Genotyping-by-Sequencing of sweet-stem and grain sorghum for linkage mapping

Nare Ngoepe

(Student Number: 12382923)

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Faculty of Natural and Agricultural Sciences

Department of Plant Sciences

University of Pretoria

Pretoria, South Africa

Supervisor: Dr B. Crampton

Dr D.A. Odeny

Dr D.J.G. Rees

Declaration

I declare that the dissertation hereby submitted to University of Pretoria for the degree of Master of Science in Genetics has not previously been submitted for the degree at this or any other university. All the assistance and contribution received have been properly acknowledged.

Signature **Date**

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Table of contents

Abstract

Advances in next generation sequencing technologies have enabled researchers to do in depth genome studies. The steadily decreasing cost of sequencing has made it possible to conduct a Genotyping-by-Sequencing (GBS) approach both in plants and animals. A reliable and efficient genotyping protocol is crucial for studying and understanding the genetics and genomics of sorghum. The current work aimed at investigating the applicability of Genotyping-by-Sequencing techniques in a sorghum mapping population generated between sweet stem and grain sorghum parents. Two methods of Genotyping-by-Sequencing, whole genome shotgun (WGS) and restriction-site associated DNA (RAD) methods were used to examine the sorghum genome in this study. A total of 921 031 and 3 119 variants (SNPs and INDELs) were identified in WGS and RAD sequencing approaches respectively using CLC Genomics Workbench 6.0.1. The Trait Analysis by aSSociation, Evolution and Linkage (TASSEL) pipeline identified a total of 2 701 814 and 17 012 in the WGS and the RAD sequencing approach respectively. The TASSEL pipeline identified 1 456 253 and 3 435 variants from the two parents in the WGS and the RAD sequencing approach respectively. The results revealed the RAD method as the better Genotyping-by-Sequencing approach for large populations and Trait Analysis by aSSociation, Evolution and Linkage as the best data analysis tool as it discovered more variations than CLC Genomics Workbench. The development of a precise and inexpensive Genotyping-by-Sequencing protocol serves as a robust framework to which sorghum populations can be

characterized. These results will contribute towards genetic mapping of the markers and subsequent identification of quantitative trait loci (QTLs) governing different traits of interest contributing towards breeding for feedstock varieties that are optimized for biofuel production from sorghum.

Abbreviations and symbols

AFLP	:	Amplified Fragment Length Polymorphism
AluI	:	*Arthrobacter luteus* restriction enzyme
ARC	:	Agricultural Research Council
bp	:	Base Pair
BCL	:	Base Call Library
BC	:	Before Christ
C	:	Control
CA	:	California
CASAVA	:	Consensus Assessment of Sequence and Variation
CGIAR	:	Consultative Group on International Agricultural Research
Cm	:	centimeters
CTAB	:	Cetyl trimethylammonium bromide
C3	:	3-carbon
C4	:	4-carbon
DH	:	Double Haploid
DNA	:	Deoxyribose Nucleic Acid
dNTP	:	Deoxynucleotide triphosphate
EDTA	:	Ethylenediaminetetraacetic acid
EST	:	Expressed Sequence Tag
F	:	Filial generation after a cross
FAO	:	Food and Agriculture Organization
Fig.	:	Figure
g	:	Gram

Gb	:	Giga Base
GBS	:	Genotyping-by-Sequencing
h	:	Hour
HpaII	*:*	*Haemophilus aegyptius* restriction enzyme
ICRISAT	:	International Crops Research Institute for the Semi-Arid Tropics
IGV	:	Intergrative Genome Viewer
INDEL	:	Insertions and Deletions
kbp	:	Kilo Base Pairs
Kg	:	Kilogram
m	:	Meter
M	:	Molar
MAB	:	Marker Assisted Breeding
MAS	:	Marker Assisted Selection
Mbp	:	Mega base pair
Mg	:	Magnesium
µl	:	Microliter
n	:	Nano
NaOH	:	Sodium hydroxide
NGS	:	Next Generation Sequencing
P	:	Phosphorus
p	:	Pico
P1	:	Parent 1
P2	:	Parent 2
Prog 1	:	Progeny 1

Prog 2	:	Progeny 2
PCR	:	Polymerase Chain Reaction
QTLs	:	Quantitative Trait Loci
RAD	:	Restriction site-associated DNA
RAPD	:	Random Amplified Polymorphic DNA
RILs	:	Recombinant Inbred Lines
RFLP	:	Restriction Fragment Length Polymorphism
SA	:	South Africa
SBS	:	Sequencing by Synthesis
SNP	:	Single Nucleotide Polymorphism
SSLP	:	Simple Sequence Length Polymorphism
SSR	:	Simple Sequence Repeat
STMS	:	Sequence-Tagged Microsatellite Sites
STR	:	Short Tandem Repeats
TAE	:	Tris-acetate-EDTA
TASSEL	:	Trait Analysis by aSSociation, Evolution and Linkage
UK	:	United Kingdom
USA	:	United States of America
WGS	:	Whole Genome Shotgun
µ	:	Micro
^{0}C	:	Degrees Centrigrade

Index of figures

Index of tables

CHAPTER1: Introduction and literature review

1.1 General introduction to sorghum

Sorghum (*Sorghum bicolor* L. Moench) is ranked as the fifth most important cereal crop in the world, after maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), and barley (*Hordeum vulgare* L.) based on the total grain production (Paterson *et al.,* 2008; Mace *et al.,* 2009). In Africa, sorghum is ranked the second most important cereal crop after maize (Borrell *et al.,* 2010). The crop is widely cultivated in warmer climates where the availability of water is limited because it's well adapted to harsh environments. The ability of sorghum to withstand drought is largely attributed to the crop uses C4 photosynthetis mechanism. C4 photosynthetic plants use complex biochemical and morphological specializations to improve carbon assimilation at high temperatures (Paterson *et al.,* 2008). In sorghum, both morphological and physiological characteristics are specialized to adapt to unfavorable conditions. For example, the crop has the ability to stop growth in periods of drought and resume when conditions become favorable (Muui *et al.,* 2013). It also has an extensive root system and a waxy bloom on the leaves that reduces water loss. However, sorghum can be grown in high rainfall areas because it is tolerant to waterlogging (Pardales *et al.,* 1991).

Sorghum is considered a primary staple food crop in the semi-arid tropics of Asia, Africa and South America and continues to play a major role in food security for millions of people. In the arid countries of northeast Africa such as Sudan and Ethiopia, sorghum contributes about 40% of calories in the human diet (Kresovich *et al.,* 2005). According to the “Investigation by the Sorghum Section 7 Committee into the South African sorghum industry” about 90% of the total sorghum produced (200 000 tons of sorghum per annum) is consumed locally as feed and food.

Sorghum grains are consumed in poor communities because of their high levels of micronutrients, which contribute towards combating malnutrition (Rao *et al.,* 2006). Paterson (2008) further emphasizes that the growing importance of sorghum is due to increasing population sizes that need more reliable feed and food. Other factors include the increasing demand for limited water supplies and global climatic concerns that affect food security. Sorghum interests farmers not only because of its wide adaptation to harsh conditions and drought tolerance, but also because of its rapid growth (Reddy *et al.,* 2005).

Besides its use as grain, sorghum is also increasingly gaining importance for its potential use in bioethanol fuel production (Reddy *et al.,* 2005; Prasad *et al.,* 2007; Laopaiboon *et al.,* 2009; Zhang *et al.,* 2010). Apart from sugarcane (*Saccharum* spp.), a close relative to sorghum (Tarpley and Vietor, 2007), which has been used traditionally for biofuel production (Limtong *et al.,* 2007; Goldemberg *et al.,* 2008), other crops like maize (Torney *et al.,* 2007) and cassava (*Manihot esculenta*) (Papong and Malakul, 2010) have been utilized as feedstock for bioethanol. However, there have been concerns over their utilization towards biofuels at the expense of food, which may escalate food insecurity concerns (Boddiger, 2007). As a result, there are global efforts to come up with integrated solutions that include both food and biofuel production in a way that does not compromise food security.

The main interest for utilizing sorghum as a source of bioethanol over sugarcane is because sugarcane is resource intensive as compared with sorghum. For example, sugarcane uses four times more water than sorghum (Reddy *et al.,* 2007). Furthermore, sugarcane takes 12-16 months before harvest, as compared to sorghum which takes only four months (Reddy *et al.,* 2005). Sorghum can also be grown on marginal land where sugarcane cannot be cultivated, but the genetic improvements of sugar content in sorghum have not been intensively studied as compared to that of sugarcane.

1.2 Origin and distribution of sorghum

It is understood that sorghum originated in Africa, due to the high genetic diversity and the wild distribution of the crop on the continent, especially in the North-Eastern quadrant of Africa (Doggett, 1988). There is evidence that the crop was first domesticated on a savanna between Chad and western Ethiopia (Doggett, 1988). From the centre of origin, sorghum was dispersed along trade and shipping routes throughout Africa and the Middle East, to India approximately 3 000 years ago. Sorghum was later introduced into eastern Africa from Ethiopia around 200 AD and subsequently, the Bantus carried it to southern African countries (de Wet and Huckabay, 1967).

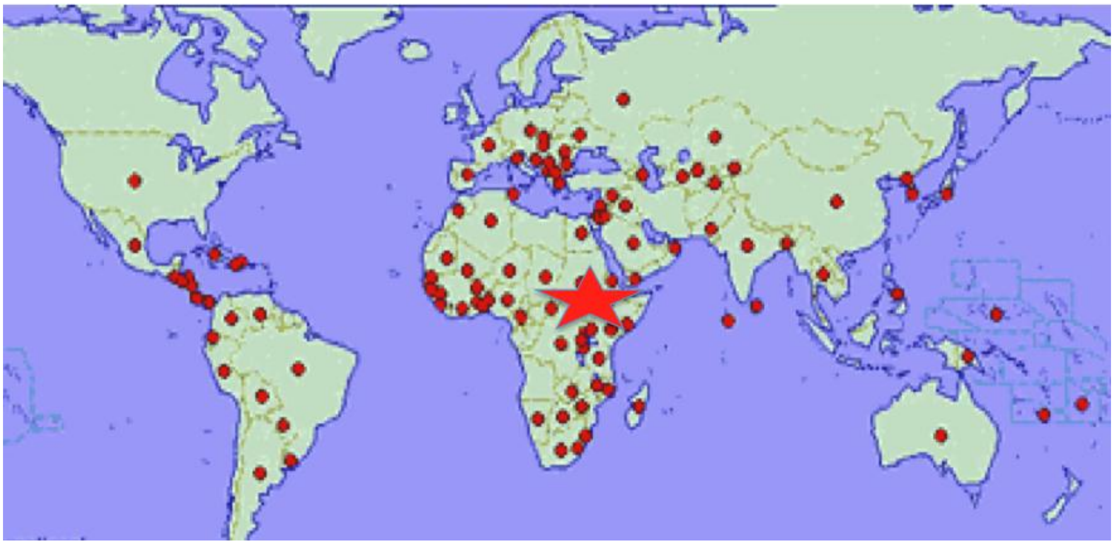


Fig. 1: A world map indicating sorghum cultivation. A red star indicates the origin of sorghum, and the red dots show sorghum-producing countries in the world (CAB International).

Currently, sorghum is cultivated for commercial farming in the drier areas of Africa (Taylor, 2003), Asia (Zerbini and Thomas, 2003), America and Australia (Stenhouse *et al.,* 1997). In South Africa, sorghum was introduced for commercial cultivation at the end of the 19th century (Balole and Legwaila, 2005), and the Department of Agriculture, Forestry and Fisheries (2010) highlights the major areas of cultivation as Gauteng, Limpopo, North West, Free State and Mpumalanga provinces. Due to great concern by the environmentalists over the use of fossil fuels, coupled with support of the government for biofuel production, sweet sorghum cultivation is expected to increase substantially in the future in South Africa (SA).

1.3 Classification and taxonomy of sorghum

Sorghum belongs to the family Poaceae, the tribe Andropogoneae and subtribe Sorghastrae. The genus Sorghum is separated into five taxonomic sections, namely: chaetosorghum, heterosorghum, parasorghum, sorghum, and stiposorghum. The section sorghum contains all the domesticated as well as cultivated sorghum races and varieties (Harlan and de Wet, 1972; Doggett, 1988). Harlan and deWet (1972) further identified five basic races of sorghum (*bicolor, guinea, caudatum, durra,* and *kafir)* and 10 intermediate races (based on panicle and spikelet morphology)*.*

The race *guinea* arose more than 2,000 years ago and is the dominant sorghum of West Africa (House, 1995). The race *caudatum* is an important agronomical race especially when combined with other races. Although the races *durra* and *kafir* are widely cultivated, *bicolor* remains the most domesticated species in the genus. *Bicolor* is a highly variable crop-weed complex and contains wild, weedy and cultivated annual forms which are fully inter-fertile (Hay *et al.,* 2013).

Sorghum variants are further grouped into five agronomic types, namely: fiber, broomcorn, forage/fodder, grain and sweet sorghum. All the variants are closely similar, however, sweet sorghum can reach up to 6 m while the other four can only attain up to 4.5 m in height. Sweet sorghum also accumulates edible sugars in the stems (Vermerris, 2011). The sugar in sweet sorghum is mainly composed of saccharose, fructose and glucose, which are similar to the sugars found in sugar beet (Capareda, 2010). Studies have shown that a mature sweet sorghum consists of approximately 75% cane, 10% leaves, 5% seeds and 10% roots by weight (Grassi *et al.,* 2002).

1.4 Uses of Sorghum

The sorghum plant is of great importance because the whole plant can be used for different purposes. From antiquity, sorghum has been used for food (Dicko *et al.,* 2006; Taylor *et al.,* 2006), beverage (McGovern, 2004; Bvochora *et al.,* 2005), feed (De Oliveira, 2007) and building materials (Reddy and Yang, 2005). For example, in other parts of the world such as Japan and the United States of America, white sorghum grains are processed into flour and snacks (Rooney, 2001). But in Africa, sorghum serves as the main food and feed especially in drought-stricken areas e.g Ethiopia (Meze-Hausken, 2004; Cavatassi, 2011) and Zambia (Van Heerden and Schönfeldt, 2004).

The grains are used for the production of traditional foods such as *ting* (a fermented porridge prepared using maize or sorghum grains), soft porridge and *pap* (a traditional porridge prepared from maize or sorghum). Additionally the grains are used for making commercial beer and non-traditional products, such as animal fodder. After harvest, the grain sorghum stems can be used for fencing and building huts while the roots are useful as fuel for cooking.

Sweet sorghum is used to make confectionery. On a commercial scale though, sweet sorghum is used for production of biofuel and alcohol (Woods, 2001; Rooney *et al.,* 2007; Yuan *et al.,* 2008; Murray *et al.,* 2008; Zhao *et al.,* 2009). The sweet juice from the stalk can be converted into sugar and syrup

(Almodares and Hadi, 2009). The sugars can be converted to biofuels (Claassen *et al.,* 2004) primarily used for transport purposes. The remaining stalk after the sweet juice is removed is called bagasse. Bagasse can be burned and gasified to produce heat and electricity (Claassen *et al.,* 2004), which can be used for cooking.

1.5 Constraints to sorghum production

The production of sorghum is affected by a variety of abiotic and biotic constraints. The main abiotic factors are low and extreme high temperatures, drought and acidic soils. Low temperatures cause poor pollen fertility and seed germination as well as retarded growth (Yu and Tuinstra, 2001). Although drought affects growth of plants (Farooq *et al.,* 2009), traits associated with various drought aspects have been studied (Harris *et al.,* 2007; Kassahun *et al.,* 2010) using different screening techniques resulting in the development of drought tolerant cultivars (Mutava *et al.,* 2011; Kapanigowda *et al.,* 2012).

According to breeders, the most damaging drought stress is that which occurs during the post-flowering stage of crop growth, called “terminal drought” (Harris et al., 2007). The genotypes considered sensitive terminal-drought are identified by reduced grain number and size, premature leaf and plant senescence; stalk collapse and lodging, and charcoal rot (Kassahun *et al.,* 2010). The “stay-green” trait, which is the ability to resist premature plant senescence, is the most effective drought tolerance mechanism (Xu *et al.,* 2000; Haussmann *et al.,* 2002; Burke et al., 2010). In sorghum particularly, stay-green properties have been associated with drought resistance trait (Mutava *et al.,* 2011; Tao *et al.,* 2000; Vinodhana and Ganesamurthy, 2010). On the other hand, aluminum toxicity in the soil has also been shown to contribute to drought stress because it damages the root system (Magalhaes *et al.,* 2007). The resultant affected plants can be vulnerable to mineral nutrient deficiencies.

Striga (*Striga asiatica*), a parasitic weed, is one of the major biotic pressures affecting sorghum productivity. It reduces photosynthesis in sorghum as it abstracts water and inorganic solutes from the host, generally affecting yields by more than 50 percent (Lendzemo *et al.,* 2007; Haussmann *et al.,* 2004). Other biotic constraints include, grain mold (Navi *et al.,* 2005) caused by a number fungi e.g. *Fusarium moniliforme* Sheld*., Curvularia lunata* etc., and leaf diseases e.g. leaf blight caused by *Exserohilum turcicum* (TeBeest *et al.,* 2004). When the rains extend beyond normal duration, grain mold develops resulting in reduced yield and seed quality (Navi *et al.,* 2005). Pedigree and backcross breeding techniques have been applied with moderate success to breed cultivars that are resistant or tolerant to the above mentioned biotic constraints (Bantilan *et al.* 2004). The germplasm lines and breeding lines tolerant to specific stress have been identified and selected.

1.6 Genetics and genomics of sorghum

Sorghum is a diploid species (2n=20) with a relatively small genome size (750 Mbp) compared to other important cereals such as wheat (16 900 Mbp) and maize (2 600 Mbp), although larger than that of rice (389 Mbp). It was the first sequenced plant genome of African origin (Paterson *et al.,* 2009) and a model crop for studying tropical grasses using C4 photosynthesis. The small genome of sorghum provides an attractive model for enhancing the understanding of the evolution, structure and function of tropical cereals. Sorghum remains an important target for plant genomics due to the high level of inbreeding in the crop and lower level of gene duplication than in many other tropical cereals such as rice (Paterson *et al.,* 2009).

Sorghum genome mapping began in the early 1990s using morphological and DNA markers, and several genetic maps have been developed. Pereira *et al.,* (1994) reported the first complete sorghum map with 10 linkage groups. Several other linkage maps have been reported since then, which Mace and Jordan (2010) recently integrated onto a complete genome map. Sorghum genetic maps have also been cross-referenced to other grass species as a step towards cloning genes linked to marker loci and for comparative genome analysis (Bhattramakki *et al.,* 2000; Kong *et al.,* 2000; Menz *et al.,* 2002).

Quantitative Trait Loci (QTLs) responsible for traits of interest have also been identified in sorghum. Quantitative traits are characters that are controlled by a combination of many genes. The regions within genomes that contain genes associated with a particular quantitative trait are termed quantitative trait loci (QTLs). Different quantitative traits have been mapped in sorghum including stay-green and drought tolerance (Xu *et al.,* 2000; Sanchez *et al.,* 2002), pest tolerance e.g. shoot fly tolerance loci (*Atherigona soccata* Rond.) (Apotikar *et al.,* 2011), parasite resistance e.g. *Striga* (Klein *et al.,* 2001; Mutengwa *et al.,*

2005) and disease resistance e.g. downy mildew caused by *Sclerophthora* (*Sclerospora*) (Gowda *et al.,* 1995). Grain quality and yield have always been areas of interest to breeders to address the issue of food security. Genomic regions controlling the grain yield and quality have been studied extensively using molecular markers (Rami *et al.,* 1998; Jordan *et al.,* 2003). Most recently, bioenergy traits (Guan *et al.,* 2011), QTLs for sugar-related traits (Shiringani *et al.,* 2010), and cold tolerance (Burow *et al.,* 2011) have been studied and mapped using molecular markers to assist in MAS. The identification of QTLs was not previously feasible using morphological characters, but the development of molecular markers (Mohan *et al.,* 1997) made this practical.

1.7 Sorghum breeding

For a long time, morphological characterization has been used to select and breed for sorghum plants with superior traits (Dahlberg *et al.,* 2002; Kayodé *et al.,* 2006). However, morphological characters are often strongly influenced by environmental factors and may not reflect true genetic composition of a plant (Mandal *et al.,* 2001; Koti *et al.,* 2005; Luzuriaga *et al.,* 2006). Moreover, morphological markers used for phenotypic characters are limited in number (Collard *et al.,* 2005). Therefore, the most suitable method of selection is molecular breeding or marker assisted selection (MAS)/marker-assisted breeding (MAB).

Molecular breeding involves the use of molecular techniques to distinguish different individuals at DNA variation level. Marker assisted selection refers to the use of DNA markers to aid in choosing the preferred plant varieties with desired traits. This is important because the main goal of plant breeding is to assemble desirable combinations of genes in new plant varieties. Breeding for desirable traits using the two methods have been exploited in important cereals including maize (Eathington *et al.,* 2007), wheat (William *et al.,* 2007) and sorghum (Vermerris et al., 2007) through a process called linkage mapping.

Linkage is when the genes that are located close to each other on a chromosome are inherited together during meiosis. Linkage maps are used to determine the position and genetic distance of genes or markers relative to each other in terms of recombination frequency. There are three main steps in creating a linkage map. The first step involves developing a mapping population, followed by identifying polymorphisms in the population, and finally, the linkage analysis of the markers.

There are different types of mapping populations and its thus vital to select the appropriate type of mapping population for the intended study. The different types of mapping populations include recombinant inbred lines (RILs), backcross (BC), double haploid (DH) and F_2 populations. The F_2 populations are derived from crossing F_1 progeny, while backcross populations are derived from crossing F_1 hybrid to one of the parents. Double haploids are developed by regenerating plants through the induction of chromosome doubling from pollen grains. RILs are derived from crossing two parents that are considered to be highly homozygous and advancing the progeny to at least F_7. An important prerequisite for choosing the two parents is the possession of distinct traits of interest. This will support achieving a segregating population for those traits.

The most common RIL population development method is called single seed descent. The single seed descent method uses a single seed from each F_2 offspring attained from crossing two parents to advance to the next generation (Borojević, 1990). For instance, a cross from the two parents results in an F_1 generation, which is then crossed (F_1 X F_1) to advance to F_2. From F_2 progeny, a single seed from each plant is randomly selected to advance to the next generation (F_3). Then from the F_3 generation a single seed is also randomly selected to advance to the next generation (F_4). This will be repeated until the seventh or eighth generation where more than 99% average homozygosity will now be expected (Scheible *et al.,* 2004).

There are drawbacks to each of the methods of creating mapping populations. Although using an F_2 or BC population is desirable because both populations are easy to construct and generating them takes a short time, the populations are ephemeral resulting in seed that will not breed true to the traits possibly observed (Rakshit *et al.,* 2012). The main disadvantage of using RILs is that it takes a lot of time to establish the mapping population because six to eight generations are required. The main advantage of DH and RILs is that they produce homozygous lines that can be multiplied and reproduced without genetic change occurring (Collard *et al.,* 2005). Different kinds of mapping populations, including F_2 (Bian et al., 2006), Back-cross (Piper and Kulakow,

1994) and largely RIL (Bhattramakki et al., 2000; Taramino *et al.,* 1997; Carrari *et al.,* 2003; Kong *et al.,* 2000; Murray *et al.,* 2008; Shiringani *et al.,* 2010; Apotikar *et al.,* 2011; Burow *et al.,* 2011; Jordan *et al.,* 2011; Zou *et al.,* 2012; Mace *et al.,* 2012; Kong *et al.,* 2013) populations have been used in sorghum for diverse studies.

Sorghum breeders' interests have always been breeding for high grain yield (Haussmann *et al.,* 2000; Patidar *et al.,* 2004; Yadav *et al.,* 2005), forage quality (Amigot *et al.,* 2006), early maturity (Baumhardt *et al.,* 2006), increased water-use efficiency and drought tolerance (Kapanigowda *et al.,* 2012; Tesso *et al.,* 2005; Ali *et al.,* 2009), and disease resistance (Chandrashekar and Satyanarayana, 2006; Nair *et al.,* 2005). Although plant breeders have made progress through conventional breeding and germplasm screening to identify sources of resistance and tolerance, and backcrossing to transfer resistant genes into elite backgrounds, the practice is highly time-consuming and labor- and cost-intensive. Advances in biotechnology have enabled breeders to follow MAB, which identifies genomic regions of a crop and makes it feasible to select specific regions in elite varieties using molecular markers.

1.8 Molecular makers

Molecular markers are polymorphisms found naturally in populations that reveal neutral sites of variation at DNA sequence level (Semagn *et al.,* 2006). The technology of molecular markers allows plant breeders and geneticists to locate and understand the basics of the numerous gene interactions determining complex traits (Haussmann *et al.,* 2000a). Gupta *et al.* (2001) broadly classified the techniques developed in the last two decades into three generations: the first generation molecular markers, which include Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNAs (RAPDs) and their modifications. Second generation molecular markers include Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) and their modifications. Finally, the third generation molecular markers include single nucleotide polymorphisms (SNPs).

a) Restriction Fragment Length Polymorphisms (RFLPs)

Botstein *et al.* (1980) work on the construction of genetic maps in human using RFLP was the first reported molecular marker technique used in the detection of DNA polymorphisms. This technique requires that DNA is first extracted and digested using restriction enzymes. The resulting restriction fragments are separated according to their lengths using gel electrophoresis and transferred on to a hybridization membrane, which later is incubated with the DNA probe (Botstein *et al.,* 1980). The unhybridized probe is washed off, and the specifically hybridized probe detected by autoradiography. The bands visible on the autoradiogram indicate the size of the digested DNA that has the sequences similar to the cloned sequences used as the probe.

Although RFLPs are relatively highly polymorphic, co-dominantly inherited and highly reproducible (Agarwal *et al.,* 2008), the technique is time consuming, costly and a large amount of DNA is required for analyses (Piola *et al.,* 1999). RFLPs have been extensively applied in sorghum (Hulbert *et al.,* 1990; Binelli *et al.,* 1992; Whitkus *et al.,* 1992; Berhan *et al.,* 1993; Tao *et al.,* 1993; Witcombe and Duncan, 1993; Bennetzen and Melake-Berhan, 1994; Chittenden *et al.,* 1994; Deu *et al.,* 1994; Pereira *et al.,* 1994; Ragab *et al.,* 1994; Vierling *et al.,* 1994; Xu *et al.,* 1994a; Cui *et al.,* 1995; White *et al.,* 1995; Ahnert *et al.,* 1996; Bennetzen *et al.,* 1996; De Oliveira *et al.,* 1996; Dufour *et al.,* 1997; Peng *et al.,* 1999). For example, Ahnert *et al.,* (1996) used RFLPs to assess the genetic diversity among elite sorghum inbred lines. In that study, different patterns of RFLP bands were observed indicating diversity amongst the lines and the data helped quantify the degree of relatedness in elite sorghum germplasm.

b) Random Amplified Polymorphic DNAs (RAPDs)

Random Amplified Polymorphic DNA (RAPD) markers, on the other hand, are the simplest version of PCR with arbitrary primers used for detecting DNA variation (Williams *et al.,* 1990). They use short synthetic oligonucleotides of about 10 bases long with random sequences as primers are used to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR (Bardakci, 2001). Amplification products are separated on agarose gels and stained with ethidium bromide. The presence or absence of bands will mark the differences between the DNA templates and this occurs because of sequence changes in the priming sites. RAPDs are useful for genetic mapping, DNA fingerprinting and plant and animal breeding (Venkatachalam *et al.,* 2008). Although the RAPD technique has a lower reproducibility and is less informative compared to other markers (Mulcahy *et al.,* 1993, Vos *et al.,* 1995), it has been used to study agronomically important traits such as grain yield and disease resistance in sorghum (Williams *et al.,* 1990; Tao *et al.,* 1993; Mutengwa *et al.,* 2005). For example, although the study of Mutengwa *et al.* (2005) found no molecular marker linked to the locus of interest, the analysis generated a molecular marker linkage map consisting of 45 markers that were distributed over 13 linkage groups.

c) Amplified Fragment Length Polymorphisms (AFLPs)

One of the second-generation markers is AFLP. This is a technique that uses selective amplification of a subset of restriction enzyme-digested DNA fragments to generate a unique fingerprint for a particular genome. Usually two restriction enzymes are used to digest the genomic DNA, and specific adapters are ligated to both ends of all resulting fragments. PCR is then performed using specific radioisotope or fluorochrome primer pairs. Another PCR is also performed after the amplification products are separated on sequencing gels. AFLPs represent the effective combination power of RFLP and flexibility of PCR-based technology (Agarwal *et al.,* 2008). Polymorphisms between two or more genotypes may arise from insertions/deletions within an amplified fragment, or due to sequence variation, or differences in the nucleotide sequences immediately adjacent to the restriction enzyme site (Vos *et al.,* 1995).

The advantage of AFLP analysis is its ability to quickly generate large numbers of marker fragments for any organism, without prior knowledge of the genomic sequence. AFLP analysis requires only small amounts of starting template and can be used for a variety of genomic DNA samples. The main disadvantage of AFLPs is the high variability that reduces similarities between distant taxa to the level of chance, hence technology is more suitable for closely related lineages (Mueller and Wolfenbarger, 1999). AFLP markers can be labor intensive, as they require an additional step of cloning into vectors. Boivin *et al.* (1999) studied the distribution of AFLP markers within the sorghum genome and their possible use in sorghum breeding. The investigated distribution of the AFLPs along the genome was found not to be uniform but the markers were used to construct a genetic linkage map.

d) Simple Sequence Repeats/Microsatellites

Microsatellites or SSRs are short DNA (2–6 base pairs) sequence motifs that occur as interspersed repetitive elements in all eukaryotic (Tautz and Renz, 1984) as well as in many prokaryotes genomes (Van Belkum *et al.,* 1998). They are also known as short tandem repeats (STR) or sequence-tagged microsatellite sites (STMS) or simple sequence length polymorphism (SSLP) (Hautea *et al.,* 2004). Microsatellite markers are widely used because in contrast to all the PCR-based techniques explained above, which are arbitrarily primed or non-specific, microsatellites-based marker techniques are sequence targeted.

Microsatellite markers are found in non-coding (genomic-SSRs), or coding (genic-SSRs or EST-SSRs) regions of the genome. Although SSRs are generally much less abundant in coding regions than in the non-coding regions (Barbará *et al.,* 2007), both types of SSR markers are widely used.

Microsatellite markers are highly reproducible and have become popular genetic markers due to their co-dominant inheritance, enormous extent of allelic diversity as well as the ease of assessing microsatellite size variation by PCR with pairs of flanking primers (Weising *et al.,* 2005; Agarwal *et al.,* 2008). Although SSRs are considered the most efficient markers, their use is still limited because of the long and laborious steps to develop them (Rakoczy-Trojanowska and Bolibok, 2004).

For a long time microsatellites were developed from partial genomic libraries of the species of interest by screening clones through colony hybridization with repeat containing probes (Song et al., 2005). Although this method is simple for microsatellite rich genomes, it is ineffective for species with low microsatellite frequencies (Zane *et al.,* 2002). Microsatellites are constantly being isolated and characterized in a wide range of plants including sorghum as genetic markers (Brown *et al.,* 1996, Taramino *et al.,* 1997). Haussmann *et al.* (2004) explored the use of microsatellites to identify the genomic regions influencing resistance to the parasitic weed *Striga hermonthica* in two recombinant inbred populations of sorghum. The QTL for resistance was found and was to be used to choose the populations for marker-assisted selection.

e) Single Nucleotide Polymorphisms (SNPs)

A Single Nucleotide Polymorphism (SNP) is a genetic change or variation in DNA sequence occurring when a single nucleotide in the genome or other shared sequence differs between members of a biological species or paired chromosomes in an individual. According to Gupta *et al.* (2001), SNPs are the most abundant molecular markers with higher frequency and far more prevalence than SSRs. This novel class of markers has a high level of polymorphism and can even be found close or within a gene.

SNPs can be used to generate ultra high-density genetic maps, for mapping traits, for phylogenetic analysis and for rapid identification of crop cultivars (Agarwal *et al.,* 2008). Although SNPs are biallelic in nature, which could make them less informative, their abundance overcomes this difficulty (Jehan and Lakhanpaul, 2006). Their usefulness has attracted scientists' interest in utilizing SNP markers to detect polymorphisms in many crops including major crops such as barley (Rostoks *et al.,* 2005), soybean (*Glycine max*) (Choi *et al.,* 2007) and also sorghum (Nelson *et al.,* 2011).

SNPs can be identified using Expressed Sequence Tag (EST) data, arrays analysis, amplicon resequencing, sequenced genomes, or next-generation sequencing technologies (Ganal *et al.,* 2009). Next-generation sequencing has increased the chances of obtaining genome and transcriptome sequences using the high-throughput technologies at relatively low costs. The short reads or assembled transcripts are mapped to the reference genome and the SNPs are then identified using different programs such as CLCBio (http://www.clcbio.com/), TASSEL (Trait Analysis by aSSociation, Evolution and Linkage) (Bradbury *et al.,* 2007) and Maq (Li *et al.,* 2008).

There are different methods used for SNP genotyping i.e Infinium® assays (Gunderson, 2009), GoldenGate® (Yan *et al.,* 2010) or TaqMan (Shen *et al.,* 2009). Giancola *et al.,* (2006) conducted a study on the model crop *Arabidopsis thaliana* using SNP genotyping methods Amplifluor and TaqMan. GoldenGate has also been fully explored on different plants e.g maize (Yan *et al.,* 2010), soybean (Hyten *et al.,* 2008) and barley (Close *et al.,* 2009). The advances in high throughput and continuously decreasing cost of sequencing technologies led to genome-wide SNP genotyping using a fairly new method called Genotyping-by-Sequencing (GBS) (Elshire *et al.,* 2011).

f) Genotyping-by-Sequencing (GBS)

Genotyping-by-Sequencing is a genome wide analysis where the sequence differences detected are used directly as markers. It is a newly developed technique that is based on high-throughput next generation sequencing of genomic subsets (Elshire *et al.,* 2011). It explores the use of reduced genome complexity for high-density SNP discovery and genotyping. It is suitable for trait mapping in diverse populations, breeding, population studies, and germplasm characterization. The advantages of using this system include reduced sample handling and fewer PCR and purification steps. This technology has been explored successfully in important cereal crops including wheat (Poland *et al.,* 2012), maize and barley (Elshire *et al.,* 2011). For example, Poland *et al.* (2012) developed high-density genetic maps for barley and wheat using an enzyme approach of Genotyping-by-Sequencing.

GBS can be performed either through a reduced-representation called restriction-site associated DNA (RAD) or a whole-genome resequencing termed whole genome shotgun (WGS) approach.

Restriction-site associated DNA (RAD)

In this method, restriction enzymes are employed to cut DNA and this allows parallel screening of millions of DNA fragments flanking individual restriction enzyme sites. This method permits over-sequencing of nucleotides next to the restriction site enabling SNP detection in those areas. The number of markers can be increased by the choice of restriction enzyme and additional enzymes can be used to increase the number of markers further (Baird *et al.,* 2008). This method has been used successfully in many plants including barley (Chutimanitsakun *et al.,* 2011), rapeseed (*Brassica napus*) (Bus *et al.,* 2012) and eggplant (*Solanum melongena* L.) (Barchi *et al.,* 2011). The RAD sequencing approach utilizes a restriction enzyme to cut the DNA into different sizes and thereafter sequencing adapters are ligated onto the pieces of the DNA for sequencing (Fig. 2). All the sequences are later pooled together, mapped and aligned simultaneously to detect variations.

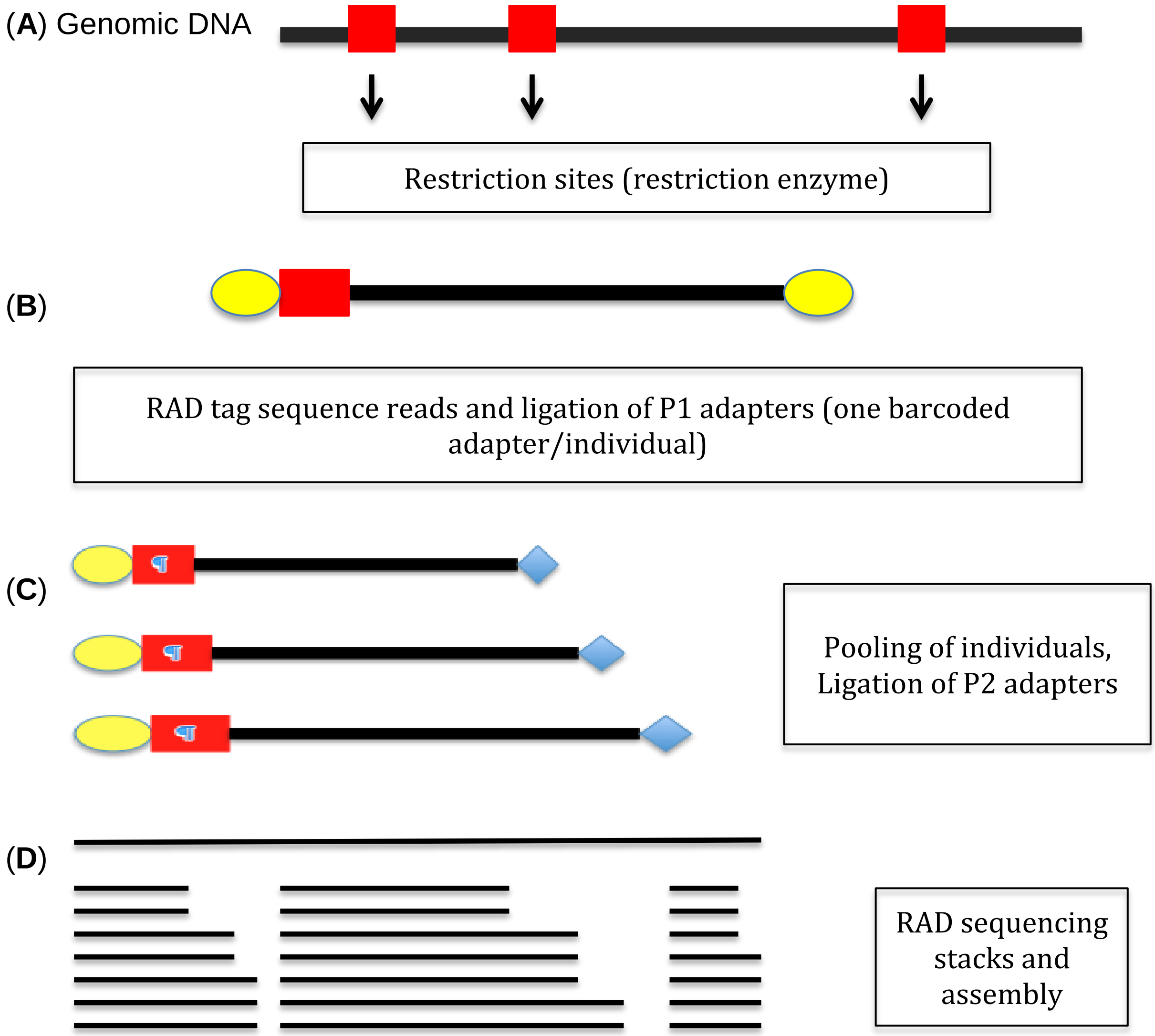

(A) Genomic DNA
Restriction sites (restriction enzyme)
(B)
RAD tag sequence reads and ligation of P1 adapters (one barcoded adapter/individual)
(C)
Pooling of individuals, Ligation of P2 adapters
(D)
RAD sequencing stacks and assembly

Fig. 2: Overview of Genotyping-by-Sequencing using restriction site associated DNA method. (**A**): DNA is digested with a restriction enzyme. Restriction sites are indicated by red-squares on the genomic DNA. (**B**): Ligation of adapter containing the Illumina P1 amplification and sequencing primer and a DNA barcode (indicated by the yellow ovals) to the DNA fragments. (**C**): Samples are pooled, sheared into 300- to 800-bp libraries (required for Illumina sequencing) and ligated to a second adapter P2 (indicated by blue structures). Sequencing is performed either as single end or paired end. (**D**): Barcoded sequences are assembled into overlapping stacks as shown in the last step.

Whole genome shotgun (WGS)

This method uses random cutting of genomic DNA by sharing DNA fragmentation or transposome, which followed by the attachment of adapters to the ends of the DNA (Fig. 3). The adapters are used for PCR amplification and later for sequencing. Fragmentation is then followed by size selection, which allows for similar sizes of DNA to be obtained from a sample for accurate sequencing and subsequent SNP discovery (Hyten *et al.,* 2010). Whole genome shotgun sequencing has been widely explored in microbial populations (Venter *et al.,* 2004), soybean (Hyten *et al.,* 2010), and bread wheat (*Triticum aestivum*) (Brenchley *et al.*, 2012).

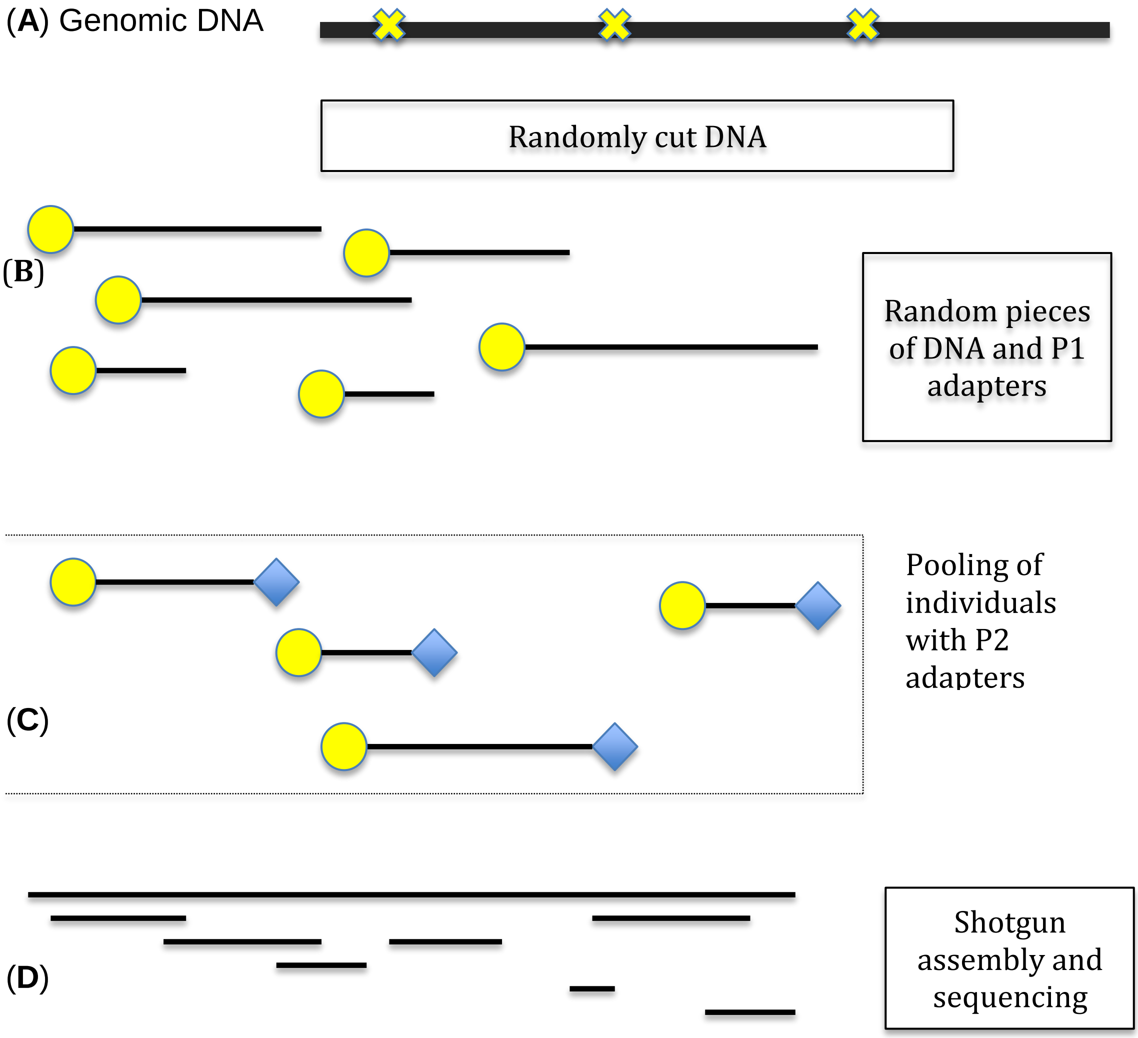

(A) Genomic DNA
Randomly cut DNA
(B)
Random pieces of DNA and P1 adapters
Pooling of individuals with P2 adapters
(C)
(D)
Shotgun assembly and sequencing

Fig. 3: Overview of Genotyping-by-Sequencing using whole genome shotgun method. (**A**): DNA is randomly sheared by a transposome which simultaneously attaches P1 adapter (indicated by yellow ovals). (**B**) and (**C**): Samples are pooled, gathered into 300- to 800-bp libraries (required for Illumina sequencing) and ligated to a second adapter P2 (indicated by blue structures). Sequencing is performed either as single end or paired end. (**D**): Barcoded sequences are assembled into overlapping stacks as shown in the last step.

1.9 Bioinformatics analysis

Bioinformatics is a set of tools used to analyze, manipulate and store biological data using algorithms and computational resources (Attwood *et al.,* 2011). The advancement in next-generation platforms has led to increased production of sequence data. Analyzing this enormous amount of data needs suitable bioinformatics tools. There is constant upgrading of software and algorithms, data storage approaches, and new computer architectures to better meet the computation requirements for NGS projects (Kumar *et al.,* 2012). Selecting the best suitable software for NGS data analysis includes the following considerations; the sequencing platform used, the availability of a reference genome, the computing and storage resources necessary, and the bioinformatics expertise available.

Once the sequence data is generated from a sequencing platform e.g Illumina, Roche 454 etc., appropriate software for bioinformatics analysis is then selected. There is both commercial and noncommercial sequence analysis software for bioinformatics analysis. The noncommercial software are usually linux based and are often free and includes Bowtie (Langmead, 2010), Bowtie2 (Langmead and Salzberg, 2012), BWA (Li *et al.,* 2009), SOAP2 (Li *et al.,* 2009) and SOAP3 (Liu *et al.,* 2012). For species that have no reference genome (*de novo* assembly), software programs such as Velvet (Zerbino and Birney, 2008), SOAPdenovo (Li *et al.,* 2010) and ABySS (Simpson *et al.,* 2009) are widely used.

Commercially available software includes CLC-Bio (http://www.clcbio.com/)

and SeqMan NGen (http://www.dnastar.com/t-sub-products-genomics-seqman-ngen.aspx). Although the programs provide a user-friendly interface, they tend to be relatively expensive. However, they are compatible with different operating systems and they are capable of performing multiple downstream analyses. The major drawback is they require locally available high computing power and have narrow customizability.

1.10 Study rationale

The increasing importance of sorghum due to the escalating need for food and the interest in utilizing the crop as a biofuel feedstock, has led to molecular research towards improving traits of interest in the crop. Although much has been achieved in sorghum improvement using traditional or conventional breeding, sorghum development still lags behind those of major cereals such as maize, rice and wheat. If sorghum is to contribute successfully to food security and as a source of alternative energy, it is important to enhance its breeding resources.

Maize and sugarcane are two major crops currently grown for both food production and as preferred sources as feedstock for the production of biofuel. The increased use of maize in particular, as an alternative source of bioethanol, has raised concerns as it threatens food security in the country. In South Africa alone, maize is a major staple food source with an average South African family feeding on maize or maize-related product at least once a day. Intensifying its use for bioethanol production is therefore likely to compromise its food security role. Sugarcane, on the other hand, is produced under intensive production systems, requires a lot of water and takes 12 to 18 months to mature, in contrast to sorghum which only requires up to 4 months to mature. The effects of climate change, for example, the increasing water scarcity due to erratic rainfall patterns discourage cultivating water intensive plants like sugarcane.

Recent advances in biotechnology and molecular breeding promise to facilitate the breeding progress through the use of cutting edge technologies, equipments and tools. Genetic linkage mapping is an example of a biotechnology tool that is considered valuable in pre-breeding but has not been fully exploited for the improvement of sorghum in SA. Global research efforts over the last decade have resulted in the complete genome sequencing of sorghum (Paterson *et al.,* 2009). Molecular markers have been particularly used in sorghum for localizing both quantitative and qualitative traits of interest (Deu *et al.,* 2005; Nagaraj *et al.,* 2005; Srinivas *et al.,* 2009; Yu *et al.,* 2009). Such molecular advances, however, have not been implemented within the breeding program initiated at the Agricultural Research Council (ARC), South Africa.

Selection of sorghum traits at the ARC has been achieved using morphological means resulting in slow cultivar development. An efficient protocol to genotype sorghum is crucial to help understand the genetic make-up of sorghum and eventually the production of a grain/sweet stem sorghum. This dual-purpose sorghum will ideally be a plant with sweet-stem to be used for biofuels and enough grains to be used for food. To enhance the value of the most recent linkage map of sorghum, there is a need to further saturate it with recent and more informative molecular markers such as SNPs. There is also need to study new state-of-the-art technologies and discover effective genotyping methods in sorghum. Effective genotyping will play a vital role in future marker-assisted selection and breeding of the crop.

The current work aimed at investigating the applicability of Genotyping-by-Sequencing techniques in a sorghum mapping population generated between sweet stem and grain sorghum parents. The outcome of this work is expected to contribute significantly towards more efficient cultivar selection in the future at the ARC and in other sorghum breeding programs elsewhere. A reliable and efficient genotyping protocol is crucial for studying and understanding the genetics and genomics of sorghum.

1.11 Aim:

To explore Genotyping-by-Sequencing (GBS) methods and establish an efficient protocol for genotyping in a sorghum mapping population, by identifying variants (SNPs and INDELs) in sorghum parental lines and the progeny.

Objectives:

- Develop a robust set of molecular markers (SNPs) for genetic characterization in F_8 sorghum RILs using Whole Genome Shotgun (WGS) and Restriction-site Associated DNA (RAD) methods.
- Assess and compare the WGS and RAD sequencing approaches.
- List variants from the parental lines for future mapping studies.

CHAPTER 2: Materials and methods

2.1 Experimental Design

The set objectives were achieved by following the experiments as outlined in Fig. 4. A RIL population generated at the ARC from crossing sweet- and grain-sorghum was advanced from F_6 to F_7 generation through single seed descend method. DNA was extracted from the two parents and two progeny lines and subjected to both the RAD and WGS sequencing methods.

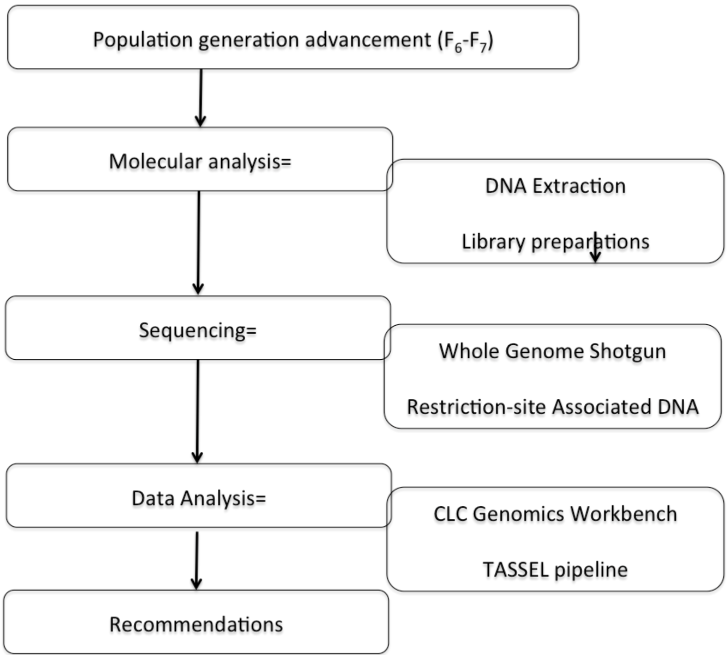


Fig. 4: A schematic representation of the experimental design followed in this project. First, the RIL population was advanced from F_6 to F_7 in the glasshouse to increase the level of homogeneity in the population. The F_7 seeds were used for molecular analysis, which comprised of DNA extraction and library preparation for sequencing. The samples were sequenced using both WGS and RAD sequencing methods. Data analyzed with CLC Genomics Workbench and TASSEL pipeline, followed by recommendations.

2.2 Plant material:

Two parental lines SS79 (sweet sorghum) and M71 (grain sorghum) were crossed to generate a 187 Recombinant Inbred Lines (RILs) mapping population. This mapping population was developed at the Agricultural Research Council (ARC) Grain Crops Institute, Potchefstroom, South Africa. The female parent (SS79) was collected from a traditional farmer in Limpopo province (South Africa). It has long internodes and is approximately 300 cm tall, and has thin but sweet juicy stalks. The male parent (M71) originates from ICRISAT-Bulawayo in Zimbabwe bred under Sorghum and Millet improvement program. It is characterized by early maturity and high grain yield, with white grains. It has short internodes and is approximately 140 cm tall, has juicy stems but the juice is not sweet.

The traits of the two parents and the two selected progeny are shown in table 1. The traits includes panicle weight, plant height, stalk weight, Brix and cane weight. A clear presentation of all traits represented by different colours in table 1.

Table 1: A range of traits represented in the sorghum RIL population generated at the ARC by crossing M71 (male) and SS79 (female). The different colours represent traits of the two progeny used alongside the parental lines to test the two methods of Genotyping-by-Sequencing

Short	Low
Medium	Medium
Tall	High

RIL #	Plant height (Cm)	Panicle weight (Kg)	Stalk weight (Kg)	Cane weight (Kg)	Brix (%/RI)
Parent 1	129.1	0.010	0.080	0.042	14.8
Parent 2	58.2	0.008	0.096	0.024	3.8
Progeny1	137.3	0.002	0.098	0.054	6.3
Progeny2	133	0.006	0.092	0.052	6.2

Out of a total of 187 RIL progeny, two randomly selected Recombinant Inbred Line (RIL) progeny were used alongside the two parental lines for Genotyping-by-Sequencing (GBS) optimization. At the start of the project, the mapping population was at the fifth generation (F_5). Further generation advancement from F_6 to F_7 was conducted in the glasshouse. Round pots (23 cm in depth and 28 cm diameter) were filled with loam soil mixed compost. Plants were watered every 48 hours, and water containing hydroponic nutrient was used with every second irrigation. The hydroponic nutrient powder used contained 6.5 % N, 2.7 % P, 13 % K, 7 % CG, 2.2 Mg, 7.5 % S, micro elements 0.15 % Fe, 0.024 % Mn, 0.024 % B, 0.005 % Zn, 0.002 % Cu, 0.001 % Mo. Three level table spoons (~10 g) were dissolved in five litres of water, stirred well and poured onto the plants. The temperature was controlled, with a minimum of 18 ^{0}C and maximum of 30 ^{0}C. Insects were controlled by spraying an insecticide Hunter spray (Cyanamid, Northern Cape, South Africa) once a week, and two ml of the insecticide was added to one litre of water. To avoid cross-pollination, plant heads were covered with a bag for two to three weeks to ensure self-fertilization.

2.3 General protocols

DNA extraction protocol

DNA extraction was performed from one-week-old sorghum leaves using standard protocol of plant DNA extraction (Macherey-Nagel®, Düren, Germany). The plant samples were homogenized using mechanical treatment, and then DNA extracted using a CTAB (Cetyl trimethylammonium bromide) based procedure designed and optimized in the kit. The DNA was bound to a silica membrane and contaminants washed away using wash buffers. Finally DNA was eluted using low salt elution buffer and stored at 4 ^{0}C. The DNA was visualized by staining with ethidium bromide following electrophoresis through 0.8 % agarose gel, and illumination with UV. DNA concentration was measured fluormetrically using a Qubit flourometer (Invitrogen®, Oregon, USA).

PCR protocol

The DNA was amplified by using PCR primer cocktail (Illumina, San Diego, USA), Nextera PCR master mix (Illumina, San Diego, USA), and index 1 primers and index primers 2 were also added to the reaction (Illumina, San Diego, USA). The thermal cycler (Applied Biosystems, Foster City, USA) was used for PCR amplification and conditions were set as follows: initiation step (98 ^{0}C for 30 seconds), followed by denaturation (98 ^{0}C for 10 seconds), annealing step (60 ^{0}C for 30 seconds), extension (72 ^{0}C for 30 seconds), and then final elongation step (72 ^{0}C for five minutes). The DNA products were then stored at 4 ^{0}C.

Gel electrophoresis protocol

A 1 % agarose gel was prepared by 1 g of agarose powder added into 500 ml flask, together with 100 ml of TAE buffer. Then 5 µl of ethidium bromide was added to the solution. The solution was poured in the casting tray where the gel combs were set and the gel was allowed to cool until it was solid. The samples were loaded onto the gel by adding 5 µl of 6X loading dye to each 2 µl DNA. Then 5 µl of the DNA ladder standard was added into at least one well of each row on the gel. The samples were electrophoried 10 volts per cm. Gels were then photographed with a Bio-Rad Gel Doc 1000 system (Bio-Rad Laboratories).

2.4 Library Preparation

2.4.1 Whole Genome Shotgun sequencing

The sequencing library was prepared following the Nextera protocol (Illumina, San Diego, USA). The Nextera protocol uses a transposome to fragment DNA while simultaneously tagging the DNA with Illumina sequencing primer sites to be used during PCR. A total of 1 µg genomic DNA of each of the two parents and the two-selected recombinant inbred lines was exposed to the transposome. A total reaction volume of 50 µl was prepared consisting of 5 µl of Nextera tagment DNA enzyme (Illumina, San Diego, USA) and 25 µl tagment DNA buffer (Illumina, San Diego, USA) and 1 µg of DNA template made to a final volume of 50 µl. It was incubated for five minutes at 55 ^{0}C and was followed by DNA purification using Qiaquick spin columns (QIAGEN, Valencia, CA) to remove the small DNA pieces. A total of 25 µl was eluted from the column. Thereafter nine cycles of PCR were performed in a total reaction of 50 µl and the PCR products were cleaned using the QIAquick PCR purification kit (QIAGEN, Valencia, CA). The PCR amplified the tagmented DNA fragments and also added specific adapters and bar codes to the sequencing library for sample identification. The index N702 (CTAGTACG) and N704 (GCTCAGGA) were used for Parent 1 and Parent 2 respectively.

The DNA fragments were then size selected for sequencing. Since the Illumina HiScanSQ, which sequences 100 bp in each direction was to be used, it was ideal to select DNA fragments from 400 to 500 bp (including the ~120-bp adaptor) for paired-end sequencing technology. The DNA fragments were separated on a 1.0 % agarose gel, using the 1 kb ladder as reference.

Four hundred to 500 bp size fragments were cut from the agarose gel using gel-excision tip, and then purified using a MiniElute gel extraction kit (QIAGEN, Valencia, CA). Libraries were normalized to 2 nM, denatured using 0.1 M of NaOH and diluted with 10 pM hybridization buffer (HT1). Individual samples (600 µl of library) were sequenced on separate lanes of an Illumina HiScanSQ DNA sequencer (Illumina, San Diego, USA). DNA templates were added to the C-bot (Illumina, San Diego, USA) for cluster generation followed by hybridization of the clusters. Sequencing by synthesis (SBS) technology was used, which uses four fluorescently-labeled nucleotides to sequence the tens of millions of clusters on the flow cell surface in parallel. During each sequencing cycle, a single labeled deoxynucleoside triphosphate (dNTP) is added to the nucleic acid chain. The nucleotide label serves as a terminator for polymerization, so after each dNTP incorporation, the fluorescent dye is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Base calls are made directly from signal intensity measurements during each cycle, reducing raw error rates compared to other technologies. The sequencer generated BCL files, then CASAVA (Illumina, San Diego, USA) was used to convert the files into fastq files and bin the sequences based on the indexing. All of these experiments were performed at the Agricultural Research Council-Biotechnology Platform (South Africa).

2.4.2 Restriction-site Associated DNA (RAD) sequencing

Optimum digestion of genomic DNA with restriction enzyme AluI was initially determined using different incubation times and enzyme concentrations. The reaction tube contained 2 µg DNA, 2 units of AluI enzyme, 10X FastDigest buffer (2 µl) (Fermentas, Inqaba, Pretoria, South Africa) and nuclease free water, in a total reaction volume of 50 µl. Then 10 µl aliquots were taken into a new tube after deactivating the reaction by incubating at 65 ^{0}C 20 minutes. Aliquots were taken at 15 minutes, 60 minutes, four hours and eight hours and stored at 4 ^{0}C. The DNA digestions were analyzed by electrophoresis. The optimized digestion time and DNA concentration was selected for the remaining experiments. A total of 2 µg of DNA from parents and selected progeny was digested for 60 minutes at 37 ^{0}C. The enzyme activity was then inactivated with a 65 ^{0}C incubation of the samples for 20 minutes. The digested DNA were separated on gel and then purified using MiniElute gel extraction kit (QIAGEN, Valencia, CA). DNA was bound to silica membrane and the contaminants washed away with a buffer. The DNA was then eluted using low salt elution buffer and concentration determined using Qubit instrument (Invitrogen®, Oregon, USA).

The 3' ends of the digested DNA were adenylated to prevent self-ligation by adding the A-tailing mix (Illumina, San Diego, USA) and incubating at 37 ^{0}C for 30 minutes. A ligation reaction was carried out at 30 ^{0}C for 20 minutes, to repair any double strand breaks of DNA. Sample purification to remove the small DNA fragments (less than 100 base pairs) was done using Qiaquick spin columns (QIAGEN, Valencia, CA). DNA fragments ranging in size from

600 bp to 800 bp (including the ~120-bp adaptor) were analyzed by electrophoresis on agarose gel and recovered from the gel and prepared for sequencing. The Illumina Miseq, which sequences up to 250 bp in each direction, was the chosen sequencing platform. The adapter/indexes were Parent 1 (ATCACG), Parent 2 (TTAGGC), Progeny 1 (ACTTGA) and Progeny 2 (GATCAG). The DNA fragments were separated on a 1.0 % agarose gel containing 0.04 µl/mL ethidium bromide in 1 X TAE electrophoresis buffer using the 1kb ladder as size reference (Fermentas, Inqaba, Pretoria, South Africa). Fragments were purified using a MiniElute gel extraction kit (QIAGEN, Valencia, CA). Libraries were normalized to 2 nM by either diluting or concentrating depending on the template, then denatured by 0.2 M NaOH and diluted with 8 pM HT1. Then 600 µl of sample was loaded on the Illumina Miseq sequencer. The Illumina Miseq uses a sequencing by synthesis method described for the Illumina HiScan instrument and the sequence data was produced within eight hours. Raw data was obtained from the machine within 24 hours.

2.5 Data analysis and SNP identification

2.5.1 CLC Genomics Workbench

The raw data was imported into the CLC Genomics Workbench software (http://www.clcbio.com) and filtered for quality. The data quality control assesses and visualizes statistics on quality scores, sequence-read lengths and base-coverages. The over-represented sequences and hints suggesting contamination events and nucleotide-contributions and base-ambiguities are checked. The data quality check was followed by adapter trimming, quality trimming and length trimming. Reads were then mapped onto the sorghum genome (www.phytozome.net) with allowance of two mismatches and the non-specific sequences were ignored. Probabilistic Variant Caller was used to call variants as it can detect variants in a wide variety of data sets with a high sensitivity and specificity. The non-specific and broken pairs were ignored in the variant calling. A minimum coverage of ten was used for the WGS and four for the RAD in calling of variants, with the 90.0 variant probability. Once the variations are detected the table files are exported into Excel where it was easier to perform SNP and INDEL counting and filtering. A diagram of the steps followed is outlined on the figure below (Fig.5).

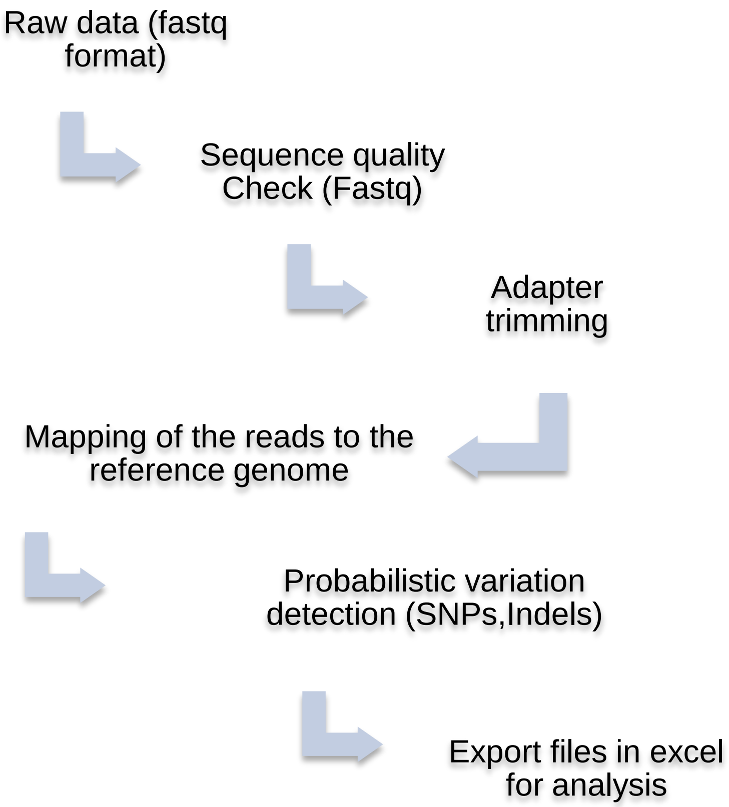

Raw data (fastq format)
Sequence quality Check (Fastq)
Adapter trimming
Mapping of the reads to the reference genome
Probabilistic variation detection (SNPs,Indels)
Export files in excel for analysis

Fig. 5: The steps followed when using CLC Genomics Workbench 6.0.1 to analyze the obtained sequence data. First, quality check of the sequence data, followed by the trimming of adapters, then mapping the reads to the sorghum reference genome. The probabilistic variant detection was used to detect variations (INDELs and SNPs).

2.5.2 TASSEL (Trait Analysis by Association Evolution and Linkage) pipeline

The TASSEL pipeline, implemented in perl programming language, was used for the processing of the sequence read data. The steps involved in the pipeline were executed in separate scripts. The pipeline uses different publicly available software tools i.e Fastq-mcf (http://code.google.com/p/ea-utils/wiki/FastqMcf), Bowtie2 (Langmead and Salzberg, 2012), SAMtools (Li *et al.*, 2009), BCFtools (Xu *et al.,* 2012) (Fig. 6). The first step involved the quality check and trimming of adapters using Fastq-mcf. Fastq-mcf detects and removes sequencing adapters and primer from the raw sequencing data.

Fastq-mcf then removes the poor quality reads (the reads that contain N's) and discard sequences that are too short (less than 50 bp). The reads were then mapped to the sorghum reference genome using Bowtie2. Bowtie2 is suitable for aligning long genomes and supports paired-end alignment modes. SAMtools was then used to view, sort and index the sequences thereof. Bcftools was then used to call for variations (SPNs and Indels). The raw SNPs that were obtained were then filtered using VCFtools based quality score of 30. The steps followed are outlined in the figure below (Fig. 6).

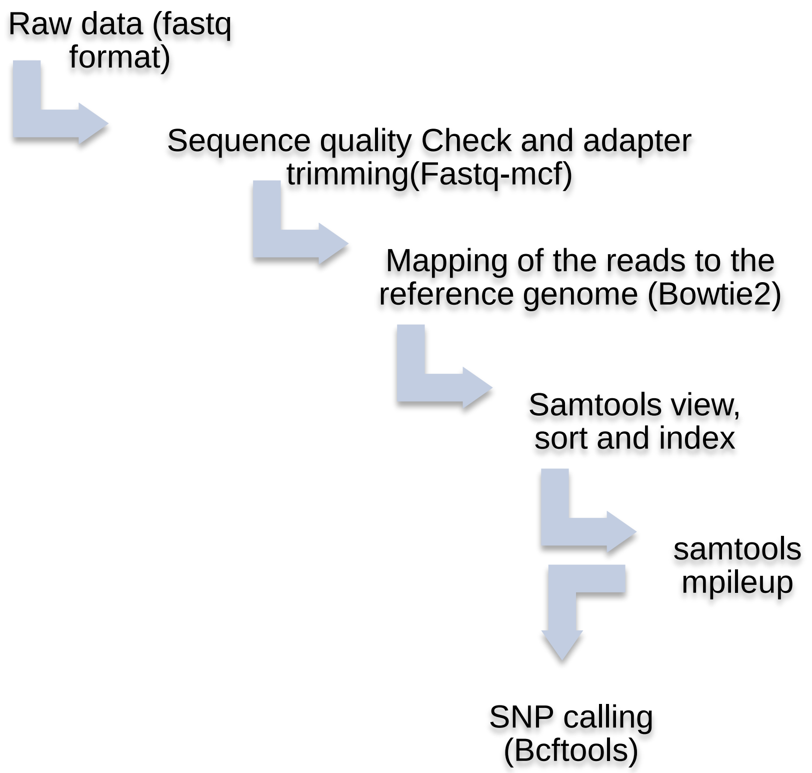

Raw data (fastq format)
Sequence quality Check and adapter trimming(Fastq-mcf)
Mapping of the reads to the reference genome (Bowtie2)
Samtools view, sort and index
samtools mpileup
SNP calling (Bcftools)

Fig. 6: The steps followed for data analysis of the obtained sequence data using the TASSEL pipeline. The raw sequence data are checked for quality and trimmed using Fastq-mcf tool. The trimmed sequences are then mapped onto the sorghum reference genome, using Bowtie2. Samtools then sort and index the sequences. The variations (SNPs and INDELs) were called with the use of BCFtools.

CHAPTER 3: Results

Genotyping-by-Sequencing (GBS) allows for portions of the genome to be sequenced and compared between different individuals and is not reliant on any previous genomic information. The selected four individuals (2 parents and 2 progeny) were sequenced using two methods of GBS (WGS and RAD). The sequence data was analyzed using CLC Genomics Workbench and TASSEL pipeline and this was followed by recommendation of the best GBS method and best data analysis method.

3.1 DNA extraction

DNA was successfully extracted from the four individuals as visualized through a 1% agarose gel (Fig. 7) following electrophoresis. The DNA concentration of different individuals were as follows: Parent 1 =141 ng/µl, Parent 2 = 104 ng/µl, Progeny 1 = 98.6 ng/µl, Progeny 2 = 94.7 ng/µl.

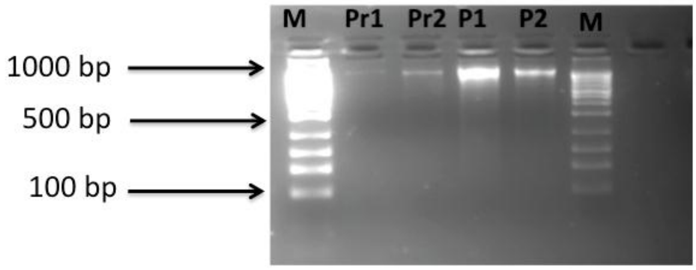


Fig. 7: Visualisation of genomic DNA extracted from sorghum plants following electrophoresis. The genomic DNA was used for Genotyping-by-Sequencing experiments. DNA was extracted from Parent 1 (P1), Parent 2 (P2), Progeny1 (Pr1) and Progeny2 (Pr2) for use in GBS. A 1-kbp molecular ladder (M) was used for size reference.

3.2 Library preparation

3.2.1 Whole Genome Shotgun

Successful whole genome shotgun (WGS) libraries of between 500−700 bp were excised from a 1% agarose gel (Fig. 8). Sequencing of the two parents for the WGS on the Illumina HiScanSQ instrument, produced 190 and 200 million paired-end reads of average 100 bp lengths respectively (Table 2). This yield is more than 20-fold depth coverage of the sorghum genome. This was determined by multiplying the number of reads obtained by average length of the reads, and then dividing by the genome length of sorghum. The expected random pattern of WGS sequences mapped to the sorghum reference genome was observed by visualizing with the Integrative Genome Viewer (IGV) in the parents and progeny (Fig. 10 a & b).

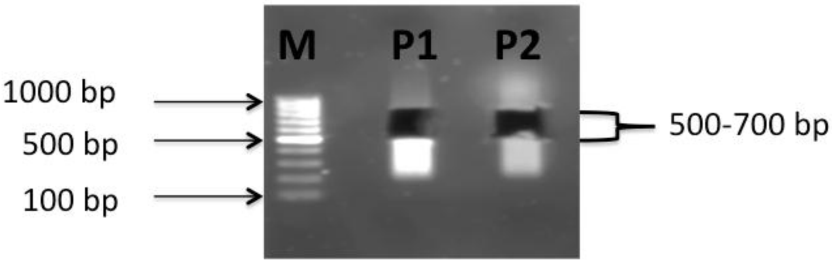


Fig. 8: Excision of 500 to 700 bp DNA fragments for library preparation of Parent 1 (M71) and Parent 2 (SS79) digested with a transposome. A 1-kbp molecular ladder (M) was used for size reference.

3.2.2 Restriction-site Associated DNA

The optimum digestion of DNA by AluI enzyme was determined to be 15 minutes with 2 units of enzyme and 1µg of DNA (Fig. 9). Each parent digested with AluI restriction enzyme and sequenced on Illumina Miseq instrument produced five million (Parent 1) and two million (Parent 2) paired-end reads respectively, within average 230 bp length (Table 2). The sequencing of the two sorghum F_7 progeny (progeny1 and progeny2) digested with AluI produced one and ten million reads respectively (Table 2). The uniform pattern of AluI digested DNA sequences mapped to the sorghum reference genome was observed when the sequences were viewed using Integrative Genome Viewer (IGV) in the parents and progeny (Fig. 11 a & b).

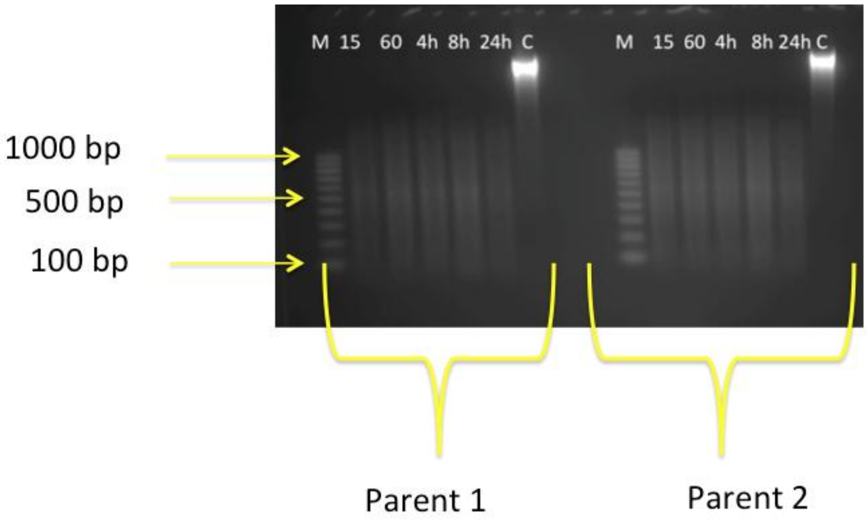


Fig. 9: Optimization of AluI digestion on two sorghum individuals (Parent 1 & 2). The following lanes represent AluI digestion times that Parent 1 and Parent 2 were exposed to (15 min, 60 min, 4 h, 8h and 24 h respectively). Lane C represents undigested genomic DNA (1 µg) used as control (C). Lane M represents DNA ladder (Fermentas).

Sequence Output

The Illumina Hiscan produced 190 and 215 million reads for the two sequenced parental lines, Parent 1 and Parent 2 respectively in the WGS sequencing approach. Validation of parental sequencing data by sequencing two F_7 progeny generated more than 200 million reads for the two sequenced progeny in WGS. The Illimuna Miseq generated five million reads from Parent 1 and two million reads from Parent 2 using the RAD sequencing approach. The progeny generated over a million sequences for each prior any processing in RAD (Table 2).

Table 2: Sequence output and genome coverage of Parent 1 (M71), Parent 2 (SS79) and the two progeny using the Whole Genome Shotgun and Restriction-site Associated DNA sequencing methods

	Sequence Output (reads)		Genome Coverage	
	WGS	**RAD**	**WGS**	**RAD**
Parent 1	190 905 080	5 829 306	25 X	6.4 X
Parent 2	215 052 184	2 774 163	29 X	3.2 X
Progeny 1	262 060 540	1 363 263	35 X	1.6 X
Progeny 2	249 308 380	11 100 989	34 X	12 X

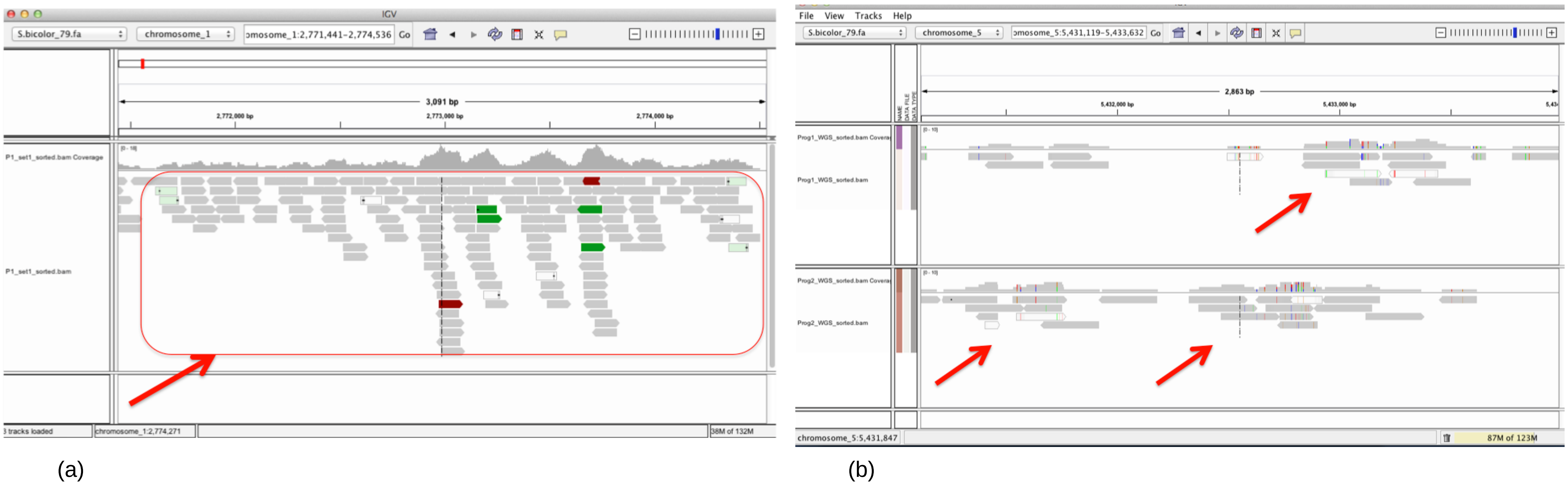


Fig. 10: The use of visual software, Integrative Genome Browser (IGV) to view the sequence data obtained using Whole Genome Shotgun (WGS) sequencing method. A random and non-specific alignment pattern of WGS was observed, as shown by the arrow, (a) from parental lines (Parent 1 and Parent 2), (b) in the progeny (Progeny 1 and Progeny 2) was observed.

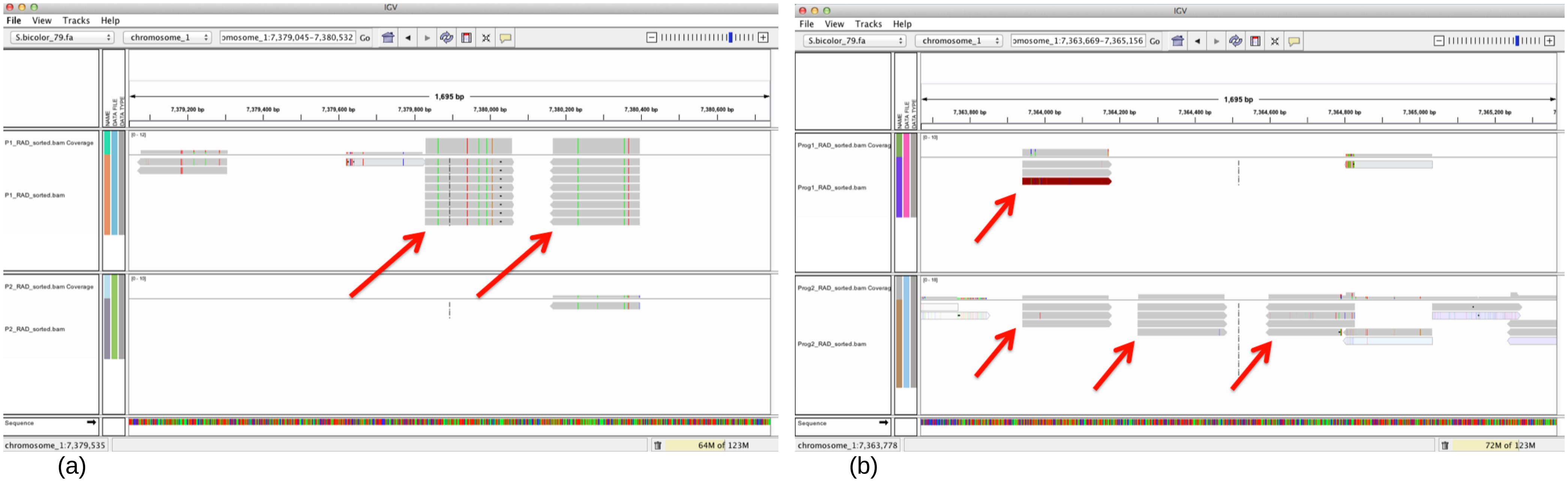


Fig. 11: The use of visual software, Integrative Genome Browser (IGV) to view the sequence data obtained using Restriction-site Associated DNA (RAD) sequencing method. An expected uniform alignment pattern of RAD method, as shown by the arrow, (a) Parent 1, (b) Progeny 2 was observed.

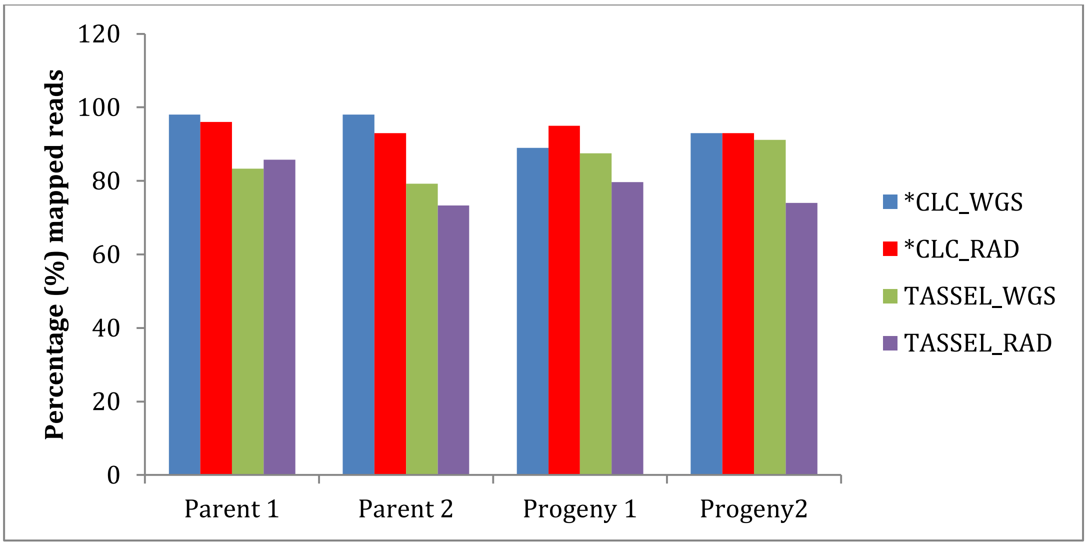
3.3 Sequence assembly

(a) CLC Genomics Workbench

After de-multiplexing all libraries and counting the number of reads assigned to each sample, the CLC Genomics Workbench quality report was created for all the individuals (Parent 1, Parent 1, Progeny 1 and Progeny 2). The report revealed the average length of the raw sequences as 100 bp. There were no ambiguous bases and the sequence duplication levels were less than 10%. The trimming of low quality sequences and the sequencing adapters removed 2% of the total sequence reads from the two parents in WGS and less than 1% in RAD. The remaining reads (100 bp length) were mapped to the sorghum reference genome. Both parents in WGS had the highest mapping percentage compared to the progeny, whilst in RAD; Parent 1 had the highest mapping percentage of all individuals (Fig. 12).

(b) TASSEL pipeline

The quality check and trimming of adapters removed between 2,5% and 17.9% of the total sequence reads in WGS and less than 1% of the reads after the de-multiplexing in RAD. The remaining reads of average 100 bp were mapped to the reference genome using Bowtie2. The mapping percentage was more than 70% in all the individuals with both the WGS and the RAD sequencing approaches (Fig. 12).



***CLC (CLC Genomics Workbench)**

Fig. 12: The percentage of each individual reads that mapped to the *Sorghum bicolor* L.Moench reference genome (www.phytozome.net). The blue and green bars indicate the whole genome shotgun sequencing method percentage of mapped reads using CLC Genomics Workbench and TASSEL respectively. The red and purple shows the restriction-site associated DNA sequencing method percentage of mapped reads using CLC Genomics Workbench and TASSEL respectively.

3.4 Variants discovery

A minimum of three reads was required to call a variant in RAD-based sequencing approach. This was because of the low sequencing coverage obtained when this method was employed, but this was considered adequate because RAD offers uniform alignment of the reads (Chutimanitsakun *et al.,* 2011). However, because WGS involves more random alignment of sequencing reads, this sequencing approach required at least ten reads to call a variant when mapped to a sorghum reference genome.

A total of 921 031 and 3 119 variants (SNPs and INDELs) were identified in WGS and RAD sequencing approaches respectively (Table 3) using CLC Genomics Workbench 6.0.1. The TASSEL pipeline identified a total of 2701814 and 17 012 in the WGS and the RAD sequencing approach respectively (Table 3). Both common and unique variations were observed between the individuals with WGS and RAD sequencing approaches (Table 4). The common variations are variations that are found in Parent 1 and Parent 2 when mapped against sorghum reference genome. The unique variations are variations found in either Parent 1 or Parent 2 but not in both. The Integrative Genome Viewer (IGV) enabled the visualisation of the identified variations from the TASSEL pipeline (Fig. 14). The variations were spread even across all the chromosomes (Fig. 15). The variations discovered using TASSEL pipeline were more than those discovered in CLC Genomics Workbench (Table 3).

Table 3: A comparison of performance of TASSEL pipeline and CLC Genomics Workbench based on the number of variants (SNPs and INDELs) identified in the parental and F_7 progeny lines

	WGS		**RAD**	
	CLC BIO	**TASSEL Pipeline**	**CLC BIO**	**TASSEL Pipeline**
Parent 1	139 967	770 518	250	2981
Parent 2	286 683	685 735	31	454
Progeny 1	195 931	544 285	117	802
Progeny 2	298 450	701 276	2721	12 684
Total	**921 031**	**2 701 814**	**3 119**	**17 012**

Table 4: The total number of variations, common variations and unique variations discovered in the parents using Whole genome shotgun (WGS) and Restricted-site Associated DNA (RAD) sequencing approaches

		All variants		**Common Variants**	**Unique Variants**	
		Parent 1	**Parent 2**	**Parent 1, Parent 2**	**Parent 1**	**Parent 2**
WGS	CLC	139 967	286 683	49 553	90 414	237 130
	TASSEL	770 518	685 735	504 305	266 213	181 430
RAD	CLC	250	31	0	250	31
	TASSEL	2981	454	215	2766	239

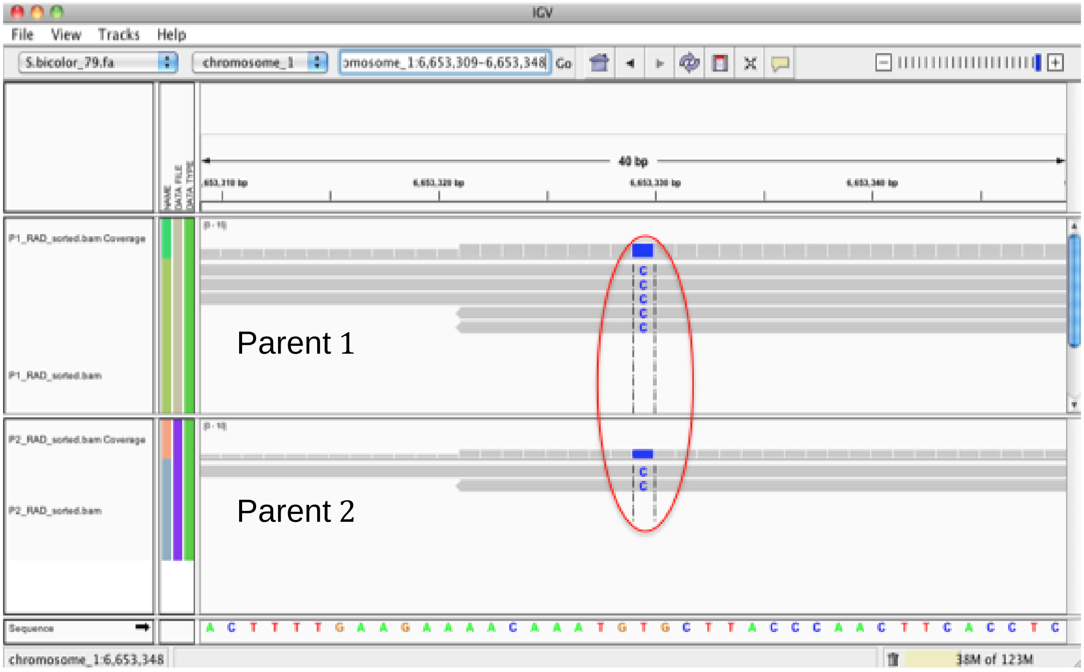


Fig. 14: A visual of parental lines mapped to the sorghum reference genome illustrating a shared SNP between the parents. The SNP differs to the sorghum reference genome as shown in a red circle above, and was visualized by using Integrative Genome Browser (IGV) software.

The unique variations between the two parents were tabulated (Appendix Table 1 and 2) and a clear diagram was drawn from the table results. The major goal was to find out from where the progeny had inherited their variations. Furthermore, to observe if there had been any recombinations that had occurred in the population. The overall picture from all chromosomes was observed as shown in Fig. 15. And one chromosome was chosen to display recombination per chromosome (chromosome 3). A single event of recombination was observed on chromosome 3.

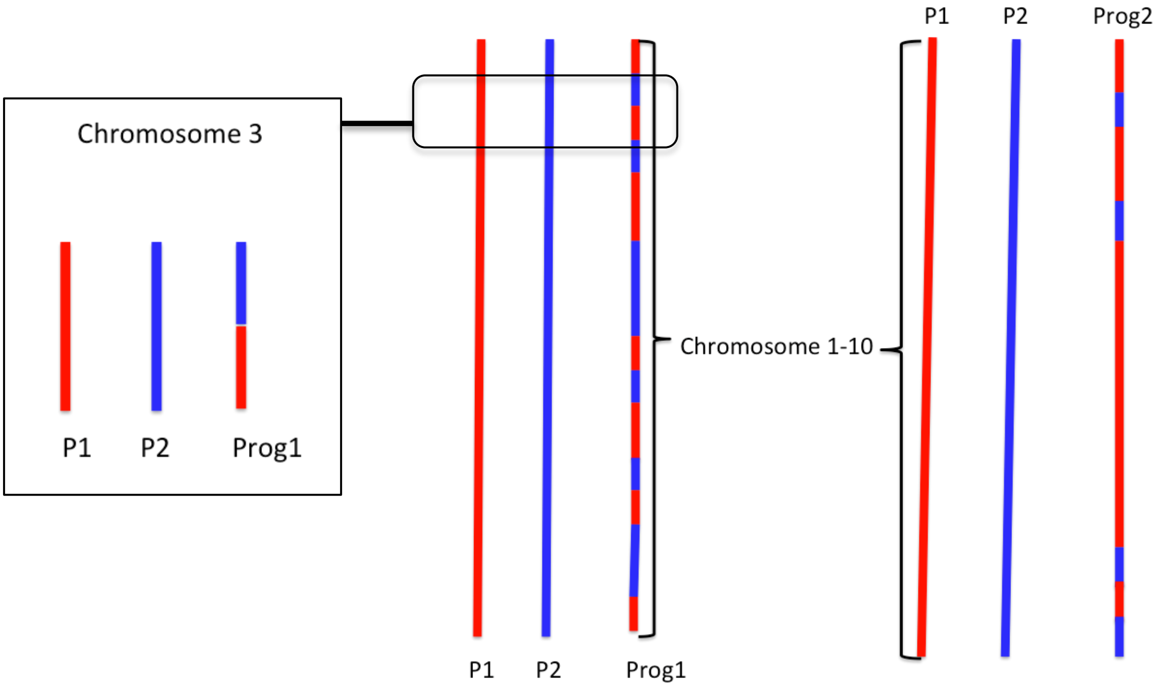


Figure 15: A schematic representation illustrating unique variations between the two parents and the two progeny on different chromosomes. The variations: red are from Parent 1 (P1) and blue Parent 2 (P2). The progeny display inheritance of variations from both PARENT 1 and P2 on different regions of the chromosome.

3.5 Sequencing Associated Costs

The costs of library preparation using the Nextera kit are higher than using the Truseq (Illumina) protocol kit (Table 5). A large proportion of data is crucial to execute a whole genome shotgun (WGS) approach at a practical certainty level, but it still proved to be cheaper than restriction-site associated DNA (RAD) per base as reflected in Table 5. Sequencing using the RAD method is expensive if more data is generated, but can be affordable at low coverage.

The data analysis methods costs were also considered. The CLC Genomics Workbench license cost is immense, but this is a once off payment and the same license is used to analyze countless genome sequencing data. For instance in the current study the software was used on four sorghum lines, which were subjected to two different sequencing methods. TASSEL pipeline data analysis method is publicly available, and in these experiments discovered more variations than CLC Genomics.

Table 5: The average cost involved in library preparation and DNA sequencing using WGS and RAD for the sorghum Parent 1 (M71) and Parent 2 (SS79) and progeny selected

Process:	**WGS (Kit)** **Illumina Hiscan**		**RAD (Truseq Kit)** **Illumina Miseq**	
	Parents	**Progeny**	**Parents**	**Progeny**
Library preparation per sample	R1 115.39	R1 115.39	R1 085.31	R1 085.31
Sequencer cost per Gb	R1 932.85	R1 932.85	R4 022.28	R4 022.28
*Average cost of sequencing	R44 793.80	R62 769.30	R19 306.94	R27 351.50
Total cost incurred per sample	**R45 909.19**	**R63 884.69**	**R20 392.25**	**R28 436.81**
*Average data generated	23.175 Gb	32.475 Gb	4.8 Gb	6.8 Gb
Sequencing cost per Gb of data	**R1 980.98**	**R1 967.19**	**R4 248.39**	**R4 181.88**

**These averages have been calculated using data from the four sequenced individuals i.e parents and progeny both in RAD and WGS*

CHAPTER 4: Discussion

This was the first study to use the blunt end restriction enzyme in a RAD-based sequencing approach. The AluI enzyme was successfully used and a smear indicating complete digestion of the DNA was obtained. After sequencing, the overlapping RAD reads were visualized and variants were detected when mapped to the reference sorghum genome. Additionally, the WGS sequencing approach was employed and directly compared to the RAD sequencing method. A random distribution of the WGS method was observed and following the sequence assembly, the variants were detected. Of the two sequencing approaches RAD emerged as a better technique for our current mapping population. This is because RAD had the potential to call variants even at a low coverage as opposed to the WGS which required deep sequencing coverage.

Genotyping-by-Sequencing (GBS) is rapidly becoming the new state-of-the-art tool commonly used by researchers as it unravels the genetic variation and diversity of individuals at the genome level (Narum *et al.,* 2013). Nevertheless, the main challenge still lies in selecting the best GBS approach for genotyping a sorghum mapping population developed at the Agricultural Research Council (Potchefstroom). In sorghum, both RAD and WGS sequencing approaches have been used to discover SNPs, but the two methods were not directly compared and the best method was not selected (Nelson *et al.,* 2011). In essence, the RAD sequencing approach in the particular sorghum study was only adopted after the WGS approach became inadequate for simultaneous SNP discovery and genotyping. The differences between the GBS methods are largely based on the potential biases and features associated with resultant GBS data (Narum *et al.,* 2013). The major advantage of GBS is the markers discovered are directly relevant to the population at hand.

The sequencing, data output and variations

The Illumina sequencing platform was selected for this study mainly because of its relatively low cost, high throughput and availability (Ansorge, 2009; Metzker, 2010; Scholz *et al.,* 2012). The cost of sequencing using the Illumina is amongst the cheapest in the sequencing industry (Hudson, 2007). This platform has been widely used in GBS studies in several plants (Elshire *et al.,* 2011; Chutimanitsakun *et al.,* 2011; Hyten *et al.,* 2010; Poland *et al.,* 2012; Spindel *et al.,* 2013; Beissinger *et al.,* 2013) including sorghum (Nelson *et al.,* 2011). The two different platforms of the Illumina (Miseq and HiScan) were used because the HiScan is suitable for producing large datasets for deep coverage sequencing and the Miseq largely used for low coverage. The MiSeq generates 1.5 Gb paired-end reads per run and each run takes one day (Coparaso *et al.,* 2012), while the Hiscan produces up to almost 30 Gb per day, with a total of 200 Gbp per run and each run takes seven days (Zhang *et al.,* 2011).

The RAD sequencing approach optimally exploits low coverage sequencing as the reads align uniformly on the reference sequence regions (Miller *et al.,* 2012; Rowe *et al.,* 2011). For instance, Chutimanitsakun *et al.* (2011)

acknowledged low sequence coverage of less than 5× could be used to accurately genotype individuals. In the current study, variations were accurately determined from an average of 3× sequence coverage under the RAD further confirming the reliability of RAD for genotyping even at low coverage. The WGS approach on the other hand, requires deep sequencing because the reads align uniquely when assembled and this might result in shallow coverage of the sequenced regions (Nelson *et al.,* 2011). In a study on cattle, an average of 16× was demonstrated to be adequate for variant identification with WGS (Zhan *et al.,* 2011), while a similar study on white spruce (*Picea glauca*) used deep coverage of 64× (Birol *et al.,* 2013). In the current study an average coverage of 30× was achieved with WGS.

High genome coverage provides the backbone for implementing approaches for individuals that are sequenced at lower genome coverage (McCouch *et al.*, 2010). The reason for that is because once the markers are identified and validated at high coverage, the lower coverage individual's markers can be scored. The WGS approach would therefore be attractive for initial marker identification and development especially for arraying in SNP chips. Generally, studies developing SNP chips take advantage of deep sequencing and this was observed both in animals, e.g chickens (Groenen *et al.,* 2011), and plants e.g rice (McCouch *et al.,* 2010). For example, the development of a 60K SNP chip in chicken was achieved at 12x genome sequencing coverage depth. The RAD sequencing approach is best suited for genotyping large population sizes as it uncovers variations with low sequence coverage. The RAD approach excels in the scoring of markers following the initial discovery phase of mining markers from a small pool of individuals. For example, the two parents in a barley mapping population were genotyped using RAD and 93 individuals of the mapping population scored at low coverage by comparing the variants to those obtained with the parents (Chutimanitsakun *et al.,* 2011). The parents were deeply sequenced at 72× and 128× respectively, while the lowest coverage on the progeny was 8×.

Different types of restriction enzymes have been used for a range of genotyping studies in sorghum. For example, Morishige *et al.,* (2013) used three different enzymes FseI, NgoMIV and HpaII in a digital genotyping study targeting the non-repetitive regions of sorghum. In the current study the choice of enzyme for the RAD sequencing approach was based on the fact that AluI*,* is a four base cutter producing numerous genomic DNA fragments, and it produces blunt ends eliminating the end-blunting step. The enzyme is predicted to cut at every 256 bases resulting in sufficient cuts for the RAD experiment. The Illumina protocol for adapter ligation requires all the DNA fragments to be blunt-ended (Son and Taylor, 2012). Enzymes producing sticky-end have been largely exploited in GBS studies (Elshire *et al.,* 2011; Miller *et al.,* 2007; Poland *et al.,* 2012). However, the sticky-end digestion requires the additional step of blunting the DNA. Although AluI has been previously used in sorghum for an RFLP study (Debener *et al.,* 1990), the current study was the first to successfully use the enzyme in a RAD-based GBS approach.

In this study, there were more variations generated using the WGS compared to the RAD sequencing approach as a result of the deep coverage accomplished in the WGS. The rate of variation discovery was 1.05 variations per Kbp on the two deeply sequenced the WGS parents. Although the current observation is similar to sweet pepper (1.0 per Kbp) (Park *et al.,* 2010), it was lower than maize (11.5 per Kbp) (Barker and Edwards, 2009) and higher than flax (*Linum usitatissimum L.*) (0.17 per Kbp) (Kumar *et al.,* 2012). The variation rate of the current study is comparable with a sorghum study (1.4 per Kbp), which looked at the Genome-wide patterns of genetic variation in sweet and grain sorghum (Zheng *et al.,* 2011). The RAD variation rate was 0.001 per Kbp, and this low rate may be a reflection of the low sequence coverage achieved using this sequence approach. Nonetheless, this rate was higher than the variation rate discovered in barley using the RAD (Chutimanitsakun *et al.,* 2011), but less than the 15× sequence coverage enzyme digested RAD variation previously obtained in sorghum (Nelson *et al.,* 2011).

Data analysis procedures

Although recent advances in next-generation sequencing have led to production of massive sequence data per run, the need for cutting-edge data analysis pipelines remains crucial to filter, sort and align the generated data (Narum *et al.,* 2013). The advantages and disadvantages of the various software often used for alignment and analysis of the next-generation data has been critically reviewed by Kumar *et al.,* (2012). The comparison of these different approaches has been demonstrated for data analysis (Zhan *et al.,* 2011). Generally, CLC Genomics Workbench discovers more variations as compared to other software (Zhan *et al.,* 2011). Zhan and co-workers (2011) used four different pipelines (SAMtools, CLC Genomics Workbench, SMALT + SAMtools and Mosaik + GigaBayes) for SNP calling, and CLC Genomics Workbench uncovered more SNPs. In contrast, in the current study on sorghum the variations discovered using CLC Genomics Workbench were less than those discovered using TASSEL pipeline in the deeply sequenced WGS individuals. Furthermore, TASSEL noticeably discovered more variations in the RAD sequencing approach, making it the preferred method over the CLC Genomics Workbench.

Recommendations for future experiments

This study demonstrates the suitability of RAD as the best Genotyping-by-Sequencing approach for large populations. RAD demonstrates applicability using low coverage data saving both cost and requires less computing. The main interest for scientists to use WGS is the even genome coverage achieved by this approach. The current study demonstrated an even distribution of variations on the genome achieved using AluI enzyme in RAD approach in sorghum (Fig. 12). Therefore RAD is a desirable method for genotyping large populations because it results in a uniform and representative reduction of the sorghum genome at a relatively low cost. The most suitable data analysis method for the analysis of large populations is TASSEL pipeline. This is because the TASSEL discovered more variations overall than CLC Genomics Workbench. Thus, the mapping population generated at the ARC will now be subjected to the RAD sequencing approach and analyzed with the TASSEL pipeline. Although the study variations were discovered by mapping with the sorghum reference genome, a future study on the sorghum mapping population would map against each consensus sequences i.e Parent 1 mapped to Parent 2 and the progeny against the parental lines.

Concluding Remarks

The main aim of the study was to explore Genotyping-by-Sequencing (GBS) and establish an efficient protocol for genotyping in sorghum. This was achieved by developing a robust set of molecular markers (SNPs) that will be used for genetic characterization in F_8 sorghum RILs using both the Whole Genome Shotgun and the Restriction-site Associated DNA sequencing approach methods. Furthermore by assessing and comparing the WGS and the RAD sequencing approaches based on the number of variations discovered, the cost and reliability of each method. The three set objectives: developing a robust set of markers (SNPs) for genetic characterization in F_8 sorghum RILs using Whole Genome Shotgun (WGS) and Restriction-site Associated DNA (RAD) methods; and to assess and compare the WGS and RAD sequencing approaches, were achieved and the results of the polymorphic markers will be explored further for mapping and QTL identification for traits of interest. The traits of interest include sugar-related traits and grain yield, which will contribute towards the biofuel industry. The development of a precise and inexpensive GBS protocol serves as a robust framework to which other sorghum populations can be characterized. Once the variations are discovered, the unique or polymorphic ones can be used as markers in genetic trait mapping, association studies, diversity analysis and marker assisted selection. The SNPs identified in this study will be specifically used on a mapping population developed at the ARC Grains Crops Institute for the genetic trait mapping study. The study will look at the traits associated with biofuel production.

The variation detection rate and accuracy are crucial quality indicators that are affected by the depth of genome sequencing. This study has only sequenced a total of four individuals, but sequencing of more individuals would increase the confidence and accuracy of the results. The methodology used here and resources generated for this study will be used as a resource for future genome sequencing studies on larger datasets. The results of the study will be applicable to the sorghum mapping population generated at the Agricultural Research Council (Potchefstroom, South Africa), of which the two parents were tested.

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Appendix

Table 1: The variations (SNPs and INDELs) found in progeny 1, which were present in either parental line using Whole genome shotgun (WGS)

Parent 1	Parent 2	Progeny 1	Position	Chromosome Number
_	C – A	C – A	69857874	Chromosome 2
_	T - -	T - -	14608491	Chromosome 3
T – A	_	T – A	74286971	Chromosome 3
_	CCGA - -	CCGA - -	809633	Chromosome 4
_	TG - -	TG - -	809647	Chromosome 4
CG - -	_	CG - -	8258372	Chromosome 4
_	A – G	A – G	23845613	Chromosome 4
_	G – A	G – A	23845929	Chromosome 4
_	A – G	A – G	27285824	Chromosome 4
_	C – T	C – T	34242223	Chromosome 4
_	G – C	G – C	53595401	Chromosome 5
GG – AT	_	GG – AT	19631846	Chromosome 5
C – G	_	C – G	50978273	Chromosome 6
C – T	_	C – T	52906010	Chromosome 6
_	T – C	T – C	7256215	Chromosome 7
C – T	_	C – T	7256230	Chromosome 7
_	G – A	G – A	9549736	Chromosome 7
G – T	_	G – T	9367858	Chromosome 8
G – T	_	G – T	42548685	Chromosome 8
_	G – A	G – A	42805164	Chromosome 8
T – C	_	T – C	43185401	Chromosome 8
_	C – A	C – A	10035218	Chromosome 10
_	G – A	G – A	38000720	Chromosome 10
G – A	_	G – A	58126244	Chromosome 10

Table 2: The variations (SNPs and INDELs) found in progeny 2, which were present in either parental line using Whole genome shotgun (WGS)

Parent 1	Parent 2	Progeny 2	Position	Chromosome Number
_	AC – GG	AC – GG	792512479	Chromosome 2
A – G	_	A – G	6011747	Chromosome 3
G – C	_	G – C	38366804	Chromosome 3
_	A – G	A – G	60338568	Chromosome 5
_	A – C	A – C	60338632	Chromosome 5
T – G	_	T – G	14403405	Chromosome 5
C – T	_	C – T	22243716	Chromosome 5
A – C	_	A – C	22243760	Chromosome 5
C – T	_	C – T	34453056	Chromosome 5
G – A	_	G – A	36778439	Chromosome 5
G – T	_	G – T	37387026	Chromosome 5
C – T	_	C – T	43231278	Chromosome 5
C – T	_	C – T	43231401	Chromosome 5
A – G	_	A – G	45203579	Chromosome 5
A – G	_	A – G	60338568	Chromosome 5
A – G	_	A – G	54535202	Chromosome 7
T – C	_	T – C	36447829	Chromosome 8
T – C	_	T – C	36447832	Chromosome 8
T – C	_	T – C	36447938	Chromosome 8
_	C – T	C – T	8804043	Chromosome 9
_	G – A	G – A	13644932	Chromosome 9
C – T	_	C – T	41871536	Chromosome 9
G – T	_	G – T	43001877	Chromosome 9
C – A	_	C – A	28228537	Chromosome 10