Implementation of marker assisted breeding in triticale

by

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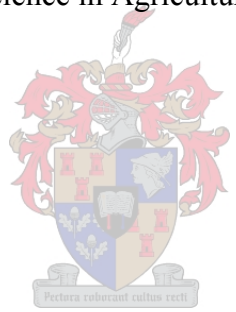

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Abstract

Research into markers for the detection of genetic diversity and cultivar identification has become an important component of the genetic improvement of crops. However, the incorporation of marker assisted selection (MAS) as a tool for the identification and characterization of breeding material has not been fully implemented in the breeding of spring triticale at Stellenbosch University's Plant Breeding laboratory (SU-PBL). The present study served as a case study in order to achieve this.

The first part of the study concerned the detection of genetic diversity in 101 newly sourced triticale cultivars, from a USDA germplasm bank, together with five local control cultivars, in order to identify possible crossing parents. Eight SSR markers, including five derived from rye and three from wheat, and five agronomic characteristics were used to assess diversity. In seedling screening the foreign cultivars showed resistance towards the stem rust isolate used, but were mostly susceptible to the leaf rust isolate. Out of the 8 SSR markers tested, 7 markers were polymorphic and revealed 140 alleles varying from 12 to 26 with an average of 17.5 alleles per locus. The observed polymorphic information content (PIC) value ranged from 0.39 to 0.88 with an average of 0.70, indicative of the good discriminatory ability of the SSR markers. The data revealed that the South African cultivars were genetically closely related to cultivars from the USA and Canada.

The second part of the study focused on the introgression of a blue aleurone layer gene (*Ba*), carried by a wheat cultivar, 'Cltr1202STR', and purple pericarp genes (*Pp1*; *Pp3*) also carried by a wheat cultivar, 'Amethyst', into a triticale background. Unfortunately the introgression of the purple pericarp genes failed. Two lines containing the blue aleurone layer, 11T023 and 11T028, were however successfully created. Molecular typing of these lines with SSR markers were able to show that BC_4F_1 line 11T023 (*Ba*) B was genetically similar to the recurrent parent 'Agbeacon'; and that the BC_4F_1 11T028 line (*Ba*) A was closest to the 'US2007' recurrent parent.

The study illustrated that MAS was a reliable tool for detecting genetic diversity in newly sourced germplasm, and assisted in making a backcross breeding effort more effective. The data generated from MAS could therefore clearly assist in making the SU-PBL breeding program more effective by moving, better informed, decision making toward data

based partly on the genotype, thereby minimizing the risks associated with purely phenotypic based decisions.

Uittreksel

Navorsing rondom die gebruik van merkers vir die bepaling van genetiese-diversiteit en kultivar identifikasie is ‘n toenemend belangriker komponent vir die genetiese verbetering van gewasse. Die inkorporering van merker-bemiddelde-seleksie (MBS) as gereedskap vir die identifikasie en karaktarisering van telingsmateriaal is nog nie ten volle geimplimenteer in die lente korogtelingsprogram van die Stellenbosch Universiteit Planteteeltlaboratorium (SU-PTL). Die studie het gedien as gevallestudie ten einde dit te bereik.

Die eerste gedeelte van die studie het gehandel oor die tipering van die genetiese diversiteit van ‘n 101 kultivars verkry vanaf ‘n USDA kiemplasmabank saam met 5 plaaslike kontroles. Dit was gedien ten einde moontlike kruisings-ouers te kon identifiseer. Agt SSR merkers, insluitend vyf afkomstig van rog en drie vanaf koring, asook vyf agronomiese kenmerke is aangewend om die materiaal se diversiteit te tipeer. Saailingtoetsing is ook gedoen en het aangetoon dat die meeste kultivars weerstandig was vir die stamroes-isolaat, maar nie die blaarroes-isolaat nie. Van die agt SSR merkers getoets het sewe getoon om polimorfies te wees en het ‘n 140 allele gegee wat gewissel het vanaf 12 tot 26 per lokus met ‘n gemiddeld van 17.5. Die waargenome polimorfiese inligtings inhoud (PII) waarde het gewissel vanaf 0.39 tot 0.88 met ‘n gemiddeld van 0.70. Die merkers kon dus suksesvol diskrimineer. Die data het aangetoon dat die Suid-Afrikaanse kultivars genetiese die naaste verwant was aan die kultivars afkomstig vanaf die VSA en Kanada.

Die tweede gedeelte van die studie het gefokus op die introgressie van ‘n blou aleuron-laag geen (*Ba*), afkomstig vanaf die koringkultivar ‘Cltr1202STR’, en twee pers-perikarp gene (*Pp1*; *Pp3*), afkomstig vanaf die koringkultivar ‘Amethyst’, na ‘n korog agtergrond. Ongelukkig het die oordrag van die pers-perikarp gene gefaal. Twee lyne wat die blou aleuron-laag bevat, 11T023 en 11T028, is egter suksesvol geskep. Tipering van die lyne met die SSR merkers het aangetoon dat die BC_4F_1 lyn 11T023 (*Ba*) B genetiese baie na aan die herhalende ouers ‘Agbeacon’ is en dat die BC_4F_1 11T028 lyn (*Ba*) A nader is aan die herhalende ouer ‘US2007’.

Die studie het dus geillustreer dat MBS gebruik kan word as ‘n betroubare manier om genetiese diversiteit te bepaal en by te dra tot die sukses van ‘n terugkruisingsprogram. Die data wat dus voortspruit uit MBS kan dus help om die SU-PTL se telingsprogram te assist-

eer in die besluitnemingsproses tydens teling deur beter genotipe gebaseerde besluite te neem wat die riskio van fenotipe gebaseerde besluite kan help verminder.

List of Abbreviations

%	Percent
˚C	Degrees Celsius
µg/ml	Microgram per millilitre
µl	Microlitre
µM	Micromolar
2n	Diploid
A	Adenine
AFLP	Amplified Fragment Length Polymorphism
Ba	Blue Aleurone
BC	Backcross
bp	Base pairs
C	Cytosine
CIMMYT	*Centro Internacional de Mejoramiento de Maíz y Trigo*
CTAB	Cetyl trimethylammonium bromide
dH_2O	distilled water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotidetriphosphate
DUS	Distinctness-Uniformity-Stability
EDTA	Ethylene-diaminetetraacetate
F	Forward
F1	First generation
FAO	Food and Agricultural Organization
g	Gram
G	Guanine
GA_3	Gibberic Acid
GD	Genetic Diversity
gDNA	Genomic Deoxyribonucleic Acid
Glu	Glutenins
H_2O	Water
ha	hectare
HWM	High Molecular Weight

Kg	Kilogram
L	Litre
$MgCl_2$	Magnesium chloride
mM	Millimolar
NaCl	Sodium chloride
ng/µl	Nanogram per microlitre
PBL	Plant Breeding Laboratory
PCR	Polymerase Chain Reaction
pH	Percentage hydrogen
PIC	Polymorphic Information Content
Pp	Purple Pericarp
R	Reverse
RAPD	Random Amplified Polymorphic DNA
RNAse	Ribonuclease
rpm	Revolutions per minute
SA	South Africa
SCM	Secale cereale Microsatellite
SDS-PAGE	Sodium Dedocyl Sulfate Polyacrylamide Gel Electrophoresis
SSR	Simple Sequence Repeats
T	Thymine
TBE	Tris-borate EDTA buffer
TE	Tris EDTA buffer
TEMED	N, N, N', N'-Tetramethylendiamine
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
U	Units
US	Stellenbosch University
USDA	United State Department of Agriculture
v/v	Volume for volume
w/v	Weight per volume
Xgwm	Gatersleben Wheat Microsatellite
Xwmc	Wheat Microsatellite Consortium

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Chapter 1: Introduction

Triticale (×*Triticosecale* Wittmack ex A. Camus) is an intergeneric hybrid between wheat (*Triticum spp*.) and rye (*Secale spp*.) (McGoverin *et al*., 2011). According to (Oettler, 2005) the first reported of triticale was in Russia in 1875. Regarding ploidy level, triticale exists in four different forms: tetraploid, hexaploid, octoploid and decaploid. It is grown in a three different types: winter, spring and facultative (Badea *et al*., 2011). Dogan *et al*. (2009) reported that Europe served as the main producer and breeder of this crop. Triticale is often considered an alternative to wheat in unfavourable growing conditions and in low input sys-tems (Dogan *et al*., 2009). Throughout the world, triticale is mainly used as animal feed, as forage and for human consumption (Badea *et al*., 2011). Additionally, over the past few years, the use of triticale has been extended as an essential industrial crop such as biomass source for bio-ethanol production and bio-composite chemicals (Mergoum *et al*., 2009; Badea *et al*., 2011).

In South Africa, spring triticale has been produced since the 1970's (Littlejohn *et al*., 1991). Current production stands at about 60 000 ha, and about 150 000 tons are produced per year. In South Africa, triticale is mainly used as animal feedstock (50%), as silage (25%) and as a cover crop (25%), mainly in vineyards (Roux *et al*., 2006). However, improving triticale through breeding is a challenge due to the narrow genetic base of the crop (Sinha and Joshi, 1985), which has lead to high levels of genotypic uniformity (Kumar and Singh, 1997). This challenge could be associated with the short breeding history of triticale, and the fact that it is a man-made hybrid crop.

Breeding of triticale has been heavily depending on the use of phenotypic (or morphological) characteristics such as grain yield, plant height, and spike colour, and thousand-kernel weight (TKW) for the identification and characterization of cultivars (Manero de Zumelzu *et al*., 2002; Kurkiev, 2006). Despite the successful use of these phenotypic characteristics in triticale breeding, there are limitations associated with the use of these traits. Some of the limitations include an overly dependence on the environment, and growth stage of the plant (Kuleung *et al*., 2006). Additionally, epistatic interactions and pleiotropic gene effects were also reported limiting the efficiency of these characteristics (Kumar, 1999; Fufa *et al*., 2005).

The past few years, however, molecular markers have been used in studying the genetic diversity of modern triticale germplasm (Tams *et al*., 2004; Kuleung *et al*., 2006), and extensively used in marker-assisted selection (MAS) of desirable traits. Lamadji *et al.* (1995) stated that genetic diversity is a prime concern for plant breeders in ensuring an effective breeding program. Despite recent developments in the use of molecular markers in triticale breeding, there are still large knowledge gaps underlying the actual levels of existing genetic diversity in many of the commercial triticale cultivars grown across the world.

The spring triticale breeding program at Stellenbosch University's Plant Breeding Laboratory (SU-PBL), Department of Genetics, like other breeding programs across the world, is always adopting new research strategies in order to maintain its sustainability, improve profitability, and to accelerate the release of new cultivars. Some of the current research strategies that are becoming increasingly important include the use of new technologies such as molecular marker techniques. These techniques could assist in the identification of cultivars and tagging useful traits that will facilitate the protection of plant breeder's rights. Ercisli *et al.* (2009) stated that an accurate identification of cultivars is important for the effective protection of plant breeder's rights in contemporary plant breeding programs. Moreover, these techniques could also help in revealing the existing genetic diversity in the SU-PBL triticale cultivars and advanced breeding material, which would be valuable for effective planning of future breeding efforts. The incorporation of new germplasm through research collaboration could be better managed. Goyal *et al.* (2011) stated that this strategy could be more useful by characterizing the genetic diversity of the newly introduced germplasm.

The introgression of phenotypic markers based on grain colour has become a key research strategy proposed for the identification of cultivars. Grain colour of wheat, blue aleurone layer and purple pericarp traits, caused by anthocyanin genes, have been intensively used as phenotypic markers in wheat breeding programs (Knievel *et al*., 2009). Measuring of gene flow and development of hybrid are some of the most common uses of these traits in wheat breeding (Zheng *et al*., 2009). Similarly in triticale breeding, the introgression of these traits could serve the same purpose.

The present study focused on two aims: firstly, determining the genetic diversity of introduced germplasm using simple sequence repeat (SSR) markers and phenotypic character-

istics; and secondly the introgression of anthocyanin genes for the phenotypic identification of triticale cultivars.

Chapter 2: Literature Review

2.1 History and development of triticale

Triticale (×*Triticosecale* Wittmack ex A. Camus) is a self-pollinating small grain crop (Vyhnanek *et al*., 2009), and one of the first synthetic amphiploids ever developed (Zhou *et al*., 2007). Triticale belongs to the family *Poaceae*, subfamily *Pooideae* and tribe *Triticeae* (Kavanagh *et al*., 2010), and resulted from crossing wheat (*Triticum aestivum* L.) with rye (*Secale cereal* L.) (Mergoum *et al*., 2009). The crop combines the higher yield potential and superior quality attributes of wheat with the biotic and abiotic tolerance attributes of rye. Wilson detected the first successful crosses between common wheat and rye in 1875 in Russia (Oettler, 2005). The resultant hybrid plant was an octoploid triticale (2n=8x=56, AABBRRDD), but was found to be sterile. According to Oettler (2005), the occurrence of seed sterility in octoploid triticale was mainly due to extreme meiotic instability.

The first fertile triticale type, a hexaploid (2n=6x=42, AABBRR), was developed in Germany by Rimpau in 1888 after many years of unsuccessful crosses between common wheat and rye (Dogan *et al*., 2009). This hybrid plant was obtained by crossing durum (a tetraploid) wheat and diploid rye, followed by chromosome doubling of the F_1 progeny (Rabiza-Swider *et al*., 2010). Decaploid (AABBDDRRRR 10x=2n=70) and tetraploid (AARR/BBRR/DDRR 4x=2n=28) triticale types were also developed, but were found to have less commercial value due to poor seed fertility (Singh, 2003). Today, hexaploids represents most triticale grown worldwide due to its superior agronomic characteristics, and stable productivity as compared to other ploidy levels (Kavanagh *et al*., 2010).

Hexaploid triticale can further be classified as either complete or substitute (Fox *et al*., 1990). Complete hexaploid triticale has all seven pairs of the rye chromosome while substitute hexaploid triticale has some of the rye chromosomes substituted with D-genome chromosomes from wheat. In addition, hexaploid triticale can further be classified according to growth habit, as with wheat, as a winter, intermediate or spring type (Mergoum *et al*., 2009). Winter trit-

icale necessitates vernalization for floral differentiation, and has a longer growth cycle than spring triticale, which does not require vernalization (Santiveri *et al*., 2004). Winter triticale also exhibits prostrate growth, which is more flat, while spring triticale has a more upright growth type. Furthermore, winter type's yields more forage than spring types due to its longer vegetative growth cycle. The intermediate types have characteristics between the winter and spring types (Mergoum *et al*., 2009).

In 2008 approximately 14 million tons of triticale was harvested globally in about 33 countries (Goyal *et al*., 2011). France, China, Hungary, Australia, Germany, Belarus and Poland were the primary producers, and breeders of triticale (Dogan *et al*., 2009; Oettler, 2005). Mergoum *et al*. (2009) reported that Europe accounted for approximately 90% of total triticale production. In South Africa, the breeding of spring triticale was started in the 1970's at the Department of Genetics, Stellenbosch University (SU) (Littlejohn *et al*., 1991).

2.2 Uses of triticale

Triticale is mainly used as a feed grain and forage crop, although small quantities enter the human food chain through mixing with other grains (Aydogan-Cifci and Yagdi, 2007; Hills *et al*., 2007). In South Africa, triticale is used as a feed grain, silage and cover crop in vineyards (Roux *et al*., 2006). When compared with other cereals triticale has a limited market internationally (Kutlu and Kinaci, 2010). However, triticale has been proven to contain higher levels of proteins, Vitamin B and essential amino acids such as lysine and methionine when compared to oats, barley, rye and corn (Roux *et al*., 2006; Mergoum *et al*., 2009). It was also reported that triticale grain contains higher levels of mineral elements such as potassium, manganese, iron and phosphorus compared to wheat grain (Gupta and Priyadarshan, 1982).

The initial objective for developing triticale was directed at developing an alternative cereal crop for human consumption. However, in recent years this has shifted to animal feed, and development as a crop for future use as bio-ethanol feedstock (Mergoum *et al*., 2009). As animal feed, it is primarily used for the feeding of pigs, poultry, and ruminants such as sheep and cattle (Kinaci and Gulmezoglu, 2007). Triticale grain contains high amounts of starch and dry matter, which promote good digestibility (Dennett *et al*., 2009, Salabi *et al*., 2010). In

an experiment conducted by Salabi *et al*. (2010), the digestibility of dry matter in triticale was found to be higher than barley grain. Moreover, good silage characteristics of triticale such as high pH, crude protein, crude lipid, crude ash, and metabolizable energy have made triticale a superior animal fodder crop (Kara and Uysal, 2009). It was observed that pigs fed on triticale grew faster than those fed on wheat (Farrell *et al*., 1983).

Recently, the proposed use of triticale has been extended to include that of a bio-ethanol feedstock. Some of the major advantages of using triticale for bio-ethanol production include a generally higher biomass and starch content, lower nitrogen requirement, softer grain and better conversion of starch into alcohol compared to other cereal crops (Davis-Knight and Weightman, 2008; Goyal *et al*., 2011). Singh *et al*. (1978) stated that the high level of amylase activity inherited from the rye genome stimulates a rapid conversion of starch to sugar during the fermentation processes. A low temperature and less synthetic enzymes required for pre-treatment and fermentation processes, compared to wheat, further makes triticale a more suitable feedstock for bio-ethanol production (Mojovic *et al*., 2009). Additionally, generally higher input cost associated with the production of fuel ethanol from wheat compared to triticale also makes the crop more attractive for bio-ethanol production (Sosulski *et al*., 1997).

Rosenberger *et al*. (2001) reported that triticale yielded higher volumes of ethanol compared to wheat and rye. It was found that winter triticale yielded an average starch content of 665.54 g kg^{-1} and bioethanol outcome of 478.14 L t^{-1} (Jansone *et al*., 2010). Tsupko (2009) reported ethanol yields ranging from 466 to 477 L t^{-1} in some of the spring triticale lines tested in his study. He further suggested ethanol yield was much dependent on grain yield, grain starch and other polysaccharides facilitating a more efficient fermentation process.

Triticale has also been suggested as having potential for the provision of nitrogen, yield improvement and protection of soil erosion when grown in a rotation with other crops (Igne e*t al*., 2007).

2.3 Breeding of hexaploid triticale

Triticale breeding programs around the world are mostly aimed at improving crop yields through improvement in tolerance to abiotic and biotic stresses (Mergoum *et al*., 2009). A-

biotic stress breeding improves the crop production in marginal areas (alkaline and acid soils), and tolerance to mineral deficiencies or toxicity such as aluminium, copper, zinc, magnesium and boron (Zhang and Jessop, 1998; Dogan *et al*., 2009). Biotic stress breeding aims to improve the crop's disease resistance against pathogens such as rusts (*Puccinia spp*.), smuts (*Ustilago* and *Urocystis spp*.), spot blotch (*Bipolaris sorokiniana*), leaf blotch (*Septoria tritici*), scab (*Fusarium spp*.), ergot (*Claviceps purpurea*) and other diseases (Haesaert and De Baets, 1994; Mergoum *et al*., 2009). In South Africa the only abiotic disease of interest for triticale breeding is currently rust.

In addition, some of the breeding programs around the world focus on the genetic improvement of grain quality traits such as nutritional quality, grain hardness, test weight and sprouting resistance. Similarly, in South Africa, Stellenbosch University's spring triticale-breeding program is still focused on the improvement of the crop for grain yield and grain quality (including starch and protein).

The improvement provided by biotic and abiotic stress breeding of triticale have mainly been achieved by wide hybridization with its breeding parents, wheat or rye, mainly wheat as donor parent for the traits of interest (Tyrka and Chelkowski, 2004; Oettler, 2005; Mergoum *et al*., 2009). Moreover, Budak *et al*. (2004) suggested that traits such as grain quality, dwarfing genes and winter hardiness in triticale could be improved through introgression of segments the D-genome chromosomes of wheat. However, the presence of genetic incompatibility between triticale and its parents, which causes embryo abortion, has lead to a delay in triticale improvement through breeding (Kapila and Sethi, 1993). This incompatibility between triticale and its parents result in poor seed set, shriveling and sterility due to chromosome imbalances (Khanna, 1990; Nkongolo *et al*., 1991). Similarly, Lukaszewski *et al*. (2001) stated the structural difference in chromosomes between wheat and triticale complicates the transfer of traits between it. In contrast, Hills *et al*. (2007) reported fertile interspecies hybrid seed when using triticale as female parent in crosses with wheat. However, Bijral *et al*. (1996) stated that the crossability between triticale and wheat is also dependent on the maternal and paternal genotypes. They found that out of the six different triticale and wheat cross combinations made; only one showed increased levels of out-crossing and viable seed.

Several techniques have been developed to overcome the incompatibility problem between different crop species; including the use of exogenous growth substances (i.e. gibberillic ac-

id) and immunosuppressant's (i.e. aeriflavin), embryo rescue, use of irradiation and stimulants (Mehetre and Aher, 2004). Backcross breeding, which is a conventional breeding method, has been extensively used in order to recover the fertility and correct chromosome imbalances resulting from wide hybridization (Bizimungu *et al*., 1998).

2.3.1 Embryo rescue

Embryo rescue has been the technique of choice in producing fertile hybrids when performing intergeneric and interspecific crosses in cereal (Sharma *et al*., 1996). Some of the uses of embryo rescue include breaking of seed dormancy, production of haploid plants and evaluating the vitality of seeds (Burun and Poyrazoglu, 2002).

Embryo rescue involves the excision of the immature embryo from the ovule and culturing it on a growth media following pollination and fertilization. In triticale breeding, embryo rescue has been used to produce fertile hybrid progenies resulting from interspecies hybridization between wheat x triticale (Kapila and Sethi, 1993). The study found that this technique resulted in average hybrid embryo recovery of 76% observed between wheat x triticale crosses.

However, there are some limitations associated with the use of embryo rescue. These include a dependence on the growing conditions of the rescued embryos for the efficient recovery of complete plantlets, growth stage and age of embryos and parental genotypes involved (Nkongolo *et al*., 1991; Kapila and Sethi, 1993). Hybrid necrosis can also occur and has been attributed to particular *Ne* genes such as *Ne1*, *Ne2*, *Ner1* and *Ner2* (Bizimungu *et al*., 1998). Despite these limitations, embryo rescue remains an effective method to facilitate viable embryos following wide hybridization (De Mesquita Dantas *et al*., 2006).

2.3.2 Backcross breeding

Backcross breeding is one of the oldest conventional breeding methods, and is aimed at recovering the genotype of the recurrent parent while allowing an addition of alleles at one or more loci from the donor parents, resulting in a superior character being contributed from the non-recurrent parents (Lewis and Kernodle, 2009). This provides for the improvement of cultivars deficient in particular traits without losing the main traits of interest.

With backcrossing donor parents are only used during the initial crosses, while the recurrent parents are used as recurrent parents throughout the crossing cycles (Allard, 1999). This

method is a form of inbreeding, as the features of the recurrent parents is automatically recovered after successive backcrosses. The process of backcrossing is often repeated four to five times, followed by self-pollination helping to establish the homozygosity of genes governing the trait of interest (Lewis and Kernodle, 2009). Allard (1999) and Morris *et al*. (2003) suggested that backcross breeding is the most efficient manner of introgressing single dominant traits such as disease resistance, insect resistance, grain and flower colour, grain and biomass yield, and adapted trait characters. It was also found to be suitable in transferring quantitative traits with moderate to high heritability (Bayles *et al*., 2005) as well as recessive genes controlling certain traits (Isleib, 1997). Vogel (2009) reported that the transfer of dominant genes involves four cycles of backcrossing while the transfer of recessive genes must also involve several generations of selfing to be fixed.

In small grain breeding, the use of the backcross method was first documented by Harlan and Pope (1920). They were successful in introgressing the smooth-awned trait into commercial barley cultivars after a two cycles of backcrossing. Later, Briggs (1938) used backcrossing to transfer bunt, stem rust and Hessian fly resistance genes into wheat. In triticale, backcross breeding has successfully been used for the improvements of genetic diversity in spring triticale by crossing with wheat (Barker *et al*., 1989). Moreover, triticale has also been used as intermediate parent for Russian wheat aphid (RWA) resistance (Lukaszewski *et al*., 2001), plant height (Kurkiev *et al*., 2006) and *Fusarium* head blight disease resistance (Oettler *et al*., 2004) in wheat breeding programs.

However, there are some limitations associated with backcross breeding. These include the difficulty of screening for recessive alleles, although the use of molecular markers if available does make this a lot more feasible (Semagn *et al*., 2006). It is also time consuming as it requires many generations to recover the gene of interest, and is less effective in transferring quantitative traits (Morris *et a*l., 2003; Benchimol *et al*., 2005). The problem of linkage drag is also present (Benchimol *et al*., 2005). Despite these limitations, Ochanda *et al*. (2009) stated that backcrossing remains the most common method to introgress genes of interest into adapted germplasm.

2.4 Different marker systems used for the identification and differentiation of triticale

Breeding programs are continually developing new cultivars with specific genotypic and phenotypic requirements. The development, validation, optimization and implementation of markers can assist in this process, as well as the maintenance of genetic variation and the protection of plant breeder's rights (Heckenberger *et al.,* 2008). Thus, interest in research surrounding markers for the detection of genetic diversity and cultivar identification has become a key component for crop improvement, and also triticale improvement (Barnnet *et al*., 2006; Salmanowicz and Nowak, 2009). Several phenotypic and molecular markers have been studied and developed over the past several years for the identification and characterizing of triticale germplasm (Kamboj and Mani, 1983; Kumar and Singh, 1997; Tams *et al*., 2004).

2.4.1 Uses of phenotypic markers in cereal breeding

Phenotypic or morphological markers are the oldest descriptors used to differentiate one cultivar from another (Adugna *et al*., 2006). These markers represent the first methods used in the taxonomic and agronomic classification of crop germplasm (Sultana *et al*., 2005). Li *et al*. (2009) stated that the advantages of using phenotypic markers are that they are simple, rapid and inexpensive to measure. Some of the most commonly used phenotypic markers in cereal breeding include: grain and flower colour (Khanjari *et al*., 2008).

These phenotypic markers have been successfully used for the assessment of genetic variability and cultivar development (Fufa *et al*., 2005), and classification and description of crops (Khanjari *et al*., 2008). Moreover, Lima-Brito *et al*. (2006) stated phenotypic markers are also used to ascertain the hybrid status in crop species.

Fufa *et al*. (2005) assessed the genetic diversity of 30 hard winter wheat cultivars using 5 morphological characteristics including days to anthesis, plant height, grain yield and grain yield component, and hectoliter mass. They found high levels of variation between the characters measured, and concluded that cultivars with shorter height yielded higher. Thirty-eight tetraploid and 161 hexaploid wheat landraces were characterized using 15 qualitative spike characters (e.g. spike shape) and 17 quantitative spike characters such as (e.g. spike width (mm)) in Oman (Khanjari *et al*., 2008). They found that all the measured spike characters were highly polymorphic, and that they could assign genotypes to their different regions of origins.

Despite the role played by phenotypic markers in cultivar development, it faces many challenges. Their limited numbers and their greater dependency on both environment and growth stage of the plant for observation hinder the application of phenotypic markers in the differentiation of germplasm. Epistatic and pleiotropic gene effects also limit the efficiency of phenotypic markers as a reliable differentiation tool (Kumar, 1999; Fufa *et al*., 2005).

Phenotypic characters such as plant height, thousand-kernel weight, glume shape, glume colour, spike shape, and grain colour have been used for the genetic improvement of wheat through breeding (Khanjari *et al*., 2008). Manifesto *et al*. (2001) stated that phenotypic characters have been used for the identification and registration of bread wheat cultivars. Among these phenotypic characters, glume colour and grain colour were reported as important taxonomic discriminators (Khlestkina *et al*., 2006), and for the determination of homogeneity between wheat cultivars due to better stability (Arbuzova and Maystrenko, 2000). These characters occur as the results of anthocyanin accumulation in tissues and anthocyanin pigmentation was among the first phenotypic traits studied (Khlestkina *et al*., 2008).

According to Khlestkina *et al*. (2008), there are two major groups of genes responsible for the anthocyanin pigmentation of different wheat tissues (Table 1). The first groups of genes are *Rc*, *Pc*, *Pan*, *Pιb* and *Pιs,* which pigments coleoptiles, culms, anthers, leaf blades and leaf sheaths respectively. These genes were located on the short arm of chromosome 7A, 7B, and 7D on the wheat genome (Blanco *et al*., 1998; Ahmadi and Nazarian, 2007; Khlestkina *et al*., 2008). The second groups of genes are *Pp* and *Ra,* leading to the pigmentation of the pericarp and auricles, and are located on chromosomes 2A, 6A and 7B of the wheat genome (Melz and Thiele, 1990; Dobrovolskaya *et al*., 2006).

Another group of genes considered of minor importance include *Ba* and *R* genes that pigments grains, and are located on the long arm of chromosomes 4A, 3A, 3B and 3D (Metzger and Silbaugh, 1970; Dubcovsky *et al*., 1996). Khlestkina *et al*. (2002) suggested that many of these anthocyanin genes are inherited as single dominant genes, and therefore have a monogenic inheritance pattern.

Table 1 : The most prominent anthocyanin genes in wheat tissues and chromosomal locations.

Gene	Chromosome location	Reference
Rc-Al	7AS	Khlestkina *et al.* 2002
Rc-B1	7BS	Khlestkina *et al.* 2002
Rc-D1	7DS	Khlestkina *et al.* 2002
Rc-D2	4D	Khlestkina *et al.*2008
Pc-A1	7AS	Khlestkina *et al.*2008
Pc-B1	7BS	Ahmadi and Nazarian, 2007
Pc-D1	7DS	Ahmadi and Nazarian, 2007
Pan-A1	7AS	Blanco *et al*. 1998
Pan-D1	7DS	Khlestkina *et al.*2008
Plb-B1	7BS	Khlestkina *et al.*2008
Plb-D1	7DS	Khlestkina *et al.*2008
Pls-B1	7BS	Khlestkina *et al.*2008
Pp1	7BL	Dobrovolskaya *et al*. 2006
Pp3	2A	Dobrovolskaya *et al*. 2006
Ra2	4B	Melz and Thiele, 1990
Ra3	6B	Melz and Thiele, 1990
Ba	4BL	Dubcovsky *et al*.1996
R1	3DL	Metzger and Silbaugh, 1970
R2	3AL	Metzger and Silbaugh, 1970
R3	BL	Metzger and Silbaugh, 1970

2.4.2 Uses of phenotypic markers in triticale breeding

Royo *et al.* (1995) used spike length, plant height, and plumpness of kernels, erect spike morphology, and kernels per spikelet as phenotypic markers to differentiate, complete and substitution, triticale types. They observed that substitution triticale have reduced spike length, shorter and plumper kernels, decreased plant height, erect spike morphology and a lower number of kernels per spikelet than complete triticale types. Furman *et al*. (1997) used spike character to group a collection of more than 3000 triticale accessions as either secondary substitute or complete hexaploid triticale type. They classified 68% of the accessions as hexaploid, made up of 38% complete and 30% as substitute, and 32% as octoploid triticale. Making use of spike character to distinguish the difference between hexaploid and octoploid triticale types similar results were reported by Zhou *et al.* (2007). Observations included that hexaploid triticale have less compact spikes, while octoploid triticale exhibited complete compact spike.

Although the use of phenotypic markers still constitutes the main breeding tool used for the genetic improvement of triticale, high levels of genetic similarity among triticale have made these markers less useful (Kamboj and Mani, 1983). Additionally, due to the shorter breeding history of the triticale, factors such as mutation and selection did not have a significant influence on generating desirable genetic variability during the early domestication of triticale (Ramirez-Calderon *et al*., 2003). This has limited the efficiency of phenotypic markers as a reliable tool in the differentiation and identification of triticale germplasm.

Therefore, identification of alternative phenotypic markers that are highly heritable, informative and stable is necessary. Anthocyanin genes are among the colour markers that can be used in cereals (Doshi *et al.*, 2007). Grain colour, as the result of anthocyanin genes, has been adopted as phenotypic marker in wheat breeding for the identification and characterization of germplasm (Zeven, 1991; Zheng *et al*., 2006; Zheng *et al*., 2009). In contrast to other phenotypic markers, this trait is not affected by genotype and environmental conditions for its expression (Abdel-Aal *et al*., 2008).

2.4.3 Blue and purple grain characters in wheat

Anthocyanin is a water-soluble pigment occurring in several plant tissues and is synthesized as part of the flavonoids and phenylpropanoid pathway (Hung *et al*., 2007). Jones *et al.*

(2003) stated that more than 300 anthocyanin related compounds have been identified in plant species. As a secondary metabolite, anthocyanin plays an essential role in the protection of plant tissues from UV damage, pollen development, flower pigmentation and defense against pathogens (Liu *et al*., 2005). Plant anthocyanins such as those leading to blue and purple grain colour in wheat have been suggested to play an essential role as antioxidants, inhibitors of systemic inflammation and cancer in humans (Guo *et al*., 2011).

The pigmentation of blue-grain wheat is caused by the deposition of anthocyanin compounds in the aleurone layer (Zheng *et al*., 2009). Major anthocyanins found includes; cyanindin-3-glucoside, pelargonidin-3-glucose and cyanidin-3-galactacide (Hu *et al*., 2007). Other anthocyanins reported occurring in quantities in blue grain includes peonidin-3-glucoside, delphinidin-3-rutinoside and delphinidin-3-glucoside (Hu *et al*., 2007; *Knievel et al.,* 2009). Purple pericarp pigmentation of grain wheat is due to the deposition of the primary anthocyanin, cyaniding-3-glycoside (Dobrovolskaya *et al*., 2006). Other anthocyanins reported in pigmentation of purple pericarp grain includes; cyanidin-3-rutinoside and cyanidin-succinyl-glucoside (Knievel *et al*., 2009).

2.4.3.1 Blue grain character in wheat

Thinopyron ponticum (previously *Agropyrum elongatum*) is a decaploid (2n=10x=70, $StStE^eE^bE^x$) perennial wild type grass species, mainly used in cereal breeding as gene donor for disease resistance, insect resistance, perennial habit and forage traits (Chen *et al.,* 2003; Morrison *et al*., 2004). Zheng *et al.* (2009) stated that the appearance of the blue-aleurone phenotype of wheat grain has occurred through crosses between wheat and *Th. ponticum*. Studies confirmed that the introgression of chromosome 4Ag of *Th. Ponticum* chromosome*,* inherited from the E-genome, carries the blue aleurone gene, *Ba(b),* leading to blue pigmentation of wheat grain (Zheng *et al*., 2006a; Zheng *et al*., 2006b).

A naturally occurring blue-aleurone layer was also reported in the wild diploid wheat type, *Triticum boeoticum* (Morrison *et al*., 2004). The responsible gene was located on the long arm of chromosome 4A in this species (Dubcovsky *et al*., 1996). The introgression of the genes responsible for the blue grain character is the result from the homeologous recombination between the long arm of chromosome 4Ag/EL of *T. boeoticum* / *Th. ponticum* and chromosome 4A of wheat (Zheng *et al.,* 2006b). The recombination occurred as either a disomic

substitution (2n=42, AABBDD–4AgIl) or disomic addition (2n=44, AABBDD + 4AgIl) of the alien chromosome of *Th. ponticum* (Zeller *et al.,* 1991; Zeven 1991; Zheng *et al.,* 2006b). In contrast, Zheng *et al.* (2006a) reported that it occurred as the result of chromosome recombination between 4D and 4Ag from *Th. ponticum*.

It was found that the blue aleurone character in wheat grain is controlled by a single dominant *Ba* gene located on chromosome 4A of the wheat genome (Zheng *et al*., 2006b; Knievel *et al.,* 2009). Mapping of *Ba* gene confirmed it to be located 0.71-0.80 (centimorgan) region on the long arm of the 4Ag chromosome of the *Th.ponticum* genome (Zheng *et al*., 2006a). In *T. boeoticum, the Ba* gene was located on the long arm of chromosome 4A (Morrison *et al.*, 2004). They further suggested that the *Ba* gene from *T. boeoticum* is more stable in expression than the *Ba* gene from *Th. ponticum* which exhibits the phenomenon of Xenia, in which the pollen parent has a zero genetic effect on the expression of a maternal trait. Zheng *et al.* (2009) stated that three dosages of blue-grained genes from *Th. ponticum* in the endosperm produced solid dark blue, two dosages produced medium blue and one dosage produced light blue pigmentation. It was also stated that expression of the blue grain trait in wheat depends on the number of genes inherited from the pollen donor who contributed this trait (Guo *et al*., 2011).

2.4.3.2 Purple grain character in wheat

Anthocyanin can also express in the pericarp leading to a purple grain colour, and has been successfully adopted as a phenotypic marker in breeding. Purple grain in hexaploid wheat originates from the tetraploid *Triticum dicoccum* (Belay *et al.,* 1995; Dobrovolskaya *et al*., 2006; Siebenhandl *et al.,* 2007).

The purple pericarp colouring is controlled by three dominant complementary genes *Pp1*, *Pp2* and *Pp3* (Dobrovolskaya *et al*., 2006; Khlestkina *et al.*, 2010). Recent mapping of these genes confirmed them as being located on chromosome 2A (*Pp3* gene), 6A (*Pp2* gene) and 7B (*Pp1*gene) (Dobrovolskaya *et al*., 2006). In contrast, Arbuzova and Maystrenko (2000) reported one dominant gene in tetraploid wheat and two dominant complementary genes in hexaploid wheat that control the purple colour.

It was suggested that both blue and purple grain characters could be used as phenotypic marker for determining the outcrossing between wheat cultivars, characterization of double

haploid plants and the production of new hybrid seed (Zeven, 1991; Zheng *et al*., 2006;). Moreover, Knievel *et al.* (2009) suggested that the blue trait could also be used to distinguish wheat for human consumption from wheat intended for bio-ethanol or animal feedstock.

2.5 DNA markers

Molecular markers reveal genetic differences (polymorphisms) in the primary structure of DNA between different individuals. Compared to protein markers, DNA polymorphisms are more stable, and can reveal very subtle changes in the genomic DNA (Horacek *et al*., 2009). Different DNA based marker techniques have been successfully used in the characterization of triticale germplasm. These techniques includes restriction fragment length polymorphism (RFLP) (Lelley *et al*., 1995; Ma *et al*., 2004), random amplified polymorphic DNA (RAPD) (Atak *et al*., 2005), amplified fragment length polymorphism (AFLP) (Tams *et al*., 2005) and simple sequence repeats (SSR) (Kuleung *et al*., 2006).

2.5.1 Restriction fragment length polymorphism (RFLP)

RFLP employs PCR amplification for the detection of different fragment sizes and has been used in many different organisms. It involves the use of restriction enzymes, which recognizes specific nucleotide motifs in DNA and cleaves DNA at these sites (Ovesná *et al*., 2002). Due to variation in the DNA sequences, caused by mutations typically, restriction enzymes will cleave DNA molecules at different sites, generating many different fragment sizes.

Therefore, different fragment sizes are detected by means of hybridizing these fragments with the suitable probes, which consists of DNA homologous to the original organism and digested with the same enzymes. Following hybridization, different sized fragments run at different rates through the gel leading to the formation of different band sizes appearing at different positions on the gel. In plant breeding, this technique has been used for cultivar identification, evaluation of genetic diversity, mapping of plant genomes and the identification of quantitative trait loci in many agricultural crop species, including cereals (Ovesná *et al*., 2002; Kumar *et al*., 2009).

In triticale, RFLP has been applied in the study of the genetic relationship between different triticale germplasm (Lelley *et al*., 1995; Ma *et al*., 2004). Lelley *et al.* (1995) analyzed the

chromosomal composition of 35 different tetraploid triticale lines using *Eco*RI- and *Hind*III restriction enzymes. Their studies observed that some triticale lines carry stable and homologous chromosomes while other lines carry homeologous chromosomes, indicating variation between these lines. Moreover, Ma *et al.* (2004) observed that over 97% of wheat coding sequences and only about 61.6% of rye coding sequences were maintained in the triticale genome of four primary triticales. This indicated that the wheat genome was generally better conserved in triticale than the rye genome. It is however important to note that low levels of polymorphism, and the complexity of this marker have limited its use for cultivar identification in wheat breeding (Manifesto *et al.*, 2001).

2.5.2 Random amplified polymorphic DNA (RAPD)

A RAPD marker involves the amplification of genomic DNA with single primers of an arbitrary nucleotide sequence (William *et al*., 1990). This single primer binds genomic DNA at the two opposite sites of the different strands of the template DNA (Ovesná *et al*., 2002). DNA polymorphisms are generated, because of the nucleotide base changes in the primer binding sites or insertion and deletion within amplified sites as detected by the presence or absence of amplified products (Tingey and Tufo, 1993; Kumar *et al*., 2009). RAPDs have been used as genetic markers for the identification of cultivars, fingerprinting of genomes, evaluation and characterization of genotypes in many crop species (Asif *et al*., 2005).

In triticale breeding, RAPD markers have been used for the characterization of triticale germplasm (Gawel *et al*., 2002; Atak *et al*., 2005). Atak *et al.* (2005) used 60 RAPD primer combinations to characterize genetic variation among 25 different triticale cultivars. They observed that out of the 60 primers used, 10 primers were highly polymorphic and reproducible. With these 10 primers, 60 amplified fragments bands were produced and 37 (61.1%) of them were found to be polymorphic, indicating the existence of high levels of genetic variation among the tested cultivars. Gawel *et al.* (2002) used 24 RAPD primers, varying from 12 to 18 bases in length, to evaluate the genetic diversity of seven triticale cultivars. Out of 24 primers used, 19 were highly polymorphic, and able to reveal genetic variation between tested cultivars.

Compared to RFLP markers, the RAPD technique requires less DNA, and do not rely on the use of radioactivity, and DNA blotting (Nagaraju *et al*., 2001). However, due to the complex-

ity of the triticale genome RAPDs are hampered with poor reproducibility that renders it less useful for the characterization of triticale's genetic variation (Gawel *et al.,* 2002).

2.5.3 Amplified fragment length polymorphism (AFLP)

The AFLP technique involves the digestion of genomic DNA with restriction endonucleases followed by the ligation of oligonucleotide adaptors to the restriction products, and the selective amplification of the resultant digested PCR products (Kumar *et al*., 2009). Restriction fragments obtained can be detected on a denaturing polyacrylamide gel or with automated sequencing analysis (Rinehart, 2004). This technique has been used successfully to analyze genetic diversity, cultivar identification, and for the characterization of accessions in many crop species including cereals (Manifesto *et al*., 2001; Aggrwarwal *et al*., 2002; Honing, 2007; Leggesse *et al*., 2007). In triticale AFLPs have been used for the assessment of genetic diversity (Tams *et al*., 2005), assigning of lines into heterotic groups (Goral *et al*., 2005), and the estimation of the evolutionary relationship between different sets of germplasm (Ma *et al*., 2004; Ma and Gustafson, 2006). Tams *et al.* (2005) tested 128 triticale cultivars and breeding lines using ten *Pst*I/*Taq*I primer combinations. A total of 344 polymorphic bands were detected, indicating genetic differences between all of the 128 entries tested.

Goral *et al.* (2005) used AFLPs to assign breeding values to six advanced breeding lines and five cultivars of winter triticale using 14 selective *Pst*I AFLP primer combinations. Ninety-one polymorphic bands were detected, and an average genetic similarity of 84.6% observed. Polyploidization-induced genome sequence variation in two hexaploid, two octoploid triticale and two sets of wheat-rye addition lines were tested using 40 *Eco*RI-*Msc*I/ *Pst*I-*Msc*I primer combinations (Ma *et al*., 2004). It was found that the *Eco*RI-*Msc*I primer combination showed a higher additive inheritance in octoploid than hexaploid triticale suggesting greater genomic sequence variation in hexaploid than octoploid triticale in the material tested. In a subsequent study the process of allo-polyploidization was studied in four triticale lines and one F_1 wheat x rye hybrid using two sets of *Eco*RI-*Msc*I/ *Pst*I-*Msc*I primer combinations (Ma and Gustafson, 2006). The results showed higher numbers for the loss in the rye parental genome than in the wheat genome in the F_1 with the *Eco*RI-*Msc*I primer combinations than *Pst*I-*Msc*I primer combinations (74.5% *vs* 68.4% and 46.3% *vs* 36.2%) than in tested triticale lines. This suggested allo-polyploidization causes genomic sequence variation in triticale and a more rapid genomic variation in the rye genome than the wheat genomes (A and B) in the

background of triticale. However, due to low levels of AFLP polymorphism in triticale, AFLPs have not been the marker of choice for genetic diversity assessment in triticale (Tams *et al*., 2005).

2.5.4 Simple sequence repeats (SSR)

SSRs are tandem repeats of short sequences of one to six nucleotides. SSRs involve the use of two unique primers composed of short length nucleotides complementary to the flanking region of the targeted sequence for amplification. The level of observed polymorphism depends on the length of the sequence repeats at each specific locus and can be detected by means of using polyacrylamide gels or an automated system (Roder *et al*., 1998; Rakoczy-Trojannowsk and Bolibok, 2004).

In plant species, Condit and Hubbell (1991) were first to report the occurrence and abundance of SSR markers. The A/T repeat-motifs were reportedly most abundant in plant genomes while A/C repeats were more abundant in mammalian genomes (Condit and Hubbell, 1991; Roder *et al.,* 1995). The occurrence of SSR markers in plant genomes is on average every 6-7 kb (Curn and Zaludova, 2007). Akkaya *et al.* (1992) stated that the high levels of polymorphism of SSR markers render them more useful as genetic markers when compared to RFLP and RAPD in many plant species.

The uses of SSR markers in self-pollinating crops ranges from the identification of genotypes, tagging of important traits, mapping of genes, and the assessment of genetic variability (Powell *et al*., 1996; Rongwen *et al*., 1995; McCouch *et al*., 1997; Honing, 2007). Furthermore, the transferability of SSR markers between closely related species made this technique the marker of choice with research and breeding programs (Yildirim *et al*., 2009).

In triticale, SSR markers have recently been used mainly for genetic diversity assessment studies (Tams *et al*., 2004; Kuleung *et al*., 2004; Kuleung *et al*., 2006; Costa *et al*., 2007; Vyhnanek *et al*., 2009). Tams *et al.* (2004) analyzed the genetic diversity of 128 European winter triticale cultivars obtained from 13 companies, using 68 wheat and 28 rye SSR markers. They observed significant genetic variation among and within germplasm from different seed companies. Their results further showed moderate genetic diversity of SSR markers with average polymorphic information content (PIC) of 0.54. Kuleung *et al.* (2004) tested 148 wheat and 28 rye SSR markers to reveal genetic diversity of 5 lines of wheat, rye and triticale

each. Their studies found that 85 (58%) of the wheat markers and 11 (39%) of the rye markers tested were transferable to triticale. However, only wheat SSR markers showed polymorphic bands in this study, and no product amplification was observed with the rye markers. Following this study, Kuleung *et al.* (2006) tested 80 hexaploid triticale accessions representing a global gene pool, including South African germplasm, with 43 wheat and 14 rye SSR markers. They found that 55% wheat and 53% rye markers were transferable to triticale, and showed an average genetic diversity value of 0.54 among tested accessions

In a study by Costa *et al*. (2007) a set of 42 wheat SSR markers were used to study the genetic diversity of 54 triticale entries (Costa *et al*., 2007). They established that out of the 42 marker sets tested, 71.42% of them were transferable to triticale, and 1% of them showed polymorphic information content (PIC) of 0.88. An average polymorphic content of 0.36 was achieved in this study, indicating low levels of genetic variability among entries tested. Additionally, Vyhnanek *et al.* (2009) tested 27 wheat and 21 rye SSR markers to study the genetic diversity of 16 triticale genotypes. They observed an average PIC of 0.48, indicating even lower levels of genetic variability between the tested genotypes.

One of the draw backs when employing SSR markers is the high cost involved in the development, hence making this technique difficult to apply to unstudied crops (Mondini *et al*., 2009; Kumar *et al*., 2009). SSR markers are also difficult to resolve on agarose gels, and require costly high resolution gels such as polyacrylamide or an automated DNA sequencer (Gupta and Varshney, 2000). The presence of null alleles further complicates the analysis of SSR data due to scoring errors (Varshney *et al*., 2005). Moreover, Kumar *et al.* (2009) and Hanlon *et al.* (1999) stated that the occurrence of homoplasy resulting from complex mutations at microsatellite loci than could lead to the underestimation of genetic diversity in certain populations. Another problem with SSR markers is replication slippage occurring due to mismatch pairing. Slippage during PCR amplification leads to the production of stutter bands, especially when using polyacrylamide gels, complicating band scoring (Park *et al*., 2009).

Chapter 3: Material and Methods

3.1 Determining genetic diversity of triticale cultivars using simple sequence repeat (SSRs) markers and phenotypic characteristics

The first aim of the project was to analyze the genetic diversity of triticale germplasm sourced via the United State Department of Agriculture (USDA). In order to complete this project aim, SSRs markers and phenotypic characteristics were used. The intended aim was separated into four objectives. Firstly, germplasm were identified and sourced. Secondly, the germplasm were phenotypically characterized in the field by assessing different agronomic traits useful for breeding. Thirdly, rust resistance was assessed. Fourthly, SSRs markers were used in typing the genetic diversity of the germplasm.

3.1.1 Phenotypic characterization

Hundred and one triticale cultivars were evaluated phenotypically by means of field planting. Two of the best performing local cultivars were included as checks.

3.1.1.1 Plant Material

Triticale cultivars were sourced from the USDA seed bank facility during 2009 and it represented material from 13 different countries (see Addendum A for detailed list). The number of different lines representing 13 countries included: 6 (6%) South African, 2 (2%) Germany, 4 (4%) Hungary, 15 (14%) Russia, 10 (9%) Ukraine, 12 (11%) Mexico, 7 (7%) Australia, 12 (11%) United State of America, 10 (9%) Canada, 2 (2%) Brazil, 2 (2%) China, 9 (8%) Bulgaria and 10 (9%) Poland (Table 2). The germplasm were planted at Mariendahl experimental farm, near Elsenburg, during June 2010. ‘AgBeacon’ and ‘US2009’ were included as local checks. Cultivars were machine planted as 3 m rows at approximately 15 cm within the row and 2 m between rows, with a Kincad (Kincad Equipment Manufacturing, Austria) sinlge row planter. There was one row per cultivar, 103 in all. Trial was planted in a check-plot design. The trial was fertilized with 40 kg N, 10 kg P, 10 kg K (4:1:1) (YARA, South Africa) during planting. This was followed by additional fertilization with 40 kg N, 6 kg P, and 18 kg K (Turbo 31) (YARA) at 40 days after planting. A mixture of herbicides consisting of Hussar at 200g/ha, MCPA at 500ml/ha, Buctrill at 375ml/ha and Ballista at 500ml/ha

(Bayer, South Africa) were also applied 40 days after planting for weed control. In addition, insecticides Mospilan at 50g/ha (Plaaskem, South Africa) was also applied 40 days after planting for protection against insects.

Field evaluation was done on a continuous basis and notes were recorded on whole row phenotyping based on growth habitat (winter or spring), plant height (cm), days to heading, grain yield (row/g) and 1000- kernel weight (g) during August 2010 to December 2010. The trial was harvested as single complete rows by hand during December 2010.

3.1.1.23.1.1.2 Phenotypic data Analysis

Days to heading were categorized into four levels as short (0 – 80 days), medium (80 – 100 days), long (100 – 120 days) and above (>120 days). Plant height as dwarf (0 – 80 cm), semi-dwarf (80 – 100 cm), normal (100 –120 cm) and above (>120 cm). Grain yield (g/row) as low (<200 g), average (200-250 g) and above (>250 g). And 1000 kernel weight (g) as low (0 – 35 g), average (35 – 40 g) and high (>40 g). All categories were chosen according the standard practices in the breeding program. The collected phenotypic data was analyzed using Microsoft Excel 2010 (Microsoft, USA) and subjected to least significant difference test at the 5% level.

3.1.2 Screening for stem and leaf rust resistance

Harvested triticale cultivars from the field trial conducted above for the phenotypic characterization were planted for rust resistance seedling screening. Due to poor seed set and germination of the phenotypically tested germplasm, some of the lines were excluded for rust resistance seedling screening. Two rust isolates harvested during the 2008 season at Welgevallen experimental farm from triticale plants were used for this purpose.

3.1.2.1 Plant Materials

A total of 96 seedlings (see Addendum E) consisting of triticale cultivars and the two local checks, ‘AgBeacon’ and ‘US2009’, were tested for stem and leaf rust resistance during March 2011 to April 2011. ‘Morocco’, highly susceptible wheat, was selected as control. Twenty-five to thirty seeds per genotype were planted in a 350 ml black plastic pot with peat. Each genotype per pot was marked with a white plastic tag and thinned back to fifteen plantlets six to eight days after emergence prior to inoculation.

Table 2: The number and percent of triticale germplasm from different countries used in the study.

Germplasm	Country												
	Australia	Brazil	China	Poland	Mexico	Canada	United States	Ukraine	Germany	Hungary	Bulgaria	South Africa	Total
Number	7	2	2	10	12	10	12	10	2	4	9	6	101
%	7	2	2	9	11	9	11	9	2	4	8	6	100

3.1.2.2 Inoculation

The stem rust isolate was suspended in a 0.700 ml of Soltrol (Chevron Phillips Chemical, USA) and sprayed onto seedlings using pressured air inside an open inoculation chamber while the pot was rotating (Figure 1). Inoculated seedlings were placed inside a tray and covered with black plastic to keep the humidity high at 25 °C for 24 hours inside a growth chamber. The black plastic bag was removed and the pots were placed in a growth chamber under artificial lighting with a constant temperature of 25 °C with 12 h day and 12 h night until disease symptoms developed. Pots were watered once a day with normal tap water. Similarly a leaf rust isolate was prepared in the same manner as the stem rust for inoculation of seedlings with a constant temperature of 18 °C with 12 h day and 12 h night until disease symptoms developed. Seedling infection typing was evaluated using the infection scale described by McIntosh *et al*. (1995) (Table 3).

Figure 1: Showing seedling inoculation chamber.

Table 3: Classification of leaf and stem rust seedlings infection types (McIntosh *et al.,* 1995).

Infection type	Host response	Symptoms
0	Immune	No visible uredia
;	Very resistant	Hypersensitive flecks
1	Resistant	Small uredia with necrosis
2	Resistant to moderately resistant	Small to medium sized uredia with green islands and surrounded by necrosis
3	Moderately resistant/moderately susceptible	Medium sized uredia with or without chlorosis
4	Susceptible	Large uredia without chlorosis
X	Resistant	Heterogeneous, similarly distributed over the leaves

3.1.3 Molecular typing of triticale

The triticale entries were typed using molecular markers. In addition, local check cultivars were also included controls. DNA extraction was done using a hexadecetyltrimethylammonium bromide (CTAB) method. Simple sequence repeats (SSRs) were used to assess the genetic diversity of the triticale lines. PowerMarker (Liu and Muse, 2005) and TreeView 1.6.6 software (Page, 1996) were used to analyze the data.

3.1.3.1 Genomic DNA extraction and quantification

Two seeds from each tested line were grown in the greenhouse. Genomic DNA was extracted from one-week-old leaves using an adapted CTAB method from Doyle and Doyle (1990) from each individual seedling. The 0.1g leaf tissue was placed inside a 2 ml microcentrifuge tube containing 3 stainless steel bearings and was ground to a fine powder using a tissuelyser (Qiagen, South Africa). The ground leaf powder was suspended in an 800 μl extraction buffer containing 100 mM [tris (hydroxymethyl) aminomethane] (Tris-HCl) at pH 8.0 (Merck, South Africa), 20 mM ethylene-diaminetetraacetate (EDTA) (Merck, South Africa), 1.4 mM

sodium chloride (NaCl) (Merck, South Africa), 2% (w/v) CTAB (Merck, South Africa) and 0.2% (v/v) β-mercaptoethanol and was incubated inside a water bath at 60 °C for one hour. An 800 µl aliquot of chloroform: isoamylalcohol (24:1 (v/v)) (Merck, South Africa) was added prior to phase separation at 12000 rpm for 10 minutes. The DNA supernatant of approximately 650 µl was transferred to a new, clean 2.2 ml tube. An equal volume of 325 µl phenol and 325 µl chloroform-isoamylalcohol was centrifuged at 12000 rpm for 5 minutes. The obtained supernatant of approximately 650 µl containing DNA was transferred to a new 2.2 ml tube containing 650 µl of chloroform-isoamylalcohol and centrifuged at 12000 rpm for 5 minutes. The obtained supernatant of approximately 650 µl was mixed with 650µ l isopropanol (Merck, South Africa) in a new 2.2 ml tube and incubated overnight inside a refrigerator at -20˚C.

The pellet was collected by centrifugation at 12000 rpm for 10 minutes at 4 °C, washed at room temperature with 1000 µl ice-cold 70% (v/v) ethanol. The pellet was collected by centrifugation at 12000 rpm for 10 minutes and air-dried for one hour at room temperature. The pellet was then re-suspended in a 50 µl TE buffer containing 40 µg/ml RNase at 37 °C for one hour, followed by precipitation with 5ul sodium acetate (pH 5.0) and 110 µl of 100% cold-ethanol.

Thereafter, the DNA was collected by centrifugation at 12000 rpm for 10 minutes at 4 °C and washed twice with ice-cold ethanol (70% v/v). The pellet was air-dried at room temperature for half an hour (1h) and re-suspended in 20-40 µl of distilled water. The DNA was stored at -20°C. The quality and quantity of the DNA was determined with a NanoDrop ® ND-1000 spectrophotometer (Thermo Scientific, South Africa) as per the manufacturer's instructions. The concentration of DNA of the different extractions were diluted and adjusted to a final concentration of 100 ng/µl for use in the PCR reaction for the SSR markers analysis.

3.1.3.2 SSRs markers

Six different polymorphic SSRs markers (either optimized or developed at the SU-PBL as part of a MSc study – unpublished data) targeting the B and R genome of rye and wheat including *Xgwm550-4B, Scm2-6RL*, *Scm152-5R*, *Scm159-4R*, *Scm109-5R* and *Scm 43-2R* were used (Addendum B). The PCR reaction conditions for the SSR markers were the same except

the annealing temperature, which varied between each individual marker (shown in Addendum B).

3.1.3.3 Polymerase chain reaction (PCR) program for SSR markers analysis

PCR reactions for all markers were performed using an Applied Biosystem 2720 Thermal cycler (Applied Biosystems, United State of America). PCR conditions used for each SSR marker amplification were as follows: 1x Bioline PCR reaction buffer, 0.2 mM dNTPs, 1.5 mM MgCl2, 0.5 µM forward primer, 0.5 µM reverse primer, 300 ng/µl DNA template and 1U Taq DNA polymerase. PCR amplification was performed with the initial denaturation at 94 °C for 3 minutes, followed by 45 cycles of 1 minute at 94 ˚C, 1 minute at each different annealing temperature, 2 minutes at 72 °C and final elongation at 72 ˚C for 10 minutes. The PCR product was then stored at -20 °C.

3.1.3.4 Polyacrylamide gel electrophoresis

A 40% acrylamide stock solution was made consisting of 76 g acrylamide, 4 g bis-acrylamide, and adjusted with distilled water to the final volume of 200 ml. The stock was then stored in a 200 ml flask covered with aluminium foil at 4ºC inside a refrigerator. A 6% sequencing gel mix was prepared from the 40% stock solution, consisting of 37.5 ml 40% stock solution, 90.09 g urea, 50 ml 5X TBE and adjusted with the distilled water to the final volume of 250 ml. The stock was stored inside a refrigerator at 4ºC, covered with aluminium foil. Dissolving 0.10 g ammoniumpersulphate with 10 ml distilled water made a 10% ammoniumpersulphate stock solution.

Plate glue stock was prepared by diluting 125 μl plate glue into 250 ml 100% ethanol. The stock was further diluted to a 1:3 ratio by adding 500 μl stock into 1500 μl 100% ethanol. Diluted plate glue stock of 1740 μl was added to 140 μl acetic acid. A long plate was prepared by wiping it with 100% ethanol, followed by distilled water until it dried out. The dried plate was then wiped with C-thru(Wynn Oil, South Africa) and left to dry for approximately 3 minutes. After the plate dried out, it was wiped down with a paper towel. The short plate was prepared by wiping it with 100% ethanol, followed by distilled water and left to dry.

The gel was prepared by adding 800 μl of ammoniumpersulphate (10% w/v) and 160 μl N, N, N', N'-Tetramethylendiamine (TEMED) to 160 ml of 6% denaturing poly-acrylamide gel. The gel was pre-run at a constant power of 700 V for 30 minutes using a Whatman Biometra Life Technologies Gibco-BRL PS 9009TC (Life Technologies, South Africa).

Each PCR product sample was mixed with an equal volume of loading buffer (98% formamide, 10mM EDTA pH 8.0, 0.05% w/v bromo phenol blue, 0.05% w/v xylene cyanol FF) and denatured at 95^{o}C inside a water bath for 5 minutes. Subsequently, the PCR sample mixtures were immediately quenched on ice before being loaded on a 6% denaturing poly-acrylamide gel. Thirteen μl of PCR sample together with 10 μl of molecular size markers were loaded on the gel and run at a constant power of 65 W for 180 minutes.

3.1.3.5 Silver Staining

The plates were separated after electrophoresis; the plate containing the gel was transferred to a plastic container filled with fixing solution (10% v/v ethanol and 0.5 % v/v acetic acid) and shaken for 20 minutes at room temperature. The fixing step was then followed by rinsing the gel plate twice with distilled water for 5 minutes. The gel plate, free of fixing solution, was placed inside the staining solution (0.1 % w/v silver nitrate) and shaken for a further 20 minutes with a shaker. The gel plate was then rinsed free of staining solution with distilled water for at least 10 seconds. This was followed by placing the gel plate inside the developing solution (1.5% w/v sodium hydroxide and 0.405% v/v formaldehyde) and shaking it until the bands appeared. The gel was left to dry after the staining processes and photographed immediately, using a digital camera. The gel plate was then sealed with a clean plastic bag to prevent it from drying out before the band sizes could be scored.

3.1.3.6 SSR marker data analysis

SSR bands sizes were scored manually and each band obtained was then entered into a scoring matrix using Microsoft Excel. The data was analyzed with PowerMarker software v3.25 (Liu and Muse, 2005) (see Addendum C), which was chosen for its ability to analyze the genetic diversity, the average of polymorphic information content for each locus, and the number of alleles per locus (Akhavan *et al*., 2009). The genetic distance was calculated using the CS Cord (1967) distance method (Cavalli-Sforza and Edwards, 1967) as it has been shown that this model can produce a correct topology regardless of the microsatellite model

(Takezani and Nei, 1996). A Phylogenetic tree was constructed based on bootstrap similarity values and the neighbour-joining cluster method found in PowerMarker software v3.25 as described by Akhavan *et al*. (2009) and Le Maitre (2010). Finally the phylogenetic tree was visualized using TreeView v1.6.6 (Page, 1996).

3.2 Introgression of anthocyanin genes for the phenotypic identification of triticale germplasm

The second aim of the study was to introgress anthocyanin genes into triticale as a phenotypic marker for the phenotypic identification of germplasm. In order to complete the project aim, two wheat sources carrying anthocyanin genes were used as the donor parents for anthocyanin genes into triticale. The intended study was separated into three objectives. Firstly, identifying phenotypic markers that are highly heritable, stable and user friendly for the phenotypic identification of triticale cultivars. Secondly, introgressing identified phenotypic markers into the triticale cultivars by means of backcrossing. Thirdly, applying molecular markers to validate introgressed lines.

3.2.1 Introgression of anthocyanin genes

Anthocyanin genes from donor parents were introgressed into selected triticale lines by means of reciprocal crosses followed by backcross breeding.

3.2.1.1 Plant material

Five triticale cultivars namely: ‘Bacchus’, ‘Tobie’, ‘US2007’, ‘Agbeacon’ and ‘US2009’ were used as recurrent parents. And two bread wheat cultivars, ‘Amethyst’ (*Pp1*, *Pp3*) and ‘Cltr1202STR’ (*Ba*) were used as donor parents carrying the anthocyanin genes responsible for grain pigmentations. Amethyst is a wheat cultivar sourced from New Zealand carrying a *Pp1* and *Pp3* genes giving the pericarp a purple pigmentation. ‘Cltr1202STR’ was sourced from the USDA, and carried the *Ba* gene giving the aleurone layer a blue pigmentation. Wheat cultivar, ‘SST88’, was used as a wheat control (Sensako, South Africa). Lines were continuously planted, from January 2009 to December 2010, in 2000 ml moistened pots containing coarse river sand in the green-house with natural day length and temperature not rising above 25°C. Planting was done twice a week and germinated plantlets inside the pots

were irrigated twice a day with a nutrient solution containing 164 g Sol-u-fert (Kynoch Fertilizers (Pty) Ltd, Milnerton, South Africa), 2 g Microplex (Ocean Agriculture (Pty) Ltd, Muldersdrift, South Africa) en 77 ml potassium nitrate in 100 liters of water (Botes, 2001). Matured plants were treated with insecticides DuettTM (BASF, South Africa) and Mospilan 20 SP (Plaaskem, South Africa) when needed.

3.2.1.2 Emasculation and pollination procedure of plant materials

The process of emasculation was done, one to two days before flowering. Selected spikes were emasculated by removing the upper and lower spikelets from the spike with forceps. The three anthers from the remaining selected spikes were carefully removed with forceps to ensure that stigmas were not damaged. Emasculated plants were then covered with a crossing bag and secured with a paper clip, thus preventing cross pollination, and the dates of emasculation were recorded on the bag. This was done twice a day, early in the morning and late afternoon when the temperature was cooler. This process was undertaken continuously from the second week of March 2009 to December 2010.

After two to three days emasculated stigmas were carefully checked for maturity. During pollination the upper portion of the glume of the emasculated spike was removed with a pair of scissors, and fresh pollen was then brushed onto the stigma of the emasculated floret within 5 to 10 minutes after the pollen was collected. This was executed in the early morning and late afternoon when the temperature was cooler and stigmas most receptive. Following pollination of the entire spike on each selected individual plant under pollination, the crossing bags were put back over the pollinated spikes, and the date and name of the cultivar/line used as pollen donor were recorded. Initially, 'Amethyst' (*Pp1*, *Pp3*) and 'Cltr1202STR' (*Ba*) were used as female parents and triticale lines as pollen donors. This was followed by the reciprocal cross whereby 'Amethyst' (*Pp1*, *Pp3*) and 'Cltr1202STR' (*Ba*) were used as a pollen donor parent and the triticale lines as a female parents. Successful seed set were followed by embryo rescue 14 to 18 days after pollination and gibberillic acid at maturity in the case of seeds

3.2.1.3 Growing F_1 plants

Working in a laminar flow hood harvested F1 seed obtained from the crosses made were surface sterilized for 30 seconds inside a McCartney bottle containing 70% ethanol (v/v). The

seeds were then transferred to a bottle containing 30% sodium hypochlorite (v/v) for about 8 minutes and rinsed in autoclaved distilled water for 2 minutes. Seeds were then placed inside sterile Petri dishes of which the tops were covered with dry filter paper and the bottoms with filter papers moistened with gibberillic acid (GA_3) at a concentration of 1000 ppm. Next, the seeds were incubated for 3-5 days inside a growth chamber with a temperature of 25ºC to stimulate germination. Germinated seeds were allowed to develop roots, and 3 to 4 days after coleoptiles emerged were trans-planted into 2000 ml moistened pots containing coarse river sand inside a greenhouse under the same regime as described previously.

3.2.1.4 Embryo culture

The immature F_1 seeds observed from the initial crosses made were also removed from the floret after 14 to 18 days of pollination. Seeds were surfaced sterilized in a similar manner as described previously. Dissection of embryos was done using a Carl Zeiss stereo-microscope (South Africa) microscope under 10x magnification. The embryos were cultured inside 100 ml jars containing 25 ml of solid MS medium (Murashige and Skoog, 1962). Bottles containing embryos were then placed in the dark at 4°C for a week and then in a growth cabinet at 23-25°C with a 14 hour light / 10 hour dark cycle (Figure 2). Germinated plantlets were subsequently removed from the jars and transferred into the moistened 350 ml pots filled with peat. The pots were covered with transparent plastic. At the three-leaf stage plantlets were transplanted into 2000 ml pots containing coarse river sand inside the greenhouse under the same regime as described previously.

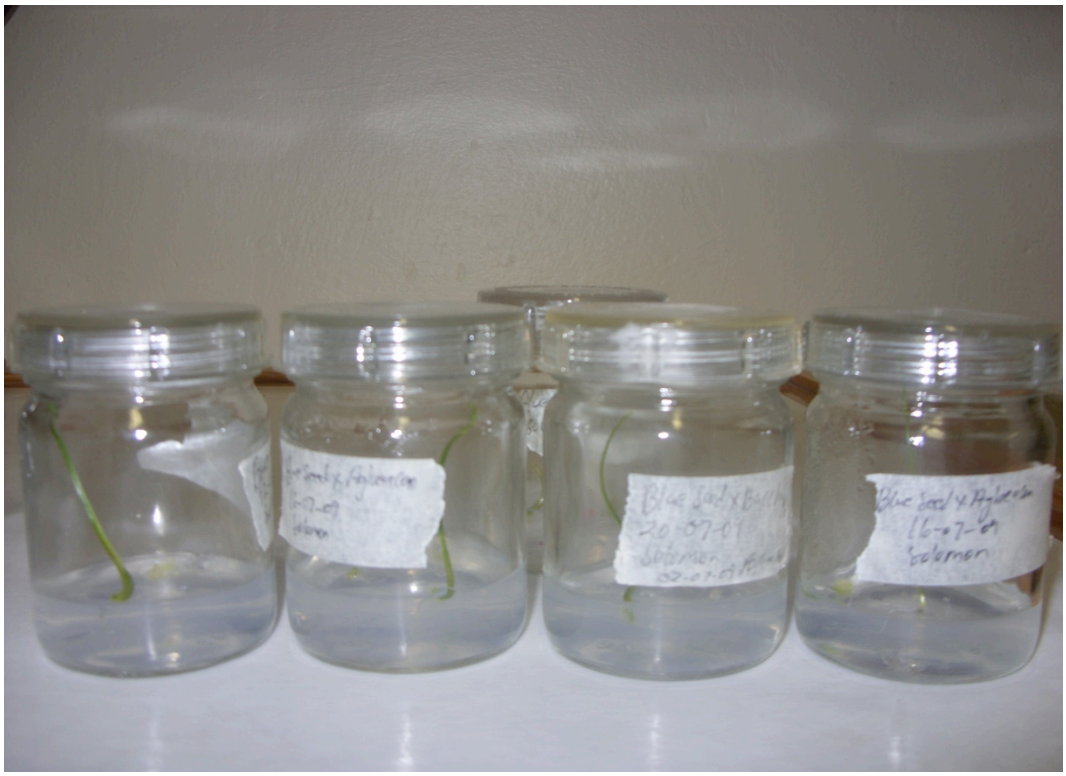

Figure 2: Germination of embryo rescue plantlets.

3.2.2 Transfer of anthocyanin genes through repeated backcrossing

Seed obtained from the plantlets grown out expressing the introgressed anthocyanin genes were selected, and planted in the greenhouse. Spikelets of these F_1-lines were emasculated and pollinated during August 2009 to March 2010. This procedure was executed manually in the greenhouse. Similarly, the same was done with the F_1-plants obtained between reciprocal crosses of 'Amethyst' (*Pp1*; *Pp3*) x 'SST88' and 'Cltr1202STR' (*Ba*) x 'SST88' crosses. Some of the obtained backcross one plants (BC_1F_1) were germinated 14-18 days after pollination using the embryo rescue technique while some of the viable BC_1F_1-plants expressing anthocyanin were germinated by directly planting them inside the pots with moistened coarse sand during August 2009 to February 2010. Selection and planting was based on expression of the anthocyanin genes until the BC_4F_1 generation (Figure 3).

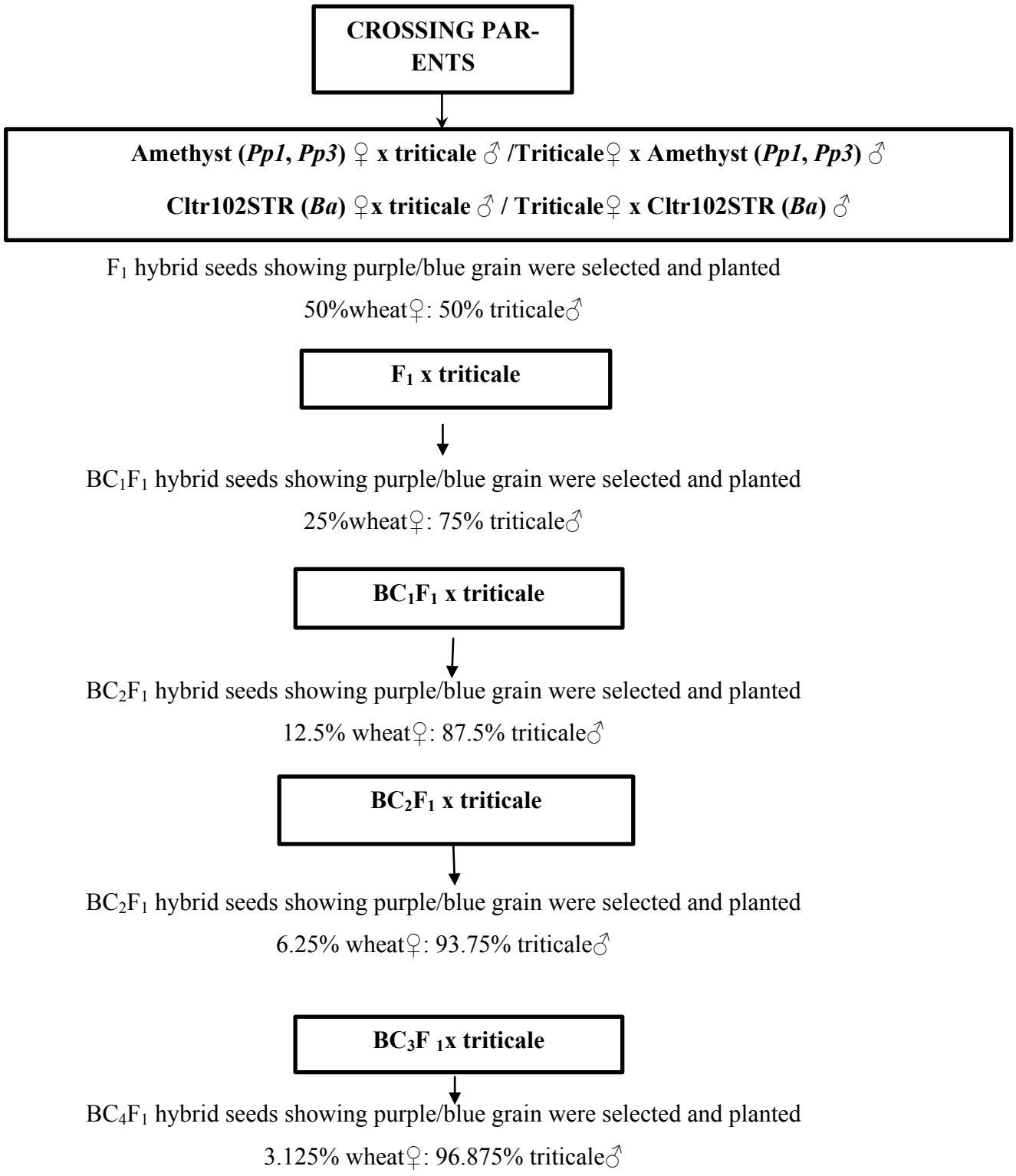

Figure 3: Schematic representation of the backcross breeding procedure used to introgress anthocyanin genes (*Pp1, Pp3* & *Ba*) from wheat sources into different triticale.

3.2.3 Molecular validation of introgressed lines

Marker assisted backcrossing was employed to type the introgressed lines genetically in order to identify lines that are the most similar to the recurrent parents. In order to complete this objective, SSRs markers analysis was done in the same manner as previously described.

3.2.3.1 Plant materials

The recurrent parents, 'US2007' and 'Agbeacon', 9 seeds of line 11T023 (*Ba*), and 6 seeds of line 11T028 (*Ba*) (see Addendum C for detailed list) were planted inside trays with peat in the greenhouse during March 2011. In addition, Cltr1202STR (*Ba*) donor parent was also planted as a control plant.

3.2.3.2 PCR amplification and SSR markers analysis of introgressed lines

DNA extraction and PCR amplification of introgressed was done in the same manner as described previously. Six SSRs markers (see list in Addendum A) were employed for the molecular validation of the introgressed lines. Polyacrylamide gel electrophoresis, silver staining and SSRs markers data analysis (see Addendum D) was done in the same manner as previously described.

Chapter 4: Results and Discussion

4.1 Determining genetic diversity of triticale cultivars using simple sequence repeat (SSRs) markers and phenotypic characteristics

4.1.1 Phenotypic characterization

Of the 101 triticale cultivars planted, 93 developed into fully matured plants. The phenotypic characteristics recorded during the field testing included plant height (cm), grain yield per 3m row (g) and thousand-kernel weight (g) (reported in Addendum D).

Entries were also classified as either spring or winter type. The results showed that spring triticale cultivars 'Currency', 'Samson', and 'Corong' originating from Australia and 'Puppy' originating from Mexico had the shortest days to heading with 92 days compared to local checks, 'AgBeacon' with 93 days and 'US2009' with 98 days respectively (Figure 4). Entry, 'AD19X01ID', originating from the Ukraine was significantly different from all the tested spring types with the longest days-to-heading of 161 days (Figure 4). The early days-to-heading showed by entries 'Currency', 'Samson' and 'Corong' compared to local checks suggest that these lines could be used as the potential crossing parents for incorporating the early heading period. Khan *et al*. (2011) stated that an earlier days-to-heading is an important trait in crops to avoid drought during late season in certain environments. As such one of the most successful local cultivar to date was the early maturing cultivar 'Tobie'. Winter type genotype showed that entry, 'OAC Wintri', originating from Canada, had the shortest days to heading with 92 days (Figure 5). The longest days to heading was observed with 'NAD 120', 'NAD 325', and 'PRAO 6/1' entries originating from Poland, and 'PRAG 41/1' originating from Russia with 162 days each. As expected the check cultivars were statistically different from all the tested entries with regard to days-to-heading.

Spring types entries, 'Currency', 'Toorf', 'Abacus' (all originating from Australia), 'Mexitol 1' (originating from Bulgaria), 'Koala' and 'Jabali' (both originating from Mexico) ranged in height between 80 - 85 cm compared to the local check cultivars ('AgBeacon' with 90 cm vs 'US2009' with 95 cm) (Figure 6). Both entries, 'Veselopodolyanskaya' originating from Russia and 'Marval' originating from the USA showed shorter plant height of 70 cm each

and the highest plant height was entry 'PRAG 31' originating from Russia with 140 cm (Figure 6). Winter type entry, 'Szalkas', originating from Hungary, was only 55 cm, and the longest plant height was observed with entry '25 AD 20', originating from Russia, with 115 cm (Figure 7). Many of the observed winter types were of similar height compared to the local check cultivars.

The results showed that spring type entry, 'AC Copia', originating from Canada had the highest grain yield of 278 g per row followed by entry, 'Joseph', originating from South Africa with a grain yield of 267 g per row. Both entries out-yielding both local check cultivars, 'AgBeacon' with 10 g/row and 'US2009' with 256.5 g/row (Figure 8). The check cultivar, 'US2009', showed a higher yield compared to all other tested entries except 'AC Copia' and 'Joseph'. Check cultivar, 'AgBeacon', showed poor yield comparable to all other tested spring type entries. The observed grain yield of winter types ranged from 0 g per row with entries, 'RAH101/3' originating from Poland, 'AD 307 XT021ID', 'AD 403' both originating from Ukraine and 'Breaker' originating from the USA to 137 g with entry 'OAC Wintri', originating from Canada (Figure 9). Compared to the check cultivars, 'AgBeacon' and 'US2009', all the winter type entries yielded poorly as to be expected under Western Cape conditions. The low grain yield of winter types was due to the failure of these genotypes to produce seeds before reaching maturity. Higher temperatures during the flowering stage of the field trial could also have contributed to the lower grain yield of winter types observed. Royo *et al*. (1995) reported similar results. They found that spikes of winter types produced few grains due to the longer flowering period, which reduced the flower fertility. Moreover, Santiveri *et al.* (2004) suggested that the lower grain yield of winter types could be due to the reduction of the assimilates (such as stored reserves) caused by an increase of temperature during the grain filling stage. Since grain yield is of primary interest for the selection of new crossing parents, entry 'AC Copia,' could be the best potential entry to be incorporated into the current breeding program for this purpose.

The TKW showed that spring type entry, 'Muir', originating from Australia had the highest yield of 47 g outperforming both local check cultivars, 'AgBeacon' with 36 g and 'US2009' with 40 g (Figure 10). Low TKW in the spring types was observed with entry, 'Toorf' originating from Australia with 17 g. The data revealed that winter type entry, 'SSKR 628', originating from South Africa had the highest TKW of 50 g out-yielding all the tested entries in-

cluding the check cultivars, ‘AgBeacon’ and ‘US2009’ (Figure 11). Except for ‘SSKR 628’ all the winter type entries yielded low TKW compared to the check cultivars. These findings were in agreement with the results reported by Santiveri *et al.* (2004). There results show that the tested spring triticale types have a generally higher seed weight than the tested winter types when planted in a spring type environment.

4.1.2 Rust screening

The different infection types resulting from the screening of the entries at seedling stage with the stem rust isolate was recorded in different categories as shown in the Addendum D. However, due to poor germination and low numbers of available seeds especially for winter types, some of these genotypes were excluded when rust screening was done. Only 69 entries were tested for rust resistance together with the two local check cultivars, ‘AgBeacon’ and ‘US2009’. The results indicated that for the stem rust isolate, 59 % of the entries showed very resistant to resistant responses, 23% showed resistant to moderately resistant responses, 15% showed moderately resistant to moderately susceptible, and 10% showed intermediate (mixture) infection types (Figure 12).

Seedling screening with the leaf rust isolate showed that the largest percentage (42%) fell into the moderately resistant/moderately susceptible category, 22% moderately resistant, 20% resistant, 15% intermediate, and 1% showed complete susceptibility (Figure 13).

The entries showed higher levels of seedling resistance towards the stem rust isolate compared to the leaf rust isolate. This was somewhat unexpected, but could perhaps be due to the emphasis on stem rust resistance breeding in most breeding programs when compared to leaf rust resistance breeding. Both check cultivars, ‘AgBeacon’ and ‘US2009’ showed complete resistance towards both isolates. This data suggest that resistant breeding of leaf rust is crucial for the future breeding efforts.

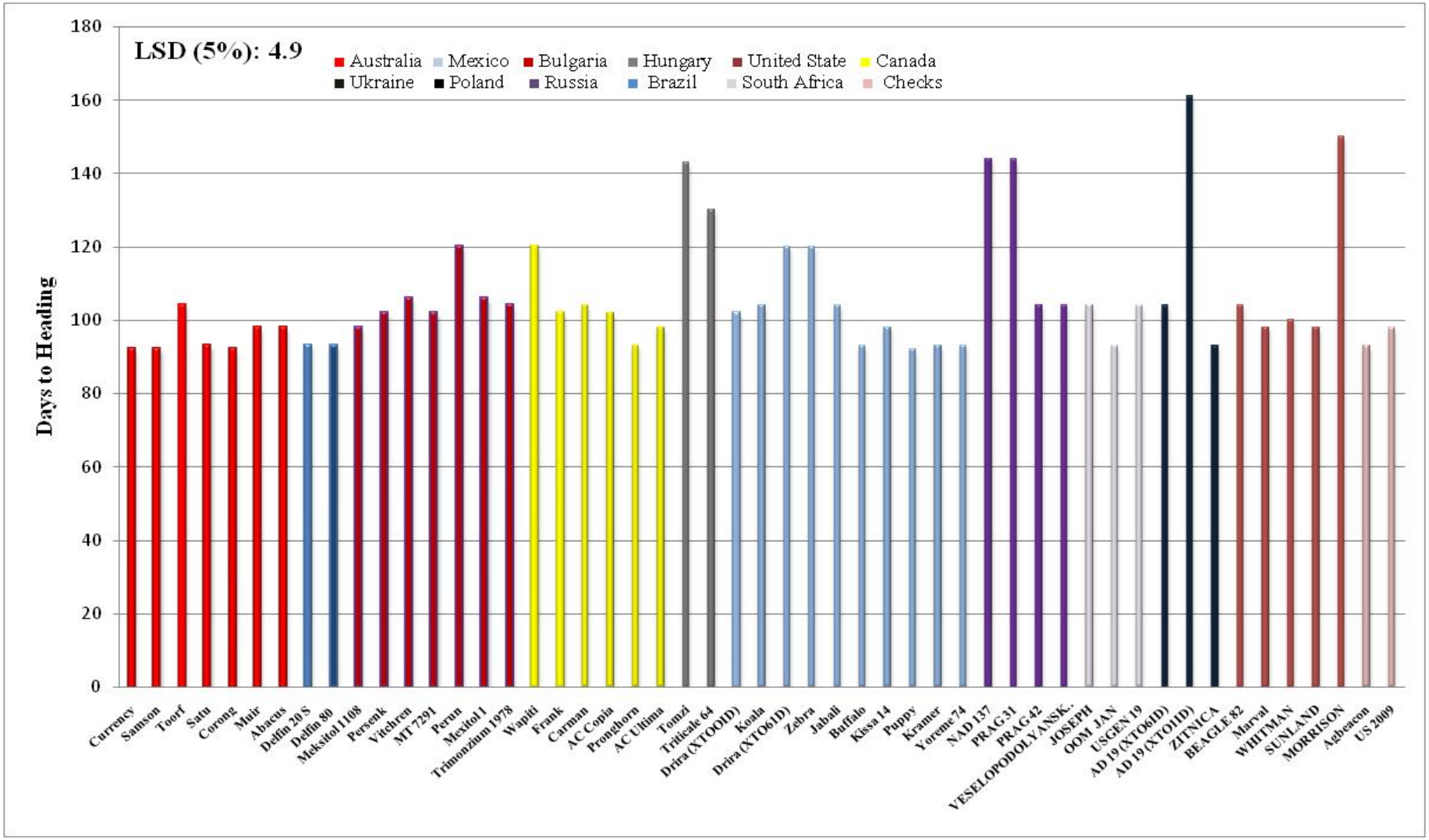

Figure 4: Graph showing the distribution of the days to heading for the spring triticale genotypes.

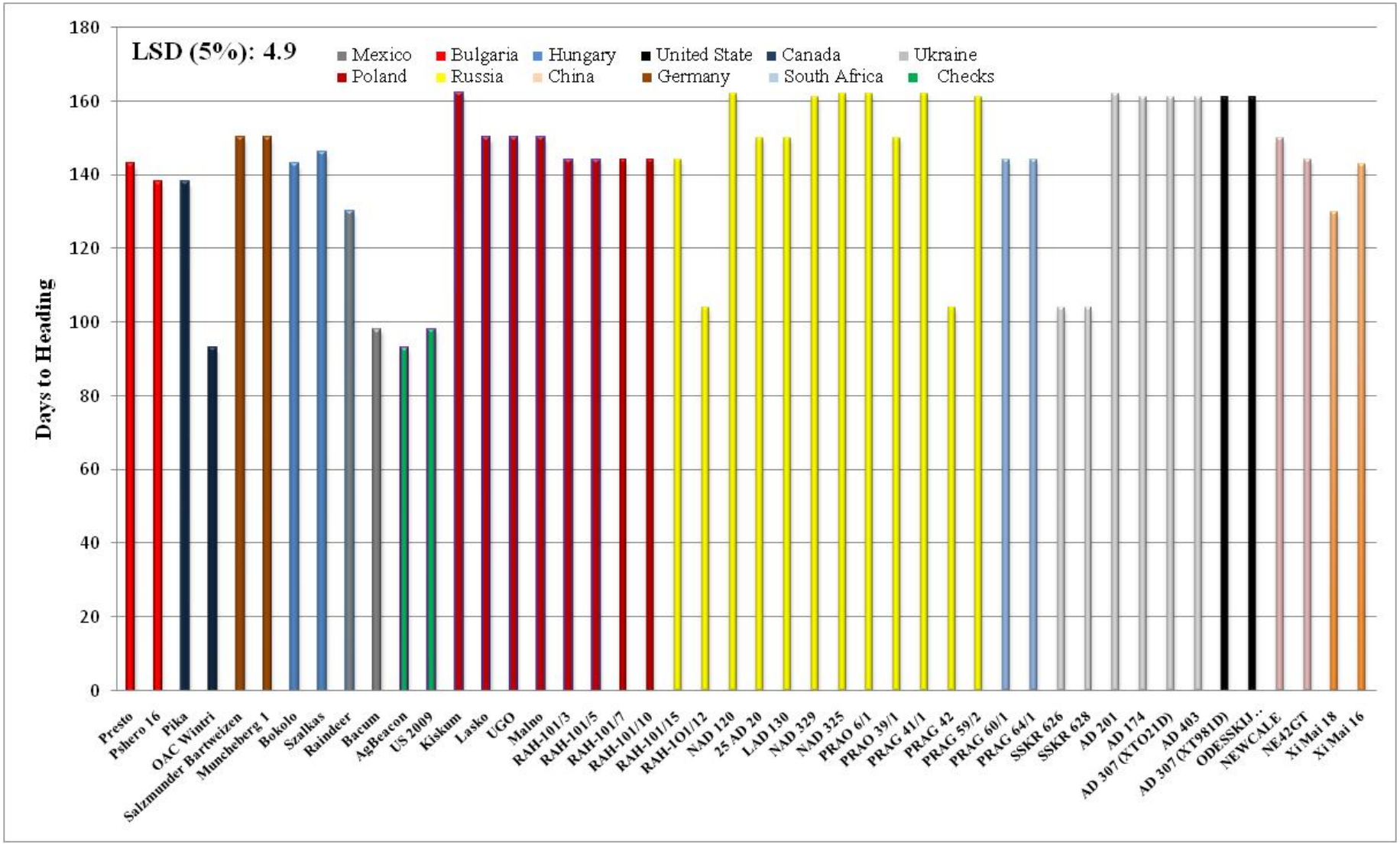

Figure 5: Graph showing the distribution of the days to heading for the winter triticale genotypes.

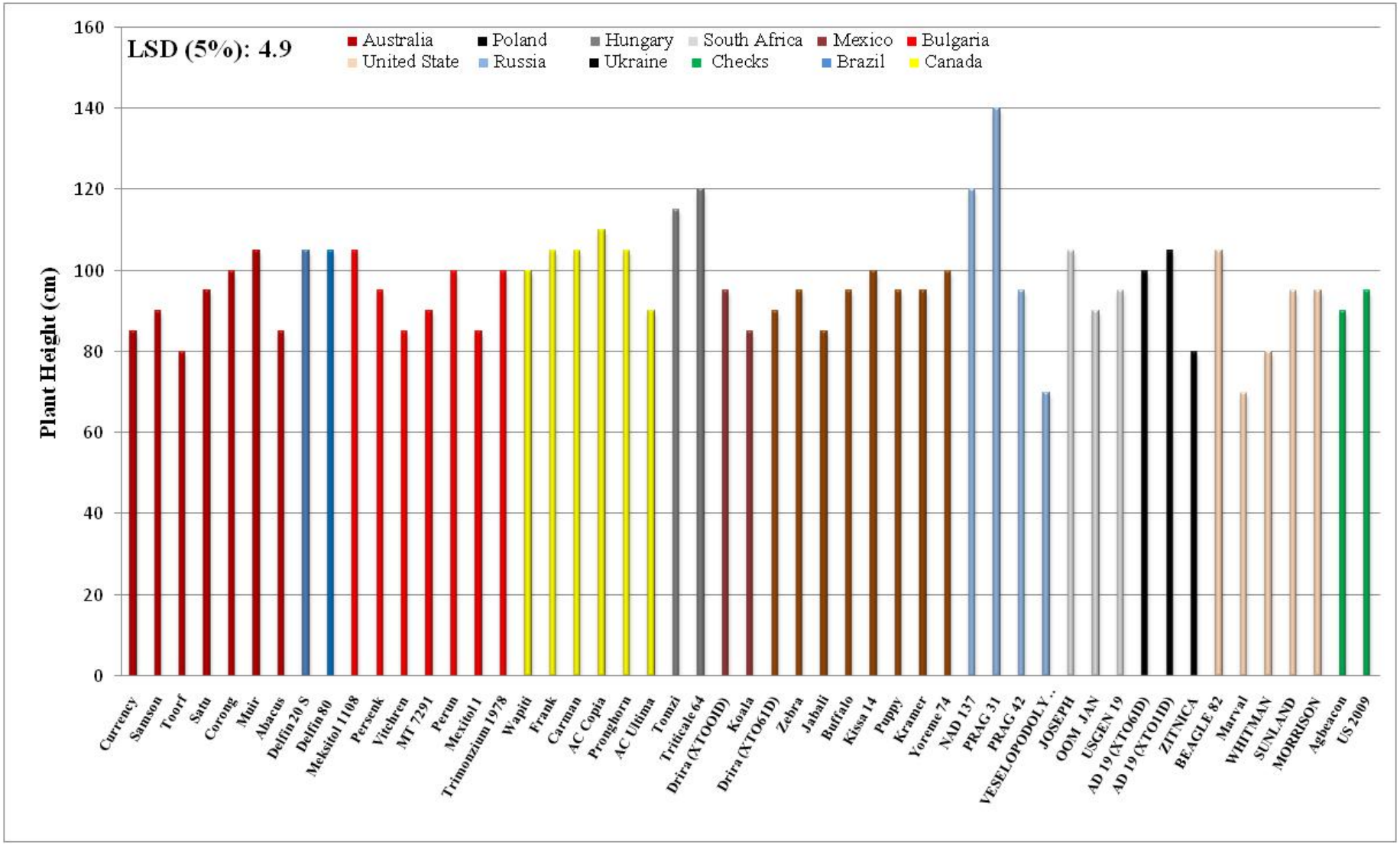

Figure 6: Graph showing the distribution of the plant height (cm) for the spring triticale genotypes.

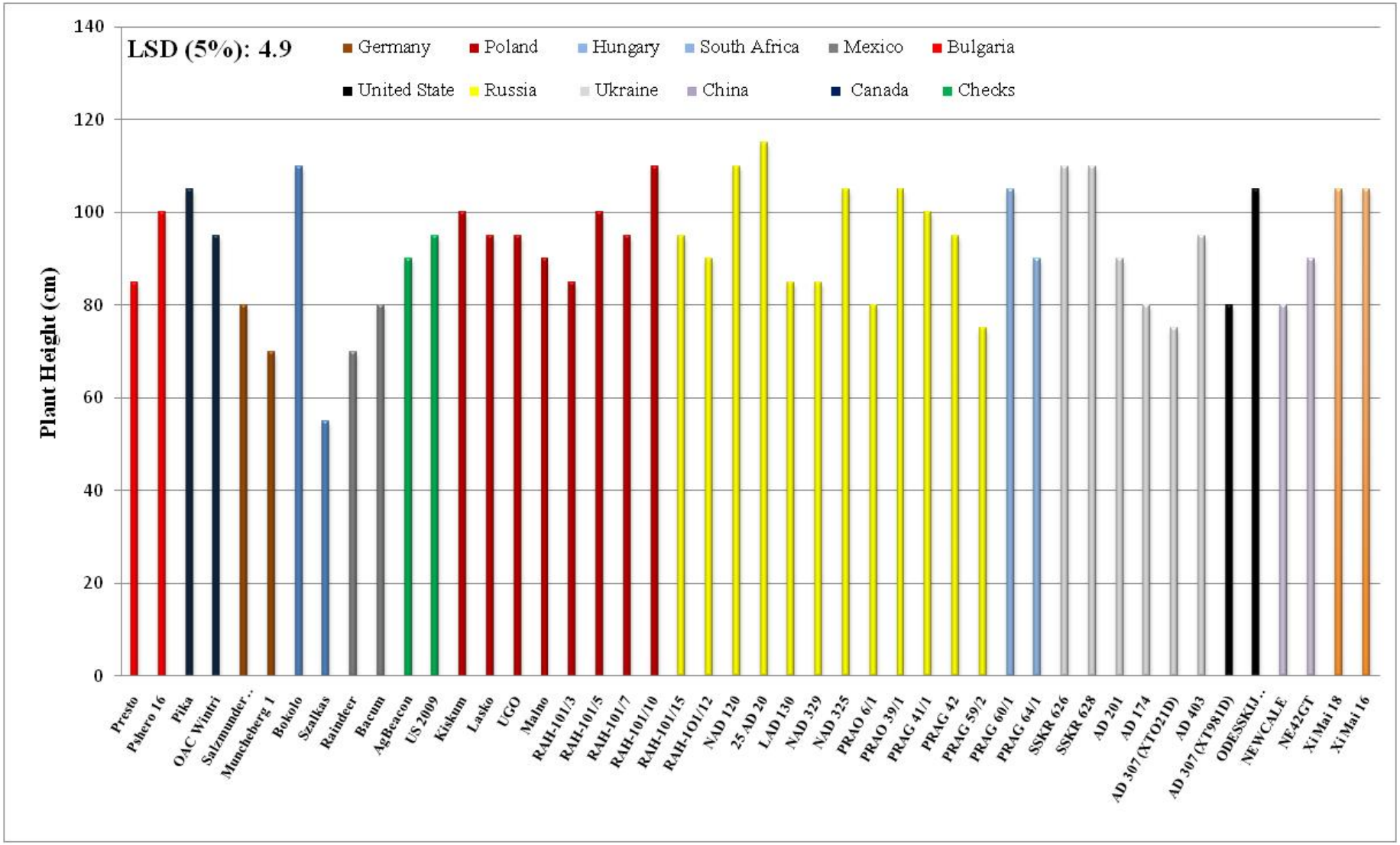

Figure 7: Graph showing the distribution of the plant height (cm) for the winter triticale genotypes.

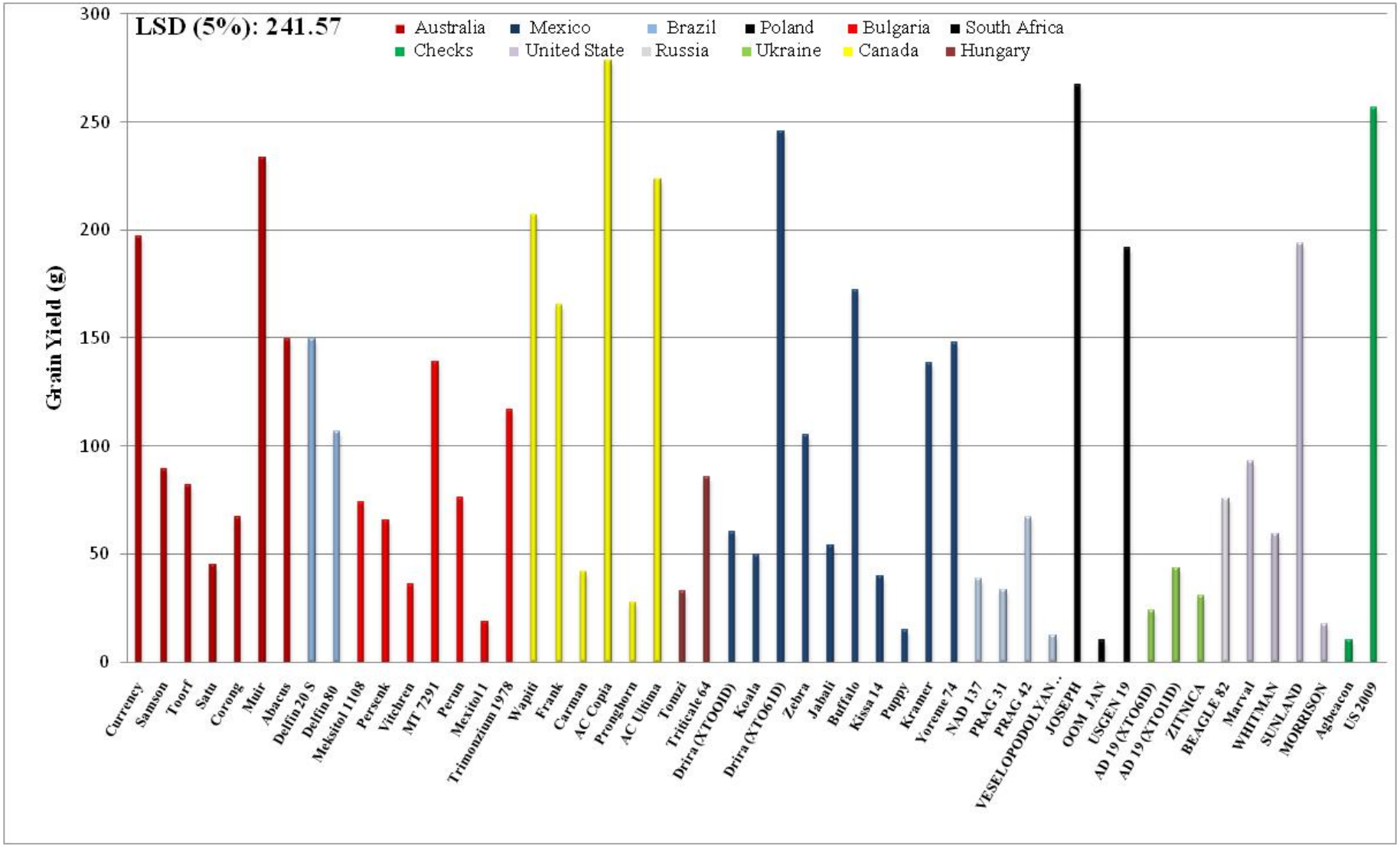

Figure 8: Graph showing the distribution of the grain yield (g/row) for the spring triticale genotypes.

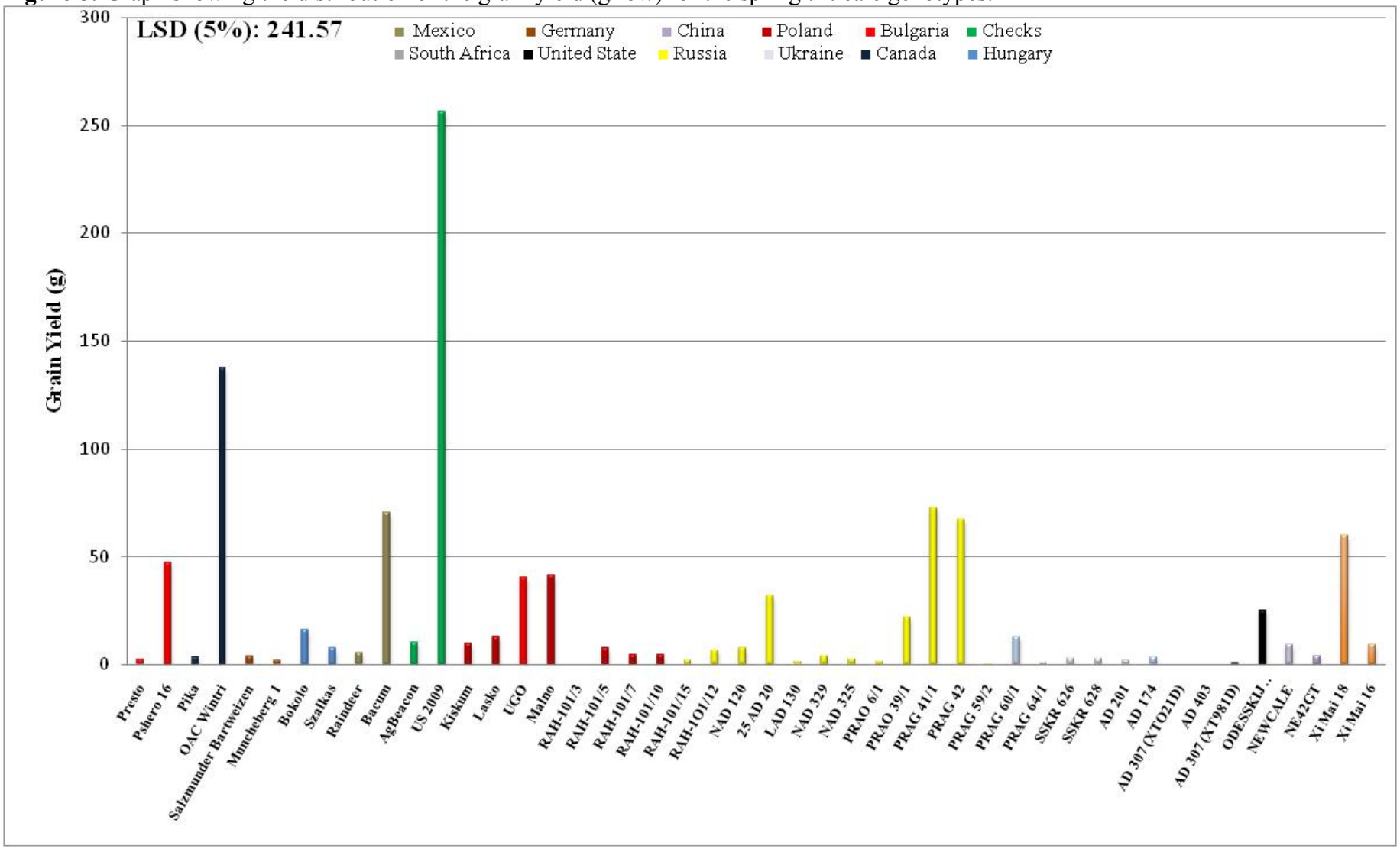

Figure 9: Graph showing the distribution of the grain yield (g/row) for the winter triticale genotypes.

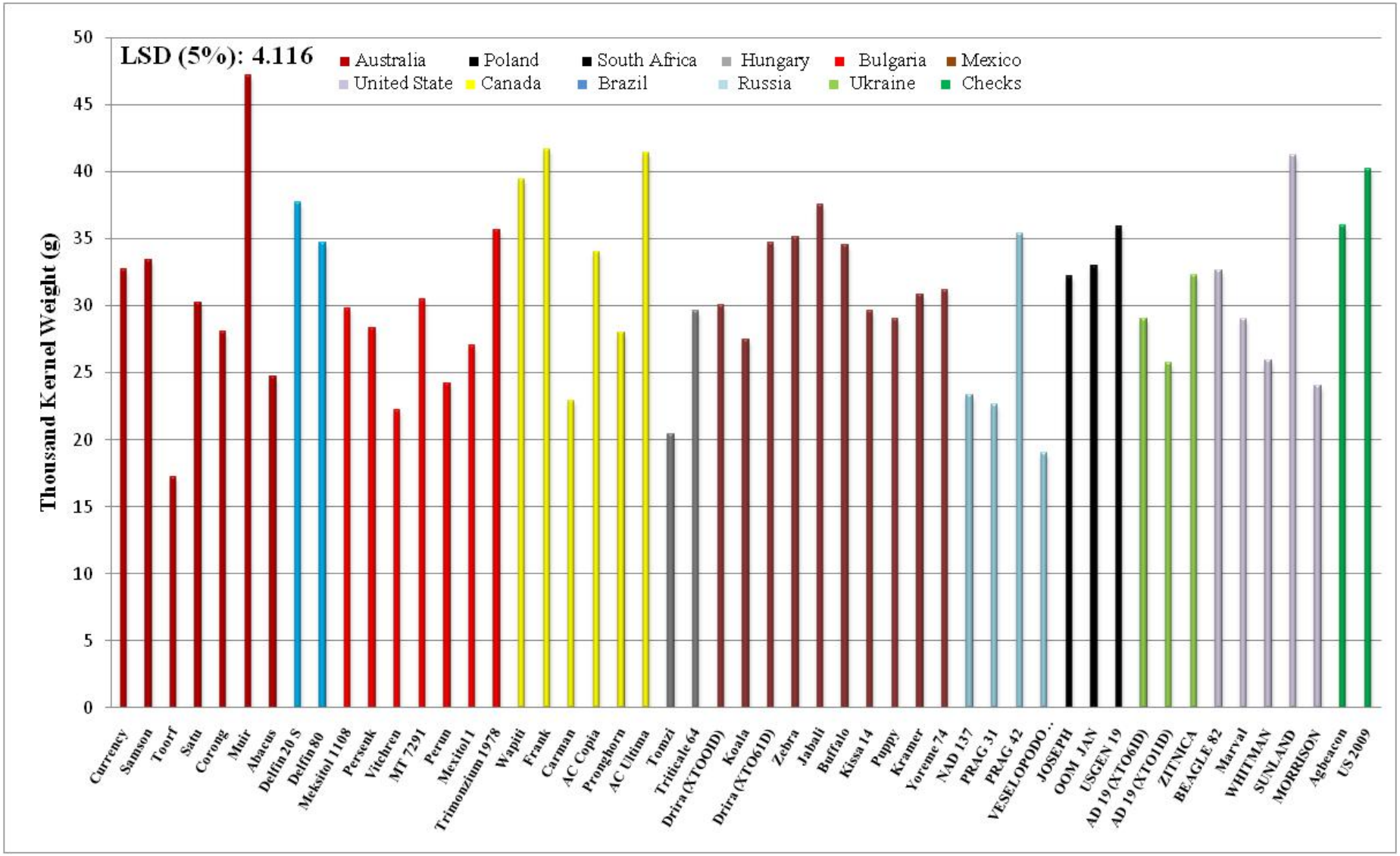

Figure 10: Graph shows the distribution of the thousand-kernel weight (g) for the spring triticale genotypes.

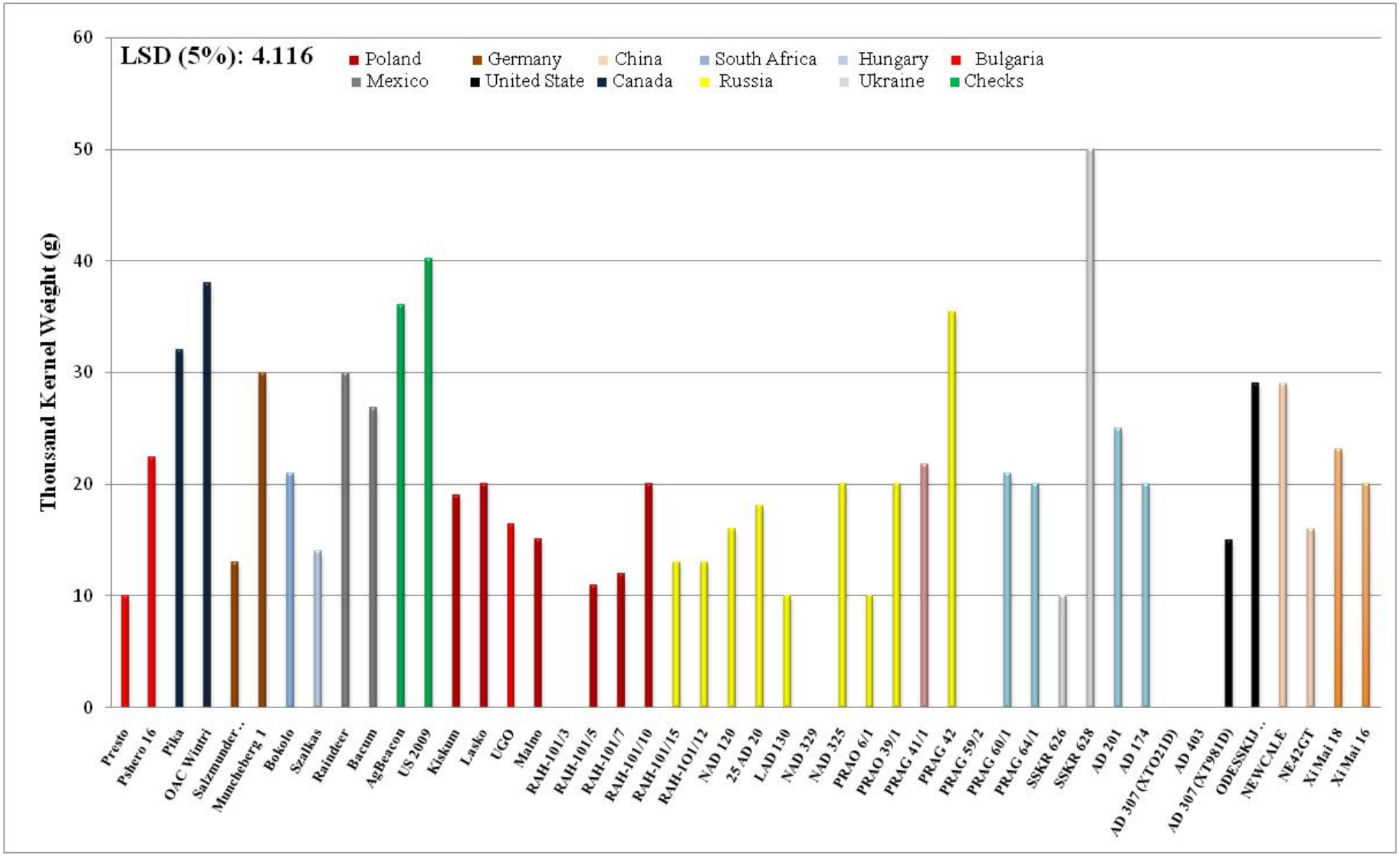

Figure 11: Graph showing the distribution of the thousand-kernel weight for the winter triticale genotype.

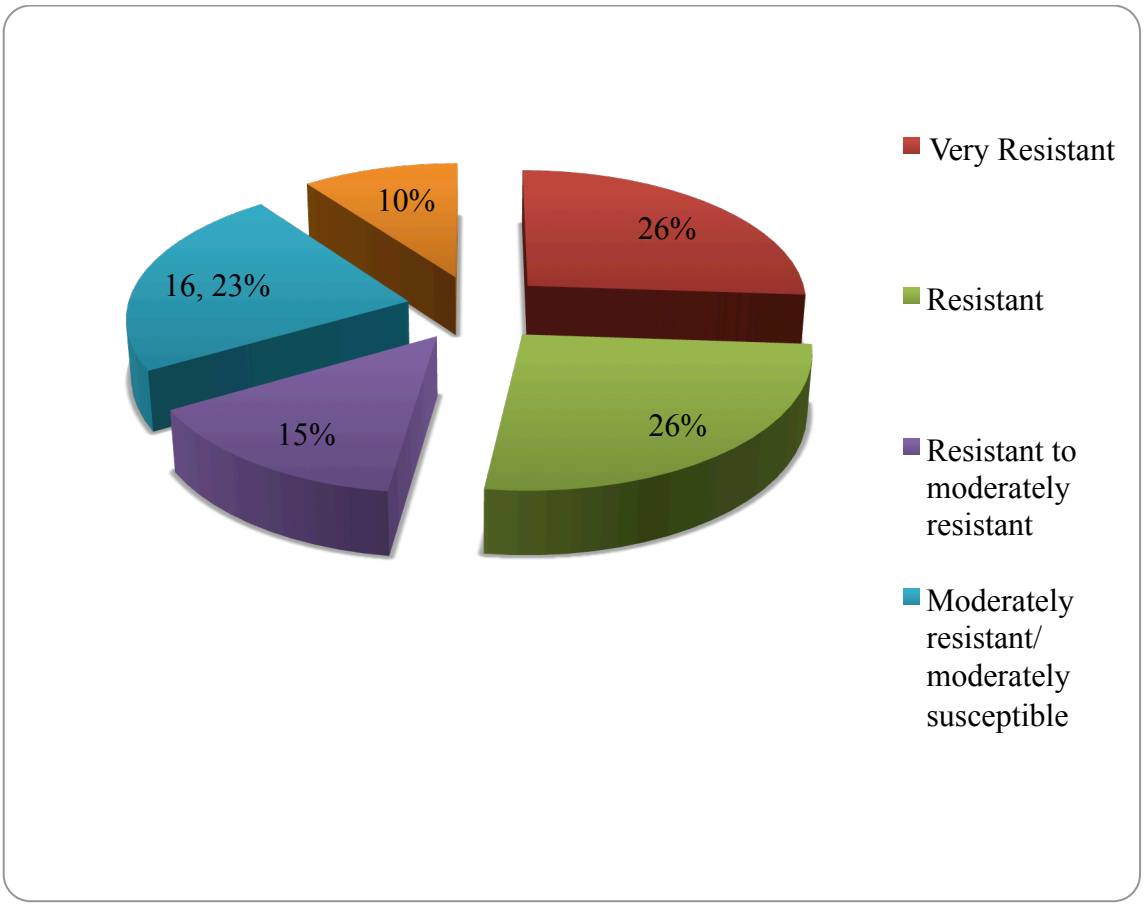

Figure 12: Pie chart showing the distribution of stem rust infection types.

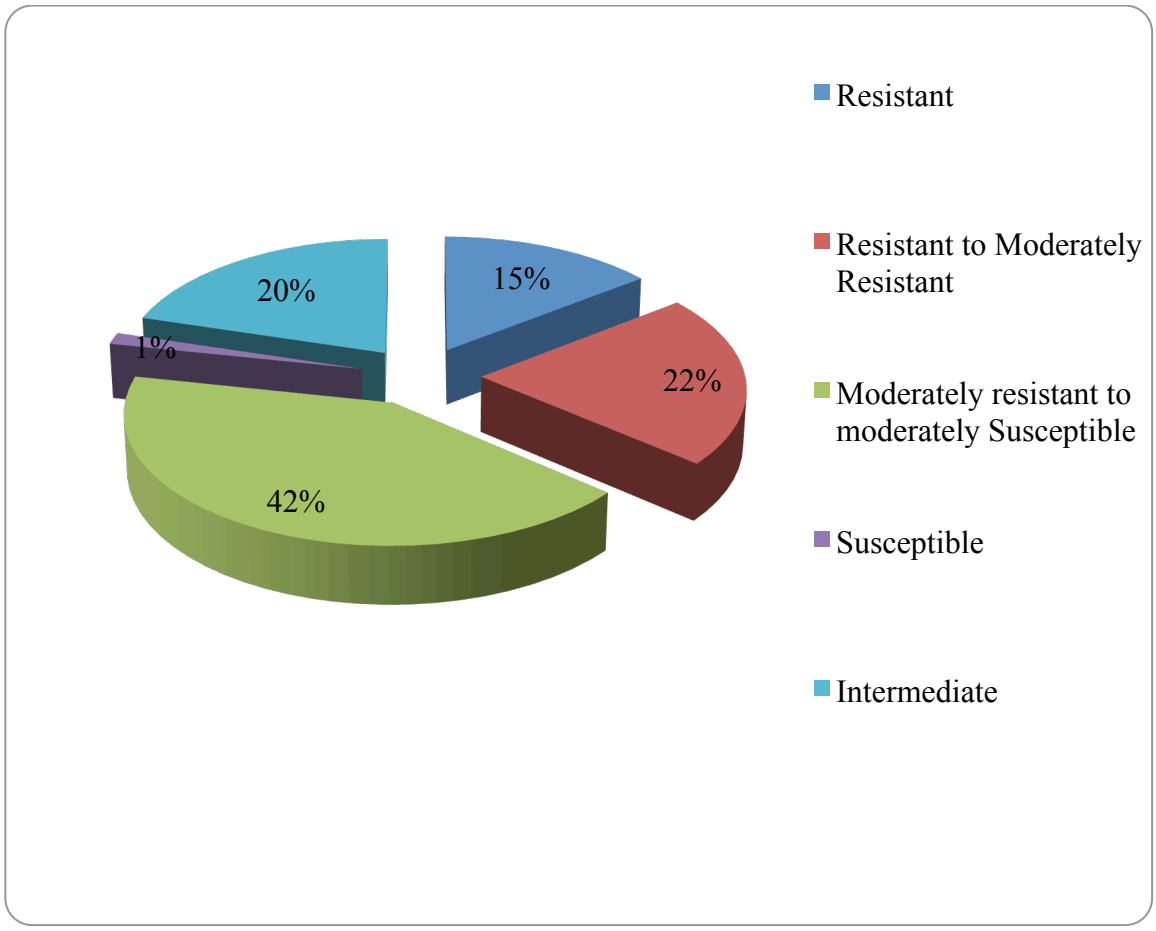

Figure 13: Pie chart showing the distribution of leaf rust infection types.

4.1.3 Molecular typing

4.1.3.1 DNA extractions

It was possible to isolate quantifiable genomic DNA from the leaf samples of all triticale cultivars using the CTAB extraction method. The DNA concentration observed ranged between 350 ng/µl to 2894 ng/µl. The quality of the extracted DNA samples was measured using A260/A280 and A260/A230 absorbance ratios with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The A260/A280 absorbance ratios observed ranged from 1.86 to 2.15 and A260/A230 absorbance ratios ranged between 2.17 and 2.47. These absorbance ratios indicated that the extracted genomic DNA was free of contaminants such as proteins, RNAs and other plant compound inhibitors, and therefore was of good quality.

4.1.3.2 Marker analysis

Eight SSR markers, five rye genome-specific and three wheat genome-specific; were used to investigate the cultivars obtained from the USDA germplasm bank (listed in Addendum A). Seven local cultivars were included as checks. Denaturing polyacrylamide gels were used for detection. The different allele sizes produced by each tested marker were scored (as listed in Addendum E) and analyzed with the PowerMarker software v3.25 (Liu and Muse, 2005) for genetic diversity assessment.

A total of 140 polymorphic alleles were produced from chromosomes *5R*, *5R*, *6R*, *2R*, *4R*, *1A*, *1B* and *4B* with an average number of 17.5 alleles per locus (Table 4). It was found that the lowest numbers of alleles were detected with markers *Scm109-5R* and *Xgwm513-4B* with 12 alleles each, and the highest with the *Scm159-4R* with 26 alleles. In a study conducted by Kuleung *et al.* (2006) a total of 241 polymorphic alleles with an average of 4.2 per locus were observed in 80 hexaploid triticale tested with 43 wheat and 14 rye SSR markers. In studies done by Vyhnanek et al. (2009), it was found that a total of 181 polymorphic alleles with an average of 3.83 alleles per locus were produced in 16 triticale genotypes tested with 27 wheat and 21 rye SSR markers. Sixty-four polymorphic alleles with an average of 2.13 alleles per locus were observed in 16 Brazilian triticale genotypes tested with 42 wheat SSR markers (Costa *et al.,* 2007). The low number of polymorphic alleles revealed by this study could suggest a low level of genetic diversity among Brazilian triticale genotypes. The high number of polymorphic alleles revealed by the current study compared to both previous studies could be due to the more diverse set of entries.

Table 4: Statistical summary of eight SSR markers used for the assessment of genetic diversity in 101 foreign and 7 local triticale accession lines.

Marker	Detected sizes of alleles (bp) range	Major Allele Frequency	Allele No	Gene Diversity	PIC
Xgwm550-1B	154 – 196	0.33	20	0.82	0.80
Scm152-5R	270 – 335	0.18	16	0.88	0.87
Scm2-6RL	109 – 147	0.47	17	0.71	0.67
Scm109-5R	97 – 127	0.58	12	0.59	0.55
Scm43-2R	106 – 177	0.36	22	0.81	0.79
Xwmc59-1A	198 – 286	0.55	15	0.66	0.64
Xgwm513-4B	154 – 190	0.76	12	0.40	0.39
Scm159-4R	114 – 167	0.28	26	0.88	0.87
Average		0.44	17.5	0.71	0.70

PIC= Polymorphic Information Content

4.1.3.3 SSR markers genetic diversity (GD) and polymorphic information content (PIC)

The study demonstrated that the observed GD with all the tested markers ranged from 0.40 with Xgwm513-4B to 0.88 with Scm152-5R and Scm159-4R each with an average GD of 0.71. The PIC obtained ranged from 0.39 with Xgwm513-4B to 0.88 with Scm152-5R and Scm159-4R each, with an average PIC of 0.70. The high levels of GD and PIC showed that the tested markers were able to differentiate the tested triticale cultivars with the exception of Xgwm513-4B which had revealed low levels of both GD and PIC value. High GD and PIC values revealed by the rye genome SSRs could suggest a more rapid change in the rye genome than in that of wheat in the background of triticale. However, Kuleung et al. (2006) observed an average GD of 0.54 in 80 hexaploid triticale revealed by 14 rye and 43 wheat SSR markers. Vyhnanek et al. (2009) reported the average GD of 0.52 and PIC of 0.48 in 16 triticale genotypes tested with 21 rye and 27 wheat SSR markers. A low average PIC of 0.36 was also observed in 54 Brazilian triticale genotypes tested with 42 wheat SSR markers by Costa et al. (2007). Low GD and PIC values observed in the previous studies could be due to

the lower number of triticale cultivars tested originating from fewer countries compared to the present study. This study showed that rye SSR markers were superior to wheat markers in assessing genetic diversity of triticale cultivars in the current study.

4.1.3.4 Phylogenetic analysis

Phylogenetic analyses grouped the entries into three main clades based on observed differences (Figure 14). Clade A consisted of a mixture of 35 spring and 35 winter type cultivars. In addition, one facultative type originating from the USA was found grouped into this clade. Three entries marked with N/A in this clade where unclassified, as they failed to germinate. The clade consisted of two sub-clusters, AI and AII. Sub-cluster AI contained 30 entries originating from South Africa, Australia, Canada, Mexico, Poland, USA, Russia, Bulgaria and Brazil. The sub-cluster was consisted of 13 spring type and 13 winter type cultivars. In addition, one facultative type originating from the USA was grouped into this sub-cluster. There were no entries originating from Germany, China and Ukraine observed in this clade. It was observed that two entries, 'Newcale' and 'NE426GT', were identical according to the analyses. Kuleung et al. (2006) likewise found the two entries clustered together. The results further demonstrated that many of the entries that dominated this clade were from the USA, (seven entries). Two of the South African entries, 'SSKR 628' and 'Oom Jan', were found to be closely related to the entries originating from Canada and the USA. It was also observed that entries from Poland showed genetic similarities to the entries from Mexico. Sub-cluster AII contained 44 entries originating from Ukraine, China, Germany, South Africa, Australia, Hungary, Poland, Canada, Russia, Bulgaria, USA and Mexico. It was observed that many of the entries found in this sub-cluster originated from Russia, with nine entries, followed by South Africa, with eight entries. Two South African entries, 'US2007' and 'AgBeacon', appeared to be genetically closely related and clustered together with 'Bacchus' also originating from South Africa. Three entries originating from South Africa, including 'Ibis', 'Rex' and 'US2009', were also confirmed to be closely related to each other, and clustered together with 'Tobie' and 'USGEN 19' also originating from South Africa. These South African entries clustered together with the entries originating from Canada and Poland as reported by Kuleung et al. (2006). This could partially be explained by the origin of the germplasm with which the South African triticale breeding program started in the 1970's.

Clade B consisted of 22 entries, a mixture of thirteen spring and nine winter types, originating from Poland, Russia, Canada, Hungary, Mexico, Bulgaria, Australia and Brazil. Many of the entries in this clade were from Mexico, with seven entries. The results showed that entries, 'AD 307 (XTO21D)' and 'AD 307 (XT981D)' (noted as AD 307 1 and AD 307 2 on the tree) originated from Ukraine and clustered together. Entries 'Marval' and 'Jenkins' originated from the USA, and were grouped together. There were no South African entries observed in this clade.

Clade C consisted of 12 entries, a mixture of seven spring and four winter types, originating from South Africa, USA, Canada, Mexico, Ukraine, Australia, Bulgaria and Russia. 'Joseph', an entry from South Africa, grouped together with 'Frosty' and 'AC Alta', entries from the USA and Canada respectively. Additionally, entries 'SSKR 626' and 'BEE SEE', both originating from South Africa, were grouped together with 'Breaker' from the USA. The results in general showed that entries originating from Mexico and the USA were the most widely spread across all the clades and sub-clusters. This could suggest that many of the triticale cultivars grown across the globe were developed with materials originating from these countries. Our findings revealed that South African entries, although clustered into one clade A, were distributed over both sub-clusters suggesting existence of genetic diversity among cultivars at the SU-PBL.

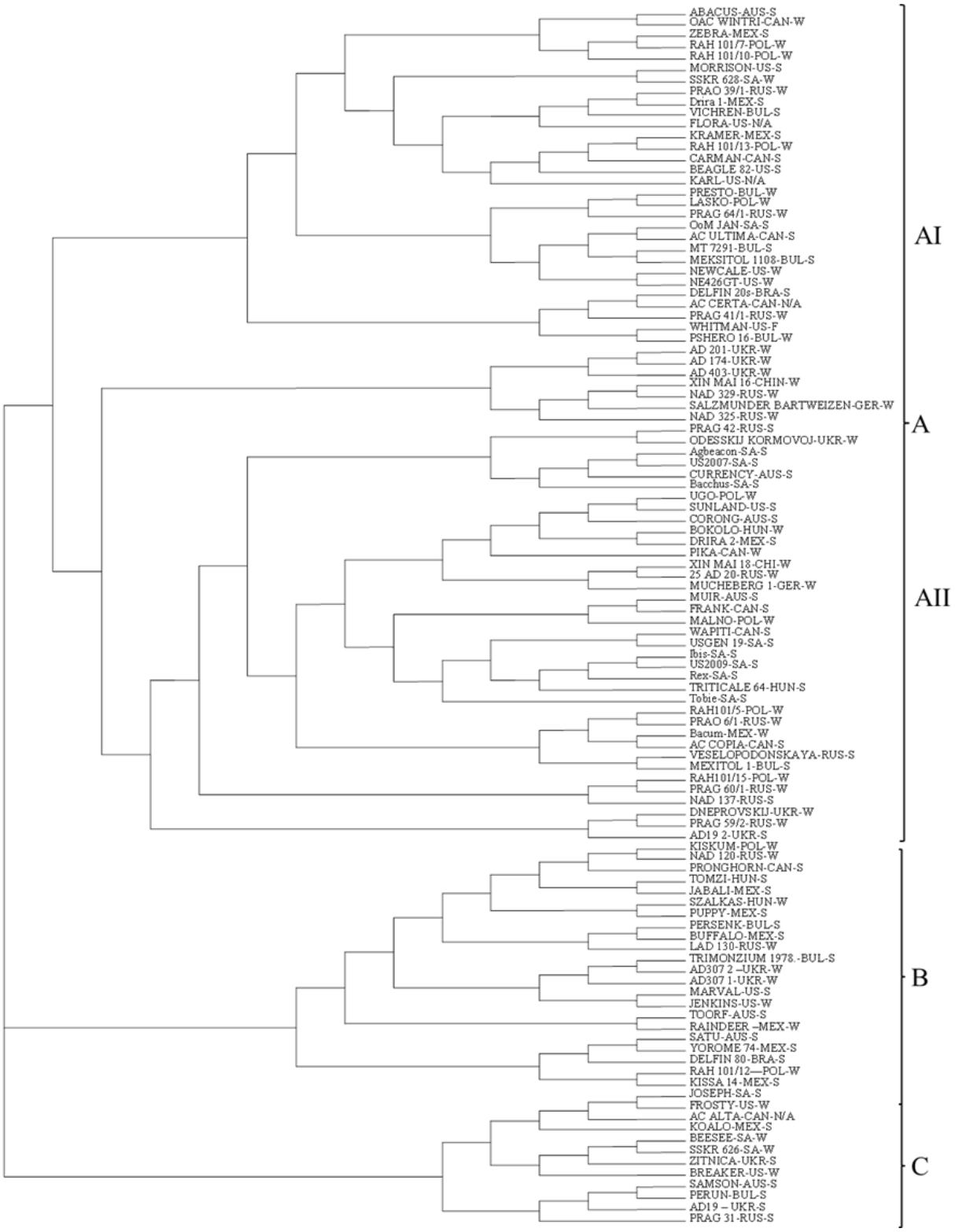

Figure 14: Phylogenetic tree of 101foreign and 7 local triticale cultivars. The analysis is based on the neighbour-joining clustering method. **(**Abbreviation is country of origin and growth habit).

4.2 Introgression of anthocyanin genes for the phenotypic identification of triticale germplasm

4.2.1 Introgression of anthocyanin genes

4.2.1.1 Purple pericarp genes (*Pp1, Pp3*)

Initially, 506 F_1-seeds were obtained by crossing 'Amethyst' with triticale cultivars 'US2007', 'AgBeacon', 'US2009', 'Bacchus', and 'Tobie' (Table 5). The results showed high numbers of F_1-seed set. The lowest seed set (30 seeds) was observed in crosses between 'Amethyst' and 'Tobie' and the highest seed set (159 seeds) was observed in crosses between 'Amethyst' and 'Bacchus' (Table 5). It thus indicates a clear genotype effect when making triticale x wheat crosses, and also validates that higher seed set will be obtained when using triticale as a male parent and wheat as female parent, results from Hills *et al.* (2007) corroborates this observation.

Twelve out of the 506 F_1-seeds observed were successfully gave fertile plants following gibberillic acid treatment. The lowest germination was observed in crosses between 'Amethyst' and 'Tobie' with only one seed germinating, and the highest germination rate was observed in crosses between 'Amethyst' and 'US2007' with six seeds germinating. The low numbers of F_1-seeds germinating were due to non-viable embryos. A similar result was reported by Khanna (1990) who concluded that that poor germination of seeds obtained by crossing wheat with triticale crosses is due to high numbers of embryos dying at the early developmental growth stages. The non-viability can be attributed to genomic incompatibility between the R and D genomes of wheat and rye. This was confirmed by the absence of seed shrivelling in the F_1 'Amethyst' x 'SST88' crosses with normal endosperm development and absence of seed shrivelling. In addition, the immature F_1 hybrid seeds observed in the study in wheat x triticale crosses had liquid to semi-liquid endosperm development. Poor endosperm development could be the reason for improper development of embryos. Nkongolo *et al.* (1991) reported similar results, and found that, despite better seed set obtained from wheat x triticale crosses, hybrid embryos appeared semi-liquid to liquid, and failed to develop. However, Hussain and Williams (2008) stated that the interaction of genome with genome and genome with cytoplasm between interspecific hybrids affects the genomic stability, which could lead to complete to partial seed sterility.

Due to the poor germination response of the F_1 hybrid seed following gibberillic acid treatment embryo rescue were done on the remaining material. Of the 64 embryos rescued from 'Amethyst' x triticale crosses, 13 gave plantlets (Table 5). The low recovery of plantlets from the rescued embryos observed in the study could be due to post-fertilization barriers such as chromosome inconsistence, poor endosperm development or embryo-endosperm incompatibility as suggested by Lu and Bridgen (1996). Nkongolo *et al*. (1991) reported a rate of between 95 and 100% of embryo necrosis when grown at temperatures between 22 °C and 30 °C. It was shown in the study that germination of embryos was also dependent on cross combinations. This was supported by higher numbers of plantlets recovered with 'US2007' in comparison with other tested triticale genotypes. Kapila and Sethi (1993) reported similar results. They found that the germination of embryos and recovery of plantlets were largely dependent on parental genotypes in crosses of wheat x triticale, and that out of 16 different cross combinations tested with embryo rescue, only one cross combination showed consistent plant development.

Subsequently, 23 seeds were obtained from crosses made between triticale cultivars as female parents and 'Amethyst' as male parent (Table 5). These were planted and resulted in the germination of 15 hybrid seeds into normal flowering plants. The results revealed low numbers of seed set as expected. Nkongolo *et al.* (1991) suggested that the poor seed set observed in triticale x wheat crosses is due to the slow growth of wheat pollen inside the style of triticale which leads to improper fertilization, resulting in fewer seeds. In contrast to the poor viability of F_1 seeds observed in 'Amethyst' x triticale crosses, the F_1 seeds of triticale crossed with 'Amethyst' had normal endosperm development, and were viable. Morphologically it displayed characteristics intermediate between the parents.

Table 5: Number of seed sets and germinated from F_1 hybrid cross

F_1 germinated with gibberillic acid (GA_3)				
Parental Crosses	**No. of florets pollinated**	**No. of seed sets**	**No. of seeds Pigmented**	**No. of F_1 seeds germinated**
11T010 (*Pp1,Pp3*)	**31**	**151**	**151 purple**	**6**
11T011(*Pp1,Pp3)*	**6**	**40**	**40 purple**	**3**
11T012 (*Pp1,Pp3*)	**15**	**62**	**62 purple**	**2**
11T013 (*Pp1,Pp3*)	**5**	**30**	**30 purple**	**1**
11T014 (*Pp1,Pp3*)	**16**	**159**	**159 purple**	**0**
11T015 (*Pp1,Pp3*) (Control)	**4**	**15**	**15 purple**	**5**
F_1 germinated with the embryo rescue				
Parental Crosses	**No. of florets pollinated**	**No. of caryop-ses rescued**	**No. of seeds Pigmented**	**No. of F_1 plantlets recovered with roots and shoots**
11T010(*Pp1,Pp3*)	**2**	**10**	**10 purple**	**5**
11T011 (*Pp1,Pp3*)	**2**	**11**	**11 purple**	**0**
11T013 (*Pp1,Pp3*)	**2**	**8**	**8 purple**	**0**
11T014 (*Pp1,Pp3*)	**5**	**35**	**35 purple**	**2**
F_1 germinated inside the pots with sand				
Parental Crosses	**No. of florets pollinated**	**No. of seed sets**	**No. of seeds Pigmented**	**No. of F_1 seeds germinated**
11T016(*Pp1,Pp3*)	**7**	**4**	**0**	**2**
11T017 (*Pp1,Pp3*)	**5**	**0**	**0**	**0**
11T018 (*Pp1,Pp3*)	**22**	**15**	**0**	**10**
11T019 (*Pp1,Pp3*)	**10**	**4**	**0**	**3**
11T020 (*Pp1,Pp3*)	**4**	**0**	**0**	**0**
11T021 (*Pp1,Pp3*) (Control)	**2**	**2**	**2**	**2**

4.2.1.2 Blue aleurone layer gene (*Ba*)

Seven hundred and thirty-two F1 hybrid seeds were obtained by crossing 'Cltr1202STR' with triticale cultivars ('US2007', 'AgBeacon', 'US2009', 'Bacchus', and 'Tobie') (Table 6). Of the 732 F_1-seeds germinated with gibberillic acid, none developed into viable plantlets. The absence of germination was due to a complete lack of viable embryos. However, 'Cltr1202STR' x 'SST88' (wheat control) crosses resulted in seed set with normal endosperm development and viability, indicating that genomic incompatibility between 'Cltr1202STR' x triticale cultivars was the main reason for production of non-viable seed.

Embryo rescues were done in the same manner as in the previous crosses between 'Amethyst' and triticale. Of the 334 embryos rescued from 'Cltr1202STR' and triticale crosses, six developed into viable plantlets (Table 6). The low germination and recovery of plantlets from the rescued embryos observed in the study could be due to the same problems as discussed previously.

In addition, crosses were made between triticale cultivars and 'Cltr1202STR' resulting in 25 seeds (Table 6). Germination of these in pots with coarse sand resulted in 14 seeds growing into complete flowering plants. Data indicated that the numbers seeds observed when using triticale as female parent in triticale x 'Cltr1202STR' crosses were comparable to triticale x 'Amethyst' crosses. This could be due to differences in compatibility of the two wheat genotypes with triticale.

Table 6: Number of seeds set and germinated from F_1 hybrid crosses.

F_1 germinated with gibberillic acid (GA_3)				
Parental Crosses	**No. of florets pollinated**	**No. of seed sets**	**No. of seeds Pigmented**	**No. of F_1 seeds germinated**
11T022(*Ba*)	**24**	**140**	**0**	**0**
11T023 (*Ba*)	**38**	**245**	**0**	**0**
11T024(*Ba*)	**11**	**129**	**0**	**0**
11T025 (*Ba*)	**10**	**97**	**0**	**0**
11T026(*Ba*)	**16**	**171**	**0**	**0**
11T027(*Ba*) (Control)	**2**	**12**	**5 blue**	**5**
F_1 germinated with the embryo rescue				
Parental Crosses	**No. of florets pollinated**	**No. of caryopses rescued**	**No. of seeds Pigmented**	**No. of F_1 plantlets recovered with roots and shoots**
11T022 (*Ba*)	**8**	**56**	**0**	**2**
11T023 (*Ba*)	**10**	**59**	**0**	**2**
11T024 (*Ba*)	**14**	**99**	**0**	**1**
11T025 (*Ba*)	**6**	**27**	**0**	**0**
11T026 (*Ba*)	**11**	**93**	**0**	**1**
F_1 germinated inside the pots with sand				
Parental Crosses	**No. of florets pollinated**	**No. of seed sets**	**No. of seeds Pigmented**	**No. of F_1 seeds germinated**
11028 (*Ba*)	**9**	**3**	**0**	**3**
11029 (*Ba*)	**7**	**2**	**0**	**2**
11030 (*Ba*)	**7**	**2**	**0**	**2**
11031 (*Ba*)	**15**	**8**	**0**	**1**
11032 (*Ba*)	**8**	**10**	**0**	**6**
11033 (*Ba*)(Control)	**2**	**12**	**0**	**7**

4.2.2 Backcrosses

4.2.2.1 Backcross of purple pericarp genes (*Pp1, Pp3*)

The progeny obtained from the reciprocal crosses made between 'Amethyst' and the triticale cultivars were used in backcrosses with the different triticale cultivars as female parents. This was repeated four times based on visual selection of lines expressing the anthocyanin genes (*Pp1*; *Pp3*) that resulted in purple pericarp pigmentation.

The first backcross (containing *Pp1* and *Pp3*) with the triticale cultivars ('US2007', 'Ag-Beacon', 'US2009', 'Bacchus', and 'Tobie') resulted in 347 BC_1F_1 rescued embryos (Table 7). Of the 347 BC_1F_1 embryos cultured, only seven germinated into complete plantlets with shoots and roots (two from 'Amethyst' x 'US2007', two from 'Amethyst' x 'AgBeacon' and three from 'Amethyst' x 'US2009') (Table 7). Most of the rescued embryos were therefore not viable and most of them produced shoots only. This could suggest the presence of meiotic irregularities resulting in low recovery of viable plantlets (Kapila and Sethi, 1993). In addition, germination of some embryos from different cross combinations confirms that genotype also plays an important role in determining the viability of embryos resulting from interspecies hybridization as previously discussed.

Matured BC_1F_1 seeds showed extreme seed shriveling with poorly developed endosperm. Barker *et al.* (1989) reported similar results. They observed a higher number of shriveled seeds with no embryos among F_1 x triticale than among triticale x F_1 crosses. Their findings further suggested that chromosome imbalances were more frequently transmitted maternally than paternally. A very important consideration when planning crosses.

Three BC_1F_1 seeds were also obtained in crosses between triticale cultivars ('US2007'; 'US2009') x 'Amethyst' (Table 7). Only one BC_1F_1 seed was observed from the cross 'US2009' x 'Amethyst' and this showed purple pericarp pigmentation.

Ten BC_2F_1 seeds were obtained in crosses of 'Amethyst' x 'US2007' (Table 7). Of the seven BC_2F_1 seeds expressing purple pericarp pigmentation planted, only one of them developed into a fertile plant. The absence of seed set in the BC_2F_1 crosses made between 'Amethyst' and triticale cultivars ('AgBeacon', 'US2009' and 'Tobie') suggest the presence of male sterility due to chromosomal imbalances. This was highlighted by severe seed shriveling of the BC_2F_1 seed.

Two hundred and fourteen BC_3F_1 'Amethyst' x 'US2007' seeds were observed (Table 7). Of the 214 BC_3F_1 seeds obtained, only 62 of them expressed anthocyanin genes giving pericarp purple pigmentation. In contrast to normal expression of anthocyanin genes in the previous generation, there was less purple pericarp pigmentation observed among the BC_3F_1 hybrid crosses. This could be due to loss of one of the two genes controlling this trait. Poor germination of planted BC_3F_1 hybrid seeds from crosses of 'Amethyst' x 'US2007' was also recorded.

The BC_4F_1 'Amethyst' x 'US2007' resulted in a total of 88 seeds (Table 7). No BC_4F_1 'Amethyst' x 'SST88' plantlets were recovered due to technical problems. The introgression of the purple pericarp colour therefore failed at the last hurdle (Figure 15).

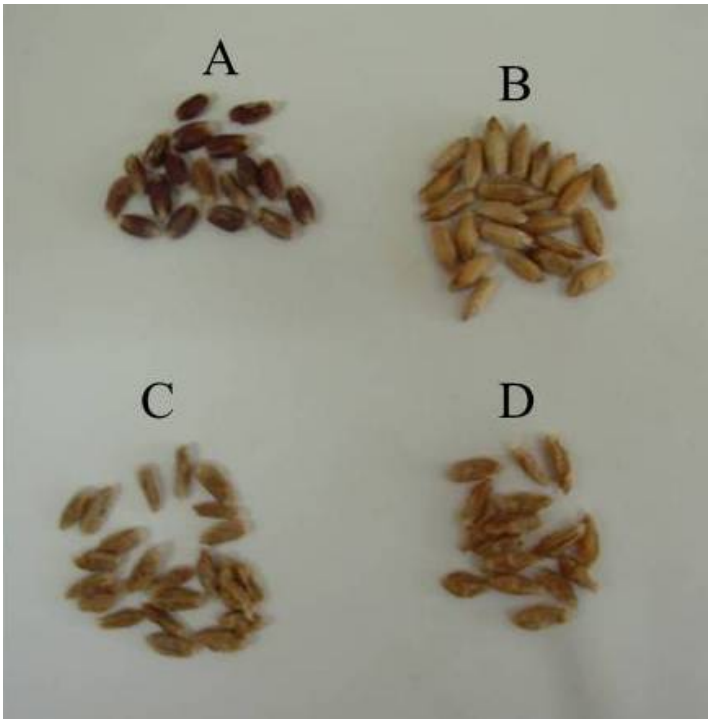

Figure 15: Mature seeds of BC_4F_1 11T010 (*Pp1, Pp3*) line without purple grain pigmentation. A represents the female wheat parent, 'Amethyst' *(Pp1, Pp3*); B represents the recurrent triticale parent, 'US2007'; C cross-pollinated shrivelled seeds of BC_4F_1 11T010 (*Pp1, Pp3*) line without purple pigmentation; and D self-pollinated seeds of BC_4F_1 11T010 (*Pp1, Pp3*) line without purple pigmentation.

Table 7: Number of seeds set and germinated from BC_1F_1, BC_2F_1, BC_3F_1 and BC_4F_1 hybrid crosses.

BC_1F_1 germinated with embryo rescue				
Parental Crosses	**No. of florets pollinated**	**No. of caryop-ses rescued**	**No. of seeds Pigment-ed**	**No. of plantlets with recovered roots and shoots**
11T010 (*Pp1,Pp3*)	18	86	86 purple	2
11T011 (*Pp1,Pp3*)	9	34	34 purple	2
11T012 (*Pp1,Pp3*)	37	176	176 purple	3
11T013 (*Pp1,Pp3*)	12	50	50 purple	0
11T014 (*Pp1,Pp3*)	1	1	1 purple	0
BC_1F_1 germinated inside the pots with sand				
Parental Crosses	**No. of florets pollinated**	**No. of seed sets**	**No. of seeds Pigment-ed**	**No. of seeds germi-nated**
11TO16 (*Pp1,Pp3*)	11	2	0	0
11T018 (*Pp1,Pp3*)	28	1	1 purple	1
11T015 (*Pp1,Pp3*) (Control)	5	21	15 purple	5
11T021 (*Pp1,Pp3*) (Control)	3	12	4 purple	4
BC_2F_1 germinated inside pots with sand				
Parental Crosses	**No. of florets pollinated**	**No. of seed sets**	**No. of seeds Pigment-ed**	**No. of seeds germi-nated**
11T010 (*Pp1,Pp3*)	36	10	7 purple	1
11T011 (*Pp1,Pp3*)	11	0	0	0
11T012 (*Pp1,Pp3*)	20	0	0	0
11T013 (*Pp1,Pp3*)	2	0	0	0
11T015 (*Pp1,Pp3*) (Control)	1	9	9 purple	5
BC_3F_1 germinated inside the pots with soils				
Parental Crosses	**No. of florets pollinated**	**No. of seed sets**	**No. of seeds Pigment-ed**	**No. of seeds germi-nated**
11T010 (*Pp1,Pp3*)	17	214	62 purple	4
11T015 (*Pp1,Pp3*) (Control)	7	43	11 purple	11
BC_4F_1 germinated inside the pots with soils				
Parental Crosses	**No. of florets pollinated**	**No. of seed sets**	**No. of seeds Pigment-ed**	**No. of seeds germi-nated**
11T010 (*Pp1,Pp3*)	13	88	20	0

4.2.2.2 Backcross of blue aleurone layer gene (*Ba*)

The F_1 obtained from the crosses made between 'C1tr1202STR' and the triticale cultivars were planted and backcrossed to different triticale cultivars as female parents in the same manner as described in the previous section.

Initially, 34 BC_1F_1 hybrid seeds were obtained from a cross between 'C1tr1202STR' and the triticale cultivars ('US2007', 'AgBeacon', and 'Bacchus') (Table 8). Of the 34 BC_1F_1 seeds, only eight presented with a blue aleurone layer pigmentation, and two of these gave viable plantlets ('Cltr1202STR' x 'US2007' and 'Cltr1202STR' x 'AgBeacon'). Chromosomal imbalances between 'Cltr1202STR' and the triticale crosses were suggested as the main reason for the low numbers of BC_1F_1 seeds expressing the blue aleurone layer trait. Kiani *et al.* (2009) suggested that the segregation of genes between interspecies crosses is due to the lower fertility of interspecies crosses. Compared to the BC_1F_1 observed in crosses of 'Amethyst' x triticale, some of the BC_1F_1 seed observed in crosses between 'C1tr1202STR' and triticale cultivars showed normal endosperm development. This improvement was observed between both cross-pollinated and self-pollinated flowers, suggesting that 'Cltrl202STR' is a more compatible genotype for making crosses with triticale.

Sixty eight BC_1F_1 seeds were obtained from crosses with the triticale cultivars ('US2007', 'AgBeacon', 'US2009', 'Bacchus' and 'Tobie') and 'Cltr1202STR' (Table 8). Eleven BC_1F_1 seeds expressed the blue aleurone pigmentation and eight seeds gave viable plantlets (seven 'US2007' x 'Cltr1202STR' and one 'Tobie' x 'Cltr1202STR'). Similar to the observation found in 'Amethyst' and triticale crosses, mature flowers of these hybrid crosses were male sterile.

Hundred and four BC_2F_1 seeds were obtained in crosses between 'C1tr1202STR' x triticale cultivars ('US2007', 'AgBeacon', and 'Tobie') (Table 8). Only four BC_2F_1 seeds expressing the blue aleurone layer (three from 'Cltr1202STR' x 'US2007' and one from 'Cltr1202STR' x 'AgBeacon') grew into viable plantlets.

A hundred and thirty six BC_2F_1 seeds were also obtained in crosses between reciprocal crosses with triticale cultivars ('US2007', 'Tobie') x 'ClTR1202STR' (Table 8). Only nine of the 18 BC_2F_1 seeds obtained expressed the blue aleurone layer pigmentation from the cross 'US2007' x 'Cltr1202STR'.

Three hundred and eighteen BC_3F_1 seeds were observed in 'Cltr1202STR' x triticale cultivars ('US2007', 'AgBeacon') crosses (Table 8). The observed seeds set resulted in the germination of 15 seeds into complete plants expressing blue aleurone pigmentation (two from 'Cltr1202STR' x 'US2007' and 13 from 'Cltr1202STR' x 'AgBeacon').

Four hundred and eighty five BC_3F_1 seeds were also observed in triticale ('US2007') x 'Cltr1202STR' crosses. Seven hundred and fifty BC_4F_1 hybrid seeds were obtained in crosses between 'Cltr1202STR' and 'AgBeacon' (Table 8). Of these 9 lines were established and designated 11T023 (Figure 16 and 17).

One hundred and fifty-six BC_4F_1 seeds were obtained in the crosses between 'US2007' and 'Cltr1202STR' (*Ba*) (Table 8). Of these 6 lines were established and designated 11T028 (Figure 18 and 19).

Figure 16: Segregation of blue grain pigmentation in immature BC_4F_1 seeds of 11T023 (*Ba)* introgressed line.

Figure 17: Mature seeds of BC_4F_1 11T023 (*Ba*) line with blue grain pigmentation. A represents the donor wheat parent, 'Cltr1202STR'; B represents the recurrent triticale parent, 'AgBeacon'; and C represents the BC_4F_1 hybrid seeds of 11T023 (*Ba*) line showing blue pigmentation.

Figure 18: Segregation of blue grain pigmentation from the immature seeds of $BC_4F_1$11T028 (*Ba*) line.

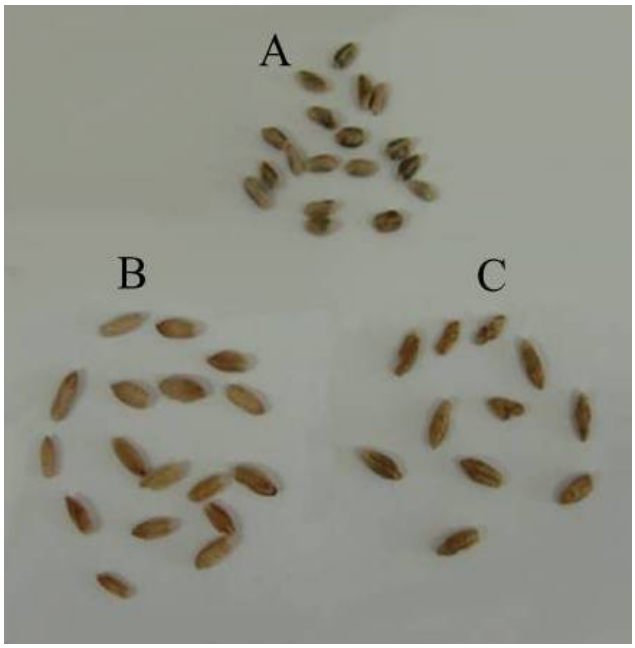

Figure 19: Appearance of matured seeds of BC_4F_1 11T028 (*Ba*) line with blue grain pigmentation. A represents wheat donor: C1tr1202STR (*Ba*), B represents the recurrent triticale parent: US2007 and C represents BC_4F_1 hybrid seeds of 11T028 (*Ba*) line showing blue pigmentation.

4.2.2.3 Molecular validation of introgressed lines

DNA extractions were performed on lines 11T023 and 11T028, the recurrent parents ('US2007' and 'AgBeacon') and donor parent ('Cltr1202STR'). Six of the tested SSR markers resulted in positive amplification between all the tested plant materials (Addendum F). The genetic diversity (GD) observed from the tested SSR markers ranged from 0.59 with *Scm43-2R* to 0.92 with *Scm159-4R,* and with an average of 0.75 (Table 9). The polymorphic information content (PIC) observed ranged from 0.51 with *Scm43-2R* to 0.92 with *Scm159-4R*, with an average of 0.71. A total number of 47 alleles were detected distributed among chromosomes *5R*, *4R*, *6R*, *2R*, *4R* and *1B* as shown in table 8. Figure 20 shows the amplification of the *Scm2-6R* SSR marker as a typical example of the visualization.

Table 8: Number of seed sets and germinated from BC_1F_1, BC_2F_1, BC_3F_1 and BC_4F_1 hybrid crosses.

BC_1F_1 germinated inside the pots with sand				
Parental Crosses	**No. of florets polli-nated**	**No. of seed sets**	**No. of seeds Pigmented**	**No. of seeds germinated**
11T022 (*Ba*)	13	15	2 blue	1
11T023 (*Ba*)	13	16	6 blue	1
11T024 (*Ba*)	21	3	0	0
11T028(*Ba*)	14	38	11 blue	7
11T029 (*Ba*)	6	12	0	0
11T030 (*Ba*)	12	11	0	0
11T031 (*Ba*)	12	13	1 blue	1
11T032 (*Ba*)	21	2	0	0
11T033 (*Ba*) (Control)	2	9	9 blue	9
11T026 (*Ba*) (Control)	3	17	2 blue	2
BC_2F_1 germinated inside pots with sand				
Parental Crosses	**No. of florets polli-nated**	**No. of seed sets**	**No. of seeds Pigmented**	**No. of seeds germinated**
11T022 (*Ba*)	6	84	8 blue	3
11T023 (*Ba*)	3	16	2 blue	1
11T025 (*Ba*)	5	14	2 blue	0
11T028 (*Ba*)	20	123	14 blue	9
11T031 (*Ba*)	5	13	4 blue	0
BC_3F_1 germinated inside the pots with soils				
Parental Crosses	**No. of florets polli-nated**	**No. of seed sets**	**No. of seeds Pigmented**	**No. of seeds germinated**
11T022(*Ba*)	13	107	3 blue	2
11T023 (*Ba*)	14	210	25 blue	13
11028 (*Ba*)	37	485	40 blue	3
BC_4F_1 germinated inside the pots with soils				
Parental Crosses	**No. of florets polli-nated**	**No. of seed sets**	**No. of seeds Pigmented**	**No. of seeds germinated**
11T023 (*Ba*)	60	750	18 blue	9
11T028 (*Ba*)	13	156	11blue	6

Table 9: Statistical summary of the six SSR markers used for the assessment of parentage similarities between BC_4F_1 hybrid crosses and recurrent parents.

Marker	Detected size of alleles (bp)	Major Allele Frequency	Allele No	Gene Diversity	PIC
Scm43-2R	106 - 177	0.44	4	0.59	0.51
Xgwm550-1B	335 - 443	0.33	9	0.82	0.80
Scm159 (F2)-4R	114 - 167	0.17	16	0.92	0.92
Scm2-6RL	119 - 147	0.33	7	0.77	0.74
Scm152 (F1)-4R	252 - 335	0.44	7	0.72	0.69
Scm109-5RL	97 - 127	0.5	4	0.64	0.59
Average		0.37037	7.83	0.75	0.71

PIC=Polymorphic Information Content

A phylogenetic tree grouped introgressed lines, recurrent parents and the donor parent into three clades based on genetic differences observed (Figure 21). Clade 1 contained 'US2007', 11T028 A, 11T028 B, 11T023 A and 11T028 C together and clade 2 contained 'AgBeacon', 11T023 B and 11T028 D. Clade 3 contained 11T028 E, 11T023 C, 11T023 D, 11T023 E, 11T023 F, 11T023 G, 11T023 H, 11T028 F and 11T023 I together, and 'Cltr1202STR' alone. Phylogenetic analyses showed that introgressed BC_4F_1 line 11T028 A was genetically similar to recurrent parent 'US2007', and that the introgressed BC_4F_1 line11T023 (*Ba*) B was genetically the best match to recurrent parent 'AgBeacon'. The information gathered from this data can be of use in selecting closely related lines to the recurrent parents for the fixing of gene *Ba* (blue aleurone layer trait) in the triticale genetic background. These results also proved the utility of the SSR markers as a selection tool for the validation of introgressed lines during the backcross process.

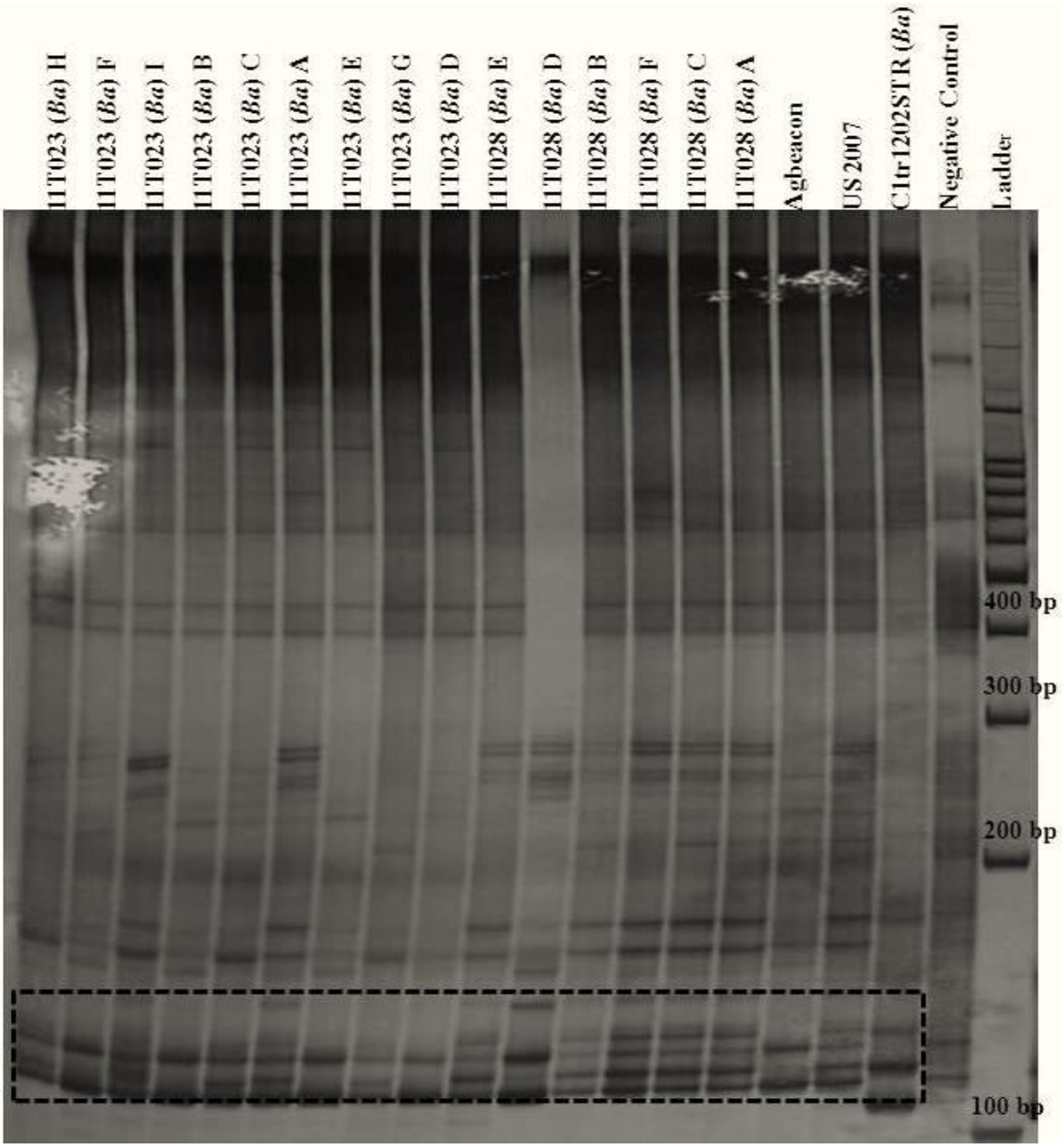

Figure 20: Amplification of *Scm2-6R* SSR marker tested between introgressed BC_4F_1 seeds of 11T023 (*Ba*) and 11T028 (*Ba*) lines using 6% polyacrylamide gel electrophoresis. Dotted black lanes indicated polymorphic alleles scored between tested materials. 'Cltr1202STR (*Ba*)' is a control donor parent.

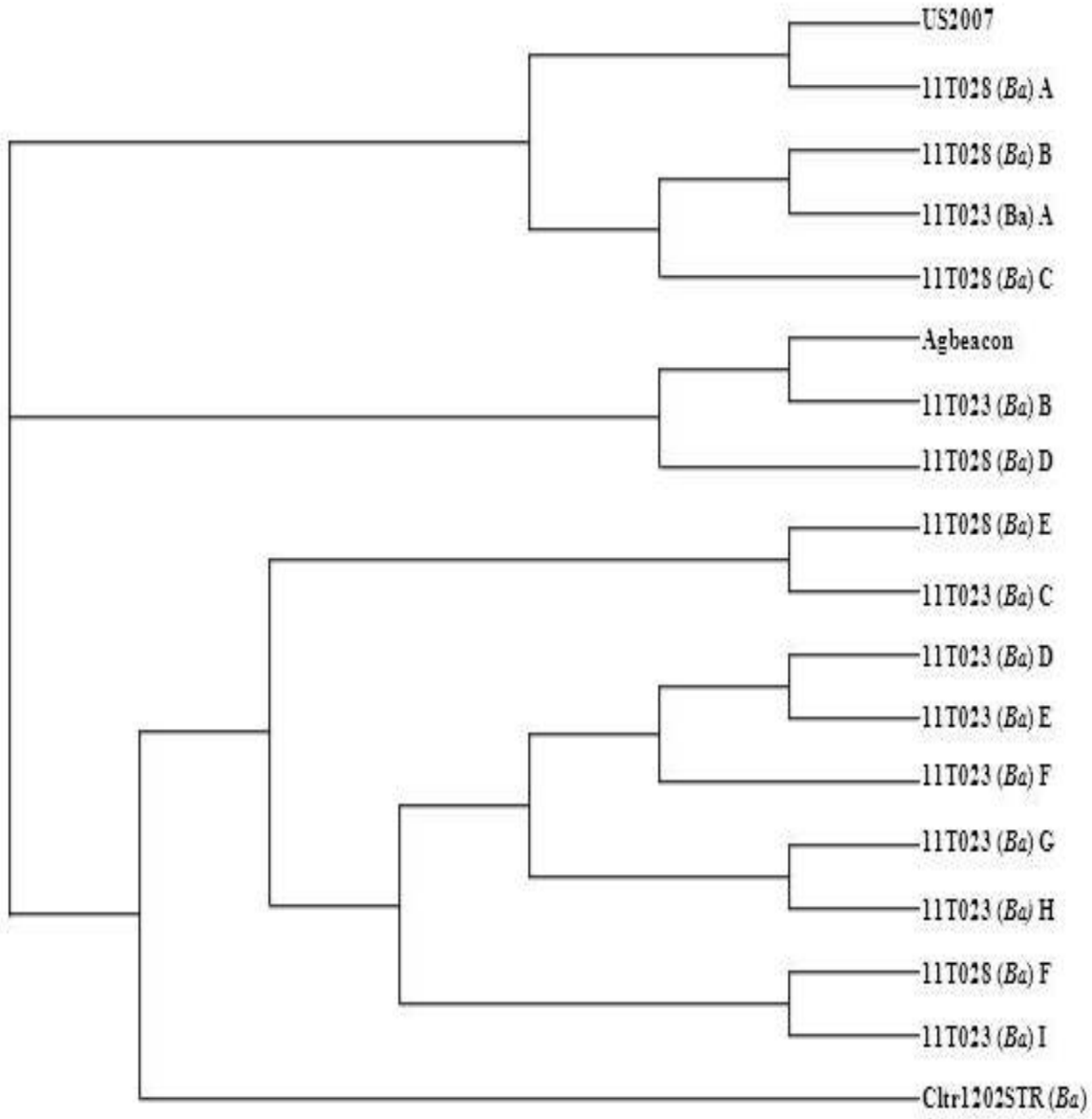

Figure 21: Phylogenetic tree of the introgressed BC_4F_1 seeds of line 11T023 (*Ba*) and line 11T028 (*Ba*). The analysis is based on the neighbour-joining clustering method using PowerMarker software v3.25. 'Cltr1202STR (*Ba*)' is a control donor parent.

Chapter 5: Conclusion and future work

The use of molecular markers as a complementary tool to phenotypic markers in evaluating the genetic diversity of different crop species has revolutionized the field of plant breeding. Molecular markers have been able to unlock knowledge about genetic diversity in different crop species, without depending on inaccurate or unavailable pedigrees. Compared to phenotypic markers, molecular markers are independent of environmental factors or the developmental stage of the plant providing an accurate estimation of the genetic diversity in the crop under study. The well-documented narrow genetic base of triticale that cause high levels of genotypic uniformity among many of its modern cultivated cultivars had hindered the genetic improvement of the crop through breeding.

Despite experimental difficulties due to technical challenges, this project has achieved most of the aims set. The first aim of the study was to assess the genetic diversity of triticale using molecular markers and phenotypic characteristics. In order to achieve this, 101 triticale cultivars were identified and sourced through the USDA Germplasm Bank (Washington DC) representing 13 different countries across the world. Phenotypic evaluation classified cultivars as spring or winter types. Phenotypic attributes measured were days to heading, plant height (cm), grain yield per row (g) and thousand-kernel weight which had showed slight variations to the local check cultivars. The results revealed that only two cultivars 'AC Copia' and 'Joseph' showed statistically significant better results in term of grain yield while cultivars, 'Muir' and 'SSKR628' showed significant higher thousand-kernel weights compared to the local check cultivars. In general, the study showed that the grain yield and thousand-kernel weights of the lines were lower compared to the local checks.

The screening of rust resistance with two isolates showed that the lines were resistant towards the stem rust and susceptible to the leaf rust isolate respectively. Representing a distinct opportunity to use as crossing parents for stem rust resistance, this is always desirable.

Molecular typing of cultivars using SSR markers showed high levels of genetic variation among the different triticale cultivars grown in different countries across the world. A lack of

genetic variation was observed among South African cultivars that could suggest that these cultivars were developed from similar parents sharing the same pedigrees mainly of Canadian and USA origin. This was supported by the clustering of cultivars originating from South Africa, the USA and Canada. The present study also revealed that SSR markers developed from the rye genome are more informative to assay the genetic diversity of triticale cultivars.

High levels of polymorphism revealed by the R-genome markers suggested that more SSR markers developed from the rye genome should be tested for future use in revealing the genetic diversity of triticale genotypes (Bitalo MSc thesis– unpublished data). The informativeness of these markers could also be used as a diagnostic tool for the future identification of triticale cultivars. The combination of phenotypic and molecular data as used in this study complemented each other well and should be used as routine for the future introduction of new germplasm in the SU-PBL's triticale breeding program. The cultivars which exhibits higher grain yield compared to local check cultivars should be planted in plots with repetitions to allow further evaluation for selection purposes.

The development of phenotypic markers that are highly heritable and stable in expression are becoming more important for cultivar identification, and assigning utility to cultivars in multi-purpose crops such as triticale. The second part of the study was aimed at the introgression of anthocyanin genes for the phenotypic identification of triticale germplasm. Two wheat sources, 'Amethyst' (*Pp1*, *Pp3*) carrying purple pericarp pigmentation and 'Ctr1202STR (*Ba*)' carrying blue aleuron grain pigmentation, were crossed with five different triticale cultivars.

Introgression of the blue aleurone layer gene (*Ba*) into 'Agbeacon' and 'US2007' was successful, but the introgression of purple pericarp genes (*Pp1*, *Pp3*) failed. This was due to the loss of these genes during the backcross breeding cycles. Molecular validation of the introgressed BC_4F_1-lines using SSR markers was successfully undertaken to select the lines more similar to the recurrent parents. The selected lines showing more genetic similarity with the recurrent parents would be further used for development of triticale lines carrying the blue aleurone pigmentation trait.

The study revealed that crossability between triticale and wheat was much dependent on the parental genotypes tested. In addition, the study revealed that germination of interspecies hy-

brid seeds with embryo rescue techniques before the embryo aborted is crucial to the recovery of the viable plantlets. The study also proved that the use of backcross breeding aided with SSR markers could facilitate the advancement and accurate selection of the introgressed lines. Therefore, full integration of molecular markers especially the use of SSR markers in the triticale backcross breeding program should be considered going forward.

Chapter 6: References

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ADDENDUM A

Triticale cultivars used in the study for the SSR and phenotypic markers analysis.

Line name	Pedigree name	Country
Salzmunder Bartweizen	Commercial Cultivar	Germany
Bokolo	Commercial Cultivar	Hungary
NAD 120	Commercial Cultivar	Russia
25 AD 20	Commercial Cultivar	Russia
LAD 130	Commercial Cultivar	Russia
NAD 137	Commercial Cultivar	Russia
NAD 329	Commercial Cultivar	Russia
NAD 325	Commercial Cultivar	Russia
BEESEE	Commercial Cultivar	South Africa
Joseph	Commercial Cultivar	South Africa
Oom Jan	Commercial Cultivar	South Africa
AD 19	Commercial Cultivar	Ukraine
AD 174	Commercial Cultivar	Ukraine
PRAO 6/1	Commercial Cultivar	Russia
AD 307	Commercial Cultivar	Ukraine
AD 403	Commercial Cultivar	Ukraine
PRAG 31	Commercial Cultivar	Russia
PRAO 39/1	Commercial Cultivar	Russia
PRAG 41/1	Commercial Cultivar	Russia
PRAG 42	Commercial Cultivar	Russia
PRAG 59/2	Commercial Cultivar	Russia
PRAG 60/1	Commercial Cultivar	Russia
PRAG 64/1	Commercial Cultivar	Russia
DNEPROVSKIJ	Commercial Cultivar	Ukraine
DRIRA	Commercial Cultivar	Mexico
VESELOPODOLYANSKAYA	Commercial Cultivar	Russia
MUNCHEBERG 1	Commercial Cultivar	Germany
TOMZI	Commercial Cultivar	Hungary
SZALKAS	Commercial Cultivar	Hungary
ZEBRA	Commercial Cultivar	Mexico
RAINDEER	Commercial Cultivar	Mexico
JABALI	Commercial Cultivar	Mexico
BUFFALO	Commercial Cultivar	Mexico
KISKUM	Commercial Cultivar	Poland
KISSA 14	Commercial Cultivar	Mexico
FLORA	Commercial Cultivar	United State
PUPPY	Commercial Cultivar	Mexico

ADDENDUM A... (Continued)

Line name	Pedigree name	Country
ZITNICA 1	Commercial Cultivar	Ukraine
AD 307	Commercial Cultivar	Ukraine
AD 201	Commercial Cultivar	Ukraine
KRAMER	Commercial Cultivar	Mexico
Beagle 82	Commerical Cultivar	United State
CURRENCY	Commercial Cultivar	Australia
SAMSON	Commercial Cultivar	Australia
MARVAL	Commercial Cultivar	United State
WHITMAN	Commercial Cultivar	United State
WAPITI	Commercial Cultivar	Canada
YOREME 74	Commercial Cultivar	Mexico
BACUM	Commercial Cultivar	Mexico
DRIRA	Commercial Cultivar	Mexico
SATU	Commercial Cultivar	Australia
CORONG	Commercial Cultivar	Australia
DELFIN 20S	Commercial Cultivar	Brazil
DELFIN 80	Commercial Cultivar	Brazil
MUIR	Commercial Cultivar	Australia
NEWCALE	Commercial Cultivar	United State
FRANK	Commercial Cultivar	Canada
JENKINS	Commercial Cultivar	United State
FROSTY	Commercial Cultivar	United State
PIKA	Commercial Cultivar	Canada
MT 7291	Commercial Cultivar	Bulgaria
SUNLAND	Commercial Cultivar	United State
MORRISON	Commercial Cultivar	United State
BREAKER	Commercial Cultivar	United State
KARL	Commercial Cultivar	United State
LASKO	Commercial Cultivar	Poland
MEKSITOL 1108	Commercial Cultivar	Bulgaria
PERSENK	Commercial Cultivar	Bulgaria
SATU	Commercial Cultivar	Australia
CORONG	Commercial Cultivar	Australia
DELFIN 20S	Commercial Cultivar	Brazil
DELFIN 80	Commercial Cultivar	Brazil
MUIR	Commercial Cultivar	Australia
NEWCALE	Commercial Cultivar	United State
FRANK	Commercial Cultivar	Canada
JENKINS	Commercial Cultivar	United State

FROSTY	Commercial Cultivar	United State

ADDENDUM A... (Continued)

Line name	Pedigree name	Country
RAH-101/3	Commercial Cultivar	Poland
RAH-101/5	Commercial Cultivar	Poland
RAH-101/7	Commercial Cultivar	Poland
RAH-101/10	Commercial Cultivar	Poland
RAH-101/15	Commercial Cultivar	Poland
RAH-101/12	Commercial Cultivar	Poland
TRITICALE 64	Commercial Cultivar	Hungary
OAC WINTRI	Commercial Cultivar	Canada
AC ALTA	Commercial Cultivar	Canada
AC CERTA	Commercial Cultivar	Canada
AC COPIA	Commercial Cultivar	Canada
ABACUS	Commercial Cultivar	Australia
USGEN 19	Commercial Cultivar	South Africa
SSKR 626	Commercial Cultivar	South Africa
SSKR 628	Commercial Cultivar	South Africa
PRONGHORN	Commercial Cultivar	Canada
PSHERO 16	Commercial Cultivar	Bulgaria
XIN MAI 16	Commercial Cultivar	China
XIN MAI 18	Commercial Cultivar	China
AC ULTIMA	Commercial Cultivar	Canada
NE426GT	Commercial Cultivar	United State
KARL	Commercial Cultivar	United State
LASKO	Commercial Cultivar	Poland
MEKSITOL 1108	Commercial Cultivar	Bulgaria
PERSENK	Commercial Cultivar	Bulgaria
PRESTO	Commercial Cultivar	Bulgaria
VICHREN	Commercial Cultivar	Bulgaria
AD-19	Commerical Cultivar	Ukraine
PERUN	Commercial Cultivar	Bulgaria
ODESSKIJ KORMOVOJ	Commercial Cultivar	Ukraine
UGO	Commercial Cultivar	Poland
MALNO	Commercial Cultivar	Poland
MEXITOL 1	Commercial Cultivar	Bulgaria
TRIMONZIUM 1978	Commercial Cultivar	Bulgaria
CARMAN	Commercial Cultivar	Canada

ADDENDUM A... (Continued)

Lines name	Pedigree name	Country
AgBeacon	Commercial Cultivar	South Africa
US 2009	Commercial Cultivar	South Africa
Tobie	Commercial Cultivar	South Africa
Bacchus	Commercial Cultivar	South Africa
US 2007	Commercial Cultivar	South Africa
IBIS	Commercial Cultivar	South Africa
Rex	Commercial Cultivar	South Africa
KOALA	Commercial Cultivar	Mexico

ADDENDUM B

Different simple sequence repeats (SSRs) markers used in the study.

Marker Names	Primer sequence	Annealing temp (^{0}C)	Repeat sequence
Xgwm513-4B	F-ATC CGT AGC ACC TAC TGG TCA R-GGT CTG TTC ATG CCA CAT TG	55^{0}C	$(CA)_{12}$
Xgwm550-1B	F-CCC ACA AGA ACC TTT GAA GA R-CAT TGT GTG TGCA AGG CAC	54^{0}C	$(CT)_8$ $(GT)_8$
Scm2-6RL	F-GAT GAC TAT GAC TAC CAG GAT GAA R-GGA GTG AGA AGG CCG AGA AG	55^{0}C	$(GT)_{10}$
Scm109-5RL	F-AAC CCC CTT TCG TAC CTT GT R-TAA AGC AAA CCA CCA GAG CC	60^{0}C	$(GT)_9$
Scm159(F2)-4R	F-CGG CCA GTG GTT CCT TGG AT R-CGG GAA GGA AAA ACA GAA AAC T	68^{0}C	$(GAAA)_5$
Scm152(F1)-4R	F-TAA AAC GAC GGC CAG TGA GCA R-ATG TAG CCG AGG ATG GTG AG	68^{0}C	$(AG)_7$
Scm43-2R	F-CTA GGG GAT TCA AGG GAG GGC A R-GTT CCC TTG TCC TAC TCG TTA CCG	60^{0}C	$(GT)_{11}$
Xwmc59-1A	F-TCA TTC GTT GCA GAT ACA CCA C R-TCA ATG CCC TTG TTT CTG ACC T	58^{0}C	$(CA)_{19}$

ADDENDUM C

Introgressed BC_4F_1 lines and crossing parents tested with SSR markers

Breeding line	Pedigrees
11T023A	C1tr1202STR(*Ba*)/4*Agbeacon
11T023B	C1tr1202STR(*Ba*)/4*Agbeacon
11T023C	C1tr1202STR(*Ba*)/4*Agbeacon
11T023D	C1tr1202STR(*Ba*)/4*Agbeacon
11T023E	C1tr1202STR(*Ba*)/4*Agbeacon
11T023F	C1tr1202STR(*Ba*)/4*Agbeacon
11T023G	C1tr1202STR(*Ba*)/4*Agbeacon
11T023H	C1tr1202STR(*Ba*)/4*Agbeacon
11T023I	C1tr1202STR(*Ba*)/4*Agbeacon
11T028A	US2007/ C1tr1202STR(*Ba2*)/*US2007
11T028B	US2007/ C1tr1202STR(*Ba2*)/*US2007
1**1T028C**	US2007/ C1tr1202STR(*Ba2*)/*US2007
11T028D	US2007/ C1tr1202STR(*Ba2*)/*US2007
11T028E	US2007/ C1tr1202STR(*Ba2*)/*US2007
11T028F	US2007/ C1tr1202STR(*Ba2*)/*US2007
US2007	
AgBeacon	
C1tr1202STR (*Ba*)	

ADDENDUM D

Phenotypic characteristics and different seedling infection type observed with stem rust pathotype UVPgt59 and leaf rust pathotype UVPrt20 of different foreign triticale genotypes.

Genotypes	Country	Growth habitat	Days to headings	Plant Height (cm)	Grain yield/row (g)	1000-kernel weight (g)	Leaf rust	Stem rust
Salzmunder								
Bartweizen	Germany	W	150	80	3.7	13	**-**	**-**
Bokolo	Hungary	W	143	110	16.2	21	3	3
NAD 120	Russia	W	162	110	7.7	16	3	2
25 AD 20	Russia	W	150	115	32.1	18.1	3	2
LAD 130	Russia	W	150	85	1	10	-	-
NAD 137	Russia	W	144	120	38.8	23.3	3	1
NAD 329	Russia	W	161	85	3.8	-	-	-
NAD 325	Russia	W	162	105	2.2	20	-	-
Joseph	South Africa	S	104	105	267	32.2	4	1
Oom Jan	South Africa	S	93	90	10.3	33	3	1
AD 19 (XT01ID)	Ukraine	S	161	105	43.5	25.7	3	2
AD 174	Ukraine	W	161	80	3.1	20	-	-
PRAO 6/1	Russia	W	162	80	1.3	10	-	-
AD 307 (XT021D)	Ukraine	W	161	75	-	-	-	-
AD 403	Ukraine	W	161	95	-	-	-	-
PRAG 31	Russia	S	144	140	33.3	22.6	2	1
PRAO 39/1	Russia	W	150	105	21.9	20	3	2

ADDENDUM D… (Continued)

Genotypes	Country	Growth habitat	Days to headings	Plant Height (cm)	Grain yield/row (g)	1000-kernel weight (g)	Leaf rust	Stem rust
DRIRA (XT06ID)	Mexico	S	120	90	245.6	34.7	-	;
SATU	Australia	S	93	95	45	30.2	3	3
CORONG	Australia	S	92	100	66.9	28.1	2	-
DELFIN 20S	Brazil	S	93	105	149.7	37.7	2-3	;
DELFIN 80	Brazil	S	93	105	106.8	34.7	3	;
MUIR	Australia	S	98	105	233.3	47.2	3	;
NEWCALE	United State	W	150	80	9.2	29	;-1	-
FRANK	Canada	S	102	105	165.4	41.7	;	
PIKA	Canada	W	138	105	42	22.9	3	;
MT 7291	Bulgaria	S	102	90	138.8	30.5	2	3
SUNLAND	United State	S	98	95	193.9	41.2	1	;
MORRISON	United State	S	150	95	17.3	24	2	2
BREAKER	United State	W	161	95	-	-	-	-

ADDENDUM D… (Continued)

Genotypes	Country	Growth habitat	Days to headings	Plant Height (cm)	Grain yield/row (g)	1000-kernel weight (g)	Leaf rust	Stem rust
MUNCHEBERG								
1	Germany	w	150	70	1.6	30	-	-
TOMZI	Hungary	S	143	115	32.9	20.4	1	2
SZALKAS	Hungary	W	146	55	7.8	14	-	1
ZEBRA	Mexico	S	120	95	105.1	35.1	3	1
RAINDEER	Mexico	W	130	70	5.3	30	-	-
JABALI	Mexico	S	104	85	53.7	37.5	2	1,3
BUFFALO	Mexico	S	93	95	172.2	34.5	-	;
KISKUM	Poland	W	162	100	9.4	19	-	1
KISSA 14	Mexico	S	98	80	70.4	29.6	1	2
PUPPY	Mexico	S	92	95	15	29	3	;-1
ZITNICA 1	Ukraine	S	93	80	30.5	32.3	3	3
AD	Ukraine	W	161	80	0.8	15	-	2
307(XT981D)								
AD 201	Ukraine	W	162	90	1.5	25	-	1
KRAMER	Mexico	S	93	95	138.5	30.8	2	2
Beagle 82	United State	S	104	105	25.2	29	2	;

ADDENDUM D… (Continued)

Genotypes	Country	Growth habitat	Days to headings	Plant Height (cm)	Grain yield/row (g)	1000-kernel weight (g)	Leaf rust	Stem rust
RAH-101/12	Poland	W	104	90	6.3	13	1-2	1-2
TRITICALE 64	Hungary	S	130	115	32.9	20.4	1	1
OAC WINTRI	Canada	W	93	95	137.7	38	2	;
AC COPIA	Canada	W	102	110	278.4	34	;-1	;
ABACUS	Australia	S	98	85	149.4	24.7	2	1
USGEN 19	South Africa	S	104	95	191.8	35.9	3	;
SSKR 626	South Africa	W	104	110	3	10	-	-
SSKR 628	South Africa	W	104	110	2.7	50	1	;
PRONGHORN	Canada	S	93	105	27.7	28	2	3
PSHERO 16	Bulgaria	W	138	100	47.2	22.4	3	2
XIN MAI 16	China	W	143	105	9.2	20	1	1
XIN MAI 18	China	W	130	105	60	23.1	2-3	3
AC ULTIMA	Canada	S	98	90	233.3	41.4	;	2-3
NE426GT	United State	W	144	90	4	16	1	3
Toorf	Australia	S	104	80	82.1	17.2	2	2
Frank	Canada	S	102	105	165.4	41.7	2-3	;
Corong	Australia	S	92	100	66.9	28.1	2	-
Koala	Mexico	S	104	85	49.7	27.5	-	-
RAH-101/3	Poland	W	144	85	-	-	-	-

ADDENDUM D… (Continued)

Genotypes	Country	Growth habitat	Days to headings	Plant Height (cm)	Grain yield/row (g)	1000-kernel weight (g)	Leaf rust	Stem rust
RAH-101/7	Poland	W	144	95	4.5	12	3	2
RAH-101/10	Poland	W	144	110	4.6	20	-	-
RAH-101/15	Poland	W	144	95	1.7	13	-	-
CARMAN	Canada	S	104	105	42	22.9	3	;
RAH-101/3	Poland	w	144	85	-	-	-	-
PRAG 42	Russia	S	104	95	67	35.4	2	3
PRAG 59/2	Russia	W	161	75	0.4	-	-	-
PRAG 60/1	Russia	W	144	105	12.8	21	1-2	3
PRAG 64/1	Russia	W	144	90	0.7	20	-	3
DRIRA (XT00ID)	Mexico	S	102	95	60	30	3	1
VESELOPODOLYANSKAYA	Russia	S	104	70	12	19	2-3	3
PRESTO	Bulgaria	W	143	85	2.2	10	-	1
VICHREN	Bulgaria	S	106	85	35.7	22.2	2-3	3
AD-19 (XT006ID)	Ukraine	S	104	100	23.8	29	3	1
PERUN	Bulgaria	S	120	100	75.9	24.2	2	;
ODESSKIJ KORMOVOJ	Ukraine	W	161	105	25.2	29	3	;
UGO	Poland	W	150	95	40.2	16.4	1	-
PRESTO	Bulgaria	W	143	85	2.2	10	-	1
VICHREN	Bulgaria	S	106	85	35.7	22.2	2-3	3
AD-19 (XT006ID)	Ukraine	S	104	100	23.8	29	3	1

ADDENDUM D… (Continued)

Genotypes	Country	Growth habitat	Days to headings	Plant Height (cm)	Grain yield/row (g)	1000-kernel weight (g)	Leaf rust	Stem rust
PERUN	Bulgaria	S	120	100	75.9	24.2	2	;
ODESSKIJ KORMOVOJ	Ukraine	W	161	105	25.2	29	3	;
UGO	Poland	W	150	95	40.2	16.4	1	-
MALNO	Poland	W	150	90	41.4	15.1	;-2	3
MEXITOL 1	Bulgaria	S	106	85	18.3	27	-	3
MARVAL	United State	S	98	70	92.7	29	3	1-2
WHITMAN	United State	F	100	80	59.3	25.9	1	1
WAPITI	Canada	S	120	100	207	39.4	3	2-3
YOREME 74	Mexico	S	93	100	148	31.2	2	;-1
BACUM	Mexico	W	98	80	70.4	26.9	3	3
RAH-101/5	Poland	W	144	100	7.5	11	1	1-2
SAMSON	Australia	S	92	90	89.1	33.4	3	;
AD 403	Ukraine	W	161	95	-	-	-	-
AD 307(XT98ID)	Ukraine	W	161	80	0.8	15	-	
Newcale	United State	W	150	80	9.2	29	;-1	-

ADDENDUM D… (Continued)

Genotypes	Country	Growth habitat	Days to headings	Plant Height (cm)	Grain yield/row (g)	1000-kernel weight (g)	Leaf rust	Stem rust
AgBeacon	South Africa	S	93	90	10	36	;	;
US2009	South Africa	S	98	95	256.5	40.2	;-1	;
TRIMONZIUM 1978	Bulgaria	S	104	100	116.9	35.6	1-2	;
CURRENCY	Australia	S	92	85	196.8	32.7	2	1
MEKSITOL 1108	Bulgaria	S	98	105	74.1	29.8	3	3
PERSENK	Bulgaria	S	102	95	65.3	28.3	2	1
PRAG 41/1	Russia	W	162	100	72.4	21.8	3	1-2
LASKO	Poland	W	150	95	12.6	20	3	;

ADDENDUM E

SSR markers alleles (in bp) size of the 108 different triticale cultivars tested.

Plant ID	Xgwm 550-1B	SCM 152-5R	SCM 2-6RL	SCM 109-5R	SCM 43-2R	SCM 159-4R
PIKA	154	300/335	109/123/147	115/97	106/121/159	114
VICHREN	154/171/183	300/335	119/132/123/137	37	140/177	-999
CURRENCY	154/171/183	300/335/270	119/132	115/97	106/121/159	114/151
FLORA	154/161	300	119/123/137	97	106/121/159	-999
RAH101/5	154/161	300/335/270	119/132	97/115	100/119/159/177	114
CORONG	-999	300/335	119/123/132/137/147	97	106/121/177	-999
NE42GT	176/196	270	119/132	97	121/140	-999
KISKUM	154/171/183	300/335	119/123/132/137/147	97	100/119/177	-999
NAD 137	154/196	298/300/335	119/132	97/115	106/121/177	-999
PRAO 6/1	154/171/183	-999	109/119/132	97/115	159	114
NAD 120	154/171/183	300/335	119/123/132/137/147	97	119/140/177	114
MUCHEBERG 1	154	300	109/123/137	97/115	106/121/177	151
UGO	154	335	119/123/132/137/147	97/115/127	106/121/121/159	-999
RAH101/15	154	270/300/335	119/132	97/115	100/119/159	-999

ADDENDUM E… (Continued)

Plant ID	Xgwm 550-1B	SCM 152-5R	SCM 2-6RL	SCM 109-5R	SCM 43-2R	SCM 159-4R
RAH 101/13	161	300/335	119/132	97	106/121/159/177	-999
TRIMONZIUM 1978	-999	-999	123/137	97	106/121/159	114/151/167
BEESEE	-999	270/300/335	109/123/137	97	106/121/159	126/167
RAH 101/12	-999	270/300	119/132	97	100/119/159	114/151
AC CERTA	154	270/300	119/132	97/115	106/121/159	129/151/167/126 /114
SZALKAS	-999	270/335	119/123/132/137/147	97	106/121/159	114/151
AD307 2	-999	-999	119/123/137/147/132	97	121/159/177	114/151
LAD 130	-999	300	109	97	177	114/151/167
AC ALTA	-999	270/300/335	119/132	97	106/121/159	114/151
SALZMUNDER BARTWEIZEN	-999	300/335	109	97/100	-999	129/151/167
JOSEPH	-999	270/300/335	119/132	97/115	106/121/159	114/151/167
25 AD 20	154	270/298/300/335	109/123/137	97/100	100/119/159	126/129/151/167
XIN MAI 16	-999	-999	109	97/100	119/127/172	114/126/167

ADDENDUM E… (Continued)

Plant ID	Xgwm 550-1B	SCM 152-5R	SCM 2-6RL	SCM 109-5R	SCM 43-2R	SCM 159-4R
AD 174	176	270/335	119/132	97/127	177	129/167
DNEPROVSKIJ	-999	270/298/300/335		97/127	-999	
MORRISON	154/196	300/335	119/132	97	106/121/159/177	114/126/167
TOORF	154	270/300	109/123/137	97	177	167
AD19 1	-999	270/335	119/132	97	106/121/159	167
KOALO	-999	270/300/335	119/132	97	-999	167
DELFIN 80	-999	270/335/300	119/132	97	-999	126/129/151/167
MARVAL	-999	300/335/270	119/123/137/132/147	97	106/121/159	114/129/151
WHITMAN	154	270/300/335	119/132	97	106/121/159	129/151/167
CARMAN	154	300/335	119/132	97	106/121/140/159/177	-999
PRAO 39/1	154	300/335	109/123/137	97	106/121/159	-999
SSKR 628	154/171	270/335/300	119/132	97	106/121/159	114/126/167
JABALI	154/171/183	270/300/335	119/123/132/137/147	97	106/121/159	167
MEXITOL 1	154/171	300/335	119/123/132/137/147	97/115	-999	114/151
FROSTY	-999	270/300/335	119/132	97/115	106/121/159	114/151

ADDENDUM E … (Continued)

Plant ID	Xgwm 550-1B	SCM 152-5R	SCM 2-6RL	SCM 109-5R	SCM 43-2R	SCM 159-4R
SAMSON	154	270/335	119/132	97	106/121/159	114/151/167
VESELOPODONSKAYA	154/161	270	119	97/115	-999	114/126/129/140
TOMZI	154/171/183	270/300/335	119/123/132/137/147	97	100/119/159	114/129
JENKINS	154/161	300/335/270	109/123/147	97	106/121/159	126/129/151/167
DRIRA 2	154	270/300/335	119/123/132/137/147	97	106/121/159	114/126/129/151/167
LASKO	-999	270/300/335	119/132	97	100/119/159	-999
ZEBRA	154/161	270/300/335	119/132	97	117	114/151
BEAGLE 82	161/171/183	300/335	119/132	97	106/121/159	-999
NEWCALE	154/171/183	300	119/132	97	121/140	-999
AD 403	154/171/183	270/300	119/132	97/100/127	177	114/151
Rex	154/171/183	270/298/300/335	119/123/132/137/147	97/115	106/121/159	-999
US2008	154/171/183	270/298/335/300	119/132	97/115	106/121/159	114/126/129/167
Tobie	-999	270/298/300/335	119/132	97/115	106/121/159	114/126/129/140/167
Elite20	154/171/183	270/298/300/335	119/123/132/137/147	97/115	106/121/159	114/126/129/167
Ibis	154/171/183	270/298/300	119/123/132/137/147	97/115	106/121/159	114/126/129/167

ADDENDUM E… (Continued)

Plant ID	Xgwm 550-1B	SCM 152-5R	SCM 2-6RL	SCM 109-5R	SCM 43-2R	SCM 159-4R
TRITICALE 64	154/161/183	270/298/300/335	119/123/132/137/147	97/115	106/121/159	114/126/129/167
AC ULTIMA	154	270/298/300	119/132	97	-999	-999
PRONGHORN	154/171/183	-999	119/123/132/137/147	97	106/121	114/151/167
BOKOLO	154	-999	119/123/132/137/147	97	100/119/159/177	114/126/129/167
KRAMER	176/196	300/335	119/132	97	-999	-999
MUIR	154/161	300/335	119/123/132/137/147	97/115	106/121/159	167
WAPITI	-999	270/335	119/123/132/137/147	97/115	106/121/159	-999
KARL	154/161	300/335	119/132	97	-999	114/151/167
PRAG 31	-999	270/300	119/132	97	106/121/159	114/151/167
NAD 325	-999	270/335	119/132	97/100	106/121/159/177	129/151/167
AC COPIA	154/183	270/300	119/123/137	97/115	106/121/159	129/151/167
SSKR 626	-999	270/300/335	109/123/137	97	-999	129/151
RAH 101/10	161/176/183	270/300/298/335	119/132	97	100/119/159/177	151
PRAG 64/1	-999	-999	119/132	97	106/121/159	-999
AD19 2	154	270/300/335	119/123/132/137/147	97/127	-999	114/151/167

ADDENDUM E… (Continued)

Plant ID	Xgwm 550-1B	SCM 152-5R	SCM 2-6RL	SCM 109-5R	SCM 43-2R	SCM 159-4R
SATU	-999	270/300	109/123/137	97	-999	126
PERSENK	-999	300	119/123/132/137/137	97	-999	114/126/167
SUNLAND	154	300/335	119/123/132/137/147	97/115	100/119	-999
PUPPY	-999	270/335	119/123/132/137/147	97	177/121/159	126/167
AD 201	-999	-999	119/132	97/127	177	129/151/167
BREAKER	-999	270/300/335	-999	97	100/119/159/177	114/129/140/167
PERUN	-999	270/335	119/132	97	-999	114/151/167
PRAG 60/1	171/183/161	270/300/335	119/132	97/115	-999	-999
ABACUS	161/176/196	335	119/132	97	106/121	114/126/129/167
ZITNICA	154/161	270/300/335	109/123/137	97	106/121/159/177	151
Bacum	154/171/183/196	270/300	119/123/137	100/97	177	114
DELFIN 20s	154	270/300	119/132	97	106/121/159	-999
PRAG 59/2	154/171	270/300	109	97/127	-999	-999
MT 7291	154/176/196	335	119/132	97	177/159	-999
MEKSITOL 1108	154/176/196	270	119/132	97	-999	-999

ADDENDUM E… (Continued)

Plant ID	Xgwm 550-1B	SCM 152-5R	SCM 2-6RL	SCM 109-5R	SCM 43-2R	SCM 159-4R
BUFFALO	-999	300	119/123/132/137/147	97	-999	114/126/151/167
NAD 329	-999	270/300/335	109	97/100	106/121/151	167
PSHERO 16	154	270/300/335	119/132	97	106/121/159/177	114/126/129/151 /167
XIN MAI 18	161	300	109/123/137	97/100	106/121/159	126/151/167/129
PRAG 41/1	154	270/300	119/132	97	-999	114/167
Bacchus	154/171/183	270/298/300/335	119/132	97/119	106/121/159/177	114/126/129/167
USgen 19	-999	-999	119/123/132/137/147	97/115	106/121/159	114/126/129/167
Drira 1	154/171/183	300/335	119/123/132/137	97	106/121/159	-999
OAC WINTRI	154/161/183	300	119/132	97	100/119/159	129/167
RAINDEER	161/176	270/300	-999	97	106/121	167
FRANK	154	335	119/123/132/137/147	97/115	106/121/159	129/151/167

ADDENDUM E … (Continued)

Plant ID	Xgwm 550-1B	SCM 152-5R	SCM 2-6RL	SCM 109-5R	SCM 43-2R	SCM 159-4R
ODESSKIJ KORMOVOJ	154/196	270/298/300/335	119/123/132/137/147	100/115	106/121/159/177	114
YOROME 74	154/176/196	-999	119/132	97	-999	114/126/129/151 /167
PRAG 42	154/196	270/298/300/335	109/123/137/147	97/100/115/127	106/121/159	-999
US2007	154/171/183	270/298/335/300	119/123/132/137/147	97/115	106/121/159	114/126/129/169
PRESTO	-999	270/300	119/132	97	106/121/159/177	-999
MALNO	-999	300/335	109/123/137	97/115	106/121/159	151
AD307 1	161/196	-999	119/123/137/147	97	177	114/129/151/167
KISSA 14	-999	270/300	119/132	97	-999	114/151
BUFFALO	-999	300	119/123/132/137/147	97	-999	114/126/151/167
OoM JAN	154/171/176	300270/300/335	119/132	97	-999	-999
RAH 101/7	161/176/183	270/300/335	119/132	97	100/119/159/177	114/151/167

ADDENDUM F

SSR markers alleles (in bp) sizes of the 15 different BC $_4F_1$ progenies and crossing parents.

Plant ID	SCM 43-2R	Xgwm550-1B	SCM159(F2)-4R	SCM 2-6R	SCM152(F1)-4R	SCM109-5RL
11T023 F	106/121/159/177	335/400/396/443	126/114/151/167	119/132	252/298/335	97/115
11T023 A	106/121/159	335/400/443	126/119/167	119/123/132/137/147	298/335	97/100/115
11T023 I	106/121/159/177	335/400/396/443	119/126/167/114	119/123/132/137/167	252/298/335	97/100/115
11T023 B	106/121/159/177	335/396/443	114/119/151/167	119/132	270/335	97/115
11T023 C	106/121/177	335/400/443	114/129/167	119/123/132/137	252/298/335	97/115
11023 G	106/121/159/177	335/396/400	114/119/126/151/167	119/123/132	252/298/335	97/115
11023 H	106/121/159/177	335/443	119/151/167	119/123/132	252/298/335	97/115/127
11T023 D	106/121/159/177	335/400/443	126/129/151/167	119/132	252/298/335	97/115
11T023 E	106/121/159/177	335/400/443	114/129/167	119/132	252/298/335	97/115/100
11T028 D	106/121/159	335/443/500	114/140/167	119/123/132/137	252/298/335	97/115/100
11T028 E	106/121/159	-999	114/129/140/167	119/123/132/147	270/298/335	97/115
11T028 F	106/121/159/177	335/400/396/443	114/129/140/151/167	119/123/132/137/147	270/335	97/100/115
11T028 B	106/121/159	335/400/443	114/129/151/167	119/123/132/137/147	252/270/298/335	97/115/127
11T028 A	106/121/159	335/400	126/129/167	119/123/132/137/147	298/300	97/115

ADDENDUM F… (Continued)

Plant ID	SCM 43-2R	Xgwm550-1B	SCM159(F2)-4R	SCM 2-6R	SCM152(F1)-4R	SCM109-5RL
11T028 C	106/121/159	335/443	114/129/167	119/123/132/137/147	300/335	97/100/115
US 2007	106/121/159	335/400/443	140/167	119/123/132/137/147	270/298/335	97/115
US2008	106/121/159	335/396/443	129/151/167	119/132	270/298/335	97/115
C1tr1202STR (*Ba*)	140/177	443/400/470	129/140/167	109/123/137	270/298/335	97/100/115