SOUTH AFRICAN SWEETPOTATO ASSESSING THE GENETIC DIVERSITY OF GERMPLASM USING DNA AND PROTEIN MARKERS

BY

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Dissertation submitted in fulfilment of the requirements for the degree of

MAGISTER TECHNOLOGIAE: BIOTECHNOLOGY

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June, 2013

The financial assistance of Hub and Spokes and National Research Foundation (NRF) of South Africa towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the author and are not necessarily to be attributed to the Hub and Spokes or NRF.

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This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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This dissertation is being submitted in partial fulfilment of the requirements for the degree of MTech Biotechnology.

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ACKNOWLEDGEMENTS

I hereby wish to express my gratitude to the following individuals who enabled this dissertation to be successfully completed:

- Firstly to God: For answering all my prayers, protecting me and sparing my life until today.
- Supervisor Prof Michael Pillay: For his kindness, patience, encouragement and continuous help and advice throughout the project.
- Co-supervisor Dr Patrick Adebola: For being my co-supervisor and providing the samples for this study.
- Hub and Spokes and NRF: For their fellowship and financial assistance.
- Vaal University of Technology: For allowing me to do my research in their laboratory.
- Lindiwe Zulu and SM Laurie: For helping in sample collection, identifying them and for providing important literature sources which assisted me in writing the dissertation.
- Samkeliso, Laurette, Steven, Tara, Dr Takaidza, Dr Okosun and Dr Grobler: For helping me with the experiments, sequencing analysis and statistical analysis.
- My mother: For taking care of Ofentse when I was at school; I would not have coped if you were not there.
- My father: For helping me collect some of the samples and for his support.
- My siblings and nephews: For their support throughout the project.
- My boyfriend Maqolo Matsoso: For his support and always being there when I needed a shoulder to cry on.
- Laboratory F 109, 305 and 310 students: For all the support and good times we had in the laboratory, especially Basadi, Samkeliso, Nnana and Doreen.

DEDICATION

I dedicate this work to my parents, Daniel and Mabel Selaocoe who God have spared to live to see the fruits of their hard work, endless love, sincere moral support and words of wisdom. To my little boy, Ofentse Karabo Selaocoe, I hope this work will be a motivation and inspiration in your future life.

ABSTRACT

Sweetpotato is one of the most important food crops in developing countries including South Africa. Currently two major types of cultivars are grown in South Africa: one is the orange-fleshed sweetpotato (OFSP) which has high β-carotene content, a precursor of vitamin A. The second type is the cream-fleshed sweetpotato (CFSP) which has low β-carotene content but is high in dry matter. Most South Africans prefer the CFSP although the OFSP offers more advantages. This presents a challenge to plant breeders to develop new varieties that will combine the desirable qualities of both the cultivars. To achieve this goal, plant breeders need knowledge about the genetic variation of the crop to develop an efficient breeding programme. This study assessed the genetic relationships of 28 orange- and cream-fleshed sweetpotato accessions by (i) examining the variation in leaf proteins, (ii) using random amplified polymorphic DNA (RAPD) and, (iii) using variation of the ITS region. The analysis of proteins, RAPD and variation of the ITS region polymorphism levels were 55.6%, 98% and 16.5%, respectively. Dendrograms generated from all the analyses generally clustered the accession according to their flesh colour and country of origin. Analysis of molecular variance (AMOVA) found a significant difference between OFSP and CFSP and a significant difference between the South African and non-South African germplasm. The high genetic diversity in the South African sweetpotato germplasm is a positive indicator for a breeding programme that has a number of targets such as breeding for nutritional improvement, disease resistance and drought tolerance.

Key words: Sweetpotato, genetic diversity, molecular markers, protein markers, RAPD, ITS, sequencing.

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CHAPTER 1
GENERAL INTRODUCTION

1.1 INTRODUCTION

Sweetpotato (*Ipomoea batatas*), which belongs to the family Convolvulaceae is regarded as "the poor man's crop" in most African countries. It is often grown and consumed by resource limited households and ranks fifth as a food crop in developing countries after rice, wheat, maize, and cassava (Elameen, Fjellheim, Larsen, Rognli, Sundheim, Msolla, Masumba, Mtunda & Klemsdals 2007: 397; Veasey, de Queiroz-silva, Rosa, Borges, Bressan & Peroni 2007: 417).

Globally, sweetpotato is grown on an estimated area of 9 million hectares with the greatest contribution coming from Asia (80%), followed by Africa (15%) and 5% from the rest of the world (Loebenstein & Thottappilly 2009: 10). Both the leaves and the roots contain large quantities of energy and substantial amounts of minerals and vitamins. The carotenoids and phenolic compounds contained in sweetpotatoes provide them with their distinctive flesh colours which could either be cream, deep yellow, orange or purple (Woolfe 1992; George Mateljan Foundation 2010).

In South Africa, consumers prefer cream-fleshed sweetpotatoes (CFSP) which have a high dry matter content, low levels of β-carotene (precursor of vitamin A) and remain dry textured after cooking (Agricultural Research Council 2006; Carey, Oyunga, Osmambo, Smit, p'Obwoya, Turyamureeba, low & Hagenimana S.a: 2- 3). On the other hand the orange-fleshed sweetpotatoes (OFSP) are high in vitamin A and have low dry matter content. The OFSP is not preferred by most South Africans because it is moist textured when cooked (Leighton, Schonfeldt & Kruger 2010: 74; Loebenstein & Thottappilly 2009: 10).

The OFSP has received renewed interest in South Africa and other Sub-Saharan countries due to its nutritional value. Currently the OFSP is being used extensively to alleviate vitamin A deficiency (VAD) which affects millions of pre-school children and pregnant/lactating

women in Africa (van Jaarsveld, Faber, Tanumihardjo, Nestel, Lombard & Benade 2005: 1080- 1081; Ndolo, Nungo, Kapinga & Agili 2007: 689; Childinfo 2009). To encourage Africans to grow and consume OFSP, plant breeders have been developing OFSP varieties that conform to Africa's distinct preference for traits such as high dry matter content (Pray, Paarlberg & Unnevehr 2007: 137).

This strategy which is called biofortification is considered the best and most cost effective approach to alleviate VAD because it is able to reach rural and low-income communities, which are more at risk. Compared to other strategies such as the administration of VAD capsules, the β-carotene that is consumed from the OFSP is converted into vitamin A in our bodies and this conversion stops when the body has sufficient vitamin A. Therefore the β-carotene does not become toxic (Office of Dietary Supplements National Institutes of Health 2006).

To effectively breed sweetpotatoes that conform to the preferences of South Africans, knowledge about the genetic diversity of the crop is required so that an efficient breeding programme can be developed. Traditionally, genetic diversity of sweetpotato is assessed by examining the morphology of the plant. This approach has certain limitations since genetic information provided by morphological characters is often limited and expression of quantitative traits is subject to strong environmental influence (Rao 2004: 138- 139).

On the other hand molecular markers are considered best for analysis of genetic diversity. Molecular markers are used as complementary strategies to traditional approaches for assessment of genetic diversity. The major advantage of molecular markers is that they are not affected by environmental conditions (Rao 2004: 138- 139). Molecular techniques include biochemical based markers such as isozymes and DNA based markers such as amplified fragment length polymorphism (AFLP), microsatellites, single nucleotide polymorphisms (SNP) and random amplified polymorphic DNA (RAPD). Each technique has its own advantages, limitations and uses (Farooq & Azam 2002: 1132).

In the present study, the genetic diversity of South African orange- and cream-fleshed sweetpotatoes were assessed by examining variation in protein profiles, with RAPD markers and variation in the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) genes. The results acquired from this research will contribute to pre-breeding knowledge of

sweetpotatoes and they will assist plant breeders to develop an efficient breeding programme for the crop.

Protein markers are biochemical markers that analyse diversity based on examining protein profiles. The advantage of protein markers is that they are able to assess co-dominance; they are easy to use and are cost effective (Mondini, Noorani & Pagnotta 2009: 22). Protein markers were used to assess the genetic diversity of *Ipomoea* species using SDS-PAGE technique. The protein markers were able to reveal the relationship of the *Ipomoea* species at the subgeneric and sectional level (Khalik, Osman & Al-Amoudi 2012).

Random amplified polymorphic DNA (RAPD) is a polymerase chain reaction (PCR) based technique which involves the random amplification of a genomic DNA sample using random primers. The advantage of this technique is its sensitivity, simplicity, speed, low cost and no prior knowledge of the sequence of the DNA is required (Bardakci 2000: 185- 186). A number of scientists have used this marker to analyse the genetic diversity of sweetpotato (Zhang, Ghislain, Huaman, Rodríguez & Cervantes 1997; Harouna 2006; He, Liu, Ishiki, Zhai & Wang 2006).

The ITS1 and ITS2 spacer regions are components of the ITS region located between the 18S and 5.8S and between 5.8S and 28S rDNA genes, respectively. These spacers exhibit a high rate of variation and have become a powerful tool for assessing genetic diversity in plants (Huang, Corke & Sun 2002; Nwakanma, Pillay, Okoli & Tenkouano 2003). The advantage of using the ITS regions is that they have a high copy number which allows for high reproducibility (Poczai & Hyvonen 2010: 1897- 1899). Analysis of the ITS region was able to group the *Ipomoea* series *Batatas* into a monophyletic clade and this proved that ITS is a powerful method to study the phylogenetic relationship of sweetpotato and its wild relatives (Huang *et al*. 2002: 547- 549).

1.2 RESEARCH AIM

The aim of this study is to determine the genetic diversity of selected orange- and cream-fleshed South African sweetpotato accessions.

1.3 RESEARCH OBJECTIVES

The specific objectives of this study are:

1. To assess the potential of using total leaf protein profiles in diversity analysis of sweetpotato.
2. To determine the genetic diversity of selected sweetpotato accessions using random amplified polymorphic DNA (RAPD).
3. To assess the utility of the internal transcribed spacer (ITS) regions in determining diversity of sweetpotato accessions.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

This chapter gives a brief overview of the origin, dispersal and botanical information of sweetpotato. The international and local (South Africa) production of sweetpotatoes is also discussed. In addition, the use of sweetpotatoes in different parts of the world and their health benefits especially that of the OFSP are also outlined. The use of different molecular markers to assess genetic diversity in sweetpotato is addressed. Lastly the computer software packages which were used for analysing genetic diversity data are briefly outlined.

2.2 ORIGIN AND HISTORY OF SWEETPOTATO

Sweetpotato is a member of the Convolvulaceae family in section *batata* (Huaman 1992: 5). Sweetpotato was originally domesticated in the New World although its exact centre of origin and domestication is not clearly defined and neither has its wild ancestor been found (O'Brien 1972: 342; Zhang, Cervantes, Huaman, Carey & Ghislain 2000: 659). Archaeological, linguistic, morphological and molecular marker studies of sweetpotato point to an origin either in the Central or South American lowlands (O'Brien 1972; Woolfe 1992; Zhang *et al*. 2000).

The archaeological evidence dated at 2000 to 1200 B.C is based on the actual remains of sweetpotato which were found at the site of Ventanilla in the Chillon Valley of Peru. In addition, there exists evidence of old sweetpotato remains dated at 1400 to 1300 B.C from Peru's Central Coast and the tubers which were dated at 1300 to 1175 B.C. Linguistic evidence suggests that sweetpotato was present in the Mayan area of America which is between 2600 to 1000 B.C (O'Brien 1972: 342- 343; Woolfe 1992: 15).

Morphological evidence comes from the study by Austin (1988) cited in Zhang *et al*. (2000) in which he postulated that the centre of origin of sweetpotato is between the Yucatán Peninsula of Mexico and the mouth of the Orinoco River in Venezuela. This might be the area where *I. trifida* crossed with another putative ancestor, *I. triloba*, and produced the wild ancestor of *I. batatas*.

A study using AFLP markers showed that sweetpotato diversity was highest in Central America. Therefore Central America was considered to be the primary centre of diversity and most likely the centre of origin while Peru-Ecuador was considered the secondary centre of origin because it had the lowest genetic diversity (Zhang *et al*. 2000).

2.2.1 Dispersal of sweetpotato

Sweetpotato spread from the New World to the Old World in different routes but the best known is the post-Columbian spread by Europeans. In 1492 Christopher Columbus introduced sweetpotato to Europe with the name 'batata' and 'padada'. This name was later changed to Spanish potato or sweetpotato to prevent the confusion with the Irish potato. The Portuguese explorers of the sixteenth century carried sweetpotato to Africa, India, Southeast Asia and the East Indies where they introduced it with the name 'batata'. In the sixteenth century the Spanish trading galleons from Mexico introduced sweetpotato to the Philippines with the name 'camote'. Then in the late sixteenth century it was introduced in China where it was used to stave off famine (O'Brien 1972: 345- 360; Rossel, Kriegner & Zhang 1999: 315- 316).

The introduction of sweetpotato to the Pacific Islands occurred in prehistoric times and this is supported by the fossil carbonized storage roots which are dated 1000 years back found in northern New Zealand. However, studies based on molecular markers show that sweetpotatoes of Peru are not closely related to those of Papua New Guinea or Mesoamerica. It was then suggested that the sweetpotatoes of Peru came from Central America through non-human dispersal (O'Brien 1972: 345- 360; Rossel *et al*. 1999: 315- 321).

2.2.1.1 Introduction of sweetpotato to Africa

According to Conklin (1963) cited by O'Brien (1972), sweetpotato arrived in Africa in two ways. The first introduction was by the Portuguese in the fifteenth and sixteenth centuries. The crop was introduced in East Africa and West Africa with the names such as 'batata', 'tata', 'mbatata' which are found in African languages such as Berber and Zulu. The second introduction was by the British in the seventeenth and nineteenth centuries with the names 'bombe', 'bambai', 'bambaira', or 'bangbe'.

Today sweetpotato is cultivated as a staple food in more than a hundred countries in the world. Sweetpotato also plays an important role in combating vitamin A deficiency (VAD)

which mostly affects children and pregnant woman especially in Asia and Sub-Saharan African countries.

2.3 BOTANICAL AND CULTIVATION INFORMATION

The sweetpotato species was first described in 1753 by Linnaeus as *Convolvulus batatas*. In 1791 Lamarck classified sweetpotato under the genus *Ipomoea* which constitutes 600- 700 species. This classification was based on the stigma shape and the surface of the pollen grains and therefore the name was changed to *Ipomoea batatas* (L.) Lam (Huaman 1992: 5- 6; Borge, Rosa, Recchia, de Queiroz-silva, Bressan & Veasy 2009: 526). Within the *batatas* section there are thirteen other species which are considered to be closely related to sweetpotato. While sweetpotato is the only hexaploid (2n=2x=90) in the section, the majority of species are diploids (2n=2x=30) with a few tetraploids (2n=2x=30) (Huaman 1992: 5- 6; Loebenstein & Thottappilly 2009: 13).

Sweetpotato is a non-woody plant whose stem varies from green to red-purple. The leaves may be green, purple or green-yellowish. The shape of the leaves ranges from rounded, reniform (kidney-shaped), cordate (heart-shaped), triangular, hastate (trilobular and spear-shaped with two basal lobes divergent), lobed and almost divided (Fig. 1). Some sweetpotato accessions produce flowers while others do not. The colour of the flowers varies from green to purple (Huaman 1992: 10).

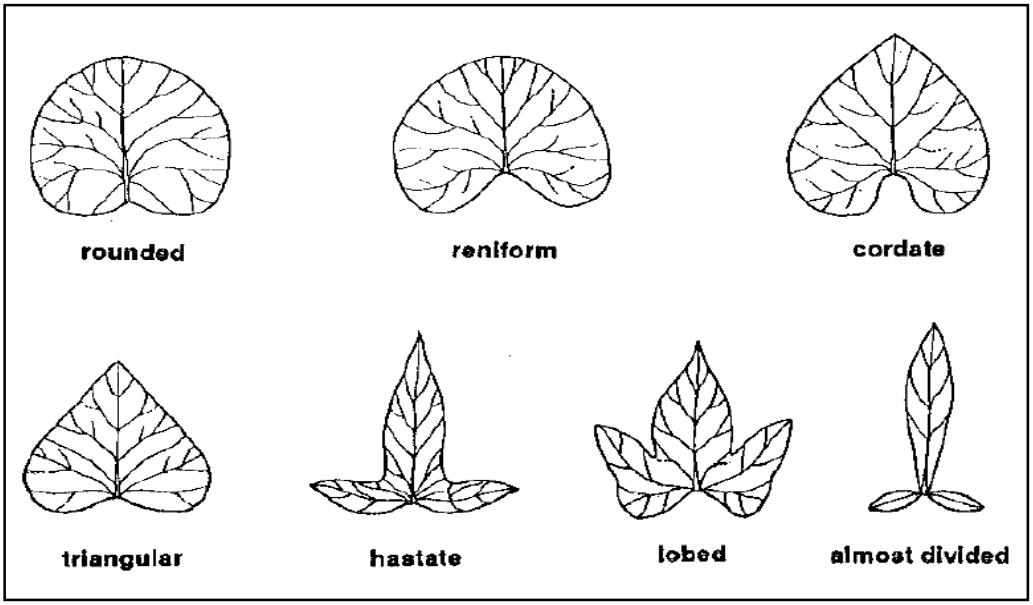

Figure 1: General outline of the sweetpotato leaf (Huaman 1992: 10).

The sweetpotato storage roots which are developed from the nodes of the stem form clusters around the stem. The formation of these clusters range from being closed if the stem is absent or short, open if the stalk is long and dispersed or very dispersed if they are formed at a distance from the stem. The surface of the storage roots is usually smooth but some may have alligator-like skin, prominent veins, horizontal constrictions or longitudinal grooves. The shape of the storage roots can be round, round-elliptic, elliptic, ovate, obovate, oblong, long oblong, long elliptic, long irregular or curved (Huaman 1992: 10- 19) (Fig. 2).

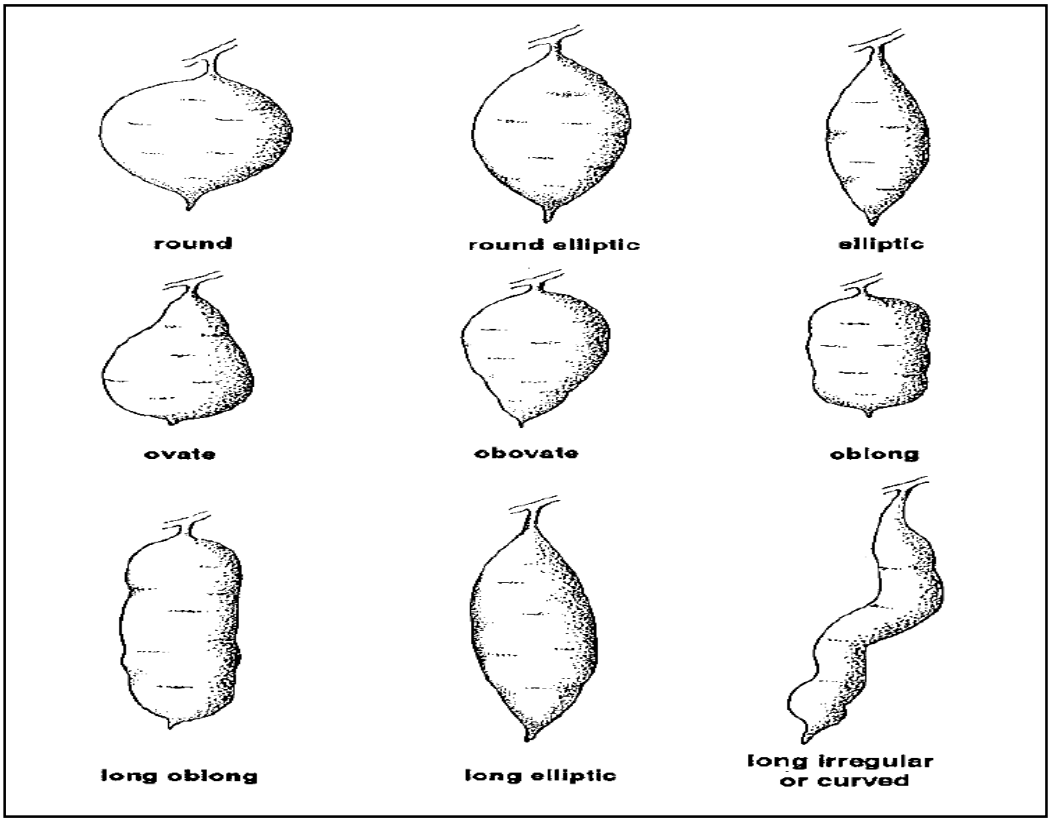

Figure 2: Types of storage root shapes in sweetpotato (Huaman 1992: 19).

The skin colour of the storage roots can be whitish, cream, yellow, orange, brown-orange, pink, red, red-purple and very dark purple. The flesh of the roots ranges from white, cream, yellow, orange or purple in colour (Fig. 3) and varies enormously in taste and texture (Huaman 1992: 10- 19). The cream- or white-fleshed sweetpotatoes yield less β-carotene (pro-vitamin A), have a high dry matter content, and are firm and mealy (Woodward 2003). The orange- and yellow-fleshed sweetpotatoes are rich in β-carotene, soft and moist. The purple-fleshed sweetpotatoes are rich in β-carotene and anthocyanins (George Mateljan Foundation 2010).

Figure 3: Sweetpotatoes with different flesh colours (North Carolina sweet Potato Commission S.a).

Sweetpotatoes are annual crops that are cultivated asexually from vine cuttings or sexually from seeds. They do not tolerate frost and grow best at an average temperature of 20 ^{o}C and higher. Sweetpotatoes require moist soil, a pH that is lightly acid or neutral to grow well and are harvested within 100- 150 days after planting (Department of Agriculture and Rural Development 2011: 20).

2.4 SWEETPOTATO PRODUCTION AND INTERNATIONAL TRADE

Sweetpotato is the seventh most important crop in the world in terms of production and ranks fifth as a food crop in developing countries after rice, wheat, maize, and cassava (Elameen *et al*. 2007: 397). Globally, sweetpotato is grown on an estimated area of 9 million hectares with the greatest contribution coming from Asia (80%), followed by Africa (15%) and the rest of the world (5%) (Loebenstein & Thottappilly 2009: 10).

China is the largest producer in Asia, producing 100 million tons of sweetpotatoes followed by Vietnam which is the second largest producer. The United States of America produce approximately 720 000 tons of sweetpotato per annum (Loebenstein & Thottappilly 2009: 10). In Africa, Uganda is the largest producer and the third-largest grower worldwide (Department of Agriculture and Rural Development 2011: 4). Rwanda and some other African countries also rank among the largest growers of sweetpotato (FAO 1990).

2.4.1 South African sweetpotato production and trade

South Africa is not a major sweetpotato producer and the main producing regions are Northern Cape, Western Cape, Limpopo, Free State, Eastern Cape and Gauteng (Department of Agriculture, Forestry and Fisheries 2010: 4). The production of sweetpotato in South Africa has not been stable between 2000- 2009 (Fig. 4). Production decreased in 2002 and 2003 while in 2004 it increased by 9.3%. In 2006, the production decreased by 14% and it was the lowest in ten years. The decrease was attributed to climatic conditions and the high cost of production. The production increased in 2007, decreased by 4% in 2008 and increased by 41% in 2009, being the highest increase over the ten years period (Department of Agriculture, Forestry and Fisheries 2010: 4).

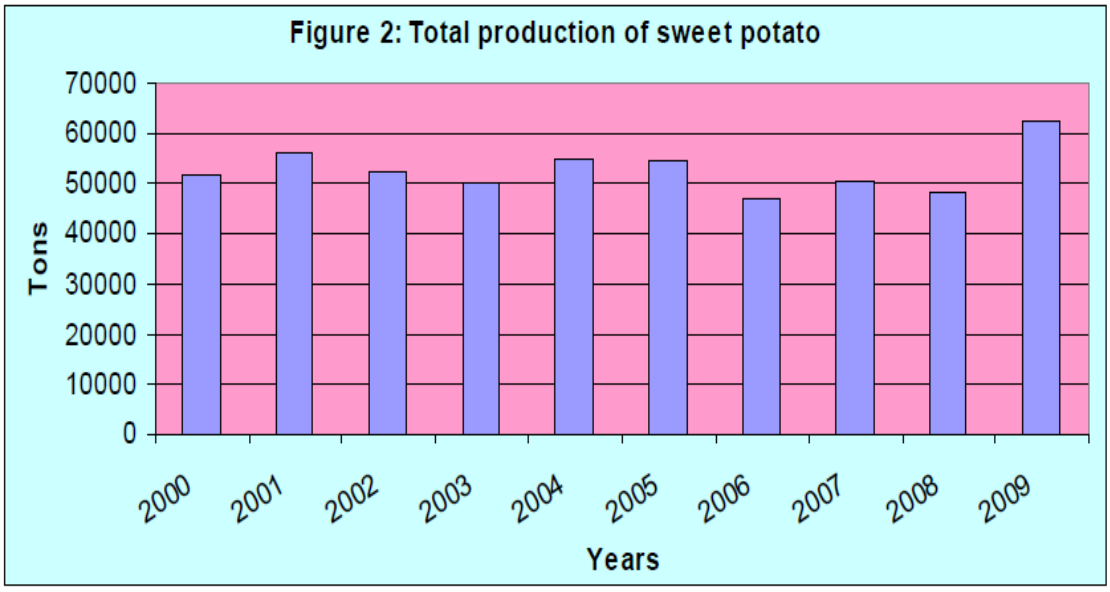

Figure 4: Total production of South African sweetpotato over a period of ten years (Department of Agriculture, Forestry and Fisheries 2010: 4).

South Africa produces more sweetpotato than what is consumed. Approximately 60 000 tons of sweetpotatoes are produced per annum of which 47 766 tons are consumed and the rest is exported. South Africa is not a major exporter of sweetpotato. It represents 1.39% of the world export and ranks fourteenth in the world. Most of the South African sweetpotatoes are mainly exported to Netherlands, United Kingdom, France, Portugal, Mozambique and Zimbabwe (Fig. 5) (Department of Agriculture, Forestry and Fisheries 2010: 4- 13).

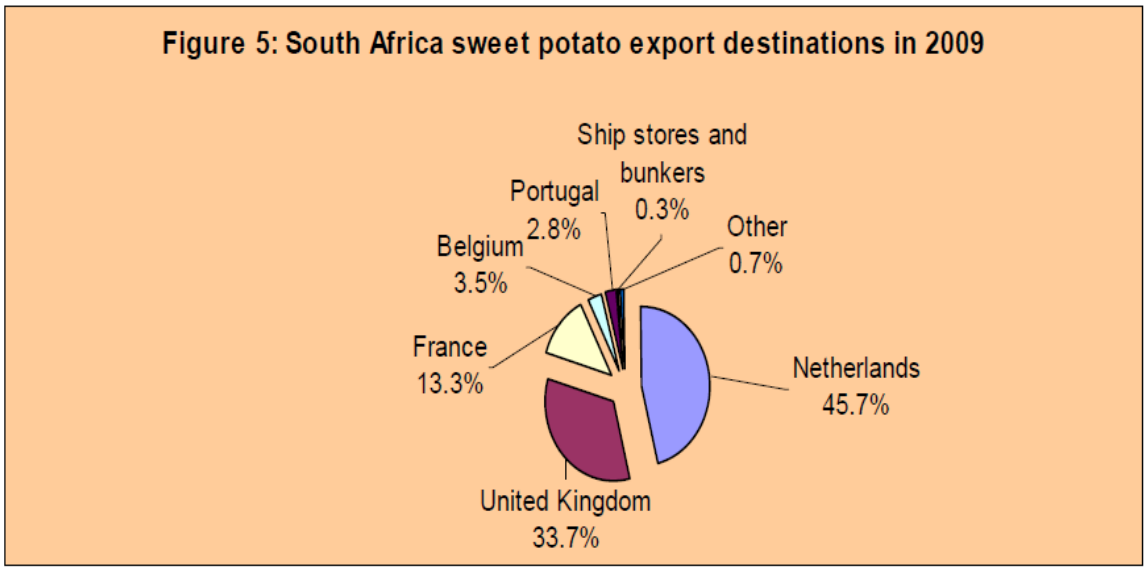

Figure 5: South Africa sweetpotato export destinations for 2009 (Department of Agriculture, Forestry and Fisheries 2010: 7).

2.5 USES AND HEALTH BENEFITS OF SWEETPOTATO

Sweetpotato has various uses including food for humans and animals. It is a cash crop and is also processed into a variety of industrial products such as starch, snacks, liquor and flour (Hu, Nakatani, Lalusin, Kuranaichi & Fajimura 2003: 297). Sweetpotato is a good source of β-carotene, vitamin B6, vitamin C, vitamin E, fiber, protein and calcium. It was ranked number one by the Centre for Science in the Public Interest (CSPI) when compared with other vegetables such as potato, spinach and cabbage in terms of dietary fiber, naturally occurring sugars, complex carbohydrates, proteins, iron, calcium, vitamin A and C (George Mateljan Foundation 2010; Food Reference.com 2009).

Sweetpotato flour is usually used in the baking industries as a 20% supplement to wheat and it is also used as a stabilizer in the ice-cream industry. Puree made from sweetpotato is used as an ingredient in sauces such as tomato sauce, baby food and in fruit-flavoured jams like pine apple, mango and orange. The juice from red sweetpotato can be combined with lime juice to make dye for clothes (Department of Agriculture, Forestry and Fisheries 2011: 19). The leaves which are a good source of amino acids, essential minerals and vitamins are consumed as leafy vegetables in West African countries and they are also used as a protein source in ruminant feeds (Woolfe 1992: 166; Adewolu 2008: 444).

In USA, sweetpotatoes are used as a Thanks Giving dish and also canned into various forms while in China they are used to produce starch which is used for making pasta and as a substrate for alcoholic drinks. In Uganda, sweetpotatoes are processed into juice, cakes, chips and chapattis. In South Africa, sweetpotatoes are dehydrated and the powder is used in instant soups and infant products (Kenyon, Anandajayasekeram & Ochieng 2006: 8; Department of Agriculture, Forestry and Fisheries 2011: 19).

The vitamin A (in the form of β-carotene) and vitamin C in sweetpotato are powerful antioxidants that eliminate free radicals that are associated with the development of conditions like atherosclerosis, diabetes, heart disease and colon cancer (George Mateljan Foundation 2010). Vitamin B assists in decreasing the risk of heart attacks and stroke (George Mateljan Foundation 2010). The high potassium content in sweetpotatoes helps to alleviate muscle cramps, which are often related to potassium deficiency, and also protect us from tension which occurs during times of stress (Green 2011). The fiber from sweetpotato can be used with other therapeutic ointments to heal wounds (Wohers Kluwer Health 2009).

2.5.1 OFSP for combating VAD

The OFSP varieties are a good source of β-carotene which is converted to vitamin A in our bodies. These varieties are used in many countries in Sub-Saharan Africa to combat VAD which may lead to blindness in children and causes diseases such as measles, diarrhoea and malaria (van Jaarsveld *et al*. 2005: 1080- 1081; Ndolo *et al*. 2007: 689; Childinfo 2009). According to World Health Organization (2009) statistics, approximately 190 million pre-school children and 19 million pregnant women do not consume enough vitamin A in their daily diet, and can be classified as vitamin A deficient. Furthermore, 5.2 million pre-school children suffer from clinical VAD especially in Sub-Saharan Africa and South East Asia (Fig. 6).

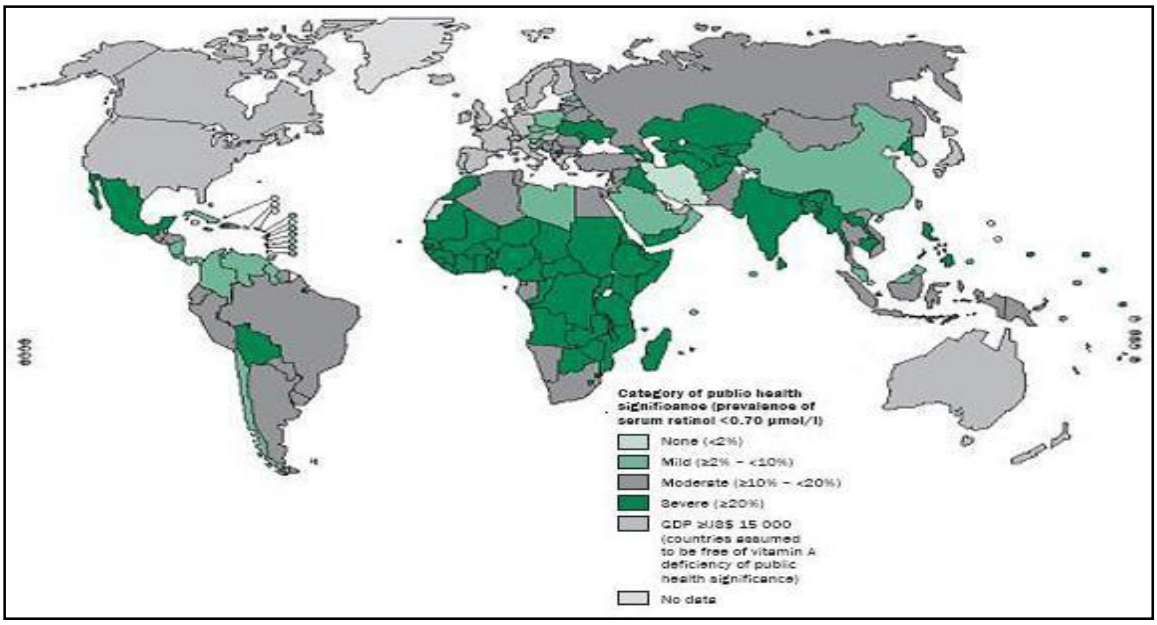

Figure 6: Global prevalence of vitamin A deficiency. Countries that are shaded in green are the most affected (World Health Organization 2009).

In South Africa, The South African Vitamin A Consultancy Group (SAVACG) (1995: 11-12) conducted a survey in 1994 for the Department of Health and it revealed that one out of three children under the age of six years in the country had poor vitamin A status. The provinces which are seriously affected by VAD were the Northern Province, Kwa-Zulu Natal, Mpumalanga, North West and Eastern Cape. It was also found that children living in rural areas and in low socio-economic environments were severely affected.

Most South Africans prefer the CFSP due to its dry texture. It is only recently that interest has been given to the OFSP and they are used in many projects to alleviate VAD (van Jaarsveld *et al*. 2005: 1080- 1081). This food-based approach which is called biofortification is considered the best and most cost effective approach when compared with other strategies such as the administration of VAD capsules because it is able to reach the rural and low-income communities (Office of Dietary Supplements National Institutes of Health 2006). On the other hand plant breeders are also developing OFSP varieties that conform to Africa's distinct preference for traits such as high dry matter content (Pray *et al*. 2007: 137).

2.6 DIVERSITY OF SWEETPOTATO

There are nearly 26,000 accessions of *Ipomoea* species that are maintained at various gene banks around the world. Amongst these accessions 8,000 are sweetpotato accessions or breeding lines (Lin, Lai, Chang, Chen, Hwang & Lo 2007: 283). The accessions vary in traits such as β-carotene content, seed size, flower colour, nutritional qualities, response to heat, cold or drought, or their ability to resist specific diseases and pests. This diversity is the result of millions of years of evolution, selection and adaptation of the crop to the environments in which they were grown. The diversity is important for the maintenance and breeding of new accessions with desired traits (Gichuki, Berenyi, Zhang, Hermann, Schmidt, Glossl & Burg 2003: 429- 430; Malik & Singh 2006: 21- 27).

Knowledge of crop genetic diversity is important for plant breeders to develop a successful breeding programme. Traditionally, diversity of sweetpotato is assessed by examining morphological characteristics. The wide diversity of morphological and phenotypic traits (Fig 1, 2 and 3), such as root size, resistance to diseases, skin colour, root shape and leaf shape in the germplasm complicates morphological classification. Morphological traits alone cannot provide a thorough assessment of genetic diversity because a plant's morphology is subject to strong environmental influence. On the other hand, the use of molecular markers is considered to be more accurate because the diversity is measured at the DNA level which is not influenced by environmental conditions (Woolfe 1992: 166; Rao 2004: 138- 139).

2.7 MOLECULAR MARKERS

Molecular markers are "genetic tags" that are used in plant, animal and microorganism studies to identify genes of a particular trait within the organism's DNA sequence. Once the gene of interest such as the gene for disease resistance has been identified using molecular marker techniques, the gene can be cloned and transferred to other species (Arif, Bakir, Khan, Al Farhan, Al Homaidan, Bahkali, Al Sadoon & Shobrak 2010: 2080; Pillay, Ashokkumar, James, Kirubakaran, Miller, Ortiz & Sivalingam 2012: 71).

Molecular markers can either be protein or DNA based. Molecular markers that reveal polymorphism at the protein level are known as biochemical markers, while the DNA based markers reveal polymorphism at the DNA level (Pillay *et al*. 2012: 71- 72). The most common protein markers are isozymes such as malate dehydrogenase (MDH), peroxidase (PRX) and galactose dehydrogenase (GDH). The most common DNA based markers include

Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and microsatellites.

2.7. 1 Protein markers

Protein markers were introduced in the 1960s as complementary methods to traditional methods for evaluating genetic differences among groups of individual plants and accessions (Rao 2004: 138- 139). The commonly used protein markers are isozyme, which are charged, separable variants of enzymes coded by genes at one or several loci (Onarici & Sumer 2003: 48; Pillay *et al*. 2012: 72).

Isozymes differ in charge and this is due to the substitution of a single amino acid of different charge at a locus. This difference in charge leads to a difference in the electrophoresis movement of the enzymes and this makes the technique an important tool for detecting variation (Onarici & Sumer 2003: 48; Rao 2004: 138- 139; Kumar, Gupta, Misra, Modi & Pandey 2009: 142).

The advantage of isozyme markers is they are inexpensive and the protocols for most of the isozymes are well established. Moreover isozyme markers can assess co-dominance making it possible to discriminate between homozygotes and heterozygotes (Farooq & Azam 2002: 1136- 1137; Onarici & Sumer 2003: 48). However, their disadvantage is the requirement of a different protocol for each isozyme system, low level of polymorphism and they are subjected to post-translational modification (Kumar *et al.* 2009: 142- 143; Pillay *et al*. 2012: 72).

Liau and Lin (2008) used chitinolytic protein markers to identify chitinolytic enzymes (enzymes responsible for defence against pathogens and they are used in the production of products such as glucosamines and other applications including mosquito control and plant pathogenic fungi control) in sweetpotato leaves of Tainong 57. In this study, new chitinolytic enzymes were identified and the highest chitinolytic enzymes were detected at 54.1 and 55.6 kDa. Protein markers were used to differentiate trypsin inhibitory activities of sweetpotato leaves and tubers. The results showed that all the accessions used in this study contained two forms of trypsin inhibitors although the leaf proteins were different from the root protein. The results also revealed that trypsin inhibitory activities in the leaf correlated with pest resistance because Tainong 34 and 65 which are resistant accessions had high quantities of trypsin

inhibitory activities and Tainong 9 and 57 which are susceptible accessions had a lower quantity (Wang & Yeh 1996: 30).

2.7.2 DNA based markers

There are two categories of DNA based markers namely, the non-PCR or hybridization based techniques and the PCR-based techniques. The hybridization techniques include restriction fragment length polymorphism (RFLP) and variable number tandem repeats (VNTR), where probes such as random genomic clones and probes for microsatellite are hybridized to filters containing DNA that has been digested with a restriction enzyme (Semagn, Bjornstad & Ndjiondjop 2006: 2543- 2544; Agarwal, Shrivastava & Padh 2008: 618- 619; Pillay *et al*. 2012: 71).

The advantage of hybridization techniques such as RFLP is that they are highly polymorphic, co-dominant and highly reproducible. The disadvantage is the requirement for a large amount of genomic DNA, it is time consuming, involves expensive and toxic reagents such as radio activity and requires prior requirement of the sequence information for probe generation (Agarwal *et al*. 2008: 618- 619).

The PCR-based techniques are relatively easy to perform and do not require prior sequence knowledge (Agarwal *et al*. 2008: 618- 619). There is a variety of PCR-based techniques. Sequence-tagged-site (STS) such microsatellites use two different site specific primers complementary to the opposite strands of the conserved DNA (Peakall 1997: 245- 248). Arbitrary-primed PCR uses random sequence primers to amplify the genomic DNA. The mostly used arbitrary-primed PCR techniques are amplified fragment length polymorphism (AFLP) and RAPD (Peakall 1997: 245- 248).

Molecular markers have been used for various reasons in the study of sweetpotato. For example, the AFLP technique has been used extensively to study the genetic diversity and relationship of sweetpotato germplasm in Tanzania, Uganda, China and Latin America (Rossel *et al*. 1999; Zhang *et al*. 2000; Huang *et al*. 2002; Elameen *et al*. 2007). These studies proved that AFLP is a powerful technique in assessing genetic diversity in sweetpotato.

AFLP markers were used to identify molecular markers associated with resistance to sweetpotato virus diseases in Kenya. From this study, four markers which gave 100% and

94% correct classification of the training set and test set, respectively, were selected using statistical methods (Miano, Labonte & Clark 2007: 15- 24).

Gibb, Padovan and Mogen (1995) conducted a study on the sweetpotato little-leaf phytoplasma disease using restriction fragment length polymorphism (RFLP). Phytoplasma disease is responsible for reduced leaf size, shoot proliferation, small tuberous roots and serious crop losses in sweetpotato. Out of the four restriction enzymes, *Alu*I showed different banding patterns and was able to identify the phytoplasmas from Northern Australia as a new subgroup.

Simple sequence repeat (SSR) markers were used to study the genetic diversity of sweetpotato in Kenya and East Africa (Karuri, Ateka, Amata, Nyende, Muigai, Mwasame & Gichuki 2010; Tumwegamire, Rubaihayo, LaBonte, Diaz, Kapinga, Mwanga & Gruneberg 2011). In both studies, the SSR markers exhibited remarkable discriminatory power of the germplasm.

2.7.2.1 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) involves random amplification of the genomic DNA using randomly constructed oligonucleotides (usually ten base pairs long) as primers. These primers detect polymorphisms in the absence of specific nucleotide sequence information and the polymorphism functions as a marker (Lynch & Milligan 1994: 91; Betal & Ratchaudhuri 2004: 450).

At an appropriate annealing temperature during the thermal cycle, the primers bind to the complementary sequences in the template genomic DNA if priming sites are within an amplifiable distance of each other and produce discrete DNA products (Fig. 7). The amplification products are separated on agarose gels, stained with ethidium bromide and viewed under UV light. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites (Bardakci 2000: 186- 187).

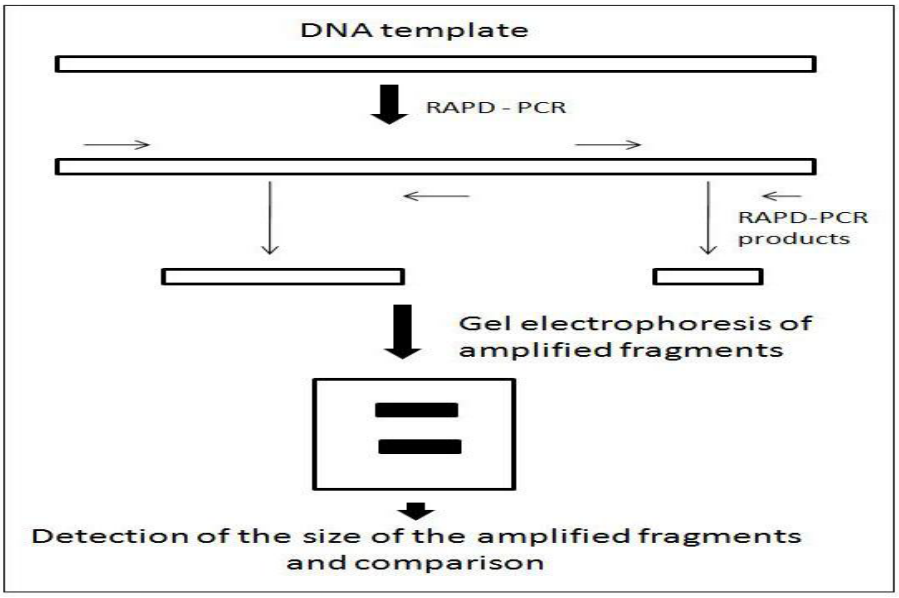

Figure 7: The principle of RAPD-PCR technique (Arif *et al*. 2010: 2085).

The advantage of RAPD is that previous knowledge of the DNA sequence is not required; the results are obtained easily especially when compared with other techniques such as RFLP and AFLP; it requires small amounts of DNA template and no radio labelling is required. The disadvantage of RAPD is that it is not reproducible and it cannot distinguish heterozygous from homozygous individuals (Connolly, Godwin, Cooper & DeLacy 1994: 332).

Random amplified polymorphic DNA markers have been widely used in sweetpotato to assess the genetic diversity of the crop (Villordon & LaBonte 1996; Taura, Abe, Onjo, Sakamaki, Yamaguchi, Tawara, Nishitani & Kawabe 2001; Das & Naskar 2008), to study diseases associated with sweetpotato (Ukoskit, Thompson, Watson & Lawrence 1997; Alajo 2009) and for identification of duplicates in the germplasm (Zhang *et al*. 1997) where it was able to identify 57 duplicates out of 66 suspected duplicates.

2.7.2.2 Internal Transcribed Spacer (ITS) Region

The ITS regions are components of the ribosomal DNA (rDNA) cistrons, which consist of 18S, 5.8S and 28S sequences (Fig. 8). The ITS1 and ITS2 spacers are non-coding regions which are positioned in the rDNA between 18S and 5.8S and between 5.8S and 28S rDNA genes, respectively (Cheng, Xia, Wu, Meng, Ji & Don, 2006: 702- 703). These regions are abundant in all eukaryotes and they evolve rapidly due to the insertions or deletions in the sequence. These rapid changes of the ITS region has made the region an important tool for

phylogenetic and systematic analysis among related species and/or among populations within a species (Liu & Schardl 1994: 775; Poczai & Hyvonen 2010: 1897- 1899).

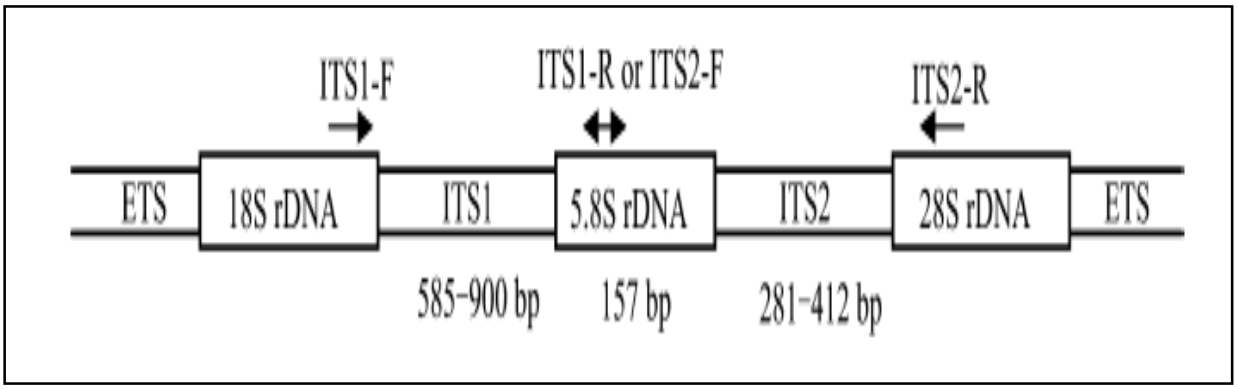

Figure 8: Schematic diagram of the nuclear ribosomal DNA showing the internal transcribed spacers in eukaryotes (Cheng *et al*. 2006: 703).

The ITS region of angiosperms is between 565- 700 bp and is longer in non-flowering seed plants (Liston, Robinson, Oliphant & Alvarez-Buylla 1996: 109- 110; Gernandt & Liston 1999: 711). The advantages of using the ITS regions for phylogenetic studies are that they are inherited biparentally compared to the maternal inheritance of chloroplast and mitochondrial DNA, the PCR amplification is easy to perform, they have a high number of copies which allow for high reproducibility and the variation makes it suitable for evolutionary studies at the species or generic level (Poczai & Hyvonen 2010: 1897- 1899).

Variation of the ITS region was used to analyse the genetic diversity and phylogenetic relationship of sweetpotato and its wild relatives in *Ipomoea* series *Batatas* (Huang *et al*. 2002: 547- 549). The study showed that all the *I.* series *Batatas* and *I. Setosa* formed a monophyletic clade. These results agreed with the taxonomy of series *Batatas* at the subgeneric level which placed *I.* series *Batatas* and *I. Setosa* in the *I. subg. Eriospermum*.

Kawamura, Sugimoto, Kakutani, Matsuda and Toyoda (2007) examined the genetic variations of the ITS1 region in sweetpotato weevil, *Cylas formicarius*, from the main infested areas in the world except Africa. The results from this study revealed that the nucleotide sequence of the ITS1 region of weevils from Georgia (USA) and St. Kitts (West Indies) were identical to those from Guangdong (China) and different from the ones in India. It was then concluded that *C. formicarius* could have been brought into these areas not from

India but from southern China. The *C. formicarius* in the Ogasawara Islands (Japan) might have been introduced with infested sweetpotato directly from southern China and/or in directly via the USA.

2.8 PROTEIN ISOLATION

Protein extraction is the first step in proteomic studies. Plant protein extraction is a difficult process due to the presence of a cell wall which is made of a complex assembly of polysaccharides and this process becomes more difficult when the plants are mature because they have a thick wall. Moreover, plant tissues have relatively low protein content when compared to bacterial or animal tissues (Balbuena, Dias, Martins, Chiquieri, Catarina, Floh & Silveira 2011: 91- 98). It is therefore vital to use an appropriate method which will be able to disrupt the cell wall, and be able to remove nucleic acids which interact with proteins and give a poor resolution.

There are several criteria which have to be considered before extracting proteins such as:

- The sample should contain a lot of proteins
- Additional components such as salts and buffer should be removed as far as possible
- The composition of the sample should be compatible with all subsequent analysis procedures.

Given all this criteria, it is therefore important to use high concentrations of chaotropic reagents like urea, thiourea and 3-[(3-cholaimdopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS) as much as possible so that the proteins are preserved and kept in solution. It is also important to remove biological contaminants such as RNA and DNA so that they do not interfere with the proteins (Lovric 2011: 21- 24).

There are different kinds of protein extraction protocols such as the trichloroethane (TCA)/acetone and sodium dodecyl sulphate (SDS) protocol but the phenol extraction method is the best known and reported method for recalcitrant plants. The advantage of the phenol method is that it is efficient in removing interfering substances, minimize protein degradation which is caused by endogenous proteolytic activity, yields a greater number of glycol proteins, acts as a dissociating agent decreasing molecular interaction between proteins and other materials, it has a high clean-up capacity and this results in high quality gels with less background and less vertical streaking. The disadvantage of this protocol is that it is time

consuming and that phenol and methanol are toxic (Thiellement, Zivy, Damerval & Mechin 2007: 416- 418).

2.8.1 Assay for protein content

There are various methods which are used to measure the concentration of proteins such as the biuret test, Lowry assay, bicinchoninic acid assay and Bradford assay. All these methods have their own advantages and disadvantages and the choice of method depends on the nature of protein, accuracy and the sensitivity of assay.

From all these methods, the Bradford assay is the commonly used method that uses Coomassie Brilliant Blue G-250 dye to determine the concentration of proteins. Under acidic conditions, Coomassie Brilliant Blue G-250 dye is red-brown in colour and it becomes blue when it is bound to a protein. This causes a shift difference in the absorbance of the dye between the absorption at 465 nm and the absorption at 595 nm. Therefore the concentration of the proteins can be measured at the absorption of 595 nm. The advantages of using the Bradford assay over other methods are its simplicity, sensitivity which is four times as sensitive as the Lowry assay and its resistance to interferences (Dennison 1999: 22- 24; Switzer & Garrity 1999: 93- 94).

2.9 DATA ANALYSIS OF PROTEINS AND RAPD MARKERS AFTER GEL ELECTROPHORESIS

After electrophoresis of the gels, the resulting banding patterns are analyzed using statistical tools. There are three steps involved in statistical analysis of genetic diversity data namely: data collection, data analysis using statistical computer software packages and interpretation of the data (Warburton & Crossa 2002: 2).

In the first step which is data collection, the resulting banding patterns from the gel are scored as present (1) and absent (0). This process can be done manually or using computer programs such as Quantity one version 4.6.9 (Bio-Rad 2000). This program detects the bands automatically and the reader can be able to count the bands. The bands are then entered into a spread sheet such as Excel and used for statistical analysis (Warburton & Crossa 2002: 2- 4).

In the second step, the data is analysed to investigate the variation between the samples. One of the statistical analyses used to study the variation between the samples is called analysis of

molecular variance (AMOVA) which partitions variation in the samples by comparing variation between and within the populations (Warburton & Crossa 2002: 4- 5). In this study Arlequin software package was used to calculate AMOVA (Excoffier, Laval & Schneider 2005: 53- 89).

Variation between the samples can also be summarized by means of dendrograms and in this study numerical taxonomy multivariate analysis system (NTSYS) was used to calculate the genetic distance. In the first step the proximity (dis/similarity) between the individuals is calculated using the simple match similarity coefficient which is suitable for measuring the proximity between closely related haploid individuals (Warburton & Crossa 2002: 6- 12; Kosman & Leonard 2005: 418- 423).

The resulting matrix is used in the SAHN programme for clustering the individuals using the unweighted pair group method of arithmetic averages (UPGMA). In this step, hierarchical algorithms cluster the entries into similar groups and the number of tree ties expected is also indicated (Warburton & Crossa 2002: 13- 20; Rohlf 2009).

In the third and last step of data analysis which is data interpretation, the clustered groups in NTSYS are presented in a form of a dendrogram. The resulting dendrogram can be viewed using the tree plot programme. The consensus clusters can be grouped by drawing circles around them or colouring the groups in the same colour (Warburton & Crossa 2002: 22), while in Arlequin the data is presented in a table form showing the percentage variation among and within the populations studied (Excoffier *et al*. 2005: 53- 89).

2.10 SEQUENCING DATA ANALYSIS

Chromas Elite is a programme that displays chromatogram files from Applied Biosystems, Amersham Mega Baceautomated sequencers and Staden SCF format. Chromas Elite displays a list of the peaks below the chromatogram from genotyping files containing information about peaks. This programme allows for the sequences to be edited, reversed and complemented, and to be exported in a plain text or FASTA format (DNA Sequencing Services 2013).

Clustal W is a sequence alignment programme which produces biologically meaningful multiple sequence alignment of divergent DNA and protein sequences. It calculates the best

match for the selected sequences, and lines them up such that their identities, similarities and differences can be seen. The evolutionary relationships are viewed via dendrograms (Bates 2006).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 SAMPLE COLLECTION

Leaf samples of OFSP and CFSP were collected from the Agricultural Research Council-Vegetable and Ornamental Plant Institute (ARC-VOPI) in Roodepoort, South Africa. The samples were preserved in liquid Nitrogen and stored at -20 ^{o}C until needed. Table 1 contains the list of accessions used in this study, their flesh colour and their place of origin.

Table 1: List of sweetpotato accessions used in this study.

Name	Flesh colour	Place of origin
1. Impilo	OFSP	South Africa
2. Beauregard	OFSP	United States of America
3. Resisto	OFSP	United States of America
4. Khano	OFSP	South Africa
5. W119	OFSP	United States of America
6. Hernandez	OFSP	United States of America
7. Purple sunset	OFSP	South Africa
8. Ejumula	OFSP	Uganda
9. Isondlo	OFSP	South Africa
10. Jewel	OFSP	United States of America
11. Bophelo	OFSP	South Africa
12. Ndou	CFSP	South Africa
13. Letlhabula	CFSP	South Africa
14. Monate	CFSP	South Africa
15. Blesbok	CFSP	South Africa
16. Phala	CFSP, slight light orange	South Africa
17. Mafutha	CFSP, with orange spots	South Africa
18. Ribbok	CFSP	South Africa
19. Mamphenyane	CFSP, slight light orange	South Africa
20. Mokone	CFSP	South Africa

21. 199062-1	BREEDING LINE (OFSP)	Peru
22. 2007-2-12	BREEDING LINE (CFSP)	South Africa
23. 2005-1-11	BREEDING LINE (OFSP)	South Africa
24. 2004-16-1	BREEDING LINE (OFSP)	South Africa
25. 99-9-4	BREEDING LINE (CFSP)	South Africa
26. 2004-5-2	BREEDING LINE (OFSP)	South Africa
27. 2007-1-3	BREEDING LINE (CFSP, with slight orange)	South Africa
28. Monate x Resisto	CROSS (OFSP)	South Africa

3.2 PROTEIN EXTRACTION

Proteins were extracted according to Hurkman and Tanaka (1986). Approximately 1 g of leaf tissue was ground to powder in liquid nitrogen. The powdered samples were suspended in 2.5 ml of extraction buffer (50 mM Tris-HCl pH 8, 25 mM Ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA), 500 mM urea and 0.5% β-mercaptoethanol which was added just before use) and 2.5 ml of acetone containing 10% TCA and 0.07% β-mercaptoethanol. The solutions were mixed with a vortex and stored at -20 ^{o}C overnight.

The samples were centrifuged at 5500 rpm at 4 ^{o}C for 15 min. The pellets were washed with 5 ml of acetone containing 0.07% β-mercaptoethanol and centrifuged at 5500 rpm at 4 ^{o}C for 15 min. The pellets were dried and resuspended in 2.5 ml buffer (0.1 M Tris pH 8, 50 mM EDTA and 2% β-mercaptoethanol) and 2.5 ml of Tris-phenol buffer. The mixture was agitated at room temperature for 10 min and centrifuged at 5500 rpm at 4 ^{o}C for 15 min.

The phenol phase was collected and 5 ml of 0.1 M ammonium acetate in methanol was added and stored at -20 ^{o}C overnight. The samples were centrifuged at 5500 rpm at 4 ^{o}C for 15 min. The pellets were washed with 5 ml of 0.1 M ammonium acetate in methanol, and then centrifuged at 5500 rpm at 4 ^{o}C for 15 min. The pellets were washed with 1% β-mercaptoethanol and centrifuged at 5500 rpm at 4 ^{o}C for 15 min. Finally the pellets were dissolved in 200 µl of isoelectric focusing (IEF) buffer (9 M urea, 2% CHAPS and 1% dithiothreitol (DTT)). The protein concentration was determined by the Bradford assay and bovine serum albumin (BSA) was used as the standard.

3.2.1 Protein quantification

Protein quantification was performed as described by Bio-Rad (S.a: 11- 14). The 1x dye reagent (Bio-Rad, Berkeley, California, USA) was removed from the fridge and left to thaw to ambient temperature. Protein standards were prepared using 2 mg/ml BSA and they were diluted using the IEF buffer according to Table 2. All the assays were performed in duplicate. Twenty microlitres of each standard and unknown sample solutions were pipetted into different tubes and 1 ml of 1x dye reagent was added into each tube and mixed by vortexing. Both the standard and the samples were incubated at room temperature for 5 min. The protein concentration was measured using NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific; Wilmington, DE; USA) and the IEF buffer was used to blank the instrument.

Table 2: Protein standard assay Bio-Rad (S.a: 30).

Tube no	**Standard volume (µl)**	**Source of standard**	**Diluents volume (µl)**	**Final protein concentration (µg/ml)**
1	70	2 mg/ml stock	0	2 000
2	75	2 mg/ml stock	25	1 500
3	70	2 mg/ml stock	70	1 000
4	35	Tube 2	35	750
5	70	Tube 3	70	500
6	70	Tube 5	70	250
7	70	Tube 6	70	125
8	Blank	0	70	0

3.2.2 SDS-PAGE electrophoresis of proteins

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure described by Laemmli (1970). A 10% separating gel was prepared by mixing 4.0 ml distilled water, 3.3 ml of 30% acrylamide, 2.5 ml of 1.5 M Tris (pH 8.8) , 0.1 ml of 10% SDS, 0.1 ml of 10% ammonium persulfate and 0.004 ml of tetramethylethylenediamine (TEMED). The solution was poured into the gel cassettes and 1 ml of cold isopropanol was poured on top of the gel to straighten the level of the gel and degas it.

The isopropanol was removed after the gel had solidified and a 0.5 cm well forming comb was inserted into the glass plates. A 4% stacking gel was prepared by mixing 3.4 ml distilled water, 0.83 ml of 30% acrylamide, 0.63 ml of 1.5 M Tris (pH 8.8), 0.05 ml of 10% SDS, 0.05 ml of 10% ammonium persulfate and 0.005 ml of TEMED. The gel was poured over the separating gel and left for 60 min to solidify.

The comb was removed and the wells were rinsed with distilled water. The cassette was inserted into the electrophoresis tank containing 1x Tris-glycine-SDS buffer (0.025 M Tris, 0.198 M glycine, 0.1% SDS and adjusted to pH 8.3). Protein samples were mixed with a loading dye (0.5 M tris-HCl (pH 6.8), 10% glycerol, 0.02% SDS and 0.1% bromophenol blue) in a ratio of 1:1. The samples were incubated at 100 ^{o}C for 5 min and then stored at -20 ^{o}C until used. The samples and an unstained protein molecular weight marker (Thermo Scientific, Rockford, USA) were loaded in the gel and the gel was run at 90 V for 90 min. The gel was stained with Oriole fluorescence stain (Bio-Rad).

3.2.3 Protein staining

After electrophoresis, the gel was placed directly into the staining solution without rinsing it. The gel was stained for 90 min then rinsed with distilled water prior to imaging. The gel was viewed using the Bio-Rad imaging system (Bio-Rad) and a picture was captured.

3.3 DNA ISOLATION

Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) procedure as described by Doyle and Doyle (1987). Approximately 1 g of the leaf tissue was ground to a fine powder in liquid Nitrogen. The powder was suspended in 4.9 ml of CTAB extraction buffer (20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB, 1% $Na_2S_2O_5$ and 0.2% β-mercaptoethanol was added just before use); the solution was mixed with a vortex and incubated at 65 ^{o}C for 45 min in a shaking water bath.

After incubation 4.9 ml of chloroform- isoamylalcohol (24:1) was added to the tubes and gently mixed for 5 min at room temperature. The solution was centrifuged at 6,000 rpm for 15 min at 4 ^{o}C. The supernatant was transferred into new tubes and 4.9 ml of chloroform-isoamylalcohol (24:1) was added and mixed gently. The solution was centrifuged at 6,000 rpm for 15 min at 4 ^{o}C and the procedure was repeated twice.

The supernatant was transferred into new tubes and the DNA was precipitated with 0.5 ml of 3 M Sodium acetate and 4.5 ml of cold isopropanol. The solution was mixed gently and centrifuged at 6,000 rpm for 15 min at 4 ^{o}C. The supernatant was discarded and the remaining DNA pellets were washed with 1.75 ml of 70% ethanol and 1.75 ml of 100% ethanol, respectively.

The pellets were dried at room temperature then resuspended in 600 µl Tris-EDTA (TE) buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and stored at 37 ^{o}C overnight. The next day, 10 µl of ribonuclease (10 mg/ml) was added to the solution and it was incubated for 60 min at 37 ^{o}C. The concentration of the DNA was measured using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and the integrity of the DNA was evaluated by electrophoresis of the samples on a 0.8% (w/v) agarose gel.

3.3.1 DNA concentration and purity

The DNA concentration and purity was estimated by fluorescence at 260 to 280 nm using a NanoDrop 2000c spectrophotometer. For the integrity of the DNA, agarose gel was prepared by mixing 0.8% (w/v) agarose gel with 1X TBE buffer [100 ml 10X TBE (0.89 M Tris base, 0.89 M Boric acid, 20 mM EDTA pH 8.0) and 900 ml distilled water]. The gel was dissolved by heating it in a microwave. Ethidium bromide (1 µg/ml) was added to the cooled mixture before casting the gel.

After complete polymerization of the gel, 2 µl of Kapa universal molecular weight marker (Kapa Biosystems, Cape Town, South Africa) was loaded into the first gel well and 8 µl of each DNA sample was mixed with 2 µl of 6X orange DNA loading dye (Fermentas life sciences, Mountain View, USA) and loaded into the remaining wells. The gel was run at 60 V for 3 hrs in 1X TBE buffer. After electrophoresis, the gel was viewed using the Bio-Rad imaging system (Bio-Rad) and a picture was captured. The DNA concentration for all the samples was adjusted to 40 ng/µl and 100 ng/µl with TE buffer and used for RAPD and ITS analyses, respectively.

3.4 RAPD REACTION AND ANALYSIS

RAPD was carried out using selected Operon 10-mer kits A and D primers (Operon Technologies, California, USA). The primers and their sequences are listed in Table 3. A 25 µl PCR reaction was set up using the Kapa Hifi HotStart kit (Kapa Biosystems, Cape Town,

South Africa) as follows: 0.2 mM RAPD primer; 10 mM dNTP; 0.5 U/reaction Kapa Hifi (HotStart) DNA Polymerase; 5 µl 5X Kapa Hifi fidelity with $MgCl_2$; 3 µl of genomic DNA template (40 ng/µl) and dH_2O.

PCR amplification was performed in a C1000 thermal cycler (Bio-Rad). For each amplification process, a preheating denaturation of DNA at 94 ^oC for 2:30 min was followed by 34 cycles consisting of 50 sec at 94 ^oC, 50 sec at 40 ^oC, 90 min at 72 ^oC and final extension for 7 min at 72 ^oC. The amplification products were analyzed by electrophoresis of 8 µl PCR product mixed with 2 µl 6X orange DNA loading dye on a 1% agarose gel (containing ethidium bromide) in 1X TBE buffer. The gel was run for 3 hrs at 60 V in 1X TBE buffer. A Kapa universal molecular weight marker (Kapa Biosystems) was used to determine the size of the amplified products.

3.5 ITS REACTION AND ANALYSIS

Two primers namely, ITSL (5'-TCGTAACAAGGTTTCCGTAGGTG-3') which is complimentary to the 18S rDNA close to the ITS1 border and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') which anneals to 26S rDNA near the ITS2 border were used to amplify the ITS region. A 50 µl PCR reaction was set up using the Kapa Taq ready mix with loading dye (Kapa Biosystems) as follows: 25 µl 2X ready mix with Mg^{2+}, 0.4 µM ITSL primer, 0.4 µM ITS4, 5 µl of genomic DNA template (100 ng/µl) and dH_2O.

Amplifications were performed in a Bio-Rad C1000 thermal cycler (Bio-Rad), using the reaction settings as follows: initial denaturation at 94 ^oC for 4 min, followed by 35 cycles of denaturing for 40 sec at 94 ^oC, annealing for 30 sec at 55 ^oC, extension for 60 sec at 72 ^oC, and a final extension for 7 min at 72 ^oC. The amplification products were analyzed by electrophoresis of 10 µl PCR product on a 1% agarose gel (containing ethidium bromide) in 1X TBE buffer. The gel was run for 3 hrs at 60 V in 1X TBE buffer. A Kapa universal molecular weight marker (Kapa Biosystems) was used to determine the size of the amplified products.

3.5.1 Restriction enzyme digestion analyses

The amplified ITS DNA fragment was digested with the following restriction enzymes: *Bam*HI, *Eco*RI, *Hae*III, *Hind*III, *Hinf*I, *Hpa*I, *Hpa*II, *Mbo*II, *Pst*I and *Xho*I (Takara, Mountain View, USA). The restriction enzyme digestion was set up in a 20 µL reaction mixture

composed of 1 µL enzyme, 2 µL 10x buffer, 10 µL amplified PCR product and 7 µL of dH_2O. The reaction mixture was incubated at 37 ^oC overnight. The restriction fragments were separated on a 2% agarose gel and electrophoresis was carried out as mentioned in 3.3.1. A Kapa universal molecular weight marker (Kapa Biosystems) was used to determine the size of the amplified products.

3.6 SEQUENCING

The ITS region was amplified as described in section 3.5. The PCR products were sent to Inqaba Biotech (Pretoria, South Africa) for sequencing.

3.7 DATA ANALYSIS

The banding patterns produced from proteins, RAPD and ITS were detected with Quantity one version 4.6.9 software package (Bio-Rad 2000) and scored as present (1) and absent (0). Both the monomorphic and polymorphic bands were scored and bands with the same mobility on the gel but different intensities were not distinguished from each other. The data was summarised in a data matrix based on both monomorphic and polymorphic fragments.

Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.2 software package (Rohlf 2009) was used to calculate genetic similarities based on simple matching similarity coefficient using the similarity of qualitative data (SIM-QUAL) programme. Cluster analyses were performed using the unweighted pair group method of arithmetic averages (UPGMA) clustering and used to construct a dendrogram using the SAHN programme of NTSYS-pc.

Analysis of molecular variance (AMOVA) was calculated using Arlequin version 3.1 programme (Excoffier *et al*. 2005). The AMOVA quantification was performed from 1000 permutations using the Euclidean method. The sweetpotato accessions were grouped into OFSP, CFSP with slight orange or orange spots and CFSP; and another analysis was grouped into South African OFSP, non-South African OFSP, South African CFSP and South African CFSP with slight orange or orange spots. The population genetic distance was analysed using the same programme.

The pDRAW32 version 1.1.115 programme (AcaClone software 2012) was used to assume the possible restriction enzymes which will cut the ITS fragments. The DNA sequences were

aligned using Clustal W from MEGA version 5 software package (Tamura, Peterson, Stecher, Nei & Kumar 2011) and data sets were checked using BioEdit version 5.0.9 (Hall 1999). The alignment was achieved through gap initiation penalty 10 and gap extension penalty 0.05. Phylogenies were estimated from aligned sequences by using MEGA version 5. Gaps were treated as missing characters. The dendrograms were obtained using the neighbour-joining, UPGMA and maximum likelihood methods, and the analyses were performed with maximum composite likelihood. Bootstrap values were calculated from 500 replicates.

CHAPTER 4
RESULTS

4.1 INTRODUCTION

This chapter describes the results obtained from protein, RAPD and ITS experiments. It also includes the results obtained from the analysis of 1- 0 matrix using NTSYS and quantification of molecular variance using AMOVA from Arlequin software package. The sequencing results which were analysed using MEGA and BioEdit software packages are also included.

4.2 SDS-PAGE PROTEIN ANALYSIS

A diagrammatic representation of the protein profiles generated from the 28 sweetpotato accessions is shown in Figure 9. A total of nine bands were detected of which five bands were polymorphic and accounted for 55.56% polymorphism. The size of the bands ranged from 11 to 110 kDa and there were no cultivar specific markers (present in the OFSP but absent in the CFSP).

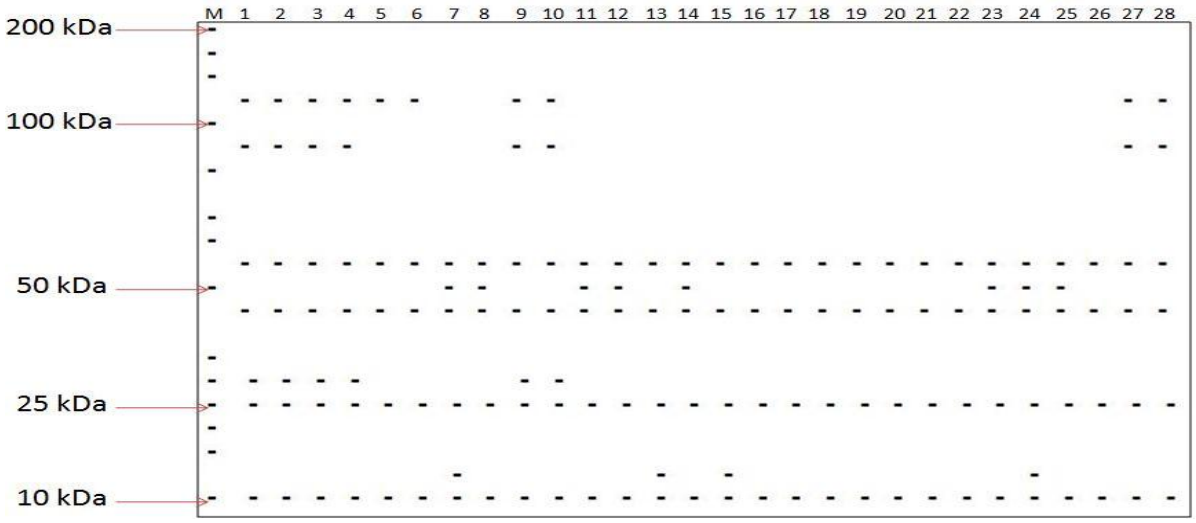

Figure 9: Leaf protein profiles of 28 sweetpotato accessions. kDa: Kilo-dalton, M: Thermo Scientific molecular weight marker. Numbering of the lanes from 1- 28 correspond to the names in Table 1.

The genetic relationship among the accessions is presented in a dendrogram (Fig. 10). The accessions were grouped into two main clusters (A and B) and all the accessions had a simple match similarity coefficient of 1.00. Cluster A had two branches and was composed only of the OFSP, except for line 2007-1-3. The first branch was composed of a mixture of South

African and non-South African OFSP accessions. The second branch consisted of line 2007-1-3 and 'Monate x Resisto' which shared a sister relationship.

Cluster B was composed of both the OFSP and CFSP and was divided into two subclusters B_1 and B_2. Subcluster B_1 had three branches; the first branch was composed of a mixture of OFSP and CFSP. The second branch consisted of 'Hernandez' and 'W119' which shared a sister relationship. The third branch consisted of the OFSP, CFSP and CFSP with slight orange or orange spots. All the accessions in the third branch are from South Africa except 199062-1 which is from Peru.

Subcluster B_2 consisted of two branches. The first branch consisted of the OFSP 'Purple sunset' and 2004-16-1 which shared a sister relationship. The second branch was composed of the CFSP 'Letlhabula' and 'Blesbok' which also shared a sister relationship. All the accessions in this subcluster originated in South Africa.

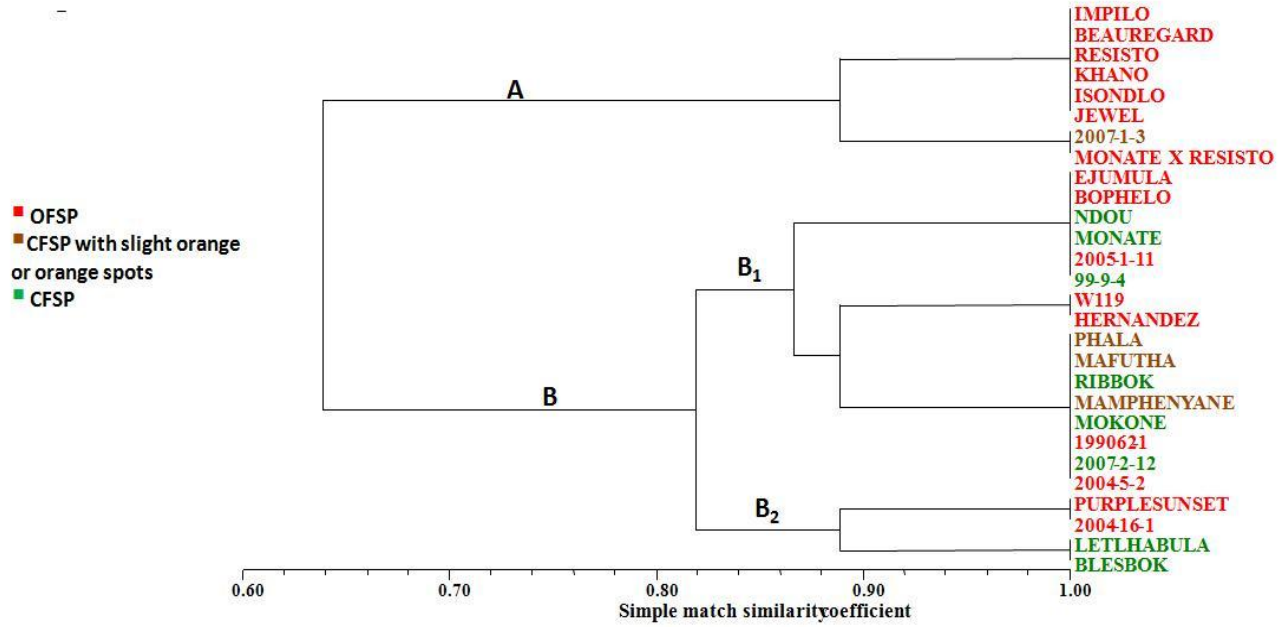

Figure 10: Unweighted pair group method analysis (UPGMA) dendrogram showing genetic diversity of 28 sweetpotato accessions based on protein profiles.

4.3 DNA ISOLATION ANALYSIS

DNA was isolated as described in section 3.3. Figure 11 shows a picture of the total DNA profiles of the 28 samples used in this study. The DNA concentrations ranged between 118.4

and 1274.4 ng/µl. The purity of the DNA calculated by determining the OD_{260}/OD_{280} ratio ranged between 1.80 and 1.96.

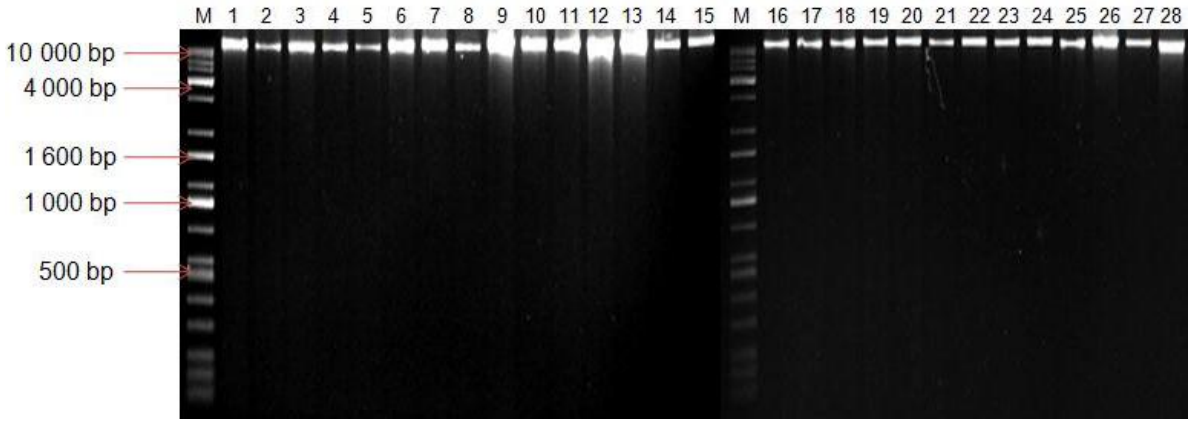

Figure 11: DNA conformation of sweetpotato accessions on a 0.8% agorose gel. M: Kapa Biosystems universal molecular weight marker. Numbering of the lanes from 1- 28 correspond to the names in Table 1.

4.4 ANALYSIS OF RAPD

The twenty RAPD primers which were used in this study produced a total of 904 bands, of which 888 were polymorphic. Typical banding patterns are shown in Figures 12 and 13. The number of amplified fragments ranged between 15 to 69 and the size of the bands ranged from 200 to 6 000 bp. The polymorphism ranged between 91 to 100% with an average of 98% (Table 3). None of the primers produced cultivar specific markers.

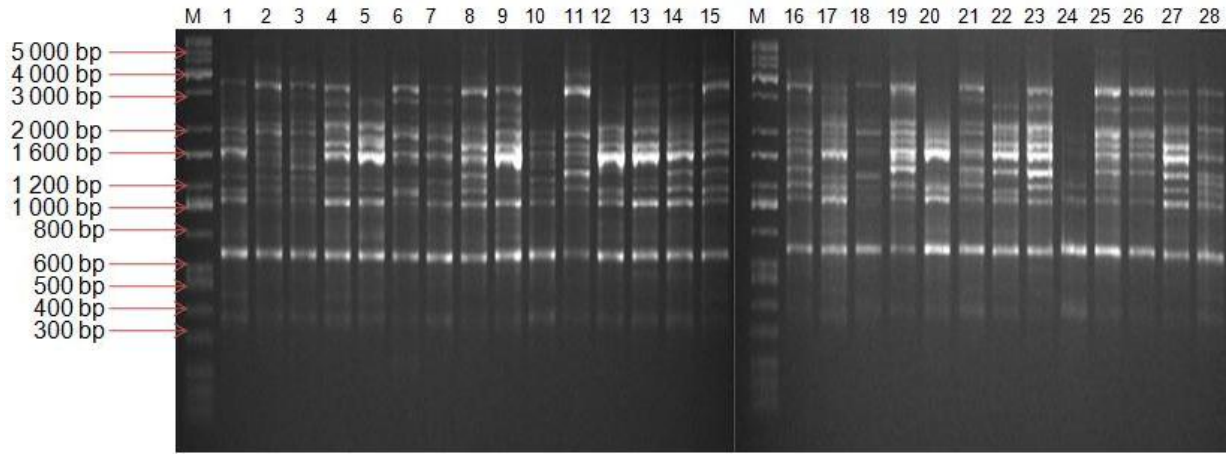

Figure 12: DNA polymorphisms of sweetpotatoes detected by amplification of total DNA using OPA 6 primer. bp: base pairs, M: Kapa Biosystems universal molecular weight marker. Numbering of the lanes from 1- 28 correspond to the names in Table 1.

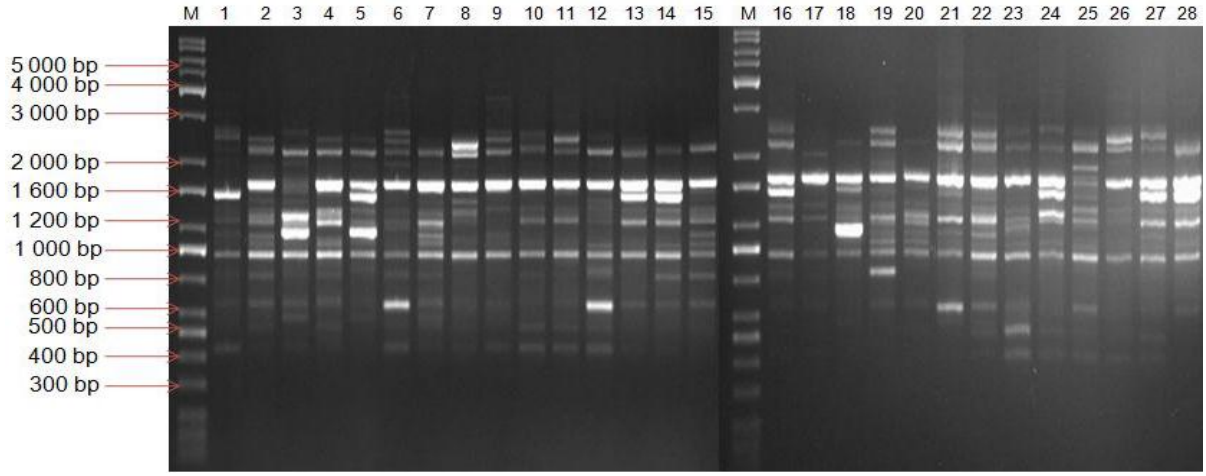

Figure 13: DNA polymorphisms of sweetpotatoes detected by amplification of total DNA using OPD 10 primer. bp: base pairs, M: Kapa Biosystems universal molecular weight marker. Numbering of the lanes from 1- 28 correspond to the names in Table 1.

Table 3: Summary of the banding patterns generated by RAPD primers.

Primer	Sequence 5'- 3'	Monomorphic bands	Polymorphic bands	Total number of bands	Polymorphism (%)
OPA 2	TGCCGAGCTG	0	39	39	100
OPA 4	AATCGGGCTG	0	52	52	100
OPA 6	GGTCCCTGAC	1	36	37	97
OPA 7	GAAACGGGTG	1	21	22	95
OPA 8	GTGACGTAGG	1	46	47	98
OPA 9	GGGTAACGCC	0	43	43	100
OPA 11	CAATCGCCGT	2	48	50	96
OPA 12	TCGGCGATAG	0	45	45	100
OPA 19	CAAACGTCGG	0	59	59	100
OPA 20	GTTGCGATCC	0	46	46	100
OPD 1	ACCGCGAAGG	1	23	24	96
OPD 2	GGACCCAACC	2	38	40	95
OPD 3	GTCGCCGTCA	0	62	62	100
OPD 4	TCTGGTGAGG	4	41	45	91
OPD 5	TGAGCGGACA	0	60	60	100
OPD 10	GGTCTACACC	1	40	41	98

OPD 12	CACCGTATCC	1	14	15	93
OPD 14	CTTCCCCAAG	0	69	69	100
OPD 16	AGGGCGTAAG	3	51	54	94
OPD 20	ACCCGGTCAC	0	55	54	100
TOTAL		17	888	904	98

The RAPD bands generated were used to determine the genetic distance between the 28 accessions. The dendrogram generated from the RAPD data is presented in Fig. 14. The dendrogram showed two main clusters A and B. Both the main clusters were divided into subclusters A_1, A_2, A_3 and B_1, B_2, B_3, respectively.

Subcluster A_1 and A_2 were composed of the OFSP only and the majority of the accessions were non-South African excluding 'Impilo', 'Khano', 'Purple sunset' and 'Isondlo'. Subcluster A_3 was a mixture of the South African OFSP and CFSP, where 'Bophelo' was the only OFSP and shared a sister relationship with 'Ndou' (simple match similarity coefficient[sm] = 0.819).

Cluster B consisted mainly of the South African accessions excluding 199062-1 which is from Peru. Subcluster B_1 consisted of two branches. The first branch was composed of the CFSP and CFSP with slight orange or orange spots which are 'Mafutha', 'Ribbok', 'Mamphenyane' and 'Mokone'. The two pairs of accessions 'Mafutha' and 'Ribbok' (sm = 0.835), and 'Mamphenyane' and 'Mokone' (sm =0.828) shared a sister relationship. The second branch consisted mainly of the OFSP except 2007-2-12 which is a CFSP. In this branch 2005-1-11 and 2004-16-1 (sm = 0.834) shared a sister relationship.

Subcluster B_2 consisted of two branches which are composed of OFSP, CFSP with slight orange or orange spots and CFSP. The first branch is made of 99-9-4 and 2004-5-2 (sm = 0.818) which shared a sister relationship and the second branch is made of a single cultivar 2007-1-3 (sm = 0.784).

Subcluster B_3 was divided into two branches, the first branch consisted of CFSP only and 'Monate' and 'Blesbok' shared a sister relationship (sm = 0.795) within this branch. The

second branch is made of the OFSP cross 'Monate x Resisto' (sm = 0.755), this cross occurs in the same Subcluster as 'Monate' which shows common ancestry.

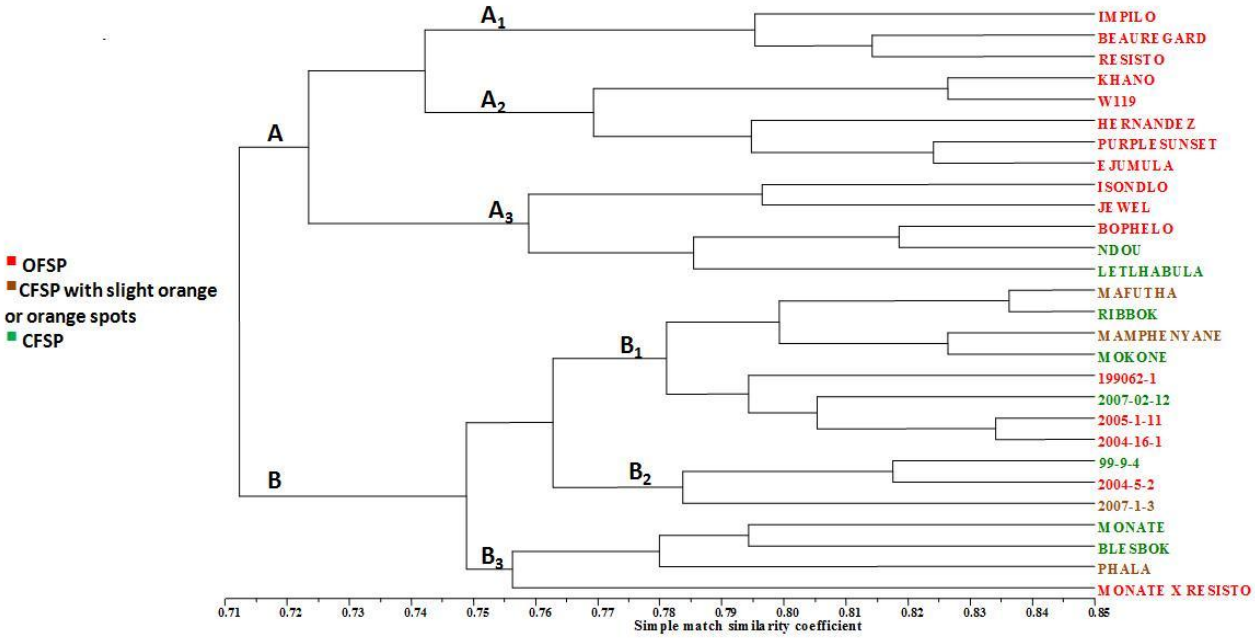

Figure 14: Unweighted pair group method analysis (UPGMA) dendrogram showing genetic diversity of 28 sweetpotato accessions based on combined RAPD data set generated with twenty primers.

Analysis of molecular variance (AMOVA) was performed to estimate the variation between the OFSP, CFSP with slight orange or orange spots and CFSP (Table 4). The difference among and between the accessions was highly significant ($p \leq 0.001$) and accounted for 4.77% and 95.23%, respectively. The genetic distance between the groups of accessions (CFSP, CFSP with slight orange or spots and OFSP) is presented in Table 5. The shortest distance was between CFSP and CFSP with slight orange or orange spots (0.013) and the largest distance was between CFSP with slight orange or orange spots and OFSP (0.070). Calculation of the mean distance revealed that OFSP had the greatest mean of 0.056.

Table 4: Analysis of molecular variance (AMOVA) among the sweetpotato accessions based on flesh colour.

Source of variation	**Sum of squares**	**Variance components**	**Percentage variation**
Among populations	323.87	5.86	4.77
Within populations	2923.34	116.93	95.23

Total	3247.21	122.79	

Significant at p= 0.001.
Groups are OFSP, CFSP with slight orange or orange spots and CFSP.

Table 5: Genetic distance among the sweetpotato accessions based on flesh colour.

	CFSP	**CFSP with slight orange or orange spots**	**OFSP**
CFSP	0.000	0.013	0.042
CFSP with slight orange of orange spots	0.013	0.000	0.070
OFSP	0.042	0.070	0.000
Mean distance	0.028	0.042	0.056

Significant at p= 0.001.
Groups are OFSP, CFSP with slight orange or orange spots and CFSP.

The second AMOVA was between the South African CFSP, South African CFSP with slight orange or orange spots, South African OFSP and non-South African OFSP accessions (Table 6). The variation among and within the accessions between the South African and non-South African accessions was highly significant ($p \leq 0.001$) and accounted for 5.36% and 94.64% of the molecular variance, respectively. The genetic distance between the groups of accessions is presented in Table 7. The shortest distance was between South African OFSP and non-South African OFSP (0.048) and the largest distance was between South African CFSP and non-South African OFSP (0.069). Calculation of the mean distance revealed that non-South African OFSP had the greatest mean of 0.062.

Table 6: Analysis of molecular variance (AMOVA) for RAPD variation between South African and non-South African sweetpotato accessions.

Source of variation	**Sum of squares**	**Variance components**	**Percentage variation**
Among population	427.50	6.65	5.36
Within population	2819.71	117.49	94.64
Total	3247.21	124.14	

Significant at p= 0.001.
Groups are South African CFSP, South African CFSP with slight orange or orange spots, South African OFSP and non-South African OFSP accessions.

Table 7: Analysis of population distance among sweetpotato accessions based on RAPD variation between South African and non- South African sweetpotato accessions.

	South Africa CFSP with slight orange or orange spots	**South African CFSP**	**Non-South African OFSP**	**South African OFSP**
South Africa CFSP with slight orange or orange spots	0.000	0.051	0.068	0.056
South Africa CFSP	0.051	0.000	0.069	0.055
Non-South African OFSP	0.068	0.069	0.000	0.048
South African OFSP	0.056	0.055	0.048	0.000
Mean distance	0.058	0.058	0.062	0.053

Significant at p= 0.001.
Groups are South African CFSP, South African CFSP with slight orange or orange spots, South African OFSP and non-South African OFSP accessions.

4.5 ANALYSIS OF ITS

The amplification of the ITS region using ITS primers L and 4 produced a 696 bp fragment (Fig 15).

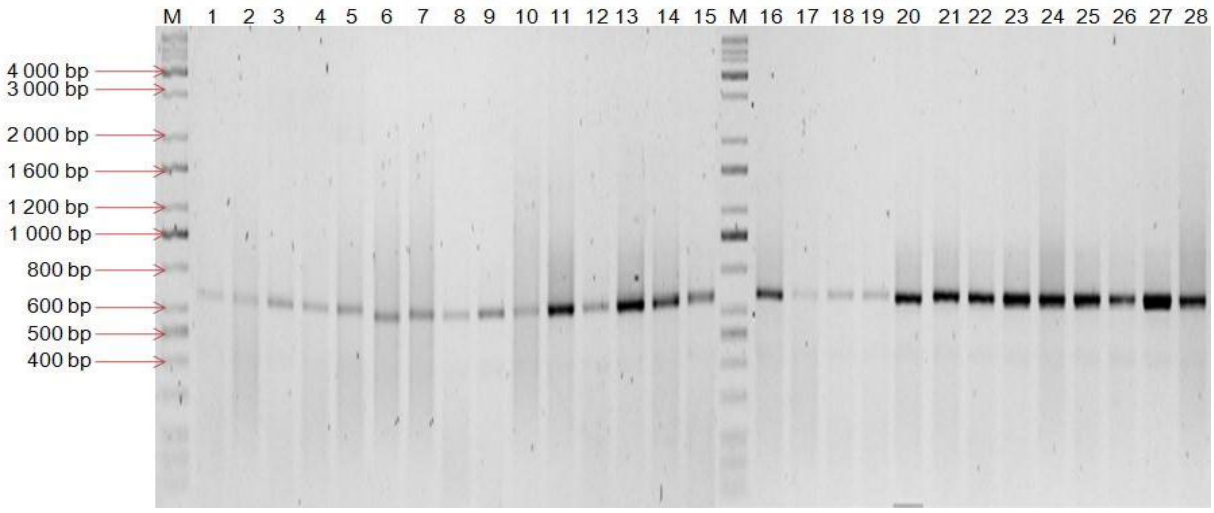

Figure 15: The fragment size of the ITS region of 28 sweetpotato accessions. bp: base pairs, M: Kapa Biosystems universal molecular weight marker. Numbering of the lanes from 1- 28 correspond to the names in Table 1.

The ITS fragment was digested with ten restriction enzymes. The restriction enzymes *Eco*RI, *Hind*III, *Hpa*I and *Pst*I did not digest the ITS fragment as shown by the presence of the entire

696 bp fragment. No polymorphisms were observed with the enzymes *Bam*HI, *Hae*III, *Hinf*I, *Hpa*II, *Mbo*I and *Xho*I. The restriction digestion patterns with *Hae*lll are shown in Fig 16.

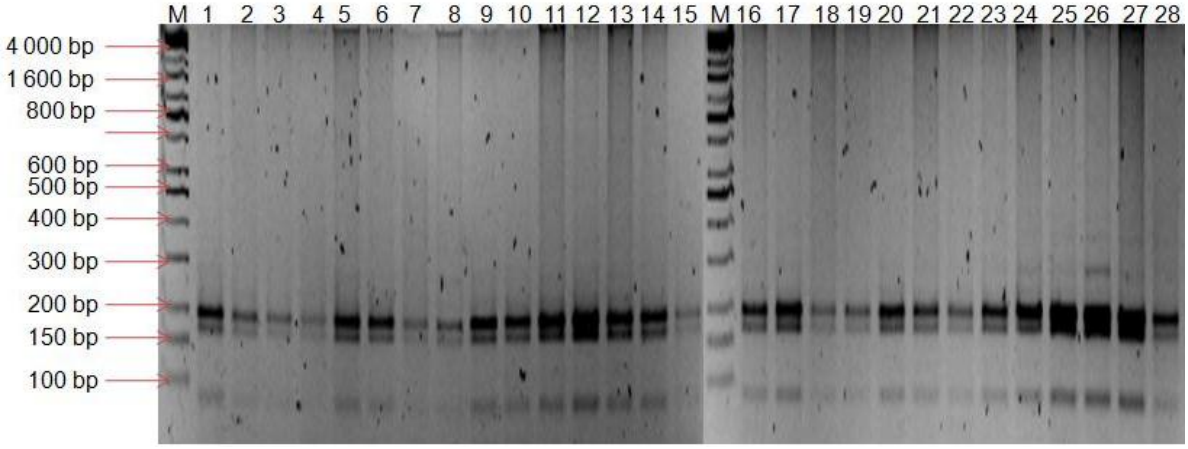

Figure 16: Restriction endonuclease of the ITS region using *Hae*III restriction enzyme. bp: base pairs, M: Kapa Biosystems universal molecular weight marker. Numbering of the lanes from 1- 28 correspond to the names in Table 1.

The banding profiles which were produced by the enzymes are summarised in Table 8. There were no polymorphic bands generated by any of the enzymes.

Table 8: Summary of the monomorphic and polymorphic banding patterns by restriction enzymes.

Restriction enzymes	**Sequence 5'- 3'**	**Monomorphic bands**	**Polymorphic bands**	**Total number of bands**	**Polymorphic %**
*Bam*HI	G/GATCC	4	0	4	0
*Eco*RI	G/AATTC	No digestion			
*Hae*III	GG/CC	3	0	3	0
*Hind*III	A/AGCTT	No digestion			
*Hinf*I	G/ANTC	5	0	5	0
*Hpa*I	GTT/AAC	No digestion			
*Hpa*II	C/CGG	4	0	4	0
*Mbo*I	/GATC	4	0	4	0
*Pst*I	CTGCA/G	No digestion			

*Xho*I	C/TCGAG	6	0	6	0
TOTAL		26	0	26	

Since the enzymes used in this study did not produce any polymorphism, the ITS regions were sequenced. The pdraw32 programme was used to assess possible restriction sites in the ITS sequences for future studies. Fig. 17 and Fig. 18 show the possible restriction enzymes for OFSP ('Impilo') and CFSP ('Monate'), respectively.

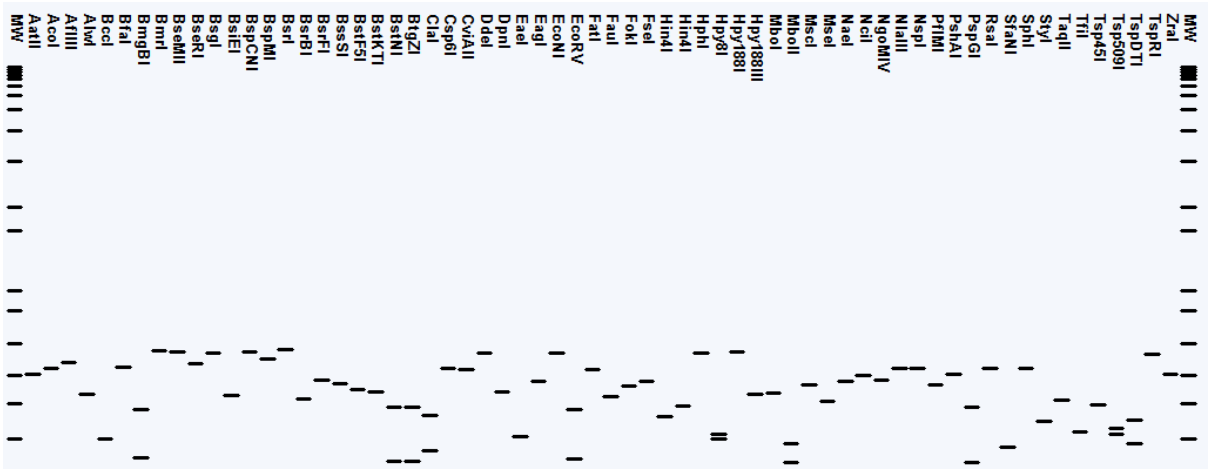

Figure 17: Possible restriction enzymes for 'Impilo' assumed by pdraw32.

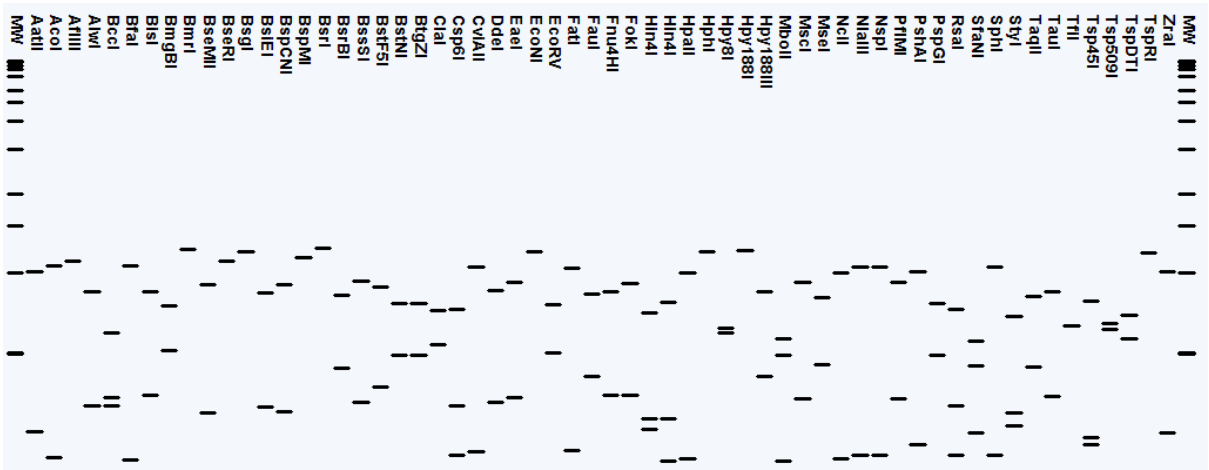

Figure 18: Possible restriction enzymes for 'Monate' assumed by pdraw32.

A summary of potential enzymes that would produce polymorphisms in the ITS region of all the sweetpotato accessions used in this study is shown in Table 8. The enzymes *Bmg*T120I, *Dpn*I, *Bse*MII, *Eag*I, *Bss*SI, *Dde*I, *Pfi*MI and *Sau*961 in Table 8 are those that will be most useful in digesting the ITS regions of the sweetpotato accessions in this study.

Table 9: Potential restriction enzymes predicted by pdraw32 computer programme.

	*Bmg*T120I	*Dpn*I	*Bse*MII	*Eag*I	*Bss*SI	*Dde*I	*Pfi*MI	*Sau*961
1. Impilo	+	+	+	+	_	+	+	+
2. Beauregard	_	+	_	+	+	_	+	_
3. Resisto	_	+	+	+	+	+	+	_
4. Khano	_	+	+	+	_	+	_	_
5. W119	_	+	_	_	+	_	+	_
6. Hernandez	+	+	_	+	_	_	_	+
7. Purple sunset	_	+	_	+	_	_	_	_
8. Ejumula	_	+	+	+	_	+	_	_
9. Isondlo	_	+	+	+	_	+	_	_
10. Jewel	_	+	+	+	_	+	_	_
11. Bophelo	_	+	+	+	_	+	_	_
12. Ndou	+	+	_	+	+	_	+	+
13. Letlhabula	_	+	_	+	+	_	+	_
14. Monate	_	+	_	+	_	_	_	_
15. Blesbok	_	+	+	+	_	_	_	_
16. Phala	_	+	+	+	_	_	_	_
17. Mafutha	_	+	+	+	_	+	_	_
18. Ribbok	_	_	+	+	_	+	+	_
19. Mamphenyane	_	+	+	+	+	+	+	_
20. Mokone	_	+	+	+	_	+	_	_
21. 199062-1	+	+	+	+	_	+	+	+
22. 2007-2-12	_	+	+	+	+	+	+	_
23. 2005-1-11	_	+	+	+	+	+	+	_
24. 2004-16-1	_	+	_	+	_	_	_	_
25. 99-9-4	_	+	+	+	_	+	_	_
26. 2004-5-2	_	+	+	+	_	+	_	_
27. 2007-1-3	_	+	+	+	+	_	_	_
28. Monate x Resisto	_	+	_	_	+	_	+	_

[+]The enzyme has a recognition site.
[-]The enzyme does not have a recognition site.

4.6 SEQUENCING ANALYSIS

Among the 28 sweetpotato accessions studied, the length of ITS1 region ranged from 175 to 184 bp and the length of ITS2 ranged from 273 to 282 bp. The GC content ranged from 57.78 to 61.33% in ITS1 and from 61.29 to 65.94% in ITS2. The AT content ranged from 35.91 to 38.07% in ITS1 and 30.11 to 34.07% in ITS2 (Table 10). The aligned data set (Annexure A) for ITS1, 5.8S and ITS2 had 640 nucleotides of which 106 were variable and for parsimony analysis 11 nucleotides were informative. DNA divergence values are shown in Table 10. The p-distance values ranged from 0 to 1.2%.

Table 10: Sequence length and the G+C and A+T content percentage of ITS1 and ITS2 region.

ACCESSIONS	ITS1			ITS2		
	Length(bp)	%(G+)	%(A+T)	Length(bp)	%(G+C)	%(A+T)
2. Impilo	177	60.03	36.72	273	64.47	34.07
2. Beauregard	176	60.23	36.93	275	65.45	33.45
3. Resisto	176	60.23	36.93	275	65.09	33.45
4. Khano	183	59.56	37.16	274	65.69	32.48
5. W119	176	59.66	38.07	276	65.94	34.06
6. Hernandez	178	60.67	37.08	276	65.22	32.97
7. Purple sunset	176	59.09	36.93	276	65.22	32.97
8. Ejumula	181	59.12	36.46	279	61.29	30.11
9. Isondlo	182	59.89	36.81	276	63.22	33.33
10. Jewel	182	59.34	37.36	276	65.58	33.70
11. Bophelo	182	58.79	37.36	277	64.26	32.49
12. Ndou	179	59.78	36.87	276	65.94	33.33
13. Letlhabula	183	60.11	37.16	276	65.58	33.70
14. Monate	176	60.80	36.93	276	65.22	32.97
15. Blesbok	175	61.14	37.14	282	62.06	32.98
16. Phala	182	58.24	36.26	276	63.77	32.61
17. Mafutha	183	58.47	36.61	276	65.22	34.06
18. Ribbok	184	58.47	36.41	276	65.58	34.06

19. Mamphenyane	176	59.09	36.93	276	65.22	33.70
20. Mokone	181	61.33	37.02	276	64.86	32.97
21. 199062-1	180	57.78	36.67	276	65.22	33.70
22. 2007-2-12	183	60.66	37.70	276	65.58	33.70
23. 2005-1-11	183	59.56	36.07	276	65.58	33.70
24. 2004-16-1	178	60.67	35.96	276	65.22	32.97
25. 99-9-4	183	59.02	37.70	276	65.22	33.33
26. 2004-5-2	182	60.99	36.81	275	65.45	32.73
27. 2007-1-3	181	59.12	35.91	276	65.22	32.97
29. Monate x Resisto	180	60.00	37.22	276	65.94	33.70

Table 11: Divergence values (p-distance) of ITS sequences between 28 sweetpotato accessions.

```
      1     2     3     4     5     6     7     8     9    10    11    12    13    14    15    16    17    18    19    20    21    22    23    24    25    26    27    28
[ 1]
[ 2]  0.000
[ 3]  0.005 0.005
[ 4]  0.003 0.003 0.003
[ 5]  0.002 0.000 0.005 0.005
[ 6]  0.003 0.003 0.007 0.008 0.003
[ 7]  0.002 0.002 0.005 0.007 0.002 0.000
[ 8]  0.005 0.005 0.010 0.009 0.005 0.009 0.007
[ 9]  0.005 0.003 0.002 0.003 0.007 0.008 0.007 0.009
[10]  0.005 0.005 0.005 0.002 0.007 0.005 0.003 0.007 0.003
[11]  0.003 0.002 0.005 0.012 0.002 0.000 0.000 0.007 0.008 0.007
[12]  0.002 0.002 0.003 0.002 0.000 0.007 0.005 0.007 0.003 0.003 0.005
[13]  0.002 0.000 0.003 0.003 0.000 0.003 0.002 0.005 0.007 0.003 0.003 0.000
[14]  0.002 0.002 0.005 0.007 0.002 0.000 0.000 0.007 0.007 0.003 0.000 0.005 0.002
[15]  0.002 0.002 0.002 0.002 0.003 0.007 0.005 0.007 0.000 0.000 0.005 0.002 0.002 0.005
[16]  0.003 0.003 0.000 0.005 0.002 0.003 0.003 0.009 0.003 0.002 0.005 0.002 0.003 0.003 0.002
[17]  0.003 0.003 0.002 0.005 0.003 0.010 0.008 0.009 0.005 0.005 0.012 0.003 0.005 0.008 0.000 0.003
[18]  0.005 0.005 0.005 0.002 0.008 0.008 0.007 0.010 0.007 0.007 0.010 0.005 0.007 0.007 0.003 0.005 0.007
[19]  0.000 0.000 0.005 0.003 0.000 0.003 0.002 0.005 0.003 0.005 0.002 0.002 0.000 0.002 0.002 0.003 0.003 0.005
[20]  0.003 0.002 0.002 0.007 0.002 0.007 0.005 0.009 0.005 0.003 0.008 0.000 0.003 0.005 0.000 0.002 0.003 0.005 0.002
[21]  0.003 0.003 0.002 0.002 0.003 0.008 0.007 0.009 0.000 0.003 0.007 0.002 0.003 0.007 0.000 0.002 0.000 0.003 0.003 0.000
[22]  0.005 0.005 0.002 0.007 0.007 0.008 0.008 0.012 0.003 0.008 0.012 0.003 0.005 0.008 0.000 0.005 0.007 0.007 0.005 0.005 0.002
[23]  0.000 0.000 0.005 0.007 0.000 0.003 0.002 0.005 0.005 0.008 0.005 0.002 0.003 0.002 0.002 0.005 0.005 0.008 0.000 0.005 0.003 0.010
[24]  0.003 0.003 0.008 0.008 0.003 0.002 0.000 0.009 0.008 0.005 0.000 0.007 0.003 0.000 0.007 0.005 0.010 0.007 0.003 0.007 0.008 0.010 0.003
[25]  0.005 0.005 0.003 0.007 0.003 0.010 0.008 0.010 0.005 0.005 0.012 0.002 0.007 0.008 0.003 0.002 0.003 0.007 0.003 0.008 0.000 0.010 0.007 0.010
[26]  0.005 0.003 0.002 0.002 0.005 0.008 0.007 0.009 0.003 0.000 0.010 0.002 0.002 0.007 0.000 0.002 0.003 0.003 0.003 0.005 0.000 0.002 0.005 0.008 0.008
[27]  0.002 0.002 0.005 0.008 0.002 0.000 0.000 0.007 0.007 0.005 0.000 0.005 0.003 0.000 0.005 0.003 0.010 0.008 0.002 0.005 0.007 0.012 0.003 0.000 0.007 0.008
[28]  0.003 0.003 0.005 0.005 0.002 0.003 0.002 0.007 0.008 0.005 0.002 0.002 0.003 0.002 0.005 0.000 0.007 0.005 0.002 0.007 0.005 0.010 0.003 0.003 0.005 0.007 0.002
```

Dendrograms were generated from the combined ITS1, 5.8S and ITS2 sequences using neighbour-joining (Fig. 19), UPGMA (Fig. 20) and maximum likelihood (Fig. 21).

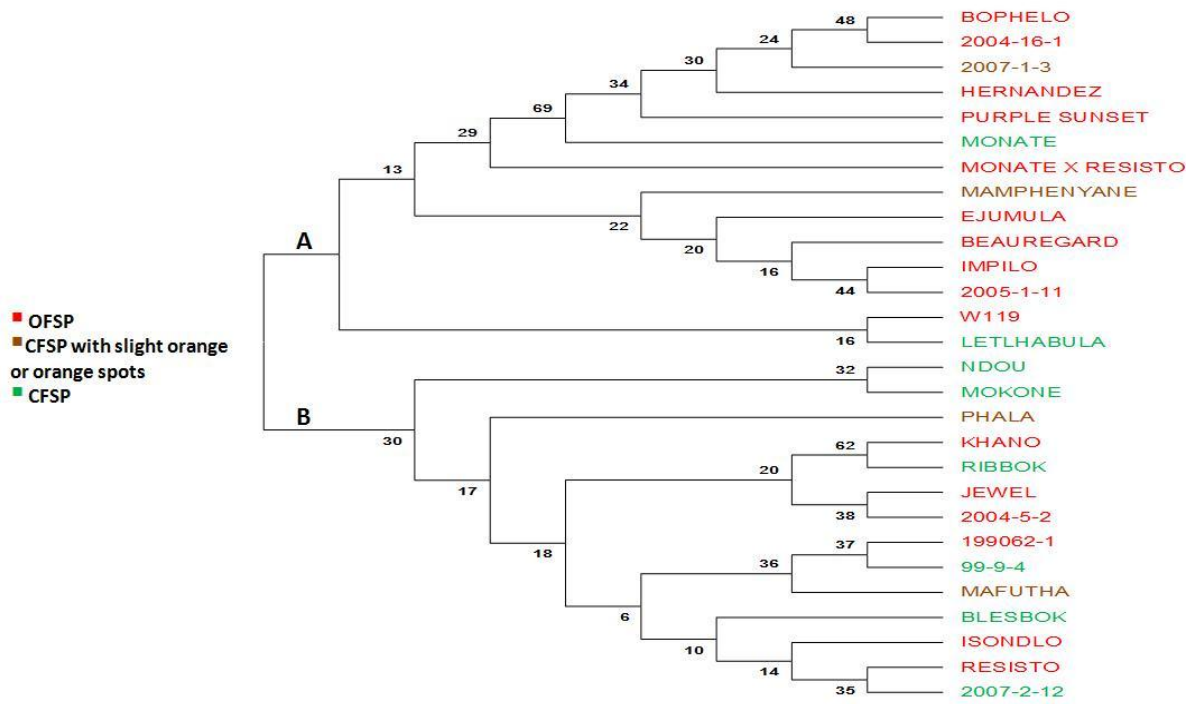

Figure 19: Neighbour-joining dendrogram showing the relationship of 28 sweetpotato accessions based on ITS1, 5.8S and ITS2. Numbers on top of the branches are bootstrap values.

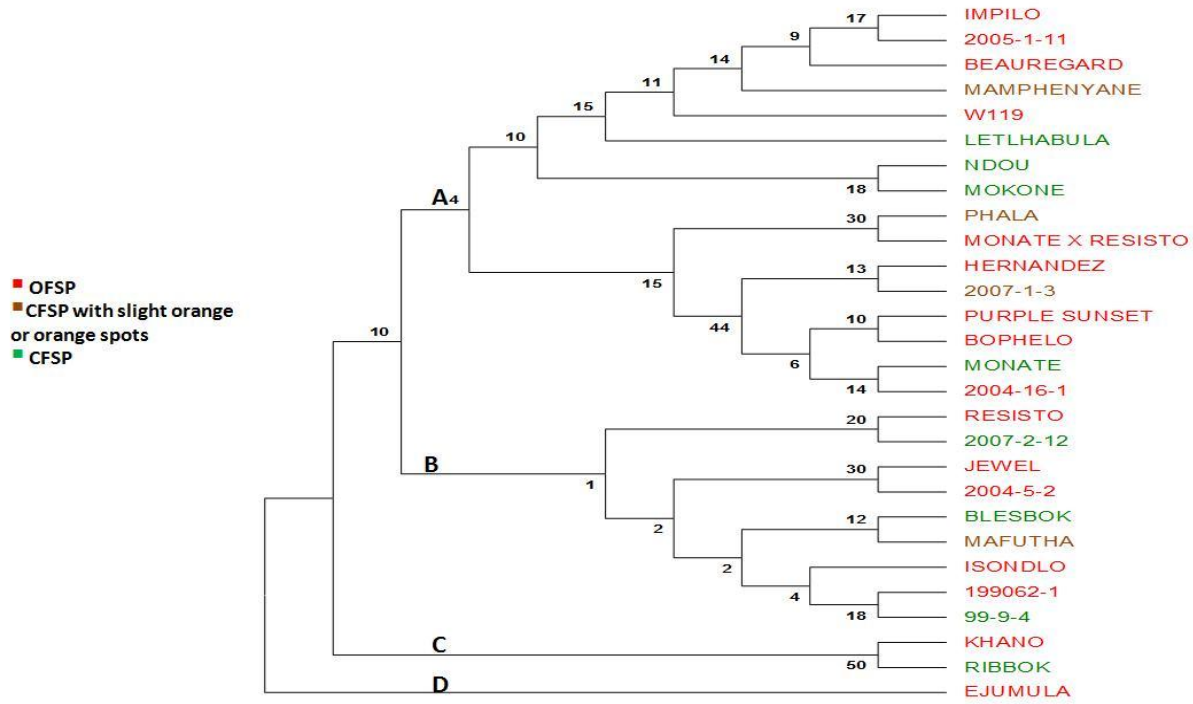

Figure 20: UPGMA dendrogram showing the relationship of 28 sweetpotato accessions based on ITS1, 5.8S and ITS2. Numbers on top of the branches are bootstrap values.

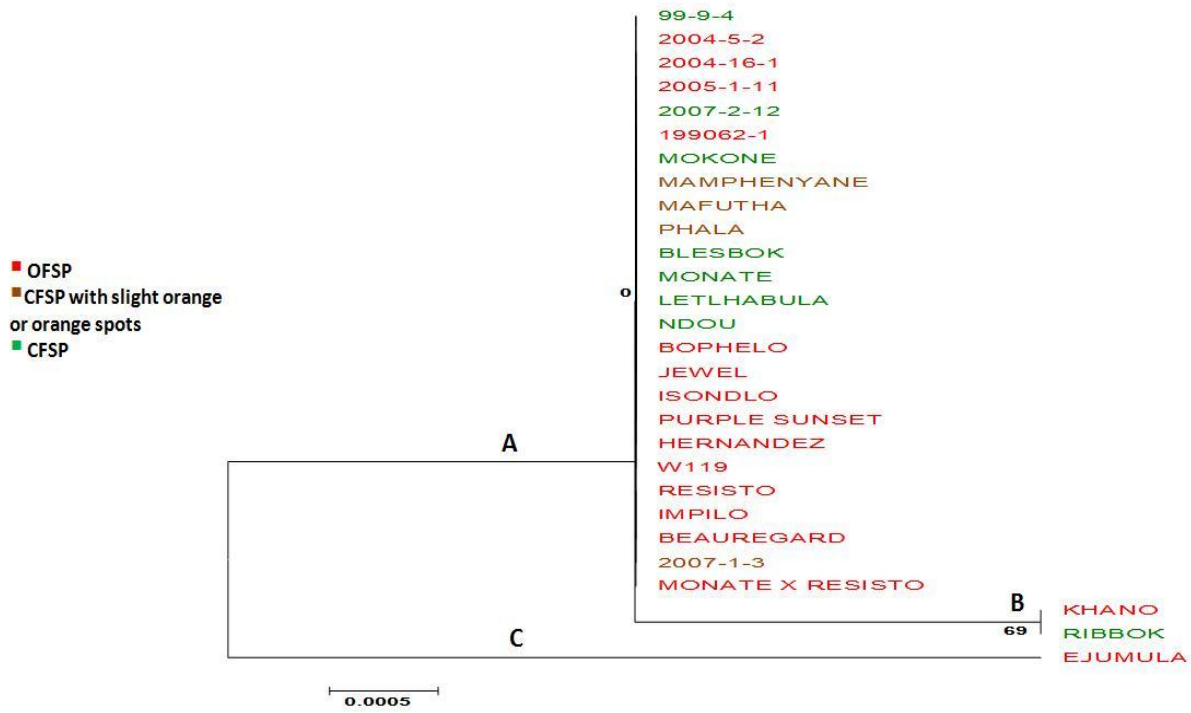

Figure 21: Maximum likelihood dendrogram showing the relationship of 28 sweetpotato accessions based on ITS1, 5.8S and ITS2**.** Numbers on top of the branches are bootstrap values.

The neighbour-joining phylogenetic tree (Fig. 19) formed two main clusters. A majority of the OFSP was found in cluster A and only two CFSP (‘Monate’ and ‘Letlhabula’) and two CFSP with slight orange or orange spots (2007-1-3 and ‘Mamphenyane’) were found in this cluster. ‘Bophelo’ and 2004-16-1, and ‘Impilo’ and 2005-1-11 shared a sister relationship at a bootstrap of 48 and 44% respectively. ‘Monate’ and ‘Monate x Resisto’ were both found in cluster A and this suggests common ancestry. The majority of the CFSP were grouped in cluster B with several accessions sharing a sister relationship and this included ‘Ndou’ and ‘Mokone’, ‘Khano’ and ‘Ribbok’, ‘Jewel’ and 2004-5-2, 199062-1 and 99-9-4, and ‘Resisto’ and 2007-2-12.

The UPGMA phylogenetic tree (Fig. 20) formed four clusters with the fourth cluster being formed by a single cultivar ‘Ejumula’. None of the clusters grouped the accessions according to their flesh colour. ‘Monate’ and ‘Monate x Resisto’ accessions still occurred in the same cluster. The sweetpotato accessions which shared a sister relationship were grouped in the same manner as in the neighbour-joining dendrogram.

The maximum likelihood dendrogram (Fig. 21) formed three clusters with ‘Ejumula’ forming a single cluster again. The majority of the accessions were found in cluster A. The sweetpotatoes were grouped in an order starting with the breeding lines, CFSP with slight orange or orange spots, CFSP and lastly the OFSP. ‘Khano’ and ‘Ribbok’ formed a sister relationship in cluster B which was supported by a strong bootstrap of 69%.

CHAPTER 5
DISCUSSION

5.1 INTRODUCTION

This chapter explains the results obtained from the protein, RAPD, ITS and sequencing results. The results obtained from this study are also compared with previous research.

5.2 SDS-PAGE PROTEIN ANALYSIS

The protein profiles of the leaves of the 28 sweetpotato accessions used in this study did not exhibit a large amount of variation. Similar findings were reported by Men (2006) from his study of the genetic diversity of leaf proteins in five sweetpotato accessions in Vietnam. Although the protein profiles did not vary greatly, a dendrogram derived from the protein profiles (Fig. 10) generally clustered the accessions according to their flesh colour and/or country of origin.

The dendrogram (Fig. 10) showed that the majority of the OFSP was found in clusters A and B_1. With the exception of breeding line 2007-1-3, cluster A was composed of entirely OFSP from the sweetpotato breeding programme in South Africa and accessions from the USA. This is not unexpected since the South African breeding programme has been using certain lines such as 'W119', 'Resisto' and 'Excel' from the USA in polycross breeding (Laurie S.a; Laurie, van Den Berg, Magoro & Kgonyane 2004: 189- 191). Another interesting aspect of the dendrogram was the grouping of the hybrid 'Resito x Monate' with one of its parents 'Resisto' in cluster A.

Further research is needed to determine the value of protein variation in analysing sweetpotato diversity and identifying a marker(s) for flesh colour. This will be valuable considering the renewed emphasis being placed on OFSP and its health benefits.

5.3 ANALYSIS OF RAPD

Unlike the protein profiles, RAPD showed high levels of genetic diversity in the sweetpotato accessions with a polymorphism level of 98%. High levels of genetic polymorphism were also reported in other studies of sweetpotato. For example, He, Prakash and Jarret (1995) reported a high level of polymorphism in sweetpotatoes in the United States with an average of 16 bands per RAPD primer. Ramisah (2001) also observed a high level of polymorphism

(98%) in Indonesian sweetpotatoes using five RAPD primers. A high level of genetic diversity (79%) was observed by Das and Naskar (2008) when assessing variability between Indian high yielding drought resistant sweetpotato by RAPD markers. Kumar, Tarafdar and Datta (2011) reported a high level of genetic diversity (88.64%) between the OFSP and CFSP when assessing the genetic relationships between Indian and exotic germplasm with 15 RAPD primers. Finally, Moulin, Rodrigues, Gonçalves, Sudre and Pereira (2012) also reported a high level of polymorphism (96.7%) in Brazilian sweetpotato.

A high genetic diversity in sweetpotato has also been reported with other markers such as sequence related amplified polymorphism, SSR, selective amplification of microsatellite polymorphic loci, DNA amplification finger printing, AFLP and morphological traits (Wang, He, Prakash & Lu 1998; Tseng, Lo & Hwang 2002; Asoro 2005; He, Liu & Wang 2005; Yada & Tukamuhabwa 2010; Wang, Li, Luo, Huang, Chen, Fang, Li, Chen & Zhang 2011).

Most vegetatively propagated crops including banana, cassava and yams seemed to have a relatively high genetic diversity (Herzberg, Muhungu, Mignouna & Kullaya 2004; Wang, Chiang, Roux, Hao & Ge 2007; Moyib, Gbadegesin, Aina & Odunola 2008; Sabir 2010). It is generally believed that there is low genetic variation in vegetatively propagated crops (Mckey, Elias, Pujol & Duputie 2010: 320- 321). The relatively high genetic variation in sweetpotato, a vegetatively propagated crop, may be due to a number of reasons including high levels of heterozygosity due to breeding, polyploidy and large genome size (Tumwegamire *et al*. 2011). The majority of the accessions and breeding lines used in this study were derived from a crossing programme involving South African and North American accessions (Laurie *et al*. 2004). This is, perhaps, one reason for the high genetic variability observed in this study.

One of the striking features of the dendrogram from the RAPD analysis is the separation of the accessions and breeding lines according to flesh colour (Fig. 14). For example, all the accessions in clusters A_1 and A_2 including ‘Impilo’, ‘Beauregard’, ‘Resisto’, ‘Khano’, ‘Hernandez’, ‘Purple sunset’ and ‘Ejumula’ were orange-fleshed varieties. From this group of accessions, ‘Beauregard’, ‘Resisto’, ‘W119’, ‘Hernandez’ and ‘Jewel’ are from North America; ‘Ejumula’ is from Uganda while the rest are South African accessions. A closer examination of cluster A showed that the North American accessions ‘Beauregard’ and ‘Resisto’ are in a sister-group relationship. The clustering patterns observed in this study are

similar to those obtained by Gichuki *et al*. (2003) who found that sweetpotato accessions grouped according to their country of origin. Asante and Offei (2003) also observed similar findings when analysing the genetic diversity of Ugandan cassava using RAPD markers. While this study did not identify a definite marker linked to flesh colour, further RAPD analysis may be able to identify the gene(s) for flesh colour in sweetpotato.

The other orange-fleshed accessions and breeding lines such as 'Bophelo', 199062-1, 2005-1-11, 2004-16-1, 2004-5-2 and 'Monate x Resisto' were found in different clusters (Fig. 14) that included cream-fleshed accessions. Similar findings were reported in the study by Tumwegamire *et al*. (2011) who assessed the genetic diversity of sweetpotatoes in East Africa using SSR markers. The sister-group relationships of white-fleshed sweetpotato (WFSP) and OFSP identified in the study by Tumwegamire *et al*. (2011) were explained by suggesting that OFSP accessions evolved from WFSP accessions as opposed to only introduced OFSP. A similar reason could account for the mixture of the OFSP and CFSP in the RAPD clusters in this study. In addition the mixture of accessions is probably due to hybridization of different sweetpotato lines with varying flesh colours.

With the exception of 199062-1, the RAPD dendrogram separated the South African germplasm that clustered in group B from the rest of the accessions. This showed a clear distinction between the South African and non-South African germplasm. These results are congruent with those obtained by Gichuki *et al*. (2003) who found that sweetpotato accessions grouped according to their country of origin. Similarly, Tumwegamire *et al*. (2011) showed that the East African sweetpotato germplasm appeared to be distinct from the non-African accessions. A RAPD study by Zhang, Ghislain, Huaman, Golmirzaie and Hijmans (1998) also found a clear distinction between accessions from South America and Papua New Guinea.

Sweetpotato breeding in South Africa started in the 1950s. Orange-fleshed sweetpotato accessions were introduced into the breeding programme in the 1980s. The relative young nature of the breeding programme perhaps accounts for the clear demarcation of the South African germplasm.

The clustering of 'Mafutha', 'Mamphenyane' and 'Mokone', is not unexpected since 'Mafutha' was involved in the parentage of 'Mamphenyane' and 'Mokone' (Laurie *et al*.

2004). It is difficult to address all the cultivar groupings in this study and identify the pedigrees of the accessions since most of them were derived from polycross breeding.

Although the RAPD dendrogram had generally separated the accession according to flesh colour, a marker(s) which distinguishes OFSP from CFSP was not obtained in this study. This is contrary to the results reported by Kumar *et al*. (2011) who found two primers (OPM-09 and RP-3) which generated markers that were able to distinguish the OFSP from the WFSP of both exotic and Indian collections. However, these markers were not clearly indicated in their publication.

The AMOVA results confirmed that there is highly significant ($p \leq 001$) variation among the OFSP, and combined CFSP and CFSP with slight orange or orange spots (Table 4). This is contrary to the results of Tumwegamire *et al*. (2011) who reported no variation among the populations of OFSP and WFSP. The within-population variability revealed a large variation which accounted 95.23% of the total molecular variance. This large variation within the accessions is perhaps due to sexual reproduction. A large within-accessions variation has also been reported in previous studies due to the same reason (Zhang *et al*. 2000; Gichuki *et al*. 2003; Tumwegamire *et al*. 2011).

The population distance (Table 5) showed that there is a short distance between the CFSP with slight orange or orange spots and the CFSP. This comparison has not been made before but it is not unexpected since the CFSP with slight orange or orange spots is more related to the CFSP rather than the OFSP. The population distance also showed that there is a large distance between the OFSP and CFSP and this confirms their genetic distinctiveness. The OFSP has the greatest mean distance indicating that the OFSP accessions have the most variation thus contributing most to the among-population variation. These results agree with the observed grouping of the majority of the OFSP in cluster A.

Analysis of molecular variance based on regions (Table 6) showed that there is a highly significant ($p \leq 0.001$) difference among the sweetpotato accessions. This is similar to previously reported studies (Zhang *et al*. 1998; Gichuki *et al*. 2003; Tumwegamire *et al*. 2011). The within-accessions variance was also significant and accounted for 94.64% molecular variance. This is similar to previous studies (Zhang *et al*. 2000; Gichuki *et al*. 2003; Tumwegamire *et al*. 2011).

The shortest population distance (Table 7) was found between the South African and non-South African OFSP confirming their close genetic relatedness. These results confirm the fact that South African OFSP evolved from non-South Africa OFSP. The large genetic distance between South African CFSP and non-South African OFSP confirms their genetic distinctiveness. The mean distance analysis showed that non-South African OFSP has the greatest mean indicating that non-South African OFSP has the most variation thus contributing most to the among-population variation. These results agree with the observed grouping of the majority of the non-South African OFSP in cluster A.

Although RAPD analysis is considered to be a relatively weak technique due to its reproducibility, this study showed that RAPD markers were generally able to separate the OFSP from the CFSP. RAPD markers were also able to separate the South African and non-South African accessions. The clustering of 'Mamphenyane' and 'Mokone' with 'Mafutha' suggests that RAPD may be able to identify pedigrees in sweetpotato since 'Mafutha' was used as a parent in the breeding of the above two varieties.

5.4 ANALYSIS OF ITS

The amplified ITS region produced a 696 bp fragment which is within a range of the ITS region of angiosperms (565- 700 bp) (Gernandt & Liston 1999: 711). This is similar to the size of the ITS region of most vegetatively propagated crops, such as banana (700 bp) (Nwakanma *et al*. 2003) and rice (591 bp) (Takaiwo, Oono & Sugiura 1985).

Digestion of the ITS fragment with the restriction enzymes did not produce any polymorphisms among the sweetpotato accessions. Restriction site analysis of some other plants such as banana provided useful information on the identification of genomes (Nwakanma *et al*. 2003). The sweetpotato ITS fragment was sequenced for further analysis.

This study used five 4-base recognition site enzymes which were unable to show polymorphism. Perhaps such enzymes should not be used in future. Pdraw32 identified several restriction enzymes namely *Bmg*T120I, *Dpn*I, *Bse*MII, *Eag*I, *Bss*SI, *Dde*I, *Pfi*MI and *Sau*961 that were able to show polymorphism between the sweetpotato accessions and these enzymes can be used in future studies of PCR-RFLP in sweetpotato.

5.5 SEQUENCING ANALYSIS

The GC content of the ITS2 is higher than that of ITS1 by 4.61 % (Table 8). This is similar to that reported by Huang *et al*. (2002) for sweetpotato. There was a small variation in the ITS region which only accounted for 16.56%. This small variation in the ITS region is due to a mechanism called concerted evolution which is responsible for assuring that the ITS intraspecific variability occurs at a lower rate (Aguilar, Rossello & Feliner 1999: 1341-1342). Although there was low variation in the ITS region, phylogenetic trees constructed from the sequences were able to compare the relationships between the sweetpotato accessions.

The neighbour-joining dendrogram (Fig. 19) grouped the majority of the OFSP in cluster A with the exception of 'Khano', 'Jewel', 2004-5-2, 199062-1, 'Isondlo' and 'Resisto' that are found in cluster B. With the exception of 'Letlhabula' and 'Monate', Cluster B was composed mainly of CFSP. This type of clustering is similar to that found in the protein and RAPD analysis of this study.

The UPGMA dendrogram (Fig. 20) was unable to cluster the sweetpotatoes according to their flesh colour and this could be attributed to the fact that the UPGMA method assumes a constant rate of evolution of the sequences in all branches of the tree (Michener & Sokal 1957). Although the UPGMA tree could not separate the accessions according to flesh colour, most of the sweetpotatoes which share a sister relationship in the neighbour-joining phylogenetic tree (Fig. 19) shared the same relationship in the UPGMA dendrogram and these include: 'Impilo' and 2005-1-11, 'Ndou' and 'Mokone', 'Khano' and 'Ribbok', 'Jewel' and 2004-5-2, 199062-1 and 99-9-4, and 'Resisto' and 2007-2-12. From these sister relationships, the most interesting one is that between 199062-1 and 99-9-4. The breeding line 99-9-4 originated from 'Rose Centennial'. The latter is a parent of 199062-1. This shows that the two accessions share a common ancestry and hence they were clustered together.

The most interesting aspect of the maximum likelihood dendrogram (Fig. 21) was the clustering of 'Khano' and 'Ribbok' which are both South African accessions. The poorly resolved relationships of the sweetpotato accessions with maximum likelihood as reflected by the low bootstrap analysis could be attributed to the low ITS divergence (Table 9). Similar findings were also reported by Huang *et al*. (2002), who reported low ITS divergence among the series *Batatas*.

One of the interesting results observed in all the dendrograms (neighbour-joining, UPGMA and maximum likelihood) is the grouping of 'Khano' and 'Ribbok'. This may suggest the OFSP cultivar, 'Khano', evolved from the CFSP 'Ribbok'. This kind of relationship was also evident in the study conducted by Tumwegamire *et al*. (2011) who studied the genetic relationship of the OFSP and WFSP East African sweetpotatoes.

Although the restriction enzymes were unable to show polymorphism, the dendrograms based on the ITS sequences were able to show the relationships between the sweetpotato accessions particularly the neighbour-joining dendrogram. The latter dendrogram was able to separate the OFSP from the CFSP. The neighbour-joining and UPGMA dendrograms were able to show the relationship of 99-9-4 and 199062-1 which share a common ancestor which is 'Rose Centennial'.

CHAPTER 6
CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The aim of this study was to assess the genetic diversity of South African sweetpotato germplasm using DNA and protein markers. Three objectives were formulated and structured to achieve the aim of the study. Three techniques which are analysis of protein profiles using SDS-PAGE, analysis of RAPD and variation in the ITS region were employed to assess the variation between the sweetpotatoes.

All the sweetpotato accessions had a simple match similarity coefficient of one when they were analysed using protein profiles. Although all the accessions had a coefficient of one, there was a clear separation of the OFSP from the CFSP and the accessions were also grouped according to their country of origin.

The RAPD results also clustered the accessions according to their flesh colour and country of origin despite the disadvantage of the RAPD technique not being reproducible. The AMOVA results showed that there is great variation among and within the sweetpotato accessions when grouped according to colour and country of origin. The analysis of the population distance showed that there is a long genetic distance between the South African CFSP and the non-South African OFSP germplasm as opposed to the South African CFSP and the South African OFSP. These population distance results showed that there is a great potential of achieving an orange-fleshed sweetpotato cultivar which will contain both high β-carotene and high dry matter content when using both the South African CFSP and non-South African OFSP accessions in the breeding programme.

Although the ITS sequence results showed low divergence values, the phylogenetic trees constructed were similar to the protein and RAPD results. The ITS sequence results generally clustered the sweetpotato accessions into OFSP and CFSP. The ITS sequence results were also able to reveal accessions which shared a common ancestor.

In conclusion, the proteins, RAPD and ITS sequencing techniques all proved to be useful in assessing the genetic diversity of South African sweetpotato germplasm. All these techniques generally separated the sweetpotato accessions according to their flesh colour and country of

origin. From these results breeders can develop an efficient breeding program with significant levels of genetic difference which is a prerequisite for improvement of sweetpotato accessions.

6.2 RECOMMENDATIONS

The following are recommendations for further research based on the results obtained from this study.

1) It is recommended that different parts of the plant such as the flowers, stem and storage roots should be analysed when using protein profiles instant of using only the leaves. This will provide a better understanding of the proteins found in the plant.

2) Further analysis for identifying a marker(s) for sweetpotato flesh colour should be done using the primers identified in Kumar *et al.* (2011). The latter study found two primers OPM-09 and RP-3 that were able to distinguish between the OFSP and CFSP.

3) Other molecular markers such as AFLP and SSR markers should be used to provide a greater understanding of the genetic diversity in sweetpotato. These markers are considered to have more advantages than the RAPD markers.

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ANNEXURE A

Sequence alignment of the entire ITS region (ITS1, 5.8S and ITS2).

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IMPILO          -------GCCCRRCA--GACGGACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 50
2005-1-11       CCAMCKKGCMRGGCA--GACG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 56
RESISTO         --------GCMRGCA-GAGCG-ACCAG-AGAACGCGTTTGTTATTCAATCTCCTCGTCCG 49
199062-1        -------GCMMRGCAAGAGCGGACCASGAGAACGYGTTTGTTATTCAATCTCCTCGTCCG 53
MAFUTHA         -TACCCTGCMMRGCA-GAGCG-ACCMG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 56
MAMPHENYANE     -------GCMMRGCA--GACG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 49
99-9-4          -GAMCAKGCACAGCA-GAGCG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 56
BEAUREGARD      -------GCCMRGCA--GACG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 49
W119            -------GCMARGCAG--ACG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 49
NDOU            -------GCMMRGCARGAACGGACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 52
LETLHABULA      GAACCKKGCMAGGCAG--ACG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 56
MONATE          -TACCKGCCA--GCAG--ACG-ACCRG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 53
ISONDLO         -GAAMGKGCMAG-CA-GAGCG-ACCAG-AGAACGCGTTTGTTATTCAATCTCCTCGTCCG 55
2004-5-2        -AACCGKGCCAG-CA-GAGCG-ACCAG-AGAACGCGTTTGTTATTCAATCTCCTCGTCCG 55
KHANO           TAAMCGKGCMMGGCA-GA-CG-ACCAG-AGAACGCGTTTGTTATTCAATCTCCTCGTCCG 56
JEWEL           TAACCKK-CMMGGCA-GA-CG-ACCAG-AGAACGCGTTTGTTATTCAATCTCCTCGTCCG 55
RIBBOK          TAMCCKGSCMMGSCM-GARCG-ACCAG-AGAACGCGTTTGTTATTCAATCTCCTCGTCCG 57
2007-2-12       GAACAKGGMCAGC-A-GAGCG-ACCAG-AGAACGCGTTTGTTATTCAATCTCCTCGTCCG 56
MOKONE          -GTCCCKGCCAG-CA-GA-CG-ACCAG-AGAACGCGTTTGTTATTCAATCTCCTCGTCCG 54
BLESBOK         -------GCCRG-CA-GA-CG-ACCAG-AGAACGCGTTTGTTATTCAATCTCCTCGTCCG 48
PHALA           AMMCCKGCMMMR-CA-GA-CG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 55
HERNANDEZ       ------GCACMRGC--AGACGGACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 51
2004-16-1       ------GCCCRRGCC-AGACG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 51
ONATE           ------GC-CMRGC--AGACG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 49
PURPLE          ------GC-CMRGC--AGACG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 49
BOPHELO         GGAMCTGC-MAGGC--AGACG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 55
2007-1-3        -GMMCKGC-MMMGC--AGACG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 54
EJUMULA         --AMCSKGCMMGGCA--GACG-ACCAG-AGAACGYGTTTGTTATTCAATCTCCTCGTCCG 54
                                 ** ***   ****** ************************

IMPILO          GGCGCATGCCTCGGGCGACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAY 110
2005-1-11       GGCGCATGCCTCGGGCGACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAY 116
RESISTO         GGCGCATGCCTCSSGCGACTAACGAACCCCCGGCGCGGAACGCGCCAAGGAATATCGTAY 109
199062-1        GGCGCATGCCTCSSGCKACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAY 113
MAFUTHA         GGCGCATGCCTCSGSCGACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAY 116
MAMPHENYANE     GGCGCATGCCTCGGGCGAYTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAY 109
99-9-4          GGCGCATGCCTCGGGCGAYTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAY 116
BEAUREGARD      GGCGCATGCCTCGGGCGACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAC 109
W119            GGCGCATGCCTCGGGCGAYTAACGAACCCCCGGCGCGGAATGCGCCAAGGAATATCGTAC 109
NDOU            GGCGCATGCCTCGGGCGACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAC 112
LETLHABULA      GGCGCATGCCTCGGGCGACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAC 116
MONATE          GGCGCATGCCTCGGGYGACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAC 113
ISONDLO         GGCGCATGCCTCGGGCGAYTWACGAACCCCCGGCGCGGAACGCGCCAAGGAATATCGTAY 115
2004-5-2        GGCGCATGCCTCGGGCGAYTWACGAACCCCCGGCGCGGAACGCGCCAAGGAATATCGTAY 115
KHANO           GGCGCATGCCTCGGGCGACAAAMGAACCCCCGGCGCGGAACGCGCCAAGGAATATCGTAY 116
JEWEL           GGCGCATGCCTCGGGCGAYTAACGAACCCCCGGCGCGGAACGCGCCAAGGAATATCGTAY 115
RIBBOK          GGCGCATGCCTCGGGTGACAAAMGAACCCCCGGCGCGGAACGCGCCAAGGAATATCGTAY 117
2007-2-12       GGCGCATGCCTCGGGCGACTAACGAACCCCCGGCGCGGAACGCGCCAAGGAATATCGTAY 116
MOKONE          GGCGCATGCCTCGGGCKACTAACGAACCCCCGGCGCGGAACGCGCCAAGGAATATCGTAY 114
BLESBOK         GGCGCATGCCTCGGGCKACTAACGAACCCCCGGCGCGGAACGCGCCAAGGAATATCGTAY 108
PHALA           GGCGCATGCCTCGGGCSACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAC 115
HERNANDEZ       GGCGCATGCCTCGGGCGACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAC 111
2004-16-1       GGCGCATGCCTCGGGCGWYTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAC 111
ONATE           GGCGCATGCCTCGGGCGACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAC 109
PURPLE          GGCGCATGCCTCSSGCKACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAC 109
BOPHELO         GGCGCATGCCTCGGGCSACTAAMGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAY 115
2007-1-3        GGCGCATGCCTCGGGCGACTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAY 114
EJUMULA         GGCGCATGCCTCGGGCGAYTAACGAACCCCCGGCGCGGAAYGCGCCAAGGAATATCGTAC 114
                ************         * ***************** ******************

IMPILO          TGAGATGGCCMGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 170
2005-1-11       TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 176
RESISTO         TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 169
199062-1        TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 173
MAFUTHA         TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 176
MAMPHENYANE     TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 169
99-9-4          TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 176
BEAUREGARD      TGAGATGGCCAGCCGCCCGTGCCCCGTSTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 169
W119            TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 169
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NDOU            TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 172
LETLHABULA      TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 176
MONATE          TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 173
ISONDLO         TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 175
2004-5-2        TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 175
KHANO           TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 176
JEWEL           TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 175
RIBBOK          TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 177
2007-2-12       TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 176
MOKONE          TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 174
BLESBOK         TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 168
PHALA           TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 175
HERNANDEZ       TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 171
2004-16-1       TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 171
ONATE           TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 169
PURPLE          TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 169
BOPHELO         TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 175
2007-1-3        TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 174
EJUMULA         TGAGATGGCCAGCCGCCCGTGCCCCGTCTTTGCGGATCGCTCGGGAGGCGTCGGCGTCTT 174
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IMPILO          ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 230
2005-1-11       ACTTAATAAAATACGACTCTCGGCAACGGATATCTYGGCTCTCGCATCGATGAAGAACGT 236
RESISTO         ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 229
199062-1        ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 233
MAFUTHA         ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 236
MAMPHENYANE     ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 229
99-9-4          ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 236
BEAUREGARD      ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 229
W119            ACTTAATAAAATACGACTCTCGGCAACGGATATCTYGGCTCTCGCATCGATGAAGAACGT 229
NDOU            ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 232
LETLHABULA      ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 236
MONATE          ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 233
ISONDLO         ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 235
2004-5-2        ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 235
KHANO           ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 236
JEWEL           ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 235
RIBBOK          ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 237
2007-2-12       ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 236
MOKONE          ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 234
BLESBOK         ACTTAAWAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 228
PHALA           ACTTAATAAAATACGACTCTCGGCMACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 235
HERNANDEZ       ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 231
2004-16-1       ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 231
ONATE           ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 229
PURPLE          ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 229
BOPHELO         ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 235
2007-1-3        ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 234
EJUMULA         ACTTAATAAAATACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGT 234
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IMPILO          AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCWTCGAGTCTTTGAACG 290
2005-1-11       AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 296
RESISTO         AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 289
199062-1        AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 293
MAFUTHA         AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 296
MAMPHENYANE     AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 289
99-9-4          AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 296
BEAUREGARD      AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 289
W119            AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 289
NDOU            ARCGAAATGCGATACTTGGTGTGAATTGCARAATCCCGTGAACCATCGAGTCTTTGAACK 292
LETLHABULA      AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 296
MONATE          AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 293
ISONDLO         AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 295
2004-5-2        AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 295
KHANO           AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 296
JEWEL           AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 295
RIBBOK          AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 297
2007-2-12       AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 296
MOKONE          AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 294
BLESBOK         ARCGAAATGCGATACTTGGTGTGAATTGCARAATCCCRTGAACCATCRAGTCTTTGAACG 288
PHALA           ARCGAAATGCGATACTTGGTGTGAATTGCARAATCCCGTGAACCATCGAGTCTTTGAACG 295
HERNANDEZ       AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 291
2004-16-1       AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 291
ONATE           AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 289
PURPLE          AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 289
BOPHELO         AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 295
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2007-1-3          AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 294
EJUMULA           AGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACG 294
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IMPILO            CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 350
2005-1-11         CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 356
RESISTO           CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 349
199062-1          CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGWCACGCATCGCGT 353
MAFUTHA           CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 356
MAMPHENYANE       CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 349
99-9-4            CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGWCACGCATCGCGT 356
BEAUREGARD        CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 349
W119              CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 349
NDOU              CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 352
LETLHABULA        CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 356
MONATE            CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 353
ISONDLO           CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGWCACGCATCGCGT 355
2004-5-2          CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 355
KHANO             CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 356
JEWEL             CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 355
RIBBOK            CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGWCACGCATCGCGT 357
2007-2-12         CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGWCACGCATCGCGT 356
MOKONE            CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 354
BLESBOK           CAAGTTGCGCCCGAAGCCGWCAGGCCGAGGGCACGTCTGCCTGGGCGTCRCGCATCGCGT 348
PHALA             CAAGTTGCGCCCGAAGCCGWCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 355
HERNANDEZ         CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 351
2004-16-1         CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 351
ONATE             CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 349
PURPLE            CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 349
BOPHELO           CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 355
2007-1-3          CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 354
EJUMULA           CAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGT 354
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IMPILO            C---CCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 407
2005-1-11         CGCCCCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 416
RESISTO           CGCCCCCCGCTCSRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 409
199062-1          CGCCCCCCGCTCSRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 413
MAFUTHA           CGCCCCCCGCTCSATCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 416
MAMPHENYANE       CGCCCCCCGCTCSRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 409
99-9-4            CGCCCCCCGCTCSRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 416
BEAUREGARD        CGCCCCCCGCTCSRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 409
W119              CGCCCCCCGCTCCGTCCTCGGTCGAGCGCGGGGGTGTGGATGTTGGCCTCCCGTGCCCCAA 409
NDOU              CGCCCCCCGCTCCGTCCTCGGTCGAGCGCGGGGGTGKGGATGTTGGCCTCCCGTGCCCCAA 412
LETLHABULA        CGCCCCCCGCTCCGTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 416
MONATE            CGCCCCCCGCTCCGTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 413
ISONDLO           CGCCCCCCGCTCSATCCTCGGTCGAGCGCGGGGGTGCGGATGTTGGCCTCCCGTGCCCCAA 415
2004-5-2          CGCCCCCCGCTCSRTCCTCGGTCGAGCGCGGGGGTGCGGATGTTGGCCTCCCGTGCCCCAA 415
KHANO             CGCCCCCCGCTCSRTCCTCGGTCGAGCGCGGGGGTGCGGATGTTGGCCTCCCGTGCCCCAA 416
JEWEL             CGCCCCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGCGGATGTTGGCCTCCCGTGCCCCAA 415
RIBBOK            CGCCCCCCGCTCSATCCTCGGTCGAGCGCGGGGGTGCGGATGTTGGCCTCCCGTGCCCCAA 417
2007-2-12         CGCCCCCCGCTCSRTCCTCGGTCGAGCGCGGGGGTGCGGATGTTGGCCTCCCGTGCCCCAA 416
MOKONE            CGCCCCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 414
BLESBOK           CSCCCCCCGCTCSATCCTCGGYCGAGCGCGGGGGTGSGGATGTTGGCCTCCCGTGCCCCAR 408
PHALA             CGCCCCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 415
HERNANDEZ         CGCCCCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 411
2004-16-1         CGCCCCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 411
ONATE             CGCCCCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 409
PURPLE            CGCCCCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 409
BOPHELO           CGCCCCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 415
2007-1-3          CGCCCCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 414
EJUMULA           CGCCCCCCGCTCCRTCCTCGGTCGAGCGCGGGGGTGYGGATGTTGGCCTCCCGTGCCCCAA 414
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IMPILO            CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 467
2005-1-11         CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 476
RESISTO           CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 469
199062-1          CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 473
MAFUTHA           CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 476
MAMPHENYANE       CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 469
99-9-4            CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 476
BEAUREGARD        CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 469
W119              CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 469
NDOU              CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 472
LETLHABULA        CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 476
MONATE            CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 473
ISONDLO           CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 475
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2004-5-2          CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 475
KHANO             CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 476
JEWEL             CTCGAGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 475
RIBBOK            CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 477
2007-2-12         CTCGTGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 476
MOKONE            CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 474
BLESBOK           CTCGWGGCGCGGCCGGSCTAAATGCRAGTCCTTGGCGACGGACRTCACGGCGAGTGGTGG 468
PHALA             CTCGWGGCGCGGCCGGCCTAAATGCRAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 475
HERNANDEZ         CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 471
2004-16-1         CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 471
ONATE             CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 469
PURPLE            CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 469
BOPHELO           CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 475
2007-1-3          CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 474
EJUMULA           CTCGWGGCGCGGCCGGCCTAAATGCGAGTCCTTGGCGACGGACGTCACGGCGAGTGGTGG 474
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IMPILO            TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCRAACRACCCCGA 527
2005-1-11         TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCGAACGACCCCGA 536
RESISTO           TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCRAACGACCCCGA 529
199062-1          TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCGAACGACCCCGA 533
MAFUTHA           TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCGAACGACCCCGA 536
MAMPHENYANE       TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCGAACGACCCCGA 529
99-9-4            TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCGAACGACCCCGA 536
BEAUREGARD        TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCGAACGACCCCGA 529
W119              TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCGAACGACCCCGA 529
NDOU              TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCGAACGACCCCGA 532
LETLHABULA        TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCGAACGACCCCGA 536
MONATE            TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCGAACGACCCCGA 533
ISONDLO           TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCGAACGACCCCGA 535
2004-5-2          TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCGAACGACCCCGA 535
KHANO             TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCGAACGACCCCGA 536
JEWEL             TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCGAACGACCCCGA 535
RIBBOK            TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCGAACGACCCCGA 537
2007-2-12         TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTTCTCGGGCGAACGACCCCGA 536
MOKONE            TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCGAACGACCCCGA 534
BLESBOK           TCGTACCTAGCGTGCATATCTTCRAGCCGTGCCCCGKCGTTCTCGGGCGAACGACCCCGA 528
PHALA             TCGTACCTAGCGTGCATATCTTCGAGCCSTGCCCCGKCGTTCTCGGGCGAACGACCCCGA 535
HERNANDEZ         TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCGAACGACCCCGA 531
2004-16-1         TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCGAACGACCCCGA 531
ONATE             TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCGAACGACCCCGA 529
PURPLE            TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCGAACGACCCCGA 529
BOPHELO           TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCRAACGACCCCGA 535
2007-1-3          TCGTACCTAGCGTGCATATCTTCGAGCCGTGCCCCGTCGTYCTCGGGCGAACGACCCCGA 534
EJUMULA           TCGTACCTAGCGTGCATATCTTCGAGCCGKGCCCCGTCGTTCTCGGGCGAACSACCCCRA 534
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IMPILO            ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 585
2005-1-11         ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 594
RESISTO           ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 587
199062-1          ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 591
MAFUTHA           ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 594
MAMPHENYANE       ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 587
99-9-4            ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 594
BEAUREGARD        ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 587
W119              ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 587
NDOU              ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 590
LETLHABULA        ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGYGGGATTACCC-G 594
MONATE            ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 591
ISONDLO           ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGYGGGATTACCC-G 593
2004-5-2          ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGYGGGATTACCC-G 593
KHANO             ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGYGGGATTACCC-G 594
JEWEL             ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGTGGGATTACCC-G 593
RIBBOK            ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 595
2007-2-12         ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGCGGGATTACCC-G 594
MOKONE            ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGYGGGATTACCC-G 592
BLESBOK           ACGAGCCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGSGGGATTACCC-K 586
PHALA             ACGAGMCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGSGGGATTACCC-G 593
HERNANDEZ         ACGAGCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGYGGGATTACCC-G 589
2004-16-1         ACGAGCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGYGGGATTACCC-G 589
ONATE             ACGAGCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGYGGGATTACCC-G 587
PURPLE            ACGAGCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGYGGGATTACCC-G 587
BOPHELO           ACGAGCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGYGGGATTACCC-G 593
2007-1-3          ACGAGCCCCCCTCAGTGCGGCTCTCCGAACGC-GACCCCAGGTCAGGYGGGATTACCC-G 592
EJUMULA           ACGARCCCCCCYTAGKGCGGCTCTCCSAAMSSCAMCCCCRGGTMRGGYGGGAATWCCCCG 594
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IMPILO          CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 616
2005-1-11       CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 625
RESISTO         CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 618
199062-1        CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 622
MAFUTHA         CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 625
MAMPHENYANE     CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 618
99-9-4          CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 625
BEAUREGARD      CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 618
W119            CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 618
NDOU            CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 621
LETLHABULA      CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 625
MONATE          CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 622
ISONDLO         CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 624
2004-5-2        CTGAGTTTAAGCATATCAATAAG-CGGAGGA------- 623
KHANO           CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 625
JEWEL           CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 624
RIBBOK          CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 626
2007-2-12       CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 625
MOKONE          CTGAGTTTAAGCATATCAATAAS-CGGAGGAA------ 623
BLESBOK         YTGAGTTTAAGCATATCAATAAG-CGGAGGAAGAGGAA 623
PHALA           CTGAGTTTAAGCWTATCAATAAS-CGGAGGAA------ 624
HERNANDEZ       CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 620
2004-16-1       CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 620
ONATE           CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 618
PURPLE          CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 618
BOPHELO         YTGAGTTTAAGCWTATCAATAARGCGGAGGAA------ 625
2007-1-3        CTGAGTTTAAGCATATCAATAAG-CGGAGGAA------ 623
EJUMULA         CTGAGTTWA-GCWTATMA-TAAGSSGGAAGGGAA---- 626
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