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Construction and analysis of a microsatellite-based database of European wheat varieties

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Abstract A database of 502 recent European wheat varieties, mainly of winter type, was constructed using 19 wheat microsatellites and one secalin-specific marker. All datapoints were generated in at least two laboratories using different techniques for fragment analysis. An overall level of >99.5% accuracy was achieved. The 199 alleles detected allowed discrimination between all of the varieties except duplicates, and varieties derived from identical parents. Approximately 25% of the varieties showed some heterogeneities, with the highest level of heterogeneity in south-eastern European material. The highest genetic diversity and the highest number of rare alleles were found in varieties from southern Europe. The relative allele frequencies varied for most microsatellites in different geographical regions.

Keywords Variety identification · SSRs · *Triticum aestivum* · Genetic diversity · Fragment analysis

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Introduction

Agriculturally important crop species mostly exist as a number of genetically distinct, but related, varieties. To-date, variety identification is usually carried out using morphological and physiological markers. These are also the type of descriptor used for registration of varieties and for awarding Plant Breeders' Rights (PBR) following distinctness, uniformity and stability (DUS) testing. New varieties have to be shown to be distinct from all existing varieties 'in common knowledge' by the expression of at least one characteristic. In addition, they have to meet certain standards with respect to uniformity and stability in the characteristics used to demonstrate distinctness. The morphological characters applied for registration purposes can also be used for identification, but this often requires the varieties to be grown to full maturity. Furthermore, many of the descriptors are multigenic, quantitative or continuous characters, the expression of which can be altered by environmental factors.

As the number of registered varieties increases over time, it becomes increasingly difficult to compare efficiently each newly submitted variety against all existing varieties. One potential approach to this problem would be to have available centrally maintained databases of DNA profiles of varieties, against which candidate varieties for testing could be checked. To-date, molecular markers are not used by the registration authorities in order to assess distinctness, uniformity and stability. A potential marker system for DUS testing or for variety identification purposes needs to be highly reproducible and to possess a high discrimination power. Marker systems which have been employed for assessing genetic diversity in wheat include RFLPs (Siedler et al. 1994; Kim and Ward 1997, 2000; Paull et al. 1998; Ward et al. 1998), RAPDs (Devos and Gale 1992; Joshi and Nguyen 1993), sequence tagged site (STS) markers (Chen et al. 1994; Burkhamer et al. 1998), AFLPs (Barrett and Kidwell 1998; Barrett et al. 1998; Law et al. 1998) and microsatellites also called simple sequence repeats (SSRs) (Plaschke et al. 1995; Röder et al. 1995; Prasad

et al. 2000; Stachel et al. 2000; Bertin et al. 2001). For high-throughput, PCR-based marker systems such as AFLPs and microsatellites (Donini et al. 1998) appear most promising and the utility of these marker systems in wheat has been directly compared (Bohn et al. 1999; Donini et al. 2000). Microsatellite markers for wheat have become available in increasing numbers (Röder et al. 1995, 1998; Bryan et al. 1997; Prasad et al. 2000) and are a potentially useful system for the construction of large molecular-marker databases for wheat varieties. SSRs generally detect high levels of polymorphism (Röder et al. 1995), can detect heterogeneity and heterozygotes, and are amenable to automated analysis, detection and recording.

The research reported here was carried out in the framework of an EU Demonstration Project. The overall objectives were to establish a microsatellite marker database for 500 recent European wheat varieties, to test the reproducibility of SSR genotyping using different detection methods in different laboratories and to determine the discrimination power of a limited number of SSR markers.

Materials and methods

Plant material

Seeds of the wheat varieties in the database were obtained from different sources and breeding companies, including Advanta Seeds UK Limited, Cebeco Zaden B.V., CIMMYT, CRA Belgium, Delley Samen und Pflanzen AG, ETS C.C. Benoist, Fa. Strube Saatzucht KG, INRA France, Institute of Field and Vegetable Crops Yugoslavia, Lochow Petkus, Ministerio di Agricultura Spain, Nickerson, PBI/Monsanto, PRI The Netherlands, Saatzucht Hans Schweiger and Co., Saatzucht Josef Breun, Svalöf Weibull, Streng's Erben GmbH, u. Co. KG, Variety Research Institute Greece and others. In total, 554 varieties were included in the database, of which 52 were duplicate accessions. Table 1 lists the number of varieties from different countries after omission of duplicates. In cases where a variety was released in several countries only the original country is listed.

Table 1 Geographical distribution of investigated varieties

Country	No. of accessions[a]	Geographical region
Austria	61	Alps
Belgium	9	Western Europe
Bulgaria	26	South-Eastern Europe
Finland	2	Northern Europe
France	95	Western Europe
Germany	83	Central Europe
Greece	29	Southern Europe
Italy	34	Southern Europe
Netherlands	12	Western Europe
Portugal	8	Southern Europe
Spain	22	Southern Europe
Switzerland	10	Alps
Turkey	20	Southern Europe
UK	41	Northern Europe
Yugoslavia	50	South-Eastern Europe

[a] No. of accessions after omission of duplicates

DNA isolation and fragment analysis

For each variety, six seeds were pooled and DNA was isolated as described in Plaschke et al. (1995). The microsatellites used for analysis are listed in Table 2. The primers amplifying the respective microsatellite loci (*Xgwm*) are from Röder et al. (1998) or not yet published. The primers for the gliadin-specific marker *Taglgap* are from Devos et al. (1995). The presence of the 1B-1R wheat-rye translocation was verified with the secalin-specific primers SecalF1 = GTAGTAGTGGTATAGGCATCGG and SecalR1 = CGTTACATTGAACACTCCATTG, which result in a PCR-product of 99 bp for the presence of the 1B-1R translocation. These primers were selected after initially testing approximately 40 wheat microsatellites on a set of 22 varieties (Vosman et al. 2001). Only primers that amplified in all laboratories with readily scorable products without significant stuttering, and for which all laboratories could agree on the allelic composition of the 22 varieties, were selected.

All data points of the database were generated in duplicate in at least two independent laboratories using different methods for fragment analysis. All datapoints were generated at the Institute for Plant Genetics and Crop Plant Research (IPK) in Gatersleben, using ALF and ALFexpress sequencers as described in Röder et al. (1998). Duplicate runs were performed at Plant Research International (PRI) in Wageningen, The Netherlands, on ALFexpress sequencers, at NIAB in Cambridge, UK, on LICOR DNA sequencers and in the Agrogene company in Moissy Cramayel, France, using conventional sequencing equipment in combination with a Molecular Dynamics 860 scanner in phosphorimager mode. In general, a PCR protocol according to Röder et al. (1998) with 45 cycles of 1 min 94 °C denaturation, 1 min 50 °C (or 55 °C or 60 °C according to primer) annealing and 1 min at 72 °C extension followed by a final extension step of 5 min at 72 °C was used in all laboratories. Where there were disagreements between two laboratories, experiments were repeated in both laboratories and DNA was exchanged between the respective partners.

Data analysis was performed using Microsoft Excel-Software, and the polymorphism information content (PIC) was calculated as described in Röder et al. (1995).

Results

Accuracy and discrimination power of the wheat data base

In total, 502 recent wheat varieties of predominantly winter type, plus 52 duplicates as controls, were fingerprinted with 19 wheat microsatellite markers and the secalin-specific marker indicating the 1B-1R wheat-rye translocation. All datapoints were generated in at least two independent laboratories using different fragment-analysis techniques. After re-checking discrepancies between different laboratories, 34 discrepancies out of 11,080 datapoints, remained unresolved, indicating an accuracy of >99.5% for the current database. The remaining discrepancies may either be due to experimental error or be a result of heterogeneity in different sample mixtures. The occurrence of null alleles was observed in 11 out of 19 markers. However, since non-amplification may also be due to experimental error, all null alleles were confirmed in a second amplification.

Although wheat is a self-pollinating plant, evidence of internal heterogeneity, defined as the identification of more than one allele for a given marker in a single variety, was found. Considering all variety/marker com-

Table 2 Description of microsatellite markers employed including the number of alleles, rare alleles and PIC-values

Locus	Repeat type	Product sizes (bp)	Chromosome	No. of alleles (rare alleles)	PIC
Taglgap	$(CAA)_{15}$	null, 209–281	1B	17 (10)	0.634
Xgwm3	$(CA)_{18}$	75–83	3D	5 (2)	0.547
Xgwm18	$(CA)_{17}GA(TA)_4$	null, 178–194	1B	10 (3)	0.750
Xgwm46	$(GA)_2GC(GA)_{33}$	null, 145–183	7B	16 (7)	0.760
Xgwm95	$(CA)_{16}$	115–134	2A	9 (2)	0.633
Xgwm155	$(CT)_{19}$	132–153	3A	9 (4)	0.680
Xgwm190	$(CT)_{22}$	null, 198–214	5D	9 (3)	0.687
Xgwm261	$(CT)_{21}$	160–209	2D	7 (3)	0.706
Xgwm325	$(CT)_{16}$	null, 133–149	6D	9 (2)	0.703
Xgwm357	$(GA)_{18}$	119–125	1A	4 (1)	0.616
Xgwm389	$(CT)_{14}(GT)_{16}$	null, 116–150	3B	15 (6)	0.829
Xgwm408	$(CA)_{>22}(TA)(CA)_7(TA)_9$	149–199	5B	11 (5)	0.705
Xgwm437	$(CT)_{24}$	null, 91–130	7D	15 (4)	0.682
Xgwm458	$(CA)_{13}$	null, 109–115	1D	5 (2)	0.624
Xgwm513	$(CA)_{12}$	140–150	4B	6 (0)	0.612
Xgwm577	$(CA)_{14}(TA)_6$	null, 126–214	7B	22 (10)	0.886
Xgwm619	$(CT)_{19}$	null, 135–173	2B	12 (2)	0.833
Xgwm631	$(GT)_{23}$	null, 187–212	7A	8 (4)	0.536
Xgwm680	$(GT)_9(GA)_{24}$imp	110–141	6B	10 (8)	0.390

Table 3 Analysis of geographical regions

Item	Northern Europe	Central Europe	Western Europe	Alps	Southern Europe	South-Eastern Europe	Total
No. of accesions	$n = 43$	$n = 83$	$n = 116$	$n = 71$	$n = 113$	$n = 76$	$n = 502$
Total no. of alleles	77	120	137	119	166	131	199
Average no of alleles per marker	4.1	6.3	7.2	6.3	8.7	6.9	10.5
No. of rare alleles ($P < 0.02$ in total set)	5	17	24	17	51	28	78
Mean of PIC-values	0.461	0.608	0.592	0.614	0.693	0.531	0.674
Heterozygosity	1.3%	1.7%	2.4%	3.8%	5.2%	10.9%	4.3%

binations, a level of 4.3% of heterogeneity was observed. Some wheat varieties were heterogeneous with respect to several markers, perhaps indicating the presence of mixtures in the seed samples. For other varieties heterogeneity was found for a single marker only. In total, more than 25% of all of the varieties analysed were non-uniform with at least one of the markers. The highest level of heterogeneity was found in south-eastern European material with a level of 10.9% regarding all variety/marker combinations (see Table 3).

The database included 41 varieties which were present as more than one accession. Some of the duplicate varieties are registered in more than one country or were obtained from different breeders or sources. In 26 out of 41 duplicate or triplicate varieties (63%) no differences between the individual samples were detected. Nine varieties (22%) showed internal heterogeneities, but agreed otherwise perfectly. In five varieties (12%), differences in one marker in duplicate samples were found which were confirmed by both laboratories. These are most-likely fixed heterogeneities between the seed samples. The varieties with such fixed heterogeneities were Estica, Genesis, Soisson, Eureka and Victo. In case of the variety Rubin, seed samples were obtained from Austria and Bulgaria. The marker analysis gave completely different patterns for the two samples, indicating that there are most-likely two different varieties of the name Rubin.

The discrimination power of the database was investigated by determining the genetic similarity of all varieties. Most varieties can be clearly discriminated from each other based on the information obtained from the 20 markers. However, because of the occurrence of internal heterogeneity between duplicate samples of the same variety the discrimination power of the database has to take into account a level of uncertainty. In practice, this means that all varieties which have a genetic similarity of 95% or more cannot be discriminated reliably from each other. This threshold ensures that most of the heterogeneities observed within duplicate samples are accepted as identical varieties. In total, 15 pairs of varieties and one quadruplet of varieties could not be discriminated, based on the similarity threshold of 0.95. Wherever we have been able to verify, the pairs of varieties which are indistinguishable from one another are derived from the same breeding company and in most

Table 4 Predominating alleles (in base pairs) and their relative frequencies

Locus	Northern Europe	Central Europe	Western Europe	Alps	Southern Europe	South-Eastern Europe	Total
Taglgap	null (0.49)	235 bp (0.67)	235 bp (0.78)	235 bp (0.75)	235 bp (0.37)	215 bp (0.37)	235 bp (0.57)
Xgwm3	75 bp (0.75)	77 bp (0.70)	77 bp (0.51)	77 bp (0.71)	77 bp (0.53)	77 bp (0.89)	77 bp (0.60)
Xgwm18	190 bp (0.53)	188 bp (0.30)	188 bp (0.63)	188 bp (0.37)	190 bp (0.41)	186 bp (0.58)	188 bp (0.36)
Xgwm46	171 bp (0.67)	171 bp (0.57)	171 bp (0.62)	171 bp (0.54)	147 bp (0.31)	175 bp (0.49)	171 bp (0.42)
Xgwm95	120 bp (0.63)	120 bp (0.60)	120 bp (0.57)	120 bp (0.67)	120 bp (0.49)	122 bp (0.50)	120 bp (0.56)
Xgwm155	143 bp (0.86)	143 bp (0.60)	143 bp (0.64)	143 bp (0.47)	145 bp (0.42)	141 bp (0.39)	143 bp (0.47)
Xgwm190	208 bp (0.47)	208 bp (0.46)	208 bp (0.47)	208 bp (0.34)	208 bp (0.39)	208 bp (0.91)	208 bp (0.50)
Xgwm261	171 bp (0.74)	161 bp (0.51)	171 bp (0.66)	193 bp (0.37)	189 bp (0.47)	189 bp (0.84)	171 bp (0.37)
Xgwm325	142 bp (0.58)	142 bp (0.39)	140 bp (0.59)	140 bp (0.41)	140 bp (0.32)	140 bp (0.50)	140 bp (0.43)
Xgwm357	123 bp (0.56)	121 bp (0.73)	121 bp (0.61)	121 bp (0.73)	123 bp (0.51)	119 bp (0.54)	121 bp (0.50)
Xgwm389	140 bp (0.35)	140 bp (0.39)	140 bp (0.28)	140 bp (0.39)	117 bp (0.34)	null (0.36)	140 bp (0.29)
Xgwm408	147 bp (0.79)	178 bp (0.40)	147 bp (0.55)	178 bp (0.45)	180 bp (0.41)	178 bp (0.57)	147 bp (0.39)
Xgwm437	91 bp (0.88)	91 bp (0.57)	91 bp (0.77)	91 bp (0.43)	91 bp (0.24)	91 bp (0.49)	91 bp (0.54)
Xgwm458	111 bp (0.51)	109 bp (0.51)	113 bp (0.40)	109 bp (0.64)	111 bp (0.69)	109 bp (0.59)	111 bp (0.43)
Xgwm513	145 bp (0.93)	145 bp (0.73)	145 bp (0.67)	145 bp (0.60)	149 bp (0.37)	145 bp (0.50)	145 bp (0.56)
Xgwm577	null (0.37)	135 bp (0.30)	160 bp (0.16)	160 bp (0.31)	160 bp (0.29)	128 bp (0.38), 160 bp (0.38)	160 bp (0.22)
Xgwm619	147 bp (0.74)	135 bp (0.33)	147 bp (0.31)	139 bp (0.41)	156 bp (0.18)	141 bp (0.38)	147 bp (0.24)
Xgwm631	200 bp (0.81)	192 bp (0.55)	200 bp (0.52)	192 bp (0.49), 200 bp (0.49)	200 bp (0.49)	192 bp (0.62)	200 bp (0.50)
Xgwm680	125 bp (0.70)	125 bp (0.71)	125 bp (0.59)	125 bp (0.74)	125 bp (0.86)	125 bp (0.83)	125 bp (0.74)

cases have identical parents in their pedigrees. It is very likely that varieties with the same pedigree differ only in a limited number of genomic regions and that such differences cannot be identified with only 20 markers.

Analysis of the database

For further analysis of the database, duplicate accessions were eliminated and the remaining 502 varieties were grouped into geographical regions comprising northern Europe, western Europe, central Europe, southern Europe, south-eastern Europe and the Alpine region (Table 1). Using the 19 wheat microsatellite markers a total of 199 alleles was found, resulting in an average allele number per marker of 10.5. The detected number of alleles for the individual markers ranged from 22 (*Xgwm577*) to 4 (*Xgwm357*) (Table 2). The highest number of alleles was found in southern European material with a total of 166 alleles (Table 3). Seventy eight of the 199 alleles detected overall occurred with a frequency of <2% in the whole dataset and thus can be considered as rare alleles. Most rare alleles, 51 out of 166 alleles in total, were observed in the wheat varieties from southern Europe, followed by those from south-eastern Europe, with 28 rare alleles out of 131 alleles in total (Table 3). The number of rare alleles also varied considerably between the different markers ranging from 10 for *Taglgap* and *Xgwm577* to 0 for *Xgwm513* (Table 2). The polymorphism information content (PIC) of the individual markers, in which the occurrence of rare alleles is taken into account, ranged from 0.886 for *Xgwm577* to 0.390 for *Xgwm680*, with an average of 0.674 for all markers. Again the highest PIC value for all markers of 0.693 was found in the southern European material and the second highest avarage PIC of 0.614 in the varieties of the Alpine region (Table 3).

The relative frequencies of alleles varied considerably in the different geographical regions (Table 4). For the marker *Taglgap*, which amplifies a microsatellite in the coding region of a gamma-gliadin pseudogene (Devos et al. 1995), a major allele of 235 bp was found with high frequency in all geographical regions, perhaps suggesting a selective advantage for the chromosomal region carrying this allele (Fig. 1). The null allele observed with *Taglgap* corresponded to the occurrence of the 1B-1R translocation and was found with the highest frequency in the northern European material. The presence of the rye chromosome was verified by amplification of the secalin-specific marker. A *Taglgap*-allele of 215 bp was detected with highest frequency in the south-eastern European material, and rare alleles of 244 bp and 265 bp were mainly observed in southern European varieties.

For the marker *Xgwm* 261 four main alleles were observed with a different predominance in the various geographical regions (Fig. 2, Table 4). *Xgwm261* is tightly linked to the dwarfing gene *Rht8* on chromosome 2DS, a chromosomal region also carrying the gene *Ppd1* for photoperiodic insensitivity (Korzun et al. 1998). It has been shown earlier that different alleles of WMS261 are diagnostic for different phenotypes of *Rht8* and also indicate to some extent the presence of the day length insensitive phenotype conferred by *Ppd1* (Worland et al. 1998). A 189-bp allele of *Xgwm261*, which corresponds to the *Rht8*-allele conferring a shortened plant height and which is often associated with day length insensitivity (Korzun et al. 1998), predominated in the southern and south-eastern European lines. A 171-bp allele of *Xgwm261* is associated with neutral plant height and day length sensitivity and was mainly found in the northern

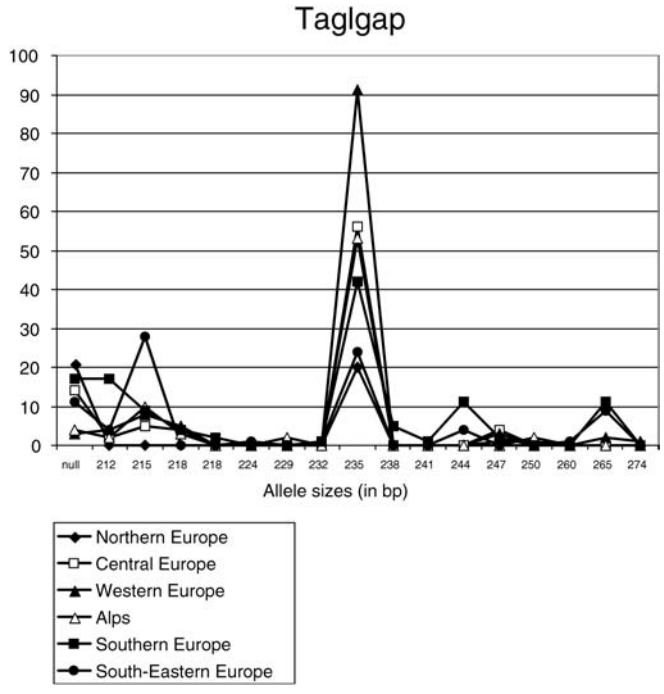

Fig. 1 Diagram representing the distribution of allele sizes for the marker Taglgap in accessions of different geographical origins. A predominance of the 235 bp allele is observed in all groups

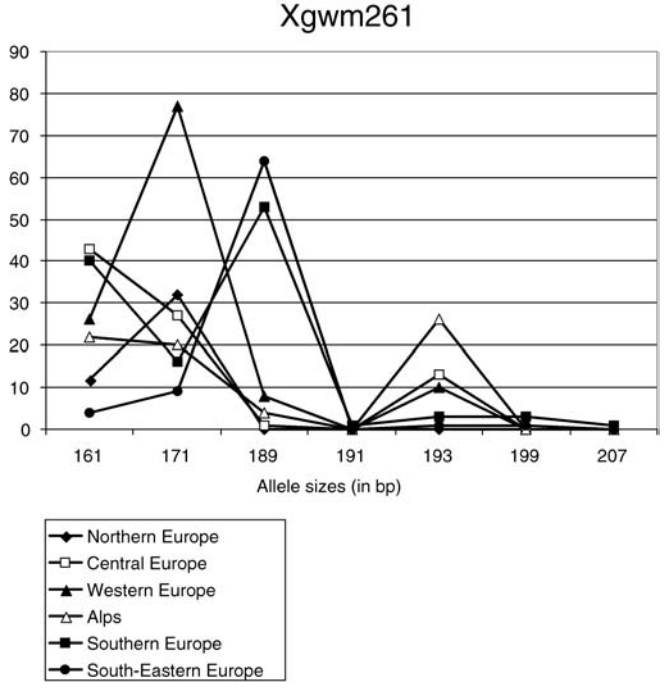

Fig. 2 Diagram representing the distribution of allele sizes for the marker *Xgwm261*. For different geographical groups different alleles predominate

and western European material. The central European varieties most frequently carried a 161-bp allele, which is associated with an increased plant height. The Alpine region is characterized by a 193-bp allele of unknown phenotype (Fig. 2).

Strong differences for the frequencies and predominance of specific alleles were also observed for other markers, such as *Xgwm18* on chromosome 1B, *Xgwm46* on 7B and *Xgwm619* on 2B. In many cases the south-eastern and southern European varieties were characterized by different predominating alleles compared to the other geographical regions (Table 4). In these cases however, the linkage of specific alleles to certain traits or phenotypes remains to be established.

Discussion

A SSR marker data base characterizing 502 recent European wheat varieties has been established. The accuracy of the database was ensured by creating all datapoints in at least two different laboratories. During the construction of the database, several important lessons were learned which helped to ensure accuracy and reproducibility of the results. For various fragment-analysis procedures including analysis on automated sequencers (ALF or ALF express), it was found that the absolute allele sizes varied by a few base pairs in different runs. For a correct allele-recognition it is therefore absolutely necessary to include a number of reference varieties in each run which amplify representative alleles and can be used as direct comparisons for naming alleles. The definition and use of reference alleles are also necessary if information from further varieties is to be added later to the database.

One of the main reasons for the occurrence of discrepancies between data from different laboratories or data for duplicate varieties seemed to be the occurrence of heterogeneity in wheat varieties. In more than 25% of all wheat varieties investigated at least one case of heterogeneity was observed. For all marker/variety combinations the level of heterogeneity was 4.3%. In most cases, it was possible to find heterogeneous loci reproducibly by investigating pooled seed samples; however, in a few cases heterogeneous alleles were differently represented in duplicate seed samples. This may cause a problem for the unequivocal identification of varieties by molecular markers, if a perfect match criterion is applied stringently. Therefore, the threshold for considering two lines as identical was set to 95%, which led to correct identification results in nearly all cases.

In this study, 19 microsatellite markers from most of the wheat chromosomes have been used. This number of markers was sufficient to distinguish between the majority of the 502 varieties. Only varieties derived from the same parents could not be distinguished. A similar observation was described in Plaschke et al. (1995) where two sister lines could not be distinguished using 23 wheat microsatellites. The differentiation of lines origi-

nating from identical parents may pose a general difficulty for the molecular identification of varieties or accessions. In such cases, only limited regions of the genome differ in their genomic composition, so that a higher number of markers from different chromosomal regions is required for detecting differences.

An average of 10.5 alleles per marker and an average PIC of 0.674 was detected in all varieties, reflecting the high level of polymorphism detectable by microsatellite markers in wheat. The highest genetic diversity was found in southern European wheat varieties which also carried most rare alleles. Overall it could be demonstrated that microsatellites are a feasible marker system for the construction of molecular databases on a large scale. In comparison to AFLPs microsatellites have some advantages. Firstly, they can detect heterogeneity and heterozygosity, which as we have shown plays an important role in registered varieties. Secondly, microsatellites are multiallelic which results in a higher information content in relation to the number of analysed fragments. Thirdly, the construction of marker data bases using mapped markers allows the assessment of the allele flow in different chromosomal regions across several generations, as has recently been demonstrated in barley (Russell et al. 2000). It is also possible to use this type of analysis for the search for highly selected chromosomal regions and the establishment of marker-trait correlations. Large differences in the frequencies of certain alleles for different European regions were observed in the present work (Table 4) and could, in the case of marker *Xgwm261*, be linked to the presence of a dwarfing gene and photoperiodic insensitivity. Other applications of molecular databases include the prediction of progeny variance from parental divergence (Burkhamer et al. 1998) and the assessment of heterotic groups based on molecular marker data (Melchinger et al. 1990; Smith et al. 1990; Messmer et al. 1992; Barbosa-Neto et al. 1996; Xiao et al. 1996).

In conclusion, we have shown that it is possible to construct large databases of DNA profiles of varieties in agricultural species and moreover that it is possible to populate these with data arising from different laboratories, if certain precautions are taken with regard to marker selection and general methodology. This may be important in the context of plant variety registration, but also for the more-general documentation and analysis of genetic resources.

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