CLASSICAL AND MOLECULAR CYTOGENETIC STUDIES FOR BREEDING AND SELECTION OF TULIPS

Aurel Popescu*, Anca Nicoleta Sutan*

*University of Pitesti, Targu din Vale street, No. 1, Pitesti, Romania
E-mail: aurel_n_popescu@yahoo.com

Abstract
Due to their extreme popularity as fresh cut flowers and garden plants, and being used extensively for landscaping, tulips undergone a continuous process of selective breeding. For almost nine decades, classical cytogenetic studies, mainly the chromosome counts, have been an important part in the breeding programme for polyploid tulips. The efficiency of breeding is greatly aided by a thorough knowledge of the occurrence of polyploidy in the plant material. While the traditional cytogenetic approaches are still highly useful in selecting polyploids and aneuploids arising from crosses involving (most often) parents of different ploidy or from the material subjected to ploidy manipulation, the new strategies for inducing polyploidy in tulips, either in vivo or in vitro, and advances in molecular cytogenetics are expected to allow a significant increase in breeding efficiency. Together with the shortening of breeding cycle, major genetic improvements could be made for specific traits. In this we review the development of cytogenetic studies in tulips, and the most relevant achievements so far, providing an overview of what we consider to be valuable tools for the processes of selective breeding .

Keywords: Tulipa, chromosome counts, polyploidy, fluorescence in situ hybridization, genomic homology, genetic improvement.

1. INTRODUCTION

Tulip is ranked third among the top ten flowers sold worldwide (Podwyszynska and Sochacki, 2010), being extremely popular for landscaping, but also as garden plants and cut flowers. Despite all appearances, creating of new tulip varieties is not an easy task. Most often, intra- and interspecific crosses are made in order to generate new combinations of specific growth characteristics, colors, flower longevity and resistance against diseases. The great majority of tulip varieties created along the centuries have derived from only two species, *Tulipa gesneriana* and *T. suaveolens* (Van Tuyl and Van Creij, 2006). Because of the differences in ploidy level and fertilization barriers which hamper breeding, a few other wild species from more than 150 belonging to the *Tulipa* genus have contributed to gene pool of the tulip world assortment. As estimated by van Scheepen (1996), from more than 3000 varieties registered, the *T. gesneriana* and so called Darwin hybrids (originating from interspecific crossing of *T. gesneriana* and *T. fosteriana*) consist of more than 1100 cultivars. Over the last decades, valuable cultivars derived from intraspecific crosses of *T. fosteriana*, *T. greigii* and *T. kaufmanniana* have also been released and introduced into commercial cultivation.

As reported by Kroon (1975), Sen (1977), Sayama et al. (1982), Kroon and Jongerius (1986), Straathof and Eikelboom (1997), Masoud et al. (2002), and Okazaki et al. (2005), the tulip assortment consists mainly of diploids with 2n = 2x = 24 (Fig. 1), but include also several dozens of triploid varieties (2n = 3x = 36) and a few tetraploid varieties (2n = 4x = 48). It was estimated that about 5% of Darwin hybrids (which are remarkable for their plant vigor and large flower, as well as for their good bulb yield) are triploid (Van Tuyl and Van Creij, 2006).

For almost nine decades, classical cytogenetic studies, mainly the chromosome counts, have been an important part in the breeding programme for polyploid tulips. The efficiency of breeding is greatly aided by a thorough knowledge of the occurrence of polyploidy in the plant material. While the traditional cytogenetic approaches are still highly useful in selecting polyploids and aneuploids arising from crosses involving (most often) parents of different ploidy or from the

material subjected to ploidy manipulation, the new strategies for inducing polyploidy in tulips, either in vivo or in vitro, and advances in molecular cytogenetics are expected to allow a significant increase in breeding efficiency.

2. CLASSICAL CYTOGENETIC STUDIES IN TULIPS

The studies carried out by De Mol (1925), who made the first attempt to investigate the genetic structure of tulips, and Newton (1927), revealed that most of the species and varieties of *Tulipa* have a diploid number of 24 chromosomes (Fig. 1). It have been shown also that some tulips are triploids (2n=36), tetraploids (2n=48), pentaploids (2n=60), or aneuploids. Newton recognized that, in such a genus, the chromosome number alone can be of little aid in helping to determine the probable relationships of species and varieties. However, he was able to separate certain groups of tulips on the basis of chromosome morphology. This was done by comparing the position of the spindle attachment constrictions and the relative lengths of the chromosomes. Later, Upcott and La Cour (1936) and Woods and Bamford (1937) have described, in addition, secondary constrictions and satellites, but only in certain tulips.

Some studies on interspecific and intervarietal hybridization in the genus *Tulipa* have revealed that certain triploid varieties and species, as well as a pentaploid species (*T. clusiana*) could be used as parents in the formation of hybrids. These hybrids were interesting in that the majority of them were aneuploids. Bamford et al (1939) reported chromosome number in a series of crosses involving diploid, triploid, tetraploid, and pentaploid tulips from which many viable hybrids were obtained, some of them aneuploids, emphasizing especially the contribution of the triploid or pentaploid parent.

Upcott and Philp (1941) found two tetraploids in a progeny of a cross between the diploid Single late tulip Bouton d'Or and the triploid Single late tulip Inglescombe Yellow.

Number of chromosome was found to be variable in tulip hybrids of interspecific origin (Hall, 1937, Bamford et al., 1939). Thus, various chromosome numbers have been reported (13, 14, 23, 25) in tulips.

The chromosomal structure and numbers of *T. gesneriana*, *T. fosteriana* and the Darwin hybrids were studied subsequently by many researchers (Upcott and La Cour, 1936, Upcott, 1937, Hall, 1937, Upcott and Philp, 1941, Ishi and Nishimura, 1963, Zeilinga and Schouten, 1968. However, the cause of their triploidy was not clearly explained.

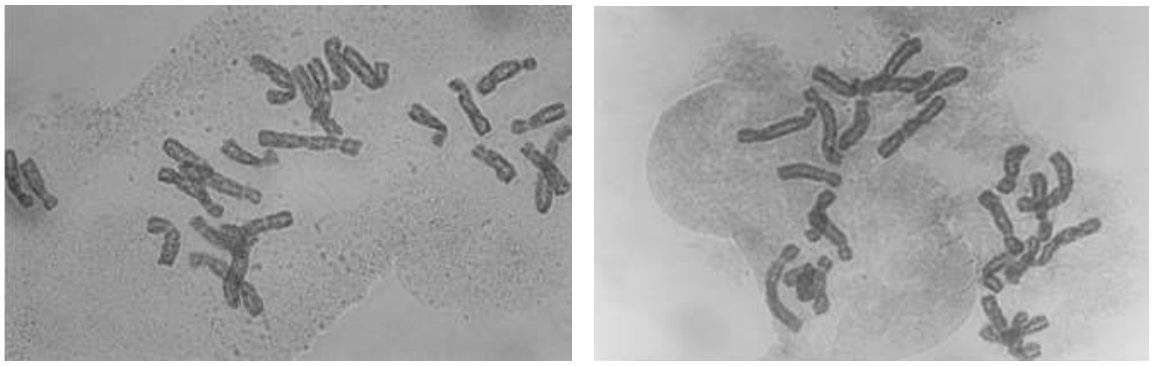

Figure 1. Metaphase chromosomes in Tulipa sp. (Masoud et al., 2002)

Traditional dyes, such as haematoxylin (Nayama et al., 1982), gentian violet (Upcott and La Cour, 1936; Upcott, 1937; Upcott and Philp, 1937), aceto-carmine (Kroon and van Eijk, 1977), aceto-orcein (Zeilinga and Schouten, 1968; Masoud et al., 2002), Feulgen (Woods and Bamford, 1937), Giemsa (Filion, 1974), have been used for staining the chromosomes in tulips. Despite the great developments of newer alternatives, these staining methods are still commonly used to visualize

mitotic chromosomes. Their main limitation is that individual chromosomes within a complement cannot be identified unless they differ morphologically (by size, centromere position, presence of secondary constrictions, etc).

2.1. Chromosome banding and karyotype analysis

The method used to band the chromosomes in tulips was a modification of the BSG (Barium hydroxide/Saline/Giemsa) technique developed by Sumner (1972). It was found that this technique yield highly reproducible banding with Giemsa (Fig. 2), concomitant with the maintenance of chromosome morphology (Filion, 1974).

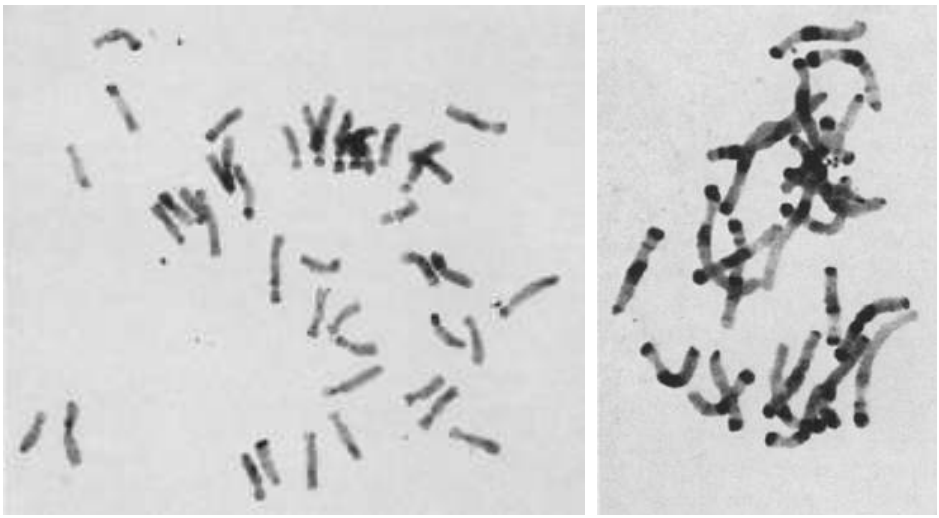

Figure 2. The Giemsa banded chromosomes of "Queen of the Night" and "Spring Song" cultivars of Tulipa gesneriana (Filion, 1974).

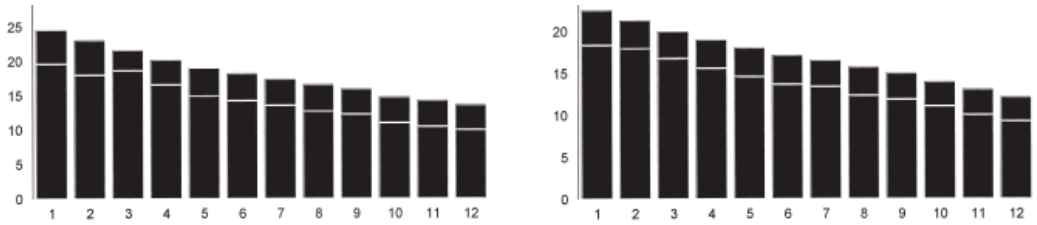

Figure 3. Representative idiograms in two genotypes of Tulipa (Masoud et al., 2002).

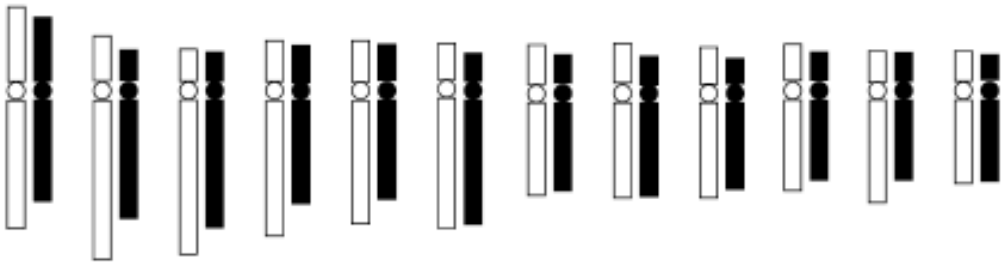

Figure 4. Idiograms of Tulipa gesneriana (white) and T. fosteriana (black) drawn based on karyotype measurements (Hanzi, 2009).

Several staining methods provide distinctive and reproducible patterns for specific chromosomes or genomes, based on the size and location of different classes of chromatin. These methods allowed idiogram drawing (Fig. 3 and 4) and comparative studies in various tulip species (Masoud et al., 2002; Hanzi, 2009). Among the classical banding methods, G-banding has been so far the only used for identification of individual chromosomes in *Tulipa* (Filion, 1974).

2.2. Cytogenetical studies for the identification of polyploids

Conventional methods of breeding are still used to a large extent for creating new varieties of tulips, which accounts for a large share within the world flower markets. Also, ploidy manipulations can be applied in order to avoid the infertility of hybrid progenies as result of differences in ploidy level (Van Tuyl et al., 2002). As reported by Okazaki et al. (2005), the tulip assortment consists mainly of diploids with 2n = 2x = 24 (Fig. 1), but include also several dozens of triploid varieties (2n = 3x = 36) and a few tetraploid varieties (2n = 4x = 48). It was estimated that about 5% of Darwin hybrids (which are remarkable for their plant vigor and large flower, as well as for their good bulb yield) are triploid (Van Tuyl and Van Creij, 2006).

Polyploids, especially triploids and tetraploids, are widely used in the breeding programs of ornamental plant species, due to their desirable traits, such as vigorous growth and large leaves and flowers. Also, on account of the sturdier flower segments, withstand transport as cut flowers better than diploids (Zeilinga and Schouten, 1968b, Podwyszynska, 2011). Therefore, the breeders are highly interested in tulip polyploids (Okazaki et al., 2005, Barba-Gonzales et al., 2006). Tetraploids are of particular interest because they probably offer better chances of success in interspecific crosses and are likely to pass their inherent characteristics better than diploids. Therefore, the availability of tetraploids would aid the breeding of new triploid varieties (Zeilinga and Schouten, 1968b).

Polyploidy in tulips has been the subject of extensive research (Newton and Darlington, 1929; Hall, 1931, 1937; Zeilinga and Schouten, 1968; Kroon and van Eijk, 1975). The setting up of an efficient breeding programme is greatly aided by a thorough knowledge of the occurrence of polyploidy in the plant material (Zeilinga and Schouten, 1968). Therefore, chromosome counts have been an important part in the modern and efficient breeding programme for polyploid tulips. Within the breeding programme carried out in The Netherland, during a decade, chromosome counts have been made in about 600 tulip varieties, resulting in identification of four tetraploids and 81 triploid varieties (Zeilinga and Schouten, 1968).

The intraspecific and interspecific crosses have been contributed to breeding of tulips (Hagiya, 1971). One of the most interesting example of interspecific crosses is Darwin Hybrid induced from *T. gesneriana* x *T. fosteriana* Red Emperor. The most of Darwin Hybrids are triploid (3x=36) in spite of the fact that both the parental cultivars used are diploid (Lefeber, 1960), Ishi and Nishimura, 1963, Zeilinga and Schouten, 1968).

2.3. Chromosome doubling for ploidy manipulation

Cytogenetical studies are very important in all the *in vivo* or *in vitro* applications for ploidy manipulation. Most likely, the triploid and tetraploid varieties raised in the past, resulted from crosses between diploid tulips, and between diploids and tetraploids. This can only be explained by the occurrence of diploid gametes in diploid (2n=24) cultivars, phenomenon which may affect both female and male gametes, and appears to be specific for certain varieties.

The doubling of chromosomes and the use of 2n gametes was proven to be suitable for ploidy manipulation, and also to overcome F1 sterility and enhance introgression of characters.

Obtaining tulip triploids by induction of 2n pollen with nitrous oxide gas, and then using such pollen for crossing with diploid cultivars (meiotic polyploidization) is one of the strategies used in the last decade. In recent years, another strategy to obtain polyploids, especially tetraploids, is the mitotic polyploidization. Thus, chromosome doubling in somatic cells using chemical antimitotic

agents, such as colchicine, oryzalin, trifluralin, and amiprophos methyl (APM) is frequently used (Podwyszynska et al., 2010, Dhooghe et al., 2011).
Chauvin et al. (2005) developed a method to obtain tetraploids from diploid genotypes using a stem-disc regeneration process *in vitro*, and the chromosome-doubling agent oryzalin, applied in different ways. Tetraploid clones were obtained from all treatments and from all cultivars tested.

3. MOLECULAR CYTOGENETICS FOR GENETIC IMPROVEMENT OF TULIPS

Fluorescence *in situ* hybridization (FISH), using different classes of DNA sequences as probes, has been recently used for cytological discrimination of specific chromosomes and individual genomes in several *Tulipa* species (Mizuochi et al., 2007; Marasek and Okazaki, 2008; Hanzi, 2009). As shown by their results, the potential of FISH approaches for identification of specific segments of individual chromosomes seems unlimited.

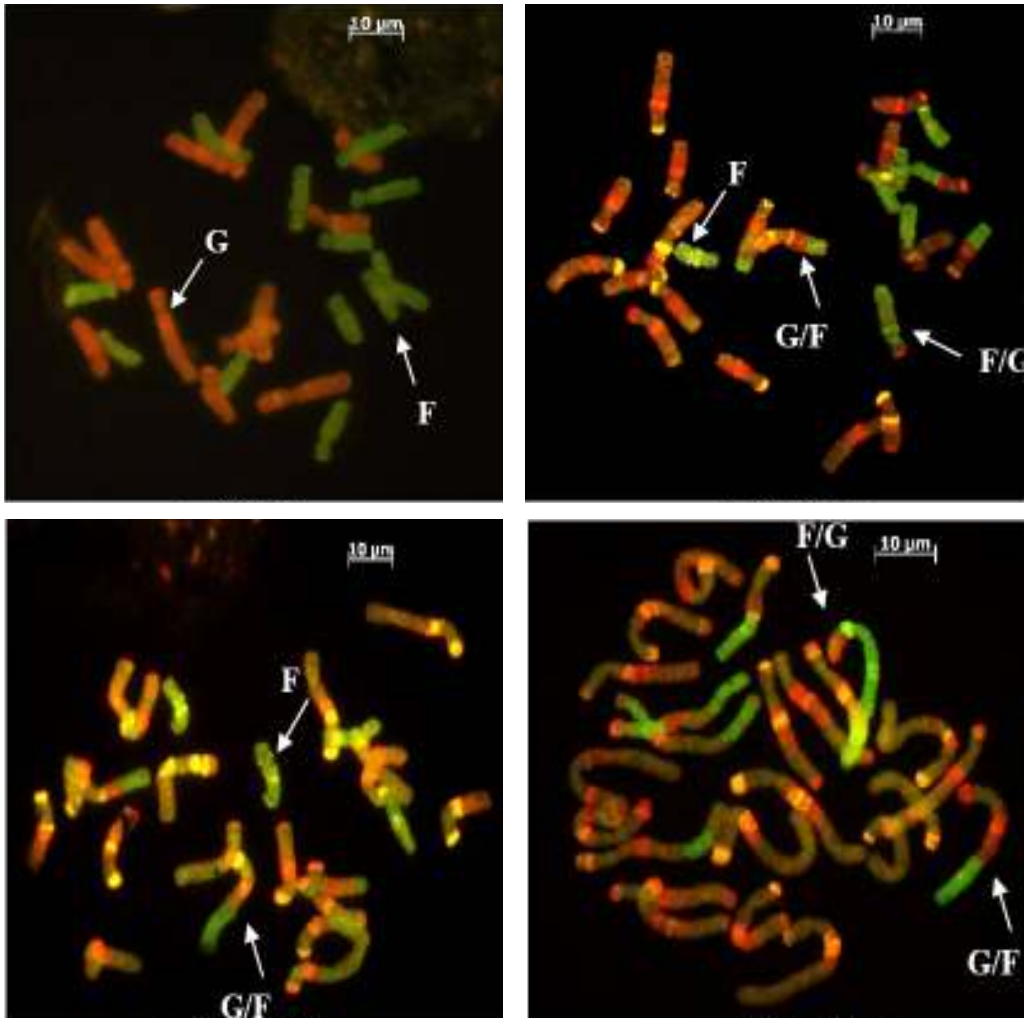

Figure 5. GISH results from different genotypes of hybrids. T. gesneriana (G) genome was labeled by biotin-16-dUTP (red), and T. fosteriana (F) genome was labeled by dig-11-dUTP (green). Recombinant chromosomes are defined as F/G and G/F indicating a T. fosteriana centromere with T. gesneriana chromosome segment(s) and a T. gesneriana centromere with T. fosteriana chromosome segment(s), respectively. Arrows indicate types of genome or recombination (after Hanzi, 2009).

FISH using total genomic DNA labelled probes (GISH), technique that is sometimes referred to as multicolor-GISH (when differentially labelled genome probes are simultaneously hybridized to

chromosome spreads) became also in recent years extremely useful for genome characterization in tulip interspecific hybrids (Marasek et al., 2006; Marasek and Okazaki, 2007; Marasek and Okazaki, 2008; Marasek-Ciolakowska et al., 2009; Hanzi, 2009). Combination of repeated and genomic probes has been demonstrated to be a great improvement in identification of particular chromosomes and chromosomal segments in hybrids originating from combinations of *T. gesneriana*, *T. fosteriana* (Hanzi, 2009).

In almost all cases, the F1 hybrids of distant related species are highly sterile as a consequence of the disturbed chromosome division during meiosis, leading to the formation of gametes with unbalanced chromosome constitution. The most widely used method of restoring fertility in interspecific hybrids is that of doubling the chromosome number in the offspring which should lead to formation of homologous chromosome pairs and, therefore, to normal meiosis. However, this approach has a great drawback, arising from the preferential pairing of chromosomes between the constituent genomes of the hybrid and, consequently, reduced possibility for homoeologous chromosome pairing and crossing-over. Since homoeologous recombination is a crucial prerequisite for introgresssion of specific desirable traits into a cultivar, chromosome doubling of the F1 hybrids is not a suitable method. Rather, backcrossing would be the appropriate method.

Backcrossing is often useful, resulting in recombination between chromosomes and thus leading to introgression of desired traits into the recipient parent. As shown in Fig. 5, this can be studied by either fluorescence *in situ* hybridization or genomic *in situ* hybridization.

GISH enabled unequivocally the identification of parental chromosomes as well as the recombinant chromosomes (Marasek-Ciolakowska et al., 2009). Southern hybridization and genomic *in situ* hybridization (GISH) have demonstrated that 'Purissima' ($2n = 2x = 24$) is an interspecific hybrid comprised of one genome of *Tulipa gesneriana* and one genome of *T. fosteriana*. Simultaneous GISH and fluorescence *in situ* hybridization (FISH) distinguished chromosomes from both parent genomes, as well as recombinant chromosomes, in interspecific hybrids and their progeny. The tulip cultivar 'Kouki' ($2n = 3x = 36$) had two genomes of the *T. gesneriana* and a single genome of the *T. fosteriana.* The number and type of recombinant chromosomes differed among cultivars. The total number of translocations ranged in the different cultivars investigated from one to six. Each was a combination of a single *T. fosteriana* fragment and a single *T. gesneriana* fragment, indicating that they resulted from a single crossover event. Sequential GISH and FISH analysis with rDNA probes yielded chromosome-specific markers that were used to identify most of the chromosomes in 'Purissima' progeny.

4. CONCLUSIONS

The above review emphasizes that the use of recent advances in molecular cytogenetics can lead to significant improvements in tulip. The cytogenetical analysis is of great importance in determining the chromosome constitutions in the offspring from the interspecific hybrid or subsequent generations. Despite some technical limitations, GISH is currently the most common tool to visualize the stable introgression of chromosome segments from wild *Tulipa* species into the chromosomes of cultivated tulips.

Since the introgression of one or a few genes into a current elite cultivar via backcrossing is a common breeding practice, methods for marker-assisted backcrosing were developed for the introgression of transgenic traits and reduction of linkage drag, where molecular markers can be used in genome scans to select those individuals that contain both the transgene(s) and the greatest proportion of favorable alleles from the recurrent parent genome. Therefore, the application of molecular genomic and cytogenetic techniques such as FISH, GISH and MAS can be of great help for fastening interspecific hybridization programs and shortening the duration of creating new varieties.

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