Molecular tagging of *Thinopyrum distichum* chromosomes involved in salt tolerance

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

ABSTRACT

Much has been written about the effects of soil salinity on plant growth. Its devastating effects have already been reported 2000 years BC. In the 21st century an alarming 80 million hectares of cultivated land area are affected by salt (Munns, 2002a) and represent a growing threat to agriculture. Salt tolerance is a complex trait moderately expressed in only a few plant genotypes (Ruiz, 2001).

An attempt to transfer salt tolerance genes from the wild grass, *Thinopyrum distichum*, to triticale and common wheat was initiated by Marais and Marais (2003). A study of *Th. distichum* x rye hybrids enabled the authors to identify chromosomes $2J_1^d$, $3J_1^d$, $4J_1^d$ and $5J_1^d$ as being involved in the determination of salt tolerance. Indirect (yet unconfirmed) evidence suggested that $7J_1^d$ might also have a role. A programme aiming to transfer regions of the critical chromosomes to homoeologous triticale chromosomes, which relies heavily on the use of molecular markers, was launched. While an RFLP marker is available for each of the *Thinopyrum* chromosomes, these are not suited for the screening of large numbers of segregates. This study therefore represents an attempt to convert the RFLP markers into less time consuming and cost-effective SCAR markers.

The published DNA sequences of the RFLP probes in question were used as templates to design PCR primers. The PCR reactions were optimised using DNA of *Th. distichum*, rye and their F_1 hybrid. When *Thinopyrum* specific amplification products were obtained, the primers were also tested on a panel of genotypes with and without the target chromosomes. Seemingly polymorphic bands were confirmed by Southern blotting and hybridisation with the corresponding RFLP probes. The primers were also tested on a panel of genotypes that included 'Rex' triticale to ensure that they would also detect a difference in a triticale genetic background during transfer. Polymorphic bands were then isolated and sequenced to further refine the markers. In certain cases, sequences of the same fragment amplified in triticale ('Rex') and *Thinopyrum* were aligned in an attempt to design more specific markers. Using this approach, it was possible to develop chromosome specific SCARs for *Thinopyrum* chromosomes $3J_1^d$ and $7J_2^d$. Three and one set(s) of PCR markers, respectively, have been developed and can be used to unequivocally detect the *Thinopyrum* chromosomes involved in salt tolerance against a triticale background. A SCAR marker was also found for chromosome 6J.

Thus, an attempt was made to convert thirteen RFLP probes to SCAR markers. Only three were successfully converted. The main reason for the low success rate is the syntenic relationships between the genomes of the different cereals that made it an arduous task to find discriminating primer sets. Based on the results obtained, an adapted procedure is suggested for future attempts to develop chromosome specific markers utilizing published sequence information that was obtained for a different species.

OPSOMMING

Baie is al geskryf oor die uitwerking van grond versouting op plantproduksie. Die vernietigende gevolge van versouting is alreeds 2000 jaar VC gerapporteer. In die 21ste eeu is `n geraamde 80 miljoen hektaar (Munns, 2002a) bewerkte land-area sout-geaffekteerd. Die ontstellende verwikkelinge verteenwoordig `n groeiende bedreiging vir die landbou. Soutverdraagsaamheid is `n komplekse kenmerk en slegs enkele plantgenotipes met matige verdraagsaamheid kon nog ontwikkel word (Ruiz, 2001).

`n Poging om soutverdraagsaamheidsgene vanaf die wilde gras, *Thinopyrum distichum*, na triticale en gewone koring oor te dra, is deur Marais en Marais (2003) geïnisieer. `n Studie van *Th. distichum* x rog hibriede het die skrywers in staat gestel om chromosome ($2J_1^d$, $3J_1^d$, $4J_1^d$ en $5J_1^d$) wat bydra to soutverdraagsaamheid te identifiseer. Indirekte (maar onbevestigde) aanduidings is gevind dat $7J_1^d$ ook `n rol mag speel. `n Program is daarna geloods om segmente van chromosome na homoeoloë triticale chromosome oor te dra, `n onderneming wat swaar steun op die gebruik van molekulêre merkers. Alhoewel daar`n RFLP merker beskikbaar is vir elk van die *Thinopyrum* chromosome, is hierdie merkers nie geskik vir die sifting van groot getalle segregate nie. Hierdie studie verteenwoordig `n poging om die RFLP merkers om te skakel na `n minder tydrowende en meer koste-effektiewe SCAR merkers.

Die gepubliseerde DNS-volgordes van die betrokke RFLP peilers is as templaat gebruik om PKR inleiers te ontwerp. Die PKR reaksies is geoptimiseer deur gebruik te maak van DNS van *Th. distichum*, rog en hulle F_1 hibried. In gevalle waar *Thinopyrum* spesifieke amplifikasie produkte verkry is, is die inleiers ook getoets op `n paneel van genotipes met en sonder die teiken-chromosoom. Skynbare polimorfiese bande is bevestig deur `n 'Southern' klad te maak en te hibridiseer met die tersaaklike RFLP peiler. Die inleiers is ook getoets op `n paneel van genotipes waarby 'Rex' triticale ingesluit was om te verseker dat dit ook verskille in `n triticale genetiese agtergrond opspoor (nodig tydens oordrag). Polimorfiese bande is verder verfyn. Dit is geïsoleer en die DNS-volgorde daarvan is bepaal. In sekere gevalle is ooreenstemmende fragmente geamplifiseer in triticale ('Rex') en *Thinopyrum*. Die volgordes is dan bepaal en met mekaar vergelyk in `n poging om meer spesifieke merkers te ontwerp. Met die gebruik van hierdie benadering was dit moontlik om chromosoom-spesifieke SCAR-merkers vir die *Thinopyrum* chromosome $3J_1^d$ en $7J_2^d$ te ontwikkel. Drie en een stel(le) PKR merkers is onderskeidelik ontwikkel en kan gebruik word om ondubbelsinnig te bepaal of die betrokke *Thinopyrum* chromosoom segregeer in `n triticale kruising. `n SCAR merker is ook gevind vir chromosoom 6J.

Dus, daar is probeer om dertien RFLP peilers na SCAR merkers om te skakel. Slegs drie van die pogings was suksesvol. Die hoofrede vir die lae sukseskoers is die hoë graad van sintenie tussen die genome van die verskillende grane wat dit `n moeilike taak gemaak het om diskriminerende inleierstelle te ontwerp. Op grond van die resultate word `n ietwat gewysigde prosedure vir die toekomstige pogings om chromosoom-spesifieke merkers te ontwerp met gebruik van gepubliseerde volgorde inligting vanaf `n ander spesie, voorgestel.

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ABBREVIATIONS USED

°C	Degree centigrade
µg	Microgram
µl	Microliter
µM	Micromolar
A	Adenine
ATP	Adenosine triphosphate
bp	Basepair
C	Cytosine
cDNA	Complementary DNA
cM	Centimorgan
cm	Centimeter
dH_2O	Distilled water
dm^3	Cubic decimeter
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
dS/m	Deci-siemens per meter
EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
EM	Electromagnetic conductivity
EST	Expressed sequence tags
EtBr	Ethidium bromide
G	Guanine
g	Gram
H^+	Hydrogen
ha	Hectares
K^+	Potassium
kb	Kilo base
kDa	Kilodalton
kg	Kilogram
LB	Luria Bertani
m^3	Cubic meter
$MgCl_2$	Magnesium chloride

Mg	Milligram
Min	Minutes
ml	Milliliter
mM	Millimolar
mm	Millimeter
mV	Millivolt
Na^+	Sodium
NaCl	Sodium chloride
ng	Nanogram
PCR	Polymerase Chain Reaction
pmol	Picomole
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SCAR	Sequence characterized amplified regions
Sec	Seconds
STS	Sequence tagged sites
T	Thymine
Ta	Annealing temperature
Taq	*Thermus aquaticus* DNA polymerase
Th.	*Thinopyrum*
Tm	Melting temperature
TBE	Tris borate EDTA buffer
UV	Ultra violet
V	Volt

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CHAPTER 1

INTRODUCTION

1.1 SOIL

Soil is the result of the continual wearing of rocks over billions of years by wind, rain and the activities of organisms. Vertical subterranean profiles tell the story of 4.5 billion years of weathering. The parental materials of soil, metamorphic rocks, are distinguishable at a shallow 120 cm. The next region, at a depth of 60 cm, consists of larger soil particles followed by a layer (10-30 cm below the surface) with more refined particles that is darker in colour attributed to the organic matter content in this region (Moore *et al*, 1998b).

Soil is the backbone of support and stability for the prosperous existence of most plant species (Wallace *et al*, 1996b). It varies in constitution (a predominate clay, sand or silt foundation) and thereby is restricted to support only specific plant species (Ghaly, 2002). Plants rely on the soil for an adequate supply of different nutrients crucial for growth and development. Soil also determines the animal population in specific regions, as they in turn are dependant on nutrition, rich in certain elements, acquired by and stored in the plants' system. Soil is therefore the initiation point of the eco-infrastructure that relays essential elements in the food chain (Moore *et al*, 1998b).

Plants are very particular to the available elements in the soil. The quantity required varies between plant species. Magnesium, potassium, oxygen and carbon are only some of the 16 elements that are required in large amounts. These elements play a crucial role in enzyme and metabolic activities and are also the building blocks of cellular units. Apart from these elements, also called macronutrients, certain elements are only required in small quantities such as chlorine, zinc, boron and iron. These elements are also referred to as micronutrients. Generally, sodium also falls into this category (Moore *et al*, 1998b).

1.2 SOIL EROSION

Soils are extensively influenced by environmental disturbances. For example the soils of the tropical rainforest have a high iron content and require a damp environment. Burgled from its shade when deforestation occurs, the soil dehydrates and it consequently hardens. This condition, induced by man, is irreversible (Moore *et al*, 1998b). Although certain forms of erosion, like the Grand Canyon, are scenically delightful, erosion is a serious problem (Moore *et al*, 1998b) especially in regions of cultivation.

1.3 SOIL SALINITY

Over the course of time environmental constraints endorsed by man disturbed the normal ambience of the soil structure, which in turn affected plant growth (Wallace *et al*, 1996b). Agriculture employed more vigorous plant species to shield against environmental disturbances but in the process they overlooked the physiological decline of the soil (Richards, 1992, as reviewed by Tester and Davenport, 2003). Soil salinity is a major environmental stress that hinders the plants' biological functioning. It has been reported in historical documents of earlier than 2000 BC and its effects have been severe, as it is believed to be the cause of the disruption of the ancient Sumerian civilization (Singh and Chatrath, 2001). The world is on a fast track of repeating history in the 21st century as the problem has escalated to the extent that a land area of about 1000 million ha (7 % of the worlds' land area) is salt-affected (Munns, 2002a). This vast area is put into perspective by Metternicht and Zinck (2003) as the authors compares it to be a tenfold equivalent to the size of the country of Venezuela.

A more eminent concern is the present degradation of approximately 80 million ha land (6 % of the worlds' cultivated land) used for cultivation (Munns, 2002a). Alarmingly, cultivated regions under irrigation make up almost two thirds of this estimate (Metternicht and Zinck, 2003). Compiled FAO (Food and Agriculture Organization of the United Nations) data of 1987, shows that more than 30 % of irrigated land in Argentina, Egypt and Iran is salt affected. In Australia and South Africa about 9 % of arable land is salt affected (Munns 2002b).

Irrigation contributes a great deal more to total production (almost double the yield) than rain-dependant land. The mere 230 million ha land under irrigation (amounts to 15 % worldwide) reinforces the global food supply by approximately 35 %. Unfortunately, more than half the existing irrigation systems worldwide, struggle with salinity and water logging (Munns, 2002a).

1.4 ECONOMICAL IMPLICATIONS OF SOIL DEGRADATION

The loss of fertile land results in an annual deprivation of the global food resources of more than one million tonnes of grain (Singh and Singh, 1995). This is only one of the many constraints that prevent the global food resource to keep pace with the progressive requirements of the world population. Agricultural capacity is approaching the danger zone of starvation as global food sources in the last decade were required to fulfil the needs of an estimated 10 % larger world population (Slafer and Araus, 1998) with wheat yields that went down by 2 % from the mid seventies to the mid nineties (Braun *et* al, 1998). Only a few decades ago (referred to as the "Green Revolution" period) genetic achievements caused excessive gain in wheat yield, which even exceeded the rapid population growth rate during this period. Over the past 10 years, the population growth increased substantially without a parallel increase in wheat yield in developed and developing countries. One of the contributing factors is environmental constraints (Slafer and Araus, 1998).

The extent to which farmers are affected by the wearing down of cultivated land reflects financially (Singh and Singh, 1995) and therefore the migration of farmers from rural to urban areas is an escalating phenomenon. As the environmental constraint becomes a reality, farmers are faced with immense problems that take on a "snowball" effect: with the decrease of arable land follows the increase in financial disabilities, follows the decrease of manpower. And as labour employment in agriculture is approaching a downhill, due to abandoned, erosive land, global poverty progresses. A study conducted in India determined a decline in labour employment of approximately 60 % and 70 % in wheat and paddy (rice), respectively, due to salinity. Water logged lands attributed similar estimates regarding to paddy with a decline of 23 % in labour in wheat fields (Singh and Singh, 1995). The economical loss is not ciphered in thousands but in millions of US dollars. The annual damage by secondary salinization in the Murray-Darling Basin in Australia is estimated at US$208 million. The damage in the Punjab and Northwest Frontier Provinces in Pakistan are perceived as US$300 million and in the Colorado River Basin in the United States the damage is on the verge of US$ 1 billion (Metternicht and Zinck, 2003). These are only examples of single regions in Australia, Pakistan and the United States.

1.5 CAUSES OF SALINIZATION

The substantial loss of wheat yield is ascribed to the exhaustion of productive land as a result of incompetent agricultural practices of irrigation. These systems were employed without an interactive system for the discharge of excess water and resulted in the accumulation of various salts in the soil (Datta *et al*, 1998).

1.5.1 Primary salinization

Irrigation systems are the media through which water from different geographic regions are channeled to cultivation and are also the inevitable transmission pathway of various solutes to the soil. The effortless flow of salt has over the years deposited considerable amounts of salt in the forms of chlorides and carbonates of sodium (most soluble), calcium and magnesium. These salts are the result of the exfoliation of small rocky particles that dissolves in the water that passes rocky matter. The amount of salt in water can vary from 200-500 mg/L, equivalent to 0.2-0.5 tonnes of salt per 1 000 m^3 (1 m^3 = 1000 L) irrigation water. Based on a maximum annual crop water requirement of 10 000 m^3 per hectare, the addition of salt amounts to approximately 5 tonnes to a single hectare. Salts, particularly sodium chloride, can also be transported through wind and rain which can deposit an average 10 kg/ha of salt at a annual rainfall rate of 100 mm, depending on the distance from the coastline. The transmission of salt by water and atmospheric conditions (wind and rain) occurs over time periods spanning thousands of years and is defined as primary or natural salinization (Munns, 2002b).

1.5.2 Secondary salinization

Man has also played a significant role in the deterioration of soil quality. An attempt to expand land cultivation resulted in substitution of the native, deep-rooted plant species (trees, shrubs) with shallow rooted plant species (crops). This re-vegetation as well as the improper regulation of water input (irrigation, rainfall) and output (drainage, plant transpiration, evaporation) disrupted the ground water level. Accompanied by the ascending ground water level, salt from the subsoil commenced to the surface where it accumulated and created a hazardous living environment for the plant. Human induced salinization is also referred to as secondary salinization (Barrett-Lennard, 2003; Munns, 2002b; McFarlane and Williamson, 2002).

1.6 RESTORATION OF SALINE LAND

Scientists predict that by 2020 the annual demand for wheat will rise to more than twice the present production of approximately 500 million tonnes (Braun *et al*, 1998). To achieve this, new wheat varieties need to endure besides biotic stresses, which cause an annual wheat loss of 20 % (Feuillet and Kellar, 1998), also abiotic stresses such as drought and salinity. To meet (let alone surpass) these requirements by the year 2020 scientists particularly need to exploit to the world's natural genetic resources, identify physiological adaptations needed to overcome abiotic stresses and implement revolutionary changes to prevent future disasters (Braun *et al*, 1998).

Reclaiming fertile land for crop production starts with the rehabilitation of saline land through advanced on-farm management. The foremost important improvements need to be the quality and quantity of water used for irrigation as variation in these two components has a direct effect on wheat yield. Literature advises the use of drip irrigation (El-Kady and El-Shibini, 2001; Munns, 2002a). Supplemented with adequate drainage at surface and subsurface level drip irrigation has proven to be effective for the discharge of excess water and leaching of excess salt (Datta *et al*, 1998, 2000; Khosla and Gupta, 1997). A case study reports the cultivation of previously fallow land in Haryana, India, with a success rate of up to 100 % in certain regions, achieved during the seven-year study period. Previously, Haryana land was not cultivated over the summer season (mid April-mid June) due to the high water table that caused water logging and salinization. The implementation of drainage enhanced crop production and promoted farming of more profitable crops (wheat exchanged for barley) that produced higher yields. Substantial increases in wheat yield (up to 200 %) created new employment for farm labourers and ensured financial gain for farmers (Datta *et al*, 2000). The reintegration of native, deep-rooted perennials (trees and shrubs) in land predominated by shallow-rooted annual crop plants can also aid in the retrogression of the water table and leaching of salt (Barrett-Lennard, 2002). However, perennial species must be selectively chosen as Slavich *et al* (1999) found that the use of the highly salt tolerant, native to clay soil, old man saltbush (*Atriplex nummularia*) succeeds to a lesser extent as a bioremedial plant species for the removal of salt from the topsoil.

Ghaly (2002) applied such a perennial replacement strategy and reported a decrease in salinity levels in northern Egypt. The salinization they encounter affects almost 500 000 feddan (an Egyptian unit of land area equivalent to 0.42 hectare) and the problem is aggravated by the clay soil structure that limits drainage. They resorted to grasses (*Phragmites communis* and *Panicum repens* –these grasses' growth pattern in drainage waterways suggest tolerance of high salt conditions) in an attempt to reclaim the land. Use of the perennial grasses was more effective in reducing soil salinity than leaching over the two-year study. Oram *et al* (2002) reports that tall wheat grass *Thinopyrum ponticum* (Podp.) and *Aegilops tauschii* Coss. have been more successful in overturning the rising ground water level in deforested grazing lands in southern Australia than the drought-resistant grass, phalaris (*Phalaris aquatica* L.). In an experiment that mimicked a saline environment equivalent to the salt strength of one quarter sea water, the reduction in dry weight of *Th. ponticum* and *Ae. tauschii* were, respectively, 4 and 2 times lower than that of phalaris (Oram *et al*, 2002).

However, reclamation of saline land to its natural state is expensive and time-consuming. For instance the wheat yield produced by remediating land is only equivalent to that of non-affected land 3-4 years after drainage installation. There is a need to develop cost-effective and less time and effort consuming methods (Ashraf, 1994; Datta *et al*, 2000).

1.7 PHYSICOCHEMICAL SOIL PROPERTIES

Overall, saline soil has abundant sodium content that threatens plant growth and agricultural sustainability (Munns and Termaat, 1986, as reviewed by Blumwald *et al*, 2000). Sodium is a positively charged monovalent ion that binds to the negatively charged clay particle of the soil complex. Prolonged periods of salt addition to the soil cause the displacement of divalent cations, which keeps the soil particle intact. Overloading of the clay particle with sodium causes slackening of the aggregates, eventually the structure breaks down and the smaller clay particles disperse. Leaching attempts with fresh water aggravates the problem as it washes the dispersed clay particles to lower soil profiles where it obstructs "pores" in the soil and hinders the permeation of water to the deeper subsurface (Munns, 2002b). This results in the steady rise of the water table and furthermore the mobilization of salt from the subsoil profiles to the root zone of the plant where it has hazardous effects. In the event that salt precipitate at soil surface it is visualized in the forms of crystals, powder or crusts with a white, black or brown colour (FAO AGL, 2000b).

1.8 MEASUREMENT OF SOIL SALINITY

Climate, shallow watertable (approximately 2 meters below the soil surface), groundwater inundation, topographical and geological conditions, the exploitation of land and impoverished soils are some of the factors that are used as indicators as to whether certain land areas are venturing into hazardous salinization levels (McFarlane and Williamson, 2002). Instruments have been developed to assist agriculturalists in determining the extent to which soils are contaminated by salt.

Small-scale measurement of soil salinity is done with an electrical conductivity (EC) meter. The level of salinity in soil is determined by preparing a sample (1 g) of the soil. First it is dehydrated, then it is hydrated again with deionised water (5 g). In the saturated solution, a hand held apparatus is placed that measures the EC. Expressed in the metric unit of dS/m, an EC=1 dS/m is equivalent to approximately 10 mM NaCl (Munns, 2002b). Saturated soils with an EC≥ 4 dS/m and pH≥ 8.5 are in general considered saline (Sairam *et al*, 2002). These soils are also characterized by a sodium chloride content exceeding 0,1 % in loam and coarser soils and 0,2 % in clay loams and in clays (McFarlane and Williamson, 2002).

The large-scale measurement of the extent of salt infestation is by means of an electromagnetic conductivity (EM) meter. Two kinds of instruments are used for the bulk measurement of soil salinity. One is ground bound and the other calculates salinity from the air. The apparatus at the ground level has the potential to calculate salinity at a vertical and horizontal depth of 1.5 m and 0.75 m, respectively. The instrument consists of a small transmitter and receiver coil. The energy unit that activates the transmitter creates a current that forms the primary magnetic field in the ground. The currents produced from this field sets off smaller secondary magnetic fields that are proportional to the soil conductivity. Both the primary and secondary fields are measured by a receiver coil nearby (Munns, 2002b).

In surveys done from the air, the transmitter is attached to the base of an aeroplane. The aeroplane creates the primary magnetic field that passes through the ground. Once conductive soil is encountered the secondary field is produced. All the data are collected by the receiver coil pulled along by the aeroplane and the EM is determined along with the values obtained from the chemical properties of the soil (Munns, 2002b).

1.9 MEASUREMENT OF SODIUM FLUXES IN THE XYLEM

Scientists have recently stumbled upon a natural "apparatus" that determines three aspects of salinity in a single action. Through this "apparatus" accurate information about the sodium and potassium content in the transpiration stream of the plant can be obtained, which in turn supplies valid information about the saline stand of the soil, and the salt exclusion ability of the plant (Watson *et al*, 2001).

To determine the sodium content in the transpiration stream, the xylem fluids need to be extracted. Direct extraction of the xylem sap was previously attempted but not with great success, due to the large negative pressure present within this tissue. Results obtained from indirect measurement methods (through root secretion) were also unreliable as the flow rate and driving forces in these regions are not a reflection of those in the highly pressurized transpiration stream. The natural "apparatus" or spittlebug overcomes all these obstacles and extracts xylem sap without any difficulty. It is an insect by the name of *Philaenus spumarius* (Cercopidae) that appears in large numbers during the summer season of the United Kingdom. It is a polyphagous insect that feeds from the full hydraulic tension of the transpiration stream of more than 500 different host plants (Watson *et al*, 2001).

In an experiment done by Watson and colleagues (2001) they permitted the insect to feed on a section of a stem perfused with artificial xylem sap and found that the potassium and sodium content in the excreta of the insect were similar to that of the xylem sap. The spittlebug was used to extract the xylem sap to determine the sodium content in two wheat species with similar transpiration rates but diverse salt tolerance. These wheat species were grown in 150 mM salt solution and the isolated excreta obtained from the insect that fed on the xylem sap of the salt tolerant wheat, *Triticum aestivum* vs. Chinese Spring, and salt-sensitive, *Triticum turgidum* vs. Langdon, showed a sodium concentration of approximately 3 mM and 10 mM, respectively. These results are supportive evidence for the effective salt exclusion ability of Chinese Spring roots and viability of the spittlebug to be used as a means to determine ion fluxes in the transpiration stream of plants (Watson *et al*, 2001).

1.10 PASSIVE MOVEMENT: ENTRY OF SODIUM

The plasma membrane of the root cells of a plant is continuously bombarded by the stealth movement of sodium ions to the interior of the plant. The inherent ability of the membrane to control the bias movement of solutes across the membrane filters 95-99.8 % of the salt from water uptake (Barrett-Lennard, 2002). The "concealed" influx of the 0.2-5 % salt is due to the chemical similarities the micronutrient, sodium, shares with the macronutrient, potassium and therefore enters the cell through transport proteins specific for potassium. The similarities (based on the hydrated ionic radii) enable sodium to "hijack" protein binding sites dependant on activation by potassium. As sodium cannot supersede the duty of potassium, it inhibits protein dependant cellular functions and protein synthesis cannot commence (Bhandal and Malik, 1998, as reviewed by Tester and Davenport, 2003). The effect that a high salt concentration has on enzymatic processes in the salt sensitive plants (glycophytes) is also evident in the salt tolerant (halophytes) plants (Flowers *et al*, 1977, as reviewed by Blumwald *et al*, 2000). The energy independent influx of sodium is encouraged by the electrochemical potential of –140 mV (Higinbotham, 1973, as reviewed by Blumwald *et al*, 2000) generated by the difference of solute concentrations across the plasma membrane. The low sodium content in the cytosol drives the influx of the element from the high exterior soil concentration. Consequently the solutes accumulate in the cytosol and cause a biochemical perturbation in the potassium/sodium ratio, which under normal physiological conditions are kept at 100-200 mM and 1-10 mM concentrations, respectively (Binzel *et al*, 1998, as reviewed by Blumwald *et al*, 2000). An increased sodium concentration of more than 100 mM suppresses the synthesis of proteins in the cell (Wyn Jones and Pollard, 1983 as reviewed by Blumwald *et al*, 2000).

1.10.1 Plasmolysis

Water uptake is limited when the solute concentration in the surrounding environment of a plant cell exceeds the solute content in the cytoplasm. In this condition the water potential is higher inside the cell and results in the efflux of water. This is a huge problem as the pressure, maintained by the water inside the cell, decreases (Yokoi *et al*, 2002). This pressure, also called turgour pressure, is vital to young cell proliferation. The continuous loss in turgour pressure results in plasmolysis, a phenomenon characterised by the shrinkage of the cytoplasm (Moore *et al*, 1998a; Wallace *et al*, 1996a) and a leaking membrane that causes the ready influx of high amounts of salt (Munns,

2002a). This adversely affects the plant and results in cellular dehydration, growth reduction and cessation.

In an attempt to avoid plasmolysis, the root zone maintains moderate sodium levels by readily extruding sodium from the roots zone and/or exporting the ion to the shoots (where sodium exclusion also occurs) via the xylem (Watson *et al*, 2001). Hereby, another problem arises as a high solute concentration in the transpiration stream has even more deleterious effects that, over time, results in the necrosis (wilt) of leaves. The lifespan of leaves depends on the rate at which sodium accumulates in the apoplast and is detoxified by the cell. Leaves are the major photosynthetic unit of the plant and injuries thereto reflect in wheat yield and productivity (Tester and Davenport, 2003; Watson *et al*, 2001).

1.11 EFFECTS ON PLANT MORPHOLOGY

Munns (2002a) investigated the morphological changes that sudden salt tension evokes on plants. The author reported that the plant system is instantly traumatized when plant cells experience an osmotic shock resulting in the irrepressible efflux of water and shrinkage of the cell (plasmolysis). Also the leaves and roots show an immediate and steep reduction in growth rate. The duration of recuperation to a more steady biochemical state is proportionate to the intensity of the osmotic shock. Gradual recovery times of 1 to 24 hours are recorded in the roots when plants are subjected to 20-90 mM and 150 mM salt solutions, respectively. Although the cell steadily regains cell volume, the elongation rate of cells (whereby the leaf and plant grow) is permanently reduced (more so in the leaves than the roots). In the following weeks lateral shoot development ceases and damage to the leaves becomes apparent as new leaves are reduced in size and older leaves start a visible decaying pattern of gradually turning yellow and subsequently dying. The rate at which the leaves die determines the lifespan of the plant. If new leaves are generated faster than older leaves die there will be enough energy to produce seeds and flowers, otherwise the plant will not proceed to the reproductive phase. In the event that fruit is produced, it seems that the detrimental effects of salinity has not even left the fruit yield untouched. Katerji *et al* (1998) reports, from a salt experiment on tomatoes, the loss of approximately 60 % tomato yield and a reduction in the weight of the fruit.

1.12 ACCLIMATIZATION TO EXTERNAL STIMULI

The evolutionary journey of plants over a time period of 400 million years from a nutrient and water rich habitat, ocean, to a nutrient and water scarce environment, land, presented them with new challenges (Bressan *et al*, 1998; Moore *et al*, 1998c). In dealing with the encounters of the new environment, plants seem to have "switched off" integral systems that dealt with high saline conditions. However, all is not lost, as gradual exposure to high salt conditions has proven the steady engagement of the cellular control systems (Bressan *et al*, 1998).

These systems are initiated under stressful conditions in an attempt to protect the plant from the adverse affects that these conditions inflict. The extensive impact that an unfavourable environment has on the plant is manifested in the wide spectrum of changes (adaptations to nearly every aspect of physiological and metabolic traits) that the plant elicits in an attempt to shield it from further onslaughts (Zhu, 2001b, Wang *et al*, 1999). The activation of tolerance mechanisms is brought about by a "sentience" generated from certain membrane receptors and absorbed through various channels to elicit an array of gene transcriptional expressions (Tester and Davenport, 2003). This complex signaling network is presumably activated by damages caused rather than detection of osmotic stress (Zhu, 2001b). The primary aim of the tolerance mechanism is to keep sodium and chloride transport to the shoots at low levels while it preferentially takes up potassium (Babourina *et al*, 2000) and exports sodium to the vacuole (Munns *et al*, 2000).

A plant is classified as salt tolerant if it successfully amends its physique when damages were inflicted, returns equilibrium in all the biochemical reactions of the plant and all the while maintains a steady growth rate (Zhu, 2001a). Another aspect that distinguishes salt tolerant plants from salt sensitive plants is its ability to prevent toxic build up of sodium in the transpiring leaves (Munns, 2002a).

1.12.1 Active movement: sodium detoxification

In an attempt to counteract sodium toxicity in the leaf apoplast (cell wall and intercellular spaces) sodium is either, by the use of the energy dependant (membrane ATPase and vacuolar ATPase and pyrophosphatase) Na^+/H^+ antiports, forced out of the cell and/or sequestered to the vacuole (Mansour *et al*, 2003). In the vacuole sodium binds with extruded chloride and is used as an (metabolically "cheap") osmoticum that functions by maintaining a ready influx of water. Although the exclusion of sodium by Na^+/H^+ antiports has been identified in a range of species, Binzel and Ratajcak, 2002, as reviewed by Mansour *et al*, 2003) speculate that it is not a feature of all plants.

When the vacuole is packed to capacity, extensive destruction follows. The volume capacity of the vacuole allows it to hold ten times more sodium than the cytoplasm. If the vacuole is saturated then the paradigm shifts, and the cytoplasm is inclined to accommodate sodium at a tenfold rate. For example, if only 5 mM per day is absorbed by a saturated vacuole then 50 mM accumulates in the surrounding cytoplasm per day. The sodium build-up in the cytoplasm will rapidly reach lethal levels (generally within two days) and the plant will soon die due to sodium poisoning and dehydration (Munns, 2002a).

1.12.2 Osmoprotectants and osmolytes

In the attempt to deal with high salinity, the plant cells make use of compatible, atoxic solutes called osmolytes and osmoprotectants to counterbalance osmotic distress. Osmolytes such as potassium have the ability to adjust the osmotic potential, while osmoprotectants are only involved in protecting against and alleviating the effects of osmotic stress (Yokoi *et al*, 2002). For example the amino acid derivative, glycine betaine, keeps the thylakoid and plasma membrane intact after exposure to saline conditions. Osmoprotectants also neutralize the harmful by-products (reactive oxygen species (ROS)) of hyperosmotic stress. The activation of atmospheric oxygen is a highly controlled process in the chloroplast and mitochondria organelles of the plant cell. Reactive oxygen species (superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\cdot$)) play a role in biosynthesis, detoxification and pathogen protection (McKersie, 1996). Under stress conditions control over the oxygen activation sites weakens and activated oxygen leaks.

Although plants do possess a detoxifying system, degenerative reactions occur when the toxic level is too excessive and cause disintegration of the cell membrane, inactivation of proteins, damage to photosynthetic pigments and mutations in DNA (McKersie, 1996; Sairam and Srivastava, 2002). In an attempt to scavenge ROS, plants employ antioxidants such as ascorbic acid and reduced glutathione as well as various enzymes such as ascorbate peroxidases (APX), catalases (CAT), superoxide dismutase (SOD), glutathione *S*-transferases (GST), and glutathione peroxidases (GPX) (Apse and Blumwald, 2002). Transgenes that overexpress the enzymes GST and GPX caused established tobacco seedlings to suffer less oxidative damage when exposed to stress environments. Transgenic plants had improved seed germination and seedling growth, higher levels of ascorbate and glutathione, higher levels of monodehydro-ascorbate reductase activity as well as larger oxidized glutathione pools. Genetic alteration of the genes regulating enzyme activities can therefore be a means to increase salt tolerance (Apse and Blumwald, 2002).

Although these compounds that accumulate in the cytoplasm have an important function in the battle against salinity, the synthesis thereof is regrettably expensive. The amount of ATP required to produce glycine betaine, mannitol or proline in leaves is 50, 34 and 41 moles, respectively. In contrast, the use of NaCl as an osmoticum in root and leaf cells requires less than 10 moles of ATP. Regardless, NaCl is not a feasible osmolyte due to its toxicity in the cytoplasm (Munns, 2002a).

1.13 TRACKING THE SIGNAL RECIPIENT: INTRICATE GENE CASCADE

The process by which physiological and biochemical traits are altered to adapt to salt stress is complex, multi-genically inherited, and a frontier to be intensively studied to tag the genes involved. Attempts to unravel the underlying mechanisms of the complex signalling pathways involved in response to salt stress are hampered by lack of appropriate study techniques at molecular and cellular level. Another obstacle is absence of an appropriate plant (or organism) model specific for ion or water stress as the response of different plant species to these constraints seems heterologous (Tester and Davenport, 2003). Recent advances have aided in the identification of the salt overly sensitive (SOS) pathway that is involved in rectifying ion homeostasis (Seki *et al*, 2003). These genes are hypersensitive to sodium and lithium and were identified in *Arabidopsis thaliana* mutants that were selected by their inability to maintain root growth when exposed to high salt concentrations. The *SOS3* genes encode for a myristoylated calcium-binding protein that is hypersensitive to high calcium concentrations in the cytosol brought on by salt stress. It reacts by activating the *SOS2* gene, a serine/threonine protein kinase. The *SOS3-SOS2* complex activates the *SOS1* gene that encodes for a plasma membrane Na^+/H^+ antiporter and thereby facilitates the export of sodium to the vacuole (Zhu, 2001b).

Overexpression of *At*NHX1, a vacuolar Na^+/H^+ antiporter in *Arabidopsis* plants, supported the normal growth of transgenic plants in NaCl–rich soils (200 mM), which is a condition hazardous to their wild type counterparts. A similar response was detected in overexpressed *At*NHX1 transgenic tomato plants (Katerji *et al*, 2001, classified tomato plants as salt sensitive). While the wild type struggled to survive in sodium chloride conditions of 200 mM (almost half the concentration of seawater), the transgenic plant (with a 50-fold enhanced tolerance capacity enforced by a single gene) thrived and produced fruit characterized by a low sodium chloride content (Zhang and Blumwald, 2001, as reviewed by Owens, 2001). The increased antiporter proteins in the tonoplast vesicles of the plant cell enhanced sodium sequestration to the vacuole and it was found that the sodium chloride content in the transgenic tomato leaves was 20 times higher than in the control plants (Zhang and Blumwald, 2001, as reviewed by Ruiz, 2001).

As previously mentioned, agriculture plans to replace shallow-rooted annual crop plants with deep-rooted perennials to restore saline land, yet, most farmers will not adopt this strategy, as it is a non-profitable approach (Barrett-Lennard, 2002). The extensive accumulation of salt in the leaves (up to 6 % of their dry weight) makes the transgenic tomato plant a favourable bioremediating component in restoration strategies as growing transgenic tomatoes will purify the soil from excess salt and, simultaneously, be remunerative to farmers (Zhang and Blumwald, 2001, as reviewed by Owens, 2001).

The introgression of increased Na^+/H^+ antiporters had also been successfully studied in unicellular organisms. A plasma membrane Na^+/H^+ antiporter isolated from a salt tolerant cyanobacterium and overexpressed in fresh-water cyanobacterium indorsed normal development of the latter species in salt water (Waditee *et al*, 2002, as reviewed by Ward *et al*, 2003).

In order to study the machinery that comprehends water deficit stress, scientists turned to yeast, *Saccharomyces cerevisiae*, as an appropriate study model. The MAPK (mitogen-activated protein kinase) cascade is the signalling pathway by which the HOG (high osmolarity glycerol) pathway is activated to produce glycerol in an attempt to rectify conditions of high osmolarity. The MAPK cascade is initiated by two transmembrane proteins, Sln1p and Sho1p, which senses unfavourable osmolarity and transmits a signal that passes along various intermediate proteins to activate the HOG pathway that controls glycerol biosynthesis (Wurgler-Murphy and Saito, 1997, as reviewed by Matsumoto *et al*, 2003). Although yeast and plants are diverse living organisms, certain genes involved in the MAPK cascade of yeast have orthologues in plants. Similar in structure to the Sln1p, a transmembrane protein, histidine kinase 1 (*AT*HK1), was isolated from *Arabidopsis*. This protein is transcribed primarily in the leaves and roots of plants under saline soil conditions and it is believed to act as an osmosensor in *Arabidopsis* (Urao *et* al, 1999, as reviewed by Matsumoto *et al*, 2003).

The non-toxic property of osmoprotectants makes it possible to interchange the transcription thereof between species (Apse and Blumwald, 2002). Plants such as tobacco and potato are suitable host plants to introduce enzymes that remediates osmotic stress as these crops accumulate osmoprotectants and osmolytes less efficient than other plants. The expression of the *betA*, a gene isolated from *Eschericia coli* that directs the conversion of choline to glycine betaine, in tobacco and potato effectuated tolerance to salt and freezing (Lilius *et al*, 1996, as reviewed by Holmberg and Bülow, 1998). As a high level of accumulation of the enzymes is required, it is questioned whether the localisation of the enzyme to the cytoplasm, which account to a small part (5 %) of the

total cell volume, is efficient for osmotic stabilisation. Using a transit peptide, a choline oxidase gene *(codA)* from *Arthrobacter globiformis* was expressed in the chloroplast (the primary site of ROS production) of Arabidopsis. This resulted in higher levels of glycine betaine that may be more efficient in rectifying high osmolarity. Salt and freezing tolerance was also reported (Hayashi *et al*, 1997, as reviewed by Holmberg and Bülow, 1998). This is another feature enrolled from the transfer of foreign salt responsive genes. Transgenic plants not only have enhanced tolerance to salt but also to cold, heat shock and drought (Zhu, 2001a). This is evident that the plants' response to these diverse stressful conditions is elicited through common reactions (Zhu, 2001b).

Previously, the complexity of the trait has led scientists to believe that the enhancement of the plants' performance under high salt conditions is only possible by pyramiding many adaptational characteristics. These preconceptions were set aside as the manipulation of a single gene (such as the overexpression of the *At*NHX1 in tomatoes) in the intricate gene cascade confers salt tolerance in the plant (Zhang and Blumwald, 2001, as reviewed by Ruiz, 2001). Mentioned in this section are only a few of the salt tolerance genes successfully employed in salt sensitive species by means of genetic transformation. As understanding of the genetics of tolerance mechanisms broadens, new salt responsive genes will be identified and added to the already long list of genes involved in osmotic and ion regulation, antioxidant protection, signalling and transcriptional control (Zhu, 2001ab; Apse and Blumwald, 2002; Morillon and Lassalles, 2002; Seki *et al*, 2003). Recent additions to the list are a further four vacuolar Na^+/H^+ antiporters in *Arabidopsis* characterized by Aharon and colleagues (2003). The authors report that these antiporters are similar to the previously discovered *At*NHX1. *At*NHX2 and *At*NHX5, like *At*NHX1, occur in all plant tissue, yet, *At*NHX5 is less abundant. The remaining transcripts, *At*NHX3 and *At*NHX4, were more pronounced in flower and root tissue (Aharon *et al*, 2003).

Salinity is a huge problem predominantly indorsed by man that needs to be corrected by man. Our time is running out to rectify this problem. FAO AGL data (2000a) indicate the loss of 3 ha arable land every 60 seconds due to soil salinity. Although there is, in the eye of the burgeoning world population, much controversy over gene engineering (Owens, 2001), this way of transfer of a specific trait is much faster and accurate than traditional breeding methods (Cushman and Bohnert, 2000; Holmberg and Bülow, 1998). In fact, years of breeding for salt tolerance has only brought forth moderate salt tolerance in a few plant species (Ruiz, 2001) while a range of salt responsive genes have been isolated and successfully transferred to host plants (Holmberg and Bülow, 1998). The elevation of plant stamina through genetic modification, in collaboration with the restoration of water logged and saline land, could be a rewarding approach as yields would increase whilst affected land is being rectified (Tester and Davenport, 2003).

1.14 NOVEL GENE DISCOVERY

Thellungiella halophilia is a useful model halophytic plant for elementary studies of salt tolerance. This plant (survives a NaCl solution of 500 mM that is fatal to *Arabidopsis*) derives from the coast of China and has the desirable 'plant model' physiological traits of self-pollination, high seed production, small size, a short life cycle and a small genome that is receptive to transformation and mutagenesis (Zhu, 2001a). Another model for studying salt resistance is the halophytic bacteria whose membrane and enzymes are protected by its salt-resistant cytoplasm. (Rose-Fricker and Wipff, 2001). Discovering the genes involved in the salt tolerance dynamics of this plant species and bacterial organism can provide additional information about the mechanism that control stress signal initiation, the stress signal recipient ports and the resulting gene expression cascade (Jaglo-Ottosen *et al*, 1998, as reviewed by Cushman and Bohnert, 2000). The *Arabidopsis* and rice genome sequencing initiatives primarily identified the "salt-responsive" genes through genome scale EST (expressed sequence tags) and genome sequencing. Gene expression analysis using these approaches has set a standard now followed by many scientists and the existing EST libraries are constantly updated with newly discovered ESTs. An extensive EST database for rice and *Arabidopsis*, hosted by Genbank, can be viewed online at http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html or at the Stress Functional Genomics Consortium website (http://stress-genomics.org/) (Cushman and Bohnert 2000).

High throughput analysis of ESTs has provided much insight in the complex gene domain. ESTs collected from salt sensitive plants have shown a 15 % increase in functional expression compared to the salt tolerant plants (Cushman *et al*, 1999, as reviewed by Cushman and Bohnert, 2000). It was also determined that upregulation of the genes involved in relieving salt stress are followed by the downregulation of genes that encode components of the photosynthetic system (Cushman and Bohnert, 2000). EST analysis in *Arabidopsis* resulted in the discovery of 15 genes that are activated during osmotic stress (Pih *et* al, 1997, as reviewed by Cushman and Bohnert, 2000) and in rice, genes were discovered that expressed certain ATP producing enzymes of the glycolysis and tricarboxylic acid pathway when salt imposes a threat (Umeda *et al*, 1994, as reviewed by Cushman and Bohnert, 2000).

A study done by Zhu (2001a) indicated that the halophytic plant, *Thellungiella halophilia*, shares more than 90 % cDNA sequence homology with *Arabidopsis* and is therefore likely to have a similar mechanism to overcome salt stress. With the extensive EST library of *Arabidopsis* it is possible to rapidly identify the orthologous genes in the halophytic plant or even to locate new genes that improve salt tolerance (Hasegawa *et al*, 2000).

cDNA microarrays can complement EST studies especially in combination with mutagenesis where different gene expression profiles between the wild type and a mutant that has a dysfunction in one of the salt stress pathways can be compared and the mutant gene identified. This approach is also referred to as the loss-of-function approach and is being implemented in transgenic *Arabidopsis* through transposon insertional mutagenesis or transfer DNA tagged lines (T-DNA) to tag the defective genes in the salt response cascade of the mutant plants (Winkler *et al*, 1998, as reviewed by Cushman and Bohnert, 2000; Cattivelli *et al*, 2002). Four hundred thousand T-DNA insertion mutants have already been generated (Maggio *et al*, 2001).

1.15 SALT TOLERANCE AND CEREALS

Until all uncertainties have been cleared about the genetic engineering of complex traits (Owens, 2001) the symptoms of salinity, such as loss of plant productivity that are inimical to agriculture, need to be tackled through traditional plant breeding methods. Fortunately, nature has provided us with grass species that are resistant to salt that can donate the relevant genes to their salt sensitive relatives.

Thinopyrum bessarabicum is a wild relative of wheat that grows on the seacoast of the Crimea. This plant, which resists scab disease caused by *Fusarium graminearum* and the Columbia root knot nematode *Meloidogyne chitwoodi*, matures in an environment that is abundant in salt and therefore has the ability to withstand saline environments (King *et al*, 1997). This characteristic makes the plant a suitable parent to use in the quest to breed salt tolerant crops and such a program was introduced by King *et al* (1997). The authors derived a wheat x *Th. bessarabicum* amphiploid with the ability to withstand saline conditions equal to half that of seawater (250 mM), a tolerance ability far better than their cultivated parents, and called the amphiploids: Tritipyrum.

A breeding program involving wheat and its wild relatives is not a novel approach as the development of the productive crop triticale (x *Triticosecale* Wittmack) resulted from hybridisation of wheat and rye (King *et al*, 1997). Certain accessions of triticale have higher salt tolerance as a result of mechanisms that extrude sodium and effectively discriminate between the K^+ and Na^+ ions. The underlying control of discrimination was found to reside on chromosome 4DL of hexaploid bread wheat (genome AABBDD) and functions through a single, incompletely dominant locus. Referred to as *Kna1*, the locus has been mapped within a 1.1-cM region on the 4D/4B map and regulates the expression of a 23-kDa protein of the oxygen evolving complex (OEC) of photosystem II in the leaf blade membrane of plants under salt stress (Gao *et al*, 2001).

Species lacking the D genome such as tetraploid durum wheat *Triticum turgidum* L. ssp. *durum* (Desf.), which only has the AABB genomes, are sensitive to highly saline conditions. This is very unfortunate as durum wheat is a sought after commodity worldwide. In the wet seasons, durum yield may exceed bread wheat yield, but in dry season transient salinity occurs which causes a huge loss of durum wheat productivity (Munns *et al*, 2003). The latter authors ascribed the steep loss to extensive sodium accumulation (at a rate five times higher than in bread wheat) in the plant cells of durum wheat.

In durum wheat disomic substitution studies where the 4A or 4B chromosomes were replaced by 4D, sodium accumulation in the leaves decreased, indicating an enhanced ability to discriminate between the sodium and potassium ions and to exclude salt. In studies that attempted the transfer of the *Kna1* locus from hexaploid bread wheat to tetraploid durum wheat a decrease in the sodium level and increase in potassium were detected. However, such recombinant lines were not agronomically successful due to the simultaneous introduction of deleterious genes (Munns *et al*, 2003).

The ability of plants to tolerate saline stress is genetically determined. The genus *Thinopyrum* is regarded as having a potent group of genes that firmly supports plant growth in times of salt stress (Wang *et al*, 2003).

1.16 *THINOPYRUM DISTICHUM*

Thinopyrum distichum (sea wheat) is a coastal grass indigenous to the western, southern and south eastern shores of South Africa. It has an inherent ability to nurture from soil characterized by a high salt content and low soil moisture. This can be of great value to cereal breeding for high salt environments if this genetic trait can be transferred to the cultivated cereals. *Th. distichum* is a segmental allotetraploid with 2n=28 (Littlejohn and Pienaar, 1994). The latter authors compiled a C-band karyotype of the 28 chromosomes and established that its two genomes, J_1^d and J_2^d, are largely homologous, the primary difference being with regard to two reciprocal translocations. The authors tentatively numbered the *Thinopyrum* chromosomes from I to XIV. Marais and Marais (2003) also produced a C-band karyotype of the *Thinopyrum distichum* chromosomes. However, the C-band procedure employed differed from that of Littlejohn and Pienaar (1994) and the two sets of results could not be related with certainty. The karyotype of Marais and Marais (2003) was based on the abilities of the homoeologues to pair during meiosis as well as the use of probes diagnostic for the different homoeologous sets of wheat chromosomes and should be more accurate.

1.17 IDENTIFICATION OF THE *THINOPYRUM DISTICHUM* CHROMOSOMES THAT ENCODE FOR SALT TOLERANCE

Marais and Marais (2003) hybridized *Thinopyrum distichum* (female parent) with tetraploid 'Henoch' rye. They used the amphiploid to construct a karyotype of *Th. distichum* and to determine which chromosomes had a role in salt tolerance. Their attempts to pollinate *Th. distichum* with diploid "Henoch' rye were unsuccessful, but they recovered two embryos when the diploid "Henoch' rye parent was substituted with tetraploid 'Henoch' rye. The resulting partially diploid hybrids had 28 chromosomes, were of genotype $J_1^dJ_2^dRR$ and the hybrid was designated 95M1. Marais *et al*, (1998) reported that these plants were characterized by a well-developed root system with continual shoot and root regeneration, were tall plants that could easily be maintained through cloning and, most important, showed the ability to withstand high salt levels in contrast to its 'Henoch' rye parent that was used as control during salt tolerance screening.

The primary hybrid, 95M1, could not be backcrossed to *Th. distichum* but an attempt to pollinate it with diploid 'Henoch" rye produced F_1 progeny with 21 chromosomes (a complete set of 14 rye chromosomes and 7 single *Thinopyrum* chromosomes). Marais and Marais (2003) argued that the 95M1 megaspore mother cells regularly produced seven rye bivalents and seven allosyndetic *Thinopyrum* bivalents. This resulted in a random, but balanced set of seven *Thinopyrum* homoeologues in each egg cell. They exposed 90-100 F_1 to a salt tolerance screening assay and identified 15 plants with excellent ability to adapt to the high salt conditions. Marais and Marais (2003) cut root tips of each and by means of C-banding could readily distinguish the seven smaller *Thinopyrum* chromosomes in each plant from the rye chromosomes, which also had distinctive telomeric bands. They arbitrarily numbered the fourteen *Thinopyrum* chromosomes and noted which subset of seven occurred in each F_1 plant. From the segregation patterns observed they could determine which were homoeologues and ordered the *Thinopyrum* chromosomes into 7 homoeologous pairs. These were subsequently characterized with RFLP probes diagnostic for the 7 homoeologous groups of wheat.

The homoeologues that were predominantly present within the fifteen selected 95M1/2x 'Henoch' hybrids had a significant role in determining salt tolerance and were allocated to the J_1^d genome. Chromosomes $2J_1^d$, $3J_1^d$, $4J_1^d$, $5J_1^d$ and $7J_1^d$ were implied most strongly in salt tolerance.

Marais and Marais (2003) then backcrossed the two most salt tolerant 95M1/2x 'Henoch' hybrids: 95M1-79 and 95M1-302 to diploid 'Henoch' rye. These two hybrids had salt tolerance similar to that of the primary amphiploid. The B_1F_1 progeny each had a complete set of 14 rye chromosomes as well as a variable number of random (derived from seven univalents) *Thinopyrum* chromosomes. A seedling salt tolerance test was done on 1040 seeds of the B_1F_1: 95M1-79/ 'Henoch' and 600 seeds of the B_1F_1: 95M1-302/ 'Henoch'. An adult plant salt tolerance assay was employed on the clones of fourty nine surviving plants of which ten had showed a high capacity to endure salt. It was found that four plants, each having 21 chromosomes, showed similar salt tolerance to their parents and originated either through reconstitution or facultative apomixis. The six remaining plants: 95M1-79-1, 95M1-79-33, 95M1-79-43, 95M1-302-1, 95M1-302-26, 95M1-302-29 had variable chromosome numbers that ranged from 16-19. Salt tolerance screening revealed them to be less tolerant than their parents. The six plants were also C-banded and RFLP analyzed. The data confirmed the putatively identified chromosomes primarily responsible for salt tolerance.

1.18 FINDING MOLECULAR MARKERS FOR *THINOPYRUM DISTICHUM* CHROMOSOMES IMPLICATED IN SALT TOLERANCE

1.18.1 Restriction fragment length polymorphism (RFLP)

Marais and Marais (2003) identified the *Thinopyrum* chromosomes encoding salt tolerance by their C-bands. They then attempted to find molecular markers for these critical chromosomes and to determine their homoeologies to the triticale and wheat chromosomes. For this purpose, they employed a set of 14 RFLP probes diagnostic for each arm of the seven homoeologous chromosome sets of wheat (*Triticum aestivum*). These probes were obtained from the Institute of Plant Science Research in Norwich, UK and were tested on *Th.distichum*, 'Henoch' rye, the primary hybrid *Th. distichum*/4x'Henoch' rye (95M1, 28 chromosomes), fifteen F_1: 95M1/ 2x'Henoch' (21 chromosomes) and six B_1F_1: 95M1/2* 'Henoch' (16-19 chromosomes).

Four of the fourteen probes (wheat) hybridized poorly to the rye and *Thinopyrum* sequences and were not useful in the analysis. However, the remaining probes allowed for RFLP analysis and a distinct polymorphism could be produced for most of the *Thinopyrum* chromosomes and each of the critical *Thinopyrum* chromosomes. While the RFLP markers will be useful in future attempts to introgress critical chromosome regions into wheat and triticale, easier to use marker systems need to be developed.

1.19 DEVELOPMENT OF HIGH THROUGHPUT MARKERS FOR THE IDENTIFICATION OF *THINOPYRUM DISTICHUM* CHROMOSOMES THAT ARE INVOLVED IN SALT TOLERANCE

RFLPs are accurate but time-consuming and laborious. It involves the restriction digestion of micrograms of genomic DNA with an appropriate endonuclease enzyme (overnight), size fractionation of the fragments produced (overnight), alkaline blotting of the separated fragments onto positively charged nylon membrane (overnight), hybridisation of DNA probes (overnight) and post-hybridisation steps to allow detection. Overall, the tedious procedure can only be completed over several days.

1.19.1 Polymerase chain reaction (PCR)

The core enzyme that facilitates the PCR reaction is acknowledged as the supreme scientific development of the 20th century and was labeled as the "molecule of the year" by the American association for the advancement of science (Guyer and Koshland, 1989). Isolated from the thermophilic bacterium, *Thermus aquaticus*, the *Taq* DNA polymerase enzyme can resist high temperatures of up to 95 °C. This is an improvement on the previous DNA polymerase (Klenow fragment of DNA polymerase 1) purified from the bacterium, *Eschericia coli* that was unstable at high temperature and needed to be replenished after the conclusion of each PCR cycle, thereby making the process prone to error (Saiki *et al*, 1988). The introduction of the thermo-stable DNA polymerase directed it towards a more automated process with more specific results, enhanced yield and improved amplification of longer fragments (Erlich *et al*, 1991).

The enzymatic reaction is a powerful and versatile DNA analytical technique as it facilitates the *in vitro* amplification of a small amount of DNA (represented by a single cell) into millions of duplicates (Guyer and Koshland, 1989). The amplification of a specific DNA segment is directed by two synthetic oligonucleotides that are complementary to the region flanking the DNA segment of interest. Also referred to as primers, their hybridisation to complementary strands is facilitated by the DNA polymerase which also facilitates the DNA synthesis of the demarcated DNA region.
The separation of the double helix into the opposing strands, primer annealing and DNA synthesis are the three essential parts of the cyclic events that compose the PCR reaction. Each successive round in the cycle (30-40) regenerates the target DNA segment produced in the previous round, thereby effectuating an exponential accumulation of the target sequence.

Automated applications produce results within hours and the genetic information amplified can be detected on an agarose gel. Polymorphic bands can be visualized more promptly by either the presence or absence of a band. The present study aimed to develop molecular markers for the critical chromosomes that will be both accurate and quick to perform. An attempt was therefore made to convert the RFLP markers employed by Marais and Marais (2003) to PCR based markers. If successful, this would reduce the time of DNA analysis from days to a few hours and would greatly accelerate throughput.

1.20 IMPEDIMENTS TO THE CONVERSION OF DNA MARKERS

The reason for the trend in genetics to convert dominant molecular markers (AFLP, RAPD) to co-dominant markers (STS, SCAR, EST) is the lower costs and high simplicity that these molecular techniques presents (Masojć, 2002). The conversion could promote future advances in especially molecular breeding (Meksem *et al*, 2001) if a means can be found to circumvent certain constraints involved with the transition of markers (Masojć, 2002).

Amplified fragment length polymorphism (AFLP) is a reliable, reproducible and efficient, marker analysis system (Shan *et al*, 1999) that allows the high-density mapping of many genes and has contributed, enormously, to the detection of genes of economical importance.

It is a great diagnostic tool that detects microsatellites, single nucleotide polymorphisms (SNPs), insertions, deletions and is widely used to construct genetic maps of various genomes, particularly those of cultivated crops species (Meksem *et al*, 2001) such as wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.) (Shan *et al*, 1999). Although AFLPs assist greatly in genome analysis, it is an expensive, methodologically complicated technique (Xu *et al*, 2001) that doesn't provide for high-throughput genotype scoring (Meksem *et al*, 2001).

The latter authors attempted to overcome this obstacle by converting AFLP bands into STS (sequence tagged sites) markers, a diagnostic tool that does allow high-throughput genotyping in an automated format. In the process of marker conversion they were confronted with many technical difficulties. The complexity of the genome marked by the occurrence of sequence replicas, the low molecular weight of the AFLP bands and locating the polymorphism within the AFLP band, set great boundaries that needed to be dealt with (Meksem *et al*, 2001). These restrictions are relevant in studies that propose marker conversions and literature reports limited success with attempts to convert AFLP markers as only a few retains the specificity of the initial AFLP marker. The reason for the impediment is uncertain and it is speculated that the nature of the AFLP polymorphism contributes largely to the difficulties (Xu *et al*, 2001; Shan *et al*, 1999). In a study done by Prins and colleagues (2001) with the aim to convert an AFLP marker of *Lr19*, a gene that confers resistance to the leaf rust pathogen: *Puccinia recondita* Rob. Ex. Desm. f.sp. *tritici* in wheat, to an STS marker they encountered many false positive clones and ascribed it to the co-segregration of different PCR (AFLP) products.

Recent literature also reports problems involved in the conversion of wheat RFLP probes into STS markers (Forsström *et al*, 2003). The authors ascribe the difficulty to the triplication of a single RFLP locus in hexaploid wheat. Although polymorphisms between homoeoloci can be detected through restriction digestion, the identification of the triplicate loci is a laborious process that involves numerous rounds of cloning and sequencing. Forsström *et al* (2003) circumvented the intensive cloning procedure by capturing all of the homoeoloci presented by a single copy cDNA through the use of derived PCR primers and the size fractionation thereof on the single-stranded conformation polymorphism (SSCP) electrophoretic system that can detect single base differences between related sequences.

The bands were recovered and sequenced and nucleotide polymorphisms were used to design locus-specific PCR primers of which one proved to be allele specific. With this robust approach they successfully marked the powdery mildew resistance on the wheat-rye translocated 2BS.2RL chromosome (Forsström *et al*, 2003).

The study aimed to convert known RFLP markers for *Thinopyrum* chromosomes into PCR based SCAR markers, as these would be more amenable to the introgression of *Thinopyrum distichum* chromosome regions into triticale and wheat.

CHAPTER 2

MATERIAL AND METHODS

Probes were obtained from the John Innes Centre, UK and their DNA sequences were found in the GrainGenes website (http://wheat.pw.usda.gov). Computer software was used to design a set of PCR primers for each probe (Table 3.1) from the DNA sequences. Amplification conditions were optimised for each primer set and the amplified fragments of positive and negative control samples were compared in an attempt to find chromosome specific markers. An attempt was made to confirm polymorphic fragments by hybridisation to the DIG labeled probe sequence. In certain instances an attempt was made to further refine promising markers. In these cases relevant bands were excised from the agarose gels and amplified in *vivo*. Following screening for positive transformants by colony PCR, the plasmids were purified and the DNA sequenced. From the sequence data polymorphic regions were identifed and new primers were designed within these regions. Following PCR optimisation the new markers were evaluated using appropriate positive and negative controls.

2.1 Plant material

Different sets of plants, primarily derived from the study of Marais and Marais (2003) were used to optimise and test newly designed primers and to confirm the chromosome location of the amplified fragments (Table 2.1).

2.2 Plant genomic DNA extraction and quantification

Plant genomic DNA was extracted according to the protocol of Doyle & Doyle (1990). The concentration of the extracted DNA was determined by gel electrophoresis. A 0.8 % agarose gel was set up in 1x TBE loading buffer. One microliter DNA was added to 4 µl dH_2O and 10 µl Ficoll Orange G loading buffer (0.1 % (w/v) Orange G, 20 % (w/v) Ficoll, 0.5 M EDTA (pH 8.0)) and loaded together with standard concentrations (0.1 and 0.3 µg/µl) of Lambda DNA. The gel was run for 45 min at 60 V and stained for 20 min in an EtBr and 1 x TBE solution (200 µl/dm^3). The DNA bands were detected under UV-light and the intensity and size compared to the Lambda bands in an attempt to estimate the concentration of the DNA. Genomic DNA sample concentrations were also determined with a Hoefer DynaQuant 200 Fluorometer as described in the manual.

Table 2.1: Genotype panels used to test primer pairs (95M1=the primary hybrid, *Th. distichum*/4x 'Henoch' rye, 2n=28; 95M1-18 through 310=salt tolerant backcross F_1: 95M1/2x 'Henoch' rye, 2n=21; 95M1-79-4 through 43 and 95M1-302-1 and 95M1-302-29 were derived by respectively backcrossing 95M1-79 and 95M1-302 to 'Henoch' rye and selecting the most salt tolerant B_1F_1 progeny). With the exception of *Th. distichum* and 'Henoch' rye, all the genotypes had a complete diploid set of rye chromosomes plus varying numbers of different *Thinopyrum* chromosomes

	DNA samples																						
Test panels	***Th. distichum***	**95M1**	**'Henoch'**	**96M2**	**'Rex'**	**95M1-18**	**95M1-21**	**95M1-79**	**95M1-95**	**95M1-300**	**95M1-301**	**95M1-302**	**95M1-303**	**95M1-304**	**95M1-305**	**95M1-306**	**95M1-307**	**95M1-308**	**95M1-310**	**95M1-79-4**	**95M1-79-33**	**95M1-79-43**	**95M1-302-1**	**95M1-302-29**
Genotype panel 1	*	*	*																				
Genotype panel 2	*			*	*																		
Genotype panel 3	*	*	*	*	*			*		*	*									*	*	*	*	
Genotype panel 4	*			*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Genotype panel 5	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

2.3 Polymerase chain reaction (PCR) primer design

The RFLP probes of which the sequences were retrieved from the GrainGenes website (http://wheat.pw.usda.gov), were only end-sequenced (Katrien de Vos, personal communication) and therefore the sequences of the larger probes: PSR 115, PSR 129, PSR 305, PSR 628, PSR 666, PSR 931, PSR 934, PSR 949, PSR 953 are given in two parts, a forward and reverse sequence. The complete sequences of the smaller sized probes: PSR 154, PSR 160, PSR 163 and PSR 167 were available. The primers were designed using PRIMER DESIGNER to fit the average criterium of 40-60 % GC content, melting temperatures (Tm) between 50 and 65 °C, a base length of between 20 and 25 and, if possible, a C or a G at the 3` ends to give a stronger primer-DNA bond.

Initial primer design was done with PRIMER DESIGNER. Subsequent attempts to design primers were based on an Internet program called PRIMER3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and homology searches between sequences were done by means of a computer software program that was downloaded from the BioEdit website: www.mbio.ncsu.edu/BioEdit/bioedit.html.

2.4 PCR optimisation

Bioline Ltd (London, UK), a manufacturer of PCR consumables recommends, a *Taq* DNA polymerase concentration of 0.02 U/µl, deoxynucleotidephosphate (dNTP) concentrations of 0.2 mM each and the use of the reaction buffer at 1/10 the total reaction volume. The optimum $MgCl_2$, primer and DNA concentration were determined on the basis of titration. In a titration series with 0.5 mM $MgCl_2$ increments starting at 1.0 mM until 3.0 mM, the $MgCl_2$ concentration was considered optimal at 3.0 mM. The forward and reverse primers were tested at concentrations ranging from 0.15-0.6 µM with 0.15 µM increments. A concentration of 0.3 µM of each of the primers (Inqaba Biotechnical Industries Ltd, Hatfield, South Africa) was considered optimal.

Initially, DNA concentrations were determined using a Hoefer DynaQuant 200 Fluorometer. Yet, it gave inconsistent results and it was decided to use agarose gel quantification instead. Generally, the concentrated DNA was diluted 10^{-1}, and 1 µl was used in the PCR reaction. This resulted in more consistent PCR results.

Initially, a basic PCR program cycle consisted of 6 min denaturing at 94 °C followed by a 36 cycle event: 45 sec at 94 °C, annealing at a temperature 5-10 °C below the melting temperature of the primers for 45 sec, 90 sec at 72 °C, and a final extension step of 6 min at 72 °C. This was a lengthy program lasting 2½ -3 hours and it was decided to shorten the time with an hour by reducing the cycles to 30 and the relevant denaturing and annealing times to 15 sec.

The PCR reactions were performed in 0.2 ml thin-walled PCR reaction tubes. In a 25 µl reaction, 1 µl of 10^{-1} diluted template genomic DNA was subjected to amplification with 0.3 µM forward and reverse primer. Buffer (Bioline Ltd, London, UK) was added to a final concentration of 1/10 the reaction volume (2.5 µl). The buffer contained 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCL (pH 8.8 at 25 °C) and 0.1 % Tween-20. Furthermore, a working solution of 3.0 mM $MgCl_2$, 0.2 mM of each deoxynucleotidephosphate (dNTP) and 0.02 U/µl Bioline *Taq* DNA polymerase (Bioline Ltd, London, UK) was added to the reaction.

Each reaction was subjected to a 4-stage PCR program. The first stage involved: 5 min at 94 °C, the second 30 cycle stage: 15 sec at 94 °C, 15 sec at x (specific for each primer) °C, 90 sec at 72 °C and the third stage: 6 min at 72 °C followed by a soak stage at 4 °C. The PCR reaction was executed in a GeneAmp ® PCR System 2700 from Applied Biosystems.

2.5 Profiling

Upon completion of the PCR program (approximately 1 hour 45 min), 10 µl of the amplification product and 2.5 µl pGem DNA marker (0.25 µg/µl) were added to 10 µl Ficoll Orange G loading buffer (0.1 % (w/v) Orange G, 20 % (w/v) Ficoll, 0.5 M EDTA (pH 8.0)) and profiled on a 1 % agarose gel. The running buffer consisted of 1 x TBE (5 x TBE: 0.5 M Tris-borate, 0.5 M Boric acid, 0.5 M EDTA (pH 8.0). The gels were run at 120 V for 2-3 hours and stained for 20 min in a container with ethidium bromide (200 µl/dm^3) and 1 x TBE. Visualization was done using a Biorad Gel Doc 1000. The sizes of the amplification products were determined by comparing it to the molecular weight marker.

2.6 Southern blotting

The PCR fragments were size fractionated on a 0.8 % agarose gel in 1 x TBE buffer for a few hours, followed by overnight alkaline blotting onto positively charged nylon membrane (Roche) according to the manufacturers' specifications. Hybridisation and detection of the relevant DIG-labeled probes were executed as described in the DIG Application Manual for Filter Hybridisation (Roche Diagnostics GmbH, Roche Molecular Biochemicals, 68298 Mannheim, Germany). However, due to the use of PCR products as DNA template, the time required to expose the blot to the X-ray film was between 10 sec-20 min as opposed to the 1-2 hours needed for a RFLP blot. The Institute of Plant Science Research in Norwich (Cambridge Laboratory, John Innes Centre, Colney, Norwich NR4 7UJ, UK) supplied the set of 15 RFLP probes, diagnostic for each of the homoeologous arms of wheat genomes.

2.7 Cloning

Polymorphic *Thinopyrum distichum* bands were isolated from the agarose gel with a sterile scalpel while visualised with an ultraviolet transilluminator (UVP Inc.). The DNA was recovered from the gel following the instructions of the QIAquick® Gel Extraction Kit (Southern Cross Biotechnology Ltd, London, UK) and quantified on a 1 % agarose gel by comparing the intensity of the band to standard Lambda DNA concentrations of 0.02, 0.05, 0.1 and 0.2 µg/µl.

Depending on the concentration of the purified PCR product, it was used as insert in either a 1:1, 2:1 or 3:1 ratio to the pGem®-T Easy Vector. The ligation reaction was executed as specified in the technical manual of the pGem®-T Easy Vector system (Promega Corporation, Madison,USA) except for the addition of only half (0.5 µl) the amount pGem®-T Easy Vector (due to the high cost of this system the positive and background controls was not done). Incubation took place overnight. A few adaptations were also made to the transformation reaction. Five microliter of the ligation reaction were added to the 50 µl DH5α competent cells. Incubation in LB media was for 45 min after which the cells were centrifuged briefly and 800 µl of the supernatant was removed.
The remaining LB media was spread onto two plates (100 µl each) and incubated overnight. Six white colonies were picked and screened for positive transformation by means of PCR. The initial primers used to amplify the DNA amplicons, used as insert in the ligation reaction, were employed to test for the transformation of the relevant amplicons.

2.8 Sequencing DNA inserts

Positive colonies were prepared for plasmid isolation by inoculating a single colony in 5ml LB medium containing 100 µg/ml ampicillin and incubated (37 °C) on a shaker to allow growth overnight. The plasmids of the positively, transformed colonies were purified using the Wizard®Plus SV Minipreps DNA purification system as specified in the technical bulletin provided by the manufacturers (Promega, USA). The concentration of the isolated plasmids were determined through agarose gel electrophoresis and adjusted to 100 ng/µl as required for DNA sequencing. Forward and reverse sequencing were done using an Abi Prism 3100 genetic analyser and primers SP6 (5'-TATTTAGGTGACACTATAG-3') and T7 (TAATACGACTCACTATAGGG-3'), which flanks the multiple cloning area of the plasmid.

2.9 Sequencing PCR products

Direct sequencing of purified PCR products were also done with an Abi Prism 3100 genetic analyser. The concentration of the PCR products was adjusted to specific concentration as required for different base pair lengths. Concentrations of 1.1 pmol/µl of relevant primers were used.

CHAPTER 3

Results and Discussion

In this study use was made of wheat RFLP probes with known chromosome location to identify homoeologous sequences in the maritime grass, *Thinopyrum distichum*. An attempt was then made to develop chromosome specific SCAR markers from these that could be used for the introgression of *Thinopyrum* chromosome regions into triticale. Generally, the following steps were involved:

a) The sequences of RFLP probes, diagnostic for the wheat homoeologous groups, were obtained from either the GrainGenes website (http://wheat.pw.usda.gov), or the John Innes Centre, and served as template for PCR primer design. As the newly designed primers were based on probes that originated from wheat cDNA, both the introns and exons of genes would be amplified when applying these primers in a PCR reaction. Thus, the amplified fragments would not necessarily be of the same size as the region inbetween the primer sites in the initial probe sequence. The three samples used for PCR optimisation were *Th. distichum*, 'Henoch' rye and the hybrid 95M1 (*Th. distichum*/tetraploid 'Henoch' rye). *Thinopyrum*-specific fragments were expected to be amplified in *Th. distichum* and 95M1 reactions but to be absent in 'Henoch' reactions. Conversely, rye specific fragments would be absent from the *Thinopyrum* reactions. In addition to these, non-specific bands were also amplified in each of the genotypes. When *Thinopyrum* specific fragments were found these were tested on genotype panel 3 (Table 2.1) in order to confirm the *Thinopyrum* chromosomes involved, and to determine whether the fragment(s) can distinguish between the homoeologues of the J_1^d and J_2^d genomes. The PCR products obtained were size fractionated and subjected to Southern blotting. This was done to confirm that the desired fragment was amplified and that the primers amplify a similar sequence/gene in the different genomes.

b) The primers were also tested on triticale 'Rex' and the primary hybrid of *Th. distichum* and triticale to determine whether it will be a useful marker in a triticale background.

c) In certain instances the amplified fragments were sequenced and an attempt made to refine the markers.

Different sample sets were used to optimise and test the newly designed primers (Table 2.1). The results obtained with primer design, optimisation of amplification reactions, evaluation of polymorphisms, Southern blot results and sequencing of selected fragments are discussed separately for each of the probes that yielded useful primer sets. The data obtained with the different probes are not necessarily presented in the same order as they appear in Table 3.1.

Table 3.1: PCR and Southern blot results with the first set of primers (product sizes are given in kb)

Chr. location	Probe	Size	Expected size of amplicon (Estimated from the published sequence following primer design)	*Thinopyrum* - specific PCR product(s)	Southern hybridisation	Fig.
1X	PSR 949	[1]2.700	2.221	No	No	-
1X	PSR 953	[1]1.500	1.496	No	No	-
2S	PSR 666	[1]1.300	1.183	Yes	Yes	3.3.1 3.3.2 3.3.3
2L	PSR 934	[1]1.800	1.772	No	No	3.7.1.1
3S	PSR 305	[1]1.300	1.200	Yes	No	3.7.2.1 3.7.2.2 3.7.2.3
3L	PSR 931	[1]1.300	1.285	Yes	Yes	3.4.1 3.4.2 3.4.3
4AS/4BL /4DL	PSR 163	[1]0.500 [2]0.425	0.451 0.376	Yes	No	3.7.4.1
4AL/5BL/ 5DL	PSR 115	[1]1.400	1.318	Yes	Yes	3.6.1 3.6.2 3.6.3
5S	PSR 628	[1]1.600	1.464	Yes	No	3.7.3.1 3.7.3.2 3.7.3.3
6S	PSR 167	[1]0.600 [2]0.848	0.496 0.744	Yes	Yes	3.5.1 3.5.2 3.5.3
6L	PSR 154	[1]0.400 [2]0.329	0.329 0.258	Yes	Yes	3.1.1 3.1.2 3.1.3
7AS/7DS/4 AL	PSR 160	[1]0.500 [2]0.389	0.471 0.360	Yes	Yes	3.7.5.1
7L	PSR 129	[1]1.200	1.088	Yes	Yes	3.2.1 3.2.2 3.2.3

[1] Sizes obtained from the John Innes Centre
[2] Sizes obtained from the GrainGenes website (http://wheat.pw.usda.gov)

3.1 PCR and Southern blot results for RFLP probe PSR 154

The RFLP probe, PSR 154, has an estimated size of 0.400 kb and hybridises to the wheat homoeologous group 6 chromosomes (long arms). The forward and reverse primers (Table 3.1.1) designed from this probe (designated as Pr-PSR 154) had melting temperatures (Tm) of 54.6 and 54.0 °C, respectively, and amplified a fragment of approximately 1.600 kb when tested on genotype panel 1 at annealing temperatures (Ta) of 54, 58 and 62 °C as shown in Figure 3.1.1. Optimisation of primer pair, Pr-PSR 154 produced a single clear band specific for *Thinopyrum* and was further tested on genotype panel 3 (Table 2.1). The amplification products produced at an annealing temperature of 54 °C are shown in Figure 3.1.2.

Table 3.1.1: Primer sequences derived from the RFLP probe sequence PSR 154

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pr-PSR 154	CCATCTGTGCGAATCAATTACT	GGAGGAATTGGTCTGAATCTG

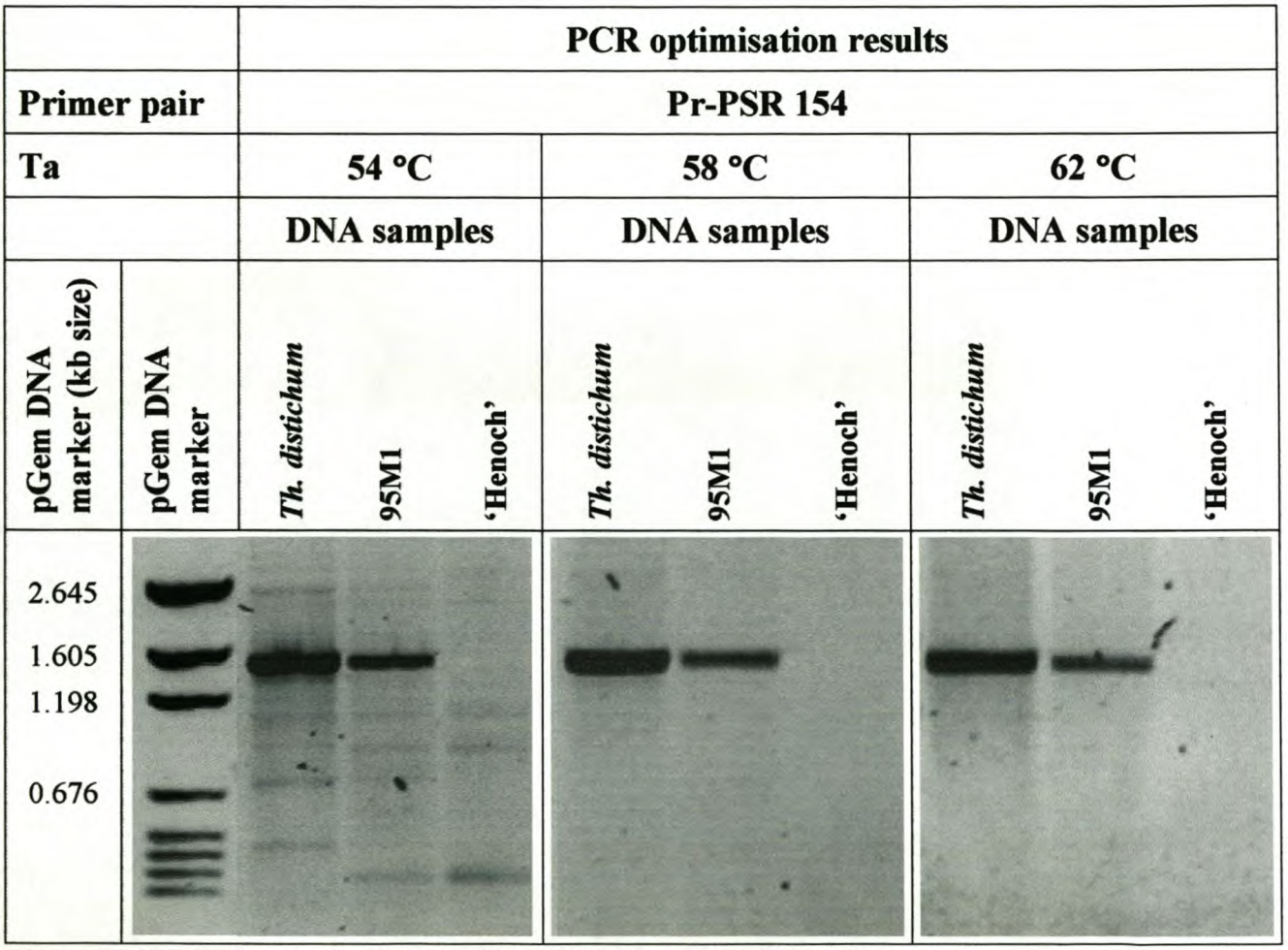


Figure 3.1.1: PCR optimisation results of primer pair Pr-PSR 154

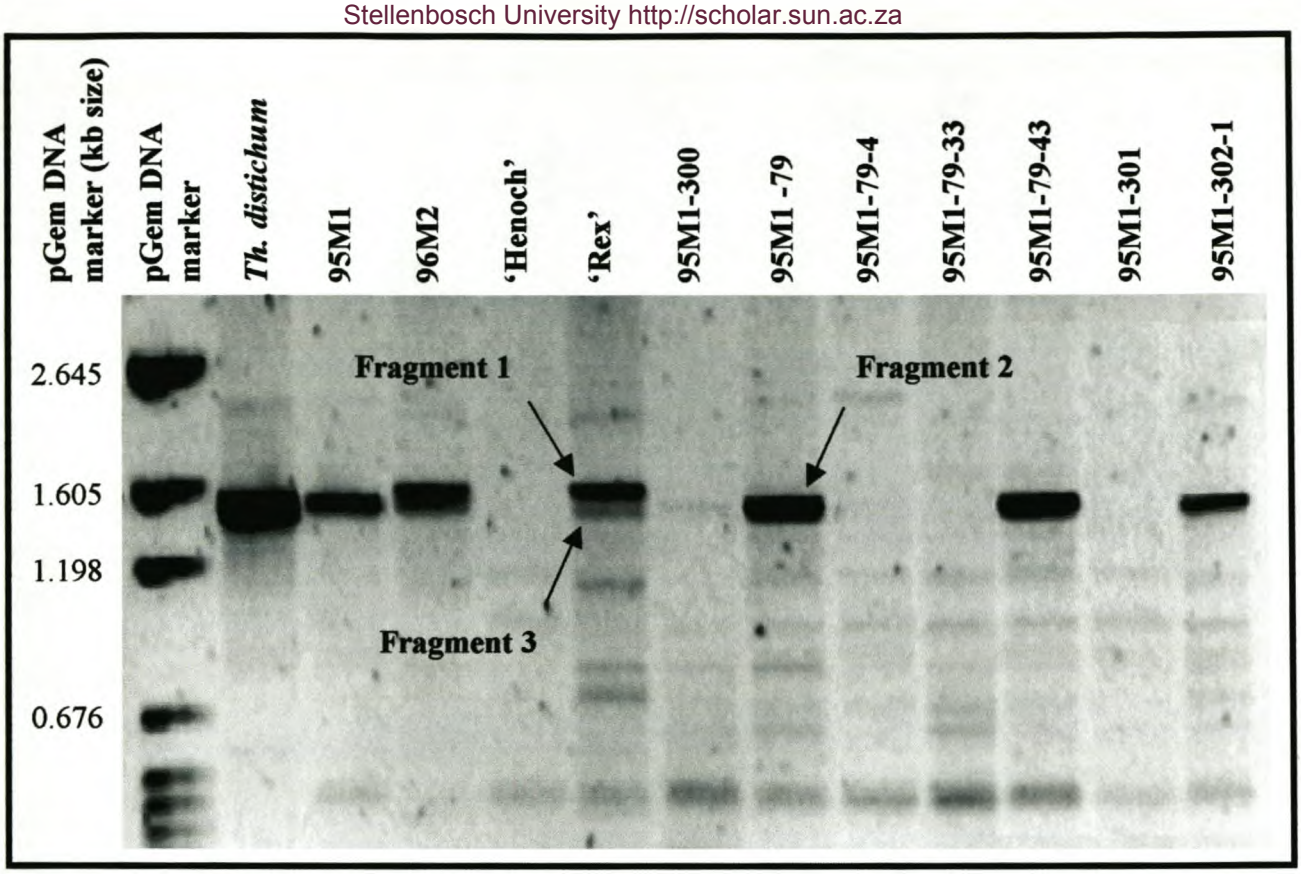


Figure 3.1.2: PCR results of primer pair Pr-PSR 154 tested on genotype panel 3

UV-light screening of an ethidium bromide stained gel identified two different sized fragments amplified within the 12 samples. The larger, designated as fragment 1, co-segregated with the 1.605 kb pGem DNA marker and the band, designated as fragment 2, was slightly smaller.

'Rex' and 96M2, which is a hybrid of the cross between *Th. distichum* and triticale, produced fragment 1 and amplification is suspected to have occurred from the triticale genome. Fragment 2 was produced by *Th. distichum* as well as samples: 95M1, 95M1-79, 95M1-79-43 and 95M1-302-1, which contain genetic material of both *Th.distichum* and 'Henoch'. The absence of this very prominent band in 'Henoch' suggests amplification occurred within the *Thinopyrum* genome. A faint band (fragment 3) similar in size to fragment 2, could also be seen in the 'Rex' lane.

Numerous other faint bands were also produced, these are believed to be the result of non-specific amplification.

The PCR gel was used for Southern blotting and probed with DIG labeled probe PSR 154 (Figure 3.1.3).

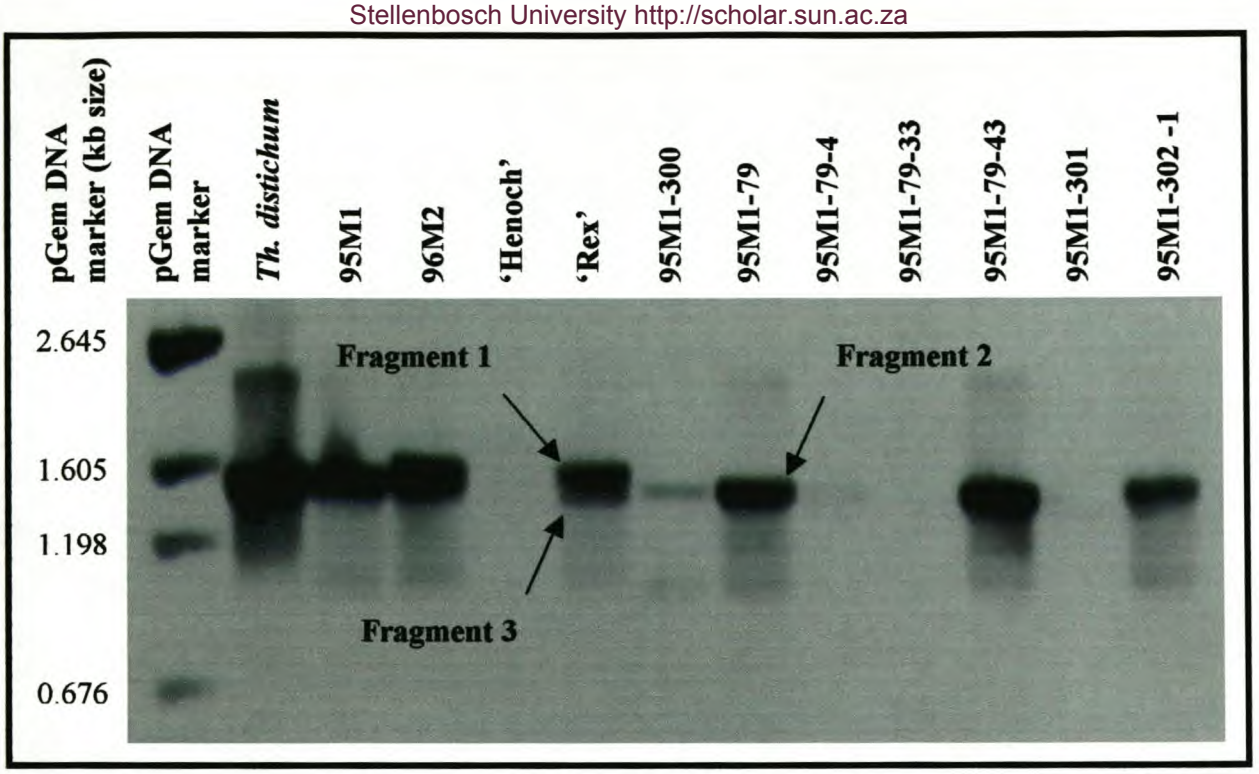


Figure 3.1.3: Southern blot results of probe PSR 154 tested on the amplification product(s) of genotype panel 3

Fragments 1, 2 and 3 appear to contain related (homoeologous) sequences since they all hybridised to probe PSR 154. Thus, the amplified products are homoeologous to anonymous loci in the group 6L arms of the common wheat from which PSR 154 derive.

Marais and Marais (2003) grouped the 14 chromosomes of *Th. distichum* into 7 homoeologous sets. However, the PSR 154 RFLPs did not relate to any of the homoeologous groups and they proposed that both group 6 chromosomes were involved in translocations with group 1 and 3 chromosomes. The present data do not suggest an association with any of the four chromosomes (Table 3.1.2). An appropriate segregation analysis will be required to determine which *Thinopyrum* chromosome(s) produces fragment 2.

The primers do not amplify a 'Henoch' rye locus and therefore are suitable for the detection of a PSR 154 related locus (loci) of *Thinopyrum* in *Thinopyrum*/rye hybrid derivatives. However, the aim of this study is to derive PCR-based markers that can be used to detect *Thinopyrum* addition chromosomes in triticale 'Rex' background. Since 'Rex' produces a faint band (3) that coincides with *Thinopyrum* band 2, Pr-PSR 154 may not always be an unambiguous marker for this background.

Table 3.1.2: Correlation of the Pr-PSR 154 band 2 with the presence/absence (+/-) of specific *Thinopyrum distichum* chromosomes in the test genotypes panel 3.

	Th. distichum	**95M1**	**96M2**	**'Henoch'**	**'Rex'**	**95M1-300**	**95M1-79**	**95M1-79-4**	**95M1-79-33**	**95M1-79-43**	**95M1-301**	**95M1-302 -1**
PCR Band 2 (Pr-PSR 154)	+	+	+	-	+	-	+	-	-	+	-	+
[1]Chromosome $6J_1^d$	+	+	+	-	-	+	+	+	-	+	-	+
[1]Chromosome $6J_2^d$	+	+	+	-	-	-	-	-	-	-	+	-
[2]Chromosome $1J_2^d$	+	+	+	-	-	-	-	-	-	-	-	+
[2]Chromosome $3J_1^d$	+	+	+	-	-	+	+	-	-	+	+	-

[1]As identified by Marais and Marais (2003)
[2]*Thinopyrum* chromosome, which may carry translocations involving the group 6 chromosomes (Marais and Marais, 2003)

3.2 PCR and Southern blot results for RFLP probe PSR 129

The RFLP probe, PSR 129, has an estimated size of 1.200 kb and hybridises to the wheat homoeologous group 7 chromosomes (long arms). The forward and reverse primers (Table 3.2.1) designed from this probe (designated as Pr-PSR 129) had melting temperatures of 56.8 and 56.1 °C, respectively, and amplified a major band of ± 1.400 kb under annealing conditions of 54, 58 and 62 °C as shown in Figure 3.2.1. The primer pair amplified a fragment in samples *Thinopyrum* and 95M1 of genotype panel 1 and was further tested (Ta=58 °C) on genotype panel 3. The results are shown in Figure 3.2.2.

Table 3.2.1: Primer sequences derived from the RFLP probe sequence PSR 129

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pr-PSR 129	AGACCTGGCGAATCTTCTGA	GACAAAGACCATCCAAGGCT

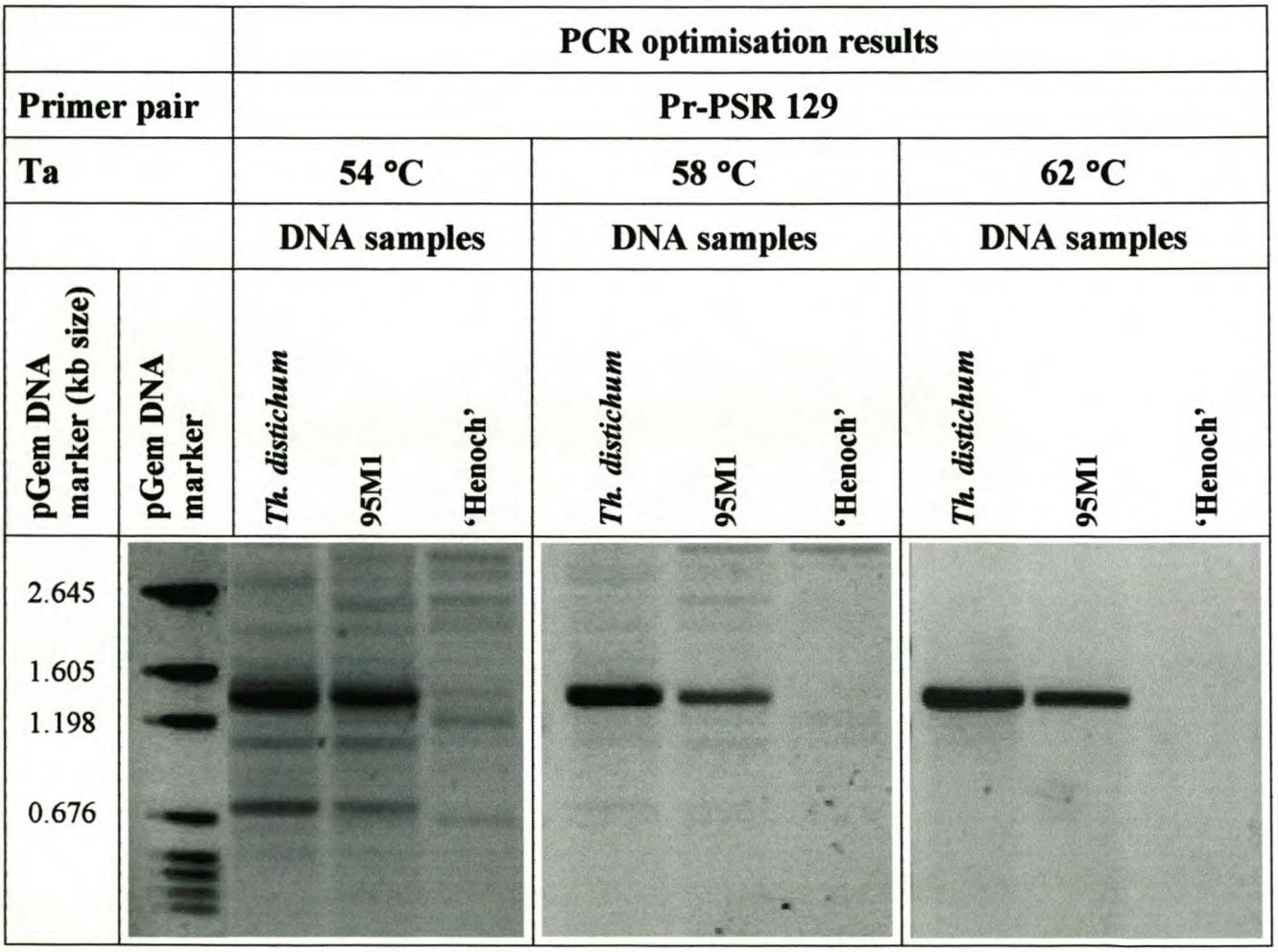


Figure 3.2.1: PCR optimisation results of primer pair Pr-PSR 129

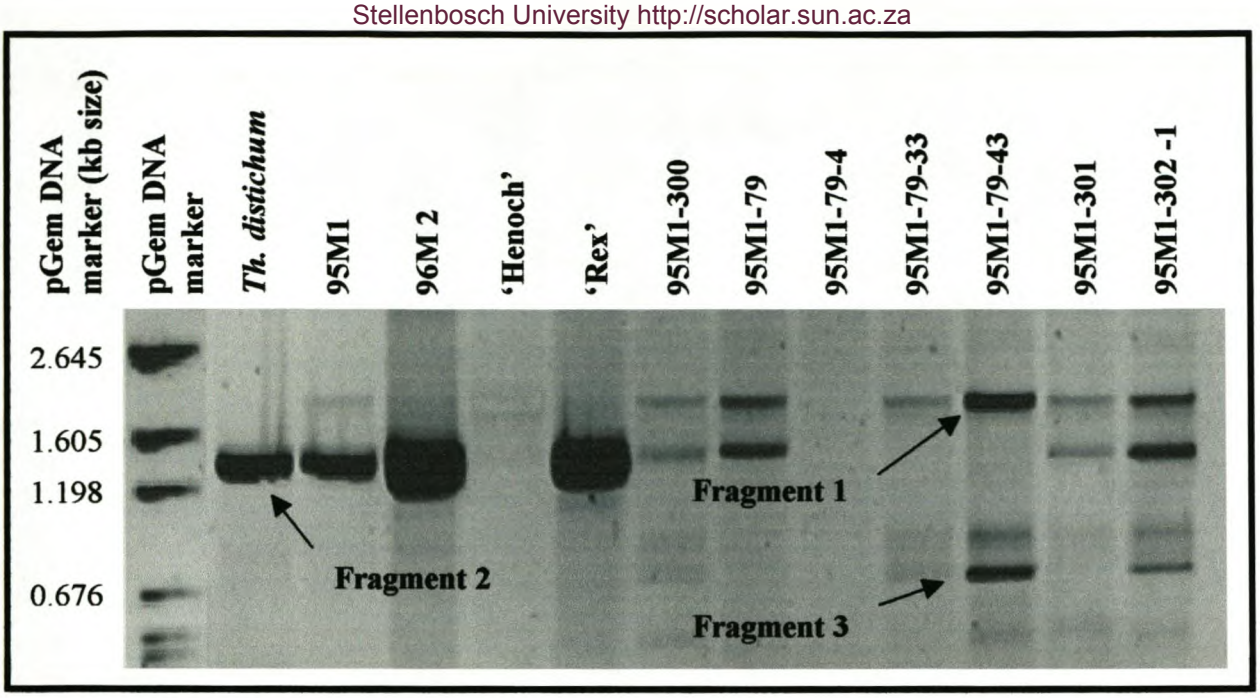


Figure 3.2.2: PCR results of primer pair Pr-PSR 129 tested on genotype panel 3

Ethidium bromide visualization showed the presence of at least 3 major fragments within the 12 samples. Fragment 3 was approximately 0.700 kb and produced by 95M1-79-43 and 95M1-302-1. These samples as well as 95M1-300, 95M1-79, 95M1-79-33 and 95M1-301 also produced a fragment of ±1.800 kb, designated as fragment 1. A smaller fragment of about ±1.400 kb, designated as fragment 2, was produced by *Th. distichum*, 95M1, 96M2, Rex, 95M1-300, 95M1-79, 95M1-301 and 95M1-302-1.

The PCR gel was then subjected to Southern blotting and probed with PSR 129. Figure 3.2.3 shows that only fragment 2 produced a strong hybridisation signal in *Th. distichum*.

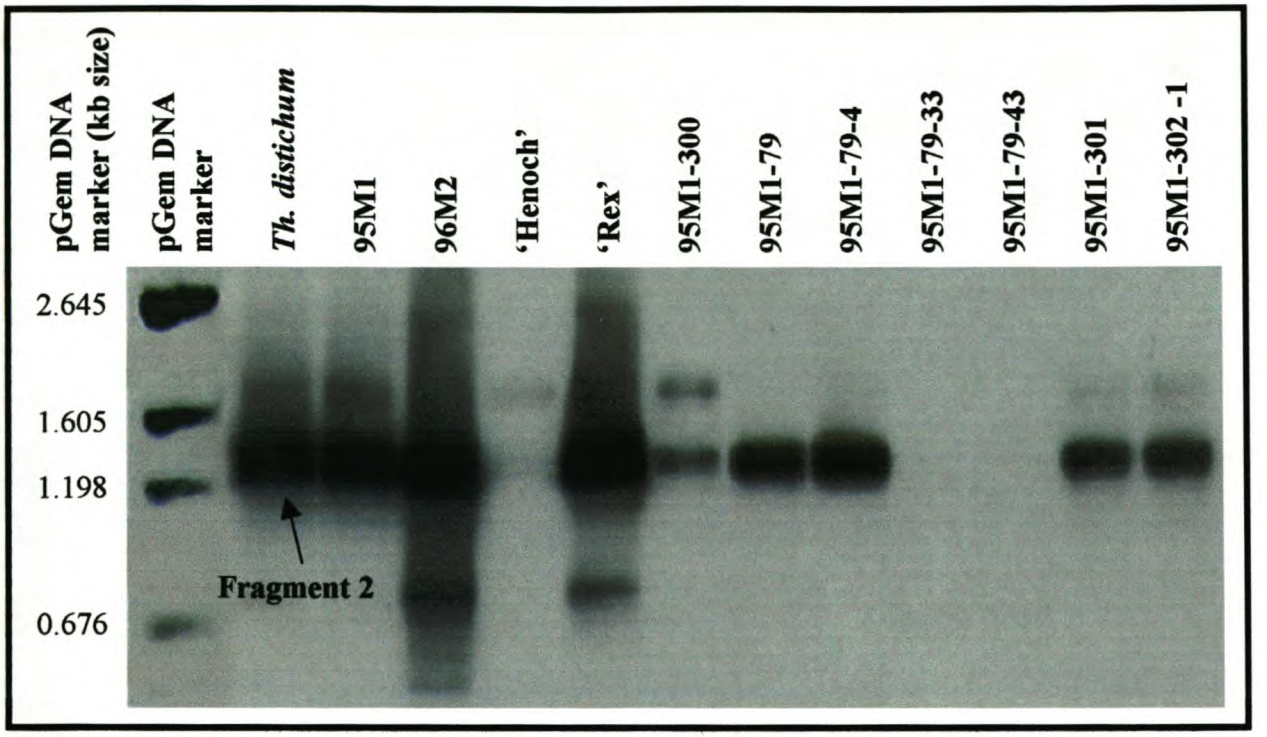


Figure 3.2.3: Southern blot results of probe PSR 129 tested on the amplification product(s) of genotype panel 3

Table 3.2.2: Correlation of the Pr-PSR 129 band 2 with the presence/absence (+/-) of a specific *Thinopyrum distichum* chromosome or RFLP polymorphism in the test genotype panel 3

	Th. distichum	95M1	96M2	'Henoch'	'Rex'	95M1-300	95M1-79	95M1-79-4	95M1-79-33	95M1-79-43	95M1-301	95M1-302 -1
Pr-PSR 129 band 2	+	+	+	-	+	+	+	+	-	-	+	+
[1]RFLP-129b	+	+	+	-	-	+	+	-[2]	-[2]	-[2]	+	-[2]
[1]RFLP-129a	+	+	+	-	-	-	-	-[2]	-[2]	-[2]	-	-

[1]RFLP (PSR129) homoeoloci detected in each of the genotypes (Marais and Marais, 2003).
[2]Does not have group 7 chromosomes from *Th.distichum*.

Marais and Marais (2003) reported that PSR 129 detects two RFLP homoeoloci in *Thinopyrum*. These segregated normally among the 95M1 testcross progeny. However, comparison of the RFLPs with the C-banded chromosomes suggested frequent recombination between the PSR 129 loci and the centromeres (i.e. the loci appeared to be located near the ends of the chromosomes). Thus, it would be more appropriate to compare the presence/absence of Pr-PSR 129 band 2 with the presence/absence of the RFLPs in the test panel (Table 3.2.2). From the results the following can be said: (1) It's not clear whether the Pr-PSR 129 primers amplify similar sized fragments from the two *Thinopyrum* homoeoloci. The test panel of genotypes does not provide an indication.
(2) Products are amplified on 95M1-79-4 and 95M1-302-1, which do not have *Thinopyrum*-derived group 7 chromosomes according to the results of Marais and Marais (2003). A possible explanation for this contradiction is the following: each of the 95M1-79 and 95M1-302 plants had a set of 21 chromosomes (14 rye chromosomes plus 7 unpaired *Thinopyrum* chromosomes). These plants were pollinated with diploid rye and the selections 95M1-79-4, 95M1-79-33, 95M1-79-43 and 95M1-302-1 were derived from the backcross progeny (the latter plants had 14 rye chromosome plus 2-4 added *Thinopyrum* chromosomes). It is well known that a high incidence of chromosome rearrangements may occur between unpaired chromosomes during meiosis. Thus, it is possible that translocations involving the *Thinopyrum* group 7 chromosome occurred during backcrossing to account for the apparent presence of a group 7 *Thinopyrum*-derived locus in the absence of a group 7 chromosome.

Reliable markers are being sought for chromosomes $7J_1^d$ and $7J_2^d$. The RFLP (PSR 129) fragment corresponding to chromosome $7J_1^d$ coincides with triticale bands and can therefore not serve as a marker. The present results have shown that the Pr-PSR 129 amplification products of triticale and *Thinopyrum* are also of similar size and the present primers are not useful either.

In a further attempt to find a suitable $7J^d$ marker, band 2 was cut from the *Thinopyrum* lane and directly sequenced. The sequence was compared to the known RFLP probe PSR 129 (wheat) sequence and the variable regions used to design new 'specific' primers (Table 3.2.3). Two forward primers were designed in a region in fragment 2, of approximately 0.270 kb, which could not be identified in the probe and thus appeared to be specific for *Thinopyrum*. Single nucleotide differences were used to design the reverse primers. The primer sequences are summarised in Table 3.2.3 and the nucleotide differences are indicated in blue.

The primers were tested on genotype panel 2 at an initial annealing temperature of 60 °C and depending on the PCR results annealing temperatures of either 4 °C increments or decrements were used to achieve optimal PCR conditions (Figure 3.2.4).

Table 3.2.3: Primer sequences derived from fragment 2 produced by Pr-PSR 129

Primer pair	Amplicon size (kb)	Name	Primer sequence (5'-3')
F-Set 1	1. 058	F-F1	GAATCTTCATCTCTTCGAATCC
		F-R1	CTTATAATTAACCGCGAGTGAG
F-Set 2	0. 855	F-F2	ATGCACCATTATGTGTGTTAAG
		F-R2	CAAATCCTCCAACCCTATAAAC

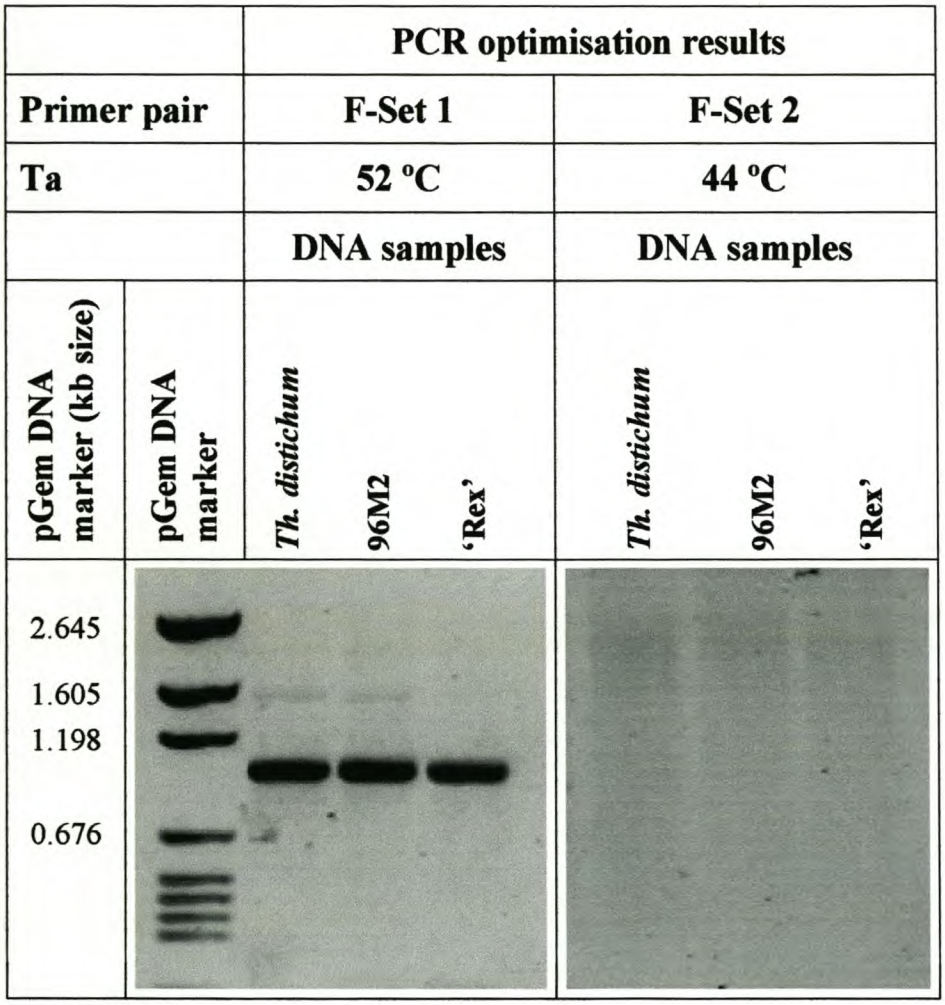


Figure 3.2.4: PCR optimisation results of primer pair F-Set 1 and F-Set 2

Figure 3.2.4 shows only the optimised conditions. *Thinopyrum* specific amplification did not occur and primer pair F-Set 1 produced strong amplification in samples *Thinopyrum*, 96M2 and Rex. Primer pair F-set 2 did not produce a product at the annealing conditions tested.

It was decided to sequence the PCR products produced by primer pair F-Set 1 in *Thinopyrum* and 'Rex'. The *Thinopyrum* sequence was examined for nucleotide(s) that differ from the 'Rex' sequence to design 'specific' markers. The three genomes of 'Rex' resulted in ambiguous sequence data and it was difficult to align certain regions to *Thinopyrum*. An overall nucleotide homology of 65 % was estimated. The forward primers of primer pair 2-FS1-Set 1 and 2-FS1-Set 2 were designed in arbitrarily chosen regions, within the first 350 bp that produced comparatively poor sequence data. The forward primer of primer pair 2-FS1-Set 3 was designed in a more 'stable' region that was characterised by good sequence data. This primer pair seemed to be a potentially valuable marker as it included a region of eleven consecutive nucleotides that were absent in 'Rex'. The reverse sequences for primer pair 2-FS1-Set 1, 2-FS1-Set 2 and 2-FS1-Set 3 were designed in regions of single nucleotide differences. The variable nucleotides included in the primers are indicated in blue (Table 3.2.4).

Table 3.2.4: Primer sequences derived from the F-Set 1 amplification products

Primer pair	Amplicon size (kb)	Name	Primer sequence (5'-3')
2-FS1-Set 1	0. 590	2-FS1-F1	GATACTGTTTTGTGCTAGCAAAGA
		2-FS1-R1	GATGTCTGACATGCCGAAC
2-FS1-Set 2	0. 511	2-FS1-F2	GCATGCACCATTATGTGTGTT
		2-FS1-R2	GCATAAGCACCGGGATCAGT
2-FS1-Set 3	0. 325	2-FS1-F3	CTTTCAGGCGTTTCCCGTT
		2-FS1-R3	GATCTGCTGCAGGTCACCA

The primers were tested at Ta=60 °C and primer pair 2-FS1-Set 1 and 2-FS1-Set 2 produced a band in *Th. distichum*, 96M2 and 'Rex' (Figure 3.2.5). These primer pairs were not tested on genotype panel 4. Primer pair 2-FS1-Set 3 produced *Thinopyrum* specific results, as expected, and an annealing temperature of 64 °C was selected to test this primer pair on genotype panel 4 (Figure 3.2.6). Amplification was not expected in the samples indicated in green (Marais 2003, personal communication). From the results in Figure 3.2.6 it can be concluded that 2-FS1-Set 3 is a homoeologue-specific marker for chromosome $7J_2^d$, as it associates with an RFLP band (PSR 129a) produced in *Thinopyrum* derivatives.

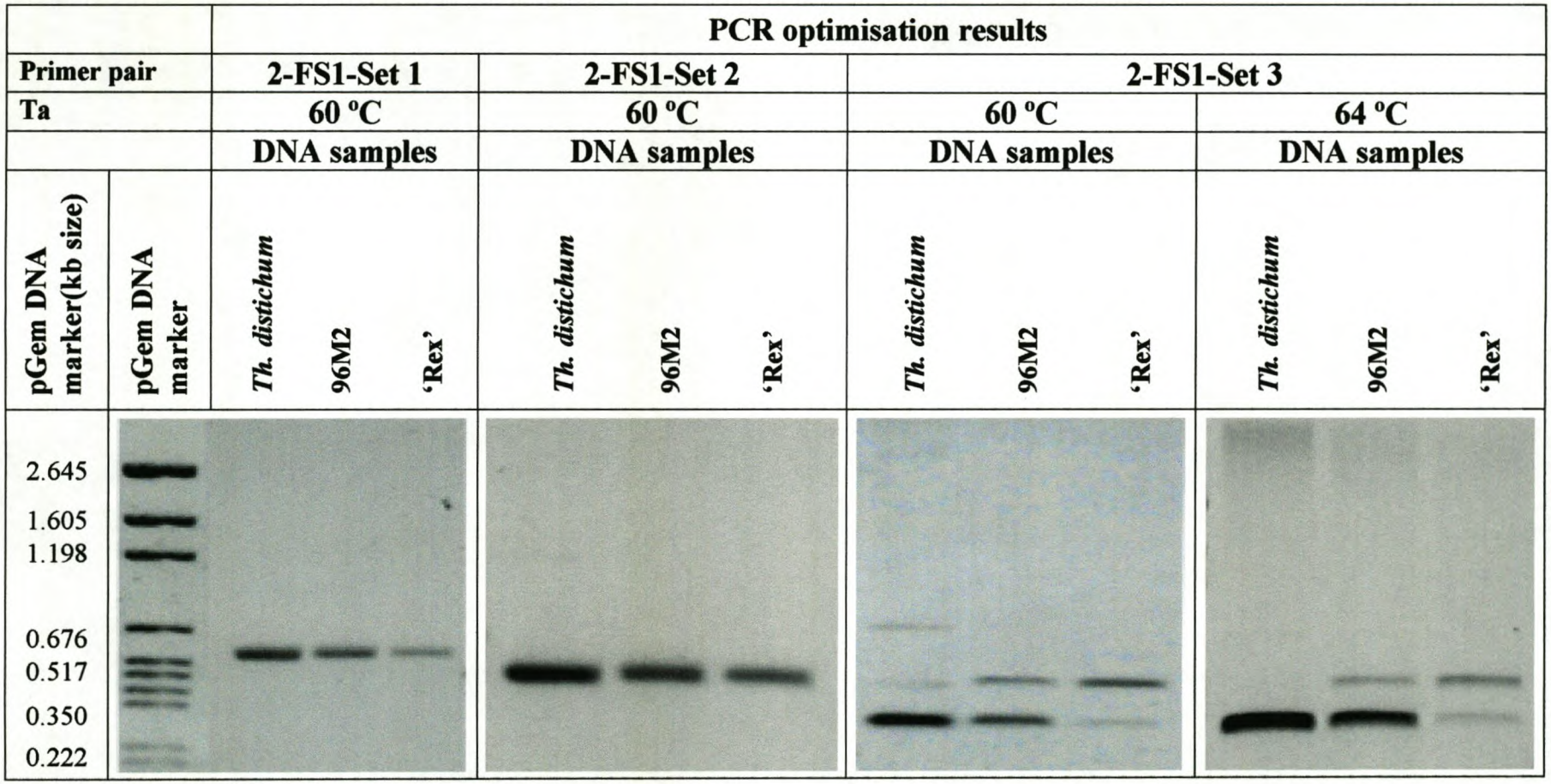
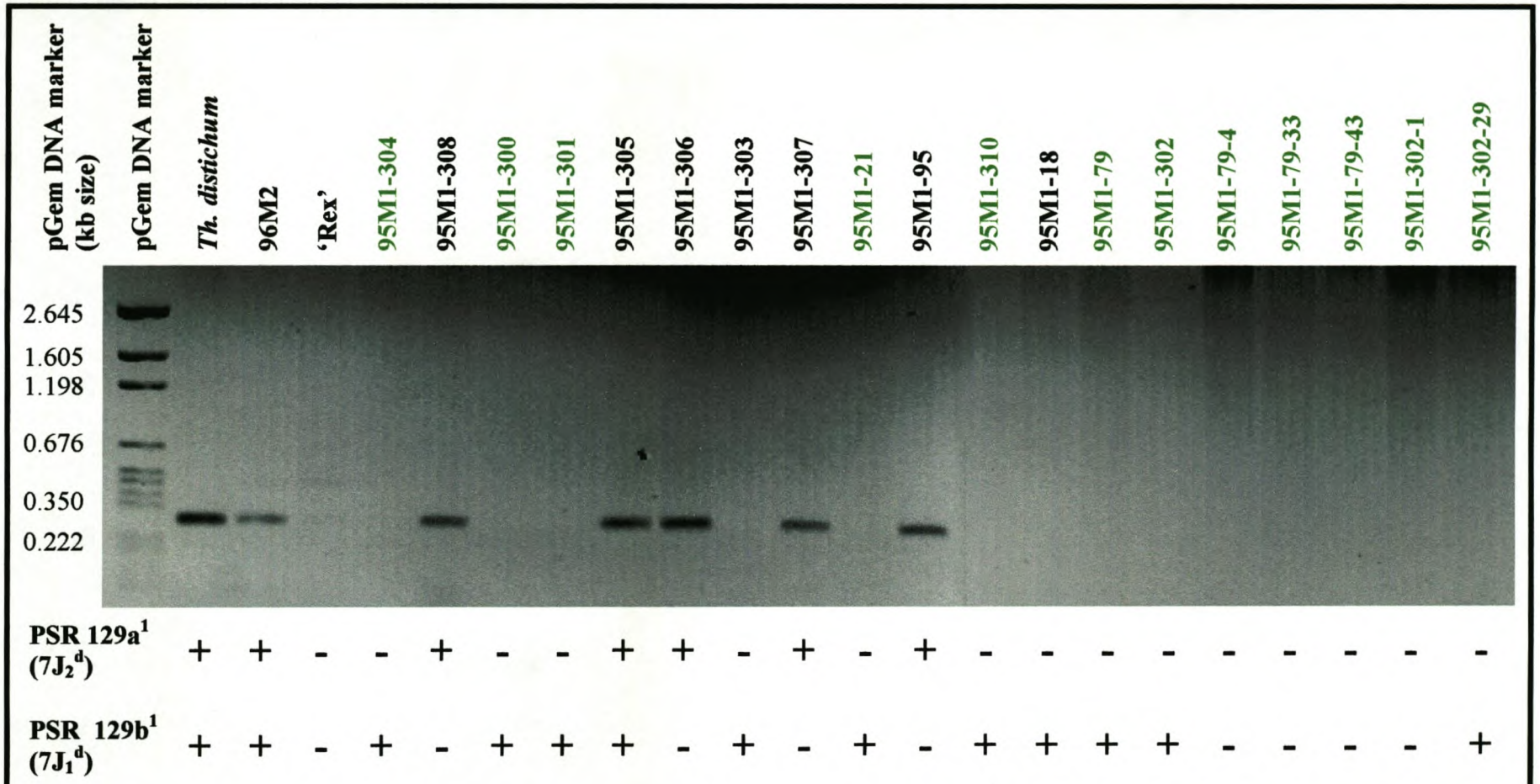


Figure 3.2.5: PCR optimisation results of primer pairs 2-FS1-Set 1, 2-FS1-Set 2 and 2-FS1-Set 3



[1]*Thinopyrum*- specific RFLP polymorphisms detected in each of the test genotypes by Marais and Marais (2003). PSR 129a represents a larger band of ± 6203 bp in size whereas PSR 129b is a smaller band of 3962 bp.

Figure 3.2.6: PCR results of primer pair 2-FS1-Set 3 tested on genotype panel 4

3.3 PCR and Southern blot results for RFLP probe PSR 666

The RFLP probe PSR 666 has an estimated size of 1.300 kb and hybridises to the wheat homoeologous group 2 chromosomes (short arms). From its sequence forward and reverse primers (Table 3.3.1) were designed and designated Pr-PSR 666. Their estimated melting temperatures (Tm) were 55.4 and 53.9 °C, respectively, and they amplified a primary fragment of ±1.000 kb in all the samples of genotype panel 1(Figure 3.3.1). More stringent annealing conditions (Ta=62 °C) produced similar results and it was decided to test the primer pair on genotype panel 3 (Figure 3.3.2).

Table 3.3.1: **Primer sequences derived from the RFLP probe sequence PSR 666**

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pr-PSR 666	CCTTCCACCTTGTCAAGTCT	TGATGTCATTCTGCATTGCT

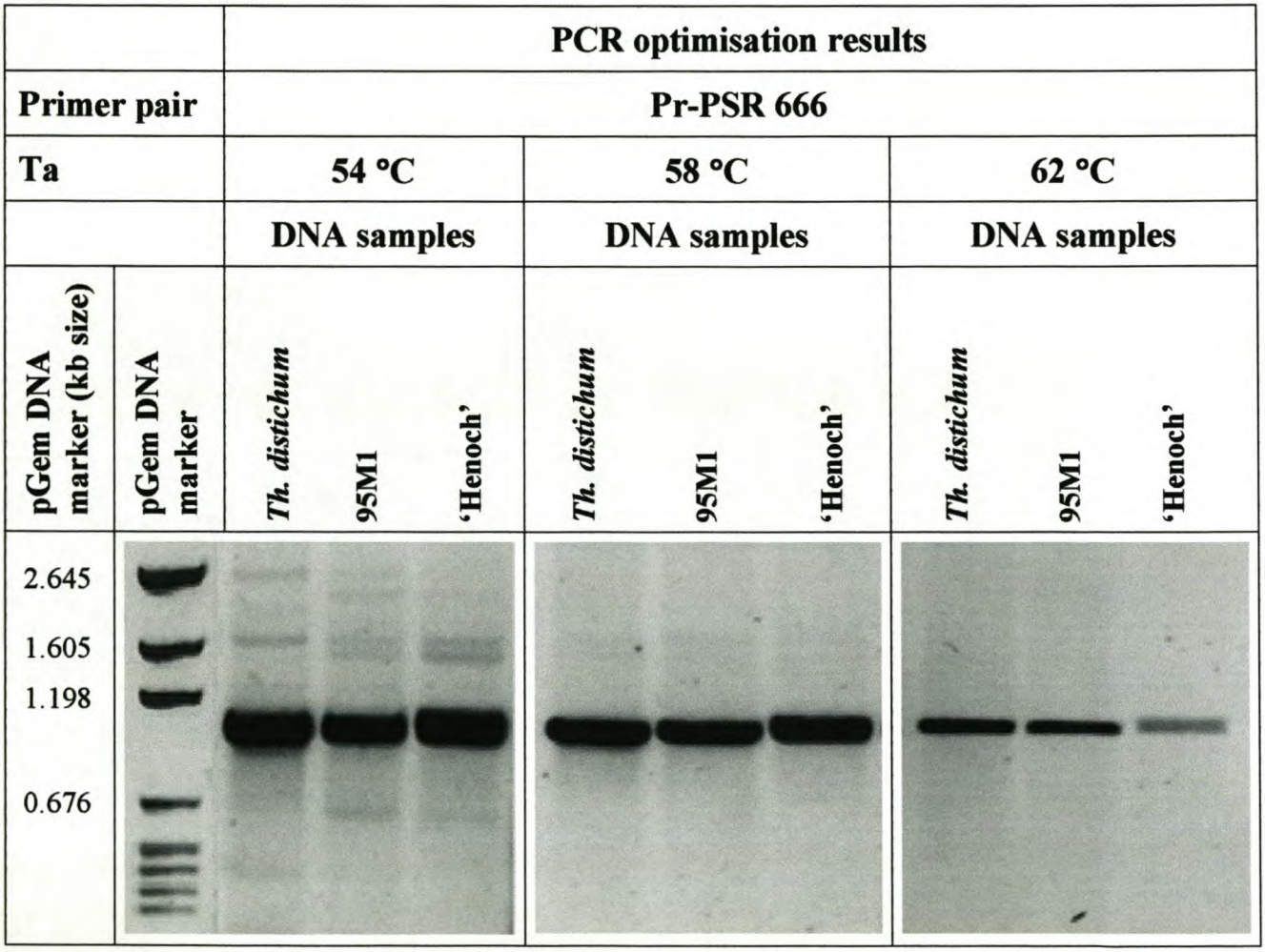


Figure 3.3.1: PCR optimisation results of primer pair Pr-PSR 666

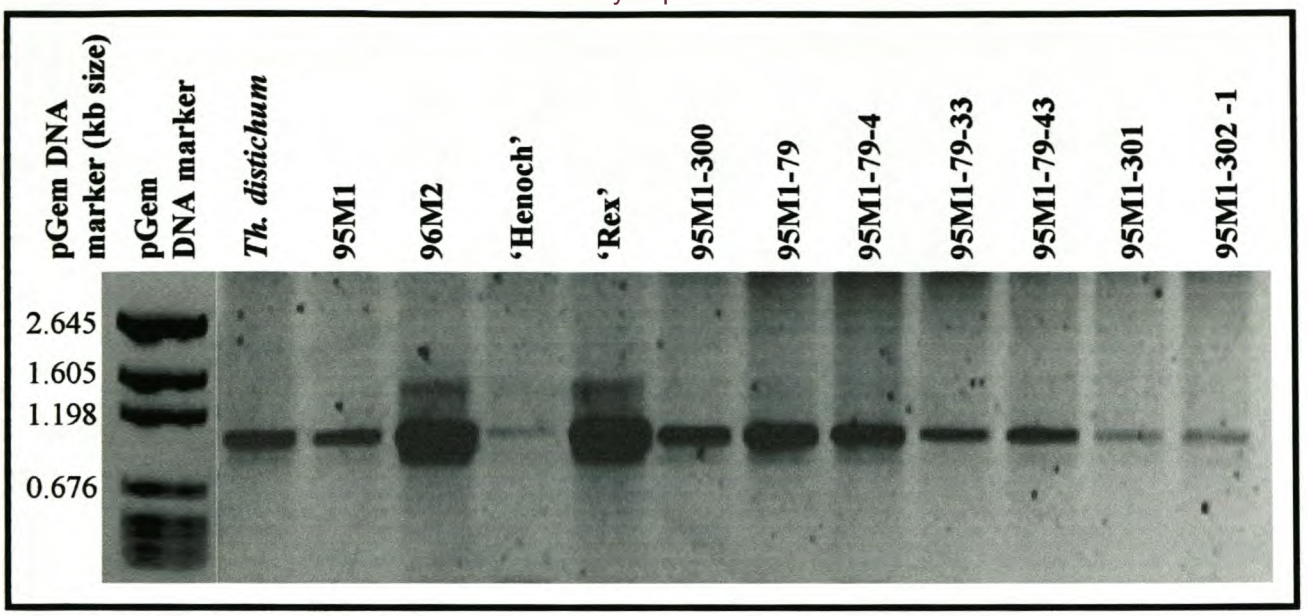


Figure 3.3.2: PCR results of primer pair Pr-PSR 666 tested on genotype panel 3

A fragment of similar size was amplified in all the DNA samples, implying that the gene sequence may be strongly conserved across species. When the DNA was transferred to a membrane and probed with PSR 666 (Figure 3.3.3) strong hybridisation was seen in all the lanes. Thus, the primers cannot differentiate between loci derived from *Thinopyrum*, rye and triticale and cannot be used as a marker.

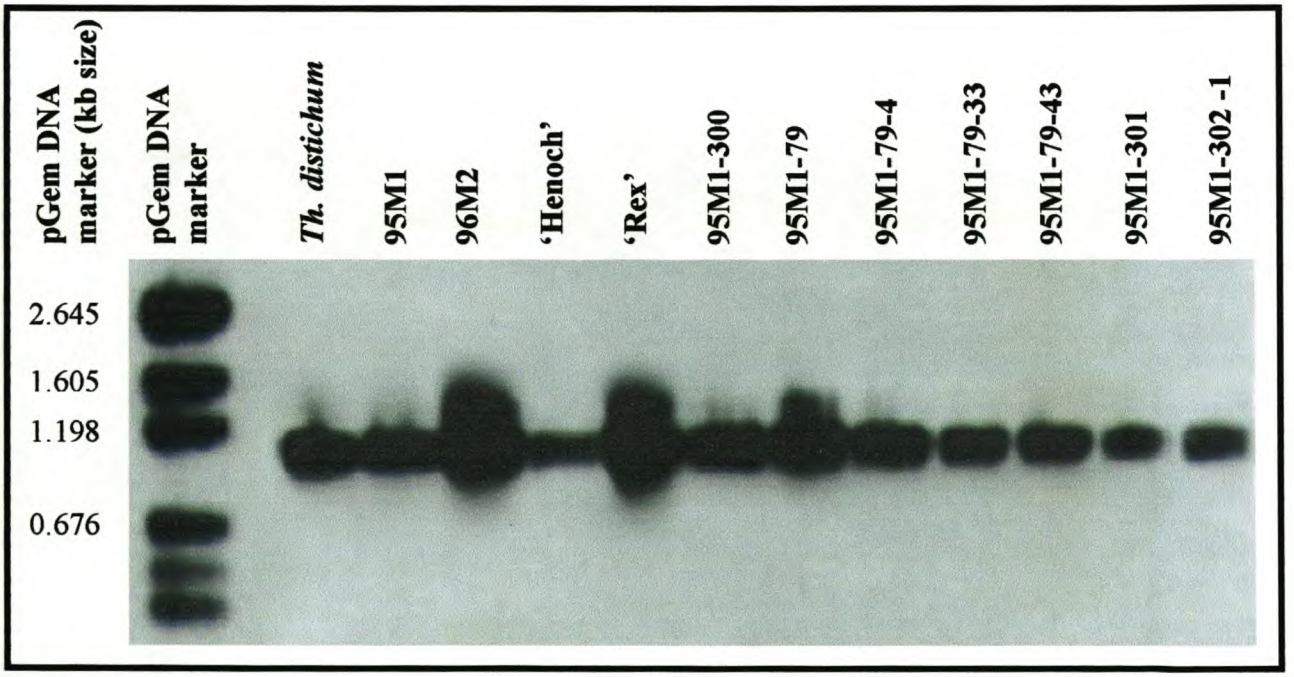


Figure 3.3.3: Southern blot results of probe PSR 666 tested on the amplification product(s) of genotype panel 3

An attempt was made to sequence the band produced by *Thinopyrum* and to derive more specific primers. Direct sequencing resulted in unsatisfactory sequence data. The band (named I) was therefore excised from the gel and purified. The purified band was ligated with the pGem®-T Easy Vector and transformed into competent cells. Primer pair Pr-PSR 666 was used to screen colonies for bands I (colony PCR) and Figure 3.3.4 shows the PCR results of positive transformed colonies.

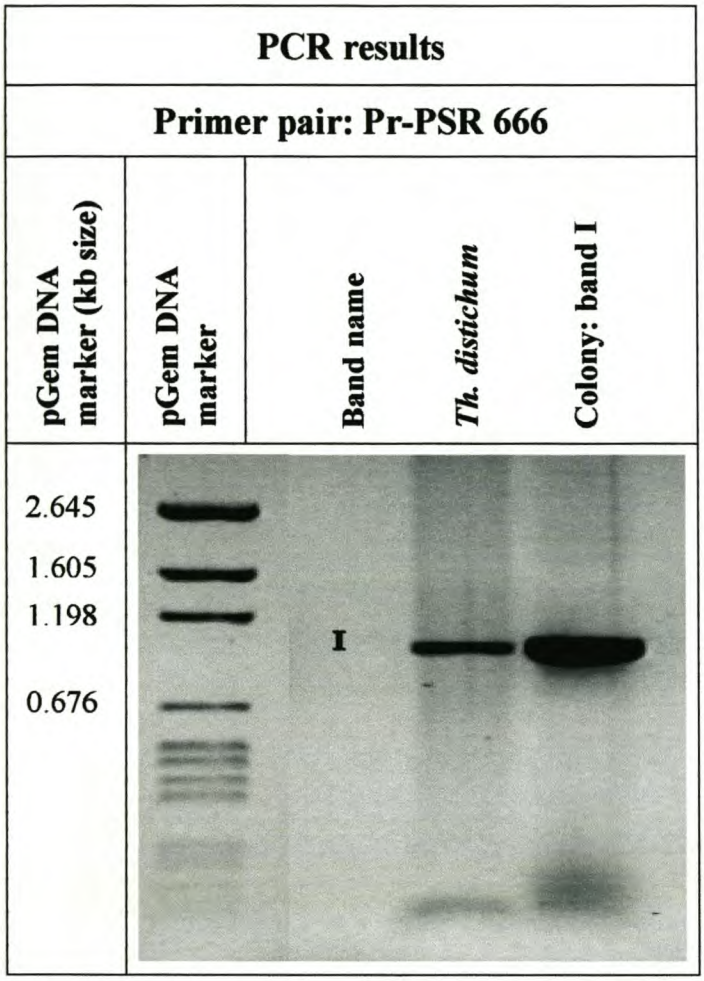


Figure 3.3.4: Isolated band I and colony PCR results

The plasmids of the transformed colonies were extracted and sequenced with primers (SP6 and T7) flanking the multiple cloning area of the vector, which made it possible to identify and examine the Pr-PSR 666 primer-binding site of the transformed amplicon. In this case the binding sites could not be identified. Also, the sequences of band I and the probe, PSR 666, did not correspond. Obviously a mistake such as switched samples might have occurred during sequencing.

Primers were designed from the suspect *Thinopyrum* sequence. The primer regions were arbitrarily chosen. The primer sequences and the size of the expected amplicon are summarized in Table 3.3.2.

Table 3.3.2 Primer sequences derived from band I produced by Pr-PSR 666

Primer pair	Amplicon size (kb)	Name	Primer Sequence (5'-3')
I-Set 1	1.163	I-F1	ATCAAAAGAATAGACCGAGATAG
		I-R1	CTGGTATCTTTATAGTCCCTGTC
I-Set 2	0.854	I-F2	CATCACCCTAATCAAGTTTTT
		I-R2	TCTGTGGATAACCGTATTACC

The primer pairs were tested at Ta=60 °C and depending on the results, also at 4 °C decrements. Figure 3.3.5 shows the results obtained at Ta=44 and 48 °C. *Thinopyrum* specific amplification did not occur and primer pairs I-Set 1 and I-Set 2 produced faint 'non-specific' bands. These primer sets were not tested on genotype panel 4.

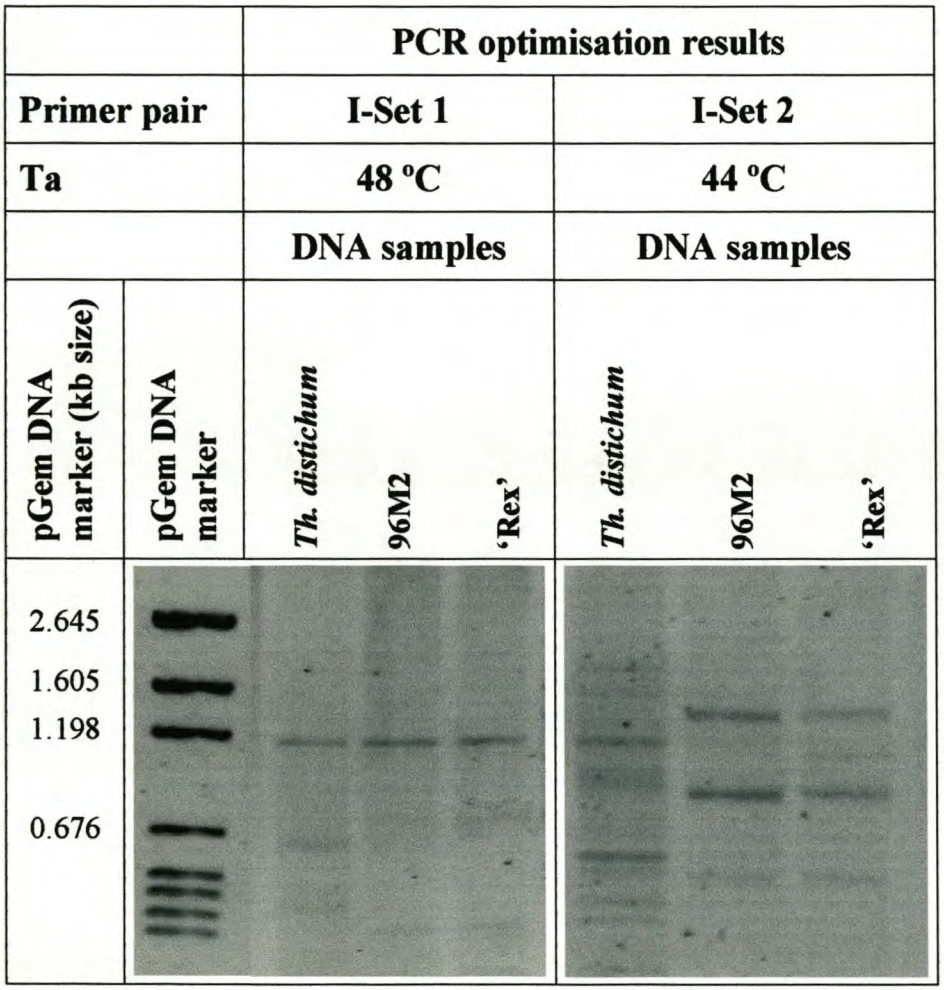


Figure 3.3.5: PCR optimisation results of primer pairs I-Set 1 and I-Set 2

Attempts to directly sequence the amplification product produced by primer pair, Pr-PSR 666, in *Thinopyrum* were repeated. The amplification product produced by this primer pair in 'Rex' was also sequenced. Ambiguous sequence data produced by Rex contributed to a poor homology count of 42 % and it is uncertain whether the regions selected for primer design were valid. The supposed single nucleotide difference used to design primer pair 2-I Set 1 and the deletion in 'Rex' used to design primer pair 2-I Set 2, are indicated in blue (Table 3.3.3).

Table 3.3.3: Primer sequences derived from band I produced by Pr-PSR 666

Primer pair	Amplicon size (kb)	Name	Primer Sequence (5'-3')
2-I Set 1	0.709	2-I-F1	GATTTCTGGAGTCCTGCTCTAAT
		2-I-R1	ACTCTTCGAGGTATAACCATGATC
2-I Set 2	0.368	2-I-F2	CATTTCGTTGGTAGTGGATGC
		2-I-R2	GCATGCATTTGCTCAGAACA

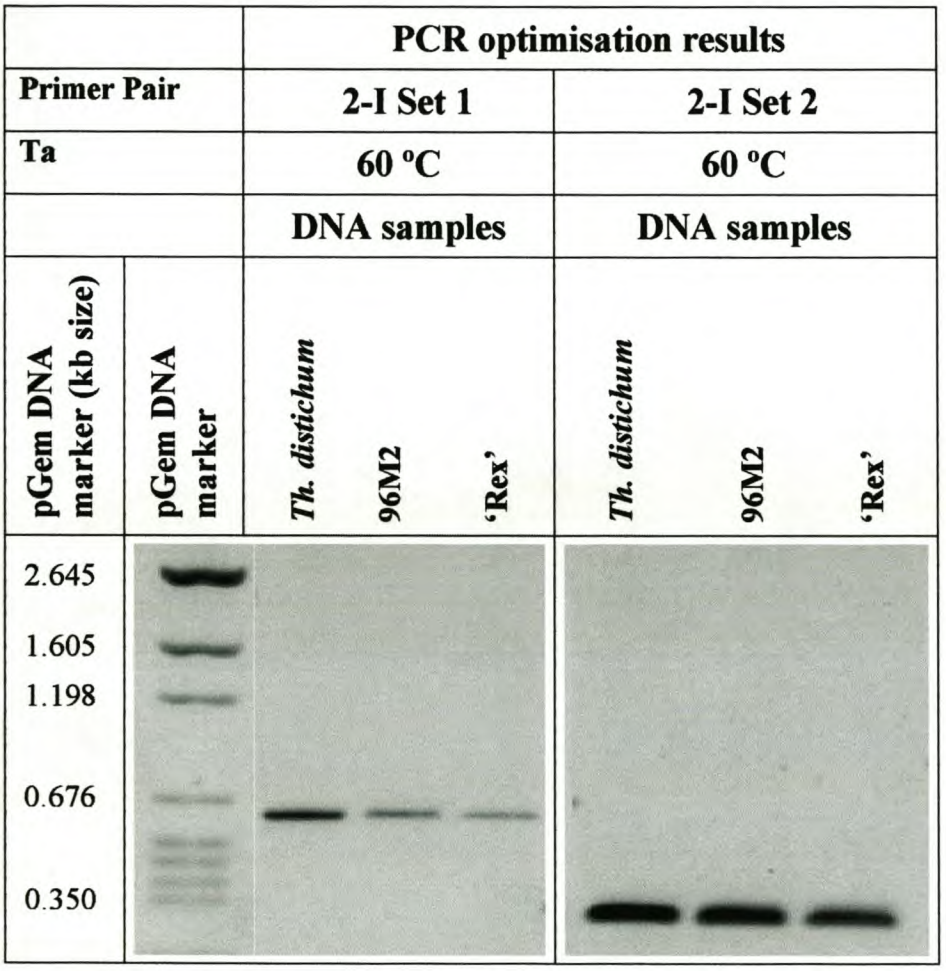


Figure 3.3.6: PCR optimisation results of primer pairs 2-I Set 1 and 2-I Set 2

None of the primer pairs tested on genotype panel 2 produced useful polymorphisms (Figure 3.3.6) and therefore are not suitable markers for the homoeologous group 2 chromosomes.

3.4 PCR and Southern blot results for RFLP probe PSR 931

The RFLP probe, PSR 931, has an estimated size of 1.300 kb and hybridises to the wheat homoeologous group 3 chromosomes (long arms). The forward and reverse primers (Table 3.4.1) designed from this probe (designated as Pr-PSR 931) had melting temperatures (Tm) of 57.9 and 57.1 °C, respectively, and amplified a major fragment of ±1.000 kb as well as numerous other fragments in genotype panel 1 when annealing temperatures of 54, 58 and 62 °C were used as shown in Figure 3.4.1. Primer pair, Pr-PSR 931, was used to do further PCR reactions (Ta=62 °C) on genotype panel 3 (Figure 3.4.2).

Table 3.4.1: Primer sequences derived from the RFLP probe sequence PSR 931

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pr-PSR 931	AAGTAGCAAGGTAGCAGGAGC	ACAGGTCATCAGCATAGCAGA

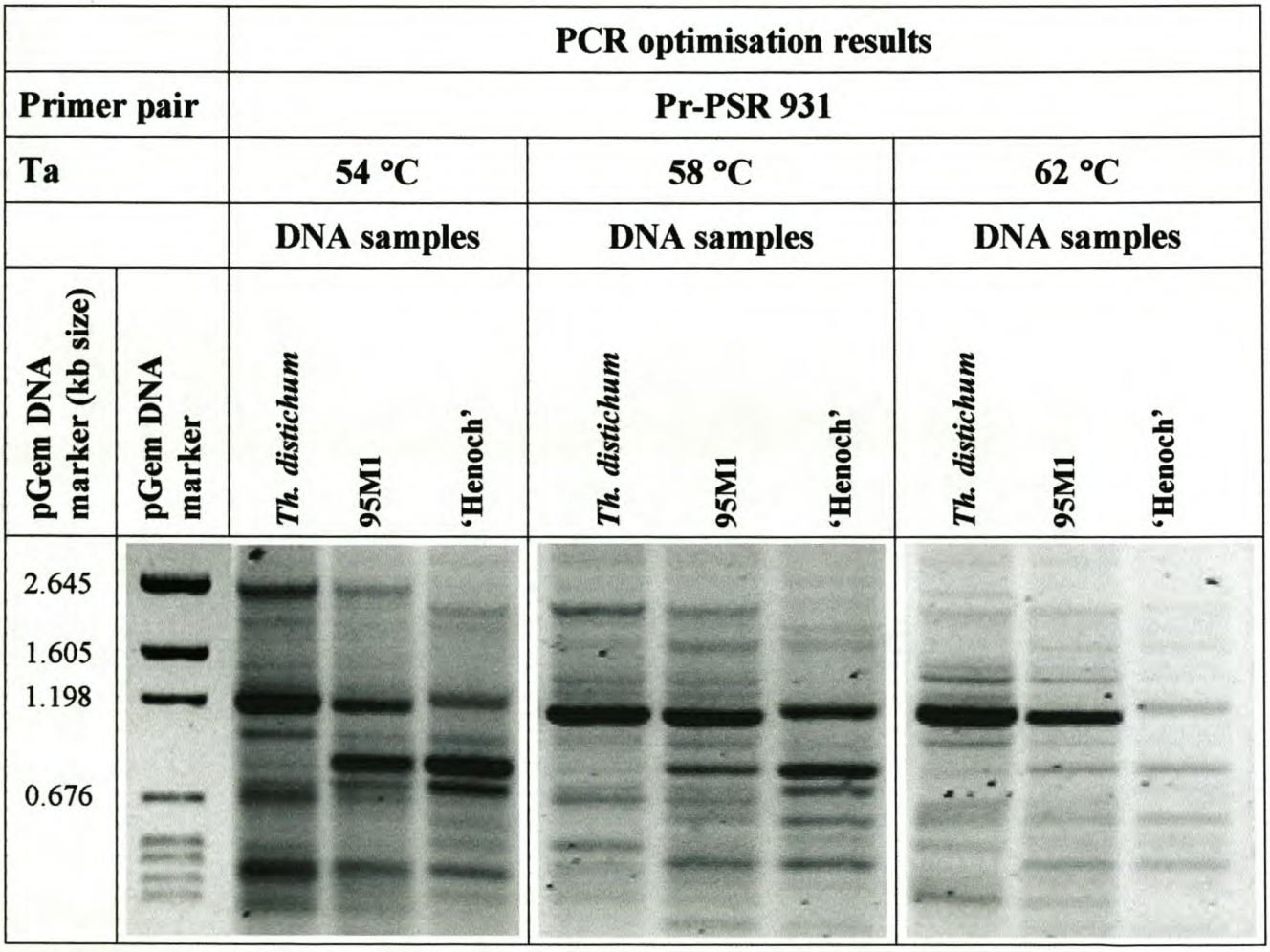


Figure 3.4.1: PCR optimisation results of primer pair Pr-PSR 931

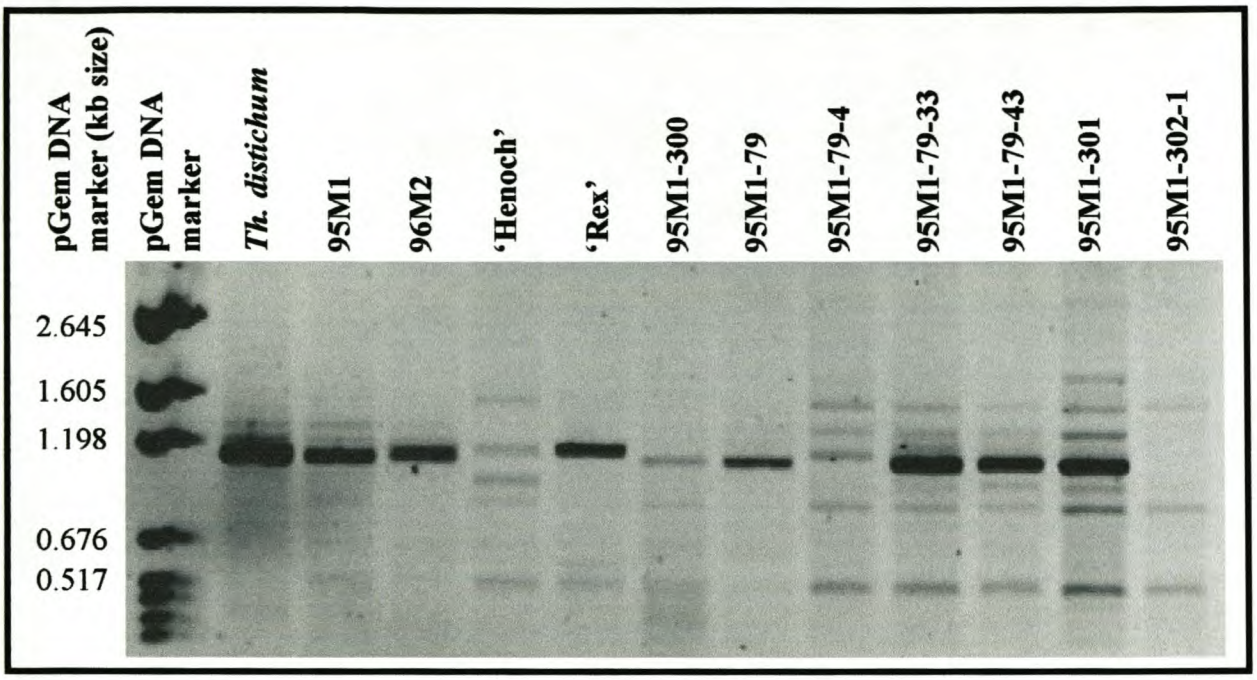


Figure 3.4.2: PCR results of primer pair Pr-PSR 931 tested on genotype panel 3

A major band was seen in all samples except in 95M1-302-1 whereas numerous fainter bands were seen in all lanes. When a Southern blot of this gel was hybridised with PSR 931 (Figure 3.4.3) it appeared that all lanes except 95M1-302-1 produced two or more bands.

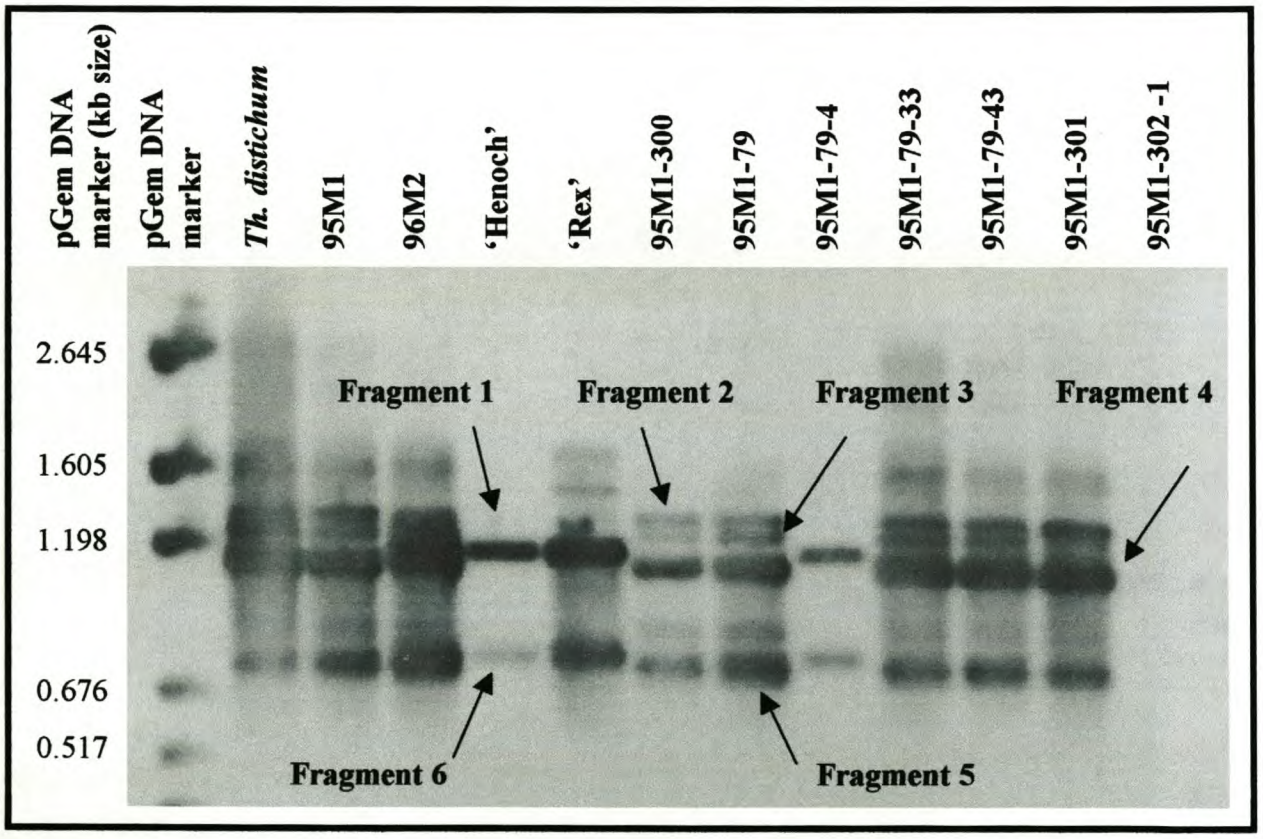


Figure 3.4.3: Southern blot results with probe PSR 931 tested on the amplification products of genotype panel 3

A total of 6 bands can be distinguished in Figure 3.4.3. Bands 1 and 6 appear to be rye-specific, but do not occur in all the genotypes that have rye genomes (these are all except *Th. distichum*). Bands 1 and 6 appear to be present in 96M2, 'Henoch', 'Rex' and 95M1-79-4 but absent in 95M1, 95M1-300, 95M1-79, 95M1-79-33, 95M1-79-43, 95M1-301 and 95M1-302-1. This result is not surprising as rye is a cross-pollinator and varieties are genetically highly heterogeneous. Bands 2, 3, 4 and 5 appear to be *Thinopyrum*-specific. It occurs in *Thinopyrum* and all the genotypes with *Thinopyrum* in their parentage, except for 95M-79-4 and 95M1-302-1. According to Marais and Marais (2003) chromosome $3J_1^d$ occur in all the 95M1 selections, except these two. It would therefore seem that the four bands are diagnostic for this chromosome. In their RFLP study, probe PSR 931 amplified 3 bands on chromosome $3J_1^d$.

However, comparison of the Figure 3.4.2 and 3.4.3 results would suggest that in order to use the markers, one would need to probe the PCR-blot with probe, PSR 931 which would detract from its usefulness. It was therefore decided to attempt a further refinement of the primers. For this purpose amplified fragments (designated as J1, J2 and J3) were excised from the *Thinopyrum* lane (Figure 3.4.4). The purified bands were ligated with the pGem®-T Easy Vector and transformed to competent cells. Colonies were screened for the different bands by means of PCR using primer pair, Pr-PSR 931, and Figure 3.4.4 shows the results of only one selected colony successfully transformed with either bands J1, J2 or J3.

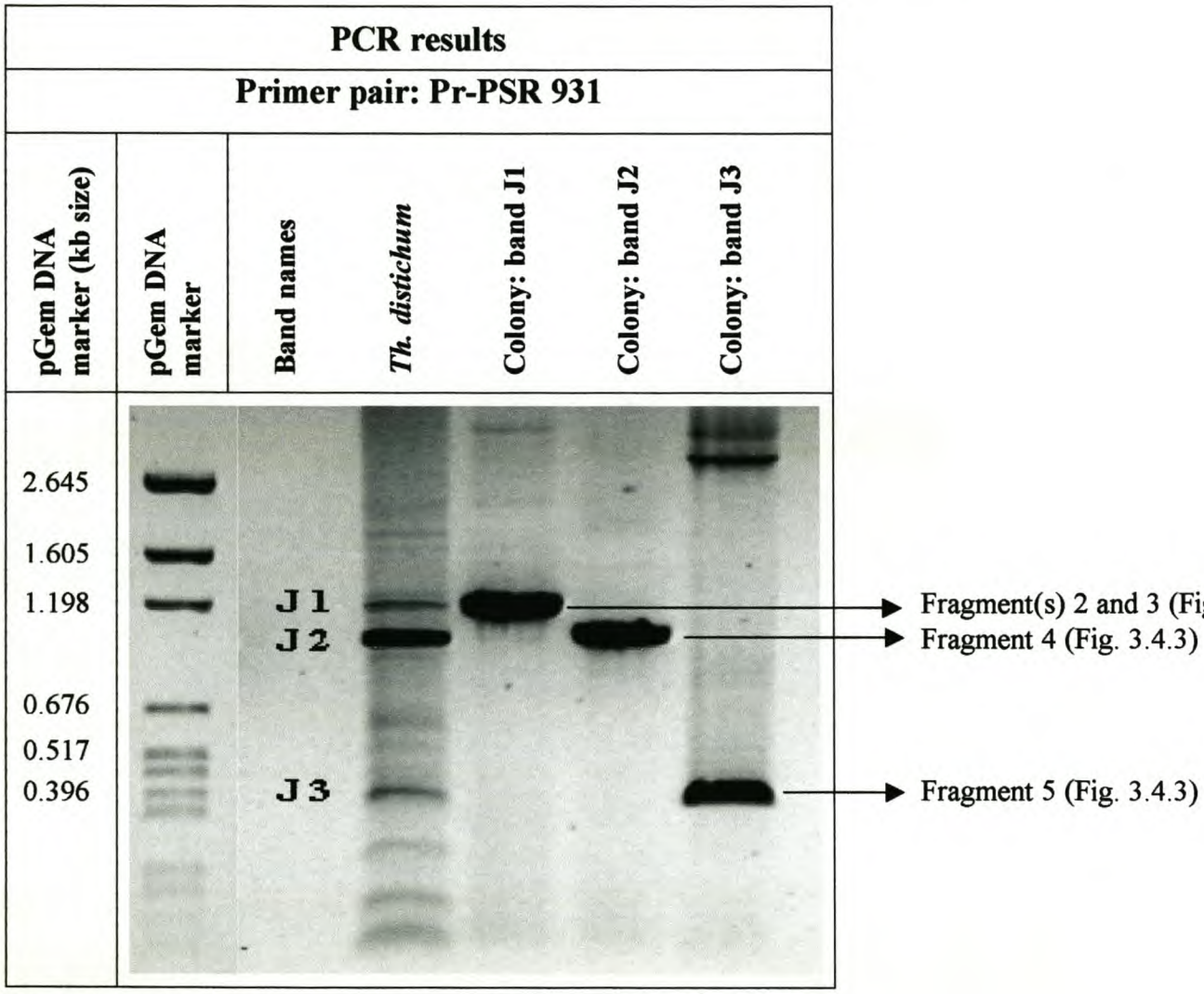


Figure 3.4.4: Isolated band J1, J2 and J3 and colony PCR results

The plasmids of these colonies were extracted and sequenced with primers (SP6 and T7) flanking the multiple cloning area of the vector. Therefore it was possible to identify the primer-binding site of primer pair, Pr-PSR 931 in the sequences of J1, J2 and J3.

Homology alignment between the sequence of J3 and the probe, PSR 931 was not possible. It is presumed that more than one amplification product occurred in band J3 and that the sequence selected for cloning did not derive from the PSR 931 locus. Otherwise, both the sequences of J1 and J2 revealed a large degree of similarity to the sequence of the probe, PSR 931. The main difference between these two bands, which also contributes to the size difference as detected on the agarose gel (Figure 3.4.4), is a region of approximately 0.200 kb downstream from the forward primer binding site on J1. This region was selected to design the forward primers of primer pairs, J1-Set 1 and J1-Set 2. Another region of approximately 60 nucleotides in the sequence of J1 did not match to J2 and was selected to design the two reverse primers of these primer pairs. Two sets of primers were also designed from the J2 sequence. The reverse primer of primer pair J2-Set 1 includes six nucleotides that were absent in the sequence of J1 and the forward primer was designed at a single nucleotide difference. The reverse primer of primer pair J2-Set 2 was also based on a single nucleotide difference and the forward primer were arbitrarily chosen. The sizes of the expected amplicons are summarized in Table 3.4.2. The variable nucleotides included in the primers are indicated in blue.

Table 3.4.2: Primer sequences derived from bands J1, J2 and J3 produced by Pr-PSR 931

Primer pair	Amplicon size (kb)	Name	Primer sequence (5'-3')
J1-Set 1	0.849	J1-F1	TTAGGATTTGTCCACTTGATTG
		J1-R1	GTTGGTGCTTGGTACCTTAAC
J1-Set 2	0.734	J1-F2	TCACTCAATGGTGTTGTGG
		J1-R2	CGACTTCATTATCGGCAAC
J2-Set 1	0.659	J2-F1	CTAGTGATTAAGTAGCAAGGTAGCA
		J2-R1	TCAATGCTTGTGCAAGAAAG
J2-Set 2	0.705	J2-F2	GTTCGATAGCTCATCATAGCAG
		J2-R2	TTCTAGTGGTTGCAATTGAAGT

The primers were optimised using samples of genotype panel 2. All the primers were tested at an initial annealing temperature of 60 °C and depending on the PCR results annealing temperatures of either 4 °C increments or decrements were used to achieve optimal PCR conditions (Figure 3.4.5 and 3.4.8). Primers that proved to be specific for *Thinopyrum* were then tested on an addition line having *Thinopyrum* chromosome $3J_1^d$, and genotype panel 4 (Figures 3.4.6, 3.4.7 and 3.4.9)

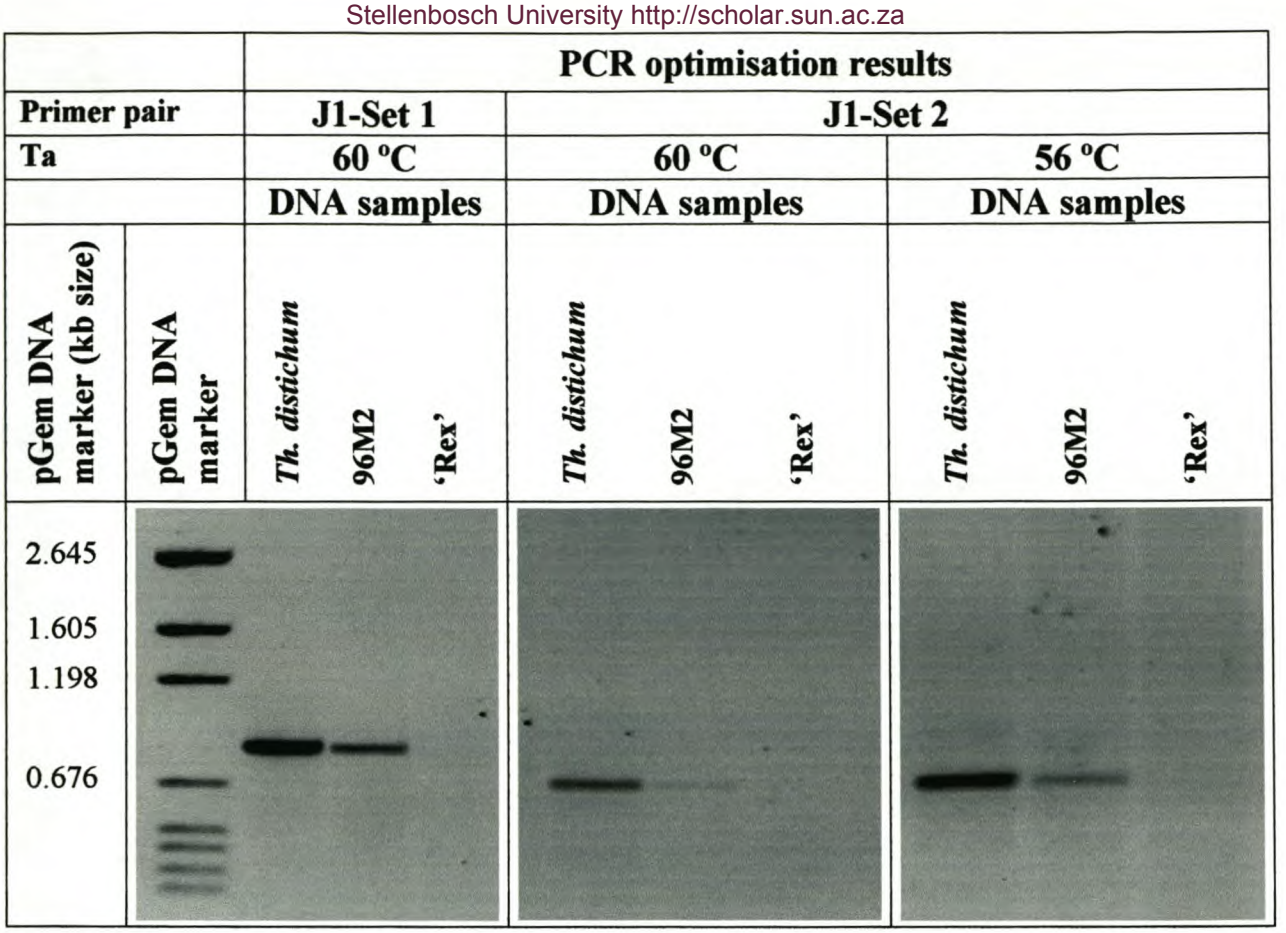


Figure 3.4.5: PCR optimisation results of primer pairs J1-Set 1 and J1-Set 2

Primer sets J1-Set 1 and J1-Set 2 were optimised at annealing temperatures of 60 and 56 °C, respectively, and each produced a single clear band that correlated with the expected amplicon size of 0.849 and 0.734 kb. Amplification was only detected in *Thinopyrum* and 96M2, thus, the target alleles are specific to *Thinopyrum* and will be valuable as markers for the group 3 chromosomes.

Primer pairs J1-Set 1 and J1-Set 2 were then tested on an addition line (addition 56) carrying one of the *Thinopyrum* chromosomes as well as genotype panel 4. The samples indicated in green (Figure 3.4.6) exclude chromosome $3J_1^d$, according to Marais and Marais (2003) and therefore an amplification product is not expected in these cases. From Figure 3.4.6

Primer pair J1-Set 1 and J1-Set 2 amplified a target fragment in all the genotypes of the test panel that have $3J_1^d$ (Figure 3.4.6 and Figure 3.4.7). The value of J1-Set 1 and J1-Set 2 as a *Thinopyrum* specific marker was confirmed by amplification in addition 56, which is a disomic addition line (2n=44) for chromosome $3J_1^d$ (Figure 3.4.6 and Figure 3.4.7).

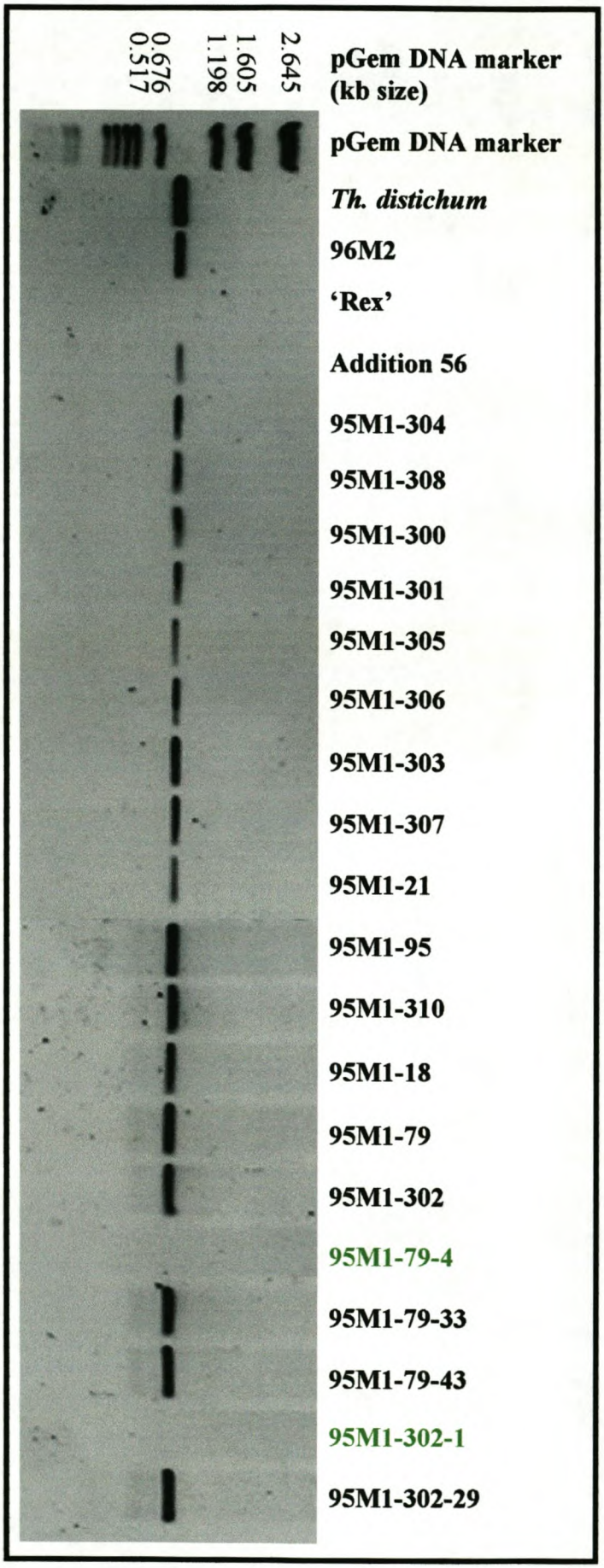


Figure 3.4.6: PCR results of primer pair J1-Set 1 tested on addition 56 and genotype panel 4

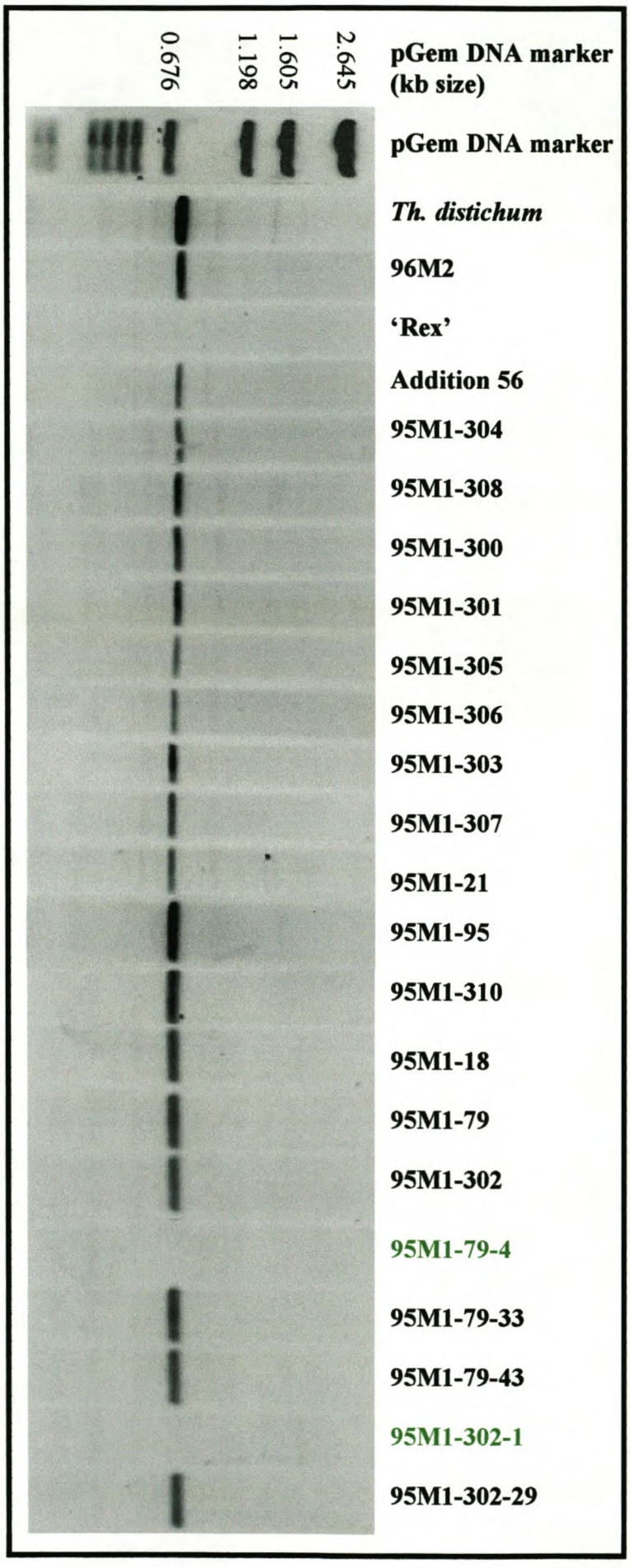


Figure 3.4.7: PCR results of primer pair J1-Set 2 tested on addition 56 and genotype panel 4

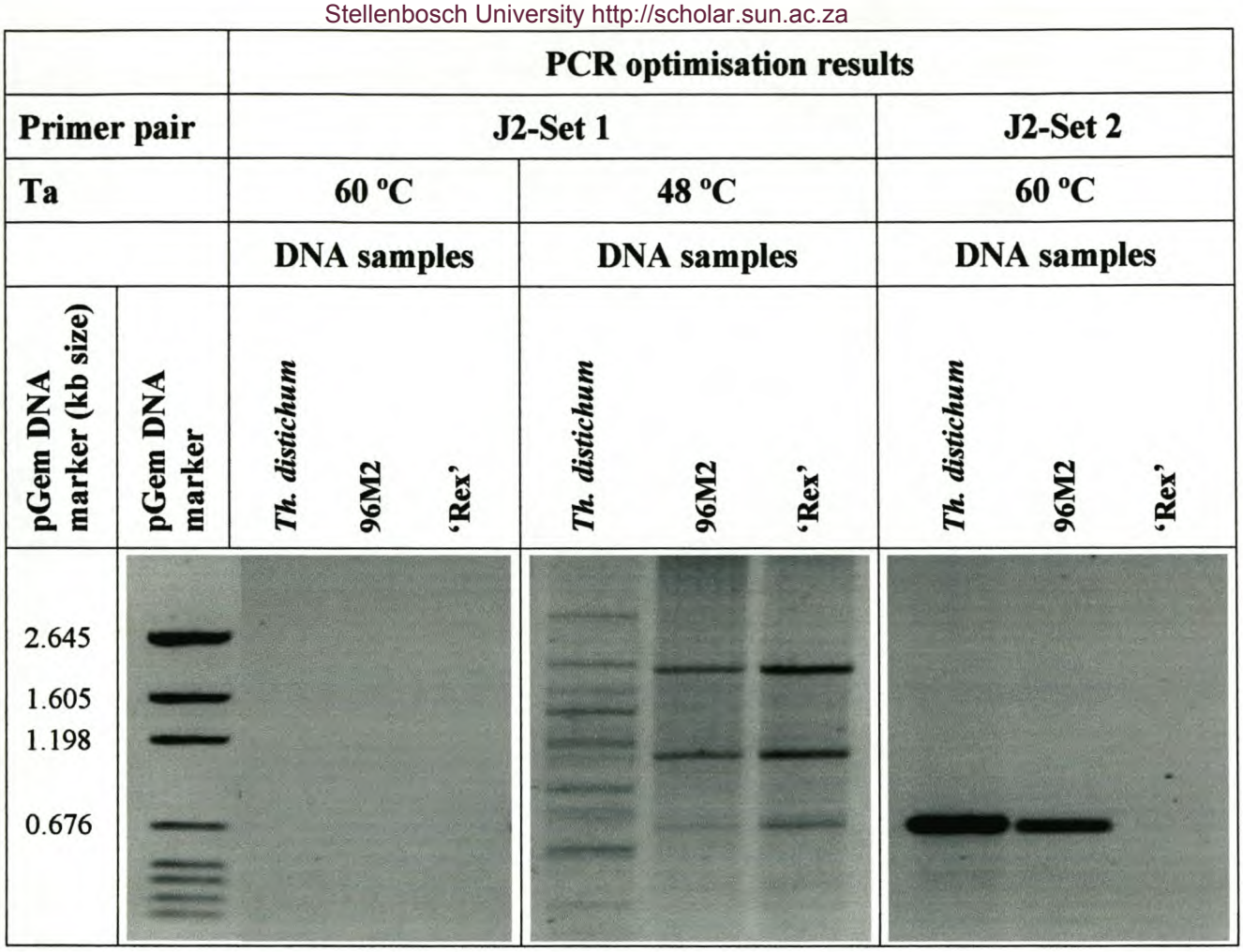


Figure 3.4.8: Primer optimisation results of primer pairs J2-Set 1 and J2-Set 2

Primer pairs J2-Set 1 and J2-Set 2 were designed from the J2 sequence and only primer pair J2-Set 2 produced *Thinopyrum* specific amplification. A band of the expected size (0.705 kb) was produced by primer pair J2-Set 2 at an annealing temperature of 60 °C and was further tested on the addition line (addition 56) and genotype panel 4. Primer pair J2-Set 1 did not produce useful results, even at very low annealing temperatures (Ta=48 °C), and was not tested further.

The PCR results obtained with primer pair J2-Set 2 on the test panel corresponded with the results obtained with the J1-set primers, and it is therefore also a *Thinopyrum* specific marker for chromosome $3J_1^d$ (Figure 3.4.9).

It can thus be concluded that primer sets J1-Set 1, J1-Set 2 and J2-Set 2 are suitable marker systems for the identification of chromosome $3J_1^d$, which is of primary importance in the determination of salt tolerance. It is not clear though, whether the J1-and J2-derived fragments derive from the same or different loci on $3J_1^d$

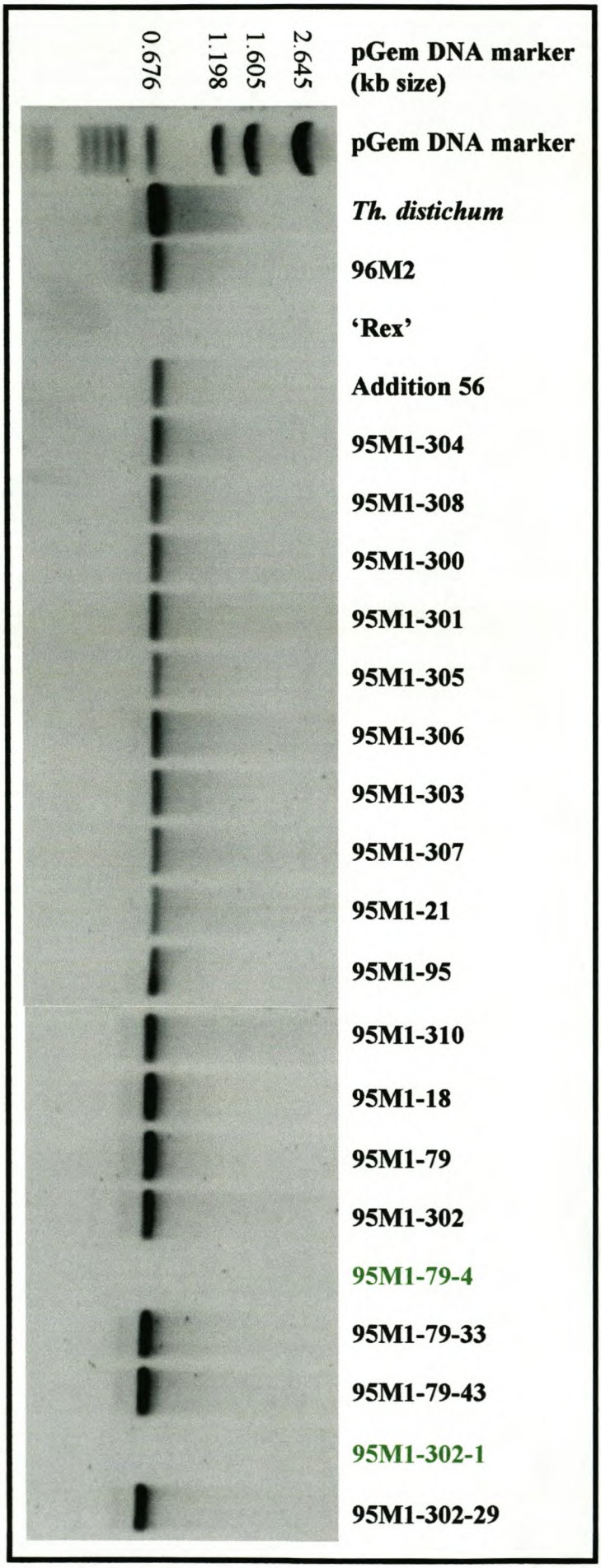


Figure 3.4.9: PCR results of primer pair J2-Set 2 tested on addition 56 and genotype panel 4

3.5 PCR and Southern blot results for RFLP probe PSR 167

The RFLP probe, PSR 167, has an estimated size of 0.600 kb and hybridises to the wheat homoeologous group 6 chromosomes (short arms). The forward and reverse primers (Table 5.1.1) designed from this probe (designated as Pr-PSR 154) had melting temperatures (Tm) of 56.3 and 52.8 °C, respectively, and amplified a fragment of ±1 300 bp when using annealing temperatures of 54, 58 and 62 °C as shown in Figure 3.5.1. Primer pair, Pr-PSR 167, produced *Thinopyrum* specific results (Ta=62 °C) when tested on genotype panel 1 and was further tested on genotype panel 3 (Figure 3.5.2).

Table 5.1.1: Primer sequences derived from the RFLP probe sequence PSR 167

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pr-PSR 167	ACTGCTACTCCATGAGGATGA	TCAGCTTATGCAAGGATGAT

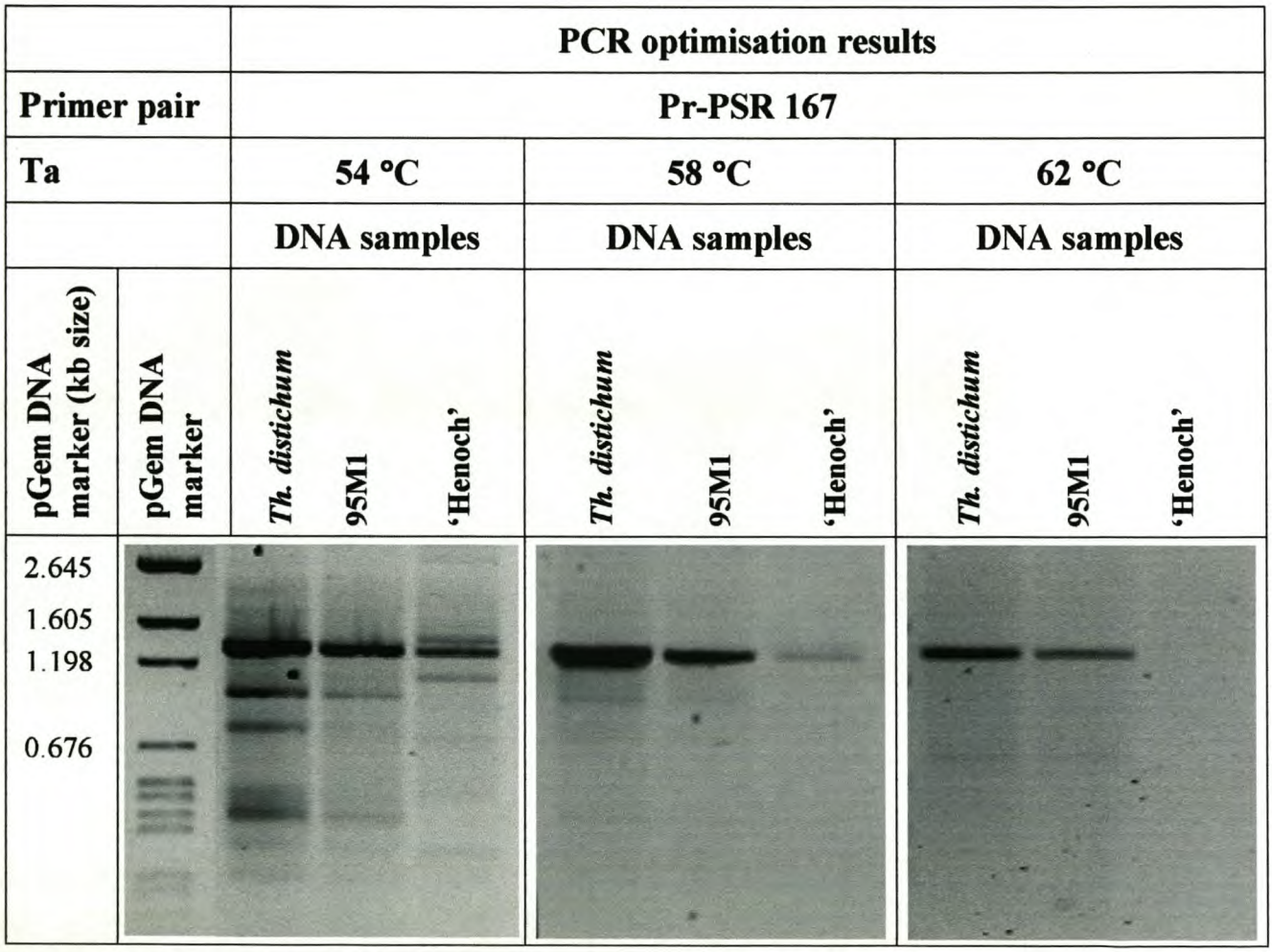


Figure 3.5.1: PCR optimisation results of primer pair Pr-PSR 167

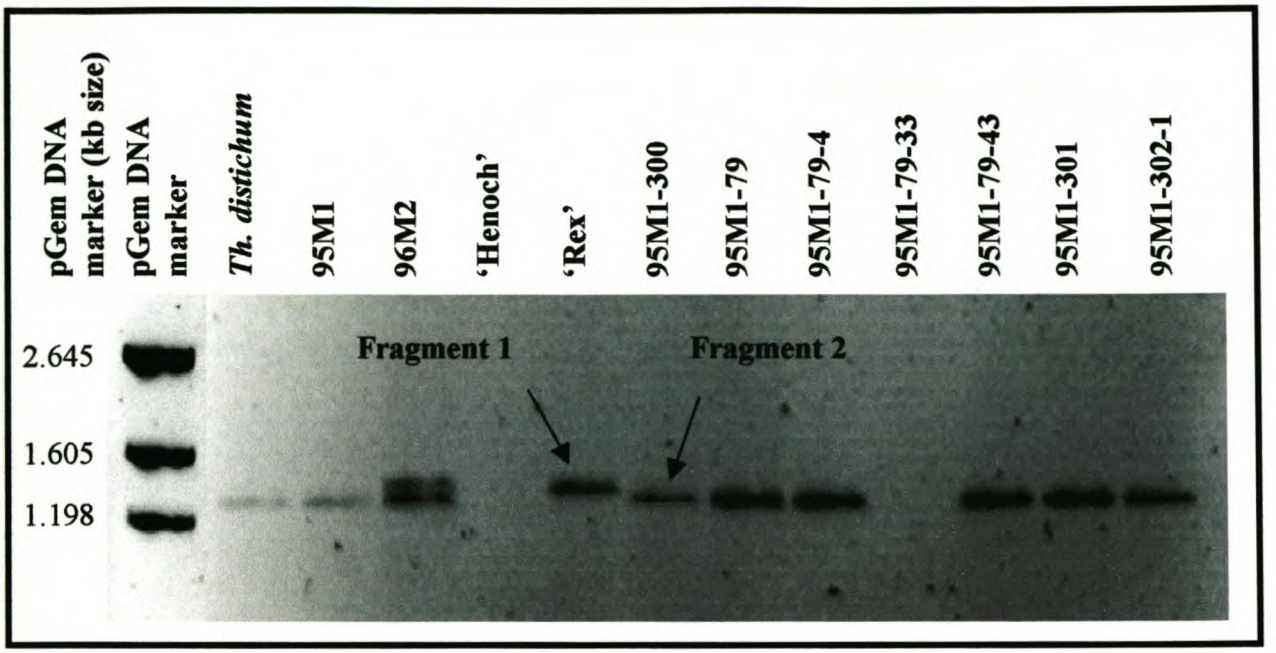


Figure 3.5.2: PCR results of primer pair Pr-PSR 167 tested on genotype panel 3

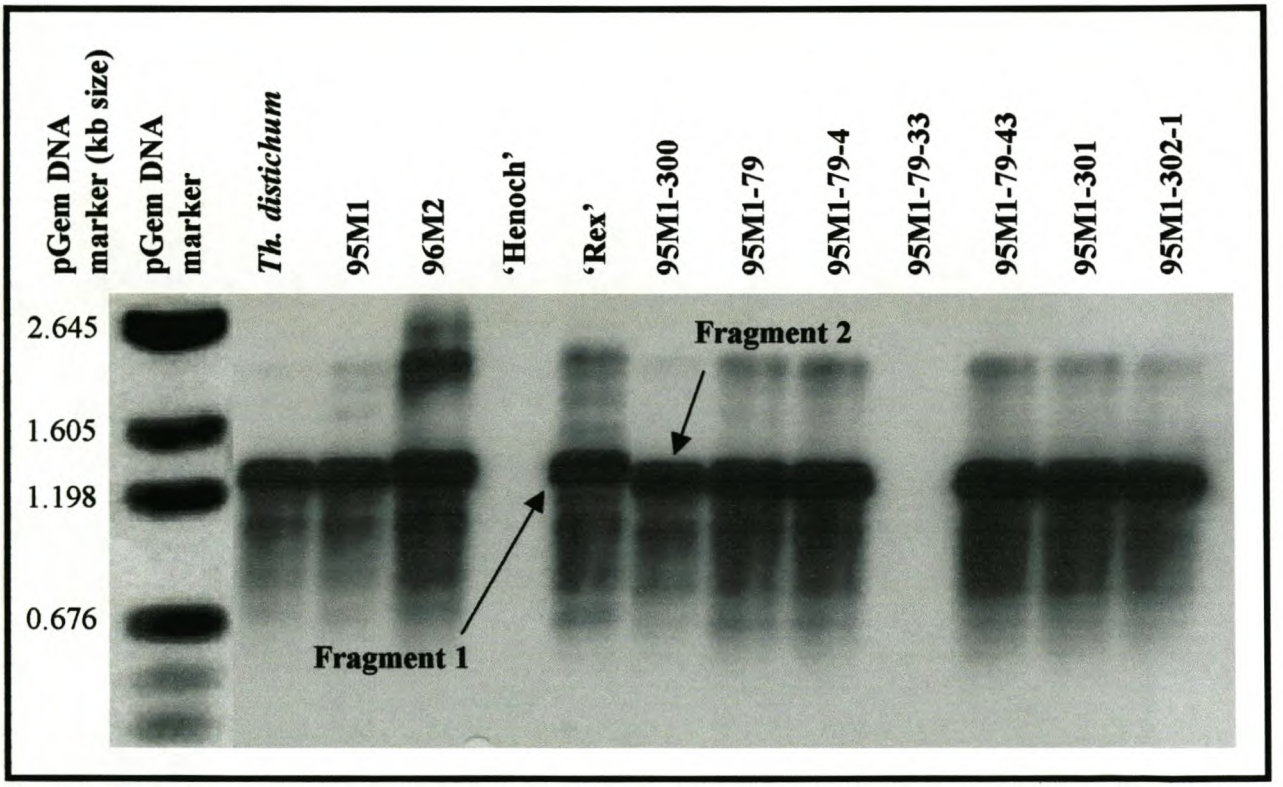


Figure 3.5.3: Southern blot results of probe PSR 167 tested on amplification product(s) of genotype panel 3

The major band (fragment 1) obtained during optimisation was seen in all samples except 'Henoch' and 95M1-79-33 (Figure 3.5.2). Similar results were obtained after hybridisation to the PSR 167 probe (Figure 3.5.3). The amplified fragment is almost 1 kb bigger than was expected (Table 3.1) and may therefore include an intron(s).

Table 3.5.2: Correlation of the Pr-PSR 167 band 1 with the presence/absence (+/-) of *Thinopyrum distichum* chromosomes 6J in the test genotype panel 3

	Th. distichum	**95M1**	**96M2**	**'Henoch'**	**'Rex'**	**95M1-300**	**95M1-79**	**95M1-79-4**	**95M1-79-33**	**95M1-79-43**	**95M1-301**	**95M1-302 -1**
PCR Band 1 (Pr-PSR 167)	+	+	+	-	-	+	+	+	-	+	+	+
[1]Chromosome $6J_1^d$	+	+	+	-	-	+	+	+	-	+	-	+
[1]Chromosome $6J_2^d$	+	+	+	-	-	-	-	-	-	-	+	-

[1]As proposed by Marais and Marais (2003)

Comparison of the Southern hybridisation results with the presence/absence of a chromosome 6J in each of the test genotypes would suggest that the marker set could differentiate 6J chromosomes from group 6 homoeologous in rye and triticale. However, it cannot distinguish between $6J_1^d$ and $6J_2^d$. It can therefore be a very useful marker to track either chromosome should it occur as an addition in a triticale background.

3.6 PCR and Southern blot results for RFLP probe PSR 115

RFLP probe, PSR 115, hybridises to the homoeologous group 5 chromosomes and is 1.400 kb in size. The forward and reverse primers (Table 3.6.1) had melting temperatures of 52.7 and 55.7 °C, respectively, and the size of the amplification product is estimated as 1.318 kb (Table 3.1). However, multiple products were produced at 50, 54 and 58 °C when the primer pairs where tested on genotype panel 1 (Figure 3.6.1) and it was decided to test (50 °C) the primer pair, Pr-PSR 115, on genotype panel 3 (Figure 3.6.2).

Table 3.6.1: Primer sequences derived from the RFLP probe sequence PSR 115

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pr-PSR 115	AGAGGCAACAATCATCAATG	CACCACCACCATCTACAACA

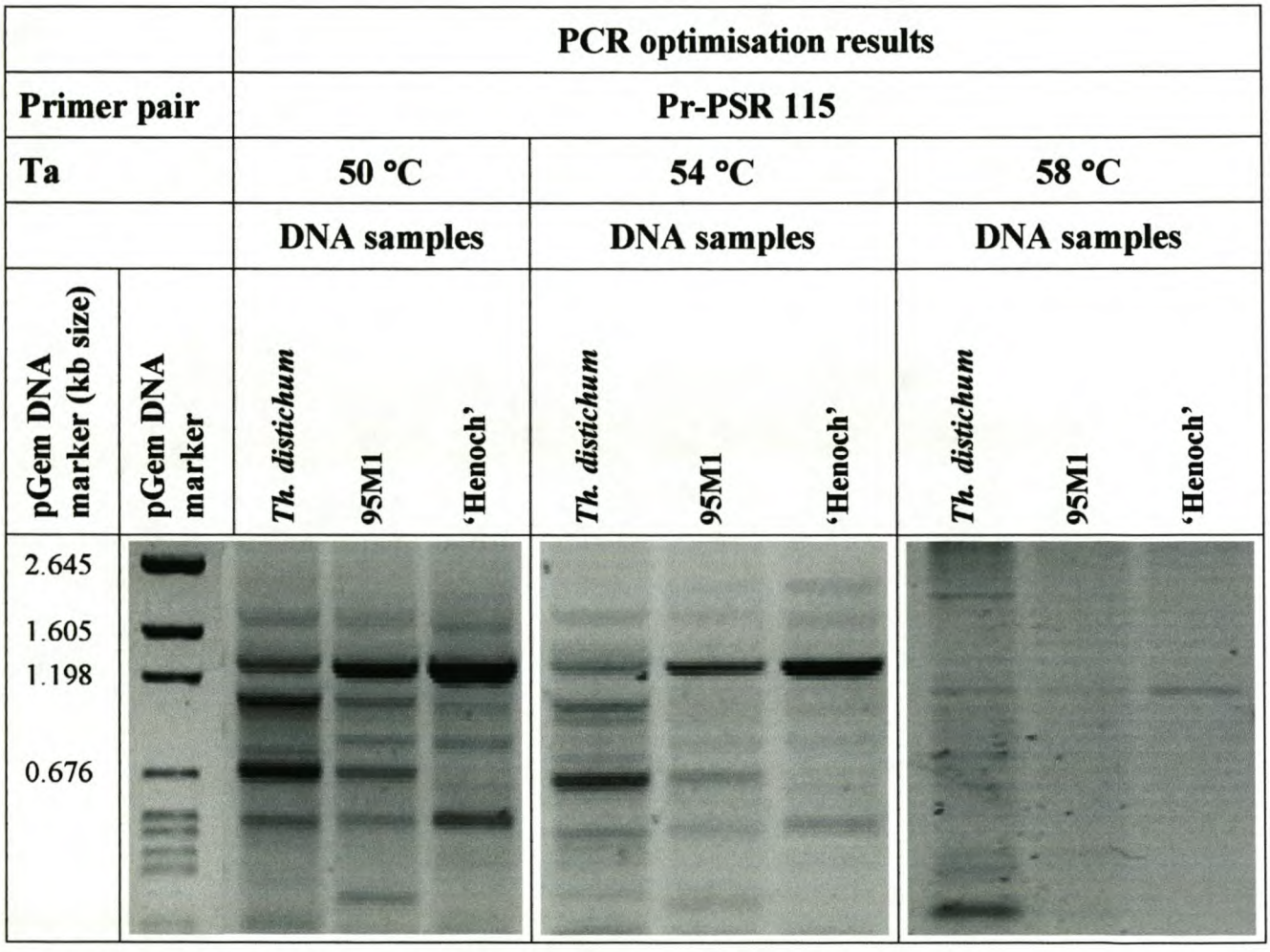


Figure 3.6.1: PCR optimisation results of primer pair Pr-PSR 115

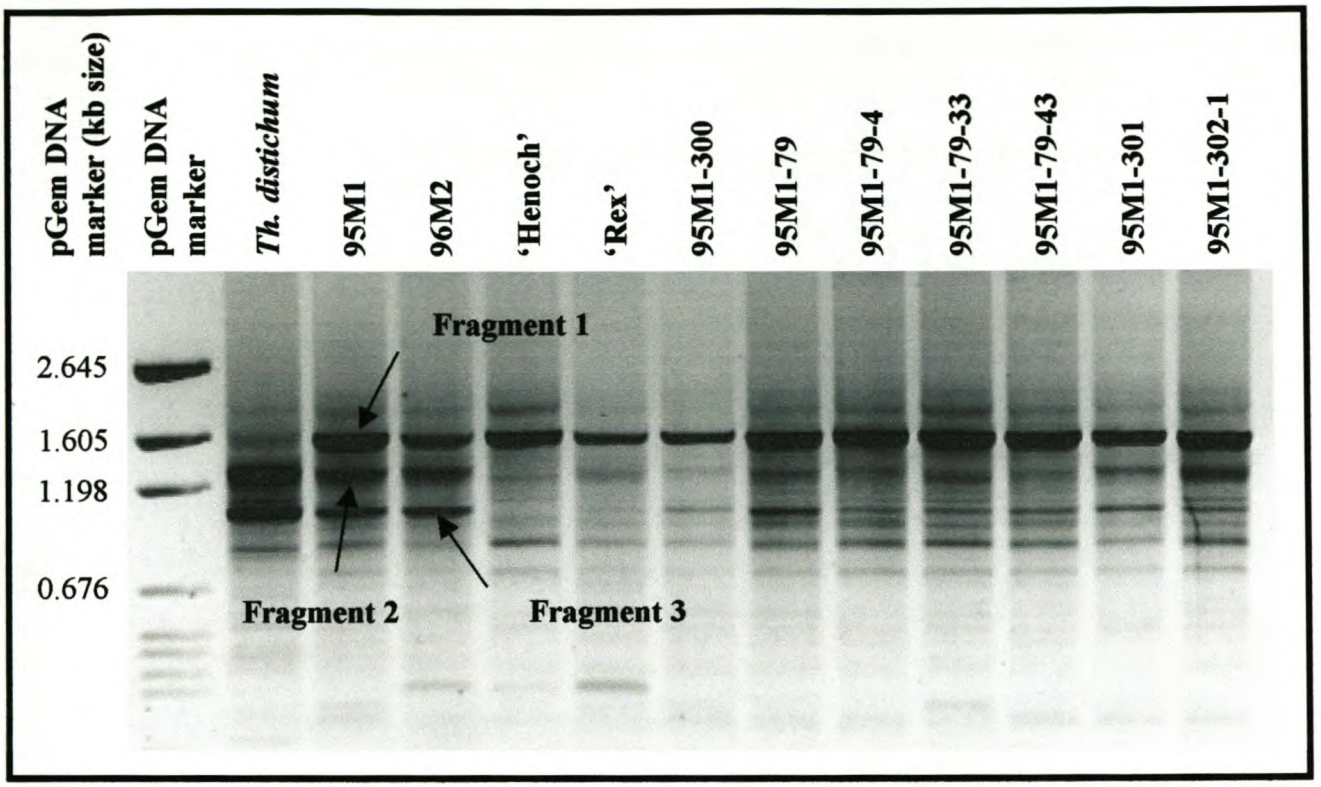


Figure 3.6.2: PCR results of primer pair Pr-PSR 115 tested on genotype panel 3

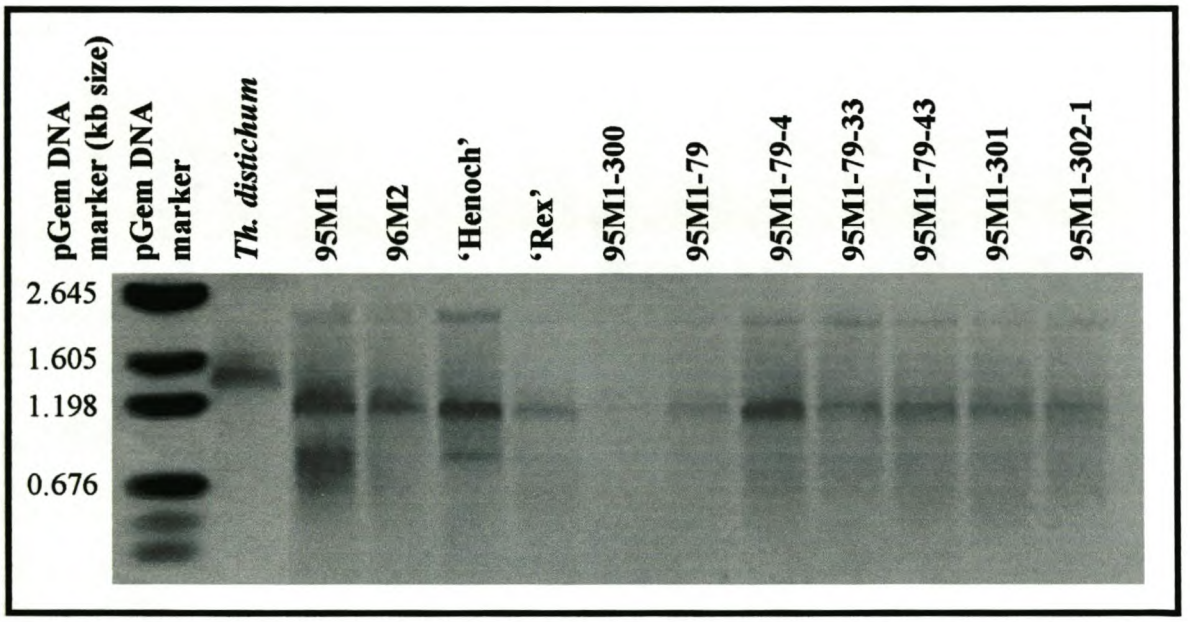


Figure 3.6.3: Southern blot results of probe PSR 115 tested on the amplification product(s) of genotype panel 3

Amplification resulted in three distinct bands within the size range 1 to 1.6 kb. Visualisation under UV-light (Figure 3.6.2) suggested that the larger band (fragment 1), produced by all the samples of genotype panel 3, was amplified from the *Thinopyrum*, rye and triticale genomes. However, probing with probe, PSR 115, suggested that this band is specific to *Thinopyrum* (Figure 3.6.3). Although the smaller bands (fragments 2 and 3) were amplified strongly in *Thinopyrum* and the primary *Thinopyrum* hybrids, 95M1 and 96M2, probing revealed no hybridisation in *Thinopyrum*. The inconsistent hybridisation patterns and the generally weak hybridisation of the probe (a second attempt produced a similar result) with the amplification products would suggest a low level of sequence homology. Thus, the locus may be poorly preserved across homoeologous genomes or, alternatively, the primers Pr-PSR 115 give poor amplification of the target loci.

A further attempt was made to develop a chromosome-specific marker. Bands (named E1 and E2) were excised and purified. The purified bands were ligated with the pGem®-T Easy Vector and transformed to competent cells. Colonies were screened for each of the bands E1 and E2 by executing a PCR reaction (colony PCR) with primer pair, Pr-PSR 115. The plasmids of these colonies were extracted and sequenced with primers (SP6 and T7) flanking the multiple cloning area of the vector. This made it possible to identify and examine the primer-binding site in the sequences. Figure 3.6.4 shows the bands isolated and the PCR results of successfully, transformed colonies.

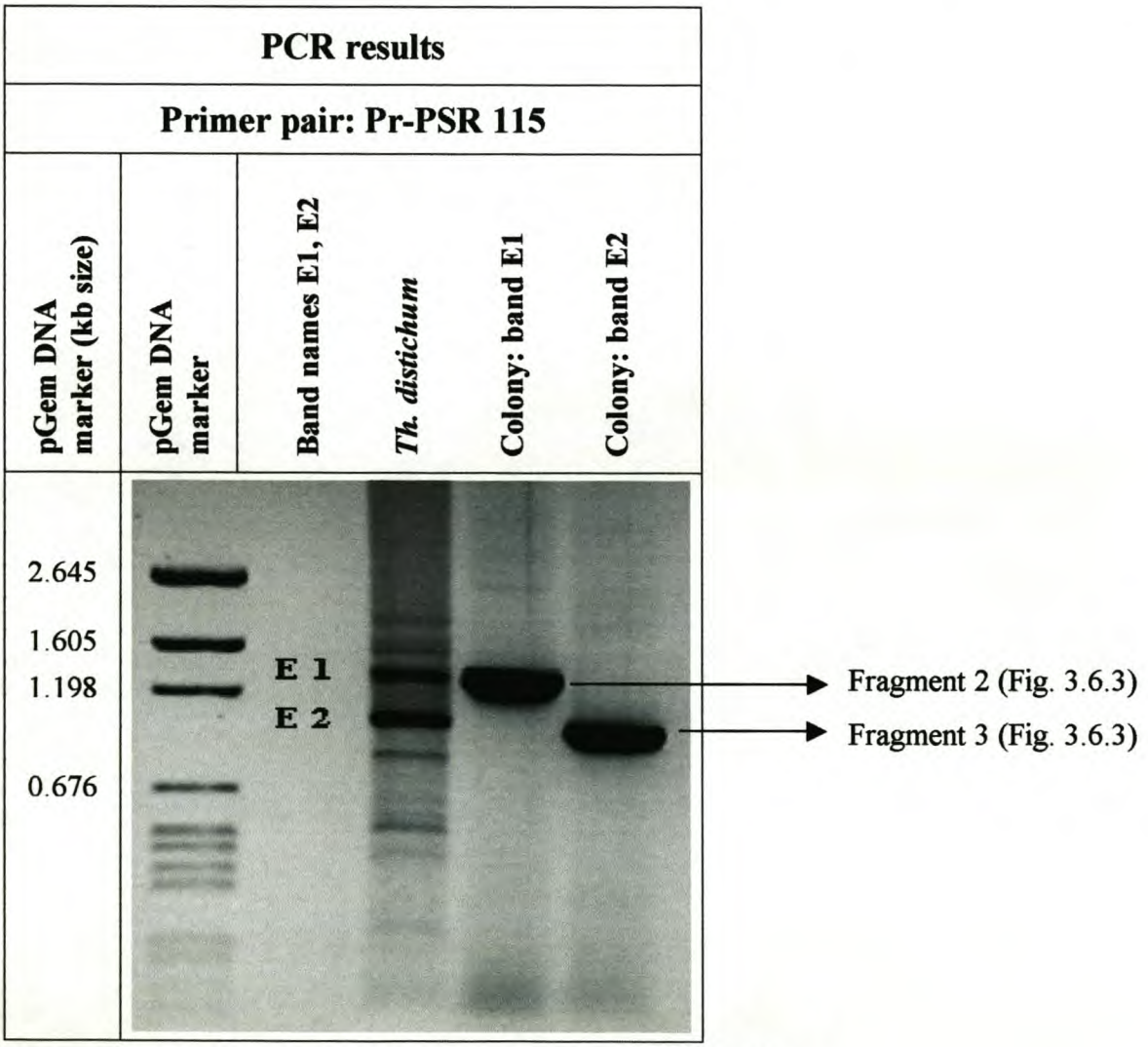


Figure 3.6.4: Isolated bands E1, E2 and colony PCR results

The sequences of E1 and E2 did show the forward and reverse primers sequences of Pr-PSR 115 but no identity match to the sequence of the PSR 115 probe could be obtained (this is not unexpected in view of the faint hybridisation observed in Figure 3.6.3) therefore regions for primer design were arbitrarily chosen. Two sets of primers were designed for each of bands E1 and E2 and are summarised in Table 3.6.2.

Table 3.6.2: Primer sequences derived from fragments E1 and E2 produced by Pr-PSR 115

Primer pair	Target Chromosome	Amplicon size (kb)	Name	Primer sequence (5'-3')
E1-Set 1	5	1.049	E1-F1	AGGAATAGCATAATGGTGAAT
			E1-R1	TCTTCTACGTGCTTTGATATG
E1-Set 2	5	0.748	E1-F2	AGAGCCAAGTACAAGAGGTG
			E1-R2	AGTGAGATCTCCCATATTGC
E2-Set 1	5	0.864	E2-F1	GTGGTACTAGCCTCATGCTC
			E2-R1	CCCTCATCAAGTCCTACAAG
E2-Set 2	5	0.609	E2-F2	GCACCAATGTAGTTGTCTTC
			E2-R2	TTCAGTGATTTCACCATCTC

The primers were optimised using samples *Thinopyrum*, 96M2 and 'Rex'. All the primers were tested at an initial annealing temperature of 60 °C and depending on the PCR results annealing temperatures of either 4 °C increments or decrements were used to determine optimal PCR conditions (Figure 3.6.5).

At an annealing temperature of 56 °C primer pair E1-Set 1 produced a band in *Thinopyrum* and 96M2 (Figure 3.6.5). This primer pair was the only primer pair, designed from sequence E1 that produced the expected polymorphism and was also tested on an addition line (addition 28), carrying *Thinopyrum* chromosomes $5J_1^d$, as well as genotype panel 4. Chromosome 5 is expected, in correspondence to Marais and Marais (2003), to be absent in the genotypes printed in green (Figure 3.6.6).

The target fragment(s) was produced in each of the test panel of genotypes including 'Rex' indicating that it is not specific for the *Thinopyrum* group 5J chromosomes (Figure 3.6.6). Primer pair E1-Set 2 was not tested on these samples as it produced a band in *Thinopyrum*, 96M2 and Rex. More stringent annealing conditions (Ta=66 °C) resulted in similar results.

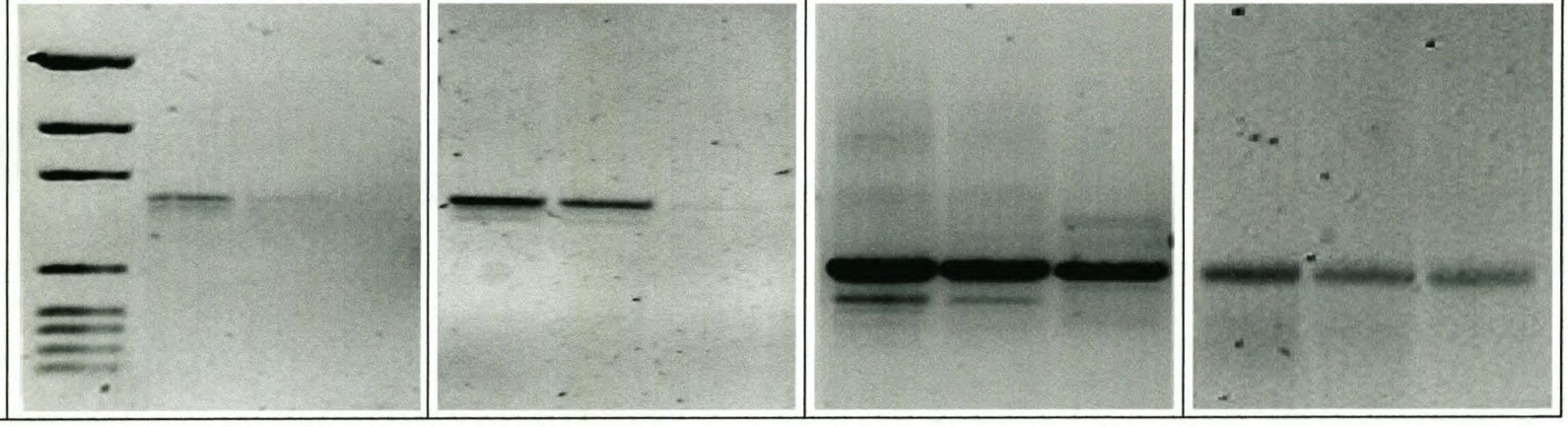
		PCR optimisation results			
Primer pair		**E1-Set 1**		**E1-Set 2**	
Ta		**60 °C**	**56 °C**	**60 °C**	**66 °C**
		DNA samples	**DNA samples**	**DNA samples**	**DNA samples**
pGem DNA marker (kb size)	**pGem DNA marker**	***Th. distichum*** **96M2** **'Rex'**	***Th. distichum*** **96M2** **'Rex'**	***Th. distichum*** **96M2** **'Rex'**	***Th. distichum*** **96M2** **'Rex'**
2.645 1.605 1.198 0.676 0.517					

Figure 3.6.5: PCR optimisation results of primer pairs E1-Set 1 and E1-Set 2

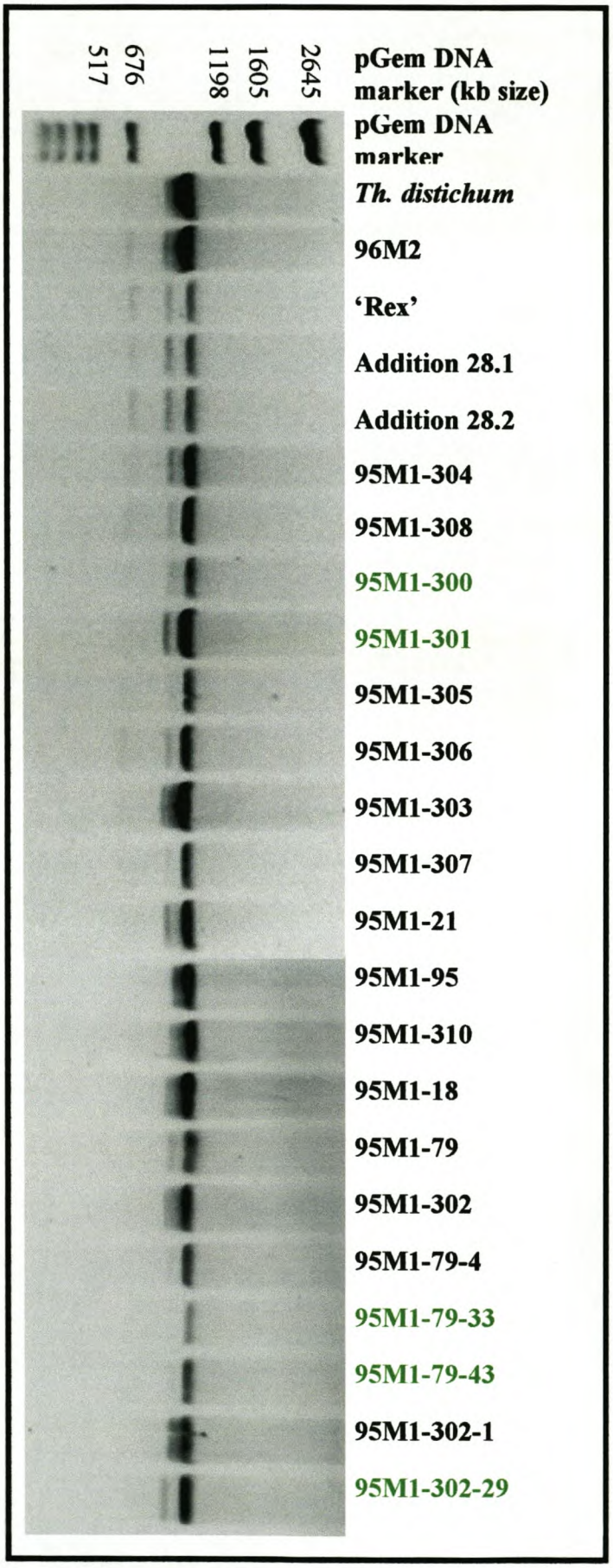


Figure 3.6.6: PCR results of primer pair E1-Set 1 tested on addition 28 and genotype panel 4

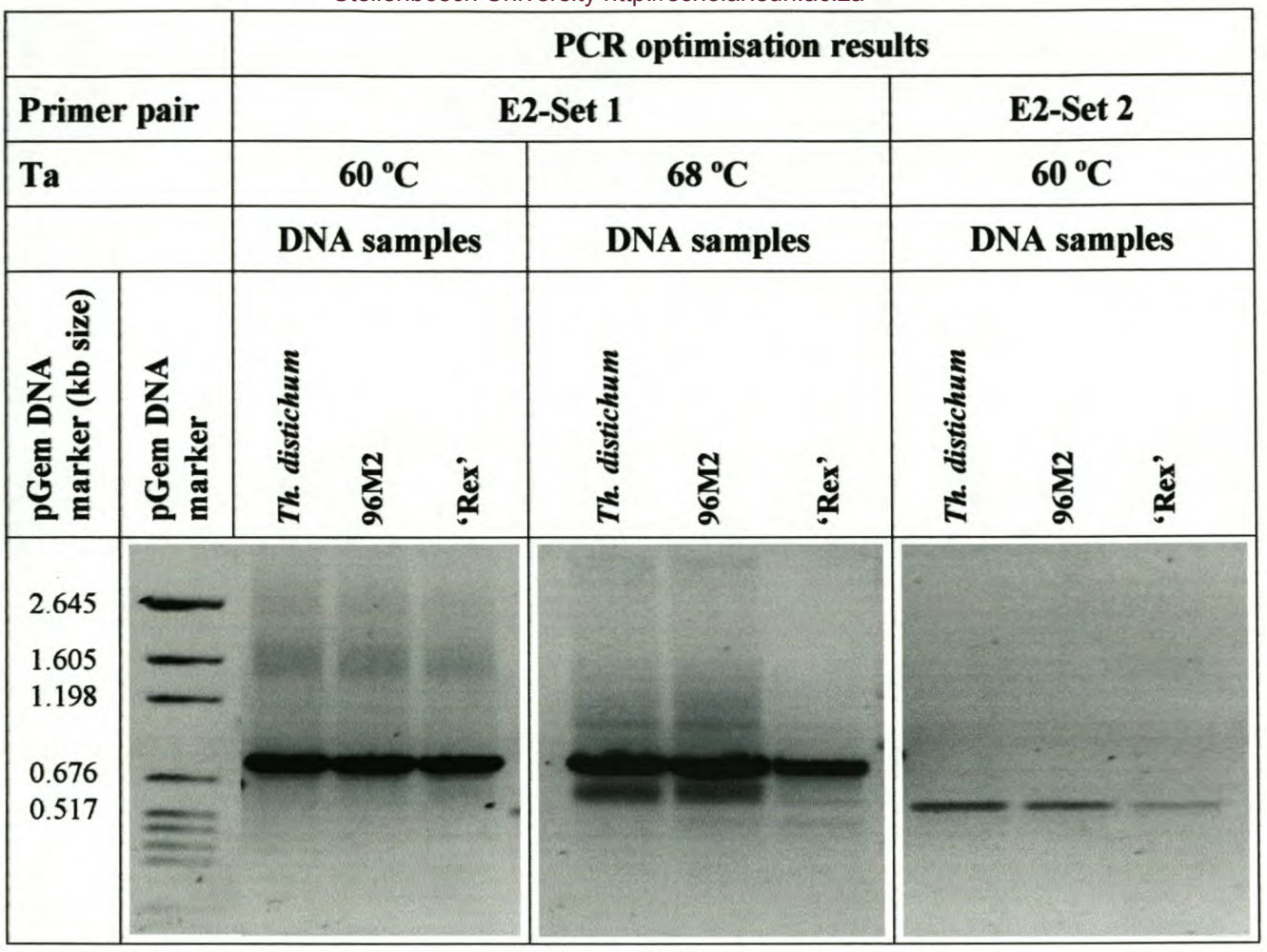


Figure 3.6.7: PCR optimisation results of primer pair E2-Set 1 and E2-Set 2

Primer pairs E2-Set 1 and E2-Set 2 were designed from sequence E2 and none produced a *Thinopyrum*-specific polymorphism. Primer E2-Set 1 was tested at annealing temperatures of 60 and 68 °C while primer pair E2-Set 2 was tested at Ta=60 °C.

It was decided to attempt further refinement of the primers and the PCR products produced by E2-Set 1 in *Thinopyrum* and 'Rex' were sequenced. Aligning of the two sequences revealed only 75 % homology. The poor correspondence was probably due to the presence of both J_1^d and J_2^d products in *Thinopyrum*. Similarly, the Rex band may have contained fragments of as many as three genomes, therefore single nucleotide differences were used to design primers (Table 3.6.3).

Table 3.6.3: Primer sequences derived from E2-Set 1 amplification product(s)

Primer Pair	Target chromosome	Amplicon size (kb)	Name	Primer Sequence (5'-3')
2-E2S1-Set 1	5	0.453	2-E2S1-F1	GACTTGTAGTTCTCGAGGAAGATA
			2-E2S1-R1	GAGCTACTACGACCTCGACAC
2-E2S1-Set 2	5	0.122	2-E2S1-F2	TGTGTGAAGCCACCATGC
			2-E2S1-R2	CTCGAGTGGGAGCGCCT

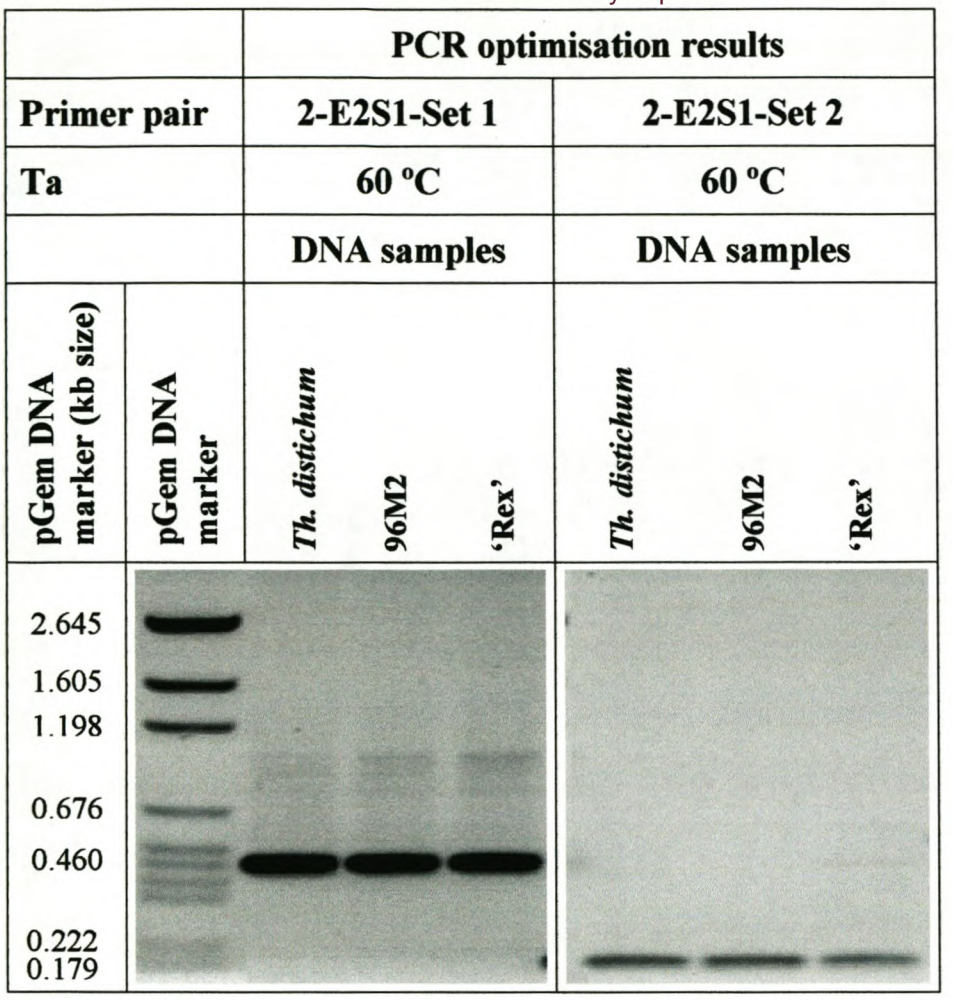


Figure 3.6.8: PCR optimisation results of primer pairs 2-E2S1-Set 1 and 2-E2S1-Set 2

Primer pairs 2-E2S1-Set 1 and 2-E2S1-Set 2 did not produce *Thinopyrum* specific bands either (Figure 3.6.8).

3.7 RFLP probes: PSR 934, PSR 305, PSR 163, PSR 628 and PSR 160

PCR primers designed to amplify the DNA regions PSR 934, PSR 305, PSR 163, PSR 628, and PSR 160 produced ambiguous results and it was therefore decided to also determine the specificity of the primers. Following PCR amplification on the parental genotypes, *Th. distichum* and Rex, the PCR products and corresponding probe were size fractionated (Figures 3.7.1.1-3.7.5.1). Southern blots were derived and hybridised with DIG-labeled probe.

3.7.1 PCR and Southern blot results for RFLP probe PSR 934

PSR 934 hybridises to the homoeologous group 2 chromosomes (long arms) and has an estimated size of 1.800 kb. The forward and reverse primers (Table 3.7.1.1) designed had melting temperatures (Tm) of 57 and 54.5 °C, respectively, and were tested at annealing temperatures ranging from 60-46 °C. The estimated size of the amplicon is 1.700 kb. However, the primer pair, Pr-PSR 934, amplified a range of bands (Figure 3.7.1.1) and from the probing results it is clear that the target locus, PSR 934, was not amplified.

Table 3.7.1.1: Primer sequences derived from the RFLP probe sequence PSR 934

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pr-PSR 934	TCTCGCTGTTGATGGTCAGA	TTGGTTTGTTTGGTGGTCTC

3.7.2 PCR and Southern blot results for RFLP probe PSR 305

Probe, PSR 305, hybridises to the homoeologous group 3 chromosomes (short arm) and has an estimated size of 1.300 kb. The forward and reverse primers (Table 3.7.2.1) designed to amplify the region, PSR 305, had melting temperatures of 55.9 and 56 °C, respectively, and were tested at annealing temperatures ranging from 60-46 °C. The estimated size of the amplification product is 1.200 kb but Figure 3.7.2.1 shows a band (*Thinopyrum* specific) of approximately 0.4 kb.

Table 3.7.2.1: Primer sequences derived from the RFLP probe sequence PSR 305

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pr-PSR 305	CGCTGGATTCTAGCAGGTATC	TCGTCTGGATGAAGGAGAGAT

Since the labeled probe did not hybridise to any of the fragments produced by Pr-PSR 305 (Figure 3.7.2.1), it is unlikely that the primer set targeted a PSR 305 homoeologous region. However, as Pr-PSR 305 produces a polymorphic band in *Thinopyrum* an attempt was made to find the chromosome marked by the polymorphic fragment. For this purpose samples of the test panel 5 (Table 2.1) were analyzed. The PCR results (Figure 3.7.2.2) suggested that the marker band occurs

in each genotype. Since the group 1 homoeologues of *Th. distichum* are the only that occur in each of the genotypes (Marais and Marais, 2003), this may be a marker for $1J_1^d$ and $1J_2^d$. Alternatively, the primers may detect *Thinopyrum*-specific repeat sequences.

Primer pair Pr-PSR 305 was also tested on a panel of triticale addition lines 1, 4, 11, 13, 17, 18, 20, 21, 22, 24, 27, 51, 70.1a, 70.2b, 70.2c (each has a disomic addition of a single, unknown *Thinopyrum* chromosome to triticale, 2n=44) (Figure 3.7.2.3). Evidently, the polymorphic band was not produced in any of the addition lines tested. It is therefore likely to be a chromosome 1J marker rather than a non-specific repeat sequence.

3.7.3 PCR and Southern blot results for RFLP probe PSR 628

Probe PSR 628 hybridises to the homoeologous group 5 chromosomes (short arm) and has an estimated size of 1.6 kb. The forward and reverse primers (Table 3.7.3.1) designed to amplify the region PSR 628 had melting temperatures of 54.8 and 55.3 °C, respectively, and was tested at annealing temperatures ranging from 46-60 °C. The estimated size of the amplification product is 1.464 kb. Figure 3.7.3.1 depicts the PCR results at Ta=54 °C as well as the Southern hybridization results. Clearly, the fragments observed were not amplified at *Xpsr 628*, the homoeo-loci.

Table 3.7.3.1: Primer sequences derived from the RFLP probe sequence PSR 628

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pr-PSR 628	**GTTGGAGATCGAGACCTGAA**	**GATGCTGGTAGTTATGGTGGA**

Primer pair, Pr-PSR 628, was also used in PCR amplification reactions with genotype panel 3 and following Southern hybridization, the DIG-labeled probe, PSR 628, did not detect homoeology with the fragments generated (results not shown). An attempt was made to assign the *Thinopyrum* bands to a chromosome by also doing PCR amplification with genotype panel 5. The results are shown in Figure 3.7.3.2, and it appears that the two bands were amplified in all samples. Thus, the polymorphisms may either be produced by $1J_1^d$ and $1J_2^d$ homoeoloci or may be the result of *Thinopyrum* – specific repeat sequences.

Primer pair, Pr-PSR 628, was subsequently tested on the panel of random triticale addition lines, 1, 4, 11, 13, 17, 18, 20, 21, 22, 24, 27, 51, 70.1a, 70.2b, 70.2c (Figure 3.7.3.3). In this case the larger band was present in only some of the additions (11, 17, 51, 70.1a, 70.1b and 70.1c) and could be a chromosome 1J marker. The smaller band occurred in all the additions and it is therefore likely to detect a repeat sequence.

When compared to the results obtained with primer pair Pr-PSR 305 (polymorphism did not occur in any of the addition lines) it is evident that at most one of the Pr-PSR 628 large fragment (amplified in some addition lines) or the Pr-PSR 305 fragment can be a chromosome 1 marker. However, it is also possible that neither is a chromosome specific marker.

3.7.4 PCR and Southern blot results for RFLP probe PSR 163

Probe, PSR 163, hybridises to the homoeologous group 4 chromosomes and has an estimated size of 0.500 kb. The forward and reverse primers (Table 3.7.4.1) designed to amplify the region PSR 163 had melting temperatures of 56.5 °C and 55.5 °C and was tested at annealing temperatures ranging from 46-60 °C. The estimated size of the amplification product is 0.451 kb. Figure 3.7.4.1 depicts the PCR results at Ta=54 °C as well as the Southern hybridisation results. The multiple bands amplified by the Pr-PSR 163 primers were unrelated to the PSR 163 sequence, and were not tested further.

Table 3.7.4.1: Primer sequences derived from the RFLP probe sequence PSR 163

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pr-PSR 163	CACAGAAGTGCCACCTTGAA	GCTGTGAATTGCTGCTGTAC

3.7.5 PCR and Southern blot results for RFLP probe PSR 160

Probe, PSR 160, hybridises to the homoeologous group 7 chromosomes and has an estimated size of 0.500 kb. The forward and reverse primers (Table 3.7.5.1)designed to amplify the region PSR 160 had melting temperatures of 54.8 and 55.1 °C, respectively, and were tested at annealing temperatures ranging from 46 to 60 °C. The estimated size of the amplification product is 0.471 kb. Figure 3.7.5.1 depicts PCR results produced at Ta = 54 °C as well as the Southern hybridisation results. In this case a target sequence was amplified only in triticale and the amplification products were therefore not studied further.

Table 3.7.5.1: Primer sequences derived from the RFLP probe sequence PSR 160

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Pr-PSR 160	ATCATCAAGAGCCTCATCTCA	AGACCATCACCTTCAAGAACA

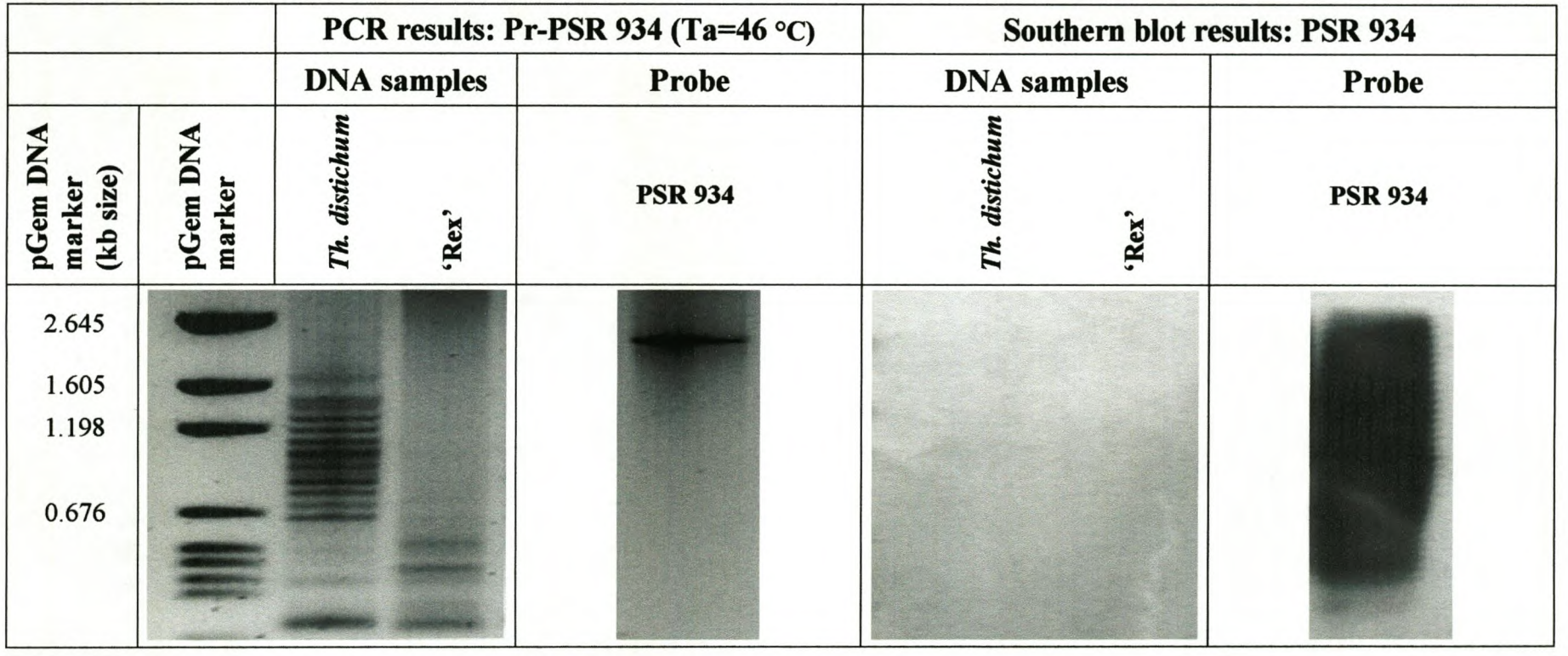


Figure 3.7.1.1: PCR and Southern blot results for RFLP probe PSR 934

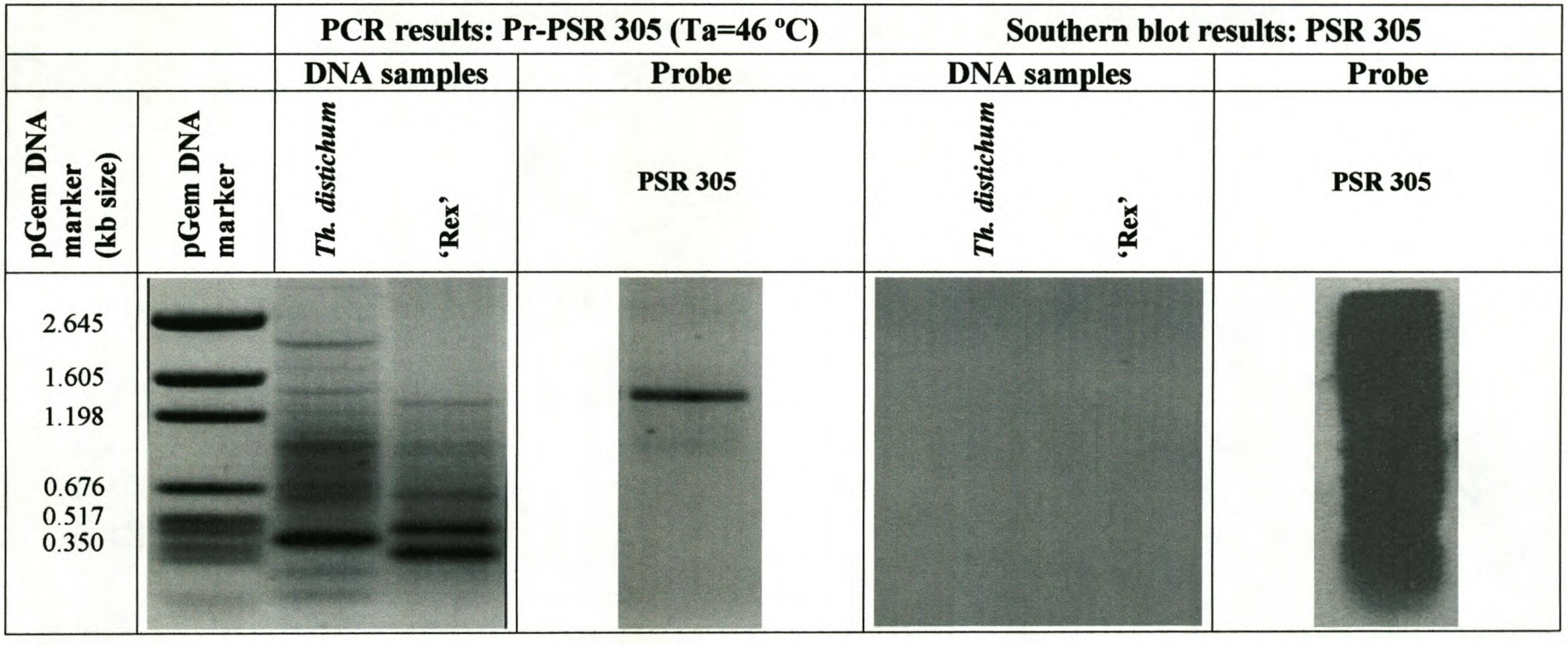


Figure 3.7.2.1: PCR and Southern blot results for RFLP probe PSR 305

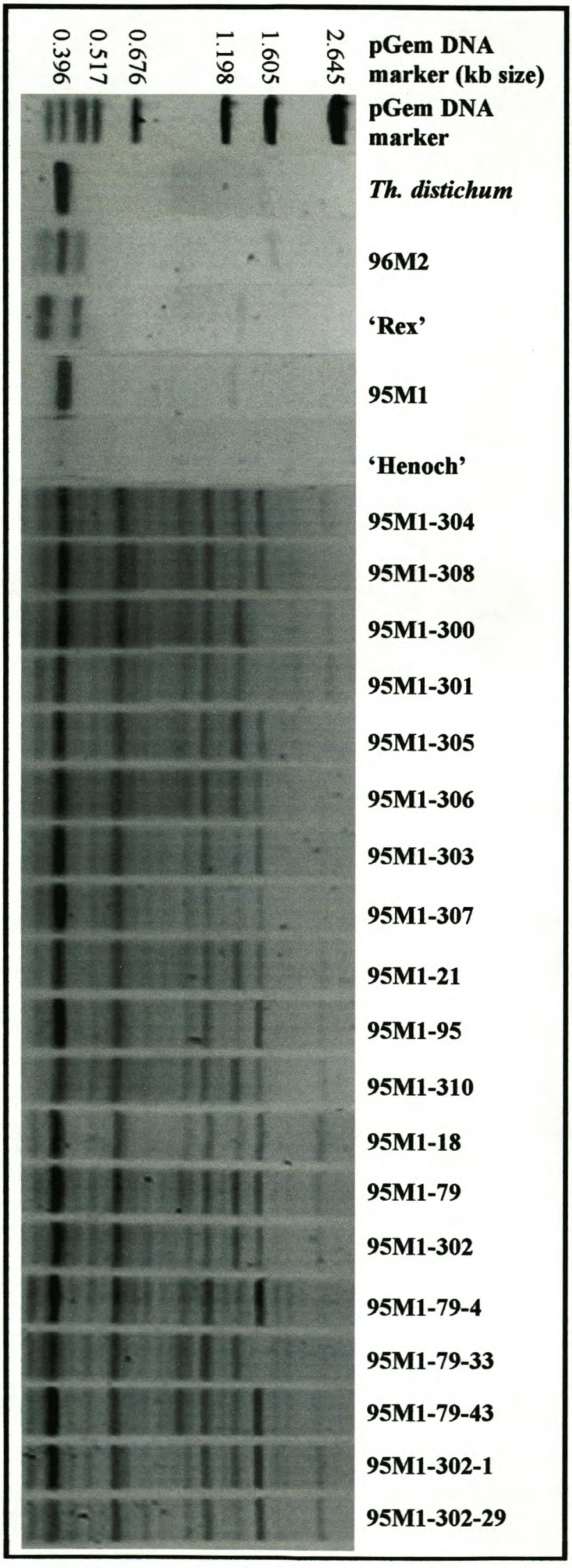


Figure 3.7.2.2: PCR results of primer pair Pr-PSR 305 tested on genotype panel 5

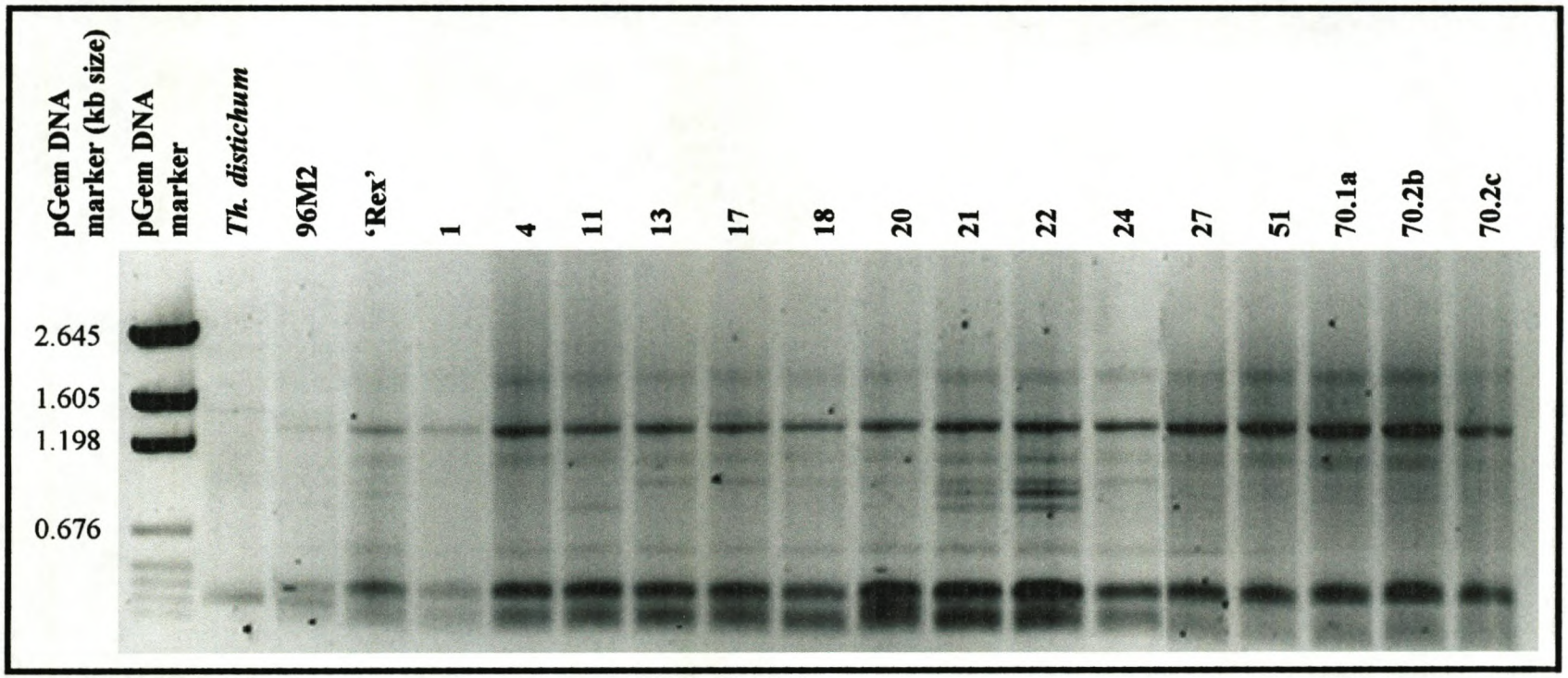


Figure 3.7.2.3: PCR results of primer pair Pr-PSR 305 tested on the panel of disomic addition lines

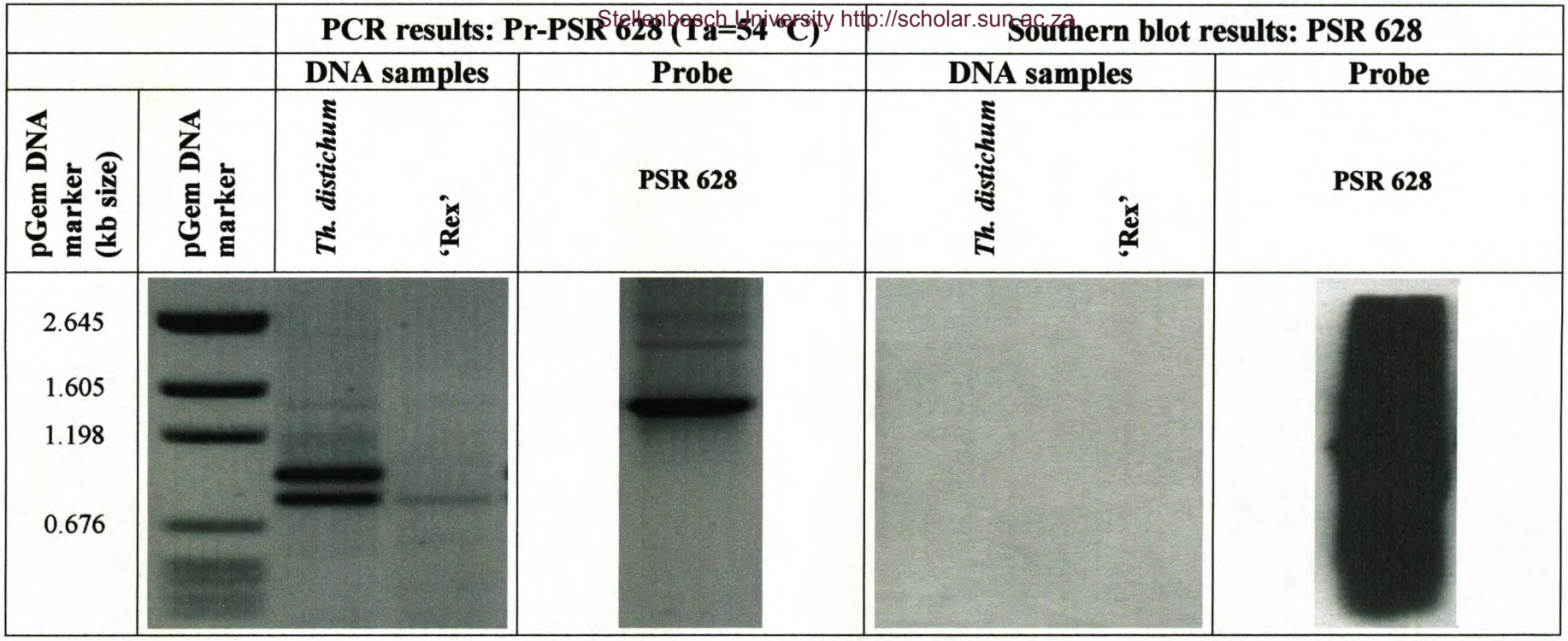


Figure 3.7.3.1: PCR and southern blot results of RFLP probe PSR 628

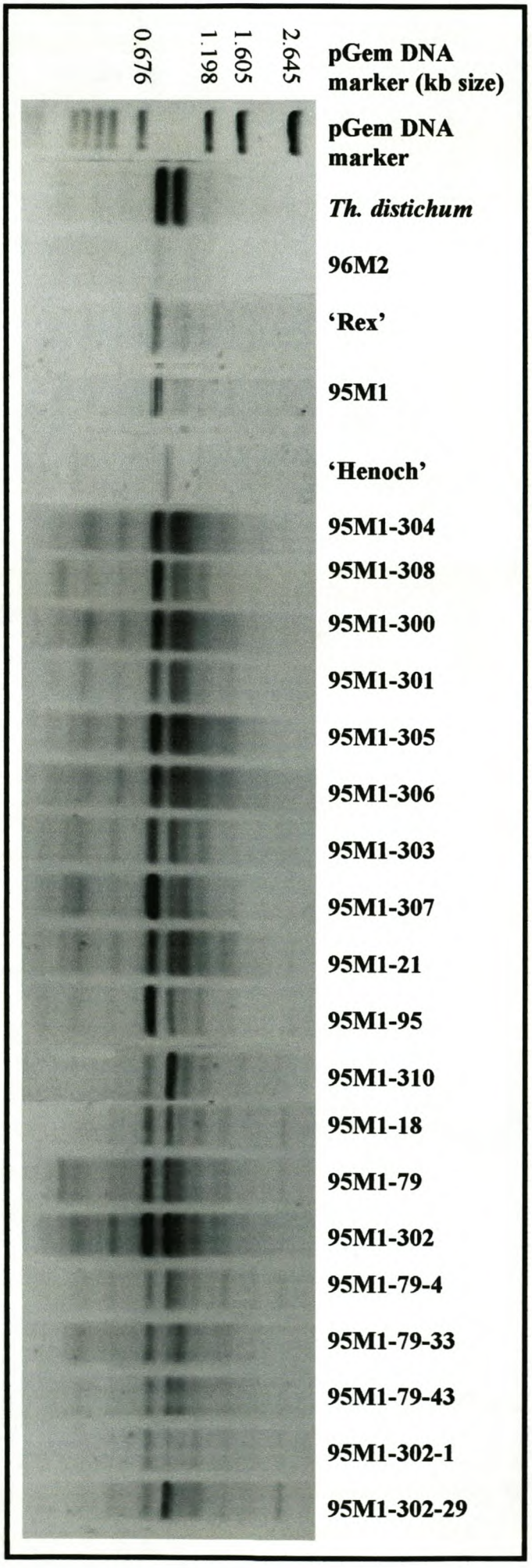


Figure 3.7.3.2: PCR results of primer pair Pr-PSR 628 tested on genotype panel 5

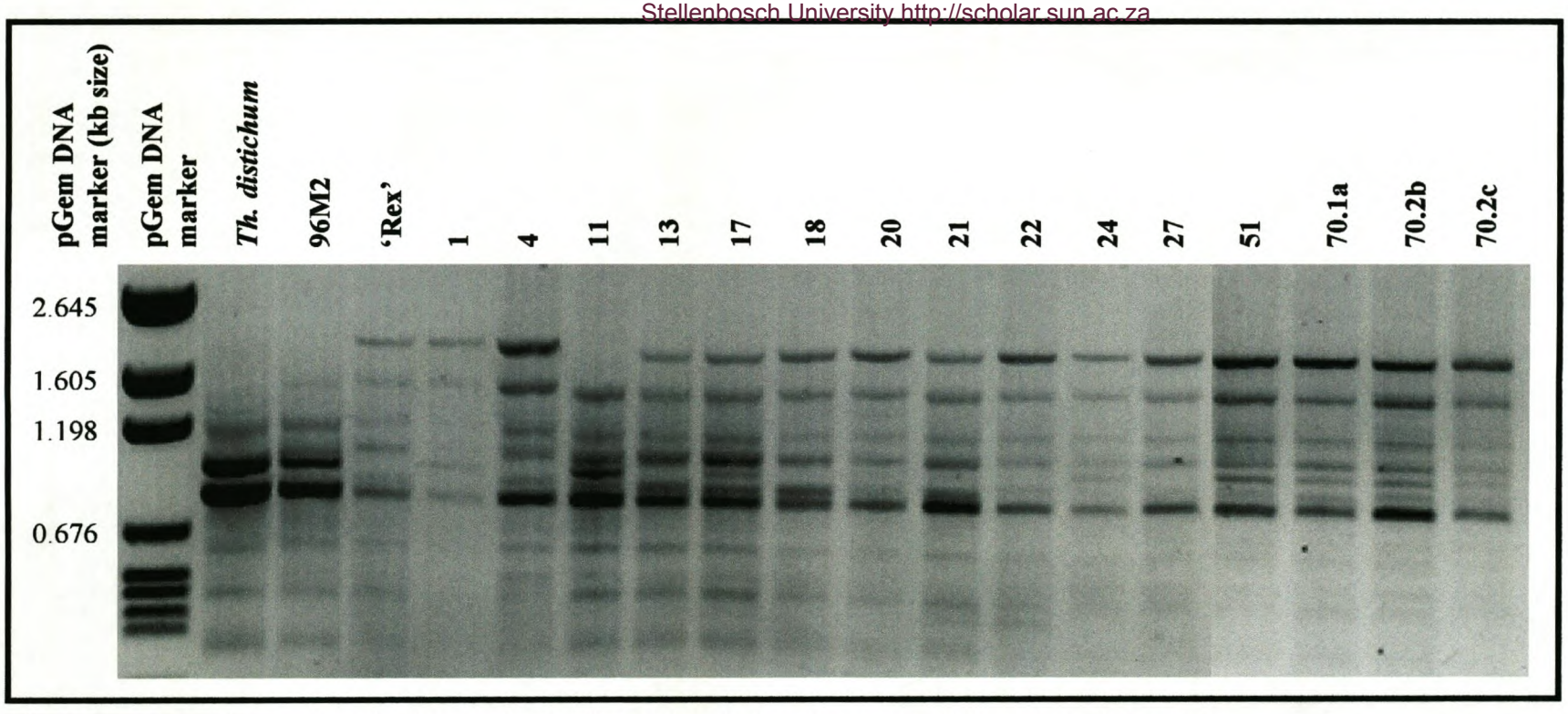


Figure 3.7.3.3: PCR results of primer pair Pr-PSR 628 tested on the panel of disomic addition lines

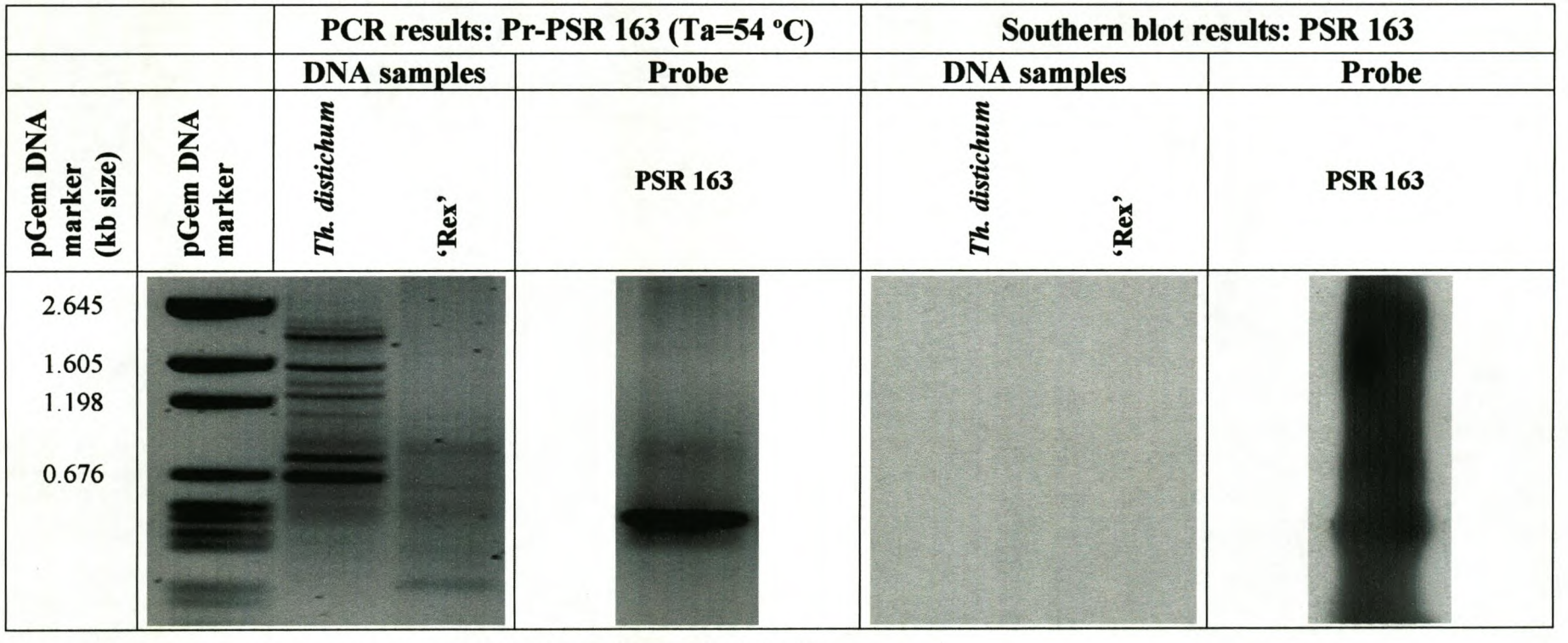


Figure 3.7.4.1: PCR and Southern blot results for RFLP probe PSR 163

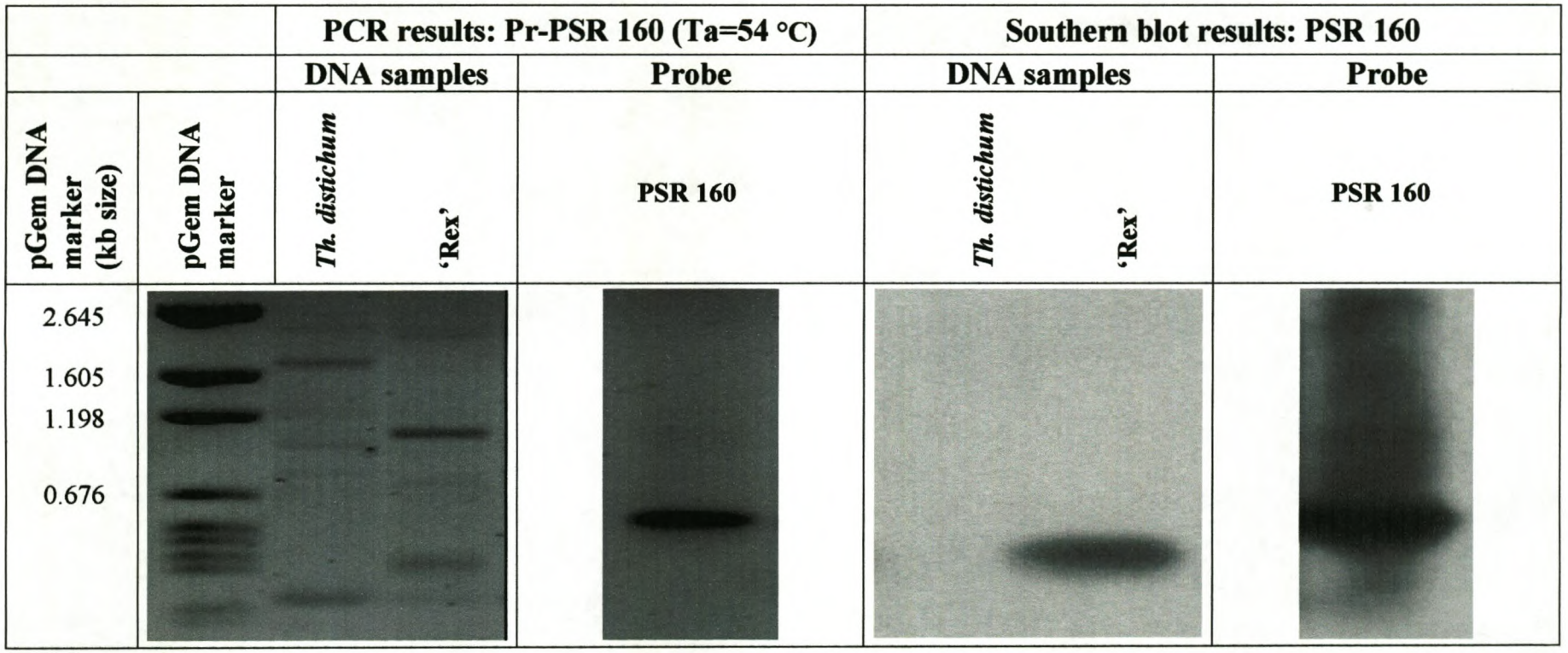


Figure 3.7.5.1: PCR and Southern blot results for RFLP probe PSR 160

CHAPTER 4

Conclusions

The aim of the study was to develop markers specific for some of the *Thinopyrum distichum* chromosomes and especially chromosomes $2J_1^d$, $3J_1^d$, $4J_1^d$, $5J_1^d$ and $7J_1^d$ that were implied in salt tolerance of this species. Reliable SCAR-markers (Table 3.1) have been developed for chromosomes 3 J_1^d and 7 J_2^d, which would facilitate the introgression of these chromosomes into triticale. A marker has also been found for the group 6 chromosomes of *Thinopyrum*. While this homoeologous group does not appear to harbour genes for salt tolerance, the markers may prove useful in further attempts to transfer other *Thinopyrum* characteristics. A putative group 1 marker has also been obtained yet needs to be verified. This marker was obtained by chance and is not related to the gene sequence that was targeted originally

Table 3.1: Summary of developed SCAR markers

RFLP	SCAR primers	*Thinopyrum* chromosome
PSR 931	J1-Set 1	$3J_1^d$ (Long arm)
	J1-Set 2	
	J2-Set 2	
PSR 129	2-FS1-Set 3	$7J_2^d$ (Long arm)
PSR 167	Pr-PSR 167	6J (Short arm)
PSR 305	Pr-PSR 305	1J?

Attempts to find markers for chromosomes 2 J_1^d, $4J_1^d$, 5 J_1^d and $7J_1^d$ have been unsuccessful. Based on present experience the following approach is suggested for future attempts to develop specific markers for these chromosomes:

(a) Identify single copy EST or cDNA loci in a wheat chromosome homoeologous to the *Thinopyrum* target chromosomes from map data

(b) Find the corresponding gene sequence and use to design more than one primer for each.

(c) Amplify sequences in *Thinopyrum*, 96M2 and 'Rex' and confirm with Southern blotting.

(d) If *Thinopyrum* specific sequences are obtained, use directly

(e) If the sequences are not *Thinopyrum*-specific, use the primers to amplify homoeologous sequences in: (i) rye (RR)

(ii) *Triticum turgidum* ssp. durum (AABB)

(iii) *Thinopyrum*-rye secondary hybrid that has only the J_1^d homoeologue of the target chromosome

(iv) *Thinopyrum*-rye secondary hybrid that has only the J_2^d homoeologue of the target chromosome

(v) Sequence and compare the different sequences to derive *Thinopyrum*-specific primers.

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