GENETIC LINKAGE MAPPING OF FIELD RESISTANCE TO CASSAVA BROWN STREAK DISEASE IN CASSAVA (*Manihot esculenta* Crantz) LANDRACES FROM TANZANIA

By

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DECLARATION

I, Heneriko Philbert Kayogoro Kulembeka, do hereby declare that the thesis hereby submitted for qualification for the degree Philosophiae Doctor in Agriculture at the University of the Free State represents my own original, independent work and that I have not previously submitted the same work for a qualification at another university.

I further cede copy right of the thesis in favour of the University of the Free State

---------- 30 November 2010

Heneriko Philbert Kayogoro Kulembeka Date

DEDICATION

This work is dedicated to my wife Joyce, my son Joel Kayogoro, my daughters Levina Mwelakale and Jocelyne Msaneza for their support, patience and for hard times they went through during my study period

To my mother Levania Mwelakale and my late father Philbert Kayogoro Kulembeka for bringing me up to who I am today. My father and my brother Yolamu Philbert Kulembeka, unfortunately passed away before seeing my PhD graduation. May God rest you in Peace!

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SYMBOLS AND ABBREVIATIONS

aa	amino acid
ABI	Applied Biosystems
ACMV	African cassava mosaic virus
AFLP	Amplified fragment length polymorphism
AMRI	Amani Research Institute
ANOVA	Analysis of variance
ARI	Agricultural Research Institute
bp	Base pairs
BC_1	Back cross generation one
BecA	Biosciences Eastern and Central Africa
BecANet	Biosciences Eastern and Central Africa Network
BSA	Bulk segregant analysis
°C	Degrees Celcius
CAD	Cassava anthracnose disease
CAPS	Cleaved amplified polymorphic sequence
CBB	Cassava bacterial blight
CBSD	Cassava brown streak disease
CBSD-RN	Cassava brown streak disease root necrosis
CBSMov	Cassava brown streak Mozambique virus
CBSUgV	Cassava brown streak Uganda virus
CBSV	Cassava Brown Streak Virus
cDNA	Complimentary DNA
CE	Capillary electrophoresis
CFSD	Cassava frog skin disease
CGM	Cassava green mite
CHZ	Chambezi
CIAT	International Centre for Tropical Agriculture/Centro International de Agricultura Tropical

CIM	Composite interval mapping
cm	Centimetre
cM	centiMorgan
CMB	Cassava mealy bug
CMD	Cassava mosaic disease
COSCA	Collaborative study of cassava in Africa
CP	Coat protection
CP	Cross-pollinated (out-breeder full-sib family)
DH	Double haploid
DM	Dry matter content
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DRC	Democratic Republic of the Congo
EAAFRO	Eastern Africa Agriculture and Forestry Research Organisation
EACMV	East African cassava mosaic virus
EDTA	Ethylene-diaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
ESSRY	Expressed Simple Sequence Repeats
EST	Expressed sequence tag
F	Forward
FAO	Food and Agricultural Organization
FRW	Fresh root weight
FSW	Fresh shoot weight
g	Gram
GA_3	Gibberellic acid
GCA	General combining ability
GCP	Generation Challenge Programme
GWAS	Genome wide association studies
h	Hour
ha	Hectare
HCl	Hydrochloric acid

HCN	Hydrogen cyanide
HI	Harvest index
Hi-Di	Highly deionised
IFAD	International Fund for Agricultural Development
IITA	International Institute for Tropical Agriculture
ILRI	International Livestock Research Institute
KARI	Kenya Agricultural Research Institute
Kcal	Kilocalorie
KCl	Potassium chloride
kg	Kilogram
LG	Linkage group
LOD	Logarithm (base 10) of odds
LSD	Least significant difference
m	metre
M	Molar
MAP	Months after planting
MAS	Marker-assisted selection
masl	Metre above sea level
Mb	Mega base pairs
mg	milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimole
mol	Mole
MS	Mean square
MXCOMP	Matrix comparison module of NTSYSpc
NAA	Naphthaleneacetic acid
NaCl	Sodium chloride
NARI	Naliendele Agricultural Research Institute

NARS	National Agricultural Research System
NEPAD	New Partnership for African Development
NDL	Naliendele
ng	Nanogram
nt	Nucleotide
NTSYS	Numerical taxonomy multivariate analysis system
PAGE	Polyacrylamide gel electrophoresis
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PDR	Pathogen-derived resistance
pg	Picogram
PROJ	Projection module of NTSYSpc
pH	Measure of acidity/basicity
PIC	Polymorphic information content
pmol	Picomole
PVP	Polyvinylpyrrolidone
QTL	Quantitative trait loci
R	Reverse
RAPD	Random amplified polymorphic DNA
REC	Recombination frequency
RFLP	Restriction fragment length polymorphism
rfu	Relative fluorescence unit
RH	Relative humidity
RNA	Ribose nucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SAHN	Sequential agglomerative hierarchical nested cluster analysis
SCA	Specific combining ability
SCAR	Sequence characterized amplified region

SDS	Sodium dodecyl sulphate
Sec	seconds
SNP	Single nucleotide polymorphism
SRI	Sugar Research Institute
ssp	Subspecie
SSR	Simple sequence repeats
t	Tonne
TAE	Tris-acetate and EDTA
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TE	Tris-HCl EDTA
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
U	Unit
UARI	Ukiriguru Agricultural Research Institute
UgV	Ugandan variant
UPGMA	Unweighted pair group method using arithmetic averages
UK	United Kingdom
USA	United States of America
US$	United States dollars
UV	Ultraviolet
V	Volt
v/v	Volume per volume
W	Watt
w/v	Weight per volume
µg	Microgram
µl	Microlitre
µM	Micromolar
µmol	Micromole

LIST OF PRESENTATIONS AND POSTERS

Kulembeka, H.P., A. Kullaya, E. Masumba, M. Labuschagne, M. Ferguson and M. Fregene. **2005**. Genetic diversity of germplasm susceptible and resistant to cassava brown streak disease. In: *Research and products development that reaches farmers*, pp. 54. Proceedings of the second general meeting on biotechnology, breeding and seed systems for African Crops, Rockefeller Foundation, January, 24-27, 2005, Nairobi, Kenya.

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CHAPTER 1

General introduction

Cassava (*Manihot esculenta* Crantz) is grown widely in tropical Africa, Asia and Latin America and is an important food security crop for many tropical and subtropical countries. The crop ranks high amongst the top ten most significant food crops produced in developing countries. In the 1990s over 130 million tonnes of fresh cassava roots were produced annually and consumed by 500 million people on a daily basis (Scott *et al*., 2000). It is the third most important source of calories in the tropics, after rice and maize (IFAD and FAO, 2001). Recently cassava has been considered as the developing world's fourth most important crop, with production in 2006 estimated at 226 million metric tonnes (FAO, 2006; African News Network, 2008). In the mid 2000s, cassava was estimated to provide the staple food for nearly one billion people in more than 105 countries where the root provides as much as a third of the daily calories (FAO, 2006). It is mainly grown by poor farmers, many of them women, often on marginal lands. It is mainly used as food and animal feed, but currently is also a source of raw material for industrial and confectionary uses. Although roots are poor in nutrition, consisting largely of carbohydrates, leaves are rich in proteins, vitamins and minerals and are an important source of vegetables in the Democratic Republic of the Congo (DRC), Tanzania, Kenya, Madagascar, Sierra Leone, Uganda and Zambia (Latham, 1980; Fresco, 1986; Nweke *et al*., 2002; Haggblade and Tembo, 2003).

In Africa, cassava is the second most important staple crop after maize and the Abuja Declaration (African Union, 2006) identified cassava as one of the crops with the greatest potential to combat poverty as well as food and nutritional insecurity (African Agriculture, 2007). About half of the world's production of cassava is found in Africa. Cassava is cultivated in about 40 African countries, stretching through a wide belt from southeast Madagascar to northwest Cape Verde. Around 75% of Africa's cassava output (storage root yield) is harvested in Nigeria, DRC, Congo, Ghana, Tanzania and Mozambique (IFAD and FAO, 2005). In Africa total production is more than 90million tonnes annually (IFAD and FAO, 2001; FAO, 2001), which is greater than any other crop, with the exception of maize. Nigeria is the largest cassava producer in Africa and the world, producing 38 million tonnes of cassava in 2005

(FAO, 2006). In east Africa, Tanzania was rated first in cassava production followed by Uganda for the period between 2006 and 2010 with figures ranging from 6.2-7.9 million tonnes respectively (FAO, 2010).

Cassava is efficient in carbohydrate production, adapted to a wide range of environments and tolerant to drought and acidic soils. In Africa, an estimated 70 million people obtain more than 500 Kcal per day from cassava (FAO, 2001). Cassava has been prioritised by the New Partnership for African Development in Africa as a 'poverty fighter' crop, which will spur industrial development in Africa (NEPAD, 2004).

Cassava is an important staple crop in more than half of Tanzania and a subsistence crop, especially in the semi-arid areas. It is sometimes, due to its drought tolerance, considered as a famine reserve crop when cereals fail. Between the 2007/8 and 2008/9 seasons, due to poor rainfall (late onset, lower amounts and poor distribution of rain), there was a decline in cereal production (maize and rice declined by 4% and sorghum by 20%) compared to an increase of 10% in cassava production (Ministry of Agriculture Food Security and Cooperatives, 2009). Eighty-four percent of the total cassava production in the country is utilised as human food while the remaining 16%is for other uses like starch production, livestock feed and export (FAO and IFAD, 2001). Both roots and leaves of cassava are of major nutritional importance in the country. Cassava is cultivated and produced in all regions of Tanzania and the main producing areas include the coastal strip along the Indian ocean (Tanga, Pwani, Dar es Salaam, Lindi and Mtwara), around Lake Victoria (Mwanza, Shinyanga, Mara and Kagera regions), Lake Tanganyika areas and along the shore of Lake Malawi (Mkamilo, 2005). The country's annual total fresh root production is estimated at 7 million tonnes, from 670000ha (FAO, 2006). Total area under cassava production increased from 604200 ha in 2001 to 761000 ha in 2005 (Nkuba and Adebayo, 2006). The average cassava yield for Tanzania is about 8 t/ha, making the country the fourth largest producer of cassava in Africa (FAO, 2001). This yield level is below the continent's average of 10t/ha and the average yield of 14 t/ha of Africa's (and the world's) largest producer, Nigeria. This low yield is caused by many factors, including susceptibility of commonly grown varieties to major diseases and pests such as cassava mosaic diseases (CMD), caused principally by the East African cassava mosaic virus

(EACMV), its Ugandan variant (UgV) as well as the African cassava mosaic virus (ACMV), cassava brown steak disease (CBSD), cassava bacterial blight (CBB), cassava green mite (CGM), cassava mealy bug (CMB) and nematodes.

Of the biotic stresses, CMD and CBSD are major constraints to cassava production in the eastern and central African region. CMD is caused by Gemini viruses that infect the foliar part of the cassava plant causing yield losses of 30-60% (Thresh *et al*., 1997) through reduction of leaf photosynthetic area. However, in severely infected plants, yield losses of up to 100% have been reported (Thresh *et al*., 1994). Equalling, if not superseding CMD in terms of yield loss, is CBSD, which unlike CMD, affects both roots and aerial parts of the plant. Prevalence of CBSD in Tanzania and the east and central African region is threatening cassava production because of its damaging nature. Knowledge of the disease dates back to the 1930s when Storey and his colleagues initiated studies on cassava virus diseases at the former Amani Research Institute (AMRI) in Tanzania (Storey, 1936).

CBSD is the most devastating disease of cassava that causes damaging losses to root production and quality in all coastal areas of Tanzania, Kenya and Mozambique and in the lakeshore areas of Malawi, where it has been thought to be confined (Nichols, 1950). CBSD reduces total yields and root quality, rendering roots useless for human consumption due to necrosis it causes to the starch storage root tissues (Hillocks *et al*., 2001). A disease survey conducted in the Tanga region of Tanzania revealed crop losses of 49-74% (Muhanna and Mtunda, 2002) but in severely affected areas, entire fields are usually destroyed, leading to 100% yield losses. In economic terms, Kanju and colleagues (Kanju *et al*., 2003; 2007) gave estimates of US$ 16.5million annual losses due to CBSD in the Tanzanian coastal lowland alone (price of fresh cassava is estimated at US$ 15 per tonne). This estimate was based onthe country's cassava production estimates of 5.65 million tonnes (FAO, 2001), of which coastal lowlands contributed 50% of the country's annual production. A conservative average yield loss estimate of about 40% was employed. Kanju *et al*. (2003) further indicated that figures will be higher for Mozambique where the disease has in recent years become devastating and seriously threatens household food security in the three major cassava producing provinces of Nampula, Zambezia and Cabo Delgado.

The disease has been thought to be confined to low altitudes of the coastal areas of the Indian Ocean (Nichols, 1950). Recent reports show that CBSD is spreading beyond coastal areas and is now found in high altitude areas where it is causing significant root yield losses in the Lake Victoria areas of Tanzania, Kenya and Uganda and has been reported in Rwanda, DRC and Congo (Alicai *et al*., 2007; Ntawuruhunga and Legg, 2007). Heavy disease pressure is now observed in all areas surrounding Lake Victoria, an area which is also known to be a centre of diversity for CMD, with all known CMD variants converging, giving rise to mixed infections (Ndunguru *et al*., 2005).

One of the efficient control measures of CBSD is the deployment of resistant varieties. The use of host-plant resistance or deployment of less susceptible cultivars has proven to be the most realistic approach to reduce yield losses caused by CBSD and CMD (Hillocks and Jennings, 2003). There are only a few cassava genotypes available as sources of resistance to CBSD but these are not suitable as varieties for farmers to leverage the effects of CBSD. They can rather be utilised as progenitors in resistance breeding strategies within a reasonable time frame (Kanju *et al*., 2003).The challenge is to efficiently introgress CBSD disease resistance into the unimproved susceptible, but farmer preferred varieties.

Conventional cassava breeding is a 7-10 year effort that involves the evaluation of tens of thousands of segregating populations in a multi-stage selection process with the eventual release of only a few varieties (Kawano *et al*., 1998; Kawano, 2003; Ceballos *et al*., 2004). This is complicated by the cumbersome nature of assaying for CBSD infection in the field. Unlike CMD whose foliar symptoms are easily identifiable, the biological assay of CBSD infected genotypes is not an easy task. In CBSD infected cassava fields, it is usually difficult to discern leaf symptoms when there are mixed infestation with CGM. The biological assay for CBSD infection in the field is further complicated by root infection. Cassava plants can grow to maturity without detectable leaf or stem symptoms, but root necrosis will only be detected when plants are uprooted. At farm level this implies that the farmer will only detect the corky, yellow-brown necrotic rot after a year when the roots are harvested. Assessment for root necrosis involves chopping of all roots from every genotype under evaluation to

appraise disease infection, a process which is cumbersome and tiresome. Use of molecular markers can overcome most of these limitations to CBSD resistance breeding. Molecular marker-assisted selection (MAS) can be used to shorten the time between population development and selection by farmers by enhancing the accuracy of simultaneous selection for a number of different biotic and abiotic stresses, including CBSD.

Molecular markers associated with genes and quantitative trait loci (QTL) controlling traits of agronomic importance provide a powerful means of increasing selection success for heritability in the early stages of cassava breeding. Molecular markers for root quality traits like dry matter content, protein and delayed post-harvest deterioration as well as disease resistance have been identified and used successfully in cassava selection at Centro International de Agricultura Tropical (CIAT) (Akano *et al*., 2002; Fregene *et al*., 2006; Egesi *et al*., 2008). These markers have been useful in introgression of useful traits from wild *Manihot* relatives into cassava through MAS, increasing efficiency in cassava breeding. Given the long breeding cycle of cassava and the cumbersome nature of assessing root necrosis in CBSD infected genotypes, mapping and identification of molecular markers tightly linked to CBSD resistance gene/s will be useful in breeding CBSD resistant cassava. This will efficiently accelerate the generation of elite cassava varieties resistant to the disease. With molecular MAS it will be possible to select at seedling stage, which will dramatically reduce the size of the working population, which would have otherwise gone through the rigorous process of assessment for root necrosis, 12 months or more after planting (MAP), thereby reducing the cost and time involved.

The damaging effects of CBSD are more on root quality than root weight (root yield). In the early resistance studies to CBSD (Nichols, 1950; Jennings, 1957), the loss in root yield of susceptible varieties was attributable more to root quality than root weight because root necrosis makes the roots inedible and unsuitable for marketing. Observations on CBSD infections in the field show that, subject to the same disease inoculum pressure, varieties show different symptom levels. Some varieties show foliar symptoms without root necrosis, others show root necrosis without foliar symptoms, while sensitive varieties exhibit both foliar and root necrosis. In some genotypes, plants get infected by the virus but show neither

foliar symptoms nor root necrosis and are field resistant to CBSD. Given these scenarios, field resistance has been used in this study because the focus was to make assessments on the relative levels of genotype reactions to CBSD infection in the field and no attempt was made to relate CBSD infection to yield losses.

The overall aim of the study was to improve cassava breeding through the use of molecular segments of the cassava genome conferring resistance to CBSD. Within this goal there were four objectives:

(a) To understand the genetic diversity among cassava germplasm resistant and susceptible to CBSD.

(b) To understand the genetic basis of CBSD field resistance in two of the resistant genotypes.

(c) Genetic mapping of molecular markers linked to CBSD field resistance in cassava.

(d) Identification of QTL controlling genes for CBSD resistance.

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CHAPTER 2

Cassava biology, production constraints and molecular marker applications

2.1 Introduction

Cassava is a major source of calories in the tropics. It is an ideal crop for subsistence agriculture as it grows well in areas with a long dry season or irregular rainfall pattern, and on poor soils. The crop can remain in the ground (in ground storage) from 8-24 or more months depending on the cultivar and growing conditions, giving flexibility to farmers with regard to harvesting time (Hershey and Jennings, 1992; El-Sharkawy, 1993). Ground storage helps to maintain a continuous food supply throughout the year making cassava an ideal famine security crop and basic component of the farming system in semi-arid areas (Nweke *et al*., 1994). Collaborative studies of cassava in Africa have shown that cassava is not only a subsistence crop, but is increasingly becoming a cash crop since small scale farmers sell cassava to rural and urban consumers (Nweke *et al*., 2002). In the Congo, for example, the percentage of cassava planted as cash crop was higher than for any other crop (Tollens, 1992) and other studies show that the crop has gained importance as a cash crop and for industrial use (Sriroth *et al*., 2000; Nkuba and Adebayo, 2006; Van der Land and Uliwa, 2007).In Nigeria, Congo and elsewhere, some small scale farmers produce five to ten hectares of cassava entirely for sale (Berry, 1993). Cassava, as an important staple crop, plays five important roles in African development as: famine reserve crop, food staple for rural people, cash crop for both rural and urban households and as raw material for animal feed and industrial products (Nweke *et al*., 2002). Cassava production figures show that Nigeria, the leading country in cassava production, produced 38 million tonnes of cassava in 2005 (FAO, 2006). In east Africa, Tanzania was rated first in cassava production followed by Uganda for the period between 2006 and 2010 (Table 2.1).

Table 2.1 Cassava production in selected east African countries in million tonnes

	Year of cassava production				
Country	**2006**	**2007**	**2008**	**2009**	**2010**
Tanzania	6.158	6.600	6.600	6.916	na
Uganda	4.926	4.456	5.072	5.179	5.292
Kenya	0.566	0.397	0.751	0.911	na
Burundi	0.571	0.559	0.630	0.660	na
Rwanda	0.588	0.700	0.700	0.680	na

Source: FAO (2010), na = not available.

2.2 Origin of cassava

All species of the genus *Manihot* are native to countries of the new world, especially Brazil and Mexico, where they form distinct centres of diversity (Nassar 1978b; 2000). Cassava was previously believed to have originated through hybridisation between two wild *Manihot* species, *M. esculenta* ssp *flabellifolia* Pohl and *M. pruinosa* Pohl followed by vegetative reproduction of the hybrid (Allem, 1999). Olsen (2004) stated that molecular markers provided strong evidence that cassava was domesticated from a single wild species. Central America, including Colombia, Venezuela, Guatemala and south Mexico were initially reported to be the centre of origin due to large numbers of varieties present in these areas (Sauer, 1952; Rogers, 1965). These areas were later referred to as the minor centre of origin and Brazil as the major centre of origin (Allem, 1994; Ekanayake *et al*., 1997). Using a molecular phylogenetic approach to understand species relationships, Hillis collected cassava species from Mesoamerica and South America, including species previously identified as potential progenitors of cassava such as *M. esculenta* ssp *flabellifolia*, to study the origin of cassava (Schaal *et al*., 2006). They noted that there was an overall similarity of DNA sequences of the glyceraldehyde 3-phosphate hydrogenase (*G3pdh*) gene between species within the genus. There was a high level of sequence similarity between the *G3pdh* gene of cassava and *M. esculenta* ssp *flabellifolia* compared to other species. This molecular data was in agreement with morphological data (Allem, 1994), which first indicated *M. esculenta* ssp

flabellifolia as a potential ancestor. In a number of studies using amplified fragment length polymorphisms (AFLP) and other DNA markers, the close similarity of this species to cassava has been noted (Fregene *et al*., 1994; Roa *et al*., 1997). *Manihot esculenta* ssp *flabellifolia* is found in the transition zone between the southern Amazon forest and the drier Cerrado region of Brazil and Peru (Allem, 1999; 2002; Schaal *et al*., 2006).To prove the ancestor theory, simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers were used to analyse populations of the species collected and it was revealed that haplotypes and microsatellite alleles of cassava were subsets of those found in *M. esculenta* ssp *flabellifolia* (Olsen and Schaal, 1999; 2001). This confirmed that cassava is derived from *M. esculenta* ssp *flabellifolia* (Schaal *et al*., 2006). The inclusion of the *M. pruinosa* population in the analysis showed no evidence of its hybridisation with *M. esculenta* ssp *flabellifolia*. The data sets confirmed that cassava was domesticated in the southern region between the lower Amazon forest and the Cerrado region of Brazil from *M. esculenta* ssp *flabellifolia* (Schaal *et al*., 2006).

Cassava was first introduced from Latin America into Africa by Portuguese traders in the 16th century, around 1550 (Jennings and Hershey, 1985; Carter *et al*., 1992). It is argued that the Portuguese learnt from the Tupinamba Indians of eastern Brazil how to process cassava into *farinha*, the flour that was used as provision for ships travelling between Africa and Brazil (Lebot, 2009). Cassava was therefore first cultivated in Africa for the sole purpose of saving slaves in ships until 1600. It was first cultivated in west Africa and then in central Africa in the Congo coast near the delta (Cabinda). By the 17th century, cassava cultivation diffused to other parts of Africa through European explorers, French Navy colonialists and African farmers (Lebot, 2009). It is argued by Jones (1969) that this development might have been the result of introduction, by freed slaves, of the processing and preparation techniques originally developed in Brazil. In 1794, cassava was introduced to east Africa via Zanzibar from the Indian Ocean Islands and reached Lake Victoria in 1862 (Lebot, 2009).

2.3 Taxonomy of cassava

Cassava is a member of the Euphorbiaceae family and belongs to the Fruticosae of the genus *Manihot* (Jennings, 1976). Other members of the Euphorbiaceae family include commercial

important plants such as rubber trees (*Hevea brasiliensis* Müll.Arg), castor oil plant (*Ricinus communis* Linnaeus) and ornamental plants (*Euphorbia* spp). About 98 species are known to belong to the genus *Manihot* (Rogers and Appan, 1973), ranging from subshrubs to trees; the majority of them produce latex and contain cyanogenic glucosides. Cultivated cassava does not grow in the wild and is the only species of the genus that is cultivated for food production and other uses (Nassar, 2000; 2005).

Experimental crosses between cassava and local wild species have frequently produced hybrids (Nichols, 1947; Jennings, 1959; Abraham, 1975). Wild *Manihot* species hybridise naturally, both with each other and with cultivated cassava (Nassar, 1984; 1989) leading to spontaneous hybrids. Through controlled crosses, Nassar (1992) obtained interspecific hybrids of cassava with several other species including *M. glaziovii* Müll.Arg, *M. pssuloglaziovii* Müll.Arg and *M. Pilosa* Pohl. Cassava is an allopolyploid and all *Manihot* species examined cytogenetically have a high chromosome number of 2n = 36 with n = 18, but in spite of this, the species behave meiotically as diploids (Jennings, 1976; Nassar, 1978a). Many isozyme analyses indicated disomic inheritance confirming the diploid behaviour of cassava (Hussain *et al*., 1987; Lèfévre and Charrier, 1993). Pachytene karyology studies of *M. esculenta* suggested that the species could be a segmental tetraploid derived from the combination of two diploid taxa having a haploid complement with six common and three different chromosomes (Jennings, 1976). Although most cassava genotypes are diploids, spontaneous polyploidy such as triploids (3n) and tetraploids (4n) for some genotypes have been reported (IITA, 1980; Hahn *et al*., 1980). Triploid and tetraploid plants differ from diploids in plant vigour, leaf shape and size with triploid plants usually growing and yielding better than tetraploid and diploid plants.

2.4 The cassava plant

2.4.1 Morphology and propagation

Cassava is a semi-woody perennial shrub mainly grown for its starchy roots. It has the ability to grow and yield on marginal lands and poor nutrient soils, where cereals and other crops cannot grow well, making it suitable for various cropping systems (Onwueme, 1978; Fregene *et al.*, 2000; Nassar, 2005). Cassava can grow over a wide range of edaphic and climatic

conditions between 30^{o}N and 30^{o}S latitudes, from sea level to an altitude up to 2300 m above sea level (masl). It can grow in areas considered marginal for other crops, with low annual rainfall from 600 mm in semi-arid tropics to 7500 mm in the subhumid and humid tropics and can tolerate temperatures of 16-38^{o}C (Wholey and Cock, 1974; Cock, 1984; El-Sharkawy *et al*., 1992; Keating and Evenson, 1979; Alves, 2002).

Cassava is propagated mainly through stem cuttings and rarely from seeds. When cassava grows from seeds, the plant develops a tap root that may become tuberous and/or fibrous. When grown from cuttings, adventitious roots develop from the base of the cuttings forming fibrous root system from which edible storage tuberous roots are formed by tuberisation (swelling) of a few fibrous roots. Depending on the level of cyanide content which is due to accumulation of cyanogenic glucosides (Du *et al*., 1995; Chiwona-Karltun *et al*., 1998), farmers classify cassava into sweet and bitter varieties in relation to safety levels of cyanogenic glucocides in the roots (Mkumbira *et al*., 2001; Chiwona-Karltun *et al.*, 2004).

If cassava is propagated from cuttings, depending on the number of nodes and type of planting (placement of the cutting in the soil), a single cutting can produce one to three or more stems. Planting cassava cuttings horizontally produces more stems. Mature stems are woody and can grow from 1-2 m tall, although some cultivars may reach up to 4 m. The main stems divide di- or tri-chotomously into secondary branches. Flowering is the consequence of branching in which some branches are transformed into flowering buds. Cassava plants therefore flower depending on their branching with some cultivars not flowering at all due to inability to branch (IITA, 1990; Alves, 2002). Leaves are formed on the terminal buds of the stem and are arranged spirally on the stem. Each leaf is subtended by three to five stipules. Lamina is simple with smooth margins but palmate or lobed with the number of lobes ranging from three to nine (IITA, 1990).

2.4.2 Reproduction in cassava

Cassava can be propagated either by stem cuttings or sexual seeds, although the former is the most common practice by farmers for multiplication and planting. For plant breeding and under natural conditions, propagation by seeds is common. Farmers are known to

occasionally use spontaneous seedlings for subsequent planting that is a starting point for generating useful genetic diversity (Alves, 2002). Cassava is monoecious with male flowers occurring near the tip of the inflorescence while female flowers occur close to the base and they open 10-14 days before the male flowers on the same branch (IITA, 1990; Ekanayake *et al*., 1997). This protogyny phenomenon favours cross pollination in cassava, but self-pollination can occur when male and female flowers on different branches on different plants of the same genotype open simultaneously (Jennings and Iglesias, 2002). Variation in flowering occurs between cultivars and in some, flowering is frequent and regular while in others it is rare or non-existent. Environmental factors such as temperature and photoperiod influence flowering. Synchronisation of flowering remains a challenge and difficult issue in cassava breeding (Ceballos *et al*., 2004).

2.4.3 Seed germination

After pollination and subsequent fertilisation, the ovary develops into young fruits, which takes about 70-90 days to mature. The fruit contains three endocarp locules, each with one seed. When the fruit is dry the locules split to release the seeds. After maturity and harvesting, cassava seeds often have a physiological dormancy period of a few months that is common in *Manihot* species. Under field conditions, cassava seeds germinate with difficulty (Nartey, 1978; Ellis and Roberts, 1979; Ellis *et al*., 1982; Iglesias *et al*., 1994). According to Nartey (1978) seeds germinate in the dark and scarification at the micropyle slightly improves the germination percentage. Acid treatments and/or alternating heat treatments employed to break seed dormancy in other crops, have no effect on cassava seeds (Evans, 1972). Research on the optimal temperature for cassava seed germination by Ellis *et al*. (1982) recommended a mean temperature of 33^{o}C or alternating 30^{o}C for 8 h and 38^{o}C for 16 h for a minimum of 21 days. This finding was based on natural habitat conditions in which cassava seeds germinate after burning. Work done at CIAT indicated that fresh cassava seeds germinated in screen houses at high temperature and humidity. Storing seeds at room temperature for two to three months in pest free and pathogen free conditions has been recommended by CIAT (2004). Since at Ibadan, Nigeria, soil temperatures of 30-35^{o}C and high soil moisture content are common, seeds are planted directly in the field at the International Institute for Tropical Agriculture (IITA) and these temperatures have been

reported to be optimum for cassava germination (IITA, 1980).

2.4.4 Cassava physiology

Cassava development is affected by temperature, photoperiod, solar radiation and water. Temperature affects sprouting, seed germination, leaf size, storage root formation and consequently the whole plant (Wholey and Cock, 1974; Mahon *et al*., 1976; 1977; Keating and Evenson, 1979). Cassava growth is favourable at 25-29^{o}C but can tolerate temperatures from 16-38^{o}C (Cock, 1984). Cassava is a short day plant with a critical photoperiod of 12-23 h (Bolhuis, 1966; Hunt *et al*., 1977). Long days promote shoot growth and decrease storage root development while short days increase storage root growth and decrease shoot growth (Veltkamp, 1985; Alves, 2002). Photoperiod may affect the hormonal balance in the plant, for example, gibberlic acid and indole acetic acid levels (IITA, 1990). There are varietal differences in sensitivity to long days with some cassava genotypes not affected at all by photoperiod (Veltkamp, 1985; Alves, 2002). Cassava is a crop that requires high solar radiation for efficient photosynthesis; shade will therefore have an effect on cassava development and production (El-Sharkawy *et al*., 1992). Shade of 20-70% reduced cassava yield by 43-80% (Okoli and Wilson, 1986). Although cassava is drought tolerant, growth and yield are reduced by prolonged dry periods. The critical period of water deficit effects in cassava is one to five MAP; this is the stage of rapid root initiation and tuberisation. Water deficit during at least two months of this period can reduce storage root yield by 32-60% (Connor *et al*., 1981).

Root development in cassava indicates that storage roots of cassava are initially physiologically inactive and they start to enlarge when the supply of assimilates exceeds the requirements of stem and leaf sinks (Tan and Cock, 1979). At seedling stage, however, starch deposition in tap roots and fibrous root cells respectively, start in the fourth and fifth week after planting (Tetteh *et al*., 1997). According to Cock *et al*. (1985) and IITA (1982) the number of storage roots that develop is genotype specific and vary from 4-20 depending on good plant management. The number and weight of storage roots are affected by moisture stress, low soil fertility and water logging (Ekanayake *et al*., 1998). Abscisic acid is believed to be responsible for the growth of storage roots by enhancing cell division and enlargement.

Higher levels of abscisic acid in young storage roots than in primary roots of the same plant are observed in the early stages of root development in cassava (Melis, 1984).

Cassava photosynthesis follows a C_3 pathway (Veltkamp, 1985; Edwards *et al*., 1990). Maximum photosynthesis rates varying from 13-14 µmol $CO_2/m^2/s^1$were observed under greenhouse or growth chamber conditions (Mahon *et al*., 1977; Edwards *et al*., 1990). For efficient photosynthesis, cassava requires an environment with high temperature and high solar radiation (Angelov, 1993).

2.4.5 Cyanide content

With the exception of seeds, all cassava organs and tissues contain cyanogenic glucosides. Cyanogen glucosides include linamarin (85%) and lotaustralin (McMahon *et al*., 1995). The glucosides are synthesised in the leaves and transported to the roots where they are broken down by the linamarase enzyme, which is also found in the tissue (Wheatley and Chuzel, 1993; Du *et al*., 1995; Anderson *et al*., 2000). Most processing methods disrupt the tissues, allowing the enzyme to act on the substrate for rapid release of free hydrogen cyanide via an unstable cyanhydrin intermediary (Wheatley and Chuzel, 1993). The oral lethal dose of hydrogen cyanide for humans ranges between 0.5-3.5mg/kg body weight. No cassava genotype is entirely free of cyanide. Farmers classify cassava into sweet when the cyanide level is low and bitter when the cyanide level is high and requires processing (Bokanga, 1994). According to Chiwona-Karltum *et al*. (2001) sweet varieties have less than 100 mg total cyanogen per kg peeled fresh roots and bitter varieties have more than 100 mg. The bitter taste is correlated with high cyanogen content although there are some sweet cassava varieties whose roots have high cyanide levels and bitter ones with low cyanide levels (Bokanga, 1994; Chiwona-Karltum *et al*., 2004). Accumulation of glucosides varies with cultivar, environmental conditions, cultural practice and plant age (McMahon *et al*., 1995).

2.5 Cassava agronomy

Cassava can grow on a wide range of soil types but optimal yields are obtained on friable soils (IITA, 1982; 1990). When cassava is grown as a food crop, it is often grown under low-

input and low-output production systems and planting materials are obtained from the plant stems available from the farmer's own or neighbouring fields (Leihner, 2002). Propagation by stem cuttings makes cassava one of the crops with a low multiplication ratio of planting materials compared to cereals. The number of stem cuttings for commercial production from a single mother plant can range from 3-30 depending on growth and soil conditions, growth habit and climate (IITA, 1990). A system of using small 2-3 node cuttings rooted in well-drained nursery beds was devised to solve the problem of low multiplication ratio (Cock *et al*., 1976; IITA, 1990). This system can produce 12000-24000 cuttings from one node-cutting after one year. These mini-stem cuttings can be established from shoot tips, semi-mature and hard-wood stems and the number of nodes on a cutting will depend on internode length, diameter, and age of the plant and weather conditions at and after planting (Leihner 1984; IITA, 1990; Kamau, 2006).

Cassava can be planted on mounds, raised beds or ridges depending on soil type and drainage conditions (Ezumah and Okigbo, 1980). Cassava stakes can be planted horizontally, vertically or inclined. Tests on the placement of the cuttings in the soil by Castro (1979) and Leihner (2002) indicated that vertical placement of stakes results in fast crop establishment and good anchorage of plants. Horizontal planting has the advantage of producing more planting material. A stem cutting of 20 cm length with 5-8 nodes is ideal for planting. When the stake is placed with about half its length in the soil at a vertical position, it is the most appropriate for planting and harvesting operations (IITA, 1990; Leihner, 2002). Depending on soil type, moisture and temperature, stakes planted horizontally are buried 5-10 cm below the soil surface and stakes planted in vertical or inclined positions are covered with soil half to two third of their length. Plant population density varies from 600-20000 plants/ha depending on the cropping system used by farmers (Enyi, 1972; Toro and Atlee, 1980; Keating *et al*., 1988). The commercial spacing for cassava production is 1.0 m x 1.0 m (IITA, 1990).

Because cassava adapts to wide ranges of ecological conditions, it holds an important position in traditional cropping systems. Cassava is intercropped with cereals, grain legumes, fruits and other root crops (Mason and Leihner, 1988; Ezumah and Lawson, 1990).

Significant root yields are obtained when using a single cropping system compared to intercropping system. In most cases, farmers plant cassava on exhausted soils and rarely use fertilisers. The crop forms mycorrhizal fungal associations with *Glomus mosseae* Gerd in the roots, enabling the crop to access fixed nitrogen and increase phosphorous uptake efficiency (CIAT, 1980). Due to this association, cassava is able to produce its potential total biomass in poor soils better than other food crops (Romanoff and Lynam, 1992).

2.6 Production constraints

Although cassava has many benefits as a crop of the tropics, it faces many constraints which limit production. Biotic and abiotic constraints include shortage of appropriate improved cultivars with high genetic potential, long growth cycle, inadequate availability of disease-free planting material, post-harvest physiological deterioration of roots, nutritional deficiency for people who solely depend on cassava, cyanide content, diseases and pests (IITA, 1990).

Late bulking associated with a long growth cycle is a major constraint to cassava production that has been identified as one of the most important reasons why farmers in Africa abandon varieties (Nweke *et al*., 1994). Post-harvest deterioration is common in production areas where processing is less common and processing machines are not available (Bokanga, 1994). Abiotic stresses such as water stress, water logging, rocks or hard soils and cold temperatures limit cassava production. In semi-arid areas drought is the most limiting factor and lack of improved genotypes worsens the situation. Although cassava is tolerant to water stress, growth and development are slowed down during stress periods. Cool temperatures, below 20^{o}C have been found to slow down cassava growth and development (IITA, 1982).

Shortage of planting material, due to the inherent low multiplication ratio of cassava, is critical in semi-arid areas where farmers harvest most of their cassava in dry months and leave stems to dry in the field. During the planting season at the beginning of the rainy season, farmers face a critical shortage or lack of planting material. This results in farmers planting any material from any variety they come across, which leads to poor yield (Lukombo *et al*., 2002). The strongly out-crossing and monoecious nature of cassava (mediated by protogyny) make it difficult to develop appropriate stocks for classical genetic

studies (Fregene *et al*., 1997). From a breeding point of view, this limits and makes it difficult to develop appropriate cultivars.

Important pests of cassava include CGM (*Monorychellus tanajoa* Bondar), CMB (*Phonecoccus manihot* Mat.Ferr) (Hahn and Williams, 1973) and stem scales (*Aonidomytilus albus* Cockerell) (Swaine, 1950). Pests, CMB and CGM are native to South America and were introduced into Africa at various times through importation of stakes of various genotypes (IITA, 1992). Yield losses due to these pests are high; CGM and CMB are estimated to cause 8-88% yield loss while scale causes 4-19% losses (Bellotti *et al*., 1985; Larbi *et al*., 1998). Other insects found in Africa are elegant and variegated grasshoppers, whiteflies (vector for CMD), coreid bug and stem borers which affect cassava products in storage (IITA, 1990).

Cassava mosaic diseases, CBSD, CBB and root rot (*Phytopthora* spp*)* are important and common diseases of cassava. Cassava anthracnose disease (CAD) caused by *Colletotrichum gloeosporoides* f. sp *manihotis* Henn (Penz) Sacc is an important stem disease in Africa, occurring in all major cassava growing areas. A sap-sucking coreid bug (*Pseudotheraptus devastans* Dist) is reported to be partly responsible for the diseases' spread. CMD occurs in all cassava growing areas in Africa and is caused by the EACMV, ACMV and UgV (Dixon *et al*., 1994; Thresh *et al*., 1994a; 1994b; Legg, 1999; Otim-Nape *et al*., 2000). Bacterial blight is common in the wet and humid areas of east, central, south and west African countries. It is also found in South America and Asia. Yield losses can be as high as 95% depending on the time of infection by any one or more of the diseases (Storey and Nichols, 1938; Brian and John, 1940; Legg, 1999; Hillocks and Thresh, 2000).

2.7 Cassava brown streak disease

CBSD is a devastating viral disease that causes severe damage to cassava roots causing significant crop losses to cassava production in affected areas. Due to brown, dry necrotic rot in the storage roots, that renders the roots useless, it is considered as the major threat to food security in worst affected areas of coastal, eastern and southern Africa. Because of the associated symptoms of root necrosis, the disease has a direct impact on both yield and root

quality (Hillocks and Thresh, 2000).

CBSD was first described and recorded by Storey at the Amani research institute at the foot of the Usambara mountains of Tanzania (formerly known as Tanganyika) in the 1930s (Storey, 1936). The disease was later reported in the east African coast and Malawi up to an altitude of 1000 masl (Storey, 1939; Nichols, 1950). Due to continuous spread of the disease along the coastal area of east Africa, CBSD was considered sufficiently important for resistance breeding to be included in the cassava improvement programme. This was supported by the British Government during the colonial time between 1940 and 1950 under the Eastern Africa Agriculture and Forestry Research Organisation (EAAFRO). A cassava breeding programme was initiated at Amani in Tanzania (Tanganyika) to develop cultivars resistant to both CMD and CBSD (Jennings, 1957). Since it was reported more than 60 years ago, the disease has spread and been reported in more areas beyond the eastern Indian Ocean coastal areas where it was thought to be endemic (Thresh *et al*., 1994b; Legg and Raya, 1998; Hillocks, 2002; Thresh, 2003; Ubwani, 2003; Alicai, 2007).

2.7.1 Causal agent

CBSD is caused by cassava brown streak virus (CBSV), a monopartite single stranded RNA encapsidated into flexous filamentous particles and has been placed in the genus *Ipomovirus,* family Potyrividae (Monger *et al*., 2001a; Mbanzibwa *et al*., 2009a) based on coat protection (CP) encoded sequences. Earlier studies at Amani by Storey (1936) on transmission and the possible causal agent demonstrated that the disease was graft transmissible and since there was no visible sign of the pathogen on the infected plants, it was assumed to be a virus. The study by Kitajima and Costa (1964) was the first to use electron microscopy to look for virus particles and it was shown that rod-shaped particles occurred in infected plants. Filamentous particles were also shown to be associated with CBSD infection in studies by Bock (1994) and these were typically 650 mm in length. The shape and size of the virion has recently been confirmed by Mbanzibwa *et al*. (2009a). The presence of pinwheels and slightly flexuous rod-shaped particles associated with CBSD symptoms was also detected in isolates from cassava samples collected from Kenya, an indication that a Potyvirus is associated with CBSD infection (Were *et al*., 2004a; 2004b; 2007).

CBSV has been fully characterised at molecular level (Monger *et al*., 2001a; 2001b; Mbazimbwa *et al*., 2009a; 2009b; Winter *et al*., 2010). The first molecular characterisation of CBSV was done by Monger *et al*. (2001a) when they produced a partial sequence of the CP. They were able to show, in the sequenced region, differences of 8% and 6% at nucleotide (nt) and amino acid (aa) level of three isolates from CBSD infected cassava samples from Kibaha (coastal area of Tanzania). The three characterised isolates showed different symptoms on the secondary host plant *Nicotiana bethamiana* Domin. The complete CP encoding sequences (1101 nt) from six CBSV isolates from Uganda and north-eastern Tanzania were compared to the partial CP of sequences (714 nt) of six CBSV isolates characterised previously from the coastal lowland of Tanzania and Mozambique and showed a similarity of only 75.8-77.5% (nt) and 87.0-89.9% (aa) (Mbanzibwa *et al*., 2009b). The authors concluded that there were two genetically different and geographically separated populations of CBSV in east Africa that were referred to as highland and coastal lowland CBSV strains. However, comparison to the coastal lowland CBSV strain was based on the partial CP sequences. In a separate study, Winter *et al*. (2010) compared the complete CP encoding sequence of isolates from the coastal lowlands of Tanzania and Mozambique to those from Uganda, Kenya and Malawi, and detected two distinct virus species. The virus found in the coastal areas of Tanzania and Mozambique was named cassava brown streak Mozambique virus (CBSMov) and the virus found in Kenya, Uganda, Malawi and northern Tanzania as cassava brown streak virus (CBSV). The two species induced totally different symptoms with CBSMov causing severe symptoms. This finding has significant implications for screening cassava genotypes for disease resistance in areas where CBSV has been characterised previously.

2.7.2 Virus transmission and spread

Earlier studies on disease transmission demonstrated that CBSV could be transmitted mechanically to indicator plants (*Petunia hybrid* Vim) and to infect cassava and reproduce similar CBSD symptoms using isolates from the indicator plants (Lister, 1959). The mechanical protocol involved maceration (in water containing aluminium powder and celite) of mature leaves from infected cassava plants, followed by rubbing the extract on the leaves of various test plants. Sap transmission was demonstrated when sap transmission to herbaceous hosts induced pin-wheeled inclusions similar to those associated with the

Potyviridae (by Harrison *et al*., 1995; 1997; Lennon *et al*., 1986). The Amani work done by Storey (1936) demonstrated that CBSV was perpetuated through vegetative propagation and transmissible through grafting. Grafting is now a standard method used for transmitting CBSV in virus studies (Were *et al*., 2004a; 2004b; Alicai *et al*., 2007; Mbanzibwa *et al*., 2009a; Winter *et al*., 2010). Work on pathological studies by Storey (1939) led to suspicions that whitefly (*Bemisia* spp) was the vector of the virus in the field. Vector transmission of CBSV by whitefly (*Bemisa tabaci* Gennadus) was confirmed by Maruthi *et al*. (2005) during their transmission studies although the transmission efficiency was low (22%). However, in a separate transmission study, efficiency was found to be 40.7% for *B. tabaci* and 25% for *B. disperses* Gennadus) (Mware *et al*., 2009).

Since CBSD was first reported at Amani in Tanzania (Storey, 1936), a number of surveys have been conducted that confirmed the presence of the disease at high incidences in the coastal areas of Kenya, Tanzania, northern Mozambique and areas close to the shore of Lake Malawi both in Malawi and Tanzania (Hillocks *et al*., 1996; Legg and Raya, 1998; Thresh and Mbwana, 1998; Munga and Thresh, 2002; Gondwe *et al*., 2003). In 1999, CBSD was for the first time reported in Mozambique and presence of the disease was detected at high incidences in some of the coastal districts of Nampula and Zambezi provinces (Hillocks, 2002; Thresh and Hillocks, 2003). As originally reported by Nichols (1950), CBSD distribution was limited to low altitude (not exceeding 1000 masl) and was further confirmed in Tanzania, Mozambique and Malawi with incidences occurring between 300-600 masl (Hillocks *et al*., 1996, Hillocks, 2002; Gondwe *et al*., 2003). However, CBSD has recently been reported in areas with altitudes above 1000 masl. Surveys conducted in Lake Victoria areas in Tanzania and western Kenya (these are high altitude areas) detected high incidences of CBSD (Alicai *et al*., 2007; Ntawuruhungu and Legg, 2007). The disease has also been reported in Burundi, Rwanda and DRC. This spread of the disease to new areas could be associated with distinct CBSD strains and poses a significant threat to cassava production.

2.7.3 Disease symptoms

Symptoms of CBSD on the leaf can take different forms depending on the cassava variety, the age of the plant and conditions under which it is growing (Nichols, 1950). Typical

symptoms on green leaves are feathery chlorosis around the secondary veins. Temperature has a marked effect on the expression and severity of the disease (Nichols, 1950). Storey (1936) recorded that a fall in temperature that is normally experienced during a cool season, significantly increased the effects of the disease leading to more obvious symptoms. Susceptible varieties died while tolerant ones survived the season although suffering varying degrees of dieback. Tolerant plants showed new growth during the onset of the hot season and symptoms disappeared in some genotypes. This observation suggested that low temperatures are unfavourable to the survival of susceptible and diseased plants. The critical temperature at which lethal effects are produced varies with individual varieties.

Detailed symptoms of CBSD were first described by Nichols (1950) and later elaborated on by Hillocks and Thresh (2000) as well as Hillocks and Jennings (2003). Symptoms described below are based on observations of Nichols (1950).

2.7.3.1 Leaf symptoms

Two types of leaf symptoms occur. In type one, leaf chlorosis appears first along the margins of secondary veins (Figure 2.1a) and later affects tertiary veins developing into blotches. In type two, circular patches of chlorosis are observed between the veins (Figures 2.1b and 2.1c). During advanced stages much of the leaf lamina may be affected. The diseased leaves remain attached to the plant for a long time. Unlike CMD in which leaf shape is distorted, the leaf shape remains intact in CBSD. The distinguishing feature for CBSD infection is that the lower, older leaves are usually affected (Nichols, 1950).

a b c

Figure 2.1 Leaves infected with cassava brown streak virus showing patterns of chlorosis on margins of leaf veins that is developing into blotches characteristic of type one leaf symptoms (a) and type two leaf symptoms showing circular patches of chlorosis (b and c).

2.7.3.2 Stem symptoms

On young, maturing stem tissue, purple brown lesions may be observed on the exterior surface of the stem, particularly on sensitive varieties (Figure 2.2a). Necrotic lesions in the leaf scars on the stem appear after the plant has shed leaves due to normal senescence. In severe infections, lesions develop to kill dormant auxiliary buds. When auxiliary buds die, a general shrinkage of the node occurs causing the death of internodes, leading to die back (Figure 2.2b).

a b

Figure 2.2 Brown lesions on young green stems of plants infected with cassava brown streak virus (a) and advanced stage of disease infection causing die back (b).

2.7.3.3 Root symptoms

The appearance of root symptoms varies with variety. However, for most varieties the most damaging symptom that renders the roots economically unmarketable is the appearance of yellow or brown, dry or corky necrotic rot in the internal tissues of the storage roots (Figures 2.3a and 2.3b). The corky necrosis sometimes appears together with blue or black streaks. The lesions seem to remain discrete, but in sensitive varieties almost the entire tissues of the starch storage root may be affected. In the advanced stages of infection, soft rot may appear due to secondary infection by secondary organisms (Nichols, 1950; Hillocks and Thresh, 2000). Symptoms on the outside of the roots are variable; they may appear as constrictions across the root (Figure 2.3c) or pits and fissures along the roof surface (Figure 2.3d). Sometimes the root appears healthy on the outside with no obvious constrictions or size reduction but upon cutting, they are found to be necrotic (Figure 2.3a).

a b c d

Figure 2.3 Roots from plants infected with cassava brown streak virus showing brown corky necrotic tissues (a), advanced necrosis (b), external constrictions (c) and external fissures (d).

2.7.3.4 Differences in symptom expression in different varieties

The degree by which varieties show stem and root symptoms and expression of foliar symptoms vary. Some varieties show marked foliar symptoms but no or delayed root necrosis while others show mild root necrosis without foliar symptoms. In more tolerant varieties, root necrosis can be apparently invisible until 12 or more MAP (Hillocks and Thresh, 2000; Kanju, unpublished data). It is therefore advisable to asses root necrosis at time of harvest or at least when the crop has been in the field for at least seven months.

2.7.4 Diagnostics

As mentioned above, Nichols (1950) provided the first detailed description of CBSD symptoms from his work at Amani in north-eastern Tanzania. Symptoms were noted on leaves, stems, fruits and tuberous roots. In sensitive varieties symptoms may be visible on all plant parts. Although disease symptoms are easily visible on diseased plants, diagnosis of CBSD infection based on visual inspection is not reliable because latent virus infections are usually missed. Uses of the enzyme-linked immunosorbent assay (ELISA) based approach is common for most virus infections and was used with limited success to detect CBSV on cassava samples from Malawi (Sweetmore, 1994). The development of a more reliable molecular diagnostic tool for CBSD by Monger *et al.* (2001b) was facilitated by the molecular characterisation of CBSV and sequencing of a portion of its RNA genome (Monger *et al*., 2001a). This diagnostic tool is based on a reverse transcriptase polymerase chain reaction (RT-PCR) protocol using specific primers that were designed from the partial CP encoding sequences. Currently, RT-PCR is a successful technique for diagnosis of CBSV since it is being used reliably by virologists to detect CBSV in screen house and field-collected cassava samples (Legg, 2003; Maruthi *et al.*, 2005, Mware *et al*., 2009; Abarsh *et al*., 2010; Monger *et al*., 2010).

The magnitude of the CBSD problem is increasingly becoming important because the disease is spreading to more new areas where it is causing high cassava yield losses. It is threatening food security in the east and central African region and if unchecked it will soon reach west Africa where the threat to food security will be even higher. Significant progress has been achieved in diagnostics and transmission studies (Nichols, 1950; Lister, 1959; Hillocks and Thresh, 2000; Monger *et al*., 2001a; 2001b; Legg, 2003; Maruthi *et al*., 2005, Alicai *et al.*, 2007; Bua and Namara, 2009; Mbanzibwa *et al*., 2009a; 2009b; Mware *et al*., 2009; Abarsh *et al*., 2010; Monger *et al*., 2010, Ogwok *et al*., 2010; Winter *et al*., 2010). Since the work done by Nichols and Jennings (Nichols, 1947; Jennings, 1957; 1959; 1960; 1975) on CBSD resistance breeding, little progress has been achieved particularly in understanding the genetics of CBSD resistance (Kanju *et al.*, 2003; Munga, 2008; Zacarias, 2008) and the transgenic approach (Patil *et al*., 2010). There is little information on the gene action and no molecular markers associated with the disease resistance have been found yet. It is

imperative that more efforts are geared towards diallel analysis to understand the genetic basis of disease resistance and to apply a molecular approach to uncover the association of molecular markers and CBSD resistance.

2.7.5 Economic importance of cassava brown streak disease

The main economically significant effect of CBSD is root necrosis. However, the relationship between leaf and root symptoms is not well known since some varieties with clear leaf symptoms may fail to show root symptoms while others not expressing leaf symptoms may produce root necrosis (Hillocks and Thresh, 2000).

Yield loss due to CBSD is more pronounced and obvious due to loss of root quality rather than root weight. Nichols (1950) argued that if yield is solely judged on harvested root weight, there appears to be a small difference between the yield of healthy and secondary infected plants of the same variety. Comprehensive studies on the effect of CBSD on yield and root quality of cassava was done in Tanzania by Hillocks *et al*. (2001). They compared root necrosis from infected plants to the control symptomless plants by removing and weighing the necrotic areas of the roots. To shorten this process, an attempt was made to remove all roots with disease scores of class three or above and taking this weight as an additional loss. However, this tended to overestimate yield losses, since at farm level women who are responsible for preparing cassava chips, sometimes manage to save some tissue from the roots having small patches of necrosis. Susceptible cultivars have been reported to sustain root yield loss of up to 70% per plant (Hillocks *et al.,* 2001).

More information on crop losses in Tanzania, Mozambique and Malawi at experimental and farm level has been gathered and reported (Muhanna and Mtunda, 2002; Gondwe *et al*., 2003; Katinila *et al*., 2003; Shaba *et al*., 2003; Mkamilo and Hillocks, 2006). A survey conducted in Tanga, Tanzania, revealed crop losses of up to 74% (Muhanna and Mtunda, 2002). Using these estimates and production data of FAO (2006), it was estimated that about 35000 tonnes of cassava is lost annually in the coastal regions due to CBSD. In economic terms it is estimated that US$ 16.5 million are lost per year due to CBSD (Kanju *et al.*, 2007). In Malawi, yield losses of up to 60% were reported and the range of loss at an average

disease incidence of 40% translated to 137000-172000 tonnes per year (Gondwe *et al*., 2003). This loss in monetary terms is estimated at US$ 6-7 million per year. All studies indicated that losses were largely due to root necrosis, and in some cases, constriction of roots. In cases where roots were severely necrotic, the quality of roots was significantly affected and roots rendered unmarketable, leading to total losses. Women are more aware of root necrosis symptoms than most men since they are involved in peeling and the subsequent processing. They throw away roots that show heavy necrosis. In Tanzania and Malawi, farmers indicated that roots with mild root necrosis were sometimes eaten but extensively damaged roots, whether processed or unprocessed, had a bad taste and were usually thrown away (Gondwe *et al*., 2003; Mkamilo and Hillocks, 2006). Using fermentation processing methods, farmers indicated that the affected parts of roots did not ferment and soften but remained brown, hardy and corky and could be easily separated for further flour making processes. Flours from heavily necrotic roots are discoloured and have a bitter taste that makes it unpalatable (Gondwe *et al*., 2003).

2.7.6 Disease management

Use of disease-free planting material has been recommended for management of CBSD (Storey, 1936; Hillocks *et al*., 2001; Hillocks and Jennings, 2003). However, this is not easy to achieve because in most cases, at farm level, the availability of planting material is not sufficient and disease diagnosis is furthermore not straightforward. This was also true during early studies (Storey, 1936; Jennings, 1960) and more importantly; visually symptomless plants may be latently infected. Field sanitation has also been advocated (Legg and Thresh, 2001; Hillocks and Jennings2003). When disease incidence is low (below 20%), rogueing infected individuals may be worthwhile. When planting material is obtained from symptomless parent stocks it may be useful to rogue since it is impossible to obtain cuttings that are 100% infection free in CBSD endemic areas. This method has been successfully used in Tanzania to produce symptomless breeding stocks from populations originally showing CBSD symptoms (Mtunda *et al*., 1998). In Uganda, rougueing was used to remove CBSD from the country that had been unknowingly introduced through planting material (Jameson, 1964).

Selection for resistance to CBSD began at Amani in Tanzania in the 1930s when the disease was first reported (Nichols, 1947; Jennings, 1957). An attempt to control CMD and CBSD was done by importing cassava germplasm from within Africa and other tropical countries. However, most of the imported varieties were found to be susceptible to CMD and CBSD when tested. Successful intraspecific crosses were done using a few collected varieties like Msitu Zanzibar, Aipin valenca, Macaxeira Aipin and C756B (b) (Jennings, 1957). It was possible to cross these varieties with interspecific hybrids because they possessed a degree of resistance to CMD. Few of the CMD resistant varieties possessed resistance to CBSD. Although Aipin valenca and Macaxeira were susceptible, they contributed factors for CBSD resistance, which were evident in the progeny that were produced. Interspecific crosses involving wild relatives of cassava *M. glaziovii, M. dichotoma* Ule, *M. melanobasis* Müll.Arg and *M. saxicols* Lanj were successfully made because intraspecific crosses did not lead to any further advance towards CBSD resistance. All species used during interspecific crosses contributed resistance to CBSD with *M. glaziovii* and *M. melanobasis* being the most promising. Three and two backcross generations were respectively required for *M. glaziovii* and *M. melanobasis* to restore root quality (Jennings, 1957). Third backcross generations of *M. glaziovii* led to selection of two hybrids 46106/27 and 4763/16, having good CBSD resistance. The strength of their resistance was shown in a trial conducted in 12 sites in Tanzania where disease pressure was high. Hybrid 46106/27 is the most successful product from the Amani breeding programme. The hybrid is known as 'Kaleso' in Kenya and 'Bwana Mrefu' in some parts of Tanzania (Hillocks and Jennings, 2003). This variety has been used successfully as a source of CBSD resistance. Molecular characterisation of CBSD resistant germplasm with SSR markers, including 46106/27 and Namikonga, revealed 90% similarity between the two varieties (Kulembeka *et al*., 2005), indicating that Namikonga is an Amani hybrid. In all these studies, resistance to CBSD was suspected to be recessive and multigenic (Jennings, 1957).

All hybrids developed at Amani were transferred to the Mtwapa Research Substation of the Kenya Agricultural Research Institute (KARI), near the Kenya coast, where they are being maintained and utilised for breeding. These materials represent a good source for future breeding for CBSD resistance. Varieties from farmers' fields are also a good source of

resistance. A tolerant local variety, Nanchinyaya, from southern Tanzania, was identified, evaluated and found to have a good tolerance level, showing leaf symptoms with no or delayed root necrosis beyond 12 MAP. Other tolerant varieties found in Tanzania are Muzege, Mfaransa, Gezaulole, Gago, Kiroba and Namikonga (Kulembeka and Kanju, unpublished data). In Mozambique, local varieties that showed promising levels of tolerance include Mundowaloya, Mulalalira and Nikwaha (Kanju and Zacarias, unpublished data).

2.8 Analysis of diallel crosses

In plant breeding different methods aid a plant breeder to perform selection. There are two ways a breeder can select parental genotypes, either through the direct method based on the performance of the genotype or the indirect method based on the performance of the progenies (Fehr, 1984). Direct selection of parents has been used by experienced breeders in maize and cassava to select parents rather than through the performance of progenies (Robertson, 1959; Fehr, 1984; Lee, 1995; Ceballos *et al*., 2004). Indirect selection methods are aided by the use of appropriate mating designs. Mating designs are used to produce progenies for direct utilisation in breeding programmes and in genetic studies. To identify the appropriate mating design, a breeder needs to understand the type and mode of pollination, pollen dissemination, aim of the breeding programme, genetic information required and the size of the progeny population (Stuber, 1970; 1980). Estimates of the components of variance, covariance and parental-offspring regression coefficients are interpreted in view of their genetic expectations. These are based on assumptions of the particular genetic model adopted. Mating design, such as the diallel (Sprague and Tatum, 1942), has been used to generate genetic information on both parents and their progenies. The diallel mating scheme is useful where three or more parental genotypes are crossed in all possible combinations. Diallel analysis and interpretation of genetic information is often done according to Hayman's (1954) and Griffing's (1956) procedures.

The diallel mating or crossing design provides genetic interpretations including combining ability and the inheritance of quantitative traits (Kang, 1994). The concept of general and specific combining ability was first defined by Sprague and Tatum (1942) when using the diallel mating design in their testing procedures. Knowledge on the general combining ability

(GCA) and specific combining ability (SCA) represent two major modes of gene action for quantitative traits and their relative importance are essential in formulating an efficient breeding strategy. GCA is important in plant and animal breeding and it is especially useful with regard to testing procedures in studying and comparing performances during hybrid production. By definition GCA of a line refers to the average value of the line based on its performance when crossed with other lines and is due largely to additive gene effects. SCA is the deviation of a cross from the average GCA of the parent lines and is due to non-additive gene effects (Sprague and Tatum, 1942; Falconer and Mackay, 1996).

According to Griffing (1956), the diallel crossing system is one in which a set of *p* inbred lines is chosen and crossed among each other to produce a maximum of *p x p* combinations. Three groups from the *p x p* combinations are obtained: (a) the *p* parental lines (b) one set of ½*p*(*p*-1) F_1s and (c) the set of ½*p*(*p*-1) reciprocal F_1s. When data from the *p x p* combinations are set out in a *p x p* table, x_{ii} represents the mean value for the i^{th} parent, x_{ij} the mean value for the F_1 resulting from crossing the i^{th} and j^{th} parents while x_{ji} represents its reciprocal. Depending on whether parental genotypes or the reciprocal F_1s or both are included in the mating design (Griffing, 1956), combinations are classified into four experimental methods: (a) parents, one set of F_1s and reciprocal F_1s are included (all p^2 combinations), (b) parents and one set of F_1s are included but no reciprocal [(½*p*(*p*+1) combinations], (c) one set of F_1s and reciprocals are included but not the parents [(*p*(*p*-1) combinations] and (d) one set of F_1s but neither parents nor reciprocal F_1s are included [(½*p*(*p*-1) combinations]. Two assumptions are considered when doing genetic interpretations: (a) the parental lines are selected randomly as samples from a population about which inferences are to be made and (b) the lines are deliberately chosen and cannot be regarded as a random sample from any population.

The diallel mating designs have been used to obtain genetic information on agronomical traits of importance in cassava (Hahn *et al*., 1989; Calle *et al*., 2005; Jaramillo *et al*., 2005; Cach *et al*., 2006). Diallel crosses were specifically devised to investigate GCA of parents and to identify superior parents for use in hybrid and cultivar development (Ortiz *et al*., 2001; Yan and Hunt, 2002). Root quality traits such as starch, dry matter content (DM) and

hydrogen cyanide (HCN) content are reported to be predominately non-additive while storage root yields show additive gene action (Rajendran, 1989; Amma *et al*., 1995). Non-additive gene action for yield components like harvest index, number of storage roots and storage root weight have also been reported by Rajendran (1989). In a diallel analysis of cassava genotypes' reactions to anthracnose disease, the combining ability analysis revealed presence of both additive and non-additive gene effects (Owolade *et al*., 2006). Very few diallel studies have been done on CBSD resistance (Kanju, unpublished data; Munga, 2008; Zacarias, 2008).

2.9 Molecular markers

Molecular markers are effective tools in genomic studies and development of new varieties in plant breeding. The development of DNA based molecular markers has removed most of the limitations associated with other types of markers like isozymes and morphological markers (Hillis *et al*., 1996; Melchinger, 1999; Semagn e*t al*., 2006a). They reveal differences at DNA level, providing a powerful tool for the assessment of genetic diversity of cultivated and wild species (Kumar, 1999; Collard *et al*., 2005). Molecular markers are important for genetic improvement of crops and can be used in various areas of plant breeding and germplasm management (Thottapilly *et al*., 2000). Applications include the assessment of genetic variation within and between plant populations and the elucidation of genetic relationships among adapted cultivars and accessions (Lee, 1995; Karp *et al*., 1996; Weissing *et al.,* 2005; Mace *et al.,* 2006). Unlike other types of markers, molecular markers occur in great abundance, can be identified at any stage of plant development, and are highly polymorphic and well distributed throughout the entire genome (Tanksley, 1983; Bohn *et al*., 1999).

According to Karp *et al.* (1996) there are two types of DNA markers namely the ones that rely on hybridisation between probe and homologous DNA segments within the genome, an example being restriction fragment length polymorphism (RFLP) (Beckmann and Soller, 1986) and those that use the polymerase chain reaction (PCR) (Mullis *et al.,* 1986).

In cassava, a number of molecular marker techniques including isozymes, random amplified

polymorphic DNA (RAPD), RFLP, AFLP, SSR and SNP have been identified and applied for genetic studies and crop improvement (Fregene *et al*., 1994; 2003; 2006; Chavariaga-Aguirre *et al.*, 1998; 1999; Nassar, 2000; Mba *et al*., 2001; Akano *et al*., 2002; Okogbenin and Fregene, 2002; 2003; 2007; Kullaya *et al*., 2004; Kizito *et al*., 2005; Kizito, 2006; Schaal *et al*., 2006). The nature and properties of molecular markers differ among each other and are described below.

2.9.1 Isozymes

Isozymes were first described by Hunter and Markert (1957) who defined them as different variants of the same enzyme having identical functions and present in the same individual. The discovery that electrophoretic gels could be used to separate allelic forms of enzymes (isozymes or allozymes) on a gel and detect them using histochemical activity stains marked the era of molecular markers in genetic research (Hunter and Markert, 1957). By the early 1980s, isozyme markers were being applied as a general tool for mapping polygenes. Compared to previous studies using morphological markers, studies using isozymes led to considerable success (Tanksley *et al*., 1982; Edwards *et al*., 1987). However, the number of available enzyme activity stains limited the number of detected available isozyme markers although genome coverage was improved (Liu, 1998). Consequently, informative isozyme markers were not sufficient to obtain complete coverage of the entire genome (Tanksley *et al*., 1982; Edwards *et al*., 1987). In cassava, isozyme markers have successfully been applied for germplasm conservation, genetic diversity and genetic mapping (Fregene *et al*., 1997; Chavarriaga-Aguirre *et al*., 1999; Cabral *et al*., 2002). In a study using four microsatellites on genotypes of a core collection of 630 cassava accessions that were originally genotyped using morphological and isozymes markers as well as agronomic characters, they detected the presence of unique alleles in the collection (Chavarriaga-Aguire *et al.*, 1999). Results obtained indicated that traditional markers have been highly effective at selecting unique genotypes for the core collection. The fact that isozymes are subjected to post-translational modifications and the insufficiency of loci has often restricted their utility (Staub *et al*., 1996).

2.9.2 Restriction fragment length polymorphism

RFLP was the first DNA profiling technique to be widely applied to study plant variation and the first to be used in human genome mapping among the various molecular marker techniques developed (Botstein *et al*., 1980; Weber and Helentjaris, 1989). The discovery of restriction enzymes and natural variation in DNA base sequences of organisms led to the discovery of the RFLP technique (Beckmann and Soller, 1986). Restriction enzymes are proteins that bind specifically to and cut (or modify) double stranded DNA at short, specific sites within or adjacent to a particular sequence known as the recognition sequence (Botstein *et al*., 1980). The principle of the RFLP technique relies on the complete digestion of genomic DNA with restriction endonucleases generating different lengths of restriction fragments that are detected and separated using gel electrophoresis (Beckmann and Soller, 1986). A labelled probe is used during Southern blot hybridisation to hybridise and detect single restriction fragments with sequence homology on a gel. Changes in the DNA due to mutations can create or destroy a restriction site, causing variations in number of restriction sites and therefore polymorphisms created between individuals. Changes of this nature in the nucleotides of the genome, even in a small proportion, yield a large number of potential markers (Paterson *et al*., 1991).

RFLP markers have been useful in studying genetic diversity within cassava and related *Manihot* species. Elias *et al.* (2000) used RFLP to assess the genetic variability in a traditional cassava (*M. esculenta* Crantz) farming system. The study revealed that the observed intra-varietal polymorphism and high level of genetic diversity was due to incorporation of volunteer seedlings produced by sexual reproduction at farm level. RFLP markers have been used to develop genetic maps in many plant species (Mohan *et al.*, 1997). A female genetic linkage map of cassava, incorporating 132 RFLP markers was constructed by Fregene *et al*. (1997) using a F_1 mapping population from a cross of heterozygous parents. It consisted of 20 linkage groups spanning 931.6 centiMorgan (cM).

RFLP markers have the advantage that they are co-dominantly inherited, which means homozygous and heterozygous loci can easily be distinguished (Jones *et al*., 1997). However, the drawback of this technique lies in its complex analysis. It requires pure and relatively

large amounts of DNA, is labour-intensive and time consuming, making its application in crop improvement limited (Yamamoto *et al*., 1994; Liu, 1998). The newer approaches based on PCR replaced the use of RFLP and are simple.

2.9.3 Random amplified polymorphic DNA

RAPD analysis is a PCR-based molecular marker technique where markers are generated using PCR amplification of random, unknown and arbitrary genomic DNA segments using single short 10 base pair oligonucleotide primers of arbitrary sequence (Williams *et al*., 1990). Amplification products are separated on agarose or polyacrylamide gels using electrophoresis. Polymorphisms are detected as DNA fragments (alleles) that amplify in one individual but not the other or due to size differences of the amplified fragments. The failure to amplify is a result of mutations in the primer-binding site due to single base substitutions and/or deletions or insertions that lead to different sizes of the amplified DNA fragments (Welsch and McClelland, 1990; Williams *et al*., 1990). Drawbacks associated with RAPDs are that of being a dominant marker and lack of reproducibility between runs and laboratories (Weeden *et al*., 1992; Jones, 1997; Liu, 1998). The advantage of the RAPD technique is that it is simple and straightforward, requires the use of small amounts of DNA, can identify large numbers of genetic polymorphisms between closely related taxa and a large set of primers can be screened within a short period (Weeden *et al*., 1992).

In cassava, RAPD analysis has been used for determining genetic diversity (Marmey *et al*., 1994; Laminski *et al*., 1997; Colombo *et al*., 1998; Asante and Offei, 2003; Zacarias *et al*., 2004), genetic mapping and identification of markers associated with a trait (Michelmore *et al*., 1991; Fregene *et al.*, 1997) and phylogenetic relationships (Mignouna and Dixon, 1997; Schaal *et al*., 1997). The first cassava linkage map that was constructed incorporated 30 RAPD markers (Fregene *et al*., 1997). In a diversity study of *Manihot* spp, RAPD markers were able to clearly distinguish between species in separate clusters consisting of accessions from *M. esculenta*, *M. caerulescens* Pohl and *M. glaziovii (*Marmey *et al*., 1994).

2.9.4 Amplified fragment length polymorphism

AFLP analysis is a molecular marker technique that is based on PCR analysis and restriction enzyme. The technique was first described by Zabeau and Vos (1993) and Vos *et al*. (1995) and later reviewed by Blears *et al*. (1998). It is able to detect non-specific but many independent loci, with reproducible amplifications. AFLP analysis involves the selective PCR amplification of restriction fragments generated by specific restriction enzymes (Vos *et al*., 1995). Two restriction enzymes, usually a rare cutter and a frequent cutter, are used to digest genomic DNA. Double stranded oligonucleotides, known as adapters, are ligated to the sticky ends of the genomic DNA at the specific restriction sites. Separate adapters are needed for each of the different restriction enzymes. The ligated DNA is then used as template for PCR reactions using primer combinations specific to the adapter and restriction site sequence. Selective amplification using primers with an additional one or three different nucleotides at the 3'prime-end reduces the number of amplified restriction fragments in the first cycle (using one selective nucleotide) as well as second PCR cycle (using three selective nucleotide). This facilitates the detection of polymorphisms. The differences in fragment lengths generated by the technique are due to mutations in restriction sites, insertions and deletions in regions of fragments between primer binding sites and differences in base pair sequences next to the restriction sites (where selective nucleotides bind) (Vos *et al*., 1995). The AFLP technique is a dominant marker system as homozygotes and heterozygotes cannot be distinguished.

The AFLP technique has been applied in cassava in different studies. In a study to compare morphological and AFLP marker techniques to determine the genetic relationships within Malawian cassava germplasm, Benesi *et al*. (2006) found that the genetic distance using morphological markers correlated well with that determined by AFLP analysis. AFLP analysis has been applied in genetic mapping of CBB disease resistance in cassava using an F_1population from a cross from TMS 30572 x CM 2177. Results showed that eight QTL were associated with disease resistance and explained 9-20% of the phenotypic variation (Jorge *et al*., 2000). Genetic diversity assessment of cassava accessions resistant and susceptible to CBB using the AFLP marker technique revealed a 79.2% level of polymorphism. The phenogram obtained indicated between 74% and 94% genetic similarity

among all cassava accessions analysed (Sanchez *et al*., 1999).

2.9.5 Simple sequence repeats or microsatellites

Plant genomes contain large numbers of SSRs of less than six base pairs (2-6 bp) that are tandemly repeated and widely scattered at many loci throughout the chromosome complement and are highly sensitive to mutation (Jones *et al*., 1997; Toth *et al*., 2000). In plants SSRs may typically be di-nucleotides $(AC)_n$, $(AG)_n$or $(AT)_n$, tri-nucleotides $(TCT)_n$, $(TTG)_n$, tetra-nucleotides $(TATG)_n$ and so on, where *n* is the number of repeating units within SSR loci (Jones *et al*., 1997). In addition to occurring at many different loci, they can also be multi-allelic and can be multiplexed and automated for high-throughput genotyping. In plants, $(AT)_n$ and $(GA)_n$ di-nucleotides and $(GAA)_n$tri-nucleotides are the most frequent repeat motifs (Hearne *et al*., 1992; Ma *et al*., 1996; Weissing *et al*., 2005). The procedure in identifying SSR at a particular locus involves the construction of a small-insert genomic library that is screened with a number of SSR probes to identify inserts carrying the SSRs. The identified inserts are sequenced and primers are designed that match unique flanking sequences for that particular locus. PCR amplification is used to generate DNA banding patterns that are separated on a gel. Polymorphism is detected based on different numbers and size of repeats at the two alleles of a locus. This type of polymorphism is highly reproducible. The SSR marker technique is fast, simple, inexpensive, co-dominant, highly polymorphic, amenable to automation, highly informative due to the number and frequency of alleles detected and can distinguish closely related individuals. However, if no SSR markers are available, development of SSR markers is time consuming, expensive, need DNA sequence information and specific primers. For some species, such as humans, mice, *Arabidopsis* and rice, a large amount of DNA sequence information has already been accumulated and searching through DNA sequence databases for sequences containing simple repeats may help identify microsatellites (Liu, 1998). The availability of the cassava genome sequence (Rounsley *et al*., 2009) will tremendously help in speeding up the identification of SSRs and designing of microsatellite primers and other genomic studies in cassava.

The discovery, inheritance and variability of 14 SSRs have been described for cassava (Chavarriaga-Aguirre *et al*., 1999). A subset of these SSR markers was used to evaluate the genetic diversity of the core collection of 521 cassava accessions of the world germplasm bank at CIAT. This study revealed that unique alleles were observed in accessions from Brazil, Colombia and Guatemala (Chavarriaga-Aguirre *et al*., 1999). The development and characterisation of 172 SSR primers and subsequent mapping of a subset of 36 SSRs in cassava have also been reported (Mba *et al*., 2001). These examples are some of the first reports of use of SSRs in cassava. Since then many SSRs have been developed in cassava and used successfully for mapping, fingerprinting, MAS and other applications.

The identification of SSRY28, SSRY169 and NS158 associated with CMD resistance paved the way for MAS introgression of the *CMD*$_2$ resistance gene into Latin American germplasm for the genetic improvement of cassava in Africa (Akano *et al*., 2002; Kullaya *et al*., 2004; Okogbenin and Fregene, 2007). An F_2 based linkage map generated by Okogbenin *et al*. (2006) consisted entirely of 122 SSRs that were assigned to 22 linkage groups spanning a genetic distance of 123.7 cM. More recently, a genetic linkage map for cassava has been constructed from a F_1 population based on 119 AFLP and 18 SSR markers (Kunkeaw *et al*., 2010). It consisted of 33 linkage groups spanning 1095 cM with an average of 7.99 cM between markers.

The SSR diversity at 67 unlinked loci in 303 accessions of cassava landraces from Tanzania, Nigeria, Brazil, Colombia, Peru, Venezuela, Guatemala, Mexico and Argentina was assessed by Fregene *et al*. (2003). The study revealed that more than 90% of the loci were polymorphic in all samples and estimates of genetic diversity and differentiation ranged widely from locus to locus.

2.9.6 Single nucleotide polymorphisms

SNPs are DNA sequence variations that occur when a single nucleotide (A, T, C or G) in the genome sequence is altered. For example a SNP might change the DNA sequence A**A**GGCTAA to A**T**GGCTAA. For a variation to be considered a SNP, it must occur in at least 1% of the population because a single nucleotide variant that occurs at a frequency of

1% or less is generally considered a point mutation (Brookes, 1999). In maize, a SNP frequency of one in every 48 bp in non-coding regions and one in every130 bp in coding regions has been reported (Rafalski, 2002a). Two of every three SNPs involve the replacement of cytosine (C) with thymine (T). SNPs can occur in coding (genes) and non-coding regions of the genome but have shown to be more commonly found in the intron regions (Rafalski, 2002a). Depending on their location in the genome and impact on expression of a gene, they are classified into non-coding SNPs, coding SNPs, exonic SNPs, cDNA SNPs and candidate SNPs (Kahl *et al*., 2004). SNPs in non-coding and coding regions have been extensively studied in a few plant species such as *Arabidopsis*, maize and soybean (Ching *et al*., 2002; Jander *et al*., 2002; Zhu *et al*., 2003).

There are many advanced, low- to high-throughput methods available for SNP discovery and detection such as pyrosequencing, mass spectrometry, denaturing gradient gel electrophoresis, Affymetrix chips, RNase mismatch cleavage, restriction of PCR products, single strand conformation polymorphism, primer extension, allele specific oligonucleotide hybridisation and allele specific oligonucleotide ligation (Gupta and Prasad, 2001; Rajesh and Muehlbauer, 2008). However, a combination of allele sequencing for SNP discovery and generation of cleaved amplified polymorphic sequence (CAPS) and derived CAPS (dCAPS) markers at SNP loci for SNP detection is a relatively inexpensive method (Rajesh and Muehlbauer, 2008).

SNP is an important tool in the development of molecular markers for important genes, traits and biodiversity assessment in crop plants. They are bi-allelic, amenable to automation, mutationally stable and are scored as co-dominant markers making them ideal for genetic analysis and applications (Gupta and Prasad, 2001). It has been shown that in some cases expressed sequence tags (EST) derived SNPs has a correlation with non-conservative changes in amino acids, which could lead to variation in the phenotype allowing for association of genotypic and phenotypic variation (Rafalski, 2002b).

SNP markers are increasingly having useful applications in crop improvement. SNPs have a wide variety of applications such as genetic diversity and phylogenetic studies, association

mapping, marker density increase, QTL mapping, high-throughput MAS and evolutionary biology (Rafalski, 2002b; Olsen, 2004; Lopez *et al.*, 2005; Rajesh and Muehlbauer, 2008). In cassava, limited information on discovery and use of SNP markers is available. In a study to monitor genetic diversity in cassava, 26 SNPs were identified and characterised using direct sequencing of diverse cassava varieties and an estimated frequency of one SNP every 121 nucleotides was observed (Kawuki *et al.*, 2009). From this study nucleotide diversity ranged from $7.8x10^{-4}$ to $8.6x10^{-3}$ and individual SNPs had lower polymorphic information content (PIC) values than haplotype-based SNPs. SNPs have been used in cassava to make inferences on the origin of cassava and its phylogenetic relationship with wild relatives (Schaal *et al.*, 1997; 2006; Olsen, 2004).

In a collaborative cassava project funded by the generation challenge programme (GCP), 2500 SNP markers have been discovered and generated (Ferguson, unpublished data) and a subset of it (1536 SNPs) have been used to genotype a CBSD mapping population from a Namikonga x Albert cross on an Illumina genotyping platform (Ferguson and Kulembeka, unpublished data). Cassava samples collected from Kenya, Mozambique, Tanzania and Uganda were screened for polymorphism at 19 SNP loci using SNaPshot technology on an ABI 3730 capillary sequencer. All 19 SNP loci showed polymorphism in samples from Mozambique and 13 of the 16 loci were polymorphic in samples from coastal Kenya (Ferguson *et al*., 2008).

2.10 Linkage mapping

In plants, advances in genetics have been achieved following the discovery of DNA and the identification and application of molecular markers, from electrophoresis assays of isozymes to more informative markers like SSRs and SNPs (Williams *et al.*, 1990; Vos *et al*., 1995; Chavarriaga-Aguirre *et al*., 1998; Mba *et al*., 2001; Semagn *et al*., 2006a). Genetic mapping has benefited as one of the applications of molecular markers. Genetic mapping is the determination of relative positions and order of markers linked to genes on the DNA molecule or chromosome and the relative distance between them (Paterson, 1996a; Collard *et al*., 2005). Genetic mapping is based on recombination of genes that is a result of crossovers and exchange of genetic information between non-sister chromatids of the homologous

chromosomes. Recombination is the process by which DNA molecules interact with one another resulting in rearrangement of genetic information in an organism. The gametes forming as a result of crossovers have new combinations of genes that differ from either of the parents and the observed recombination frequency between two loci is an estimate of half the number of crossover events between two loci (Jones and Karp, 1994). This process produces two types of gametes, parental and recombinant gametes when a crossover does not occur and when it occurs respectively. Thus genetic mapping is based on the principle that genes (markers linked to genes) or loci segregate via chromosome recombination during meiosis in the parents, allowing their analysis in the progeny (Paterson, 1996a). Depending on the location of genes, recombination can occur between genes on the same chromosome or different chromosomes. If genes are located on different chromosomes, they will assort independently (unlinked) and their recombination frequency, which is the proportion of the segregating progeny that are recombinants, will be 50% while genes on the same chromosome have a less than 50% recombination frequency depending on their relative distances apart. Genes that are closer together undergo fewer crossing over events and parental gametes will exceed the number of recombinant gametes while genes right next to each other on the same chromosome will rarely show a crossover event (Jones and Karp, 1994; Semagn *et al*., 2006b).

Assuming that the probability of crossovers is proportional to the distance between loci, recombination frequency gives an estimate of the distance between two loci on a chromosome (Liu, 1998). Geneticists have a standard system by which 1% recombination is equivalent to one map unit. The level of crossing over is a function of how close genes are to each other on the chromosome. If two genes are far apart, for example at opposite ends of the chromosome, chances of double crossovers happening is high. Due to double crossovers and interference, recombination events in adjacent regions of a chromosome are not independent (Stam, 1979). This makes recombination fraction non-additive along a chromosome which means an increase in distance between loci will increase the departure from additivity (Liu, 1998). When genes are closely linked (between one and ten map units), estimates of the frequency of crossing over are most reliable. In estimating actual genetic distances, Haldane and Kosambi mapping functions were developed to make recombination frequencies additive

(Liu, 1998) and are used to convert recombination fractions into map units (cM). When the recombination fraction is small, map distances using Haldane's mapping function and recombination fraction are approximately equal (Liu, 1998). As the size of the segment increases, the expected number of double crossovers increases and the map is adjusted for double crossovers through Haldane's mapping function (Haldane, 1919). Haldane's mapping function works for situations with absence of crossover interference (where coefficient of coincidence C = 1). However, experimental evidence has been found to support that crossover interference exists and crossovers occur non-randomly in genomes (Muller, 1916). The Kosambi mapping function (Kosambi, 1944) which assumes positive interference (i.e. fewer double recombinants compared to no interference (C < 1) was developed. The rationale behind Kosambi's (1944) function is that the crossover interference depends on the size of a genome segment and the interference increases as the segment decreases (e.g. C $\rightarrow$1 when r $\rightarrow$ 0.0). The relationship between the size of the segment and the crossover interference is C = 2r (Liu, 1998).

The steps for the construction of a genetic linkage map have been reviewed by Semagn *et al*. (2006b). They include (a) development of an appropriate mapping population and deciding on sample size. Parents for the mapping population should have sufficient variation for the trait of interest at both phenotypic and DNA sequence levels, variation at DNA level being essential for tracing the recombination events. A population size of 50-250 individuals have been used in mapping, but to have high resolution mapping and a statistical power to detect linkage, a large population is required (Mohan *et al*., 1997); (b) decide on the type of molecular marker(s) to use for genotyping the mapping population; (c) screen parents for marker polymorphism followed by genotyping the mapping population together with the parents and (d) linkage analysis of the data. This involves screening each marker to test conformity to Mendelian segregation patterns, calculating pair wise recombination frequencies between markers and testing existence of linkage, establishing linkage groups, estimating map distance and determining map order. In linkage mapping the null hypothesis being tested is the absence of linkage between two markers whose recombination frequency (r) is 50% (H_0: r = 0.5). There are statistical computer packages that aid in the analytical computation during linkage mapping (Cheema and Dicks, 2009). The most widely used

include MAPMAKER (Lander *et al.*, 1987), GMendel (Echt *et al*., 1992), LINKAGE (Suiter *et al*., 1983), MapManager QTX (Manly *et al*., 2001) and JoinMap (Van Ooijen, 2006).

Detailed genetic linkage maps with high genome coverage facilitate the introgression of desirable genes through MAS and allow comparative mapping between different species to evaluate similarity between gene orders and function (Paterson *et al*., 2000). MAS for CMD resistance has successfully been applied to introgress resistance to CGM and CMD in local Tanzanian varieties (Kullaya *et al.*, 2004). Maps allow detailed genetic analysis of qualitative and quantitative traits that enable localisation of QTL (Mohan *et al*., 1997) and constitute a step towards map based cloning of genes responsible for economically important traits (Mohan *et al*., 1997). The CMD_2 gene for resistance to CMD has been mapped using a linkage mapping approach (Akano *et al.*, 2002). Identification of markers associated with resistance to CBB in cassava has been achieved through genetic linkage mapping (Jorge *et al*., 2000). A genetic linkage map for cassava incorporating 119 AFLP and 18 SSR markers have been constructed from 58 F_1 progenies based on AFLP and SSR markers (Kunkeaw *et al*., 2010).

2.11 Quantitative trait loci linkage and analysis

Many traits of agricultural importance in plants, such as yield, drought, quality and some forms of disease resistance are controlled by many genes and are known as quantitative traits, also referred to as polygenic, multi-factorial or complex traits. These quantitative traits are most often difficult to measure (Paterson, 1996b) and markers offer a potential value in their diagnostics. According to Paterson (1996b), one of the examples of such traits is nematode resistance that requires one to dig up the plant to examine the damage on the roots. This is also true for CBSD that requires uprooting of the whole plant and chopping of all roots to asses root necrosis. One of the first molecular markers developed in plants were markers linked to nematode resistance (Paterson, 1996b). Regions within genomes that contain genes associated with a particular quantitative trait are referred to as QTL. Identification of QTL based on phenotypic evaluation alone is almost impossible, but the development of molecular markers in the 1980s was a major breakthrough in the characterisation of complex traits, which created opportunities to select for QTL. One of the main uses of DNA markers in

agricultural research has been in the construction of linkage maps for various crops. These maps have been applied in the identification of chromosomal regions that contain genes controlling quantitative traits as well as simple traits controlled by single genes (Mohan *et al*., 1997; Collard *et al*., 2005).

QTL mapping is dependent on the construction of genetic linkage maps as described above. Since the resulting map can be used in QTL mapping, the mapping population must be phenotypically evaluated for the trait of interest before the subsequent mapping of QTL is done.

Mapping of QTL involves three basic steps: (a) selection of appropriate genetic material (mapping population) and evaluation to generate phenotypic and genotypic data, (b) building a genetic linkage map and (c) detection of QTL and estimation of their number, location, effects and interactions. QTL analysis is based on the principle of detecting an association between trait values (phenotype) and the variants (genotypes) of a marker because genetic markers which tend to be transmitted together with a specific value of the trait are likely to be close to a QTL affecting that trait. Based on the presence and absence of a particular marker locus, the mapping population is partitioned into different genotypic groups (either two or three) and the existence of significant differences between groups for the trait being measured is determined (Tanksley, 1983; Collard *et al*., 2005). Depending on the marker system and type of population used, the observed significant differences between phenotypic means of the groups indicate that the marker locus being used to partition the mapping population is linked to a QTL controlling the trait (Collard *et al*., 2005).

In the analysis of QTL, there are various methods used in detecting QTL. They include single-marker analysis, simple interval mapping and composite interval mapping (CIM) (Tanksley, 1983; Liu, 1998). Single-marker analysis uses the statistical procedures of t-tests, analysis of variance (ANOVA) and linear regression analysis and is the simplest method for detecting QTL associated with single markers. The coefficient of determination (R^2) obtained by linear regression explains the phenotypic variation arising from QTL linked to a marker. Simple and composite interval methods use linkage maps to analyse the interval between

adjacent pairs of linked markers along the linkage group or chromosome simultaneously instead of one marker at a time (Lander and Botstein, 1989). In addition, CIM combines interval mapping and linear regression by including additional markers in the statistical model in addition to adjacent pairs of linked markers for interval mapping (Jansen, 1993; Jansen and Stam, 1994). The interval mapping method is widely used for mapping of QTL in segregating generations derived from crosses between inbred lines. When linked QTL are involved, CIM is more precise and effective at mapping QTL compared to single point analysis and interval mapping. Like in linkage mapping there are software tools that aid in the computation and analysis of QTL. QGene and MapManager QTX/QTL are commonly used computer programmes to perform single marker analysis and simple interval mapping (Nelson, 1997; Manly *et al*., 2001) while QTL Cartographer, MapManager QTX and PLABQTL are used for CIM (Utz and Melchinger, 1996; Basten *et al*., 2001; Manly *et al*., 2001). MapQTL (Van Ooijen, 2004) is software which uses interval mapping and is designed to accommodate population types from cross pollinated crops (CP)

Apart from linkage maps, identification of genes or QTL associated with traits of interest is possible through use of the bulk segregant analysis (BSA) technique. BSA was first described by Michlemore *et al.* (1991) for tagging molecular markers linked to major disease and pest resistance genes. The method provides a quick way for identification of markers in specific regions of the genome. The technique is well suited for detecting molecular markers linked to QTL with major effects. In this technique, two pooled DNA samples of individuals from a segregating population are compared and the two bulks contrasting for a trait (e.g. resistant and susceptible) or a marker allele in a previously mapped population is analysed to identify markers distinguishing between bulks. In each bulk individuals are identical for the trait or gene of interest but arbitrary for other genes. The target genomic region of interest is studied against a randomised genetic background of unlinked loci (Giovannoni *et al*., 1991). Markers polymorphic between bulks should be genetically linked to loci determining the trait employed to construct the bulks (Michlemore *et al*., 1991).

In cassava, laudable achievements have been reached in searching for QTL affecting agronomically important traits. QTL have been identified for traits for pest and disease

resistance such as CMD, CBB, CGM (Jorge *et al*., 2000; Akano *et al*., 2002; Macea *et al.*, 2008), early bulking and high dry matter content (Okogbenin and Fregene, 2002; 2003; Kizito, 2006), protein content, carotene content and delayed physiological post-harvest deterioration (Akinbo *et al*., 2008; Egesi *et al.*, 2008; Marin and Fregene, 2008; Morillo *et al*., 2008). The discovery of markers associated with these QTL has improved the efficiency of cassava breeding through MAS by which important traits are being introgressed into farmers' varieties effectively.

The review of literature has provided information on cassava biology, production and marker applications that can be applied for improvement of cassava. Cassava is an important security crop and its demand as an industrial raw material and source of bio-fuel is increasing. Ravages of biotic stresses such as CMD and CBSD viral diseases are limiting the expansion of acreage and production of cassava. CBSD that was originally confined to coastal areas is now expanding to high altitude areas where it is causing significantly high yield losses to cassava. Since the disease was reported more than 60 years ago, some progress has been achieved in CBSD resistance breeding using conventional approaches. More significant progress can be achieved if biotechnological approaches are followed. One of the biotechnological tools is the application of molecular markers to tag genes for CBSD resistance through linkage mapping. Cassava is highly heterozygous with high genetic load. Production of classical mapping populations from inbred parents is tasking given the fact that the crop has a long growing cycle. Use of a pseudo-test cross in a full-sib cross is the quickest way to produce mapping populations for linkage mapping in a heterozygous crop like cassava. In this study the main task was to search for SSR markers associated with CBSD resistance for MAS as discussed in the following chapters.

2.12 References

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CHAPTER 3

Genetic diversity of cassava germplasm susceptible and resistant to cassava brown streak disease in Tanzania

3.1 Introduction

During many years of crop cultivation, farmers have used genetic variation in wild and cultivated plants to develop their crops. In order to accelerate and optimise plant breeding, screening and evaluation of genetic variability of available germplasm is critical. In domesticated crop species, genetic diversity provides a source of variation, which is the raw material for the improvement of agricultural crops while decreasing vulnerability to biotic and abiotic stresses. It ensures long term selection gain and promotes rational use of genetic resources (Martin *et al*., 1991; Smith and Smith 1992; Messmer *et al*., 1993; Barrett and Kidwell, 1998). Of great importance to plant geneticists, breeders, taxonomists and other scientists is the genetic variability within a taxon (Prince *et al*., 1992). Diversity within a population is a product of interplay of biotic factors, artificial selection, physical environment and plant characteristics such as size, mating system, mutation, migration and dispersal (Frankel *et al*., 1995). The pattern of genetic diversity seen in crop plants results from the interaction of five important factors: gene mutation, migration, recombination, selection and genetic drift. The first three of these factors all enhance variation in natural populations, whereas selection and genetic drift tend to reduce variation (Heywood, 1991).

Conservation and utilisation of genetic resources require knowledge of the existing variation, which can be studied using a number of techniques including molecular tools. Molecular tools provide valuable information on genetic diversity through their ability to detect variation at DNA level. A variety of molecular markers have been used in different crop species for assessment of genetic diversity among cultivars and wild relatives in specific germplasm collections (Lowe *et al.*, 1996; Karp *et al.*, 1997; Jeong *et al*., 1999; Karp, 2000; Weissing *et al*., 2005; Semagn, *et al*., 2006; Zambrano *et al*., 2007). However, SSRs have been considered as the marker of choice for this application in many crop species (Gupta and Varshney, 2000; Perera *et al*., 2000; Fregene *et al*., 2003; Bajracharya *et al.*, 2006). SSR markers have also been useful in a study of genetic diversity to broaden the genetic base of

USA rice varieties, genetic analysis of Indian aroma and quality of rice and to study the genetic diversity in maize, cassava and groundnuts (Enoki *et al*., 2002; Jain *et al*., 2004; Xu *et al.*, 2004; Mace *et al*., 2006; Thomson *et al*., 2007). SSR markers have an advantage of being co-dominant, being able to distinguish between homozygous and heterozygous loci. SSRs are easy to perform, highly polymorphic, amenable to automation, highly informative due to the number and frequency of alleles detected and able to distinguish closely related individuals (Paterson *et al*., 1991; Jones, 1997; Jones *et al*., 1997; Weissing *et al*., 2005).

Genetic improvement of cassava productivity is a major concern for cassava breeders in order to increase and stabilise storage root yield for resource poor farmers who grow cassava cultivars with low genetic potential. This improvement requires a precise understanding of the genetic structure and variation underlying cassava varietal differences in a number of agronomic traits of importance. Various markers for morphological and agronomic traits have been used for divergence and characterisation studies of cassava cultivars and phylogenetic relationships within *Manihot* species (Charrier and Léfèvre, 1987; Léfèvre and Charrier, 1993; Perera *et al*., 2000; Elias *et al*., 2001a; Benesi *et al.*, 2006; Zambrano *et al*., 2007; Zacarias, 2008). Unfortunately most agronomical and morphological traits are influenced by environmental conditions in which they are grown and may in some cases offer less accuracy in genetic distance estimation (Wanyera *et al*., 1992; Jorge, 1995; Simwambana *et al*., 1996). The development of molecular marker techniques such as RFLP, RAPD, AFLP and SSR has revolutionised genetic studies in cassava and other crop species. They are not influenced by the environment and it is now possible to accurately locate QTL, identify and characterise varieties at molecular level, conduct MAS in early stages of the crop cycle, apply DNA fingerprinting to crop species, etc. (Angel *et al*., 1992; Beeching *et al*., 1994; Fregene *et al.*, 1994; Colombo *et al*., 2000; Elias *et al*., 2000; Gupta and Varshney, 2000). Molecular markers can overcome some of the difficulties encountered and can be used as an alternative for cassava genotype identification and characterisation (Marmey *et al*., 1994; Roa *et al*., 1997). Studies of genetic diversity in cassava have shown that SSR markers are highly polymorphic and with the application of few highly polymorphic SSR markers it is possible to do genetic variation studies in cassava (Fregene *et al*., 2003; Moyib *et al*., 2007).

Genetic diversity allows crop species to adapt to changing environments, including new pests and diseases and new climatic conditions. The interaction of various factors such as biotic stress (particularly pests and diseases), artificial selection at farm level, environmental factors and plant characteristics such as mating system, dispersal, spontaneous hybridisation leading to volunteer seedlings, the heterozygous nature and vegetative propagation of cassava have contributed to shaping the genetic structure in cassava (Frankel *et al*., 1995; Elias *et al*., 2001a; 2001b; Fregene *et al*., 2003). Biotic stresses such as pests like CMB and CGM and diseases such as CMD, CBSD and CBB cause enormous yield losses at farm level and in some cases lead to losses of susceptible genotypes (Storey and Nichols, 1938; Bellotti *et al*., 1985; Larbi *et al*., 1998; Legg, 1999; Hillocks *et al*., 2002; Kizito *et al*., 2005). In the last six years, CBSD has spread to regions in which it was not observed before where it is causing devastating yield losses to cassava production as well as extinction of susceptible varieties (Hillocks *et al*., 2001; Ntawuruhunga and Legg, 2007). However, there exists variation in the way different genotypes react to disease infection and genotypes with varying levels of resistance to CBSD are now available for genetic crosses to produce resistant cassava varieties (Hillocks and Thresh, 2000; Hillocks and Jennings, 2003; Kanju *et al*., 2003; 2007a; 2007b). For genetic improvement of cassava, knowledge of the extent of genetic diversity among cassava accessions with varying levels of disease resistance is crucial (Sanchez *et al*., 1999; Fregene *et al*., 2000). An understanding of the genetic structure of this species through molecular markers is therefore important for guiding parental choices in hybridisation programmes.

In the strategy to address the ravages of CBSD, field resistant cassava landraces have been identified and are being deployed to seek and produce resistance varieties. The objective of this study was twofold: (a) to analyse the genetic distance and relationship among 15 cassava accessions which are being used as potential parents in cassava improvement and (b) to select, based on genetic distances, four genetically dissimilar parents for diallel genetic studies and generating CBSD mapping populations for genetic linkage mapping.

3.2 Materials and methods

3.2.1 Plant material

Cassava genotypes from the Tanzanian Root and Tuber Crops Research programme, whose fresh young leaves were harvested and collected from the cassava germplasm bank being conserved at Kibaha Sugar Research Institute (SRI), were used for this study. Genotypes were from the cassava field gene bank that was grown at Allavi farm estate near Kibaha. All genotypes except Amani (46106/27), TMS30001 and UKG93/041 were collections from the Mtwara region, southern Tanzania. They consisted of ten resistant and five susceptible genotypes of which four were improved varieties obtained through research and 11 were farmer varieties (Table 3.1). All selected genotypes had been previously screened for CBSD (data not presented) and thus had known disease reactions. Screening was done on a scale of 1-5, where 1 represents no symptoms and 5 severe disease symptoms (Hillocks *et al.*, 1996; Anonymous, 2003). For the present study, genotypes with a disease reaction of < 3 were grouped as resistant while those with disease reaction of ≥ 3 were classified as susceptible. One of the four improved varieties (TMS30001) was introduced from IITA and it contains both CBSD and CMD resistance (Table 3.1). All materials were originally selected for genetic crosses in order to generate data for genetic studies on CBSD resistance, including estimation of combining abilities and gene actions for disease resistance. But the use of all the above parents was not feasible given the costs and space of such an experiment and selection of fewer parents was required. In order to select all possible sources of resistance for gene tagging and genetic studies, SSR markers were utilised to identify suitable parents based on their genetic relationship.

3.2.2 DNA extraction

DNA was extracted using the modified mini preparation extraction protocol of Delaporta (Dellaporta *et al*., 1983). Approximately 0.5-2.0 g of freshly harvested leaf samples from young half mature leaves was grounded in liquid nitrogen with a mortar and pestle into a fine powder. The powdered tissue was transferred to a 1.5 ml frozen Eppendorf tube, then 800 µl extraction buffer and 50 µl 20% (w/v) sodium dodecyl sulphate (SDS) was added. The extraction buffer contained 100 mM Tris (hydroxymethyl) aminomethane hydrochloride

(Tris-HCl), 50 mM ethylene-diaminetetraacetate (EDTA) and 500 mM sodium chloride (NaCl) at pH 8.0 plus 1% (w/v) polyvinylpyrrolidone (PVP) 40000 and 0.2% (v/v) β-mecarptoethanol.

Table 3.1List of Tanzanian cassava genotypes included in the study and their CBSD reaction

Name	Pedigree	Origin	Disease reaction
Kigoma-Red	Local landrace	Kigoma	Resistant
Nanchinyaya	Local landrace	Mtwara	Resistant
Kiroba	Local landrace	Kibaha	Resistant
Kalolo	Local landrace	Bagamoyo	Resistant
Kitumbua	Local landrace	Mtwara	Resistant
Namikonga	Local landrace	Mtwara	Resistant
Amani (46106/27)	BC_3*M. glaziovii*	Amani hybrid	Resistant
NDL90/034	Kibaha half sib	NARI	Resistant
UKG93/041	Kibaha half sib	UARI	Resistant
TMS30001	Breeding line	IITA	Resistant
Albert	Local landrace	Mtwara	Susceptible
Cheupe	Local landrace	Temeke	Susceptible
Kibaha	Local landrace	Kibaha	Susceptible
Kibangameno	Local landrace	Muheza	Susceptible
Mreteta	Local landrace	Masasi	Susceptible

NARI = Naliendele Agricultural Research Institute, UARI = Ukiriguru Agricultural Research Institute, IITA = International Institute for Tropical Agriculture.

The mixture in the tube was shaken vigorously until the tissue became dispersed in the buffer. Mixing was continued for 1 min and the homogenate mixture transferred to a water bath at 65^{o}C. The mixture was left in the water bath for 15 min and vortexed intermittently 5-6 times. The mixture was removed from the water bath and allowed to cool at room temperature for 2 min when 250 µl of ice-cold 5 M potassium acetate was added and mixed gently by inverting the tubes 5-6 times followed by incubation on ice for 20 min. This was followed by centrifugation at 12000 rpm for 10 min. The supernatant was transferred to a

new 1.5 ml Eppendorf tube and one volume of ice-cold isopropanol (approximately 700 µl) was added then mixed by gentle inversion 8-10 times. This was followed by incubation at -80^{o}C for 1 h and centrifugation at 12000 rpm for 10 min. The supernatant was poured off and the last drops of isopropanol removed by placing the Eppendorf tube face down on paper towels. The pellet was re-suspended in 500 µl of 50 mM Tris-HCl/10 mM EDTA by incubating at 65^{o}C for 10-15 min with constant gentle shaking. One volume of ice cold isopropanol (about 500 µl) was added and mixed by gentle inversion 8-10 times, followed by incubation at -80^{o}C for1 h and centrifugation at 12000 rpm for 10 min.

The supernatant was poured off and the last drops of isopropanol removed by placing the Eppendorf tube face down on paper towels. The pellet was allowed to air dry by leaving it on the paper towel for 1 h. About 100-200 µl of 10 mM Tris-HCl/1 mM EDTA containing 10 mg/ml RNase A was added and stored overnight at 4^{o}C to dissolve the pellet. The DNA was stored at -20^{o}C for long term purposes or at 4^{o}C for temporary storage.

3.2.3 Determination of DNA quantity and quality

Yield and quality of isolated DNA samples were determined using a spectrophotometer and agarose gel electrophoresis, respectively. The DNA concentration of each of the samples was determined by taking the reading directly from a Cecil CE3021 spectrophotometer (Cecil Instruments Cambridge, UK). The A260/A280 ratio was used to provide an estimate of DNA purity. The quality and purity of DNA was verified by running samples on a 0.8% (w/v) agarose gel in 1x Tris-acetate EDTA (TAE) (40 mM Tris-HCl and 1 mM EDTA at pH 8.3 adjusted with acetic acid) buffer containing ethidium bromide (10 mg/ml) at 80 V for 1 h. The simultaneous loading of standard λ-DNA at concentrations of 50, 100, 150 and 200 ng/µl also allowed the determination of DNA concentration. To visualise DNA under ultraviolet (UV) illumination, the Syngene Bioimaging System (Sony Corporation Tokyo) fitted with analysis software was used. After determination of DNA concentration and quality, each of the samples was diluted to a working concentration of 10 ng/µl by adding double distilled water.

3.2.4 SSR analysis

SSR markers used in this study are listed in Table 3.2 and were supplied by CIAT and consisted of 36 primer pairs, which were selected from a subset of 186 SSR markers developed at CIAT (Chavariagga-Acquire *et al*., 1998; Mba *et al*., 2001). The 36 primer pairs (Table 3.2) were selected based on being polymorphic, having clear reproducible allele patterns, high PIC, even spread across the cassava genome (16 of the 18 linkage groups were represented by at least one SSR marker each) and complete lack of duplication in the genome (Mba *et al*., 2001; Hurtado *et al*., 2008). In assessing the genetic diversity in cassava, a minimum of 30 SSR markers are considered enough to obtain the maximum amount of information on allelic diversity (Fregene *et al*., 2003).

Amplification of DNA samples was carried out on a GeneAmp®PCR System 9700 Base Module (Applied Biosystems Inc) PCR machine as described by Mba *et al*. (2001). PCR reactions were carried out in 25 µl volumes containing 50 ng genomic DNA, 1x reaction buffer (10 mM Tris-HCl at pH 8.3 and 50 mM KCl), 0.2 µM of each forward and reverse primer, 1.5 or 2.0 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTP) and 0.3 U *Taq* DNA polymerase. The thermocycling profile consisted of an initial denaturation step for 2 min at 95°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C or 45°C for 1 min and primer extension at 72°C for 1 min and a final extension cycle of 5 min at 72°C. Before amplification products were resolved using polyacrylamide gel electrophoresis (PAGE), it was necessary to confirm whether amplification occurred or not. About 3 µl of the PCR products were electrophoresed on 1.5% (w/v) ethidium bromide-stained agarose gels for 30 min at 150 V.

Table 3.2 SSR primers, sequences, annealing temperature and linkage group for each SSR locus used for assessing cassava parental genotypes from Tanzania

Locus	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Annealing. temperature (^{0}C)	Linkage group[a]
SSRY4	CTAACGCACACGACTACGGA	ATAGAGCAGAAGTGCAGGCG	GA(16)TA GA(3)	55	A
SSRY5	CGCCTACCACTGCCATAAAC	TGATGAAATTCAAAGCACCA	GA(38)	55	J
SSRY9	CCGTTATTGTTCCTGGTCCT	ACAATTCATCATGAGTCATCAACT	GT(15)	55	D
SSRY12	CCACAATTTCTACAT	AACTTCAAACCATTCTACTTC	CA(19)	55	H
SSRY19	TCTCCTGTGAAAAGTGCATGA	TGTAAGGCATTCCAAGAATTATCA	CT(8)CA(18)	55	V
SSRY21	CAACAATTGGACTAAGCAGCA	CCTGCCACAATATTGAAATGG	GA(26)	55	B
SSRY34	TTCCAGACCTGTTCCACCAT	ATTGCAGGGATTATTGCTCG	GGC(5)GGT GGC GGT(2)	55	M
SSRY38	GTAGTTGAGAAAACTTTGCATGAG	GGCTGTTCGTGATCCTTATTAAC	CA(17)	55	G
SSRY45	TCCAGTTCACATGTAGTTGGCT	TGAAACTGTTTGCAAATTACGA	CT(27)	55	C
SSRY47	TTGGAACAAAGCAGCATCAC	GGAGCACCTTTTGCTGAGTT	CA(17)	55	J
SSRY49	TGAAAATCTCACTGGCATTATTT	TGCAACCATAGTGCCAAGC	GA(25)	55	C
SSRY51	GGATGCAGGAGTGCTCAACT	AGGTTGGATGCTTGAAGGAA	CT(11)CG CT(11) CA(18)	55	N
SSRY61	GGCTGCTTTACCTTCTACTCAGA	CAAGAACGCCAATATGCTGA	CA(12)	55	B
SSRY63	AAGACAATCATTTTGTGCTCCA	TCAGAATCATCTACCTTGGCA	GA(16)	55	H
SSRY64	GCAGAGGTGGCTAACGAGAC	CGACAAGTCGTATATGTAGTATTCACG	CT(13)CG CT(6)	55	J
SSRY69	CACTCCGTTGCAGGCATTA	CGATCTCAGTCGATACCCAAG	CT(18)ATT AT(2) CTTTCTTCTTT(2)CCTTCT	55	I
SSRY79	CAGCATCAGAAAGACAAAAACAA	CAAACCAATGGTCATGCTGT	CT(19)T CT(7)	55	na
SSRY82	CACCATCGGCATTAAACTTTG	TGTGACAATTTTCAGATAGCTTCA	GA(24)	55	B
SSRY102	TTGGCTGCTTTCACTAATGC	TTGAACACGTTGAACAACCA	GT(11)	55	M
SSRY110	ATCCACCTTAAAACA	TTATTAATCAAA	T(12)	55	L
SSRY135	AACATGTGCGACAGTGATTG	CCAGAAACTGAAATGCATCG	CT(16)	45	G
SSRY164	TGAGATTTCGTAATATTCATTTCACTT	TCAAACAAGAATTAGCAGAACTGG	GA(29)	45	H
SSRY175	GCTAACAGTCCAATAACGATAAGG	TGACTAGCAGACACGGTTTCA	GA(38)	55	K
SSRY177	ACCACAAACATAGGCACGAG	CACCCAATTCACCAATTACCA	CCT(6)CTN(65)CT(4)AT CT(18)	45	O
SSRY179	GCGAAAGTAAGTCTACAACTTTTCTAA	CAGGCTCAGGTGAAGTAAAGG	GA(28)	55	F
SSRY181	CAATCGAAACCGACGATACA	GGTAGATCTGGATCGAGGAGG	GA(22)G(3)C GA(3) GGAA GA(4)	55	K
SSRY182	TTCCTTTACAATTCTGGACGC	GGAATTCTTTGCTTATGATGCC	CA(17)N(31)GAGG GA(8)	55	M

na = linkage group not available

[a]Linkage groups according to Mba *et al*. (2001).

3.2.5 Polyacrylamide gel electrophoresis and silver staining

Amplification products were resolved on 6% (w/v) denaturing PAGE [19:1 acrylamide:bis-acrylamide, 7 M urea and 1x TBE (89 mM Tris-HCl, 2 mM EDTA and 89 mM anhydrous boric acid) and visualised by silver staining. Prior to loading, 5 µl of PCR products were mixed with an equal volume of formamide loading buffer [95% (v/v) de-ionised formamide, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 20 mM EDTA pH 8.0 and 10 mM NaCl] and denatured at 95°C for 5 min. After denaturing, the mixture was immediately placed on ice and after cooling an aliquot of 7 µl from each sample was electrophoresed in 1x TBE buffer at 80 W for 2 h on a Sequi-Gen GT Nucleic acid Sequencing cell. A molecular marker size standard was run together with samples to determine allele sizes.

PAGE gels were visualised by silver staining according to Promega's manufacturing guide (Caetano-Anollés and Gresshoff, 1994; Mba *et al*., 2001). After staining, gels were left in the open overnight to air dry. Unambiguous SSR fragments were scored manually. A di-allelic model of inheritance was adhered to in which markers giving only two bands for each individual sample was scored and the rest were eliminated. Nine markers were eliminated in the final analysis because they were either monomorphic, did not amplify all samples or gave amplification patterns which were difficult to score.

3.2.6 Data analysis

All unambiguous SSR fragments from each of the 27 SSR markers were scored using binary numbers as presence (1) and absence (0) of an allele across all 15 genotypes. The genotype NDL90/034 was also eliminated because of too many missing data. The SSR marker data was analysed using Numerical Taxonomy Multivariate Analysis System (NTSYSpc) version 2.20e (Applied Biostatistics). The binary data matrix was converted into a genetic similarity matrix using Dice similarity coefficient (Dice, 1945) and clustered using the unweighted pair group method using arithmetic averages (UPGMA) (Sokal and Michener, 1958). UPGMA clustering was performed using the SAHN programme of NTSYSpc to construct the dendrogram. Using the MXCOMP programme of NTSYSpc, coefficient of cophenetic correlation between the genetic

similarity (original distances) and cophenetic distances were computed for the dendrogram. Using the Mantel correspondence test (Mantel, 1967), significance was determined to test the goodness of fit between the genetic similarity and the cophenetic matrices. Clustering of genotypes by principal component analysis (PCA) was performed using the PROJ module of NTSYSpc to produce ordination of accessions. A scatter plot of cassava accessions in two dimensions was produced using a MatrixPlot module.

Allele frequency, gene diversity and PIC were calculated using PowerMarker V3.25 software (Liu and Muse, 2005). Gene diversity (H) is the probability that two randomly chosen alleles are different in the sample while PIC gives informativeness of a marker (Weissing *et al*., 2005). Gene diversity and PIC as implemented in PowerMarker were calculated using the following formulas:

$$H_l = 1 - \sum_{i=1}^{k} P_i^2$$

where H_l is Nei's gene diversity, l = 1, 2, 3... number of loci, P_i is the frequency of i^{th} allele and k is the number of alleles.

$$PIC_l = 1 - \left(\sum_{i=1}^{k} P_i^2\right) - \sum_{i=1}^{k-1} \sum_{j=1+1}^{k} 2P_i^2 P_j^2$$

where PIC is polymorphic information contentment for l = 1, 2, 3.... number of loci, P_i and P_j are the frequencies of i^{th} and j^{th} alleles and k is the number of alleles (Botstein *et al*., 1980; Liu, 2005).

3.3 Results

3.3.1 SSR amplification

A total of 36 SSR loci were used in this study to provide information on genetic diversity and genetic distance among the 15 cassava parental genotypes chosen for CBSD resistance studies in Tanzania. Of the 36 SSR markers used in this study, 27 primers equivalent to 75%, were polymorphic and one (SSRY171) was monomorphic and not informative. Three markers (SSRY52, SSRY59 and SSRY103) did not generate good PCR amplification and were excluded.

Five markers (SSRY100, SSRY106, SSRY108, SSRY155 and SSRY169) showed selective amplification because less than 50% of the samples gave amplification products and they were finally excluded from further analysis. One cassava genotype, NDL90/034 was eliminated from further analysis due to a large number of missing data.

The observed number of alleles at each locus, gene diversity and PIC is presented in Table 3.3. A total of 103 clear and scorable DNA fragments were detected among the 14 genotypes with an average of 3.81 alleles per primer pair. The number of alleles observed at each locus in the data set ranged from two to six alleles per locus (Table 3.3). Three or more alleles were found in 81.5% of the 27 studied marker loci. The highest number of alleles was generated by SSRY175, SSRY177 and SSRY179.

The average allele frequency of the most predominant alleles across loci was 0.75 with the highest frequency of 0.88 observed in marker SSRY5 and the lowest was 0.64 (SSRY51). PIC values give information on what each marker contributes to the study. It is the measure of the usefulness of each marker in distinguishing one individual from another and PIC values are influenced by both the allele number and allele frequency (Liu *et al*., 2000). PIC values ranged from as low as 0.15 and 0.16 for SSRY5 and SSRY9 to as high as 0.34 for SSRY51. The allele frequency of the predominating allele observed from the data has a direct correlation with the PIC value (Table 3.3). Marker SSRY5 which had the highest predominant allele frequency had the lowest PIC and the converse was true for marker SSRY51 (Table 3.3). PIC values were lower than the estimates of unbiased Nei's gene diversity (H) across all loci (Table 3.3). Generally PIC values were low but markers SSRY4, SSRY19, SSRY51, SSRY61, SSRY79, SSRY102 and SSRY179 had higher values for both PIC and gene diversity than the rest of the markers (Table 3.3).

3.3.2 Genetic relationship among the genotypes

The similarity indices generated by the 27 SSR markers among the cassava genotypes were determined by calculating Dice coefficients (Dice, 1945). The genetic similarities among genotypes ranged between 0.38 and 0.90 (Table 3.4).

Table 3.3 Number of alleles, allele frequency, gene diversity and polymorphic information content for each SSR marker used to asses genetic diversity within cassava parental genotypes from Tanzania

Marker	Number of alleles	Major allele frequency	Gene diversity	PIC [a]
SSRY4	3	0.67	0.40	0.31
SSRY5	3	0.88	0.18	0.15
SSRY9	2	0.86	0.20	0.16
SSRY12	4	0.75	0.34	0.28
SSRY19	5	0.74	0.37	0.30
SSRY21	5	0.83	0.24	0.20
SSRY34	4	0.81	0.26	0.22
SSRY38	4	0.83	0.23	0.19
SSRY45	4	0.84	0.27	0.23
SSRY47	4	0.79	0.32	0.26
SSRY49	3	0.77	0.33	0.27
SSRY51	4	0.64	0.44	0.34
SSRY61	2	0.69	0.41	0.33
SSRY63	2	0.78	0.30	0.24
SSRY64	4	0.68	0.38	0.30
SSRY69	5	0.71	0.37	0.30
SSRY79	4	0.70	0.40	0.32
SSRY82	5	0.70	0.38	0.30
SSRY102	2	0.68	0.40	0.31
SSRY110	3	0.69	0.37	0.29
SSRY135	4	0.80	0.30	0.24
SSRY164	3	0.71	0.36	0.28
SSRY175	6	0.80	0.28	0.23
SSRY177	6	0.77	0.32	0.26
SSRY179	6	0.70	0.40	0.32
SSRY181	2	0.77	0.31	0.25
SSRY182	4	0.73	0.37	0.29
Mean	3.81	0.75	0.33	0.27

[a] PIC = Polymorphic information content.

Table 3.4 Dice similarity coefficients for SSR characterisation of 14 cassava varieties

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Kigoma-Red	1.00													
Nanchinyaya	0.51	1.00												
Kiroba	0.74	0.48	1.00											
Amani (46106/27)	0.64	0.52	0.67	1.00										
Kalolo	0.53	0.60	0.51	0.56	1.00									
Namikonga	0.56	0.53	0.58	0.90	0.51	1.00								
Kitumbua	0.67	0.48	0.63	0.41	0.52	0.38	1.00							
TMS30001	0.46	0.41	0.51	0.50	0.44	0.46	0.47	1.00						
UKG93/041	0.46	0.52	0.47	0.44	0.46	0.51	0.48	0.59	1.00					
Albert	0.62	0.63	0.53	0.56	0.54	0.57	0.44	0.60	0.48	1.00				
Cheupe	0.68	0.49	0.62	0.55	0.54	0.58	0.55	0.51	0.54	0.55	1.00			
Kibaha	0.77	0.45	0.69	0.62	0.58	0.60	0.60	0.57	0.57	0.63	0.66	1.00		
Kibangameno	0.56	0.55	0.48	0.56	0.55	0.56	0.48	0.52	0.46	0.54	0.57	0.66	1.00	
Mreteta	0.63	0.48	0.65	0.64	0.51	0.66	0.55	0.52	0.48	0.61	0.60	0.61	0.62	1.00

1 = Kigoma-Red, 2 = Nanchinyaya, 3 = Kiroba, 4 = Amani (46106/27), 5 = Kalolo, 6 = Namikonga, 7 = Kitumbua, 8 = TMS30001, 9 = UKG93/041, 10 = Albert, 11 = Cheupe, 12 = Kibaha, 13 = Kibangameno, 14 = Mreteta.

The highest genetic similarity was recorded between Amani (46106/27) and Namikonga while the lowest was between Namikonga and Kitumbua (Table 3.4). The Dice similarity coefficient matrix was used to construct hierarchical clusters and the resulting UPGMA dendrogram is presented in Figure 3.1.The Mantel test of association of similarity coefficients and cophenetic values were applied to measure the goodness of fit of the dendrogram (Mantel, 1967). The matrix correlation obtained was 0.80, which showed that the goodness of fit of the dendrogram was good. The dendrogram obtained showed genetic similarity that ranged between 0.49 and 0.90. At 0.62 genetic similarity four clusters were defined among the genotypes. With the exception of cluster II, which contained Amani (46106/27) as an improved hybrid, all improved breeding lines from research, TMS30001 and UK92/041 clustered together in cluster IV. This cluster was entirely separated from the rest of the clusters which consisted of local landraces.

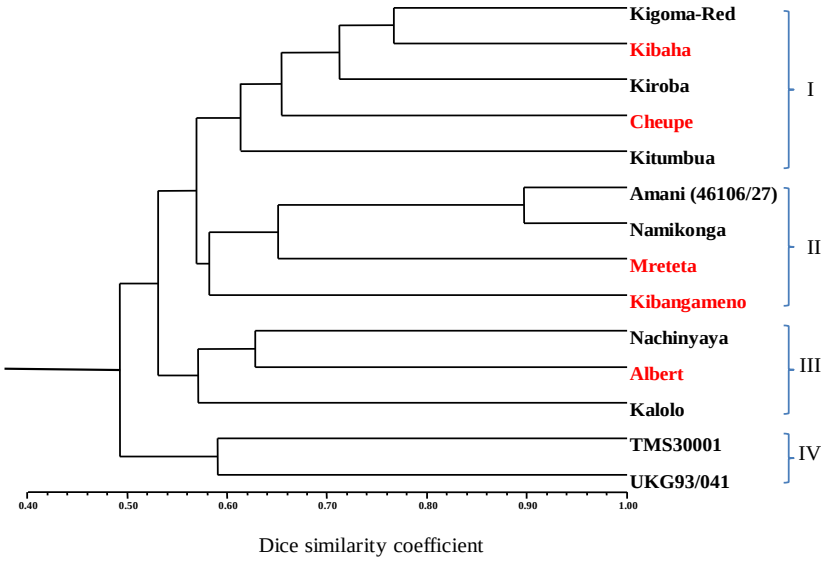

Figure 3.1 UPGMA dendrogram showing the genetic relationship among resistant (black) and susceptible (red) cassava genotypes as revealed by Dice similarity coefficients.

Clustering of genotypes by ordination is shown in Figure 3.2 which shows projections of the accessions in two dimensions, so called principal components (PC). The first PC explained 14.76% of the total variation between accessions while the second accounted for 13.84%. The ordination of cassava accessions by PCA showed relatively similar patterns of relationships as the clustering observed using cluster analysis although some differences were observed. As observed in the dendrogram, Namikonga and Amani (46106/27) clustered closely together. They were almost identical, separated by 0.01 units with respect to PC-1 but slightly further apart (0.03 units) with respect to PC-2. Kitumbua, which formed a subcluster within cluster I containing Cheupe, Kiroba, Kibaha and Kigoma-Red in the dendrogram (Figure 3.1), was also clearly separated from the group using PCA (Figure 3.2), with PC-1 explaining much of the variation within the cluster compared to PC-2. Kibangameno which clustered together with Mreteta, Namikonga and Amani (46106/27) in cluster II of the dendrogram was clearly separated from the group with respect to PCA and fell in quadrant I while the rest were in quadrant II (Figure 3.2). Kibangameno formed a subcluster within cluster II of the dendrogram, which indicated that although it clustered with Amani (46106/27), Namikonga and Mreteta, the association was not that strong. This was confirmed using PCA. PCA indicated a strong correlation with Namikonga and Amani (46106/27) based on PCA-2 but a weaker correlation to all three genotypes based on PCA-1. Nanchinyaya, Kalolo and Kibangameno grouped in different clusters, III and II, of the dendrogram (Figure 3.1) but showed a closer relationship based on PCA.

A similar pattern of clustering of resistant varieties was observed between the dendrogram and PCA. Distribution of resistant varieties into four clusters in the scatter plot was still evident as was observed by the dendrogram. Resistant genotypes were distributed in all four quadrants of the scattergram, with Nanchinyaya and Kalolo grouping close together in the same quadrant (Figure 3.2, quadrant I). Namikonga and Amani (46106/27) grouped close together in quadrant II. They clustered together in the same quadrant with only one susceptible variety, Mreteta. The resistant varieties, Kigoma-Red, Kiroba and Kitumbua clustered together in quadrant III, together with susceptible varieties Kibaha and Cheupe. Although Kitumbua was in the same quadrant as Kigoma-Red and Kiroba, it was separated from the other two when the plots of accessions were viewed with respect to PC-1. This

confirmed results obtained using cluster analysis where Kitumbua formed a subcluster within the main cluster containing Kigoma-Red, Kibaha, Kiroba, Cheupe and Kitumbua. The improved varieties UKG93/041 and TMS30001, as observed from the dendrogram, grouped together in the quadrant IV and were separated from the other genotypes (Figure 3.2).

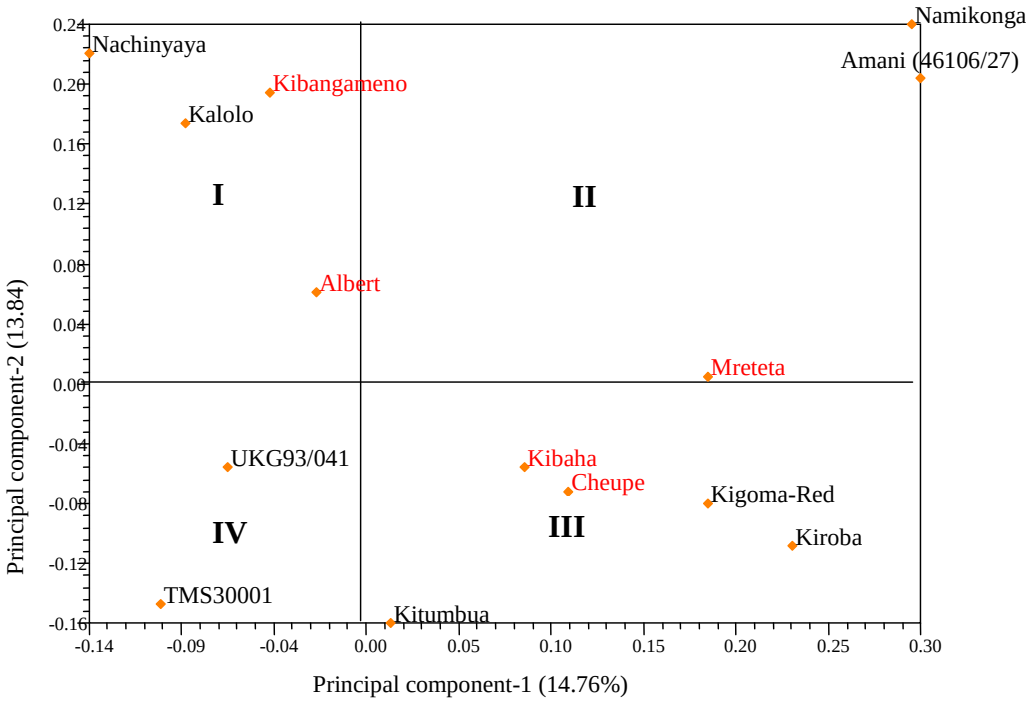

Figure 3.2 Scatter gram based on principal component analysis of ten resistant (black) and five susceptible (red) parental genotypes assessed for their genetic similarity using 27 SSR primer pairs.

3.4 Discussion

3.4.1 Genetic relationships among genotypes

The ravages of CBSD to cassava production in the affected regions of east, central and southern Africa is threatening cassava production and it calls for concerted efforts in its control through a reliable resistance breeding strategy. As a prerequisite to developing the mapping population for tagging gene(s)/QTL linked to CBSD resistance, SSR analysis was applied to a collection of resistant and susceptible Tanzanian cassava accessions. The aim was to use molecular markers to determine possible genetic differences between CBSD resistant genotypes in order to select genetically dissimilar parents for genetic crosses. As it

has been shown for resistance to CMD, clusters of resistant/tolerant germplasm based on molecular markers, may represent different sources of resistance genes (Fregene *et al*., 2000). Analysis using 27 polymorphic SSRs among 15 potential cassava parental lines defined four major clusters and indicated that at a 62% truncation point of genetic similarity, the 10 CBSD resistant lines were distributed in all four clusters. A local variety Nanchinyaya from Mtwara, southern Tanzania, which grouped together with Kalolo in cluster III of the dendrogram and quadrant I of the scattergram (Figures 3.1 and 3.2), exhibited different patterns of resistance to the disease. This variety showed delayed or no root necrosis but showed clear shoot symptoms when infected (Hillocks and Thresh, 2000). This is different from Namikonga (cluster II and quadrant II of Figures 3.1 and 3.2), which exhibited resistance in the shoot with no observed root necrosis in the roots. Apart from other factors, separation of resistant varieties into different clusters may be related to their different ways of responding to CBSD infection.

The separation between local landraces and improved lines suggested intense selection for adaptation to biotic stress traits during the breeding process where certain alleles are favoured (Fregene *et al*., 2000). UKG93/041 and TMS30001 were selected for adaptation to biotic stresses such as CMD and CBSD resistance. Even after years of testing against CMD in different locations with high disease pressure, they have continued to exhibit high levels of CMD resistance (Mahungu *et al*., 2004). Even though they clustered separately from the rest of the genotypes, they were still 49% similar to the rest of the genotypes that showed an overall similarity of 53%. This indicated that although intense selection took place, they still showed a high level of similarity to the landraces.

A notable observation was the high genetic similarity between Namikonga and Amani (46106/27). They shared a genetic similarity of 0.90 and clustered even closer together in the scattergram (Figure 3.2), implying a similar ancestry. They were furthermore the only two genotypes with a genetic similarity higher than 0.80 (Table 3.3). Amani (46106/27) is resistant to CBSD and is a third backcross derivative of a resistance breeding programme at Amani in Tanzania with *M. glaziovii* as the donor parent (Nichols, 1947; Childs, 1957; Jennings, 1960). Although the current data did not reveal 100% similarity between the two

genotypes, they have similar morphological characteristics and it has been postulated throughout the CBSD screening period in Tanzania that the two varieties are the same and could have been diffused to farmers (E. Kanju, person communication).

Diffusion of varieties from research to farm level is a result of variety's success with farmers and they become widespread leading to being assigned different names and considered different varieties. This change of variety composition at farm level has been traced to active farmer participatory involvement in evaluation and selection of elite varieties, which are adapted to farmers' agro-ecologies, cropping systems and new market demands (Nweke *et al*., 1994; 2002). In Kenya, where all hybrids have been transferred following the termination of the cassava breeding programme at Amani in 1956, Amani (46106/27) acquired the name 'Kaleso' (Hillocks and Jennings, 2003; Njeru and Munga, 2003). The variety Kaleso became popular with Kenyan farmers due to its CBSD resistance and high yield. The variety is presently available to farmers and its CBSD resistance has persisted for many years in farmers' fields and has acquired different names in different places. Recent SNP analysis of cassava accessions consisting of different CBSD mapping populations, including Namikonga and Kaleso, revealed a 100% similarity between the two varieties, suggesting they are duplicates of one variety (M. Ferguson, IITA unpublished data)

Nanchinyaya and Namikonga clustered into different clusters and showed different CBSD symptoms. Differences in observed disease symptom expression may suggest that these two lines have different disease resistance mechanisms. It had been pointed out by Jennings (1960), one of the first researchers on CBSD, that severity of root and shoot symptoms (leaf and stem) may independently vary from each other and there may be some recovery during periods of rapid plant growth. According to Jennings (1960) a common form of disease resistance in the highly resistant varieties is mild brown streaks in the roots with the stem and leaves remaining symptomless. This observation does not hold for varieties such as Nanchinyaya which expressed mild or no root necrosis but showed stem and leaf symptoms. The resistance form of Kiroba, which clustered in group I, is simply based on early maturity since it can be harvested 6-9 months before root necrosis sets in (Hillocks, 2003).
Kigoma-Red, clustering with Kitumbua, Kiroba, Kibaha and Cheupe, was collected from

southern Tanzania alongside Nanchinyaya. Although the two varieties were obtained from the same locality, they grouped into different clusters independent of their geographical origin. Using AFLP analysis, Nanchinyaya and Kigoma-Red (known as Kigoma mafia in Mozambique) also grouped in different clusters (Zacarias, 2008). The absence of clear division between cassava genotypes according to their origin has also been reported by Benesi (2005).

Although the small number of accessions selected for this study was just a subset of cassava germplasm found in Tanzania, findings suggested broad genetic diversity within cassava populations in the country. Most of the accessions studied were collected from the eastern and southern coastal areas of Tanzania where CBSD has been endemic for many years. Due to effects of selection and adaptation for resistance to CBSD one might have expected a loss of local varieties and reduction of genetic diversity within the cassava gene pool of Tanzania. It has also been observed that the severe outbreaks of CMD in Uganda, coupled with large scale multiplication of some varieties, had no effect on the genetic diversity of cassava in Uganda (Kizito *et al*., 2005). The observed genetic diversity from this study is comparable to the broad genetic diversity of Tanzanian cassava germplasm that was observed by Fregene *et al*. (2003). This finding has an implication for the hybridisation programme for CBSD resistance breeding, because genetic gain for the trait of importance depends on the identification of recombinant individuals in various populations carrying favourable alleles. The analysis has shown that all resistant accessions were represented in all four clusters of the dendrogram, suggesting that CBSD resistance could be present in genetically diverse cassava accessions, as opposed to being concentrated in one source or few descendants/ancestries.

3.4.2 Selection of parents

Molecular markers have been useful in defining genetic variation structures in populations. It has been recommended that future improvements for responses to biotic stress, agro-ecological niches, other traits of agronomical importance and different end uses will be far more efficient if parents for breeding programmes are carefully selected based on existing genetic structures (Fregene *et al*., 2000).

Molecular markers have the advantage of generating quantitative and additive data, which are useful in selecting parents for development of breeding populations in an out-crossing species to maximise heterosis. Selection of parents based on heterotic patterns in maize has enabled exploitation of heterosis which has been the basis of a successful maize hybrid industry (Shull, 1952; Keeratinijakal and Lamkey, 1993). Selection of parents for genetic hybridisation from accessions between clusters will maximise hybrid variability while crosses of individuals within the same clusters reduces hybrid variability and should be avoided (Bhatt, 1970; Chauhan and Singh, 1982; Aruanachalam *et al*., 1984; Ariyo, 1987, Zongh-hu, 1991; Benesi, 2005).

Analysis of the 14 cassava accessions produced four divergent groups in which all resistant genotypes were distributed. No single fragment or group of fragments was observed to be unique and associated with resistance against CBSD. On the basis of the genetic structure observed from this study, four genetically contrasting genotypes were selected as parents for genetic crosses. Namikonga and Kalolo were selected as resistant parents and Kibaha and Albert as susceptible parents. Kalolo shows moderate resistance to CBSD compared to Namikonga and its source of resistance is unknown. It is a variety which is commonly grown in the coastal areas of the Indian Ocean, particularly in the district of Bagamoyo in the coastal region of Tanzania. Namikonga is a successful variety with famers in the districts of Newala and Mtwara rural areas and it shows a high level of CBSD resistance both in the shoots and roots. Because of the similarity of Namikonga with Amani (46106/27), the source of CBSD resistance in Namikonga is believed to have been obtained from the wild cassava progenitor, *M. glaziovii.* The two selected resistant varieties exhibit a branching habit of growth and therefore have high flowering ability, a trait which is important for breeding programmes. The high level of CBSD resistance exhibited by Namikonga is however compromised by its high susceptibility to CMD, which makes it difficult to select against the two viral diseases simultaneously. Although the genotypes Kibaha and Albert are susceptible to CBSD, they are very popular with farmers in the coastal areas of Tanzania. Kibaha is commonly grown in the districts of Kibaha and Rufiji in the coastal region while Albert is popular in the Mtwara region, southern coast of Tanzania. Another important feature which

was observed during screening for virus resistance is the high level of resistance to CMD of Albert despite its susceptibility to CBSD. Kibaha also showed a good level of reaction towards CMD resistance compared to other genotypes. Since they are popular with farmers due to their high yield and cooking qualities, they were incorporated in the project on MAS and participatory evaluation of varieties for farmers' preferred traits (Kullaya *et al.*, 2004) to improve their resistance to CMD and CBSD. Although Kibaha is regarded as a local landrace, it is suspected to be among the products of the former Amani cassava breeding programme in the 1940s (E. Kanju, person communication). Kibaha is an officially released variety in the coastal lowland agro-ecology with root yield potential up to 30t/ha with a dry matter content of 29.9% and is a good leaf vegetable source (Kibaha SRI, unpublished data). The two selected susceptible varieties exhibit branching habit and they have good flowering ability but Albert gives more flowers than Kibaha. All four parents were selected for genetic crosses for the diallel analysis of CBSD resistance while Namikonga and Albert were used to develop a mapping population for gene tagging for CBSD resistance.

3.5 Conclusions

The present study has shown that SSR analysis is a useful tool in studying genetic diversity for utilisation of germplasm for crop improvement. Using 27 SSR loci, a total of 103 polymorphic fragments were generated with an average of 3.8 fragments per loci.

The study has indicated that there existed wide genetic diversity within cassava germplasm from Tanzania. The highest Dice similarity coefficient was observed between Namikonga and Amani (46106/27), suggesting a common ancestry or that they are duplicates. The lowest similarity coefficient was observed between Namikonga and Kitumbua. All accessions were grouped into four main clusters with the resistant genotypes being represented in each of the four clusters. Two of the three improved varieties clustered separately in one cluster away from the local landraces.

Based on genetic relationships among genotypes, flowering ability, level of resistance and susceptibility, four genotypes, Namikonga, Kalolo, Kibaha and Albert were selected as contrasting parents for genetic crosses. Namikonga and Kalolo are resistant while Kibaha and

Albert are susceptible. The four parents were selected for genetic crosses to study the genetic basis of resistance against CBSD. In addition Namikonga and Albert were selected for the development of the genetic mapping population for gene tagging of CBSD resistance. It is recommended that any extensive hybridisation programme should be preceded by analysis of genetic structure within cassava that will aid selection of parents and benefit crop improvement as well as germplasm conservation.

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CHAPTER 4

Diallel analysis of field resistance to cassava brown streak disease in cassava germplasm from Tanzania

4.1 Introduction

The tropical root crop cassava is the third most important source of calories for human food in the tropics after rice and maize and over 600 million people depend on cassava in Africa, Asia and Latin America (Owolade *et al*., 2006). Cassava is a rustic crop that grows well in conditions where few other crops could survive. It can give appreciable yield in degraded soils, is tolerant to drought, is naturally tolerant to acid soils and offers flexibility, in time of harvesting, to farmers when they need it (Calle *et al*., 2005). Cassava requires minimum inputs making it an ideal crop for areas prone to drought in tropical and sub-tropical Africa, Asia and the Americas (El-Sharkawy, 2003). In spite of the importance of this crop as a famine and food security crop, it is constantly threatened by production constraints such as drought, low yields of local varieties, diseases and pests and shortage or lack of good quality planting materials. Virus diseases, CMD and CBSD particularly, have been a threat to cassava production.

Since CBSD was reported by Storey (1936) at the foothills of mount Usambara at AMRI in Tanganyika (now Tanzania), the disease has been known to be endemic to cassava growing areas in the east African coastal areas of Kenya, Tanzania, Mozambique and in the lakeshore areas of Malawi. In these areas the disease is causing huge losses in cassava root production and quality (Nichols, 1950). In recent years it has spread to many areas in other countries such as northern Mozambique, Uganda, DRC, Burundi and Rwanda where it is threatening cassava production and food security (Legg and Raya, 1998; Hillocks *et al*., 2002; Alicai *et al*., 2007; Ntawuruhunga and Legg, 2007). More concerted efforts are required to control the disease. As for CMD, selection of resistant varieties and continuous breeding appears to be the most efficient and sustainable strategy of controlling CBSD (Legg *et al*., 1999). It has been advocated that the development and use of resistant varieties could potentially form the

basis of a sustainable management strategy for cassava diseases (Asiedu *et al.*, 1994; Mahungu *et al.*, 1994; De Vries and Toenniessen, 2001). Achievements in developing and use of resistant varieties will require an understanding of genetics and inheritance of CBSD resistance.

The genetics of CBSD is not well known and is the least studied among cassava diseases. Despite some previous screening work on CBSD at former AMRI and KARI in the 1950s, only limited information is available on the inheritance of CBSD resistance. The first attempt in understanding the inheritance and gene action of CBSD was done at AMRI in Tanzania when breeding for resistance to CBSD and CMD were initiated in the 1940s (Nichols, 1947; Jennings and Iglesias, 2002). During that time, important sources of resistance to CMD and CBSD were *M. glaziovii* and *M. melanobasis*, which were used in interspecific hybridisation with cassava varieties leading to the production of F_1 hybrids with improved resistance (Jennings, 1957; 1960a; 1960b). In the series of resistance studies at Amani, it was shown that like for CMD, resistance to CBSD was multigenic and recessive in inheritance (Jennings, 1960a; 1978; Jennings and Iglesias, 2002). Kanju and colleagues (2003) reported a zigzag stem trait that was controlled by recessive genes associated with resistance/tolerance to the disease such that homozygous recessive and heterozygous zigzag genotypes were tolerant to CBSD. Some initiatives to produce cassava cultivars with broad resistance to CBSD through genetic transformation have been underway at IITA (Ingelbrecht *et al*., 2005). This is based on the concept of pathogen-derived resistance (PDR) which postulates that nucleic acid sequences derived from a pathogen can be used to engineer virus resistance in many crops.

Management of cassava diseases through resistance breeding will improve cassava productivity. One of the most important strategies in a breeding programme is the identification of suitable parents, which dictates the success of breeding. According to Banziger and Paterson (1992), selection of parental genotypes is usually done on the basis of their performance or the performance of their progeny. In cassava breeding, breeders traditionally did selection based on the performance *per se* of parental genotypes to be included in the breeding programmes (CIAT, 2004). In the current study on CBSD, parental

genotypes were selected based on their performance *per se* for their reaction to CBSD infection. Through combining ability analysis, diallel analysis is a technique widely used to aid in the selection of better performing parents (Hayman, 1954; Griffing, 1956).

The diallel mating design has frequently been used by plant breeders to obtain genetic information from crosses (Sprague and Tatum, 1942; Griffing, 1956; Eberhart and Gardner, 1966). Diallel studies are important and have been useful in studying inheritance of resistance to diseases and pests and other traits of agronomic importance in plants (Griffing 1956; Riggs and Hayter, 1972; Orangel and Borges, 1987; Kang *et al*., 1999). In cassava, a number of diallel studies have been conducted to study the inheritance of useful agronomic traits but few articles are available on disease tolerance/resistance inheritance. In the study of inheritance of useful traits in cassava grown in sub-humid conditions, GCA and SCA and their interaction with the environment were found to be significant (Cach *et al*., 2006). In other diallel studies, significant GCA and SCA have been observed for agronomic traits such as fresh root yield, harvest index and root dry matter content (Calle *et al*., 2005; Jaramillo, 2005; Perez *et al*., 2005a; 2005b). In a diallel analysis of cassava genotypes to anthracnose disease the combining ability analysis revealed presence of both additive and non-additive gene effects in control of disease resistance (Owolade *et al*., 2006). Results further showed that the presence of intermediate disease reactions to anthracnose among the genotypes from a resistant and susceptible cross, suggested polygenic control of disease resistance. It was shown by Zacarias (2008) that non-additive gene effects made a contribution in CBSD resistance control. In the present search for resistance to CBSD, parental genotypes and F_1 progenies were screened for their reaction to the disease and diallel analysis was performed with the objective of studying the genetics of resistance to CBSD disease within cassava germplam from Tanzania.

4.2 Materials and methods

4.2.1 Selection of parents

Parents used for this study were selected on the basis of their range of severity to CBSD infection (different in levels of field resistance to CBSD), genetic distance and ability to

flower as described in section 3.3.4. The four parents used in genetic crosses were, Namikonga and Kalolo, which showed field resistance to CBSD and Albert and Kibaha, which were susceptible. These parents were crossed in a 2 x 2 half diallel mating design (Griffing, 1956) to produce six families for genetic studies of CBSD resistance (Table 4.1).

Table 4.1 List of parents and F_1 progeny used in the half diallel study

	Entry codes	Pedigree	CBSD reaction	Remarks
Parents	1	Kalolo	Resistant	Landrace
	2	Namikonga	Moderate resistant	Landrace
	3	Kibaha	Moderate susceptible	Landrace
	4	Albert	Susceptible	Landrace
F_1	1 x 2	Kalolo x Namikonga	n/a	F_1
	1 x 3	Kalolo x Kibaha	n/a	F_1
	1 x 4	Kalolo x Albert	n/a	F_1
	2 x 3	Namikonga x Kibaha	n/a	F_1
	2 x 4	Namikonga x Albert	n/a	F_1
	3 x 4	Albert x Kibaha	n/a	F_1

CBSD = cassava brown streak disease, n/a = not available.

4.2.2 Genetic crosses

A crossing block consisting of the four parents was established at Chambezi experimental station in Tanzania in the 2005 growing season. Varieties were crossed in a half diallel mating design and resistant parents were used as females and susceptible parents as pollen donors. Genetic crosses were performed by hand pollination according to Kawano (1980) and IITA (1990). Flowering may take place any time of the year depending on the weather and age of plants but the main flowering period in the area is between May and October and it is during this time that the highest number of fruits was obtained. Plants started to flower six MAP.

Mature pollen grains (maturity of pollen was determined by change of colour - anthers change from green to yellow) were collected in the morning and mature unopened female flowers were bagged with white muslin bags to prevent honey bees or other insects from

pollinating opened female flowers. Pollination was performed in the afternoon by rubbing anthers with pollen on the stigma of female flowers. After pollination, bags were replaced on the pollinated flowers to prevent unwanted pollen grains landing on the stigma. Pollination of mature unopened female flowers was assured through emasculation by removing the perianth. Pollinated flowers were labelled to indicate each of the cross combinations. After 5-6 days muslin bags were substituted with mosquito netting bags to allow air and light flow to the developing fruit. It was essential to enclose the fruit in these bags because when fruits are mature, they dehisce explosively, scattering the seeds. Mature seed were harvested and collected 70-90 days after pollination. The average number of seeds produced for each cross was 353, ranging from 184-509.

4.2.3 Seed germination and establishment of seedlings

Cassava seeds have low rates of germination and poor rates in some varieties and require three months of dormancy to germinate (Jennings, 1963; IITA, 1990). Due to problems of seed germination, more than 100 seeds from each of the six families were sown to ensure an appropriate sample size. In February 2006 seeds were germinated and grown in seed trays containing sterilised forest soil in the screen house at SRI-Kibaha. Since temperatures up to 35^{o}C are required for seed germination of cassava (IITA, 1980; Ellis *et al*., 1982), seed trays were put in the screen house to ensure maximum temperature for seed germination. During the rainy season in March 2006, 44 days after sowing the seeds, seedlings were transported and transplanted in the field at Chambezi experimental station. Seedlings and mature stakes (about 25 cm long) from each of the four parents were planted in a single row at spacing of 1.0 m x 0.50 m. No fertiliser was applied and 12 MAP (April 2007) plants were harvested and about five to six cuttings from each of the 60 genotypes of each of the F_1 families were obtained. Cuttings were replanted three days later in order to produce more planting material for replicated and multi-location field screening for CBSD resistance. During this season NPK (15:15:15) fertiliser was applied to boost production of planting materials. At harvest time, 10 MAP, enough vegetative stakes from each of the genotypes were obtained for replicated trials at two locations, Chambezi and Naliendele. Based on the availability of enough vegetative cuttings, 35 genotypes for each cross were finally selected from each family for CBSD screening and this number of genotypes represented each F_1 family.

4.2.4 Field screening of CBSD infection

4.2.4.1 Locations

Screening of CBSD infection in the field was conducted in the 2008/9 season at two locations, Chambezi and Naliendele which are hot spots for CBSD infection. Both locations lie on the coastal belt of the Indian Ocean. Chambezi is located 06^0 33' 32"S, 38^0 54' 37"E and 48 masl while Naliendele is 10^o 22' 20"S, 40^o 10' 34"E and 111 masl. Chambezi receives bimodal rainfall with yearly variation but peaks are between September-January and March-May (Figure 4.1). Naliendele receives rainfall from December-May with scattered showers in August-October (Figure 4.2). Soils at Chambezi are characterised as very deep, excessively drained, with brownish black, poorly structured loamy sand, moderately acidic topsoil over a yellowish brown to brown, very strongly acidic, poorly structured subsoil. Naliendele soils are characterised by very deep, well drained, weak structured, dark reddish brown loamy sand topsoil over a reddish brown moderately structured sandy loam to sandy clay loam subsoil (Mugogo and Njapuka, 2007).

4.2.4.2 Field layout, planting and data collection

A randomised complete block design with four replications was used. Mature cassava cuttings (25 cm long) of each of the 35 F_1 genotypes from each family and the four parents were planted at the beginning of the rainy season at Chambezi and Naliendele (March 2008). Each replication consisted of ten entries (six crosses and four parents) that constituted main plots and each cross was represented by 35 F_1 genotypes. All 35 F_1 genotypes constituting a cross were planted together in the main plot and three cuttings of each was planted at a spacing of 1.0 m between plants and 1.0 m between genotypes/rows to give a plant population of 10000 plants/ha. In order to increase CBSD inoculum pressure, three cuttings from a known susceptible and infected genotype were planted after every seven rows of the test genotypes to act as a disease spreader. In addition, the first and last rows and first and last plants within each row were also planted with infected cuttings. This configuration ensured that every plant was exposed to similar high inoculum pressure and no plant escaped infection.

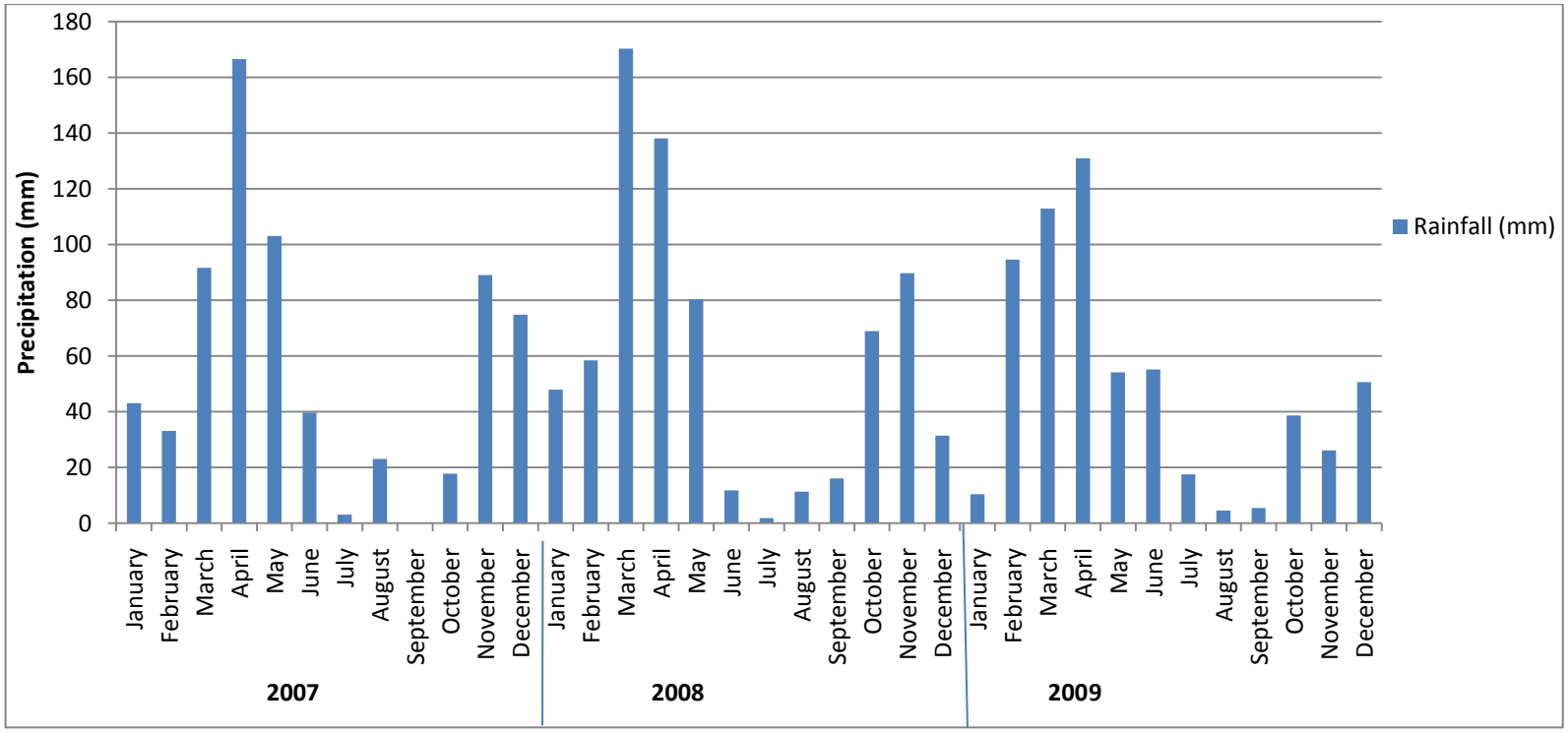

Figure 4.1 Precipitation (mm) collected at Chambezi during the growing seasons from January 2007 to December 2009. Temperature and relative humidity (RH) was not collected at Chambezi. (Source: Tanzania Meteorological Agency, 2009).

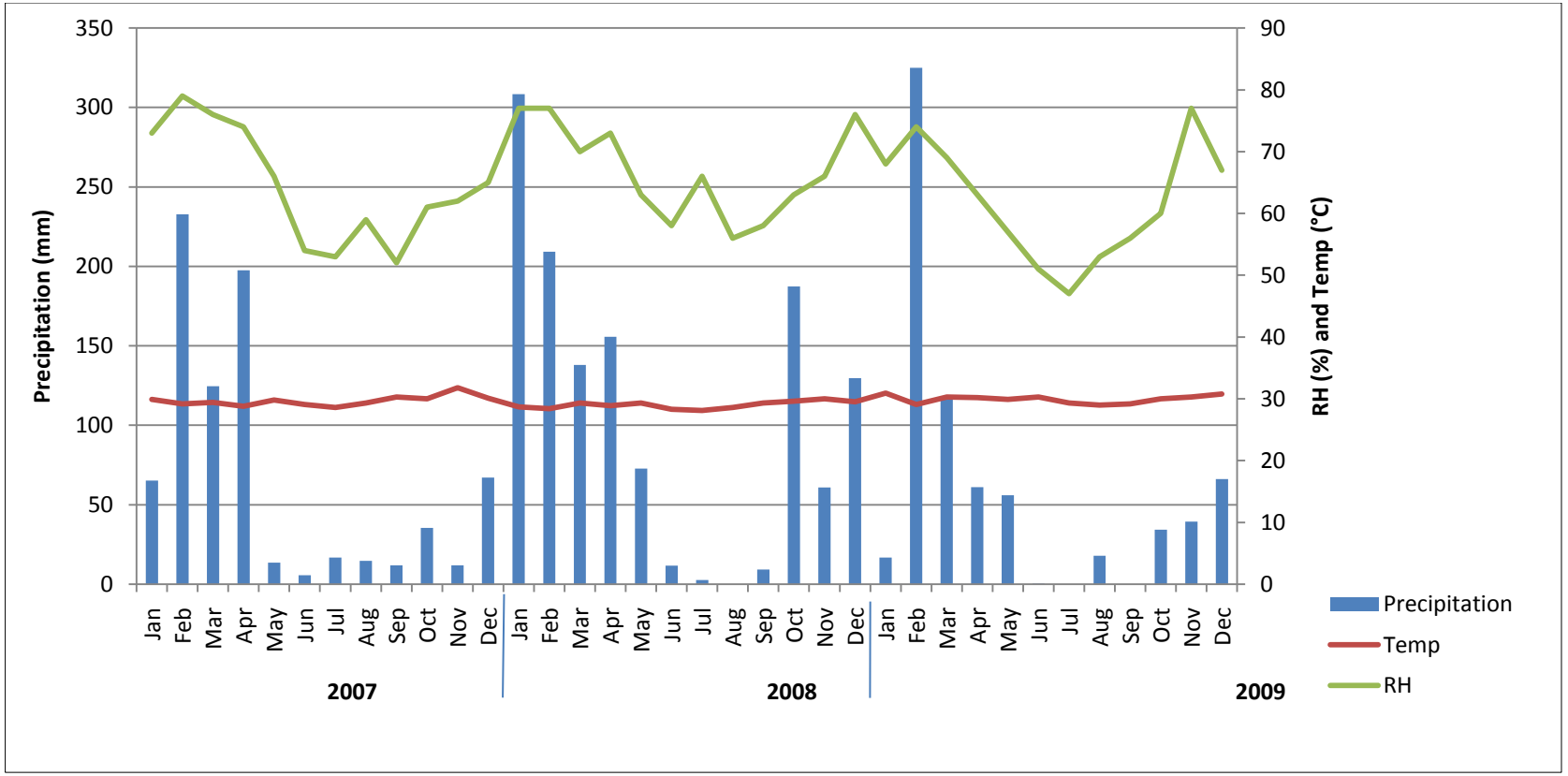

Figure 4.2 Rainfall, temperature and relative humidity (RH) observed at Naliendele during the growing seasons from January 2007 to December 2009 (Source: Tanzania Meteorological Agency, 2009).

Neither fertiliser nor irrigation was applied and the field was rain fed throughout the growing period. The field was kept weed free throughput the growing period. During the growing period plants were assessed for their reaction to CBSD infection in the shoot (stem and leaf). The trial was harvested by hand 12 MAP. Individual plants were assessed for root necrosis, number of storage roots and root yield per plant. Shoot weight was taken by weighing the shoot (stems and leaves) of each of the genotypes. Data on number of roots and yield per plant as well as shoot weight per plant for each genotype was averaged over the number of plants harvested in each plot. Harvest index (HI) was calculated as the ratio of root biomass to the total biomass (root and shoot weight) of each genotype on a fresh weight basis:

HI% = (root weight/total biomass) x 100

4.2.4.3 Field screening for CBSD

Screening of individual genotypes for their reaction to CBSD infection in the shoot was done at 3, 6 and 9 MAP and in the roots at harvest. Severity of CBSD infection on the shoot was done by assessing individual plants of each of the genotypes. Assessment involved scoring each plant individually for CBSD symptom severity on the shoot (leaf and stem) on a 1-5 scale according to Hillocks *et al*. (1996). A score of 1 means no visible leaf chlorosis/blotches or stem lesions and 5 means foliar chlorosis/blotches and/or severe stem lesions including severe die back. In addition, severity of CMD was also assessed on a scale of 1-5 where 1 means no visible symptoms and 5 means severe leaf distortion (IITA, 1990).

The main damage to the cassava crop by CBSD is caused by root symptoms (root necrosis). At harvest, roots were examined for root symptoms. Roots from each of the genotypes were chopped longitudinally and transversely to look for the presence of necrotic patches on the starch bearing tissues. The severity of root necrosis of each of the genotypes was scored on a scale of 1-5 based on a classification used by IITA (Anonymous, 2003; McSween, 2004, Figure 4.3). A score of 1 means no visible necrosis and 5 means that more than 30% of the root is necrotic. A score for each genotype was derived by taking the maximum scorefrom individual root scores.

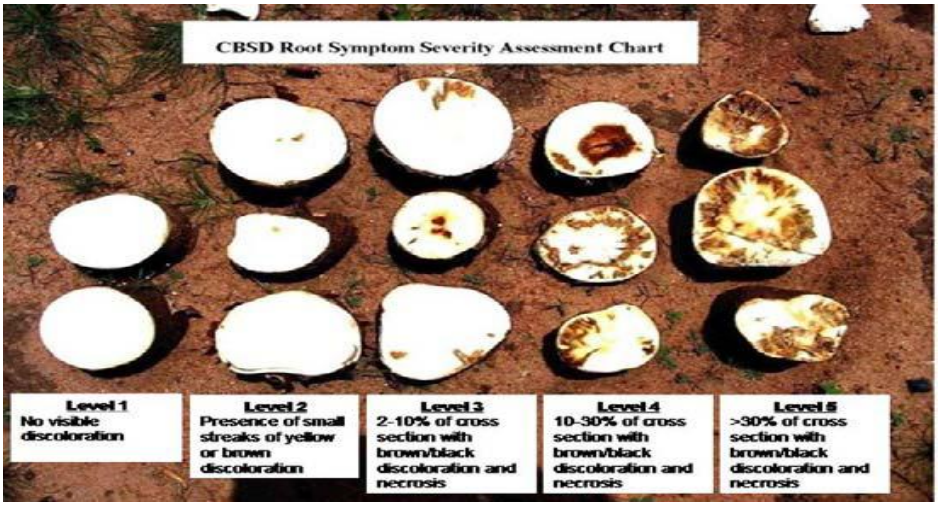

Figure 4.3 Classification of root symptom severity due to cassava brown streak disease used for assessing root necrosis. (Courtesy of: Save the Children illustration of IITA's root symptom severity classification using on-farm cassava samples, http://www.savethechildren.org).

4.2.5 Data analysis

Genotypes were partitioned into variation due to parents and crosses. A mean disease score of 35 F_1 individuals constituting each of the six crosses was used for variance and diallel analysis. ANOVA was based on Griffing's method 2, model 1 for fixed genotypes (Griffing, 1956). The ANOVA and diallel analysis was performed on individual environments using Agrobase (2005). Analysis of GCA and SCA for individual environments was done to determine the breeding value of parents and gene actions. The general linear model for each environment used to estimate GCA and SCA was:

$$Y_{ijk} = \mu+g_i+g_j+S_{ij}+R_k+E_{ijk}$$

Where: Y_{ijk} was the observed value for a cross between the i^{th} and j^{th} parents in k^{th} replication, μ was the general mean, g_i and g_j were GCA of the i^{th} and j^{th} parents, S_{ij} was the SCA of i^{th} and j^{th} cross, R_k was the replication effect and E_{ijk} experimental error. GCA and SCA variances provide respectively an indication of levels of additive and non-additive variances in a population (Falconer and Mackay, 1996).

Estimates of genetic components of variation due to GCA and SCA effects were obtained from their expected mean squares. The ratio of the components was computed to estimate

the relative importance of GCA in predicting progeny performance. The GCA:SCA ratio indicates whether the characteristic is controlled by additive or non-additive (dominant) genes. GCA and SCA effects were estimated by Agrobase (2005) and according to Singh and Chaudhary (1985). Pearson's phenotypic correlation coefficients were calculated between root necrosis and shoot infection, CBSD infection (shoot) and CMD infection as well as CBSD with other yield traits. Phenotypic correlation was calculated from the combined analysis data based on genotype values over environments and replications and the following formula was used:

$r_p = cov_{xy}/(\sigma_x\sigma_y)$

where: r_p is phenotypic correlation, cov_{xy} is phenotypic covariance between characters x and y and σ_x and σ_y are the phenotypic variance of x and y respectively. Genetic correlations were computed from the GCA effects to produce a genetic correlation matrix.

The genetic parameters were obtained by Agrobase (2005) and calculated to determine the relative contribution of each component. Estimates of GCA variance (δ^2_{GCA}) and SCA variance (δ^2_{SCA}) for each trait were derived from mean squares generated by Agrobase (2005). Additive (V_A) and dominance (V_D) variances were estimated as $V_A = 2(\delta^2_{GCA})$ and $V_D = (\delta^2_{SCA})$ where $\delta^2_{GCA} = (MS_{GCA}\text{-}MS_{SCA})/p\text{-}2$ and $\delta^2_{SCA} = MS_{SCA}\text{-}MS_E$. Estimates of phenotypic (V_P) and genotypic variance (V_G) were derived as $V_G = V_A + V_D$, $V_P = V_G + V_E$ and were used to estimate heritability values.

According to Falconer and Mackay (1996) broad sense heritability indicates the proportion of the total variance that is due to the average effects of genes and is estimated as $h^2_b = V_G/V_P$ and narrow sense heritability is the proportion of the total variance which is attributable to additive genetic variance and is given as

$h^2_n = V_A/V_P$

4.3 Results

4.3.1 Weather at trial sites

The diallel experiment was conducted at Chambezi and Naliendele whose weather patterns are relatively similar. Temperature, rainfall, and relative humidity data for

Naliendele are presented in Figure 4.2. Only rainfall data was recorded at Chambezi because of lack of equipment for measuring temperature and relative humidity and is presented in Figure 4.1. Weather data were collected throughout the experimentation phase between 2007-2009 at the two locations and showed variations in amount of rainfall received across years. The highest amount of rainfall was recorded in 2008 at both locations with Naliendele receiving a higher amount (1285.2 mm) than Chambezi (968.7 mm) (Figures 4.1 and 4.2). Evaluation of genotypes at both locations was conducted in 2008 and the amount of rainfall was sufficient to support cassava growth. Cassava is commonly grown in areas receiving less than 800 mm amount of rainfall and is sufficient for growth and development (IITA, 1990; Alves, 2002) but it is critical that water is available during 1-5 MAP. Rainfall received during this period was adequate to support cassava growth. There was variation in the amount of rainfall received across months in each year. In March 2008, the highest amount of rainfall (170.3 mm) was received at Chambezi while in March and April 138.0 and 155.6 mm was received at Naliendele, respectively. These were the months when the experiments were initiated at both locations. At both locations, from June to September there was a dry spell when the lowest amount of rainfall was received at both locations. The lowest total rainfall was received in 2009 when Chambezi received 613.5 mm compared to Naliendele which received 733.5 mm. Temperature and relative humidity were only recorded at Naliendele and showed a similar trend across years and on average the temperatures observed were 29.7°C, 29.0°C and 29.9°C in 2007, 2008 and 2009 respectively.

4.3.2 Analysis of variance

The coefficients of variation indicated that the experimental errors in this study were relatively low across both locations for most of the traits studied with the exception of number of roots per plant and fresh root weight (FRW) per plant (Table 4.2). The two traits had relatively high coefficients of variation (CV) (30.45% and 32.59% for number of roots per plant and 43.12% and 30.26% for FSW at Chambezi and Naliendele respectively). This could have been due to CBSD infection, since some genotypes had severe root necrosis which resulted in rotting of a number of cassava roots with a consequent reduction in root weight per plant.

Table 4.2 Mean squares of genotypes, GCA, SCA and GCA:SCA ratios for CBSD severity mean score and other traits in a 2 x 2 diallel evaluation of cassava F_1 and parental genotypes at Chambezi and Naliendele in 2008

Source of variation	Df	CBSD3MAP		CBSD6MAP		CBSD9MAP		CMD3MAP		CMD6MAP		CMD9MAP	
		CHZ	NDL	CHZ	NDL	CHZ	NDL	CHZ	NDL	CHZ	NDL	CHZ	NDL
Replication	3	0.045	0.220**	0.029	0.356**	0.073	0.122	0.081	0.154*	0.038	0.518*	0.124	0.670*
Crosses	9	2.387***	2.610***	3.128***	2.500***	3.611***	2.523***	2.199***	2.054**	1.460***	1.512***	1.420***	1.095***
GCA	3	1.609***	1.68***	2.175***	1.631***	2.487***	1.676***	1.308***	1.209***	0.927***	0.928***	0.919***	0.623***
SCA	6	0.090***	0.139***	0.086***	0.122***	0.111***	0.108*	0.171**	0.166***	0.084	0.103*	0.073	0.099
Residual	27	0.006	0.007	0.010	0.012	0.016	0.046	0.050	0.009	0.055	0.153	0.062	0.055
GCA:SCA		17.88:1	12.09:1	25.29:1	13.37:1	22.41:1	15.52:1	7.65:1	7.28:1	11.04:1	9.01:1	12.59:1	6.29:1
CV (%)		5.889	7.216	8.496	9.768	9.971	17.960	18.827	8.807	19.963	17.119	20.525	19.469
% SS due to GCA		89.91	85.78	92.70	86.98	91.82	88.53	79.30	78.51	84.69	81.89	86.26	75.78
% SS due to SCA		10.09	14.20	7.30	13.03	8.18	11.47	20.70	21.49	15.31	18.11	13.74	24.22

		CBSD-RN		Number of roots/plant		FRW (kg/plant)		FSW (kg/plant)		Harvest index	
Replication	3	0.104	0.116	1.817	1.334*	0.327	0.57*	0.375	0.827**	0.000	0.026*
Crosses	9	4.132***	3.733***	2.182**	0.283	3.133***	0.101***	6.160***	0.096	0.013***	0.043***
GCA	3	2.779***	2.666***	0.796**	0.066	0.822**	0.040***	2.626**	0.012	0.007***	0.019***
SCA	6	0.160**	0.067	0.42	0.073	0.764**	0.018*	0.997***	0.03	0.001	0.007***
Residual	27	0.048	0.037	0.188	0.103	0.153	0.004	0.134	0.017	0.001	0.001
GCA:SCA		17.36:1	39.79:1	1.89:1	0.90:1	1.08:1	2.22:1	2.63:1	0.4:1	7.0:1	2.7:1
CV (%)		15.098	14.710	30.449	32.589	43.117	30.264	23.715	27.424	14.836	22.34
% SS due to GCA		89.66	95.20	48.65	30.97	34.98	52.19	56.83	17.13	73.33	58.76
% SS due to SCA		10.34	4.80	51.35	69.03	65.02	47.81	43.17	82.41	26.67	41.24

CBSD 3, 6 and 9 MAP = symptom severity scores for CBSD shoot infection at 3, 6 and 9 months after planting, CMD 3, 6, 9 = symptom severity scores for CMD at 3, 6 and 9 months after planting, CBSD-RN = CBSD root necrosis, FRW = fresh root weight, FSW = fresh shoot weight, CHZ = Chambezi, NDL = Naliendele, GCA = general combining ability, SCA = specific combining ability, Df = degree of freedom, and *, **, *** = P < 0.05, P < 0.01 and P < 0.001, CV = Coefficient of variation, SS = sum of squares.

Results indicated that mean squares were highly significant (P<0.001) for crosses at all locations for CBSD root necrosis (CBSD-RN), shoot infection and other traits, except number of roots per plant and fresh shoot weight per plant, which were non-significant at Naliendele and number of roots per plant at Chambezi that were significant. GCA effects mean squares were highly significant (P < 0.001) for all traits except number of roots per plant (both locations), FRW per plant (at Chambezi) and fresh shoot weight (FSW) per plant at Naliendele (Table 4.2). GCA effects mean squares for number of roots per plant, FRW per plant and FSW per plant were significant (P < 0.01) at Chambezi but non-significant at Naliendele. SCA effects mean squares were highly significant (P < 0.001) for CBSD symptom severity in the shoot at 3 MAP and 6 MAP at both locations. SCA effects were also highly significant for CBSD symptom severity in the shoot 9 MAP and FSW per plant at Chambezi and CMD symptom severity 3 MAP and harvest index (HI) at Naliendele. SCA effects mean squares were highly significant (P < 0.01) for CMD 3 MAP, root necrosis and FRW per plant at Chambezi while the effects were only significant (P < 0.05) for CBSD infection 9 MAP, CMD infection 6 MAP and FRW per plant at Naliendele (Table 4.2). SCA effects were not significant for CMD infection 9 MAP and number of roots per plant (both locations), CMD infection 6 MAP and HI at Chambezi as well as CBSD-RN and FSW per plant at Naliendele.

For all traits except number of roots per plant, FRW per plant and FSW per plant, GCA sum of squares accounted for more than 73% of the sum of squares of crosses across all locations. The highest sum of squares accounted for by GCA were observed for CBSD infection 6 MAP at Chambezi (92.70%) while the lowest was 17.13% for FSW per plant at Naliendele (Table 4.2). For CBSD-RN and shoot infection at 3, 6 and 9 MAP the GCA accounted for between 85.78% and 92.70% of the total sum of squares of crosses. The highest proportion of variation accounted for by SCA was only 82.41%, observed for FSW per plant at Naliendele (Table 4.2). This was the same trait which had the lowest GCA contribution to the total sum of squares of crosses. The sum of squares accounted for by SCA ranged from 4.80% (CBSD-RN at Naliendele) to 82.41% (FSW per plant at Naliendele).

The relative importance of GCA and SCA were also determined by computing the

GCA:SCA ratio which is presented in Table 4.2. The ratio of GCA:SCA for most traits was larger than one except for number of roots per plant and FSW per plant at Naliendele where SCA was higher than GCA and the ratios were 0.90:1 and 0.4:1 respectively. The highest GCA:SCA ratio was 39.79:1 observed for CBSD-RN at Naliendele and the lowest was 1.08:1 for FRW per plant at Chambezi. With exception of CBSD-RN and FRW per plant higher GCA:SCA ratios were observed at Chambezi than Naliendele. Ratios showed that GCA was more important than SCA in predicting the progeny performance and explaining the breeding value of the parents.

4.3.3 Combining ability

Combining ability estimates for CBSD mean scores and other traits consisted of both GCA and SCA estimates.

4.3.3.1 General combining ability

Lower disease scores indicate disease resistance and hence low GCA are desirable for CBSD resistance. Namikonga, a CBSD resistant parent had a negative and significantly lower GCA for CBSD shoot infection (at 3, 6 and 9 MAP) and CBSD-RN at both locations (Table 4.3). Negative and lower GCA effects were also observed for number of roots per plant and FRW per plant at all locations. However, positive and significantly higher GCA effects for Namikonga were observed for CMD infection (3, 6 and 9 MAP) at both locations than other parents. Kalolo, which was also employed in crosses as a moderately resistant parent to CBSD infection, exhibited negative and non-significantly lower GCA effects for CBSD shoot infection (3, 6 and 9 MAP), root necrosis and FRW per plant at both locations than the susceptible parents (Table 4.3) except for CBSD infection 3 MAP which was positive and non-significant at Chambezi. Positive and lower GCA effects were also observed for Kalolo for CMD infection at Chambezi. Positive and significant GCA effects for Kalolo were recorded for HI and FRW per plant at both locations while for the number of roots per plant GCA effects were non-significant but positive and negative at Naliendele and Chambezi respectively. Results indicated that Namikonga and Kalolo had excellent performance towardsCBSD resistance at both locations.

Table 4.3 Estimates of GCA effects in a diallel analysis of CBSD severity mean score and other traits for cassava F_1 and parental genotypes evaluated at Chambezi and Naliendele in 2008

Source of variation	CBSD3MAP		CBSD6MAP		CBSD9MAP		CMD3MAP		CMD6MAP		CMD9MAP	
	CHZ	NDL	CHZ	NDL	CHZ	NDL	CHZ	NDL	CHZ	NDL	CHZ	NDL
Kalolo	0.030	-0.214	-0.266	-0.182	-0.289	-0.202	0.202	-0.249	0.171	-0.148	0.135	-0.011
Namikonga	-0.718	-0.624	-0.679	-0.627	-0.731	-0.643	0.564	0.466	0.439	0.392	0.455	0.152
Kibaha	0.182	0.254	0.237	0.231	0.280	0.307	-0.357	0.280	-0.139	0.237	-0.123	0.302
Albert	0.506	0.583	0.707	0.579	0.739	0.538	-0.409	-0.497	-0.471	-0.482	-0.468	-0.444
SE (Gi)	0.027	0.029	0.036	0.038	0.044	0.076	0.788	0.034	0.083	0.069	0.088	0.083
SE (Gi-Gj)	0.043	0.048	0.059	0.062	0.072	0.124	0.129	0.056	0.136	0.113	0.144	0.133
LSD (P≤0.05)	0.089	0.099	0.121	0.128	0.148	0.254	0.264	0.115	0.279	0.231	0.296	0.277

Source of variation	CBSD-RN		Number of roots/plant		FRW (kg/plant)		FSW (kg/plant)		Harvest index	
	CHZ	NDL	CHZ	NDL	CHZ	NDL	CHZ	NDL	CHZ	NDL
Kalolo	-0.472	-0.454	-0.203	0.137	0.251	0.104	-0.327	-0.050	0.050	0.076
Namikonga	-0.689	-0.660	-0.072	-0.083	-0.175	-0.021	0.099	0.059	-0.029	-0.024
Kibaha	0.504	0.369	-0.258	0.026	-0.435	-0.093	-0.651	-0.014	-0.004	-0.055
Albert	0.657	0.745	0.534	-0.080	0.359	0.009	0.879	0.006	-0.016	0.003
SE (Gi)	0.078	0.068	0.153	0.114	0.138	0.023	0.129	0.046	0.009	0.012
SE (Gi-Gj)	0.127	0.111	0.251	0.186	0.226	0.037	0.211	0.075	0.015	0.021
LSD(P≤0.05)	0.259	0.228	0.514	0.381	0.463	0.076	0.433	0.155	0.032	0.041

CBSD 3, 6 and 9 MAP = symptom severity scores for CBSD shoot infection at 3, 6 and 9 months after planting, CMD 3, 6, 9 = symptom severity score for CMD at 3, 6 and 9 months after planting, CBSD-RN = CBSD root necrosis, FRW = fresh root weight, FSW = fresh shoot weight, CHZ = Chambezi, NDL = Naliendele, SE(Gi) and SE(Gi-Gj) = standard error and standard error of difference, LSD = least significant difference.

The susceptible varieties, Kibaha and Albert had positive GCA effects for CBSD shoot infection and root necrosis across both locations. The GCA effects were significant for Albert but not Kibaha which recorded low values except for root necrosis in which all the susceptible parents had positive and significant GCA effects. Similarly, Kibaha and Albert had more or less a uniform response to CBSD infection at both locations towards susceptibility. Kibaha and Albert had negative GCA effects for CMD infection (3, 6 and 9 MAP) at Chambezi and Naliendele with the exception of Kibaha that had positive GCA effects for CMD infection (3, 6 and 9) MAP at Naliendele. The GCA effects observed for CMD infections for Albert were significant while for Kibaha it were lower and non-significant.

With the exception of number of roots per plant whose GCA effects were positive but lower at Naliendele, Kibaha had negative but lower GCA effects for number of roots per plant, FRW per plant, FSW per plant and HI at both locations. On the other hand, Albert had higher and positive GCA effects than Kibaha for CBSD infection 3, 6 and 9 MAP, CBSD-RN, FRW per plant and FSW per plant across all the locations. Number of roots per plant and HI had negative GCA effects at Naliendele and Chambezi respectively. A notable observation for Albert is the significantly higher and positive GCA effects for number of roots per plant, FRW per plant and FSW per plant at Chambezi. Although Albert is very susceptible to CBSD, it grows vigorously and has a tendency of producing high root yield explaining why the higher values were observed for the three traits (ARI Naliendele, unpublished data).

4.3.3.2 Specific combining ability

There were significant variations among F_1'sfor SCA effects for some traits and certain cross combinations (Table 4.4). For a cross involving Namikonga and Kibaha SCA effects were found to be positive and significantly higher than other crosses for CBSD shoot infection and root necrosis but the effects were negative for CMD infection and FSW per plant at both locations. For this cross, negative and non-significantly lower SCA effects were observed at Chambezi for number of roots per plant, FRW per plant and HI. F_1 progenies from a cross involving Namikonga and Albert performed consistently better for resistance to CBSD shoot infection and root necrosis at both locations because it recorded significant and highly

negative SCA effects. The cross, however, had consistently positive SCA effects for CMD infection. This was also true for the Kibaha x Albert cross which also had negative and significantly lower SCA effects for CBSD shoot infection and root necrosis. Surprisingly, the cross Kalolo x Namikonga involving CBSD resistant parents had negative SCA effect values only for CBSD infection 3 MAP at Chambezi and CBSD infection 9 MAP at Naliendele while the rest of the SCA effects were positive. However, SCA effects for CMD infection were negative. For this cross the lowest disease scores were not necessarily associated with negative SCA effects, it was also contrary to the observed higher negative GCA values for these CBSD resistant parents.

It is therefore reasonable to conclude that GCA effects were more important than SCA effects. For CBSD shoot infection and root necrosis additive rather than non-additive effects control most of the reaction to the disease. These results gave extra weight to those observed from the ANOVA in Table 4.2 where SCA sum of squares accounted for less than 12.0% of the sum of squares due to crosses compared to more than 70.0% accounted for by GCA. For the other traits, number of roots per plant, FRW per plant, FSW per plant and HI, all crosses had negative SCA effects at Chambezi and positive SCA effects at Naliendele although some exceptions were observed particularly for FSW per plant and HI (Table 4.4).

Table 4.4 Specific combining ability effects for a diallel analysis of CBSD severity mean score and other traits for cassava F_1 and parental genotypes evaluated at Chambezi and Naliendele in 2008

	CBSD3MAP		CBSD6MAP		CBSD9MAP		CMD3MAP		CMD6MAP		CMD9MAP	
Cross	**CHZ**	**NDL**	**CHZ**	**NDL**	**CHZ**	**NDL**	**CHZ**	**CHZ**	**CHZ**	**NDL**	**CHZ**	**NDL**
Kalolo x Namikonga	-0.002	0.247	0.203	0.070	0.185	-0.022	-0.086	0.029	-0.073	-0.023	-0.062	0.065
Kalolo x Kibaha	-0.301	-0.164	-0.261	-0.259	-0.252	-0.379	0.532	0.093	0.315	0.082	0.355	-0.010
Kalolo x Albert	-0.312	-0.401	-0.134	-0.380	-0.162	-0.207	0.261	0.457	0.321	0.331	0.287	0.202
Namikonga x Kibaha	0.378	0.246	0.224	0.157	0.244	0.074	0.090	-0.600	-0.143	-0.483	-0.175	-0.398
Namikonga x Albert	-0.149	-0.368	-0.356	-0.325	-0.341	-0.340	0.153	0.095	0.189	0.058	0.096	0.156
Kibaha x Albert	-0.085	-0.277	-0.229	-0.234	-0.361	-0.186	0.349	-0.296	0.153	-0.251	0.118	-0.417
SE± (S_i)	0.047	0.053	0.064	0.068	0.079	0.136	0.141	0.061	0.149	0.124	0.158	0.477
SE± (S_i-S_j)	0.061	0.068	0.083	0.088	0.102	0.175	0.182	0.079	0.192	0.159	0.204	0.191
LSD ($P \leq 0.05$)	0.097	0.108	0.132	0.140	0.162	0.279	0.289	0.126	0.305	0.254	0.324	0.979

	CBSD-RN		Number of roots/plant		FRW (kg/plant)		FSW (kg/plant)		Harvest index	
Cross	**CHZ**	**NDL**	**CHZ**	**NDL**	**CHZ**	**NDL**	**CHZ**	**NDL**	**CHZ**	**NDL**
Kalolo x Namikonga	0.594	0.478	0.266	0.350	-0.338	0.1340	-0.197	0.208	-0.010	-0.010
Kalolo x Kibaha	-0.203	-0.060	-0.237	0.029	-0.166	0.104	0.197	0.130	-0.015	0.017
Kalolo x Albert	0.038	-0.010	-0.893	0.115	-0.052	0.031	-1.425	0.039	-0.017	-0.017
Namikonga x Kibaha	0.047	-0.176	-0.005	0.096	-0.182	0.136	-0.371	-0.249	-0.003	0.149
Namikonga x Albert	-0.083	-0.218	-0.516	0.083	-0.756	0.018	-0.765	-0.096	-0.047	0.030
Kibaha x Albert	-0.512	-0.067	-0.423	0.258	-0.483	0.034	-0.512	0.067	-0.031	0.013
SE± (S_i)	0.139	0.122	0.274	0.203	0.247	0.041	0.231	0.083	0.017	0.022
SE± (S_i-S_j)	0.171	0.157	0.354	0.262	0.319	0.527	0.299	0.107	0.022	0.029
LSD ($P \leq 0.05$)	0.285	0.249	0.563	0.417	0.507	0.084	0.475	0.169	0.035	0.045

CBSD 3, 6 and 9 MAP = symptom severity scores for CBSD shoot infection at 3, 6 and 9 months after planting, CMD 3, 6 and 9 = symptom severity score for CMD at 3, 6 and 9 months after planting, CBSD-RN = CBSD root necrosis, FRW = fresh root weight, FSW = fresh shoot weight, CHZ = Chambezi, NDL = Naliendele, SE(Si) and SE(Si-Sj) = standard error and standard error of difference, LSD = least significant difference.

4.3.4 Phenotypic and genotypic correlation

Highly and significant ($P < 0.001$) phenotypic correlation were found between root necrosis and CBSD infection in the shoot (Table 4.5).Values ranged from $r = 0.7377$ for CBSD infection 3 MAP to $r = 0.8601$ for CBSD infection 6 MAP. This was expected because disease severity scores showed that genotypes were either resistant or susceptible to both root necrosis and shoot infection. No single progeny, including the parents, had root necrosis without shoot infection and vice versa. Genotypes which were resistant against root necrosis and shoot infection had lower disease scores while susceptible genotypes showing root necrosis and shoot infection had higher scores for CBSD infection. It should be pointed out that lower scores for CBSD meant better (resistant) reaction to the disease. Root necrosis and CBSD shoot infection were also positively correlated with number of roots per plant, FSW per plant and FRW per plant but were negatively correlated with HI. CBSD infection 3 MAP was positively correlated with CBSD infection 6 and 9 MAP and HI. Negative and significant ($P < 0.05$) phenotypic correlation was observed between CBSD infection (root necrosis and shoot) and CMD infection. This was also expected since most genotypes under evaluation that were resistant to CBSD were susceptible to CMD. This was also true for the CBSD resistant parents used in this study. Unlike CBSD which had positive correlations with cassava yield components, CMD infection had a negative and significant ($P < 0.05$) correlation with number of roots per plant, FSW per plant and FRW per plant. There were highly significant ($P < 0.001$) positive phenotypic correlations for number of roots per plant with FSW per plant and FRW per plant as well as HI and FRW per plant and also between FRW per plant and FSW per plant.

The genetic correlations presented in Table 4.5 (above the diagonal) showed patterns similar to phenotypic correlations but some exceptions were observed. Unlike phenotypic correlation, there were negative genetic correlations for HI with number of roots per plant and FSW per plant but a positive genetic correlation with FRW per plant. Like phenotypic correlation, highly significant ($P < 0.001$) positive genetic correlation was found between CBSD-RN and shoot infection at 3, 6 and 9 MAP, respectively ($r = 0.9187$, 0.9636 and 0.9963). Significant ($P < 0.05$) negative genetic correlation was observed between CBSD and CMD infection as shown for phenotypic correlation.

Table 4.5 Phenotypic correlation (below diagonal) and genetic correlation (above diagonal) of CBSD and CMD severity mean scores and other traits for cassava genotypes under a diallel evaluation at Chambezi and Naliendele in 2008

	No. roots per plant	FSW (kg/plant)	FRW (kg/plant)	HI	CBSD-RN	CBSD3MAP	CBSD6MAP	CBSD9MAP	CMD3MAP	CMD6MAP	CMD9MAP
No. roots/plant		0.0968	0.6450	-0.0655	0.2853	0.2983	0.4483	0.4485	-0.3174	-0.4585	-0.4320
FSW (kg/plant)	0.7262***		0.6544	-0.2690	0.1679	0.1483	0.2894	0.2779	-0.0803	-0.2808	0-0.3246
FRW (kg/plant)	0.6255***	0.8067***		0.3626	-0.0386	0.2471	0.1997	0.1771	-0.1437	-0.3045	-0.3412
Harvest index	0.2253	0.0987	0.6139***		-0.2794	0.0018	-0.0975	-0.1288	-0.3556	-0.2863	-0.2099
CBSD-RN	0.2852	0.1876	0.0346	-0.1558		0.9187**	0.9636***	0.9663***	-0.7541*	-0.7725*	-0.7335*
CBSD3MAP	0.3402*	0.2518	0.3082*	0.2319	0.7377***		0.9648***	0.9605**	-0.7831*	-0.8157*	-0.7601*
CBSD6MAP	0.3992**	0.3474*	0.2030	-0.0057	0.8601***	0.9099***		0.9974***	-0.8276*	-0.868*	-0.8138*
CBSD9MAP	0.3598*	0.3067*	0.2023	0.0399	0.8355***	0.9031***	0.9761***		-0.8057*	-0.8396*	-0.7845*
CMD3MAP	-0.3195*	-0.3264*	-0.3785*	-0.1482	-0.7756*	-0.7605***	-0.7483***	-0.7563*		0.9721***	0.9115**
CMD6MAP	-0.4474*	-0.4153*	-0.4126*	-0.1127	-0.6855***	-0.7510***	-0.7385***	-0.7420*	0.8859***		0.9545**
CMD9MAP	-0.5108*	-0.3835*	-0.3859*	-0.1181	-0.6496***	-0.7438***	-0.7191*	-0.7200***	0.8154***	0.9597***	

CBSD 3, 6 and 9 MAP = symptom severity scores for CBSD shoot infection at 3, 6 and 9 months after planting, CMD 3, 6 and 9 = symptom severity score for CMD at 3, 6 and 9 months after planting, CBSD-RN = CBSD root necrosis, FRW = fresh root weight, FSW = fresh shoot weight , HI = harvest index, *, **, *** = P < 0.05, P < 0.01 and P <0.001 respectively.

4.3.5 Genetic parameters

The relative importance of GCA effects over SCA effects observed for CBSD root necrosis and shoot infection in the ANOVA was also indicated by estimates of GCA and SCA variances (Table 4.6). Higher GCA than SCA variance estimates were obtained for CBSD infection (root necrosis and shoot infection). GCA variance estimates were generally higher at Chambezi than Naliendele. Additive and dominance (with few exceptions) variance estimates were also higher at Chambezi than Naliendele. Mean scores for CMD infection at 6 MAP showed negative values for SCA and dominance variances at Naliendele. At the same location, number of roots per plant and FSW per plant had negative values for GCA, SCA (except FSW per plant), genetic variance, additive and dominance variances as well as broad and narrow sense heritability. Plants at this location were affected by termite damage during the growing season leading to poor performance and loss of some genotypes. This could explain why negative variance values were observed at this location. HI recorded much lower variances estimates for all genetic parameters than other traits. High heritability (broad and narrow sense) was obtained for CBSD-RN, CBSD shoot infection and CMD infections. Broad heritability for these traits ranged from 91.17% for CMD shoot infection to 99.63% for CBSD shoot infection while root necrosis was 98.27%. Narrow sense heritability ranged from 84.11% for CMD infection to 97.49% for root necrosis. The heritability estimates appear to be excessively high, which could be due to the inaccuracy of phenotyping which needs to be improved to incorporate phenotyping methods which are more quantitative than the present qualitative approaches. For the other traits, number of roots per plant, FRW per plant, FSW per plant and HI no particular pattern was evident with respect to heritability values. The lowest narrow sense heritability (7.06%) was obtained for FRW per plant and the highest was 75.0% for HI.

Table 4.6 Genetic parameter estimates for CBSD symptom severity mean score and other traits under diallel evaluation of cassava F_1 and parental genotypes at Chambezi and Naliendele in 2008

Trait	Location	δ^2_{GCA}	δ^2_{SCA}	δ^2_G	δ^2_A	δ^2_D	δ^2_E	δ^2_P	$H^2_{(b)}$ (%)	$h^2_{(n)}$ (%)
CBSD3MAP	CHZ	0.760	0.084	1.603	1.519	0.084	0.006	1.609	99.63	94.41
	NDL	0.771	0.132	1.673	1.541	0.132	0.007	1.680	99.58	91.73
CBSD6MAP	CHZ	1.045	0.076	2.165	2.089	0.076	0.010	2.175	99.54	96.05
	NDL	0.755	0.110	1.619	1.509	0.110	0.012	1.631	99.26	92.52
CBSD9MAP	CHZ	1.188	0.095	2.471	2.376	0.095	0.016	2.487	99.36	95.54
	NDL	0.784	0.062	1.630	1.568	0.062	0.046	1.676	97.26	93.56
CMD3MAP	CHZ	0.569	0.121	1.258	1.137	0.121	0.050	1.308	96.18	86.93
	NDL	0.522	0.157	1.200	1.043	0.157	0.009	1.209	99.26	86.27
CM6MAP	CHZ	0.422	0.029	0.872	0.843	0.029	0.055	0.927	94.07	90.94
	NDL	0.413	-0.050	0.775	0.825	-0.050	0.153	0.928	83.51	88.90
CMD9MAP	CHZ	0.423	0.011	0.857	0.846	0.011	0.062	0.919	93.25	92.06
	NDL	0.262	0.044	0.568	0.524	0.044	0.055	0.623	91.17	84.11
CBSD-RN	CHZ	1.310	0.112	2.731	2.619	0.112	0.048	2.779	98.27	94.24
	NDL	1.300	0.030	2.629	2.599	0.030	0.037	2.666	98.61	97.49
Number of roots/plant	CHZ	0.188	0.232	0.608	0.376	0.232	0.188	0.796	76.38	47.24
	NDL	-0.004	-0.030	-0.037	-0.007	-0.030	0.103	0.066	-56.06	-10.61
FRW (kg/plant)	CHZ	0.029	0.611	0.669	0.058	0.611	0.153	0.822	81.39	7.06
	NDL	0.011	0.014	0.036	0.022	0.014	0.004	0.040	90.00	55.00
FSW (kg/plant	CHZ	0.815	0.863	2.492	1.629	0.863	0.134	2.626	94.90	62.03
	NDL	-0.009	0.013	-0.005	-0.018	0.013	0.017	0.012	-41.67	-15.00
Harvest index	CHZ	0.003	0.001	0.007	0.006	0.001	0.001	0.008	87.50	75.00
	NDL	0.006	0.006	0.018	0.012	0.006	0.001	0.019	94.74	63.16

CBSD 3, 6 and 9 MAP = symptom severity scores for CBSD shoot infection at 3, 6 and 9 months after planting, CMD 3, 6 and 9 = symptom severity score for CMD at 3, 6 and 9 months after planting, CBSD-RN = CBSD root necrosis, FRW = fresh root weight, FSW = fresh shoot weight, GCA = general combining ability, SCA = specific combining ability, δ^2_{GCA}, δ^2_{SCA}, δ^2_G, δ^2_A, δ^2_D, δ^2_P, δ^2_E, $H^2_{(b)}$ and $h^2_{(n)}$= variances for GCA, SCA, genetic, additive, dominance, phenotypic effects, error variance, broad and narrow sense heritability respectively.

4.4 Discussion

4.4.1 Analysis of variance

There was a high level of variation among the parents and F_1 crosses for most traits studied. Disease scores for CBSD resistant parents were lower than scores for susceptible parents. Crosses displayed variation for CBSD ranging from resistance to susceptibility. There were no F_1 crosses which had higher or lower disease score values than parents which ruled out transgressive inheritance for CBSD resistance. Highly significant variations were found for parents and crosses for most traits. Based on disease scores it was clear that the correct resistant and susceptible parental lines were selected and selection was done based on both known phenotypic and genotypic data from Chapter 3.

Partitioning of sum of squares of crosses into GCA and SCA components helped to understand the relative contribution of each. The proportion of sum of squares for crosses explained by GCA components gives an estimation of the relative importance of additive and non-additive effects in the expression of variables or traits (Calle *et al.*, 2005). The sum of squares accounted for by GCA were larger than SCA suggesting the importance of additive gene effects for controlling CBSD resistance as well as CMD resistance. For most of the measured traits, GCA effects accounted for more than 58% of the total variation for crosses. For the FRW per plant and number of roots per plant, GCA and SCA effects made similar contributions to total variance. Field observation agreed with these findings and showed that the reaction of genotypes to CBSD showed a continuous variation ranging from susceptibility to high resistance indicating CBSD to be polygenic trait. This finding has an implication in breeding for CBSD resistance in cassava because for the traits controlled by additive gene effects, the recurrent mass selection method will be an efficient method in cassava. SCA effects made a low contribution to the total variation for most traits, the highest proportion being observed for FSW per plant at Naliendele. This is the same trait which had the lowest GCA contributions to the total sum of squares. For this trait, number of roots per plant and fresh root weight per plant non-additive gene effects were important in predicting the performance of the progeny in the family. The importance of additive gene effects for HI and dry matter content was also reported by Jaramillo *et al*. (2005) and Cach *et al*. (2006).

4.4.2 General and specific combining ability

Determination of additive and non-additive gene effects which are reflected in GCA and SCA are important in defining the appropriate breeding methods and in gaining knowledge on the inheritance of traits with agronomic relevance in cassava (Easwari Amma and Sheela, 1995; Catch *et al.*, 2006). According to Falconer and Mackay (1996) GCA is the average value of all F_1s having the line as one parent, the value which is expressed as a deviation from the overall mean of crosses. SCA is the deviation from the expected value from a cross, which is the sum of the GCA of two parental lines involved in a cross. In statistical terms GCA is the main effect and SCA is an interaction (Falconer and Mackay, 1996).

Both positive and negative GCA effects were found in this study. The resistant parents tended to have negative GCA values while susceptible parents had positive values for CBSD. Negative GCA effect values of parental genotypes indicated a contribution towards resistance while positive values represented contribution towards susceptibility. This finding was also observed by Owolade *et al.* (2006) in their diallel study of cassava genotypes for anthracnose resistance. Results from the current study indicated that GCA effects of the resistant parent, Namikonga were significantly lower and negative for CBSD root necrosis and shoot infection indicating its high breeding value for CBSD resistance. This parent produced progeny that showed resistance to CBSD root necrosis and shoot infection. However, Namikonga had poor combining ability for CMD resistance since high positive GCA effects were obtained. Namikonga, although resistant to CBSD, is susceptible to CMD. Kalolo, which showed moderate resistance to CBSD as well as CMD, had negative GCA effects for both the diseases, highlighting its ability to transfer CBSD resistance and CMD to its progeny. The two susceptible varieties, Kibaha and Albert, had positive GCA effects for CBSD root necrosis and shoot infection and Albert had significantly higher positive values than Kibaha, an indication that Albert had a notoriously high capability to transfer susceptibility to its progeny. However, Albert showed good resistance to CMD infection as indicated by significantly higher negative GCA values for CMD infection than SCA values. Kibaha also had negative GCA effects for CMD at all locations. From this finding the magnitude and sign (positive or negative) of GCA effects of each parent were generally in

agreement with their individual performance in terms of their observed reaction to CBSD and CMD. Kalolo had negative GCA effects for CBSD shoot and CMD infection at one or both locations. This suggests that for a given trait, the magnitude of GCA and SCA effects depend on the environment and genotypes involved (Ojulong, 2006)

Lower values of SCA effects than values for GCA effects were generally recorded for CBSD root necrosis and shoot infection for this study. These results reinforced the finding from the ANOVA which showed higher contributions of GCA variances to total sum of squares of crosses than SCA. Again this stresses the importance of additive gene effects in controlling resistance to CBSD and CMD infection implying that resistance to these diseases is quantitatively controlled by polygenes and therefore recurrent mass selection will be appropriate method for breeding. Although progeny from the cross involving resistant parents (Kalolo x Namikonga) were consistently giving low CBSD scores suggesting good resistance to CBSD, their SCA effects were only negative for CBSD infection 3 MAP at Chambezi but they were generally low for CBSD infection 6 and 9 MAP. However the SCA values for Kalolo x Namikonga were significantly higher for CBSD root necrosis, indicating that non-additive effects contributed less in controlling resistance to CBSD root necrosis. It is also surprising that the cross involving susceptible parents (Kibaha x Albert) had negative SCA effects suggesting that specific combinations of genes may be important in conferring resistance to the disease. In addition Kibaha was not as susceptible as Albert; it showed moderate susceptibility and therefore could have contributed genes for resistance in this cross.

The relative importance of GCA effects for CBSD severity observed in this study agreed with results obtained by Munga (2008) who demonstrated that GCA effects were important in controlling resistance to CBSD root necrosis. However results from this study differed from Zacarias (2008) which suggested that resistance to CBSD root necrosis is contributed more by non-additive gene effects than additive gene effects. This disparity could have been due to the fact that in the present study the primary goal was to screen for CBSD infection as opposed to the study by Zacarias (2008) whose primary focus was on diallel analysis of root yield and yield related traits in cassava. Findings from the current study suggest that the

genetic control of CBSD resistance is inherited in an additive way. This correlates with the findings of Jennings (1957) who was the first to suggest that CBSD is additively and polygenically inherited. On the other hand, CMD resistance was reported to be controlled by polygenic factors, also inherited in an additive way (Hahn and Howland, 1972).In this study the relatively high contribution of GCA effects for CMD, indicating additive gene effects, have an implication for CBSD resistance breeding in the sense that selection for CMD resistance may not necessary lead to CBSD resistance just because resistance to both diseases are additively controlled. The progeny's performance towards resistance to both diseases may be based on the parent's performance *per se*. As expected, progeny from a cross involving resistant parents had better performance in terms of CBSD resistance than from susceptible parents.

4.4.3 Correlation and genetic parameters

Although some cassava genotypes exhibit shoot infection without root necrosis and vice versa (ARI Naliendele, unpublished data) results from this study showed highly significant positive correlation of CBSD root necrosis and shoot infection. Most genotypes evaluated were either showing resistance both against root necrosis and shoot infection or were susceptible to both. This is the first attempt to correlate CBSD root necrosis and shoot infection in a diallel evaluation where positive correlation was detected. In a CBSD field survey by Hillocks *et al*. (1996) to determine the relationships between above-ground and below ground (root necrosis) symptoms of CBSV, symptoms were found in 62 of the 64 fields sampled and 79% of plants with above-ground CBSV symptoms also exhibited root necrosis, compared with only 18% of plants with no visible symptoms of CBSV at all. In Hillock's surveyan attempt was made to determine if there was any association between root necrosis and fungal or virus infection. No evidence was found to indicate a cause of root necrosis other than CBSV.

The positive correlation of root yield components with CBSD infection (root necrosis and shoot infection) and negative correlation with CMD found in this study can be explained based on the nature of infection of the two diseases. While CMD mainly affects the shoot, CBSD largely affects roots due to root necrosis. CMD infection affects young growing

shoots causing leaf chlorosis accompanied by leaf deformation, distortion, reduction in leaf size and general stunting of the plant (Storey and Nichols, 1938; IITA, 1990; Hillocks and Thresh, 2000). Since CMD infects cassava plants as early as one MAP, leading to reduction in size of leaves, it reduces photosynthetic area with consequent reduction of shoot and root development and growth (El-Sharkawy, 1993). This leads to reduction in root yield and yield losses up to 90% have been reported (Hahn *et al*., 1980b). CBSD infection also causes leaf chlorosis, but unlike CMD there is no reduction in leaf size, leaves remain intact and foliar symptoms appear on older leaves and not young leaves like CMD (Nichols, 1950; Hillocks and Thresh, 2000). There is therefore less reduction in shoot growth than with CMD infection. With CBSD infection root necrosis is more important than shoot infection and as Nichols (1950) put it, losses due to CBSD are more on loss of root quality than root weight. In most susceptible cultivars, root necrosis is visible at five or more MAP during which the plant had undergone root development and tuberisation.

CBSD infection (root necrosis and shoot infection) had high and negative correlation with CMD infection, for reasons mentioned previously. The observed disease score for CBSD and CMD were inversely correlated, suggesting absence of association of genes controlling resistance to these two viral diseases. During field screening of the genotypes reactions to the two diseases, no single progeny was found to show resistance to both diseases. This was also true for the parental genotypes, for example Namikonga that showed a high level of resistance to CBSD was very susceptible to CMD while Albert was very susceptible to CBSD but resistant to CMD. These findings are in line with screening results of the parental genotypes obtained in CBSD endemic areas of southern Tanzania (ARI, Naliendele, 2003 unpublished data).Positive genetic correlation between resistance to CMD and CBB (another important foliar disease of cassava) was reported by Hahn *et al.* (1980b) which implies that selection for CMD resistance in a breeding programme also may result in genetic gain in CBB resistance and vice versa. The observed negative genetic correlation between CBSD and CMD resistance suggests that genes controlling the resistance against these diseases are not linked and improvement of resistance to CBSD may not necessarily lead to resistance to CMD and vice versa. The observed negative correlation between these two diseases means that pyramiding resistance genes in one variety will prove difficult unless molecular markers

linked to resistance are used. Currently markers linked to CMD resistance are available (Akano *et al*., 2002) and the putative markers linked to CBSD resistance that have been identified from this study will help cassava breeders to actively breed for both resistances in one genotype. Markers will enable breeders to screen many offspring at seedling stage to find one or more progeny that contain both CMD and CBSD resistance.

Phenotypic correlation obtained between FRW per plant and HI was high. High phenotypic correlation was also obtained between fresh root yield per plant and FSW per plant as well as with number of roots per plant. The genetic correlation between FRW per plant and HI was positive. A positive association of FRW and HI have also been reported in literature (Kawano *et al.*, 1998; Calle *et al*., 2005; Jaramillo *et al*., 2005; Cach *et al*., 2006). The high association between FRW and HI obtained from this study indicated that selection for HI will result in positive gain in fresh root yield. Kawano *et al*. (1998) and Kawano (2003) demonstrated the importance of HI in cassava during early stages of selection. Because of a limitation of planting materials in cassava, selection in early stages is based on single plants or single row plots. In non replicated trials, selection based on FRW will suffer an environmental influence compared to HI which is not affected by environment, hence the importance of using HI in selecting superior genotypes. The importance of using HI in selection was also emphasised by Jennings and Iglesias (2002) who reported high correlation ($r = 0.763$) of HI with fresh root yield. They also indicated that harvest index had high heritability with the harvest indices of progenies being highly correlated with the parental means. High correlation and regression coefficients for harvest index with root yield were also reported by Kawano and Thung (1982) who demonstrated the effectiveness of using HI at all stages of selection as an indirect selection criterion for root yield.

High heritability (both broad and narrow sense) was obtained for CBSD root necrosis as well as CMD infection. Broad sense heritability ranged from 98.27% for root necrosis to 99.63% for shoot infection and narrow sense heritability were 91.73% to 94.24% for shoot and root necrosis respectively. The broad sense heritability estimates for CMD ranged from 83.51% to 99.60% and narrow sense was 84.11% to 92.06%. These estimates were in agreement with high genetic variances observed and the importance of additive genetic effects respectively.

The relative importance of additive genetic effects over non-additive effects suggested that the most resistant progeny might be derived from crosses involving parents with the largest negative GCA effects. This was the case of Namikonga which had the largest negative GCA effects and most of the progeny from this parent had good performance for CBSD resistance when combined with certain parents. The high GCA effects detected for CBSD and CMD infection may suggest that a mass recurrent selection would be the most efficient approach for improving resistance to the diseases as was suggested by Cach*et al.* (2006) on dry matter content, reaction to thrips and height of first branching.

The high heritability values recorded suggest that the control of CBSD and CMD resistance is mediated by polygenes with major effects or could result from the effects of some few major genes within the polygene. In a 7 x7 diallel study, Hahn *et al.* (1980a) found that CMD resistance was controlled by a recessive gene with heritability of about 60%. Control of CMD by recessive genes was also confirmed by Jennings (1994) in a 6x6 parental diallel cross but a major dominant CMD_2 gene was found to control CMD resistance and has been mapped on linkage group R of the CM2477-2 derived linkage map (Fregene *et al*., 1997; Akano *et al*., 2002) and the gene has been useful in marker-assisted breeding for CMD resistance in cassava. The findings on the genetics of CBSD resistance observed from the current study will make a significant contribution towards the improvement of cassava breeding for CBSD resistance.

4.5 Conclusions

This study has indicated that GCA effects were more important than SCA in the genetic control of resistance to CBSD in cassava. These findings therefore have indicated that additive gene effects were more important than dominance effects and therefore CBSD resistance genetic control is quantitative in nature. This has an important implication in breeding for disease resistance in cassava. A mass recurrent selection will be the most efficient approach for CBSD resistance breeding in cassava. Selection of parents with good GCA effects will be important for success in CBSD resistance breeding. Due to the quantitative nature of genetic control of CBSD resistance revealed in this study, it will be important to look for molecular markers associated with the disease resistance to increase the

selection efficiency for future cassava breeding work. Because of the importance of additive gene effects, the introduction of inbreeding in resistant parental genotypes will facilitate the assembly and concentration of resistance genes into resistant genotypes leading to durable resistance.

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CHAPTER 5

Genetic linkage mapping in a full-sib cassava (*Manihot esculenta* Crantz) family from Tanzania

5.1 Introduction

Cassava is an important food security crop for many tropical and subtropical countries. The crop is a source of calories for more than 500 million people in tropical and subtropical Africa, Asia and Latin America (El-Sharkawy, 2004). Cassava is increasingly becoming an important source of raw materials for different industrial uses. Some of the most important industrial uses of cassava include its use as a raw material in the feed industry, a source of starch and starch derived products such as high fructose-glucose syrup and for the production of ethanol (Ceballos *et al.,* 2007; Kunkeaw *et al*., 2010a). In Africa cassava yield an average of 10 t/ha. Compared to yields achieved in some south Asian countries such as China (16.8 t/ha), Indonesia (15.9 t/ha), Thailand (15.6 t/ha) and India (27.9 t/ha), yield in Africa are low (FAO, 2006). The increasing importance of cassava as source of calories and industrial raw materials; prevalence of biotic and abiotic stresses as well as an increasing demand for both quality and quality traits call for increased efforts in genetic improvement of cassava. One strategy for increasing the efficiency of selection in cassava breeding programmes is the use of molecular markers. MAS allows for the selection of a marker or markers known to be linked to, and inherited with, a trait(s) of interest.

Cassava is a challenging crop to work with due to its genetic nature. It is vegetatively propagated allowing transmission of viruses from year to year through stem cuttings. It is an out-crossing and heterozygous crop that suffers from inbreeding depression as a result of selfing. It has a long growing cycle (9-18 months), unpredictable flowering that is highly influenced by the environment, unpredictable seed set (a maximum of three per pollination) and seed germination is low. Combining several important genes into a single variety is extremely difficult using conventional breeding techniques alone and requires a large number of progenies and acreage. Use of molecular markers offers an opportunity to efficiently accelerate the genetic improvement of cassava through MAS. MAS allows for selection to take place at seedling stage, it allows for the reduction of population sizes and in the time

taken to deliver a new variety. A pre-requisite for finding markers associated with traits of interest is a framework genetic linkage map on which variation in the phenotype can be mapped. Alternative methods such as the use of association mapping approaches also exist (Marques *et al*., 2002).

The first classic map of cassava with 20 linkage groups spanning 931.6 cM and an average marker density of 7.9 cM was developed by Fregene *et al*. (1997) using predominantly RFLP markers. The map provided insights into the organisation of the genome and provided an important tool for cassava genetic improvement. It also consisted of RAPD and isozyme markers and a few SSR markers. SSR markers are generally more useful than RFLP, RAPD and isozyme markers as they are co-dominant and generally show a higher level of polymorphism in intraspecific crosses. Since the generation of the first cassava linkage map, many SSR markers, including EST derived SSRs, have been developed. These are slowly being added to the cassava map. As marker of choice for genetic mapping of cassava due to their high number of allelic bridges, SSR markers are an important genetic tool for genetic improvement of cassava. Since the construction of the first map a number of cassava maps incorporating SSRs have been published (Mba *et al*., 2001; Okogbenin *et al*., 2006; Chen *et al*., 2010; Kunkeaw *et al*., 2010a; 2010b).

Cassava is an out-crossing crop generally considered as a diploid with a haploid chromosome number of n = 18 although an allopolyploid with basic chromosome number x = 9 and segmental allotetraploidy have been postulated (Hahn *et al*., 1990). It has a DNA content 1.67 pg per cell nucleus (Awoleye *et al*., 1994). This value corresponds to 772 mega base pairs in the haploid genome and puts cassava's genome size at the lower end of the range for higher plants (Bennett and Smith, 1991). The relatively small size of the cassava genome favours the development of a saturated genetic map that would contribute to an understanding of the inheritance of important agronomic traits despite the crop's heterozygous nature (Fregene *et al*., 1997).

Like other out-crossing species, linkage analysis in cassava using molecular markers is performed using F_1 individuals from a full-sib family which is considered to be the progeny

of a cross between non-inbred plants of an out-crossing diploid species. Compared to crosses derived from inbred lines, in linkage analysis of a full-sib family from out-breeding species up to four segregating alleles per locus can be observed with unknown linkage phase (Maliepaard *et al*., 1997; Van Ooijen, 2006). In segregating populations from fully homozygous diploid crosses, all segregating loci will segregate for only two alleles and the alleles from the same parent are in coupling phase in F_1 individuals. Because of the difference between inbred and out-crossing species, different strategies in linkage analysis are used. Linkage analysis in inbred lines is generally more straightforward than out-breeders. In out-crossing species, due to differences in meiosis between the two parents, one strategy is to use double or two-way pseudo-test crosses by which linkage analysis is performed separately for each parent (Grattapaglia and Seedorf, 1994; Grattapaglia *et al*., 1995). Integration of the individual parental linkage maps for important alleles segregating in both parents may be a problem for some marker types such as dominant markers (Maliepaard *et al*., 1997). Creating backcross progeny resembling a BC_1 population in order to simplify the segregation analysis may be another strategy although the linkage phase may be unknown. However, a BC_1 population may not be a practical solution for crop species with a long growth cycle like tree species. The development of the JoinMap® software programme however permits the construction of integrated linkage maps (Stam, 1993).

Linkage analysis in crosses involving out-breeders is described in number of papers (Ritter *et al*., 1990; Arus *et al*., 1994; Ritter and Salamini, 1996; Alves *et al.*, 2010). Linkage analysis in full-sib families essentially tries to detect recombination events between loci during meiosis of both parents. For each homologue or haplotype of every individual in the F_1 progenies a reconstruction of events can be done to determine which of the two recombination events has occurred. Recombination occurs if an allele at a locus is from one homologue of a parent and the allele at the next locus is from the other parent (Ritter and Salamini, 1996). The segregation types of a locus e.g. *ab x cd* describing alleles present in the parents of a full-sib family, give four possible genotypes of the progeny (Maliepaard *et al*., 1998; Van Ooijen, 2006).

Although linkage analysis is dealt with independently in each parent in full-sib families of out-crossing species, co-dominant markers such as RFLPs and SSRs are useful in integrating homologous linkage groups of respective parents. These types of markers are multi-allelic and powerful in linkage analysis and have been recommended for mapping studies in out-breeding progenies (Grattapaglia and Seedorf, 1994; Ritter and Salamini, 1996). These markers provide availability of allelic bridges which make it possible to construct a single map rather than two separate maps for the parents of a cross. In cases where the *ab x cd* type of markers are present, differences in recombination between the male and female parents can be estimated directly, which makes co-dominant markers useful. It was suggested by Maliepaard *et al.* (1997) that if sufficient *ab x cd* markers are available or if differences in recombination frequency are not too large an integrated map from out-breeding crops can be constructed. The two approaches, either construction of separate maps for each of the two parents or an integrated map, are available within JoinMap® 4 software (Van Ooijen, 2006). Using multi-allelic markers such as SSRs, it is therefore possible to create a consensus map from crosses between non-inbred parents of a crop like cassava.

Construction of a molecular genetic map is the first step toward marker-assisted analysis of traits of agronomic importance. The analysis permits association of molecular markers with traits of agronomic importance. In cassava, using molecular genetic maps, the genetics of resistance of two important diseases, CBB and CMD, have been studied and markers associated with the resistance trait identified (Mba *et al*., 2001; Akano *et al*., 2002). It is expected that the construction of a genetic linkage map using a CBSD mapping population will help in gaining insights into the genetics of CBSD resistance and identification of molecular markers linked to CBSD resistance for use in MAS. The present study constitutes one of the objectives within the framework of the project titled "*Molecular marker-assisted and farmer participatory improvement of cassava germplasm for farmer/market preferred traits in Tanzania*" implemented since 2004 by the Tanzania National Agricultural Research System (NARS) in collaboration with IITA and CIAT. Subsequently the work was funded through a BecANet (Biosciences Eastern and Central Africa network) project entitled 'A high-density genetic linkage map for cost-effective mapping in cassava', implemented by Tanzania agricultural research institutes (ARIs) and IITA. The objectives of the present study

were:

(a) to screen parents from a mapping population for marker polymorphism and genotype the mapping population using genomic SSR and ESSR (expressed simple sequence repeats) markers;

(b) to conduct linkage analysis in order to assign markers to a molecular genetic linkage map based on their segregation in the mapping population.

5.2 Materials and methods

5.2.1 Plant material

Two contrasting parents, Namikonga (CBSD field resistant) and Albert (CBSD susceptible) were selected (based on results presented in Chapter 3) to produce an F_1 segregating population used as CBSD mapping population. F_1 progenies were produced by an intraspecific cross between Namikonga (female parent) and Albert (male parent) as explained in section 4.2.1 (Chapter 4). Resistance to CBSD is thought to have been introgressed into Namikonga from *M. glaziovii* (Nichols, 1947).

5.2.2 Embryo rescue at CIAT

To enhance germination, a total of 420 cassava seeds generated from the Namikonga x Albert cross were shipped in two batches (300 and 120 seeds in first and second batch respectively) from Tanzania to CIAT for embryo rescue. According to protocol at CIAT (2003), seeds from the Namikonga x Albert cross were first tested for viability by soaking in water. After the viability test, seeds were treated with concentrated sulphuric acid for 50 min, thoroughly washed with water and soaked in water for 30 min. Seeds were then surface-sterilised by immersion in 70% (v/v) alcohol for 5 min and followed with immersion in 5% (v/v) sodium hypochlorite and Tween-20 for 20 min before being rinsed three times with sterile water. Under aseptic conditions, each seed was split along the longitudinal axis and embryos removed using a sterile forceps and scalpel. Excised embryos were cultured *in vitro* in 17N growth medium with their radicles down. The 17N culture medium used for embryo rescue mainly contained the mineral salts of Murashige and Skoog (MS) (Murashige and Skoog, 1962; Roca, 1984). The culture medium was supplemented with 0.01 mg/l naphthaleneacetic acid (NAA), 0.01 mg/l gibberellic acid (GA_3), 1.0 mg/l thiamine-HCL,

100 mg/l inositol, 2% (w/v) sucrose, 0.7% (w/v) agar (Sigma Co.) and 25 mg/l of a commercial fertiliser containing NPK (10:52:10) at pH of 5.7-5.8 (Roca, 1984).Embryo cultures were incubated in darkness for three days to promote radicle growth and then transferred to growth chambers with a 12 h photoperiod. Plantlets remained in the growth chamber for six weeks before they underwent *in vitro* multiplication to generate enough copies from each of the F_1 progenies. Five to eight plantlets of each progeny were shipped back to Tanzania for field evaluation and molecular analysis.

5.2.3 Acclimatisation and hardening in the screen house

Plantlets were delivered in polystyrene boxes at ARI-Mikocheni, removed from the boxes and placed in the growth room (30ºC, 12/12 h photoperiod and 50% relative humidity) for acclimatisation before hardening. After one week of acclimatisation, *in vitro* plantlets were transferred to KibahaSRI for the hardening process. Plantlets were carefully removed from the tissue culture bottles, washed thoroughly with sterile distilled water to remove media before planting in 10-15cm diameter plastic pots containing a sterilised mixture of peat moss sieved forest soil containing sand in a ratio of 1:3 by volume. Plastic pots containing the plantlets were kept in the screen house and covered with a transparent polythene bag to maintain high humidity. The temperature in the screen house was maintained between 30ºC and 35ºC with 12/12 h photoperiod throughout the acclimatisation period. Spray of 0.2% (w/v) Dethane M-45 (Mancozeb, 80%; Manganese, 16%; Zinc, 2%; Ethylenebisdithiocarbamate, 62%) fungicide solution was applied in the humidity chamber to control fungal infection. The screen house was thoroughly sprayed with an insecticide (Actellic 50 EC) to eliminate insects. Plantlets were kept in the humidity chamber for 14 days after which two to three holes were punctured on the humidity chamber to help plantlets adapt to the screen house environment. More holes were created after 21 days in the humidity chamber. Watering was done at least once daily for eight to ten weeks, then reduced to once every two days just before transplanting to the field.

After three to four weeks a water soluble NPK foliar fertiliser (Plantex 10:50:10 + micronutrients) was applied at 2mg/plant and the rate was doubled after eight weeks. Plants were transplanted to the field after three months in the screen house. Alongside *in vitro*

plantlets, 37 seedlings that were raised by direct sowing of seeds in seed trays at ARI Mikocheni were transplanted to the field. In the field they were planted at a spacing of 1.0 m between rows and 0.5 m within rows and were allowed to grow before leaf samples were collected from 192 plants six months after transplanting.

5.2.4 DNA extraction

Extraction of DNA from each of the 192 leaf samples was done at ARI Mikocheni as described in section 3.2.2 (Chapter 3). However, due to the need to have large quantities of DNA for genotyping, a modified maximum preparation extraction protocol of Dellaporta *et al.* (1983) was applied. In this case approximately 3-4 g leaf material was used. DNA samples were shipped from Tanzania and purification was done at IITA Nairobi campus using the Biosciences Eastern and Central Africa (BecA) biotechnology laboratory based at the International Livestock Research Institute (ILRI), Nairobi, Kenya. Each of the DNA samples was purified with ethanol:sodium acetate (20:1) solution and washed twice with 70% ethanol.

Yield and quality of DNA samples were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific State, USA) and agarose gel electrophoresis respectively. The A260/A280 ratio values were automatically generated and used to provide an estimate of DNA purity. The quality and purity of DNA was verified by running the samples on standard 0.8% (w/v) agarose gel in 1x TBE buffer containing ethidium bromide (10 mg/ml) at 100 V for 1 h.

5.2.5 Genotyping of mapping population

Genotyping of the CBSD mapping population for genetic linkage analysis was conducted at the BecA biotechnology laboratory in Nairobi. A total of 190 F_1 progenies plus the two parents were genotyped using SSR markers. During SSR marker analysis it became evident that DNA from four individuals did not amplify well and 66 individuals were outcrosses, meaning that the mother was known but pollen sources unknown. In addition, SSR analyses suggested that nine individuals were triploids while 51 were selfs (S_1) obtained from selfing of the female parent (Namikonga). In total 79 individuals were therefore excluded for linkage

analysis and 60 F_1s and 51 S_1s were used in the final linkage analysis.

5.2.5.1 Optimisation of SSR primer pairs

PCR conditions for cassava SSR primers used were optimised prior to the parental screen for polymorphisms. Locus-specific SSR primers used in this study were either directly fluorescently-labelled or labelled using a universal tail. Locus-specific SSR primers from the cassava genomic library (Mba *et al*., 2001; Fregene and Puonti-Kaerlas, 2002) were optimised in this study while 150 ESSRs (M. Ferguson, IITA, unpublished data) were optimised by Kathurima (2009) and Mzena (2009). All SSR primer pairs used in this study are given in Appendix I. Primer pairs for each directly labelled SSR were synthesised by Eurofins MWG Operon, Ebersberg, Germany while universally labelled SSRs were synthesised by Bioneer (South Korea). The majority of the 605 SSR primer pairs screened in this study were directly labelled (501 primer pairs) while the rest and all 150 ESSRs were universally labelled. Primers were reconstituted with TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) to obtain a stock solution of 100 pmol/µl. For each marker, primer stock solutions were diluted to a working solution of 1 pmol/µl for each forward (F) and reverse (R) primer.

The first step to PCR optimisation involved determination of the annealing temperatures for each of the primers. Amplification reactions for optimising annealing temperature consisted of 0.4 pmol/µl F and R primers and only one DNA sample. The 10 µl reaction mixture contained 9 µl of amplification mixture [1 x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.375 U *Taq* DNA-polymerase (New England, Biolabs)] and 1 µl of the diluted DNA sample as template. PCR amplifications were performed on a Techne TC-512 Thermal Cycler (Global Medical Instrumentation (GMI), USA). The thermocycler was set for a gradient programme from 52°C to 62°C. The temperature profile consisted of one initial cycle of DNA denaturing at 95°C for 2 min and then 30 cycles of denaturing at 95°C for 30 s, annealing at 52°C to 62°C for 1 min, extension at 72°C for 1 min and one cycle of final extension at 72°C for 30 min. PCR amplified products were visualised on 2% (w/v) electrophoresis grade agarose gel (Invitrogen, UK) stained with ethidium bromide (10 mg/ml). The appropriate optimum annealing temperature was selected visually based on the

strength of amplified fragment and the temperature of the sharpest fragment was selected. Optimisation of primer, $MgCl_2$ and dNTP concentrations were carried out according to the protocol from IITA at BecA laboratory as shown in Table 5.1.

Table 5.1 Optimisation conditions of PCR as developed by IITA at BecA laboratory for cassava genotyping[a]. Primer, magnesium chloride ($MgCl_2$) and dNTP concentrations varied across conditions A, B and C

Component	Condition A	Condition B	Condition C
Primer F and R	0.4 pmol/µl	0.8 pmol/µl	1.2 pmol/µl
$MgCl_2$	1.5 mM	2.0 mM	2.5 mM
dNTP	0.15 mM	0.20 mM	0.25 mM

[a]IITA, unpublished data.

5.2.5.2 Labelling of ESSR and other unlabelled SSR primers

For high throughput, fragments for analysis in capillary electrophoresis (CE) need to be fluorescently labelled with a suitable dye for detection on the Applied Biosystems (ABI) 3730 analysis platform. The directly labelled SSR primers used in the study were synthesised with an added 5' labelled tail on the F primer. Primers were labelled with fluorescent dyes, which were either FAM (Blue), VIC (Green), NED (Yellow) or PET (Red) (Applied Biosystems, dye set G5). Indirect labeling of primers was performed by adding a universal unlabelled 'tail' (5' GCTACAGAGCATCTGGCTCACTGG 3') to the 5' end of the F primer. Four separate aliquots of the primer each with the same sequence complementary to the tail were labelled with FAM (Blue), VIC (Green), NED (Yellow) or PET (Red). During PCR the fluorescent complementary sequence that bound to the universal tail, was incorporated in the PCR mixture and enabled visualisation on the ABI platform. The decision on which specific dye to add to unlabelled SSR primer pairs was based on allele size ranges of amplified products. Loci with overlapping or close allele sizes were differently labelled and up to four marker loci could be co-loaded when running PCR products on the ABI platform. Amplification reactions were carried out using the optimised conditions for each

marker and 0.175 pmol/µl of the tail. All polymorphic SSRs and ESSRs were respectively run at optimised annealing temperatures of 57°C and 62°C.

5.2.5.3 Screening for polymorphic markers and high throughput genotyping

To identify polymorphic markers, 605 SSRs and 150 ESSRs were screened against the two parental genotypes, Namikonga and Albert. Amplification reactions were set using the optimised PCR conditions for each marker and the diluted parental DNA samples in 96 PCR plates. PCR amplifications were carried in an auto-Lid Dual 384-Well GeneAmp® PCR System 9700 (Applied Biosystems Inc).

Before PCR products were run on the ABI 3730 automated sequencer for fragment analysis, marker panels comprising of SSRs with no overlapping allele sizes or being differently labelled were used to design co-loading primer sets for high throughput genotyping. Co-loading markers were selected and put into groups of four based on their dye label, fragment size and relative fluorescence unit (rfu). For each co-loading group, empirical proportions of PCR products from each SSR to be pooled together were worked out based on the rfu on the ABI 3730 and the relative intensities of each individual SSR PCR product when resolved on a 2 % (w/v) TBE agarose gel.

Between 1 and 2 µl of each fluorescently-labelled PCR product (i.e. 4-8 µl total products for four PCR products) were combined in one new plate. Products were briefly vortexed and centrifuged at 3500 rpm for 30 s. Nine µl formamide-standard mix (0.11 µl GS500 LIZ and 8.89 µl Hi-Di Formamide, Applied Biosystems) was added into each well of a new, empty PCR plate. The standard, which allows alignment of peaks for analysis, was labelled with an orange dye (LIZ) (dye set "G5", Applied Biosystems). One µl of the PCR product mixture was added to 9 µl formamide-standard mix. The pooled plate was vortexed and centrifuged at 3500 rpm for 60 s. Samples were denatured at 95°C for 5 min, placed on ice for 5 min, centrifuged, and then loaded into the ABI 3730. Capillary electrophoresis separates DNA fragments based on their size-dependant mobility when passing through a sieving matrix. Following separation, DNA fragments were analysed for fluorescent signals as well as fragment size to check for polymorphisms and polymorphic markers between the parents

were selected.

5.2.5.4 Genotyping of F_1 progenies

SSR genotyping of the F_1 progeny of the cassava mapping populations was performed using the identified polymorphic markers. The optimised PCR conditions were annealing temperatures of 57°C and 62°C for SSRs and ESSRs respectively containing 9 µl of amplification mixture [1x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.375 U *Taq* DNA-polymerase, 0.8 pmol/µl each of F and R primer] and 1 µl of DNA template for each of the progenies and parents. The thermocycler programme as well as fragment analysis was performed as described in sections 5.2.5.1 and 5.2.5.3 above.

5.2.6 Data scoring, coding and linkage analysis

Sizing of fragments, allele calling and scoring of polymorphic markers were performed using Genemapper Version 3.7 software (Applied Biosystems). Markers which failed to amplify, those with non-specific amplifications and/or which fell below the range of ABI automated allele sizing of 400 rfu, were discarded. Markers homozygous between parents were eliminated from further analysis while individuals with ambiguous genotypes were treated as missing data. All markers were tested for expected segregation ratios of 1:1 or 1:1:1:1. Linkage analysis was done using the pseudo-testcross method and performed by the 'Create Maternal and Paternal Population Node' function of JoinMap® 4 (Gratapaglia and Seedorf, 1994; Maliepaard *et al*., 1997; Rongling *et al*., 2002; Van Ooijen, 2006). With the cross-pollinated (CP) population type that was used, the function permitted separation into maternal and paternal populations. Using this function, markers segregating in a ratio of 1:2:1 (particularly the *ab x ab* segregation type) were ignored for both maternal and paternal populations.

Linkage maps were constructed using the JoinMap® 4 software package, which permits linkage analysis in out-breeding progenies involving markers with different segregation types (Table 5.2). Marker grouping into linkage groups was evaluated using the independence logarithm of odds (LOD) threshold value of 3.0. The independence LOD was used as opposed to linkage LOD which is affected more by linkage distortion than the former

(Maliepaard *et al*., 1998; Van Ooijen, 2006; Cavalcanti and Wilkinson, 2007).

Table 5.2 Segregation types and marker genotype codes of the SSR markers observed in linkage analysis of Namikonga x Albert full-sib family

Segregation type	Marker genotypes for F_1 progenies
<nn x np>	nn, np, --
<lm x ll>	lm, ll, --
<hk x hk>	hh, hk, kk, h-, k-, --
<ef x eg>	ee, ef, eg, fg
<ab x cd>	ac, ad, bc, bd

Source: Van Ooijen (2006), - = unknown allele

The regression mapping function was employed using the following default parameters: recombination frequency (REC) threshold of 0.4, a jump threshold value of 5.0 and ripple 1 function. Jump in the goodness-of-fit represented the difference in goodness-of-fit chi-square values before and after adding a locus to the map (Van Ooijen, 2006). A large jump (disparity) indicates a poor fit for the added marker and may warrant its removal from the map. Map distances were calculated using the Kosambi mapping function (Kosambi, 1944). The position of markers was developed using a sequential map build-up (Stam, 1993) and the most informative pair of markers was selected, followed by sequential addition of other markers. The best fitting position of an added marker was examined on the basis of the goodness-of-fit test (chi-square) for the resulting map. The marker was removed when a marker generated a negative map distance, or a large shift (jump) in goodness-of-fit and map construction was continued as a first-round map. After the first-round marker ordering, the previously removed markers were added back and again subjected to the goodness-of-fit testing. The marker ordering was continued to a third round until an optimum order of markers was found.

5.3 Results

5.3.1 Embryo rescue

Embryo rescue of mature cassava sexual seeds is a key technique for establishing breeding populations *in vitro*. For cassava seeds that require about a three month seed dormancy period before germination, embryo rescue hastens the development of breeding populations.

Of the 420 sexual seeds that were sent to CIAT for embryo rescue, 286 seeds were successfully germinated and micropropagated (CIAT, 2003). This represented a success rate of 68.1%. Due to flooding that affected the growth room at CIAT, many genotypes were lost and only 146 genotypes from the first batch and 74 from the second batch were finally shipped back to Tanzania. All *in vitro* plants were hardened and together with seedlings that were raised in the screenhouse in Tanzania, a total of 234 genotypes were finally established in the field from which 190 were selected to form the CBSD mapping population.

5.3.2 Labelling of SSR primers and screening for polymorphism

DNA isolation from the mapping population resulted in acceptable DNA yields from leaf samples ranging from 160 to 3172 ng/µl. The DNA quality was acceptable with A260/A280 ratios ranging from 1.67 to 1.94 which allowed amplification using most markers.

Labelling of primers with different fluorescent dyes for co-loading was necessitated by overlapping allele sizes. Allele sizes of the ESSR markers overlapped between 166-174 bp and 166-223 bp which resulted in a minimum of 9 bp and maximum of 57 bp differences. These small bp differences did not allow visualisation or good co-separation of allele peaks in the co-loading sets without dye labelling. For the universally labelled SSR primers, markers having a maximum of 100 bp differences were co-loaded. There were no directly labelled SSR primers with the same fluorescent dye that were multiplexed on the ABI 3730 capillary sequence.

Up to eight types of segregation involving up to four alleles were observed (Table 5.3).A total of 382 SSR and 223 ESSR markers were initially screened for polymorphism between the parental genotypes. Results of the parental screen indicated that 44.2% of SSR and 26.5% of ESSR markers showed informative polymorphic patterns. These polymorphic markers were used to genotype F_1 progenies of the mapping population. The number of alleles observed from the polymorphic markers ranged from two to four and showed heterozygosity in either or both of the parents. Of the 213 SSR and 164 ESSR markers that were not informative, 161 SSR and 126 ESSR markers were monomorphic for either a single or double fragment while 52 SSR and 38 ESSR markers failed to give clear amplification

products.

Table 5.3 Segregation types of SSR markers observed during parental screen for polymorphic markers used in the construction of linkage maps

Category [a]	Female	Male
1	aa	bb
2	aa	ab
3	ab	aa
4	ab	ab
5	ab	ac
6	aa	bc
7	bc	aa
8	ab	cd

[a]Category 1 markers were not used for map construction.

5.3.3 Genotyping of F_1 progenies and marker segregation

A total of 228 polymorphic SSR and ESSR markers were used to genotype the mapping population. Eight percent of these markers (i.e. 12 SSRs and 8 ESSRs) gave PCR amplification products but were difficult to resolve on the ABI 3730 capillary sequence analyser. The number of markers with scorable fragments were reduced to 208 but 12 of these were found to amplify more than one region of the genome (duplicate loci) and therefore the total number of polymorphic markers was increased to 220. These duplicate loci did not co-segrergate.

The segregation type and number of markers observed for each type are presented in Table 5.4. Markers exhibited different segregation patterns ranging from 1:1, 1:2:1 to 1:1:1:1. Segregation patterns and their codes (Van Ooijen, 2006) that were used in the present study were: (a) *lm x ll*, this type of segregation showed maternal polymorphism and most fragments segregated in a expected Mendelian ratio of 1:1, (b) *nn x np* showed a segregation ratio of 1:1 as a result of heterozygosity and homozygosity in male and female parents respectively; (c) *ef x eg* had two unique alleles, one in each of the heterozygous parents and segregated in a ratio of 1:1:1:1; (d) *ab x cd* in which four different alleles segregated per locus in a ratio of 1:1:1:1 and (e) *hk x hk* in which both alleles were in common and heterozygous in each

parent and segregated in a ratio of 1:2:1. The last type of segregation was less informative than the others.

Table 5.4 Segregation types and expected ratios corresponding to number of SSR and ESSR markers observed in the parents and the segregating population of Namikonga x Albert [a]

Segregation type	<lm x ll>	<nn x np>	<ef x eg>	<ab x cd>	<hk x hk>	Total
SSR	39	47	66	11	2	165
ESSR	22	11	16	4	2	55
Total number of markers	**61**	**58**	**82**	**15**	**4**	**220**
Expected segregation ratio	1:1	1:1	1:1:1:1	1:1:1:1	1:2:1	

[a] Segregation types as explained in Van Ooijen (2006).

5.3.4 Genetic linkage map construction

Using JoinMap® 4 software, 165 SSR and 55 ESSR polymorphic markers segregating in gametes of female and male parents were tested for linkage. Three maps, one from each of the two parents and an integrated map were generated from the full-sib population. This was performed using the *Create maternal and paternal node* and *Create population node* functions of JoinMap® 4. In addition, a pseudo F_2 map was constructed from a self-pollinated population of the female parent Namikonga (Namikonga x Namikonga population). Detailed information of all maps is presented in Table 5.5 and Figures 5.1-5.4. Of the 157 markers (markers segregating in the female and allelic bridges) used for linkage analysis in the female parent, 131 markers mapped to 17 linkage groups (N1-N17) of the female (Namikonga) linkage map (Table 5.5 and Figure 5.1). The length of the map spanned 779.5 cM and the number of markers for each linkage group varied from 3-17 with an average distance of 5.95 cM between markers. The most densely populated linkage group (N1) consisted of 17 markers spanning a distance of 95.29 cM while the least populated group (N3) had three markers spanning 12.18 cM which happened to be the shortest of all linkage groups. Twenty six markers (16.56%) remained unmapped.

The male framework map was constructed using 155 markers from which 123 markers (79.35%) defined 18 linkage groups spanning 854.26 cM. The average distance between

markers was 6.94 cM and the number of markers per linkage group ranged from 2 to 19. The most densely populated linkage group (A11) had 19 markers (Table 5.5 and Figure 5.2). The length of the linkage groups ranged from 4.82 cM (A12) to 143.34 cM (A1) and 32 markers (20.65%) could not be assigned to any linkage group and remained unmapped. The average map length per linkage group was 47.46 cM.

Table 5.5 Details of the female (Namikonga), male (Albert), integrated and Namikonga-S_1 genetic linkage maps

Details	Map			
	Female	**Male**	**Integrated**	**Namikonga-S_1**
Number of linkage groups	17	18	23	17
Number of markers used	157	155	219	162
Number of mapped markers	131	123	174	124
Number of unmapped markers	26	32	45	38
Range of marker number per linkage group	3-17	2-19	3-20	2-15
Average map length per linkage group (cM)	45.85	47.46	43.28	30.86
Total map length (cM)	779.48	854.26	995.46	524.58
Mean map distance between markers (cM)	5.95	6.94	5.72	4.23

A total of 219 markers segregating in both gametes of the male and female parents of the CBSD mapping population were employed for construction of an integrated linkage map. One hundred and seventy four markers (79.45%) were assigned to 23 linkage groups spanning a length of 995.46 cM (Table 5.5 and Figure 5.3). The largest linkage group (C11) contained 20 markers while the three smallest groups (C3, C12 and C20) each had only three markers. The shortest linkage group (C2) was 4.82 cM while the longest (C4) spanned 99.08 cM. The number of markers which remained unlinked was 45 which accounted for 20.54%. This map had an average map size per linkage group of 43.28 cM. The presence of markers with segregation types (*ef x eg*) and (*ab x cd*) served as allelic bridges (homologous loci) in combining the female and male maps into an integrated linkage map. Markers segregating in both parents were combined with markers segregating in one or the other parent to form an integrated linkage map (Stam, 1993; Maliepaard *et al*., 1998; Van Ooijen, 2006).

Only markers segregating in the female parent and those segregating in both parents (*ef x eg* and *ab x cd*) were employed to construct a pseudo F_2 framework map. The map was generated as a result of linkage analysis of markers in a population developed from selfing Namikonga (the female parent). Considering this type of population, markers segregating in the male parent but homozygous in the female parent (*nn x np*) were not used. All markers were converted into F_2 segregation codes and the resulting map consisted of 17 linkage groups spanning a distance of 524.58 cM (Table 5.5 and Figure 5.4). A total of 162 markers were employed to construct the map and 124 markers could be assigned to 17 linkage groups. The remaining 38 markers (23.46%) could not be assigned to any linkage group and remained unmapped. The average marker interval was 4.23 cM while the average map size per linkage groups was 30.86 cM. The number of markers per linkage group ranged from two (S3) to 15 (S11). The 17 linkage groups have been named S1-S17.

During the construction of all four types of maps, no suspected linkages were observed. The level of markers that showed distorted segregation at $P \leq 0.05$ varied among maps. Only 11.46% of all markers showed genetic distortion in the Namikonga (female) map while 29.03% of the markers had genetic distortion in the Albert map. The levels of distorted segregation in the integrated and Namikonga-S_1maps were 23.74% and 24.69% respectively.

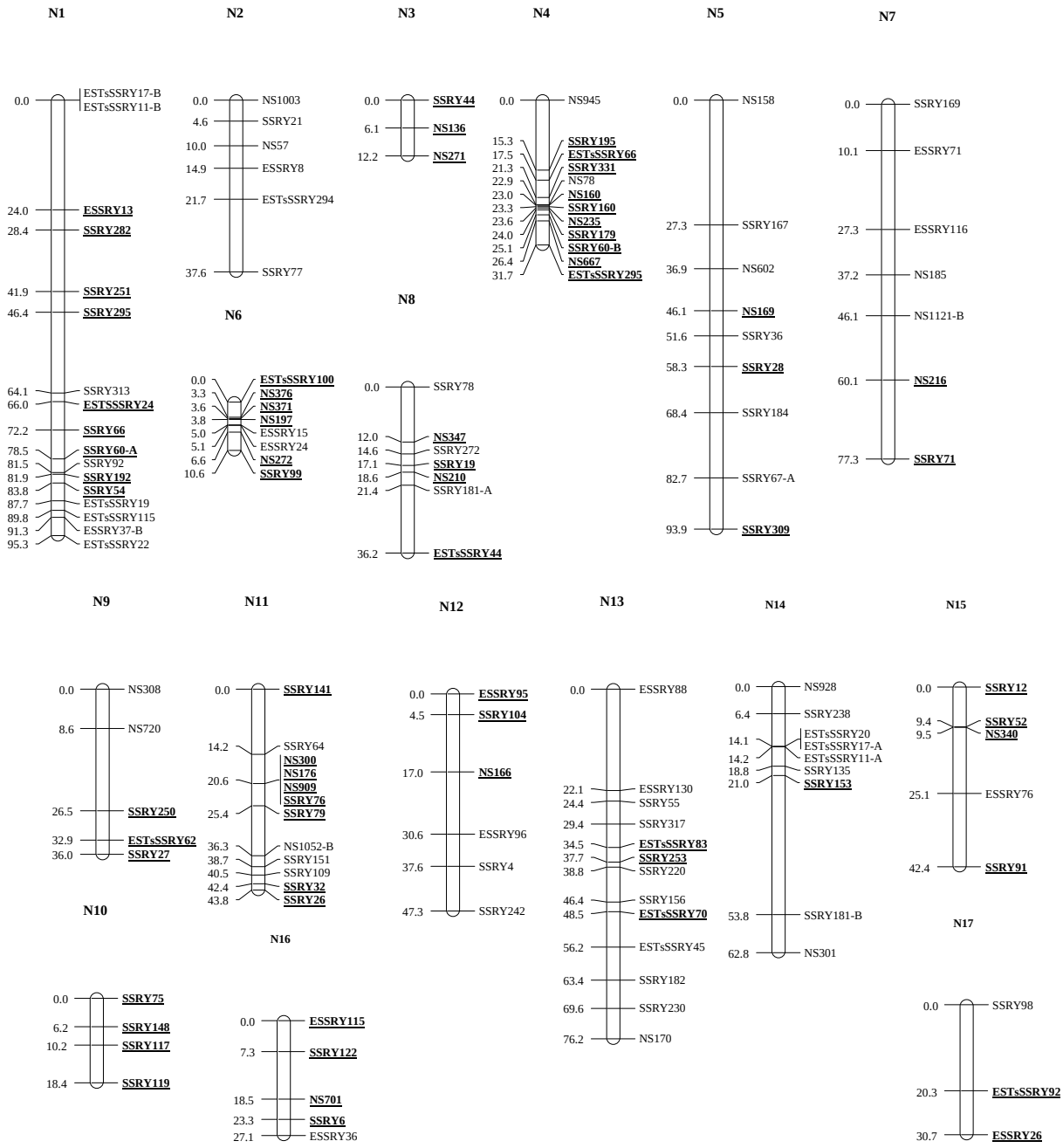

Figure 5.1 Female (Namikonga) genetic linkage map of cassava from a cross between a CBSD resistant (Namikonga) and susceptible genotype (Albert) indicating 17 (N1-N17) linkage groups. The map shows the linear order and relative distance of markers (cM) on the left with markers placed on the right. Markers common in both parents are underlined and in bold.

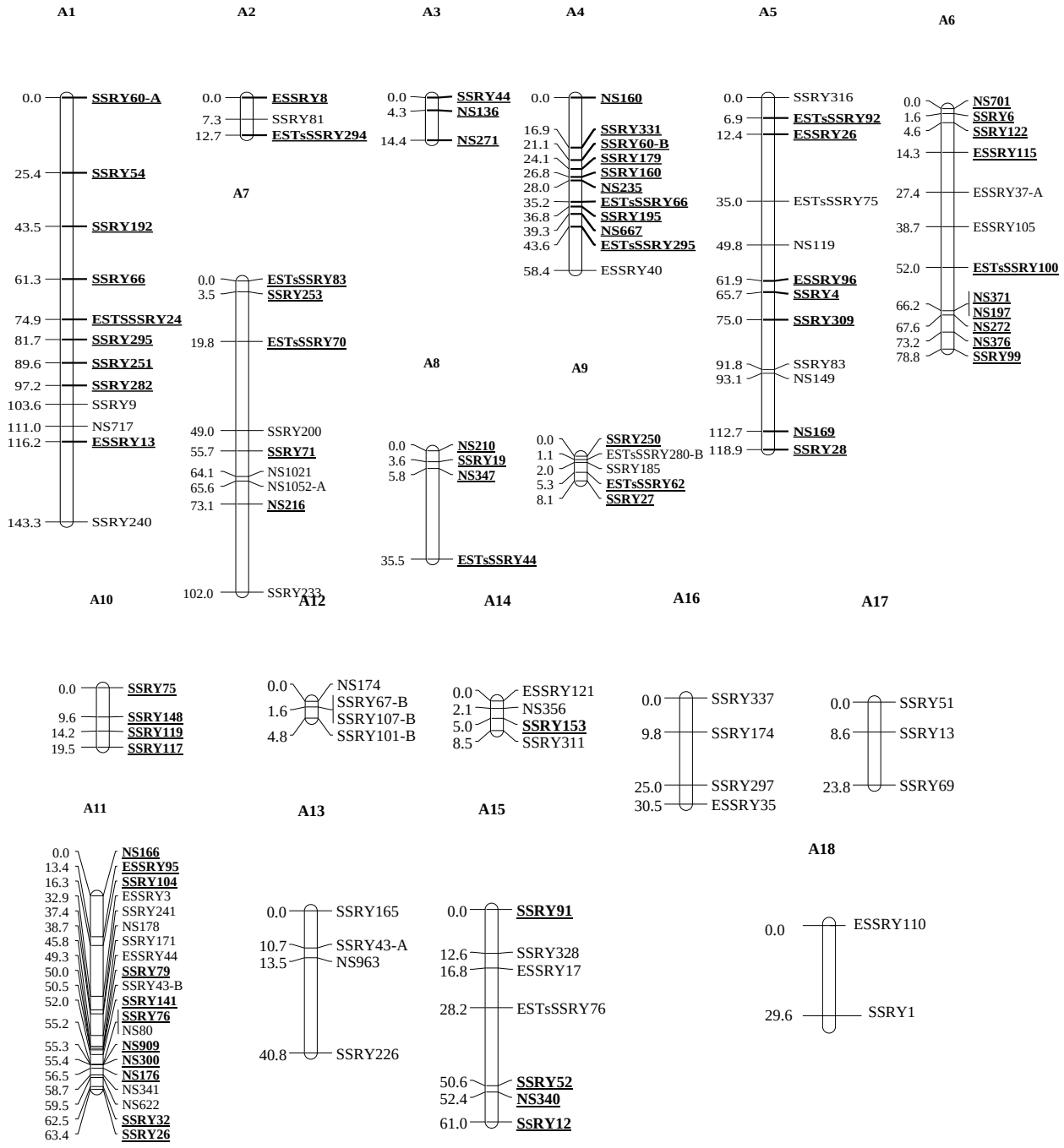

Figure 5.2 Male (Albert) genetic linkage map of cassava from a cross between a CBSD resistant (Namikonga) and susceptible genotype (Albert) showing 18 (A1-A18) linkage groups. The map shows the linear order and relative distance of markers (cM) on the left with markers placed on the right. Markers common in both parents are underlined and in bold.

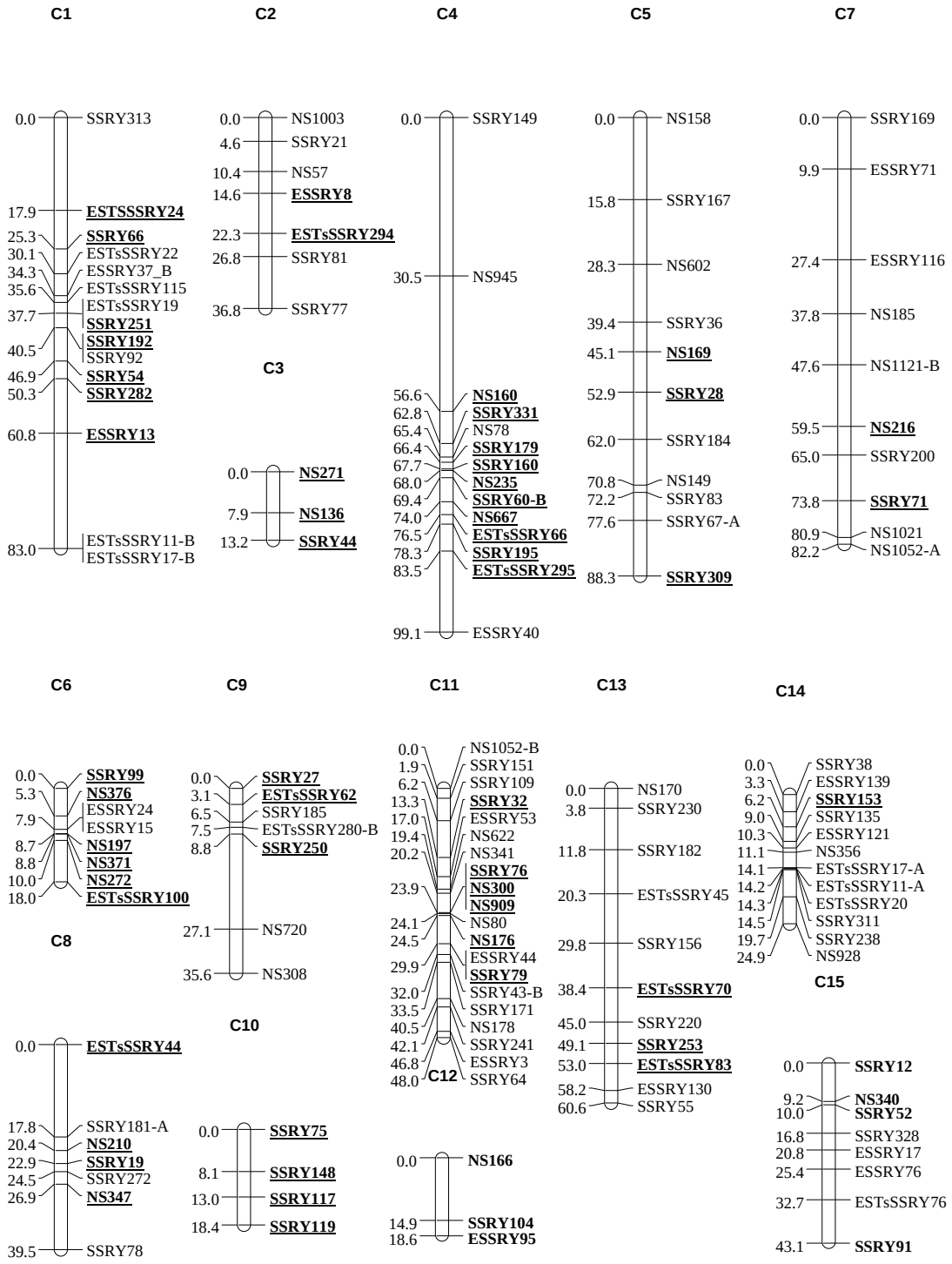

Figure 5.3 An integrated genetic linkage map of cassava from a cross between a CBSD resistant (Namikonga) and susceptible genotype (Albert) showing 23 (C1-C23) linkage groups. The map shows linear order and relative distance of markers (cM) on the left with markers placed on the right. Markers common in both parents are underlined and in bold.

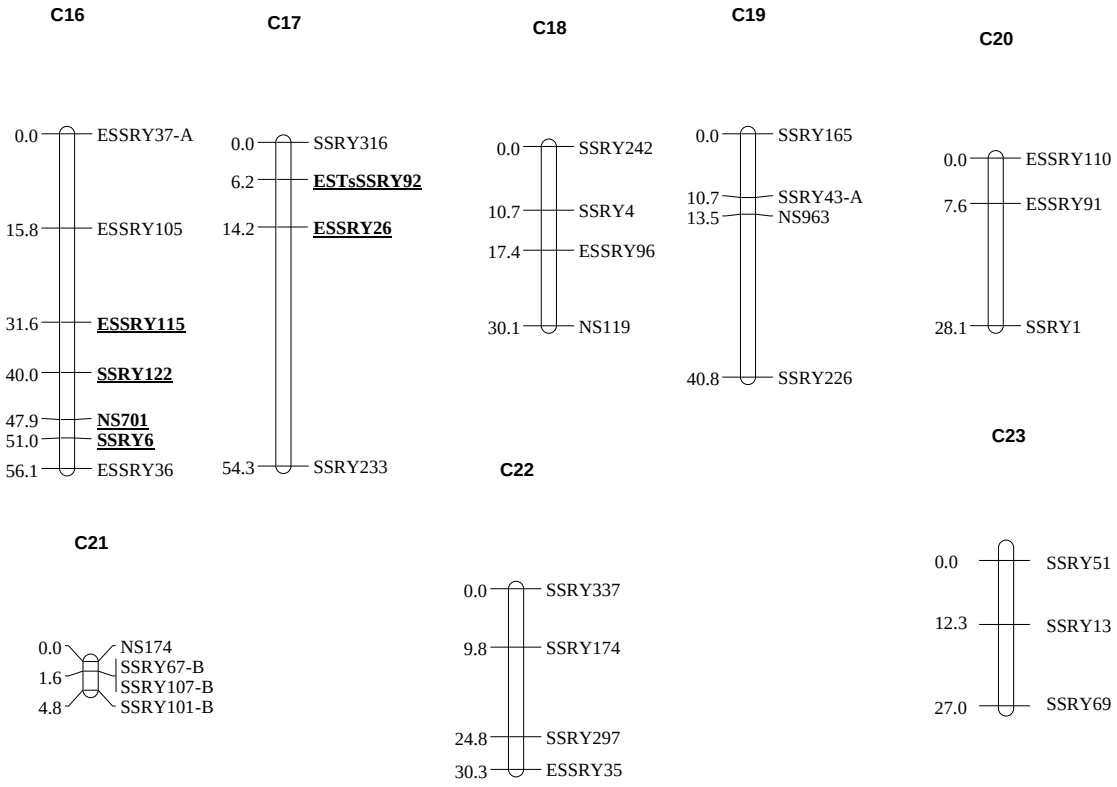

Figure 5.3 Continued.

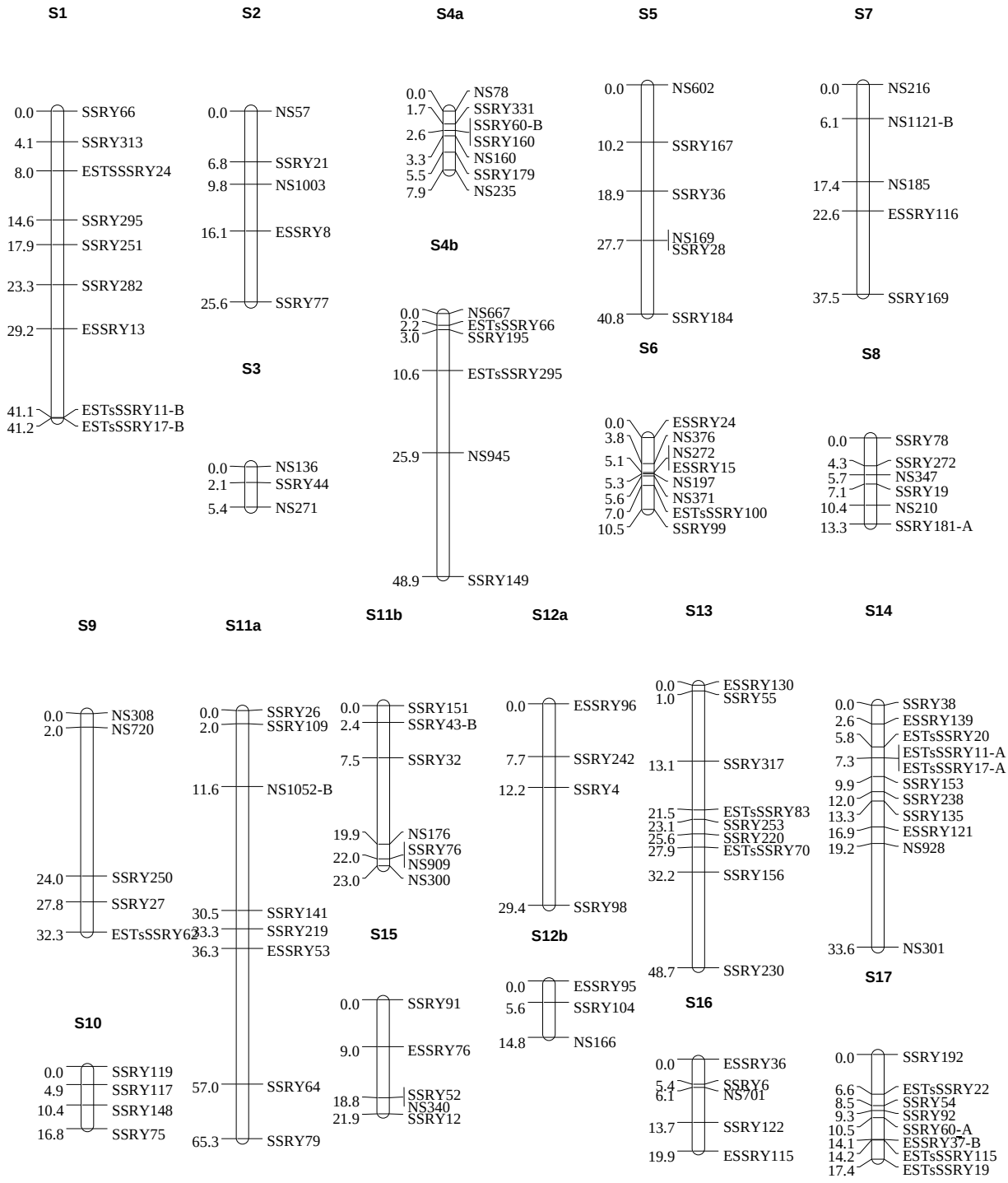

Figure 5.4 Namikonga-S_1 genetic linkage map of cassava from a self pollination cross of the CBSD resistant female parent genotype (Namikonga x Namikonga population) showing 17 (S1-S17) linkage groups. The map shows the linear order and relative distance of markers (cM) on the left with markers placed on the right.

5.3.5 Comparison of female, male, integrated and Namikonga-S_1 maps

There were variations in different aspects of the four maps generated. The integrated map showed the largest total map length (995.46 cM) while the shortest map length was recorded for the Namikonga-S_1 map. Excluding the Namikonga-S_1 map, the integrated map contained the highest number of markers, highest number of linkage groups and the smallest mean map interval between markers (Table 5.5).

The comparison of female and male maps based on common markers present in both parents is highlighted in Table 5.6. Common markers (SSR and ESSR) which saved as allelic bridges in generating an integrated map were identified. These makers are important in the identification of homologous linkage groups. The number of common markers varied between linkage groups and ranged from 1-10 (Table 5.6). In total there were 84 common markers in both female and male maps and they allowed the identification of homologous linkage groups. Homologies with at least one bridging locus were established between all 17 linkage groups of the female map and 13 of the male map. Homology between linkage group N4 (female) and A4 (male) showed the highest number of bridging loci (10) while groups N14 and A14 shared just a single common marker (Table 5.6). Most of the bridging loci found in both female and male maps were also present in the integrated and Namikonga-S_1maps. The highest number of common markers was found in linkage groups C4 and S4 of an integrated and Namikonga-S_1 maps respectively (Table 5.6). The lowest number of common markers was observed in linkage groups C14, S2, S7 and S14. In total there were respectively 76 and 67 common markers in the integrated and Namikonga-S_1maps that were also present in linkage maps of both female and male parents.

Table 5.6 Homologous linkage groups observed in female and male maps as identified by common markers. The number of common markers in both parents, which are also present in an integrated and Namikonga-S_1 maps, are shown

Linkage group in female map[a] (Namikonga)	Linkage group in male map (Albert)	Number of bridging common markers	Linkage group and number of common markers in integrated map		Linkage group and number of common markers in Namikonga-S_1 map[b]	
N1	A1	9	C1	7	S1	6
N1	A1	9	C1	7	S17	3
N2	A2	2	C2	2	S2	1
N3	A3	3	C3	3	S3	3
N4	A4	10	C4	10	S4	10
N5	A5	5	C5	3	S5	2
N6	A6	6	C6	6	S6	6
N7	A7	2	C7	2	S7	1
N8	A8	4	C8	4	S8	3
N9	A9	3	C9	3	S9	3
N10	A10	4	C10	4	S10	4
N11	A11	8	C11	6	S11	8
N12	A11	3	C12	3	S12	3
N12	A5	2	C18	2	S12	2
N13	A7	3	C13	3	S13	3
N14	A14	1	C14	1	S14	1
N15	A15	4	C15	4	S15	4
N16	A6	4	C16	4	S16	4
N17	A5	2	C17	2	-	-
-	A12	-	C19	-	-	-
-	A13	-	C20	-	-	-
-	A16	-	C21	-	-	-
-	A17	-	C22	-	-	-
-	A18	-	C23	-	-	-

[a] N1-N17, A1-A15, C1-C18 and S1-S16 refer to linkage groups of Namikonga, Albert, integrated and Namikonga-S_1 maps respectively, - = common markers absent.
[b] These are the markers that were mapped in Namikonga-S_1 map but also mapped in the female, male and integrated map.

5.3.6 Comparison with other published maps

Table 5.7 presents information on comparison of the integrated linkage map from this study with the two published linkage maps by Okogbenin *et al*. (2006) and Kunkeaw *et al.* (2010b). Of the 20 linkage groups of the F_2 based linkage map of cassava, 14 linkage groups were

similar in having common markers with the integrated map from this study with a total of 34 markers commonly mapped between the two maps. The EST-SSR map by Kunkeaw *et al*. (2010b) had 20 linkage groups of which 12 were similar to integrated map with 42 markers common to both maps.

Table 5.7 Comparison of integrated map with other published maps

Linkage group of integrated map	Linkage group of F_2 map[a]	Number of common markers with integrated map	Linkage group of EST-SSR map[b]	Number of common markers with integrated map
C1	4	1	1	5
C2	14	2	18	2
C3	-	-	-	-
C4	-	-	4	5
C5	2	5	12 and 11	5
C6	7	2	9	1
C7	13	1	-	-
C8	11	5	-	-
C9	18	1	13	3
C10	-	-	7	3
C11	8	3	3	9
C12	-	-	10	2
C13	6	2	2	4
C14	3	3	-	-
C15	9	4	-	-
C16	-	-	-	-
C17	-	-	-	-
C18	-	-	-	-
C19	-	-	-	-
C20	21	1	-	-
C21	-	-	-	-
C22	10	1	17	3
C23	5	3	-	-
Total	**14**	**34**	**12**	**42**

[a] Okogbenin *et al*. (2006), [b] Kunkeaw *et al*. (2010b).

5.4 Discussion

5.4.1 Embryo rescue

Embryo rescue is a technique employed by plant breeders to rescue inherently weak immature or hybrid embryos to prevent degeneration. Successful embryo culture was demonstrated in 1929 for the first time by Laiback to obtain interspecific crosses between *Linum perene* x *L. austriacum* (Sharma *et al*., 1996). Since then embryos from different crops including cassava that are otherwise unviable or immature can be reared and grown into mature plants using tissue culture. At present embryo rescue is important not only for effective wide crosses, but also for obtaining plants from inherently weak embryos, obtaining haploid plants and shortening the breeding cycle. In cassava, fertility and seed viability are frequently low which can slow down efforts in production of improved varieties. Breeding and selection programmes in cassava can be restricted by low fertility, seed set and rates of germination in some varieties (Jennings, 1963). Embryo culture has been used to recover plants from wild cassava species known to have poor seed germination (Roca, 1984). In the present study, 68.1% of the seeds from the CBSD mapping population were cultured into mature plantlets (CIAT, 2003). The aim of the embryo rescue was to improve seed germination but more importantly to rapidly propagate each of the genotypes (F_1 progenies) (*in vitro*) in order to obtain sufficient plants within the shortest possible time for phenotyping for response to CBSD field resistance. The number of plants recovered through embryo culture was much higher than those recovered from conventional seed germination (Biggs *et al*., 1986). Cassava is propagated vegetatively and has a low multiplication ratio. Embryo rescue coupled with *in vitro* propagation was an effective strategy in ensuring an effective number of plants for each F_1 genotype within a short time frame. However, flooding which occurred at CIAT leading to a loss of about 50% of the genotypes caused a delay in field establishment of the mapping population.

5.4.2 Screening markers for polymorphism

The discovery of molecular markers has revolutionised crop improvement in modern plant breeding programmes. Prior to molecular discovery, morphological markers played an essential role in crop improvement as they assisted in selection. However, their application

has been limited due to their low abundance and the fact that they are influenced by the environment. Molecular markers which detect variation at DNA level are abundant in number and are not influenced by the environment and can increase the accuracy and efficiency of selection. Molecular markers have been useful in MAS for different crops (FAO, 2007). For a crop like cassava, which has a long growing cycle, the application of MAS complements conventional selection in plant breeding programmes. A MAS programme for different traits helps to speed up genetic gain, increases heritability through increased selection efficiency and reduces population sizes at early growth stage. Because of the increasing need for high throughput genotyping, the need for automation for accuracy, rapidity and quality has increased (Mansfield *et al.*, 1995). Advances in high throughput DNA extraction, PCR multiplexing, fluorescent detection systems, softwares for fragment size analysis and the availability of numerous microsatellite markers which are easily amenable to automation, have contributed significantly to the ability to genotype large sample sizes rapidly and accurately (Hall *et al*., 1996; Ghosh *et al*., 1997; Schuelke, 2000; Pan *et al*., 2007).

In the present study the AB1 3730 fluorescent-based capillary detection system was used for visualisation of PCR products (Applied Biosystems). With this system high throughput genotyping of up to four SSR markers per run was performed due to post-PCR multiplexing using four different dye labels. Both fluorescently-labelled and universally labelled primers were used in this study. Universally labelled primers, mostly ESSRs' allele sizes were overlapping between 166 and 174 bp making it difficult to co-load on the ABI platform. Co-separation was achieved through labelling with spectrally resolvable fluorescent dyes (fluorophore) with different emission wavelengths. On the basis of colour and size, the analysis of up to four loci in the same capillary injection was possible and detection complications due to spectral overlap were avoided. The accurate alignment of peaks was possible using LIZ-labelled size standard in the loading buffer. Automated scoring of alleles by means of software like Genemapper (Applied Biosystems) makes this system more accurate compared to other techniques like silver staining.

Microsatellites have become the molecular markers of choice for a wide range of molecular applications including genetic mapping and genome analysis due to high levels of polymorphism, co-dominance, multi-allelism, ease of assay, wide transportability across different mapping populations and ease of automation (Li *et al*., 2000). EST derived SSRs have been useful for unravelling the complexities of eukaryotic organisms because they are directly linked to genic regions of the genome (Akagi *et al.*, 1997). ESTs are useful in cloning encoding genes and for mapping functional genes (Akkaya *et al.*, 1992).Since ESTs represent coding regions of the genome, direct association of traits and markers can lead to identification of QTL underlying traits of interest (Rudd, 2003).In this study, 38% of the amplified SSRs and ESSRs showed polymorphic patterns in the parents and were informative in the reference population. The number of informative polymorphic ESSRs (26.4%) was lower than the informative genomic SSRs (44.2%). Results are similar to a 39.4% polymorphic level detected using SSRs and ESSRs in cassava by Kunkeaw *et al.* (2010b). Kunkeaw *et al.* (2010b) also observed lower polymorphic ESSRs (30.9%) than SSRs (45.2%). This low level of polymorphism observed with ESSRs may be due to the conserved nature of ESTs since they are generated from expressed regions that are more conserved than non-coding regions of the genome (Dreisigacker *et al*., 2004; Varshney *et al*., 2005).

5.4.3 Marker segregation and duplicated loci

Prior to linkage analysis the genotypic frequency of each locus was analysed to study the possibility of segregation distortion. Segregation was tested against the normal Mendelian expectation ratios using the chi-square test (Van Ooijen, 2006). Marker segregation type provided information on the number of segregating alleles present in the parents of a full-sibfamily and possible genotypes of the progeny (Maliepaard *et al.*, 1997). In full-sib families, it is obvious that segregation types in which at least one of the parents is heterozygous are relevant in linkage analysis. Five segregation types, <*lm x ll*>, <*nn x np*>, <*ef x eg*>, <*ab x cd*> and <*hk x hk*> were used in this study. The highest percentage of markers (82 of 220) were of <*ef x eg*> type, which together with <*ab x cd*>were useful in providing allelic bridges between female and male maps. These two segregation types gave four possible offspring genotypes. Only two offspring genotypes were possible for segregation types<*lm x ll*> and <*nn x np*> and meiosis in the second and first parent in each

segregation type was respectively non-informative. Initially markers with type <*hk x hk*> segregation were excluded during parental screen but when all individuals were genotyped four <*hk xhk*> markers were observed. This explains why very low frequency of these markers was observed in our mapping population. This type of marker contributes little information to the linkage map and recombination frequency estimates obtained using these markers are normally less accurate (Maliepaard *et al*., 1997).

In this study markers with distorted segregation patterns were observed. Segregation distortions, which are deviations from normal Mendelian genetic ratios happen due to various reasons and have been reported in various studies (Liebhard *et al*., 2003; Myburg *et al*., 2003; Cavalcanti and Wilkinson, 2007). Reasons for distortions include technical problems in genotyping and scoring errors, genetic drive, locus duplication and chromosome rearrangement (Cavalcanti and Wilkinson, 2007). Biological factors such as the expression of deleterious alleles that cause either death of gametes or offspring, chromosome loss, isolation mechanism and genetic load also contribute to distorted ratios (Bradshaw and Stettler, 1994; Liebhard *et al*., 2003; Xian-Liang *et al*., 2006). In out-crossing species, high proportions of segregation distortions are frequent (Gan *et al*., 2006). Cassava, being an out-crossing species has high genetic load, suffers from severe inbreeding depression upon selfing (Okogbenin *et al*., 2006) and is therefore affected by segregation distortions.

In the present study 11-29% segregation distortion was observed. This was within the range and comparable to the distortion of 27% reported by Okogbenin *et al*. (2006). A slightly higher distortion of 29% was observed in the male map compared to the female, integrated and Namikonga-S_1maps which showed less than 27% distortion. In the construction of the maps no attempt was made to remove distorted markers as has been suggested by other researchers (Marques *et al*., 1998; Weber *et al*., 2003). However, some researchers argue that exclusion of distorted markers seems unnecessary and a waste of valuable data since such markers provide important information (Marques *et al*., 2002; Liebhard *et al*., 2003; Myburg *et al*., 2003). In this linkage analysis, of the 18 markers showing segregation distortion in the female map, only three markers remained unlinked and could not be mapped. For the male map, of the 45 distorted markers, 17 of them mapped to linkage group A11 and only 12 were

unlinked. Since no marker was excluded during linkage analysis, the presence of unlinked markers suggests the presence of some regions of the cassava genome which have not been mapped. The use of independent LOD, which is calculated in JoinMap®4 based on chi-square tests for independence of segregation, could have resulted in linkage and consequent mapping of markers with distorted segregation. The usual LOD score (linkage LOD) is affected by distorted segregation while independence LOD is not, which lead to less incidences of spurious linkages (Maliepaard *et al*., 1998; Van Ooijen, 2006).

The presence of duplicated loci in the cassava genome has been reported by Fregene *et al*. (1997) and Chavarriagga-Aguirre*et al*. (1998). Twelve microsatellites (eight SSRs and four ESSRs) were found to amplify two loci in cassava. These duplicated loci of each marker did not co-segregate but mapped to different linkage groups of each of the different maps constructed. Duplicated microsatellite and RFLP loci are present in the cassava genome as random genomic duplication or due to polyploidisation events that are thought to have occurred (Chavariagga-Aguirre *et al*., 1998). Recently available cassava genome sequence information indicated the presence of duplicated parts of the genome. Compared to an estimated genome size of 760 Mb, sequence information indicated that the assembly (cassava 1) consisted of 11243 scaffolds spanning 416 Mb. It is believed that the 416 Mb represents nearly the entire genome genic regions and that the missing portion could be repetitive sequences (Rounsley *et al*., 2009).

5.4.4 Construction of linkage map

For genetic improvement of crops, MAS is an efficient strategy and is based on linkage relationships between markers and traits. A molecular marker linkage map is an essential intermediary for MAS breeding in crops. Different types and sizes of mapping populations are used in the construction of genetic linkage maps. In out-crossing species like cassava, full-sib families, derived from heterozygous parents, are used for linkage analysis and genetic mapping of markers segregating independently in the female and male gametes leading to two autonomous maps (Maliepaard *et al*., 1997). Mapping population sizes of 50-300 individuals have been used to construct linkage maps and the larger the mapping population size the better because populations less than 50 individuals provide little mapping resolution

(Young, 1994; Ferreira *et al*., 2006; Semagn *et al*., 2006).

In the present study, a full-sib family from a cross of two heterozygous parents, Namikonga and Albert, with a population size of 60 individuals was used to construct maps using SSR and ESSR markers. The use of threshold LOD score of 3.0 produced 17 and 18 linkage groups for female and male maps respectively. An integrated map with 23 linkage groups was constructed from the two linkage maps using JoinMap® 4 (Van Ooijen, 2006). An additional pseudo F_2 (Namikonga-S_1) map consisting of 17 linkage groups was constructed from a Namikonga-S_1 population. With the exception of the integrated map, the number of linkage groups observed was close to the expected number of 18 linkage groups (n = 18) for a comprehensive map of cassava (2n = 36). Assuming the polyploidy origin of cassava, the nature of chromosome assortment would determine the expected number of linkage groups and between 18 and 36 linkage groups are expected where there is a mixture of preferential and random pairing (Fregene *et al*., 1997).

The map distance of 995.5 cM observed for the integrated map was comparable to the distance of the map by Fregene *et al*. (1997) (931.6 cM) but less than other maps (Okogbenin *et al*., 2006; Chen *et al*., 2010; Kunkeaw *et al*., 2010a; 2010b). This short distance could have been due to the small population size used. The 174 markers mapped to the current integrated map is higher than the 100 markers that mapped to the F_2 map of Okogbennin *et al.* (2006) and comparable to the recent map constructed by Kunkeaw *et al*. (2010b) who mapped 211 out of 307 markers. In addition, the observed average map size per linkage group, average marker interval and range of number of markers per linkage group were similar to published maps (Fregene *et al*., 1997; Okogbenin *et al*., 2006; Kunkeaw *et al*., 2010b).

Molecular markers uniformly distributed throughout the genome are a precondition for the detection of QTL associated with a trait of interest (Tanksley *et al*., 1992). One of the important components of this is the mean map distance between markers. In the female map mean separation between markers was 5.95 cM compared to 6.94 cM in male map, 5.72 cM in the integrated map and 4.23 cM in the Namikonga-S_1 map. Compared to other published maps of cassava, these averages represent good marker coverage of the genome. In Fregene

et al. (1997) and Okogbenin *et al*. (2006) map averages of 5.5 cM and 12.4 cM were respectively observed, while 7.99 cM, 5.6 cM and 4.8 cM have respectively been reported by Kunkeaw *et al*. (2010a; 2010b) and Chen *et al*. (2010). In other crops a range of 6.0-17.0 cM has been reported in citrus eucalyptus, European pears and *Pinus* (Myburg *et al*., 2003; Shephered *et al*., 2003; Weber *et al*., 2003; Yamamoto *et al*., 2003). Clustering of markers separated by intervals of more than 20 cM on some linkage groups such as N5, N9, N13, N14 and N17; A1, A5, A7 and A8; C4, C17, C19 and C20 and S4, S9 and S11 suggest that recombination events are not uniformly occurring and distributed across the genome. Also it might indicate that not enough markers were screened and that these linkage groups were underrepresented with markers leading to big map distances between markers. Adding more markers might reduce the map distance between markers in these linkage groups.

5.4.5 Comparison between maps

The presence of allelic bridges was important for comparison of homologous linkage groups between female and male maps. Allelic bridges are markers that are heterozygous in the gametes of both female and male parents and share common alleles. They have been useful in reconciling the separate maps constructed on the basis of segregation from female and male gametes of female and male parents (Stam, 1993; Fregene *et al*., 1997; Maliepaard *et al*., 1998). In this study homologies were found between 17 linkage groups of the female map and 13 linkage groups from the male map based on 84 common markers. From these results it was evident that homology was identified in all the 17 linkage groups of the female map compared to 13 linkage groups of the male map. Since SSR markers have known sequences, this high level of homology is advantageous in that these markers can act as points of anchorage for other mapping work and QTL identification. Most of the markers common to the female and male maps also mapped to the integrated and Namikonga-S_1 map.

A higher total map length for the female map compared to the male map was observed. Marker order and interval was furthermore different between the two maps. Differences between map distances between sexes are a common phenomen and have been documented. In flowering plants, females appear to have higher genomic map lengths than males due to variation in the rate of meiotic recombination whereas in gymnosperms greater meiotic

recombinations occur in males than in females (Graner *et al*., 1991; Groover *et al*., 1995). Cassava falls in the flowering plants groups and hence the observed higher female map compared to the male map.

For a few linkage groups, analogous markers were not uniformly distributed and in some instances, some minor differences in the estimated marker order between the two parental maps were observed. These could be due to differences in recombination frequencies or chromosome rearrangements of one parental genotype relative to the other in the group. However, if enough common co-dominant markers with the same order is available in chromosomes of both parents, with information of more markers from different populations, it is still possible to combine the available map with other published maps to generate a comprehensive consensus cassava map.

5.4.6 Comparison with other published maps

Since the publication of the first cassava map, which consisted of 132 RFLP, 30RAPD, three SSR and three isozyme markers by Fregene *et al*. (1997) and then by Mba *et al*. in2001, four more cassava linkage maps have been generated (Okogbenin *et al*., 2006; Chen *et al.*, 2010; Kunkeaw *et al*., 2010a; 2010b). The map by Mba *et al*. (2001) consisted of only 36 additional markers that were placed on the cassava RFLP framework map in order to saturate it. This resulted in reduction of two linkage groups from 20 to 18. The integrated map of the present study spanned a distance of 995.46 cM, similar to the distance of the first map (931.6 cM) but was shorter than subsequent maps. The estimated genome length of cassava is 1610 cM. The total map length of the integrated map observed from this study was 995.46 cM, which represents 62% coverage of the cassava genome. This is similar to what was reported by Fregene *et al*. (1997) who estimated 60% genome coverage.

Compared to the F_2 map (Okogbenin *et al*., 2006) consisting of 100 mapped markers, our integrated map consisted of 174 markers, including the 36 SSRs mapped by Mba *et al*. (2001). Only 47 markers were common between both our integrated map and the F_2 map. Despite the low number of common markers between the two maps, there were 14 linkage groups in total from the F_2 map that were similar to our integrated linkage map. All common

markers in linkage groups 2, 3, 5, 9 and 11 of the F_2 map were respectively mapped to linkage groups C5, C14, C14, C23, C15 and C8 of our integrated map. Most of the linkage groups of the F_2 map contained fewer markers than our integrated map which made it difficult to compare the two maps. For example, linkage groups 17, 18, 19, 20, 21 and 22 of the F_2 map consisted of only two markers with one or none markers common to our map whereas only two linkage groups of our integrated map had a minimum of three markers.

In terms of average map distance between markers and number of markers per linkage group, our maps were comparable to published maps. The mean distance of our maps ranged from 5.95-6.94 cM which was comparable to the published maps ranging from 5.5 cM (Fregene *et al.,* 1997; Kunkeaw *et al*., 2010a; 2010b) to 12.4 cM observed by Okogbenin *et al*. (2006). In most aspects, maps produced from our mapping population were similar to published maps of cassava. However, the marker order between published maps and our maps is not similar which could be due to differences in sample sizes used in the maps construction.

5.5 Conclusions

This study has been able to screen a total of 605 SSR and ESSR markers in two cassava varieties, Namikonga and Albert which were used as female and male parental lines respectively. A total of 174 markers were successfully mapped to the integrated linkage map spanning a map length of 995.46 cM which was an improvement of the previously published linkage maps (RFLP framework and F_2 based maps). Despite a low population size of 60 individuals, the strategy of using microsatellites enabled successful mapping of 79.5% of all polymorphic markers in a CBSD mapping population and most markers mapped to linkage groups similar to previously published maps. The low population size was due to pollen contamination and seed mixing during harvesting which was discovered during genotyping. Based on the current results, three CBSD mapping populations, involving Namikonga and other sources of CBSD resistance, with large sizes are being established and more SSR markers from the large number of available cassava SSRs will be screened.

The primary aim of generating the mapping population was to provide a mapping resource to enable the genetic mapping of an important trait, resistance to CBSD. This study has

successfully achieved this objective by generating four different types of maps that will be a resource for tagging CBSD resistance. We hope that the population can also be used for other traits since it also segregates for diseases like CMD.

5.6 References

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CHAPTER 6

Identification of quantitative trait loci controlling resistance to cassava brown streak disease

6.1 Introduction

Cassava represents a valuable subsistence and cash crop in many countries. As a cash crop it is providing a source of raw material for industrial uses and biofuel in Asian and other countries (Chen *et al*., 2010). It is a staple crop for over 800 million people around the world and plays an important food security role in Africa as it provides a cheap source of carbohydrate (Nweke, 1996, Nweke *et al*., 2002) and has a flexible harvesting which permits farmers to keep the storage roots in the ground until needed.

More cassava is produced in Africa than the rest of the world combined and the largest producing countries in 2005 were Nigeria (40%), DRC (19%), Ghana (10.1%), Tanzania (7%) and Mozambique (6%). Total cassava production increased from 35 million to over 100 million tonnes between 1965 and 2005 (FAO, 2006). Despite these production increases, a wide range of production constraints limit its full production potential in Africa. Some of these include diseases particularly CMD, CBSD, CBB, anthracnose and root rot (*Phytopthora*) which cause different levels of yield losses as high as 100% (Storey and Nichols, 1938; Brian and John, 1940; Dixon *et al*., 1995; Legg, 1999, Otim-Nape *et al*., 2000; Hillocks, 200; Jennings and Iglesias, 2002).CMD and CBSD are the most economically damaging of these diseases and have been present in sub-Saharan Africa for many years. A recent surge in the severity of symptoms and spread of CBSD has increased its impact during the early part of the millennium. New outbreaks of CBSD have been reported in previously unaffected areas of the Great Lakes region (Alicai *et al*., 2007). The disease is causing high yield losses in many parts of central, eastern and southern Africa (Thresh *et al*., 1994; 1997; Hillocks *et al*., 2001; Hillocks and Jennings, 2003). CBSD is increasingly becoming more important, perhaps even more than CMD due to the severe necrotic effects it causes on cassava roots.

Until recently CBSD was thought to be caused by a single virus, CBSV (Monger *et al*., 2001). Recently however it has been recognised that CBSD is caused by two different virus species (Winter *et al*., 2010). These have been called CBSV [referring to the original species first described by Monger *et al*., (2001)], and a second species, CBSUgV which predominates in, but is not exclusive to, the Lake Zone of Tanzania and Uganda. Both of these viruses are monopartite RNA viruses of the genus *Ipomovirus* family Potyviridae (Monger *et al*., 2001). Since the discovery of CBSD in 1930s in the Tanzanian coastal areas of Indian Ocean (Storey, 1936) the disease has spread to areas in north-western Tanzania, Kenya, Uganda, Burundi, Rwanda, Mozambique, Malawi and DRC where it is causing severe root yield and quality losses (Legg and Raya, 1998; Gondwe *et al*., 2003; Alicai *et al*., 2007; Ntawuruhunga and Legg, 2007). Studies have shown that yield losses of up to 74% have been observed due to CBSD infection but in severely infected susceptible varieties yield losses up to 100% have been recorded (Hillocks *et al*., 2001; Muhana and Mtunda, 2002; Gondwe *et al*., 2003).Since the emergence of CBSD in the 1930s, progress has been made in understanding and controlling the disease through resistance breeding using conventional methods (Nichols, 1947; 1959; Jennings, 1960; 1975; Kanju *et al*., 2007). Significant progress has been achieved in applying molecular approaches to understand the viruses (Monger *et al*., 2001; Mbazimbwa *et al*., 2009; Winter *et al.*, 2010) but little or no progress has been made in developing molecular tools for breeding for CBSD resistance. Unlike CMD resistance for which markers have been identified for MAS (Akano *et al*., 2002), no markers associated with CBSD resistance have so far been identified.

Genetic markers are fundamental tools in understanding inheritance and diversity of natural variation. In cassava, morphological markers and isozymes were the earliest markers to be used in genetic studies (Hussain *et al*., 1987; Hershey and Ocampo, 1989; Ocampo *et al*., 1992). Since then a number of DNA markers have been applied to cassava, including RFLP, RAPD, AFLP, SSR and SNP. Markers have been used to study cassava genes, genetic diversity, genome analysis, linkage mapping and QTL analysis. Currently SSRs are the marker system of choice for cassava (Fregene *et al*., 1997; Chavarriaga-Aquirre *et al*., 1998; Elias *et al*., 2000; Mba *et al*., 2001; Okogbenin and Fregene, 2002).MAS can be used as a tool within a plant breeding programme to improve the accuracy of selection and thereby the

effective heritability of a trait. It is particularly useful when traits are difficult to score, or can only be scored at maturity, which is the case of cassava that is generally scored at 12 months. In addition it allows for selection in the absence of the trait. Marker-assisted breeding can reduce the time needed to develop new cultivars and can dramatically reduce population sizes in the early stages. The availability of molecular markers can facilitate introgression of resistance genes into elite varieties' backgrounds in a cost effective way and less time consuming way than conventional approaches (Tanksley *et al*., 1989; Das *et al*., 2006).

Several approaches can be used to identify molecular markers associated with traits of interest. They include genetic linkage mapping, BSA and Genome Wide Association Studies (GWAS) (Tanksley *et al*., 1989; Michelmore *et al*., 1991, http://www.stats.ox.ac.uk/~mcvean/gwa4.pdf). The BSA technique enables breeders to rapidly identify genetic markers based on minimal classic genetic information and has been developed as a rapid procedure for identifying markers in specific genome regions for traits expressing variation at a single locus of large effect (Giovannoni, 1991; Michelmore *et al*., 1991). On the other hand, genetic linkage mapping uses genetic analysis of segregating populations to construct maps that provide a direct method of selecting genes via their easily detectable markers (Tanksley *et al*., 1989). The basic methodology for mapping QTL involves arranging a cross between two parents differing substantially in the quantitative trait of interest. Segregating progeny are then scored both for the trait of interest and for a number of genetic markers (Lander and Botstein, 1989). It is important that parents are chosen to maximise the chance that they segregate for QTL having relatively large phenotypic effects, thereby allowing mapping with a manageable number of progeny (Lander and Botstein, 1989). The traditional approach to QTL mapping is carried out by looking for associations between genotypes at individual markers and phenotypic traits of interest (Kruglyak and Lander, 1995). A powerful approach of interval mapping was introduced by Lander and Botstein (1989) in which the presence of a QTL is tested at every location in a genome by exploiting the full power of a complete genetic linkage map.

QTL associated with two putative genes, CMD_1 and CMD_2 conferring resistance to CMD from TME3 have been identified. Three SSR markers (SSRY28, NS158 and SSRY40)and

one sequence characterised amplified region (SCAR) marker, RME1, define these QTL and have been placed on the cassava linkage map using the linkage mapping approach (Fregene *et al*., 2001; Akano *et al*., 2002, GCP Molecular marker Toolkit). In addition, two SSR markers (NS1099 and NS346) have been identified linked to green mite resistance (CIAT, 2006). At present no markers associated with CBSD resistance have been identified. The aim of the present study was to identify molecular markers linked to QTL of genes controlling resistance to CBSD using genetic linkage mapping analysis of a F_1 family derived from a cross of unrelated CBSD resistant and susceptible varieties.

6.2 Materials and methods

6.2.1 Plant materials

The plant material consisted of a segregating F_1 population consisting of 190 F_1 progeny obtained by crossing two contrasting parents, the female parent, Namikonga, resistant to CBSD, and the male parent, Albert, susceptible to CBSD. Selection of parents and the development of the CBSD mapping population for linkage mapping and QTL analysis are explained in sections 3.4.2 and4.2.2 respectively.

6.2.2 Isolation of genomic DNA and SSR analysis

Young cassava leaves were collected from each of the 190 individuals and two parental lines of the CBSD mapping population and DNA extraction was performed as described in sections 3.2.2 and 5.2.4. Genotyping of the CBSD mapping population using 169 polymorphic SSRs and 59 polymorphic ESSR polymorphic is described in section 5.2.5.

6.2.3 Linkage analysis and map construction

Construction of four genetic linkage maps for QTL analysis is described in section 5.2.6.

6.2.4 Phenotypic evaluation of CBSD field resistance

The 190 individuals and two parental lines constituting the CBSD mapping population were evaluated for their reaction to CBSD over two seasons, 2007 and 2008. During the first season, due to limitation of planting material, evaluation was done at one site, Chambezi.

During the second season the mapping population was evaluated at two locations, Chambezi and Naliendele in Tanzania (elevation, bearings etc. for these locations are given in section 4.2.4.1). At each location, the materials were planted in a randomised complete block design with three replications. Each of the individuals of the mapping population in each replication was represented by three plants making a total of nine plants for each genotype across the three replications. Plants were planted at a spacing of 1.0 m between plants and 1.0 m between rows. To insure high inoculum pressure within the experimental field, cuttings from infected susceptible genotypes were planted after every ten rows of test genotypes and each replication was surrounded by infected plants to act as a spreader of the disease. No fertiliser or irrigation was applied during the growing season. Evaluation for response to CBSD infection involved screening the genotypes by scoring each individual plant on a scale of 1-5 for root necrosis as described in detail in section 4.2.4. The mean disease severity score for root necrosis for each genotype was obtained by averaging over three replications.

Statistical analysis of CBSD reactions in the progeny was performed using ANOVA to test the significance of the genotypic effect. In addition, correlation of root necrosis across locations and seasons and estimation of genetic and error variance components was done using GenStat Discovery Edition 3 (VSN International Ltd, 2008) statistical software. Broad sense heritability was calculated using the formula $H_b^2 = \delta_g^2 / (\delta_g^2 + \delta_e^2/3)$ where δ_g^2 and δ_e^2 are the genetic and error variances (Wricke and Weber, 1986; Singh, 2005). The error variance was divided by three corresponding to the number of clonal replicates for each genotype. Mean squares for genotypes and error were employed to compute δ_g^2 and δ_e^2 variances. The genotypic variance was computed from the ANOVA table as δ_g^2 = (Mean square (MS) due to genotype – MS due to error)/r (r = number of replications) and δ_e^2 was the MS due to error (Singh, 2005). Frequency distributions of CBSD reaction of the entire mapping population in each of the three locations was performed using Microsoft Excel 2007.

6.2.5 QTL analysis

QTL associated with CBSD resistance were of interest in this study. QTL analyses were conducted using MapQTL version 6.0 (Van Ooijen, 2009) using phenotypic data from each

of the three evaluation trials (Chambezi 2007, Chambezi 2008 and Naliendele 2008). Analyses were performed using the two parental maps (Namikonga and Albert using the double haploid (DH) population type option available in MapQTL (Van Ooijen, 2009) and using the integrated map as well as Namikonga-S_1 map (see Chapter 5 for different maps). The parental maps were analysed separately mainly to enable detection of QTL present in Namikonga, the source of CBSD resistance, completely independently of the other parent and in order to avoid the potential differences in the rates of recombination between the two parents. Interval mapping (Lander and Botstein, 1989; Jung *et al*., 1996) was used to estimate the map location, LOD score and phenotypic effect of potential QTL, in terms of the percentage of phenotypic variance explained. Estimates of QTL positions were obtained at the point where the LOD score assumes its maximum.

The non-parametric, single marker-based Kruskal-Wallis analysis was conducted to independently identify significant marker-trait association. The test ranks all individuals according to their quantitative traits while classifying them according to their genotype classes. The presence of a segregating QTL with large effect will result in a large difference in average rank. The test is performed on each locus separately with no use of the linkage map order. For this analysis, a genome wide $P \leq 0.0005$ was chosen based on a comparison-wise P-value of 0.05 divided by the approximate number of markers used in the construction of each of the maps.

Significant LOD thresholds were determined by deciding which P-value to use and whether or not individual threshold per linkage group or genome wide threshold is used (Van Ooijen, 2009). Group-wide (chromosome-wide) threshold significance is the probability of obtaining a LOD above the threshold somewhere on the single chromosome just by chance while genome-wide is the probability of obtaining a LOD above the threshold somewhere in the whole genome just by chance (Van Ooijen, 1999). Group-wide LOD thresholds for QTL detection at $P < 0.05$ were estimated using the permutation test as implemented in MapQTL version 6.0 with 1000 iterations. Specifically, for each of the combinations of trait and linkage map, the 5% group-wide threshold was determined. In summary, a QTL was considered as significant only if it exceeded group-wide 5% threshold, and/or had significant

P-value in the Kruskal-Wallis analysis. Markers that were consistently a significant at $P < 0.01$ via Kruskal-Wallis analysis over locations were presented for illustrative purposes, despite having LOD scores lower than the group-wide threshold.

6.3 Results

Phenotypic screening of individuals of the two CBSD mapping populations, Namikonga x Albert and Namikonga x Namikonga conducted at Chambezi and Naliendele during the 2008 growing season showed significant variation in their response to CBSD infection in cassava roots. Results of the analysis of variance showing phenotypic values (mean disease scores), standard error, mean squares, coefficient of variation and broad sense heritability values at both Chambezi and Naliendele for CBSD root necrosis reaction of the F_1progenies of the Namikonga x Albert mapping population are presented in Table 6.1. Results showed highly significant ($p < 0.001$) variation among progenies in their reaction to CBSD root necrosis at both locations. Higher mean CBSD scores and mean squares were observed at Chambezi than at Naliendele but higher coefficient of variations were obtained at Naliendele than Chambezi. Field observations indicated that some F_1 progenies which had a score of 1 showed high levels of resistance which were regarded as being field immune compared to the resistant parent. This was also true for the susceptible genotypes, some of them showed high levels of susceptibility leading to die back and death of plants. Estimates of broad sense heritability values for CBSD root necrosis at Chambezi and Naliendele were 87.85% and 70.08% respectively.

Table 6.1 General statistics for root necrosis reaction measured on a scale of 1-5 for F_1 genotypes of the Namikonga x Albert mapping population screened against CBSD resistance at Chambezi and Naliendele during the 2008 growing season

Location	Mean + SE	$MS_{genotype}$	MS_{error}	F value	P	CV%	H_b (%)
Chambezi	2.926 ± 0.6357	3.3274	0.4042	8.23	0.001	21.7	87.85
Naliendele	2.706 ± 0.9286	2.8820	0.8622	3.34	0.001	34.3	70.08

SE = standard error, MS = mean square, F value = variance ratio, P = probability significance level, CV(%) = coefficient of variation (in %), H_b = broad sense heritability.

Table 6.2 presents results of analysis of variance for CBSD root necrosis of S_1 progenies of the Namikonga x Namikonga population screened at Chambezi and Naliendele including CBSD mean scores, mean squares, coefficient of variation and heritability estimates. Highly significant ($P < 0.001$) variation was observed among S_1progenies in their reaction to CBSD root necrosis, showing different levels of CBSD infection but mean scores across locations were similar. At Chambezi higher mean scores as well as mean squares for genotypes were obtained than at Naliendele but at the latter location coefficients of variation were higher. Some plants at Naliendele were affected by termite damage explaining the high coefficients of variation observed. Field observations indicated that most genotypes from this population indicated high levels of resistance compared to the resistant parent. Estimates of broad sense heritability for CBSD root necrosis were higher at Chambezi (94.63%) than at Naliendele (86.57%).

Table 6.2 General statistics for root necrosis reaction of S_1 genotypes of the Namikonga x Namikonga mapping population screened against CBSD resistance at Chambezi and Naliendele in 2008 season

Location	Mean + SE	$MS_{genotype}$	MS_{error}	F value	P	CV%	H_b (%)
Chambezi	2.361 ± 0.5156	4.9517	0.2658	18.63	0.001	21.8	94.63
Naliendele	2.327 ± 0.6847	3.4895	0.4688	7.44	0.001	29.4	86.57

SE = standard error, F value = variance ratio, P = probability significant level, CV (%) = Coefficient of variation (in %), H_b = broad sense heritability.

The frequency distribution of CBSD root necrosis reaction of individuals of the Namikonga x Albert mapping population is presented in Figure 6.1. With exception of CBSD screening at Chambezi in 2007 which showed slightly skewed distribution towards disease resistance, possibly due to low inoculum pressure as it was the first year of screening at this location, CSBD root necrosis of individuals of the Namikonga x Albert mapping population at both Chambezi and Naliendele in 2008 showed a normal distribution around the mean. The reaction of the progeny against CBSD infection at two locations in 2008 showed normal distribution patterns. The genotypes reaction to CBSD exhibited continuous variation with the majority having an intermediate level of resistance. Few progeny showed CBSD resistance like the resistant parent while the other part of the normal distribution had few progeny showing susceptibility level like the susceptible parent.

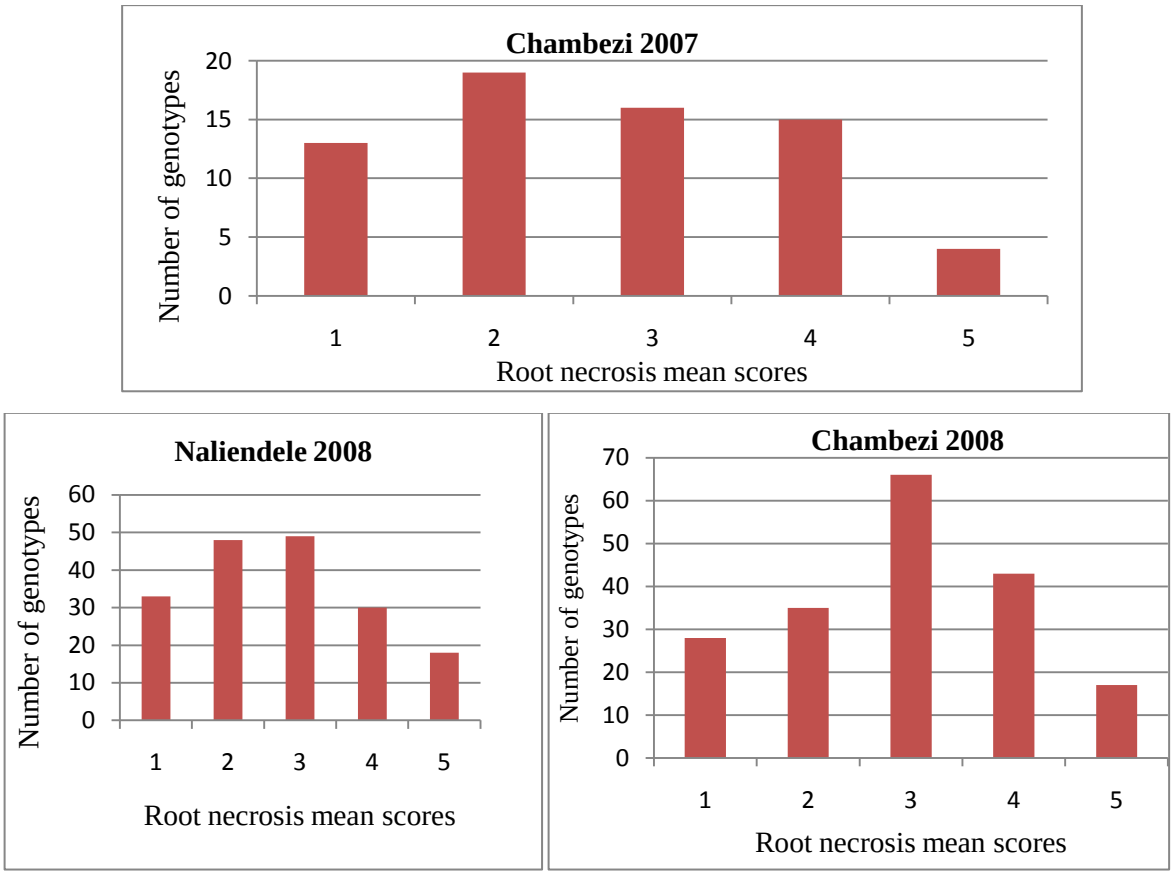


Figure 6.1 Frequency distribution of CBSD phenotypic data from two locations (Chambezi 2008 and Naliendele 2008) based on CBSD root necrosis mean scores of each of the F_1 genotypes of the Namikonga x Albert mapping population.

The frequency distribution of CBSD root necrosis reaction of individuals produced from self pollination of the resistant parent, Namikonga (Namikonga x Namikonga population) produced totally different distributions which were skewed from normality (Figure 6.2). The distribution of the individual CBSD root necrosis scores were skewed towards the resistant phenotype with more genotypes scoring class 1 and 2 and less classes 4 and 5. Even genotypes with intermediate CBSD root necrosis score of 3 were less. Similar frequency distribution patterns were observed at both locations. Most genotypes (S_1 progeny) from this population had a score of 1 and 2 similar to the resistant parent Namikonga. The broad sense heritability estimates for root necrosis at Chambezi and Naliendele were 94.63% and 86.57% respectively.

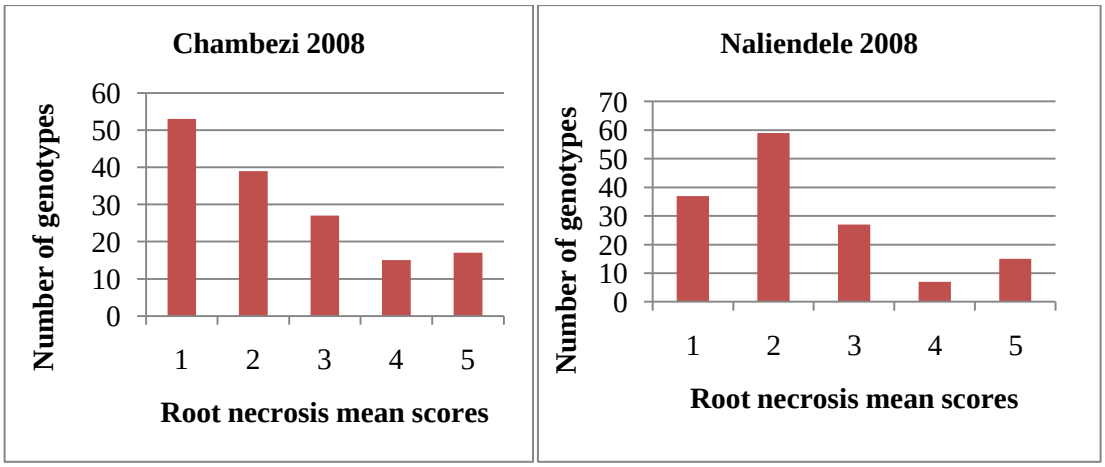


Figure 6.2 Frequency distribution of CBSD phenotypic data from three locations (Chambezi 2007, Chambezi 2008 and Naliendele 2008) based on CBSD root necrosis mean scores of each of the S_1 genotypes of the Namikonga x Namikonga mapping population.

The correlation of CBSD root necrosis mean scores obtained at Chambezi in 2008 and Naliendele in 2008 produced a correlation coefficient of r = 0.83 while CBSD root necrosis for Chambezi 2007 showed relatively weak correlation to either Chambezi 2008 (r = 0.63) or Naliendele 2008 (r = 0.61). The correlation of root necrosis mean scores obtained at Chambezi in 2008 and Naliendele in 2008 for the Namikonga x Namikonga population was r = 0.91, which suggested a stronger correlation of results from the two locations.

6.3.1 QTL linked to CBSD root necrosis

Root necrosis phenotypic data for CBSV infection in cassava roots of the F_I and S_1 populations at both Chambezi in 2007 and 2008 and Naliendele in 2008 were subjected to QTL analysis using the interval mapping procedure. QTL analysis was done separately for each location and QTL were detected using three linkage maps namely the integrated, Namikonga and Namikonga-S_1 maps. Results of QTL mapping using interval mapping and Kruskal-Wallis analysis from the three maps are presented in Table 6.3. Multiple QTL were detected at each location with more than two potential genomic regions in each of the locations. Using the integrated linkage map three QTL were detected on linkage groups C4, C16 and C18 for root necrosis at Chambezi 2007.

These QTL had LOD scores ranging from 2.59 to 2.80 with the percentage of the phenotypic variance explained ranging from 17.3% to 18.5%. Markers SSRY4, ESSRY96 and ESSRY105 were close to these QTLs. One putative QTL on linkage group C5 was also detected but with weaker effects and LOD score was 2.9 less than the LOD threshold (2.80). The QTL with the highest LOD and percentage of phenotypic variance explained was located on linkage group C4 of the integrated map (Table 6.3). This QTL was observed in Chambezi 2008 and its LOD score was 3.56 and explaining 22.9% phenotypic variance. This QTL explained 19.2% of the phenotypic variance at Naliendele 2008 and had a LOD score of 2.82. The LOD plots of the major QTL detected on linkage groups C4, C16 and C18 of the integrated map are indicated in Figure 6.3 and the names of the QTL are designated as *cbsd_rn_c4_ch08*, *cbsd_rn_c16_ch07* and *cbsd_rn-c18_ch07* respectively.

Table 6.3 Summary of QTL detected for resistance to CBSD root necrosis based on interval mapping in the Namikonga x Albert and Namikonga x Namikonga mapping populations

Trait[a]	Map	Linkage group[b]	Position (cM)	Marker[c]	Kruskal-Wallis	Interval mapping[d]		
					P-value	LOD	LOD threshold α	% variance
CBSD-RN_CHZ2007	Integrated	C5	70.81	NS149 [e]	0.005	2.61	2.80	17.40
		C5	72.23	SSRY83 [e]	0.01	2.63	2.80	17.50
		C5	77.63	SSRY67-A	0.01	2.69	2.80	17.80
		C18	10.67	SSRY4	0.05	2.59	2.40	17.30
		C18	17.40	ESSRY96	0.1	2.80	2.40	18.50
		C16	15.79	ESSRY105	ns	2.69	2.50	17.80
CBSD-RN_CHZ2008	Integrated	C4	30.48	NS945	0.01	3.56	3.00	22.90
CBSD-RN_NDL2008	Integrated	C4	30.48	NS945 [e]	0.05	2.82	3.00	19.20
CBSD-RN_CHZ2008	Namikonga	N4	15.32	SSRY195	0.05	1.76	1.60	12.10
		N4	26.38	NS667	0.01	1.90	1.60	13.00
CBSD-RN_CHZ2007	Namikonga-S_1	S4a	0.00	NS78	0.05	2.00	1.90	16.50
		S4b	2.16	ESTsSSRY66	0.05	2.06	1.90	17.00
		S4a	2.63	SSRY60-B	0.05	2.07	1.90	17.10
		S4a	2.63	SSRY160	0.05	2.07	1.90	17.10
		S4a	7.88	NS235	0.05	2.33	1.90	19.00
CBSD-RN_CHZ2008	Namikonga-S_1	S1	14.62	SSRY295	0.05	2.68	2.20	21.50
		S12a	0.00	ESSRY96	0.05	2.45	2.00	19.80
		S12a	12.20	SSRY4	0.005	2.75	2.00	22.00
		S8	5.70	NS347	0.05	2.19	2.00	17.90
		S8	7.12	SSRY19	0.05	2.21	2.00	18.10
CBSD-RN_NDL2008	Namikonga-S_1	S1	14.62	SSRY295	0.01	2.31	2.20	19.50
		S17	8.53	SSRY54	0.05	2.1	2.00	17.90
		S4a	7.88	NS235	0.01	2.06	1.90	17.60

[a] Combination of trait and location, CBSDRN = cassava brown streak disease root necrosis, CHZ2007, 2008 = Chambezi in 2007 and 2008 seasons, NDL2008 = Naliendele in 2008 season

[b] Linkage group in which QTL was detected

[c] Name of the marker most closely linked to QTL in question

[d] For interval mapping, the LOD score, LOD thresholds and percentage of phenotypic variance explained is indicated. LOD thresholds were derived by permutation analysis for each trait and linkage map. The α indicates a 5% LOD group-wide threshold for all linkage groups in each linkage map

[e] These are potential QTL since their LOD scores did not exceed the threshold but were included for of illustrative purposes, SSRY83 and NS149 were detected using BSA.

On the Namikonga map, two possible QTL were detected at Chambezi 2008 on linkage group N4. Markers SSRY195 and NS667 were the closet to these QTL and explained 12.1% and 13.0% phenotypic variance. However, the QTL close to NS667 was stronger than the one close to SSRY195 (Table 6.3 and Figure 6.4). The LOD profile of the QTL is presented in Figure 6.4 showing the *cbsd_rn_n4_chz08-QTL* position. Using the Namkonga-S_1 linkage map, three genomic regions had effects at Chambezi in 2008 and three at Naliendele in 2008. One of the QTL (*cbsd_rn_S1_chz08)* was detected on linkage group S1 at both Chambezi and Naliendele in 2008 explaining 21.5% and 19.5% of the phenotypic variance at the two locations respectively (Table 6.3) with marker SSRY295 closely linked to the QTL. Two QTL were detected on linkage groups S8 and S12a. The QTL (*cbsd_rn_chz08_qtl* on linkage S8 was close to two markers NS347 and SSRY19 and it explained 18.0% of the phenotypic variance. QTL on linkage group S12a was close to SSRY4 and explained 22.0% of the phenotypic variance. At Naliendele in 2008 another QTL was detected on linkage group S17 that was close to marker SSRY54 and it explained 17.9% of the phenotypic variance. The LOD plots of linkage group S1, S8, S12a and S17 on which QTL were detected are shown in Figure 6.5 where QTL *cbsd_rn_S1_chz08, cbsd_rn_S1_nd108, cbsd_rn_S8_chz08, cbsd_rn_S12_chz08* and *cbsd_rn_S17_ndl08* are indicated. Minor QTL were also detected on linkage groups S4a and S4b at Chambezi 2007 and one on S4a at Naliendele 2008 (Table 6.3). For all QTL the non-parametric, single marker-based Kruskal-Wallis values were significant except for QTL on linkage group C16 close to ESSRY105.

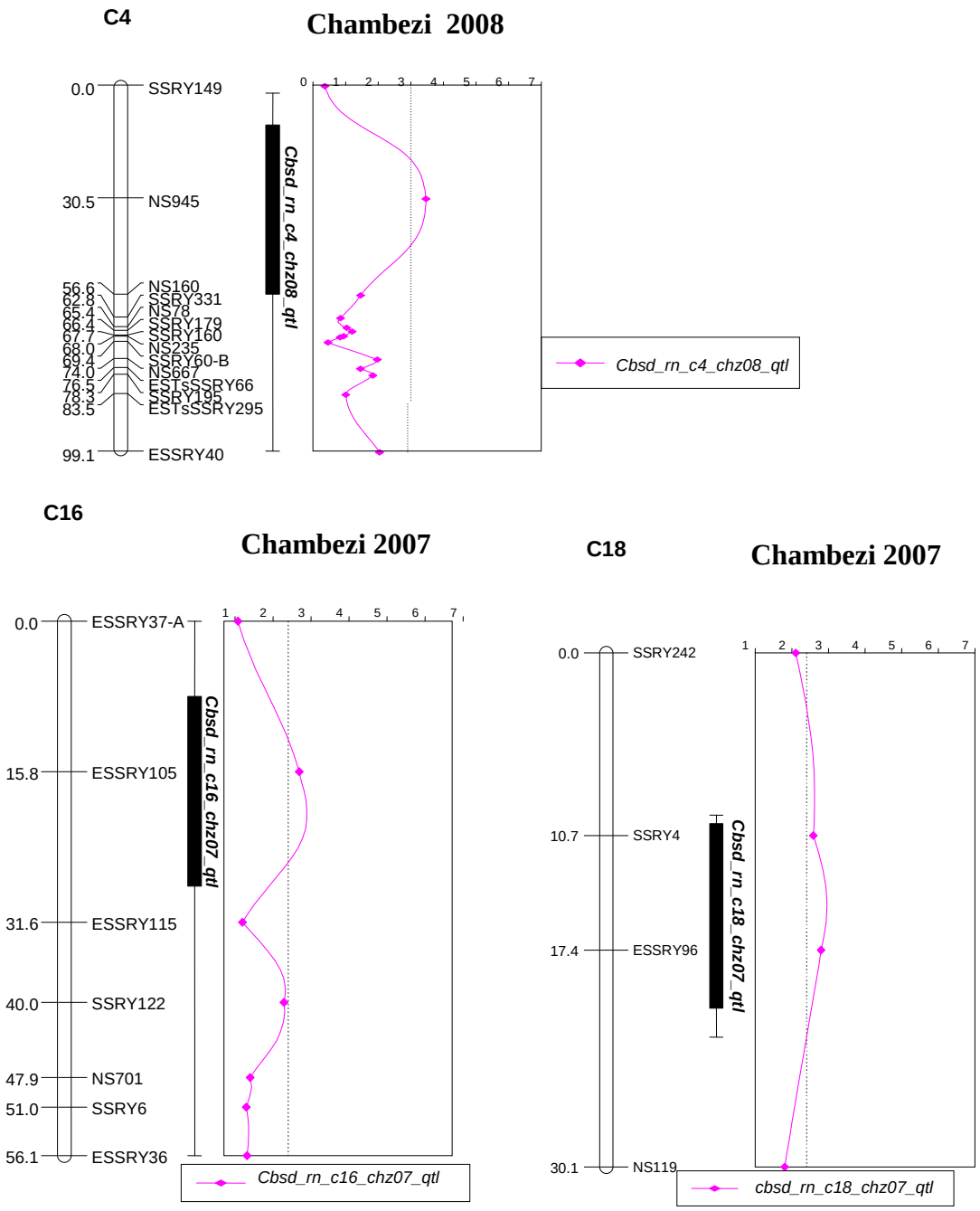


Figure 6.3 LOD score profiles of QTL for resistance against CBSD root necrosis, detected from screening of F_1 progenies of the CBSD mapping population at Chambezi in 2007 and 2008 based on mean disease scores for each genotype and interval mapping analysis. C4, C16 and C18 are linkage groups of the integrated map where QTL were detected. Left: Linkage maps of different linkage groups (LG) with genetic distances (cM) and marker names at the left and right sides of the LG. Right: QTL likelihood profile for CBSD root necrosis. LOD score and map distance are indicated on the x and y axes respectively. The bar graph in the middle of the figure represents the standard LOD (shaded region) and/or 2 LOD support intervals for the different QTL.

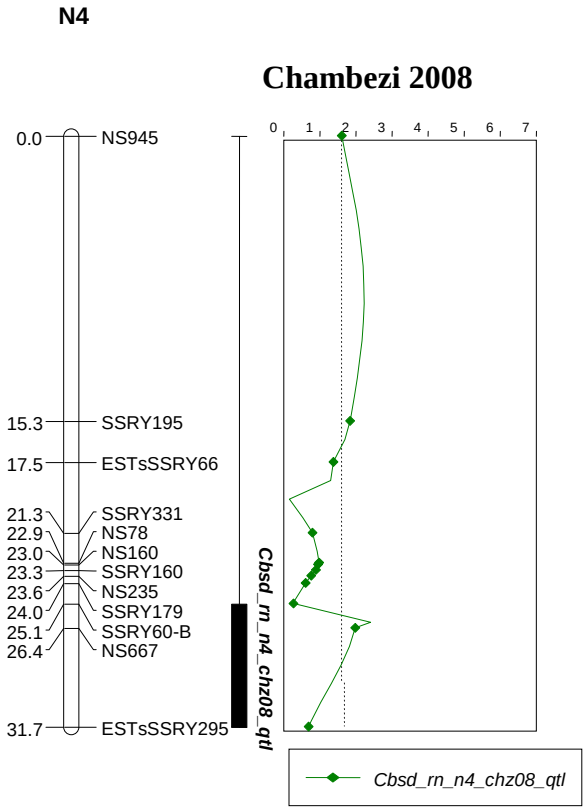
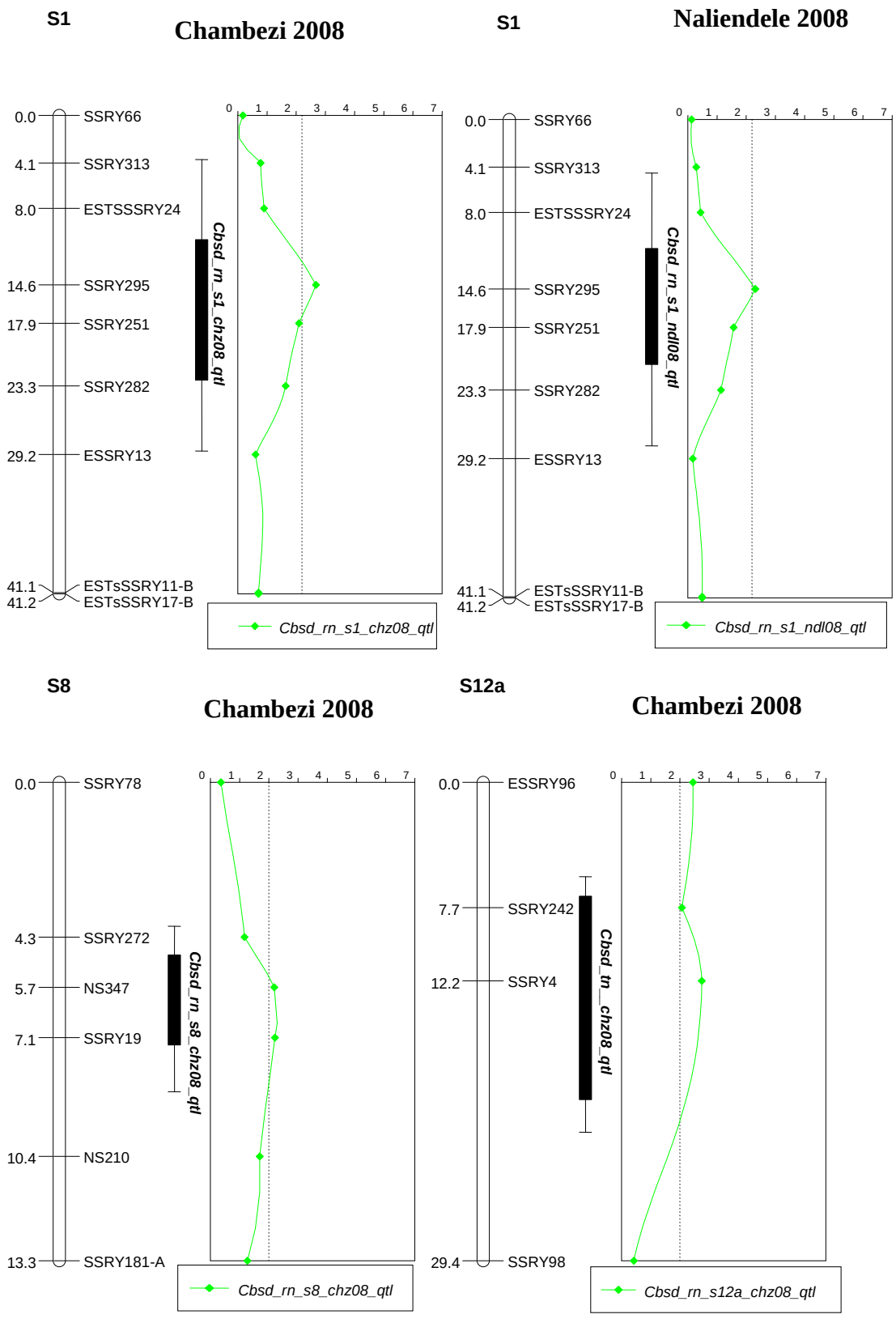


Figure 6.4 LOD score profile of QTL for resistance against CBSD root necrosis, detected from screening of F_1 progenies of the CBSD mapping population at Chambezi in 2008 based on mean disease scores for each genotype and interval mapping analysis. N4 is the linkage group of the Namikonga map where QTL was detected. Left: Linkage maps of different linkage groups (LG) with genetic distances (cM) and marker names at the left and right sides of the LG. Right: QTL likelihood profile for CBSD root necrosis. LOD score and map distance are indicated on the x and y axes respectively. The bar graph in the middle of the figure represents the standard LOD (shaded region) and/or 2 LOD support intervals for the different QTL.



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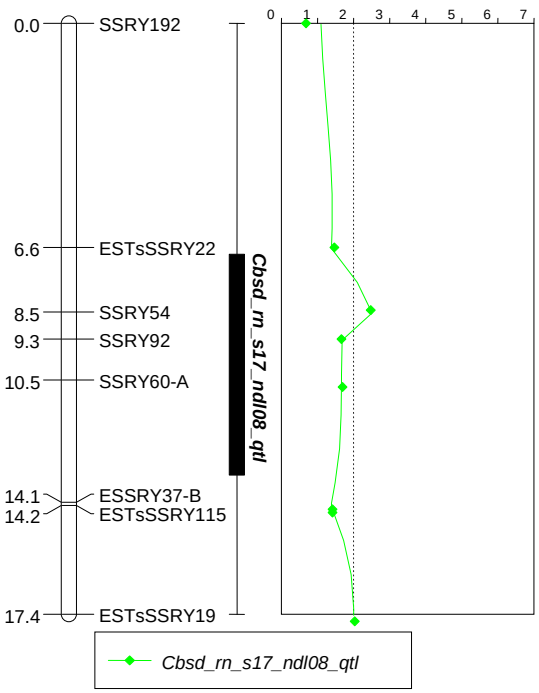

Figure 6.5 LOD score profile of QTL for resistance against CBSD root necrosis, detected from screening of S_1 progenies of the CBSD mapping population at Chambezi in 2008 based on mean disease scores for each genotype and interval mapping analysis. S1, S8 and S12a are the linkage groups of the Namikonga-S_1 map where QTL were detected. Left: Linkage maps of different linkage groups (LG) with genetic distances (cM) and marker names at the left and right sides of the LG. Right: QTL likelihood profile for CBSD root necrosis. LOD score and map distance are indicated on the x and y axes respectively. The bar graph in the middle of the figure represents the standard LOD (shaded region) and/or 2 LOD support intervals for the different QTL.

6.4 Discussion

The F_1 individuals of the CBSD mapping population screened for their reaction to CBSD root necrosis showed different levels of reaction to CBSD. Analysis of variance of phenotypic data for CBSD infection showed significant variation among genotypes in their response to CBSD infection from resistance to susceptibility. Variation in response to CBSD infection in the roots of the Namikonga x Albert mapping population was quantitative with genotypes reaction ranging from complete resistance to high susceptibility. This quantitative response to CBSD infection was confirmed by the frequency distribution of CBSD root necrosis scores which showed a normal distribution. Although the frequency distribution of CBSD necrosis in the Namikonga x Namikonga mapping population showed skewed distribution towards CBSD resistance, there was no evidence to suggest qualitative inheritance of CBSD as has been observed for CMD resistance (Akano *et al*., 2002).There were no defined classes of resistant and susceptible individuals from this frequency to suggest qualitative inheritance of CBSD root necrosis. In fact the majority of S_1 progeny showed a high level of resistance to root necrosis, which was considered as field immunity to CBSD infection compared to the resistant parent. Some of these progeny failed to become diseased at all and this showed 100% resistance. This improvement can be explained by the fact that during the selfing process, some loci formerly in the heterozygous state in the parents were concentrated and brought into the homozygous state in the S_1 progeny. If one or more QTL linked to CBSD resistance were in the heterozygous state in Namikonga, they may be brought into the homozygous state during selfing, allowing the full expression of the resistance gene. This phenomenon was also reported by Jennings (1975) for CMD resistance where immunity was found by inter-crossing of *M. glaziovii* hybrids to concentrate resistance genes which had been dispersed during backcrossing of *M. glaziovii* hybrids to cassava. The high heritability estimates observed for CBSD root necrosis indicated that phenotypic variation for this trait is contributed largely by genetic components but the question of ambiguity on accuracy of phenotyping could have inflated the heritability estimates. . This observation suggested that the mapping populations used were suitable for QTL mapping. High heritability also implies that since genotypic effects played a strong role and the trait was not influenced that much by the environment, this is the good trait to be used in a breeding programme. This high heritability indicated that cassava breeders will be able to

successfully breed for CBSD resistance to root necrosis in their cassava breeding programmes.

QTL mapping is an important approach for dissecting the genetic factors affecting traits of physiological and agronomic importance. Rapid progress in the development of DNA-based genetic linkage maps has made QTL mapping a practical and widely used approach. QTL mapping in this study has showed that resistance for CBSD infection (CBSD root necrosis) in cassava is controlled by several genes with minor effect. In the present study more than four putative QTL for CBSD root necrosis explaining 13.0-22.9% and 16.5-22% of phenotypic variation in Namikonga x Albert and Namikonga x Namikonga populations were detected respectively. These multiple number of QTL observed in response to CBSD infection and the observed continuous frequency distribution of the phenotypic data (CBSD root necrosis) confirmed the polygenic nature of CBSD resistance in cassava. The polygenic nature of CBSD resistance was first reported by Jennings (1957) who suggested that CBSD resistance is controlled by polygenic and recessive genes.

Genomic regions potentially associated with CBSD resistance in cassava were detected in the integrated, Namikonga and Namikonga-S_1 maps. No QTL were detected in the male derived linkage map (Albert map). The failure to detect QTL in the Albert map could suggest that most of the QTL detected were contributed by the resistant parent (Namikonga). This was consistent to field observations where Namikonga always exhibited field resistance against CBSD infection at both locations. It is important to note that the QTL detected on linkage groups C4, N4 and S4 of the integrated, Namikonga and Namikonga-S_1 maps respectively may be common among the maps because these linkage groups had 10 SSR markers in common (see section 5.3.5). This may suggest that regardless of which map is used in QTL analysis these QTL will still be detected. The QTL detected on linkage group C4 linked to NS945 was detected in both environments suggesting its stability across environments. The relative distance of markers from the QTL is crucial since markers tightly linked to QTL will be useful in breeding programmes because chances of recombination between the marker and QTL will be minimal thereby increasing accuracy of selection in MAS.

Generally the proportion of phenotypic variance explained by the detected QTL was low with some being less than 18%. Low LOD scores for the detected QTL were also observed from this study probably due to the use of group-wide LOD thresholds compared to the stringent genome-wide threshold. Low LOD scores were also reported by Curley *et al.* (2005) who also used group-wide LOD thresholds in the detection of QTL for resistance to gray leaf spot in ryegrass. These low values observed in this study could be due to the small (60) population size used in the study, which could had some limitation in the detection of QTL with larger effects. It is argued that as the variance explained by QTL decreases the number of progeny which must be analysed in order to detect QTL must be increased (Lander and Botstein, 1989).With a small population size, only QTL with sufficient effects are detected while those with minor or much smaller effects may go unnoticed (Paterson *et al.*, 1991). In addition, the accuracy with which a QTL can be detected increases with increased population size. With a small population size, the standard deviation associated with any QTL will be large. It is important to note that in this study originally a population of 190 F_1 individuals was developed but it was reduced to 60 individuals because during genotyping it was discovered that some individuals were outcrosses (from unintended parents) and some were as a result of self pollination. This happened because not only pollinations involving Namikonga x Albert was done but other cross combinations was being handled at the time. The current efforts under the project "*Biotechnology applications to combat cassava brown streak disease*" may help to circumvent the problem of low population size that was faced in this study. Under this project, CBSD mapping populations involving other sources of CBSD resistance are being established. In addition, more individual progenies of the Namikonga x Albert mapping population are being added for the purpose of embarking on fine mapping for CBSD resistance. The use of a bigger population size will therefore improve the detection of QTL but it will also be important that phenotyping is done in more locations over more years.

Another reason for the relatively low proportion of variance explained by detected QTL could be the fixation of major QTL in the population. As QTL with large effects are fixed in a population, it increasingly becomes possible to detect QTL with smaller effects (Paterson *et al.*, 1990). Such genes of smaller effects could prove useful for crop improvement where

QTL of large effects have been fixed in a population. Another reason for the low phenotypic variance explained could be due to accuracy of phenotyping. A quantitative measure of root necrosis would have given greater accuracy in the phenotypic data used in the QTL mapping. In addition, the fact that there are still gaps in the linkage maps used in this study for QTL analysis leading to some markers being unlinked lead to low variance of phenotype explained. For example on linkage group S12a there seems to be two QTL but the one near marker ESSRY96 is at position 0.0 cM and the LOD profile peak is therefore not very sharp. More markers need to be screened and availability of more markers will improve resolution of QTL mapping for CBSD resistance. It has been argued by Tanksley *et al*. (1992) that molecular markers uniformly distributed throughout the genome is the precondition for detection of quantitative loci associated with a trait of interest. Thus more markers are required to saturate the Namikonga-S_1 map in order to link the two arms of linkage group S12a and S12b.

There exists a relationship between the heritability of a trait and the power to detect QTL which shows that high heritability corresponds to an increase in the power to detect QTL (Kearsey and Pooni, 1996). The high heritability obtained for root necrosis in the Namikonga x Namikonga population corresponded to a higher number of QTL being detected in this population than in the Namikonga x Albert population.

6.5 Conclusions

The primary objective of generating the mapping populations was to provide a mapping resource to enable the genetic mapping of CBSD resistance in cassava. This objective has been achieved through the identification of putative genomic regions for CBSD resistance. This was the first initiative to map CBSD resistance in cassava and so far no QTL and markers have been detected elsewhere. However, these detected QTL need to be confirmed through validation studies involving independent populations constructed from the same parental genotypes or closely related genotypes used in the detection of QTL in the present study. Currently there are on-going efforts in the development of CBSD mapping populations involving Namikonga and Albert and other sources of CBSD resistance. These mapping populations that are being developed will help with the verification of the current QTL and in

detection of new genomic regions conferring resistance to CBSD. The current QTL will also benefit from the available cassava sequence information which will help to locate candidate genes. Currently efforts to sequence Namikonga and Albert that were used to generate populations for QTL mapping (M. Ferguson, personal communication) are underway. This will help to identify the actual genes behind the QTL controlling CBSD resistance on the cassava genome.

The detected QTL will have an important implication for the current breeding efforts for CBSD resistance. Markers will allow selection for resistance at seedling stage, thereby dramatically reducing the size of the population that needs to be phenotyped. It will reduce the time taken to deliver varieties and allow for pre-emptive breeding in regions where the disease is not currently present, but that are seriously threatened by the disease. During selection a cassava breeder has often been obliged to determine phenotypically the presence of a particular resistant gene in the background of other genes which may mask its detection in the population. A DNA marker associated with CBSD resistance will greatly improve selection efficiency for CBSD resistance. Such facility will eliminate the need for the search of phenotypic expression of the gene, which always involves expensive phenotyping of many individuals in order to detect it. In this study the following microsatellite markers NS945, NS347, NS667, SSRY295, SSRY4 and ESSRY96 were detected as putative markers associated with QTL for CBSD field resistance.

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CHAPTER 7

General conclusions and recommendations

Cassava is an important crop which provides a cheap source of carbohydrate and is the staple food crop for more than 800 million people around the world. In Africa more than 600 million people depend on cassava as a source of calories. In Tanzania cassava is the second most important food crop after maize but in many areas of the country it is the staple food and most important food security crop. However, the production of cassava in Tanzania and in the great Lakes region is affected by CBSD which causes high yield losses of up to 100% in susceptible varieties. In some areas where cassava is a staple food and farmers grow and depend on a single or few varieties, CBSD has caused food insecurities. An example is Ukerewe Island in Lake Victoria in Tanzania where cassava is a staple food. In this region Lwakitangaza and Lwabakanga were the most common varieties grown by farmers but new outbreaks of CBSD in the Lake region has wiped out all varieties leading to a total loss of the crop. New outbreaks of CBSD in previously unaffected areas is a potential threat to cassava production and food security in areas like west Africa where cassava is important and they are yet to be affected by CBSD. In these areas pre-emptive CBSD resistant breeding is important to provide varieties that will offset the impact of the disease. Biotechnological approaches may be one of the quickest and efficient ways to address this problem. Molecular markers linked to traits of interest have been one of the effective ways to introgress genes controlling important traits into new crop varieties because they increase the efficiency of selection. No marker associated with CBSD resistance have been identified yet and cassava breeding for CBSD resistance can also benefit from the use of molecular markers. It was this realisation which led to the initiation of the present study with the goal of improving cassava breeding through the use of molecular markers linked to CBSD resistance to aid in selection for CBSD resistant varieties.

The present study was the first attempt to search for markers associated with CBSD resistance, an important virus disease threatening cassava production in the east, central and southern Africa. The study also aimed to understand the genetics of disease resistance. In

order to elucidate the importance of the disease (host-pathogen interaction), available cassava germplasm from Tanzania were utilised. A collection of resistant and susceptible genotypes was selected from a cassava germplasm collection in Tanzania and genotyped using 36 SSR markers with the objective of understanding their genetic structure in relation to CBSD. Diversity analysis of the germplasm was important to understand the genetic distance among cassava genotypes in order to select appropriate parents for genetic crosses to produce a population segregating for CBSD. Findings indicated that cassava germplasm from Tanzania is genetically diverse which implies that cassava breeders have a broad genetic base to exploit for cassava breeding. For CBSD resistance breeding this finding gave a genetic resource to help breeders to make informed decisions in selecting parents to use for breeding resistant varieties. In the present study results from fingerprinting of the germplasm facilitated selection of parents for genetic crosses to attain maximum CBSD segregation among the F_1 progeny. The choice of SSR markers was appropriate and helpful in selecting parents because the technique was able to separate parental genotypes into distinct groups. The diversity analysis results lead to the selection of Namikonga and Kalolo as resistant parents and Kibaha and Albert as susceptible parents for genetic analysis. The higher the polymorphism between parents for a particular trait the higher is the chance of obtaining a highly segregating population for genotyping, genetic linkage analysis and detection of QTL. Fingerprinting was therefore important in order to obtain two diverse and contrasting parents for development of the CBSD mapping population. Apart from disease reaction and genetic distance, parents were selected on the basis of their flowering ability. Flowering is an important trait in cassava because it is influenced by the environment and for successive cassava breeding careful selection of parents and synchronisation of flowering is important. Although a number of CBSD resistant cassava varieties are available, flowering has become a problem in some of them making it difficult to transfer this important trait to most farmer preferred varieties. An example is a variety Nanchinyaya, which is resistant to CBSD but unfortunately has a sterile male flower which makes it impossible to transfer its resistance to other varieties.

Diallel crosses and combining ability analyses were done in order to understand the genetic control of CBSD resistance in cassava. This analysis was important because it was not well

known whether CBSD resistance is controlled by major gene or many genes whose gene actions were unknown. The analysis of variance, GCA:SCA ratio and percentage contribution of GCA and SCA effects to the total sum of squares of genotypes indicated that GCA effects were larger than SCA effects for CBSD roots necrosis, CBSD severity in the shoot at three, six and nine MAP, CMD severity at three, six and nine MAP and HI indicating the importance of additive gene effects in controlling these traits. For improvement of CBSD resistance and other traits controlled by additive gene effects recurrent mass selection will be an appropriate method to use in cassava breeding.

Low disease scores for disease resistance including CBSD are desirable and therefore low or negative GCA values as well as SCA signify contribution of additive and non-additive effects towards disease resistance. Parents with low and negative GCA effects as well as a combination of parents with low and negative SCA effects were identified for CBSD shoot infection, root necrosis and CMD. Parents like Namikonga and Kalolo had low and negative GCA effects indicating significantly higher disease resistance than Kibaha and Albert which were susceptible to CBSD. Kalolo x Kibaha, Kalolo x Albert and Namikonga x Albert were crosses with low and negative SCA values compared to others, indicating that SCA was important in predicting progeny's performance towards CBSD resistance. Namikonga x Kibaha and Kalolo x Namikonga crosses had low and negative SCA for CMD resistance. High heritability estimates observed for CBSD resistance also indicated that CBSD is a highly heritable polygenic trait although the subjective nature of phenotyping methods used, as discussed above, could have caused high heritability estimates. The high heritability values obtained implies that it is easy to transfer CBSD resistance to other varieties. The observed polygenic nature of CBSD means that fixing the genes/QTL in one variety will be easier using a biotechnological approach than using conventional methods. It will take more effort and time to pyramid these genes into one variety using conventional breeding compared to use of molecular markers linked to these QTL.

One of the major aims of this study was to search for molecular markers linked to CBSD resistance. As a prerequisite to QTL analysis, a CBSD mapping population was developed, genotyped using SSR markers and linkage analysis lead to production of four linkage maps,

Namikonga (Female), Albert (Male), integrated (female and male) and Namikonga-S_1. These maps spanned a distance of 779.48 cM, 854.26 cM, 995.46 cM and 524.58 cM comprising of 131, 123, 174 and 124 mapped SSR markers respectively. The genetic distances and number of markers mapped on these maps were comparable to published maps. The genetic linkage maps produced using 220 polymorphic SSR markers indicated that on some linkage groups markers were spaced at big map distances, suggesting that recombination events were not uniformity distributed throughout the cassava genome as was reported elsewhere. This means that more markers need to be screened and added to the maps to reduce the map distance between markers in some of these linkage groups. One problem which might have contributed to the big map distance between markers in some of these linkage groups was the low population size (60 individuals) used during linkage mapping. This problem originated during genetic crosses and was caused by out-crossing (due to pollen from unintended parents) and seed mixing during harvest due to handling of many cross combinations simultaneously. These problems were discovered during genotyping of the original 190 individuals of the mapping population. Based on the genotyping results, the population was reduced to 60 individuals representing individuals from the true Namikonga x Albert cross. If an inexperienced breeder is handling the full-sib genetic crosses in cassava, it is recommended that pollinations and seed harvest should be carefully undertaken and fewer cross combinations should be handled at a time. Furthermore, whenever resources permit, before field evaluation of any population resulting from full-sib crosses, offspring from the crosses should be genotyped with few markers to determine true crosses and/or self pollinated progeny. This will avoid unnecessary genotyping and phenotyping of self-pollinated progeny.

This small population size could also have affected the detection of QTL with stronger effects because only QTL with relatively low effects were detected. With the exception of QTL which were detected on linkage group 4 of the female parent, Namikonga (N4), linked to NS945, the group-wide instead of genome-wide LOD threshold was used for the detection of QTL. The LOD score of most of the detected QTL were below the genome-wide LOD threshold which necessitated use of group-wide LOD threshold. Use of group-wide LOD threshold has been reported elsewhere. Another problem that might have contributed towards

the detection of QTL with low LOD values could have been lack of appropriate phenotyping methods for CBSD resistance. As much as the genotypic data need to be of high quality, phenotypic data even more so need to be of good quality in order to accurately detect QTL. The current method for CBSD screening is based on an ordinal disease scoring scale of 1-5, which most of the times may be subjective leading to phenotypic data which may not be as quantitative as required for QTL analysis. More objective screening approaches like imaging of necrotic areas of the root affected by CBSD or scoring of each of the individual roots in a plot and finding the average, may be more appropriate than assigning a CBSD severity score per plot for each genotype, an approach which is currently in use and employed in this study. In this way quantitative phenotypic data will be obtained leading to improved QTL detection. The heterozygous nature of cassava produces heterogeneous F_1 populations which make detection of QTL more complex and less efficient compared to inbred lines. Another general problem encountered in cassava breeding is limited availability of planting materials which limits quick multi-location screening for CBSD. In this study embryo rescue coupled with *in vitro* multiplication was used to bulk planting materials for CBSD screening in two locations. Although plantlets were affected by flooding, the intention was to obtain enough plants for each of the F_1 progeny as soon as possible. However, embryo rescue is expensive and unaffordable for most national research systems in the developing countries. It is recommended that cassava mini cuttings from shoots of young plants from the age of 3-6 MAP should be used to improve the accelerated availability of planting materials for phenotyping in more than one location as quickly as possible. Currently, it takes one year to raise seedlings and another year of multiplication of planting materials before phenotyping is done in the third year. This delays the whole process of obtaining data as quickly as possible and the use of mini cuttings can therefore reduce the time by one year in the most effective and affordable way.

Despite these problems, putative QTL for CBSD were found linked to SSRY and ESSR markers NS945, NS347, NS667, SSRY295, SSRY4 and ESSRY96. QTL were detected on ten linkage groups (LGs) of the different linkage maps but important ones were LG4 of the female map (N4); LG4, LG16 and LG18 of the integrated map (C4, C16 and C18) and LG1, LG8, LG12 and LG17 of the Namikonga-S_1 map (S1, S8, S12 and S17). The detected

markers linked to CBSD resistance need to be tested in other populations apart from the one used in this study over years and locations. The present initiatives in development of CBSD mapping populations from different sources will provide resources to test these candidate markers. The different markers on different linkage groups found to be associated with CBSD resistance in the present population may indicate that several genes/QTL are involved in the same population. This is also supported by the diallel analysis results which detected large contribution of additive gene effects in the control of CBSD resistance.

The phenotypic distribution of CBSD severity scores of F_1 individuals from the Namikonga x Albert mapping population showed a normal distribution, suggesting that CBSD resistance is controlled by polygenes. This agreed with findings from diallel analysis and QTL data which showed that CBSD is controlled by additive gene effects. Therefore, the use of molecular markers linked to CBSD resistance will be useful in improving selection efficiency for this trait. The discovered markers linked to CBSD resistance will be useful to cassava breeders in Tanzania and other countries affected by CBSD for MAS. It will be even more important for pre-emptive breeding in cassava growing areas where CBSD is not yet a problem but is a potential threat to cassava production, for example west Africa where cassava is important and CBSD pose a potential threat. The present study is the first to report the detection of molecular markers linked to CBSD resistance and has confirmed that CBSD resistance show polygenic inheritance in an additive way. This study has contributed important genetic information towards improving CBSD resistance breeding for cassava improvement in areas currently affected by CBSD and where the disease is a potential threat to cassava production.

SUMMARY

Cassava brown streak disease (CBSD) is an important biotic constraint to cassava production in cassava growing areas of east, central and southern Africa. A study was initiated to study the genetics of CBSD resistance and search for molecular markers associated with disease resistance. Two resistant and two susceptible cassava parents were selected based on their flowering ability, genetic distance and phenotypic reaction to CBSD. They were crossed in a half diallel to produce six families and F_1 progeny plus parents were screened for their reaction to CBSD and evaluated for yield traits at two locations. Results indicated that general combining ability (GCA) was higher than specific combining ability (SCA) for CBSD root necrosis, CBSD severity in the shoot at three, six and nine months after planting (MAP), cassava mosaic disease (CMD) severity at three, six and nine MAP and harvest index indicating the importance of additive gene action in controlling CBSD resistance. Results indicated that CBSD resistance was quantitatively controlled by polygenic genes. Higher SCA than GCA was detected for number of roots per plant, fresh root weight per plant and fresh shoot weight per plant indicating the importance of non-additive gene effects in controlling yield traits.

The Namikonga x Albert cross was used to generate a segregating mapping population comprising of 190 F_1's which was planted and screened for CBSD reaction at two locations and genotyped using 605 SSR markers. A total of 220 polymorphic SSR markers, tested on 60 F_1 and 51 S_1, were finally used for linkage analysis and four genetic linkage maps were produced. The maps produced included integrated (male and female), Namikonga (female), Albert (male) and Namikonga-S_1maps which spanned a distance of 995.46cM, 779.48 cM, 854.26 cM and 524.58 cM consisting of 174, 131,123 and 124 mapped SSR markers respectively. Mean map distance between markers ranged from 4.23 cM in Namikonga-S_1 to 6.94 cM in Albert map while average map length per linkage group was 43.23 cM, 45.85 cM, 47.46 cM and 30.86 cM for integrated, Namikonga, Albert and Namikonga-S_1 maps respectively.

The linkage maps were used for QTL analysis and three putative QTL were detected on the integrated map on linkage groups C4, C16 and C18 linked to NS945, ESSRY105 and two

flanking markers SSRY4 and ESSRY96 respectively. One QTL linked to NS667 was detected on Namikonga map on linkage group N4 while four putative QTL were detected on the Namikonga-S_1 map on linkage groups S1 (at Chambezi and Naliendele in 2008), S8 and S12a at Chambezi 2008 and on S17 at Naliendele 2008. These QTL were respectively found to be linked to SSRY295, NS347 and SSRY4. These findings will have an implication in breeding for CBSD resistance. The observed relative importance of additive gene effects will mean that a mass recurrent selection will be the most efficient approach for CBSD resistance breeding in cassava. Markers associated with CBSD resistance will greatly improve selection efficiency for CBSD resistance in which selection for resistant genotypes can efficiently be done at seedling stage.

Key words: Cassava, *Manihot esculenta* Crantz, cassava brown streak disease, genetic diversity, diallel analysis, general combining ability, specific combining ability, linkage mapping, QTL analysis

OPSOMMING

Cassava bruin streep siekte (CBSD) is ‘n belangrike biotiese stremming vir cassava produksie in cassava verbouingsareas van oos, sentraal en suidelike Afrika. Hierdie studie is onderneem om die genetika van CBSD weerstand te bestudeer en om molekulêre merkers geassosieer met siekteweerstand op te spoor. Twee weerstandbiedende en twee vatbare cassava ouers is op grond van hul blomvermoë, genetiese afstande en fenotipiese reaksie tot CBSD gekies. Half-dialleel kruisings is gedoen om ses families te produseer. Die F_1 nageslag asook die ouers se CBSD weerstandsreaksies asook opbrengseienskappe op twee lokaliteite is getoets. Resultate het aangetoon dat algemene kombineervermoë (GCA) ‘n groter rol as spesifieke kombineervermoë (SCA) vir CBSD wortelnekrose, CBSD felheid in die lote drie, ses en nege maande na aanplanting (MAP), cassava mosaïek siekte (CMD) felheid in die lote drie, ses en nege MAP en oesindeks gespeel het, wat die belangrikheid van additiewe geen aksie vir die beheer van CBSD weerstand aangetoon het. Resultate het aangetoon dat CBSD kwantitatief deur poligene beheer word. Hoër SCA as GCA is vir aantal wortels per plant, vars wortelmassa per plant en vars lootmassa per plant waargeneem, wat op die belangrikheid van nie-additiewe effekte vir die beheer van opbrengseienskappe gedui het.

Die Namikonga x Albert kruising is gebruik om ‘n segregerende karteringspopulasie bestaande uit 190 F_1’s te ontwikkel. Hierdie populasie is geplant en in twee lokaliteite vir CBSD weerstand geëvalueer en hul genotipes is met 605 mikrosatelliet merkers bepaal. ‘n Totaal van 220 polimorfiese mikrosatelliet merkers, getoets op 60 F_1 en 51 S_1 individue, is vir koppelingsanalises gebruik en vier genetiese koppelingskaarte is saamgestel. Hierdie kaarte het uit ‘n geïntegreerde (manlik en vroulik), Namikonga (vroulik), Albert (manlik) en Namikonga-S_1 kaart bestaan wat onderskeidelik ‘n afstand van 995.46 cM, 779.48 cM, 854.26 cM en 524.58 cM beslaan het en uit 174, 131, 123 en 124 gekarteerde mikrosatelliet merkers bestaan het. Die gemiddelde kaartsafstand tussen merkers het van 4.23 cM in die Namikonga-S_1 kaart tot 6.94 cM in die Albert kaart gewissel terwyl die gemiddelde kaartafstand per koppelingsgroep onderskeidelik 43.23 cM, 45.85 cM, 47.46 cM en 30.86 cM vir die geïntegreerde, Namikonga, Albert en Namikonga-S_1 kaarte was.

Hierdie koppelingskaarte is vir kwantitatiewe eienskap lokus (QTL) analises gebruik. Drie moontlike QTL is op die geïntegreerde kaart op koppelingsgroepe C4, C16 en C18, onderskeidelik gekoppel aan NS945, ESSRY105 en twee aangrensende merkers SSRY14 en ESSRY96, opgespoor. Een QTL gekoppel aan NS667 is op koppelingsgroep N4 van die Namikonga kaart gekarteer terwyl vier moontlike QTL op die Namikonga-S_1 kaart op koppelingsgroepe S1 (vir Chambezi en Naliendele in 2008), S8 en S12a vir Chambezi 2008 en op S17 vir Naliendele 2008 gekarteer is. Hierdie QTL was onderskeidelik aan SSRY295, NS347 en SSRY4 gekoppel. Bevindinge van hierdie studie sal ‘n impak hê op teling vir CBSD weerstand. Die waargenome relatiewe belangrikheid van additiewe geen effekte beteken dat massa herhalende seleksie die effektiefste benadering vir CBSD weerstandsteling in cassava sal wees. Merkers geassosieer met CBSD weerstand sal die effektiwiteit van seleksie vir CBSD weerstand baie verbeter deurdat seleksie vir weerstandbiedende kultivars reeds op saailinge gedoen sal kan word.

Sleutelwoorde: Maniok, *Manihot esculenta* Crantz, cassava bruin streep siekte, genetiese diversiteit, dialleel analise, algemene kombineervermoë, spesifieke kombineervermoë, koppelingskaarte, QTL analise

Appendix I Features of fluorescently labelled cassava loci screened during parental survey; their primer pairs, type of repeat motif and fragment sizes in bp in the two parents (Namikonga and Albert)

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
ESSR1	TCGCAGCAAACCTCTCCCCA	TCTGCTCAGCTGCCCAGCCA	(AG)11	186	186
ESSR2	AAAACACCAGCAAAATTGCACAGGAC	TGGAAATGCTGAAAGTGAACGCTTGA	(AC)12	186	186
ESSR3	CAGCGGCTGCTCCCATTCCT	GCAACAGGTGCCCGATGTGTAGC	(CT)11	223	213, 223
ESSR4	GGTCACGTCAAGTACCTGTCAAGGCA	TCTCTCACAGGTCGCCCAACACA	(CT)8	181, 187	181, 187
ESSR5	ACCCAATGGAGCCGTAACAAATTCA	TGCCACAACGCCTGTGTAGAATCG	(TA)8	190	190, 205
ESSR6	GGAACGATTTTCTCAACCAAAATGCGA	TCCACCATTTCATTCATCAAGGCCA	(CT)8	NA	NA
ESSR7	GCTCAATCAGGTGCCACAGCG	AGCACTCTAATCATGCAACTCCTTCGG	(CT)11	181	181
ESSR8	TCGACTTGTTTTCAAGTGCATCCCA	TTCTGCCGAGCACGAATATTACCCC	(CT)13	166, 176	169, 179
ESSR9	TCCCAATGTAACCAGCACCACCG	CTCTAGCCTGGAGCTCGTGACGACATT	(TC)9	176	176
ESSRI0	TCCAGACGCTGCATTTGCCA	AGCCACCACACACACCAAACGC	(CT)11	NA	NA
ESSR11	GGAGGGTGGCTGTGAATCCCG	GAGGAGGTTTGGGACCCTCCCTG	(CT)8	187	187
ESSR12	GCATCGTGACTTTTCTTGATAGGCCAG	TGTCAATACACTGTCAGACACGTTCGC	(TA)8	NA	NA
ESSR13	CCCTTCTCTGAGGCCAGTCCCA	TCAGAATTCGAGCTGAGAGTGTTGAGG	(TC)8	184, 188	178, 184
ESSR14	TGCTCAGAGAATCCCAAAGGCACA	ATGGGGTTCTCACAGTGACGGTTCC	(AT)12	199	199
ESSR15	GCTTTCTCTTCAAGCCAAAAGCGTCC	GTCAGCCGTCATCCGGCCAT	(TC)8	186, 189	186
ESSR16	GGCACCTGGGGACCTGTAATCAGTC	TTGCCAGCATTGATACTGCACAAGC	(AGC)8	NA	NA
ESSR17	CCACTCGCATGCTCCTCAAGCA	CTATTGGATGTGGGCTGGCGCT	(TAT)6	183	180, 183
ESSR18	CAAGGGTGACGTCCACTAAATCGACA	CACCGGATCCCACGTGCAAGA	(ATT)5	194	194
ESSR19	CAAATGGACAACATCAACGATCACAGG	ACGGTAGTGCCCTTGAGGTTGGG	(TTA)8	184, 190	190
ESSR20	AGCAAGCACATGCCATTCTTTTCTTTC	TGTCAATTTGGGTCCAATTGCAACAGT	(ATT)5	197	197
ESSR21	CACAAATGGTGAAGACACAGAAAACGC	TACAGGATTGACGTTGCTGTTGCATGT	(AAT)6	183	183
ESSR22	TGCATAATGAGGTCAAATGTTTGGGG	TTGGAATGCACTGAAACTCATTTGGGA	(TTAT)5	203	203
ESSR23	TGGAGGGGAAGGAGAGATTTTTCAGA	GGTTGATGGGAATGTTGTTTGGCTC	(AT)6	214	214
ESSR24	GGTTTCTGAGAGAAAGCATGCGCAGA	GGGACGCGTGAATTCTTGCTTTTG	(TA)6	174, 176	176
ESSR25	GGTCAGGCAAAGCAATTGGGC	TCCTCGTCTTCAAAACCCACAAGGC	(ATA)6	180, 184	180, 184
ESSR26	TTTCTGCTGCTTTCGAAGCTCTCTGTT	GCGTGGAAGCAAGGCAATACTGAAT	(ATA)10	190, 206	194, 203

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
ESSR27	TTCTGGACGTCCTCTTCAGAGCCA	AGTTGCTGGGTCCTGCGTTTAAGG	(AT)7	204	204, 206
ESSR28	GAGCGAGCGAGACCGAGCGA	TTCCTATTGACCGACATCCCTCTCCC	(CT)6	170	170
ESSR29	GCCGGTGTGAGTCGCGAGAA	TTCGCGTCTTCAATCCGTAGCCA	(TC)6	203	203
ESSR30	CCTTCGAAGTACAACCAAAGCCATGA	CGTGTTGTGCATCTGGGCCG	(TG)6	193	193
ESSR31	TGCCTCTTGGCGGGGGTCTT	CGGCCGCTGCATCAGAGCTT	(TC)6	NA	NA
ESSR32	TGGATCATCGGAGACCCCTCG	GGGGAAATCACAAACTCCAAGCCA	(AG)6	202	202
ESSR33	GGAATATCAACGGTGATGCCGGA	CCGCAAGCAACGGCCAAGA	(CT)7	215, 217	215
ESSR34	ACTGCAGCCGCTCCTCCCAA	TCACAGGCTGGAGTTTATGAAGGCG	(TA)7	206, 208	206, 208
ESSR35	TGCTCCCGGTAACCAGTGGTGG	GGGTCCTGAGCCACCTGCATC	(TA)7	208, 210	208, 212
ESSR36	TGAGACAACACAGGTGGATTGCAGC	ACGATGTTTGTCTTTGAGGATTGGTGG	(CT)7	172, 176	176
ESSR37	TGCATCCCAAGCAAGAGAGGAGAA	TGGAGGCAGGGCCTTCTTTGC	(AT)6	178	174, 180
ESSR38	AAACTCATGCCCCTCGTGAAAACAA	TGATCATAAAGCTGGAGCAGAGGCTGA	(AT)6	194, 196	194, 196
ESSR39	GGAGGCTCCACTCCCCACTGA	GTTGCAGCAAAGCTTGCTATCCAATCA	(AG)6	186, 188	186, 188
ESSR40	ATCGCATGCCTCTGCGTGGA	GGGAGTACCTCGAGTACAACGAAGCAA	(TC)6	178	176, 178
ESSR41	TCCAATTCCATTTTCATCACCAGCA	TCCGCGAAAACAATTTGGCACA	(GA)6	200	200
ESSR42	GCCTTCGTCAGGCAAGGAGCA	TCATTCTTTCCCTGTTTTGCCTTCG	(TA)7	193	193
ESSR43	TCACGAGCTGACACGTTGCCG	TTTGCTCACCAGCACCAGCGA	(CT)6	189	189
ESSR44	GAGAAAACGCAATTCCGAGCCAA	TTCCTCGTTAACGCTGGCCTTGTG	(AT)6	204	204, 206
ESSR45	TCGCCTTCCTCTTCTTTCTGTGTCCA	GTCTCAGTCCCTGCCAGACCCG	(TC)6	177	177
ESSR46	GCCTTGGTTCTAAGAGGGTGGGC	TCCGTCAACTCTCTCACTCTGCGTTG	(AG)7	197	197
ESSR47	TGCAGAGTCCATGGTTTGGCGA	TTCGCTTCTTGACATCTTCCGCC	(GA)6	181	181
ESSR48	CACGGAAAGCTTGGTGTTTTTGGC	TCTCCGCCCTTCCCCCATCT	(TC)6	175	175
ESSR49	GCTGTGAAAATGAACTGCATGCCAC	CTGGCACAAAGTGCAGTTGGAGTTG	(AG)7	NA	NA
ESSR50	GGCCAAAATCTTTGCAACGTGGT	ACGCCAACTAGCCTCTGATTTCTCACA	(AG)6	200	200
ESSR51	CACTTGTTCCTGTGCTTAACCCACCTT	AGATGGAGAGGCAATGCTGGGC	(AT)6	196	196
ESSR52	CCCCAATTGCAGCAAGGCGT	ATGGGTGTCCTTGTGCCTACTGGA	(AG)6	NA	NA
ESSR53	TCGCCTATGCCGACGGAGGA	TCCCACTTCCCAGTCAACGCC	(TC)7	200, 216	200, 216
ESSR54	TGGTAAAACTCATGCCCCTCGTGA	TGATCATAAAGCTGGAGCAAAGGCTG	(AT)6	176	176
ESSR55	TGGTGTAAGCGGCTCACCATTCTC	AGGGTTGGAGGCTGAGCTGGC	(AGC)6	NA	NA

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
ESSR56	GGAGGTACCGGCTTGAAGGGGA	TTGGGTGACGATGACGCCGA	(GCC)5	183	183
ESSR57	GGCACATCATCTTCTCCAATCATAGCC	TCCATGAGCAGTGAAGGAGCTTCAAGT	(AAC)5	212	212
ESSR58	GGATGATCACCATCTTGCAAGCCTAA	TGGATCTGATGAGGAAGGGGATCA	(TGA)5	188	188
ESSR59	CCAGTGATGATTGGGCTTCATGGTC	GGAATGGTTGAAACGGGAAAGCC	(TCT)7	175	175
ESSR60	CCCACTAAGCTGATTGGTTGCTGCT	CGTTTCCCTTGCGCTCTCCG	(AGC)5	175	175
ESSR61	CAAGCAGCCCTGCAATCCTCTTTC	CAGCAGCAGCAACAACATCCGC	(GCA)5	219	219
ESSR62	TGGGTCGACACCAAATCTACCATTCA	AGCATGAGCGCATGTCTGTGAGC	(AGA)6	198	198
ESSR63	AAGGTCATGATGAACGACTGGAGCA	CCACCAACATCCTCATCATGGAAGAC	(CAT)5	NA	NA
ESSR64	TGCACCACTCGCTCGTTCACC	GCTACGGGGGATTACACGACCTTTG	(TGC)5	NA	NA
ESSR65	TGATCGCCGCTTCGACGACTT	GATGGAGCCGCTGACCTCCG	(CTC)5	215	215
ESSR66	GACGGAGCAAATTATCATCATCGAACC	CGTCTCTCCGGTGACGTTGTCG	(TCT)10	182, 184	182, 184
ESSR67	GGTGCATCTGGCGGAATGTCAA	TATGATCCAGCGCCCAGCGG	(AGA)5	NA	NA
ESSR68	GCTGGAGGGTATGCTGCAGTGG	CGCCGCCCTCGCTTAGCC	(CTT)7	188	188
ESSR69	TGGGGACAAGAGGACCAAATCCC	TGGAGGCTGTAATGGCTTGCTGG	(TGA)5	NA	NA
ESSR70	TGGTCTTCAGTCAGGGGAACAGGA	TTGACAGGCCCGCAGCTGGT	(CCA)7	195	195
ESSR71	TGCTCCGCCAATTCAAGCCG	GGTGGAGGCGGAGGGCAGTA	(CCA)5	207, 210	210
ESSR72	TCATCACCTCTCGCAAAACAAAAGGC	GCAACATCCTGGCTTTAGGAGTGTCC	(GAA)5	190, 214	190, 214
ESSR73	ACGATCGAGCTGCAACCCCAA	CGGTGTAGAAGTTGAAGCCGTTGAACC	(TCA)5	191	191
ESSR74	TGGCAAAGACATGGGCGGTC	TGCGAGGCGTGTTACAGCTCTAATTG	(GGT)6	180, 194	180
ESSR75	TTGCAGCTTCCAAATGATGAGTGTTCA	CATCTCTTGCTCATCGAAGTCAAGGC	(ATG)5	201, 203	179
ESSR76	ACAGCTGGTCCTTTGCTCCGTTG	TGAAGCGCAGTGATTTTGGCTCC	(TCT)7	177, 180	177, 192
ESSR77	GAGCGTCTCTCCGGTGACGTTG	CGGAGCAAATTATCATCATCGAACCA	(TCT)10	173, 182	173, 182
ESSR78	TTCCTTCAATTCTTGGCCATCCCC	GCATGTTGAGCCTGGGCTTGC	(GCA)5	180	180
ESSR79	CTACCTCAAGGACAAAAAGTGAGGCCA	CGGGGAACTTCTCAATGTTTTTCTTGG	(GCA)5	192	192
ESSR80	CATCAATACCGGCGCTAATTCTTCCA	TCCACTACAGACGCCTCTCCGTTC	(GAT)5	190	190
ESSR81	CTGCTTCCTCCGTCTTCCAAACGC	ATCGGCGACGCTGAGATTCCC	(AGA)5	174	174
ESSR82	TGAAGAGAATGAGGAGGGTGGATTGA	TCCTCCTTAGGTGCATCCTTCTTTGG	(TGA)5	147, 157	147, 157
ESSR83	TCCTGGAGTTTGCTCCATTTGTTTGA	CCATGCCATGTCGATAACGATGC	(TTC)5	76	76
ESSR84	TGAAGGGAAGGCTCAAATGAAGAGCA	CACTCCTCTCTGCACCACCACCC	(GGA)5	191	191

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
ESSR85	AAGTCACCACCCCCACCCCC	CACTGCATCAACGTTCGATCTCGTCT	(CCA)5	174	174
ESSR86	CAAAAGATGGGTGTTGGAGGAACTTTG	CATTCTGAACCTTGAGCTCACAGCCAT	(AAG)5	105	105
ESSR87	AAATCTGTGGCCTCTACGATTCACCC	CCCGGGTATTCTTGTCAACAAAGACAG	(TCT)5	181, 190	190
ESSR88	TCAGAGCCTTTTGGGTAAAACCAAGC	GGGCCTGGACTGGAACAGAAGAGAA	(ATC)5	198, 201	198, 204
ESSR89	TGGAACTGGTTTCGGCGGTG	TCACGTCCTCTCTTTTGTCCAATCCAG	(TCT)6	NA	NA
ESSR90	TGGCTGACCTGCCAATGGTTTTG	GCTAGCTCAGTCACCCATGTCTCCAC	(AGA)5	NA	NA
ESSR91	TTCATTTCCAGGAGACCACCCTTTCA	CCAAAGGAACACTCTCTGGGCCG	(CTC)7	196, 206	198, 206
ESSR92	TTGCAATGAGCTCCCTGATTATGCTG	GCCTTCACATGGCACGGTCG	(TGA)6	166, 215	215
ESSR93	CTTGGAGCTGCCTCTCACTACGGC	GGGTTGACGACTGGGTGGCG	(CCA)6	191	191
ESSR94	AAGGTGGCAACAGCAAATGGATGG	GCAAAAGGGTGGATAGTTTTGGTTTGG	(TCT)8	186	186
ESSR95	CCATCTCCAGAGCTCTAAACCGCCA	CGAGGAAGCATTCGAGACGCA	(CTT)6	197, 215	197, 206
ESSR96	TTTTTCCCTTTTCACCGCTCTGGTT	TTGAATCACCGCTGCTCTGATGG	(AGA)8	166, 178	161, 170
ESSR97	CAAACCCTTTTCTCCCATCGCCT	GCGTCAACGGTTGCAGATTCTGACT	(TCT)6	223	223
ESSR98	CGAAGTTGGTGTCACAGAAGATCACGA	TTGGAGGAGGACCACCTTCTTTGC	(GAA)6	182	182, 194
ESSR99	TCACTCATGCAGCTAACAAGGGCG	TGCTTCCACTTCCACCTGAGGTTTACA	(TTC)5	NA	NA
ESSR100	CTACCTTCAAAGCTTCAGCCTTCAGCA	AGACCTGCAGCTGTTTTGAGGCCTTT	(AAG)5	NA	NA
ESSR101	GAAGGAGCAACGTGATTACAGGGCA	CGAGGAGGCATTGCTTGGCG	(AGA)8	188, 194	188, 194
ESSR102	TGGAATTGTTGGACGCCGGA	CCGCGTAGAATCCTGAATCGCC	(ACC)6	NA	NA
ESSR103	TCCCATGTTGGAGACCCCATCG	CCTCTGGTGGCTGTTGCCCA	(GAA)6	NA	NA
ESSR104	TGCCTCCTCCTCCTCCACTGC	GGATCCGGAAGAGAGAAAAGAGGACC	(TTC)11	190	190
ESSR105	CCGACGGCAAGGCTCAGCTC	CCCAGCATCATCTTGAAAGCGAGG	(TTC)7	182	182, 194
ESSR106	CGAGCTCCGCCTCTTTCCCA	TGCCTGAAGCTCTGTAGCATCGC	(GCA)5	186, 190	186, 190
ESSR107	GGGGCTGGGTTTTGGGGGA	TGGGCTTCATGGCCAAAGGA	(TCT)5	NA	NA
ESSR108	GGTGGAGCTGGAGGGAGTCGTG	AACTCAATCCCAGGCGGCGG	(GCT)5	177	177
ESSR109	TGCAACCCCAAAGATCAGGGC	GCATCTCGGTGTAGAAGTTGAAGCCG	(TCA)5	189	189
ESSR110	TTTATAAATACTCCGCCCTTCCCATCG	CGCCGCTTTGGACGAGTCAAC	(TCG)5	191	182, 191
ESSR111	GATGAAGCTGTTGAAACTGCAAAGCAA	GCTCGCCGAAGCTCGGGAATA	(GCT)5	121, 109	121, 136
ESSR112	TTCCGTCGTTCAAATGCTTCCG	CGGCGGTTGCTTGACAGCTC	(CTT)5	NA	NA
ESSR113	TCAAGCGAAGAGCATCAGAGCAAATCT	TGTCAGGATTGTAGGGATTGACGGC	(GCA)9	185, 194	185, 194

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
ESSR114	GGGATCTGCGCCCATCACAGA	CAGCTGCACGCTCTTCCGCA	(TGA)5	173, 179	173
ESSR115	TACGAGCATCGCTCTCTTCCCTCA	CGTGTAGTGCTCCGCAAACTACCGA	(CAA)6	188, 194	188, 196
ESSR116	CGCCTCTTTCCCACCTCCGA	GAATGGCTTTCGCCTGAAGCTCTG	(GCA)7	190, 196	202, 209
ESSR117	CTTTGAAAGAACTCCAAATGGCCACC	TGGAGGTGGTGGGTTTGAGGAAAA	(CAC)8	102, 170,	102, 118
ESSR118	GTTGTGAAAGCCGAGAAGACGCC	TCTCCGGCAATGCAATTCTACTGCTT	(AGA)7	NA	NA
ESSR119	GATGGAGAGGATGCTTATGATATGCGG	GCTTTTGCCTCTGATTTTGGGTCTGG	(CAT)6	173	173
ESSR120	AACGGCGGGTGTCTCACCTCC	GGTTGATGATAGGAGCAGGAGCAGC	(CTT)5	190	102
ESSR121	GAAGGACCAAGGGTTTTGCAGATGG	TCCTCAAAGATTCCTCTGCCTGTCTTG	(TCT)8	199	199, 209
ESSR122	CCATTCTTTGCTACACATGGAGGGC	TCAGGAGTACCCTTCTGAAAGGGGAAA	(TTC)5	175, 216	175, 216
ESSR123	AACCCTTTTGCCGGTGGTGG	GGCTAGAAGGGCATGGCAGAGCTT	(AAG)7	178	178
ESSR124	GCAGCTGCCGTTTTATCCTCTTGC	TGCTCATCGACAAGCACCCTTTG	(CTT)5	NA	NA
ESSR125	TTCTCCTGGATATCAAGCTCCGCA	ACGACCACGTCCTCGGCCAT	(CAG)6	257	257
ESSR126	GGGGATGAGAGCGGTCTTGGTTTT	TGGTGCTGAGGTAATGAAGGCATGG	(TTC)5	175, 194	175, 194
ESSR127	CTCGCCAACTCTTTGGCCGC	TGCCATTGCTGGGAACTTGTCC	(ATC)5	198	198
ESSR128	ACGAGGTACGGCCGGGGAGA	GGGAAGGTTGGTAAGCAGTTGCATGA	(TCT)5	124	124
ESSR129	CCAACACATGTTTACATGGCTCAGCA	TCAACGGTTGCAGATTCTGACTGGA	(TCT)6	184, 187	184, 187
ESSR130	GCAAGCAACAACGTTTCTGAGAGCTG	GTTTCTTGGCCGTCCTGGCG	(TTC)10	174, 177	1174
ESSR131	GTTGACTCTGGGGTGACCAAGATTCCT	AACCCGCTTACCATAACTTTCAATGCC	(CAG)5	194, 197	194, 197
ESSR132	CTCCCATACCACTGGAACAGCCTCA	TCCCAGTTGGCCGGTGGTTG	(AGC)7	103, 201	103, 201
ESSR133	TGTCCGCGGAGGGATCGTCT	CGCTATTCTTTCCGATGGACGACG	(AGG)6	178, 184	178,184
ESSR134	TACGAGTTCATTGGCTGCAGTTATGGC	AAGGATGAAGGTGGTTTGGTTACAGCA	(ATG)5	176, 179	176, 179
ESSR135	AAGGCTGAGGACCAAGGTGCTGAAT	CGCAAGATAAAAATCCTCCAAACCCA	(GAA)8	216	216
ESSR136	GCTCATCGGGTCAACAACCGC	CTCGGGTCCTCTGGGCCCTC	(GAA)6	201	201
ESSR137	CCGAGAATGCTTCCAGGACCCC	TGAGCTCGGCCCTTTTGCCA	(GAG)5	307	307
ESSR138	TGCCTGTGTGCACTGAGCGG	GCAACTCCAACAAGAACAAGCCCA	(GGT)5	NA	NA
ESSR139	GGAAATTGCATTAAAGCGTGTGTTTGG	GCCTGGCTCCATCCTGTCTGC	(TC)8	169, 181	181
ESSR140	GGGTCCCCATGTAAATTGTAAAACCCC	TGGCTAGAACCACTCCCTGTGAGCA	(TTC)10	168	168
ESSR141	ACGGGTTACTGAGTTTTGATTGCATGG	GATCATCGTCACCGTCCAATAATCCA	(AGA)9	NA	171, 174
ESSR142	GGGGATTCATGGGTCGTGCC	TCCTGTTGAAAGAAAAACCGACCCAA	(GAA)6	192	192

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
ESSR143	CATTCCCGTCAACCAACCCTTTCTC	TCCATGCAAAGTCCATCCGCA	(CT)6	195, 207	195, 207
ESSR144	AGAAGAACATTCAAAATGCAAAGGGGG	TCTGCAGGAGGGAGGCCAGC	(GA)7	NA	NA
ESSR145	GTCCCTCAAACCCCAAAACTCCCC	TTCTTCTGCCTGGGTCGCCA	(TC)6	101	101
ESSR146	CCCCAATTTGCAGCAGCAAGGA	GTGGCAAACCCGCCAAGCAT	(CAG)6	202	202
ESSR147	GAGAATTATGGCTATGCCCCTGCTCC	TCATCAGTTGAAAGCACAAGAATGCCA	(CAG)9	122	122
ESSR148	TTGGATCTCTTGCTTTGCAATTTGAGG	ACAGCCAACAAAACCAAACAGCTCC	(TTC)6	NA	NA
ESSR149	TGGGCTTGAAGAAGGATGGCAA	TCCCTGGCCCTTACTCATGGAAAA	(GAA)6	NA	NA
ESSR150	GCGGAGGGATCGTCTGCGTC	TGACTCTCTCCCCGACAGATCGC	(AGG)6	200	200
ESTSSRY5	CCAAGGGAAGTCCAGAAGAA	AATGCAATTTGCTGCTTCCT	ATGG(5)	251	251
ESTSSRY11	TTGGGGTTCTCCCTTAATCC	AAGAGCTTTAGGCGGTCACA	GAA(5)	204, 207	204
ESTsSSRY12	TTGGGGCCCGATAATAAGAT	AAAAATGATCCAACATCTAGCAA	TTC(5)	181	181
ESTsSSRY13	AGCATAGGAACCTGCGTCTC	TCCAGCTGTAGCTGTTGTGG	GCA(5)	NA	NA
ESTsSSRY14	GGCAGAGCTGGATTCATCA	TCGATGCCCTTATTGGTAGG	AAG(5)	199, 203	199, 203
ESTsSSRY15	CGGAATGGTCAATACCCTTG	GAGCAATTTCCACCACCATC	GAT(5)	283	283
ESTsSSRY16	TCATCCATAAGATCATCTCTGTTACTG	TCGAAGCATTCTGTGTGTCC	AGA(5)	264	264
ESTsSSRY17	CACCCCATTGTTCTTTGACA	GGCCATGCTCGTTTTAGAAG	CCT(5)	206, 208	206
ESTsSSRY18	ACCTCCAGAAGGTCGTCGT	AGTAGGTTTTCGCGGTCTCC	TCA(5)	200	200
ESTsSSRY19	GAGAAGTGGTTGGGGTTTCA	TCAAGAGCTGGACTTGAGGAA	GAT(5)	235, 238	235
ESTsSSRY20	GCAAAGACAAAGAGGCATCC	CGTCAGCCCATGAAATCTCT	TCC(5)	204, 207	204
ESTsSSRY21	AGTGCCCGATTATTGGAGTG	CAAAGCATAACCGCGAATTT	AAC(5)	202	202
ESTsSSRY22	AATCCAATCCCCTTGGACTC	CCAACCCACAACCTTCTGAT	CAT(5)	206, 209	206
ESTsSSRY24	AGGAAAAAGAGGCTGGAGGA	ACCTACGGAATCCCAGAAGC	AAG(5)	99, 106	99, 109
ESTsSSRY28	ACAGCCACTTGCCTCATCTT	GACACGATCGTTGCGAGATA	TCT(6)	NA	NA
ESTsSSRY32	TCAGGCTCAATCACAAGCAC	TGCATGCTCTGTTCTGCTTT	TTA(6)	232	232
ESTsSSRY37	GAGGGAGTCTGCTTCTGCTT	AAGCTCAGAATACAACGGAAAAA	TTC(7	160	160
ESTsSSRY44	CATAAACGCGGTCCAAAAAT	ACGAAAATGCGGATTACAGC	TTC(7)	230, 239	227, 230
ESTsSSRY45	AATCAAAGAGACGGCGAAGA	GCCGGTAAACGGTAAAATCA	TTA(7)	207, 210	204, 210
ESTsSSRY47	TCATCAGATCACCACCATCAA	GTGTCGCTGCTAGATCTTGACT	TCT(7)	252, 258	252, 258
ESTsSSRY48	GAGAGCAAACAACCAAAGTTGA	GCCCCCATAAAAATCACCAT	TCC(8)	180, 186	180,186

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
ESTsSSRY49	TGCCACGACATCTTCTTCTG	GACCACGTCTCGTCGTTTTA	TC(8)	207	210
ESTsSSRY50	TCTCCCCACACACTCTCTCTC	GGACCGTATCCAAGCTCATC	TC(8)	208, 210	208, 210
ESTsSSRY53	GGTGCTGCAGAAGAAGTGCT	CCCCTTTCTTCCACTTTCCT	AT(8)	NA	NA
ESTsSSRY54	AAGACACGAAGACGGTTGCT	GCAAATTGGGGGAATGTTTT	GA(8)	NA	NA
ESTsSSRY55	TTGGAAGATTCCTTTTCCATAGAC	TGCCCTCAAATTTTCTCCAT	TG(8)	91, 133	91, 133
ESTsSSRY58	AGCCATCCAATCCAATCTGA	TATGCGAGGATAGCAGCACA	TC(8)	152, 156	152
ESTsSSRY61	TCGCAGCACTAAACATCTCG	TTCAATGATGGCTGAGCAAG	TGA(8)	146	146
ESTsSSRY62	TGGGCGTAGGACAGTAAGAGA	CCATGGCACAATAACATTGG	TG(8)	214, 220	214, 218
ESTsSSRY63	CCTGTATCCAAGCTGCCATAA	CCCTTCCCGAGCAAGAAC	TC(8)	159	159
ESTsSSRY65	CATCATTCACAACCCCATGA	GCACCAGTCAACATTCCTGA	ATG(9)	158	158
ESTsSSRY66	TGACGTCCTTAGCCATCCTC	CGCTTACAACACCACCTTCA	AG(9)	122, 126	114, 122
ESTsSSRY68	TCTCCAGGTTAAGGGGGAAG	CCTCCCTTCCATCCTCCTAC	GA(9)	210	210
ESTsSSRY70	GCTGATGAACCCTTCACGTT	GCCAATTTTGCTGGGTTTAC	CT(9)	109, 113	109, 111
ESTsSSRY72	CGAACTCGATCAGATTCCACT	TACGGCCTATCCTCTGATGG	TC(9)	189	189
ESTsSSRY73	GTCAGAGCGGACACAACAAC	AGGAGTCCACCGAGGAAGTT	CT(9)	NA	NA
ESTsSSRY75	TGCGCTTCTCAGGGACTATT	TTCCCCGATTGTTTGTAGGA	AGA(10)	280	286, 304
ESTsSSRY76	GAAACGCTCGATCCTACTCG	AGCAAGGAGGGTAACGACAA	TA(10)	236	236, 240
ESTsSSRY77	ATCTCAGGGTGGTCGACAGA	TGCCAAAGGAGGAGAAAATG	AG(10)	211, 219	211, 219
ESTsSSRY81	CCCACATGTACTTGGAAGAGA	GTTGGTGGACTCGTGGATCT	CT(11)	118, 130	118, 130
ESTsSSRY83	TGGACCTGTTTTCTTTTCCTG	GCACCCACCACTGAAGTACA	TA(11)	198, 214	204, 213
ESTsSSRY92	GGAAATTTCAATTCCAGACAGG	ACGGCGGCACACTAAATATC	ATA(13)	262, 278	265, 274
ESTsSSRY98	CCTCAAGCAAAGCAAAGGAC	CGGAGTGCTCCTCTCCATTA	CT(16)	NA	NA
ESTsSSRY100	GCGCTTTACAGGCGTTTTTA	GGTCTTTGCTCCGTCGTTAC	AG(17)	181, 187	177, 181
ESTsSSRY106	TGTGGTCGATGAACAAGATA	GGAAGCTTCTAAACTGGTCA	GA(5)	226	234
ESTsSSRY110	CGAGAGAAGTGGTACAGAGG	AAGATCTCGAGTCTGACGAA	GCA(5)	194	194
ESTsSSRY115	TGGACTTGAGGAAGAGACAT	AGCACAGGTTTCATGCTAAT	GA(5)	254, 257	254
ESTsSSRY117	CAGAGCTCCGTAGCTTATGT	CAGAGAGAGAGCCTGAAAGA	AG(5)	261	261
ESTsSSRY264	CTCTTTGGTTCTGGAAAATG	TCCACTTCAAATCTTCTGCT	TTC(6)	NA	NA
ESTsSSRY266	GTCATCAGAATCCTCCTCTG	CACGATCATCTAAACCAACC	GAA(6)	249, 257	249, 257

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
ESTsSSRY272	TGTATACCGAGCCTTTGACT	CACTCCTTTTCCACAAAACA	CT(7)	268, 280	268, 280
ESTsSSRY273	CACAAAACCGAACACCATA	GGCAATACAGAAGAGGACAC	CT(7)	215	215
ESTsSSRY274	AGTACGACAACCCACATCTC	CTGAGCTTCTCCTCCTCTTT	TC(7)	238	238
ESTsSSRY275	GCATAGCAGAGAGAAGAAGG	CGAAGTGGATTTATGGGTAA	GAAAG(7)	236, 241	236, 241
ESTsSSRY277	ATTGAATGGTGAGGATGTGT	GAGTTTGACATTGCAGGTCT	TG(7)	280	280
ESTsSSRY278	CTTTCTTTCTCTCTCTTTGTAGAGC	GAAATCGGGGAAGAGGAG	TA(7)	NA	NA
ESTsSSRY279	TACACTCAAATGGGTTTTCC	GAGACCAGTGATTGTGGTTT	CT(7)	283	283
ESTsSSRY280	AGAAGCGGATTGTAAGATCA	GGTGAATTTTGGGGTTAGAT	TC(7)	259, 265	257, 259
ESTsSSRY281	AAGAACCTGTGGTTCCTTTT	ATGTGGTGGTGATGGTTACT	GT(7)	NA	NA
ESTsSSRY283	GTCGCTGCTAGATCTTGACT	ATCATCAGATCACCACCATC	TCT(7)	239, 245	239, 245
ESTsSSRY284	CTTTCCAAGCAAACTGGTAG	GAGAAGTTTTGCCACCATAG	GA(7)	246	246
ESTsSSRY287	AGAAACCCCTAGAGTGAAGG	GAGAACTTGGCAACACACTT	TG(8)	223, 229	NA
ESTsSSRY288	TGGTACTGAATCTCCTGGAC	ACGATTGTCCATTCTTTGTG	TG(10)	245, 249	245, 249
ESTsSSRY289	CTTTAAAGACGCGAGAACTG	CACCTCCGAAATCACTAAAA	AG(10)	236, 246	236, 246
ESTsSSRY290	TCATCTCTCACAGGAACACA	TGAAGAAGGAAATCAACACC	AG(12)	289	289
ESTsSSRY293	ATCTCCAGGCTCTCCTGCT	CAACTTGCAACTCTTGTTCA	CT(21)	416, 428	418, 428
ESTsSSRY294	TCCTCTCAATCCCTACCTCT	TTATCGGGCCATATGTTATC	(CT)5, (TTTC)6	316, 330	312, 316
ESTsSSRY295	CTTCAAGCTCACAAAACACA	CACGGTAGAAAGACCATAGC	(GA)6, (AG)9, (AG)6	226, 230	218, 226
ESTsSSRY296	AGATCACAAGGATCACAAGG	GCAGTTGTCAAACACTAGCA	(AAG)6, (AGC)5	403	403
ESTsSSRY297	TTATTTTCCCACTCACCAAC	GACTCATCATTGGCAGAGTT	(CT)5, (TTTC)6	293	293
ESTsSSRY300	AACTTGGGTAAATCAACTGG	GGATTAGAGCAGACATTTGG	(AT)9, (AC)5	302	302
ESTsSSRY301	GTTGTCCTCCAAAGATCGTA	GAAAGTAGTCCACCCATCAA	(TA)5, (AGA)5	435, 439	NA
ESTsSSRY292	TCTCTCTTCCTTGAACTCTCC	CATCATAAACTGGTGAGACG	TC(15)	253, 263	253, 263
NS6	GCTCGCATATTCCCATCAAT	TTCCATCCAAGCTCCTTCAG	n/a	268	266
NS10	CGATTTTGCGAATTACCACC	GTCATTACGGCGAATCTGCT	n/a	202	202, 204
NS22	CAAGCCCCATCATCATTTTC	CAGCCAAATCAACATCCCTT	n/a	289	295
NS33	TCACCTCACAGCCAAGAGAA	GAGCTTGCTCACCGGACTTA	n/a	250, 256	250, 256
NS40	GATGTCTGAAATCCCTCTCTTT	GCTGTTACGGCCAGAGTAGA	n/a	173	173
NS53	CCTAAGTTGTTAGCCAGTGATTAGA	CCAACGTATGGAATGTGCTG	n/a	219, 239	239

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
NS57	CAACAATTGGACTAAGCAGCA	ACCAAAATCTCCACACCCTG	n/a	160, 170	160
NS59	CTCCTGCCAAAATTGACCAC	CATGAGCATATCCCTCCTCA	n/a	117	119
NS74	TAGTGTTGGGGACTCTTTCG	TCGCTGTATGCAATACTTCGTT	n/a	206, 228	206, 228
NS76	ATGCAGTCAACTGTCCAACG	TGCCTCTGCAATATGAGCTG	n/a	NA	NA
NS78	AAGATGGCAATTCAAGCAAG	AGCAATGCCTTGATCTTGAG	n/a	375, 383	369
NS80	GTGTGAAGCCACGCTGTAAA	ATCAGGTCCATTGTTTGCCT	n/a	251	247, 271
NS82	AATTGAATTTTTCTCAGCACTGT	TGTCGCCCACTTACATTTCA	n/a	NA	NA
NS92	AGCGTTTAGCACGTCAGAGC	ACTTCATTGGTGCTGGTGCT	n/a	262	262
NS97	GATACCCACAAGCCCAAAGA	TTAAAAGCACCTGTGGGTCC	n/a	242	242
NS109	TCCTTGGCATAGCCAAAATC	AACTGCAAACAAAGCCGAAG	n/a	162	162
NS119	GATGGTGGTGATGATGATGC	GGGAAGTGAGCAGAGACTGG	n/a	198,	186, 188
NS124	CCCACGCTTCTGCTCTTTTA	AGGCATACCGCCATGATTAG	n/a	341	341
NS128	AAAGGACAGCGCTACCAGAA	GATCGCTTCACCTTCCTCAG	n/a	264	256
NS136	GACTATTTGTGATGAAGGCTTGC	GGTTCAAGCATTCACCTTGC	n/a	132, 139	139, 141
NS149	TTTGATTCCACGAAATCTAGAGAA	TCTTGCTCAAGGGCTCAAAT	n/a	300	300
NS158	GTGCGAAATGGAAATCAATG	TGAAATAGTGATACATGCAAAAGGA	n/a	184, 198	168
NS159	TATGGAGCAAAGTCAGCCCT	GTCCATGCACATGCCACTAC	n/a	251, 261	259
NS160	ATGCATCTTCCTGGTTTTGT	CAATAAGAGTATAACCATTACCTGTG	n/a	110, 134	124, 130
NS166	TTGTGCACCATGAAACCATT	ACAAAAGCAATCAGGCAAGC	n/a	276, 306	273, 276
NS169	GCCTTCTCAGCATATGGAGC	GTGCGAAATGGAAATCAATG	n/a	320, 334	302, 320
NS170	TTCCTTTACAATTCTGGACGC	AGTCGGGAGTTGGAACCTCT	n/a	308, 316	308
NS174	TTAGGAGGTGTGTCCCATCC	CTCAATTCATTAAAGCGCGG	n/a	288	288, 294
NS176	TGTGATACAGGGTGCTTTGC	AACTAGGGAAAGTTCTGAAAGTAGAGA	n/a	274, 288	262, 292
NS178	CGTTCGAGTTGCATTCATTC	TTACAGGTGCCCGATGTGTA	n/a	168	158, 168
NS185	CCTTTCTGATGTTCTCTCTGCAT	AGTTAAGGGCCAATTCCTGC	n/a	155, 169	155
NS189	CATGAGTTTAAAAATTATCACATCCG	TGGGCTGTTCGTGATCCTTA	n/a	91	91
NS190	TTCATGTGTGGGTTTCCTCA	CCAAGCAACCATCATTCAGA	n/a	NA	NA
NS193	AAAGCCCATCCCCTCTATGT	TTGGGGGCTTTAAGTTGTTG	n/a	250	250, 256

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
NS194	TGGTAAGGTTCTTATTTTTTGAG	TCCTTCATTTCAGCAATCTT	n/a	NA	NA
NS197	ATGGCATTTCTGCAGCTCTT	TGTAAAATTTAAGACTTTCCATCTTCA	n/a	138, 178	170, 178
NS207	AGTCGGGAGTTGGAACCTCT	TTCCTTTACAATTCTGGACGC	n/a	301, 309	NA
NS210	TGGCTTTGGTTGTATGTGGA	AAAGGGAGATTTGCAGAGCA	n/a	236, 238	236, 252
NS216	TTTTGAACAGAACACCATCCC	CCCCTTCAGGTCGTCACTAA	n/a	170, 182	170, 174
NS217	AGGGAAGGAACACCTCTCCTA	TCAGCTGTATGTTGAGTGAGCA	n/a	218	218
NS235	AATTATGGGCAGGAGAAGCC	CCAAAACATAGGGAGCGAAA	n/a	173, 195	175, 217
NS260	AGGGAAGGAACACCTCTCCTA	TCAGCTGTATGTTGAGTGAGCA	n/a	220, 226	220, 226
NS267	CCGCTCCAGGTGCTTTTAC	ACACGCAACAAATCAACCAA	n/a	151	151
NS271	AAACCACCAATCAGTCCAGC	ATGTCTAATTGAAGGAGAGGATTC	n/a	230, 236	234, 236
NS272	ATGGCATTTCTGCAGCTCTT	TGTAAAATTTAAGACTTTCCATCTTCA	n/a	134, 176	168, 176
NS300	TGAGAATTGTTGATTCCCAAGTT	GCCAGGCTTTCCTGTGATAG	n/a	304, 318	292, 322
NS301	TGGGCTGTTCGTGATCCTTA	CATGAGTTTAAAAATTATCACATCCG	n/a	116, 124	116
NS306	CAATTTCCAATGTGGTCTCAAA	AAGACCCACCAGAAAGCTGA	n/a	302	302
NS308	CGCATTGGACTTCCTACAAA	GGAAATTGGTTATGTCCTTTCC	n/a	143, 155	143
NS319	AGGGAAGGAACACCTCTCCTA	TTCTAAAGGTTGATTAAAGCTCTGTG	n/a	161	161
NS323	CCTGGCAGAGAACTGGAGAC	ATGGTGCATGCTCAAATGTC	n/a	278	278
NS340	TTGCTAAAAATCCCTGGACC	GCCAGCAAGGTTTGCTACAT	n/a	244, 248	238, 248
NS341	TCTTTGATGAGACCAAGCCA	GCACAGACAAACAACCAAGC	n/a	138	138, 150
NS346	TCTCCATCAGCAGTGCAAAC	CTGCAAAGTCGATGCCTACA	n/a	289	289
NS347	TGGCTTTGGTTGTATGTGGA	AAAGGGAGATTTGCAGAGCA	n/a	234, 236	234, 250
NS349	GCAAAAGTGAAAAGGACGGA	CACTGCGTACAAGCAACACC	n/a	NA	NA
NS350	GGTGTTGCTTGTACGCAGTG	TCTGGAGCATCAAACTGCTG	n/a	216	216
NS356	CGCATTTTGCTTGCAGATTA	CAATAGTATTACATGTCCTGCATACG	n/a	249	249, 263
NS371	CCAGAGCTATGTGCAGGCTT	TGTAAAATTTAAGACTTTCCATCTTCA	n/a	113, 153	143, 153
NS376	GGACTATCAAGGCGCAAAAG	TCAAGACCCTTGCTTTGGTT	n/a	188, 196	196, 204
NS391	ATTTCCTGCACCCGGATAA	TCTCAACCTCAGAATGTTCCAA	n/a	132	132
NS576	GAGAGGAAGGAAGTTAGAAATCCA	ATGAGTGAGAAATCTGCCGC	n/a	145	145
NS584	TTTTATATGCGAGCGTATACGTG	CAGATAGGCGTTCCCCAATA	n/a	184, 188	180, 196

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
NS602	GCCTTTGTCCAGTCCATAGG	AGTGGATGTATTTGTGTTTTG	n/a	118, 120	120
NS622	AGCCCACAACAACTGTGTGA	TAAGTCGCGCAAATCCTTCT	n/a	138	138, 150
NS656	TGCCTCTAAGAAGATTGGAAGC	AAGAACCCAACGCATTTGTC	n/a	181	181
NS658	TCGTTGGAGCCATTACATTTC	CATGATGGCCCGAAGATAGT	n/a	160, 188	160, 188
NS664	GGTGAGAGCCTAACCTGTGC	GGGTGCCAAACTCTCATTGT	n/a	296	296
NS667	GAATGCATAACATGAAAACAGG	TGGAGGTGGAGCTTAGGAGA	n/a	203, 250	238, 250
NS701	TCCATTTGAGCCAAAATTTTATT	TCTCTTGTTCATTTGTTGCGTT	n/a	296, 362	343, 362
NS717	GGTGAGTGATAAGGTTACGGC	GCCAAATCGCCAAGGTAATA	n/a	212	212, 222
NS720	GGAAATTGGTTATGTCCTTTCC	CCATTACTTACACATTGGACTTCCT	n/a	152, 162	152
NS725	AAAACGAAAATCATGCCCAG	TGCTATTCCAAGTGTTTGCC	n/a	137	137
NS774	TCTCTTGCTTCTGTCACAACG	AACCCGCAGAGAATCATGG	n/a	127	127
NS847	TTGGCCTGTAAGGTTCCATC	CAAACTTAAACTCCGTCCGC	n/a	270, 280	270, 280
NS890	TGCTTACTCTTTGATTCCACG	TAAATTGGGGGTTCTTGCTC	n/a	321	289, 321
NS898	GCATCTTCCTGGTTTTGTCC	CAATAAGAGTATAACCATTACCTGTG	n/a	127	127
NS905	TTGGCCTGTAAGGTTCCATC	CAAACTTAAACTCCGTCCGC	n/a	269, 277	269, 277
NS909	TTGGCCTGTAAGGTTCCATC	CAAACTTAAACTCCGTCCGC	n/a	334, 348	322, 352
NS911	TTGAAGCAGTTATGAACCGT	TGTTGTTCAGACGATGTCCAA	n/a	123	123
NS928	GACCCACCCATCCACTAGAA	GATACCCACAAGCCCAAAGA	n/a	256, 272	272
NS945	TGTTTGAAATAGTGTTGCTTCTTGA	GCAAGGCTCCATTAAAAGTCC	n/a	375, 399	381, 399
NS963	GAAGAAACCACCCAAGTGGA	TTTTTGTCTGCTGCATATGTTT	n/a	80	74, 80
NS964	TGGTTAAAATTTCTTTTGTGAACTG	AAGGGACACGACTTGGTCAC	n/a	113	113
NS977	GACCCTTTGCCGTTACTCAT	TTCCTCGCATGGCAGAAG	n/a	302	302
NS978	TTGAACACACTTGGCCAGAA	TGTTGGCCATATTTCCCATT	n/a	239	239
NS980	TGATCCCAAGGATCTTCCAG	TTTTGCCTTCCTCTTAGCCA	n/a	234	234
NS983	TCTGCAGATGCAACAAATCC	GCTTCAAACATCAAACCCTAAC	n/a	NA	NA
NS995	GGGCTGTTCGTGATCCATA	CATGAGTTTAAAAATTATCACATCCG	n/a	91	91
NS1003	AATTTGGAGCTCAAGCGATG	TGCAATTGTAAGGGC CAAAT	(GA)5(AAGAGA)3 (GA)3	267, 273	267
NS1006	AACTTGCGTCCCAAAGTGTT	TCCTATACAGTGCTGTCCGC	(GA)3N4(GC)3	268	268
NS1008	AACCCACTCAAGTGTCTCCG	AAATGGCCTAGAAATCCATGA	(TC)8N2(CT)2N(CT)5	258, 262	258, 262

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
NS1014	TTTTCCAATTGCTTTGGTGA	TCCCTCTATTTTGACGATCCA	(CTT)4N9(CT)2	225	225
NS1019	AAGCAGTGGCTCAGTTTGGT	CTGGAGAAGACCACCCAGAA	(TTG)2N8(TAA)5	215, 225	215, 225
NS1020	GAGAAAGACTCAGGTTCGGC	TCCAAGCCTCAAAACTTGCT	(CGG)2	255	255
NS1021	TTGTATCTGAGCCTTGCGTG	TCACAATGAAGCCCAGTGAA	(CT)2N3(CA)2N2(CTT)9	318,	321, 330
NS1025	TGGATGTTTACGGACTCAAAA	AGCACTCCAAACAAGACCAAA	(AT)6	NA	NA
NS1028	GGGGATGGAGTAAATACGCA	TTCCCCAAATATCACTTCTGC	(GGA)3	357	357
NS1035	TGCTCCTGCTGTACTGGTTG	TTCCTTGCTTCTCAAGGCAT	(CAA)2(AG)2N2(GCT)6N3(GCT)3	215	215
NS1039	CGGTCGCAAATAACGAAAAC	TATTCAAGAACCGGGCAGAC	(ATTT)2N2(TA)2N6(ATT)4	249	249
NS1040	CGCCTCTCAACCCAATAAGA	TAGAAATCTGAGCCACCGCT	(GA)2N8(CT)6(CTT)2N5(TC)2	293, 295	293, 295
NS1043	AAAGCCTGCAAAGAAAACCA	CTCACCATGGCTCATTCTCA	(GA)5(GGGA)2	285	285
NS1045	GTGTTGTGCCCCTTGCTTAG	AAAAAGGGTGGCGGATAATG	(CT)3(TTCTCT)2N(TC)3(TG)4	142	142
NS1046	CCTTCCTACCCATCAAGCCT	AATAAATTGGTGATGGCTGAA	(TGA)2N(TA)5N7(TA)4	416	416
NS1047	AACTCAACCCCTCCCATTTC	TATGCACATTGCCTCCAAAA	(GA)2N2(GA)5	308	308
NS1052	CCATTCCATGGGTTTTGTTT	GAGTGTTGTCCGGCAGTTTC	(CA)8(TA)5(TGTA)3(TA)2	362, 366	366, 368
NS1054	ACCCAACCATGAGAAGCAAC	CTGGTGATGGTGGGAAAAAT	(TA)7	180	180
NS1056	TTCCTTTGGCTCTCCCTGTA	GCTTAAACTCATTGGGCTGC	(CT)2N(CT)5	251	251, 253
NS1063	AAGTTTGGGAATGCAAACTG	TGCATACAAAACTGCCCTCA	(TA)10N4(ATCCAA)2	198	198
NS1070	GGTCTGTAATCAAGTTTAGGTTTGTG	TCATGGCTTTGACTAACATCTTTC	(TTA)4	NA	NA
NS1072	GCCTATTCAAATTGCATCCC	GGCATTTGAAGAATGGAGGA	(TAA)3N6(CA)2	NA	NA
NS1075	AAGGGCGATGAGGAACTGTA	TTTCTCAAAACATTTATTGCATTC	(TA)12	NA	NA
NS1077	CCTTGGCTACTTTTCTGTCACC	TGCCTTTGAGTTAACTTCTTATTCG	(CA)2N5(TGG)4	NA	NA
NS1082	AAGGCTTCCCAAGAATTAAACC	AGAGGACACAATGGAGGTGA	(CTT)2N3(CTT)5N2(CTT) 3N5(CTT)2	275	275
NS1087	GTTCCACAGCATGGGCTACT	TGGGCATCAAAGTTTACATCA	(CT)2N2(CT)7N(TA)4	374	374
NS1099	ATTTCTTTCTGCGCAAGCAT	GAGTTCGAGAATGTGCGTGA	(CCA)3N7(CT)7N(TA) 2N4(AAT)2N3(TAA)2	250	216
NS1109	CACACGCAATTCAATCCATC	TGTGCATGGTACGAGGGTTA	(GA)3N2(GA)12	NA	NA
NS1111	ATATGTGCCTGTGGTGGGTT	TCAAATTCGATACCTCGCAA	(CTTTC)2(TC)5	NA	NA
NS1113	GGTGGGGACTGCTATTCTGA	ACCATTCCATTCTGGGCATA	(CT)5	324	324

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
NS1116	TGGCCAGCCACTATAAAAGG	AAACATGCATTCTACCCCCA	(GA)2(GAC)2	322	322
NS1119	CTCCACTCCTGCCACGTATT	GGGACAGTGATGGTCTTCGT	(TC)6(TA)3(TC)2	425	425
NS1121	TGCTGTTGAGGAGCTGTTGT	TTTTGCTCCCTCACATGAAA	(TA)6	398	398
NS1123	CTCACTGATGCTCATTCGCT	ACAGAGAAAATCCGCCATTG	(TC)2N5(CA)2(GA)6N5(TG)2	NA	NA
NS1128	CCAGGATCTTATGGACCCAA	TGATCTCGGTGCAGAATGAG	(TAA)3N6(TTA)2N3(TTA)2	200	200
NS1141	TTGGTGCATAAAGGGGAGAG	CTGATGATTGCAAGGTGTGG	(GA)2N3(GAGGA)2N8 (GGA)2N3(GA)2N7(GA)2	348	348
SSRY1	CCAAGAGATTGCACTAGCGA	GCAGCTGCCGCTAATAGTTT	GCC(6)	190, 193	193
SSRY2	TGATGAAATTCAAAGCACCA	CGCCTACCACTGCCATAAAC	CT(18)CC CT(16)	106	106
SSRY3	TTAGCCAGGCCACTGTTCTT	GCGAGGTTCAAATATGCGAT	CA(17)	247	247
SSRY4	CTAACGCACACGACTACGGA	ATAGAGCAGAAGTGCAGGCG	GA(16)TA GA(3)	266, 284	278, 284
SSRY5	CGCCTACCACTGCCATAAAC	TGATGAAATTCAAAGCACCA	GA(38)	298, 364	348, 364
SSRY6	AACAAATCATTACGATCCATTTGA	TTTGTTGCGTTTAGAAAGGTGA	CA(7)N(51)CA(17) N(47)CA(15)	298	298
SSRY7	TGCTAAGCTGGTCATGCACT	TGCCTAAGGAAAATTCATTCAT	CT(26)	217	217
SSRY9	CCGTTATTGTTCCTGGTCCT	ACAATTCATCATGAGTCATCAACT	GT(15)	262	262, 274
SSRY11	TTCTTGTGTCGTGCAACCAT	TGTAACAAGGCAAATGGCAG	GA(19)	249	249
SSRY12	CCACAATTTCTACAT	AACTTCAAACCATTCTACTTC	CA(19)	261, 265	255, 265
SSRY13	CAATGATGGTAAGATGGTGCAG	GCAAGAATTCCACCAGGAAG	CT(29)	221	211, 221
SSRY14	TTGACCTTAGCACATTTAAGGATTC	TTTGCATCGATTCCATCATC	CA(6)	300	300
SSRY15	TGATGCAGGTAGCAAGGATG	TGAAAGCCTGCATTCAAACA	GA(24)	214	214
SSRY16	CTGGAAAGATGGGACGTGTT	GCACTGCAAAAATATCATCTTGA	GAAGAGG GA(13) CA GA(3)TGAATA GA(4)	223, 227	223, 227
SSRY17	CTTAGAAAAGAAATTGCATGTGAG	TGTCTGATCAAGCTGGTGACA	GT(13)N(69)GT(15)	242, 280	242, 280
SSRY18	GTGCTGCAAGGCGATTAAGT	GCTACAACTGATAGTTGCATGCTT	CA(13)N(90)GA GAA(2)CAGG GA(2)AGA(2)N(6) GA(5)	NA	NA
SSRY19	TCTCCTGTGAAAAGTGCATGA	TGTAAGGCATTCCAAGAATTATCA	CT(8)CA(18)	198, 200	198, 214
SSRY20	TGATGGAAAGTGGTTATGTCCTT	CATTGGACTTCCTACAAATATGAAT	GT(14)	144	144
SSRY21	CAACAATTGGACTAAGCAGCA	CCTGCCACAATATTGAAATGG	GA(26)	184, 194	184
SSRY22	CTTGCCACTAGAACAGCCAC	GGCGTGGACTAACCTGTTCT	GT(13)	281	281

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
SSRY23	GCGAGGTTCAAATATGCGAT	TTAGCCAGGCCACTGTTCTT	CA(17)	234, 254	248, 257
SSRY25	CGCATGGTTTGTCTCGTTTA	TGGCTACATGATAGCAACATCAA	GA(27)	292	292
SSRY26	TGCTAATTGCAGGAAATAGGAT	GCAGCTTTTTAGCATAACAATCAA	GA(18)	124	124
SSRY27	CCATTGGAGAACTTGGCAAC	CCATGATTGTTTAAGTGGCG	CA(14)	262, 268	262, 266
SSRY28	GCTGCGTGCAAAACTAAAAT	TTGACATGAGTGATATTTTCTTGAG	CT(26)AT(3) AC AT(2)	164, 180	154, 180
SSRY29	TGCCAACCAAACCATTATAGAC	TGGTAGCTTTTGAATATCTGATGG	CT(18)	264	264
SSRY30	CCATCCACTAGAAACTTTAAAAGCA	CAACTCAGCGGAGCTTTTTC	CT(22)	NA	NA
SSRY31	ATTGTTGTGGTTGCAGGACA	CTTCATCACGTGTTAATACCAATC	GA(21)	167	199
SSRY32	CAAATTTGCAACAATAGAGAACA	TCCACAAAGTCGTCCATTACA	CA(11)	286, 308	290, 302
SSRY33	CATGATTACCGCCAAGGCT	AACTCTTTTGACTGAAGATGCTGA	CT(18)	NA	NA
SSRY35	GCAGTAAAACCATTCCTCCAA	CTGATCAGCAGGATGCATGT	GT(3)GC GT(11) GA(19)	283	283
SSRY36	ATTCTCGTGAACTGCTTGGC	CAACTGTTTCAACCAACAGACA	CT(15)	128, 134	128
SSRY37	GGCCAGTAATTCCTCAAGGC	ATGGCAAAAGATCGAGCAAC	CT(33)	NA	NA
SSRY38	GTAGTTGAGAAAACTTTGCATGAG	GGCTGTTCGTGATCCTTATTAAC	CA(17)	104, 114	104
SSRY39	AATGAAATGTCAGCTCATGCT	TCAATGCATAGGATTTTGAAAGTA	CT(24)AT CT(3)AT(3)	292, 302	292, 302
SSRY40	TGCATCATGGTCCACTCACT	CATTCTTTTCGGCATTCCAT	GA(16)	NA	243, 249
SSRY41	TTTTCCAACAATCTGATACTCGT	TATCACAATCGAAACCGACG	CT(4)TC CT(3)CGCC CT(20)	268	268
SSRY42	TTCCTCCAAAGTTATCTAGAACCA	CAATCCTTGTAGTAGCCAGTCTCA	CACACG(3)CACGCGCACACG(3) CACGCG CA(4)CG(2)CA(12) GA(3)G(12 GA(9)	NA	NA
SSRY43	CCAGAGCATGGTCTTTCTGA	TCAGACGTTGATACCTCACTTCA	CT(25)	242, 254	238, 254
SSRY44	GACTATTTGTGATGAAGGCTTGC	GGTTCAAGCATTCACCTTGC	GA(28)	105, 110	105, 112
SSRY45	TCCAGTTCACATGTAGTTGGCT	TGAAACTGTTTGCAAATTACGA	CT(27)	208	196
SSRY46	CGCTAAAGAAGCTGTCGAGC	TCAGGAACAATACTCCATCGAA	CT(19)	265	265
SSRY47	TTGGAACAAAGCAGCATCAC	GGAGCACCTTTTGCTGAGTT	CA(17)	217	240
SSRY48	TCATAAAGCTCGTGATTTCCA	AGCTGCCATGTCAATTGTTG	CA(11)	287	287
SSRY49	TGAAAATCTCACTGGCATTATTT	TGCAACCATAGTGCCAAGC	GA(25)	266	266
SSRY50	CAAGTGGATGAGCTACGCAA	CCGCTTAACTCCTTGCTGTC	CA(6)N(6)GA(31)	256	256
SSRY51	GGATGCAGGAGTGCTCAACT	AGGTTGGATGCTTGAAGGAA	CT(11)CG CT(11) CA(18)	260, 278	260, 298

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
SSRY52	AACTGTCAAACCATTCTACTTGC	GCCAGCAAGGTTTGCTACAT	GT(19)	263, 267	258, 267
SSRY53	ATTTTCACCAACCGCAACTC	CCATGCAGTAGTGCCATCTTT	CT(8)CC CT(8)	139, 141	122, 141
SSRY54	TGCAAATGACAAATAACCATCTC	GCGACTTTCTGGATGGATTC	GT(18)GA(18)	127, 143	116, 127
SSRY55	TGTGGAGCTTGATTTTGCAG	GCAATTTGCAAAGACATACCA	GA(16)	136, 144	136
SSRY56	TTTTAGTTTAGTTTAGTTAGTTGCGCT	AACTCTTAATGGCTAAAATTATTGATG	GA(21)G GA(5)	123	123
SSRY57	TGTCATTGTCTGTTGACCATTT	TAACCTGCCAAGAACAAGGC	GA(21)	325	322, 325
SSRY58	TGGAATCCAATATTGATGACTAAGA	GAAGGACAAGCAAAGAAGCAA	GA(6)AA GA(31)	173, 218	173, 218
SSRY59	CGTTTGTCCTTTCTGATGTTC	GCAATGCAGTGAACCATCTTT	CA(20)	148	148
SSRY60	TTGCAATGATATCAACGGCT	CGGCCACCAACTCAAATAAC	CT(20)	111, 124	117, 124
SSRY62	CATTCTCCAGGAAAGTCATTTTG	AGCTCATGCCATACAAGCAA	GT(11)GA GT(4)GA GT(5)GA(9)AA GA(5)	276	276
SSRY63	AAGACAATCATTTTGTGCTCCA	TCAGAATCATCTACCTTGGCA	GA(16)	285, 295	285
SSRY64	GCAGAGGTGGCTAACGAGAC	CGACAAGTCGTATATGTAGTATTCACG	CT(13)CG CT(6)	198, 200	190, 198
SSRY66	ATCTCAGCTTCCAACTCTTTCAGT	CGAAATGCTTGGAGACAGGTATAG	GA(19)AAGA	269, 275	275
SSRY67	AGTTTGCACCACCTTTTTCC	TGTCAAGTGATGAGCTGCTG	GA(20)TGA	268, 274	274
SSRY68	CAGCTGGAGGACCAAAAATG	GCTGCAGAATTTGAAAGATGG	CT(12)CC CT(17)	264, 283	283
SSRY69	CACTCCGTTGCAGGCATTA	CGATCTCAGTCGATACCCAAG	CT(18)ATT AT(2) CTTT CTT CTTT(2)CCTTCT	234	216, 226
SSRY70	CGCTTGTTGTATCCATTGGC	CGCTATTAGAATTGCCAGCAC	GT(18)	234, 242	234, 242
SSRY71	TGAAAGCCTGCATTCAAACA	TGATGCAGGTAGCAAGGATG	CTT(5)N(10)CT(9)TT CT(15)CCT	220, 222	220, 224
SSRY72	TTTTGCTGTGCTATTTCTGAGC	AAGCATCAGTGGCTATCAACA	CA(10)	136, 144	136, 144
SSRY73	AAGTTGATGGTTCTGAATCTGGA	ACAGTGATTGAGCGAGGCTT	CT(30)CCT	NA	NA
SSRY74	GGTCAGGTGAGTAATAAAGAACAGTG	TTGCTCGAATTCCACACAAT	CT(21)	111, 113	111, 113
SSRY75	TTCATGCACGTCCTGATACA	TCTGGTAAACCTACTAGTGCTCCA	GA(23)	264, 286	284, 286
SSRY76	CATGATTTGGATTTTGGAATGA	AAAGGAAGCAACCTTCAGCA	GT(17)GA(21)	264, 278	252, 282
SSRY77	GCATGTTCCACCTGCATAAG	CAGGAGGTGGCAGATTTTGT	GT(20)CT GT(2)	268, 276	276
SSRY78	TGCACACGTTCTGTTTCCAT	ATGCCTCCACGTCCAGATAC	CT(22)	241, 251	251
SSRY79	CAGCATCAGAAAGACAAAAACAA	CAAACCAATGGTCATGCTGT	CT(19)T CT(7)	198, 212	208, 212

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
SSRY80	TTCCTGGAAATGTCCTTAGATG	TGGCACATGCAACAATTAGC	GA(25)	NA	NA
SSRY81	TGATTTTCTGCGTGATGAGC	GGCGATTTCATGTCATGCTT	GA(22)	204	184, 204
SSRY82	CACCATCGGCATTAAACTTTG	TGTGACAATTTTCAGATAGCTTCA	GA(24)	192, 210	192, 210
SSRY83	TGCTTACTCTTTGATTCCACG	TGGCTAGATGGTGATTATTGCTT	GTCGTG GT(22)CT GT(2)	242	208, 242
SSRY84	AGAACTTCATGCACACAAGTTAAT	TTCCTTTCATTCATCCTGGC	GA(24)	200, 202	174
SSRY85	AAGAATACTATACGGACTACATGCCA	AAGGTGGCAGCACTTTTCTG	CT(27)	281, 293	273, 293
SSRY86	GACACCTGCTGATTCCGAG	TTGCCACATAGCAGAATCCTT	GT(4)GCGTGG GA(15)GG GA(2))	NA	NA
SSRY87	AGAGCACGCATTGTGCATTT	CTCATCTCATGAAGAACTTGTGC	GA(18)	91, 99	91, 99
SSRY88	CCAAGTCCTCACCTCCAAAG	CCTTGATGTGGCCAAAGTG	CA(7)TACATA GA(22)	263, 265	263, 265
SSRY89	AGTTGAGAAAACCTTGCATGAG	GGCTGTTCGTGATCCTTATTAAC	GT(19)	NA	NA
SSRY90	GCGATTTTGCGAATTACCAC	AGGTTATGGCGGTGGCAG	GGA(5)TAT GGA(2)	195	195
SSRY91	TGCCTGCTTCATATGTTTTTG	GTCTGCATGGCTCGATGAT	GA(16)	302, 306	294, 302
SSRY92	TCGGCTTAAGGTATGAACGC	CCAATGCTCAGTTTGACAACTC	GT(14)	158, 287	158
SSRY93	CAGATTTCTTGTGGTGCGTG	TTTGTTGCTCACATGAAAACG	CT(25)	283	264, 283
SSRY94	GGTGGAAGTAAGGCTGTTAGTG	AGGATGGACTTGGAGATGGA	CA(23)TC CA(3)CT(3) TCCTTCCAT CCT(2)CT(2)	275	236, 275
SSRY95	CATGATTTGGATTTTGGAATGA	CAAAAGAAGCAACCTTCAGCA	CT(19)CA(16)CC CA(2)CC CA(3)	286, 300	286, 300
SSRY96	CTCCATGTTATCCAAGGTTGC	CTTTACCTGCATGCCATTGA	GT(12)	152, 156	152, 156
SSRY97	GAGCAATCAAATTCAACAGCA	AAGCCGAAGCTTATGAAGGA	GT(12)	217, 220	217, 220
SSRY98	GTGATTGGTAGTGGTGGCCT	ACCAATCCAAGCTGCAAATC	GT(11)	213, 221	209
SSRY99	CTTGCTTTGGTTCCAATTATTTA	ATCAAGGCGCAAAAGTCAAT	GT(15)GA(13)AA GA(3)	180, 186	180, 184
SSRY100	ATCCTTGCCTGACATTTTGC	TTCGCAGAGTCCAATTGTTG	CT(17)TT CT(7) CCCT	190, 200	200
SSRY101	ACACACAATCACCATTTC	AAATACCACCACAA	CT(13)	217	211, 217
SSRY102	TTGGCTGCTTTCACTAATGC	TTGAACACGTTGAACAACCA	GT(11)	178, 180	178, 180
SSRY104	TTCTTGATATGCGCAACAGC	AGGCCATGGCAATTACTGAA	CA(4)GA CA(2)C CA(4)TAG A CA(16)GA(2)GATA(3)GA(21)	251, 255	245, 251
SSRY106	CACAAACCATCACCATTT	AAACTCTTCACAAAA	CT(24)	245, 269	245, 269
SSRY107	TTTTAATCCTATAAAATCCTT	CCATTTTCTCTTCTTCTTCA	CT(23)	89, 93	89, 115

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
SSRY109	TGCTAATTGCAGGAAATAGGAT	GCAGCTTTTTAGCATAACAATCAA	CT(20)CCT	148, 168	148
SSRY110	ATCCACCTTAAAACA	TTATTAATCAAA	T(12)	248	248
SSRY111	GCATCTTACATCCAGAATACTGCT	GAAGGAATGCCTGGCTTAAA	GA(29)	218	218
SSRY112	ACAATCAAAGGAGTCGTGTAATC	CGCAAGGTAAATCGGAGCTA	CT(15)C CT(3)	NA	NA
SSRY113	TCAACAATTACTAACAC	TTTCTACCTCCACAATA	A(19)	194, 204	194
SSRY115	TGCCATCACAATTTTGCCTA	CAACCGCTTTCGATGGTATT	GA(8)	NA	NA
SSRY116	CGTTTTCCTGTTAAATCTTGCAT	TAGAGCAGCTGCAAAGCAAA	GC(5)A CA(7)	NA	NA
SSRY117	GCAAATGTGTTTTCAATATAAGGC	TAAAGTTTGGCATGCCTGTG	GA(12)GTCA GA(3)N(32)GA(5)	143, 157	143, 157
SSRY119	CAAATTTTTTCAATATAAC	AACATACATTAAATTTCA	GT(2)ATGTGC GT(3)N(123)GA(5) A(8)(3)A(3)N(4)A (3)N(32)A(5)T(2)ATTC T(3)	169, 171	157, 171
SSRY120	TCACCGTTAATTGTAGTCTGCG	GCGAGGTTCAAATATGCGAT	CA(7)AA GA(8)GGA	NA	NA
SSRY122	TCTTTTTATCCTT	AACCAATTTTTATTC	A(12)T A(3)T A(3)T A(6)N(41)A(14)	275, 283	281, 289
SSRY123	TTCAACAATAAAGCTCAGAAAGAG	AGCAGATCCAAATCACTGAAA	CT(14)	135, 143	131, 135
SSRY124	TGGCATCAATTTTTGCTTCA	CTGCTGGACGGAGGATTCTA	GA(9)A GA(2)AGA	141, 143	141, 143
SSRY125	GCATGTTAGAAGTTTTTGCAATTT	CAGGACATGACGCAATTCTG	GT(2)T(8)GT(6)	248	248
SSRY126	TTGAAATACGGCTCAAGCTC	AATGGATCATGTTCAATGTCTTC	GT(2)T GT(5)GC(4)	250	250
SSRY127	CTTCGGCCTCTACAAAAGGA	GCTGAACTGCTTTGCCAACT	GTGC GT(8)GC(5)	NA	NA
SSRY129	AATGGATCATGTTCAATGTCTTC	CTTTTTGCCAGTCTTCCTGC	GC(3)G CA(6)A CA(3)	202	202
SSRY130	CTTTTTGCCAGTCTTCCTGC	GGTCCCTGATAGTTGATAATGGAT	GT(2)T GT(5)GC(3)	227	227
SSRY132	TGTCCAATGTCTTCCTTTCCTT	CTTTTTGCCAGTCTTCCTGC	CA(6)ACA(2)	194	194
SSRY134	GCAAGTTCAAAAGGAGCAGC	TCCACAAAGATAAGCTAAGCG	CAGA CA(4)GA(4)	211	211
SSRY135	AACATGTGCGACAGTGATTG	CCAGAAACTGAAATGCATCG	CT(16)	234, 248	248
SSRY136	AGCATGTCATTGCACCAAAC	CGACTGCATCAGAACAATGC	CAA CA(2)TA CA(4) CAAA(2)CAA	294	294
SSRY137	TTTTGATAGATCAAGAGAGTTTTTGAA	TAGTTAGCTCGGTTCGTCCG	ATT(3)ATTT ATT(2) T(9)N(9)AT AAT(4)ATAAAT	159	159
SSRY138	TTCAGGAAACATGCACAAACA	AGAATGTCTCTTTATTCTTGACAATTT	TA TTA(8)CT(5)	NA	NA

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
SSRY140	GGCACTTTGGAAAGGAAGAG	CAGTGAGCAGAAACTAAAAACATTG	ATT(4)CATTATTTA TTATTT ATTTT(3)ATTT	NA	NA
SSRY141	TCCAAAATCTTGGTCATTTTGA	TGCTGTGATTAAGGAACCAACTT	CT(8)CG CT(2)	278, 282	272, 282
SSRY143	AGCAGATCCAAATCACTGAAA	GCTCATGAACTGAGCCTTCA	GA(12)	156, 164	152, 156
SSRY144	GCTGATAGCACAGAACACAG	TAATGTCATCGTCGGCTTCG	n/a	125	116, 125
SSRY145	CGATGATGCTCTTCGGAGGG	GATTCCTCTAGCAGTTAAGC	n/a	136	136
SSRY147	GTACATCACCACCAACGGGC	AGAGCGGTGGGGCGAAGAGC	n/a	100	115
SSRY148	CAATGCTTTACGGAAGAGCC	GGCTTCATCATGGAAAAACC	n/a	112, 118	110, 112
SSRY149	TGTGGAGTTAAAGGTGTGAATG	AGCAGAGCATTTACAGCAAGG	n/a	150, 180	180, 182
SSRY150	AGGGTGCTCTTCAGAGAAAGG	CAATGCAGGTGAAGTGAATACC	n/a	164	164
SSRY151	CCCATAATTGATGCCAGGTT	AGTGGAAATAAGCCATGTGATG	n/a	215, 221	184, 186
SSRY152	CTCTAGCTATGGATTAGATCT	GTAGCTTCGAGTCGTGGGAGA	n/a	NA	NA
SSRY153	CTCAACTACTGCACTGCACTC	TTCCAGAAAGACTTCCGTTCA	n/a	95, 113	113, 117
SSRY155	CGTTGATAAAGTGGAAAGAGCA	ACTCCACTCCCGATGCTCGC	n/a	148	148, 156
SSRY156	GAGCCACATCTACTCGACACC	TTCAAGGAAGCCTTCAGCTC	n/a	162, 172	158, 162
SSRY158	CCAAGTCCTCACCTCCAAAG	CCTTACTTGTGTTTCTTACTGACAAG	CT(23)ATGTAT GT(7)	162	162
SSRY159	GACAATTGCATAGGAAGCACA	CTTATCCTGTCCCCTCCACC	CT(4)TT CT(16)TCT	154	154
SSRY160	GGCAAGAGAAGCCATAAAGC	CTGGCTCTTCCAGACACCTT	CT(30)	106, 128	108, 152
SSRY161	CCAGCTGTATGTTGAGTGAGC	AAGGAACACCTCTCCTAGAATCA	CT(11)TT CT(21)CA(19)	215, 221	215, 221
SSRY162	AACTCTTAATGGCTAAAATTATTGATG	TTTAGTTAGTTGCGCTAGCTTCC	CT(28)	NA	NA
SSRY163	AGGCCTCCAACAATTAGCCT	TCATGATGCTATTCCAAGTGTG	CTTCTCC CT(21)CA(19)	NA	NA
SSRY164	TGAGATTTCGTAATATTCATTTCACTT	TCAAACAAGAATTAGCAGAACTGG	GA(29)	166	166
SSRY165	GGTAAACAAATGATGTGGTGTTC	AAATGAGTTGCAAAGGCCAA	GT(17)GA(18)	247	247, 249
SSRY166	TATCCATGACTGTGATGCGG	AATAACAACAAGAGTTGTGGAAAAA	GA(16)GGGA(2)GA(2)	249	249
SSRY167	AAGGAAAGGGAGAAATCAAAGA	AAAATTGGATGGGACCGTTT	CT(27)	142, 158	158, 196
SSRY169	AACGTAGGCCCTAACTAACCC	ACAGCTCTAAAAACTGCAGCC	GA(19)A(3)GAA(2)	99, 101	101
SSRY170	TCTCGATTTGGTTTGGTTCA	TCATCCTTGTTGCAGCGTTA	TA(5)N(71)CT(24)	305	305
SSRY171	TCATGAGTGTGGGATGTTTTTATG	ACTGTGCCAAAATAGCCAAATAGT	TA(5)CATA GATA(8) GC GA(23)GTGA(2)	290, 292	290, 292

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
SSRY172	TTTAGTTTTTGAAACAATGATGAAA	TCCAACTGGCTTAACTTGAGG	CT(17)	179	206, 284
SSRY173	TACCTTTGGTGGAGTTTGCC	TGTAAATATGCAAAGAAGCACGA	GT(3)GC GT(2) GA(20)GG GA(2))	NA	NA
SSRY174	TTGCATACTCATCTCCATCTTCA	AACAAAACCATTTTCATGTTGA	GA(16)	125	123, 125
SSRY175	GCTAACAGTCCAATAACGATAAGG	TGACTAGCAGACACGGTTTCA	GA(38)	85, 97	85, 97
SSRY176	TTTTTCAAAATAGAGGGACCAA	TGGCTAAATTATTGATGTTTTAGTGT	GA(19)	NA	NA
SSRY178	CTGCAAAAACACGATCCCTT	GGCCCGTAAGGTTTACAGAG	GA(20)N(123)GA(6)	178	178
SSRY179	GCGAAAGTAAGTCTACAACTTTTCTAA	CAGGCTCAGGTGAAGTAAAGG	GA(28)	182, 204	184, 225
SSRY180	GGGGCATTCTACATGATCAATAA	CCTTGGCAGAGATGAATTAGAG	GA(16)G(4) GA(5)	116, 120	120, 130
SSRY181	CAATCGAAACCGACGATACA	GGTAGATCTGGATCGAGGAGG	GA(22)G(3)C GA(3) GGAA GA(4)	187, 191	191
SSRY182	TTCCTTTACAATTCTGGACGC	GGAATTCTTTGCTTATGATGCC	CA(17)N(31)GAGG GA(8)	226, 234	226, 230
SSRY183	TTAACTTTTTCCAGTTCTACCCA	TGCTGTGATTAAGGAACCAACTT	GAGC GA(8)	212, 218	208, 218
SSRY184	CTCCGACAAGCATGTGAATG	TCATCCCAAAAATACCTCTAACA	ATT(4)T ATT(3)T(7)	160, 169	160
SSRY185	ATGCCAGTTTGCTATCCAGG	GAAGAAGACGGTTAAAGCAAGTT	GC(3)AC GC(2)A CA(2)N(3)CA(10) GA(8)	239	239, 241
SSRY186	AATGACCATGCCAACACAAG	GCTTTGTGTAAACAACCTCGC	CA(13)	105	105
SSRY191	TTTCATGAACAACTTTTCTGGGTA	TGTTGGCTGAGAAAGCAAGA	(GA)8GTGA(GGGA)2GGA	202, 204	202, 204
SSRY192	GCCAATGCTCCATTTTCAAT	TTTCCAATCCACCATTTGCT	(CT)12	198, 216	208, 216
SSRY195	CGCTTACAACACCACCTTCA	GCTTGATCTCAGCCATGTCA	(GA)6CA(GA)6CA(GA)3	223, 227	215, 223
SSRY200	CCAACTCACCTTTCAACCAGA	GAAGCCAACCCATCATCTTC	CT(CTT)4N6(CTCTT)2CT	244	234, 244
SSRY203	AGGAAGCTGTCCCCTATTCA	TACCAGCAAGACCATCACCA	(CT)19	257, 281	257, 281
SSRY204	TCTTTGCTGGTGAGTTTTGC	GAATGCCTCCCAAACATCTC	(GA)11	178	178, 184
SSRY205	CTGTCTTGATTCCGGCAACT	GCAAGTCGTTGCCTACCTTG	(CT)11	225, 247	225, 233
SSRY207	TGCCCTTCACCAGCTAGTTT	ACAATTCCTTTCGCCTTGAA	(GCA)8A(CAA)2	NA	NA
SSRY209	ACGAGGATGAAGACGGTGAA	TCCTCTCAATAGAGCCTCAACC	(GAT)6	NA	NA
SSRY210	GACCAGTTGCTGTTCGTCAA	TTGCCGGCTTTTCTGATTAC	(CA)4(TA)4	217	217
SSRY219	CGAGAACAACAGGGTTCTACA	GCTCTCTTGGGGAGGTGTCT	(CA)6(GA)6TA(GA)10	195, 201	195
SSRY220	TGCTCTCTGATCTTGCACTAGC	GTTGCATCAATGCCTTCAGT	(TA)6(N)8(GTTGT)3GT	194, 196	194
SSRY222	TAAGGCAACGGGAAACAGAG	GCAATATTCTTCCCAGCGAAT	(CT)5CG(CT)2CG(CT)2CG(CT) 2CG(CT)2CGCT	NA	NA

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
SSRY223	GCATCTGTTTCCTGATTGTTTG	CGGGGTTTGGAAATCAGTAA	(CT)8(C)2(CT)4	192	192
SSRY225	GAAAATTGCAGAGGCTGCT	TTGCACCTGAGTTCCATCAG	(GA)20	232	226, 232
SSRY226	AACTGGAGTAGAGAAACTGGAGGA	AACGTTTCGCCGATTACAAG	(GA)8(N)7(GA)5	220	217, 220
SSRY227	CCAAGGTTGTTGGGTAAGGA	CCCATCAATGGAAACCTCAC	(GCA)4(N)3GCA	200	200
SSRY228	TGGCAGAGATTTTGAGACGA	TTTCTTATCCTCCGCTATCCA	(CT)21	NA	NA
SSRY229	AGTCCAGCCTCTTCCTTCGT	TGATCAGCGAAATCGTGGTA	(CT)9TT(CT)5	182, 188	188
SSRY230	CCATCTCTCTCCCTCTGCAA	AAATGGCAGGAGATTGATGC	(CT)19	178, 184	180, 184
SSRY231	CCACAGAAGAACACCAAACTG	GGGCGCCTATTACTGTGAAA	(GA)7(G)2(GA)4	288	288
SSRY233	CGAAACGATCGAAGTTCCAC	TGTGGCCATCACACTCATTT	(CT)9	206	206, 210
SSRY235	CAGCTTTGCCATCCAATTTT	CAGCAAAATGACATGAGTGTATCTC	(CA)8(N)13CAGA(CA)6(GA)2	222	222
SSRY236	TGAAAAACGTTCCTTCCCTTT	CCGTATCCTATGGCAACACC	(CT)2T(CT)13	185	185
SSRY238	GGCCTTAAGCCACCATTCTA	GCTTCTGCGAATTCGTTTCT	(CA)4CC(CA)7	208, 216	216
SSRY239	TGCATTTCCCTGGGTGTAAG	TTTCTCAATAGACAGACGAGCA	(GA)15GG(GA)2	190, 202	190, 202
SSRY240	TCGGCTTTTAACATCCTTCG	AGCTAGGAGCAACGCAGTTC	(GA)22	160	166, 272
SSRY241	GTAGGGCAATGGTGATTGGT	ACCCAGCCATACACTAGCAA	(CT)13	227	225, 227
SSRY242	TGGGTTCGAAAACAGCAAAC	TAATGCCTGGAGGGTAATGG	(CT)5AT(CT)16	297, 309	309
SSRY243	GAGAGAGTACGTCACAGAGATCG	TCCAGCAGGATAAACATCCA	(GAA)4(GA)2GAA	NA	NA
SSRY246	TCCCCTCAGATGCTCAAGAC	TGTAGGGCTGAGCAAGTTCC	(TA)6(TTA)2(TA)3	198	198
SSRY248	TGTTTGCTGAGTGCAGTCCT	CCTCGTACAGAAAACTCAAGCA	(GA)12	237	237
SSRY250	GATCGGATGTCTGAGGAGGA	AATTGGAAGGGAAAGCCAAA	(CT)18(ATCT)2CTAT(CT)2	175	175, 177
SSRY251	AGTCTCTACAGCCAAGTAGCATCT	GCGTTGACGACGTCCATAAT	(CT)8	230, 234	230, 232
SSRY252	GACGCGAGAACTGACAAGTTT	CCCACCTCCGAAATCACTAA	(GA)5AA(GA)10	206, 214	206, 214
SSRY253	TCCAGAGGGAGAGATCTGACA	GCTGATGGAGGAGTAGCAGTG	(CT)11	182, 186	182, 184
SSRY254	GACGCATCTCCTGCACAATA	TGTCAGATCTCTCCCTCTGGA	(GT)6	225	225
SSRY255	GAAGAGATGCGACGATGGTT	ACCAGCACTGTTGTGTCCAG	(GAA)6GA	183	183
SSRY256	AGAACAAAGGGCTCCATTCA	GGTGGTGGAGGTCCTGATTA	(GAT)7	202	202
SSRY261	CAGAGACTTCTCCGCCTGTT	TCAAAAGAGAGACGCACAGGT	(CT)9(N)81(CT)14	193	173
SSRY262	TTCAGTTTCAGGAGACAGAAAGG	CTCGACATTCCCTTCACTTTG	(GA)11	126, 128	126
SSRY265	GCTGCTGGTCAAAAGAGTCC	CTGCTCCACGCTGTTTATCA	(TA)8(CA)9	209, 213	209, 213

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
SSRY266	TCTGGTTTTCCACAGGGAAC	TCGTTAGGCCCAATTCGTAG	(CT)5	190, 200	190, 200
SSRY268	CCAATATAAGCGGCGTCATT	GCAATATCTTCTTGGAGTTCAATTCT	(CT)18	NA	NA
SSRY269	AATAGTTTCAGGCAAGGGTGA	TCAATCACAAGCCAGACACA	(GA)55(N)22(GA)36	158, 172	158, 172
SSRY270	CGAAGCTCCGCCTCAGTAT	CCAGAAGTGGACCAAGTGAGA	(CT)10TT(CT)3	228, 236	228, 236
SSRY271	CTGGTAACCGATTCCACACC	GCAAGCTCTCCAATGACCTT	(GGT)4(GGA)6	298	298
SSRY272	ACTCGTGGGATCTTCCCTTT	CTGGAACCTCTGGCTCTCAC	(GA)18	188, 192	192
SSRY274	TTCTTCCCCGTTCACGAAT	TCAGCGACATCTCTCTTCCA	(CT)17A(CT)2	227	227
SSRY277	CTGAGTGGTGATTCCGCTAA	GGTTTGGGTCCGGATTCTAT	(GAA)4GA	188	188
SSRY279	GGTCCTGCCTTGCACTGTAT	ACGCGTGGGTTTTATGCTT	(CT)5TT(CT)2	NA	NA
SSRY280	AAGTCGTTTATTGCCGATGC	TGTGCATGGAGAGATTGACAG	(CT)3CC(CT)2CC(CT)2CC(CT)7	NA	NA
SSRY282	CCATCCCCAACAAACAAGAT	TCCAACACTACGCTGTGTTTCT	(CT)10TT(CT)5	193, 205	193, 195
SSRY283	TGGAACTCAGTGAAGCGATG	AAAAGCGTGGGCTTTTGA	(GA)11	NA	NA
SSRY284	TCCTTCCAAAGCCAGACTTG	GGAACATTTTAGCGGTCAGG	(CT)12	201	201
SSRY286	TGGGCCCAGAGATAGAGTGT	ATCCACAATCCTCCCTACCC	(CT)5(N)3(CT)5	203	203
SSRY287	GGCTTTGTTGTGGTTGTTGA	CATGAATTAGAAACCAAGGGAAG	GAA(GA)2GAAGA	205, 207	205, 207
SSRY288	TCAGGCATCCCTAATATTTCTTTC	TGGTTTAAGGTTTGGGCGTA	(TA)3TTAAATA	178	178
SSRY289	ATCTTCTTGGTGGGTCATGG	AACACCCAACAGCAACATCA	(CCA)3ACA(CCA)6	188	188
SSRY291	AAAGGACCCTCCCTCTTTCC	TGGGTTCTGGATTTGTTCAAG	(CT)3CC(CT)8	NA	NA
SSRY295	GCTTAGCTTATCCTTGGACCTTG	AGACAAGCACCCACCAGAAT	CTC(CT)17	185, 197	195, 197
SSRY296	TCAATTTGTTTTTCCCTGCTG	TTTTCTGAGGCTGGTGTCCT	(CT)3TT(CT)2(GT)7GG(GT)3GGT	292, 294	292, 294
SSRY297	TTGTGCTCCAATGAAAATGG	CCCAGGCACAGGTACTCTCA	(GA)19	190	190, 204
SSRY298	GCCTTGTTCTGATCCATGCT	TGCCTGAATCAGTGTCTTCAA	(GT)9	181, 207	181, 207
SSRY299	ATGCGAAACAGAGAGGAGGA	TCTTTTTCACAGAACCAACTGAA	(CT)10	207	207
SSRY300	CCATTTCCAGGCATTTCATC	ATAGAAGGGCCCGAGACTGT	(GAA)5GA	294	294
SSRY301	GAACGCTTCAACGGCATAAT	CCAATGCCAACAACACTTCTT	(CT)10	324	324
SSRY302	CAAACCCAAACCCAGAGCTA	CGCTTGCCTTCTCTTTCTCT	(CT)19	240, 242	240, 242
SSRY303	GCATCATCACCATTTTTCTTTG	TGGAAGGTGTTAGCAACTGTG	(CA)34(N)7(GT)6	217	217, 231
SSRY305	AAGCTTGGCTAAGGTTCTGC	AAAACACAATCAAATCCCTCA	(CT)22(CCT)7	209	209
SSRY306	GGACAGCCTCGTCATTTCAC	CGGAGTGCTCCTCTCCATTA	(CT)3C(CT)17CG(CT)2	296, 298	296, 298

Appendix I Continued.

Marker	Left primer (Forward 5' - 3')	Right primer (Reverse 5' - 3')	Repeat motif	Namikonga	Albert
SSRY309	TCCTCCTCCCTCTTCAGATTC	GCCACAGGCTAAGGAAACAA	(CT)11CCT	152, 198	196, 198
SSRY311	GCCAACAGGAATCCTTCTGTA	GAAACCAATGCAGTTTCACAA	(TA)2(T)3(GT)9A(TA)10(T)2 (TA)2(TGTA)2(TA)3	203	195, 203
SSRY312	TGATTGCAGCAGAAAGCAAG	ATGGAAGGCTTGAAGTGGTG	(CT)13	204	204
SSRY313	ATAGCACCCCACCACCTGTA	GCGAACTTTGCAGCTATTGA	(CT)14	212, 232	230, 232
SSRY314	CAAACATTGGCAAAGCTTCA	GCTCTCTCAAGCGCAGATTT	(CAT)7	189, 193	189, 193
SSRY316	ACATCGTGAGGTCCAAGAGG	GAGCTTTACGGTCTCTGTCTCTG	(GT)12	195	183, 195
SSRY317	CGAAGAAGCCTCATTCCTGA	CCTCTGCTACTGGCTATTTTCA	(CT)12	220, 224	224, 226
SSRY319	CCAATCCATCCTTCCTCTCA	AGGAAGCAAAGACGACCTGA	(CT)9	202	202
SSRY320	GTTGATGAGCTGTGGCATTG	CCTAGACGAAGTGGGTCGAA	CTT(CT)2(CCTCT)2TCT	NA	NA
SSRY323	GCTTGCTCAGCCTTGAGTATT	TTGCCATGGCTACAAAAGAA	(CT)14	NA	NA
SSRY325	GCTATTGCTGTGTGGTCCAG	AGCCAAAAACCATACCCACA	(GT)2AT(GT)7	234	234
SSRY328	GCCCAATTACCAAAAGCTGA	CTGCTGCCACCACTCTGATA	(GA)26	229, 237	237, 249
SSRY330	TTCTTCTTCTTCCCCATTGC	CCACCATCATCATCGTCATC	(CT)2TCTC(CT)8	NA	NA
SSRY331	TCAGATCCCTCGGTTCTCAG	TGCTTTGCTTCTCAAGTCCA	(CT)11	280, 296	286, 296
SSRY332	CCACCCCATCTTCAACATTT	CAGGCTCGAGGTCTTCTTTG	(GA)11	239, 241	239
SSRY333	GGTTGGAGGAGCCATAGGTT	TACTTTGGGCCTTCCTTTCA	(CT)11	NA	NA
SSRY336	CATTTTCAGCAGCCTTTTCC	CAGTCTCTAACGATCCCCTCA	(CT)17	NA	NA
SSRY337	AGATCTTTGCACGTTCACCA	TCGCTCACAAAAACAATCCA	(CT)11	212	210, 212
SSRY338	CTCTACTCGGCATGGATTGG	AGCTCCGCTAAAACACATGC	(GT)6TT(GT)4	NA	NA
SSRY339	CGCACCAACCTCATTTATCC	GGCTTCACAGCCGTAAAAGT	(CT)17CCT	221, 233	221, 229
SSRY342	GGTTCGAAGCATTCTATGGTTA	TCTGCTTTTGGCTGGAATTT	(CT)18TTCT	194	194
SSRY343	CTCAATCCGCTCCATCTCTC	CACTCTTGTGCAGAACTTTGCT	(CT)16 (N)26(GA)11(N)30(GA)20	NA	NA

n/a = not available, NA = no PCR amplification