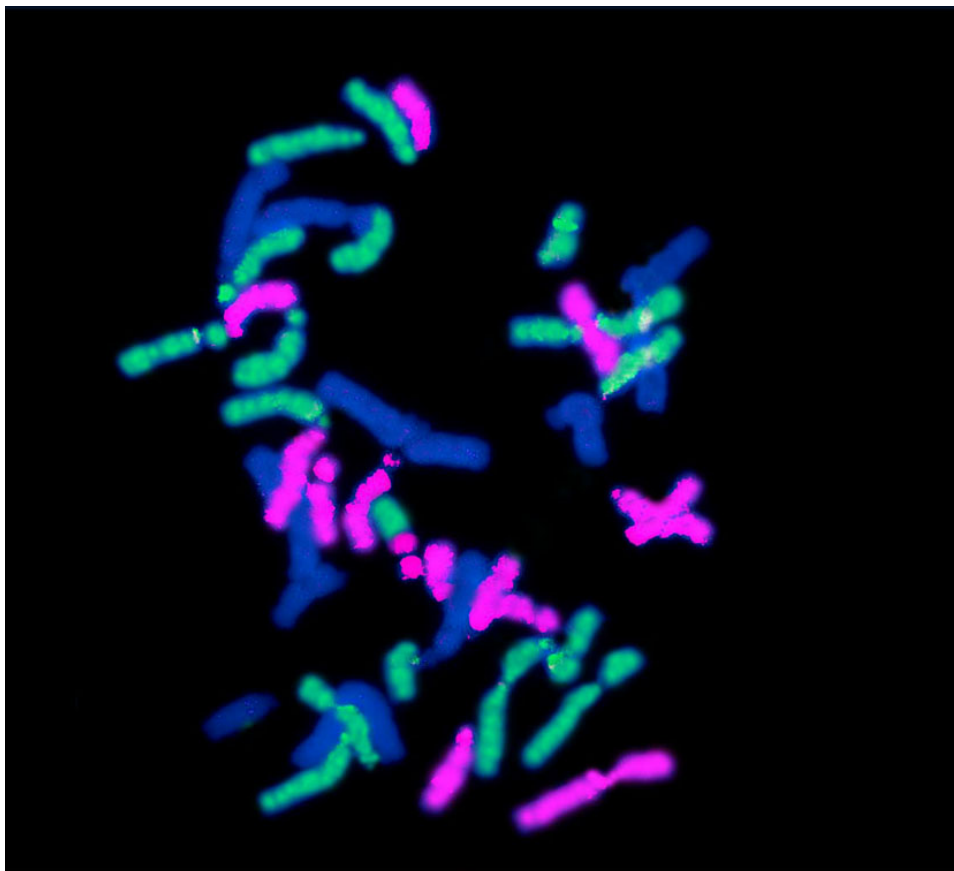# Recombination in hybrids of lily and tulip studied by GISH

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Preface

This is a master thesis report focussing on examining genome composition and recombination in interspecific hybrids of both tulip and lily with the cytological technique GISH. The work for this thesis was done between September 2012 and February 2013. I would like to thank Mengli Xi for her constant help in explaining GISH to me. Some of the tables and pictures I used in this report are made by her. Furthermore I would like to thank my supervisors Jaap Van Tuyl and Paul Arens for their guidance in the, sometimes difficult, process of completing this thesis. I would like to thank everyone from the ornamental breeding group and all others who have helped me to understand the complex processes of GISH and interspecific hybrids.

Nadine Rijk

Abstract

Lilies are part of the *Liliaceae* family and belong to the genus *Lilium*. The lily family can be subdivided in 7 sections: *Sinomartagon, Martagon, Archelirion, Leucolirion, Pseudolirium, Oxypetalum and Lilium*. Each section can be distinguished based on differences in morphology and physiology. Most modern lily cultivars are interspecific hybrids that are made between the different sections. The most important hybrid groups are the Longiflorum (L), Oriental (O), Trumpet (T) and Asiatic (A) hybrid groups. Tulips are also part of the *Liliaceae* family and belong to the genus *Tulipa*. The two most important families are *Tulipa gesneriana (G)* and *T. fosteriana (F)*. One of the most important cultivated group consists of hybrids between *T. fosteriana* and *T. gesneriana,* this group is called the Darwin hybrid group. Creating interspecific hybrids gives the opportunity of combining the valuable characteristics of each specific section but the creation of hybrids is difficult and laborious due to sexual crossing barriers between the different sections. Introgression breeding, which is the introduction of specific traits from a species into a cultivar, can be potentially valuable in creating new lily and tulip cultivars. Studying recombination in meiotic polyploids and segregation of chromosomes in mitotic polyploids or triploids, in order to obtain information about specific traits in neo-polyploids can be very useful in introgression breeding. How the parental genomes are dispersed in the offspring is important for the effectiveness of the introgression breeding. In this research the genome composition and recombination frequency are investigated in both lily and tulip crosses. Several crosses are made between different lily hybrid groups. Tulip crosses consist of hybrids between Gesneriana and Fosteriana parents and a backcross to the Gesneriana parent. In lily, in two plants originated from a hybrid cross , a recombinant chromosome was found. This originated during meiotic recombination in gametes of one of the parents. In tulip, different crosses between a Gesneriana parent and a Gesneriana and Fosteriana hybrid are used. In all tulip genotypes analysed using GISH in this study, there was lack of the typical 'chromosome painting' along the chromosomes. The banding pattern of higher fluorescent bands can be explained by the binding of the probe to repetitive DNA sequences. Further research will be necessary to gain more insight in the behaviour of interspecific hybrids of lily and tulip and the cytological processes behind interspecific crosses. This knowledge can improve the efficiency of introgression breeding

Introduction

Lily

Lilies are part of the Liliaceae family and belong to the genus *Lilium*. There are about 80 lily species known. The lily family can be subdivided in 7 sections: *Sinomartagon, Martagon, Archelirion, Leucolirion, Pseudolirium, Oxypetalum* and *Lilium* (Comber, 1949). Each section can be distinguished based on differences in morphology and physiology. Most modern lily cultivars are interspecific hybrids that are made between the different sections. Figure 1 shows a crossing polygon of the genus *Lilium* and visualizes all successful crosses made within and between the different lily sections. These intersectional cultivars are commercially interesting because the individual strong characteristics of different lily species, like forcing ability, disease resistance or beautiful flower shape and colour, are combined. The four most important cultivar groups are *Longiflorum* hybrids, Asiatic hybrids, Oriental hybrids and Trumpet hybrids. *Longiflorum* hybrids originate from interspecific crosses between species of the *Leucolirion* section. They have trumpet-shaped white flowers and have a strong year-round forcing ability. Asiatic hybrids are the result of interspecific crosses within the *Sinomartagon* section. They have a wide variation in color and in some species in this group resistance against *Fusarium oxysporum* and viruses is observed. The Oriental hybrids group originates from interspecific crosses within the *Archelirion* section. These hybrids are characterized by big and fragrant flowers, combined with a fair degree of resistance against *Botrytis elliptica* (Barba-Gonzalez et al., 2005)*.* Lily species are predominantly diploid (2n=2x=24) and have a set of 12 chromosomes (Lim, 2000).
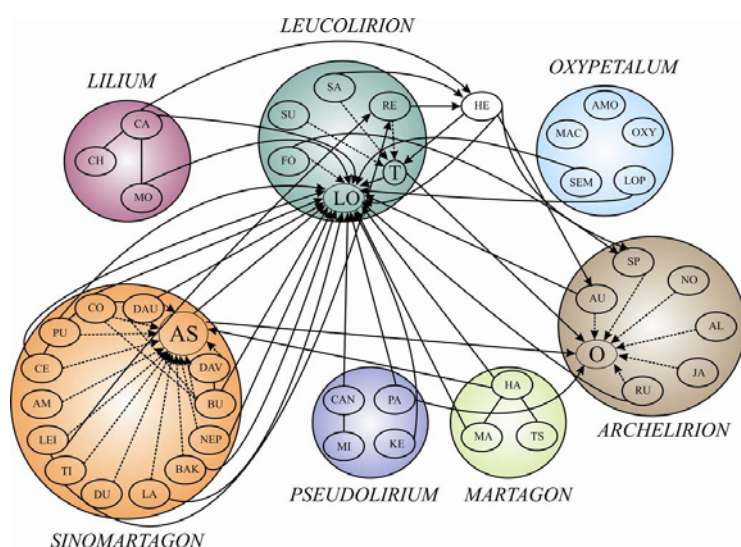

Figure 1. Crossing polygon of the *Lilium* genus as developed by Plant Research International, Wageningen University and Research Centre. The colored balls indicate the seven different sections. The different arrows between the sections indicate all successful crosses that have been made between different sections. Dotted arrows indicate crosses between the Asiatic, Trumpet, and Oriental hybrid groups. Abbreviations: AL: *L. alexandrae*; AM: *L. amabile*; **AS**:Asiatic hybrids; AU: *L. auratum*; BAK: *L. bakerianum*; BU: *L. bulbiferum;* CA: *L. candidum*; CAN: *L. canadense*; CE: *L. cernuum*; CH: *L. chalcedonicum*; CO: *L. concolor*; DAU: *L. dauricum;* DAV: *L. davidii*; DU: *L. duchartrei*; FO: *L. formosanum*; HA: *L. hansonii*; HE: *L. henryi*; JA: *L. japonicum*; KE: *L. kelloggii*; LA: *L. lankongense*; LEI: *L. leichtlinii*; **LO**: *L. longiflorum*; MA: *L. martagon*; MI: *L. michiganense*; MO: *L. monadelphum*; NEP: *L. nepalense*; NO: *L. nobilissimum*; **O**: Oriental hybrids; PA: *L. pardalinum*; PU: *L. pumilum*; RE: *L. regale*; RU: *L. rubellum*; SA: *L. sargentiae;* SP: *L. speciosum*; SU: *L. sulphureum*; **T**: trumpet hybrids, TI: *L. tigrinum*; TS: *L. Tsingtauense (*Van Tuyl and Arens, 2011)

Creating intersectional hybrids gives the opportunity of combining the valuable characteristics of each specific section. For example, creating a lily cultivar that combines *Fusarium oxysporum* resistance found in Asiatic hybrids and the strong forcing ability of the *Longiflorum* hybrids would be commercially very interesting. Creating an intersectional hybrid has many advantages but is not easily obtained. The creation of hybrids is difficult and laborious due to sexual crossing barriers between the different lily sections (Asano, 1982). These sexual barriers prevent successful interspecific hybridization and can be divided into pre- and post-fertilization barriers (Van Tuyl et al., 1991). In many interspecific crosses, pollen tube inhibition in the style was found (Ascher and Peloquin, 1986). Overcoming the pre-fertilization barriers can be achieved by using a good pollination method. Research showed that using the cut-style technique is the best technique for bypassing the pre-fertilization barrier (Van Tuyl et al., 1988). This technique consists of placing pollen on the stylar, after cutting the style with stigma. This avoids the stylar barrier that will block proper pollen tube growth (Van Tuyl et al., 1991). A disadvantage of this method is the impaired pollen tube growth, resulting in a lower fertilization rate. Another technique developed to bypass the pre-fertilization barrier is the grafted-style method. This technique places pollen on a compatible stigma and is after one day, subsequently connected to the ovary of an incompatible species (Van Tuyl et al., 1991). Post-fertilization barriers result in embryo abortion (Dowrick and Brandram, 1970; Asano and Myodo, 1977). Embryo rescue techniques have been developed that can bypass the post-fertilization problems. In vitro pollination and embryo rescue techniques have been developed to overcome incongruity barriers. In vitro-techniques for preventing embryo abortion include ovary-slice culture, ovule culture and embryo culture (Kanoh et al., 1988; Van Tuyl et al., 1991; Okazaki et al., 1994). Combining (in vitro) pollination and embryo rescue techniques has been successful in bypassing pre- and post-fertilization barriers and resulted in the development of interspecific hybrids (Van Creij et al, 1992).

The development of commercial cultivars started around fifty years ago. It took a few decennia before lily became an commercially important ornamental crop. In the early stages of lilium breeding, crosses were made only within sections. One of the first commercially very successful cultivars was the Mid-Century Hybrid 'Enchantment', an intrasectional hybrid which was introduced in 1944. The most important hybrid group in the market shifted throughout the years from the Asiatic hybrids to the Oriental hybrids (Van Tuyl and Arens, 2011) . 'Star gazer' has been the most popular cultivar for over 25 years but has been replaced by other cultivars in the last few years. Oriental hybrid cultivars 'Sorbonne' and 'Siberia' were developed in the early 1990's and are nowadays still the two most grown cultivars in The Netherlands (Table 1) (Van Tuyl and Arens, 2011) . The first intersectional hybrids were developed in the first years of the 1990s and were the result of the development of new techniques that made it possible to overcome the pre- and post-fertilization barriers that prevented hybridization occurring in nature. The first intersectional hybrid group resulted from a cross between *Longiflorum* and Asiatic hybrids, also called the LA hybrid group. The LA hybrid group originates from a backcross between a doubled F1 LA hybrid with the Asiatic parent. This intersectional hybrid group became so popular that they almost completely replaced the Asiatic hybrid group. Subsequently other hybrid groups like LO, OA and OT were developed and became commercial (Van Tuyl and Arens, 2011). Nowadays, the most popular intersectional hybrids are the OT and LA hybrid group (Table 1). Main goals in breeding are characteristics, like a sturdy stem, big and upward facing flowers and forcing ability. Also obtaining resistance against *Fusarium oxysporum* is important (Khan, 2009).

Table 1. An overview of the top 10 most widely grown lily cultivars in The Netherlands in 2010. Group stands for hybrid group, Ploidy for ploidy level, Production area stands for hectares grown each year, Breeder stands for the breeding company that developed the cultivar and Intro stands for year of introduction of the cultivar on the market. Abbreviations: A=Asiatic, O=Oriental, L=*Longiflorum*, LA=*Longiflorum* x Asiatic, LO=*Longiflorum* x Oriental, OT=Oriental x Trumpet, VH=Vletter and Den Haan BV, M=Makbreeding, IM=Imanse, RVZ=Royal van Zanten, WB=World Breeding (Van Tuyl and Arens, 2011)

Cultivar	Group	Ploidy	Production area (ha)		Breeder	Intro.
			2010	1994		
Sorbonne	O	2	200		VH	1994
Siberia	O	2	193	6	Mak	1992
Robina	OT	3	84		Marklily	2004
Tiber	O	2	71		VH	1994
Conca dór	OT	3	67		VH	2002
Brindisi	LA	3	67		VH	2002
Rialto	O	2	63		VH	2002
Santander	O	2	61		VH	2007
Litouwen	LA	3	59		VH	2005
Tresor	A	4	56		VH	1997

Tulip

Tulips are part of the *Liliaceae* family and belong to the genus *Tulipa*. Tulips are predominantly diploid (2n=2x=24). There are about 100 tulip species known (Stork, 1984). The tulip family can be partly divided into commercial groups. The commercially most important cultivated group is the *T. gesneriana L.* group (Killingback, 1990). The group has very diverse morphological and physiological characteristics, ranging widely in color, shape and forcing ability (De Jong, 1974). The second important cultivated group consists of hybrids between the *T. fosteriana* and *T. gesneriana,* also called the Darwin hybrid group. This hybrid group is getting more important because it combines a large flower and plant size with strong forcing ability and resistance to among others *Fusarium oxysporum* and Tulip Breaking Virus (Marasek-Ciolakowska et al., 2012). Most cultivated Darwin hybrids are triploids, for example the cultivar 'Apeldoorn' but the Darwin hybrid group also includes cultivars that are diploid or tetraploid, like 'Tender Beauty' (Marasek et al., 2006). The ploidy level of the Darwin hybrid used in this research, which is backcrossed to a *gesneriana* parent, is diploid. The triploidy of the hybrids is caused by one of the parents that supplies an unreduced gamete. Some species of tulip seem to be able to produce both n and 2n gametes in a more frequent manner than other crops. For instance, in crosses where the *T. fosteriana* 'Red Emperor' is used as a male parent, the resulting progeny is triploid. This indicates that Red Emperor seems to provide the unreduced gametes (Kroon and Van Eijk, 1977). Nowadays, the Darwin hybrid cultivars obtained 10 % of the Dutch Tulip market. The main breeding goals of tulip are physiological characteristics like flower shape and colour, short forcing period and long vase life. Also resistance against Tulip Breaking Virus, *Botrytis tulipae* and *Fusarium oxysporum*. are important traits but are more difficult to select for. (Marasek-Ciolakowska et al., 2012). Multiple interspecific crosses have been made between the two tulip sections Eichleres and Tulipa by conventional breeding methods (Figure 2).
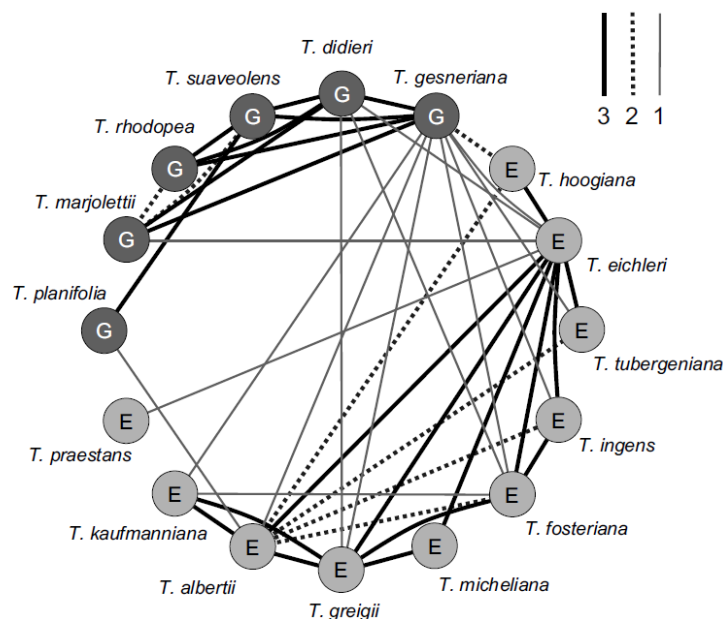
Figure 2. Crossing polygon of species of subgenera Eichleres ('E') and subgenera *Tulipa* ('G')*. 1: several successful attempts, low effectivity; 2: one successful attempts, effectivity high; 3: several successful attempts, effectivity high. Low effectivity: less than 5 F1 bulbs per seed pod; high effectivity: more than 15 F1 bulbs per seed pod. The data shown are pooled results of all crosses carried out per combination. (Van Raamsdonk et al., 1995; Marasek-Ciolakowska et al., 2012)

Introgression breeding

Introgression breeding, which is the introduction of specific traits from a species into another species, can be potentially valuable in creating new lily and tulip cultivars. Genetic variation is necessary for creating new and improved cultivars. This variation is present in segregating populations by recombination or mutation, but can also be newly established in hybrid progeny through genetic changes, like recombination between the different genomes of a hybrid (Xie, 2011). Recombination in plants is the main generator for genetic variation in newly synthesized interspecific hybrids. Studying recombination in meiotic polyploids in order to obtain information about specific traits in neo-polyploids can be very useful in introgression breeding. If and how frequently the parental genomes are recombined in the offspring is important for the effectiveness of the introgression breeding.

New developments that improve the efficiency of introgression breeding are molecular cytogenetic techniques (Lim, 2000; Barba-Gonzalez, 2005; Zhou, 2007; Khan, 2009) and molecular assisted breeding (Shahin et al., 2009) (Van Tuyl and Arens, 2011). The use of molecular markers and the use of molecular and physical maps are important developments in molecular assisted breeding. Markers that are linked to traits-of-interest can be used in marker assisted selection (MAS) to speed up the process of creating new cultivars with the desired traits-of-interest. Markers can aid selection for target alleles that are not easily assayed in individual plants, also linkage drag is minimized and the number of generations needed to obtain a high percentage of the recurrent parent genetic background. Different markers systems can be used to construct genetic maps, up until a few years ago the most frequently used techniques were DArT, AFLP and NBS profiling (Shahin et al., 2009). Nowadays, only SNP markers are used. SNP markers are single nucleotide polymorphic markers that can detect polymorphisms in a population

For better and more accurate maps, more markers with a link to a trait of interest need to be developed and tested. The use of molecular markers in ornamental crops is relatively modest compared to seed crops where markers are used much more frequently. Within ornamental crops, lily is one of the more elaborately studied crops. Molecular marker techniques have been applied on lily for example for the identification of interspecific hybrids (Yamagishi, 1995; Yamagishi et al., 2002), finding genetic diversity (Arzate-Fernández et al., 2005) and identification of RAPD-markers linked to Fusarium resistance (Straathof et al., 1996). The obtained knowledge obtained through the above mentioned techniques can help to improve the efficiency in introgression breeding.

Research done by Shahin et al., (2010) resulted in two genetic maps of an Asiatic lily population that were published and contained 6 QTLs for *Fusarium*-resistance. There are several maps known that each give different types of information. Linkage maps (also called recombination maps) are maps that show the position of genes or markers relative to each other in terms of recombination frequency (Khan, 2009). Chromosome maps (also called cytogenetic or cytomolecular maps) are maps that are drawn based on the karyotype of the genome. The chromosomes are defined at the cytogenetic level according to their size and banding pattern. The location and order of markers on a chromosome map is determined in relation to structures on the chromosomes, like centromeres and telomeres. One way of obtaining a chromosome map is by in situ hybridisation. This technique uses specific DNA probes to directly visualize the location of the corresponding sequence on a chromosome. Studying recombination with molecular cytogenetic techniques like GISH can provide possible landmarks for the mapping of markers (Khan, 2009). Knowing where the traits of interest are located makes it easy to scan a population for the presence of the wanted genes of interest but is not necessary for marker assisted breeding. This information can be obtained by analyzing outcrossing populations, like recombinant in bred lines (RILs) or doubled haploids (DH) and subsequently mapping the gene or QTL of interest. The use of markers speeds up the breeding process. Physical maps are based on direct analysis of DNA and contain the DNA sequence. The distances between loci are measured in base pairs. Both chromosomal, linkage and physical maps give information on the order of the markers or traits of interest but the distances within each map are calculated differently. A cytogenetic map contains information obtained by cytogenetic techniques, like recombination sites that are detected by GISH and provides a visual overview of the chromosomes and banding patterns on those chromosomes that are obtained by direct observation of stained chromosomes. Using some kind of labeling, where your gene or sequence of interest is located on your chromosome is visualized. Integrating the different maps and thereby combining the information they offer can be valuable by assisting the marker-assisted selection (Khan, 2009).

Polyploidization

In lily most of the intersectional hybrids are sterile due to problems at meiosis caused by the difference between the genomes. These differences result in association failure and abnormal segregation (Asano, 1982). In order to use hybrids in introgression breeding, the fertility of hybrids needs to be restored. This can be done by formation of polyploid plants (Van Tuyl et al., 1992). There are two ways of polyploidization, namely meiotic polyploidization and mitotic polyploidization. Polyploidization of plants can have both negative and positive effects. Polyploid plants are said to have an increased vigor due to heterosis and are to be less vulnerable for the effect of mutations due to gene redundancy (Comai, 2005). The development of polyploid lines is more difficult, the neopolyploids can be less stable due to among others genome enlargement. (Papp et al., 1996). Also,

there are problems at mitosis and meiosis (Comai, 2005). Spindle formation can be disrupted, which is essential for the formation of bivalents and subsequently a stable meiosis. Polyploidy in plants has a relation to the number of aneupolyploids formed, the number of aneupolyploids influences genetic and epigenetic stability (Papp et al, 1996). Summarized, polyploidy in plants can be both advantageous and disadvantageous. The main advantages of polyploidization of hybrids are the abolishing of F1 sterility but also results in plants with increased vigour and bigger flowers.

Mitotic polyploidization is an approach to restore the fertility of hybrids by somatic chromosome doubling, which is obtained by colchicine or oryzalin treatment of mitotic cells (Van Tuyl et al., 1992). These substances inhibit spindle formation (Marasek-Ciolakowska et al., 2012). The use of oryzalin or colchicine as spindle inhibitor has been successfully applied in crops like *Lilium* (Van Tuyl et al., 1992; Barba-Gonzalez et al., 2006; Xie, 2012), *Nerine* (Van Tuyl et al., 1992) and *Nicotiana* (Marubashi and Nakajima, 1985). In lily, chromosome doubling has been proven effective in enabling further crossing with the material, when using for example *Longiflorum* x Asiatic hybrids (Lim et al. 2000; 2001) that produced 2n gametes. Also research done on doubling of Oriental x Asiatic hybrids provided fertile offspring and subsequently the possibility to use hybrids in further breeding purposes (Barba-Gonzalez et al, 2004). Mitotic polyploidization with colchicine in tulip is more difficult compared to other crops and is not applied frequently. This is partly because the meristems of tulip are not easy to reach (Van Tuyl et al., 1992; Marasek-Ciolakowska et al., 2012). This problem can be solved by injection of colchicine into the ovary , in vitro treatment with oryzalin or colchicine or treatment with oryzalin during in-vitro stem-disc regeneration (Marasek-Ciolakowska et al., 2012). The disadvantage of this asexual method of polyploidization is that there is no recombination between the different genomes. This is because the different genomes will pair with their homologs, also called 'autosyndetic chromosome pairing'. Recombination will occur but between identical chromosomes therefore no new genetic variation is generated. As a result, the hybrids will be 'permanent hybrids' and will not be able to contribute any genetic variation (Lim et al., 2000; Van Tuyl et al., 2002; Ramanna and Jacobsen, 2003). The crossing polygon of lily shows lines indicating successful crosses within and between different sections. The crosses between sections are enabled by mitotic and meiotic polyploidization

Meiotic polyploidization involves the formation of unreduced gametes. Unreduced gametes occur in nature at a low rate, unreduced gamete formation is present in plants with a frequency of 0.5% per gamete. This rate can be increased by certain conditions like high temperature and the selection of genotypes that have a high rate of unreduced gamete formation (Schatlowski and Kohler, 2012). In most modern lily hybrid cultivars spontaneous meiotic polyploidization took place (Van Tuyl and Arens, 2011). LA, OA and OT intersectional hybrid groups are all obtained through meiotic polyploidization. In the interphase, before the meiosis starts, DNA replication takes place. The cells contain at this phase of the cell cycle two copies of each chromosome, one from the father and one from the mother. Recombination takes place between the different chromosomes. The cell cycle of meiosis consists of two reduction phases, each phase consists of a Metaphase , Anaphase and Cytokinese phase. The first reduction phase separates the two chromosomes from each other and is called a 'reductional division' . The second reduction phase separates the two sister chromatids from one chromosome from each other, as also happens during mitotis, and is called an 'equational division'(Comai, 2005). Unreduced gametes can arise through multiple meiotic abnormalities like blocking of a proper first or second reduction division, problems with spindle formation or cytokinesis (Xie et al., 2011). In the case of lilies the unreduced gametes are the result of first division

restitution (FDR), second division restitution (SDR) or Indeterminate meiotic restitution (IMR)(Mok & Peloquin. 1972; Ramanna, 1979, Lim et al., 2000). In FDR-originated progeny, the chromosomes have been separated but the chromatids have not been separated. The 2n gametes consist of non-sister chromatids, therefore indicating a maximum of heterozygosity (Lim, 2000). In SDR-originated progeny, the two chromosomes have not been separated but the chromatids are separated. The originating 2n-gametes are genetically different from each other and also from the parental genotype. In IMR-originated progeny the univalents, which are the unpaired chromosomes, divide equally but the bivalents, which are two homologous chromosomes paired at meiosis, disjoin reductionally (Schatlowski and Kohler, 2012). In interspecific hybrids, hybrid sterility is a consequence of FDR. The different genomes are too diverse and can therefore not recognize each other and will not form a bivalent (Blanco et al, 1983). For introgression breeding, polyploidization should happen with unreduced gametes in order to allow intergenomic recombination (Karlov et al., 1999a; Lim et al., 2001; Ramanna et al., 2003). The use of unreduced gametes in breeding has been successfully applied, for example in *Primula* (Skiebe, 1958), *Solanum* (Mendiburu and Peloquin, 1971; Mendiburu et al.,1974), *Medicago* (Bingham, 1980; Veronesi et al., 1986) ,*Alstroemeria* (Kamstra et al., 1999; Ramanna et al., 2003) and *Lilium* (Lim and Van Tuyl, 2002; Van Tuyl et al., 2002; Van Tuyl and Lim, 2003).

Cytogenetics

Cytogenetics is the part of genetics focussing on the structure and function of the cell, especially on the chromosomes. The *Liliaceae* family, including the *Lilium* and *Tulipa* genus, has been frequently used for chromosome analysis, it is very suitable for analysis of chromosomes because of the large size of the chromosomes (Khan, 2009). The chromosome studies on *Liliaceae* include subjects like chromosome morphology (Stewart. 1947, Marasek and Orlikowska, 2001), meiotic stages (Bennett and Stern 1975), cross-overs (Lim et al., 2003;Khan, 2009) and chromosome banding (Lim et al., 2001). Chromosome analysis of interspecific hybrids can provide information about the distribution of the parental genomes, the number of chromosomes and the frequency and location of recombination (Lim, 2000; Khan, 2009; Zhou, 2007; Xie, 2009).

Plant breeding has been changed majorly in the last few decades, partly due to the development of new cytogenetic techniques and an increased understanding of chromosome behaviour during meiosis. One of the key changes in plant breeding was the development of marker assisted-breeding. The use of high-throughput markers enables fast screening of vast numbers of cross progenies (Wijnker and De Jong, 2008). Molecular genetic markers like RFLP, AFLP and RAPD's have been developed to analyse allopolyploid crops. Another new breeding technique that has been developed is called 'reverse breeding' and has been applied in *Arabidopsis thaliana (*Wijnker et al, 2012*)*. This technique is 'reversed' from normal breeding because the breeding process does not start with selecting good quality parents but with selecting a good performing hybrid individual plant. By silencing DMC1, a gene that encodes a protein involved in meiotic recombination, cross-over recombination is suppressed. With this approach homozygous parental lines can be generated from a hybrid individual, through segregation of the chromosomes at meiosis and subsequent doubling of the DNA of the un-recombined parental chromosomes. Homozygous diploid lines that each contain half of the DNA from the original selected hybrid plant are obtained. From the resulting group of homozygous lines, the lines that, when combined, deliver the same hybrid that was used as starting material are selected (Wijnker et al., 2012). The homozygous lines are also very useful in mapping

traits. Other techniques like DNA in situ hybridisation and molecular mapping are developed to aid in improving systematic breeding approaches (Ramanna and Jacobsen, 2003). Karyotyping, which is mapping the chromosomes during metaphase, provides an image of all chromosomes and can be used to count the number of chromosomes and detect any anomalies in chromosome number. For analysing the whole genome, molecular cytogenetic techniques like GISH are deployed.

GISH

Genomic DNA *in situ* hybridization (GISH) is a frequently used cytogenetic technique to analyze genome composition. It is a technique that can visualize the different genomes when those are differentiated enough, and subsequently the (possible) recombination sites in chromosomes of different parental genomes. GISH is a technique using the total genomic DNA from a species as a fluorescent probe. It differs with FISH, another variation on the in-situ hybridization technique, where only a particular part of the DNA is used. The goal with FISH is to locate a specific part of DNA on a chromosome. GISH uses the complete genomic DNA and therefore labels the whole DNA. To identify hybrid with three or more genome origins, multicolor GISH can be used (Xie, 2012). It is a technique that can be used to analyze polyploid hybrid plants and their progenies to verify their origin, genome composition and intergenomic recombination (Ali et al, 2004). When using GISH for analyzing genome composition in hybrids or polyploid cultivars, the technique is valuable because it can clearly visualize the distinction between the different parental genomes. Lily is a suitable species for GISH analysis because the chromosomes are large and the genomes of the different groups are very different, making it easier to accurately identify structural arrangements (Khan, 2009). In tulip, GISH analysis is harder because the genomes, and subsequently the chromosomes are less distinct from each other because their length and morphology is similar and they lack distinct chromosomal landmarks. Only a few chromosomes can be identified by measuring their length and centromere position (Marasek-Ciolakowska et al., 2012). The GISH technique is a valuable tool for analyzing inter-genomic recombination.

The GISH technique has been successfully used in crops like *Lilium* (Karlov et al., 1999b; Khan, 2009; Lim, 2000; Barba Gonzalez, 2005; Zhou, 2007), *Tulipa gesneriana x T. fosteriana* (Marasek and Okazaki, 2008) ,potato (Jacobsen et al., 1995) and *Alstroemeria aurea x A. inodora* (Ramanna et al., 2003). Multiple studies of GISH in lily were performed. Research done by Lim (2000) provided insight in the principle of how *2n* gametes originate and what the genetic influence is of the different ways of originating of the *2n* gamete. GISH can be applied to visualize the difference between hybrids obtained by mitotic and meiotic polyploidization (Xie, 2012). The occurrence and use of *2n* gametes and their possible use on introgression breeding has been researched by, among others, Barba-Gonzalez (2005); Zhou (2007) and Khan (2009). Research done by Khan et al. (2007) showed the opportunities of using functional *n* and *2n* gametes in introgression breeding by putting the recombination data into chromosomal recombination maps. Making a chromosomal recombination maps is a tool to clearly analyze the possible contribution of parental genomes and the amount of inter-genomic recombination between different cross types. In a recombination map, the chromosomes are identified and ordered based on their total length and arm ratios. The recombination sites are identified and the distance of the recombination site to the centromere is calculated. The pattern of recombination, so the distribution and number of observed recombination sites, can provide an overview of how suitable the cross is for introgression breeding. The advantages of creating a recombination map over creating other maps are the fact that an overview of the whole

genome is given and that the recombination sites that are found can serve as permanent cytological land marks on chromosomes that can be used for mapping molecular markers or genes of interest (Lim, 2000). In interspecific hybrids, it is possible to determine the recombination sites with GISH. In order for the GISH to properly distinguish between the different parental genomes, the genomes need to be differentiated and not too closely related. The amount of recombination that is found also depends on the genome divergence between the parental genomes. The oriental hybrid group and the Asiatic hybrid group are more divergent than the *Longiflorum* hybrid group and the Asiatic hybrid group. Less recombination is expected in hybrids from Oriental and Asiatic descent than in hybrids from *Longiflorum* and Asiatic descent (Khan et al., 2007). Also, the recombination frequency between chromosomes can be different (Khan et al., 2009). The amount of recombination influences the efficiency of introgression breeding. When the aim is introgression a certain gene of interest, the location of the gene of interest is important to know because the recombination frequency and therefore the introgression efficiency is different for each chromosome

Meiotic recombination is essential in plant breeding, as it is one of the sources of generating genetic diversity. Breeders rely on meiotic recombination for generating new allele combinations. Understanding and controlling recombination is important to improve efficiency in plant breeding programmes (Wijnker et al., 2012). Recombination can be influenced by several factors like targeted disruption of a gene by using transgenes and application of physical stress, for example heat- or cold shock, chemicals and UV-radiation (Wijnker, 2013). Wijnker (2013) shows the critical role of CDKA;1, a master regulator that influences among others recombination. Modifying the expression of this gene can also influence recombination in interspecific hybrids. Influence can be exerted on recombination cross-over frequency, position and allocation to homoeologous regions (Wijnker et al., 2012).

Aim of the thesis

Introgression breeding is a very useful way of introducing new traits from a donor species into a recipient species. Interspecific hybridization is considered to be the best tool for obtaining this result. For example the year-round forcing ability of the *Longiflorum* hybrids can be combined with the good characteristics of the Asiatic hybrids, like a wide range of colours and upright facing flowers. In this research, different lily and tulip genotypes were cytologically analyzed using GISH. The plant material used has not been investigated before and is originating from meiotic or mitotic polyploidization. In this thesis research, the plant material consists of intersectional hybrids between the *Longiflorum* hybrid group, Oriental hybrid group and the Trumpet hybrid group. Special attention is paid to the genome composition, the purpose of this research is to find out the genome composition of the hybrids. Another main topic of interest is the presence of recombinant chromosomes and the frequency and location of recombination. The amount of recombination in the interspecific hybrid material gives information about the suitableness of the genotypes for introgression breeding.

Material and Methods

Plant Materials

The *Lilium* plant material used in this research consists of polyploid progeny derived from interspecific crosses between three groups of cultivars, namely *Longiflorum* (L), Oriental (O) and Trumpet (T) (Table 2). The parental material of the crosses is shown in Table 3. The progeny is derived from 4 different crosses. The first cross is made between an allotriploid (LLO) and an allotetraploid hybrid cultivar, namely TTTT. A second cross was made between an allotriploid (LLO) and a diploid OT cultivar. The third cross has an diploid (OT) hybrid parent and a diploid Oriental parent (OO). The last cross is made between two tetraploid (OT) hybrid cultivars. For each different genotype two plants are analyzed; one whole bulb and one bulb that has been disbanded in 5 separated scales. The plant bulbs are put on medium and stored at 20° C until 5-10 mm long roots have been developed.

Table 2. Overview of the lily crosses used in this research.

CrosNumber	Mother	Father	Genomes	No. of genotypes analyzed
117005	Triumphator	Angel Gabriel	LLO x TTTT	1
117029	Triumphator	Nymph	LLO x OT	1
117031	Triumphator	Nymph	LLO x OT	3
117033	Triumphator	Ice Folies	LLO x TTTT	1
117056	Triumphator	Ice Caves	LLO x TTTT	1
116613	Gluhwein	Santander	OT x OO	5
116622	Gluhwein	Tessa	OT x OO	2
106945	091078	Yeloween	OT x OT	4
106954	061037	101064	OT x OT	1

Table 3 The parent material of lily cultivars used in the present research.

Cultivars	No. of chromosomes	Genome composition		
		L	O	T
Triumphator	36	24	12	
Angel Gabriel	48			48
Ice Folies	48			48
Ice Caves	48			48
Nymph	24		12	12
Gluhwein	24		12	12
Pontiac	36		24	12
Belladonna	36		24	12
101064	24		12	12
111017	24		24	

Tessa	24		24	

The tulip plant material used in this research consists of a pool of 8 GG x GF BC1 progeny groups (Table 2). From each BC1 cross a number of bulbs ranging from 13 to 55 has been used in this study. The progeny studied with GISH is the result of diploid (*T. gesneriana* x *T. fosteriana* or GF) hybrids that are backcrossed as male parents to a *T. gesneriana* parent. From each of the 8 BC1 genotypes, one plant is analyzed. The bulbs underwent a cold treatment at 4° C for 6 weeks. When roots started developing, the bulbs were planted in the greenhouse. The bulbs are stored until 5-10 mm roots have been developed.

Table 4. Overview of the tulip crosses used in this research. The abbreviation G stands for *Tulipa gesneriana.* The abbreviation F stands for *Tulipa fosteriana*.

Number	Cross number BC1 (GGxGF)	BC1 parent (G)	Genotype code F1 hybrid (GF)	Mother F1 (G)	Father F1(F)	No. of plants analyzed
1111336	90017	Sachi	20193-7	Bellona	148 Cantata x Princeps	10
1111337	90018	Sensitive	20171-8	Bellona	113 Juan x Cantata	10
1111368	80057	Candy Price	20254-1	Pax	141 Mad. Lef x Princeps	10
1111377	80055	Royal Virgin	20193-2	Bellona	148 Cantata x Princeps	10
1111431	60058	Piet Paulusma	20221-3	Ile de France	137 Cantata x Mad. Lef	10
1111675	90009	Bellona	20259-12	Pax	155 Princeps x Cantata	10

Chromosome preparations

The roots are pretreated with 0.7 M cyclohexamide (lily) or 0.1% colchicine (tulip) and stored in Carnoy's solution. Subsequently the roots are digested in an enzyme buffer consisting of 1% pectolyase Y23 and 1% cellulose RS. The cells are squashed by a cover slip onto a glass slide with a drop of 45% acetic acid. The slide is heated on a slide heater for 30 seconds, washed with Carnoy's solution and air-dried at room temperature. The best slides are selected under a phase contrast microscope. The slides are stored at -20° C until further use

Probe and Block DNA

For GISH analysis, total genomic DNA is extracted from the young roots of tulip and lily as carried out as described in (Fulton et al, 2005). For the genotypes resulting from the crosses LLO x TTTT and LLO x OT, the probe DNA used for lily is DNA from White Fox, a *Longiflorum* cultivar. Block DNA consists of DNA from 'Sorbonne', an Oriental cultivar and 'Royal Gold', a Trumpet cultivar. The probe DNA for the genotypes resulting from the crosses OT x OO and OT x OT is DNA from 'Royal Gold'. For block DNA, DNA of 'Sorbonne', an Oriental lily cultivar is used. The block DNA used for GISH on tulip consists of Herring Sperm DNA and *Tulipa saxatilis* DNA. The probe DNA used for tulip consists of DNA of the cultivar 'Princeps' (*T. fosteriana)* and 'Ile de France' (*T.gesneriana*). The probe DNA is labeled with Digoxigenin-11-dUTP and Biotin-16-dUTP respectively by Nick translation in accordance with the manufacturer's instruction (Roche Diagnostics GmbH, Mannheim, Germany).

GISH procedure

The GISH procedure for the lily hybrids is carried out as is described in (Khan et al, 2009) (See appendix). The GISH procedure for the tulip hybrids is carried out as is described by Marasek-Ciolakowska et al., 2012. After the probes are labeled, hybridization and subsequent washing steps are carried out. The slides are treated with RNase A (100 μg/ml) and incubated in a humid chamber for 1 hour at 37°, incubated in 0.01 M HCl for 2 minutes at room temperature and subsequently incubated in a pepsin solution (100 μg/ml) in a humid chamber for 10 minutes at 37°. The slides were fixated with a 4% paraformaldehyde solution for 10 minutes at room temperature. The hybridization mixture consists of 50% deionised formamide, 50% dextransulfate, 20x SSC, 10% SDS and 150 ng of each probe and block DNA type. The hybridization mix is denatured for 10 minutes at 70° C and placed on ice for 5 minutes. After the hybridization mix is added to the slides, the slides are denatured for 5 minutes at 80° C and stored for hybridization overnight in a humid chamber at 37° C. Washing steps with 2xSSC at room temperature and 0.1xSSC for 30 minutes at 42° C and another step with 2xSSC are carried out. The probes are detected by anti-digoxigenin, with FITC for a green color, for the probes labeled with Digoxigenin-11-dUTP and by Cy3-streptavidin systems with a red color for the probes labeled with Biotin-16-dUTP. The slides were counterstained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) in Vectashield. The slides with the stained chromosomes are then photographed and analyzed with a Axiophot microscope and a Canon camera.

Results

Chromosome analysis of lily hybrids

From the initial eleven hybrid lily genotypes, six genotypes produced good quality GISH pictures. The other genotypes had problems with obtaining metaphases on the slides and obtaining metaphases that were good enough to analyse and photograph. The genotypes 117005, 117033 and 117056 were analysed to obtain knowledge about the behaviour of the Oriental, Trumpet and *Longiflorum* genomes in a LLO x TTTT cross. The genotypes 117029 and 117031 were analysed to obtain knowledge about the behaviour of the Oriental, Trumpet and *Longiflorum* genomes in a LLO x OT cross. For analysis of the OT x OO cross, genotype 116613 was used. GISH analysis provided proof that the crosses had indeed yielded hybrid lily genotypes. In most cases, the distinction between the different parental genomes was possible through multi-coloured staining of the chromosomes.

The genome analysis of genotypes resulting from a LLO x TTTT cross, including number of chromosomes, genome composition and number of recombinant chromosomes is summarized in Table 5. The chromosome number ranged between 41 and 42. The number of *Longiflorum* and Trumpet chromosomes was stable for each genotype, namely 24 chromosomes of the Trumpet genome and 12 chromosomes of the *Longiflorum* genome. The number of Oriental chromosomes varied between 5 and 6. This is due to the irregular univalent formation at the meiosis. The univalents of the *Longiflorum* and Trumpet chromosomes pair regularly while the univalents of the Oriental chromosomes are randomly distributed into the gametes. There were no recombinant chromosomes found.

Genotype code	Female parent	Male parent	No. of chromosomes	Genome composition			No. of recombinant chromosome
				L	T	O	
117005-1	Triumphator	Angel Gabriel	41	12	24	5	0
117033-1	Triumphator	Ice Folies	42	12	24	6	0
117033-2	Triumphator	Ice Folies	41	12	24	5	0
117033-3	Triumphator	Ice Folies	42	12	24	6	0
117056-1	Triumphator	Ice Caves	40	12	24	4	0

Table 5 The genome composition of the progeny derived from crossing allotriploid (LLO) ×autotetraploid (TTTT)

The genome analysis of genotypes resulting from a LLO x OT cross, including number of chromosomes, genome composition and number of recombinant chromosomes is summarized in Table 6. The chromosome number ranged between 42 and 44. For each genotype, a stable number of twelve chromosomes of the *Longiflorum* genome were found. Both in genotype 117031-1 and genotype 117031-2 one recombinant chromosome is found (Picture 4). The chromosome has a centromere of a Trumpet chromosome and a recombinant segment of an Oriental chromosome.

Table 6. Chromosome analysis of the Lily hybrid cross LLO x OT.

Genotype code	Female parent	Male parent	No. of chromosomes	Genome composition			No. of recombinant chromosome
				L	T (T/O)*	O	
117029-1	Triumphator	Nymph	42	12	12	18	0
117029-2	Triumphator	OTevans	44	12	12	20	0
117031-1	Triumphator	OTevans	42	12	13(1)	17	1
117031-2	Triumphator	OTevans	42	12	12	18	0
117031-3	Triumphator	OTevans	43	12	13(1)	18	1

*Chromosomes of the T-genome possessing an recombinant segment of the O-genome

Figure 3 and 4 show GISH results for the genotypes presented in Table 5 and 6. For each genotype the number of analysed metaphases ranged from 1 to 9. The most representative picture of each genotype is shown here.

Figure 3. GISH photos of lily genotypes 117005-1 (A), 117033-1 (B) and 117056-1 (C), all genotypes originated from a cross between LLO x TTTT. A blue color (DAPI) indicates a *Longiflorum* chromosome, a green color (digoxigenin-11-dUTP) indicates an Oriental chromosome and a red color (Biotin-16-dUTP) indicates a Trumpet chromosome.
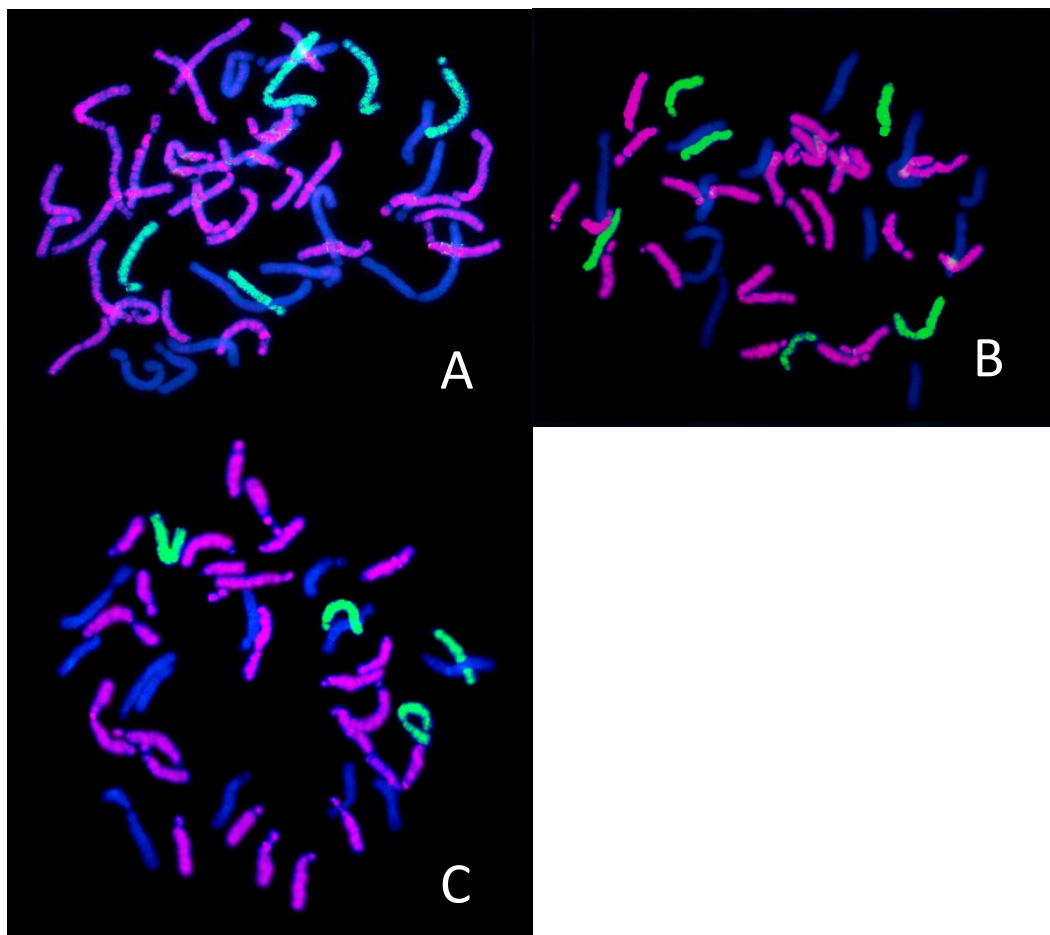
Figure 4. GISH photos of lily genotypes 117029-1 (A) and 117031-1 (B), all genotypes originated from a cross between LLO x OT. A blue color (DAPI) indicates a *Longiflorum* chromosome, a green color (digoxigenin-11-dUTP) indicates an Oriental chromosome and a red color (Biotin-16-dUTP) indicates a Trumpet chromosome. In figure 4B, a recombinant (T/O) chromosome is visible and indicated with a white arrow
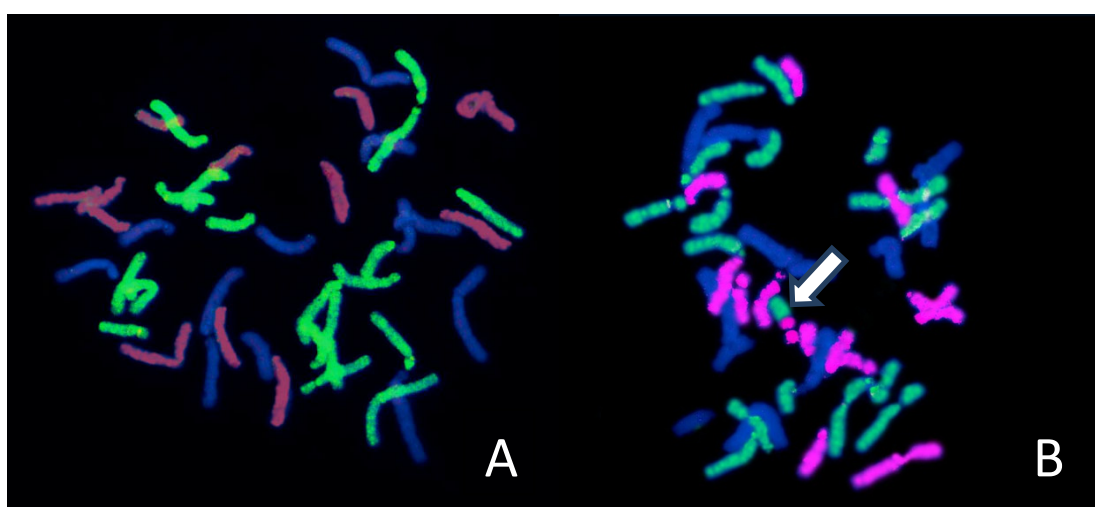
The cross OT x OO could only be analysed with one genotype, namely 116613. The genome analysis of the OT x OO cross is summarized in Table 3. Although clear distinction between the two genomes was difficult. The colour of the chromosomes seemed to change when different settings were tested

with the microscope. Therefore results were difficult to interpret. The GISH pictures showed a total of 36 chromosomes (Figure 7) with 24 Oriental chromosomes and 12 Trumpet chromosomes. This indicates that in this cross unreduced gametes were produced by a reduction restitution at meiosis. This result was as expected and shown by previous research (Luo et al, 2012). No recombinant chromosomes were found.

Table 7. Chromosome analysis of the lily hybrid cross OT x OO

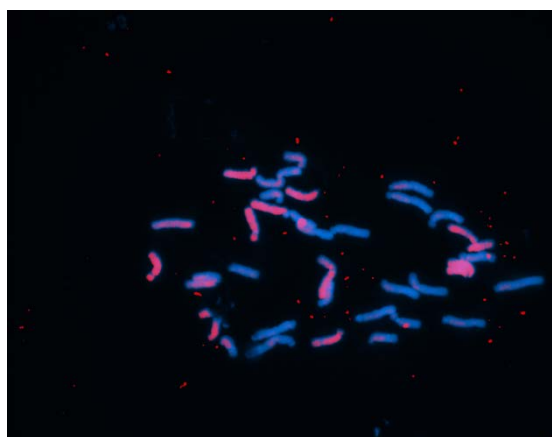| Genotype	Nr. Of chromosomes	Genome composition		Nr of recombinant chromosomes
		O-genome	T-genome	
116613	36	24	12	0

Figure 5. GISH photo of lily genotype 116613, resulting from the cross between OT x OO. A red color (Biotin-16-dUTP) indicates a Trumpet chromosome and a blue color (DAPI) indicates an Oriental chromosome.

GISH analysis of tulip hybrids

GISH pictures of tulip were more difficult to obtain. Due to time limitations, only one round of GISH on Tulip hybrids was performed on the genotypes 1111377, 1111675, 1111368 and 1111336. For one genotype, good quality GISH pictures were obtained. This genotype, 1111377-30, possesses 24 chromosomes (Figure 6). The Ile de France cultivar (*T. gesneriana*) was labeled with Biotin-16-dUTP and was red fluorescent. Cantata (*T. fosteriana*) was labeled with dig-oxigenin-11-dUTP and was green fluorescent.
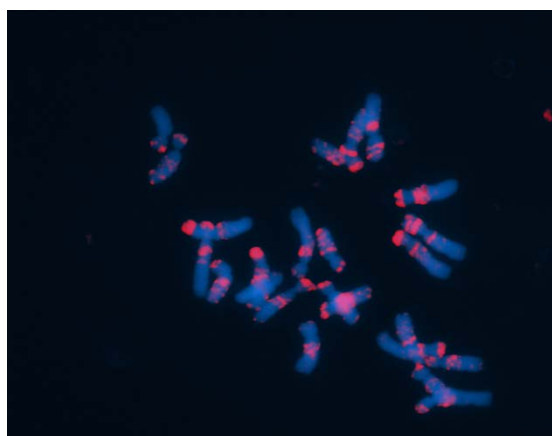
Figure 6. Tulip hybrid 1111377-30. The slides are counterstained with DAPI. *T. gesneriana* is stained with Biotin-16-dUTP (red). *T. fosteriana* is stained with Digoxigenin-11-dUTP (green) but is not visible in this picture.

In all tulip genotypes analysed using GISH in this study, there was lack of the typical 'chromosome painting' along the chromosomes. Only some segments of chromosomes exhibited stronger fluorescence intensity, therefore a banding pattern was visible in the GISH slides. Signals were predominately localized at subtelomeric/telomeric and intercalary chromosomal regions. There was only red hybridisation visible, no green fluorescence was observed. Probably there was a problem with labelling the *T. fosteriana* with, of with the detection of, the Digoxigenin-11-dUTP because there is no fluorescent signal present in any of the GISH pictures taken from the different genotypes.

Discussion

GISH analysis of lily

Because the polyploidization in the crosses LLO x TTTT is the result of mitotic polyploidization in both the parents there is no recombination expected between the parental genomes. The somatic doubling by oryzalin happened in mitotic cells. The homologous chromosomes that arose will only pair with each other. Similarly no recombinant chromosomes were found by Xie et al. (2012) and Van Tuyl et al. (2002). In two cases of a LLO x OT cross, namely in 117031-1 and 117031-3, a recombinant chromosome is found. This is in the gametes of the OT-parent where recombination takes place. The OT parent underwent meiotic polyploidization, the LO parent is mitotically doubled. Research done by Luo et al. (2012) shows GISH analysis of backcross progenies of OT hybrids, the article shows the presence of 2n gametes in OT hybrids, due to first division restitution. Meitotic polyploidization allows for recombination and can therefore explain the recombinant chromosomes found in genotypes 117031-1 and 11703133. The number of recombinant chromosomes found for a BC1 of OT x OO ranges from 0 to 9 chromosomes and the number of recombination sites per chromosome ranged from 0 to 4 (Luo et al., 2012). The BC2 progeny contained far less recombinant chromosomes. This was as expected because of the segregation that will occur in the triploid BC1 parent. These results indicate that OT hybrids can be well used for introgression breeding.

The polyploid genotypes obtained with meiotic polyploidization should show multiple recombinations. In this type of polyploidization, intergenomic recombination can be expected. The unreduced gametes that are the result of abnormal meiosis can recombine, unlike the case of the 'permanent hybrids' obtained by mitotic polyploidization. There were many recombinations to be expected, as shown in previous research by Van Tuyl et al., 2002 and Luo et al., 2012. Unfortunately, the hybrid genotypes that resulted from meiotic polyploidization, namely OT x OO and OT x OT did not produce enough results to be able to draw a conclusion about the recombination frequency of the chromosomes. The only slide with OT x OO or OT x OT background that yielded a GISH picture was a slide with the genotype number 116613 and a OT x OO background. This picture showed a distinction between the two different parental genomes but did not show any recombination sites.

There are multiple reasons that could have caused the GISH slides not to be of sufficient quality. The preparation of the slides is a vital element of the GISH technique. Proper enzymatic digestion of the root tips should take place before good metaphase chromosomes can be found. The main element of the GISH slide is the spread of the chromosomes in metaphase. If no good metaphase chromosomes are found, for example when the chromosomes are too close together or they are too wide spread, it is not possible to take a good picture. Getting proper metaphases was a challenge in this research. In order to get enough slides with good metaphases the most important thing is repetition. Getting good metaphases on the slides is influenced by experience and needs to carried out in bigger volumes, to filter out only the best slides for GISH analysis. For some genotypes, it was not possible in the limited time schedule to get slides with metaphases. Maybe the root tips of these genotypes needed a longer digestion time in the enzyme buffer in order for the chromosomes to be released out of the nucleus. Another reason preventing good metaphase spreading on the slide could be insufficient root top growth, the lack of a sufficient amount dividing cells makes it very hard to get good metaphases on the slides.

Another element of the GISH protocol that could explain the problems with obtaining a proper fluorescent picture is the hybridization step where the slides are incubated with the appropriate hybridization mix, including the labelled probe DNA. Almost all slides did not show a strong fluorescent image. Only the DAPI colour was shown, there was no other fluoresce visible. This can be explained by the insufficient addition of the labelled probe DNA. In one of the executions of the GISH protocol, there was not enough labelled probe DNA present. Instead of the required amount of DNA, only about half of that amount was added. If not enough labelled DNA was present to hybridise with the DNA on the slides, this could result in an insufficient fluorescent GISH image. This was probably one of the main reasons for the improper result of the slides with genotypes OT x OT and OT x OO.

It is difficult to clearly visualize the distinction between the two different genomes in the OT x OO cross. The difference between the two genomes is made by using one of the parent's DNA as a labelled probe and the other parent's DNA as a (unlabelled) block. In this case the Oriental parent was labelled with Biotin-16-dUTP and made visual with Cy3 (red). The other parent, a Trumpet cultivar, was used as a block. In research done by (Luo et al., 2012), the GISH method is slightly different. To enhance the visual discrimination possibility, both parents are used as labelled probes. No block DNA was used. In further research, this adjustment could be tried to improve the discrimination between the two parental genomes. Another method could be to use other, less related DNA as block, for example DNA from Asiatic hybrids. This might improve discrimination between the target genomes.

GISH analysis of Tulip

The banding pattern that was found in the GISH slides was previously reported after in situ hybridization of total genomic DNA of 'Queen of Night' to somatic metaphase chromosomes of 'Queen of Night' (Marasek et al., 2006). Signals were predominately localized at telomeric and intercalary chromosomal regions. The level of fluorescence is determined by the amount of hybridisation of the fluorescent probe to the DNA of the hybrid. Hybridisation occurs mainly to repetitive DNA sequences, therefor the banding pattern of higher fluorescent bands can be explained by the binding of the probe to repetitive DNA sequences. This pattern is also found and explained in other research (Marasek et al., 2006; Marasek-Ciolakowska et al., 2012). In this research of Darwin hybrid cultivars, in both *T. gesneriana* and *T. fosteriana* chromosomes, some segments in the telomeric and intercalary regions were found that exhibited a higher intensity of fluorescence. These segments were located in heterochromatin and were repetitive DNA. The uneven distribution of the fluorescent signals shows that repetitive DNA is mostly present at the end of the chromosome arms and in the pericentromeric regions. This banding pattern has been found in other crops like *Brassica* (Snowdon et al., 1997) and *Alstroemeria* (Kuipers et al., 1997). The presence of repetitive DNA in the heterochromatin and on the locations that show higher fluorescence is proven by in situ hybridisation with probes such as 5S and 45S. These probes have been used in various crops (Marasek-Ciolakowska et al., 2012a). The pattern of repetitive DNA is distinguishable between *T. gesneriana* and *T. fosteriana* genomes, as found in research by (Marasek-Ciolakowska et al., 2012). In future research, the banding patterns can be used to distinguish between the parental genomes of interspecific hybrids.

Repetitive DNA is present in heterochromatin. Heterochromatin has been found to influence the recombination frequency in plants (Stack, 1984). *Lilium* is known to contain a relative low amount of heterochromatin compared to *Tulipa*, which contains relatively a lot of heterochromatin. If heterochromatin indeed influences the recombination frequency, further research could be done on comparing the amount of recombination between lily and tulip and whether or not the locations of the recombination sites are similar with the locations of repetitive DNA. This research would require a method that can clearly distinguish heterochromatin in chromosomes and a method that can show the location of recombination, like GISH. Combining results that show the location of heterochromatin and the location of the cross-overs give a decisive answer of the possible connection between the two and the possible difference between lily and tulip.

In this research, the GISH technique did not detect differences between parental genomes. In further research, another technique that can be applied to visualize the genome composition of interspecific tulip hybrids and the subsequent differences between the parental genomes is karyotyping. Karyology provides a good overview of the chromosomes and can be used to analyse chromosome number and C-banding patterns in order to distinguish between parental genomes . This research is already done on tulip with chromosome number and C-banding (Marasek-Ciolakowska et al., 2012b) Karyotyping can detect if there are morphological differences between chromosomes of the parental genomes. Hybrids have successfully been identified, for example in *Lilium* (Okazaki et al., 1994; Marasek and Orlikowska, 2001). Karyotyping of tulip can be used to check for big morphological differences between chromosomes, like chromosome size that can make discrimination between the parental genomes possible. Chromosomes of *T. gesneriana* and *T. fosteriana* are highly similar, only the median chromosomes are distinguishable (Marasek-Ciolakowska et al., 2012a). If there are no

clear differences present between the genomes, as has been shown by previous research, discriminant analysis of chromosome size can be applied in order to detect small differences between chromosomes (Marasek et al., 2006). Karyotyping can be a tool for identifying recombinant chromosomes by visualizing for example the degree and distribution of heterochromatic regions. If there is a clear difference in heterochromatic regions between the parental genomes, karyotyping of the heterochromatic regions can be used to distinguish the possible presence of both parental genomes in a chromosome, and therefore can detect the presence of recombinant chromosomes. The research shows that identifying recombinants in tulip is very difficult compared to lily, because of the small differences between the parental genomes.

The use of *Tulipa saxatilis* DNA instead of DNA of *Tulipa tarda* as block can make a difference in the obtained results. *T. tarda* is used as block DNA in most GISH studies with tulip and is less closely related to *T. gesneriana and* T. fosteriana compared with *T. saxatilis* (see the tulip crossing polygon in Figure 2). To obtain good quality results, further research with tulip GISH can include a comparison between results obtained with *T. tarda* as block and with *T. saxatilis* as block, to see if there is a possible difference in GISH results.

One of the goals in breeding for good tulipcultivars is obtaining virus resistance. This research used multiple tulip backgrounds and has therefore more virus resistance potential compared with research done by Marasek-Ciolakowska et al., 2012, where only tulip cultivar 'Purissima' is used as background material. The chance of finding a cultivar that has a good virus resistance and after that focusing on introducing the resistance in another cultivar, is higher when a lot of different cultivars are investigated.

 In further research for obtaining virus resistance, a broad background of multiple cultivars can be advantageous for obtaining virus resistance faster. A broad background is conform with a higher heterozygosity for the resistance trait. Using cultivars from different background and different sections and crossing those cultivars can be a way in order to obtain

Obtaining good GISH results with tulip genotypes is more challenging than obtaining good GISH results with lily genotypes because the chromosomes parents in the tulip crosses are less distinctive. Gaining more experience in executing the GISH technique and having a longer time to establish a good database of GISH slides will be the most important elements in obtaining good GISH results in further research. Further research involving GISH analysis of recombination in hybrids is important to acquire knowledge about recombination frequency between specific crosses for a more efficient way of using lily or tulip hybrids in introgression breeding. Information on the amount of recombination is a good indication of the suitableness for a genotype for introgression breeding. A high recombination frequency enhances the possibility for combining the desirable traits of two different backgrounds into one cultivar. Also, the recombination sites that are found can be used for developing maps with the location of (markers linked to) specific traits of interest. Another way of checking the possible use of cultivars in introgression breeding could be the use of markers. Development of markers for the traits-of-interest of different cultivars would be very useful, knowledge would be obtained about the presence of the wanted traits resulting in a faster selection process in the breeding program (Shahin et al., 2009). Knowledge of the location of genes can be obtained through FISH and aid in the process of developing of markers and ultimately development of an extensive map containing many markers linked to genes-of-interest (Khan, 2009).

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Appendix

Propagation Protocol

Lily propagation Medium (per liter) pH 5,8 for autoclaving

- MS salts 2.2 grams
- Sucrose 50 grams
- Gelrite 4 gram

Make medium and poor petri-dishes and small tubes (with red lids)

For each genotype:
 Cut 5 scales for 1 petri-dish and place the scales with cutted ends in the medium. Place 1 small bulb in a tube with a red cap.

Store at room temperature until roots appear (2-4 weeks).

Chromosome preparation Protocol

Pre-treatment solution for Lily: 0.7 mM Cyclohexamide
Treat the roots for 5,5 hours with the cyclohexamide solution.
Then put the roots in Carnoy's solution (3:1 Ethanol: Acetic Acid) and store the roots at 4° until use.

Pre-treatment solution for Tulip: 0.1% Colchicine
Put the roots in 0.1% Colchicine for 4 hours at room temperature.
Then fix in Carnoy's solution for 12 hours and store in freezer at -20° until use .

Enzyme mixture:

2% Cellulose RS:
- 400 mg Cellulose RS
- 20 ml Citrate buffer (2 mM)

2% Pectolyase Y23:
- 400 mg Pectolyase Y23
- 20 ml Citrate buffer (2 mM)

Citrate buffer :
- Citric acid (10 mM): Dissolve 1.05 g citric acid in 500 ml distilled water
- Sodium citrate (10 mM): Dissolve 1.47 g sodium citrate in 500 ml distilled water
- Mix 440 ml of the citric acid solution (10mM) with 360 ml of the sodium citrate solution (10 mM) to form the citrate buffer solution (2 mM)
- Adjust the pH of the citrate buffer to 4.5

Mix equal volumes of the Cellulose RS solution and the Pectolyase Y23 solution

Roots are incubated in enzyme mixture for 90 minutes at 37° C.

Fulton DNA isolation protocol

PMB Rep. 13 (3) 1995; 207-209

Works well with tomato, poplar, cabbage

50 - 200 mg is sufficient

Starting material is lyophillized, grinded leaf (small leaves or leaf disc, grinded with glass pearls)

All centrifugations are at maximum speed.

- Switch waterbath on at 60°C
- Prepare fresh microprep buffer 2,5 parts extraction buffer (EB) (+ RNAse 100μg/ml), 2.5 parts lysis buffer and 1.0 part 5% sarcosyl (w/v). Add 0.38 g sodium bisulfite/100 ml buffer immediately before use.
- Add 750 μl microprep buffer to leaf powder, mix well
- Incubate in 60°C waterbath for 30-60 min.
- Extract with 800 μl chloroform (mix well)
- Spinn 5 min.
- Pipet off 400-600 μl aqueous phase
- Add equal volume cold isopropanol and invert tubes repeatedly untill DNA precipitates.
- Spinn 5 min.
- Wash with 500 μl 70% ethanol (pellet often very loose), spin 5 min. pour off.
- Dry pellet
- Resuspend DNA in TE^{-4}

Caution: chloroform is carcinogenic: wear gloves. Waste choroform in cat. cans

DNA extraction buffer:

Per liter:

- 0.35 M Sorbitol   - 63.7 g ⎫
- 0.1 M Tris-HCl   - 12.1 g` ⎬ pH 7.5
- 5 mM EDTA   - 1.7 g ⎭

Lysis buffer:

- 0.2 M Tris   ⎫
- 0.05 M EDTA   ⎬ pH 7.5
- 2 M NaCl   ⎪
- 2% CTAB   ⎭
-
Sarcosyl ⟶ Do not autoclave!

TE^{-4} : 10 mM Tris/0,1 mM EDTA pH 8.0

GISH protocol Lily

Day 1

Pre-treatment

1- Dry the chromosome preparations in an incubator at 37° C overnight
2- Dilute RNase A stock to a concentration of 100 µg/ml (1:100) in 2x SSC. Add to each slide 100 µl of the diluted solution. Cover the slide with a 24x50 coverslip and incubate the slides in a prewarmed humid chamber f or 1h at 37° C. Then, remove the coverslip
3- Wash the slides in 2x SSC three times for 5 minutes
4- Incubate the slides in 0.01 HCl for 2 minutes
5- Dilute pepsin-stock to a concentration of (1:100) in 0.01 HCl. To each slide add 100 µL of the diluted pepsin solution. Cover the slides with a coverslip and incubate the slides in a humid chamber for 10 minutes at 37° C. Then, remove the coverslip
6- Wash the slides in Milli-Q water for 2 minutes
7- Wash the slides in 2x SSC two times for 5 minutes
8- Incubate the slides in 4% paraformaldehyde for 10 minutes (use the fume hood)
9- Wash the slides in 2x SSC three times for 5 minutes
10- Dehydrate the slides 3 minutes each in 70%, 90% and absolute ethanol and then, airdry at 37° C

Hybridization

11- Prepare the hybridization mix (40 µL per slide)
   - 20 µl formamide
   - 8 µl 50% dextransulfate (DS)
   - 4 µL 20X SSC
   - 1 µl 10% SDS
   - x µl probe DNA (25 ng/slide)

- y µl block DNA
- z µl water

12- Denature the hybridization mix for 10 minutes at 70° C and directly place on ice for at least 5 minutes.
13- Add the appropriate hybridization mix to each slide and cover with a plastic 24x24 coverslip.
14- Denature the slides for 5 minutes at 80° C on the slide heater
15- Place the slides in a prewarmed humid chamber and incubate overnight at 37° C.

Stringency washing

16- After removing the coverslips, wash the slides with 2x SSC for 5 minutes
17- Wash the slides in 0.1x SSC for 30 minutes at 42° C.
18- Wash the slides in 2x SSC for 5 minutes

Day 2

(From here on, work in the dark)

Detection

19- Wash the slides in buffer 1
20- Add 100 µl blocking reagent in buffer 1 (block buffer) to each slide. Cover the slides with a 24x50 coverslip and incubate for 5 minutes at room temperature
21- Dilute appropriate antibidoes in 100 µl blockbuffer per slide. See table
22- Remove coverslips, drain the slides and add the diluted antibodies to the slide. Cover the slides with coverslips and place the slides in a humid chamber for 1 hour at 37° C.
23- Remove coverslips and wash in buffer 1 three times for 5 minutes at 37° C
24- Perform 2nd and 3rd detection as above. Start from step 20
25- Add 100 µl DAPI or PI solution (1:100 solution in SSC) on slides. Cover slides with coverslip and incubate for 5 minutes at room temperature
26- Wash briefly three times in Milli-Q water and dry of the slides at 37° C.
27- Add a drop of Vectashield to each slide and apply a coverslip
28- The slides can be stored at 4° C in dark in a slide box.

	Red	Green
Label	**Biotin-16-Dutp**	**Dig-11-UTP**
1st detection	Streptavidin-CY3 1:100 0.4 µl per slide	
2nd detection	Biotinylated antistreptavidin 1:200 2 µl per slide	Anti-dig-FITC 1:100 2 µl per slide
3rd detection	Streptavidin-CY3 1:200 0.4 µl per slide	Anti-sheep-FITC 1:200 2 µl per slide

Buffer 1: 0.1 M TRIS HCl, 0.15 M NaCl, pH 7.5

20x SSC: 100.2 g Sodium Citrate, 175 g Sodium Chlorite, 1000 ml DDW.

Autoclave and store at room temperature