Chromosomal characterization and physical mapping of the 5S and the 18S-5.8S-25S ribosomal DNA in *Helianthus argophyllus*, with new data from *Helianthus annuus*

T. Cuéllar, J. Orellana, E. Belhassen, and J.L. Bella

Abstract: The characterization of the mitotic chromosomes of *Helianthus argophyllus* by means of Feulgen staining, Giemsa C-banding, and the usual DNA sequence-specific fluorochromes allows a chromosomal classification of this species, and shows that two kinds of heterochromatin co-exist equilocally in its chromosomes. One type is confined to the pericentromeric areas of all the chromosomes and the other is associated with the secondary constrictions of the satellite chromosomes. This species is evolutionarily very close to *H. annuus* with which it is involved in introgression breeding programs. Since these two species show no intra- or interspecific differences with the above treatments, we have used C-banding followed by DAPI, chromomycin A_3 or Acridine Orange, and the fluorescent in situ hybridization (FISH) with 5S and 18S-25S ribosomal DNA probes to characterize further the chromosomes of both species in an attempt to find practically applicable chromosomal markers. Our results confirm the heterogeneity of the heterochromatin in these species and show that neither its distribution nor its response to distinct fluorochrome treatments allows better discrimination of the chromosomes within or between the species. On the other hand, the 18S-5.8S-25S rDNA arrays are located in the secondary constrictions of the satellited SM7, SM10, and ST13 pairs and the 5S-rDNA genes are interstitially placed on the short arm of the SM7 and SM11 chromosomes in both species. This permits these chromosomes to be distinguished and provides markers which may be helpful for further physical mapping of DNA sequences in these species.

Key words: chromosome banding, sunflower cytogenetics, heterochromatin, ribosomal DNA mapping, FISH.

Résumé : La caractérisation des chromosomes mitotiques de l'*Helianthus argophyllus* par coloration Feulgen, par révélation des bandes C ainsi que par coloration avec les fluorochromes spécifiques de certaines séquences a permis de classifier les chromosomes chez cette espèce et de démontrer l'existence de deux types d'hétérochromatine. Un type est confiné aux régions péricentromériques de tous les chromosomes alors que l'autre est associé aux constrictions secondaires des chromosomes satellites. L'*Helianthus argophyllus* est très proche de l'*H. annuus* sur le plan évolutif et elle est mise à contribution dans des programmes d'amélioration génétique par introgression. Puisque ces deux espèces ne montrent aucun polymorphisme intra- ou interspécifique à l'aide des techniques mentionnées plus haut, les auteurs ont eu recours à la révélation des bandes C suivie de coloration au DAPI, à la Chromomycine A, à l'acridine orange de même qu'à l'hybridation in situ en fluorescence (FISH) au moyen de sondes d'ADN ribosomique 5S et 18S-5.8S-25S afin de caractériser davantage les chromosomes des deux espèces en vue d'identifier des marqueurs chromosomiques. Les résultats confirment l'hétérogénéité de l'hétérochromatine chez ces deux espèces et montrent que ni la distribution ni la réponse aux différents fluorochromes ne procure une meilleure discrimination chromosomique parmi et entre les espèces. Par ailleurs, les régions d'ADNr 18S-5.8S-25S sont situées au niveau des constrictions secondaires des paires de chromosomes à satellite SM7, SM10 et SM13 chez ces deux espèces. Les gènes d'ADNr 5S sont pour leur part situés dans des régions intermédiaires des bras courts des chromosomes SM7 et SM11 chez les deux espèces. Cela permet de distinguer les chromosomes et fournit des marqueurs qui pourront aider à la cartographie physique de séquences d'ADN chez ces espèces.

Mots clés : révélation des bandes chromosomiques, cytogénétique du tournesol, hétérochromatine, cartographie des ADN ribosomiques, FISH.

[Traduit par la Rédaction]

Corresponding Editor: G. Jenkins.

Received May 13, 1998. Accepted July 1, 1998.

T. Cuéllar and J.L. Bella.[1] Departamento de Biología, Unidad de Genética, Facultad de Ciencias, Universidad Autónoma de Madrid, E-28049 Madrid, Spain.
J. Orellana. Departamento de Biotecnología, ETSI Agrónomos, Universidad Politécnica de Madrid, E-28040 Madrid, Spain.
E. Belhassen. Génétique et Amélioration des Plantes, INRA, Place Pierre Viala, 2. F-34060 Montpellier, France.

[1]Author to whom all correspondence should be addressed (e-mail: bella@uam.es).

Introduction

The 2*n* = 34 chromosome number of *Helianthus argophyllus* was determined by Heiser in 1948. Since then, chromosomal characterization of this species has been restricted to the work of Kulshreshtha and Gupta (1981), in spite of its evolutionary importance as the closest relative of *H. annuus*, the cultivated sunflower (Schilling and Heiser 1981), its economic importance as an ornamental plant, and its exploitation as a drought tolerant parent in sunflower breeding programs (Belhassen et al. 1994, 1995).

As in other sunflowers, cytogenetical work in *H. argophyllus* has been little developed because of the intrinsic difficulties of a material characterized by a relatively high number of small similar chromosomes enclosed in oily hard-walled cells (see Chandler 1991). In a previous paper (Cuéllar et al. 1996*a*) we reported that the use of pectinase allows mitotic chromosome preparations of *H. annuus* of sufficiently good quality to be obtained. By this means, the amount and distribution of the heterochromatin and its response to commonly used fluorochromes with DNA-base pair specificity were evaluated. Fluorescent in situ hybridization (FISH) with an rDNA probe and silver staining allowed us to map the rDNA sites on the chromosomes and to determine the cytological activity of the nucleolar organizer regions (NORs) in this species.

In this paper we report a similar characterization carried out in the chromosomes of *H. argophyllus* in order to compare them with those of *H. annuus*, and also in an attempt to find chromosomal markers of interest in the developing programs by which economically useful traits are introgressed into these species. This study also includes a further characterization of the heterochromatin of *H. annuus* by C-banding combined with fluorochrome staining techniques which has also been assayed in *H. argophyllus*, and the number and distribution of the 5S-rDNA in both species, as determined by FISH.

Materials and methods

The cultivated lines RHA-274, RHA-265, AL5–4, 89B1, 89B2, and the inbred line HA89 of *H. annuus*, and the ecotypes HAR585, 92, 93, and 1095 of *H. argophyllus*, all from the INRA sunflower collection, were used.

Seeds from different individuals were germinated and the roots pretreated to obtain mitotic chromosomes according to Cuéllar et al. (1996*a*). A minimum of 5 plants and 15 cells per line or ecotype were studied with each technique.

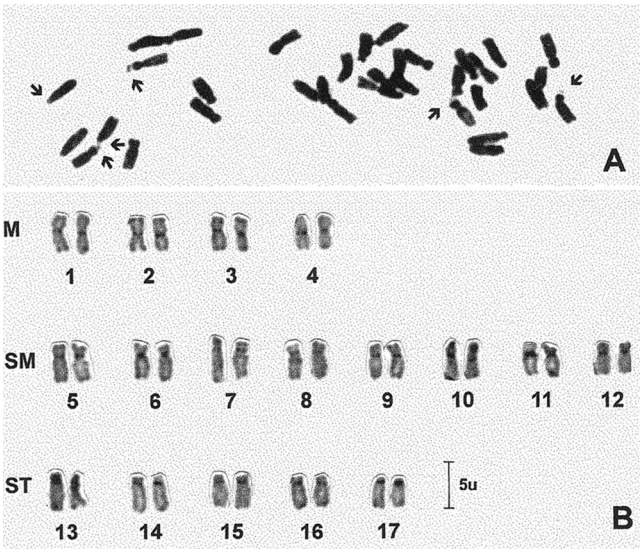
Feulgen, C-banding, 4′-6-diamidino-2-phenylindole (DAPI) or chromomycin A_3 (CMA_3) staining, counterstained or not with distamycin A (DA) or actinomycin D (AD), were carried out as described by Cuéllar et al. (1996*a*).

DAPI, CMA_3, and Acridine Orange (AO) staining after C-banding were performed according to Bella and Gosálvez (1991).

Two probes were in situ hybridized following Fernández-Calvín et al. (1995): a 9-kb *Eco*RI rDNA fragment of wheat containing the 18S, 5.8S, and 26S rRNA coding regions and the spacers (rDNA probe), obtained from the pTA71 clone (Gerlach and Bedbrook 1979), and the 362-bp rDNA fragment of flax containing the 5S-rRNA coding regions plus the spacers (5S-rDNA probe) obtained from the 36pBG13 clone, the latter having kindly been supplied by Dr. Sossey-Alaoui (INRA). Both cloned DNA sequences were labelled by nick translation with biotin-11-dUTP for the rDNA probe and with digoxigenin-11-dUTP for the 5S-rDNA probe, following the manufacturer's standard protocol (Boehringer-Mannheim). Hybridization sites were detected with rhodamine-streptavidin and antidigoxigenin antibody conjugated with fluorescein isothiocyanate. The slides were mounted in Vecta Shield antifade solution (Vector Laboratories).

Preparations were examined at 100× magnification under a Zeiss Axiophot fluorescence microscope, equipped with the appropriate single and double filters for the observation of these fluorescent stains. Light field photographs were taken with Kodak Imagelink HQ film, whilst Fuji Sensia 400 film was used for fluorescence and in situ hybridization pictures.

Fig. 1. Feulgen-stained mitotic metaphase chromosomes (A) and Giemsa C-banded karyotype (B) of *Helianthus argophyllus*. Three pairs of chromosomes show secondary constrictions (arrows). The heterochromatin is present in the centromeric areas of all the chromosomes and in the secondary constrictions of the satellite chromosomes (pairs SM7, SM10, and ST13).



Results

Feulgen staining

On account of the minor differences in size, and following the convention for *H. annuus* (as stated in the following section), we classify the 2*n* = 34 chromosomes of *H. argophyllus* on the basis of the position of their primary constrictions as eight metacentric (pairs M1–M4), sixteen submetacentric (pairs SM5–SM12), and 10 subtelocentric (pairs ST13–S17) chromosomes. The two submetacentric and one subtelocentric chromosome pairs bearing secondary constrictions (satellite chromosomes) will henceforth be denominated as SM7, SM10, and ST13 (Fig. 1).

C-banding

All the chromosomes of *H. argophyllus* differentially stain around the primary constrictions, indicating small bands of pericentromeric constitutive heterochromatin. The three pairs of satellite chromosomes show further heterochromatin associated with their secondary constrictions (Fig. 1B).

Fluorochrome staining

DAPI, DA-DAPI, or AD-DAPI treatments uniformly stain the chromosomes of *H. argophyllus*. The heterochromatin

Fig. 2. The mitotic metaphase chromosomes of *Helianthus argophyllus* after DA-DAPI (A) or DA-CMA_3 staining (B). Only the heterochromatin associated with the secondary constrictions shows positive response to one of these treatments (arrows in B). The C-banded chromosomes of *H. annuus* (C and D) and *H. argophyllus* (E) stained either with DAPI (C), CMA_3 (D), or AO (E) show the same pattern, wherein only the heterochromatin associated with the secondary constrictions appears highly fluorescent (arrows). Simultaneous in situ hybridization with differentially labelled rDNA and 5S-rDNA shows 18S-5.8S-25S rDNA clusters in the SM7, SM10, and ST13 pairs (arrowheads) and 5S-rDNA in the short arm of the SM7 and SM11 pairs (arrows) both in *H. annuus* (F) and *H. argophyllus* (G).

associated with the secondary constrictions of the satellite chromosomes yields a positive response to DA-CMA_3 staining. Pericentromeric heterochromatin is not distinguishable with these stains (Fig. 2A and B) and none of these treatments differentiates non-Giemsa-C-banded heterochromatic regions.

C-banding and fluorochrome staining

The chromosomes of *H. annuus* and *H. argophyllus* show the same pattern after staining C-banded slides either with DAPI, CMA_3, or AO. The heterochromatin associated with the secondary constrictions (pairs SM7, SM10, and ST13 in both cases) appears highly fluorescent, in contrast with the rest of the chromatin (including the pericentromeric heterochromatin) which stains more weakly and uniformly (Fig. 2C, 2D, and 2E). No further heterochromatic regions are revealed by these treatments.

In situ hybridization

The double target in situ hybridization with the differentially labelled rDNA and 5S-rDNA probes reveals three sites of rDNA and two sites of 5S-rDNA at the same place in both species. The 18S-5.8S-25S clusters are located on the three pairs of satellite chromosomes, and are associated with their secondary constrictions (SM7, SM10, and ST13 pairs). The 5S-rDNA genes are located interstitially on the short arm of the SM7 and SM11 chromosomes (Fig. 2F and 2G). Interphase nuclei show hybridization signals consistent with these observations (result not shown).

Discussion

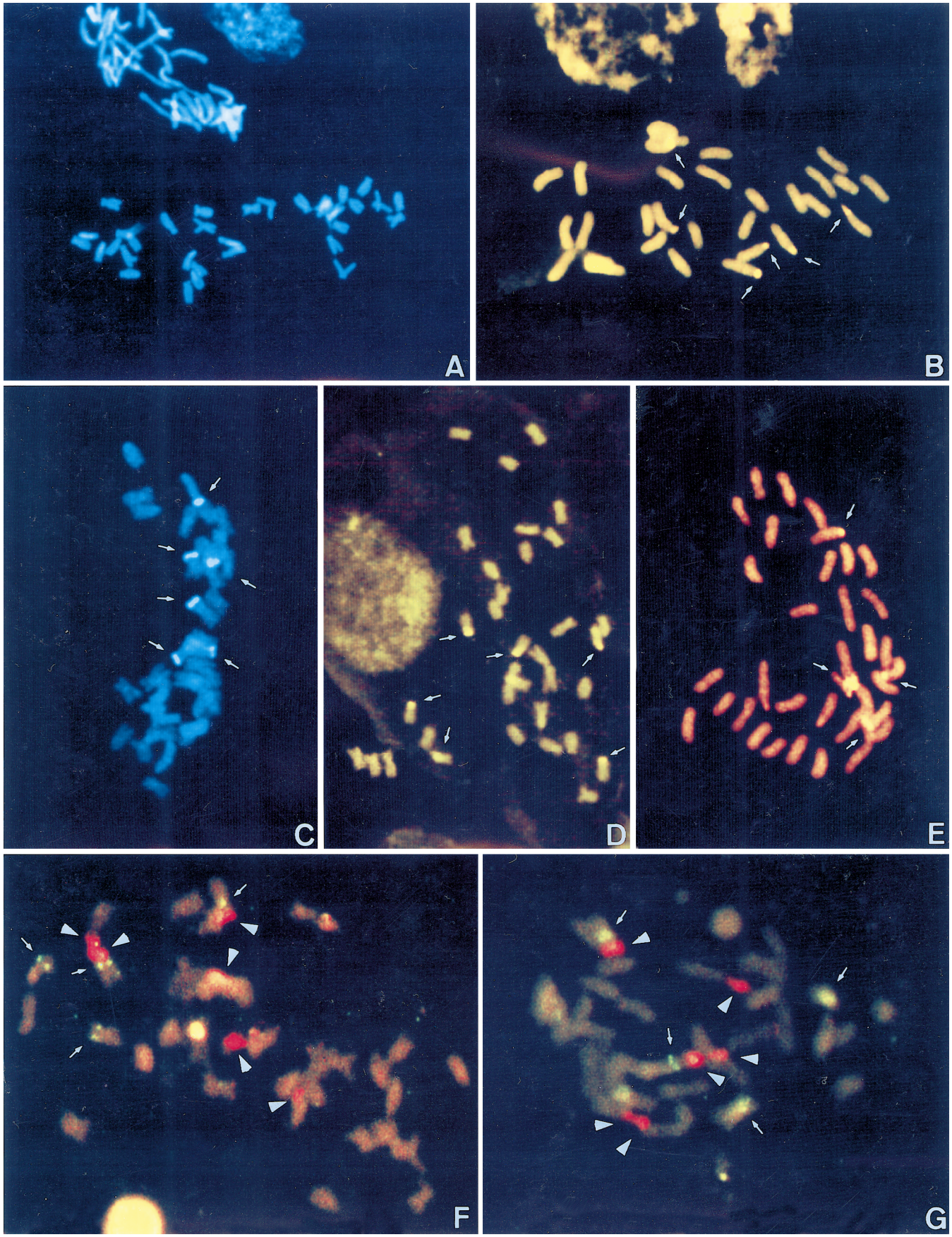
In a previous paper, we characterized the chromosome complement of *H. annuus* by means of Feulgen staining, C-banding, standard fluorochrome staining, silver impregnation, and FISH with a rDNA probe (Cuéllar et al. 1996*a*). This karyotype classification is consistent with that previously described by Al-Allaf and Godward (1979). Our present study shows the results obtained using the same techniques on the chromosomes of *H. argophyllus*, and extends the cytogenetical characterization of these species by using alternative fluorochrome staining methods and a further 5S-rDNA probe.

After analyzing a considerable number of cells and individuals from a total of 10 cultivated and inbred lines and ecotypes from both species, we have found a very close similarity between *H. annuus* and *H. argophyllus* in karyological terms. Both have an equal number of chromosomes, which share an identical ranking in size, display concordant morphological features, identical number and distribution of ribosomal sequence sites. For these reasons, we propose the same karyotype classification for *H. argophyllus* as for *H. annuus*, following the established convention for the latter species. This differs from that of Kulshreshtha and Gupta (1981), who used a distinct root tip pretreatment and stained the chromosomes with a different dye. As these authors state, their karyological descriptions are significantly affected by the high degree of contraction of the chromosomes. However, this does not invalidate the main finding of their work concerning asymmetry among the karyotypes of twelve sunflower species.

The Giemsa C-banding technique did not show differences among the individuals and ecotypes of *H. argophyllus* analyzed. Heterochromatin is present as small pericentromeric bands and also associated with the secondary constrictions. This is the same pattern as found in *H. annuus* (Cuéllar et al. 1996*a*) where differences between individuals or lines were also not observed. A further study involving more varieties, ecotypes, and lines from these species would clarify the extent to which this is their standard pattern of heterochromatin distribution. Although the existence of polymorphisms for the distribution and size of these heterochromatic regions in other natural or cultivated populations cannot be discounted, the absence of variation noticed here is indeed remarkable. No data on the heterochromatin distribution in other species of this genus are available. However, this pattern is also present in other species and genera of the same family (Schweizer and Ehrendorfer 1983; Guerra and Nogueira 1990) which may indicate an ancient origin.

The behaviour of *H. argophyllus* heterochromatin after DAPI, DA-DAPI, AD-DAPI, CMA_3, or DA-CMA_3 reveals the existence of two types of heterochromatic regions in its chromosomes: one is pericentromeric and undifferentiable from the rest of the chromatin, and the other is associated with the secondary constrictions which, being DA-CMA_3 positive, can be interpreted as being relatively rich in GC-pairs. This pattern was also found in *H. annuus* chromosomes (Cuéllar et al. 1996*a*).

Since neither standard Giemsa C-banding nor fluorochrome staining allows us to discriminate among chromosomes and reveals the same patterns in *H. argophyllus* and *H.annuus*, we performed C-banding followed by fluorochrome staining in both species. This technique can reveal heterochromatin that is cryptic to other techniques or differences between heterochromatic regions that are undetectable with standard treatments (Bella et al. 1986; Bella and Gosálvez 1991; 1995). Both species show the same pattern with this technique after staining with DNA sequence-specific fluorochromes such as DAPI or CMA_3 or with the non-specific AO. The pericentromeric heterochromatin which would otherwise be revealed by Giemsa C-banding is undetectable when any of the fluorochromes are applied after C-banding. However, the heterochromatin associated



with the secondary constrictions, is revealed with all of the assayed fluorochromes following C-banding.

The consistency of the neutral response of the pericentromeric heterochromatic regions to fluorochromes and C-banding with fluorochromes in these two species may be the result of either a balanced AT/GC composition of the involved repeat or the existence of alternating repetitive sequences of opposite base pair composition in these heterochromatic areas, as we have already proposed for *H. annuus* (Cuéllar et al. 1996*a*). Molecular analyses indicate the coexistence of distinct DNA repetitive sequences that occur in both species. In terms of composition, the repetitive sequences analyzed do not have a pronounced base pair bias and they seem to hybridize on the pericentromeric regions of some of the chromosomes of *H. annuus* (Sossey-Alaoui 1995; K. Sossey-Alaoui, personal communication). This may explain our results for these regions. However, the behaviour of the heterochromatin associated with the secondary constrictions is remarkable, since its negative response to DA-DAPI and positive response to DA-CMA_3 treatments imply that it is GC-rich, but C-banding followed by staining with the same fluorochromes or with the nonspecific AO reveals this heterochromatin in all cases. Heterochromatic regions associated with secondary constrictions, where rDNA sequences are located, are usually GC-rich (Schweizer 1980) so we assume that the DA-CMA_3 also reveals this feature of *H. annuus* and *H. argophyllus* satellite heterochromatin. The unspecific response of these regions to C-banding with fluorochrome staining treatments may be related to a compositional or structural rearrangement of this heterochromatin induced by the C-banding. This technique substantially alters the chromatin and seems simultaneously to involve differential extraction of DNA and proteins, DNA depurination and differential levels of denaturation or reannealing of chromatin (Sumner 1990; Bella and Gosálvez 1995). These may affect the standard staining behaviour of the fluorochromes used.

Our results confirm the existence of two types of heterochromatin in these species and demonstrate that the equilocal behaviour of *H. annuus* heterochromatin (Cuéllar et al. 1996*a*) is also characteristic of *H. argophyllus*. This equilocality is interspecific since not only do equilocal heterochromatic regions show the same response to DNA base-pair-specific fluorochromes (either upon direct application or following C-banding) within the complement of each species, but also between the two species. The other conclusion from our characterization of the heterochromatin of *H. annuus* and *H. argophyllus* is that neither its distribution nor its response to distinct fluorochrome treatments allows better discrimination of the chromosomes within or between the species.

The in situ hybridization with the appropriate double probe allows us simultaneously to locate the 18S-5.8S-25S (rDNA) and the 5S-rDNA in both species, which show the same pattern of distribution of these clusters. The rDNA genes are placed in the secondary constrictions of the three pairs of satellite chromosomes. Both the number and the distribution of the nucleolar organizer regions (NORs) found in these sunflowers are common in other plants (Lapitan 1992; Risueño and Testillano 1994). Silver staining revealed remnants of the activity of all these NORs in mitotic cells of the root tips of *H. annuus* (Cuéllar et al. 1996*a*) as also observed in *H. argophyllus* (not shown). An RFLP analysis of the rDNA in these species detected intraindividual heterogeneity which allows *H. annuus* and *H. argophyllus* to be differentiated. The co-existence of different rDNA units within single individuals has been proposed to explain this variability, which may be involved in the regulation of rDNA (Cuéllar et al. 1996*b*). As might be expected, this heterogeneity is not reflected at the chromosomal level.

The 5S-rDNA probe reveals that the 5S-rRNA genes are interstitially located on the short arm of the satellite SM7 and the non-satellite SM11 chromosome pairs. The number of chromosomes bearing 5S-rDNA sequences is relatively low: up to six pairs of chromosomes carrying these genes are common in plants (Shaw et al. 1993). 5S-rDNA genes may be present in the same chromosomes as the other rDNA sequences as occurs in *Aegilops umbellata* (Castilho and Heslop-Harrison 1995) and Pineaceae species (Lubaretz et al. 1996). Alternatively, they may be physically separated on other chromosomes, as in the soybean and the common bean (Shi et al. 1996), or in an intermediate condition in which some chromosomes bear both types of rDNA and others carry only one type, such as in many species of cereals (see Cuadrado et al. 1995) or *H. annuus* and *H. argophyllus*. The physical independence of the repeated arrays of 5S-rRNA genes in eukaryotes seems to be evolutionarily favoured and may indicate a further biological role for 5S-rRNA that extends beyond a possible structural role in cross-linking the small and large ribosomal units (Appels and Honeycutt 1986; Kellog and Appels 1995).

A recent paper by Schrader et al. (1997) re-analyzes the karyotype of *H. annuus* with similar techniques and has served to confirm most of our previous results on this species (Cuéllar et al. 1996*a*). However, their karyotype classification differs in that they found only four acrocentric chromosome pairs and 13 meta- to submetacentric pairs. They subdivided these groups by computer-aided image processing on the basis of the banding patterns produced by an HKG technique, but were unable to demonstrate further differentiation according to size and morphology. The results of the FISH they performed with a 18S/25S-rDNA probe are consistent with our previous (Cuéllar et al. 1996*a*) and current results, except for their reported extra fourth, smaller and apparently inactive, ribosomal locus. This was seen in 70% of an unstated number of metaphase plates and individuals of only one inbreeding line (HA89) of *H. annuus*. Their FISH with a species-specific 5S-rDNA probe in this species gave the same results as we present here for *H. annuus* and *H. argophyllus*.

The number and distribution of the 5S-rDNA loci do not permit the two species of sunflowers studied to be distinguished. However, the in situ hybridization with the double probe we have used serves to label the chromosome pair SM7, making it differentiable from the SM10 pair, which belongs to the same group of submetacentric chromosomes and only carries the other rDNA clusters. The in situ hybridization with a 5S-rDNA probe also provides a marker to identify a further chromosome pair, the SM11, which only has the 5S genes.

Our results show the very close resemblance of *H. annuus* and *H. argophyllus* at the chromosomal level. The lines, genotypes, and ecotypes considered from both species share all

the chromosomal characteristics analyzed. Therefore, although our results are informative from the evolutionary point of view, they do not offer markers suitable for current breeding programs involving these two species of sunflower and their hybrids. This highlights the necessity to search for suitable non-chromosomal markers. However, we have found some reference points along the chromosomes that are valuable both for chromosome identification and for physical mapping of other DNA sequences. Further analyses of other natural or cultivated populations of these and other species of this genus would clarify the extent to which the patterns of chromosomal differentiation outlined here are shared by other sunflowers.

Acknowledgements

We are indebted to Drs. E. Benavente, J. Gosálvez, and P.L. Mason, and to E. Sánchez-Morán for their valuable assistance. This work was partially supported by the Spanish-French program of "Integrated Actions," by the Spanish CICYT grant AGF96–0443, and the help of the UAM-Cytogeneticists group.

References

Al-Allaf, S., and Godward, M.B.E. 1979. Karyotype analysis of four varieties of *Helianthus annuus* L. Cytologia, **44**: 319–323.

Appels, R., and Honeycutt, R.L. 1986. rDNA: Evolution over a billion years. *In* DNA systematics II. Plant DNA. *Edited by* S.K. Dutta. CRC Press, Boca Raton Flor. pp. 81–125.

Belhassen, E., Augé, G., Ji, J., Billot, C., Fernández-Martínez, J., Ruso, J., and Varès, D. 1994. Dynamic management of genetic resources: First generation analysis of sunflower artificial populations. Genet. Sel. Evol. **26**: 241–253.

Belhassen, E., This, D., and Monneveux, P. 1995. L'adaptation génétique face aux contraintes de sécheresse. Cahiers Agric. **4**: 251–261.

Bella, J.L., and Gosálvez, J. 1991. C-banding with specific fluorescent DNA-ligands: A new approach to constitutive heterochromatin heterogeneity. Biotech. Histochem. **1**: 44–52.

Bella, J.L., and Gosálvez, J. 1995. C-banding plus fluorochrome staining shows differences in C-, G-, and R-bands in human and mouse metaphase chromosomes. Genome, **38**: 864–868.

Bella, J.L., García de la Vega, C., López-Fernández, C., and Gosálvez, J. 1986. Changes in acridine orange binding and its use in the characterization of heterochromatic regions. Heredity, **57**: 79–83.

Castilho, A., and Heslop-Harrison, J.S. 1995. Physical mapping of 5S and 18S-25S rDNA and repetitive DNA sequences in *Aegilops umbellulata*. Genome, **38**: 91–96.

Chandler, J.M. 1991. Chromosome evolution in sunflower. *In* Chromosome engineering in plants: Genetics, breeding, evolution. Part B. *Edited by* P. Tsuchiya and P.K. Gupta. Elsevier, Amsterdam. pp. 229–249.

Cuadrado, A., Jouve, N., and Heslop-Harrison, J.S. 1995. Physical mapping of the 5S rRNA multigene family in 6x triticale and rye: Identification of a new rye locus. Genome, **38**: 623–626.

Cuéllar, T., Belhassen, E., Fernández-Calvín, B., Orellana, J., and Bella, J.L. 1996*a*. Chromosomal differentiation in *Helianthus annuus* var. *macrocarpus*: Heterochromatin characterization and rDNA location. Heredity, **76**: 586–591.

Cuéllar, T., Bella, J.L., and Belhassen, E. 1996*b*. Intraindividual heterogeneity of rDNA allows the distinction between two closely related species in the genus *Helianthus*. Theor. Appl. Genet. **93**: 794–800.

Fernández-Calvín, B., Benavente, E., and Orellana, J. 1995. Meiotic pairing in wheat-rye derivatives detected by genomic "in situ" hybridization and C-banding. A comparative analysis. Chromosoma, **103**: 554–558.

Gerlach, W.L., and Bedbrook, J.R. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. **7**: 1869–1885.

Guerra, M.D.S., and Nogueira, M.T.M. 1990. The cytotaxonomy of *Emilia* spp. (*Asteraceae*: *Senecioneae*) occurring in Brazil. Plant Syst. Evol. **170**: 229–236.

Heiser, C.B. 1948. Taxonomic and cytological notes on the annual species of *Helianthus*. Bull. Torrey Bot. Club, **75**: 512–515.

Kellog, E.A., and Appels, R. 1995. Intraspecific and interspecific variation in 5S RNA genes are decoupled in diploid wheat relatives. Genetics, **140**: 325–343.

Kulshreshtha, V.B., and Gupta, P.K. 1981. Cytogenetic studies in the genus *Helianthus* L. II. Karyological studies in twelve species. Cytologia, **46**: 279–289.

Lapitan, N.L.V. 1992. Organization and evolution of higher plant nuclear genomes. Genome, **35**: 171–181.

Lubaretz, O., Fuchs, J., Ahne, R., Meister, A., and Schubert, I. 1996. Karyotyping of three Pinaceae species via fluorescent "in situ" hybridization and computer-aided chromosome analysis. Theor. Appl. Genet. **92**: 411–416.

Risueño, M.C., and Testillano, P.S. 1994. Cytochemistry and inmunocytochemistry of nucleolar chromatin in plants. Micron, **25**: 331–360.

Schilling, E.E., and Heiser, C.B.Jr. 1981. Infrageneric classification of *Helianthus* compositae. Taxon, **30**: 393–403.

Schrader, O., Ahne, R., Fuchs, J., and Schubert, I. 1997. Karyotype analysis of *Helianthus annuus* using Giemsa banding and fluorescence in situ hybridization. Chromosome Res. **5**: 451–456.

Schweizer, D. 1980. Fluorescent chromosome bands in plants: applications, mechanisms and implications for chromosome structure. *In* The plant genome. *Edited by* D.R. Davies and R.A. Hopwood. John Innes Charity, Norwich, U.K. Proceedings of the 4th John Innes Symposium, pp. 61–72.

Schweizer, D., and Ehrendorfer, F. 1983. Evolution of C-band patterns in Astereceae-Anthimideae. Biol. Zentralbl. **102**: 637–655.

Shaw, P., Rawlins, D., and Highett, M. 1993. Nuclear and nucleolar structure in plants. *In* The chromosome. *Edited by* J.S. Heslop-Harrison and R.B. Flavell. John Innes Centre, Norwich, U.K. pp. 161–171.

Shi, L., Zhu, T., and Keim, P. 1996. Ribosomal RNA genes in soybean and common bean: Chromosomal organization, expression, and evolution. Theor. Appl. Genet. **93**: 136–141.

Sossey-Alaoui, K. 1995. Applications des marqueurs RAPD au genre *Helianthus* pour une classification moléculaire et pour révéler des origines allopolyploïdes dans le genre. Thèse de Doctorat. Université Montpellier II (France).

Sumner, A.T. 1990. Chromosome banding. Unwin Hyman Ltd. London, U.K.