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ORIGINAL PAPER

Analysis of genetic structure in a panel of elite wheat varieties and relevance for association mapping

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Abstract During the last decades, with the intensification of selection and breeding using crosses between varieties, a very complex genetic structure was shaped in the elite wheat germplasm. However, precise description of this structure with panels and collections is becoming more and more crucial with the development of resource management and new statistical tools for mapping genetic determinants (e.g. association studies). In this study, we investigated the genetic structure of 195 Western European elite wheat varieties using the recent development of high throughput screening methods for molecular markers. After observing that both microsatellites and Diversity Array Technology markers are efficient to estimate the structure of the panel, we used different complementary approaches (Genetic distances, principal component analysis) that showed that the varieties are separated by geographical origin (France, Germany and UK) and also by breeding history, confirming the impact of plant breeding on the wheat germplasm structure. Moreover, by analysing three phenotypic traits presenting significant average differences across groups (plant height, heading date and awnedness), and by using markers linked to major genes for these traits (*Ppd-D1, Rht-B1, Rht-D1* and *B1*), we showed that for each trait, there is a specific optimal Q matrix to use as a covariate in association tests.

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Introduction

From the first cultivated landraces bred under local selection pressures to the use of foreign germplasm in plant breeding programmes, cultivated plants have been recurrently selected and crossed between one another. All this genome shuffling and selection, notably intensified during the last century, have led to a very complex structure of genetic diversity in cultivated plants. In fact, geographic structure and impact of plant breeding on crop genetic diversity have been reported in numerous crops such as maize, barley and oat (Camus-Kulandaivelu et al. 2006; Koebner et al. 2003; Fu et al. 2003).

Knowledge on the levels and distribution of genetic diversity in the existing crop germplasm allows the development of strategies for genetic resources management and exploitation. Moreover, the recent development of association genetics based on linkage disequilibrium to identify loci underlying complex traits has also resulted in an increased interest in the studies of population structure (Pritchard et al. 2000b; Balding 2006). Indeed, the presence of undetected population structure can lead to spurious associations between a phenotype of interest and unlinked candidate loci (Pritchard and Rosenberg 1999).

Wheat is a worldwide cultivated crop and one of the most important cereals in terms of production and cultivated area [Food and Agriculture Organisation of the United Nations (FAO) http://www.fao.org]. Studies on

wheat population structure have reported a geographic differentiation based on molecular marker polymorphism among worldwide accessions (Hai et al. 2007; Balfourier et al. 2007) or European accessions (Röder et al. 2002; Roussel et al. 2005). Other studies on panels of historical wheat lines, from 1800 to 2000, have demonstrated the impact of plant breeding on crop genetic diversity, reaching sometimes opposite conclusions on the trend in diversity over the period of modern plant breeding (Donini et al. 2000; Roussel et al. 2005; White et al. 2007). However, little information is available on the structure of diversity among European elite wheat varieties, although mainly elite germplasm is used by plant breeders in their breeding programs.

Molecular markers are reliable tools to assess genetic diversity and the recent development of high-density methods gives a new dimension to population structure analysis. However, not all types of markers have the same ability to reveal underlying population structure. Different studies in plants (Williams et al. 1990; Melchinger et al. 1991; Plaschke et al. 1995; Prasad et al. 2000) revealed that Simple Sequence Repeats (SSRs) have the best discriminating power compared to restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) markers. Single nucleotide polymorphism (SNP) markers also received high attention because of the development of high throughput screening methods and because they occur at much higher frequency in the genome than SSRs (Van Inghelandt et al. 2010). A comparison between SSRs and SNPs has given similar results in estimating the structure and the diversity of a population in maize, although more SNP are needed to reach the same precision (Van Inghelandt et al. 2010). Diversity Array Technology (DArT) markers have recently been shown appropriate for the estimation of the genetic diversity of wheat and barley populations (Zhang et al. 2009; White et al. 2007). DArT technology enables the typing of several hundred polymorphic loci spread over the genome without the need of prior sequence information (Wenzl et al. 2006). To our knowledge, no previous study has compared SSR and DArT markers to study the structure of a panel, especially in wheat.

The objectives of this work were (1) to compare the genetic structure of a panel of 195 Western European elite wheat varieties using 159 microsatellites markers and 634 DArT markers, and (2) to evaluate the effect of the population structure in association tests for three major genes involved in plant height, heading date and awnedness variability.

Materials and methods

Plant material

A set of 195 elite wheat (*Triticum aestivum L.*) varieties was provided by Arvalis-Institut du vegetal (Table S1). These genotypes originated from 18 breeding companies and have all been registered for cultivation in France. They were bred between 1969 and 2008 and originated principally from France, UK and Germany. All the seeds used for DNA extraction were obtained from self-pollinated ears.

Genetic mapping

A biparental doubled haploid (DH) population of 176 lines, from a cross between the varieties Brigadier and Alcedo, was used to map all the markers. The marker positions were estimated using the Mapmaker software (Lander et al. 1987).

Microsatellite markers

DNA was extracted from young leaf material using a modified CTAB method (Saghai-Maroof et al. 1984). A set of 159 microsatellite markers was selected for genotyping on the basis of their chromosomal location. Primer sequences were obtained from the Graingenes website (http://wheat.pw.usda.gov/GG2/index.shtml). Forward primers were modified during synthesis with the addition of Fam/Ned/Hex labelled sequences. PCR amplifications were performed in a total volume of 7.5 µl containing: 2.5 µM forward + reverse primer, 10× PCR buffer, 50 mM $MgCl_2$, 1.2 mM dNTP, 5 U Taq DNA polymerase (Promega, www.promega.com) and 10 ng/µl template DNA. PCR was performed on a GeneAmp PCR system 9700 (Applied Biosystems, www.Appliedbiosystems.com) using the following thermal cycling conditions: 94°C for 5 min, then 8 cycles at 94°C for 1 min, 65–51°C dropping 2°C/cycle for 30 s, 72°C for 1 min, followed by 27 cycles at 94°C for 1 min, annealing temperature for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min. Amplified fragments were sized using the internal molecular weight standard GeneScan-500 ROX and GeneMapper v3.7 software (Applied Biosystems). When markers produced more than one band, the bands with clearly separated size ranges were scored independently as a different locus. For each microsatellite locus and for the whole set of accessions, the total number of alleles observed and the number of rare alleles (frequency lower than 0.05) were recorded.

DArT markers

Diversity Array Technology are dominant markers, based on SNPs or INDELs at restriction enzyme cutting sites or based on large INDELs within restriction fragments (White et al. 2007). Development of the markers is described by Akbari et al. (2006). DArT marker data were provided by Triticarte Pty. Ltd, Yarralumla, ACT, Australia (http://www.triticarte.com.au). The technology is protected by patent No. WO 01/73119.

A total of 634 DArT markers was generated, genotyped on the panel and mapped on the Brigadier-Alcedo mapping population.

Markers for *Ppd-D1*, *Rht-D1*, *Rht-B1* and *B1* genes

All of the varieties of the panel were also genotyped with four markers tightly linked with major genes involved in heading date, plant height and awnedness. For heading date, the assay developed by Beales et al. (2007) in *Ppd-D1*, a major photoperiod response locus in hexaploid wheat located on chromosome 2D, was used. For plant height, two SNP were used: one from the *Rht-B1* gene, located on chromosome 4B, and one from the *Rht-D1* gene, located on chromosome 4D, as described in Ellis et al. (2002). These two SNP markers were combined to form a unique locus in further association analysis. Finally a SSR marker (*gwm291*, http://www.shigen.nig.ac.jp/wheat/komugi/genes/symbolClassList.jsp), located on chromosome 5A and close to the *B1(tipped 1)* gene (Sears 1954; Jagathesan et al. 1961) was used to study the awnedness trait.

Phenotypic data

Evaluations of heading date and awnedness were obtained from field experiments conducted in Ouzouer le Marché (France lat: 47.9111, long: 1.5275) in 2008 and 2009 and in Mondonville (France lat: 43.672, long: 1.288) in 2010. Plants were sown the 06/11/2007, the 29/11/2008, and the 18/11/2009, respectively, for the 3 years. Evaluations for plant height were obtained in Ouzouer le Marché in 2008 and 2009. Data in Ouzouer le Marché were obtained from two replicates of a field experiment in a randomized complete block design, with each of the 195 lines sown in two rows of 1.5 m. In Mondonville, varieties were sown in a two replicate randomized block design, with varieties sown in a single row of 1 m. Heading date was noted when 50% of flowers were visible on the whole plot and converted in °C/day after January 1st. Plant height was recorded in cm, and awnedness was noted between 1 and 9 (9 for the longest awns). For plant height and awnedness, 50 plants, located in the middle of the plot were measured. Each year, the arithmetic means of phenotypic data were used and broad sense heritability was estimated as $h^2 = 1 - (1/F)$, where $F = \text{MSc/MSe}$ (MSc and MSe are the mean squares of cultivars and error, respectively). Adjusted means for each trait were estimated using the R software v 2.9.2 (R Development Core Team (2009).

Genetic diversity assessment

The Nei's diversity (1973) was calculated for each locus according to the formula: $\text{he} = 1 - \sum_{j=1}^{k} p_j^2$ where p_j is the frequency of the jth allele and k is the total number of alleles. This value provides an estimation of the discriminatory power of each marker.

Model-based cluster analysis

The genetic structure among the 195 wheat genotypes was explored using a model-based method implemented in the software *Structure* v2.2 (Pritchard et al. 2000a). Different numbers of genetic groups (K) ranging from 1 to 10 were tested using the linkage and the correlated allele frequency models (Falush et al. 2003). For each K, five independent runs were achieved. The replication number was set to 50,000 both for the burn-in and the Markov chain Monte Carlo (MCMC)) periods. We used neighbour-joining tree based on Euclidian distance between outputs to evaluate structure stability according to Camus-Kulandaivelu et al. (2007) . Finally, to compare the results obtained by SSRs and DArTs, we performed Fisher's exact test using R software v 2.9.2 to check that both types of markers provide the same picture of genetic structure among the lines.

Genetic distance-based cluster analysis

We used the Rogers distance (1972) to calculate pairwise genetic distances among all of the genotypes using *PowerMarker* v3.25 software (http://www.powermarker.net). A tree based on the unweighted pair-group method with arithmetic average (UPGMA) was built using the S-PLUS 8.0 software (Copyright 1988, 2007 Insightful Corp).

Principal component analysis

Principal component analysis (PCA) was performed to visualize the relative dispersion of the subpopulations in a three-dimensional space using S-PLUS 8.0. In the PCA, missing data were replaced by the corresponding mean allele frequency.

Marker–trait associations

A fixed effects linear model was used to test for associations between major gene polymorphisms and phenotypic

variation. The model included main gene effect and covariates to take into account the structure of the panel. Different population structures were evaluated using the covariates from the *Q* matrix obtained with the *Structure* software.

Results

Microsatellite polymorphism

From the 159 SSR markers analysed, a total of 1,191 alleles were detected which represent an average allele number of 7.49 per locus. This number is reduced to 569 alleles (3.58 alleles per locus) when alleles with a frequency lower than 0.05 were excluded (Table S2). The mean frequency of missing data was 8.98% and the total number of alleles varied from 2 (gdm129) to 29 (wmc050). The SSR markers were not evenly distributed among the chromosomes, as the number of markers per chromosome ranged from 4 (Chr1B) to 12 (Chr5A) with an average distance of 16.56 cM between two consecutive markers. Markers of chromosome 5B were united with those of chromosome 7B because a common translocation between these two chromosomes was observed in the mapping population.

The Nei's diversity values (he) ranged from 0.11 (gwm547) to 0.89 (wmc754) with a mean value of 0.61. Diversity values were not significantly different among the chromosomes (*P* value Kruskall–Wallis = 0.07).

Dart polymorphism

A total of 634 DArT markers were mapped in the DH recombinant population and genotyped in the panel. The number of markers per chromosome varied tremendously from 2 (Chr4D) to 158 (Chr1B) with an average distance between markers of 9.56 cM. However, due to a high amount of clustering on some chromosomes (particularly chromosome 1B), already observed by Wenzl et al. (2006) and Zhang et al. (2009), and the elimination of rare alleles and markers with more than 10% of missing values, only 252 non-redundant DArT markers were kept for the analysis (Table S3). The mean frequency of missing data was 7.35% in this subset of DArT markers.

The Nei's diversity values were computed for the 252 DArT markers and ranged from 0.06 to 0.5, with a mean value of 0.38. Diversity values were computed for the different chromosomes and no significant difference was observed among chromosomes (*P* value Kruskall–Wallis = 0.96).

Comparison of the genetic structure obtained with SSRs and DArTs

To compare the genetic structure obtained with SSR and DArT markers among the 195 wheat varieties, we used the *Structure* software package and performed runs for each value of *K*. With the 46 output matrices (5 replicates for *K* varying from 2 to 10, as well as the no genetic structure model), neighbour-joining trees were built for each of the two marker types (Figs. 1, 2).

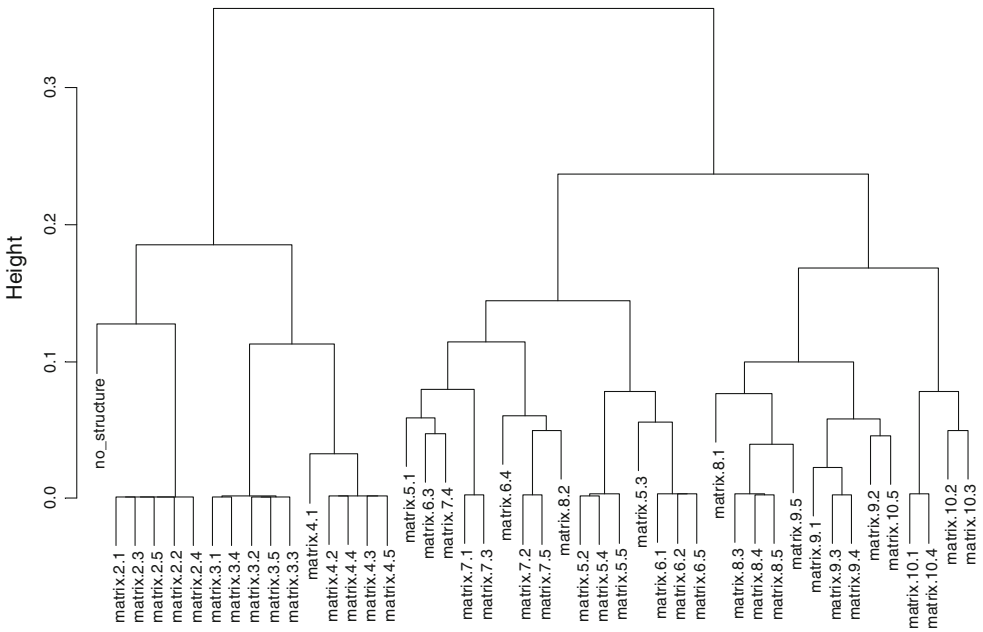

Fig. 1 Neighbour-joining tree for 46 *Structure* outputs, including the no genetic structure model, obtained with the 159 SSR markers on the panel of 195 varieties. For each output, the group number *K* is indicated at leaf positions

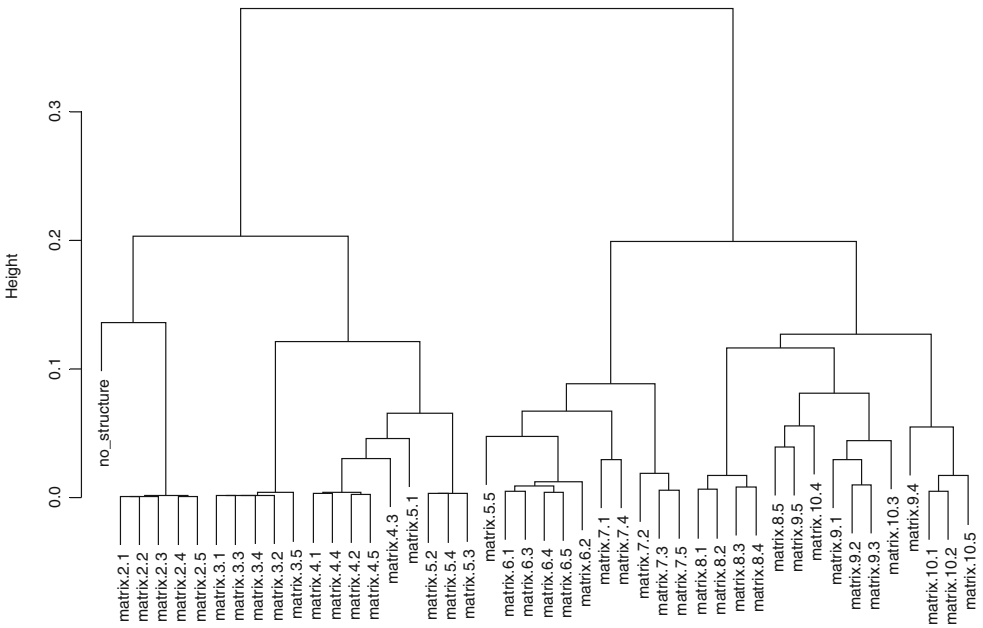

Fig. 2 Neighbour-joining tree for 46 *Structure* outputs, including the no genetic structure model, obtained with the 252 DArT markers on the panel of 195 varieties. For each output, the group number K is indicated at leaf positions

In the two trees, output matrices are divided in two clear clusters. The first cluster contains the matrices from group 1 to group 4, and the second cluster contains the matrices from group 6 to group 10. Matrices of the group 5 are mixed between the two clusters. With the SSR markers, group 5 belong to the second cluster, whereas it is distributed between the two clusters with DArT. Concerning matrices with identical K, a clear clustering is observed until group 4 for the two types of markers. For higher groups (group 5 to group 10), matrices are mixed together. Thus, we decided to focus on the $K = 2$ to $K = 4$ matrices for further analyses, as they correspond to the most stable groups among different repetitions of the *Structure* results.

In order to compare the different groups defined by *Structure* with the two markers types, the varieties assigned to each of the groups (from group 2 to group 4 for a threshold of 0.7) were examined (Table 1).

For $K = 2$, 49 individuals were assigned to group 1 and 81 to group 2 with the SSR markers, while 53 and 69 varieties fell in group 1 and group 2, respectively, with the DArT markers.

By comparing the assignation of the varieties into each of the two groups, we observed a good match between the groups defined with DArT markers and the groups defined with SSR markers (p value of Fisher's exact test $= 9.2 \times 10^{-31}$, 8.07×10^{-23}, 8.33×10^{-23} for $K = 2$, 3 and 4, respectively. At $K = 3$, SSR-based groups correspond to DArT-based groups, except for group 2 defined with DArT markers, which corresponds to group 1 and group 2 defined with the SSR markers. At $K = 4$, the same four groups are found by the two sets of markers, although the number of common varieties assigned to each group is low (4 for group 1, 8 for group 2 and 10 for group 3) and the number of non-assigned varieties became high (113 with DArT and 101 with SSR markers).

Structure analysis of the panel

Although grown in France, varieties from the panel have been developed in different countries. Based on the group membership estimated by *Structure* (threshold >0.7) for each variety, we observed that each group formed from $K = 2$ to $K = 3$ (Fig. 3) corresponds to a geographic origin. The $K = 2$ partition separated varieties originating from the UK from French and German varieties. At $K = 3$, French varieties are separated from the German varieties, whereas the UK group remained stable. Finally at higher levels ($K = 4$), the French varieties are divided into two subgroups.

Another way of analysing the structure of diversity in a panel is to estimate genetic distance for each pair of genotypes. In the panel, the pairwise genetic distances computed with SSR markers ranged from 0.02 (PP231 and PP198) to 0.65 (PP245 and PP1), with an average of 0.51. Distance-based UPGMA cluster analysis (Fig. 4) first divided the 195 wheat varieties into two main groups. Interestingly, these two groups separate UK varieties from French and German varieties, which is consistent with the separation obtained with *Structure* for $K = 2$. The average distance between the two groups is 0.44. Moreover, the division suggested by *Structure* at higher K levels ($K = 3$ and $K = 4$) into two French and one German group could also be identified on the dendrogram.

A third representation of the genetic diversity within the panel can be obtained using PCA. In the PCA of the 195 varieties with SSR markers, varieties are represented with

Table 1 Comparison of the assignation of varieties to groups by each of the two types of markers (DArT and SSR) calculated by *STRUCTURE* software for *K* varying from 2 to 4

$K = 2$	Assign. Thresh. 0.7	Grp1 DArT 53	Grp2 DArT 69	Not assign. 73
Grp1 SSR	49	45	0	4
Grp2 SSR	81	0	60	21
Not assign.	65	8	9	48

$K = 3$	Assign. Thresh. 0.7	Grp1 DArT 14	Grp2 DArT 40	Grp3 DArT 40	Not assign. 101
Grp1 SSR	31	12	9	0	10
Grp2 SSR	32	0	14	0	18
Grp3 SSR	37	0	0	32	5
Not assign.	95	2	17	8	68

$K = 4$	Assign. Thresh.0.7	Grp1 DArT 11	Grp2 DArT 15	Grp3 DArT 24	Grp4 DArT 32	Not assign. 113
Grp1 SSR	7	4	0	0	0	3
Grp2 SSR	21	2	8	0	0	11
Grp3 SSR	29	0	2	10	0	17
Grp4 SSR	37	0	0	0	30	7
Not assign.	101	5	5	14	2	75

For each assignation of varieties to groups, we used an arbitrary threshold of 0.7

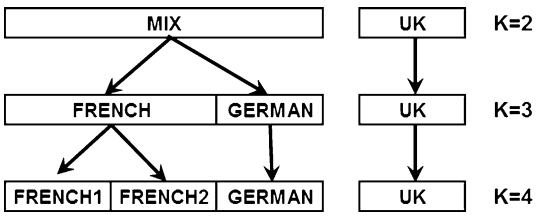

Fig. 3 Geographic origin of each *K* level calculated from *Structure* v2.2 software between $K = 2$ and $K = 4$. For each assignation of varieties in groups, we used an arbitrary threshold of 0.7

different colours according to the classification from *Structure* ($K = 4$), based on an assignment threshold of 0.7 (Fig. 5). Unassigned varieties are also represented. The first, second and third principal components (PC) explained 7.3, 4 and 3.1% of the genetic variance, respectively. PC1, PC2 and PC3 clearly separated the varieties from France, the UK and Germany.

Thus, the different analyses obtained with the SSR markers are consistent with the groups formed by the *Structure* software with the two set of markers (DArT and SSR), run independently. We then used this information on the structure of the panel to investigate the correlation between three phenotypic traits (Heading Date, Plant Height and Awnedness) and the groups defined by *Structure*.

Influence of the panel structure on three phenotypic traits

Heading Date (HD) and Awnedness (AW) were recorded over 3 years on the panel and Plant Height (PH) was recorded over 2 years. Variations for HD ranged from between 1,359 and 1,693°C/day in 2008, between 1,025 and 1,311°C/day in 2009 and between 845 and 1,426°C/day in 2010, variations for AW ranged from between 1 and 8 in 2008 and 2010 and between 2 and 8 in 2009 and variations for PH ranged from between 64 and 112 cm in 2008 and between 69 and 120 cm in 2009.

Pearson correlation coefficients for the three traits between 2 years were high and highly significant: for HD, $r = 0.90$ ($P < 0.001$) between 2008 and 2009, $r = 0.85$ ($P < 0.001$) between 2008 and 2010 and $r = 0.86$ between 2009 and 2010; for AW, $r = 0.95$ ($P < 0.001$) between 2008 and 2009, $r = 0.94$ ($P < 0.001$) between 2008 and 2010 and $r = 0.96$ ($P < 0.001$) between 2009 and 2010 and finally for PH, $r = 0.84$ ($P < 0.001$) between 2008 and 2009. High heritabilities were also obtained: 0.90 for HD, 0.98 for AW and 0.92 for PH.

Adjusted means were calculated for each variety of the panel, using the following model: $P_{ijk} = \mu + G_i + A_j + R_{ijk,}$ where G_i is the genotypic effect, A_j is the year effect and R_{ijk} is the residual effect.

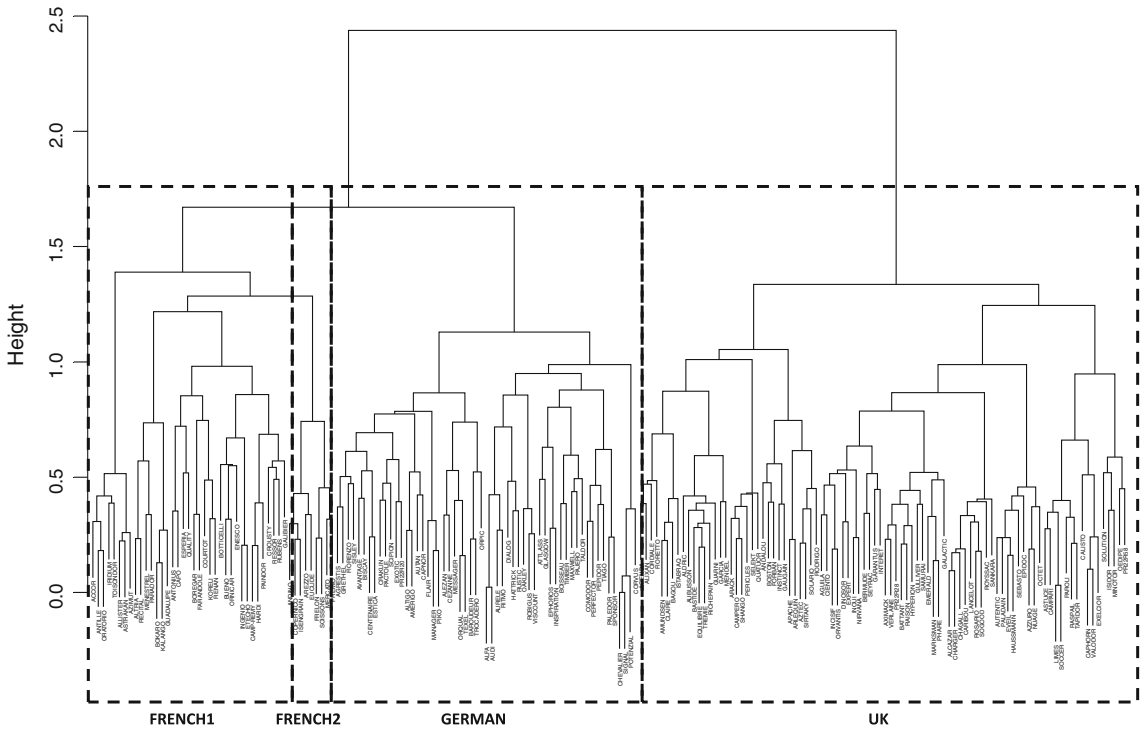

Fig. 4 Dendrogram of 195 winter wheat elite lines with SSR markers based on Rogers' genetic distance (1972) using UPGMA method

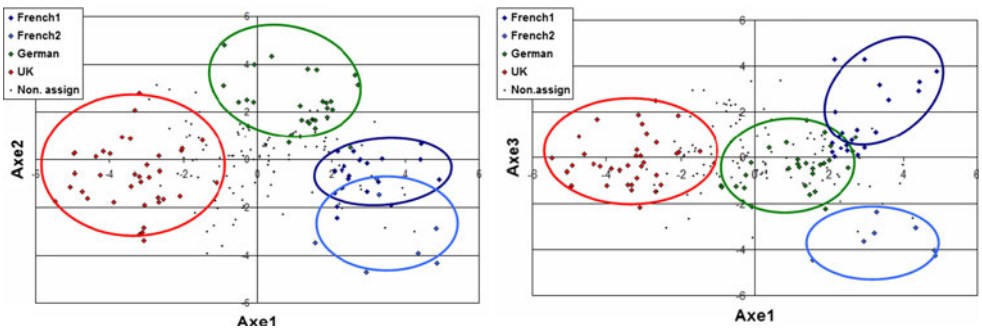

Fig. 5 Principal Component Analysis of the 195 wheat varieties with *Structure* v2.2 based on SSR marker information. The subdivision of the varieties, assuming $K = 4$, separates the four groups (two French groups, one German and one from the UK). Inertia values are 7.3, 4 and 3.1% for factorial coordinate axes 1, 2 and 3, respectively

Newman–Keuls tests were performed to compare the means for the three traits within the four subgroups obtained with *Structure* at $K = 4$ (Fig. 6). This test revealed that the four subgroups were significantly differentiated for the three traits. For HD, both German and UK groups are later than the two French groups. For PH, the German group shows significantly taller varieties than those of the other groups. Finally, varieties of the French2 group have the longest awns compared to the varieties of the other groups.

Association analysis

For these analyses, we selected four well known major genes (*Ppd-D1*, *Rht-B1*, *Rht-D1* and *B1*) involved in the three traits (HD, PH, AW), as described in "Materials and methods".

The analysis of the mean values of the related trait of each allele of the four genes (Table 2) allowed to identify allele effects. For the SNP in the *Ppd-D1* gene, the two alleles had a different effect on HD. Varieties carrying the *Ppd-D1* sensitive allele head later than varieties carrying *Ppd-D1a,* corresponding to the insensitive allele as expected. For the *Rht* genes, only three combinations from the two SNP markers have been detected in our panel. *Rht-B1a_Rht-D1a*, corresponding to the double wild type-recessive combination, was associated with taller plants than the single dwarfing combinations (*Rht-B1b_Rht-D1a* and *Rht-B1a_Rht-D1b*). Varieties carrying single dwarfing combinations have the same mean height, underlying a similar effect on the trait of the two dwarfing alleles (*Rht-D1b and Rht-B1b*). For the SSR marker *gwm291*, tightly linked to the *B1* gene, five alleles were detected in the panel. Varieties with allele_5 have the longest awns with a

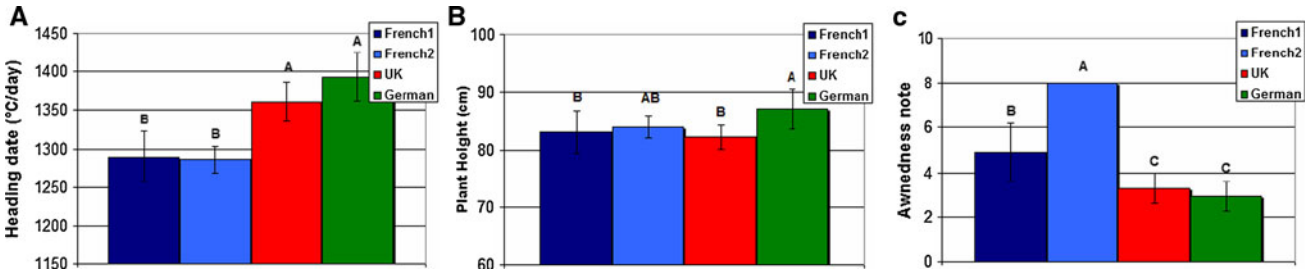

Fig. 6 Adjusted means for **a** heading date, **b** plant height and **c** awnedness with *Structure* v2.2 clustering information assuming $K = 4$. *Different letters* indicate a significant difference according to Newman–Keuls tests

mean awn note of 7.98. Allele_1, allele_2 and allele_4 have the same moderate effect on awn length, and allele_3 occurs more frequently in the varieties with very short awns.

The distribution of each allele of the four genes in the four groups (French1, French2, German and UK) (Fig. 7) defined by the *Structure* software showed clear differences among groups. *Ppd-D1a*, the photoperiod insensitive allele (Table 2), is highly frequent in the French material (100% in the French2 group and 70% in the French1 group), whereas 81% of the varieties of German group and 82% of the varieties of the UK group carry the sensitive allele, *Ppd-D1*. Concerning the *Rht genes*, the double wild type-recessive combination (*Rht-B1a_Rht-D1a*) is only present in the German group (42%) and in the French1 group (18%). It is interesting to note that all the varieties of the French2 group carry the same single dwarf allele (*Rht-B1b*) and that the varieties of the UK group only carry the other single dwarf allele (*Rht-D1b*). Finally, allele_5 of *gwm291*, responsible for longer awns (Table 2), is fixed in the French2 group (100%). Allele_1 of *gwm291* is only present in the French1 group (22%) and in the UK group (28%) and allele_4 and allele_3 only in the German group.

Table 2 Mean values of the different traits [heading date (HD), plant height (PH) and awnedness (AW)] for each allele of the three major genes (*Ppd-D1* for HD, *Rht* combination s for PH, *gwm291* for AW)

Alleles	Nbr varieties	Mean values (°C/day)	N–K test
Ppd-D1			
Ppd-D1a	70	1,278.49	A
Ppd-D1	112	1,380.96	B
Alleles	**Nbr varieties**	**Mean values (cm)**	**N–K test**
Rht combinations			
Rht-B1b_Rht-D1a	25	81.36	A
Rht-B1a_Rht-D1b	80	82.03	A
Rht-B1a_Rht-D1a	19	95.66	B
Alleles	**Nbr varieties**	**Mean values (Awn note)**	**N–K test**
Gwm291 (*B1*)			
Allele_3 (140 bp)	13	2.23	A
Allele_2 (118 bp)	89	2.85	B
Allele_1 (116 bp)	24	2.96	B
Allele_4 (165 bp)	9	3.17	B
Allele_5 (171 bp)	27	7.98	C

Different letters indicate significant difference between mean values according to the Newman–Keuls test

Results of association studies between the markers (*Ppd-D1*, *Rht* combinations and *gwm291*) and the phenotypic traits (HD, PH and AW), using different covariates ($K = 2$ to $K = 10$) are presented in Fig. 8. For all the markers tested, we obtain very significant P values, confirming that the four markers are associated with their corresponding trait. Moreover, for K levels superior than $K = 4$, results are less homogeneous between each other, confirming the clustering of matrices with identical K observed only from $K = 2$ to $K = 4$ (Figs. 1, 2).

For each marker the $-\log10$ (P values) results show the same profile: after a slight decrease, they reach a minimum value and slightly increase for higher K levels. Markers related to HD (*Ppd-D1*) and PH (*Rht* combinations) reached the minimum value at $K = 3$, whereas markers related to AW (*gwm291*) reached the minimum value at $K = 4$.

Discussion

SSR and DArT polymorphisms

A set of 159 SSR and 252 DArT markers were used to characterize 195 Western European elite varieties of hexaploid wheat. In the first part of the analysis, the two marker types permitted an independent evaluation of the genetic diversity of the panel. Concerning the SSR loci, there were an average number of 7.41 alleles per locus. Similarly to these findings, Zwart et al. (2008) found an average of 8.0 alleles per SSR locus in a panel of 144 Western European winter wheat cultivars with a set of 47

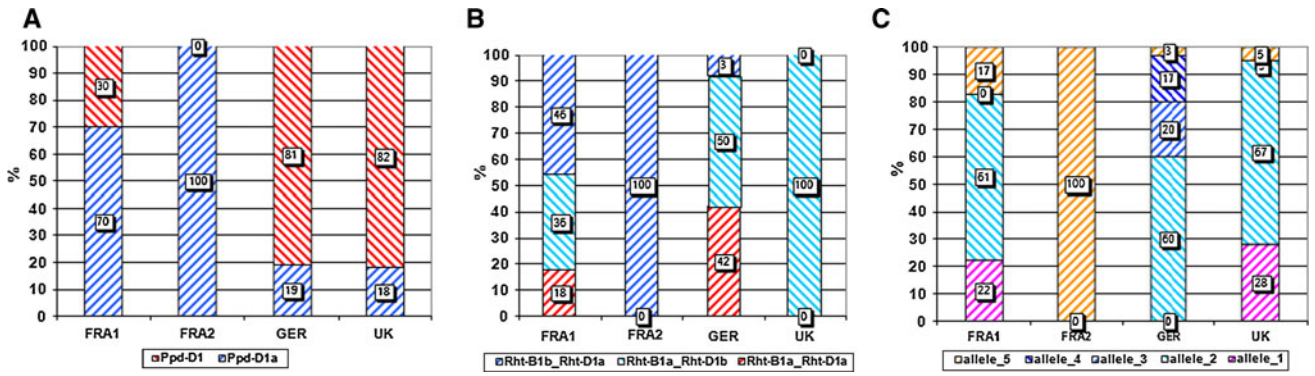

Fig. 7 Distribution of the different alleles of the three genes (**a** *Ppd-D1*, **b** *Rht*combinations, **c** *gwm291*) in the different groups obtained with *Structure* v2.2 clustering information assuming $K = 4$

SSR markers and Hai et al. (2007) found 7.81 alleles per locus by using a set of 52 SSR markers in a panel of 69 European spring wheat accessions. This result is also consistent with other results obtained in panels of different diversity levels showing means between 4.8 alleles per locus (Eastern European varieties) (Stachel et al. 2000) and 18.1 alleles per locus (worldwide varieties) (Röder et al. 2002). Likewise, the gene diversity of the SSR markers (0.61 on average) was within the range of mean Nei diversity values previously found: 0.54 for Western European varieties, (Zwart et al. 2008), 0.580 in Southern European accessions and 0.68 for spring accessions (Hai et al. 2007).

Studies using DArT markers are fewer in the literature than studies using SSR markers. However, the studies with DArT markers show that this kind of marker is suitable for genetic studies pertaining to diversity structure, QTL detection or association genetics (Zhang et al. 2009; Semagn et al. 2007; Crossa et al. 2007). In the panel considered in this study, the mean diversity value obtained with DArT markers was 0.38, much lower than that for the SSR markers, however, DArTs are biallelic markers and the maximum possible gene diversity is therefore 0.5.

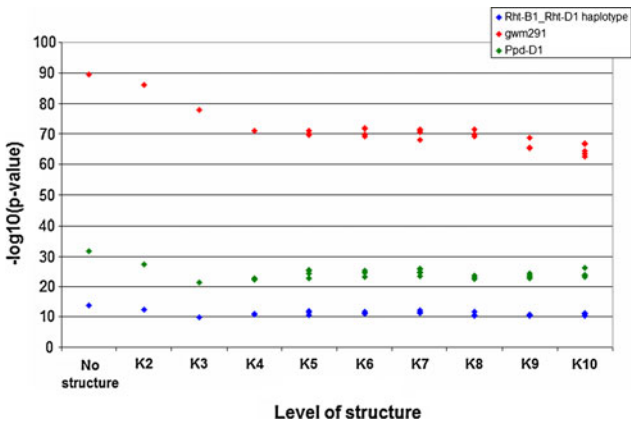

Fig. 8 Results of association tests between three major genes (*Ppd-D1*, *Rht* combinations, *gwm291*) and their related traits (heading date, plant height and awnedness) taking into account different *K* level of *Structure* v2.2. For each *K* level, five repetitions were tested

Information provided by DArT markers is consistent with that provided by SSR markers and both types of markers portray a similar global organization of wheat diversity (Fig. 1; Table 1). This result confirms the potential of DArT markers for structure analysis as they are easier and quicker to use than SSR markers. In this study however, the comparison was based on 159 SSR and 252 DArT markers, which corresponds to a ratio of 1.6 DArT markers for one SSR marker. In a panel of maize lines, Van Inghelandt et al. (2010) estimated that one has to use seven times more SNP than SSR markers to reach the same estimation of genetic structure. This result is quite different from ours. However, it is based on a comparison between sampling variances associated with SNP and SSR pairwise genetic distances, whereas in this study, the 1.6/1 DArT/SSR ratio is only based on a comparison between the DArT and SSR assignments of varieties into groups (Table 1). Moreover, the difference of diversity observed between SSRs and SNPs (0.37) by Van Inghelandt et al. (2010) is notably superior to that observed in this study between SSRs and DArTs (0.23), which may partly explain the difference between ratios.

Population structure

Structure of wheat populations is not well known. There are no heterotic groups, unlike the situation found in maize, and the pedigree records of elite varieties are often difficult to obtain. Studies on historical panels selected during the twentieth century report a trend of temporal and geographical variation linked to breeding practices and agricultural policies (Roussel et al. 2004; Roussel et al. 2005). However, no study analysed the structure in modern panels, although the increase in germplasm exchange among breeding programs may have changed the historical

structure of genetic diversity. For an assessment of the structure of our elite panel, we used both a model-based clustering method and a distance-based method. The results of the two approaches were consistent between each other and allowed us to identify distinct groups originating from the UK, France and Germany. The consistency between both approaches was already observed in previous studies (Breseghello and Sorrells 2006; Somers et al. 2007; Tommasini et al. 2007) but a complementary PCA analysis also allowed us to confirm the structure results. The structure obtained with genetic markers could also be compared to the pedigree information available. For example, Altria and Recital, Courtot and Renan, Camp-Remy and Hardi, Isengrain and Soissons, Texel and Orqual either share common ancestors or are themselves in the pedigree of each other (Table S4) and are found close to each other on the classification (Fig. 4).

The subdivision of our panel appears to be the result of a geographical separation. At $K = 2$ in the *Structure* software output and in the classification (Fig. 4), varieties originating from the UK are clearly separated from the other varieties originating from France and Germany. At $K = 3$ and $K = 4$, two groups originating from France and one from Germany are separated. Whereas the separation between French, German and UK varieties can be explained by geographical specificity, the separation between the two French groups is not due to geographic origin or linked to the breeding companies (data not shown). The analysis of the varieties of these groups showed that in each group, a variety with great success in France could be found: Oratorio for the French1 group and Soissons for the French2 group. Moreover, the specificity of the French2 group in our panel for a non agronomic selected trait (awnedness) and the common alleles of *PpD-D1*, *Rht-B1* and *Rht-D1* shared by all of the varieties of this group, reinforced the idea that varieties of this group share a common genetic origin, potentially originating from a unique elite variety extensively used as progenitor.

Use of the structure information in association studies

Many studies have stressed the importance of taking the structure of a panel into account in association studies to avoid "spurious associations" (Pritchard et al. 2000b; Marchini et al. 2004; Freedman et al. 2004). In fact, the presence of related subgroups in the panel could create covariances among individuals that generate bias in the estimates of allele effects (Kennedy et al. 1992; Freedman et al. 2004). As previously described, four genetic groups have been identified in our panel. One solution, proposed by Garris et al. (2003), is to conduct the analysis within subpopulations, but this approach significantly reduced the power of the analysis. The more common method is to take account of the relationships among varieties by including a structure covariate in the model (Pritchard et al. 2000b). However, if the majority of genome wide analyses only use a common structure covariate for the different analysed traits (Breseghello and Sorrells 2006; Crossa et al. 2007; Miedaner et al. 2010), we observed with our results for the major genes (*Ppd-D1*, *Rht-B1*, *Rht-D1* and *B1*) that for each trait a specific number of covariates are needed to reach the optimal power in the analysis ($K = 3$ for HD and PH and $K = 4$ for AW). To determine this optimal association model, we investigated the influence of different structure covariates on association tests. The optimal model seems to correspond to the best correlation between substructure level and allele differentiation (the K3 sublevel corresponding to the isolation of the French varieties which mainly carry the *Ppd-D1* allele, the K4 sublevel corresponding to the isolation of the varieties of the French2 group which mainly carry the allele_5 at the *gwm291* locus). However, these are particular cases where the genes studied are major components explaining a very large part of the variation in the trait and mainly underline the fact that no single structure covariate matrix can be used for all traits. In our study, each trait has to be considered independently to identify the optimal structure. Previous studies (Aranzana et al. 2005; Yu et al. 2006; Zhao et al. 2007) have highlighted this point and more work is needed to validate this approach, especially with traits not controlled by major loci and less correlated to the structure.

Heading date

The transition from vegetative to reproductive growth is a key adaptive trait in wheat. Indeed, long growing seasons allow cereal crops to flower later in the year and thus exploit an extended vegetative period for resource capture and storage (Cockram et al. 2007). It is well documented that the flowering time of winter wheat varieties are largely dependent on genes determining the plant's response to the length of the photoperiod. Although complex, this genetic control is determined primarily by a homoeologous series of genes *Ppd1*, *Ppd2* and *Ppd3*, respectively, renamed as *Ppd-D1*, *Ppd-B1* and *Ppd-A1* (McIntosh et al. 2003) located on group 2 chromosomes 2D, 2B and 2A, respectively (Law et al. 1978). The degree of photoperiod insensitivity of these three genes has been ranked in the order *Ppd-D1a* > *Ppd-B1a* > *Ppd-A1a* (Worland 1996). In our analysis, we focused mainly on the *Ppd1* gene whose benefits to the adaptability of European winter wheat varieties have been elucidated by Law (1966). The highly significant *P* value for the association tests between the *Ppd-D1* locus and HD confirmed its influence in European wheat germplasm.

The *Ppd-D1a* allele, insensitive to photoperiod, was introduced into European wheats from a Japanese variety "Akakomugi", to reduce flowering time (Strampelli 1932). In fact, the photoperiod insensitive genotypes can immediately develop their floral primordia and flower whilst photoperiod sensitive genotypes have their floral initiation delayed until lengthening days satisfy their photoperiod requirements (Worland 1996). The predicted adaptive advantage of *Ppd-D1* was rapidly recognized by European wheat breeders and used according to the environmental conditions prevailing during the period from grain development to maturity. Insensitive varieties were generally used to avoid the effects of hot dry summer conditions encountered in Southern Europe (Worland et al. 1998). A survey of 120 European wheat varieties for the presence of photoperiod insensitivity, revealed that French varieties were in majority photoperiod insensitive, UK varieties were photoperiod sensitive and German varieties were also photoperiod sensitive despite the predicted yield advantage of insensitivity (Worland et al. 1994).

The analysis of the allele composition of each of our genetic groups clearly shows that French varieties (French1 group and French2 group) possess in majority the day length insensitive *Ppd-D1a* allele (Fig. 7), whereas the day length sensitive *Ppd-D1* allele is highly frequent in the German group and in the UK group, consistent with the results of Worland et al. (1994). Table 2 confirms that varieties possessing the *Ppd-D1a* allele will be earlier than varieties carrying the *Ppd-D1* allele (1,278 vs. 1,380°C/day), consistent with Börner et al. (1993) who showed that under European conditions, this gene delays flowering by 4–10 days.

Plant height

Reducing plant height in wheat has been of great importance because it increased lodging resistance, and thus stability of yield (Borlaug 1968). Several genes for plant height have been identified, but the *Rht-D1* and *Rht-B1* genes have been the most widely used in breeding programs over the last 40 years due to the lack of negative effects on yield potential (Sourdille et al. 1998). *Rht-B1* and *Rht-D1* act by reducing internode length without altering spike morphology (Allan et al. 1968) and have similar effects on plant height (reduction of about 20 cm), even though *Rht-D1* seems to have a stronger effect than *Rht-B1* (Börner et al. 1993). Numerous alleles for each of these genes have been identified (http://www.shigen.nig.ac.jp/wheat/komugi/genes/symbolClassList.jsp): seven for *Rht-B1* and four for *Rht-D1*. In our study, only two alleles of each gene were identified: *Rht-B1a* (tall), *Rht-B1b* (dwarf), *Rht-D1a* (tall) and *Rht-D1b* (dwarf). Consistent with Allan (1983), additive effect between the two loci could be observed (Table 2). The combination of *Rht-B1b* and *Rht-D1b* in the same genotype produces a strong reduction of plant height which decreases the agronomical value of the variety. Indeed, most of the modern varieties carry a single dwarfing allele (Sourdille et al. 1998), as exemplified in our panel in which 85% of the varieties carried either *Rht-B1b or Rht-D1b* and where no *Rht-B1b-Rht-D1b* combination were observed.

According to allele frequencies (Fig. 7), 42% of the German group is composed of the double wild type-recessive *Rht-B1a_Rht-D1a* combination, whereas the other groups are composed of at least 80% of varieties carrying one dwarfing gene. In spite of some disadvantages, notably their susceptibility to lodging, tall varieties are still cultivated, for example in Germany, because of the environmental conditions (long winter and short spring) that limit plant height and because of the use of plant growth regulators. Although the two single dominant combinations give the same height effect (Fig. 6), UK varieties possess the *Rht-B1a_Rht-D1b* combination whereas French varieties possess in majority the *Rht-B1b_Rht-D1a* combination. This geographic use of the two dwarfism alleles *Rht-B1b* and *Rht-D1b* seems thus to be the result of different breeding practices in each country.

Awnedness

Awnedness was not reported to have specific agronomical effects, although Sourdille et al. (2002) reported a role of the awn in the elaboration of yield in wheat under drought conditions. The genetic control of awnedness was found to be simple, involving three dominant inhibitors genes: (McIntosh et al. 1998); *Hd* (Hooded), *B1* and *B2* (tipped 1 and 2). We chose to follow the *B1* gene as its effect on awnedness was easier to observe, compared to the other genes (Sourdille et al. 2002). Three alleles have been identified for *B1*: *B1a*, *B1b* and *B1c* (http://www.shigen.nig.ac.jp/wheat/komugi/genes/symbolClassList.jsp) but the gene has not been cloned yet. As no molecular markers on *B1* itself were available, we used an associated SSR: *gwm291*, which was confirmed to be very close to the *B1* gene according to the association test results (Fig. 8). Phenotypic effects of each allele of *gwm291* (Table 2) revealed that one allele is responsible for longer awns (allele_5) corresponding to the *B1a* allele. We could not identify the two others alleles (*B1b* and *B1c*) among the four others alleles of *gwm291*, although allele_3 revealed a stronger effect on awn length compare to allele_2, allele_1 and allele_4. According to Sourdille et al. (2002), *B1* alone has insufficient effect to induce complete awn inhibition, as observed in Table 2. Even with allele_3, the mean awn note is 2.23. Concerning the distribution of the *gwm291* alleles in the genetic groups, 100% of the varieties assigned to the

French2 group carried allele_5, responsible for longer awns, and this morphological trait is characteristic of this group. On the other hand, allele_3 and allele_4 were only found in the German group and could therefore be considered as diagnostic for German germplasm. Moreover, these alleles can be associated with different effects on awnedness (Table 2), showing that this material brings diversity to our panel for association studies related to this trait.

Conclusions

Information on panel structure is essential for association studies. First we observed that both DArT and SSR markers can be used to efficiently estimate the structure of a panel. Secondly analysis of this structure by different complementary approaches (Dendrogram, PCA) showed that the groups created by the *Structure* software are based on geographical origin (French, German and UK), and on breeding programs (French1 and French2). By analysing three traits presenting significant differences among groups, and by using markers on major genes for these traits, we showed that there is an optimal Q matrix for each trait ($K = 3$ for HD and for PH, and $K = 4$ for AW) that will be useful for carrying out association tests.

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