ISSN 1479-2621
Plant Genetic Resources: Characterization and Utilization (2017) **15**(1); 36–44
doi:10.1017/S1479262115000349

Molecular characterization of winter durum wheat (*Triticum durum*) based on a genotyping-by-sequencing approach

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Received 18 March 2015; Revised 22 June 2015; Accepted 22 June 2015 – First published online 5 August 2015

Abstract

Durum wheat (*Triticum durum*) is predominantly grown as spring type and depending on the production area autumn or spring sowing is used. For the durum production in Austria and Germany, autumn sowing has several advantages, such as yield increase and stability, but this requires the selection for winter hardiness including a good frost tolerance. The aim of this study was to support breeding of winter durum and to facilitate genomic approaches by molecularly characterizing a panel of 170 diverse winter and 14 spring durum lines employing a genotyping-by-sequencing approach. We obtained an unprecedentedly high number of 30,611 polymorphic markers covering the entire genome. The principal coordinate analysis and the cluster analysis revealed the absence of a major population structure but a tendency of lines to group according to their country of origin. Linkage disequilibrium was found to decay within a short distance of approximately 2–5 cM and also showed variable patterns along chromosomes. In summary, our results can assist breeding of durum wheat and pave the way for genomic approaches towards knowledge-based winter durum breeding.

Keywords: allelic diversity; genetic diversity; genotyping-by-sequencing; linkage disequilibrium; population structure; *Triticum durum*; winter durum

Introduction

Durum wheat (*Triticum durum*) is the main source for pasta due to its special kernel characters and quality composition (Elias, 1995). Owing to the climate, favouring durum cultivation, South Europe, West Asia and North Africa (Royo *et al.*, 2009) are the main growing areas of durum. In these regions with mild winters, spring rain and dry summers, the spring-type durum is sown in autumn. Germany and Austria, by contrast, are characterized by harsh winters with recurring frost periods below −10°C, and warm and dry summers often with rain at the end of the season. However, owing to the lack of winter durum varieties with sufficient winter hardiness, combined with high quality, spring sowing of the spring type is most widely used. While this prevents harvest losses due to frost damage, autumn sowing would prolong the growing time, extend the vegetative phase and result in a higher yield potential.

Many of today's winter durum lines trace back to material bred in the Ukraine and Russia. To obtain winter durum, interspecific crosses with *Triticum aestivum* were initially made coupled with subsequent cycles of backcrossing to durum wheat (Palamarchuk, 2005). The achieved winter hardiness, however, was still unsatisfactory and the productivity was lower compared to available bread wheat. Consequently, crosses between *T. aestivum* lines selected for their high winter hardiness and spring durum were made and the resulting lines are the founders of many current winter durum varieties (Palamarchuk, 2005). First efforts on winter durum breeding in Germany and Austria started in the 1960s, when breeders were looking for lines with sufficient winter hardiness in addition

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to the required quality. Such programmes started with available material from gene banks, which likely contained the aforementioned lines generated earlier in the Ukraine and Russia (Walther, 1978). In recent years, the continuous selection for quality, grain yield and frost tolerance has resulted in first winter durum varieties widely accepted by farmers, millers and the pasta industry (Sieber *et al.*, 2014).

The rapid advances in genotyping technologies nowadays enable genotyping-by-sequencing (GBS) approaches also in crops such as durum wheat, generating a previously unprecedented density of genome-wide markers. These can for example be used for genomic approaches and molecular characterization. In the past, population structure, genetic diversity and linkage disequilibrium (LD) patterns were analysed in spring durum with different molecular markers (Sorrells *et al.*, 1995; Maccaferri *et al.*, 2005; Maccaferri *et al.*, 2006; Ren *et al.*, 2013; van Poecke *et al.*, 2013). These studies were, however, only based on spring durum and on a comparably low number of markers. To date, no study is available on the molecular characterization of winter durum.

The aim of this study was to employ a genotyping-by-sequencing approach to analyse a unique and diverse panel of winter durum lines. In particular, our objectives were to (1) assess the extent of population structure and genetic relationships among winter durum, (2) evaluate allelic diversity on a genome level, (3) determine the extent and the pattern of LD across the durum genome and (4) discuss consequences for winter durum breeding.

Materials and Methods

Plant materials

In this study, a diverse set of 184 durum wheat lines (*T. durum*) was used. Fourteen of these lines are spring types, while the remaining 170 are winter types (Fig. S1, available online).

Molecular markers

DNA was extracted from young leaves following standard procedures. GBS was conducted by Diversity Arrays Technology (DArT) Pty Ltd (Yarralumla, Australia) using the DArTseq assay. This analysis yields dominant silico-DArTs scored as presence–absence variation (PAV) and, in addition, co-dominant single nucleotide polymorphism (SNP) markers that can be scored in fragments that are present in all genotypes. For quality assurance, markers that were monomorphic, had more than 20% missing values or a minor allele frequency (MAF) of $< 5\%$ were not considered for further analyses. This resulted in 23,541 PAV and 7,070 SNP markers. As both marker types yielded similar results, all 30,611 markers were jointly used for the subsequent analyses (Fig. S2, available online). For 13,431 of these markers, a map position was known.

Molecular analyses

Relationships among the 184 genotypes were analysed by applying a principal coordinate analysis (PCoA) and a cluster analysis (Gower, 1966) based on Rogers' distances among the individuals (Wright, 1978). To assess the genetic diversity, the polymorphic information content (PIC) was calculated for every marker as follows:

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

where p_i^2 is the squared frequency of allele i at each locus.

LD was assessed by the LD measure r^2 (Weir and Cockerham, 1996). The association between LD and genetic map distance was assessed by fitting a curve by locally weighted regression to the r^2 values that were plotted against the genetic map distance. To obtain a threshold of r^2 above which LD was likely to be caused by genetic linkage, 986 equidistantly spaced markers were randomly sampled across all chromosomes and the 95th percentile derived from the distribution of the r^2 values of unlinked loci was taken as a population-specific critical value (Breseghello and Sorrells, 2006). To assess the LD along chromosomes, a sliding window approach with 5 cM windows at 500 positions along the chromosomes was used.

Calculations were performed with the open source programming language and statistical software 'R' (R Development Core Team, 2011). LD and PCoA computations were performed using the software package Plabsoft (Maurer *et al.*, 2008).

Results

In this study, a panel of 170 diverse winter durum lines of different origins and release dates, complemented by 14 spring durum lines, were genotyped by a GBS approach. After quality checks, a total of 30,611 polymorphic markers remained for subsequent analyses. These high-quality markers were equally distributed among the A and B genomes of durum with 5,849 markers on the A genome and 7,582 markers on the B genome (Table 1). Also, the 14 chromosomes were approximately equally covered with markers and the average marker density was 1.4 cM.

The principal coordinate analysis based on the Rogers' distances of the individuals showed that 20.5 and 9.7% of

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Table 1. Distribution of presence–absence variation (PAV) and single nucleotide polymorphism (SNP) markers and their average genetic map distance (in cM) on the 14 chromosomes

	A genome						
Chr	1	2	3	4	5	6	7
PAVs	489	622	567	695	438	601	708
SNPs	254	240	296	174	271	192	302
All	743	862	863	869	709	793	1010
Average distance	2.1	1.5	1.1	1.8	1.3	0.9	1.2
	B genome						
Chr	1	2	3	4	5	6	7
PAVs	935	1297	950	339	633	827	748
SNPs	332	374	309	118	246	255	219
All	1267	1671	1259	457	879	1082	967
Average distance	2.5	1.3	1.4	0.8	1.5	0.6	1.5

the total genetic variation could be explained by the first and second principal coordinates, respectively (Fig. 1(a)). The spring types grouped together, but nevertheless, the two growth types, spring and winter, were not clearly separated with regard to the first two principal coordinates. We defined five groups based on the country of origin of the lines: Central Europe (Austria and Germany), France, Northern America (Canada and United States), the Mediterranean countries (Italy and Spain) and Eastern Europe with the Black Sea area (Hungary, Bulgaria, Romania, Russia, Slovakia, Turkey and Ukraine; in the following Eastern Europe) (Fig. 1(b); for later discussions, Hungary is depicted separately). The Central European and the Eastern European groups were separated especially with regard to the first principal coordinate. In general, there appeared to be no major population structure as revealed by the violin plot visualizing the density distribution of the first ten principal coordinates (Fig. 1(c)). The cluster analysis, which depicts the genetic relationships based on the full molecular variance, confirmed the trend observed by the principal coordinate analysis that the Central European and the Eastern European lines are genetically distinct (Fig. 1(d) and Fig. S1, available online). However, this separation is not strict and some Eastern European lines also cluster among the Central European lines. Especially lines from Hungary build a large branch within the Central European group. Within the Eastern European lines, genotypes from one country tended to cluster together.

The allelic diversity was assessed by calculating the PIC for all markers (Fig. 2 and Table 2). The mean PIC value across all genotypes was 0.35 and chromosome 3B showed the lowest PIC value (0.31) and chromosome 2B the highest PIC value (0.38). For the A and B genomes, average PIC values of 0.34 and 0.35 were calculated. The winter durum lines had a mean PIC value of 0.34, whereas the spring types showed a slightly lower mean PIC value of 0.32. The PIC values also showed some variation along each of the chromosomes (Fig. 2).

LD was analysed across the genome, as well as along chromosomes. The 95th percentile of LD between unlinked markers was used as a population-specific threshold for LD due to linkage and equalled $r^2 = 0.07$ (Fig. 3). The LD decay was rather similar between all 14 chromosomes and intersected the threshold after approximately 2–5 cM. The extent of LD along chromosomes was analysed by assessing the LD between adjacent markers using a sliding window approach. This revealed that LD is variable on each of the 14 chromosomes (Fig. 4).

Discussion

The economic interest in an autumn-sown winter durum for Austria and Germany poses some challenges for plant breeding as winter hardiness including a good frost tolerance has to be combined with high quality and yield. Breeding of winter durum can profit from a molecular characterization of winter durum material. We therefore employed a GBS approach to analyse a diverse set of winter durum lines with regard to their genetic relatedness, population structure, allelic diversity and the extent of LD and discuss the consequences for durum breeding and future genomic research.

Genotyping-by-sequencing in winter durum

The utility of DArT markers for the analysis of genetic diversity has been shown in several crops, like bread wheat (Nielsen *et al.*, 2014), triticale (Badea *et al.*, 2011),

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barley (Zhang *et al.*, 2009), as well as spring durum (Laidò *et al.*, 2013; Ruiz *et al.*, 2013). Here, we employed a GBS approach that yields presence–absence variation markers like the classical DArTs and, in addition, SNP markers. The major difference, however, lies in the number of obtained polymorphic markers and thus in the coverage of the chromosomes. While this has only a small effect for some analyses, like genetic relationship analyses, it is a critical factor for others, for example, for association mapping. An advantage when compared with SNP arrays is that this

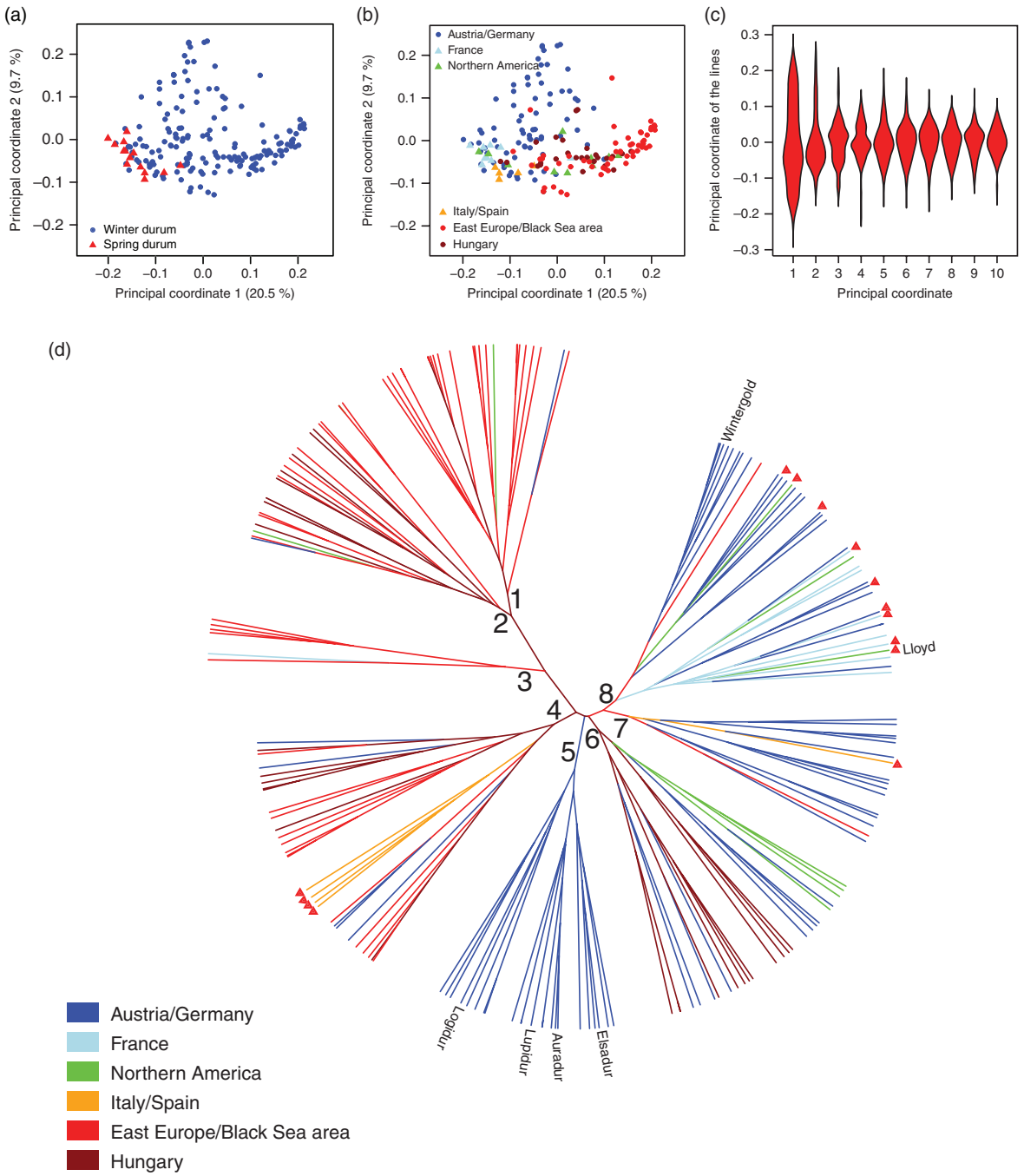

Fig. 1. Population structure and genetic relatedness assessed in the diverse durum wheat panel. (a and b) Principal coordinate analysis based on the Rogers' distances among all genotypes. Percentages in parentheses refer to the proportion of variance explained by the principal coordinate, (a) lines according to their growth type, (b) lines according to their country of origin, (c) violin plot showing the density distribution of the first ten principal coordinates, (d) dendrogram based on the genetic distances among all lines (spring durum lines are indicated by a triangle; population groups (dendrogram clades) are indicated by numbers and current varieties important for Austria and Germany are indicated by names).

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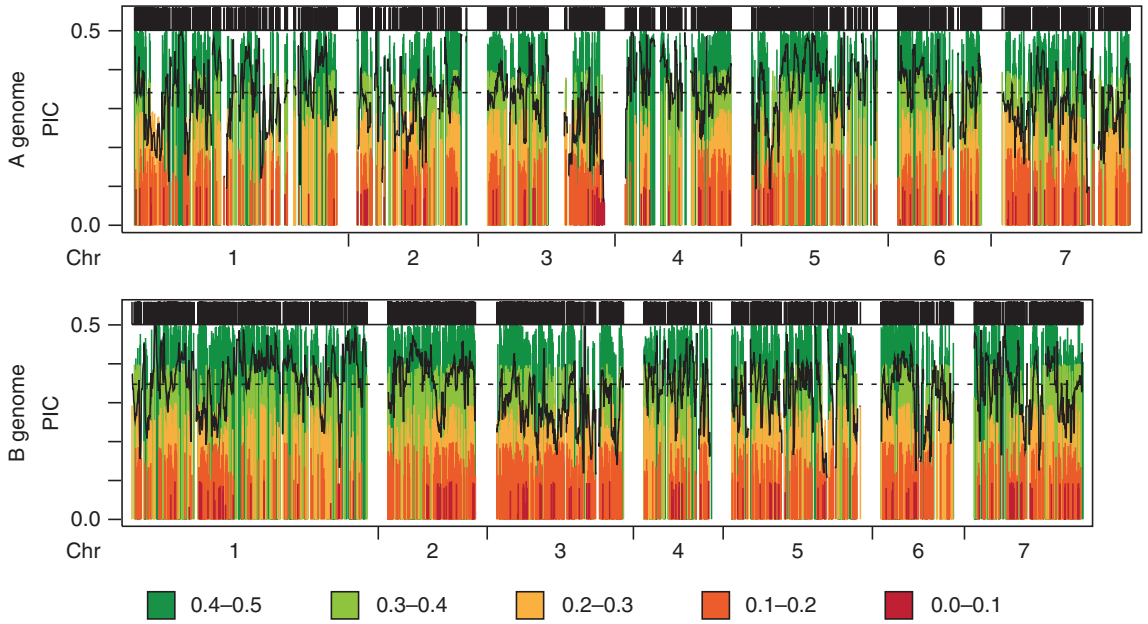

Fig. 2. Polymorphic information content (PIC) values along chromosomes (Chr) in winter durum wheat. Different colours indicate varying levels of the PIC. Black lines indicate the mean PIC value assessed by a sliding window approach and the dashed line represents the mean PIC value across all chromosomes. The black bars above each plot indicate the positions of the markers used for the analysis.

approach does not suffer from ascertainment biases, i.e. a prevalence of SNPs on the array that are based on a discovery panel of a small number of individuals from selected populations but which may not be representative and informative in the studied population. The same GBS approach has recently been used to characterize bread wheat (Würschum *et al.*, 2015) and Central European soybean germplasm yielding promising results (Hahn and Würschum, 2014).

We observed an equally high number of markers for both the A and the B genomes (Table 1). This is in agreement with the results from wheat showing a reduced degree of polymorphism for the D genome but no major difference between the A and the B genomes (Akhunov *et al.*, 2010). Furthermore, the chromosomes were all covered with markers without major gaps that would indicate monomorphic regions. Thus, winter durum does not appear to have gone through a bottleneck that would have resulted in a severe reduction of the degree of polymorphism and consequently in monomorphic chromosomal regions. Taken together, the high coverage of the entire genome with polymorphic markers makes this dataset ideally suited for a molecular analysis of winter durum.

Genetic diversity and implications for breeding programmes

The principal coordinate analysis revealed no separation of winter and spring types (Fig. 1(a)). This is in contrast to bread wheat and triticale where the two growth types were clearly separated by the first principal coordinate (Chao *et al.*, 2010; Alheit *et al.*, 2012). Our findings can have several reasons. First, the number of spring types included in this study was low and might not be representative. Second, the lack of winter and spring grouping may indicate frequent crosses and thus the exchange of diversity between spring and winter types in durum wheat. Third, it may reflect that spring durum was initially used to establish the founder lines in combination with the rather short breeding history of winter durum which has not yet resulted in a clear divergence of winter from spring types.

Table 2. Polymorphic information content (PIC) values for each chromosome shown for all lines, winter types (WD) and spring types (SD)

	A genome						
Chr	1	2	3	4	5	6	7
All lines	0.34	0.33	0.32	0.36	0.37	0.37	0.32
WD	0.34	0.33	0.32	0.36	0.37	0.37	0.32
SD	0.30	0.35	0.32	0.34	0.29	0.33	0.30
	B genome						
Chr	1	2	3	4	5	6	7
All lines	0.36	0.38	0.31	0.35	0.35	0.35	0.35
WD	0.35	0.37	0.31	0.35	0.34	0.35	0.33
SD	0.32	0.31	0.30	0.29	0.33	0.29	0.37

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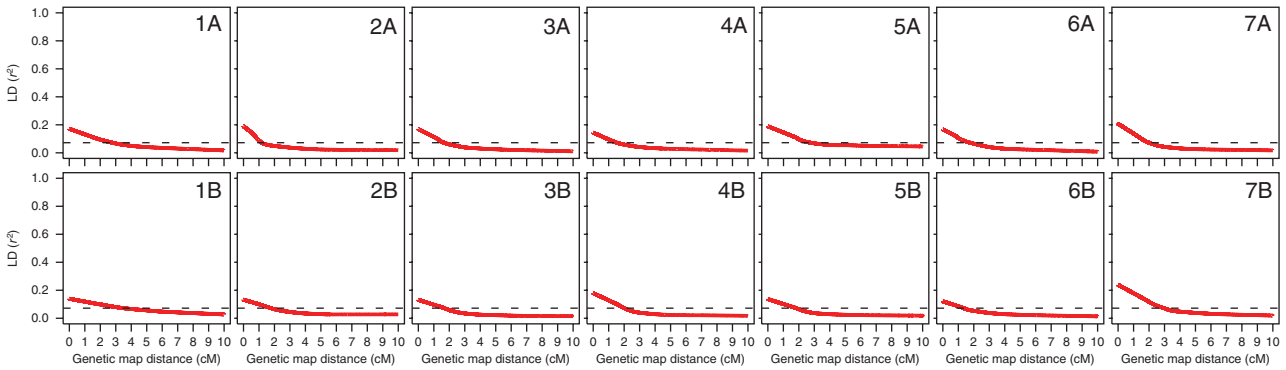

Fig. 3. Extent of linkage disequilibrium (LD) in winter durum wheat. The red curve was fitted by robust locally fitted regression and shows the decay of LD with genetic map distance for all 14 chromosomes. The dashed horizontal line indicates the population-specific threshold for LD due to linkage.

In general, the principal coordinate analysis revealed absence of a major population structure. This was also observed in bread wheat, which can be explained by the breeding history of bread wheat and the constant exchange of breeding lines between breeders and countries preventing the formation of subgroups (Chao *et al.*, 2010; Würschum *et al.*, 2013). Consequently, in a bread wheat analysis based on SNP markers, the first principal coordinate explained only 5.1% of the genetic variance. By contrast, in our winter durum analysis, the first principal coordinate explained 20.5% of the variance indicating a certain grouping of lines in subpopulations. An analysis based on elite spring durum material also reported a certain grouping of lines according to their origin (Maccaferri *et al.*, 2005).

This conclusion was substantiated by the cluster analysis, which in contrast to single principal coordinates exploits the entire genetic variation. This analysis revealed a clustering of the lines with regard to their origin into eight dendrogram clades, i.e. eight groups (Fig. 1(d) and Fig. S1, available online). Roughly, groups 1–3 reflect old varieties from Eastern Europe with a slight separation into Ukrainian or Russian origin (group 1) and old Hungarian origin (group 2). Most of this material has a very good frost tolerance but a rather poor quality. Modern elite winter durum

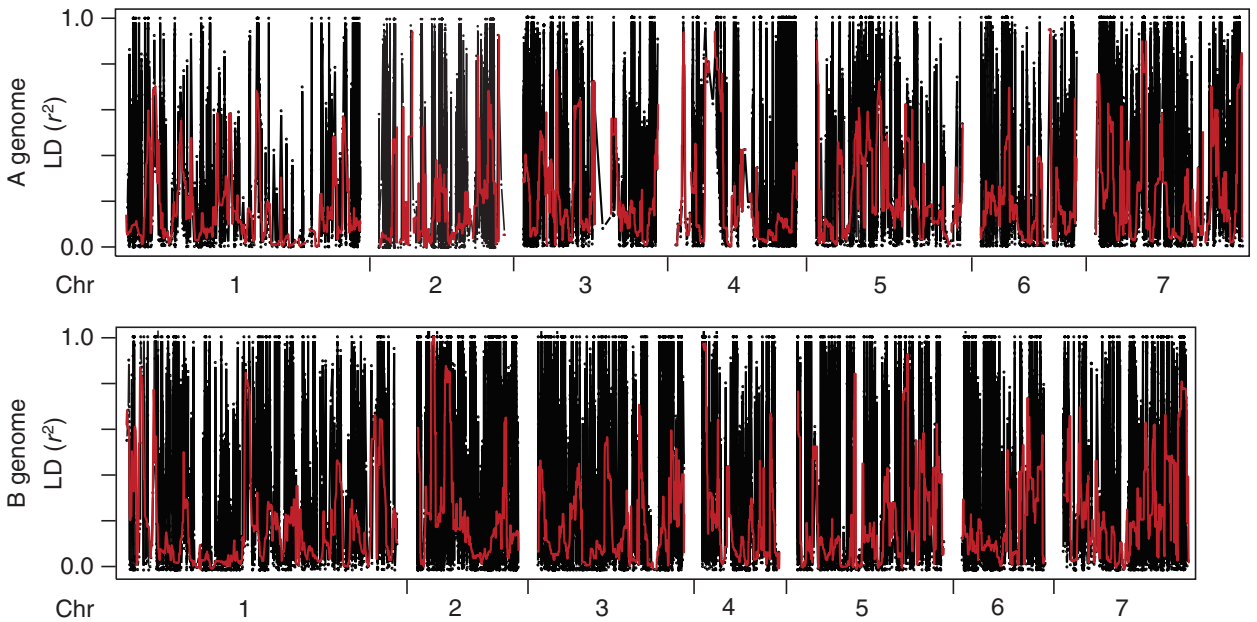

Fig. 4. Distribution of linkage disequilibrium (LD) along the chromosomes in winter durum wheat. Black points show the LD of each locus with its neighbouring locus. The red line represents the median of r^2 values calculated by a sliding window approach within a 5 cM window at 500 positions along the chromosomes.

https://doi.org/10.1017/S1479262115000349 Published online by Cambridge University Press

breeding material can mostly be found in groups 5–8. In group 8, mostly German elite winter durum breeding lines cluster together with elite spring durum varieties. This is not surprising, because the breeding strategy of the University of Hohenheim was to introgress quality from elite spring durum into winter durum material followed by strict selection on winter hardiness and quality. As a result, the only yet registered winter durum variety in Germany 'Wintergold' clusters in this group. Clearly separated from this group are the elite winter durum varieties from Austria found in group 5. Thus, it would be of high interest to perform crosses between groups 5 and 8, which have already been started resulting in the lines of group 6. In addition to German and Austrian elite durum lines, recent elite breeding lines from Hungary are also in group 6. For several decades, the University of Hohenheim (Germany), Saatzucht Donau (Austria) and the Hungarian Academy of Science in Martonvasar (Hungary) intensively exchanged durum material, which resulted in the lines clustering in group 6. Group 7 is breeding material from the University of Hohenheim, which based on pedigree records traces back to the old French spring durum variety 'Eurodur' (not included in this study). Also mentioned as an important founder line should be 'Hordeiforme 1/4' (also known as 'Kharkovskaya 1'; not included in this study). This non-registered variety from the Ukraine is characterized by good winter hardiness and has been used for many pedigrees (groups 1–4). Other important founder lines are 'Parus' and the younger variety 'Alyi Parus', both including 'Hordeiforme 1/4' (Fig. S1, available online).

Based on the short breeding history and the strict selection for winter hardiness, it could have been suspected that winter durum possesses only a small genetic basis and consequently a reduced diversity. This, however, does not seem to be the case. While the limited number of spring types does not allow drawing conclusions on the diversity in winter durum when compared with that in spring durum, but the analysis nevertheless illustrates the availability of a substantial diversity in winter durum. This indicates that there is a high potential for crosses within winter durum to recombine and exploit the available diversity.

The identified diverse subgroups might also be of interest for hybrid breeding. In bread wheat, one major research attempt is currently to evaluate the potential shift from line towards hybrid breeding (Longin *et al.*, 2012). To facilitate hybrid breeding, distinct heterotic groups of lines are of high importance. Thus, before further mixing the elite winter durum material, it should be decided whether to continue line breeding, where mixing of groups is advantageous, or if the existing groups should be exploited for hybrid breeding. For Central Europe, our cluster analysis would suggest a heterotic pattern of group 5 and group 8, which are clearly separated and both consist of latest elite winter durum lines. Although groups 1–4 would be more distant, their poor quality hinders their use as heterotic group for Central European hybrid breeding. However, in regions, where quality requirements are not that high, a heterotic pattern consisting of groups 1 and 2 versus groups 4–8 would be of high interest.

Patterns of allelic diversity and LD in winter durum

The mean PIC value across all genotypes was 0.35 and, for individual chromosomes, ranged from 0.31 to 0.38 (Table 1). Similar mean PIC values were found for bread wheat (0.37), triticale (0.37), rye (0.34) and barley (0.38), also using DArTs (Wenzl *et al.*, 2004; Badea *et al.*, 2011). A much higher mean PIC value of 0.61 was observed by Mondini *et al.* (2010) for Ethiopian durum wheat landraces assessed with SSR markers. It must be noted, however, that PIC values have a different range for multi-allelic markers like SSRs compared with biallelic markers and are therefore not comparable. The slight differences in the PIC values between the winter and spring types might reflect different breeding goals in the two groups but may also be an artefact due to sample size and thus require further research. The variable PIC values along the chromosomes as visualized by the sliding window approach (Fig. 2) may reflect the effects of adaptation and selection as well as the introgression of diversity from other germplasm.

A detailed knowledge on the LD present in a population is a prerequisite for association mapping, which relies on LD between markers and the QTL. The decay of LD with genetic map distance thereby indicates the mapping resolution that can be achieved but also the marker density that is required to cover the genome. A fast decay of LD is advantageous in that it enables a higher mapping resolution but also requires more markers to cover the genome without gaps (Myles *et al.*, 2009; Würschum, 2012). We found that in winter durum, LD decayed below the population-specific threshold after approximately 2–5 cM (Fig. 3). Similar results were found in spring durum wheat by Somers *et al.* (2007). By contrast, bread wheat showed a slightly slower decay within 5–10 cM (Würschum *et al.*, 2013), which may indicate a broader diversity in winter durum when compared with elite bread wheat panels. The variability of LD along chromosomes has also been reported in other crops such as sugar beet (Würschum *et al.*, 2011), wheat (Würschum *et al.*, 2013) and soybean (Hahn and Würschum, 2014) and can, for example, reflect the effects of selection or the introgression of favourable genes from

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other germplasm sources. Our results are promising for association mapping in winter durum and suggest that, with the high marker density provided by the GBS approach in combination with the observed extent of LD, high-resolution association mapping is feasible.

Conclusions

The Central European market for durum wheat would profit from an autumn sowing as this is beneficial for plant development and maturity resulting in an increased yield and, in addition, increases quality as rains before harvest can often be avoided. Breeding of winter durum is still at its beginning and can therefore profit from a molecular characterization of winter durum wheat. In this study, we used a panel of 170 diverse winter durum lines and employed a GBS approach generating an unprecedentedly high number of genome-wide markers. We show that while no major population structure exists in this panel of winter durum, lines from different origins tend to cluster together which is in line with their different breeding history. The results on the genetic relationships may assist targeted crosses, for example to further improve the winter hardiness in the Central European lines. Our results further revealed that winter durum possesses a substantial genetic diversity that can be exploited by breeding. The analysis of LD showed that high-resolution association mapping is possible in this panel, which is promising for the identification of QTL for important agronomic traits towards a knowledge-based breeding of winter durum.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1479262115000349

Acknowledgements

This research was funded by the Deutsche Forschungsgemeinschaft (Grant ID: LO 1816/2-1). We thank Dr Olga Liapounova (Vavilov Research Institute of Plant Industry, St. Petersburg, Russia), Dr Carl A. Griffey (Virginia Tech Inc.), Dr Julia Lafferty (Saatzucht Donau), Edgar Müller (Südwestdeutsche Saatzucht) and Dr Gyula Vida (Hungarian Academy of Sciences) for seeds as well as Angela Harmsen for excellent technical assistance in the laboratory and Bernd Habeck, Bianca Yildirim, Verena Till, Sabine Frey-Tippelt, Sabit Recaj, Ortwin Schildmann, Marcel Mosdorf and Martina Schnieder for outstanding work in the field.

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