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Review

DNA-based methods for identification and quantification of small grain cereal mixtures and fingerprinting of varieties

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Abstract

The composition of cereal-based foods is a key factor in determining the quality and safety of the final product while the reliable identification of cereal species and cultivars are essential for the handling, marketing and processing of grain and for the protection of plant breeders' rights. Analytical methods have therefore been developed and applied to identify and quantify cereal species in food products and also to fingerprint and identify grain at the genotype and variety levels. DNA-based methods for the detection and quantification of mixtures of small grain cereals are reviewed, together with the recent development of molecular markers for varietal fingerprinting.
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1. Introduction

Five major cereal species (bread and durum wheats, maize, rice and barley) satisfy two thirds of the human food requirements, either directly or indirectly, with total annual yields exceeding 2000 million tonnes (FAO, 1999). Cereal crops are therefore essential for human welfare. The end use quality of cereals for food and feed is determined by their composition. In general, cereal grains contain high levels of carbohydrates (65–75%), mainly represented by starch, low lipid contents (2–9%) and protein contents, ranging from about 7–15%. Nevertheless, they currently provide over 200 million tonnes of protein for human and livestock nutrition. Furthermore, the functional properties of the grain proteins are key determinants of the final utilisation of the grain in food processing. In addition, other classes of grain component, such as arabinoxylans (pentosans), $(1\rightarrow3,1\rightarrow4)$-β-D-glucans, antioxidants and vitamins, have potential importance for the production of functional foods and have been shown to vary in amount and composition between species, genotypes and, to a lesser extent, with growth environment. Consequently, the species composition of a cereal-based food is a key factor in determining the quality and safety of the final product and the reliable identification of species and cultivars is essential for the handling, marketing and processing of grain (Ko et al., 1994).

Two strategies have been developed in response to the need to maintain and strengthen consumers' trust in the quality and origin of foods introduced into the market. One is to control all the production steps, with the application of Hazard Analysis Critical Control Point (HACCP) systems from field to table. The second is to develop analytical tools to monitor the composition of raw materials and processed food and feed. With this aim, DNA-based analytical methods have been developed and applied to identify and quantify cereal species and to fingerprint and identify genotypes and varieties to determine authenticity (Popping, 2002).

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2. DNA-based detection and quantification of mixtures of small grain cereals

DNA profiling is widely applied to identify small grain cereals in raw materials and in processed food and feed. In fact, despite a high level of gene conservation between grasses (Devos and Gale, 2000), cultivated cereal species show different end-use characteristics which are mainly related to their storage protein fractions. For example, the high content of globulin storage proteins in oat grain contributes to its high nutritional value and explains the widespread use of oats for livestock feed (Cuddeford, 1995). Similarly, the malting quality of barley is related to differences in grain composition and structure that affect the modification of the grain and digestion of starch during malting and brewing, including storage protein composition (Shewry and Darlington, 2002). In wheats, the prolamin storage proteins are the major components of the gluten protein fraction that forms a viscoelastic network in doughs and is largely responsible for the ability to process wheat to form bread, pasta and many other food products (Shewry and Halford, 2002). As a consequence, pasta made from durum wheat is considered superior in several qualitative aspects to that manufactured from bread wheat or from a mixture of the two species. Several regulations, including Italian law, currently prohibit the manufacture for sale of pasta containing more than 3% bread wheat, and require the correct labelling of imported pasta products to show the proportions of each wheat species in the final product. The incentive for the adulteration of pasta with bread wheat comes from the lower price at which bread wheat is usually traded compared with durum wheat.

Several analytical procedures are currently available to detect the presence of wheat in food and to test pasta for authenticity, as summarized by Autran et al. (1994). Most of these methods are based on the detection of specific proteins, separated by electrophoresis or high-performance liquid chromatography or identified immunochemically (Durotest, Rhône Diagnostic Technologies, France). The main limitation to the use of these analytical methods is the occurrence of protein denaturation during the technological processes involved in manufacturing food and pasta. In particular, for pasta, the use of faster drying processes characterised by high temperatures can lead to extensive protein denaturation. In contrast, the thermostability of DNA molecules, together with the use of PCR techniques, can provide molecular markers to identify raw materials and trace them through the manufacturing steps. Analytical tools have been developed to detect the presence of wheat species in flour and pasta based on end-point and real-time PCR. A common feature of this PCR approach is the use of sequences related to the D genome of bread wheat. Bread and durum wheats are in fact polyploid species containing three (AABBDD) and two (AABB) related genomes, respectively. Bryan et al. (1998) exploited this difference to design a set of PCR primers based on the 2.2 kb Dgas44 sequence (McNeil et al., 1994) and determined their specificity for a range of samples including wheat species, bread and durum wheat cultivars and chromosome substitution lines. Four primers (Table 1) showed very strong PCR amplification and a very high degree of genome specificity when used in any of the possible forward–reverse combinations. An alternative end point PCR method for detection of bread wheat recently been developed by Arlorio et al. (2003) using the sequence of the the puroindoline b gene (which is present on chromosome 5D) as a target for primer design (Table 1). Universal ribosomal primers based on the Internal Transcribed Spacers region (ITS) were also used to monitor the quality of genomic DNA extracted from food. The limit of sensitivity of the detection method was given as 0.2% bread wheat contamination. Alary et al. (2002) used the same puroindoline b sequence to develop a real-time PCR system (Table 1) for the detection and quantification of bread wheat adulteration of durum wheat pasta. They analysed a range of pasta types (dried at low, high and very high temperatures) containing 3% bread wheat and determined a mean value of $3\% \pm 0.4$ at a 95% confidence limit. The work of Terzi et al. (2003) was directed toward the development of analytical systems for the qualitative and quantitative detection of specific cereals in food. More specifically, the primary aim of the work was to develop analytical tools based on end-point and real-time PCR to detect the presence of *Triticum* species in flour and food. Furthermore, qualitative and quantitative PCR-based methods were evaluated to detect hexaploid wheat adulteration in pasta. Seed storage protein sequences were used to design primer pairs and probes and the genus and species-specificity of the two systems were tested on a panel of grass genotypes, on hexaploid and tetraploid wheats and on standard spaghetti made with 100, 98, 96, 93 and 85% of durum wheat. GLUD primers/probe (based on consensus sequences of low molecular weight glutenin storage proteins) detected *Triticum* and *Triticosecale* (triticale) species, whereas GLIA primers/probe (based on gliadin sequences) detected only *Triticum aestivum* (Table 1).

This type of analysis can be used not only to determine food authenticity but also to determine food safety for those who need to avoid the consumption of wheat and related cereal products. In particular, several cereal species (wheat, barley, rye and triticale) contain proteins that are toxic to individuals affected by coeliac disease (Kasarda, 2000; Shan et al., 2002). Consequently, analytical methods are required to check that products are free from gluten and related proteins from other species. Various immunochemical methods are available for prolamin identification and their specificity and sensitivity have been evaluated, including the effects of heat treatments of products (Denery-Papini et al., 1999; Ellis et al., 1998; Sorell et al., 1998). These methods are widely used and have the advantages that they often target the actual proteins that are toxic to individuals affected by coeliac disease

Table 1
Species-specific primer pairs and probes

Species detected	Forward primer (5′–3′)	Reverse primer (5′–3′)	Probe (5′–3′)	Reference
Bread wheat	GTCCTATATCTTGAGGCC GCAAG	AACCCACTGTACCTGAG TATATATC		Bryan et al. (1998)
Bread wheat	CTTCTGACGGGTCAGG GGCAC	CTGAATGCCCCTGCGG CTTAAG		Bryan et al. (1998)
Bread wheat	GGGAAATGGGTAGCAAA GCAACC	GCGAGCCAACAGAAA TGGTGC		Arlorio et al. (2003)
Bread wheat	TCGGCTGGTACATCAT CCTCCC	GCGAGCCAACAGAAA TGGTGC		Arlorio et al. (2003)
Bread wheat	AGCACTTCTCCCGA ACCTCA	CAGTACCTGGCCCACAAA	CTCACAGCCGCCCTT CCACCA	Alary et al. (2002)
Wheats	CGTTGGCACCGGAGTTG	TCACCGCTGCATCGACAT	ATTGGGTCACCGTTGT TTAGTCGATGGA	Terzi et al. (2003)
Bread wheat	TGGTCTCATCCCTCT GGTCAA	GCTGCTGAGGAATC TGTGCTA	TGAGGCAACAATGC TGCCAACAA	Terzi et al. (2003)
Wheats	GCGGCGTGTGCCACG TACGTGGTTT	GAACGGGCTGTACGTG GACACGGGA		Allmann et al. (1993) and Köppel et al., 1998
Wheats, barley, rye	GGTAACTTCCAAATTCA GAGAAAC	TCTCTAATTTAGAAT TAGAAGGAA		Dahinden et al. (2001)
Wheats	CAGAAAGCGAGTGGA AAGATGAAAG	GCAAGGAGGACAAAGA TGAGGAA		Sandberg et al. (2003)
Rye	TTTTTCAGAAAGCGAGT TCAATGATG	CGAGGACAAAGATGAGG AAGGTCT		Sandberg et al. (2003)
Barley	ATTAATTCCCAAACTGAA CGACTA	CATGGCGAACAATG TGAAC		Sandberg et al. (2003)
Oats	CGCTCAGTGGCTTC TAAGA	TTTTATTTTATTTGTCAC CGCTAC		Sandberg et al. (2003)
Oats	TGGGAAGTCCTCGTG TTGCA	TTTAGTGCTGGTATGA TCGC		Köppel et al. (1998)
Rye, triticale	TAGTCACCTTCCAT CATCCA	CATCATCACCATATCC GTAG		Terzi et al. (2004)
Rye, triticale	TTACATGATAATATCCTT GCAAACATAGG	TTTAGTACCCTTTCAG TGTGCTTTATTT	TTTTTCTTCAGCAAAG CAAACCATCCATACA	Terzi et al. (2004)
Bread wheat	GCAGCAAGAACAACAA GAACAA	CGGCGACTACGCTGGA		von Büren et al. (2001)
Bread wheat, spelt	GCAACCACAACAACAA TTTTCT	GATATAGTGGCAGCA GGATATG		von Büren et al. (2001)

(Sandberg et al., 2003). The Codex Alimentarius Standard for gluten-free food states that immunological methods should be used and an immunoassay based on a monoclonal antibody directed against omega-gliadins has been validated by inter-laboratory tests (AOAC 1995). However, DNA-based methods have also been proposed as confirmatory tests for the analysis of gluten-free foods, based on the observation that the products contain both wheat DNA and the toxic proteins (Dahinden et al., 2001; Sandberg et al., 2003). The GLUD primers/probe discussed above (Terzi et al., 2003) can therefore be applied to specifically detect wheat and triticale in flour and food, because of the high sensitivity of real-time approaches. A 109-bp segment of the major repeat unit of the intergenic region between the 25S and 18S ribosomal RNA genes of wheat (Table 1) was selected by Allmann et al. (1993) to develop a wheat-specific PCR marker because of the low level of homology with other cereals. Primers specific for wheat, barley and rye (Table 1) were also designed based on the intron of the chloroplast gene for leucine tRNA (*trnL*) and used in a quantitative competitive PCR system to detect contamination of these three cereals in gluten-free food for coeliac patients (Dahinden et al., 2001). In addition, an internal DNA standard was constructed by adding a 20-bp sequence to the original PCR product. This standard was calibrated to 0.02 and 0.2% wheat DNA, corresponding to 10 and 100 ppm gliadin, respectively. Sandberg et al. (2003) developed a real-time PCR method for the specific discrimination of wheat, rye, barley and oats in food samples, using melting curve analysis for product identification. Primers based on cereal prolamin genes were chosen for the amplification (Table 1). Five or fewer genome equivalents could be detected, corresponding to less than 50 pg genomic DNA. The sensitivity of this approach is therefore comparable to that of immunochemical methods, with the advantage that PCR also discriminates between different cereal species. However, a reference gene is required to normalise the results obtained with the different species and enable the method to be used in quantitative assay. Oats are considered to be tolerated by most coeliac patients, so a quantitative assay to detect wheat in oat products has been developed by Köppel et al. (1998), using primer pairs based on 25S and 18S

ribosomal genes (Table 1). The quantitative detection of rye and triticale by real-time PCR amplification has been reported by Terzi et al. (2004) using the Sybr Green and TaqMan probe approaches with a chloroplast *trnL* reference gene. The primer pair and probe are based on rye prolamin (secalin) and EST sequences (expressed sequence tags, corresponding to the sequences expressed mRNAs) sequences (Table 1) and were tested for their species specificity on a panel of grass genotypes and foods. The sensitivity of the approach was evaluated using dilutions

Table 2
The main characteristics of the different molecular approaches for cereal species detection are reported

Species detected	Method	Notes	Sensitivity	Reference
Bread wheat	End-point PCR for the detection of 286 bp fragment from multi-copy Dgas44 sequence	Tested for specificity on a panel of 74 *Triticum durum*, 64 *Triticum aestivum* cultivars, on 3 amphiploid lines, 3 secondary hexaploids, 3 primary octaploids and 3 hexaploid wheat ancestors	Below 1% (500 pg bread wheat in 50,000 pg DNA template extracted from seeds and pasta)	Bryan et al. (1998)
		Qualitative method for bread wheat presence detection	1% (100 pg bread wheat in 100,000 pg DNA template extracted from five-day-old bread)	Tilley (2004)
Bread wheat	End-point PCR for the detection of 874 bp and 551 bp fragments from single copy puroindoline b sequence	Tested for specificity on a panel of 6 *Triticum durum* and 6 *Triticum aestivum* cultivars and on corn, rice, barley, oat and soybean	0.2% (1,000 pg bread wheat in 500,000 pg DNA template extracted from seeds, bakery products and dried pasta)	Arlorio et al. (2003)
	Multiplexed with end point PCR for the detection of 310 bp fragment from high repeated ribosomal genes	Multiplex qualitative method for bread wheat presence detection with an inner positive control to avoid false-negative responses		
Bread wheat	Real-time quantitative PCR for the detection of 63 bp portion of single-copy puroindoline b gene	Multiplex quantitative method for determining bread wheat content with a reference gene for total DNA determination	3% (220 pg of bread wheat DNA in 7,360 pg of total DNA extracted from pasta). The real time duplex PCR efficiency is of 104% for both genes	Alary et al. (2002)
	Multiplexed with real time quantitative PCR for the detection of 61 bp portion of a low-copy number lipid transfer protein gene			
Wheat/bread wheat	Real-time quantitative PCR for the detection of 101 bp portion of glutenin gene	Tested for specificity on a panel of 39 *Triticum durum,* 29 *Triticum aestivum* and 15 *Triticosecale* cultivars and on *Triticum dicoccum, dicoccoides, spelta*, corn, sorghum, rice, barley, oat, rye	1% (1,000 pg of bread wheat DNA in 100,000 pg of total DNA extracted from pasta or seeds)	Terzi et al. (2003)
	Real-time quantitative PCR for the detection of 101 bp portion of gliadin gene	Quantitative method for determining wheats and bread wheat content		
Wheats	End-point PCR for the detection of 109 bp segment in the major repeat unit of the intergenic region between the 25S and 18S ribosomal RNA genes	Tested for specificity on 5 wheat cultivars and on corn, rice, barley, oat, rye, millet and bakery products in comparison with ELISA assay	0.03% of wheat flour in food	Allmann et al. (1993)
		Qualitative method for wheat presence detection	0.001% of wheat flour in milled oat flakes	Köppel et al. (1998)
Wheats, barley, rye	Quantitative competitive PCR for the detection of 196–201 fragments of a non-coding region of chloroplast *trnL* gene	Quantitative method for determining wheat, barley and rye content in food	0.02% of wheat flour in food (40 pg of wheat DNA in 200,000 pg total DNA)	Dahinden et al. (2001)
Wheats	Real-time PCR for the detection of portions of cereal prolamin genes based on melting curve analysis	Qualitative method for wheat, barley, oat and rye detection	0.1–0.01% (between 5 and 50 pg cereal DNA in 50,000 pg total DNA)	Sandberg et al. (2003)
Rye, triticale	Real-time quantitative PCR for the detection of 101 bp portion of a rye EST	Tested for specificity on a panel of 14 *Secale cereale* and 15 *Triticosecale* cultivars and on bread and durum wheats, sorghum, rice, barley and oat	150 pg of rye DNA, with efficiency of about 90%	Terzi et al. (2004)
	Quantitative method for determining rye and triticale content in flour and food			
Bread wheat, spelt	Quantitative competitive PCR for the detection of fragments of GAG56D gliadin gene	Quantitative method for determining bread wheat and spelt content in flour	0.01% (20 pg of wheat DNA in a background of 200,000 pg spelt DNA)	von Büren et al. (2001)

of rye DNA and mixtures of rice and rye flours, in comparison with a commercially available enzyme immunoassay kit.

Wheats cultivated in former times, like spelt and emmer, have recently gained renewed interest as niche-market products, in relation to the demand for natural or organically grown foods. The lower yields of these crops in comparison with durum and bread wheats and the requirement for additional technological processing (e.g. dehulling for spelt) result in higher market prices for these products. To monitor purity of spelt products, a PCR-RFLP system consisting of a GAG56D-specific PCR system with subsequent restriction was used to estimate the relative proportions of wheat and spelt in DNA mixtures (Von Büren et al., 2001).

Finally, Henterich et al. (2003) developed an assay for gliadin quantification by real-time immunopolymerase reaction (iPCR), as a bridge technology between immunodetection and qPCR detection. In this technique, immunological detection of gliadin by a monoclonal antibody conjugated with an oligonucleotide is amplified by PCR. For quantification, iPCR was performed as real time PCR in one step, without changing the reaction vessels.

Table 2 summarises the main characteristics of the different molecular approaches for cereal species detection above mentioned.

3. DNA-based variety identification of small grain cereals

Classical and marker assisted selection (MAS) based breeding are being used to develop new value-enhanced varieties in response to the increased emphasis on quality and to tailor products for specific markets. In fact, many qualitative characteristics of raw materials and food can be related to genetically controlled traits in specific genotypes. For example, it has been demonstrated that allelic variation in the composition of the HMW subunits of wheat glutenin is strongly correlated with differences in breadmaking quality (reviewed by Shewry and Halford, 2002). Similarly, in barley the genotype significantly influences the amount and composition of nutritionally important antioxidants, such as tocols (Cavallero et al., 2004). In a competitive market, these new value-added genotypes need to be tracked in response to the demands of farmers, importers, exporters and consumers and to protect plant breeders' rights. High-throughput fingerprinting methods are therefore required, characterized by low cost and high levels of automation in handling and processing samples. Both genomic and proteomic approaches have therefore been developed for varietal description based on distinctness, uniformity and stability.

Protein profiling determines the composition, abundance, modification and subcellular localization of proteins in a cell or tissue and can be applied to cultivar characterization. Variety ID based on seed storage proteins patterns is now facilitated by the use of Matrix Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS), which provides accurate molecular weight determination and is amenable to automation. The procedure includes seed grinding, protein extraction, generation of protein profiles, conversion of the results into a database-compatible format and interrogation of the database.

Chmelik et al. (2002) identified several proteins extracted from barley grains using a combination of gel electrophoresis, MALDI-TOF MS and bioinformatics. However, the application of proteomics to varietal fingerprinting is limited by the level of variation in storage protein composition.

The development of high-throughput sequencing and bioinformatics has shifted the focus in plant genetic analysis from single genes to whole genomes, with the complete genome sequencing providing the ultimate level of information that can be exploited to design new markers. As a consequence, a variety of different techniques have been developed in recent years for the analysis of genetic variation at the genome-wide level, with the use of markers dispersed throughout the genome improving the molecular description of genotypes (Gupta and Roy, 2002; Manifesto et al., 2001; Terzi et al., 2001). Amplified Fragment Length Polymorphisms (AFLPs) are an example of PCR-based multilocus markers with high efficiency in detecting polymorphisms (Almanza-Pinzón et al., 2003; Vos et al., 1995) and have been widely used for assessing variability in germplasm (Donini et al., 2000). Moreover, AFLP-based DNA profiling can be performed on processed food such as malt as well as on plants and seeds. Faccioli et al. (1999) evaluated AFLP markers for their effectiveness and reproducibility for fingerprinting malt and concluded that it was possible to determine the variety of an unknown malt by referencing to a collection of the AFLP patterns of the most common feed and malting varieties.

However, the working group on Biochemical and Molecular Techniques (BMT) of the International Union for the Protection of New Varieties of Plants (UPOV) recently concluded that microsatellites (also called Simple Sequence Repeats or SSRs) are the most widely used marker system for varietal characterization (UPOV, 2002). SSRs are currently the markers of choice because of their ability to follow multi-allelic loci and co-dominant alleles and therefore provide wide genomic coverage. In addition, they are amenable to automation and a wide range of primers is available through collaborative efforts of the Wheat Microsatellite Consortium.

The availability of centrally maintained databases of DNA microsatellite profiles of varieties, generated according to an agreed protocol, therefore provides a useful tool for distinctiveness, uniformity and stability (DUS) testing of small grain cereals (Cooke et al., 2003). A database of 502 recent European wheat varieties was constructed using 19 wheat microsatellites and one secalin-specific marker by Röder et al. (2002). The 199

alleles that were detected allowed discrimination between all of the varieties with an accuracy level >99.5%, except for duplicates and varieties derived from identical parents. An average of 10.5 alleles per marker and an average PIC of 0.674 was detected in this work, which was similar to the study of Prasad et al. (2000) which showed an average of 7.4 alleles per locus and a PIC (Polymorphism Information Content) of 0.71 in 55 elite wheat genotypes. Holland et al. (2001) showed that 21% of SSR markers and 50% of intron markers were polymorphic among 22 hexaploid oat varieties and two diploid species, while Li et al. (2003) developed and mapped 127 new microsatellites in barley. In addition, new classes of SSRs have been recently developed based on EST data from small grain cereals. For example, Holton et al. (2002) searched the International *Triticeae* Cooperative EST database for the presence of microsatellites, finding 388 dinucleotide repeats and 978 trinucleotide repeats among 24,344 ESTs. These EST-derived-microsatellites were then tested for polymorphism in wheat and barley and showed a high level of transferability between the two species. An assessment of the impact and performance of EST-derived microsatellite markers vis-à-vis their genomic counterpart was made by Leigh et al. (2003) on wheat. Their results showed that genic SSRs based on EST sequence are less efficient in discriminating bread wheat varieties compared to genomic SSRs. However, the main advantages of EST-based markers are that they are inexpensive and rapidly identified, give high quality profiles and reveal variation in transcribed genes, thus allowing functional fingerprinting. For this purpose, Gao et al. (2004) developed 478 functional EST-SSRs from 71,000 wheat ESTs, providing a starting point for the construction of a candidate gene map of the genomes of bread wheat using genic markers. Furthermore, the transferability of genic SSRs between bread and durum wheats has been demonstrated by Eujayl et al. (2002), supporting the hypothesis that “most microsatellites reside in regions pre-dating the recent genome expansion in many plants” (Morgante et al., 2002). Eujayl et al. (2002) identified nine SSRs as providing the best combination for genotyping 64 durum wheat accessions, whereas Perry (2004) developed a multiplexed set of seven genic and genomic SSRs to uniquely identify all 18 durum wheat varieties registered in Canada for commercial production. SSRs developed from wheat and rye have also been shown to be suitable for estimating of diversity in the composite genome of triticale (Tams et al., 2004). A comprehensive overview of public data for EST-SSRs is available at http://wheat.pw.usda.gov/pgpages/ITMI/EST-SSR/, which collects Uni-EST-SSRs from wheat, barley, rice, maize and sorghum, while http://wheat.pw.usda.gov/ggpages/genomics.shtml provides links to marker development and cooperative projects, EST projects, genomics and comparative mapping in *Triticeae*.

Table 3
Web addresses of the most important integrated databases for small grain cereal genomics

Database name	web address
Gramene	http://www.gramene.org
GrainGenes	http://graingenes.org
Barley DB	http://ukcrop.net/perl/ace/search/BarleyDB
BarleyBase	http://barleypop.vrac.iastate.edu/BarleyBase/
Barley genomics	http://barleygenomics.wsu.edu/
Plant GDB	http://www.plantgdb.org/
CereGeneDB	http://kosh-348-001.berkeley.edu/BarleyTag/unigene_result.pl
Komugi	http://www.shigen.nig.ac.jp/wheat/komugi/top/top.jsp

The web addresses of the most important integrated databases for small grain cereal genomics are given in Table 3.

DNA sequence information is also the starting point for the identification of Single Nucleotide Polymorphisms (SNPs). The frequent occurence of such markers provides a large source of variation that can also be used for genotyping. SNPs are often identified from ESTs: Somers et al. (2003) proposed this approach for hexaploid wheat and estimated that it would be possible to develop a diagnostic SNP test from every contig (continuous nucleotide sequence) with 10–60 EST members. The Wheat SNP Development project aims to help the wheat breeding and research community by co-ordinating efforts to develop SNPs and make them freely available. Pyrosequencing is a promising, high-throughput, sequence-by-synthesis technique for SNP detection in which a series of enzymatic reactions yields detectable light, which is proportional to the incorporated nucleotides. As example, in the work of Pacey-Miller and Henry (2003) a universal biotinylated primer technique has been developed and applied in plants as more cost-effective and less labour intensive alternative to more traditional methods of single-stranded template preparation. Several different approaches have been proposed for SNP validation, such as re-sequencing the locus, MALDI-TOF MS analysis, real time PCR assay with TaqMan probe, PCR-based approaches with two forward SNP primers (one for each allele of the sequence) that have different lengths or different level of mismatch. Advantages and disadvantages of the different techniques in SNPs detecting are reviewed by Jander et al. (2002).

High-throughput genotyping methods such as SNP detection will probably change conventional breeding perspectives and procedures, leading to the possibility to conduct larger breeding programs (Koebner and Summers, 2003; Tost and Gut, 2002), as well as facilitating varietal fingerprinting.

A further emerging technology for varietal fingerprinting is microarray analysis. This has been successfully applied to detect genetic polymorphisms and Jaccoud et al. (2001)

proposed a new array-platform technology as a low-cost, high-throughtput, robust system that covers the entire genome even in the absence of detailed information on the DNA sequence.

Finally, the problem of the paucity of information about the uniformity of varieties, on a plant by plant basis, with respect to molecular markers is very well addressed by Cooke et al. (2003). They analysed 45 wheat varieties at 7–9 different SSR loci and found variation both between varieties and between microsatellites in their degree of polymorphism and identified possible sources of non-uniformity. Akkaya and Buyukunal-Bal (2003) also reported variation within bread wheat varieties grown in Turkey, which could possibly have originated from the selection of non-uniform seeds when developing the cultivar or from mixing during propagation.

The ability to identify varieties in a milled sample is crucial for analysis of purity, and for marketing and quality control and Cooke et al. (2003) conclude that the future of molecular markers for DUS testing depends on exactly how markers are used. In fact, if they are used in the same way as morphological traits, analysis of individual seeds is required. However, a change in testing philosophy to use distance estimation opens up the possibility of using bulked samples of seeds or plants to produce an overall profile of the variety. Bioinformatics can therefore help in data analysis. A format for databasing and comparison of AFLP fingerprint profiles has been developed by Hong and Chuah (2003) and tested for its effectiveness to evaluate the novelty of a new cultivar by comparing its AFLP fingerprint profile with those of related genotypes in the database. However, it is a more difficult task to evaluate the varietal composition of mixtures. This has been demonstrated for tomato by a consortium of five laboratories who constructed a microsatellite database containing more than 500 tomato varieties (Bredemeijer et al., 2002). 'Blind testing' showed that identification was easy for homogeneous varieties, but was less successful with heterogeneous varieties.

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