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Breeding and Cytogenetics in the Genus *Tulipa*

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ABSTRACT

Tulip (*Tulipa*) is one of the most important ornamental bulbous plants, which has been cultivated for cut flower, potted plant, garden plant and for landscaping. Species from the different sections display complementary agronomic characteristics and breeding techniques are used to combine desired features. The main goals of modern tulip breeding are the introgression of resistance against Tulip Breaking Virus (TBV), *Botrytis tulipae* and *Fusarium oxysporum* (bulb-rot), and also characteristics such as a short forcing period, good flower longevity and new flower colours and flower shapes into the commercial assortment of *T. gesneriana*. *T. gesneriana* has been crossed successfully with only 12 out of the approximately 55 tulip species by using conventional breeding methods. Many successful crosses have been made between *T. gesneriana* cultivars and TBV resistant *T. fosteriana* cultivars resulting in highly resistant Darwin hybrids tulips. The majority of tulip cultivars are diploid ($2n = 2x = 24$) however, there have been many attempts to obtain polyploid tulips. The production of tetraploids was described in the late sixties when young ovaries were treated, under pressure, with laughing gas (N_2O). In breeding of polyploid tulip laughing gas has also been used to induce 2*n* gametes. Several new tetraploids were also obtained by making crosses between tetraploid lines. Polyploids have been derived from interploidy crosses between diploid, triploid, and tetraploid cultivars. Several other polyploids have resulted from 2*n* gametes, spontaneously produced by diploid F1 hybrids. Molecular cytogenetic tools such as FISH and GISH permitted detailed studies of genome composition and chromosome recombination in the progenies of interspecific hybrids. In this context, tulip breeding and the use of cytogenetic techniques for genome analysis of hybrids are discussed.

Keywords: breeding, chromosome analysis, GISH, intergenomic recombination, polyploidization, *Tulipa*
Abbreviations: BC, back cross; **FISH**, fluorescence *in situ* hybridization; **GISH**, genomic *in situ* hybridization; **G**, *T. gesneriana*; **F**, *T. fosteriana*; **GF**, *T. gesneriana* × *T. fosteriana*; **PMC**, pollen mother cell; **TBV**, *Tulip breaking virus*; **rDNA**, ribosomal DNA

CONTENTS

INTRODUCTION

General information on the genus *Tulipa*, systematics, species and main cultivated groups

Tulip originated from the Pamir Alai and Tien Shan mountain ranges in Central Asia (Hoog 1973). It was introduced into Europe from Turkey in the 16th century (Killingback 1990) and with the passage of time become one of the most important ornamental crops. The genus *Tulipa* belongs to the Liliaceae family. The number of species reported in literature range from about 40 (Stork 1984) to more than 100 (Hall 1940; Botschantzeva 1962). According to taxonomic classification by Van Raamsdonk and De Vries (1995) and Van Raamsdonk *et al*. (1997), the genus *Tulipa* is divided into two subgenera *Tulipa* and *Eriostemones* (**Fig. 1**). Subgenus *Tulipa* comprises of about 55 species which are arranged into five sections, including the cultivated *T. ges-*

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Invited Review

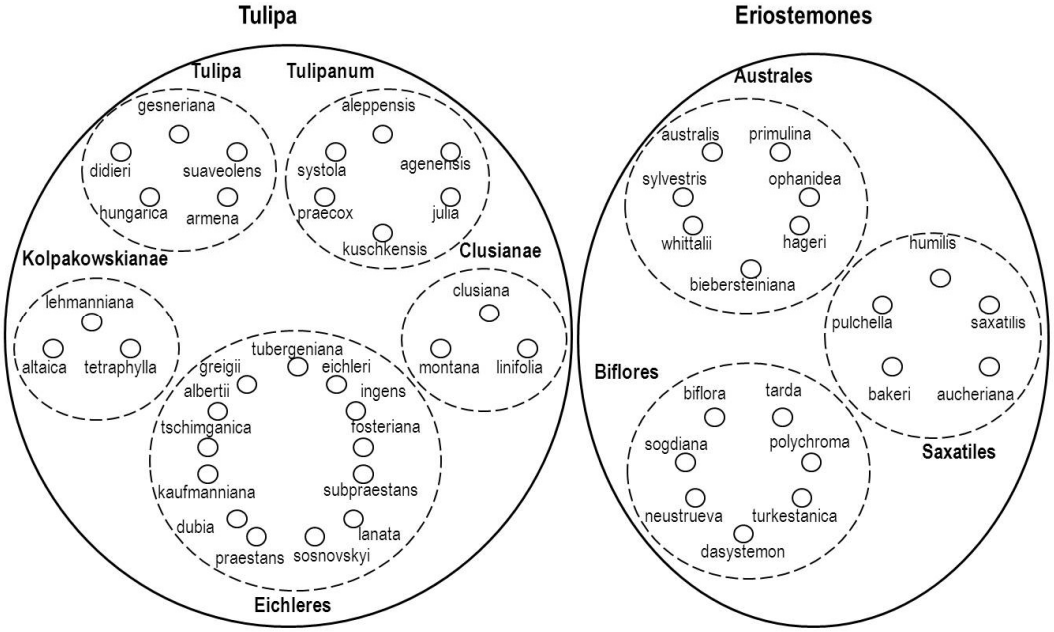

Fig. 1 The systematics of the genus *Tulipa* L. according to Van Raamsdonk *et al.* (1997).

neriana. Subgenus *Eriostemones* comprises about 20 species arranged in three sections (Van Raamsdonk and De Vries 1992).

Many tulip varieties have been developed mainly in the Netherlands and more than 8,000 of them are included in the list of 'tulips names' (Van Scheepen 1996). Of the primary cultivars distributed to the commercial markets consisting of more than 1100 cultivars (Van Scheepen 1996), the majority of them belong to *T. gesneriana* L from the section *Tulipa* which is the collective name given to a large number of varieties of unknown origin (Killingback 1990). The second commercial group is Darwin hybrid tulips, which have been obtained from interspecific crosses between cultivars of *T. gesneriana* and *T. fosteriana* Hoog ex W. Irving genotypes of the section *Eichleres* (Van Tuyl and Van Creij 2007).

INTERSPECIFIC HYBRIDIZATION

Crossability and the main goals of tulip breeding

Interspecific crosses are usually made between genotypes of *T. gesneriana* and other *Tulipa* species to enrich the commercial assortment with desirable traits from those species. *T. gesneriana* has been crossed successfully with only 12 out of the approximately 55 tulip species by using conventional breeding methods (Van Eijk *et al*. 1991; Van Raamsdonk *et al*. 1995). Several hybrids have been obtained from crosses between *T. gesneriana* and species of the section *Eichleres*; like the hybrids obtained between *T. gesneriana* and *T. fosteriana* Hoog, *T. kaufmanniana* Regel, *T. greigii* Regel, *T. eichleri* Regel, *T. ingens* Hoog, *T. albertii* Regel (formerly *T. vvedenskyi*) and *T. didieri* Jord (**Fig. 2**). In many other interspecific crosses hybrid development was prevented by crossing barriers. Crosses between *T. gesneriana* and species from the *Eriostemones*, like *T. tarda* Stapf, *T. pulchella* Fenzl and *T. turkestanica* Regel have never been successful (Van Eijk *et al*. 1991; Van Raamsdonk *et al*. 1995).

Since tulip can be affected by several diseases, among them *Fusarium oxysporum*, *Botrytis tulipae* and *Tulip breaking virus* (TBV) that cause economic losses (Van Tuyl and Van Creij 2007) (**Fig. 3**), the main goal of modern tulip breeding is introgression of disease resistance into commercially successful cultivars (Van Eijk *et al*. 1983, 1986; Van Creij 1997). Several tests have been developed to screen tulip plants for resistance (Romanow *et al*. 1991; Straathof *et al*. 1996). Within the *T. gesneriana* assortment

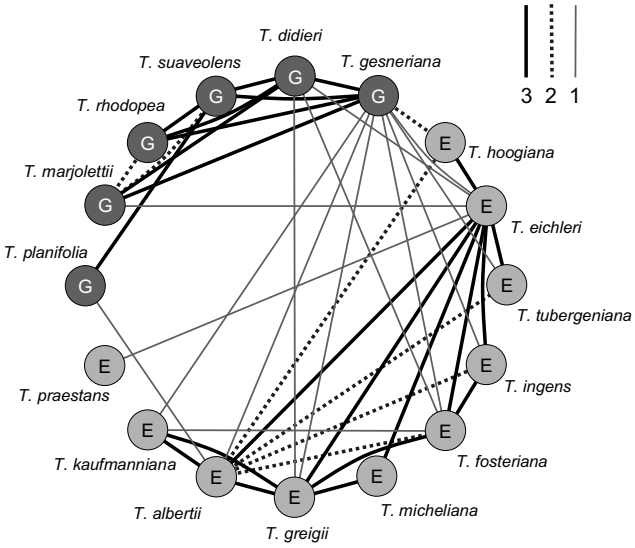

Fig. 2 Crossing polygon of species of section *Eichleres* ('E') and *Tulipa* ('G'). Meaning of lines: 1: several successful attempts, effectivity low; 2: one successful attempt, effectivity high; 3: several successful attempts, effectivity high. Low effectivity: less than $5F_1$ bulbs per seed pod; high effectivity: more than 15 F_1 bulbs per seed pod. The data shown are pooled results of all crosses carried out per combination. Modified from Van Raamsdonk *et al*. (1995).

the resistance against TBV has not been found thus far. Screening other *Tulipa* species identify several genotypes with high levels of resistance against TBV, especially among *T. fosteriana* cultivars (Romanow *et al*. 1991; Eikelboom *et al*. 1992). Therefore, many crosses have been made between TBV resistant *T. fosteriana* cultivars and *T. gesneriana* cultivars (Van Tuyl and Van Creij 2007) which generated highly TBV resistant genotypes called Darwin hybrid tulips (Eikelboom *et al*. 1992; Van Tuyl and Van Creij 2007). Other important goals for tulip breeding are introgression of short forcing period, improved flower longevity, and new flower shapes and flower colours (Hagiya 1971; Kho and Baër 1971; Van Eijk *et al*. 1991; Van Raamsdonk *et al*. 1995; Van Creij *et al*. 1997a, 1997b, 1999).

Fig. 3 The most important pathogens threatening tulip production. (**A**) *Fusarium oxysporum* (bulb-rot), (**B**) *Botrytis tulipae* and (**C**) *Tulip breaking virus* (TBV).

Incompatibility problems

In interspecific crosses in tulips, various kinds of barriers preventing fertilization or normal embryogenesis have been identified (Van Creij *et al*. 1997a). Pre-fertilization barriers were analyzed in crosses between *T. gesneriana* and 12 other tulip species from all eight sections of the genus *Tulipa*. Depending on the cross e.g. low pollen germination on stigma, low pollen tube growth in style and low pollen tube penetration of ovules were observed (Van Creij *et al*. 1997a). Similarly, Kho and Baër (1971) observed abnormal growth of the pollen tubes in the embryo sac regions which resulted in low numbers of seeds in crosses between cultivars of *T. gesneriana* and *T. fosteriana*, between *T. gesneriana* and *T. greigii* and between *T. gesneriana* and a hybrid of *T. kaufmanniana* × *T. greigii*. They established that environmental conditions, particularly the temperature at which pollinations were carried out, played an important role on seed production. The highest seed yield was achieved at 14°C (Kho and Baër 1971). In tulip, pre-fertilization barriers were bypassed for some crosses, through the use of the cut-style method and *in vitro* pollination (Van Creij 1997).

Methods for bypassing post-fertilization barriers focus on the survival of hybrid embryos and on restoring the fertility of F1-hybrids (Van Tuyl and De Jeu 1997). The application of embryo rescue techniques in tulip breeding has been reported by Van Tuyl *et al*. (1990), Custers *et al*. (1992, 1995) and Van Creij *et al*. (1999, 2000a, 2000b). Custers *et al.* (1995) obtained hybrids from the cross *T. gesneriana* × *T. kaufmanniana* after both embryo culture and ovule culture. However the later technique was more successful in rescuing small abortive embryos. Similarly, the culture of embryos and ovules have been successfully used for hybrids obtained from the interspecific crosses of *T. gesneriana* × *T. fosteriana* 'Red Emperor', *T. gesneriana* × *T. eichleri* 'Exelsa' and *T. gesneriana* × *T. greigii* (Okazaki 2005). By using ovary-slice culture followed by ovule culture, hybrids have been obtained from the interspecific crosses of *T. gesneriana* × *T. praestans* and *T. gesneriana* × *T. agenesis* (Van Creij *et al*. 1999).

POLYPLOIDIZATION

Mitotic polyploidization

Most interspecific hybrids of tulip are highly sterile which can be caused by the lack of chromosome pairing during meiosis. The fertility of hybrids can be restored by artificial chromosome doubling using spindle inhibitors such as colchicine and oryzalin which have been successfully used for polypoidization in many crops e.g., *Lilium* (Van Tuyl 1989; Van Tuyl *et al*. 1992; Barba-Gonzalez *et al*. 2006), *Nerine* (Van Tuyl *et al*. 1992) and *Nicotiana* (Marubashi and Nakajima 1985). In tulip, mitotic polyploidization with colchicine is difficult, because the meristems are hidden in noses inside bulbs, and colchicine is harmful to bulbous plants (Van Tuyl *et al*. 1992). Plavcová (1985) obtained polyploid tulip plants after the application of a 0.05% colchicine solution in the form of a capillary injection into the ovary at the first zygote division. Tetraploid tulip cultivars have also been produced after treating tulip stems from bulbs *in vitro* with either oryzalin or colchicine (Van Tuyl *et al*. 1992; Eikelboom *et al*. 2001). Oryzalin was successfully used with *in vitro* chromosome-doubling in tulip *T. gesneriana* during a stem-disc regeneration process (Chauvin *et al*. 2006).

Meiotic polyploidization

Sexual polyploidization is another alternative to obtain polyploid cultivars. In this case, diploid genotypes that produce 2*n* gametes can give rise to polyploid progenies (Van Tuyl 1997). The 2*n* gametes can transmit a large amount of heterozygosity to the polyploid offspring and thus contribute to the vigor of the progeny (Okazaki 2005). The spontaneous production of 2*n* gametes in low frequencies by interspecific hybrids was observed e.g., in *Alstroemeria* and *Lilium* (Van Tuyl 1989; Lim *et al.* 2001; Ramanna and Jacobsen 2003). According to Kroon and Van Eijk (1977) triploid and tetraploid tulips are likely to have arisen as a result of the occurrence of diploid gametes in diploid cultivars. This phenomenon appears to be restricted to certain varieties and there is as yet no information on the origin of these diploid gametes. Upcott and Philp (1939) found two tetraploids in the progeny of a cross between the diploid Single late tulip 'Bouton d'Or' and the triploid Single late tulip 'Inglescome Yellow'. Among Darwin hybrid tulips resulting from interspecific crosses between *T. gesneriana* and *T. fosteriana*, diploid ($2n = 2x = 24$), triploid ($2n = 3x = 36$) e.g., 'Apeldoorn', 'Ad Rem', 'Pink Impression' and some tetraploid ($2n = 4x = 48$) hybrids such as 'Tender Beauty' can be found, in spite of the fact that both of the parental cultivars are diploid ($2n = 2x = 24$) (Van Scheepen 1996). Tetraploid varieties such as 'Riant', 'Beauty of Canada' and 'Peerless Yellow' originated after a cross between diploid and tetraploid varieties where the former provided diploid egg cells (Kroon and Van Eijk 1977). By studying karyotypes, Marasek *et al*. (2006) demonstrated that the triploid Darwin Hybrid tulip 'Yellow Dover' has two copies of the *T. gesneriana* genome and one copy of the *T. fosteriana* genome which suggest that *T. gesneriana* has supplied the diploid gamete. Marasek and Okazaki (2008) observed spontaneous giant pollen formation in Darwin hybrid tulip 'Purissima' (2.8%, estimated on 850 grains). Similar in our study on 'Purissima' a low percentage of abnormal large pollen grains were observed (**Fig. 4**). As a result of the occurrence of spontaneously produced diploid gametes by diploid parents, the polyploid progenies have been obtained (Marasek *et al.* 2006; Marasek and

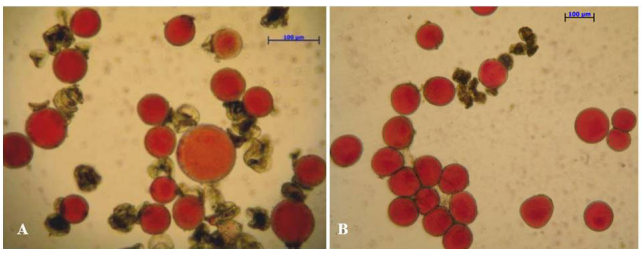

Fig. 4 Pollen samples stained with 3% aceto-carmine with abnormal large pollen grains indicating 2n gametes. (**A**) Diploid cultivar 'Purissima' (Darwin hybrid) ($2n = 2x = 24$), (**B**) Diploid F1 hybrid 222-2 ($2n = 2x = 24$) resulted from cross 'Ile de France' × ('Cantata' × 'Madame Lefeber').

Fig. 5 Darwin hybrid tulips resulting from a 'Golden Melody' × 'Purissima' cross. (**A**) Diploid BC1 hybrid ($2n = 2x = 24$); (**B**) tetraploid BC1 'Purissima' hybrid 99345-37 ($2n = 4x = 48$).

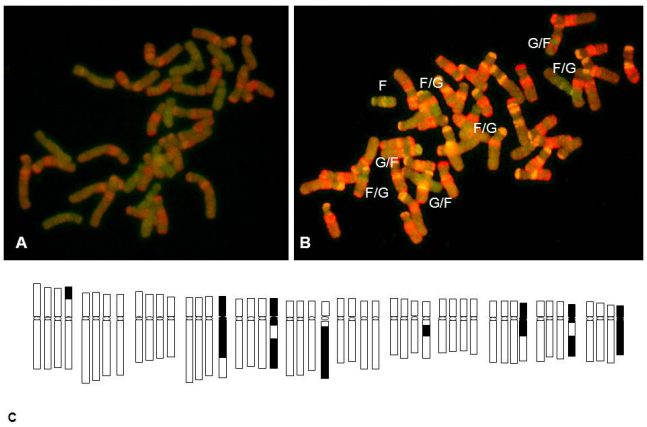

Fig. 6 Chromosome differentiation in a triploid and a tetraploid Darwin hybrid using GISH. (**A**) A chromosome complement of triploid BC1 hybrid ($2n = 3x = 36$) without recombinant chromosomes. (**B**) A chromosome complement of tetraploid BC1 'Purissima' hybrid 99345-37 ($2n = 4x = 48$), with seven recombinant chromosomes. The biotin-labeled *T. gesneriana* DNA was detected with the Cy3-streptavidin system (red fluorescence) and *T. fosteriana* chromosomes were detected with FITC (green fluorescence) (**C**) Diagrammatic representation of BC1 hybrids 99345-37 ($2n = 4x = 48$). The black spaces represent the chromatin of *T. fosteriana*.

Okazaki 2008) (**Fig. 5**). The most important advantage of meiotic polyploidization is that homoeologous recombination occurs between parental chromosomes during meiosis. **Fig. 6** show the chromosome complement of tetraploid BC1 hybrids ($2n = 4x = 48$) resulting from a cross between 'Golden Melody' and 'Purissima' with 7 recombinant chromosomes revealed by GISH analysis. The amount of introgressed *T. fosteriana* genome (11.54%) may indicate the contribution of $2n$ gametes from both parents in development of this genotype.

Interploidy crosses (ploidy of progenies in crosses between diploids, triploids and tetraploids)

Interploidy crosses were used to get polyploid progenies. Crosses between diploid ($2n = 2x = 24$), triploid ($2n = 3x = 36$) and tetraploid ($2n = 4x = 48$) varieties were conducted. By making crosses between tetraploids, new tetraploids were obtained of which the best known is 'Judith Leyster' (Straathof and Eikelboom 1997). Crossing tetraploids with diploids ($4x \times 2x$) can result in vigorously growing triploids e.g., 'World's Favourite' originating from a tetraploid seedling 'Denbola' × 'Lustige Witwe' crossed with a diploid *T. fosteriana* seedling (Straathof and Eikelboom 1997). Triploid varieties such as 'Lady Margot', 'Benny Neyman' and 'Sun Child' have been obtained by crossing diploid varieties with those that are tetraploid ($2x \times 4x$) (e.g., 'Mrs. John T. Scheepers') (Van Scheepen 1996). Upcott and Philip (1939) in diploid-triploid crosses observed progenies with chromosomes numbers from 24 to 48 while aneuploids having 25 chromosomes were most common (37%). According to Bamford *et al.* (1939), 50% of progenies resulting from the $2x \times 3x$ crosses had 25 chromosomes while the chromosome number in other genotypes ranged from 24 to 31. In contrast, Okazaki and Nishimura (2000) reported that, in the $2x \times 3x$ crosses 92.6% were diploids and 7.4% were aneuploids, while in the $3x \times 2x$ crosses 60.0% were diploids and 40% were aneuploids.

Induction of polyploids by nitrous oxide gas treatment

As it was mentioned before interploidy crosses in tulip are a possible method to obtain polyploids however it is not a very effective method. A method with a higher chance to obtain tetraploids is the treatment of young ovaries with nitrous oxide gas (N_2O) under pressure (Zeilinga and Schouten 1968b). Plavcová *et al.* (1976) obtained tetraploids with N_2O treatment of seed buds. Optimal condition for induction of tetraploids included gas pressure ranging from 5.5 to 8 atmospheres for 20-24 h over an application time of 7-14 days after pollination. Similarly, tetraploid seedlings were also induced by placing young seed buds, which were pollinated a week before, for one day in a cylinder with laughing gas (N_2O) at five to six atmospheric pressure (Straathof and Eikelboom 1997). The attempt to obtain hexaploid individuals failed when N_2O was applied to bulbs (4-9 cm) of triploid cultivars in the period of daughter bulb setting. The cytological analysis in the subsequent year showed that obtained plants, having hexaploid tissues, resumed their triploid character (Plavcová *et al.* 1976). The gas pressure used in the trial ranged between 6-9 atm for a period of 7-30 days.

Laughing gas (N_2O) treatment is also a promising method for $2n$ gamete induction in tulip (Okazaki 2005; Okazaki *et al.* 2005; Barba-Gonzalez *et al.* 2006). Okazaki *et al.* (2005) reported $2n$ pollen induction by the treatment of bulbs, when meiosis in anthers reached metaphase I, with N_2O for 24–48 h at 6 atm. The subsequent use of pollen containing a relatively high proportion of giant pollen grains in crosses with diploids tended to yield larger numbers of triploids in the progeny.

CYTOGENETIC ANALYSIS IN GENUS *TULIPA*

Genomes, chromosome numbers of *Tulipa* species and varieties

All *Tulipa* species possess large genomes with 2C DNA values ranging for the diploids from 32 pg for *T. clusiana* (the Clusius tulip) to 69 pg for *T. gesneriana* (Zonneveld 2009). Chromosome numbers have been reported for 63 *Tulipa* species (Kroon and Jongerius 1986) and over 600 varieties (Zeilinga and Schouten 1968a). The basic chromosome number is $x = 12$. The majority of tulip species and cultivars is diploid ($2n = 2x = 24$), however, triploids ($2n = 3x = 36$), tetraploids ($2n = 4x = 48$) and even some pentaploids ($2n = 5x = 60$) and hexaploid ($2n = 2x = 72$) occur (Holitscher 1968; Kroon 1975; Zeilinga and Schouten 1968a, 1968b; Kroon and Jongerius 1986; Van Scheepen 1996). Polyploid series occur in both the subsections *Eriostemones* and *Leiostemones* (Upcott and La Cour 1936). Among 600 tulip varieties analyzed by Zeilinga and Schouten (1968a), four tetraploids and 81 triploids were found. The highest chromosome number determined so far in either wild species or garden varieties was in hexaploid *T. polychroma* Stapf. ($2n = 2x = 72$) (Kroon and Jongerius 1986). In many *Tulipa* species or subspecies several ploidy

levels may occur. Out of 63 species analyzed by Kroon and Jongerius (1986) 13 were found to include different ploidy levels, for example in *T. bifloriformis* diploid, triploid and tetraploid forms were observed. Similarly, Wafai and Koul (1986) reported that *T. clusiana* exists in 2*x*, 3*x*, 4*x* and 5*x* cytotypes with chromosome numbers ranging from 24-60.

Chromosomes morphology and identification

Tulips are favourable material for cytological study due to the large size of their chromosomes. In triploid *T. lanata* Regel chromosomes are 13 to 24 µm long (Wafai and Koul 1973) whereas in diploid *T. gesneriana* cultivar 'Queen of Night' chromosomes size range from 9.2 to 16.4 µm (Marasek *et al*. 2006). Karyotypes have been analysed for many *Tulipa* species and varieties (Upcott and La Cour 1936; Wafai and Koul 1981a, 1981b; Sayama *et al*. 1982; Wafai and Koul 1983, 1986; Van Raamsdonk and De Vries 1995). In spite of their large size, most tulip chromosomes are not distinguishable from each other, because they are very similar in shape and lack distinct chromosomal characteristics. Secondary constrictions seem to be much more pronounced and more frequent in the chromosomes of the *Leiostemones* than in the *Eriostemones* (Woods and Bamford 1937). In addition only median chromosomes are easily recognizable based on their morphology (Marasek *et al*. 2006). Southern (1967) analyzed the relationships of diploid and polyploidy species belonging to the subsection *Eriostemones* from the point of view of chromosome morphology. He observed remarkable similarity of the karyotype morphology among the sixteen species studied. Karyology has also been employed in exploring species interrelationships within subsection *Clusianae* by Wafai and Koul (1981a, 1981b, 1986).

Chromosomes morphology - length and centromere position is highly conserved within and between species, therefore only a few chromosomes are recognizable on the basis of above traits. Chromosome identification has been improved by using techniques enabling longitudinal differentiation of chromosomes such as Giemsa staining (C-banding) revealing heterochromatic regions on chromosomes (Filion 1974; Blakey and Vosa 1981, 1982; Van Raamsdonk and De Vries 1995). Rejon and Rejon (1985) reported on the existence and behaviour of a heterochromatic supernumerary segment located terminally on the short arm of a submetacentric chromosome in natural population of *T. australis* Link. Filion (1974) revealed chromosomal polymorphism for two tulip varieties 'Queen of Night' (2*n* = 24) and 'Spring Song' (2*n* = 24) and *T. turkestanica* (2*n* = 48) using Giemsa staining. The most pronounced bands were observed in 'Queen of Night' with less pronounced ones in *T. turkestanica*. All bands revealed in these varieties had either terminal or interstitial localization and no centromeric bands have been observed. The species relationships, applying the C-banding technique, in subg. *Eriostemones* and subg. *Leiostemones* were analyzed by Blakey and Vosa (1981, 1982). In their studies several chromosome types were recognized with respect to chromosome morphology and heterochromatin distribution leading to the identification of groups of species with common chromosome characteristics.

Chromosome analysis by fluorescence *in situ* hybridization (FISH)

The introduction of molecular cytogenetic tools such as fluorescence *in situ* hybridization (FISH) has greatly advanced chromosome analysis in tulip by providing markers for chromosome identification. Mizuochi *et al*. (2007) observed variation in size, number and chromosome distribution of 5S rDNA and 45S rDNA loci between *T. gesneriana* and *T. fosteriana* cultivars but also among *T. gesneriana* cultivars. 5S rDNA and 45S rDNA probes enabled discrimination of four different chromosome hybridization patterns in 'Christmas Dream' (*T. gesneriana*), seven types

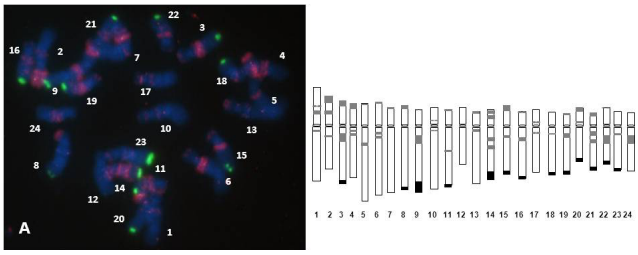

Fig. 7 (**A**) Double-target fluorescence *in situ* hybridization of 45S rDNA (green) and 5S rDNA (red) probes to the metaphase plate of F1 20191-4 hybrid resulting from the cross 'Bellona' × ('Princeps' × 'Madame Lefeber'). (**B**) Diagrammatic representation of F1 20191-4 chromosomes with marked position of 45S rDNA (black) and 5S rDNA (gray) loci.

in 'Queen of Night' (*T. gesneriana*) and nine types in 'Red Emperor'(*T. fosteriana*) (Mizuochi *et al*. 2007).

In situ hybridization with 5S rDNA and 45S rDNA probes can also provide molecular cytogenetic markers for chromosome identification in hybrids. For example, **Fig. 7A** shows the chromosome complement of F1 Darwin hybrid 20191-4 ('Bellona' × ('Princeps' × 'Cantata')) in which FISH with 45S rDNA and 5S rDNA probes revealed the presence of 48 loci of 5S rDNA and 13 loci of 45S rDNA. 45S rDNA loci were localized exclusively in the telomeric position of the long arm of chromosomes, whereas strong 5S rDNA signals were predominately localized in the telomeric position on the short arm of chromosomes. The remaining 5S rDNA sites were intercalative positions on the long arms. The hybridization pattern provided markers for chromosomes identification (**Fig. 7B**).

Marasek and Okazaki (2008) have used genomic *in situ* hybridization (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 the 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 situ hybridization with 5S rDNA and 45S rDNA probes has been used to evaluate karyotype rearrangements and stability of rDNA in off-type plants selected from long term *in vitro* culture plants of *T. gesneriana* cultivar 'Prominence' (Marasek and Podwyszynska 2008). The study focused on the polymorphism of number, appearance and chromosomal localization of rDNA sites. Karyotype comparison exhibited variation in the number of 45S and 5S rDNA loci and in the size of hybridization signals both between standard 'Prominence' and somaclones as well as among somaclones (Marasek and Podwyszynska 2008).

Cytogenetic analysis of meiosis in *Tulipa*

Until recently, quite a number of studies have been carried out to study the different meiotic stages in *Tulipa* species. Many attempts have been made to analyze the nature of polyploidy in tulip (Upcott 1937, 1939; Wafai and Koul 1973, 1981a, 1982, 1984). Wafai and Koul (1973) analyzed chromosome associations in *T. lanata* Regel (3*x* = 36) and suggested that the taxon was either autoploid or a segmental allotriploid. They observed a high frequency of closely associated trivalents in the PMC with a high number of chiasmata which reflected on the homology of constituent genomes. In contrast, analysis of meiotic behavior in triploid *T. clusiana* var. *stellata* (3*x* = 36) revealed trivalent, bivalent and univalent formation at metaphase I and irregular segregation of chromosomes in anaphase I. All cells had laggards and some had a dicentric bridge as well as a chromatin fragment (Wafai and Koul 1981a). Upcott (1939) showed that tetraploid tulip species have a lower chiasma frequency at meiosis then diploids and triploids and fewer changes of partner exchange at pachytene than the latter;

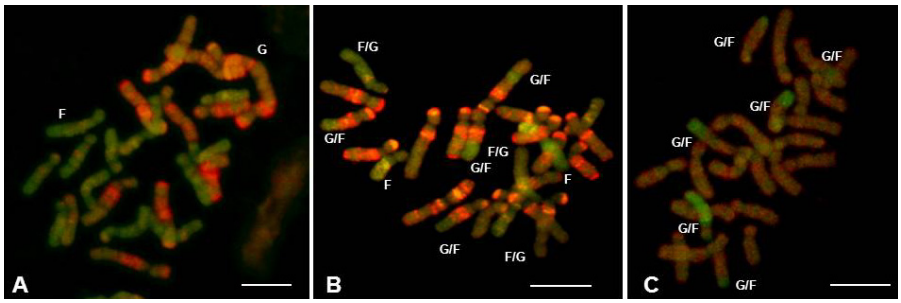

Fig. 8 GISH pictures represent diploid (2*n* = 2*x* = 24) Darwin hybrids. (**A**) F1 hybrid resulting from 'Generaal de Wet' × '104 Juan × Cantata' cross. (**B**) Chromosome complement of 99343-6 BC1 hybrid resulting from 'Christmas Marvel' × 'Purissima' cross, with 7 recombinant chromosomes, (**C**) Chromosome complement of BC2 hybrid 083275 resulting from 070018 × 99343-6 cross showing 6 recombinant chromosomes. *T. gesneriana* DNA is detected with Cy3-streptavidin system (red) and *T. fosteriana* with FITC (green). Example 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. Bar = 10 μm.

consequently, they formed a few quadrivalents and are sexually fertile. Sporogenesis and gametogenesis have been analyzed in tetraploid *T. clusiana* var. 'Typical' (Wafai and Koul 1982). Meiosis turned out to be regular in both the sexes resulting in the formation of balanced gametes and high fertility of this genotype. Bivalent type of chromosomes pairing was dominant and multivalent associations were formed at a significantly low frequency. Similarly, Koul and Wafai (1981) observed regular meiosis in diploid *T. aitchisonii*. The comparison of sporogenesis and gametogenesis in diploid *T. aitchisonii* with tetraploid *T. clusiana* indicated that polyploidy influence the size of the megasporangium, the megaspore mother cell and the embryo sac (Wafai and Koul 1984).

Couzin and Fox (1974) analyzed the variation in chiasma frequency during the development of tulip anthers. It was shown that meiosis starts at the basis of anthers and progresses to the tip. The earlier a cell enter meiosis the lower its total chiasma score is and chromosome length is positively correlated with mean cell chiasma frequency (cells with lower chiasma frequency have lower total chromosome length). U-type exchange between sister chromatids has been described in some plants of *T. hageri* (Couzin and Fox 1973).

Chromosome analyses in interspecific tulip hybrids by genomic *in situ* hybridization (GISH) technique

The genome composition of tulip can be investigated in detail using genomic *in sit*u hybridization (GISH). This technique utilizes genomic DNA of one parental genotype as a probe and excessive fragmented DNA of another parent as blocking DNA. GISH enables the discrimination of parental genomes in hybrids and polyploid forms. This technique also detects chromosome recombination between chromosomes from different genomes and can be used to visualize the level of introgression in backcrossed progenies (**Fig. 6**).

1. GISH analysis in Darwin hybrid tulips

In tulip GISH has been used to clarify genome compositions in diploid (2*n* = 2*x* = 24), triploid (2*n* = 3*x* = 36) and tetraploid (2*n* = 4*x* = 48) cultivars (Marasek *et al*. 2006; Marasek and Okazaki 2007, 2008; Marasek-Ciolakowska *et al*. 2009). In diploid cultivar 'Shirayukihime' (2*n* = 2*x* = 24) GISH with total genomic DNA of 'Queen of Night' (*T. gesneriana*) and 'Red Emperor' (*T. fosteriana*) revealed that twelve chromosomes originated from *T. gesneriana* and twelve from *T. fosteriana* (Marasek and Okazaki 2007). Similarly, GISH analysis showed that 'Purissima', an old cultivar showing high crossability with other *Tulipa* cultivars, and formerly classified to the Fosteriana group, is an interspecific *T. gesneriana* and *T. fosteriana* hybrid without recombinant chromosomes (Marasek and Okazaki 2008). In triploid cultivars 'Diplomate', 'Pink Impression', 'Come Back' and 'Oxford' (2*n* = 3*x* = 36) classified to Darwin hybrid tulips, GISH revealed that 24 chromosomes were delivered from *T. gesneriana* and 12 chromosomes originated from *T. fosteriana* indicating that *T. gesneriana* has supplied the diploid gamete (Marasek and Okazaki 2007). The same genome composition without intergenomic recombination has been demonstrated in Darwin hybrid tulip 'Yellow Dover' by Marasek *et al*. (2006) based on karyotype and GISH analysis. Similarly, no recombinant chromosomes were observed in tetraploid cultivar 'Ollioules' (2*n* = 4*x* = 48) (Darwin hybrid tulips) which genome comprised of 36 chromosomes of *T. gesneriana* and 12 chromosomes of *T. fosteriana* (Marasek and Okazaki 2007).

2. Chromosome recombination in interspecific BC1 hybrids as revealed by GISH

Through GISH analysis, the presence of intergenomic recombination has been proved in Purissima' hybrids (Marasek and Okazaki 2008; Marasek-Ciolakowska *et al*. 2011). Marasek and Okazaki (2008) detected non-recombinant and recombinant chromosomes in 'Hatsuzakura', 'Kikomachi' and 'Momotaro', the diploid progenies of 'Purissima'. The number of the *T. gesneriana* and *T. fosteriana* recombinant chromosomes in these cultivars detected by GISH ranged from 1 to 6. Similarly, recombinant chromosomes have been distinguished in progenies of *T. gesneriana* cultivars such as 'Bellona', 'Christmas Marvel', 'Debutante', 'Golden Melody', 'Ile de France' and 'Pax' crossed with 'Purissima' (Marasek-Ciolakowska *et al*. 2011). In contrast, the triploid cultivar 'Kouki' (2*n* = 3*x* = 36; a cross between the diploid *T. gesneriana* cultivar 'Paul Richter' and diploid 'Purissima') comprises of 24 chromosomes of *T. gesneriana*, and 12 chromosomes of *T. fosteriana* without recombinant chromosomes (Marasek and Okazaki 2008). The occurrence of recombinant chromosomes has been shown in tetraploid cultivar 'Judith Leyster' (2*n* = 4*x* = 48) (Triumph Group). In this cultivar 4 chromosomes possessed *T. fosteriana*/*T. gesneriana* recombinations, whereas 36 chromosomes represented *T. gesneriana* and 8 chromosomes *T. fosteriana* genomes, respectively (Marasek and Okazaki 2008). Marasek-Ciolakowska *et al*. (2009) analyzed genome composition and extent and position of intergenomic recombination in diploid BC1 plants (2*n* = 2*x* = 24) resulting from crossing *T. gesneriana* cultivar 'Yellow Flight' with GF hybrids. The number of recombinant chromosomes differed among hybrids from six to ten. Most recombinant chromosomes contained a combination of a single *T. gesneriana* and a single *T. fosteriana* fragment. An example of GISH analysis in F1, BC1 and BC2 Darwin Hybrid tulips is shown in **Fig. 8**. The presence of recombinant chromosomes proves the possibility of transmitting important agricultural traits such as TBV resistance into the *T. gesneriana* assortment *via* breeding.

CONCLUSIONS AND FUTURE PERSPECTIVES

Introgression of important agricultural traits is one of the main goals in interspecific hybridization. Many crosses have been made to introgress the resistance to TBV present in *T. fosteriana* germplasm into *T. gesneriana* cultivars. The Darwin hybrids resulting from these crosses turned out to be very useful intermediate parents for introgressing the *T. fosteriana* germplasm into the *T. gesneriana* assortment. In genus *Tulipa*, GISH enables not only the monitoring of the hybridity of progenies resulting from interspecific hybridization, but also the analysis of the introgression of chromosomes and chromosome segments into hybrids. Through GISH it is also possible to trace the mode of origin of polyploid tulips and the role of 2*n* gametes in polyploidization. It was found that some tulip F1 hybrids not only produced *n* gametes but also 2*n* gametes. This provides unique opportunities to generate polyploid as well as diploid BC1 progenies from backcrossing GF hybrids (Darwin hybrids) to *T. gesneriana* parents. The identification of individual chromosomes of tulip has been improved by the application of FISH with repetitive DNA probes. In future the FISH method can be applied for the physical mapping of resistance genes or molecular markers of virus resistance on tulip chromosomes and to trace their inheritance in progenies.

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