An unmöglichen Dingen soll man selten verzweifeln, an schweren nie.

Johann Wolfgang von Goethe

Cytogenetic mapping of BAC contigs assigned to barley chromosome 3H and comparative subchromosomal analysis within the genus *Hordeum*

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Alexa 488	Alexa Fluor 488 dye, a bright green-fluorescent dye
Atto 550	Atto 550 dye, is a novel cationic orange-fluorescent dye manufactured by Atto-TEC GmbH in Siegen, Germany
BAC	bacterial artificial chromosome
cDNA	complementary DNA
d-UTP	deoxyuridine triphosphate
DAPI	4´, 6´-diamidino-2-phenylindole
fl-cDNA	full-length complementary DNA
FISH	fluorescence *in situ* hybridization
FPcontig	finger printed contig
gDNA	genomic DNA
mya	million years ago
NOR	nucleolus organizer region
PCR	polymerase chain reaction
rDNA	ribosomal DNA
TexasRed	sulforhodamine 101 acid chloride, a red-fluorescent dye

1. Introduction

1.1 Barley as a crop and model

Barley *(Hordeum vulgare)* has very long cultivation history and is known to humanity since ancient times. Several archaeological evidences indicate that this important crop was domesticated already 10,000 years ago (Badr *et al.* 2000). Although barley has lost its importance in the human diet, the popularity of it today is higher than ever.

Barley has become an important animal fodder and has found a wide application in the malting, brewing and distilling industries. Additionally to the role as an agricultural crop, barley has gained a model character for other Triticeae genomes and is frequently used for cytological and genomic studies. Due to the high application levels and important features, including tolerances to water stress, cold and alkali, the grain of barley is one of the best-studied materials in cereal crops (Gubatz *et al.* 2007). Barley mutation breeding programs started already in 1927 with ionizing radiation and chemical mutagen treatments. Later, new cultivars have been developed by conventional crossing and resulted in reduced diversity of commercially used cultivars (Horvath *et al.* 2001).

Barley is a large genome species (> 5.1 Gbp) which belongs to the Triticeae tribe together with rye (*Secale cereale*), bread wheat (*Triticum aestivum)* and various wild species. The genus *Hordeum* is very particular among Triticeae as it has both annual plants, such as *H. vulgare* and *H. marinum* and perennial species, such as *H. bulbosum and H. pubiflorum* (Fig. 1). *Hordeum* genus contains 32 species, all having x=7 as a basic number of chromosomes. Cultivated species and its ancestor, *H. vulgare* and *H. vulgare spp. spontaneum,* are diploid and possess 2n=14 chromosomes. Two Mediterranean species, *H. marinum* and *H. murinum*, are annual and closely related to each other, but seem to be different from other *Hordeum* genomes (Blattner 2004) (Figure1).

Hordeum genus has three different gene pools, which were used for expanding of the reduced genetic diversity in different approaches. The primary gene pool includes *H. vulgare spp. vulgare* and wild barley (*H. vulgare ssp. spontaneum*) that has been crossed with cultivated barley to achieve disease resistance and tolerance to abiotic stress. The secondary gene pool is represented by *Hordeum bulbosum.* The only member of the genus included in production of doubled haploid barley. The tertiary gene pool consists of 30 *Hordeum* species, but crossing attempts with cultivated barley were not successful.

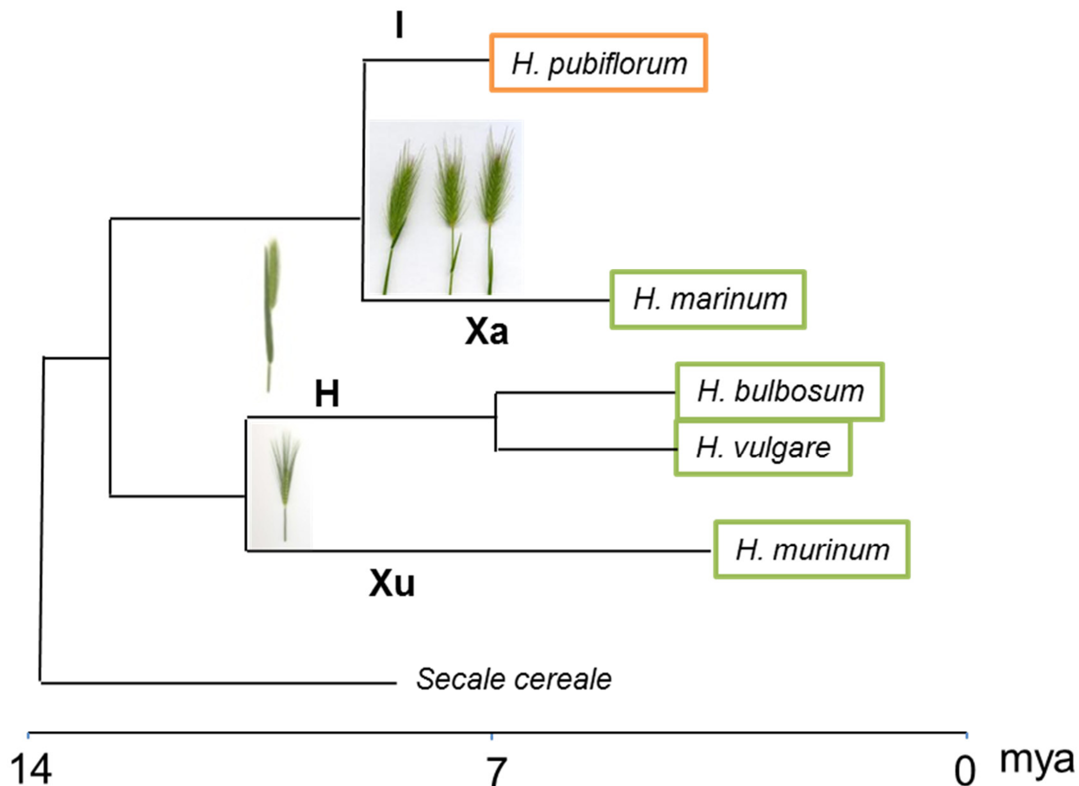


Figure 1 Phylogenetic tree of the genus *Hordeum* showing four subclades with a single representative species each, respectively. Patagonian species (orange square), Mediterranean and Western Eurasia species (green square), (Brassac and Blattner, 2015, modified).

Barley can be reproduced in two primary ways: vegetative and generative (sexual) reproduction. Vegetative spread of barley is however limited. One possibility is a production from rooted tillers (Morrall and Briggs 1978). Sexual reproduction, connected to flowering, depends on several factors. Firstly it is necessary to initiate the floral development by vernalisation (cold stimulus) particularly in winter barley. Spring barley flowers without vernalisation. The second factor is the day length with long days advancing floral development and the third is the optimal growth temperature that can vary within different cultivars between 15°C - 30°C. Flowering starts from the generative apical meristem and takes place before head emergence from the boot, leading to the inflorescence – to spike. Barley cereals include both, closed-flowering and open-flowering. The closed-flowering type has anthers that remain inside each floret, so that self pollination occurs. In open-flowering types, anthers are not completely inside the floret, hence out-crossing is possible. Most of the barley varieties are self-pollinating types.

1.2 Organization of the barley genome

H. vulgare is diploid (2n=14) and possesses relatively large chromosomes, which is quite typical across the genus. Chromosomes are predominantly metacentric. Arm ratios vary from 1 to 1.5 (Marthe and Kunzel 1994). Two satellite chromosomes (5H and 6H) carry the nucleolus organizer regions (NORs). Long and short arms are distinguishable according to their physical lengths and abbreviated as l (long) and s (short). Current chromosome numbering system was established in course of the barley chromosome homoeology study in the wheat genome (Powling *et al.* 1981).

The chromosome staining technique called Giemsa C-banding makes areas of constitutive heterochromatin visible. Using C-banding all seven pairs of chromosomes of barley were identified (Kakeda *et al.* 1991). Eight tandem array trinucleotides also known as SSR markers ($(AAG)_5$, $(AGG)_5$, $(AAC)_5$, $(ACT)_5$, $(CAT)_5$, $(GTG)_5$, $(ACG)_5$ and $(CAG)_5$) were tested by FISH analysis showing a distribution similar to C-banding sites of barley, excluding $(ACT)_5$ that revealed an euchromatic distribution (Cuadrado and Jouve 2007). FISH signals on barley chromosomes were first obtained by hybridization of 45S rDNA (pTa71), 5S rDNA (pTa794), HvT01 probes and Afa-family repetitive sequences (pHvA14) (Heslop-Harrison *et al.* 1992, Leitch and Heslop-Harrison 1992, Leitch and Heslop-Harrison 1993, Schubert *et al.* 1998, Tsujimoto *et al.* 1997). Later application of FISH unveiled further genomic information. Other repetitive DNA probes (pHv-365, pHv-177, pHv-1112, pHv-689, pHv-1476, pHv-1889 and pHv-1972) revealed by sequencing analysis of barley BACs resulted in specific patterns on barley chromosomes. Clones pHv-38 (5S rDNA), pHv-365, pHv-961 (HVT01) together with two microsatellites, $(GAA)_5$ and $(TAG)_5$, allowed a distinct recognition of all chromosomes in different cultivars of barley (Kato 2011). Repetitive DNA probes of barley with corresponding signals are summarized in Table 1. Barley chromosome-specific low- and single-copy sequences are described in chapter 1.4 (different strategies of physical mapping) and are summarized in Table 2.

Table 1 Repetitive DNA probes of barley with corresponding chromosomal positions

Probe	Chromosomal position	Chromosome	Reference
pTa71 (45S rDNA)	intercalar	1H, 2H, 5H, 6H, 7H	(Leitch and Heslop-Harrison 1992)
pTa794 (5S rDNA)	subterminal, intercalar	2Hl, 3Hl, 4Hl, 7Hs	(Leitch and Heslop-Harrison 1993)
HvT01	subtelomeric	1H to 7H on short and long arms	(Schubert *et al.* 1998)
pHvA14 (Afa-family)	distal	1H to 7H	(Tsujimoto *et al.* 1997)
GAA microsatellite	pericentromeric	1H to 7H	(Pedersen *et al.* 1996)
pHv-365		1H, 2H, 4H, 6H	(Kato *et al*. 2011)
pHv-177	distal and centromeric	1Hs	(Kato *et al*. 2011)
pHv-1112	distal	1Hl	(Kato *et al*. 2011)
pHv-689	pericentric	1H to 7H	(Kato *et al*. 2011)
pHv-1476	pericentric	1H to 7H	(Kato *et al*. 2011)
pHv-1889	pericentric	1H to 7H	(Kato *et al*. 2011)
pHv-1972	pericentric	1H to 7H	(Kato *et al*. 2011)
$(TAG)_5$	polymorphic subtelomeric	4H, 6H, 5H 2Hl, 3Hl	(Cuadrado and Jouve 2007)
$(AAG)_5$	pericentromeric	1H to 7H	(Cuadrado and Jouve 2007)
$(AGG)_5$	pericentromeric	1H to 7H	(Cuadrado and Jouve 2007)
$(AAC)_5$	pericentromeric	1H to 7H	(Cuadrado and Jouve 2007)
$(ACT)_5$	intercalar/ telomeric/ subtelomeric	2H, 3H, 4H, 5H, 6H	(Cuadrado and Jouve 2007)
$(CAT)_5$	pericentromeric	4H and 5H	(Cuadrado and Jouve 2007)
$(GTG)_5$	centromeric	1H to 7H	(Cuadrado and Jouve 2007)
$(ACG)_5$	centromeric	1H to 7H	(Cuadrado and Jouve 2007)
$(CAG)_5$	centromeric	3H and 4H	(Cuadrado and Jouve 2007)

The genome of barley is 12 times larger than the rice genome and possesses over 80% of repetitive DNA (Sandhu and Gill 2002). Sequencing of the entire genome as performed in small-genome species is not feasible in barley, because both the high content of repeats and the large genome size would disturb the analysis. A significant contribution to the knowledge of the genome organization has been delivered by the combination of chromosome sorting and next generation sequencing techniques (NGS) to establish a virtually ordered gene inventory of 1,987 anchored genes of chromosome 1H (Mayer *et al.* 2009). Additionally, it was shown that the collinearity among homoeologous chromosomes in barley, rice and sorghum was about 36.2% on the gene level. In 2012, the International Barley Sequencing Consortium made a big step forward, establishing a physical map of 4.98 Gb with 4,556 BAC contigs anchored to the genetic map of barley representing a cumulative length of 3.9 Gb. 48% or 1.9 Gb of the physical map was assigned to the region exhibiting reduced recombination frequency standing for centromeric and pericentromeric regions (International Barley Genome Sequencing *et al.* 2012). The study confirmed that 84% of the genome contains mobile elements or other repeat sequences. 76% is represented by retrotransposons. LTR (long terminal repeat) retrotransposons (99.6%) strongly outnumber non LTR-retrotransposons (0.3%). The LTR *Gypsy* retrotransposon superfamily is of higher abundance than the *Copia* superfamily. *Copia* elements were frequently found in gene-bearing BACs, in contrast, *Gypsy* retroelements were absent in recombinogenic regions. One of the centromere enriched repeats is a *Ty3/Gypsy*-like element known as *cereba* element (centromeric retroelement of barley). Estimates suggested about 200 copies of *cereba* elements per centromere of each barley chromosome totaling at least 1.4 Mb of the centromeric DNA sequence (Hudakova *et al.* 2001, Presting *et al.* 1998).

Comparison against genes of *Arabidopsis thaliana* indicates that the barley genome encodes approximately 30,400 genes. Parallel analysis based on homology to other grass species allowed identifying 26,159 as high-confidence genes, which are associated and anchored to the physical map. 53,220 transcript loci lacked a homology support from one of the reference genomes and were considered as low-confidence genes. Terminal parts of the chromosomes are more gene-rich, but a large number (>30%) of functional genes are also present in recombinationally cold genomic regions (IBSC, 2012).

Like in all eukaryotic organisms DNA of barley is folded into chromatin, which is post-translationally modified. Most of the histone modifications are similarly distributed among different plant species, while others can differ (Fuchs *et al.* 2006). Histone H3K4me1, 2, 3 and H3K36me1, 2, 3 are euchromatic histone marks conserved in plants. In contrast, modifications of H3K9, H4K20 and H3K27 are heterochromatin specific and can vary in distribution between different plant species. In *H. vulgare* H3K4me 1, 2, 3 modifications correlate with euchromatic

pole of the nuclei in interphase and label gene-rich terminal parts of metaphase chromosomes. Distribution of some histone marks can be genome size dependent, as for instance, a di-methylation of H3K9. In species with large genomes H3K9me2 signals are uniformly distributed over the entire interphase nucleus (Houben *et al.* 2003), in contrast in small-genome species this mark is restricted to constitutive heterochromatin. In barley H3K9me2 shows the typical `heterochromatin-specific` distribution of large genome species, staining interphase chromatin and metaphase chromosomes uniformly.

1.3 Genetic maps of barley

The first genetic map was constructed in 1913 by the outstanding scientist Alfred Sturtevant. This map was based on the recombination events between different traits in the genome of *Drosophila melanogaster* (Sturtevant and Morgan 1923). Standard linkage analysis is illustrated by the three-point testcross approach, where a triple heterozygote is crossed with a triply recessive homozygote. All phenotypes of the progeny are analysed. The most frequent phenotypes are parental and the least common are double-crossover phenotypes. To determine the order and distances of loci on the map, percentage of offsprings with different traits is calculated. Originally, markers taken for the map analysis were visual on the phenotypic level. In contrast, markers referring to non coding DNA such as microsatellites and SNP markers are frequently used nowadays. Recombination frequency is usually measured with the aid of the statistical test called LOD score, logarithm of the odds (Morton 1996). This test compares the status of likelihood of the test data of two loci in case of the linkage to the status of the likelihood of the same data occurred randomly. LOD score that is positive is interpreted as a linkage, negative LOD shows an absence of linkage between loci. Equation for the calculating of LOD score is following:

$$LOD= \log_{10} \frac{\theta^{R} (1- \theta)^{NR}}{(\Theta=0,5)^{R+NR}}$$

Θ - recombination factor
R - recombinant
NR - non recombinant

One type of the genetic linkage mapping is so called quantitative trait loci (QTL) mapping that involves identification of quantitative trait loci. Data of genetic markers are used to construct a linkage map. Identification of genes controlling QTLs is a result of combination between

marker and phenotypic data. Questions corresponding to the quantity of QTLs, to the location and interaction of loci are of the interest for such kind of mapping. Aim of the QTL map is to group loci into candidate chromosome segments and arrange the loci within linkage groups.

Three points of construction, referring to the arrangement of loci are: grouping, ordering, and distance estimation (Kahl 2005). Ordering of locus is a central point of mapping, which can be achieved by different approaches. Simplest algorithms starts with most tightly linked locus pair and put other loci outward of it. One of the well-established algorithms for this purpose is applied for computer package MAPMAKER (Lander and Green 1987). Other used computer software products are e.g. Map Manager QTX, CarthaGene or MultiPointULD. The development of new strategies for algorithms' updating for new computer programs is ongoing.

While constructing the genetic linkage map additional criteria such as epistasis of genes, recombination suppression and chromosomal aberrations are not taken into consideration. The effect of double crossover mainly results in no recombination and therefore leads together with mentioned points to underestimation of distances between two traits. Various genetic maps were constructed for barley in the last two decades and genetic information from different maps was used for the anchoring to the physical map. There are more than ten genetic (Becker and Heun 1995, Close *et al.* 2009, Komatsuda and Mano 2002, Nasuda *et al.* 2005, Poland *et al.* 2012, Qi and Lindhout 1997, Sato *et al.* 2009, Stein *et al.* 2007, Varshney *et al.* 2006) maps of barley, constructed with the help of different molecular markers. First incomplete barley restriction fragment polymorphism map was published in 1988 (Melzer *et al.* 1988).

A 1000 loci transcript map of the barley genome has been generated using 1,032 EST (expressed sequence tags)-based markers. 179 EST-based markers could be arranged into seven barley linkage groups (Stein *et al.* 2007). This study has been applied on three double haploid (DH) populations. Two years later 2,890 PCR-based ESTs were mapped using a single double haploid mapping population (Sato *et al.* 2011) only. This work involved about 9% of barley genes. In 2011 an improved consensus linkage map of barley was published (Munoz-Amatriain *et al.* 2011) reporting 2,994 single nucleotide polymorphism (SNP) markers mapped to 1,163 unique positions. These results were gained using ten mapping populations. The most recently developed map is so called high-density genetic map. A relatively new method known as genotyping-by-sequencing (GBS) was used in the course of construction. Bi-parental barley population was genotyped by mapping of 34,000 SNPs and 240,000 tags onto the Oregon Wolfe barley reference map (Poland *et al.* 2012). And finally, numerous contigs of barley have been ordered along the "iSelect" map (Comadran *et al.* 2012). Applying recently developed POPSEQ approach 498,856 contigs with a cumulative length of 927 Mb

were anchored and ordered in a *de novo* NGS contig assembly (Mascher *et al.* 2013). Few other maps are yet in the process of development.

1.4 Different strategies of physical mapping

In the last two decades different approaches of physical mapping were applied on barley. Mapping through the application of chromosome deletion lines (Serizawa *et al.* 2001), physical mapping by application of reciprocal translocation lines (Kunzel *et al.* 2000, Marthe and Kunzel 1994, Sorokin *et al.* 1994) or utilization of wheat-barley addition lines (Islam and Shepherd 2000, Naseda *et al*. 2005) are among some strategies used to physically map the barley genome. However, these strategies are limited to only same regions of the barley genome. This is because not all chromosomal regions such as centromeric regions are covered by these approaches (e.g. application of deletion lines).

To overcome this limitation FISH could be used. Indeed, for some so-called small genome species (<1Gb) such as *Arabidopsis* (Fransz *et al.* 1998), *Brachypodium* (Hasterok *et al.* 2006) or rice (Cheng *et al.* 2002, Gong *et al.* 2011, Jiang *et al.* 1995) the integration of physical and genetic maps was successfully performed with the aid of FISH. The best optimization of FISH was reached in the area of repeat sequence localization and determination of landmarks on the chromosome pairs. Single-copy hybridization remains however a challenge, since short sequences (<5 Kb) such as cDNA are not always detectable and too long sequences (BACs) contain repeats. As small genome species harbor only small amounts of repetitive sequences it is possible to avoid these sequences efficiently by blocking (e.g. unlabelled Cot-100 fraction) (Szinay *et al.* 2008). Attempts to map bacterial artificial chromosomes (BACs) in large genome species by FISH has led to the cross-hybridization with all chromosomes, however unlabelled *Cot* fractions were not applied in this study (Suzuki and Mukai 2004, Zhang *et al.* 2004).

In barley, till now only nine preselected BAC clones and twenty seven cDNA clones were effectively mapped and integrated with the linkage map of barley (Karafiatova *et al.* 2013, Lapitan *et al.* 1997, Ma *et al.* 2010, Phillips *et al.* 2010, Stephens *et al.* 2004). Stephens et al. (2004) applied ultrasensitive FISH for hybridization of single-copy cDNA probes. Indirect tyramide signal amplification (TSA) enhanced the intensity of the hybridization signals. Fourteen cDNA probes were distinguishable after FISH. Eleven of these probes (Table 1) were assigned to a unique position on homologous chromosomes. In cytogenetic study that has been undertaken by Phillips et al. 2010 fifteen genetically anchored and 90 unanchored BACs were identified by a Southern dot blotting as BACs with a low repeat content. All 105 BACs were used as FISH probes. These experiments yielded in four anchored and three unanchored BACs having a single locus in genome while remaining 98 probes landed on

multiple loci or were not detectable. BACs were assigned to five out of seven chromosome pairs. Recently, Karafiatova et al. (2013) determined the relative order of thirteen cDNAs via FISH mapped on mitotic pro-metaphase chromosome 7H of barley. In addition, during the study of synteny relationship between barley and *Brachypodium, a* so called “bridge species”, serving as a model to analyze more complex cereals, two out of thirteen *Brachypodium* BACs revealed FISH hybridization signals on barley chromosomes (Ma *et al.* 2010). Barley chromosome-specific low- and single-copy sequences used for FISH are summarized in Table 2.

Table 2 Barley-specific low- and single-copy sequences

Probe	Position	Chromosome ID	reference
Adh	subtelomeric	3Hs, 4Hs, 5Hs	Stephens *et al.* 2004
Ale	intercalar	5Hl	Stephens *et al.* 2004
Amy 1	distal	6Hl	Stephens *et al.* 2004
Amy 2	intercalar	7Hl	Stephens *et al.* 2004
Brz	subtelomeric	7Hs	Stephens *et al.* 2004
Chi	subtelomeric	7Hl	Stephens *et al.* 2004
Chs	distal	2Hs	Stephens *et al.* 2004
Dhn 6	subtelomeric	4Hs	Stephens *et al.* 2004
Glb 3	subtelomeric	3Hs	Stephens *et al.* 2004
Glx	subtelomeric	7Hs	Stephens *et al.* 2004
His 3	subtelomeric	4Hs, 6Hs, 7Hs	Stephens *et al.* 2004
Nar 1	subtelomeric	6Hs	Stephens *et al.* 2004
Nar 7	subtelomeric	6Hl	Stephens *et al.* 2004
Ubi	subtelomeric	6Hs, 7Hl	Stephens *et al.* 2004
BAC 0356N21	distal	1Hs	Phillips *et al.* 2010
BAC 0146M01	distal	6Hl	Phillips *et al.* 2010
BAC 026D09	subtelomeric	2Hl	Phillips *et al.* 2010
BAC 0047K17	distal	4Hl	Phillips *et al.* 2010
FLbaf140k15	pericentromeric	7Hs	Karafiatova *et al.* 2013
FLbaf67j12	pericentromeric	7Hs	Karafiatova *et al.* 2013
FLbaf140c21	pericentromeric	7Hs	Karafiatova *et al.* 2013
FLbaf104j18	pericentromeric	7Hs	Karafiatova *et al.* 2013
FLbaf151b16	centromeric	7Hs	Karafiatova *et al.* 2013
FLbaf169o18	centromeric	7Hl	Karafiatova *et al.* 2013
FLbaf125j04	pericentromeric	7Hl	Karafiatova *et al.* 2013
FLbaf54a18	pericentromeric	7Hl	Karafiatova *et al.* 2013
FLbaf148b24	pericentromeric	7Hl	Karafiatova *et al.* 2013
FLbaf24d09	pericentromeric	7Hl	Karafiatova *et al.* 2013
FLbaf25l12	pericentromeric	7Hl	Karafiatova *et al.* 2013
FLbaf129g09	pericentromeric	7Hl	Karafiatova *et al.* 2013
FLbaf89h06	pericentromeric	7Hl	Karafiatova *et al.* 2013
FLbaf107j09	pericentromeric	7Hl	Karafiatova *et al.* 2013
FLbaf175h04	pericentromeric	7Hl	Karafiatova *et al.* 2013

BAC 58H2	subtelomeric	7Hs	Ma *et al.* 2010
BAC 63E11	distal	7Hl	Ma *et al.* 2010
FPc678	distal	2Hl	Schmutzer *et al.* 2014
FPc38863	distal	2Hl	Schmutzer *et al.* 2014

In silico extraction of unique genomic sequences of large genomic fragments suitable for FISH is a new tool to overcome the problem of cross-hybridization in large genome species like barley (Schmutzer *et al.* 2014) (Figure 2). Eight predefined fragments derived from contigs of chromosome 2H were successfully attached to a single locus respectively. Unique hybridization signals were detected on the short arm of 2H chromosome of *Hordeum vulgare* cv. Morex. Extracted genomic single copy fragments were at least 4 Kb long (Ma *et al.* 2010). However, an optimal length for FISH is about 7 Kb. The method of *in silico* extraction of single copy sequences can be widely used for the integration of genetic and cytogenetic maps in species with large and complex genomes if the sequence of the target sequence (e. g BACs, sequence contigs) and a 4-fold coverage of unassembled genomic sequences of the species of interest is available. Kmasker-web has been made publically available as web-access using the address: http://webblast.ipk-gatersleben.de/kmasker. Genetically mapped barley BAC clones (or BAC contigs) are available too (Schulte *et al.* 2011, Ariyadasa *et al.* 2014).

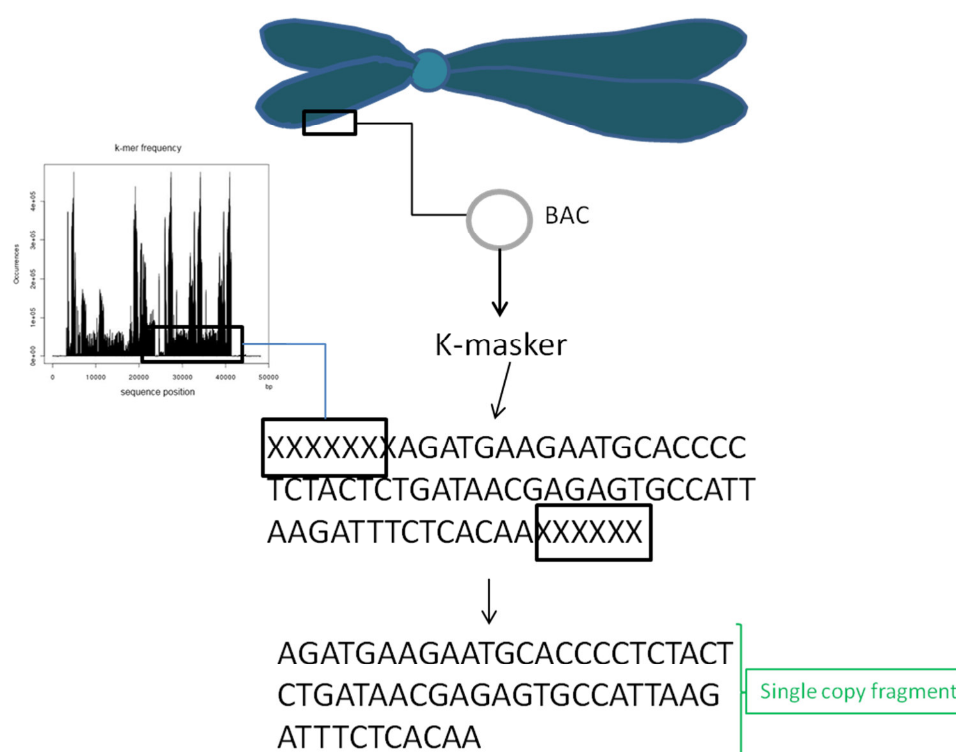


Figure 2 Schematic illustration of the K-masker analysis, showing a processed BAC sequence and an output sequence with masked repeats (X nucleotide).

Using *in silico* predefined single copy sequences Ma et al. (2010) demonstrated clear FISH signals on the short arm of barley chromosome 2H. However, due to the high degree of chromatin condensation of mitotic chromosomes only unique sequences with a distance of at least 2 Mb from each other can be distinguished by this method. Therefore, to physically map sequences with a distance from each other below 2 Mb it will be required to apply high-resolution light microscopy (SIM) and/or to employ chromosomes with a lower degree of compaction, like pachytene chromosomes.

1.5 Integration of physical and genetic maps

The anchoring of physical and genetic maps in small genome species (Cheng *et al.* 2002) and in barley (Kunzel *et al*. 2000; Ma *et al.* 2010) has visualized the discrepancies between two of these maps. It is generally known that genetic maps often do not represent actual physical distances. The reason is the variable recombination frequency along the chromosome. Level of recombination in the area of heterochromatin is much lower than in the euchromatin regions. Small distances in the genetic map here corresponds with a large distances on the chromosome that results in the mentioned discrepancy.

An integrated physical-genetic map is a powerful tool that has been applied frequently for gene cloning and could support genome sequencing projects. In case that the genetic map places a trait (e.g. disease or resistance) between two markers, it is important to know the distance in base pairs (bp) to define the likely numbers of genes or regulatory regions.

Several plant genomes of the species such as *A. thaliana* (Fransz *et al.* 1998), sorghum (Kim *et al.* 2002), rice (Jiang *et al*. 1995) or tomato (Szinay *et al*. 2008) have an available integrated map or are about to possess one. There are several methods that have been used to correlate genetic and physical maps in plants. One of the first successful methods involves the construction of contigs formed by clones with large inserts and ordering the definite markers relative to contigs. This method was applied for the physical mapping of small genomes such as *A. thaliana* (Schmidt et al. 1995) and sorghum (Klein *et al.* 2000), but could not be efficiently used in large genomes with a high content of repetitive sequences. Combination of flow-sorting of chromosomes and PCR with marker-specific primers is a second mapping technique. This approach is supported at the first place by the ability to purify large amounts of chromosomes, sort them and map high numbers of sequences. This method allows the mapping of even very short single-copy targets. Contaminations by other chromosomes could occur and should be considered while mapping. Using this combined method, linkage groups IV and VII of garden pea have been integrated with physical chromosomes (Neumann *et al.*

2002). To correlate the genetic and physical map of wheat a set of chromosome deletion lines has been used (Qi and Gill 2001). Southern hybridization of restriction fragment length polymorphism (RFLP) clones located near to single gene or QTL was undertaken. Finally fluorescent *in situ* hybridization presents a method, which allows to map specific sequences most directly and visible on the chromosomes. Cytogenetics was rediscovered in the view of physical mapping and appears to be very promising for this field of research (Lapitan *et al.* 1997; Stephens *et al.* 2004; Phillips *et al.* 2010).

1.6 Cytogenetic characterization of barley chromosomes

The possibility to identify each of the seven barley chromosomes by FISH is an indispensable prerequisite for further cytogenetic analysis. By application of two probes derived from repeated DNA this task was stably achieved (Fukui *et al.* 1994, Ma *et al.* 2010). A combination of a 5S rDNA probe that was checked on *H. vulgare* cultivars Golden Promise and Morex, and a subtelomeric probe known as HvT01 were primarily used to specifically label and thus identify each barley chromosome.

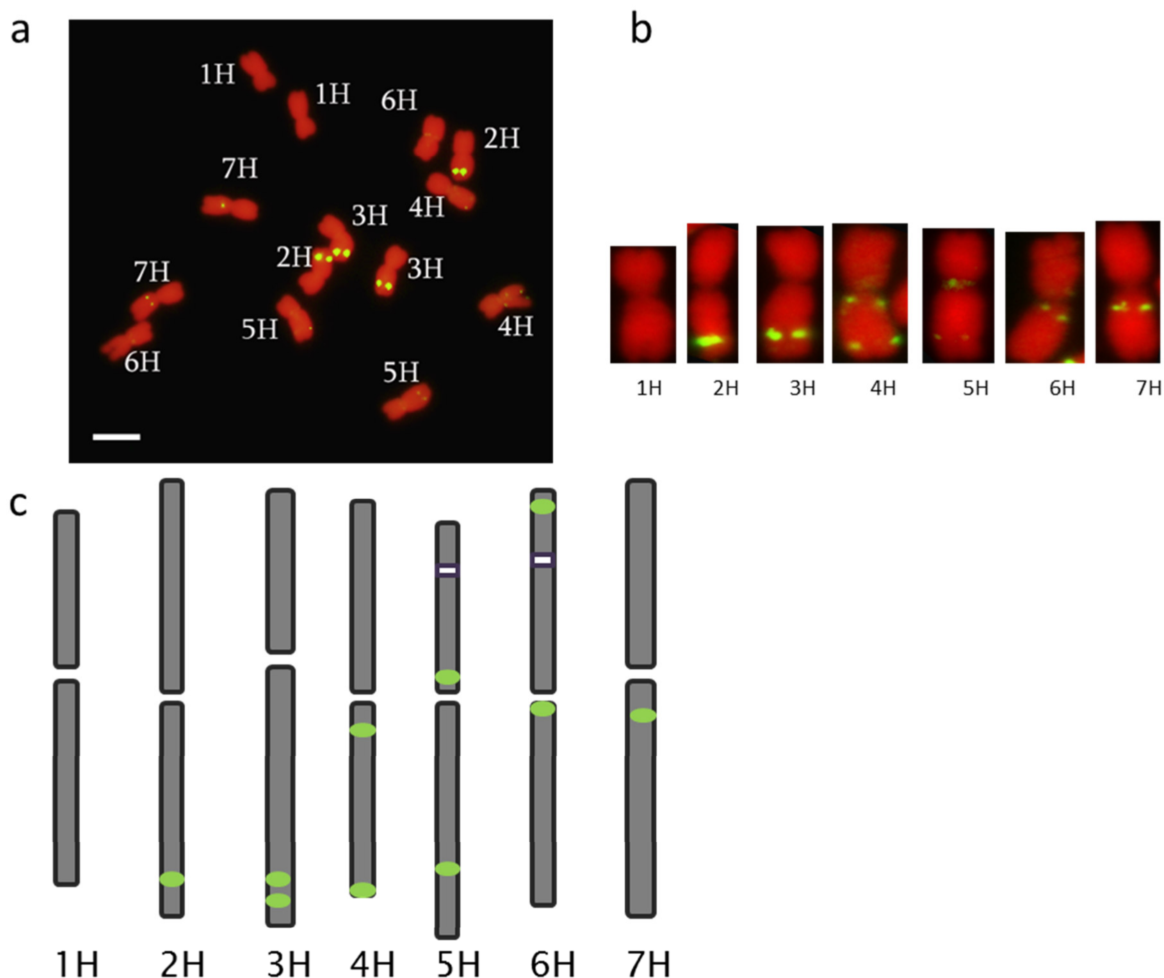


Figure 3 Mitotic metaphase spread **(a)** of barley chromosomes showing signals of the 5S rDNA probe (green). Scale = 5 µm, **(b)** Selected barley chromosomes identified by 5S rDNA specific signals (green), **(c)** Ideogram of barley chromosomes with 5S rDNA (green) and the two NOR positions (orange).

Identification of chromosome 2H and 3H is not easy, since both chromosomes possess intense 5S rDNA signals on the long arms, but the different long arm/short arm ratios between both chromosomes allows an exact differentiation (Figure 3).

2. *Scope of this study*

Physical mapping of genetically predefined sequences is important for comparative genomics as well as for the sequence characterization of large genome species like barley (*H. vulgare*). The aim of this research was to apply FISH to resolve the order of BAC contigs genetically assigned to a 5.5 cM bin around the centromere of chromosome 3H and to use selected FISH probes to demonstrate syntenic relationships and chromosomal rearrangements within the genus *Hordeum* and *Secale cereale*. Generally, centromeres are poor in meiotic recombination and based on this fact the positions of physically and genetically mapped sequences may differ.

At first, repeat-free sequences suitable for FISH of preselected FPcontigs (BACs) were identified by in *silico* mathematically defined repeat (MDR) analysis using the Kmasker tool. Next, the identified sequences were used to produce FISH probes, which were mapped on chromosome 3H to determine the physical length of the genetic centromere and to allocate the corresponding FPcontigs in this region. In addition to mitotic metaphase chromosomes, pachytene chromosomes were employed to achieve a higher resolution of the cytogenetic mapping. Chromosomes at the pachytene stage are less condensed in comparison to mitotic metaphase chromosomes and allow therefore a higher resolution of physically mapped sequences. Finally, I compared chromosome 3H of barley with the corresponding chromosomes of *H. bulbosum, H. marinum, H. murinum, H. pubiflorum* and rye by application of single-copy FISH probes and established cytogenetic maps that are allowing a better understanding of the extent of synteny among these species. In addition, the homoeologous chromosomes to barley chromosome 3H have been characterized for each analysed species.

3. Results and Conclusions

3.1 Cytogenetic mapping of FP contigs assigned to the genetic centromere of chromosome 3H

Genetic map distances, based on the frequency of recombination events, do not always directly correlate with physical distances between molecular markers or genes. For example in centromeres, regions with reduced or even suppressed recombination, small genetic distances can be translated into large physical distances. As a consequence, marker loci anchored to the genetic centromere remain unresolved.

3.1.1 Sizing genetic and physical centromere of barley chromosome 3H

In 2012, as this study started, the physical order of BAC contigs associated with the genetic centromere of barley chromosomes was almost at random. However affiliation of FPcontigs to the respective chromosome arm was known, determined by flow-sorting of barley chromosome arms (Mayer *et al.* 2011). FPcontigs were sorted into chromosome arm bins that obtained the same genetic position like the barley centromeres.

The question arose whether FPcontigs genetically mapped to a 5.5 cM bin around the centromere of chromosome 3H would show a similar position and order on the physical chromosome. Moreover, it was asked whether the chromatin landscape differs between chromosomal regions characterized by low or high frequency of meiotic recombination.

To determine the chromosomal position and order of 65 FPcontigs mapped to a 5.5 cM bin around the genetic centromere of chromosome 3H of barley a fluorescence *in situ* hybridization (FISH) strategy was employed, which allowed the microscopic detection of *in silico* defined single-copy sequences derived from BAC contigs. In many cases, contigs containing up to 90% of repetitive sequences were interrupted by short single-copy regions. These 65 out of 291 available FPcontigs were selected based on the presence of at least a 2.5 kb long single copy sequence region. The workflow of the experiment included four consecutive steps: 1. *in silico* prediction of single-copy regions by the “Kmasker” tool (Schmutzer *et al*. 2014) for probe generation, 2. amplification and pooling of unique PCR fragments, obtaining a total probe size of up to 5 - 7 kb, 3. labelling of the probes with fluorochromes, 4. hybridization of the probes to the chromosomes of barley and microscopic analysis (Figure 4).

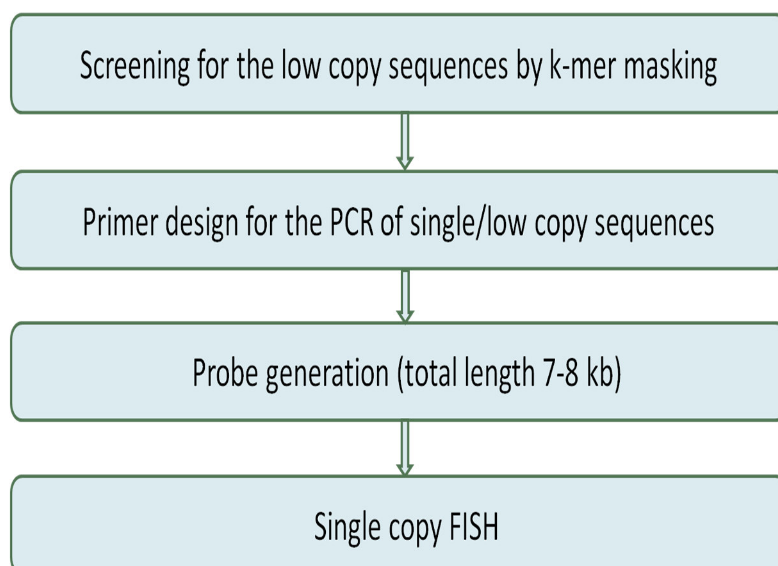


Figure 4 Workflow, showing the steps needed for the generation of contig-derived single-copy FISH probes of barley.

All single-copy probes yielded in highly specific signals on chromosome 3H with no background. Parallel hybridization with a differentially labelled 5S rDNA-specific probe was used to identify chromosome 3H. The position of the contig-derived chromosome 3H specific probe signals varied slightly between different metaphases and sometimes even between sister chromatids. Therefore, the hybridization position of 5 to 10 chromosomes was measured to determine an average position of each analysed probe (Figure 5).

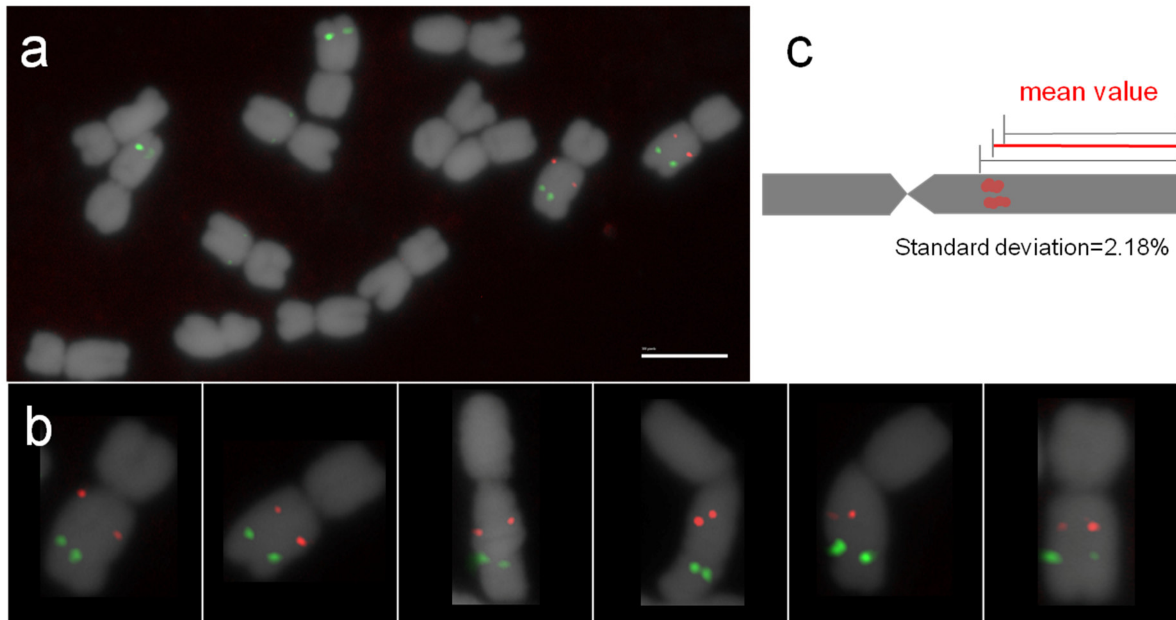


Figure 5 FISH mapping of an FPcontig-derived single-copy probe on barley chromosome 3H. **(a)** Mitotic metaphase chromosomes hybridized with the diagnostic 5S rDNA probe (in green) and single-copy gene-containing probe 43477* (in red). **(b)** Examples of barley chromosome 3H hybridized with the same single-copy probe (red) showing positional deviation of 2.18% and 5S rDNA probe (in green). **(c)** Schematic chromosome 3H showing 6 different positions for the same probe with the indicated average position (scale bar=10 µm).

Overall, seventy single-copy probes derived from 65 FPcontigs were generated. FISH mapping of the low-copy probes revealed that the physical dimension of the centromeric 5.5 cM bin of chromosome 3H comprised 58% of the mitotic metaphase chromosome length. Twenty five probes were mapped on the short arm and 45 on the long arm of chromosome 3H. Eight probes mapped very close to the primary constriction (physical position of the centromere) of the chromosome (Figure 6). The remaining 62 probes were distributed along both chromosome arms. To analyse whether genetic centromere contains coding sequences, eighteen gene-containing probes were generated (Figure 6 (indicated in red)).

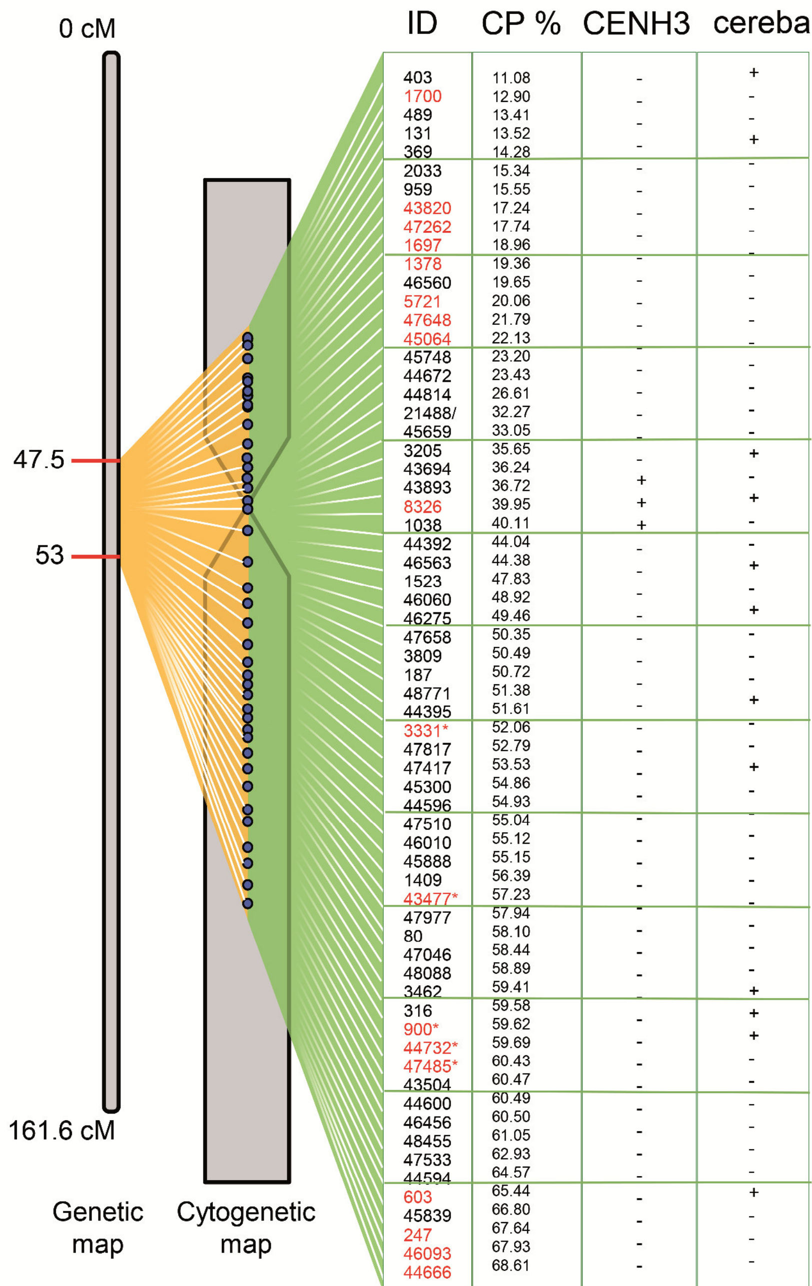


Figure 6 Correlating the genetic and cytogenetic maps of barley chromosome 3H. Genetic positions of 65 FPcontigs range from 47.5-53.0 cM (IBSC, 2012). Cytogenetic positions of 70 deduced single-copy probes were determined by FISH and represented on the schematic chromosome. Cytogenetic position (%) of FPcontigs and corresponding positions of CENH3 and centromeric *cereba* repeat are indicated by + (right panels). Probes containing a coding sequence are highlighted in red. For contigs labelled by asterisk two independent probe pools were generated, one with and another without a coding sequence.

FPcontigs 900, 3331, 43477, 47485 and 44732 were represented by two probes with differences in length and sequence composition, one with and one without a gene coding sequence. FISH signals for either double-tested contig-derived probes yielded similar

hybridization positions, proposing that neither the length of the sub-probes nor the probe positions within a contig, has any strong influence on the chromosomal hybridization position. Gene-coding probe 8326 was localized in close proximity to the primary constriction. Co-hybridization of this probe with a barley centromeric repeat confirmed the centromere position of this probe (Figure 7).

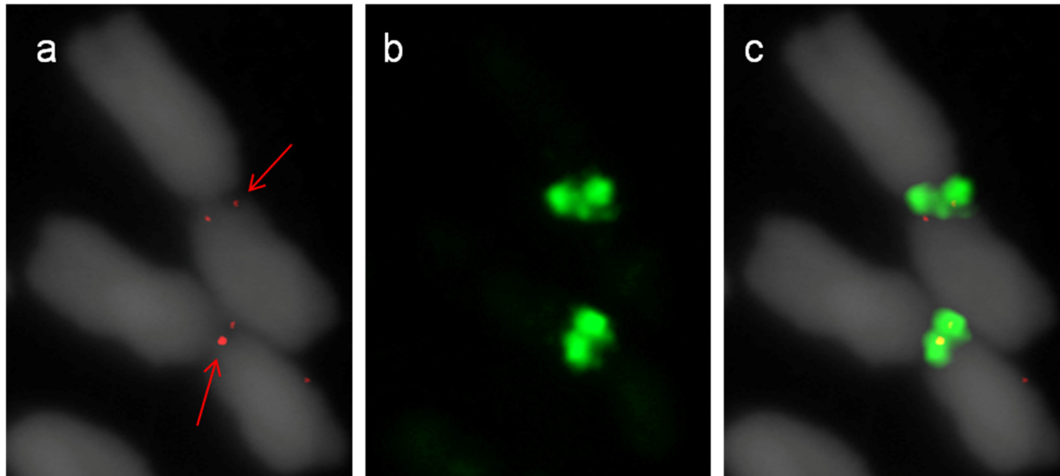


Figure 7 The centromeric regions of 3H encode genes. Colocalization of **(a)** the single-copy probe 8326 (in red) containing a high-confidence gene and **(b)** the barley centromeric repeat $[AGGGAG]_5$ (in green). **(c)** Merged image showing overlapped signals; chromosomes were counterstained with DAPI (grey).

3.1.2 Application of pachytene chromosomes for increased resolution

Ma et al. (2010) demonstrated distinct FISH signals in barley using the approach described above. However, due to the high degree of condensation of mitotic chromosomes only unique sequences with a distance of at least 2 Mbp from each other will be distinguished by FISH. To overcome the optical limitations of small sized mitotic chromosomes, pachytene chromosomes have been used for FISH mapping in selected species e.g. tomato (Tang *et al.* 2008), papaya (Wai *et al.* 2012), *Arabidopsis (Lysak et al. 2005)* and maize (Sadder *et al.* 2000). During the early steps of meiosis in most species (including barley) the chromosomes are largely extended in length. Spatial resolution of neighbouring loci in the euchromatic region was improved down to 60 -120 Kbp (in heterochromatic region 0.14 -1.2 Mbp) as compared to 5-10 Mbp after FISH on mitotic chromosomes (Valarik *et al.* 2004). Therefore, the physical distance even between closely linked sequences can be determined using pachytene chromosomes (Figure 8).

The main prerequisite for the analysis of extended chromosomes was the preparation of pachytene chromosomes suitable for single-copy FISH experiments. Several protocols for the preparation of meiotic chromosomes are known for plants (de Jong *et al.* 1999, Iacia and Pinto-Maglio 2013). Three different protocols were tested to obtain high-quality pachytene chromosomes of barley. Best results were obtained by a squashing method, using an enzyme mix (0.7% Cellulase Onozuka R10 (Duchefa, C8001), 0.7% Cellulase (CalBioChem, 319466), 1% Pectolyase (Sigma, P3026) and 1% Cytohelicase (Sigma, C8274) in 0.01 M citrate buffer and incubation time of 2.5 h at 37°C. Anthers were squashed between slide and cover slip in a drop of 45% acetic acid. To reduce the amount of cytoplasm the slides were shortly heated over a Bunsen burner flame (de Jong *et al.* 1999). Subsequently, the order of partly overlapping probes on mitotic metaphase chromosomes after FISH was figured out for the following eight contig pairs: 1409 - 45888, 3331- 44399, 43477 - 44600, 900 - 3462, 44594 - 45839, 46010 - 44596, 316 - 900, 187 – 48771. Probe 80 was applied as a reference probe (Figure 9 b, e-h). These probe combinations were labelled with Atto550 and with a 1:1 mixture of Texas Red and Alexa488. Cytological positions of the overlapping probes were determined by the comparison of ten different nuclei.

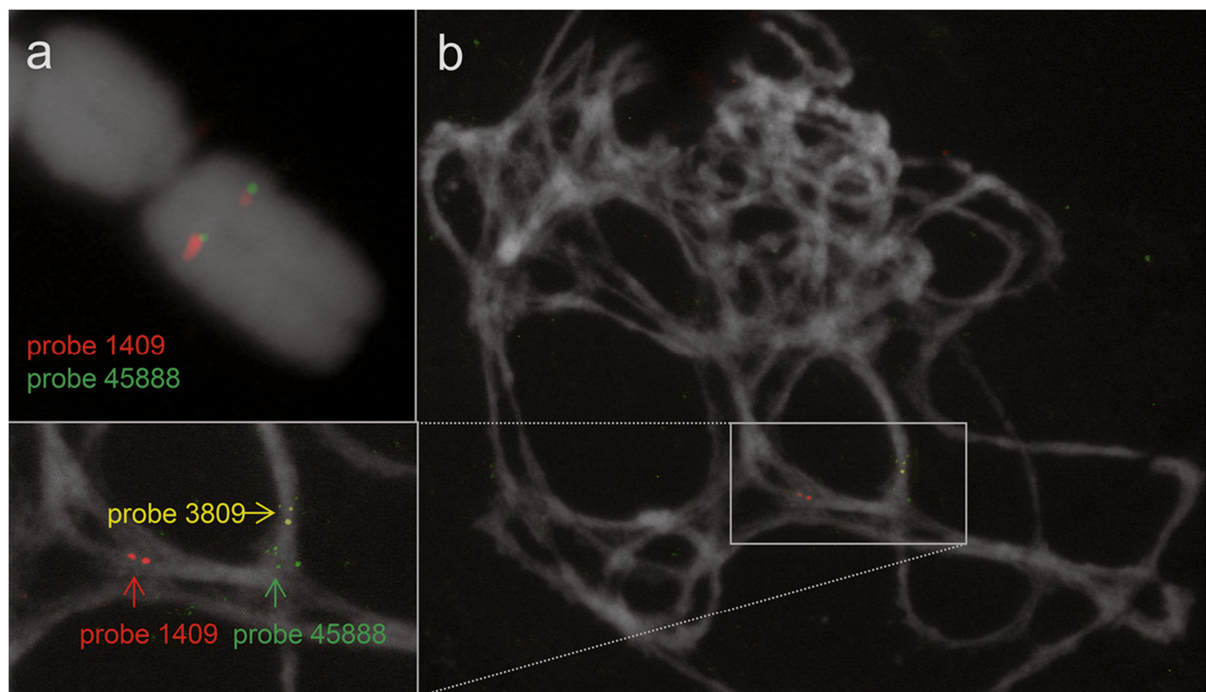


Figure 8 Comparison of single-copy FISH at resolution of (a) mitotic metaphase and (b) meiotic pachytene chromosomes of barley. **(a)** After FISH on metaphase chromosomes 3H overlapping position for probes 1409 and 45888 were found. **(b)** In contrast, FISH on pachytene chromosomes revealed distinct FISH signals of the differentially labelled single-copy probes 1409, 45888 and 3809. Probes 1409, 45888 and 3809 are labelled with Texas Red-12-dUTP, Alexa488-dUTP and TexasRed-12-dUTP/Alexa488-dUTP, respectively.

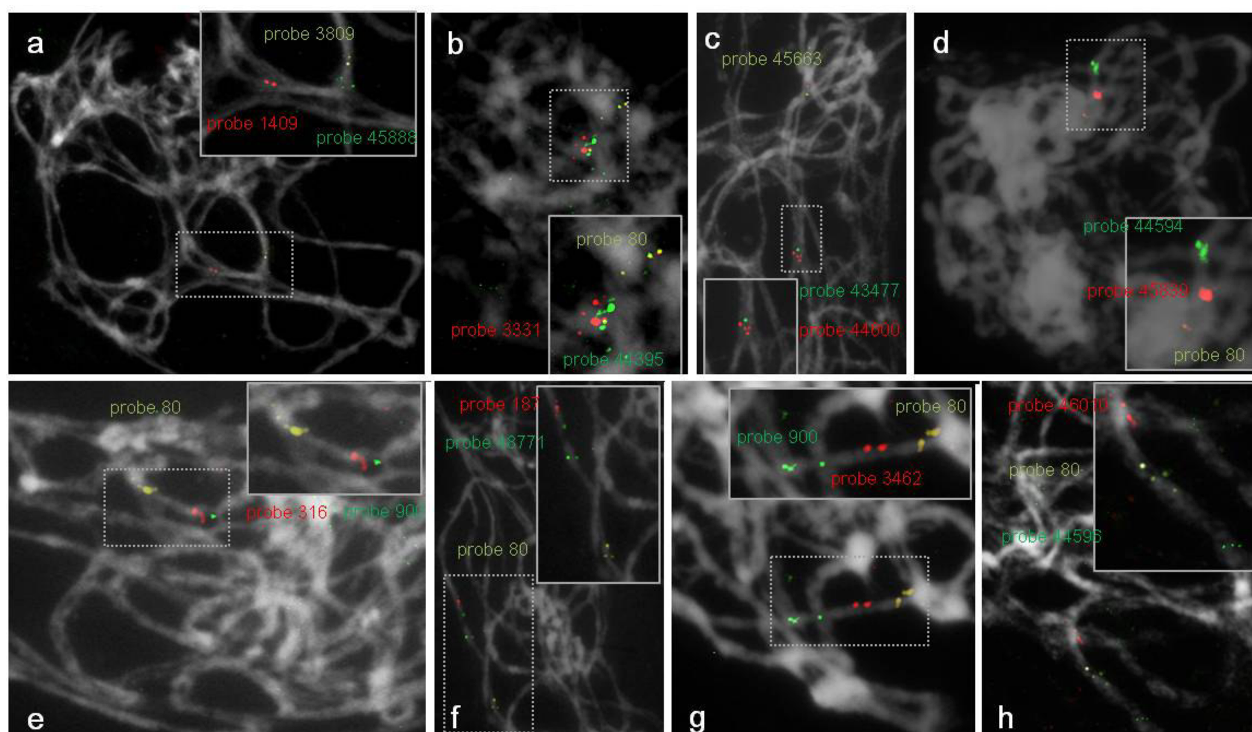


Figure 9 Identification of the correct order of overlapping probes by FISH using barley pachytene chromosomes. **(a-h)** Probes 1409, 3331, 44600, 45839, 316, 187, 3462, 46010 were labelled with Texas Red-12-dUTP, probes 45888, 44399, 43477, 44594, 900, 48771 and 44596 were labelled with Alexa488-dUTP **(a-g)** Probes 3809, 80 and 45663 were labelled with TexasRed-12-dUTP/Alexa488-dUTP mixture (pseudo coloured). **(h)** Probe 80 is labelled with Atto 550 for the orange signal. Chromosomes were counterstained with DAPI (grey).

3.1.3 FISH with the *cereba* repeat reveals additional extracentromeric signals

The centromere of barley is enriched in a Ty3/gypsy-like repeat known as *cereba* element (*ce*ntromeric *re*troelement of *ba*rley). Each centromere of barley chromosomes is estimated to contain around 200 *cereba* elements representing the centromeric DNA of at least 1.4 Mb (Hudakova *et al*. 2001). An interaction between CENH3-containing centromeric nucleosomes, *cereba* and GC-rich satellites was shown previously by Houben *et al.* (2007).

To analyse whether the presence of *cereba* elements would facilitate a proper assignment of contigs to the functional centromere, we performed BLAST analysis against centromeric *cereba* sequence on all 65 previously applied FPcontigs. Only contigs showing alignment hits longer than 1,000 bp and over 90% sequence identity were considered as *cereba* positive. Twelve FPcontigs carried sequences with at least 90% sequence identity over a region longer

than 1,000 bp to the centromeric Ty3/gypsy-like element *cereba*. Six of the *cereba*-containing probes (21488, 3205, 8326, 46563, 46275 and 48771) showed signals in the proximity of the centromere on chromosome 3H. Co-hybridization of probe 8326 with the barley centromeric $[AGGGAG]_5$ minisatellite confirmed the centromere position (Figure 7). In contrary to the expectations, *cereba*-positive probes were not concentrated at the functional centromere only, but hybridized additionally outside the centromere. The existence of extracentromeric *cereba* sites was confirmed cytogenetically after identification of weak interstitial *cereba* signals along both arms in addition to accumulated FISH signal at the centromeres of barley (Figure 10). The detection of extra-centromeric *cereba* repeats indicates that physical map-based prediction of the position of the centromere based exclusively on the presence of *cereba* sequence motifs is risky and non-reliable. Indeed, it was assumed before by Miller et al. (1998) that only 95% of this Ty3/gypsy-type mobile element clustered in centromeres, while 5% of this repeat might be present outside of this region.

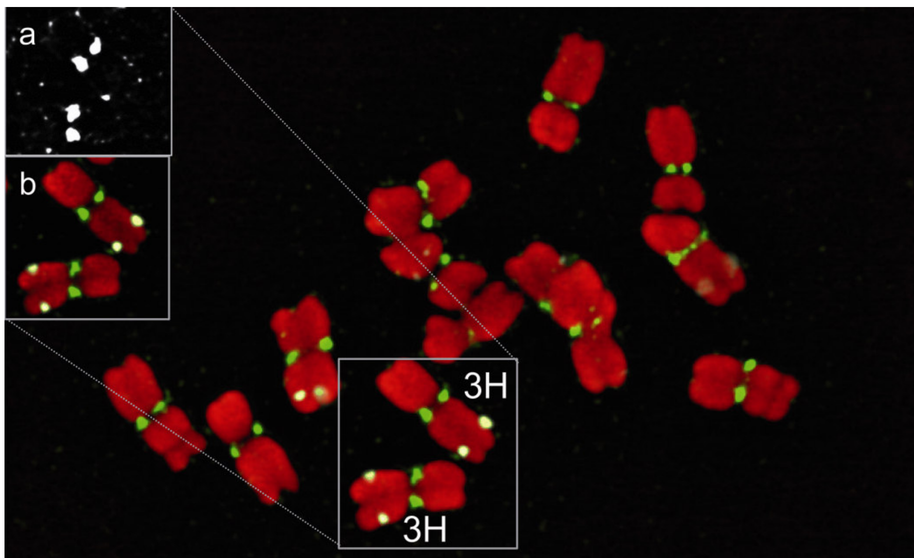


Figure 10 BAC-FISH with the centromeric *cereba* Ty3/gypsy retroelement (green) and the 5S rDNA (yellow) probe on metaphase chromosomes of barley. **(a)** *Cereba* element reveals strong signals in centromeres and additional small signals outside centromere after extended signal detection. **(b)** Merged images of *cereba* (in green) and 5S rDNA signals (in yellow) stained with DAPI (red).

3.1.4 Posttranslational histone H3 modifications in the regions of suppressed recombination

As the 5.5 cM bin around the genetic centromere comprised 58 % of the entire length of barley chromosome 3H (Fig. 2), the question arose whether this region of reduced recombination frequency would correlate with distinct chromatin fractions, specific for eu- or heterochromatin. Dimethylated lysine 9 of histone H3 (H3K9me2), a prominent mark for constitutive heterochromatin, showed a uniform distribution throughout the entire length of the chromosome, as it is typical for plants with genomes larger than 500 Mbp (Houben *et al.* 2003; Fuchs *et al.* 2006). In contrast, H3K4me2 and H3K4me3 (Litt *et al.* 2001, Strahl *et al.* 1999), typically associated with transcriptionally active euchromatin were strongly enhanced in the terminal parts and represented about 37 % of the length of chromosome 3H (Figure 11). A similar region was stained by H3K27me3, a mark for transcriptionally inactive gene-containing chromatin (Bennett and Leitch 2005, Plath *et al.* 2003). As these modifications may differ between mitosis and meiosis (Oliver *et al.* 2013), we performed immunostaining on flow-sorted mitotic metaphase chromosome 3H as well as on meiotic metaphase I chromosomes. The centromere of metaphase I chromosomes was visualized with the help of a CENH3-specific antibody. Immunostaining on meiotic bivalents showed labeling at terminal parts and lack of staining in centromeric and pericentromeric regions (Figure 11c - e, right panel). As next, the cytogenetically-defined pericentromere of barley was determined using antibodies specific for the phosphorylated histone H3 at positions serine 10 (H3S10ph) and 28 (H3S28ph) (Figure 11f, g) (Gernand *et al.* 2003, Houben *et al.* 1996a, Houben *et al.* 1996b). Out of 70 contig-derived probes, twenty one (44672, 44814, 21488, 45659, 3205, 43694, 43893, 8326, 1038, 44392, 1523, 46060, 46275, 47658, 3809, 187, 48771, 44395, 3331, 47817, 47417) were located within this pericentromeric region, comprising the interval of 51.34-51.62 cM on the genetic map (Figure 6). Thus, the region low in recombination, comprising 58% of the physical length of the respective chromosome, is located in a region enriched in transcriptionally less active chromatin. This region is flanked by transcriptionally active chromatin correlating in contrast only with 37% of the physical chromosome 3H. Aegilops and rye revealed a comparable distribution of histone marks shown previously by Oliver et al. (2013). Thus the recombination frequency correlated with a distinct chromatin structure and supported the earlier established link between euchromatin and recombinationally active regions (Tanksley *et al.* 1992, Higgins *et al.* 2012, Higgins *et al.* 2014).

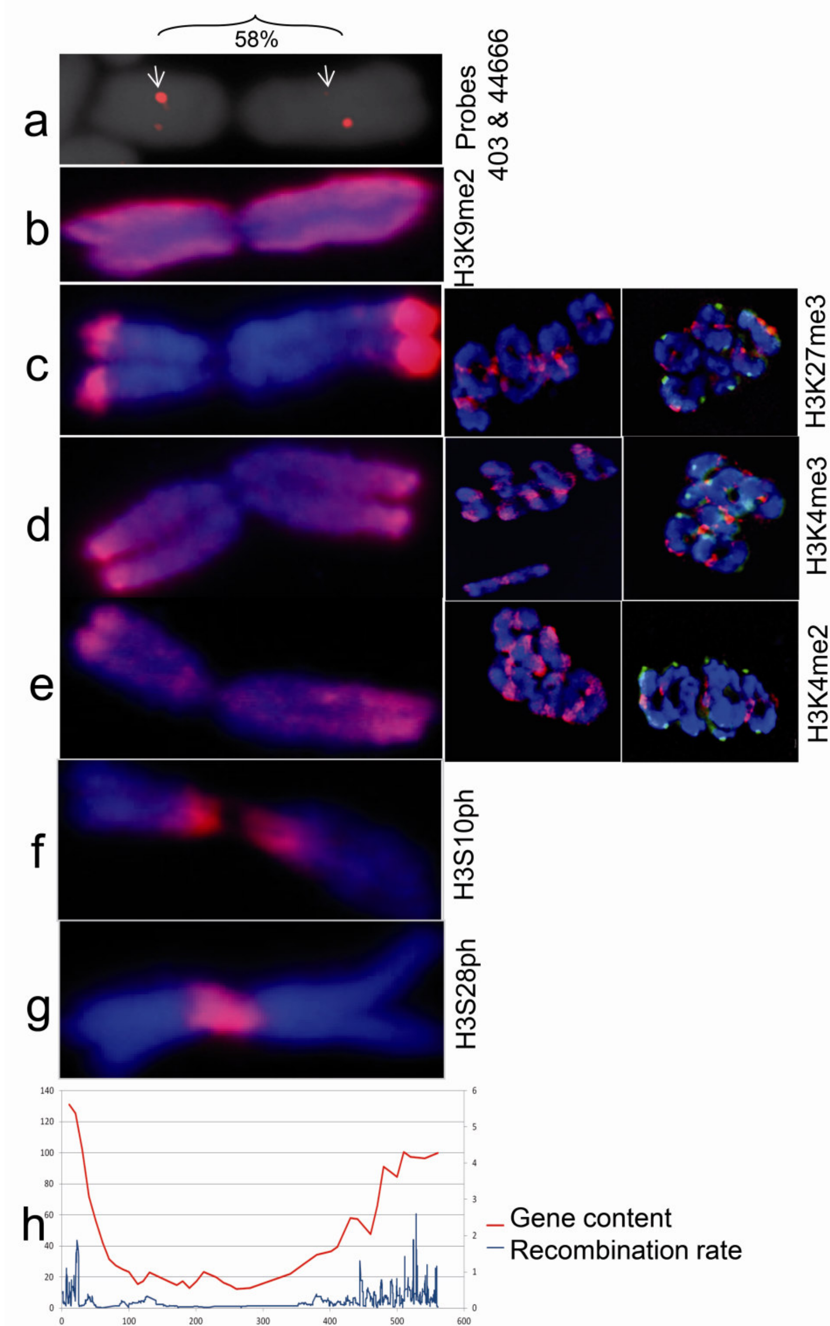


Figure 11 Characterization the chromatin landscape of chromosome 3H. **(a)** FISH with probes 403 and 44666 marks the outer boarders of the 5.5 cM bin around the genetic centromere of 3H. Immunostaining (in green) of flow sorted chromosome 3H with antibodies recognizing **(b)** heterochromatin-typical mark H3K9me2 as well as the euchromatin-typical marks **(c)** H3K4me2, **(d)** H3K4me3 and **(e)** H3K27me3. Corresponding meiotic metaphase I chromosomes are shown right to it. The pericentromeric regions are stained with antibodies recognizing H3 either phosphorylated at serine position 10 **(f)** or **(g)** 28 **(h)** Schemata on the distribution of genes and meiotic recombination rates along the physical map of chromosome 3H (based on data from IBSC, 2012).

3.2 Collinearity of homoeologous group 3 chromosomes in the genus *Hordeum* and *Secale cereale* as revealed by 3H-derived FISH analysis

Wild relatives of agriculturally important crop plants are considered as important genetic resources of allelic diversity for domesticated crop species. Their utilization in breeding programs, however, is often limited due to crossing barriers and genome incompatibilities. Wild relatives of barley possess attractive properties for adapting barley better to changing environmental conditions. Therefore, gaining a better knowledge about genomic synteny between cultivated barley and wild relatives of the same genus is an important task.

3.2.1 Cytological characterization of chromosomes homoeologous to chromosome 3H of barley

The genus *Hordeum* can be divided into three gene pools in relation to barley (Jacobsen and von Bothmer 1995). Different varieties of cultivated barley and *H. vulgare* subsp. *spontaneum* represent the primary gene pool. *H. bulbosum* is the only species of the secondary pool and the tertiary gene pool including all the other *Hordeum* species, which do not cross with barley.

FISH mapping applied with repeat-free, chromosome type-specific probes was shown to be a powerful tool in detecting homoeologous relationships between chromosomes of wheat and its related wild species (Danilova *et al.* 2014). It has been asked whether single-copy probes derived from the genetic centromere of chromosome 3H of barley and especially eighteen probes derived from coding sequences, which are likely to be conserved among species of the same tribe, could represent adequate markers for the study of genome collinearity in different *Hordeum* and Triticeae species.

To investigate the syntenic relationships between the homoeologous group 3 chromosomes of the H, Xa, Xu and I genomes of the genus *Hordeum* and the R genome of a related Triticeae species, rye (*Secale cereale*) 3H-derived single-copy FISH markers and cDNA markers were employed (Table 3). The H genome was represented by *H. bulbosum*. *H. marinum* and *H. murinum* were selected as representative species for the Xa and Xu genomes, respectively. *H. pubiflorum* belongs to the I-genome species. To test the extent of synteny between species of the same tribe, analysis on chromosomes of rye was included.

Table 3 Probe-IDs (ID) of the used probes and corresponding FISH probe-IDs (FP-contig). `g´ is derived from genomic DNA and `c´ is derived from cDNA

ID	FP-contig
g1	1700
g2	489
g3	47262
g4	5721
g5	47648
g6	8326
g7	46563
g8	187
g9	3331
g10	45300
g11	46010
g12	43477
g13	80
g14	900
g15	47485
g16	44600
g17	46456
g18	47533
g19	603
g20	45839
g21	247
g22	44666
g23	43820
g24	1697
g25	1378
g26	45064
g27	44732
g28	46093
c1	AK251893
c2	AK248957
c3	tplb0001g16
c6	AK251014
c4	AK251696
c11	AK249216
c5	AK336104
c7	AK248727
c9	AK248230
c8	tplb0014n06
c10	tplb0045e08
c12	AK251227

One of the important prerequisites of the study was the possibility to identify each of the chromosomes of analysed species from the genus *Hordeum* and each of the rye chromosomes using chromosome-specific marker sequences like microsatellites or tandem repeats in conjunction with morphological characteristics (Carmona *et al.* 2013b, Cuadrado *et al.* 2013, Cuadrado and Jouve 2007). Labelled $(CTT)_{10}$ oligonucleotides and the repetitive sequence pSc119.2 were used to distinguish the chromosomes of *H. bulbosum* as previously recommended (Pickering *et al.* 2006). FISH pattern of pSc119.2 in combination with morphological features such as the centromere index or the position of nucleolus organizer region (NOR), as described earlier by Cuadrado et al. (1995), allowed a preliminary identification of all rye chromosomes (Figure 12). Finally, characterization with the probe $(CTT)_{10}$ (Carmona *et al.* 2013a, Carmona *et al*. 2013b, Cuadrado, Carmona and Jouve 2013) allowed the differentiation of all seven chromosome pairs in *H. pubiflorum, H. marinum and H. murinum.*

FISH mapping resulted in detection of 22 gDNA and 12 cDNA probes on one and the same *H. bulbosum* type of chromosome, confirming $3H^{b}$ as being homoeologous to chromosome 3H of barley. $3H^{b}$ is characterised by a centromere-specific prominent $(CTT)_{10}$ signal (Figure 13 b, g).

Detection of 12 gDNA and 5 cDNA probes on the same chromosome of rye allowed the identification of chromosome 3R. Hence, the rye homoeologue to barley chromosome 3H was distinguished as a metacentric chromosome with two band-like pSc119.2-specific signals at both termini (Figure 13 f, g).

Eighteen single-copy probes, each carrying a high-confidence gene, were detectable in all three species identifying barley 3H homoeologous chromosomes. Chromosome $3H^{mar}$ is characterised by $(CTT)_{10}$ signals in the proximity of the centromere and in the distal region of the long arm (Figure 13 c, g). Chromosome $3H^{pub}$ can be identified as the only chromosome showing no cross-hybridization with $(CTT)_{10}$ (Figure 13 d, g). Metacentric chromosome $3H^{mur}$ can be distinguished by means of three diagnostic signals as shown in Figure 13 e, g.

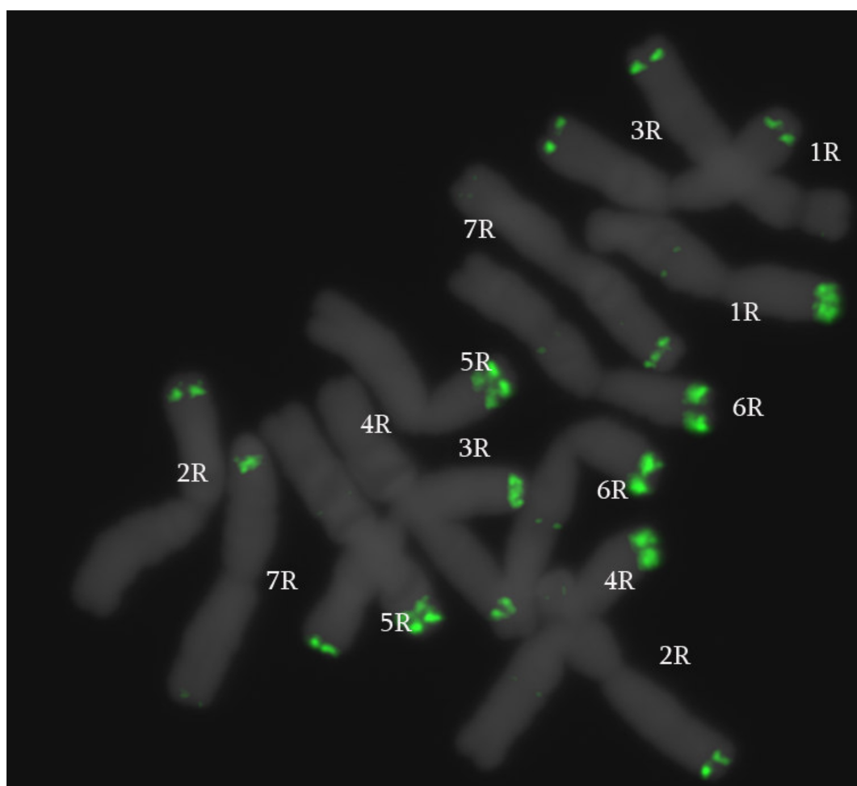


Figure 12 Identification of *S. cereale* chromosomes based on pSc119.2-specific hybridization patterns (green). Chromosomes are counterstained with DAPI (grey). Differentiation of all chromosome pairs was described earlier by Cuadrado et al. (1995) using the different repetitive DNA families.

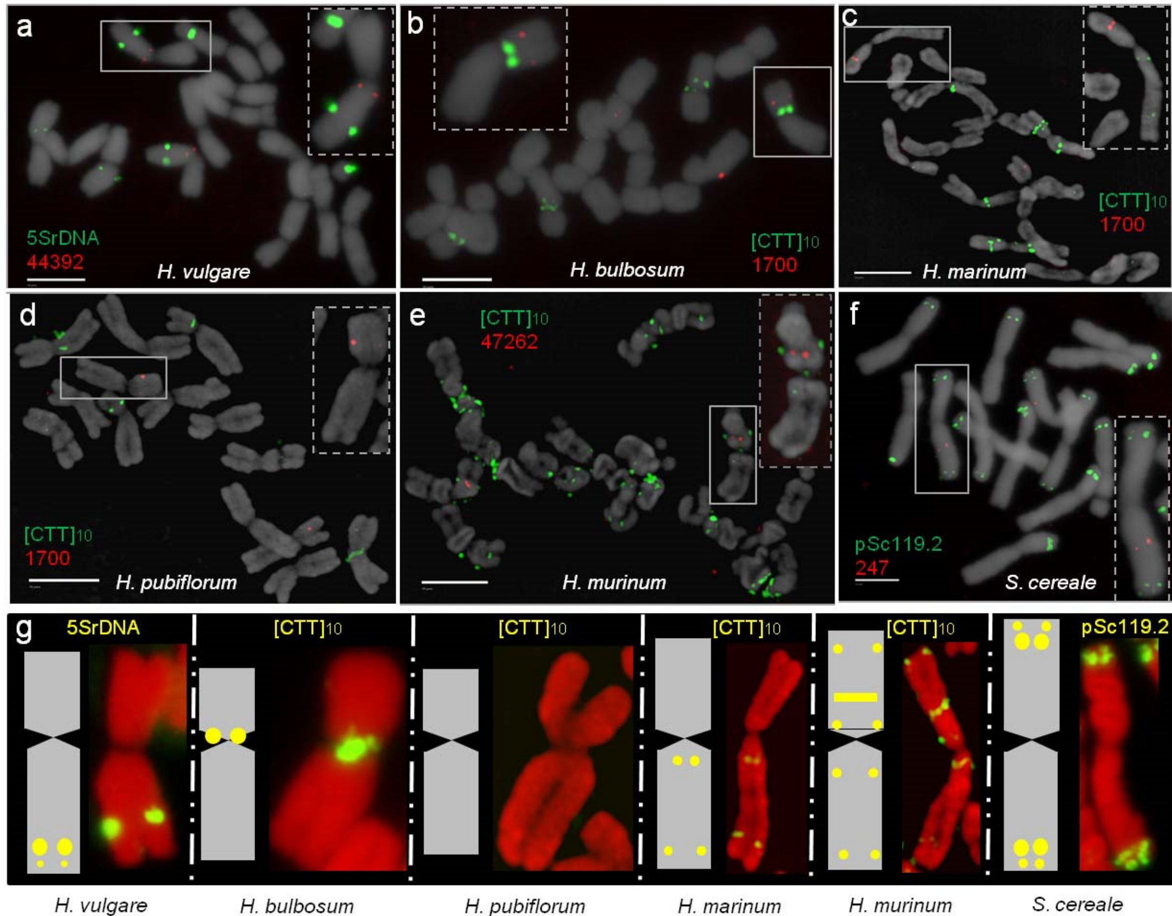


Figure 13 Single-copy FISH on metaphase chromosomes of the five species from *Hordeum* and of *S. cereale***.** **(a)** The insets show further enlarged chromosomes with specific signals. Hybridization of a single-copy FISH probe (red) on chromosome 3H of *H. vulgare* and 5SrDNA (green) applied as a diagnostic probe. (b) Hybridization of a single-copy FISH probe (red) on chromosome 3H of *H. bulbosum* and $[CTT]_{10}$ microsatellite (green) applied as a diagnostic probe. **(c, d, e)** Hybridization of a single-copy FISH probe (red) on chromosome 3H of *H. marinum, H. pubiflorum and H. murinum* and $(CTT)_{10}$ microsatellite (green) applied as a diagnostic probe. **(f)** Hybridization of a single-copy FISH probe (red) on chromosome 3R of *S. cereale* and pSc119.2 (green) applied as a diagnostic probe. **(g)** Characterization of the homoeologous chromosomes of chromosome 3H of *H. vulgare* revealed by FISH mapping with 18 single-copy probes. Chromosome 3H of *H. vulgare* and homoeologous chromosomes from the other species are represented by the diagnostic bands (green) of the characterising probes: 5SrDNA for *H. vulgare*, $[CTT]_{10}$ microsatellite for *H. bulbosum*, *H. pubiflorum*, *H. marinum* and *H. murinum* and pSc119.2 for *S. cereale.* Scale bar= 10 µm.

3.2.2 Syntenic relationship between barley and wild *Hordeum* species from the secondary and tertiary gene pools

In order to compare synteny between homoeologous group 3 chromosomes of related species of the genus *Hordeum,* 3H-chromosome-specific single copy genomic DNA- and cDNA-derived probes for FISH were employed. Outcoming results revealed very good synteny between *H. vulgare* and *H. bulbosum* group 3 chromosomes, as 28 out of 34 3H-derived probes cross-hybridised with $3H^b$, in a collinear order (Figure 14). All gene-containing genomic probes were showing an identical distribution like on chromosome 3H, confirming collinearity between *H. bulbosum* and cultivated barley. However, five of the analysed noncoding genomic sequences changed the position on *H. bulbosum*. This observation is in line with results obtained by Wicker et al. (2011) on conserved, but non-collinear sequences among Triticeae. The sequence-movement was shown to be likely mediated by adjacent transposable elements (Wicker *et al.* 2011). Two additional cDNA signals on chromosomes 2H and 4H of barley were detected on the orthologous chromosomes of *H. bulbosum* ($2H^b$ and $4H^b$), while no signals were detectable on $3H^b$. Thus, the absence of signals on $3H^b$ could be explained either by an insertion within *H. vulgare*, which did not occur in *H. bulbosum* or to a deletion event in this species. Comparison of FISH mapping between cultivated barley and three species from the tertiary gene pool of the genus *Hordeum* asserted the close relationship between those species. Besides a generally efficient cross-hybridization of almost all probes with the respected homoeologous chromosome, the conserved arm affiliation documented the high extent of synteny among *H. vulgare*, *H. pubiflorum, H. marinum* and *H. murinum* (Figure 15). While the order of the most gDNAs was conserved, their relative chromosomal positions revealed deviations, which could be explained by differences in abundance and distribution of repetitive sequences that are known to differ between those species (Cuadrado *et al.* 2013; Cuadrado and Jouve, 2007). One probe (g6) revealed a non-centromeric position in *H. marinum,* intercalary position on a different chromosome of *H. murinum* and additional signals on other chromosomes of *H. pubiflorum*.

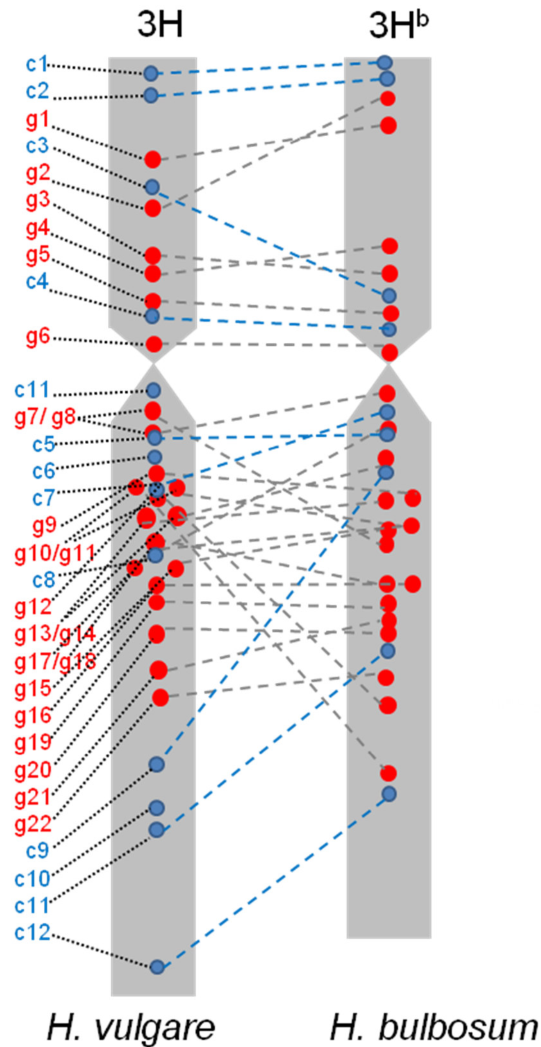
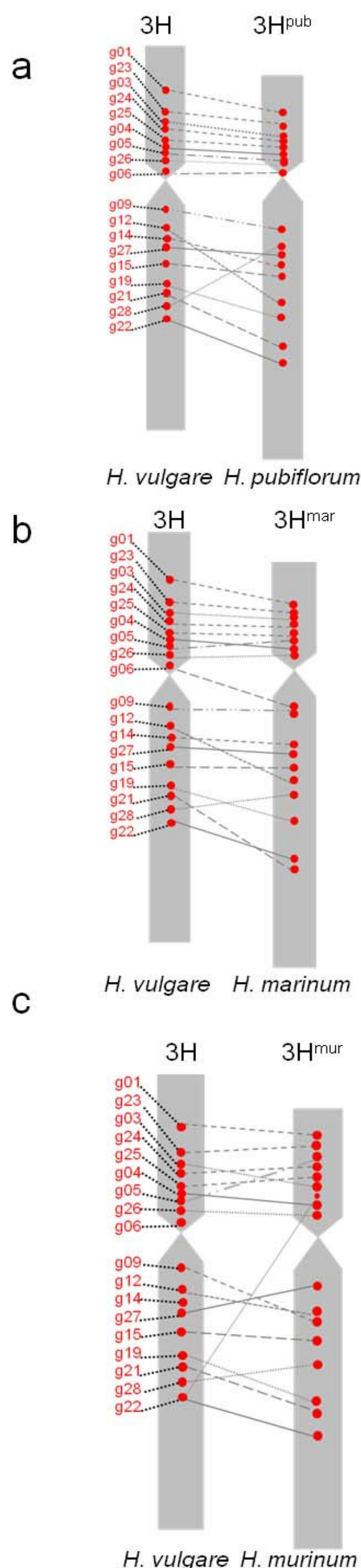


Figure 14 Comparative FISH mapping of single-copy probes between barley and *H. bulbosum*, showing 22 single-copy gDNA (red dots) and 12 cDNA markers (blue dots) mapped on chromosome 3H of *H. vulgare* and hybridized on homoeologous chromosome $3H^b$ of *H. bulbosum.*

The positional change of this probe from the centromere in barley to a non-centromeric region in two wild species might indicate a possible shift of the centromere within the genus *Hordeum* as was shown previously for the centromere of rice chromosome 8 (Ma *et al.* 2007). A discrepancy was observed in the physical length of the chromosomal interval comprising all tested probes. In *H. marinum* and *H. murinum* syntenic chromosomal interval showed a barley-like dimension, whereas this interval was physically shorter in *H. bulbosum* and *H. pubiflorum*. Interestingly, these latter species have a considerably smaller genome size (4.342 Mbp/1C and 4.220 Mbp/1C) than *H. vulgare* (5.809 Mbp/1C) and the two other *Hordeum* species (Jakob *et al.* 2004). Previous cytogenetic studies demonstrated that deviations in



marker position together with decreased hybridization intensity are indicative for a phylogenetically more distant relationship (Hasterok *et al.* 2006). Based on this, positional deviations between barley and its wild species were counted, showing two severe deviations in probe position in *H. pubiflorum*, three in *H. marinum* and in *H. murinum*. The latter has shown the absence of specific signals for probe g6 on $3H^{mur}$ and intercalar signal on a different chromosome. These few changes point out that all three species are phylogenetically very close. Both, positions of the signals relatively to the total length of the chromosome as well as signal intensity of the probes allowed assuming that *H. pubiflorum* and *H. marinum* are closer to each other. This relationship would agree with previous results from phylogenetic analyses postulating that Xa genome is the sister group of the I genome (Blattner, 2009; Brassac and Blattner, 2015) (Figure 1).

Figure 15 Comparative FISH mapping of single-copy probes in barley and related species, showing 18 gDNA probes hybridized to the homoeologous chromosomes of **(a)** *H. pubiflorum*, **(b)** *H. marinum* and **(c)** *H. murinum*.

3.2.3 Syntenic relationship between barley and rye as revealed by FISH

In total, twenty four probes were hybridized to mitotic metaphase chromosomes of rye, comprising 12 cDNAs and 12 gene-containing gDNA probes. All but one of the 12 genomic probes produced collinear signals on the respective arms of chromosome 3R compared to barley (Figure 16). In contrast to gDNA, only seven of the 12 cDNAs revealed specific signals on rye chromosomes. Five of these probes produced a 3R-specific signal. The remaining probes were detected on either arms of chromosome 6R (Figure 16). cDNA c11, representing a member of a zinc knuckle DNA-binding gene family, yielded, besides a signal on chromosome 3R, a second signal on the short arm of chromosome 6R (Figure 17). Syntenic analysis between the chromosomes of

wheat subgenomes based on FISH revealed similar results, showing that 92 % of markers hybridized to all three homoeoloci (Danilova et al., 2014). This is comparable to the data of the current analysis between chromosomes 3H and 3R estimating a level of 91 % collinearity for the gDNA derived signals. cDNA markers, in contrast, revealed three differences in order between chromosomes 3R and 6R. The rearrangement between the terminal part of the long arms of 3R and 6R is in agreement with the comparative genome zipper analysis of rye and barley describing a reciprocal translocation event (Martis *et al.* 2013). Signals of the barley cDNA probe c12 at the subtelomeric part of 3R long arm supports the proposed translocation, indicating that a small portion of 3R could be deleted and added onto chromosome 6R.

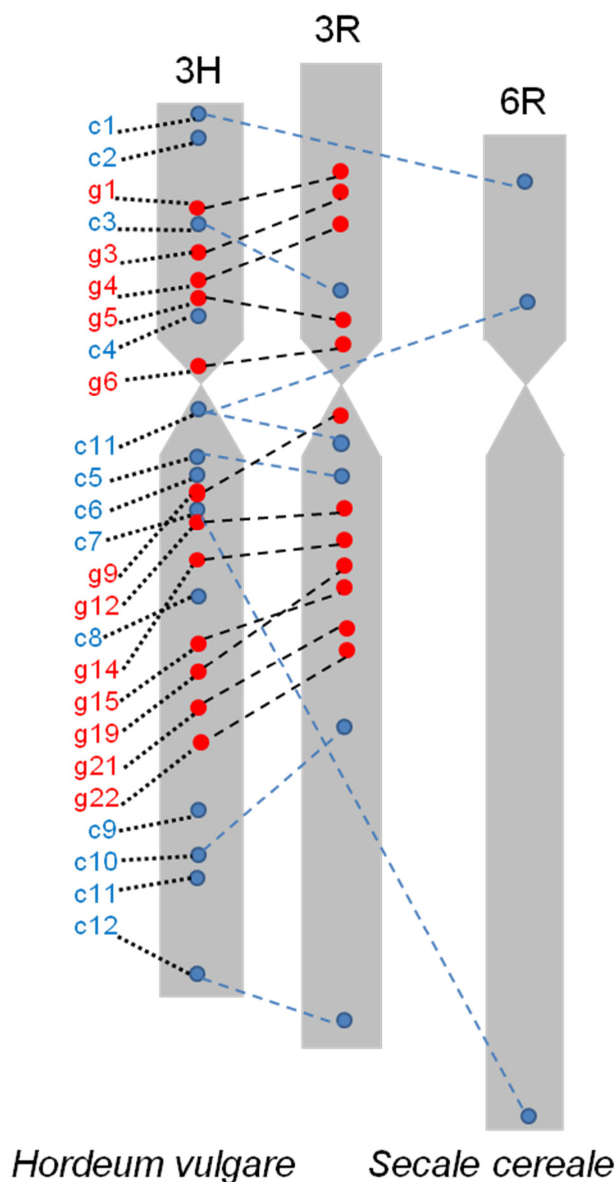


Figure 16 Comparative FISH mapping of single-copy probes in barley and related species, showing 12 single-copy (gDNA, red dots) and 12 cDNA markers (blue dots) hybridized and detected on chromosomes 3R and 6R of *S. cereale.*

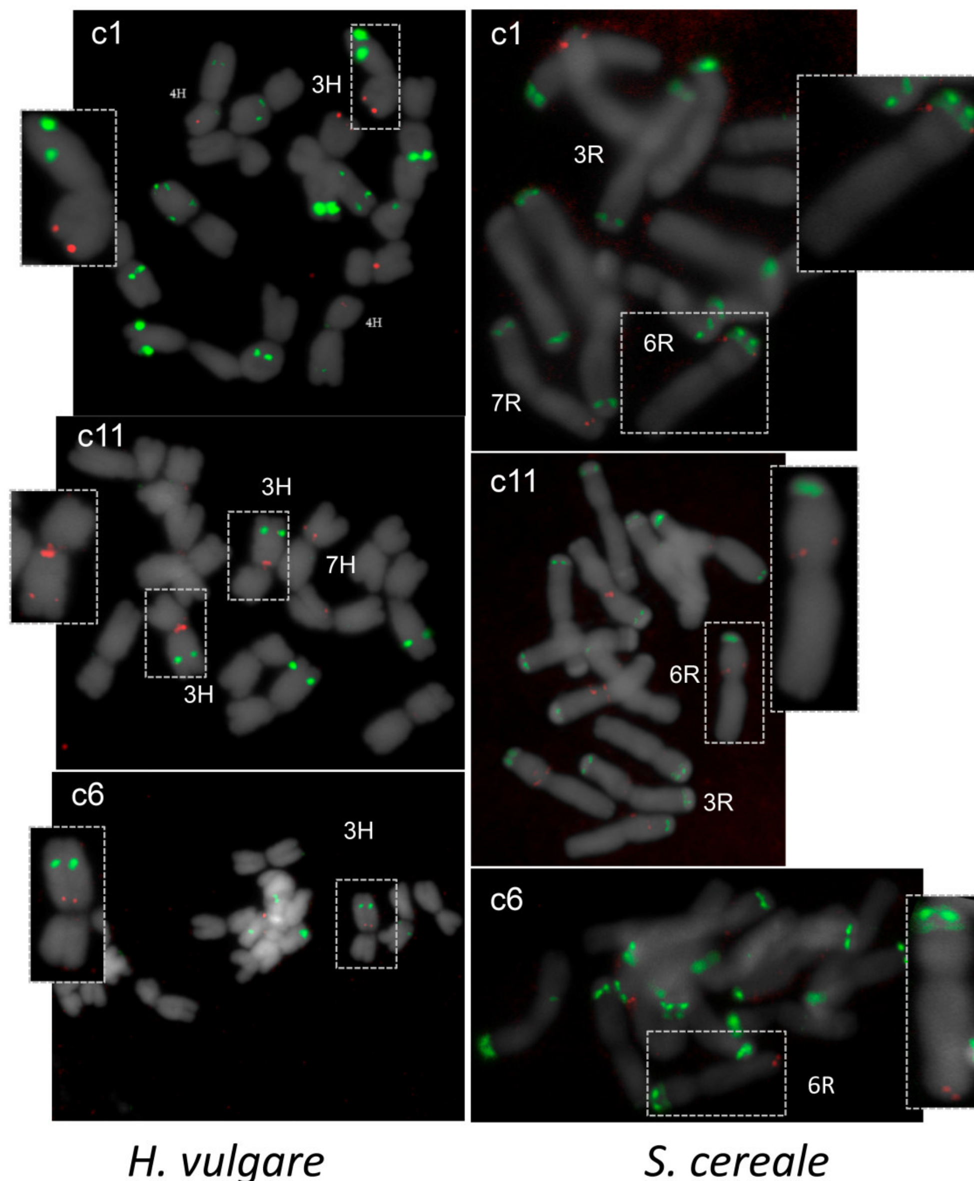


Figure 17 Comparative FISH mapping on metaphase chromosomes showing signals of selected cDNA probes (red) on *H. vulgare* and *S. cereale*. The insets show chromosome spreads and enlarged chromosomes with specific signals for three cDNA probes hybridized to barley (left) and rye (right). 5SrDNA (green) and pSc119.2 (green) were used as diagnostic probes for *H. vulgare* and for *S. cereale*, respectively. Chromosomes are counterstained with DAPI (grey).

To determine the most suitable probes for comparative studies three types of probes were tested: 1) repeat-free genomic probes carrying a coding sequence; 2) repeat-free non-coding probes and 3) cDNA probes originated from barley or wheat for FISH mapping. Genomic gene-containing probes produced single, distinct dot-like signals. Non-coding single copy

sequences performed a higher positional divergence on the comparative analysis between barley and *H. bulbosum* and were therefore omitted for the analysis on rye and on three *Hordeum* species from the tertiary pool. cDNA probes, while being coding sequences, often resulted in smaller signals, with a higher hybridization background and occasionally additional signals on other chromosomes**.** The reason for additional signals could be the high sequence similarity of members of the same gene family. For instance, cDNA AK 249216 encodes a putative zinc knuckle DNA-binding protein belonging to one of the largest gene-families and known to be expanded throughout the genome by duplication events (Tadepally *et al.* 2008). This probe revealed multiple signals not only in barley, but also in rye and *H. bulbosum*. Overall comparison between repeat free genomic probes and cDNA probes showed that gene containing single copy sequences derived from the genomic contigs is a better alternative for FISH-based mapping.

Outlook

This study demonstrates that cytogenetic mapping enables an increase in resolution of the physical map in the region associated with the genetic centromere and emphasizes the importance of alternative approaches for resolving the physical order of markers/sequences in regions with suppressed recombination. The provided cytogenetic map is currently used to reconcile the order of the analysed FPcontigs on the physical assembly of the chromosome 3H. Furthermore, centromeric sequences obtained in this study may facilitate future studies on the structure of centromeres and chromatin. For instance, differently labeled single-copy probes derived from the known physical distance (in bp) could be applied to analyse the DNA packaging degree along chromosomes to compare recombination `cold´ and ´hot` spots.

A promising technique as novel mapping tool for highly repetitive and complex genomes is optical mapping (Schwartz *et al.* 1993, Shearer *et al.* 2014, Tang *et al.* 2015). This mapping strategy results in high-resolution restriction maps prepared from DNA molecules immobilized on a slide. Newly introduced modifications of this approach include nanofluidic devices equipped with series of microchannels allowing easy movement and subsequent analysis of DNA molecules. Though, optical maps were originally constructed from large-insert DNA clones, recent studies confirmed that flow-sorted chromosomes are suitable as a DNA source and could simplify the entire mapping procedure (Doležel *et al.* 2012). Major advantage of this approach is the possibility to use various fragment lengths (20 Kb to 3Mb) in a single molecule analysis and order them along the linearized chromosomal DNA (Tang *et al*. 2015). Optical mapping can be applied in several different ways: for the de novo construction of optical maps, for the scaffolding assistance of the existing contigs (Stankova *et al.* 2016) or assembly corrections by the identification of the genome assembly errors.

Discrepancy between genetic and physical maps exists not only in the chromosome regions of low recombination, but also in regions with increased recombination -`hot spots´. In future, probes derived from distal parts of the physical map could be applied to chase the region of cytogenetic “hot spots”. Currently, the generation of probes for each chromosome arm of 3H derived from these regions of the physical map is in progress.

A similar FISH approach could be very useful when addressing questions relevant for breeding research, for instance, confirmations of successful introgressions, especially when the site and position of the introgression is not known. In addition, single-copy sequences can be easily generated from other chromosomes of barley and applied for establishing of FISH maps for related wild species to study rearrangements and collinearity of other chromosomes. Additionally, reasons for pairing disturbance within species of the primary and tertiary gene pools of the genus *Hordeum* might be elucidated.

4. Summary

I. Cytogenetic mapping of FP contigs assigned to the genetic centromere of chromosome 3H

In order to improve the resolution of the physical map of barley in the centromeric and pericentromeric regions fluorescence *in situ* hybridization (FISH) was used to order 70 repeat-free BAC-derived single-copy sequences that were genetically assigned to a 5.5 cM bin around the centromere of 3H of *Hordeum vulgare.*The total physical distribution of the analysed genetic centromere of barley mitotic metaphase chromosome 3H comprises 58% of the entire length of the chromosome. Hence 5.5 cM represented 58% of the chromosome length, demonstrating the high discrepancy between genetic and physical map. The borders of this region are represented by probe 403 on the short arm of the chromosome and probe 44666 on the long arm possessing cytogenetic positions of 11.08 % and 68.61 %, respectively. The frequency of the gene-containing probes increased as expected toward the distal parts of the physical chromosome 3H. The analysis of posttranslational histone H3 modifications makes clear that recombination frequency correlates with a distinct chromatin structure supporting the earlier established link between euchromatin and recombinationally active regions. More than the half of the physical length of chromosome 3H is shown to be associated with reduced recombination, which is the main cause for the imprecise location of most contigs in the region around the genetic centromere.

II. Subchromosomal evolution of chromosome 3H within genus *Hordeum* visualized by chromosome 3H derived probes

Comparative FISH mapping of chromosome-specific single copy probes in related species allowed the identification of homoeologous group 3 chromosomes of barley and demonstrated a high degree of similarity at the chromosomal level among *Hordeum* species from different subgenome groups. Comparison of gDNA probes to chromosome 3H of *H. vulgare* has shown 100% synteny for *H. bulbosum*, of 88% for *H. pubiflorum* (16 of 18 probes), of 83% for *H. marinum* (15 of 18 probes) and 78% for *H. murinum* (14 of 18 probes). Detected positional differences within *Hordeum* occurred either due to conserved, but non-collinear genes among Triticeae, which by far outnumber the syntenic genes in model grass genomes, or to small-scale chromosomal rearrangements. Single-copy genomic gene containing probes emerged as superior to cDNA for the studies at the chromosomal level. Examination of the extent of synteny between homoeologous chromosomes of barley and rye complemented the results of previous studies and delivered additional insight into the dynamics of grass synteny at the subchromosomal level.

5. Zusammenfassung

I. Zytogenetische Kartierung des genetischen Zentromers mit Chromosomen 3H zugeordneten FPcontigs

Die physische Karte der Gerste im Zentromer verfügt nur über eine geringe Auflösung. Um diesen Problem zu lösen, wurden 70 Einzelkopie-Sonden des Chromosoms 3H aus dem 5,5 cM langen Zentromerbereich abgeleitet und mittels Fluoreszenz *in situ* Hybridisierung kartiert. Die physikalische Länge des genetischen Zentromers vom mitotischen Metaphasechromosom 3H der Gerste umfasst 58% des physischen Chromosoms. Somit entspricht ein 5,5 cM langer Abschnitt 58% der gesamten Chromosomenlänge, was eine hohe Diskrepanz zwischen den genetischen und physikalischen Karten darstellt. Die Grenzen dieser Region sind durch die Einzelkopie-Sonde 403 auf dem kurzen Chromosomenarm und der Sonde 44666 auf dem langen Arm repräsentiert. Diese Positionen entsprechen den zytogenetischen Positionen 11,08% bzw. 68,61%. Die Frequenz genenthaltenden Sequenzen nimmt in den distalen Chromosomenregionen zu. Mehr als die Hälfte der physikalischen Länge von 3H liegt in einem Bereich mit stark reduzierter Rekombinationfrequenz, was die ungenaue Zuordnung von Contigs im genetischen Zentromer erklärt. Die Analyse von posttranslationalen Histon H3-Modifikationen auf chromosomaler Ebene zeigt, dass die Rekombinationshäufigkeit mit einer bestimmten Chromatinstruktur korreliert. Eine Wechselbeziehung zwischen Euchromatin und rekombinationsaktiven Regionen wird postuliert.

II. Subchromosomale Evolution des Chromosoms 3H innerhalb der Gattung *Hordeum* untersucht mit Hilfe 3H-spezifischer Sonden

Die FISH-basierte Kartierung von chromosomenspezifischen Einzelkopie-Sonden der Gerste in verwandten Arten der kultivierten Gerste erlaubte die Identifizierung von homöologen Chromosomen. Auf chromosomaler Ebene wurde eine hohe Ähnlichkeit zwischen den zu vier verschiedenen Subgenomen zugehörenden untersuchten Arten der Gattung *Hordeum* nachgewiesen. Der Vergleich von gentragende Einzelkopie-Sonden auf Chromosom 3H von *H. vulgare* zeigte 100% Syntenie für die homöologen Chromosomen von *H. bulbosum*, 88% für *H. pubiflorum* (16 von 18 Sonden), 83% für *H. marinum* (15 und 18 Sonden) und 78% Syntenie für *H. murinum* (14 von 15 Sonden). Die Unterschiede in Markerpositionen zwischen untersuchten Arten können entweder auf die konservierten, jedoch nicht kollinearen Gene im Tribus Triticeae zurückgeführt werden, oder auf subchromosomale Translokationen innerhalb *Hordeum* hinweisen.

Gentragende genomische single-copy Sonden erwiesen sich im Vergleich zu den cDNA-Sonden als bessere Alternative für die FISH-basierte Analyse. Die Untersuchung der Syntenie zwischen den homöologen Chromosomen von Gerste und Roggen liefert zusätzliche Einblicke in die Dynamik subchromosomaler Genomabschnitte.

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7. Curriculum vitae

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10/2006 - 09/2011	Technical University of Dresden, Dresden (Germany)
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10/2011-04/2012	Research assistant at the Institute of Plant Cell- and Molecular Biology, Technical University of Dresden (Germany).
Since 04/2012	PhD student at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben in the group Chromosome Structure and Function of Dr. habil. Andreas Houben.

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Publications

Aliyeva-Schnorr, L., Beier, S., Karafiatova, M., Schmutzer, T., Scholz, U., Dolezel, J., Stein, N. and Houben, A. (2015a) Cytogenetic mapping with centromeric bacterial artificial chromosomes contigs shows that this recombination-poor region comprises more than half of barley chromosome 3H. *Plant J*, **84**, 385-394.

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Poster presentations

Lala Aliyeva, Lu Ma, Thomas Schmutzer, Frank Blattner, Nils Stein, Andreas Houben. Title: Physical mapping of barley BACs using fluorescent *in situ* hybridization. 04.06 – 07.06.2012, 8th Plant Science Student Conference, IPK Gatersleben, Germany

Lala Aliyeva, Lu Ma, Thomas Schmutzer, Frank Blattner, Nils Stein, Andreas Houben. Title: Physical mapping of barley BACs using fluorescent *in situ* hybridization. 03.09 – 05.09.2012, The International PhD Student Conference on Experimental Plant Biology, Brno, Czech Republic

Lala Aliyeva, Thomas Schmutzer, Nils Stein, Andreas Houben. Title: Barley gets physical: Physical mapping of barley BACs using fluorescent *in situ* hybridization. 28.05. – 31.05.2013, 9Th Plant Science Student Conference, Leibniz Institute of Plant Biochemistry Halle, Halle (Saale), Germany

Lala Aliyeva, Thomas Schmutzer, Nils Stein, Andreas Houben. Title: Barley gets physical: Physical mapping of barley BACs using fluorescent *in situ* hybridization. 02.09. – 06.09.2013, The 19th International Chromosome Conference, Bologna, Italy

Lala Aliyeva-Schnorr, Steven Dreissig, Thomas Schmutzer, Sebastian Beier, Uwe Scholz, Jörg Fuchs, Nils Stein, Andreas Houben. Title: Single copy FISH and single pollen genotyping - two novel approaches to study regions of repressed and increased recombination in barley. 07.10 – 09.10.2014, Institutes day IPK Gatersleben, Gatersleben, Germany

Lala Aliyeva-Schnorr, Fernanda Bustamante, Sebastian Beier, Miroslava Karafiatova, Thomas Schmutzer, Uwe Scholz, Jaroslav Doležel, Nils Stein, Andreas Houben. Title: Cytogenetic mapping of contigs assigned to barley chromosome 3H. 14.10 – 16.10.2015, Institutes day IPK Gatersleben, Gatersleben, Germany

Oral presentations (only talks presented by L. Aliyeva-Schnorr)

Lala Aliyeva, Lu Ma, Thomas Schmutzer, Nils Stein, Andreas Houben. Title: Sizing the genetic and physical centromere of barley chromosomes as a prerequisite of understanding genome evolution in the genus *Hordeum.*10.01 - 15.01.2014, Plant and Animal Genome XXII (PAG 2014), San Diego, USA

Lala Aliyeva-Schnorr, Lu Ma, Thomas Schmutzer, Nils Stein, Andreas Houben. Title: Sizing the genetic and physical centromere of barley chromosomes. 02.06. – 05.06.2014, 10th Plant Science Student Conference, IPK Gatersleben, Germany

Lala Aliyeva-Schnorr, Lu Ma, Thomas Schmutzer, Nils Stein, Andreas Houben. Title: FISH to support resolving physical map order in centromeres. 22.09 – 23.09.2014, IBSC MTP Sequencing workshop IPK Gatersleben, Gatersleben, Germany

Attended conferences

23.04 – 25.04.2012, Gatersleben Research Conference 2012, IPK Gatersleben, Germany

04.06 – 07.06.2012, 8th Plant Science Student Conference, IPK Gatersleben, Germany

03.09 – 05.09.2012, The International PhD Student Conference on Experimental Plant Biology, Brno, Czech Republic

28.05 – 31.05.2013, 9Th Plant Science Student Conference, Leibniz Institute of Plant Biochemistry Halle, Halle (Saale), Germany

02.09 – 06.09.2013, The 19th International Chromosome Conference, Bologna, Italy

10.01 - 15.01.2014, Plant and Animal Genome XXII (PAG 2014), San Diego, USA

02.06 – 05.06.2014, 10th Plant Science Student Conference, IPK Gatersleben, Germany

Stays in research centres

10.11 - 10.12. 2013, Institute of Experimental Biology, Centre of the Region Hana for Biotechnological and Agricultural Research, Olomouc, Czech: Supervisor: Prof. J. Doležel

05.07 – 24.07.2014, Institute of Experimental Biology, Centre of the Region Hana for Biotechnological and Agricultural Research, Olomouc, Czech Republic: Supervisor: Prof. J. Doležel

8. Eidesstattliche Erklärung / Declaration under Oath

Hiermit erkläre ich, Lala Aliyeva-Schnorr, an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.
I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

Gatersleben, 29.02.2016
(Lala Aliyeva-Schnorr)

9. Erklärung über bestehende Vorstrafen und anhängige Ermittlungsverfahren / Declaration concerning Criminal Record and Pending Investigations

Hiermit erkläre ich, Lala Aliyeva-Schnorr, dass ich weder vorbestraft bin noch dass gegen mich Ermittlungsverfahren anhängig sind. / *I hereby declare that I have no criminal record and that no preliminary investigations are pending against me.*

Gatersleben, 29.02.02016
(Lala Aliyeva-Schnorr)

10. Print-outs of the publications on which this thesis is based on and declaration on the contribution to these publications

Aliyeva-Schnorr, L., Beier, S., Karafiatova, M., Schmutzer, T., Scholz, U., Dolezel, J., Stein, N. and Houben, A. (2015a) Cytogenetic mapping with centromeric bacterial artificial chromosomes contigs shows that this recombination-poor region comprises more than half of barley chromosome 3H. *Plant J*, **84**, 385-394.

Most experimental work except preparation of pachytene chromosomes (performed together with Dr. Miroslava Karafiatova) and flow-sorting of chromosome 3H (performed by Dr. Jan Vrána) was done by me. Kmasker analysis was performed by Sebastian Beier. My contribution to the work corresponds approximately 80%.

Gatersleben, 29.02.2016 Gatersleben, 29.02.2016
(L. Aliyeva-Schnorr) (A. Houben)

Aliyeva-Schnorr, L., Ma, L. and Houben, A. (2015b) A fast air-dry dropping chromosome preparation method suitable for FISH in plants. *Journal of visualized experiments : JoVE*, **106**, DOI:10.3791/53470

Most experimental work was done by me. My contribution to the work corresponds approximately 80%.

Gatersleben, 29.02.2016 Gatersleben, 29.02.2016
(L. Aliyeva-Schnorr) (A. Houben)

Aliyeva-Schnorr, L., Stein, N. and Houben, A. (2016) Collinearity of homoeologous group 3 chromosomes in the genus *Hordeum* and *Secale cereale* as revealed by 3H-derived FISH analysis. *Chromosome Res*. DOI: 10.1007/s10577-016-9518-8

Most experimental work was done by me. My contribution to the work corresponds approximately 80%.

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Cytogenetic mapping with centromeric bacterial artificial chromosomes contigs shows that this recombination-poor region comprises more than half of barley chromosome 3H

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SUMMARY

Genetic maps are based on the frequency of recombination and often show different positions of molecular markers in comparison to physical maps, particularly in the centromere that is generally poor in meiotic recombinations. To decipher the position and order of DNA sequences genetically mapped to the centromere of barley (*Hordeum vulgare*) chromosome 3H, fluorescence *in situ* hybridization with mitotic metaphase and meiotic pachytene chromosomes was performed with 70 genomic single-copy probes derived from 65 fingerprinted bacterial artificial chromosomes (BAC) contigs genetically assigned to this recombination cold spot. The total physical distribution of the centromeric 5.5 cM bin of 3H comprises 58% of the mitotic metaphase chromosome length. Mitotic and meiotic chromatin of this recombination-poor region is preferentially marked by a heterochromatin-typical histone mark (H3K9me2), while recombination enriched subterminal chromosome regions are enriched in euchromatin-typical histone marks (H3K4me2, H3K4me3, H3K27me3) suggesting that the meiotic recombination rate could be influenced by the chromatin landscape.

Keywords: physical map, BAC FISH, centromere, recombination, chromatin, pachytene chromosomes, post-translational histone modifications.

INTRODUCTION

Barley (*Hordeum vulgare* L.) is an important crop cultivated in the temperate zones and the fourth most widely grown small-grain cereal in the world. Along with its agronomic importance, barley has played an extensive role in genomic, genetic and cytogenetic studies of Triticeae tribe (Ashida *et al.*, 2007; Mayer *et al.*, 2011). The genome of cultivated diploid barley comprises 5.1 Gbp/1C and belongs therefore to 'large-genome' species with a very high portion (>80%) of repetitive sequences. Previous studies indicated the presence of closely linked genes, forming gene islands that are interrupted by large regions of repetitive DNA (Rostoks *et al.*, 2002; Wicker *et al.*, 2009b). Gene density alongside the chromosome in cereals is known to be heterogeneous with the majority of genes clustering in highly recombinogenic distal chromosome regions. However, recent studies showed that a considerable proportion of genes are also located outside of these regions. For instance, in barley >30% of genes are located in chromosomal regions exhibiting reduced recombination (Mayer *et al.*, 2011), and genes could even be verified in regions around centromeres in wheat and barley (International Barley Genome Sequencing Consortium *et al.*, 2012; Choulet *et al.*, 2014). To fully exploit the information of the barley genome for crop improvement, the analysis of a significantly larger portion of the Triticeae genomes is needed (Stein *et al.*, 2007).

Different mapping strategies were developed over years helping to understand the organization of plant genomes. In barley, genetic maps have been constructed using various molecular markers, including restriction length polymorphism (RFLP) markers (Siedler and Graner, 1991), (Devaux *et al.*, 1993), amplified fragment length polymorphism (AFLP) markers (Qi and Lindhout, 1997), single-nucleotide polymorphism (SNP) markers (Close *et al.*,

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2009) and high-density genotyping-by-sequencing (GBS) markers (Poland *et al.*, 2012). However, genetic map distances, determined on the basis of recombination events, do not necessarily directly correlate with physical distances between molecular markers or genes. For example, in regions with reduced or even suppressed recombination small genetic distances can translate into large physical distances. Such discrepancy in the resolution of gene/marker loci can be corrected by physical or cytological mapping.

Successful integration of a physical map into genetic maps of barley has been achieved first by PCR analysis of DNA obtained from microdissected, cytologically characterized translocation chromosomes (Marthe and Künzel, 1994) using primers derived from genetically mapped sequences (Sorokin *et al.*, 1994; Künzel *et al.*, 2000; Künzel and Waugh, 2002). It was found that regions with highest recombination frequency (less than or equal to 1 Mb/cM) correspond to only 5% of the total barley genome and harbour around 50% of the 429 markers of the studied RFLP map. The mean physical to genetic ratio in this study was 1.5 Mb/cM in the distal portion of the chromosome arms and 89 Mb/cM near the centromere (Künzel *et al.*, 2000). A set of chromosome deletion lines enabled the PCR-based physical allocation of AFLP and STS markers (Serizawa *et al.*, 2001), as well as ESTs to different barley chromosomes (Ashida *et al.*, 2007; Joshi *et al.*, 2011; Nasuda *et al.*, 2005; Sakai *et al.*, 2009; Sakata *et al.*, 2010). Similar to meiotic recombination, the distribution of deletions is not randomly distributed throughout the barley chromosomes and again, the centromeric regions are less represented. The centromere of barley is enriched in a Ty3/gypsy-like repeat known as *cereba* element (*ce*ntromeric *re*troelement of *ba*rley). Each chromosome of barley is estimated to have about 200 *cereba* elements that represent the centromeric DNA of at least 1.4 Mb (Hudakova *et al.*, 2001). An interaction between centromeric nucleosomes and *cereba* as well as GC-rich satellites exists (Houben *et al.*, 2007).

In species with small genomes, such as rice (Jiang *et al.*, 1995), Arabidopsis (Fransz *et al.*, 1998), *Sorghum* (Kim *et al.*, 2002), *Brachypodium* (Hasterok *et al.*, 2006), cotton (Wang *et al.*, 2007) or tomato (Szinay *et al.*, 2008) efficient integration of a physical map into genetic maps has been achieved by fluorescence *in situ* hybridization (FISH) of labelled bacterial artificial chromosomes (BACs). This mapping approach, however, is feasible only in small genome species harbouring small amounts of repetitive sequences. In these species repetitive DNA present on BACs can be efficiently blocked (e.g. unlabelled *Cot*-1-100 fractions) (Szinay *et al.*, 2008).

Although this method provides the most direct way of physically mapping DNA sequences on chromosomes, BAC FISH as a routine method for barley and other large-genome species was not available until recently. This is because most BACs of large-genome species contain a high amount of different types of repetitive sequences and a low density of unique sequences (Steuernagel *et al.*, 2009; Wicker *et al.*, 2009a).

To decipher the position and order of sequences genetically mapped to a 5.5 cM bin around the centromere of chromosome 3H of barley we performed FISH with 70 genomic single-copy probes derived from 65 FP contigs (fingerprinted contigs) consisting of overlapping BAC clones and genetically assigned to this challenging chromosome region (Ariyadasa *et al.*, 2014; Colmsee *et al.*, 2015). Chromosome 3H was selected, because it contains a number of agronomically important genes such as *Rps1.α, Hsdr4, Sdw1* and *Btr1, Btr2* (I) (Komatsuda and Mano, 2002; Suprunova *et al.*, 2007; Yan and Chen, 2007; Jia *et al.*, 2009). More importantly, genomic sequence resources are currently more advanced for chromosome 3H than for other barley chromosomes. The employed workflow combined a tool for *in silico* detection of unique sequences suitable as FISH probe, along with a low-copy FISH technique allowing the detection of 2.5–10 kb long DNA fragments.

Our work discovered that contigs assigned to only 5.5 cM on the genetic map (3.5% of the genetic map) overlapping the centromere, comprise 58% of the physical length of mitotic metaphase chromosome 3H. The chromatin of this recombination-poor region was preferentially marked by a heterochromatin-typical histone mark (H3K9me2), while recombinationally active subterminal regions were enriched in euchromatin-typical histone marks (H3K4me2, H3K4me3, H3K27me3).

RESULTS

A 5.5 cM bin around the genetic centromere comprises 58% of the physical length of barley chromosome 3H

To determine the chromosomal position and order of 65 out of 291 sequence contigs genetically mapped to a 5.5 cM bin around the genetic centromere of chromosome 3H of barley we employed a FISH strategy, which allowed the chromosomal detection of *in silico* defined single-copy sequences derived from BAC contigs. The selection criteria for the contigs were their genetic positions within the 47.5–53.0 cM region. The overall length of the corresponding genetic map (Comadran *et al.*, 2012) is 161.6 cM. The tool Kmasker (Schmutzer *et al.*, 2014) was used to *in silico* predict single-copy regions for probe-generation. To obtain a total probe size of 2.5–10 kb, several unique fragments located up to 50 kb apart were PCR amplified from each contig of interest and pooled (Table S1). BAC sequence analysis revealed that short single-copy regions ranging from 500 to 3700 bp were often intermingled with regions containing up to 90% of repetitive sequences. 98% of all

single-copy probes yielded specific FISH signals on chromosome 3H (Figure 1a and Figure S1).

Eighteen of the 70 probes contained gene-coding sequences (Table S2). FP contigs 900, 3331, 43 477, 47 485 and 44 732 were represented by two probes each with difference in length and sequence composition, one with and one without a gene-coding sequence. However, FISH yielded identical hybridization positions, showing that neither the length of the subprobes nor the probe positions within an FP contig strongly influenced the chromosomal hybridization position. The hybridization position of the same contig-specific probe varied 0.4–0.6 μm between different metaphases and even sister chromatids. Therefore, the hybridization position of 5 to 10 chromosomes was measured to determine an average position of each probe used (Figure 1b, c and Table S2). All single-copy probes revealed specific signals on chromosome 3H (Figure 2). Simultaneous FISH with a differentially labelled 5S rDNA-specific probe was used to identify chromosome 3H (Fukui *et al.*, 1994).

FISH mapping of the low-copy probes revealed that physical dimension of the centromeric 5.5 cM bin of chromosome 3H comprised 58% of the mitotic metaphase chromosome length. The borders of this region were represented by probe 403 on the short arm and probe 44666 on the long chromosome arm, possessing the cytogenetic positions of 11% and 69%, respectively (Figures 2 and 4a). Both arms of the cytogenetic map revealed 5–8% long gaps due to the absence of suitable single-copy probes. The frequency of gene-containing probes increased towards the distal parts of chromosome 3H (Figure 2, in red).

Twelve FP contigs carried sequences with at least 90% sequence identity over a region longer than 1000 bp to the centromeric Ty3/gypsy-like element *cereba.* Six of the *cereba*-containing probes (21488, 3205, 8326, 46 563 46275 and 48 771) showed signals in the proximity of the primary constriction on chromosome 3H. Co-hybridization of probe 8326 with the barley centromeric $[AGGG]_{30}$ minisatellite confirmed the centromere position (Figure S2). The remaining *cereba*-positive probes hybridized outside the centromere. The existence of extracentromeric *cereba* sites was confirmed after identification of weak interstitial *cereba* signals along both arms in addition to the strong centromeric FISH signal (Figure S4).

To test whether naturally extended pachytene chromosomes could be used to decipher the order of probes partly colocalizing at the resolution of mitotic metaphase chromosomes (Figure 3a), we co-hybridized pachytene chromosomes with differentially labelled FP contig 1409 and 45 888 specific probes. Hybridization resulted in clearly separated signals (Figure 3b). Orientation on the pachytene chromatin fiber and proper positioning were assured by the application of a third probe, the reference probe.

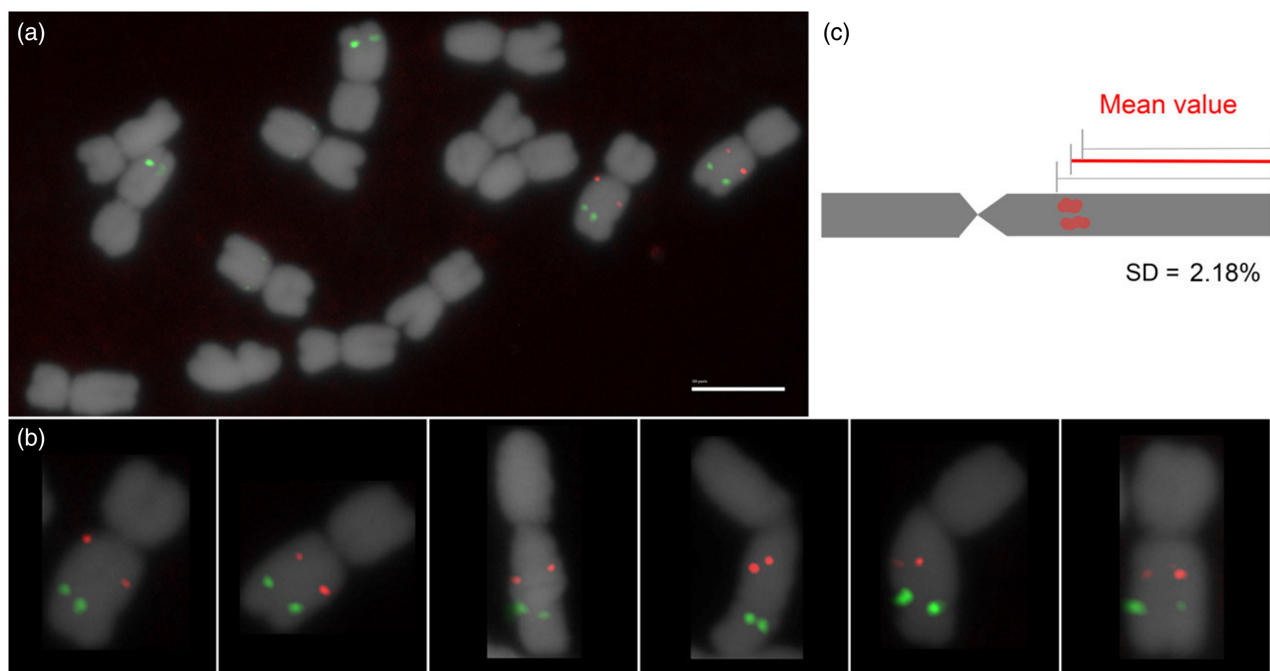

Figure 1. Fluorescence *in situ* hybridization (FISH) mapping of an FP contig-derived single-copy probe on barley chromosome 3H.
(a) Mitotic metaphase chromosomes hybridized with the diagnostic 5S rDNA probe (in green) and single-copy gene-containing probe 43477* (in red).
(b) Examples of barley chromosome 3H hybridized with the same single-copy probe (red) showing positional deviation of 2.18% and 5S rDNA probe (in green).
(c) Schematic model of chromosome 3H showing six different positions for the same probe with the indicated average position. Scale bar represents 10 μm.

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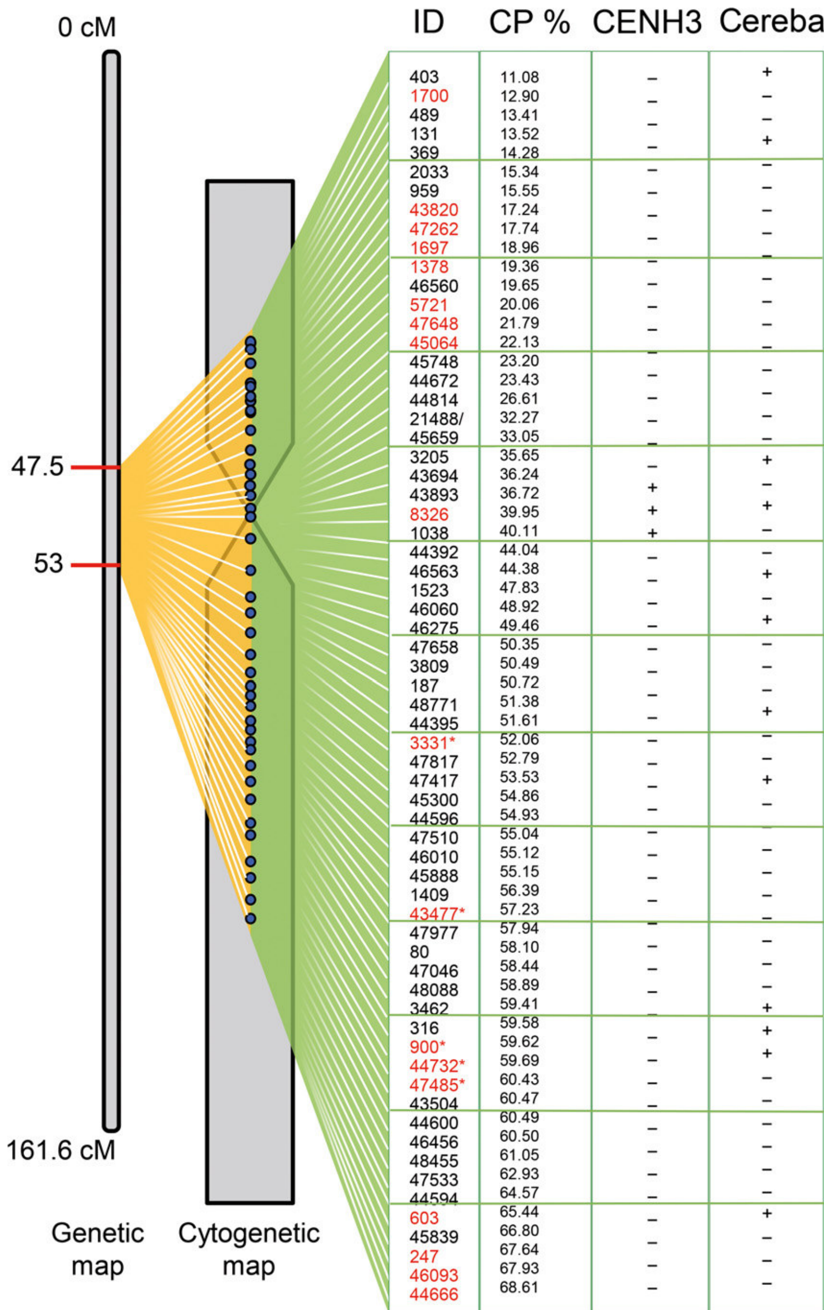

ID	CP %	CENH3	Cereba
403, 1700, 489, 131, 369	11.08, 12.90, 13.41, 13.52, 14.28	– – – –	+ – – +
2033, 959, 43820, 47262, 1697	15.34, 15.55, 17.24, 17.74, 18.96	– – – –	– – – – –
1378, 46560, 5721, 47648, 45064	19.36, 19.65, 20.06, 21.79, 22.13	– – – –	– – – –
45748, 44672, 44814, 21488/45659	23.20, 23.43, 26.61, 32.27, 33.05	– – – – –	– – – –
3205, 43694, 43893, 8326, 1038	35.65, 36.24, 36.72, 39.95, 40.11	– + + +	+ – + –
44392, 46563, 1523, 46060, 46275	44.04, 44.38, 47.83, 48.92, 49.46	– – – –	– + – +
47658, 3809, 187, 48771, 44395	50.35, 50.49, 50.72, 51.38, 51.61	– – – –	– – – +
3331*, 47817, 47417, 45300, 44596	52.06, 52.79, 53.53, 54.86, 54.93	– – – –	– – + –
47510, 46010, 45888, 1409, 43477*	55.04, 55.12, 55.15, 56.39, 57.23	– – – –	– – – – –
47977, 80, 47046, 48088, 3462	57.94, 58.10, 58.44, 58.89, 59.41	– – – – –	– – – +
316, 900*, 44732*, 47485*, 43504	59.58, 59.62, 59.69, 60.43, 60.47	– – – –	+ + – –
44600, 46456, 48455, 47533, 44594	60.49, 60.50, 61.05, 62.93, 64.57	– – – –	– – – –
603, 45839, 247, 46093, 44666	65.44, 66.80, 67.64, 67.93, 68.61	– – – –	+ – – –

Figure 2. Correlating the genetic and cytogenetic maps of barley chromosome 3H.
Genetic positions of 65 FP contigs range from 47.5 to 53.0 cM (IBSC, 2012). Cytogenetic positions of 70 deduced single-copy probes were determined by fluorescence *in situ* hybridization (FISH) and represented on the schematic chromosome. Cytogenetic position (%) of FP contigs and corresponding positions of CENH3 and centromeric *cereba* repeat are indicated by + (right panels). Probes containing a coding sequence are highlighted in red. For contigs *-labelled two independent probe pools were generated, one with and another without a coding sequence.

After first confirmation, pachytene chromosomes were used to clarify the order of the following seemingly overlapping contig pairs: 3331–44 395, 43 477–44 600, 900–3462, 44 594–45 839, 46 010–44 596, 316–900, 187–48 771, and the reference probe 80 (Figure S5).

Differences in recombination frequency are reflected by distinct sub-chromosomal distribution of posttranslational histone H3 modifications

To elucidate whether the chromosome region characterized by reduced recombination frequency correlates with distinct chromatin modifications, we determined the chromosome-wide distribution of posttranslational histone modifications, typical for eu- or heterochromatin (Jenuwein, 2001; Jenuwein and Allis, 2001). As these modifications may differ between mitosis and meiosis (Oliver *et al.*, 2013), we immunostained flow-sorted mitotic metaphase chromosome 3H as well as meiotic metaphase I chromosomes. The centromere of metaphase I chromosomes was visualized with the help of a CENH3-specific antibody.

Dimethylated lysine 9 of histone H3 (H3K9me2), a prominent mark for constitutive heterochromatin, showed a uniform distribution throughout the entire length of the chromosome, as it is typical for plants with genomes

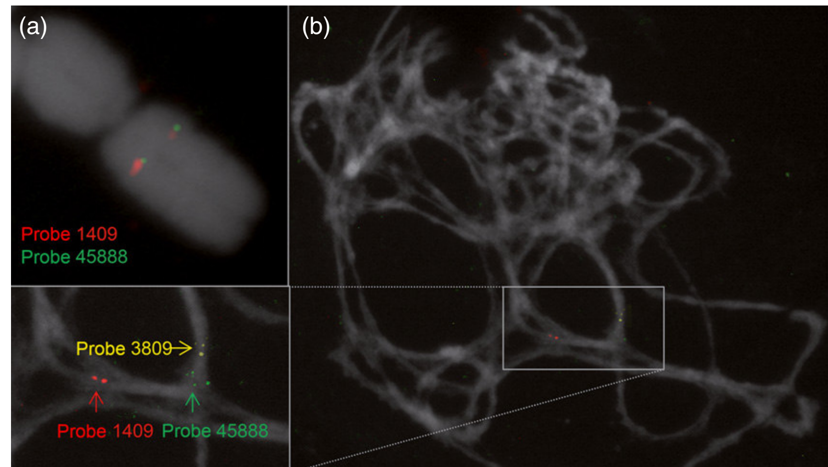

Figure 3. Comparison of single-copy fluorescence *in situ* hybridization (FISH) at resolution of (a) mitotic metaphase and (b) meiotic pachytene chromosomes of barley.
(a) After FISH on metaphase chromosomes 3H overlapping position for probes 1409 and 45 888 were observed.
(b) In contrast, FISH on pachytene chromosomes revealed distinct FISH signals of the differentially labelled single-copy probes 1409, 45 888 and 3809 (inset). Probes 1409, 45 888 and 3809 are labelled with Texas Red-12-dUTP, Alexa488-dUTP and TexasRed-12-dUTP/Alexa488-dUTP, respectively.

larger than 500 Mbp (Houben *et al*., 2003; Fuchs *et al*., 2006). In contrast, marks, such as H3K4me2 and H3K4me3 typically associated with transcriptionally potent euchromatin (Strahl *et al*., 1999; Litt *et al*., 2001), were strongly enhanced in the subterminal regions of mitotic and meiotic chromosomes (Figure 4), representing about 37% of the length of chromosome 3H (Figure 4d, e). A comparable subterminal region was detected with H3K27me3-specific antibodies, a mark for transcriptionally inactive gene-containing chromatin (Plath *et al*., 2003), (Figure 4c). Immunostaining of meiotic bivalents showed labelling at terminal parts and lack of staining in centromeric and pericentromeric regions (Figure 4c–e, right panel).

To determine the cytogenetically defined pericentromere of mitotic barley 3H, we used antibodies specific for the phosphorylated histone H3 at positions serine 10 (H3S10ph) and 28 (H3S28ph) (Figure 4f, g and Figure S3) (Houben *et al*., 1996; Gernand *et al*., 2003). The position of 21 probes (44 672, 44 814, 21 488, 45 659, 3205, 43 694, 43 893, 8326, 1038, 44 392, 1523, 46 060, 46 275, 47 658, 3809, 187, 48 771, 44 395, 3331, 47 817, 47 417) were located within the pericentromeric region, comprising the interval of 51.34–51.62 cM on the genetic map (Figure 2). Thus, the region low in recombination, comprising 58% of the physical length of the respective chromosome, is located in a region enriched in transcriptionally less active chromatin. This region is flanked by transcriptionally more potent chromatin correlating, in contrast, only with 37% of the physical length.

DISCUSSION

Sizing the genetic and physical centromere of chromosome 3H

Our cytogenetic mapping revealed that physical map BAC contigs anchored to only 5.5 cM around the genetic centromere of barley chromosome 3H represented 3.2% of a framework genetic map (Comadran *et al*., 2012), but 58% of the entire physical length of the respective chromosome. This finding is in line with earlier results showing that approximately 1.9 Gb corresponding to 48% of the genetically anchored physical map (3.9 Gb) was anchored to the genetic centromere [The International Barley Genome Sequencing Consortium *et al* (IBSC, 2012), Künzel *et al*., 2000]. Unequal distribution of recombination frequency along the chromosome is the main reason for non-linear translation of genetic distance into physical distances, hence short genetic distances may translate into shorter or longer physical distances depending on the chromosomal region. Suppressed recombination in the centromeric region is a typical feature for large-genome Triticeae (Hordeae) species, first observed for barley (Holm, 1960), followed by similar results for wheat (Dvorak and Chen, 1984; Gill and Gill, 1994) and rye (Wang *et al*., 1992). Similar discrepancies between genetic and physical maps have been earlier reported for barley (Karafiátová *et al*., 2013) as well as for other plant species such as maize (Lamb *et al*., 2007), cucumber (Yang *et al*., 2012) and tomato (Shearer *et al*., 2014) underling the importance of cytogenetic confirmation of genetic maps.

The detection of extracentromeric *cereba* repeats in addition to those allocating to a centromeric position indicates that a physical map-based prediction of the position of the centromere only based on the presence of *cereba* sequence motifs is risky and non-reliable. Indeed, it was assumed that only 95% of this Ty3/gypsy-type mobile element clustered in centromeres, while 5% of this repeat might be present outside the centromeres (Miller *et al*., 1998). Consistent with previous findings (IBSC, 2012) a number of functional genes was revealed in the less-recombining region of chromosome 3H. Though, the reason for suppressed recombination is not fully understood, it is assumed that reduced recombination might protect evolutionarily selected and co-adapted gene structures (Stevison

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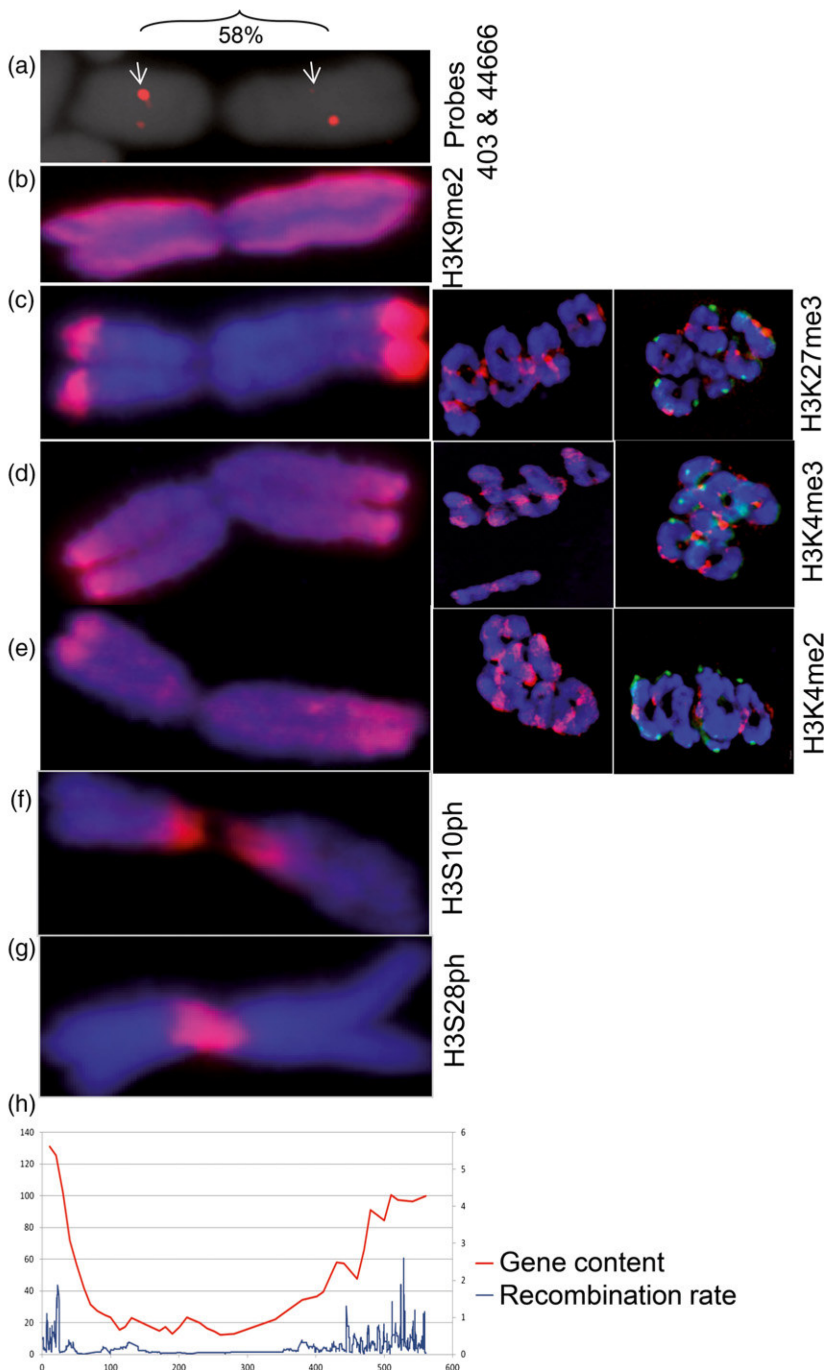

Figure 4. Characterization of the epigenetic landscape of chromosome 3H.
(a–e) (a) Fluorescence *in situ* hybridization (FISH) with probes 403 and 44 666 marked the outer boundaries of the 5.5 cM bin around the genetic centromere of 3H. Immunostaining (in green) of flow-sorted chromosome 3H with antibodies recognizing (b) the heterochromatin-typical mark H3K9me2 as well as the euchromatin-typical marks (c) H3K4me2, (d) H3K4me3, and (e) H3K27me3. Corresponding meiotic metaphase I chromosomes are shown in the right panel.
(f, g) The pericentromeric regions were stained with antibodies recognizing H3 either phosphorylated at serine position (f) 10 or (g) 28.
(h) Schemata of the distribution of genes and meiotic recombination rates along the physical map of chromosome 3H, based on data from IBSC (2012).

et al., 2011). Moreover, preferential occurence of cross overs in the distal parts of barley chromosomes was shown to be associated with the timing of the recombination initiation that is delayed in the proximal and interstitial regions (Higgins *et al.*, 2012). Hence, such regions may remain concealed for crop improvement by conventional breeding.

Detection of eu- and heterochromatic histone marks revealed that the physical distribution of the 5.5 cM region anchored to the genetic centromere correlated with chromatin containing preferentially the heterochromatin-typical posttranslational histone modification H3K9me2. In contrast, antibodies recognizing the euchromatic marks H3K4me2, H3K4me3 and H3K27me3 stained mainly distal regions of mitotic metaphase chromosomes, representing 37% of the entire length of chromosome 3H. A similar staining was detected for meiotic metaphase I chromosomes, confirming a stable chromosomal distribution of the employed posttranslational histone modifications in mitosis and meiosis. A comparable distribution of histone

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marks was described for *Aegilops* and rye (Oliver *et al.*, 2013). Thus the recombination frequency correlates with a distinct chromatin structure and supports the earlier established link between euchromatin and recombinationally active regions (Tanksley *et al.*, 1992; Higgins *et al.*, 2012, 2014).

Design and selection of probes suitable for FISH mapping of repeat-rich genomic contigs: assets and drawbacks

Physical mapping of genomic contigs of the large-genome species barley applied here was based on cytogenetic tools along with the *in silico* extractions of repeat-free sequences suitable for FISH. The probe length sufficient for unambiguous mapping was reduced from 7 kb in the previous study (Poursarebani *et al.*, 2013) to 5 Kb. Probes below this range resulted only in faint signals and required moderately condensed high quality chromosome spreads free of any cytoplasm and avoiding enzymatic overdigestion. The shortest sequence that could be mapped reproducibly in this study was 2.2 kb in length (Figure S1). The correct hybridization position of this probe was confirmed by applying a 7.7 kb long probe derived from the same contig. The selection of fluorescent dye was another factor influencing strongly the intensity of FISH signals. The brightest signals were produced with Texas Red labelled probes confirming previous observations (Kato *et al.*, 2006; Karafiátová *et al.*, 2013). The second best label was the fluorochrome Atto 550 Cy3. Alexa 488 signals were less intense and often produced background noise. This observation can be explained by the lower labelling efficiency, because differences in the refraction index would have a reverse effect, benefiting Alexa 488 in comparison with other fluorochromes (Baddeley *et al.*, 2009). Application of three different fluorochromes facilitated the mapping procedure on pachytene chromosomes; hence the use of a Texas Red/Alexa488 fluorochrome mixture could be used for additional probe.

Overall, our study produced a cytogenetic map for 65 physical map contigs anchored to a 5.5 cM interval including the genetic centromere of chromosome 3H. The cytogenetic mapping approach delivered an increase in resolution for the physical map in the region associated with the genetic centromere and emphasized the importance of alternative approaches for resolving physical map order in regions with suppressed recombination. Our mapping of centromeric sequences may facilitate future studies on the structure of centromeres. Moreover, we established a protocol for the preparation of barley pachytene chromosomes suitable for low-copy FISH, allowing a better resolution of signals on the chromosome and therefore a more precise mapping of contig positions. Immunodetection of typical eu- and heterochromatic histone marks on mitotic and meiotic metaphase chromosomes of barley demonstrated that recombination-reduced regions of 3H were mainly composed of heterochromatin, while the recombination enriched subterminal regions were enriched in euchromatin, underlining the fact that meiotic recombination rate is likely to be influenced by the chromatin landscape.

Using barley as a model we were able to show that cytogenetic mapping of BAC contigs is feasible in large-genome species with a high portion of repetitive sequences. Even though single-copy FISH is not a high-throughput technique, it asserted itself as a powerful tool in ordering of contig positions derived from non-recombining regions having a low resolution on the genetic map. Therefore, cytogenetic map can be used to improve the genome assembly, particularly in heterochromatic regions with low or suppressed recombination.

EXPERIMENTAL PROCEDURES

Plant material and preparation of mitotic cells for FISH

Hordeum vulgare cv. Morex (2n = 2x = 14) seeds were germinated under dark conditions and young roots were treated with ice-water for 20 h to increase the frequency of metaphase cells. Then root tips were fixed in 3:1 (v/v) ethanol: acetic acid mixture at room temperature (RT) for 2 days and stored at 4°C. Chromosome preparation was performed using the dropping technique (Kato *et al.*, 2004). Post-fixation of slides was carried out according to Ma *et al.* (2010).

Preparation of pachytene chromosome spreads for FISH

Plants were grown under a 16 h photoperiod at 20°C during the day and 15°C at night in the green house. Anthers of barley cv. Morex at pachytene stage are light green and around 0.8–1 mm long. Staged spikes were collected and fixed in freshly prepared 3:1 (ethanol: acetic acid) for at least 1 week at 4°C. Anthers were isolated and rinsed twice in distilled water followed by rinsing twice in 0.01 M sodium citrate buffer (0.01 M citric acid, 0.01 M sodium citrate, pH 4.8) before incubation in an enzyme mix (0.7% cellulase Onozuka R10 (Duchefa, C8001, https://www.duchefa-biochemie.com), 0.7% cellulase (CalBioChem, 319466, http://www.merckmillipore.com), 1% pectolyase (Sigma, P3026) and 1% cytohelicase (Sigma, C8274, http://www.sigmaaldrich.com/catalog) in 0.01 M citrate buffer for 2.5 h at 37°C. After enzyme treatment anthers were gently washed in distilled water and kept on ice. Anthers were squashed between slide and coverslip in a drop of 45% acetic acid. To reduce the amount of cytoplasm the slides were shortly heated over a Bunsen burner flame (de Jong *et al.*, 1999). After, the slides were transferred into liquid nitrogen and coverslips were removed and dehydrated in 70, 90 and 99% ethanol for 2 min each. The quality of all slides was checked under a phase-contrast microscope and well spread pachytene chromosomes were selected.

Generation of single-copy FISH probes

FP contigs were sequenced by the IBSC to generate a map-based sequence of barley using the minimal tiling path provided by the physical map (IBSC, 2012). FP contigs anchored to the genetic centromere of 3H chromosome were selected for the design of single-copy FISH probes. To identify unique sequences, contigs were processed by Kmasker (Schmutzer *et al.*, 2014). For each of the *in silico* extracted single-copy sequences PCR primers were designed using the program Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/, Table S1). Following parameters were set: optimal Tm = 57°C

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(range 55–60°C with max. difference of 2°C between forward and reverse primers), optimal GC content = 50% (range 40–60%). Single-copy fragments with a length between 1800 and 6500 bp were amplified by touchdown PCR using the corresponding BAC DNA as template. The PCR program comprised of 36 cycles with the first seven cycles having an annealing temperature of 63°C. The annealing temperature was gradually decreased (1°C/every cycle) until it reached 56°C. The 5S ribosomal DNA probe was generated from genomic DNA of barley by PCR as described earlier (Fukui *et al.*, 1994). Obtained PCR fragments were purified and labelled by nick translation with Texas Red-dUTP, Alexa-488-dUTP (Invitrogen, Life Technologies, https://www.lifetechnologies.com) or Atto 550 CY3, according to Kato *et al.* (2004). Probe combinations for the pachytene chromosomes were labelled by Texas Red for the first probe, Alexa488 for the second probe and the reference probe was labelled either by Atto550 or by a 1:1 mixture of Texas Red and Alexa488. Synthesized DNA oligonucleotide $[AGGG]_{30}$ was labelled by FITC and applied as a centromeric repeat (Eurofins MWG Operon, Ebersberg, Germany). *Cereba* probe was generated from BAC 7 isolated using Qiagen Plasmid Mini Kit (100) and labelled directly by Texas Red-dUTP. The PCR program used for amplification comprised 30 cycles with the annealing temperature of 60°C. Detailed probe information is shown in Table S1.

FISH procedure and quantification of single-copy FISH signal positions

Single-copy FISH was performed as described earlier (Kato *et al.*, 2006; Ma *et al.*, 2010) with minor alterations: specimen were washed in 2× SSC and treated with 45% acetic acid for 10 min at RT before FISH. For post-fixation, slides were incubated for 10 min in 4% formaldehyde and subsequently washed in 2× SSC for 3 × 5 min, then slides were dehydrated in a series of increasing ethanol concentration (70, 90 and 96%, v/v), and air dried. The hybridization mixture contained 50% deionized formamide, 25% 20× SSC, 1 M Tris–HCL pH 8.0, 1.5–3 μl (135 ng μl^{-1}) single-copy probe, 10 $\mu g\ ml^{-1}$ salmon sperm DNA, and 0.5 M EDTA. Hybridization mixture was denatured together with the chromosomal DNA on a hot plate at 80°C for 2 min. Hybridization at 37°C was performed for 12–20 h in a moist chamber. Subsequently, slides were washed in 2× SSC for 20 min at 58°C and dehydrated in an ethanol series (70, 90 and 96%). Finally, the slides were air dried at RT and counterstained with 1 $\mu g\ ml^{-1}$ 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories, http://vectorlabs.com/). Images were acquired with an epifluorescence microscope BX61 (Olympus, http://www.olympus.fi/medical/en/microscopy) using a cooled charge coupled device (CCD) camera (Orca ER, Hamamatsu, www.hamamatsu.com). Pictures were processed and merged using Adobe Photoshop (Adobe Systems Incorporated, USA, http://www.adobe.com).

For each single-copy probe six to 10 metaphase 3H chromosomes showing a specific hybridization signal were measured. The length of the chromosome, the length of the long arm and the position of the hybridization signal from the end of the long arm were measured with the help of Photoshop and/or Image J (Image Processing, http://imagej.net). The relative cytological position (CP) was determined as: $CP = (S/T) \times 100\%$. Where T is the total length of the chromosome and S is the distance from the end of the long arm to the signal (Table S2).

Flow sorting of chromosomes for immunostaining

Seeds of *H. vulgare* cv. Morex were germinated and young seedlings were used to prepare suspensions of intact mitotic metaphase chromosomes from synchronized root tips according to Lysák *et al.* (1999). Chromosome fractions highly enriched for chromosome 3H were obtained following the approach of Vrána *et al.* 2015). Briefly, the composite peak on barley flow karyotype, which represents chromosomes 2H-7H was divided into small sections, and the section with the highest proportion of chromosome 3H was used as sort window. The chromosomes were sorted into a drop of 7.5 μl LB01 buffer (Doležel *et al.*, 1989) on microscopic slides and left dry at RT overnight. Afterwards, slides were transferred into 100% glycerine and stored at 4°C until use.

Preparation of meiotic chromosomes and indirect immunostaining

Plants were grown under a 16 h photoperiod at 23°C during the day and 17°C at night in the green house. Anthers at metaphase I stage are light green and around 1–1.2 mm long. Anthers were isolated and fixed in 4% ice-cold paraformaldehyde dissolved in 1 × phosphate-buffered saline (PBS, pH 7.3) for 30 min. After, anthers were washed three times for 5 min in ice-cold 1 × PBS before the treatment in 0.1% enzyme mix (stock enzyme solution in 1 × PBS: 0.7% cellulase Onozuka R10 (C8001, Duchefa), 0.7% cellulase (319466, CalBioChem), 1% pectolyase (P3026, Sigma) and 1% cytohelicase (C8274, Sigma) for 0.5 h at 37°C. After enzyme treatment anthers were gently washed in 1 × PBS and kept on ice. Digested anthers were then squashed between the glass slide and the cover slip in a drop of 1 × PBS with 0.01% Tween20. Slides were either used immediately or stored in 99% ethanol at −20°C.

Slides with flow-sorted mitotic chromosomes were washed in 1 × PBS to remove glycerine. Meiotic metaphase I chromosome slides were used fresh after preparation. Then, slides were incubated for 30 min in 1 × PBS containing 3% BSA (w/v) and 0.1% TritonX-100 in the humid chamber at RT. After two washes in 1 × PBS for 5 min each, slides were incubated with primary antibodies. All primary histone antibodies [(anti-H3K4me2 (CN.07-030, Merck Millipore, http://www.merckmillipore.com), H3K4me3 (CN.07-473, Merck Millipore), H3K27me3 (CN.07-449, Merck Millipore), H3K9me2 (CN.39239, Active Motif, http://www.activemotif.com/), H3Ser10ph (CN.09-797, Merck Millipore), H3Ser28ph (CN.07-145, Merck Millipore)] and an Atto488 directly labelled anti-grass CENH3 (Sanei *et al.*, 2011) using a labelling kit (LK01-10, Dojindo EU, http://www.dojindo.eu.com/) were diluted 1:100 in 1 × PBS, 1% BSA. Incubation lasted for 12 h at 4°C. After three washes in 1 × PBS for 10 min each, slides were incubated with FITC-conjugated anti-rabbit antibodies, diluted 1:400 in 1 × PBS, 1% BSA, for 1 h at 37°C. Finally, slides were washed in 1 × PBS and counterstained and analysed as described for FISH.

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SUPPORTING INFORMATION

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Figure S1. Selected metaphase barley 3H chromosomes after FISH with 65 single-copy probes derived from FPcontigs.

Figure S2. Immunolocalization of different histone methylation marks on flow-sorted chromosome 3H of barley.

Figure S3. The centromeric regions of 3H encode genes.

Figure S4. FISH with the centromeric *cereba* Ty3/gypsy retroelement (green) and the 5S rDNA (yellow) probe on metaphase chromosomes of barley.

Figure S5. Identification of the correct order of overlapping probes by FISH using barley pachytene chromosomes.

Table S1 Subprobes and primer pairs designed for the generation of single-copy probes derived from 65 FPcontigs.

Table S2 List of used single-copy FISH probes with genetic position, probe length and the determined cytogenetic position (CP) as well as the standard deviation for CP.

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Video Article

A Fast Air-dry Dropping Chromosome Preparation Method Suitable for FISH in Plants

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Abstract

Preparation of chromosome spreads is a prerequisite for the successful performance of fluorescence *in situ* hybridization (FISH). Preparation of high quality plant chromosome spreads is challenging due to the rigid cell wall. One of the approved methods for the preparation of plant chromosomes is a so-called drop preparation, also known as drop-spreading or air-drying technique. Here, we present a protocol for the fast preparation of mitotic chromosome spreads suitable for the FISH detection of single and high copy DNA probes. This method is an improved variant of the air-dry drop method performed under a relative humidity of 50%-55%. This protocol comprises a reduced number of washing steps making its application easy, efficient and reproducible. Obvious benefits of this approach are well-spread, undamaged and numerous metaphase chromosomes serving as a perfect prerequisite for successful FISH analysis. Using this protocol we obtained high-quality chromosome spreads and reproducible FISH results for *Hordeum vulgare, H. bulbosum, H. marinum, H. murinum, H. pubiflorum* and *Secale cereale*.

Video Link

The video component of this article can be found at http://www.jove.com/video/53470/

Introduction

Fluorescence *in situ* hybridization (FISH) is an effective tool for the physical mapping of single and high copy sequences at the chromosomal level. Prerequisite is the preparation of high quality chromosome spreads. There is no general chromosome preparation protocol that would be equally suitable for animal and plant cells. Preparation of plant chromosomes is particularly challenging due to the rigid cell wall and various cytoplasm consistency within different species. One of the favorable methods for the preparation of plant chromosomes is a so-called drop technique also known as drop-spreading technique and air-drying technique [1,2]. This method was first introduced in 1958 by Rothfels and Siminovitch for *in vitro* grown mammalian cells [3]. Later Martin *et al.* [4] and Kato *et al.* [5] adapted this method for plants.

More recently, a method named 'SteamDrop' was developed which used water steam for the preparation of non-overlapping chromosomes [6]. Although, the positive influence of high humidity was observed earlier [7], 'SteamDrop' delivers a controlled workflow of high-quality chromosome preparations [6]. The steam treatment causes stretching of chromosomes probably connected to some modifications of chromosomal proteins. The quality of resulting metaphase spreads is very high, although retaining of sufficient number of complete metaphase spreads for subsequent FISH experiments demands technical expertise.

Here we present a protocol for the preparation of mitotic cereal chromosomes suitable for the FISH detection of single and high copy probes [5,8]. This method is an improved variant of the air-dry dropping method described by Kato [9] performed under relative humidity of 50%-55% (**Figure 1**). This protocol comprises a reduced number of washing steps making its application easy, efficient and reproducible. Using this protocol we obtained high-quality chromosome spreads and FISH results for *Hordeum vulgare, H. bulbosum, H. marinum, H. murinum, H. pubiflorum* and *Secale cereale*.

Protocol

1. Chromosome Preparation

1. Seed germination and fixation of root tips
    1. Germinate 10-20 barley seeds on two layers of moist filter paper in a Petri dish under dark conditions for 2 days at 22-24 °C. Cut off vigorous roots with the length of 1-2 cm from the seed by using a razor blade.

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2. Prepare ice-cold water by placing a 500 ml glass bottle containing cold tap water into crushed ice-water. Aerate the ice-cold water and immerse root tips for 20 hr to increase the frequency of metaphase cells.
3. Transfer roots from water to 50 ml of ethanol: acetic acid (3:1) fixative to fix them at RT for 2 days. Store roots in a freshly prepared ethanol: acetic acid (3:1) fixative at 4 °C until use up to a year.

2. Washing and enzyme treatment
    1. Wash the 10-20 roots with 30 ml ice-cold tap water for 5 min twice using a 50 ml glass beaker. Use binocular microscope. Transfer roots one by one into 30 ml 0.01 M citrate buffer (0.01 M citric acid + 0.01 M sodium citrate, pH 4.8) using forceps and wash by shaking the glass beaker for 5 min twice. Place roots on filter paper to remove the liquid completely and cut-off undesired non-meristematic tissue using a razor blade.
    2. Incubate up to 20 root tips in 1 ml enzyme mixture at 37 °C for about 50 min to soften the plant tissue (**Table 1**) in a watch glass. Enzyme mixture contains 0.7% cellulase R10, 0.7% cellulase, 1% pectolyase and 1% cytohelicase diluted in 0.01 M citrate buffer. Store the enzyme mixture at -20 °C and reused up to five times.
    3. Remove the enzyme by pipetting and wash the root tips on ice with 5 ml 0.01 M citrate buffer twice to replace the residual enzyme.

3. Root maceration
    1. Wash root tips with 1 ml 96% ethanol twice carefully in the same watch glass. Replace ethanol with freshly prepared fixative (75% acetic acid : 25% ethanol). Use 10-15 µl fixative per root tip.
    2. Transfer root tips together with the fixative into a 2 ml tube and disintegrate root meristems with a dissecting needle or forceps. Tap the tube 20 times to re-suspend cells to obtain a cell suspension. Store the cell suspension at -20 °C up to two months.

4. Dropping of the cell suspension
    1. Place 2-3 layers of water-soaked paper tissue on a hot plate at 50 °C. Immerse microscopic slides in ice-cold tap water in the fridge for 30 min. And place slides on top of the moist paper tissue.
    2. Pipette 7-10 µl of cell suspension and drop it from a distance of 20 cm onto the cooled slide placed on the hot plate. Pipette 10 µl of acetic acid-ethanol mixture on the same place as cell suspension on the slide and keep the slide on the hot plate for additional 2 min. Place the slide on the hot plate without the wet tissue and let it dry for 1 min.

5. Quality control and storage of slides
    1. Check slides using a phase-contrast microscope to control the quality of the chromosome spread. Use slides either the same day or store by immersing in 96% ethanol in a Coplin jar at -20 °C.

6. Pretreatment of slides before FISH; all steps are carried out at RT
    1. Place slides in a Coplin jar containing 50 ml of 2x SSC (20x SSC contains 3 M NaCl and 300 mM trisodium citrate) for 5 min. Using forceps, transfer slides to a Coplin jar containing 50 ml of 45% acetic acid for 3-10 min.
    2. Transfer slides to a Coplin jar containing 50 ml of 2x SSC for 10 min. Transfer slides to a Coplin jar containing 50 ml of 4% formaldehyde (in 2x SSC) and immerse slides for 10 min to fix chromosomes.
    3. Remove formaldehyde by rinsing the slides 3 times for 4 min each, in a Coplin jar containing 50 ml 2x SSC. Dehydrate slides in a Coplin jar for 2 min in series of 70%, 90% and 100% ethanol, respectively and dry slides in a vertical position.

2. Fluorescent *In Situ* Hybridization (FISH)

1. For each slide, prepare a hybridization solution of 20 µl in total using 10 µl of deionized formamide, 5 µl of 4x hybridization buffer (200 µl buffer contains 80 µl 20x SSC, 8 µl 1 M Tris-HCl pH 8.0, 1.6 µl 0.5 M EDTA, 11.2 µl 10 µg/ µl salmon sperm and 99.2 µl DNase-free water), 3 µl of the probe and 2 µl of DNase-free water.
2. Add 20 µl of hybridization solution per slide and cover with a 24 x 32 mm cover slip and arrest the cover slip with rubber cement. Denature slides with probes simultaneously at 80 °C for 2 min on a hot plate.
3. Transfer slides to a moist chamber and incubate slides at 37 °C O/N avoiding light. Remove cover slips by rinsing the slides in a Coplin jar with 2x SSC. Place slides in a Coplin jar containing 55-60 °C 2x SSC and incubate for 20 min.
4. Place the slides to 2x SSC in a Coplin jar for 2 min at RT. Dehydrate slides in a Coplin jar for 2 min in series of 70%, 90% and 100% ethanol, respectively.
5. Air-dry the slides and counterstain with 1 µg/ml 4',6-diamidino-2-phenylindoline (DAPI) in antifade mounting medium, avoid intense light.

3. Microscopic Analysis and Storage

1. Analyze the slides using an epifluorescence microscope. The selection of filter depends on the fluorochrome used for probe labeling. If necessary, store slides at 4 °C under dark conditions up to a year.

Representative Results

Microscopic slides with the mitotic metaphase spreads were prepared by the fast air-dry dropping chromosome preparation method described above (**Suppl. Figure 1**). FISH analysis was carried out using both, repetitive and single-copy sequences. Images were obtained by a epifluorescence microscope with a set of filters enabling excitation of corresponding fluorophores and captured by a high-sensitivity CCD monochrome camera. For the image acquisition we used a computer with an image acquisition software. Results of the FISH experiments on mitotic metaphase chromosomes using 5S rDNA, $[CTT]_{10}$, and single-copy probes were distinct and of a high quality for *Hordeum vulgare* (**Figre 2A, B**), *H. bulbosum* (**Figure 2C**), *H. marinum, H. murinum, H. pubiflorum* and *Secale cereale* (**Figure 2D**). Obvious benefits of this approach are well-spread, undamaged and numerous metaphase chromosomes serving as a perfect prerequisite for successful FISH analysis. It is possible to store the cell suspension at -20 °C up to two months and to prepare the chromosome spreads on the day of the FISH experiment. Freshly

prepared slides can be also stored at -20 °C in 96% ethanol, though we observed that the quality of hybridization signals on such chromosomes is reduced compared to the freshly-prepared metaphase spreads. The methods can be used to prepare high-quality chromosome spreads in cereals in an easy, efficient and reproducible way and most likely can be used in other plant species too.

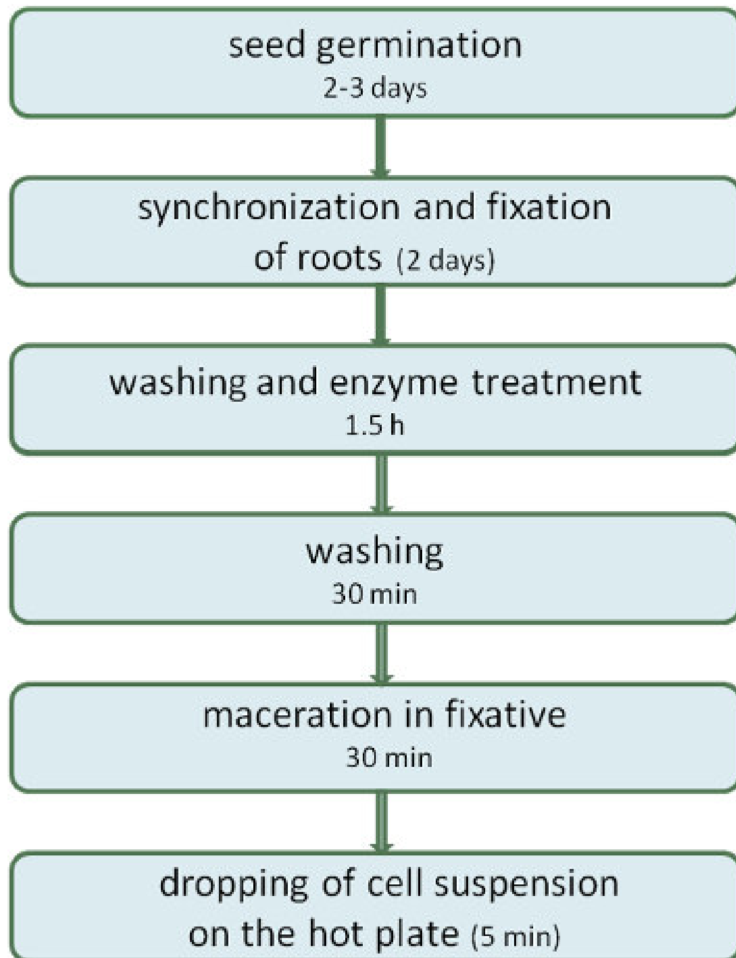


Figure 1. A scheme describing the procedure of the air-dry dropping plant chromosome preparation method.

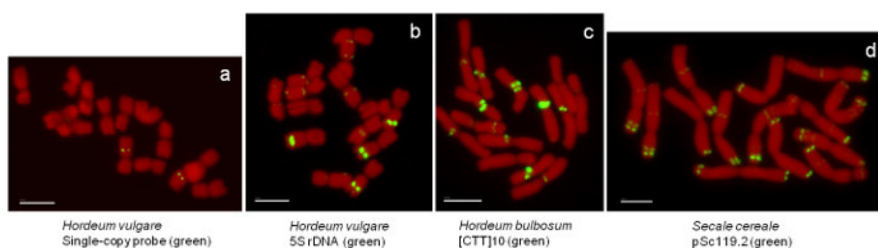
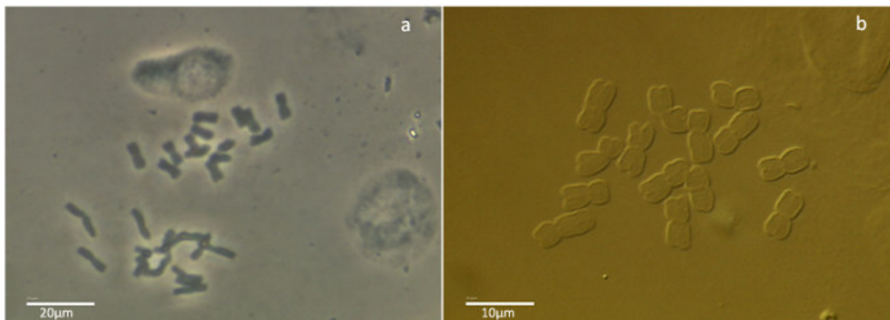


Figure 2. FISH on mitotic metaphase chromosome spreads of *Hordeum vulgare*, *H. bulbosum* and *Secale cereale* prepared by the air-dry dropping method. (**A**) *H. vulgare* with a single copy probe (FPct_40752) labeled with a red fluorescent dye. (**B**) *H. vulgare* with 5S rDNA probe labeled by a green fluorescent dye. (**C**) *H. bulbosum* with CTT-microsatellite labeled by a green fluorescent dye and (**D**) *S. cereale* with pSc119.2 repeat labeled by a green fluorescent dye. All chromosomes were counterstained with DAPI (in red). FISH signals are shown in yellow. Scale bar = 10 µm. Please click here to view a larger version of this figure.

Species	Enzyme treatment time in [min]
Hordeum vulgare	50
Hordeum bulbosum	55
Hordeum marinum	50
Hordeum murinum	55-60
Hordeum pubiflorum	60-65
Secale cereale	55-60

Table 1. Incubation time of enzyme treatment for different species.



Suppl. Figure 1. Phase-contrast and differential interference contrast (DIC) images of mitotic metaphase chromosome spreads of the air-dry dropping plant chromosome preparation method on the example of *Hordeum vulgare*. (**A**) Phase-contrast image taken at 200X magnification and (**B**) Differential interference contrast image taken at 630X magnification. Please click here to view a larger version of this figure.

Discussion

The chromosome preparation experiment has been carried out using young roots of cereals belonging to the grass family (Poaceae). All analyzed species have 14 relatively long mitotic metaphase chromosomes (11-15 µm) in the diploid genome set and belong to large-genome species (5.1-7.9 Gbp).

Length of germinated roots was not more than 2 cm to obtain a maximum of meristematic tissue. Synchronization of dividing cells was achieved by a 20 hr long ice-water treatment that improved the quantity of mitotic metaphase spreads [10].

Two steps are important for the preparation of high-quality chromosome preparations: (I) the relative humidity of 50%-55% and (II) duration of the enzyme treatment. The first point was achieved by placing wet paper tissues on a hot plate in proximity of the glass slides. The relative humidity was measured with a hygrometer. The optimal humidity for the preparation of plant chromosomes was similar to the humidity reported by Kirov *et al.* [6]. The positive effect on the chromosome quality at optimal relative humidity occurs by swelling of the cytoplasm and cell wall hydrolysis.

The duration of enzyme treatment is species dependent (**Table 1**). The period of enzyme treatment also depends on the time span of root fixation in ethanol/acetic acid and the size of the roots. The longer roots were stored in the fixative (up to 1 year at 4 °C), the longer it takes to digest roots to the proper grade. Insufficiently digested root material is difficult to macerate and will increase the total time of preparation as a result of long lasting maceration. Moreover, metaphase chromosomes remain embedded into cytoplasm that could hamper ensuing probe penetration during the FISH experiment. On the other hand over-digested material can influence the structure of the chromosomes themselves, and damage target DNA for the FISH analysis.

An additional factor for the improvement of the preparation is the use of the second drop of fixative (3:1, acetic acid/ethanol). High concentration of acetic acid in this mixture stimulates the digestion of cytoplasm and promotes chromosome spreading in species with large chromosomes. Cytoplasm reduction can also take place after the immobilization of the chromosomes on slides. For this purpose microscope slides carrying the chromosome spreads can be incubated in 45% acetic acid at RT for 2-10 min depending on cytoplasm level. Quality check of chromosome spreads was performed with a phase-contrast microscope without any supplementary staining (*e.g.,* 1% aceto-carmin). Normally more than 25 slides containing high-quality chromosome spreads can be obtained from 20 roots using the method above.

Results of the FISH experiments on mitotic metaphase chromosomes using 5S rDNA, $[CTT]_{10}$, and 6 kb long single-copy probe (FPct_40752) were distinct and of a high quality for all species described above (**Figure 2**). Obvious benefits of this approach are well-spread, undamaged and numerous metaphase chromosomes serving as a perfect prerequisite for successful FISH analysis. It is possible to store the cell suspension at -20 °C up to two months and to prepare the chromosome spreads on the day of the FISH experiment. Freshly prepared slides can be also stored

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at -20 °C in 96% ethanol, though we observed that the quality of hybridization signals on such chromosomes is reduced compared to the freshly-prepared metaphase spreads.

Chromosome spreads prepared by the fast air-dry dropping technique were suitable for FISH and were reproduced a number of times. Combination of this chromosome preparation method with FISH could be widely applied to explore the genome organization in plants, for instance, for karyotyping[11], chromosomal mapping [12], in synthetic studies, and for the integration of physical and genetic maps[13].

Disclosures

The authors have nothing to disclose.

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Collinearity of homoeologous group 3 chromosomes in the genus *Hordeum* and *Secale cereale* as revealed by 3H-derived FISH analysis

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Abstract Crop wild relatives are considered as important genetic resources of allelic diversity for domesticated crop species. Their utilization in breeding programs, however, is often limited due to crossing barriers and genome incompatibilities. Wild relatives of barley possess attractive properties and hence allelic diversity for adapting barley better to changing environmental conditions. Therefore, gaining a better knowledge about genomic synteny between cultivated barley and wild relatives of the same genus is an important task. To visualize genomic collinearity in related species, 22 genomic single-copy and 14 complementary DNA (cDNA) chromosome 3H-specific probes were mapped to the chromosomes of *Hordeum bulbosum*, *Hordeum marinum*, *Hordeum pubiflorum*, *Hordeum murinum*, and *Secale cereale* by fluorescent in situ hybridization (FISH). Most probes showed reliable signals confirming homoeology between cultivated barley and related species. Differences in order and position of FISH markers demonstrated sequence movements or small-scale chromosomal rearrangements within genus *Hordeum* and confirmed interchromosomal rearrangements between barley and rye. Comparison between repeat-free genomic and cDNA probes showed that gene-containing single-copy genomic DNA (gDNA) probes are performing more reliably for FISH-based analysis of synteny.

Keywords FISH · synteny · chromosome 3H · genus *Hordeum* · *Secale cereale* · Triticeae

Abbreviations

Alexa 488	Green-fluorescent dye
cDNA	Complementary DNA
Cy3	Cyanine dye
dUTP	Deoxyuridine triphosphate
DAPI	4′,6′-Diamidino-2-phenylindole
fl-cDNA	Full-length complementary DNA
FISH	Fluorescence in situ hybridization
FPcontig	FingerPrinted Contig
gDNA	Genomic DNA
NOR	Nucleolus organizer region
rDNA	Ribosomal DNA

Introduction

The genus *Hordeum* comprises one of the important crop species worldwide—barley (*Hordeum vulgare* subsp. *vulgare*). Besides barley, the genus *Hordeum* includes further 32 species, all of them being morphologically characterized by an inflorescence (spike) bearing three sessile single-flowered spikelets per node. The wild relatives of barley possess a potential to increase

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genetic diversity and therefore agricultural performance of barley under changing environmental conditions (Chalmers et al. 1992; Jacobsen and von Bothmer 1995).

Early cytogenetic studies of interspecific *Hordeum* hybrids suggested the existence of four basic genomes, called H, I, Xa, and Xu (following the nomenclature of Blattner 2009), based on the average ratio of meiotic crossing-over events (Lange 1971; Thomas and Pickering 1988; von Bothmer et al. 1983, 1988, 1989). *H. vulgare* together with *H. bulbosum* possess the H genome. The *H. marinum* group is known to have the Xa genome. The Xu genome is common for *H. murinum* and its subspecies, and the I genome occurs in the wild *Hordeum* species from North and South America. The genus *Hordeum* can also be divided into three gene pools in relation to barley (Jacobsen and von Bothmer 1995). Different varieties of cultivated barley and *H. vulgare* subsp. *spontaneum* represent the primary gene pool. *H. bulbosum* is the only species of the secondary pool and the tertiary gene pool includes all the other *Hordeum* species, which do not cross with barley.

Exact estimations of the age of the genus and the time of divergence between individual species are not available due to the lack of a fossil record for the tribe Triticeae including the genus *Hordeum*. Nevertheless, approximate ages were calculated based on the divergence time of 15 million years (my) between barley and wheat (Marcussen et al. 2014). According to this, the genus *Hordeum* started to diversify about 9 million years ago (mya) and the split between primary and secondary gene pools likely occurred about 4 mya (Blattner et al. 2004).

Phylogenetic relationships in *Hordeum* have been studied intensively using different molecular markers such as amplified length polymorphisms (AFLP), random amplified polymorphic DNA (RAPD), sequence-tagged sites (STS), nuclear ribosomal DNA (nrDNA), ribosomal DNA (rDNA), and chloroplast regions (Blattner 2004; Jakob and Blattner 2010; Terzi et al. 2001; Vos et al. 1995). A recent study on the phylogeny of *Hordeum* applied second-generation sequencing using amplicons of chloroplast and nuclear single-copy loci to clarify relationships of all diploid and polyploid taxa of *Hordeum* (Brassac and Blattner 2015). The results of these studies provided insights into the relationships among *Hordeum* species, although not all conclusions were unanimous.

Another method for studying genome evolution including chromosome structure and organization within a genus or tribe is fluorescence in situ hybridization (FISH) (Danilova and Birchler 2008; Ma et al. 2010). FISH mapping applied with repeat-free, chromosome type-specific probes can be a powerful tool in detecting homoeologous relationships between chromosomes of related species (Danilova et al. 2014). Chromosome-specific marker sequences like microsatellites or tandem repeats in connection with morphological characteristics enabled the identification of individual chromosomes of barley and some wild relatives (Cuadrado et al. 2008; Komuro et al. 2013). The identification of each chromosome of diploid *H. bulbosum* (bulbous barley), *H. murinum* (wall barley), *H. marinum* (sea barley), and *H. pubiflorum* became possible and enabled the comparison of the homoeologous chromosomes between barley and related species (Carmona et al. 2013b; Cuadrado et al. 2013; Cuadrado and Jouve 2007).

In our previous work, we allocated the chromosomal position of 70 single-copy probes derived from the genetic centromere of chromosome 3H of barley. Eighteen probes were directly derived from coding sequences, which were likely to be conserved among species of the same tribe and thus could be adequate markers for the study of genome collinearity in different *Hordeum* and Triticeae species (Aliyeva-Schnorr et al. 2015a).

In the current study, we investigated the syntenic relationships between the homoeologous group 3 chromosomes of the H, Xa, Xu, and I genomes of the genus *Hordeum* and the R genome of a related Triticeae species, rye (*Secale cereale*), based on barley chromosome 3H-derived single-copy FISH markers. The H genome was represented by barley's closest relative *H. bulbosum*. *H. marinum* and *H. murinum* were selected as representative species for the Xa and Xu genomes, respectively. *H. pubiflorum* belongs to the I genome species. In addition, we included rye, which diverged from barley around 14 mya (Huang et al. 2002). We observed the expected strong collinearity; however, depending on species, a certain level of identified non-collinearity likely due to intrachromosomal gene movements of homoeologous sequences or small-scale chromosomal rearrangements was observed within genus *Hordeum* and interchromosomal rearrangements between barley and rye.

Materials and methods

Plant material and preparation of mitotic chromosomes

Hordeum vulgare L. cv. Morex (2n = 2x = 14), *Hordeum marinum* L. (BCC 2006, 2n = 2x = 14), *Hordeum murinum* L. subsp. *glaucum* (Steud.) Tzvelev (BCC 2002, 2n = 2x = 14), and *S. cereale* L. (self-fertile inbred line 7415, 2n = 2x = 14) seeds were germinated under dark conditions. To stimulate root growth of *H. bulbosum* L. (HB2032, 2n = 2x = 14) and *H. pubiflorum* Hook f. (BCC 2028, 2n = 2x = 14), plants were cold treated by 4 °C for 2 weeks and then transferred to 24 °C. Roots of all species were treated with ice water for 20 h to increase the frequency of metaphase cells. Then, root tips were fixed in 3:1 (*v*:*v*) ethanol:acetic acid mixture at room temperature (RT) for 2 days and stored at 4 °C up to a year. Chromosome preparation was performed using the dropping technique as described previously (Kato et al. 2006). Post-fixation of slides was carried out according to Ma et al. (2010) and Aliyeva-Schnorr et al. (2015b).

Applied FISH probes

All genomic single-copy probes were derived from FingerPrinted contigs (FPcontigs) (International Barley Genome Sequencing et al. 2012) anchored to the genetic pericentromere of chromosome 3H of barley. To identify unique sequences suitable for FISH, contigs were processed by "K-masker" (Schmutzer et al. 2014). Details of genomic probe generation are described in Aliyeva-Schnorr et al. (2015a) and Table S2. The 5S ribosomal DNA probe was generated from genomic DNA of barley by PCR as described (Fukui et al. 1994). The labeled oligonucleotide probe $(CTT)_{10}$ was generated by Cu(I) catalyzed azide-alkyne cyclo addition using FAM-azide (Baseclick GmbH, Tutzing, Germany). pSc119.2 was nick labeled with Alexa 488 (Bedbrook et al. 1980). Inserts of full-length complementary DNA (fl-cDNA) of barley (Sato et al. 2009) and wheat assigned to chromosome 3H (Danilova et al. 2014) were amplified by PCR using T3/T7 primers (Table S3). Obtained PCR fragments were purified and directly labeled by nick translation with Texas red-dUTP, Alexa-488-dUTP (Invitrogen, Life Technologies), or Atto 550 Cy3 according to Kato et al. (2004).

FISH and microscopical analysis

Single-copy FISH was performed as described earlier (Kato 2006; Ma 2010) with minor alterations (Aliyeva-Schnorr et al. 2015b). Specimens were washed in 2× saline sodium citrate (SSC) and treated with 45 % acetic acid for 2–10 min. For post-fixation, slides were incubated for 10 min in 4 % formaldehyde and subsequently washed in 2× SSC for 3× 5 min, then slides were dehydrated in a series of increasing ethanol concentration (70, 90, and 96 %, *v*/*v*) and air dried. The hybridization mixture contained 50 % deionized formamide, 20× SSC, 1 M Tris–HCl pH 8.0, 1.5–3 μl (135 ng μl^{-1}) single-copy probe, 10 μg/ml salmon sperm DNA, and 0.5 M EDTA. Hybridization mixture was denatured together with the chromosomal DNA on a hot plate at 80 °C for 2 min. Hybridization at 37 °C was performed for 20 h in a moist chamber. After slides were washed in 2× SSC for 20 min at 58 °C and dehydrated in an ethanol series (70, 90, and 96 % *v*/*v*), the slides were air dried at room temperature and counterstained with 1 μg 4′,6′-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories). Images were acquired with an epifluorescence microscope BX61 (Olympus) using a cooled CCD camera (Orca ER, Hamamatsu). Pictures were processed and merged by Adobe Photoshop software (Adobe Systems Incorporated, USA).

Quantification of single-copy FISH signal positions

For each probe, four to six metaphase chromosomes showing specific hybridization signals were measured. The relative cytological position (CP) was determined as $CP = (S/T) \times 100$ %. The length of the chromosome (*T*) and the position of the hybridization signal from the end of the long arm (*S*) were measured with the help of Photoshop and/or Image J.

Results

Identification and characterization of chromosome 3H homoeologs in related species

To analyze the relationships between the genomes of cultivated barley, wild *Hordeum* species, and rye, we selected a set of barley chromosome 3H-specific single-copy genomic DNA probes (gDNAs) for FISH (Table S1a, b). Besides genomic probes, full-length

cDNAs derived from barley and wheat were tested as 3H-specific probes (Table 1). Eight and four cDNAs of barley and wheat, respectively, yielded 3H-specific signals (Fig. 1 (blue dots); Fig. S2c, d; and Table S3). Probe c1 (AK251893) showed an additional but smaller signal on 3H. The barley cDNA probes AK249253, AK251893, AK250402, AK248174, and AK248167, by BLAST analysis (http://webblast.ipk-gatersleben.de/barley/) preassigned to chromosome 3H, revealed

Table 1 Probe IDs and corresponding FISH probe IDs

ID	Fpcontig
g1	1700
g2	489
g3	47262
g4	5721
g5	47648
g6	8326
g7	46563
g8	187
g9	3331
g10	45300
g11	46010
g12	43477
g13	80
g14	900
g15	47485
g16	44600
g17	46456
g18	47533
g19	603
g20	45839
g21	247
g22	44666
g23	43820
g24	1697
g25	1378
g26	45064
g27	44732
g28	46093
c1	AK251893
c2	AK248957
c3	tplb0001g16
c6	AK251014
c4	AK251696
c11	AK249216
c5	AK336104
c7	AK248727
c9	AK248230
c8	tplb0014n06
c10	tplb0045e08
c12	AK251227

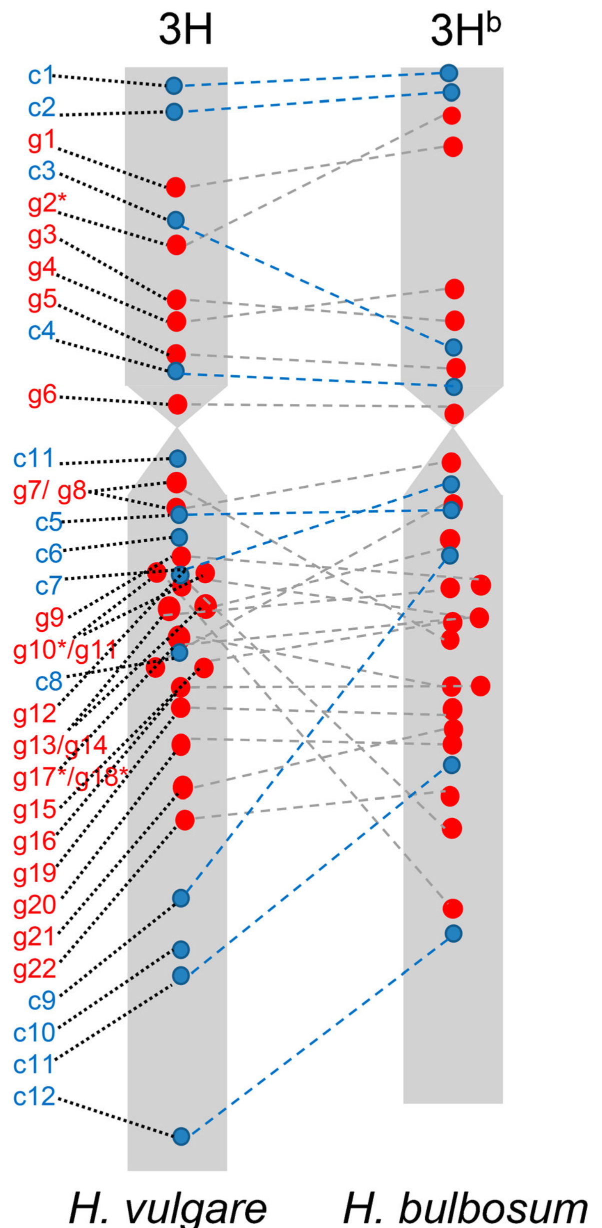

Fig. 1 Comparative FISH mapping of single-copy probes between barley and *H. bulbosum*, showing 22 single-copy (gDNA, *red dots*) and 12 cDNA markers (*blue dots*) mapped on chromosome 3H of *H. vulgare* and hybridized on homoeologous chromosome $3H^b$ of *H. bulbosum*

distinct signals only on chromosome 1H. Moreover, cDNA probes c1 and c6 (AK251014 and AK251893; Table 1) of barley displayed signals on 4H and 2H, respectively.

Next, we tested whether single-copy genomic probes of barley could be used to trace the evolution of the corresponding homoeologous group 3 chromosomes in *H. bulbosum* and *S. cereale*. Although the distribution of highly repetitive sequences differs between both barley species (Blattner 2004), *H. bulbosum* was selected, because it represents the closest relative to cultivated barley outside the primary gene pool, with a divergence time of 3.7 my between both species (Blattner 2004). Differences in genome organization are also visible in Giemsa C-banding pattern that consists of more interstitial and centromeric bands in *H. vulgare*, whereas the C-banding pattern of *H. bulbosum* is essentially centromeric with some small terminal bands (Linde-Laursen et al. 1990).

Labeled $(CTT)_{10}$ oligonucleotides and repetitive sequence pSc119.2 were used to distinguish the chromosomes of *H. bulbosum* as previously recommended (Pickering et al. 2006). Detection of 22 gDNA and 12 cDNA probes on one and the same *H. bulbosum* type of chromosome confirmed $3H^b$ as being homoeologous to chromosome 3H of barley. $3H^b$ is characterized by a centromere-specific prominent $(CTT)_{10}$ signal (Fig. 2b, g) (Pickering et. al. 2006; Carmona et al. 2013a).

Rye is the most distant relative of barley tested in this work. Rye shared an ancestor with barley around 14 mya (Huang et al. 2002). Identification of rye chromosomes is difficult due to frequent polymorphisms of high-copy sequences (Mukai et al. 1992). However, the FISH pattern of pSc119.2 in combination with morphological features such as centromere index or the position of nucleolus organizing region (NOR), as described earlier by Cuadrado et al. (1995), allowed a preliminary identification of all rye chromosomes (Fig. S1). Detection of 12 gDNA and 5 cDNA probes on the same chromosome allowed the identification of chromosome 3R. Hence, the rye homoeolog to barley chromosome 3H was distinguished as a metacentric chromosome with two band-like pSc119.2-specific signals at both termini (Fig. 2f, g).

Next, characterization with probe $(CTT)_{10}$ (Carmona et al. 2013a, b; Cuadrado et al. 2013) allowed the differentiation of all seven chromosome pairs in *H. pubiflorum*, *H. marinum*, and *H. murinum* (Fig. 2c–e). Eighteen single-copy probes, each carrying a high-confidence gene, identified barley 3H homoeologous chromosomes in all three species. Chromosome $3H^{mar}$ is characterized by $(CTT)_{10}$ signals in the proximity of the centromere and in the distal region of the long arm (Fig. 2c, g). Chromosome $3H^{pub}$ can be identified as the only 3H homoeologous chromosome showing no cross-hybridization with $(CTT)_{10}$ (Fig. 2d, g), and metacentric chromosome $3H^{mur}$ can be distinguished with the help of three diagnostic signals as shown in Fig. 2e, g. This chromosome displayed the same pattern as "chromosome B" of *H. murinum* described previously by Carmona et al. (2013b).

Synteny between barley and *H. bulbosum* revealed by 3H chromosome probes

The collinearity of chromosome 3H probes was tested between barley and *H. bulbosum*. All 22 genomic probes resulted in chromosome $3H^b$-specific signals (Fig. 1). The arm affiliation between both species was conserved; thus, the relative position of centromere did not differ between both species. Seventeen out of 22 gDNA probes were detected at collinear sites in both species (Table S1a). Whereas all gene-containing gDNA probes allocated to collinear positions between *H. vulgare* and *H. bulbosum*, five of ten probes (g7, g10, g13, g18, and g20) lacking a coding sequence displayed a non-collinear order of signals on chromosome $3H^b$ (Fig. 1), indicating intrachromosomal sequence movements or small-scale chromosomal rearrangements in both arms of 3Hb. Moreover, four of these probes produced additional signals on other chromosomes of *H. bulbosum* (Fig. 1, asterisks).

Nine out of 12 barley cDNA probes mapped to the expected chromosome of *H. bulbosum* in collinear order except for two closely linked barley probes c5 and c7 (Fig. 1). Probes c1 and c6 exhibited additional hybridization positions on barley chromosomes 4H and 2H and also produced clear signals on $4H^b$ and $2H^b$, respectively, yet no signal was found for probe c6 on chromosome $3H^b$. Remarkable differences between individual positions were observed for three barley cDNAs (c9, c11, and c12) and one wheat cDNA, tplb001g16 (Fig. 1 and Fig. S2c). Two of the four (c8 and c10) wheat cDNAs did not result in a hybridization signal at all (Fig. 1, blue dots).

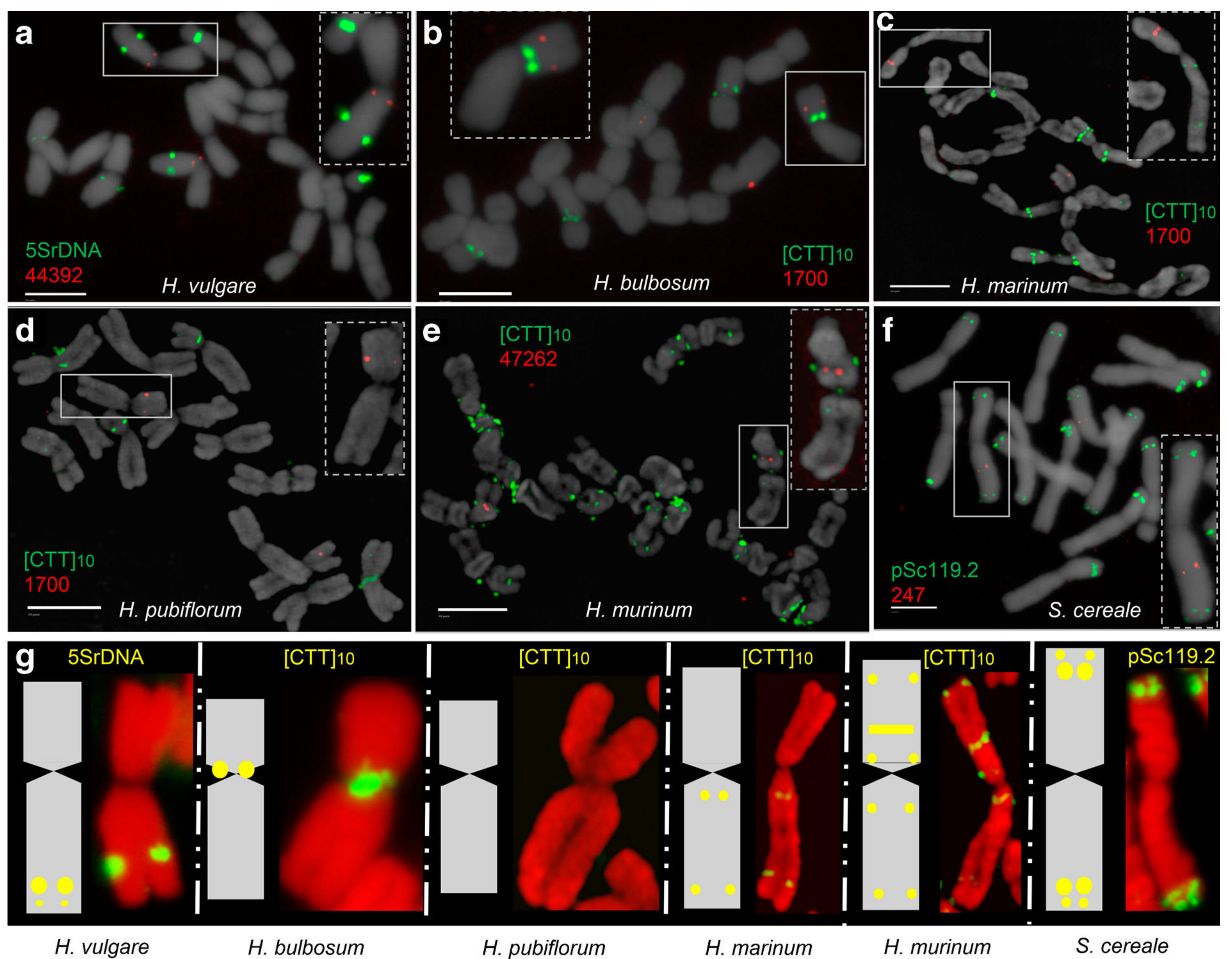

Fig. 2 Single-copy FISH on metaphase chromosomes of the five species from *Hordeum* and of *S. cereale*. **a** The *insets* show further enlarged chromosomes with specific signals. Hybridization of a single-copy FISH probe (*red*) on chromosome 3H of *H. vulgare* and 5SrDNA (*green*) applied as a diagnostic probe. **b** Hybridization of a single-copy FISH probe (*red*) on chromosome 3H of *H. bulbosum* and $(CTT)_{10}$ microsatellite (*green*) applied as a diagnostic probe. **c**–**e** Hybridization of a single-copy FISH probe (*red*) on chromosome 3H of *H. marinum*, *H. pubiflorum*, and *H. murinum* and $(CTT)_{10}$ microsatellite (*green*) applied as a diagnostic probe. **f** Hybridization of a single-copy FISH probe (*red*) on chromosome 3R of *S. cereale* and pSc119.2 (*green*) applied as a diagnostic probe. **g** Characterization of the homoeologous chromosomes of chromosome 3H of *H. vulgare* revealed by FISH mapping with 18 single-copy probes. Chromosome 3H of *H. vulgare* and homoeologous chromosomes from the other species are represented by the diagnostic bands (*green*) of the characterizing probes, 5SrDNA for *H. vulgare*, $(CTT)_{10}$ microsatellite for *H. bulbosum*, *H. pubiflorum*, *H. marinum*, and *H. murinum*, and pSc119.2 for *S. cereale*. *Scale bar* = 10 μm

Syntenic relationship between barley and wild *Hordeum* species from the tertiary gene pool

Homoeologous group 3 chromosome of *H. pubiflorum*, *H. marinum*, and *H. murinum* was characterized with a set of 18 coding gDNA probes. Seventeen FISH probes displayed signals on chromosome $3H^{pub}$ of *H. pubiflorum*. Only probe g25 did not reveal clear signals in some of the chromosome spreads (Fig. 3a). Distribution of the short-arm probes demonstrated collinearity. Like in barley, probe g6 resulted in a centromeric signal (Fig. 3a and Fig. S3a). However, this probe disclosed additional signals on other chromosomes as well as high background noise, too. Six out of nine long arm-specific probes revealed similar physical positions compared to chromosome 3H. A positional deviation was detected for the probe g28 that is more interstitial on the chromosome $3H^{pub}$ than in barley. An inverted order was revealed for g12 and g28 (Fig. 3a). Notably, the physical interval comprising all 18 probes was significantly reduced in *H. pubiflorum* compared to the same interval in barley (Mann–Whitney test,

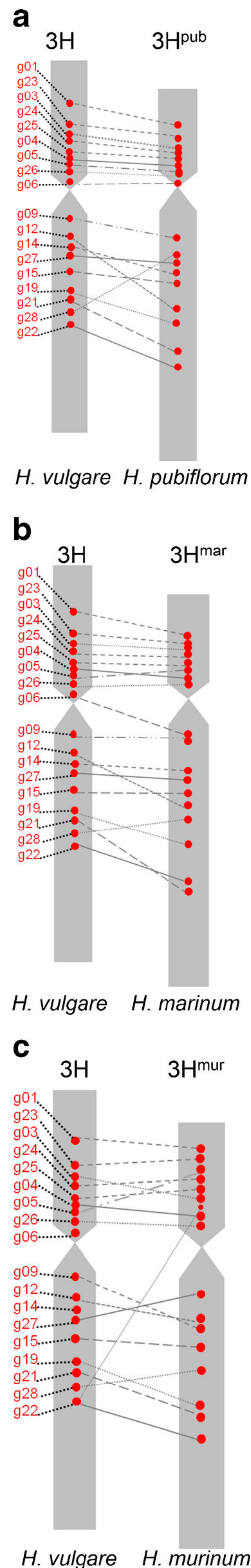

Fig. 3 Comparative FISH mapping of single-copy probes in barley and related species, showing 18 single-copy gene-containing probes hybridized to the homoeologous chromosomes of **a** *H. pubiflorum*, **b** *H. marinum*, and **c** *H. murinum*

$p < 0.0006$). The position of the short- and long-arm probes shifted toward the centromere. Hybridization with the two boundary probes (g1 and g22) marked an interval of only 49.6 % of the total physical length (Fig. S5). In barley, the same probe pair marked a region of 58 %.

The same probes were used to analyze homoeologous group 3 chromosomes of the Xa genome species *H. marinum*. All but two probes (probes g25 and g26) resulted in distinguishable signals on chromosome $3H^{mar}$ (Fig. S3b). Fifteen out of 18 probes used were found in the same order like in barley. In contrast, probe g6 showing a centromeric position in *H. vulgare*, *H. bulbosum*, and *H. pubiflorum* localized on the long arm of $3H^{mar}$, colocalizing with a $(CTT)_{10}$ signal (Fig. S3b). In addition, two of the long-arm probes (g28 and g21) changed position (Fig. 3b). The chromosomal interval comprising all 18 probes is comparable to the region on chromosome 3H of barley and represents 53.6 % of the chromosome length.

Similar distribution of probes as in the H genome was found in the Xu genome species *H. murinum* (Fig. 3c and Table S1b). Only probe g6 was absent on chromosome $3H^{mur}$, but revealed an intercalary position on a different chromosome of *H. murinum*, characterized by one centromeric $(CTT)_{10}$ band. However, the hybridization signals were weaker and often present only on one chromatid. In addition, several probes resulted in background noise. Probe g22 showed a second weak signal on the same chromosome (Fig. S3c). The relative distance between the boundary probes used was comparable to the region on chromosome 3H of barley and represented 58.2 % of the chromosome length.

Syntenic relationship between *H. vulgare* and *S. cereale* revealed by 3H chromosome-specific probes

Altogether, 24 probes were hybridized to mitotic metaphase chromosomes of rye, comprising 12 cDNAs and 12 gene-containing gDNA probes (Fig. 4). All except two of the 12 genomic probes produced collinear signals on the respective arms of chromosome 3R compared to barley (Fig. 4 and Fig. S2b, d). Only probes g19 and g15 changed their relative order. In contrast to gDNA, seven of the 12 cDNAs revealed specific signals on rye chromosomes and five probes producing a 3R-specific signal. The remaining probes were detected on either arms of chromosome 6R, indicating possible translocation events between chromosomes 3R and 6R of rye

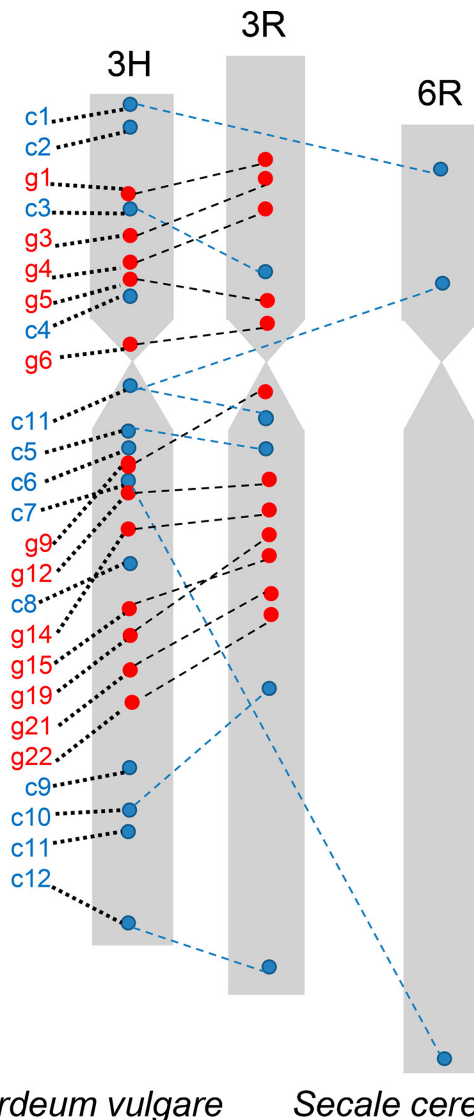

Fig. 4 Comparative FISH mapping of single-copy probes in barley and related species, showing 12 single-copy (gDNA, *red dots*) and 12 cDNA markers (*blue dots*) hybridized and detected on chromosomes 3R and 6R of *S. cereale*

(Fig. 4 and Fig. S2d). cDNA c11, representing a member of a zinc knuckle DNA-binding gene family, yielded, besides a signal on chromosome 3R, a second signal on the short arm of chromosome 6R (Fig. 4 and Fig. S4).

In summary, we used both cDNA and gene-containing gDNA probes to reveal the extent of synteny between distantly related species within a genus but also between species belonging to the same tribe. Genomic single copy probes resulted generally in stronger hybridization signals than cDNAs and therefore should be favored as FISH probes. Positions and order of the majority of analyzed sequences were conserved in all three *Hordeum* species, namely 78 % in *H. murinum*, 83 % in *H. marinum*, and 88 % of the probes in *H. pubiflorum*. Observed non-collinear signals may indicate intrachromosomal gene movements or small-scale chromosomal rearrangements within the genus *Hordeum*. gDNA probes have shown gross collinearity between chromosomes 3H and 3R of barley and rye, respectively, whereas cDNA probes confirmed the previously detected relationship between chromosomes 3R and 6R (Martis et al. 2013).

Discussion

FISH mapping reveals extensive collinearity of group 3 chromosomes in the genus *Hordeum* and the tribe Triticeae

This study focused on chromosome 3H that possesses agronomically useful genes such as *rps1* (Yan and Chen 2007), *Hsdr4* (Suprunova et al. 2007), *sdw1* (Chloupek et al. 2006), and *btr1* and *btr2* (Komatsuda and Mano 2002). In order to compare synteny between homoeologous group 3 chromosomes of related species of the genus *Hordeum* and *S. cereale*, we employed 3H chromosome-derived single-copy genomic DNA- and cDNA-derived probes for FISH. Our results revealed very good synteny between *H. vulgare* and *H. bulbosum* group 3 chromosomes, as 28 out of 33 3H-derived probes cross-hybridized with $3H^b$, in collinear order, and thus supported the previous identification of chromosome $3H^b$ (Pickering et al. 2004) as homoeologous to barley chromosome 3H. All gene-containing genomic probes were showing identical distribution like on chromosome 3H, confirming collinearity between *H. bulbosum* and cultivated barley. However, five of the analyzed non-coding genomic sequences changed the position in *H. bulbosum*. This observation is in line with results obtained by Wicker et al. (2011) on conserved but non-collinear sequences among Triticeae. The sequence movement was shown to be mediated by adjacent transposable elements (Wicker et al. 2011). Two additional cDNA signals on chromosomes 2H and 4H of barley were detected on the orthologous chromosomes of *H. bulbosum* ($2H^b$ and $4H^b$), while no signals were detectable on $3H^b$. Thus, the absence of signals on

$3H^b$ could be explained either by an insertion within *H. vulgare*, which did not occur in *H. bulbosum* or to a deletion event in this species.

We were able to assert the close relationship between cultivated barley and three species from the tertiary gene pool of the genus *Hordeum*. Besides a generally efficient cross-hybridization of almost all probes with the respected homoeologous chromosome, the conserved arm affiliation documented the high extent of synteny among *H. vulgare*, *H. pubiflorum*, *H. marinum*, and *H. murinum*. While the order of most gDNAs was conserved, their relative chromosomal positions revealed deviations, which could be explained by differences in abundance and distribution of repetitive sequences that are known to differ between those species (Carmona et al. 2013b; Cuadrado et al. 2013; Cuadrado and Jouve 2007). Probe g6 revealed a non-centromeric position in *H. marinum*, intercalary position on a different chromosome of *H. murinum*, and additional signals on other chromosomes of *H. pubiflorum*. The positional change of this probe from the centromere in barley to a non-centromeric region in two wild species might indicate a possible shift of the centromere within the genus *Hordeum* as was shown previously for the centromere of rice chromosome 8 (Ma et al. 2007). However, the use of additional centromeric markers will be required to better resolve the nature and occurrence time of this possible shift or translocation.

Additionally, a discrepancy was observed in the physical length of the chromosomal interval comprising all tested probes. In *H. marinum* and *H. murinum*, we determined a barley-like dimension of the syntenic chromosomal interval, whereas this interval was physically shorter in *H. bulbosum* and *H. pubiflorum*. Interestingly, these latter species have a considerably smaller genome size (4.342 Mbp/1C and 4.220 Mbp/1C) than *H. vulgare* (5.809 Mbp/1C) and the other analyzed *Hordeum* species (Jakob et al. 2004). Previous cytogenetic studies demonstrated that deviations in marker position together with decreased hybridization intensity are indicative of a phylogenetically more distant relationship (Hasterok et al. 2006). Based on this, we counted the positional deviations between barley and its wild species, showing two deviations in probe position in *H. pubiflorum* and three in *H. marinum* and in *H. murinum*. The latter has also shown the absence of probe g6-specific signals on $3H^{mur}$. These few changes point out that all three species are phylogenetically very close. Moreover, positions of the signals relatively to the total length of the chromosome as well as signal intensity of the probes allowed for the assumption that *H. pubiflorum* and *H. marinum* are closer to each other. This relationship would agree with previous results from phylogenetic analyses postulating that the Xa genome is the sister group of the I genome (Blattner 2009; Brassac and Blattner 2015).

Expressed sequence tag (EST) bin maps for the wheat chromosomes 3A, 3B, and 3D have shown a large collinearity between those chromosomes, ranging from 91 to 96 % (Akhunov et al. 2003), as well as some deviation in the order of ESTs, indicating putative rearrangements. Another syntenic analysis between the chromosomes of wheat subgenomes based on FISH revealed similar results, showing that 91 % of markers hybridized to all three homoeoloci (Danilova et al. 2014). This is comparable to our analysis between chromosomes 3H and 3R estimating a level of 92 % collinearity for the gDNA-derived signals. cDNA markers, in contrast, revealed three differences in order between chromosomes 3R and 6R. The rearrangement between the terminal part of the long arms of 3R and 6R is in agreement with the comparative in silico analysis of rye and barley describing a reciprocal translocation event (Martis et al. 2013). Signals of the barley cDNA probe c12 at the subtelomeric part of 3R long arm supports the proposed translocation, indicating that a small portion of 6R could be deleted and added onto chromosome 3R.

Effectivity of single-copy genomic and cDNA probes for the analysis of synteny differs

We tested repeat-free coding genomic probes, repeat-free non-coding probes, and cDNA probes originating from barley or wheat for FISH analysis to determine the most suitable probe for comparative studies. Genomic gene-containing probes produced single, clear dot-like signals. Probes without a gene-coding sequence were omitted for the analysis of rye and the three *Hordeum* species from the tertiary gene pool, because non-coding single-copy sequences seemed to be more susceptible for divergence between *H. vulgare* and its close relative, *H. bulbosum*, and therefore less suitable to trace synteny in related species. Surprisingly, also cDNA probes often resulted in less intense signals, with a higher hybridization background and occasionally additional signals on other chromosomes. The better performance of repeat-free gene coding genomic probes compared to cDNA is correlated with the difference in probe length that is ~3500 bp for cDNA

and ~5000 bp for gDNA probes. Furthermore, the specificity of gDNA probes compared to other applied probes could be favored by the presence of introns, which however are known to be less conserved among different species. Syntenic conservation of introns in gDNA probes could therefore indicate a possible regulatory role of these sequences within the genome (Kim et al. 2006; Rippe et al. 1989). The origin of additional cDNA signals could be explained by the cross-hybridization to paralogous genes with high sequence similarity. For instance, cDNA c11 (Table S3) encodes a putative zinc knuckle DNA-binding protein belonging to one of the largest gene families and known to be expanded throughout the genome by duplication events (Tadepally et al. 2008). This probe revealed multiple signals not only in barley but also in rye and *H. bulbosum*.

FISH helped also to correct the annotation of cDNA clones. Six cDNAs previously assigned to chromosome 3H using genetically mapped ESTs (http://www.shigen.nig.ac.jp/barley/) hybridized instead to chromosome 1H and 5H. Sequence comparison against the assembly WGS Morex (http://webblast.ipk-gatersleben.de/barley/) confirmed the FISH results showing a significant hit of 95 % for the respective chromosomes.

In summary, comparative FISH mapping of chromosome-specific single-copy probes in related species enabled the identification of homoeologous group 3 chromosomes of barley and demonstrated a high degree of similarity at the chromosomal level among *Hordeum* species from different subgenome groups. Comparison of gDNA probes to chromosome 3H of *H. vulgare* has shown 100 % synteny for *H. bulbosum*, of 88 % for *H. pubiflorum* (16 of 18 probes), of 83 % for *H. marinum* (15 of 18 probes), and 78 % for *H. murinum* (14 of 18 probes). Detected positional differences within *Hordeum* occurred either due to conserved but non-collinear genes among Triticeae, which by far outnumber the syntenic genes in model grass genomes, or to small-scale chromosomal rearrangements. Single-copy genomic gene-containing probes are superior to cDNA for the studies at the chromosomal level. Examination of the extent of synteny between homoeologous chromosomes of barley and rye complemented the results of previous studies and delivered additional insight into the dynamics of grass synteny at the subchromosomal level.

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