Journal of Applied and Natural Science
16(2), 768 - 776 (2024)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

Molecular and cytogenetic techniques (FISH and GISH) for rapid hybrid confirmation to hasten the breeding pace of ornamental geophytes

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Article Info
https://doi.org/10.31018/jans.v16i2.5544
Received: March 9, 2024
Revised: May 21, 2024
Accepted: May 27, 2024

How to Cite
Jamja, T. *et al.* (2024). Molecular and cytogenetic techniques (FISH and GISH) for rapid hybrid confirmation to hasten the breeding pace of ornamental geophytes. *Journal of Applied and Natural Science*, 16(2), 768 - 776. https://doi.org/10.31018/jans.v16i2.5544

Abstract
Ornamental geophytes, also called ornamental bulbous plants, are botanically classified as true bulbous, rhizomatous, corm, or tuberous and are some of the most economically important floricultural crops. In addition to the physico-chemical hindrances in reproductive parts, a prolonged juvenile and vegetative period in ornamental geophytes is a hurdle to their breeding process, making it painstaking and costly efforts to introduce a new variety into the market. The molecular cytogenetic breeding techniques, fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) provide avenues for fast and precise hybrid verification, introgression breeding, localization and discrimination of genes to analyse the recombination patterns, ploidy levels, evolution of the genome and parental identification of these species and to establish their relationships. These techniques have been widely and successfully used for the breeding and hybrid development of numerous ornamental geophytes. Despite significant improvements and shifts in plant breeding and genetics with the advent of new and sophisticated technologies, FISH and GISH are still used for hybrid verification and confirmation of genomic constitution and ploidy levels in hybrids. However, their application has been limited to only a few ornamental geophytes, viz., tulips, lilium, narcissus and hippeastrum. Their applicability has not been explored or exploited in other ornamental geophytes. This review provides insight into the application of FISH and GISH techniques in breeding ornamental geophytes and related achievements.

Keywords: Bulbous flower, Fluorescence *in situ* hybridization (FISH), Genomic *in situ* hybridization, (GISH), Molecular cytogenetics, Geophytes, Ornamental geophytes

INTRODUCTION

Ornamental geophytes are bulbous plants that are botanically classified as true bulbous, corm, rhizomatous, or tuberous and are widely grown in gardens as herbaceous perennials. Geophytes are popular cut flowers

worldwide and are used as bedding materials, border plants and potted plants (Marasek *et al*., 2021). There are approximately 800 botanical genera of ornamental geophytes (Bryan 1995). However, not all of these geophytes are economically important, as only a dozen of them have been identified as economically highly important crops, namely, lilies, narcissi, gladioli, tulips and hippeastrum (Marasek *et al*., 2021). According to Royal Flora Holland (2021), tulips and lilies were among the top five cut flowers sold in Dutch flower auctions, with values of approximately 243 million euros and 144 million euros, respectively in 2020. Thus, ornamental geophytes hold a significant position in global floricultural industries worldwide, and their demands have increased rapidly in recent decades.

Purposeful breeding is necessary for developing a variety of decorative flowers in ornamental geophytes to meet market demands and for adapting plants to changing environmental conditions. However, breeding ornamental geophytes is time-consuming, particularly due to their long juvenile phase and often low natural vegetative propagation rates of propagules (Marasek *et al*., 2021). Introducing new ornamental geophytes into the market may take 20-25 years for tulips (Orlikowska et al., 2018) and 15-25 years for narcissi (Hanks, 2002). In addition, from the juvenile stage to flowering, plants can take as long as 3-8 years in narcissi (Rees, 1972 and Erhardt, 1993), 4-7 years in tulips (Rees, 1972), and 2-3 years in lilies (Fortanier, 1973). However, hybrid lilies (Anderson *et al*., 2013) and hippeastrum (Tombolato and Matthes, 1998) reported to flowers earlier than nonhybrid plants.

Therefore, the pace of breeding in ornamental geophytes is crucial for introducing new cultivars (Marasek *et al*., 2021). Several studies have been conducted to reduce the juvenile phase of ornamental geophytes, viz., Van Eijk *et al*. (1983) in tulipa and Anderson (2005) and Anderson *et al*. (2009) in lilium, and Sochacki and Orlikoswa (2005) in narcissi. Nevertheless, most of these reports are based on physiological factors, and limited studies have been conducted concerning molecular and genetic aspects (Marasek *et al*., 2021). Shahin *et al*. (2012) speculated that such deficits in molecular and genetic studies might be attributed to the large genomic size of geophytes, which ranges from 25 GB in lilium to 36 GB in tulipa.

Nonetheless, molecular and cytogenetic techniques (FISH and GISH) have been widely used to hasten the breeding process in ornamental geophytes (Younis *et al*., 2015). Although the application of these techniques has decreased in recent years with the advancement of new and more sophisticated breeding techniques, they are still used for the analysis of genomic structure, functions, chromosome constituents and recombination patterns, alien gene introgression, genome evolution, and ploidy level (Younis *et al*., 2015). These techniques have been efficiently and effectively used for identifying the parental genome, genetic recombination, determining, discriminating genomes, and localizing chromosomes (Marasek *et al*., 2004; Barba-Gonzalez *et al*., 2005; Hwang *et al*., 2015 and Xi *et al*., 2015) and for hybrid verification and confirmation in general in numerous ornamental geophytes. Nevertheless, these techniques have been limited and widespread to only a handful of ornamental geophytes, viz., tulips, lilium, narcissus and hippeastrum. Their applicability and application have not been explored for most ornamental geophytes. Therefore, this review provides insight into the application of FISH and GISH in breeding ornamental geophytes and their achievements.

Background of *in situ* hybridization

Mendelian genetics is considered the guiding principle for the genetic movement of chromosomes from one generation to another generation. There has been a significant shift in genetics studies from Mendelian to plant breeding and modern genetics; the latter is mostly based on molecular and cytogenetic studies. With advancements in science and technology, how researchers observe chromosomes has changed drastically, and chromosome profiles have become increasingly clear and more pronounced. In plant science, molecular cytogenetics was introduced after the mid-1950s (Ramzan *et al*., 2017) to obtain better chromosome spread in the metaphase stage of cells (Younis *et al*., 2015). Later, different cytogenetic techniques were developed, such as G-, R-, C-, and NOR banding and sister chromatid exchange (Kannan and Zilfalil, 2009). These conventional banding techniques involve a narrow approach, are limited to the metaphase stage and cannot help identify chromosomal aberrations or rearrangements (Tonnies, 2002). Classic cytogenetic techniques were subsequently developed, primarily based on the study of chromosome morphology, viz., the size of arms, placement of secondary constrictions, position of centromeres, chromosome number, and alterations in chromosome numbers, in the 1970s (Silva and Souza, 2013).

In the 1980s, in situ hybridization was first developed by John *et al*. (1969) and Gall and Purdue (1969). First, the FISH technique was developed, followed by the GISH technique; GISH is a modified version of FISH. These techniques have become the most powerful tools in molecular cytogenetic studies. The combined use of GISH and FISH in molecular cytological studies provides accurate and additional information on the mechanism of gamete formation (Barba-Gonzalez *et al*., 2005 and Chung *et al*., 2013). These tools help decipher the genomic constitution and gene localization and aid in their precise discrimination (Xi *et al*., 2015) for efficient and effective breeding. With breakthroughs in cytogenetics and 3D-structured resolution imaging

technology, the application of FISH and GISH has become widespread in chromosome and genome analysis (Figueroa and Bass, 2010).

Applications of FISH and GISH techniques in hybrid confirmation of ornamental geophytes

FISH technique and its mechanism

FISH (fluorescent *in situ* hybridization) is a traditional (Liu *et al*., 2016) and powerful (Younis *et al*., 2015) cytomolecular technique that enables the mapping of specific repetitive or single-copy sequences on chromosomes (Marasek *et al*., 2021). This technique was first developed in the early 1980s (Shwarzacher *et al*., 1989) and has now become the most common way of detecting low copy numbers and individual DNA sequences and gene locations (Guzzo *et al*., 2000). This approach allows researchers to ascertain the distribution and precise identification of specific DNA sequences within chromosomes (Younis *et al*., 2015) with 5S and 45S rDNA sequences (Lim, 2001; Mizouchi *et al*., 2007; Hwang *et al*., 2011 and Zeng *et al*., 2020), which are used as probes. Moreover, FISH karyotype analysis can also be pivotal in mapping and detecting cloned DNA sequence positions on chromosomes (Younis *et al*., 2015).

The first step in the FISH technique is to prepare a probe containing short sequences of single-stranded DNA that match the genes the researchers are looking for. The probe is subsequently labelled with a fluorescent dye, digoxin, fluorescein or biotin, which emits visible fluorescence when induced by UV light (genome.gov, 2023). These DNA probes are subsequently hybridized with chromosomes fixed on slides or nylon membranes (Liu *et al*., 2016). The intended single-stranded DNA (researchers' probes) subsequently binds to the complementary DNA strand. Fluorescence signals can be detected from probes attached to target genes (Younis *et al*., 2015) using fluorescence microscopes, and different genes can be analysed simultaneously (Lysak and Mandàkovà, 2013), as shown in Fig. 1.

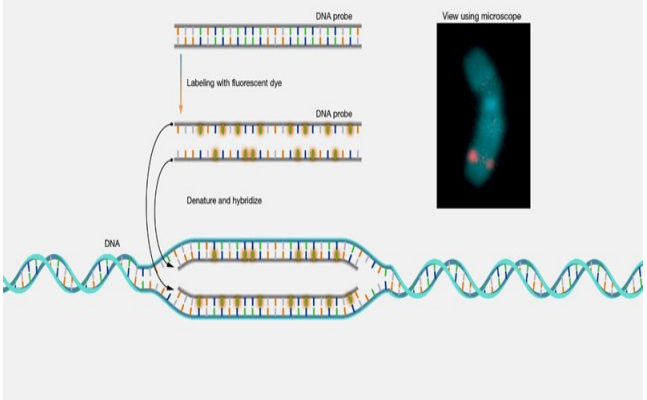

Fig. 1. *Mechanism of the FISH technique. Picture source: genome.gov (Source: https://www.genome.gov/genetics-glossary/Fluorescence-In-Situ-Hybridization.)*

The FISH technique has been successfully and widely employed in breeding numerous ornamental geophytes for hybrid confirmation. The application of FISH to confirm the hybrids of ornamental geophytes reported by different researchers is presented in Table 1.

Analysis of genomic constitution using the FISH technique

The FISH technique has been widely used to analyse the genomic constitution of ornamental geophytes to characterize chromosomal sequences (De Jeu *et al*., 1997 and Lim *et al*., 2001). The FISH technique facilitated the identification of different genomic compositions (Hwang *et al*., 2011) and the presence of DNA probes (5S and 45S) in tulips (Mizuochi *et al*., 2007) and *Lilium species* (Hwang *et al*., 2015 and Wang *et al*., 2015). Additionally, Mizuochi *et al*. (2007) distinguished the chromosomes of *Tulipa gesneriana* and *T. fosteriana* based on their size and signal pattern using FISH.

Recently, Zeng *et al*. (2020) used FISH with 5S and 45S rDNA probes to clarify the process of tetraploid cultivar formation in narcissus. They identified five genomes of 16 different cultivars of narcissus, which they called A, B, C, D and E, as well as localized rDNA loci on the chromosome. They confirmed that most of the 16 tetraploid cultivars analysed were autotetraploids resulting from chromosome doubling and were allotetraploids.

Hybrid verification using FISH

The FISH technique is used to determine the hybrid status of different ornamental geophytes (Marasek *et al*., 2004; Mizuochi *et al*., 2007 and Marasek and Okazaki, 2008; Wang *et al*., 2015 and Tang *et al*., 2020). The use of different probes enables researchers to quickly and easily verify the hybrid status of plants (Marasek *et al*., 2004). In addition, the FISH technique with probes also enables the determination of the different ploidy levels of hybrids and the comparison of the genetic composition of hybrids with parental genotypes to confirm the true hybrid status of progenies and their hybrid authenticity (Wang *et al*. 2015). Mizuochi *et al*. (2007) in tulips and Marasek *et al*. (2008), Wang *et al*. (2015) and Tang *et al*. (2020) in lily, distinguished the parental genotypes from the hybrid progenies and confirmed their interspecific hybrid status.

Introgression breeding using the FISH technique

The FISH technique determines intergenomic recombination and genetic variation in populations (Barba-Gonzalez *et al*., 2005). This technique has been successfully used in the introgression breeding of ornamental geophytes (Barba-Gonzalez *et al*., 2005; Marasek and Okazaki, 2008 and Tang *et al*., 2020). This

Table 1. Application of FISH in the breeding of ornamental geophytes

Geophytes	Chromosome Number	Genomic DNA Probe type	Work	References
Asiatic hybrid cv. Renoir, Gironde, Navona, Detroit, Loreto, and Tresor	24, 24, 36, 48, 48 and 48	45S rDNA	Detection of ploidy level of crossed breed Asiatic lily using karyotype and FISH	Tang *et al*. (2020)
15 cultivars of Narcissus; Dutch Master, Las Vegas, Stadium, Ice Follies, Accent, Mount Hood, Pink Charm, Pink Parasol, Bridal Crown, Easter Born, Eline, Flower Parade, Flower Surprise, Queen's Day and Replete	Autotetraploid 28	5S rDNA	To analyse the genome composition of Narcissus on their metaphase chromosome	Zeng *et al*. (2020)
Asiatic hybrid lily cv. Renoir', 'Gironde', 'Navona', 'Loreto', 'Detroit' and 'Tresor' and 'Freya' (Longifolium lily×Asiatic lily)	NA	45S rDNA	Lily cross breeding affinity, identify the ploidy level of hybrids, and distribution of 45S rDNA sequence	Wang *et al*. (2015)
L. distichum	24	5S and 45S	Physical mapping and karyotype analysis	Hwang *et al*. (2015)
L. lancifolium (2x), *L. lancifolium* (3x), *L. amabile*, *L. cernuum*, *L. dauricum*, *L. callosum*, *L. pumilum* and *L. concolor*	NA	5S and 45S rDNA	To elucidate the chromosomal diversity of eight *Lilium species* using FISH	Lee *et al*. (2014)
Wild *Lilium* spp. (*L. lancifolium* (2x), *L. lancifolium* (3x), *L. maximowiczii*, *L. amabile* Palibin, *Lilium callosum*, *L. cernuum, L. concolor*, *L. dauricum, L. distichum L. hansonii*, and *L. tsingtauense*)	NA	45S rDNA	To detect the variability in the rRNA gene loci and to analyse the interspecific relationships among the species	Sultana *et al*. (2012)
L. tigrinum	24, 36	FISH using 25S rDNA and 5S rDNA	Karyotype study of diploid and triploid *L. tigrinum*	Hwang *et al*. (2011)
Tulipa gesneriana cv. Queen of Night and *T. fosteriana* cv. Red Emperor	24	5S and 45S rDNAs from *T. fosteriana*	To evaluate the cytological diversity within *T. gesneriana*, and between *T. gesneriana* and *T. fosteriana*	Mizuochi *et al*. (2007)
Lilium (Oriental x Asiatic)	24	clone pTa71 and synthetic telomeric probe	Intergenomic recombination in BC1	Barba-Gonzalez *et al*. (2005)
Lilium spp. cv. Henry	24	25S rDNA and 5S rDNA	Hybrid verification	Marasek *et al*. (2004)
L. longiflorum cv. Snow Queen, and *L. rubellum*	NA	5S rDNA (pScT7 probe) and 45S rDNA (pTa71 probe)	Comparative karyotype analysis of *L. longiflorum* and *L. rubellum*	Lim *et al*. (2001)
Alstromeria aurea	16	*A. aurea* A001-I	Repetitive DNA sequences localization and characterization in ornamental Alstromeria	De Jeu *et al*. (1997)

approach enables breeders to identify and distinguish the chromosomes of progenies, the parent genome and the genomic recombination of different cultivars. The FISH technique also allows for deciphering the parental ploidy level and its influence on hybridization affinity (Tang *et al*., 2020).

GISH technique and its mechanism

The GISH (genomic *in situ* hybridization) technique is a modification of the FISH technique (Shwarzacher *et al*., 1989) that follows the same protocols as FISH. The only difference is that genomic DNA blocking is used in GISH analysis (Ramzan *et al*., 2017). GISH was first used to discriminate the genomes of *Hordeum chilense* and *Secale africanum*, which are integeneric hybrids between the parental genomes (Schwarzacher *et al*., 1989). Since then, it has become one of the most powerful tools for analysing natural polyploids, hybrid plants and their backcross progenies for alien gene introgressions, genomic composition, intergenomic rearrangements and the integration of chromosomes and recombination maps (Ramzan *et al*., 2017; Khrustaleva *et al*.,

Table 2. Application of GISH in the breeding of ornamental geophytes

Ornamental geophytes	Chromosome Number	Genomic DNA Probe type	Genomic blocked DNA	Work	References
Progenies of LLO × TTTT	24-48	For LLOTT and LLOOT hybrids, DNA of oriental cv. Sorbonne, and Trumpet cv. Royal Gold was used as probe. For MAD, DNA from Asiatic cv. Connecticut King was used as probe For MAD x AA hybrids, DNA from L. martagon was used as probe	For LLOTT and LLOOT hybrids, DNA of longiforum cv. White Fox was used as block. For MAD, DNA from *L. martagon* was used as block. For MAD x AA hybrids, DNA from Asiatic cv. Connecticut King was used as block	Introgression breeding of lily, analysis of genome composition, intergenomic recombination of 10 progenies derived from LLO x TTTT/ OTOT	Xi *et al*. (2015)
Gloriosa superba cv. Lutea, Marron Gold, *Littonia modesta,* and *S. aurantiaca*	22, 44, 22, 24	Total DNA of one parent was used as probe	50-fold excess salmon sperm DNA was used as blocking DNA	Analysed the meiotic chromosome pairing in diploid and triploid intergeneric hybrids to establish relationship among *Gloriosa*, *Littonia* and *Sandersonia*	Kishimoto *et al*. (2014)
L. aurantium x L. henryi hybrid	24, 24	Genomic DNA from *L. henryi* was used	Sheared herring sperm DNA was used to block nonhenryi specific DNA sequences	Analysis of composition of parental chromosome of *L. aurantum* x *L. henryi* hybrid and their BC1 progeny produced	Chung *et al*. (2013)
Tulipa gesneriana and *T. fosteriana*	24	Both parental DNA was used as probe	Excessive fragmented DNA of unrelated species	Genomic information of interspecific hybrids	Marasek *et al*. (2012)
Three groups of lilies; Longiflorum, Asiatic, and Oriental	24	Genomic DNA of longiflorum cv. White Fox and Oriental cv. Sorbonne was used as probe	DNA of longiflorum and Oriental	Cytological maps of interspecific hybrids lilies base on the recombination sites identified in BC progeny populations	Khan *et al*. (2009)
T. fosteriana & *T. gesneriana*	24, 36	DNA from leaves of Tulip cv. Red Emperor and Queen of Night	Genomic DNA block was not used	Analysis of Darwin hybrid origins	Marasek *et al*. (2006)
Lilium (Oriental x Asiatic)	24	Sonicated genomic DNA (1-10 kb) from Oriental cv. Sorbonne	DNA (100-500 bp) from Asiatic cv. Connecticut King	Intergenomic recombination and its significance genetic variation in BC1	Barba-Gonzalez *et al*. (2005)
Lilium henryi	24	DNA from *L. henryi*	DNA from paternal lilium cv. Marco Polo and Expression	Confirmation of hybrids	Marasek *et al*. (2004)

2019). This approach allows us to distinguish ploidy levels effectively (Wong and Murray, 2014) and distinguish distinct alien species with mosaic chromosomal rearrangements (Sun *et al*., 2014).

An important feature that makes GISH one of the most efficacious techniques for distinguishing between the parental genomes of interspecific plant hybrids is that no sequence information is needed to distinguish the genomes of interspecific hybrids (Khrustaleva *et al*., 2019). Thus, GISH has become one of the most important tools in molecular cytogenetics for investigating the evolutionary relationships of crops to help identify alien species from their parents. Such genome characterization facilitates the selection of hybrids or species with potentially desirable characteristics during the early hybridization process and expedites the detection of introgressed chromosomes (Ramzan *et al*., 2017). The mechanism of GISH is shown in Fig. 2.

Analysis of genomic constitution using GISH

Confirming ploidy levels can help determine plants' genomic composition and evolutionary history (Liu *et al*., 2011). Through GISH, unambiguous genomic distinctions of plants are possible. It has been used in many

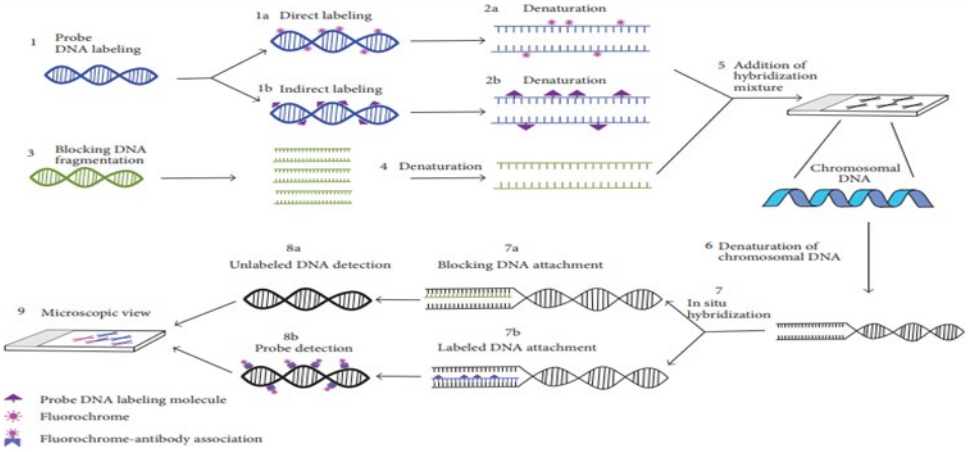

Fig. 2. *Mechanism of GISH. Source: Ramzan et al. (2017)*

ornamental geophytes, particularly in tulip (Marasek *et al*., 2006 and 2012), begonia (Marasek *et al*., 2010), and lilium (Barba-Gonzalez *et al*., 2006; Khan *et al*., 2009; Chung *et al*., 2013; Xiao *et al*., 2021), to confirm the presence of recombinant chromosomes. This distinction helps in the identification of parental chromosomes and the determination of important chromosomal compositions (Yamashita *et al*., 2002).

In the early 2000s, Marasek and Okazaki (2007) confirmed the ploidy level of interspecific crosses between *T. gesneriana* and *T. fosteriana* through GISH; they found an equal distribution of the parental genome, i.e., one of each set of chromosomes from both parents in the case of diploids (2n=24). In contrast, under triploid conditions (3n), 24 chromosomes from one parent (*T. gesneriana*) and 12 from the other parent (*T. fosteriana*) were observed. Similarly, in the case of the tetraploid (4n) strain, 36 chromosomes were derived from *T. gesneriana,* and 12 were derived from *T. fosteriana*.

Hybrid confirmation using GISH

For any parental discrimination, visualization in somatic hybrids requires a hybridization mixture of stringency, posthybridization washes and, most importantly, the ratio of genomic to blocking DNA (Schwarzacher and Heslop-Harrison, 2000), which is used in GISH. The GISH technique is important for hybrid analysis and verification (Bennett, 1995). It has proven to be the most effective and exact way to confirm hybrids (Barba-Gonzalez *et al*., 2005), as it remarkably distinguishes donor parents and alien genomes in hybrids (Czernicka *et al*., 2010) and describes modifications in chromosome number and constitution of functional gametes (Chung *et al*., 2013).

The GISH technique has been successfully used to verify hybrids in different ornamental geophytes, viz., *Clivia miniata* (Ran *et al*., 2001), *Lycoris species* (Chung *et al*., 2013), *Lilium species* (Marasek *et al*., 2004; Chu *et al*., 2013 and Xie *et al*., 2014), and *Tulipa species* (Marasek *et al*., 2012). It has been observed that variation and recombination of genomes result from combining and complementary abilities of the species (Chung *et al*., 2013) and that progeny may carry a phenotype intermediate to that of the parents (Chu *et al*., 2013). GISH enables the identification of recombinant chromosomes in offspring (Marasek *et al*., 2012), which easily facilitates hybrid confirmation in ornamental geophytes.

Introgression breeding using GISH

Intergenomic recombination is one of the most important attributes of sexual polyploidization (Barba-Gonzalez *et al*., 2005). It is a breeding technique in which useful horticultural traits are successfully transferred into progeny (Ramzan *et al*., 2017). In ornamental geophytes, numerous hybrids between distantly related species that enable the production of fertile 2n gametes have been reported in nature and are used inadvertently to produce polyploidy in narcissus (Brandham, 1986) and alstroemeria (Ramanna, 1992). This technique is extensively used by plant breeders.

Researchers have successfully obtained intergenomic recombinant plants via GISH, as these methods enable convenient measurement of the nature and extent of intergenomic recombination (Ramzan *et al*., 2017). This breeding technique has been successfully employed mostly in tulips (Marasek *et al*., 2011, 2012) and lilium (Zhou *et al*., 2014 and Marasek *et al*., 2011). Chung *et al*. (2013) investigated intergenomic hybrids generated by crossing *Lilium auratum* with *L. henryi* using the GISH technique. The authors confirmed the presence of chromosomes from both parents in F1. They observed the production of a relevant frequency of 2n gametes, which was subsequently used for hybrid production of oriental hybrids.

Conclusion

The use of molecular cytogenetic breeding techniques such as FISH and GISH has successfully developed numerous hybrids of ornamental geophytes. They are

powerful tools for confirming the genomic constitution, verifying hybrids, breeding introgressed tracts, and locating and discriminating chromosomal genes efficiently and effectively. An arduous breeding process in ornamental geophytes can be accelerated by by passing plants' long juvenile and vegetative phases or without waiting for the plants to reach an adult stage to confirm the hybrid or its genomic constitution and recombination. Additionally, FISH and GISH can also be used in combination to obtain more effective and precise results. Nonetheless, their application remains limited to a handful of important ornamental geophytes, viz., lilium, tulip, hippeastrum, and narcissus, and few on alstroemeria. This may be because only a few ornamental geophytes have significant market value. As the market value and market size of ornamental geophytes have increased rapidly and manifold in recent decades, considering hurdles in their breeding due to unusually long juvenile phase, and reproduction obstacles, FISH and GISH cytogenetic techniques have the potential to gap the bridge in the breeding of ornamental geophytes. Although FISH and GISH became unpopular in recent years, they can be successfully used to hasten the processing pace in ornamental geophytes, which are different from other ornamental plants.

Conflict of interest

The authors declare that they have no conflicts of interest.

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