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REVIEW

# FISH and GISH: molecular cytogenetic tools and their applications in ornamental plants

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**Abstract**
***Key message*** **The innovations in chromosome engineering have improved the efficiency of interrogation breeding, and the identification and transfer of resistance genes from alien to native species.**
*Abstract* Recent advances in molecular biology and cytogenetics have brought revolutionary, conceptual developments in mitosis and meiosis research, chromosome structure and manipulation, gene expression and regulation, and gene silencing. Cytogenetic studies offer integrative tools for imaging, genetics, epigenetics, and cytological information that can be employed to enhance chromosome and molecular genomic research in plant taxa. In situ hybridization techniques, such as fluorescence in situ hybridization (FISH) and genomic in situ hybridization (GISH), can identify chromosome morphologies and sequences, amount and distribution of various types of chromatin in chromosomes, and genome organization during the metaphase stage of meiosis. Over the past few decades, various new molecular cytogenetic applications have been developed. The FISH and GISH techniques present an authentic model for analyzing the individual chromosome, chromosomal segments, or the genomes of natural and artificial hybrid plants. These have become the most reliable techniques for studying allopolyploids, because most cultivated plants have been developed through hybridization or polyploidization. Moreover, introgression of the genes and chromatin from the wild types into cultivated species can also be analyzed. Since hybrid derivatives may have variable alien chromosome numbers or chromosome arms, the use of these approaches opens new avenues for accurately identifying genome differences.

**Keywords** Banding techniques · Chromosome characterization · In situ hybridization · Physical mapping · Polyploidization

## Introduction

In the 1950s, various technical developments, such as colchicine addition to capture cells in metaphase and the use of hypotonic solutions to obtain better chromosome spreads, were established. Then various cytogenetic techniques, e.g., banding techniques, i.e., G-, R-, C- and NOR-banding, and sister chromatid exchange (SCE), were developed (Kannan and Zilfalil 2009). Classical cytogenetics was the only chromosome analysis technique until the start of the 1970s and it was primarily based on the study of chromosome morphology, i.e., chromosome arm size, placement of the secondary constrictions, centromere position, and chromosome number, including alterations in chromosome number (Silva and Souza 2013). The chromosomal nucleolar organizer regions (Ag-NORs) are very important in chromosome characterization, but this approach had limited reproducibility and was time consuming. The conventional cytogenetic banding approaches

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were very narrow in their application, were restricted to the metaphase stage, and could not identify chromosome aberrations or chromosomal rearrangements, which can occur in chromosome regions with similar band analyses (Tonnies 2002).

Recently, advancements in cytogenetic techniques, such as fluorescence in situ hybridization (FISH), and genomic in situ hybridization (GISH) have widely improved the scope of its applications, which now range from karyotype analysis to gene localization. Modified nucleotides, which bind with fluorescent moieties—biotin or digoxigenin, are used to make in situ hybridization probes. These probes allow microscopic imaging and localization of complementary sequences in cells, nuclei, and single chromosomes (Shen et al. 1987; Xu and Earle 1994; Hwang et al. 2011). Over the last 25 years, direct and indirect FISH analysis has been applied to the chromosome characterization of various plant taxa (Jiang and Gill 2006). During its initial development, in situ hybridization had some drawbacks, such as limited reproducibility and sensitivity (Gall and Pardue 1969). The recent breakthroughs in cytogenetics and 3D-structured high-resolution imaging technology have overcome the inconsistencies of in situ hybridization, and have also closed the gap between the cytological and molecular approaches to chromosome and genome analysis in plants (Wang et al. 2009; Figueroa and Bass 2010; Hwang and Kim 2014). Cytogenetics tools have now been developed that can offer an integrated approach to a wide range of studies ranging from functional and structural genomics to chromosome engineering and comparative evolutionary biology (Ananiev et al. 2009; Harper et al. 2012).

## FISH (fluorescence in situ hybridization)

FISH is a powerful, consistent tool that is used in plant molecular cytogenetics to detect changes in chromosomes and to ascertain the distribution of specific DNA sequences. The FISH technique was established in 1981 using DNA fragment labeling by fluorochromes, which are detected by ultraviolet light (Schwarzacher et al. 1989). The FISH technique, which has led to the development of other innovative molecular cytogenetic methods, identifies particular DNA sequences at the interphase or metaphase stages of an organizationally preserved cell. The DNA paired double-stranded characteristic is the basis of the FISH technique. This technique results in the hybridization of particular DNA probes through annealing or binding of the complementary DNA sequence. Molecular cytogenetic analysis is carried out using a chromosome-specific probe labeled with fluorescent dyes and after the probe has been attached to the target, a fluorescent signal can be detected (Fig. 1). This targeted approach produces more precise and accurate molecular cytogenetic results compared to classical banding techniques, such as probe labeling, and detection with fluorescence microscopy allows the simultaneous analysis of different genes (Mühlmann 2002; Lysak and Mandáková 2013).

The cytogenetic techniques, based on molecular level radioactively labeled probes, were used initially because low energy radiation, such as isotope tritium [3H], ensured better resolution of the probe. Currently, non-radioactive DNA probes are used, such as biotin and digoxigenin, as labeling agents. Detection of labeling molecules is by indirect staining, i.e., an antibody–fluorochrome conjugate, or by direct staining, i.e., fluorochromes. When fluorochromes are used, the technique is termed fluorescent in situ hybridization (FISH) (Guerra 2004). FISH has proved to be an excellent tool for studying copy numbers at various locations, the DNA sequence distribution on the chromosome, and for observing the evolutionary variations to their physical structure in the genome (Harrison and Heslop-Harrison 1995). Chromosome size, secondary constriction, and centromere position analyses by conventional staining techniques were unable to distinguish between individual chromosomes that had a similar morphology and size (Song 1987). Fluorescence in situ hybridization (FISH) can improve resolution so that copy numbers and DNA sequences at various chromosome locations can be identified (Harrison and Heslop-Harrison 1995). (Heslop-Harrison et al. 1991) also reported that FISH analysis gives more NOR detail when ribosomal DNAs are used as a probe. FISH analysis of *Lilium* Sinomartagon showed that there was a divergence in number, size, and the distribution pattern of 5S and 45S rDNA gene loci, and the nontranscribed spacer (NTS) and internal transcribed spacer (ITS) regions analysis clearly showed ribosomal DNA evolution and species interrelationships among Sinomartagon lilies (Lee et al. 2014). This technique can also visualize large-size chromosome segments, chromosome arms, and whole chromosomes via chromosome painting (Lysak and Mandáková 2013). During chromosome painting, contigs of bacterial artificial chromosomes from target species are generally used as painting probes. This can help trace and identify any specific chromosome section or an entire chromosome during the meiotic stage, and this also enables structural chromosome rearrangements and meiotic-pairing to occur (Lysak and Mandáková 2013).

The FISH technique enables researchers to identify various ribosomal probes that can establish phylogenetic interrelationships when comparing plant species and their genetics (Jiang et al. 1995). Furthermore, this powerful tool has been used for mapping multi-copy gene families and repetitive DNA sequences (Jiang and Gill 2006), and for precisely locating gene's position on chromosomes. This

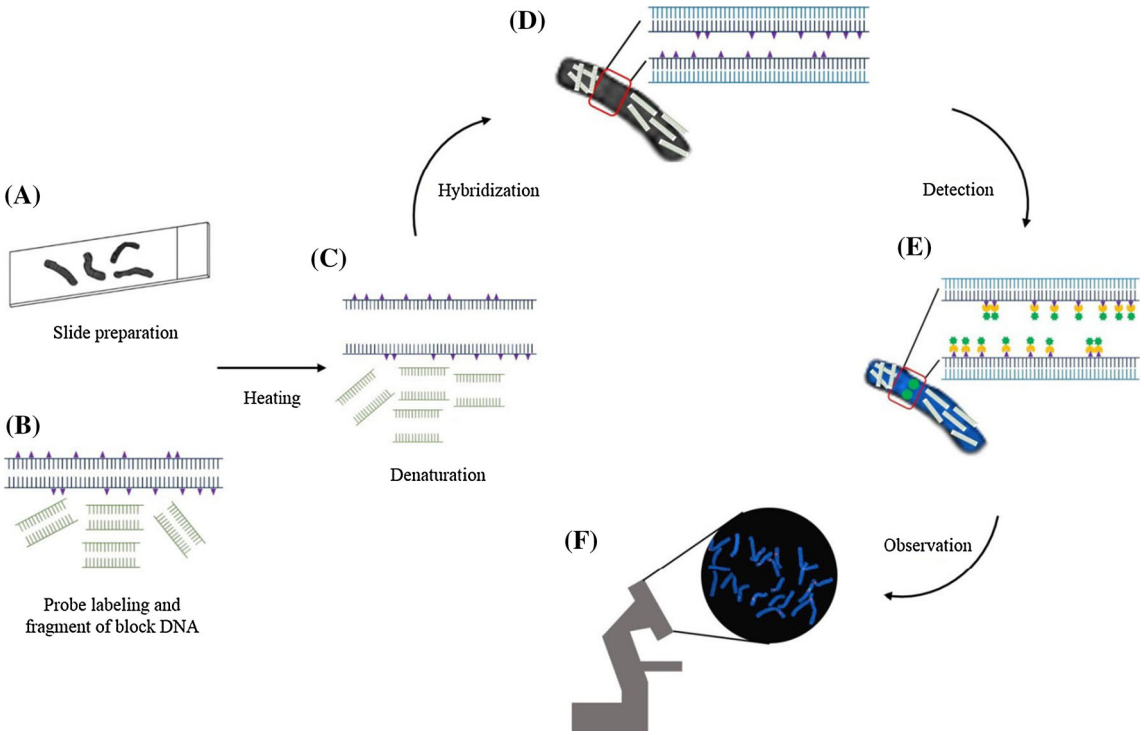


**Fig. 1** Concept of fluorescence in situ hybridization (FISH). **a** Somatic metaphase chromosomes were obtained from root tips and squashed on the slides. **b** Target DNAs were labeled with haptens and non-hybridization positions were blocked with fragmented DNAs. **c** For denaturing, hybridization mixtures containing probes and block DNA were incubated at 70 °C for 10 min. **d** In situ hybridization of target sequences on the chromosome. **e** Diluted antibody added to the hybridization area. **f** The target signals can be visualized as fluorochromes under fluorescence microscope

**Fig. 2** FISH karyotype analysis of *Lilium bakerianum* using 5S (*green fluorescence*) and 45S (*red fluorescence*) rDNAs. Homologous chromosomes were classified according to their centromere position, rDNA signal patterns, and chromosome shape. Chromosomes are arranged in order of descending short arm length

method is now the most common way of detecting low copy and individual DNA sequences, and gene locations on chromosomes (Guzzo et al. 2000; Fransz et al. 1996). FISH karyotype analysis could play a key role in mapping and detecting the cloned DNA sequence positions on chromosomes (Fig. 2). This analysis has become a common method for mapping, localizing, and positioning the genes in many different genomes (Table 1). The information is considered vital when attempting to narrate molecular sequences and genetic loci, and the morphological sequences of chromosomes.

## Physical mapping

The mapping of ribosomal DNA through FISH is a targeted and effective tool for accurately characterizing diverse groups of germplasm materials and breeding lines. The FISH analysis technique provides ribosomal DNA

**Table 1** Application of cytogenetic techniques in various ornamental plants

| Plant | Species | Chromosome# | Technique | Probe type | Working | References |
|---|---|---|---|---|---|---|
| *Alstroemeria* | *A. aurea* | 16 | FISH | *A. aurea* A001-I<br>*A. aurea* A001-IV | Repetitive DNA sequences localization and characterization in ornamental *Alstroemeria* | De Jeu et al. (1997) |
| *Antirrhinum* | *A. majus* | 16 | FISH | CentA1, TAC clones | Mapping of pachytene chromosome | Zhang et al. (2005) |
| *Chrysanthemum* | *C. boreale* | 18, 20 | FISH | 5S and 45S<br>Ribosomal DNAs | Karyotype analysis of wild *chrysanthemum* of Korean habitat | Hwang et al. (2013) |
| *Lilium* | *L. henryi*<br>'Marco Polo'<br>'Expression' | 24 | FISH and GISH | FISH using 25S rDNA and 5S rDNA | Confirmation of hybrids | Marasek et al. (2004) |
| | *L. tigrinum* | 24, 36 | FISH | FISH using 25S rDNA and 5S rDNA | Karyotype study of diploid and triploid *L. tigrinum* | Hwang et al. (2011) |
| | *L. distichum* | 24 | FISH | 5S and 45S<br>Ribosomal DNAs | Physical mapping and karyotype analysis | Hwang et al. (2015b) |
| | *L. longiflorum*<br>*L. rubellum* | 24 | FISH | Clone pTa71 holds a 9-kb *Eco*RI fragment of the 45S rDNA (wheat) and pScT7 holds a 462-bp *Bam*HI fragment of the 5S rDNA from rye | Karyotype study of *Lilium longiflorum* and *L. rubellum* | Lim et al. (2001b) |
| | Asiatic cultivar (Golden Horn and Pollyanna) | 24, 36 | FISH | Cloned 45S rDNA from rice | Cytogenetic study of Asiatic cultivars and their $F_1$ hybrids | Gao et al. (2014) |
| | *L. regale*, *L. duchartrei L. brownii* var. *viridium* and *L. leucanthum*<br>var. *centifolium* | 24 | FISH | 45S rDNA, from clone pTa71 and 5S rDNA from clone pScT7 | Ribosomal gene mapping and chromosome study | Wang et al. (2012) |
| | *L. longiflorum* and Asiatic hybrid (LA hybrid) | 24, 36 | GISH | Genomic DNA of *longiflorum* cultivar 'White Fox' | Karyotype structure and intergenomic recombination | Khan et al. (2009b) |
| *Paphiopedilum* | *P. delenatii* | 26 | FISH and GISH | GISH using genomic DNA of parental specie *P. delenatii* and FISH using 45S rDNA | Chromosome pairing behavior at the meiotic MI stage by GISH and homeologous pairing tracing by FISH | Lee et al. (2011) |
| | *P. micranthum* | 28, 42 | | | | |
| | *P. bellatulum* | 30–37 | | | | |
| | *P. othschildianum* | | | | | |
| | *P. callosum* | | | | | |
| | *P. glaucophyllum* | | | | | |
| | *P. moquetteanum* | | | | | |
| *Petunia* | *P. linearis* | 18, 14 | FISH | 18S-5.8S-25S rDNA | Analysis of chromosome structure and distribution in hybrid and wild petunia | Benabdelmouna and Abirached-Darmency (1997) |
| | *P. integrifoliaa* | | | | | |
| | *P. hybrid* | | | | | |
| | *P. parviflora* | | | | | |
| | *P. parodii* | | | | | |
| | *P. axillaris* | | | | | |
| | *P. inflata* | | | | | |

**Table 1** continued

| Plant | Species | Chromosome# | Technique | Probe type | Working | References |
|---|---|---|---|---|---|---|
| *Passiflora* | *P. cacaoensis*<br>*P. gardneri*<br>*P. gibertii* | 18 | FISH | 45S ribosomal DNAs | Cytological preparations for in situ hybridization protocol | Souza et al. (2010) |
| *Rosa* | *R. willmottiae*<br>*R. rugosa*<br>*R. marretti*<br>*R. foliolosa* | 14 | FISH | 45S ribosomal DNA clone from wheat and 5S ribosomal DNA clone from *R. multiflora* | Wild rose species karyotype analysis | Akasaka et al. (2003) |
| *Tecoma* | *T. garrocha Hieron*<br>*T. stans Juss.* | 18 | FISH and GISH | FISH using 18S rDNA and GISH using Dig-labeled *T. garrocha* | Hybridity confirmation by GISH and 18S rDNA copy number determination by FISH in *Tecoma* hybrids | Contreras et al. (2012) |
| *Tulip* | *T. fosteriana*<br>*T. gesneriana* | 24, 36 | FISH and GISH | Leaves of Tulip 'Red Emperor' and 'Queen of Night' | Analysis of Darwin hybrid origins | Marasek et al. (2006) |

locations, and physical mapping is an important procedure that has been used to obtain an image of the *Chrysanthemum* genome and the Anthemideae-related genera genomes. It has shown that in a few gymnosperms, there is localization of 45S and 5S ribosomal DNA (Abd El-Twab and Kondo 2012). The 45S and 5S rDNAs, combined with the FISH technique, can produce unique information that can be used in *Chrysanthemum* breeding and in genome detection. Moreover, the discovery of wide colocalization in *Argyranthemum*, *Artemisia*, *Nipponanthemum*, *Tanacetum*, and *Leucanthemella* has produce valuable information about the interrelationships between species and the evolution of ribosomal DNA.

In *Iris*, FISH analysis was used to detect phylogenetic and genetic associations based on chromosome markers, plastid sequences, and the number of chromosomes. The different intensities of the fluorescence signals showed that loci 5S ribosomal DNA and 45S ribosomal DNA had variable numbers of repeats. In *Iris tingitana*, analysis of in situ hybridization showed the occurrence of a pair of metacentric chromosomes that had a high intensity of 5S ribosomal DNA signals. The use of the 5S ribosomal DNA probe identified eight low-intensity signal locations, but the results depended upon metaphase quality. Four locations containing 45S ribosomal DNA gene bands that described the minor contractions and signals related to NOR were observed in two chromosome pairs. Four major gene clusters of 45S rDNA were also observed in two chromosome pairs showing the secondary constrictions that were satellite linked with the NORs (Martınez et al. 2010).

## GISH (genomic in situ hybridization)

Since the first appearance of DNA sequences in situ hybridization, various changes to have been made to this technique. One of these changes is the genomic in situ hybridization (GISH) technique where the targeted DNA of an organism is bound to the genomic DNA of another organism, which is the probe (Valente et al. 2009).

GISH is a sophisticated tool that can discriminate between parental genomes, show an interspecific hybrid's genome organization, and consequently the (promising) recombination locations in chromosomes of allopolyploid and interspecific introgression lines (Jiang et al. 1995). The GISH technique uses the total genomic DNA of a species, in contrast to FISH, where a specific part of the DNA is used as a probe (Xie 2012). In GISH analysis, more DNA is available, and therefore, specific probes are easier to identify and amplify (Peñaloza and Pozzobon 2007). However, the ratio of probe/blocking DNA should be sufficient to inhibit the chromosome labeling of both genomes together (Brammer et al. 2009). The role of blocking DNA is important in hybrids derived from closely related

species because there is high possibility that homology can be appear during hybrid production (Parokonny et al. 1997; Xie et al. 2010).

This extensively applied cytogenetic method offers a direct visual analysis for differentiating parental genomes and can be used to investigate genome association allopolyploid species, interspecific introgression lines, and interspecific hybrids (Jiang and Gill 1994). This tool can also be an effective and precise technique for finding alien chromatin stages and integration positions. Furthermore, in recent years, GISH has been successfully used to determine several intergenomic, intersectional, and interspecific *Lilium* hybrid cultivars (Lim et al. 2000, 2001a, 2003; Zhou et al. 2008; Khan et al. 2010). Moreover, the GISH technique offers a new tool for better parental genome analysis in somatic and sexual hybrids. This analysis can identify translocation-related chromosomes from various genomes and also makes it possible to analyze painted-chromosomes during the complete cell cycle (Kenton et al. 1993; Zhao et al. 2013).

GISH can be used to analyze meiotic behavior, i.e., chromosome behavior during the meiotic cycle, the chromosome pairing process, the meiotic behavior of artificial and natural hybrids, and homologous and homeologous chromosome pairing (Xie et al. 2010). The GISH technique can also quantify the multivalent, bivalent, and univalent formation rates in hybrids by connecting them to genitor genomes, and the chiasma frequency between homeologous chromosomes. Another basic benefit is that when using this technique, we can see which factors cause irregular meiosis, and how this may affect fertility (Silva and Souza 2013). GISH also confirms the existence of hybrids derived from apomictic species, which can reproduce without fertilization. The occurrence of apomixis has already been observed in some species of the genus *Lilium*. Consequently, it is essential to find out if the hybrids resulting from the cross between these species are true hybrids or not. This can be unequivocally identified using GISH. Due to a clear and definite difference between genomes, GISH can be used to confirm allopolyploid species and hybrid plants (Table 1). When there is no initial information on chromosome morphology, this method can be used to identify the genomic origin during prophase and metaphase chromosome spreads, and in interphase nuclei (Marasek et al. 2004). The GISH technique is the method with the ability to identify intergenomic recombination, genome composition and the origin of polyploid hybrids and their progeny (Ali et al. 2004; Chung et al. 2013).

## Chromosomal analysis

The individual chromosome identification abilities of FISH can help to detect the interspecific hybrids and backcross progenies that have been produced for various plant species (Iwano et al. 1998; Khan et al. 2009a, b, 2010; Luo et al. 2012). Furthermore, gene mapping on chromosome arms and the tracing of chromosome segments in successive backcross individuals can mostly be achieved by karyotype analysis. Wang et al. (2012) carried out a karyotype analysis of four *Lilium* species (*L. brownii* var. viridium, *L. duchartrei*, *L. leucanthum* var. centifolium, and *L. regale*) using the FISH technique banding with 45S and 5S rDNA probes, together with morphometric information. Their findings revealed that the comparative length of four genomes were 300 μm and the karyotype analysis confirmed two pairs of (sub) metacentric and ten pairs of (sub) telocentric chromosomes. In *L. regale*, two 5S rDNA and six 45S rDNA signals were detected in four pairs of homologous chromosomes, while in *L. duchartrei*, four 5S rDNA and twelve 45S rDNA signals were observed in seven pairs of homologous chromosomes. However, four 45S rDNA and two 5S rDNA loci were found in three pairs of homologous chromosomes of *L. leucanthum*. Furthermore, seven 45S rDNA gene loci, and four 5S rDNA loci were identified in five pairs of homologous chromosomes and one on chromosome 2. Heterozygosity in *L. leucanthum* var. centifolium was confirmed by the odd number of 45S rDNA signals. This technique has been successfully used in *Lilium* breeding and FISH karyotype investigations of *L. hansonii*, *L. tsingtauense L. distichum*, *L. lancifolium* (2x), *L. callosum*, and *L. concolor* that used ribosomal DNA probes for the 5S rDNA and 45S rDNA. The modifications in the 45S rDNA gene loci in the *Lilium* genome revealed microstructural variations in the 45S rDNA sequence, which may have appeared during their evolutionary development. Physical mapping of the 45S rDNA signals from chromosome loci showed the tandem repeats coding 45S rRNA that had developed independently. The occurrence of repetitive sequences is the result of molecular processes, such as gene transfiguration, rearrangement, and irregular crossing-over, which change the Mendelian segregation percentage and induce a specific pattern of evolution. Additionally, tandem repetitive arrangements and the 45S signal rDNA proved a classic model to explain the DNA sequence's organization in the genus *Lilium*.

Chromosomal organization, distribution, and 5S rDNA spacer interspecific variation were analyzed in seven *Petunia* species using FISH (Benabdelmouna and Abirached-Darmency 1997). The species study divided into two groups, based on the amplified 5S rDNA variant size. One group contained *Petunia integrifolia* ($2n = 14$) and *Petunia linearis* ($2n = 18$) with a 460-bp repeat unit, whereas the second group included *Petunia* hybrid plants and *Petunia* wild type species ($2n = 14$) with a 350-bp repeat unit. After amplified fragments cloning, these were used in the FISH analysis to quantify the 5S rDNA location and number. The 5S rDNA chromosomal organization meant that it was possible to differentiate between a colored

flower species group that had one locus on chromosome 2 next to the 8S-25S rDNA loci, and a white flowered species that had a surplus locus on metacentric chromosome IV in the centromeric region. The FISH analysis of the majority of the studied *Petunia* species showed four hybridization locations for 18S-5.8S-25S rDNA loci. Two hybridization locations were only found in *P. parviflora* and *P. linearis* ($2n = 18$). The expression patterns of the hybridization locations of 18S-5.85-25S rDNA in interphase nuclei proved that these locations are transcriptionally active. The FISH results, together with the RFLP and PCR amplification results for rDNA loci, provided new information about the phylogenetic interactions among *P. hybrida* and *Petunia* wild type species (Benabdelmouna and Abirached-Darmency 1997). Microdissection and microcloning was carried out on *L. tigrinum* by Hwang et al. (2015a) to study the evolutionary patterns of repetitive DNA. They constructed a chromosome library of repetitive DNAs and considered this a novel tool for complete genome sequencing.

The *Antirrhinum* genus has been widely studied in classical plant genetics. Many different aspects, such as transposon-induced mutation, flower development biology, and gametophytic self-incompatibility have been investigated. Zhang et al. (2005) studied the cytogenetic characterization of *Antirrhinum majus*. The tandem repetitive sequences, CentA1 and CentA2, were isolated from the centromeric points of *Antirrhinum* chromosomes. FISH analysis was performed by attaching these repetitive sequences to the meiotic pachytene chromosome. According to the FISH pattern, CentA2 was the centromeric portion of chromosome 2 while CentA1 acts as the centromeric part for the other five chromosomes. Heterochromatin distribution has been described by developing an ideogram based on the DAPI (4′,6-diamidino-2-phenylindole) staining pattern of the pachytene chromosomes. Furthermore, TAC clones (transformation-competent artificial chromosome) were used as a probe in the FISH analysis. After hybridization of these clones with *A. majus*, the pachytene chromosomes showed correlations between linkage groups (LGs) and chromosomes in the *Antirrhinum* genome.

In various tulip species, cytological discrimination of single genomes and specific chromosomes can be determined using FISH and probes belong to a number of different classes of DNA sequences (Mizuochi et al. 2007; Hanzi 2009). The chromosomes of parent genomes, recombinant chromosomes of interspecific hybrids, and their progeny can be differentiated by the combined use of FISH and GISH analysis techniques. FISH analysis of tulip cultivar Kouki, which has 36 chromosomes, revealed that it had one genome from *Tulipa fosteriana* and two from *Tulipa gesneriana* (Popescu and Sutan 2012). Among the cultivars studied, the type and the number of recombinant chromosome varied and the number of translocations sorted in the examined tulip cultivars ranged from 1 to 6. The results showed that a single cross event occurred in each cultivar because every cultivar was a combination of a single *T. gesneriana* fragment and a *T. fosteriana* fragment. Consecutive analysis by FISH and GISH using ribosomal DNA probes produced chromosome-specific markers that were used to identify Purissima progeny chromosomes (Popescu and Sutan 2012). The FISH and GISH analysis facilitated tulip crop improvement and decreased the time required for producing new varieties by accelerating interspecific hybrid verification.

## Cytological improvements

Recent development and modifications in cytogenetic techniques has improved the molecular analysis of plant taxa (Zhang et al. 2007). In the *Lilium* Sinomartagon section, *L. tigrinum* is a native species of Korea and is recognized as a polyploidy-complex containing diploid (i.e., $2n = 2x = 24$) and triploid (i.e., $2n = 3x = 36$) types of plants. Natural interspecific hybridization in diploid *L. tigrinum* and other similar diploid species, e.g., *L. leichtlinii*, can produce allotriploid plants, whereas diploid individuals can produce auto-triploid plants when a functional, unreduced 2*n*-gamete is crossed with a reduced gamete (Noda 1978, 1986). Hwang et al. (2011) explained the differences between diploid (2*n*) and triploid (3*n*) *L. tigrinum* by using chromosome morphological and FISH data. It was revealed that the range of the metaphase chromosome length in diploid *Lilium* was from 15.92 to 30.18 μm with a genome that had a total length of 250.46 μm, whereas in triploid *Lilium*, the range was from 15.24 to 28.16 μm with a genome that had a total length of 233.42 μm. A karyotype analysis classified the *Lilium* chromosomes based on centromere position. In diploid plants, two pairs of metacentric, four pairs of sub-telocentric, and six pairs of telocentric chromosomes were found while in triploids, two pairs of metacentric, six pairs of sub-telocentric and four pairs of telocentric chromosomes were identified. In diploid chromosomes, 10 (5 pairs) or 12 (6 pairs) loci of 45S rDNA were identified, whereas, in triploid chromosomes 15 45S rDNA loci were detected. There was no difference between diploid and triploid chromosomes with regard to 5S rDNAs localization, i.e., both 5S rDNAs were detected at chromosome 3. The karyotype study of 2*n* and 3*n* *L. tigrinum* described an approach that identified differences in the same species (Hwang et al. 2011).

The somatic metaphase and interphase nuclei DNA sequences have been in situ localized by complementary nucleic acid probes labeled with fluorochrome during FISH

analysis (Pedrosa-Harand et al. 2009). Labeling of probes is usually indirectly completed using digoxigenin or biotin. Digoxigenin is a steroid obtained from *Digitalis purpurea* (foxglove), while biotin is basically vitamin H. These molecules are combined with the DNA probe using standard molecular labeling techniques. During hybridization, the cytoplasm may act as a physical barrier. It can obstruct the imagining of a signal in the chromosomes or prevent the DNA probe from reaching the target DNA. Poor target results for hybridization may be caused by dense cytoplasm between the nuclei or distorted chromosomes (Schwarzacher and Haslop-Harrison 2000).

A metaphase stage that is cytoplasm free and with suitable chromosome spreading using various enzymes can be achieved in *Passiflora* species. Souza et al. (2010) optimized the FISH protocol for *Passiflora* and proceeded using a 45S rDNA as a probe with biotin labeling and with fluorescence detection in isothiocyanate. The $F_1$ hybrid progeny (*P. gardneri* × *P. gibertii*), *P. gardneri*, and *P. cacaoensis* confirmation was successfully achieved, and the "Pectinex SP ULTRA" enzyme produced the best results, probably because the Pectinex enzyme is very efficient at cell wall digestion compared to other enzymes. However, the incubation time for Pectinex was long. The use of 1.0 M HCl along with Pectinex significantly decreased incubation time and improved cytological preparation quality. The methodological changes pioneered by this research have improved FISH analysis of *Passiflora* (Souza et al. 2010).

The genus *Silene* can be used to study sex chromosome evolution as this genus contains species that are dioecious and hermaphroditic, but contain a stable number of chromosomes, i.e., $2n = 24$. There is a possibility of producing interspecific hybrids through *Silene* interspecific crossing. Markova et al. (2006) undertook a karyomorphological analysis study of a hybrid where the maternal parent was dioecious *S. latifolia* and the paternal parent was hermaphroditic *S. viscosa.* Analysis by the genomic in situ hybridization (GISH) technique clearly distinguished the parental genomes and revealed that they were mostly found in different nuclear domains. FISH and molecular GISH markers mapped the somatic cells, which were stable, hybrid genomes. In addition, from each parent, 12 chromosomes originally came from a gene that contained the *S. latifolia* X chromosome. Meiotic analysis showed that even allied, relevant chromosomes from the parent only partially paired or did not pair, which caused many defects in the chromosomes, such as irregular non-disjunctions and bridges. The FISH and GISH markers proved that in *S. latifolia*, the larger genome, as well as its largest chromosome constituent, the X chromosome, were frequently engaged in chromosome misdivision and lagging (Markova et al. 2006).

Chromosomal characterization is a dynamic tool that is used in phylogenetic studies, evolutionary biology, the classification of plant species, and for distinguishing their physiological and morphological features. The discrimination of individual chromosomes is difficult in horticultural plants where metaphase chromosomes are comparatively small compared to other plant species. Akasaka et al. (2003) carried out a karyotype analysis of four wild *Rosa* species. The 5S rDNA probe was obtained from *R. multiflora* and 45S rDNA was obtained from WHEAY (pWRRN). According to the analysis, chromosome 7 was the shortest among the 14 chromosomes. In the four rose species, the relative length of the chromosome varied from 10 to 20 %. On a centromere basis, the chromosomes of all species were sub-telocentric or metacentric, while the largest two chromosomes were median or metacentric. In *R. foliolosa*, three 45S rDNA loci were detected, while one pair of 45S rDNA was found in *R. rugosa*, *R. marretii*, and *R. willmottiae*. In *R. foliolosa* and *R. willmottiae*, 5S rDNA was detected on the long-arm of chromosome 4, whereas two 5S rDNAs were found in chromosome 4 of *R. marretii* and in chromosome 5 of *R. rugosa*. This study proved that horticultural species with small metaphase chromosomes can be used in cytological studies (Akasaka et al. 2003).

## Hybrid confirmation

Chromosome analyses, such as distribution of the parental genomes, location and frequency of recombination, and the number of chromosomes of interspecific hybrids, are important applications of FISH and GISH (Zhou 2007; Lim 2000; Khan 2009; Xie 2012). In *Tecoma*, interspecific hybridization was carried out by Contreras et al. (2012) to produce new varieties. Fertile hybrids were obtained after crossing *T. garrocha* as the pistillate parent with *T. stans*. The $F_1$ hybrids were confirmed by GISH, were backcrossed with both parents, and then self-pollinated to produce BC (backcross) and $F_2$ progeny. Three hybrid species were produced when the $F_1$ hybrids were crossed with *T. guarume* 'Tangelo' and *T. capensis*. FISH analysis of the $F_1$ hybrids confirmed that there were four copies of the 18S internal transcribed spacer (ITS) region. Additionally, FISH has also given insights into Bignoniaceae family evolution and the potential role of polyploidy in plant improvement.

Hybrids obtained from distant relative crosses are not true hybrids because the development of the embryo is due to apomixis. These types of plants contain maternal parent genetic material. Marasek et al. (2004) used cytological and molecular cytogenetic methods to verify true hybrids obtained from *Lilium* 'Expression' × *L. henryi* and *Lilium* 'Marco Polo' × *L. henryi* crosses. Cytogenetic analysis

showed that all the genotypes were diploid, i.e., $2n = 2x = 24$. During hybrid verification, the GISH technique distinguished the maternal and parental chromosomes at the somatic metaphase and prophase stages. Maternal DNA was utilized as blocks, whereas paternal DNA was used as a probe. After GISH, further hybrid verification was carried out by FISH analysis using 5S rDNA and 25S rDNA probes. Locus selected chromosome markers were created, based on genome-specific localization of rDNA, for $F_1$ hybrid analysis. For every parental genotype, the presence of a marker chromosome's features confirmed that true hybrids were obtained from these crosses.

Hybrid verification in the genus *Lilium* has been achieved based on the C-banding pattern, chromosome morphology, genome size comparison, utilization of RAPD markers and in situ hybridization by total genomic DNA. Consequently, in situ hybridization with 5S and 45S rDNA probes is a precise method for chromosomes analysis (Lee et al. 2014). The GISH can discriminate between all maternal and paternal chromosomes at the somatic prophase and metaphase stages. The paternal genome DNA could be used as probe DNA, while maternal DNAs as blocking DNA. Further verification is also possible using FISH, and 25S rDNA and 5S rDNA probes. The results of Marasek et al. (2004) showed that using 25S rDNA as a probe detected the location of a secondary constriction and this was enough for hybrid confirmation. However, GISH offered more reliable and consistent ''monotonous markers'' compared to FISH. This proved the superiority of a GISH approach for quick and consistent hybrid identification in intersectional lily hybrids.

### Intergenomic recombination investigation

Gene flow and introgression are facilitated by sexual polyploidization (Watanabe et al. 1991), whereas first division restitution (FDR) and second division restitution (SDR) are two processes that are used for 2*n*-gamete formation (Younis et al. 2014). During meiosis, each mechanism has various genetic effects on recombination. All *Phalaenopsis* species contain similar numbers of chromosomes (38); however, genome size varied considerably. The *Phalaenopsis* species relationships and their genome organization were studied using GISH and seven interspecific hybrids that had the same or different genome sizes. The *P. aphrodite* × *P. sanderiana* hybrid parents had small-sized genomes. The two parental chromosome sets could not be differentiated due to the distribution and strength of the hybridization signals. The same results were produced from a *P. mannii* × *P. violacea* cross whose parents have large genome sizes. This revealed that the parents of hybrids have the same types of genomes, which is in contrast to hybrids that have one large genome size parent and a parent with a small size genome, e.g., *P. amboinensis* × *P. stuartiana*. GISH analysis, with or without the application of blocking DNA, we can efficiently identify the two sets of parental chromosomes. According to one hybridization signals study by Lin et al. (2005), species containing small genomes have their own particular sequences, but large genome containing species have considerably more repetitive sequences, based on amount and type. This proved that plant species relationships and genome organization can be examined more efficiently by GISH analysis, particularly when the meiotic behavior analysis is technically difficult (Lin et al. 2005).

In *Lilium* interspecific hybrids, the recombination level can be determined by GISH analysis because it facilitates the chromosome discrimination of two distinct genomes. Therefore, this technique was used to examine one $F_1$-hybrid, one trigenomic hybrid and four backcross hybrid genome compositions (van Tuyl et al. 2002). It also described the 2*n*-gametes production mechanism in hybrids produced by crossing *L. longiflorum* with Asiatic hybrids. This study showed a perfect diversity among *L. longiflorum*. The presence of Oriental and Asiatic hybrid chromosomes, i.e., one OLA-hybrid and four ALA-hybrids, was proved by GISH analysis. Three and five recombinant chromosomes with five and ten crossover loci, respectively, in each hybrid were present in two ALA-hybrids and their positions randomly occurred on the chromosomes. In OLA- and ALA-hybrids, the existence of a first division restitution (FDR) mechanism for 2*n*-pollen development was confirmed in LA-hybrids based on the absence of homologous chromosomes in *L. longiflorum*. The GISH technique revealed that $F_1$-hybrids producing 2*n*-gametes could potentially be used for introgression breeding (Karlov et al. 1999). In interspecific hybrids, the GISH technique allows for variation between parental chromosomes (Schwarzacher et al. 1992). This technique, in some lily hybrids, has improved awareness about the different modes of origin and the intergenomic features of 2*n*-gametes (van Tuyl et al. 2002).

The potential value of intergenomic *Lilium* $F_1$ hybrids (*L. auratum* × *L. henryi*) and their backcross progeny was investigated by GISH (Chung et al. 2013). This study showed that the 2*n* pollen development in total $F_1$ AuH hybrids was derived from a first division restitution (FDR) mechanism, in which the all the BC1 plant genome compositions had 12 Oriental chromosomes, 12 *L. auratum* chromosomes, and 12 *L. henryi* chromosomes. The findings of this analysis show that important horticultural traits can be created by the production and investigation of $F_1$ AuH hybrids and their progeny through sexual polyploidization.

## Conclusions

Recent innovations in cytogenetics will improve the understanding of chromosome behavior and genetic regulation, and the mechanisms that control chromosomal dynamics at the gene level. These developments will open new avenues that will allow the identification of non-homologous chromosomes within an organism and closely related genomes. Furthermore, cytogenetic approaches and innovations in chromosome engineering will improve the efficiency of interrogation breeding, and the identification and transfer of resistance genes from alien to native species.

**Author contribution statement** A.Y. and F.R. wrote and revised the manuscript; Y.J.H. made the figures and provided technical assistance in cytogenetic methods; K.B.L. gave the concept of manuscript and reviewed it methodically.

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**Conflict of interest** The authors declare that they have no competing interests of this manuscript.

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