Plant Syst Evol (2012) 298:887–899
DOI 10.1007/s00606-012-0598-4

ORIGINAL ARTICLE

Assessment of intergenomic recombination through GISH analysis of F1, BC1 and BC2 progenies of *Tulipa gesneriana* and *T. fosteriana*

A. Marasek-Ciolakowska · H. He · P. Bijman · M. S. Ramanna · P. Arens · J. M. van Tuyl

Received: 21 September 2011 / Accepted: 22 January 2012 / Published online: 12 February 2012

Abstract Using 23 F1 hybrids, 14 BC1 and 32 BC2 progenies, the genome composition of Darwin hybrid tulips was analysed through genomic in situ hybridisation (GISH) of somatic chromosomes. All plants were diploids ($2n = 2x = 24$) with the exception of one tetraploid BC1 ($2n = 4x = 48$) and one aneuploid BC2 ($2n = 2x + 1 = 25$) hybrid. Morphometric analysis in F1 hybrids revealed a difference in the total length of chromosomes representing genomes of *T. gesneriana* and *T. fosteriana*, where the percentage of each genome equaled 55.18 ± 0.8 and 44.92 ± 0.6% respectively. GISH distinguished chromosomes from both parent genomes although there was a lack of consistent chromosome labelling in some cases. In both *T. gesneriana* and *T. fosteriana* chromosomes some segments of heterochromatin in the telomeric and intercalary regions exhibited a higher intensity of fluorescence. In situ hybridisation with 5S rDNA and 45S rDNA probes to metaphase chromosomes of F1 hybrids showed that these regions are rich in rDNA. A notable feature was that, despite genome differences, there was a considerable amount of intergenomic recombination between the parental chromosomes of the two species as estimated in both BC1 and BC2 offspring. The number of recombinant chromosomes ranged from 3 to 8 in BC1 and from 1 to 7 in BC2 progenies. All recombinant chromosomes possessed mostly a single recombinant segment derived from either a single crossover event or in a few cases double crossover events. This explains the fact that, unlike the situation in most F1 hybrids of other plant species, certain genotypes of Darwin hybrid tulips behave like normal diploid plants producing haploid gametes and give rise to mostly diploid sporophytes.

Keywords Genome differentiation · Interspecific hybrids · In situ hybridisation · Recombinant chromosomes · *Tulipa*

A. Marasek-Ciolakowska
Department of Physiology and Biochemistry,
Research Institute of Horticulture,
Konstytucji 3 Maja Str. 1/3, 96-100 Skierniewice, Poland

A. Marasek-Ciolakowska (✉) · H. He · P. Bijman · M. S. Ramanna · P. Arens · J. M. van Tuyl
Wageningen UR Plant Breeding, Wageningen University and Research Center, P.O. Box 16, 6700 AA Wageningen, The Netherlands
e-mail: agnieszkamarasek@wp.pl

A. Marasek-Ciolakowska · H. He · P. Bijman · M. S. Ramanna · P. Arens · J. M. van Tuyl
Droevendaalse steeg 1, 6708 PB Wageningen, The Netherlands

Introduction

The genus *Tulipa* of the Liliaceae family consists of about 40 (Stork 1984) to more than 100 species (Bryan 2002). The tulip was introduced into Western Europe more than 400 years ago and has been extensively subjected to selection and hybridisation. The most widely cultivated group belongs to *T. gesneriana* L., which is the collective name given to a large number of varieties derived from the crossing of closely related species in the section *Tulipa* (Killingback 1990). The primary cultivars of this group sold in the commercial markets consist of more than 1,100 cultivars with a large variation in flower colour, flower form, flowering time and forcing ability (Van Scheepen 1996). The second commercial group is the Darwin hybrid group, which has been obtained from interspecific crosses between cultivars of *T. gesneriana* and *T. fosteriana* Hoog ex W. Irving of the section *Eichleres* (Van Eijk et al. 1991; Van Tuyl and Van Creij 2007).

Tulips have been subjected to considerable cytogenetic studies with regard to chromosome number (Bamford et al.

1939; Sayama et al. 1982; Van Raamsdonk and De Vries 1995), chromosome morphology (Upcott and La Cour 1936; Sayama et al. 1982; Wafai and Koul 1981a, b, 1983, 1986; Van Raamsdonk and De Vries 1995; Marasek et al. 2006) and chromosome banding (Filion 1974; Blakey and Vosa 1982; Van Raamsdonk and De Vries 1995). The basic chromosome number in the genus *Tulipa* is $x = 12$. The majority of tulip species and cultivars is diploid ($2n = 2x = 24$) but also triploids, tetraploids and even some pentaploids have been found (Hall 1937; Holitscher 1968; Kroon 1975; Zeilinga and Schouten 1968a, b; Kroon and Jongerius 1986; Van Scheepen 1996). Some species have been described to exist at different ploidy levels. For instance, Wafai and Koul (1986) reported that *T. clusiana* DC. exists in $2x$, $3x$, $4x$ and $5x$ cytotypes with chromosome numbers ranging from 24 to 60.

Karyotypes have been analysed for many *Tulipa* species and varieties (Upcott and La Cour 1936; Sayama et al. 1982; Wafai and Koul 1981a, b, 1983, 1986; Van Raamsdonk and De Vries 1995). However, the similarity of the length and morphology and lack of distinct chromosomal landmarks make chromosome identification difficult. Only a few chromosomes, e.g. median chromosomes, are recognisable on the basis of length and centromere position (Marasek et al. 2006). Southern (1967) analysed the relationships between diploid and polyploid species belonging to the subgenus *Eriostemones* from the point of view of chromosome morphology. He observed remarkable similarity of the karyotype morphology among the 16 species studied. Karyology has also been employed in exploring species' interrelationships within the section Clusianae by Wafai and Koul (1981a, b, 1986). The introduction of Giemsa staining (C-banding) revealed heterochromatic regions on chromosomes (Filion 1974; Blakey and Vosa 1981, 1982; Van Raamsdonk and De Vries 1995). The species relationships applying the C-banding technique in subg. *Eriostemones* and subg. *Leiostemones* were analysed by Blakey and Vosa (1981, 1982). In their studies several chromosome types were recognised with respect to chromosome morphology and heterochromatin distribution, and groups of species with common chromosome characteristics could be identified. Filion (1974) revealed a chromosomal polymorphism for two tulip varieties, 'Queen of Night' ($2n = 24$) and 'Spring Song' ($2n = 24$), and *T. turkestanica* Regel ($2n = 48$) using Giemsa staining. Fluorescence in situ hybridisation (FISH) has greatly advanced chromosome analysis in the tulip providing markers for chromosome identification. In situ hybridisation with 5S rDNA and 45S rDNA probes provided molecular cytogenetic markers for chromosome identification both in cultivars (Mizuochi et al. 2007) and in hybrids (Marasek and Okazaki 2008).

From the breeding point of view, Darwin hybrid tulips, which resulted from crosses between *T. gesneriana* (G) and *T. fosteriana* (F), are becoming increasingly interesting since they combine the desirable horticultural traits from two sections viz., *Tulipa* and *Eichleres*, such as good forcing quality, resistance to *Fusarium oxysporum* (bulb-rot) and resistance or partial resistance to Tulip Breaking Virus (TBV). Although Darwin hybrid tulips have been obtained from interspecific crosses at the diploid level, most of the commercial cultivars are triploids (Van Scheepen 1996); however, most breeding lines are diploids (Marasek-Ciolakowska et al. 2009, 2011). F1 Darwin hybrid tulips GF genotypes are usually sterile or show low fertility. However, through large-scale screening it is possible to select genotypes of GF hybrids with reasonably high frequencies of fertile pollen that could be used for backcrossing. An important feature of diploid Darwin hybrid tulips is that they can produce not only functional n gametes, but also $2n$ gametes. This provides the opportunity to generate diploid and polyploid progenies from backcrossing FG hybrids to *T. gesneriana* parents, where the latter are excellent hybrids due to their large flower, sturdy stem and bigger plant size as compared to diploids.

The genome composition of tulip hybrids can be investigated in detail using genomic in situ hybridisation (GISH). This technique utilises genomic DNA of both parental genotypes as probes and excessive fragmented DNA of unrelated species as blocking DNA. In tulip GISH enables the discrimination of parental genomes in hybrids and polyploid forms (Marasek et al. 2006). This technique also detects chromosome recombination between chromosomes from different genomes and can be used to visualise the level of introgression in backcross progenies (Marasek-Ciolakowska et al. 2009, 2011). Marasek and Okazaki (2008) have used GISH with genomic DNA of *T. gesneriana* and *T. fosteriana* and subsequent FISH with 45S rDNA and 5S rDNA probes for chromosome identification in Darwin hybrid 'Purissima' and its BC1 progenies. They recorded differences in the distribution of rDNA signals between *T. gesneriana* and *T. fosteriana* chromosomes in 'Purissima', which allowed some chromosomes bearing rDNA sites to be distinguished in 'Purissima' BC1 hybrids.

In the present study, we have cytologically investigated the F1, BC1 and BC2 progenies of Darwin hybrids by backcrossing to *T. gesneriana* and assessed the extent and nature of intergenomic recombination between the parental species through GISH and FISH.

Materials and methods

Plant material

The plant material used for chromosome analysis in F1, BC1 and BC2 progenies is shown in Table 1. Diploid

($2n = 2x = 24$) *T. gesneriana* cultivars and different breeding lines of *T. fosteriana* were used for producing F1 hybrids. A BC1 population consisting of 14 plants resulted from interspecific crosses between *T. gesneriana* cultivars and 'Purissima', which is a Darwin hybrid tulip representing genomes GF (Marasek and Okazaki 2008). Five BC1 progenies were backcrossed as male parents with *T. gesneriana* cultivars. In total 23 F1, 14 BC1 and 32 BC2 plants were analysed by GISH. Bulbs of all hybrids were subjected to cold treatment at 4°C for 4 weeks before planting in the greenhouse. The mitotic chromosomes of F1, BC1 and BC2 plants were analysed in this experiment.

Chromosome preparation

Root tips were pre-treated with 0.1% colchicine for 4 h and then fixed in 3:1 ethanol:glacial acetic acid solution for at least 12 h and stored at −20°C until use. The roots were subjected to enzymatic digestion in a mixture comprising 1% (w/v) pectolyase Y23, 1% (w/v) cellulase RS at 37°C for about 2 h. Meristems were squashed in a drop of 45% acetic acid. After freezing in liquid nitrogen, cover slips were removed by using a razor blade, and the preparations were dehydrated in absolute ethanol and air dried. The best slides were selected under a phase contrast microscope (Leica Dialux 20 EB) and stored at −20°C until use.

Table 1 Darwin hybrid tulips selected for GISH analysis

Generation	Cross[a]	Genotype code	Parents		No. of plant analysed
			Female	Male	
F1	GF	20161-5	Bellona	103 Juan × Cantata	1
	GF	20179-1	Bellona	121 Cantata × Juan	1
	GF	20170-4	Bellona	112 Juan × Cantata	2
	GF	20176-1	Bellona	118 Cantata × Juan	1
	GF	20231-1	Gen. de Wet	102 Juan × Cantata	1
	GF	20232-2	Gen. de Wet	104 Juan × Cantata	1
	GF	20249-1	Pax	123 Cantata × Juan	1
	GF	20233-1	Gen. de Wet	104 Juan × Cantata	1
	GF	20241-2	Pax	102 Juan × Cantata	1
	GF	20222-4	Ile de France	138 Cantata × Mad. Lef	1
	GF	20253-1	Pax	137 Cantata × Mad. Lef	1
	GF	20190-3	Bellona	143 Princeps × Mad. Lef	1
	GF	20171-1	Bellona	113 Juan × Cantata	1
	GF	20196-3	Bellona	136 Cantata × Mad. Lef	1
	GF	20189-1	Bellona	141 Mad. Lef × Princeps	1
	GF	20165-5	Bellona	117 Juan × Cantata	1
	GF	20181-1	Bellona	123 Cantata × Juan	1
	GF	20259-11	Pax	155 Princeps × Cantata	1
	GF	20251-2	Pax	135 Cantata × Mad. Lef	1
	GF	20185-5	Bellona	135 Cantata × Mad. Lef	1
	GF	20208-2	Ile de France	114 Juan × Cantata	1
	GF	20190-4	Bellona	143 Princeps × Mad. Lef	1
BC1	GGF	99342	Bellona	Purissima	3
	GGF	99343	Chr. Marvel	Purissima	1
	GGF	99344	Debutante	Purissima	2
	GGF	99345	Golden Melody	Purissima	5
	GGF	99346	Ile de France	Purissima	2
	GGF	99347	Pax	Purissima	1
BC2	GGGF	083272	Freeman	99346-9	7
	GGGF	083275	Snowboard	99343-6	6
	GGGF	083508	Target	99342-2	4
	GGGF	083568	Target	99342-47	6
	GGGF	083569	Target	99345-25	9

[a] G and F denote the parents, *Tulipa gesneriana* and *T. fosteriana* respectively

GGF indicates the backcross involving *T. gesneriana* and the F1, GF hybrid

GGGF indicates the backcross involving *T. gesneriana* and the BC1, GGF parents

Preparation of probes and block DNA

For GISH, total genomic DNA was extracted from young leaves according to Fulton et al. (1995). Sonicated DNA (1–10 kb) of cultivars 'Princeps' (*T. fosteriana*) and 'Ile de France' (*T. gesneriana*) was used as a probe and labelled by nick translation with either Digoxigenin-11-dUTP or Biotin-16-dUTP, in accordance with the manufacturer's instruction (Roche Diagnostics GmbH, Mannheim, Germany). Block DNA was obtained by autoclaving genomic DNA of *T. tarda* (subgenus *Eriostemones*) for 5 min to a fragment size of 100–500 bp.

For FISH, 45S rDNA and 5S rDNA isolated from the clone pTa71 containing the 9-kb *Eco*RI fragment of 45S ribosomal DNA from wheat (Gerlach and Bedbrook 1979) and pScT7 containing the 462-bp *Bam*HI fragment of 5S rDNA from rye (Lawrence and Appels 1986), respectively, were labelled with Digoxigenin-11-dUTP or Biotin-16-dUTP, in accordance with the manufacturer's instruction (Roche Diagnostics GmbH, Mannheim, Germany).

GISH and FISH

DNA denaturation and in situ hybridisation steps were performed according to Marasek and Okazaki (2008) with minor modifications. Slides were pre-treated with RNase A (100 μg/ml) for 1 h at 37°C, treated with 10 mM HCl at 37°C for 2 min followed by incubation in pepsin solution (5 μg/ml) for 10 min and post-fixed in 1% formaldehyde in PBS buffer for 10 min. For GISH the hybridisation mixture consisted of 50% deionised formamide, 10% dextran sulphate, 2× SSC, 1% SDS, 150 ng of each probe of DNA per slide and block DNAs (*T. tarda* DNA and herring sperm DNA) in 30- to 60-fold excess of labelled probe. Chromosome preparations and pre-denatured probes (incubation at 75°C for 10 min) were denatured at 70°C for 4.5 min and allowed to hybridise overnight in a humid chamber at 37°C. The post-hybridisation washes were carried out for 15 min in 2× SSC at room temperature, followed by washes in 0.1× SSC at 42°C for 30 min (73% stringency) and 2× SSC for 15 min at room temperature. Digoxigenin-labelled DNA was detected with antidigoxigenin-FITC (sheep) (Boehringer, Mannheim, Germany) and amplified with anti-sheep-FITC (rabbit) (Vector Laboratories). Biotin-labelled DNA was detected with CY-3 conjugated streptavidin and amplified with biotinylated goat-anti-streptavidin (Vector Laboratories). The chromosomes were counterstained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma) in Vectashield (Vector Laboratories). Procedures for hybridisation using 45S and 5S rDNA probes, post-hybridisation washing and signal detection were the same as those for GISH. The hybridisation mixture consisted of 50% deionised formamide, 10% dextran sulphate, 2× SSC, 1% SDS, 100 ng per slide of DNA isolated from pTa71 and pScT7 and 2 mg of sheared herring sperm DNA (GIBCO BRL). Images of fluorescently stained chromosomes were acquired using a Canon digital camera attached to an Axiophot microscope with an appropriate filter and then processed using software (Axio Vision 4.2).

Chromosome identification and karyotyping

Whole chromosome and arm lengths were measured using the computer program MicroMeasure (http://www.colostate.edu/Depts/Biology/MicroMeasure). From these data, the relative lengths (percentage of the total length of all chromosomes), centromeric index (percentage of short arm length to the total length of chromosome) and arm index (ratio of long arm to short arm lengths) were determined. Nomenclature for the centromeric position on the chromosome was based on the arm index (1.0–1.7: median chromosomes; 1.7–3.0: submedian; 3.0–7.0: subterminal; 7.0–∞: terminal) (Levan et al. 1964). *Tulipa gesneriana* and *T. fosteriana* chromosomes are arranged in the sequence of decreasing length of short arm length according to Marasek et al. (2006). Some of the chromosomes in the karyotype could be identified based on the total chromosome length and the arm ratio. Furthermore, the centromeric index (the ratio of the length of the short arm of the chromosome to that of the total chromosome; expressed as a percentage) and relative chromosome length [(length of the individual chromosome/total length of all chromosomes) × 100%]. were used as additional criteria for identification.

Results

Chromosome analysis in tulip hybrids

The GISH technique was used to confirm the hybrid status in 23 F1 Darwin hybrid tulips obtained in a breeding programme. Simultaneous application of differentially labelled total genomic DNA of *T. gesneriana* cultivar 'Ile de France' and *T. fosteriana* 'Princeps' enabled the discrimination of the parental genomes in Darwin hybrid genotypes. In all F1 Darwin hybrid tulips, 12 chromosomes from each parental genome of *T. gesneriana* and *T. fosteriana* (GF hybrids) were distinguished. Morphometric analysis in 23 F1 hybrids revealed a difference in the total length of chromosomes representing the genomes of *T. gesneriana* and *T. fosteriana*. The percentage of *T. gesneriana* and *T. fosteriana* genomes in these hybrids equaled 55.18 ± 0.78 and 44.92 ± 0.6% respectively. Figure 1a, b shows GISH-painted chromosome complement of diploid GF hybrid 20208-2, whereas detailed morphometric data of

Table 2 Chromosome characteristics in F1 Darwin hybrid tulip 20208-2

Genome	Chr. no.	*p* (μm)	*q* (μm)	*p* + *q* (μm)	RL (%)	CI (%)	*q*/*p*	Type
T. gesneriana	1	7.0	11.3	18.3	11.1	38.2	1.6	M
	2	4.1	13.3	17.4	10.6	23.6	3.2	St
	3	3.8	12.2	16.0	9.7	23.8	3.2	St
	4	3.6	11.7	15.3	9.3	23.5	3.2	St
	5	3.3	11.5	14.8	9.0	22.1	3.5	St
	6	3.6	9.9	13.5	8.2	26.9	2.7	Sm
	7	3.2	8.7	11.9	7.3	27.0	2.7	Sm
	8	3.3	8.4	11.7	7.1	28.1	2.5	Sm
	9	3.3	8.5	11.8	7.2	27.8	2.6	Sm
	10	3.6	8.0	11.6	7.1	31.1	2.2	Sm
	11	3.5	7.4	10.9	6.7	32.1	2.1	Sm
	12	3.2	7.2	10.4	6.4	31.2	2.2	Sm
Total				163.7				
T. fosteriana	1	5.2	9.1	14.3	10.5	36.5	1.7	M
	2	2.7	11.0	13.7	10.1	19.9	4.0	St
	3	3.0	10.4	13.4	9.9	22.8	3.4	St
	4	2.4	10.4	12.8	9.4	18.8	4.3	St
	5	3.1	8.7	11.8	8.7	26.4	2.8	Sm
	6	3.4	8.1	11.5	8.5	29.7	2.3	Sm
	7	2.0	8.1	10.1	7.5	20.0	4.0	St
	8	2.5	7.5	10.0	7.4	25.3	2.9	Sm
	9	2.8	6.8	9.6	7.1	29.1	2.4	Sm
	10	2.2	7.5	9.7	7.1	22.4	3.4	St
	11	2.4	7.0	9.4	6.9	25.6	2.9	Sm
	12	2.5	6.7	9.2	6.8	27.6	2.6	Sm
Total				135.5				

p = Short arm, *q* = long arm, RL = relative length [(length of the individual chromosome/total length of all chromosomes) × 100%], CI = centromeric index [(*p*/*p* + *q*) × 100%], M = median chromosomes (*q*/*p* = 1.0–1.7), Sm = submedian chromosomes (*q*/*p* = 1.7–3.0), St = subterminal chromosomes (*q*/*p* = 3.0–7.0)

its chromosomes are shown in Table 2. In this hybrid the difference of 28.2 μm in the total length of all metaphase chromosomes between *T. gesneriana* and *T. fosteriana* genomes was observed. All chromosomes representing the *T. gesneriana* genome were bigger as compared to *T. fosteriana* (Fig. 1a, b; Table 2). According to Levan et al. (1964) the chromosomes within each genome could be classified to median, submedian and subterminal chromosomes. In F1 hybrid 20208-2 (Table 2), variation between submedian and subterminal types of chromosomes was observed for some chromosomes. The other F1 hybrids comprised one pair of median chromosomes and a variable number of submedian and subterminal chromosomes, which ranged from 3–9 submedian and 2–8 subterminal chromosomes in the *T. fosteriana* genome and from 5–8 and 2–6 subterminal in the *T. gesneriana* genome.

The difference in chromosome length between *T. fosteriana* and *T. gesneriana* genomes makes the karyotyping more complicated, especially in the backcross progenies. In tulip hybrids chromosome classification based on decreasing length of the short arms or the total length of chromosomes, could not be the only criterion for positioning within the idiograms. In F1 hybrids the final decisions on the positioning of chromosomes on the idiograms were made on the basis of the decreasing length of the short arm, decreasing relative chromosome length and arm ratio. Chromosomes representing the *T. gesneriana* genome were positioned first in each pair in the karyotype.

Distribution of GISH signals

An interesting aspect of in situ hybridisation in Darwin hybrids tulips is the lack of uniform chromosome labelling along entire somatic chromosome arms where telomeric and certain blocks of intercalary regions of chromosomes showed stronger fluorescence intensity (Fig. 1). In situ hybridisation with 5S rDNA and 45S rDNA probes to metaphase chromosomes of F1 hybrids showed that certain regions of chromosomes exhibiting more intensive fluorescence after GISH were rich in rDNA (Fig. 1). Figure 1b shows the chromosome complement of F1 Darwin hybrid 20208-2 [Bellona × (Princeps × Cantata)] with enlarged median chromosomes (inset). 45S rDNA loci were localised exclusively in the telomeric position of the long arm of chromosomes (green fluorescence), whereas strong 5S rDNA signals were localised in the telomeric position on

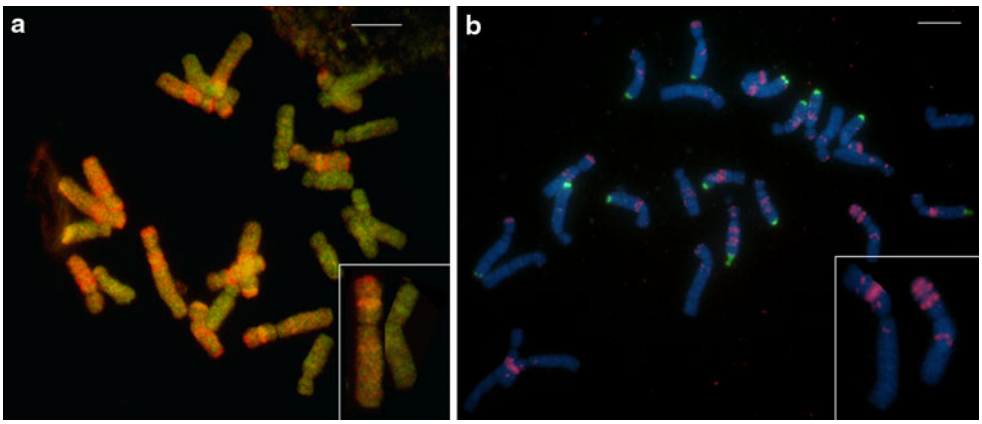


Fig. 1 Chromosome labelling in diploid F1 hybrid 20208-2 ($2n = 2x = 24$). **a** Genomic in situ hybridisation to somatic metaphase chromosome complement showing 12 F and 12 G chromosomes. *Tulipa gesneriana* DNA is detected with Cy3-streptavidin system (*red*) and *T. fosteriana* with FITC (*green*); **b** double target fluorescence in situ hybridisation of 45S rDNA (*green*) and 5S rDNA (*red*) to somatic metaphase chromosome complement. Insets show enlarged median chromosomes. *Bar* 10 μm

the short arm of chromosomes and in intercalary positions on the long arms (red fluorescence) with the exception of median chromosomes having additional strong intercalary positions of 5S rDNA locus on the short arm. Thus, the banding pattern of FISH following GISH revealed additional information, which allowed identification of a few individual chromosomes.

Genome composition of BC1 progenies

The results of GISH analysis in 14 BC1 progenies derived from crosses between *T. gesneriana* cultivars (G) and 'Purissima' (GF) hybrid are summarised in Table 3. All BC1 plants were diploids ($2n = 2x = 24$) with the exception of one tetraploid ($2n = 4x = 48$) genotype, 99345-37. Because the Gesneriana cultivars were used for backcrossing, the number of G genome chromosomes (chromosomes with centromere of *T. gesneriana* genome) predominated in the BC1 progenies, and their number varied from 18 to 20 per diploid complement, whereas the total number of chromosomes with the centromere of *T. fosteriana* genome ranged from 4 to 6 (Table 3). Taking the total length of both G and F chromosomes in the BC1 progenies, the percentage of each genome present in BC1

Table 3 The genome composition of BC1 hybrids derived from backcrossing 'Purissima' (GF) to *T. gesneriana* cultivars (the number of recombinant chromosomes are in brackets)

Generation	Genotype no.	Parents		Ploidy level	Genome composition		No. of break points	% of F-genome
		Female	Male		G(G/F)[a]	F(F/G)[b]		
BC1	99342-2	Bellona	Purissima	2*x*	19 (4)	5 (3)	8	18.9
	99342-47	Bellona	Purissima	2*x*	20 (3)	4 (2)	7	20.4
	99342-60	Bellona	Purissima	2*x*	19 (5)	5 (2)	9	21.3
	99343-6	Chr. Marvel	Purissima	2*x*	19 (4)	5 (0)	5	21.4
	99344-5	Debutante	Purissima	2*x*	19 (3)	5 (5)	11	20.0
	99344-15	Debutante	Purissima	2*x*	19 (5)	5 (2)	8	24.4
	99345-25	Golden Melody	Purissima	2*x*	18 (3)	6 (2)	8	22.1
	99345-37	Golden Melody	Purissima	4*x*	42 (2)	6 (5)	9	11.5
	99345-102	Golden Melody	Purissima	2*x*	18 (3)	6 (1)	5	24.7
	99345-108	Golden Melody	Purissima	2*x*	20 (5)	4 (3)	12	18.5
	99345-123	Golden Melody	Purissima	2*x*	20 (3)	4 (0)	3	17.7
	99346-7	Ile de France	Purissima	2*x*	19 (5)	5 (3)	9	18.1
	99346-9	Ile de France	Purissima	2*x*	19 (5)	5 (3)	9	17.8
	99347-2	Pax	Purissima	2*x*	19 (2)	5 (1)	5	22.3

[a] Chromosomes with a *T. gesneriana* centromere possessing *T. fosteriana* recombinant segment

[b] Chromosomes with a *T. fosteriana* centromere possessing *T. gesneriana* recombinant segment

progenies was estimated (Table 3). The percentage of F genome in diploid genotypes varied from 17.7% (99345-123) to 24.4% (99344-15) with an expected value of 22.5%.

GISH clearly identified the presence of recombinant chromosomes in all BC1 progenies tested. In all genotypes, with the exception of 99343-6 and 99345-123, there were two distinct types of recombinant chromosomes. Chromosomes with a *T. gesneriana* centromere possessing the *T. fosteriana* recombinant segment are indicated as G/F, whereas chromosomes with a *T. fosteriana* centromere possessing *T. gesneriana* recombinant segment were indicated as F/G. An example of GISH with two types of recombinant chromosomes is shown in Fig. 2a. The numbers of these two types of recombinant chromosomes varied in different BC1 genotypes, and the total ranged from 3 to 8 (Table 3). Regardless of G/F or F/G, all recombinant chromosomes possessed mostly a single recombinant segment derived from either a single crossover event or in a few cases double crossover events (Fig. 3). The numbers of break points were counted for individual chromosomes, and they varied from 1 to 3 per chromosome. The total number of break points per BC1 genotype varied from 3 to 12 (Table 3; Fig. 3). Of the total number of 84 recombinant chromosomes that were found in 14 BC1 plants, 57 (67.85%) were the results of single crossover events. The break points were distributed along the entire length of the chromosomes, and their positions ranged from highly proximal to distal. However, only 18 recombination sites were found on the short arm of *T. gesneriana* and *T. fosteriana* genomes.

GISH analysis of the tetraploid progeny, 99345-37 ($2n = 4x = 48$), resulting from a cross between 'Golden Melody' and 'Purissima', revealed that its karyotype consists of 42 chromosomes of *T. gesneriana* (2 G/F) and 6 chromosomes of *T. fosteriana* (5 F/G) (Fig. 2b; Table 3), where the amount of introgressed *T. fosteriana* genome was 11.5%. The chromosome composition of the exceptional tetraploid has obviously resulted from the functioning of 2*n* gametes from both parents.

Genome composition of BC2 progenies and transmission of recombinant chromosomes

The genome compositions determined through GISH in 32 BC2 progenies are given in Table 4, and some are illustrated in Figs. 4 and 5. With the exception of one BC2 plant 083275-4, which was an aneuploid ($2n = 2x + 1 = 25$), all others BC2 genotypes were diploids. BC2 progeny is expected to contain 11.25% of the *fosteriana* genome. It was found that the amount of *fosteriana* genome transmitted to BC2 progenies varied from 1.1% (083272-6) to as high as 12.7% (083568-3) (Table 4). The scarcity of *fosteriana* genome chromosomes in some BC2 progenies might have resulted from non-inclusion of F genome chromosomes during gamete formation or due to the process of selection of gametes during fertilisation. As compared to BC1 parents the frequencies of recombination events were higher among BC2 progeny of 99342-47, 99343-6 and 99345-25 hybrids, whereas they were lower in the progenies of 99342-2 and 99346-9 (Table 4). The total number of recombinant chromosomes found in BC2 hybrids was 130, of which 43 represented chromosomes with *fosteriana* centromeres. The total number of recombinant chromosomes per BC2 genotype ranged from 1 to 7, and breaking points varied from 2 to 11. Break points occurred both in the long and short arm; however the former ones predominated (128 out of 165). Six recombinant chromosomes were, for example, found in one BC2 plant, 083569-4 (Table 4; Fig. 5), of which one was the same as in the BC1 parent, whereas three were new recombinant chromosomes. In this genotype two original recombinant chromosomes were involved in the second cycle of homoeologous recombination. Out of 130 recombinant chromosomes found in 32 BC2 hybrids, 42 were the same as in BC1 and 20 resulted from the second cycle of homoeologous recombination, whereas 68 were new types of recombinant chromosomes.

Discussion

Chromosome number

In contrast to many ornamental crops, e.g. *Narcissus* and *Lilium* where diploid cultivars have been replaced by polyploids, in the genus *Tulipa* the majority of *T. gesneriana* and *T. fosteriana* cultivars are diploids ($2n = 2x = 24$); only a small number of cultivars are triploids ($2n = 3x = 36$) and about 100 registered tulips are tetraploids ($2n = 4x = 48$) (Holitscher 1968; Kroon 1975; Zeilinga and Schouten 1968a, b; Kroon and Jongerius 1986; Van Scheepen 1996). Most of the commercial Darwin hybrids are triploids; however, 95% of crossing populations are diploid (Marasek-Ciolakowska et al. 2009, 2011).

In general, hybrids between distantly related species have disturbed chromosome pairing, and when used as parents, if they are 'fertile', they give rise to either aneuploid or polyploid progenies (due to functioning of 2*n* gametes) (Ramanna et al. 2003). In this respect, tulips seem to be an exceptional crop in which interspecific hybrids can produce functional n gametes and the majority of interspecific crosses can be done at the diploid level. Similarly in our study, all F1 hybrids and the majority of BC1 and BC2 progenies (Tables 3, 4) are diploids.

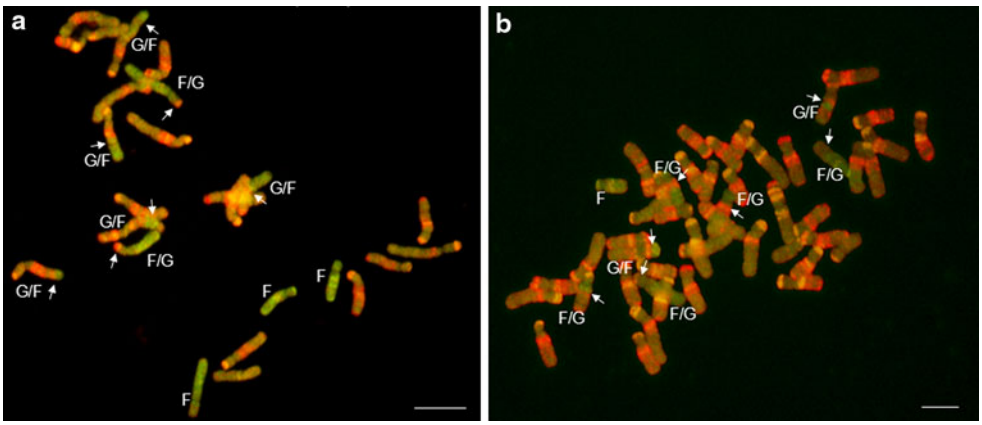


Fig. 2 The representative GISH results for BC1 progenies. **a** Diploid BC1 hybrid 99344-15 ($2n = 2x = 24$) with 19 G chromosomes (5 G/F) and 5 F chromosomes (2 F/G). **b** Chromosome complement of tetraploid BC1 hybrids 99345-37 ($2n = 4x = 48$) with 42 G chromosomes (2 G/F) and 6 F chromosomes (5 F/G). *Tulipa gesneriana* DNA is detected with Cy3-streptavidin system (*red*) and *T. fosteriana* with FITC (*green*). Recombinant chromosomes are defined as F/G and G/F indicating a *T. fosteriana* centromere with *T. gesneriana* chromosome segment(s) and a *T. gesneriana* centromere with *T. fosteriana* chromosome segment(s), respectively. The *arrows* indicate the recombinant segment. *Bar* 10 μm

Polyploid tulips are likely to have arisen as a result of the occurrence of diploid gametes in diploid cultivars (Kroon and Van Eijk 1977; Marasek et al. 2006). For instance, triploid Darwin hybrid tulips ($2n = 3x = 36$) 'Apeldoorn', 'Ad Rem', 'Pink Impression' and tetraploid 'Tender Beauty' ($2n = 4x = 48$) resulted from hybridisation at the diploid level (Van Scheepen 1996). The diploid Darwin hybrid tulip 'Purissima' ($2n = 2x = 24$), used in our study to generate BC1 hybrids can produce $2n$ pollen in low percentage (8%) (data not shown); however the number of polyploids obtained when 'Purissima' was used as a pollen donor is highly limited. In the present work, out of 69 Darwin hybrids analysed using the GISH technique, only one tetraploid BC1 ($2n = 4x = 48$) and one aneuploid BC2 ($2n = 2x + 1 = 25$) 'Purissima' hybrid was found. Similarly in the study of Marasek and Okazaki (2008) all 'Purissima' hybrids were diploids, except for one triploid cultivar 'Kouki'.

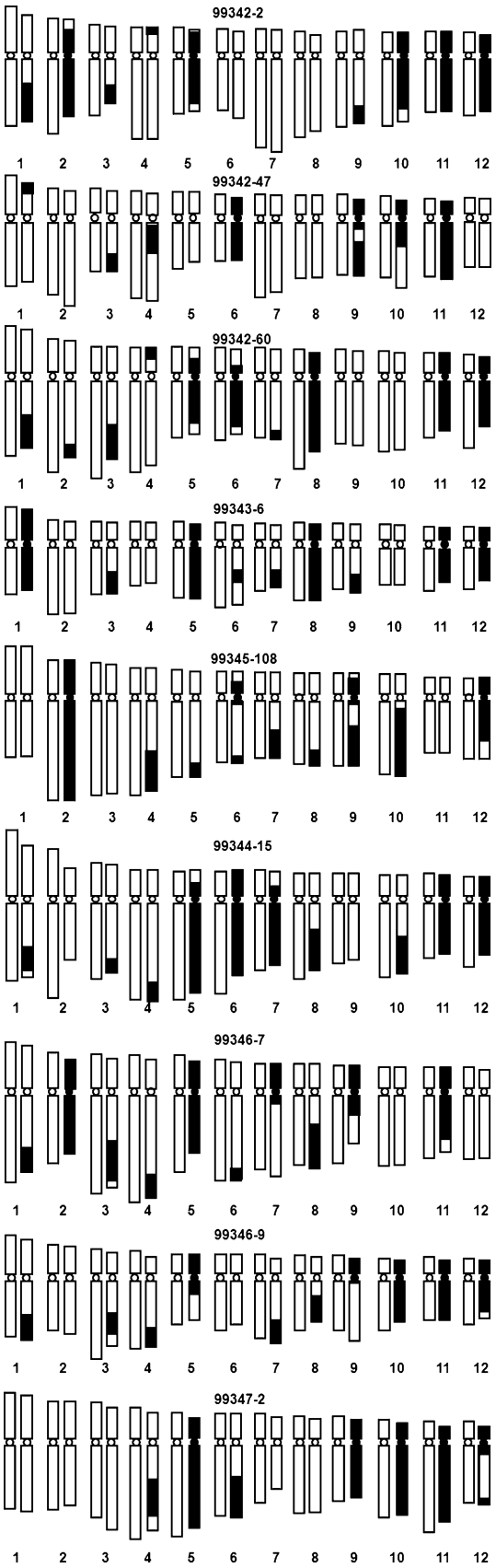
Karyotype similarities and differences between *T. gesneriana* and *T. fosteriana* genomes

Due to their large size, tulip chromosomes are convenient for cytological study. However, the absence of distinct chromosomal landmarks such as secondary constructions and the similarity of size and morphology of most of the chromosomes in karyograms makes discrimination of tulip chromosomes difficult. Detailed analysis of chromosome morphology using discriminant analysis made by Marasek et al. (2006) revealed the difference in size of median chromosomes between *T. gesneriana* and *T. fosteriana* cultivars, which was successfully used as a criterion in the analysis of genome constitution of triploid Darwin hybrid tulips. The difference in chromosome size between *T. gesneriana* and *T. fosteriana* chromosomes was also observed in 'Purissima' and its hybrids (Marasek and Okazaki 2008). Similarly, in the present work morphometric analysis made for 23 F1 hybrids revealed a difference in the total length of chromosomes representing genomes of *T. gesneriana* and *T. fosteriana*. In spite of the difference in length within each parental species, some chromosomes are very similar in morphology and, based on the system proposed by Levan et al. (1964), they could be classified as median, submedian and subterminal.

Chromosome differentiation in tulips

The two main species involved in the origin of the important group of tulips, viz., Darwin hybrids, are *T. gesneriana* and *T. fosteriana*. As with the taxonomic distinction, these two species are also genetically differentiated, which is exemplified by the fact that the F1 hybrids are generally sterile and only in rare cases some amount of fertility is noticed in some hybrids.

Although chromosome morphology is similar, cytological differentiation is evident from Giemsa C-banding of somatic metaphase chromosomes of the two species (Blakey and Vosa 1982) as well as differential staining of the two genomes through FISH with 5S rDNA and 45S rDNA probes (Mizuochi et al. 2007; Marasek and Okazaki 2008) and GISH (Marasek et al. 2006; Marasek and Okazaki 2007; Marasek-Ciolakowska et al. 2009, 2011). A notable feature is that the banding pattern is also evident in GISH preparations (see Figs. 1a, 2, 3), which appear to be concurrent with the position of rDNA loci (see Fig. 1b) and C-banding. The FISH patterns differed between



◄ **Fig. 3** A diagrammatic representation of chromosomes in diploid ($2n = 2x = 24$) BC1 hybrids. In this figure the *black* colour represents the *Tulipa fosteriana* genome, while *white* represents *T. gesneriana* one

T. gesneriana and *T. fosteriana* cultivars with respect to size, number and chromosome distribution of some of the rDNA FISH signals. Although the general chromosome localisation of 45S rDNA and 5S r DNA loci is similar for *T. gesneriana* and *T. fosteriana* chromosomes, in the study of Mizuochi et al. (2007) only four pairs of *T. fosteriana* chromosomes shared the same pattern of rDNA sites with chromosomes of *T. gesneriana*. In our study using FISH with 45S rDNA (Fig. 1b) as well as in the study of Mizuochi et al. (2007) and Marasek and Okazaki (2008), both *T. fosteriana* and *T. gesneriana* chromosomes possess numerous loci of 45S rDNA exclusively located on the long arm at the telomeric positions. Chromosomal localisation of the 5S rDNA sites in tulip cultivars exhibits more variation in which loci can be located at pericentromeric, intersticial and telomeric regions of chromosomes. The C-banding patterns of the two species display certain characteristic differences. In the case of *T. gesneriana*, prominent blocks of C-heterochromatin are observed on the proximal positions of long arms of all the chromosomes except chromosome 12. And all of the short arms of these chromosomes terminate with a block of C-heterochromatin, whereas only seven chromosomes have blocks of C-heterochromatin at the terminal ends of the long arms. On the other hand, in the case of *T. fosteriana* only 7 of the 12 chromosomes of the long arms possess blocks of C-heterochromatin in the proximal positions and 7 of the short arms terminate in blocks of C-heterochromatin (Blakey and Vosa 1982).

Distribution of recombinant sites

It is well established that heterochromatin can influence chiasma formation and crossing over in plants (Stack 1984). It would be instructive to compare the recombination pattern in the F1 hybrids or BC progenies of species that possess large amounts of C-banded heterochromatin, such as tulip, with those that possess much less of such heterochromatin, such as *Lilium*, both of which we have investigated extensively (Lim et al. 2003; Barba-Gonzalez et al. 2005, 2006; Zhou et al. 2008; Khan et al. 2009, 2010; Xie et al. 2010). Both *Tulipa* and *Lilium* have many biological characteristics in common: both belong to the family Liliaceae; both species possess huge amounts of nuclear DNA with 2C DNA values ranging from 65.5 to 86.4 pg in *Lilium* (Bennett 1972) and from 32 to 69 pg in *Tulipa* (Zonneveld 2009), and consequently very large chromosomes. In both cases interspecific hybridisation

Table 4 The genome composition of five BC1 hybrids and their BC2 derivatives analysed by GISH (the numbers of recombinant chromosomes are in brackets)

Generation	Genotype no.	Parents		Ploidy level	Genome composition		No. of breaking points	% of F-genome
		Female	Male		G (G/F)	F (F/G)		
BC1	99342-2	Bellona	Purissima	2*x*	19 (4)	5 (3)	8	18.9
BC2	083508-1	Target	99342-2	2*x*	22 (0)	2 (2)	3	3.9
	083508-2	Target	99342-2	2*x*	22 (1)	2 (2)	3	3.8
	083508-4	Target	99342-2	2*x*	23 (1)	1 (1)	3	4.6
	083508-5	Target	99342-2	2*x*	22 (0)	2 (2)	3	5.3
BC1	99342-47	Bellona	Purissima	2*x*	20 (3)	4 (2)	7	20.4
BC2	083568-1	Target	99342-47	2*x*	23 (3)	1 (1)	5	7.1
	083568-3	Target	99342-47	2*x*	21 (4)	3 (3)	10	12.7
	083568-4	Target	99342-47	2*x*	23 (5)	1 (1)	6	10.5
	083568-5	Target	99342-47	2*x*	21 (2)	3 (3)	5	10.7
	083568-8	Target	99342-47	2*x*	23 (3)	1 (1)	6	6.3
	083568-10	Target	99342-47	2*x*	23 (4)	1 (1)	7	8.6
BC1	99343-6	Chr. Marvel	Purissima	2*x*	19 (4)	5 (0)	5	21.4
BC2	083275-4	Snowboard	99343-6	2*x* +1	25 (4)	0	5	4.5
	083275-5	Snowboard	99343-6	2*x*	23 (3)	1 (1)	4	5.4
	083275-6	Snowboard	99343-6	2*x*	23 (3)	1 (1)	5	7.3
	083275-7	Snowboard	99343-6	2*x*	22 (3)	2 (2)	5	9.3
	083275-8	Snowboard	99343-6	2*x*	23 (4)	1(1)	5	7.0
	083275-9	Snowboard	99343-6	2*x*	24 (5)	0	5	7.3
BC1	99345-25	Golden Melody	Purissima	2*x*	18 (3)	6 (2)	8	22.1
BC2	083569-1	Target	99345-25	2*x*	21 (2)	3 (2)	5	12.3
	083569-2	Target	99345-25	2*x*	23 (3)	1 (0)	3	7.8
	083569-3	Target	99345-25	2*x*	22 (3)	2 (2)	7	6.9
	083569-4	Target	99345-25	2*x*	21 (3)	3 (3)	11	8.2
	083569-5	Target	99345-25	2*x*	23 (3)	1 (1)	8	3.6
	083569-6	Target	99345-25	2*x*	23 (3)	1 (1)	4	6.3
	083569-7	Target	99345-25	2*x*	22 (1)	2 (2)	4	6.9
	083569-9	Target	99345-25	2*x*	23 (3)	1 (1)	4	6.8
	083569-10	Target	99345-25	2*x*	24 (2)	0	4	2.4
BC1	99346-9	Ile de France	Purissima	2*x*	19 (5)	5 (3)	9	17.8
BC2	083272-1	Freeman	99346-9	2*x*	23 (3)	1 (1)	5	4.9
	083272-3	Freeman	99346-9	2*x*	22 (2)	2 (2)	6	6.2
	083272-5	Freeman	99346-9	2*x*	23 (3)	1 (1)	6	5.5
	083272-6	Freeman	99346-9	2*x*	24 (1)	0	2	1.1
	083272-7	Freeman	99346-9	2*x*	22 (2)	2 (2)	6	8.0
	083272-8	Freeman	99346-9	2*x*	23 (2)	1 (1)	5	5.7
	083272-9	Freeman	99346-9	2*x*	22 (2)	2 (2)	5	6.3

followed by selection has been practiced and both are cultivated as bulbous ornamental crops. In the case of tulips, there appear to be one or two crossovers per chromosome, and in the case of *Lilium* multiple crossovers occur per chromosome (Khan et al. 2009). Regarding the localisation of crossovers in tulip, they appear to be randomly distributed on the chromosome arms and on different chromosomes of the genomes. Contrary to this, in *Lilium* the crossovers are non-randomly distributed on the chromosomes and some chromosomes are almost devoid of crossovers, but others are replete with multiple crossovers.

Tulip belongs to a group of crop plants that have been subjected to extensive (interspecific) hybridisation followed by selection and are comparable in this respect to other bulbous crops like *Crocus* (Ørgaard et al. 1995), *Narcissus* (Brandham and Kirton 1987), *Lilium* (Van Tuyl

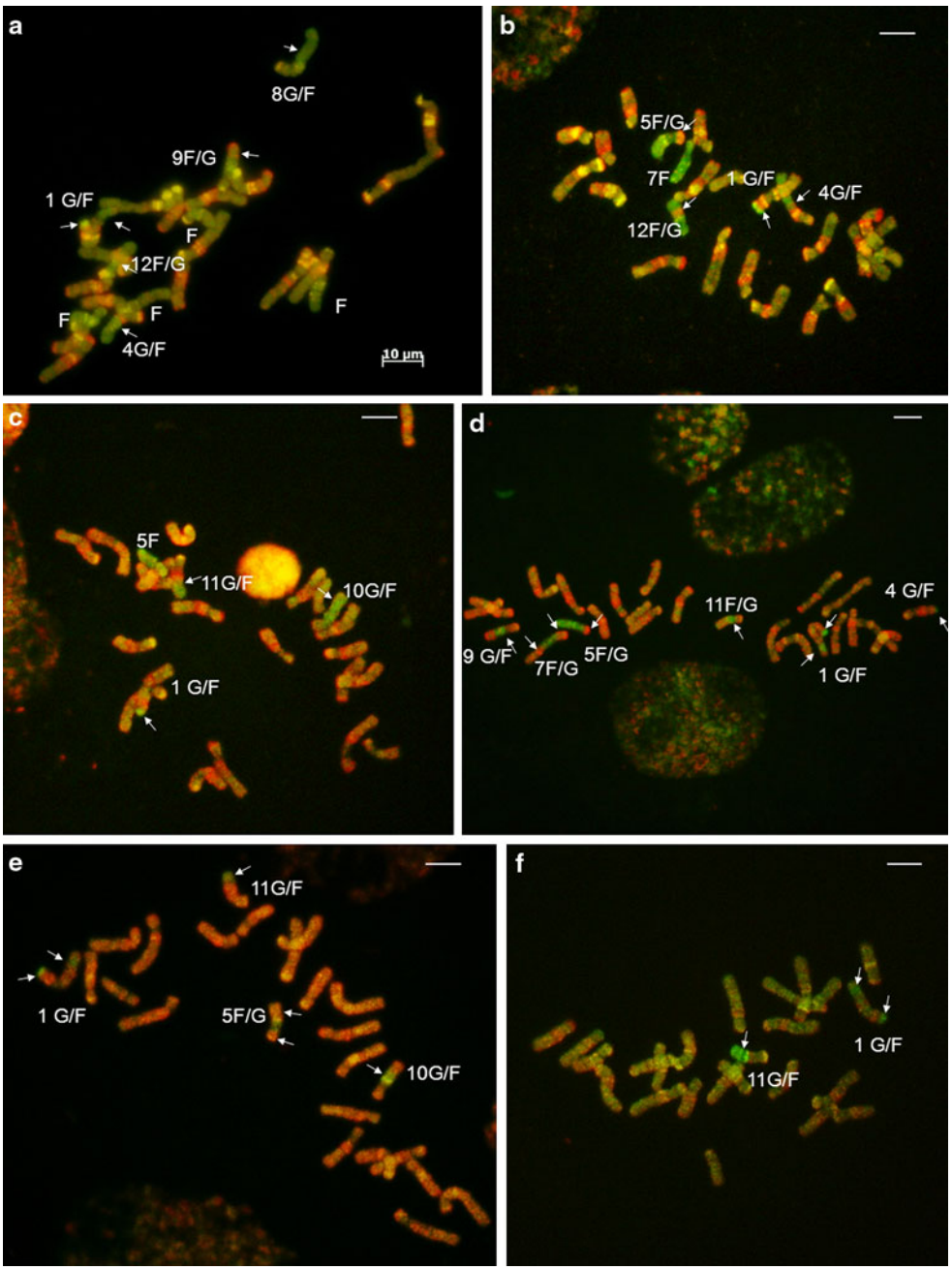


Fig. 4 GISH results for BC1 diploid GGF hybrid and its representative BC2 progenies. **a** Chromosome complement of diploid BC1 hybrids 99345-25 ($2n = 2x = 24$) showing 6 F chromosomes (2 F/G) and 18 G chromosomes (3 G/F); **b** BC2 progeny 083569-1 ($2n = 2x = 24$) with 3 F chromosomes (2 F/G) and 21G chromosomes (2 G/F); **c** BC2 progeny 083569-2 ($2n = 2x = 24$) with 1 F chromosomes and 23 G chromosomes (3 G/F); **d** BC2 progeny 083569-4 ($2n = 2x = 24$) with 3 F chromosomes (3 F/G) and 21 G chromosomes (3 G/F); **e** BC2 progeny 083569-5 ($2n = 2x = 24$) with 1 F chromosomes (1 F/G) and 23 G chromosomes (3 G/F); **f** BC2 progeny 083569-10 ($2n = 2x = 24$) with 0 F chromosomes and 24 G chromosomes (2 G/F). *Tulipa gesneriana* DNA is detected with Cy3-streptavidin system (*red*) and *T. fosteriana* with FITC (*green*). Recombinant chromosomes are defined as F/G and G/F indicating a *T. fosteriana* centromere with *T. gesneriana* chromosome segment(s) and a *T. gesneriana* centromere with *T. fosteriana* chromosome segment(s), respectively. The *arrows* indicate the recombinant segment. *Bar* 10 µm

et al. 2002) and *Alstroemeria* (Ramanna 1992). In these cases, intergenomic recombination, spontaneous polyploidisation and unconscious selection by the breeders have played a role in their evolution. Initial efforts have been made in these cases to unravel chromosomal changes, if any, during the course of their development of cultivars through the use of GISH analyses, and valuable information has emerged in some cases. Results from these studies

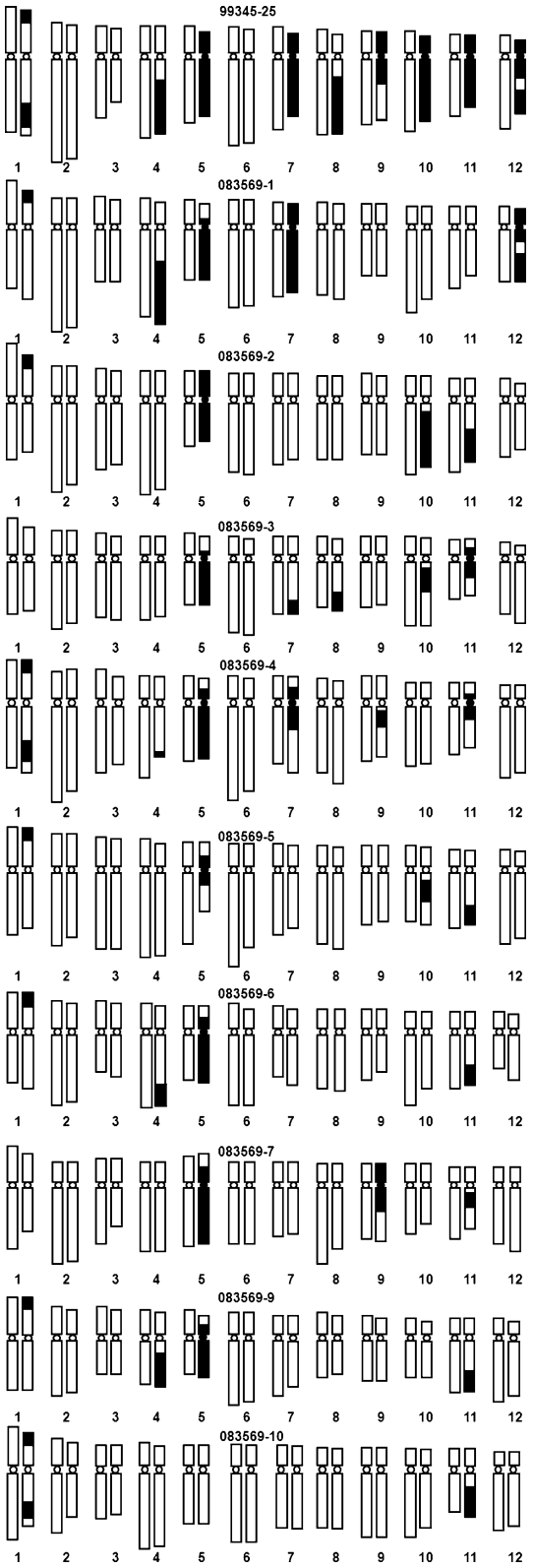


Fig. 5 A diagrammatic representation of chromosomes in 99345-25 BC1 hybrids and its BC2 progenies. In this figure the *black* colour represents the *Tulipa fosteriana* genome while *white* represents *T. gesneriana* one

are not merely of interest for breeding processes but also from an evolutionary point of view because such studies can shed light on the source of genetic variation, introgression and polyploidy, which are highly relevant to plant breeding.

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