Evaluation of simple sequence repeat (SSR) markers associated with soybean resistance to *Sclerotinia sclerotiorum* (Lib.) de Barry in commercial South African soybean cultivars.

By

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DECLARATION

I, Pfano Princess Mbedzi, declare that the dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: ____________________

Date: ____________________

TABLE OF CONTENTS

ABSTRACT

Sclerotinia sclerotiorum (Lib.) de Barry, commonly known as Sclerotinia stem rot is one of the pathogens that have a potentially devastating impact on the growth of soybean industry in South Africa. Several quantitative trait loci (QTL) that play a role in soybean resistance to Sclerotinia stem rot have been identified and mapped on the soybean's integrated genetic linkage map. However, none of these QTL and their underlying markers have been evaluated in any of the commercial soybean cultivars grown in South Africa. The aim of this study was to screen and characterise SSR markers linked to QTL that are reported to have an association with stem rot resistance on South African commercial soybean [*Glycine max* (L.) Merr] cultivars. Twenty SSR markers that have been reported to have an association with stem rot resistance on soybean were evaluated in 29 commercial soybean cultivars that are currently planted in South Africa. The genetic diversity of markers and the genetic relationships across the cultivars were evaluated using the GenAlEX software package. Five markers were not polymorphic, while twelve markers were polymorphic across all cultivars. Evaluation of genetic relationships across cultivars showed that 10 South African cultivars share alleles with a cultivar with known resistance (M.A) to Sclerotinia stem rot. We propose that the shared alleles between 10 South African cultivars and M.A could indicate shared resistance to Sclerotinia stem rot and indicates the presence of a genetic rather than environmental mechanism of resistance in South African soybean cultivars. The results from this study will aid South African soybean breeders in knowing which markers they can use to screen for Sclerotinia stem rot resistance within the context of South African soybean cultivars.

DISSERTATION COMPOSITION

Section 1 of this dissertation contains a background on the *Sclerotinia sclerotiorum* pathogen and the diseases caused by the pathogen with a focus on its impact on the production of soybean. The pathogen's classification, biology and its physiological interaction with soybean is briefly described. A brief history of soybean production in South Africa is given, as well as management strategies, including soybean's resistance against the pathogen. The last part of this chapter contains the main aim of the study as well as the objectives.

Section 2 is composed of the materials and methods that were used to achieve the study aim. These include the selection methods that were used to select SSR markers that are linked with soybean resistance to Sclerotinia stem rot, the growth and maintenance of plant materials, DNA isolation, multiplex PCR as well as SSR data analysis. The marker diversity across South African soybean cultivars as well as the genetic relationships between South African cultivars and resistance/ susceptible cultivars was evaluated.

Section 3 contains the results that were obtained from the initial selection of markers as well as the key results that were obtained from the final SSR marker data analysis.

Section 4 discusses the results obtained from the study as well as the implications of these results. The **Appendix** section contains tables of allele frequencies in each locus, across the soybean samples representing South African commercial soybean cultivars; the allele diversity over loci, as well as the matrices of Nei pairwise genetic distance and Pairwise Shannon diversity index.

The list of literature cited in the study is contained under the **References** section.

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ABBREVIATIONS AND SYMBOLS

%	Percent
µl	micro litre
˚C	degree Celsius
ARC	Agricultural Research Council
BC	before Christ
bp	base pairs
cfu	colony forming unit
cm	centimetre
CTAB	cetyl trimethylammonium bromide
DNA	deoxyribonucleic acid
ETI	effector mediated immunity
gDNA	genomic DNA
GenAlEX	genetic analysis in Excel
GM	*Glycine max* (chromosomes in *Glycine max*)
H_2O_2	hydrogen peroxide
Ha	hectares
Kb	kilobase
LG	linkage group
M.A	Maple Arrow
mA	milliampere

mg	milligram
ml	millilitre
mM	millimolar
M	molecular weight marker
Mt	million tonnes
ng	nanogram
NTC	non-template control
O_2^-	superoxide
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
PGPR	plant growth promoting Rhizobacteria
PIC	polymorphic information content
QTL	quantitative trait locus
rfu	relative fluorescence unit
RNA	ribonucleic acid
rpm	rotations per minute
sec	seconds
SNP	single nucleotide polymorphism
SSR	simple sequence repeats
TAE	tris-acetate-EDTA
US	United States

USDA	United States Department of Agriculture
UV	ultraviolet
W82	Williams 82

INDEX OF FIGURES

INDEX OF TABLES

1. INTRODUCTION

1.1. Soybean production

1.1.1. Brief history on soybean cultivation

Soybean [*Glycine max* (L.) Merr] breeding started as early as 2 838 BC in China, before it became a commonly cultivated crop (Hymowitz, 1970). As ship trade became popular, soybean was later introduced to the United States around the late 1700s. It wasn't until the 1920s when the USDA (United States Department of Agriculture) and agricultural experiment stations initiated formal soybean research

For three decades during the 20th century, the largest producers of soybean were China, Indonesia, Japan, and Korea, until the United States (US) overtook China in the 1950s (Hymowitz, 1970). In the 1960s, soybean was produced over 25 million hectares of farmland in over 25 countries with the US and China contributing 76% and 17% respectively. Currently soybean is grown over 76 million hectares in five continents. In 2013 the world soybean production was 278 million tonnes (Mt) which was 70% of the world's grain legume produced. The US was the largest producer, followed by Brazil, Argentina, China, and India (Foyer et al., 2016).

1.1.2. Production in South Africa

South Africa is not one of the world's largest soybean producers; however, soybean production has increased over the years. In 1976, the area planted for soybean in South Africa was approximately 22 000 hectares with an output of 17 900 tons a year. By 2012, these numbers had increased by 450 000 hectares and 632 100 tons of area planted and production output respectively (Dlamini et al., 2014). The current soybean production estimates show that approximately 573 950 hectares of soybean were planted in summer of 2017 with a production forecast of 1 233 130 tons. (Department of Agriculture Forestry and Fisheries, 2016) (Table 1.1). Soybean is produced in all nine provinces of South Africa, with Mpumalanga, Free State, and Kwazulu-Natal province having the highest soybean production output (Dlamini et al., 2014). Production of soybean is mainly based on dryland farming, with only four production regions in the North West and Limpopo provinces that produces soybean under irrigation farming (de Beer and Bronkhorst, 2016).

Table 1.1 Soybean production from 1976 to 2017 and current output estimates in South Africa (Dlamini et al, 2014; Department of Agriculture Forestry and Fisheries, 2016).

Year	**Area Planted (Ha)**	**Output/Yield (Ton)**
1980	28 000	39 900
1985	23 000	39 900
1990	61 000	119 000
1994	55 000	63 100
2000	93 790	153 925
2005	150 000	272 500
2010	418 000	708 750
2015	687 300	1 059 850
2017	573 950	1 316 370

* The 2017 figures are a forecast of area planted in hectare and amount produced in tonnes.

1.1.3. Soybean importance

Soybean is among the most important crops that are consumed by humans and animals alike. The soybean seeds are an important source of protein as well as oil (http://ncsoy.org/media-resources/uses-of-soybeans/, Accessed 13 March 2016). Soybean seed contains on average 18% oil and 38% protein; of the 18% oil, 95% is refined and used as edible oil while the remaining oil is used for industrial purposes (Hartman et al., 2011). Soybean is widely used in China because of its oil's popular use as a liquid, powder, or curd to make miso (fermented soy paste), shoyu (soy sauce), and soy milk (Hymowitz, 1970). Cultivars that are grown in the US are used to produce oil, which is used to make margarine, shortening, mayonnaise, and sandwich spread (Hymowitz, 1970).

1.2. Soybean production limitation by Sclerotinia stem rot

Sclerotinia sclerotiorum (Lib.) de Bary is a soil-borne, necrotic fungal pathogen (Purdy, 1979) which infects over 370 plant species in over 225 genera and 64 plant families (Bolton et al., 2006). *S. sclerotiorum* is a non-host specific pathogen because it has a wide range of host plants including both monocotyledonous and dicotyledonous plants (Purdy, 1979; Boland and Hall, 1994). The pathogen infects various crops; causing cottony rot, drop, crown rot, blossom blight and, stem rot

(Yousefdoost and Ghosta, 1993; Bolton et al., 2006). Dicotyledonous plants that are infected by the pathogen include, soybean, sunflower, oilseed rape, edible dry bean, chickpea, and lentils (Boland and Hall, 1994); onion and tulip are two monocots that are also infected by this pathogen (Bolton et al., 2006). Among the cultivated crops that are affected by Sclerotinia stem rot, soybean is one of the crops whose production has been greatly impacted (Hartman et al., 2011). Infection of soybean and the response of soybean cultivars have long been studied in countries with large scale soybean production; however, similar studies are limited in South African soybean cultivars.

1.2.1. *S. sclerotiorum* biology

S. sclerotiorum is classified under the kingdom Fungi, phylum Ascomycota, order Heliotiales and family Sclerotiniaceae (Sharma et al., 2015). Microscopic and macroscopic identification of *S. sclerotiorum* involves the use of characteristics that are species specific. *S. sclerotiorum* species have inoperculate asci (sexual spore containing body) which are produced from a stipitate apothecium (Sharma et al., 2015). The apothecia which appear as a light yellow-brown concave structure (Figure 1.1 A, B) can form from sclerotial stomata or be associated with the infected plant (Whetzel, 1945). The pathogen has hyaline hyphae that are multinucleate and septate (Figure 1.1C) (Bolton et al., 2006). *In planta* and *in vitro*, mycelia appear as white (Figure 1.1 D) or tan cottony structures. The pathogen can survive harsh conditions for long periods through the formation of black pigmented multi-hyphal structures known as sclerotia (Figure 1.1 E). Under favourable conditions sclerotia germinates into mycelia (Ordonez-Valencia et al., 2015). Ascospores are produced by meiosis and are released from spore containing bodies known as asci (Bolton et al., 2006; Sharma et al., 2015).

S. sclerotiorum has been reported across five continents (Badet et al., 2015); however, the pathogen has a wide range of hosts and is probably found in every continent which gives it a worldwide distribution (Purdy, 1979).

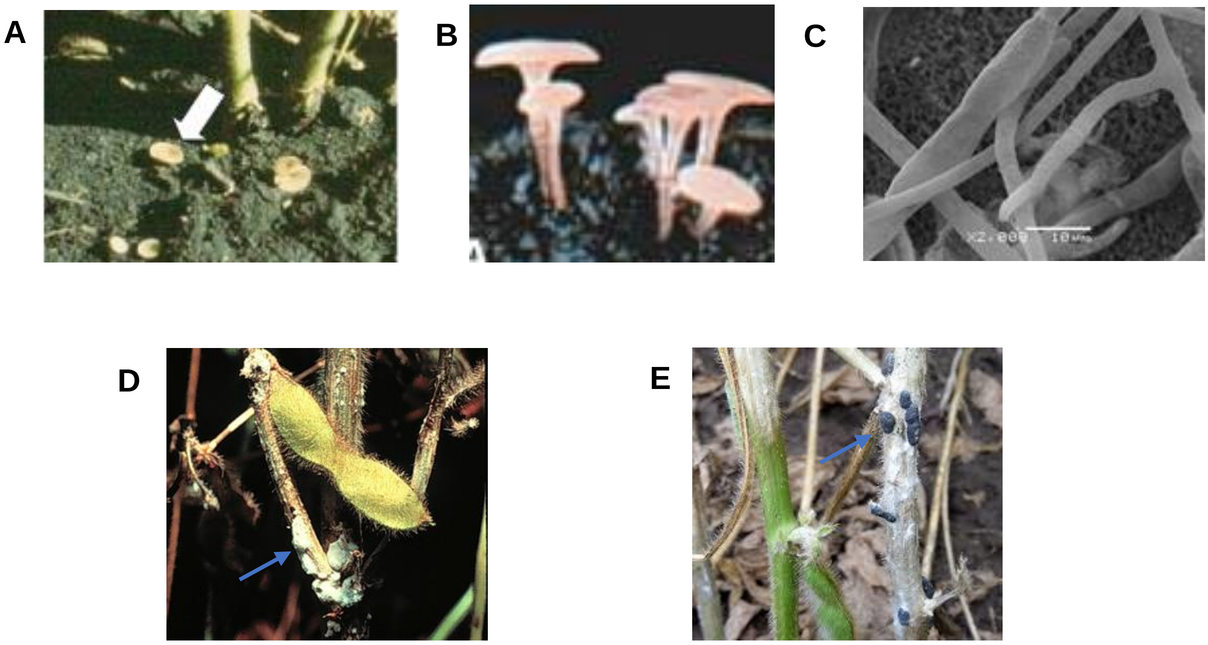


Figure 1.1 Fungal structures that are used to characterise *S. sclerotiorum*, apothecia (A and B), hyphal extensions (C), mycelia and sclerotia on infected plants (D and E respectively) (Bolton et al., 2006; Ordonez-Valencia et al., 2015).

1.2.2. Disease epidemiology

S. sclerotiorum has a large reproductive potential and the ability to survive long term unfavourable conditions through the production of sclerotia (Willetts and Bullock, 1992). The production of spores and cell wall degrading enzymes (Bolton et al., 2006) are also characteristics that make it successful as a pathogen. The fungus is classified as a necrotrophic pathogen because it lives off dead or dying host plant tissue (Amselem et al., 2011; Sharma et al., 2015);

Sclerotia can germinate in two ways depending on the environmental conditions (Figure 1.2). It can either germinate myceliogenically, where sclerotium germinate directly into mycelia, which attack plant tissues Figure 1.2 stages 1, 6 and 7) (Bardin and Huang, 2001), or carpogenically where it produces fruiting bodies known as apothecia (Figure 1.2 stages 2 to 6). Apothecia produce ascospores which infect above ground plant parts (Figure 1.2 stages 3 and 4) (Bolton et al., 2006; Amselem et al., 2011). Hyphal germination infects the plant first by colonizing non-living organic matter. This is followed by mycelium formation as an intermediate step for mycelial infection. When sclerotium germinates into apothecia, the apothecia release air-borne ascospores which infect the plant through a wound or by penetrating the stomatal opening (Sharma et al., 2015). When conditions are favourable, mycelia form a black sclerotia which subsequently develops on the external surfaces of the stem, or within the stem of the infected plant (Figure 1.2 stages 5 and 6) (Purdy, 1979). When sclerotia eventually meet the soil surface, they become buried in the soil until conditions are favourable and it can germinate again (Bolton et al., 2006).

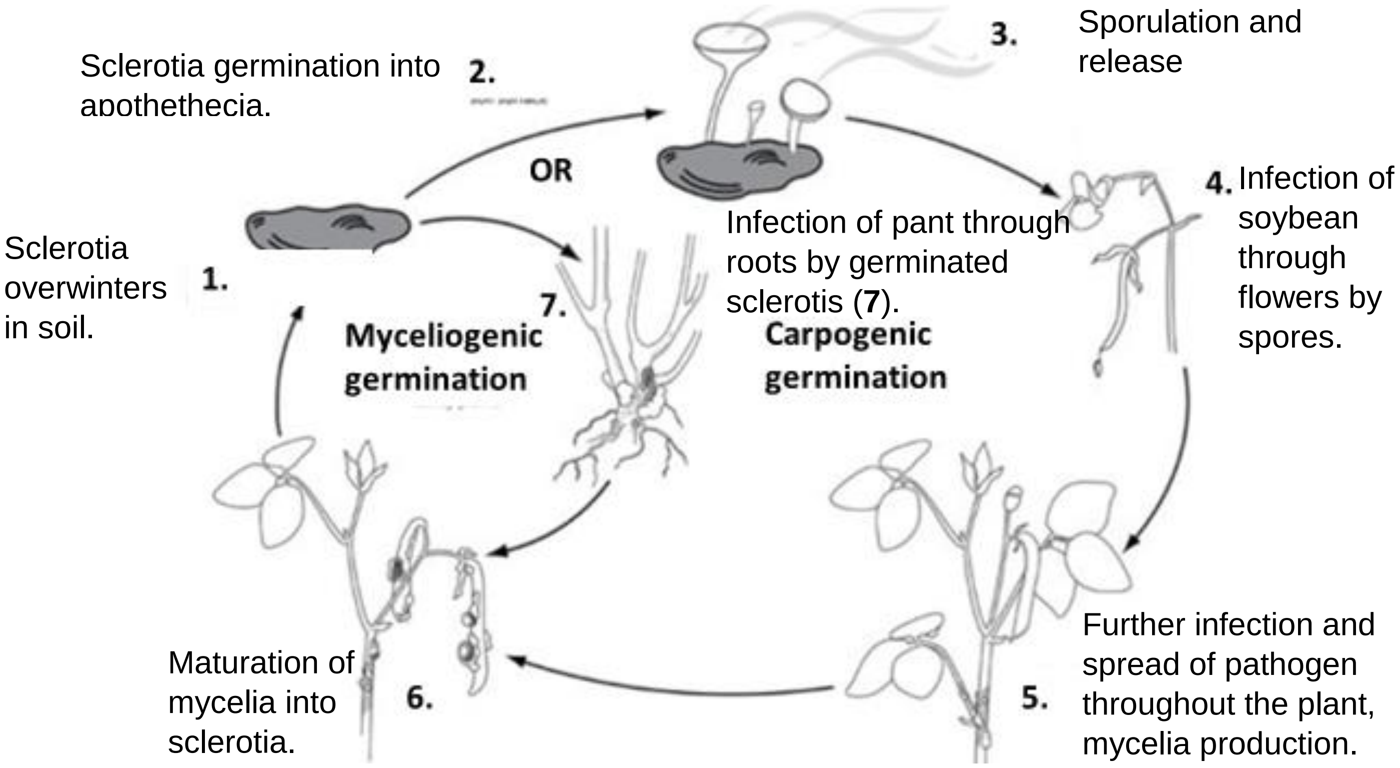


Figure 1.2 The disease cycle of *S. sclerotiorum* depicting the stages of myceliogenic and carpogenic germination on a soybean plant (Link and Johnson, 2007).

Growth of *S. sclerotiorum,* disease initiation and disease development depend on environmental conditions among other factors. Generally, *S. sclerotiorum* occurs in regions of the world that are relatively cool and moist (Purdy, 1979); however, *S. sclerotiorum* has also been reported in hot, dry as well as in semi-arid regions where it may seem that conditions are unfavourable (Sharma et al., 2015). *S. sclerotiorum* prevails in temperatures between 0^{o}C and 32^{o}C but does not grow on the outside of either extreme (Purdy, 1979). Even though hot temperatures may be unfavourable for pathogen growth as well as for disease development; irrigation provides moisture and therefore a favourable condition for germination of sclerotia in soil and disease development.

Sclerotia development has been reported to coincide with the production of oxalic acid and it is suspected that oxalic acid might be one of the metabolites required for initiation of sclerotia (Donaldson et al., 2001). Sclerotia initiation and development also depends on the activities of polyphenol oxidases, tyrosinases and lactases which provide organic sources for its development (Cooke, 1971; Willetts and Bullock, 1992). Once sclerotia have matured, apothecia germination begins with the emergence of a stalk-like stipe from the soil (Merriman, 1976). Apothecia are produced when the soil has more than 50% field capacity moisture content (Sharma et al., 2015) and soil temperatures are between 10^{o}C and 20^{o}C. There must be a non-living food base in rhizosphere just two centimetres below the soil surface (Abawi and Grogan, 1975; Fernando et al., 2004). The stipe grows up to approximately one centimetre above the soil surface and when exposed to UV-light, it differentiates into apothecia (Sharma et al., 2015). When the apothecia have matured, two to 30 million spores are ejected into air over a given number of days, depending on the environmental conditions. The timing at which spore production starts as well as the phototropic nature of the asci are important adaptations which maximise spore dispersal potential (Wu, 1988). Freshly produced spores can naturally survive for five to 21 days depending on the relative humidity; but in laboratory conditions, they can survive for years when freeze-dried (Wu, 1988). All these factors make *S. sclerotiorum* successful as a fungal pathogen.

1.2.3. Soybean infection by *S. sclerotiorum*

The infection of soybean by *S. sclerotiorum* primarily occurs through eruptive germination of soil-borne sclerotia; but, can also occur through air-borne spores under favourable environmental conditions (Bolton et al., 2006). Sclerotia can persist in the soil for more than ten years during unfavourable conditions (Purdy, 1979). The pathogen is then transferred to healthy soil through planting seeds that are infested with sclerotia (Wegulo et al., 1998). It is therefore important to screen soybean seeds thoroughly before planting. Infection can still occur through air-borne ascospores that are released from apothecial fruiting body when conditions are favourable (Hoffman et al., 1998; Wegulo et al., 1998). Once spores have colonized the dying tissue of the plant where they are established, they germinate to form mycelium. Upon colonising the soybean plant; the pathogen releases oxalic acid and lytic enzymes or appressoria which helps it penetrate living tissue that has not yet been infected (Fernando et al., 2004; Sharma et al., 2015). When the invaded tissues die, the mycelia aggregate into pigmented sclerotia (Sharma et al., 2015).

Pathogenicity or virulence factors include extracellular lytic enzymes like cellulase, hemi-cellulase and pectinase, which degrade cell walls and cause tissue maceration (Fernando et al., 2004). Production of oxalic acid provides acidic conditions under which cell wall degrading enzymes are more active. It also has toxic effects on the cells of the host plant (Bolton et al., 2006; Sharma et al., 2015). Oxalic acid also reduces defence of the host plant by supressing the oxidative burst (Cessna et al., 2000). It has therefore been established that cell wall degrading enzymes and oxalic acid are two of the main virulence factors of *S. sclerotiorum*.

Sclerotinia stem rot symptoms in soybean initially appears as water-soaked stains on the leaves (Figure 1.3 A), stems and pods that results from the ascospores mediated infection of flowers (Fernando et al., 2004; Chen and Wang, 2005). As the disease progresses, the infected stems start dying off and become tan or bleached as they dry off (Bolton et al., 2006; Ramusi and Flett, 2014). Necrotic lesions may also develop on the infected leaves and stems. In moist conditions, white cottony mycelia can be seen on the leaves and stems of infected plants (Figure 1.3 B). These further develop into melanised compact structures (sclerotia) that appear black, and can be found on seeds, stem, as well as inside the stem of infected plants (Purdy, 1979).

Sclerotinia stem rot, like most pathogens of soybean, attack all parts of the plant which destroys the crop (Hartman et al., 2011). Infected plants wilt (Figure 1.3 C), leaves become withered and eventually the plant dies. Seeds of infected pods may also appear shrivelled or the grain becomes completely replaced with sclerotium in the pod (Ramusi and Flett, 2014).

Figure 1.3 Appearance of infected soybean plants as disease develops. Symptoms are shown at the beginning as water-soaked leaf lesions (A), mycelia infestation on stems and leaves (B) and late infection stage is indicated by plant wilt and drying out of leaves (C) (Chen and Wang, 2005).

1.3. Economic and agricultural impact

Sclerotinia stem rot has a devastating economic impact on the soybean production industry. Dead infected tissues within stems clog and prevent transportation of water and minerals, further slowing down pod development and seed production (Hartman et al., 2011). Losses due to Sclerotinia stem rot can occur directly through yield loss as the crop is destroyed, or indirectly, through reduced grain quality (Ramusi and Flett, 2014).

Wrather et. al. (2003) reported soybean yield suppression by Sclerotinia stem rot in the US and Ontario between 1996 and 2002; the study was based on estimates from nine states. The lowest yield suppression was estimated at 54.43 tonnes, while the highest suppression was approximately 1.6 million tonnes (Wrather et al., 2003). Even though authors suspect that other factors as well as pathogens may be involved in this loss, it was reported that Sclerotinia stem rot is among the top ten diseases that resulted in yield suppression over the given number of years in the US. In South Africa Sclerotinia stem rot was first recognised in Lydenburg district during the late 70s (Thompson and Van der Westhuizen, 1979) but has lately spread across all local production regions (Botha and McLaren, 2009). Yield loss, due to Sclerotinia stem rot in South Africa has been estimated to be as high as 60% in certain planting seasons and regions (Ramusi and Flett, 2014).

1.4. Sclerotinia stem rot management strategies in soybean

It is important to understand or have a background in the host plant and pathogen interaction before implementing strategies to control any pathogen in general. *S. sclerotiorum* is a necrotrophic pathogen, which might pose a challenge to strategies that aim to control it. There are several strategies that are currently employed to control and manage Sclerotinia stem rot in soybean.

1.4.1. Pathogen and host plant interaction

S. sclerotiorum has been reported to produce several pathogenic factors that supress the plant's natural defence system (Cessna et al., 2000). In general, there are two phases of interaction between plants and microbes. The first phase is where the plant recognises certain classes of microorganisms including non-pathogen organisms. The second phase is where the pathogen produces effectors which are either recognised by the host plant recognition system which results in effector triggered immunity (ETI), or where the pathogen's effectors may supress the effector recognition system resulting in effector triggered susceptibility (Jones and Dangl, 2006). Whether the plant is successful in mounting defences that result in resistance depends on the specific pathogen; for example, it's usual for the plant to have successful defences against biotrophic pathogens but unusual to have defence against necrotrophic pathogens as these often kill the plant tissue and weakens its defence (Dangl and Jones, 2001).

Fungal pathogens usually infect the plant cells directly by entering epidermal cells and indirectly by producing hyphae, which either extends through the cell walls or between cells (Jones and Dangl, 2006). Plants produce various metabolites and proteins when they sense colonisation by fungal pathogens. The salicylic acid pathway, which results in the increase of salicylic acid and activation of downstream defence processes, is common against biotrophic pathogens; however, the jasmonic acid and ethylene pathways are important in defence against necrotrophic pathogens like *S. sclerotinia* (Glazebrook, 2005; Guo and Stotz, 2007). In their 2007 study, Guo and Stotz reported that jasmonic acid may delay or even inhibit *S. sclerotiorum* pathogen growth *Arabidopsis* mutants. Further investigation may however be required to see if there are no other pathways involved. There are however, limited studies in metabolic or physiological profile changes that happen with soybean infection by *S. sclerotiorum*. This might therefore present an important step towards solving Sclerotinia stem rot disease in soybean.

An oxidative burst which involves the production of reactive oxygen species (O_2^-, H_2O_2) is usually the first process mediating plant defence against necrotrophic pathogens in almost all plants (Wojtaszek, 1997). The production of oxalic acid as a pathogenicity factor, however, supresses this oxidative burst and therefore inhibits downstream defences which are mediated by the oxidative burst (Cessna et al., 2000). The exact mechanism by which oxalic acid supresses the host's oxidative burst is not well understood and requires further study. It has been reported that oxalic acid produced by *S. sclerotiorum* also creates an acidic environment which increases the activity of the pathogen's cell wall degrading enzymes (Sharma et al., 2015). This is expected to further weaken the plant's natural defences; it is therefore tempting to conclude that studies that aim to increase the plant's defence system should focus on oxalic acid production by the pathogen. The levels of lignin as well as soluble pigments of the soybean stem are associated with the production of oxalic acid by the pathogen (Peltier et al., 2009; Zhao et al., 2015); these may thus be important response pathways to pathogen infection in soybean and should be the target in controlling stem rot of soybean.

1.4.2. Chemical control

Chemical control of a pathogen mainly focuses on the use of fungicides which either eliminates or prevents the growth of fungal pathogens. This reduces the spread of pathogens thereby reducing disease effects on the plant. Fungicides may be applied as foliar sprays to reduce the infection through ascospores (Mueller et al., 2002). In South African fields, fungicides are also applied as a seed treatment before planting the seed. This prevents soil-borne infection from sclerotia when seeds are planted (Ramusi and Flett, 2014). It is however, important to understand that seed treatment by a fungicide will not be effective if the soil is already infested with sclerotia.

In soybean, benomyl and thiophanate methyl are considered the most effective foliar fungicides (Boland, 1997; Mueller et al., 2002; Johnson and Atallah, 2006). In South Africa, Benomyl 500 WP® containing benomyl as the active ingredient is used as a foliar fungicide as well as in seed treatment. Sclerotinia stem rot does not only colonise soybean leaves; it also infects other parts of the plant and therefore a combination of both foliar and seed treatment might be recommended for managing Sclerotinia stem rot.

In soybean, ascospore infection occurs through the blossoms (Bolton et al., 2006), it is therefore important to spray the plants as they flower, before the environment favours ascospores release. In his study, Boland (1997) found that fungicide treatment becomes less effective when the environment favours disease development even though in some environments, disease suppression may still occur. It is important to ensure that all the blossoms are covered, which can be a problem especially in dense canopies where plants are cropped in narrow rows (Morton and Hall, 1989; Mueller et al., 2002).

The main disadvantage of using chemical control to manage *S. sclerotiorum* is that most of the constituents of fungicides are not environmentally friendly and may have non-target effects on the plant (Paul et al, 1998), on the beneficial soil micro-flora (Chen et al., 2001; Ingham, 1985), or even the surrounding fauna. It is therefore important to consider strategies, which may not have a direct negative or non-target impact on the plant or the environment.

1.4.3. Biological control

Biological control refers to an environmentally friendly means by which pest populations are reduced using other organisms that are natural enemies of the pest. Bio-control agents reduce their antagonist through competition for nutrients, parasitism, or antibiosis (production of growth inhibiting compounds) (Boland, 1997). According to Sharma et. al. (2015) there are more than 185 studies reported on the bio control of Sclerotinia stem rot. Bio control strategies in Sclerotinia stem rot focus on three key aspects; the reduction of primary and secondary inoculum by preventing germination of sclerotia, or by killing it, to reduce infection of the rhizosphere and phyllo sphere; and lastly, reduction of the pathogen's virulence (Kamal et al., 2016).

There are organisms that have been identified as potential bio control organisms for *S. sclerotiorum*. According to Kamal et. al. (2016), there are more than 100 fungal and bacterial species identified as potential bio control agents for Sclerotinia stem rot. Fungal antagonists of *S. sclerotiorum* that have been described include *Alternaria alterrnata*, *Claviceps cladosporioides*, *Epicoccum nigrum*, *Myrothecium verrucaria*, *Trichoderma viride, Streptomyces* sp., the most recently described *Tricoderma harzianum*, and the most commonly known *Coniothyrium minitans* (Boland, 1997; Ooijkaas et. al., 2000; Derbyshire and Denton-Giles, 2016; Kamal et. al., 2016; Zhang et al., 2016).

C. minitans is a well-studied organism and has been formulated to produce the commonly used commercial biological control Contans® WG (Derbyshire and Denton-Giles, 2016). *C. minitans* parasitize sclerotia in soil and prevents carpogenic germination there by reducing the spread of the pathogen through carpogenic germination (Ooijkaas et al., 2000). The mycoparasite has also been reported to reduce virulence of *S. sclerotiorum* by breaking down oxalic acid which also affects the activity of cell wall degrading enzymes that are produced by the pathogen as virulence factors (Ren et al., 2007; Zeng et al., 2014).

T. harzianum is one of the most promising potential bio control agent against *S. sclerotiorum* (Zhang et al., 2016). The fungus has been reported to reduce linear growth of mycelium and production of apothecia (Agosin et al., 1997). Zhang et al (2016) reported a three-way interaction on *T. harzianum*, *S. sclerotiorum* and *G. max* (soybean) plants. The mycoparasite was reported to inhibit the growth of *S. sclerotiorum* with an efficiency of 56.3 %. In addition to parasitizing the pathogen; *T. harzianum* also reduced the plant stress associated with pathogen infection (Zhang et al., 2016).

1.5. Soybean resistance to Sclerotinia stem rot in soybean

1.5.1. Brief history of soybean breeding and domestication

Plant breeding is one of the oldest agricultural practices in history. This strategy has been applied in several important crops including maize, wheat, and soybeans. The goal of any breeding programme is usually to introduce new superior varieties, which are then commercialized. Any experienced plant breeder would agree that one of the most important steps in a plant breeding programme is the introduction of genetic material (germplasm) that can be used as a base population to start the breeding programme (Acquaah, 2010).

Even though there have been many published studies on evaluating resistance of soybean to *S. sclerotiorum*, there is still a need to discover more germplasms as sources of resistance to the pathogen. More studies should therefore focus on finding more sources of resistance. There has not been enough research done on finding soybean cultivars that display resistance to *S. sclerotiorum* in South Africa compared to other countries where soybean is being produced. Among those soybean cultivars that are grown commercially in South Africa, less than 50% of the cultivars have been screened for resistance to Sclerotinia stem rot. It is therefore important to screen commercially grown soybean cultivars for resistance in South Africa and establish germplasms that can be selectively crossed to increase resistance or introduce resistance in susceptible cultivars.

1.5.2. Mechanisms of soybean resistance to Sclerotinia stem rot

Soybean resistance has been reported as partial and quantitative (Arahana et. al., 2001; Kim and Diers, 2000; Kim et. al., 2000) meaning that a given number of genes contribute a level of resistance resulting in the overall resistance of the plant. In 1987, Boland and Hall reported that soybean employed an “escape” mechanism of resistance, which involves cultivar maturity (some cultivars may mature faster before disease onset or before the disease becomes significant).

Resistance of soybean to stem rot might also be related to environmental conditions. This is evident in studies where little to no correlation in the response of greenhouse soybean and field planted soybean to infection by *S. sclerotiorum* was found (Wegulo et al., 1998; Kim et al., 2009; McLaren and Craven, 2008). This implies that environmental conditions or the regional climate is an important aspect in determining whether a specific soybean cultivar will be resistant to *S. sclerotiorum*. Cultivar selection may also influence the response to infection by *S. sclerotiorum* in correlation to environmental conditions; for example, a drought tolerant cultivar may have a better chance of surviving infection by *S. sclerotiorum* than a drought susceptible cultivar (Mueller et al., 2002).

1.5.3. Identifying resistant soybean cultivars

Soybean resistance to Sclerotinia stem rot has been reported as quantitative and partial (Kim and Diers, 2000; Kim et al., 2009). Cultivars showing partial resistance to Sclerotinia stem rot have been identified in countries with major soybean production (Wegulo et al., 1998; Peltier et al., 2009; Zhang and Xue, 2014; Vuong et al., 2008). However, none of these studies have ever reported a complete resistance of soybean to Sclerotinia stem rot.

1.5.3.1. Physiological and physical traits

The identification of resistant soybean cultivar can be based on disease severity, where the leaf (for foliar disease) or stem area covered by lesions or lesion length is compared and rated per the disease rating guidelines such as the ones provided by Vincelli and Hershman (2011) for maize and soybean diseases.

The different mechanisms of soybean resistance to *S. sclerotiorum* are some of the factors that pose a great challenge in evaluating the resistance of soybean cultivars to Sclerotinia stem rot. There are only two methods reported so far for evaluation of soybean cultivar resistance to stem rot, whose results might not be influenced by environmental conditions. One of these methods is the oxalic acid assay, which has been well described by Vasic et al. (2002) as well as by Wegulo et al. (1998). It appears, however, that the oxalic assay method is not as common as field and greenhouse evaluations. It is therefore important to keep in mind that cultivar resistance should be reported with consideration of the climate of a production region. Another strategy that can be used together with field, greenhouse of oxalic acid assay is the implementation of molecular markers that can distinguish between Sclerotinia stem rot resistant and susceptible soybean cultivars.

In soybeans, resistance to Sclerotinia stem rot has also been reported based on stem soluble pigment content (Wegulo et al., 1998) which is believed to be related to physiological pathways that are involved in plant defence. It is widely accepted that *S. sclerotiorum* releases oxalic acid as a virulence factor, which results in production of defence compounds that are believed to be associated with the anthocyanin pathway (Li et al., 2010; Zhao et al., 2015). Oxalic acid has therefore been utilised to screen for resistant cultivars based on the concentration of soluble pigments that are produced by soybean stems when they are exposed to oxalic acid (Wegulo et al., 1998). This test thus provides an alternative method of screening for resistance which might not necessarily be influenced by environmental factors.

Another trait that has been reported to be associated with partial resistance of soybean to Sclerotinia stem rot is the amounts of pre-formed lignin in parts of the plant as this is believed to be one of the secondary metabolites produced in defence against pathogen attack (Peltier et al., 2009). In their study, Peltier et al. (2009) noted that plants that are known as resistant to Sclerotinia stem rot contain less lignin in their stems. This has however, not been extensively studied and opens a gap for further research. A metabolomics approach to study various compounds or metabolites that are produced by soybean in response to infection and disease progression may be a good first step before identification of quantitative trait loci involved in resistance.

1.5.3.2. Molecular traits: QTL and molecular markers

Resistance that is identified based on physical symptoms can vary between greenhouse and field experiments as well as across different environments as other factors may influence disease development. The observed lack of disease or low disease may therefore not be necessarily associated with the plant’s physiological or genetic resistance. A molecular approach should therefore be used to indicate the genetic basis of resistance together with disease response phenotypes.

Eleven major QTL regions and two minor QTL regions that are significantly associated with stem rot resistance have been reported (Guo et al., 2008; Bastien et al., 2014; Vuong et. al., 2008; Li et al., 2010; Zhao et al., 2015). The reported QTL are linked to lesion length, lignin and soybean stem soluble pigments (Guo et al., 2008, Li et al., 2010; Guo et al., 2008). SSR markers that are genetically linked (which means thy segregate together with a given resistance QTL) with QTL mentioned above have been reported as stem rot resistance markers on soybean. (Guo et al., 2008, Li et al., 2010). The different QTL that have been reported and mapped so far are Qsp-1, Qsp-2, Qsp-3, Qswm13-1 which control plant response to infection measured by soluble stem pigment content (Li et al., 2010; Zhao et al., 2015); as well as QTL at specific positions of chromosomes GM15, GM01, GM20, GM19 which control plant response to infection when measured as lesion length (Bastien et al., 2014). Other additional QTL controlling stem rot resistance on soybean were reported as linkage groups, LGE, LGF, LGM, LGO (Guo et al., 2008) as well as those linked to SSR markers, Sat_138 and Satt126 (Vuong et al., 2008). There might be many more QTL involved in resistance that are still unexplored.

Molecular markers are an important part of QTL studies as most of these are linked to many important QTL and can therefore be used as an indirect approach to identify resistant cultivars. Soybean is one of the crops with a growing database for genomic resources. In 2010, the initial soybean linkage map was completed through the collaborative efforts of several researchers, (Cregan et al., 1999; Song et al., 2004; Song et al., 2010) and made available in the soybean breeder's tool database, known as Soy Base (www.soybase.org). Soybean researchers contributed over the years in to the development of this database. Approximately 210 990 SSR markers with di-, tri-, and tetranucleotide repeats that are locus-specific were developed from the soybean whole genome sequence and published on the Soy Base database in 2010 (www.soybase.org) (Song et al., 2010).

1.5.3.3. Choice of molecular markers

In most QTL identification and mapping studies, molecular markers that are commonly used are single nucleotide polymorphism markers, (SNP) (Bastien et al., 2014; Zhao et al., 2015) as well as, microsatellites or simple sequence repeats (SSR) (Guo et al., 2008; Li et al., 2010). SSR and SNP are also co-dominant markers, a feature which would be particularly useful in soybean markers data analysis because the soybean genome is regarded as a partially diploidised tetraploid (Shultz et al., 2006). SNP are good candidate markers in QTL mapping and breeding studies as they are highly polymorphic and can therefore separate different cultivars (Spooner et al., 2005). SNP are also abundant which makes their discovery relatively less challenging (Abdel-Mawgood, 2012). SNP databases have been developed for soybean (Cregan et al., 1999; Song et al., 2004; Song et al., 2010) which reduces the amount of work as one will not need to necessarily go through the whole process of SNP discovery and development. Even though SNP may be ideal for plant breeding, their analysis which includes the development of a SNP chip is still relatively expensive compared to that of SSR (Butler et al., 2007). SSR provide most of the benefits that SNP markers do and are relatively low in analysis cost, making them more ideal in routine studies of a breeding program.

1.6. <u>Problem statement</u>

The soybean industry has grown since the first introduction of soybean in South Africa (Department of Agriculture Forestry and Fisheries, 2016). With the growth of soybean industry in South Africa, several factors pose challenges to soybean farmers, including drought, pathogens, and pests. There have been a small number of publications on the study of soybean interaction with biotic or abiotic factors that threaten the soybean industry in South Africa, compared to other soybean producers in the world. There is therefore a crucial need in understanding especially the interaction between soybean and a given biotic or abiotic stress at a molecular level. There are several factors that may determine whether a certain cultivar is resistant to Sclerotinia stem rot. In separate publications, low correlations in resistance between field and controlled environment were reported (Mc Larren and Craven, 2008; Kim et al. 2009; Wegulo et al. 1998). This low correlation, which is suspected to be related to environmental effects thus pose a challenge when evaluating the resistance of soybean cultivars to Sclerotinia stem rot based only on phenotyping. It is suspected that the form of resistance that some soybean varieties display is an “escape” mechanism where short growing cultivars mature before the onset of disease (Boland and Hall, 1987; Mueller et al., 2002). Markers that are genetically linked with the agronomic traits that allow the plant to “escape” disease would therefore provide a feasible indirect way of screening for Sclerotinia stem rot resistance on soybean. In this study, we evaluate and characterise SSR markers that are genetically linked to stem rot resistance QTL and their feasibility for use as an alternative method of screening for resistance in South African cultivars. The SSR markers evaluated in this study will provide useful information that South African soybean breeders can use as an initial step to assess the potential of genetic resistance of cultivars to Sclerotinia stem rot before phenotyping the given cultivars. Soybean cultivars grown in South Africa have not been extensively studied as far as Sclerotinia stem rot is concerned. This opens a gap to therefore find an alternative and relatively non-expensive first step to identify partially resistant soybean cultivars.

1.7. Research Aims and objectives

The main aim of this study was to screen and characterise simple sequence repeat (SSR) markers linked to QTL of soybean stem rot resistance in South African cultivars.

To achieve this aim, the study had the following objectives:

1. To identify quantitative trait loci (QTL) from literature that have previously been reported to be associated with Sclerotinia stem rot resistance in soybean.
2. To select SSR markers that are linked to the selected QTL based on their applicability in South African cultivars.
3. To evaluate the diversity of the selected SSR markers in South African soybean germplasm.
4. To identify South African soybean cultivars sharing resistance alleles with a cultivar that has known resistance.
5. To estimate the potential of the current commercial soybean cultivars for use as genetic material in future breeding programs against Sclerotinia stem rot.

2. MATERIALS AND METHODS

2.1. The selection of SSR markers from literature and database (www.soybase.org)

Markers were selected within the regions of QTL that have been previously reported to be significantly associated with soybean response to Sclerotinia stem rot. Eleven major QTL regions and two minor QTL regions that are significantly associated with stem rot resistance on soybean were identified from literature (Table 2.1); the corresponding positions of each QTL was identified with the aid of the soybean composite Interval maps on the Soy Base database (www.soybase.org). The selection of SSR markers (Table 2.1) was done by identifying the positions of a given QTL on the composite interval map and selecting markers that are have either been reported as linked to a resistant QTL position or are identified close to a given QTL. Twelve of these are markers that have been previously reported in QTL that are associated with Sclerotinia stem rot resistance on sobean.

Table 2.1 QTL regions previously reported to be involved in soybean resistance to *S. sclerotiorum* and SSR markers that are associated with each region (Guo et al., 2008; Bastien et al., 2014; Vuong et al., 2008; Li et al., 2010; Zhao et al., 2015)

Chromosome no	Associated QTL Name	Approximate QTL position (cM)	Associated Markers	Motif	Position (cM)	Reference
1	QSP-1	49.084 to 50.008	Satt502*	(TTTA)3agttttaaact (ATA)16	49.084	Bastien et al., 2014 and Li et al., 2010
			Satt169	(AAT)16	50.054	
			Satt323*	(ATA)17	60.004	
8	QSP-3	96.097 to 100.008	Satt233*	(ATA)16	100.008	Vuong et al., 2008, Guo et al., 2008 and Li et al., 2010
			Satt133	(AAT)10	125.037	
			Satt525*	(TTA)15	96.097	
			Sat_233	(TA)14	86.041	
10	Unknown QTL	106.000	Satt581*	(TAA)11	106.002	Guo et al., 2008
	Unknown QTL	118.014	Satt153*	(TTG)4	118.013	
11	QSP-2	36.048-56.013	Satt251*	(TAT)15	36.048	Li et al., 2010
			Satt638	(ATA)13	37.079	
	AW186493	11.004	Satt269*	(ATA)31	11.037	Guo et al., 2008 and Zhao et al., 2015
			Satt145	(AAT)4c(ATA)7	10.065	
			Satt149*	(AAT)16	18.012	
			Satt252	(TAT)23	16.008	
14	Unknown QTL	27.062	Satt126*	(AAT)18	27.062	Vuong et al., 2008
15	Unknown QTL	56,002	Satt369*	(TAT)17	56.027	Bastien et al., 2014 Guo et al., 2008
	Unknown QTL	12.009	Satt411*	(TAT)11	12.092	
	Unknown QTL	50.89	Satt685	(AAT)14	56.007	
			Sat_124	(TA)35	15.086	

* Markers that are marked with an asterisk are those that have been previously reported for Sclerotinia stem rot resistance on soybean cultivars

* Markers that are marked with an asterisk have been previously reported for stem rot resistance on soybean before.

2.2. Plant Material growth and maintenance

2.2.1. Plant growth and maintenance

Twenty-Nine South African commercial soybean cultivars (Table 2.2) were selected based on the results from the national soybean cultivar trials done by Annelie de Beer at the Agricultural research council (ARC Grain Crop Institute, South Africa). Two more cultivars were grown together with 29 cultivars that were selected for the study; these two were used as controls in the study. Maple Arrow (M.A) cultivar was used as a reference for cultivars that are partially resistant to stem rot, while Williams 82 (W82) was used as a reference for susceptible cultivars. Three individual plants per cultivar sampled in South African cultivars and W82 while only two individual plants could be sampled for MA. This sampling procedure was done to increase the population being screened and to increase statistical significance for downstream marker analysis.

Two seeds were planted in each pot [18 cm x 2 °Cm diameter (top) and 13 cm (bottom)] using sand-coir mixture as a growth medium. Each seed was inoculated with *Bradirhizobium japonicum* (strain WB 74-1) powder at 109 CFU g^{-1} (Soygro bio-fertilizer Limited, South Africa) and planted in an approximately 0.5 cm depth, to promote a faster germination. Plants were grown under artificial lighting in a phytotron with lighting up to a 13 hr photoperiod at 600 mM $m^{-2-}s^{1}$ photosynthetically active radiation (PAR) and 60% relative humidity; the temperature in the phytotron was kept at $25^{°C}$/ $16^{°C}$ day/ night. Plants were watered alternatively with distilled water and nitrogen free Hoagland’s solution.

Table 2.2 Twenty-Nine South African soybean cultivars that were studied, M.A and W82 as reference cultivars for resistance and susceptibility respectively, with their respective maturity groups and resistance status (De Beer and Bronkhorst, 2016).

Cultivar No	Cultivar Name	Maturity Group	Resistance status
1	LS 6240 R	4,0	Unknown
2	LS 6444 R	4,0	Unknown
3	PAN 1454 R	4,4	Unknown
4	LS 6146 R	4,4	Unknown
5	PHB 94 Y 80 R	4,8	Unknown
6	LS 6248 R	4,8	Unknown
7	NS 5009 R	5,0	Unknown
8	DM 5.1i RR	5,1	Unknown
9	PHB 95 Y 20 R	5,2	Unknown
10	PAN 1583 R	5,2	Unknown
11	PAN 1664 R	5,3	Unknown
12	DM 5953 RSF	5,3	Unknown
13	LS 6453 R	5,3	Unknown
14	PAN 1521 R	5,7	Unknown
15	PAN 1500 R	5,7	Unknown
16	NS 5909 R	5,9	Unknown
17	PAN 1513 R	6,0	Unknown
18	LS 6466 R	6,0	Unknown
19	PAN 1666 R	6,1	Unknown
20	PAN 1623 R	6,1	Unknown
21	LS 6261 R	6,1	Unknown
22	DM 6.2i RR	6,2	Unknown
23	LS 6164 R	6,2	Unknown
24	LS 6161 R	6,3	Unknown
25	PAN 1614 R	6,4	Unknown
26	NS 6448	6,4	Unknown
27	DM 6.8i RR	6,8	Unknown
28	NS 7211 R	7,2	Unknown
29	PAN 1729 R	7,3	Unknown
30	W82	3	Completely susceptible
31	M.A	0	Partially resistant

* The higher the maturity group rating of the plant, the longer it takes to reach maturity and vice versa. MA is the fastest maturing cultivar in this case. M.A cultivar was selected as a reference for Sclerotinia stem rot resistance while W82 was selected as a reference for completely susceptible cultivars

Plant material for DNA extractions were collected by harvesting the youngest leaves of three week old plants; the specific period of harvest had no relevant to the study as these materials was used for DNA extractions, and thus the time of harvest would not make any difference. The harvested leaves were stored inside Eppendorf tubes from which DNA would later be extracted and stored for short term in liquid nitrogen until the -80 ˚C storage became available.

2.3. DNA extraction and quantification

Deoxyribonucleic acid (DNA) was extracted from plant samples representing each of the 29 commercial soybean cultivars that are used in South Africa, as well as from W82 and M.A reference cultivars. DNA extraction was done following the 2 X cetyl trimethylammonium bromide (CTAB) (Sigma-Aldrich, Germany) method developed by Doyle and Doyle (1990), which is based on phenol-chloroform. Leaf tissue was ground in liquid nitrogen using clean autoclaved mini-pestles, 0.5 mg of tissue was transferred to a sterile 1.5 ml Eppendorf tube. Five hundred micro-litres of 2 x CTAB buffer containing polyvinylpyrollidone (Sigma-Aldrich, Germany) and 2-Mercaptoethanol (Merck, Germany) was added to plant tissue; the tubes were incubated for approximately an hour at 55 ˚C in a heating block. Incubation was done with occasional mixing every 30 min to make sure that the plant tissue does not clump down at the bottom of the tube. Ribonucleic acid (RNA) was removed from the DNA samples by treating the incubated samples with one microliter of RNase A (Thermo fisher Scientific, United States) and incubating the samples at 37 ˚C for 15 min. Following incubation, 500 µl of chloroform was added to the samples and these were mixed by inverting the tubes ten times. Proteins and lipids were separated from DNA by centrifuging the samples at 14 000 rpm for 13 min. The top aqueous layer was removed and transferred to new sterile tubes which were labelled with the respective sample names. The volume of the aqueous phase obtained was estimated, Ammonium acetate (Merck, Germany) and Isopropanol (Merck, Germany) were added to the samples as 0.08 and 0.54 times the estimated sample volumes respectively. DNA samples were kept at -20℃ overnight and removed the next day. DNA precipitate was obtained from the suspension by centrifuging at 14 000 rpm for three min. DNA pellets were washed with 700 µl of 70 % ethanol followed by 700 µl of 95 % ethanol. Samples were centrifuged at 14 000 rpm for one minute after the addition of ethanol; the supernatant was removed, and samples were dried by inverting the tubes upside down on a clean Kim wipe for 15 min. Excess ethanol was further removed from the samples by letting them air dry upright for an additional 10 min before re-suspending in 30 µl of double distilled water. DNA quantity measured by evaluating the amount in nanograms per microliter while DNA purity was evaluated by measuring the absorbance 260/280 and 260/230 ratios using Nano drop spectrophotometry (Thermo fisher scientific, United States). DNA sample quality was further analysed by running the samples on a 1 % Agarose gel, in a one times tris-acetate-EDTA (TAE) (Sigma-Aldrich, Germany) buffer for one hour at a constant current of 400 mA and a voltage of 100 Volts.

2.4. SSR marker primer testing and multiplex polymerase chain reaction (PCR) design

2.4.1. Single marker PCR and further selection of SSR markers

The primer sequences (Table 2.3) for each marker were obtained from the BARCSOYSSR database (https://soybase.org/BARCSOYSSR/index.php) (Song et al., 2010). Cultivar W82 was used as a positive control for initial selection because most of the SSR markers of soybean had been developed using whole genome sequence of this cultivar. Primers for all SSR markers were tested across eight different annealing temperatures as suggested by Song et al. (2010). A single plex PCR involving the amplification of one marker locus at a time was set up using the OneTaq® PCR master mix kit per manufacturer's instructions (New England Biolabs, United States), using the genomic DNA of the cultivar W82 as a template. A non-template control (NTC) which contained water instead of template genomic DNA was included in each PCR. Each PCR reaction contained 1 X OneTaq master mix, 0.25 µM forward and reverse primer each, 50 ng template DNA and deionized water up to a final volume of 10 µl. The primers were optimised by changing the annealing temperatures using the temperature range of 55 ˚C to 62 ˚C. The PCR cycle reaction was conducted in a BOECO TC-PRO thermocycler (Germany), under the following conditions: an initial denaturation step at 94 ˚C for five min, denaturation step at 94 ˚C for 30 seconds, a primer annealing step using the temperature range 55 ˚C -62 ˚C for 30 seconds, an extension step at 72 ˚C for one minute; the denaturation, primer annealing, and extension steps were repeated for 30 cycles in each PCR , followed by a final extension step at 72 ˚C for 10 min. The resulting PCR products were stored at four degrees Celsius and analysed using agarose gel electrophoresis. All markers that could not be amplified in single PCR reactions were removed from the study; all markers that amplified at the same annealing temperature were kept and used in future experiments.

Table 2.3 Primer sequences that were used for PCR of the selected SSR markers, (Cregan et al., 1999; Song et al., 2004; Song et al., 2010).

Marker name	Forward Primer Sequence	Reverse Primer Sequence	Reference
Satt502	5’ GCGCAACTCCCCTTCTAGTGTAATT3GT 3’	5’ GCGACTCAACTCCCCTATAATGTATAT 3’	Cregan et al., 1999
Satt233	5’ AAGCATACTCGTCGTAAC 3’	5’ GCGGTGCAAAGATATTAGAAA 3’	Cregan et al., 1999
Satt369	5’ AACATCCAAAGAAATGTGTTCACAA 3’	5’ GCGAGTTCGAATTTCTTTTCAAGT 3’	Cregan et al., 1999
Satt323	5’ GCGGTCGTCCTATCTAATGAAGAG 3’	5’ TGTGCGTTTAAATTGCAGCTAAAT 3’	Cregan et al., 1999
Satt581	5’ CCAAAGCTGAGCAGCTGATAACT 3’	5’ CCCTCACTCCTAGATTATTTGTTGT 3’	Cregan et al., 1999
Satt153	5’ GGGTTATATCAGTTTTTCTTTTTGTT 3’	5’ CCATCCTCGTTAGCATCTAT 3’	Cregan et al., 1999
Satt169	5’ GGCAAGTTTATGAAAGTTTTAAATTTACTA 3’	5’ GATTGCTTTTACAAAGTCTAGAGAACTATA 3’	Song et al., 2010
Satt251	5’ CCTCCACCCCCTTCCCACCCAAAA 3’	5’ GGTGATATCGCGCTAAAATTA 3’	Cregan et al., 1999
Satt525	5’ GCGCATAGCTTTCAGAGAAGTTT 3’	5’ CATTCACCAATCCTCATTAGA 3’	Cregan et al., 1999
Satt411	5’ TGGCCATGTCAAACCATAACAACA 3’	5’GCGTTGAAGCCGCCTACAAATATAAT 3’	Cregan et al., 1999
Satt269	5’ GCGTGCCAGGTAGAAAAATATTAG 3’	5’ GCGGTTTTTCACTTTTCAAAATTC 3’	Cregan et al., 1999
Satt133	5’ GCAAATGAAGAAAAGATGGATT 3’	5’ TAAAGCGATGGTTGAAGAAAG 3’	Cregan et al., 1999
Satt145	5’ AGCATATGGGATACAAGTGATTAG 3’	5’ CGGTGTTGGTGTGGTATGT 3’	Cregan et al., 1999
Satt685	5’ ATCGTGGCATGTCTCACTAC 3’	5’ GAGGCGGAAGGAAATCTAAT 3’	Song et al., 2004
Satt126	5’ GCTTGGTAGCTGTAGGAA 3’	5’ ATAAAACAAATTCGCTGATAT 3’	Cregan et al., 1999
Satt149	5’ TTGCACATTCTTTTTGGTAAACAGTCATAA 3’	5’ GTTGGAGGCCATAGTCACATTAATCTTAGA 3’	Cregan et al., 1999
Satt638	5’ GCGGATCCTATGAGCCTATCTGTATTT 3’	5’ GCGGCAAGCATTATACTATTTATACGG 3’	Song et al., 2004
Sat_233	5’ GCGGGGAGAACACTCAAAACTCTCTAAAGAA 3’	5’ GCGCCAGTCATTTTTTTTATGATATGTAGAT 3’	Song et al., 2004
Sat_124	5’ GGGTCCATTCCACTTTTTGTACAATAT 3’	5’ GGGAGTTCAAACATCCATTAGTGGTATA 3’	Cregan et al., 1999
Satt252	5’ GCGAATTTGGATTAATTAAATTTATG 3’	5’GCGCTCGGTCCTCTCAAATAAGGTCTC 3’	Cregan et al., 1999

2.4.2. Multiplex PCR design and primer labelling

The markers were grouped into multiplex panels (a group of markers that could be amplified together in a single PCR reaction) of seven markers per panel. An *in silico* multiplex experiment was designed using the multiplex manager software version 1.0 (Holleley and Geerts, 2009). The software allowed a simulation of a multiplex PCR for the selected markers as if it was done in the lab and showed the best marker combinations that could be used in a multiplex PCR. Each panel that had previously been a combination of seven markers was further divided into two sub-panels to avoid marker overlap during agarose gel analysis; each of these sub-panels consisted of either four or three markers per panel. A single forward primer for each marker was labelled with fluorescent dye. One of each of four different fluorescent dyes was used per marker; the dyes that were used to label markers were Ned, Pet, 6-Fam, and Vic. Once a fluorescent dye was assigned to each marker, single forward primers of each marker were sent for physical labelling at Life technologies labs, South Africa.

2.5. SSR marker polymorphism pre-screening

Four cultivars were randomly selected from 29 commercial soybean cultivars that are grown in South Africa, for marker polymorphism pre-screening test. The selected cultivars were initially used as a representative of the 29 cultivars, cultivar W82 was included as a reference for soybean cultivars that are susceptible to Sclerotinia stem rot while M.A was used as a reference for cultivars that have partial resistance to Sclerotinia stem rot. SSR marker polymorphism across the selected cultivars was evaluated by doing a multiplex PCR using the marker combinations that were created in section 2.4.2. A PCR was done using the Platinum Multiplex PCR Kit from Applied Biosystems according to manufacturer’s instructions (Applied Biosystems, Life Technologies, South Africa). A single PCR reaction contained a combination of primers for either four or three markers at a final concentration of 0.5 mM each, 1 X Platinum Multiplex PCR Master Mix, 50 ng genomic DNA as a template, and nuclease free water up to a final volume of 12.5 µl. The PCR cycle reaction was conducted using the BOECO TC-PRO thermocycler (Germany), using the same PCR conditions as in section 2.4.1 above. The resulting PCR products were stored at 4 ˚C and analysed using agarose gel electrophoresis.

2.6. Screening markers on soybean cultivars and genotype data analysis

2.6.1. Fragment length analysis

Three individual plants were sampled in each cultivar; and two individuals for M.A; one plant sample from each cultivar was used to optimise multiplex PCR for fragment and genotype analysis.

Multiplex PCR was first done on the designed subpanels using the platinum multiplex PCR master mix per manufacturer's instructions (Applied Biosystems, Life technologies, South Africa) and 58 ˚C as the optimal annealing temperature. The products were analysed on a three percent agarose gel (Sigma-Aldrich, Germany) at 80 Volts for two hours and 30 min. A genotype test run was done to identify the best PCR product dilution factor before. Four different dilution factors were tested for PCR products of all twenty markers, on M.A as the partially resistant cultivar and W82 the most susceptible cultivar. The following dilution factors were tested; a 10 X, a 25 X, a 50 X, as well as a 100 X. The optimal primer concentration that was used in multiplex PCRs was 1.2 µM. Fragment analysis was done using GeneScan™ 500 LIZ™ dye Size Standard Liz (Applied Biosystems, Life technologies, South Africa).

Marker genotype assessment

2.6.1.1. Allele calling

The genotypes for potential stem rot resistance SSR markers were analysed using Gene mapper software version 5.0 (Applied Biosystems, Life technologies, South Africa). The allele size range on W82 cultivar and the recommended relative fluorescent unit (rfu) were used as the basis for calling alleles present for a specific marker locus in each sample. A genotype plot peak was regarded as a true allele if the peak height was above 400 rfu to avoid scoring false alleles. All individuals showing peaks that fell outside the expected allele range or alleles that had not been observed before in other cultivars were not scored. All SSR marker loci that could be scored were used in further analysis while those that were difficult to score were discarded.

2.6.2. Genotype data analysis in genetic analysis on Excel (GenAlEx)

2.6.2.1. Allele frequency, allele patterns and gene diversity

Allele frequency and allelic patterns were calculated for each SSR locus using all samples from South African commercial cultivars; this was done as the first step to predict if there is genetic differentiation among South African cultivars. The graphs of allele frequency per locus in each cultivar was also used to visualise the overall allele diversity in each locus and the overall genetic diversity in South African soybean cultivars. The genetic relatedness and genetic diversity between cultivars was estimated by calculating fixation indices within cultivars and the total fixation index. The polymorphic information content (PIC) was calculated to see which of the selected markers were polymorphic enough to distinguish among the selected South African cultivars. PIC was calculated according to a formula developed by Guo and Elston (1999). Other genetic diversity parameters namely, the percentage of polymorphic loci per cultivar, heterozygosity per population and heterozygosity per locus indicating allele diversity per cultivar as well as allele diversity per locus over South African commercial soybean population were also analysed.

2.6.2.2. Genetic relationship analysis

Ninety-two individual samples (three samples from each South African cultivar, W82 and two from M.A) were assessed, and heterozygous individuals were used to estimate the ploidy of each microsatellite locus, all loci displayed diploidy and were thus analysed as diploid loci. Loci with only individuals that showed one allele were scored as homozygous diploids. After allele calling, the genotype data was further used for distance based analysis on GenAlEx software version 6.5 (Peakall and Smuse, 2012). SSR marker genotype data from each plant sample was used to evaluate the genetic relationships between South African soybean cultivars and the reference cultivars.

To evaluate the genetic relationships, the pairwise Nei's genetic distance (NeiP) and Nei genetic identity, pairwise Fixation index (FstP), as well as pairwise Shannon's index of diversity (SHuaP) matrices were constructed. The three matrices were used to specifically evaluate the genetic relatedness across South African cultivars and their relatedness with W82 and M.A (Sclerotinia stem rot resistance reference cultivars). Pairwise Nei's genetic distances were used to compare the genetic relatedness between South African cultivars and either M.A or W82 as resistant and susceptible cultivar respectively; if the genetic distance between a cultivar and M.A was smaller than the distance between the same cultivar and W82, the cultivar was considered to be more related to M.A than W82 and was therefore grouped under stem rot resistant cultivars that might be resistant to Sclerotinia stem rot. A principal coordinate analysis (PCoA) was done to visualise the results of genetic relatedness between the stem rot resistance reference cultivars and South African cultivars, as well as to visualize the cultivar's genetic clustering in a multidimensional space. The information from PCoA was also used to see which of the previously reported stem rot resistance markers are diverse enough in South African cultivars and would therefore be useful in providing an indirect method of screening for stem rot resistant varieties in South African soybean cultivars.

3. RESULTS

3.1. DNA extraction and quantification

DNA was extracted from W82, M.A and 29 South African cultivars that were selected for the study. The quality of isolated DNA was analysed on a 1% agarose gel (Sigma-Aldrich, Germany) (Figure 3.1) by gel electrophoresis, samples were analysed on gel for 1hr at 100V and visualized under UV-light thereafter. DNA samples were analysed together with a 1kb molecular marker as well as a low mass molecular weight marker to estimate the approximate amount of DNA obtained from each sample. DNA quantity was analysed using the Nano drop spectrophotometer (Thermo Fisher Scientific, United States) and this is shown on Table 3.1.

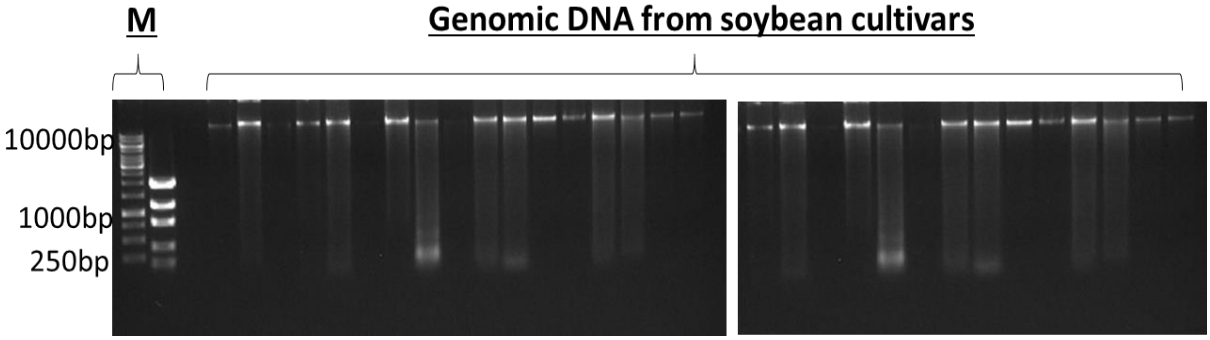


Figure 3.1 Assessment of genomic DNA quality using a 1% agarose gel. A 1Kb molecular marker and a low mass molecular weight marker (M) were used to estimate the amount of DNA in each sample.

3.2. SSR marker primer testing and multiplex PCR design

3.2.1. Single marker PCR and further selection of SSR markers

Single marker PCR analysis was done to characterise each marker as a single locus; the characteristics that were assessed include the optimal temperature at which the primer for each marker binds to genomic DNA template, the number of bands produced by each marker when analysed by agarose gel (Sigma-Aldrich, Germany) electrophoresis and finally, the approximate size of each marker on an agarose gel profile. Twenty markers were further characterised by PCR, using W82 genomic DNA as a template; these markers were then assessed using agarose gel electrophoresis (Figure 3.2). The estimated size ranges from agarose gel electrophoresis analysis was between 90 base pairs (bp) and 350bp. All markers showed a single defined band on agarose gel, except two markers which also showed a second but very faint band (Figure 3.2 Satt_124 and Satt525).

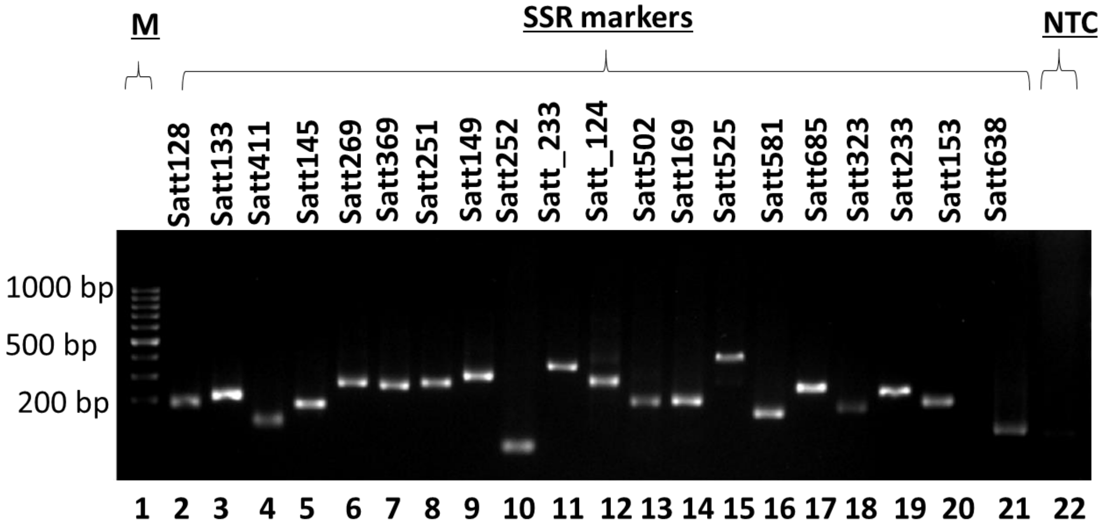
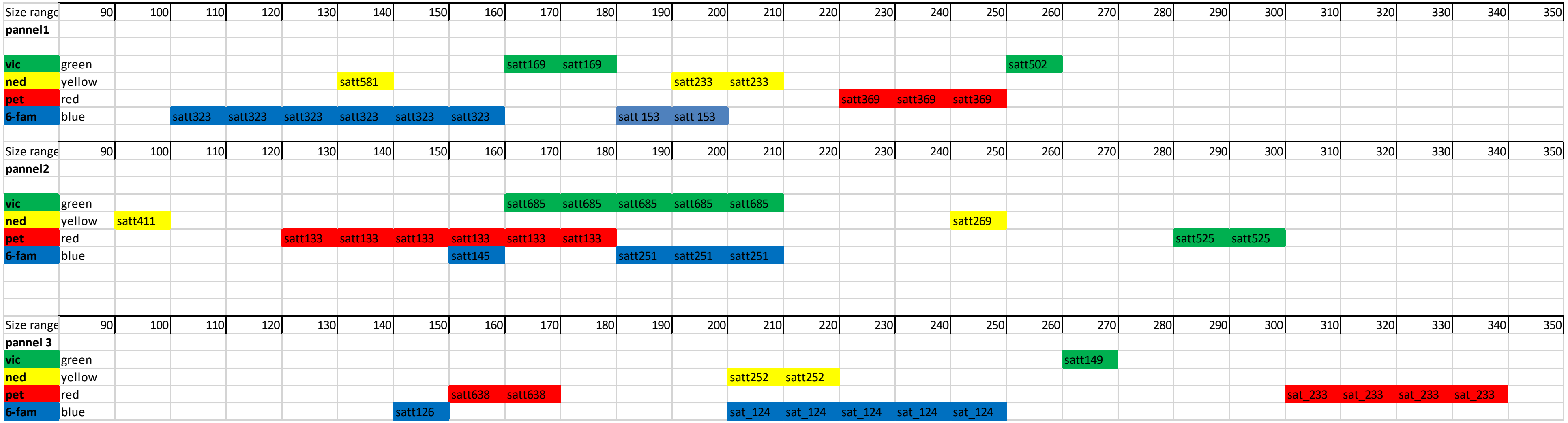


Figure 3.2 PCR amplification analysis of 20 SSR markers on a 3% agarose gel. A 100bp molecular size marker (M) was used to estimate the size of SSR markers; a negative control which includes no DNA template (NTC) was also included in the PCR analysis.

3.2.2. Multiplex PCR design and primer labelling

An *in silico* multiplex PCR allowed for a visualisation of how the amplification of each marker locus would look when done in a physical PCR in the lab. This was showed as the number of markers that would overlap as well as markers whose primers would anneal to each other resulting in primer dimerisation and less PCR product. Markers were divided into three multiplex PCR panels (Figure 3.3), two consisting of seven markers, and one consisting of six markers. Some markers showed an overlap when a multiplex PCR combination was simulated; each of the three panels were therefore divided into two subpanels (Figure 3.3 B and C) to avoid marker profiles with overlapping bands on agarose gel electrophoresis analysis. Two of the subpanels consisted of four markers, while the remaining three subpanels had a combination of three markers each.

A



Size range	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350
pannel1																											
vic	green								satt169	satt169								satt502									
ned	yellow					satt581						satt233	satt233														
pet	red														satt369	satt369	satt369										
6-fam	blue		satt323	satt323	satt323	satt323	satt323	satt323			satt 153	satt 153															

Size range	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350
pannel2																											
vic	green								satt685	satt685	satt685	satt685	satt685														
ned	yellow	satt411															satt269										
pet	red				satt133	satt133	satt133	satt133	satt133	satt133											satt525	satt525					
6-fam	blue							satt145			satt251	satt251	satt251														

Size range	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350
pannel 3																											
vic	green																		satt149								
ned	yellow												satt252	satt252													
pet	red							satt638	satt638														sat_233	sat_233	sat_233	sat_233	
6-fam	blue						satt126						sat_124	sat_124	sat_124	sat_124	sat_124										

B

Size ranges	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350
pannel1.1																											
vic	green								satt169	satt169																	
ned	yellow											satt233	satt233														
pet	red														satt369	satt369	satt369										
6-fam	blue		satt323	satt323	satt323	satt323	satt323	satt323																			

Size ranges	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350
pannel2.1																											
vic	green																										
ned	yellow																satt269										
pet	red				satt133	satt133	satt133	satt133	satt133	satt133											satt525	satt525					
6-fam	blue										satt251	satt251	satt251														

Size ranges	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350
pannel 3.1																											
vic	green																										
ned	yellow																										
pet	red																						sat_233	sat_233	sat_233	sat_233	
6-fam	blue						satt126						sat_124	sat_124	sat_124	sat_124	sat_124										

C

Size ranges		90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350
Dye	**pannel1.2**																											
green	**vic**																		satt502									
yellow	**ned**						satt581																					
red	**pet**																											
blue	**6-fam**											satt 153	satt 153															

Size ranges		90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350
Dye	**pannel2.2**																											
green	**vic**									satt685	satt685	satt685	satt685	satt685														
yellow	**ned**		satt411																									
red	**pet**																											
blue	**6-fam**								satt145																			

Size ranges		90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350
Dye	**pannel3.2**																			satt149								
green	**vic**																											
yellow	**ned**													satt252	satt252													
red	**pet**								satt638	satt638																		
blue	**6-fam**																											

Figure 3.3 A multiplex PCR design showing 20 SSR markers that are divided into three PCR panels (A), six sub-panels (B and C) resulting from a split of three multiplex panels (all markers had an optimal annealing temperature of 58°C) .

3.3. SSR marker polymorphism pre-screening

A polymorphism pre-screening was done (Figure 3.4) using four South African soybean cultivars (SA) that were randomly selected from the study; this was done to ensure that the selected markers can distinguish across South African cultivars. Two cultivars [M.A (MA) and W82 (W)] that have previously been reported as resistant and susceptible to stem rot were also included as a positive and negative control for resistance respectively. All mentioned cultivars were screened by multiplexing either three or four markers in a PCR reaction which was later analysed by agarose gel electrophoresis. A three percent agarose gel showed size polymorphism on twenty SSR markers (Figure 3.4, m1 to m20) that were multiplexed either as four or three individual markers. Of these markers that showed size polymorphism during pre-screening, four markers were from panel 1 (Figure 3.4 A and B), three from panel 2 (Figure 3.4 C and D), while the remaining four were from panel 3 (Figure 3.4 E and F). From the gel image (Figure 3.4) slight differences in the profiles of different cultivars were observed, however this was not enough to make a final distinction of different cultivars, a different method of screening the remaining cultivars was therefore followed.

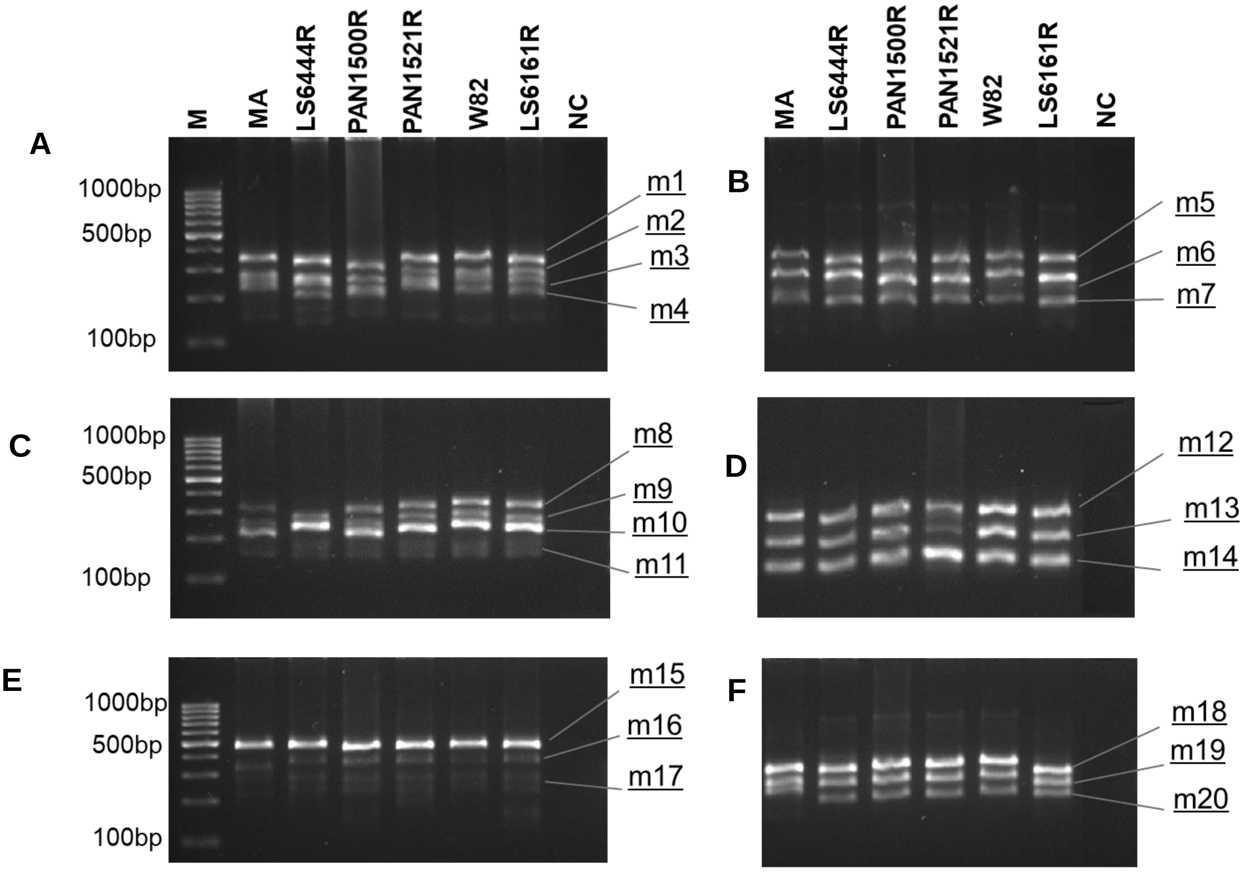


Figure 3.4 A 3% agarose gel analysis showing size polymorphism of 20 SSR markers (m1 to m20) on four South African soybean cultivars (SA) as well as a reference cultivar for stem rot resistance (MA) and a reference cultivar for stem rot susceptibility (W). A molecular size marker (M) and a non-template PCR (NTC) control are also shown. ^{oC}

3.4. **Screening markers on soybean cultivars and genotype data analysis**

3.4.1. Genotype data analysis in genetic analysis on Excel (GenAlEx)

3.4.1.1. Allele diversity per cultivar over all loci

In this study, there were only three samples evaluated per cultivar and any genetic differences that were found between samples of the same cultivar as measured by allele diversity per cultivar may only be used as the first step in estimating parentage (i.e., whether the seedling originates from selfing or from outcrossing). These statistics also provided the first step in understanding whether the cultivar was inbred or not, based on the overall allele diversity. The average observed heterozygosity (Table 3.1) (H_o) was 0.070 which is lower than expected heterozygosity (H_e) (0.083) under Hardy-Weinberg equilibrium which can be attributed to some inbreeding in this sample set. The unbiased estimate of expected heterozygosity (uH_e) was 0.100 which still shows very low heterozygosity or gene diversity in this sample set which represent current commercial South African cultivars. The cultivar with the highest allele diversity over all loci was PAN1500R; this cultivar also had the highest heterozygosity and was possibly the most outbred cultivar compared to the rest, a bigger sample size would however be required to make a conclusion on this. The average percentage polymorphic loci which was calculated from allele frequency was 19.24%; PAN1500R also had the highest percentage polymorphic loci of 52.63%. The three seedlings that were tested for PAN1500R were most likely a result of outcrossing as opposed to selfing. PHB94Y80R, LS6453R, LS6466R, LS6161R and NS7211R had the lowest gene diversity measured by heterozygosity and percentage loci polymorphism; the seedlings representing these cultivars are most likely a result of selfing and clonal.

Table 3.1 Allele diversity evaluated using three samples in each cultivar reflects parentage of the soybean cultivars evaluated in the study.

Cultivar	**Percentage of Polymorphic Loci per cultivar**	**H_e**	**uH_e**	**H_o**
LS 6240 R	36.84%	0.146	0.175	0.035
LS 6444 R	5.26%	0.023	0.028	0.000
PAN 1454 R	31.58%	0.140	0.168	0.000
LS 6146 R	36.84%	0.193	0.239	0.000
PHB 94 Y 80 R	0.00%	0.000	0.000	0.000
LS 6248 R	36.84%	0.164	0.196	0.000
NS 5009 R	5.26%	0.015	0.018	0.018
DM 5.1i RR	21.05%	0.094	0.112	0.000
PHB 95 Y 20 R	10.53%	0.041	0.049	0.035
PAN 1583 R	15.79%	0.053	0.063	0.035
PAN 1664 R	5.26%	0.023	0.028	0.000
DM 5953 RSF	15.79%	0.070	0.084	0.000
LS 6453 R	0.00%	0.000	0.000	0.000
PAN 1521 R	5.26%	0.014	0.018	0.018
PAN 1500 R	52.63%	0.225	0.270	0.018
NS 5909 R	15.79%	0.070	0.084	0.000
PAN 1513 R	15.79%	0.070	0.084	0.000
LS 6466 R	0.00%	0.000	0.000	0.000
PAN 1666 R	26.32%	0.117	0.140	0.000
PAN 1623 R	42.11%	0.178	0.214	0.018
LS 6261 R	31.58%	0.140	0.168	0.000
DM 6.2i RR	15.79%	0.061	0.074	0.088
LS 6164 R	10.53%	0.038	0.046	0.018
LS 6161 R	0.00%	0.000	0.000	0.000
PAN 1614 R	10.53%	0.029	0.035	0.035
NS 6448	42.11%	0.190	0.228	0.018
DM 6.8i RR	36.84%	0.164	0.196	0.000
NS 7211 R	0.00%	0.000	0.000	0.000
PAN 1729 R	31.58%	0.140	0.168	0.000
Average	19.24%	0.083	0.100	0.070

3.4.1.2. *Allele diversity, numbers, patterns, and polymorphism per locus*

One (Sat_124) of 20 markers was removed from further analysis (from here onwards) because of ambiguity during scoring resulting in 19 of 20 final markers. The evaluated SSR markers had 55 alleles in total when evaluated in 29 South African soybean cultivars as well as W82 and M.A. The average number of alleles was 2.87 per locus. The loci containing the highest number of alleles per locus were Satt369 and Satt252, both with an average of 5.0 alleles per locus. The allele sizes from the evaluated markers ranged from 93bp to 360bp when evaluated across South African commercial cultivars (Table 3.2). These allele sizes had previously been reported by Song et al (2010). The marker locus with the highest allele size range was sat_233 with a difference of 118bp. The percentage polymorphic information content was calculated (Table 3.2) to evaluate the usefulness of these markers specifically to South African cultivars; five out of the remaining 19 markers were not polymorphic in South African cultivars. The marker with the highest percentage polymorphic information content was Sat_323, when PIC was calculated using allele frequencies of South African soybean cultivars (Appendix, Table 5.1).

Allele diversity per locus over all cultivars (Table 3.3) was measured by heterozygosity (Appendix, Table 5.2) as well as Wright's F-statistics. Satt323 had the highest allele diversity per locus (0.202) while Satt133, Satt411, Satt685, Satt126, and Satt638 had the lowest allele diversity over all South African cultivars in this study. Satt323 however, also had a positive inbreeding coefficient (Fis) which may indicate the allele relationships on Satt323 locus, for this specific sample set of soybean genotypes. The overall genetic diversity and gene flow was determined using the mean heterozygosity and F-statistics. The gene flow estimate (Nm) in this case could be used to estimate the exchange of genetic material across cultivars and not the exchange of genetic material between soybean populations. The mean gene diversity over all cultivars was 0.079, the inbreeding coefficient as well as estimate of gene flow over all cultivars was 0.567 and 0.077. Finally, the Fixation index which also measures heterozygosity and genetic diversity was 0.791. All of the above statistics were calculated to predict if any of the evaluated cultivars would be a good candidate to include in a base population for Sclerotinia stem rot resistance breeding (provided that some of the cultivars show any form of resistance after several screening tests).

Table 3.2 Average allele sizes range, numbers, and percentage polymorphism on stem rot resistance SSR markers determined from average allele frequency from selected South African commercial soybean cultivars.

Marker locus	**Size range (bp)**	**Number of alleles per locus**	**Percentage polymorphism (%)**
Satt323	145-170	4	59
Satt502	251-260	2	38
Satt233	186-207	4	50
Satt369	221-251	5	53
Satt581	132-146	4	44
Satt153	188-209	3	51
Satt169	185-224	4	37
Satt251	204-211	3	43
Satt525	302-304	2	8
Satt411	93-96	2	0
Satt269	251-258	2	10
Satt133	181-190	2	0
Satt145	142-146	3	47
Satt685	213	1	0
Satt126	149	1	0
Satt149	251-275	3	46
Satt638	176	1	0
Sat_233	242-360	4	70
Satt252	207-224	5	64

*Only 19 markers that were further analysed after the difficulty in scoring one marker are included in this table. All the non-polymorphic markers were discovered after further screening with capillary electrophoresis and fragment length analysis because of the lack of exact size length polymorphism.

Table 3.3 Allele diversity per locus, over 29 South African commercial soybean cultivars.

Locus	**Ho**	**He**	**UHe**	**Fis**	**Fit**	**Fst**	**Nm**
Satt323	0.022	0,168	0,202	0,872	0,963	0,712	0,101
Satt502	0,011	0,081	0,097	0,867	0,973	0,797	0,064
Satt233	0,032	0,134	0,161	0,760	0,934	0,723	0,096
Satt369	0,032	0,167	0,200	0,806	0,940	0,688	0,113
Satt581	0,000	0,072	0,086	1,000	1,000	0,859	0,041
Satt153	0,000	0,145	0,176	1,000	1,000	0,715	0,100
Satt169	0,032	0,124	0,148	0,739	0,916	0,679	0,118
Satt251	0,022	0,090	0,108	0,760	0,951	0,794	0,065
Satt525	0,000	0,043	0,052	1,000	1,000	0,577	0,183
Satt411	0,000	0,000	0,000	#N/A	1,000	1,000	0,000
Satt269	0,022	0,032	0,039	0,333	0,846	0,768	0,075
Satt133	0,032	0,016	0,019	-1,000	-0,016	0,492	0,258
Satt145	0,000	0,115	0,138	1,000	1,000	0,745	0,086
Satt685	0,000	0,000	0,000	#N/A	1,000	1,000	0,000
Satt126	0,000	0,000	0,000	#N/A	1,000	1,000	0,000
Satt149	0,011	0,082	0,101	0,870	0,977	0,822	0,054
Satt638	0,000	0,000	0,000	#N/A	1,000	1,000	0,000
Sat_233	0,000	0,100	0,120	1,000	1,000	0,839	0,048
Satt252	0,032	0,127	0,153	0,746	0,951	0,808	0,059
Mean	0.013	0.079	0.095	0.567	0.918	0.791	0.077

* Ho= observed heterozygosity, He= expected heterozygosity under hardy Weinberg equilibrium, uHe= unbiased expected heterozygosity, Fis= inbreeding coefficient, Fit=overall inbreeding coefficient in this sample relative to total soybean population, Fst= fixation index, Nm= gene flow estimation.

3.4.1.3. Genetic relatedness of W82, M.A with South African cultivars, and genetic relatedness across South African cultivars

The genetic relationships between M.A or W82 and South African cultivars were analysed to get a better understanding of whether stem rot resistance markers can separate or group the cultivars into different groups. Nei pairwise genetic distance (Appendix, Table 5.3) and genetic identity were the first parameters evaluated to understand genetic relatedness among cultivars, and these were calculated as explained in Nei (1972). Pair-wise Nei genetic distance ranged between 0.027 and 1.189. The highest genetic distance and the lowest genetic identity was between W82 and M.A, while the lowest genetic distance and highest genetic identity was between cultivar NS6448 and LS6248R. The Shannon index (Appendix, Table 5.4) showed the pair-wise differentiation across cultivars and this was also compared between W82, M.A and South African cultivars. The lowest genetic differentiation was 0.037 between NS6448 and LS6248 while the highest genetic differentiation was 0.682 between W82 and M.A. The genetic distances between South African cultivars and W82 were compared with the genetic distances between South African cultivars and M.A (Table 3.4). Based on the pair-wise Nei genetic distance comparison, cultivars were divided into two groups; one group representing South African cultivars that genetically closer to W82 than M.A and the second group representing cultivars that are closer to M.A than W82. There were more South African cultivars that group closer to W82 than M.A, and only ten cultivars that are closer to M.A than W82. Using the groupings from genetic distances, a principal coordinate analysis (PCoA) (Figure 3.5) was constructed to visualise how cultivars would cluster in a two-dimensional space. PCoA showed two main groups, Samples representing South African cultivars that clustered towards the direction of M.A (Figure 3.5 green points) as well as South African cultivars that are clustered more towards W82 (Figure 3.5 red points). Ten South African cultivars that were clustered towards M.A than W82 had also been classified as Sclerotinia stem rot tolerant cultivars in a detached leaf assay experiment (van der Hoven, Unpublished).

Table 3.4 Average pair-wise Nei's genetic distance between W82, M.A and South African cultivars.

Cultivar	Average genetic distance between W82 and South African cultivars (NeiP)	Average genetic distance between M A and South African cultivars (NeiP)
Will 82	0.000	1.189
LS 6453 R	0.111	0.887
LS 6240 R	0.226	0.576
PAN 1623 R	0.244	0.545
PAN 1521 R	0.286	0.738
NS 6448	0.287	0.549
LS 6444 R	0.328	0.576
PAN 1500 R	0.318	0.413
NS 5909 R	0.343	0.430
LS 6248 R	0.370	0.565
NS 5009 R	0.372	0.630
PAN 1614 R	0.378	0.566
PHB 94 Y 80 R	0.379	0.655
DM 5953 RSF	0.396	0.459
PAN 1513 R	0.423	0.767
PAN 1729 R	0.441	0.768
DM 6.2i RR	0.442	0.556
NS 7211 R	0.460	0.588
LS 6146 R*	0.470	**0.389**
PAN 1454 R	0.471	0.481
LS 6164 R*	0.512	**0.329**
LS 6466 R*	0.547	**0.385**
DM 6.8i RR	0.552	0.637
DM 5.1i RR*	0.560	**0.371**
LS 6261 R*	0.566	**0.309**
PAN 1666 R*	0.580	**0.559**
PHB 95 Y 20 R*	0.588	**0.475**
PAN 1664 R*	0.597	**0.484**
LS 6161 R*	0.642	**0.556**
PAN 1583 R*	0.684	**0.454**
M.A	1.189	0.000

* the asterisk indicates cultivars that are more related to MA than W82 due to shared Sclerotinia stem rot tolerance alleles. The genetic distances that indicate shared tolerance alleles between MA and South African cultivars are written in bold.

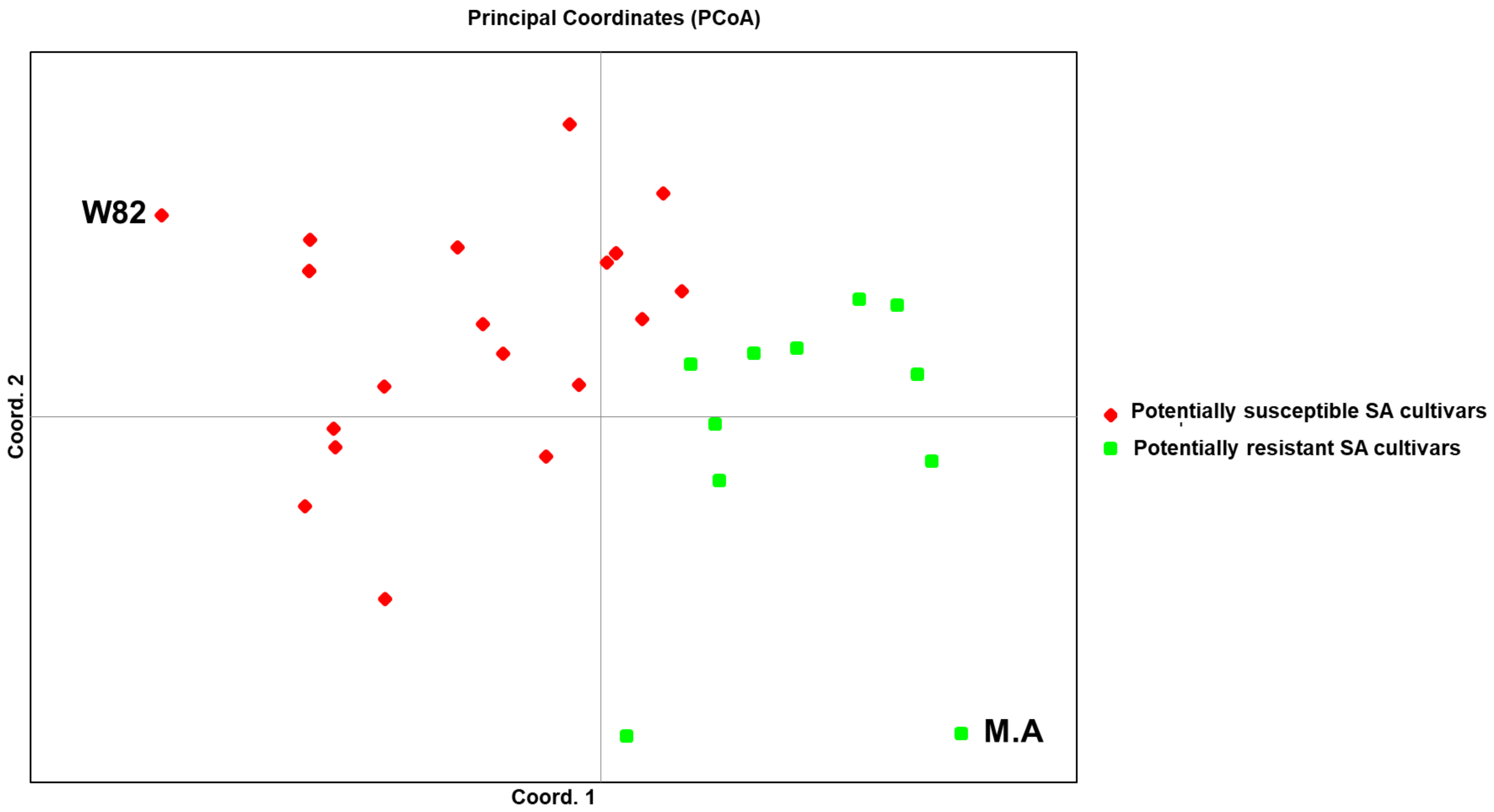


Figure 3.5 A scatter plot of principal coordinate analysis (PCoA) showing South African cultivars that cluster towards W82 (red points) and those that cluster towards M.A (green points) The first and the second axes explained 21.75% and 16.04% variation respectively. This figure is based on the average genetic distance of three samples representing each cultivar.

4. DISCUSSION

In this study 20 SSR markers that are associated with soybean resistance to *Sclerotinia sclerotiorum* were selected using literature and SoyBase (www.soybase.org) database. Twelve of 20 markers had previously been reported as Sclerotinia stem rot resistance markers (Guo et al., 2008; Bastien et al., 2014; Vuong et al., 2008; Li et al., 2010; Zhao et al., 2015), while the remaining markers were linked to either of eleven major and two minor quantitative trait loci that have been reported to have an association with soybean resistance to Sclerotinia stem rot. One of 20 markers showed ambiguous results during allele scoring on gene Mapper and therefore, only 19 of 20 markers were characterised further on the rest of South African soybean cultivars.

All the markers that were used in this study had previously been reported as polymorphic markers being able to distinguish across soybean cultivars or genotypes (Cregan et al., 1999; Song et al., 2004; Song et al., 2010); however, it is important to understand that while most of the markers in the BARCSOYSSR database (https://soybase.org/BARCSOYSSR/index.php) may have been reported as polymorphic, there is also a possibility that this polymorphism may not apply on all soybean genotypes and the reasons for this deviation might be due to lack of diversity in a population.

A pre-polymorphism screening study was done to confirm polymorphism of the selected markers across four randomly selected South African cultivars as well as W82 and M.A. W82 has previously been reported as a completely susceptible cultivar while M.A was previously reported as a partially resistant cultivar (Li et al., 2010); using this knowledge, M.A and W82 cultivars were therefore used as references for resistance and susceptibility to Sclerotinia stem rot respectively. A 3% agarose gel showed that some of the markers that distinguish W82 from M.A are not polymorphic across South African commercial soybean cultivars; however the resolution of this was too low and therefore all South African cultivars were screened further by fragment length analysis using a Gene scan™ Liz 500™ size standard.

Seven markers had the lowest polymorphic information content and allele diversity; five of these, namely, Satt411, Satt133, Satt685, Satt126, and Satt638 were not polymorphic across South African cultivars at all. Satt411 however, was polymorphic between W82 and M.A. The lack of polymorphism of Satt411 and the other four markers in South African cultivars, gives an insight that these markers might not be useful as stem rot resistance markers for South African cultivars. Lesion length difference across soybean cultivars that are infected with *S. sclerotiorum* has been used in many studies to measure soybean resistance to the pathogen; while satt411 is linked to this trait it is important to consider that Satt411 might also be associated with other traits that are specific in South African cultivars and are not necessarily linked to soybean resistance to Sclerotinia stem rot. A study by Holla et al. (2014) showed that markers that appear monomorphic on a given set of genotypes were more linked to or were found in regions of functional genes that control important characteristics; this restricts any change that could otherwise make a given marker polymorphic while disrupting the functionality of the gene region involved. The markers that were not polymorphic in South African cultivars might therefore be involved in a functional trait that is only conserved in South African cultivars.

Allele diversity per cultivar was estimated using three samples per cultivar; because of the number of samples per each cultivar, allele diversity of each cultivar only gives us an insight on the parentage of the cultivars used in this study. All the seeds from this study were harvested from the same plant for each cultivar and therefore any allele difference between samples of the same cultivar might reflect either the heterozygosity of a parent, in cases where the loci only has two alleles, or a result of bi-parental cross in cases where there are more than two alleles per locus. A few cultivars contained rare alleles in eight loci, however these alleles might not necessarily be related to stem rot resistance.

The overall gene diversity on all marker loci was high with an average allele number of five alleles per locus. Fourteen (Table 4.1) markers were the most polymorphic across South African cultivars. Of the 14 polymorphic markers, 12 have been previously reported to either be associated with soluble stem pigments (Li et al., 2010; Zhao et al., 2015) or lesion length (Guo et al., 2008; Vuong et al., 2008; Bastien et al., 2014), while the remaining reported for the first time from QTL regions involved in Sclerotinia stem rot resistance in this study. The ability of these markers in distinguishing resistant cultivars from susceptible ones was only based on their linkage to QTL that are involved in soybean resistance to Sclerotinia stem rot, and because these are reported for the first time here, it is crucial to further study the association of these markers to soybean resistance by phenotyping the response of cultivars to infection. These markers also gave an insight on which South African cultivars might have partial genetic based resistance to Sclerotinia stem rot as evaluated using the genetic relationships between W82, M.A, and South African cultivars. Heterozygosity measurements per locus showed that three loci that had the highest percentage polymorphism also had the highest allele diversity in South African cultivars.

The mean allele diversity over all cultivars was closer to zero while both the inbreeding coefficient and fixation index, calculated over all cultivars indicated a deficiency in heterozygotes in these cultivars. This might not be directly related to the resistance status of these cultivars but gives us an insight on the genetic structure of this specific group of South African cultivars. The observed heterozygosity is also lower than what would be expected under Hardy Weinberg equilibrium. The low genetic diversity of these cultivars might be due to inbreeding and this has important implications on the use of these cultivars as sources of resistance in breeding programs against Sclerotinia stem rot; if these cultivars are the results or excessive inbreeding, there will not be enough diversity to use them as the founding population in a breeding program. Most of these cultivars have also been subjected to artificial selection of other agronomic traits which might be the reason for the reduced genetic diversity.

The overall fixation index is very close to one, which further implies the lack of genetic diversity across the cultivars that are currently used commercially; this has important implications regarding the use of these cultivars as sources of resistance in soybean breeding. The challenge of reduced genetic diversity in domestic soybean genotypes is not a problem only in South Africa but a world-wide challenge for breeders. Genetic diversity studies have often found that generally wild soybeans have more diversity than cultivated soybeans (Kuroda et al., 2009; Hwang et al., 2008; Zhang et al., 2016). The improvement of cultivated crop varieties using their wild relatives has been explored in rice (Marjee et al., 2004), wheat (Peleg et al., 2009), and soybean (Ji et al., 2010; Kim et al., 2009) and this option might be worth exploring for soybean resistance to Sclerotinia stem rot. Low heterozygosity in this study also implies that any separation that was found is due to population structure.

In this case the separation might be related to some cultivars containing Sclerotinia stem rot resistance alleles while others contain susceptible alleles.

Using genetic relationships, the relatedness between South African cultivars and W82 (a stem rot susceptible cultivar) as well as between South African cultivars and M.A (a stem rot resistant cultivar), we could estimate which South African cultivars have shared alleles with either M.A or W82. The comparison of genetic distances between W82, M.A and South African cultivars revealed that 10 South African cultivars are more related to M.A than to W82. We propose that due to the shared alleles which might be stem rot resistance alleles, South African cultivars that are closely related to M.A than to W82 have some genetic based resistance. Other traits that are believed to be related to stem rot resistance on soybean include stem lignin content (Peltier et al., 2009) as well as short time to maturity, which is also described as “escape” mechanism (Kim et al., 2009).

M.A is one of the cultivars with the shortest time to maturity which could act as an “escape” mechanism of resistance; however, the results obtained from this study would indicate that even though M.A uses the “escape” strategy, there are also other traits including soluble stem pigment content as well as lesion length, which the cultivar uses as resistance strategies. Another point to consider is that all South African Cultivars that shared more alleles with M.A did not have a short time to maturity, suggesting that the resistance traits shared between these cultivars and M.A are not linked to the “escape” mechanism of resistance.

It is tempting to also propose that cultivars that share more alleles with W82 are completely susceptible; however, it is crucial to consider that perhaps these cultivars harbour other traits of resistance besides those that were evaluated in this study. More cultivars expressing other Sclerotinia stem rot resistance traits strongly, should therefore be considered as reference cultivars in future studies where the resistance status of the cultivars in question is unknown. Using markers that are linked to resistance traits other than those that were studied here would also provide more information on those South African cultivars sharing more alleles with W82 than M.A. The comparison between the detached leaf assay results (Article in preparation) showed that all the cultivars that are closely related to M.A are also among cultivars that respond better to *in vitro* infection than some of the South African cultivars that are closely related to W82 which further emphasises that, these cultivars could have some level of genetic resistance to Sclerotinia stem rot.

Considering the results of this study, Satt638, Satt133, Satt411, Satt685 and Satt323 would not be recommended for use as an indirect approach to screen Sclerotinia stem rot resistance in soybean cultivars that are currently commercially grown in South Africa, due to their low genetic diversity in these cultivars. Markers that were polymorphic in the South African cultivars include Satt502, Satt233, Satt525, Satt251, Satt369, Satt269, Satt153, Satt581, Satt149, Satt323, Satt252, Satt169, Satt145, and Sat_233; these markers are good candidate markers to use as a first step (before field and greenhouse assessments) to screen for soybean resistance to Sclerotinia stem rot specifically in South African soybean cultivars. Satt233 and Satt525, however, had low polymorphic information content and could not distinguish W82 from M.A.

The marker-based approach is beneficial because it is not subject to environmental conditions and will therefore produce unbiased results. Based on the results obtained from shared resistance alleles between South African cultivars and M.A , we estimated which South African cultivars potentially have genetic resistance to Sclerotinia stem rot. This is the first study to explore the possible genetic resistance to Sclerotinia stem rot in cultivars LS6146R, LS6261R, LS6164R, LS6161R, DM5.1IRR, PHB95Y20R, PAN1583R, PAN1664R, PAN1666R and LS6466R which are grown in South Africa; providing one of the important steps towards South African soybean breeding for Sclerotinia stem rot resistance. These cultivars were also found to form reduced lesions after infection by *S. sclerotiorum* in a detached leaf assay test (van der Hoven, Unpublished)

The genetic diversity of stem rot resistance SSR markers data that was generated from this study can further be applied as a first step in screening more South African cultivars for Sclerotinia stem rot resistance. The results of marker-assisted screening can be used together with results from greenhouse assays as well as lto corroborate field trials.

This is the first study providing information on which markers (among those that have been reported as stem rot resistance markers) are applicable for soybean selection, with concerns to Sclerotinia stem rot resistance traits in South African cultivars that are currently grown commercially. The study also revealed four additional SSR markers that have not been reported as stem rot resistance markers before. The data on genetic resistance to stem rot in South African cultivars provides information that can be used to test for environmental conditions which would allow optimal expression of Sclerotinia stem rot resistance traits. The cultivars harbouring genetic resistant traits as found in this study, can be planted across various environments or localities to determine the optimum genetic-environmental interaction which gives a cultivar its resistance to Sclerotinia stem rot.

In summary, the study screened 20 SSR markers that are linked to QTL associated with Sclerotinia stem rot resistance on soybean; however, due to difficulty of scoring one of the markers, only 19 markers were analysed further in the study. Further studies that can be done should involve generating the first and second generation of soybeans resulting from a cross between a resistant and a susceptible cultivar. The inheritance of the given marker loci among progeny will provide information on which loci contain resistance alleles which can then facilitate mapping of more QTL that are involved in stem rot resistance of soybean. Even though the twelve markers had been reported before, none of them had previously been reported in South African soybean cultivars. This provided an opportunity to assess the markers in cultivars that are currently grown commercially in South Africa. Using polymorphic markers to evaluate the genetic relationships between South African cultivars and a cultivar with known resistance provided an insight on which South African cultivars contain SSR alleles that are associated with soybean resistance to Sclerotinia stem rot.

The overall aim and objectives of the study were achieved, with some insight on the possible genetic basis of resistance in commercial South African soybean cultivars. Given the number of studies that have been published on finding quantitative trait loci involved in soybean resistance to Sclerotinia stem rot, there is still a great need for understanding the genetic basis of soybean resistance to stem rot. The anthocyanin pathways which is related to soluble stem pigments content trait as proposed by Li et al. (2010) should be explored further especially at the genetic level. Perhaps a holistic step like the metabolomics approach to assess the differential accumulation of metabolites during infection between susceptible and resistant cultivars would be a good starting point towards finding more QTL and biological markers that can be used to study soybean resistance to Sclerotinia stem rot.

5. APPENDIX

Table 5.1 Allele frequency of 19 SSR marker loci that were screened across 29 commercial South African soybeans.

Locus/Marker name	Allele ID	Allele frequency
Satt323	145	0.126
	158	0.270
	161	0.034
	170	0.569
Satt502	251	0.741
	260	0.259
Satt233	186	0.034
	192	0.034
	198	0.638
	207	0.293
Satt369	221	0.006
	224	0.011
	245	0.149
	248	0.190
	251	0.644
Satt581	132	0.092
	138	0.011
	142	0.667
Satt269	251	0.948
	258	0.052
	146	0.230
Satt153	188	0.011
	194	0.523
	209	0.466
Satt169	185	0.218
	188	0.764
	221	0.006
	224	0.011
Satt251	204	0.701
	207	0.011
	211	0.287
Satt525	302	0.057
	304	0.943
Satt411	93	0
	96	1
Satt133	181	1
	190	0
Satt145	142	0.023
	143	0.287
	146	0.690

Table 5.2 Allele frequency of 19 SSR marker loci that were screened across 29 commercial South African soybeans (continues from page 67).

Locus/Marker name	Allele name	Allele frequency
Satt685	213	1
Satt126	149	1
Satt149	251	0.287
	261	0.034
	275	0.678
Satt638	176	1
Sat_233	342	0.276
	344	0.517
	348	0.161
	360	0.046
Satt252	207	0.046
	210	0.454
	218	0.011
	221	0.371
	224	0.126

*Markers with an allele frequency of 0 on some alleles contain alleles that were present on Williams 82 and Maple arrow but were not present in South African cultivars. Some of these markers were monomorphic in South African cultivars but could distinguish W82 from M.A.

Table 5.3 Allele diversity per locus in each cultivar as well as the average allele diversity for each locus over all cultivars.

Cultivar	Satt323	Satt502	Satt233	Satt369	Satt581	Satt153	Satt169	Satt251	Satt525	Satt411	Satt269	Satt133	Satt145	Satt685	Satt126	Satt149	Satt638	Sat_233	Satt252
LS 6240 R	0,533	0,000	0,533	0,533	0,000	0,533	0,000	0,333	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,333	0,000	0,533	0,000
LS 6444 R	0,000	0,000	0,444	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
PAN 1454 R	0,533	0,000	0,533	0,533	0,000	0,000	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,533	0,000	0,000	0,533
LS 6146 R	0,800	0,000	0,533	0,000	0,000	0,667	0,000	0,533	0,000	0,000	0,000	0,000	0,533	0,000	0,000	0,667	0,000	0,000	0,800
PHB 94 Y 80 R	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
LS 6248 R	0,533	0,533	0,533	0,533	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,533	0,000	0,000	0,000	0,000	0,533	0,533
NS 5009 R	0,333	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
DM 5.1i RR	0,000	0,000	0,000	0,000	0,533	0,533	0,000	0,000	0,000	0,000	0,533	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,000
PHB 95 Y 20 R	0,000	0,000	0,600	0,000	0,000	0,000	0,000	0,333	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
PAN 1583 R	0,333	0,000	0,333	0,000	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
PAN 1664 R	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,533	0,000	0,000	0,000
DM 5953 RSF	0,000	0,000	0,000	0,533	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,000
LS 6453 R	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
PAN 1521 R	0,000	0,000	0,333	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
PAN 1500 R	0,000	0,333	0,533	0,533	0,000	0,533	0,533	0,533	0,000	0,000	0,000	0,000	0,533	0,000	0,000	0,533	0,000	0,533	0,533
NS 5909 R	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,533	0,000	0,000	0,000	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,000
PAN 1513 R	0,000	0,000	0,000	0,000	0,000	0,533	0,533	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
LS 6466 R	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
PAN 1666 R	0,533	0,000	0,000	0,533	0,533	0,533	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,533	0,000
PAN 1623 R	0,533	0,533	0,000	0,533	0,000	0,000	0,533	0,533	0,000	0,000	0,333	0,000	0,533	0,000	0,000	0,000	0,000	0,533	0,000
LS 6261 R	0,000	0,533	0,000	0,533	0,533	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,533	0,000	0,000	0,533
DM 6.2i RR	0,000	0,000	0,000	0,533	0,000	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,333
LS 6164 R	0,533	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,333
LS 6161 R	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
PAN 1614 R	0,000	0,000	0,000	0,333	0,000	0,000	0,333	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
NS 6448	0,533	0,533	0,533	0,533	0,000	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,533	0,000	0,000	0,000	0,000	0,533	0,600

DM 6.8i RR	0,533	0,000	0,000	0,533	0,533	0,533	0,533	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,533
NS 7211 R	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
PAN 1729 R	0,533	0,000	0,000	0,000	0,533	0,533	0,533	0,000	0,533	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,533	0,000
M.A	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,333	0,600	0,000	0,000	0,000	0,000	0,000	0,000	0,000
W 82	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Mean	0,202	0,097	0,161	0,200	0,086	0,176	0,148	0,108	0,052	0,000	0,039	0,019	0,138	0,000	0,000	0,101	0,000	0,120	0,153
Fixation	0,800	0,800	0,693	0,775	1,000	1,000	0,700	0,657	1,000	N/A	0,200	1,000	1,000	N/A	N/A	0,800	N/A	1,000	0,659

Table 5.4 Pairwise Nei genetic distance matrix across South African cultivars, W82 and M.A.

Cultivar	LS 6240 R	LS 6444 R	PAN 1454 R	LS 6146 R	PHB 94 Y 80 R	LS 6248 R	NS 5009 R	DM 5.1i RR	PHB 95 Y 20 R	PAN 1583 R	PAN 1664 R	DM 5953 RSF	LS 6453 R	PAN 1521 R	PAN 1500 R	NS 5909 R	PAN 1513 R	LS 6466 R	PAN 1666 R	PAN 1623 R	LS 6261 R	DM 6.2i RR	LS 6164 R	LS 6161 R	PAN 1614 R	NS 6448	DM 6.8i RR	NS 7211 R	PAN 1729 R	M.Arrow	Will 82	
LS 6240 R	0,000																															LS 6240 R
LS 6444 R	0,131	0,000																														LS 6444 R
PAN 1454 R	0,175	0,327	0,000																													PAN 1454 R
LS 6146 R	0,131	0,110	0,205	0,000																												LS 6146 R
PHB 94 Y 80 R	0,157	0,318	0,183	0,198	0,000																											PHB 94 Y 80 R
LS 6248 R	0,203	0,173	0,332	0,158	0,343	0,000																										LS 6248 R
NS 5009 R	0,369	0,469	0,381	0,432	0,554	0,534	0,000																									NS 5009 R
DM 5.1i RR	0,247	0,486	0,307	0,294	0,305	0,428	0,475	0,000																								DM 5.1i RR
PHB 95 Y 20 R	0,201	0,297	0,193	0,162	0,411	0,349	0,313	0,335	0,000																							PHB 95 Y 20 R
PAN 1583 R	0,335	0,228	0,145	0,128	0,302	0,298	0,515	0,538	0,234	0,000																						PAN 1583 R
PAN 1664 R	0,272	0,169	0,226	0,074	0,318	0,228	0,590	0,486	0,273	0,072	0,000																					PAN 1664 R
DM 5953 RSF	0,143	0,234	0,154	0,178	0,222	0,141	0,518	0,244	0,272	0,312	0,234	0,000																				DM 5953 RSF
LS 6453 R	0,250	0,181	0,412	0,352	0,379	0,316	0,372	0,560	0,556	0,490	0,420	0,293	0,000																			LS 6453 R
PAN 1521 R	0,082	0,331	0,218	0,269	0,359	0,287	0,502	0,310	0,254	0,477	0,348	0,204	0,438	0,000																		PAN 1521 R
PAN 1500 R	0,190	0,236	0,270	0,158	0,265	0,125	0,325	0,325	0,330	0,278	0,236	0,145	0,239	0,306	0,000																	PAN 1500 R
NS 5909 R	0,106	0,103	0,357	0,154	0,318	0,148	0,298	0,311	0,256	0,382	0,331	0,179	0,245	0,298	0,114	0,000																NS 5909 R
PAN 1513 R	0,392	0,594	0,530	0,465	0,480	0,280	0,375	0,421	0,489	0,715	0,594	0,315	0,510	0,430	0,255	0,333	0,000															PAN 1513 R
LS 6466 R	0,180	0,181	0,254	0,107	0,379	0,240	0,438	0,330	0,110	0,290	0,203	0,135	0,379	0,286	0,214	0,157	0,451	0,000														LS 6466 R
PAN 1666 R	0,282	0,305	0,294	0,210	0,426	0,056	0,534	0,466	0,349	0,290	0,255	0,183	0,547	0,297	0,203	0,256	0,298	0,317	0,000													PAN 1666 R
PAN 1623 R	0,181	0,160	0,348	0,161	0,219	0,098	0,336	0,336	0,318	0,316	0,257	0,159	0,244	0,354	0,137	0,120	0,215	0,244	0,190	0,000												PAN 1623 R
LS 6261 R	0,296	0,318	0,186	0,197	0,384	0,313	0,391	0,422	0,185	0,191	0,226	0,201	0,441	0,391	0,189	0,276	0,385	0,139	0,322	0,300	0,000											LS 6261 R
DM 6.2i	0,382	0,375	0,311	0,325	0,644	0,405	0,220	0,561	0,173	0,360	0,416	0,329	0,310	0,478	0,255	0,299	0,458	0,172	0,483	0,372	0,148	0,000										DM 6.2i

RR																																RR
LS 6164 R	0,187	0,151	0,153	0,175	0,440	0,305	0,283	0,391	0,101	0,183	0,228	0,203	0,347	0,303	0,276	0,170	0,553	0,102	0,342	0,292	0,138	0,145	0,000									LS 6164 R
LS 6161 R	0,409	0,420	0,206	0,298	0,379	0,398	0,858	0,560	0,467	0,187	0,181	0,318	0,547	0,385	0,404	0,639	0,639	0,460	0,343	0,495	0,230	0,610	0,413	0,000								LS 6161 R
PAN 1614 R	0,157	0,177	0,306	0,195	0,303	0,221	0,603	0,468	0,383	0,312	0,315	0,246	0,365	0,295	0,163	0,152	0,569	0,233	0,324	0,266	0,223	0,399	0,248	0,459	0,000							PAN 1614 R
NS 6448	0,166	0,123	0,341	0,146	0,368	0,027	0,409	0,444	0,333	0,295	0,225	0,160	0,236	0,258	0,097	0,098	0,313	0,224	0,080	0,094	0,288	0,334	0,257	0,426	0,166	0,000						NS 6448
DM 6.8i RR	0,348	0,278	0,323	0,254	0,316	0,348	0,768	0,609	0,515	0,238	0,228	0,343	0,398	0,512	0,335	0,421	0,597	0,398	0,415	0,364	0,323	0,584	0,393	0,265	0,310	0,337	0,000					DM 6.8i RR
NS 7211 R	0,498	0,476	0,635	0,470	0,547	0,240	0,438	0,528	0,655	0,666	0,597	0,318	0,305	0,722	0,214	0,293	0,269	0,460	0,343	0,195	0,471	0,400	0,558	0,865	0,532	0,236	0,621	0,000				NS 7211 R
PAN 1729 R	0,324	0,292	0,395	0,384	0,600	0,362	0,559	0,699	0,465	0,459	0,400	0,385	0,357	0,377	0,467	0,405	0,486	0,384	0,369	0,430	0,318	0,444	0,315	0,330	0,402	0,311	0,276	0,600	0,000			PAN 1729 R
M.A	0,576	0,576	0,481	0,389	0,655	0,565	0,630	0,371	0,475	0,454	0,484	0,459	0,887	0,738	0,413	0,430	0,767	0,385	0,559	0,545	0,309	0,556	0,392	0,556	0,466	0,549	0,637	0,588	0,768	0,000		M.Arrow
Wi82	0,226	0,318	0,471	0,470	0,379	0,370	0,372	0,560	0,588	0,684	0,597	0,396	0,111	0,286	0,318	0,343	0,423	0,547	0,580	0,244	0,566	0,442	0,512	0,642	0,378	0,287	0,552	0,460	0,441	1,189	0,000	Will 82

Table 5.5 Pairwise cultivar Matrix of Mean Shannon (sHua) Values Over Loci (Using Log Base = 2).

Cultivar	LS 6240 R	LS 6444 R	PAN 1454 R	LS 6146 R	PHB 94 Y 80 R	LS 6248 R	NS 5009 R	DM 5.1i RR	PHB 95 Y 20 R	PAN 1583 R	PAN 1664 R	DM 5953 RSF	LS 6453 R	PAN 1521 R	PAN 1500 R	NS 5909 R	PAN 1513 R	LS 6466 R	PAN 1666 R	PAN 1623 R	LS 6261 R	DM 6.2i RR	LS 6164 R	LS 6161 R	PAN 1614 R	NS 6448	DM 6.8i RR	NS 7211 R	PAN 1729 R	M.Arrow	Will 82	
LS 6240 R	0,000																															LS 6240 R
LS 6444 R	0,136	0,000																														LS 6444 R
PAN 1454 R	0,136	0,279	0,000																													PAN 1454 R
LS 6146 R	0,128	0,135	0,187	0,000																												LS 6146 R
PHB 94 Y 80 R	0,156	0,273	0,180	0,193	0,000																											PHB 94 Y 80 R
LS 6248 R	0,197	0,164	0,281	0,170	0,303	0,000																										LS 6248 R
NS 5009 R	0,299	0,378	0,305	0,359	0,426	0,407	0,000																									NS 5009 R
DM 5.1i RR	0,251	0,399	0,299	0,278	0,283	0,369	0,380	0,000																								DM 5.1i RR
PHB 95 Y 20 R	0,179	0,246	0,175	0,156	0,337	0,286	0,266	0,304	0,000																							PHB 95 Y 20 R
PAN 1583 R	0,264	0,197	0,150	0,130	0,251	0,250	0,396	0,430	0,207	0,000																						PAN 1583 R
PAN 1664 R	0,233	0,154	0,212	0,096	0,273	0,204	0,445	0,399	0,233	0,082	0,000																					PAN 1664 R
DM 5953 RSF	0,136	0,212	0,151	0,181	0,202	0,140	0,395	0,234	0,239	0,265	0,212	0,000																				DM 5953 RSF
LS 6453 R	0,237	0,168	0,342	0,313	0,316	0,279	0,316	0,441	0,419	0,379	0,340	0,255	0,000																			LS 6453 R
PAN 1521 R	0,088	0,273	0,184	0,252	0,298	0,266	0,385	0,275	0,220	0,378	0,292	0,184	0,350	0,000																		PAN 1521 R
PAN 1500 R	0,154	0,210	0,211	0,170	0,248	0,129	0,281	0,313	0,280	0,239	0,210	0,132	0,218	0,252	0,000																	PAN 1500 R
NS 5909 R	0,114	0,107	0,285	0,173	0,269	0,145	0,251	0,282	0,219	0,313	0,279	0,164	0,216	0,251	0,126	0,000																NS 5909 R
PAN 1513 R	0,331	0,437	0,400	0,359	0,374	0,252	0,303	0,345	0,381	0,490	0,437	0,269	0,388	0,342	0,245	0,275	0,000															PAN 1513 R
LS 6466 R	0,170	0,168	0,236	0,126	0,316	0,222	0,350	0,293	0,103	0,255	0,182	0,135	0,316	0,245	0,210	0,150	0,360	0,000														LS 6466 R
PAN 1666 R	0,242	0,279	0,255	0,213	0,360	0,095	0,399	0,392	0,291	0,256	0,240	0,182	0,441	0,270	0,200	0,242	0,251	0,279	0,000													PAN 1666 R
PAN 1623 R	0,185	0,166	0,290	0,171	0,227	0,086	0,292	0,304	0,269	0,271	0,233	0,154	0,227	0,300	0,139	0,116	0,198	0,223	0,193	0,000												PAN 1623 R
LS 6261 R	0,267	0,289	0,187	0,194	0,328	0,275	0,313	0,380	0,168	0,186	0,212	0,208	0,360	0,342	0,187	0,250	0,313	0,145	0,281	0,255	0,000											LS 6261 R
DM 6.2i	0,317	0,318	0,284	0,296	0,484	0,346	0,194	0,451	0,171	0,318	0,350	0,301	0,265	0,385	0,246	0,261	0,378	0,173	0,398	0,315	0,156	0,000										DM 6.2i

RR																																RR
LS 6164 R	0,182	0,144	0,162	0,182	0,368	0,265	0,252	0,346	0,095	0,176	0,206	0,198	0,292	0,260	0,246	0,164	0,423	0,097	0,301	0,255	0,143	0,144	0,000									LS 6164 R
LS 6161 R	0,338	0,340	0,212	0,270	0,316	0,342	0,579	0,441	0,367	0,177	0,168	0,283	0,421	0,320	0,329	0,465	0,465	0,368	0,311	0,399	0,218	0,474	0,340	0,000								LS 6161 R
PAN 1614 R	0,154	0,173	0,267	0,212	0,268	0,216	0,456	0,393	0,319	0,274	0,278	0,223	0,316	0,254	0,173	0,154	0,430	0,215	0,283	0,242	0,244	0,350	0,230	0,373	0,000							PAN 1614 R
NS 6448	0,164	0,123	0,275	0,169	0,319	0,037	0,328	0,379	0,282	0,252	0,206	0,156	0,214	0,240	0,092	0,105	0,263	0,218	0,116	0,078	0,255	0,293	0,227	0,358	0,175	0,000						NS 6448
DM 6.8i RR	0,313	0,256	0,307	0,255	0,285	0,321	0,528	0,469	0,419	0,230	0,214	0,297	0,323	0,408	0,288	0,343	0,417	0,342	0,360	0,310	0,277	0,451	0,334	0,232	0,292	0,294	0,000					DM 6.8i RR
NS 7211 R	0,395	0,378	0,457	0,380	0,421	0,218	0,350	0,413	0,472	0,480	0,445	0,269	0,263	0,508	0,219	0,255	0,230	0,368	0,303	0,175	0,370	0,336	0,431	0,579	0,421	0,210	0,457	0,000				NS 7211 R
PAN 1729 R	0,312	0,265	0,366	0,342	0,451	0,327	0,451	0,511	0,395	0,376	0,327	0,332	0,303	0,332	0,370	0,337	0,359	0,332	0,319	0,341	0,297	0,388	0,295	0,289	0,348	0,282	0,199	0,447	0,000			PAN 1729 R
M.A	0,409	0,429	0,378	0,325	0,472	0,417	0,454	0,310	0,365	0,359	0,377	0,358	0,577	0,506	0,338	0,344	0,516	0,314	0,425	0,397	0,273	0,429	0,324	0,419	0,371	0,413	0,459	0,442	0,526	0,000		M.Arrow
W 82	0,222	0,273	0,370	0,380	0,316	0,317	0,316	0,441	0,442	0,484	0,445	0,322	0,105	0,245	0,275	0,283	0,336	0,421	0,451	0,227	0,441	0,360	0,397	0,474	0,320	0,253	0,418	0,368	0,356	0,682	0,000	Will 82

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