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A new approach to extending the wheat marker pool by anchored PCR amplification of compound SSRs

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Abstract A study was undertaken to determine the utility in bread wheat of anchored PCR for the development of single locus SSR markers targeted at compound repeat motifs. In anchored PCR, microsatellite amplification is achieved using a single primer complementary to the flanking sequence, and one which anchors to the repeat junction of the compound SSR. The recovery rate of useable markers was found to be similar (43%) to that reported for conventionally generated SSRs. Thus, anchored PCR can be used to reduce the costs of marker development, since it requires that only half the number of primers be synthesised. Where fluorescence-based platforms are used, marker deployment costs are lower, since only the anchoring primers need to be labelled. In addition, anchored PCR improves the recovery of useful markers, as it allows assays to be generated from microsatellite clones with repeat sequences located close to their ends, a situation where conventional PCR amplification fails as two flanking primers cannot be designed. Strategies to permit the large-scale development of compound SSR markers amplified by anchored PCR are discussed.

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Introduction

Microsatellites, or simple sequence repeats (SSRs), have a range of applications in agricultural research, such as genotype identification, management of breeding populations, genome mapping, marker assisted breeding and population genetic studies (Gupta and Varshney 2000; Prasad et al. 2000). Based on tandem repeats of 1–6 nucleotides, SSRs are abundant and widely distributed throughout most eukaryotic genomes, although their frequency and type varies among species (Litt and Luty 1989; Lagercrantz et al. 1993). As molecular markers, SSRs combine many desirable marker properties including high levels of polymorphism, co-dominance, rapid genotyping assays, and amenability to automation. Moreover, the exchange of SSR assays between laboratories requires only the transfer of primer sequences, thus providing an ideal tool for collaborative research.

In many plant species, the availability of SSRs is hampered by the considerable cost and effort involved in their development. Conventionally, this requires the sequencing of clones containing microsatellite repeats in order to allow the design of PCR primers that are able to amplify the target sequence. The recovery rate of functional SSRs is generally low, due to a variety of causes, including lack of polymorphism, failure of primers to amplify, and lack of sufficient flanking sequence suitable for primer design. As a consequence, large-scale isolation of SSR markers has only been undertaken for commercially important crop species, such as the cereals maize, rice and wheat, and soybean (Cregan et al. 1998; Röder et al. 1998; Senior et al. 1996).

Several strategies have recently been described to improve the efficiency of SSR marker development. These approaches have attempted to target the development of SSRs to specific regions of the genome (Cregan et al. 1999; Hayden and Sharp 2001b; Li et al. 2001), and reduce the cost of marker development by utilising strategies that reduce the number of primers required (Fisher et al. 1996; Hayden and Sharp 2001a). An example of the latter approach is anchored PCR, in

which microsatellite amplification is achieved with one primer complementary to the flanking sequence, and one that is specific for the repeat motif. In principle, this technique halves the cost of marker development compared to conventional SSR amplification, which is achieved using a pair of specific primers flanking the repeat. It may also serve to improve the marker recovery rate since it can exploit SSRs located so close to the ends of cloned DNA fragments that insufficient flanking sequence is available for a conventional primer to be designed (Varghese et al. 2000). However, a disadvantage of anchored PCR is the low frequency of single locus amplification when the technique is used to develop markers for simple tandem repeats in plant species with large and complex genomes, such as bread wheat (Hayden and Sharp 2001a) and pine (Fisher et al. 1998). The purpose of the present study was to determine whether the recovery of single locus markers in anchored PCR could be improved by targeting compound SSRs, which consist of two or more repeat motifs arranged in tandem. More broadly, the research was undertaken to establish whether anchored PCR is suitable as a generic procedure to develop a large number of functional SSRs in bread wheat.

Materials and methods

Plant materials and mapping populations

The wheat varieties used to screen for polymorphic SSRs were CD87, Cranbrook, Egret, Halberd, Janz, Katewpa, Kukri, Opata85, Sunco, Sunstar, Tasman and W7984. All varieties, except Opata85 and W7984, represent important lines for the Australian wheat industry and included the parents of the Australian doubled haploid populations: Cranbrook×Halberd, Sunco×Tasman, Egret×Sunstar, CD87×Katepwa and Kurki×Janz (Kammholz et al. 2001). The genetic mapping of SSRs was performed on the Cranbrook×Halberd and Sunco×Tasman (161 and 180 lines, respectively) crosses, and the recombinant inbred population (114 lines) of the International Triticeae Mapping Initiative (ITMI) W7984×Opata85 cross. The latter population was derived by single seed descent from the cross of *Triticum aestivum* cv. Opata85 with W7984, a synthetic hexaploid derived from the wide cross *T. durum* (AABB) cv. Altar84×*T. tauschii* (DD), as described by van Deynze et al. (1995). The nullisomic-tetrasomic lines of cv. Chinese Spring (Sears 1966) were used to determine the chromosomal location of SSRs.

Source of compound SSR sequence data

Compound SSRs were extracted from the sequences of microsatellite clones isolated from $(AC)_n$ and $(AG)_n$-enriched genomic DNA libraries according to Edwards et al. (1996). These sequences were obtained from the Wheat Microsatellite Consortium (WMC) (Agrogene), and cannot be released publicly before 2004 without permission from the individual members to whom the clones belong. All primer sequences reported in the present study are published with the consent of the appropriate WMC members.

Primer design and PCR analysis

A specific primer matching the conserved sequence flanking the compound SSR was designed with NetPrimer (Premier Biosoft International) using the following parameters: primer length 18–24 nt, 3′-end stability –5.5 to –9.0 kcal/mol, oligomer T_m 55–65°C, GC content 40–70% and primer rating >90. Compound SSRs were amplified using a 10 μl reaction mixture containing 0.2 mM dNTPs, 1× PCR buffer (20 mM $(NH_4)_2SO_2$, 0.01% Tween-20, 75 mM Tris-HCl, pH 8.5), 1.5 mM $MgCl_2$, 0.2 μM each of flanking (forward) and microsatellite anchoring (reverse) primers, 50 ng genomic DNA, and 0.5 U *Taq* DNA polymerase. PCR was performed for 37–47 cycles with the touchdown profile: 60 s at 92°C, 60 s at 62°C and 30 s 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 were mixed with an equal volume of gel loading buffer (98% formamide, 10 mM EDTA, 0.25% xylene cyanol as tracking dye), heated at 95°C for 3 min, chilled quickly on ice and separated using a 6% sequencing gel (Sambrook et al. 1989). SSR amplicons were detected by silver staining (Bassam et al. 1991). For fluorescent-based SSR detection, PCR was performed using microsatellite anchoring primers labelled at their 5′-ends with hexachloro-6-carboxylfluorescein (HEX). The separation and detection of PCR products was performed on a Gel Scan 2000 DNA fragment analyser (Corbett Research) using a 5% sequencing gel.

Chromosomal assignment of SSRs

The chromosomal location of SSRs was determined by integrating (P<0.001) polymorphic markers into the genetic linkage maps of appropriate mapping populations using Map Manager QTX (Manly 1998) or by PCR amplification using DNA from the set of 21 nullitetrasomic lines. The genetic maps of the Cranbrook×Halberd and Sunco×Tasman crosses were published by Chalmers et al. (2001). Map data for the doubled haploid populations was kindly provided by R. Appels (WA Department of Agriculture), and the data for the genetic map of the recombinant inbred population was provided by P. Leroy (INRA).

Polymorphism information content

Polymorphism information content (PIC) values were calculated following Anderson et al. (1993), assuming that the wheat lines were homozygous: $\mathrm{PIC}_i = 1 - \sum_{j=1}^{n} P_{ij}^2$, where P_{ij} is the frequency of the jth pattern for marker i and the summation extends over n patterns. This value provides an estimate of the discriminatory power of a SSR locus by taking into account not only the number of alleles per locus, but also their relative frequencies in the germplasm studied.

Results

Characterisation of compound SSRs

Compound SSRs present in the sequences of microsatellite clones obtained from the WMC were identified by searching for all permutations of $(AC)_n$ and $(AG)_n$ repeats directly adjacent to other dinucleotide tandem repeats, where each perfect tandem repeat was composed of at least four repeat units. This approach identified compound SSRs in which the two tandem repeats were directly adjacent to each other, with no region of imperfect repeat sequence between them. Searches for compound SSRs composed solely of $(AT)_n$ and $(CG)_n$ repeats were not performed as the occurrence of these sequences was expected to be low due to the enrichment of the genomic DNA libraries for clones containing $(AC)_n$

Table 1 Size distribution of repeat sequences observed in ten classes of compound SSRs

SSR motif	Total number of dinucleotide repeats in compound SSRs[a]												
	8–12	13–16	17–20	21–24	25–28	29–32	33–36	37–40	41–44	45–48	≥49	Total	%
$(AT)_m(AG)_n$	–	–	–	–	–	–	–	–	–	–	1	1	0.3%
$(AC)_m(AT)_n$	–	–	3	3	4	2	2	1	–	–	–	15	3.9%
$(AC)_m(AG)_n$	–	3	11	7	1	3	5	–	1	–	1	32	8.3%
$(AG)_m(AT)_n$	–	–	–	–	–	–	–	1	–	–	–	1	0.3%
$(AG)_m(AC)_n$	2	–	2	2	–	–	–	–	–	–	–	6	1.6%
$(CA)_m(CT)_n$	–	1	2	4	1	–	–	–	–	–	–	8	2.0%
$(CT)_m(CA)_n$	33	63	35	56	26	25	11	6	4	–	1	260	67.5%
$(CT)_m(CG)_n$	–	–	–	1	–	–	–	–	–	–	–	1	0.3%
$(CG)_m(CA)_n$	16	10	1	1	–	1	1	–	–	–	–	30	7.7%
$(GC)_m(GT)_n$	9	8	3	5	1	2	2	1	–	–	–	31	8.1%
Total	60	85	57	79	33	33	21	9	5	0	3	385	
%	15.6%	22.1%	14.8%	20.5%	8.6%	8.6%	5.5%	2.3%	1.3%	0.0%	0.8%		

[a] Compound SSRs include perfect and imperfect sequences

and $(AG)_n$ repeats. After accounting for redundant compound SSR sequences (e.g. the same microsatellite was identified by searching for $(CT)_4(CA)_4$ or by searching for $(TC)_4(AC)_4$) and sequence complementarity resulting from the non-directional cloning of DNA fragments in the plasmid vector, a total of 385 microsatellite sequences comprising ten classes of repeat motifs were identified (Table 1).

As a proportion of the total number of clone sequences searched, 14.7% (288/1957) contained at least one compound SSR. Whilst this proportion has not been adjusted for redundant clones (i.e. clones sequenced more that once), this result indicates that compound SSRs have a relatively low abundance in the bread wheat genome. There was a disproportionate representation of the different classes of compound SSRs, with $(CT)_m(CA)_n$ motifs accounting for 67.5% of the total number of sequences identified (Table 1). The next most abundant class was $(AC)_m(AG)_n$, which represented 8.3% of the compound SSRs. The average length of the dinucleotide tandem repeats in the compound SSRs was comparable for AC, AG, TC and TG motifs (12.0, 11.9, 9.9 and 12.2 repeat units, respectively). In contrast, the average length of AT and GC repeat motifs was about half that of the other dinucleotide repeat classes (5.8 and 5.9, respectively).

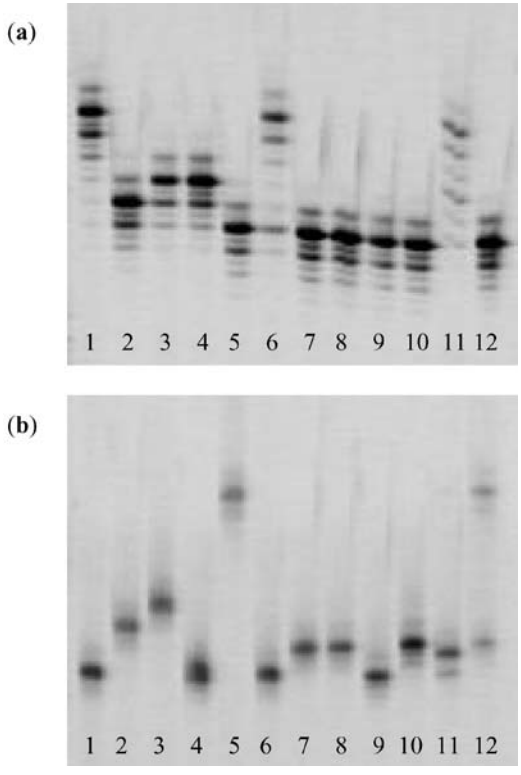

Fig. 1a, b Anchored PCR amplification of compound SSRs from a set of 12 bread wheat varieties: **a** stm7tcac and **b** stm506tgag. Samples from left to right: *1* Opata85, *2* W7984, *3* Halberd, *4* Cranbrook, *5* Sunco, *6* Tasman, *7* Egret, *8* Sunstar, *9* CD87, *10* Katepwa, *11* Kukri, *12* Janz

Primer design and polymorphism

Overall, 281 (98%) of the clones containing compound SSRs were considered useful for anchored PCR as they contained sufficient flanking sequence for the design of a primer complementary to one side of the repeat motif. Of these, 58 (21%) were reported to possess insufficient flanking sequence to allow the design of a specific primer pair for conventional SSR amplification (P. Isaac, personal communication).

Primers complementary to the conserved sequences flanking the compound SSRs could be designed for 180 (64%) of the clone sequences that were considered suitable for anchored PCR. In 111 (62%) instances, a flanking primer could be designed for either side of the repeat motif, indicating that both tandem repeats of the compound SSR could potentially be assayed for polymorphism. For the remaining 69 (38%) clones, insufficient flanking sequence for one side of the repeat motif meant that only one half of the compound SSR could be amplified by anchored PCR.

Table 2 Primer sequences, chromosomal locations, number of alleles and polymorphic information content (*PIC*) of anchored compound SSRs

Primer set[a]	WMC clone	Sequence (5′–3′)	Observed allele size range (bp)	Chromosome location	Number of alleles	PIC
stm1actc	14e4	fwd ATTGCATGTTGATAACAGACTCG rvs ACACACACACACACTCTCTCTC	175–204	7D	3	0.40
stm2tcac	8a6	fwd TAGCAGCCAAAAAAAACATACTCG rvs TCTCTCTCTCTCTCACACACAC	98–118	3D	7	0.48
stm3tgag	13a3	fwd ACCTAGCTCTGATACCAAATGTTA rvs TGTGTGTGTGTGTGAGAGAGAG	163–180	6B	4	0.65
stm5tcac	13f6	fwd CATACAAATGCCAGGAGAGAT rvs TCTCTCTCTCTCTCACACACAC	91–101 83–87	7D 7B	3 5	0.40 0.61
stm7tcac	21a3	fwd GGAAGGTTAGTCGTCTCTTGGAAA rvs TCTCTCTCTCTCTCACACACAC	106–118	5B	4	0.65
stm15tgag	4c7	fwd GAGTAGGTCAAGCACCAATGAGG rvs TGTGTGTGTGTGTGAGAGAGAG	128–137 112–124	6D 6B	5 4	0.53 0.58
stm17tcac	10f1	fwd CAGAGGTCCCGAAGCACGAAA rvs TCTCTCTCTCTCTCACACACAC	172–174 184–190	2A 2B	5[#] 2	0.76 0.40
stm22tcac	14c5	fwd GGGCACTGCACAAGAAGCAAA rvs TCTCTCTCTCTCTCACACACAC	74–92	3B	5[#]	0.67
stm25tgag	26e3	fwd GCATTCTACTTCTAGGATCTTCTG rvs TGTGTGTGTGTGTGAGAGAGAG	129–135	5B	4[#]	0.76
stm400tcac	4c11	fwd CCAGGATGAATGGAAAGGAGAT rvs TCTCTCTCTCTCTCACACACAC	160–166	1D	3	0.49
stm405tgag	2h1.2	fwd ATGAGTTTCTAACTGGGTGTTTG rvs TGTGTGTGTGTGTGAGAGAGAG	124–138	3B	3	0.29
stm503acag	13f1.2	fwd CTTTCCTTATAAATAGGCTCGC rvs ACACACACACACACAGAGAGAG	70–98	7A	6	0.82
stm503tctg	24c7	fwd GACAAGACAATAGATCCAACAACTG rvs TCTCTCTCTCTCTCTGTGTGTG	140–142	2A	2	0.38
stm504acag	24c7	fwd TGGACTAGAATTGTGTGCTCG rvs ACACACACACACACAGAGAGAG	114–126	2A	3	0.49
stm506tgag	1h5	fwd CTACTAGGAATAGGAATGAGGGAAG rvs TGTGTGTGTGTGTGAGAGAGAG	160–178	4A	6	0.78
stm509tgag	5h3	fwd GAGCCAGAGGAAAAACTCCGCATC rvs TGTGTGTGTGTGTGAGAGAGAG	216–227	1B	3	0.63
stm512tcac	13e1	fwd CCTCGTGTTTCTCCTCGTGATTCC rvs TCTCTCTCTCTCTCACACACAC	228–232	1A	2	0.26
stm522tcac	1b3	fwd CCTCGTGTTTCTCCTCGTGATTCC rvs TCTCTCTCTCTCTCACACACAC	122–124	1B	3[b]	0.54
stm535tcac	3e3	fwd CAACGTGTATGGACCAACGACTAGC rvs TCTCTCTCTCTCTCACACACAC	165–171	5B	4[b]	0.68
stm535tgag	22f10	fwd GACTCACACCACTAACATTACTCTA rvs TGTGTGTGTGTGTGAGAGAGAG	232–240	4B	7	0.76
stm556tcac	7b3	fwd CAGAAGGGTTAGAGAAAATGAGT rvs TCTCTCTCTCTCTCACACACAC	116–124	2B	5	0.64

[a] This table is limited to those microsatellite clones for which permission to publish primer sequences was obtained
[b] Indicates the presence of null alleles

To determine whether compound SSRs could be amplified by anchored PCR using a primer specific to the conserved sequence flanking one side of the repeat motif in combination with a primer anchoring to the repeat junction of the SSR, primers were designed from the sequences of 96 microsatellite clones. These clones were selected to contain a compound SSR for which primers had not previously been designed, or could not be amplified in our hands using conventional PCR and the primer pair designed by the WMC (50 and 46, respectively). Fifty-nine (62%) of the primer sets amplified a scorable SSR fragment of the expected size in anchored PCR, of which 41 exclusively amplified the expected fragment, 16 amplified mostly one or, in a few cases, several additional fragments, and two amplified multiple additional fragments. The remaining 37 primers gave no amplification, or non-specific fragments that were unscorable. Importantly, the amplification of inter-simple

Table 3 Sequences of primers used to amplify published compound SSRs by anchored and conventional PCR

Primer set	Sequence (5′–3′)	Type of PCR
WMC31	fwd GTTCACACGGTGATGACTCCCA rvs CTGTTGCTTGCTCTGCACCCTT	Conventional
stm551tcac	fwd GTTCACACGGTGATGACTCCCA rvs TCTCTCTCTCTCTCACACACAC	Anchored
stm549tgag	fwd CTGTTGCTTGCTCTGCACCCTT rvs TGTGTGTGTGTGTGAGAGAGAG	Anchored
WMC112	fwd TGAGTTGTGGGGTCTTGTTTGG rvs TGAAGGAGGGCACATATCGTTG	Conventional
stm544tgag	fwd TGAGTTGTGGGGTCTTGTTTGG rvs TGTGTGTGTGTGTGAGAGAGAG	Anchored
stm4tcac	fwd TGAAGGAGGGCACATATCGTTG rvs TCTCTCTCTCTCTCACACACAC	Anchored
WMC116	fwd ACGGCACCCCAGAACCTAGTCG rvs GCGTGCGTCTGTGCTGCATCGG	Conventional
stm545tgag	fwd ACGGCACCCCAGAACCTAGTCG rvs TGTGTGTGTGTGTGAGAGAGAG	Anchored
stm548tcac	fwd GCGTGCGTCTGTGCTGCATCGG rvs TCTCTCTCTCTCTCACACACAC	Anchored
WMC208	fwd ATTGCCTTTGGACTTCCTCACC rvs CACAAGCTTGCTTCTTCTTCAT	Conventional
stm550tcac	fwd ATTGCCTTTGGACTTCCTCACC rvs TCTCTCTCTCTCTCACACACAC	Anchored
stm548tgag	fwd CACAAGCTTGCTTCTTCTTCAT rvs TGTGTGTGTGTGTGAGAGAGAG	Anchored
WMC245	fwd GCTCAGATCATCCACCAACTTC rvs AGATGCTCTGGGAGAGTCCTTA	Conventional
stm551tgag	fwd GCTCAGATCATCCACCAACTTC rvs TGTGTGTGTGTGTGAGAGAGAG	Anchored
stm590tcac	fwd AGATGCTCTGGGAGAGTCCTTA rvs TCTCTCTCTCTCTCACACACAC	Anchored
WMC296	fwd GAATCTCATCTTCCCTTGCCAC rvs ATGGAGGGGTATAAAGACAGCG	Conventional
stm591tcac	fwd GAATCTCATCTTCCCTTGCCAC rvs TCTCTCTCTCTCTCACACACAC	Anchored
stm552tgag	fwd ATGGAGGGGTATAAAGACAGCG rvs TGTGTGTGTGTGTGAGAGAGAG	Anchored
WMC331	fwd CCTGTTGCATACTTGACCTTTTT rvs GGAGTTCAATCTTTCATCACCAT	Conventional
stm605tgag	fwd CCTGTTGCATACTTGACCTTTTT rvs TGTGTGTGTGTGTGAGAGAGAG	Anchored
stm589tcac	fwd GGAGTTCAATCTTTCATCACCAT rvs TCTCTCTCTCTCTCACACACAC	Anchored
WMC332	fwd CATTTACAAAGCGCATGAAGCC rvs GAAAACTTTGGGAACAAGAGCA	Conventional
stm509acag	fwd CATTTACAAAGCGCATGAAGCC rvs ACACACACACACACAGAGAGAG	Anchored
stm508tctg	fwd GAAAACTTTGGGAACAAGAGCA rvs TCTCTCTCTCTCTCTGTGTGTG	Anchored
WMC357	fwd TAGTGGGTGACCGGTCAAGA rvs TGGACGGATTTGGTCATTTC	Conventional
stm562tcac	fwd TAGTGGGTGACCGGTCAAGA rvs TCTCTCTCTCTCTCACACACAC	Anchored
stm550tgag	fwd TGGACGGATTTGGTCATTTC rvs TGTGTGTGTGTGTGAGAGAGAG	Anchored
WMC537	fwd CTAGGATCTTCTGTACATTGAACAAC rvs GATGCAGAACCGTGATAGGATGT	Conventional
stm516tgag	fwd CTAGGATCTTCTGTACATTGAACAAC rvs TGTGTGTGTGTGTGAGAGAGAG	Anchored
stm523tcac	fwd GATGCAGAACCGTGATAGGATGT rvs TCTCTCTCTCTCTCACACACAC	Anchored

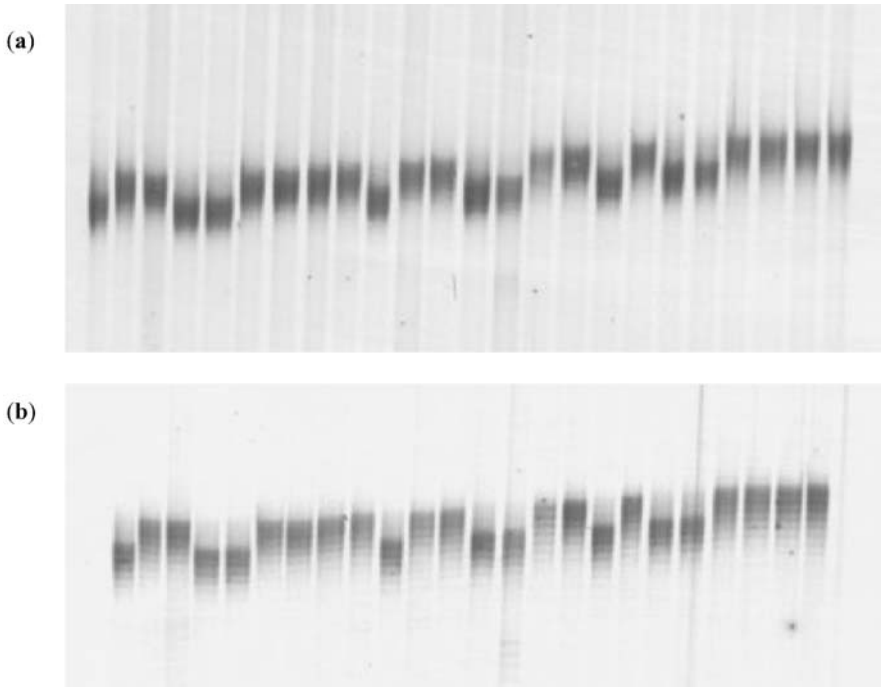

Fig. 2 Identical segregation patterns generated in a Cranbrook×Halberd doubled haploid population by **a** conventional (primed with WMC112) and **b** anchored (primed with stm544tgag) PCR

sequence repeat fragments by the microsatellite anchoring primers was not observed.

Forty one (70%) of the primer sets amplifying a scorable SSR revealed polymorphism between the 12 wheat varieties (Fig. 1, Table 2). In total, 57 SSR loci were amplified, generating a mean of 1.39 loci per primer set. A mean of 4.8 alleles per locus were detected, with PIC values ranging from 0.26 to 0.82 (mean 0.56, when monomorphic loci were omitted from the calculation). The chromosomal location of all loci was determined by a combination of genetic mapping and aneuploid assignment.

Anchored SSR amplification using published primer sequences

Anchored PCR should provide substantial cost savings for fluorescent-based SSR detection because only a single dye-labelled microsatellite anchoring primer is needed to detect markers targeting the same type of compound repeat sequence. Cost savings would be maximised if existing primers for published SSRs based on compound repeats could also be used in anchored PCR. To investigate this possibility, ten primer sets designed by the WMC that amplified polymorphic SSRs were tested (Table 3), using either the forward or reverse published primer as the flanking primer, in combination with an appropriate anchoring primer. On the basis that the amplification product was of the expected size, and that it either could be assigned by aneuploid analysis to the same chromosome as the published marker, or co-segregated with the published marker (Fig. 2), all ten SSRs were successfully amplified in anchored PCR. In only one instance did both published primers fail to amplify the target locus (see *Xwmc296–2A*, Table 4).

To determine whether anchored SSR amplification revealed the same power of discrimination as conventional PCR, each of the published SSRs were screened for polymorphisms on the set of 12 bread wheat varieties. In all cases where the target SSR was amplified in anchored PCR by the published forward and reverse primers, both PCR techniques revealed the same power of discrimination between the parental cultivars of each cross (Table 4). For example, polymorphism revealed by conventional PCR at the *Xwmc332–2B* locus between the cultivars Halberd and Cranbrook, and Egret and Sunstar was also detected by anchored PCR using either the forward or reverse published primer in combination with an appropriate anchoring primer (Fig. 3 lanes 3–4, 7–8). However, the forward and reverse published primers in anchored PCR did not always reveal the same power of discrimination. In these cases, it was inferred that the repeat length variation giving rise to the polymorphism was contained in the portion of compound microsatellite sequence that was excluded from the PCR amplicon. Of particular note were the profiles detected for cv Sunco (Fig. 3, lanes 5), which would be interpreted as null alleles using either the conventional or stm509acag primer sets, but which generated a typical SSR amplicon with stm508tctg. This clearly demonstrates how a null allele at a SSR locus can come about through the deletion/mutation of the one of the flanking primer sites. For four SSRs, anchored PCR showed a better level of discrimination between the parents of the mapping populations than did conventional PCR. Thus, the latter could not differentiate between the parents of any mapping population at the *Xwmc31–1B*, *Xwmc31–1D* and *Xwmc245–2D*

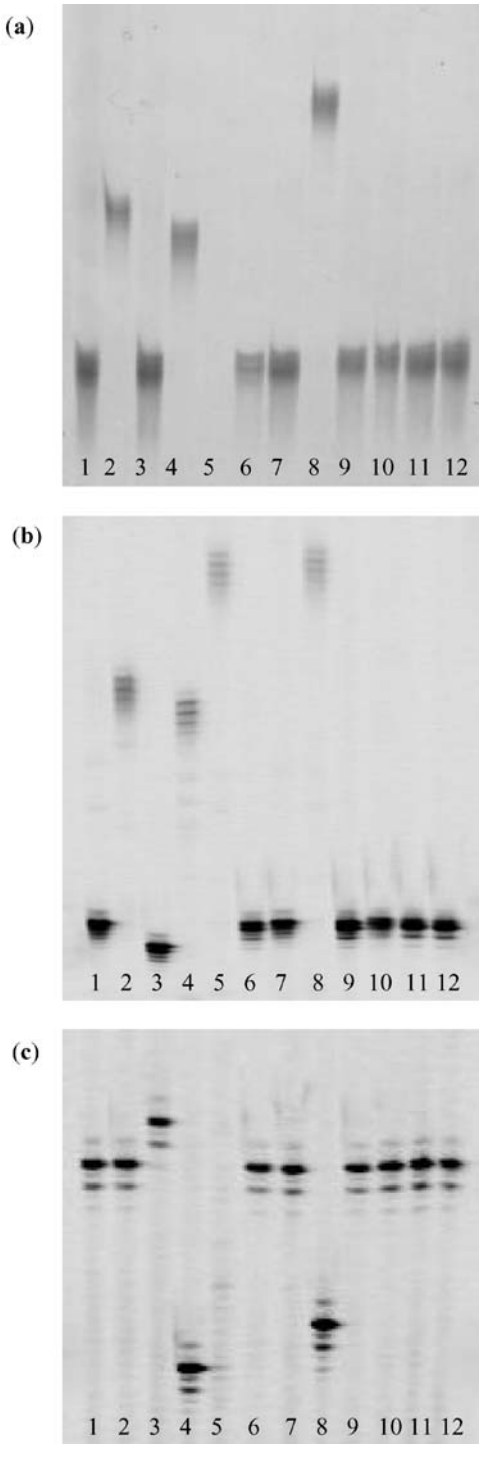

Fig. 3a–c Amplification of the *Xwmc332–2B* locus from a set of 12 bread wheat varieties. PCR primed with **a** the conventional WMC332 primer set, **b** primer set stm509acag or **c** primer set stm508tctg. Samples *1–12* as in Fig. 1

loci, but anchored PCR revealed allelic differences between the parental cultivars of several crosses at these loci (Table 4, Fig. 4). This indicated that anchored PCR is able to reveal microsatellite repeat length variation that

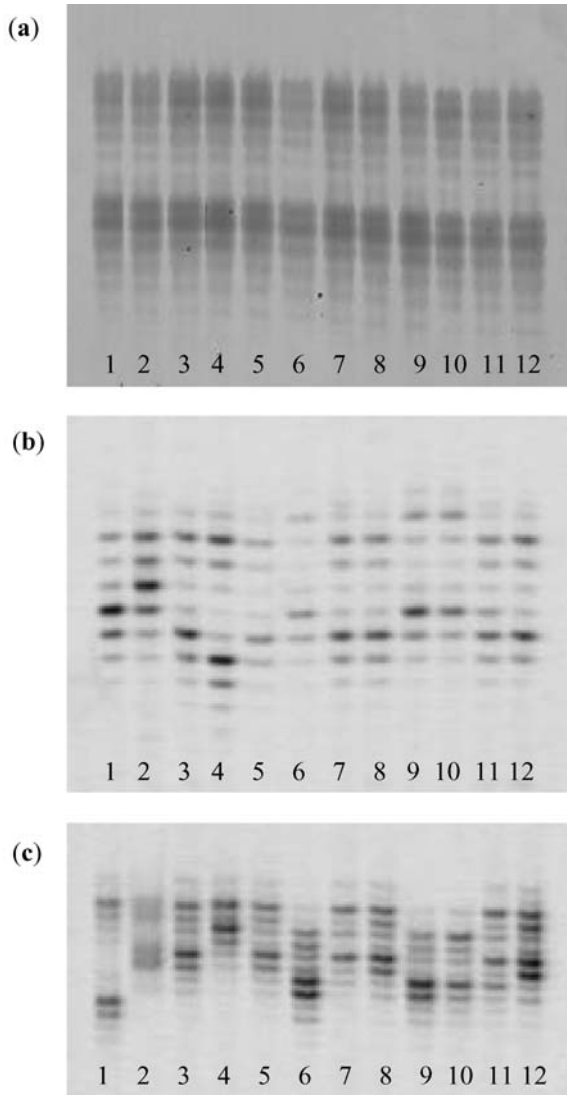

Fig. 4a–c Amplification of the *Xwmc31–1B* and *Xwmc31–1D* loci from a set of 12 bread wheat varieties. PCR primed with **a** the conventional WMC31 primer set, **b** primer set stm551tcac or **c** primer set stm549tgag. Samples *1–12* as in Fig. 1

causes no net change to the size of the PCR amplicon amplified using conventional PCR.

Discussion

The present study has shown that compound SSRs can be amplified by anchored PCR using a primer complementary to the conserved sequence flanking one side of the repeat motif in combination with a primer that is anchored to the tandem repeat junction of the compound SSR. The quality of the compound SSR markers was typical of published wheat SSRs amplified by conventional PCR using a pair of primers flanking the microsatellite sequence (Fig. 1). The recovery rate (43%) of polymorphic markers in anchored PCR was similar to that

Table 4 Allelic discrimination of anchored and conventional PCR. *Bold* entries indicate where anchored PCR amplification improved the power to differentiate between the mapping parents. *na* No amplification of a scorable SSR fragment

Locus	Primer set	Opata 85 *x* W7984	Cranbrook *x* Halberd	Sunco *x* Tasman	Egret *x* Sunstar	CD87 *x* Katepwa	Kukri *x* Janz
Xwmc31–1B	WMC31	**–**	**–**	**–**	–	–	–
	stm551tcac	**+**	**+**	**+**	–	–	–
	stm549tgag	**+**	**+**	**+**	–	–	–
Xwmc31–1D	WMC31	–	–	**–**	–	–	–
	stm551tcac	–	–	**+**	–	–	–
	stm549tgag	–	–	**+**	–	–	–
Xwmc112–2D	WMC112	+	+	–	–	–	–
	stm544tgag	+	+	–	–	–	–
	stm4tcac	+	–	–	–	–	–
Xwmc116–7A	WMC116	+	+	–	–	+	+
	stm545tgag	+	+	–	–	+	+
	stm548tcac	–	–	–	–	–	–
Xwmc208–5B	WMC208	+	+	+	–	+	–
	stm550tcac[a]	+	+	+	–	–	–
	stm548tgag	–	+	+	–	+	–
Xwmc245–2D	WMC245	**–**	**–**	**–**	**–**	**–**	–
	stm551tgag	**+**	**+**	**+**	**+**	**+**	–
	stm590tcac	**+**	**+**	**+**	**+**	**+**	–
Xwmc296–2A	WMC296	+	–	–	–	–	–
	stm591tcac	+	–	–	–	–	–
	stm552tgag	na	na	na	na	na	na
Xwmc331–4D	WMC331	+	+	+	–	+	+
	stm605tgag	+	+	+	–	–	+
	stm589tcac	+	–	–	–	+	+
Xwmc332–2B	WMC332	+	+	+	+	–	–
	stm509acag	+	+	+	+	–	–
	stm508tctg	–	+	+	+	–	–
Xwmc357–5D	WMC357	+	–	+	–	+	**–**
	stm562tcac	+	–	–	–	–	**+**
	stm550tgag	–	–	+	–	+	**+**
Xwmc537–5B	WMC537	+	+	+	–	+	–
	stm516tgag	–	+	+	–	+	–
	stm523tcac	+	–	+	–	+	–

[a] Indicates the amplification of a large number of non-target fragments

reported for published wheat SSRs. For example, Byran et al. (1997), Röder et al. (1998) and Pestova et al. (2000) reported that on average 39% (32, 41 and 44%, respectively) of all primer pairs developed from the sequences of wheat microsatellite clones were functional. Moreover, the average PIC of the compound SSRs revealing polymorphism among the Australian cultivars was similar to that reported by Harker et al. (2001) for published SSRs screened on the same set of cultivars (0.56 and 0.57, respectively). These results demonstrate that anchored PCR is suitable for the development of polymorphic compound SSR markers in bread wheat.

During the development of SSR markers, microsatellite clones are often encountered in which the repeat sequence is located close to the end of the cloned DNA fragment, leaving insufficient DNA for primer design. In many instances, these clones are discarded because a primer pair for conventional SSR amplification cannot be designed. The present study has shown that when these clones contain a compound SSR, anchored PCR can be used to develop useful DNA markers, thereby increasing the efficiency of marker development. In the present study, 21% (58/281) of the WMC clones containing a compound SSR fell into this category, and all of these were considered to be suitable for amplification by anchored PCR.

The amplification of compound SSRs by anchored PCR offers the potential for substantial cost savings during the development of microsatellite markers and for fluorescent-based SSR analysis. The requirement for a single primer complementary to the conserved sequence flanking a compound SSR reduces the cost of primer synthesis by half compared to conventional SSR amplification. Similarly, the use of a primer anchoring to the repeat junction of the compound SSR significantly reduces the cost of fluorescent-based DNA detection because dye-labelling of only a single microsatellite anchoring primer is needed to detect all markers based on the same type of repeat sequence. The findings of the present study demonstrate that the cost savings provided by anchored PCR for fluorescent-based SSR detection also extend to published markers based on compound repeat sequences. In all instances, the amplification of a target SSR was achieved using a published primer in

combination with an appropriate microsatellite anchoring primer. This result indicates that any laboratory could benefit from these cost savings, as the design and synthesis of new primers for anchored PCR amplification of published SSRs would not normally be required. The capacity to utilise published primers in anchored PCR will be particularly useful to laboratories that do not have access to the sequence data of published SSRs.

Of particular interest was the finding that anchored PCR often revealed a better level of allelic discrimination than conventional SSR amplification. Anchored PCR may therefore be particularly useful for genotyping purposes, since it often allows alleles that appear identical in conventional PCR to be further resolved. Examples in the present study include the revelation of an allelic series at the *Xwmc31–1B*, *Xwmc31–1D* and *Xwmc245–2D* loci that appeared to contain single alleles in conventional PCR (Fig. 4). Similarly, amplification of the *Xwmc332–2B* locus by anchored PCR using either the stm509acag or stm508tctg primer sets revealed that the cvs Halberd and Egret possessed different alleles that were not discriminated as size variants by conventional SSR amplification (Fig. 3, lanes 3, 7). Hence, anchored PCR is useful for adding confidence that alleles which appear to be the same by conventional PCR are resolved. In addition, anchored PCR can help to resolve problems associated with the relatively high frequency of null alleles in bread wheat. Null alleles are problematic in genotyping because they cannot always be differentiated from failed reactions. In the present study, the null allele detected for cv Sunco using the conventional or stm509acag primer sets was amplified with stm508tctg (Fig. 3, lanes 5). This finding clearly demonstrates that in some instances it will be possible to generate assays that are capable of resolving null alleles revealed by conventional PCR, and thus eliminate allele assignment uncertainty that can be arise from failed reactions.

A potential limitation to the development of compound SSR markers using anchored PCR is the relatively low abundance of these repeat sequences in the bread wheat genome. In the present study, only 14.7% of the microsatellite clones contained a compound SSR. This finding is unlikely to be a consequence of the enrichment procedure used to construct the genomic libraries from which the microsatellite clones were isolated, as Röder et al. (1998) found a similar frequency (16.9%) of compound SSRs in clones isolated from unenriched libraries. Hence, the capacity to develop a large number of compound SSR markers is likely to be determined by the procedure that is used to isolate the microsatellite sequences. Techniques utilising clone screening, or enrichment procedures, based on the hybridisation of oligonucleotide SSR probes are not specifically designed to enrich for compound SSRs, and are therefore expected to yield only relatively small numbers of these markers. In contrast, strategies employing PCR-based procedures for library enrichment are more likely to yield a larger number of compound SSRs. Inoue et al. (1999) described an approach based on the hybridisation of biotinylated SSR-specific oligonucleotide probes to PCR amplified DNA fragments. The SSR probes were then extended by DNA polymerase and the biotinylated DNA fragments were recovered by affinity capture using streptavidin-coated magnetic beads. The use of oligomers targeting compound repeat motifs would permit the construction of genomic DNA libraries highly enriched for compound SSRs. Another approach, called sequence tagged microsatellite profiling (STMP) (Hayden and Sharp 2001a), is based on the PCR amplification of SSRs contained within a pool of DNA fragments. As SSR enrichment of the fragment pool is achieved using a microsatellite-specific primer, the use of a primer anchoring to the tandem repeat junction of compound SSRs would also facilitate the generation of a DNA library that was highly enriched for compound SSRs. We are currently using STMP to develop compound SSR markers in bread wheat.

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