Chromosomal distribution patterns of the (AC)10 microsatellite and other repetitive sequences, and their use in chromosome rearrangement analysis of Avena species

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Chromosomal distribution patterns of the (AC)$_{10}$ microsatellite and other repetitive sequences, and their use in chromosome rearrangement analysis of *Avena* species

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Abstract

Fluorescence in situ hybridization (FISH) was used to determine the physical location of the \((\text{AC})_{10}\) microsatellite in metaphase chromosomes of six diploid species (AA or CC genomes), two tetraploid species (AACC genome), and five cultivars of two hexaploid species (AACCDD genome) of the genus *Avena*, a genus in which genomic relationships remain obscure. A preferential distribution of the \((\text{AC})_{10}\) microsatellite in the pericentromeric and interstitial regions was seen for both the A- and D-genome chromosomes, while in C-genome chromosomes the majority of signals were located in the pericentromeric heterochromatic regions. New large chromosome rearrangements were detected in two polyploid species: an intergenomic translocation involving chromosomes 17AL and 21DS in *A. sativa* cv. Araceli, and another involving chromosomes 4CL and 21DS in all the analyzed cultivars of *A. byzantina*. The latter 4CL-21DS intergenomic translocation differentiates clearly between *A. sativa* and *A. byzantina*. Searches for common hybridization patterns on the chromosomes of different species revealed chromosome 10A of *A. magna* and 21D of hexaploid oats to be very similar in terms of the distribution of 45S and Am1 sequences. This suggests a common origin for these chromosomes and supports a CCDD rather than an AACC genomic designation for this species.

**Keywords:** *Avena*, chromosome structure, fluorescence in situ hybridization (FISH), repetitive DNA.
Introduction

The genus *Avena* contains approximately 30 species, two of which are important crop species: *Avena sativa* L. and *A. byzantina* C. Koch. These species are also described as homotypic subspecies (Rodionova et al. 1994; Diederichsen 2008). *A. strigosa* Schreb is also cultivated in some areas, and is an important resource of resistance for economically important diseases of oats such as cereal cyst nematode (caused by *Heterodera avenae* Woll) and crown rust (caused by *Puccinia coronata*) (Loskutov and Rines 2011; Rines et al. 2007). Many wild *Avena* species are also of economic interest given their possession of genes potentially useful in crop improvement (Loskutov and Rines 2011). Research into the differentiation of chromosome structure may provide important information for the management of useful *Avena* genes via interspecific hybridization.

*Avena* species can be divided into diploid, tetraploid and hexaploid groups with a base chromosome number of seven. Diploid species have either an A or C genome, tetraploids have either A, B or A, C genomes, and hexaploids have three genomes i.e., A, C and D. A-genome diploid species have five minor genomes - As, Al, Ad, Ap and Ac - whereas C genome diploids have two minor genomes - Cv and Cp (Thomas 1992; Loskutov and Rines 2011). Information on genome structure differences among related species has been provided by C-banding (Fominaya et al. 1988a, 1988b; Linares et al. 1992; Jellen et al. 1993a, 1993b; Shelukhina et al. 2007; Shelukhina et al. 2008a, 2008b; Badaeva et al. 2011; Chaffin et al. 2016), genomic in situ hybridization (GISH) (Chen and Armstrong, 1994; Jellen et al. 1994; Leggett and Markhand, 1995; Katsiotis et al. 1996), and fluorescence in situ hybridization (FISH) of homologous repetitive

The cytogenetic positions of specific repetitive sequences have been useful in revealing the structural evolution of chromosomes in *Avena* species. For example, information on the relative locations of ribosomal 45S and 5S DNA in diploid and polyploid species has produced evidence regarding the structural evolution of the chromosomes carrying these sequences (Linares et al. 1996; Badaeva et al. 2010, 2011; Tomás et al. 2016), while nucleotide similarity among diploid and polyploid rDNA copies has revealed some of their phylogenetic and genomic relationships (Peng et al. 2010). A small number of satellites, such as As120a in *A. strigosa*, and Am1 in *A. murphyi*, have also been useful in revealing chromosome structure. As120a is dispersed on the chromosomes of the A genome (Linares et al. 1998) while obvious signals of Am1 repeats have been detected on those of the C genome (Fominaya et al. 1995). Other tandem repeats appear in more restricted locations in pericentromeric positions. For example, CCS1 *Avena*-700 appears all around the centromeric regions (Tomás et al. 2016) whereas A336 (isolated from chromosome 18D) appears in the centromeric regions of A- and D-genome chromosomes (Luo et al. 2015). Low copy genes represented by families of resistance gene analogues (Sanz et al. 2012), or which code for endosperm proteins (Fominaya et al. 2016), have also been located on specific chromosomes of diploid and hexaploid species, even allowing for the identification of certain homeologous regions in hexaploids. However, the above findings are insufficient to clarify the complex homology relationships within *Avena*. New sequences with chromosome distributions that give further clues in this respect are much needed.
Microsatellites, or simple sequence repeats (SSRs), are tandemly repeated sets of a few base pairs (1-6) that can vary extensively in terms of the number of times they are repeated. They form a large part of non-coding regions, but are also found in transcribed regions (see a review by Kalia et al. 2011). Their repeated nature has facilitated in situ hybridization studies, and many papers report differences in their density, distribution and composition (Pinto et al. 2003; Oliveira et al. 2006; Fuentes et al. 2009; Cuadrado et al. 2008; Cuadrado and Jouve, 2010; Molnár et al. 2011; Carmona et al. 2013; Kejnovský et al. 2013; Han et al. 2015). In oats, SSRs have been used in: (i) the construction of genetic maps (Pal et al. 2002; Jannink and Gardner 2005; Becher 2007; Wight et al. 2010; Oliver et al. 2010, 2011a, 2011b; Song et al. 2015), (ii) to examine the association of microsatellites with other repetitive elements (Li et al. 2000), (iii) in population genetics studies and genotyping analyses (He and Bjørnstad 2012), (iv) to identify relationships among oat species (Li et al. 2000; 2009), and (v) to determine the transferability of *A. sativa* SSR to *A. strigosa* germplasm (Da-Silva et al. 2011). However, no report discusses the use of FISH to determine the distribution of any microsatellite on the *Avena* chromosomes.

Since the (AC)_{10} motif has been described as common in oats (Li et al. 2000; Pal et al. 2002; Becher 2007; He and Bjørnstad 2012), diploid and polyploid species of *Avena* were subjected to (AC)_{10}-FISH to reveal the overall distribution of this repetitive element in their chromosomes. The resulting patterns were compared in order to detect homology among the examined species. Repetitive sequences such as120a and Am1, in combination with 45S and 5S rDNA sequences, were also used to identify chromosomes. Karyotypes for the different *Avena* species, including all the above
sequences, were analyzed, and differences in the chromosome structure of polyploid species examined.

**Material and Methods**

**Plant materials**

Table 1 shows the *Avena* species used in the present study. These include wild diploid and tetraploid forms, as well as several cultivars of two hexaploid species (*A. sativa* and *A. byzantina*). These were kindly provided by different germplasm resource centres.

**Mitotic chromosome preparation**

Mitotic chromosomes were prepared as described in earlier work (Fominaya et al. 1995) with some modifications. Briefly, seeds of all the examined species/cultivars were germinated on moistened filter paper for 48 h at 25° C. To synchronize cell division and accumulate metaphases, the germinated seed were then exposed to a temperature of 4° C for 72 h followed by 24 h at 25° C. Oat root tips were excised and placed in ice-cold water for 24 h and fixed for at least a further 24 h in ethanol-acetic acid (3:1). They were then kept in that mixture at -20° C until use. Chromosomes were prepared using enzymatically (cellulose, peptinase) digested root tips prior to squashing the meristematic cells in a drop of 60% acetic acid on a clean microscope slide. After removal of the coverslips by freezing, the slides were air dried for further processing.

**DNA probes**
The microsatellite sequence (AC)\textsuperscript{10}, with biotin-11-dUTP incorporated at both ends, was produced (automated synthesis) by TIB MOLBIOL. Four repetitive DNA probes were used for chromosome identification: (1) pAs120a, a satellite DNA sequence specific to the oat A genome, containing an insert of 114 bp isolated from \textit{A. strigosa} (Linares et al. 1998); (2) pAm1, a satellite DNA sequence specific to the oat C genome, containing an insert of 464 bp isolated from \textit{A. murphyi} (Solano et al. 1992); (3) p45S, a ribosomal probe derived from \textit{A. strigosa} (Sanz et al. 2010), and (4) pTa794, a satellite DNA sequence containing a 410 bp 5S rDNA gene and the intergenic spacer isolated from \textit{T. aestivum} (Gerlach and Dyer 1980). These clones were amplified and labelled by PCR with digoxigenin11-dUTP or biotin16-dUTP (Roche). These repetitive probes were used in FISH both simultaneously and sequentially after hybridization with the (AC)\textsuperscript{10} probe.

**Fluorescence in situ hybridization**

Chromosome preparations were treated with RNase, fixed with 4% (w/v) paraformaldehyde, and dehydrated in an ethanol series before air drying, according to Fominaya et al. (1995). Between the RNase and paraformaldehyde treatments, the preparations were washed in 2xSSC (saline-sodium citrate). Hybridization mixture (30 \(\mu\)l) containing 50% (v/v) formamide, 2 x SSC, 10% (w/v) sodium dodecyl sulphate (SDS), 10% (w/v) dextran sulphate, 50 \(\mu\)g/ml of \textit{Escherichia coli} DNA, and 2.6 pmol of the microsatellite (AC)\textsuperscript{10} sequence, was denatured for 15 min at 75\(^\circ\) C and then applied to each chromosome preparation. The preparations were then covered with plastic coverslips and the chromosomes and probe denatured at 75\(^\circ\) C for 7 min using a programmable thermal controller (PT-100, M.J. Research Inc.). Hybridization was
performed at 37\(^{\circ}\) C in a humidity chamber overnight. Post-hybridization washing was
performed in 4 x SSC/0.2% Tween-20, agitating for 10 min at room temperature (RT).
The detection of biotin was performed at 37\(^{\circ}\) C in a humidity chamber, incubating the
chromosome preparations for 1 h in streptavidin-Cy3 (Sigma) in 5% (W/V) BSA. The
preparations were then rinsed for 10 min in 4 x SSC/0.2% Tween-20 at RT. 4′,6′-
diamidine-2′-phenylindole dihydrochloride (DAPI) was used as a counterstain. The
preparations were the mounted in Vectashield (Vector). Reprobing FISH and probe
detection were performed as described in Linares et al. (1998).

Images were obtained using a Zeiss Axiophot microscope equipped with a
fluorescent lamp and appropriate filters, and recorded using a CCD camera (Nikon DS).
The images were pseudo-coloured in blue (chromosomes) and pink or green (signals),
merged, and optimized for brightness and contrast using Adobe Photoshop.

**Results**

\(\text{(AC)}_{10}\)-FISH signal patterns in diploid species

The karyotypes of the present diploid species were previously described in studies
involving conventional staining (Rajhathy and Thomas 1974) and C-banding (Fominaya
et al. 1988a, 1988b; Badaeva et al. 2005; Shelukhina et al. 2008a, 2008b). FISH has
previously been used to reveal the number and physical locations of 45S and 5S
ribosomal loci in the chromosomes of the examined diploid species (Linares et al.
1996; Shelukhina et al. 2008a; Tomás et al. 2016). The nomenclature used in the above
papers is followed here to designate both the A-genome and C-genome chromosomes.
To identify individual chromosomes labelled with \((AC)_{10}\) repeats, the slides were reprobed with 45S and 5S ribosomal sequences.

When the \((AC)_{10}\) oligonucleotide probe was hybridized with metaphases of the diploid species, two signals patterns were clearly identified (Fig. 1). One showed discrete, well-defined bands in the pericentromeric and interstitial regions of the A-genome chromosomes (Fig. 1a, 1c, 1e, 1g, and 1m); the other showed very strong bands in the pericentromeric regions and dispersed signals throughout the arms of the C-genome diploid species chromosomes (Fig. 1i, 1k, and 1m).

In *A. strigosa*, the \((AC)_{10}\) probe produced signal patterns in each chromosome (Fig. 1a and 1m) except for chromosome 2. Distinct signal patterns were observed in the pericentromeric regions of each chromosome, with chromosomes 1, 3, and 4 showing very strong signals. In chromosomes 1 and 4 they covered a large region around the centromeres. Chromosome 1 also showed a strong signal in the distal region of the long arm. Chromosomes 6 and 7 showed only weak signals in the pericentromeric region, while 5 and 6 showed very strong signals in the interstitial region of the long arms. Chromosomes 2 and 3 were identifiable by their 45S and 5S rDNA signals (Fig. 1b and 1m).

For *A. damascena*, all chromosomes possessed obvious \((AC)_{10}\) signals except for 3 and 5 (Fig. 1c and 1m). Chromosomes 1, 4, and 6 showed signals on both sides of the centromeric region, while 2 and 7 showed signals on only one side. 45S and 5S rDNA was used to identify chromosomes 3 and 6 (Fig. 1d and 1m).

For *A. longiglumis*, the \((AC)_{10}\) probe produced signals in the interstitial region of the long arms as well as close to the pericentromeric region in all chromosomes except...
3 and 6 (Fig. 1e and 1m). Chromosomes 1 and 3 showed signals with the 45S and 5S rDNA probes (1f and 1m). For *A. canariensis*, the (AC)$_{10}$ probe produced strong signals in the interstitial regions of all chromosomes except number 6 (Fig. 1g and 1m). One strong signal on chromosome 1 was produced near the 45S rDNA loci (Fig. 1h and 1m). In contrast, no signals were detected near the 45S rDNA loci of chromosome 3. Chromosome 7 also returned a strong signal at the end of the short arm. On the whole, signals in *A. longiglumis* and *A. canariensis* were less intense than those observed in the other two A-genome species.

*A. eriantha* (Fig. 1i and 1m) and *A. ventricosa* (Fig. 1k and 1m) showed strong similarity in their signal patterns. The (AC)$_{10}$ probe produced clear, bright signals in the pericentromeric regions of all the chromosomes of these C-genome species. Scattered signals were evident in most of the chromosomes, especially on the short arms of chromosomes 6 and 7.

**(AC)$_{10}$-FISH signal patterns in tetraploid species**

Sequential FISH analysis was performed on the AACC-tetraploid species *A. magna* and *A. murphyi*, first using the (AC)$_{10}$ probe on mitotic metaphase chromosomes (Fig. 2a and 2c), and then using the 45S and Am1 probes simultaneously on the same cells (Figs. 2b and 2d). This allowed the chromosome of these two species to be assigned to either the A or C genome. The chromosomes were numbered 1-14 according to the nomenclature of Linares et al. (1996). This was based on the chromosome arm ratio, relative length, and FISH patterns for the four repetitive probes. The resulting karyotype was taken as the reference karyotype (Fig. 2e).
In both *A. magna* and *A. murphyi*, the A-genome chromosomes showed discrete (AC)$_{10}$ signals in the interstitial regions, whereas the C-genome chromosomes showed strong (AC)$_{10}$ signals in the pericentromeric regions. Comparison of the A-genome chromosomes of these tetraploid species revealed differences in the number of signals and in the intensity of some of these. In the karyotype of *A. magna* more discrete signals were seen than in that of *A. murphyi*. Several chromosomes, such as 6A, 11A and 13A, each showed two signals of similar intensity. In contrast, no A-genome chromosome of the *A. murphyi* showed more than one signal, and the intensity of these signals was highly variable across chromosomes. Very conspicuous signals were recorded in the short arm of chromosomes 12A and 13A. It should be noted that the signal patterns of the A-genome chromosomes of these two species are more similar in their intensity to those of *A. longiglumis* and *A. canariensis* than to those of *A. strigosa* and *A. damascena*. Very strong signals were seen on all C-genome chromosomes of both species, covering a large area of the pericentromeric regions. Clear signals were also seen at the ends of the long arms of chromosomes 2C and 4C of *A. magna*, and on those of 2C and 6C of *A. murphyi*. These chromosomes also showed strong Am1 signals (related to heterochromatin) in similar positions.

(AC)$_{10}$-FISH signal patterns in hexaploid species

The usefulness of the (AC)$_{10}$ probe in karyotyping the two AACCDD hexaploid species of cultivated oats - i.e., three cultivars of *A. sativa* (Figs. 3a, 3d, and Fig. 4) and two of *A. byzantina* - was tested (Fig. 4). Both simultaneous and sequential FISH was then performed using on the same cells, As120a or Am1, in combination with a ribosomal
probe (45S or 55) (Fig. 3b, 3e, 3f and 4). This allowed all the chromosomes to be assigned to the A, C or D genome. The nomenclature used for the reference hexaploid karyotype (Fig. 4) was that proposed by Sanz et al. (2010) who used the same repetitive probes as in the present work.

Most of both the A- and D-genome chromosomes showed discrete signals of (AC)\textsuperscript{10} sequences in the interstitial regions. The signals in the C-genome chromosomes, however, covered a large region either side of the centromeres. For all the cultivars analyzed, the (AC)\textsuperscript{10} hybridization signals were less intense on the D-genome chromosomes than the A-genome chromosomes (Fig. 4). Indeed, the hybridization of this chromosome marker differentiated every chromosome of each cultivar (Fig. 4). The chromosomes of all five cultivars were further examined in an attempt to identify diagnostic signals common to them all. In A. sativa, chromosomes 8A, 13A, 15A, 16A and 19A showed either interstitial, pericentromeric or terminal signals common to all three of its cultivars, although differences in intensity were evident (e.g., in the interstitial signal on chromosome 8A, and in the pericentromeric signal on chromosome 15A). Chromosomes 11A, 15A and 19A of A. byzantina showed identical signal patterns in both the cultivars analyzed. Moreover, the signals seen on chromosomes 15A and 19A were the same in both A. byzantina and A. sativa.

The C-genome chromosomes showed strong (AC)\textsuperscript{10} signals in the pericentromeric region of every chromosome (Fig. 4). In addition, distinct signals were observed in the interstitial and terminal regions of the long arms of some chromosomes, with some intra- and interspecific variation observed. Common signals in the interstitial regions of the long arms appeared on chromosome 5C of all five
analyzed cultivars, and more faintly on chromosome 4C of the A. sativa cultivars. The (AC)\textsubscript{10}–FISH distribution pattern on the D-genome chromosomes was similar to that observed on the A-genome chromosomes (Fig. 4), although the signals were less intense. Among the three A. sativa cultivars, similar patterns were found on chromosomes 10D, 14D, and 20D. The two A. byzantina cultivars showed similar patterns for chromosomes 10D, 20D and 21D. A comparison of the A. sativa and A. byzantina signal patterns revealed strong similarities between chromosomes 10D and 20D. By contrast, an important difference between the two species affected chromosome 21D. A. byzantina showed a conspicuous signal on the short arm of this chromosome which was absent in A. sativa. Other minor differences affecting other chromosomes were also seen between the two species; for example, hybridization signals were terminally located on chromosome 9D of A. byzantina but interstitially located on that of A. sativa.

**Discussion**

The (AC)\textsubscript{10} regions in the diploid and polyploid species of Avena mainly appeared as sharp, thin bands in the pericentromeric and interstitial positions of the A- and D-genome chromosomes. These regions have been characterized as euchromatin (Fominaya et al. 1988a; Linares et al. 1992; Jellen et al. 1993a, 1993b; Shelukhina et al. 2008b; Badaeva et al. 2011). This agrees with observations in barley and Rumex acetosa, in which the distribution of (AC)\textsubscript{10}-FISH signals and euchromatic regions appear closely correlated (Cuadrado et al. 2008; Kejnovský et al. 2013). This suggests that blocks of this microsatellite lie within regions containing single copy sequences. In the present work, however, the location of the (AC)\textsubscript{10} hybridization signals in the C-
genome chromosomes was very similar to the C-banding of the centromeric and pericentromeric regions. This indicates that (AC)_{10} is either a component of these regions or of similar sequences of C-banded heterochromatin (Fominaya et al. 1988b; Linares et al. 1992; Jellen et al. 1993a, 1993b; Shelukhina et al. 2008b; Badaeva et al. 2011). However, no strict correlation was seen between (AC)_{10} and the C-bands in terminal positions. Thus, the terminal C-bands of 3C and 5C in hexaploid species must be composed of sequences others than (AC)_{10}.

The somatic chromosomes of *A. sativa* and *A. byzantina* have been characterized by C-banding (Linares et al. 1992; Jellen et al. 1993a, 1993b; Badaeva et al. 2011). FISH and GISH techniques have also been used to make detailed descriptions of them (Chen and Armstrong 1994; Jellen et al. 1994; Leggett and Markhand 1995; Linares et al. 1998; Linares et al. 2000, 2001; Sanz et al. 2010). However, these studies failed to detect chromosome polymorphism among cultivars, except where large chromosome arrangements had occurred (Jellen and Beard 2000; Sanz et al. 2010). The existence of intra-cultivar variation detected could raise doubts on the validity of using information provided by SSR-FISH to make inferences on inter-species differentiation. Only highly conserved signal patterns in chromosomes of different cultivars of one species should be used for detect interspecific polymorphism. In the present study, contrasted interspecific polymorphisms were observed fairly frequently (Fig. 4). The (AC)_{10} pattern on chromosome 21D, with a strong signal in the terminal region of the short arm in the cultivars of *A. byzantina* but absent from those of *A. sativa*, differentiates these species. In addition, this chromosome showed a unique (AC)_{10} pattern in the non-terminal region of the short arm of *A. sativa* cv. Araceli. Moreover, changes were evident in the position of several hybridization signals in *A.*
byzantina cv. Kanota compared to A. sativa. These seem to follow a pattern in which the Kanota signals lie in a terminal position, whereas those of the others cultivars are in interstitial positions (e.g., on the long arm of chromosome 17A and both arms of 8A and 9D chromosomes). The polymorphisms observed could have been caused by changes in the number of copies of the microsatellite (e.g., via amplifications or deletions) during the differentiation of the species and less likely in that affecting cultivars (Kalia et al. 2011). However, determining the chromosome distribution of SSRs in different cultivars allowed to evaluate the cytogenetic diversity within each Avena species. This is especially significant in species where chromosome rearrangements have played an important role in the differentiation within and between species (Jellen and Beard 2000; Sanz et al. 2010).

The existence of translocations and inversions between parents used in genetic mapping have complicated the obtention of a robust consensus genetic map in oats (Oliver et al. 2013; Chaffin et al. 2016). Detection of changes in signal position of SSRs in those parental lines, might contribute to establish a more complete assignments of linkage groups to specific chromosomes and explain some uneven distribution of markers along the linkage groups. The present work using (AC)$_{10}$-FISH provides evidence of new large translocations. A. sativa cv. Araceli carries a translocation involving the long arm of chromosome 17A and the short arm of 21D (Figs. 3e, 3f, and 4). This translocation was detected using C-genome specific sequences (Am1) and ribosomal 45S, but the location of the (AC)$_{10}$ hybridization signals helped to outline the interchange (Figs. 4 and 5). This cultivar has not taken part of any mapping population but if a similar translocation affected to a parental line the consensus linkage groups merged 18 and merged 28 would be likely altered (Chaffin et al. 2016; Yan et al. 2016).
A second intergenomic translocation detected by (AC)_{10}-FISH affected the satellite of chromosome 21D and the long arm of chromosome 4C in the two cultivars of *A. byzantina* (Fig. 6). This 4CL-21DS translocation might explain the lack of markers and the short genetic distances observed for chromosome 21D in the map for the cross between *A. sativa* cv. Ogle and *A. byzantina* cv. Kanota (Oliver et al. 2013). Thus, it is noteworthy that (AC)_{10}-FISH allowed the detection of the 4CL-21DS intergenomic translocation, but the detection of other putative chromosome rearrangements between these two cultivars is no less interesting. For example, that likely affecting chromosome 9D might explain the reduced genetic distance observed in the corresponding linkage group KO17 (Oliver et al. 2013).

Sanz et al. (2010) classified the different types of intergenomic translocations seen in the cultivars of *A. byzantina* and *A. sativa* into three classes: (i) common translocations, present in homologous chromosomes of all closely related hexaploid oat species; (ii) species-specific translocations involving certain chromosomes, exclusive to each hexaploid species; and (iii) cultivar-specific, exclusive to some cultivars of crop oats. Since the new intergenomic translocation 17AL-21DS (Fig. 5) is seen only in *A. sativa* cv. Araceli, its origin must lie within the breeding programme for this cultivar, and should therefore be identified as cultivar-specific. In contrast, the intergenomic translocation 4CL-21DS detected in *A. byzantina* should be identified as species-specific.

Based on molecular cytogenetic studies, a genomic designation of CCDD has been suggested for the tetraploid species involved in the formation of the AACCDD-genome hexaploid species (see Fominaya et al. 2006). The change was proposed since
the species thought to be the donor of the A genome, *A. strigosa*, had repetitive sequences that failed to hybridize with the chromosomes of AACC tetraploids, but did hybridize with those of the AACCDD hexaploids (Linares et al. 1998). In the present work, the similar degree of (AC)$_{10}$ signalling among chromosomes of the reference A genome of *A. magna* and the D-genome chromosomes of the hexaploid species may indicate close proximity between these genomes, even though no individual chromosome maintained an (AC)$_{10}$ pattern in common with those seen in the tetraploid and hexaploid species. This was likely due to the many rearrangements that have occurred during the evolution of the genus (Chen and Armstrong 1994; Jellen et al. 1994; Leggett and Markhand 1995; Linares et al. 1998; Linares et al. 2000, 2001; Sanz et al. 2010). However, the hybridization patterns observed for the 45S and Am1 repetitive sequences on chromosome 10A of the tetraploid species *A. magna* (Fig. 2) were identical to those on chromosome 21D of the hexaploid oat species, reinforcing the idea that a close relationship exists between the A genome of the reference *A. magna* genome and the D genome of the reference *A. sativa* genome (Fig. 4). In contrast, no similar hybridization patterns were observed in the other tetraploid species *A. murphyi*. The above observation regarding *A. magna* is supported by comparisons of molecular linkage maps (Oliver et al. 2011b). For example, a region containing several crown rust resistant QTLs, which are anchored to chromosome 9D of hexaploid oats (Jackson et al. 2009), was found in the genetic map constructed from an *A. magna* recombinant inbred line population. One *A. magna* SNP has been mapped to chromosome 20D of the physically-anchored consensus map of hexaploid oats (Oliver et al. 2013).
Indirect observations argue against the involvement of *A. strigosa* in the formation of the ancestral tetraploid AACC. Molecular studies have revealed the dissimilarity between the *A. strigosa* genome and the A genome of tetraploid oat species (Linares et al. 1998; Peng et al. 2010; Oliver et al. 2011a). Chromosome pairing studies of the hybrid between a synthetic allotetraploid (involving *A. strigosa* and *A. clauda*) and *A. magna* showed highly irregular pairing, indicating that the two *A. magna* genomes differed considerably from those of the diploid species involved in the tetraploid (Ladizinsky 2012). Genotypic by sequencing (GBS) has recently returned a large SNP dataset for numerous accessions belonging to species with different genomes and levels of ploidy. Comparing genetic distances between *in silico* tetraploids (obtained by combining information for the *A. strigosa* SNPs and two C-genome species) and *A. magna*, Chew et al. (2016) raised serious doubts about the involvement of *A. strigosa* in the ancestral tetraploid AACC. If it is not involved, the D-genome donor of the hexaploid species must have been the tetraploid species *A. magna* or a closely related species. Thus, the hexaploid forms might have come into existence via the allopolyploid convergence of species with the CCDD genome and others with the A genome. In addition, Chew et al. (2016) came to the conclusion that both the tetraploidization and hexaploidization events may have occurred in northwest Africa, followed by the radiation of the hexaploid oats to their current worldwide distribution. The results of the above authors’ analyses revealed strong genetic similarity between the *in silico* and extant hexaploids, suggesting an ancestral hexaploid oat participated in the creation of different hexaploid *Avena* species. More recently, Yan et al. (2016) extent substantially the *Avena* GBS studies and present strong evidences to justify the CCDD genome identity of the tetraploids species *A.*
insularis, A. magna (A. maroccana) and A. murphyi. These authors examine the genetic similarities of fifty-one possible combinations of diploids with tetraploids using GBS markers and demonstrate that all in silico hexaploid comprised of CCDD genome tetraploid plus A-genome diploids clustered closely with the extant hexaploids. The closest in silico matches to the extant hexaploids included the CCDD tetraploids plus A. longiglumis which led to the authors to suggest a possible role of these species in the formation of the hexaploids. The similarity of intensity in (AC)_{10}-FISH signals of the A. longiglumis accession studied here and the A (D) genome of the tetraploids would not agree with that proposal. However, more A. longiglumis accessions should be studied study to clarify this.

In conclusion, (AC)_{10}-FISH patterns have allowed differentiate all the chromosomes of the Avena species analyzed. This has resulted in more complete and specific karyotypes than the obtained using C-banding and FISH with the so far available repetitive probes. However, it will be interesting to extent this study to several accessions of diploid and tetraploid species for quantifying the magnitude of of intra- and interspecific variation. Moreover, the analysis carried out on hexaploid species shows the utility of this SSR to identify new translocations undetected by the other karyotyping methods. Increasing the number of microsatellites used in FISH-based studies on oats might provide valuable information on the structure of the genomes and chromosomes of this genus, as well as help explain the incongruities of genetic oat maps. The economical and easy applicability of this technique compared to conventional FISH involving cloned sequences will facilitate this task.
Acknowledgements

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E., and Jackson, E.W. 2013. SNP discovery and chromosome anchoring provide the first physically anchored hexaploid oat map and reveal synteny with model species. PLoS ONE 8: e58068.


**Figure 1.** FISH microphotographs showing the distribution of repetitive sequences on metaphase chromosomes of diploid species. *A. strigosa* (a, and b), *A. damascena* (c, and d), *A. longiligumis* (e, and f), *A. canariensis* (g, and h), *A. eriantha* (i, and j), and *A. ventricosa* (k, and l), after DAPI staining and in situ hybridization with biotin-labelled probes (pink) or digoxigenin-labelled probes (green): (a), (c), (e), (g), (i) and (k) fluorescence in situ hybridization (FISH) with (AC)₁₀ probe; (b), (d), (f), (h), (j), and (l) simultaneous FISH with p45S and p5S probes. (m) Karyotypes of each diploid species analysed showing a single chromosome of each homologous group chosen from the metaphases in a-l. Note that chromosomes carried signals from p45S and p5S are shown in duplicate on karyotypes at the right side of chromosomes with (AC)₁₀ signals. Scale bar = 10 µm.

**Figure 2.** FISH performed on mitotic metaphase of tetraploid species *A. magna* (a, and b), and *A. murphyi* (c, and d). (a) FISH of biotin-labelled (AC)₁₀ (pink) probe. (b) The same cell as in a after simultaneous FISH with the biotin-labelled pAm1 (pink) and digoxigenin-labelled p45S (green) probes. (c) FISH of biotin-labelled (AC)₁₀ (pink) probe. (d) The same cell as in c after simultaneous FISH with the biotin-labelled pAm1 (pink) and digoxigenin-labelled p45S (green) probes. (e) Karyotypes showing a single chromosome of each homologous group chosen from the metaphases in a and c. The reference karyotypes was made from the metaphases b and d for *A. magna* and *A. murphyi*, respectively. They are based on the hybridization patterns of Am1 (pink) and 45S (green) sequences and following the nomenclature of Linares et al. (1996). Scale bar = 10 µm.
Figure 3.- FISH performed on metaphase chromosomes of hexaploid species *A. sativa* cvs. Ogle (a) and Araceli (d) using (AC)$_{10}$ probe (pink). Chromosomes are counterstained with DAPI. (b) The same cell as in a after double FISH with pAs120a (green) and p45S (pink) probes. (c) The same cell as in a after rehybridizing with pAm1 (green) and p5S (pink) probes. (e) The same cell as in d after double FISH with pAs120a (green) and p5S (pink) probes. (f) The same cell as d after rehybridizing with pAm1 (pink) and p45S (green) probes. In e, arrows indicate the translocated chromosome pair 21D; asterisks indicate the translocated chromosome pair 17A. Scale bar = 10 µm.

Figure 4.- (AC)$_{10}$-FISH (pink) karyotypes from each of three cultivars of hexaploid species *A. sativa* and two cultivars of hexaploid species *A. byzantina*. Each chromosome probed with (AC)$_{10}$ was identified by the hybridization patterns of repetitive sequences after simultaneous and sequential FISH with pAs120a, pAm1, p45S and p5S probes and reprobing of the same cell. According to Sanz et al. (2010), the reference karyotype was based on the hybridization patterns of pAs120a (green), p45S (pink) and p5S (pink) probes which identified the A-genome chromosomes; the hybridization patterns of both pAm1 (green) and p5S (pink) which identified the C-genome chromosomes; and the hybridization patterns of pAm1 (green), p45S (pink) and p5S (pink) probes which identified the D-genome chromosomes. Chromosomes of reference karyotype were chosen from the same metaphase and corresponding to cv. Ogle (Figs. 3 b, and 3c).

Figure 5.- Diagram showing the formation of an intergenomic translocation detected in cv. Araceli compared to *A. sativa* cv. Ogle and affecting to chromosomes 17A and 21D.
FISH signals using different probes are illustrated. In (a) probes pAS120a, pAm1 and p45S. In (b) probe (AC)$_{10}$.

**Figure 6.-** Diagram showing the formation of the intergenomic translocation in *A. byzantina* (compared to *A. sativa*). (AC)$_{10}$ signals are illustrated.
Table 1. *Avena* species used in this study, including ploidy level, genomic composition, accession numbers (or cultivar names) and provider.

<table>
<thead>
<tr>
<th>Species</th>
<th>Ploidy</th>
<th>Genomic composition</th>
<th>Accession</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. strigosa</em> Schreb</td>
<td>2x</td>
<td>AsAs</td>
<td>PI 258727</td>
<td>National Small Grains Collection, Betsville, USA.</td>
</tr>
<tr>
<td><em>A. damascena</em></td>
<td>2x</td>
<td>AdAd</td>
<td>CAV 0258</td>
<td>Plant Research Centre, Ottawa, Canada.</td>
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<tr>
<td><em>A. longiglumis</em> Dur.</td>
<td>2x</td>
<td>AIAI</td>
<td>PI 367390</td>
<td>National Small Grains Collection, Betsville, USA.</td>
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<tr>
<td><em>A. canariensis</em> Baum</td>
<td>2x</td>
<td>AcAc</td>
<td>WIR-292</td>
<td>N.I. Vavilov Research Institute, Russia.</td>
</tr>
<tr>
<td><em>A. eriantha</em></td>
<td>2x</td>
<td>CpCp</td>
<td>PI 657579</td>
<td>National Small Grains Collection, Betsville, USA.</td>
</tr>
<tr>
<td><em>A. ventricosa</em></td>
<td>2x</td>
<td>CvCv</td>
<td>PI 657338</td>
<td>National Small Grains Collection, Betsville, USA.</td>
</tr>
<tr>
<td><em>A. magna</em> Murph et Terr</td>
<td>4x</td>
<td>AACC</td>
<td>PI 659402</td>
<td>National Small Grains Collection, Betsville, USA.</td>
</tr>
<tr>
<td><em>A. murphyi</em> Ladiz.</td>
<td>4x</td>
<td>AACC</td>
<td>PI 657382</td>
<td>National Small Grains Collection, Betsville, USA.</td>
</tr>
<tr>
<td><em>A. sativa</em> L. cv Ogle</td>
<td>6x</td>
<td>AACCDD</td>
<td></td>
<td>Plant Research Centre, Ottawa, Canada.</td>
</tr>
<tr>
<td><em>A. sativa</em> L. cv Cobeña</td>
<td>6x</td>
<td>AACCDD</td>
<td>Cobeña</td>
<td>National Institute of seeds, Madrid, Spain.</td>
</tr>
<tr>
<td><em>A. sativa</em> L. cv Araceli</td>
<td>6x</td>
<td>AACCDD</td>
<td>Araceli</td>
<td>National Institute of seeds, Madrid, Spain.</td>
</tr>
<tr>
<td><em>A. byzantina</em> C Koch cv Culta</td>
<td>6x</td>
<td>AACCDD</td>
<td>WIR 5206</td>
<td>N.I. Vavilov Research Institute, Russia.</td>
</tr>
<tr>
<td><em>A. byzantina</em> C Koch cv Kanota</td>
<td>6x</td>
<td>AACCDD</td>
<td>2265</td>
<td>Plant Research Centre, Ottawa, Canada.</td>
</tr>
</tbody>
</table>
Reference Karyotype

A. sativa cv Ogle

A. sativa cv Cobaña

A. sativa cv Araceli

A. byzantina cv Culta

A. byzantina cv Kanota

245x163mm (300 x 300 DPI)
a

\[ A. \text{sativa} \]
\[ \text{cv Ogle} \]
\[ 17A \]
\[ 21D \]
\[ A. \text{sativa} \]
\[ \text{cv Araceli} \]
\[ 17AL-21DS \]
\[ 21DS-17AL \]

b

\[ A. \text{sativa} \]
\[ \text{cv Ogle} \]
\[ 17A \]
\[ 21D \]
\[ A. \text{sativa} \]
\[ \text{cv Araceli} \]
\[ 17A \]
\[ 21D \]

Legend:
- 120a
- Dapi
- 45S
- Am1
- (AC)\text{10}
A. sativa

A. byzantina

4C

21D

\((AC)_{10}\)

Dapi

21DS-4CL