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The phylogenetic relationships of species and cytotypes in the genus *Hordeum* based on molecular karyotyping

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Abstract

Unravelling the relationships between the members of *Hordeum* is no easy task since many of its member taxa, including most of the tetra- and hexaploids, is of hybrid origin. *Hordeum* is one of the largest genera of the family Triticeae with more than 30 species dispersed all over the world. In order to analyze the phylogenetic relationships, intraspecific diversity and origin of polyploids, our group reported detailed ND- FISH, FISH and GISH based karyotypes of more than 50 accessions of different species and cytotypes of the four genomes (**H**, **Xa**, **Xu** and **I**) of the genus *Hordeum*. This review will compile all this information with new accessions not reported previously. Each chromosome pair in all the accessions analyzed was identified and subgenomes distinguished in polyploids. The results allowed the assessment of the chromosomal diversity within species and the identification of possible homeologous relationships. A scheme of probable evolution shows the intricate pattern of relationships among species: 1) Genome **H**: *H. vulgare* subsp. *vulgare* (2x) and subsp. *spontaneum* (2x) and *H. bulbosum* (2x and 4x). 2) Genome **Xa**: *H. marinum* subsp. *marinum* (2x) and subsp. *gussoneanum* (2x and 4x). 3) Genome **Xu**: *H. murinum* subsp. *glaucum* (2x and 4x), subsp. *murinum* (4x and 6x) and subsp. *leporinum* (4x and 6x). 4) Genome **I**: The diploids *H. californicum*, *H. intercedens*, *H. cordobense*, *H. chilense*, *H. roshevitzii*, and *H. bogdanii*, and 4x *H. brachyantherum* and 5) Allopolyploids with **Xa** and **I** genomes: *H. secalinum* (4x), *H. capense* (4x) and 6x *H. brachyantherum*.

Keywords: Allopolyploids, Barley; Evolutionary History; FISH; GISH; *Hordeum,* ND-FISH; satDNA; SSRs

Introduction

The genus *Hordeum*, a member of the agronomically important tribe Triticeae, comprises about 31 species (45 taxa and 51 cytotypes) including diploid, tetraploid and hexaploid cytotypes with a basic chromosome number of x=7 [1]. Due to the economic importance of cultivated barley (*H. vulgare* L.) as one of the most important crops, the suitability as a model for Triticeae genetics and genomics, and the potential use of its wild relatives to improve barley and other Triticeae such as bread wheat, *Hordeum* has been object of an intensive study from all these perspectives [2]. In spite of the numerous analyses carried out during the last 50 years to clarify species relationships, the phylogeny has been not resolved and there is disagreement in the taxonomic handling of *Hordeum* [3-5]. In general, the majorities of molecular phylogenies show great conformity about relationships of diploid species. However, when the polyploid species (about 50% of the taxa) are included in phylogenetic analyses the results are complex and difficult to infer in part due to the hybrid origin of most of the polyploid forms [6-10]. New attempts to solve the evolution of polyploid cytotypes are indispensable to advance in the knowledge of phylogeny and taxonomy of the genus *Hordeum* [11]. Anyway, the genus offers an excellent material for investigating the patterns of polyploidy evolution in plants. Cytogenetic studies, based on chromosome structural analysis made it possible to lay the foundations for the evolutionary understanding of the genus *Hordeum*. Löve based on the similarities in C-banding patterns, which reveals the position of the constitutive heterochromatin, divided the genus into two groups: the genus *Hordeum*, with *H. vulgare* L. as a unique member, and the genus *Critesion*, including all other species [12]. Dewey, also based on karyological analysis, supported this classification but moved to the genus *Hordeum* the species *H. bulbosum* L. [13]. Even so, Bothmer and Jacobsen retook the classical view of as a single genus, *Hordeum* L., with four sections: *Hordeum*, *Anisolepis*, *Stenostachys* and *Critesion*, primarily according to morphological criteria [14]. Although Bothmer and co-workers maintain this classification in their last review of the genus, they established new phylogenetic relationships on the base of data obtained from the analysis of the chromosomal pairing in interspecific hybrids [15]. Blattner proposed the most recent classification up to now as a single genus *Hordeum* L., with two subgenus: *Hordeum* with two sections: *Hordeum* and *Tricostachys* and subgenus *Hordeastrum* with three sections: *Marina*, *Stenostachys* and *Nodosa* citation [4]. Thus, in *Hordeum* would exist at least four basic genomes designated by the symbols **I** (*H. vulgare*, *H. bulbosum*), **Y** (*H. murinum*), **X** (*H. marinum*) and **H** (the rest of species of the genus). Subsequently, the genome **Y** was renamed as **Xu** and the genome **X** changed to **Xa** [16]. After establishing the homeologous relationships between the chromosomes of the basic genomes of wheat *Triticum* sp., and barley *Hordeum*, it was proposed using the symbol **H** for the cultivated barley and therefore were exchanged the names of the genomes **H** and **I** [17]. In this paper, we will use the most accepted nomenclature: **H** for *H. vulgare* and *H. bulbosum*, **Xu** for *H. murinum*, **X**a for *H. marinum* and **I** for the rest of species of the genus [3]. In the last decades, the cytogenetic studies have been enriched with numerous analyses on DNA sequences, both in chloroplasts and nuclear genome, which have contributed interesting data on the phylogeny of the genus *Hordeum*. However, the different works contribute contradictory results, depending on the type of molecular markers or analyzed sequences (as nuclear genes of unique copy, SSRs or DNA of chloroplasts), the chosen criteria to build the phylogenetic trees, the number of accessions by taxa or the source of the analyzed species. In general, when these techniques are applied, the phylogenetic trees separate the diploid species of *Hordeum* in four groups agreeing with the genomic constitution proposed cytogenetically [18-20]. At this point, is necessary the integration of new techniques on the distribution of certain sequences of DNA in order to add more details over the physical maps of chromosomes of the genus *Hordeum*. This would facilitate an advance in the knowledge on the origin and phylogenetic relationships of the polyploid cytotypes and species of *Hordeum*. Our group recently reported detailed fluorescence in situ hybridization (FISH)-based karyotypes of several *Hordeum* species that helped clarify their relationships and the origin of their polyploids [21-28]. Fluorescence *In Situ* Hybridization (FISH) has played an important role in modern molecular cytogenetics providing a useful tool for chromosome identification and evolution. Thus, the ribosomal 45S rDNA and 5S rDNA probes are useful as molecular markers to identify the *H. vulgare* chromosomes [29]. However, only a few chromosome pairs can be identify using bicolour FISH with rDNA-targeted probes in wild barleys [30]. Moreover, other probes detecting different families of tandemly repeated sequences have been used to analyze and increase the number of FISH landmarks in chromosomes of different species of *Hordeum*. However, these probes do not provide enough information for the confident identification of all chromosomes [31]. In 2010, the authors reported a highly efficient non-denaturing FISH (ND-FISH) technique useful to detect SSRs-enriched chromosome regions with synthetic oligonucleotides as probes [23]. This molecular cytogenetic technique is of considerable interest since it can provide valuable information to complement the results of molecular phylogenetic analyses and help test the different evolutionary hypotheses proposed in the genus *Hordeum*. The aim of the present study was to take advantage of ND-FISH technique to add SSRs markers in the karyotype maps that could be used to spot possible homologous chromosome relationships between different subsepecies and species of *Hordeum*, including diploid, tetraploid and hexaploid cytotypes, and hence provide information on species evolution within *Hordeum*.

The satDNA as tools useful for the analyses of karyological evolution in Triticeae

Unlike observed in other model plants, like Arabidopsis or rice, the barley has a complex genome of 5.1 Gb of size. It contains about 26,000 genes, which are organized in genic islands separated by long repetitive DNA sections that altogether suppose more than 84% of the whole genome [32]. The first draft of the genome obtained by the International Barley Sequencing Consortium (IBSC) shows a physical map of 4.9 Gb, from which 1.13 Gb have been sequenced. A great part is constituted by repetitive DNA of which, excluding mobile elements (transposons and retrotransposons), or multigene families, which have been moderately well characterized, the greater percentage corresponds to simple sequences repeats or satDNA (satellite DNA) families repeated in tandem (micro-, mini- and satellites), classified normally on the basis of the size of the repetition unit [33, 34]. In barley, as in the majority of the genomes of model organisms “totally” sequenced, do not exist sufficient information on the structure and molecular organization of the extensive regions where the satDNA is accumulated, which continue being “black holes” of the genomes [35, 36]. The microsatellites or SSRs (Short Sequences Repeated in tandem in which DNA motifs of repetition ranges in length from 1- 6 base pairs) represent a very important percentage of the DNA repeated fraction in the genome of barley, as in general in all eukaryotes [37, 38]. The study of chromosome distribution of the different repeated nucleotide sequences of the genome has a double utility. In the first place, it allows characterizing the chromosomes of the species that include them, connecting the molecular and chromosomal level. Secondly, the comparative analysis of the dispersion of the different satDNA families in the chromosomes of different species and cytotypes could facilitate the study of the phylogenetic relationships of the *Hordeum* species. In this way, the presence, position and intensity of these sequences in the chromosomes allow solving the intra- and interspecific diversity in each taxa and their evolutionary trends. It could help also to identify the genomes of the diploid species that have contributed to the formation of the polyploid species and cytotypes. It is thought, that structural changes in chromosomes have played an important role in evolution. However, in general the karyotypes of all the species of *Hordeum*, which were first characterized in the decade of the 60s based on their morphology, are rather symmetrical with predominantly metacentric and relatively great chromosomes [39, 40]. After this, the C-banding technique was used to mark the position of the constitutive heterochromatin, which allowed a more complete chromosomal analysis within and between the species of the genus *Hordeum* [41]. C-bands are located mainly in pericentromeric regions, which permit the distinction of the seven chromosome pairs. Nevertheless, some chromosomes having a similar size and morphology show also a similar pattern of C-bands, which makes its individualized identification difficult. Therefore, the unequivocal and individualized identification of all the chromosomes of barley was not possible until the FISH techniques using probes of repetitive DNA arrived. The addition to the physical maps of new landmarks using ND-FISH to localize SSRs and other repeat sequences would allow to identify possible structural variations and to relate the possible homology between the chromosomes of different species.

The application of a selected set of repetitive DNA sequences in *Hordeum*

Probes and *in situ* hybridization techniques

Multiple-target *in situ* hybridization was performed combining two selected probes to analyze the localization of repetitive DNA sequences by FISH followed by ND-FISH, or vice versa, in chromosomes of accessions belonging to subspecies and cytotypes representing the four genomes of *Hordeum*. GISH (Genomic *In Situ* Hybridization) was also used in identifying the subgenomes present in the polyploids. For chromosome preparations, root tips were obtained from seedlings and exceptionally from plants grown in a greenhouse as previously described [22, 23]. A strategy of successive multiple-target hybridizations on good spread metaphase cells was followed for chromosome identification and karyotype analysis. Figure 1 exemplifies the used methodology. Five probes were used in FISH: pTa71, pTa794 (45S rDNA and 5S rDNA repeat sequences from *Triticum aestivum* L. respectively), pSc119.2 and pAs1 (containing respectively tandem repeat sequences obtained from *Secale cereale* L. and *Triticum tauschii* Coss), and pHch950 (disperse repetitive sequence derived from *H. chilense* Roem. & Schult.). Full probe descriptions, probe labeling procedures and the FISH conditions used have been reported previously [21]. The synthetic oligonucleotide probes $(AG)_{10}$, $(AAC)_5$, $(AAG)_5$ and $(ACT)_5$ were also used to reveal the presence of SSRs in the chromosomes by ND-FISH (non-denaturing FISH) as previously reported [23]. To detect telomeric repeats the oligonucleotide $(5'\text{-CCCTAAA-}3')_3$ was used according to our previous description [42]. GISH analyses, performed following the hybridization, washing and detection procedure reported previously, were carried out to reveal the genome or subgenome identification in 4x and 6x materials. Thus, total genomic DNA from subsp. *gussoneanum* was used in the analyses of *H. capense*, *H. secalinum* and the 6x cytotype of *H. brachyantherum*, allowing distinguish between the subgenomes **I** and **Xa** in these polyploids. In addition, total genomic DNA from subsp. *californicum* (used as probe) and *H. roshevitzii* (used as DNA block) was used at ratio 1:50 to analyses the 4x cytotype of subsp. *brachyantherum*, however GISH was unable to distinguish between their two subgenomes **I**. For image analysis, metaphase cells were examined using a Zeiss Axiophot epifluoresecence microscope and images were recorded with each filer set using a cooled CCD camera (AxioCamMRc). The images were processed using Adobe Photoshop, employing only those functions that are applied equally to all pixels in the image.

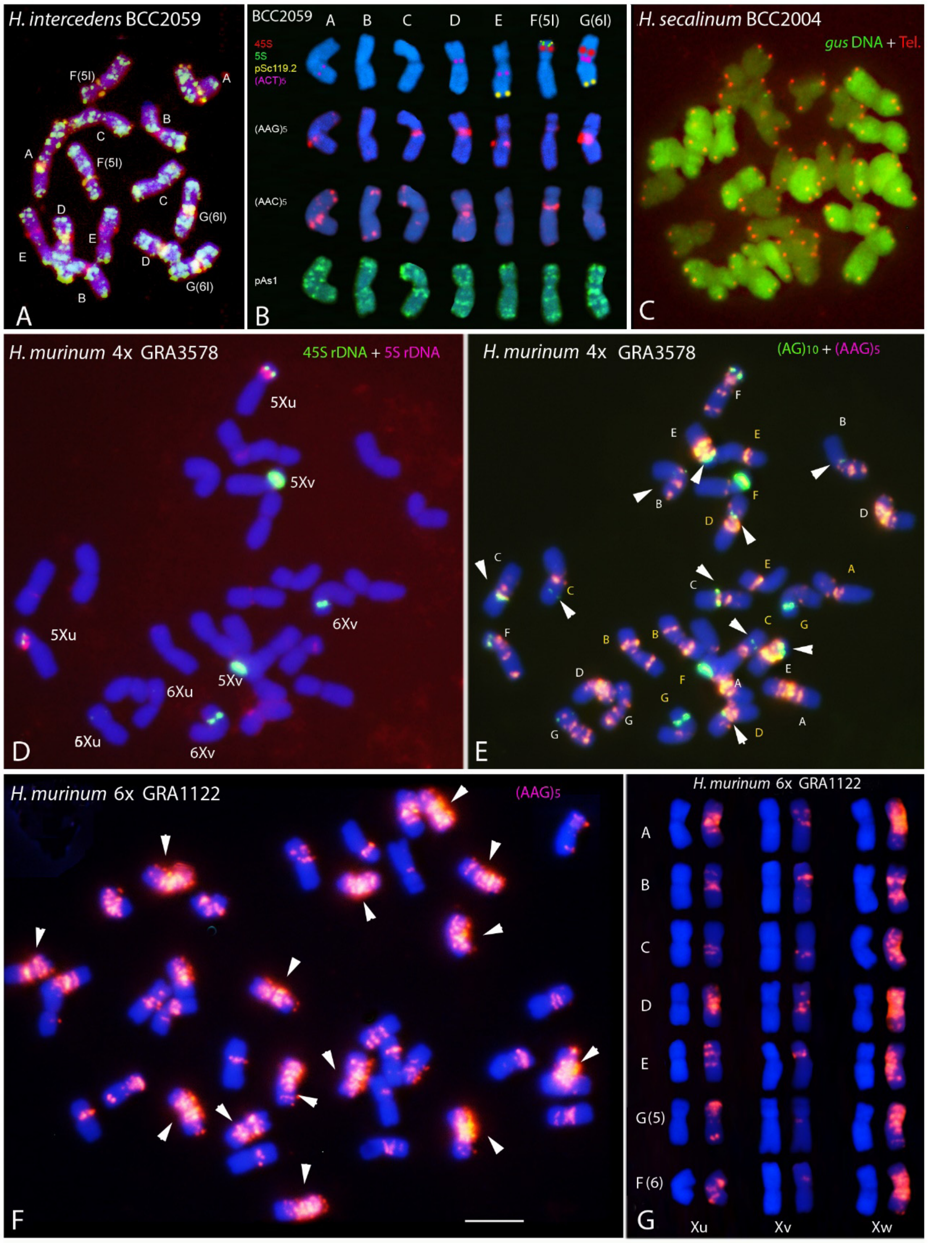


Figure 1: Multiple-target in situ hybridization in different materials with different probes as indicated. pTa71, pTa794, pSc119.2, pAs1 by FISH; $(AG)_{10}$, $(AAC)_5$, $(AAG)_5$, $(ACT)_5$ and the telomeric probe (Tel.) by ND-FISH and GISH with genomic DNA from subsp. *gussoneanum* (*gus* DNA). A. Metaphase cell of *H. intercedens* that shows the superposition of the images of multiple-target obtained with the probes indicated in B. B. The karyotypes of the same cell with the probes indicated. To facilitate the visualization and localization of 45S rDNA, 5S rDNA, pSc119.2 and $(ACT)_5$, these signals were painted over the blue DAPI-stained chromosomes (top row). C. GISH revealing the presence of two different genomes, **I** (dark green) and **Xa** (brilliant green), in a cell of *H. secalinum*. D-E. Chromosomes of a 4x cell of *H. murinum* after using a successive hybridization with the probes pTa71, pTa794 (FISH) and $(AG)_{10}$, $(AAG)_5$ (ND-FISH). Arrows indicate the signals (green) of the probe $(AG)_{10}$. In E, chromosomes identities are indicated in white (subgenome **Xu**) and yellow (subgenome **Xv**). F. A metaphase of 6x *H. murinum* after hybridization with $(AAG)_5$. The arrows point the 14 chromosomes belonging to subgenome **Xw**, enriched with clusters of AAG repeats. G. The chromosomes of the haploid complement chosen from the cell shows in F. Chromosome identification and the distinction of the subgenomes (**Xu**, **Xv** and **Xw**) in 4x and 6x forms of *H. murinum* according with [24]. Scale bar 10μm.

Plant Material

The plant material used in this search included 59 accessions representing all genomes and cytotypes of the genus *Hordeum* covering the species' entire geographic distribution (Table 1). Genome **H**: including *H. vulgare* and *H. bulbosum*. Genome **X**a: including *H. marinum*. Genome **Xu**: including *H. murinum*. Genome **I**: including 2x and 4x cytotypes of *H. brachyantherum*, *H. roshevitzii*, *H. intercedens*, *H. chilense*, *H. cordobense* and *H. bogdanii*. And the allopolyploids combining **I** and **Xa** genomes: *H. capense*, *H. secalinum* and the 6x cytotype of *H. brachyantherum*.

Species	Haploid Genome/s	Ploidy	Origin	Cultivar/Accession
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	Germany	'Alexis'
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	France	'Barberousse'
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	France	'Beka'
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	France	'Dobla'
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	France	'Gaelic'
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	Great Britain	'Golden Promise '
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	Great Britain	'Graphic'
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	France	'Hispanic'
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	USA	'Morex'
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	México/Spain	'Orria'
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	France	'Plaisant'
Hordeum vulgare L. subsp. *vulgare*	**H**	2x	Germany	'Triumph'
H. spontaneum K. Koch var. *spontaneum*	**H**	2x	Iran	HOR2680
H. spontaneum K. Koch var. *spontaneum*	**H**	2x	Turkmenistan	HOR4873
H. spontaneum K. Koch var. *spontaneum*	**H**	2x	Turkmenistan	HOR4894
H.spontaneum K. Koch var. *spontaneum*	**H**	2x	USA	HOR8538
H.spontaneum K. Koch var *ischnatherum (Coss.)Thell*	**H**	2x	USA	HOR8543
H.spontaneum K. Koch	**H**	2x	Israel	HOR22052
H.spontaneum K. Koch var. *spontaneum*	**H**	2x	Israel	HOR22053
H. bulbosum L.	**H**	2x	Italy	BCC2061
H. bulbosum L. subsp. *bulbosum*	**HH**	4x	Georgia	GRA1094
H. bulbosum L. subsp. *nodosum* (l.) *Baum*	**H**	2x	Italy	GRA1154
H. bulbosum L. subsp. *bulbosum*	**HH**	4x	Tajikistan	GRA1193
H. murinum L. subsp. *glaucum* (Steud.) Tzvelev	**Xu**	2x	Tunisia	BCC2002
H. murinum L. subsp. *leporinum* (Link) Arcang.	**XuXv**	4x	Spain	BCC2007
H. murinum L. subsp. *leporinum* (Link) Arcang.	**Xu Xv**	4x	Italy	GRA1021
**H. murinum* L. subsp. *leporinum* (Link) Arcang.	**XuXvXw**	6x	Armenia	GRA1122***
H. murinum L. subsp. *leporinum* (Link) Arcang.	**Xu Xv**	4x	France	GRA1144
H. murinum L. subsp. *murinum*	**XuXvXw**	6x**	Germany	GRA1183
H. murinum L. subsp. *glaucum* (Steud.) Tzvelev	**XuXv**	4x**	Portugal	GRA2735

H. murinum L. subsp. *murinum*	**XuXv**	4x	Spain	GRA2894
**H. murinum* L. subsp. *murinum*	**XuXv**	4x	Germany	GRA3578***
H. marinum Huds. subsp. *marinum*	**Xa**	2x	Greece	BCC2001
H. marinum Huds. subsp. *marinum*	**Xa**	2x	Spain	GRA963
H. marinum Huds. subsp. *marinum*	**Xa**	2x	Italy	GRA1078
H. marinum subsp. *gussoneanum* (Parl.) Thell.	**XaXa**	4x	Turkey	BCC2011
H. marinum subsp. *gussoneanum* (Parl.) Thell.	**Xa**	2x	Bulgaria	BCC2012
H. marinum subsp. *gussoneanum* (Parl.) Thell.	**Xa**	2x	Italy	GRA1077
H. brachyantherum Nevski subsp. *brachyantherum*	**II**	4x	USA (Cal.)	BCC2045
H. brachyantherum Nevski subsp. *brachyantherum*	**IIXa**	6x	USA (Cal.)	BCC2046
H. brachyantherum Nevski subsp. *brachyantherum*	**II**	4x	USA (Alaska)	BCC2050
H. brachyantherum Nevski subsp. *brachyantherum*	**II**	4x	Canada	BCC2056
H. brachyantherum Nevski subsp. *californicum* (Cov. & Steb.) Both. & al.	**I**	2x	USA (Cal.)	BCC2057
H. brachyantherum Nevski *subsp. californicum* (Cov. & Steb.) Both. & al.	**I**	2x	USA (Cal.)	BCC2058
H. roshevitzii Bowden	**I**	2x	China	BCC2015
H. roshevitzii Bowden	**I**	2x	Russia	BCC2069
**H. intercedens* Nevski	**I**	2x	USA	BCC2044
**H. intercedens* Nevski	**I**	2x	USA	BCC2053
**H. intercedens* Nevski	**I**	2x	Mexico	BCC2059
**H. chilense* Roem. & Schult.	**I**	2x	unknown	H1
**H. cordobense* Bothmer et al.	**I**	2x	Argentina	BCC2067
**H. cordobense Bothmer et al.*	**I**	2x	Argentina	BCC2039
**H. bogdanii Wilensky*	**I**	2x	Pakistan	BCC2063
H. capense Thumb	**IXa**	4x	South Africa	BCC2062
H. capense Thumb	**IXa**	4x	South Africa	H335
H. secalinum Schreb.	**IXa**	4x	Spain	BCC2004
H. secalinum Schreb.	**IXa**	4x	Sweden	H231
H. secalinum Schreb.	**IXa**	4x	UK	H3121
H. secalinum Schreb.	**IXa**	4x	Sweden	GRA1147

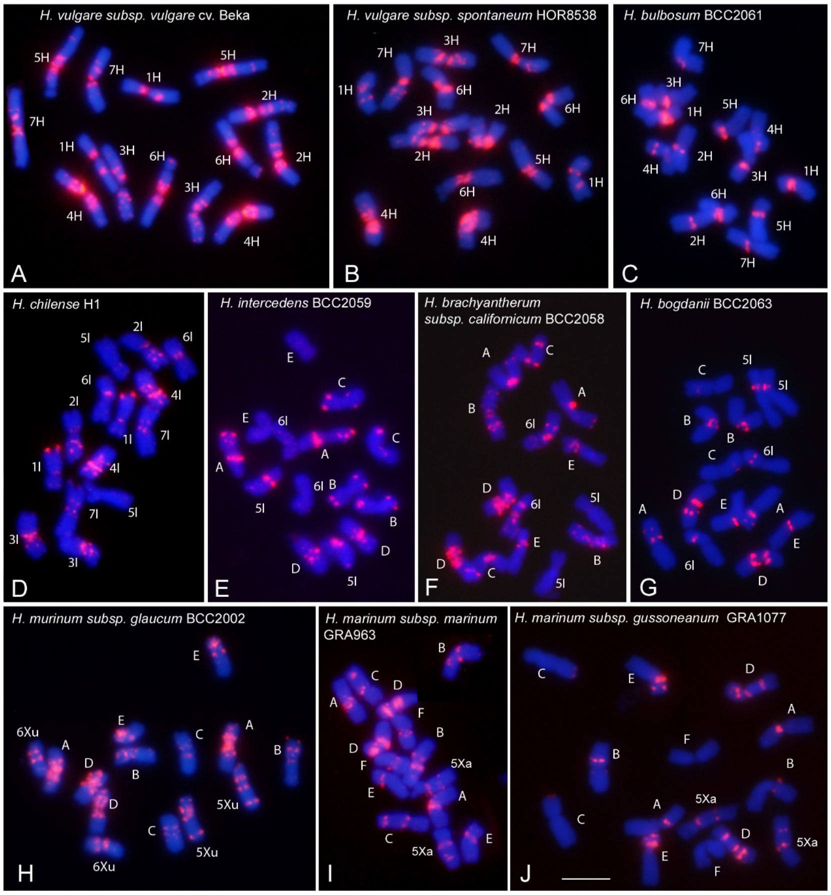
Note:- Most of the seeds of the wild Hordeum accessions examined in this study were obtained from the Germplasm Bank at IPK (Gatersleben, Germany); some accessions belonged to the Barley Core Collection (BCC). H. capense accession H335 and H. secalinum H231 and H3121 from the Nordic Genetic Resource Center (NordGen, Scandinavia); The H. chilense accession H1 from the Germp. Coll. IAS - CSIC, Córdoba (Spain). (*) Accessions not studied previously; (**) Disagreement with the ploidy level hoped; (***) Originally misidentified as H. secalinum. These accessions has been botanically reclassified as H. murinum by the morphology of spikes and spikelets and chromosome composition.

Table 1: The Plant material studied.

Chromosome recognition and karyotyping

$(AAG)_5$

The most clear, intense and rich pattern of signals was obtained with the probe $(AAG)_5$, which permits the identification of homologous chromosome pairs in all the plants analysed. In fact, the use of $(AAG)_5$ displays a hybridization pattern that resembles the C-banding in barley and other grasses [43]. The identification of the chromosomes with this probe facilitated a chromosome-to-chromosome recognition in combination with the hybridization patterns of the remaining probes in the multiple target *in situ* analysis. Figure 2 displays a panel of metaphase cells of diploid species representing the four genomes of *Hordeum* after ND-FISH with this probe. The designation of the chromosomes of Triticeae has been object of different numbering proposals in the last years. For *H. vulgare*, *H. bulbosum* and *H. chilense* chromosomes are designated by numbers according to their known homeologous relationships with other Triticeae. As a reference for *H. vulgare* and *H. bulbosum* homeologies see Carmona et al. (2013) [26] and for *H. chilense* homeologies see Prieto et al. (2004) [44] (Figure 2A-D). Following the classical nomenclature used for Triticeae chromosomes with unknown homeology for the rest of species, chromosomes were designated by letters and chromosomes in the karyotypes (haploid complement) were arranged in order of decreasing length (A to G) with the satellitized (SAT) chromosomes at the end (Figure1B). For the karyotyping of polyploid species, the chromosomes of the haploid complement were arranged as two or three subgenomes of seven chromosomes, which were designated by numbers or letters following the nomenclature used for diploids. The chromosomes were designated as A to N in the cases in which the differentiation as belonging to different subgenomes was not possible (**I** subgenomes of 4x and 6x in *H. brachyantherum*). This system simply provides a means of identifying chromosomes in each species; assignment of the same letter should not be interpreted as representing homeologous relationships among chromosomes of different species.


H. vulgare subsp. vulgare cv. Beka

H. vulgare subsp. spontaneum HOR8538

H. bulbosum BCC2061

H. chilense H1

H. intercedens BCC2059

H. brachyantherum subsp. californicum BCC2058

H. bogdanii BCC2063

H. murinum subsp. glaucum BCC2002

H. marinum subsp. marinum GRA963

H. marinum subsp. gussoneanum GRA1077

Figure 2: ND-FISH with the probe $(AAG)_5$ in somatic metaphases of diploid species representing the four genomes of the genus *Hordeum*. Note that this probe was enough to distinguish the seven pairs of chromosomes in all species. A-C. Genome **H**: Note that both subspecies of *H. vulgare* share the same pattern, however, *H. vulgare* and *H. bulbosum* showed great differences. D-G. Genome **I**: the four species of the panel display pericentromeric and intercalary signals of different intensity. H. Genome **Xu**. I-J. Genome **Xa**. Note that subsp. *marinum* and subsp. *gussoneanum* although similar revealed some differences for $(AAG)_5$. Scale bar 10µm.

pTa794 and pTa71

The use of FISH with the rDNA-targeted probes, pTa794 and pTa71 (Figure 1D) reveals the position of 5S rDNA and 45S rDNA respectively in the SAT chromosomes 5H and 6H of *H. vulgare* and *H. bulbosum* and 5I and 6I of *H. chilense*. Thus, the combination of the ribosomal DNA probes allows recognizing the homeologous SAT chromosomes in the rest of the species and genomes. These probes permit to identify the chromosomes 5I and 6I in all species with the genome **I** as is shown for *H. intercedens*, *H. brachyantherum* subsp. *californicum* and *H. bogdanii* (Figure 2E-G). The same probes allowed identifying the 5Xa in both subspecies of *H. marinum* (Figure 2I-J) and 5Xu and 6Xu in diploids of *H. murinum* subsp. *glaucum* (Figure 2H).

$(AAC)_5$, pAs1 and pHch950

$(AAC)_5$ has been found predominantly associated with $(AAG)_5$ in the heterochromatin of chromosomes. It produces similar though slightly less diagnostic patterns than $(AAG)_5$. On the other hand, a rich pattern of *in situ* signals was obtained with pAs1 on all chromosome arms in wild species (Figure 1B). Although these two probes returned similar patterns for different chromosomes in all material analyzed, as here shown for the first time in *H. intercedens*, after reprobing with the $(AAG)_5$ was possible the characterization of the chromosomes (compare patterns in Figure 1B). The presence and distribution of $(AAC)_5$ and pAs1 increased the number of physical markers to the karyotypes of numerous *Hordeum* which had been previously reported. Nevertheless, the only use of these probes was not enough effective to elucidate the variability between species and to establish relationships between them. For that reason, $(AAC)_5$ and pAs1 have been discarded for the intergenomic relationhips analysis in *Hordeum* in the present work. pHch950 was not useful neither to distinguish chromosomes nor subgenomes in the polyploid forms with the only exception of the 4x and 6x cytotypes of *H. murinum*. This probe hybridized in a dispersed fashion in only seven chromosome pairs of allopolyploid cytotypes of this species (data not showed). This result was particularly important for identifying the chromosomes of two subgenomes joined in the tetraploids: The **Xu**, with dispersed signals of pHch950, and a different subgenome for which we proposed the designation of **Xv**, without pHch950 signals. The **Xu** subgenome showed sizes, morphology, and hybridization patterns similar to those seen in the diploid *H. murinum* subsp. *glaucum* (compare Figure1E and 2H). This subgenome was also present in the hexaploid cytotype of *H. murinum*. Ones the subgenome **Xu** was identified in polyploids with probe pHch950, the easy identification of all chromosome pairs after FISH with $(AAG)_5$ allowed chromosome-by-chromosome identification without needing to use pHch950 for subgenome distinction see (Figure1D-G).

$(ACT)_5$

Figure 3 shows the distinctive hybridization patterns obtained with $(ACT)_5$. This probe produced a rich pattern of clear intercalary signals of different intensity similar in all the cultivated (subsp. *vulgare*) and the related wild barley (subsp. *spontaneum*) lines analysed (Figure 3 A-C). The multiple sites observed in metaphase cells allowed the identification of all chromosome pairs using the distribution pattern previously established in *H. vulgare* subsp. *vulgare* cv. 'Plaisant'. A karyotype ideogram showing the positions of $(ACT)_5$ is very useful as anchoring references in the physical maps of *H. vulgare* (Figure 3C). In contrast, clusters of $(ACT)_5$ were exclusively observed near the centromeres of some chromosome pairs in *H. bulbosum* (Figure 3D). In the rest of species and cytotypes analysed with the **Xu**, **Xa** and **I** genome, $(ACT)_5$ only revealed signals of weak intensity that were very little suitable as diagnostic markers (Figure 3E-G). Nevertheless, the identification of chromosomes carrying clusters of $(ACT)_5$ as showed in Figure 3G for subsp. *californicum* was possible after reprobing the same metaphase with $(AAG)_5$ (Figure 2F).

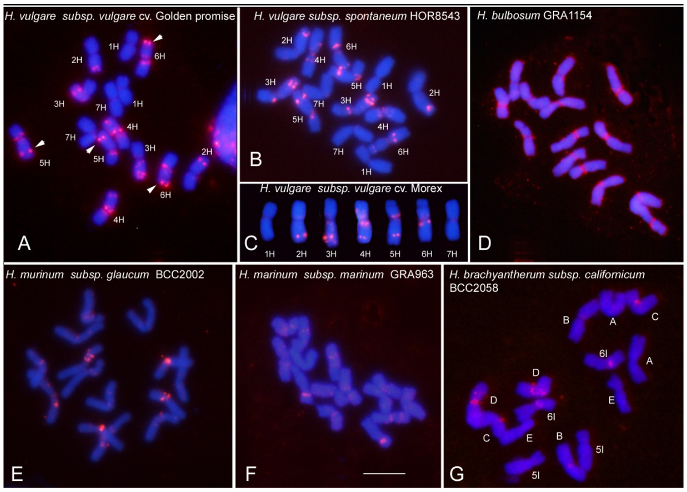


Figure 3: ND-FISH with the probe $(ACT)_5$ in metaphases of representative diploid samples of the four *Hordeum* genomes of the genus *Hordeum*. A-D. Genome **H**. Note that both subspecies of *H. vulgare* share similar pattern but *H. bulbosum* showed remarkable differences with *H. vulgare*. In C, karyotype of cultivar 'Morex'. E. Genome **Xu**. Poor presence of $(ACT)_5$ clusters in *H. murinum* subsp. *glaucum* chromosomes. F. Genome **Xa**. Weak intense signals in *H. marinum*. G. Genome **I**. The same metaphase cell of *H. brachyanthrum* subsp. *californimum* after rehybridization with $(AAG)_5$ is showed in Figure 2F. Scale bar 10μm.

$(AG)_{10}$

The results of application the probe $(AG)_{10}$ are shown in Figure 4. This probe returned strong signals of generally similar intensity at all centromeres of the chromosomes for all the cultivars and lines analysed of *H. vulgare* subsp. *vulgare* and subsp. *spontanteum* (Figure 4A-B). No interstitial signals were observed with this probe. Nevertheless, a weak terminal cluster of AG repeats on 4HL was observed in all cultivars after increasing the CCD camera exposure time [26]. In contrast, signals of $(AG)_{10}$ were observed only after increasing the exposure time of the CCD camera, and then only poorly, in *H. bulbosum* carrying also the **H** genome (Figure 4C). No clusters of AG repeats were observed in chromosomes of the genome **Xa** present in *H. marinum* subsp. *marinum* and subsp. *gussoneanum*. By contrast, well defined signals of different intensity near the centromeres were observed with $(AG)_{10}$ in four chromosomes pairs in the **Xu** genome of the diploid accession of *H. murinum* subsp. *glaucum* analysed (Figure 4D). The identification of chromosomes carrying clusters of AG was possible after reprobing the same metaphase with $(AAG)_{5}$ (see Figure 2H). As in the diploid forms, no clusters of AG repeats were observed in the chromosomes of tetraploids of *H. marinum* subsp. *gussoneanum*. Clusters of AG were also present in chromosomes of subgenomes **Xv** and **Xw** in polyploids of *H. murinum* (Figure 1E). Indeed, signals of $(AG)_{10}$ in *H. murinum*, intensely present in the subgenome **Xw** of *H. murinum* subsp. *leporinum* were previously reported. No signals of $(AG)_{10}$ were observed in the species with the genome **I**, with the only exception of *H. roshevitzii*, which presents a cluster of AG repeat close to the 45S rDNA signal on chromosome E (Figure 4E). Interestingly, this signal was also present in only one chromosome pair in the tetraploids (Figure 4F) and hexaploids (Figure 4G) cytotypes of *H. brachyantherum*. It is noticeable that in both polyploid cytotypes of subsp. *brachyantherum* the AG cluster was located close to the 45S rDNA locus on the long arm of the SAT chromosomes L and M, respectively. The physical map of the 45S rDNA locus and $(AG)_{10}$ of the chromosome E of *H. roshevitzii* is coincident with the L and M chromosomes of the tetraploid and hexaploid forms, respectively (compare Figure 4E to G).

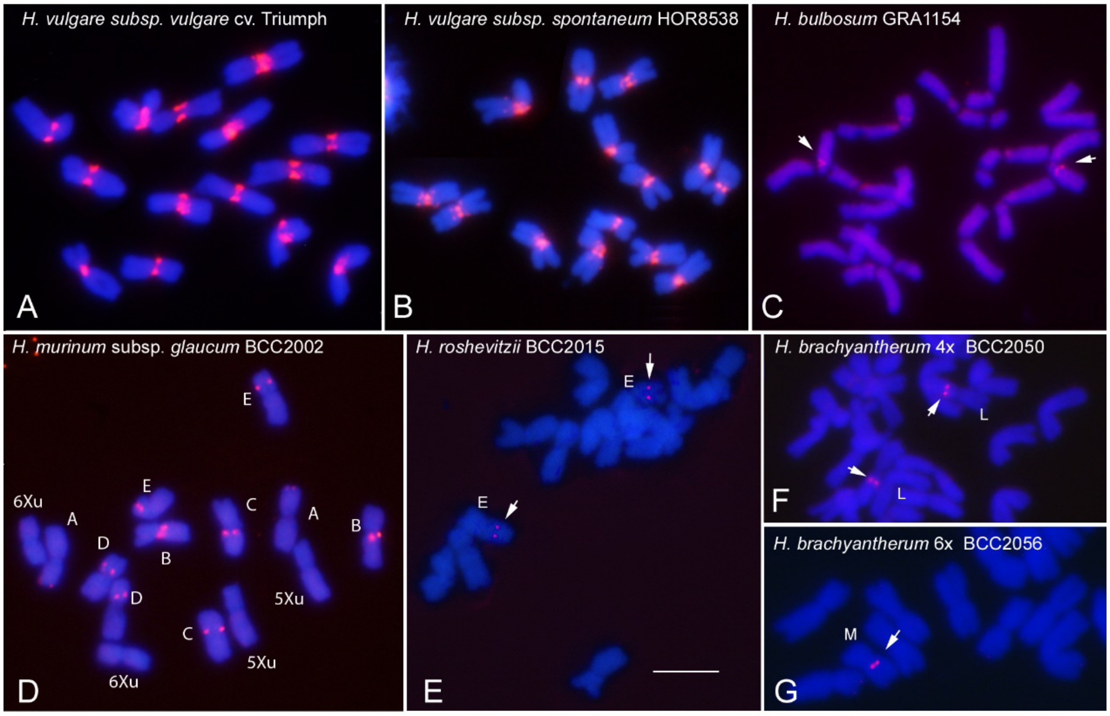


Figure 4: ND-FISH with the probe $(AG)_{10}$ in metaphases of representative samples of genus *Hordeum*. A-C. Genome **H**. Both subspecies of *H. vulgare* share the same centromeric pattern. Note that *H. bulbosum* showed remarkable differences with *H. vulgare*. D. Genome **Xu**. Presence of AG clusters in *H. murinum*. E-G. Genome **I**. Note only a pair of chromosomes in *H. roshevitzii* (E), 4x subsp. *brachyantherum* (F) and 6x subsp. *brachyantherum* (G) carry a cluster of this microsatellite. Scale bar 10µm.

pSc119.2

The probe pSc119.2 revealed subtelomeric signals of different intensity in *Hordeum* chromosomes with the only exception of the genomes **H** of *H. vulgare* and **Xu** and **Xw** of *H. murinum*. The pattern of localization of these signals varied in number, from a few to nearly all the chromosome arms, depending on the species. pSc119.2 was present in the genome **H** of *H. bulbosum* (Figure 5A), the genome **I** of all specie analyzed (Figure 5B) and the subgenome **Xv** of the tetraploid and hexaploid cytotypes of *H. murinum* (Figure 5C). pSc119.2 showed also subtelomeric signals in both subspecies of *H. marinum* carrying the **Xa** genome (Figure 5D-F). It was especially diagnostic the presence of an interstitial site on the long arm of the most submetacentric chromosome pair (chromosome E) in diploid and tetraploid cytotypes of *H. marinum* subsp. *gussoneanum* (Figure 5E-F). This provided a marker of choice for distinguishing this chromosome in all the allopolyploids carrying the **Xa** genome, as revealed after GISH, including the hexaploid cytotype of *H. brachyantherum* (Figure 5G), *H. secalinum* (Figure 5H) and *H. capense* (Figure 5I). A set of 14 chromosomes showing the same size, morphology and hybridization patterns with the set of probes investigated to the seven chromosome pairs present in the diploid forms of subsp. *gussoneanum* was found in the tetraploid cytotype of *H. marinum* subsp. gussoneanum as well as in the allopolyploids carrying the **I** and **Xa** genomes.

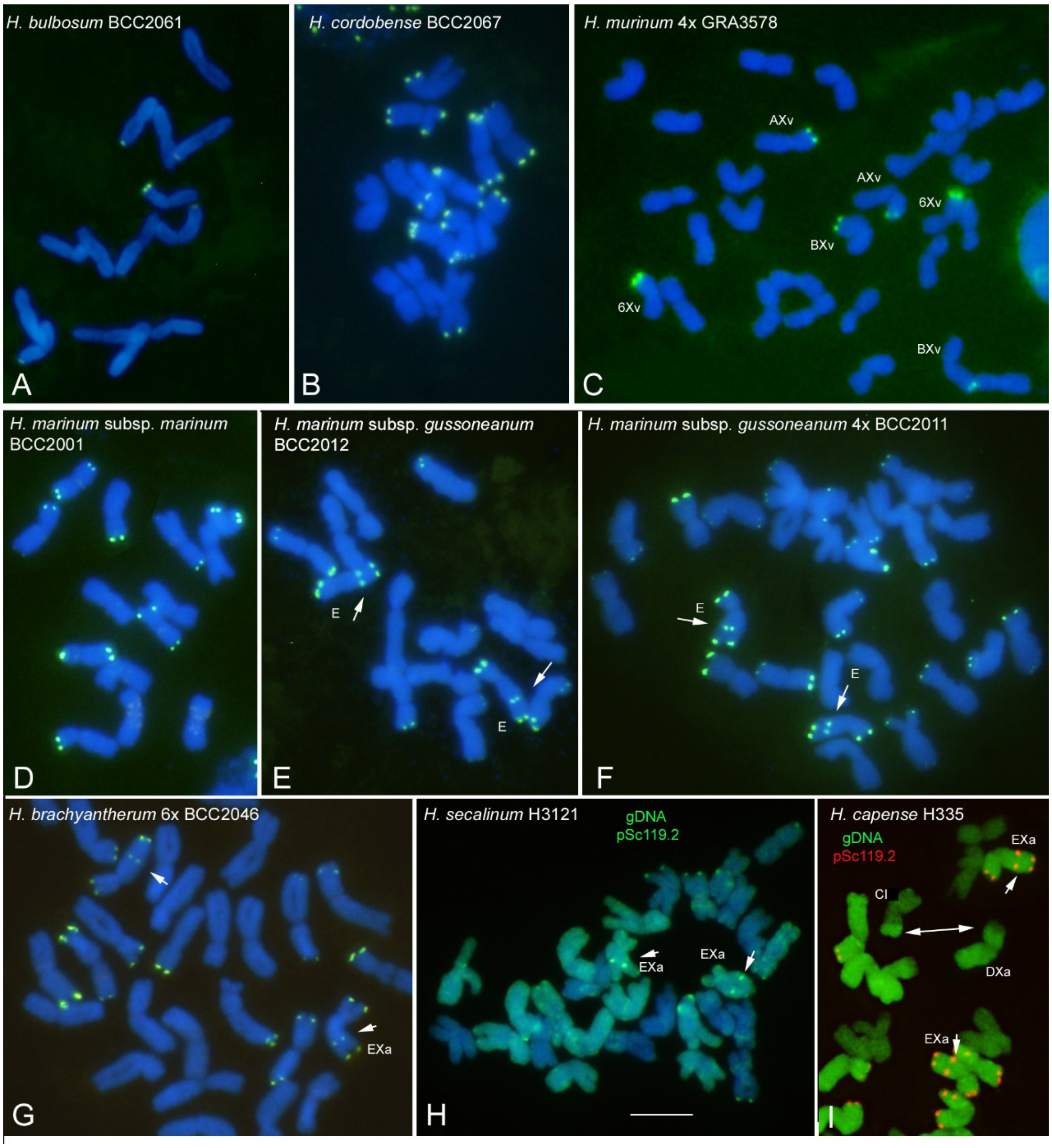


Figure 5: The presence and distribution of the probe pSc119.2 in metaphase cells of different genomes or subgenomes of *Hordeum*. A. Genome **H**. *H. bulbosum*. B. Genome **I**. *H. cordobense*. C. Subgenome **XU**. *H. murinum* 4x. D-F. Genome **Xa**. subsp. *marinum* (D), 2x subsp. *gussoneanum* (E), and 4x subsp. *gussoneanum* (F). G-I. Subgenomes **I** and **Xa**. 6x *H. brachyantherum* (G), *H. secalinum* (H) and *H. capense* (I). Arrows point to the interstitial loci found on chromosome E of 2x and 4x subsp. *gussoneanum* (E) as well as all the allopolyploids carrying the **Xa** subgenome (F-I). Note the presence of an intergenic translocation in *H. capense* accession H335 (arrow with two ends in I). Scale bar 10µm.

(CCCTAAA)$_3$

The telomeric probe (CCCTAAA)$_3$ (Tel.) marks usually the ends of all chromosome arms as is shown in Figure 6 for *H. vulgare* (Figure 6A) and subsp. *californicum* (Figure 6B). In addition, one interstitial telomeric signal was found in one SAT chromosome pair in all cytotypes of *H. murinum* (arrows in Figure 6C and E-F). This SAT chromosome carries both 5S and 45S rDNA loci and hence was identified as chromosome 5Xu see (Figure 6C-D).

It is noticeable that chromosome 5Xu identified in the tetraploid and hexaploid cytotypes of *H. murinum* using the ribosomal rDNA probes revealed the same interstitial telomeric signal (Figure 6E-F).

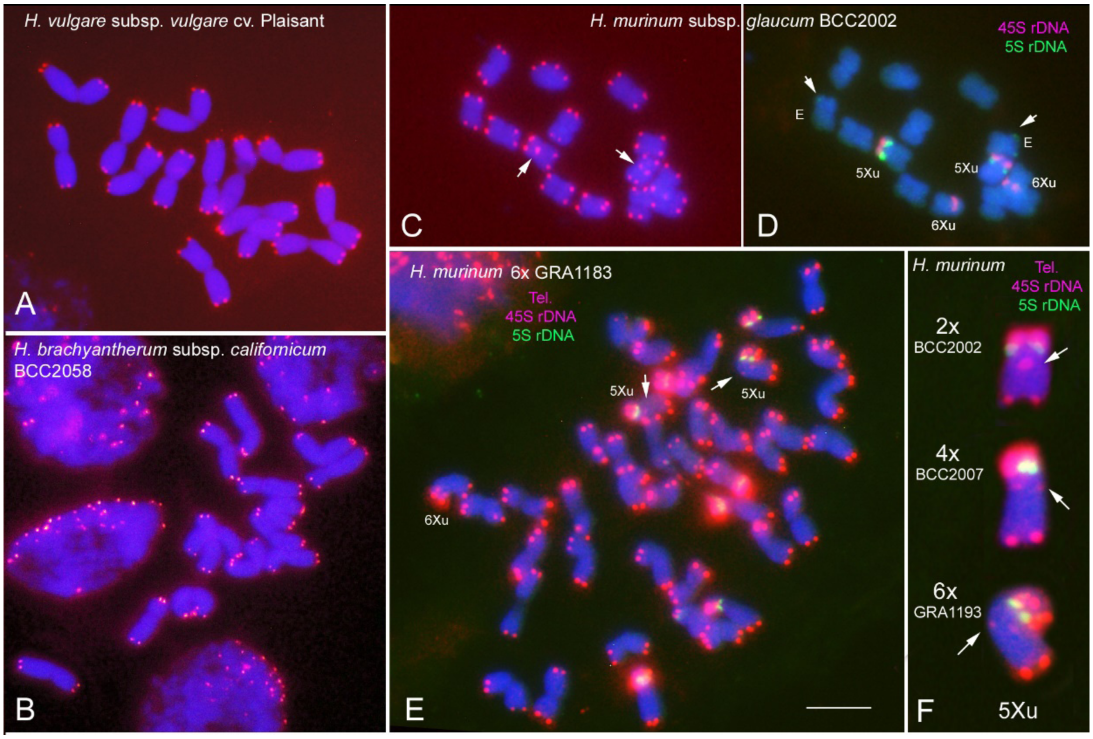


Figure 6: Telomeric signals (Tel.) in somatic metaphases of species representing the genus *Hordeum*. A. Genome **H**. *H. vulgare* cv. ‘Plaisant’. B. Genome **I** subsp. *californicum*. C-D. Genome **Xu**. Combining the use of (CCCTAAA)$_3$ (Tel.) with pTa794 and pTa71 on the same metaphase of 2x forms of subsp. *glaucum* allowed the recognition of the pairs 5Xu and 6Xu, and the presence of an interstitial telomeric signal in 5Xu (arrowed in C). Arrows in D points the minor 5S rDNA locus present in chromosome E. E. The combination of these three probes also permitted to distinguish the presence of only one chromosome pair (5Xu) carrying the interstitial telomeric sequence in the polyploid cytotypes of *H. murinum*. F. Amplified chromosome 5Xu of the indicated 2x, 4x and 6x accessions of *H. murinum* with the same combination of probes to easy visualizate the interstitial telomeric signals (arrows). Note that chromosome 5Xu of 2x and 6x accessions were chosen from metaphases showed in C and E respectively. Scale bar 10µm.

Use of physical maps after FISH and ND-FISH for the intergenomic relationhips analysis in *Hordeum*

By combining the SSR motifs analyzed by ND-FISH with other cloned probes of repetitive DNA by FISH was possible to enrich the physical maps by adding new landmarks to the karyotypes of an important number of species of *Hordeum*. Some probes analyzed in combination with GISH were especially useful to establish the origin of the genomes present in the polyploidy cytotypes. Figures 7 and 8 summarize the phylogenetic relationships of 19 species and cytotypes of *Hordeum* based on the chromosome position of a selected set of repetitive DNA. The total phylogenetic tree is split in two branches. The first branch includes the results of genomes **H** and **Xu** (Figure 7) and the second correspondes to the genomes **Xa** and **I** (Figure 8). It has been included the complete karyotype of the genomes **H** of *H. vulgare* and *H. bulbosum* and **I** of *H. chilense* (nomenclature acoording the homeology with other Triticeae). For the rest of species and genomes only have been included the chromosomes with diagnostic markers allowing the follow up from the diploid species to the polyploids ones.

Genomes H and Xu (Figure 7)

The physical map of homeologous chromosomes (1H to 7H) of all cultivars of *H. vulgare* and the accessions of *H. spontaneum* analyzed was identical after ND-FISH and FISH with the same probes. This was also supported by comparative analyses of DNA sequences [45]. Nevertheless, the comparison of abundance and distribution of each SSR analysed, between *H. vulgare* (both subspecies) and *H. bulbosum*, suggest that these species do not share the same **H** genome. A significant difference between the chromosomes of *H. vulgare* and *H. bulbosum* was observed with different SSRs, as for example with (AG)$_{10}$, (AAG)$_5$ and (ACT)$_5$. For example, the probe (AG)$_{10}$, which returned strong signals of similar intensity at all the centromeres of the cultivars and accessions of *H. vulgare* studied, was not observed in the centromeres of *H. bulbosum* (Figure 4A-C). Other differences were the distinct patterns of distribution of 5S and 45S rDNA among species and the absence of pSc119.2 in *H. vulgare*. These results support the idea that *H. vulgare* and *H. bulbosum* are not very closely related. This could be probably due to their separation from a common ancestor at a relatively early stage. Assuming that the 120-bp is an old and constant repetitive DNA family present in the Triticeae, its absence in *H. vulgare* could be explained supposing a reduction of this sequence after separation of the genome **H** of *H. bulbosum*. Similarly, it is therefore reasonable to assume that specific SSR clusters came about via massive amplification of preexisting SSR sequences in *H. vulgare*, or by their deletion in *H. bulbosum*, after both species were separated from their common ancestor. A similar phenomenon has been observed previously in other Triticeae. For example, in the genus *Secale*, clusters of AAG and AAC have been amplified in cultivated rye after the divergence of *S. sylvestre* [46].

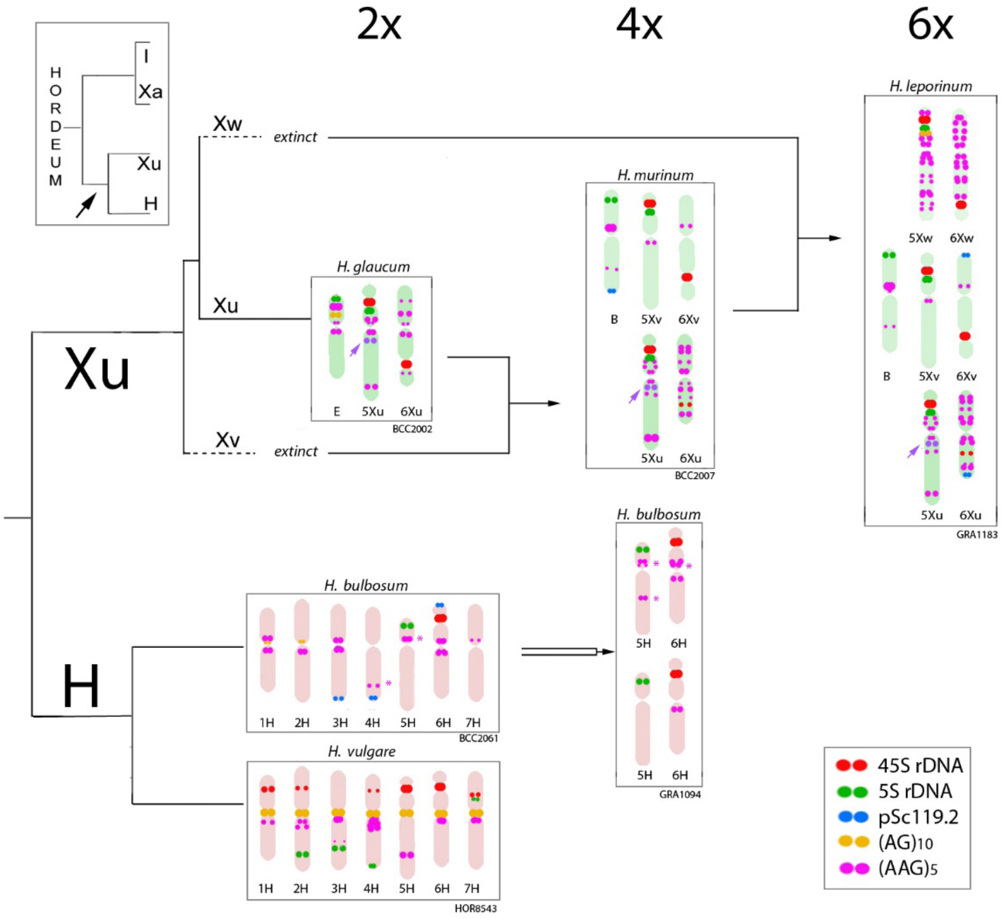


Figure 7: Phylogenetic tree branch (see left upper inset) reflecting relationships among the species of *Hordeu*m carrying the genomes **H** and **Xu**. The ideograms include the distribution of five repetitive sequences (see color key) in the accessions indicated. Arrows indicate the diagnostic interstitial telomeric signal (in purple) found in 5Xu in all *H. murinum* taxa. Lines (arbitrary lengths) to their inferred progenitors connect polyploid taxa (right in the tree). Double lines indicate autopolyploid origin of 4x *H. bulbosum*. Extinct denotes probably extinct taxa. Asterisks indicate diagnostic polymorphic signals within and/or between *H. bulbosum* accessions.

H. murinum is a species complex composed of the subspecies *glaucum* (2x and 4x), *murinum* (4x and 6x) and *leporinum* (4x and 6x). Allopolyploidy has been strongly supported for the polyploid forms of this species by molecular and phylogenetic analysis that differentiates 2x, 4x and 6x cytotypes [47, 48]. FISH and ND-FISH using the selected set of repetitive DNA sequences permitted the identification of three subgenomes involved in the accessions of *H. murinum* here examined. All tetraploids of the three subspecies shared two subgenomes of which one was found to be very similar (though not identical) to the set of seven chromosome pairs present in the diploid forms of subsp. *glaucum*. It is interesting to note the differences between the three subgenomes (**Xu**, **Xv** and **Xw**) present in the *H. murinum* complex. For example, subgenome **Xu** with stronger dispersed signals of pHch950 than subgenomes

Xv and **Xw**. pSc119.2 was absent in the diploid genome **Xu** of subsp. *glaucum* and in the **Xw** subgenome of the hexaploids. This subgenome, **Xw**, was also distinguished in the hexaploid forms by the intense presence of signals with the $(AAG)_5$ (Figure 1F-G). The absence of pSc119.2 signals in (sub)genomes **Xu** and **Xw** could be explained by a reduction of the 120-bp repetitive DNA family in the branch of subsp. *glaucum* and the unidentified extinct diploid donors of the subgenome **Xw** present in the hexaploids forms of *H. murinum* after their divergence of the extinct diploid donor of the subgenome **Xv**. The intersticialization of the telomeric signal in 5Xu (this signal is absent in both, the 5Xv and 5Xw) clearly must be happened only in genome **Xu** after the diversification of the three diploid donors of the allopolyploids and before the hybridization which originated the 4x and 6x forms of *H. murinum* (purple arrows in Figure 7). As an example of genome changes associated with polyploidisation at the chromosome level, the presence of minor 45S rDNA locus in the long arm of 6Xu of polyploids at the same position of the SAT chromosome 6Xu of *H. glaucum*. This result suggests the deletion or inactivation of ribosomal genes after polyploidization. These results suggest that the members of the *H. murinum* complex could be divided into three species according to their ploidy level: 2x-*murinum* with the haploid genome **Xu**, including the diploid form of subsp. *glaucum*; 4x-*murinum*, with the haploid genome formula **XuXv**, including the currently recognized subsp. *murinum* and the tetraploid cytotypes of subsp. *glaucum* and subsp. *leporinum*, and 6x-*murinum*, with the haploid genome formula **XuXvXw**, including the hexaploid cytotypes of subsp. *murinum* and subsp. *leporinum*. As named in Figure 7, we propose the existence of three cryptic species according to ploidy level: *H. glaucum* for 2x, *H. murinum* for 4x and *H. leporinum* for 6x forms.

Genomes Xa and I (Figure 8)

H. marinum subsp. *marinum* and the 2x and 4x forms of *H. marinum* subsp. *gussoneanum*, in the tree named *H. marinum* and *H. gussoneanum* respectively were characterized by multiple-target *in situ* hybridization (Figure 8). The chromosomes E, F and the SAT chromosome 5Xa exhibited a series of diagnostic landmarks useful in the identification of diploid forms involved in the origin of tetraploids of *H. gussoneanum,* with the haploid genome formula **XaXa**, and other allopolyploid species which share the genomes Xa and I. The tetraploids *H. secalinum* and *H. capense,* with the haploid genome formula **IXa**, and the hexaploid cytotype of *H. brachyantherum*, with the haploid genome formula **IIXa**. It has been assumed that *H. secalinum* and *H. capens*e share a hybrid origin involving subsp. *gussoneanum* as the donor of genome **X**a and an unidentified **I** genome progenitor. Both genomes were distinguished using genomic DNA of subsp. *gussoneanum* as a probe in GISH (see Figure 5H-I). 14 chromosomes of *H. secalinum* and *H. capense* showed fluorescence signals with GISH confirming the presence of the **Xa** genome in both tetraploid species. The probe pSc119.2 gave some significant differences between the diploid genome **Xa** of *H. marinum* and *H. gussoneanum*. This probe produced mainly subtelomeric signals of different intensity on a variable number of chromosome pairs. In addition, pSc119.2 produced a diagnostic signal in an interstitial site on the long arm of the chromosome E of 2x *H. gussoneanum* that was not in the chromosome E of *H. marinum*. This interstitical pSc119.2 signal on chromosome E was also present in 4x *H. gussoneanum* and in *H. secalinum*, *H. capense* and 6x *H. brachyantherum* (blue arrows in Figure 8). Similarly, the probe pTa794 produced a specific signal near the telomeres of the long arm of the SAT chromosome 5Xa of 2x *H. gussoneanum* that was not present in the chromosome 5Xa of *H. marinum*. However, this diagnostic signal appears in one chromosome pair (5Xa) of 4x cytotype of *H. gussoneanum*, and again in *H. capense*, *H. secalinum* and 6x *H. brachyantherum* (green arrows in Figure 8). There is a general coincidence of the physical map of the genome **Xa** of *H. secalinum* and some accessions of *H. capense* with *H. gussoneanum* (see chromosomes E, F and 5Xa in Figure 8). The presence of an extra 45S rDNA locus in the short arm of the SAT chromosome C in some accessions of *H. capense* (red asterisk), not observed in *H. secalinum,* and other minor differences in others **Xa** chromosomes of *H. capense* accession BCC2062 with respect to *H. gussoneanum* and *H. secalinum* were observed. These differences could be explained by diversification of genome **Xa** in an extinct diploid species before hybridization to generate at least some samples of *H. capense*, or by genome remodeling after polyploidization during evolution of *H. capense* from *H. secalinum* (discontinuous lines in the tree towards *H. capense*). These results demonstrate the participation of the genome **Xa** of *H. gussoneanum* in the allopolyploid origin of *H. capense*, *H. secalinum* and 6x *H. brachyantherum* after hybridization with an unidentified diploid species carrying the genome **I**. The maintenance of the physical map for the repetitive DNA clusters investigated in the genome **Xa** of *H. gussoneanum* present in all these species could be explained as a consequence of the relatively recent formation of the allopolyploids. *H. brachyantherum* is a complex taxon with three cytotypes usually considered representing two subspecies, the diploid subsp. *californicum* (also treated as *H. californicum*, as named in the tree), and the polyploid subsp. *brachyantherum*, which consists of tetraploid and the hexaploid cytotypes. Of all diploid species carrying the genome **I** investigated, the Asiatic *H. roshevitzii* is the one that displays a physical map more similar to genome **I** present in subsp. *brachyantherum*. The probe $(AG)_{10}$ gave a diagnostic cluster of AG repeats close to the 45S rDNA signal on the long arm of chromosome E of the genome **I** of *H. roshevitzii* (see brown arrows in Figure 8). These signals were absent in the Asiatic *H. bogdanii*, the tetraploid *H. brevisobulatum* (not included in the present work) and the American diploid species having the genome **I** investigated, including *H. californicum*, the other putative donor of one of the subgenomes **I** in polyploids of *H. brachyantherum*. However, the AG cluster and the 45SrDNA signals of the chromosome E of the genome **I** of *H. roshevitzii* appear in the chromosome pairs L and M of the tetraploid and hexaploid cytotypes of *H. brachyantherum*, respectively (see Figure 4F-G). This result suggests that *H. roshevitzii* or a very close related extinct species is the Asian parental that took part in the origin of the *H. brachyantherum* 4x and 6x as we largely discussed in a previous work [28]. However, no set of seven chromosome pairs was seen in these forms to show the same *in situ* patterns as those seen for *H. roshevitzii*. Similarly, it cannot be confirmed that *H. californicum* to be the other progenitor because significant FISH-markers differences had been found. These differences would have been accumulated since polyploidization. How much chromosomal variation was contributed by the ancestors of 4x subsp. *brachyantherum*, or as a consequence of the evolution of the allopolyploid, is unknown (the ambiguity of the origin of the genomes **I** is represented by discontinuous lines in the phylogenetic tree). Moreover, if we assume that 4x *H. brachyantherum* to be with 2x *H. gussoneanum* the parents of 6x *H. brachyantherum* forms, this seems to demonstrate that the genome **I** of *H. bracyantherum* 4x and 6x has undergone significant changes since the hybridization and polyploidization processes. This does not happen with the chromosomes of the **Xa** genome present in the 6x *H. brachyantherum* that does not display any difference with respect to the **Xa** genome of *H. gussoneanum*.

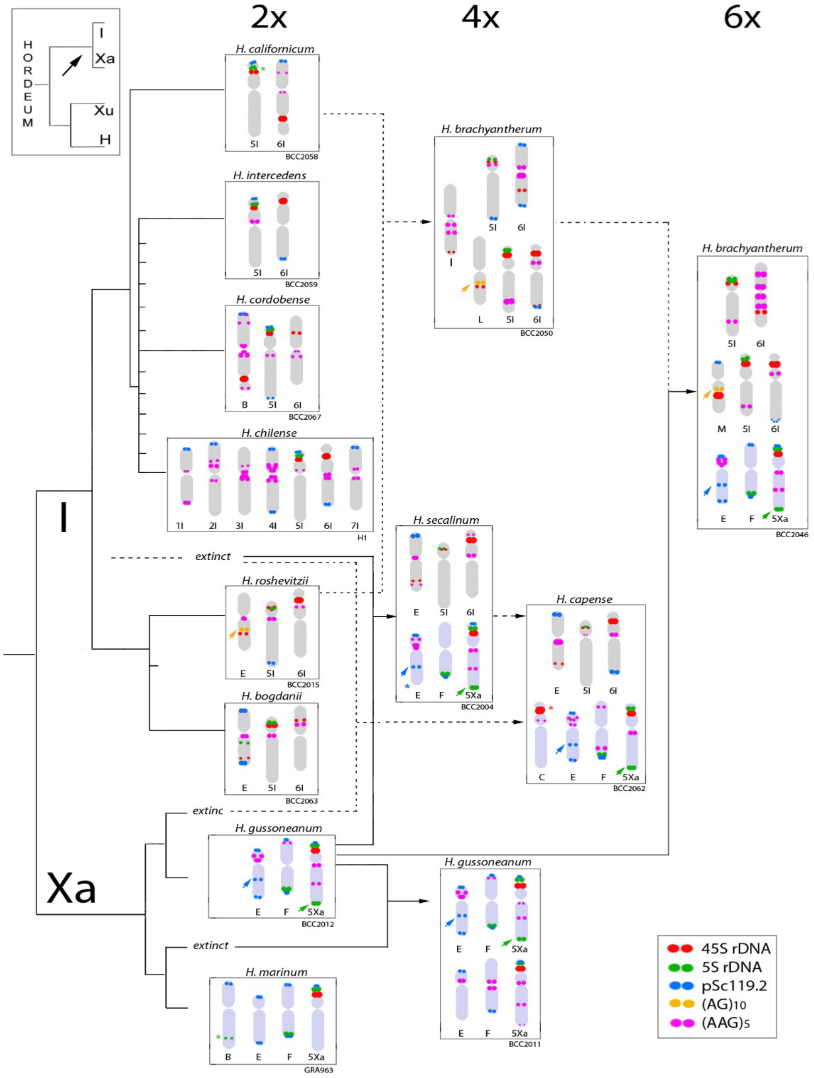


Figure 8: Phylogenetic tree branch (see left upper inset) reflecting relationships among the species of *Hordeum* carrying the genomes **Xa** and **I**. The ideograms include the distribution of five repetitive sequences (see color key) in the accessions indicated. Asterisks indicate diagnostic polymorphic signals between accessions of the same species. Green and blue arrows (see color key) indicate the diagnostic signals found in chromosomes of 2x and 4x *H. gussoneanum*, maintained in the subgenome **Xa** of *H. secalinum*, *H. capense* and 6x *H. brachyantherum*. An AG cluster (brown arrows) and the 45SrDNA signal on the long arm of the chromosome E (red color) of *H. roshevitzii* (genome I) appear in the chromosome pairs L and M of 4x and 6x *H. brachyantherum*, respectively. Dashed lines indicate the possible polyphyletic origin of *H. capense* and the ambiguity over the origin of both cytoypes of *H. brachyantherum*.

Conclusion

The huge differences in the abundance and distribution of the repeats sequences analyzed in *H. vulgare* and *H. bulbosum* suggest that these species do not share the same **H** genome. In contrast, there were not differences between the two subspecies of *H. vulgare* (subsp. *vulgare* and subsp. *spontanum*). With respect to the *H. murinum* complex, the results support the idea that *H. glaucum* and two extinct species were respectively the diploid donors of the subgenomes **Xu**, **Xv** and **Xw** present in polyploids. Assuming that the three progenitors have a monophyletic origin, the FISH-karyotype differences suggest that their separation from a common ancestor have occurred at a relatively early stage. With respect to *H. marinum*, the genome **Xa** present in all taxa was very similar, but distinguishable after FISH-karyotype comparison. The 4x forms of subsp. *gussoneanum* should be originated through a cross between 2x subsp. *gussoneanum* and a second, related, but unidentified, ancestor. Karyotyping showed differences in the genome **I** of diploid species. The presence of a marker chromosome in *H. roshevitzii* and polyploids of *H. brachyantherum* supports that *H. roshevitzii*, or a form closely related, must be involved in the origin of 4x *H. brachyantherum*. *H. brathyantherum* subsp. *californium* or a species closely related should be involved as the other progenitor. The results show a wide divergence between the genome **I** of 4x *H. brachyantherum* and those of 6x *H. brachyantherum* not supporting the general accepted involvement of 4x *H. brachyantherum* as the genome **I** donor of 6x forms. The diploid cytotype of subsp. *gussoneanum* must be the **Xa** genome donor, without undergoing any chromosomal changes after hybridization/polyploidisation, of 6x *H. brachyantherum*, *H. secalinum* and *H. capense*. *H. capense* underwent significant genomic and chromosomal modification during its diversification. Alternatively, this species could be a polyphyletic origin involving an extinct **Xa** progenitor closely related to subsp. *gussoneanum*. Finally, the donor of genome **I** involved in the origin of both *H. secalinum* and *H. capense* remains unknown.

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