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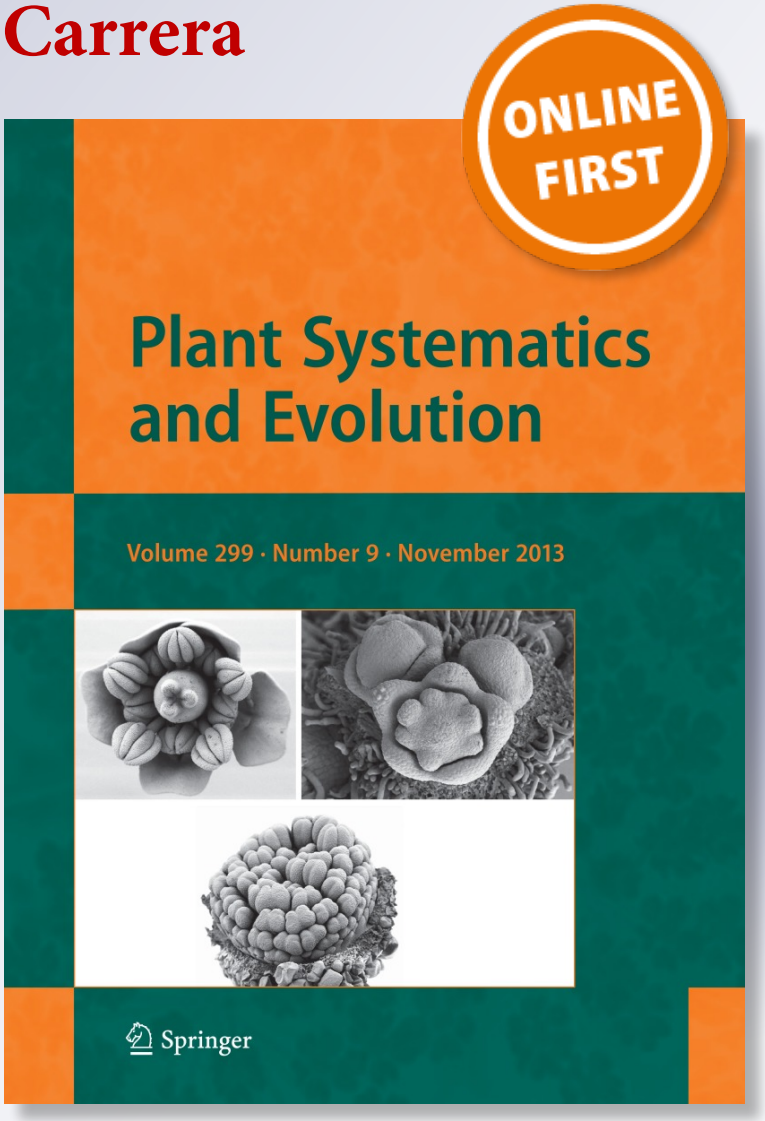

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ORIGINAL ARTICLE

Genomic relationships between hexaploid *Helianthus resinosus* and diploid *Helianthus annuus* (Asteraceae)

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Abstract Genus *Helianthus* comprises diploid and polyploid species. An autoallopolyploid origin has been proposed for hexaploid species but the genomic relationships remain unclear. Mitotic and meiotic studies in annual *Helianthus annuus* ($2n = 2x = 34$) and perennial *Helianthus resinosus* ($2n = 6x = 102$) as well as the F_1 hybrids between both species were carried out. Chromosome counting confirmed the hybrid origin of the latter plants and their tetraploid condition. Bivalents in hybrids ranged from 12 to 28 ($\bar{x} = 20.8$). Univalents, trivalents and quadrivalents were also observed. Meiotic products comprised dyads, triads and normal tetrads and pollen grains were heterogeneous in size. These observations suggest the occurrence of $2n$ pollen in addition to the expected n. Genomic in situ hybridization (GISH) of total *H. annuus* DNA on *H. resinosus* chromosomes rendered weak but uniform signals; similar hybridization pattern was observed using three other annual species. Hybridization with *H. annuus* probe performed on root tip cells of F_1 *H. annuus* × *H. resinosus* hybrids revealed 17 chromosomes with a strong hybridization signal. GISH in hybrid meiocytes distinguished chromosomes from parental species and revealed autosyndetic pairing of *H. resinosus* chromosomes, allosyndetic pairing in bivalents, trivalents and quadrivalents, and the presence of univalents derived from parents, *H. annuus* and *H. resinosus*. Results obtained from classical and molecular cytogenetics do not support *H. annuus* as a direct ancestor of *H. resinosus*. The occurrence of allosyndetic pairing and the relatively high fertility of the F_1 hybrids point to the possibility that useful genes could be transferred from *H. resinosus* to cultivate sunflower, although the effective rate of recombination has not been evaluated. GISH method proved effective to recognize parental chromosomes in *H. annuus* × *H. resinosus* progeny.

J. Miranda Zanetti (✉) · M. Poverene · A. Carrera
Laboratorio de Biotecnología Vegetal CERZOS-CONICET/UNS, CCT Bahía Blanca Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina
e-mail: jmirandazanetti@criba.edu.ar

E. Greizerstein · L. Poggio
Departamento de Ecología, Genética y Evolución Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria, UBA Intendente Güiraldes 2160, C1428EGA Buenos Aires, Argentina

E. Greizerstein
Facultad de Ciencias Agrarias, Universidad Nacional de Lomas de Zamora, Ruta 4, Km 2 Llavallol, 1846 Buenos Aires, Argentina

E. Camadro · M. Echeverría
Estación Experimental Agropecuaria (EEA) Balcarce Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina

E. Camadro · M. Echeverría
Facultad de Ciencias Agrarias (FCA), Universidad Nacional de Mar del Plata (UNMdP), C.C. 256, 7620 Balcarce, Buenos Aires, Argentina

E. Camadro
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Balcarce, Buenos Aires, Argentina

M. Poverene · A. Carrera
Departamento de Agronomía, Universidad Nacional del Sur, San Andrés 800, 8000 Bahía Blanca, Argentina

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Abbreviations

CTAB	Cetyltrimethylammonium bromide
ETS	External Transcribed Spacer
FISH	Fluorescence in situ hybridization
GISH	Genomic in situ hybridization
ITS	Internal Transcribed Spacer

Introduction

Genus *Helianthus* consists of 51 species native to North America, distributed in five sections and six series (Schilling and Heiser 1981; Jan and Seiler 2007). It comprises diploid, tetraploid and hexaploid species with a basic chromosome number of $n = 17$. *Helianthus annuus* is a diploid annual species belonging to sect. *Helianthus*, from which the cultivated sunflower was derived, and *Helianthus resinosus* is a hexaploid perennial tuberous species, placed in sect. *Atrorubens*.

The identity of the direct ancestor of modern polyploid species in *Helianthus* has long been disputed and remains unresolved. Meiotic observations in hybrids obtained from hexaploid *Helianthus tuberosus* (a species closely related to *H. resinosus*) and *H. annuus* suggested that the polyploid species would possess two chromosome stocks: the A genome, which comes from the perennial *Atrorubens* section, and the B_t genome, that is related to the *H. annuus* Ba genome, being its genomic constitution $A_1A_1A_2A_2B_tB_t$ (Kostoff 1939). Espinasse et al. (1995) examined the patterns of chromosome pairing and proposed a similar genomic formula, in which *H. tuberosus* shares the B genome with cultivated sunflower, but the other two genomes seem to be genuine homologs rather than segmental homologs. Homology between the chromosomes from *H. resinosus* and those from *H. annuus* has been suggested based on the high number of bivalents and pollen viability observed in F_1 hybrids (Atlagic 1996). On the other hand, Heiser et al. (1969) analyzed the variability in the root type—either rhizomes or tubers—and postulated that all the *H. tuberosus* genomes proceed from perennial species; they also suggested that the diploid perennials *Helianthus giganteus* and *Helianthus mollis* contributed two of the three possible genomes to hexaploid *H. resinosus*. In accordance with this observation, some hybrids between perennial diploid and hexaploid species have shown more regular pairing than those from annual diploid x hexaploid species (Chandler 1991).

Different molecular characters have been used to investigate *Helianthus* phylogeny. Restriction patterns of chloroplast DNA (Schilling 1997) suggested the existence of four distinct lineages: two of those contained a single annual species (*Helianthus agrestis* and *Helianthus porteri*, respectively); the remaining annual species collectively formed a third lineage (sect. *Helianthus*) and the fourth lineage included all perennial species. Based on RAPD markers, Sossey-Alaoui et al. (1998) postulated the hypothesis that four types of basic genomes exist and they are differently combined in *Helianthus.*

Distinction between perennial and annual species has been observed using ITS ribosomal region (Schilling et al. 1998), dehydrin-encoding sequences (Giordani et al. 2003) and retrotransposon-based markers (Vukich et al. 2009). A more comprehensive study based on ribosomal ETS was able to detect most of the morphologically recognized perennial groups and pointed to perennial species of sect. *Atrorubens* as putative parents of polyploid *H. resinosus* (Timme et al. 2007).

In situ genomic hybridization (GISH) is a powerful tool that has been successfully used to unravel the genomic origins of a number of polyploid taxa, including grasses such as *Milium montianum* (Bennett et al. 1992) and *Festuca arundinacea* (Humphreys et al. 1995). The aim of this study was to examine genome affinity and chromosome pairing between some annual species, particularly diploid *H. annuus* and hexaploid *H. resinosus* by means of cytogenetic studies performed in mitotic and meiotic cells. GISH allowed description of meiotic configurations in the hybrids and hypotheses about the origin of *H. resinosus* are discussed on that base.

Materials and methods

Plant material

The material studied comprised diploid *H. annuus* inbred line HA89 (CMS RES 1) (Echeverría et al. 2003) and hexaploid *H. resinosus* accession PI435864 (USDA), as well as three F_1 hybrid plants obtained by conventional crossing techniques at INTA Balcarce Experimental Station of Argentina. Samples of wild *H. annuus* collected in Argentina (Garayalde et al. 2011), and three additional annual close relatives, *Helianthus argophyllus*, *Helianthus petiolaris* and *Helianthus anomalus*, were included in GISH experiments. These three taxa are cross-compatible with *H. annuus* in both crossing directions (Rogers et al. 1982) and the latter is a homoploid hybrid species derived from *H. annuus* and *H. petiolaris* (Rieseberg 1991).

Meiotic studies

Immature heads were fixed in Farmer solution (3:1, v:v, ethanol:acetic acid) for 24 h and stored in 70 % ethanol at 4 °C until use. Anthers were dissected and squashed in 2 %

acetic haematoxylin. Chromosome number and meiotic configurations were determined at diakinesis and meiotic products were described. Pollen diameter was registered using the Image-Pro Plus 5.1 program, which allows to count cells and to establish comparative size measures. All preparations were scanned with 40× optical lens and images were captured at the 20× zoom option of the software. Four hundred and three cells were measured and sorted according to a relative size unit (RSU) generated by the software.

In situ hybridization

Genomic DNA was isolated from young leaves of the annuals *H. annuus, H. anomalus, H. argophyllus* and *H. petiolaris* according to the CTAB method (CIMMYT 2005). DNA was labeled either by nick translation with biotin (BioNick Labeling System Invitrogen) or by random priming with digoxigenin (DIG-High Prime, Roche). The first protocol generates small (50–500 bp) biotin-labeled DNA probes by nick translation. In the case of DIG-protocol, DNA was fragmented prior to label by rapidly passing through a 17-gage needle. Hybridization parameters were set to occur with >85 % sequence homology. For hybridization, root tips of *H. resinosus* and *H. annuus* × *H. resinosus* F_1 plants were treated with 0.05 % colchicine, fixed in Farmer solution and stored at −20 °C until use. Root tips were treated with cellulase 2 % (w/v) plus pectinase 20 % (v/v) for 3 h at 37 °C and then squashed.

Sunflower cDNA clone EF235 (GenBank accession No. BU671882), containing sequences with similarity to the large subunit ribosomal RNA gene (Fernandez et al. 2003) was used as probe for detecting rDNA locations on mitotic chromosomes.

For meiotic analyses, anthers from F_1 plants were fixed in Farmer solution, and squashed in 45 % acetic acid. The same *H. annuus* probe as for mitotic studies was utilized. Hybridization step was carried out according to Poggio et al. (1999). Differentially labeled chromosomes were classified as either R if they belonged to *H. resinosus* complement, or A if they corresponded to the *H. annuus* complement. Chromosome associations observed in the hybrids were registered as follows: R–R autosyndetic pairing if only *H. resinosus* chromosomes were involved and A–R allosyndetic pairing between *H. annuus* and *H. resinosus* chromosomes.

To detect digoxigenin and biotin-labeled probes, slides were treated with 2.5 % bovine serum albumin (BSA) in detection buffer and subsequently treated with anti-dig-FITC (green) or streptavidin-Cy3 conjugate (red). Slides were counterstained with 1 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) and then mounted in antifade solution (Vector Labs). Images were captured with a Leica DFC 350 FX camera and analyzed with the Adobe Photoshop CS3 program.

Results

Meiotic configurations

Meiotic behavior in *H. resinosus* was regular and 99 % of the final products consisted of typical tetrads. In *H. annuus* × *H. resinosus* F_1 hybrids, multivalents and univalents were observed along with bivalents at diakinesis (Fig. 1a). Chromosome counting confirmed the hybrid origin of the analyzed plants and their tetraploid condition ($2n = 4x = 68$). Although the large number of small-size chromosomes hindered the precise determination of the frequency of each meiotic configuration, meiotic figures could be accurately recorded in 12 cells out of the 57 analyzed. The number of univalents ranged from 2 to 9 (mean 5.1) whereas the total number of bivalents varied from 12 to 28 (mean 20.8). The remaining chromosomes were involved in multivalent configurations: the number of trivalents varied from 0 to 4 (mean 2.3) and quadrivalents from 0 to 9 (mean 3.7). Both, chain and ring types of multivalent were observed. These configurations were similarly visualized at metaphase I, with bivalents and multivalents presenting mainly equatorial arrangement and some unpaired chromosomes placed out of the metaphase plate (Fig. 1b).

Meiotic products

Meiocytes containing two (dyads) and three (triads) cells were observed along with the expected four microspores (tetrads) at the tetrad stage (Fig. 1c, d). Dyads and tetrads consisted of equal-size cells whereas triads comprised two small equal-size cells and a larger third cell. Frequencies for each type of meiocytes were computed for both the parental *H. resinosus* and the F_1 plants (Table 1). Single cells with fourfold size compared to normal cells were also observed and interpreted as monads. Pollen grain size was heterogeneous (Fig. 1e) and measures of diameters revealed the existence of two sub-populations with peaks at 19 and 24 rsu (Fig. 2). Size distribution deviated significantly from normality (Shapiro–Wilks test, $p = 0.002$).

Molecular cytogenetics

GISH experiments using labeled *H. annuus* DNA on mitotic chromosomes of hexaploid *H. resinosus* produced a weak scattered signal over the 102 chromosomes (Fig. 3a). Similar results were obtained using labeled DNA from

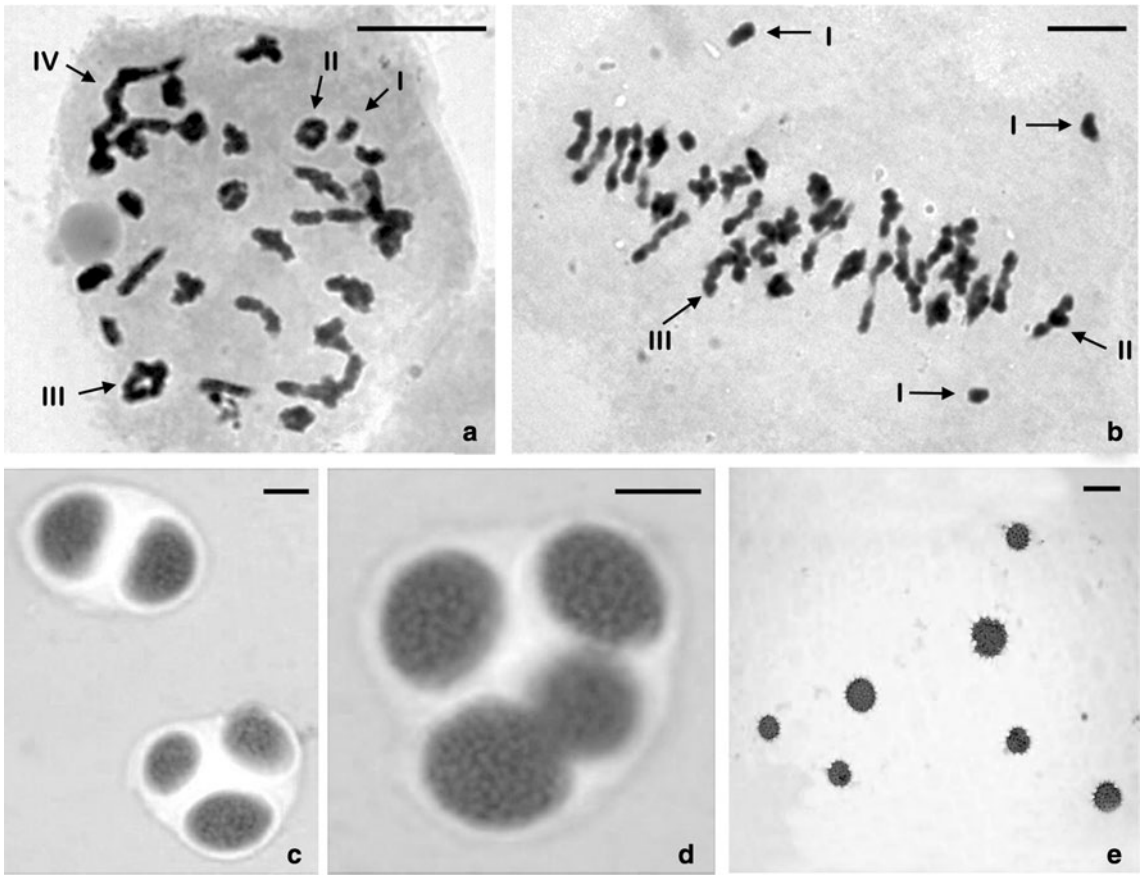

Fig. 1 Meiotic phases and microspores of *Helianthus annuus* × *H. resinosus* F_1 hybrids: **a** diakinesis, **b** metaphase I, **c** dyad and triad, **d** tetrad, **c** and **d** belong to the same anther, **e** pollen grains. *I* univalent, *II* bivalent, *III* trivalent and *IV* quadrivalent. *Bar* corresponds to 5 μ

Table 1 Number of total cells, dyads, triads and tetrads in *Helianthus resinosus* and in *H. annuus* × *Helianthus resinosus* F_1 plants

Genotype	Meiocytes	Dyads	Triads	Tetrads
H. resinosus				
Plant 1	577	0	6	571
Plant 2	1,043	0	2	1,041
Total	1,620	0	8	1,612
H. annuus × *H. resinosus*				
Plant 1	257	25	77	155
Plant 3	649	92	167	390
Total	906	117	244	545

either cultivated or wild *H. annuus,* and also after hybridization with DNA from the close relatives *H. petiolaris*, *H. anomalus* and *H. argophyllus*. Assays performed on root tip cells of *H. annuus* × *H. resinosus* hybrids using *H. annuus* DNA as probe revealed 17 chromosomes with a strong hybridization signal (Fig. 3b). FISH studies on mitotic chromosomes using 26S rDNA probe revealed six signals in *H. annuus,* ten signals in *H. resinosus*, and eight in the hybrids (Fig. 3c).

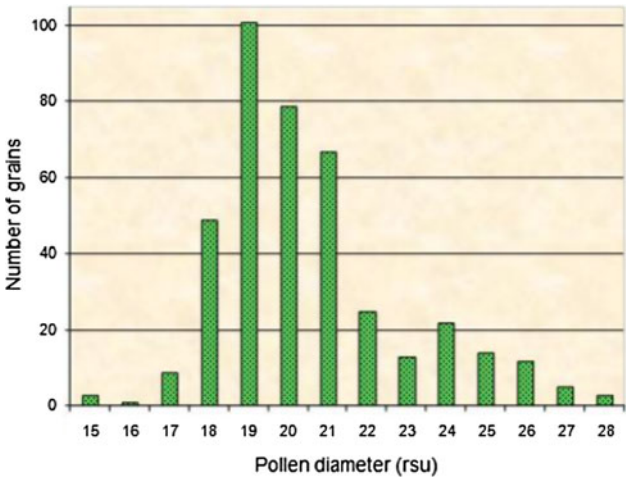

Fig. 2 Pollen diameter distribution of *Helianthus annuus* × *H. resinosus* hybrids. *rsu* relative size unit generated by the Image-Pro Plus 5.1 program

Hybridization studies carried out on meiotic cells of the F_1 hybrids using *H. annuus* DNA as probe confirmed the results of the mitotic studies, with the presence of 17 labels, and allowed the description of chromosome associations at

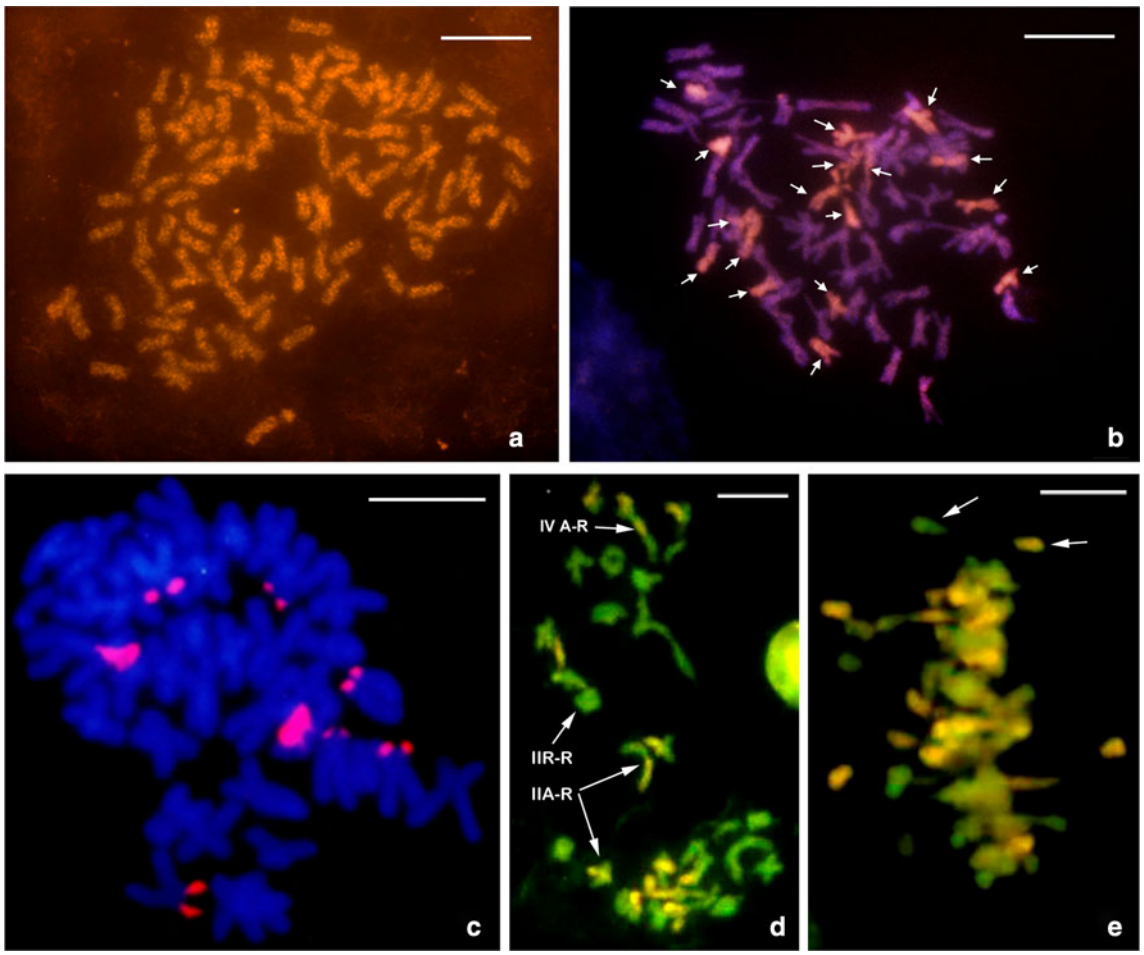

Fig. 3 **a** *Helianthus resinosus* (6*x*) mitotic chromosomes hybridized with *H. annuus* DNA probe and detected with Cy3 (*red*). **b–e** *H. annuus* × *Helianthus resinosus* hybrids: **b** Mitotic chromosomes hybridized with *H. annuus* probe (*red*) with DAPI counterstain (*blue*). *Arrowheads* indicate 17 labeled *H. annuus* chromosomes. **c** Mitotic chromosomes after FISH with EF235 sequence corresponding to a 26S rDNA probe. Hybridization signals were detected with Cy3 (*red*); **d–e** diacinesis and metaphase I showing *H. annuus* (*yellow*) and *Helianthus resinosus* (*green*) chromosomes. *II R–R* autosyndetic *Helianthus resinosus* bivalent; *II A–R*, *IV A–R* allosyndetic bivalents and quadrivalents; *arrows* in **e** show univalents from each genome. *Bars* correspond to 5μ

diakinesis and metaphase I (Fig. 3d–e). Bivalents mainly revealed autosyndetic R–R pairing but allosyndetic A–R bivalents were also found. Autosyndetic A–A bivalents were not observed, and univalents belonged either to *H. annuus* A complement or to *H. resinosus* R complement. Furthermore, allosyndetic A–R trivalents and quadrivalents were observed. In summary, meiotic GISH analysis in F_1 hybrid plants revealed autosyndetic pairing of *H. resinosus* chromosomes, allosyndetic pairing in bivalents and multivalents, and univalents derived from both *H. annuus* and *H. resinosus*.

Discussion

Characteristics of meiosis

F_1 plants were tetraploid composed of three *H. resinosus* genomes ($n = 3x = 51$) and one *H. annuus* genome ($n = x = 17$). Chromosome pairing was complex, comprising bivalent and multivalent configurations. Bivalent number per meiocyte ranged from 12 to 28, with $\bar{x} = 20.8$, a lower value in comparison with the accession reported by Atlagic (1996) ($\bar{x} = 27$). Considering strictly autosyndetic pairing, chromosomes from *H. resinosus* could form up to 17 bivalents; a maximum of 28 bivalents—as observed—and the presence of quadrivalents, are evidence of both autosyndetic pairing of *H. resinosus* chromosomes and allosyndetic pairing between *H. resinosus* and *H. annuus* chromosomes. Autosyndetic pairing in the hybrids is also supported by isozyme tetrasomic inheritance observed in *H. resinosus* (Carrera et al. 2004).

Microsporogenesis

The presence of dyads and triads in addition to normal tetrads in meiocytes of the F_1 hybrids reveals the occurrence of

meiotic abnormalities related to nuclear restitution. Restitution mechanisms can include failure of pairing or chromatid separation, alterations in spindle morphology or defects in cytokinesis (Ramanna and Jacobsen 2003). Sala and Echarte (1996) observed fused spindles in *Helianthus* interspecific polyploid hybrids. When anaphase II spindles are parallel oriented, dyads of two 2*n* microspores are formed; alternatively, spindle fusion at one extreme leads to the formation of a triad of one 2*n* and two *n* microspores (Mok and Peloquin 1972; Camadro et al. 2008). In *Helianthus,* these mechanisms seem to be specific of genotypes since crosses between *H. resinosus* and others *Helianthus* annual species produce hybrids exhibiting different meiotic abnormalities (unpublished data).

Size heterogeneity in pollen of interspecific hybrids is a clear indication of disturbed meiosis (Prabakaran and Sujatha 2004). When meiotic products include 2*n* microspores, pollen size displays a bimodal diameter distribution (Veilleux 1985). The observed dyads and triads were interpreted as evidence of the formation of numerically unreduced gametes that mature in 2*n* pollen grains. The 23–26 RSU section in pollen diameter distribution should contain the population associated with the formation of 2*n* microspores; approximately 15 % pollen grains were included in this segment and in that order, the expected 2*n* ratio according to the meiotic observations (Table 1) also sums up to 15 %. Pollen grains at the rightmost side of the distribution may be assigned to the monad class and they are explained by the omission of both meiotic divisions (Taschetto and Pagliarini 2003). The production of 2*n* gametes might stand as an important mechanism in *Helianthus* evolution in which 13 polyploid species (4*x* and 6*x*) have been identified. Polyploids are frequently found among perennial species in which successive reproductive cycles increase the chances of generating viable and fertile progeny (Otto and Whitton 2000).

The occurrence of allosyndetic pairing and the relatively high fertility of the F_1 plants (Echeverría et al. 2003) are indications that useful genes could be transferred from *H. resinosus* to cultivated sunflower. *H. resinosus* exhibits defense mechanisms for *Sclerotinia sclerotiorum* (Mondolot-Cosson and Andary 1994) and *Alternaria helianthi* (Sujatha and Prabakaran 2006), two important fungal diseases of the crop. In addition, high oleic acid content has been reported for this species (Thompson et al. 1981). However, there are no examples of transferred genes from *H. resinosus* to sunflower beside mitochondrial male-sterility factors (Echeverría et al. 2003).

In situ hybridization experiments

In situ hybridization techniques have been utilized for the characterization of *Helianthus annuus* complement (Cuellar et al. 1996; Ceccarelli et al. 2007; Talia et al. 2010) and to identify wild-introgressed fragments into crop genomic background (Liu et al. 2009). Recently, BAC/BIBAC clones containing specific-linkage group markers were used as FISH probes to align the genetic and cytogenetic *H. annuus* maps (Feng et al. 2013). To our knowledge, this is the first report about GISH techniques applied to *H. resinosus*. When *H. resinosus* mitotic chromosomes were hybridized with *H. annuus* DNA probe, differentially labeled chromosomes were not found and therefore subgenomes could not be identified. Similar patterns of hybridization were observed with probes obtained from the annuals *H. anomalus*, *H. argophyllus* and *H. petiolaris.* These results are in agreement with Cavallini et al. (2010) who observed that only minor changes at repetitive DNA levels have occurred among annual species after their divergence. Taken together, these observations render hardly plausible the hypothesis of an annual species being a parental of this polyploidy and reinforce the hypothesis that *Helianthus* hexaploid genomes originated from perennial species (Heiser et al. 1969; Chandler 1991).

GISH methods predominantly rely on dispersed repetitive sequences for subgenome differentiation. Annual and perennial *Helianthus* species share *gypsy* and *copia* retrotransposon superfamilies indicating that sequence amplification occurred prior to their divergence (Vukich et al. 2009; Cavallini et al. 2010). Moreover, all insertions are within the age estimates for the origin of genus *Helianthus* (Staton et al. 2012). Hybridization of F_1 mitotic cells with *H. annuus* DNA probe showed strong signals in 17 chromosomes, interpreted as GISH capacity to identify *H. annuus* parental complement. Six ribosomal signals found in *H. annuus* were in accordance to Talia et al. (2010). Eight signals in chromosomes of the F_1 plants seemed to correspond to three plus five ribosomal zones inherited from each parent.

Concerning meiosis, the occurrence of some A–R bivalents and multivalents in the F_1 hybrids reflected partial localized homology between parental complements although these pairings were often restricted to chromosome ends. Bivalents have been already observed in hybrids of *H. annuus* with several diploid perennial species (Atlagic et al. 1995; Espinasse et al. 1995). Allosyndesis is in accordance with the genomic structure proposed by Sossey-Alaoui et al. (1998) for genus *Helianthus*; these investigators assigned a CPA formula for sect. *Atrorubens*, and a CH genome constitution for sect. *Helianthus*. According to this model, R–R pairing could be attributable to PA genome interactions, whereas R–A pairing could be due to the common C genome. Interspecific chromosome pairing was interpreted within the context of a relatively recent divergence of the genus (Schilling 1997).

The results of this study are indications that the *H. annuus* complement is not present in the *H. resinosus* genome and agree with the proposed ribosomal ETS phylogeny (Timme et al. 2007), in which hexaploid *H. resinosus* and *H. tuberosus* are placed within a well-supported perennial clade, close to putative diploid parental species. We did not obtain evidence that the formula $A_1A_1A_2A_2B_tB_t$ proposed for hexaploid species (Kostoff 1939; Espinasse et al. 1995) could be applicable to *H. resinosus*. The only scenario that would allow retaining *H. annuus* as a candidate parental species for *H. resinosus* entails a mechanism of subgenomes homogenization at repetitive DNA following hybridization. This mechanism implies intergenomic concerted evolution, and amplification and loss of repetitive DNA sequences such as retrotransposons (Chester et al. 2010). Examples have been found in *Nicotiana,* in which allopolyploids that were formed more than 5 million years ago have lost the genomic parental signature, rendering GISH ineffective to differentiate subgenomes (Lim et al. 2007); this is principally due to the replacement of parental sequences with newly evolved or massively amplified subsets of repetitive DNA. There is no evidence about this mechanism operating in genus *Helianthus* but, if this hypothesis is true, GISH could provide scarce information about polyploidy evolution in this genus. However, based on the observed ability of GISH to reliably distinguish chromosomes from the perennial specie and the cultivated sunflower, the method could be still successfully utilized on the newly available progenies from *H. resinosus* and *H. annuus* crosses (Liu et al. 2013) to facilitate sunflower breeding for *Sclerotinia* diseases.

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