Genetic analysis of the *Puccinia graminis* f. sp. *tritici* and *Puccinia triticina* populations in southern Africa

by

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Submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

In the Faculty of Natural and Agricultural Sciences

Department of Plant Sciences

University of the Free State

Bloemfontein

South Africa

2015

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Declaration

I, Tonny Ion Selinga, declare that the thesis that I herewith submit for the Masters Degree qualification Magister Scientiae Botany at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

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"Science at its best is an open-minded method of inquiry, not a belief system." Rupert Sheldrake

"For by Him all things were created that are in heaven and that are on earth, visible and invisible, whether thrones or dominions or principalities or powers. All things were created through Him and for Him. And He is before all things, and in Him all things consist." NKJV

"In the beginning was the Word, and the Word was with God, and the Word was God. He was in the beginning with God. All things were made through Him, and without Him nothing was made that was made. In Him was life, and the life was the light of men. And the light shines in the darkness, and the darkness did not comprehend it." NKJV

"Any time a science avoids coming to grips with numbers it somehow immersing itself in perhaps an avoidable but certainly an unattractive miasma." David Berlinski

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Acknowledgements

I am greatly indebted to the following people:

- Dr. Botma Visser, study leader, thank you for your guidance and support throughout the study.
- Prof. Zakkie Pretorius, co-study leader, for your input to make this study a success.
- Dr. Tarekegn Terefe and the ARC–Small grain institute for providing samples.
- Prof. Schall thank you for helping with the statistics.
- My friends (especially Mpho Mafa and Khotso Mokgeseng), colleagues in lab 32, Cornel Bender and the Department of Plant Sciences, thank you for your help and friendship.
- Prof. Herselman, thank you for your help with the population genetics programs.
- My brethren in Christ, Wayne and Mariaan Pohorille, thank you for your support.
- My girlfriend, Masabata Mokgesi, thank you for your support.

I am greatly indebted to the following institutions:

- The Department of Plant Sciences and the University of the Free State, for providing the facilities and resources necessary to complete the study.
- The NRF for financial support.

List of abbreviations

	A
AMOVA	Analysis of molecular variance
AFLPs	Amplified fragment length polymorphisms
ARC-SGI	Agricultural Research Council-Small Grain Institute
	B
bp	Base pair(s)
	C
CI	Confidenc interval
CTAB	Cetyltrimethylammonium bromide
	E
EDTA	Ethylene-diaminetetraacetate
	F
Fam	6-Carboxy-fluorescein
	H
Hex	6-Carboxy-2,4,4,5,7,7-hexachlorofluorescein succinidyl ester
HWE	Hardy-Weinberg equilibrium
	K
K	Number of unknown populations/genetic clusters

L

LE	Linkage equilibrium

M

MCMC	Monte Carlo Markov Chain

N

NTSYSpc	Numerical taxonomy and multivariate analysis system
NJ	Neighbour-joining
NSW	Northern South Wales
Nm	Rate of migration

P

PCR	Polymerase chain reaction
Pg	*Puccinia graminis*
Pgt	*Puccinia graminis* f. sp. *tritici*
Pst	*Puccinia striiformis*
Pt	*Puccinia triticina*

Q

qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait loci

R

r	Cophenetic correlation coefficient
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism

S

SAHN	Sequential agglomerative hierarchical nested
SAS	Statistical Analysis System
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeats

T

TAE	Tris, Acetate, EDTA
TBE	Tris, Borate, EDTA
TE	Tris-EDTA
Tris	Tris (hydroxymethyl)-aminomethane hydrochloride

U

UPGMA	Unweighted pair-group method with arithmetic averages

SI units

cm	Centimetres
g	Gravitational acceleration
h	Hour(s)
ha	Hectare(s)
M	Molar
mM	Millimolar
mm	Millimetre
µM	Micromolar
µm	Micrometre
mg	Milligram(s)
ml	Millilitre(s)
min	Minute(s)
ng	Nanogram(s)
pH	Measure of acidity/basicity
r/s	revolutions per second
sec	Second(s)
V	Volts
v/v	Volume/volume
w/v	Weight/volume
W	Wattz
µl	Microlitre(s)
%	Percentage
^{0}C	Degrees centigrade

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Pretorius Z.A., Visser B., Terefe T., Herselman L., Prins R., Soko T., Siwale J., Mutari B., **Selinga T.I.** and Hodson D.P. 2014. Races of *Puccinia triticina* detected on wheat in Zimbabwe, Zambia and Malawi and regional germplasm responses. *Australasian Plant Pathology* 44:217-224.

Terefe T, Visser B, Herselman L, **Selinga T** and Pretorius ZA. 2014. First report of *Puccinia triticina* (leaf rust) race FBPT on wheat in South Africa. *Plant Disease* 98: 1001.

Chapter 1:

Introduction

Wheat was first introduced to South Africa in 1652 when Jan van Riebeeck settled in Cape of Good Hope (du Plessis, 1933). Wheat production then spread from the Mediterranean climate region to other agro-ecological regions with summer rainfall as the settlers moved to the rest of the country (du Plessis, 1933).

Since then wheat production reached an average of 1 852 800 tons per year during the period 2003-2014 (SAGL, 2014). During the 2013/2014 season, 505 500 ha of wheat was planted (grainsa.co.za, accessed on 17/03/2015). Wheat in South Africa is used as a food source for humans and animals, absorbing agent and for the production of alcohol (nda.agric.za, accessed on 17/03/2015). However, wheat production is limited by both abiotic and biotic factors.

Rusts are able to limit wheat production because they produce large numbers of urediniospores that are potentially damaging in different agro-ecological regions. Wheat rusts usually have many different races and can be transported long distances through wind dispersal.

Of the two modes of reproduction that are known to contribute to the diversity of wheat leaf rust (*Puccinia triticina* Eriks., *Pt*) and stem rust (*Puccinia graminis* Pers. f. sp. *tritici* Eriks and Henn., *Pgt*) where wheat is cultivated, only one is known to occur in South Africa. The sexual reproduction stage has not been detected in South Africa for both leaf and stem rust and therefore is not known to contribute to the diversity of the rust populations. However, the asexual stage has contributed by means of mutation to the genetic variation of the two rust species in South Africa.

Rust surveys are important to show which races are present in the country. This can help to develop strategies to reduce the rust inoculum. Rust surveys are conducted annually and have helped to show the prevalence of different stem rust and leaf rust races in South Africa (Terefe *et al.*, 2009; 2010). They have also helped to show which resistance genes/quantitative trait loci (QTL) were overcome by specific virulent races. The best method to control rusts is using

resistance genes/QTL because they are economically and environmentally safe. Rust samples collected during surveys are usually diagnosed by infection type analysis (phenotyping) which requires differentiating lines (differentials) with single resistance genes.

In South Africa, the first rust epidemics were detected in 1726 (du Plessis, 1933). However, the first phenotyping of races of leaf and stem rust in South Africa was pioneered in the 1900s (Verwoerd, 1931; 1935; 1937). In the early 1980s annual rust surveys were initiated to detect race shifts that occurred and the reaction of newly released varieties to different races (Pretorius *et al.*, 2007).

Since then several stem rust races have been detected using phenotyping. A new stem rust race, Ug99, was first detected in 1999 in East Africa, and has a broad virulence and adaptive ability (Pretorius *et al.*, 2000; Wanyera *et al.*, 2006; Jin *et al.*, 2007). Race 2SA88 was the first race detected in South Africa belonging to a group of races that evolved from Ug99 and was first detected in the 2000 - 2001 season (Boshoff *et al.*, 2002b). In South Africa 2SA88 was the dominant stem rust race by 2010 threatening wheat cultivated in all agro-ecological regions (Terefe and Pretorius, 2011a; b).

One non-Ug99 stem rust race that was formed by single step mutation from 2SA102 in South Africa is 2SA105. It increased to significant proportions in the last decade.

New races of leaf rust also pose a threat to wheat. From 1989 to 2008 the adaptation of *Pt* was low in South Africa except for the occurrence of race 3SA144 which most likely developed through single-step mutation possibly from 3SA132 (Pretorius and Bender, 2010). Races such as 3SA145, 3SA146 and 3SA147 were detected from 2009 - 2010 and are thought to be introduced into South Africa (Terefe *et al.*, 2011; 2014a; b). Race 3SA145 and 3SA146 pose a threat to wheat production because a reasonable number of wheat entries are susceptible to them. This is with

the exception to wheat entries containing *Lr34* which has been a source of durable leaf rust resistance for many years in South Africa.

DNA genotyping has been used minimally to confirm the identity of new races in South Africa. It has never been used to identify isolates from an entire survey. Pretorius *et al.* (2007) stated that surveys and phenotyping could be supported and improved by incorporating molecular methods.

The main aim of the study was to determine whether genotyping of rust isolates complements phenotyping. The hypothesis will be tested by race identification using a large sample size, analysis of population structure of leaf and stem rust, and analysis of exchange of leaf rust inoculum in southern Africa.

Chapter 2:

Literature review

2.1 Introduction

Wheat is regarded as an important food crop because of its adaptability to different environments, high yield and special dough features which are determined by the gluten protein fraction (Shewry, 2009). Most of the wheat grown worldwide is hexaploid bread wheat (*Triticum aestivum* L.) while less than 5% is durum wheat (*Triticum turgidum* L.) (Shewry, 2009). Durum wheat is more widely adapted to the dry Mediterranean region. The major use of bread wheat is to produce flour for leavened and flat breads, cookies, pastries and cakes, while semolina, flour produced from durum wheat, is used for pasta making (Peña, 2002). Durum wheat has also been used to make bulgar and couscous in North Africa (Shewry, 2009).

Bread is an important source of nutrients. It is a good source of vitamins B and E, carbohydrates and proteins (Pomeranz, 1987). The starch content in white flour and whole wheat grain is usually between 65-75% and 60-70%, respectively while the protein content is between 8-15% (Shewry, 2000; 2009). Wheat contributes up to 44% of the daily intake of zinc and 25% of iron (Henderson *et al.*, 2007).

In South Africa wheat was first grown in the 1600s after Jan van Riebeeck settled in the Cape of Good Hope (present Cape Town) (du Plessis, 1933). The main wheat grown in South Africa is bread wheat with the major irrigated areas being located in Mpumalanga, Northern Cape and KwaZulu-Natal provinces (Terefe *et al.*, 2014a). The predominant dry land wheat producers are the Free State and Western Cape provinces. South Africa produced 1.87 million tons of wheat in the 2013 and 2014 season with a ten year average of 1.85 million tons per year (SAGL, 2014). The province which produced the most wheat is the Western Cape. However wheat production in South Africa is also declining.

Wheat yield can be reduced by abiotic and biotic stress (different pests and pathogens, including the obligate biotrophic wheat rusts). Biotrophs infect the host by forming feeding structures

within the host plant cells (Green *et al.*, 1995; Heath and Skalamera, 1997). Wheat rusts are taxonomically classified in the phylum Basidiomycota, class Urediniomycetes, order Uredinales, family Pucciniaceae and genus *Puccinia* (Leonard and Szabo, 2005; Bolton *et al.*, 2008). Included in the genus *Puccinia* are *Pgt, Pt* and *Puccinia striiformis* Erikss (*Pst*, stripe rust). *Puccinia* species of cereals are heteroecious and macrocyclic. Heteroecious organisms go through different stages of their life cycle on different hosts. Macrocyclic rust fungi produce basidiospores, teliospores pycniospores, aeciospores and urediniospores during their life cycle (apsnet.org, accessed on 15/10/2015).

2.2 *Puccinia graminis* Pers. f. sp. *tritici*

2.2.1 Characteristics of *Pgt*

2.2.1.1 Signs and symptoms of *Pgt* infection on wheat and barberry

Pgt is the causal agent of stem rust of wheat (Roelfs, 1985; Leonard and Szabo, 2005). Uredinia burst open the epidermal walls of the gramineous host 7 - 15 days post-infection as stem rust infection appears on the leaf sheaths, stems and occasionally on leaves (apsnet.org, accessed on 11/15/2014). The uredinia bear urediniospores on the surface of the epidermis which make the uredinia appear brick-red (Figure 2.1). The uredinia can reach up to 10 mm in length and are long and narrow or diamond shaped. Teliospores formed at the end of the wheat growing season make the telia to appear black on the gramineous host (apsnet.org, accessed on 15/10/2015).

Clusters of pycnia appear on top of the leaf blade of the alternate barberry (*Berberis*) host during spring in the northern hemisphere. Yellow cup-shaped aecia form on the lower surface of the leaf which can grow up to 5 mm from the epidermis of the leaf. Orange-yellow aeciospores have a slightly rough surface and can infect the gramineous host but not barberry (apsnet.org, accessed on 15/10/2015).

Figure 2.1: Uredinia of *Puccinia graminis* f. sp. *tritici* on wheat stems. Photos by Prof. Zakkie Pretorius.

2.2.1.2 Infection process of *Pgt* on wheat

The urediniospores of *Pgt* germinate once in contact with a leaf sheath and stem of the wheat host that is covered by a film of water (Wiethölter *et al.*, 2003). The growth of the germ tube is orientated perpendicular to the leaf surface (Maheshwari and Hildebrandt, 1967; Dickinson, 1969; Wynn, 1976; Collins and Read, 1997). When the germ tube encounters a stomatal pore (Allen, 1923) it develops an appressorium over the stoma. This is followed by the migration of the two nuclei from the urediniospore through the germ tube into the appressorium, where they then undergo mitosis and are isolated from the germ tube. The formation of septa compartmentalizes the two nuclei in the appressorium (Leonard and Szabo, 2005).

According to earlier studies by Yirgou and Caldwell (1968) the *Pgt* fungus forms the appressorium at night, when there are drops of water on the epidermal surface where after the appressorium growth is stopped by the presence of CO_2. In the morning, a penetration peg grows from the appressorium into the substomatal space to form a substomatal vesicle and the fungal nuclei undergo mitosis. Infection hyphae grows from the substomatal vesicle below the epidermal cells in contact with the intercellular space, until it reaches a mesophyll cell (Allen, 1923). The nuclei migrate into the infection hyphae. On the mesophyll cell surface a haustorial mother cell develops which is separated from the hypha by a septum. The haustorial mother cell of *Pgt* houses 2-4 nuclei. The haustorial mother cell then produces a penetration peg which enters the host mesophyll cell to form a haustorium inside the periplasmic space. The haustorium that has two nuclei (Allen, 1926) absorbs nutrients from the cytoplasm of the host cell (Szabo and Bushnell, 2001). This infection process repeats many times to form a network of fungal mycelia which will form uredinia and urediniospores. Severe *Pgt* infection leads to a disruption of nutrient flow (Leonard and Szabo, 2005). This in turn causes the wrinkling and contraction of the wheat grain, and weakening of the stem.

2.2.2 The primary and alternate hosts of *Pgt*

Pgt is a heteroecious fungus, which means it needs more than one host plant to complete its life cycle. The primary hosts of *Pgt* are grass species like *T. aestivum* L. (bread wheat) and *T. turgidum* L. var. *durum* (tetraploid durum wheat), *X Triticosecale* Wittmack. ex A. Camus. (triticale) and *Hordeum vulgare* L. (barley) (Roelfs *et al*., 1992). The alternate hosts of *Pgt* include several *Berberis* and *Mahonia* spp. (Roelfs 1985; Roelfs and Groth, 1988; Roelfs *et al*., 1992).

2.2.3 Life cycle

2.2.3.1 Asexual reproduction of *Pgt*

The asexual reproduction process is initiated when aeciospores infect the wheat host to form dense hyphal mats below the epidermis (Figure 2.2). The hyphae produce sporophores which form uredinia and eventually urediniospores. Single-celled dikaryotic urediniospores are wind dispersed until they reach a moist leaf sheath or stem of a nearby wheat host and infect it. The urediniospores can re-infect the wheat host. The hyphae of two individuals sometimes conjugate to allow asexual recombination of nuclei. Since this stage allows mutation and selection on the susceptible hosts, it is the most important driving force for population change (Singh *et al*., 2008).

2.2.3.2 Sexual reproduction of *Pgt*

Stem rust teliospores are two-celled spores each with two haploid nuclei (Boehm *et al*., 1992) that are produced at the end of the wheat growing season (Figure 2.2). After undergoing karyogamy to produce diploid nuclei they become dormant to germinate in spring, only in the northern hemisphere. The germinating teliospore produces a promycelium and four haploid nuclei are formed through meiosis.

The haploid nuclei migrate into four promycelial cells. Sterigma forms from each promycelium

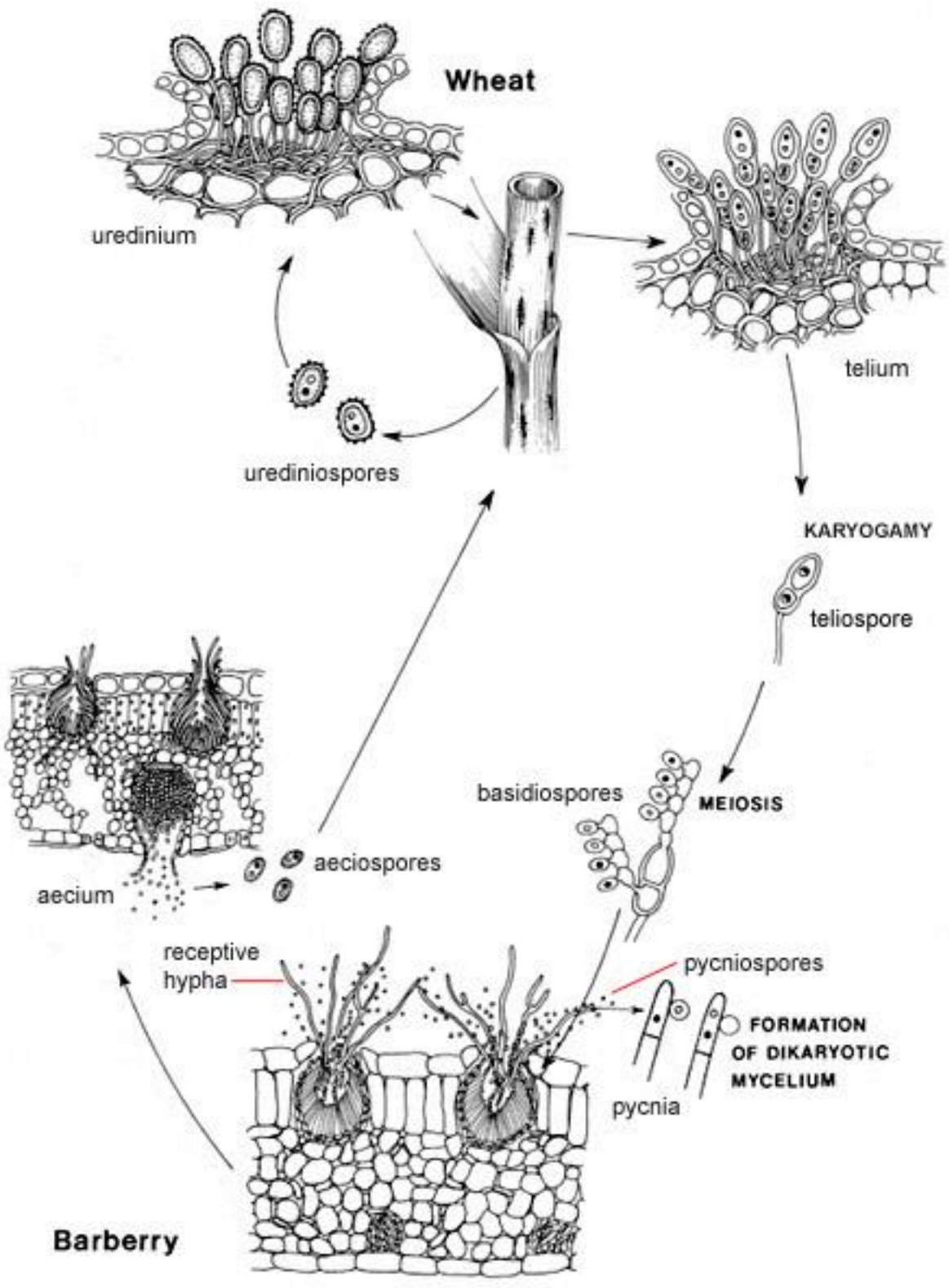

Figure 2.2: Life cycle of stem rust (apsnet.org, accessed on 04/06/2015).

cell and basidiospores expand at the tip of the sterigma. Each haploid nucleus migrates into the basidiospore via the sterigma. Mitosis takes place in the basidiospore and the result is single celled basidiospores with two identical nuclei.

Mature basidiospores travel via air currents from the primary host to infect the alternate host. A flask-shaped pycnium develops after infection on the upper side of the leaf. Haploid pycniospores then develop from the pycnium. There are two mechanisms used by *Pgt* to disperse pycniospores to other pycnia. During the first, pycniospores are disseminated by rain splash. For the second, the pycnium produces nectar to attract insects and the pycniospores stick to the insects which spread them to other pycnia. During mating the pycniospores act as male gametes and can only fertilize an opposite flexuous hyphae, which acts as the female gamete. Fertilization takes place on the upper leaf surface and results in a dikaryotic state. An aecium then establishes on the lower leaf surface directly below the pycnium to produce chains of single-celled dikaryotic aeciospores. The aeciospores then infect a primary host.

The sexual cycle of *Pgt* on *Berberis* has caused problems in the past in North America and northern and eastern Europe, because *Berberis* is a source of inoculum and new recombinants (Singh *et al*., 2008). At least three species of *Berberis* have been found in South Africa (Keet *et al.* 2015). However, before 2015 *Pgt* asexual stage has been the only mode of reproduction known to occur in South Africa for over 90 years (de Jager, 1980; Le Roux and Rijkenberg, 1987; Le Roux, 1989; Boshoff *et al*., 2000; Pretorius *et al*., 2007; Figlan *et al*., 2014). The South African stem rust population is thus believed to be an asexually reproducing population.

2.2.4 Global history of *Pgt*

2.2.4.1 The origin of *Pg*

Puccinia graminis (*Pg*) was proposed to have originated from central Asia on species of Berberidaceae (Leppik, 1959, 1961; Anikster and Wahl, 1979). The ancestor of stem rust of

cultivated wheat is considered to be *P. graminis* ssp. *graminicola* which evolved into *P. graminis* ssp. *graminis*. Studies have shown that *Pg* is most probably a monophyletic compound species and therefore has a single common ancestor (Zambino and Szabo, 1993; Abbasi *et al.*, 2005). The entire heteroecious cycle of *Pg* evolved because the fungus has existed long enough with *Berberis* species and grasses from the subfamily Festucoideae. This allowed *Pg* to have an expanded grass host range (70-80 genera) and a limited host range for alternate hosts (*Mahonia* and *Berberis*) (Wahl *et al.*, 1984). Most potential host species are found in the northern hemisphere (Leppik, 1961). The pathogen has spread to different parts of Asia, through the Mediterrenian region to Europe, Africa, the Americas and Australia in association with the alternate hosts and wheat cultivation, although the initial spread is untraceable (Wahl *et al.*, 1984).

2.2.4.2 Impact of *Pgt* on wheat yield

Pgt has caused yield losses in many different wheat growing areas over the last century. Severe losses can occur on susceptible wheat when there is adequate inoculum. The disease is capable of destroying an entire crop in a region or field (Singh *et al.*, 2008).

A *Pgt* epidemic caused 5 - 20% yield losses in east European countries in 1932 (Zadoks, 1963). It started in Bulgaria but was distributed to eastern and northern Europe (Zadoks, 2008). The yield losses increased to between 9 - 33% in 1951 in Scandinavia, northern Europe (Zadoks, 1963).

Occasional yield losses also occurred in the warmer areas of Queensland and northern New South Wales, Australia in the mid-20th century (Rees, 1972). In south-eastern Australia, a stem rust epidemic caused between 25-30% loss in production in 1973 (Park, 2007). The 1973 stem rust epidemic was thought to be the most intense in the history of the Australian wheat industry. The southern states of Australia were again struck with a severe epidemic in 1974 (Watson, 1981). Stem rust severity levels decreased from 1969 till 2006 in Australia probably due to the availability of cultivars with the *Sr2*, *Sr24* and *Sr26* resistance genes (Park, 2007). In the period from 1998-1999 to 2007-2009 yield losses caused by stem rust were less than 1% for three

production regions of Australia, covering southern Queensland to Western Australia (Murray and Brennan, 2009).

Pgt has caused severe problems in the southern parts of India during the years with warm weather usually in January and February (Joshi and Palmer, 1973). China has experienced *Pgt* epidemics in 1948, 1951, 1952 and 1956 (Roelfs, 1977). The epidemics occurred due to frequent rainfall and higher than normal temperatures and the vulnerability of northern China and Inner Mongolian spring wheat to infection.

Epidemics have also occurred in the southern Great Plains, USA. In 1935 more than 50% yield was lost because of *Pgt* in North Dakota and Minnesota, USA (Leonard, 2001). From 1920 to 1960 10% of yield was lost over an eight year period and more than 20% yield was lost over a five year period in the spring wheat region of Minnesota, North Dakota and South Dakota.

In Africa, stem rust has been a problem in North, East and southern Africa before 2000 (Roelfs *et al*., 1992). Algeria lost 20% in yield trials in 1979 (Saari and Prescott, 1985). Stem rust caused devastating epidemics in Ethiopia in 1993 and 1994 (Shank, 1994). This resulted in 65-100% yield losses on cultivar Enkoy. The Ethiopian highlands experienced 42% reduction in yield due to this epidemic (Dubin and Brennan, 2009). Despite the stem rust epidemic in Ethiopia, there were no major epidemics for over three decades in other parts of the world (Singh *et al*., 2008). However in 2013-2014 period an epidemic caused mainly by race TTKTTF hit Ethiopia again on the cv. 'Digalu' (Olivera *et al*., 2015). The race caused devastating yield losses (92%) especially in Agarfa, Garesa and Sinana districts. The last recorded epidemic in South Africa was at Albertinia, Western Cape, in 1985 (Boshoff *et al*., 2000; Figlan *et al*., 2014). The epidemic, caused by races 2SA100 and 2SA101, affected wheat cultivars with *Sr24* resistance (cultivars SST44 and Gamka) resulting in yield losses ranging between 17-75% (Le Roux and Rijkenberg, 1987).

2.2.4.3 The emerging threat of race Ug99

Pgt race Ug99 first appeared in Uganda in 1998 and was documented in 1999 (Pretorius *et al.*, 2000). Initially called race TTKS (Wanyera *et al.*, 2006) according to the North American nomenclature system (Roelfs and Martens, 1988), it was later renamed TTKSK using a larger differential set (Jin *et al.*, 2008). Race TTKSK threatened wheat worldwide because it is virulent to *Sr5, 6, 7a, 7b, 8a, 8b, 9a, 9b, 9d, 9g, 10, 11, 12, 15, 16, 17, 18, 19, 20, 23, 30, 31, 34, 38* and *Wld-1* (Jin *et al.*, 2007). The development of virulence to *Sr24* by TTKST, which was detected in 2006, caused stem rust to increase to epidemic proportions on variety Mwamba in 2007, which was planted over 30% of wheat area in Kenya (Jin *et al.*, 2008; Singh *et al.*, 2008).

Ug99 threatens about 25% of wheat area across the globe and an estimated 19% of global wheat production (117 million tons) (Reynolds and Borlaug, 2006). This could disturb the food production of approximately 1 billion people who live in the affected areas.

Since then TTKSK spread from Uganda to eight other countries (Kenya, Ethiopia, Sudan, Yemen, Iran, Tanzania, Eritrea and Rwanda) (Singh *et al.*, 2011; rusttracker.cimmyt.org; accessed on 27/11/2014). Ug99 has also evolved into eleven different variants which are known to occur in 10 countries (South Africa, Zimbabwe, Uganda, Eritrea, Tanzania, Ethiopia, Rwanda, Kenya, Sudan, Iran, Egypt, Yemen and Mozambique) (Bhardwaj *et al.*, 2014; rusttracker.cimmyt.org; accessed on 22/06/2015).

2.2.5 The situation of *Pgt* in South Africa

2.2.5.1 Development of *Pgt* races

Twenty-five *Pgt* races have been characterized in South Africa since the early 1980s. Genotype analysis of the South African *Pgt* races indicated two distinct *Pgt* lineages, namely the non-Ug99 and Ug99 lineages (Visser *et al.*, 2009).

2.2.5.1.1 Traditional non-Ug99 South African races

A possible evolutionary pathway for the development of the non-Ug99 lineage in South Africa was proposed by Pretorius *et al.* (2007). The first races described in South Africa were 21 and 34 found in 1922 and 1929 respectively (de Jager, 1980). The specialization of these two races gave rise to a plethora of new races in South Africa during the 1900s (Figure 2.3). Only non-Ug99 races were detected during the 1980s and 1990s with five of these races being detected between 2000 and 2010 (Le Roux, 1989; Le Roux and Rijkenberg, 1987; Boshoff *et al.*, 2000; Komen 2007; Terefe *et al.*, 2010; Terefe and Pretorius, 2011a;b).

2SA100 was first detected in 1984 in the northern parts of South Africa (Gauteng, Limpopo, Mpumalanga and North West) (Le Roux and Rijkenberg, 1987). 2SA100 developed from race 222 (Figure 2.3). 2SA4 and 2SA100 differ for virulence to *Sr9E*, *24* and *30* (Table 2.1) and share a common ancestor (Figure 2.3).

2SA102 was detected for the first time in South Africa in 1988 (Smith and Le Roux, 1992). It is thought to have been the result of specialization from race 16 (Figure 2.3). 2SA102 shares a common ancestor with 2SA55 but differs from 2SA55 with virulence to *Sr9G*, *Sr27* and *SrKw* (Table 2.1). 2SA102 has at least 96% genetic similarity with 2SA4 and 2SA100 (Figure 2.4).

2SA103 was detected for the first time in South Africa in 1988 (Smith and Le Roux, 1992). The acquisition of virulence to *Sr27* in race 326 produced 2SA103. Race 2SA102 is another possible ancestor of 2SA103. They differ with virulence to *Sr9G* and also have a high (84%) genetic similarity (Figure 2.4). 2SA103 differs from 2SA55 with virulence to *Sr7B*, *Sr9D, Sr11*, *Sr27*, *Sr30*, *SrEm*, *SrKw* and *SrSatu* (Table 2.1).

Race 2SA104 was identified for the first time in South Africa in 2003 and was previously identified as 2SA102K (Komen, 2007; Terefe *et al.*, 2010; Figure 2.3). Race 2SA104 evolved from

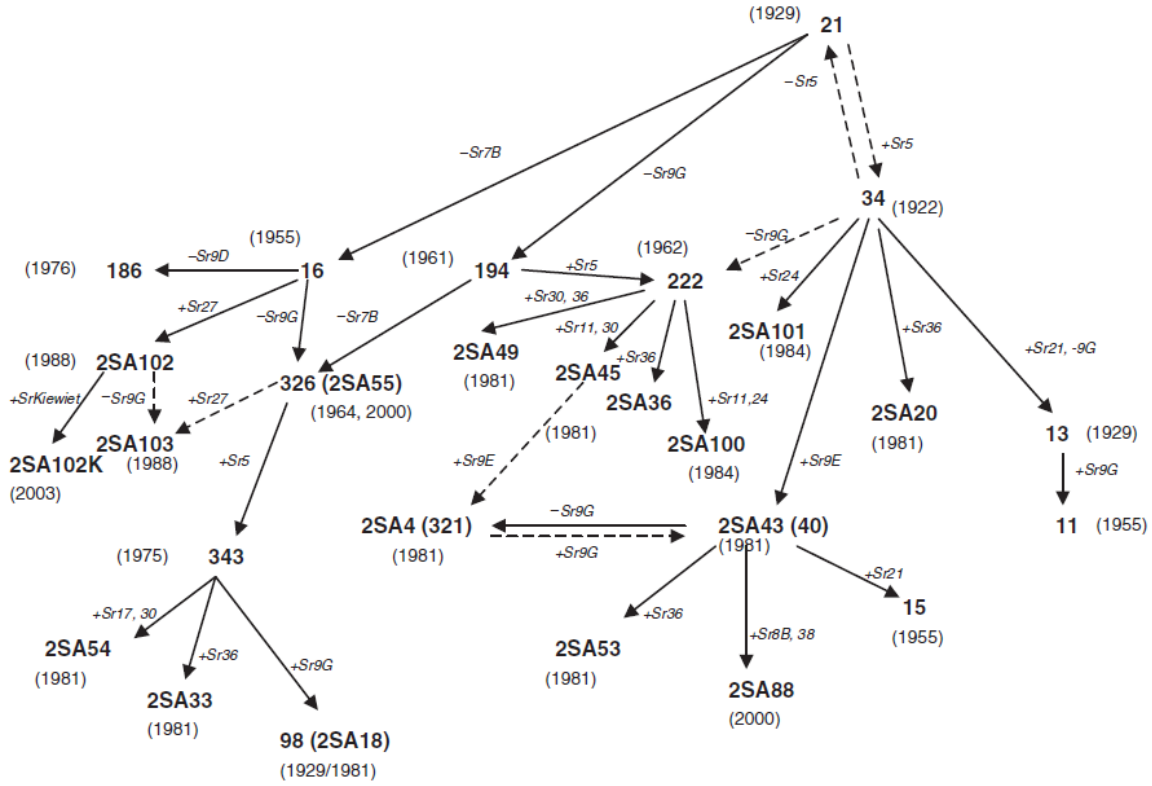

Figure 2.3: The evolution of South African *Pgt* as hypothesized by Pretorius *et al.* (2007). The years show first discovery of the race. The broken lines show other pathways of specialization that are likely to have occurred.

Table 2.1: The avirulence/virulence profiles of *Pgt* races detected between 2000 and 2010 in South Africa.

Races	Avirulence/ virulence profile	Reference
2SA4	*Sr8b, 9g, 21, 24, 27, 31, 36, 38, Tmp, Kw, Satu/5, 6, 7b, 8a, 9a, 9b, 9d, 9e, 10, 11, 17, 30, Wld-1, McN*	Komen, 2007
2SA55	*Sr5, 6, 7b, 8b, 9b, 9e, 9g, 17, 21, 24, 27, 30, 31, 36, 38, Em, Kw, Satu, Tmp/8a, 9a, 9d, 10, 11, 44, McN*	Terefe *et al.*, 2010
2SA88	*Sr21, 24, 27, 31, 36, 44, Em, Kw, Satu, Tmp/5, 6, 7b, 8a, 8b, 9a, 9b, 9d, 9e, 9g, 10, 11, 17, 30, 38, McN*	Terefe *et al.*, 2010
2SA88+ (TTKSF+)	*Sr21, 24, 27, 31, 36, 44, Em, Kw, Satu, Tmp/5, 6, 7b, 8a, 8b, 9a, 9b, 9d, 9e, 9g, 10, 11, 17, 30, 38, Web, McN*	Pretorius *et al.*, 2012
2SA99	*Sr5, 6, 9b, 9e, 21, 24, 27, 31, 36, 38, Kw, Satu/7b, 8a, 9g, 11, 17, 30*	T. Terefe, unpublished data
2SA100	*Sr8b, 9e, 9g, 21, 27, 30, 31, 36, 38, Tmp, Satu/5, 6, 7b, 8a, 9a, 9b, 9d, 10, 11, 17, 24, Wld-1, McN*	Komen, 2007
2SA102 (race 16)	*Sr5, 6, 7b, 8b, 9b, 9e, 17, 21, 24, 30, 31, 36, 38, Em, Satu, Tmp/8a, 9a, 9d, 9g, 10, 11, 27, 44, Kw, McN*	Terefe *et al.*, 2010
2SA103	*Sr5, 6, 8b, 9b, 9d, 9e, 9g, 11, 17, 21, 24, 31, 36, 38, Tmp/7b, 8a, 9a, 10, 27, 30, Wld-1, McN*	Komen, 2007
2SA104 (2SA102K)	*Sr5, 6, 7b, 8b, 9b, 9e, 17, 21, 24, 30, 31, 36, 38, Em, Kw, Satu, Tmp/8a, 9a, 9d, 9g, 10, 11, 27, 44, McN*	Terefe *et al.*, 2010
2SA105	*Sr5, 6, 7b, 8b, 9b, 9e, 17, 21, 24, 30, 31, 36, 38, Em, Tmp/8a, 9a, 9d, 9g, 10, 11, 27, 44, Kw, Satu, McN*	Terefe *et al.*, 2010
2SA106	*Sr21, 27, 31, 36, 44, Em, Kw, Satu, Tmp/5, 6, 7b, 8a, 8b, 9a, 9b, 9d, 9e, 9g, 10, 11, 17, 24, 30, 38, McN*	Terefe *et al.*, 2010
2SA107	*Sr21, 27, 36, 44, Em, Tmp, Satu/5, 6, 7b, 8a, 8b, 9a, 9b, 9d, 9e, 9g, 10, 11, 16, 17, 24, 30, 31, 34, 38, 41, McN*	Pretorius *et al.*, 2010

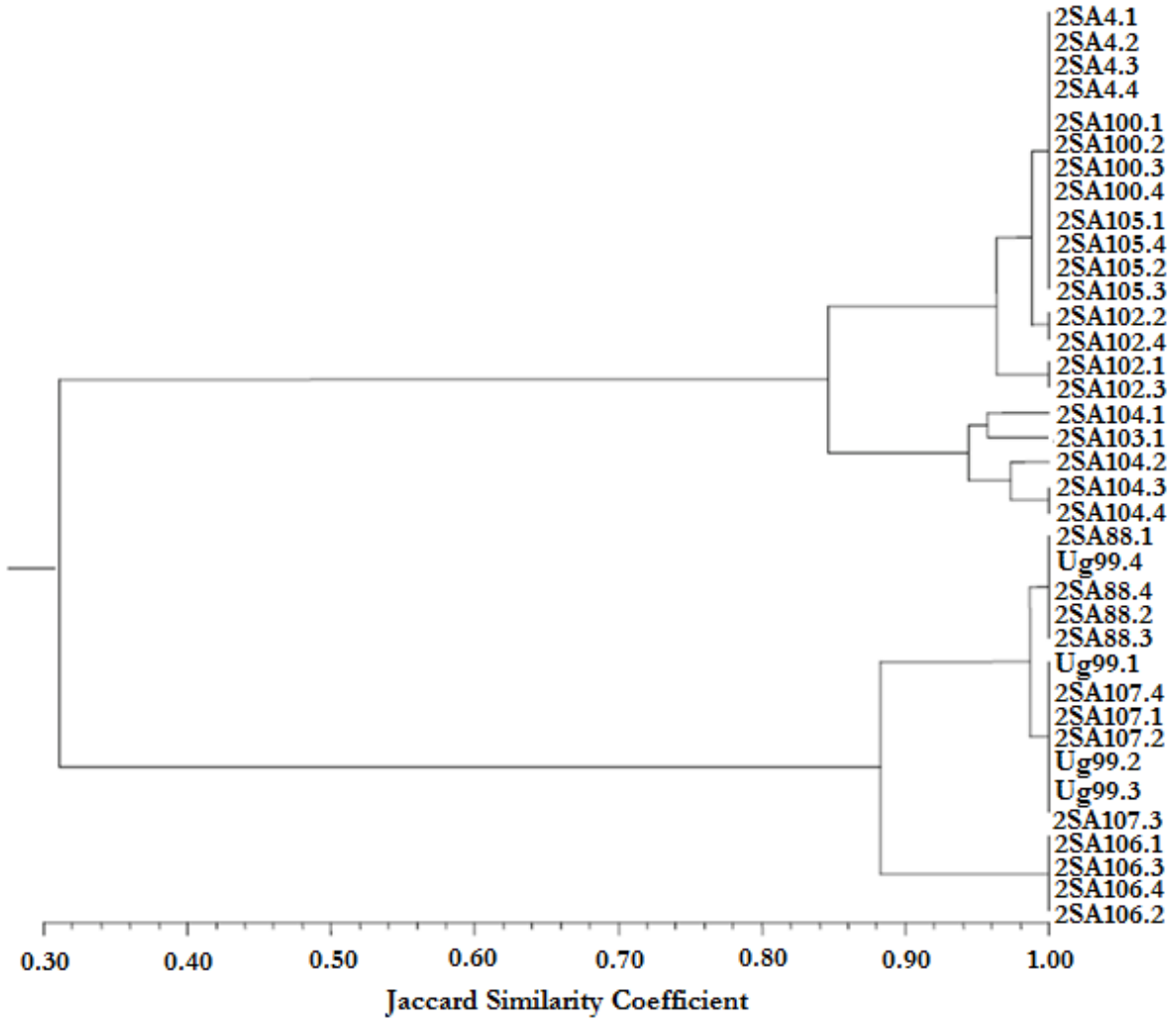

Figure 2.4: Dendrogram showing genotypic similarities between South African *Pgt* races (Visser *et al.*, 2011).

2SA102 (Figure 2.3). The races differ by virulence to *SrKw* (Table 2.1). Race 2SA104 had the highest (94%) genetic similarity with 2SA103 (Figure 2.4).

2SA105 was detected for the first time on triticale in the Western Cape in 2005 (Roux *et al.*, 2006). The ancestor of 2SA105 is thought to be 2SA102 (Terefe *et al.*, 2010). Race 2SA105 differs from 2SA102 by avirulence to *SrKw* and *SrSatu* (Table 2.1). They had at least 96% genetic similarity (Figure 2.4). Races 2SA104 and 2SA105 differ by virulence to *SrSatu* and they also clustered together with 84% a genetic similarity (Figure 2.4).

2.2.5.1.2. The Ug99 lineage

Since 2000 a new lineage of *Pgt* races was detected in South Africa. The first Ug99 variant detected in South Africa was 2SA88 (TTKSF) during the 2000 to 2001 season (Boshoff *et al.* 2002b). The race was found to have an avirulence/virulence profile similar to TTKSK (Ug99) except for avirulence to *Sr31* (Pretorius *et al.*, 2007). Ug99 had a high genetic similarity with 2SA88 (Figure 2.4) confirming that 2SA88 is a member of the Ug99 lineage (Visser *et al.*, 2009). Komen (2007) thought that it was improbable that race 2SA88 developed by asexual reproduction from a pre-existing race because it did not have virulence/avirulence profiles common to those races that were found in South Africa at the time. It was thus postulated that 2SA88 was introduced from eastern Africa to South Africa (Komen, 2007; Visser *et al.*, 2009) and did not develop by physiologic specialization within South Africa as postulated by Pretorius *et al.* (2007). Race 2SA88 was speculated to have mutated to produce TTKSK (Visser *et al.*, 2009). Isolates similar to 2SA88 have also been detected in 2010 in Zimbabwe and Mozambique (Mukoyi *et al.*, 2011).

Race 2SA106 (TTKSP) was first detected in 2007 in the Western Cape (Terefe *et al.*, 2010; Visser *et al.*, 2011). High genetic similarity (Figure 2.4) and virulence/avirulence profile similarities between this race and Ug99 confirmed that 2SA106 was a variant of the Ug99 lineage (Visser *et al.*, 2011). Race 2SA106 has also been speculated to be a foreign introduction. It differed from

2SA88 with virulence to *Sr24* (Table 2.1) and is thought to have developed from 2SA88 by a single step mutation (Terefe *et al.*, 2010).

Race 2SA107 (PTKST) was first detected in 2009 in South Africa at Greytown and Cedara in KwaZulu-Natal (Pretorius *et al.*, 2010). It was later found to have 100% genetic similarity with most of the isolates of Ug99 (Figure 2.4). It has also been hypthesized to be an exotic introduction (Visser *et al.*, 2011). PTKST has also been detected in Ethiopia (2007), Kenya (2009) (Abebe *et al.*, 2013) and Zimbabwe (2010) (Mukoyi *et al.*, 2011). It differs from 2SA88 with virulence to *Sr24* and *Sr31* (Table 2.1).

2SA88+ was first detected in South Africa in the 2010 survey at Afrikaskop in eastern Free State (Pretorius *et al.*, 2012). When analyzed, it could not be differentiated from 2SA88 unless cv. Matlabas was used. 2SA88+ is virulent to *SrWeb* which is now known as *Sr9h* (Rouse *et al.*, 2014) and it is possible that 2SA88+ defeated *SrWeb* in cv. Matlabas. Race 2SA88+ probably developed from 2SA88.

2.2.5.2. Occurrence of *Pgt* races in South Africa

Only non-Ug99 *Pgt* races were detected when *Pgt* surveys first started in South Africa in 1980. In 2000, only races 2SA55 and 2SA88 were detected (Table 2.2). 2SA88 predominated the population from 2001-2004 while non-Ug99 races like 2SA4, 2SA55, 2SA99 and 2SA103 started to disappear. By 2010 the latter four races were no longer detected. 2SA105 was detected as the predominant race in 2007 after which there was a change in the population of *Pgt* from 2007-2010. 2SA105 started to decrease while 2SA88 became the dominant race in South Africa from 2008-2010. From 2007-2009, 2SA104 declined and the new Ug99 variants, 2SA106 and 2SA107, increased to significant frequencies. 2SA102 increased from 2001-2004, then decreased to a low frequency in 2007 and then increased again till 2009. 2SA106 and 2SA107 declined to their lowest frequencies in 2010. Most of the non-Ug99 races disappeared in 2010 with only 2SA102,

Table 2.2: Occurrence of *Pgt* races from 2000 to 2010. The frequencies of races are given in percentages.

	Komen, 2007					Terefe *et al.*, 2010		Terefe and Pretorius, 2011a; b	
Race	**2000**	**2001**	**2002**	**2003**	**2004**	**2007**	**2008**	**2009**	**2010**
2SA004	0	0	0	1	0.8	0	0	0	0
2SA055	65	0	0	0	3.3	3.3	4.5	0	0
2SA088 (TTKSF)	35	68.8	81	85	69.7	26.1	38.4	39	78
2SA099	0	18.8	3	1	0.8	0	0	0	0
2SA100	0	0	0	1	0.8	0	0	0	0
2SA102	0	12.4	10	12	23.8	1.1	8.9	12	1
2SA103	0	0	6	0	0.8	0	0	2	0
2SA104	0	0	0	0	0	14.1	9.8	8	3
2SA105	0	0	0	0	0	53.2	20.5	21	15
2SA106 (TTKSP)	0	0	0	0	0	2.2	17.9	14	2
2SA107 (PTKST)	0	0	0	0	0	0	0	4	1
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

2SA104 and 2SA105 being detected (Table 2.2).

2.3 *Puccinia triticina* Eriks

2.3.1 Characteristics of *Pt*

2.3.1.1 Symptoms of *Pt* infection

Pt is the causal agent of leaf rust in wheat. *Pt* infection is evident as orange to brown coloured uredinia on the upper and lower surfaces of leaves where the maximum diameter of uredinia is 1.5 mm. The uredinia grow vigorously and are spherical to ovoid in shape (Figure 2.5). The globular orange-brown urediniospores have an average diameter of 20 μm with spiky walls that have a maximum of eight germ pores. *Pt* differs from *Pgt* in that *Pt* only infects the leaves of the wheat host (Section 2.2.1.1) (Bolton *et al*., 2008; Figure 2.2, 2.5).

2.3.1.2 Infection process of *Pt* on wheat

Pt urediniospores germinate and produce germ tubes on the wheat leaf surface when there is 100% humidity at 25^{0}C (Hu and Rijkenberg, 1998; Zhang and Dickinson, 2001; Zhang *et al*., 2003). Germ tubes keep growing until a stoma is reached or until the food supply is exhausted (Dickinson, 1969). *Pt* reaches a stoma without the need for light or influence of CO_2 concentration (Wynn and Staples, 1981). The germ tubes are only able to detect the stomata of host plants. Once a stoma is reached, an appressorium is produced. However, should this not happen within 24 h of germination, the germinated urediniospore will not undergo further development and eventually dies (Zhang *et al*., 2003). From the appressorium a penetration peg is formed which can penetrate a closed stoma to form a substomatal vesicle from which infection hyphae develop (Allen, 1926). From the infection hyphae, haustorial mother cells and haustoria can extract food reserves from the host cell. The haustorial mother cell of *Pt* possesses three nuclei and forms 12-24 h after appressorium penetration (Hu and Rijkenberg, 1998). Multiplication and development of infection hyphae give rise to the fungal mycelium. At six days

Figure 2.5: Uredinia of *Pt* on leaves of wheat. Photos by Prof. Zakkie Pretorius.

after inoculation, cells of a susceptible host do not show any signs of alteration by infection. Seven to ten days after inoculation, uredinia form from the mycelium developing in the leaf tissue. At 16 days after inoculation *Pt* has killed not more than 1% of the susceptible host cells (Allen, 1926).

2.3.2 The hosts and alternate hosts of *Pt*

The primary hosts of *Pt* include *T. aestivum*, *T. turgidum* var. *durum* and X *Triticosecale* (Roelfs *et al.*, 1992). Other primary hosts include *T. dicoccum* Schrank ex Schübl., *T. dicoccoides* Körn., *T. monococcum* L., and *T. speltoides* L. The alternate hosts are *Thalictrum speciosissimus* Loefl., *Th. flavum* L., *Th. foetidum* L., *Th. japonicum* Thunb., *Isopyrum fumaroides* L., *Clematis mandshurica* Rupr. and *Anchusa italic* Retz. Accessory hosts are non-crop grasses which can be infected by *Pt* in nature. They include wild or weedy species of *Aegilops* (e.g. *Aegilops cylindrical* L., *Aegilops speltoides* Tausch.), *Triticum* related species of *Agropyrum* and *Secalis* (e.g. *Agropyron repens* L.).

2.3.3 The life cycle of *Pt*

2.3.3.1 Asexual cycle of *Pt*

The asexual cycle of *Pt* is similar to that of *Pgt* (section 2.2.3.1). The fact that urediniospore re-infection requires water on the leaf surface with temperatures of between 10-25^{o}C, and that the alternate hosts have not been detected in South Africa probably contribute to why the fungus is frequently observed in winter rainfall and irrigated areas (Pretorius and Le Roux, 1988; Pretorius *et al.*, 2007; Terefe *et al.*, 2009). In South Africa the *Pt* population can exist indefinitely as uredinial infections on the telial hosts (Figure 2.6), thereby contributing to its low level of genetic diversity (Terefe *et al.*, 2014a).

2.3.3.2 Sexual cycle of *Pt*

The sexual cycle of *Pt* is also similar to that of *Pgt* (section 2.2.3.2), with only minor differences.

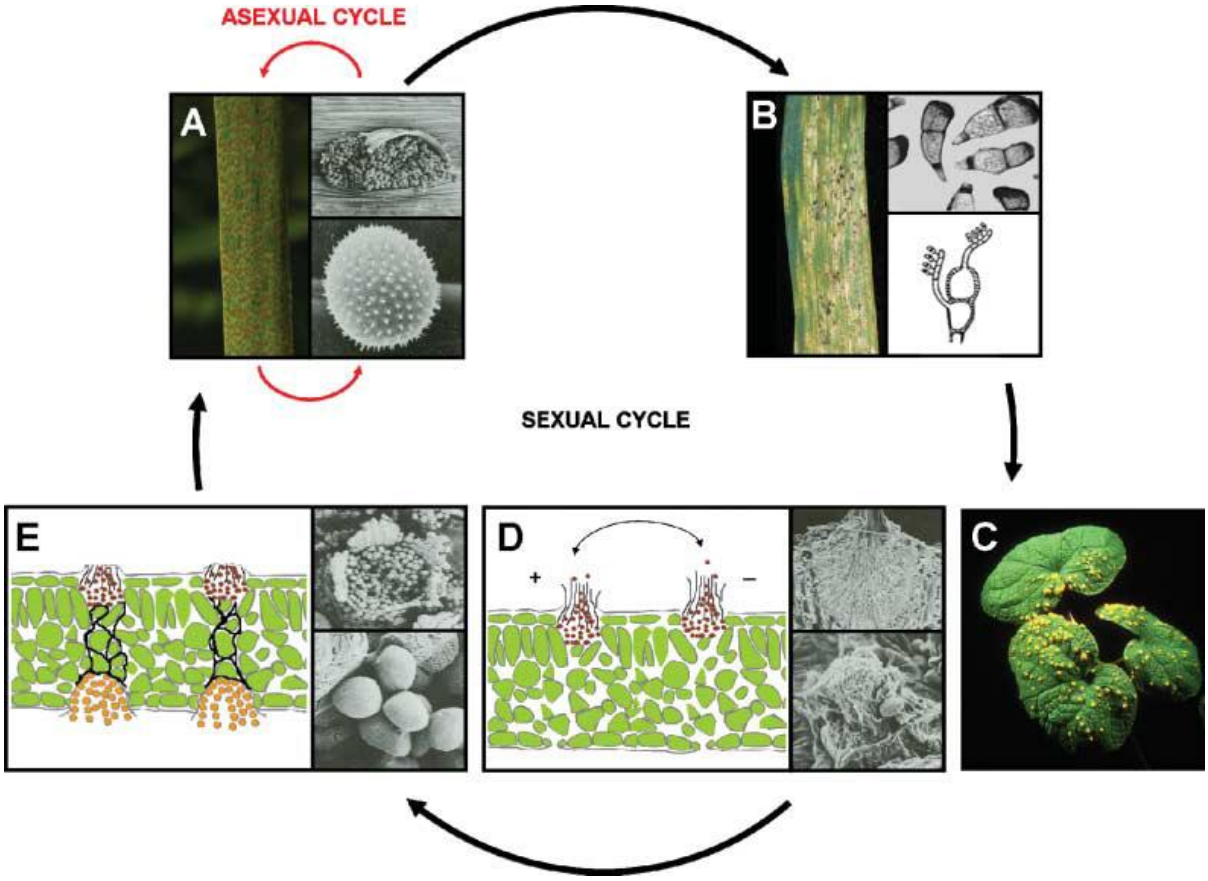

Figure 2.6: Life cycle of leaf rust (Bolton *et al.*, 2008). **A**: The asexual cycle of *Pt* dipicted by the urediniospore (bottom inset), uredinia (top inset) and wheat leaf infection; **B**: The sexual cycle beginns when telia grows below the leaf and give rise to teliospores (top inset) below the epidermis; mature teliospores undergo karyogamy and meiosis, and germinate to form promycelium which give rise to four haploid basidiospores; **C**: Basidiospores produce pycnia which appears yellow on the upper leaf surface of *Thalictrum* leaves; **D**: The pycnia (top inset) produce pycniospores and flexuous hyphae (bottom inset). The pycniospores fuse with a compatible pairs of opposite mating types ; **E** A dikaryotic aecium forms after fertilization (top inset). The aecium give rise to dikaryotic aecispores.

The *Pt* teliospores are able to endure hot and dry summers in Mediterranean climates and only infect the alternate host in autumn. Teliospore germination of *Pt* requires water and a temperature between 12-20^{0}C (Anikster, 1986).

2.3.4 History of *Pt* globally

2.3.4.1 The impact of *Pt*

Wheat leaf rust is one of the most common diseases of wheat. It has caused significant yield losses and contributed to epidemics in different regions of the world. *Pt* reduces yield by decreasing the number of kernels per head and the weight of individual kernels (Bolton *et al.*, 2008). Early infections of leaf rust on wheat may account for 7 to over 50% yield loss depending on how early the infection starts (Huerta-Espino *et al.*, 2011).

In the USA, yield losses to leaf rust were estimated to be over three million tons during the period 2000-2004 (Singh *et al.*, 2004). Cultivars Atil C2000 and Altar C84 had losses of 27.5% and 29.7%, respectively in northwestern Mexico in 2001-2002. At least $32 million worth of grain yield was lost during 2000-2003 because of the epidemics caused by race BBG/BN on durum wheat. In 1997, an epidemic in Canada on three spring cultivars had yield losses ranging from 5-20% (Kolmer, 1999). In southern Sonora Mexico, leaf rust caused an estimated $40 million yield loss during 2008-2009 (Huerta-Espino *et al.*, 2011). However, leaf rust has not caused any devastating epidemic on bread wheat for the past two decades because of the use of cultivars with slow rusting resistance in Mexico (Huerta-Espino *et al.*, 2011).

In South America, the annual fungicide costs to control leaf rust were estimated to be more than $50 million (Huerta-Espino *et al.*, 2011). In the southern Cone region of South America, yield losses can potentially be greater than 50% in areas with conclusive wheather conditions if fungicides are not applied. The yield losses attributed to leaf rust in South America between 1996-2003 were estimated to be $172 million (Germán *et al.*, 2004).

Leaf rust has historically been a major problem in Central, South, South-East and West Asia (Roelfs *et al.*, 1992). It has been estimated that leaf rust causes three million tons yield loss annually in China (Huerta-Espino *et al.*, 2011). Severity of leaf rust in China is usually between 10-30%, but can be greater than 60% in some regions. Severity in Pakistan has varied between 40-50%, with 100% severity on susceptible hosts (Hassan *et al.*, 1973). Pakistan had 10% production loss in 1978 due to an intense leaf rust epidemic leading to $86 million loss (Hussain *et al.*, 1980). Leaf rust has the potential to cause approximately 30% damage to 21 million ha of wheat grown in West Asia (Huerta-Espino *et al.*, 2011).

Leaf rust in Australia has been a constant problem. Leaf rust was estimated to reduce yield by more than 10% (Keed and White, 1971; Rees and Platz, 1975). Leaf rust often occurs in northern New South Wales (NSW) and Queensland (Huerta-Espino *et al.*, 2011). Western Australia experienced irregular epidemics between 1990-2000. Varieties Triller (*Lr26*) and Marombi (*Lr37*) had localized sudden occurrences of leaf rust in 1998 and 1999, and 2005 respectively. Leaf rust was estimated to cause $12 million in yield losses annually in Australia (Murray and Brennan, 2009).

Leaf rust was a major problem mainly in north Africa (Roelfs *et al.*, 1992), causing up to 50% yield losses in Egypt and 30% yield losses in Tunisia (Adbel-Hak *et al.*, 1980; Deghais *et al.*, 1999). In the 1980s in South Africa, localised leaf rust epidemics occurred in the Western Cape province and on irrigated wheat (Pretorius *et al.*, 1987). It has been predicted that *Pt* could cause more than 50% yield losses on susceptible cultivars (Boshoff *et al.*, 2002a).

2.3.4.2 Origin of *Pt*

Pt is thought to have originated from the Fertile Crescent region of southwest Asia where there is an overlap of alternate and primary hosts (D'Oliveira and Samborski, 1966; Wahl *et al.*, 1984). Southwest Asia is where the origin of wheat overlaps with the geographical range of *T. speciossissimum* (Chester, 1946). Marková and Urban (1977) proposed that *Pt* is heteroecious on

various hosts, but can live without an alternate host in some countries. This led to the supposition that the ancestor of modern leaf rust originated at the center of hexaploid wheat evolution, which is the Near East within the Mediterranean region.

2.3.5 The situation of *Pt* in South Africa

2.3.5.1 Development of the *Pt* population in South Africa

Twenty *Pt* races have been identified in South Africa since the 1980s (Pretorius *et al*., 2007; Terefe *et al*., 2014a; b). Nine races were initially detected when leaf rust surveys were initiated in 1980. Since then, the composition of the leaf rust population changed due to both foreign introductions into the country, as well as the development of new races by asexual reproduction.

Races 3SA126, 3SA132 and 3SA133 have been present in South Africa since the early 1980s (Pretorius *et al*., 1987). 3SA126 has also been detected in Zambia and Zimbabwe (Pretorius and Purchase, 1990). Race 3SA133 has also been detected in Malawi (Pretorius and Purchase, 1990). Races 3SA126 and 3SA132 had a similar virulence/avirulence profile. These races differed in that 3SA132 had virulence to *Lr24* (Table 2.3). At genetic level 3SA133 is different from 10 other South African races (Figure 2.7). Race 3SA133 has been speculated to be a foreign introduction (Visser *et al*., 2012).

Race 3SA140 was first detected in South Africa in 1987 (Pretorius and Le Roux, 1988) and was similar to 3SA132, save for virulence to *Lr26* (Table 2.3). On genetic level, they were 100% similar (Figure 2.7) suggesting that 3SA140 probably developed in South Africa from 3SA132.

Race 3SA137 was first detected in South Africa in 1988 (Pretorius *et al*., 1990; Terefe, *et al*., 2014a), as well as in Zambia and Zimbabwe (Pretorius and Purchase, 1990). 3SA137 differed from 3SA126 with added virulence for *Lr24* and *Lr26*. Race 3SA126 was proposed to have given

Table 2.3: Virulence/avirulence profiles of *Pt* races which impacted the composition of the *Pt* population in South Africa detected between 2006 and 2010.

Race	Avirulence/ virulence profile	Reference
3SA122 (3SA61)=3SA57	*Lr1, 2a, 2b, 3ka, 11, 15, 17, 20, 24, 26, 30/Lr2c, 3a, 3bg, 10, 14a, 16*	Pretorius *et al.,* 1990
3SA126	*Lr3a, 3bg, 3ka, 9, 11, 16, 18, 24, 26, 30/1, 2a, 2b, 2c, 10, 14a, 15, 17, 20, 23, 28, B*	Terefe *et al.*, 2014a
3SA132	*Lr3a, 3bg, 3ka, 9, 11, 16, 18, 26, 30/1, 2a, 2b, 2c, 10, 14a, 15, 17, 20, 23, 24, 28, B*	Terefe *et al.*, 2014a
3SA133	*Lr2a, 2b, 9, 15, 16, 17, 18, 23, 26/1, 2c, 3a, 3bg, 3ka, 10, 11, 14a, 20, 24, 28, 30, B*	Terefe *et al.*, 2014a
3SA134	*Lr3a, 3bg, 3ka, 10, 11, 16, 20, 24, 26, 30/Lr1, 2a, 2b, 2c, 14a, 15, 17*	Pretorius and Le Roux, 1988
3SA137	*Lr3a, 3bg, 3ka, 9, 11, 16, 18, 24, 30/1, 2a, 2b, 2c, 10, 14a, 15, 17, 20, 23, 26, 28, B*	Terefe *et al.*, 2014a
3SA140	*Lr3a, 3bg, 3ka, 9, 11, 16, 18, 30/1, 2a, 2b, 2c, 10, 14a, 15, 17, 20, 23, 24, 26, 28, B*	Terefe *et al.*, 2014a
3SA144	*Lr3a, 3bg, 3ka, 9, 10, 11, 16, 18, 26, 30/1, 2a, 2b, 2c, 14a, 15, 17, 20, 23, 24, 28, B*	Terefe *et al.*, 2014a
3SA145	*Lr1, 2a, 2b, 2c, 9, 11, 16, 18, 20, 23, 24/3a, 3bg, 3ka, 10, 14a, 15, 17, 26, 28, 30, B*	Terefe *et al.*, 2014a
3SA146	*Lr 2a, 2b, 2c, 3ka, 9, 11, 16, 18, 20, 24, 28, 30/1, 3a, 3bg, 10, 14a, 15, 17, 23, 26, B*	Terefe *et al.*, 2014a
3SA147	*Lr1, 2a, 9, 11, 16, 24, 26/ 2c, 3, 3ka, 10, 14a, 17, 18, 30, B*	Terefe *et al.*, 2014b

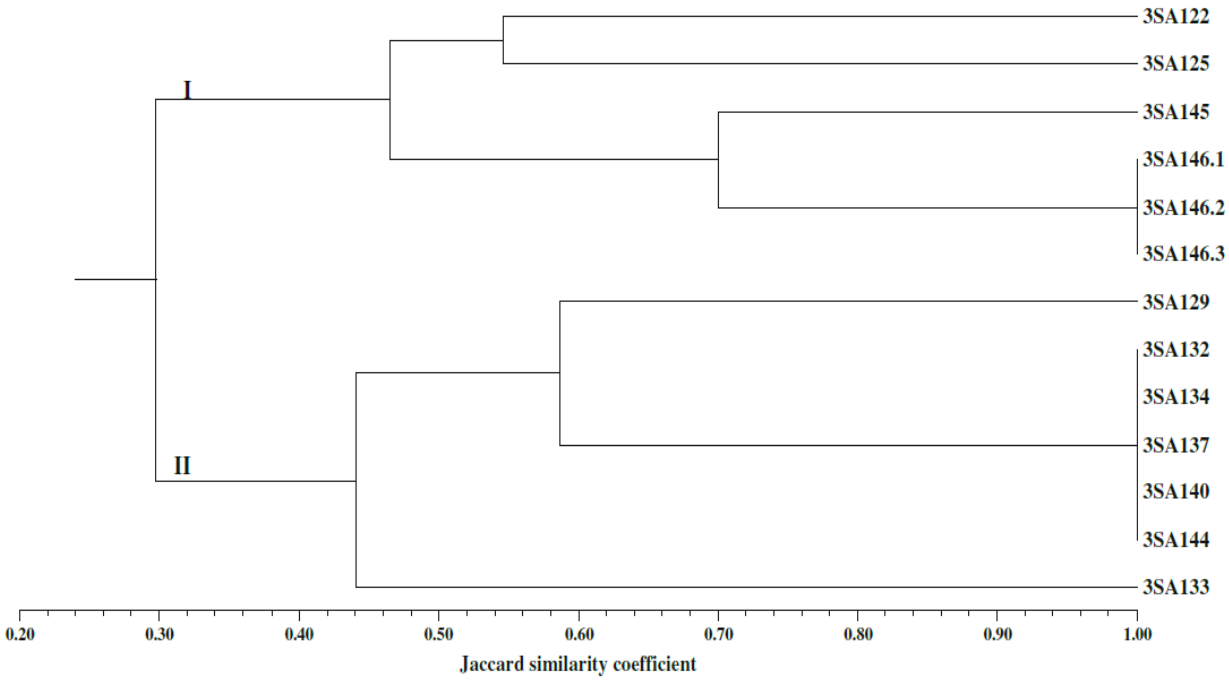

Figure 2.7: Dendrogram showing the genetic similarity of South African *Pt* races (Terefe *et al.*, 2014a).

rise to 3SA137 (Visser *et al.*, 2012).

Race 3SA144 was first detected in South Africa in 2005 in the Western Cape province (Pretorius and Bender, 2010). It was genetically identical to 3SA132, 3SA134, SA137 and 3SA140 (Figure 2.7) which correlates with the fact that they share virulence to 11 *Lr* genes (Terefe *et al.*, 2014a). Race 3SA144 was proposed to have originated from race 3SA132, 3SA134, 3SA137 or 3SA140 (Visser *et al.*, 2012).

Race 3SA145 was detected in the eastern and western Cape in 2009 (Terefe *et al.*, 2011). It shared a genetic relationship with race 3SA146 (Figure 2.7). It became the dominant race in 2010 (Terefe *et al.*, 2014a). The race is proposed to be an exotic introduction sharing genetic similarity with several European races.

Race 3SA146 was detected in South Africa in 2010 in KwaZulu-Natal, eastern Cape and Free State (Terefe *et al.*, 2014a). Race 3SA146 shared 70% genetic similarity with 3SA145 (Figure 2.7) but differed from 3SA145 with *Lr1*, *Lr3ka*, *Lr23*, *Lr28* and *Lr30* virulence (Table 2.3) suggesting a common ancestor. As with 3SA145, 3SA146 was considered to be a foreign introduction.

Finally, race 3SA147 was detected in South Africa in 2010 in the western Cape (Terefe *et al.*, 2014b). Similar races have been detected in Gwebi, Zimbabwe (Pretorius *et al.*, 2015). Race 3SA147 is thought to be a foreign incursion that shared high genetic similarity with 3SA145 and 3SA146 (Terefe *et al.*, 2014b).

2.3.5.2 The impact and frequencies of *Pt* races in South Africa

The year 2009 marked a change in the *Pt* population when the newer South African races were detected in significant proportions, while some of the older races disappeared (Table 2.4). Races 3SA126, 3SA132 and 3SA137 decreased until they were no longer detected in 2010, while 3SA144 decreased to less than 1% in 2010 (Table 2.4). While 3SA133 was dominant from 1997-

Table 2.4: Occurrence of *Pt* races in South Africa from 2006 to 2010. The prevalence is presented in percentages.

	Terefe, 2007	Terefe *et al.*, 2009	Terefe *et al.*, 2014a		
Race	**2006**	**2007**	**2008**	**2009**	**2010**
3SA126	0	11	5.1	2.7	0
3SA132	0	3.7	3.9	2	0
3SA133	54.1	76.8	78.2	33.8	25.7
3SA137	0	1.2	5.1	20.9	0
3SA140	45.8	7.3	7.7	6.8	5.2
3SA144	0	0	0	10.8	0.7
3SA145	0	0	0	23	47.1
3SA146	0	0	0	0	21.3
Total	**99.9**	**100**	**100**	**100**	**100**

2007 (Terefe, 2007; Terefe *et al.*, 2009), its prevalence decreased with 3SA145 and 3SA146 becaming the dominant races in 2010 (Table 2.4). Races 3SA1345 and 3SA146 poses a risk to 27% of South African wheat breeding lines and cultivars which were resistant to 3SA133 (Terefe *et al.*, 2014a). This is because the breeding lines are susceptible to 3SA145 and 3SA146.

2.4 Monitoring fungal pathogen populations

2.4.1 Surveys

During annual surveys investigators travel through the country to search for rust infected wheat to collect samples (Le Roux and Rijkenberg, 1987; Komen, 2007). They identify isolates on a set of wheat differentials and keep a collection of these races so that they can use them to determine the resistance genes in the breeders' lines and test cultivars. From the data the occurrence and distribution of races can be determined. *Pgt* and *Pt* surveys are conducted annually in South Africa and the most recent published surveys were done during 2008 to 2010 (Terefe and Pretorius, 2010; Terefe *et al.*, 2014a).

2.4.2 Phenotyping: identification of races using virulence phenotypes

Phenotyping of fungal pathogens is done by determining the infection type of isolates on a defined wheat differential set. The wheat differentials are either near-isogenic lines each having a single resistance gene or they can have more than one resistance gene for a particular rust pathogen. The differentials used for both *Pgt* and *Pt* phenotyping are indicated in Table 2.5. The wheat differential set is infected with urediniospores of each isolate. After 14 days, the response of each differential to the isolate is scored using the 0-4 scale which depends on the sizes of uredinia (Roelfs *et al.*, 1992, Pretorius Z.A., personal communication). A score of 0 indicates that no symptoms were observed, 1 indicates a small uredinium with necrosis, 2 indicates medium sized uredinia surrounded by chlorosis or necrosis, 3 indicates medium sized uredinia without

Table 2.5: The recently published differential set used to detect stem rust and leaf rust races (Terefe *et al.*, 2010; 2014a).

Stem rust differential set		Leaf rust differential set	
Name of line	**Resistance gene**	**Name of line**	**Resistance gene**
ISr5-Ra	*Sr5*	**RL6003**	*Lr1*
ISr6-Ra	*Sr6*	**RL6016**	*Lr2a*
ISr7b-Ra	*Sr7b*	**RL6047**	*Lr2c*
ISr8a-Ra	*Sr8a*	**RL6002**	*Lr3a*
Barleta Benvenuto	*Sr8b*	**RL6010**	*Lr9*
ISr9a-Ra	*Sr9a*	**RL6005**	*Lr16*
W2402	*Sr9b*	**RL6064**	*Lr24*
ISr9d-Ra	*Sr9d*	**RL6078**	*Lr26*
Vernal	*Sr9e*	**RL6007**	*Lr3a*
Acme	*Sr9g*	**RL6053**	*Lr11*
W2691Sr10	*Sr10*	**RL6008**	*Lr17*
ISr11-Ra	*Sr11*	**RL6049**	*Lr30*
Renown	*Sr27*	**RL6019**	*Lr2b*
Einkorn	*Sr21*	**RL6042**	*Lr3bg*
Gamka	*Sr24*	**RL6004**	*Lr10*
Coorong triticale	*Sr27*	**RL6013**	*Lr14a*
Festiquay	*Sr30*	**RL6052**	*Lr15*
Gamtoos	*Sr31*	**RL6009**	*Lr18*
Zaragoza	*36*	**RL6092**	*Lr20*
***Agropyron intermedium* derivative**	*Sr44*	**RL6012**	*Lr23*
Entrelargo de Montijo	*SrEm*	**RL6079**	*Lr28*
Triumph 64	*SrTmp*	**RL6051**	*LrB*
McNair 701	*SrMcN*		
Kiewiet triticale	*SrKw*		
Satu triticale	*SrSatu*		

chlorosis and 4 large uredinia without chlorosis. The scoring system also includes characters like X which indicate variable sized uredinia dispersed randomly on a single leaf, Y which indicates variable sized uredinia increasing in size towards the leaf tip, and Z which indicates variable sized uredinia increasing in size towards the leaf base. The (;) indicates that no uredinia were present but hypersensitive necrotic or chlorotic flecks were visible. The (+) and (-) respectively indicate larger and smaller uredinia than usual. A new race is identified when its infection type profile differs from previously described races.

2.4.3 Genotyping

If the genetic variation of fungal pathogens is continuously monitored, the co-evolutionary race between the pathogen and the host, and the genetic diversity of the fungal pathogen can be understood (Aradhya *et al*., 2001).

There are two commonly used techniques to genotype fungal pathogens. Simple sequence repeats (SSRs) are DNA elements in eukaryotes with short sequences which appear in the genome as tandem repeats (Beckmann and Weber, 1992). SSRs are codominant, PCR-based markers that can be used to characterize the population. New SSR markers are continuously developed for studies on *Pt* and *Pgt* (Duan *et al*., 2003; Szabo, 2007; Szabo and Kolmer, 2007; Karaoglu *et al.,* 2013). They have previously been valuable in understanding the *Pgt* and *Pt* populations in South Africa (Visser *et al*., 2009; 2011; 2012a).

Single nucleotide polymorphisms (SNP) analysis is another valuable PCR-based tool to discriminate between individuals that are genetically similar. A SNP is a DNA locus in which a single nucleotide is different between two individuals (Kwok and Gu, 1999).

By early 1990, new technologies like TaqMan® probe chemistry were developed to be used in Real-time or quantitative PCR (qPCR) (Holland *et al*., 1991). The qPCR reaction requires allele specific fluorescently-labelled TaqMan® probes, as well as PCR primers that will amplify the

particular locus. The probe is a short oligonucleotide labelled with a 5' reporter fluorophore and a 3' quencher. During the qPCR reaction, the probe binds to its specific allele. At this stage, no fluorescence is detected since fluorescence emitted by the reporter is absorbed by the quencher. However, during the subsequent extension of the primers, the 5'-3' exonuclease activity of the Taq DNA polymerase cleaves the probe thereby releasing the fluorophore which emits fluorescence when excited. The emission can be measured using a combined thermal cycler-fluorescence reader system.

2.5 Objectives of the research

The objectives of the study are:

2.5.1 To determine whether *Pgt* and *Pt* phenotypes correlate with genotypes and whether genotypes can be used to identify races. This will provide new information to breeders about the current status and population dynamics of *Pgt* and *Pt*.

2.5.2 To determine the population genetic structure of *Pgt* and *Pt* populations.

2.5.3 To determine whether SSR genotypes can show that there is an exchange of *Pt* inoculum between South Africa, Zimbabwe, Zambia and Malawi, since the exchange in inoculum between the four countries has been shown only on phenotypic level.

Chapter 3:

Materials and methods

3.1 Materials

3.1.1 Infected wheat tissue

Stem rust infected wheat leaf sheaths (2010-2012) and leaf rust infected wheat leaves (2013) collected during annual surveys in South Africa were provided by Dr T. Terefe (Agricultural Research Council-Small Grain Institute (ARC-SGI), Bethlehem, South Africa). The 2010 *Pgt* isolates were collected from the Western Cape (Tygerhoek, Langgewens, Koringberg, Malmesbury, Panorama, Riversdal, Porterville, Nietvoorbij, Oude Nektar, Dankbaar), Gauteng (Kleinfontein), Free State (Clarens, Harrismith, Reitz), Eastern Cape (Elliot) and KwaZulu-Natal (Winterton) provinces (Appendix 1, Table 1). The 2011 *Pgt* isolates were from the Western Cape (Tygerhoek, Langgewens, Loskop, Koringberg, Rietpoel/Alpha, Roodebloem, Voorstekop), KwaZulu-Natal (Makhathini, Cedara), and Eastern Cape (Langkloof) provinces (Appendix 1, Table 2). The *Pgt* isolates of the 2012 survey were all collected from the Western Cape regions. The 2013 leaf rust samples were collected in the Western Cape (Blydskap, Bredasdorp, De Vlei, Langgewens, Melkboem, Panorama, Riversdal, Tygerhoek, Voorstekop) (Appendix 1, Table 4). Single pustule *Pt* isolates were collected in Zimbabwe (Zim001-12; Z13-1), Zambia (Zam12-003) and Malawi (Mal1/1; Mal2/1). All controls represented single pustule isolates for *Pgt* (Visser *et al*., 2011) and *Pt* (Visser *et al*., 2012a).

3.2 Methods

3.2.1 Infection type determination

The infection type analysis of leaf and stem rust isolates was done by Dr. Terefe (Terefe *et al*., 2010; 2014a). It is included here as background information. A standard set of wheat differentials was used for both *Pgt* and *Pt*. The standard differential set for stem rust included (Table 2.5): ISr5-Ra (*Sr5*), ISr6-Ra (*Sr6*), ISr7b-Ra (*Sr7b*), ISr8a-Ra (*Sr8a*), Barleta Benvenuto (*Sr8b*), ISr9a-Ra (*Sr9a*), ISr9d-Ra (*Sr9d*), Vernal (*Sr9e*), Acme (*Sr9g*), W2691Sr10 (*Sr10*), ISr11-Ra (*Sr11*), Renown

(*Sr17*), Gamka (*Sr24*), Coorong triticale (*Sr27*), Festiquay (*Sr30*), Gamtoos (*Sr31*), Zaragoza (*Sr36*), VPM1 (*Sr38*), *Agropyro intermedium* derivative (*Sr44*), Entrelago de Montijo (*SrEm*), Triumph 64 (*SrTmp*), McNair 701 (*SrMcN*), Kiewiet triticale (*SrKw*), Satu triticale (*SrSatu*), W2691 (*Sr9b*), Cns_T_Mono_deriv (*Sr21*) and Matlabas (*SrWeb*) (Terefe *et al*., 2010; T. Terefe, personal communication). The leaf rust standard differential set included the following near isogenic lines: RL6003 (*Lr1*), RL6016 (*Lr2a*), RL6047 (*Lr2c*), RL6002 (*Lr3a*), RL6007 (*Lr3ka*), RL6010 (*Lr9*), RL6053 (*Lr11*), RL6005 (*Lr16*), RL6008 (*Lr17*), RL6064 (*Lr24*), RL6078 (*Lr26*), RL6049 (*Lr30*) (Long and Kolmer, 1989), RL6019 (*Lr2b*), RL6042 (*Lr3bg*), RL6004 (*Lr10*), RL6013 (*Lr14a*), RL6052 (*Lr15*), RL6009 (*Lr18*), RL6092 (*Lr20*), RL6012 (*Lr23*), RL6079 (*Lr28*) and RL6051 (*LrB*) (Kolmer *et al*., 2009; McCallum and Seto-Goh, 2009; Huerta-Espino *et al*., 2011). The infection types were determined on seedlings according to the 0-4 scale (Roelfs *et al*., 1992).

3.2.2 DNA extraction and testing of DNA quality

DNA was extracted from infected freeze dried wheat tissue according to Saghai-Maroof *et al*. (1984). Rust infected plant tissue was homogenized to a fine powder in 2-ml microcentrifuge tubes containing two metal balls using a TissueLyzer (Qiagen, Venlo, Netherlands) for 30 sec at a speed 30 r/s. To each sample, 750 µl Cetyltrimethylammonium bromide (CTAB) buffer [100 mM Tris (hydroxymethyl)-aminomethane hydrochloride (Tris-Cl) pH 8.0, 20 mM Ethylene-diaminetetraacetic acid (EDTA) pH 8.0, 1.4 M NaCl, 2% (w/v) CTAB and 0.2% (v/v) β-mercapto-ethanol] was added and the samples incubated at 65^{0}C for 1 h. This was followed by extraction with 500 µl chloroform/isoamylalcohol (24:1 v/v). The samples were centrifuged for 10 min at 4^{0}C at 12 000 *g*. The aqueous phase was transferred to another microcentrifuge tube and the DNA precipitated with 500 µl isopropanol at room temperature for 20 min. After centrifugation for 10 min at 12 000 *g* at 4^{0}C, pellets were washed with 500 µl 70% (v/v) ice-cold ethanol at room temperature for 20 min. Samples were then centrifuged at 4^{0}C for 5 min at 12 000 *g* and pellets air-dried for 1 h. The DNA was resuspended overnight at 4^{0}C in 200 µl Tris-

Cl EDTA (TE) buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA). RNA was removed with RNase A (0.1 mg/ml) at 37^{0}C for 30 min. The DNA was then extracted with 0.68 M ammonium acetate and 200 µl chloroform/isoamylalcohol (24:1 v/v) as described. The DNA was finally precipitated from the aqueous phase with 500 µl 100% (v/v) ice-cold ethanol by incubating it overnight at -20^{0}C. Samples were centrifuged for 15 min at 12 000 *g* at 4^{0}C and the pellet washed once with 500 µl 70% (v/v) ice-cold ethanol and centrifuged for 10 min at 12 000 *g* at 4^{0}C. The DNA was finally dissolved overnight in 50 µl TE buffer at 4^{0}C. The DNA concentration was determined using the Nanodrop-2000 spectrophotometer (Thermo Scientific, Massachusetts, United States).

3.2.3 Gel electrophoresis of extracted DNA

The integrity of isolated DNA was confirmed on a 2% (w/v) agarose gel prepared with 0.5 x TAE buffer (20.14 mM Tris-Cl, 0.058% (v/v) glacial acetic acid, 0.5 mM EDTA) (Sambrook *et al.*, 1989). Each 100 ng genomic DNA sample was mixed with loading buffer [0.25% (w/v) Bromophenol blue, 15% (w/v) Ficoll] and loaded on the agarose gel. The DNA was separated for 30 min at 10 V/cm in 0.5 x TAE running buffer and the gel photographed with a gel documentation system (Bio-Rad, California, United States).

3.2.4 Genetic analysis of *Pgt* isolates

Stem rust isolates were analyzed using a two-step procedure to first distinguish between Ug99 and non-Ug99 isolates. The Ug99 isolates were then identified using a qPCR based SNP assay, while non-Ug99 isolates were identified with SSRs.

3.2.4.1 qPCR analysis to distinguish between Ug99 and non-Ug99 *Pgt* isolates

Ug99 isolates were identified using four (5111, 9406, 13470 and 18022) experimental primer/probe combinations developed by Dr. L.J. Szabo (L.J. Szabo, USDA-ARS, USA,

unpublished data). All four probes were designed using *Pgt* genome sequencing data to detect SNP differences between Ug99 and non-Ug99 isolates. Each 10 µl reaction contained 40 ng genomic DNA from experimental samples or 10 ng genomic DNA from control samples, 0.4 µM of each Taqman probe, 1 µM of each primer and 1 x concentration of Kapa Probe Fast Mix (Kapa Biosystems, Cape Town, South Africa). The primer sequences were not included because they were not published at the time of the study. Included in each reaction was the experimental probe labelled with Fam (6-carboxy-fluorescein) and an actin reference gene probe labelled with Hex (6-Carboxy-2,4,4,5,7,7-hexachlorofluorescein succinidyl ester) at the same concentrations. The amplification regime was 95^{0}C for 1 min, followed by 40 cycles of 95^{0}C for 10 sec, 60^{0}C for 1 min and 72^{0}C for 1 min in a C1000 thermal cycler with CFX96 real time attachment (Bio-Rad). Three control samples were included for each experiment, namely genomic DNA from Ug99 race TTKSF (2SA88), non-Ug99 race 2SA4 and a no template control.

3.2.4.2 qPCR identification of Ug99 positive isolates

Putative Ug99 positive isolates were genotyped using eight SNP primer/probe combinations (A003, A005, A007, A010, A012, A021, A022 and A031) that were developed by Dr. L.J. Szabo (L.J. Szabo, USDA-ARS, USA, unpublished data). Each 10 µl reaction contained 40 ng genomic DNA for experimental samples or 10 ng genomic DNA for control samples, 0.4 µM of each Taqman probe, 1 µM of each primer and 1 x Kapa Probe Fast Mix. Each reaction mix contained two different probes labelled with Fam and Hex, respectively. The amplification regime was 95^{0}C for 10 min, followed by 40 cycles of 95^{0}C for 3 sec, 51^{0}C, 54^{0}C, 55^{0}C or 59^{0}C for 1 min and 72^{0}C for 10 sec. Different annealing temperatures where used to allow effective and clear amplification of the fragments. The genotype of each isolate was compared to five control samples which were 2SA88 (TTKSF), 2SA106 (TTKSP), 2SA107 (PTKST), 2SA88+ (TTKSF+), Ug99 (TTKSK) and a no template control.

Table 3.1: SSR primer pairs used in the study for genotyping non-Ug99 *Pgt* isolates (Karaoglu *et al.*, 2013).

Locus	Repeat motif	Primer sequences (5'-3')	Annealing temperatures (°C)
PgSUN7	$(ACA)_{18}(CAA)_{11}$	GCACCCCAAAGCAATACTAC CCCCTTTTTTTATTAACTTC	55
PgSUN11	$(AAAG)_5(A)_{26}$	GCGGTGCTCGTAGTTTAGAC ACCTCCCATTGTGATTTGATA	55
PgSUN20	$(AAAG)_9$	TTTGGGCAAGATCAGACCTA AGCGAAAAACAGGGTAAGGA	54
PgSUN27	$(AAAAG)_6$	GGGGGGTGACTGAAAAATA CCCTTTTCTACCTCCTTACTT	55
PgSUN30	$(TTCTG)_6$	ATTCGGGATGGCTAGAGACG CCCCCATAAAACAATCAATA	55
PgSUN31	$(TCCAT)_6(A)_{10}$	AGCCAACACGGTCGAATAAA GGACGGGAAAGAAAAGGAAA	55
PgSUN33	$(AAAAT)_8$	ATCCGCTGGTAAAGGTGAAA GGGGGGTGACTGAAAGATAA	54
PgSUN34	$(TTTTG)_8$	ATCCCCTCTCAGTCCAACAG AATCCCGCTCGGCAAGAAGT	54
PgSUN42	$(TTTGG)_{12}$	GCTGGGAATGGTTTATCTAA CACCAGAAGGGACTGATTAT	55
PgSUN45	$(ACAAC)_{13}$	ATCACATCCCTAACTACACA GCCCTTTTATTATTTCATTT	55

3.2.4.2 SSR analysis of non-Ug99 *Pgt* isolates and genotype naming system

Non-Ug99 isolates were genotyped using ten published SSR primer combinations developed by Karaoglu *et al.* (2013) (Table 3.1). The primer combinations included PgSUN7, PgSUN11, PgSUN20, PgSUN27, PgSUN30, PgSUN31, PgSUN33, PgSUN34, PgSUN42 and PgSUN45. Each 10 µl reaction contained 10 ng DNA, 1 µM of each primer and 1 x Kapa Taq Ready Mix.The temperature regime was 94^{0}C for 1 min, followed by 34 cycles of 94^{0}C for 30 sec, 54^{0}C or 55^{0}C for 30 sec, 72^{0}C for 30 sec and a final 72^{0}C step for 5 min. Control DNA samples that were included were 2SA4, 2SA100, 2SA102, 2SA88, 2SA104, 2SA105, 2SA103 and a no template control. The amplified DNA was first separated by agarose gel electrophoresis to confirm amplification (section 3.2.3).

All the non-Ug99 isolates in the study were renamed according to their genotypes. The naming system was based on the following: eg. SAG1 were SA=South Africa, G=genotype, 1= genotype numbering which started with the 2010 isolates and control isolates. The control isolates were named in the following manner: SAG1=2SA4, SAG3=2SA100/2SA105, SAG5=2SA102, SAG7=2SA103/2SA104 and SAG9=2SA88. The individual *Pgt* isolates were numbered differently e.g. S128-10, where S=stem rust, 128=sample number and 10=year the sample was collected. Individual isolates with the same genotype were given the same name eg. S52-10, S60-10, S103-10 and S119-10 was collectively named SAG6.

3.2.5 Genetic analysis of *Pt* isolates

3.2.5.1 SSR analysis of *Pt* races and isolates

Leaf rust isolates were genotyped using 18 described SSR primer combinations (Szabo and Kolmer, 2007; Wang *et al.*, 2010) (Table 3.2). The primers included PtSSR50, PtSSR55, PtSSR61, PtSSR76, PtSSR91, PtSSR92, PtSSR152, PtSSR158, PtSSR161, PtSSR164, PtSSR173, PtSSR186,

PtSSR0083, PtSSR5594, PtSSR0801, PtSSR0243, PtSSR0481 and PtSSR0639. Control DNA samples used were races 3SA122, 3SA125, 3SA129, 3SA132, 3SA133, 3SA134, 3SA137, 3SA140, 3SA144, 3SA145, 3SA146 and 3SA147. Each 10 µl reaction mix contained 45 ng genomic DNA for experimental samples and 25 ng for control samples, 1 µM of each primer and 1 x Kapa Taq Ready Mix. The temperature regime was 94^{0}C for 10 min, followed by 34 cycles of 94^{0}C for 10 sec, 58^{0}C or 60^{0}C for 1 mi and 72^{0}C for 10 sec. A final step of 72^{0}C for 5 min was included. The amplicons were first separated on an agarose gel to confirm succeful amplification (section 3.2.3).

3.2.6 Polyacrylamide gel electrophoresis and silver staining

All SSR samples were separated on a 5% (w/v) denaturing polyacrylamide gel [19:1 acrylamide : bisacrylamide, 7 M Urea, 1 x TBE buffer (89 mM Tris-borate pH 8.0, 2.0 mM EDTA)] for 1.5 h at 80 W using 1 x TBE as running buffer (Sambrook *et al.*, 1989). The 10 µl PCR reactions were mixed with 5 µl loading buffer [98% (v/v) de-ionized formamide, 10 mM EDTA pH 8.0, 0.5 mg/ml Bromophenol blue, 0.5 mg/ml Xylene cyanol]. Samples were denatured for 5 min at 95°C and placed on ice till 5 µl of each reaction mix was separated on the gel. After separation the gel was stained using the protocol provided by the manual of the Silver Sequence™ DNA Sequencing System (Promega, Madison, WI, USA). The stained gels were left to dry overnight and photographed using an Illford Multigrade IV RC paper by exposing it in light for 16 sec. A 25-bp DNA ladder (Promega) was used to determine the fragment lengths. The SSR amplicons generated for each sample were recorded in a data matrix as present (1) or absent (0).

3.2.7 Data analysis

3.2.7.1 Analysis of genetic diversity

The genetic relationships among non-Ug99 *Pgt* and *Pt* isolates were investigated separately using rooted dendrograms. The Ug99 isolates were not included in this investigation. The SIMQUAL

Table 3.2: SSR primer pairs used for genotyping *Pt* isolates (Szabo and Kolmer, 2007; Wang *et al.*, 2010).

Locus	Repeat motif	Primer sequences (5'-3')	Annealing temperature (°C)
PtSSR50	$(TC)_n$	CATCGGAATGGTCTGTCTCC CCAAATGCTATGAGTGGAAAA	60
PtSSR55	$(TC)_n$	AGCTTACGGTCCTCAATCG AGTGAAAGGGGCTGGGAGT	58
PtSSR61	$(TG)_n$	CGAACTGGTACAACGCACTG CGCAAAAAGGCTGATCTCTG	60
PtSSR76	$(TG)_n$	GGCGTCGTATTTCTCGTAGC TTCGGACTACTGGGTAAGCA	58
PtSSR91	$(TG)_n$	ATCTTGCGTCTCAGCCATCT CGCCGCTCTTCATCTCTTAC	60
PtSSR92	(TG)n	CCAAGGAACAGTCCACCAAG GAGTCGGGTAAGCCATCTGA	60
PtSSR152	$(TG)_n+(TG)_n+(TG)_n$	CTCCGTTCCTCTTTCTGTCG CCATCGCAACCAACAAACA	60
PtSSR158	$(AAC)_n$	GACGACTTCGTCACTGCTGA GAGGAGAAGCCGTTCTGTTG	60
PtSSR161	$(TC)_n$	ACTGCCTCCTGTGCCTTCT TAGTCCGAGGGTGACGAAGT	60
PtSSR164	$(TC)_n$	GTGGAAGTGAGCGGAAGAAG GGAGATGGGCAGATGAGGTA	60
PtSSR173	$(TC)_n+(TC)_n$	CTCAGCGACCTCAAAGAACC GAGACGACGGATGTCAACAA	58
PtSSR186	$(AAC)_n$	GCCACGAGAAATACATAGAAATAAA GGTTGTTGATGGGCTTGAGT	60
PtSSR0083	$(GA)_n$	ATGGATTTGGAGACCAGTCG GTTGAAAGATCTGGGGGTGA	60
PtSSR5594	$(GAT)_n$	CGGACCAAACACAAAGGAAA CCCTGCGTTTAACACCTTGT	60
PtSSR0801	$(TG)_n$	CAATGGTAGTGGCAAGCAAA GCACCTCTCACGCTCTTAGC	60
PtSSR0243	$(CA)_n+(AT)_n$	CTCACTCGCTCGCTTGTTCT GACGAAAAGATCGGGTTTGA	60
PtSSR0481	$(TTT)_n$	CCACAATCCTCCGTTCTGAT CGAAAGCAAAACACATGAGG	58
PtSSR0639	$(GAA)_n$	TCTCCGCCTACCAACACTG AAAGGAGGGAGAGGGGAGG	60

program was used to generate a similarity matrix according to Jaccard's similarity coefficient (Jaccard, 1908) using a numerical taxonomy and multivariate analysis system (NTSYSpc) version 2.20e software (Rohlf, 2000). The unweighted pair-group method with arithmetic averages (UPGMA) was used to perform a cluster analysis and draw a dendrogram using the sequential agglomerative hierarchical nested (SAHN) clustering method (Sokal and Michener, 1958). The dendrogram shows the genetic relationship between control and unknown DNA samples based on the difference in SSR alleles. To determine the goodness of fit of the data matrices, the cophenetic correlation coefficients were calculated using Matrix comparison (MXCOMP and COPH procedures) in NTSYSpc. The cophenetic correlation coefficient (*r*) is interpreted as very poor if $r < 0.7$, poor if $0.7 \leq r \leq 0.8$; good if $0.8 \leq r \leq 0.9$ or very good if $r \geq 0.9$. The correlation between SSR genotypes and virulence data was determined by comparing the similarity matrices of the genotypic and phenotypic data using MXCOMP.

The genetic relationship of non-Ug99 *Pgt* isolates was also investigated using an unrooted tree. The unrooted dendrogram was generated based on the unweighted neighbour-joining cluster analysis using DARwin 5.0.158 software (Perrier *et al.*, 2003). The multilocus allelic data for non-Ug99 isolates was used to calculate the simple matching genetic dissimilarity matrix according to:

$$dij = 1 - \frac{1}{L}\sum_{l=1}^{L}\frac{Ml}{\pi}$$

where *dij* indicates dissimilarity between genotype *i* and *j*, *L* indicates the number of loci, π indicates ploidy and *Ml* indicates the number of matching alleles for locus *l*.

3.2.7.2 Analysis of molecular variance and F-statistics

The ARLEQUIN 3.11 software was used to further investigate the structure of the *Pgt* and *Pt* populations using analysis of molecular variance (AMOVA) (Excoffier *et al.*, 2005). AMOVA is a DNA-based analysis of variance framework used to investigate the division of molecular

variation within a species. It was designed by Excoffier *et al.* (1992) and developed further by Michalakis and Excoffier (1996). It can be used to determine the correlation of haplotype diversity at variable levels of genetic and non-genetic hierarchical sub-populations by estimating the variance component within and between populations, and F-statistics analogues. AMOVA removes the necessity of the normality assumption because the significance of the variance components and F-statistics analogues is determined using permutation approaches.

F-statistics is defined as the measure of genetic differentiation in distinct sub-populations of a larger hierarchically structured population (Wright, 1951). A hierarchically structured population is one that can be separated into sub-populations that can be grouped into gradually nested levels in which, at each group, each level is included within a higher one (Hartl and Clark, 1997). The F-statistics explain the level of heterozygosity and thus differentiation in a population when compared to Hardy-Weinberg equilibrium (HWE) of random mating of gametes. FST is the total gene diversity or expected heterozygosity in the total population as estimated from the pooled allele frequencies. If the F_{ST} value is 0 there is no genetic variation between subdivided populations but when the F_{ST} value is equal to 1 then there is 100% genetic variation between different alleles of subdivided populations. The F_{ST} is affected by the balance between gene flow and random genetic drift, when it is assumed that mutation pressure in differentiated populations is similar and low. In this case the determined level of genetic differentiation, as measured by F_{ST}, reflects the expected level of population variation caused by both genetic drift and gene flow. The ARLEQUIN 3.11 software package and 16 000 permutations were used to test the significance of the F-statistics parameters and the division of the genetic variance components. F_{ST} is interpreted as low if $0.0 \leq F_{ST} \leq 0.05$, moderate when $0.05 \leq F_{ST} \leq 0.15$; high when $0.15 \leq F_{ST} \leq 0.25$ and very high when $F_{ST} \geq 0.25$ (Wright, 1951).

3.2.7.3 Determination of population structure

The genetic structure of the non-Ug99 *Pgt* and *Pt* populations in South Africa was determined according to the Bayesian model-based clustering method implemented in STRUCTURE 2.23 software. The exact number of sub-populations (K) that best fit the data and the number that minimizes the HWE and Linkage Equilibrium (LE) in those sub-populations were calculated by sampling genotypes and assuming HWE and LE in loci. With no prior information on population sampling design, the number of sub-populations can be calculated in STRUCTURE. STRUCTURE can be used to assign isolates to populations and identify admixed and migrated isolates. The admixture model was used for simulations in the study. The admixture model permits some individuals to have a mixture of ancestors and determines the fraction of the individual's genome that can be traced to the estimated K populations.

The basic admixture model was used to calculate the exact number of sub-populations. The standard method of Evanno *et al.* (2005) was used to accurately determine the 'true K' for successive K values (K=2 to K=10) using the *ad hoc* statistic Δ*K*. This serves as a basis for the second order rate of change P(X | K), the probability of the data with reference to the given K. Evanno *et al.* (2005) suggested that the real number of groups in a population is best detected by using the modal value of the distribution of Δ*K*. A plot showing the Δ*K* values against successive K values was drawn by using the 'true K', which was indicated by the highest P(X | K).

The correlated allele frequencies and basic admixture model with separated loci were used to draw the barplot. The assumed number of populations ranged from one to 10. The Δ*K* was calculated using ten replicate runs per *K* value. A total of 50 000 Monte Carlo Markov Chain (MCMC) iterations and burn-in period length were used. The analysis was repeated with the calculated Δ*K* using both burn-in period and MCMC replications of 900 000. The alpha and allele frequency model were inferred using the data. There was correlation of allele frequencies and lambda was set to one.

3.2.7.4 Inference of occurrence of ancestral and evolutionary trends

The genetic structure of the non-Ug99 isolates was analyzed by constructing a minimal spanning network using the median joining procedure in Network 4.5.0.0 software. To deduce the ancestral evolutionary events, the genetic distances separating individuals and the population increases can be detected by using the minimum spanning network (Bandelt *et al.*, 1995; 1999). The median joining network procedure warrants construction of higher-resolution networks from allelic data. To determine the ancestral node of the network, the network nodes were compared with out-groups.

3.2.7.5 Analysis of agreement of the genotypic and phenotypic classification for the identification of stem and leaf rust races

The accuracy of identifying *Pgt* and *Pt* isolates was assessed through analysis of conditional probabilities similar to the sensitivity and specificity of diagnostic tests. Procedure FREQ of the

Statistical Analysis System (SAS) was used for all analyzes.

The estimation of certain conditional probabilities is a standard statistical method used to assess the agreement between two methods of classification. Normally, one of the two methods, for instance Methodology 1, is thought to be the Gold standard or the established method. The conditional probabilities are calculated that Methodology 2 race classification agrees with Methodology 1 race classification, on the condition that Methodology 1 race classifications are correct. The conditional probabilities are universal and are therefore independent of the prevalence of the Methodology 1 races. Thus other researchers are able to check study results expressed in terms of those conditional probabilities using their own sample of specimens, even if that sample has a different prevalence of Methodology 1 races. In contrast, the simple (unconditional) probability that the Methodology 1 classification agrees with Method 2

classification, depends on the prevalence of the Methodology 1 races in the particular sample of specimen.

In the present study the phenotypic classification (infection type analysis method) was chosen as the Gold standard since it is a well-established method. The following conditional probabilities were calculated (together with 95% confidence intervals for those probabilities to indicate the reliability of the estimates):

Prob (genotype = race X|phenotype = race X)

Prob (genotype = not X|phenotype = not race X)

The two conditional probabilities could be referred to as sensitivity and specificity, of genotypic classification. It must be noted that isolates were included in the statistical analysis only if both their genotypic and phenotypic classifications detected only one race. Samples which were not included were those that had two race phenotypes, that were not phenotyped, failed to be phenotyped or failed to be genotyped.

Chapter 4:

Results

4.1 The integrity of extracted genomic DNA

Genomic DNA extracted from wheat infected with *Pgt* and *Pt* field isolates was separated on agarose gels to check its quality and integrity. All samples had fragmented DNA (Figure 4.1). The reason for this fragmentation was probably because samples were dried and stored before extraction, resulting in breakdown. The genomic DNA yields for leaf rust were 12.5-718 ng/µl while the OD260 values ranged between 0.251-14.366 units. The concentration for stem rust was between 3-498.4 ng/µl and OD260 ranged between 0.058-15.46 units.

4.2 DNA-based identification of *Pgt* isolates collected during the 2010-2012 surveys

4.2.1 Discrimination of Ug99 isolates from non-Ug99 isolates

To distinguish the Ug99 *Pgt* field isolates from the non-Ug99 isolates, the qPCR based detection procedure using four primer/probe combinations developed by Dr. L.J. Szabo was used. When all four experimental amplicons showed unambiguous amplification in each reaction for a specific isolate, it was taken as positive for Ug99. This amplification was detected from the fluorescence emitted by the degraded probe in the reaction (Figure 4.2a). When one or more of the amplicons failed to amplify, the isolate was scored as non-Ug99 (Figure 4.2b). The positive amplification of the internal actin control confirmed that each PCR reaction worked.

4.2.1.1 Discrimination between Ug99 and non-Ug99 isolates collected during 2010-2013 surveys

A total of 86 *Pgt* field isolates collected during the 2010 *Pgt* survey were characterized. Of these, one isolate failed to amplify (Table 4.1), while 64 were characterized as Ug99 and 21 as non-Ug99.

There were 58 *Pgt* samples collected during the 2011 survey (Table 4.2). One DNA extraction

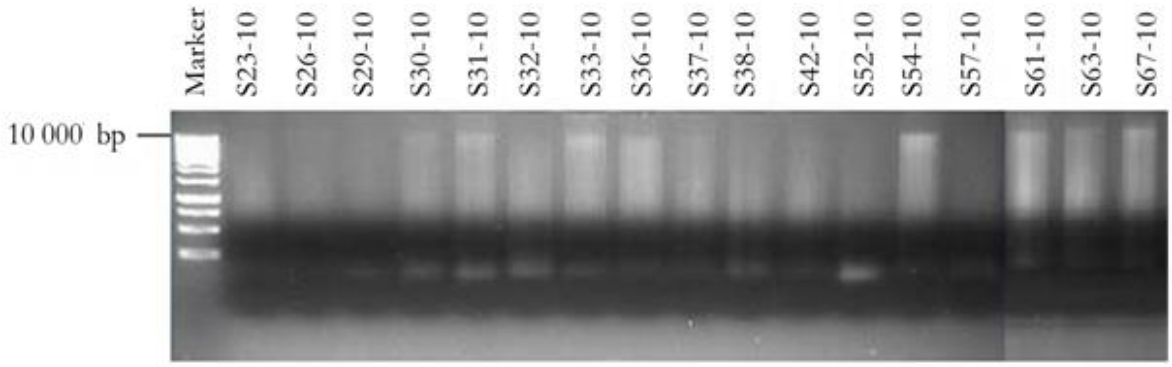

Figure 4.5: Genomic DNA extracted from 17 South African *Pgt* field isolates. The DNA ladder is indicated to the left.

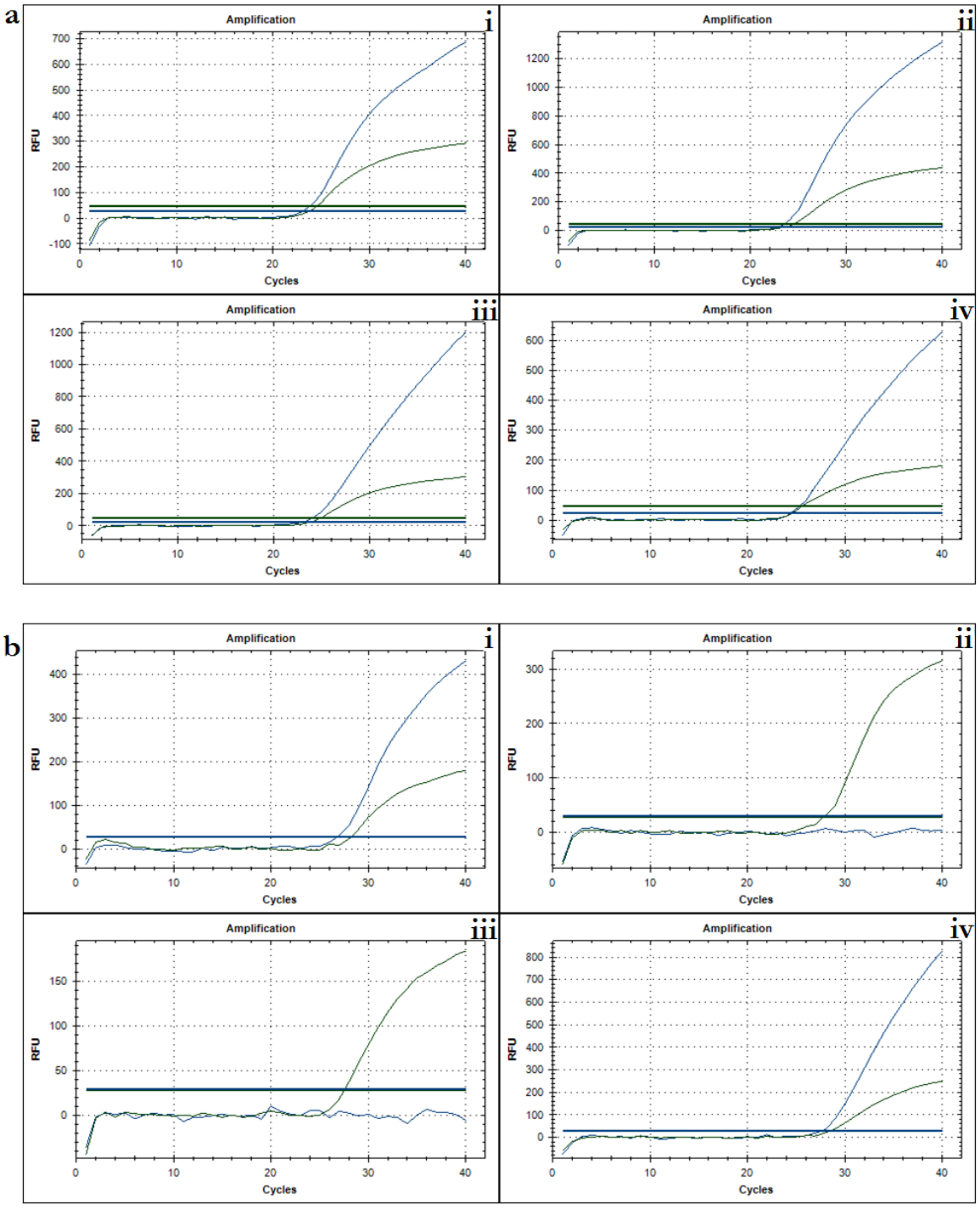

Figure 4.26: The amplification profiles of a Ug99 positive (a) and Ug99 negative isolate (b). The experimental amplicons for primer/probe combinations P5111 (i), P9406 (ii), P13470 (iii) and P18022 (iv) are indicated with a blue line and the actin control with green. RFU=relative fluorescence units.

Table 4.1: Discrimination between Ug99 and non-Ug99 *Pgt* field isolates collected during the 2010 survey

Isolate name	qPCR identification	Isolate name	qPCR identification	Isolate name	qPCR identification
S1-10	Ug99	**S57-10**	Non-Ug99	**S118-10**	Non-Ug99
S2-10	Ug99	**S58-10**	Ug99	**S119-10**	Non-Ug99
S4-10	Ug99	**S60-10**	Non-Ug99	**S120-10**	Ug99
S5-10	Ug99	**S61-10**	Non-Ug99	**S121-10**	Ug99
S6-10	Ug99	**S63-10**	Non-Ug99	**S122-10**	Ug99
S7-10	Ug99	**S64-10**	Non-Ug99	**S123-10**	Non-Ug99
S10-10	Ug99	**S65-10**	Ug99	**S124-10**	Ug99
S11-10	Ug99	**S66-10**	Ug99	**S127.1-10**	Ug99
S12-10	Ug99	**S67-10**	Ug99	**S128-10**	Non-Ug99
S18-10	Ug99	**S68-10**	Ug99	**S130-10**	Non-Ug99
S22-10	Ug99	**S70-10**	Non-Ug99	**S132-10**	Ug99
S23-10	Ug99	**S72-10**	Ug99	**S133-10**	Ug99
S24-10	Ug99	**S75-10**	Ug99	**S134-10**	Ug99
S25-10	Ug99	**S76-10**	Ug99	**S135-10**	Ug99
S26-10	Ug99	**S81-10**	Ug99	**S137-10**	Ug99
S27-10	Ug99	**S83-10**	Ug99	**S138-10**	Ug99
S29-10	Ug99	**S86-10**	Non-Ug99	**S139-10**	Ug99
S30-10	Ug99	**S91-10**	Non-Ug99	**S142-10**	Ug99
S31-10	Ug99	**S92-10**	Ug99	**S144-10**	Ug99
S32-10	Ug99	**S93-10**	Ug99	**S146-10**	Ug99
S33-10	Ug99	**S94-10**	Ug99	**S148-10**	Non-Ug99
S34-10	Non-Ug99	**S96-10**	Ug99	**S153-10**	Ug99
S35-10	No amplification	**S99-10**	Ug99	**S154-10**	Ug99
S36-10	Ug99	**S100-10**	Ug99	**S155-10**	Ug99
S37-10	Ug99	**S103-10**	Non-Ug99	**S156-10**	Ug99
S38-10.1	Ug99	**S105-10**	Ug99	**S157-10**	Ug99
S41-10	Non-Ug99	**S108-10**	Ug99		
S42-10	Ug99	**S109-10**	Non-Ug99		
S52-10	Non-Ug99	**S110-10**	Non-Ug99		
S54-10	Ug99	**S114-10**	Non-Ug99		

Table 4.2: Discrimination between Ug99 and non-Ug99 *Pgt* field isolates collected during the 2011 survey

Isolate name	qPCR identification	Isolate name	qPCR identification
S4-11	Ug99	**S33-11**	Ug99
S5-11	Ug99	**S34-11**	Ug99
S6-11	Ug99	**S35-11**	Ug99
S7-11	Non-Ug99	**S36-11**	Ug99
S8-11	Non-Ug99	**S37-11**	Ug99
S9-11	Ug99	**S38-11**	Ug99
S10-11	Non-Ug99	**S39-11**	DNA extraction failed
S11-11	Non-Ug99	**S39-11.2**	Ug99
S12-11	Non-Ug99	**S40-11**	Non-Ug99
S13-11	Non-Ug99	**S41-11**	Ug99
S14-11	Non-Ug99	**S42-11**	Ug99
S15-11	Ug99	**S43-11**	Non-Ug99
S16-11	Non-Ug99	**S44-11**	Ug99
S17-11	Ug99	**S45-11**	Ug99
S18-11	No amplification	**S46-11**	Ug99
S19-11	Ug99	**S47-11**	Ug99
S20-11	Non-Ug99	**S48-11**	Ug99
S21-11	Ug99	**S49-11**	Ug99
S22-11	No amplification	**S51-11**	Non-Ug99
S23-11	Non-Ug99	**S52-11**	Ug99
S24-11	Non-Ug99	**S53-11**	Ug99
S25-11	Non-Ug99	**S54-11**	Ug99
S26-11	Non-Ug99	**S55-11**	Non-Ug99
S27-11	Ug99	**S56-11**	Ug99
S28-11	Non-Ug99	**S57-11**	Ug99
S29-11	Non-Ug99	**S58-11**	Ug99
S30-11	Ug99	**S59-11**	Ug99
S31-11	Ug99	**S61-11**	Ug99
S32-11	Ug99	**S62-11**	Ug99

failed while two DNA samples failed to amplify. Fifty five isolates were successfully characterized of which 36 were Ug99 positive and 19 non-Ug99.

There were 105 samples collected in the 2012 survey (Table 4.3). Of these, 103 isolates were successfully characterized while two samples failed to amplify. Fifty two isolates were genotyped as Ug99 and 51 as non-Ug99.

4.2.2 Race specific identification of Ug99 positive isolates

To identify the Ug99 positive isolates using eight different SNP primer/probe combinations developed by Dr. L.J. Szabo, the genotype of each isolate was compared with that of the control races (Tables 4.4, 4.5, 4.6). Since stem rust produces dikaryotic urediniospores, each locus resulted in two alleles, one for each haploid chromosome. A heterozygote showed both Fam and Hex fluorescence (Figure 4.3a). Homozygotes were detected by either Fam or Hex fluorescence (Figure 4.3b). To be identified as a specific Ug99 race, the SNP genotype pattern for a field isolate had to be 100% similar with that of the control race. There were no SNP genotypes that did not match the genotypes of the control races.

4.2.2.1 SNP based race specific identification of Ug99 positive field isolates collected during the 2010-2012 surveys

The 64 Ug99 positive isolates collected during 2010 resulted in the detection of three different Ug99 race group members (Table 4.4). Genotyping of S105-10 failed because of insufficient DNA. A total of 44 Ug99 positive isolates had a similar genotype to 2SA88, 18 were similar to 2SA88+ and one was similar to 2SA106. The phenotypic data generated by Dr. Terefe in 2010 showed that of the 64 isolates, 53 were identified as 2SA88, four as 2SA105, five were mixed while two samples could not be identified (Table 4.4). The 2SA88+ phenotype was not detected phenotypically in 2010.

Of the 36 Ug99 positive isolates collected in 2011, 19 had a genotype similar to 2SA88, 16

Table 4.3: Discrimination between Ug99 and non-Ug99 *Pgt* field isolates collected during the 2012 survey

Isolate name	qPCR identification	Isolate name	qPCR identification	Isolate name	qPCR identification
S11-12	Non-Ug99	**S51-12**	Ug99	**S88-12**	Non-Ug99
S12-12	Non-Ug99	**S52-12**	Ug99	**S89-12**	Ug99
S13-12	Non-Ug99	**S53-12**	Ug99	**S90-12**	Ug99
S14-12	Non-Ug99	**S54-12**	Ug99	**S91-12**	Non-Ug99
S16-12	Non-Ug99	**S55-12**	Ug99	**S92-12**	Non-Ug99
S17-12	Non-Ug99	**S56-12**	Ug99	**S93-12**	Non-Ug99
S19-12	Non-Ug99	**S57-12**	Ug99	**S94-12**	Ug99
S20-12	Non-Ug99	**S58-12**	Non-Ug99	**S95-12**	Ug99
S21-12	Ug99	**S59-12**	Non-Ug99	**S96-12**	Non-Ug99
S22-12	Non-Ug99	**S60-12**	Ug99	**S97-12**	Non-Ug99
S23-12	Non-Ug99	**S61-12**	Non-Ug99	**S98-12**	Ug99
S24-12	Non-Ug99	**S62-12**	Non-Ug99	**S99-12**	Ug99
S25-12	Non-Ug99	**S63-12**	Ug99	**S100-12**	Ug99
S27-12	Non-Ug99	**S64-12**	Ug99	**S101-12**	Ug99
S28-12	Ug99	**S65-12**	Non-Ug99	**S102-12**	Ug99
S31-12	Non-Ug99	**S66-12**	Ug99	**S103-12**	Ug99
S32-12	Ug99	**S67-12**	Non-Ug99	**S104-12**	Ug99
S33-12	Non-Ug99	**S68-12**	Ug99	**S105-12**	Ug99
S34-12	Non-Ug99	**S69-12**	Ug99	**S106-12**	Ug99
S35-12	Non-Ug99	**S71-12**	Non-Ug99	**S107-12**	Ug99
S36-12	Ug99	**S72-12**	Non-Ug99	**S108-12**	Non-Ug99
S37-12	No amplification	**S73-12**	Ug99	**S109-12**	Non-Ug99
S38-12	Ug99	**S75-12**	Ug99	**S110-12**	Non-Ug99
S39-12	Non-Ug99	**S76-12**	Non-Ug99	**S111-12**	Non-Ug99
S40-12	Ug99	**S77-12**	Non-Ug99	**S112-12**	Non-Ug99
S41-12	Non-Ug99	**S78-12**	Ug99	**S113-12**	Ug99
S42-12	Ug99	**S79-12**	Non-Ug99	**S114-12**	Ug99
S43-12	Ug99	**S80-12**	Non-Ug99	**S115-12**	Non-Ug99
S44-12	Ug99	**S81-12**	Non-Ug99	**S116-12**	Non-Ug99
S45-12	Ug99	**S82-12**	Ug99	**S117-12**	Non-Ug99
S46-12	Ug99	**S83-12**	Ug99	**S118-12**	Non-Ug99
S47-12	Ug99	**S84-12**	No amplification	**S119-12**	Non-Ug99
S48-12	Ug99	**S85-12**	Ug99	**S120-12**	Non-Ug99
S49-12	Non-Ug99	**S86-12**	Ug99	**S121-12**	Non-Ug99
S50-12	Ug99	**S87-12**	Ug99	**S122-12**	Ug99

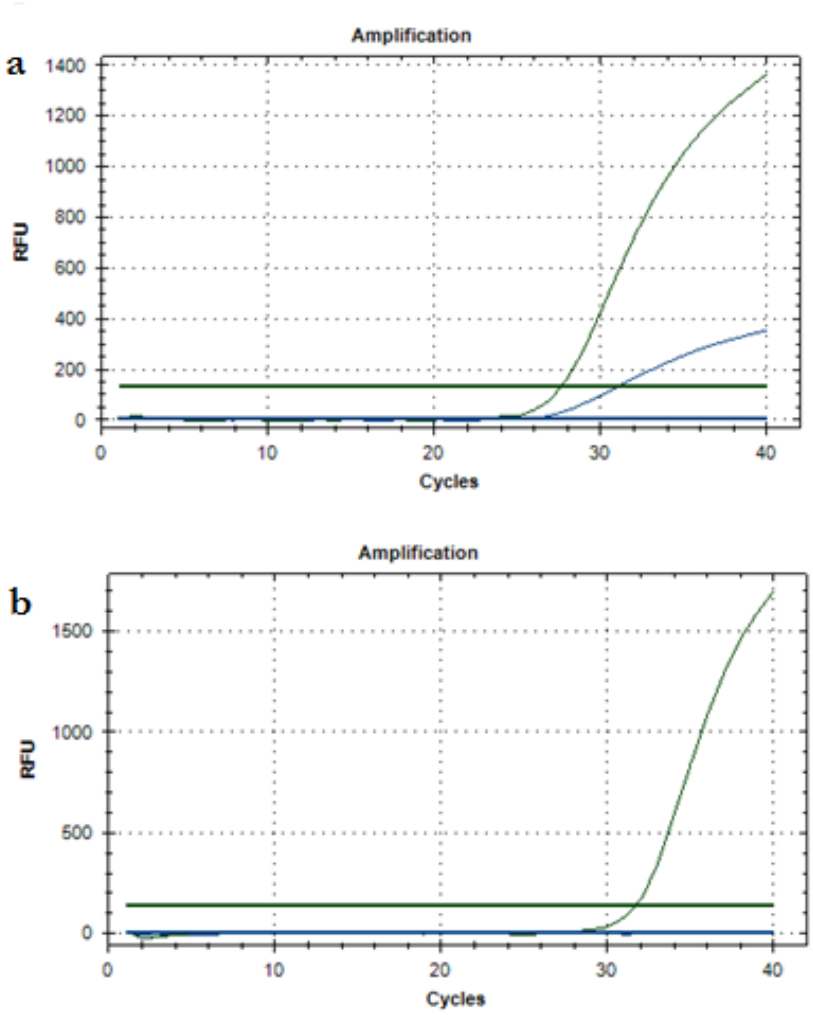

Figure 4.3: Genotyping of Ug99 positive field isolates using SNPs. Indicated in (a) is the amplification profile of a heterozygous isolate for locus A003 and in (b) the amplification of a homozygous isolate for locus A003. The amplification profile of allele 1 (Fam) is indicated in blue and that of allele 2 (Hex) in green. RFU=relative fluorescence units.

Table 4.4: Race specific identification of field isolates identified as Ug99 positive collected during the 2010 survey using SNP genotyping and infection type phenotyping

	Race identification results									
Isolate	**Phenotyping**	**Genotyping**	**SNP genotypes**							
			A003	A005	A007	A010	A012	A021	A022	A031
Control		2SA88	CC	GG	GG	TT	TT	AA	TT	GG
Control		2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
Control		2SA106	CC	GG	GG	TT	TG	AA	TT	GG
Control		2SA107	CT	GG	GG	TT	TT	TT	CT	GT
S1-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S2-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S4-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S5-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S6-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S7-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S10-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S11-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S12-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S18-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S22-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S23-10	2SA88/2SA105	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S24-10	2SA105	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S25-10	2SA88/2SA105	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S26-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S27-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S29-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S30-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S31-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S32-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S33-10	2SA106/2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S36-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S37-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S38-10.1	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S42-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S54-10	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S58-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S65-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S66-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S67-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S68-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG

Table 4.4: continued

	Race identification results								
Isolate	**Phenotyping**	**Genotyping**	**SNP genotypes**						
			A003	A005	A007	A010	A012	A021	A022	A031
S72-10	2SA88/2SA104	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S75-10	2SA105	2SA106	CC	GG	GG	TT	TG	AA	TT	GG
S76-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S81-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S83-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S92-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S93-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S94-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S96-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S99-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S100-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S105-10	2SA88	Unknown	CC	GG	GG	TT	TT	AA		GG
S108-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S120-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S121-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S122-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S124-10	2SA105	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S127.1-10	2SA105	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S132-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S133-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S134-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S135-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S137-10	2SA88/2SA107	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S138-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S139-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S142-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S144-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S146-10	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S153-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S154-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S155-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S156-10	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S157-10	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG

Table 4.5: Race specific identification of field isolates identified as Ug99 positive collected during the 2011 survey using SNP genotyping and infection type analysis

	Race identification results		SNP genotypes							
Isolate	**Phenotyping**	**Genotyping**								
			A003	A005	A007	A010	A012	A021	A022	A031
Control		2SA88	CC	GG	GG	TT	TT	AA	TT	GG
Control		2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
Control		2SA106	CC	GG	GG	TT	TG	AA	TT	GG
Control		2SA107	CT	GG	GG	TT	TT	TT	CT	GT
S4-11	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S5-11	2SA88/2SA105	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S6-11	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S9-11	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S15-11	Unknown	2SA106	CC	GG	GG	TT	TG	AA	TT	GG
S17-11	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S19-11	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S21-11	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S27-11	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S30-11	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S31-11	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S32-11	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S33-11	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S34-11	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S35-11	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S36-11	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S37-11	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S38-11	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S39-11.2	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S41-11	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S42-11	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S44-11	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S45-11	2SA88/2SA102	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S46-11	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S47-11	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S48-11	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S49-11	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S52-11	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S53-11	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG

Table 4.5: Continued

	Race identification results								
Isolate	**Phenotyping**	**Genotyping**	**SNP genotypes**						
			A003	A005	A007	A010	A012	A021	A022	A031
S54-11	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S56-11	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S57-11	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S58-11	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S59-11	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S61-11	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S62-11	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG

Table 4.6: Race specific identification of field isolates identified as Ug99 positive collected during the 2012 survey using SNPs and infection type analysis

	Race identification results								
Isolate	**Phenotyping**	**Genotyping**	**SNP genotypes**						
			A003	A005	A007	A010	A012	A021	A022	A031
Control		2SA88	CC	GG	GG	TT	TT	AA	TT	GG
Control		2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
Control		2SA106	CC	GG	GG	TT	TG	AA	TT	GG
Control		2SA107	CT	GG	GG	TT	TT	TT	CT	GT
S21-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S28-12	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S32-12	2SA105	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S36-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S38-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S40-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S42-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S43-12	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S44-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S45-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S46-12	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S47-12	2SA105/ 2SA104	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S48-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S50-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S51-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S52-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S53-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S54-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S55-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S56-12	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S57-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S60-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S63-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S64-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S66-12	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S68-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S69-12	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S73-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S75-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S78-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG

Table 4.6: Identification

	Race identification results								
Isolate	**Phenotyping**	**Genotyping**	**SNP genotypes**						
			A003	A005	A007	A010	A012	A021	A022	A031
S82-12	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S83-12	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S85-12	Unknown	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S86-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S87-12	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S89-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S90-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S94-12	2SA88/2SA104	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S95-12	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S98-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S99-12	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S100-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S101-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S102-12	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S103-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S104-12		Not genotyped								
S105-12	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S106-12	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S107-12	2SA88	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S113-12	2SA104	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG
S114-12	Unknown	2SA88	CC	GG	GG	TT	TT	AA	TT	GG
S122-12	2SA88	2SA88+	CC	GG	GG	TT	TT	AA	CT	GG

isolates gave a similar genotype to 2SA88+, while one isolate was identified as 2SA106 (Table 4.5). From these 36 isolates, 15 were phenotyped as 2SA88 by Dr. Terefe, two samples were mixed while the rest did not provide viable spores that could be phenotyped. No 2SA88+ phenotypes were detected.

Out of 52 Ug99 positive isolates collected in 2012, 37 had a 2SA88 genotype and 14 the 2SA88+ genotype (Table 4.6). Only 39 isolates were phenotyped as 2SA88 (Table 4.6) while 2SA88+ was not detected, two of the samples were mixed, one isolate was detected as 2SA105 and one isolate detected as 2SA104.

4.2.2.2 Statistical analysis of agreement between genotypes and phenotypes of Ug99 positive isolates

Table 4.7 shows the genotype X phenotype cross-classification of 111 Ug99 isolates collected during all three years. Samples used for statistical analysis only included samples with single race phenotypes, and those that were successfully genotyped and phenotyped. Only samples which were not included were those that did not have two race phenotypes, were phenotyped, did not fail to be phenotyped or failed to be genotyped were included in the analysis. Of the 105 isolates with a 2SA88 phenotype, 77 had a matching 2SA88 genotype, resulting in a conditional probability of agreement of Prob (genotype = 2SA88|phenotype = 2SA88) = 77/105 = 73.3% (95% CI 63.8% to 81.5%). CI refers to the confidence interval. This shows that with confidence it can be said that the probability lies between 63.8% and 81.5%. This conditional probability suggested that SNPs have a relatively high sensitivity to identify 2SA88. The sample size was too small to calculate the conditional probability of agreement of Prob (genotype = not 2SA88|phenotype = not 2SA88) with a meaningful confidence interval.

Conditional probabilities for the other Ug99 race phenotypes were not calculated because their frequency was too low. As can be seen in Table 4.7, in five samples phenotyping detected

Table 4.7: Genotype X phenotype cross-classification of 111 Ug99 positive isolates sampled during 2010-2012 period. The diagonal cells of the cross-table show numbers of isolates where genotype and phenotype agree. Only samples which were not included were those that did not have two race phenotypes, were phenotyped, did not fail to be phenotyped and did not fail to be genotyped were included in the analysis.

		Phenotypes					
	Frequency	**2SA104**	**2SA105**	**2SA106**	**2SA88**	**2SA88+**	**Total**
Genotypes	**2SA104**	0	0	0	0	0	0
	2SA105	0	0	0	0	0	0
	2SA106	0	1	0	0	0	1
	2SA88	0	3	0	77	0	80
	2SA88+	1	1	0	28	0	30
	Total	1	5	0	105	0	111

2SA105 whereas SNPs detected 2SA106, 2SA88 and 2SA88+, respectively. In one of the samples, phenotyping identified 2SA104 whereas SNPs detected 2SA88+. Since 2SA88+ phenotypes were not identified, it did not seem appropriate to estimate the sensitivity and specificity of detecting 2SA88+ statistically. However it seems like the current SNPs were not specific enough to allow for correct identification of 2SA88+ genotypes or phenotypes could not differentiate between 2SA88 and 2SA88+.

4.2.3 Identification of non-Ug99 isolates using SSRs

4.2.3.1 Polymorphism of SSR markers and genotypes

Of the 10 SSR markers used in the study, only seven consistently amplified reproducible fragments. These seven primer combinations amplified 19 alleles of which 17 were polymorphic and two monomorphic (Table 4.8). SSR genotypes were assigned so as to track the changes of the populations from year to year.

Genotypes SAG3 and SAG6 were present from 2010 to 2012 and were detected 54 and 14 times (Table 4.9). Genotypes SAG1, SAG7 and SAG9 were not detected in the field isolates collected from 2010 to 2012. SAG2 and SAG8 were detected only in 2010 each with a frequency of one, while SAG10 was detected in 2011 ones and SAG11, SAG12, SAG13 and SAG14 only in 2012 each with a frequency of one.

Six new genotypes (SAG4, SAG10, SAG11, SAG12, SAG13 and SAG14) were found that differed from all the control race genotypes and differed from SAG3 and SAG6 by two alleles at most. SAG4 and SAG11 differed from SAG3 by two alleles in loci PgSUN27 and PgSUN33, while SAG10 and SAG12 differed from SAG3 by one allele in PgSUN27 and PgSUN33 respectively (Table 4.9). SAG13 differed from SAG3 by two alleles in loci PgSUN27 and PgSUN30. Genotype SAG14 differed from SAG6 by one allele in PgSUN33. There were no new

Table 4.8: Characteristics of SSR loci amplified in *Pgt* field isolates collected in South Africa during the 2010-2012 surveys. The underlined allele sizes represent polymorphic alleles.

Locus	N_a	N_b	N_p	Allele sizes
PgSUN7	2	1	2	291, 305
PgSUN20	4	3	3	300, 306, 343, 424
PgSUN27	3	2	3	310, 316, 324
PgSUN30	2	1	2	358, 363
PgSUN33	3	2	3	283, 289, 295
PgSUN42	1	1	0	248
PgSUN45	4	1	4	349, 353, 357, 364

N_a total number of alleles

N_b number of alleles specific to reference races

N_p number of polymorphic alleles

Table 4.9: Allele sizes at seven SSR loci for 14 multilocus genotypes of stem rust detected during 2010 to 2012 in South Africa

Name of genotype	Simple sequence repeat locus and allele sizes (bp)							Year[a]	Number of times the genotype was detected
	PgSUN7	PgSUN20	PgSUN27	PgSUN30	PgSUN33	PgSUN42	PgSUN45		
SAG1 – Control 2SA4	291	424/300	316	358	289	248	349[b]	Not	0
SAG2	291	424/300	316	358	289	248	357	2010	1
SAG3	291	424/300	316	358	289	248	353	All	59
SAG4	291	424/300	316/310	358	289/283	248	353	2010/2011	3
SAG5	291	424/306	316	358	289	248	353	2011/2012	2
SAG6	291	424/300	310	358	283	248	353	All	14
SAG7 – Control 2SA103 and 2SA104	291	424/306	324	358	295	248	353	Not	0
SAG8	291	424/300	324	358	295	248	353	2010	1
SAG9 – Control 2SA88	305	424/343	324	363	295	248	364	Not	0
SAG10	291	424/300	316/310	358	289	248	353	2011	1
SAG11	291	424/300	310	358	295	248	353	2012	1
SAG12	291	424/300	316	358	295/289	248	353	2012	1
SAG13	291	424/300	310	363	289	248	353	2012	1
SAG14	291	424/300	310	358	289	248	353	2012	1

[a]Year of detection of the unknown isolates. "Not" indicates that the genotype was not detected in the field but used as a control genotype from 2010 to 2012, "All" indicates that the genotype was detected in all three years.

[b] Alleles highlighted in dark grey indicate alleles that make the genotype unique from others. They are also found only in one or two genotypes.

alleles present in these new genotypes, suggesting the new genotypes were just combinations of previously existing alleles. However there were new alleles present in loci PgSUN27 (310bp), PgSUN33 (283) and PgSUN45 (357bp).

4.2.3.2 Correlation and clustering between non-Ug99 *Pgt* race SSR genotypes and virulence phenotypes

Two dendrograms were drawn using infection type (Table 4.10) and SSR marker (Figure 4.4) to determine whether correlation existed between genotypes and phenotypes of non-Ug99 *Pgt* races in South Africa. The phenotypic data was obtained from the avirulence/virulence profiles of six reference races to 23 *Sr* genes with the exception of 2SA4 and 2SA100 where only 21 genes were available, and 2SA103 where 22 genes were available (Table 4.10). The genotypic dendrogram was drawn based on 12 alleles generated using seven SSR markers for six reference races (appendix 1, Table 5). The cophenetic correlation values showed that there was a good fit (r = 0.93, 0.99) between the Jaccard coefficient matrix and the symmetrical matrix based on the genotypic and phenotypic data, respectively (Figure 4.4). There was a low correlation (r = 0.14) between the genotypes and phenotypes of the non-Ug99 races.

Races 2SA105 was most similar to 2SA102 with 82% phenotypic similarity (Figure 4.4A) while 2SA100 was most similar to 2SA4 with 87% phenotypic similarity, but 2SA100 was 100% genetically similar to 2SA105 and 2SA104 was 100% genetically similar to 2SA103 (Figure 4.4B). Race 2SA102 had the highest genetic similarity (78%) to 2SA4, 2SA100 and 2SA105. Race 2SA104 was phenotypically most similar to 2SA102 and 2SA105 with a similarity of 70% and 73% respectively (Figure 4.4A). Race 2SA103 was phenotypically most similar to the latter three races with a phenotypic similarity of 55% for 2SA102 and 50% for 2SA104 and 2SA105. The data suggested that a high genetic relationship with one race did not necessarily imply a high phenotypic relationship.

Table 4.10: Avirulence/virulence profiles of South African *Pgt* races to *Sr* genes. The highlighted resistance genes were not used for the construction of the phenotypic dendrogram, because information on them was not available for all races.

Races	Avirulence/virulence profile	Reference
2SA4	*Sr8b, 9g, 21, 24, 27, 31, 39, 38, Tmp,* ***Kw, Satu****/Sr5, 6, 7b, 8a, 9a, 9b, 9d, 10, 11, 17, 30, McN*	Komen, 2007 (ARC data)
2SA100	*Sr8b, 9e, 9g, 21, 27, 30, 31, 36, 38, Tmp,* ***Satu****/Sr5, 6, 7b, 8a, 9a, 9b, 9d, 10, 11, 17, 24, McN*	Komen, 2007 (ARC data)
2SA102	*Sr5, 6, 7b, 8b, 9b, 9e, 17, 21, 24, 30, 31, 36, 38,* ***Em, Satu****, Tmp/Sr8a, 9a, 9d, 9g, 10, 11, 27, 44,* ***Kw****, McN*	Terefe *et al.*, 2010
2SA103	*Sr5, 6, 8b, 9b, 9d, 9e, 9g, 11, 17, 21, 24, 31, 36, 38, Tmp/Sr7b, 8a, 9a, 10, 27, 30, McN*	Komen, 2007 (ARC data)
2SA104	*Sr5, 6, 7b, 8b, 9b, 9e, 17, 21, 24, 30, 31, 36, 38,* ***Em, Kw, Satu****, Tmp/Sr8a, 9a, 9d, 9g, 10, 11, 27, 44, McN*	Terefe *et al.*, 2010
2SA105	*Sr5, 6, 7b, 8b, 9b, 9e, 17, 21, 24, 30, 31, 36, 38,* ***Em****, Tmp/Sr8a, 9a, 9d, 9g, 10, 11, 27, 44,* ***Kw, Satu****, McN*	Terefe *et al.*, 2010

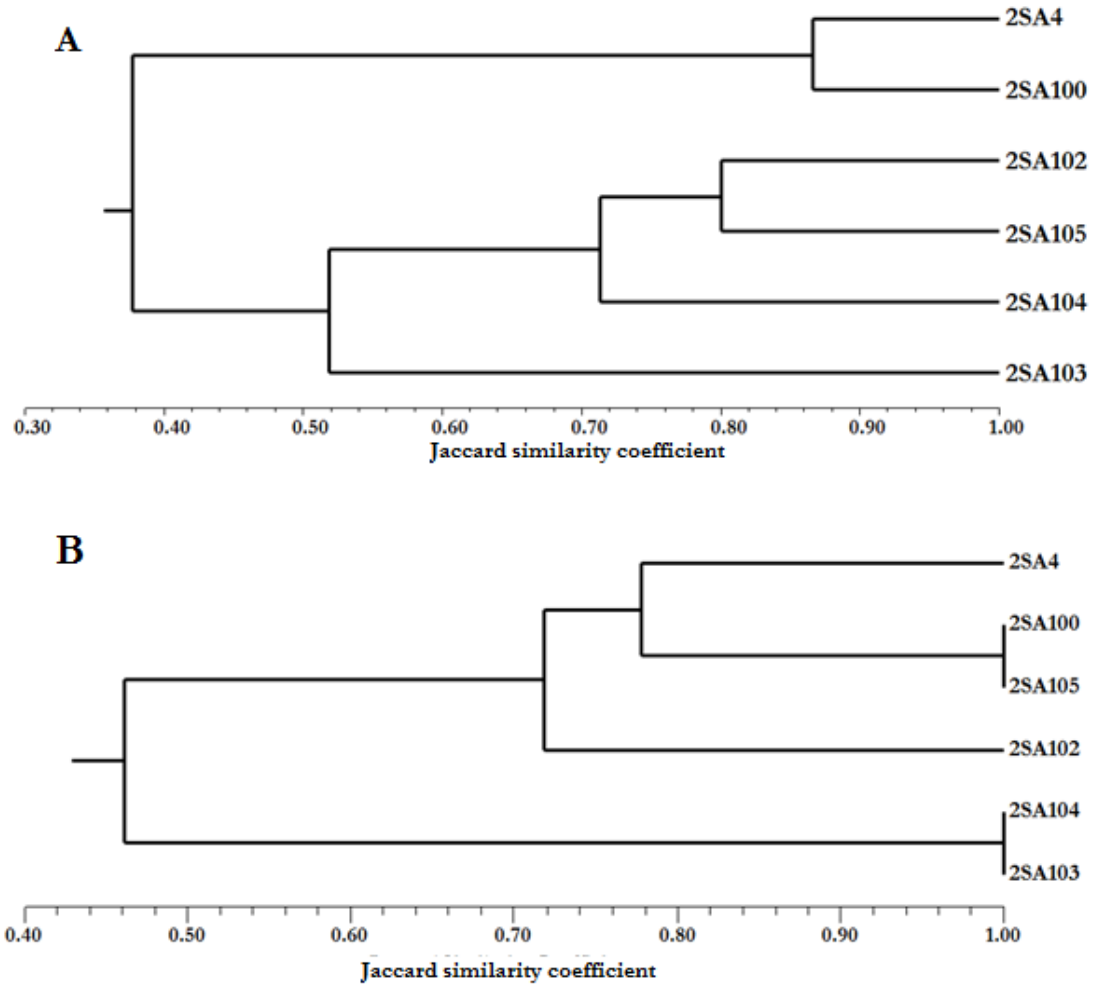

Figure 4.4: Genotypic and phenotypic dendrograms constructed using the UPGMA cluster analysis based on Jaccard similarity coefficient. (a) Phenotypic relationship of different stem rust races based on avirulence/virulence reaction profile of races to 23 resistance genes. (b) The genotypic relationship between different genotypes of South African *Pgt* races generated with seven SSR markers.

4.2.3.3 Genetic diversity and identification of 2010-2012 survey non-Ug99 isolates using SSRs

To determine the racial identity of genotyped non-Ug99 field isolates, dendrograms were generated for each year. The cophenetic correlation (*r* values) for the 2010-2012 dendrograms were 0.969, 0.983 and 0.984 respectively, indicating very good fits between the Jaccard coefficient and symmetrical matrixes for each dendrogram.

For isolates collected during the 2010 survey, SSR analysis divided the *Pgt* isolates into the single Ug99 control used as outgroup (2SA88; genotype SAG9) and cluster A containing all the non-Ug99 isolates (Figure 4.5). The non-Ug99 isolates were subdivided into group **i** composed of genotypes SAG1 to SAG6 and group ii containing genotypes SAG7 and SAG8. The field isolates were all included into genotypes SAG2, SAG3, SAG4, SAG6 and SAG8. In subgroup ii, isolate S70-10 (SAG8) shared 78% genetic similarity with races 2SA103 and 2SA104 that were 100% similar based on the used SSR primer combinations, identification showed that the isolate was most probably 2SA103 or 2SA104. Subgroup i was further subdivided into groups a and b. In group b, SAG6 isolates (S52-10, S60-10, S103-10 and S119-10) did not have close genetic relationships with any of the reference isolates making it impossible to identify them through genotyping alone. Group a contained the following control races: 2SA4, 2SA100, 2SA105 and 2SA102. No isolate with close similarity to 2SA102 was detected during the 2010 season. Genotype SAG2 (isolate S128-10) shared 78% genetic similarity with 2SA4, classifying it as closely related to 2SA4. Genotype SAG3 contained races 2SA100 and 2SA105 which were 100% similar to eleven field isolates with the SAG3 genotype. Isolates S110-10 and S118-10 (genotype SAG4) were 80% similar to SAG3 isolates, including 2SA105 and 2SA100. Since 2SA100 was last detected in the field in 2006, it was concluded that SAG3 and SAG4 were all probably 2SA105 or closely related to SAG4.

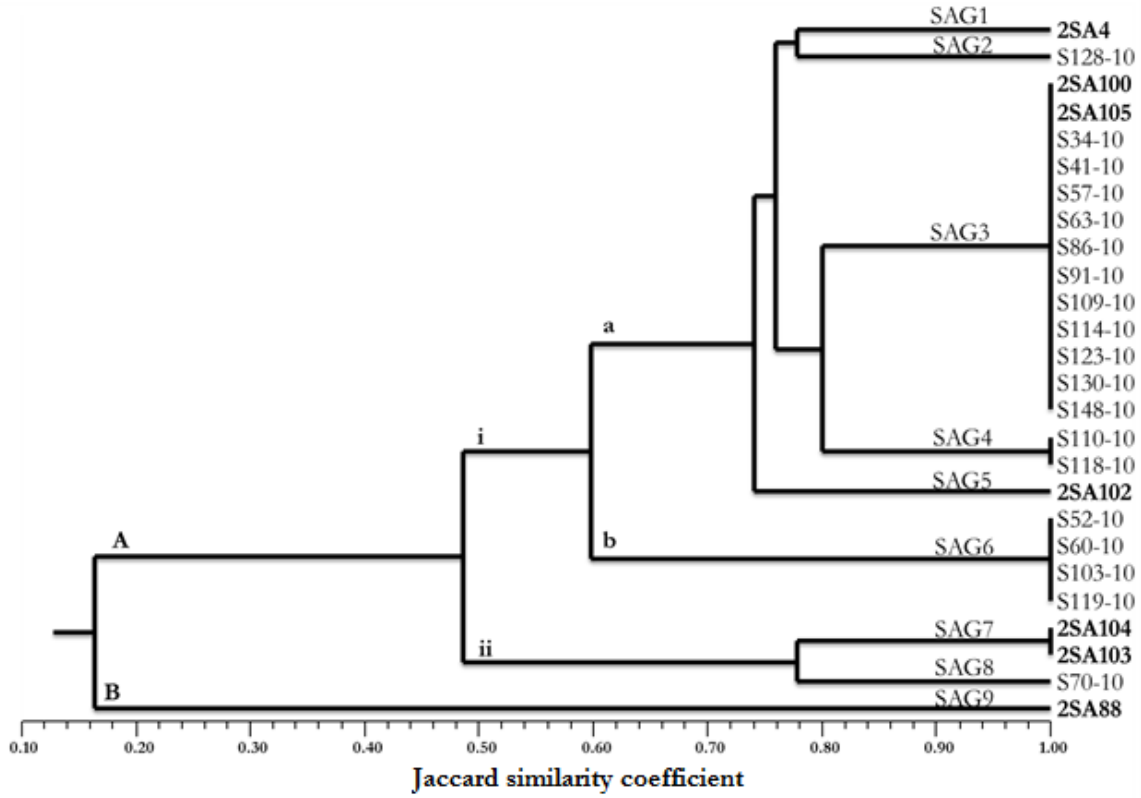

Figure 4.5: Genotyping of non-Ug99 field isolates collected in South Africa during the 2010 survey. The dendrogram was constructed using UPGMA cluster analysis based on the Jaccard similarity coefficient. The control isolates are indicated in bold and are given the 2SA notation (e.g. 2SA102), the individual isolates collected in 2010 are indicated with the S-notation (e.g. S34-10) and the genotypes are indicated with SAG notation (e.g. SAG1).

The genotypic identification of field isolates was compared to their respective phenotypes (Table 4.11). While all isolates analyzed with SSRs were supposed to be non-Ug99 based on qPCR results, several (S57-10, S63-10, S119-10, S123-10, S118-10, S130-10) were phenotyped as Ug99 positive. Most of the SAG3 isolates were both genotyped and phenotyped as 2SA105. The two members of SAG4, namely S110-10 and S118-10 were phenotyped as 2SA105 and 2SA106 respectively. This gives more evidence that S110-10 were most probably be closely related to 2SA100 or 2SA105 since S110-10 had a high genetic similarity (80%) to both 2SA100 and 2SA105. While genotype SAG6 did show 60% genetic similarity with 2SA100 and 2SA105, three of the isolates were phenotyped as 2SA105. Showing that there is a possibility that the three isolates with SAG6 could be closely related 2SA105.

A similar dendrogram was found for the 2011 field isolates (Figure 4.6). While isolates with genotypes SAG3, SAG4, SAG5 and SAG6 were again found, a new genotype (SAG10) was detected for isolate S28-11. Genotypes SAG4 (isolate S51-11) and SAG10 (S28-11) were 90% similar could be classified as closely related to 2015. Field isolates (11) containing SAG3, were all classified as 2SA105. S11-11 was 100% to and classified as 2SA102. No isolates closely related to 2SA4, 2SA103 and 2SA104 were found in 2011 using genotyping.

A total of 17 field isolates collected in 2011 was genotyped of which only four were succesfully phenotyped (Table 4.12). Three of these (S20-11, S24-11, S51-11) confirmed that SAG3 and SAG4 genotypes were indeed closely related to 2SA105. Even though they were not phenotyped, genotyping identified S11-11 (SAG5) as 2SA102. Isolates containing SAG6 had 60% genetic similarity and are most similar to 2SA105. However further investigation of SAG6 is needed.

During the 2012 season, isolates that were collected in the field included genotypes SAG3 (37 isolates), SAG5 (S120-12), SAG6 (seven isolates) and four new genotypes namely SAG11, SAG12, SAG13 and SAG14 (Figure 4.7). The 37 SAG3 isolates were identified as 2SA105, while S120-12 was identified as 2SA102. Genotypes SAG11 (S24-12) and SAG12 (S97-12) were 84%

Table 4.11: Genotypic based identification of 2010 *Pgt* field isolates and their respective phenotypes. Rows highlighted in light grey indicate a match between phenotypes and genotypes. Dark grey rows show isolates for which the racial identity could not be determined using genotyping. White rows show no match between genotypes and phenotypes.

Isolate name	Genotype	Race identification using SSR genotyping	Race identification by phenotyping
S34-10	SAG3	2SA105	2SA105
S41-10	SAG3	2SA105	2SA102
S52-10	SAG6	Unknown	2SA88/2SA105
S57-10	SAG3	2SA105	2SA88
S60-10	SAG6	Unknown	2SA105
S61-10	Not genotyped		2SA105/2SA88
S63-10	SAG3	2SA105	2SA88/2SA104
S70-10	SAG8	2SA104	2SA104
S86-10	SAG3	2SA105	2SA105
S91-10	SAG3	2SA105	2SA105
S103-10	SAG6	Unknown	2SA105
S109-10	SAG3	2SA105	2SA105
S110-10	SAG4	2SA105	2SA105
S114-10	SAG3	2SA105	2SA105
S118-10	SAG4	2SA105	2SA106
S119-10	SAG6	Unknown	2SA88
S123-10	SAG3	2SA105	2SA88
S128-10	SAG2	2SA4	2SA105
S130-10	SAG3	2SA105	2SA107
S148-10	SAG3	2SA105	2SA105

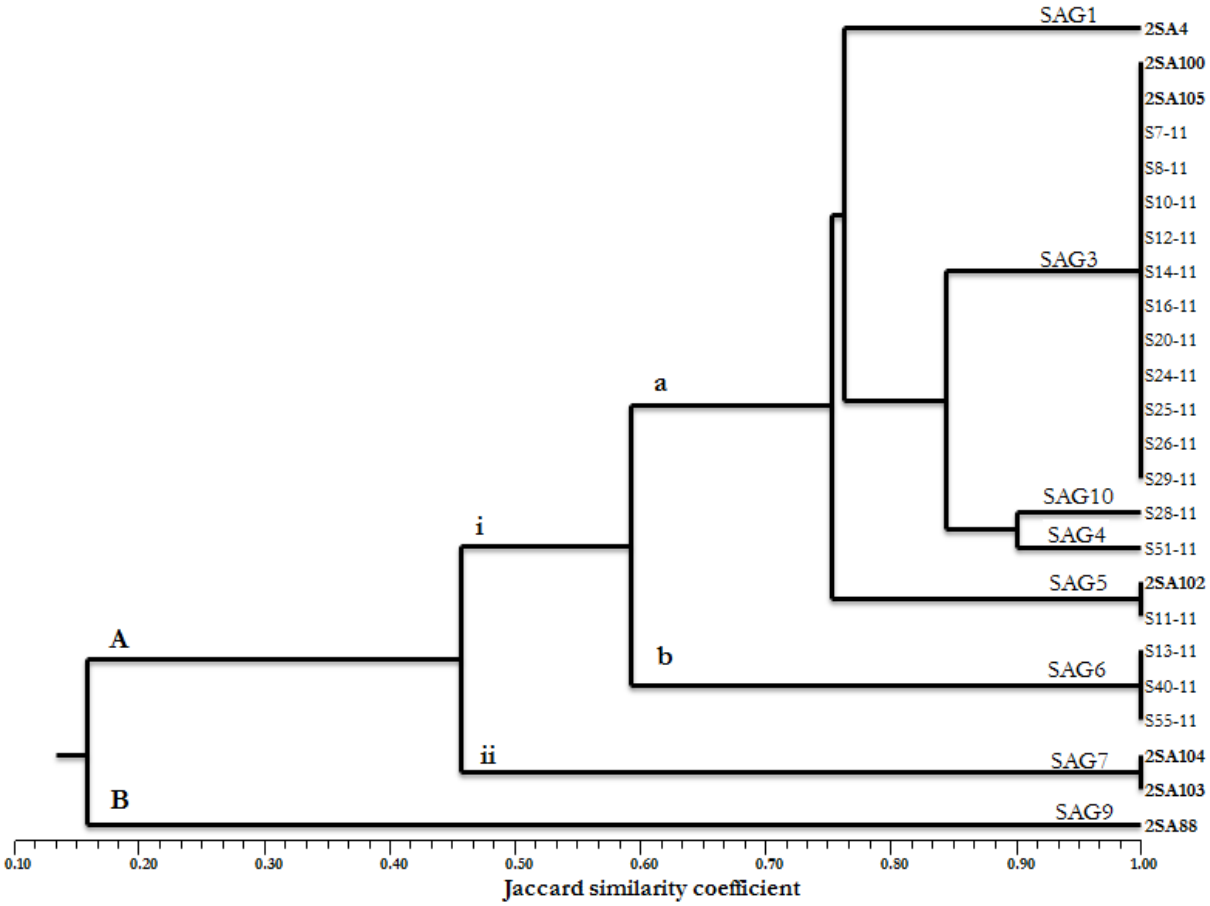

Figure 4.6: Genotyping of non-Ug99 field isolates collected in South Africa during the 2011 survey. The dendrogram was constructed using UPGMA cluster analysis based on the Jaccard similarity coefficient. The control isolates are indicated in bold and are given the 2SA notation (e.g. 2SA102), the individual isolates collected in 2011 are indicated with the S-notation (e.g. S29-11) and the genotypes are indicated with SAG notation (e.g. SAG1).

Table 4.12: Genotypic based identification of 2011 *Pgt* non-Ug99 field isolates and their respective phenotypes. Rows highlighted in light grey indicate a match between genotypes and phenotypes. Dark grey rows show isolates for which the racial identity could not be shown using genotyping, phenotyping or both. White rows show no match between genotypes and phenotypes.

Isolate name	Genotype	Race identification using SSR genotyping	Race identification by phenotyping
S7-11	SAG3	2SA105	Not phenotyped
S8-11	SAG3	2SA105	Not phenotyped
S10-11	SAG3	2SA105	Not phenotyped
S11-11	SAG5	2SA102	Not phenotyped
S12-11	SAG3	2SA105	Not phenotyped
S13-11	SAG6	Unknown	Not phenotyped
S14-11	SAG3	2SA105	Not phenotyped
S16-11	SAG3	2SA105	Not phenotyped
S20-11	SAG3	2SA105	2SA105 and 2SA104
S23-11	Failed		2SA88
S24-11	SAG3	2SA105	2SA105
S25-11	SAG3	2SA105	Not phenotyped
S26-11	SAG3	2SA105	Not phenotyped
S28-11	SAG10	2SA105	Not phenotyped
S29-11	SAG3	2SA105	Not phenotyped
S40-11	SAG6	Unknown	Not phenotyped
S43-11	Failed		Not phenotyped
S51-11	SAG4	2SA105	2SA105 and 2SA88
S55-11	SAG6	Unknown	Not phenotyped

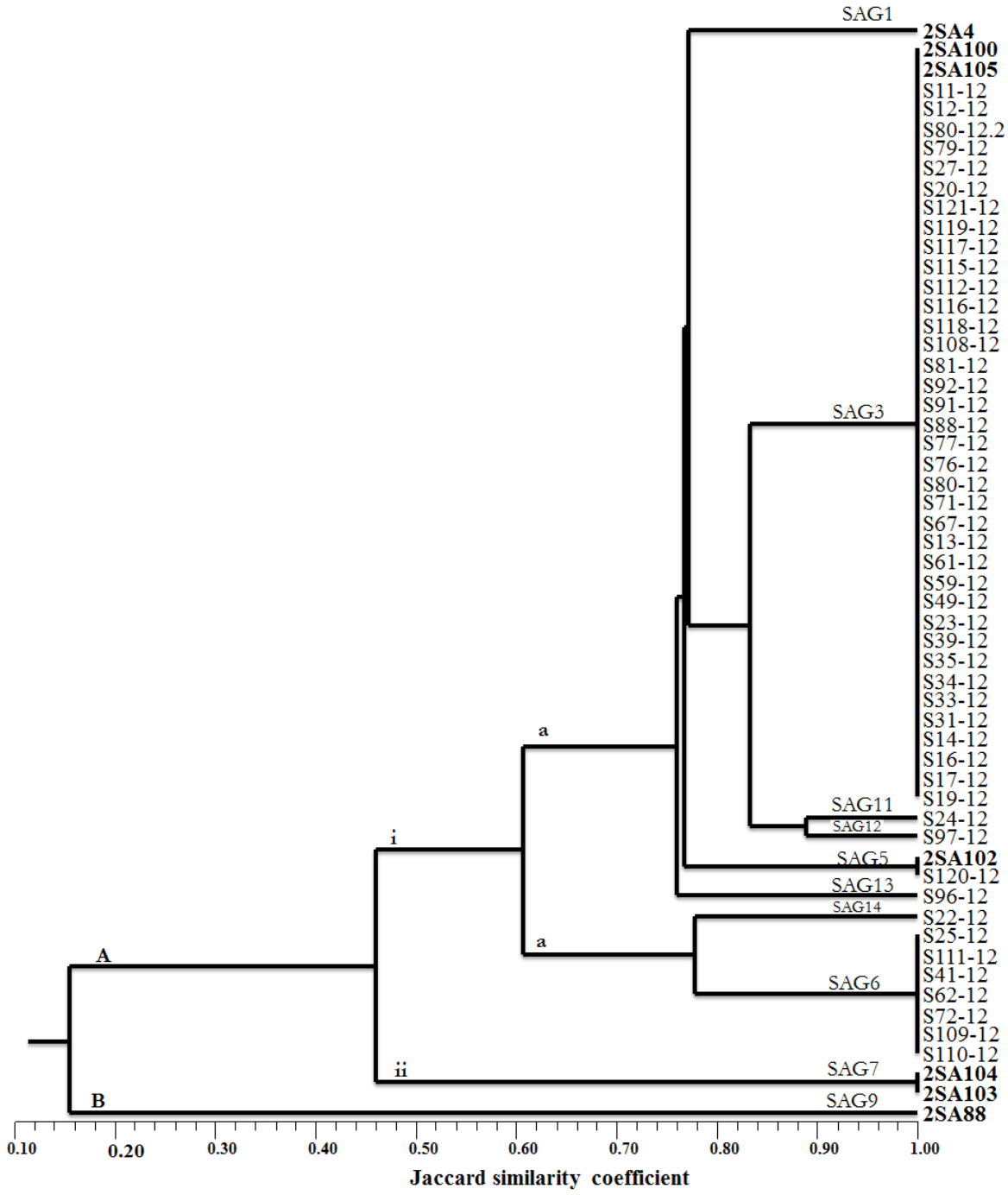

Figure 4.7: Genotyping of non-Ug99 *Pgt* field isolates collected in the Western Cape during the 2012 survey. The dendrogram was constructed using the UPGMA cluster analysis based on the Jaccard similarity coefficient. The control isolates are indicated in bold and are given the 2SA notation (e.g. 2SA102), the individual isolates collected in 2012 are indicated with the S-notation (e.g. S72-12) and the genotypes are indicated with SAG notation (e.g. SAG1).

similar to genotype SAG3, suggesting that both are most similar to 2SA105. Genotype SAG13 (S96-12) was most similar to 2SA105 with 78% genetic similarity. Genotype SAG14 (S22-12) shared 78% genetic similarity with the isolates of SAG6, suggesting that it was closely related to SAG6. SAG14 was most similar to 2SA105 with 78% genetic similarity.

The genotypes of forty-two collected in 2012 isolates could be compared with their respective phenotypes (Table 4.13). A total of 13 SAG3 isolates were confirmed to be 2SA105 through phenotyping, as well as one isolate of SAG6 (S41-12), SAG11 (S24-12) and SAG14 (S22-12). No phenotype was available for SAG13 (S96-12). Most of the genotyped isolates were phenotyped as 2SA88. Race phenotypes 2SA102 and 2SA104 were detected each with one isolate.

4.2.4 Statistical analysis of agreement between non-Ug99 phenotypes and genotypes

Table 4.14 shows the genotype X phenotype cross-classification of 47 non-Ug99 isolates collected during 2010-2012 period. Only samples which were not included were those that did not have two race phenotypes, were phenotyped, did not fail to be phenotyped and did not fail to be genotyped were included in the analysis. The conditional probability of agreement of Prob (genotype = 2SA105|phenotype = 2SA105) = 21/22 = 95.5% (95% CI = 77.2% to 99.9%). This suggests that the SSR genotypes were highly sensitive for identifying 2SA105 phenotypes. The conditional probability of agreement Prob (genotype = not 2SA105|phenotype = not 2SA105) = 2/25 = 8.0% (95% CI 1.0% to 26.0%), indicating a very low specificity for detecting 2SA105. This suggests that the SSR genotypes might not be specific to identify 2SA105. The conditional probability of agreement for detection of phenotypes 2SA102 and 2SA104 was not evaluated statistically because they had a low frequency (Table 4.14).

Table 4.13: Genotypic based identification of 2012 non-Ug99 *Pgt* field isolates and their respective phenotypes. Rows highlighted in light grey indicate a match between phenotypes and genotypes. Dark grey row shows isolates for which the racial identity could not be determined using genotyping, phenotyping or both. White rows show no match between genotypes and phenotypes.

Isolate name	Genotype	Race identification using SSR genotyping	Race identification by phenotyping
S11-12	SAG3	2SA105	2SA88
S12-12	SAG3	2SA105	2SA88
S13-12	SAG3	2SA105	2SA88
S14-12	SAG3	2SA105	2SA105
S16-12	SAG3	2SA105	2SA105
S17-12	SAG3	2SA105	Not phenotyped
S19-12	SAG3	2SA105	2SA105
S20-12	SAG3	2SA105	Not phenotyped
S22-12	SAG14	Unknown	2SA105
S23-12	SAG3	2SA105	Not phenotyped
S24-12	SAG11	2SA105	2SA105
S25-12	SAG6	Unknown	2SA88
S27-12	SAG3	2SA105	Not phenotyped
S31-12	SAG3	2SA105	2SA105
S33-12	SAG3	2SA105	2SA105
S34-12	SAG3	2SA105	2SA88
S35-12	SAG3	2SA105	2SA88
S39-12	SAG3	2SA105	2SA105
S41-12	SAG6	Unknown	2SA105
S49-12	SAG3	2SA105	2SA88
S59-12	SAG3	2SA105	2SA88
S61-12	SAG3	2SA105	2SA88
S62-12	SAG6	Unknown	2SA88/2SA105
S67-12	SAG3	2SA105	2SA105
S71-12	SAG3	2SA105	2SA104
S72-12	SAG6	Unknown	2SA102
S76-12	SAG3	2SA105	2SA104/2SA105
S77-12	SAG3	2SA105	2SA105
S79-12	SAG3	2SA105	2SA88
S80-12	SAG3	2SA105	2SA105
S80-12.2	SAG3	2SA105	2SA105
S81-12	SAG3	2SA105	Not phenotyped
S88-12	SAG3	2SA105	2SA88
S91-12	SAG3	2SA105	2SA105
S92-12	SAG3	2SA105	2SA105
S96-12	SAG13	Unknown	2SA88

Table 4.13: Continued

Isolate name	Genotype	Race identification using SSR genotyping	Race identification by phenotyping
S97-12	SAG12	2SA105	2SA88
S108-12	SAG3	2SA105	2SA88
S109-12	SAG6	Unknown	2SA88
S110-12	SAG6	Unknown	2SA88
S111-12	SAG6	Unknown	Not phenotyped
S112-12	SAG3	2SA105	2SA102/2SA105
S115-12	SAG3	2SA105	2SA88
S116-12	SAG3	2SA105	2SA88
S117-12	SAG3	2SA105	Not phenothyped
S118-12	SAG3	2SA105	2SA88
S119-12	SAG3	2SA105	2SA88
S120-12	SAG5	2SA102	2SA88
S121-12	SAG3	2SA105	2SA105

Table 4.14: Genotype X phenotype cross-classification for 46 non-Ug99 isolates sampled during 2010-2012 period. The diagonal cells of the cross-table show the number of isolates where phenotype and genotype agree. Only samples which were included were those that did not have two race phenotypes, were phenotyped, did not fail to be phenotyped or fail to be genotyped were included in the analysis.

		Phenotypes							
		2SA4	**2SA102**	**2SA104**	**2SA105**	**2SA106**	**2SA107**	**2SA88**	**Total**
Genotypes	**2SA4**	0	0	0	1	0	0	0	1
	2SA102	0	0	0	0	0	0	1	1
	2SA104	0	0	1	0	0	0	0	1
	2SA105	0	1	1	21	1	1	18	43
	2SA106	0	0	0	0	0	0	0	0
	2SA107	0	0	0	0	0	0	0	0
	2SA88	0	0	0	0	0	0	0	0
	Total	0	1	2	22	1	1	19	46

4.3 Genetic analysis of all non-Ug99 *Pgt* field isolates

4.3.1 Analysis of genetic variation

SSR data from all non-Ug99 field isolates (85) collected between 2010 and 2012 was combined to construct an unrooted tree (Figure 4.8). The unrooted tree was divided into four groups based on genetic dissimilarity. Group A contained all isolates with the SAG3 genotype, while group D contained genotypes SAG4 (S51-11, S110-10 and S118-10), SAG6 and SAG14 (S22-12). Group C contained genotypes SAG7 (2SA103 and 2SA104), SAG8 (S70-10), SAG9 (2SA88) and SAG11 (S24-12). Isolates with intermediate genotypes which did not fall in either Group A, C or D were assigned to group B. Isolates with genotype SAG5 (2SA102, S11-11 and S120-12) were the most dissimilar isolates in group B. Group C was the most dissimilar group from group A.

There were a few isolates that grouped differently from the previous dendrograms (Figures 4.5, 4.6 and 4.7). S24-12 and S97-12 were more dissimilar (0.28) to each other than previously shown in Figure 4.7 and S24-12 was more dissimilar (0.25) to group A (2SA105), suggesting that both isolates are not closely related to each other and S24-12 is most probably not related to 2SA105. S22-12 was less dissimilar (0.073) to SAG4 isolates (S118-10, S110-10 and S51-11) than to the SAG6 isolates (0.25) in group D, confirming that S22-12 and SAG4 are probably genetically closely related. However S97-12 seems to be closely related to 2SA105 with a dissimilarity distance of 0.111. S24-12 grouped together with S70-10, 2SA103, 2SA104 and 2SA88 (group C) while it did not in the 2012 dendrogram (Figure 4.7). S28-11 and S51-11 were more dissimilar to each other than previously shown (Figure 4.6). This was probably because of the use of a larger sample size in the unrooted tree and indicates S28-11 and S51-11 are not closely related. The dendrograms showed that SAG4 was more related to SAG3 but the unrooted tree showed that it is closely related to SAG6, this indicates that SAG4 and SAG6 might be new races since they were not grouped to any of the control isolates.

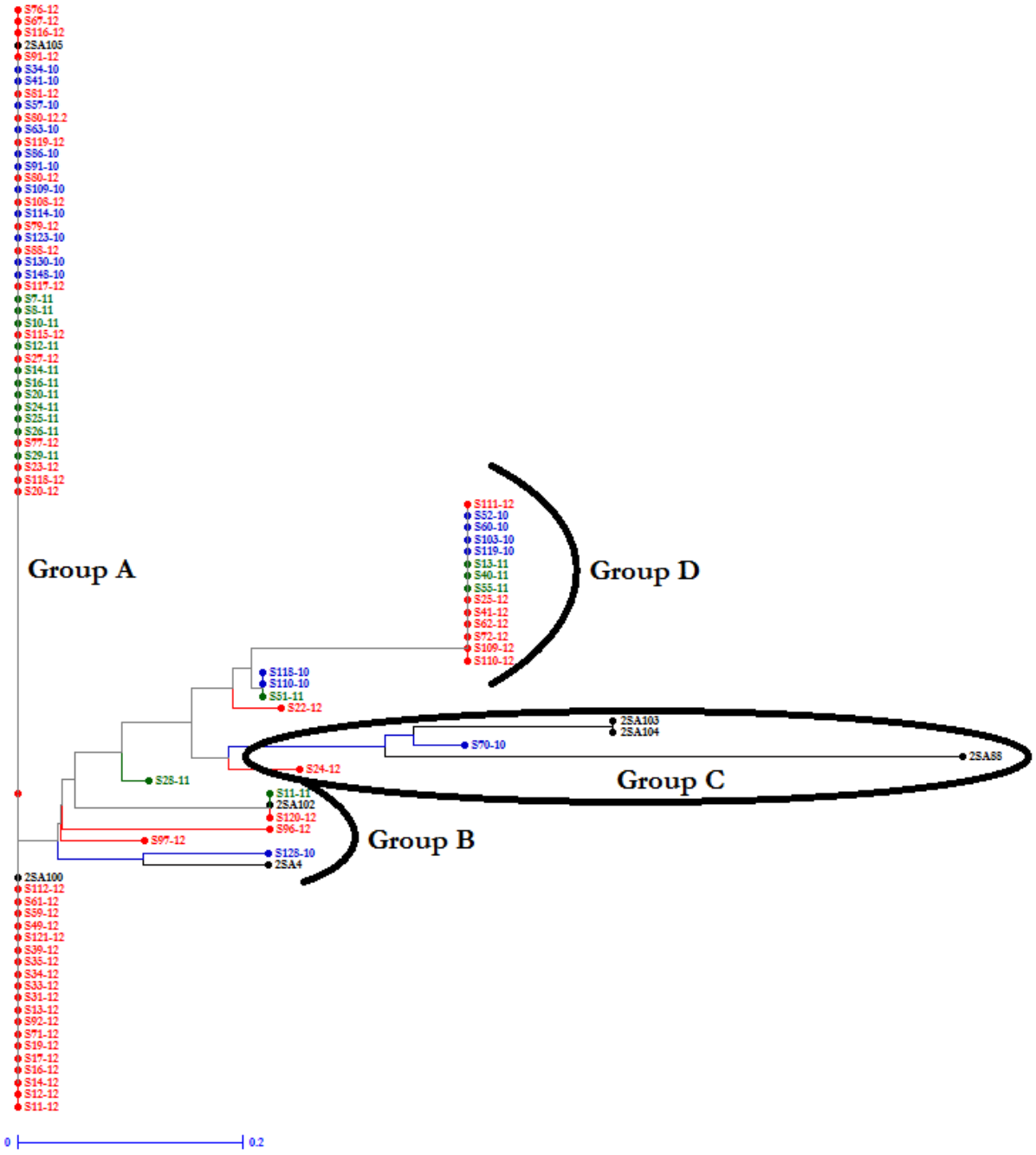

Figure 4.8: Unrooted neighbour-joining (NJ) tree representing 85 non-Ug99 *Pgt* field isolates collected from 2010 - 2012 and seven control races. Black represents control isolates, blue 2010 isolates, green 2011 isolates and red 2012 isolates.

4.3.2 Population structure analysis of the combined SSR data

STRUCTURE analysis is a Bayesian model-based approach which is used to infer population structure, assign individuals to populations and identify admixture in individuals within populations based on their multi-locus genotypic data. An admixture is observed from the barplot as a mixture of genetic composition from at least two populations. The optimum number of non-Ug99 populations in South Africa was determined to be K=3 (Figure 4.9).

Reference races 2SA100 and 2SA105 were assigned to population 1, while races 2SA4, 2SA88, 2SA102, 2SA103 and 2SA104 formed part of population 3 (Figure 4.10). Included in population 1 were isolates from SAG3 and SAG10 (S28-11). Only S28-11 was admixed having a mixture of all three populations confirming that Population 1 was mostly a pure population. Population 2 contained isolates from group D (SAG4, SAG6 and SAG14 (S22-12)) (Figure 4.8). S22-12 and SAG4 isolates were admixed with composition of all three populations being found while SAG6 isolates (14 in total) showed almost no admixture indicating population 2 was by a large extent also a pure population. Population 3 included all isolates in groups B and C, excluding S28-11. Population 3 was thus the only population which had isolates with more than three different genotypes (SAG1, SAG5, SAG7, SAG8, SAG9, SAG11, SAG12 and SAG13), making it the most diverse population in South Africa.

AMOVA was used to determine the sources of genetic variation between the three non-Ug99 *Pgt* populations in South Africa. For the three populations, 75.19% of the variation could be attributed to variation between populations and 24.81% to variation within populations with the major contributor to the variation being population 3 (Figure 4.10, Table 4.15). The F_{ST} of 0.75 ($P<0.0001$) indicated high genetic differentiation between the three populations.

4.3.3 Network analysis of non-Ug99 *Pgt* isolates

Network analysis of non-Ug99 *Pgt* field isolates collected during the 2010-2012 surveys

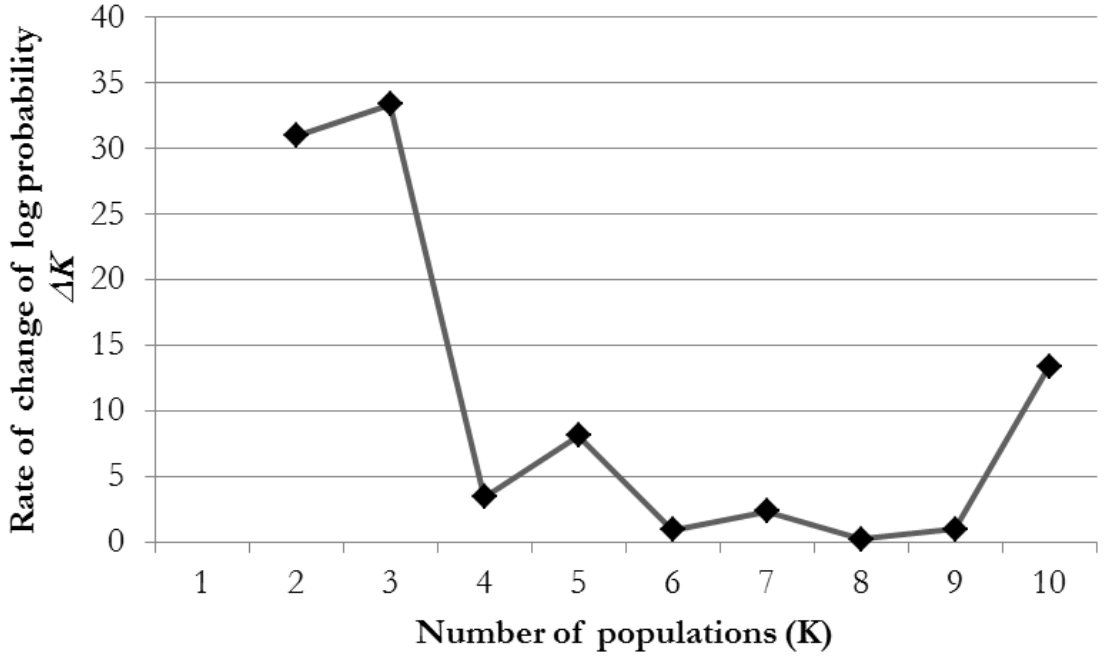

Figure 4.9: A plot representing an *ad hoc* Δ*K* statistics (Evanno *et al.*, 2005) for the 2010 - 2012 non-Ug99 *Pgt* field isolates. Analysis was based on 50 000 burn-in and MCMC replications for K=2 to 10 and 10 replications per run.

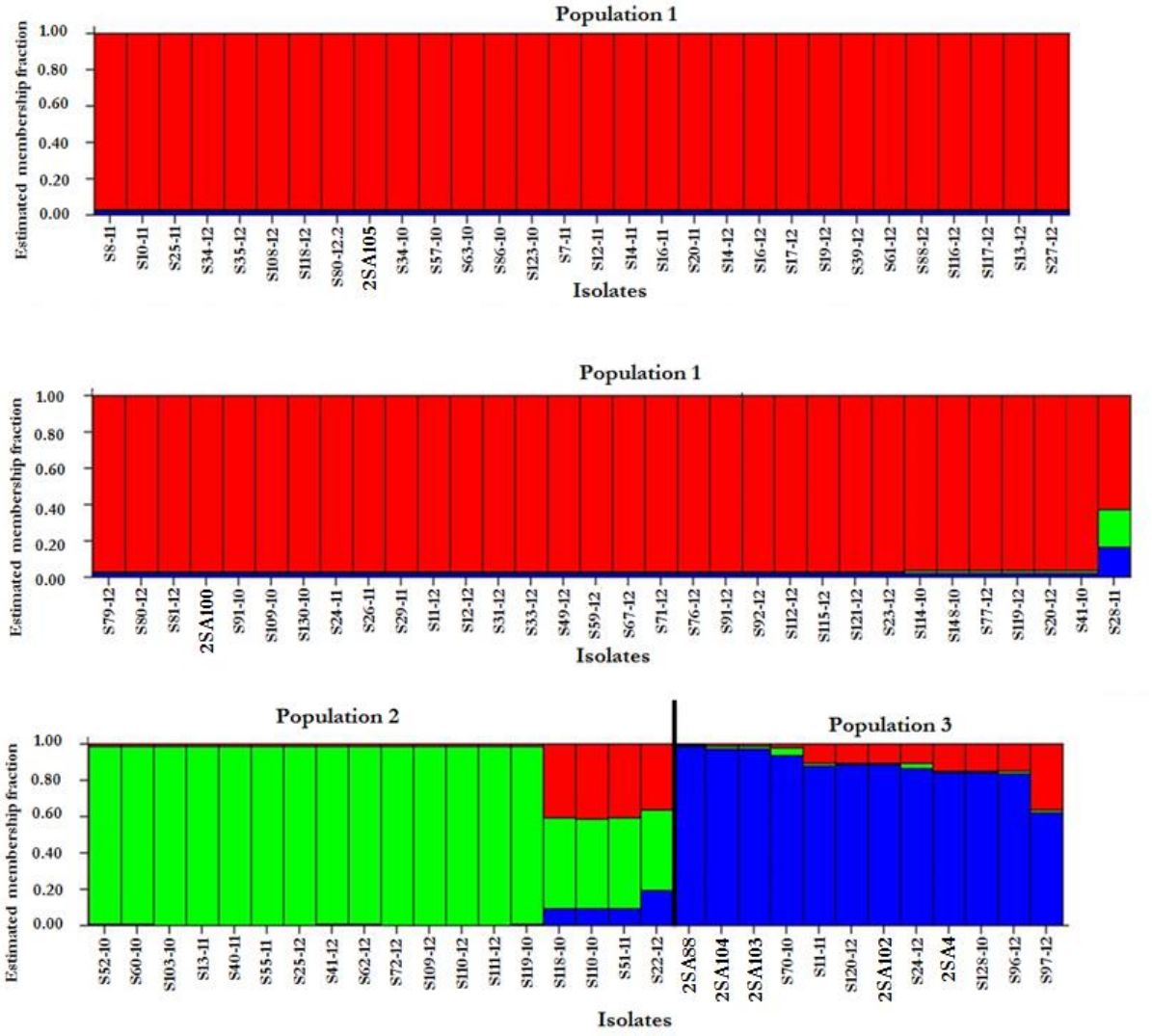

Figure 4.10: Population structure analysis of non-Ug99 *Pgt* field isolates from 2010-2012 surveys when K=3. The colours blue, green and red show the genetic composition of the isolates in different populations. An isolate was assigned to a specific population based on its highest genetic contribution.

Table 4.15: Genetic variation of the combined non-Ug99 *Pgt* population in South Africa from 2010 - 2012 surveys when K=3.

Source of variation	df	Sum of squares	Variation components	% variation	P-value	F_{ST}
Between populations	2	55.82	1.22	75.19	0.00	0.75
Within populations	91	35.79	0.40	24.81		
Total	93	91.61	1.62			

confirmed the structure of the unrooted dendrogram (Figure 4.11). S70-10 was separated from 2SA104 and 2SA103 by two mutational events confirming their genetic relationship. S70-10 was also separated by two mutational events from S24-12 was separated by one mutational event from S97-12, which in turn was separated from genotype SAG3 with one mutational event. Both S96-12 and SAG5 (S11-11, S120-12 and race 2SA102) was separated from SAG3 by two mutational events. 2SA4 and S128-10 were separated by two mutational events from each other and one more from SAG3.

The fact that most of the isolates fell within SAG3 or were separated by two mutational events at most from SAG3, and that 2SA100 and 2SA105 (both SAG3) had the same genotype, suggest that most of the collected isolates were closely related to 2SA100 and 2SA105. A possible recombination event was suggested between S28-11, S22-12, and genotype SAG4 (S110-10, S118-10 and S51-11). This was consistent with the population structure data which showed that these isolates were admixed mostly with genetic composition from population 1 and 2 (Figure 4.9). Network analysis also showed that SAG3 and SAG6 were closely related confirming the relationship between population 1 and 2. SAG4 and S22-12 fall between SAG3 and SAG6 confirming the unrooted dendrogram (Figure 4.8). SAG4 and SAG6 genotypes were not identical to any of the reference races while S22-12 and S28-11 were isolates with new genotypes that were detected for the first time.

4.4 Genetic analysis of *Pt* isolates collected during the 2013 survey

4.4.1 Correlation between known *Pt* race phenotypes and genotypes

To determine whether there existed a correlation between the phenotypes and genotypes of known South African *Pt* races, two dendrograms were drawn using infection type (Table 4.16) and SSR marker data (Figure 4.12). The infection type data was obtained from the response of races to 17 *Lr* resistance genes (Table 4.16) while 51 alleles were used ofor the SSR marker data

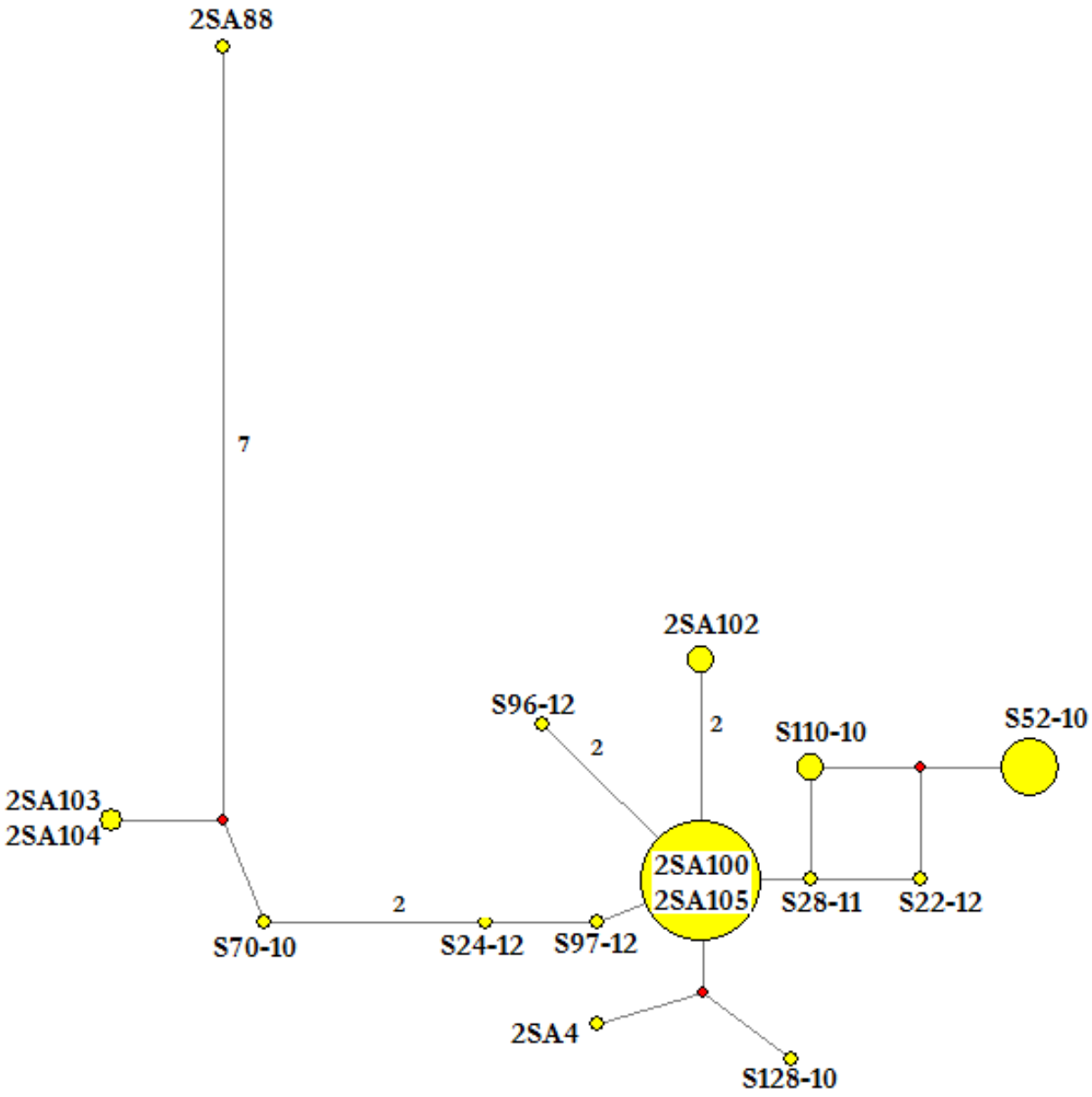

Figure 4.11: Minimum spanning network for 85 non-Ug99 *Pgt* field isolates collected during the 2010-2012 surveys. Closed squares indicate a possible recombination event. Red circles indicate hypothetical intermediates. Yellow circles indicate isolates and the diameter is proportional to the number of isolates with the genotype. Numbers next to the lines indicate the number of proposed mutational events between individuals and where none is indicated, one mutation is implied. 2SA102 forms part of SAG5; 2SA100 and 2SA105 form part of SAG3; S110-10 forms part of SAG4 and S52-10 forms part of SAG6.

Table 4.16: Avirulence/virulence profiles of *Pt* races in South Africa. The highlighted resistance genes were not used for the construction of the phenotypic dendrogram because information on them was not available for all races.

Races	Avirulence/ virulence profile	Reference
3SA122	*Lr1, 2a, 2b, 2c, 3ka, 11, 15, 16, 17, 20, 24, 26, 30/Lr3a, 3bg, 10, 14a*	Visser *et al.*, 2012a
3SA125	*Lr1, 2a, 3bg, 10, 11, 14a, 15, 16, 17, 24, 26/Lr2b, 2c, 3a, 3ka, 20, 30*	Visser *et al.*, 2012a
3SA129	*Lr3a, 3bg, 3ka, 10, 11, 14a, 16, 17, 20, 24, 26, 30/Lr1, 2a, 2b, 2c, 15*	Visser *et al.*, 2012a
3SA132	*Lr3a, 3bg, 3ka,* ***9****, 11, 16,* ***18****, 26, 30/Lr1, 2a, 2b, 2c, 10, 14a, 15, 17, 20,* ***23****, 24,* ***28****,* ***B***	Terefe *et al.*, 2014a
3SA133	*Lr2a, 2b,* ***9****, 15, 16, 17,* ***18****,* ***23****, 26/Lr1, 2c, 3a, 3bg, 3ka, 10, 11, 14a, 20, 24,* ***28****, 30,* ***B***	Terefe *et al.*, 2014a
3SA134	*Lr3a, 3bg, 3ka, 10, 11, 16, 20, 24, 26, 30/Lr1, 2a, 2b, 2c, 14a, 15, 17*	Visser *et al.*, 2012a
3SA137	*Lr3a, 3bg, 3ka,* ***9****, 11, 16,* ***18****, 24, 30/Lr1, 2a, 2b, 2c, 10, 14a, 15, 17, 20,* ***23****, 26,* ***28****,* ***B***	Terefe *et al.*, 2014a
3SA140	*Lr3a, 3bg, 3ka,* ***9****, 11, 16,* ***18****, 30/Lr1, 2a, 2b, 2c, 10, 14a, 15, 17, 20,* ***23****, 24, 26,* ***28****,* ***B***	Terefe *et al.*, 2014a
3SA144	*Lr3a, 3bg, 3ka,* ***9****, 10, 11, 16,* ***18****, 26, 30/Lr1, 2a, 2b, 2c, 14a, 15, 17, 20,* ***23****, 24,* ***28****,* ***B***	Terefe *et al.*, 2014a
3SA145	*Lr1, 2a, 2b, 2c,* ***9****, 11, 16,* ***18****, 20,* ***23****, 24/Lr3a, 3bg, 3ka, 10, 14a, 15, 17, 26,* ***28****, 30,* ***B***	Terefe *et al.*, 2014a
3SA146	*Lr2a, 2b, 2c, 3ka,* ***9****, 11, 16,* ***18****, 20, 24,* ***28****, 30/Lr1, 3a, 3bg, 10, 14a, 15, 17,* ***23****, 26,* ***B***	Terefe *et al.*, 2014a
3SA147	*Lr1, 2a, 2b,* ***9****, 11, 15, 16, 20, 24, 26,* ***28****/Lr2c, 3a, 3bg, 3ka, 10, 14a, 17,* ***18****,* ***23,*** *30,* ***B***	Terefe *et al.*, 2014b

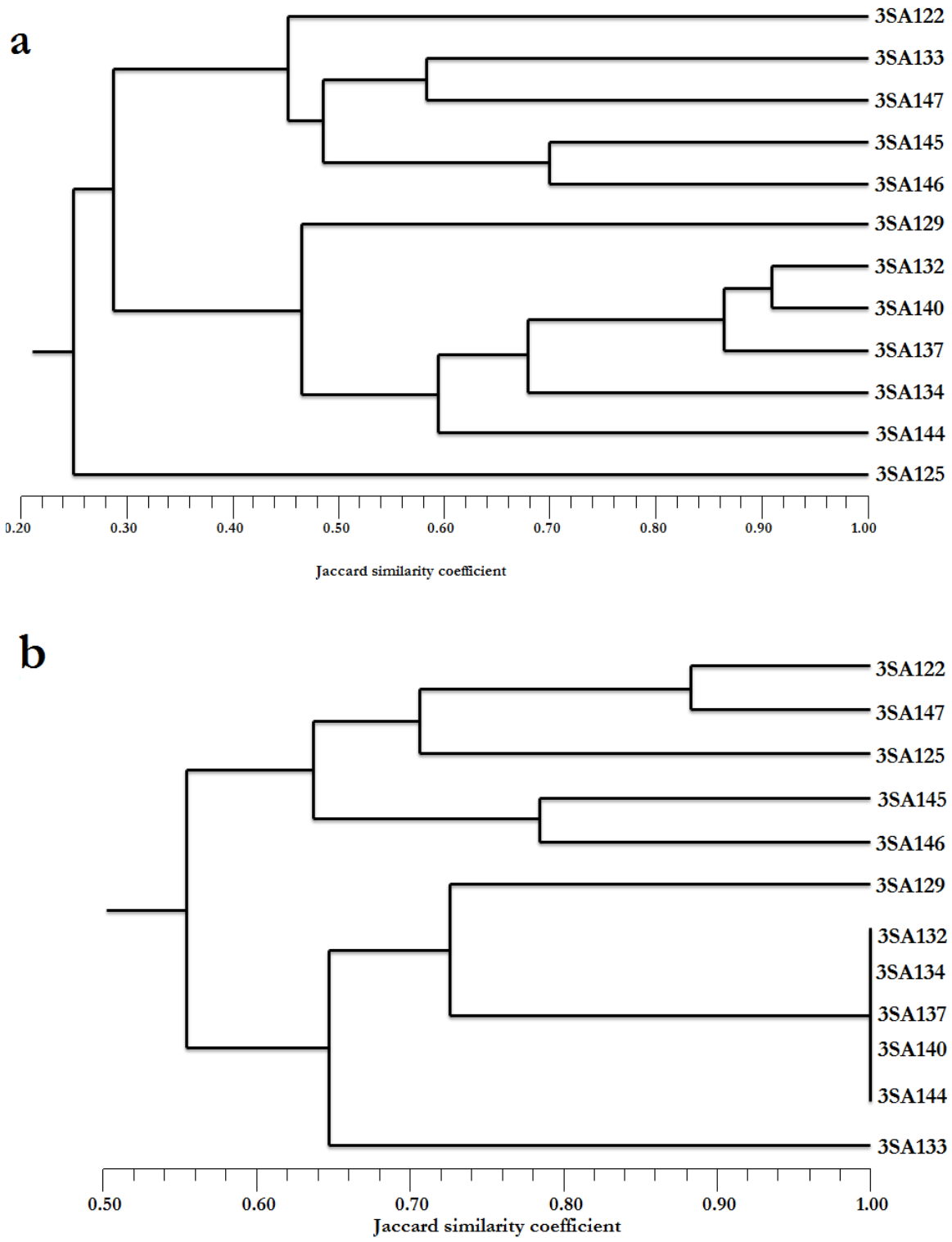

Figure 4.12: Dendrograms constructed using UPGMA cluster analysis based on the Jaccard similarity coefficient for 12 known South African *Pt* races. The phenotypic dendrogram (A) was constructed using avirulence/virulence reaction profiles of isolates to 17 *Lr* resistance genes while the genotypic dendrogram (B) was constructed based on 51 alleles from 20 SSR primers.

(Appendix 1, Table 6). The cophenetic correlation values indicated that there was a good fit (r=0.94 and 0.84) between the Jaccard similarity coefficient and symmetrical matrixes produced based on genotypic and phenotypic data, respectively (Figure 4.12). There was also a high correlation (r=0.71) between the phenotypic and genotypic data.

The phenotypic dendrogram showed that 3SA132, 3SA134, 3SA137, 3SA140 and 3SA144 were different, while on genotypic level they were 100% similar for markers used (Figure 4.12). Both phenotyping and genotyping grouped 3SA129, 3A132, 3SA134, 3SA137, 3SA140 and 3SA144 races together while 3SA122, 3SA145, 3SA146 and 3SA147 formed a second group. Race 3SA133 was phenotypically more similar to 3SA147 while genetically it was more similar to 3SA129, 3SA132, 3SA134, 3SA137, 3SA140 and 3SA144. Phenotyping thus showed three groups while genotyping showed two (Figure 4.12).

4.4.2 Identification of unknown *Pt* field isolates

In order to identify the *Pt* field isolates collected during the 2013 survey, a dendrogram was constructed using Jacquard similarity coefficient and UPGMA clustering (Figure 4.13). The co-phonetic correlation coefficient indicated that there was a good fit (r =0.98) between the Jaccard similarity coefficient matrix and the symmetrical matrix produced from the dendrogram.

All isolates and reference races were divided into two main (A and B) groups sharing 48% genetic similarity (Figure 4.13). None of collected isolates grouped with races 3SA122, 3SA125, 3SA129, 3SA146 or 3SA147. The majority of isolates grouped with *Pt* race 3SA145 with 82% genetic similarity in group a. A total of nine different genotypes sharing different similarities were represented in this group.

Only four *Pt* field isolates did not group with 3SA145 (Figure 4.13). They were L12-13 and L15-13 that shared 71% genetic similarity with 3SA133 in group ii and isolates L22-13 and L8-13 that shared 78% genetic similarity with a cluster of reference races (3SA132, 3SA134, 3SA137,

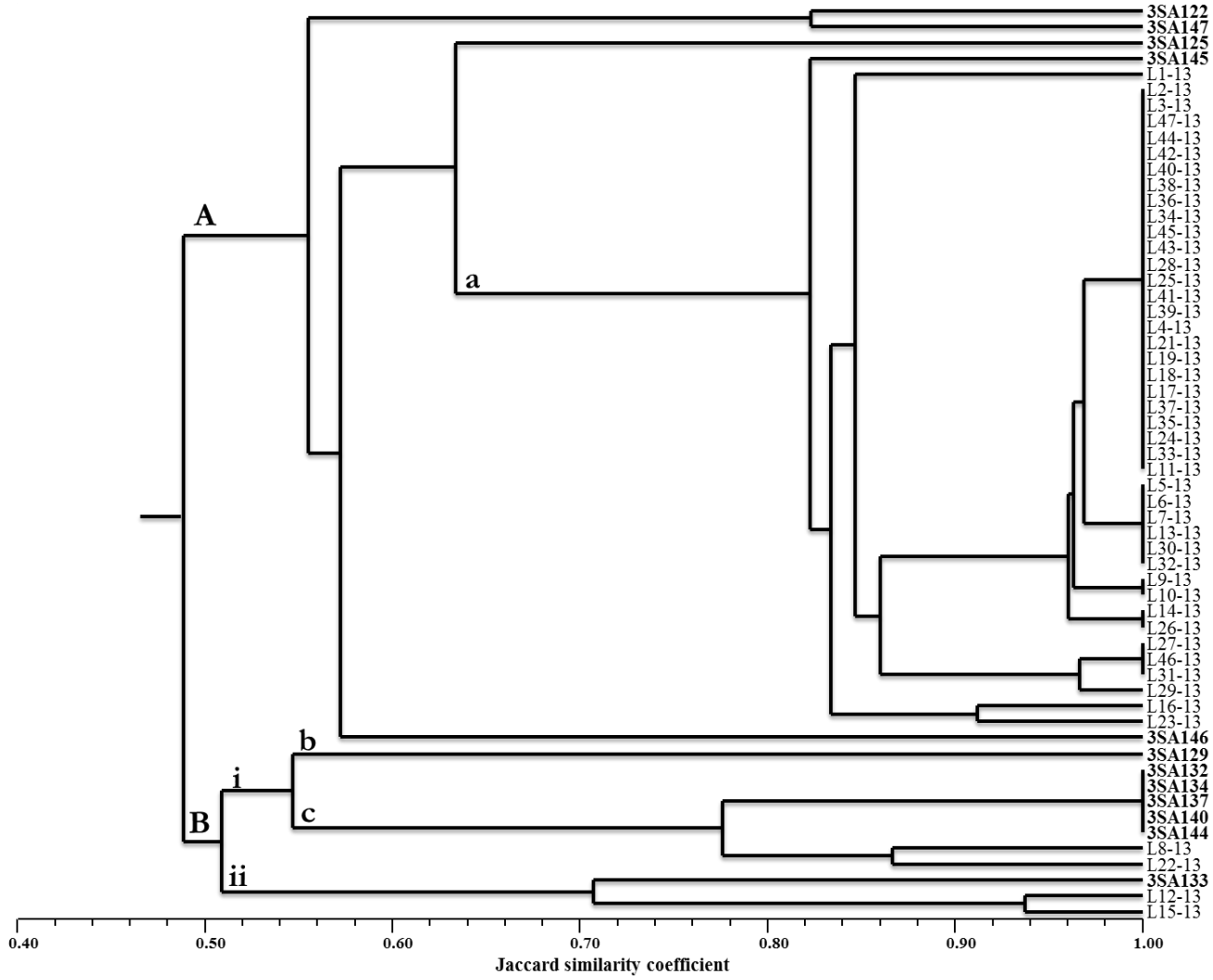

Figure 4.13: Dendrogram representing *Pt* isolates collected during the 2013 survey. The dendrogram was constructed using SSR markers developed by Szabo and Kolmer (2007) and Wang *et al.* (2010) which generated 58 alleles. The reference races are indicated in bold.

3SA140 and 3SA144) in group c. Despite several attempts, these races could not be separated from each other, making the identification of L22-13 and L8-13 inconclusive.

The predominant race genotype and phenotype was 3SA145 (Table 4.17). There were two unknown genotypes while one sample failed to amplify. There was no agreement between the genotype and phenotypes of samples S29-13, S31-13, S45-13 and S47-13. This was probably because there was no reference race for 3SA115 included in the genotypic analysis. However there was agreement between the phenotypes and genotypes in 35 samples. Only one 3SA133 phenotype was correctly identified by genotyping while the other 3SA133 genotype was phenotyped and therefore could not be checked for agreement. One isolate phenotyped as race 3SA147 was identified by genotyping as 3SA145. Three samples were identified by phenotyping as 3SA115 while genotyping detected them as 3SA145.

4.4.3 Statistical analysis of comparison between genotypes and phenotypes of *Pt* isolates collected during the 2013 survey

Table 4.18 shows the genotype X phenotype cross-classification of 39 *Pt* isolates. Only samples which had been identified by both phenotyping and genotyping were included in the statistical analysis. The conditional probability of agreement of Prob (genotype = 3SA145|phenotype = 3SA145) = 34/34 = 100% (95% CI 89.72 to 100%). This indicates that there was perfect agreement and high sensitivity for identification of isolates with the 3SA145 phenotype. The probability of agreement Prob (genotype = not 3SA145|phenotype = not 3SA145) was not calculated because their frequency was too low. It was not possible to meaningfully estimate the sensitivity and specificity of 3SA115, 3SA133 and 3SA147 phenotypes because they had too low frequencies.

Table 4.17: The identification of *Pt* field isolates collected in 2013 using genotypes and phenotypes. Rows highlighted in light grey indicate a match between phenotypes and genotypes. Dark grey rows show isolates for which the racial identity could not be determined using genotyping, phenotyping or both. White rows show no match between genotypes and phenotypes.

Sample	Genotype	Phenotype	Sample	Genotype	Phenotype
L1-13	3SA145	3SA145	**L25-13**	3SA145	Not phenotyped
L2-13	3SA145	3SA145	**L26-13**	3SA145	3SA145
L3-13	3SA145	3SA145	**L27-13**	3SA145	3SA145
L4-13	3SA145	Not phenotyped	**L28-13**	3SA145	3SA145
L5-13	3SA145	3SA145	**L29-13**	3SA145	3SA115
L6-13	3SA145	3SA145	**L30-13**	3SA145	3SA145
L7-13	3SA145	3SA145	**L31-13**	3SA145	3SA115
L8-13	Unknown	Not phenotyped	**L32-13**	3SA145	3SA145
L9-13	3SA145	3SA145	**L33-13**	3SA145	3SA145
L10-13	3SA145	3SA145	**L34-13**	3SA145	3SA145
L11-13	3SA145	3SA145	**L35-13**	3SA145	3SA145
L12-13	3SA133	3SA133	**L36-13**	3SA145	3SA145
L13-13	3SA145	3SA145	**L37-13**	3SA145	3SA145
L14-13	3SA145	3SA145	**L38-13**	3SA145	3SA145
L15-13	3SA133	Not phenotyped	**L39-13**	3SA145	3SA145
L16-13	3SA145	3SA145	**L40-13**	3SA145	3SA145
L17-13	3SA145	3SA145	**L41-13**	3SA145	3SA145
L18-13	3SA145	Not phenotyped	**L42-13**	3SA145	Not phenotyped
L19-13	3SA145	3SA145	**L43-13**	3SA145	3SA145
L20-13	Not genotyped	3SA145	**L44-13**	3SA145	3SA145
L21-13	3SA145	3SA145	**L45-13**	3SA145	3SA147
L22-13	Unknown	Not phenotyped	**L46-13**	3SA145	3SA145
L23-13	3SA145	3SA145	**L47-13**	3SA145	3SA115
L24-13	3SA145	3SA145			

Table 4.18: Genotype X phenotype cross-table of 39 *Pt* isolates. The diagonal cells of the cross-table show the number of isolates where phenotype and genotype agree.

		Phenotypes				
	Frequency	**3SA115**	**3SA133**	**3SA145**	**3SA147**	**Total**
Genotypes	**3SA115**	0	0	0	0	0
	3SA133	0	1	0	0	1
	3SA145	3	0	34	1	38
	3SA147	0	0	0	0	0
	Total	3	1	34	1	39

4.4.4 Population structure analysis of *Pt* field isolates collected during the 2013 survey

The STRUCTURE program was used to determine the number of *Pt* population groups in South Africa. The optimum number of *Pt* populations in South Africa was K=2 (Figure 4.14). The two populations were clearly evident in Figure 4.15. In population 1 most of the isolates were not genetically mixed with only 3SA146, 3SA147, 3SA122 and 3SA125 having genetic components from both populations. Population 1 contained all the reference races excluding 3SA145. Only 3SA145 of the reference races which had a mixed genetic component, was present in population 2. Population 2 contained all isolates that were genotyped as genetically similar to 3SA145. L1-13, L23-13 and L16-13 had a mixed genetic component from population 1. Structure analysis confirmed the groupings obtained in the rooted dendrogram.

AMOVA was used to determine how differentiated the two populations were (Table 4.19). The F_{ST} value of 0.543 ($P<0.0001$) showed that two populations were highly differentiated. Most of the genetic variation was due to variation between the populations (54.3%) with less variation from within populations (45.7%).

4.4.5 Network analysis of *Pt* isolates collected during the 2013 survey

A minimum spanning network constructed to detect the development of *Pt* races and isolates collected in South Africa (Figure 4.16) correlated with the genetic dendrogram and STRUCTURE analysis results (Figures 4.13 and 4.15). This was evident from the fact that the network divided the isolates into two groups (groups A and B) that were separated by 10 mutational events in a similar manner as the dendrogram and STRUCTURE results. Reference races 3SA125, 3SA122 and 3SA147 were the most distantly related isolates to sub groups a and b in group A (Figure 4.16). L29-13 that was phenotyped as 3SA115, was separated by one mutation from L27-13, L31-13 and L46-13, with L31-13 also being phenotyped as 3SA115

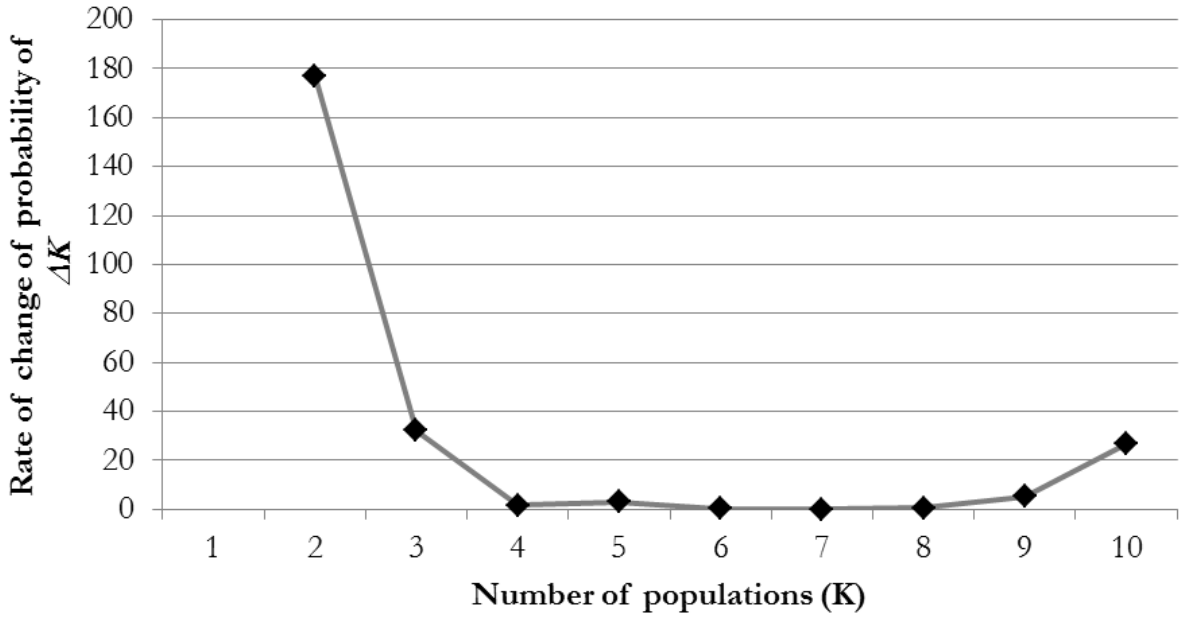

Figure 4.14: A plot representing an *ad hoc* ΔK statistics (Evanno *et al.*, 2005) for 2013 *Pt* isolates. Analysis was based on 50 000 burn-in and MCMC replications for K=2 to 10 and 10 replications per run.

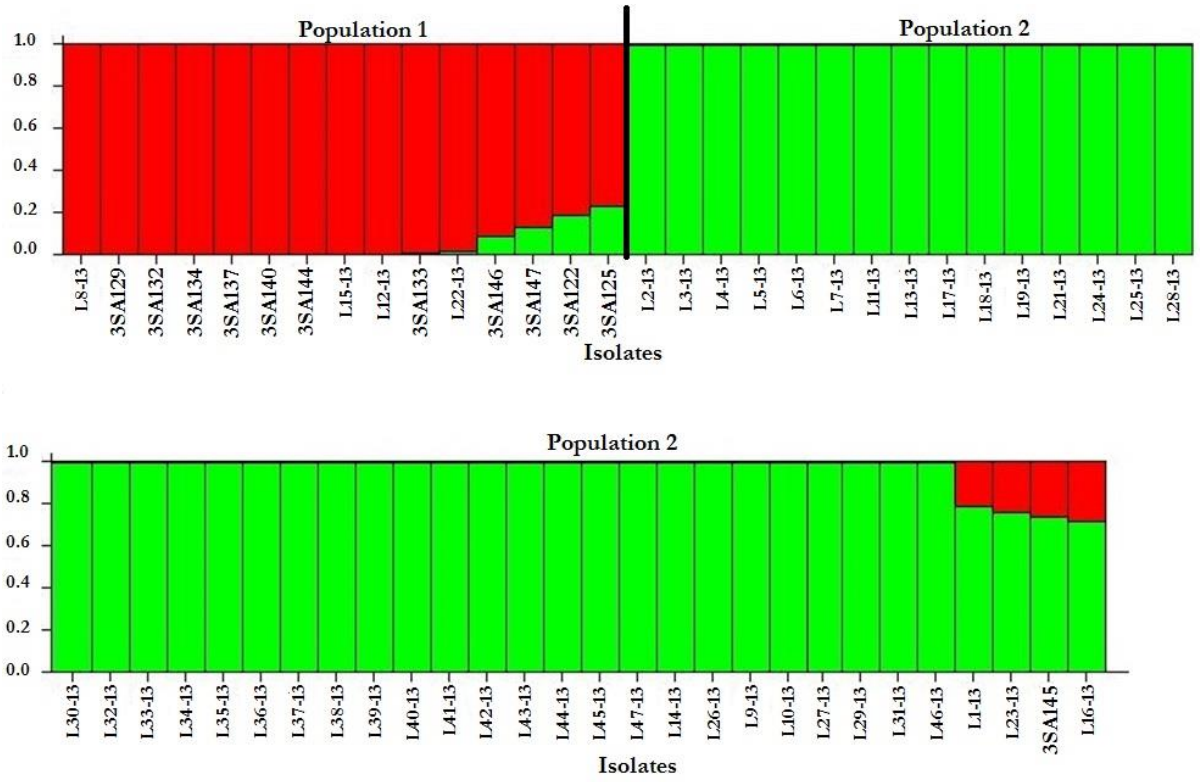

Figure 4.15: Population structure analysis of *Pt* isolates collected in 2013 when K=2. The green and red colours show the genetic composition of each isolate. Each isolate was assigned to a specific population based on its highest genetic contribution.

Table 4.19: Genetic variation of the 2013 survey *Pt* populations in South Africa when K=2.

Source of variation	df	Sum of squares	Variation components	% variation	P-value	F_{ST}
Between populations	1	92.7	4.0	54.3	0.00	0.543
Within populations	57	189	3.4	45.7		
Total	58	281.7	7.4			

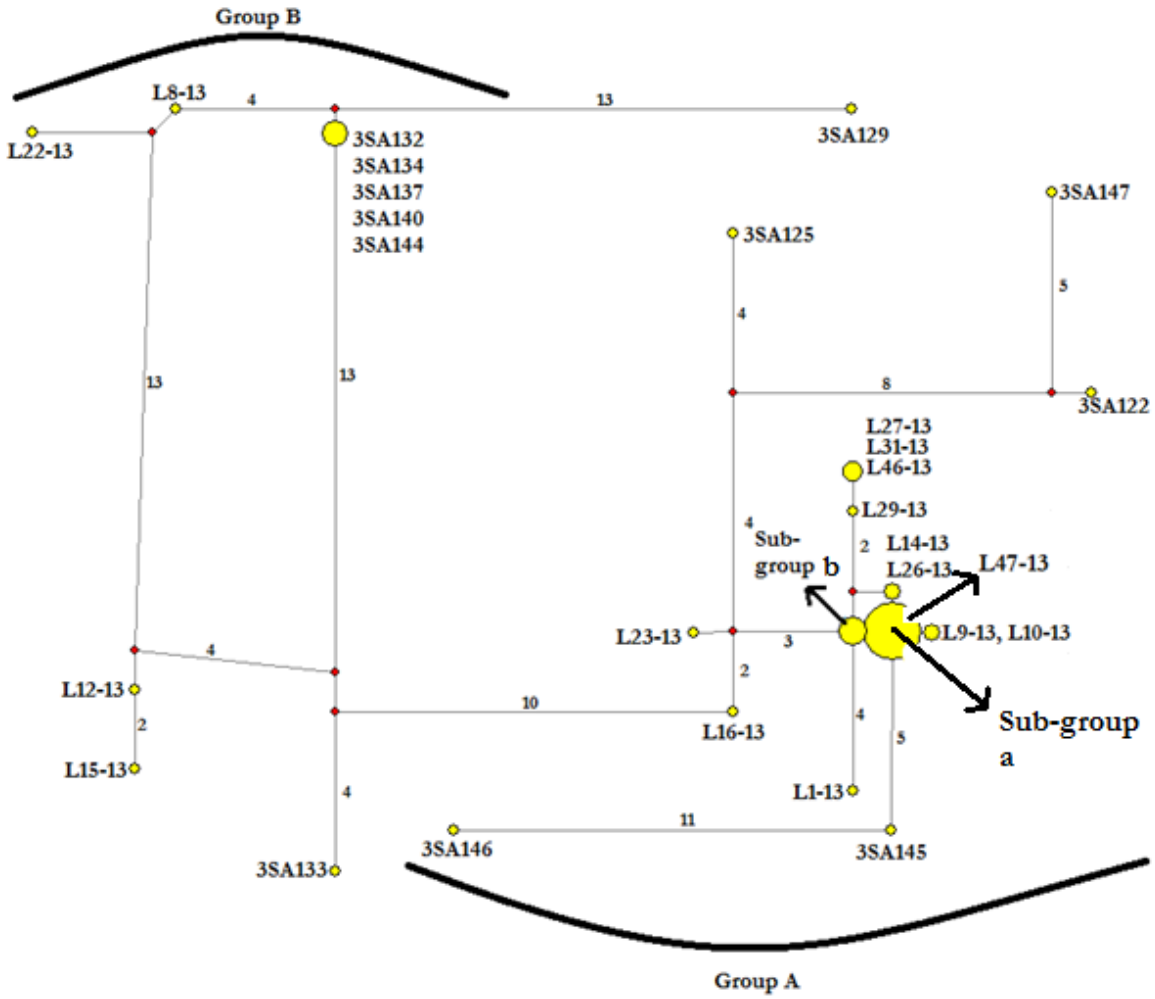

Figure 4.16: Minimum spanning network for 2013 *Pt* survey isolates. Closed squares indicate possible recombination events. Yellow circles indicate genotypes of the isolates and the size of the circle is proportional to the number of isolates. Red circles indicate hypothetical intermediates. Numbers next to the lines indicate the number of proposed mutational events and where no number is given, one mutational event is implied.

(Table 4.17). L27-13, L31-13 and L46-13 isolates were separated by four mutational events from the L14-13 and L26-13, and sub group b isolates and a further five mutational events from sub group a. These three isolates were separated by 10 mutational events from 3SA145. Isolate L47-13 which belongs to sub group a was phenotyped as 3SA115. The isolates which were distantly related to sub group a and b were admixed in population 1 (3SA122, 3SA125, 3SA146 and 3SA147) (Figure 4.15) because they were separated by at least 12 mutational events from sub group a. Isolates L1-13, L16-13, L23-13 and race 3SA145 which were admixed in genetic population 2 (Figure 4.15) were separated by more than four mutational events from sub groups a and b isolates that were genotyped as 3SA145. Therefore isolates which had a mixed genetic component in population 2 (L1-13, L16-13, L23-13 and 3SA145) were more closely related to sub groups a and b. Race 3SA146 was separated from 3SA145 by eleven mutational events. In group B, there was a possible recombination event between races 3SA132, 3SA133, 3SA134, 3SA137, 3SA140 and 3SA144 and isolates L8-13 and L12-13. Isolates in Group B were mainly isolates from population 1 (Figure 4.15).

4.5 Genetic analysis of southern African *Pt* isolates

4.5.1 Analysis of genetic variation in southern African *Pt* isolates

Five *Pt* isolates were collected in Zambia (Zam12_003), Zimbabwe (Zim001_12 and Z13-1) and Malawi (Mal1 and Mal2). To determine the genetic relationship of these isolates with South African *Pt* races, a dendrogram was constructed using SSR data (Figure 4.17). There was a good (r=0.927) fit between the Jaccard coefficient and symmetrical matrixes.

Two major groups (A and B) were found (Figure 4.17). All four Zim001_12 single pustule isolates clustered with 100% genetic similarity with 3SA147, suggesting that these two are identical. Two Z13-1 single pustule isolates grouped with 3SA146 with 89% genetic similarity while the two Mal isolates clustered with 3SA132, 3SA134, 3SA137, 3SA140 and 3SA144 (83%

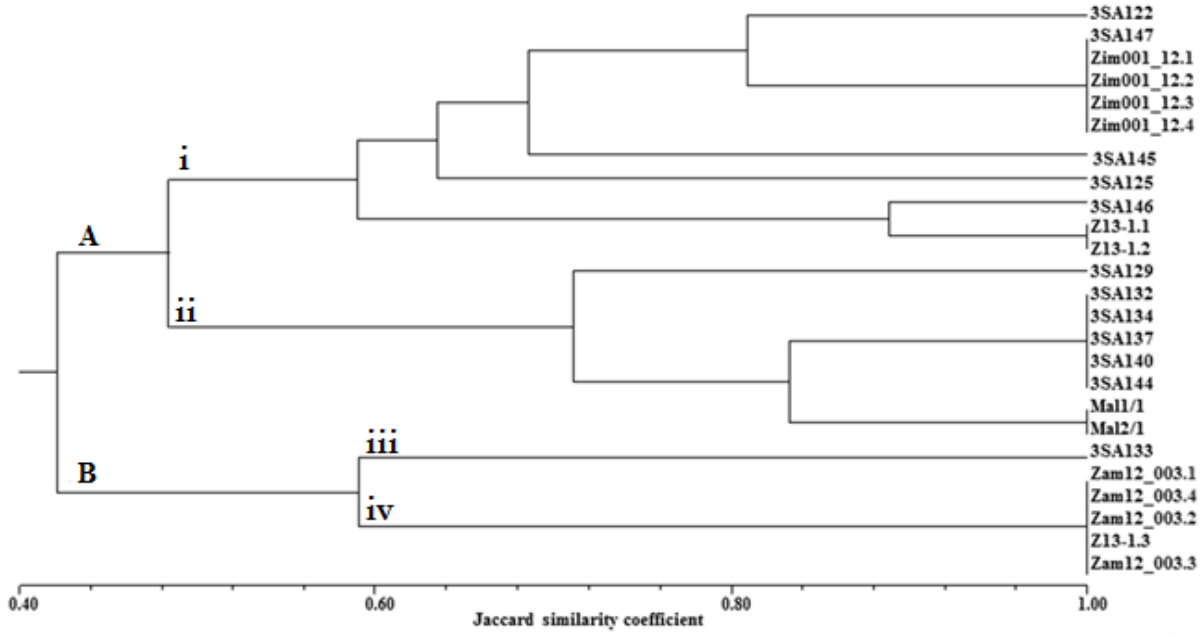

Figure 4.17: Dendrogram representing *Pt* races and isolates from South Africa (3SA), Malawi (Mal), Zimbabwe (Zim001_12 and Z13) and Zambia (Zam12).

genetic similarity). The Zam12_003 isolates clustered with Z13-1.3 with 59% genetic similarity to 3SA133 in group B.

4.5.2 Population structure analysis of southern African *Pt* isolates

To determine the number of *Pt* populations present in southern Africa, STRUCTURE was used. The analysis showed that the optimum number (ΔK) of populations was K=3 and therefore three populations were found (Figure 4.18). Population 1 contained both South African races and the Zim001_12 isolates (Figure 4.19). Races 3SA122 and 3SA145 contained genetic components from population 3, while 3SA125 had a genetic component from population 2. Population 2 included the Z13-1.3 and Zam12_003 isolates. Isolates in population 2 which formed part of group B (Figure 4.17) were clearly different from the other isolates in the dendrogram and the genetic component was almost 100% only from population 2. Population 3 contained the Malawian isolates, as well as several South African races and two Zimbabwean (Z13-1.1 and Z13-1.2) isolates. Isolates without a mixed genetic component in population 3 were mostly from sub group ii in the dendrogram (Figure 4.17). Isolates Z13-1.1 and Z13-1.2 had a mixed genetic component mainly from population 1 and 3, confirming the relatedness between Zimbabwean isolates. The genetic components of 3SA133 and 3SA129 in population 3 were admixed with those of population 2. There was a correlation between the 2013 *Pt* data survey results (Figure 4.15) and the southern African *Pt* population (Figure 4.19). Population 1 (Figure 4.19) partially corresponded with South African population 2 (Figure 4.15) and population 3 (Figure 4.19) with South African population 1 (Figure 4.15).

AMOVA analysis was used to determine the genetic differentiation within and between the three populations of southern African *Pt* isolates (Table 4.20). The AMOVA results showed that there was a high F_{ST} value of 0.67 ($P<0.0001$) indicating a high differentiation between the three populations. Most of the genetic variation was attributed to variation between populations (67%) with a smaller component variation coming from within populations (33%).

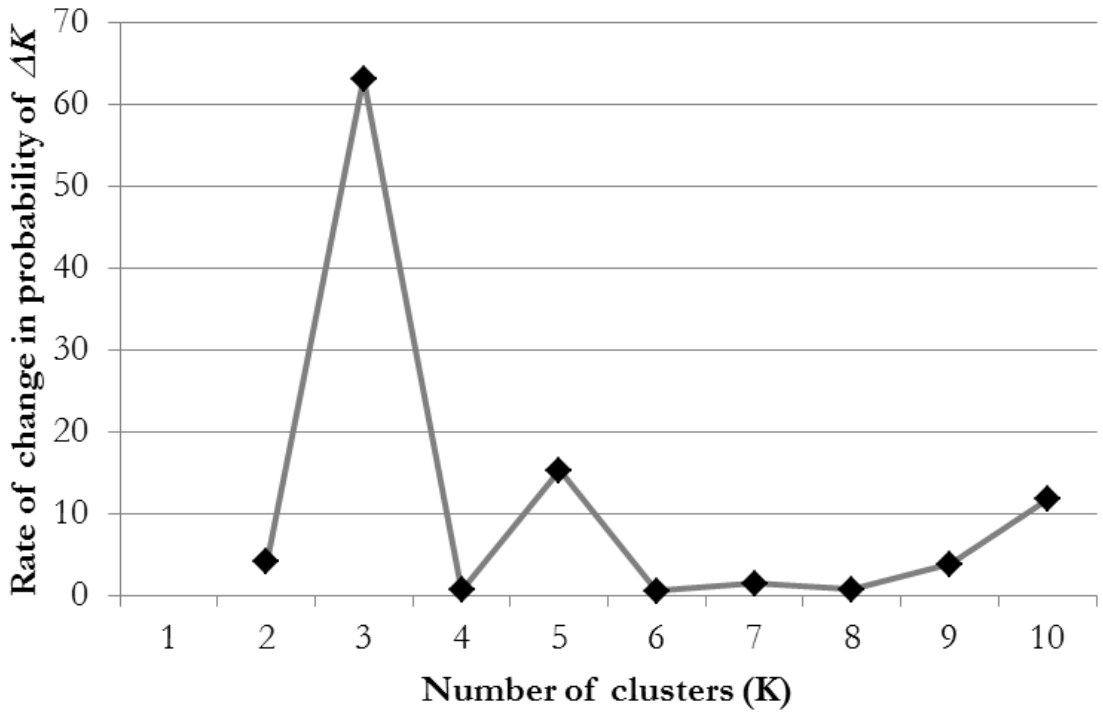

Figure 4.18: A plot representing an *ad hoc* Δ*K* statistics (Evanno *et al.*, 2005) for the southern African *Pt* isolates. Analysis was based on 50 000 burn-in and MCMC replications for K=2 to 10 and 10 replications per run.

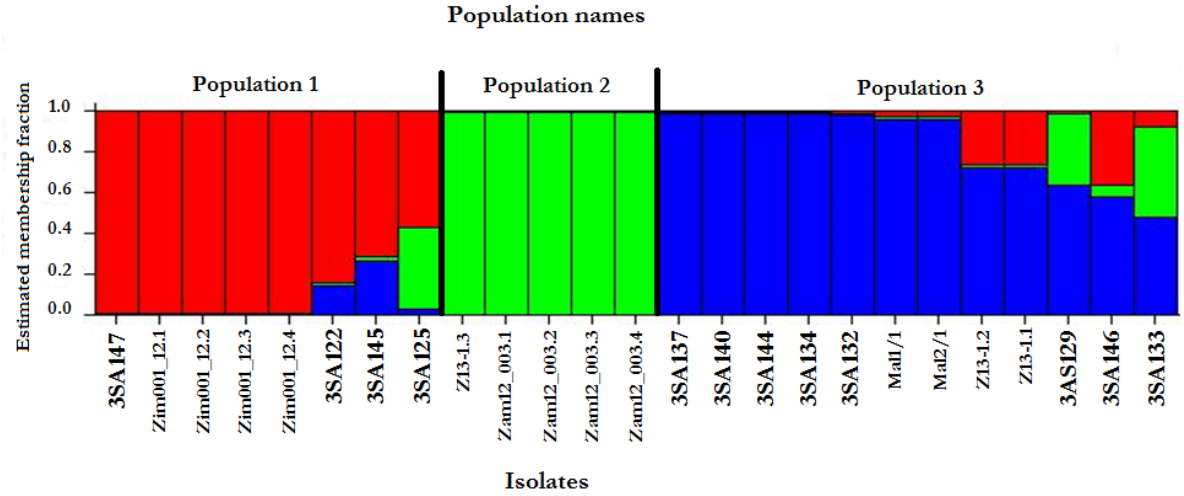

Figure 4.19: Population structure analysis of *Pt* isolates representing the isolates from South Africa (3SA), Zimbabwe (Zim001_12 and Z13), Zambia (Zam12) and Malawi (Mal). The blue, green and red colours represent the genetic composition of each isolate from the contribution of three populations. Isolates were assigned to specific populations based on their highest genetic contribution.

Table 4.20: Genetic variation of the three *Pt* populations collected from South Africa, Zimbabwe, Zambia and Malawi.

Source of variation	df	Sum of squares	Variation components	% variation	P-value	F_{ST}
Between populations	2	78.040	4.55314	67	0.00	0.67
Within populations	24	49.080	2.23089	33		
Total	26	127.120	6.78402			

Chapter 5:

Discussion

5.1 Stem rust survey from 2010 to 2012

The correlation between stem rust races and genotypes has not been investigated on a population level in South Africa. Genetic variation between races has however been investigated (Visser *et al*., 2009; 2011). This was the first study to investigate the efficiency of detection of South African stem rust race phenotypes using genotypes on a large scale.

Infection type analysis (phenotyping) for identification of races can be labour intensive while the non-viability of spores can make it impossible to identify isolates. A two stage protocol was used to distinguish between Ug99 and non-Ug99 isolates collected during the 2010-2012 surveys. Visser *et al*. (2012b) confirmed the effectiveness of the protocol. The Ug99 races were then identified using SNPs while the non-Ug99 races were identified using SSRs (Karaoglu *et al*., 2013). For comparison, the isolates were first identified by phenotyping by Dr. T. Terefe from the ARC-SGI.

5.1.1 The possibility of mixed races and the confidence of the classification methods

The stem rust isolates which were used in the current study were collected from all South African agro-ecological regions by sampling whole plants. Genotyping was performed on single pustule isolates while phenotyping was done using spores collected from the entire plants. This was the reason why only one race was genotyped for each sample (Tables 4.4, 4.5, 4.6, 4.9, 4.11, 4.12, 4.13). For the same reason, two different races were frequently identified in some samples using phenotyping. However, some Ug99 genotyped samples had non-Ug99 phenotypes while some non-Ug99 genotyped samples had Ug99 phenotypes. This indicates the possibility that samples where a single race was identified using phenotyping, could contain more races that were not identified using phenotyping. It was speculated that some samples used in the current study contained multiple races (Visser *et al*., 2012b) that probably caused differences between genotyping and phenotyping results.

Phenotyping and genotyping showed that the predominant race was a Ug99 race (2SA88) followed by a non-Ug99 race (2SA105) while other races were present in low levels. A small proportion of non-Ug99 phenotypes where identified as Ug99 genotypes (Table 4.7) while a large proportion of Ug99 phenotypes were genotyped as non-Ug99 (Table 4.14). These were prevalent in samples where single races were identified by phenotyping. This pattern suggests mixed races within samples.

The problem of mixed races was also observed when 19 Ug99 phenotyped races seemed to be present in samples which had non-Ug99 genotypes (Table 4.14). One might interpret this as that both Ug99 and non-Ug99 races had the same genotype, but observations cast doubt on that idea. Different phenotypes with the same genotype are present in South Africa (Visser *et al.*, 2011). Ug99 races like 2SA88 have always been different from non-Ug99 races with at least 63% genetic dissimilarity in terms of SSR loci (Visser *et al.*, 2009). Even in the current study the reference 2SA88 genotype was not similar to any of the non-Ug99 reference genotypes (Figures 4.5, 4.6, 4.7).

Keiper *et al.* (2006) found a *P. graminis* f. sp. *avenae* race (94+Pg13) in Australia which isolates had significantly large (44%) genetic variation. In Ethiopia, another non-Ug99 race (RRTT) had variants with 69.6% genetic dissimilarity (Admassu *et al.*, 2009; 2010). However, two Ethiopian isolates with a similar phenotype belonging to the Ug99 lineage only differed by one out of 20 loci. Ug99 races in South Africa were genetically also similar to one another (Tables 4.4, 4.5, 4.6, Visser *et al.*, 2009; 2011). This confirms that isolates which seemingly had Ug99 phenotypes but were similar to non-Ug99 isolates when genotyped, were probably mixed cultures (Figure 4.5, 4.6, 4.7).

The SSR genotyping was able to distinguish Ug99 isolates from non-Ug99 isolates (data not shown) with some isolates which were genetically similar to 2SA88. These isolates were characterized as Ug99 during the first stage of genotyping. These isolates were not included in

the results because the point of using SSR markers was to identify non-Ug99 isolates. Results showed that some samples which had Ug99 phenotypes, non-Ug99 genotypes were detected (Table 4.11, 4.12, 4.13, 4.14). Therefore this confirms that the presence of Ug99 phenotypes amongst non-Ug99 genotypes was because phenotyping and genotyping detected two different races. This made the sampling method followed for phenotyping not definitive to allow comparison of one race phenotype to its genotype.

For stem rust samples the problem of mixed races could not be solved during the current study. In future, field isolates collected by Dr. Terefe will be single pustule isolates in order to solve the problem.

5.1.2 Sensitivity and specificity of identifying races

To test the accuracy of genotyping to identify races, calculations of sensitivity and specificity of genotyping were done. Sensitivity referred to the probability that isolates known to have a specific race phenotype will be detected by genotyping as the same race. Sensitivity shows the percentage of isolates which have genotypes whose identity matches with race phenotypes. Sensitivity can be used to rule out the condition of a specimen if tested negative for a particular condition (Sackett *et al.*, 2000). For example, a pustule identified as 2SA88 will be the specimen and 2SA88 the condition of the pustule. That means sensitivity will be helpful in ruling out a race if an isolate does not have the genotype of the race. High values for sensitivity were useful in this regard. It showed the proportion of isolates that can be identified using genotyping. Specificity was the probability that isolates not phenotyped as a certain known race will not be genotyped as that race. Specificity was used to confirm a condition of a specimen which was tested positive for a particular condition. That means for this study, high specificity of genotyping will be helpful in 'ruling in' race X if an isolate had the genotype of race X. It also means that if specificity is high, then the genotype(s) is mostly to be found in only one race phenotype. If the specificity is low,

then the genotype will be found in other race phenotypes thereby giving inaccurate results. The confidence interval covers the value of the parameter.

5.1.2.1 SNP genotyping for identification of Ug99 races

SNPs have a lower discriminatory power (heterozygosity = 0.2 to 0.4 for 10 to 20 loci) than multi-allelic markers like microsatellites (heterozygosity = 0.6 to 0.8 for four to six loci) (Morin *et al.*, 2004). This makes SNPs more suitable for the identification of races, since a race is a group of individuals with similar virulence characteristics. Lower discrimination is helpful because it will show less variation between the isolates which belong to a single race thereby solving the problem of unknown genotypes. qPCR is known to have high sensitivity and specificity for species identification (Blanco-Meneses and Ristiano, 2011; Boutigny *et al.*, 2013).

It was previously shown that the Ug99 races in South Africa were closely related in terms of SSR loci (Visser *et al.*, 2011). This made it difficult to differentiate between the Ug99 races and SSRs were therefore unsuitable for identification. Dr. L.J. Szabo developed SNP markers which can differentiate between Ug99 races. This prompted the need to test the use of a qPCR based SNP diagnostic method to identify Ug99 races in order to complement phenotyping.

Results showed that SNPs were highly sensitive to detect 2SA88 and therefore indicated that in a group of isolates phenotyped as 2SA88, most of the isolates had a 2SA88 genotype (section 5.1.2.). Due to the fact that sensitivity can be used to rule out the condition of a specimen if tested negative for a particular condition (Sackett *et al.*, 2000), it can therefore be said that a pustules not identified as 2SA88 using these SNPs in the future can be regarded as not 2SA88. The false identification of 2SA88+ based on genotypes lowered the sensitivity value to 77%. The use of SNP marker A022 influenced the sensitivity, since it was included to distinguish 2SA88 from 2SA88+. There was a high frequency of isolates which were falsely identified as 2SA88+ using SNPs (Table 4.7). This is because marker A022 was not 100% able to distinguish between 2SA88 and 2SA88+. Dr. Szabo is in the process to find a new locus which can accurately

distinguish between them. 2SA88+ (TTKSF+) was first detected on cv. Matlabas in South Africa and Zimbabwe in 2010 (Pretorius *et al.*, 2012). The race could only be differentiated from 2SA88 by using cv. Matlabas and virulence to *Srweb* gene. SSRs were able to show that it belongs to the Ug99 lineage. Accurate determination of genotype specificity in identifying these two Ug99 races can be improved finding a new marker that can distinguish between 2SA88 and 2SA88+. Increasing the sample size of isolates which were phenotyped as 2SA88+ and isolates which were not phenotyped as 2SA88+ can help to determine specificity.

Due to low frequencies, sensitivity and specificity for identifying 2SA106 and 2SA107 were not calculated. The sample sizes of 2SA106 and 2SA107 have to be increased because to analyze the sensitivity and specificity of genotypes to identify races, a large number of samples are needed.

There seems to be little research done on the identification of rust races using DNA/molecular based techniques, however there were several articles which showed that plant pathogens can be identified in this manner. Mumford *et al.* (2006) pointed out that it was theoretically possible to design qPCR assays to identify plant pathogens and their races. However, it seems like the practical application of molecular techniques to identify isolates particularly in rusts like *Pgt* and *Pt* is rare, as researchers seem to mainly focus on the identification of species rather than races (Barnes and Szabo, 2007). The high sensitivity to identify 2SA88 is a promising discovery and might be a landmark in the identification of Ug99 races using SNPs. One advantage of molecular techniques is the identification of non-viable rust spores and the speed of identification which makes it easier to identify preserved pustules.

5.1.2.2 Identification of non-Ug99 races

5.1.2.2.1 Correlation between phenotypes and SSR genotypes

In essence, correlation shows whether phenotypes can be associated with genotypes to allow the prediction of identity of unknown isolates. Low correlation between virulence phenotypes and

molecular genotypes is common among plant pathogens. Inconsistent correlation was found between SSR genotypes and phenotypes of *Pgt* races in populations with high gene flow (Admassu *et al.*, 2010). McCallum *et al.* (1999) also found low correlation between random amplified polymorphic DNA (RAPDs) or isozymes and virulence phenotypes in *Pgt*. In asexually reproducing. Low correlation was also detected in races from asexual populations of *P. graminis* f. sp. *avenae* (Keiper *et al.*, 2006). Races with two different genotypes were detected in *P. graminis* f. sp. *avenae*, where race 94+Pg13 had 13 SSR genotypes in Australia, some of which substantially differed from each other. Isolates were collected from different parts of Australia, especially in New South Wales. In other species, little or no correlation between genotypes and phenotypes were detected in *Pst* using double stranded RNA (Dickinson *et al.*, 1990), RAPDs (Cheng *et al.*, 1993) and amplified fragment length polymorphism (AFLPs) (Steele *et al.*, 2001), as well as in *Puccinia coronata* f. sp. *avenae* using DNA amplification fingerprinting (Brake *et al.*, 2001). Correlation was also not detected in Australian *Uromyces appendiculatus* isolates using restrictive fragment length polymorphism (RFLP) or RAPD markers (Braithwaite *et al.*, 1994).

In the same way there was low correlation (0.18) with major differences between dendrograms of virulence phenotypes and SSR genotypes of non-Ug99 races (Figure 4.4). For good correlation, it was expected that a race will have a genotype(s) which differed from that of other races. The fact that there was 100% genetic similarity between 2SA100 and 2SA105, and 2SA103 and 2SA104 (Figure 4.4) probably gave low correlation values between SSR genotypes generated from seven SSR markers and avirulence/ virulence phenotypes. One way to improve this is to increase the sample size and find SSR markers that can separate 2SA100 from 2SA105 and 2SA103 from 2SA104.

5.1.2.2.2 SSR genotyping to identify non-Ug99 isolates

This study detected several new SSR genotypes (SAG6, SAG11, SAG12, SAG13 and SAG14) in South Africa (Table 4.8, Figures 4.5, 4.7). This was probably due to SSR loci having high

mutation rates caused by DNA replication slippage (Levinson and Gutman, 1987; Tautz, 1989), DNA recombination (Harding *et al.*, 1992) and a higher resolving power to identify individuals compared to SNPs (Morin *et al.*, 2004). SSRs also mainly target non-coding region of the genome and wil thus detect difference that might not give rise to a change in phenotype that can be seen or expressed.

Isolates were classified by comparing their genotypes to SSR loci of reference races using dendrograms of which the results were then compared with the phenotyping results. High sensitivity (95.5%) of SSR genotypes (SAG3, 4, 10, 11, 12) to detect the 2SA105 phenotype was confirmed from the comparison. However this does not give any information about the sensitivity of SAG6 to 2SA105.

Since SAG6 could not be used for calculating sensitivity, it was impossible to rule it out during the identification of 2SA105 because it will still be possible that it is 2SA105. The detection of high sensitivity might also indicate that the correlation determined in the study does not seem to give the whole picture about the identification of *Pgt* isolates but this will be prevalent when the true specificity is determined. Introduction of bootstrap values to the dendrograms can also help in making the dendrogram more reliable by revealing significant clusters, thereby reducing the number of mistakes (Felsenstein, 1985).

Previous studies have shown that 2SA100 and 2SA105 were genetically identical for many SSR loci (Visser *et al.*, 2009; 2011). Since 2SA100 and 2SA105 as well as 2SA104 and 2SA103 could not be separated using seven SSR primer combinations, the number of primer combinations must be increased to separate them because that affects the specificity of the SSR genotypes. In order to compensate for this, S70-10 which had high genetic similarity to 2SA103 and 2SA104 was classified as 2SA104 because 2SA103 has not been detected in the field since 2007 (Terefe *et al.*, 2010; Terefe and Pretorius, 2011a; b). Samples which were identical or similar to 2SA100 and 2SA105 were identified as 2SA105 because 2SA100 was not detected in South Africa since 2007.

5.1.3 The possible identity of unknown genotypes

SAG6 and SAG14 were two unknown genotypes. These two genotypes could either be mixed with two or more known races present in one sample, two other 2SA105 genotypes, or two new different races. The unrooted tree showed that SAG14 (S22-12) and SAG4 (S110-10, S118-10 and S51-11) isolates grouped with SAG6 (Figure 4.8). Most of the samples had a 2SA105 phenotype. These genotypes can thus not be regarded as two different races because isolate S22-12 was also phenotyped as 2SA105, as was most of the SAG6 and SAG4 (S110-10) samples. No new races were detected by phenotyping of SAG6 and SAG14 genotypes. Therefore the best explanation was that all SAG6 and SAG14 genotyped isolates were most probably race 2SA105, only with new genotypes. The identity of isolates with these genotypes must be further investigated on an extended differential set to determine whether they might represent new races.

5.1.4 *Pgt* non-Ug99 population structure

The South African *Pgt* population has not been evaluated for genetic structure before. Three populations were found for the non-Ug99 races. Although 2SA88 formed part of the third population in the STRUCTURE analysis, it is proposed that Ug99 races form part of a fourth population in South Africa and because there was only one Ug99 isolate used for the analysis, the population could not be seen. This is possible because Ug99 and non-Ug99 isolates are very different from one another (Figures 4.5, 4.6, 4.7, Visser *et al.*, 2011). Further investigations are needed to confirm this proposition. The Network and STRUCTURE results showed that there was a possible recombination event which gave rise to some of the isolates collected in the 2010 (S118-10, S110-10), 2011 (S28-11, S51-11) and 2012 (S22-12) seasons (Figures 4.10, 4.11). The recombination was probably an asexual recombination event which is a rare form in rusts (Singh *et al.*, 2008). The fact that 2SA100 and 2SA105 were both in population 1 with the same genotype indicated that 2SA100 possibly gave rise to 2SA105. In population 3 the similarity of

2SA103 and 2SA104 supports the hypothesis that both races have a common ancestor (Pretorius *et al.*, 2007).

The high fixation index (F_{ST} = 0.75) of the South African non-Ug99 *Pgt* populations indicated that most (75%) genetic variation was between populations while 25% of variation was between isolates within populations. This could be seen by small admixtures in eight isolates in population 3, a large admixture in four isolates in population 2 and one admixture in an isolate from population 1. It shows that the variation within populations was mostly contributed by isolates from populations 2 and 3 (Figure 4.10) which was composed of older races (population 3) and new isolates (population 2). The STRUCTURE results indicated that population 1 did not significantly change genetically from 2010-2012, and that only four out of eighteen isolates in population 2 changed. Isolates in population 3 also showed that there were no significant changes in the genetic component of the population within three years. Many isolates with the same genotype were detected in the study thereby explaining the high F_{ST} value which confirms asexual reproduction. These findings support the view that *Pgt* sexual reproduction does not occur in South Africa (Figlan *et al.*, 2014) because the alternate host had not been described. Insignificant changes in the population are good evidence for asexual reproduction because the association between virulence and genetic background is maintained while the genetic characteristics are kept similar in asexual populations (Liu and Kolmer, 1998).

Asexual populations are detected by the absence of the alternative host, the presence of clonal groups and often lower genetic variation. The *Pgt* population in Great Plains, North America was an asexual population which was less diverse than European and South American populations (Roelfs *et al.*, 1997; McCallum *et al.*, 1999). It had 11 clonal groups with each race per group having the same isozyme polymorphisms and similar virulence phenotypes. During the 2004 season there was only one predominant race (QFCS) which comprised almost the entire population, confirming that there was still low variation at the time (Kolmer *et al.*, 2007). The

Australian *Pgt* population was also found to be homozygous and uniform in all isozyme investigated loci (Burdon *et al.*, 1983) confirming that it was an asexual population. Park (2007) pointed out that from 1987-2007 every race that was detected in Australia was a mutational derivative, confirming that an asexual reproducing *Pgt* population existed in 2007.

5.2 Leaf rust survey in 2013

5.2.1 Correlation between *Pt* genotypes and phenotypes

The correlation between *Pt* genotypes and phenotypes was determined using a similarity matrix of genotypes and phenotypes using only reference isolates. A good correlation (r=0.70) was detected between *Pt* phenotypes and genotypes in South Africa. It also gives a degree of interdependence between genotypes and phenotypes. If the two methods have a high correlation, then there is a good possibility that they will have matching results in most experiments.

Wang *et al.* (2010) found moderate correlation (r=0.46) between expressed sequence tag SSRs and virulence phenotypes in Canadian isolates. Significant correlation was also found between SSR genotypes and virulence to *Lr2a*, *Lr2c* and *Lr17a*. In some *Lr* genes like *Lr3*, *Lr3ka*, *Lr9*, *Lr10*, *Lr11*, *Lr16* and *Lr30* no correlation was detected indicating that correlation was not the same for all virulence phenotypes. Similar results were found by Ordoñez and Kolmer (2009) with a moderate correlation (r=0.43) between virulence phenotypes and SSR genotypes in North American isolates. Kolmer and Ordoñez (2007) found correlation between SSR genotypes and virulence phenotypes among individual isolates and at population level in Central Asia and the Caucasus. These results also showed that there was a significant correlation (r=0.46, P=0.001) between SSR genotypes and the *Lr2a* and *2c* genes.

High correlation (r=0.74, P=1.0) was detected between SSR genotypes and virulence phenotypes of *Pt* isolates in Italy (Mantovani *et al.*, 2010). Since identification was based on SSR genotypic

relationship of isolates, it can therefore be concluded that correlation can indicate whether isolates of different races can be identified.

Goyeau *et al.* (2007) indicated that determination of correlation of genotypes and virulence phenotypes can allow confident extrapolation of multilocus genotypes from isolates which have not been genotyped. This showed that there was a possibility that phenotypes could be used to predict the genotypic identity of isolates.

5.2.2 Sensitivity and specificity of SSR genotypes in detecting 3SA145

To date, South African *Pt* isolates have not been evaluated for genetic diagnostics of races on a large scale. 3SA145 was the dominant race in 2010 (Terefe *et al.*, 2014a). Most field isolates collected in 2013 had a high genetic similarity to 3SA145. This made it possible to study the sensitivity of detecting 3SA145 using SSR genotypes. Single pustule isolates were used to identify isolates by genotyping and phenotyping, with few isolates which had genotypes that did not match their phenotypes. Compared to the *Pgt* results, the use of single pustules improved results and allowed a more accurate identification of isolates using genotyping.

The perfect sensitivity to detect 3SA145 indicated that isolates genetically different to 3SA145 can be ruled out as not being 3SA145 (section 5.1.2). Specificity was unable to be determined with a high confidence interval and therefore isolates which had unknown genotypes (Table 4.17) could not be ruled in as part of the 3SA145 race. An isolate (L45-13) which had a 3SA147 phenotype genetically differed from 3SA147 race by 46.3% and had a similar genotype as 3SA145 (Figure 4.13, Table 4.17). This could pose some difficulty in distinguishing 3SA145 from 3SA147 using SSR genotypes. The fact that isolates with high genetic similarity to 3SA145 had 3SA115 and 3SA147 phenotypes and 3SA147 phenotype differed significantly from 2SA145 phenotype indicated that the SSR markers possibly did not have a high specificity to detect 3SA145 because the genotypes closely related to 3SA145 were not found in one race phenotype as explained in section 5.1.2. These findings suggest that not all isolates which were genetically

dissimilar to 3SA145 could be regarded as a different race and therefore SSRs have a possibility of giving false positives.

3SA115 is a new race which differed phenotypically from 3SA145 with virulence to *Lr15*, *Lr17* and *Lr26* (T. Terefe, unpublished data). The dendrogram showed that isolates L29-13 and L31-13, which were phenotyped as 3SA115, clustered with L46-13 and L27-13 which were both genotyped as 3SA145. L47-13, also phenotyped as 3SA115, clustered together with other 3SA145 genotyped isolates. The close relationship between L29-13, L27-13, L31-13 and L46-13 in the Network and dendrogram (Figure 4.13, 4.16) results suggests that isolates phenotyped as 3SA115 could have developed from 3SA145, but this made genotypic identification of 3SA115 difficult. Inclusion of 3SA115 as control could improve the results in the study.

5.2.3 South African *Pt* populations

To date, the South African *Pt* population was not evaluated for genetic structure using a large population size. Visser *et al.* (2012a) showed that the *Pt* population was genetically divided into two populations. Results in the present study confirmed the two genetic populations. The genetic variation between the *Pt* populations was higher than that within each of the populations (Table 4.19). Similar results were detected when the *Pt* races were genetically analyzed (Visser *et al.*, 2012a). The F_{ST} was high (0.54) suggesting that populations were probably not randomly mating but the presence of an admixture event between populations 1 and 2 (Figure 4.15) suggests that random mating might be present at a low rate. Results indicate that the *Pt* populations were asexually reproducing which was in agreement with the phenotypic results. Since population 2 was a pure population with mostly 3SA145 isolates and was highly differentiated from population 1, it can be concluded that the population arose from one ancestral individual isolate through mutation.

In total, 130 North American *Pt* isolates collected between 1990s and 2008 were evaluated based on SSR polymorphisms and a high fixation index (F_{ST}=0.228), heterozygosity and linkage

disequilibria were found (Ordoñez *et al.*, 2010). This indicated that the pathogen was reproducing asexually in the region. Evidence for asexual reproduction in the *Pt* population in Europe was indicated by a high fixation index (F_{ST}=0.317) and higher than expected heterozygosity (Kolmer *et al.*, 2012). Even in France, a large fraction of genotypic clones, significant linkage disequilibrium, a strongly negative F_{IS} value and a high level of heterozygosity were taken as a clear indication that the population was clonal and therefore asexually reproducing (Goyeau *et al.*, 2007). Therefore the high F_{ST} in the South African *Pt* population and the detection of a population made up mostly out of isolates with similar genotypes was typical of asexually reproducing populations.

5.3 Genetic analysis of leaf rust in Malawi, Zambia and Zimbabwe

Three *Pt* populations were detected in southern Africa. The fixation index (F_{ST}=0.67) for the southern African populations was high which suggests that there was significant differentiation between the three southern African populations (Figure 4.19, Table 4.20). The fact that South African races were assigned to the same population as the Malawian (Mal1 and Mal2), and Zimbabwean (Zim001_12 and Z13) isolates (Figure 4.19) using Bayesian model cluster analysis suggests that there was inoculum exchange between South Africa, Malawi and Zimbabwe.

There was evidence of possible recombination within the leaf rust isolates in southern Africa. Recombination was found between the older (3SA122, 3SA125 and 3SA133) and newer (3SA145 and 3SA146) South African and Zimbabwean races. This was revealed by the admixture of the races (Figure 4.19) which showed that each have a genetic contribution from at least two populations. Recombination plays a role in increasing variation in a population and therefore acts as a source of new virulence. Determining the extent of this recombination will be valuable because it will show the contribution of recombination to variation in the Zimbabwean *Pt* population.

Migration of inoculum of rust pathogens was shown by the similarity in race phenotypes and molecular genotypes between different regions. Isolates of *P. graminis* f. sp. *avenae* from distant locations in Australia were identical and were taken as evidence for long-distance dispersal (Keiper *et al.*, 2006). Similarity in AFLP banding patterns of isolates from Australia and the United Kingdom strengthened the view that *Pst* was dispersed from Europe to Australia (Steele *et al.*, 2001). Genetic similarities in isozyme and RAPD banding patterns were taken as evidence for migration of *Pgt* isolates from Europe to South America (McCallum *et al.*, 1999). Migration of *Pt* inoculum between North America and South America was shown to possibly occur using similarity in SSR groups (Ordoñez *et al.*, 2010).

Migration plays a role in changing the population structure of pathogens and increasing variation. Sometimes the migrating races are aggressive, capable of causing epidemics and contributing to loss of effective resistance genes from newly released cultivars. In Uruguay, the introduction of MCDSS and similar race phenotypes caused epidemics in 1999 (Ordoñez *et al.*, 2010). These epidemics occurred over a large area on cultivars which contained *Lr17* (Estanzuela Pelon 90 and INIA Mirlo). The arrival of a new *Pt* race in Australia in 1984 with virulence to *Lr16*, *Lr27* and *Lr31* changed the whole population structure of *Pt* within 20 years because the race gave rise to new variants which dominated agro-ecological regions (Park *et al.*, 1995; Ordoñez and Kolmer, 2009). A new race in Canada and the United States contributed virulence to *Lr3bg*, *Lr17* and *LrB* in 1996 (Ordoñez and Kolmer, 2009). It was speculated that the race migrated into North America because it was different in AFLP polymorphism and virulence phenotypes to the local Manitoba and Saskatchewan isolates (Kolmer, 2001). Kolmer *et al.* (2005) speculated that races with virulence to *Lr14a* and *Lr20* migrated into the United States because they were new races with new phenotypes. Therefore it would be helpful to conduct regular surveys within southern Africa rather than just South Africa to anticipate races that might migrate into the country.

Epidemiological regions are characterized based on the anticipated airborne spread of urediniospores by wind during the wheat growing season (Saari and Prescott, 1985), diversity in the pathogen population (Kolmer, 1989) and in some geographical regions according to mountain barriers (Kolmer and Ordoñez, 2007). Saari and Prescott (1985) considered sub-Saharan Africa, combined with the south-western Arabian peninsula, as an epidemiological zone for cereal rust fungi.

Pretorius and Purchase (1990) showed by phenotyping that races similar to South African races were present in Zimbabwe, Zambia and Malawi indicating that there was possible movement of inoculum between these countries. The SSR dendrogram results (Figure 4.17) were consistent with this as Mal1/1 and Mal2/1 were similar to 3SA137 while Zim001_12 was phenotyped similar to 3SA147 (Pretorius *et al*., 2014). Zam12_003 and Z13-1.3 were designated TCPS which had not been previously identified in South Africa. Since isolates Zam12_003 and Z13-1.3 were genetically identical but different from the closest South African race (3SA133), it indicates that TCPS could possibly be identified using genotyping.

It will thus be difficult to breed for durable resistance in South Africa without considering the neighboring countries as they contribute variation in the *Pt* population. Calculation of the rate of migration (N_m) will help in understanding the influence of migration in the population.

5.4 Closing remarks

The hypothesis for the study was that genotyping of *Pgt* and *Pt* can supplement phenotyping. The hypothesis was tested when identifying field isolates, when analysing the population structure of *Pgt* and *Pt* and when *Pt* was analyzed to show that southern Africa is a single epidemiological region.

The specificity of genetic markers for uniquely identifying *Pgt* and *Pt* isolates was poor. Since specificity is important for ruling in isolates which have a similar genotype of a race, it gives an

indication of how often the genotype(s) are present in a particular race alone. Although good sensitivity was detected, it was difficult to confidently indicate the proportion of isolates with genotypes that could be exclusively found in a single race. Therefore, there is no way of confidently ruling in isolates which had genotypes 2SA105, 2SA88 or 3SA145. This is because there is a possibility that these races share genotypes with different races. The specificity of 2SA88 must be re-tested because the one calculated in the study was unreliable as there were mixed races. Therefore the genotypes can supplement phenotypes by ruling out isolates which are not 2SA105, 2SA88 and 3SA145.

The main lesson learned from the study is that results of *Pt* race identification were much better than that of *Pgt*. The *Pt* results were improved because the *Pt* samples were single pustule isolates, indicating that genotyping is best investigated using single pustule isolates as opposed to whole plants that might contain mixed isolates.

The fact that isolates classified as TCPS (Zam12_003 and Z13-1.3) could be differentiated from other races is a good illustration of the potential of genotyping to identify unknown races. However, since the sensitivity and specificity of genotypes to identify the race is unknown, future research will focus on determining them.

New genotypes were detected for *Pgt*. Genotyping can also be used to detect populations that cannot be detected by phenotyping. Asexual reproduction in *Pgt* and *Pt* was confirmed using genotyping. Therefore this shows that genotyping did complement phenotyping in population genetic analysis of *Pgt* and *Pt*. Migration of *Pt* isolates was confirmed using genotyping and phenotyping. Genotyping also showed possible recombination between isolates which was not observed by phenotyping.

To improve the current study, future work can include the determination of mode of reproduction by calculation of heterozygote excess. It can also be made better by determining

whether geographical distance or location affects the genetic diversity of the South African populations. The accuracy of the study can be improved by collecting larger numbers of *Pgt* and *Pt* isolates from all over South Africa in one year because it makes more sense to define a population in one year than to define it over three years. The polymorphic information content can also be used to characterize the efficiency of each primer for detecting polymorphic loci (Shete *et al.*, 2000).

Chapter 6:

Conclusion

In conclusion, the high sensitivity of SNP and SSR genotyping revealed that genotypes complemented phenotyping in South Africa. It is still not clear whether genotyping is specific in the identification of races of *Pgt* and *Pt*. In addition four *Pgt* and two *Pt* populations were confirmed in South Africa, while three *Pt* populations were found in Southern Africa. Since inoculum exchange occurs within Southern Africa, it indicates that these populations are dynamic with frequent changes occurring.

Chapter 7:

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Summary/ opsomming

The hypothesis of whether genotyping complements phenotyping of *Puccinia graminis* f. sp. *tritici* (*Pgt*) and *P. triticina* (*Pt*) was tested. Phenotyping (infection type analysis) has been the method of choice for the identification of races, determination of single-step mutations which amount to asexual reproduction and evaluation of inoculum exchange. However, phenotyping can be labour intensive while the non-viability of spores makes it impossible to identify isolates. Genotyping has been used to confirm the identity of specific races but it has never been used to identify isolates on a large scale. Movement of inoculum has been shown by phenotyping to occur in southern Africa, but not by genotyping.

A two stage protocol was used to identify stem rust Ug99 and non-Ug99 isolates collected during the 2010 - 2012 surveys. Generally there was a large agreement between genotypes and phenotypes. The SNP genotypes had high sensitivity for identification of 2SA88 phenotypes [73.3% (95% CI = 63.8% to 81.5%)]. There was a poor correlation (r=0.14) between the SSR genotypes and 26 *Pgt* resistance genes of the non-Ug99 races. The SSR genotypes had a high sensitivity for identification of 2SA105 [95.5% (95% CI = 77.2% to 99.9%)].

However there was strong correlation (r=0.71) between genotypes and 17 *Pt* resistance genes for *Pt* isolates. There was a high sensitivity for genotypes to identify 3SA145 [100% (95% CI = 89.72 to 100%)]. It is still not clear whether genotyping is specific in the identification of *Pgt* and *Pt* races. Although there was good sensitivity detected, it was difficult to confidently indicate the proportion of isolates with genotypes that could be exclusively found in a single race.

Three highly differentiated (F_{ST}=0.75) non-Ug99 *Pgt* and a single Ug99 population were identified in South Africa. Two highly differentiated (F_{ST}=0.543; P<0.0001) *Pt* populations were identified in South Africa. Both the *Pgt* and *Pt* populations in South Africa were asexually reproducing. Three *Pt* populations were detected in southern Africa The fixation index (F_{ST}=0.67; P<0.0001) for the southern African populations was high which suggests that there was significant differentiation between the three southern African populations. The Bayesian

model cluster analysis results suggested that there was inoculum exchange between South Africa, Malawi, Zambia and Zimbabwe.

Keywords: Stem rust, leaf rust, genotypes, phenotypes, population genetics

Tydens die studie is die volgende hipotese getoets: die fenotipering van *Puccinia graminis* f. sp. *tritici* (*Pgt*) en *P. triticina* (*Pt*) word deur genotipering gekomplimenteer. Fenotipering (infeksie tipe analise) was voorheen die mees algemene metode om rasse te identifiseer, enkelstap mutasies weens ongeslagtelike voortplanting te bevestig en inokulum uitruiling te evalueer. Fenotipering is egter arbeidsintensief. Nie-lewensvatbare spore kan verder die identifikasie van isolate verhoed. Hoewel genotipering gereeld gebruik word om rasse te identifiseer, was dit nog nooit voorheen op groot skaal gebruik nie. Hoewel fenotipering vroeër die beweging van inokulum in suider-Afrika bevestig het, is genotipering nog nie gebruik nie.

Stamroes isolate wat tydens die 2010 - 2012 opnames versamel is, is met 'n twee-ledige protokol geïdentifiseer. Oor die algemeen het die genotipes en fenotipes van isolate ooreengestem. Die SNP genotipes het 'n hoë sensitiwiteit gehad om 2SA88 fenotipes te identifiseer [73.3% (95% CI = 63.8% tot 81.5%)]. Hoewel die nie-Ug99 rasse 'n swak korrelasie (r=0.14) tussen SSR genotipes en die fenotipes van 26 *Pgt* gene getoon het, het die SSR genotipes met hoë sensitiwiteit 2SA105 geïdentifiseer [95.5% (95% CI = 77.2% tot 99.9%)].

Daar was 'n sterk korrelasie (r=0.71) tussen die genotipes en fenotipes van 17 *Pt* weerstandsgene vir *Pt* isolate, asook 'n hoë sensitiwiteit van genotipering om 3SA145 te identifiseer [100% (95% CI = 89.72 tot 100%)]. Dit is egter nog nie duidelik of genotipering spesifiek vir die identifikasie van *Pgt* en *Pt* rasse is nie. Hoewel hoë sensitiwiteit gevind is, was dit moeilik om met sekerheid die gedeelte van die isolate met genotipes wat eksklusief in binne 'n bepaalde ras voorkom, aan te dui.

Drie hoogs gedifferensieerde (F_{ST}=0.75) nie-Ug99 en 'n enkele Ug99 *Pgt* populasie is in Suid-Afrika gevind. Verder is daar ook twee hoogs gedifferensieerde (F_{ST}=0.75; P<0.0001) *Pt* populasies gevind. Beide die *Pgt* en *Pt* populasies plant aseksueel voort. In suider-Afrika is drie *Pt* populasies gevind met 'n fiksasie indeks van 0.67 (P<0.0001). Dit was 'n aanduiding dat die drie populasies betekenisvol van mekaar verskil. Die Bayesian model groeperings analise het aangedui dat inokulum tussen Suid-Afrika, Malawi, Zambië en Zimbabwe uitgeruil word.

Sleutelwoorde: Stamroes, blaarroes, genotipes, fenotipes, populasie genetika

Appendix 1

Table 1: *Pgt* isolates collected in South Africa during the 2010 annual survey conducted by Dr. T. Terefe.

Name of sample	[a]Bulk	Source
S1-10	2SA88	Tygerhoek
S2-10	2SA88	Tygerhoek
S4-10	2SA88	Tygerhoek
S5-10	2SA88	Tygerhoek
S6-10	2SA88	Tygerhoek
S7-10	2SA88	Tygerhoek
S10-10	2SA88	Tygerhoek
S11-10	2SA88	Tygerhoek
S12-10	2SA88	Tygerhoek
S18-10	2SA88	Tygerhoek
S22-10	2SA88	Kleinfontein
S23-10	2SA88/2SA105	Langgewens
S24-10	2SA105	Langgewens
S25-10	2SA88/2SA105	Langgewens
S26-10	2SA88	Langgewens
S27-10	2SA88	Kleinfontein
S29-10	2SA88	Kleinfontein
S30-10	2SA88	Kleinfontein
S31-10	2SA88	Kleinfontein
S32-10	2SA88	Kleinfontein
S33-10	2SA106/2SA88	Kleinfontein
S34-10	2SA105	Kleinfontein
S35-10	2SA88	Kleinfontein
S36-10	2SA88	Kleinfontein
S37-10	2SA88	Kleinfontein
S38-10	2SA88	Kleinfontein
S41-10	2SA102	Kleinfontein
S42-10	2SA88	Kleinfontein
S52-10	2SA88/2SA105	Koringberg
S57-10	2SA88	Koringberg
S58-10	2SA88	Langgewens
S60-10	2SA105	Langgewens
S61-10	2SA105/2SA88	Langgewens
S63-10	2SA88/2SA104	Malmesbury

S64-10	2SA88	Malmesbury
S65-10	2SA88	Malmesbury
S66-10	2SA88	Panorama
S67-10	2SA88	Panorama
S68-10	2SA88	Panorama
S70-10	2SA104	Panorama
S72-10	2SA88/2SA104	Panorama
S75-10	2SA105	Panorama
S76-10	2SA88	Panorama
S81-10	2SA88	Panorama
S83-10	2SA88	Riversdale
S86-10	2SA105	Riversdale
S91-10	2SA105	Porteville
S92-10	2SA88	Tygerhoek
S93-10	2SA88	Tygerhoek
S94-10	2SA88	Tygerhoek
S96-10	2SA88	Tygerhoek
S99-10	2SA88	Tygerhoek
S100-10	2SA88	Tygerhoek
S103-10	2SA105	Tygerhoek
S105-10	2SA88	Tygerhoek
S108-10	2SA88	Nietvoorbij
S109-10	2SA105	Nietvoorbij
S110-10	2SA105	Oude Nektar
S114-10	2SA105	Oude Nektar
S118-10	2SA106	Langgewens
S119-10	2SA88	Langgewens
S120-10	2SA88	Langgewens
S121-10	2SA88	14km South of Langgewens
S122-10	2SA88	14km South of Langgewens
S123-10	2SA88	14km South of Langgewens
S124-10	2SA105	14km South of Langgewens
S127-10	2SA105	33km South of Langgewens
S128-10	2SA105	4km South of Langgewens
S130-10	2SA107	Reitz
S132-10	2SA88	Reitz

S133-10	2SA88	Winterton
S134-10	2SA88	Winterton
S135-10	2SA88	Winterton
S137-10	2SA88/2SA107	Winterton
S138-10	2SA88	Winterton
S139-10	2SA88	Winterton
S142-10	2SA88	Winterton
S144-10	2SA88	Elliot
S145-10	2SA88	Elliot
S146-10	2SA88	Elliot
S147-10	2SA105	Elliot
S148-10	2SA105	Elliot
S153-10	2SA88	Harrismith
S154-10	2SA88	Harrismith
S155-10	2SA88	Harrismith
S156-10	2SA88	Clarens
S157-10	Non-viable spores	

[a]The race identified from the urediniospores collected from infected stems

Table 2: *Pgt* isolates collected in South Africa during the 2011 annual survey conducted by Dr. T. Terefe.

Name of sample	Bulk	Source
S4-11	2SA88	Makhathini
S5-11	2SA88/2SA107	Makhathini
S6-11	Not phenotyped	Makhathini
S7-11	Not phenotyped	Tygerhoek
S8-11	Not phenotyped	Tygerhoek
S9-11	Not phenotyped	Tygerhoek
S10-11	Not phenotyped	Tygerhoek
S11-11	Not phenotyped	Tygerhoek
S12-11	Not phenotyped	Tygerhoek
S13-11	Not phenotyped	Voorstekoop
S14-11	Not phenotyped	Voorstekoop
S15-11	Not phenotyped	Tygerhoek
S16-11	Not phenotyped	Tygerhoek
S17-11	Not phenotyped	Langkloof
S18-11	Not phenotyped	Koringberg
S19-11	2SA88	Langkloof
S20-11	2SA105/2SA104	Langgewens
S21-11	2SA88	Langgewens
S22-11	2SA88	Langkloof
S23-11	2SA88	Koringberg
S24-11	2SA105	Langgewens
S25-11	Not phenotyped	Tygerhoek
S26-11	Not phenotyped	Tygerhoek
S27-11	Not phenotyped	Tygerhoek
S28-11	Not phenotyped	Tygerhoek
S29-11	Not phenotyped	Rietpool/ Alpha
S30-11	Not phenotyped	Roodebloem
S31-11	Not phenotyped	Roodebloem
S32-11	Not phenotyped	Tygerhoek
S33-11	2SA88	Roodebloem
S34-11	Not phenotyped	Roodebloem
S35-11	Not phenotyped	Tygerhoek
S36-11	2SA88	Roodebloem
S37-11	2SA88	Roodebloem

S38-11	2SA88	Rietpool/ Alpha
S39-11	2SA88	Rietpool/ Alpha
S40-11	Not phenotyped	Tygerhoek
S41-11	Not phenotyped	Tygerhoek
S42-11	Not phenotyped	Tygerhoek
S43-11	Not phenotyped	Tygerhoek
S44-11	Not phenotyped	Tygerhoek
S45-11	2SA88/2SA102	Tygerhoek
S46-11	2SA88	Tygerhoek
S47-11	2SA88	Tygerhoek
S48-11	Not phenotyped	Tygerhoek
S49-11	Not phenotyped	Tygerhoek
S51-11	2SA105/2SA88	Tygerhoek
S52-11	2SA88	Tygerhoek
S53-11	2SA88	Tygerhoek
S54-11	2SA88	Tygerhoek
S55-11	Not phenotyped	Tygerhoek
S56-11	2SA88	Marblehall
S57-11	Not phenotyped	Marblehall
S58-11	2SA88	Vaalharts
S59-11	Not phenotyped	Vaalharts
S61-11	Not phenotyped	Cedara
S62-11	Not phenotyped	Cedara
S63-11	2SA88	Loskop
S64-11	2SA88	Loskop
S65-11	2SA88	Loskop

Table 3: *Pgt* isolates collected in the Western Cape during the 2012 annual survey conducted by Dr. T. Terefe.

Sample	Race 2SA-	Remark
S11-12	2SA88	
S12-12	2SA88	
S13-12	2SA88	
S14-12	2SA105	
S16-12	2SA105	
S17-12	Not pathotyped	
S19-12	2SA105	
S20-12	Not phenotyped	
S21-12	2SA88	
S22-12	2SA105	
S23-12	Not phenotyped	
S24-12	2SA105	
S25-12	2SA88	
S27-12	Not phenotyped	
S28-12	2SA88	
S31-12	2SA105	
S32-12	2SA105	
S33-12	2SA105	
S34-12	2SA88	
S35-12	2SA88	
S36-12	2SA88	
S37-12	2SA105	
S38-12	2SA88	
S39-12	2SA105	
S40-12	2SA88	
S41-12	2SA105	
S42-12	2SA88	
S43-12	Not phenotyped	
S44-12	2SA88	
S45-12	2SA88	
S46-12	2SA88	
S47-12	2SA105/2SA104	2 single pustules yielded different races
S48-12	2SA88	
S49-12	2SA88	
S50-12	2SA88	
S51-12	2SA88	

S52-12	2SA88	
S53-12	2SA88	
S54-12	2SA88	
S55-12	2SA88	
S56-12	2SA88	
S57-12	2SA88	
S58-12	2SA88	
S59-12	2SA88	
S60-12	2SA88	
S61-12	2SA88	
S62-12	2SA88/2SA105	2 single pustules yielded different races
S63-12	2SA88	
S64-12	2SA88	
S65-12	2SA88	
S66-12	Not phenotyped	
S67-12	2SA105	
S68-12	2SA88	
S69-12	2SA88	
S70-12	Not phenotyped	
S71-12	2SA104	
S72-12	2SA102	
S73-12	2SA88	
S74-12	2SA104	
S75-12	2SA88	
S76-12	2SA104/2SA105	2 single pustules yielded different races
S77-12	2SA105	
S78-12	2SA88	
S79-12	2SA88	
S80-12	2SA105	
S81-12	Not phenotyped	
S82-12	2SA88	
S83-12	2SA88	
S84-12	2SA88/2SA104	2 single pustules yielded different races
S85-12	Not phenotyped	
S86-12	2SA88	
S87-12	Not phenotyped	
S88-12	2SA88	
S89-12	2SA88	
S90-12	2SA88	
S91-12	2SA105	

S92-12	2SA105	
S93-12	2SA105	
S94-12	2SA88/2SA104	2 single pustules yielded different races
S95-12	2SA88	
S96-12	2SA88	
S97-12	2SA88	
S98-12	2SA88	
S99-12	Not phenotyped	
S100-12	2SA88	
S101-12	2SA88	
S102-12	Not phenotyped	
S103-12	2SA88	
S104-12	Not phenotyped	
S105-12	2SA88	
S106-12	Not phenotyped	
S107-12	2SA88	
S108-12	2SA88	
S109-12	2SA88	
S110-12	2SA88	
S111-12	Not phenotyped	
S112-12	2SA102/2SA105	2 single pustules yielded different races
S113-12	2SA104	
S114-12	Not phenotyped	
S115-12	2SA88	
S116-12	2SA88	
S117-12	Not phenotyped	
S118-12	2SA88	
S119-12	2SA88	
S120-12	2SA88	
S121-12	2SA105	
S122-12	2SA88	

Table 4: *Pt* isolates collected in South Africa during the 2013 annual survey conducted by Dr. T. Terefe.

Sample	Leaf rust race	Locality
L1-13	3SA145	Tygerhoek
L2-13	3SA145	Tygerhoek
L3-13	3SA145	Tygerhoek
L4-13	Not phenotyped	Tygerhoek
L5-13	3SA145	Tygerhoek
L6-13	3SA145	Tygerhoek
L7-13	3SA145	Tygerhoek
L8-13	Not phenotyped	Tygerhoek
L9-13	3SA145	Tygerhoek
L10-13	3SA145	Tygerhoek
L11-13	3SA145	Tygerhoek
L12-13	3SA133	Tygerhoek
L13-13	3SA145	Tygerhoek
L14-13	3SA145	Bredasdorp
L15-13	Not phenotyped	Bredasdorp
L16-13	3SA145	Bredasdorp
L17-13	3SA145	Blydskap
L18-13	Not phenotyped	De vlei
L19-13	3SA145	De vlei
L20-13	3SA145	De vlei
L21-13	3SA145	De vlei
L22-13	Not phenotyped	De vlei
L23-13	3SA145	De vlei
L24-13	3SA145	De vlei
L25-13	Not phenotyped	De vlei
L26-13	3SA145	Voorstekop
L27-13	3SA145	Melkboem
L28-13	3SA145	Melkboem
L29-13	3SA115	Riversdale
L30-13	3SA145	Riversdale
L31-13	3SA115	Riversdale
L32-13	3SA145	Panorama
L33-13	3SA145	Langgewens
L34-13	3SA145	Langgewens
L35-13	3SA145	Langgewens
L36-13	3SA145	Langgewens

L37-13	3SA145	Langgewens
L38-13	3SA145	Langgewens
L39-13	3SA145	Langgewens
L40-13	3SA145	Langgewens
L41-13	3SA145	Langgewens
L42-13	Not phenotyped	Langgewens
L43-13	3SA145	Langgewens
L44-13	3SA145	Langgewens
L45-13	3SA147	Langgewens
L46-13	3SA145	Philadelphia
L47-13	3SA115	Langkloof

Table 5: Allelic data used for generating dendrogram of *Pgt* reference races. 1=allele was present, 0=allele was absent. 2SA= *Pgt* reference race, PgSUN=SSR marker with allele

	2SA4	2SA100	2SA102	2SA104	2SA105	2SA103
PgSUN7.2	**1**	**1**	**1**	**1**	**1**	**1**
PgSUN20.1	**1**	**1**	**1**	**1**	**1**	**1**
PgSUN20.4	**0**	**0**	**1**	**1**	**0**	**1**
PgSUN20.5	**1**	**1**	**0**	**0**	**1**	**0**
PgSUN27.1	**0**	**0**	**0**	**1**	**0**	**1**
PgSUN27.2	**1**	**1**	**1**	**0**	**1**	**0**
PgSUN30.2	**1**	**1**	**1**	**1**	**1**	**1**
PgSUN33.1	**0**	**0**	**0**	**1**	**0**	**1**
PgSUN33.2	**1**	**1**	**1**	**0**	**1**	**0**
PgSUN42.1	**1**	**1**	**1**	**1**	**1**	**1**
PgSUN45.3	**0**	**1**	**1**	**1**	**1**	**1**
PgSUN45.4	**1**	**0**	**0**	**0**	**0**	**0**

Table 6: Allelic data used for generating dendrogram of *Pt* reference races. 1=allele was present, 0=allele was absent. 3SA= *Pt* reference race, Pt=SSR marker with allele

	3SA122	3SA125	3SA129	3SA132	3SA133	3SA134	3SA137	3SA140	3SA144	3SA145	3SA146	3SA147
Pt3.1	0	1	0	0	1	0	0	0	0	1	1	1
Pt3.2	1	1	1	1	1	1	1	1	1	0	1	1
Pt4.2	1	1	1	1	1	1	1	1	1	1	1	1
Pt5.1	0	0	1	1	0	1	1	1	1	0	0	0
Pt5.2	0	0	1	0	0	0	0	0	0	0	1	1
Pt5.3	1	1	0	1	1	1	1	1	1	1	0	0
Pt7.1	0	0	0	0	1	0	0	0	0	0	1	0
Pt7.2	1	1	1	1	1	1	1	1	1	1	1	1
Pt8.1	1	1	1	1	1	1	1	1	1	1	1	1
Pt9.1	1	1	0	0	1	0	0	0	0	0	0	1
Pt9.3	1	0	0	0	1	0	0	0	0	1	1	1
Pt9.4	0	1	1	1	0	1	1	1	1	0	0	0
Pt11.1	1	0	0	1	0	1	1	1	1	1	1	1
Pt11.2	1	1	1	1	1	1	1	1	1	1	1	1
Pt13.2	1	1	1	0	0	0	0	0	0	1	0	1
Pt13.3	0	0	1	1	1	1	1	1	1	1	1	1
Pt14.1	1	1	1	1	1	1	1	1	1	1	1	1
Pt15.1	1	1	0	0	0	0	0	0	0	1	0	1
Pt15.2	0	0	0	0	1	0	0	0	0	0	0	0
Pt15.3	1	1	1	1	1	1	1	1	1	1	1	1
Pt16.1	1	1	0	0	0	0	0	0	0	1	1	1
Pt16.3	0	0	0	1	1	1	1	1	1	0	0	0
Pt16.4	1	1	1	1	1	1	1	1	1	0	1	1

Table 6: Continued

	3SA122	3SA125	3SA129	3SA132	3SA133	3SA134	3SA137	3SA140	3SA144	3SA145	3SA146	3SA147
Pt17.1	1	1	0	0	0	0	0	0	0	1	1	1
Pt17.2	1	0	0	0	0	0	0	0	0	0	0	1
Pt17.3	0	0	1	0	1	0	0	0	0	0	1	0
Pt17.4	0	1	1	1	1	1	1	1	1	0	0	0
Pt18.1	0	0	0	0	1	0	0	0	0	0	0	0
Pt18.2	1	1	0	0	0	0	0	0	0	1	0	1
Pt18.3	1	0	0	0	0	0	0	0	0	0	0	1
Pt18.4	0	1	1	1	1	1	1	1	1	1	1	0
Pt19.1	0	0	0	1	1	1	1	1	1	1	1	0
Pt19.2	1	1	1	1	1	1	1	1	1	1	1	1
Pt21.1	0	0	0	0	1	0	0	0	0	0	0	0
Pt21.2	1	1	1	1	0	1	1	1	1	1	1	1
Pt21.3	0	1	1	1	1	1	1	1	1	0	0	0
Pt21.4	1	0	0	0	0	0	0	0	0	0	0	1
Pt21.5	0	0	0	0	0	0	0	0	0	1	1	0
Pt23.1	1	1	0	0	1	0	0	0	0	1	0	1
Pt23.2	1	1	1	1	1	1	1	1	1	1	1	1
Pt25.1	1	1	0	1	1	1	1	1	1	1	1	1
Pt25.2	1	1	1	0	1	0	0	0	0	1	0	1
Pt25.3	0	0	0	1	0	1	1	1	1	0	0	0
Pt26.1	0	0	0	0	0	0	0	0	0	1	1	1
Pt26.2	1	1	1	1	1	1	1	1	1	0	0	0
Pt26.4	1	1	0	1	1	1	1	1	1	1	1	1
Pt27.2	0	1	0	1	0	1	1	1	1	0	0	0
Pt27.3	1	0	1	0	1	0	0	0	0	1	1	1
Pt28.1	0	1	1	0	1	0	0	0	0	1	1	0

Pt28.2	0	0	1	1	1	1	1	1	1	1	1	0
Pt28.3	1	1	0	0	0	0	0	0	0	0	0	1