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ORIGINAL PAPER

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Sequence tagged microsatellites for the *Xgwm533* locus provide new diagnostic markers to select for the presence of stem rust resistance gene *Sr2* in bread wheat (*Triticum aestivum* L.)

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Abstract The stem rust resistance gene *Sr2* has provided durable broad-spectrum, adult-plant resistance to the fungal pathogen *Puccinia graminis* Pers. f. sp. *tritici* throughout wheat-growing regions of the world for more than 50 years. The ability to select for *Sr2* in wheat breeding programs was recently improved by the identification of a tightly linked microsatellite marker *gwm533*. This marker typically amplifies a 120-bp polymerase chain reaction fragment from wheat lines carrying *Sr2*. In instances where the 120-bp fragment is not associated with the presence of *Sr2*, DNA sequence analysis has shown that a second allele was amplified, differing in the structure of the microsatellite repeat. To discriminate this allelic homoplasy (alleles identical in size, but not identical by descent), sequence-tagged microsatellites (STM) markers were developed for the *Xgwm533* locus. These markers were shown to be diagnostic for the presence of *Sr2* in a wide range of germplasm, representative of all major wheat varieties historically grown in Australia. The STMs will be particularly useful for marker-assisted selection in Southern Australian breeding programs, where the use of the marker *gwm533* is often precluded by the presence of the non-*Sr2*-associated 120-bp allele in the pedigree of current breeding germplasm. The STMs also revealed a high incidence of previously undetected allelic homoplasy at the *Xgwm533* locus and may have broader utility in genetic research and breeding, as this locus is also reported to be strongly associated with a major gene conferring resistance to Fusarium head blight.

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Introduction

Stem rust resistance conferred by the *Sr2* gene located on the short arm of chromosome 3B is an important disease resistance gene in many wheat breeding programs around the world. For more than 50 years, this adult plant resistance gene has provided effective broad-spectrum resistance to wheat stem rust caused by the fungal pathogen *Puccinia graminis* Pers. f. sp. *tritici*. The importance of *Sr2* in modern wheat breeding is reflected by its presence in many current wheat varieties (McIntosh 1988; Rajaram et al. 1988; Roelfs 1998).

Resistance conferred by *Sr2* is characterized by a slow-rusting response and variable levels of disease symptoms on field grown, adult plants (McIntosh et al. 1995). This resistance gene tends to be non-specific and is currently effective against all isolates of *P. graminis* throughout wheat-growing regions of the world (Sunderwith and Roelfs 1980; McIntosh et al. 1995). The *Sr2* resistance provides a desirable genetic background for the deployment of other effective, but less durable, stem rust resistance genes (McIntosh 1988). Consequently, the use of *Sr2* remains a major factor in the control of wheat stem rust in Australia (McIntosh 1992). It is also a key gene deployed in the CIMMYT wheat breeding program (Rajaram et al.1988; Rajaram and Ceccarelli1988), and is therefore important in the developing world spring wheat areas.

As *Sr2* resistance is recessively inherited, the phenotype can be difficult to select in breeding programs. The severity of disease symptoms can also be influenced by the presence of other stem rust resistance genes and environmental effects (Brown 1993; McIntosh et al. 1995). Moreover, the resistance phenotype is expressed only at the adult plant stage, which delays the classification of

progeny (Roelfs 1988). Morphological markers tightly linked to *Sr2*, such as pseudo-black chaff (PBC) and high-temperature-induced seedling chlorosis (HTISC) have been used to indirectly select the resistance phenotype in breeding programs (Eagles et al. 2001), but these markers have limitations. PBC, a dark pigment that develops on the glumes and below stem internodes, is partially dominant and has variable levels of expression in different genetic backgrounds and environments (Bhowal and Narkhede 1981). High PBC expression is also thought to reduce yield (Hare and McIntosh 1979). Similarly, HTISC is recessively inherited (Brown 1997) and is therefore not ideal for marker-assisted breeding despite being tightly linked to *Sr2*.

The deployment of *Sr2* in wheat breeding programs was recently accelerated by the identification of a tightly linked microsatellite marker, *gwm533*. In a survey of a representative but unrelated set of Australian cultivars that differed for the presence-absence of *Sr2*, Speilmeyer et al. (2003) showed that a 120-bp polymerase chain reaction (PCR) fragment was amplified from all wheat lines carrying the resistance gene, while a 155-bp or null allele was amplified from wheat lines lacking *Sr2*. The strong association of the 120-bp allele with the presence of *Sr2* was also confirmed in a set of international cultivars. In only a few instances was the 120-bp PCR fragment amplified from Australian wheat varieties that were assessed to not carry the resistance gene. DNA sequence analysis of the 120-bp fragments amplified from wheat varieties known to carry *Sr2* and those without the resistance gene revealed that the marker *gwm533* amplified at least two 120-bp alleles in Australian germplasm that differed in the structure of their microsatellite repeat sequences (Speilmeyer et al. 2003). These results showed that the 120-bp fragment amplified by the marker *gwm533* provided a useful tool to select for the presence *Sr2* in most genetic backgrounds, providing that the *Sr2* status and pedigree of the parental cultivars used for crossing was known.

In Southern Australian wheat breeding programs, the use of the marker *gwm533* for the introgression of *Sr2* is often complicated by the presence of wheat varieties, namely from the Aroona family, in the pedigree of current breeding germplasm that amplify the non-*Sr2* associated 120-bp microsatellite allele. In these instances, marker-assisted selection of *Sr2* is precluded by the absence of a detectable polymorphism that can be assayed using the published microsatellite marker. To overcome this limitation, two sequence-tagged microsatellite (STM) markers (Hayden et al. 2004) were developed to exploit the microsatellite sequence variation in the two 120-bp *gwm533* alleles reported by Speilmeyer et al. (2003). The ability of these markers to discriminate the reported allelic homoplasy (alleles identical in size, but not identical by descent, Grimaldi and Crouau-Roy 1997) at the *Xgwm533* locus demonstrated that the two STMs were diagnostic for the presence of *Sr2* in Australian wheat germplasm. Moreover, an extensive historical survey of Australian wheat varieties and lines important for breeding purposes revealed additional allelic homoplasy, demonstrating that the STMs provide a high-level allelic discrimination power at the *Xgwm533* locus.

Materials and methods

Plant material

A set of 12 unrelated wheat varieties differing for the presence-absence of *Sr2* but carrying the 120-bp PCR fragment amplified by the microsatellite marker *gwm533* were used to confirm that the two STMs could differentiate the *Sr2* and non-*Sr2* associated microsatellite alleles reported by Speilmeyer et al. (2003). The wheat varieties used for the historical survey of Australian wheat were derived from the set of cultivars used by Paull et al. (1998). These cultivars were selected by Paull et al. (1998) on the basis of: (1) being of historical significance to wheat breeding and production in Australia, (2) each accounting for more than 2% of deliveries to silos in any Australian state since 1982, (3) recently released varieties carrying agronomic traits of interest and (4) significant parents in current breeding programs. The subset of wheat varieties used in the present study was selected based on the availability of phenotypic assessment for *Sr2* status, as determined by the Australian National Rust Control Program (Bariana et al. 1998), and is presented in Table 1. Genetic mapping of SSRs was performed in the Cranbrook × Halberd doubled haploid population, composed of 161 progeny (Kammholz et al. 2001). This population was previously shown to segregate for the *Sr2* resistance gene, with Cranbrook being the donor parent (Bariana et al. 2001). DNA was extracted from the bulked leaf material of six 2-week-old seedlings (Rogowsky et al. 1991).

PCR amplification and analysis of the Xgwm533 locus

PCR amplification of the *Xgwm533* locus was performed in a 10-μl reaction mixture containing 0.2 mM dNTP, 1× Qiagen PCR buffer, 0.2 μM of each primer, 0.25 U *Taq* DNA polymerase (Qiagen) and 50 ng genomic DNA. Primer sequences for the microsatellite marker *gwm533* (Roder et al. 1998) and STMs are given in Fig. 1. The reverse primers of the markers *gwm533*, *stm559tgag* and *stm598tcac* were dye-labeled with HEX, FAM and NED, respectively (Applied Biosystems). Following an initial denaturation step of 3 min at 92°C, PCR was performed for 47 cycles with the touchdown profile: 30 s denaturation at 92°C, 30 s annealing at 62°C and 30 s extension at 72°C. Following the first cycle, the annealing temperature was reduced by 1°C per cycle for the next seven cycles. The PCR products for each set of markers were mixed together in a ratio of 1:1:5 for FAM:NED:HEX. The fluorophore dye HEX was pooled at a higher ratio due to its weaker signal compared to the other fluorophores. The

Table 1 Wheat varieties, *Sr2* status and allelic constitution of the *Xgwm533* locus as determined using the microsatellite markers *gwm533*, *stm559tgag* and *stm598tcac*

Variety	*Sr2* status	Observed allele size (bp)		
		gwm533	*stm559tgag*	*stm598tcac*
Batavia	+	120	83	56
Baxtar	+	120	83	56
Blade	+	120	83	56
Cranbrook	+	120	83	56
Diamondbird	+	120	83	56
Dollarbird	+	120	83	56
Goldmark	+	120	83	56
Hartog	+	120	83	56
Lowan	+	120	83	56
Pelsart	+	120	83	56
Rowan	+	120	83	56
Siete Cerros	+	120	83	56
Sunbrook	+	120	83	56
Suneca	+	120	83	56
Sunstate	+	120	83	56
Banks	−	120	81	58
Bindawarra	−	120	81	58
Worrakatta	−	120	81	58
Amery	−	120	79	60
Condor	−	120	79	60
Gutha	−	120	79	60
Mawson	−	120	79	60
Sunco	−	120	79	60
Tammin	−	120	79	60
Vectis	−	120	79	60
Angus	−	120	77	62, 56
Aroona	−	120	77	62, 56
Barunga	−	120	77	62, 56
Krichauff	−	120	77	62, 56
Molineux	−	120	77	62, 56
Schomburgk	−	120	77	62, 56
Tatiara	−	120	77	62, 56
Yarralinka	−	120	77	62, 56
Excalibur	−	141	95	66
Bungulla	−	141	105	56
Avocet	−	149	95	74
Dagger	−	149	95	74
Halberd	−	149	95	74
Kalgarin	−	149	95	74
Silverstar	−	149	95	74
Stiletto	−	149	95	74
Sunbri	−	149	95	74
Sunelg	−	149	95	74
Sunland	−	149	95	74
Sunvale	−	149	95	74
Frame	−	149, 141	95	74, 66
Trident	−	149, 141	95	74, 66
Braewood	−	Null	Null	Null
Cocamba	−	Null	Null	Null
Currawong	−	Null	Null	Null
Giles	−	Null	Null	Null
Grebe	−	Null	Null	Null
Harrier	−	Null	Null	Null
Janz	−	Null	Null	Null
Meering	−	Null	Null	Null
Rosella	−	Null	Null	58
Vulcan	−	Null	Null	58
Corella	−	Null	Null	58

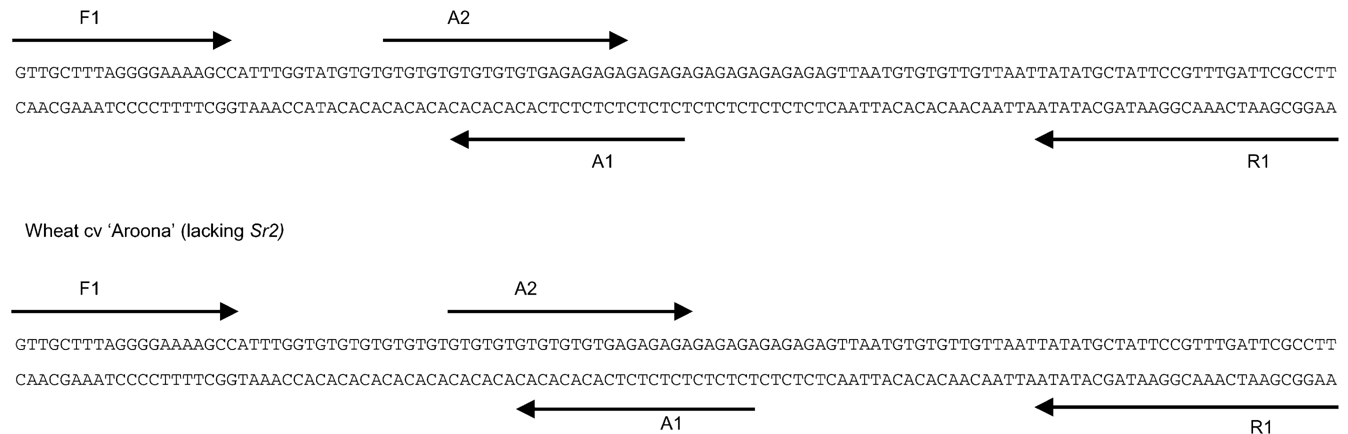
mixed PCR products were separated by capillary electrophoresis, using an ABI3700 instrument and ROX-labeled Genescan400HD as an internal size standard (Applied Biosystems). Allele sizes were determined using GeneScan, version 3.7, software (Applied Biosystems). Graphical images of the SSR fragments detected in the ABI3700 traces were generated using the publicly available software Genographer, version 1.6.0 (Benham et al. 2001). All PCR reactions were performed in duplicate to confirm cases of null alleles.

Results and discussion

Development of STMs for the *Xgwm533* locus

Speilmeyer et al. (2003) showed by DNA sequence analysis that the two 120-bp PCR fragments amplified by the microsatellite marker *gwm533* from wheat lines known to carry *Sr2*, and those without the resistance gene differed by the number of dinucleotide repeat units that formed the compound microsatellite motif. The 120-bp alleles amplified from wheat lines carrying *Sr2* and those without *Sr2* contained microsatellite sequences with composition $(TG)_{10}(AG)_{13}$ and $(TG)_{13}(AG)_{10}$, respectively (Fig. 1). To exploit the DNA sequence variation within the microsatellite repeat and generate markers that could distinguish the two 120-bp homoplasic alleles, a pair of STM markers was developed as illustrated in Fig. 1. F1 and R1 are the published forward and reverse primers of the microsatellite marker *gwm533*, respectively, and A1 and A2 are primers that anchor to the repeat junction of the compound microsatellite. The ability of the STMs to exploit the allelic homoplasy at the *Xgwm533* locus results from only one half of the compound repeat being assayed by each marker, i.e. the forward STM assay (marker *stm559tgag*), using the primer combination F1/A1, amplifies only the $(TG)_m$ portion of the microsatellite repeat, while the reverse STM assay (marker *stm598tcac*), using the primer combination R1/A2, amplifies the $(AG)_n$ portion of the repeat motif. In contrast, the conventional SSR assay using the F1/R1 primers for the marker *gwm533* assays the entire repeat sequence, and is therefore unable to detect cryptic variation in the number of dinucleotide repeats forming each portion of the compound microsatellite when no net

Wheat cv ‘Hope’ (with *Sr2*)



Marker	Forward Primer Sequence	Reverse Primer Sequence	Expected PCR fragment size (bp)	
			Hope	Aroona
gwm533	F1 GTTGCTTTAGGGGAAAAGCC	R1 AAGGCGAATCAAACGGAATA	120	120
stm598tcac	F1 GTTGCTTTAGGGGAAAAGCC	A1 TCTCTCTCTCTCTCACACACAC	61	67
stm559tgag	R1 AAGGCGAATCAAACGGAATA	A2 TGTGTGTGTGTGTGAGAGAGAG	85	79

Fig. 1 DNA sequences of the 120-bp alleles amplified by the microsatellite marker *gwm533* from genomic DNA of wheat varieties Hope (*Sr2*) and Aroona (lacking *Sr2*) (from Speilmeyer et al. 2003)

change in the overall length of the repeat motif is generated (Fig. 1).

Based on the number of dinucleotide repeat units reported by Speilmeyer et al. (2003) to be present in each portion of the compound SSR of the two 120-bp alleles amplified by the marker *gwm533*, the marker *stm559tgag* was expected to generate a PCR fragment from wheat lines carrying *Sr2* that was 6 bp smaller in size than from wheat lines lacking the resistance gene. In contrast, the marker *stm598tcac* was predicted to amplify a PCR fragment that was 6 bp larger from wheat lines without *Sr2*.

Validation of the STM markers

To determine if the two STMs could differentiate the *Sr2* and non-*Sr2*-associated 120-bp microsatellite alleles reported by Speilmeyer et al. (2003), a set of unrelated wheat varieties differing for the presence-absence of *Sr2* but carrying the 120-bp PCR fragment amplified by the marker *gwm533* were tested (Fig. 2). In all instances, the two STMs amplified a single PCR fragment from wheat lines carrying *Sr2*. The marker *stm559tgag* amplified an 83-bp fragment (Fig. 2b), while *stm598tcac* amplified a 56-bp fragment (Fig. 2c). However, two different PCR banding patterns were observed in wheat lines that lacked the stem rust resistance gene. For the wheat variety Bindawarra, 81-bp and 58-bp PCR fragments were amplified by the markers *stm559tgag* and *stm598tcac*, respectively (Fig. 2b, c). In contrast, a 77-bp PCR fragment was amplified by *stm559tgag* from the remaining varieties (Fig. 2b), and two PCR fragments of 56-bp and 62-bp were amplified by the marker *stm598tcac* (Fig. 2c). Based on the predicted and observed STM fragment sizes, it is likely that the 77-bp and 62-bp PCR fragments amplified by *stm559tgag* and *stm598tcac* from wheat lines without *Sr2* were allelic and corresponded to the 120-bp allele amplified by the marker *gwm533*. Similarly, it is likely that the 81-bp and 58-bp fragments amplified from the wheat cv. Bindawarra (lacking *Sr2*), and the 83-bp and 56-bp fragments amplified from wheat lines carrying *Sr2* by *stm559tgag* and *stm598tcac*, respectively, were allelic and corresponded to the 120-bp *gwm533* allele. These results indicated that both STMs discriminated the 120-bp allelic homoplasy at the *Xgwm533* locus, as the expected 6-bp difference in PCR fragment sizes was observed between wheat lines that differed for the presence-absence of *Sr2* when the $(TG)_m$ and $(AG)_n$ portions of the compound microsatellite repeat were assayed (Table 2). These results also indicate that the wheat cv. Bindawarra carried a third, and previously uncharacterized, homoplasic 120-bp *gwm533* allele. Further demonstrating that the STMs amplified the *Xgwm533* locus was the cosegregation of the two STMs and microsatellite marker *gwm533* in the Cranbrook × Halberd doubled haploid population (Fig. 3), a population previously shown to segregate for the *Sr2* resistance gene (Bariana et al. 2001).

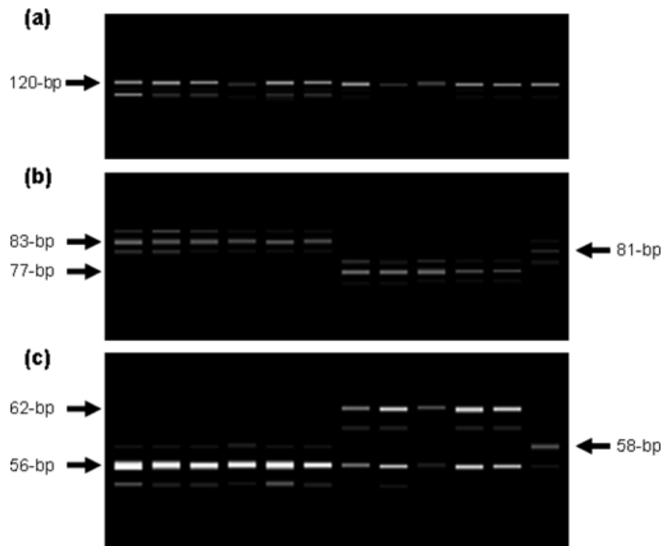


Fig. 2 Polymerase chain reaction amplification of the *Xgwm533* locus in an unrelated set of Australian wheat varieties differing for the presence of *Sr2* using the markers **a** *gwm533* **b** *stm559tgag* and **c** *stm598tcac*. The wheat varieties containing *Sr2* were Cranbrook, Dollarbird, Hartog, Pelsart, Rowan and Suneca (*lanes 1–6*), and those without *Sr2* were Aroona, Barunga, Molineux, Schomburgk, Yarralinka and Bindawara (*lanes 7–12*)

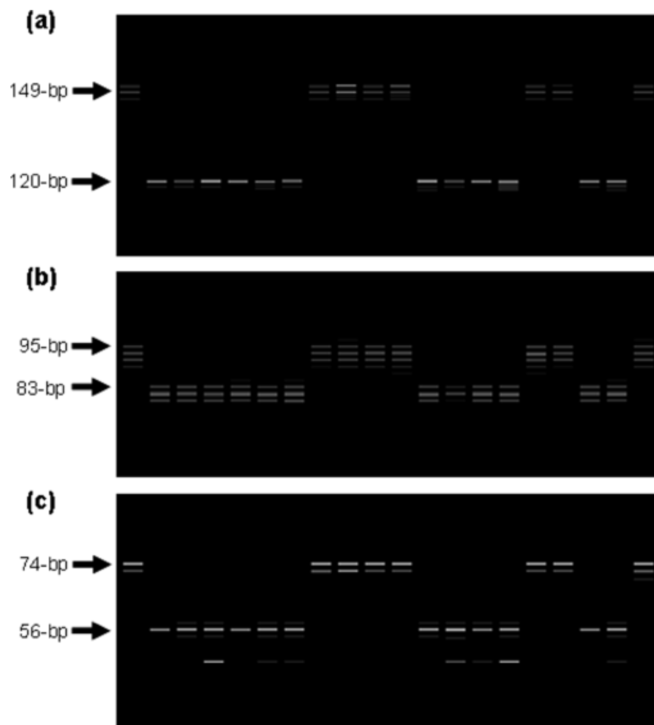


Fig. 3 Cosegregation of the markers **a** *gwm533*, **b** *stm559tgag* and **c** *stm598tcac* in the Halberd × Cranbrook doubled haploid population. From *left to right*: Halberd (lacking *Sr2*), Cranbrook (*Sr2*) and 18 progeny

The amplification of two PCR fragments from several wheat lines not carrying the stem rust resistance gene indicated that the marker *stm598tcac* also amplified a second microsatellite locus (Fig. 2c). However, the chromosomal origin of this SSR could not be determined due to the absence of a mapping population in which the locus segregated. Furthermore, this SSR could not be assigned to a chromosome by aneuploid analysis, as the second locus was not amplified in the wheat variety Chinese Spring (data not shown).

Table 2 Sizes of polymerase chain reaction (PCR) fragments amplified from wheat varieties differing for the presence-absence of *Sr2* and the predicted composition of the compound SSR for each allele at the *Xgwm533* locus

Observed PCR fragment size (bp)			*Sr2* status	Diff.[a] (bp)	Predicted SSR composition
gwm533	*stm559tgag*	*stm598tcac*			
120	83	56	+	NA[b]	$(TG)_{10}(AG)_{13}$
120	77	62	−	6	$(TG)_{13}(AG)_{10}$
120	81	58	−	2	$(TG)_{11}(AG)_{12}$

[a]Size difference between PCR fragments amplified from wheat lines carrying *Sr2* and those without *Sr2*
[b]*NA* Not applicable

Allelic homoplasy at the *Xgwm533* locus in Australian germplasm

The observation that the wheat cv. Bindawarra carried a third 120-bp allele amplified by the marker *gwm533* indicated the presence of additional, and previously unreported, allelic homoplasy at the *Xgwm533* locus in Australian germplasm. To investigate the extent of allelic homoplasy at this locus, a survey of 60 major wheat varieties historically grown in Australia was undertaken using the marker *gwm533* and two STMs (Table 1). In total, four alleles were detected using the marker *gwm533*, and nine alleles were revealed using the two STMs (Table 3).

A 120-bp PCR fragment was amplified from 33 (55%) of the wheat varieties surveyed, using the microsatellite marker *gwm533*. The 120-bp PCR fragments were resolved as four homoplasic alleles, using the STM markers. The 120^a allele reported by Speilmeyer et al. (2003) and found in the present study to be completely associated with the presence of *Sr2* (Table 1) was the most (45.5%) common allele observed, followed by the 120^d, 120^c and 120^b alleles with frequencies of 24.2, 21.2 and 9.1%, respectively (Table 3). These results show that the 120^a allele revealed by the STM markers was diagnostic for the presence of *Sr2* in the germplasm tested. Moreover, the relatively frequency of the four homoplasic alleles in the germplasm surveyed indicated that the STMs would have particular utility in marker-assisted breeding to select for the presence of *Sr2*, as an allele size difference of at least 4 bp would differentiate progeny segregating for the presence-absence of the resistance gene. This allele size difference can be routinely resolved using high-resolution agarose and polyacrylamide gel electrophoresis.

Allelic homoplasy was also observed among wheat lines that carried the 141-bp and null alleles amplified by

Table 3 Allelic diversity and homoplasy detected at the *Xgwm533* locus in a survey of wheat varieties historically grown in Australia

Allele code	Allele size (bp)			No. of alleles observed	Allele frequency (%)	Relative frequency of 120-bp variants	*Sr2* status
	gwm533	*stm598tcac*	*stm559tgag*				
120[a]	120	56	83	15	25.0	45.5%	+
120[b]	120	58	81	3	5.0	9.1%	−
120[c]	120	60	79	7	11.7	21.2%	−
120[d]	120	62, 56	77	8	13.3	24.2%	−
141[a]	141	56	105	1	1.7	NA	−
141[b]	141	66	95	3	5.0	NA	−
149	149	74	95	12	20.0	NA	−
Null[a]	Null	58	Null	3	5.0	NA	−
Null[b]	Null	Null	Null	8	13.3	NA	−

the marker *gwm533*. Within the four wheat varieties carrying the 141-bp allele, two homoplasic alleles were detected using the STM markers (Table 3). Similarly, two homoplasic alleles were detected among wheat lines that revealed a null allele with the marker *gwm533*. The amplification of a 58-bp PCR fragment by *stm598tcac* from 28% (3/11) of wheat varieties that produced the null *gwm533* allele (null[a], Table 3) would be particularly useful in marker-assisted selection, as it would eliminate the uncertainty that can arise when null alleles must be discerned from failed PCR reactions. The absence of detectable allelic homoplasy was only observed for the 149-bp PCR fragment amplified by the marker *gwm533* (Table 3). Heterogeneity at the *Xgwm533* locus was observed in the wheat varieties Frame and Trident (Table 1). However, it is unclear from the present results if this was due to residual heterozygosity within the wheat varieties or the presence of mixtures in the seed samples. The apparent lack of allele fixation among wheat varieties without *Sr2* suggests that there has been no other selection for the chromosomal region containing the *Xgwm533* locus during the history of wheat breeding in Australia.

Conclusion

Marker-assisted breeding requires robust markers that are effective across different genetic backgrounds. The results of the present study show that the two STMs developed for the *Xgwm533* locus are diagnostic for the presence of *Sr2* in a broad range of Australian germplasm, representative of all major cultivars historically grown in Australia. In particular, the STMs clearly differentiated the allelic homoplasy reported by Speilmeyer et al. (2003) that prevents the selection of *Sr2* in certain genetic backgrounds, using the microsatellite marker *gwm533*. The present results show that either STM marker could be deployed in breeding to select for the presence of *Sr2*. These markers are expected to have wide utility in Southern Australian breeding programs where the use of the marker *gwm533* is often precluded. Moreover, the high allelic-discrimination power provided by the STMs may provide broader application for their use in genetic research and breeding, as the *Xgwm533* locus is also strongly associated with a major gene conferring resistance to Fusarium head blight (Buerstmayr et al. 2003; Zhuo et al. 2003; Anderson et al. 2001).

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