Molecular genetic study of wheat rusts affecting cereal production in the Western Cape

by
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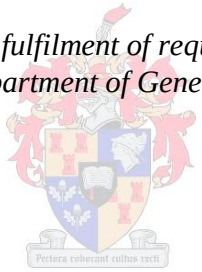

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Declaration

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Abstract

Microsatellites were used to differentiate Leaf (*Puccinia triticina* Eriks.) and Yellow rust (*Puccinia striiformis* Westend. f. sp. *tritici* Eriks.) pathotypes. There was sufficient diversity in the Leaf rust microsatellite markers to differentiate the pathotypes and create a phylogenetic tree of Leaf rust. Three of the microsatellite markers were sufficient to differentiate all the Leaf rust pathotypes. Sufficient diversity in the Yellow rust microsatellite markers was also observed which made it possible to differentiate the pathotypes. Only three pathotypes were used so no phylogenetic inference was made. Two microsatellite markers were sufficient to differentiate all the yellow rust pathotypes.

Microsatellite and Amplified Fragment Length Polymorphisms (AFLP) markers were used to differentiate Stem rust (*Puccinia graminis* f. sp. *tritici* Eriks. and Henn.) pathotypes, and the data was combined for phylogenetic analysis. AFLP bands unique to each Stem rust pathotype were converted to Sequence Characterised Amplified Region (SCAR) markers. A single specific SCAR marker was created for UVPgt52. A second SCAR marker amplified four of the eight pathotypes. None of the other SCAR markers were specific.

A 270 basepair fragment of the ITS1 region of the rDNA gene of all the *Puccinia spp*. was also sequenced in order to develop pathotype specific primers that could be used in a Real Time-PCR to determine relative levels of pathogen inoculum in a sample. Unfortunately insufficient diversity in the sequences of the ITS1 region of the rDNA gene did not allow unique primers to be designed for each pathotype making it impossible to proceed with the relative quantification using Real Time-PCR.

Following marker development ninety one field isolates were collected from eleven sites in the Overberg and Swartland regions during 2008 and 2009. In the field isolates, four different Leaf rust pathotypes were identifiable. UVPgt13 and UVPgt10 were most prevalent. The most prevalent Stem rust pathotypes were UVPgt50, UVPgt52, UVPgt54 and UVPgt57. Only 6E16A- was identifiable in the Yellow rust isolates.

There were no apparent patterns in the distribution of Leaf, Stem or Yellow rust. Leaf and Stem rust were widely distributed, while Yellow rust was confined to three sites in the central South Cape, the only sites where climatic conditions were favourable for its development during the sampling period. The low levels of diversity found in the rust population when compared to international populations are probably due to the relatively small population size, the lack of a host for sexual reproduction, the small sample size, the effective monoculture and the strong selective pressure created by artificial control methods.

Opsomming

Mikrosatellietmerkers is gebruik om Blaar- (*Puccinia triticina* Eriks.) en Geelroes-(*Puccinia striiformis* Westend. f. sp. *tritici* Eriks.) patotipes te onderskei. Daar was genoeg diversiteit in die Blaarroesmerkers om verskillende patotipes te kon onderskei en om ‘n filogenetiese-boom te kon saamstel. Met drie van die mikrosatellietmerkers was dit moontlik om al die Blaarroespatotipes te kon onderskei. Daar was genoeg diversiteit in die Geelroesmerkers om al die patotipes te kon skei en met twee van die mikrosatellietmerkers kon al drie Geelroespatotipes van mekaar onderskei word.

Mikrosatelliet- en ge-Amplifiseerde-Fragment-Lengte-Polimorfismes (AFLP) is gebruik om die Stamroes- (*Puccinia graminis* f. sp. *tritici* Eriks. and Henn.) patotipes te skei. AFLP-fragmente uniek aan ‘n spesifieke patotipe is omgeskakel na Volgorde-Spesifieke-ge-Amplifiseerde-Streek (SCAR) merkers. ‘n Spesifieke SCAR-merker is gemaak vir UVPgt52. ‘n Tweede SCAR-merker het vier van die patotipes geidentifiseer. Nie een van die ander SCAR-merkers was spesifiek t.o.v. ‘n spesifieke patotipe nie.

Die volgorde van ‘n 270 basispaar fragment van die ITS1-streek van die rDNS-geen van al die *Puccinia spp.* is bepaal om patotipe spesifieke inleiers te kon ontwerp. Hierdie inleiers kan gebruik word om ‘n Intydse-Polimerase-Ketting-Reaksie (RT-PCR) te ontwerp om sodoende die relatiewe vlakke van die patogeen besmetting in ‘n monster te bepaal. Daar was nie genoeg diversiteit in die bepaalde volgordes om die spes1fieke inleiers te kon identifiseer nie en dus is RT-PCR laat vaar.

Na die ontwikkeling van die merkers was een-en-negentig veldmonsters ingesamel afkomstig van elf lokaliteite in die Overberg en Swartland gedurende 2008 en 2009. Vier Blaarroespatotipes was uitkenbaar. Blaarroespatotipes UVPrt10 en UVPrt13 was die mees algemeenste. UVPgt50, UVPgt52, UVPgt54 en UVPgt57 was die mees algemene Stamroespatotipes. Net 6E16A- is geidentifiseer by die Geelroes-isolate.

Daar was geen patroon in die verspreiding van Blaar-, Stam- of Geelroes patotipes. Blaar- en Stamroes was die wydste versprei, maar Geelroes het net by drie lokale in die sentrale Suid-Kaap voorgekom. Die lokaliteite is die enigste waar die weersomstandighede gunstig was vir Geelroes ontwikkeling gedurende die periode van monsterneming. Die lae vlakke van diversiteit wat in die roespopulasie gevind was is in teenstelling met internasionale populasies. Dit mag moontlik wees as gevolg van die relatief beperkte populasie grootte, die afwesigheid van ‘n gasheer vir seksuele voortplanting, die beperkte hoeveelheid monsters wat ingesamel is en die sterk selektiewe druk weens kunsmatige beheer.

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List of Abbreviations

°C	Degrees Centigrade
A_{600}	Absorbance at a wavelength of 600 nanometres
AFLP	Amplified Fragment Length Polymorphism
$AgNO_3$	Silver Nitrate
bp	Basepair
$CaCl_2$	Calcium Chloride
cm	Centimetre
CTAB	N-Cetyl-N, N, N-trimethyl Ammonium Bromide
dH_2O	distilled, autoclaved water
DNA	Deoxyribonucleic Acid
EDTA or NA2EDTA	Ethylenediamine tetra acetic acid disodium salt dihydrate
f. sp.	Forme special
FSU-12	Countries of the Former Soviet Union
g	gravitation force exerted by the Earth
g	Gram
g/l	grams/litter
H_2O	Water
ha	Hectare
Hz	Hertz
IGC	International Grains Council
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS1	Internal Transcribed Spacer 1
LB	Luria Bertani
LB (amp)	Luria Bertani agar or medium containing ampicillian
m	meter
M	Molar
mg/ml	milligrams/millilitre
$MgCl_2$	Magnesium Chloride
min	minute(s)
ml	Millilitre
mm	Millimetre
mM	milliMolar
mmhos/cm	millimhos/centimetre
NaCl	Sodium Chloride
ng/µl	nanograms/microliter
nM	nanoMolar
nm	Nanometre
OD_{600}	Optical Density at a wavelength of 600 nanometres
PCR	Polymerase Chain Reaction
RAPD	Random Amplification of Polymorphic Differences
rDNA	ribosomal Deoxyribonucleic Acid

RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rpm	revolutions per minute
RT-PCR	Real-Time Polymerase Chain Reaction
SA	South Africa
SAM	Selectively Amplified Microsatellite
SCAR	Sequence Characterised Amplified Region
sec	second(s)
spp.	*species pluralis*
SSCP	Single Strand Conformation Polymorphism
STMP	Sequence-Tagged Microsatellite profiling
TBE	Tris-borate EDTA buffer
TE	Tris EDTA buffer
TEMED	N, N, N', N'-Tetramethylethylenediamine
TRIS-HCl	Tris-Hydrocholoric Acid
U/µl	Units/microliter
UPGMA	Unweighted Pair Group Method with Arithmetic mean
USA	United States of America
UVPgt	Universiteit Vrystaat *Puccinia graminis* f. sp. *tritici*
UVPrt	Universiteit Vrystaat *Puccinia recondita* f. sp. *tritici*
V	Volt
W	Watt
X-gal	X-galactose or bromo-chloro-indolyl-galactopyranoside
µg/ml	microgram/millilitre
µg	microgram
µl	microliter
µm	micrometer
µM	microMolar

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1. Introduction

1.1 Wheat Production

Agricultural production of grains feeds the global population. Grain is not only a staple food but also a vital feedstock for livestock. According to the International Grains Council (IGC) Grain Market Report (International Grains Council, 2006), in 2005/6 the global production of grain was around 2016 million tonnes, with wheat accounting for ~31% (621 million tonnes) of the total. South Africa is a minor player in the global wheat market, producing less than 0.4% of the global total.

Table 1: Global wheat production by country/region [Major regional producer] (International Grains Council, 2006)

Country/Region	Production (million tonnes)
European Union [France, Germany, United Kingdom]	114.39
United States	57.28
Canada	25.75
Australia	25.17
Argentina	14.5
China	97.45
FSU-12 [Russia, Ukraine, Kazakhstan]	83.2
India	68.64
Pakistan	21.61
Turkey	18.5
South Africa	1.89

Wheat surpluses, produced primarily by the Western countries (see **Table 1**), are essential to global food security. Without these surpluses, the populations of nett importers of wheat such as the countries of the Former Soviet Union (FSU-12) and China would be unable to feed their massive populations. Furthermore, these surpluses are used as food aid for famine-stricken countries. In short, wheat surpluses prevent famine on a global scale. However wheat production has been declining over the last ten years and in 2005/2006 global reserves stood at fifty days, which is their lowest level for several decades (International Grains Council, 2006).

There are many factors that influence the production of wheat. These include:

1.1.1 Climate

Wheat is in general a very adaptable plant that will grow under a wide range of conditions. It has been successfully cultivated from the equator to north of the 60th parallel. Its altitude range is also large, growing from sea level to 3300m. The optimum temperature range is in the region of 15-20°C. Spring wheat is more susceptible to frost damage than winter wheat; therefore areas free of frost are preferred for spring wheat cultivation. An annual rainfall of 450-650mm (dependant on the length of the growth period) is necessary; irrigation maybe required if the annual rainfall is too low (Shellenberger, 1971).

1.1.2 Soil

Wheat prefers soils of a medium texture and peaty soils should be avoided. The pH should be between 6 and 8. Wheat will tolerate some soil salinity but it should have an Electrical Conductivity measurement of less than four mmhos/cm during germination (Evans and Peacock, 1981).

1.1.3 Fuel and fertilizer

Wheat production is a highly mechanized process that requires large amounts of fuel and fertilizer. The price of fertilizer is also directly linked to the oil price. If fertilizers are not used then the actual yield per hectare can be drastically reduced. Use of fertilizers also increases the protein content of the wheat (Evans and Peacock, 1981).

The volatility of the international oil price is a problem, because it is almost impossible to predict trends and formulate long term financial plans.

1.1.4 Financial

To produce wheat profitably, large areas of land and expensive machinery are required. Additionally, imports of cheap wheat, due to the subsidization of production, from foreign countries can drive the prices down to such low levels that it is no longer economically viable to produce wheat. Other factors such as drought can drive the production price per ton up, exacerbating the situation. For example, in 2005, South African farmers could expect to make a profit of around R800/ha while in the drought-stricken Swartland production costs were around R2380/ha (South African Department of Agriculture, 2005).

1.1.5 Pathogens

Wheat is vulnerable to a wide range of pathogens. It is the attacks by these pathogens that create a significant percentage of the difference between the absolute yield (based on the genetic potential) and the actual yield (Cook and Veseth, 1991). A variety of wheat pathogens exist ranging from the Russian wheat aphid to fungi such as the *Fusariam spp.* (for a complete list see Wiese, 1987). The most problematic of these are the obligate parasites such as the wheat rusts, since these have the greatest evolutionary potential and complex disease management.

1.2 Wheat Rusts

Puccinia triticina Eriks, *Puccinia graminis* f. sp. *tritici* Eriks. and Henn. and *Puccinia striiformis* Westend. f. sp. *tritici* Eriks. are the causative agents of Leaf, Stem and Yellow Rust on wheat, respectively (see **Figure 1**). The lifecycles, morphology and optimal growth conditions differ between the *Puccinia spp.* (Wiese, 1987). Wheat rusts are one of the primary biotic restrictors of wheat production globally (Keiper, *et al.*, 2006). They are obligate pathogens of living tissue and thus require a host as a "green bridge" in order to survive until the next growing season (Staples, 2003). In South Africa the green bridge is provided by Lesotho, where due to the altitude it is possible to grow wheat in summer (Pretorius, *et al.*, 2007). The rusts may also survive as dormant mycelia on self-sown wheat during the offseason (Killian and Burger, 2008).

Leaf and Yellow rust mostly occur on the leaves of wheat plants while Stem rust occurs on both the stem and the leaves. When sporulation occurs, the epidermis of the plant bursts open to release the spores. The damage to the epidermis reduces the ability of the plant to photosynthesise and increases the rate of transpiration and respiration. This reduces the yield from the plant. The degree to which any plant becomes infected is dependent on a variety of factors including cultivar, pathotype and chemical control methods (Wiese, 1987; Russel, 1978).

In South Africa, the first documented rust epidemic occurred in 1726 (du Plessis, 1933) and since then, wheat breeding in South Africa has simply been trying to stay one step ahead of the rusts (Pretorius, *et al.*, 2007). Intermittent surveys (regular surveys only started in the 1980's) and lack of any standard system of nomenclature has prevented the elucidation of the

evolution of the *Puccinia spp.* in South Africa. Since South Africa created a unified standard system whereby new races are identified by a alpha-numerical code consisting of three digits with the first digit being the rust type (2SA is Stem Rust and 3SA is Leaf Rust) and the following two digits being a sequential record number for the pathotype, the code is also accompanied by a virulence/avirulence formula, the situation has improved (Le Roux and Rijkenberg, 1987a). Before this system was in place, most followed the system of Verwoerd (Verwoerd, 1931; Verwoerd, 1937) where Leaf rust was designated as Universiteit Vrystaat *Puccinia recondita* f. sp. *tritici* (UVPrt) with a number to identify the pathotype and Stem rust as "Universiteit Vrystaat" *Puccinia graminis* f. sp. *tritici* (UVPgt) with a number to identify the pathotype. Yellow rust follows an international system whereby races are identified by an alphanumeric code e.g. 6E16A-, which contains all the virulence/avirulence data (Johnson, *et al.*, 1972).

Figure 1: (A) Leaf Rust (B), Stem Rust (C) and Yellow Rust (photos courtesy of Dr I. Paul, ARC)

1.2.1 Life cycle of the *Puccinia spp*.

The *Puccinia spp.* have an intricate lifecycle that consists of both sexual and asexual reproduction (see **Figure 2**). Asexual reproduction occurs primarily on wheat, while sexual reproduction takes place on an alternative host. For Leaf rust this alternative host is species of the genus *Thalictrum*, for Stem rust it is *Barberis vulgaris* and the alternative host for Yellow rust has yet to be identified. None of these alternative hosts occur in South Africa, and the wheat rusts have not been noted on indigenous species. Therefore it is believed that the rusts cannot undergo sexual reproduction in South Africa (Wiese, 1987; Knott, 1989).

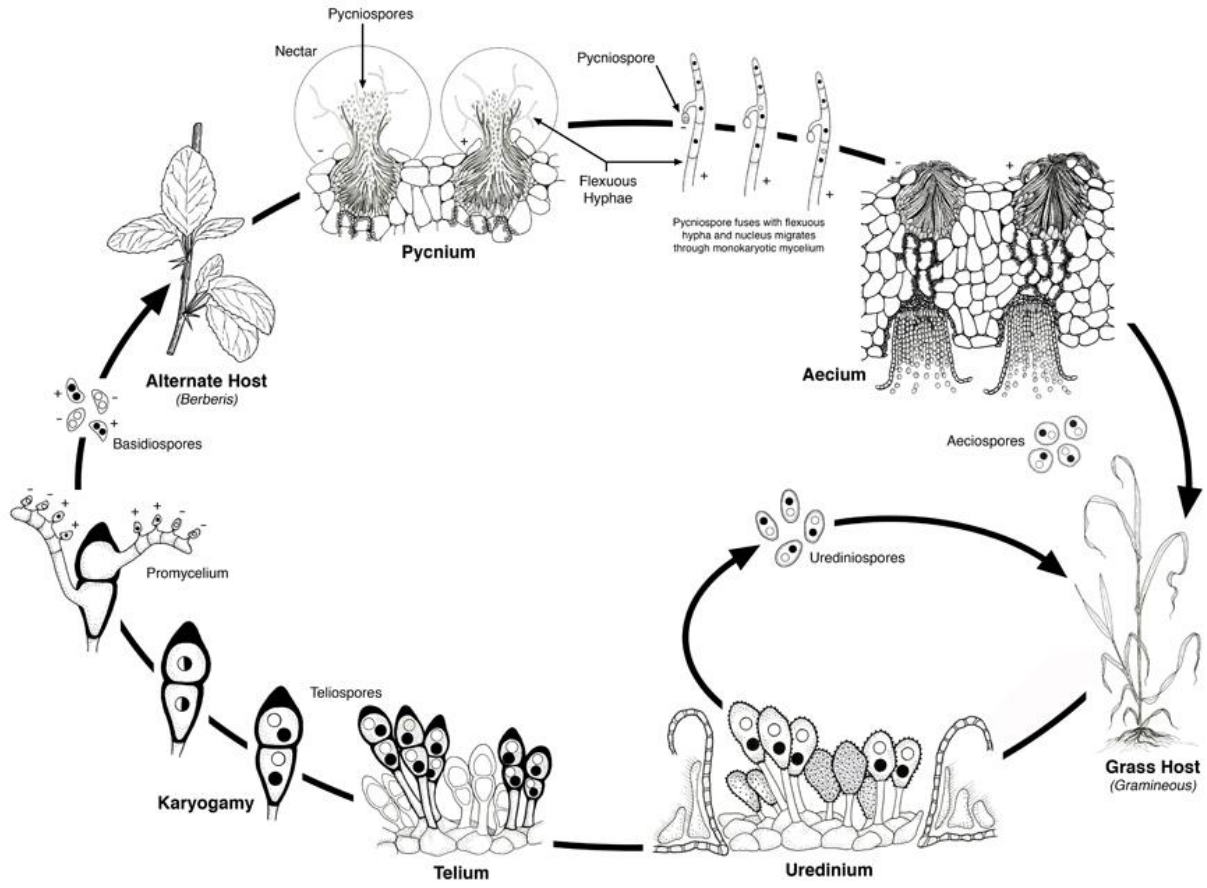

Figure 2: Wheat rust life cycle (US Department of Agriculture)

Urediniospores, formed by the uredinium on infected plants, are tiny single celled, dikaryotic spores that are released by the million. They are dispersed by wind and water action and can spread the infection over vast distances (Keiper, *et al.*, 2006). Germination of the urediniospore on a susceptible host plant requires water, and so is usually started by rain or heavy dew. Germ-tubes grow from the infection site until they reach the stomata, where an apressorium is formed. The formation of a structure bordering the membrane inside the

stomata is indicative of a successful infection. This structure then forms haustorium mother cells as well as mycelia. The speed at which the infection proceeds is dependent on the rust pathotype, cultivar as well as the environmental conditions such as temperature and can vary between five and eight days. The urediniospores can reinfect the wheat plant and asexual reproduction can continue indefinitely (Wiese, 1987; Knott, 1989).

Sexual reproduction, which allows the pathogen to survive periods of environmental stress and introduces variation, can occur after urediniospore formation is completed. Dikaryotic teliospores form. These teliospores are more robust than urediniospores. The teliospores germinate, fusing the dikaryon into a single nucleus which then undergoes meiosis producing a pro-mycelium that consists of four haploid basidiospores. These basidiospores can only infect the alternative host and not wheat. Once the host has been infected by the basidiospores, positive or negative pycnia are formed. Positive pycnia fuse with negative pycnia to form a dikaryon again. The dikaryon develops to form an aecium which produces aeciospores that can infect wheat (Wiese, 1987; Knott, 1989).

1.2.2 Leaf rust

Leaf rust flourishes in regions such as the Western Cape, Northern America and Eastern Europe, where wheat becomes ripe late in the season (Wiese, 1987; Murray, *et al.*, 1998). In the Western Cape, around 300 000 hectares of spring wheat is at risk of Leaf rust infection. Leaf rust grows optimally with temperatures of between 15°C and 22°C and high humidity. New spores are produced every seven to ten days allowing the rapid spread of the infection via wind and water. Leaf rust infections can result in yield losses of as much as 63% (Murray, *et al.*, 1998; Boshoff, *et al.*, 2002c).

One of the typical signs of Leaf rust infection is the formation of orange uredia on the dorsal surface of the leaf. The uredia are up to 1.5mm in diameter and produce vast numbers of spherical spores of around 20µm diameter. Telia develop as the growing season ends and produce black teliospores (Wiese, 1987; Knott, 1989; Murray, *et al.*, 1998). Leaf rust occurs on wheat and triticale.

The study of Leaf rust in South Africa has been sporadic, the first survey was conducted in 1937 (Verwoerd, 1937) and described five races based on their physiological characteristics. Subsequent surveys started in 1983 and continued until 1988 (Pretorius, *et al.*, 1987; Pretorius and Le Roux, 1988; Pretorius, *et al.*, 1990). At this time, most of the commonly used cultivars were susceptible to Leaf Rust and epidemics were common. Fifteen different pathotypes have been detected in field isolates (Pretorius, *et al.*, 2007) and one, with

virulence to *Lr41*, was detected in a greenhouse (Pretorius, 1997). Variation between the pathotypes occurs mostly at the *Lr10, -14a, -17, -24* and *-26* loci (Pretorius, *et al.*, 2007). Most of the variance is thought to come from either introduction of species or mutation as it appears that the rate of sexual reproduction is low to nonexistent (Bengtsson, 2003; Goyeau, *et al.*, 2007). Regular spraying of fungicides and the introduction of cultivars with the *Lr34/Yr18* genes has lead to a decline in Leaf Rust prevalence. No new Leaf Rust pathotypes have been reported in South Africa since 1988 (Pretorius, *et al.*, 2007).

1.2.3 Stem rust

Stem rust has been problematic in South Africa with studies showing average yield losses of 35% for a range of different cultivars (Pretorius, *et al.*, 2007). This can increase to a total loss dependant on when in the growth cycle the infection starts. Stem rust grows between 15°C and 40°C with an optimal growth occurring at 26°C (Murray, *et al.*, 1998).

The brown uredia occur on both the leaves and stem and are quite large, about 3mm by 10mm in size. The urediniospores are oval and red-orange in colour; they are 15-20 µm by 40-60 µm in size (Wiese, 1987; Knott, 1989). As the uredia age, they cause the formation of grey-black teliospores (Murray, *et al.*, 1998). Stem rust is not restricted to wheat, it also occurs on rye and triticale (Pretorius, *et al.*, 2007).

Internationally, Stem rust had largely been removed as a threat by the slow rusting approach used since the last outbreak of Race 15-B in the 1950's (Rodenheiser and Moore, 1951) and the start of the Green Revolution (Saari and Prescott, 1985). Thus research until recently had focused on breeding resistance to leaf and stripe rusts.

In South Africa the first pathotype, #34, was identified in 1922, followed by #21 in 1929 (de Jager, 1980). In 1960, interest in Stem rust was renewed, leading to improvements in the differential set and more regular surveys. The improved differential sets showed that well established Stem rust epidemics were in fact caused by separate pathotypes. In 1980 the Agricultural Research Council (ARC) initiated annual rust surveys. This coincided with the mandatory inclusion of Stem rust resistance in all new cultivar releases (Pretorius, *et al.*, 2007). The new system of nomenclature was only implemented in 1987 (Le Roux and Rijkenberg, 1987a), the lack of a unified system of nomenclature before 1987 has prevented the reconstruction of a clear history of Stem Rust. Stem rust has acquired virulence to many resistance genes, including virulence to resistance genes prevalent in triticale (Smith and Le Roux, 1992). The widespread use of a single cultivar has lead to the rapid increase of those pathotypes that are virulent to it, for example: SST44 was widely used in the 1980's and the

prevalence of 2SA100, which is virulent to it, increased dramatically. Since then however new cultivars have been introduced and Race #34 has acquired new virulences and become UVPgt55 (2SA88) (Boshoff, *et al.*, 2002d) which is now the dominant pathotype in South Africa (Pretorius, *et al.*, 2007).

In 1999, a new Stem rust pathotype, Ug99, arose in Uganda and spread through Kenya eventually reaching Ethiopia in 2004. Most of the cultivars grown in Kenya and Ethiopia are susceptible to Ug99 and the potential for devastation is great (Expert Panel on the Stem Rust outbreak in East Africa, 2005; Singh, *et al.*, 2006). Global production is also at risk if Ug99 spreads rapidly by international air travel. The Global Rust Initiative (www.globalrust.org) has been launched in response and includes an emergency crossing programme to concentrate effective resistance genes accompanied by a massive testing of advanced lines in the affected areas (Jin and Singh, 2006). The South African pathotype, UVPgt55, was compared to Ug99 using molecular markers and it was found to resemble Ug99 closely (Visser, *et al.*, 2009). UVPgt55 avirulence/virulence composition is also identical to Ug99 except that it lacks virulence for *Sr24* and *Sr31*. Initially Ug99 was not a great threat in South Africa as cultivars with *Sr31* are not common, as they have not found favour with the baking and milling industries (Pretorius, *et al.*, 2007). Ug99 has subsequently acquired virulence for *Sr24* and *Sr36*, making it much more of a threat (Jin and Szabo, 2009). An Indian rust pathotype, 58G13-3 or PKTSC, has recently become virulent to *Sr25* (Jain, *et al.*, 2009), which was one of the few remaining unbroken resistance genes. If Ug99 acquires *Sr25* virulence as well, it will combine virulence to most of the major rust resistance genes in use globally.

1.2.4 Yellow rust

While Leaf- and Stem rust are able to tolerate a wide temperature range, Yellow rust cannot. Its optimum temperature for infection is between 9°C and 11°C and optimum development occurs at temperatures below 23°C. Therefore it only occurs in cooler regions where the temperature range is favourable and the humidity is high (Murray, *et al.*, 1998). A new Australian pathotype, Jackie, has emerged; it requires temperatures of less than 18°C for infection but, once it has successfully infected a wheat plant it is able to survive brief periods with temperatures as high as 40°C (Hollaway, 2009).

Characteristic yellow stripes of uredia occur between the veins of the leaves in infected plants. Yellow rust is a chronic infection, infecting all plant organs. Urediniospores are yellow and about 20µm-30µm in diameter (Wiese, 1987; Knott, 1989). Telia form in necrotic tissue and produce black teliospores in the late season, causing black stripes on the

leaves (Knott, 1989; Murray, *et al.*, 1998). No alternative host for sexual reproduction has been identified in South Africa (Pretorius, *et al.*, 2007), and therefore Yellow rust reproduces asexually by repetitive cycles of uredia formation (Wiese, 1987; Knott, 1989).

Yellow rust was first recorded in South Africa in 1996 (Pretorius, *et al.*, 1997). There is an unsubstantiated report of Yellow rust occurring in the former Transvaal in 1935, but this is not in any official South African plant disease record (Pretorius, *et al.*, 2007). The first Yellow rust pathotype found in South Africa was 6E16A-, a common Middle Eastern pathotype. With the addition of *Yr25* virulence 6E16A- has become 6E22A- . Yellow rust commonly occurs in the Western Cape, KwaZulu-Natal and eastern Free State which are cooler and wetter. A third pathotype, 7E22A-, which has virulence to *Yr1,* has been found in Lesotho. This new pathotype should not threaten South African wheat as no local cultivars have *Yr1*.

Yellow rust has been costly to the industry, as farmers now have to cope with an additional threat and many commercial cultivars are not yellow rust resistant. Wheat breeding programmes have also been harmed, loosing up to 60% of early generation breeding material (Boshoff, *et al.*, 2002a). The many of the Yellow rust resistant cultivars available in South Africa are not resistant to Stem rust and this has lead to an increase in Stem rust (Pretorius, *et al.*, 2007).

1.3 Identification of Pathotypes

Any population genetic study requires a method of uniquely identifying each particular sample, or in this case, pathotype. Conventional plant pathology techniques for pathotype identification follow a complex protocol. Firstly isolates are created from single spore pustules, increased and then inoculated onto a differential set that consists of various cultivars that have a range of resistance genes. Depending on the reaction of a specific isolate on each of the cultivars in the differential set it is possible to determine what the avirulence/virulence composition of the isolate is and therefore which pathotype it is. See **Table 2A**, **Table2B** and **Table 2C** for the virulence/avirulence composition of the South African rust pathotypes in this study; see **Addendum 1** for the virulence/avirulence composition of South African rust pathotypes excluded from this study. This technique requires a lengthy period of time to identify the pathotype, usually in the region of five weeks in order to ensure the fidelity of the results, and also requires the skills of a highly trained and experienced pathologist. Modern

molecular techniques have the potential to aid the current conventional methodology with fast, accurate tests that can provide same day results. Several different molecular methods exist, each with their own advantages and disadvantages (McCartney, *et al*., 2003)

1.3.1 Southern Blots

Also known as DNA Fingerprinting, the genomic DNA is digested by a restriction enzyme that cuts at a common site. The DNA fragments are separated on a gel, and then hybridized with a single stranded DNA/RNA probe. The probe is labeled with a fluorescent dye or radioactive isotope. The probe is complementary to a DNA sequence that occurs widely in the genome. The DNA-probe complex is then transferred to a membrane and visualized by exposure of the membrane to X-ray plates, in the case of a radioactive label, or by eye, in the case of a fluorescent label. This technique is quite robust and simple but can require much labour and time due to the numerous controls that are needed. It also requires some prior knowledge of the DNA sequence being studied, in order to develop specific probes (Brown, 1996). Southern Blots cannot show co-dominant markers. Southern Bolts have been used to differentiate Yellow rust pathotypes (Zhan, *et al.*, 1998).

1.3.2 Random Amplification of Polymorphic Differences (RAPD)

RAPD uses arbitrary ten base primers to amplify random DNA sequences. The amplified sequences are separated on an agarose or polyacrylamide gel. This produces a characteristic banding pattern that should be unique to each sample. It is a very simple and fast technique, that requires no prior knowledge of the DNA sequence, but reproducibility is often a problem. The amplified fragments can be excised from the gel and cloned into a vector. The vector can be sequenced and specific primers designed for the fragment. The presence and/or absence of the fragment can then be ascertained with a simple PCR amplification using the desired primers. Usually it provides much more reproducible results. The fragment is then known as a Sequence Characterized Amplified Region or SCAR marker (Brown, 1996; Williams, *et al.*, 1990; McCartney, *et al.*, 2003; Razavi and Huges, 2004). RAPD markers are unable to show co-dominance. RAPD markers have been used to assess the link between virulence and molecular diversity (Kolmer, *et al.*, 1994) and population diversity (Park, *et al.*, 2000) in Leaf rust.

Table 2 A: Avirulence/virulence composition of South African Stem rust pathotypes in this study (Pretorius, 1983; Le Roux and Rijkenberg, 1987a; Le Roux and Rijkenberg, 1987b; Le Roux, 1989; Le Roux and Rijkenberg, 1989; Le Roux and Rijkenberg, 1989; Marais and Pretorius, 1996; Boshoff, *et al.*, 2000; Pretorius, *et al.*, 2000; Boshoff, *et al.*, 2002d; Roux, *et al.*, 2006; Pretorius, *et al.*, 2007; Jin and Szabo, 2009; Marais, *et al.*, 2009; Visser, *et al.*, 2009)

Stem Rust	Avirulence genes	Virulence genes
UVPgt50 (2SA4)	*Sr8b, Sr9g, Sr13, Sr15, Sr21, Sr22, Sr24, Sr25, Sr26, Sr27, Sr29, Sr31, Sr32, Sr33, Sr35, Sr36, Sr38, Sr39, Sr43, SrEm, SrKiewiet, SrSatu*	*Sr5, Sr6, Sr7a, Sr7b, Sr8a, Sr9a, Sr9b, Sr9d, Sr9e, Sr9f, Sr10, Sr11, Sr12, Sr14, Sr16, Sr17, Sr18, Sr19, Sr20, Sr23, Sr28, Sr30, Sr34, Sr37, SrGt, SrLc*
UVPgt51 (2SA36)	*Sr8b, Sr9e, Sr9g, Sr13, Sr15, Sr21, Sr22, Sr23, Sr24, Sr25, Sr26, Sr27, Sr29, Sr30, Sr31, Sr32, Sr33, Sr35, Sr37, Sr38, Sr39, Sr43, SrAgi, Srdp2, SrEm, SrGt*	*Sr5, Sr6, Sr7a, Sr7b, Sr8a, Sr9a, Sr9b, Sr9d, Sr9f, Sr10, Sr11, Sr12, Sr14, Sr16, Sr17, Sr19, Sr20, Sr28, Sr34, Sr36, SrLc*
UVPgt52 (2SA100)	*Sr8b, Sr9e, Sr9g, Sr13, Sr15, Sr21, Sr22, Sr25, Sr26, Sr27, Sr29, Sr30, Sr31, Sr32, Sr33, Sr35, Sr36, Sr37, Sr38, Sr39, Sr43, SrAgi, Srdp2, SrEm, SrGt, SrKiewiet, SrSatu*	*Sr5, Sr6, Sr7a, Sr7b, Sr8a, Sr9a, Sr9b, Sr9d, Sr9f, Sr10, Sr11, Sr12, Sr14, Sr16, Sr17, Sr18, Sr19, Sr20, Sr23, Sr24, Sr28, Sr34, SrLc*
UVPgt53 (2SA102)	*Sr5, Sr6, Sr7b, Sr8b, Sr9b, Sr9e, Sr11, Sr13, Sr15, Sr17, Sr21, Sr22, Sr23, Sr24, Sr25, Sr26, Sr29, Sr30, Sr31, Sr32, Sr33, Sr35, Sr36, Sr37, Sr38, Sr39, Sr43, SrEm, SrGt, SrKiewiet, SrSatu*	*Sr7a, Sr7b, Sr8a, Sr9a, Sr9d, Sr9f, Sr9g, Sr10, Sr12, Sr14, Sr16, Sr19, Sr20, Sr27, Sr30, Sr34, SrLc, SrTobie*
UVPgt54 (2SA55)	*Sr5, Sr6, Sr7b, Sr8b, Sr9b, Sr9e, Sr9g, Sr13, Sr15, Sr21, Sr22, Sr23, Sr24, Sr25, Sr26, Sr27, Sr29, Sr30, Sr31, Sr32, Sr33, Sr35, Sr38, Sr39, Sr43, SrEm, SrGt*	*Sr7a, Sr8a, Sr9a, Sr9d, Sr9f, Sr10, Sr11, Sr12, Sr14, Sr16, Sr19, Sr20, Sr34, SrLc*
UVPgt55 (2SA88)	*Sr13, Sr15, Sr21, Sr22, Sr24, Sr25, Sr26, Sr27, Sr29, Sr31, Sr32, Sr33, Sr35, Sr36, Sr39, Sr43, SrAgi, SrEm, SrKiewiet, SrSatu*	*Sr5, Sr6, Sr7a, Sr7b, Sr8a, Sr8b, Sr9a, Sr9b, Sr9d, Sr9e, Sr9f, Sr9g, Sr10, Sr11, Sr12, Sr14, Sr16, Sr17, Sr19, Sr20, Sr23, Sr30, Sr34, Sr38, SrLc*
UVPgt56 (2SA102K)	*Sr5, Sr6, Sr7b, Sr8b, Sr9b, Sr9e, Sr11, Sr17, Sr21, Sr24, Sr30, Sr31, Sr36, Sr38, SrEm, SrSatu, SrTobie*	*Sr8a, Sr9g, Sr27, SrKiewiet*
UVPgt57 (2SA105)	*Sr5, Sr6, Sr7b, Sr8b, Sr9b[b], Sr9e Sr17, Sr21, Sr24, Sr30, Sr31, Sr36, Sr38, SrEm*	*Sr8a, Sr9g, Sr11, Sr27, SrGt, SrKiewiet, SrSatu*
Ug99[a] (TTKS)	*Sr21, Sr22, Sr25, Sr26, Sr27, Sr29, Sr32, Sr33, Sr35, Sr39, Sr40, Sr42, Sr43, SrAgi, SrEm*	*Sr5, Sr6, Sr7b, Sr8a, Sr8b, Sr9b, Sr9e, Sr9g, Sr11, Sr15, Sr17, Sr24, Sr30, Sr31, Sr36, Sr38*

[a]Ug99 does not occur in South Africa (as of November 2009) and is only included to show its relatedness to UVPgt55.

[b]UVPgt57 is virulent to *Sr9b* in a W2691 background, but avirulent to *Sr9b* in a W2402 background which contains *Sr9b and Sr7b*

Table 2 B: Avirulence/virulence composition of South African Leaf rust pathotypes in this study (Pretorius, *et al.*, 1987; Pretorius and Le Roux, 1988; Pretorius, *et al.*, 1990; Marais and Pretorius, 1996; Boshoff, *et al.*, 2002c; Roux, *et al.*, 2006; Pretorius, *et al.*, 2007; Marais, *et al.*, 2009)

Leaf Rust	Avirulence Genes	Virulence genes
UVPrt2	*Lr1, Lr2a, Lr2b, Lr3ka, Lr11, Lr15, Lr17, Lr20, Lr24, Lr26, Lr30*	*Lr2c, Lr3a, Lr3bg, Lr10, Lr14a, Lr16*
UVPrt3 (3SA123)	*Lr3a, Lr3bg, Lr3ka, Lr10, Lr11, Lr14a, Lr16, Lr17, Lr20, Lr26*	*Lr1, Lr2a, Lr2b, Lr2c, Lr15, Lr24*
UVPrt4	*Lr1, Lr2a, Lr2b, Lr3bg, Lr11, Lr15, Lr16, Lr17, Lr24, Lr26*	*Lr2c, Lr3a, Lr3ka, Lr10, Lr14a, Lr20, Lr30*
UVPrt5	*Lr1, Lr2a, Lr3bg, Lr10, Lr11, Lr14a, Lr15, Lr17, Lr24, Lr26*	*Lr2b, Lr2c, Lr3a, Lr3ka, Lr16, Lr20, Lr30*
UVPrt8 (3SA132)	*Lr3a, Lr3bg, Lr3ka, Lr11, Lr16, Lr20, Lr26, Lr30*	*Lr1, Lr2a, Lr2b, Lr2c, Lr10, Lr14a, Lr15, Lr17, Lr24*
UVPrt9 (3SA133)	*Lr2a, Lr2b, Lr3bg, Lr15, Lr16, Lr17, Lr26*	*Lr1, Lr2c, Lr3a, Lr3ka, Lr10, Lr11, Lr14a, Lr20, Lr30*
UVPrt10 (3SA126)	*Lr3a, Lr3bg, Lr3ka, Lr11, Lr16, Lr20, Lr24, Lr26, Lr30*	*Lr1, Lr2a, Lr2b, Lr2c, Lr10, Lr14a, Lr15, Lr17*
UVPrt13 (3SA140)	*Lr3a, Lr3bg, Lr3ka, Lr11, Lr16, Lr20, Lr30*	*Lr1, Lr2a, Lr2b, Lr2c, Lr10, Lr14a, Lr15, Lr17, Lr24, Lr26*
UVPrt19	*Lr3a, Lr3bg, Lr3ka, Lr10, Lr11, Lr16, Lr20, Lr26, Lr30*	*Lr1, Lr2a, Lr2b, Lr2c, Lr14a, Lr15, Lr17, Lr24*

Table 2 C: Avirulence/virulence composition of South African Yellow rust pathotypes in this study (Pretorius, *et al.*, 1997; Boshoff, *et al.*, 2002a; Boshoff, *et al.*, 2002b; Boshoff, *et al.*, 2003; Pretorius, *et al.*, 2007)

Yellow Rust	Avirulence Genes	Virulence genes
6E16A-	*Yr1, Yr3a, Yr4a, Yr4b, Yr5, Yr9, Yr10, Yr15, Yr25, Yr27, YrA, YrCle, YrCv, YrHVII, YrMor, YrSd, YrSp, YrSu*	*Yr2, Yr6, Yr7, Yr8, Yr17*
6E22A-	*Yr1, Yr3a, Yr4a, Yr4b, Yr5, Yr9, Yr10, Yr15, Yr27, YrA, YrCle, YrCv, YrHVII, YrMor, YrSd, YrSp, YrSu*	*Yr2, Yr6, Yr7, Yr8, Yr17, Yr25*
7E22A-	*Yr3a, Yr4a, Yr4b, Yr5, Yr9, Yr10, Yr15, Yr27, YrA, YrSp*	*Yr2, Yr6, Yr7, Yr8, Yr17, Yr25*

1.3.3 Restriction Fragment Length Polymorphism (RFLP)

RFLP uses the presence or absence of restriction enzyme cut sites in amplified fragments to differentiate between samples. This technique requires prior knowledge of the DNA sequence in order to identify restriction sites in the DNA. While the technique itself is relatively simple, finding markers can be very time consuming as polymorphic restriction sites have to be identified. Furthermore, it can suffer from reproducibility problems and the cost of restriction enzymes is high (Brown, 1996; McCartney, *et al.*, 2003). RFLP can show co-dominant markers. This technique has been successfully used in studies of diversity in fungal pathogens of wheat (Chen, *et al.*, 1994; Keller, *et al.*, 1997a; Keller, *et al.*, 1997b; Zhan, *et al.*, 1998).

1.3.4 Amplified Fragment Length Polymorphism (AFLP)

The process of generating AFLP markers is a complex technique. A number of frequent and rare cutter restriction enzymes (RE) are used to cut the genomic DNA into fragments. These fragments have 5' and 3' overhangs. Two different double stranded adapter molecules are used, one for each RE. Each adapter molecule is designed to bind to the overhang left by its specific RE. The fragments are ligated to the double stranded adapter molecules. The fragments then undergo two rounds of amplification. In the first round two non-selective primers are used, one complementary to the one adapter molecule and the other complementary to the second adapter molecule. Therefore only those fragments that were cut by both REs should be amplified. In the second round of amplification, selective primers are used. These are primers that are complementary to the 3' end of the adapter molecule, with a short 3' overhang of two to three nucleotides. This results in the amplification of only those fragments that were cut with both REs and have 5' sequences complementary to the 3' end of the specific primers. The use of several rounds of amplification as well as non-selective and selective primers reduces the number of fragments drastically and increases the reproducibility of the technique. Unique banding patterns should be identifiable for each sample. This technique is very effective and requires no prior knowledge of the DNA sequence but it is very time consuming requiring around three days before a result can be seen (Vos, *et al.*, 1995; Brown, 1996; McCartney, *et al.*, 2003). It is possible to convert single bands to SCAR markers, by direct sequencing of the bands excised from the gel rather than by cloning the fragment into a vector as the primers that amplified that specific fragment are known (Williams and Kane, 1993; Khorana, *et al.*, 1994; Brugmans, *et al.*, 2003). The SCAR marker can then be amplified directly using PCR. AFLP cannot show co-dominance. AFLP has been used to differentiate Stem rust pathotypes (Visser, *et al.*, 2009).

1.3.5 Single Strand Conformation Polymorphism (SSCP)

Primers are designed to amplify a region of interest in the sample DNA. The double stranded DNA is denatured and placed in a buffer that maintains it in a single strand conformation. Single base pair differences between fragments that are otherwise identical are enough to sufficiently alter the fragments electrophoretic mobility that it is possible to discern the differences visually with the use of gel electrophoresis. This technique suffers from poor reproducibility, low specificity and requires prior knowledge of the DNA sequence which makes it impractical (McCartney, *et al.,* 2003). SSCP can show co-dominant markers.

1.3.6 Microsatellites

These are stretches of DNA where a specific nucleotide motif of a few bases in length is repeated several times. Microsatellites are very polymorphic and generally high in copy number. They are also widely dispersed throughout the genome. All these characteristics make microsatellites particularly suited for population genetic and diversity studies (McCartney, *et al.*, 2003). Using a panel of several different microsatellites it is possible to indentify a unique haplotype for each sample. Microsatellites can show co-dominant markers. Several techniques have been developed to isolate microsatellites from a particular genome, without prior knowledge of its sequence. These are Selectively Amplified Microsatellite analysis (SAM; Hayden and Sharp, 2001b) and Sequence-Tagged Microsatellite Profiling (STMP; Hayden and Sharp, 2001a). Both these techniques have been used to generate microsatellite markers in *Puccinia spp.* (Keiper, *et al.*, 2006). Microsatellite markers have been developed and characterized in Leaf (Szabo and Kolmer, 2007), Stem (Szabo, 2007 and Zhong, *et al.*, 2009) and Yellow rust (Enjalbert, *et al.*, 2002).

1.3.7 Real Time-PCR (RT-PCR)

This technique uses a DNA binding dye that binds preferentially to double stranded DNA generated during a PCR. The dye fluoresces when bound to the double stranded DNA. The change in fluorescence is measured and plotted in real-time on a graph. Relative quantification of the amount of initial template DNA is determined by comparing the data captured to a standard curve. Relative quantification requires unique primers for each pathotype. Absolute quantification is also possible but requires fluorescently labeled TaqMan probes that are unique to each pathotype. The fungal rDNA genes are usually good areas to target for unique sequences as the Internal Transcribed Spacer 1 (ITS1) region is very variable (Zambino and Szabo, 1993; Barnes and Szabo, 2007). The DNA is melted at the end of the

PCR and the change in fluorescence is measured. This data is used to generate melting curves which can be used to differentiate the amplified fragments. RT-PCR is suited to a high throughput system as it requires no post-PCR processing. The initial capital investment is high as the RT-PCR cyclers are very expensive, however the reaction components are relatively cheap and the lack of a post-PCR processing step means that the running costs are not significantly higher than those of other techniques (McCartney, *et al.*, 2003). RT-PCR can show co-dominant markers. RT-PCR has been used in differentiation of rust pathotypes (Schena, *et al.*, 2004; Barnes and Szabo, 2007).

1.4 Selection of a marker system

In selecting the marker system that will be used, three criteria need to be taken into consideration (Brown, 1996). Firstly, the marker system used must find variation in samples. If almost all the samples appear identical the system is unlikely to have sufficient statistical power. Likewise, if almost every sample appears different it will be impossible to find differences between populations. Secondly, the chosen marker system must fit the genetic assumptions that provide the foundation of the method by which the data will be analyzed. Thirdly, the practicality of the system, that is: the length of time required to develop, optimize and implement the markers system, as well as the costs involved must also be taken into account.

1.4.1 Genetic assumptions

A marker system is just another form of a tool used to investigate one or more groups of organisms. Therefore it is important that one choose the correct tool for the task. The first set of assumptions concerns the identification of genotypes, phenotypes and hetero- and homozygosity.

The assumption that the unambiguous identification of each genotype is possible: Many marker systems, for instance RAPD, assume that fragments of the same size are also homologous. A second assumption is also made: phenotypes are unambiguously identifiable. This is also not always true, especially with RAPD markers where reproducibility is poor. The third assumption applies to organisms with a higher ploidy level, is that heterozygotes are distinguishable from homozygotes. Again this is a problem with RAPD markers as heterozygotes are indistinguishable from homozygotes. The use of codominant markers, such

as microsatellites is therefore favoured as they validate the assumptions made. Co-dominant markers also yield greater statistical power meaning that smaller sample sizes can still give significant results (Brown, 1996).

The second set assumptions concerns the independence of the markers used. If multiple markers are used, they must also be independent of one another. If they are not independent, it will result in the correlation of results from what are supposed to be separate markers, and leads to overestimation of certain parameters. A facet of the independence requirement is that within a subpopulation the marker must not be in linkage disequilibrium or undergoing selection (Brown, 1996).

The third set of assumptions is concerned with natural selection. When subdivisions are found within a population there are two possible causes. One is that the difference in allele frequencies is as a result of little to no gene flow. The second possible cause is selection acting on that particular locus but only in one population. The subdivision is therefore due to other outside forces and not due to a lack of gene flow. Therefore the markers selected for the study must not be undergoing selection. They must also not be in linkage disequilibrium with genes that are being selected for or against (Brown, 1996).

The ideal marker system is therefore in which: "(a) no band maps to more than one locus; (b) all bands are completely reproducible; (c) all alleles at a locus can be identified, and, for diploids and dikaryotes, there are no null alleles; (d) markers are in linkage equilibrium within each sub-population; (e) no marker is selected, linked to a selected gene or is in linkage disequilibrium with a selected gene in any other sub-populations; (f) most alleles are at intermediate frequencies. (Brown, 1996)"

1.4.2 Practical considerations

The ease of use, the time required to implement, the cost and the time required to analyze each sample are also important in the choice of which marker system to use in the study (Brown, 1996).

It is difficult, if not impossible to find a maker system that fulfill all of the genetic criteria and is still practical to use. The system chosen will have to be a compromise between the genetic and practical requirements.

1.5 Population genetics

Molecular marker based studies of fungal pathogens of wheat have only recently become common with the first review published by Brown (1996). The delay in switching from conventional techniques, i.e. differential testing, to more modern techniques is probably due to the recent development of many of the molecular techniques and the initially high costs of applying these molecular techniques. Also, until virulence genes are cloned and sequenced, allowing direct identification, there is always the possibility that a linked marker will segregate from the gene and no longer be useful.

Molecular marker based population genetic studies can be used to guide resistance breeding programmes. These studies attempt to understand the genetic structure of a population. Genetic structure can be defined as the degree and of distribution of genetic variation on both an intra- and inter-population level. Genetic structure of a population is created by the complex interactions between five evolutionary forces. The evolutionary history of a population can be inferred from its current genetic structure as its structure is a product of that history. It is also possible to derive insights into the evolutionary potential of a pathogen from an understanding of its current genetic structure (McDonald and Linde, 2002).

The five evolutionary forces shaping the genetic structure of a population are Mutation, Population Size, Migration, Reproduction and Selection.

1.5.1 Mutation

Mutation is the source of new alleles in a population. It creates new virulent pathotypes, breaking resistance genes. It even gradually breaks down quantitative resistance. According to the "Gene-for-Gene" hypothesis proposed by Flor (1971); avirulence genes in the pathogen are recognised by the plant and elicit an immune response. If the avirulence gene is lost or changed sufficiently through mutation, the plant is no longer able to recognise the pathogen and becomes infected (Brown and Simpson, 1994). Most such mutations are created by errors in the replication of DNA during cell division (Bengtsson, 2003). There are also rare events of recombination during replication; for instance when gene conversion occurs, it can have a drastic effect on the reshuffling of genetic material (Bengtsson, 2003). Mutation on its own however is not enough to completely break the effectiveness of a resistance gene. It is only when the virulence mutation occurs in combination with directional selection that it will increase substantially in frequency to break a resistance gene completely. If the pathogen reproduces clonally, it can increase the frequency of the fittest genotypes and can cause the

emergence of new genotypes and pathotypes (McDonald and Linde, 2002). This increase in frequency of the fittest genotypes occurs at a rate much greater than would be achieved by mutation alone (McDonald and Linde, 2002; Awadalla, 2003; Razavi and Huges, 2004).

It was thought that the evolution of virulence genes would have a general negative effect on pathogen fitness. A study by Kolmer (Kolmer, 1993) of Leaf rust has found that there is no general linkage between virulence genes and a loss of fitness but rather that some specific virulence genes carried a fitness penalty. This is unfortunate as it means that newer more virulent pathotypes, that have no fitness disadvantage, will even more easily out-compete the less virulent pathotypes.

1.5.2 Population size

Mutations occur at a very low rate in a population. Logically, therefore larger populations have a greater chance of developing mutants. With a sufficiently large population it is a virtual certainty that a mutation that creates virulence will occur. It is also less likely that the mutation will be lost from a larger population as they are less affected by processes like genetic drift than smaller populations. Genetic bottlenecks where the population is drastically reduced in size, with a concurrent loss of diversity, create less adaptable populations that are a lower disease risk as they have less potential for rapid evolution (McDonald and Linde, 2002). These genetic bottlenecks and the founder effect also lead to a closer linkage between pathotype and genotype (Kolmer, *et al.*, 1994; Sujkowski, *et al.*, 1994; Goyeau, *et al.*, 2007). This linkage is due to the lack of time for differentiation to occur. The linkage can be used to track the virulence/avirulence composition of a pathogen, but it will reduce in strength over long periods of time.

1.5.3 Migration

The horizontal transfer of genetic information between pathogens that have some degree of geographical separation is known as gene flow. Either single alleles or whole genotypes can be exchanged (McDonald and Linde, 2002). If rates of gene flow between populations are high, it prevents differentiation of the subpopulations and leads to a more diverse population overall (Keller, *et al.*, 1997a; Keller, *et al.*, 1997b; Rosewich Gale, *et al.*, 2002). Populations with high levels of gene flow have a greater potential to spread virulence. Spread of whole genotypes by asexual spores would seem to pose a greater threat as these whole genotypes contain a great degree of diversity and have already undergone selection (Limpert, *et al.*, 1999; McDonald and Linde, 2002).

1.5.4 Reproduction

There are three mating systems that occur: sexual, asexual and a combination of both. Sexually reproducing populations have a high degree of genotypic diversity where as asexually reproducing populations have lower genotypic diversity. Populations that reproduce asexually show a much greater association between genotype and virulence phenotype than those that reproduce sexually (Liu and Kolmer, 1998b; McDonald and Linde, 2002). Sexual reproduction, through meiosis, causes changes at a much faster rate than clonal reproduction where changes occur due to mutations. Recombination during meiosis can lead to offspring that carry the virulence genes for a combination of resistance genes (McDonald and Linde, 2002). Pathogens that reproduce via a combination of sexual and asexual reproduction pose perhaps the greatest threat as they are able to combine mutations that confer virulence through mating and then increase their frequency greatly by asexual reproduction.

1.5.5 Selection

The major force altering the frequency of mutant alleles and thereby creating new pathotypes is selection (McDonald and Linde, 2002). In plant pathogens such as the wheat rusts, selection is primarily an artificial construct created more by the use of control methods such as fungicides and the introduction of less susceptible cultivars. Chen *et al.* (1994) found that the allele frequencies in a population remain stable over time if the host plants remain susceptible. Selection is caused by the introduction, over a wide area, of cultivars that are not susceptible to the current pathotype population (Kolmer, 1999; Park, *et al.*, 2000; Harvey, *et al.*, 2001). This directs selection of those alleles in the population that are virulent to the new cultivar and increase their frequency (McDonald and Linde, 2002). Only those pathotypes that are able to successfully infect a host plant are able to reproduce, tending to make the population homogenous. In populations where there is less selective pressure the population tends to be very diverse (McDonald and Linde, 2002).

Internationally, the use of country or region specific wheat cultivars has created unique selection pressures on the fungal pathogens occurring in each area. These unique selection pressures make it possible to identify distinct regional groupings when comparing isolates of a pathogen from international collections (Kolmer and Liu, 2000).

Once an understanding of the forces shaping a pathogen's genetic structure has been arrived at it may be possible to find methods of reducing the effect of these forces. It is currently impossible to reduce the rate at which mutations occur so the focus must move to the remaining four forces.

Reducing population size by crop rotations or application of fungicides reduces the genetic diversity available for the creation of new virulent pathotypes (McDonald and Linde, 2002).

The limitation of gene flow between populations results in the formation of smaller, less diverse populations with reduced potential for causing disease (McDonald and Linde, 2002).

Preventing sexual reproduction reduces the possibility that virulences will be combined. Preventing asexual reproduction can slow the increase in frequency of virulent pathotypes (McDonald and Linde, 2002).

The effect of selection can be reduced by several methods.

Pyramiding several major resistance genes and breeding for durable resistance based on the amassing of additive minor genes through nonspecific pathotype (slow rusting) resistance is a successful strategy. With slow rusting, disease progression is not prevented but rather slowed. The result is intermediate to low levels of disease with all the pathotypes of the particular pathogen (Duvellier, *et al.*, 2007).

Rotation of genes, i.e. where different major resistance genes are rotated in time and space or cultivars with different combinations of resistance genes are grown has also been shown to disrupt selection (Zhu, *et al.*, 2000; McDonald and Linde, 2002). The use of quantitative resistance is also effective as it does not rely on gene-for-gene resistance, but rather on the additive effect of many minor genes and appears to work against all pathotypes of a pathogen. Such quantitative resistance cannot be rapidly broken and will only be gradually eroded (McDonald and Linde, 2002).

Lastly, it is sometimes possible to infer from the population structure where the pathogen originated, which will help to focus the search for sources of resistance (Park, *et al.*, 2000; Jurgens, *et al.*, 2006; Keiper, *et al.*, 2006).

1.6 Rust control in South Africa

A variety of methods for controlling rusts are in use in South Africa. Fungicides have been proved to be effective in controlling the wheat rusts and reducing population sizes. However, in South Africa, the prohibitively high costs have prevented their use in the most effective manner (Boshoff, *et al.*, 2003).

Crop rotation can be effective if the sequence of crops chosen does not provide alternative hosts for the rusts. If crops are not rotated or rust susceptible crops are planted before or after a wheat crop then care must also be taken to remove stubble and regrowth as these can allow the rusts to survive between crops (Anonymous, 2008). Removal of the stubble is expensive because it requires intensive tilling practices; these tilling practices also increase soil erosion which is far from ideal.

The rusts are wind dispersed over such wide areas that both fungicides and crop rotation can be ineffective in reducing the pathogens population size unless one or both methods are practiced over the entirety of the cultivated area in a region or country. An Australian study by Keiper *et al.* (2006) found identical genotypes that had been wind dispersed over the entire wheat growing area which is approximately twelve times larger than South Africa (International Grains Council, 2006). It is also believed that the rusts have a 'green bridge' in Lesotho, where wheat is also grown continuously throughout the year (Pretorius, *et al.*, 2007). It may be that even if the entire rust population is virtually wiped out in South Africa, sufficient quantities will survive in Lesotho to infect the next South African crop.

Fortunately for South African farmers it appears that no suitable host for rust sexual reproduction is found in South Africa. The rusts must therefore reproduce clonally, meaning that any new pathotypes must develop by mutation which is usually a slower process and pathotypes are less likely to be combined into "super" pathotypes (McDonald and Linde, 2002).

Introduction of resistant cultivars has been shown to be an effective method of reducing rust infections (Pretorius, *et al.*, 2007). However the use of these resistant cultivars must be carefully managed because if they are introduced and used too widely (effectively creating a monoculture) the rusts will quickly develop new virulences due to the very high selective pressures being placed upon them (McDonald and Linde, 2002). Breeding a new resistant cultivar requires extremely long timeframes; sometimes upwards of fifteen years can pass between the initial cross made for a cultivar and the first release of commercial seed. For this reason it is necessary to have an accurate picture of the pathotype composition of the current and future rust population. In order to gain this knowledge and make predictions as to the future pathogen population structure we shall have to conduct population genetic studies of the wheat rusts.

1.7 Study Objective

The objective of this study is to conduct a population genetic study of the *Puccinia spp.* on cereals in the Western Cape Province of South Africa.

This shall be achieved by means of the following:

- The optimization of DNA extraction techniques: conventional techniques will be compared to commercial kits;
- The development of molecular markers that are able to differentiate between the major pathotypes of the *Puccinia spp.;*
- Maintenance and multiplication of the major pathotypes of *Puccinia spp.* on a differential set in order to ensure the purity of the samples;
- Applying developed markers in order to type major pathotypes; and
- Field isolates will be collected, typed, assigned to specific pathotypes, and data analyzed to determine the population structure and other population genetic factors such as the influence of selection.

The results of this analysis will assist in the better understanding of the population structure of the *Puccinia spp.* leading improvements in the strategies for disease control (Brown, 1996; Harvey, *et al.*, 2001; McDonald and Linde, 2002). An accurate knowledge of the plant pathogen's population structure and epidemiology is the key to the successful management of the pathogen (Chakraborty, *et al.*, 2006). By using this knowledge it will be possible to better direct current resistance breeding programmes, and thereby improve yields.

2. Materials and Methods

Isolates of the common pathotypes of the *Puccinia spp.* in South Africa were used in this study. Pure cultures were maintained on a differential set or were stored as frozen urediniospores. DNA was extracted from the spores using either a N-Cetyl-N, N, N-trimethyl Ammonium Bromide (CTAB) protocol or a commercial kit. Microsatellite markers were used to differentiate between the pathotypes of Leaf and Yellow rust. The Stem rust pathotypes were differentiated using AFLP markers. Some AFLP bands that appeared to be pathotype specific were cloned and sequenced, and appropriate primers designed, completing their conversion to SCAR markers. The ITS1 region of the nuclear rDNA gene was sequenced in order to develop pathotype specific primers in order to relatively quantify the amount of each pathotype present in each sample. Field isolates were collected from twelve sites throughout the Swartland and Overberg regions of the Western Cape.

2.1 Pathotypes

All the pure samples of pathotypes used in this study were provided by L Snyman (University of Stellenbosch). Professor Z A Pretorius of the University of the Free State provided the original samples. The following pathotypes were used in this study:

- Stem rust: UVPgt50 (2SA4), UVPgt51 (2SA36), UVPgt52 (2SA100), UVPgt53 (2SA102), UVPgt54 (2SA55), UVPgt55 (2SA88), UVPgt56 (2SA102K), UVPgt57 (2SA105)
- Leaf rust: UVPrt2, UVPrt3 (3SA123), UVPrt4, UVPrt5, UVPrt8 (3SA132), UVPrt9 (3SA133), UVPrt10 (3SA126), UVPrt13 (3SA140) and UVPrt19
- Yellow rust: 6E16A-, 6E22A-, 7E22A-

2.2 Inoculations of multiplications

Isolates of the Leaf rust races UVPrt2, UVPrt3, UVPrt9 and UVPrt19 were multiplied on the cultivars Zaragosa, Agent, Karee and Bacchus respectively. The other races, UVPrt4, UVPrt5, UVPrt8, UVPrt10 and UVPrt13 were obtained as pure, frozen urediniospores from L Snyman at Stellenbosch University.

Isolates of the Stem rust races UVPgt50 (2SA4), UVPgt53 (2SA102), UVPgt55 (2SA88), UVPgt56 (2SA102K) and UVPgt57 (2SA105) were multiplied on the cultivars SST66, Coorong, McNair, Kiewiet and Tobie respectively. The other stem rust races, UVPgt 51, UVPgt 52 and UVPgt 54 were obtained as pure, frozen urediniospores from L Snyman at Stellenbosch University.

Isolates of the Yellow rust races used were 6E16A-, 6E22A- and 7E22A-. These races were obtained as pure, frozen urediniospores from L Snyman at Stellenbosch University.

The plants were grown under artificial lighting supplied via Osram High Pressure Mercury (400 W HQL (MBF-U)) lights, in a growth chamber. The plants were inoculated with 300 µl of spores in a Saltrol 170 (Chevron Phillips Chemical Company LP) oil suspension seven days after emergence and then placed in a dew cabinet (S.M.C.) overnight at 22°C. The plants were transferred to an isolation cabinet (Scientific Manufacturing cc. 20x26x35cm) at 22°C eight days after emergence. Spores were collected after sporulation which occurs between days 18 and 24 after inoculation. Seed stocks of the cultivars used for the multiplications were maintained by planting the seeds in an artificially cooled greenhouse. Cross-pollinations were prevented by enclosing the spikes in glycine bags just before anthesis.

2.3 DNA extractions

DNA was extracted from 40ug of urediniospores taken from -80°C storage using the modified CTAB protocol of Liu and Kolmer (1998a).Three commercial DNA extraction kits from Zymo Research (Inqaba Biotec), Qiagen (Southern Cross Biotechnology) and PeqLab (OptimaScientific) were also evaluated. The quality and quantity of the DNA was verified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific).

2.3.1 CTAB extractions

CTAB extractions from urediniospores: 40µg spores were placed in a 1.5ml Eppendorf tube and ground with carborundum. 600µl extraction buffer (0.165M Tris-HCl, pH 8.0; 66mM EDTA, pH 8.0; 1.54M NaCl; 1.1% CTAB; Proteinase K at 50µg/ml) was added to each tube and the tubes were then vortexed briefly. 66µl 20% SDS was added, followed by incubation in a waterbath at 65°C for 90 minutes. Tubes were inverted every 20 minutes. An equal volume of 24:1 chloroform:isoamyl alcohol was added and the tubes were then shaken gently for 20 minutes. The tubes were then centrifuged at 13 000g for 5 minutes. The aqueous layer was removed and placed in a clean tube. 0.6 volumes of isopropanol were added. The tubes were incubated at -20°C overnight. Then the tubes were centrifuged at 13 000 g for 5 minutes to precipitate the DNA. The pellet was washed with 70% ethanol and then dried. The pellet was then resuspended in distilled, autoclaved water (dH_2O).

2.3.2 Commercial Kits

40µg spores were used, and the kits' instructions were followed. All the kits followed the same principle:

A small tube open at one end and with a membrane at the other is placed inside a microcentrifuge tube. The membrane is washed with a solution to give it a positive charge so that it will bind DNA. The spores are placed in the tube with an extraction buffer and vortex for a short time to break open the spores. The DNA binds to the membrane. The microcentrifuge-tube assembly is then centrifuged for 5 minutes at around 12 000g. The flow-through is discarded and the membrane is washed with a second solution to remove any remaining proteins and cell debris. The flow through is discarded again. The membrane is then washed with a third solution to alter the charge to negative and the flow-through is discarded. The DNA is then eluted using either dH_2O or a buffered solution.

2.4 Primers

All primers used were at a working concentration of 10ng/µl. The volume of each primer used for the PCRs is shown in the relevant table.

Microsatellite primers: Labelled and unlabelled primers were used. Labelled primers, obtained from Applied Biosystems (USA), were tagged with fluorescent dyes (NED, VIC,

PET and 6-FAM). Multiplex PCRs were done using "Primer Sets". Each "Primer Set" was composed of as many as four different labelled primer pairs.

Unlabelled primers obtained from IDT were also used. The unlabelled primer pairs were used in simplex PCRs.

Table 3 A: Multiplex primer sets of labelled primer pairs used for Leaf rust (Szabo and Kolmer, 2007)

	Name	Label	Sequence	Volume (µl)
Primer Set 1	PtSSR3-F	PET	5' – TTC AAT TTG CCC CTT GAC TC- 3'	0.250
	PtSSR3-R		5' – AGG TAG CAT TGC CAGT GGC A- 3'	0.250
	PtSSR13-F	6 - FAM	5' – CGA ATT CGC GTT TTA TGT CC- 3'	0.150
	PtSSR13-R		5' – TGA TCC AAT CGAA CCT AGC C- 3'	0.150
	PtSSR50-F	6 - FAM	5' – CAT CGG AAT GGT CTG TCT CC- 3'	0.250
	PtSSR50-R		5' – CCA AAT GCT ATG AGT GGA AAA- 3'	0.250
	PtSSR55-F	VIC	5' – AGC TTA CGG TCC TCA ATC G - 3'	0.275
	PtSSR55-R		5' – AGT GAA AGG GGC TGG GAG T- 3'	0.275
Primer Set 2	PtSSR61-F	6 - FAM	5' – CGA ACT GGT ACA ACG CAC TG- 3'	0.200
	PtSSR61-R		5' – CGC AAA AAG GCT GAT CTC TG- 3'	0.200
	PtSSR68-F	NED	5' – GAC TCA GCC CAC TGC TAA CC- 3'	0.250
	PtSSR68-R		5' – GAT GGC GAC GTA TTT GGT CT- 3'	0.250
	PtSSR76-F	6 - FAM	5' – GGC GTC GTA TTT CTC GTA GC- 3'	0.250
	PtSSR76-R		5' – TTC GGA CTA CTG GGT AAG CA- 3'	0.250
	PtSSR91-F	NED	5' – ATC TTG CGT CTC AGC CAT CT- 3'	0.225
	PtSSR91-R		5' – CGC CGC TCT TCA TCT CTT AC - 3'	0.225
Primer Set 3	PtSSR92-F	VIC	5' – CCA AGG AAC AGT CCA CCA AG - 3'	0.250
	PtSSR92-R		5' – GAG TGC GGT AAG CCA TCT GA- 3'	0.250
	PtSSR151A-F	VIC	5' – TCA TCG CAC TCC ACT CAG AC- 3'	0.225
	PtSSR151A-R		5' – ATG CTG CCC AAC CTG CTC- 3'	0.225
	PtSSR152-F	PET	5' – CTC CGT TCC TCT TTC TGT CG- 3'	0.225
	PtSSR152-R		5' – CCA TCG CAA CCA ACA AAC A- 3'	0.225
	PtSSR154-F	NED	5' – ACG GTC AAC AGC CAA CTA CC- 3'	0.225
	PtSSR154-R		5' – CCT CGT CAT CCT GGT TGA GT- 3'	0.225
Primer Set 4	PtSSR158-F	6 - FAM	5' – GAC GAC TTC GTC ACT GCT GA- 3'	0.300
	PtSSR158-R		5' – GAG GAG AAG CCG TTC TGT TG- 3'	0.300
	PtSSR161-F	NED	5' – ACT GCC TCC TGT GCC TTC T- 3'	0.300
	PtSSR161-R		5' – TAG TCC GAG GGT GAC GAA GT- 3'	0.300
	PtSSR164-F	PET	5' – GTG GAA GTG AGC GGA AGA AG - 3'	0.300
	PtSSR164-R		5' – GGA GAT GGG CAG ATG AGG TA - 3'	0.300
	PtSSR173-F	VIC	5' – CTC AGC GAC CTC AAA GAA CC- 3'	0.300
	PtSSR173-R		5' – GAG ACG ACG GAT TGT CAA CAA- 3'	0.300
Primer Set 5	PtSSR184-F	VIC	5' – GGT CTG GCG AAT CTT TCC TT- 3'	0.275
	PtSSR184-R		5' – CAT TTT TAG TTG TGA GCC CTT G- 3'	0.275
	PtSSR186-F	PET	5' – GCC ACG AGA AAT ACA TAG AAA TAA AA- 3'	0.150
	PtSSR186-R		5' – GGT TGT TGA TGG GCT TGA GT - 3'	0.150

Table 3 B: Multiplex primer sets of labelled primer pairs used for Stem rust (Szabo, 2007)

	Name	Label	Sequence	Volume (µl)
Primer Set 1	PgtSSR1–F	VIC	5' - CCC TCA ACA TAC CAA ATT GTC C - 3'	0.150
	PgtSSR1–R		5' - CGG TAG TGA AGG AGC AGA GG - 3'	0.150
	PgtSSR3–F	PET	5' - GGA CCA AAA CCA GAA CCA GA - 3'	0.150
	PgtSSR3–R		5' - CCC ACT CCT AAT CCT CAC GA - 3'	0.150
	PgtSSR4–F	6 - FAM	5' - CCA AGA GCG GCT AAC AAA AG - 3'	0.150
	PgtSSR4–R		5' - CAA ACC AAT CTT GCC GAA AT - 3'	0.150
	PgtSSR6–F	NED	5' - CCA GCC AAG GAA TGG TTA GA - 3'	0.125
	PgtSSR6–R		5' - AAT GCC ACT ACC CAA CTT CG - 3'	0.125
Primer Set 2	PgtSSR11–F	VIC	5' - AGT TCG GCA TAG GGA ATC CT - 3'	0.150
	PgtSSR11–R		5' - GAT TTG CTG GCT TCG GTT AG - 3'	0.150
	PgtSSR12–F	PET	5' - GGA CTA CTT CAT CAG CAT TAC CA - 3'	0.150
	PgtSSR12–R		5' - TTC CTC TGT TTT CTC TCT CTC TCT C - 3'	0.150
	PgtSSR13–F	6 - FAM	5' - TGA GTT TGA CAT GTT GCC GTA - 3'	0.150
	PgtSSR13–R		5' - CAG TTC CCT TTT CCC CAT TT - 3'	0.150
	PgtSSR14–F	NED	5' - TTC CAC ATT TCG AAC AAC GA - 3'	0.150
	PgtSSR14–R		5' - GCT TGT GTC CCA AGA GCT TC - 3'	0.150
Primer Set 3	PgtSSR20–F	VIC	5' - CTA GAT GAG GGG CAG CGA AT - 3'	0.200
	PgtSSR20–R		5' - TTC TCT CTC CTT CAT CCT AA - 3'	0.200
	PgtSSR21–F	PET	5' - AAA ATG ATG GTC TCC TTG GCT A - 3'	0.150
	PgtSSR21–R		5' - CGT CGC CGA CCT TAT CTA AT - 3'	0.150
	PgtSSR47–F	6 - FAM	5' - GAC TAC TGG TGG CGG TCC T - 3'	0.150
	PgtSSR47–R		5' - AAT CAG GTT GAC CAG GAT GG - 3'	0.150
	PgtSSR68–F	NED	5' - AAC CAG GGA ACC AAA GGT CT - 3'	0.150
	PgtSSR68–R		5' - GAT TGA CTC GGC AGT TGG AG - 3'	0.150
Primer Set 4	PgtSSR90–F	VIC	5' - GTC GTC CAC CAT CCT CAA CT - 3'	0.150
	PgtSSR90–R		5' - TCA AGA GCA ATT GAA ATG GAA - 3'	0.150
	PgtSSR119–F	PET	5' - AGA GAT CAT GCT CAT TGA TGG A - 3'	0.150
	PgtSSR119–R		5' - TCC ACT CAC CAT GTT CTT GC - 3'	0.150
	PgtSSR129–F	6 - FAM	5' - CGT GAC AGT TCT CAC CAA AAA - 3'	0.150
	PgtSSR129–R		5' - CTG GCA CAA AAC CTA CAG CA - 3'	0.150
	PgtSSR133–F	NED	5' - CGT TCC TTT TTC CCC ATT TT - 3'	0.200
	PgtSSR133–R		5' - CGC TAT CGG ATG TCA CTT CA - 3'	0.200
Primer Set 5	PgtSSR134–F	VIC	5' - ATC GGG CTC CCT TTT GTA TC - 3'	0.150
	PgtSSR134–R		5' - TTG GTC TGT TCG ATT GCT TG - 3'	0.150
	PgtSSR140–F	PET	5' - TTT GGA ATC TAT GCG GTT ATT T - 3'	0.175
	PgtSSR140–R		5' - CCT TCC GCT CTT CCT TTC AC - 3'	0.175
	PgtSSR147–F	6 - FAM	5' - GGA TTC CGA GTG AGA ATT GG - 3'	0.150
	PgtSSR147–R		5' - CTC ACC TCT CGC ACA GTC AA - 3'	0.150
	PgtSSR149–F	NED	5' - CAG TTC CCT TTT CAC CCA TT - 3'	0.175
	PgtSSR149–R		5' - GAC TAC CGA TGA GTT AGA CAT GTT G - 3'	0.175

Continue … Table 3 B: Multiplex primer sets of labelled primer pairs used for Stem rust (Szabo, 2007)

Primer Set 6	PgtSSR151–F	VIC	5' - CTT TCC CCC ACA CCA TTC C - 3'	0.150
	PgtSSR151–R		5' - AAT TTG GTT GTG GAA AGA GAA C - 3'	0.150
	PgtSSR162–F	PET	5' - TGG ACT GGC TTG AAC TTG TG - 3'	0.200
	PgtSSR162–R		5' - ATT CGC GCT CGT CTC GTT - 3'	0.200
	PgtSSR164–F	6 - FAM	5' - GCT CTT TAT CGC GTT CGT A - 3'	0.150
	PgtSSR164–R		5' - AGT TAG TGG GCG GAC AAT TT - 3'	0.150
	PgtSSR180–F	NED	5' - CGA CTA GCT TGA ACG GGA AC - 3'	0.150
	PgtSSR180–R		5' - CTA GTC CCA CCC AAA CTT CG - 3'	0.150

Table 3 C: Multiplex primer sets of labelled primer pairs used for Yellow rust (Enjalbert, *et al.*, 2002)

	Name	**Label**	**Sequence**	**Volume (µl)**
Primer Set 1	RJ3-F	6 - FAM	5' - GCA GCA CTG GCA GGT GG - 3'	0.300
	RJ3-R		5' - GAT GAA TCA GGA TGG CTC C - 3'	0.300
	RJ4-F	PET	5' - GTG GGT TGG GCT GGA GTC - 3'	0.300
	RJ4-R		5' - GCT AAT CCA TTC CAC GCA CC - 3'	0.300
	RJ12-F	NED	5' - ATC ATT CCG ATT TCT TTC TCA CC - 3'	0.300
	RJ12-R		5' - TCA CAC TGA TCC CAA TAG ATC AG - 3'	0.300
	RJ13-F	VIC	5' - CAG GTT CGT TGT GGT GAG TGG - 3'	0.300
	RJ13-R		5' - CGG ACC CAG TCC ACC CAA C - 3'	0.300
Primer Set 2	RJ15-F	PET	5' - ATC GAG CAC GTC CAA ATC G - 3'	0.400
	RJ15-R		5' - CAC TGG ACA GAC GAC GGT TG - 3'	0.400
	RJ17-F	VIC	5' - TGG TGA GTG GAT GAG CTG G - 3'	0.275
	RJ17-R		5' - ACA GCA ACA AAC TCA CCC ATC - 3'	0.275
	RJ20-F	NED	5' - AGA AGA TCG ACG CAC CCG - 3'	0.275
	RJ20-R		5' - CCT CCG ATT GGC TTA GGC - 3'	0.275
	RJ22-F	6 - FAM	5' - CCC TTC GTC TGT CAT CCG - 3'	0.350
	RJ22-R		5' - ATC AAG AAG ATT CCT GGG TGA G - 3'	0.350
Primer Set 3	RJ18-F	PET	5' - CTG CCC ATG CTC TTC GTC - 3'	0.400
	RJ18-R		5' - GAT GAA GTG GGT GCT GTC G - 3'	0.400
	RJ21-F	VIC	5' - TTC CTG GAT TGA ATT CGT CG - 3'	0.300
	RJ21-R		5' - CAG TTC TCA CTC GGA CCC AG - 3'	0.300
	RJ24-F	6 - FAM	5' - TTG CTG AGT AGT TTG CGG TGA G - 3'	0.300
	RJ24-R		5' - CTC AAG CCC ATC CTC CAA CC - 3'	0.300
	RJ27-F	NED	5' - CGT CCC GAC TAA TCT GGT CC - 3'	0.300
	RJ27-R		5' - ATG AGT TAG TTT AGA TCA GGT CGA C - 3'	0.300

Table 4: Simplex unlabelled primer pairs used for Stem rust (Zhong, *et al.*, 2009)

Name	Sequence	Volume (µl)
Pgestssr098-F	5’ - CAG TGG GAG GGA GAA TAA CG – 3’	1.0
Pgestssr098-R	5’ - GCC TCT TCG AAG TTG TTG CT – 3’	1.0
Pgestssr101-F	5’ - CTC AAC CGC AAT AAC AGC AA – 3’	1.0
Pgestssr101-R	5’ - GCG ACC CAA TCA TGA ATC TT – 3’	1.0
Pgestssr121-F	5’ - CGG AAA ATT GAG GGA AGA CA – 3’	1.0
Pgestssr121-R	5’ - CGG CGT CCT AGA AAC AGA AC – 3’	1.0
Pgestssr124-F	5’ - TCC TGA CAT GCA ATT TGG TT – 3’	1.0
Pgestssr124-R	5’ - GAG CCT AAC AAT CCC CAC AA – 3’	1.0
Pgestssr131-F	5’ - AGC TGG GGG AAA CAA AAG TT – 3’	1.0
Pgestssr131-R	5’ - GAC CAT TCC ATC CAT CGT TT – 3’	1.0
Pgestssr149-F	5’ - GGG GAG AAG CAC AAT CAC AT – 3’	1.0
Pgestssr149-R	5’ - CGG TTC CCA ATG ACA AAA AC – 3’	1.0
Pgestssr194-F	5’ - GGG GGA TAA GGA AAC CAG AT – 3’	1.0
Pgestssr194-R	5’ - ATC TCT GGC CAC TCG GTA TG – 3’	1.0
Pgestssr196-F	5’ - TGA CGA TGA TCC AGA AGC AG – 3’	1.0
Pgestssr196-R	5’ - TGG GAA GGG AAG TTT GAC TG – 3’	1.0
Pgestssr231-F	5’ - TGA AAG CGA AAA CTT CAC ACA – 3’	1.0
Pgestssr231-R	5’ - ACG ACC CAT CAA AAA CAA GC – 3’	1.0
Pgestssr269-F	5’ - TGG TTT GTT TGT GGT GAT GG – 3’	1.0
Pgestssr269-R	5’ - TCG CTC CAT ACT TTC CTT CG – 3’	1.0

Sequencing primers: The primer pair was obtained from IDT.

Table 5: Primer pair used for sequencing of the ITS1 region of the *Puccinia spp.* (Barnes and Szabo, 2007)

Name	Sequence	Volume (µl)
ITS1rustF10d	5' - ACC TGC AGA AGG ATC ATT A - 3'	0.5
ITS1rustR3c	5' - TGA GAG CCT AGA GAT CCA TTG TTA - 3'	0.5

AFLP: All primer pairs and adapter molecules were obtained from Applied Biosystems, except the four unlabelled *EcoRI* primers which were obtained from IDT.

Table 6: Primers used for AFLP analysis of the *Puccinia spp.* (Visser, *et al.*, 2009)

Name	Label	Sequence	Volume (µl)
EcoRI Adapter[a]		5'- CTC GTA GAC TGC GTA CC -3' 3'- CAT CTG ACG CAT GGT TAA -5'	1.00
MseI Adapter[a]		5'- GAC GAT GAG TCC TGA G -3' 3'- TAC TCA GGA CTC AT -5'	1.00
EcoRI Primer+0		5'- GAC TGC GTA CCA ATT C -3'	1.50
EcoRI Primer+1.0	NED	5'- GAC TGC GTA CCA ATT CA -3'	0.25
EcoRI Primer+2.1	VIC	5'- GAC TGC GTA CCA ATT CAA -3'	0.25
EcoRI Primer+2.2	6-FAM	5'- GAC TGC GTA CCA ATT CCC -3'	0.75
EcoRI Primer+2.3	PET	5'- GAC TGC GTA CCA ATT CTG -3'	0.75
MseI Primer+0		5'- GAT GAG TCC TGA GTA A -3'	1.50
MseI Primer+2.1		5'- GAT GAG TCC TGA GTA AAT -3'	1.00
MseI Primer+2.2		5'- GAT GAG TCC TGA GTA AAG -3'	1.00
MseI Primer+3.0		5'- GAT GAG TCC TGA GTA ACA T -3'	1.00

[a]Adapters were obtained as double stranded molecules

Table 7: Primers used for conversion of AFLP to SCAR markers

Name	Sequence	Volume (µl)
EcoRI Primer+1.0	5'- GAC TGC GTA CCA ATT CA -3'	1.0
EcoRI Primer+2.1	5'- GAC TGC GTA CCA ATT CAA -3'	1.0
EcoRI Primer+2.2	5'- GAC TGC GTA CCA ATT CCC -3'	1.0
EcoRI Primer+2.3	5'- GAC TGC GTA CCA ATT CTG -3'	1.0
MseI Primer+2.1	5'- GAT GAG TCC TGA GTA AAT -3'	1.0
MseI Primer+2.2	5'- GAT GAG TCC TGA GTA AAG -3'	1.0
MseI Primer+3.0	5'- GAT GAG TCC TGA GTA ACA T -3'	1.0

Table 8: SCAR primers designed from AFLP fragment sequences

Name	Sequence	Volume (µl)	Annealing temperature
UVPgt50/12/256 F	5’ - ACA CTG GTC AAT GGA ATG GAG G – 3’	0.5	59°C
UVPgt50/12/256 R	5’ - GCC TTC TTC TCC GCT CAC AGT – 3’	0.5	59°C
UVPgt51/8/284 F	5’ - GGC AAC ACC AGA ATA GAA CT – 3’	0.5	49°C
UVPgt51/8/284 R	5’ - CAT CAG TCA GAT TGT GCG C – 3’	0.5	49°C
UVPgt52/5/264.1 F	5’ - TTC CAA TCT CGT TGG TTG TG – 3’	0.5	63°C
UVPgt52/5/264.1 R	5’ - ACC AGG ACC GGT GTG AGA T – 3’	0.5	63°C
UVPgt52/5/264.2 F	5’ - CGT GGT CAC AAA GTC AGA GG – 3’	0.5	63°C
UVPgt52/5/264.2 R	5’ - TGC GAG TGC ATA AAG AGC TG – 3’	0.5	63°C
UVPgt53/8/105 F	5’ - TAA AGC CCA ATG GAA GTT G – 3’	0.5	47°C
UVPgt53/8/105 R	5’ - CCC AGA AAA ACA CAA ATT TG – 3’	0.5	47°C
UVPgt54/10/201 F	5’- GCA AAC TCA ATA ATA AAT CAT – 3’	0.5	54°C
UVPgt54/10/201 R	5’ - TGC CCC AGT TCA GTC AA – 3’	0.5	54°C
UVPgt55/8/214 F	5’ - ATT GGC AGA GAC ACA GCA T – 3’C	0.5	59°C
UVPgt55/8/214 R	5’ - CAC CTG AAA CGA CCC TCT A – 3’	0.5	59°C
UVPgt56/7/448.1 F	5’ - GCT TCA AGC GTT TTT CCT TG – 3’	0.5	55°C
UVPgt56/7/448.1 R	5’ - CAC CAT TAC TGG GGG ACA CT – 3’	0.5	55°C
UVPgt56/7/448.2 F	5’ - AGT CCT CCG ATC ACA TTT GC – 3’	0.5	55°C
UVPgt56/7/448.2 R	5’ - TAG ATC TCG CCA CTC CGT CT – 3’	0.5	55°C
UVPgt57/10/280 F	5’ - CCC CCA TCA AAT ACA TGT CAC – 3’	0.5	62°C
UVPgt57/10/280 R	5’ - GGC TGT CAT GCA AGG AAA AT – 3’	0.5	62°C

2.5 Microsatellites

All PCRs were run in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, USA).

2.5.1 Multiplex PCRs to amplify microsatellites

Identical PCR conditions (**Table 9**) were used for the Leaf, Stem and Yellow rust multiplex PCRs using the labelled primers. The PCRs required optimization primarily of the concentration of primer pairs within a Primer Set to ensure the fidelity of the reaction.

Table 9: PCR conditions for multiplex PCRs to amplify microsatellites in Leaf, Stem and Yellow rust

Duration (min)	Temperature (°C)	
2.0	95	
0.5	95	35 cycles
0.5	60	
0.5	72	
10	72	
Soak	4.0	

Identical (excepting the volumes of the primers in the Primer Set) reaction mixes were used for Leaf, Stem and Yellow rust.

Table 10: Reaction mix for multiplex PCR to amplify microsatellites in Leaf, Stem and Yellow rust

Reagent	Volume (µl)	Stock Concentration	Supplier
Kapa Buffer A	1.0	10 X	KapaBiosystems
$MgCl_2$	2.0	25mM	KapaBiosystems
Kapa dNTP mix	0.8	10mM	KapaBiosystems
Primer Set	See **Table 3A**, **Table 3B** or **Table 3C**	10nM	Applied Biosystems
KapaTaq	0.042	5 U/µl	KapaBiosystems
dH_2O	Sufficient to make the final volume 10µl		
Sample DNA	1.0	10 ng/µl	

The PCR products were analyzed on an Applied Biosystems 3130xl Genetic Analyzer which has a resolution of less than one basepair (Coburn, *et al.*, 2002). The analysis of the electropherograms was done in GeneMapper 4.0 (Applied Biosystems).

2.5.2 Simplex PCRs to amplify Stem rust microsatellites

Ten unlabelled primer pairs were used. The primer sequences are in **Table 4** (Zhong, *et al.*, 2009).

Table 11: PCR conditions to amplify Stem rust microsatellites

Duration (min)	Temperature (°C)	
5.0	94	
0.5	94	30 cycles
0.5	52	
0.5	72	
10	72	
Soak	4.0	

Table 12: Reaction mix for PCR to amplify Stem rust microsatellites

Reagent	Volume (µl)	Stock Concentration	Supplier
Kapa Buffer A	2.00	10 X	KapaBiosystems
$MgCl_2$	2.00	25mM	KapaBiosystems
Kapa dNTP mix	0.80	10mM	KapaBiosystems
Primer Forward (**Table 4**)	1.00	10nM	IDT
Primer Reverse (**Table 4**)	1.00	10nM	IDT
KapaTaq	0.20	5U/µl	KapaBiosystems
dH_2O	12.8		
Sample DNA	1.00	100ng/µl	

2.6 Gel Electrophoresis

2.6.1 Agarose gel electrophoresis

Agarose gels of 1, 1.5 and 2% were made. Gels were made using 1X TBE buffer (12.11g Tris (hydroxymethyl) Aminomethane, 0.744g Ethylenediamine tetra acetic acid disodium salt dihydrate (EDTA) and 6.183g Boric Acid in 1l dH_2O).

Gels were run in a 1X TBE buffer at the voltages and for the durations as indicated in the text.

2.6.2 Polyacrylamide gel electrophoresis

An acrylamide stock solution of 40% was made. It contained 76g acrylamide, 4g bis-acrylamide and sufficient distilled water to make the final volume 200ml. The stock solution was placed in a foil covered flask.

From the 40% stock solution a 6% sequencing gel mix was prepared. It contained 37.5ml of the 40% acrylamide stock solution, 90.09g urea, 50ml 5X TBE and sufficient distilled water to make the final volume 250ml.

A 10% solution of ammonium persulfate was prepared by dissolving 0.1g of ammonium persulfate in 1ml of distilled water.

The gel was prepared by adding 800µl 10% ammonium persulfate and 160µl of N, N, N', N'-Tetramethylethylenediamine (TEMED) to 160ml of 6% gel mix and mixed thoroughly.

Plate preparation: The plate glue stock was prepared by adding 125µl γ-Methacryloxypropyltrimethoxysilane to 25ml 100% ethanol. This stock was further diluted to a 1:3 solution by adding 500µl plate glue stock to 1500µl 100% ethanol. Diluted plate glue (1740µl) was added to 140µl acetic acid.

The long glass plate was cleaned with 100% ethanol then the entire plate wiped with Wynn's C-thru (Wynn's Oil South Africa) and left to dry for 3 minutes. After the plate dried it was polished with paper towel.

The short glass plate was cleaned with 100% ethanol then the plate was wiped with the plate glue and left to dry for 30 seconds. The plate was then wiped with a paper towel.

The gel was cast using 1mm spacers.

AFLP loading dye (10µl; 98% formamide, 10mM EDTA pH 8.0, 0.05% w/v bromo phenol blue, 0.05% w/v xylene cyanol FF) was added to 10µl of PCR sample. Samples were denatured at 95°C for 5 min and then quenched on ice.

The gel was prerun at 700V for 30 min using a Whatman Biometra Life Technologies Gibco-BRL PS 9009TC. Electrophoresis was performed at a constant 65W for 200 minutes.

Silver staining: The fixing solution was prepared by adding 210ml 100% ethanol to 10.5ml acetic acid and 1879.50ml distilled water. The staining solution was prepared by dissolving 2.1g of $AgNO_3$ in 2100ml distilled water. The developing solution was prepared by dissolving 31.5g of NaOH to 2100ml distilled water; 8.505ml of formaldehyde was added to the developing solution just before use.

Staining procedure: The spacers and the short plate were removed and the gel was placed in the fixing solution for 20 minutes on a shaker. The gel was then rinsed twice, for 5 minutes, in distilled water on a shaker. It was then placed in the staining solution for 20 minutes on the shaker. The gel was then rinsed in distilled water for 10 seconds. The gel was then placed in the developing solution on the shaker until the bands were obvious. The gel was then rinsed in distilled water a final time.

2.7 Sequencing

A 270bp fragment from the ITS1 region of the nuclear rDNA gene of each pathotype was amplified using primers from Barnes and Szabo (2007) (see **Table 5**).

Table 13: PCR conditions to amplify the ITS1 region for sequencing

Duration (min)	Temperature (°C)	
2.0	95	
0.25	95	45 cycles
0.5	60	
0.5	72	
10	72	
Soak	4.0	

Table 14: Reaction mix for PCR to amplify the ITS1 region

Reagent	Volume (µl)	Stock Concentration	Supplier
Kapa Buffer A	2.00	10X	KapaBiosystems
$MgCl_2$	4.00	25mM	KapaBiosystems
Kapa dNTP mix	1.00	10mM	KapaBiosystems
ITS1rustF10d	0.50	10nM	IDT
ITS1rustR3c	0.50	10nM	IDT
KapaTaq	0.20	5U/µl	KapaBiosystems
dH_2O	10.8		
Sample DNA	1.00	10ng/µl	

The amplicon was sequenced using direct sequencing on an Applied Biosystems 3130xl Genetic Analyser. The sequence data was aligned using Bioedit 7.0.9.0. (www.mbio.ncsu.edu/BioEdit/bioedit.html)

2.8 AFLP

The Stem, Leaf and Yellow rust pathotypes were analysed using AFLP. The AFLP protocol used was from Honing (Honing, 2007). The primers and adapter sequences that were used were obtained from Visser *et al.* (2009) (see **Table 6**). The *Puccinia spp.* genomic DNA was restriction enzyme digested and adapters were ligated to the fragments.

Table 15: Reaction mix for the restriction digestion of genomic DNA and ligation of adapters

Reagent	Volume (µl)	Stock Concentration	Supplier
Sample DNA	6.0	50ng/µl	
dH_2O	6.6		
One-Phor-All Buffer	2.0	10X	USB
ATP	2.0	10mM	New England Biolabs
EcoRI adapter	1.0	10nM	Applied Biosystems
MseI adapter	1.0	10nM	Applied Biosystems
EcoRI Restriction Enzyme	0.5	10U/µl	Roche
MseI Restriction Enzyme	0.5	10U/µl	New England Biolabs
Bovine Serum Albumin	0.2	10mg/ml	New England Biolabs
T4 DNA Ligase	0.2	5U/µl	USB

The reaction was mixed, centrifuged and incubated overnight at room temperature.

Fifteen microlitres of the reaction was diluted with 135µl 1x TE buffer (10mM TRIS-HCl and 0.1mM Na_2EDTA at pH 8.0). The digestion and ligation reaction was verified by running the remaining 5µl of undiluted reaction with 6µl Ficoll loading dye on a 1.5% agarose gel (1x TBE buffer) at 100V for 45min.

The diluted, digested and ligated genomic DNA was then used in a pre-selective amplification reaction. All PCR amplifications were performed using an Applied Biosystems 2720 Thermal Cycler.

Table 16: Pre-selective amplification reaction mix

Reagent	Volume (µl)	Stock Concentration	Supplier
Diluted restriction-ligation DNA	5.2		
dH_2O	9.5		
Kapa Buffer A	2.0	10X	KapaBiosystems
Kapa dNTP mix	0.4	10mM	KapaBiosystems
EcoRI Primer +0	1.5	10nM	Applied Biosystems
MseI Primer +0	1.5	10nM	Applied Biosystems
$MgCl_2$	1.2	25mM	KapaBiosystems
KapaTaq	0.2	5U/µl	KapaBiosystems

Table 17: Pre-selective amplification PCR conditions

Duration (min)	Temperature (°C)	
5.0	72	
0.5	94	30 cycles
1.0	56	
1.0	72	
5.0	72	
Soak	4.0	

Fifteen microlitres of the reaction was diluted with 135µl 1x TE buffer (10mM TRIS-HCl and 0.1mM Na_2EDTA at pH 8.0). The pre-amplification reaction was verified by electophoresing the remaining 5µl of undiluted reaction with 6µl Ficoll loading dye on a 1.5% agarose gel (1x TBE buffer) at 100V for 45min.

The selective amplification of the samples was done as three separate multiplex reactions. Each reaction was identical except that each reaction contained a unique unlabelled *MseI* Primer as well as all four labelled *EcoRI* primers (see **Table 18**).

Table 18: Primer combinations for multiplex selective amplification reaction

Unlabelled *MseI* Primer	**Labeled *EcoRI* Primer**			
MseI Primer+2.1	*EcoRI* Primer+1.0	*EcoRI* Primer+2.1	*EcoRI* Primer+2.2	*EcoRI* Primer+3.0
MseI Primer+2.2	*EcoRI* Primer+1.0	*EcoRI* Primer+2.1	*EcoRI* Primer+2.2	*EcoRI* Primer+3.0
MseI Primer+2.3	*EcoRI* Primer+1.0	*EcoRI* Primer+2.1	*EcoRI* Primer+2.2	*EcoRI* Primer+3.0

Table 19: Multiplex selective amplification reaction mix

Reagent	Volume (µl)	Stock Concentration	Supplier
Diluted Pre-selective DNA	1.50		
dH_2O	3.65		
Kapa Buffer A	1.00	10X	KapaBiosystems
Kapa dNTP mix	0.20	10mM	KapaBiosystems
Unlabelled *MseI* Primer	1.00	10nM	Applied Biosystems
EcoRI Primer+1.0	0.25	10nM	Applied Biosystems
EcoRI Primer+2.1	0.25	10nM	Applied Biosystems
EcoRI Primer+2.2	0.75	10nM	Applied Biosystems
EcoRI Primer+2.3	0.75	10nM	Applied Biosystems
$MgCl_2$	0.60	25mM	KapaBiosystems
KapaTaq	0.05	5U/µl	KapaBiosystems

Table 20: Touchdown PCR conditions for selective amplification

Duration (min)	Temperature (°C)	
0.5	94	13 cycles
0.5	65 (less 0.7°C/cycle)	
1.0	72	
0.5	94	23 cycles
0.5	56	
1.0	72	
Soak	4.0	

The PCR products were loaded on an Applied Biosystems 3130xl Genetic Analyzer. The analysis of the electropherograms was done in GeneMapper 4.0 (Applied Biosystems).

2.9 SCAR Markers

Stem rust AFLP markers were converted to SCAR markers. The AFLP fragments targeted for conversion were unique to a pathotype and differed in size with respect to the nearest fragment by five or more basepairs.

2.9.1 AFLP amplification

The diluted pre-selective DNA from the AFLP markers was used. Each pathotype was amplified in a simplex PCR using all twelve possible primer pair combinations (see **Table 21**) with a reaction mix as in **Table 22** and conditions as in **Table 20**.

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Table 21: Primer pair combinations used for conversion of AFLP markers to SCAR markers

	Primer			
	EcoRI Primer 1.0	*EcoRI* Primer 2.1	*EcoRI* Primer 2.2	*EcoRI* Primer 2.3
MseI Primer 2.1	1	2	3	4
MseI Primer 2.2	5	6	7	8
MseI Primer 3.0	9	10	11	12

Table 22: Reaction mix to amplify targeted AFLP markers

Reagent	**Volume (µl)**	**Stock Concentration**	**Supplier**
Diluted Pre-selective DNA	5.0		
dH_2O	9.3		
Kapa Buffer A	2.0	10X	KapaBiosystems
Kapa dNTP mix	0.4	10mM	KapaBiosystems
MseI Primer	1.0	10nM	Applied Biosystems
EcoRI Primer	1.0	10nM	Applied Biosystems
$MgCl_2$	1.2	25mM	KapaBiosystems
KapaTaq	0.1	5U/µl	KapaBiosystems

The amplified products were separated on a 6% polyacrylamide gel that was prepared and stained using silver staining as for the microsatellites.

The selected bands were excised from the polyacrylamide. The excised pieces were then placed in 20µl dH_2O and incubated in a water bath at 80°C for 20 minutes. They were then centrifuged at 14,000 RPM for 10 minutes. A further 20µl dH_2O was added and the tubes were incubated at 80°C for 20 minutes and centrifuged again at 14,000rpm for 10 minutes.

The supernatant was removed and used in a PCR reaction with conditions as in **Table 20** and reaction mix as in **Table 23**.

Table 23: Reaction mix to amplify AFLP markers excised from polyacrylamide gel

Reagent	**Volume (µl)**	**Stock Concentration**	**Supplier**
Supernatant	10		
dH_2O	2.3		
Kapa Buffer A	2.0	10X	KapaBiosystems
Kapa dNTP mix	0.4	10mM	KapaBiosystems
MseI Primer	2.0	10nM	Applied Biosystems
EcoRI Primer	2.0	10nM	Applied Biosystems
$MgCl_2$	1.2	25mM	KapaBiosystems
KapaTaq	0.1	5U/µl	KapaBiosystems

The PCR products were then run on a 1% agarose gel at 120V for 60min to confirm that they were the correct size. Clear bands of the correct size were excised from the gel and purified using a Promega Wizard® SV Gel and PCR Clean-Up system and a Sigma-Aldrich

GenElute™ Gel Extraction Kit. The eluted DNA from the purification process was then used in a PCR reaction with conditions as in **Table 20** and reaction mix as in **Table 24**.

Table 24: Reaction mix to amplify AFLP markers excised and purified from agarose

Reagent	Volume (µl)	Stock Concentration	Supplier
Eluted DNA	10		
dH_2O	2.3		
Kapa Buffer A	2.0	10X	KapaBiosystems
Kapa dNTP mix	0.4	10mM	KapaBiosystems
MseI Primer	2.0	10nM	Applied Biosystems
EcoRI Primer	2.0	10nM	Applied Biosystems
$MgCl_2$	1.2	25mM	KapaBiosystems
KapaTaq	0.1	5U/µl	KapaBiosystems

Direct sequencing of the bands purified from the agarose was not successful due to excessive background.

2.9.2 Cloning

It was decided to clone the bands into *E.coli*. Several media and solutions were prepared.

Table 25: Media and solutions used for cloning

Media	Concentration	Sterilization
Luria Bertani (LB) medium	30g/l	autoclave
LB agar	45g/l	autoclave
Ampicillin	100mg/ml	filter
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	0.1M	filter
X-galactose (X-gal)	20mg/ml	
$MgCl_2$	100mM	autoclave
$CaCl_2$-15% Glycerol	100mM	autoclave the $CaCl_2$ before adding the glycerol
Glycerol	50%	filter

The Lysogeny Broth (LB) agar was cooled to less than 60°C and 500µl ampicillin (100mg/ml) was added per 500 ml agar, making LB (amp) agar. The LB was then poured onto sterile petri dishes which were stored at 4°C.

Competent cells were prepared by defrosting frozen *E. coli* cells [supplied by Aletta Eksteen (University of Stellenbosch)] on ice, and streaking them out on to LB agar plates. The inverted plates were incubated over night at 37ºC. A single colony of *E.coli* DH5α was selected and placed in 5ml of LB medium at 37°C on a shaker at 225rpm overnight. The culture obtained was diluted to 1:100 and again incubated at 37°C on a shaker at 225rpm. After two hours an OD_{600} reading was taken using a Nanaodrop ND1000, if the A_{600} was

between 0.5 and 0.6 then the cells were at the optimal density. If the A_{600} was less than 0.5 the cells were incubated longer. The culture (50ml) was transferred to round bottom centrifuge tubes and incubated for 5 min on ice. The tubes were then centrifuged at 5000rpm at 4°C for 10 min in a Beckman Coulter Allegra™ X-22R centrifuge. The supernatant was poured off and the culture was kept on ice. The culture was resuspended in ice-cold $MgCl_2$ and incubated for 20 min on ice. The culture was again centrifuged at 4°C for 10 min at 4000rpm. The supernatant was poured off and the culture was kept on ice. The culture was resuspended in 2ml of ice-cold $CaCl_2$-15% Glycerol. The culture (100µl) was added to 1.5ml Eppendorf tubes and flash-frozen in 100% isopropanol pre-cooled to -80°C. The competent cells were stored at -80°C.

The Promega pGEM® T-Easy Vector System I was used. The pGEM vector and Control Insert DNA tubes were centrifuged and the 2x Rapid Ligation Buffer was vortexed prior to use.

Table 26: Ligation reaction mix

Reagent	Volume (µl)	Stock Concentration	Supplier
Fragment DNA	3.0		
pGEM®-T Easy vector	1.0	50ng/µl	Promega
2x Rapid Ligation Buffer	5.0	2X	Promega
T4 DNA ligase	1.0	3U/µL	Promega
Control Insert DNA (added to positive control only)	3.0	4ng/µL	Promega
H_2O (added to negative control only)	3.0		

The mixture was prepared by adding each component in descending order (as in **Table 26**) and mixed by gentle pipetting. The ligation mixture was then incubated at 4°C overnight.

The ligation mix was added to 100µl competent cells and mixed by gentle pipetting and incubated on ice for 20 min. It was then heatshocked by placing it in a waterbath at 42°C for 1 min and was placed immediately on ice for 2 min. 900µl LB medium was added and the tubes were incubated at 37°C, slanted, on a shaker at 115rpm for 90 min. The LB (amp) agar plates were prepared by warming them up to room temperature and spreading 100µl of 0.1M Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 20µl X-galactose or bromo-chloro-indolyl-galactopyranoside, (Whitehead Scientific) onto the plates. The transformed culture (100µl) was plated out onto the LB (amp) agar. The remaining transformed culture was centrifuged at 2000rpm for 5 minutes and 700µl supernant was removed. The cells were resuspended in the remaining medium and 100µl was plated out onto LB (amp) agar plates.

The plates were incubated, inverted, at 37°C overnight. The plates were then wrapped in Parafilm and stored at 4°C.

A colony PCR was performed, with a mix as described in **Table 27** and conditions as in **Table 28**. One third of each of three white colonies and one blue colony were transferred from a plate, using a pipette tip, to the PCR tubes containing the reaction mix.

Table 27: Colony PCR mix

Reagent	Volume (µl)	Stock Concentration	Supplier
0.3 colony			
dH_2O	7.90		
Kapa Buffer A	1.00	10X	KapaBiosystems
Kapa dNTP mix	0.20	10mM	KapaBiosystems
T7 Primer	0.25	10mM	KapaBiosystems
Sp6Primer	0.25	10mM	KapaBiosystems
$MgCl_2$	0.60	25mM	KapaBiosystems
KapaTaq	0.10	5U/µl	KapaBiosystems

Table 28: Colony PCR conditions

Duration (min)	Temperature (°C)	
5.0	94	
0.5	94	30 cycles
0.5	55	
1.0	72	
7.0	72	
Soak	4.0	

Colonies that tested positive were transferred to McCartney bottles containing 5ml LB medium and 5µl of 100mg/ml ampicillin. The bottles were incubated, at a slant, overnight at 37°C on a shaker at 225rpm.

Plasmid extraction was performed using the Promega Wizard® *Plus* SV minipreps DNA Purification System Protocol. Once the plasmids had been extracted and purified they were diluted to 100ng/µl and submitted for automated sequencing.

The sequences were analysed using BioEdit and primers were designed using the online Primer 3 software at http://frodo.wi.mit.edu/primer3/. Primers were checked for self annealing and hairpin loops using Oligo Analyser (www.uku.fi/~kuulasma/OligoSoftware). Primer sequences are shown in **Table 8**.

Primers were then optimised and tested against a panel of all the stem rust pathotypes used in this study, UVPgt50 to UVPgt57, to confirm specificity.

The same optimised PCR mix (**Table 29**), was used with all the primers (**Table 8**). PCR conditions are shown in **Table 30**.

Table 29: SCAR marker reaction mix

Reagent	Volume (µl)	Stock Concentration	Supplier
Kapa Buffer A	2.00	10X	KapaBiosystems
Kapa dNTP mix	1.00	10mM	KapaBiosystems
Forward Primer	0.50	10mM	KapaBiosystems
Reverse Primer	0.50	10mM	KapaBiosystems
$MgCl_2$	2.00	25mM	KapaBiosystems
KapaTaq	0.20	5U/µl	KapaBiosystems
dH_2O	12.8		
Sample DNA	1.00	100ng/µl	

Table 30: SCAR marker PCR conditions

Duration (min)	Temperature (°C)	
5.0	94	
0.5	94	30 cycles
0.5	See **Table 8**	
0.5	72	
10	72	
Soak	4.0	

It was not possible to multiplex the reactions due to the differences in annealing temperatures and similarity in product sizes.

2.10 Field Isolates

Field isolates were collected from eleven localities in the Overberg and Swartland regions of the Western Cape (see **Figure 3**) between February 2008 and November 2009. The isolates were of Leaf, Stem and Yellow rust and were collected from wheat and triticale commercial cultivars as well as from advanced breeding material. Full details of each isolate are described in **Addendum 2**.

Sections of infected plant tissue were cut finely (less than $1mm^2$) and placed in a 2ml microcentrifuge tube. Three stainless steel ball bearings (2mm diameter) were placed in each tube and the tubes were frozen at -80°C overnight. The tubes were then placed in a Qiagen Tissue Lyser for three cycles of 1.5 min at 30Hz. Then the CTAB protocol was followed as in section **2.3.1**.

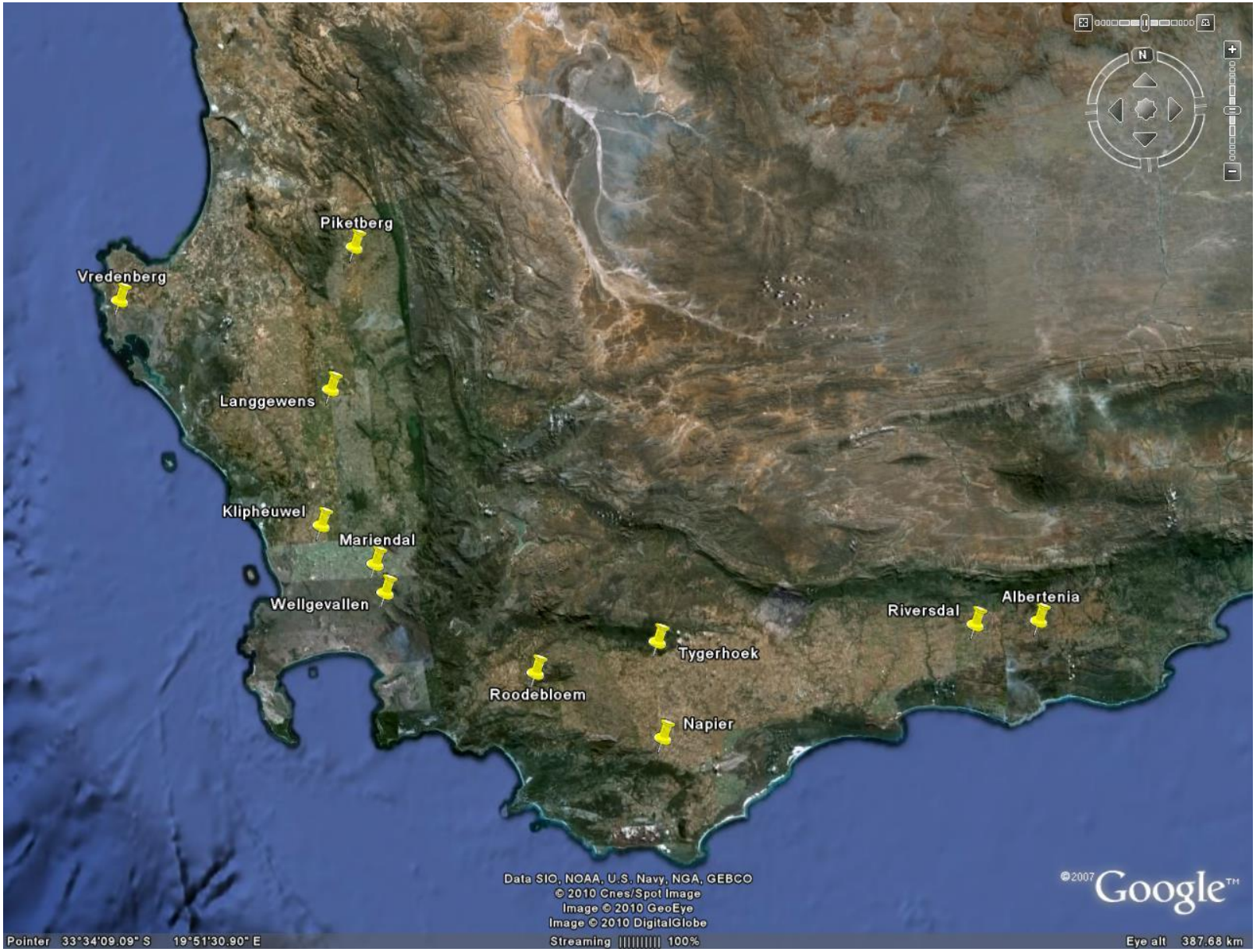

Figure 3: Localities in the Western Cape from which field isolates were collected

2.11 Data analysis

2.11.1 Microsatellites

The haplotype of each pathotype was entered into a matrix that recorded the size of each specific microsatellite fragment in Excel. Data analysis was performed using PowerMarker v3.25 (Liu and Muse, 2005; http://statgen.ncsu.edu/powermarker/). PowerMarker was chosen because of its ability to handle microsatellite data as well as perform all the required calculations including: allele frequencies, genetic distance and Neighbour-Joining clustering with no additional software required. The data was imported into PowerMarker from a text file and the allele frequencies were calculated. Genetic distances were calculated from the frequency data using the CS Cord 1967 distance calculation (Cavalli-Sforza and Edwards, 1967) as it has been shown that this model can produce true tree topology irrespective of the microsatellite mutation model used (Takezaki and Nei, 1996). Cladograms were generated in PowerMarker using the Neighbour-Joining clustering method, as this method is more suited to determining tree topology from CS Cord distance than the Unweighted Pair Group Method with Arithmetic mean (UPGMA) method (Takezaki and Nei, 1996). Cladograms were visualized in TreeView v1.66 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.11.2 AFLP

A binary matrix for each AFLP fragment was constructed in Excel and the data was analysed in Power Marker v3.25. The allele frequencies were calculated and the genetic distances determined using the CS Cord 1967 distance calculation. Dendrograms were generated using the Neighbour-Joining clustering method. The cladograms were visualised in Tree View v1.66.

No estimates of genetic diversity or testing for Hardy-Weinberg equilibrium was done, see section **4.5**.

3. Results

3.1 DNA extractions

The CTAB method was found to provide the best compromise between yield (1043.51ng/µl) and purity (2.14 A260nm/A280nm) while the yield from the PeqLab (8.32ng/µl, 1.58 A260nm/A280nm), Qiagen (5.86ng/µl, 1.18 A260nm/A280nm) and Zymo (21.94ng/µl, 1.24 A260nm/A280nm) commercial kits was too low for practical use (**Table 31**). DNA extracted using the Zymo Research kit was successfully used in PCRs.

Table 31: Results of DNA extractions using a conventional protocol as well as commercial kits

Method	Average Yield (ng/ul)	Absorbance ratio (260nm/280nm)	Cost/ Sample	Time required	Hazardous reagents
CTAB	1043.51	2.14	< R10	24 hours	Yes
PeqGold Fungal DNA Minikit (PeqLab)	8.32	1.58	R51.20	25 min	Yes
DNeasy Plant Mini Kit (Qiagen)	5.86	1.18	R43.05	35 min	No
ZR Fungal/Bacterial DNA Kit (Zymo Research)	21.94	1.24	R25	20min	No

3.2 Marker polymorphism

It is possible to distinguish between Leaf and Stem rust as the microsatellite markers are unique to each species, some of the Yellow rust markers do cross amplify Leaf Rust (Enjalbert, *et al.*, 2002; Szabo and Kolmer, 2007; Szabo, 2007). Electropherograms and photos of the microsatellite markers used in the differentiation of Leaf, Stem and Yellow rust pathotypes are in **Addendum 3**.

3.2.1 Microsatellites and AFLP

Leaf Rust: Eighteen microsatellite primer pairs were obtained from Szabo and Kolmer (2007). Of the eighteen, eight pairs either did not amplify or were monomorphic across all the pathotypes. The remaining ten of the primers pairs amplified twenty nine alleles (including six null alleles) across the pathotypes. Null alleles were only scored if the allele completely failed to amplify while the other alleles in the same primer set amplified successfully. Genetic distances as calculated in Power Marker are given in **Table 32**.

Table 32: Table of genetic distances between the pathotypes of Leaf rust

Pathotype									
	UVPrt2	UVPrt3	UVPrt4	UVPrt5	UVPrt8	UVPrt9	UVPrt10	UVPrt13	UVPrt19
UVPrt2	0	0.57293	0.65478	0.57293	0.49108	0.49108	0.49108	0.32739	0.49108
UVPrt3	0.57293	0	0.24554	0.49108	0.40924	0.49108	0.49108	0.32739	0.40924
UVPrt4	0.65478	0.24554	0	0.49108	0.65478	0.57293	0.57293	0.57293	0.65478
UVPrt5	0.57293	0.49108	0.49108	0	0.40924	0.49108	0.57293	0.57293	0.49108
UVPrt8	0.49108	0.40924	0.65478	0.40924	0	0.65478	0.32739	0.16369	0.08185
UVPrt9	0.49108	0.49108	0.57293	0.49108	0.65478	0	0.57293	0.49108	0.65478
UVPrt10	0.49108	0.49108	0.57293	0.57293	0.32739	0.57293	0	0.16369	0.32739
UVPrt13	0.32739	0.32739	0.57293	0.57293	0.16369	0.49108	0.16369	0	0.16369
UVPrt19	0.49108	0.40924	0.65478	0.49108	0.08185	0.65478	0.32739	0.16369	0

A cladogram was constructed based on the genetic distances between the pathotypes (**Figure 4**). The analysis grouped the pathotypes into two major groups with UVPrt 2, UVPrt 13, UVPrt 10, UVPrt 8 and UVPrt 19 together; UVPrt 5, UVPrt 4, UVPrt 3 together and UVPrt 9 alone. It was possible to distinguish between the pathotypes using a subset of the microsatellites, namely PtSSR68, PtSSR151A and PtSSR154 (**Table 33**).

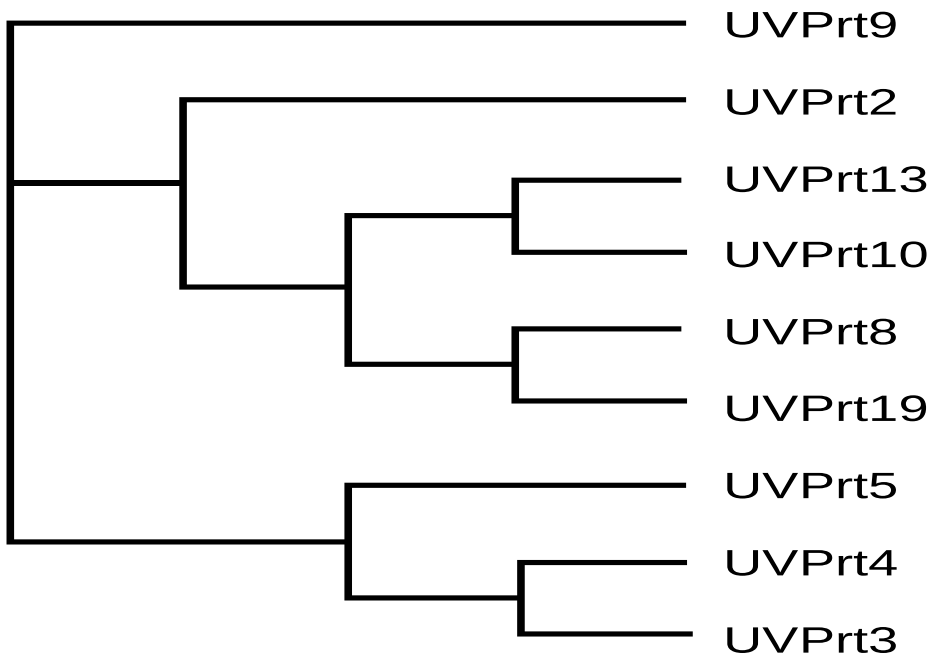

Figure 4: Cladogram of Leaf rust pathotypes based on the Neighbour-Joining tree clustering method

Table 33: The unique haplotype of each Leaf rust pathotype as amplified using a subset of three microsatellites

Leaf Rust Pathotype	Microsatellite		
	PtSSR68	PtSSR151A	PtSSR154
2	1	5	8
3	2	5	8
4	2	5	9
5	1	6	9
8	3	6	8
9	4	5	10
10	3	5	9
13	3	5	8
19	3	7	8

Stem Rust: Twenty four microsatellite markers were obtained from Szabo, (2007) and ten from Zhong *et al.*, (2009). Of the thirty four, twenty four either did not amplify or were monomorphic across all the pathotypes. The remaining ten amplified twenty two alleles (including three null alleles).

Twelve different AFLP primer combinations were used. The twelve combinations yielded 1926 reproducible fragments. A binary data matrix for each AFLP fragment was created in Excel. The microsatellite data was also included in the Excel data matrix, but not in binary format. The microsatellite data was in fragment size format. The Excel file was exported to Power Marker and the data was analysed as outlined in section **2.11**. The microsatellite and AFLP data was combined to generate a table of genetic distances (see **Table 34**).

A cladogram was constructed based on the genetic distances between the pathotypes (see **Figure 5**). There were two main groups, UVPgt50, UVPgt52, UVPgt54, UVPgt56 and UVPgt57 in one group, UVPgt51 and UVPgt53 in a second and UVPgt 55 alone. It was not possible to distinguish between the pathotypes using only the microsatellite markers as there was insufficient allelic diversity found.

Yellow rust: Twelve microsatellite primer pairs were obtained from Enjalbert *et al.* (2002). Of the twelve, nine did not amplify or were monomorphic across all the pathotypes. The other three primers (RJ3, RJ22 and RJ27) amplified nine alleles (including three null alleles) across the pathotypes. It was possible to differentiate between the pathotypes using only two markers, RJ22 and RJ27 (**Table 35**).

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Table 34: Table of genetic distances between the pathotypes of Stem rust

Pathotype								
	UVPgt50	UVPgt51	UVPgt52	UVPgt53	UVPgt54	UVPgt55	UVPgt56	UVPgt57
UVPgt50	0	0.40665	0.27684	0.36338	0.29313	0.45830	0.40200	0.28056
UVPgt51	0.40665	0	0.42759	0.27033	0.44015	0.32337	0.34989	0.44062
UVPgt52	0.27684	0.42759	0	0.38339	0.19681	0.45877	0.38572	0.21868
UVPgt53	0.36338	0.27033	0.38339	0	0.37734	0.33035	0.39130	0.37688
UVPgt54	0.29313	0.44015	0.19681	0.37734	0	0.45272	0.38246	0.18379
UVPgt55	0.45830	0.32337	0.45877	0.33035	0.45272	0	0.36804	0.43643
UVPgt56	0.40200	0.34989	0.38572	0.39130	0.38246	0.36804	0	0.38944
UVPgt57	0.28056	0.44062	0.21868	0.37688	0.18379	0.43643	0.38944	0

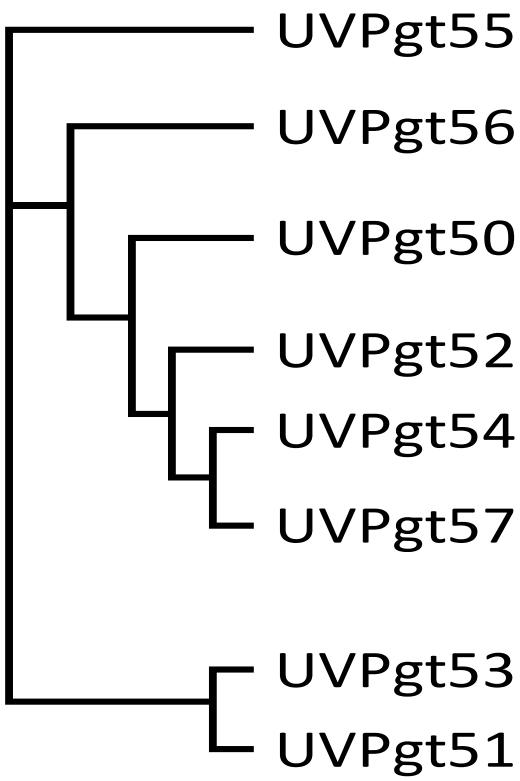

Figure 5: Cladogram of Stem rust pathotypes based on the Neighbour-Joining tree clustering method.

Table 35: The unique haplotype identified for each pathotype of Yellow rust

Yellow Rust Pathotype	Microsatellite			
	RJ22		RJ27	
	Allele 1	Allele 2	Allele 1	Allele 2
6E16A-	1	2	4	6
6E22A-	1	2	5	6
7E22A-	1	3	5	6

3.3 SCAR markers

Of the 1926 AFLP fragments, 186 fragments were unique. Of these unique fragments, 138 were possible candidates for conversion to SCAR markers as the adjacent fragments were more than five basepairs distant. Fourteen were successfully cut out of polyacrylamide gels and re-amplified. Direct sequencing of the bands failed due to the high levels of background. Eight bands (see **Addendum 4**), one band per pathotype, were selected and cloned into the pGEM® T-Easy Vector System I. The insert sizes were verified, and it was found that there were two different sized bands for UVPgt52 and UVPgt56. All the inserts were sequenced

and ten sequences were obtained. Primers were designed for each sequence and tested against all the Stem rust pathotypes. Only one primer, UVPgt52/5/264.2 amplified a single pathotype, UVPgt52. UVPgt52/5/264 amplified four pathotypes, UVPgt50, UVPgt52, UVPgt 54 and UVPgt 57 (see A**ddendum 4**). All the other primers amplified all the Stem rust pathotypes.

3.4 Sequencing of the ITS1 region

The sequences of all the pathotypes were too similar to allow unique primers to be designed for each pathotype. It was possible to distinguish Stem- and Leaf- from Yellow rust, but not from one another. The sequence alignments are shown in **Addendum 5**.

3.5 Field Isolates

Ninety one field isolates were collected from a total of eleven sites, five sites in the Overberg and six sites in the Swartland from February 2008 until November 2009. Fifty six were Leaf rust, thirty seven were Stem rust and three were Yellow rust. Some isolates were infected by a combination of rust species and /or pathotypes. The distribution of the pathotypes across the sites is shown in **Figure 6**.

3.5.1 Leaf rust isolates

The Leaf rust isolates were typed using the PtSSR68, -151A and -154 microsatellite primers. Two novel alleles and three existing alleles were amplified with PtSSR68. Three novel alleles and three existing alleles were amplified by PtSSR154. These novel alleles do however, fall within the size ranges found by Szabo and Kolmer (2007). Several isolates were infected by more than one pathotype. Due to the novel alleles and the multiple pathotypes present in each sample it was not possible to assign a specific pathotype to some field isolates.

Table 36: Leaf rust isolates by pathotype

Leaf rust pathotype	Number
UVPrt8	1
UVPrt9	4
UVPrt10	27
UVPrt13	23
Unknown	52

Leaf rust was found at all eleven of the localities (**Table 37**).

Table 37: Leaf rust pathotypes by locality

Locality	Pathotype				
	UVPrt8	UVPrt9	UVPrt10	UVPrt13	Unknown
Albertenia	0	0	4	5	3
Klipheuwel	0	1	2	2	3
Langgewens	0	0	2	0	2
Mariendal	0	0	0	0	1
Napier	0	0	3	1	11
Piketberg	1	1	0	0	3
Riversdal	0	0	2	4	4
Roodebloem	0	0	1	1	4
Tygerhoek	0	0	5	2	10
Vredenburg	0	0	1	1	1
Wellgevallen	0	2	7	7	10

3.5.2 Stem rust field isolates

Stem rust isolates were typed using the UVPgt52/5/264.2, UVPgt52/5/264.1 and UVPgt55/8/214 primers. These primers allowed the differentiation of the isolates into three groups: UVPgt52; UVPgt51, UVPgt 53, UVPgt 55 and UVPgt 56; and UV50, UVPgt 54 and UVPgt 57. Several isolates were infected by more than one pathotype.

Table 38: Stem rust isolates by pathotype

Stem rust pathotype	Number
UVPgt52	23
UVPgt51, -53, -55, -56	3
UVPgt50, -54, -57	11

Stem rust was found at seven of the eleven localities (**Table 39**).

Table 39: Stem rust pathotypes by locality

Locality	Pathotype		
	UVPgt52	UVPgt51, -53, -55, -56	UVPgt50, -54, -57
Langgewens	1	0	0
Napier	20	1	6
Riversdal	1	1	1
Roodebloem	0	0	1
Tygerhoek	0	1	2
Vredenburg	1	0	0
Wellgevallen	0	0	1

3.5.3 Yellow rust field isolates

Yellow rust field isolates were typed using the RJ22 and RJ27 primers. One novel alleles was amplified by the RJ27 primer. This novel allele does however, fall within the size range found by Enjalbert *et al.* (2002). Several isolates were infected by more than one pathotype. Due to the novel allele and the multiple pathotypes present in each sample it was not possible to assign a specific pathotype to some field isolates.

Table 40: Yellow rust isolates by pathotype

Yellow rust pathotype	Number
6E16A+	2
Unknown	1

Yellow rust was found at three of the eleven localities (**Table 41**).

Table 41: Yellow rust pathotypes by locality

Locality	Pathotype	
	6E16A+	Unknown
Napier	1	0
Tygerhoek	0	1
Wellgevallen	1	0

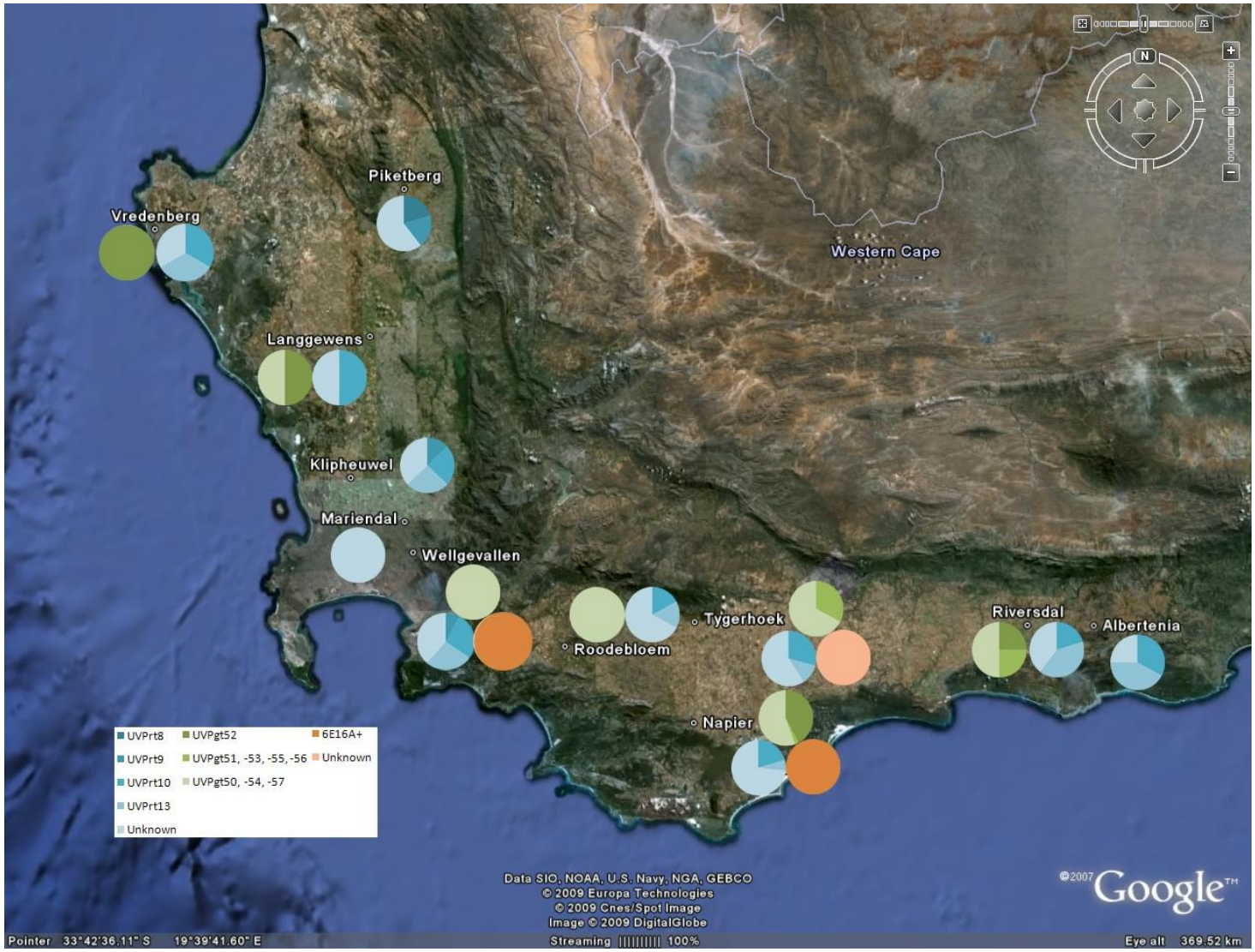

Figure 6: Map of the Western Cape showing the distribution of rust pathotypes found during this study

4. Discussion

4.1 DNA extractions

The yield and purity of the DNA isolated using the commercial kits was less than ideal, with only the Zymo kit extracting more than the 10 ng/µl required for the PCR amplifications. The Qiagen and Zymo kits do not contain hazardous reagents allowing the kit to be used outside of a fumehood whereas the CTAB protocol (Liu and Kolmer, 1998a) uses chloroform and the PeqLab kit uses 2-mecaptoethanol and the extractions must be performed in a fumehood. The commercial kits required much less time per sample. When the higher cost per sample (between 2 and 5 times more expensive per sample than the CTAB method) is taken into account it is not possible to justify the use of the commercial kits over the conventional CTAB extraction protocol even though the commercial kits require significantly less time per sample and, in the case of the Qiagen and Zymo kits, do not require the use of a fumehood.

The PeqLab kit was the only kit which did not contain all the required reagents, 2-mercaptoethanol, Isopropyl alcohol, ethanol and RNaseA have to be supplied by the user.

The DNA extracted using the Zymo kit was of sufficient quantity and quality to allow PCRs. It is therefore possible that this kit could be used in a high throughput environment where the cost disadvantages are outweighed by the reduced time required or where a fumehood is not available. The poor performance of the commercial kits could possibly be because they are designed for plant DNA extractions, with the exception of the Zymo kit which is designed for use with fungi; rather than extractions from spores, which are far more robust than plant cells.

4.2 Selection of a marker system

Initially it was planned to use RAPD markers in this study, however these markers are far from ideal, they are not co-dominant and they are difficult to reproduce. With the publication of the microsatellite markers for Leaf, Stem and Yellow rust it was decided to rather use these as they satisfy far more of the conditions outlined in **1.4**. Microsatellites are co-dominant i.e. it is possible to distinguish homozygotes from heterozygotes, and they are reproducible.

Furthermore they are very simple to use and can be used to rapidly type large numbers of samples, if fluorescently labelled primers are used in combination with an automated sequencer. It was only with the discovery that there was insufficient diversity in the Stem rust microsatellite markers that a new approach had to be taken. AFLP markers were chosen as even though they are not co-dominant they are very reproducible, allowing the absence of a band to be reliably scored (Brown 1996). The typing of field isolates where there is a combination of DNA from two or more species is however not possible using AFLP markers on their own as the process used to amplify the markers will amplify fragments of all the DNA present, not just that of a single species. Therefore the conversion to of certain AFLP bands to SCAR markers is necessary. This conversion also avoids the problems associated with fragments bands that are identical in size but not in sequence. However as SCAR markers are only representative of the presence of a specific sequence unique to a pathotype, i.e. an allele, it is then not possible to determine genotype frequencies (Brown, 1996, McCartney, et al., 2003).

4.3 Marker polymorphism

4.3.1 Genetic Diversity in Leaf Rust pathotypes

The diversity in the alleles present at each locus in the index samples (twenty nine alleles across all loci) is sufficient to allow each pathotype to be uniquely identified using a subset of three primer pairs namely: PtSSR68, PtSSR151A and PtSSR154 (see **Table 33**). The use of the marker subset will allow rapid typing of field samples.

Table 33: The unique haplotype of each Leaf rust pathotype as amplified using a subset of three microsatellites

Leaf Rust Pathotype	Microsatellite PtSSR68	PtSSR151A	PtSSR154
2	1	5	8
3	2	5	8
4	2	5	9
5	1	6	9
8	3	6	8
9	4	5	10
10	3	5	9
13	3	5	8
19	3	7	8

The cladogram of the Leaf rust pathotypes based on the microsatellite marker data (see **Figure 4**) showed two major groupings: UVPrt9 alone; UVPrt2, UVPrt13, UVPrt10, UVPrt8 and UVPrt19; and UVPrt5, UVPrt4 and UVPrt3.

No suitable outgroup could be indentified for comparison but the positioning of UVPrt9 in a separate group to the other pathotypes would seem to imply that it was introduced into South Africa relatively recently, whereas the other two groups appear to have been present in South Africa for some time, allowing differentiation to occur. This study is this first molecular marker based study of South African Leaf rust. Previous studies (Szabo and Kolmer, 2007) of other collections of Leaf rust, using the same markers have found more diversity at each of the loci tested. Szabo and Kolmer (2007) found about four alleles per locus where as this study found about two alleles per locus. There are two possible causes for these discrepancies. It may be due to 1) the limited number of samples tested initially to characterise each pathotype, only one sample per pathotype, or 2) because South African Leaf rust has not developed as much genetic diversity. Szabo and Kolmer (2007) do not state if the samples they tested are only from the United States or if they are from an international collection. An international collection would tend to be much more diverse as the rusts in each country or region are subject to very different selection pressures to rusts from another continent. Therefore, if the samples are from an international collection it could explain the differences observed between the results of this study and that of Szabo and Kolmer (2007).

4.3.2 Genetic Diversity in Stem Rust

Twenty four of the tested microsatellite markers either did not amplify or were monomorphic across all the tested pathotypes. The other ten microsatellite markers amplified twenty two alleles across the pathotypes. Due to the lack of diversity found in the microsatellite markers tested, it was not possible to distinguish between the South African Stem rust pathotypes. This finding replicates that of Visser *et al.* (2009), who were also unable to detect significant differences between the South African pathotypes, except that Visser *et al.* (2009) were able to distinguish UVPgt55. The studies by Szabo (2007) and Zhong *et al.*, (2009) found much greater diversity in these same markers, but, as with the study of Leaf rust by Szabo and Kolmer (2007) it is not specified if the samples tested are from a national or international collection. Again, if the samples are from an international collection, rather than a national or regional collection, it could explain why Szabo (2007) found so much more diversity.

AFLP markers were used, and were found to be highly diverse. This finding again replicates that of Visser *et al.* (2009) who also used AFLP markers. There was sufficient diversity in the AFLP markers to distinguish each pathotype. This study found significantly more AFLP fragments than Visser *et al.* (2009) but this can be attributed to the use of fluorescently labelled primers and the analysis using the automated sequencer, which is much more sensitive than the analysis of polyacrylamide gels.

The cladogram of the Stem rust pathotypes based on AFLP and microsatellite data showed two major groups: UVPgt55 alone; UVPgt56, UVPgt50, UVPgt52, UVPgt57 and UVPgt54; and UVPgt53 and UVPgt51 (see **Figure 5**).

Pretorius *et al.* (2007) created a putative Stem rust phylogeny based on the avirulence/virulence composition of the South African Stem rust pathotypes. Broadly, it assumed a common ancestor for all South African Stem rust pathotypes and divided them in to two groups: UVPgt53, UVPgt54 and UVPgt56; and UVPgt51, UVPgt50, UVPgt52 and UVPgt55. However, the results of Visser *et al.* (2009), and those of this study support the conclusion that UVPgt55 is an introduction into South Africa rather than a descendant of a South African pathotype. Visser *et al.* (2009) did not test all the pathotypes used in this study, and excluded UVPgt51 and UVPgt54, such that not all the results can be compared directly. But their phylogenic analysis did however group some isolates of UVPgt53 separately from the other South African Stem rust pathotypes, a result which was also verified by this study.

4.3.3 Genetic Diversity in Yellow Rust

A study by Enjalbert *et al.,* (2002), of ninety six yellow rust isolates from France and China found low levels of diversity at the loci tested. As Yellow rust has only been recorded in South Africa since 1996 (Pretorius, *et al.*, 1997), the expectation was that South African Yellow rust would be even less diverse as it has not had sufficient time to diversify to the same extent as the Yellow rust found internationally.

There was sufficient polymorphism in the loci tested (nine alleles across all pathotypes) to allow the pathotypes to be differentiated.

This study found two alleles per locus at the RJ3, RJ17 and RJ21 loci similar to what was found by Enjalbert *et al.*, (2002). The RJ20, RJ21, RJ22, RJ24 and RJ27 loci all had fewer alleles than were found by Enjalbert *et al.*, (2002). Seven novel alleles were found, two at the RJ3 locus and one at each of the RJ17, RJ20, RJ22, RJ24 and RJ27 loci. Of the sixteen alleles found by this study, seven have not been identified previously. Enjalbert *et al.*, (2002),

tested the 6E16 pathotype but did not give the haplotypes for the pathotypes they tested, so it is not possible to compare the South African 6E16 pathotype to the French 6E16 pathotype.

The South African pathotypes are less diverse than the international samples tested by Enjalbert *et al.*, (2002), but this was expected due to the lack of time for differentiation to occur. The novel alleles would seem to indicate that the South African Yellow rust pathotypes did not originate in France or China but it is not possible to say this categorically as Enjalbert *et al.*, (2002), do not give the haplotypes of the pathotypes they tested.

Two primers, RJ22 and RJ27, (see **Table 35**) are required to distinguish between the three Yellow rust pathotypes. Phylogenetic analysis of the pathotypes was omitted as there are too few pathotypes for meaningful results.

Table 35: The unique haplotype identified for each pathotype of Yellow Rust

Yellow Rust Pathotype	Microsatellite			
	RJ22		RJ27	
	Allele 1	Allele 2	Allele 1	Allele 2
6E16A-	1	2	4	6
6E22A-	1	2	5	6
7E22A-	1	3	5	6

4.3.4 SCAR markers in Stem rust

Insufficient diversity was found in the microsatellite markers to distinguish between the pathotypes, and therefore another technique was needed. AFLP markers have been shown to be diverse enough to allow pathotype differentiation by Visser *et al.* (2009). However, the typing of samples that contain a mixture of DNA from multiple species is not possible as the process of amplifying AFLP markers is nonspecific. AFLP markers will only work if pure samples are used. Therefore it was necessary to convert AFLP fragments unique to each pathotype to SCAR markers. Fourteen different fragments were isolated from the eight pathotypes and cloned and sequenced. A short coming of AFLP markers is that fragments that differ in size may contain common sequences. Therefore an AFLP marker that appears to be unique to a pathotype may, in fact, not be unique. Thus, when the AFLP marker is converted to a SCAR marker and amplified; it may amplify more than one pathotype as happened with this study.

It was not possible to multiplex the PCR of the SCAR markers due to the differences in annealing temperature, but it was possible to combine the reaction products of several

reactions and run them on a single gel. In this way, it is possible to discern if a sample contains, for instance, UVPgt52 and one of UVPgt51, UVPgt53, UVPgt55 or UVPgt56. If a SCAR marker is developed for each pathotype, including those of Leaf and Yellow rust, it would allow rapid, inexpensive diagnostic testing of field isolates.

4.4 Sequencing

The lack of polymorphism in the sequence data (99.05% homology between the sequences) is insufficient to allow unique primers to be designed for each pathotype (see **Addendum 5**). This means that it will unfortunately be impossible to conduct quantification analyses using RT-PCR. It was possible to distinguish Yellow rust from Leaf and Stem rust, but it was not possible to distinguish between Leaf and Stem rust.

The inability to distinguish between pathotypes of the same species was not completely unexpected as previous studies of the same region had indicated that while it was possible to distinguish between the rust species, it was sometimes not possible to distinguish smaller differences such as those between pathotypes (Zambino and Szabo, 1993; Barnes and Szabo, 2007). However the lack of differences between Leaf and Stem rust is surprising as these species are very different in both physiology and pathology.

4.5 Field Isolates

Ninety one field isolates were collected from eleven locations in the Swartland and Overberg regions of the Western Cape (see **Figure 6**). Many samples were infected by more than one rust species and most were infected by several pathotypes. Considerably more diversity was found in the microsatellite markers used to distinguish the Leaf and Yellow rust field isolates than had been found in the pathotype samples used initially. These alleles found are novel, in a South African context, but have already been identified by Szabo and Kolmer (2007), Zhong, *et al.*, (2009) and Enjalbert *et al.* (2002) who developed the markers. These novel alleles were found in most of the field isolates, and together with the presence of multiple pathotypes on a single sample proved a confounding factor, made it impossible to definitively

assign a specific pathotype to each field isolate. This reiterates that molecular marker based testing is not sufficient on its own but should rather be used in combination with conventional techniques such as differential testing.

The presence of the novel alleles and the multiple pathotypes present in most of the isolates prevented the determination of genotype frequencies. This unfortunately precludes the estimation of genetic diversity and the use of goodness of fit calculations such as testing for Hardy-Weinberg equilibrium, that require both allele and genotype frequencies to be known. The Hardy-Weinberg test establishes whether a population is in equilibrium or not. This is done by comparing the observed genotype frequencies to the expected genotype frequencies, which are calculated from the observed allele frequencies. If the observed genotype frequencies differ significantly to the expected genotype frequencies then the population is not in equilibrium and is subject to a disturbing force such as selection. In a species like the *Puccinia spp.* that is subject to artificial control methods, such as the use of fungicides, it would be expected that the population would not be in Hardy-Weinberg equilibrium, it would be undergoing selection as a result of the use of fungicides and disease resistant crops.

One of the requirements of a marker used in a population genetic study is that the marker must not be undergoing selection; it must be in equilibrium (Brown 1996). The inability of this study to test for equilibrium at any of the loci used precludes the further use of these markers in such a study until it is possible to establish whether or not the markers are in equilibrium. The markers can still be used diagnostically to determine which pathotypes are present in a sample. To this end, work must be done to determine if the novel alleles represent existing or novel pathotypes.

4.5.1 Leaf rust field isolates

The most prevalent identifiable Leaf rust pathotype found was UVPrt10, followed by UVPrt13 (see **Table 36**). This contrasts with a 2007 survey (Terefe, *et al.*, 2009) conducted by the Small Grain Institute of the Agricultural Research Council (SGI-ARC) which found that UVPrt9 was the most prevalent (76.8%) followed by UVPrt10 (11.0%) and UVPrt13 (7.3%). The survey by Terefe *et al.* (2007) concentrated on rust on wheat cultivars, whereas this survey included both wheat and triticale cultivars, which can explain most of the differences. It is also possible that some of the field isolates that were not assignable to a

specific pathotype may in fact be UVPrt9. In other words UVPrt9 may have more than one haplotype. This may also be true of the other pathotypes.

Leaf rust was found at all eleven of the localities (see **Figure 6**). This distribution is not unexpected as previous surveys have found it widely distributed throughout the Western Cape (Pretorius, *et al.*, 2007). There does not appear to be a pattern to the distribution of the pathotypes with UVPgt10 and UVPgt13 present at almost all of the sites. UVPgt9 was only found in the Swartland and at Wellgevallen, though it is not possible to determine whether this is a true representation of its distribution or merely the inability of this study to assign a specific pathotype to all the Leaf rust haplotypes. Two other factors may have contributed to the even distribution of the Leaf rust pathotypes are: the effective wheat monoculture in the Western Cape, most farmers grow identical or closely related cultivars and the small geographical area with strong prevailing winds that aid the spread of rust spores over the entire area. Sampling rust from areas outside of the Western Cape where different cultivars are grown and climatic differences are more pronounced may produce different results.

The most prevalent pathotypes, UVPgt9, UVPgt10 and UVPgt13, are all avirulent to *Lr3bg* and *Lr16* (see **Table 2B** for a more complete list of virulence and avirulence genes) therefore new cultivars introduced should carry at least one, preferably both of *Lr3bg* and *Lr16*. New cultivars should avoid the genes to which UVPgt9, UVPgt10 and UVPgt13 are all virulent, genes such as *Lr1*, *Lr10* and *Lr14a*.

4.5.2 Stem rust field isolates

UVPgt52 was the most commonly identified isolate followed by the group of UVPgt50, UVPgt54, and UVPgt57 (see **Table 38**). This again contrasts with SGI-ARC findings of 2004 (Pretorius, *et al.*, 2007) which found that UVPgt55 was the most prevalent, followed by UVPgt53 and then UVPgt52. However, the SGI-ARC survey concentrated on wheat cultivars, whereas this survey included both wheat and triticale cultivars. Furthermore five years have elapsed since those findings were made and it is possible that the prevalence of specific pathotypes can have changed in the intervening years. Stem rust was found at seven of the eleven localities (see **Figure 6**), not entirely unexpectedly as previous studies have found it widely distributed throughout the Western Cape (Pretorius, *et al.*, 2007). The distribution of Stem rust does not appear to follow a pattern, as with that of Leaf rust. The inability of this study to identify each specific pathotype rather than groups will have contributed to the lack

of observable differences between the sampling sites. Also the monoculture, small area and strong winds will have helped to even the distribution of Stem rust across the Western Cape.

The most prevalent pathotypes, UVPgt50, UVPgt52, UVPgt54 and UVPgt57, are all avirulent to *Sr8b*, *Sr21*, *Sr31*, *Sr38* and *SrEm* (see **Table 2A**). Therefore breeding programmes should concentrate on lines that have contain as many of those genes as possible. The breeding programmes should avoid *Sr8a* as UVPgt50, UVPgt52, UVPgt54 and UVPgt57 are all virulent to it.

4.5.3 Yellow rust field isolates

One novel allele was found for the RJ27 marker, this allele has previously been identified by Enjalbert *et al.*, (2002). Only 6E16A- was identifiable in the Yellow rust isolates, and so no conclusions can be drawn about its prevalence relative to other pathotypes. No 7E22A- was found, as was expected as this pathotype is confined to Lesotho where susceptible cultivars are grown (Pretorius, *et al.*, 2007). Therefore it must be assumed, as with Leaf rust, that the pathotypes used initially are not representative of the diversity found in South African Yellow rust. Therefore work must be done to assign the novel alleles to specific pathotypes.

Yellow rust was confined to just three of the twelve localities, those of the central Southern Cape. Yellow rust has a smaller range of optimal growth conditions than those of the other rust species and the central region of the Southern Cape was the only region where these conditions are found during the sampling period. With only 6E16A- identifiable, it is not possible to identify any pattern in the distribution of the Yellow rust across the sampling sites.

4.6 Resistance breeding programmes

Resistance breeding programmes must concentrate on developing wheat cultivars that combine resistance to the most prevalent pathotypes found by this study, namely UVPrt10, UVPrt13, UVPgt50, UVPgt52, UVPgt54, UVPgt57 and 6E16A-. Quantitative resistance should be preferred over single major resistance genes as these will rapidly be overcome due to the excessive selective pressure place on the population. More emphasis must also be placed on physical control methods, such as crop rotation, so that the rust population size is reduced as much as possible without the use of fungicides or other chemical controls. Alternating different cultivars year by year may also be effective as the rusts will not have as long to develop virulence to the genes present in the cultivars. By combining resistant

cultivars and physical control methods it should be possible to reduce the yield losses caused by the *Puccinia spp.*

5. Conclusions and Future Work

This study has established that it is possible to conduct molecular marker based surveys of rust pathotype prevalence and distribution. It established that if time is not a constraint then the use of conventional DNA extraction techniques should be favoured over and above those of commercial kits.

The lack of diversity in the tested markers was initially surprising, but only when it is considered in a South African context. The relatively small scale production of wheat and the lack of diversity in commercial cereals in South Africa have not driven the diversification of the rust species to the same extent as in the United States where a much larger area is cultivated and more cultivars are used.

The differences observed between the expected and observed prevalence of certain pathotypes are interest provoking. However, most of the differences can be explained by the different focus of this survey when compared to previous surveys that focused on wheat. It is also possible that the pathotypes used initially are not completely representative of the diversity within their phenotype.

The lack of diversity found in the rust population is probably due to a combination of small population size, small sample size, the lack of a host for sexual reproduction and the strong selective pressure created by artificial control methods. The strong selective pressure makes it highly probable that new rust pathotypes will arise, if new major resistance genes are introduced.

Resistance breeding programmes should concentrate on introducing cultivars that have quantitative resistance, where the plant exhibits low to intermediate levels of infection across all the pathotypes of a pathogen, as this resistance is not broken easily or quickly. The use of these cultivars in conjunction with carefully planned policies of physical control should reduce the yield losses caused by rust.

This study should perhaps been seen as a pilot study for future work. This will entail the differential testing of Leaf and Yellow rust isolates with novel alleles to establish if these represent new pathotypes and if they are not, to which species they can be assigned. Further work should be done to create pathotype specific SCAR markers for the Stem rust pathotypes to allow unambiguous differentiation of pathotypes. The development of SCAR markers for Leaf and Yellow rust should also be done. This will allow rapid, cheap diagnostic typing of

isolates. Each of the markers identified must be confirmed with differential testing to ensure that the genotype identified is indeed linked to the correct phenotype. More isolates should be collected from a wider area to ensure that the full spectrum of rust diversity is captured.

The completion of this future work will allow a comprehensive study of the *Puccinia spp.* in South Africa to be done.

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Addendum 1: Avirulence-virulence composition of South African rust pathotypes

Stem Rust Pathotypes

	UVPgt50 (2SA4)	UVPgt51 (2SA36)	UVPgt52 (2SA100)	UVPgt53 (2SA102)	UVPgt54 (2SA55)	UVPgt55 (2SA88)	UVPgt56 (2SA102K)	UVPgt57[a] (2SA105)	UVPgt58	UVPgt59	2SA2	2SA4	2SA6	2SA10	2SA18	2SA20	2SA32	2SA33	2SA36	2SA39	2SA43	2SA45	2SA48	2SA49	2SA51	2SA52	2SA53	2SA54	2SA103	Ug99[b]
Avirulence genes				*Sr5*	*Sr5*		*Sr5*	*Sr5*	*Sr5*		*Sr5*						*Sr5*			*Sr5*									*Sr5*	
				Sr6	*Sr6*		*Sr6*	*Sr6*	*Sr6*		*Sr6*			*Sr6*			*Sr6*												*Sr6*	
				Sr7b	*Sr7b*		*Sr7b*	*Sr7b*	*Sr7b*					*Sr7b*	*Sr7b*		*Sr7b*	*Sr7b*											*Sr7b*	
	Sr8b	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*		*Sr8b*	*Sr8b*	*Sr8b*		*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	*Sr8b*	
				Sr9b	*Sr9b*			*Sr9b*	*Sr9b*		*Sr9b*			*Sr9b*	*Sr9b*		*Sr9b*			*Sr9b*					*Sr9b*				*Sr9b*	
		Sr9e	*Sr9e*	*Sr9e*	*Sr9e*		*Sr9e*	*Sr9e*	*Sr9e*		*Sr9e*		*Sr9e*	*Sr9e*	*Sr9e*	*Sr9e*	*Sr9e*	*Sr9e*	*Sr9e*	*Sr9e*		*Sr9e*	*Sr9e*	*Sr9e*	*Sr9e*	*Sr9e*		*Sr9e*	*Sr9e*	
	Sr9g	*Sr9g*	*Sr9g*		*Sr9g*				*Sr9g*			*Sr9g*					*Sr9g*	*Sr9g*	*Sr9g*			*Sr9g*	*Sr9g*	*Sr9g*	*Sr9g*			*Sr9g*	*Sr9g*	
				Sr11																									*Sr11*	
	Sr13	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*				*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	*Sr13*	
	Sr15	*Sr15*	*Sr15*	*Sr15*	*Sr15*	*Sr15*				*Sr15*																			*Sr15*	
				Sr17			*Sr17*	*Sr17*	*Sr17*		*Sr17*			*Sr17*			*Sr17*	*Sr17*		*Sr17*					*Sr17*				*Sr17*	
	Sr21	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*	*Sr21*
	Sr22	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*				*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*	*Sr22*
		Sr23		*Sr23*	*Sr23*																								*Sr23*	
	Sr24	*Sr24*		*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*		*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	*Sr24*	
	Sr25	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*				*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*	*Sr25*
	Sr26	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*				*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*	*Sr26*
	Sr27	*Sr27*	*Sr27*		*Sr27*	*Sr27*				*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*	*Sr27*		*Sr27*
	Sr29	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*				*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*	*Sr29*
		Sr30	*Sr30*	*Sr30*	*Sr30*		*Sr30*	*Sr30*	*Sr30*		*Sr30*			*Sr30*	*Sr30*	*Sr30*	*Sr30*	*Sr30*	*Sr30*	*Sr30*			*Sr30*		*Sr30*	*Sr30*				
	Sr31	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	*Sr31*	
	Sr32	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*				*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*	*Sr32*
	Sr33	*Sr33*	*Sr33*	*Sr33*	*Sr33*	*Sr33*				*Sr33*																			*Sr33*	*Sr33*
	Sr35	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*				*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*	*Sr35*
	Sr36		*Sr36*	*Sr36*		*Sr36*	*Sr36*	*Sr36*	*Sr36*	*Sr36*	*Sr36*	*Sr36*	*Sr36*	*Sr36*	*Sr36*		*Sr36*			*Sr36*	*Sr36*	*Sr36*	*Sr36*		*Sr36*	*Sr36*		*Sr36*	*Sr36*	*Sr36*
		Sr37	*Sr37*	*Sr37*																									*Sr37*	
	Sr38	*Sr38*	*Sr38*	*Sr38*	*Sr38*		*Sr38*	*Sr38*																					*Sr38*	
	Sr39	*Sr39*	*Sr39*	*Sr39*	*Sr39*	*Sr39*				*Sr39*																			*Sr39*	*Sr39*
																														Sr40
																														Sr42
	Sr43	*Sr43*	*Sr43*	*Sr43*	*Sr43*	*Sr43*				*Sr43*																			*Sr43*	*Sr43*
		SrAgi	*SrAgi*			*SrAgi*				*SrAgi*																				*SrAgi*
		Srdp2	*Srdp2*								*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*	*Srdp2*		
	SrEm	*SrEm*	*SrEm*	*SrEm*	*SrEm*	*SrEm*	*SrEm*	*SrEm*		*SrEm*																			*SrEm*	*SrEm*
		SrGt	*SrGt*	*SrGt*	*SrGt*																								*SrGt*	
	SrKiewiet		*SrKiewiet*	*SrKiewiet*		*SrKiewiet*			*SrKiewiet*	*SrKiewiet*																				
	SrSatu		*SrSatu*	*SrSatu*		*SrSatu*	*SrSatu*		*SrSatu*	*SrSatu*																				
							SrTobie																							
											SrTt3	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*	*SrTt3*		
Virulence genes	*Sr5*	*Sr5*	*Sr5*			*Sr5*				*Sr5*		*Sr5*	*Sr5*	*Sr5*	*Sr5*	*Sr5*		*Sr5*	*Sr5*		*Sr5*	*Sr5*	*Sr5*	*Sr5*	*Sr5*	*Sr5*	*Sr5*		*Sr5*	
	Sr6	*Sr6*	*Sr6*			*Sr6*				*Sr6*		*Sr6*	*Sr6*		*Sr6*	*Sr6*		*Sr6*	*Sr6*	*Sr6*	*Sr6*	*Sr6*	*Sr6*	*Sr6*	*Sr6*	*Sr6*	*Sr6*		*Sr6*	
	Sr7a	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*				*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	*Sr7a*	
	Sr7b	*Sr7b*	*Sr7b*	*Sr7b*		*Sr7b*				*Sr7b*	*Sr7b*	*Sr7b*	*Sr7b*			*Sr7b*			*Sr7b*	*Sr7b*	*Sr7b*	*Sr7b*	*Sr7b*	*Sr7b*	*Sr7b*	*Sr7b*	*Sr7b*		*Sr7b*	*Sr7b*
	Sr8a	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*	*Sr8a*
				Sr8b						*Sr8b*																				*Sr8b*
	Sr9a	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*				*Sr9a*		*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	*Sr9a*	
	Sr9b	*Sr9b*	*Sr9b*			*Sr9b*				*Sr9b*		*Sr9b*	*Sr9b*			*Sr9b*		*Sr9b*	*Sr9b*		*Sr9b*	*Sr9b*	*Sr9b*	*Sr9b*		*Sr9b*	*Sr9b*	*Sr9b*		*Sr9b*
	Sr9d	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*				*Sr9d*		*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	*Sr9d*	
	Sr9e					*Sr9e*				*Sr9e*		*Sr9e*									*Sr9e*						*Sr9e*			*Sr9e*
	Sr9f	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*				*Sr9f*		*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	*Sr9f*	
				Sr9g		*Sr9g*	*Sr9g*	*Sr9g*		*Sr9g*	*Sr9g*		*Sr9g*	*Sr9g*	*Sr9g*	*Sr9g*				*Sr9g*	*Sr9g*			*Sr9g*	*Sr9g*					*Sr9g*
	Sr10	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*				*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	*Sr10*	
	Sr11	*Sr11*	*Sr11*		*Sr11*	*Sr11*				*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*	*Sr11*		*Sr11*
	Sr12	*Sr12*	*Sr12*	*Sr12*	*Sr12*	*Sr12*				*Sr12*																			*Sr12*	
	Sr14	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*				*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	*Sr14*	
																														Sr15
	Sr16	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*				*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	*Sr16*	
	Sr17	*Sr17*	*Sr17*			*Sr17*				*Sr17*		*Sr17*	*Sr17*		*Sr17*	*Sr17*			*Sr17*		*Sr17*	*Sr17*	*Sr17*	*Sr17*		*Sr17*	*Sr17*	*Sr17*		*Sr17*
	Sr18		*Sr18*								*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*	*Sr18*		
	Sr19	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*				*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	*Sr19*	
	Sr20	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*				*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	*Sr20*	
	Sr23		*Sr23*			*Sr23*				*Sr23*																				*Sr24*
			Sr24							*Sr24*																				
				Sr27			*Sr27*	*Sr27*	*Sr27*																				*Sr27*	
	Sr28	*Sr28*	*Sr28*								*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*	*Sr28*		
	Sr30			*Sr30*		*Sr30*				*Sr30*		*Sr30*	*Sr30*								*Sr30*	*Sr30*		*Sr30*			*Sr30*	*Sr30*	*Sr30*	*Sr30*
																														Sr31
	Sr34	*Sr34*	*Sr34*	*Sr34*	*Sr34*	*Sr34*				*Sr34*																			*Sr34*	
		Sr36														*Sr36*		*Sr36*	*Sr36*					*Sr36*			*Sr36*			
	Sr37																													
						Sr38				*Sr38*																				*Sr38*
	SrAgi			*SrAgi*	*SrAgi*																								*SrAgi*	
	SrGt							*SrGt*																						
							SrKiewiet	*SrKiewiet*																						
	SrLc	*SrLc*	*SrLc*	*SrLc*	*SrLc*	*SrLc*				*SrLc*																			*SrLc*	
								SrSatu																						
				SrTobie																										

[a] UVPgt57 is virulent to *Sr9b* in a W2691 background, but avirulent to *Sr9b* in a W2402 background which contains *Sr9b and Sr7b* (Personal communication from Prof. ZA Pretorius, University of the Free State)

[b] Ug99 does not occur in South Africa (as of November 2009) and is only included to show its relatedness to UVPgt55.

Leaf rust Pathotypes

	UVPrt2	UVPrt3 (3SA123)	UVPrt4	UVPrt5	UVPrt8 (3SA132)	UVPrt9 (3SA133)	UVPrt10 (3SA126)	UVPrt13 (3SA140)	UVPrt16	UVPrt18	UVPrt19	3SA57	3SA58	3SA60	3SA61	3SA62	3SA63	3SA64	3SA68	3SA73	3SA77	3SA78	3SA79	3SA86	3SA120	3SA121	3SA122	3SA124	3SA125	3SA127	3SA128	3SA129	3SA134	3SA137	3SA141	3SA142	3SA143
Avirulence genes	*Lr1*		*Lr1*	*Lr1*					*Lr1*			*Lr1*			*Lr1*												*Lr1*	*Lr1*	*Lr1*							*Lr1*
	Lr2a		*Lr2a*	*Lr2a*		*Lr2a*			*Lr2a*	*Lr2a*		*Lr2a*		*Lr2a*	*Lr2a*			*Lr2a*			*Lr2a*				*Lr2a*		*Lr2a*	*Lr2a*	*Lr2a*		*Lr2a*				*Lr2a*	*Lr2a*
	Lr2b		*Lr2b*			*Lr2b*			*Lr2b*	*Lr2b*															*Lr2b*		*Lr2b*	*Lr2b*			*Lr2b*				*Lr2b*	*Lr2b*
		Lr3a			*Lr3a*		*Lr3a*	*Lr3a*			*Lr3a*		*Lr3a*			*Lr3a*	*Lr3a*		*Lr3a*	*Lr3a*	*Lr3a*	*Lr3a*	*Lr3a*	*Lr3a*	*Lr3a*	*Lr3a*			*Lr3a*		*Lr3a*	*Lr3a*	*Lr3a*	*Lr3a*	*Lr3a*	
		Lr3bg	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*		*Lr3bg*		*Lr3bg*	*Lr3bg*		*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*		*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*	*Lr3bg*
	Lr3ka	*Lr3ka*			*Lr3ka*		*Lr3ka*	*Lr3ka*	*Lr3ka*		*Lr3ka*	*Lr3ka*	*Lr3ka*		*Lr3ka*	*Lr3ka*	*Lr3ka*		*Lr3ka*	*Lr3ka*	*Lr3ka*	*Lr3ka*	*Lr3ka*	*Lr3ka*	*Lr3ka*	*Lr3ka*	*Lr3ka*			*Lr3ka*		*Lr3ka*	*Lr3ka*	*Lr3ka*	*Lr3ka*	*Lr3ka*	*Lr3ka*
		Lr10		*Lr10*					*Lr10*		*Lr10*															*Lr10*			*Lr10*			*Lr10*	*Lr10*			*Lr10*
	Lr11	*Lr11*	*Lr11*	*Lr11*	*Lr11*		*Lr11*	*Lr11*	*Lr11*		*Lr11*	*Lr11*	*Lr11*		*Lr11*	*Lr11*	*Lr11*		*Lr11*	*Lr11*	*Lr11*	*Lr11*	*Lr11*	*Lr11*	*Lr11*	*Lr11*	*Lr11*	*Lr11*	*Lr11*		*Lr11*	*Lr11*	*Lr11*	*Lr11*	*Lr11*	*Lr11*
		Lr14a		*Lr14a*					*Lr14a*																	*Lr14a*			*Lr14a*			*Lr14a*				*Lr14a*
	Lr15		*Lr15*	*Lr15*		*Lr15*			*Lr15*	*Lr15*		*Lr15*		*Lr15*	*Lr15*			*Lr15*			*Lr15*				*Lr15*		*Lr15*	*Lr15*	*Lr15*		*Lr15*				*Lr15*	*Lr15*	*Lr15*
		Lr16	*Lr16*		*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*		*Lr16*	*Lr16*		*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*		*Lr16*		*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*	*Lr16*
	Lr17	*Lr17*	*Lr17*	*Lr17*		*Lr17*			*Lr17*	*Lr17*		*Lr17*		*Lr17*	*Lr17*	*Lr17*		*Lr17*	*Lr17*	*Lr17*	*Lr17*	*Lr17*	*Lr17*		*Lr17*	*Lr17*	*Lr17*	*Lr17*	*Lr17*		*Lr17*	*Lr17*			*Lr17*	*Lr17*	*Lr17*
	Lr20	*Lr20*			*Lr20*		*Lr20*	*Lr20*	*Lr20*		*Lr20*	*Lr20*	*Lr20*		*Lr20*	*Lr20*	*Lr20*		*Lr20*	*Lr20*	*Lr20*	*Lr20*	*Lr20*	*Lr20*	*Lr20*	*Lr20*	*Lr20*			*Lr20*		*Lr20*	*Lr20*	*Lr20*	*Lr20*	*Lr20*	*Lr20*
	Lr24		*Lr24*	*Lr24*			*Lr24*					*Lr24*			*Lr24*		*Lr24*			*Lr24*	*Lr24*	*Lr24*			*Lr24*	*Lr24*	*Lr24*	*Lr24*	*Lr24*			*Lr24*	*Lr24*	*Lr24*		
	Lr26	*Lr26*	*Lr26*	*Lr26*	*Lr26*	*Lr26*	*Lr26*		*Lr26*	*Lr26*	*Lr26*																					*Lr26*	*Lr26*	*Lr26*	*Lr26*	*Lr26*
	Lr30				*Lr30*		*Lr30*	*Lr30*			*Lr30*	*Lr30*	*Lr30*		*Lr30*	*Lr30*	*Lr30*		*Lr30*	*Lr30*	*Lr30*	*Lr30*	*Lr30*	*Lr30*	*Lr30*	*Lr30*	*Lr30*			*Lr30*		*Lr30*	*Lr30*	*Lr30*			
Virulence genes		*Lr1*			*Lr1*	*Lr1*	*Lr1*	*Lr1*		*Lr1*	*Lr1*		*Lr1*	*Lr1*		*Lr1*	*Lr1*	*Lr1*	*Lr1*	*Lr1*	*Lr1*	*Lr1*	*Lr1*	*Lr1*	*Lr1*	*Lr1*				*Lr1*	*Lr1*	*Lr1*	*Lr1*	*Lr1*	*Lr1*	*Lr1*	
		Lr2a			*Lr2a*		*Lr2a*	*Lr2a*			*Lr2a*		*Lr2a*			*Lr2a*	*Lr2a*		*Lr2a*	*Lr2a*		*Lr2a*	*Lr2a*	*Lr2a*		*Lr2a*				*Lr2a*		*Lr2a*	*Lr2a*	*Lr2a*			
		Lr2b		*Lr2b*	*Lr2b*		*Lr2b*	*Lr2b*			*Lr2b*															*Lr2b*			*Lr2b*	*Lr2b*		*Lr2b*	*Lr2b*	*Lr2b*			
	Lr2c	*Lr2c*	*Lr2c*	*Lr2c*	*Lr2c*	*Lr2c*	*Lr2c*	*Lr2c*	*Lr2c*	*Lr2c*	*Lr2c*																					*Lr2c*	*Lr2c*	*Lr2c*	*Lr2c*	*Lr2c*	*Lr2c*
	Lr3a		*Lr3a*	*Lr3a*		*Lr3a*			*Lr3a*	*Lr3a*		*Lr3a*		*Lr3a*	*Lr3a*			*Lr3a*								*Lr3a*	*Lr3a*	*Lr3a*		*Lr3a*						*Lr3a*
	Lr3bg											*Lr3bg*			*Lr3bg*											*Lr3bg*										
			Lr3ka	*Lr3ka*		*Lr3ka*								*Lr3ka*				*Lr3ka*									*Lr3ka*	*Lr3ka*		*Lr3ka*						
	Lr10		*Lr10*		*Lr10*	*Lr10*	*Lr10*	*Lr10*		*Lr10*															*Lr10*		*Lr10*	*Lr10*		*Lr10*	*Lr10*			*Lr10*	*Lr10*	*Lr10*	
						Lr11				*Lr11*				*Lr11*				*Lr11*												*Lr11*						
	Lr14a		*Lr14a*		*Lr14a*	*Lr14a*	*Lr14a*	*Lr14a*		*Lr14a*	*Lr14a*														*Lr14a*		*Lr14a*	*Lr14a*		*Lr14a*	*Lr14a*		*Lr14a*	*Lr14a*	*Lr14a*	*Lr14a*	
		Lr15			*Lr15*		*Lr15*	*Lr15*			*Lr15*		*Lr15*			*Lr15*	*Lr15*		*Lr15*	*Lr15*		*Lr15*	*Lr15*	*Lr15*		*Lr15*				*Lr15*		*Lr15*	*Lr15*	*Lr15*			
	Lr16			*Lr16*								*Lr16*			*Lr16*												*Lr16*		*Lr16*							
					Lr17		*Lr17*	*Lr17*			*Lr17*		*Lr17*				*Lr17*							*Lr17*						*Lr17*			*Lr17*	*Lr17*			
			Lr20	*Lr20*		*Lr20*				*Lr20*				*Lr20*				*Lr20*										*Lr20*	*Lr20*		*Lr20*					
		Lr24		*Lr24*	*Lr24*			*Lr24*	*Lr24*		*Lr24*		*Lr24*	*Lr24*		*Lr24*		*Lr24*	*Lr24*				*Lr24*	*Lr24*						*Lr24*	*Lr24*				*Lr24*	*Lr24*	
								Lr26																									*Lr26*			
			Lr30	*Lr30*		*Lr30*			*Lr30*	*Lr30*				*Lr30*				*Lr30*										*Lr30*	*Lr30*		*Lr30*				*Lr30*	*Lr30*	*Lr30*

Yellow rust Pathotypes

	6E16A-	6E22A-	6E22A+	7E22A-
Avirulence genes	*Yr1*	*Yr1*	*Yr1*	
	Yr3a	*Yr3a*	*Yr3a*	*Yr3a*
	Yr4a	*Yr4a*	*Yr4a*	*Yr4a*
	Yr4b	*Yr4b*	*Yr4b*	*Yr4b*
	Yr5	*Yr5*	*Yr5*	*Yr5*
	Yr9	*Yr9*	*Yr9*	*Yr9*
	Yr10	*Yr10*	*Yr10*	*Yr10*
	Yr15	*Yr15*	*Yr15*	*Yr15*
	Yr25			
	Yr27	*Yr27*	*Yr27*	*Yr27*
	YrA	*YrA*	*YrA*	*YrA*
	YrCle	*YrCle*	*YrCle*	
	YrCv	*YrCv*	*YrCv*	
	YrHVII	*YrHVII*	*YrHVII*	
	YrMor	*YrMor*	*YrMor*	
	YrSd	*YrSd*	*YrSd*	
	YrSp	*YrSp*	*YrSp*	*YrSp*
	YrSu	*YrSu*		
Virulence genes				*Yr1*
	Yr2	*Yr2*	*Yr2*	*Yr2*
	Yr6	*Yr6*	*Yr6*	*Yr6*
	Yr7	*Yr7*	*Yr7*	*Yr7*
	Yr8	*Yr8*	*Yr8*	*Yr8*
	Yr17	*Yr17*	*Yr17*	*Yr17*
		Yr25	*Yr25*	*Yr25*
			YrA	

Addendum 2: Table of Field Isolates

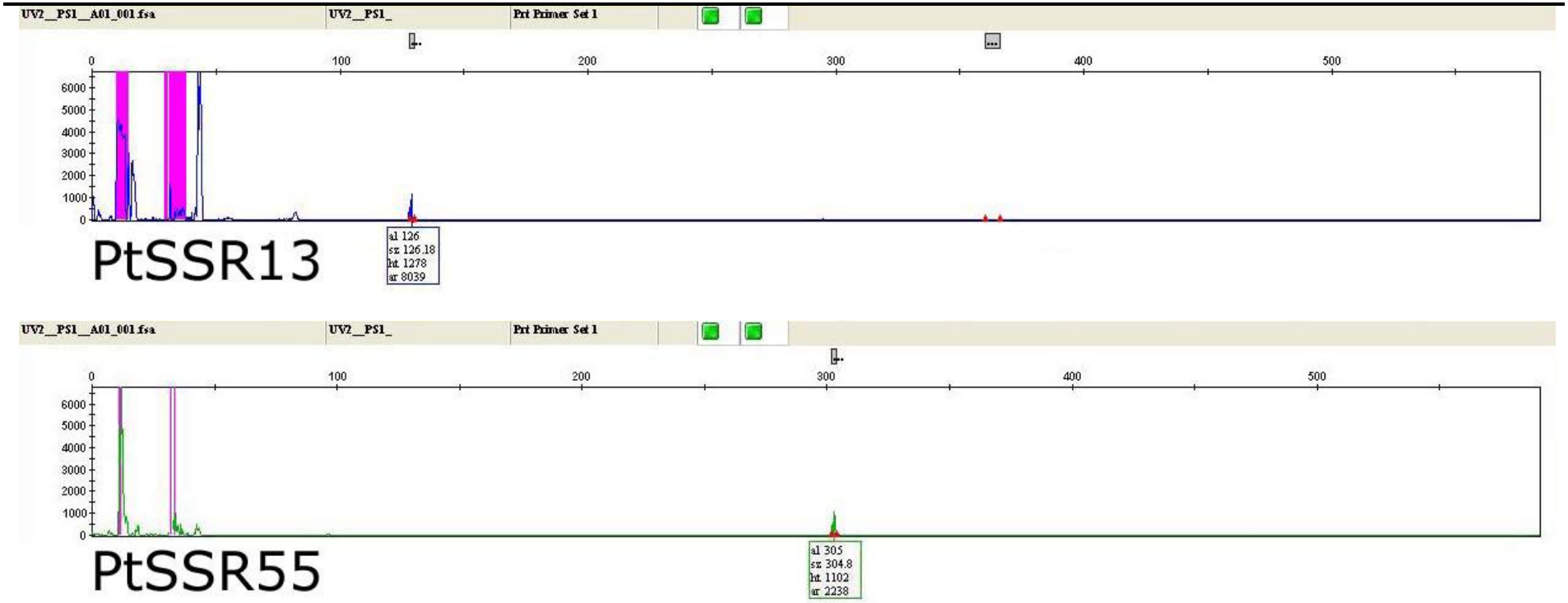
Number	Rust Type: Stem rust	Rust Type: Leaf rust	Rust Type: Yellow rust	Location	Cultivar	Wheat/Triticale	UVPgt52	UVPgt51/53/55/56	UVPgt50/54/57	UVPgt8	UVPgt9	UVPgt10	UVPgt13	Unknown LR	6E16A	Unknown YR
1	S			Riversdal	Bacchus	T		1								
2		L		Riversdal	Ibis	T								1		
3	S	L		Napier	Tobie	T	1							1		
4		L		Napier	Rex	T								1		
5		L		Napier	Ibis	T								1		
6		L		Wellgevallen	40ITYN02	T						1	1	1		
7		L		Wellgevallen	40ITYN03	T						1	1	1		
8		L		Wellgevallen	40ITYN04	T						1	1	1		
9		L		Wellgevallen	40ITYN05	T								1		
10		L		Wellgevallen	40ITYN08	T						1	1	1		
11		L		Wellgevallen	40ITYN19	T						1	1	1		
12		L		Wellgevallen	07US-038	T						1	1	1		
13		L		Wellgevallen	07US-40	T						1	1	1		
14		L		Wellgevallen	Kariega	W					1			1		
15		L		Wellgevallen	07US-108	W					1			1		
16	S	L		Langgewens	Bacchus	T	1					1		1		
17		L		Piketberg	O5T133	W								1		
18	S	L		Vredenburg	Tobie	T	1					1	1	1		
19		L		Klipheuwel	SST88	W						1	1	1		
20		L		Piketberg	Bacchus	T				1				1		
21		L		Langgewens	SST88	W						1		1		
22		L		Piketberg	97KI-4-2	W					1			1		
23		L		Klipheuwel	Bacchus	T						1	1	1		
24	S			Tygerhoek	Tobie	T			1							
25		L		Tygerhoek	Ibis	T								1		
26		L		Mariendal	SST88	W								1		
27		L		Roodebloem	SST88	W								1		
28	S			Napier	Bacchus	T			1							
29			Y	Napier	Bacchus	T									1	
30		L		Napier	SST88	W								1		
31	S			Napier	SST88	W	1									
32			Y	Wellgevallen	Marocco	W									1	
33	S			Tygerhoek	Tobie	T		1								
34		L		Tygerhoek	03H86-8	W								1		

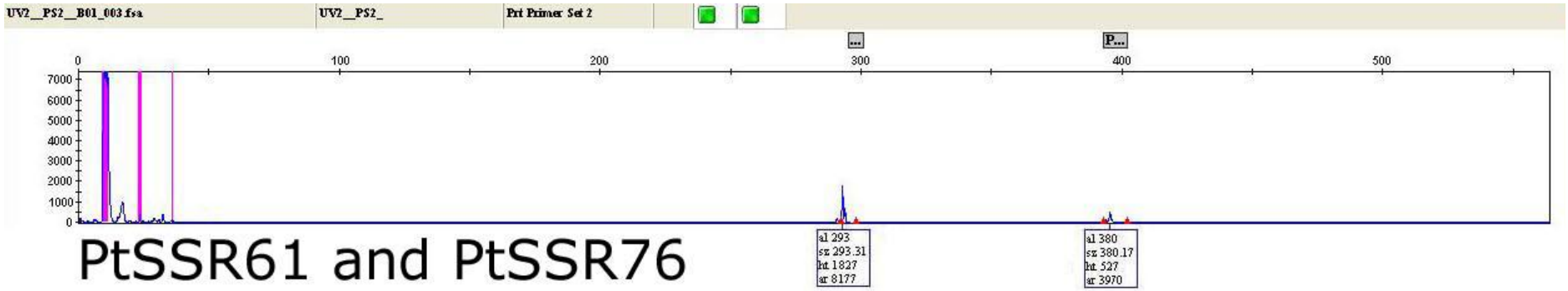
Number	Rust Type: Stem rust	Rust Type: Leaf rust	Rust Type: Yellow rust	Location	Cultivar	Wheat/Triticale	UVPgt52	UVPgt51/53/55/56	UVPgt50/54/57	UVPgt8	UVPgt9	UVPgt10	UVPgt13	Unknown LR	6E16A	Unknown YR
35		L		Tygerhoek	03H86-8	W								1		
36		L		Tygerhoek	00K 60-1	W						1	1	1		
37		L		Tygerhoek	Bacchus	T								1		
38		L		Tygerhoek	SST88	W								1		
39		L		Albertenia	Tobie	T						1	1			
40		L		Albertenia	07 US 038	W							1	1		
41		L		Albertenia	Rex	T						1	1			
42		L		Riversdal	00T 207	T						1	1			
43		L		Riversdal	Tobie	T							1			
44		L		Riversdal	SST027	W								1		
45	S			Wellgevallen	McNair	W			1							
46		L		Riversdal	Tobie	T							1	1		
47	S			Riversdal	Tobie	T			1							
48		L		Roodebloem	Unknown	W								1		
49		L		Napier	SST027	W								1		
50		L		Napier	SST027	W								1		
51		L		Roodebloem	Bacchus	T						1	1	1		
52	S			Riversdal	SST027	W	1									
53		L		Riversdal	Ibis	T						1	1	1		
54		L		Albertenia	Rex	T						1	1	1		
55		L		Albertenia	37th ITSN 43	T						1	1	1		
56		L		Tygerhoek	Bacchus	T						1		1		
57		L		Tygerhoek	00T 207	T						1		1		
58		L		Tygerhoek	27th ITYN 36-1	T						1	1	1		
59		L		Tygerhoek	Tobie	T						1		1		
60	S			Tygerhoek	Bacchus	T			1							
61		L		Napier	Tobie	T						1		1		
62		L		Napier	987 376	T						1		1		
63		L		Napier	27th ITYN 39-1-8	T						1	1	1		
64		L		Napier	SST88	W								1		
65		L		Napier	US2007	T								1		
66			Y	Tygerhoek	981 376-1	T										1
67	S	L		Roodebloem	SST88	W			1					1		
68	S			Napier	SST88	W	1									
69	S			Napier	SST88	W	1									
70	S			Napier	SST88	W		1								
71	S			Napier	SST88	W	1									

Number	Rust Type			Location	Cultivar	Wheat/Triticale	UVPgt52	UVPgt51/53/55/56	UVPgt50/54/57	UVPgt8	UVPgt9	UVPgt10	UVPgt13	Unknown LR	6E16A	Unknown YR
	Stem rust	Leaf rust	Yellow rust													
72	S			Napier	SST88	W	1									
73	S			Napier	SST88	W	1									
74	S			Napier	SST88	W	1									
75	S			Napier	SST88	W	1									
76	S			Napier	SST88	W	1									
77	S			Napier	SST88	W	1									
78	S			Napier	SST88	W	1									
79	S			Napier	SST88	W	1									
80	S			Napier	SST88	W	1									
81	S			Napier	SST88	W			1							
82	S			Napier	SST88	W	1									
83	S			Napier	SST88	W	1									
84	S			Napier	SST88	W	1									
85	S			Napier	SST88	W			1							
86	S			Napier	SST88	W	1									
87	S			Napier	SST88	W	1									
88	S			Napier	SST88	W			1							
89	S			Napier	SST88	W			1							
90	S			Napier	SST88	W			1							
91	S			Napier	SST88	W	1									

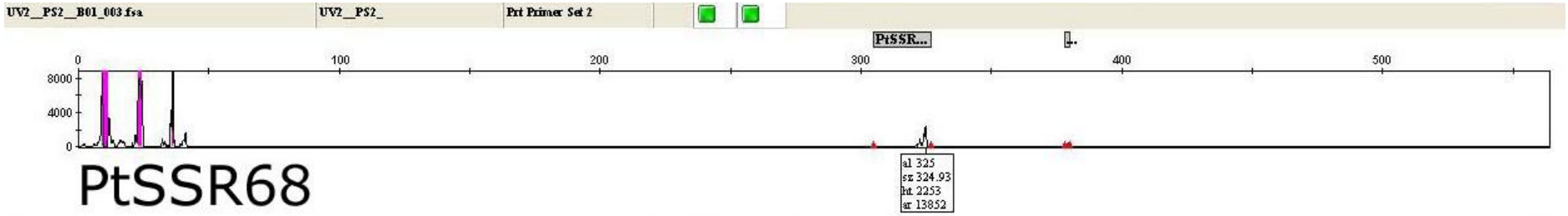
Addendum 3: Rust microsatellite markers

Leaf Rust
UVPrt2

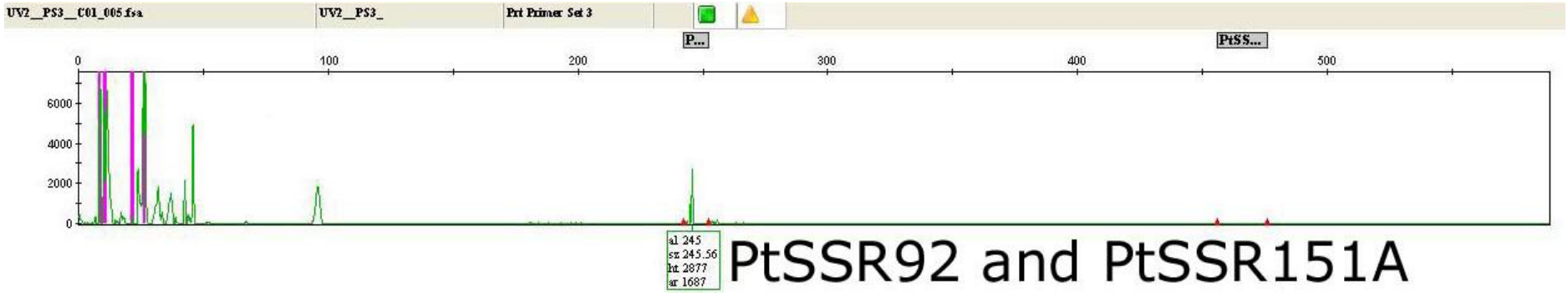




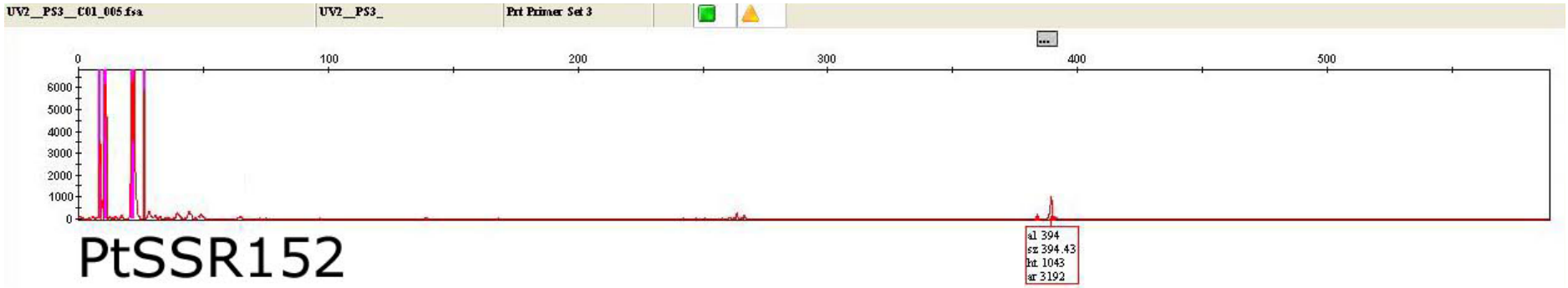
PtSSR61 and PtSSR76



PtSSR68



PtSSR92 and PtSSR151A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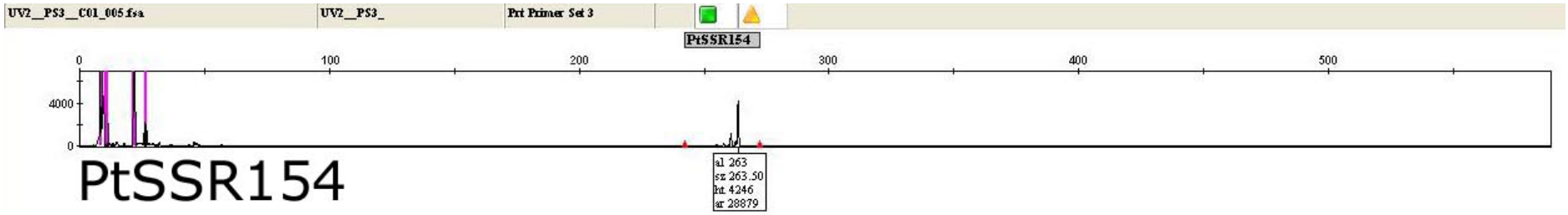
PtSSR152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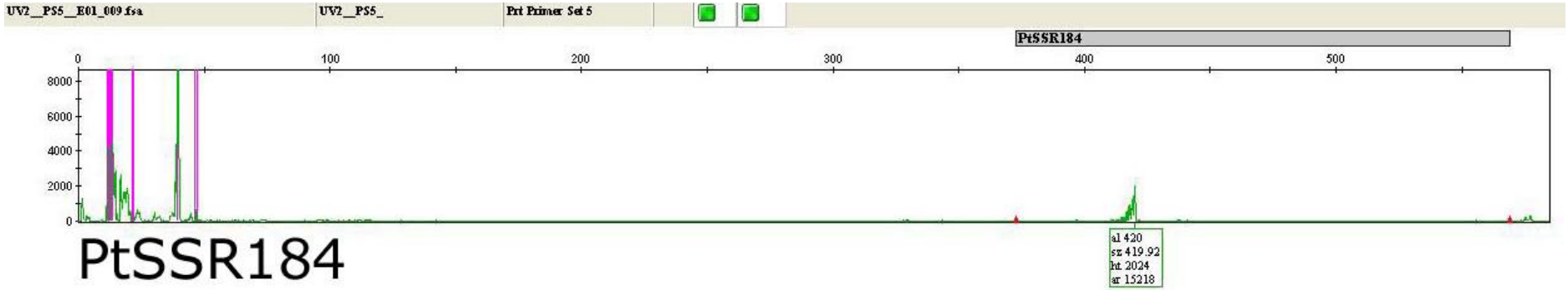
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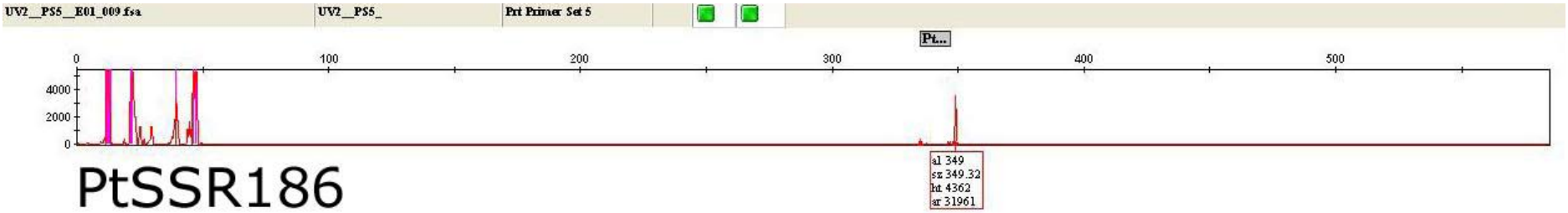
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ar 31961

PtSSR186

UVPrt3


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6000
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2000
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al 126
sz 126.19
ht 2101
ar 3798
PtSSR13
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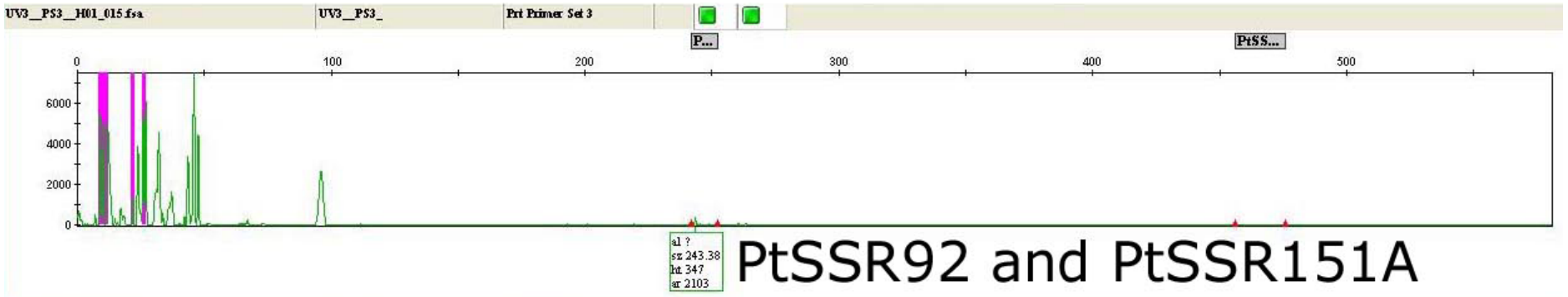
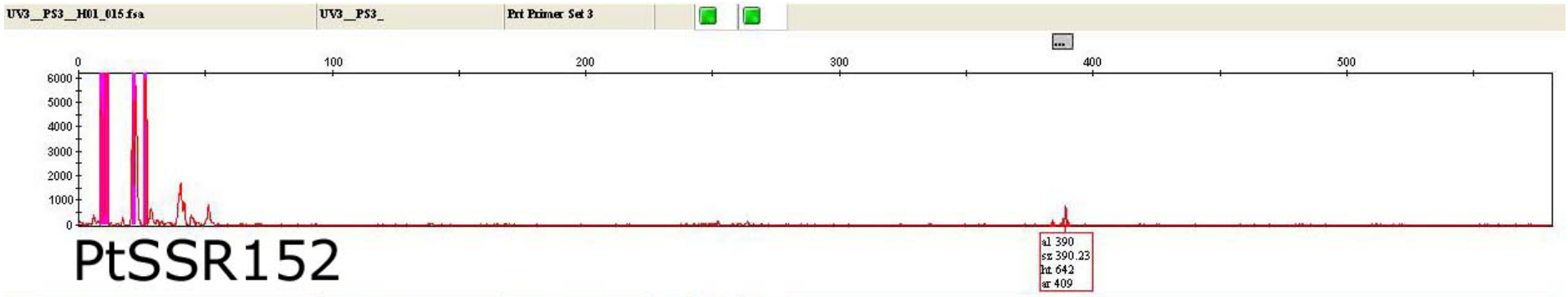
PtSSR55

PtSSR61 and PtSSR76

PtSSR68



PtSSR92 and PtSSR151A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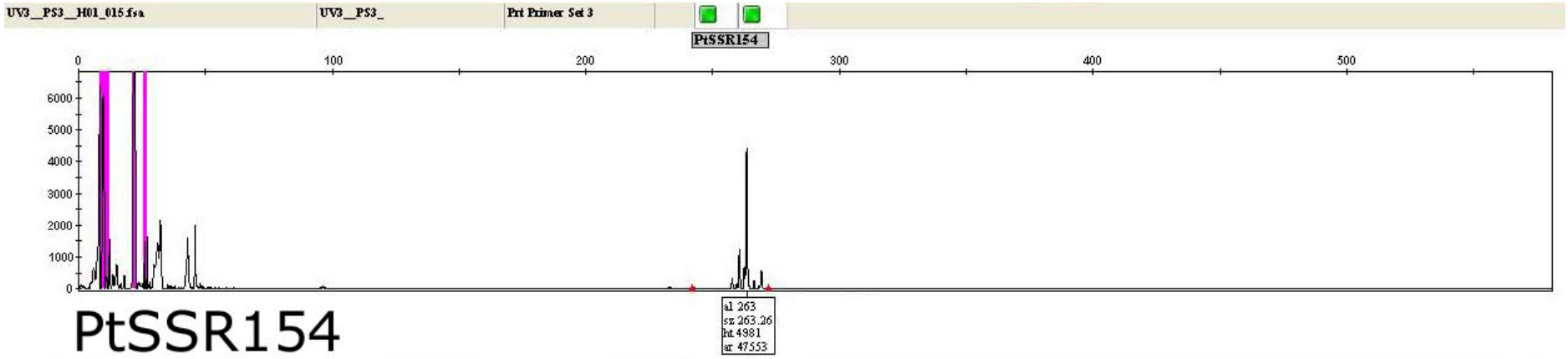
ar 409
PtSSR152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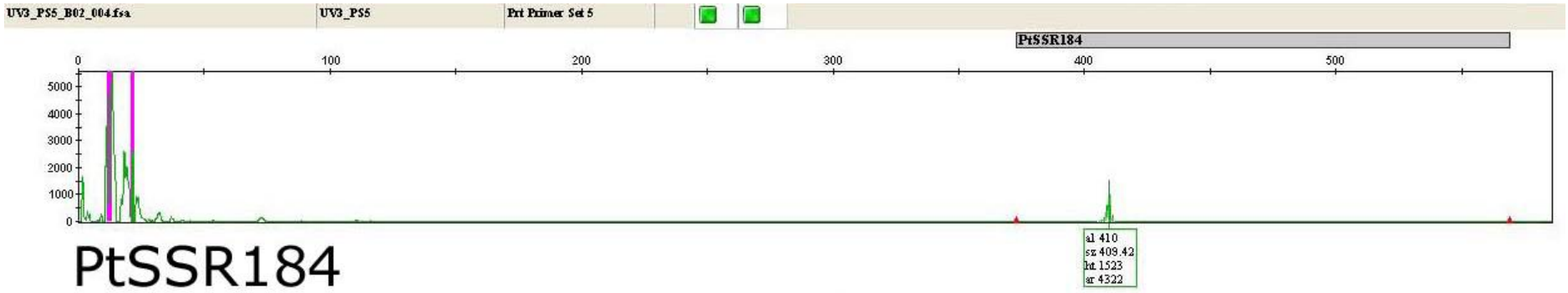
ar 47553
PtSSR154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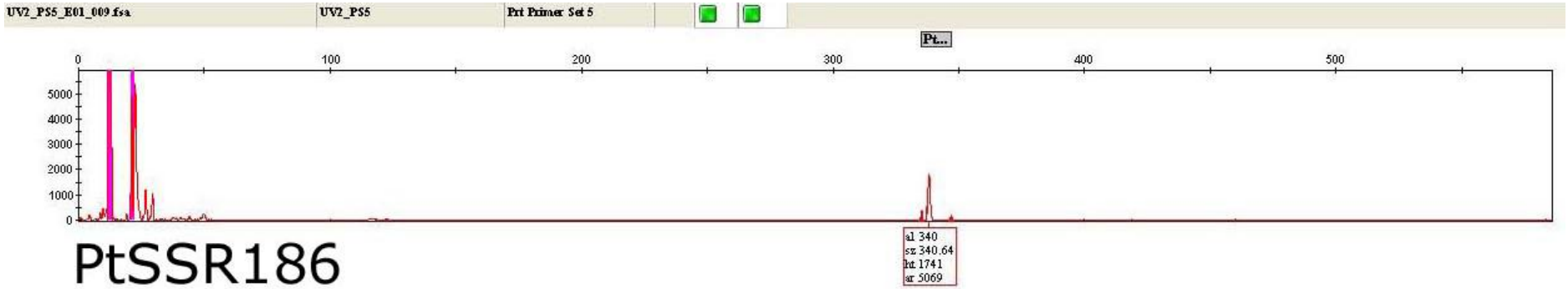
PtSSR184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ar 5069
PtSSR186

UVPrt4


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6000
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2000
1000
0
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sz 126.13
ht 943
ar 442
PtSSR13
UV4_PS1_C02_006.fsa
UV4_PS1_
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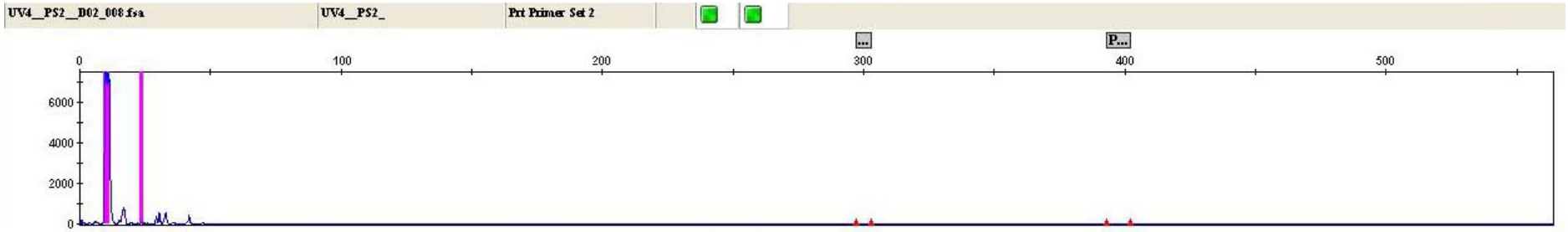
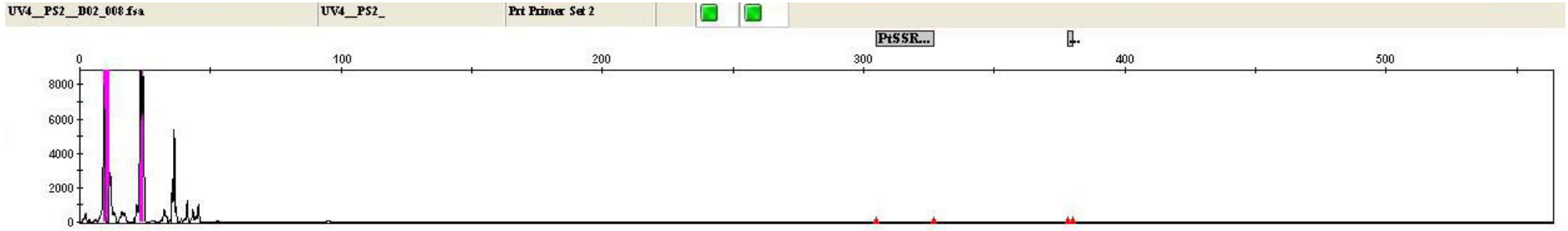
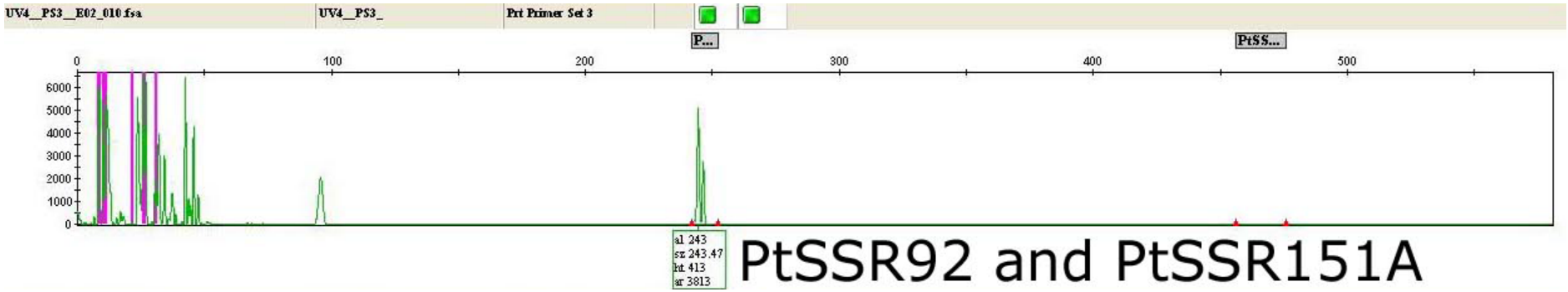
PtSSR55



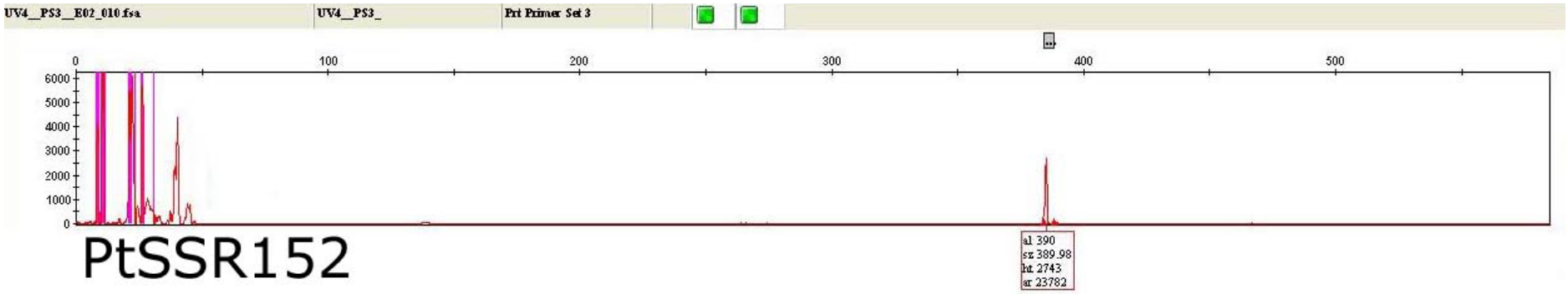
PtSSR61 and PtSSR76



PtSSR68



PtSSR92 and PtSSR151A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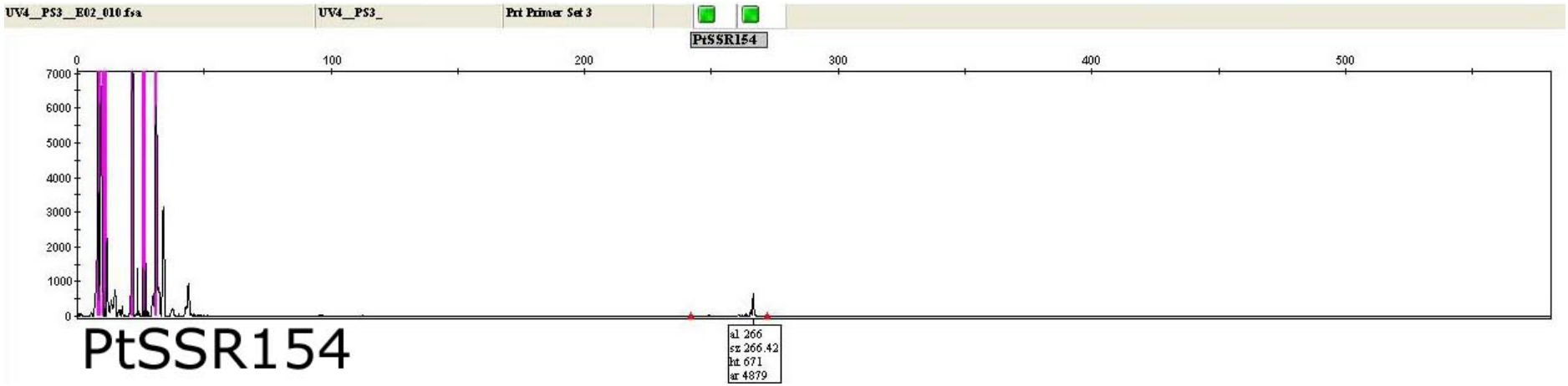
ar 23782
PtSSR152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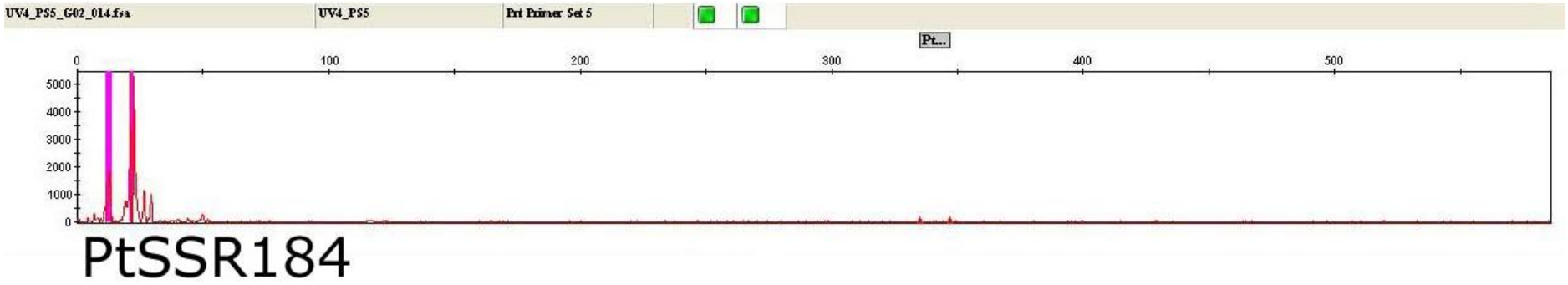
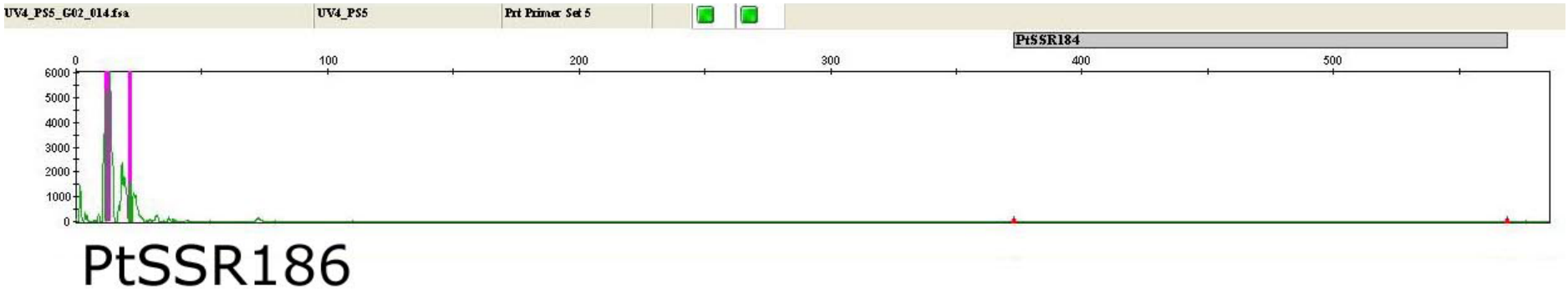
PtSSR154

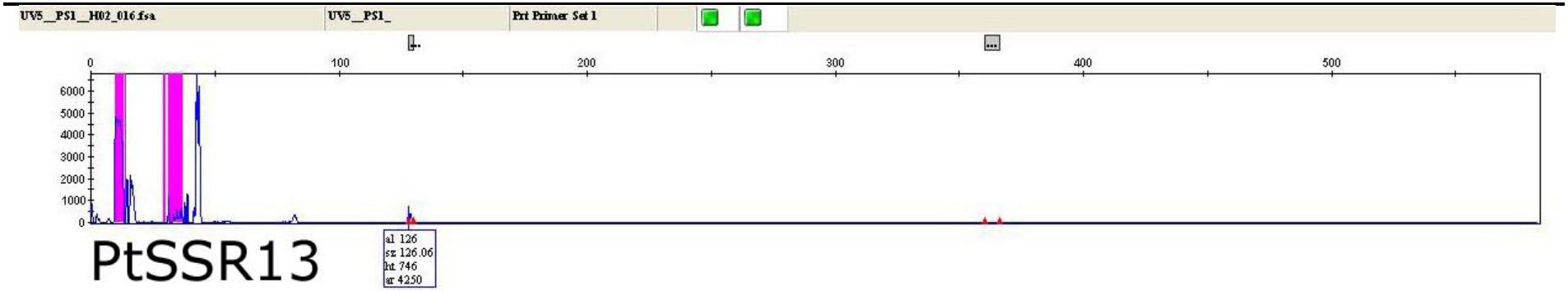


PtSSR184



PtSSR186

UVPrt5


UV5_PS1_H02_016.fsa
UV5_PS1_
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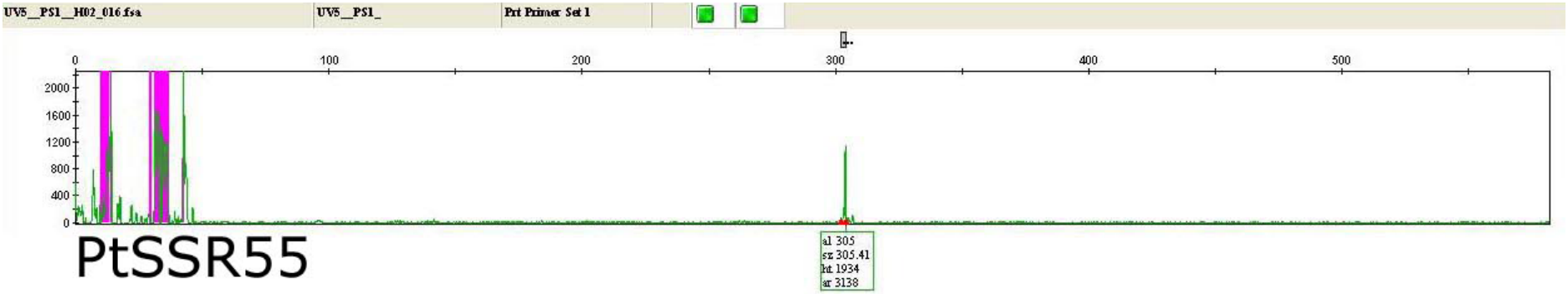
al 126
sz 126.06
ht 746
ar 4250


UV5_PS1_H02_016.fsa
UV5_PS1_
Prt Primer Set 1
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1200
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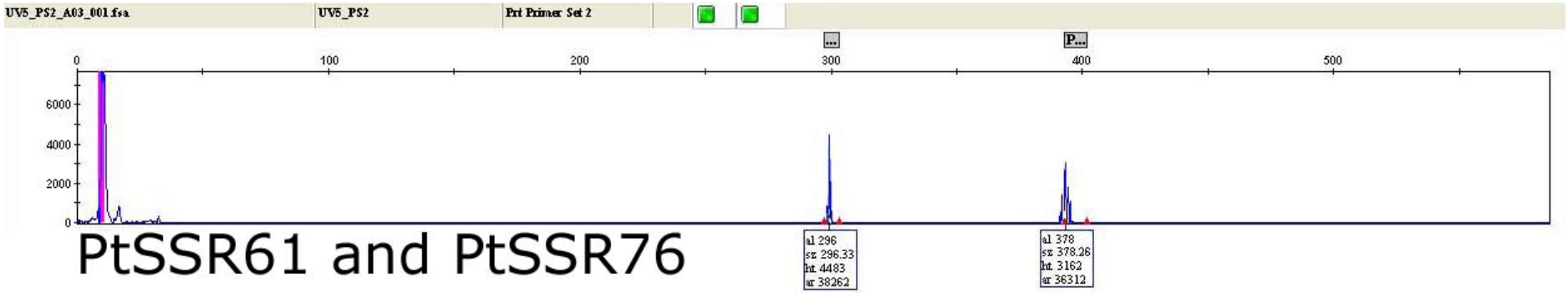
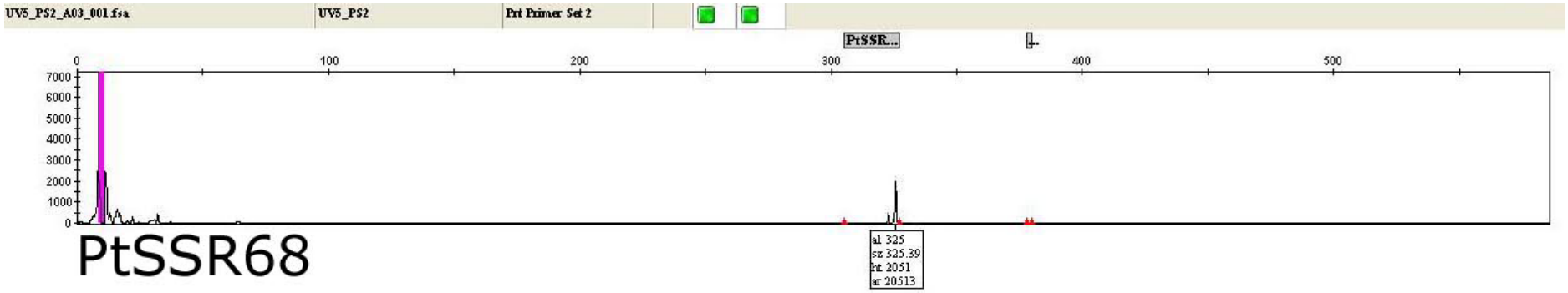
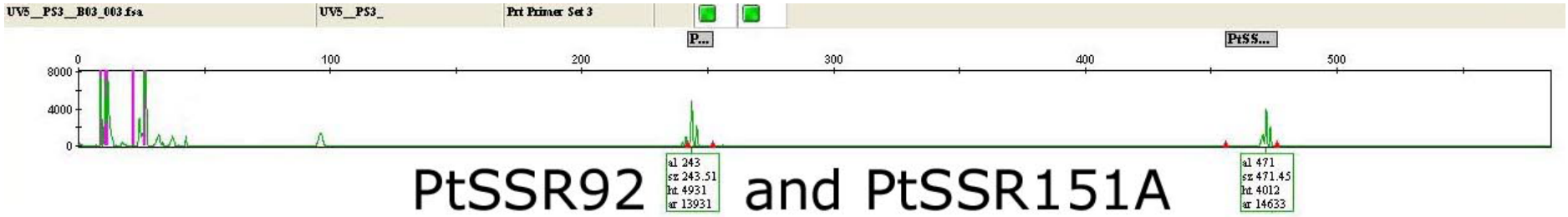
PtSSR55
al 305
sz 305.41
ht 1934
ar 3138



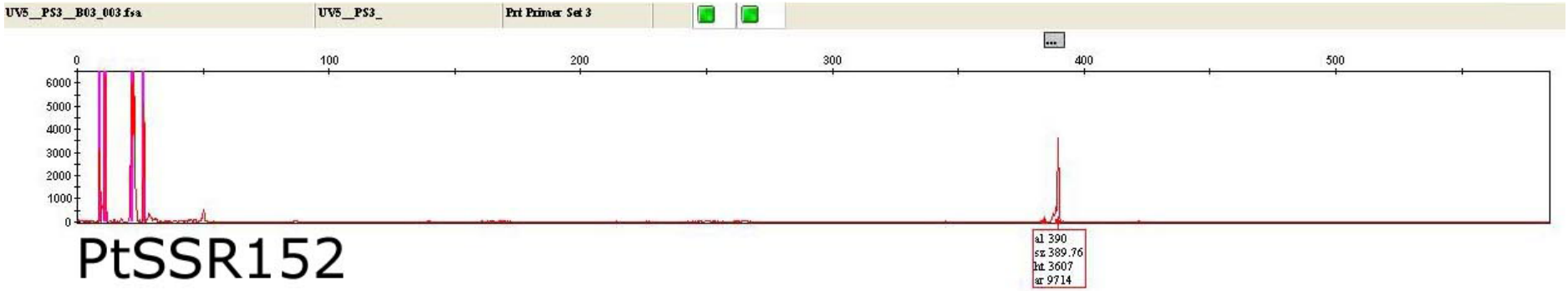
PtSSR61 and PtSSR76



PtSSR68



PtSSR92 and PtSSR151A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UV5__PS3_
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1000
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al 390
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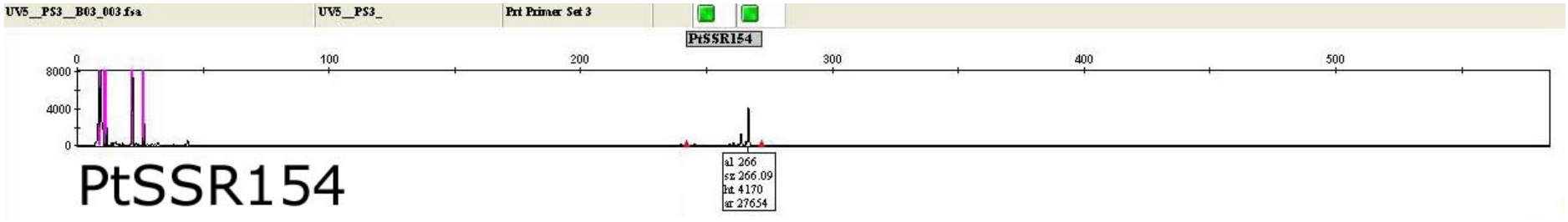
ar 9714
PtSSR152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UV5__PS3_
Prt Primer Set 3
PtSSR154
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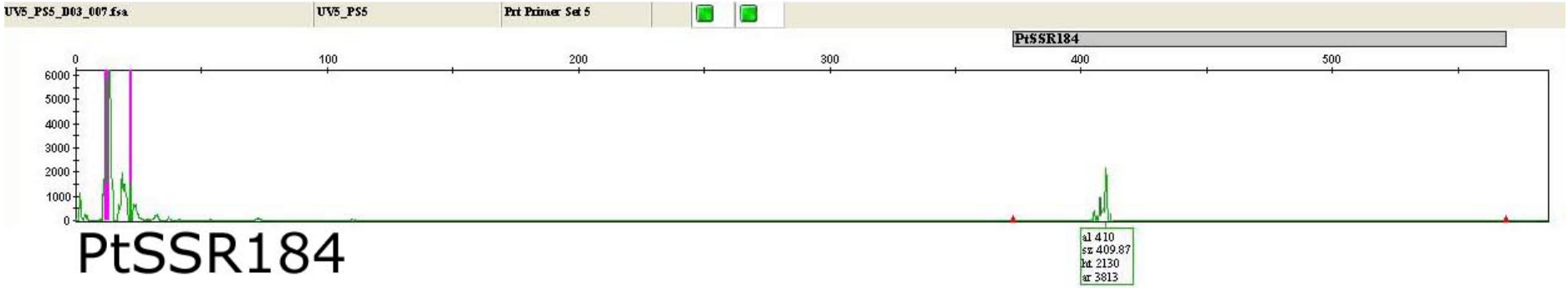
ht 4170
ar 27654
PtSSR154


UV5_PS5_D03_007.fsa
UV5_PS5
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PtSSR184
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100
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3000
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1000
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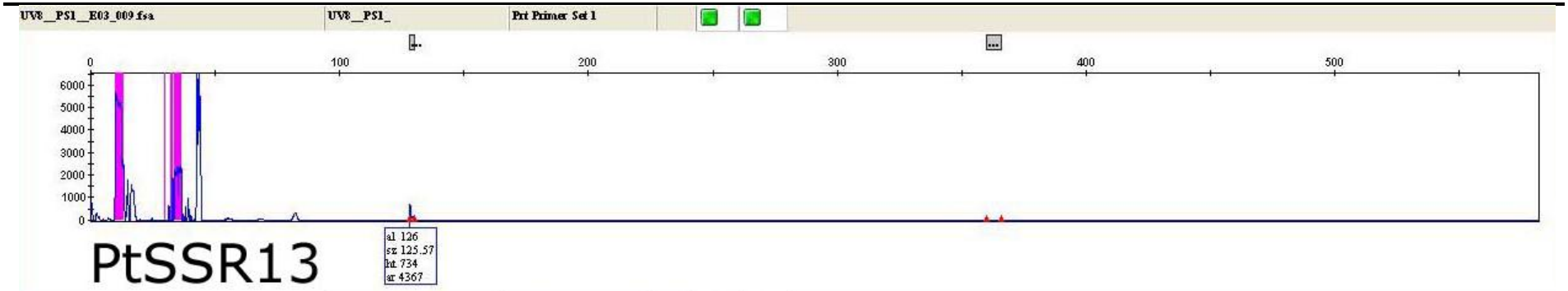
ht 2130
ar 3813
PtSSR184

UVPrt8


UV8__PS1__E03_009.fsa
UV8__PS1_
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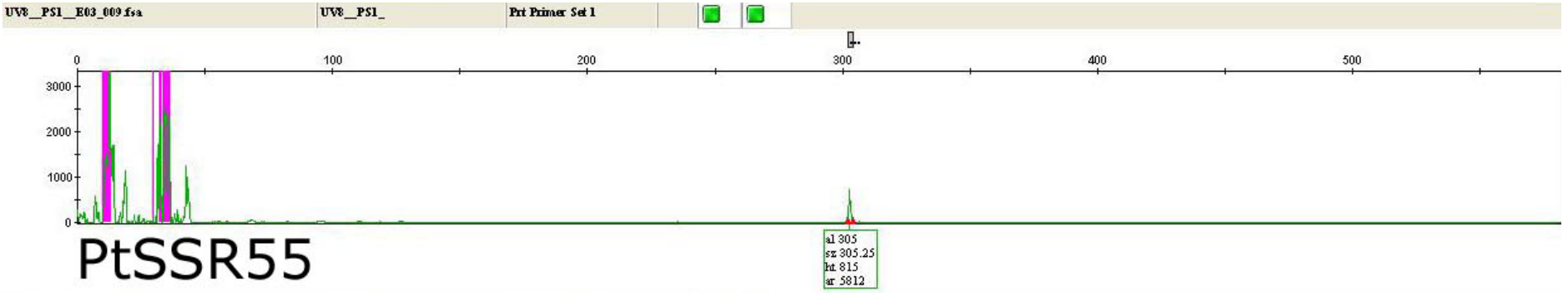
ar 4367
PtSSR13


UV8__PS1__E03_009.fsa
UV8__PS1_
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1000
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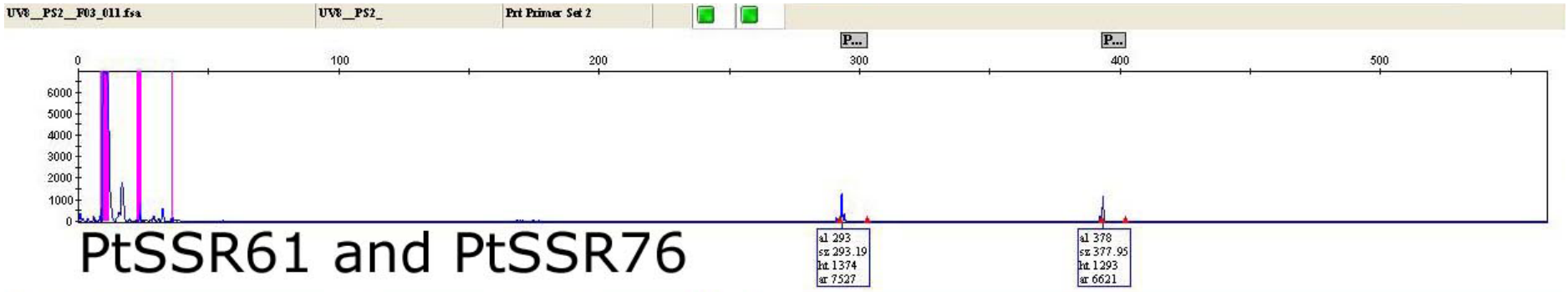
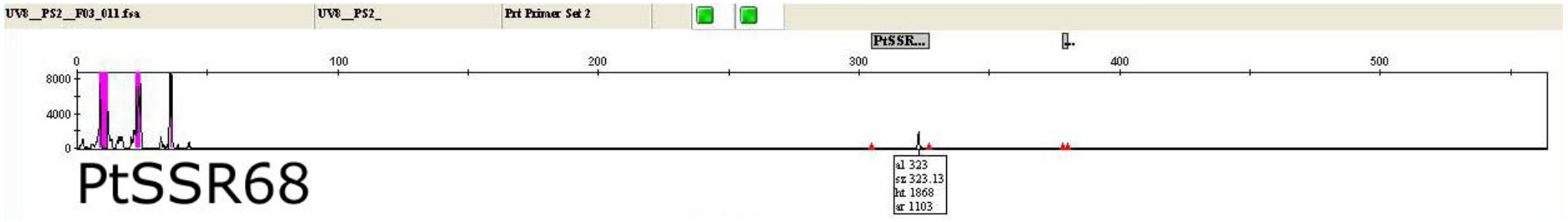
al 305
sz 305.25
ht 815
ar 5812
PtSSR55


UV8__PS2__F03_011.fsa
UV8__PS2_
Prt Primer Set 2
P...
P...
0
100
200
300
400
500
6000
5000
4000
3000
2000
1000
0
al 293
sz 293.19
ht 1374
ar 7527
al 378
sz 377.95
ht 1293
ar 6621
PtSSR61 and PtSSR76


UV8__PS2__F03_011.fsa
UV8__PS2_
Prt Primer Set 2
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8000
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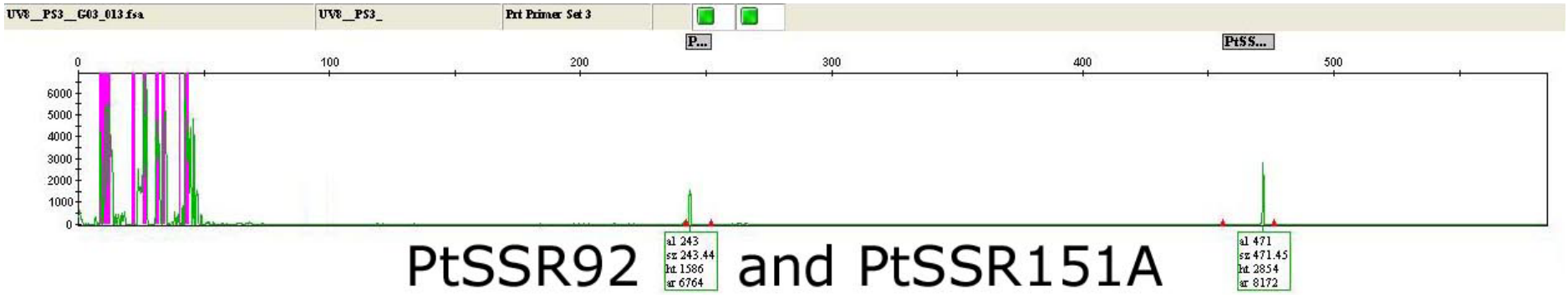
ar 1103
PtSSR68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PtSS...
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200
300
400
500
6000
5000
4000
3000
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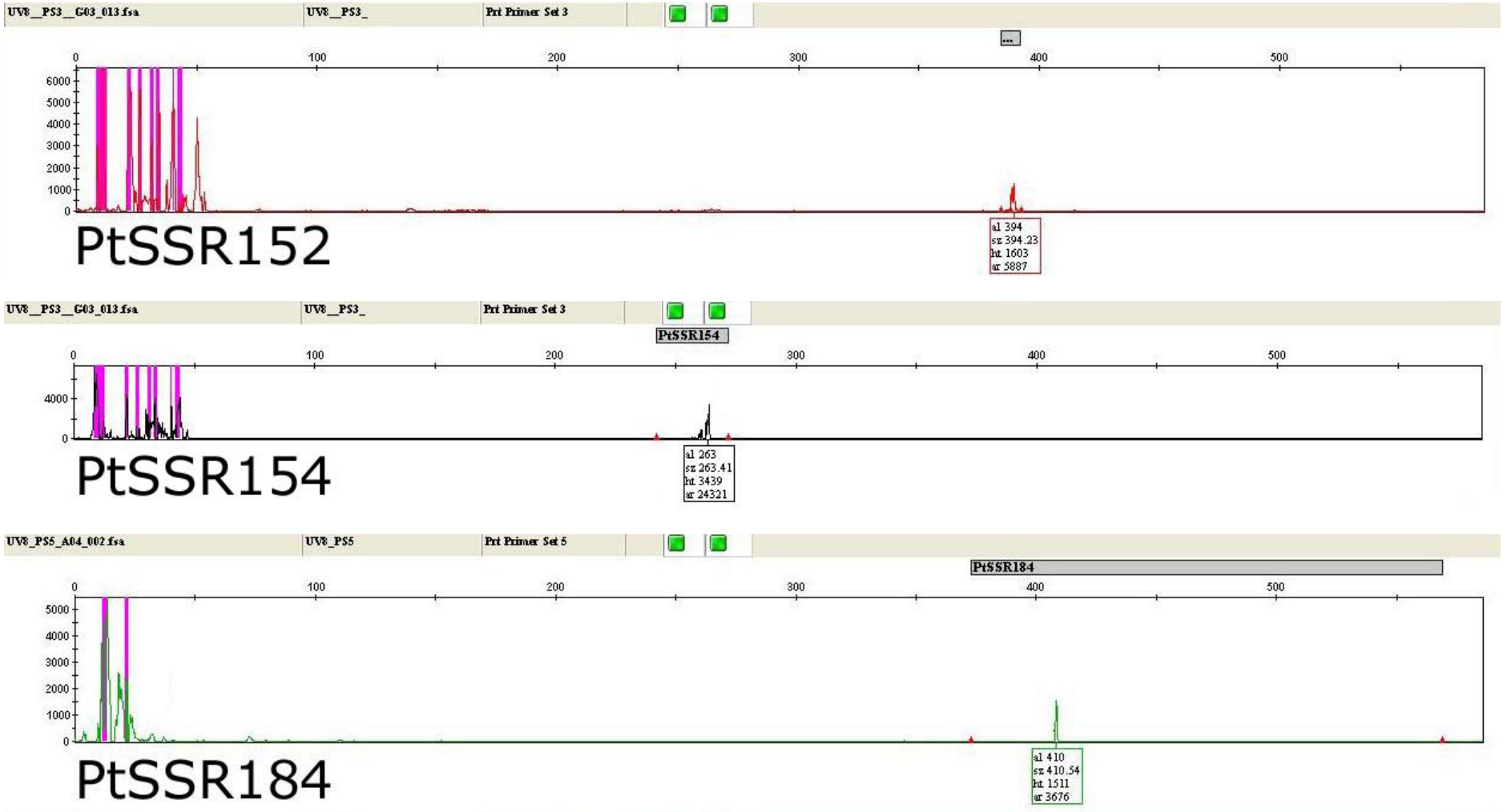
sz 243.44
ht 1586
ar 6764
al 471
sz 471.45
ht 2854
ar 8172
PtSSR92 and PtSSR151A


UV8__PS3__G03_013.fsa
UV8__PS3_
Prt Primer Set 3
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400
500
6000
5000
4000
3000
2000
1000
0
al 394
sz 394.23
ht 1603
ar 5887
PtSSR152
UV8__PS3__G03_013.fsa
UV8__PS3_
Prt Primer Set 3
PtSSR154
0
100
200
300
400
500
4000
0
al 263
sz 263.41
ht 3439
ar 24321
PtSSR154
UV8_PS5_A04_002.fsa
UV8_PS5
Prt Primer Set 5
PtSSR184
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3000
2000
1000
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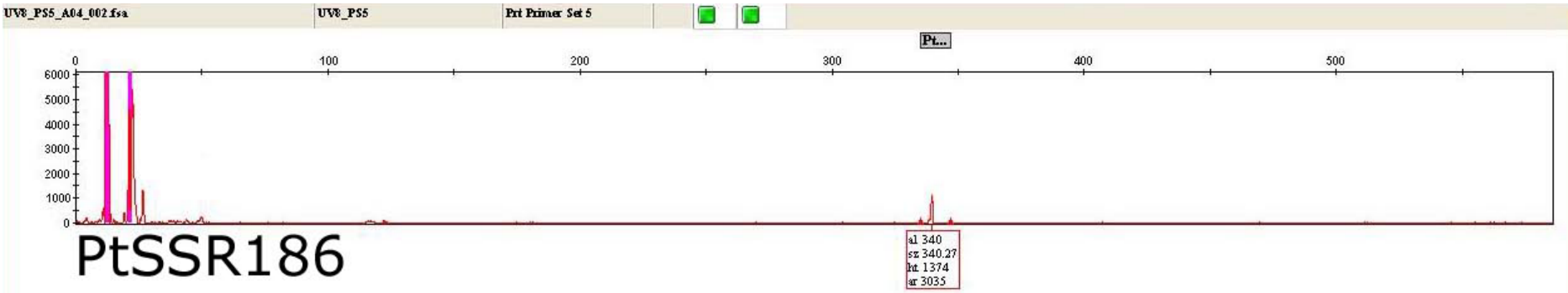
al 410
sz 410.54
ht 1511
ar 3676
PtSSR184


UV8_PS5_A04_002.fsa
UV8_PS5
Prt Primer Set 5
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0
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1000
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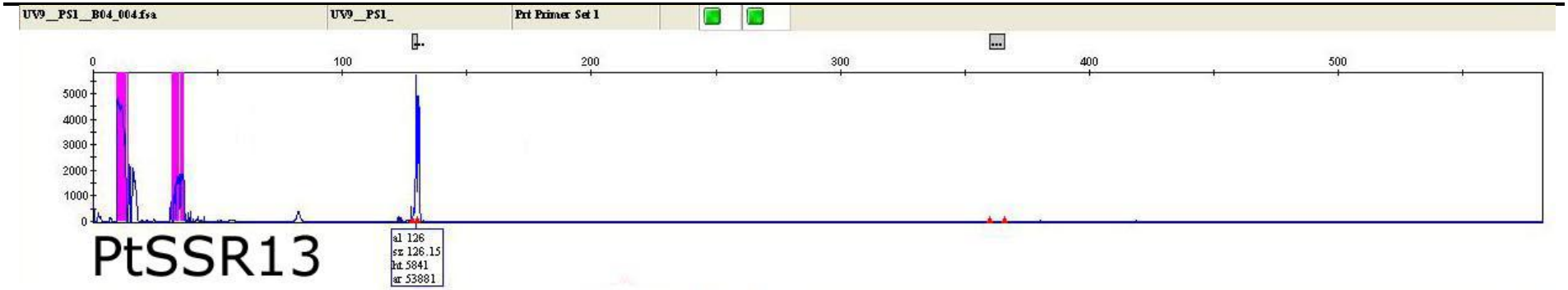
al 340
sz 340.27
ht 1374
ar 3035

UVPrt9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UV9__PS1_
Prt Primer Set 1
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1000
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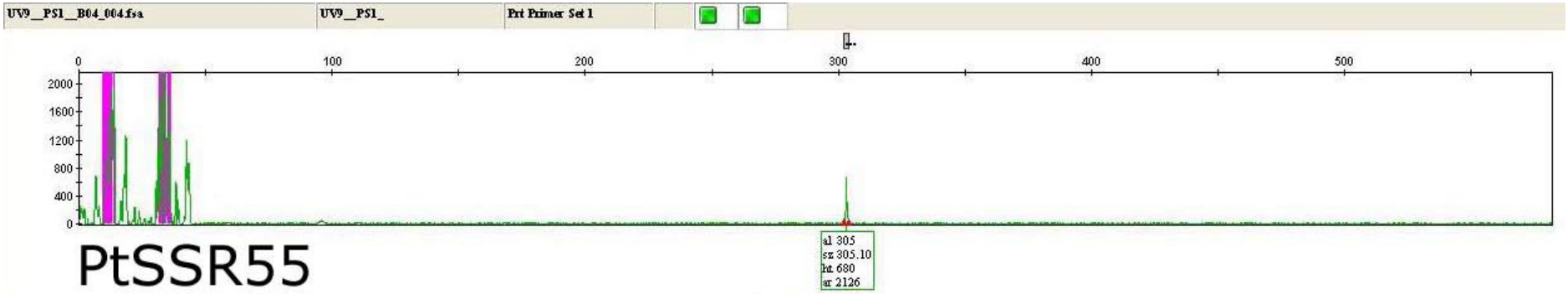
sz 126.15
ht 5841
ar 53881


UV9__PS1__B04_004.fsa
UV9__PS1_
Prt Primer Set 1
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400
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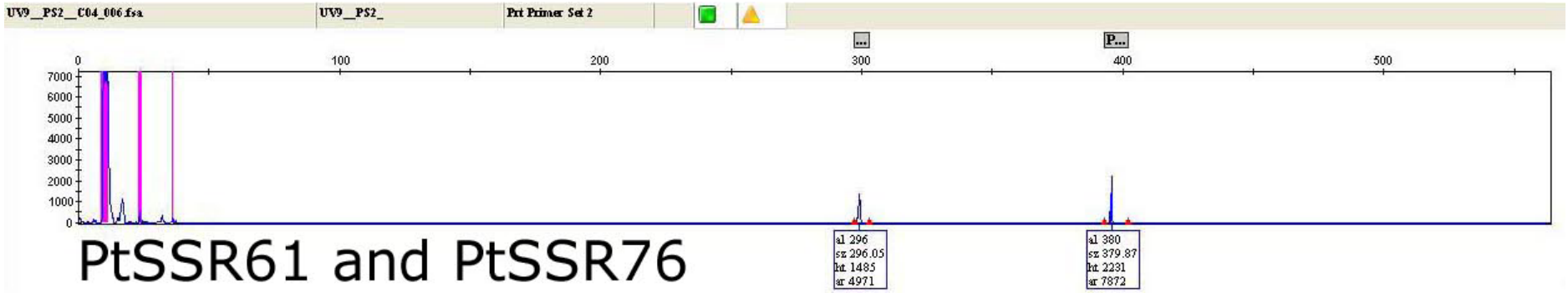
sz 305.10
ht 680
ar 2126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UV9__PS2_
Prt Primer Set 2
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P...
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200
300
400
500
7000
6000
5000
4000
3000
2000
1000
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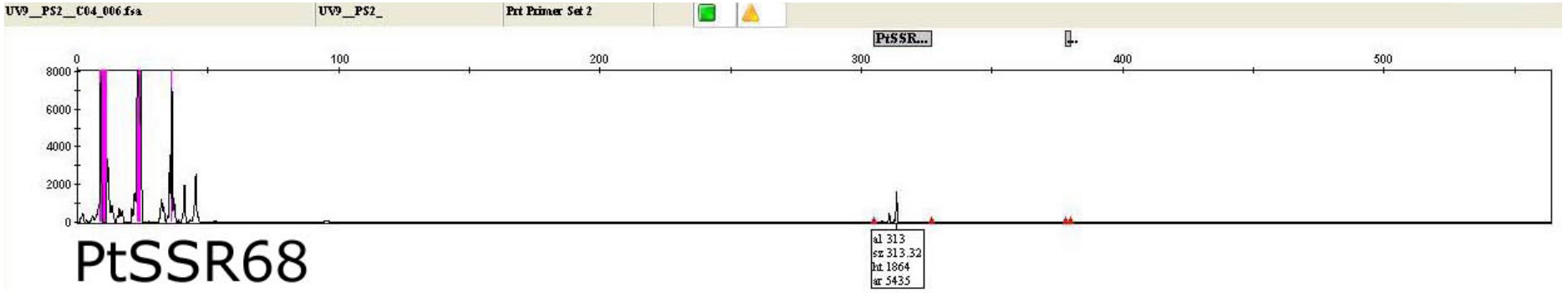
sz 296.05
ht 1485
ar 4971
al 380
sz 379.87
ht 2231
ar 7872
PtSSR61 and PtSSR76


UV9__PS2__C04_006.fsa
UV9__PS2_
Prt Primer Set 2
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8000
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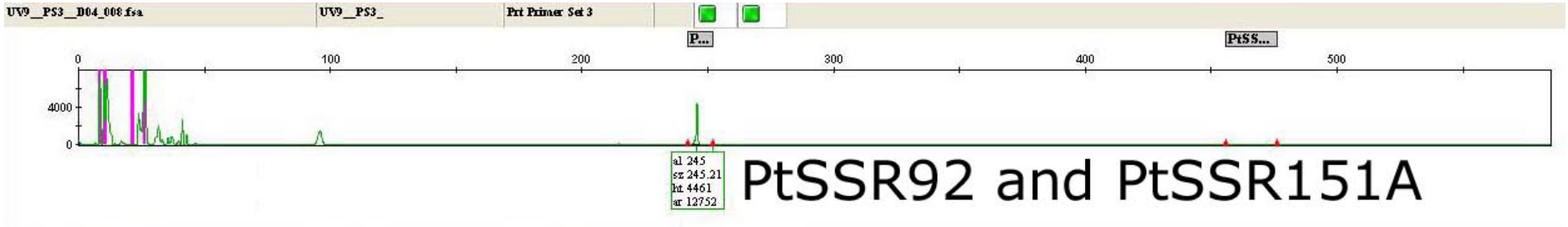
0
al 313
sz 313.32
ht 1864
ar 5435
PtSSR68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Prt Primer Set 3
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PtSS...
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al 245
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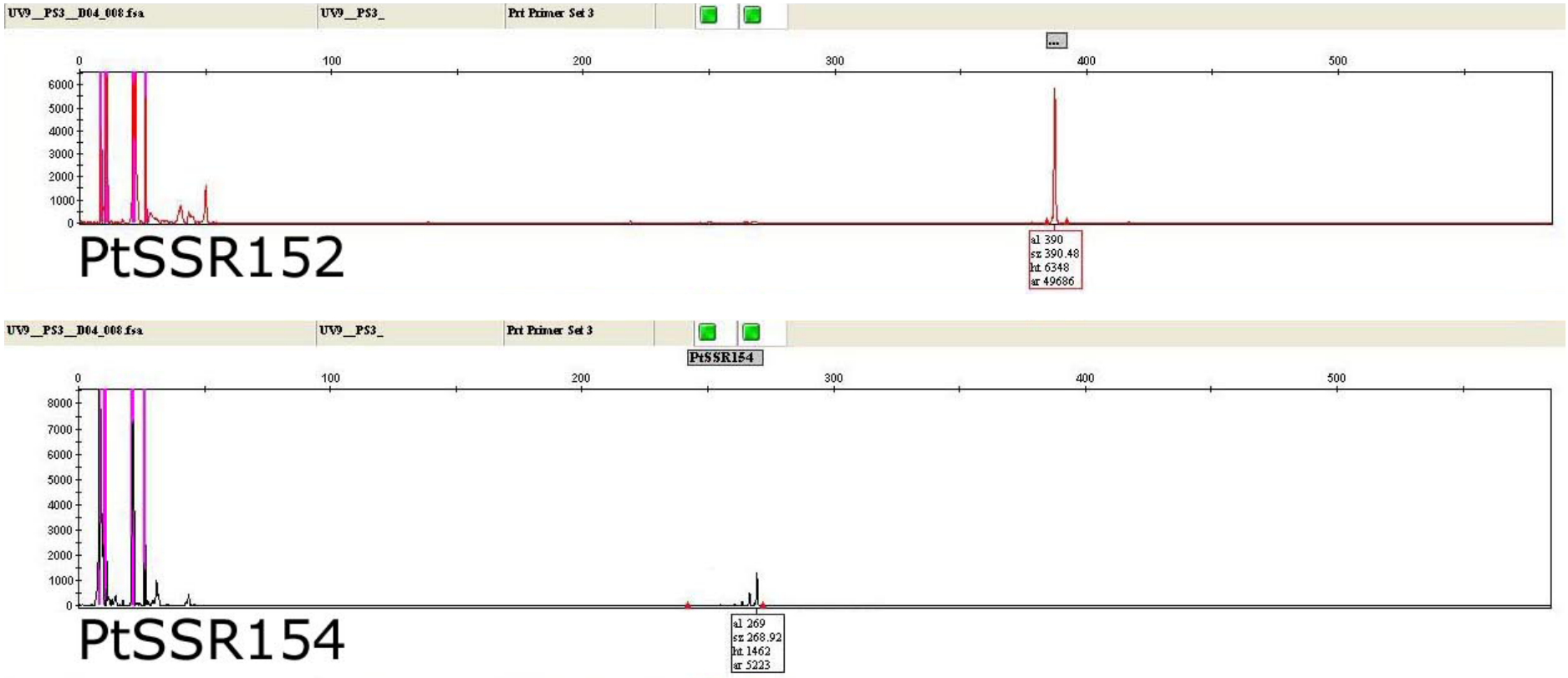
ht 4461
ar 12752
PtSSR92 and PtSSR151A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1000
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al 390
sz 390.48
ht 6348
ar 49686
UV9__PS3__D04_008.fsa
UV9__PS3_
Prt Primer Set 3
PtSSR154
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400
500
8000
7000
6000
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4000
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2000
1000
0
PtSSR154
al 269
sz 268.92
ht 1462
ar 5223


UV9_PS5_F04_012.fsa
UV9_PS5
Prt Primer Set 5
PtSSR184
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200
300
400
500
5000
4000
3000
2000
1000
0
al 410
sz 409.76
ht 1227
ar 6533
PtSSR184
UV9_PS5_F04_012.fsa
UV9_PS5
Prt Primer Set 5
PtS...
0
100
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ht 1512
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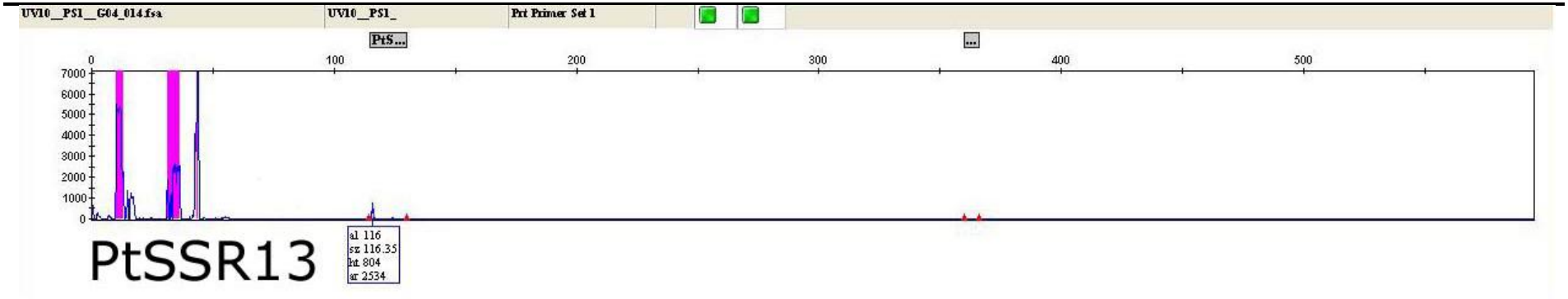
PtSSR186

UVPrt10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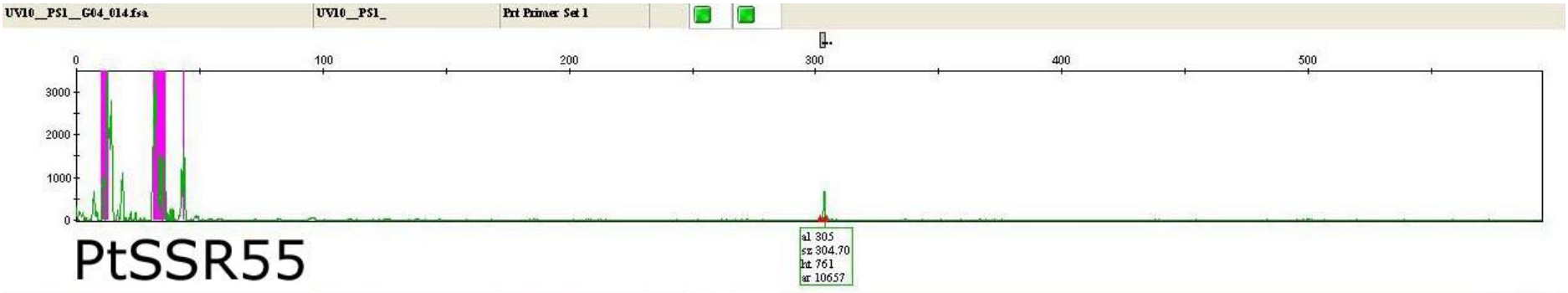
PtSSR13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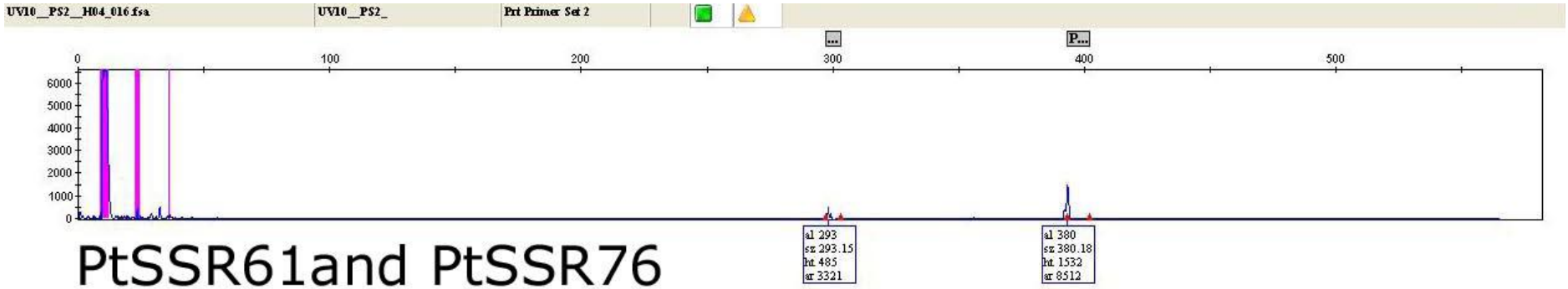
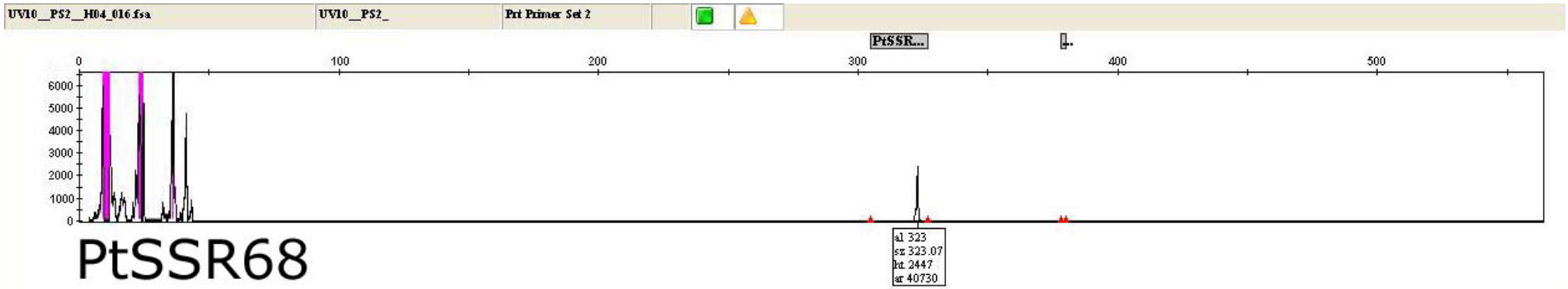
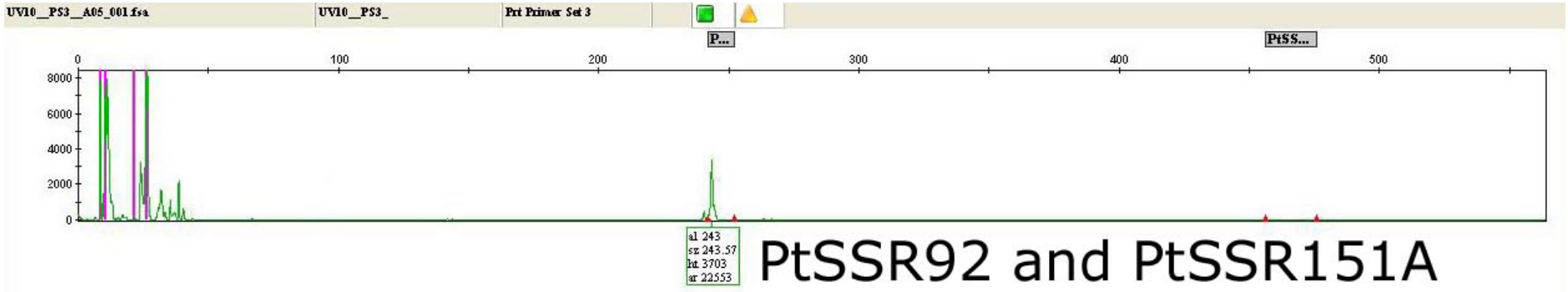
sz 304.70
ht 761
ar 10657
PtSSR55



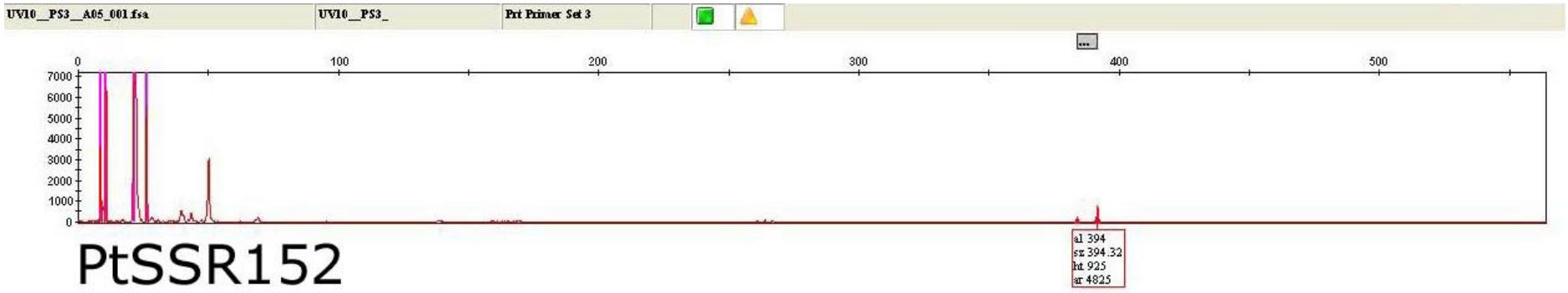
PtSSR61and PtSSR76



PtSSR68



PtSSR92 and PtSSR151A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UV10__PS3_
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6000
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3000
2000
1000
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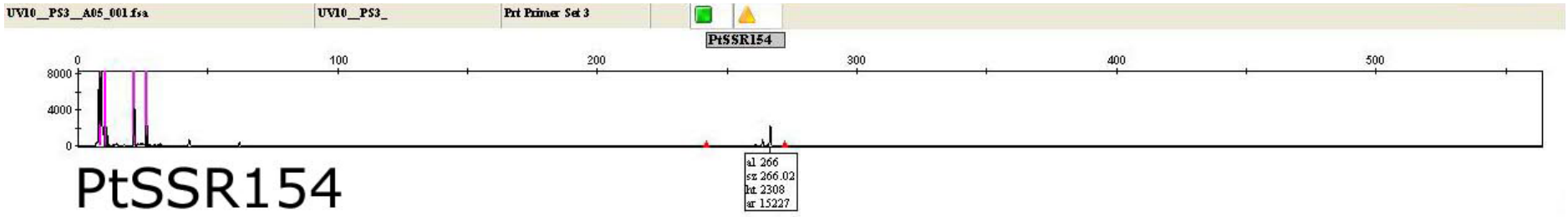
sz 394.32
ht 925
ar 4825
PtSSR152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UV10__PS3_
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300
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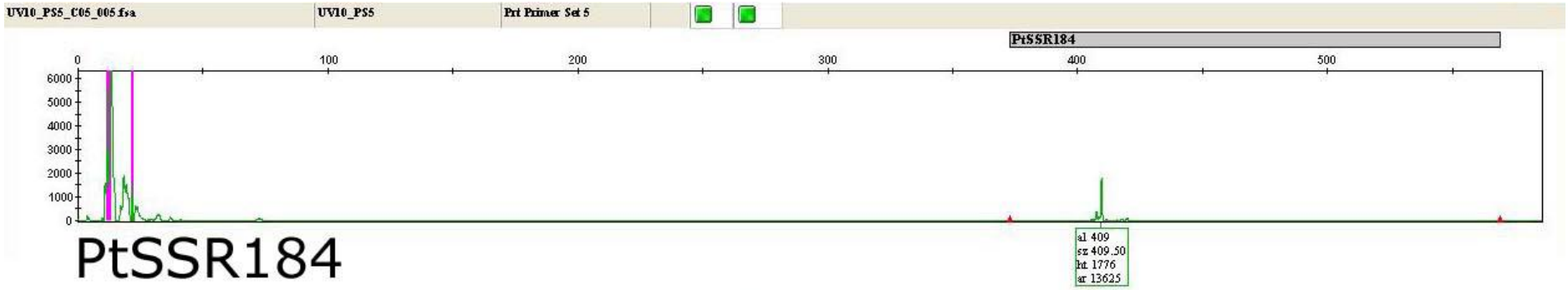
sz 266.02
ht 2308
ar 15227
PtSSR154


UV10_PS5_C05_005.fsa
UV10_PS5
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500
6000
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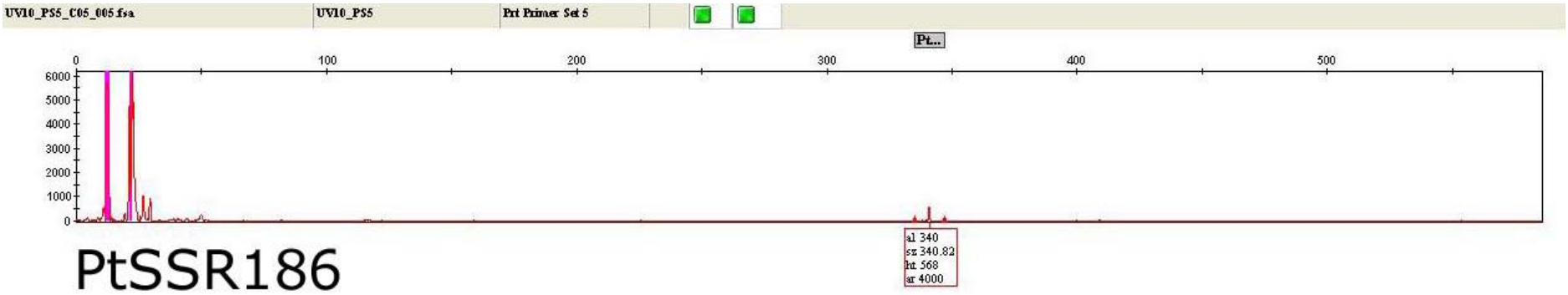
3000
2000
1000
0
al 409
sz 409.50
ht 1776
ar 13625
PtSSR184


UV10_PS5_C05_005.fsa
UV10_PS5
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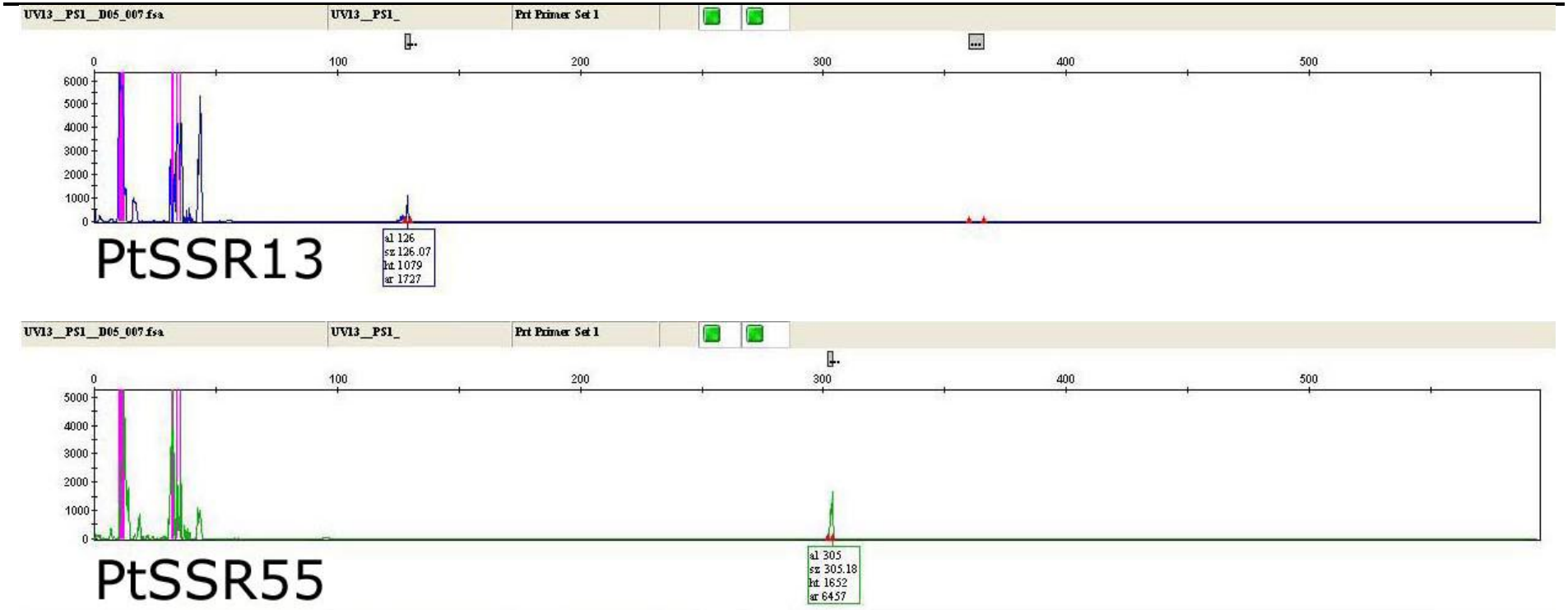
ht 568
ar 4000
PtSSR186

UVPrt13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UV13__PS1_
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1000
0
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sz 126.07
ht 1079
ar 1727
PtSSR13
UV13__PS1__D05_007.fsa
UV13__PS1_
Prt Primer Set 1
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400
500
5000
4000
3000
2000
1000
0
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sz 305.18
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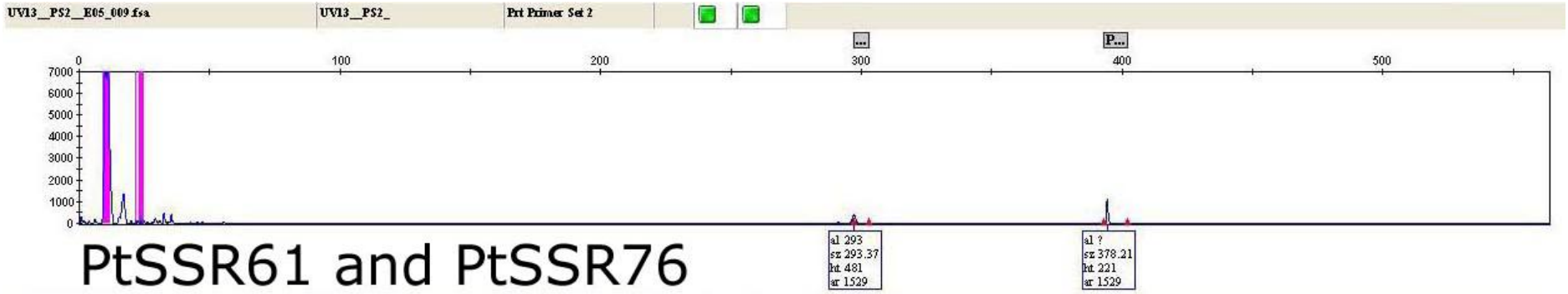
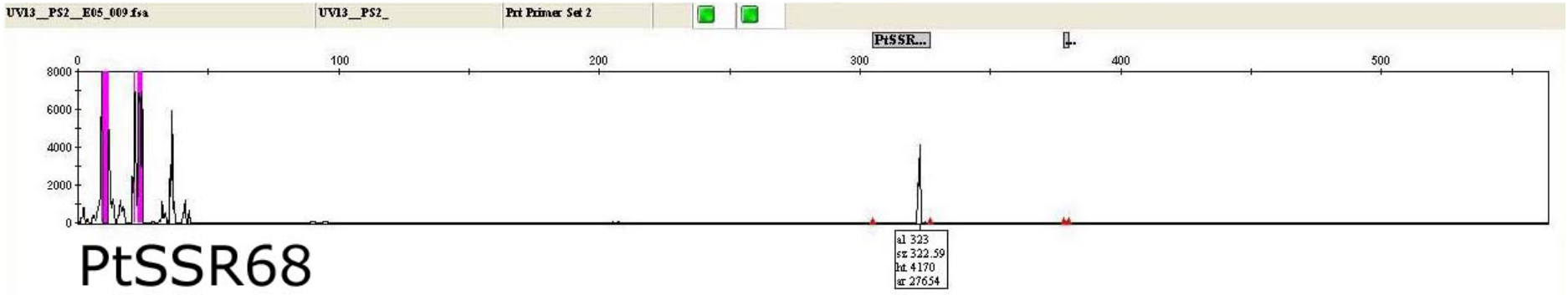
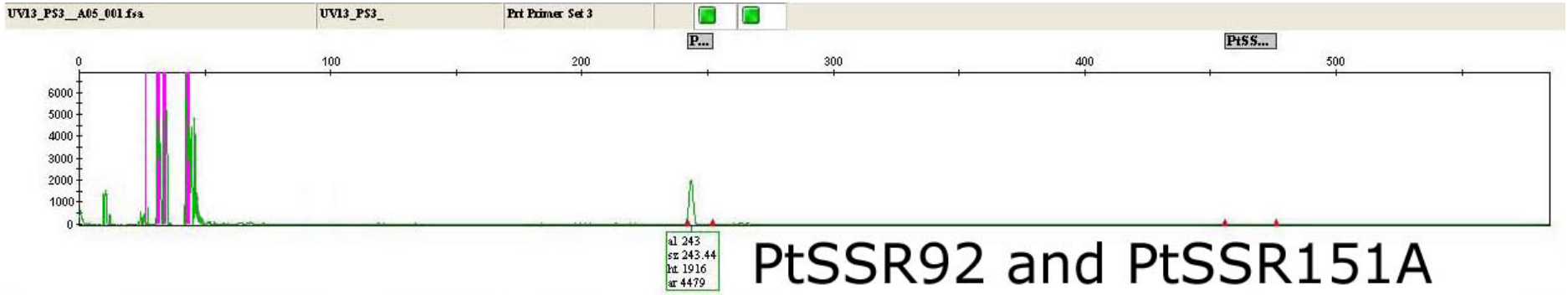
ar 6457
PtSSR55



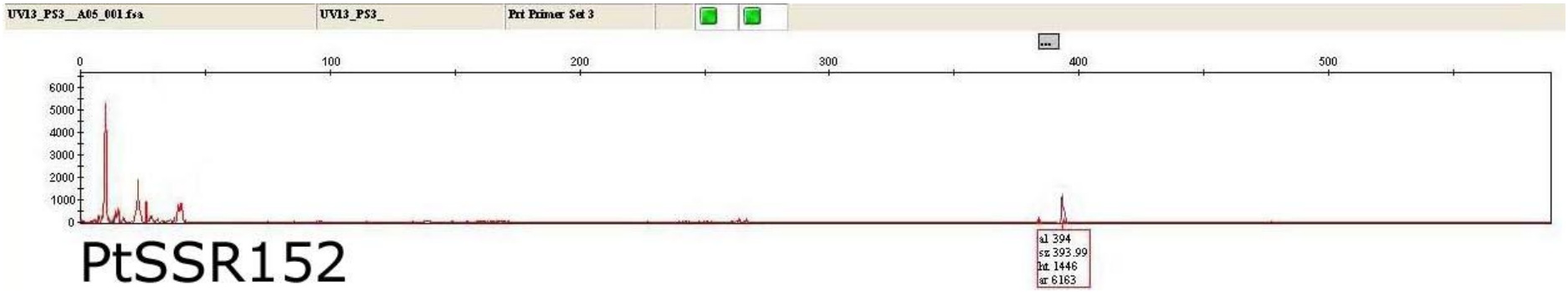
PtSSR61 and PtSSR76



PtSSR68



PtSSR92 and PtSSR151A


UVI3_PS3__A05_001.fsa
UVI3_PS3_
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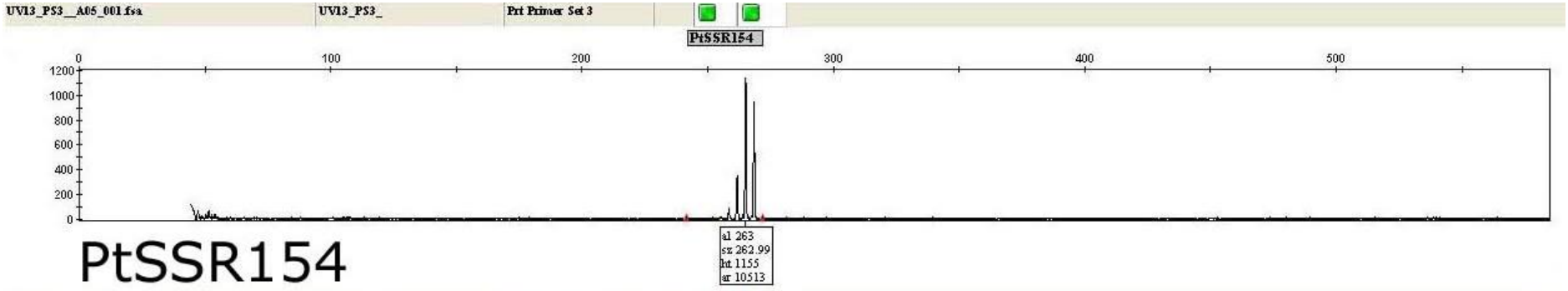
100
200
300
400
500
6000
5000
4000
3000
2000
1000
0
al 394
sz 393.99
ht 1446
ar 6163
PtSSR152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UVI3_PS3_
Prt Primer Set 3
PtSSR154
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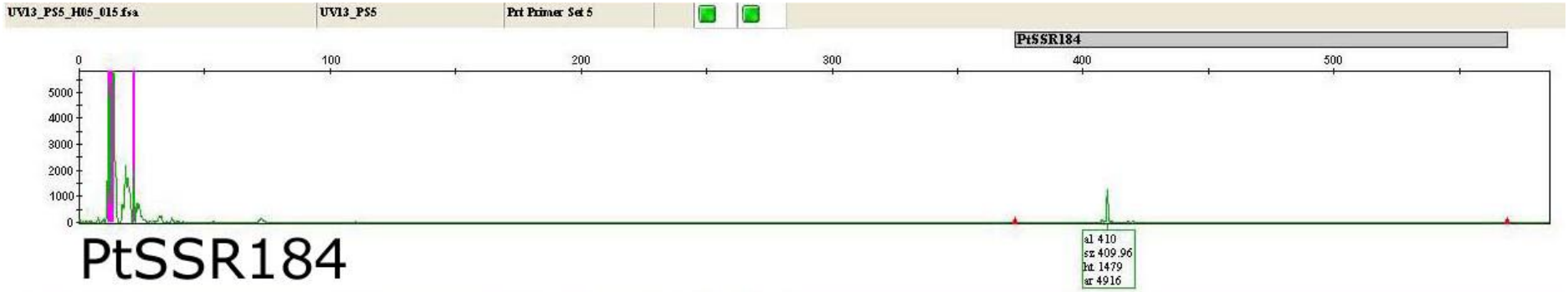
800
600
400
200
0
al 263
sz 262.99
ht 1155
ar 10513
PtSSR154


UVI3_PS5_H05_015.fsa
UVI3_PS5
Prt Primer Set 5
PtSSR184
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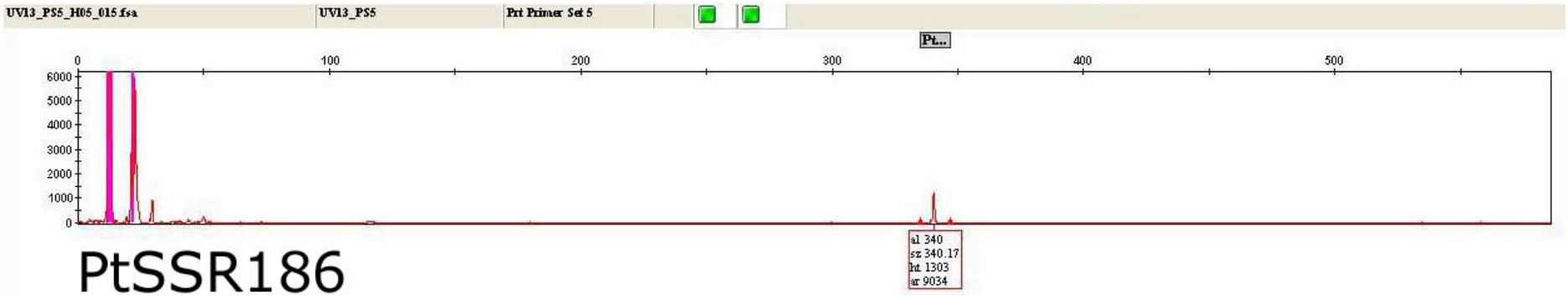
300
400
500
5000
4000
3000
2000
1000
0
al 410
sz 409.96
ht 1479
ar 4916
PtSSR184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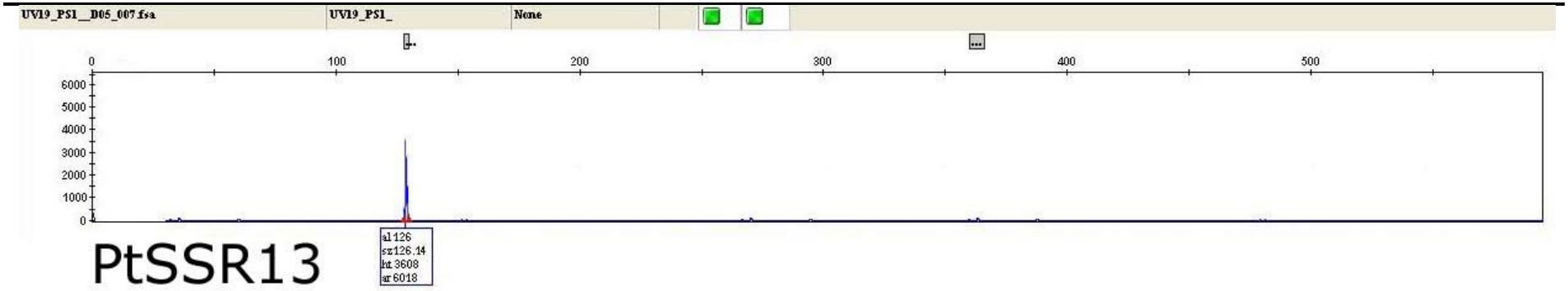
UVI3_PS5
Prt Primer Set 5
Pt...
0
100
200
300
400
500
6000
5000
4000
3000
2000
1000
0
al 340
sz 340.17
ht 1303
ar 9034
PtSSR186

UVPrt19


UV19_PS1__D05_007.fsa
UV19_PS1_
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200
300
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5000
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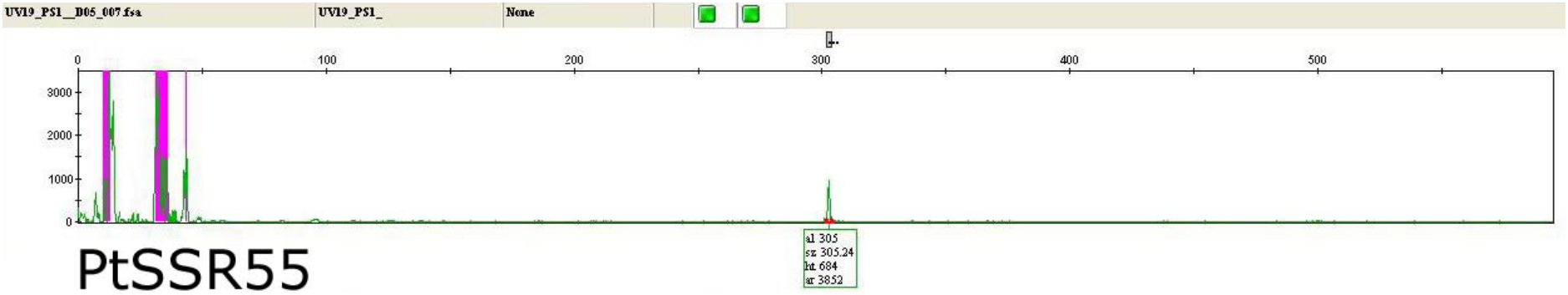
3000
2000
1000
0
al 126
sz 126.14
ht 3608
ar 6018
PtSSR13


UV19_PS1__D05_007.fsa
UV19_PS1_
None
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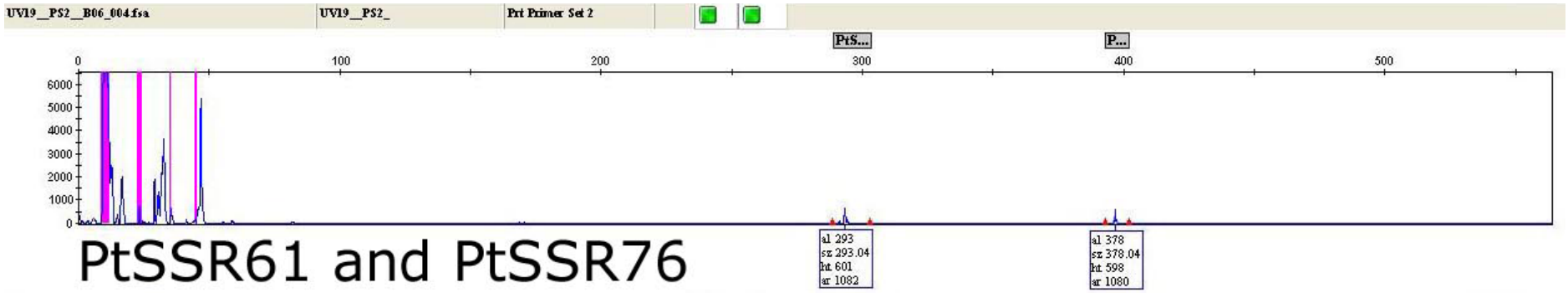
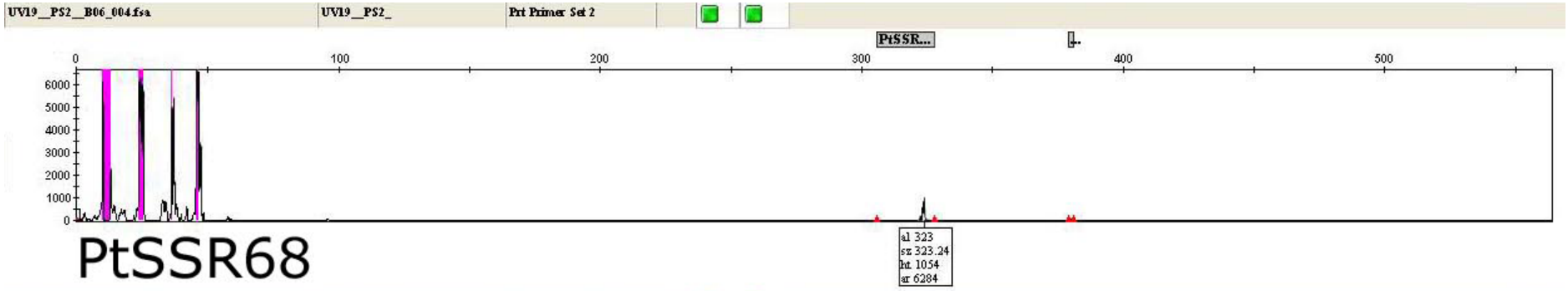
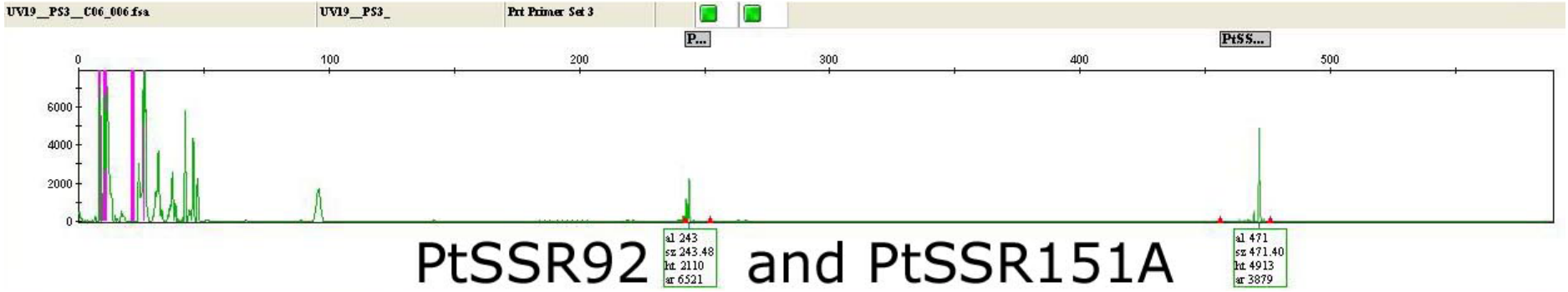
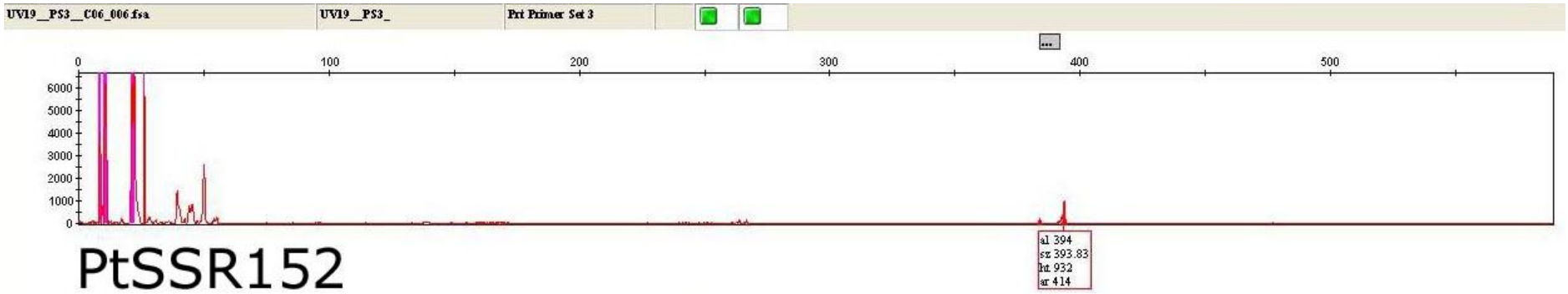
200
300
400
500
3000
2000
1000
0
al 305
sz 305.24
ht 684
ar 3852
PtSSR55


UV19__PS2__B06_004.fsa
UV19__PS2_
Prt Primer Set 2
PtS...
P...
0
100
200
300
400
500
6000
5000
4000
3000
2000
1000
0
al 293
sz 293.04
ht 601
ar 1082
al 378
sz 378.04
ht 598
ar 1080
PtSSR61 and PtSSR76


UV19__PS2__B06_004.fsa
UV19__PS2_
Prt Primer Set 2
PtSSR...
0
100
200
300
400
500
6000
5000
4000
3000
2000
1000
0
al 323
sz 323.24
ht 1054
ar 6284
PtSSR68


UV19__PS3__C06_006.fsa
UV19__PS3_
Prt Primer Set 3
P...
PtSS...
0
100
200
300
400
500
6000
4000
2000
0
al 243
sz 243.48
ht 2110
ar 6521
al 471
sz 471.40
ht 4913
ar 3879
PtSSR92 and PtSSR151A


UV19__PS3__C06_006.fsa
UV19__PS3_
Prt Primer Set 3
0
100
200
300
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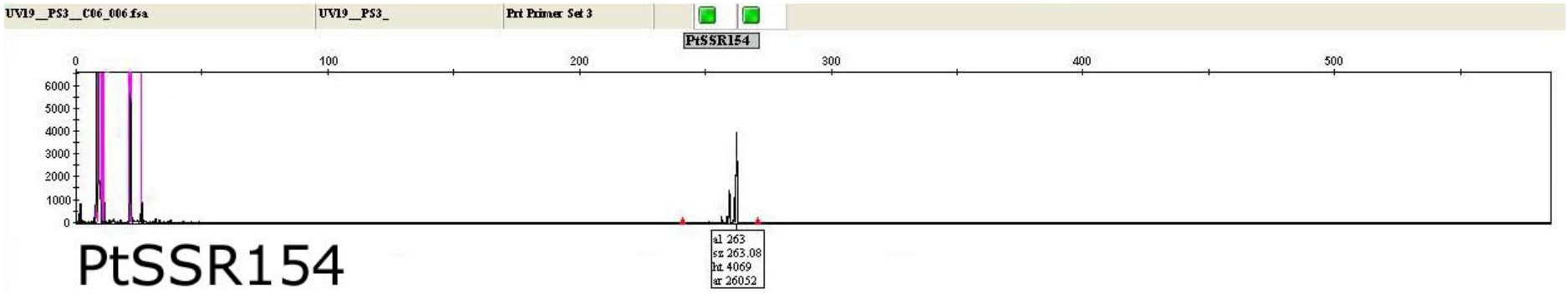
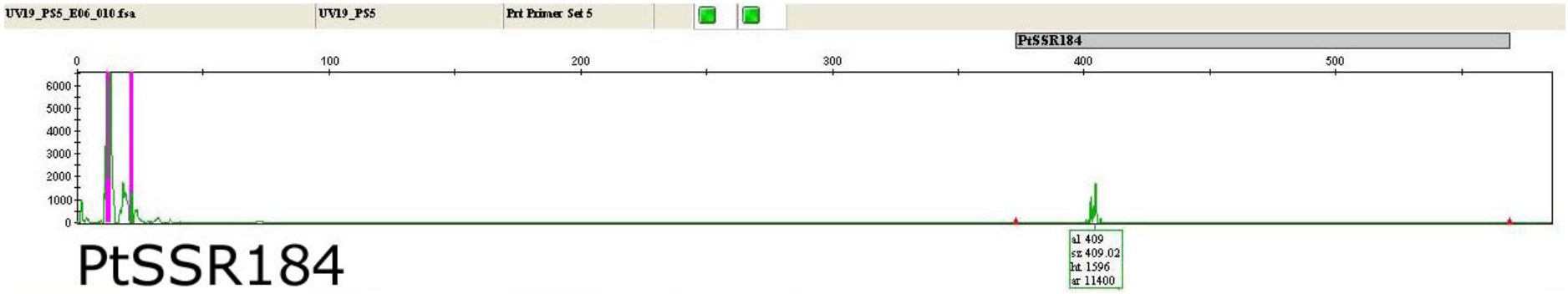
500
6000
5000
4000
3000
2000
1000
0
al 394
sz 393.83
ht 932
ar 414
PtSSR152


UV19__PS3__C06_006.fsa
UV19__PS3_
Prt Primer Set 3
PtSSR154
0
100
200
300
400
500
6000
5000
4000
3000
2000
1000
0
al 263
sz 263.08
ht 4069
ar 26052
PtSSR154


UV19_PS5_E06_010.fsa
UV19_PS5
Prt Primer Set 5
PtSSR184
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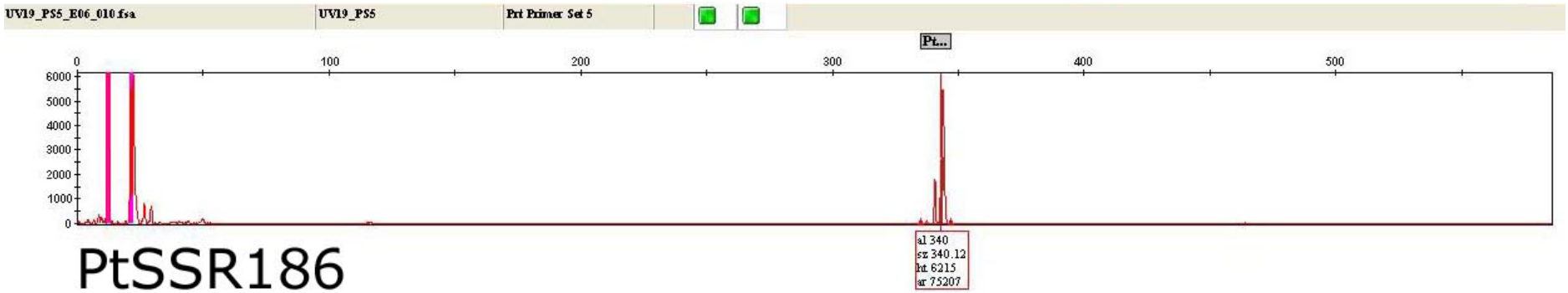
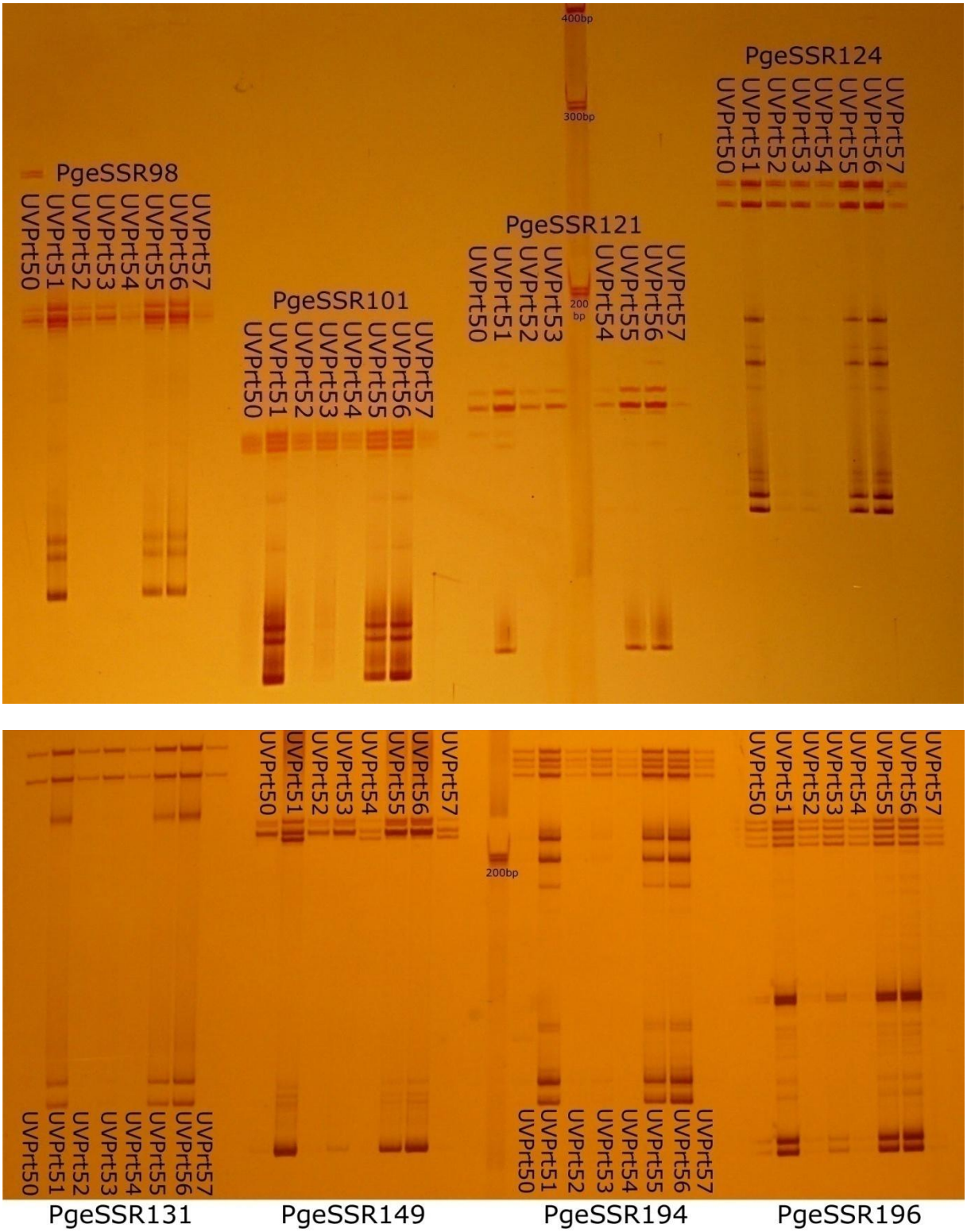
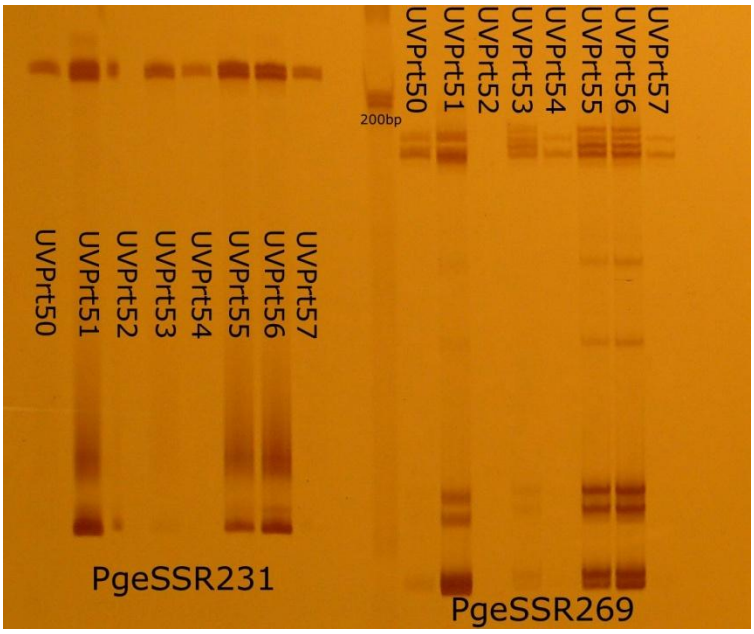
100
200
300
400
500
6000
5000
4000
3000
2000
1000
0
al 409
sz 409.02
ht 1596
ar 11400
PtSSR184


UVI9_PS5_E06_010.fsa
UVI9_PS5
Prt Primer Set 5
Pt...
0
100
200
300
400
500
6000
5000
4000
3000
2000
1000
0
al 340
sz 340.12
ht 6215
ar 75207

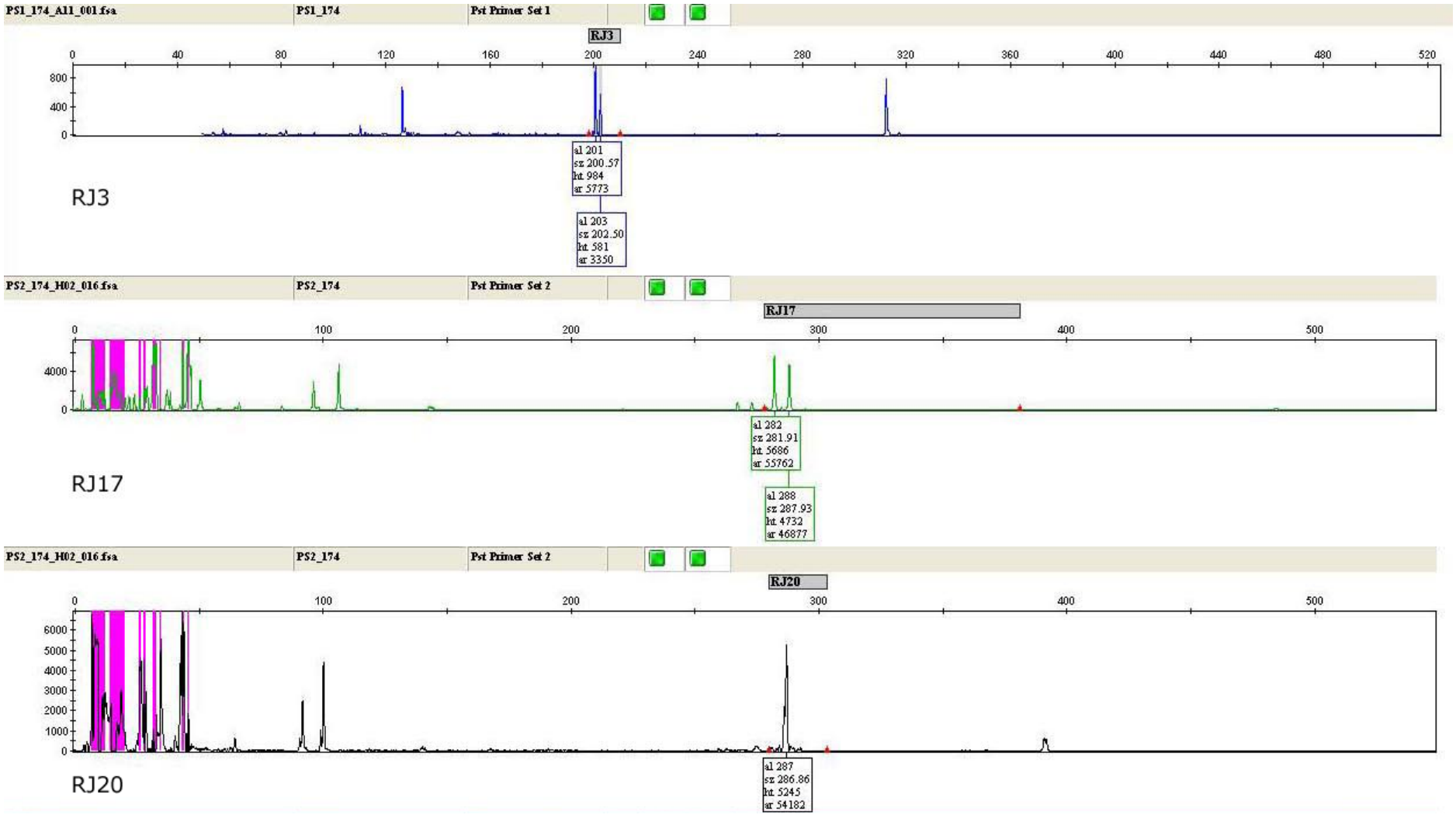
PtSSR186

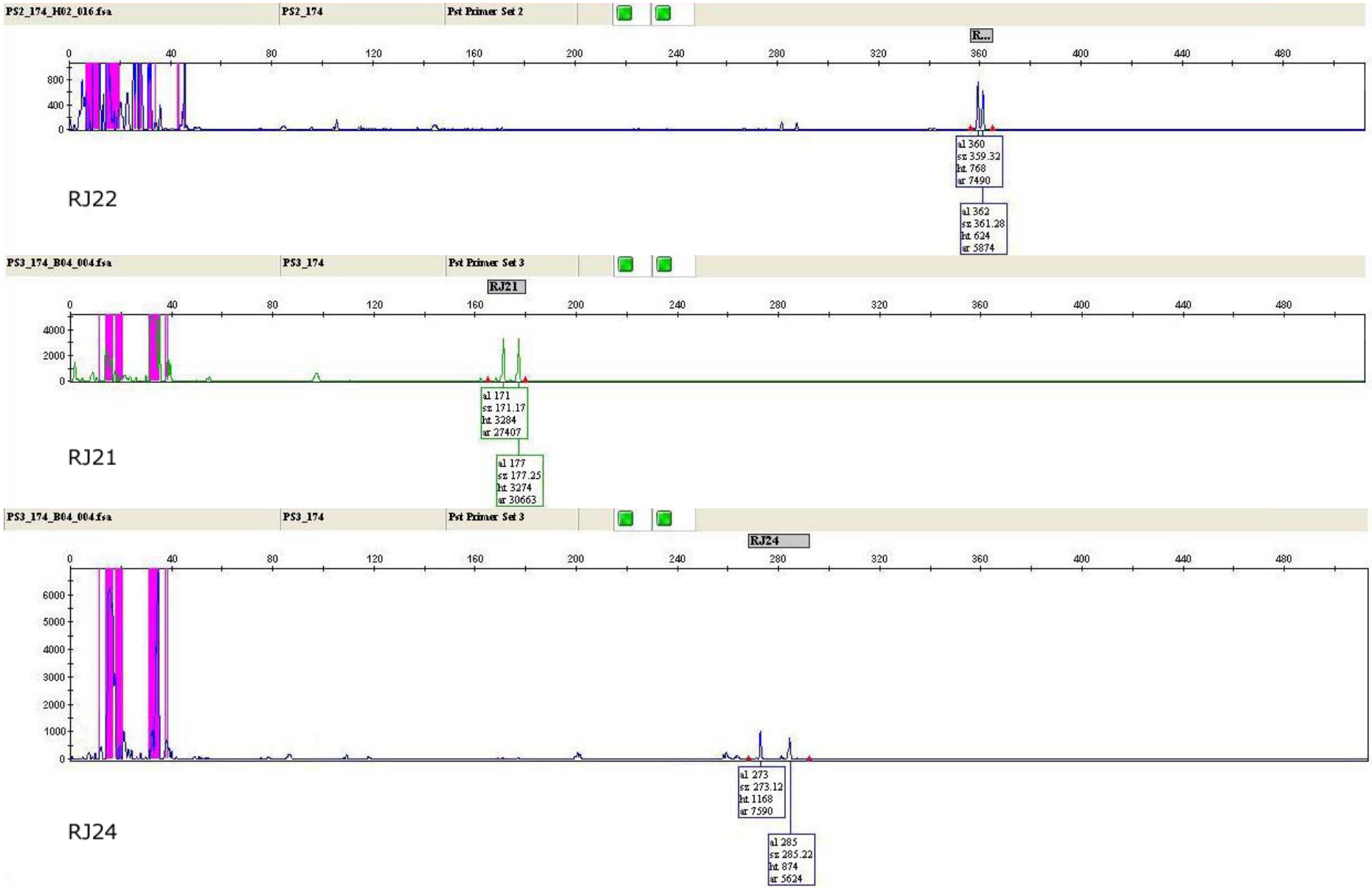
Stem rust

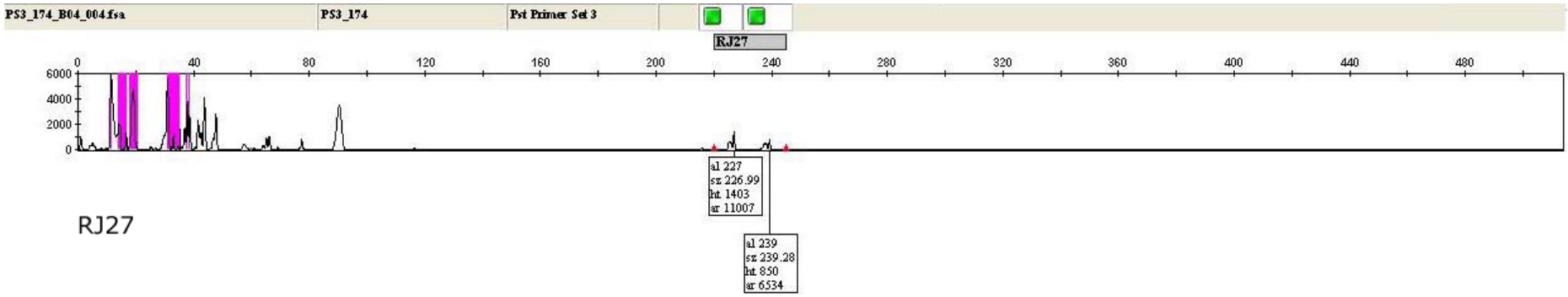

400bp
300bp
200 bp
PgeSSR98
PgeSSR101
PgeSSR121
PgeSSR124
UVPrt50
UVPrt51
UVPrt52
UVPrt53
UVPrt54
UVPrt55
UVPrt56
UVPrt57
200bp
PgeSSR131
PgeSSR149
PgeSSR194
PgeSSR196


UVPrt50
UVPrt51
UVPrt52
UVPrt53
UVPrt54
UVPrt55
UVPrt56
UVPrt57
PgeSSR231
200bp
UVPrt50
UVPrt51
UVPrt52
UVPrt53
UVPrt54
UVPrt55
UVPrt56
UVPrt57
PgeSSR269

Yellow rust
6E16A-

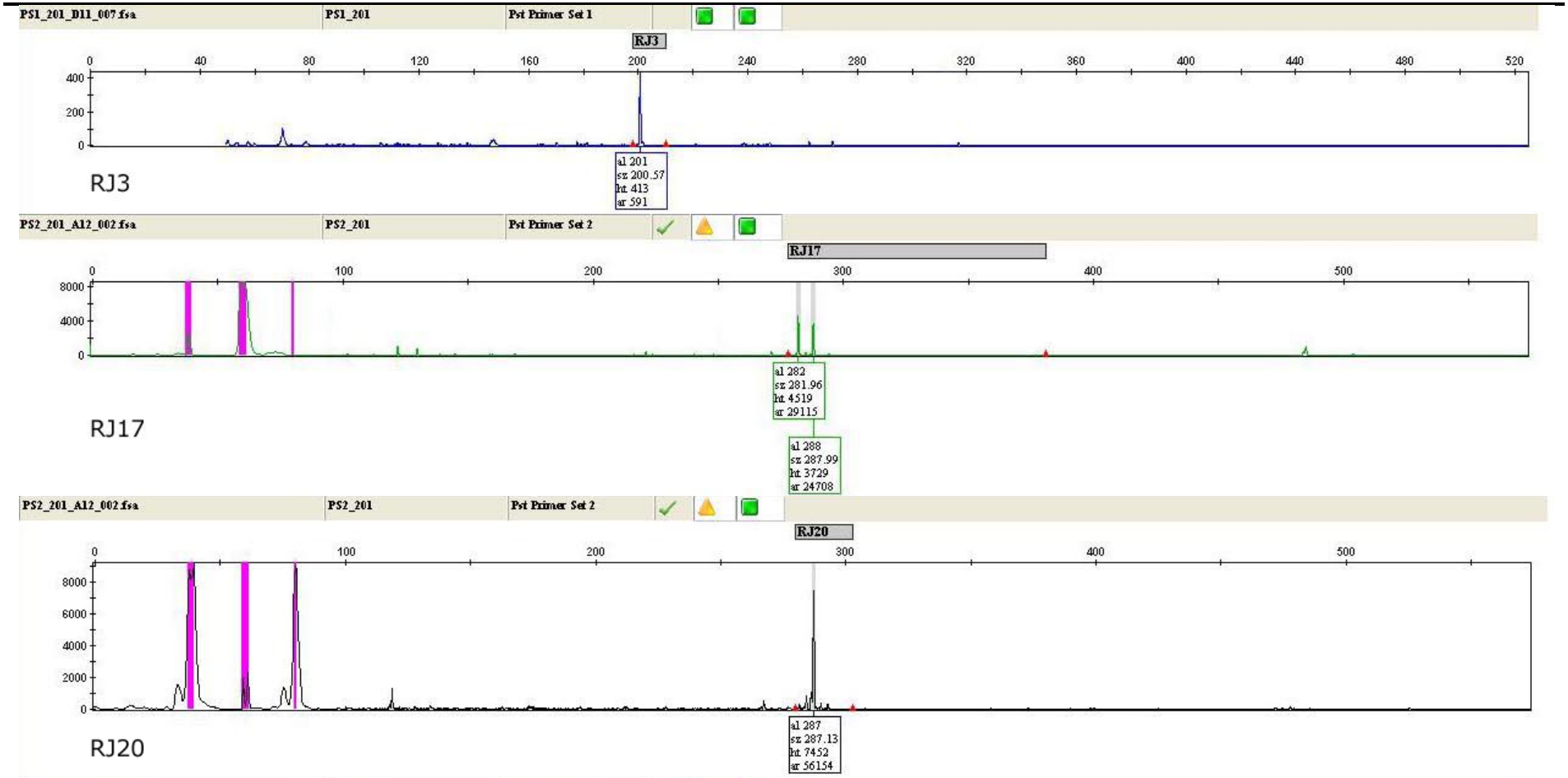


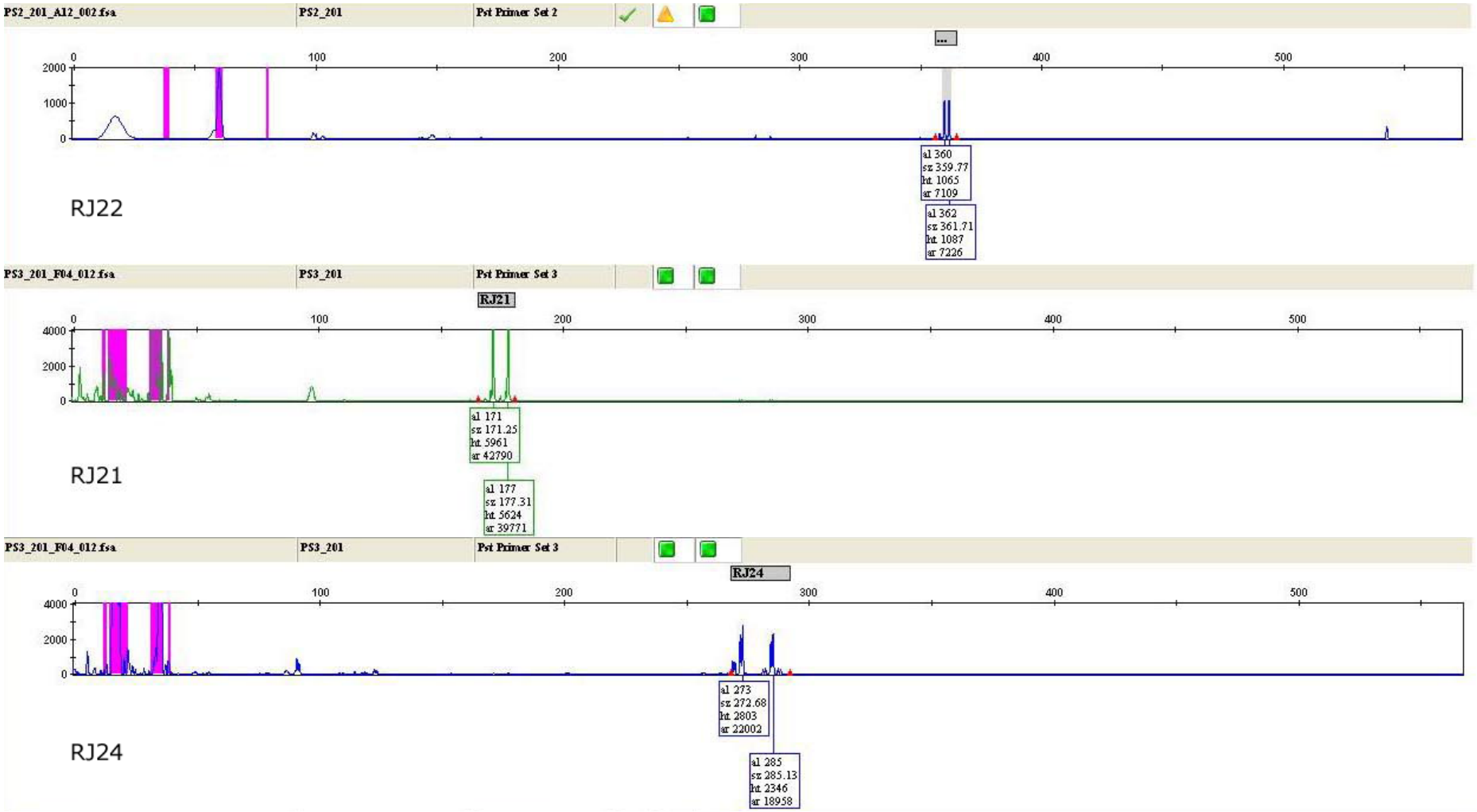

PS2_174_H02_016.fsa
PS2_174
Pst Primer Set 2
R...
al 360
sz 359.32
ht 768
ar 7490
al 362
sz 361.28
ht 624
ar 5874
RJ22
PS3_174_B04_004.fsa
PS3_174
Pst Primer Set 3
RJ21
al 171
sz 171.17
ht 3284
ar 27407
al 177
sz 177.25
ht 3274
ar 30663
RJ21
PS3_174_B04_004.fsa
PS3_174
Pst Primer Set 3
RJ24
al 273
sz 273.12
ht 1168
ar 7590
al 285
sz 285.22
ht 874
ar 5624
RJ24

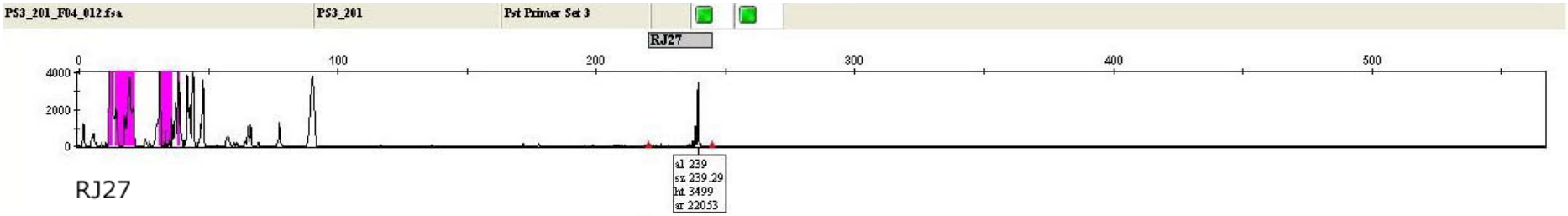

PS3_174_B04_004.fsa
PS3_174
Pst Primer Set 3
RJ27
0
40
80
120
160
200
240
280
320
360
400
440
480
6000
4000
2000
0
al 227
sz 226.99
ht 1403
ar 11007
al 239
sz 239.28
ht 850
ar 6534

RJ27

6E22A-


PS1_201_D11_007.fsa
PS1_201
Pst Primer Set 1
RJ3
al 201
sz 200.57
ht 413
ar 591
RJ3
PS2_201_A12_002.fsa
PS2_201
Pst Primer Set 2
RJ17
al 282
sz 281.96
ht 4519
ar 29115
al 288
sz 287.99
ht 3729
ar 24708
RJ17
PS2_201_A12_002.fsa
PS2_201
Pst Primer Set 2
RJ20
al 287
sz 287.13
ht 7452
ar 56154
RJ20


PS2_201_A12_002.fsa
PS2_201
Pst Primer Set 2
al 360
sz 359.77
ht 1065
ar 7109
al 362
sz 361.71
ht 1087
ar 7226
RJ22
PS3_201_F04_012.fsa
PS3_201
Pst Primer Set 3
RJ21
al 171
sz 171.25
ht 5961
ar 42790
al 177
sz 177.31
ht 5624
ar 39771
RJ21
PS3_201_F04_012.fsa
PS3_201
Pst Primer Set 3
RJ24
al 273
sz 272.68
ht 2803
ar 22002
al 285
sz 285.13
ht 2346
ar 18958
RJ24


PS3_201_F04_012.fsa
PS3_201
Pst Primer Set 3
RJ27
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100
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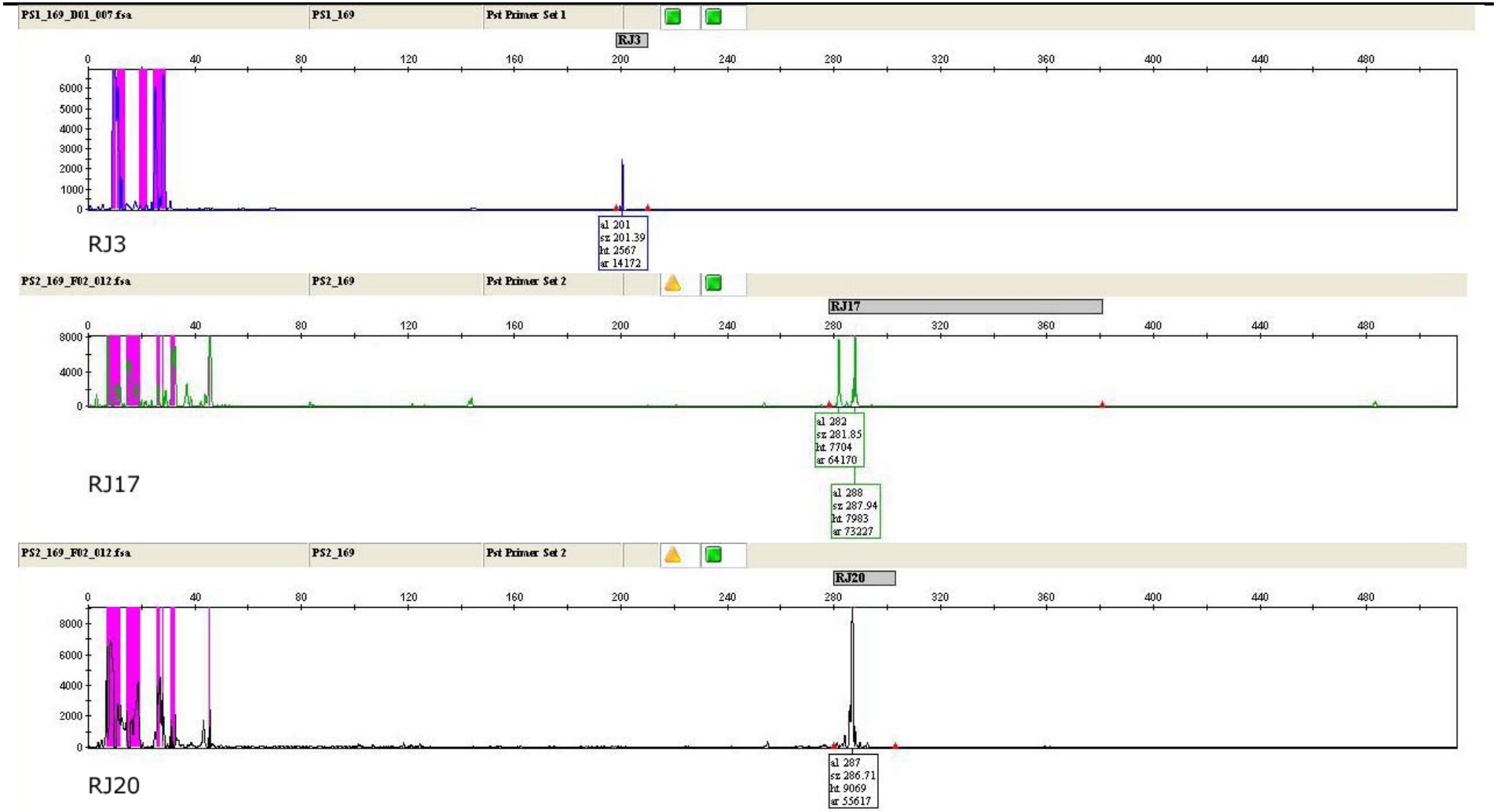
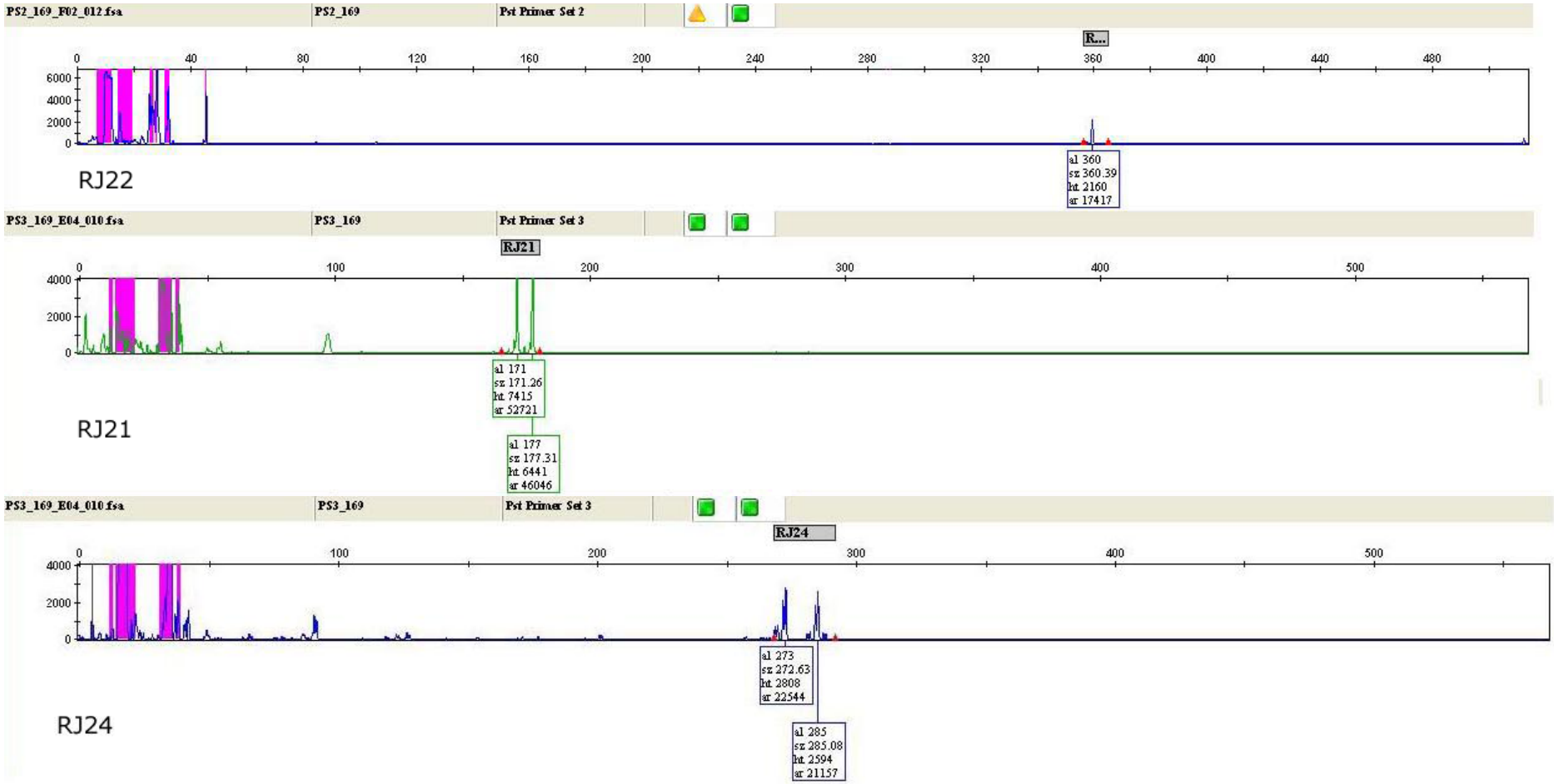
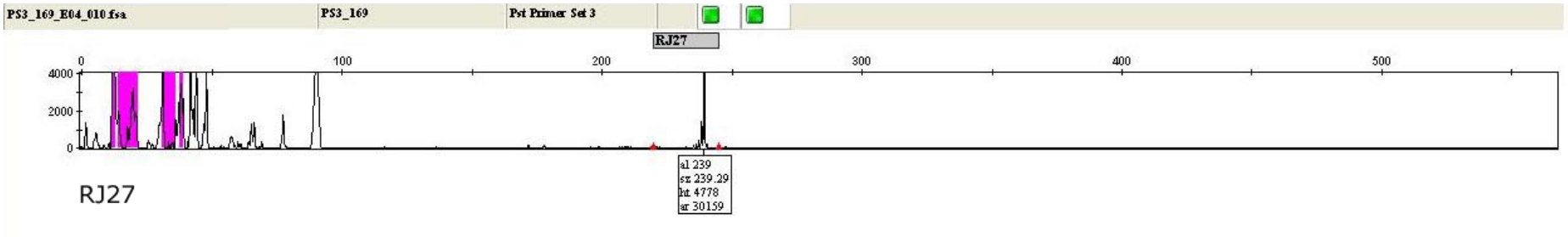
300
400
500
4000
2000
0
al 239
sz 239.29
ht 3499
ar 22053
RJ27

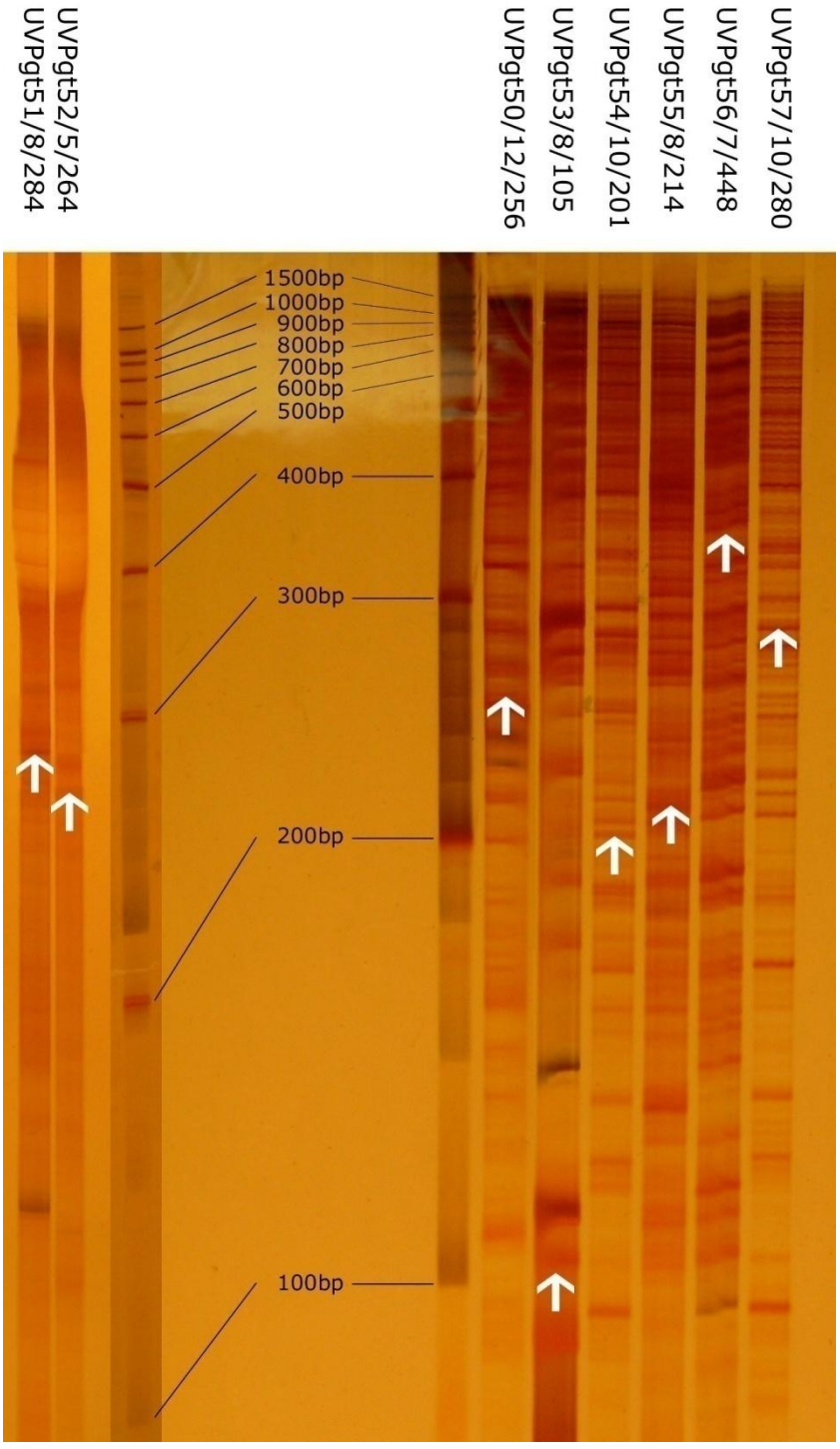
7E22A-

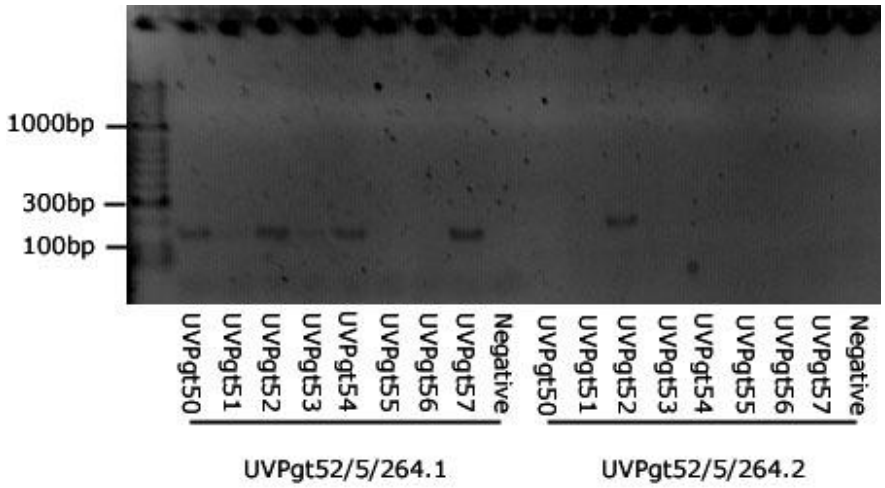

PS1_169_D01_007.fsa
PS1_169
Pst Primer Set 1
RJ3
al 201
sz 201.39
ht 2567
ar 14172
RJ3
PS2_169_F02_012.fsa
PS2_169
Pst Primer Set 2
RJ17
al 282
sz 281.85
ht 7704
ar 64170
al 288
sz 287.94
ht 7983
ar 73227
RJ17
PS2_169_F02_012.fsa
PS2_169
Pst Primer Set 2
RJ20
al 287
sz 286.71
ht 9069
ar 55617
RJ20


PS2_169_F02_012.fsa
PS2_169
Pst Primer Set 2
R...
al 360
sz 360.39
ht 2160
ar 17417
RJ22
PS3_169_E04_010.fsa
PS3_169
Pst Primer Set 3
RJ21
al 171
sz 171.26
ht 7415
ar 52721
al 177
sz 177.31
ht 6441
ar 46046
RJ21
PS3_169_E04_010.fsa
PS3_169
Pst Primer Set 3
RJ24
al 273
sz 272.63
ht 2808
ar 22544
al 285
sz 285.08
ht 2594
ar 21157
RJ24


PS3_169_E04_010.fsa
PS3_169
Pst Primer Set 3
RJ27
0
100
200
300
400
500
4000
2000
0
al 239
sz 239.29
ht 4778
ar 30159
RJ27

Addendum 4: AFLP bands excised for the conversion to SCAR markers and specific SCAR markers




1000bp
300bp
100bp
UVPgt50
UVPgt51
UVPgt52
UVPgt53
UVPgt54
UVPgt55
UVPgt56
UVPgt57
Negative
UVPgt50
UVPgt51
UVPgt52
UVPgt53
UVPgt54
UVPgt55
UVPgt56
UVPgt57
Negative
UVPgt52/5/264.1
UVPgt52/5/264.2

Addendum 5: Sequences of the ITS1 region of the *Puccinia spp.*