Cytological characterization of sunflower by in situ hybridization using homologous rDNA sequences and a BAC clone containing highly represented repetitive retrotransposon-like sequences

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Abstract: In the present work we report new tools for the characterization of the complete chromosome complement of sunflower (*Helianthus annuus* L.), using a bacterial artificial chromosome (BAC) clone containing repetitive sequences with similarity to retrotransposons and a homologous rDNA sequence isolated from the sunflower genome as probes for FISH. The rDNA signal was found in 3 pairs of chromosomes, coinciding with the location of satellites. The BAC clone containing highly represented retroelements hybridized with all the chromosome complement in FISH, and used together with the rDNA probe allowed the discrimination of all chromosome pairs of sunflower. Their distinctive distribution pattern suggests that these probes could be useful for karyotype characterization and for chromosome identification. The karyotype could be subdivided into 3 clear-cut groups of 12 metacentric pairs, 1 submetacentric pair, and 4 subtelocentric pairs, thus resolving previously described karyotype controversies. The use of BAC clones containing single sequences of specific markers and (or) genes associated with important agricultural traits represents an important tool for future locus-specific identification and physical mapping.

Key words: karyotype, BAC-FISH, rDNA, repetitive elements, sunflower, *Helianthus annuus*.

Résumé : Dans le présent travail, les auteurs rapportent de nouveaux outils pour la caractérisation du jeu chromosomique complet chez le tournesol (*Helianthus annuus* L.) au moyen d'analyses FISH avec une sonde BAC contenant des séquences répétées présentant de l'homologie avec des rétrotransposons et avec une séquence homologue d'ADNr isolée du génome du tournesol. Des sites chromosomiques de l'ADNr ont été observés sur trois paires de chromosomes, lesquels coïncidaient avec l'emplacement de satellites. Le clone BAC contenant des rétroéléments très présents dans le génome a hybridé avec tous les chromosomes en analyse FISH et, employé conjointement avec la sonde d'ADNr, a permis de distinguer toutes les paires de chromosomes du tournesol. La distribution distinctive de ces séquences répétées suggère qu'elles pourraient servir pour la caractérisation du caryotype et pour l'identification des chromosomes. Ainsi, le caryotype se diviserait en trois groupes nets formés de 12 paires métacentriques, une paire submétacentrique et quatre paires subtélocentriques. Cela permettrait ainsi de mettre fin à des controverses au sujet du caryotype. De plus, l'emploi de séquences BAC représente un outil important en vue d'une future identification de locus spécifiques et de la cartographie physique à l'aide de clones BAC contenant des séquences uniques pour certains marqueurs ou gènes spécifiques liés à des caractères agronomiques importants.

Mots-clés : caryotype, BAC-FISH, ADNr, éléments répétés, tournesol, *Helianthus annuus*.

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Introduction

Cultivated sunflower (*Helianthus annuus* L.) is one of the most important sources of vegetable oil in the world. Reliable cytological techniques for chromosome identification are necessary for efficient genome research and germplasm utilization (Paniego et al. 2007). Sunflower is a diploid plant with $2n = 2x = 34$ chromosomes that are very similar in size and morphology, making identification of chromosome pairs a difficult task.

Fluorescence in situ hybridization (FISH) is a powerful tool for chromosome identification. This technique is widely used for karyotype analysis, construction of physical maps, comparative genome mapping, localization of genes, and examination of transgenic insertions. The use of genomic DNA cloned in large-insert BAC (bacterial artificial chromosome) vectors as probes in FISH experiments is called BAC-FISH (Zhang et al. 2004). Many BAC clones contain repetitive sequences (Schwarzacher 2003). Thus, when these sequences are used in BAC-FISH, it is common to observe FISH signals widely distributed across the genome. In sunflower, BAC-FISH offers an alternative to classical cytogenetics for chromosome identification, which can be difficult owing to the lack of specific chromosomal characteristics at the morphological level and the limited identification provided by classical banding patterns for some of the chromosomes.

Although previous works have identified and classified the sunflower chromosome pairs, the results have been contradictory with respect to both the specific number of chromosomes within each morphological class and the chromosome pairs with satellites and (or) rDNA sites. This is mainly due to the large number of chromosomes, their relatively small size and, most of all, the striking similarity of the chromosome complement. Previous cytogenetic studies in sunflower, based on chromosome length and arm ratios (long/short) and using Feulgen staining, allowed the identification of 10 metacentric, 3 submetacentric, and 4 acrocentric chromosome pairs in the haploid complement (Raicu et al. 1976; Schrader et al. 1997). In addition, Al-Allaf and Godward (1979) and Cuellar et al. (1996) proposed a chromosome classification of 4 metacentric, 8 submetacentric, and 5 acrocentric chromosome pairs. Recently, Ceccarelli et al. (2007) characterized the chromosome complement by in situ hybridization using a tandemly repeated DNA sequence. According to these authors, the chromosome complement can be subdivided into two groups: 4 acrocentric and 13 meta- to submetacentric pairs. Furthermore, contradictory results have been reported regarding the number of chromosome pairs with satellites. Bohorova and Georgieva-Todorova (1987) found only 1 pair, Kulshreshta and Gupta (1981) found 2 pairs, and Raicu et al. (1976), Cuellar et al. (1996), Schrader et al. (1997), and Ceccarelli et al. (2007) reported 3 pairs of satellite chromosomes.

Using different techniques such as C-banding, silver staining, and FISH with heterologous rDNA probes, different authors found 6 to 8 rDNA signals in different sunflower accessions (Cuellar et al. 1996; Schrader et al. 1997; Vanzela et al. 2002). Recently, Ceccarelli et al. (2007) detected signals in 4 chromosome pairs using heterologous rDNA probes for in situ hybridization, 3 pairs showing strong signals and 1 showing a weaker signal.

BAC-FISH signals could serve as excellent cytological markers for locus-specific chromosome or genomic segment identification (Hanson et al. 1995; Gómez et al. 1997; Dong et al. 2000; Fransz et al. 2000; Islam-Faridi et al. 2002; Kim et al. 2002). Prior to this, a reliable characterization of the sunflower chromosome complement is necessary for future physical identification of single tag sequences corresponding to molecular markers or physical mapping of specific interesting genes. We report here the unambiguous characterization of the complete chromosome complement of sunflower using two complementary FISH strategies: BAC-FISH using a BAC probe containing repetitive sequences with similarity to retrotransposon sequences, and rDNA-FISH using a specific homologous sunflower rDNA sequence. The obtained karyotype represents an important tool for future locus-specific identification using BACs containing single sequences, overcoming previous uncertainties in sunflower chromosome identification.

Materials and methods

Plant materials

The inbred line HA89 was used as a template for probe amplification and for chromosome preparations. HA89 was released by the US Department of Agriculture – Agricultural Research Service and the Texas Agricultural Experiment Station as a maintainer line in 1971. It has been extensively used as a parental genotype in breeding programs as well as to develop mapping populations (Gentzbittel et al. 1995; Jan et al. 2002; Langar et al. 2003; Burke et al. 2004). HA89 was multiplied and seeds were provided by EEA INTA Balcarce, Buenos Aires, Argentina.

rDNA probe isolation and characterization

The sunflower cDNA clone EF235 (GenBank accession No. BU671882), containing sequences with similarity to the large subunit ribosomal RNA gene (Fernández et al. 2003), was used as probe for detecting the chromosomal location of rDNA in sunflower. Amplification with specific forward (5′-ATGACGAGGCATTTGGCTAC-3′) and reverse primers (5′-TCCCAGGAAACCAGCTAATG-3′) was achieved in a total volume of 50 μL containing 10 mmol/L *Taq* polymerase buffer, 0.25 μmol/L each primer, 0.2 mmol/L dNTPs, 100 ng of plasmid DNA, and 0.75 U of *Taq* polymerase (Roche, France). A touch-down PCR protocol was applied (95 °C for 3 min; 10 cycles of 95 °C for 1 min, 60 °C (–0.5 °C/cycle) for 1 min, and 72 °C for 1 min; 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; 72 °C for 3 min; hold at 15 °C). Amplified DNA fragments were resolved by electrophoresis on 1% agarose gel, stained with ethidium bromide, and photographed under UV light (302 nm). The corresponding bands were excised and purified with a QIAEX II gel extraction kit (QIAGEN, Germany) for FISH applications.

Repetitive DNA probe isolation and characterization

A highly repetitive DNA probe (GenBank acc. No. GF100475), hereafter named Ha785, originally identified from a genomic sunflower DNA library (Paniego et al. 2002) and similar to a highly repetitive sequence of *H. annuus* (GenBank acc. No. AJ009965), was used to character-

Table 1. Relevant repetitive sequences present in the BAC probe.

Location	BLASTN similarity (acc. No.)	BLASTX similarity (acc. No.)	Similarity to repetitive element	*E* value
Contig GU074382				
3–2441	EF469194.1		*copia*-like retrotransposon	0.0
Contig GU074383				
2453–3189	FJ791046.1		Retrotransposon HA2	0.0
17324–17699	**AJ009965**		***Helianthus annuus* highly repetitive sequence**	**1e–151**
24154–24977	EF469194.1		*Helianthus annuus copia*-like retrotransposon	4e–36
15560–16639		ABF67947	Opie2 pol protein (integrase core domain)	2e–65

Note: The sunflower DNA BAC insert used as probe for FISH was subcloned in sequencing vectors and sequenced, and the resulting sequences were submitted to bioinformatic analysis. These sequences could be grouped into two contigs of 3 082 (GU074382) and 30 545 (GU074383) nucleotides. BLASTN and BLASTX analysis allowed the identification of similarities to relevant repetitive sequence element motifs. The specific target sequence with homology to the probe originally used to characterize and identify this specific BAC clone (called Ha785) is shown in boldface.

ize a set of clones from the commercial sunflower BAC library HA_HBa (Clemson University Genomics Institute BAC/EST Resource Center) to select a clone containing repetitive sequences (HaBACr) to be used in BAC-FISH studies.

In addition, two partial nucleotide sequences with similarity to a *gypsy*-like retrotransposon (GenBank acc. No. DQ229838.1), hereafter named HaRetro3, and a *copia*-like retrotransposon (GenBank acc. No. AJ009967), hereafter named HaRep1, were used for characterization of clone HaBACr. Probes were amplified from sunflower line HA89 with specific forward (5′-AGGGCATTCAAATGGCTATG-3′ and 5′-TCTCAGAACCTCGGCAATCT-3′) and reverse primers (5′-GTCTCATCCGGAAGATCCAA-3′ and 5′-GGCAGCAAAAGAGAAAATG-3′), respectively (Santini et al. 2002; Tang et al. 2006).

PCR amplification was performed using an Eppendorf thermocycler in a reaction containing 100 ng of HA89 DNA in 10 mmol/L buffer, 0.25 µmol/L each specific primer, 0.2 mmol/L dNTPs, and 0.75 U of *Taq* polymerase (Roche, France), with an initial denaturing step of 3 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 30 s at 72 °C, a final extension step of 10 min at 72 °C, and a hold at 15 °C. PCR amplifications were run at 80 V on 1% agarose and stained with ethidium bromide. The corresponding bands were excised and purified with a QIAEX II gel extraction kit (QIAGEN, Germany). The probes were labelled with [α-^{32}P]dCTP by random priming according to the manufacturer's recommendations (Promega Biotech, USA).

Clone HaBACr, used in this study for FISH hybridization, was originally identified from a commercial genomic sunflower BAC library (HA_HBa; Clemson University Genomics Institute BAC/EST Resource Center) by screening for germin-like protein sequences. Positive clones were isolated and purified using a QIAGEN Plasmid Midi Kit (QIAGEN, Germany), digested with restriction enzyme *Eco*RI, electrophoresed in 0.8% agarose gels, and blotted on Hybond N$^+$ nylon membranes (GE Healthcare, UK) as described by Sambrook et al. (1989). The filters were prehybridized using a buffer containing polyanethol sulfonic acid at 65 °C for 16 h. Denatured ^{32}P-labelled Ha785 probe was added and allowed to hybridize overnight at 65 °C. After hybridization, filters were washed in a series of buffers with increasing stringency: 2× SSC (0.3 mol/L NaCl, 0.03 mol/L sodium citrate) and 0.1% SDS at room temperature for 20 min, 1.5× SSC and 0.1% SDS at room temperature for 15 min, and 0.5× SSC and 0.1% SDS at 65 °C for 15 min (Sambrook et al. 1989). The filters were sealed in plastic bags, exposed for 3 days to X-ray-sensitive screens, and then scanned with a Typhoon Trio imager (Amersham Biosciences, UK). The same procedure was applied to characterize HaBACr using HaRep1 and HaRetro3 in separate assays.

Clone HaBACr was further characterized by shotgun sequencing (Macrogen DNA sequencing services, South Korea; http://dna.macrogen.com/eng/), and two contigs representing the assembly of 304 partial sequences were obtained (GenBank acc. Nos. GU074382 and GU074383). These two contigs were 3 082 and 30 545 bp, respectively, giving a total combined contig length of 33 627 bp. Different repetitive sequences were detected within clone HaBACr by sequence comparison using BLASTN and BLASTX (Table 1).

Chromosome preparations

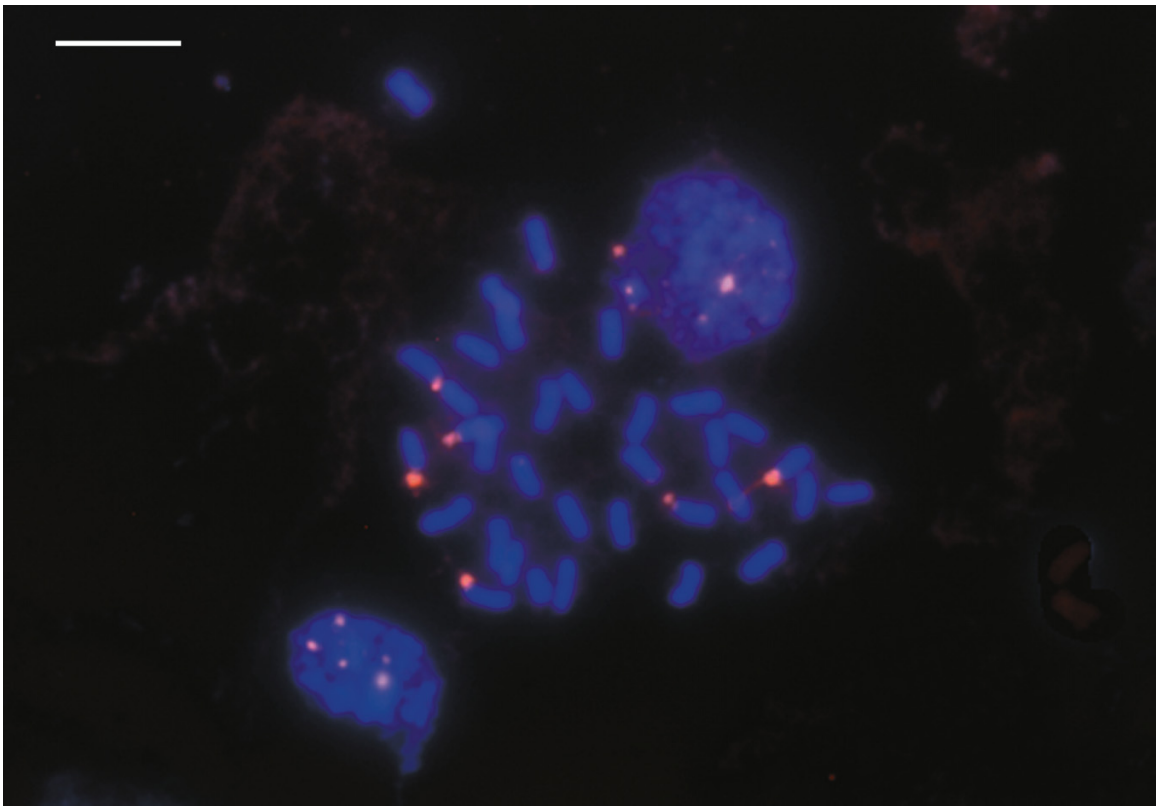
Root tips, obtained from germinating seeds on moist paper, were treated with 0.05% colchicine for 2 h at room temperature, fixed in absolute ethanol : acetic acid (3:1, *v*/*v*), and stored at 4 °C for several weeks. For cell wall digestion, root tips were treated with an enzymatic solution (cellulose 2% [*w*/*v*] plus pectinase 20% [*v*/*v*]) for 3 h at 37 °C and stored in 0.01 mol/L citric acid – sodium citrate buffer at 4 °C for 24 h. Digested material was transferred to a drop of 45% acetic acid previously applied to a slide and then flamed before squashing.

Fluorescence in situ hybridization

The FISH technique was conducted according to protocols previously described by Cuadrado and Jouve (1994). Slides were incubated in absolute ethanol : acetic acid (3:1, *v*/*v*) for 10 min and then washed in absolute ethanol twice for 10 min each.

Clone HaBACr was purified using NucleoBond Xtra Midi Plus (Machery-Nagel, Germany). This BAC clone and the EF235 rDNA sequences were labelled with digoxigenin-11-dUTP by random priming labelling (Boehringer, Mannheim,

Fig. 1. Mitotic metaphase chromosomes of *Helianthus annuus* after FISH with sequence EF235, corresponding to a 26S rDNA probe. Hybridization signals were detected with Cy3. Bar represents 10 μm.



Germany) and biotin-14-dUTP by nick translation (BioNick labelling system, GIBCO BRL, USA), respectively, and used as probes for FISH.

Chromosome slide preparations were pretreated with RNase for 1 h at 37 °C and fixed with 4% paraformaldehyde. The hybridization mixture consisted of 50% deionized formamide, 10% dextran sulphate, 2× SSC, 0.1% SDS, 0.3 mg/mL sheared salmon sperm DNA (Sigma, USA), and 3.3 μg/mL probe. After chilling on ice for 5 min, 30 μL of the denatured hybridization mixture was applied to each slide (100 ng probe per slide), and hybridization was performed using a thermocycler (Eppendorf, Mastercycler, Germany) at 75 °C for 7 min, 55 °C for 30 s, 45 °C for 5 min, 38 °C for 5 min, and 37 °C for 10 min, and then in a humid chamber at 37 °C overnight.

After overnight hybridization at 37 °C, washes were in 2× SSC at 42 °C for 10 min, 20% formamide plus 0.1× SSC at 42 °C for 10 min, 0.1× SSC at 42 °C for 5 min, 2× SSC at 42 °C for 5 min, 4× SSC plus 0.2% Tween 80 (polyoxyethylene sorbitan monooleate) at 42 °C for 5 min, and 4× SSC plus 0.2% Tween 80 at room temperature for 5 min, twice. Slides were blocked for 5 min at room temperature with 5% (*w*/*v*) BSA in 4× SSC plus 0.2% Tween 80.

Detection of digoxigenin at hybridization sites was carried out by incubating the slides in a solution of 4 μg/mL anti-digoxigenin-FITC (fluorescein isothiocyanate, Boehringer, Mannheim, Germany) in 5% (*w*/*v*) BSA in 4× SSC plus 0.2% Tween 80 for 1 h at 37 °C. The biotin-labelled probe was detected with streptavidin-Cy3 conjugate (Sigma, USA). Afterwards, the slides were washed in 4× SSC plus 0.2% Tween 80 three times for 10 min each. DNA was immediately stained with 4 μg/mL DAPI (4′,6-diamidino-2-phenylindole) and washed briefly with 4× SSC plus 0.2% Tween 80. After rinsing, Vectashield antifade solution (Vector Laboratories, USA) was applied.

Image analysis

Photographs were taken with a Leica DFC 350 FX camera (Germany) and analyzed with Adobe Photoshop CS2 informatics software. Lengths of chromosome arms and FISH bands were determined using MicroMeasure 3.01 software (Reeves and Tear 1997).

Construction of idiograms

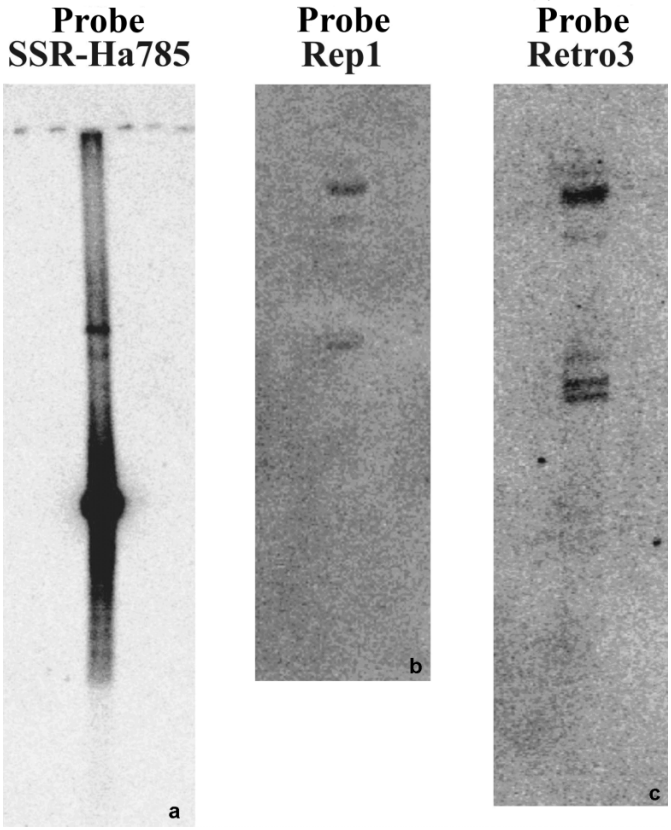
Five complete metaphases were carefully analyzed. Relative length and arm ratio (long arm / short arm) were calculated for each chromosome. Additionally, size and position of FISH banding were measured.

Chromosome morphology was assigned following Levan et al. (2009), who classified chromosomes according to their centromeric index (length of the short arm × 100 / total length of the chromosome) as metacentric, submetacentric, subtelocentric, or telocentric.

Results and discussion

Although BAC-FISH is currently one of the most widely used techniques for chromosome characterization in plants

Fig. 2. (*a*) Southern blot hybridization of BAC clone HaBACr digested with *Eco*RI and hybridized with Ha785 probe. (*b* and *c*) Southern blot hybridization of clone HaBACr digested with *Eco*RI and hybridized with HaRep1 and HaRetro3 probes, respectively.

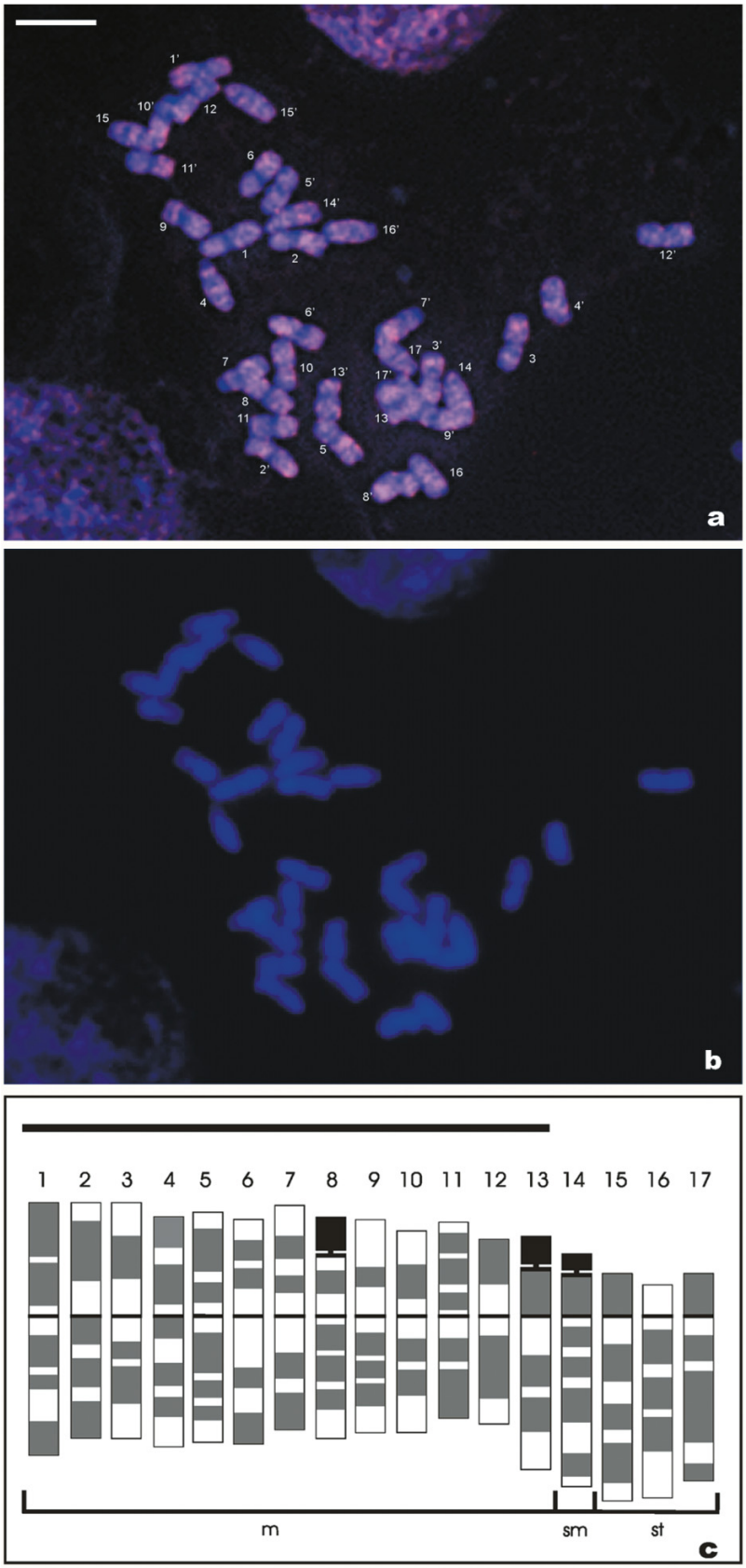


(Zhang et al. 2004), it has not previously been applied in sunflower. Here we assayed this approach for studying the chromosome complement of cultivated sunflower on the basis of size, morphology, arm ratio, and FISH using a BAC clone containing different representative homologous repetitive DNA sequences with similarity to retrotransposons, in combination with conventional FISH but using a homologous rDNA probe instead of the more widely used pTa71 probe from wheat.

Using an rDNA probe corresponding to the 26S rDNA of *H. annuus* (cDNA clone EF235, which is a specific sequence isolated from the sunflower genome; GenBank acc. No. BU671882), instead of heterologous monocot probes, 3 chromosome pairs showed strong hybridization signals of different intensity and size at their satellite region (Fig. 1). These differences in intensity and size could be due to variation in the number of tandemly repeated copies of this sequence in each of the chromosomes. This could also explain the variation in the number and intensity of signals reported by different authors, i.e., 3 pairs according to Cuellar et al. (1996) and 4 pairs according to Schrader et al. (1997), Vanzela et al. (2002), Ceccarelli et al. (2007), and Natali et al. (2008). In addition, this variation could be attributed to differential resolution of the assays and (or) nonspecific hybridization of heterologous probes containing wheat ribosomal spacer sequences, which are very different from their sunflower counterparts. The EF235 probe seems to be a more reliable tool to detect rDNA regions than the pTa71 probe owing to its smaller size, the possibility of PCR amplification, and the larger extent of homologous sequences in the case of sunflower. Also, it is interesting to point out that using this probe, superior signals were obtained compared with wheat rDNA probes not only in cultivated and wild sunflower (*H. annuus* and *H. resinosus*) but also in other non-related species such as *Amaranthus* spp. and *Allium cepa* (data not shown).

In parallel, this study explored the use of BAC clones with a high content of repetitive sequences as probes for chromosome identification using FISH technology. A set of BAC clones originally isolated from a sunflower BAC library was analyzed by Southern blotting using ^{32}P-labelled Ha785 as probe. Strong hybridization signals were observed for only a single BAC clone (HaBACr), as shown in Fig. 2*a*. This positive clone was further analyzed by Southern blotting using HaRep1 and HaRetro3 as probes. These experiments led to strong hybridization signals, thus indicating that this BAC clone also contains sequences with similarity to *copia*- and *gypsy*-like retrotransposons (Figs. 2*b* and 2*c*). Altogether, these results indicate that HaBACr contains sequences with similarity to *gypsy*-like and *copia*-like retrotransposons and repetitive sequence Ha785 (GenBank acc. No. GF100475). These results were confirmed by partial shotgun sequencing of HaBACr, as described in the Materials and methods. Similarity analysis using the BLASTX and BLASTN programs (Altschul et al. 1990) confirmed that clone HaBACr contains sequences with similarity to *copia*-like retrotransposons and sunflower repetitive elements (GenBank acc. No. AJ009965), among other repetitive sequences (Table 1). In the present work, 12 metacentric, 1 submetacentric, and 4 subtelocentric chromosome pairs were clearly identified. BAC-FISH performed using HaBACr as probe showed distinct hybridization patterns on *H. annuus* chromosomes, allowing the identification of each chromosome pair (Fig. 3*a*). The deduced idiogram of the haploid chromosome complement of *H. annuus* is presented in Fig. 3*c*. These results largely agree with those of Ceccarelli et al. (2007), who classified the chromosomes into two groups: 13 meta- to submetacentric pairs and 4 acrocentric pairs. Interestingly, these authors characterized the chromosome complement of sunflower using a different probe consisting of tandemly repeated DNA and not retroelement-rich repetitive DNA like that used here. In both works a single probe allowed the identification of the total chromosome complement of sunflower; the present work is the first report of the application of a full repetitive BAC clone as probe for this purpose. As expected from the differences in DNA sequences used as probes, the chromosome banding patterns obtained in this work differ from those reported by Ceccarelli et al. (2007). In our study, hybridization signals were found at the end of both chromosome arms in 2 pairs and at the end of only one arm in 7 pairs. Moreover, there were signals in the intercalary regions in all the pairs (in both chromosome arms of 12 pairs and in only one arm in 5 pairs) and in centromeric regions in 6 pairs. On the other hand, Ceccarelli et al. (2007) found hybridization signals at the end of both chromosome arms in 4 pairs, at the end of only one arm in 9 pairs, and in the intercalary regions in all the pairs (in both chromosome arms of 8 pairs and in only one arm of 9

Fig. 3. (*a*) Mitotic metaphase chromosomes of *H. annuus*: hybridization using a repetitive BAC clone (HaBACr). The numbers indicate the members of each chromosome pair. (*b*) Chromosomes counterstained with DAPI. (*c*) Idiogram of the 17 sunflower chromosomes showing the hybridization sites with the repetitive BAC clone in grey and sites with rDNA in black. m, metacentric; sm, submetacentric; and st, subtelocentric. Bars represent 10 μm.



pairs). In addition, these authors found hybridization signals at the centromeric regions in only 1 pair. Thus, the results presented here and those reported by Ceccarelli et al. (2007) turned out to be complementary for the characterization of the sunflower chromosome complement.

In conclusion, the use of two different repetitive probes isolated from the *H. annuus* genome for FISH studies — a BAC probe containing repetitive sequences and an rDNA sequence — allowed the characterization of the sunflower karyotype, an important advance in sunflower chromosome identification. This karyotype characterization represents a key tool for future physical mapping and locus-specific identification using BAC clones containing single sequences of specific markers and (or) genes associated with important agricultural traits.

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