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M. Stachel · T. Lelley · H. Grausgruber · J. Vollmann

Application of microsatellites in wheat (*Triticum aestivum* L.) for studying genetic differentiation caused by selection for adaptation and use

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Abstract For studying genetic differentiation caused by selection for adaptation and end-use, the allele frequencies of 42 microsatellites (MS), representative of the three wheat genomes, were analysed in a total of 60 wheat cultivars. The cultivars originate from three agroecological areas (AEAs) – Germany, Austria and Hungary – and represent equal numbers of 'quality wheats' and 'feed wheats' for each country. For the 42 loci, 202 alleles were detected using PAGE and silver staining. The average number of alleles per locus was 4.8, including four monomorphic loci. For 16 loci, null alleles were detected. Cluster analysis clearly differentiated the varieties according to the three AEAs and, within each AEA, into quality wheats from feed wheats. Analysis of variance revealed highly significant differences of distance data between AEAs as well as between quality groups. The correlation between genetic distance (GD) and pedigree data (coefficient of diversity, COD) was r_s=0.45. The results have proven the excellent resolving power of MS in varietal differentiation, which arises through breeding under specific environmental conditions, and for different end-use.

Key words DNA markers · Genetic diversity · Agroecological area · Quality · Adaptation

Introduction

Genetic-distance data reflect the level of genetic diversity among cultivars. Genetic diversity is the ultimate basis for genetic improvement. Genetic diversity can be quantified indirectly, by the estimation of genetic distance using pedigree information, or directly, using molecular markers which compare DNA-sequence variation among genotypes. In wheat, DNA markers represent an exciting new tool for studying genetic relationships between species, populations and cultivars (Siedler et al. 1994; Plaschke et al. 1995; Röder et al. 1995; Kim and Ward 1997; Barbosa-Neto et al. 1998; Barrett and Kidwell 1998; Barrett et al. 1998; Burkhamer et al. 1998; Donini et al. 1998). For this purpose different marker types have been used with varying results. Recently, amplified fragment length polymorphisms (AFLPs, Vos et al. 1995; Barrett and Kidwell 1998) and microsatellites (MS, Röder et al. 1998), also referred to as sequence tagged microsatellite sites (STMS) or simple sequence repeats (SSRs), have found increasing application because of their known advantages over other systems.

At present, microsatellites are one of the most promising molecular-marker types able to identify or differentiate genotypes within a species. Their co-dominant inheritance, high level of polymorphism and easy handling make them extremely useful for many different applications (Devos et al. 1995; Plaschke et al. 1995; Röder et al. 1995; Korzun et al. 1997). Apart from the developmental work to create the primers, MS represent a very economic and time-saving technique.

The purpose of the present work was to study the genetic differentiation caused by selection for adaptation and end-use. Allele frequencies of a sample of MS, representative of the three wheat genomes, were analysed in a total of 60 wheat cultivars which originate from the three agroecological areas (AEAs), Germany, Austria and Hungary, and which represent the categories 'quality wheat' and 'feed wheat' of each country.

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M. Stachel · T. Lelley (✉)
Institute for Agrobiotechnology,
Department of Plant Biotechnology, Konrad Lorenz Strasse 20,
A-3430 Tulln, Austria
e-mail: lelley@ifa-tulln.ac.at

H. Grausgruber · J. Vollmann
University of Agricultural Sciences Vienna,
Department of Plant Breeding, Gregor Mendel Strasse 33,
A-1180 Vienna, Austria

Materials and Methods

Microsatellite origin

For each chromosome arm one wheat microsatellite (WMS) marker was selected to guarantee an even coverage of the total wheat genome. The primer pairs were made available by IPK Gatersleben, Germany (for designation and chromosomal location see Table 1). All these MS are di-nucleotide repeats. Sequence data and the fragment size of 38 out of the 42 MS primer pairs in the wheat variety 'Chinese Spring' are listed in Röder et al. (1998). Information on WMS375–4B is published in Korzun et al. (1997). Sequence information of the remaining three microsatellite primers (WMS241–7B, WMS603–1D, WMS638–3A) is available upon request from Dr. M. Röder (roder@ipk-gatersleben.de).

Plant material

The origin and quality category of the 60 wheat cultivars are given in Fig. 1. The choice of cultivars was based partly on their classification in the respective national list of cultivars, and partly on HMW glutenin subunit composition. Cultivars with subunits 7+9 and 5+10 were basically quality wheats; those with 6+8 and 2+12 were feed wheats.

Molecular analysis

DNA was isolated from seeds according to Benito et al. (1993) with an additional proteinase K and RNase A treatment. DNA was precipitated with ethanol and re-disolved in TE buffer.

The PCR reaction and amplification were performed according to Röder et al. (1995). After separation of the fragments in a 12% non-denaturing polyacrylamide gel (PAGE), bands were visualised by silver- staining. Visual allele identification followed a conservative approach, i.e. only clearly different bands were accepted as to be different. In case of doubt, e.g. null alleles, experiments were repeated.

Statistical analysis

Average linkage cluster analysis (UPGMA) was used to group cultivars according to their genetic relationship with the SPSS software package. Genetic distances (GDs) were estimated according to Nei and Li (1979) with RAPDistance Programs, vers. 1.03 (Armstrong et al. 1994). Polymorphism information content (PIC) values, assuming homozygosity, were calculated according to the formula

$$\mathrm{PIC} = 1 - \sum_{i=1}^{n} f_i^2,$$

where f_i is the frequency of the ith allele. This value provides an estimate of the discriminatory power of an MS locus by taking into account not only the number of alleles per locus but also their relative frequencies in the studied population (Anderson et al. 1993). For visualising diversity, the MS distance matrix was subjected to a multi-dimensional scaling analysis using NTSYS-pc software, vers. 1.8 (Rohlf 1993).

Pedigree information of the cultivars was mainly obtained from GRIP I (Mackay et al. 1996). In case of doubt the pedigrees were verified with the respective breeders. The coefficient of parentage (COP) was computed for all pairwise combinations of cultivars with complete pedigree records to summarise genealogical similarities between them. Pedigree records of cultivars were considered complete when they could be followed back at least to known great-grandparents. According to this definition 43 of the 60 wheat cultivars had complete pedigree records. Pedigrees were followed back four or five generations and COP values were calculated using the KIN program, vers. 1.0.5, devised by Tinker and Mather (1993). Complete homozygosity was assumed as the inbreeding level of the ancestors. A coefficient of diversity (COD) was calculated according to the definition of Souza et al. (1994) as 1 – COP. The Spearman rank correlation coefficient between COD- and GD-values was calculated using the statistical package SAS, vers. 6.12 (SAS Institute Inc. 1988).

The effects of selection for different agroecological areas and for different quality categories within an AEA on the MS-derived genetic distance between cultivars were evaluated statistically using a general linear fixed-effects model (SAS Institute Inc. 1988). In this analysis, genetic distances were separated into six fixed classes of genotype pairs, i.e. pairs within Hungary, within Austria and within Germany, and pairs between Hungary and Austria, between Hungary and Germany, and between Austria and Germany. The two seed-quality categories were nested within AEAs.

Results

For the set of 42 MS loci on the 60 wheat cultivars, a total of 202 alleles were detected, resulting in an average number of 4.8 alleles per locus. The highest number of

Table 1 Designation of the wheat microsatellites (WMS), PIC-values calculated for the 42 MS loci and mean PIC-values for the A, B and D genomes as well as for the long (L) and short (S) chromosome arms

Location	A		B		D		Total means
	WMS	PIC	WMS	PIC	WMS	PIC	
1L	135	0.42	259	0.64	232	0.50	
1 S	136	0.78	18	0.62	603	0.38	
2L	294	0.80	120	0.73	157	0.00	
2 S	95	0.70	148	0.75	261	0.69	
3L	638	0.56	340	0.67	383	0.69	
3 S	369	0.69	389	0.70	161	0.64	
4L	160	0.56	375	0.59	194	0.50	
4 S	4	0.00	107	0.00	608	0.00	
5L	595	0.57	408	0.80	272	0.67	
5 S	304	0.72	234	0.56	192	0.03	
6L	169	0.49	219	0.70	325	0.67	
6 S	334	0.76	518	0.73	469	0.55	
7L	282	0.87	241	0.68	437	0.71	
7 S	60	0.60	43	0.51	44	0.65	
Mean		0.61		0.62		0.48	0.57
Mean/long arm		0.61		0.69		0.53	0.61
Mean/short arm		0.61		0.55		0.42	0.53

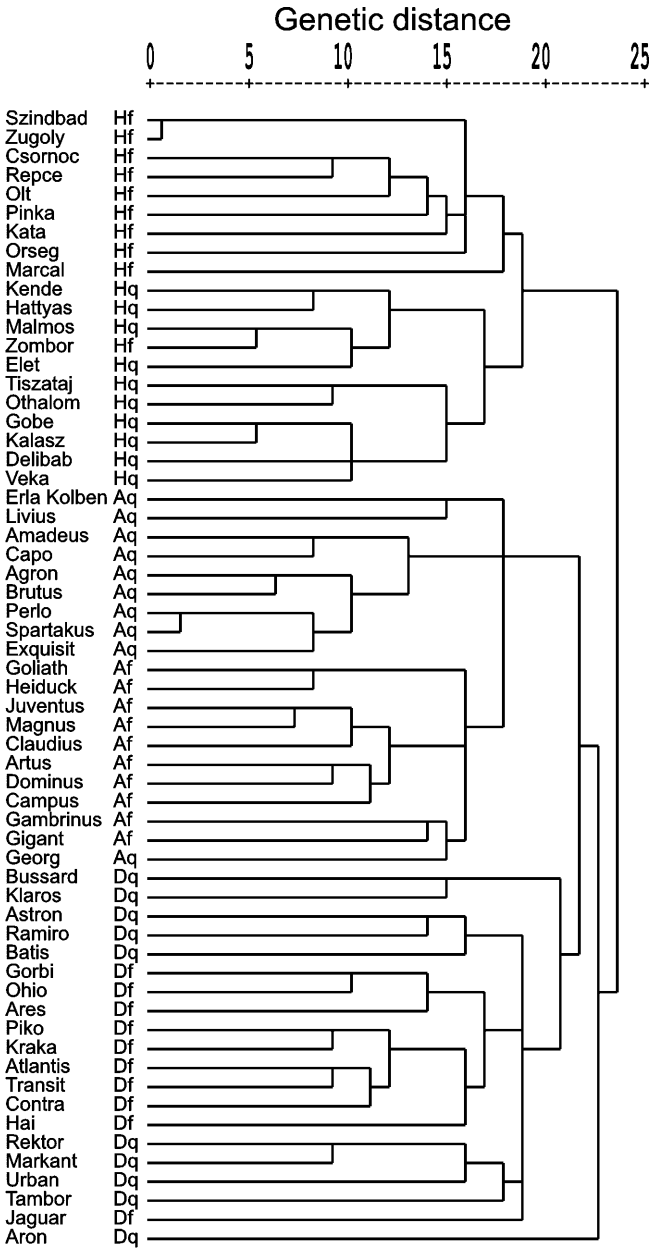

Fig. 1 Dendrogram resulting from a cluster analysis of 60 wheat cultivars originating from Hungary (*H*), Austria (*A*) and Germany (*D*). Each country is represented by ten quality (*q*) and ten feed (*f*) wheats. Data obtained with 42 microsatellite primer pairs

alleles occurred in the A genome (36%), closely followed by the B genome (35%). In the D genome, the total number of alleles was substantially lower (29%). Using the present set of primers the most polymorphic MS-locus with 11 alleles was found on the long arm of chromosome 7 A. Four loci remained monomorphic. A null allele occurred at 16 loci with a frequency per locus from 1 to 42. Two primer pairs produced only a single band. Usually more than one band occurred, with the smallest fragment as the major band of expected size. These "shadow bands" were of clearly different molecular weights, deviating from the main band in a range of

Table 2 Allele frequencies of genomes, quality categories, agroecological areas and quality categories per agroecological area

Genome		Quality category		AEA		Quality category per AEA	
						Q	F
A	5.2	Q	4.3	Hungary	3.30	2.5	2.6
B	5.1	F	3.9	Austria	2.97	2.4	2.5
D	4.0			Germany	3.45	3.0	2.5

Table 3 Genetic distance within and between quality categories (Q, quality wheats; F, feed wheats) and agroecological areas (A, Austria; D, Germany; H, Hungary)

Item	AQ	AF	DQ	DF	HQ	HF
AQ	*0.42*					
AF	**0.49**	*0.41*				
DQ	0.62	0.62	*0.52*			
DF	0.57	0.56	**0.54**	*0.44*		
HQ	0.64	0.65	0.65	0.66	*0.42*	
HF	0.60	0.62	0.69	0.63	**0.52**	*0.44*

Table 4 Average genetic distance between and within AEAs

approximately 50 to 400 bp. They disappeared in cases of null alleles.

PIC-values were ranging ranged from 0.00 to 0.87 for each of the MS loci (Table 1) with an average of 0.57. Mean PIC-values for the three genomes A, B and D were 0.61, 0.62 and 0.48, respectively. PIC-values for the long and short chromosome arms averaged 0.61 and 0.53, respectively. These values changed when chromosomes with a monomorphic MS were omitted from the calculation. Then, PIC-values for the A, B and D genome were 0.66, 0.68 and 0.55, respectively, and 0.66 and 0.61, respectively, for the long and short arms.

Figure 1 gives information about the relationships between cultivars based on the allele distribution of the 42 MS loci. The dendrogram demonstrates that cultivars bred in Hungary, Austria or Germany are differentiated into three distinct groups, and that these differences in GD data for Austria (0.46±0.08), Germany (0.51±0.08) and Hungary (0.48±0.09) are highly significant ($P<0.001$, see Table 4). Moreover, the allele distribution of MS not only separated the cultivars according to the three agroecological areas but also according to the quality category. With three exceptions (Zombor in Hungary, Georg in Austria and Jaguar in Germany), quality and feed wheats represent distinct groups within the respective AEAs (Fig. 1). Allele frequencies per MS locus were the highest in Germany followed by Hungary and Austria (Table 2). Mean genetic distances of the two quality categories in the three AEAs are shown in Table 3. A three-dimensional representation of the relative genetic distances between AEAs and quality classes is presented in Fig. 2.

Genetic distances were re-calculated using data obtained from only one genome, i.e. A, B or D. With respect to geographical distribution these analyses with on-

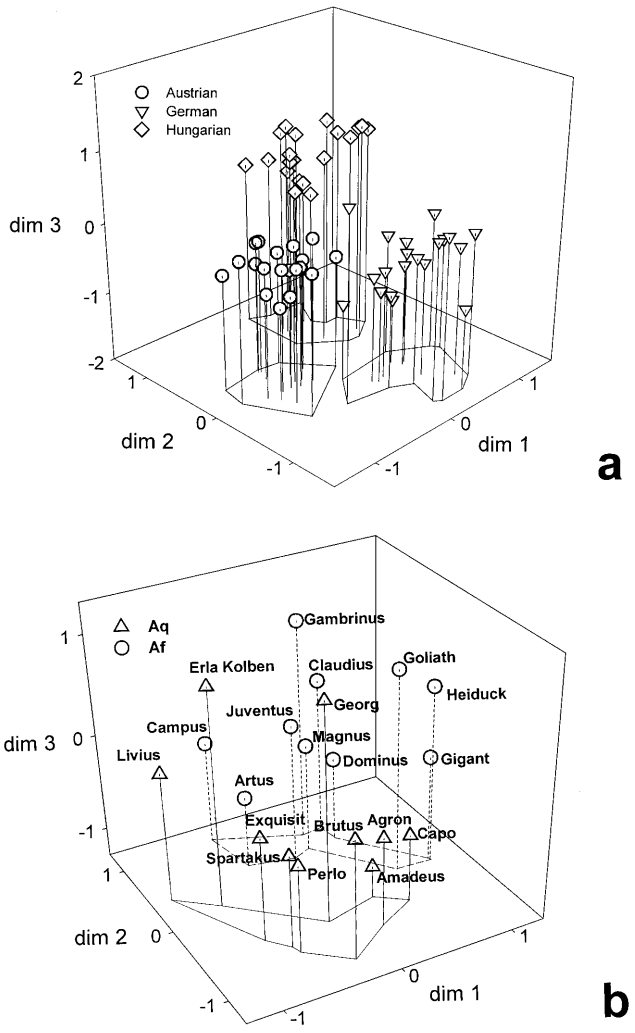

Fig. 2 Multi-dimensional scaling of (**a**) the 60 cultivars from three different AEAs, and (**b**) the 20 Austrian cultivars of the two quality categories

ly 14 MS loci gave results comparable to those obtained by using the complete set of MS (data not shown). The rank correlation coefficient between COD- and GD-values was r_s=0.45 (P<0.001).

Both the selection within different AEAs and breeding for different quality categories had statistically significant effects (P<0.001) on the genetic distance between the investigated cultivars. In an analysis-of-variance model based on AEAs only, 49% of variation in MS-based genetic distance could be explained, whereas in a model based on AEAs and quality categories within AEA, 56% of the variation in genetic distance could be explained.

Discussion

In this work 42 MS primer sets were used to study 60 wheat cultivars. For an even coverage of the total wheat genome we selected one MS for each chromosome arm. The selection of the 60 wheat cultivars from three different geographic regions and according to their end-use was made with the purpose of studying the influence of (1) natural selection, i.e. agroecological areas (AEAs), and of (2) purposeful selection, i.e. end-use, on forming distinguishable gene pools. The relatively high allele-frequencies of the microsatellites combined with their even distribution throughout the whole genome made them most suitable for procuring such information.

Using 23 MS in 40 genotypes from 12 different countries of origin Plaschke et al. (1995) found 6.2 alleles per locus distributed on 15 chromosomes. Röder et al. (1995) studying 15 MS in 12 breeding lines found an average of 3.2 alleles, while Bryan et al. (1997) in ten wheat varieties with 49 MS found an average of 3.5 alleles. The average number of alleles in our study is 4.8. Considering the conservative mode of allele differentiation using PAGE and silver staining, this value is probably an under-estimation of the real allele frequency in this material. The higher variation in Plaschke's material can be explained partly by the extended geographic distribution of the investigated genotypes, and partly by the higher resolution power of their detection system using automated laser fluorescence (Plaschke et al. 1995). Röder et al. (1995) used ethidium bromide for staining the polyacrylamide gels, which is comparable to silver-staining as far as resolution power is concerned. As in case of Bryan et al. (1997), the low level of allele frequency might have been caused by the restricted sample size. When excluding the four monomorphic MS loci from the calculation, the average number of alleles in our study increases to 5.2. This appears to be an adequate allele frequency to obtain relevant information on genetic diversity and on the change of allele distribution following selection.

In the present material we found null alleles at 16 loci. It is interesting to note that, apart from the monomorphic loci, it is a null allele (WMS 169) that occurs with maximum frequency, i.e. in 42 out of the 60 varieties. Null alleles for MS in wheat were previously described by Devos et al. (1995), Plaschke et al. (1995) and Donini et al. (1998). While alleles of MS are the result of a change in the number of repeats, null alleles are the consequence of polymorphism in the primer-binding site. Nevertheless, while for RAPDs and AFLPs null alleles are the usual alternatives, in MS they represent just one of a series of multiple alleles.

Changes in the repeat numbers creating new alleles of MS are thought to be caused by a DNA-replication error called slippage (Tautz et al. 1986). Considering the average allele frequency of 4.8 in this material, the complete lack of variation in four loci over the 60 cultivars is remarkable. This suggests that they are not selectively neutral and may have a functional role (Gupta et al. 1994).

Bryan et al. (1997) described additional amplification products which, however, they found generally smaller in size than the expected fragment. We also observed additional fragments which were clearly not stutter bands.

In contrast to the findings of Bryan et al. (1997), however, they were always larger than the expected one. In most cases their polymorphism pattern followed that of the main band. Since they disappeared in cases of null alleles, we do not believe that they were from homoeologous chromosomes. Analogous to Röder et al. (1995) we found no correlation between the length of the respective microsatellite repeat and the level of polymorphism, either estimated by the number of alleles or by PIC-values, as was described by Bryan et al. (1997).

Agroecological areas

The almost identically high allele frequencies in the German and Hungarian gene pools imply a wide genetic basis of the Hungarian breeding material. This is worth emphasising since the Hungarian cultivars originated from only one breeding company, while the 20 German cultivars came from several breeding stations and included cultivars from the former German Democratic Republic. Via these cultivars several MS alleles of completely different origin became incorporated into the German gene pool. The 14% lower level of polymorphism in the Austrian breeding material might be taken as a warning sign for a too narrow genetic base for further varietal improvement. The fact that even the highest average allele frequency, which was found in the German varieties (3.45), represents only 72% of the total European average (4.8) is a strong indication that the alleles among the three AEAs are differentially distributed. This is also clearly reflected in the clustering of the cultivars (Fig. 1) and by the highly significant differences between genetic distance classes. The differential distribution of MS alleles can be expected because of the large ecological divergence of the environments leading to different adaptations. In Szeged, Hungary, a considerable portion of the genetic diversity is derived from Yugoslavian and Russian breeding material adapted to continental conditions. Cultivars adapted to more maritime environments in Western Europe may have influenced the genetic make-up of the German gene pool. The average genetic distance within an AEA reveals the greatest diversity in the German cultivars, followed by the Hungarian and Austrian cultivars (Table 4).

The distance data between the gene pools show the greatest divergence between the German and Hungarian pools, the lowest between the German and Austrian pools (Table 4). In fact, in the Austrian national list of registered cultivars approximately 40% are of German origin and no cultivar derives from Hungary. A number of key varieties, e.g. Cappelle Desprez, Carstens 8, Heines 7, Kronjuwel and Maris Huntsman, were used both in Austrian and German breeding programs, whereas only one key variety, Bezostaja 1, is common for the two AEAs of Austria and Hungary.

The moderate correlation coefficient between COD- and GD-values (r_s=0.45) is comparable to other studies (Plaschke et al. 1995; Barrett et al. 1998; Burkhamer et al. 1998). As the frequency distribution of COD- and GD-values shows (Fig. 3a), GD-values are normally distributed whereas the distribution of the COD-values is strongly biased. This is most certainly due to the imperfect estimation of the relationship between two cultivars which is provided by the COD-value. In fact, its accuracy is affected by selection, genetic drift, the heterogeneous nature of ancestors (e.g. landraces) and unknown relationships among supposedly unrelated ancestors. If the distribution of GD data for unrelated cultivars is redrawn (COD>0.95), they still show a distribution within the same range of GD frequencies (Fig. 3b). This result is a clear indication of the high information value of MS data for plant breeders compared with pedigree information, and explains the generally weak correlation between these two measures.

Area	A	D	H
A	**0.46**±0.08		
D	0.59±0.07	**0.51**±0.08	
H	0.63±0.07	0.66±0.06	**0.48**±0.09

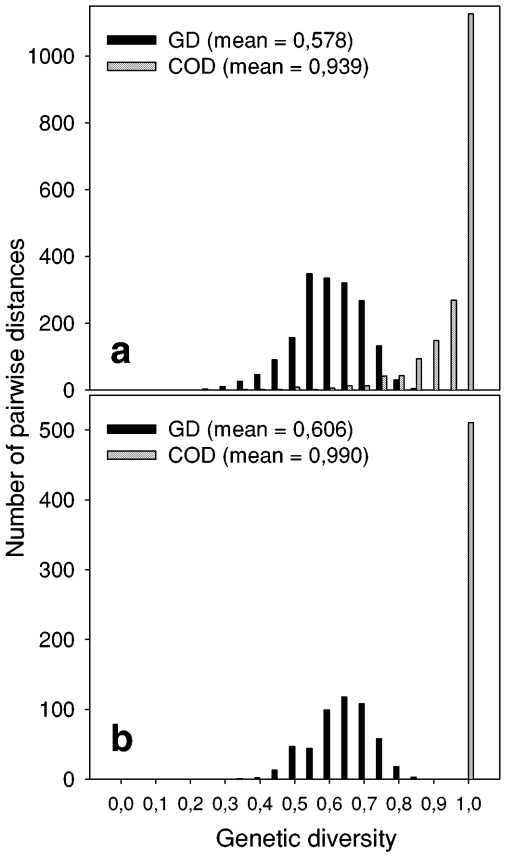

Fig. 3 Frequency distribution of (**a**) the genetic diversity (*GD*) and coefficient of diversity (*COD*) values based on MS and pedigree data, respectively, and (**b**) restricted to COD≥0.95

Quality categories

A further proof of the differentiating power of MS is demonstrated by clustering the cultivars according to their quality categories. As can be seen in the dendrogram (Fig. 1) quality and feed wheats were separated into distinct groups in all three AEAs, indicating their different genetic make-up. Data for GD between the two quality categories are not significantly different from each other across all AEAs (Austria 0.49, Germany 0.54 and in Hungary 0.52; shown bold in Table 3). Within each AEA the difference between the average distances of these two categories is highly significant ($P<0.001$; given in italics in Table 3, see also Fig. 2b). Thereby, the power of selection for these two different uses becomes evident.

Between Hungarian and Austrian cultivars there is only a slight difference in the allele frequency between the two quality categories, 2.5 versus 2.6 and 2.4 versus 2.5, respectively (Table 2). In Germany the difference in allele frequencies between the categories is much larger, 3.0 versus 2.5. This difference can be explained by the cultivars from the former Eastern Germany (Aron, Ramiro, Tambor) and by the only spring wheat which was included in the study (Klaros). Since all these four cultivars are quality wheats representing a different gene pool, the genetic diversity within this category is much higher than within the Austrian and/or Hungarian quality wheats.

The clear reduction of allele frequency in the specific groups of cultivars in comparison with the total allele frequency of their respective AEA (Table 2) demonstrates how breeding for specific targets narrows down the genetic variation.

Genomes and chromosome arms

The choice of this set of MS allows quantification of the genetic variation between the three wheat genomes. Microsatellite data, like that of RFLPs, have already shown the lower diversity of the D genome (Bryan et al. 1997; Langridge and Chalmers 1998). Our data are in good agreement with these observations. Average PIC values show highly significant differences between D and the two other genomes which are not significantly different from each other (Table 1). The clearly lower level of polymorphism in the D genome is most likely due to its more recent incorporation into wheat.

Genetic diversity is closely related to recombination frequency, which in turn is correlated with the physical length of a chromosome arm. Long arms accommodate more cross-overs than short ones. Average PIC values of short and long chromosome arms were not significantly different in this study. However, when long and short arms for the three genomes are compared separately, the largest, though still not significant, difference was found for the chromosomes of the B genome (Table 1). Lukaszewski and Curtis (1993) reported an "overwhelming" difference in the distribution pattern of recombination between the short and long chromosome arms of wheat. While on the short arms recombination was concentrated in terminal regions and was virtually absent in proximal and interstitial positions, on the long arms a low, though consistently detectable, level of interstitial recombination was observed. Lukaszewski and Curtis in their study refer to eight chromosome arms all belonging to the B genome. Higher PIC values for the long arms of the B genome chromosomes in our case indicate a higher recombination frequency in these chromosome arms, which is in line with the occurrence of interstitial chiasmata on the long chromosome arms. In long arms, physical distance reduces interference to a point where a second cross-over can occasionally occur (Lukaszewski and Curtis 1993).

Finally, it is encouraging that a reduced number of MS is able to clearly differentiate between AEAs and even between quality categories within AEAs. A selected number of highly polymorphic MS markers, providing good coverage of the total wheat genome, could be used for varietal identification or differentiation, as already proposed by Plaschke et al. (1995).

Our results suggest that selection for both adaptation and end-use quality will affect the whole of the wheat genome, as one would expect considering the polygenic nature of these two characters. Moreover, microsatellites should be a highly valuable tool for establishing a complete phylogenetic structure of the entire present-day European wheat gene pool for the benefit of plant breeding.

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