Theor Appl Genet (2009) 119:939–951
DOI 10.1007/s00122-009-1101-7

ORIGINAL PAPER

Development and assessment of simple PCR markers for SNP genotyping in barley

Matthew J. Hayden · T. Tabone · D. E. Mather

Received: 9 May 2009 / Accepted: 21 June 2009 / Published online: 12 July 2009

Abstract Simple molecular marker assays underpin routine plant breeding and research activities in many laboratories worldwide. With the rapid growth of single nucleotide polymorphism (SNP) resources for many important crop plants, the availability of routine, low-tech marker assays for genotyping SNPs is of increased importance. In this study, we demonstrate that temperature-switch PCR (TSP) supports the rapid development of robust, allele-specific PCR markers for codominant SNP genotyping on agarose gel. A total of 87 TSP markers for assessing gene diversity in barley were developed and used to investigate the efficacy for marker development, assay reliably and genotyping accuracy. The TSP markers described provide good coverage of the barley genome, are simple to use, easy to interpret and score, and are amenable to assay automation. They provide a resource of informative SNP markers for assessing genetic relationships among individuals, populations and gene pools of cultivated barley (*Hordeum vulgare L.*) and its wild relative *H. spontaneum* K. Koch. TSP markers provide opportunities to use available SNP resources for marker-assisted breeding and plant genetic research, and to generate information that can be integrated with SNP data from different sources and studies. TSP markers are expected to provide similar advantages for any animal or plant species.

Communicated by P. Hayes.

M. J. Hayden and T. Tabone contributed equally to this work.

M. J. Hayden (✉) · T. Tabone · D. E. Mather
Molecular Plant Breeding Co-operative Research Centre,
Glen Osmond, SA 5064, Australia
e-mail: matthew.hayden@dpi.vic.gov.au

M. J. Hayden · T. Tabone · D. E. Mather
School of Agriculture, Food and Wine,
The University of Adelaide, Glen Osmond, SA 5064, Australia

Present Address:
M. J. Hayden
Primary Industries Research Victoria, Victorian AgriBioscience Center, La Trobe Research and Development Park,
Bundoora, VIC 3082, Australia

Present Address:
T. Tabone
Ludwig Institute for Cancer Research, Royal Melbourne Hospital, PO Box 2008, Parkville, VIC 3050, Australia

Introduction

Single nucleotide polymorphisms (SNPs) are the smallest unit of genetic variation and represent the most common type of sequence polymorphism in plant and animal genomes. SNPs are usually discovered in silico from preexisting datasets of genomic sequences or expressed sequence tags (Kota et al. 2003; Rostoks et al. 2005), and through sequencing or re-sequencing of candidate genes or anonymous PCR products (van Orsouw et al. 2007; Ponting et al. 2007). When compared with other types of markers, such as RFLPs, RAPDs, AFLPs and SSRs, SNPs offer higher levels of genotyping throughput owing to their amenability to simpler and quicker processes that collectively facilitate automation (Rafalski 2002). SNP markers are biallelic (in diploids), provide exact allele information that can be described in a binary alphanumeric manner according to the nucleotide present, and enable the direct comparison of data collected across time and in different laboratories, and using different assay chemistries and platforms.

Rapid advances in DNA sequencing and genotyping technologies make SNP markers an ideal tool for applications in plant genetics and breeding. As a consequence,

large-scale identification and mapping of SNPs has been initiated in many crop plant species including, barley, grape, soybean, sugar beet and forest trees (Hayes and Szucs 2006; Lijavezky et al. 2007; Choi et al. 2007; Schneider et al. 2007; Pavy et al. 2008). The generation of dense genetic linkage maps based on the SNP markers will provide a genomic resource to further support applications, such as the assessment of genetic relationships between individuals and populations, understanding of genome architecture, mapping of key traits and marker-assisted breeding.

Numerous methods have been developed to genotype SNPs, ranging from single marker assays to specialized systems that enable thousands of SNPs to be scored in parallel (see Kwok 2001; Gupta et al. 2008 for reviews). These methods are based on a range of assay chemistries, have different requirements for specialized equipment and reagents, and vary in suitability for use in different types of studies. Two widely used methods, cleaved amplified polymorphic sequences (CAPS) and allele-specific PCR, allow for simple SNP genotyping by analysis of size polymorphisms of PCR products separated on agarose gel (Konieczny and Ausubel 1993; Bundock et al. 2005). However, because CAPS technology relies upon the cleavage of PCR products at restriction sites, it can be costly (depending on the restriction enzyme used) and unreliable (due to partial digestion of PCR products) and it is not applicable to all SNPs. Allele-specific PCR methods can lack robustness and typically require two assays per sample for codominant SNP scoring.

Temperature-switch PCR (TSP) is a newly reported method for developing allele-specific PCR markers for rapid, codominant SNP genotyping (Tabone et al. 2009). TSP marker assays are performed under standardized reaction conditions and the genotyping products can be detected by gel electrophoresis or high-resolution melt analysis, making them easy to deploy. Application of this technology for SNP genotyping in plant genomes has the potential to provide simple PCR markers that can be deployed in laboratories with widely differing technical capabilities and used to generate data that can be integrated into, and interpreted in, global contexts. TSP could allow the growing resource of SNP information for crop species to be used by any laboratory for marker-assisted breeding, and to assess genetic relationships among individuals, populations and gene pools for the efficient management and utilization of genetic diversity and germplasm resources.

The aim of this study was to investigate the efficacy of TSP for developing robust markers to genotype SNPs in barley. To achieve this objective, we used published SNPs to develop 87 TSP markers providing good genome coverage, as a first step towards producing an informative set of gene-based markers for distinguishing genotypes of cultivated barley (*Hordeum vulgare* L.) and its wild relative *H. spontaneum* K.Koch.

Materials and methods

Plant materials

Barley lines used for SNP genotyping were based on a collection of released varieties, breeding materials, mapping populations, and wild relatives of cultivated barley. Forty-eight cultivated varieties and advanced breeding lines of barley obtained from the University of Adelaide barley breeding program, and 40 accessions of *H. spontaneum*, obtained from the Australian Winter Cereal Collection, Tamworth NSW, Australia, were used to assess TSP markers for amplification and SNP diversity. The *H. spontaneum* accessions were collected in diverse geographical regions of the world including Afghanistan, China, the former Soviet Union, Iran, Israel, Nepal and Tajikistan. A subset of 48 highly informative doubled haploid lines, identified using the software MapPop (Howad et al. 2005), from a cross between varieties Chebec and Harrington (Karakousis et al. 2003) was used for genetic mapping. Ninety-six F_3 progeny derived from a cross between two doubled haploid lines from the Chebec × Harrington population (unpublished data) and tissue samples from 48 F_1 plants, obtained from the University of Adelaide barley breeding program, were used to confirm the ability of TSP markers to detect heterozygous SNP loci. DNA for each barley line was isolated from a single plant as described by Rogowsky et al. (1991).

Primer design and marker nomenclature

Temperature-switch PCR markers were designed to amplify SNPs that Rostoks et al. (2005) had identified from the re-sequencing of EST unigenes in eight barley lines. The sequence and SNP information used is available at http://bioinf.scri.ac.uk/. Each TSP marker comprised a set of three primers: a pair of locus-specific primers flanking the SNP and designed to amplify the unigene sequence, and an allele-specific primer designed to assay the SNP harbored within that sequence (Fig. 1). Primers were designed using Primer3 (Rozen and Skaletsky 2000) and NetPrimer (Premier Biosoft International) software according to Tabone et al. (2009) and are provided in Table 1. Briefly, the locus-specific primers reported by Rostoks et al. (2005) were redesigned to increase the primer melting temperature at 60–65°C and to amplify a PCR fragment greater than 400 bp in length. The locus-specific primers were positioned such that the forward primer, designed to the same template DNA strand as the allele-specific primer, was located at least 100 nucleotides from the SNP. The allele-specific primer was designed with a melting temperature of 43–48°C and to have a 3′ nucleotide complementary to one of the SNP alleles present at the locus. A short (2 or 3 bp)

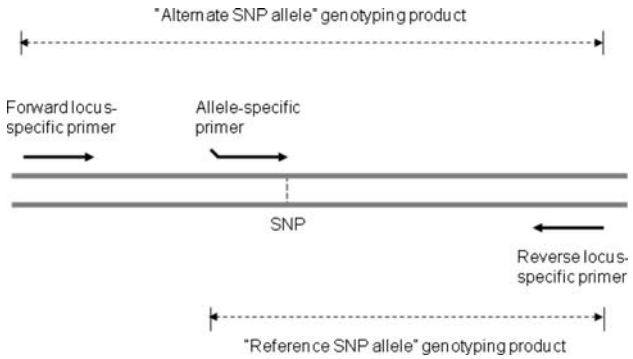

Fig. 1 Diagram illustrating the position and orientation of PCR primers for three primer, allele-specific TSP markers relative to matching template DNA

arbitrary nucleotide sequence, non-complementary to the target DNA, was added to the 5′ end of the allele-specific primer. This 5′-tail sequence was designed to increase the melting temperature of the allele-specific primer to about 53°C once the non-complementary sequence was incorporated into PCR product.

Temperature-switch PCR markers were named using the nomenclature of Rostoks et al. (2005) and consisted of the prefix 'scsnp', followed by the contig number from the Affymetrix Barley Gene Chip assembly (HarvEST assembly #21, http://www.harvest-web.org). Additional information linked to each TSP marker name indicates the position (bp) of the SNP in the resequencing data, nucleotide variants at the SNP, and the designation 'top' (top) or 'bottom' (bot) to indicate the DNA strand in relation to the resequencing data for which the allele-specific primer was designed. For example, scsnp00388_768[G/A]bot indicates an allele-specific primer was designed to the bottom DNA strand of contig ABC00388 for an SNP located at nucleotide position 768 in the resequencing data, with a guanosine to adenosine transition.

In addition, three TSP markers were developed for nucleotide sequence variation in the endosperm-specific β-amylase gene (*Bmy1*) located on the long arm of chromosome 4H. The SNPs corresponded to reported substitutions in the deduced protein sequence at amino acid positions 165, 347 and 430 (Kaneko et al. 1998), which are located at nucleotide positions 1,137, 2,856 and 3,281 in the genomic DNA sequence of cv. Haruna Nijo (GenBank D49999), respectively. Primer sequences for the TSP markers are provided in Table 1.

TSP assay

Temperature-switch PCR assays were performed in a 4 µl reaction mixture containing 0.2 mM dNTP, 1× ImmoBuffer (Bioline) (16 mM $(NH_4)_2SO_2$, 0.01% Tween-20, 100 mM Tris–HCl, pH 8.3), 1.5 mM $MgCl_2$, 100 ng/µl bovine serum albumin Fraction V (Sigma Aldrich), 0.1 µM each locus-specific primer, 0.5 µM allele-specific primer, 0.15 U Immolase DNA polymerase (Bioline), and about 20 ng dried-down genomic DNA. To dry-down the genomic template, 2 µl of diluted DNA was added to the PCR well and completely desiccated by heating at 80°C for 10 min. Following an initial denaturation step of 10 min at 95°C to heat activate the DNA polymerase, PCR was performed for a total of 35 cycles with the profile: 30 s at 94°C, 30 s at 58°C, 60 s at 72°C for 15 cycles. The next five cycles were with 10 s at 94°C, 30 s at 45°C, followed by 15 cycles with 30 s at 94°C, 30 s at 53°C, 5 s at 72°C. The PCR products were mixed with an equal volume of gel loading buffer (40% sucrose, 10 mM EDTA and 0.5% bromophenol blue as tracking dye) and the entire reaction mixture was separated by electrophoresis on a 2% agarose gel. TSP genotyping products were visualized using ethidium bromide staining.

Marker scoring

Temperature-switch PCR markers amplify two genotyping products that differ by at least 100 bp in size (Fig. 1). The genotyping products amplified from homozygous DNA samples correspond to the 'reference SNP allele' (smallest PCR product) or 'alternate SNP allele' (largest PCR product) depending on the SNP allele present. An SNP allele complementary to the allele-specific primer produced the 'reference SNP allele' product, while those without complementarity produced the 'alternate SNP allele' product. Both types of TSP product were amplified from heterozygous DNA samples.

Polymorphism information content (SNP diversity)

The polymorphism information content (PIC), of each SNP was calculated as $1 - \sum p_i^2$ where p_i is the frequency of the *i*th allele for a given locus (Nei 1987).

Genetic mapping

A "minimapping" subset of 48 doubled haploid lines from the Chebec × Harrington population was used for genetic mapping of TSP markers. MapManager QTX (Manly et al. 2001) was used to integrate SNP loci into the published Chebec × Harrington genetic map (Karakousis et al. 2003) using the distribute function with $P = 0.0001$. Final map distances were calculated using the dataset for all 120 doubled haploid lines of the mapping population.

Genotyping accuracy

Genotyping accuracy was assessed by comparing SNP datasets generated using TSP markers with those produced

Table 1 Marker name, chromosome, map position, SNP alleles and primer sequences for 87 SNPs

SNP name	Chromosome[a]	Pos. (cM)[a]	SNP alleles[a]	Locus-specific primers		ASPE Primer[b,c]	SNP allele assayed
scsnp21640_136[T/C]top	1H	0.00	T/C	Fwd	CCCACTGACCCCTACGAACG	**GC**TGATTACCGTTGCTATTC	C
				Rvs	CCGCTTCGTCTTGGCAAACT		
scsnp21640_328[T/C]top	1H	0.00	T/C	Fwd	CCCACTGACCCCTACGAACG	**CC**GCATTGGGTGGTCTACT	T
				Rvs	CCGCTTCGTCTTGGCAAACT		
scsnp07301_416[G/A]top	1H	47.22	G/A	Fwd	AACGGAAACTCCAATGGCGA	**CG**CGTCTTTCGAAGACTCG	G
				Rvs	AGCCACAGCCATAGGGCAAA		
scsnp13561_727[G/A]bot	1H	61.82	G/A	Fwd	CCCTGAGCAAGGGGACACAT	**GG**CCAAACTTGCTTCGTC	G
				Rvs	TGCCAACAAGCTCCACCGTA		
scsnp03346_170[C/G]top	1H	68.95	C/G	Fwd	CTTCTTAGCCCGGGGCAACT	**GGC**TTAGCTCATGCACTCTG	G
				Rvs	TGCAACTTGCGAAACGAACC		
scsnp04853_317[C/T]top	1H	99.36	C/T	Fwd	CCCGTGGGTGTTGAAGGTCT	**CCG**ATGTAATGTACTCCCTCC	C
				Rvs	GCAATTGCAGATGCTGCTGG		
scsnp04853_522[A/G]top	1H	99.36	A/G	Fwd	CCCGTGGGTGTTGAAGGTCT	**CG**TGAATTGATTGATTTTGG	G
				Rvs	GCAATTGCAGATGCTGCTGG		
scsnp06274_166[C/T]bot	1H	105.91	C/T	Fwd	TCGAAACAAAACTGCGTGGC	**CC**AAATGGGAACATAGGG	C
				Rvs	TGGCTCACAGTGCCATCCAT		
scsnp06274_318[A/T]top	1H	105.91	A/T	Fwd	TGGCTCACAGTGCCATCCAT	**CG**ATAATGCGAACAAAACA	A
				Rvs	TCGAAACAAAACTGCGTGGC		
scsnp01812_678[T/C]bot	1H	140.07	T/C	Fwd	TTGATAAGACAAGACTC	**GC**ATTGATAAGACAAGACTCG	C
				Rvs	TCCTGTGCGGGAACAAGGTT		
scsnp07434_260[G/A]bot	1H	150.40	G/A	Fwd	TGGAATGGCTTGAACCAGCA	**CC**GGAACAAATGTCTCTTGC	G
				Rvs	GGTTGTCCGAGAATGGTGCC		
scsnp17647_248[T/C]top	1H	170.12	T/C	Fwd	CTGTTCTGGGCCTTCTGCGT	**CG**GAAACTTGTAAATGTGGAT	T
				Rvs	AGAATCGAGCCAGCGATTGG		
scsnp02622_674[T/C]bot	2H	3.03	T/C	Fwd	TGGACGGCTCAATGGAACAA	**GC**ATCTCGAAACAAGAACG	C
				Rvs	GCGATACGACGCCGAGAAAG		
scsnp02329_170[A/G]top	2H	13.43	A/G	Fwd	GGGGAAAACGTGAAGAGCCC	**GC**CGTCATCCTGTTGTACTTA	A
				Rvs	CATGGCCACGAAGCTCAATG		
scsnp01327_275[C/T]bot	2H	41.73	C/T	Fwd	TCGTGCGATCCGTTTTAGCA	**GG**GTCTTCGGAGCACGA	T
				Rvs	GAAGTCGACGCTGATGGCAA		
scsnp05033_332[G/A]bot	2H	52.56	G/A	Fwd	CAAGCGCCGTATGGTGTGTC	**GC**CTCCAGGTTGCCCAC	G
				Rvs	TCGAGGGTCAGATGCTGTCG		
scsnp01644_323[A/G]top	2H	58.12	A/G	Fwd	CGAGGATTGGCTCAAGACGC	**GG**CCAGAGTAAGTTGCTGAA	A
				Rvs	GCAGCGTTCTTAGGACTGGCA		

Table 1 continued

SNP name	Chromosome[a]	Pos. (cM)[a]	SNP alleles[a]	Locus-specific primers		ASPE Primer[b,c]	SNP allele assayed
scsnp01644_557[T/C]bot	2H	58.12	T/C	Fwd	GCAGCGTTCTTAGGACTGGCA	**CG**AATGGATTCTTCAGAAAAG	C
				Rvs	CGAGGATTGGCTCAAGACGC		
scsnp02403_54[T/C]top	2H	58.37	T/C	Fwd	GGGAGGAACAGTGCCTGCAA	**GCC**TTAGTACTGTTGCTATTGAT	T
				Rvs	CCAGTCCTGGCACAACCACA		
scsnp15266_311[C/T]bot	2H	65.42	C/T	Fwd	GAGCTCTGCTACCGGCCTCA	**CC**CGTCACTCAAGTGACACA	T
				Rvs	ACGTGAAGTGCGCAAAGCAG		
scsnp15266_493[G/A]top	2H	65.42	G/A	Fwd	ACGTGAAGTGCGCAAAGCAG	**GC**ATACATCCGTATCTAGACAA	A
				Rvs	GAGCTCTGCTACCGGCCTCA		
scsnp03181_210[C/T]top	2H	69.61	C/T	Fwd	GCCCATTCGTTTGATCAGGG	**GC**GCAAAATTTTAGTGTAACT	T
				Rvs	CCTTTTCTTGGCGGTGATGC		
scsnp04861_524[T/C]top	2H	83.89	T/C	Fwd	GCAAGGGTGGAAAGCGAGAA	**GC**GCTCAACTGAAGAAGATT	T
				Rvs	CCGAACACCTGTCCTGGGAG		
scsnp04861_655[T/G]top	2H	83.89	T/G	Fwd	GCAAGGGTGGAAAGCGAGAA	**CGC**TGGCACTCTCTTCATAT	T
				Rvs	CCGAACACCTGTCCTGGGAG		
scsnp06130_208[C/T]bot	2H	93.81	C/T	Fwd	TTGGCCGGGAACTTATGGTG	**CG**CCAAACATCTTTTACAAG	C
				Rvs	GACGTCCCTCGCGTAAATGG		
scsnp14531_165[G/A]top	2H	96.98	G/A	Fwd	TGGGCTCTCAGATTCCACGG	**GC**CAACTACTAAGTTAGTAATGCTA	A
				Rvs	TTCCATGCAAATGCCTGCTG		
scsnp06766_249[G/A]top	2H	143.10	G/A	Fwd	AGCTCCCATCGAGCTTGTGC	**GC**TCGTCGAGAAGTTCCA	A
				Rvs	GTTCAGCGACAGCCAACGAA		
scsnp05814_321[A/G]top	2H	152.46	A/G	Fwd	AGGCACTGCTGTCATGCTGG	**CC**CTGCATCTACAGTACCTTA	A
				Rvs	TTTTCAATCGGGCGTCTTCC		
scsnp05814_98[G/A]top	2H	152.46	G/A	Fwd	AGGCACTGCTGTCATGCTGG	**GC**CGGCATAAACTTTGTACTA	A
				Rvs	TTTTCAATCGGGCGTCTTCC		
scsnp01404_680[T/C]bot	3H	6.87	C/T	Fwd	CTTCTGGGTGCACAACACGG	**AC**GGCTTCGCTCATCAG	C
				Rvs	AAGCCGCCTCTGTCAAGTGC		
scsnp00495_502[G/T]bot	3H	48.73	G/T	Fwd	ATGGCAAATTCACATCGGGC	**CG**ATGCAGAACTGTGGC	G
				Rvs	GGCTCTGCTCTCGCTCAAGG		
scsnp04006_344[T/C]top	3H	55.06	T/C	Fwd	CAGCCGTGACACCATCCTTC	**GC**TGGAAGAAAACATAACCAC	C
				Rvs	CAATGAACAGCCCCCACCTC		
scsnp03835_396[A/G]top	3H	56.02	A/G	Fwd	GGGCGGTATCAGAGGTGCAG	**GC**GTCATACAACACCCGG	G
				Rvs	GCATGCACGCAGCAAGACTC		
scsnp03835_399[A/G]bot	3H	56.02	A/G	Fwd	GCATGCACGCAGCAAGACTC	**CC**GGGTCACTACTTCCAATC	G
				Rvs	GGGCGGTATCAGAGGTGCAG		

Table 1 continued

SNP name	Chromosome[a]	Pos. (cM)[a]	SNP alleles[a]	Locus-specific primers		ASPE Primer[b,c]	SNP allele assayed
scsnp05580_438[T/A]top	3H	77.65	T/A	Fwd	GCCCCGGTGAAGGGAGTAAG	**CG**AAGACTTATATTTAGGAACGA	A
				Rvs	CCGCGATTCTCGTCCCTCT		
scsnp03814_188[A/G]top	3H	94.64	A/G	Fwd	GTGATGGGACTCGCTTCGGT	**GG**AGATAGCGACACAGTAACAA	A
				Rvs	GCTCTTTGCGTACCCATGCC		
scsnp03814_307[A/G]top	3H	94.64	A/G	Fwd	GTGATGGGACTCGCTTCGGT	**GC**CAACCACAGGAGAAGG	G
				Rvs	GCTCTTTGCGTACCCATGCC		
scsnp14307_683[T/C]top	3H	106.40	T/C	Fwd	TGGAGCAGCTGTTCTCGGTG	**CC**CTGAGCGTTTGATGAAT	T
				Rvs	CCAAGAGGCCAAAACTGTGGA		
scsnp19616_322[G/A]top	3H	111.05	G/A	Fwd	AGGCCATCGTCGACACCTTC	**GC**GGTGCGTCTCCCAG	G
				Rvs	ATGCGGTGATCCTCCAGCTC		
scsnp19616_617[C/T]bot	3H	111.05	C/T	Fwd	ATGCGGTGATCCTCCAGCTC	**GCG**ACTGTGATATTGCCTG	C
				Rvs	AGGCCATCGTCGACACCTTC		
scsnp06381_514[G/A]top	3H	117.99	G/A	Fwd	CGGAGATCCTTTCAACCCGA	**GGC**ATTTTGTAACATGTTCAG	G
				Rvs	TCGGATGTCCGTCCAGATCA		
scsnp06381_726[T/G]top	3H	117.99	T/G	Fwd	CGGAGATCCTTTCAACCCGA	**CC**TGGTCGTTAATTAGAGCTT	T
				Rvs	TCGGATGTCCGTCCAGATCA		
scsnp05754_646[G/A]top	3H	122.55	G/A	Fwd	CGCATACCACACCCGGTACA	**CG**ACTTACAGTTAAGGAGCTCA	A
				Rvs	GGCCCAACCAGGAAATCTCA		
scsnp05754_714[T/C]bot	3H	122.55	T/C	Fwd	GGCCCAACCAGGAAATCTCA	**CG**ATGATCCGCCCTGA	T
				Rvs	CGCATACCACACCCGGTACA		
scsnp06172_369[G/A]bot	4H	26.69	G/A	Fwd	GAATCTACCACCGCTCCAGCA	**GC**GGATTGTTGCCCTAC	G
				Rvs	GGGACATGCATGGTGGCATA		
scsnp05351_422[G/A]bot	4H	33.32	G/A	Fwd	GCAACTAAACACCCCTGCCG	**CG**AATAGTCTTCAGTGAAGCC	G
				Rvs	CGGCCCTCAGACCAGACCTA		
scsnp07112_598[T/C]bot	4H	35.81	T/C	Fwd	TTGGAATAGAAGCGGGCACC	**TCT**CAACGCCAGTGAGGA	T
				Rvs	ACCAGGACTTCGGCATGGAC		
scsnp03465_115[T/C]top	4H	53.15	T/C	Fwd	GCGTCGCAAAGACAAGCTGA	**CG**GATATGTTTGGGTATCATT	T
				Rvs	CCGCAGGCGAACCTTTACAT		
scsnp03465_158[T/A]bot	4H	53.15	T/A	Fwd	CCGCAGGCGAACCTTTACAT	**CG**TGCATACTGCACAAATT	A
				Rvs	GCGTCGCAAAGACAAGCTGA		
Bmy1_A430V_3281[T/C]top	4H	129.97	T/C	Fwd	CCTGAGCACAAGCTGTTTGGA	**GC**GAGGGACAAAACTATGT	T
				Rvs	CAGGTTGGCATGCATTCTGTC		
Bmy1_E165D_1137[G/C]bot	4H	129.97	G/C	Fwd	TGTATGCCGATTACATGACAAGC	**CG**AGGGAGAACATGAAAGAC	G
				Rvs	CGTGGCTCTGAGGATATGATGG		

Table 1 continued

SNP name	Chromosome[a]	Pos. (cM)[a]	SNP alleles[a]	Locus-specific primers		ASPE Primer[b,c]	SNP allele assayed
Bmy1_S347L_2856[T/C]top	4H	129.97	T/C	Fwd	TGATAGAGACGGCTACAGAACCA	**GC**CGGAGATGAGGGATTC	C
				Rvs	CTAGTTCTTCTGGTGCGCTCAT		
scsnp00388_768[G/A]bot	5H	1.73	G/A	Fwd	GCCAAACTGCATCACCAACG	**CG**ACGGACGATTACAGTAGC	G
				Rvs	CCGATACACTCTGAGCGGCA		
scsnp01741_596[C/T]top	5H	26.00	C/T	Fwd	TGAGGCTGGCACAACTGGTC	**GC**AAAGTATGGATGGCTCTC	C
				Rvs	GCTGCAAAAGCAAGCAAGCA		
scsnp07010_126[A/C]top	5H	56.18	A/C	Fwd	GACTCTGTCGGGCAGCATGA	**CG**CAGTCAAACTCTACTTTCTAC	C
				Rvs	CCCATAAATTGCGAGCGTGG		
scsnp07010_217[G/A]top	5H	56.18	G/A	Fwd	GACTCTGTCGGGCAGCATGA	**CC**AACACAAGATAATCAGTCG	G
				Rvs	CCCATAAATTGCGAGCGTGG		
scsnp05926_188[C/G]top	5H	57.21	C/G	Fwd	CCAACACTTGGTGTGAGGCG	**CC**GAAGATGTCGCAAAGG	G
				Rvs	GCTAACCATCAGCCCCATGC		
scsnp05926_55[T/C]top	5H	57.21	T/C	Fwd	CCAACACTTGGTGTGAGGCG	**CG**GACAAATCTAAATGCCTTC	C
				Rvs	GCTAACCATCAGCCCCATGC		
scsnp02265_354[A/G]bot	5H	59.87	A/G	Fwd	GCTGGTCCTAGCCGTTCCCT	**GG**CCTGGTGACCTAGAGAAC	G
				Rvs	TGTCAAGAACAACCCGGCAG		
scsnp03594_515[T/C]top	5H	87.13	T/C	Fwd	TGGGGCTGTTTGAGACGGAT	**GG**GGTTATAGATGCTCTTATGTT	T
				Rvs	AGAGGAAGTTCGGCGTGTCG		
scsnp02739_543[T/C]bot	5H	100.59	T/C	Fwd	CCGGGCTTATTCATGCATCC	**GG**CATGGAGGAAGCCG	C
				Rvs	TTGGGTCATCAACACCGTGG		
scsnp02739_586[G/A]bot	5H	100.59	G/A	Fwd	CCGGGCTTATTCATGCATCC	**CG**GAGAATAGGCCTTGCC	G
				Rvs	TTGGGTCATCAACACCGTGG		
scsnp00177_689[T/C]bot	5H	124.38	T/C	Fwd	GCACTCCCAGGAAGAACGGA	**GCG**TTTTCAGTTGGTTGATG	C
				Rvs	GGCTATGTCATCCGCAACCC		
scsnp00314_166[T/C]top	5H	149.73	T/C	Fwd	TTGGCAAGTGCTGTGGCAAT	**CG**AAGCGAACACGATCAC	C
				Rvs	TATCGCAGGCGGGTGTTTTT		
scsnp03683_406[A/C]top	5H	199.15	A/C	Fwd	CAACGGCGCCACCTTCTACT	**AC**GACTTGGCCGTGGA	A
				Rvs	CACATACCCCACTGCCATGC		
scsnp07305_298[T/A]bot	6H	1.70	T/A	Fwd	TCCGACCATCGACCTGAACA	**CG**AGAGGATCTTTTCCGAT	A
				Rvs	TCAACGTTGCAAGGAAGCCA		
scsnp07351_407[A/C]bot	6H	10.45	A/C	Fwd	CCCAGCTAGCAGCAAGGGAA	**GGC**ATAGCTGGAACAGCG	C
				Rvs	TACTCCCCCGGCGGACTTAT		
scsnp06204_159[C/T]bot	6H	35.61	C/T	Fwd	ATCATGACCCGATGCGGTG	**GCG**AGCTCTTGCATTCTCCG	C
				Rvs	TCAAAGTGGGCAGGCATCAA		

Table 1 continued

SNP name	Chromosome[a]	Pos. (cM)[a]	SNP alleles[a]	Locus-specific primers		ASPE Primer[b,c]	SNP allele assayed
scsnp06204_63[A/G]top	6H	35.61	A/G	Fwd	TCAAAGTGGGCAGGCATCAA	**CG**TGGCAAAAGACCAACA	A
				Rvs	ATCATGACCCGATGCGGTG		
scsnp02895_268[A/G]bot	6H	63.80	A/G	Fwd	GGAATCGCAAGCACTACGGG	**CG**GATGGAGAAAGAAAGTCC	G
				Rvs	TGATCGGTCCAGTTCACCCA		
scsnp02895_423[T/C]bot	6H	63.80	T/C	Fwd	TGATCGGTCCAGTTCACCCA	**CCA**GAGCTTGACATCCTCG	C
				Rvs	GGAATCGCAAGCACTACGGG		
scsnp03149_168[C/A]top	6H	133.30	C/A	Fwd	CCGAAGAGGAAGCCATCGAA	**GC**TGATTTCCGGTGACC	C
				Rvs	TGTCCACAAAACCCATGCCA		
scsnp03149_617[G/A]top	6H	133.30	G/A	Fwd	CCGAAGAGGAAGCCATCGAA	**GC**GCTCCGGCTAACTAG	G
				Rvs	TGTCCACAAAACCCATGCCA		
scsnp04273_107[G/A]bot	6H	137.79	G/A	Fwd	ACCACACAAGCTGCCCAACA	**CG**GTATAAGGATGAAATAGAGAGAT	A
				Rvs	ACGTGCCCATCGTTCCAAAT		
scsnp04220_436[C/T]bot	6H	143.14	C/T	Fwd	CAGCTTTCCTCACCGCGTCT	**GC**GCCTATTATGTCTGACAAG	C
				Rvs	TACAAGCCGCTAGCCCCGTA		
scsnp04220_508[G/T]bot	6H	143.14	G/T	Fwd	CAGCTTTCCTCACCGCGTCT	**GC**AGCCCTCTGATGAAGC	G
				Rvs	TACAAGCCGCTAGCCCCGTA		
scsnp04135_235[T/C]top	6H	149.78	T/C	Fwd	GCTGAGCAACCTTGGTGCAA	**GC**CAAAAGTTCACAGTCAAAT	T
				Rvs	TCATCTTGCATGTCGGCACA		
scsnp04135_67[C/G]top	6H	149.78	C/G	Fwd	GCTGAGCAACCTTGGTGCAA	**GC**ACCACCGATCTTAGTTC	C
				Rvs	TCATCTTGCATGTCGGCACA		
scsnp02493_292[G/C]bot	7H	13.02	G/C	Fwd	TCATCTCGCCAAGAACCCAA	**CGG**GATCAATCAATGTGATC	G
				Rvs	CCCTCAGGGTGTTGGTGGAC		
scsnp02493_317[C/T]bot	7H	13.02	C/T	Fwd	TCATCTCGCCAAGAACCCAA	**GG**CATCCATGAGGGAGG	C
				Rvs	CCCTCAGGGTGTTGGTGGAC		
scsnp00823_425[G/A]top	7H	20.31	G/A	Fwd	GCCTTTGTGCAGCCTGCTTT	**CC**GTCTTCCTACCTTTGCA	A
				Rvs	CCCTCAACAGCCAATGGGAC		
scsnp00694_225[T/A]top	7H	34.70	T/A	Fwd	CTCCAGGGAATGCGCTATGG	**GC**CGATCCTCTTGTGTAAAA	A
				Rvs	AAAGAGGCAAGCGGCACAAC		
scsnp00694_248[T/C]top	7H	34.70	T/C	Fwd	CTCCAGGGAATGCGCTATGG	**GC**CATCTTGTTCATCCTGGT	T
				Rvs	AAAGAGGCAAGCGGCACAAC		
scsnp06931_64[A/G]top	7H	40.74	A/G	Fwd	GCCCCTCTACAGCCTCGTCA	**CGG**CTAGCTACAACGCTG	G
				Rvs	AGATCCAGTGCCCTTGCAGC		
scsnp00610_670[A/G]bot	7H	98.30	A/G	Fwd	ACCAGGCTGTTGGGGATTCA	**CCG**TTTGATATACTTACGTATGTATAC	G
				Rvs	AGGCCTTCACTGTCCGCAAC		

Table 1 continued

SNP name	Chromosome[a]	Pos. (cM)[a]	SNP alleles[a]	Locus-specific primers		ASPE Primer[b,c]	SNP allele assayed
scsnp00610_69[C/T]top	7H	98.30	C/T	Fwd	AGGCCTTCACTGTCCGCAAC	**GC**TTGGATCGGCCATT	T
				Rvs	ACCAGGCTGTTGGGGATTCA		
scsnp05362_340[C/G]bot	7H	142.55	C/G	Fwd	TACACGCCGGCCTTCATCTT	**GG**CACAGCTCCGAACG	C
				Rvs	AAAAGGGGGCAAATCAAGCG		

[a] As reported by Rostoks et al. (2005)

[b] A SNP allele complementary to the allele-specific primer produces the 'reference SNP allele' (smallest) PCR product, whereas those without complementarity produce the 'alternate SNP allele' (largest) product. Heterozygous samples produce both TSP genotyping products

[c] The arbitrary 5′-nucleotide sequence indicated in underlined, bold font is non-complementary to the target DNA

using an independent genotyping method. Allele-specific primer extension assays were performed as described by Hayden et al. (2009) on the BioPlex™ microsphere suspension array platform (BioRad) to generate independent genotyping data using the same DNA samples and SNP loci.

Results

Marker development

A total of 87 TSP markers were tested on single plants for 16 barley varieties and advanced breeding lines to assess PCR amplification and SNP allele specificity. Each marker produced TSP genotyping products of the expected size indicating that the unigene sequences were successfully amplified. As all of the lines were highly inbred, homozygosity at most loci was expected for each plant making it possible to assess SNP allele specificity. The amplification of a single TSP genotyping product from most or all of the individual plants was used to infer that only the target SNP allele was amplified by the allele-specific primer (Fig. 2a). Markers consistently amplifying two TSP genotyping products from individual plants were considered to have poor SNP allele specificity, probably due to mis-extension of the allele-specific primer by DNA polymerase. Of the 87 TSP markers, six showed poor SNP allele specificity. To improve the specificity of these markers, the allele-specific primers were redesigned. This process involved substituting the 3′-nucleotide of the allele-specific primer with the nucleotide variant for the alternate SNP allele. Retesting on the same barley lines using the six new allele-specific primers found all markers were SNP allele specific. Thus, a 100% success rate was achieved for the development of the TSP markers.

To ensure the markers assayed the targeted SNP loci and behaved in a Mendelian fashion, 42 TSP markers revealing polymorphism between the varieties Chebec and Harrington were genotyped on 48 doubled haploid lines of a mapping population derived from a cross between these varieties. Integration of the marker loci into the published genetic map for this cross showed that the SNP loci were located on the expected chromosomes and at approximately the same map positions reported by Rostoks et al. (2005). Forty markers segregated at the 1:1 expected ratio ($\chi^2_{1:1}$ non-significant at $P = 0.05$ and 1 df). Two markers scsnp01644_323[A/G]top and scsnp06130_208[C/T]bot showing skewed segregation mapped to a region on chromosome 2H, which also contained RFLP and SSR markers showing significant segregation distortion.

Genotyping efficacy

To assess the success rate of the assays and the accuracy of SNP allele calling, subsets of the TSP markers were scored on a diverse collection of barley plants with different levels of zygosity. First, 67 markers were scored using genomic DNA extracted from individual plants of 48 barley varieties and advanced breeding lines. These plants were expected to be homozygous at almost all SNP loci. Next, all 87 markers were genotyped on 48 unrelated F_1 individuals and 40 markers, known to be polymorphic between two doubled haploid lines from the Chebec × Harrington mapping population, were scored on 96 F_3 progeny derived from a cross between those two lines (Fig. 2b). The F_1 and F_3 plants were expected to contain both homozygous and heterozygous SNP loci. Overall, 11,232 TSP marker assays were performed, of which 98.5% produced TSP genotyping products. Missing data (1.5%) arose solely from occasional PCR failure of individual assays (i.e. absence of PCR product), rather than the amplification of TSP genotyping products of unexpected size. Comparison of the TSP genotyping results with a dataset produced using an independent method (see "Materials and methods") for the same DNA samples and SNP loci found 100% of genotypes called in both datasets were concordant.

Marker transferability and applications

Having established that TSP markers provide robust genotyping from a technical point of view, we tested the ability for 16 randomly chosen TSP markers to distinguish among 48 cultivated barley lines and 40 *H. spontaneum* accessions. Each marker tested in the *H. spontaneum* accessions produced the expected TSP genotyping products. The PIC (SNP diversity) of the SNPs ranged from 0.00 to 0.50, with an average of 0.36 across all lines and accessions. The average SNP diversity was 0.37 in the *H. spontaneum* accessions, which was slightly higher than that observed (0.35) for the barley lines. All 16 markers revealed an SNP diversity greater than 0.20 in at least one of the germplasm sets. Fifteen and 13 markers showed a diversity greater than 0.20 in only the *H. spontaneum* and barley sets, respectively. Principal component analysis of the 88 genotypes revealed a clear separation between cultivated and wild barley, and separation of most genotypes within each type of germplasm (Fig. 3).

Discussion

Simple molecular marker assays that do not rely upon specialized equipment or reagents underpin routine plant breeding and research activities in many laboratories worldwide. With the rapid growth of genomic resources for many crop plant species, the availability of robust, simple molecular marker assays for genotyping SNPs has become increasingly important.

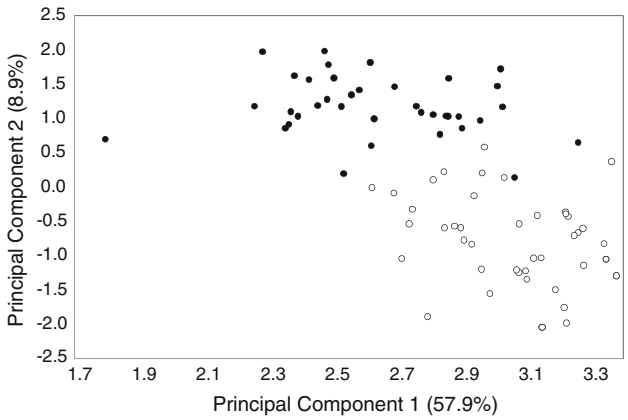

Fig. 3 Principal component analysis of 88 barley genotypes based on 16 TSP markers. Cultivated barley varieties (*open circle*) and *H. spontaneum* accessions (*solid circles*)

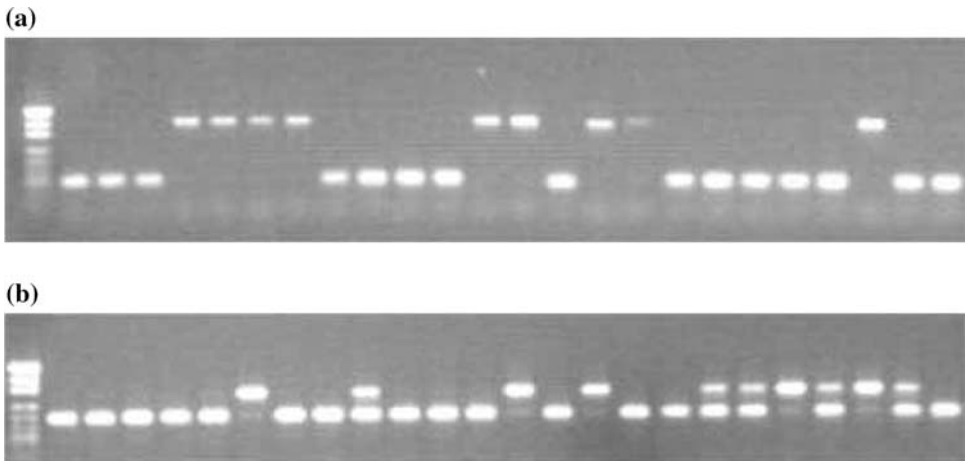

Fig. 2 TSP genotyping assay performed using **a** marker scsnp05814_98[G/A]bot in 24 barley varieties, and **b** marker scsnp06172_369(G/A)bot in 24 F_3 progeny of the Chebec × Harrington mapping population. Lane 1 contains pUC19/*Hpa*II as size standard

Temperature-switch PCR is a new method for rapid, low-tech SNP genotyping (Tabone et al. 2009). It differs from existing methods by partitioning the amplification and detection of an SNP into two distinct PCR phases that are separated by primer annealing temperature. In the first PCR phase, a target locus harboring the polymorphism is enriched from genomic template, while in the second phase, the SNP allele harbored within the enriched sequence is interrogated. This biphasic mechanism permits SNP genotyping in a single-step, closed-tube reaction that is performed under standardized reaction conditions for all markers. TSP markers provide an opportunity for laboratories with differing technical capabilities to utilize available SNP resources for marker-assisted breeding and to assess genetic relationships among individuals, populations and gene pools for the efficient management and utilization of germplasm resources. Our goal was to investigate the efficacy for TSP marker development in barley, to assess TSP assay reliability and accuracy for SNP genotyping, and to develop a resource of useful markers for distinguishing genotypes of cultivated barley cultivated barley and its wild relative *H. spontaneum*.

Marker development and genotyping efficacy

A 93% (81/87) success rate was achieved for developing robust TSP markers in the first round of primer design. Because TSP marker assays are performed under standardized reaction conditions, primer design should be the only factor affecting marker development. Failure of locus-specific primers to amplify a target sequence is easily recognized by the detection of no PCR product, or by the detection of TSP genotyping products of unexpected size. Similarly, poor specificity of an allele-specific primer for the target SNP allele is easily recognized by the consistent amplification of both types of TSP genotyping products from materials that are expected to be homozygous. However, this study found poor SNP allele specificity was readily resolved by targeting the alternate SNP allele. This was achieved by substituting the 3′ nucleotide of the allele-specific primer with the alternate nucleotide for the SNP variant. Using this strategy, we were able to achieve a 100% success rate for marker development. In difficult cases, an allele-specific primer could also be designed for the opposite template DNA strand. It should be noted, however, from our initial studies (Tabone et al. 2009) that the use of a secondary mismatch in the design of allele-specific primers (Chiapparino et al. 2004) had a negative impact on SNP genotyping accuracy, caused by the over-amplification of the 'alternate SNP allele' (largest) TSP genotyping product.

We also found that TSP markers produce reliable and accurate codominant SNP genotyping. In the present study, an SNP allele call rate of 98.5% was achieved across 11,232 assays. Occasional failure of individual reactions, resulting in the absence of PCR product, was the sole cause of missing data. Complete (100%) concordance for SNP allele calling between the TSP markers and an independent genotyping method indicated that the TSP genotypes were accurate. The technical reliability of molecular marker assays, in terms of allele calling rate and accuracy, is rarely addressed in the scientific literature, but it is known that genotyping error rates can be relatively high and variable, and that errors can affect biological inferences made from the data (Bonin et al. 2004). Average allele call rates for marker systems, which assay one locus at a time have been reported to be about 90% for SSRs (Hayden et al. 2008; Jones et al. 2008) and about 97% for SNPs (Jones et al. 2008). The reproducibility (accuracy) of genotypic data from these assays has been reported to be about 90–95% for SSRs (Jones et al. 2008; Dreisigacker et al. 2004), and 97–100% for SNPs (Jones et al. 2008). In this context, TSP markers assays can be considered to provide efficient and accurate SNP genotyping.

With the ongoing identification of SNPs in many crop species, TSP offers opportunities for the rapid development of markers to assay polymorphisms of interest. Robust, simple PCR marker assays are especially desirable for marker-assisted breeding, as they allow the direct selection of favorable alleles and allele combinations for key traits in crop improvement, without the need for specialized equipment. Increasing genomic resources will increase the availability of trait-linked SNPs that will be useful for marker-assisted breeding. TSP markers can potentially be developed for any SNP providing that sufficient flanking sequence is available. The amount of flanking sequence required depends on the resolution of the separation matrix used, with about 400 bp required for agarose gel. Only a small (>150 bp) amount of flanking sequence is required to detect TSP genotyping products by high-resolution melt analysis, as described by Tabone et al. (2009).

Marker transferability and utility

Temperature-switch PCR markers developed in this study were highly transferable from cultivated barley to its wild relative *H. spontaneum*. A subset of 16 markers tested on a group of 88 cultivated varieties and wild accessions showed that most barley lines could be uniquely distinguished (Fig. 3). These results indicate the utility of TSP markers for assessing genetic diversity and germplasm characterization. All 16 markers tested revealed polymorphism among the cultivated varieties and among the wild barley accessions, with an average SNP diversity of 0.36. Because the TSP markers developed in this study were designed for SNPs known to be polymorphic in diverse barley

germplasm, which included seven cultivated lines and one *H. spontaneum* accession (Rostoks et al. 2005), it is expected that all 87 TSP markers will be useful for assessing genetic relationships among individuals, populations and germplasm collections. The markers described here provide good coverage of the barley genome, with 12, 17, 15, 8, 13, 13 and 9 loci distributed over chromosomes 1H to 7H, respectively (Table 1).

Similar to other SNP genotyping assays, a major advantage of TSP markers is that the nucleotide variant itself is interrogated. This allows results across time, laboratories and different germplasm to be readily compared without the need for inclusion of reference genotypes, as is needed for comparative sizing of SSR alleles. Similarly, TSP marker data can be directly compared with SNP data collected using different assay chemistries and platforms. By providing unequivocal genotyping, TSP markers allow information to be produced that can be directly integrated with SNP data from different sources and interpreted in a global context. Importantly, TSP markers provide opportunities for any laboratories to develop germplasm databases that are meaningful across different organizations and that will not become redundant as further improvements to SNP genotyping technologies are made.

The application of SNP markers has mostly been reported for diploid organisms, where the presence–absence of either one or both of the alternate nucleotides indicates homozygosity or heterozygosity. Their application in polyploid organisms is generally lacking owing to complications caused by the requirement to detect allele dosage at an SNP locus, which varies depending on the ploidy of the genome. For example, allohexaploid wheat contains three homoeologous copies of each gene and therefore up to six copies of an SNP allele. To reduce this complexity to a diploid situation, SNP genotyping in polyploid organisms usually relies on methods that involve a two-step process: genome-specific amplification (usually by PCR) of the target sequence harboring a polymorphism, followed by SNP detection. Two-step processes are a bottleneck for high throughput genotyping, especially in marker-assisted breeding. A perceived advantage and use of TSP marker assays is for genome-specific SNP genotyping in polyploid crop plants, because the biphasic PCR mechanism of temperature-switch PCR should allow genome-specific SNP detection to be reduced to a single-step assay (Tabone et al. 2009). The application of TSP markers for genome-specific SNP genotyping in bread wheat is currently being investigated.

Conclusion

In summary, temperature-switch PCR was shown to support the rapid development of robust allele-specific PCR markers for codominant SNP genotyping in barley. The TSP markers described represent an informative set of SNP markers that are useful for assessing genetic relationships among individuals and populations of cultivated barley and its wild relative *H. spontaneum*. Moreover, they are simple to use, easy to interpret and score, and are amenable to assay automation and automated data acquisition. The TSP markers developed are suited for routine deployment on agarose gel, but can easily be adapted for the detection on high-throughput systems, such as high-resolution melt analysis. These technological features that provide advantages for SNP genotyping in barley are expected to provide similar advantages for other plant and animal species.

References

Bonin A, Bellemain E, Bronken-Eidesen P, Pompanon F, Brochmann C, Taberlet P (2004) How to track and assess genotyping errors in population genetics studies. Mol Ecol 13:3261–3273

Bundock PC, Cross MJ, Shapter FM, Henry RJ (2005) Robust allele-specific polymerase chain reaction markers developed for single nucleotide polymorphisms in expressed barley sequences. Theor Appl Genet 112:358–365

Chiapparino E, Lee D, Donini P (2004) Genotyping single nucleotide polymorphisms in barley by tetra-primers ARMS-PCR. Genome 47:414–420

Choi IY, Hyten DL, Matukumalli L, Song Q, Chaky JM, Quigley CV, Chase K, Lark KG, Reiter RS, Yoon MS, Hwang EY, Yi SI, Young ND, Shoemaker RC, van Tassell CP, Specht JE, Cregan PB (2007) A soybean transcript map: gene distribution, haplotype and SNP analysis. Genetics 176:685–696

Dreisigacker S, Zhang P, Warburton ML, van Ginkel M, Hoisington D, Bohn M, Melchinger AE (2004) SSR and pedigree analyses of genetic diversity among CIMMYT wheat lines targeted to different mega-environments. Crop Sci 44:381–388

Gupta PK, Rustgi S, Mir RR (2008) Array-based high-throughput DNA markers for crop improvement. Heredity 101:5–18

Hayden MJ, Nguyen TM, Waterman A, McMichael GL, Chalmers KJ (2008) Application of multiplex-ready PCR for fluorescence-based SSR genotyping in barley and wheat. Mol Breed 21:271–281

Hayden MJ, Tabone TL, Nguyen TM, Coventry S, Keiper FJ, Fox RL, Eglinton JK (2009) An informative set of SNP markers for molecular characterization of Australian barley germplasm. Crop and Pasture Science (submitted)

Hayes P, Szucs P (2006) Disequilibrium and association in barley: thinking outside the glass. Proc Natl Acad Sci USA 103:18385–18386

Howad W, Yamamoto T, Dirlewanger E, Testolin R, Cosson P, Cipriani G, Monforte AJ, Georgi L, Abbott AG, Arús P (2005) Mapping with a few plants: using selective mapping for microsatellite saturation of the prunus reference map. Genetics 171:1305–1309

Jones ES, Sullivan H, Bhattramakki D, Smith JSC (2008) A comparison of simple sequence repeat and single nucleotide polymorphism marker technologies for the genotypic analysis of maize (*Zea mays* L.). Theor Appl Genet 115:361–371

Kaneko T, Kihara T, Ito K (1998) Genetic variation of β-amylase thermostability among varieties of barley. *Hordeum vulgare* L., and relation to malting quality. Plant Breed 117:425–428

Karakousis A, Gustafson JP, Chalmers KJ, Barr AR, Langridge P (2003) A barley consensus map integrating SSR, RFLP and AFLP markers. Aust J Agric Res 54:1173–1185

Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific markers. Plant J 4:403–410

Kota R, Rudd S, Facius A, Kolesov G, Thiel T, Zhang H, Stein N, Mayer K, Graner A (2003) Snipping polymorphisms from large EST collections in barley (*Hordeum vulgare* L.). Mol Genet Genomics 270:24–33

Kwok PY (2001) Methods for genotyping single nucleotide polymorphisms. Annu Rev Genomics Hum Genet 2:235–258

Lijavezky D, Cabezas JA, Ibanez A, Rodriguez V, Martinez-Zapater JM (2007) High throughput SNP discovery and genotyping in grapevine (*Vitis vinifera* L.) by combining a re-sequencing approach and SNPlex technology. BMC Genomics 8:424

Manly KF, Cudmore RH, Meer JN (2001) Map Manager QTX, cross-platform software for genetic mapping. Mamm Genome 12:930–932

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York

Pavy N, Pelgas B, Beauseigle S, Blais S, Gagnon F, Gosselin I, Lamothe M, Isabel N, Bousquet J (2008) Enhancing genetic mapping of complex genomes through the design of highly-multiplexed SNP arrays: application to the large and unsequenced genomes of white spruce and black spruce. BMC Genomics 9:21

Ponting RC, Drayton MC, Cogan NIO, Dobrowolski MP, Spangenberg GC, Smith KF, Forster JW (2007) SNP discovery, validation, haplotype structure and linkage disequilibrium in full-length herbage nutritive quality genes of perennial ryegrass (*Lolium perenne* L.). Mol Genet Genomics 278:585–597

Rafalski JA (2002) Application of single nucleotide polymorphisms in crop genetics. Curr Opin Plant Biol 5:94–100

Rogowsky PM, Guidet FLY, Langridge P, Sheperd KW, Koebner RMD (1991) Isolation and characterization of wheat-rye recombinants involving chromosome arm 1DS of wheat. Theor Appl Genet 82:537–544

Rostoks N, Mudie S, Cardle L, Russell J, Ramsay L, Booth A, Svensson JT, Wanamaker SI, Walia H, Rodriguez EM, Hedley PE, Liu H, Morris J, Close TJ, Marshall DF, Waugh R (2005) Genome-wide SNP discovery and linkage analysis in barley based on genes responsive to abiotic stress. Mol Genet Genomics 274:515–527

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, pp 365–386

Schneider K, Kulosa D, Soerensen TR, Mohring S, Heine M, Durstewitz G, Polley A, Weber E, Lein JJ, Hohmann U, Tahiro E, Weisshaar B, Schulz B, Kock G, Jung C, Ganal M (2007) Analysis of DNA polymorphisms in sugar beet (*Beta vulgaris* L.) and development of an SNP-based map of expressed genes. Theor Appl Genet 115:601–615

Tabone TL, Mather DE, Hayden MJ (2009) Temperature Switch PCR (TSP): amplification and detection of SNPs in a single-step, closed-tube system. BMC Genomics (under review)

van Orsouw NJ, Hogers RCJ, Janssen A, Yalcin F, Snoeijers S, Verstege E, Schnieders H, van der Poel H, van Oeveren J, Verstegen H, van Eijk MJT (2007) Complexity reduction of polymorphic sequences (CRoPS^{TM}): a novel approach for large-scale polymorphism discovery in complex genomes. PLoS ONE 2:e1172