

## Determination by GISH and FISH of hybrid status in *Lilium*

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In the genus *Lilium*, plants obtained from crosses, especially between distant relatives, are not always hybrids because embryos can develop as a result of apomixis. These plants constitute genetic material of the maternal parent only. In this study, verification of hybrid status of plants which have been obtained from the crosses 'Marco Polo' × *Lilium henryi* and 'Expression' × *L. henryi* was performed through the use of cytological and molecular cytogenetic methods. According to cytological analyses, all genotypes tested had  $2n = 2x = 24$  chromosomes. Genomic in situ hybridisation (GISH) was used for hybrid verification. In hybrid plants, this method distinguished all paternal and maternal chromosomes at the stage of somatic metaphase and prophase. For GISH, paternal genomic DNA was used as a probe and maternal DNAs were used as blocks. Fluorescence in situ hybridisation (FISH) with 5S rDNA and 25S rDNA probes was used as the second method of hybrid verification. Selected chromosome markers based on genome-specific localisation of rDNA loci were used for analysis of the  $F_1$  hybrids obtained from the crosses 'Marco Polo' × *L. henryi* and 'Expression' × *L. henryi*. The presence of marker chromosomes characteristic for each of the paternal genotypes was a confirmation that the plants obtained were hybrids.

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Wide hybridisation enables the introduction of desired genes, such as those encoding resistance to pathogens and adverse climatic conditions, to many plant crops (METWALLY et al. 1996) and ornamentals (VAN TUYL et al. 1997; VAN TUYL et al. 2000). It is well known that not all plants obtained from wide crosses are hybrids. They may also arise apomictically from unfertilised maternal cells. This process may be induced by incompatible or sterile pollen (GEORGI 1985; NASSAR et al. 1998). In the genus *Lilium*, the apomictic means of propagation was reported in *Lilium regale* (NORTH and WILLS 1969), *L. speciosum*, *L. canadense*, *L. szovitsianum*, *L. longiflorum*, *L. superbum* and *L. pumilum* (GEORGI 1985). Apomixis and the possibility of uncontrolled pollination make it necessary to confirm whether or not seedlings obtained from distant crosses are indeed desired hybrids. Different methods of hybrid verification based on morphological, cytological and molecular markers are commonly applied; the most useful are those which provide reproducible results and which may be used at the earliest possible stage of seedling development.

In the genus *Lilium*, hybrid verification has been performed on the basis of chromosome morphology (UHRING 1968; NORTH and WILLS 1969; OKAZAKI et al. 1994; FERNANDEZ et al. 1996; OBATA et al. 2000; MARASEK and ORLIKOWSKA 2001), C-banding pattern (SMYTH and KONGSUWAN 1980; SMYTH 1991;

MARASEK and ORLIKOWSKA 2001), comparison of genome size (VAN TUYL and BOON 1997), using RAPD markers (OBATA et al. 2000; WIEJACHA et al. 2001) and in situ hybridisation with total genomic DNA (KARLOV et al. 1999; LIM et al. 2000).

So far in *Lilium*, in situ hybridisation with 5S rDNA and 45S rDNA probes has been used exclusively for chromosomes analysis of particular species (LIM et al. 2000). This study is the first attempt to use these markers for hybrid identification. We demonstrate the usefulness of chromosomal markers obtained by fluorescence in situ hybridisation with total genomic DNA (GISH) or with simultaneously applied 25S rDNA and 5S rDNA probes. These markers allow the unambiguous verification of the status of lily hybrids obtained from crosses 'Marco Polo' × *Lilium henryi* and 'Expression' × *L. henryi*.

## MATERIAL AND METHODS

### *Plant material*

The following plant material was used: *Lilium henryi* Baker (Chinese species – section *Leucolirion*) and two lily cultivars – 'Marco Polo' and 'Expression'. Cultivars used in this study belong to the horticultural group of Oriental hybrids. This group originated from hybridization within section *Archelirion* – *L. auratum*,

*L. speciosum*, *L. japonicum*, *L. rubellum*, *L. nobilissimum*, *L. alexandre* (WITHERS 1967; FELDMAIER and MCRAE 1982).

The seedlings were obtained in vitro by ovule culture. Chromosomal markers were sought in  $F_1$  seedlings from crosses between 'Marco Polo'  $\times$  *Lilium henryi* (4 plants) and 'Expression'  $\times$  *L. henryi* (10 plants). The aim of these crosses was to obtain phenotypic forms which were morphologically as close to Oriental hybrids as possible, but with greater resistance to disease and winter conditions. Parental genotypes and putative hybrids were propagated in vitro on MS medium (MURASHIGE and SKOOG 1962) without growth regulators, through adventitious bulblets. Roots were obtained in a medium supplemented with 60 g l<sup>-1</sup> sucrose and 5 g l<sup>-1</sup> charcoal.

#### Preparation of chromosomes

Roots of 0.5–1.5 cm length were treated with 0.1% colchicine for 4 hours in the dark at room temperature, then fixed in 3:1 ethanol-glacial acetic acid for 4 hours and stored at –20°C until required. For further preparation, fixed root tips were washed in 0.01 M enzyme buffer (citric acid-sodium citrate, pH 4.8) for 20 min and digested in a mixture of enzymes consisting of 20% pectinase (Sigma) and 4% cellulase (Sigma) for 5 h at 37°C. Root meristems were squashed in a drop of 45% acetic acid. After freezing, the coverslips were quickly removed and the slides were dehydrated in pre-chilled absolute ethanol for 15 min, air-dried and kept at 4°C until use.

#### DNA probes

For the GISH procedure, total genomic DNA from *L. henryi* was used as a probe. DNA was isolated from young leaves obtained in vitro as described by ALJANABI and MARTINEZ (1997) with slight modifications. Isolated DNA was cut with DNase I (0.05 U  $\mu$ l<sup>-1</sup>) to 1 to 10 kb fragments and labelled by nick translation with rhodamine-4-dUTP (Amersham Pharmacia).

The 5S rDNA probe was obtained from the wheat clone pTa794 (GERLACH and DYER 1980) by PCR amplification and labelled with rhodamine-5-dUTP (Roche) as described in HASTEROK et al. (2002). The 25S rDNA probe was obtained by nick translation with digoxigenin-11-dUTP (Roche) of a 2.3 kb subclone of the 25S rDNA coding region of *A. thaliana* (UNFRIED and GRUENDLER 1990).

#### Fluorescence in situ hybridisation

The fluorescence in situ hybridisation procedure was adopted from HASTEROK et al. (2001). Chromosome preparations were pre-treated with DNase-free RNase

(100  $\mu$ g ml<sup>-1</sup>) in 2  $\times$  SSC for 1 h at 37°C, then washed in 2  $\times$  SSC for 15 min, post-fixed in 1% aqueous formaldehyde in PBS buffer for 10 min, washed again in 2  $\times$  SSC for 15 min and finally dehydrated in an ethanol dilution series and air-dried. The hybridisation mixture consisted of 50% deionised formamide, 10% dextran sulphate, 2  $\times$  SSC, 1% SDS and probe DNA (100 ng per slide). Chromosome preparations and pre-denatured (75°C for 10 min) probes were denatured at 70–72°C for 5 min and allowed to hybridise overnight at 37°C in a moist chamber. After hybridisation, slides were washed for 10 min in 20% formamide in 0.1  $\times$  SSC at 42°C, followed by several washes in 2  $\times$  SSC. Digoxigenated probes were immunodetected by FITC-conjugated anti-digoxigenin antibodies (Roche). The chromosomes were mounted and counterstained in Vectashield (Vector Laboratories) containing 2.5  $\mu$ g ml<sup>-1</sup> 4',6-diamidino-2-phenylindole (DAPI; Sigma).

Genomic in situ hybridisation was performed as described by JENKINS and HASTEROK (2001). Total genomic DNA isolated from maternal parents 'Marco Polo' and 'Expression' was cut with DNase I (0.05 U  $\mu$ l<sup>-1</sup>) to 100–1 000 bp fragments and used as a block. Blocking DNA was used at a ratio 1:30 (probe:block).

#### Photographic documentation

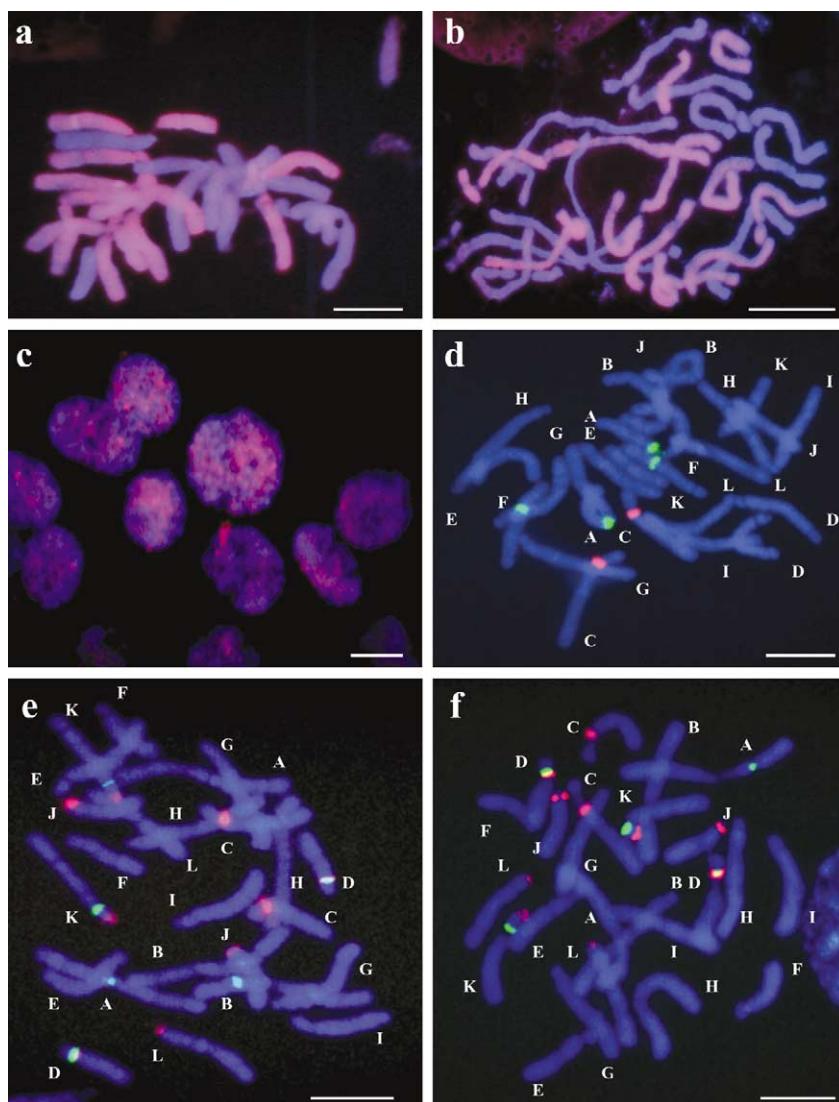
For each genotype, the four best slides were analysed. At least 10 metaphase and interphase cells were examined for each plant. Selected chromosome spreads and interphase nuclei were photographed with a camera attached to a Leica DMRB epifluorescence microscope using UV excitation for DAPI visualisation, blue light excitation for FITC-conjugated antibodies and green excitation for rhodamine-dUTP labelled probes on Fujicolour 400 ISO colour negative film. Images were scanned and processed uniformly using Micrografx Picture Publisher software.

Chromosomes were identified and classified from A to L as described by STEWART (1947).

## RESULTS

#### Genomic in situ hybridisation

Figure 1a–b show the results of hybridisation with total genomic DNA of *L. henryi* to somatic chromosomes of plants obtained from crosses 'Marco Polo'  $\times$  *L. henryi* (a) and 'Expression'  $\times$  *L. henryi* (b), respectively. In both cases, 12 chromosomes of the paternal form are uniformly painted red along their entire length. Remaining (maternal) chromosomes do not hybridise at all or show an insignificant level of cross-hybridisation with the probe. In interphase nuclei of



**Fig. 1a–c.** Fluorescence in situ hybridisation with total genomic DNA (GISH) of *L. henryi* as a probe (red). ‘Marco Polo’ × *L. henryi* (a and c) and ‘Expression’ × *L. henryi* (b). (d–f) Double-target FISH of 25S rDNA (green) and 5S rDNA (red) probes to somatic metaphase chromosomes of *L. henryi* (d), ‘Marco Polo’ (e) and ‘Expression’ (f). The chromosomes are counter-stained with DAPI (blue). Bars represent 10 µm.

‘Marco Polo’ × *L. henryi*, there are large and distinct sectors of pink-labelled paternal chromatin visible (Fig. 1c).

#### Fluorescence in situ hybridisation

Figures 1d–f and 2b–c show the results of simultaneous fluorescence in situ hybridisation with 5S rDNA and 25S rDNA probes to metaphase chromosomes of *L. henryi*, ‘Marco Polo’, ‘Expression’ and hybrids obtained from crosses ‘Marco Polo’ × *L. henryi* and ‘Expression’ × *L. henryi*, respectively.

In *L. henryi* (Fig. 1d) the presence of four sites of 25S rDNA and two sites of 5S rDNA on metaphase

chromosomes was revealed. 25S rDNA loci are located at the secondary constriction in the long arms of A chromosomes close to the centromere and at an intercalary site on the long arm of F chromosomes. The large hybridisation signals of 5S rDNA are observed in the long arms of the C chromosomes in close proximity to the centromere.

In ‘Marco Polo’ (Fig. 1e) 6 sites of 25S rDNA and 9 sites of 5S rDNA were observed. Two pairs of 25S rDNA loci are located at the secondary constrictions in the short arms of chromosome pair D in close proximity to the centromere, and at an intercalary site in the long arms of chromosome pair K, while the two

remaining signals localise to the secondary constriction on one out of two homologous chromosomes A and B. 25S rDNA sites are located close to the centromere on chromosome A and at an intercalary site on chromosome B. Two sites of 5S rDNA are located pericentromerically in the long arm of chromosome pair C, and the remaining seven signals are visible on chromosomes D, J, K and one of the chromosomes L. The 5S rDNA loci borne by the chromosomes J, K and on one of the chromosomes L were located in the distal part of their short arms. Both kinds of rDNA sites are in close apposition or co-localised in the short arm of chromosome D. In contrast to this, on telocentric chromosomes K the two classes of rRNA genes, though both on the same chromosome, occupy different chromosome arms.

In 'Expression' (Fig. 1f) fluorescence *in situ* hybridisation revealed the presence of 5 sites of 25S rDNA which were roughly uniform in size, and 10 sites of 5S rDNA which, as in 'Marco Polo', differed in size. The former sites occupy secondary constrictions in different arms of chromosomes D and K and on the short arm of one of the chromosomes A, whilst the latter are located pericentromerically or terminally. The two largest 5S rDNA sites occupy pericentromeric regions of the long arms of chromosomes C. The smaller signals are seen in the short arms of chromosomes D, close to the centromere, and distally on the chromosomes J, K and L. On the chromosome pair D, like in 'Marco Polo', 25S rDNA and 5S rDNA loci are closely linked or co-localised in their short arms. In the small loci, the number of hybridisation signals observed on metaphase spreads differed slightly depending on the metaphase plate analysed.

Simultaneous fluorescence *in situ* hybridisation with 5S rDNA and 25S rDNA probes provided more markers for identification of the maternal genotypes – 'Marco Polo' and 'Expression' than for *L. henryi*, where a relatively low number of chromosomes carries rDNA loci. Fig. 2a shows all rDNA bearing chromosomes extracted from metaphase complements. Some of them have been chosen as the markers for hybrid verification. For the paternal form (*L. henryi*), chromosome F having the 25S rDNA signal in the long arm was chosen as the primary marker for identification of this genome in hybrids because both in 'Marco Polo' and 'Expression' neither 25S rDNA nor 5S rDNA hybridisation signals were detected on this chromosome. For the same reason, chromosomes D, J and K were chosen as markers for 'Marco Polo' and 'Expression'. In all three parental forms some 25S rDNA signals were located in similar positions on chromosomes A, and the largest signals of 5S rDNA

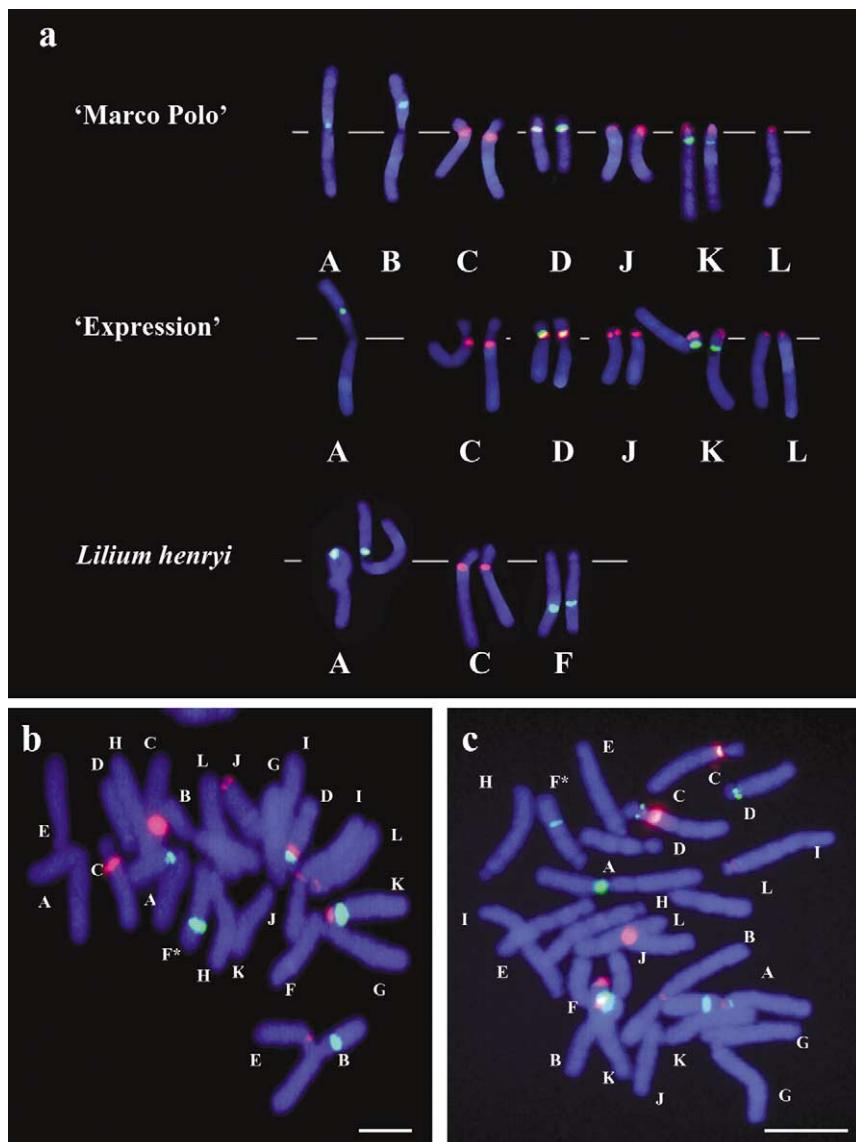
were always present on chromosomes C making them useless for the purpose of genome identification.

On metaphase plates of 'Marco Polo' × *L. henryi* and 'Expression' × *L. henryi*, chromosomes having signals of 5S and 25S rDNA in the positions characteristic for each parental genome were visible, which confirmed that the seedlings tested were hybrids. In 'Marco Polo' × *L. henryi* preparations, 5 clearly distinguishable 25S rDNA signals, and 4 large and from 4 to 5 smaller 5S rDNA signals were observed (Fig. 2b). On metaphase spreads of 'Expression' × *L. henryi*, 7 hybridisation sites of 25S rDNA as well as 3 large and 4 smaller 5S rDNA sites were found (Fig. 2c). In all cases, hybrid status of the plants tested was confirmed by the presence of the paternal marker chromosome F having a unique distribution of 25S rDNA locus in its long arm. In images of the hybrids, this chromosome has been marked with the white asterisk.

## DISCUSSION

Genomic *in situ* hybridisation is a very useful method for the identification of plant hybrids and allopolyploid species because it usually gives clear and unambiguous distinction between genomes. Theoretically, even with no initial knowledge of chromosome morphology, genomic *in situ* hybridisation offers the possibility of determining the genomic origin on metaphase and prophase chromosome spreads as well as in interphase nuclei. In the genus *Lilium*, GISH was successfully used for verification of *F*<sub>1</sub> hybrid obtained from crosses between *L. longiflorum* and *L. rubellum* (LIM et al. 2000) and to trace recombination events in BC<sub>1</sub> (LIM et al. 2000) and BC<sub>2</sub> progenies (KARLOV et al. 1999; LIM et al. 2000). The quality of hybridisation of total genomic DNA probe to target chromosomes was satisfactory in our study for the verification of *F*<sub>1</sub> hybrids both on somatic prophase and metaphase chromosomes and in interphase nuclei. Optimisation of the conditions of GISH allowed distinct and uniform "painting" along paternal chromosomes and a low level of probe cross-hybridisation to maternal chromosomes. The best results were achieved when the concentration of blocking DNA was about 30 times higher than the concentration of the genomic probe, which seemed to be sufficient to prevent from cross-hybridisation for a wide range of repeated sequences families, common to both parental genomes.

Most other cytological methods used for lily hybrid identification is based upon the characteristics of single chromosomes, which requires karyotype analyses and selection of markers which would be easily



**Fig. 2a–c.** (a) Extracted rDNA-bearing chromosomes of 'Marco Polo', 'Expression' and *L. henryi*. Double-target FISH of rDNA probes to somatic metaphase chromosomes of 'Marco Polo' × *L. henryi* (b) and 'Expression' × *L. henryi* (c). In hybrids, the primary marker chromosome characteristic for the paternal form is distinguished by the white asterisk. Colour codification the same as in the Fig. 1d–f. Bars represent 10 µm.

recognisable in hybrid cells. Although GISH requires more sophisticated equipment, in many cases it may replace time-consuming studies of the karyotype. In this paper we have proved that the FISH technique can also assist hybrid identification. Probes used in this study determined the chromosomal localisation of rRNA genes, which provided markers for hybrid verification.

Multicolour fluorescence *in situ* hybridisation using two or more repetitive DNA sequences as the probes was used for chromosome identification in numerous plant species. Frequently these were the species with

numerous and morphologically uniform chromosomes. For instance, the use of 5S rDNA and 25S rDNA probes identified most chromosomes in some of the diploid and tetraploid species of *Brassica* (Hasterok et al. 2001; KULAK et al. 2002) as well as in *Sinapis alba* and *Raphanus sativus* (SCHRADER et al. 2000). Furthermore, in *Hordeum vulgare* the use of 5S and 18S-5.8S-25S rDNA probes enabled unambiguous identification of all chromosomes in this genome (LEITCH and HESLOP-HARRISON 1993). In the genus *Lilium*, both kinds of rDNA probes were successfully used for chromosome identification in *L. rubellum*

and *L. longiflorum*, where they provided useful landmarks for identification of 6 to 8 out of the 24 chromosomes (LIM et al. 2000). A comparison of the results obtained using Feulgen staining and FISH indicates that the latter provides significantly more reliable markers for chromosome identification. In *L. henryi* however, C-banding offers more markers for individual chromosome identification than FISH with rDNA probes (MARASEK and ORLIKOWSKA 2001). In case of cultivars, the number of useful markers for chromosomes identification is comparable. Such a difference in the number of rDNA sites between *L. henryi* and cultivars 'Marco Polo' and 'Expression' may reflect the fact that they belong to different sections of the genus *Lilium*. The parental forms which were used to obtain the cultivars are unknown. However, the analysis of a crossing polygon of the genus *Lilium* (VAN TUYL et al. 1997) and the similar position of 5S rDNA sites on C chromosomes of *L. henryi* and cultivars may indicate that *L. henryi* could possibly be one of their ancestors (FELDMAIER and MCRAE 1982).

The results obtained in this study show that simultaneously applied rDNA probes establish markers which are easily recognisable in cells, offering quick and reliable confirmation of hybrid status. Although this method provided only one marker chromosome specific for the parental genotype, it was sufficient to enable verification of the hybrids 'Marco Polo' × *L. henryi* and 'Expression' × *L. henryi*. In fact, for the purpose of this study, the use of only the 25S rDNA probe that marked the position of secondary constrictions would be sufficient for hybrid status verification.

On the other hand, the comparison of signals in cultivars 'Marco Polo' and 'Expression' clearly shows that this method can not be used for verification of hybrids from such relatives due to very similar localisation of rDNA signals. GISH, although providing more "monotonous" chromosome markers in comparison to FISH, seems to be superior for the purpose of rapid and reliable hybrid identification of intersectional lily hybrids. The question for further study is whether GISH will be more useful also for the purpose of cultivar hybrids identification.

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