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ORIGINAL PAPER

Cytogenetic diversity of SSR motifs within and between *Hordeum* species carrying the H genome: *H. vulgare* L. and *H. bulbosum* L.

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Abstract Non-denaturing FISH (ND-FISH) was used to compare the distribution of four simple sequence repeats (SSRs)—$(AG)_n$, $(AAG)_n$, $(ACT)_n$ and $(ATC)_n$—in somatic root tip metaphase spreads of 12 barley (*H. vulgare* ssp. *vulgare*) cultivars, seven lines of their wild progenitor *H. vulgare* ssp. *spontaneum*, and four lines of their close relative *H. bulbosum*, to determine whether the range of molecular diversity shown by these highly polymorphic sequences is reflected at the chromosome level. In both, the cultivated and wild barleys, clusters of AG and ATC repeats were invariant. In contrast, clusters of AAG and ACT showed polymorphism. Karyotypes were prepared after the identification of their seven pairs of homologous chromosomes. Variation between these homologues was only observed in one wild accession that showed the segregation of a reciprocal translocation involving chromosomes 5H and 7H. The two subspecies of *H. vulgare* analysed were no different in terms of their SSRs. Only AAG repeats were found clustered strongly on the chromosomes of all lines of *H. bulbosum* examined. Wide variation was seen between homologous chromosomes within and across these lines. These results are the first to provide insight into the cytogenetic diversity of SSRs in barley and its closest relatives. Differences in the abundance and distribution of each SSR analysed, between *H. vulgare* and *H. bulbosum*, suggest that these species do not share the same H genome, and support the idea that these species are not very closely related. Southern blotting experiments revealed the complex organization of these SSRs, supporting the findings made with ND-FISH.

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Introduction

Microsatellites, or simple sequence repeats (SSRs), which are found in both coding and non-coding regions, are among the most abundant and widely dispersed types of tandemly repetitive DNA sequences in eukaryotic genomes (Tautz and Renz 1984). Composed of short, repeated units about 1–6 nucleotides (nt) in length, these sequences are organized in tandem arrays usually under 100 nt long. One of the most remarkable properties of SSRs is their ability to produce variants in terms of the numbers of repeats at a given locus. Such polymorphism, easily detected by PCR, makes SSRs the molecular markers of choice for many species in linkage mapping, gene tagging, DNA fingerprinting and genetic evolution studies (Morgante and Olivieri 1993; Röder et al. 1998). SSRs, or at least SSR-based markers, show wide variation between individuals, however, the extent to which SSR variation at the chromosome level may contribute to adaptive divergence among populations and species is still to be determined.

In situ hybridization techniques can be used to detect SSRs in both animal and plant chromosomes. However, over the last three decades, only a few reports have described the physical organization of SSRs (Nanda et al. 1991; Cuadrado and Schwarzacher 1998). Some species of the family *Triticeae* are among the best characterized in terms of the chromosomal distribution of SSRs.

The synthetic GAA oligomer was the first to be used for localising the classic GAA-rich satellite sequence in barley and wheat (Pedersen et al. 1996; Pedersen and Langridge 1997). Our group later reported the physical mapping of all possible classes of di- and trinucleotide SSRs by FISH in the chromosomes of wheat, rye and barley (see review by Cuadrado et al. 2008). These reports contributed to the characterization of the genome of these important cereal crops. Moreover, they showed that, as well as containing SSRs uniformly dispersed throughout the chromosomes, the genomes of these species also contain a large proportion of clusters of different SSRs producing specific and motif-dependent in situ hybridization patterns. The variation reported between related species in terms of the abundance and chromosomal distribution of SSR clusters is not surprising. However, some of the reported specific chromosomal locations might simply reflect genotype differences among lines. The polymorphisms detected by in situ hybridization affecting chromosomal SSR landmarks between different accessions and genotypes has yet to be examined.

Cultivated barley, *Hordeum vulgare* ssp. *vulgare*, with its large proportion of clusters of different SSRs, provides a model for studying the dynamics of SSR distribution at the chromosome level. This species has a diploid chromosome constitution and the identification of all seven pairs (1H–7H) in mitotic metaphase by FISH, employing several DNA probes including SSR probes, is reliable (Cuadrado and Jouve 2007b). The aim of the present study was to characterize the cytogenetic diversity of 4 SSRs in 12 varieties of cultivated barley representing a broad range of genetic diversity. This plant material included cv. 'Morex', which has been selected as a reference genotype for genome sequencing by the International Barley Genome Sequencing Consortium (IBSC) (Schulte et al. 2011). Characterization analyses were performed using non-denaturing FISH (ND-FISH), a method that quickly and efficiently allows the detection of SSR-enriched chromosome regions, and which is an excellent tool for comparative analysis (Cuadrado and Jouve 2010). Four SSR systems were analysed, chosen for their characteristic pattern of chromosomal location revealed by FISH in cv. 'Plaisant' (Cuadrado and Jouve 2007a, b): AG (exclusively concentrated at centromeres in the latter cultivar), AAG (preferentially associated with pericentromeric heterochromatin), ATC (particularly concentrated in the pericentromeric region on 4H) and ACT (found in diagnostic intercalary locations of chromosomes 2H, 3H, 4H, 5H and 6H). The ND-FISH analysis was extended to the closest relatives of cultivated barley, which also carry the H genome. This material included seven lines of the subspecies *H. vulgare* ssp. *spontaneum*, considered to be the wild progenitor of cultivated barley, and four of *H. bulbosum*, including diploid and tetraploid cytotypes. The molecular organization of the SSR clusters was also studied by Southern blotting.

Materials and methods

Plant material

The plant material used covered all species and cytotypes of the genus *Hordeum* with the H genome, including samples of 12 improved cultivars of barley (*Hordeum vulgare* L. ssp. *vulgare*) with diverse agronomic characteristics (spring or winter; two or six rows; malt or feed types), 7 wild barley accessions (*H. vulgare* L. ssp. *spontaneum* (C. Koch) Thell.) and four accessions of bulbous barley grass (*H. bulbosum* L.) (diploid and tetraploid forms). Cultivated barley is here defined as *H. vulgare* ssp. *vulgare*; wild barley is defined as *H. vulgare*. ssp. *spontaneum*. The wild materials were obtained from the Germplasm Bank at the Leibniz-institut für planzengenetik und kulturplanzenforschung (IPK) Gatersleben. Detailed information on these materials is provided in Table 1.

Chromosome preparation

Root tips were obtained from seedlings and exceptionally from older plants (grown in pots in a greenhouse). Metaphases were prepared as previously reported (Cuadrado and Jouve 2007b).

Probes and in situ hybridization

Four SSR probes were used: $(AG)_{10}$, $(ATC)_5$, $(ACT)_5$ and $(AAG)_5$, labelled with biotin or digoxigenin (Roche Applied Science) at either end. ND-FISH was performed as previously described by Cuadrado and Jouve (2010). Three other probes, pTa71, pTa794 and pSc119.2, respectively containing 45S rDNA and 5S rDNA from *Triticum aestivum* and a 120 bp repeat sequence from *Secale cereale*, were also used after ND-FISH to identify *H. bulbosum* chromosomes by FISH as described by de Bustos et al. (1996).

Fluorescence microscopy and imaging

Slides were examined using a Zeiss Axiophot epifluorescence microscope. The images captured from each filter were recorded separately using a cooled CCD camera (Nikon DS) and the resulting digital images processed using Adobe Photoshop.

Southern blot assays

Genomic DNA was isolated from young leaves following standard protocols. 12.5 µg of DNA were digested with *Alu*I and *Rsa*I restriction endonucleases (with recognition sites for four bases) and separated by electrophoresis in

Table 1 List of the *Hordeum* species studied

Cultivar	Types	Rows	Country of origin	Pedigree
Hordeum vulgare L. ssp. *vulgare*				
Alexis	Spring	2	Germany	St.1622d5 × Trumpf
Barberousse	Winter	6	France	Ager × [W.259-711 × (Hatif × Ares)]
Beka	Spring	2	France	BethgeXIII × Kneifel
Dobla	Winter	6	France	Union × Nymphe
Gaelic	Winter	2	France	Marinka × (Flika × Lada)
Golden Promise	Spring	2	UK	Maythorpe (Gamma-ray mutant)
Graphic	Spring	2	UK	Casino × Dandy
Hispanic	Winter	2	France	Mosar × (Flika × Lada)
Morex	Winter	6	USA	Cree × Bonanza
Orria	Winter	6	Mexico/Spain	{[(Api x Kristina) × M66.85] × Sigfrido's} × 79w40762
Plaisant	Winter	6	France	Ager × Nymphe
Triumph	Spring	2	Germany	Diamant × 14029-64/6
Accession number	Country of origin		Scientific name	
Hordeum vulgare L. ssp. *spontaneum*				
HOR2680	Iran		*Hordeum spontaneum* K. Koch var. *spontaneum*	
HOR4873	Turkmenistan		*Hordeum spontaneum* K. Koch	
HOR4894	Turkmenistan		*Hordeum spontaneum* K. Koch var. *spontaneum*	
HOR8538	USA		*Hordeum spontaneum* K. Koch var. *spontaneum*	
HOR8543	USA		*Hordeum spontaneum* K. Koch var. *ischnatherum* (*Coss.*) *Thell.*	
HOR22052	Israel		*Hordeum spontaneum* K. Koch	
HOR22053	Israel		*Hordeum spontaneum* K. Koch var. *spontaneum*	
Accession number	Ploidy	Country of origin	Scientific name	
Hordeum bulbosum L.				
BCC2061	2×	Italy	*Hordeum bulbosum* L.	
GRA1094	4×	Georgia	*Hordeum bulbosum* L. ssp. *bulbosum*	
GRA1154	2×	Italy	*Hordeum bulbosum* L. ssp. *nodosum* (L.) *Baum*	
GRA1193	4×	Tajikistan	*Hordeum bulbosum* L. ssp. *bulbosum*	

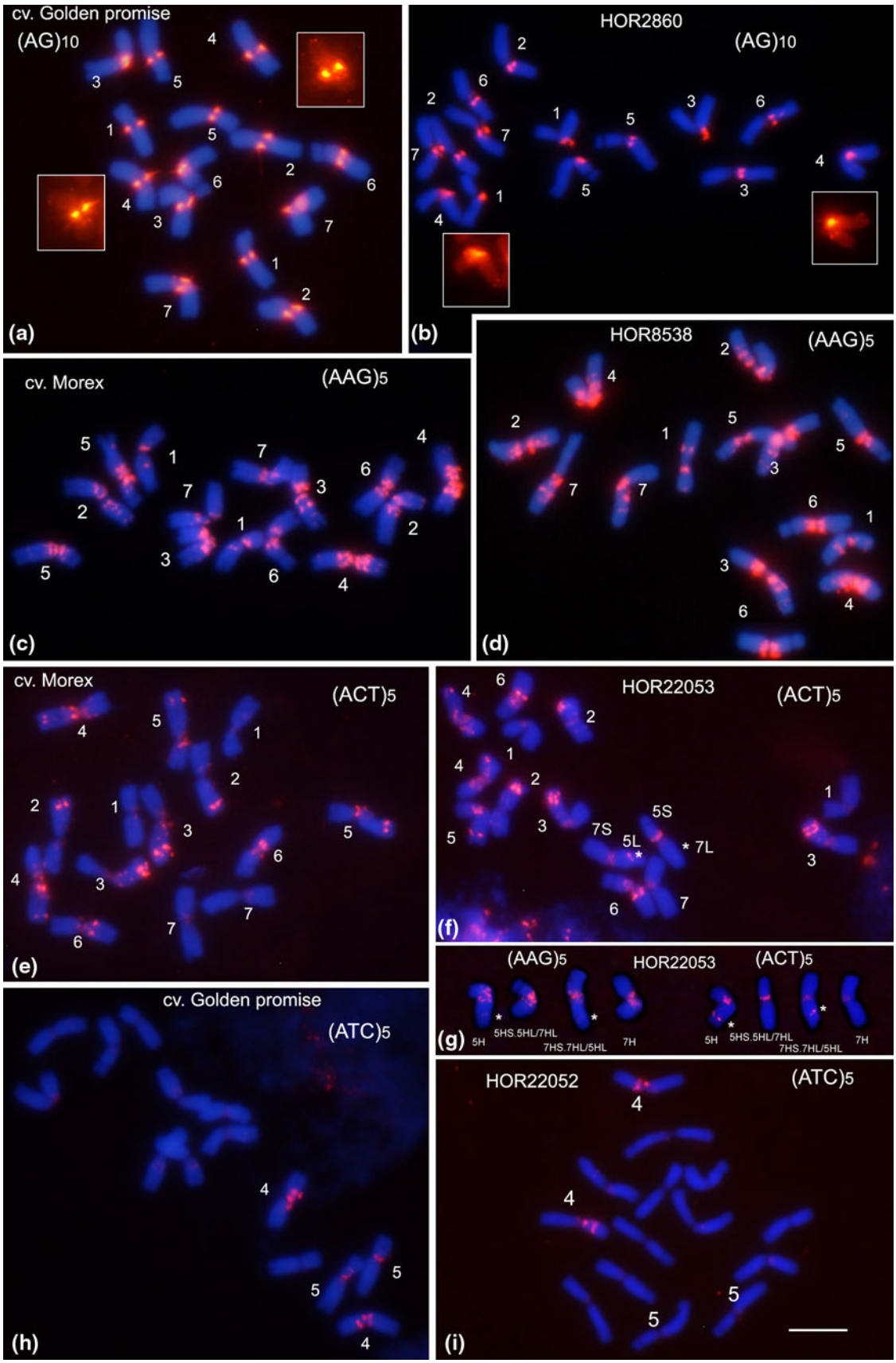
0.8 % agarose gels. Fragments were blotted onto nylon membranes (Biodyne, Pall Corporation) under alkaline conditions following standard techniques. The same filters were successively hybridized with the different digoxigenin-labelled oligonucleotides as probes. Hybridization was performed overnight with 10 pm of chosen probe in 5× SSC, 0.02 % SDS, 0.01 % LSS and 0.3 % blocking reagent (Roche) at 5 °C below the respective duplex stability temperature (T_m): 31.2 °C for $(ACT)_5$, $(AAG)_5$ and $(ATC)_5$, and 49.3 °C for $(AG)_{10}$. The T_m was calculated for each probe following the GC content rule. Before detection, using anti-digoxigenin-AP, Fab fragments (Roche) and CDP-Star (Roche) according to the manufacturer's recommendations, the filters were washed with 0.15× SSC and 0.1 %SDS at the corresponding hybridization temperature for 30 min.

Results

Chromosomal localization of SSRs in *H. vulgare*

Figure 1 shows the physical map for four SSR probes—$(AG)_{10}$, $(AAG)_5$, $(ACT)_5$ and $(ATC)_5$—in samples of both subspecies of *H. vulgare*. Figures 1S, 2S, 3S and 4S (online supplementary material) show metaphase spreads for all the lines investigated, revealing the chromosomal distributions of the AG, AAG, ACT and ATC arrays, respectively.

The $(AG)_{10}$ probe returned strong signals of similar intensity at all centromeres for all the barley cultivars analysed (Fig. 1a and online Fig. S1a-l), No interstitial signals were observed with this probe. Nevertheless a weak terminal cluster of AG repeats was observed in all cultivars


cv. Golden promise
(AG)10
(a)
HOR2860
(AG)10
(b)
cv. Morex
(AAG)5
(c)
HOR8538
(AAG)5
(d)
cv. Morex
(ACT)5
(e)
HOR22053
(ACT)5
5S
7S
5L
* 7L
(f)
(AAG)5
HOR22053
(ACT)5
5H
5HS.5HL/7HL
7HS.7HL/5HL
7H
(g)
cv. Golden promise
(ATC)5
(h)
HOR22052
(ATC)5
(i)

◀ **Fig. 1** Photomicrographs showing the motif-dependent chromosomal distribution of $(AG)_n$, $(AAG)_n$, $(ACT)_n$ and $(ATC)_n$ in metaphases of representative samples of *H. vulgare* ssp. *vulgare* (*left*) and *H. vulgare* ssp. *spontaneum* (*right*) after ND-FISH and DAPI staining. A metaphase of individuals of each line examined, a set of 12 cultivars and 9 wild lines, with $(AG)_{10}$, $(AAG)_5$, $(ACT)_5$ and $(ATC)_5$ as probes, is shown in supplementary Figs. S1, S2, S3 and S4, respectively. Chromosome identification was performed according to Cuadrado and Jouve (2007b). **a**, **b** $(AG)_{10}$. In *square insets*, chromosome 4H showing less intense subtelomeric AG signals on the long arm, **c**, **d** $(AAG)_5$, **e**, **f** $(ACT)_5$ and **h**, **i** $(ATC)_5$, **g** Chromosomes involved in the reciprocal translocation 5HS. 5HL–7HL detected in accession HOR22053 via relocated $(AAG)_5$ and $(ACT)_5$ signals chosen from metaphases shown in Fig. S2 s and (**f**), respectively. *Scale bar* = 10 μm

after increasing the CCD camera exposure time. Two colour ND-FISH or reprobing with $(ACT)_5$ (see below) allowed the detection of the signal on chromosome arm 4HL (Fig. 1a insert). The physical map of $(AG)_{10}$ in all the accessions of *H. spontaneum* analysed was identical to that of cultivated barley (Fig. 1b and online Fig. S1 m–s).

Of the four SSRs analysed, $(AAG)_5$ produced the most intense and rich patterns of signals. The strongest signals were observed in the pericentromeric regions of all chromosomes. The similar patterns observed for some morphologically similar chromosomes hindered their identification; however, individual chromosomes (1H–7H) were unequivocally identified in high quality metaphases of each cultivar (Figs. 1c, S2a-l). No differences were observed between homologous chromosomes even between plants of the same cultivar and a similar—though not identical—pattern was seen among different cultivars. To facilitate the comparison between the homologous pairs among tested materials, karyotypes showing one chromosome of each type (1H–7H) were constructed from the metaphases shown in supplemental Fig. S2. Figure 2 (left) shows the karyotypes of cultivated barleys arranged alphabetically. The karyotypes were comparable, although variations were very noticeable, especially the discrepancy in the number of bands on chromosome 3H. For example, some barley cultivars such as cv. 'Plaisant' or cv. 'Morex' carried two bands on chromosome arm 3HL compared to only one observed in cvs. 'Dobla', 'Golden Promise' and 'Triumph'. No strong signals were seen on 3HL observed in cv. 'Orria'.

The physical maps of $(AAG)_5$ for the *H. vulgare* ssp. *spontaneum* lines were very similar to those recorded for cultivated barley (Figs. 1d, S2 m–s). Also polymorphism was detected comparing the karyotypes of different wild accessions, especially on chromosome arms 3HL and 5HL (Fig. 3 left, arranged in numerical order). No heterozygosity for the presence/absence or variation in intensity of the $(AAG)_5$ signals was observed between individuals within wild barley lines, except for HOR22053 which showed segregation for a reciprocal translocation involving chromosomes 5H and 7H, resulting in two new chromosomal combinations 5HS.5HL-7HL and 7HS.7HL-5HL, with these being more metacentric and submetacentric than the normal 5H and 7H, respectively (Figs. 1g, S2 s).This change in morphology indicates that the sizes of the translocated fragments involved in the reciprocal translocation are different.

Probe $(ACT)_5$ produced a rich pattern of clear intercalary signals of different intensity similar in all the cultivated and wild barley lines analysed (Figs. 1e, f, S3). The multiple sites observed in metaphase cells allowed the identification of all chromosomes pairs following the distribution pattern previously established in cv. 'Plaisant' (Cuadrado and Jouve 2007b). No variations between homologous chromosomes or between plants of the same line were observed. However, despite the general coincidence of the hybridization pattern, some quantitative and qualitative differences were seen. Karyotypes of the 12 barely cultivars and 7 wild barley lines were constructed from the metaphases shown in supplemental Fig. S3 in order to mark polymorphisms for the presence/absence or intensity of hybridization signals (Figs. 2, 3, right). A detailed analysis revealed several polymorphic signals. For instance, only some genotypes showed the interstitial bands on 5HL and the terminal bands in the satellite of 6HS. The absence of the signal found distally on 4HL in some wild accessions is noteworthy. The $(ACT)_5$ probe also confirmed the 5H/7H translocation observed in some plants of accession HOR22053 (Fig. 1f–g).

Despite the polymorphism observed among genotypes, the use of the $(ACT)_5$ probe in combination with the morphology of the DAPI-stained chromosomes was enough to quickly and easily distinguish the seven pairs of chromosomes in all barleys (cultivated and wild). Given the usefulness of $(ACT)_5$ as a physical marker (see "Discussion"), a standard ideogram of the barley chromosomes was prepared (Fig. 4).

Finally, all the cultivated barley varieties and wild accessions analysed showed similar patterns after ND-FISH with $(ATC)_5$ (Fig. 1h–i). As previously reported for cv 'Plaisant' when using FISH with $(CAT)_5$ (Cuadrado and Jouve 2007b), the ATC repeats showed the greatest variation in intensity among the chromosomes, clustering mostly in the pericentromeric region of chromosome 4H (Fig. S4).

Chromosomal localization of SSRs in *H. bulbosum*

H. bulbosum is thought to be the species most closely related to *H. vulgare*; this was why the analysis of the chromosomal distribution of the SSR made for the two subspecies of *vulgare* was extended to four lines of *bulbosum* [two with $2n = 14$ (BCC2061 and GRA1154)

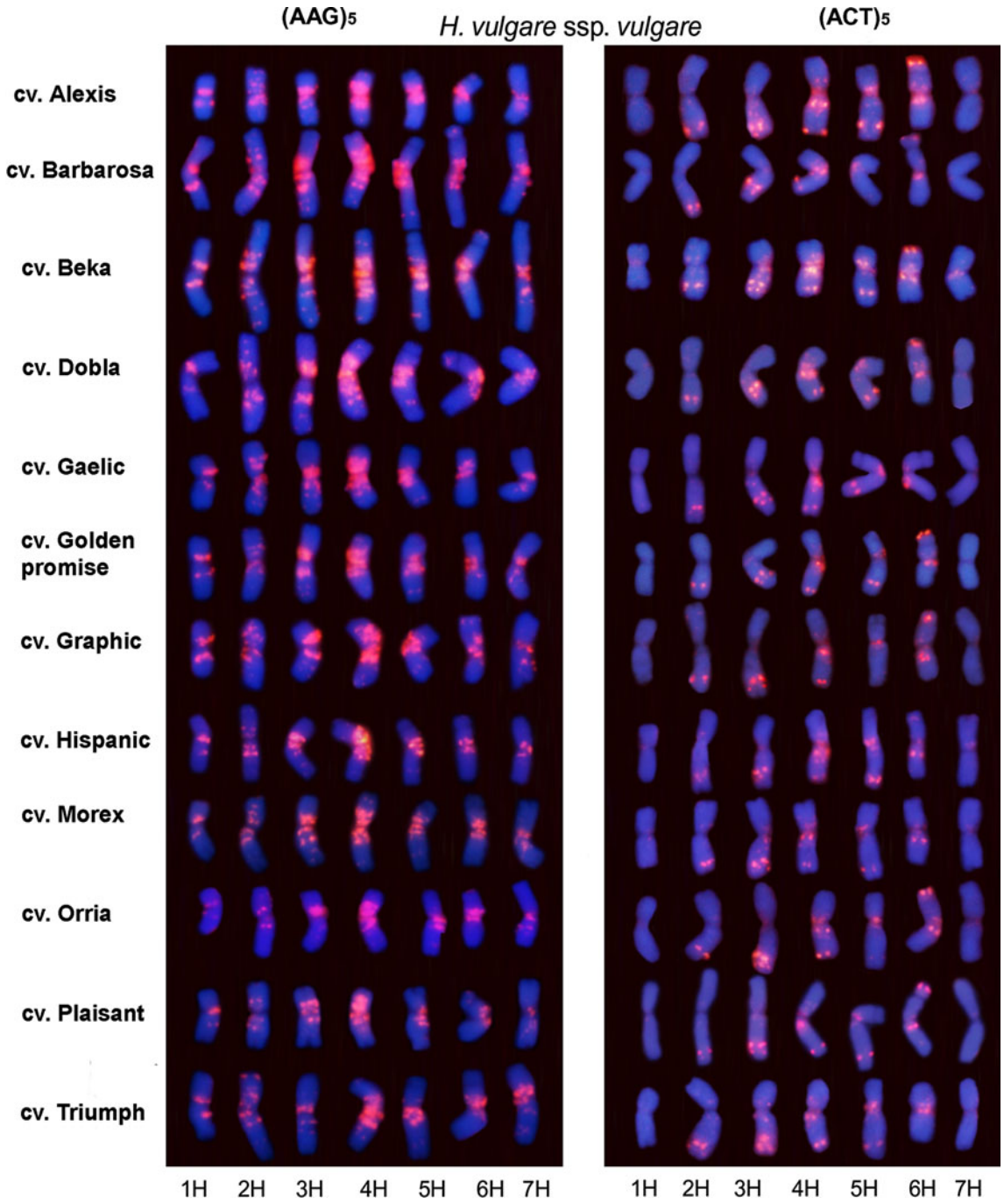


Fig. 2 $(AAG)_5$ (*left*) and $(ACT)_5$ (*right*) karyotypes of individuals from a set of barley cultivars showing one chromosome of each homologous group (1H–7H) chosen from metaphases shown in supplementary Figs. S2 and S3, respectively

and two with $2n = 28$ (GRA1094 and GRA1193)]. The $2n = 28$ lines revealed a pattern that corresponded to their double chromosomal constitution (compared to their diploid forms), in accordance with the autoploid nature assumed for the tetraploid cytotypes (Fig. 5).

To compare results among the homologous chromosomes of *H. vulgare* and *H. bulbosum*, efforts were made to identify *H. bulbosum* chromosomes ($1H^b$–$7H^b$) in the diploid accessions. Thus, after ND-FISH with the SSRs probes, FISH was performed with three probes (pTa794, pTa71 and pSc119.2), the pattern of localization of which has been well established (Pickering et al. 2006) in two genotypes of *H. bulbosum*. The only chromosomes easily identified were $5H^b$ (carrying 5S rDNA on the short arm) and the satellitized $6H^b$ (showing 45S rDNA at the secondary constriction on the short arm). In contrast, the

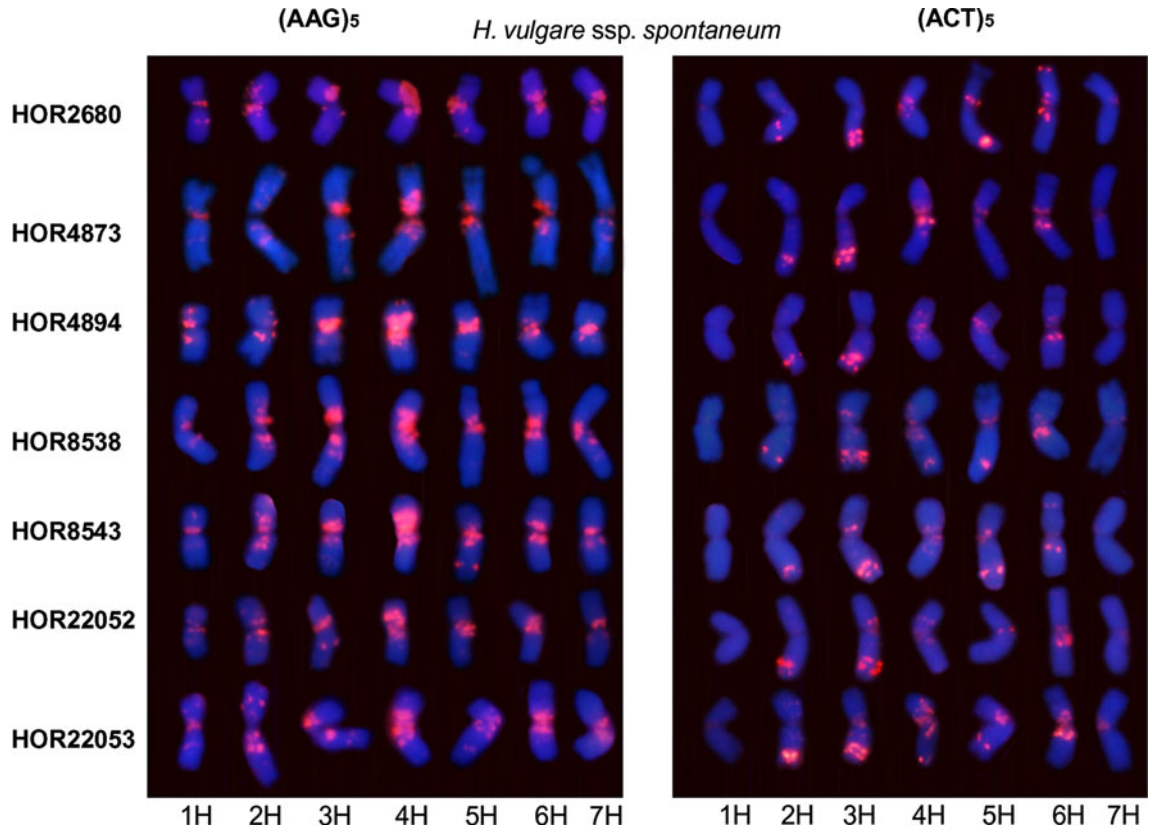


Fig. 3 $(AAG)_5$ (*left*) and $(ACT)_5$ (*right*) karyotypes of wild barley *lines* showing one chromosome of each homologous group chosen from metaphases shown in supplementary Figs. S2 and S3, respectively

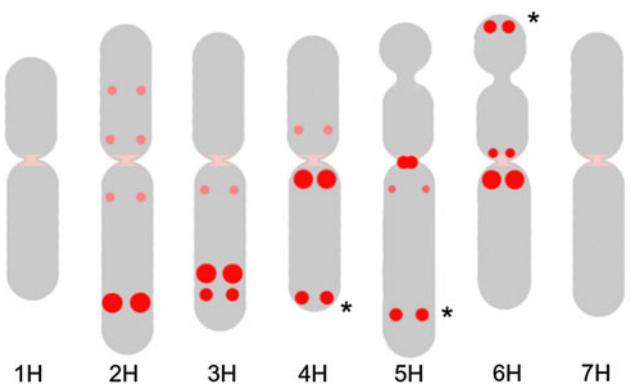
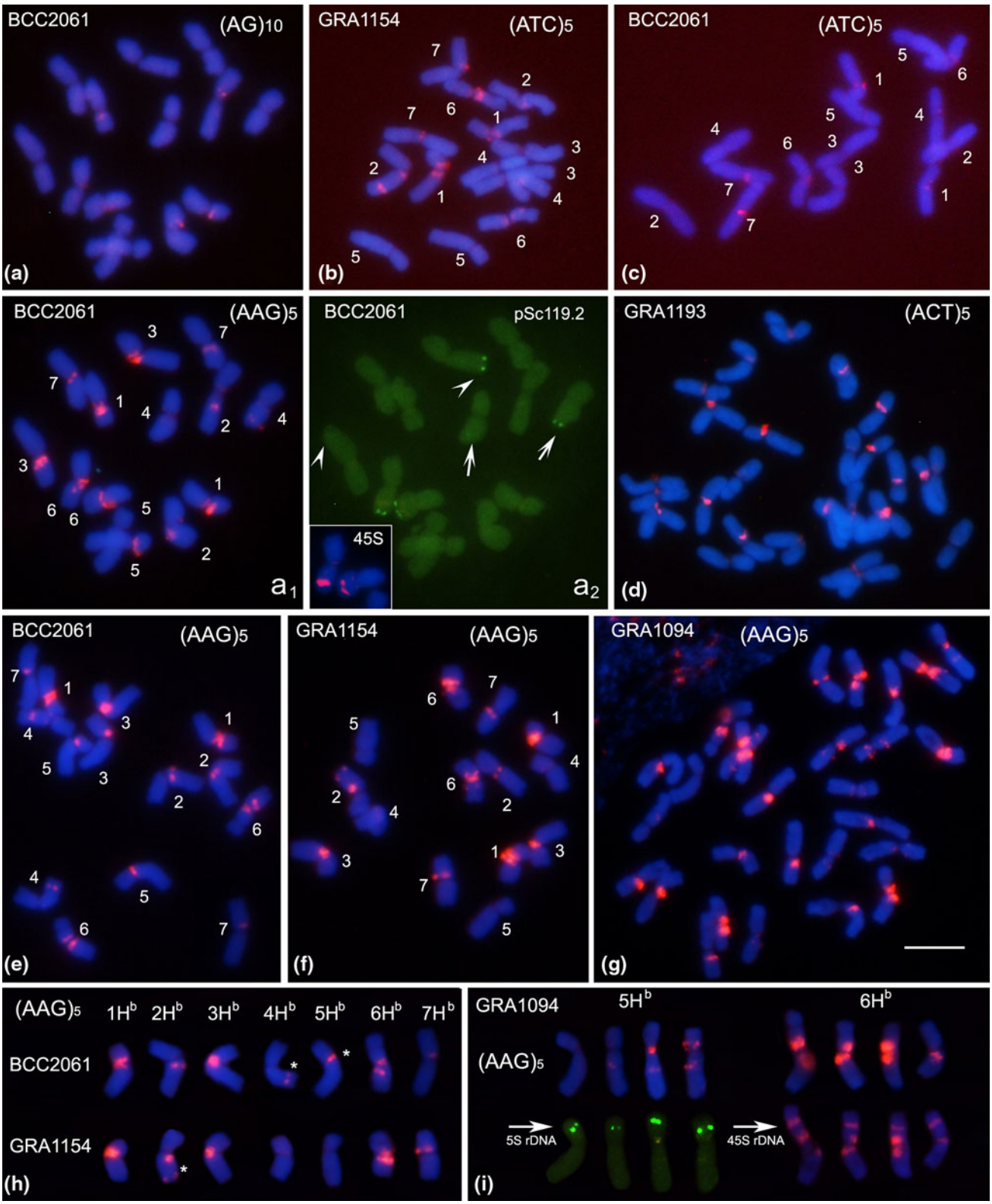


Fig. 4 Diagram of $(ACT)_5$ distribution pattern on barley chromosomes. Intense sites observed in all *lines* are drawn in *dark grey/red*. *Asterisks* indicate polymorphism at sites not observed in some *lines*. Weak, inconsistent sites observed in some genotypes are shown in *light grey/pink* (colour figure online)

presence of pSc119.2 signals, previously localized near the telomeres of chromosome arms $3H^bL$, $4H^bL$, $6H^bS$ and $7H^bL$, polymorphic among and even within the individuals of the genotypes analysed, was not so effective for identifying these individual chromosomes (Fig. $5a_2$). Nevertheless, in combination with the distribution pattern of $(AAG)_5$, and using the C-band ideograms reported by Linde-Laursen et al. (1992) as a reference, each individual chromosome was distinguished (Fig. $5a_1$, a_2).

Well defined and intense signals that gave a distinct distribution pattern on *H. bulbosum* chromosomes were observed only for $(AAG)_5$. Chromosome clusters for the other SSRs were observed only after increasing the exposure time of the CCD camera, and then only poorly. Indeed, clusters of AG repeats located near the centromere of chromosomes $1H^b$ and $2H^b$ (Fig. 5a) were only observed in high quality spread metaphases after long exposure times in accession BCC2061. $(ATC)_5$ signals were localized mostly near the centromeres in some chromosomes in all four lines analysed. In addition, a polymorphic intercalary signal was observed in one chromosome in accession GRA1154, identified as $2H^b$ (Fig. 5b). Differences in the presence/absence and intensity of the signals were observed among lines and even between homologous chromosomes within genotypes (compare Fig. 5b, c). All the *H. bulbosum* chromosomes analysed showed hybridization of $(ACT)_5$ exclusively near the centromeres. Polymorphism in the intensity of the signals was clearly observed between lines and between individual chromosomes of the same lines (Fig. 5d).


BCC2061
(AG)10
(a)
GRA1154
(ATC)5
(b)
BCC2061
(ATC)5
(c)
BCC2061
(AAG)5
a1
BCC2061
pSc119.2
45S
a2
GRA1193
(ACT)5
(d)
BCC2061
(AAG)5
(e)
GRA1154
(AAG)5
(f)
GRA1094
(AAG)5
(g)
(AAG)5
1Hb 2Hb 3Hb 4Hb 5Hb 6Hb 7Hb
BCC2061
GRA1154
(h)
GRA1094
5Hb
6Hb
(AAG)5
5S rDNA
45S rDNA
(i)

◀ **Fig. 5** Photomicrographs showing the motif-dependent chromosomal distribution of $(AG)_n$, $(AAG)_n$, $(ACT)_n$ and $(ATC)_n$ in metaphases of diploid (BCC2061 and GRA1154) and tetraploid (GRA1094 and GRA1193) lines of *H. bulbosum* after ND-FISH and DAPI staining. The distribution pattern of pSc119.2, pTa794 and pTa71 after FISH was used for chromosome identification, according to Pickering et al. (2006). **a–a$_2$** The same metaphase after successive ND-FISH with $(AG)_{10}$ (**a**) and $(AAG)_5$ (**a$_1$**), before FISH with probes pSc119.2 and pTa71 (*inset*), in *green* and *red*, respectively (**a$_2$**). *Arrows* and *arrowheads*, pointing chromosome arms 3HL and 4HL, respectively, indicate polymorphism pSc119.2 signals **b**, **c** $(ATC)_5$, **e–g** $(AAG)_5$, **h** $(AAG)_5$ karyotype from an individual of each diploid genotype studied showing one chromosome of each homologues group ($1H^b$–$7H^b$) chosen from metaphases shown in **e** (*top*) and **f** (*bottom*). *Asterisks* indicate polymorphism sites observed both within and among individuals of each genotype. For example, compare homologous chromosomes 4 between the individuals shown in **a$_1$** and **e,** or homologous chromosomes 2 and 5 in **f** and **a$_1$**; **i** Chromosomes $5H^b$ (*left*) and $6H^b$ (*right*) chosen from metaphase shown in **g** after FISH with pTa794 (5S rDNA) and pTa71 (45S rDNA) probes unambiguously identify this chromosomes and illustrate the high degree of SSR variations among homologous chromosomes in the tetraploid *H. bulbosum* cytotypes. *Scale bar* = 10 μm (colour figure online)

$(AAG)_5$ produced the most intense and the richest patterns of signals on *H. bulbosum* chromosomes, mostly confined to pericentromeric positions. A few weak signals were also present in other chromosome locations (intercalary and subtelomeric bands) (Fig. 5a$_1$, e–g). Regardless of the polymorphism caused by variation in band size and/or the presence/absence of signals at certain positions with $(AAG)_5$, karyotypes could be produced for the two diploid cytotypes (BBC2061 and GRA1154) (Fig. 5a$_1$, e, f, h). No attempt was made to identify chromosomes other than $5H^b$ and $6H^b$ in the tetraploid *H. bulbosum* cytotypes (Fig. 5g–i).

Molecular organization of SSRs

To characterize the molecular organization of the SSR clusters observed by in situ hybridization, Southern blotting was performed using genomic DNA from individuals representing each of the 23 plant materials. This DNA was digested separately with the restriction enzymes *Alu*I and *Rsa*I, followed by hybridization with digoxigenin-labelled SSR probes. Both enzymes have specific recognition sites for four base pairs and make no cleavage within the four SSR sequences analysed. Thus, the entire $(AG)_n$, $(AAG)_n$, $(ACT)_n$ and $(ATC)_n$ tracts could be isolated with minimal flanking sequences. As expected, most of the genomic DNA was digested into small fragments with both enzymes, as observed in the gel before transfer of the DNA to filters (Fig. 6a, f).

The hybridization patterns detected, independent of the restriction enzyme or SSR probe used, revealed similarities in terms of the profusion and size of fragments for all materials of both subspecies of *H. vulgare*. These patterns varied significantly from those observed for the four *H. bulbosum* accessions, which revealed the same hybridization patter. Figure 6b–e and g–j show the Southern blotting results obtained after digestion of the genomic DNA of samples of all three taxa with *Rsa*I and *Alu*I, respectively, and successive hybridization with $(AG)_{10}$, $(ACT)_5$, $(ATC)_5$ and $(AAG)_5$. The strongest hybridization patterns obtained with the DNA samples of the *H. vulgare* subspecies were more intense than those obtained with *H. bulbosum.* Differences in signal intensity were less patent with $(ATC)_5$. Notable differences were seen when using different probes to compare the restriction fragment patterns of *Alu*I and *Rsa*I digests of cultivated and wild barley DNAs. $(AG)_{10}$ and $(AAG)_5$ returned a continuous smear with some stronger bands including high molecular weight fragments cleaved with both enzymes. This indicates the existence of long arrays of these SSRs. $(ACT)_5$ and $(ATC)_5$ revealed multiple and well defined hybridization bands over a range of molecular weights that differed significantly for the *Alu*I and *Rsa*I digests. $(ATC)_5$ revealed a number of bands that were particularly strong and constant over all barleys analysed.

Discussion

SSRs as chromosomal markers revealed by ND-FISH

In *H. vulgare* ssp. *vulgare*, $(AG)_{10}$, $(AAG)_5$, $(ACT)_5$ and $(ATC)_5$ returned different hybridization patterns that provide cytogenetic landmarks for chromosome identification. In recent years, SSR motifs have notably increased the number of physical markers of barley chromosomes (see review Houben and Pickering 2009), improving on the use of 45S rDNA and 5S rDNA ribosomal multigene families and other barley- or Triticeae-specific repetitive DNA probes that have been commonly used over the last 20 years. FISH allows the visualization of clusters of SSR sequences (Pedersen et al. 1996; Cuadrado and Jouve 2007b; Kato 2011). However, ND-FISH was used in the present work. This is a new technique that is simpler, faster and more efficient than any previously reported FISH method for analysing the chromosome distribution of SSRs (Cuadrado and Jouve 2010, 2011). For all barley cultivars/lines, a distal signal on the long arm of chromosome 4H was detected with $(AG)_{10}$ (see insets in Fig. 1a, b) that was never detected by FISH in cv. 'Plaisant' (see Cuadrado and Jouve 2007a). This result shows that ND-FISH is more sensitive than FISH for the analysis of SSR-enriched chromosome regions.

The SSR probe $(ACT)_5$ revealed the most intercalary diagnostic sites, allowing the easy identification of all chromosomes in the 19 materials of *H. vulgare* examined.

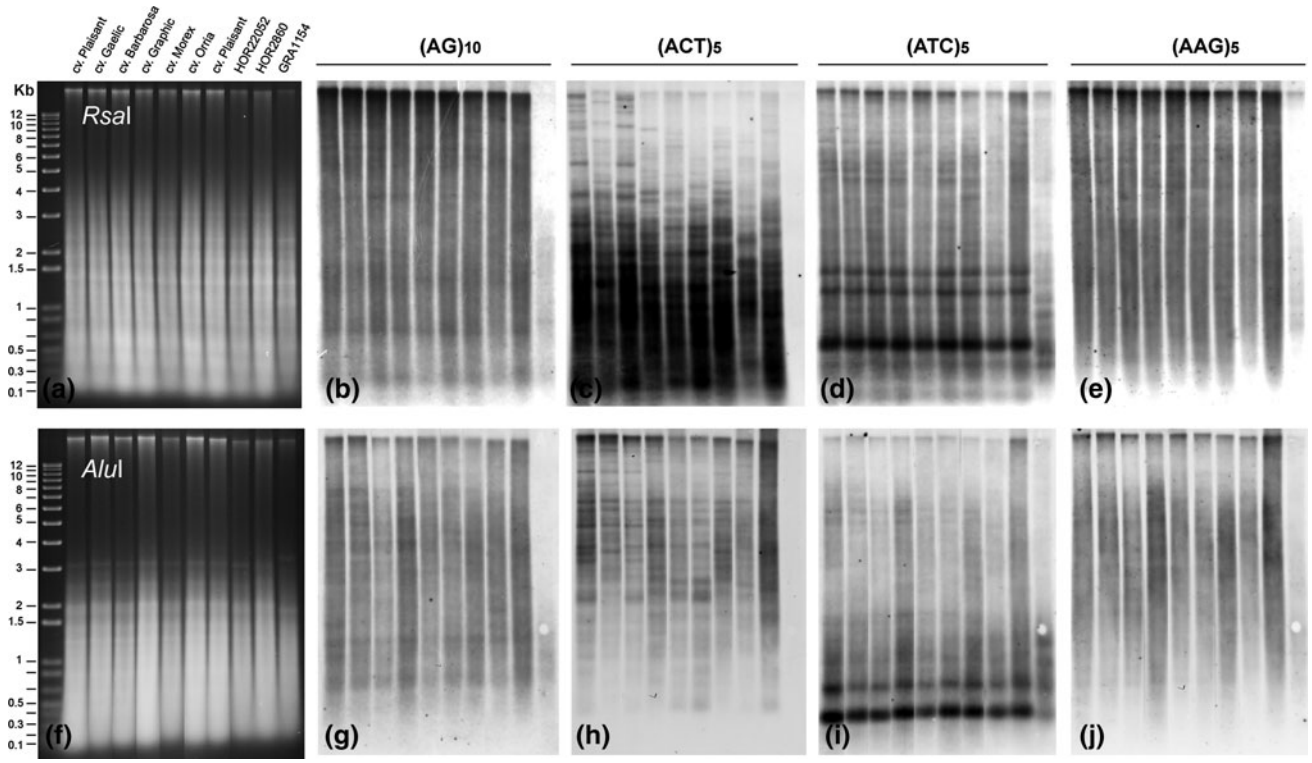


Fig. 6 Southern blot patterns of genomic DNA from representative samples of *H. vulgare* ssp. *vulgare*, *H. vulgare* ssp. *spontaneum* and *H. bulbosum* digested by *Rsa*I (*top*) and *Alu*I (*bottom*). **a**, **f** Gels, **b–e**, **g–j** the two filters transfer sequentially hybridized with the digoxigenin labelled probes $(AG)_{10}$, $(ACT)_5$, $(ATC)_5$ and $(AAG)_5$ as indicated. Size markers (*M*) are given in kb

The ideogram of the distribution of ACT repeats shown in Fig. 4, which includes the polymorphic sites, provides a ideogram in which the positions of these markers can be very useful as anchoring references in the physical maps of barley. Thus, any other DNA sequence to be physically located may be mapped on the same metaphase. Another practical application is the detection of alien material or chromosome rearrangements in breeding lines. Indeed a translocation was identified in accession HOR22053, involving the long arms of chromosomes 5H and 7H, via the relocated ACT landmarks on these chromosomes (Fig. 1f, g).

Even though some SSRs did not permit the identification of many barley chromosomes, they could be the probes of choice in other situations. The species-specific localization of the AG repeats in the centromeres may provide a means of easily distinguishing barley chromosomes in hybrids, for example, in *H. vulgare* × *H. bulbosum*, and of following uniparental chromosome elimination (Sanel et al. 2011). In contrast, no reliable AG and ATC repeat clusters were observed in *H. bulbosum* chromosomes, ACT repeats were little obvious, and then exclusively seen at centromeric sites. The reliable signals observed with $(AAG)_5$ varied between the chromosomes of each homologous pair, as reported for $(CTT)_{10}$ signals in other genotypes of *H. bulbosum* (Pickering et al. 2006). However, since clusters of SSRs are very abundant in *Triticeae* (Cuadrado and Schwarzacher 1998; Cuadrado et al. 2008), other SSR probes might provide more information about *H. bulbosum*.

Structure of SSR clusters

To produce consistent SSR hybridization signals in metaphase chromosomes, (such as those observed in the present work) several kilobases of target sequence are required, but this does not mean that the targets must be long interrupted stretches of SSRs. The present Southern blot hybridization results revealed clusters of AG and AAG repeats to share significant similarity in their genomic organization, and that this was notably different from the genomic organization of tracts of ACT and ATC repeats. Indeed, the smear of fragments with $(AG)_{10}$ and $(AAG)_5$ observed probably reflects the intimate association of SSRs with retrotransposons and other dispersed repetitive elements (Ramsay et al. 1999). Clear differences were seen in the size of the ACT fragments among the 19 varieties and wild accessions of barley analysed, correlating with the extensive polymorphism observed at the chromosomal level by ND-FISH. This reveals the informative capacity of this SSR, which is suitable for barley DNA fingerprinting. Moreover,

no obvious differences were detected with respect to the ATC probe among the samples of both subspecies of *H. vulgare*, according to the unvarying in situ hybridization pattern observed. The presence of intense DNA fragments with molecular weights ranging from 0.3 to under 1.5 kb suggests that ATC arrays are organized in shorter tandem repeat stretches involved in complex repeated structures, and that these are conserved in both cultivated and wild barleys.

SSRs and ND-FISH for assessing the cytogenetic diversity of barley

The ability to analyse genetic diversity has long been a major goal of evolutionary biologists and plant breeders. Over the last two decades, genomic sequence diversity in many species, including barley and its wild relatives, has been assessed by analysing different molecular markers, including the extraordinarily polymorphic SSRs (Saghai-Maroof et al. 1994; Casas et al. 1998). However, no cytogenetic diversity studies have been carried out based on the presence and extension of SSRs visualized by FISH—until now, the cytogenetic diversity of barley and plants in general has been carried out using Giemsa C-banding techniques (Friebe and Gill 1994; Taketa et al. 2009). Unfortunately, with plants, FISH can be time-consuming, and for many species there is a lack of probes that provide diagnostic in situ patterns for chromosome identification. Information on cytogenetic diversity is therefore poor, even for species for which chromosome characterization is of special interest (Schneider et al. 2003; Albert et al. 2010).

The present results show the similar abundance and chromosomal distributions of $(AG)_{10}$ and $(ATC)_5$, indicating that these SSRs are inappropriate as chromosome markers for assessing cytogenetic diversity in barley. However, each genotype can be distinguished based on the in situ pattern by using two more SSRs: $(AAG)_5$ and $(ACT)_5$. Wide chromosomal variation is then revealed both within and among the two subspecies of *H. vulgare*. Our experience suggests that SSRs and ND-FISH together afford an excellent tool for analysing chromosomes reproducibly in different laboratories. Differences in signal intensity obtained with oligonucleotide probes of the same length (and hence the same amount of labelling) at different laboratories could only reflect differences in the size of the target. The ND-FISH protocol might therefore provide a better "standard" technique of in situ hybridization for examining the cytogenetic diversity of germplasm collections (for example the Barley Core Collection).

Examination of the $(AAG)_5$ and $(ACT)_5$ karyotypes revealed the cytogenetic diversity among the barley cultivars with similar agronomic characteristics—and even between wild barley lines of similar geographic origin. Differences were also observed when comparing karyotypes of closely related cultivars of barley, for example, cvs. 'Dobla' and 'Plaisant', which share cv. 'Nymphe' as a parental. Further, no greater similarity was seen within each *H. vulgare* subspecies than between the two subspecies. Indeed, these subspecies were indistinguishable in terms of their patterns of distribution of the SSRs analysed. This result agrees with the interfertility seen between wild and cultivated forms, strongly indicating that they in fact belong to the same species.

Cytogenetics and species relationships

H. vulgare and *H. bulbosum* share the H genome defined by classical cytological studies based on chromosome pairing in hybrids, chromosome morphology and Giemsa C-banding patterns (Linde-Laursen et al. 1997). This is supported by the results of sequence-based molecular inspections (von Bothmer and Jacobsen 1985; Blattner 2009; Terasawa et al. 2012). This suggests that *H. vulgare* and *H. bulbosum* have a monophyletic origin, constituting a cluster that became isolated early from other *Hordeum* species and which are grouped into three divergent clusters corresponding to the other three basic *Hordeum* genomes. However, the results of the present work seem to demonstrate the existence of substantial differences in the abundance and distribution of AG, AAG, ACT and ATC repeats between *H. vulgare* and *H. bulbosum*. This contradicts the supposedly close genomic relationship between them. Rather, the present results agree with those of previous cytogenetic investigations using other repetitive DNA sequences as chromosome-specific markers, which suggest a less close relationship (Xu et al. 1999; Svitashev et al. 1994; de Bustos et al. 1996). Even assuming that *H. vulgare* and *H. bulbosum* have a monophyletic origin (Blattner 2009), and that their separation from a common ancestor occurred at a relatively early stage, the present results reopen the discussion about whether these species have a common genomic nature or represent two groups—*H. bulbosum* and *H. vulgare*, with different genome designations—as previously proposed (Shcherban and Vershinin 1992; Svitashev et al. 1994).

Many phylogenetic studies have been conducted to clarify the relationships in *Hordeum* because of the importance of barley as a crop, and given the importance of its wild relatives as a potential source of material for genetic improvement programs. In our opinion, cytogenetic data provide indispensable complementary information; ND-FISH using SSRs can define different karyotypes or karyotype groups that can be used to test phylogenetic hypotheses.

Evolutionary trends followed by SSRs during speciation

Changes in the amount and distribution of tandem repetitive DNA components are one of the driving forces of genome evolution and speciation. However, the molecular mechanisms by which genomes change are unknown; neither do we know whether these changes are subject to selection (Plohl et al. 2002). The conservative pattern of distribution shown by AG and ATC repeats among the cultivated and wild barleys suggests that the clusters observed on the chromosomes were present before the domestication of barley from ssp. *spontaneum* about 10,000 years ago in the Fertile Crescent (Badr et al. 2000). ACT and AAG showed a chromosome similar—but not identical—distribution pattern showing polymorphism for the presence/absence of hybridization signals at different sites. This suggests these sequences are more predisposed to being amplified or deleted as a consequence of independent events in particular cultivars or related wild forms. It is noteworthy that *H. bulbosum* differs from *H. vulgare* in terms of the abundance of the four SSR sequences analysed. It is therefore reasonable to assume that specific SSR clusters came about via massive amplification of pre-existing SSR sequences in *H. vulgare*, or by their deletion in *H. bulbosum,* after both species separated from their common ancestor. A similar phenomenon has been observed in other graminae. For example, in the genus *Secale*, clusters of AAG and AAC have been amplified in cultivated rye after the divergence of *S. sylvestre* (Cuadrado and Jouve 2002). However, given the lack of information about the SSR contents of other *Hordeum* taxa, the evolutionary trends of each SSR during speciation in *Hordeum* cannot be deduced.

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