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Comparison among available marker systems for cereal introgression breeding: A practical perspective

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Summary

There is an increasing amount of public sequence information for the main cultivated cereals, such as wheat and barley. It is not foreseeable that comparable efforts or resources could be devoted to related wild species. However, wild species are interesting sources of genetic variation through introgression breeding. Comparative genomics can be a helpful approach to make use of the available genomic resources. In this context, the potential of the wild barley species *Hordeum chilense* has been explored in recent years. It exhibits great levels of polymorphism and high crossability with different cereal genera. In addition, interesting biotic and abiotic stress resistance genes, and important quality traits like carotene content and seed storage protein variability shown in the species are also expressed in wheat backgrounds, and are the basis of a breeding program. Different approaches have been undertaken for tagging *H. chilense* genomic regions in a wheat background. The search for the most suitable DNA marker system started with the development of RAPD and SCAR markers due to a lack of sequence information from the wild species. Transferability of markers from wheat and barley (like STSs or SSRs) have also been useful approximations. More recently, SNP development is being accomplished for the species. In this work, the situation and prospects with the available molecular tools are considered from a practical point of view.

Introduction

The wild South American barley species *Hordeum chilense* Roem. et Schult. has an indirect but interesting potential in agriculture through introgression breeding (Martin et al., 1998). It belongs to a heterogeneous group of South American *Hordeum* species (Sec. Anisolepsis Nevski). It is very polymorphic and has been hybridized with species from the genera *Aegilops*, *Agropyron*, *Dasypyrum*, *Hordeum*, *Secale*, and *Triticum* (Fedak, 1992). It contains interesting genes for biotic and abiotic stress resistance, as well as important quality traits such as carotene content and seed storage proteins, many of which are expressed in a wheat background (Martin et al., 1998). *Tritordeum*, the barley-wheat amphiploid, is the basic genetic material for using *H. chilense* genetic variability in wheat breeding (Martin et al., 1996). The use of this wild species to increase wheat genetic resources will be greatly facilitated by marker-assisted introgression. To do this, molecular markers that enable tracking of *H. chilense* chromatin in a wheat background are needed.

For the breeding aims outlined above, a series of tools, from molecular to genomic markers, have become available. In the present paper, their use, possibilities, and limitations are discussed from a practical point of view.

Materials and methods

Plant material

H. chilense lines H1 and H7, *H. vulgare* cv 'Betzes' and bread wheat cv. 'Chinese Spring' were used for initial RAPD screening and SSR and STS marker

transferability studies. *H chilense* lines H1 and H7 belong to two very distinct ecophysiological and molecular groups (Martin et al., 1998; Vaz Patto et al., 2001). To assign markers to chromosomes, six wheat (cv. 'Chinese Spring')/*H. chilense* accession H1 addition lines (Miller et al., 1982) including a monotelodisomic $1H^{ch}S$ addition, a ditelosomic addition for $2H^{ch}$ alpha arm, and disomic addition lines for chromosomes $4H^{ch}$, $5H^{ch}$, $6H^{ch}$, and $7H^{ch}$ were used.

DNA extraction

DNA was extracted from young frozen leaf tissue using the CTAB method of Murray and Thompson (1980) with modifications. The concentration of each sample was estimated by comparing their band intensities with lambda DNA controls of known concentrations after ethidium bromide staining of 0.8% agarose gels subjected to electrophoresis.

PCR amplification and marker analysis

Methods for RAPD (Random Amplified Polymorphic DNA) analysis and SCAR (Sequence Characterized Amplified Region) development are described by Hernández et al., 1996, 1999. As a modification, the Stoffel Fragment from the *Taq* polymerase (Applied Biosystems) was used for RAPD amplification. SSR (Simple Sequence Repeat) and STS (Sequence Tagged Site) amplification conditions are also detailed elsewhere (Hernández et al., 2002). SSR fragments were resolved in an automated ABI 310 capillary system (Applied Biosystems) using fluorescently labelled primers.

Real-time PCR for SCAR and SNP (Single Nucleotide polymorphism) detection, and melt-curve analysis, were performed on a iCycler IQ Detection System (Biorad). 0.2× SYBR Green I® (Molecular Probes) was added to the PCR reaction.

Results and discussion

Simplifying molecular protocols for breeding applications

The practical application of molecular markers in a breeding program requires simple and economic methods, due to the high numbers of individuals that need to be characterized every generation. The advent of PCR-based molecular markers has made molecular tools accessible for breeder use. Shortening and simplification of methods is always desirable. For example, a RAPD profile run usually takes 5–6 h. Starting from the standard RAPD PCR cycling method (Hernández et al., 1996), the temperature profile was optimized in order to obtain a high level of polymorphism, reducing as much as possible the time of the run. In order to maintain the RAPD profiles, a slow ramp between annealing and polymerization temperature (1 °C per s) was needed, but it was possible to reduce denaturation, annealing, and polymerization times without affecting the degree of polymorphism obtained. Using such thermal profile and thin-walled tubes, the time for each run can be reduced by 2 h. The cycling conditions are: 94 °C/3 min, followed by 40 cycles of amplification (94 °C/20 s, 35 °C/20 s, then increase to 72 °C/1 min with a ramp of 1 °C per s), and by a 7 min final extension. Amplification is performed in a System 9600 Cycler (Applied Biosystems). This cycling profile (Hernández, 1998) has been successfully used later for RAPD amplification in olive (Belaj et al., 2001) and miscanthus (Atienza et al., 2002).

A further development on the search of faster and cost-effective systems for the use of molecular markers in breeding, is a method to transform RAPDs in SCAR markers by direct sequencing of the RAPD products, thus avoiding the costs on labor and consumables derived from cloning (Hernández et al., 1999). Additionally, PCR primer designs are optimized when possible for shorter 2-step-PCR amplification runs in a search for high specificity to avoid electrophoresis (Hernández et al., 2002). This has been the starting point to the development of markers suitable for automation using real-time PCR detection systems.

RAPD markers

The information obtained using a total of 125 RAPD primers and 345 primer pairs is summarized in Table 1.

Table 1. Information generated from RAPDs banding profiles using single primers and pairwise combinations

	1 Primer	2 Primers
Non-informative	41 (32.8%)	177 (51.3%)
Informative (A, B, AB)	86 (68.2%)	168 (48.7%)
A[a]	5 (6%)	15 (9%)
B[b]	48 (56%)	104 (62%)
A, B	33 (38%)	49 (29%)
Total	125	345

[a]A: marker/s located on chromosomes.
[b]B: polymorphism H1–H7.

The percentage of useful primers (69% for single and 49.5% for primer pairs) was quite good. Nevertheless, dependence of banding patterns on wheat and *H. chilense* genetic backgrounds limited their practical application. Therefore, their transformation into SCAR markers was undertaken (Hernández et al., 1999).

SCARs

The transformation of chromosome-specific *Hordeum chilense* RAPD markers that could be detected in a wheat background, into more specific SCAR assays (Hernández et al., 1999) produced the first molecular tools to be applied routinely in a breeding program. The development of real-time PCR technology has allowed the development of very cost-effective assays. They are based on a fine-tuning of the PCR reaction conditions (preferably a 2-step PCR with a 72 °C annealing phase) and the addition of SYBR Green I® to the PCR mixture. This leads to a simple and economic in-tube assay, ready in 2 h 30 min, avoiding agarose gel electrophoresis. Results are obtained by melt-curve analysis (see Figure 1 for an example). Melt-curve analysis measures the melting temperature (T_m) of double-stranded DNA molecules that can be visualized by the incorporation of SYBR Green I® binding dye. As the temperature (T) is raised towards the T_m of duplex, the fluorescence (F) will decrease at a constant rate. At the T_m, however, there is a dramatic decrease in the fluorescence with a noticeable change. The rate of this change is determined by plotting the negative first derivative ($-\mathrm{d}F/\mathrm{d}T$) versus temperature. The temperature at which a peak occurs in this plot corresponds to the T_m of the double-stranded DNA complexes. Figure 1 shows the melt-curve analysis of SCAR IAS-pHc6.4, specific for *Hordeum chilense* chromosome **6H**^{ch}. The presence of a DNA duplex of 86.5 °C T_m (corresponding to the marker) is detected in *Hordeum chilense* and in its chromosome **6H**^{ch}-wheat addition line. Any product is detected in the wheat sample. This analysis relies on a very specific PCR amplification of the target marker, and the absence of primer-dimers or other non-specific products. This can be achieved (like in Figure 1) using the original SCAR primers. In other cases, non-specific products may appear and new primer designs are necessary.

Microsatellite markers

Microsatellite markers have become the marker of choice for cereal molecular breeders (see Korzun et al., 1997; Pestsova et al., 2000, 2001 for examples on cereal introgression applications). Simple sequence repeat (SSR or microsatellite) markers are potentially superior to RAPDs and SCARs because of their codominant nature and suitability for semiautomation. SSRs can be identified using DNA database searches (Akkaya et al., 1992; Devos et al., 1995) but this is not feasible for wild germplasm like *H. chilense*, where relatively few sequences are currently available. High development costs also make it impractical to develop them directly from wild species. However, if SSR markers developed in related crop species can be used, genetic analysis of wild germplasm could benefit from existing resources. This has been the case for *H. chilense*, and SSR markers developed for barley (*H. vulgare*) and wheat (*T. aestivum*) are useful for mapping, introgression, and variability studies in this wild species (Hernández et al., 2002).

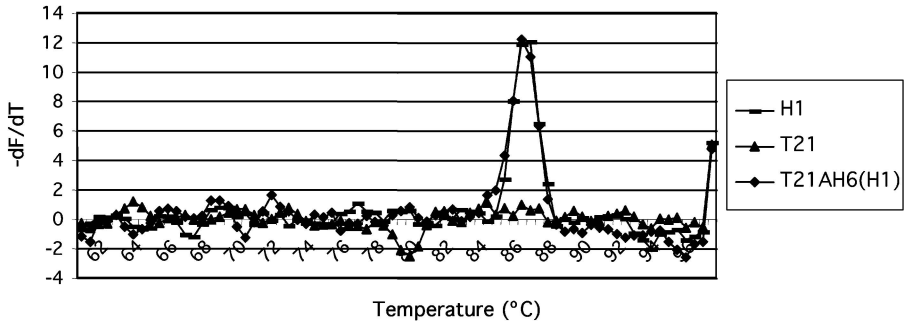

Figure 1. Melt-curve analysis of SCAR marker IAS-pHc6.4, for the detection of introgression of *Hordeum chilense* chromosome **6H**^{ch} in wheat. H1: *H. chilense* line 'H1'; T21: *Triticum aestivum* cv 'Chinese Spring'; T21AH6(H1): *H. chilense* chromosome **6H**^{ch} in *Triticum aestivum* cv 'Chinese Spring' disomic addition line.

Unfortunately, methods for microsatellite analysis rely on electrophoretic separation of the amplified products. Semiautomated systems, based on capillary gel electrophoresis using fluorescently labelled primers are preferred. First of all, because they reduce labor, time, and costs compared to sequencing gel systems. At the same time, they offer ca. 2 bp resolution for allele sizing and effective detection limits. This is particularly important for the study of introgression. Due to the competitive nature of the PCR reaction, alleles of homoeologous loci are amplified at different efficiencies. This is shown in Figure 2. When the *H. chilense* genome (H1) is amplified alone using primer Xgwm165 (Röder et al., 1998) it yields an intense peak with stuttering (Figure 2). But if the *H. chilense* genome is in the wheat background as in tritordeum (HT21) or in the **4H**^{ch} addition line (T21AD4H^{ch}), the *H. chilense* allele has the competition of the wheat alleles of the A, B, and D genomes. Although the intensity of the *H. chilense* peak is significantly decreased, it is still detectable using the capillary system (Figure 2a). It cannot be detected in metaphor agarose (Figure 2b). Thus, for this marker, the technique (i.e. capillary electrophoresis) allows the simultaneous detection of four alleles corresponding to the **4A**, **4B**, **4D**, and **4H**^{ch} chromosomes.

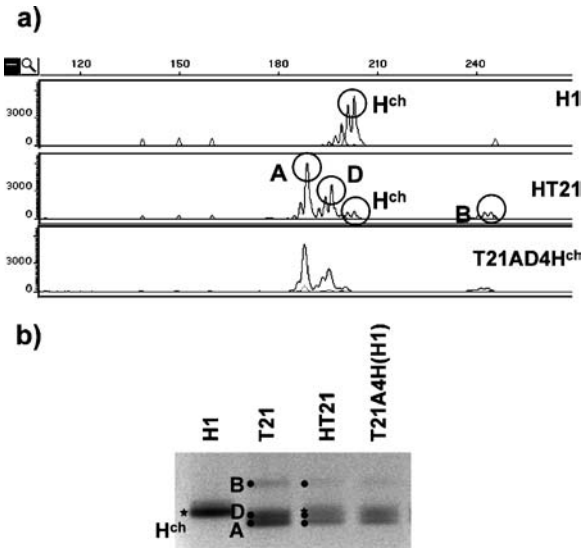

Figure 2. Genotypic analysis for the detection of introgression of *Hordeum chilense* chromosome **4H**^{ch} in wheat, using the microsatellite marker Xgwm165. (a) Fluorograms using an automated capillary system; (b) 3.5% metaphor agarose gel. H1: *H. chilense* line 'H1'; T21: *Triticum aestivum* cv 'Chinese Spring'; HT21: tritordeum amphiploid (H1 × T21); T21AD4H^{ch}: *H. chilense* chromosome **4H**^{ch} in *Triticum aestivum* cv 'Chinese Spring' disomic addition line.

SNP markers

Two approaches are being undertaken for the development of SNP markers: tagging candidate genes by comparative mapping, and conversion of SCAR markers. Whenever possible, direct sequencing of PCR products is preferred to cloning. Cloning requires an additional cost and is a time consuming procedure. Additionally, *Taq* errors corresponding to individual cloned DNA molecules make necessary to sequence several clones in order to avoid false SNPs. By directly sequencing the PCR products, the *Taq* errors are diluted in the PCR-amplified population of DNA molecules.

Particularly interesting because of its simplicity, is the SNP detection using melt-curve analysis. Figure 3 shows an example using the original SCAR primer IAS-pHc7.2. The two alleles corresponding to H1 and H7 lines differ by three SNPs: two G$->$A and one T$->$C transitions. This corresponds to a T_m shift of 3 °C and can be detected by melt-curve analysis. This provides a simple and economic tool for SNP detection, and does not require the design of allele-specific primers.

Comparison among marker types

Although the highest polymorphism per primer is achieved by a RAPD analysis (49.7% of primers tested), this technique is very dependent on the wheat background (i.e. the wheat line used for synthesizing tritordeum or as recurrent parent) where the *H. chilense* markers are amplified. Therefore, to be useful for introgression breeding, these markers should be transformed into SCARs, or, better, SNP markers. Microsatellite transfer from wheat and barley, though fewer in number (30%) yields very useful codominant markers. It should be noted that transferability is higher (ca. 50%, work in progress) for wheat D-genome SSRs and EST-SSRs. SSRs have the additional advantage of detecting intergeneric homoeologous loci in the same reaction (Figure 2). Finally, SNP markers are the most polymorphic and allow for a binary (±) detection. Preliminary results are within the range of 1 SNP every 115 bps for SCAR markers, and 1 SNP every 54–134 bp for candidate genes (depending on the locus). Table 2 summarizes the available techniques for tracking alien introgression from *Hordeum chilense* into wheat.

Table 2. Comparison of the available techniques for tracking alien introgression from *Hordeum chilense* into wheat

	Development cost	Polymorphism detected	Practical convenience	Loci per assay	Relative cost per marker (%)[a]	Labor (relative)	Total time (h)	Resolution	SA[d]	A
RAPD	Low	Good	–	1–5	24.6–4.9	High	7	Low	No	No
SCAR/AG	Medium	Good	Good	1–3[b]	24.6–8.2	High	5	Low	No	No
SCAR/EB	Medium	± (Binary)	–	1	21.4	Low	3	Low	No	No
SCAR/RT-PCR	Medium	Good	Very good	1	24	Very low	2.5	Very good	Yes	Yes
SSR/HRAG	Low (TA)	Good	Good	1	32.8	High	6	Good	No	No
SSR/CE	Low (TA)	Very good	Very good	1–4[c]	100–25	Intermediate	3	Very good	Yes	No
SNP/RT-PCR	Medium	± (binary)	Very good	1	24	Very low	2.5	Very good	Yes	Yes

[a]100% cost assigned to the most expensive technique, costs calculated in Spain, March 2005.
[b]Multiplexed PCR.
[c]Either multiplexed or simultaneous detection of orthologous loci by the same marker.
[d]SA: semiautomation; A: automation; AG: agarose gel; EB: direct ethidium bromide detection in the PCR tube; HRAG: high resolution agarose gel; CE: capillary electrophoresis using fluorescently labelled primers; TA (transferability approach).

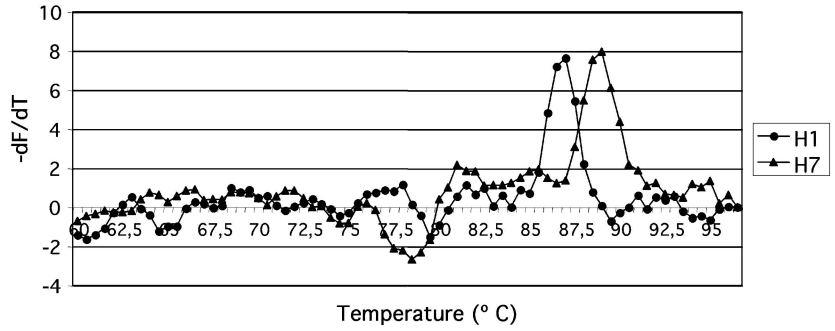

Figure 3. Melt-curve analysis of SCAR marker IAS-pHc7.2, for the detection of introgression of *Hordeum chilense* chromosome **7H**^{ch} and SNPs. H1: *H. chilense* line 'H1'; H7: *H. chilense* line 'H7'.

Microsatellite markers are at the moment the marker of choice for cereal molecular breeders, due to their availability and codominant nature. The semi-automated capillary sequencing systems, combine a resolution power of *ca.* 2 bp with semi-automation, thus avoiding the set up of sequencing gels and manual sample loading. This is the preferred system for microsatellite analysis. Nevertheless, requirements for a high throughput genotyping system amenable for MAS include an increased level of automation both for set up and scoring. Markers based on allele-specific PCR and real-time detection systems or allele-specific oligonucleotides also amenable for microarrays meet these automation requirements and deserve the attention of breeders.

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