Euphytica (2006) 151:279–290
DOI 10.1007/s10681-006-9147-y

The origin of Darwin hybrid tulips analyzed by flow cytometry, karyotype analyses and genomic *in situ* hybridization

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Received: 26 November 2005 / Accepted: 19 March 2006

Abstract Chromosome morphology was studied in diploid cultivars of *Tulipa fosteriana* and *T. gesneriana* ($2n = 2x = 24$) and triploid Darwin hybrids ($2n = 3x = 36$) developed from interspecific crosses of *T. gesneriana* and *T. fosteriana*. Chromosomes were arranged in the karyotype according to decreasing total length. Based on our karyotypic analysis, we propose that median chromosomes may serve as markers for diploid genotypes. Discriminant analysis with respect to total chromosome length and short arm length showed a significant difference between the size of the larger median chromosomes of *T. gesneriana* and *T. fosteriana.* Comparison of median chromosome length in Darwin hybrid tulips showed that two larger chromosomes and one smaller chromosome were derived from *T. gesneriana* and *T. fosteriana*, respectively. This finding was clearly and unambiguously confirmed by simultaneous hybridization of differentially labeled genomic probes of *T. fosteriana* and *T. gesneriana* to metaphase chromosomes of the triploid cultivar 'Yellow Dover', thereby enabling us to distinguish between the 24 chromosomes derived from *T. gesneriana* and 12 chromosomes derived from *T. fosteriana*. Thus, genomic *in situ* hybridization and median chromosome analyses can be useful to identify the genome constitution of triploid Darwin hybrid tulips. In addition, their hybridity was readily verified by flow cytometry using vegetative tissue of Darwin hybrid tulips. Our results clarify the process of *Tulipa* cultivar formation and will be useful for interspecific hybridization breeding.

Keywords Chromosome morphology · Flow cytometry · Genomic *in situ* hybridization · Triploid Darwin hybrid tulips · *Tulipa*

Introduction

Most cultivated tulips belong to *T. gesneriana* and are classified into 15 horticultural groups based on morphological traits and flowering characteristics (Van Scheepen, 1996). Darwin hybrid tulips, one of the cultivar group, was spontaneously developed by an interspecific cross of a Darwin tulip and *T. fosteriana* Hoog ex W. Irving (Doorenbos, 1954; Anonymous, 1968; Bryan, 2002; Van Tuyl & Van Creij, 2005). Darwin tulips, named after Charles Darwin, is an old horticultural group of *T. gesneriana*; this group is now included in the Single Late group (Bryan, 2002). Most of the Darwin hybrid tulips are triploid, whereas one

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is diploid and a few are tetraploid. Triploid Darwin hybrid tulips are excellent cultivars due to their large flower, sturdy stem and plant size, and their appearance on the tulip market was a revolution (Kho & Baer, 1971; Kroon & Van Eijk, 1997). Most Darwin hybrid tulips have red or yellow flowers, but there are also orange and pink varieties. New varieties with differently colored speckles and stripes have recently become available. Such ornamental characteristics make these varieties very popular both for cut flowers and in the garden. In Holland, Darwin hybrid tulips constitute about 10% of the total tulip production area (http://www.bloembollenkeuringsdienst.nl/). In Toyama prefecture, Japan—one of the country's major tulip bulb production areas—about 350 cultivars are produced, and four cultivars of Darwin hybrid tulips rank in fourth-tenth in bulb production (T.Tsuji, personal communication). In the past 50 years, more than 50 cultivars of Darwin hybrid tulips have been developed (Van Scheepen, 1996). It seems that *T. fosteriana* cv. 'Red Emperor' (='Madame Lefeber') was used exclusively as the male parent in these crosses, whereas different Darwin tulip cultivars were used as the female parent (Doorenbos, 1954; Kroon & Van Eijk, 1977). Despite the horticultural importance of Darwin hybrid tulips, both their genomic constitution and the mechanism by which they became triploid through interspecific hybridization are unknown (Sayama et al., 1982).

In plant breeding, there is a need to discriminate between genomes of closely related species in order to identify parents of hybrid plants or ancestors of allopolyploid species. Several complementary methods, such as karyotyping and banding pattern analyses, can be used for identification. Species in the genera *Alstroemeria* (Rustanius et al., 1991) and *Clivia* (Ran et al., 1999) have been distinguished by morphological features of their chromosomes. Based on differences in chromosomal morphology between parental genotypes, hybrids were verified in the genera *Anemone* (Heimburger, 1962), *Allium* (Keller et al., 1996) and *Lilium* (Nassar et al., 1998; Okazaki et al., 1994; Obata et al., 2000). Staining techniques that create bands on chromosomes have been used to characterize somatic hybrids of *Solanum* (Srebniak et al., 2002), *Clivia* (Ran et al., 2001) and cultivars of *Tulipa* (Filion, 1974). In *Lilium,* banding patterns have been used to identify genotypes (Smyth et al., 1989; Smyth, 1999; Lim et al., 2001a) and verify interspecific hybrids (Marasek et al., 2004a).

Fluorescence *in situ* hybridization (FISH) is another technique that produces chromosomal markers, thereby permitting chromosomal identification. Employing a probe to label repetitive rDNA sequences, this method was used to discriminate somatic chromosomes (Lim et al., 2001a; Kato et al., 2004), identify chromosomes in allopolyploids species (Hasterok et al., 2005) and verify hybrids (Marasek et al., 2004b). When parental species cannot be distinguished using this analysis, genomic *in situ* hybridization (GISH) using labeled whole-genomic DNA can be applied. Generally, this method can unambiguously distinguish between genomes, making it very useful for identifying plant hybrids and allopolyploid species (Orgaard et al., 1995; Lim et al., 2000; Hasterok et al., 2004; Maluszynska & Hasterok, 2005); moreover, chromosome rearrangements can be detected (Karlov et al., 1999; Lim et al., 2001b; 2003; Barba-Gonzales et al., 2004).

In our present study, we analyzed the genomic constitution of triploid Darwin hybrid tulips using flow cytometry, karyotype analyses and GISH. Our results will help clarify the process of *Tulipa* cultivar formation and will be useful for the disciplines of systematic botany and interspecific hybridization breeding.

Material and methods

Plant material

The cultivars used in this study were as follows: 'Christmas Dream', 'Ile de France', 'Negrita', 'Preludium' and 'Queen of Night' for *T. gesneriana*; 'Cantata', 'Easter Parade', 'Princeps', 'Red Emperor' and 'Zombie' for *T. fosteriana*; and 'Come Back', 'Diplomate', 'Pink Impression', 'Red Matador', 'Spring Song' and 'Yellow Dover' for Darwin hybrid tulips. Bulbs were obtained from the Toyama Bulb Growers Association and the Toyama Agricultural Research Center Vegetable and Ornamental Plants Experiment Station, Japan.

Flow cytometry

The ploidy of cultivars was analyzed by flow cytometry (Partec PA-I, Partec GmbH, Munster, Germany) using samples of chopped internal bulb buds in an adequate aliquot of Nuclei Extraction Buffer and dilution with 4, 6-diamidino-2-phenylindole (DAPI) Staining

Buffer (High Resolution DNA kit, CyStain UV Precise P, Partec, Germany). Five bulbs were examined for each cultivar. DAPI solution containing nuclei of *Hosta sieboldiana* was prepared from the leaves using the same method and then used as an internal standard. The value of the relative *Hosta* DAPI fluorescence was adjusted to 50 (Okazaki et al., 2005). Relative DNA cotents of tulips were expressed as DAPI fluorescence values.

Chromosome preparation

Actively growing root tips were pre-treated with 0.05% colchicine at 18 °C for 2.5 h and then excised from bulbs and transferred to 3:1 ethanol-glacial acetic acid to be fixed overnight. Fixed root tips were washed in 0.01 M enzyme buffer (sodium citrate, pH 4.8) for 20 min and digested in an enzyme mixture containing 2% (w/v) cellulase Onozuka-RS and 1% (w/v) pectolyase Y-23 for 2–2.5 h at 37 °C (Kato, 1997). For GISH, squash preparations of meristems were made in a drop of 45% acetic acid. Cover slips were removed with a razor blade after freezing on dry ice; slides were dehydrated in absolute ethanol and air-dried.

Karyological study

For the karyological study, chromosomes were stained in 1% (w/v) acetic orcein. Well-spread metaphases were acquired using a camera (VB-7000, Keyence, Japan) attached to a microscope (Nikon Optiphot, Japan). The short arm, long arm and total chromosome length were measured using computer software (Image-Pro Plus, Media Cybernetics, Inc., US). 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 from these values. Nomenclature for the centromeric position on the chromosome was according to Levan et al. (1964) based on arm index (1.0–1.7 – median chromosomes; 1.7–3.0 – submedian; 3.0–7.0 – subterminal; 7.0–∞ – terminal). Positioning of *T. gesneriana* and *T. fosteriana* chromosomes within the karyotype was based on the total chromosome length and centromeric index. Darwin hybrid tulips chromosomes were ordered within the karyotype according to the total length. To identify which parents provide the largest three median chromosomes to Darwin hybrid tulips (Fig. 2), a discriminant function were calculated using data of the largest median chromosome exsiting in *T. gesneriana* and *T. fosteriana* (Hair et al., 1984) by statistics computer software (ESUMI Co. Ltd., Japan). Data of short arm length and total chromosome length of the largest median chromosome were collected from five plants per cultivar of *T. gesneriana*, *T. fosteriana* and Darwin hybrid tulips (except for 'Spring Song') listed in Table 1.

DNA probes

For GISH, total genomic DNA from 'Red Emperor' and 'Queen of Night' was extracted from the leaves using the standard cetyltrimethylammonium (CTAB) method (Murray & Thompson, 1980). DNA was sonicated to 1–10-kb fragments and labeled by nick translation with either tetramethyl-rodamine-5-dUTP or digoxigenin-11-dUTP (Roche).

GISH

The *in situ* hybridization protocol was carried out according to Hasterok et al. (2001) with minor modifications. Slides were pre-treated with RNase A (100 μg/ml) for 1 h at 37 °C, then washed in 2× SSC, post-fixed in 1% formaldehyde in PBS buffer for 10 min, washed again in 2× SSC and dehydrated in absolute ethanol. The hybridization mixture consisted of 50% deionized formamide, 10% dextran sulphate, 2× SSC, 1% SDS and probe DNA (200 ng of each per slide). No unlabeled genomic DNA was used as a block in the GISH experiment on Darwin hybrid tulip 'Yellow Dover' (we found that 30–75× excess of *T. gesneriana* DNA did not affect the GISH result with Darwin hybrid 'Yellow Dover'). Chromosome preparations and pre-denatured probes (incubation at 75 °C for 10 min) were denatured at 70 °C for 10 min and allowed to hybridize overnight in a humid chamber at 37 °C. The post-hybridization washes were carried out for 10 min in 20% deionized formamide in 0.1× SSC at 42 °C, followed by washes in 2× SSC. The digoxigenin probe was immunodetected using the standard protocol with FITC-conjugated anti-digoxigenin (Roche). The chromosomes were counterstained with 1 μg/ml DAPI (Sigma) in Vectrashield (Vectra Laboratories). At least ten metaphases were examined for hybridization. Images of fluorescently stained chromosomes were acquired using a digital camera attached to

Table 1 Quantitation of tulip nuclear DNA by flow cytometry

Species	Cultivars	Ploidy	Relative DAPI fluorescent values (2C)	SE
T. gesneriana	Queen of Night	2x	153	1.0
	Christmas Dream	2x	144	0.7
	Negrita	2x	151	0.9
	Preludium	2x	146	1.0
	Ile de France	2x	148	1.1
	Mean		148	3.5
T. fosteriana	Red Emperor	2x	116	0.7
	Cantata	2x	120	1.4
	Easter Parade	2x	120	0.8
	Princeps	2x	119	1.2
	Zombie	2x	116	0.8
	Mean		118	3.5
	Spring Song	2x	127	1.1
Darwin hybrid tulips	Come Back	3x	210	3.0
	Diplomate	3x	201	1.4
	Pink Impression	3x	210	1.4
	Red Matador	3x	211	0.6
	Yellow Dover	3x	211	0.8
	Mean[a]		209	4.2

[a]Mean was calculated using values of triploid Darwin hybrid tulips.

a microscope (BX51N, Olympus, Japan) with an appropriate filter and then processed using software (DP Manager, Olympus, Japan).

Results

Relative DNA content and karyological analysis

DAPI fluorescent values obtained by flow cytometric analysis were used to assess relative DNA content of tulips (Table 1). *T. gesneriana* cultivars had higher DNA content compared with *T. fosteriana* cultivars. Mean DAPI fluorescent value (relative to *Hosta* DNA content, which was defined as 50; Okazaki et al., 2005) of *T. gesneriana*, *T. fosteriana* and Darwin hybrid tulips was 148.4 ± 3.5, 118.2 ± 3.5 and 208.7 ± 4.2, respectively. The diploid cultivar 'Spring Song', obtained from the cross between *T. gesneriana* and *T. fosteriana* 'Red Emperor', had relative DAPI fluorescent value of 127 ± 1.1.

Figure 1 (panels a–c) shows karyotypes of *T. gesneriana* 'Queen of Night', *T. fosteriana* 'Red Emperor' and Darwin hybrid tulip 'Yellow Dover'. Detailed morphometric analysis of diploid cultivars revealed a difference in the total length of all metaphase chromosomes ranging from 297.9 μm for 'Queen of Night' to 262.1 μm for 'Red Emperor' (Tables 2 and 3). Between these genotypes, differences in chromosome size (length and width) of particular chromosomes were also observed (Fig. 1a,b). For instance, the difference in the length of the longest matching chromosomes between genotypes was 2.5 μm, and the difference in the length of the shortest matching chromosomes was 0.6 μm. This reflects the relative DNA content of 152.9 ± 1.0 for 'Queen of Night' and 116.4 ± 0.7 for 'Red Emperor'. The karyotypes of *T. gesneriana* and *T. fosteriana* were very similar in shape, consisting of median, submedian and subterminal chromosomes (Levan et al., 1964) (Table 4), and most chromosomes did not have the marker features e.g. secondary constrictions required to distinguish one from another without physical measurements. In both genotypes, homologous chromosome polymorphism was observed, which was reflected in slight differences in the arm length. Only the median chromosomes were easily recognizable. Discriminant analysis using the length of the short arm and the total chromosome length of the two largest median chromosomes of *T. gesneriana* and *T. fosteriana* revealed a significant difference in this factor between these genotypes; the percentage correctly classified the two groups was 83.3% (Fig. 2).

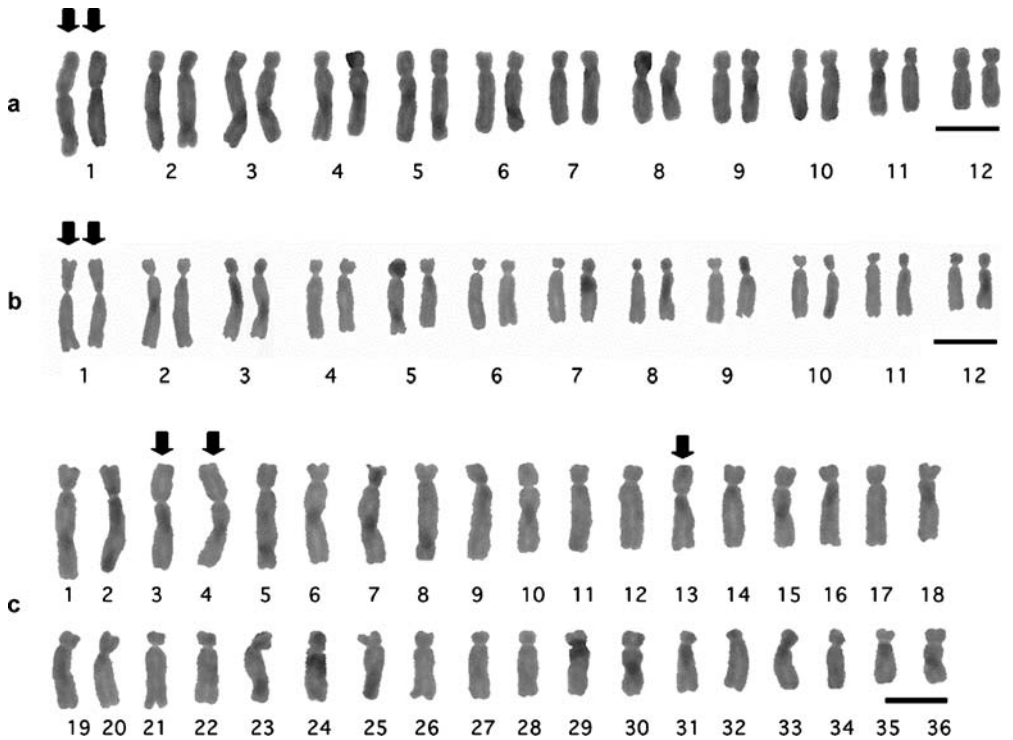

Fig. 1 Karyotypes of *T. gesneriana* 'Queen of Night'(a), *T. fosteriana* 'Red Emperor' (b), and triploid Darwin hybrid tulip 'Yellow Dover' (c). Arrows indicate median chromosomes subjected to discriminant analysis. All bars represent 10 μm

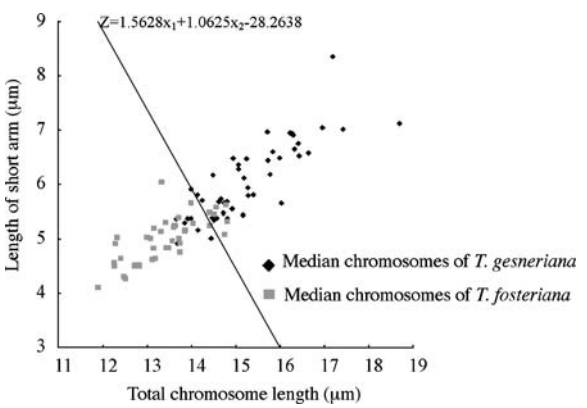

Fig. 2 Discriminant analysis regarding the median chromosomes of *T. gesneriana* and *T. fosteriana* using short arm length and total chromosome length of the two median chromosomes in 10 cultivars of *T. gesneriana* and *T. fosteriana* shown in Table 1. Z represents the discriminant function

Darwin hybrids karyotyped in this report had chromosome number $3x = 36$. The diploid Darwin hybrid 'Spring Song' was only analyzed by flow cytometry. Among the Darwin hybrids analyzed, only the morphometric characteristics for 'Yellow Dover' are presented (Table 4, Fig. 1c). Although the total length, relative length, centromeric index and arm ratio were compared and the karyograms were arranged for each cultivar, it was difficult to match the chromosomes unambiguously. In hybrids, the total length of all metaphase chromosomes ranged from 430 μm for 'Pink Impression' to 446.0 μm for 'Come Back'. The difference in chromosome size was 1.5 μm between the longest matching chromosomes and 0.6 μm between the shortest matching chromosomes. The longest chromosomes of 'Come Back', 'Diplomate' and 'Pink Impression' were highly similar in length (16.2 μm 16.5 μm and 16.2 μm, respectively)**.** The chromosome types in the triploid cultivars are presented in Table 4. Like in *T. gesneriana* and *T. fosteriana*, only the median chromosomes were easily recognizable in Darwin hybrids. Fig. 3 shows the relationship between short arm length and total chromosome length of the three largest median chromosomes of Darwin hybrid tulips in three metaphase plates. For each Darwin hybrid tulip, there are two groups of chromosomes markedly different in

Table 2 Chromosome characteristics in *T. gesneriana* 'Queen of Night'

Chr. No	p[a] (μm)	q[b] (μm)	p+q (μm)	R L[c] (%)	Cen. index	p/q	Type
1	6.7	9.7	16.4	5.5	40.9	1.4	m[d]
2	5.7	9.0	14.7	4.9	38.8	1.6	m
3	3.1	12.2	15.3	5.1	20.3	3.9	st[f]
4	3.3	11.7	15.0	5.0	22.0	3.5	st
5	2.9	11.5	14.4	4.8	20.1	4.0	st
6	3.0	11.4	14.4	4.8	20.8	3.8	st
7	3.9	10.2	14.1	4.7	27.6	2.6	sm[e]
8	4.0	9.9	13.9	4.7	28.8	2.5	sm
9	3.0	10.9	13.9	4.7	21.6	3.6	st
10	2.7	10.3	13.0	4.4	20.8	3.8	st
11	2.8	9.8	12.6	4.2	22.2	3.5	st
12	2.8	9.2	12.0	4.0	23.3	3.3	st
13	2.7	8.7	11.4	3.8	23.7	3.2	st
14	2.5	8.7	11.2	3.8	22.3	3.6	st
15	4.1	7.0	11.1	3.7	36.9	1.7	m
16	3.2	8.0	11.2	3.8	28.6	2.5	sm
17	2.7	8.6	11.3	3.8	23.9	3.2	st
18	2.6	8.5	11.1	3.7	23.4	3.3	st
19	3.0	8.0	11.0	3.7	27.3	2.7	sm
20	2.4	8.4	10.8	3.6	22.2	3.5	st
21	3.1	7.4	10.5	3.5	29.5	2.4	sm
22	2.6	7.3	9.9	3.3	26.3	2.8	sm
23	3.1	6.4	9.5	3.2	32.6	2.1	sm
24	2.4	6.8	9.2	3.1	26.1	2.8	sm
Total		297.9					

[a]Short arm, [b]Long arm, [c]Relative length, [d]Median chromosomes, [e]Submedian chromosomes, [f]Subterminal chromosomes.

size. One group consists of three smaller chromosomes and the other group from six bigger chromosomes, suggesting the smaller chromosomes and the bigger ones were derived from *T. gesneriana* and *T. fosteriana*, respectively. In addition, the discriminant function $Z = 1.5628x_1 + 1.0625x_2 - 28.2623$ obtained from Fig. 2 was used to analyze the data for the short arm length and the total chromosome length obtained from the three largest median chromosomes of Darwin hybrid tulips (Z = discriminant score, variable x_1 = total chromosome length, variable x_2 = short arm length). The resulting discriminant values confirmed that two larger chromosomes and one smaller chromosome in Darwin hybrid tulips were derived from *T. gesneriana* and *T. fosteriana*, respectively, suggesting that all of the Darwin hybrid tulips used have two copies of the *T. gesneriana* genome and one copy of the *T. fosteriana* genome.

GISH in triploid hybrids

Figure 4a shows the results of hybridization of total genomic DNA from 'Queen of Night' to somatic chromosomes of the same cultivar (green fluorescence). Hybridization signals were observed for each chromosome, localized in the telomeric and pericentromeric regions. A few bands were also observed at intercalar positions on the chromosomes. In contrast, hybridization of total genomic DNA from 'Red Emperor' to somatic chromosomes of 'Red Emperor' yielded uniform "painting" of the entire length of all 24 chromosomes (data not shown). Fig 4b shows two-colour GISH on metaphase chromosomes of Darwin hybrid tulip 'Yellow Dover'. Simultaneous application of 'Queen of Night' (green) and 'Red Emperor' (red) total genomic DNA probes enabled us to distinguish the parental genotype. As expected from the karyological analysis, triploid Darwin hybrid tulips contained the diploid genome of *T. gesneriana* and the single genome of *T. fosteriana.* Of the 36 Darwin hybrid tulip chromosomes, twelve chromosomes derived from 'Red Emperor' were uniformly painted red, whereas the remaining chromosomes showed a hybridization pattern similar to that observed with 'Queen of Night'.

Table 3 Chromosome characteristics in *T. fosteriana* 'Red Emperor'

Chr. No	p (μm)	q (μm)	p+q (μm)	R L (%)	Cen. index	p/q	Type
1	5.2	8.7	13.9	5.3	37.4	1.7	m
2	5.2	8.5	13.7	5.2	38.0	1.6	m
3	2.6	11.1	13.7	5.2	19.0	4.3	st
4	2.6	11.0	13.6	5.2	19.1	4.2	st
5	2.5	10.2	12.7	4.8	19.7	4.1	st
6	2.5	9.9	12.4	4.7	20.2	4.0	st
7	2.3	9.9	12.2	4.7	18.8	4.3	st
8	2.8	8.8	11.6	4.4	24.1	3.1	st
9	2.2	9.0	11.2	4.3	19.6	4.1	st
10	2.2	8.7	10.9	4.2	20.2	3.9	st
11	2.3	8.3	10.6	4.0	21.7	3.6	st
12	1.9	8.4	10.3	3.9	18.4	4.4	st
13	2.0	8.2	10.2	3.9	19.6	4.1	st
14	2.6	7.4	10.0	3.8	26.0	2.8	sm
15	2.1	7.8	9.9	3.8	21.2	3.7	st
16	2.1	7.8	9.9	3.8	21.2	3.7	st
17	1.6	8.2	9.8	3.7	16.3	5.1	st
18	2.1	7.6	9.7	3.7	21.6	3.6	st
19	2.2	7.5	9.7	3.7	22.7	3.4	st
20	2.8	6.8	9.6	3.7	29.2	2.4	sm
21	2.2	7.4	9.6	3.7	22.9	3.4	st
22	1.9	7.6	9.6	3.7	19.8	4.0	st
23	1.9	6.8	8.7	3.3	21.8	3.6	st
24	2.2	6.4	8.6	3.3	25.6	2.9	sm
Total		262.1					

Table 4 Number and type of chromosomes observed in analyzed genotypes[a]

Species	Cultivars	Type of chromosome		
		Median	Submedian	Subterminal
T. gesneriana	Queen of Night	3	8	13
	Christmas Dream	3	8	13
	Negrita	3	12	9
	Preludium	3	14	7
	Ile de France	3	8	13
T. fosteriana	Red Emperor	2	3	19
	Cantata	1	5	18
	Easter Parade	2	7	15
	Princeps	2	5	17
	Zombie	1	2	21
Darwin hybrid tulips	Come Back	3	12	21
	Diplomate	4	18	14
	Pink Impression	4	9	23
	Red Matador	5	10	21
	Yellow Dover	3	8	25

[a]According to Levan, 1964

Table 5 Chromosome characteristics in Darwin hybrid tulips 'Yellow Dover'

Chr. No	p (μm)	q (μm)	p+q (μm)	R L (%)	Cen. index	p/q	Type
1	3.9	13.8	17.7	4.0	22.0	3.5	st
2	5.0	12.1	17.1	3.8	29.2	2.4	sm
3	6.2	10.3	16.5	3.7	37.6	1.7	m
4	6.3	10.2	16.5	3.7	38.2	1.6	m
5	4.1	12.3	16.4	3.7	25.0	3.0	sm
6	2.7	12.8	15.5	3.5	17.4	4.7	st
7	3.1	12.3	15.4	3.5	20.1	4.0	st
8	3.0	12.3	15.3	3.4	19.6	4.1	st
9	3.4	11.8	15.2	3.4	22.4	3.5	st
10	3.6	10.5	14.1	3.2	25.5	2.9	sm
11	3.0	10.7	13.7	3.1	21.9	3.6	st
12	2.7	10.6	13.3	3.0	20.3	3.9	st
13	4.9	8.3	13.2	3.0	37.1	1.7	m
14	3.1	9.9	13.0	2.9	23.8	3.2	st
15	3.1	9.6	12.7	2.9	24.4	3.1	st
16	2.6	10.1	12.7	2.9	20.5	3.9	st
17	3.0	9.6	12.6	2.8	23.8	3.2	st
18	2.8	9.6	12.4	2.8	22.6	3.4	st
19	2.5	9.9	12.4	2.8	20.2	4.0	st
20	3.5	8.8	12.3	2.8	28.5	2.5	sm
21	2.1	9.9	12.0	2.7	17.5	4.7	st
22	2.6	8.9	11.5	2.6	22.6	3.4	st
23	2.5	8.9	11.4	2.6	21.9	3.6	st
24	2.6	8.2	10.8	2.4	24.1	3.2	st
25	2.3	8.4	10.7	2.4	21.5	3.6	st
26	2.4	8.1	10.5	2.4	22.6	3.4	st
27	1.9	8.6	10.5	2.4	18.1	4.5	st
28	2.3	8.1	10.4	2.3	22.1	3.5	st
29	2.5	7.9	10.4	2.3	24.0	3.2	st
30	2.6	7.7	10.3	2.3	25.2	3.0	sm
31	1.7	7.8	9.5	2.1	17.9	4.6	st
32	1.8	7.6	9.4	2.1	19.1	4.2	st
33	2.4	6.9	9.3	2.1	25.8	2.9	sm
34	2.3	6.9	9.2	2.1	25.0	2.9	sm
35	1.8	6.8	8.6	1.9	20.9	3.8	st
36	2.3	6.2	8.5	1.9	27.1	2.7	sm
Total		444.4					

Discussion

Polyploidization in Darwin hybrid tulips

Spontaneously occurring triploids resulting from unreduced gametes have been recorded in many species, e.g., *Hordeum* (Sandfacer, 1975), *Malus* (Chyi & Weeden, 1984), *Lilium* (Noda, 1986), tomato (Kagan-Zur et al., 1991) and *Crocus* (Ørgaard et al., 1995). In tulips, natural polyploidization occurs in crosses of *T. gesneriana* × *T. fosteriana* when *T. fosteriana* 'Red Emperor' is primarily used as the male parent; moreover, 'Red Emperor' seems to supply the unreduced gametes (Kroon & Van Eijk, 1977). In the present study, however, we demonstrated that all of the Darwin hybrid tulips tested have two copies of the *T. gesneriana* genome and one copy of the *T. fosteriana* genome, indicating that *T. gesneriana* has supplied the diploid gamete. Lefeber (1960) reported that 'Lefeber's Favourite' and 'Windsor' were generated from the crosses of the diploid Darwin tulip 'Copland' × *T. fosteriana* 'Red Emperor' and diploid 'Pride of Haarlem' × 'Red Emperor', respectively, but the parents of other Darwin hybrid tulips are unknown. It

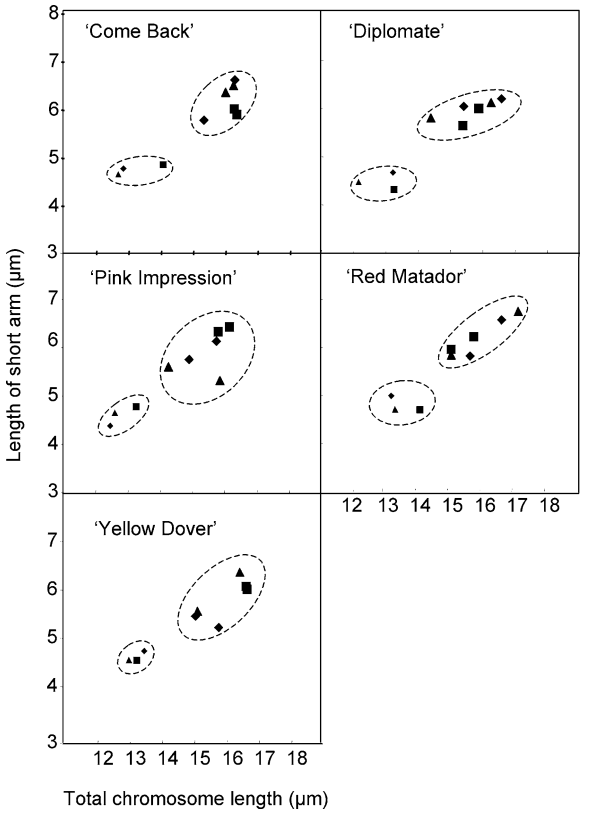

Fig. 3 Relationship between short arm length and total chromosome length of the three median chromosomes of Darwin hybrid tulips. ■, ▲ and ♦ represent data for the median chromosome for three different methaphases

is thought that diploid cultivars of *T. gesneriana* have been used as the parent of other Darwin hybrid tulips because most *T. gesneriana* cultivars are diploid and only a few are tetraploid. However there is a need to determine if tetraploid cultivars (e.g., 'Mrs. John T. Scheepers') were used to develop Darwin hybrid tulips.

Relative DNA content

Flow cytometry has been frequently used for hybrid identification (Nakamura et al., 2005; Trucco et al., 2005). In this study, the relative DNA content of diploid Darwin hybrid tulip 'Spring Song', obtained from the cross of *T. gesneriana* and *T. fosteriana*, was within the intermediate range between *T. gesneriana* and *T. fosteriana*. Since triploid Darwin hybrid tulips have two copies of the *T. gesneriana* genome and one copy of the *T. fosteriana* genome, the theoretical relative DNA content of triploid Darwin hybrid tulips can be calculated by adding the 2C value (148) of *T. gesneriana* and the 1C value (59) of *T. fosteriana*, resulting in a value of 207. This value is very close to the 2C value of Darwin hybrid tulips obtained by flowcytometry (208.7 ± 4.2).

Karyology of tulips

Karyology of tulips is well-documented with respect to chromosome number (Sayama et al., 1982; Van Raamsdonk & De Vries, 1995) and C-banding (Filion, 1974; Van Raamsdonk & De Vries, 1995). The latter technique was used to analyze diploid Darwin hybrid 'Spring Song'; all banding patterns, however, occur as homologous pairs (Filion, 1974), and thus this technique could not be used to analyze phylogenetic relationships. Similarly, Van Raamsdonk & De Vries (1995) could not establish a relationship between C-band position in different species, although the pattern appeared to be unique for each species.

In the present work, karyotypes were presented both for *T. gesneriana* and *T. fosteriana* cultivars and triploid Darwin hybrid tulips after aceto-orcein staining. We observed no remarkable chromosome landmarks, such as secondary construction, in the two species. In addition, FISH of 5S and 45S rDNA failed to discriminate chromosomes of *T. gesneriana* and *T. fosteriana* (data not shown). However, detailed analysis of chromosome morphology using discriminant analysis revealed the difference between *T. gesneriana* and *T. fosteriana* cultivars when applied to their median chromosomes (Fig. 1). This finding was used to identify the genome constitution in Darwin hybrid tulips. In other studies, when there were morphological differences in single-chromosome morphology between parental genotypes, hybrids were readily verified in genus *Allium* (Keller et al., 1996) and *Lilium* (North & Wills, 1969; Okazaki et al., 1994; Fernandez et al., 1996; Obata et al., 2000; Marasek & Orlikowska, 2001). Chromosome size also served as a trait for verification of hybrids between *Cucurbita pepo* L. and *Cucurbita martinenzii* (Metwally et al., 1996) and somatic hybrids *Oriza sativa* + *Hordeum vulgare* (Kisaka et al., 1998). On the other hand, when no large morphological differences between parental chromosomes are observed, as in the case of Darwin hybrid tulips, we have shown that discriminant analysis of chromosome size is useful to identify small differences in chromosome size.

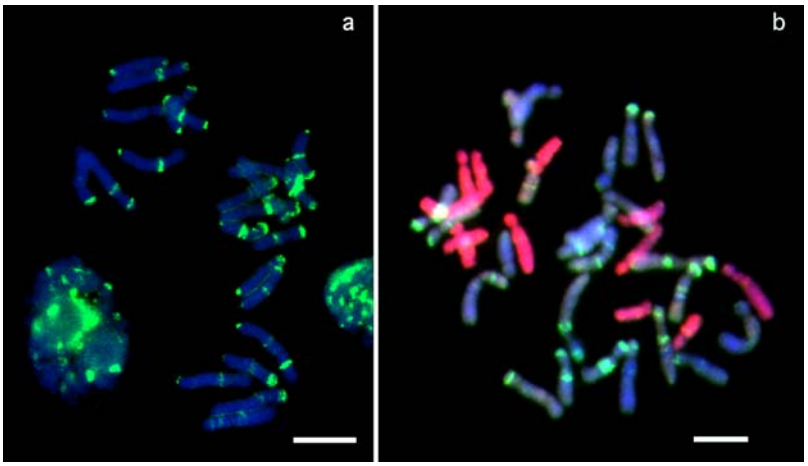

Fig. 4 *In situ* hybridization of total genomic DNA of 'Queen of Night' (green fluorescence) to somatic metaphase chromosomes of 'Queen of Night' (a). Double-target *in situ* hybridization of total genomic DNA of 'Queen of Night' (green fluorescence) and 'Red Emperor' (red fluorescence) to somatic metaphase chromosomes of Darwin hybrid 'Yellow Dover' ($3x = 36$) (b). The chromosomes were counterstained with DAPI (blue). Bar represents 10 μm

GISH

GISH is a powerful method that has been used to identify many hybrids and allopolyploid species. We have now demonstrated that GISH is a suitable technique to differentiate chromosomes, because simultaneously applied total genomic DNA probes from two cultivars clearly distinguished the parental origin of chromosomes in Darwin hybrid tulips. Cross-hybridization between genomes comprising the hybrid was not observed even though no blocking DNA was used in the experiments, indicating that *T. gesneriana* and *T. fosteriana* may be distantly related species. Similarly, discrimination between genomes in the absence of blocking DNA was also possible in interspecific hybrids of *Nicotiona rustica* L. × *N. tabaccum* L. (Kitamura et al., 1997), in *Brassica juncea* (Maluszynska & Hasterok, 2005) and in *Brachypodium* polyploid species (Hasterok et al., 2004). In our experiment with Darwin hybrids, the uniform distribution of the fluorescent probe DNA along entire chromosomes was specific for 12 chromosomes derived from 'Red Emperor'. The remaining 24 chromosomes showed strongly labelled bands that resembled *in situ* hybridization with genomic DNA of 'Queen of Night' to somatic chromosomes of the same cultivar. In both 'Queen of Night' and Darwin hybrid tulips, signals were predominantly concentrated in telomeric and pericentromeric regions of chromosomes. Since GISH relies largely on hybridization of the probe to repetitive DNA sequences, the uniform labeling of chromosomes may indicate that these sequences are dispersed uniformly throughout the genome of 'Red Emperor'. In contrast, it seems that repetitive DNA families are not evenly distributed along chromosomes in 'Queen of Night' but rather highly concentrated in distal parts of the chromosome arms and in pericentromeric regions. However, further studies are necessary to confirm this hypothesis. Similarly, non-uniform hybridization of total genomic DNA was reported primarily in species with small genomes; e.g., in *Brassica* (Snowdon et al., 1997; Maluszynska & Hasterok, 2005), rice (Fukui et al., 1997; Li et al., 2001), and *Brachypodium* (Hasterok et al., 2004) but also in *Alsroemeria* (Kuipers et al., 1997).

Since Darwin hybrid tulips have been only spontaneously generated by interspecific crosses between Darwin tulips and *T. fosteriana*, it has been impossible to breed them systemically. Our findings suggest that the unreduced gamete must be supplied from *T. gesneriana* to develop Darwin hybrid tulips. Thus, it is necessary to screen *T. gesneriana* cultivars producing unreduced gametes or to apply 2n pollen of *T. gesneriana* induced with N_2O treatment, as reported by Okazaki et al. (2005), in interspecific breeding of Darwin hybrid tulips.

Acknowledgements This work was partially supported by the Japanese Society for the Promotion of Science (JSPS fellowship award to Agnieszka Marasek, 2005–2007).

References

Anonymous (1968) Kruisingouderslijst tulpen. Koninklijke Algemeene Vereeniging voor Bloembollencultuur (KAVB). Haarlem

Barba-Gonzales R, Lokker AC, Lim KB, Ramanna MS, Van Tyul JM (2004) Use of 2n gametes for the production of sexual polyploids from sterile Oriental × Asiatic hybrids of lilies (*Lilium*). Theor Appl Genet 109:1125–1132

Bryan JE (2002) Bulbs. Timber Press, Inc. US

Chyi YS, Weeden NF (1984) Relative isozyme band intensities permit the identification of the 2n gamete parent of triploid apple cultivars. Hort Sci 19:818–819

Doorenbos J (1954) Notes on the history of bulb breeding in the netherlands. Euphytica 3:1–11

Fernandez AM, Nakazaki T, Tanisaka T (1996) Development of diploid and triploid interspecific hybrids between *Lilium longiflorum* and *L. concolor* by ovary slice culture. Plant Breed 115:167–171

Filion WG (1974) Differential giemsa staining in plants. I. Banding patterns in three cultivars of *Tulipa*. Chromosoma 49:51–60

Fukui K, Shishido R, Kinoshita T (1997) Identification of the rice D-chromosomes by genomic *in situ* hybridization. Theor Appl Genet 95:1239–1245

Hair JF, Anderson RE Jr, Tatham RL, Grablowsky BJ (1984) Multivariate data analysis. Macmillan Publishing Co. US

Hasterok R, Jenkins G, Longdon T, Jones RN, Maluszynska J (2001) Ribosomal DNA is an effective marker of *Brassica* chromosomes. Theor Appl Genet 103:486–490

Hasterok R, Draper J, Jenkins G (2004) Laying the cytotaxonomic foundations of a new model grass, *Brachypodium distachyon* (L.) Beauv. Chromosome Res 12:397–403

Hasterok R, Wolny E, Kulak S, Zdziechowicz A, Maluszynska J, Heneen WK (2005) Molecular cytogenetic analysis of *Brassica rapa-Brassica oleracea* var. *alboglabra* monosomic addition lines. Theor Appl Genet 111:196–205

Heimburger M (1962) Comparison of chromosome size in species of *Anemone* and their hybrids. Chromosoma 13:329–340

Kagan-Zur V, Mizrahi Y, Zamir D, Navot N (1991) A tomato triploid hybrid whose double genome parent is the male. J Amer Soc Hort Sci 116:342–345

Karlov GI, Khrustaleva LI, Lim KB, Van Tuyl JM (1999) Homoeologous recombination in 2n-gametes producing interspecific hybrids of *Lilium* (Liliaceae) studied by genomic in situ hybridization (GISH). Genome 42:681–1999

Kato A (1997) An improved method for chromosome counting in maize. Biotech Histochem 72:249–252

Kato A, Lamb JC, Birchler JA (2004) Chromosome painting using repetitive DNA sequences as probes for somatic chromosome identification in maize. Proc Natl Acad Sci USA 101:13554–13559

Keller ERJ, Schubert I, Fuchs J, Meister A (1996) Interspecific crosses of onion with distant *Allium* species and characterization of the presumed hybrids by means of flow cytometry, karyotype analysis and genomic *in situ* hybridization. Theor Appl Genet 92:417–424

Kho YO, Baer J (1971) Incompatibility problems in species crosses of Tulips. Euphytica 20:30–35

Kisaka H, Kisaka M, Kanno A, Kameya T (1998) Intergeneric somatic hybridization of rice (*Oriza sativa* L.) and barley (*Hordeum vulgarae* L.) by protoplast fusion. Plant Cell Rep 17:362–367

Kitamura S, Inoue M, Ohimido N, Fukui K (1997) Identification of parental chromosomes in the interspecific hybrids of *Nicotiana rustica* L. × *N. tabacum* L. and *N. gossei* Domin × *N. tabacum* L., using genomic in situ hybridization. Breed Sci 47:67–70

Kroon GH, Van Eijk JP (1977) Polyploidy in tulips (*Tulipa* L.). The occurrence of diploid gametes. Euphytica 26:63–66

Kuipers AGJ, Van Os DPM, de Jong JH, Ramanna MS (1997) Molecular cytogenetics of *Alstroemeria*: identification of parental genomes in interspecific hybrids and characterization of repetitive DNA families in constitutive heterochromatin. Chromosome Res 5:31–39

Lefeber DW (1960) Raising and introducing new tulips. Daffodil and Tulip, Year Book, pp 16–24

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position of chromosomes. Hereditas 52:201–220

Li CB, Zhang DM, Ge S, Lu BR, Hong DY (2001) Identification of genome constitution of *Oriza malampuzhaensis*, *O. minuta*, and *O. punctata* by multicolour genomic in situ hybridization. Theor Appl Genet 103:204–211

Lim KB, Chung JD, Van Kronenburg BCE, Ramanna MS, de Jong JH, Van Tuyl JM (2000) Introgression of *Lilium rubellum* Baker chromosomes into *L. longiflorum* Thunb.: a genome painting study of the F1 hybrid, BC1 and BC2 progenies. Chromosome Res 8:119–125

Lim KB, Wennekes J, de Jong JH, Jacobsen E, Van Tyul JM (2001a) Karyotype analysis of *Lilium rubellum* and *Lilium longiflorum* by chromosome banding and fluorescence in situ hybridization. Genome 44:911–918

Lim KB, Ramanna MS, de Jong JH, Jacobsen E, Van Tyul JM (2001b) Intermediate meiotic restitution (IMR): a novel type of meiotic nuclear restitution mechanism detected in interspecific lily hybrids by GISH. Theor Appl Genet 103:119–130

Lim KB, Ramanna MS, Van Tyul JM (2003) Homoeologous recombination in interspecific hybrids of *Lilium*. Korean J Breed 35:8–12

Maluszynska J, Hasterok R (2005) Identification of individual chromosomes and parental genomes in *Brassica juncea* using GISH and FISH. Cytogenet Genome Res 109:310–314

Marasek A, Hasterok R, Orlikowska T (2004a) The use of chromosomal markers linked with nucleoli organisers for F1 hybrid verification in *Lilium*. Acta Horticulturae 651:77–82

Marasek A, Hasterok R, Wiejacha K, Orlikowska T (2004b) Determination by GISH and FISH of hybrid status in *Lilium*. Hereditas 140:1–7

Marasek A, Orlikowska T (2001) Distant lily hybrids identification based on chromosomal markers. Biotechnologia 3:243–248 (In Polish)

Metwally EI, Haruon SA, El-Fadly GA (1996) Interspecific cross between *Cucurbita pepo* L. and *Cucurbita martinenzii* through *in vitro* embryo culture. Euphytica 90:1–7

Murray MG, Thompson WF (1980) Rapid isolation of high-molecular-weight plant DNA. Nucleic Acid Res 8:4321–4325

Nakamura T, Kuwayama S, Tanaka S, Oomiya T, Saitou H, Nakano M (2005) Production of intergeneric hybrid plants between Sandersonia aurantiaca and Gloriosa rothschildiana via ovule culture (Colchicaceae). Euphytica 142:283–289

Nassar NMA, Vieira MA, Vieira C, Grattapaglia D (1998) A molecular and embryonic study of apomixis in cassava (*Manihot esculenta* Crantz). Euphytica 102:9–13

Noda S (1986) Cytogenetic behavior, chromosomal differentiations, and geographic distribution in *Lilium lancifolium* (Liliaceae). Plant Spec Biol 1:69–78

North C, Wills AB (1969) Interspecific hybrids of *Lilium lankongense* Franchet produced by embryo culture. Euphytica 18:430–434

Obata Y, Niimi Y, Nakano M, Okazaki K, Miyajima I (2000) Interspecific hybrids between *Lilium nobillissimum* and *L. regale* produced via ovules with placenta culture. Scientia Horticulturae 84:191–204

Okazaki K, Asano Y, Oosawa K (1994) Interspecific hybrids between *Lilium* 'Oriental' hybrid and *L.* 'Asiatic' hybrid produced by embryo culture with revised media. Breed Sci 44:59–64

Okazaki K, Kurimoto K, Miyajima I, Enami A, Mizuochi H, Matsumoto Y, Ohya H (2005) Induction of 2n pollen in tulips by arresting the meiotic process with nitrous oxide gas. Euphytica 143:101–114

Ørgaard M, Jacobsen N, Heslop-Harrison JS (1995) The hybrid origin of two cultivars of *Crocus* (Iridaceae) analyzed by molecular cytogenetics including genomic southern and in situ Hybridization. Ann Bot 76:25–262

Ran Y, Murray BG, Hammett KRW (1999) Karyotype analysis of the genus *Clivia* by Giemsa and fluorochrome banding and in situ hybridization. Euphytica 106:139–147

Ran Y, Keith R, Hammett W, Murray BG (2001) Hybrid identification in *Clivia* (Amaryllidaceae) using chromosomes banding and genomic in situ hybridization. Ann Bot 87:457–462

Rustanius P, Griesbach RJ, Gross KC, Line M (1991) Chromosome analysis of *Alstroemeria ligtu* hybrids. HortScience 26:902–904

Sandfacer J (1975) The occurrence of spontaneous triploids in different barley varieties. Hereditas 80:149–153

Sayama H, Moug T, Nishimura Y (1982) Cytological study in *Tulipa gesneriana* and *T. fosteriana*. Jpn J Breed 32:26–34

Smyth DR, Kongsuwan K, Wisudharomn S (1989) A survey of C-band patterns in chromosomes of *Lilium*. Plant Syst Evol 163:53–69

Smyth DR (19990. *Lilium* chromosomes. The Lily Yearbook NALS 52:66–76

Snowdon RJ, Köhler W, Friedt W, Köhler A (1997) Genomic in situ hybridization in *Brassica* amphidiploids and interspecific hybrids. Theor Appl Genet 95:1320–1324

Srebniak M, Rasmussen O, Maluszynska J (2002) Cytogenetic analysis of asymetric potato hybrid. J Appl Genet 43:19–31

Trucco F, Jeschke MR, Rayburn AL, Tranel PJ (2005) *Amaranthus hybridus* can be pollinated frequently by *A. tuberculatus* under field conditions. Heredity 94:64–70

Van Raamsdonk LWD, De Vries T (1995) Species relationships and taxonomy in *Tulipa* subg. Tulipa (*Liliaceae*). Plant Syst Evol 195:13–44

Van Scheepen J (1996) Classified list and international register of tulip names. General Bulbgrowers' Association, Holland

Van Tuyl JM, Van Creij MGM (2005) *Tulipa gesneriana* and *Tulipa* hybrids. In: Flower Breeding and Genetics: Issues, Challenges and Opportunities for 21st Century, Chapter 23, Springer Verlag, pp 613–637