CSIRO PUBLISHING
www.publish.csiro.au/journals/ajar *Australian Journal of Agricultural Research*, 2006, **57**, 953–959

New eSSR and gSSR markers added to Australian barley maps

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Abstract. To enhance genetic maps of barley previously developed in Australia for identifying markers useable in molecular breeding, a new set of simple sequence repeat (SSR) and indel markers was added to the maps. These markers were developed through (*i*) database mining of barley expressed sequence tag (EST) sequences, (*ii*) comparative barley-rice genome analysis, and (*iii*) screening of a genomic library with SSR probes. The primer set selected for this study comprised 216 EST-SSR (eSSR) and 25 genomic SSR (gSSR) markers, which were screened for polymorphism on 4 doubled haploid (DH) or recombinant inbred line (RIL) populations. In total, 81 new markers were added to the maps, with good coverage on all 7 chromosomes, except 6H, which only had 2 new markers added. The marker order of previously published maps was re-evaluated by comparing recombination fractions calculated by 2 methods to discover the best position for each marker. The new SSR markers were then added to the updated maps. Several of these new markers are linked to important barley disease resistance genes such as those for cereal cyst nematode, spot form of net blotch, and leaf scald resistance, and are readily useable for marker-assisted barley breeding. The new maps are available on-line at www.genica.net.au.

Additional keywords: microsatellite, *Hordeum vulgare*, genetic mapping, marker-assisted selection.

Introduction

Barley (*Hordeum vulgare* L.) is the world's fourth major cereal crop, and is an important export crop for Australia. Many molecular genetic linkage maps of barley have been created, first based on RFLP markers (Graner *et al*. 1991; Heun *et al*. 1991) and latterly on PCR-based markers (Ramsay *et al*. 2000). Adapted and improved barley varieties are now being released more rapidly than before through the use of marker assisted selection (MAS), with more than 27 loci being tracked with molecular markers in Australian barley breeding programs (Langridge and Barr 2003). MAS has 2 critical requirements: knowledge of the genetic loci controlling critical traits; and the availability of informative, inexpensive molecular markers linked to these loci. Simple sequence repeat (SSR) markers have become the most commonly used tool for MAS in barley breeding programs, and also the community standard marker system for genetic mapping studies. New SSR markers are developed by groups within the international cereal research community to enhance gene discovery and MAS programs. These markers require screening in barley germplasm to verify their location and accurately determine their linkage with key traits. Markers used in this project have been primarily developed through the increasingly used method of database mining of expressed sequence tag (EST) sequences in search of SSRs (Thiel *et al*. 2003; R. K. Varshney, unpublished). Compared with genomic SSR (gSSR) markers (Ramsay *et al*. 2000; Li *et al*. 2003), development of EST-derived SSR (eSSR) markers is very cost effective and in addition, eSSR markers possess a higher level of transferability for use across species (Varshney *et al*. 2005).

Over the past decade, genetic mapping has been used to identify many agronomic, quality, and disease resistance loci in Australian barley varieties (Langridge and Barr 2003). Accurate localisation of genes is dependent on having a high-quality genetic map with evenly spaced molecular markers at an appropriate density (Varshney *et al*. 2004). If these markers are also useable in marker-assisted breeding programs, the transfer of the technology and consequent genetic gains will be more rapidly achieved.

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10.1071/AR05384 0004-9409/06/090953

This paper describes improvement of the quality and accuracy of 4 barley genetic maps through incorporation of new eSSR and gSSR markers as an aid to MAS and the future characterisation, mapping, and validation of marker trait associations for key traits that are as yet poorly or incompletely understood.

Methods

Plant materials

Four Australian barley populations with existing genetic maps were chosen for this study (Barr *et al*. 2003*a*, 2003*b*; Karakousis *et al*. 2003*a*, 2003*b*). These maps were developed from 3 doubled haploid (DH) populations: Galleon*Haruna Nijo (479 markers), Chebec*Harrington (372 markers), and Clipper*Sahara (285 markers), and 1 DH/recombinant inbred line (RIL) population, Sloop*Alexis (312 markers).

SSR genotyping and analysis

In total, 216 primer pairs for eSSR markers (Thiel *et al*. 2003; R. K. Varshney, unpublished data) and 25 primer pairs for gSSR markers (Li *et al*. 2003) were used in the present study. Nineteen EST-derived SSR and indel markers were used (Rostoks *et al*. 2006). Standard PCR conditions and subsequent electrophoresis of amplified fragments were used (Karakousis *et al*. 2003*c*). All markers were tested for polymorphism on 8 parental lines. Subsequent genotyping of each DH or RI line was achieved by visually scoring each marker in accordance with the corresponding parental alleles.

Comparative marker discovery

A comparative approach to exploit the synteny between grass genomes was used to develop eSSRs linked to traits of interest. The primers of 2 eSSRs (gbm 1174 and gbm 1006) that flanked the *Rpt4* gene (Williams *et al*. 1999) on chromosome 7H were used to identify the EST sequences from which they were derived (HU02H07 and bags37j12, respectively), which were aligned against the rice genome to identify rice chromosome 6 BAC clones AP005395 (89% identity, e-value 6^{e-89}) and AP003634 (83% identity, e-value 6^{e-21}). The rice BACs AP005445, AP003621, and AP004324, which were flanked by AP005395 and AP003634, were then used to search the barley EST collection. The 50 most significant barley ESTs for each search were then selected and searched for SSR motifs using a search tool at: http://hornbill.cspp.latrobe.edu.au/cgi-binpub/brassica/indexssr.pl. One SSR was selected from each set of barley ESTs that were derived from the rice clones except for the set from AP005445 in which 2 SSRs were selected. The primers and their sequences are as follows.

AP005395-1F: ATGAACCGAACCTCTACTAC
AP005395-1R: GCAACTTAGCATCACACACA

AP005445-2F: ATTCCAACATCAATACAAGGA
AP005445-2R: AGGGCGACATCAGCAAGT

AP003621-1F: GCTGGTCGCTCGTAAAGG
AP003621-1R: TGTATGTATGTGTCGTCGTTG

AP005445-1R: TGTATGTATGTGTCGTCGTTG
AP005445-1F: GCTGGTCGCTCGTAAAGG

AP004324-1F: AAATCAGTTGCCATCCGT
AP004324-1R: CTGCTGTTGCTGTTGCTG

AP003634-1F: CCTCCTCTCACACCCTCTAC
AP003634-1R: ATCACACGACACACCACAC

Curation of original maps

The original maps (Barr *et al*. 2003*a*, 2003*b*; Karakousis *et al*. 2003*a*, 2003*b*) were evaluated to establish a firm foundation of quality control and data curation using the approach of Lehmensiek *et al*. (2005). The procedure involved applying computer code using S-Plus (Insightful Corp.) to the genotypic data, which calculates the recombination fractions between all pairs of markers, regardless of the assignment of markers to chromosomes. These were compared with the recombination fractions produced using Map Manager and subsequent differences between marker pairs were evaluated to discover the best position for each marker. These improved maps formed the basis of this study.

Addition of markers to maps

The original maps were imported into Map Manager QTX (version QTXb20; Manly *et al.* 2001), using the Kosambi mapping function (Kosambi 1944; Lander *et al*. 1987), with threshold values of $P = 0.05$–0.001. New genotypic data from the DH and RI populations were integrated into each chromosome using the 'Links report' function, then in conjunction with the 'Ripple' function and published maps to retain the established alignment and order, the marker was positioned. The number of double recombinants was evaluated and data were rechecked if necessary. Maps were drawn using MapChart (Voorrips 2002).

Results

The mapping populations Clipper*Sahara (C*S), Chebec*Harrington (C*H), Galleon*Haruna Nijo (G*H), and Sloop*Alexis (S*A) were obtained and screened for polymorphism with all of the 241 SSR markers. eSSR markers had a polymorphism rate of 40%, compared with 60% polymorphism for gSSR markers. In total, 81 polymorphic markers were mapped on these populations, with coverage on all chromosomes; however, 6H was the least represented with only 2 new markers mapping to that chromosome (Fig. 1). These markers were previously unmapped in Australian germplasm, but chromosomal locations for some of the markers were known (Li *et al*. 2003; Thiel *et al*. 2003). From this study, the location of 56 previously unmapped eSSRs was determined and, in addition, 25 eSSR and gSSR markers were added to Australian maps for the first time. The new maps are available on-line at www.genica.net.au. The comparative marker discovery approach yielded 6 potential new SSRs, but only 2 (AP005395-1 and AP003621-1) out of the 6 eSSRs gave a clear polymorphism in a mapping population and were mapped to chromosome 7H, near the target gene *Rpt4*.

The overall length of the maps was compared before and after curation and addition of extra markers (Table 1). Even with the addition of extra markers, the overall map lengths decreased substantially in 3 of the 4 maps. In particular, the

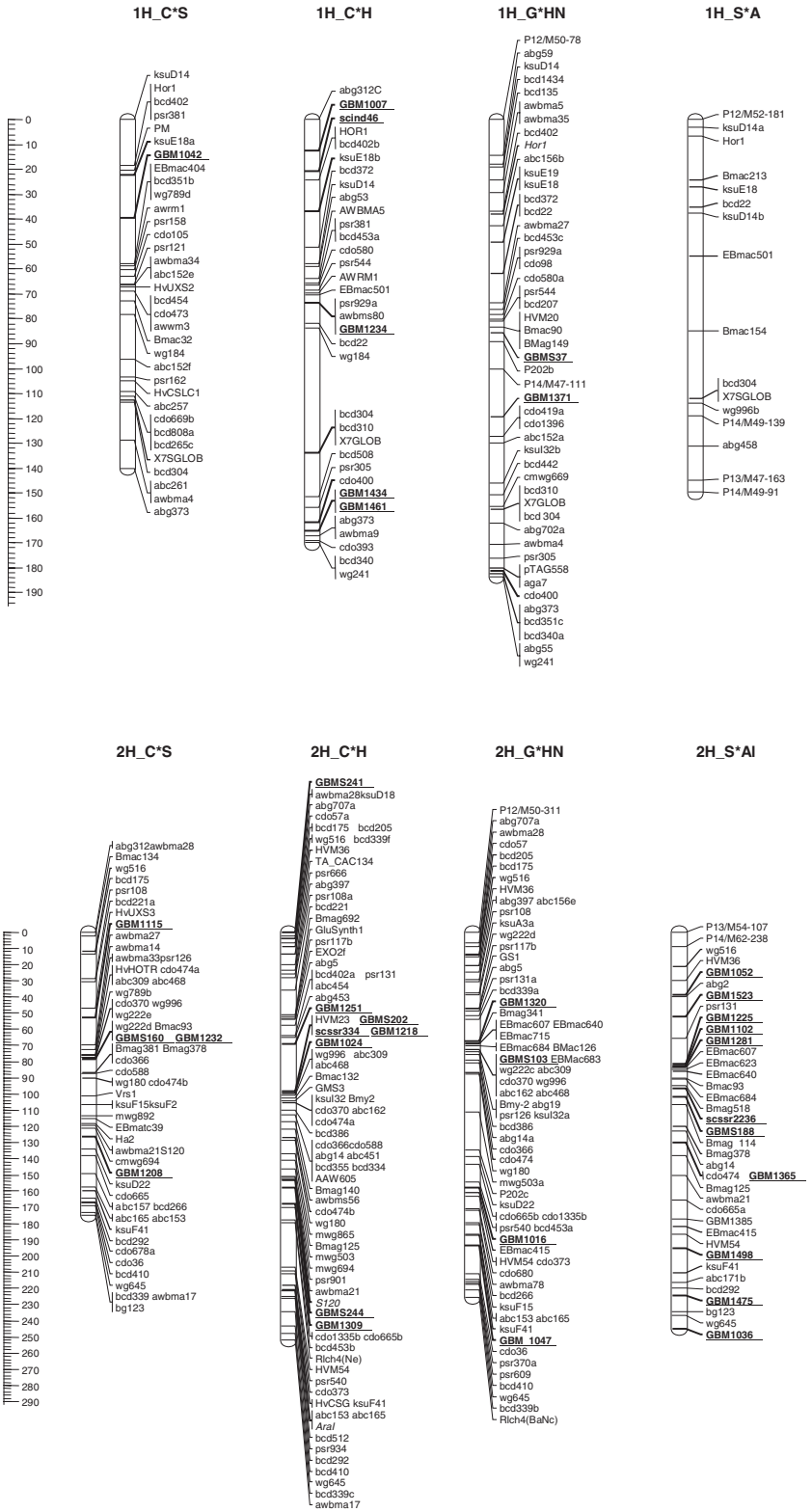

Fig. 1. Updated chromosomes 1H–7H of Australian mapping populations: Clipper*Sahara (C*S), Chebec*Harrington (C*H), Galleon*Haruna Nijo (G*HN), Sloop*Alexis (S*A). New SSR markers are **bold** and underlined. Scale bar indicates centimorgan distances.

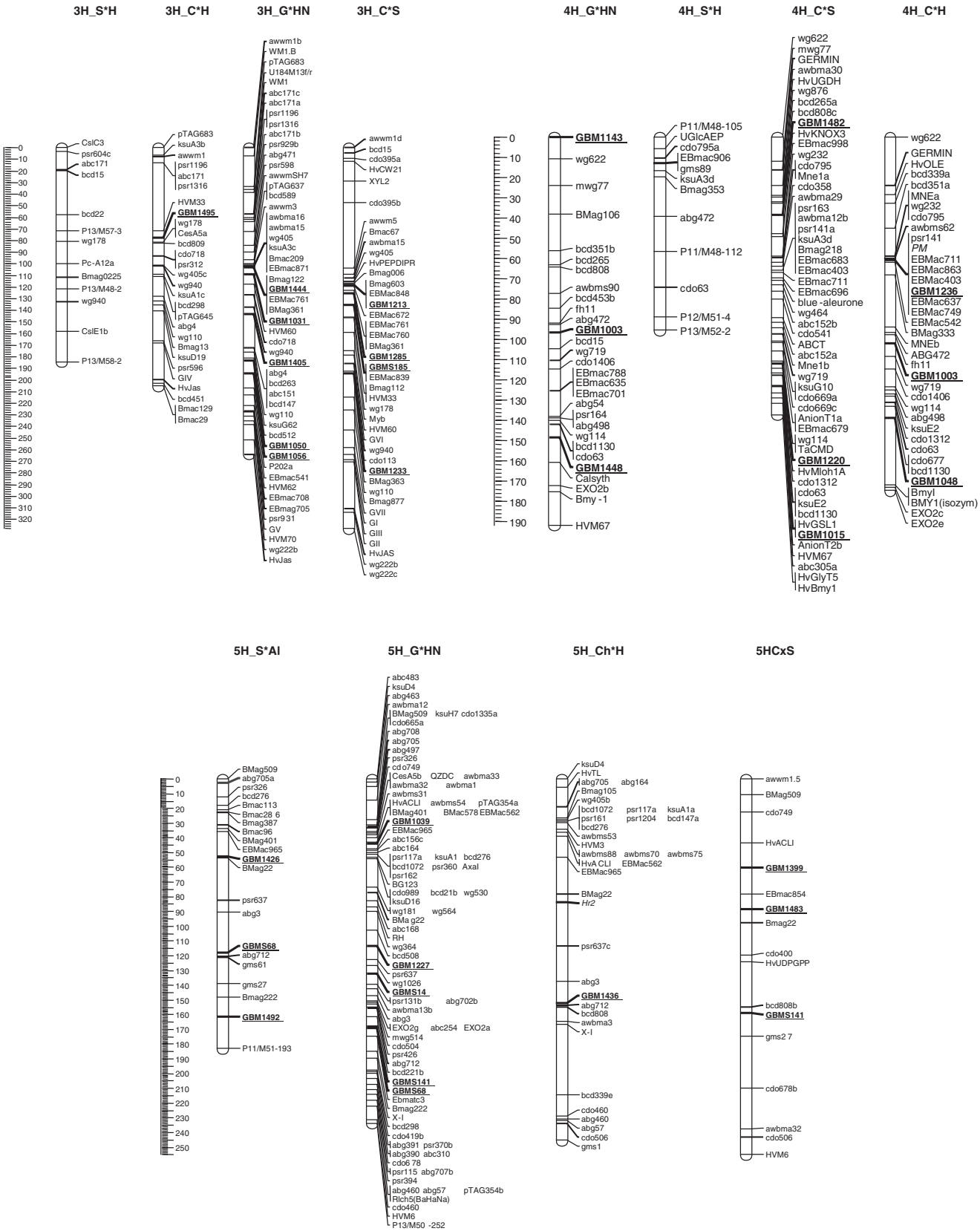

Fig. 1. (*continued*)

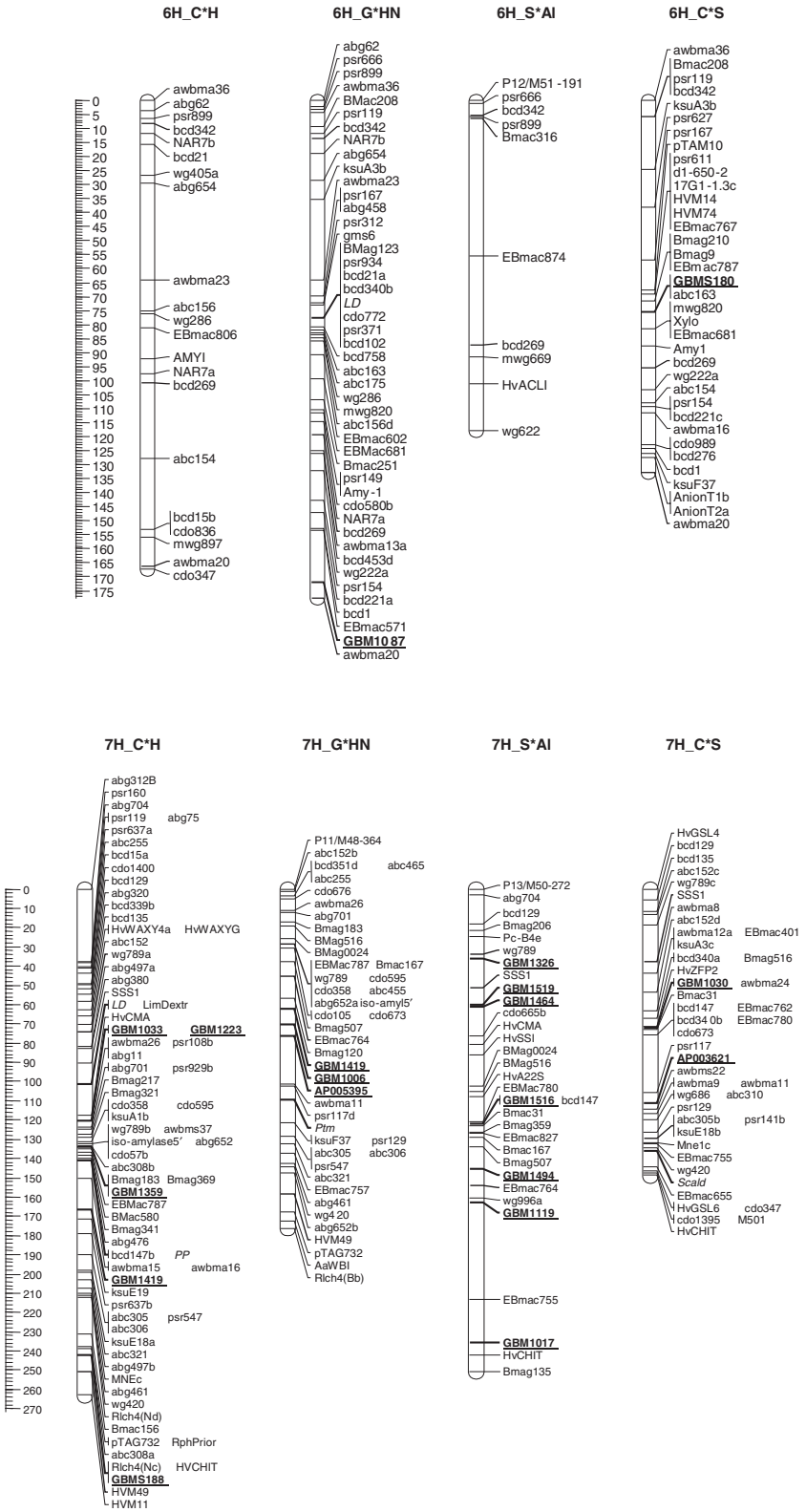

Fig. 1. (*continued*)

Table 1. Total genome length (cM) for each Australian barley mapping population before and after addition of new markers

	Chebec*Harrington	Galleon*Haruna Nijo	Clipper*Sahara	Alexis*Sloop
Before curation and addition of new markers	1618.4	1570.5	1816.2	1241.2
After curation and addition of new markers	1214.5	1452.8	950.9	1247.0
Number of new markers added	23	22	16	27

length of the Clipper*Sahara map decreased by over 800 cM, highlighting the benefits of the curation process.

Discussion

The addition of these new SSR markers to genetic maps of barley serves dual purposes: improving chromosome coverage and marker density, and replacing expensive and complex marker technologies with readily implementable, inexpensive and robust SSR technology. This provides barley breeders with a greater number of potentially useable markers to use for tracking a locus, overcoming the low levels of marker polymorphism between parents in breeding programs, which is still the greatest impediment to MAS (Varshney *et al*. 2004). Greater coverage of the distal ends of chromosome maps with SSRs has been achieved with the new markers, and this is very helpful in terms of marker order and map alignment.

Marker order has been established using a combination of techniques, aiming to minimise chromosome length and the number of double recombinants whilst maximising LOD scores. In addition, the approximate location and order of markers has been checked using a comparative map viewer (www.genica.net.au), although a greater emphasis has been placed on maintaining high LOD scores with the least number of crossovers between adjacent markers. Even though it is possible to continue to increase marker density further, researchers and breeders will determine when these maps have obtained a degree of saturation suitable for effective MAS, gene exploration, and quantitative trait loci detection.

Random marker screening to improve barley maps rarely finds new markers linked to important loci. Markers were selected from the set of gSSR markers (Li *et al*. 2003) as having potential links to important genes and were mapped in those locations accordingly. The eSSR markers (Varsheny *et al*., unpublished) also mapped to regions of interest; however, the random development of the markers from EST databases can result in the positioning of markers to chromosome locations with no known genes. However, bioinformatics-based marker discovery, based on synteny between triticeae species, may be the most effective method to produce highly informative and locus-specific SSR markers. This approach was used in this study to tag the *Rpt4* gene (Williams *et al*. 1999) on chromosome 7H with 2 new eSSR markers, and many extra *in-silico*-derived eSSR markers are also available to fine-map this gene.

The polymorphism rate for EST-derived SSR markers was 44% relative to 60% for markers identified from genomic libraries. Karakousis *et al*. (2003*c*) found that SSRs derived from random genomic sequences were twice as polymorphic in Australian germplasm as those derived from ESTs, and Pillen *et al*. (2000) generated low PIC values for EST-derived SSR markers when screened against European and North American barley cultivars. However, the inexpensive discovery of potentially large numbers of genetically or functionally targetted SSRs via ESTs may outweigh their lower informativeness.

Currently, these barley genetic maps provide important marker-trait associations to breeding programs for disease resistance and tolerance genes, quality traits for malting, and agronomic traits for improved yield potential, frost tolerance, and maturity. Several of the new SSR markers mapped in this study are linked to genes for resistance to diseases such as cereal cyst nematode, net blotch, and scald and are ready for immediate implementation, as well as for use in future mapping studies.

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Manuscript received 8 November 2005, accepted 13 April 2006