 \times 100 \times F}{\text{Sample weight in (mg)}}$$

Where, T = Titre volume in sample

B = Titre volume for control

N = Normality of Acid to 4 decimal points

F = Factor 6.25.

The conversion factor used was 6.25 which is specific for cassava plant sample (Bradbury *et al.,* 1988*).* The whole process was replicated three times with a control consisting of all the above reagents and conditions except the experimental samples.

3.4.2 Zinc and iron test

The two micronutrients, iron and zinc were determined four weeks after sampling as follows: Three plants per variety were harvested at twelfth month after planting. Three cassava roots of different sizes (large, medium and small) were randomly selected per variety and labelled appropriately. The roots were then washed in tap water to remove soil and dirt, and air dried on a chux-lined surface. The roots were peeled with stainless steel knife and rinsed in distilled water and sliced thinly (0.5 cm^3) before drying. All samples were dried in an air oven at 60^0C for about 72 hours, then cooled to ambient temperature, milled by means of a hammer mill (coated with Teflon) and sieved through a mesh of 1mm diameter. The milled samples were stored in airtight plastic containers until required for analysis. Finely ground and dried cassava root samples (0.3 g) were weighed in a dry clean digestion tube. A digestion mixture (4 mls of selenium-sulphuric acid mixture) was added and allowed to react

at room temperature for at least 2 hours. The digestion tubes were heated to 200^{o}C in a block digester, allowed to cool and 3 successive portions of 1 ml of hydrogen peroxide added, waiting at least 10 seconds between additions. The tubes were returned to the block digester and temperatures adjusted to 330^{0}C. The digestion was complete when the digest became colourless or light yellow. The tubes were removed from the block digester and cooled to room temperature then the contents transferred into a 50ml volumetric flask and made up to the mark with deionised water.The digested samples were analyzed for trace metals (iron and zinc), using the Atomic Absorption Spectrophotometer, (Shimadzu Model AA-6300, Tokyo-Japan) (Figure 3). The instrument was calibrated using standard serial dilutions of iron and zinc ranging in concentration of 1.25 ppm, 2.5 ppm, 5 ppm, 7.5 ppm and 10 ppm. A calibration curve was drawn using the standards. Iron and zinc were measured by atomic absorption as they were absorbing radiations from element-specific hollow cathode lamps at a wavelength of 248.3 nm and 213.9 nm, respectively. The absorbencies obtained were used to estimate the concentrations of the metal ions from the curve in the different samples (Okalebo *et al.,* 2002).

Figure 3: Analysis of cassava accessions using Atomic Absorption Spectrophotometer (Shimadzu Model AA-6300, Tokyo-Japan).

3.5 Molecular Assay

3.5.1 DNA isolation

Two DNA isolation techniques described in sections 3.5.1.1 and 3.5.1.2 were tested for 69 cassava accessions as shown in Table 1b. Initially DNA was isolated from healthy young sampled leaf stored under two different conditions; silica gel dried and frozen (at -80^{o} C)

3.5.1.1 Macro DNA extraction using modified Cetyltrimethylammonium Bromide (CTAB) method.

Genomic DNA was isolated using the 2X CTAB method as described by IAEA (2002).The leaves (500 ***µg)*** were quickly ground in a pre chilled mortar using liquid nitrogen. Using a clean spatula that had been immersed in liquid N, the ground leaf material from the mortar was collected and transferred to the 500 ***µl*** preheated CTAB extraction buffer (2% CTAB, 100*mM* Tris HCl pH 8, 1.4*M* NaCl, 20*mM* EDTA, 1% DDT) in a tube. The sample was incubated with gentle agitation at 60^{o}C for 15 minutes in water bath. One volume (500 ***µl***) of 24:1 chloroform: isoamyl alcohol (CIA) was added to the sample and the two gently mixed at 45 rpm for 5 minutes. The sample was centrifuged at 13,000 rpm for 15 minutes at room temperature. The aqueous phase was transferred to a fresh tube using a pipette with wide opening. One volume (500 ***µl***) of 24:1 CIA was added again to the remaining contents of the earlier tube and mixed gently. The sample was then centrifuged at 13,000 rpm for 10 minutes at room temperature. The aqueous phase was transferred to the eppendorf tube containing the aqueous aliquote from the previous step. A 2/3 volume (350 ***µl***) of ice cold isopropanol was added and mixed gently. The sample was then stored overnight at 4 ^{o} C and centrifuged at 13,000 rpm for 20 minutes to collect the precipitate. The liquid was drained out and the pellet washed thrice with 70% ethanol (ETOH), centrifuged for 5 minutes at 13,000 rpm and 4^{o}C. The tube was inverted to drain out the ethanol and the pellet air dried and thereafter suspended in 100 ***µl*** 1 x TE buffer (10*mM* Tris HCl pH 7.5; 1*mM* EDTA).

3.5.1.2 Mini preparation DNA extraction protocol of plant DNA: version II.

DNA was isolated using the method of Dellaporta *et al.,* (1983). Leaf samples (500 μg) were ground using a mortar and pestle in liquid nitrogen. 500*ml* of extraction buffer (150μl of mercaptoethanol, 5*ml* of 1 *M* NaCl, 5*ml* of 1*M* Tris HCL pH8, 5*ml* of 0.5 EDTA pH 8 and 34*ml* of double distilled water) was added then mixed with the pestle. 33ml of 20% lauryl sulphate was added to remove high molecular mass protein. The contents were mixed by gentle invertion of the tube and then incubated at 65^{o}C for 10 minutes. 160*ml* 5*M* potassium acetate was added to precipitate salts. The sample was then kept on ice for 10 minutes and then centrifuged at 13,000 rpm for 5 minutes. The supernatant was removed and transferred in to a new tube. Cold isopropanol (600 μl) was added and tubes gently inverted in order to precipitate DNA. The sample was centrifuged at 13,000 rpm for 5 minutes to pellet the DNA. The pellet was washed thrice with 70% ETOH and centrifuged at 13000 rpm for 5 minutes to remove soluble ions (organic). The pellet was air dried for 15-20 minutes and then resuspended in 100 μl 1x TE buffer.

3.5.2 Purification of isolated DNA

The isolated DNA was purified as described. 500 µl CIA was added to the tube containing DNA and shaken gently for 15 min, followed by centrifugation at 12,000 rpm for 15 min. The aqueous phase was transferred to a new tube. 200 μl 1*M* NaCl-TE (1*M* NaCl, 10 *mM* Tris-HCL (pH 8.0), 1 *mM* EDTA) was added to the previous tube and shaken for 15 min. The previous tube was centrifuged for 15 min at 12,000 rpm. The aqueous phase was transferred to the new tube (the one containing aqueous phase) and mixed, followed by centrifugation at 12,000 rpm for 15 min. The supernatant was then transferred to a new tube. Ice cold isopropanol (700 μl) was added to the sample and mixed gently, and then sample centrifuged at 10,000 rpm for 5 min and the supernatant removed. Cold 75% ethanol (1000 μl) was added to wash the pellet, and sample centrifuged at 5,000 rpm for 5 min. The ethanol was poured out and the pellet air dried. The pellet was resuspended in 200 μl sterile distilled water and then incubated overnight at 55 °C and then stored at 4 ^{0}C.

3.5.3 Determination of DNA concentration, purity and integrity

To determine the intactness (integrity) and further confirm the quantity of the isolated DNA, a 3 *µl* aliquot of the DNA and 3 *µl* of gel loading buffer [50% Glycerol, 250*mM* EDTA (pH 8.0), 0.01% Bromophenol blue] were mixed. The mixed sample was electrophoresed at 50 V for 45 minutes on 1% molecular biology grade agarose gel (Sigma, UK) in 1X TBE [89*mM* Tris-HCL, pH 8.3; 89*mM* Boric Acid; 2.5*mM* EDTA] buffer alongside some uncut unmethylated lambda (λ) DNA standards (750 *ng*, 500 *ng*, 250 ng, 125 *ng*, 100 *ng*, and 83 *ng*), (Sigma, UK). The gel was stained in ethidium bromide (10 *ug/ml*), visualised on a ultra violet transilluminator at 254 *nm* and photographed. The band size and staining intensity of the isolated and electrophoresed DNA samples from cassava were compared to those of the λ DNA standards to determine concentration. Inergrity of the DNA was determined by absence of smears.

3.6 DNA Amplification by Polymerase Chain Reaction (PCR) and Selection of Primers

3.6.1 Optimization of SSR-PCRs

PCR optimization experiments were carried out using five DNA samples by varying the concentration of the template DNA, *Taq* DNA polymerase, annealing temperature, number of cycles and the Mg^{2+} salt concentration. Each optimized 10*µ*l PCR contained 1 *µl* 10XPCR buffer, 2.5 *mM* $MgCl_2$, *0.25 µl* of 100*nM* dNTPs (d ATP, d CTP, d GTP and d TTP, Sigma chemicals), 0.1 *µl* of 5 units of Taq DNA polymerase (New England Biolabs, UK), 3 *µl* of 1 *nM* primer pair, 1 *µl* of 25 *ng/µl* DNA template and 3.65 *µl* of Sterile distilled water (SDW).

3.6.2 SSR amplification

Optimised SSR assays were performed using fifteen pairs of oligonucleotide primer sequences (Table 3) obtained from Operon Technologies Inc. (USA). Using the optimized PCR assay the 15 oligonucleotide pairs were screened on a sub set of 5 samples from the entire collection to reveal those that would generate unambiguous polymorphic SSR alleles. The primers which gave scoreable amplicons were then used in the analysis of all the 69 test

cassava accessions. Following the initial screening, 7 SSR primers that amplified clear and reproducible SSR allele profiles were selected to study SSR variation in the samples. A negative control was also set in which sterile distilled water was used to replace template DNA. Amplification reactions were performed in a DNA thermocycler machine (Mastercycler) with a heated lid (94^0C) programmed as follows *;* one hot start cycle of 94^0C for 2 minutes followed by 30 cycles of 94^0C for 1 minute; 56^0C for 1 minute (DNA annealing); 72^0C for 1 minute and a final extension cycle of 72^oC for 10 minutes. The samples were then maintained at 4^o C.

3.6.3 Gel electrophoresis

The generated SSR amplicons by amplification were separated according to size by electrophoresis on high resolution 3% metaphor agarose gels run in 1XTBE (89*mM* Tris-HCL, pH 8.3; 89*mM* boric acid ,2.5*mM* EDTA) for 2 hrs at 100V. A 100 base pair DNA ladder (Sigma,UK) was used to estimate the sizes of amplification products. Gels were stained in ethidium bromide and visualised on a UV light trans illuminator at 254 *nm*.

3.6.4 Scoring of SSR segments

SSR alleles were scored from the reproducible PCRs set using different primer pairs. The size of SSR alleles was estimated from the gel photograph by comparison with 100 base pair ladder marker. Allele profiles were manually scored and compiled into a binary matrix. Positive amplification was treated as separate characters and was scored for the presence (1) or absence (0) of alleles. Only intensely stained unambiguous alleles were scored and used for statistical analysis.

Table 3: Properties of cassava SSR loci and their primer pairs

SSR locus	Type of repeat	Left primer 5'- 3'	Right primer 5'– 3'
SSRY9	$(GT)_{15}$	ACAATTCATCATGAGTCATCAACT	CCGTTATTGTTCCTGGTCCT
SSRY13	$(CT)_{29}$	GCAAGAATTCCACCAGGAAG	CAATGATGGTAAGATGGTGCAG
SSRY35	$(GT)_3GC(GT)_{11}(GA)_{19}$	GCAGTAAAACCATTCCTCCAA	CTGATCAGCAGGATGCATGT
SSRY51	$(CT)_{11}CG(CT)_{11}(CA)_{18}$	AGGTTGGATGCTTGAAGGAA	GGATGCAGGAGTGCTCAACT
SSRY66	$(GA)_{19}AAGA$	ATCTCAGCTTCCAACTCTTTCAGT	CGAAATGCTTGGAGACAGGTATAG
SSRY78	$(CT)_{22}$	TGCACACGTTCTGTTTCCAT	ATGCCTCCACGTCCAGATAC
SSRY106	$(CT)_{24}$	GGAAACTGCTTGCACAAAGA	CAGCAAGACCATCACCAGTTT

3.7 Data Analysis

Biochemical quality data on cyanide, protein, zinc and iron content was analyzed using the SAS package version 3.1. Two way Analysis of Variance (ANOVA) was carried out and means separated using LSD (SAS Institute, Inc., 1995). Pooled biochemical quality data from the three test sites was used to derive dendograms depicting the levels of genetic similarity between the cassava accessions based on the quality traits. The dendograms were derived using the software Minitab (1996). Stability of the biochemical quality traits was estimated by regression of genotype biochemical performance on the mean biochemical performance for each environment (Chekroun *et al.,* 1990). Sensitivity to environmental change (SE_i^2) was also computed to describe stability using the method described by Wachira *et al*., (2001).

$$SE_i^2 = \frac{\sum_{j=1}^{n} (\bar{y}_{ij} - \bar{y}_i)^2}{(n-1)}$$

Where for y_{ij}, the mean biochemical of the i^{th} genetic entry (i = 1, 2 m) in the j^{th} environment (j=1,2n).

For the molecular diversity study, the scored molecular data in a binary form (1=allele presence, 0=allele absence) was configured as an input file and analyzed with POPGENE version 1.31. (Yeh *et.al*., 1997). Proportion of polymorphic alleles (P) was derived as;

$$P = n_{pj}/n_{total}$$

Where n_{pj} is the number of polymorphic alleles and n_{total} is the total number of amplified alleles. Single population descriptive statistics were derived. Genetic variability within the test cassava accessions was determined through derivative of average expected heterozygosity (He) of the accessions using the POPGENE software assuming Hardy-Weinberg equilibrium and no population structure. The index proposed by Nei and Li (1979) was used to calculate genetic identity (S_{ij}) between cultivars (i) and (j) as;

$$S_{ij}=2N_{ij}/(N_i+N_j)$$

Where N_{ij}= the number of bands (alleles) in common between cultivars $_i$ and $_j$; N_i and N_j are the number of alleles for cultivars $_i$ and $_j$ respectively. The similarities were used to derive genetic diversity trees by average linkage cluster analysis (POPGENE version 1.31).

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Results

4.1.1 Biochemical characterization of cassava accessions

Biochemical data of the 14 test accessions is presented on Table 4. The analysis of variance (ANOVA) showed that genotypes (G) at the Baringo site were significantly (p<0.05) different in mean values for leaf and root cyanide, root protein and root zinc. At this site, accession MM96/3868 recorded the highest concentration of cyanide in leaves (71.25 ppm) while the local land race Mucericeri had the lowest concentration of cyanide (33 ppm).

For the roots, cultivar Migyera had the highest concentration of cyanide (112.14 ppm) with accession KME 1 having the lowest (40 ppm) followed by Mucericeri (43.75 ppm). Root Protein content at this site was above 2 percent only for the local land race Migyera and accessions SS4, MM96/7151 and MH95/0183. Accession MH95/0183 had the highest level at 3.47 percent. There were no significant (p>0.05) differences in the levels of iron in the roots although all accessions had levels that were above 20 ppm. Root zinc concentration ranged from 110.76 ppm (accession MH95/0183) to 39.39 ppm (Serere) with a farmer preferred accession Migyera at 104.76 ppm.

Table 4: Means for leaf and root cyanide, root protein, iron and zinc of cassava accessions tested at Baringo, Kericho and Nakuru sites of the central Rift Valley, Kenya.

Cassava Germplasm (Accessions)	Leaf Cyanide (ppm)			Root Cyanide (ppm)			Root Protein (%)			Root Iron (ppm)			Root Zinc (ppm)		
	Baringo	**Kericho**	**Nakuru**	**Baringo**	**Kericho**	**Nakuru**	**Baringo**	**Kericho**	**Nakuru**	**Baringo**	**Kericho**	**Nakuru**	**Baringo**	**Kericho**	**Nakuru**
Migyera	55.42ad	70.42a	64.17cf	112.14a	40.56bc	56.67ef	2.13cd	1.27	2.05	25.59	36.80cd	25.85	104.76b	64.56f	59.51bd
SS4	47.92ce	31.82c	53.33ef	73.33be	53.13bc	115.56ad	2.56bc	1.49	2.56	23.56	29.89d	22.49	49.67d	80.21bd	86.12ac
Mucericeri	33.00e	31.82c	64.58be	43.75de	23.89bc	75.56de	.	1.45	3.43	27.8	42.06c	17.81	69.63cd	86.23ac	91.88ab
KME-1	40.00ce	38.33c	53.75ef	40.00e	56.11bc	75.63de	1.96ce	1.52	2.85	26.75	37.10cd	34.17	58.50cd	93.07a	87.75ab
MM96/4466	54.58ad	44.04bc	42.92f	63.33be	21.67c	93.33ce	1.25e	1.4	3.19	34.18	37.93cd	19.72	87.14ac	84.19ad	54.46cd
MM96/7151	57.08ac	50.00abc	78.75ad	77.78ad	54.44bc	139.38ab	3.01ab	1.38	3.09	23.28	39.53cd	26.44	44.87d	78.47be	46.78d
MM96/4884	47.50ce	40.42c	74.17ae	66.11be	41.67bc	104.44bd	1.27e	1.15	3.08	28.55	41.19c	22.49	58.76cd	74.13cf	69.23bd
MM96/3868	71.25 a	42.92bc	62.50cf	89.44ac	51.67bc	92.22ce	1.62ed	1.54	2.29	29.24	43.33bc	23.85	62.58cd	72.04ef	82.71ad
MM96/1871	51.25ae	50.45abc	85.83ab	76.67bd	55.00bc	114.29ad	1.57ed	1.38	2.98	30.39	46.15bc	26.19	48.50d	76.48bf	69.67bd
MH95/0183	69.17ab	49.13bc	82.92ac	54.29ce	40.00bc	154.44a	3.47a	1.41	2.58	37.51	41.09c	23.76	110.76a	80.32ad	75.32bd
Serere	70.00ab	70.42a	90.83a	75.00be	58.33bc	20.83f	1.29e	1.57	2.66	26.05	40.99c	27.16	39.39d	89.13ab	95.81ab
990067	35.42de	40.00c	58.18def	93.13ab	96.67a	78.00de	1.98ce	1.49	3.02	34.37	44.59bc	30.48	62.83cd	67.04ef	86.38ac
990072	49.58be	49.58bc	78.33ad	88.33ac	66.11ab	132.50ac	1.98c-e	1.43	2.72	31.69	52.97ab	27.85	71.74bd	75.70cf	82.20ad
KME-61	55.00ad	62.00ab	82.50ac	77.50ad	63.57ab	82.22de	1.35e	1.4	2.53	.	59.69a	24.44	.	72.93df	118.10a
Site Mean	**52.94**	**45.17**	**69.55**	**74.64**	**51.79**	**98.28**	**1.97**	**1.42**	**2.78**	**29.22**	**42.38**	**25.23**	**67.82**	**78.17**	**79.69**
LSD (p=0.05)	0.003	0.03	0.0001	0.03	0.05	0.0001	0.0001	n/s	n/s	n/s	0.001	n/s	0.002	0.004	0.05

Means with the same letters are not significantly (p>0.05) different according to LSD test.

At the Kericho site, significant (p<0.05) cultivar differences were recorded for mean leaf and root cyanide as well as root iron and zinc contents (Table 4). The farmer prefered Migyera and Serere recorded the highest leaf cyanide values (70.42 ppm) while Mucericeri and SS4 recorded the lowest (31.82 ppm). Root cyanide was highest in accession 990067 (96.67 ppm). The lowest level of root cyanide was recorded in accession MM96/4466 (21.67 ppm) while Mucericeri which is popular with farmers was at 23.89 ppm. For both leaf and root cyanide quality traits, the other accessions were either above or below the critical level of consumption (40 ppm) as shown in Table 4. The fourteen test accessions were not significantly (p>0.05) different from each other. The iron concentration was highest in accession KME 61 (59.69 ppm) and lowest in accession SS4 (29.89 ppm). The zinc levels were high in KME 1 (93.07 ppm) and lowest in the local land race Migyera (64.56 ppm).

There were also significant quality differences among the test cassava germplasm at the Nakuru site in mean leaf and root cyanide and root zinc content (Table 4). Serere had the highest levels of cyanide in leaves (90.83 ppm). Accession MH95/0183 had the highest root cyanide concentration (154.44 ppm) while Serere had cyanide root levels below the 40 ppm threshold (20 ppm). This study revealed no significant (p>0.05) differences among the accessions for root protein content at the Nakuru test site although all accessions recorded values that were over 2% with Mucericeri, MM96/4466, MM96/7151, MM96/4884 and 990067 all having three percent and above. There were no significant (p>0.05) differences among the test accessions for root iron content at the Nakuru site. Significant (p>0.05) differences were however observed for zinc in roots with KME 61 having highest concentration (118.10 ppm) and MM96/7151 the lowest (46.78 ppm).

The three test sites (S) were significantly (p<0.05) different and overall, the site means for leaf and root cyanide, root protein and root zinc contents were all higher at the Nakuru site when compared to the other two sites whereas mean root iron content was higher in Kericho when compared to the other two sites (Table 4). Pooled data for the

combined sites revealed that levels of cyanide concentration in leaves and roots were significantly (p<0.05) different among each other. In the roots, which is the most consumed part of cassava plant, accession 990072 had the highest level of cyanide (94.23ppm) while Mucericeri recorded the lowest (50.68 ppm) as other preferred farmer accessions Migyera and KME 1 having 67.73 ppm and 57.20 ppm respectively (Table 5).

From the mean biochemical data for all the test sites, five dendograms were derived depicting the levels of genetic similarity between cassava accessions based on biochemical traits. (Appendix 1-5). For leaf cyanide, at a 70 % similarity coefficient, four cluster groups were delineated (Appendix 1). Cluster one comprised of accession MM96/3868. Cluster two with accessions SS4, Mucericeri, KME-1 and 990067 formed a low leaf cyanide group. Medium leaf cyanide content accessions formed cluster three which comprised accessions Migyera, MM96/4884, MM96/7151, MM96/1871, KME-61 and MH95/0183. Cluster group four included the high leaf cyanide accession Serere. A dendogram derived from the root cyanide data is presented in appendix 2. At a 70 % similarity coefficient, five cluster groups were delineated with cluster one comprised of accession Migyera. All other test accessions clustered in group 2 apart from accession KME-1 which formed cluster three, while cluster groups four and five comprised of accessions Serere and 990067 (Appendix 2). At a 60 % similarity coefficient, the root protein trait formed three major cluster groups with groups one and three having accessions Migyera and MM96/7151 respectively and group two containing all the other varieties (Appendix 3). A dendogram derived from the root Zinc data is presented in Appendix 4. At a 65 % similarity coefficient, accession Migyera formed a distinct out-group, with groups two and three comprised of accessions MM96/4466 and MH95/0183, respectively. The other accessions were in group four. At a similarity coefficient of 70 %, root iron content clustered the fourteen test accessions into three major groups with accession Mygyera forming a distinct out group with group two comprised of accessions MM96/4466 and MH95/0183. The rest of the accessions clustered in group three (Appendix 5).

Table 5: Combined sites data for cassava quality traits (cyanide in leaves and roots, protein in roots, iron and zinc in roots) in fourteen accessions tested at Baringo, Kericho and Nakuru sites of central Rift Valley, Kenya.

Cassava Germplasm (Accessions)	Leaf Cyanide (ppm)	Root Cyanide (ppm)	Root Protein (%)	Root Iron (ppm)	Root Zinc (ppm)
Migyera	50.86def	67.73cdef	1.77	29.86	78.37
SS4	44.71f	81.73abc	2.18	25.53	74.79
Mucericeri	44.09f	50.68f	2.35	29.23	82.58
KME-1	44.14f	57.20def	2.10	33.41	82.43
MM96/4466	47.18ef	55.21ef	1.79	31.99	77.86
MM96/7151	61.94bcd	88.65abc	2.50	30.16	56.71
MM96/4884	54.03cdef	70.74bcdef	1.83	30.74	67.37
MM96/3868	58.89bcde	77.78abcd	1.81	32.50	73.68
MM96/1871	62.86bc	79.40abc	1.92	34.24	64.88
MH95/0183	67.07ab	85.20abc	2.49	34.12	88.80
Serere	77.08a	55.21ef	1.74	31.93	72.15
990067	44.14f	91.14ab	1.92	36.74	73.24
990072	59.17bcd	94.23a	2.00	37.50	76.55
KME-61	67.50ab	75.00abcde	1.88	42.07	95.52
Site mean	56.02	73.89	2.02	32.71	75.56
LSD(p=0.05)	0.0001	0.0005	n/s	n/s	n/s

Means with the same letters are not significantly ($p_{>}0.05$) different according to LSD test.

To differentiate the test sites in terms of their influence on biochemical quality traits of cassava, dendograms for the three sites were also derived. The first dendogram showed that the three test sites were identical at a similarity coefficient of 78.38 % with regard to expression of the leaf cyanide trait (Appendix 6). For the expression of root cyanide, two major groups were differentiated at a similarity coefficient of 46 % with one forming a sub group of Nakuru and Baringo sites at 70 % which represented sites with higher levels of hydrogen cyanide. The Kericho site formed an independent cluster which represented a low root hydrogen cyanide site (Appendix 7). For root protein content, the sites were differentiated into two major groups at a similarity coefficient of a 43 % with the Kericho site isolated. The site elicited expression of low levels of root protein whereas the Nakuru and Baringo sites elicited expression of relatively higher levels of root protein content (Appendix 8). For root zinc content, at a 36 % similarity coefficient, the test sites differentiated in to two major groups with Baringo and Kericho forming a sub group at 62 % similarity (Appendix 10). The Nakuru site expressed higher levels of root zinc in the test samples. Iron root content classified the test sites into two major groups at a 59 % similarity coefficient with Nakuru and Baringo sites forming one sub group at 72 % (Appendix 9). Kericho elicited expression of high levels of root iron.

4.1.2 Genetic stability of biochemical quality characteristics

There were significant site (S) x genotype (G) interactions for root cyanide, root protein and root zinc which indicated that biochemical quality traits in the test accessions varied differently due to environment (Appendix 11). Stability estimates for the assayed quality traits are presented in Table 6. The regression of mean accession trait values on environmental index resulted in significantly (p<0.05) heterogeneous regressions, with regression coefficients (b_i) ranging from 0.56 to 1.44 for leaf cyanide; -2.21 to 2.29 for root cyanide; 0.31 to 1.57 for root protein; 0.48 to 2.23 for root iron and -0.33 to 1.04 for root zinc.

Accessions which had the smallest environmental variance (SE_i^2) were; accessions MM96/4466 for leaf cyanide, KME 61for root cyanide, MM96/3868 for root protein, SS4 for root iron and 990072 for root zinc (Table 6). Farmers preferred

accessions Migyera, SS4 and KME 1 had at least two parameters with low SE_i^2. Additionally, the following accessions had the largest SE_i^2; MM96/1871 for leaf cyanide, MH95/0183 for root cyanide, 990072 for root protein, KME 61 for root iron and Serere for root zinc.

Table 6: Measures of stability estimated by the regression coefficient (b_i) and sensitivity to environmental changes (SE_i^2) statistic for five biochemical quality traits in fourteen cassava accessions tested at three sites in the central Rift Valley of Kenya.

Cassava Germplasm (Accessions)	**LC (ppm)**		**RC (ppm)**		**RP (%)**		**RI (ppm)**		**RZ (ppm)**	
	b_i	SE_i^2	b_i	SE_i^2	b_i	SE_i^2	b_i	SE_i^2	b_i	SE_i^2
Migyera	-0.36	56.76	0.12	1410.02	1.08	0.22	1.36	40.93	-0.26	614.84
SS4	0.98	124.81	0.71	1014.81	0.89	0.37	2.23	15.99	0.32	382.69
Mucericeri	1.43	343.17	0.88	679.49	0.68	0.98	0.72	148.25	0.55	133.75
KME-1	-0.56	71.6	0.72	318.34	1.00	0.46	0.91	28.45	0.33	346.49
MM96/4466	-0.56	31.46	0.64	1295.11	0.56	1.16	0.75	92.45	-0.23	326.75
MM96/7151	0.82	224.37	0.51	1925.68	0.58	0.93	0.95	74.22	0.15	356.13
MM96/4884	0.69	316.74	0.72	1001.09	0.59	1.16	0.93	91.02	0.74	61.63
MM96/3868	0.43	210.41	0.91	513.09	1.57	0.16	0.89	101.17	0.58	101.42
MM96/1871	0.58	408.02	0.76	900.02	0.74	0.76	0.85	110.73	0.41	212.9
MH95/0183	0.69	318.73	0.34	3888.45	0.31	1.06	0.79	83.69	-0.33	367.93
Serere	0.98	140.26	-0.57	769.75	0.77	0.52	1.03	69.27	0.20	950.31
990067	0.90	144.91	-2.21	99.83	0.87	0.60	1.23	53.11	0.37	157.72
990072	0.71	275.03	0.67	1142.05	1.05	2.21	0.66	183.09	1.04	27.88
KME-61	0.73	204.25	2.29	94.01	0.92	0.43	0.48	310.63	0.03	510.07

Correlation coefficients between cassava biochemical quality traits were not statistically significant (p>0.05) (Table 7). All the traits were nonetheless positively correlated except for leaf cyanide versus root protein; root cyanide versus root zinc and root protein versus root iron.

Table 7: Phenotypic correlation coefficient for cassava biochemical quality traits (leaf cyanide, root cyanide, root protein, root iron and zinc across the three test sites.

	Leaf Cyanide	**Root cyanide**	**Root protein**	**Root iron**	**Root zinc**
Leaf cyanide	1.000	0.137	-0.001	0.334	-0.003
Root cyanide	-	1.000	0.269	0.277	-0.248
Root protein	-	-	1.000	-0.233	0.002
Root iron	-	-	-	1.000	0.440
Root zinc	-	-	-	-	1.000

4.1.3 Molecular Characterization

The two extraction methods revealed that the method of leaf storage i.e (silica gel and frozen at 80^0C) impacted on quality of extractable DNA. Irrespective of leaf storage treatment, all DNA extracted by the CTAB method were degraded and the isolation efficiency was not repeatable between samples. The Dellarporta method yielded high quality DNA from fresh leaf samples (refrigerated) but degraded DNA from silica gel preserved samples. For the Dellarporta protocol, the DNA extracted from silica preserved samples showed variation in DNA yields. The variations could have been attributed to the different levels of secondary metabolites in the different accessions which resulted to the variations in efficiency of extraction of DNA observed. Since the fresh leaf samples exhibited minimal variations in quantity and quality of extracted DNA, this type of tissue was chosen in subsequent DNA isolation activities.

The concentration of isolated cassava DNA was estimated by comparing the band size of 3 *ul* of isolated DNA with that of uncut, unmethylated lambda (λ) DNA standards (750*n*g, 500*n*g, 250*n*g, 125*n*g, 100*n*g and 83*n*g) (Figure 4). The extracted DNA ranged in concentration from 27.8 *ng/µl* to 250 *ng/µl*. The isolated DNA was also high in molecular weight and was intact. Based on this, PCR optimization of cassava DNAs was carried out using diluted samples with a DNA concentration of 100 *ng/µl*, 50 *ng/µl*, 25 *ng/µl* and 18 *ng/µl*. This study established the 25 *ng/µl* DNA sample to be optimal for

PCR assays. All primers were diluted to 1 *nm* whereas $MgCl_2$ in the PCR was optimized using five serial dilutions of 2.5 *mM*, 3.5 *mM*, 4.0 *mM*, 4.5 *mM* and 5.0 *mM* per PCR. The 5.0 *mM* $MgCl_2$ concentration gave best observable amplicons. After trying different types of agarose, metaphore agarose was chosen for subsequent use due to its ability to resolve alleles that differed only in a few base pairs.

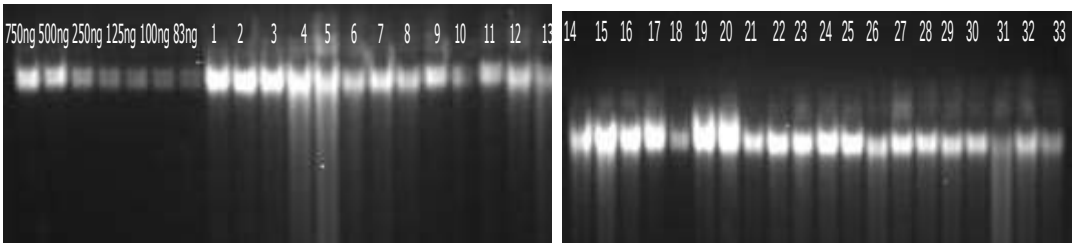

Figure 4: Lambda (λ) DNA standards (83 *ng* – 750 *ng*) and DNA samples isolated from 33 cassava accessions elecrtophoresed on 1 % molecular grade agarose gel in TBE buffer.(lane numbers represent in the same order the accessions in Table 1b).

The molecular size of SSR amplicons (alleles) differed with the selected primers and ranged from 230 bp to 310 bp with primer SSRY 13, SSRY 78 and SSRY 51, respectively. The smallest difference between the highest and lowest values of allele size was 10 bp at locus SSRY 13, and the largest difference (40 bp) was detected at locus SSRY 35. The allele sizes scored at the other remaining loci presented differences between 20 and 30 bp. A representative SSR profile of 19 cassava accessions with primer SSRY35 is presented in Figure 5. The seven SSR primers that were screened amplified a total of 39 alleles. The average number of alleles per primer pair ranged from 4 to 8. The number of polymorphic alleles ranged from 2 to 4. Percentage polymorphism ranged from 42.8 percent to 75 percent (Table 8 a). In most cases, the number of unique alleles (i.e., amplified products in just one individual) was positively correlated with the total number of alleles per locus and their size differences. A binary data matrix based on 69 accessions and 21 polymorphic alleles from seven primer pairs was generated (Appendix 12). This binary matrix was used for statistical analysis.

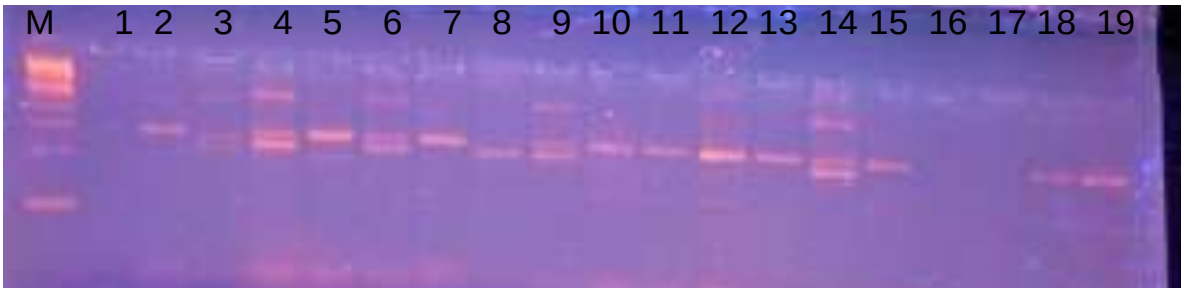

Figure 5: SSR alleles of 19 cassava accessions amplified by primer SSRY 35. M=100 bp ladder, lane numbers represent the accessions number in the same order as Table 1b.

Table 8a: Number of alleles amplified by SSR primers in test cassava germplasm

Primer Name	Total No. of alleles	No. of polymorphic alleles	Percent polymorphism (P)
SSRY9	7	3	42.8
SSRY13	4	2	50
SSRY35	8	4	50
SSRY51	6	3	50
SSRY66	4	3	75
SSRY78	5	3	60
SSRY106	5	3	60
Mean	**5.6**	**3**	**55.4**

The mean Nei's gene diversity index (H_e) and shannons information index (I) estimates of heterrozygosity, for the 69 accessions of cassava are presented in Table 8b.

Table 8b: Genetic heterozygosity (H_e) and Shannons index (I) of 69 cassava accessions.

Heterozygosity (H_e)	Shannons index (I)
0.36 ± 0.15	0.53 ± 0.19

A genetic identity and distance matrix based on the proportion of shared (common) alleles (Nei, 1972) was derived using Popgene version 1.31 that was used to

establish the level of relatedness between the 69 accessions (Table 9). Others like Mue, a land race and 990072-B an IITA introduction were found to be the only one having the lowest genetic identity of 0.1 although there were nine pairs of accession, pairs with low genetic identity of 0.2 such as Nyamambakaya and Nyakatanegi-2. Estimation of molecular identity ranged from 0.2 between the following pairs of accessions; Nyakatanegi 3 and Nyamambakaya; Kamisi and Obarodak; Opondo and Kiringis; MM96/7688 and Nyakatanegi 3; Kapchetuya and Nyakatanegi 2; Ex-Mariakani and Kiringis; Mue and Obarodak; Mue and 990072 among others to 1.0 between accessions Agriculture and CK-9, Tamisi and Sifros, Nabwire and Sifros, Bumba and MM96/1871 among others. Pairs of accessions with an identity of 1.0 could not be distinguished by the 21 polymorphic alleles. This clearly suggests that these paired accessions could be identical genetically and possibly have only been given different names. The observation points to possible genetic redundancy of some accessions conserved in the national repository centers in Kenya. The collection held in these centres need to be rationalized to remove the genetically redundant accessions in order to contribute to generation of a truly core collection. Other accessions which are popular in Kenya and had high genetic identity of 0.9 included Kaleso and Kibandameno, the popular landraces of the Kenyan coast; CK-8 and CK-9 together with Nabwire and Bumba which are western province land races; Kapchelelyo, Kapchetuya and Marakwet land races which are exclusively grown at Kerio valley of Kenya among others mainly found in cluster IV.

Table 9: Nei's genetic identity matrix for 69 cassava accessions based on 21 polymorphic SSR alleles. Above diagonal is identity and below diagonal is distance. Names of accessions are as indicated by table 1b.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
1	****	0.6	0.5	0.5	0.5	0.5	0.5	0.4	0.6	0.6	0.4	0.4	0.4	0.3	0.5	0.3	0.5	0.6	0.6	0.6	0.8	0.9	0.5
2	0.4	****	0.4	0.3	0.5	0.5	0.5	0.3	0.5	0.6	0.5	0.4	0.5	0.2	0.6	0.3	0.5	0.5	0.5	0.4	0.7	0.6	0.5
3	0.6	0.9	****	0.9	0.8	0.8	0.7	0.6	0.8	0.7	0.5	0.4	0.4	0.6	0.5	0.3	0.5	0.7	0.4	0.5	0.6	0.6	0.5
4	0.8	1.1	0.1	****	0.9	0.8	0.8	0.6	0.7	0.7	0.6	0.6	0.5	0.7	0.4	0.5	0.5	0.7	0.4	0.6	0.6	0.5	0.5
5	0.7	0.7	0.2	0.1	****	0.7	0.9	0.6	0.7	0.8	0.7	0.6	0.6	0.6	0.5	0.4	0.6	0.9	0.5	0.7	0.6	0.7	0.6
6	0.7	0.8	0.2	0.2	0.4	****	0.7	0.6	0.8	0.7	0.4	0.4	0.4	0.6	0.4	0.3	0.5	0.7	0.4	0.5	0.6	0.6	0.7
7	0.8	0.6	0.4	0.2	0.1	0.4	****	0.6	0.5	0.7	0.6	0.6	0.5	0.5	0.4	0.5	0.5	0.7	0.4	0.6	0.6	0.5	0.5
8	1	1.1	0.5	0.4	0.5	0.5	0.4	****	0.6	0.7	0.5	0.6	0.5	0.6	0.5	0.4	0.2	0.7	0.5	0.5	0.5	0.4	0.4
9	0.6	0.7	0.2	0.3	0.4	0.2	0.6	0.4	****	0.9	0.5	0.5	0.5	0.6	0.6	0.4	0.6	0.8	0.6	0.5	0.6	0.6	0.5
10	0.5	0.5	0.3	0.4	0.2	0.4	0.4	0.4	0.1	****	0.6	0.6	0.7	0.6	0.7	0.4	0.6	1	0.7	0.6	0.7	0.8	0.6
11	1	0.7	0.6	0.5	0.3	0.9	0.5	0.8	0.7	0.5	****	0.8	0.8	0.5	0.6	0.5	0.5	0.7	0.7	0.8	0.5	0.6	0.7
12	0.9	0.9	0.8	0.5	0.5	0.9	0.5	0.5	0.7	0.5	0.2	****	0.8	0.6	0.6	0.7	0.4	0.7	0.8	0.9	0.6	0.6	0.8
13	1	0.6	0.8	0.8	0.5	1	0.8	0.7	0.6	0.4	0.2	0.3	****	0.6	0.8	0.7	0.5	0.7	0.8	0.6	0.5	0.5	0.6
14	1.4	1.4	0.5	0.3	0.5	0.6	0.6	0.4	0.5	0.5	0.7	0.5	0.5	****	0.6	0.8	0.4	0.6	0.4	0.5	0.4	0.4	0.4
15	0.7	0.5	0.7	0.9	0.6	0.9	0.9	0.6	0.5	0.3	0.5	0.5	0.3	0.5	****	0.6	0.4	0.7	0.7	0.5	0.6	0.6	0.6
16	1.1	1.1	1.3	0.8	0.9	1.2	0.8	0.9	1	0.9	0.7	0.4	0.4	0.3	0.5	****	0.4	0.5	0.5	0.6	0.4	0.4	0.5
17	0.8	0.6	0.8	0.7	0.6	0.7	0.7	1.8	0.6	0.6	0.8	1	0.7	1	1	0.9	****	0.6	0.4	0.4	0.4	0.5	0.4
18	0.6	0.6	0.3	0.3	0.1	0.4	0.3	0.4	0.2	0	0.4	0.4	0.3	0.5	0.4	0.8	0.4	****	0.7	0.6	0.7	0.7	0.5
19	0.5	0.7	0.8	0.9	0.6	0.9	0.9	0.7	0.5	0.3	0.3	0.2	0.3	0.9	0.3	0.7	1	0.4	****	0.7	0.6	0.8	0.8
20	0.6	0.8	0.7	0.5	0.4	0.8	0.5	0.6	0.8	0.5	0.2	0.1	0.5	0.6	0.7	0.6	1	0.5	0.4	****	0.8	0.7	0.7
21	0.2	0.4	0.5	0.5	0.5	0.5	0.5	0.6	0.5	0.3	0.6	0.5	0.7	0.9	0.5	0.9	0.9	0.4	0.5	0.3	****	0.9	0.7
22	0.2	0.5	0.5	0.6	0.4	0.6	0.6	0.9	0.5	0.3	0.5	0.5	0.6	1	0.5	1	0.7	0.3	0.3	0.3	0.1	****	0.6
23	0.8	0.7	0.7	0.6	0.5	0.4	0.6	0.9	0.6	0.5	0.4	0.3	0.5	1	0.5	0.8	1	0.6	0.3	0.3	0.4	0.5	****
24	0.7	0.8	0.5	0.5	0.3	0.5	0.5	0.9	0.5	0.5	0.3	0.3	0.4	0.9	0.6	0.9	0.7	0.4	0.3	0.3	0.5	0.4	0.1
25	0.7	0.9	0.4	0.4	0.5	0.5	0.7	0.9	0.5	0.6	0.3	0.3	0.4	0.7	0.6	0.7	0.7	0.5	0.3	0.4	0.6	0.5	0.3
26	0.9	0.8	0.7	0.5	0.5	0.5	0.5	0.9	0.7	0.6	0.3	0.2	0.4	0.9	0.6	0.7	0.9	0.5	0.3	0.3	0.5	0.5	0
27	0.7	0.8	0.6	0.5	0.5	0.4	0.5	0.7	0.5	0.5	0.6	0.5	0.7	0.7	0.5	0.7	1	0.5	0.5	0.4	0.3	0.4	0.1
28	0.6	1	0.7	0.6	0.7	0.3	0.6	0.9	0.6	0.7	0.7	0.5	0.8	1	0.9	0.8	0.9	0.8	0.5	0.4	0.5	0.6	0.2

29	0.7	0.8	0.6	0.5	0.5	0.4	0.5	0.7	0.5	0.5	0.5	0.3	0.5	0.9	0.6	0.9	1	0.5	0.3	0.3	0.3	0.4	0
30	1.4	1.2	0.6	0.4	0.6	0.4	0.7	1	0.6	0.9	0.7	0.5	0.5	0.5	1	0.9	0.7	0.7	0.7	0.5	0.9	1	0.4
31	0.9	1.2	0.6	0.4	0.5	0.5	0.4	0.8	0.8	0.7	0.5	0.4	0.6	0.8	1	0.7	0.7	0.6	0.6	0.4	0.7	0.8	0.3
32	0.7	0.9	0.5	0.4	0.3	0.5	0.4	0.6	0.7	0.5	0.3	0.2	0.4	0.7	0.6	0.7	0.9	0.4	0.3	0.1	0.3	0.4	0.1
33	0.9	0.9	0.7	0.5	0.5	0.7	0.5	0.9	0.9	0.6	0.5	0.3	0.3	0.5	0.6	0.5	0.9	0.5	0.5	0.3	0.5	0.5	0.3
34	0.8	0.9	0.5	0.5	0.4	0.4	0.5	0.7	0.6	0.4	0.4	0.3	0.5	0.8	0.5	1	1	0.5	0.3	0.3	0.4	0.5	0.1
35	0.6	1.1	0.4	0.3	0.5	0.3	0.6	0.6	0.5	0.5	0.5	0.4	0.6	0.6	0.7	0.8	1	0.6	0.4	0.3	0.4	0.5	0.2
36	0.7	0.9	0.5	0.4	0.3	0.5	0.4	0.6	0.7	0.5	0.3	0.2	0.4	0.7	0.6	0.7	0.9	0.4	0.3	0.1	0.3	0.4	0.1
37	0.5	0.7	0.5	0.5	0.5	0.5	0.5	0.6	0.7	0.5	0.6	0.5	0.7	0.7	0.3	0.7	1.3	0.5	0.5	0.4	0.2	0.4	0.3
38	0.7	1	0.5	0.4	0.3	0.7	0.4	0.6	0.9	0.6	0.5	0.3	0.7	0.7	0.6	0.7	1.5	0.5	0.6	0.2	0.3	0.5	0.4
39	0.6	0.9	0.6	0.5	0.7	0.6	0.8	0.7	0.6	0.7	0.5	0.5	0.6	0.6	0.7	0.8	1.2	0.6	0.5	0.5	0.5	0.5	0.6
40	0.6	0.7	0.5	0.5	0.7	0.3	0.8	0.5	0.3	0.5	0.7	0.7	0.8	0.6	0.7	1.4	1.3	0.6	0.7	0.5	0.4	0.6	0.5
41	0.6	0.9	0.5	0.5	0.6	0.5	0.8	0.7	0.4	0.6	0.7	0.7	0.7	0.6	0.8	1.6	1.2	0.6	0.7	0.5	0.6	0.6	0.6
42	1.2	0.7	0.6	0.5	0.4	0.4	0.3	0.7	0.6	0.5	0.4	0.4	0.6	0.8	0.7	0.9	1.3	0.6	0.5	0.5	0.7	0.8	0.2
43	1.4	0.8	0.6	0.4	0.5	0.3	0.6	0.8	0.5	0.5	0.8	0.7	0.6	0.6	0.6	1	1.1	0.6	0.7	0.9	0.7	0.9	0.4
44	1	0.7	0.5	0.5	0.5	0.7	0.7	1.1	0.7	0.6	0.5	0.6	0.4	0.4	0.3	0.4	1	0.7	0.6	0.7	0.8	0.7	0.5
45	1.2	1.1	0.5	0.6	0.5	0.8	0.9	0.8	0.6	0.5	0.7	1	0.5	0.3	0.4	0.8	1.1	0.5	1	0.9	0.9	0.9	1.1
46	1.2	1.5	1	0.8	0.8	0.9	0.8	1.4	1.3	1.1	1.2	1.1	0.6	0.8	1.1	0.7	0.9	0.9	1.1	1.1	1.3	1.1	0.9
47	1	0.8	0.7	0.7	0.4	0.7	0.7	1	0.7	0.4	0.7	0.7	0.4	0.7	0.4	0.9	0.9	0.5	0.5	0.7	0.6	0.5	0.4
48	1	0.6	0.6	0.6	0.4	0.8	0.6	1	0.8	0.5	0.4	0.5	0.3	0.5	0.3	0.4	1	0.6	0.5	0.6	0.7	0.6	0.5
49	0.8	0.6	0.5	0.7	0.5	0.9	0.7	0.7	0.7	0.5	0.6	0.8	0.5	0.4	0.4	0.7	1.2	0.5	0.8	0.5	0.5	0.5	0.9
50	0.5	0.7	0.7	0.7	0.6	0.8	0.7	0.9	0.7	0.6	0.6	0.7	1	0.7	0.6	0.6	0.8	0.7	0.7	0.5	0.6	0.5	0.8
51	0.9	0.6	0.8	1	0.6	1.2	1	1.1	0.9	0.6	0.7	0.7	0.6	0.7	0.4	0.9	1	0.6	0.7	0.5	0.5	0.5	0.7
52	0.7	0.6	0.5	0.5	0.6	0.5	0.9	0.9	0.4	0.6	0.5	0.6	0.7	0.5	0.5	0.7	1	0.7	0.6	0.5	0.6	0.5	0.5
53	0.7	0.7	1	1	0.8	1.1	1	1.4	1	0.8	0.9	1	0.5	0.5	0.6	0.4	0.5	0.7	1	0.7	0.8	0.7	1.1
54	0.8	0.7	0.7	0.7	0.9	0.8	1.3	1.2	0.6	0.9	0.7	0.7	0.5	0.7	0.7	1.2	0.7	0.7	0.7	0.5	0.6	0.7	0.7
55	1.1	0.5	0.6	0.6	0.4	0.6	0.6	1.1	0.5	0.4	0.5	0.7	0.3	0.6	0.5	0.8	0.4	0.3	0.7	0.8	0.7	0.6	0.6
56	0.6	0.5	0.9	0.8	0.7	0.7	0.8	1.6	0.6	0.7	1	1.1	0.8	0.8	1	0.7	0.5	0.8	1.1	0.8	0.7	0.6	1
57	0.4	0.2	0.7	0.9	0.5	0.7	0.9	1.2	0.5	0.4	0.8	0.9	0.6	1.1	0.4	1.4	0.5	0.4	0.6	0.6	0.2	0.3	0.6
58	0.6	0.6	0.6	0.6	0.7	0.5	0.6	1.1	0.8	0.7	1.4	1.1	0.8	0.8	0.7	0.7	0.7	0.8	1.1	1.1	0.5	0.8	0.7
59	0.7	0.9	0.1	0.1	0.2	0.2	0.2	0.3	0.4	0.3	0.6	0.6	0.8	0.5	0.7	1.1	1	0.3	0.8	0.5	0.4	0.5	0.5
60	1.1	1.1	0.6	0.4	0.5	0.5	0.4	0.8	0.8	0.7	0.7	0.7	0.6	0.4	0.9	0.6	1.2	0.8	0.9	0.6	0.9	1	0.6

61	0.9	0.9	0.9	0.8	1	0.7	0.8	1.1	1.1	1	1	0.8	0.6	0.8	0.7	0.7	1.1	1.1	0.8	0.8	0.7	1.1	0.5
62	1	0.8	0.5	0.3	0.3	0.4	0.3	0.5	0.6	0.4	0.2	0.3	0.5	0.6	0.5	0.8	1.3	0.5	0.4	0.3	0.5	0.6	0.2
63	1.1	0.6	0.4	0.4	0.5	0.4	0.4	0.8	0.7	0.7	1.1	1	1	0.8	1	0.9	0.7	0.7	1.4	1.2	0.8	1.2	0.7
64	1.1	0.7	0.7	0.8	0.5	1	0.8	1.3	0.8	0.5	0.7	0.8	0.8	1.2	0.8	1.8	0.8	0.5	0.8	0.8	0.5	0.5	0.8
65	0.9	0.5	0.5	0.5	0.6	0.5	0.5	1	0.8	0.8	0.9	0.8	0.8	1	0.8	0.7	0.7	0.8	1.2	1	0.7	1	0.6
66	0.9	0.9	0.5	0.5	0.6	0.6	0.5	1	1	0.8	1.3	1	0.8	0.7	1	0.6	0.7	0.8	1.4	1	0.7	1	0.8
67	0.9	0.9	0.4	0.3	0.4	0.3	0.5	0.4	0.5	0.4	0.5	0.5	0.5	0.5	0.5	0.9	1	0.5	0.5	0.5	0.5	0.6	0.3
68	0.8	1	0.5	0.4	0.5	0.4	0.5	0.6	0.5	0.5	0.5	0.4	0.5	0.7	0.6	1	1	0.5	0.4	0.4	0.5	0.5	0.2
69	0.9	0.8	0.6	0.6	0.4	0.5	0.4	0.8	0.6	0.4	0.6	0.6	0.5	0.7	0.6	1.2	1.2	0.5	0.5	0.6	0.6	0.6	0.4

Table 9: Continued

	24	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**
1	0.5	0.5	0.4	0.5	0.6	0.5	0.2	0.4	0.5	0.4	0.4	0.6	0.5	0.6	0.5	0.5	0.6	0.5	0.3	0.2	0.4	0.3	0.3
2	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.4	0.4	0.4	0.3	0.4	0.5	0.4	0.4	0.5	0.4	0.5	0.5	0.5	0.3	0.2
3	0.6	0.7	0.5	0.5	0.5	0.5	0.6	0.5	0.6	0.5	0.6	0.7	0.6	0.6	0.6	0.5	0.6	0.6	0.5	0.6	0.6	0.6	0.4
4	0.6	0.7	0.6	0.6	0.6	0.6	0.7	0.7	0.7	0.6	0.6	0.7	0.7	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.6	0.5	0.5
5	0.7	0.6	0.6	0.6	0.5	0.6	0.5	0.6	0.7	0.6	0.7	0.6	0.7	0.6	0.7	0.5	0.5	0.5	0.7	0.6	0.6	0.6	0.5
6	0.6	0.6	0.6	0.7	0.7	0.7	0.7	0.6	0.6	0.5	0.7	0.7	0.6	0.6	0.5	0.6	0.7	0.6	0.7	0.7	0.5	0.4	0.4
7	0.6	0.5	0.6	0.6	0.6	0.6	0.5	0.7	0.7	0.6	0.6	0.5	0.7	0.6	0.7	0.4	0.5	0.4	0.7	0.5	0.5	0.4	0.5
8	0.4	0.4	0.4	0.5	0.4	0.5	0.4	0.5	0.5	0.4	0.5	0.5	0.5	0.5	0.6	0.5	0.6	0.5	0.5	0.4	0.3	0.4	0.2
9	0.6	0.6	0.5	0.6	0.6	0.6	0.5	0.4	0.5	0.4	0.5	0.6	0.5	0.5	0.4	0.5	0.7	0.7	0.5	0.6	0.5	0.5	0.3
10	0.6	0.5	0.5	0.6	0.5	0.6	0.4	0.5	0.6	0.5	0.7	0.6	0.6	0.6	0.5	0.5	0.6	0.5	0.6	0.6	0.5	0.6	0.3
11	0.7	0.7	0.7	0.5	0.5	0.6	0.5	0.6	0.7	0.6	0.7	0.6	0.7	0.5	0.6	0.6	0.5	0.5	0.7	0.5	0.6	0.5	0.3
12	0.7	0.7	0.8	0.6	0.6	0.7	0.6	0.7	0.8	0.7	0.7	0.7	0.8	0.6	0.7	0.6	0.5	0.5	0.7	0.5	0.5	0.4	0.3
13	0.7	0.7	0.7	0.5	0.5	0.6	0.6	0.6	0.7	0.8	0.6	0.5	0.7	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.7	0.6	0.5
14	0.4	0.5	0.4	0.5	0.4	0.4	0.6	0.4	0.5	0.6	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.7	0.7	0.5
15	0.5	0.5	0.5	0.6	0.4	0.5	0.4	0.4	0.5	0.5	0.6	0.5	0.5	0.7	0.5	0.5	0.5	0.4	0.5	0.5	0.7	0.6	0.3
16	0.4	0.5	0.5	0.5	0.4	0.4	0.4	0.5	0.5	0.6	0.4	0.5	0.5	0.5	0.5	0.4	0.3	0.2	0.4	0.4	0.7	0.5	0.5
17	0.5	0.5	0.4	0.4	0.4	0.4	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.4	0.3	0.4
18	0.7	0.6	0.6	0.6	0.5	0.6	0.5	0.6	0.7	0.6	0.6	0.5	0.7	0.6	0.6	0.5	0.5	0.6	0.5	0.5	0.5	0.6	0.4
19	0.7	0.7	0.7	0.6	0.6	0.7	0.5	0.6	0.7	0.6	0.7	0.7	0.7	0.6	0.5	0.6	0.5	0.5	0.6	0.5	0.5	0.4	0.3

20	0.8	0.7	0.8	0.7	0.7	0.8	0.6	0.7	0.9	0.8	0.7	0.7	0.9	0.7	0.8	0.6	0.6	0.6	0.6	0.4	0.5	0.4	0.3
21	0.6	0.5	0.6	0.7	0.6	0.7	0.4	0.5	0.7	0.6	0.7	0.7	0.7	0.8	0.7	0.6	0.7	0.6	0.5	0.5	0.5	0.4	0.3
22	0.7	0.6	0.6	0.7	0.6	0.7	0.4	0.4	0.7	0.6	0.6	0.6	0.7	0.7	0.6	0.6	0.5	0.5	0.5	0.4	0.5	0.4	0.3
23	0.9	0.8	1	0.9	0.9	1	0.7	0.7	0.9	0.8	0.9	0.8	0.9	0.8	0.7	0.5	0.6	0.5	0.8	0.7	0.6	0.3	0.4
24	****	0.8	0.9	0.8	0.8	0.9	0.7	0.7	0.9	0.8	0.8	0.8	0.9	0.7	0.7	0.5	0.6	0.6	0.7	0.5	0.5	0.4	0.5
25	0.2	****	0.8	0.6	0.7	0.7	0.8	0.8	0.8	0.7	0.7	0.9	0.8	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.4	0.5
26	0.1	0.2	****	0.8	0.8	0.9	0.7	0.8	0.9	0.8	0.8	0.8	0.9	0.7	0.7	0.6	0.6	0.6	0.8	0.6	0.5	0.3	0.5
27	0.2	0.5	0.2	****	0.8	0.9	0.6	0.6	0.8	0.7	0.8	0.8	0.8	0.9	0.7	0.5	0.6	0.5	0.7	0.6	0.6	0.5	0.4
28	0.2	0.4	0.2	0.2	****	0.9	0.6	0.8	0.8	0.7	0.8	0.9	0.8	0.7	0.6	0.4	0.7	0.5	0.7	0.5	0.5	0.2	0.4
29	0.1	0.3	0.1	0.1	0.1	****	0.7	0.7	0.9	0.8	0.9	0.9	0.9	0.8	0.7	0.5	0.7	0.6	0.8	0.6	0.5	0.4	0.4
30	0.4	0.2	0.3	0.5	0.4	0.4	****	0.8	0.7	0.7	0.7	0.8	0.7	0.5	0.5	0.6	0.6	0.6	0.6	0.7	0.5	0.4	0.6
31	0.3	0.3	0.3	0.5	0.2	0.3	0.3	****	0.8	0.7	0.8	0.8	0.8	0.6	0.6	0.5	0.6	0.5	0.7	0.5	0.4	0.3	0.7
32	0.1	0.2	0.1	0.2	0.2	0.1	0.3	0.2	****	0.9	0.9	0.9	1	0.8	0.8	0.6	0.6	0.6	0.7	0.6	0.5	0.4	0.5
33	0.2	0.3	0.2	0.3	0.4	0.2	0.3	0.3	0.1	****	0.8	0.8	0.9	0.7	0.7	0.5	0.5	0.5	0.6	0.6	0.6	0.5	0.7
34	0.2	0.3	0.2	0.2	0.3	0.1	0.3	0.2	0.1	0.2	****	0.8	0.9	0.8	0.7	0.5	0.6	0.5	0.8	0.7	0.6	0.4	0.5
35	0.3	0.1	0.3	0.3	0.2	0.1	0.2	0.3	0.1	0.3	0.2	****	0.9	0.8	0.7	0.6	0.7	0.6	0.6	0.6	0.6	0.4	0.5
36	0.1	0.2	0.1	0.2	0.2	0.1	0.3	0.2	0	0.1	0.1	0.1	****	0.8	0.8	0.6	0.6	0.6	0.7	0.6	0.5	0.4	0.5
37	0.3	0.5	0.3	0.1	0.4	0.2	0.6	0.5	0.2	0.3	0.2	0.3	0.2	****	0.8	0.5	0.6	0.5	0.6	0.6	0.6	0.5	0.4
38	0.3	0.5	0.3	0.3	0.5	0.3	0.7	0.5	0.2	0.3	0.4	0.4	0.2	0.2	****	0.5	0.5	0.5	0.6	0.5	0.5	0.4	0.4
39	0.7	0.4	0.5	0.7	0.8	0.7	0.6	0.6	0.5	0.7	0.6	0.5	0.5	0.7	0.6	****	0.6	0.7	0.4	0.6	0.5	0.3	0.4
40	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.6	0.5	0.7	0.5	0.3	0.5	0.5	0.7	0.5	****	0.9	0.6	0.6	0.4	0.5	0.2
41	0.5	0.5	0.6	0.7	0.6	0.6	0.5	0.7	0.6	0.7	0.7	0.5	0.6	0.7	0.8	0.4	0.1	****	0.5	0.4	0.3	0.4	0.3
42	0.4	0.5	0.3	0.4	0.4	0.3	0.5	0.4	0.4	0.5	0.2	0.5	0.4	0.5	0.5	0.8	0.5	0.6	****	0.7	0.6	0.4	0.3
43	0.6	0.5	0.4	0.4	0.6	0.4	0.4	0.7	0.5	0.5	0.4	0.4	0.5	0.5	0.7	0.6	0.5	0.8	0.4	****	0.7	0.5	0.5
44	0.6	0.5	0.6	0.5	0.8	0.6	0.6	0.8	0.6	0.5	0.5	0.5	0.6	0.5	0.6	0.7	0.9	1.1	0.5	0.4	****	0.7	0.5
45	0.9	0.9	1.1	0.7	1.4	1	1	1.4	0.9	0.6	0.9	0.9	0.9	0.6	0.9	1.1	0.7	0.8	1	0.7	0.3	****	0.3
46	0.8	0.6	0.8	0.9	0.8	0.9	0.6	0.4	0.6	0.4	0.7	0.8	0.6	0.9	0.8	0.8	1.6	1.3	1.1	0.7	0.6	1.2	****
47	0.4	0.5	0.5	0.4	0.8	0.4	0.6	0.7	0.4	0.3	0.3	0.5	0.4	0.4	0.6	0.9	1	1.3	0.6	0.4	0.3	0.6	0.3
48	0.5	0.5	0.5	0.4	0.8	0.5	0.7	0.8	0.5	0.4	0.5	0.6	0.5	0.4	0.5	0.8	1	1.3	0.5	0.4	0	0.4	0.6
49	0.7	0.9	0.9	0.6	1.3	0.8	1.1	1.2	0.7	0.5	0.7	0.9	0.7	0.5	0.7	0.9	0.6	0.7	0.8	0.8	0.4	0.2	1
50	0.8	0.7	1	0.6	0.7	0.8	1.1	0.6	0.8	1.1	0.6	0.7	0.8	0.6	0.9	0.7	0.7	0.9	0.7	1.1	0.5	0.8	1.1
51	0.6	0.9	0.7	0.5	1.3	0.7	0.9	1.3	0.7	0.7	0.7	1	0.7	0.5	0.8	1	0.7	0.8	0.9	0.9	0.5	0.5	1.7

52	0.6	0.5	0.6	0.5	0.7	0.6	0.7	1	0.8	1	0.7	0.5	0.8	0.6	0.8	0.4	0.4	0.5	0.5	0.6	0.3	0.6	1.6
53	0.8	0.7	1	0.8	1	1.1	0.8	0.6	0.8	0.6	0.9	1	0.8	0.8	1.2	0.7	1	0.9	1.3	1.1	0.5	0.5	0.4
54	0.5	0.4	0.6	1	0.9	0.7	0.3	0.7	0.6	0.6	0.7	0.5	0.6	0.9	0.9	0.6	0.3	0.2	1	0.8	0.9	0.8	1.2
55	0.5	0.7	0.5	0.7	1.1	0.7	0.8	1	0.7	0.5	0.7	1.1	0.7	0.9	1	0.9	0.8	0.8	0.6	0.6	0.5	0.5	0.8
56	1	1.1	1.1	1	0.9	1	1.1	1.1	1.3	1	1	1.1	1.3	1.3	1.4	1.2	0.8	1.1	0.8	1.1	0.6	0.9	0.9
57	0.6	0.9	0.7	0.6	1	0.6	0.8	1.1	0.7	0.7	0.6	0.9	0.7	0.5	0.8	0.9	0.5	0.7	0.9	0.7	0.7	0.8	1.4
58	1	0.8	0.8	0.7	0.6	0.7	0.7	0.5	0.7	0.5	0.5	0.6	0.7	0.5	0.7	1.2	0.8	1.2	0.8	0.7	0.6	0.9	0.4
59	0.5	0.5	0.5	0.5	0.6	0.5	0.6	0.5	0.4	0.5	0.4	0.4	0.4	0.4	0.4	0.6	0.5	0.6	0.5	0.6	0.7	0.7	1
60	0.7	0.7	0.7	0.7	0.4	0.5	0.5	0.4	0.5	0.4	0.4	0.4	0.5	0.7	0.7	1.1	0.6	0.9	0.4	0.5	0.4	0.6	0.5
61	0.7	0.6	0.6	0.7	0.4	0.5	0.4	0.3	0.5	0.3	0.3	0.4	0.5	0.5	0.7	1.2	0.6	0.9	0.6	0.7	0.6	0.9	0.4
62	0.4	0.4	0.3	0.4	0.4	0.3	0.4	0.3	0.3	0.4	0.1	0.3	0.3	0.4	0.4	0.6	0.5	0.6	0.1	0.3	0.4	0.7	0.9
63	1	0.8	0.7	0.8	0.7	0.8	0.7	0.7	0.8	0.8	0.8	0.8	0.8	0.8	0.9	1.1	0.8	1.1	0.5	0.4	0.7	1	1
64	0.8	1.2	0.8	0.8	1.8	0.8	1	1.7	0.8	0.8	0.7	1.2	0.8	0.8	0.9	1.4	1.2	1.2	1	1	1	0.8	2.2
65	0.8	0.7	0.6	0.7	0.6	0.7	0.7	0.7	0.7	0.7	0.8	0.7	0.7	0.7	0.8	0.9	1	1.4	0.6	0.5	0.6	1.2	1
66	1	0.8	0.8	0.8	0.7	0.8	0.7	0.5	0.7	0.5	0.6	0.7	0.7	0.7	0.8	1.3	1.2	1.6	0.8	0.8	0.6	0.8	0.4
67	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.3	0.4	0.5	0.6	0.3	0.5	0.3	0.4	0.5	0.6	0.6
68	0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.2	0.2	0.3	0.1	0.1	0.2	0.3	0.5	0.5	0.4	0.6	0.3	0.5	0.6	1.1	0.7
69	0.4	0.6	0.5	0.5	0.5	0.3	0.6	0.5	0.3	0.3	0.3	0.4	0.3	0.5	0.7	0.9	0.6	0.6	0.3	0.5	0.6	0.7	0.6

Table 9: Continued

	47	**48**	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**
1	0.4	0.4	0.4	0.6	0.4	0.5	0.5	0.5	0.3	0.5	0.6	0.5	0.5	0.3	0.4	0.4	0.3	0.3	0.4	0.4	0.4	0.4	0.4
2	0.5	0.5	0.5	0.5	0.6	0.5	0.5	0.5	0.6	0.6	0.8	0.5	0.4	0.3	0.4	0.4	0.5	0.5	0.6	0.4	0.4	0.4	0.5
3	0.5	0.5	0.6	0.5	0.4	0.6	0.4	0.5	0.5	0.4	0.5	0.5	0.9	0.5	0.4	0.6	0.7	0.5	0.6	0.6	0.7	0.6	0.6
4	0.5	0.5	0.5	0.5	0.4	0.6	0.4	0.5	0.5	0.4	0.4	0.6	0.9	0.7	0.4	0.7	0.7	0.5	0.6	0.6	0.7	0.7	0.6
5	0.6	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.7	0.5	0.6	0.5	0.8	0.6	0.4	0.8	0.6	0.6	0.6	0.6	0.7	0.6	0.7
6	0.5	0.5	0.4	0.5	0.3	0.6	0.3	0.4	0.6	0.5	0.5	0.6	0.8	0.6	0.5	0.7	0.7	0.4	0.6	0.6	0.7	0.7	0.6
7	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.3	0.5	0.4	0.4	0.6	0.8	0.7	0.4	0.7	0.7	0.5	0.6	0.6	0.6	0.6	0.7
8	0.4	0.4	0.5	0.4	0.3	0.4	0.3	0.3	0.3	0.2	0.3	0.3	0.7	0.5	0.3	0.6	0.4	0.3	0.4	0.4	0.6	0.6	0.4
9	0.5	0.5	0.5	0.5	0.4	0.7	0.4	0.5	0.6	0.6	0.6	0.4	0.7	0.5	0.3	0.5	0.5	0.5	0.4	0.4	0.6	0.6	0.5
10	0.6	0.6	0.6	0.6	0.5	0.5	0.4	0.4	0.7	0.5	0.7	0.5	0.7	0.5	0.4	0.7	0.5	0.6	0.4	0.4	0.7	0.6	0.7

11	0.5	0.7	0.5	0.5	0.5	0.6	0.4	0.5	0.6	0.4	0.5	0.2	0.5	0.5	0.4	0.8	0.3	0.5	0.4	0.3	0.6	0.6	0.6
12	0.5	0.6	0.4	0.5	0.5	0.5	0.4	0.5	0.5	0.3	0.4	0.3	0.5	0.5	0.4	0.8	0.4	0.5	0.4	0.4	0.6	0.7	0.5
13	0.7	0.7	0.6	0.4	0.5	0.5	0.6	0.6	0.7	0.4	0.5	0.4	0.4	0.6	0.6	0.6	0.4	0.5	0.4	0.4	0.6	0.6	0.6
14	0.5	0.6	0.7	0.5	0.5	0.6	0.6	0.5	0.5	0.4	0.3	0.4	0.6	0.7	0.4	0.5	0.4	0.3	0.4	0.5	0.6	0.5	0.5
15	0.6	0.8	0.7	0.6	0.7	0.6	0.6	0.5	0.6	0.4	0.6	0.5	0.5	0.4	0.5	0.6	0.4	0.5	0.4	0.4	0.6	0.5	0.6
16	0.4	0.7	0.5	0.5	0.4	0.5	0.7	0.3	0.4	0.5	0.2	0.5	0.3	0.6	0.5	0.5	0.4	0.2	0.5	0.5	0.4	0.4	0.3
17	0.4	0.4	0.3	0.4	0.4	0.4	0.6	0.5	0.7	0.6	0.6	0.5	0.4	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.4	0.4	0.3
18	0.6	0.5	0.6	0.5	0.5	0.5	0.5	0.5	0.7	0.4	0.6	0.4	0.7	0.5	0.3	0.6	0.5	0.6	0.4	0.4	0.6	0.6	0.6
19	0.6	0.6	0.4	0.5	0.5	0.5	0.4	0.5	0.5	0.3	0.5	0.3	0.4	0.4	0.4	0.7	0.2	0.5	0.3	0.2	0.6	0.7	0.6
20	0.5	0.5	0.6	0.6	0.6	0.6	0.5	0.6	0.4	0.4	0.5	0.3	0.6	0.6	0.4	0.7	0.3	0.5	0.4	0.4	0.6	0.7	0.6
21	0.5	0.5	0.6	0.6	0.6	0.5	0.4	0.5	0.5	0.5	0.8	0.6	0.7	0.4	0.5	0.6	0.4	0.6	0.5	0.5	0.6	0.6	0.5
22	0.6	0.5	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.6	0.7	0.4	0.6	0.4	0.3	0.5	0.3	0.6	0.4	0.4	0.5	0.6	0.6
23	0.6	0.6	0.4	0.4	0.5	0.6	0.3	0.5	0.5	0.4	0.5	0.5	0.6	0.6	0.6	0.8	0.5	0.5	0.6	0.4	0.7	0.8	0.7
24	0.6	0.6	0.5	0.4	0.5	0.5	0.4	0.6	0.6	0.4	0.5	0.4	0.6	0.5	0.5	0.7	0.4	0.5	0.4	0.4	0.7	0.7	0.7
25	0.6	0.6	0.4	0.5	0.4	0.6	0.5	0.7	0.5	0.3	0.4	0.4	0.6	0.5	0.6	0.7	0.4	0.3	0.5	0.4	0.7	0.8	0.6
26	0.6	0.6	0.4	0.4	0.5	0.5	0.4	0.5	0.6	0.3	0.5	0.4	0.6	0.5	0.6	0.8	0.5	0.5	0.6	0.4	0.7	0.8	0.6
27	0.6	0.7	0.5	0.6	0.6	0.6	0.4	0.4	0.5	0.4	0.5	0.5	0.6	0.5	0.5	0.7	0.4	0.5	0.5	0.4	0.7	0.7	0.6
28	0.5	0.5	0.3	0.5	0.3	0.5	0.4	0.4	0.3	0.4	0.4	0.5	0.5	0.7	0.7	0.7	0.5	0.2	0.5	0.5	0.7	0.8	0.6
29	0.6	0.6	0.4	0.4	0.5	0.5	0.3	0.5	0.5	0.4	0.5	0.5	0.6	0.6	0.6	0.8	0.4	0.5	0.5	0.4	0.8	0.8	0.7
30	0.6	0.5	0.3	0.3	0.4	0.5	0.5	0.8	0.5	0.3	0.4	0.5	0.6	0.6	0.6	0.6	0.5	0.4	0.5	0.5	0.7	0.8	0.6
31	0.5	0.4	0.3	0.5	0.3	0.4	0.5	0.5	0.4	0.3	0.3	0.6	0.6	0.7	0.7	0.7	0.5	0.2	0.5	0.6	0.7	0.8	0.6
32	0.6	0.6	0.5	0.4	0.5	0.5	0.4	0.5	0.5	0.3	0.5	0.5	0.7	0.6	0.6	0.8	0.4	0.5	0.5	0.5	0.8	0.8	0.7
33	0.7	0.7	0.6	0.3	0.5	0.4	0.6	0.5	0.6	0.4	0.5	0.6	0.6	0.7	0.7	0.7	0.4	0.5	0.5	0.6	0.8	0.7	0.7
34	0.7	0.6	0.5	0.5	0.5	0.5	0.4	0.5	0.5	0.4	0.6	0.6	0.7	0.6	0.7	0.9	0.5	0.5	0.5	0.5	0.8	0.9	0.8
35	0.6	0.5	0.4	0.5	0.4	0.6	0.4	0.6	0.3	0.3	0.4	0.6	0.7	0.7	0.7	0.7	0.4	0.3	0.5	0.5	0.8	0.9	0.7
36	0.6	0.6	0.5	0.4	0.5	0.5	0.4	0.5	0.5	0.3	0.5	0.5	0.7	0.6	0.6	0.8	0.4	0.5	0.5	0.5	0.8	0.8	0.7
37	0.6	0.7	0.6	0.6	0.6	0.5	0.4	0.4	0.4	0.3	0.6	0.6	0.7	0.5	0.6	0.7	0.4	0.5	0.5	0.5	0.7	0.7	0.6
38	0.6	0.6	0.5	0.4	0.4	0.4	0.3	0.4	0.4	0.2	0.5	0.5	0.7	0.5	0.5	0.7	0.4	0.4	0.5	0.5	0.6	0.6	0.5
39	0.4	0.4	0.4	0.5	0.4	0.7	0.5	0.6	0.4	0.3	0.4	0.3	0.5	0.3	0.3	0.5	0.3	0.2	0.4	0.3	0.5	0.6	0.4
40	0.4	0.4	0.5	0.5	0.5	0.7	0.4	0.7	0.4	0.4	0.6	0.4	0.6	0.6	0.6	0.6	0.4	0.3	0.4	0.3	0.7	0.7	0.6
41	0.3	0.3	0.5	0.4	0.4	0.6	0.4	0.8	0.5	0.3	0.5	0.3	0.6	0.4	0.4	0.5	0.3	0.3	0.3	0.2	0.6	0.6	0.6
42	0.5	0.6	0.4	0.5	0.4	0.6	0.3	0.4	0.5	0.4	0.4	0.4	0.6	0.7	0.6	0.9	0.6	0.4	0.6	0.4	0.7	0.7	0.8

43	0.7	0.6	0.5	0.3	0.4	0.5	0.3	0.4	0.6	0.3	0.5	0.5	0.6	0.6	0.5	0.7	0.6	0.4	0.6	0.4	0.7	0.6	0.6
44	0.7	1	0.7	0.6	0.6	0.7	0.6	0.4	0.6	0.6	0.5	0.6	0.5	0.7	0.6	0.7	0.5	0.4	0.6	0.6	0.6	0.5	0.6
45	0.6	0.7	0.9	0.5	0.6	0.5	0.6	0.4	0.6	0.4	0.4	0.4	0.5	0.5	0.4	0.5	0.4	0.4	0.3	0.4	0.5	0.3	0.5
46	0.7	0.5	0.4	0.3	0.2	0.2	0.7	0.3	0.4	0.4	0.2	0.7	0.4	0.6	0.7	0.4	0.4	0.1	0.4	0.6	0.5	0.5	0.6
47	****	0.8	0.6	0.5	0.6	0.4	0.6	0.4	0.6	0.5	0.6	0.6	0.5	0.6	0.6	0.6	0.4	0.4	0.4	0.6	0.7	0.7	0.7
48	0.2	****	0.7	0.6	0.7	0.7	0.6	0.4	0.6	0.6	0.5	0.6	0.5	0.7	0.6	0.7	0.5	0.5	0.6	0.6	0.6	0.6	0.6
49	0.5	0.3	****	0.6	0.8	0.6	0.7	0.5	0.6	0.5	0.6	0.4	0.6	0.5	0.4	0.5	0.4	0.5	0.3	0.5	0.6	0.4	0.6
50	0.8	0.5	0.5	****	0.6	0.8	0.7	0.5	0.3	0.7	0.5	0.4	0.4	0.5	0.4	0.6	0.3	0.2	0.3	0.4	0.4	0.5	0.4
51	0.6	0.4	0.2	0.4	****	0.7	0.6	0.6	0.5	0.5	0.8	0.3	0.4	0.3	0.3	0.5	0.3	0.6	0.3	0.3	0.4	0.4	0.4
52	0.9	0.4	0.5	0.3	0.4	****	0.6	0.6	0.5	0.6	0.5	0.3	0.5	0.4	0.3	0.6	0.4	0.3	0.4	0.2	0.5	0.5	0.4
53	0.5	0.5	0.3	0.3	0.4	0.6	****	0.6	0.5	0.7	0.5	0.5	0.3	0.5	0.5	0.3	0.3	0.2	0.3	0.5	0.4	0.4	0.4
54	0.8	1	0.7	0.8	0.5	0.5	0.5	****	0.5	0.5	0.7	0.4	0.4	0.4	0.5	0.4	0.3	0.4	0.3	0.3	0.5	0.6	0.4
55	0.5	0.5	0.5	1.2	0.7	0.7	0.6	0.8	****	0.6	0.6	0.5	0.5	0.4	0.4	0.5	0.5	0.6	0.5	0.5	0.5	0.4	0.5
56	0.7	0.6	0.6	0.4	0.7	0.5	0.4	0.8	0.5	****	0.6	0.6	0.4	0.5	0.5	0.4	0.4	0.4	0.4	0.6	0.4	0.4	0.3
57	0.5	0.6	0.5	0.7	0.3	0.6	0.7	0.4	0.5	0.4	****	0.6	0.5	0.3	0.5	0.5	0.4	0.7	0.4	0.4	0.5	0.5	0.4
58	0.4	0.6	0.9	0.9	1.3	1.3	0.6	0.9	0.7	0.5	0.5	****	0.6	0.7	0.9	0.5	0.7	0.4	0.7	0.9	0.6	0.6	0.5
59	0.7	0.6	0.5	0.9	0.8	0.7	1.2	0.9	0.6	1	0.7	0.5	****	0.6	0.5	0.7	0.7	0.6	0.7	0.7	0.8	0.7	0.6
60	0.5	0.4	0.6	0.7	1.3	0.9	0.7	1	0.8	0.6	1.2	0.4	0.5	****	0.8	0.8	0.6	0.2	0.5	0.7	0.8	0.6	0.7
61	0.4	0.6	0.9	0.9	1.3	1.3	0.6	0.7	1	0.8	0.8	0.1	0.7	0.2	****	0.6	0.5	0.2	0.5	0.7	0.7	0.7	0.6
62	0.4	0.3	0.6	0.6	0.7	0.5	1.1	0.9	0.6	1	0.7	0.7	0.3	0.3	0.5	****	0.6	0.5	0.6	0.5	0.8	0.8	0.8
63	1	0.7	0.9	1.2	1.1	1	1.2	1.2	0.6	0.8	0.8	0.4	0.3	0.5	0.7	0.5	****	0.4	0.9	0.8	0.6	0.5	0.5
64	0.8	0.8	0.7	1.7	0.5	1.2	1.7	1	0.4	1	0.3	1	0.6	1.8	1.7	0.8	0.9	****	0.4	0.4	0.4	0.4	0.4
65	1	0.6	1.1	1.2	1.1	0.8	1.2	1.2	0.8	0.8	0.8	0.4	0.4	0.6	0.7	0.6	0.1	0.9	****	0.8	0.5	0.5	0.4
66	0.5	0.6	0.8	1	1.1	1.4	0.7	1.2	0.8	0.5	0.8	0.1	0.4	0.4	0.3	0.7	0.3	0.9	0.3	****	0.6	0.5	0.5
67	0.4	0.5	0.5	0.8	0.9	0.7	0.8	0.6	0.6	1	0.7	0.5	0.2	0.2	0.3	0.2	0.6	1	0.7	0.6	****	0.9	0.9
68	0.4	0.5	0.9	0.6	0.8	0.6	0.9	0.6	0.9	1	0.7	0.5	0.3	0.4	0.3	0.2	0.8	0.9	0.8	0.6	0.1	****	0.7
69	0.4	0.5	0.6	0.9	1	1	0.9	0.9	0.7	1.1	0.8	0.7	0.5	0.3	0.5	0.3	0.8	0.9	0.9	0.8	0.2	0.3	****

The accessions with similar names among the accessions showed varied levels of genetic identity (Table 9). Varieties Serere, MH95/0183 and Migyera showed high genetic identity of 0.7. This shows that these collections with similar names could actually be the same. This was followed closely by accessions MM96/1871 and KME-1 each with 0.6 and 0.5 genetic identity respectively.

Among the Kenyan land races, genetic identity ranged from 0.2 to 1 (for example Mue and Kitwa with Sifros and Tamisi respectively) while among the IITA lines, identity ranged from 0.2 to 0.9 (for example MM96/1871 and 990072 with 990183 and 990056 respectively). The distance matrix was used to derive a dendogram using unpaired group mean linkage cluster analysis (UPGMA) (Figure 6).The dispersion of cassava accessions into various groups appeared to be random. The wild accessions collected at Kerio Valley clustered into different groups i.e Arror-1 clustered into group II whereas Arror-2, Arror-3 and Arror-4 clustred in group IV. The two wild accessions, Arror 2 and 4 were very close to each other and formed a tight cluster in group IV. The three wild types of cassava found in Kenya were spread in the dendogram. This wild type cassava accessions may be the only ones to be found in Africa as has previously been reported by Halsey *et al.*, (2008).

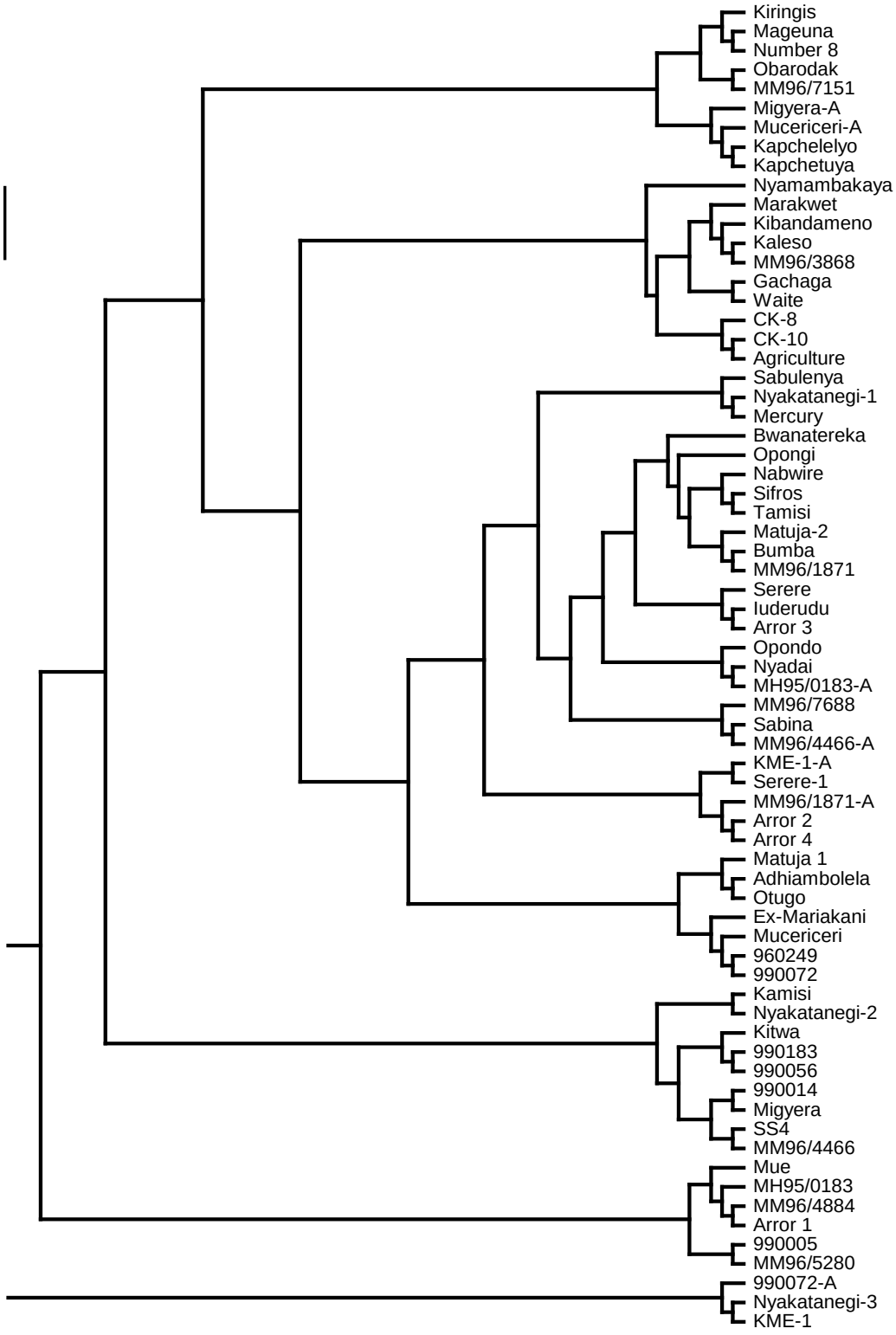

Figure 6: Dendogram of 69 cassava accessions based on SSR analysis of seven primers based on Nei's (1979) genetic distance.

Table 10: Distribution of 69 cassava accessions into clusters based on SSR data

Cluster No	No. of accessions	Name of accessions	Origin
Group I	3	Nyakatanegi-3	Landrace
		KME-1	Landrace
		990072-A	IITA
Group II	6	Mue	Landrace
		MM96/4884	IITA
		Arror -1	Landrace
		MH95/0183	IITA
		990005	IITA
		MM96/5280	IITA
Group III	9	Kamisi	Landrace
		Nyakatanegi -2	Landrace
		990183	IITA
		990056	IITA
		Kitwa	Landrace
		990014	IITA
		Migyera	IITA
		SS4	IITA
		MM96/4466	IITA
Group IV	42	Kaleso	Landrace
		MM96/3868	IITA
		Kibandameno	Landrace
		Marakwet	Landrace
		Gachaga	Landrace
		Waite	Landrace
		CK-8	Landrace
		CK-9	Landrace
		Agriculture	IITA
		Nyamambakaya	Landrace
		Sabulenya	Landrace
		Nyakatanegi-1	Landrace
		Mercury	Landrace
		Sifros	Landrace
		Tamisi	Landrace
		Nabwire	Landrace
		Matuja -2	Landrace
		Bumba	Landrace
		MM96/1871	IITA
		Opongi	Landrace
		Bwanatereka	Landrace
		Serere	CIAT
		Iuderudu	Landrace

		Arror-3	Landrace
		Nyadai	Landrace
		MH95/0183-A	IITA
		Opondo	Landrace
		Sabina	Landrace
		MM96/4466	IITA
		MM96/7688	IITA
		KME-1-A	Landrace
		Serere-1	CIAT
		MM96/1871-A	IITA
		Arror -2	Landrace
		Arror-4	Landrace
		Adhiambolela	Landrace
		Otugo	Landrace
		Matuja-1	Landrace
		Ex-Mariakani	Landrace
		960249	IITA
		990072	IITA
		Mucericeri	Landrace
Group V	9	Kiringis	Landrace
		Mageuna	Landrace
		Number 8	Landrace
		Obarodak	Landrace
		MM96/7151	IITA
		Migyera-A	IITA
		Kapchelelyo	Landrace
		Kapchetuya	Landrace
		Mucericeri-A	Landrace
Total	**69**		

At a distance of 68 %, the 69 accessions were clustered together into five marker based groups (Table 10). Cluster group IV had the largest number of accessions (42) comprised of two accessions originating from CIAT, 9 lines originating from IITA and 32 accessions originating from local land races. It is important to note that most land races were clustered into this group. Group I formed a distinct cluster group with accessions 990072 from IITA and landraces Nyakatanegi-3 and KME 1 appearing to be most distantly related from all others.

4.2 Discussions

4.2.1 Biochemical quality traits

Biochemical assays on the test cassava accessions revealed that quality traits varied significantly among the accessions and between test sites. At the Baringo site, accession MM96/3868 expressed the highest leaf cyanide above the safe limit of 40 ppm while the local land race Mucericeri expressed the lowest concentration (33 ppm) that is considered safe for human consumption without being processed. At the Kericho site, accessions Migyera and Serere expressed the highest leaf cyanide at 70.42 ppm whereas accessions Mucericeri and SS4 had the lowest levels of 31.82 ppm. Leaf cyanide levels for the Nakuru site for all accessions was way above the safe limit of 40 ppm.

Accession Migyera had the highest concentration of root cyanide (112.14 ppm) at Baringo site. This level of cyanide is highly poisonous if consumed raw. Accession KME 1 had the lowest and safest level of cyanide in roots at 40 ppm and therefore together with Mucericeri seemed to be the best accessions overall at below 40 ppm at this site in both cyanide in leaves and roots. Among the breeding lines, 990067 expressed the highest concentration of root cyanide (96.67 ppm) which was way above the safe critical level of consumption of 40ppm. Accession 990067 was the only one with very high levels of root cyanide at 96 ppm at the Kericho site that warranted thorough processing. At the Nakuru site, accession MH95/0183 had the highest root cyanide concentration and therefore could be highly poisonous when consumed fresh. Only Serere had root cyanide levels below the 40 ppm threshold which is the safe level for human and animal consumption. Variations in cyanide similar to those observed in this study for both leaves and roots have also been reported in a study of cassava cultivars used for consumption in the Sao Paulo state of Brazil (Lorenzo *et al*., 1993). Those accessions with above critical levels of cyanide in roots must be subjected to various methods of processing like fermentation, drying, boiling, soaking and grating in order to reduce the cyanide levels to safe limits. Similar suggestions have been made by Chiwona-Karltun *et al*., (1998) to Tanzanian and Malawian farmers that tuberous roots from bitter cultivars need to be processed before consumption.

The Protein values in the roots at Kericho site were all below the 2 percent threshhold value. High levels of root protein (> 3%) were elicited at the Nakuru site and on a few accessions at the Baringo site. These high levels of root protein content have also been reported in some land races and improved accessions of cassava by Chavez *et al*., (2005). Another study by Ceballos (2006) reported large differences in protein content of roots ranging from 0.95 % to 6.42 %. He suggested that a considerable proportion of these differences are genetic in nature and therefore are excellent possibilities for exploiting these differences and further increasing them by traditional breeding methods. However, our study suggest use of a different analytical method which is not based on the use of crude nitrogen since the cassava samples that are analysed in most studies even though dry, still have locked nitrogen in hydrogen cyanide that gives false high levels of protein.

All the accessions at Kericho site were relatively high in zinc and were all above 64 ppm in content. CIAT (2006) suggested these high levels of zinc may improve conversion of beta carotene to vitamin A, when high carotenoid cassava cultivars are consumed. An important aspect in enhancing micronutrient levels in cassava roots is ensuring a good agronomic background of micro nutrient-enriched genotypes (Dixon *et al*., 2005).

It is notable that all the test accessions in the three test sites gave higher or equal values for iron and zinc but not less than values observed by Chavez *et al*., (2005) for cassava collections from Meso America and Dixon *et al.*,(2005) for collections from Nigeria. CIAT (2006) suggested that the variation in zinc concentrations in cassava roots is due mostly to soil available zinc level and soil pH. Chavez *et al*., (2005 has also suggested that there is genotype variation for zinc in cassava.

The site means for the assayed biochemical quality parameters suggest that an environment suitable for the expression of high protein and zinc content, in this case Nakuru, may also elicit expression of high levels of leaf and root cyanide content. This calls for judicious selection of germplasm for such a site to ensure that positive quality

traits are maintained and negative traits such as high cyanide levels are selected against. The CIAT variety Serere-1 could be recommended for this site. However, there appears to be no obstacle to combining low HCN content with desirable root quality contents as reported by Hahn (1984) who produced a low HCN population of cassava by continuous selection and recombination where the protein contents were high.

Except for root iron content, the Nakuru site elicited expression of high levels of cyanide, protein and zinc in the tested cassava genotypes. This site is a non traditional growing site for cassava. Low root cyanogenic genotypes like Serere-1 (20 ppm) and Migyera (56.67 ppm) are recommended for this site. Choice of high cyanogenic content genotypes at this site could result in HCN poisoning of consumers and therefore processing is recommended before consumption. At the Kericho site, all the cultivars except 990067 (96.67 ppm) were relatively safe. At the Baringo site, except for accessions Migyera (112.14 ppm), and 990067 (93.13 ppm), some could be considered relatively safe for consumption while for others, simple processing methods like soaking and boiling could be applied. Processing has been shown to lower the levels of HCN in cassava roots (Nambisan, 1993).

From the pooled data on leaf and root cyanide, it was apparent that all the accessions were above the accepted critical level of 40 ppm. The local landrace Mucericeri however seemed to have faired well for both the traits with the lowest average cyanide concentration in ppm. This therefore indicates that proper processing needs to be applied for all cassava cultivated in the Central Rift Valley before human consumption.

Farmer preferred cultivars were superior in some biochemical quality traits. For example, the landrace Mucericeri had the lowest concentration of root cyanide (50.68 ppm) (Table 4). The farmer preferred landraces Mucericeri and KME 1 had over 2 % root protein content. Some improved accessions like MM96/7151, MH95/0183 and 990072 also had acceptable levels of over 2 % root protein content. Both farmer preferred and improved cultivars were high in root iron ad zinc content with KME 61 having highest levels for both quality traits at 32.7 ppm and 75.6 ppm for the two micronutrients,

respectively. This observation shows that local landraces could also serve as useful germplasm in any biofortification programmes.

4.2.2 Stability of biochemical quality traits

The significance of sites (S) x genotypes (G) effects demonstrates that genotypes responded differently to variations in environmental conditions. These variations could be attributed to the different climatic and edaphic conditions at the different sites (S). The significant (p<0.05) interaction of sites and genotypes indicated the necessity of testing cassava accessions at multiple locations over time for accurate characterization of genotype biochemical quality performance over divergent geographical regions.

The significant heterogeneity of regressions was also an indication of effective genotype X environment (G x E) interactions in cassava biochemical quality traits. Large variations in b_i indicate large differences in genotype responses to different environments. Regression values above 1.0 describe genotypes with higher sensitivity to environmental change (below average stability) and greater specificity of adaptability to high trait value environments. Regression coefficients decreasing below 1.0 provide a measure of greater resistance to environmental change (above average stability), and therefore increasing specificity of adaptability to low trait value environments (Chekroun *et al.*, 1990). Accession MM96/3868 for root protein and accessions SS4 and 990067 for root iron had large b_i values indicating their greater sensitivity to environmental changes for these biochemical quality traits. These accessions had relatively large biochemical quality trait values in environments that favored such large trait values but also expressed low trait values in low trait value environments when compared to the other genotypes. The inability of these genotypes to maintain their biochemical quality trait values under poor growing conditions may presumably be because of their lesser ability to tolerate stresses relative to others. All the other genotypes with low b_i values indicated their resistances to environmental changes and were therefore more adapted to low trait value environments. For the negative biochemical quality traits of leaf and root cyanide contents, accessions Mucericeri and KME 61 had large b_i values, respectively. These accessions exhibited great trait sensitivity to environmental change and therefore were

unstable with regard to expression of the cyanogenic glucosides and therefore could potentially contribute to HCN poisoning if they are grown in environments which favor expressions of high levels of cyanogenic glucosides. Accession 990067 had a regression coefficient of -2.2 for root cyanide content and cyanogenic glycosides decreased in this accession as the environmental index increased. Negative b_i indicates that as the environmental index increases, the performance of that trait decreases. This is not favorable for positive parameters like yield, nutrients etc but it is favorable for negative traits like hydrogen cyanide. This accession could therefore be ideal for high cyanogens environments. This agrees with the findings by Grace, (1977); Bruijn, (1971) and TRIP, (1993) who found that movement of a cassava genotype from one location to another could alter its cyanogenic potential because of differences in climate and soil characteristics and that different genotypes of cassava do not react the same way with the changing environmental conditions with regard to hydrogen cyanide content.

Environmental variances (SE_i^2) derived for all the biochemical quality traits assayed were heterogeneous an indication that the cassava accessions exhibited different levels of sensitivity to changes in the environment.

Accessions with the lowest SE_i^2 values were MM96/4466 for leaf cyanide; 990072 for root cyanide; MM96/3868 for root protein content; SS4 for root iron content and 990072 for root zinc content. These accessions exhibited greater stability in the expression of respective biochemical traits across the test sites.

The parametric approaches used in this study did not however seem to provide an overall picture of the individual genotype quality responses to the environment. Some genotypes exhibited stability for one type of measure and instability to the other i.e sensitivity to environmental change (SE_i^2) which is a type 1 stability measure and regression coefficient (b_i) which is a type 2 stability measures. Examples includes MM96/3868 for leaf cyanide, MH95/0183 and KME-61 for root cyanide, Migyera and MH95/0183 for root protein, SS4 and KME-61, 990072 and KME-61 for root zinc. This

is a problem that has been identified in G x E interaction studies (Wachira *et al*., 2001; Lin *et al*., 1986 ;).

Phenotypic correlation coefficients between biochemical cassava quality traits indicated that they are not linked and it is therefore possible to enhance one trait without adversely affecting the other. This completely agrees with work done on the same by Hershey (1982) and Iglesias (1994).

4.2.3 Molecular diversity of cassava accessions

Genetic diversity in cassava has previously been studied using DNA molecular markers. Among the molecular tools that have been used include isozymes (Sarria *et al.*, 1992), RFLPs (Angel *et al.,* 1992), RAPDs (Tonukari *et al.,* 1997) and SSRs (Fregene *et al*., 2001; Moyib *et al.,* 2007). In most studies low to medium genetic diversity has been observed. In the present study, also, there was generally medium genetic diversity among the land races and between the improved (introductions) accessions from IITA and Kenyan land races, as shown by the dendogram. This might be as a result of a common source of collection (IITA) from which the Kenyan cassava breeders and farmers choose their common desirable traits of cassava, such as potential for high yields and disease resistance. Because of the wide variability in biochemical quality traits, it is feasible to use some of the accessions assayed in this study as progenitors to introgress useful genes into improved cassava lines. This agrees with the study by Moyib *et al.,* (2007) on Nigerian collections.

SSR primers have shown high levels of polymorphism in many important crops including *Sorghum bicolor* (Smith *et al.,* 2000), *Triticum aestivum* L. (Ahmad, 2002), and *Cucumis melon* L. (Danin-Poleg *et al.,* 2001). SSR primers were also polymorphic in the Kenyan cassava cultivars assessed in this study. The results of this study showed that each of the 7 primer pairs detected polymorphisms among the 69 cassava accessions studied. Results of this study, therefore, established a collection of these 7 polymorphic SSR primers, SSRY 9, SSRY 13, SSRY 35, SSRY 51, SSRY 66, SSRY 78 and SRRY 106, that could readily be used for genotype identification and genetic diversity studies in

Kenyan cultivated cassava. One of these SSR markers, SSRY 51 is located at position N on genetic linkage map of cassava (Fregene *et al.,* 2003). A few highly polymorphic SSR markers like SSR 66 with PIC of 75 %, SSR 78 and SSR 106 all together with PIC of 60 %, can be used in genetic studies of cassava. This would reduce the necessity for applying many SSR primers for the identification of cassava cultivars in Kenya and, hence, contributes to saving time and also cut the cost of research studies for genotype identification and genetic diversity studies in the species.

The genetic identity of Kenyan land races ranged from 0.2 to 1 while for IITA introductions from 0.2 to 0.9. This shows that the Kenyan landraces are a rich source of diversity as compared to improved IITA introductions. Nonetheless, the relatively high genetic identity values for some landraces indicate close relatedness. Cassava is routinely propagated vegetatively and it is likely for two similar accessions to be assigned different names. This might also stem from the fact that the Kenyan landraces were domesticated in the same ecological zones with narrow genetic base while the improved were obtained from different exotic sources that might have diverse ecological ranges. The diversity index which is the probability that two randomly selected alleles in a given accession are different, estimated by (H_e) was 0.36 and the Shannon's index (I) was 0.53, indicating the average genetic diversity of cassava. Genetic diversity index (H_e) of cassava landraces and introductions was different from the Neotropics and Africa (0.46 to 0.62) but similar to that found in Guatemala as reported by Fregene *et al.,* (2003). The reliability of estimates for genetic variation such as (H_e) and (I) and genetic distances depends more on the number of loci than on the number of individuals sampled (Fregene *et al.,* 2003). Estimates of genetic differentiation ranged widely from locus to locus, underscoring the danger of assessing SSR diversity with a small set of SSR markers.

In the distribution of cassava into clusters, it is important to note that most land races were clustered into group IV. This may indicate a common ancestry for the local landraces. Since IITA is a secondary centre of diversity for cassava, this helps to explain why accessions from there were found in every cluster. This is due to the movement of cassava from centre of origin to other places within the region. Land races Nyakatanegi 3

and KME 1 together with accession 990072-A clustered together in group I and diverged from other accessions. This may be due to common ancestry or could be an indicator of duplicates. However, similarities in accessions can also arise due to convergent evolution, selection or sharing of common parentage.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Results obtained from this study revealed that there is great variation in different biochemical quality parameters in cassava grown in central Rift Valley studied which can be attributed to genotypes and environments that were used. The observed values for root protein and micronutrient contents suggest the potential for improving the nutritive value of cassava in Kenya exists.

The variation observed in cyanide concentrations calls for careful release of low cyanogenic cultivars to these non-cassava traditional areas. More caution is needed when handling cassava programmes in Nakuru district because of the very high levels of hydrogen cyanide recorded. For Baringo district, average processing among the varieties would drastically reduce hydrogen cyanide in the roots whereas at Kericho district, minimal processing would be required for the same. On the issue of cyanide detoxification, the high levels of protein at Nakuru site will be ideal in breaking down HCN to harmless biomolecules when cassava is consumed as staple or snack. On average, higher levels of micronutrients which is favorable for the human diet were recorded at Kericho district compared to Nakuru and Baringo sites. The site specificity requires that material is tested before release in each specific environment rather than observations on general performance of the selection.

The molecular study has also proved that SSR markers can be useful in breeding programmes of cassava allowing for the identification of new cultivars as well as assessment of genetic similarity/diversity among different genotypes. The average level of genetic diversity observed in the Kenyan landraces will benefit cassava germplasm conservation and enhancement efforts in Kenya, and contribute to the elucidation of forces that shape genetic differentiation in this asexually propagated allogamous crop.

5.2 Recommendations

The following recommendations can be drawn from this study:

1. The high micronutrient content landraces Mucericeri, KME-1 and KME-61 should be adapted for wide use by farmers and also for use in improvement programmes.
2. The following high root protein content landraces Mucericeri and KME-1 should be adapted for wide use by farmers and also for use in improvement programmes.
3. Only low root cyanogenic landraces Mucericeri and KME-1 should be introduced into the central Rift Valley. These accessions should also be incorporated in cassava improvement programmes.
4. Cassava tubers harvested from the Nakuru area should be processed before consumption because of high levels of hydrogen cyanide.
5. The landraces should be collected, conserved and maintained at the national cassava repository centre.
6. Further introduction of IITA improved lines should be rationalized on the basis of their distance from the local landraces.

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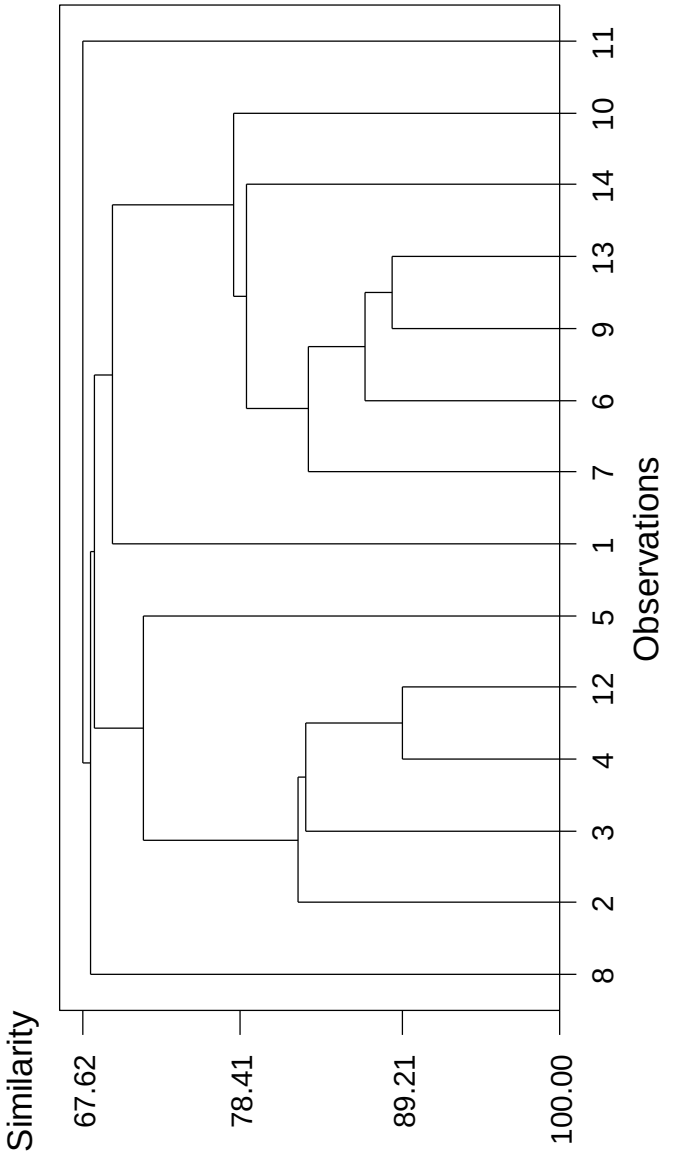
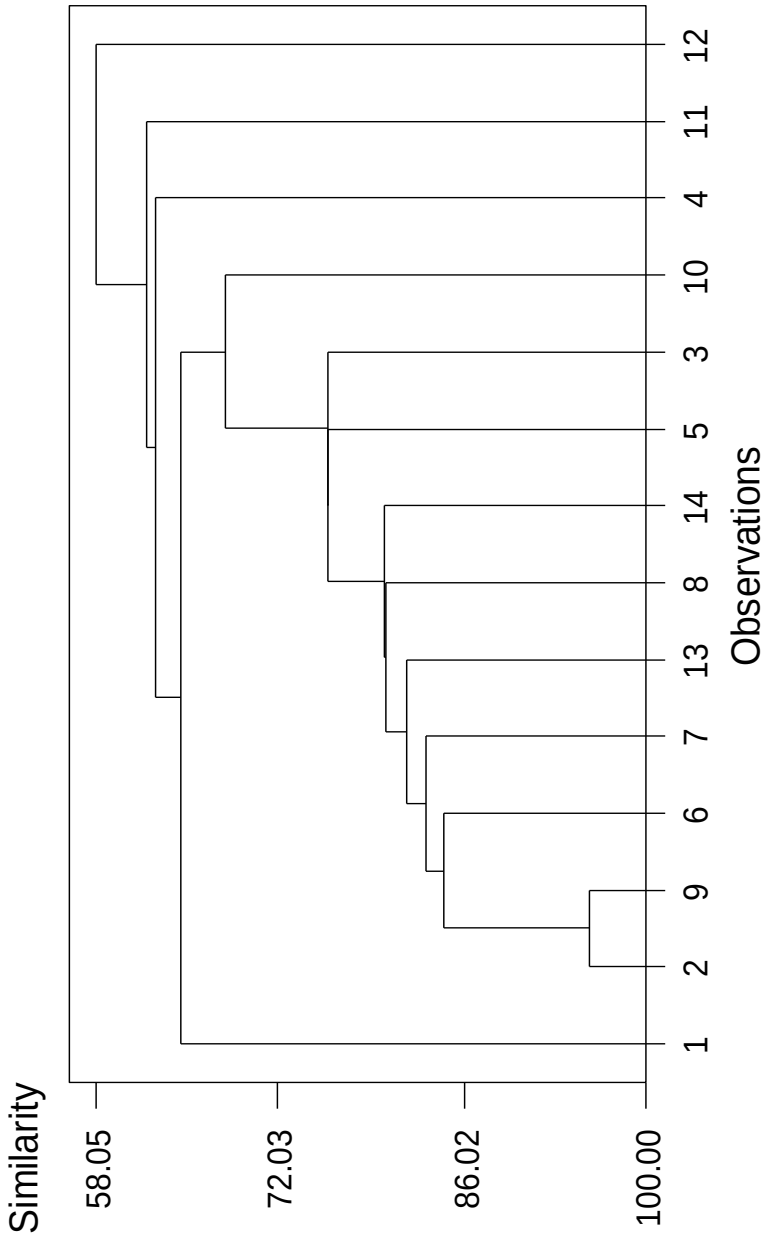
APPENDICES:

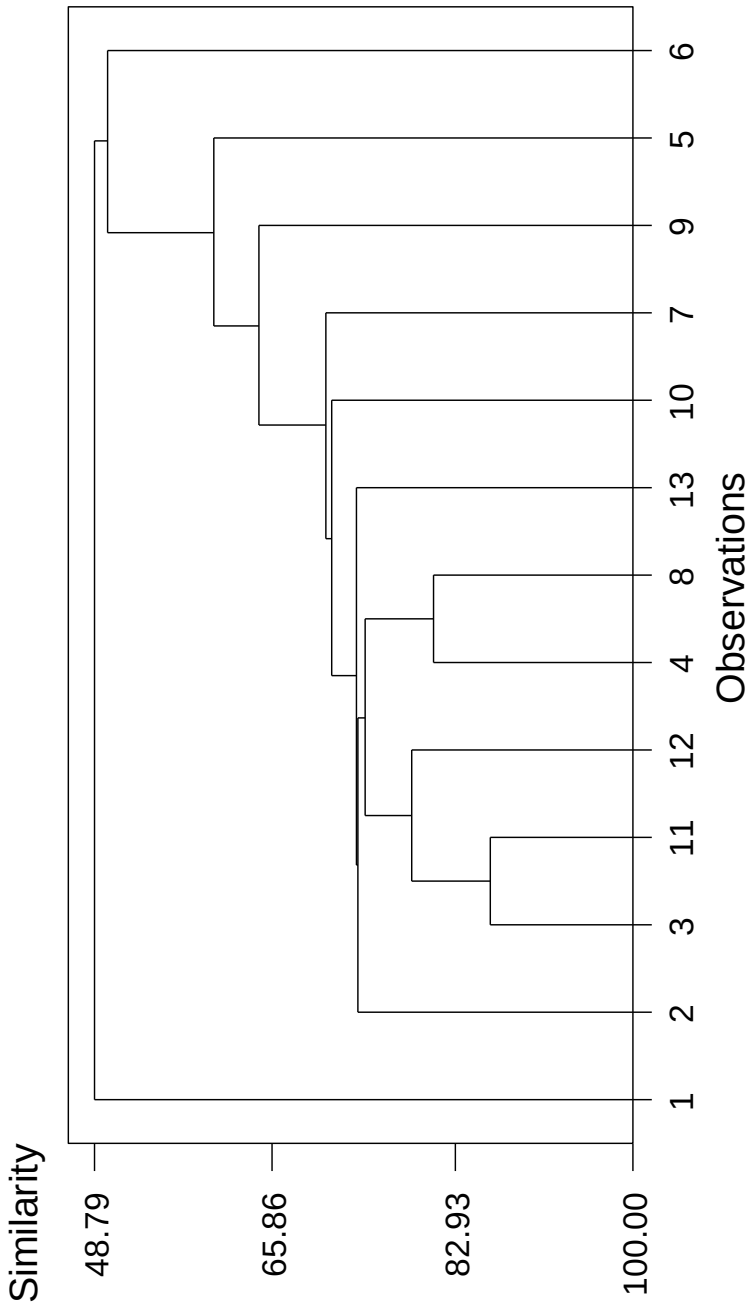
Appendix 1: Dendogram of standardized leaf cyanide data. Names of accessions are as presented in Table 1.



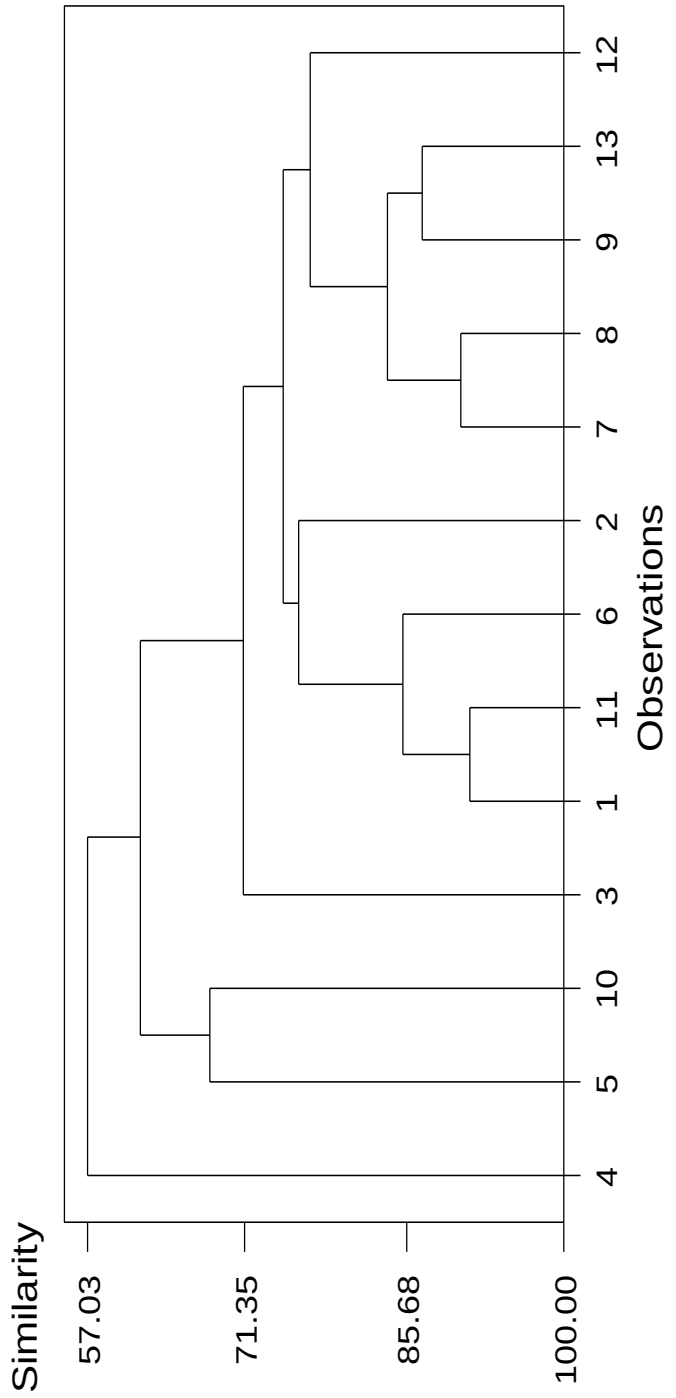
Appendix 2: Dendogram of standardized root cyanide data. Names of accessions are as presented in Table 1.



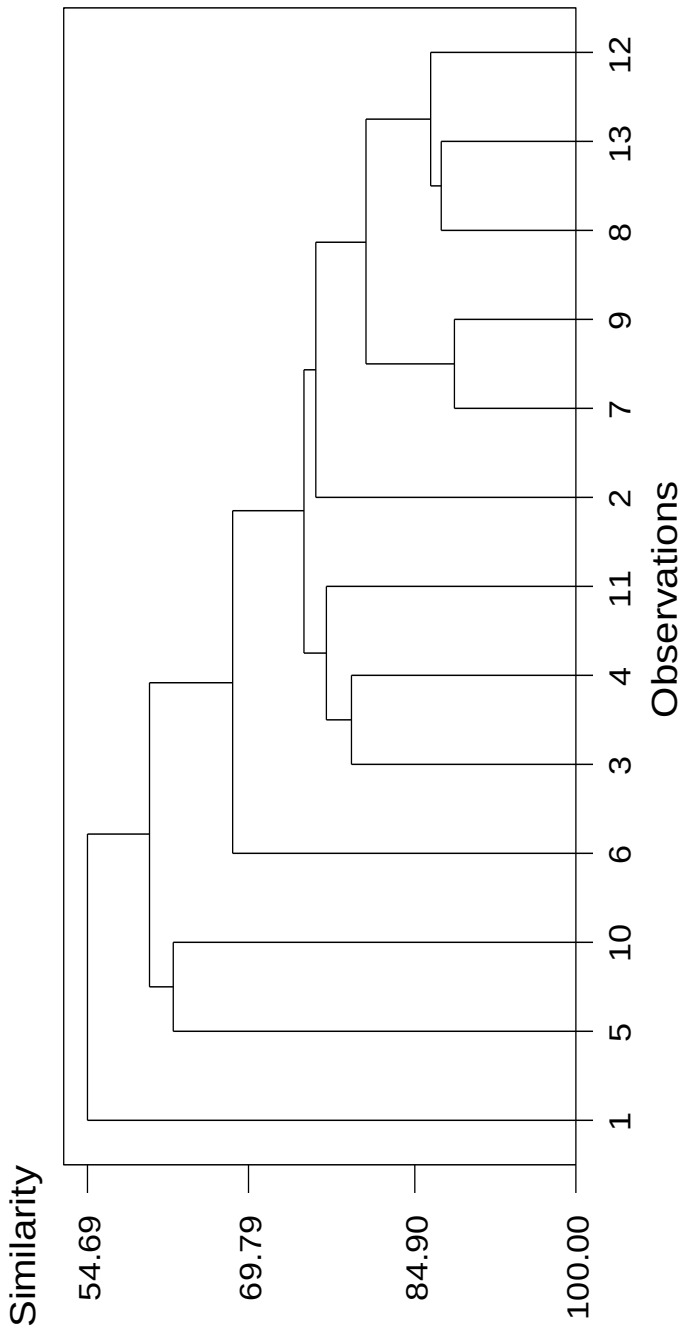
Appendix 3: Dendogram of standardized root protein data. Names of accessions are as presented in Table 1.



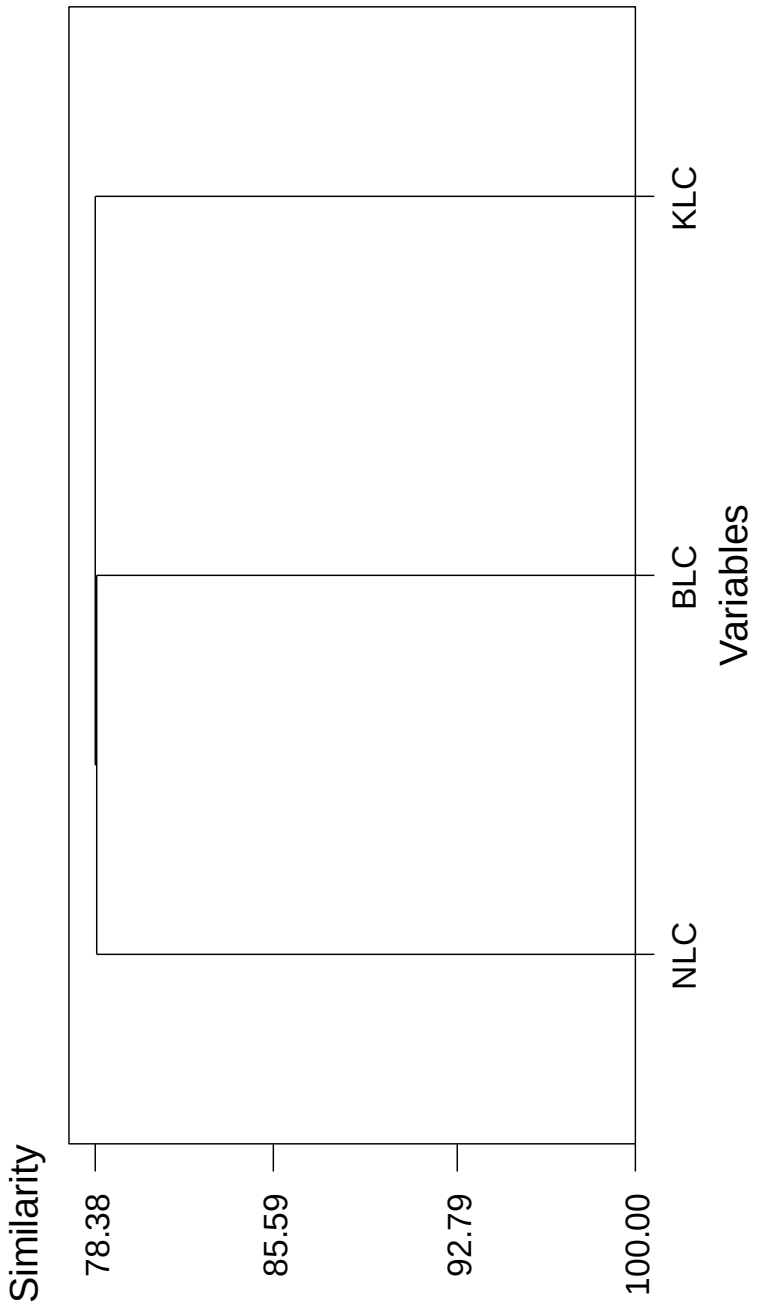
Appendix 4: Dendogram of standardized root iron data. Names of accessions are as presented in Table 1.



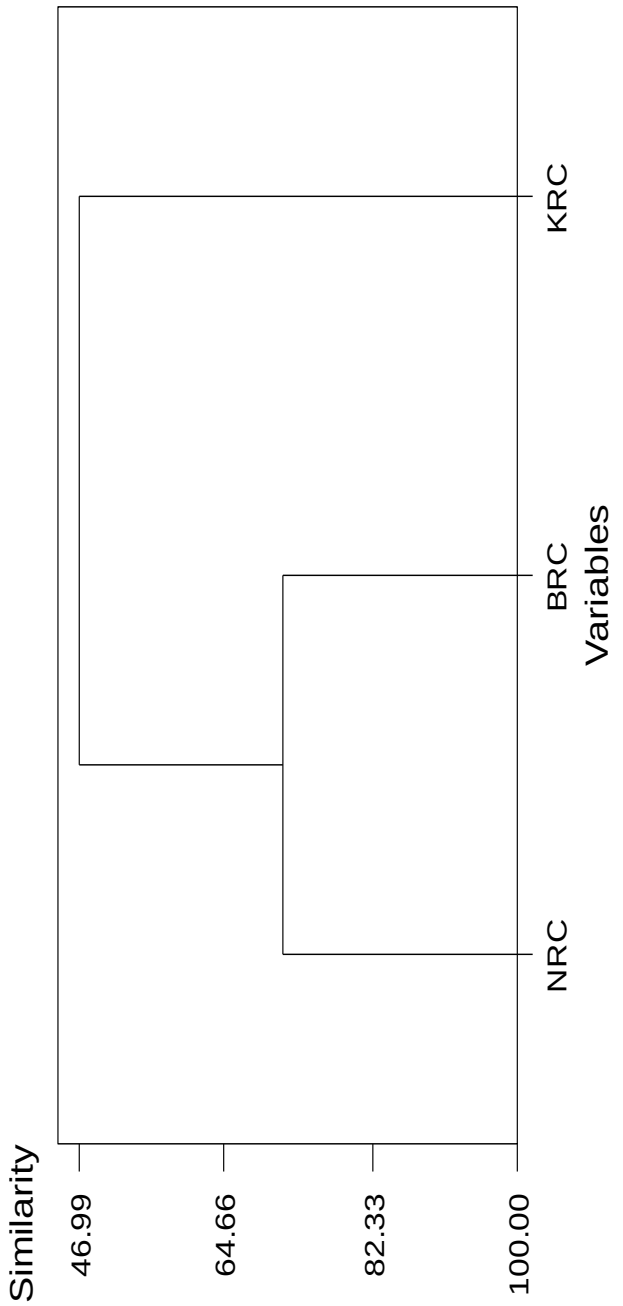
Appendix 5: Dendogram of standardized root zinc data. Names of accessions are as presented in Table 1.



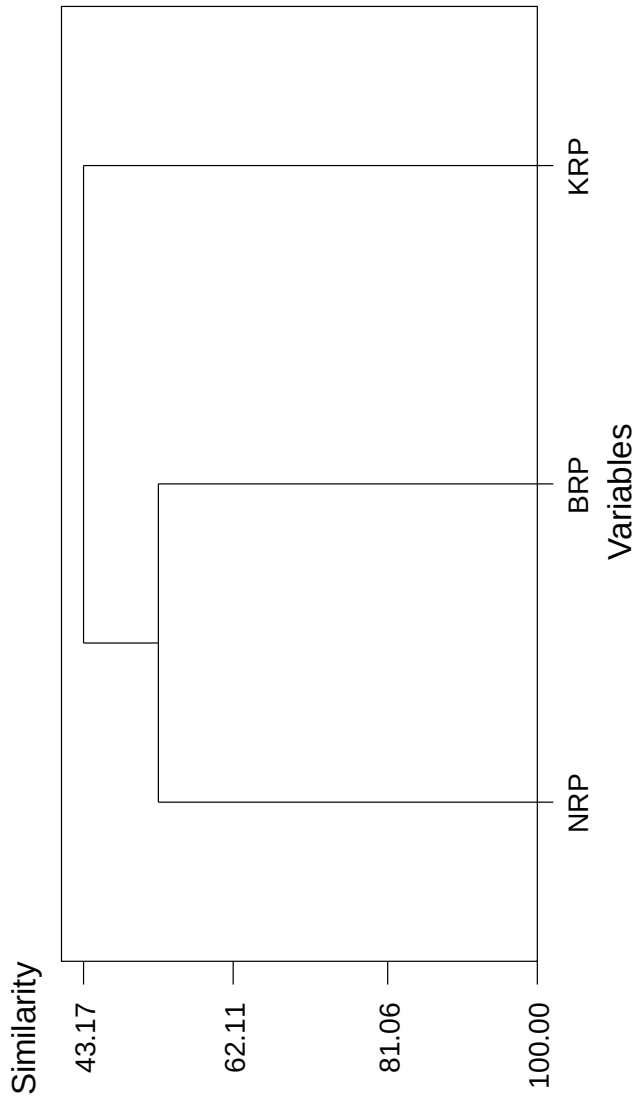
Appendix 6: Dendogram of leaf cyanide (LC) across sites. (N-Nakuru, B-Baringo, K-Kericho)



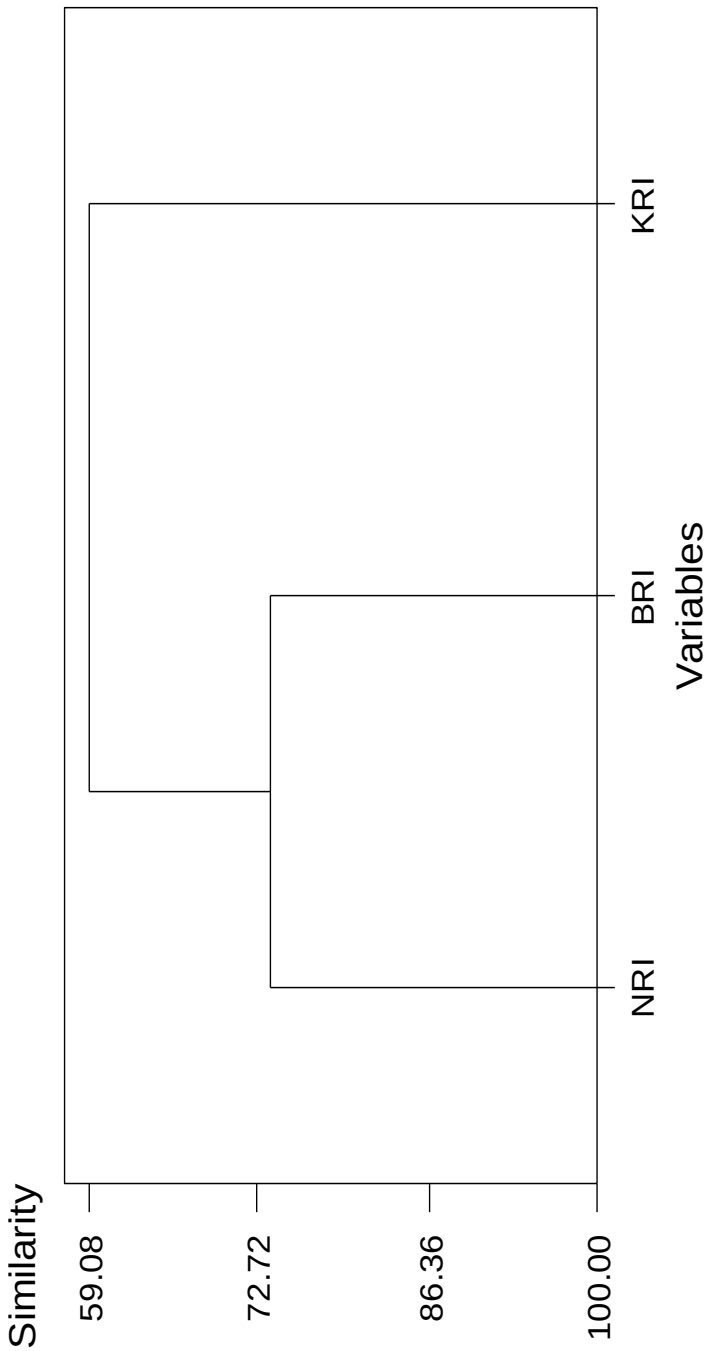
Appendix 7: Dendogram of root cyanide (RC) across sites. (N-Nakuru, B-Baringo, K-Kericho)



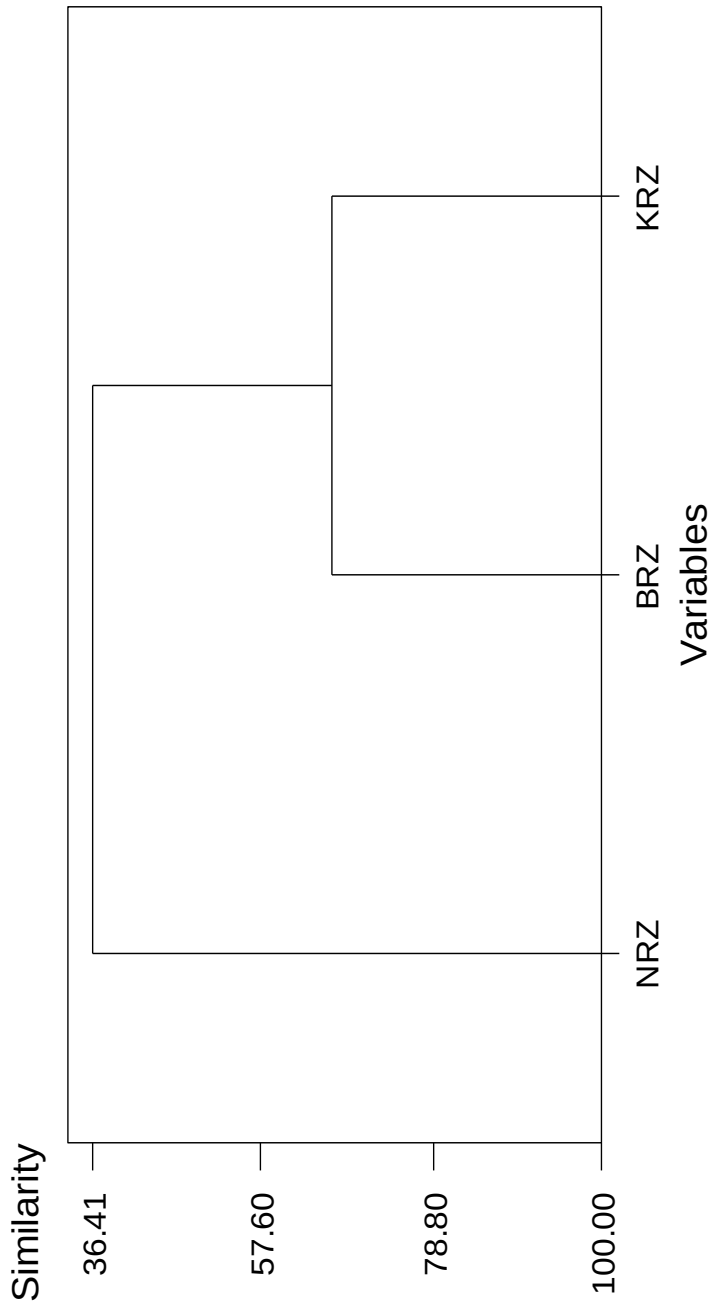
Appendix 8: Dendogram of root protein (RP) across sites. (N-Nakuru, B-Baringo, K-Kericho).



Appendix 9: Dendogram of root iron (RI) across sites. (N-Nakuru, B-Baringo, K-Kericho).



Appendix 10: Dendogram of root zinc (RZ) across sites. (N-Nakuru, B-Baringo, K-Kericho).



Appendix 11: ANOVA table for cyanide roots, cyanide leaves, protein, iron and zinc.

Cyanide Roots	**DF**	**SS**	**MS**	**F-value**	**Pr > F**
Site	2	393.57	196.79	39.51	0.0001
Variety	13	211.43	16.26	3.27	0.0002
Site * Variety	26	412.06	15.85	3.18	0.0001
Cyanide leaves					
Site	2	246.44	121.22	48.22	0.0001
Variety	13	203.39	15.65	6.22	0.0001
Site * Variety	26	95.87	3.69	1.47	0.07
Protein					
Site	2	71.31	35.66	63.49	0.0001
Variety	13	11.54	0.89	1.58	0.1
Site * Variety	25	30.86	1.23	2.2	0.002
Iron					
Site	2	6367.51	3183.76	71.5	0.0001
Variety	6	1490.68	114.67	2.58	0.0036
Site * Variety	13	1726.88	69.08	1.55	0.08
Zinc					
Site	2	2701.51	1350.76	5.24	0.0076
Variety	13	8600.00	661.54	2.56	0.006
Site * Variety	25	22579.41	903.18	3.5	0.0001

Appendix 12: Data matrix for 69 cassava accessions based on 7 SSR markers. '1' indicates presence and '0' indicates absence of the marker. Code '?' is for missing values. Names of accessions are as indicated by table 3.

	SSRY9 (300)	SSRY9(280)	SSRY9(270)	SSRY51(310)	SSRY51(300)	SSRY51(290)	SSRY13(230)	SSRY13(240)	SSRY66(280)	SSRY66(270)
1	0	0	1	0	0	1	0	1	0	0
2	0	0	1	0	0	1	1	1	0	0
3	0	1	0	?	?	?	1	1	0	0
4	0	1	0	1	1	0	1	0	0	0
5	0	1	0	1	1	0	1	1	0	0
6	0	1	0	0	1	1	1	0	0	0
7	0	1	0	1	1	0	1	0	0	0
8	1	0	0	?	?	?	1	0	?	?
9	1	0	0	0	1	1	1	1	0	0
10	1	0	0	0	1	1	1	1	0	0
11	?	?	?	1	1	0	1	1	0	1
12	1	0	0	1	1	0	1	0	0	1
13	1	0	0	0	1	0	1	1	0	1
14	1	0	0	1	1	0	1	0	1	0
15	1	0	0	0	0	1	1	1	1	0
16	1	0	0	1	1	0	1	0	1	0
17	?	?	?	0	1	0	1	1	0	0
18	1	0	0	0	1	0	1	1	0	0
19	1	0	0	0	1	1	0	1	0	1
20	0	0	1	1	1	0	1	0	0	1
21	0	0	1	0	0	1	1	0	0	0
22	0	0	1	0	1	1	0	1	0	0
23	0	1	0	0	1	1	1	0	0	1
24	0	1	0	0	1	0	1	1	0	1
25	0	1	0	0	1	0	1	1	0	1
26	0	1	0	0	1	0	1	0	0	1
27	0	1	0	0	1	1	1	0	1	0
28	0	1	0	0	1	1	1	0	0	1
29	0	1	0	0	1	1	1	0	0	1

30	**0**	**1**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**1**
31	**0**	**1**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**1**
32	**0**	**1**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**1**
33	**0**	**1**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**1**
34	**0**	**1**	**0**	**0**	**1**	**1**	**1**	**0**	**0**	**1**
35	**0**	**1**	**0**	**0**	**1**	**1**	**1**	**0**	**0**	**1**
36	**0**	**1**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**1**
37	**0**	**1**	**0**	**0**	**0**	**1**	**1**	**0**	**1**	**0**
38	**0**	**1**	**0**	**1**	**0**	**0**	**1**	**0**	**?**	**?**
39	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**0**	**?**	**?**
40	**0**	**0**	**1**	**0**	**0**	**1**	**1**	**0**	**0**	**1**
41	**0**	**0**	**1**	**0**	**0**	**0**	**?**	**?**	**0**	**1**
42	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**0**	**0**	**1**
43	**0**	**1**	**0**	**0**	**1**	**1**	**1**	**0**	**?**	**?**
44	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**0**
45	**?**	**?**	**?**	**?**	**?**	**?**	**1**	**1**	**1**	**0**
46	**0**	**1**	**0**	**0**	**1**	**0**	**?**	**?**	**?**	**?**
47	**0**	**1**	**0**	**0**	**1**	**1**	**1**	**1**	**?**	**?**
48	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**0**
49	**0**	**0**	**1**	**?**	**?**	**?**	**1**	**1**	**1**	**0**
50	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**
51	**0**	**0**	**1**	**?**	**?**	**?**	**1**	**1**	**1**	**0**
52	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**
53	**0**	**0**	**1**	**0**	**1**	**0**	**1**	**1**	**1**	**0**
54	**0**	**0**	**1**	**0**	**0**	**0**	**1**	**1**	**0**	**1**
55	**?**	**?**	**?**	**0**	**1**	**0**	**1**	**1**	**0**	**0**
56	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**0**
57	**0**	**0**	**1**	**0**	**0**	**1**	**1**	**1**	**0**	**0**
58	**0**	**1**	**0**	**0**	**0**	**1**	**1**	**0**	**0**	**0**
59	**0**	**1**	**0**	**?**	**?**	**?**	**1**	**0**	**0**	**0**
60	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**0**	**0**	**1**
61	**0**	**1**	**0**	**0**	**0**	**1**	**1**	**0**	**0**	**1**
62	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**0**	**0**	**1**
63	**0**	**1**	**0**	**?**	**?**	**?**	**1**	**0**	**0**	**0**

64	**?**	**?**	**?**	**?**	**?**	**?**	**?**	**?**	**0**	**0**
65	**0**	**1**	**0**	**?**	**?**	**?**	**1**	**0**	**0**	**0**
66	**0**	**1**	**0**	**?**	**?**	**?**	**1**	**0**	**0**	**0**
67	**0**	**1**	**0**	**0**	**1**	**1**	**1**	**0**	**0**	**1**
68	**0**	**1**	**0**	**0**	**1**	**1**	**1**	**0**	**0**	**1**
69	**0**	**1**	**0**	**0**	**1**	**1**	**?**	**?**	**0**	**1**

Appendix 2: Continued

	SSRY66(260)	SSRY78(250)	SSRY78(245)	SSRY78(230)	SSRY106(280)	SSRY106(270)	SSRY106(260)	SSRY35(300)	SSRY35(280)	SSRY35(270)	SSRY35(260)
1	1	0	1	0	0	0	1	?	?	?	?
2	1	?	?	?	0	1	0	0	1	0	0
3	1	0	1	0	1	0	0	1	0	1	1
4	1	0	1	0	1	0	0	1	0	1	1
5	1	0	1	0	1	0	0	0	0	1	0
6	1	0	1	1	1	0	0	1	0		1
7	1	0	1	0	1	0	0	0	1	0	0
8	?	0	1	0	1	0	0	0	0	0	1
9	1	0	1	1	1	0	0	1	0	1	1
10	1	0	1	0	1	0	0	0	0	1	0
11	0	0	1	0	0	1	0	0	0	1	0
12	0	0	1	0	0	1	1	0	0	1	0
13	0	1	0	0	0	1	0	0	0	1	0
14	0	1	0	0	1	0	0	1	0	1	1
15	0	1	1	0	0	1	0	0	0	1	0
16	0	1	0	0	0	1	1	?	?	?	?
17	1	?	?	?	?	?	?	?	?	?	?
18	1	0	1	0	1	0	0	0	0	1	0
19	0	0	1	0	0	1	1	0	0	1	0
20	0	0	1	0	0	0	1	0	0	1	0
21	1	0	1	0	0	0	1	0	0	1	0
22	1	0	1	0	0	0	1	0	0	1	0
23	0	0	1	1	0	1	1	0	0	1	0
24	0	0	1	1	0	0	1	0	0	1	0
25	0	0	1	0	0	1	1	1	0	1	1
26	0	0	1	1	0	1	1	0	0	1	0
27	0	0	1	1	0	0	1	0	0	1	0
28	0	0	1	1	0	0	1	?	?	?	?
29	0	0	1	1	0	0	1	0	0	1	0

30	0	?	?	?	?	?	?	1	0	1	1
31	0	0	1	0	?	?	?	?	?	?	?
32	0	0	1	0	0	0	1	0	0	1	0
33	0	1	0	0	0	0	1	0	0	1	0
34	0	0	1	0	?	?	?	0	0	1	0
35	0	0	1	0	0	0	1	1	0	1	1
36	0	0	1	0	0	0	1	0	0	1	0
37	0	0	1	0	0	0	1	0	0	1	0
38	?	0	1	0	0	0	1	0	0	1	0
39	?	0	1	0	0	1	0	1	0	1	1
40	0	0	1	1	1	0	0	1	0	1	1
41	0	0	1	1	1	0	0	1	0	1	1
42	0	0	1	1	1	1	0	0	1	1	0
43	?	?	?	?	1	1	0	1	0	1	0
44	0	1	0	0	0	1	0	1	0	1	0
45	0	1	0	0	1	0	0	1	0	1	0
46	?	1	0	0	?	?	?	?	?	?	?
47	?	1	0	0	?	?	?	0	0	1	0
48	0	1	0	0	0	1	0	0	0	1	0
49	0	1	0	0	1	0	0	0	0	1	0
50	0	0	1	0	?	?	?	?	?	?	?
51	0	?	?	?	?	?	?	0	0	1	0
52	0	0	1	1	0	1	0	1	0	1	1
53	0	1	0	0	?	?	?	?	?	?	?
54	0	?	?	?	?	?	?	1	0	1	1
55	1	1	0	1	1	1	0	0	0	1	0
56	1	1	0	1	?	?	?	?	?	?	?
57	1	?	?	?	?	?	?	0	0	1	0
58	1	1	0	0	?	?	?	?	?	?	?
59	1	0	1	0	1	0	0	0	0	1	1
60	0	1	0	0	1	0	0	?	?	?	?

61	0	1	0	0	?	?	?	?	?	?	?
62	0	0	1	0	1	1	0	0	0	1	0
63	1	?	?	?	1	1	0	?	?	?	?
64	1	?	?	?	?	?	?	0	0	1	0
65	1	?	?	?	0	1	0	?	?	?	?
66	1	1	0	0	?	?	?	?	?	?	?
67	0	1	1	0	1	0	0	0	0	1	1
68	0	0	1	0	?	?	?	0	0	1	1
69	0	1	1	0	1	0	0	0	1	1	0