Identification of Parental Chromosomes and Detection of Ribosomal DNA Sequences in Interspecific Hybrids of *Lilium* Revealed by Multicolor in Situ Hybridization

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Abstract

In wide interspecific crosses of lily, different hybrid groups were used viz., *L. longiflorum* (L), Asiatic hybrids (A) and Oriental hybrid (O). Two BC_1 plants, OLA and OLO, were derived from crossing Oriental hybrids as a mother to LA and LOLO hybrids as male parent. LA and LOLO F_1 hybrids were known to produce 2n-gametes (<9.6%) and 2x-gametes (>40%), respectively. GISH enabled unequivocally the identification of parental chromosomes. Both BC_1 progeny, OLA and OLO, possessed three genome sets with 36 chromosomes (2n=3x=36) without any intergenomic recombinations. 45S rDNA probe was successfully hybridized on NOR bearing chromosomes of *L. longiflorum* and Oriental hybrid. *L. longiflorum* possessed 3 rDNA signals on chromosome 3, 4 and 7. Chromosome 3 of *L. longiflorum* showed one signal near to the secondary constriction on the long arm. 9 chromosomes of Oriental hybrid showed one or two hybridization signals indicating some extent of polymorphism. This polymorphism confirmed the fact that Oriental hybrids are mixed with several species in the section Archelirion. Based on result of the polymorphism of the rDNA signal, the origin of some chromosome number of Oriental hybrid could be assumed.

INTRODUCTION

Lilium, a large genus with over 80 species, is classified into seven taxonomic sections (Comber 1947). Because of its large genome size (*L. longiflorum* = 141.1 pg/cell, Bennett and Smith 1976), the genus *Lilium* has furnished material for cytological research since Strasburger (1880). All species are endemic to Asia, North America and Europe of the Northern Hemisphere.

Identification of recombinant chromosome or chromosome segments in interspecific hybrid progenies would be beneficial for introgression breeding. Furthermore, it is important to identify the exact chromosome number and breakpoint of the recombinant chromosomes for the probable introgressed genes and localization of gene(s) on the chromosome along with phenotypic characters.

In order to identify the parental chromosomes of the interspecific hybrid, genomic in situ hybridization (GISH) is the most powerful tool.

Physical localization of rDNA genes was used as a useful marker to distinguish between NOR containing chromosomes and provides valuable evidence about the genome evolution at molecular cytogenetic point of view (Gerlach and Dyer 1980; Kamstra et al., 1997). 5S and 18S-25S rDNA probes corresponding to the site of ribosomal RNA genes were detected through fluorescence in situ hybridization in many crops (Mukai et al., 1991). Species specific sequence together with FISH is also useful markers to identify the rest of chromosomes, which can not be distinguished through banding techniques (Kamstra et al., 1997).

The aim of the present study was identification of individual chromosomes of *L.*

longiflorum and Oriental hybrid by means of GISH and FISH.

MATERIAL AND METHODS

Plant Material

OLA-hybrid '942653-1' was derived from Oriental hybrid 'San Marco' (**O**) × **LA**-hybrid '88542-69'. F_1 **LA**-hybrid was known to produce fertile 2n-pollen by FDR about 9.6 % and was used as male parent for BC plant production. Since the **LO**-hybrid was sterile, an amphidiploid '950232' (**LOLO**) was made by doubling the chromosome number of the **LO**-hybrid. The triploid '972729-4'(2n=3x=36; **OLO**), the interspecific hybrid of Oriental hybrid 'Mero Star' (**O**) × **LO**-hybrid, was used for GISH and FISH analysis. Interspecific hybrids were produced through integrated pollination and embryo rescue methods (Van Tuyl et al.,. 1991). Plants were grown in a greenhouse at 20 – 25°C during the day and 14 – 18°C during the night.

Chromosome Preparation

For the study of mitotic metaphase complements, the fast growing root tips were collected in the morning and pretreated in saturated α-bromonaphtalene solution for 2 hours at 20 °C followed by an overnight treatment at 4 °C. The material was fixed in Carnoy's solution (acetic acid:ethanol = 1:3) and stored at –20 °C until use. Before making squash preparations, root tips was incubated in a pectolytic enzyme mixture containing 0.3% pectolyase Y23, 0.3% cellulase RS and 0.3% cytohelicase in 10mM citrate buffer (pH 4.5) for about 1 – 1.5 hour at 37 °C. Squash preparations were made in 60% acetic acid. Slides were frozen in liquid nitrogen and the cover slips were removed by using a razor blade. Slides were finally dehydrated in absolute ethanol for a few minutes, dried and stored at –20 °C until use.

DNA Probes Preparation

Total genomic DNA of *L. longiflorum* 'Snow Queen' was used as a probe. The probe DNA was labeled with digoxigenin-11-dUTP by nick translation according to the manufacturer's instructions (Boehringer Mannheim). Clone pTa71 contains the 9 kb EcoRI fragment of 45 S ribosomal DNA from wheat (Gerlach and Bedbrook 1979). Isolated DNA of 45 S rDNA sequences from pTa71 was labeled with either biotin-16-dUTP by nick translation for in situ hybridisation according to the manufacturer's manual (Boehringer Mannheim, Germany). Blocking DNA was obtained by autoclaving herring sperm DNA for 5 min at 121°C. The size of blocking DNA ranged from 100 to 500 bp. Because of Oriental hybrids are derived from at least 3 species of Archelirion section, 45S ribosomal DNA sequences were hybridized to the mitotic chromosome complement of **OLO**-hybrid.

Fluorescence in Situ Hybridization (FISH)

The in situ hybridization protocol was carried out according to Kuipers et al., (1997) with minor modifications. Briefly, slides were pretreated with RNase A (100 µg/mL) for 1 hour and pepsin (5 µg/mL) for 10 min, both at 37°C, followed by formaldehyde (4%) for 10 min at 20°C, dehydration with 70%, 90% and absolute ethanol for 3 min and air dried. Hybridization followed using a mixture consisting of 2x SSC, 50% formamide, 10% sodium dextran sulfate, 0.25% SDS, 2.0 ng/µL digoxigenin-11-dUTP labeled total genomic DNA of *L. longiflorum* 'Snow Queen' and 30-40 ng/µL herring sperm DNA for blocking. DNA was denatured by heating the hybridization mixture at 70°C for 10 min and then placed on ice for 10 min. For each slide 40 µL hybridization mixture was used. The preparations were denatured at 80°C for 10 min. After overnight hybridization at 37°C in humid chamber, slides were washed at room temperature in 2x SSC for 15 min, 0.1x SSC at 42°C for 30 min. The digoxigenin labeled probe DNA was detected with 20 µg/mL anti-dig-FITC (fluorescein isothiocyanate; Boehringer Mannheim) and 20 µg/mL rabbit-anti-sheep-FITC (Vector Laboratory).

Slides were counterstained with DAPI (4´,6-Diamidino-2-phenylindole) or PI (propidium iodide) and examined under the Zeiss Axiophot microscope equipped with epi-fluorescence illumination and single band filters for DAPI, FITC and PI. Images were photographed on 400 ISO color negative film and scanned at 1200 dpi for digital processing in Photoshop (Adobe Inc.).

RESULTS AND DISCUSSION

Multicolor GISH enabled to discriminate trigenomic chromosomes (2n=3x=36) viz., Oriental, *Longiflorum* and Asiatic hybrid genomes. The **OLA**-hybrid proved to have in total 2n=36 chromosomes, one genome set (x=12) derived from Oriental female parent and other two genome sets from F_1 **LA**-hybrid without any homoeologous recombination (Fig. 1a). The triploid BC_1 plant with three different genome sets from Oriental, Longiflorum and Asiatic hybrid showed normal growth habit and phenotypic character are intermediate of trigenomes. Because of no homoeologous recombinations it seems that the genes lying on each chromosome for certain characters are active as in normal diploid plants. 2n-gametes by FDR from F_1-hybrids often contain homoeologous recombinations between parental genomes, which have been shown in other BC_1 plants (Karlov et al., 1999). The frequency of homoeologous recombination in several crosses using 2n-gametes (FDR) producing F_1-hybrids was more than 50 % (unpublished data).

Oriental hybrids are late flowering (100 – 120 days), however, *L. longiflorum* has relatively early flowering (about 90 – 100 days). On the other hand, Oriental hybrids have large flower size, strong growth habit and are resistant to *Botrytis*. To circumvent the disadvantages of both species, introgression of specific genes or characters into recipient by interspecific hybridization is preferred. Another BC_1 analyzed in the present study was an **OLO**-hybrid, which showed exceptionally strong growth habit with large flower-size (about 40 cm in diameter). Because of the F_1 **LO**-hybrid showed absolute sterility, chromosome number was doubled by artificial chromosome doubling. The fertility of the amphidiploid was about 40% and this pollen was used successfully for further crossing.

There was no evidence of homoeologous recombination in **OLO**-hybrid between parental chromosomes as expected in the case where amphidiploid F_1-hybrid was used as parent (Lim et al.,. 2000). As Lim et al.,. (2000) made clear, the frequency of homoeologous recombination is dramatically reduced from the gametes in amphidiploids or even no chance to pair between homoeologous chromosomes. GISH revealed clearly the parental chromosomes of the **OLO**-hybrid without any homoeologous recombinations.

NORs are often useful to identify the parental species chromosomes. The same preparation of the **OLO**-hybrid was used for the detection of 45S rDNA sequence (Fig. 1c). FISH unequivocally revealed the NOR bearing chromosomes of both *L. longiflorum* and Oriental chromosomes. *L. longiflorum* chromosomes 3, 4 and 7 showed one rDNA signal and Oriental hybrid revealed on the chromosomes 1, 2, 3, 4 and 12. Two Oriental chromosome sets showed different rDNA signals, for example, chromosomes possess the rDNA signal in chromosome #1 (2x), #2 (1x), #4 (1x), #5 (2x) and #12 (2x). This indicates that the Oriental chromosomes are derived from several species (Table 1). Based on various references (Stewart 1947; Noda 1991; Ogihara 1968), the possible origin of the nucleolar chromosomes of the **OLO**-hybrid was postulated as shown in Table 1.

In conclusion, this study demonstrates the successful multicolor GISH (FISH) and the discrimination of parental chromosomes with 45S rDNA in two triploid genotypes of lily.

Literature Cited

Bennett, M.D. and Smith, J.B. 1976. Nuclear DNA amounts in Angiosperms. Philosophical Transactions of the Royal Society B. 274:227–274.

Comber, H.F. 1947. A new classification of the *Lilium*. Lily Yearbook, Royal Horti. Soc., London. 15:86–105.

Gerlach, W.L. and Bedbrook, J.R.1979. Cloning and characterization of ribosomal RNA

genes from wheat and barley. Nucl. Acids Res. 7:1869–1885.
Gerlach, W.L. and Dyer, T.A. 1980. Sequence organization of the repeating units in the nucleus of wheat, which contain 5 S rRNA genes. Nucl. Acids Res. 8:4851–4865.
Kamstra, S.A., Kuipers, A.G.J., De Jeu, M.J., Ramanna, M.S. and Jacobsen, E. 1997. Physical localisation of repetitive DNA sequences in *Alstroemeria*: karyotyping of two species with species specific and ribosomal DNA. Genome 40:652–658.
Karlov, G.I., Khrustaleva, L.I., Lim, K.B., and van Tuyl, J.M. 1999. Homoeologous recombination in 2n-gamete producing interspecific hybrids of *Lilium* (Liliaceae) studied by genomic *in situ* hybridization (GISH). Genome 42:681–686.
Kuipers, A.G.J., Van Os, D.P.M., de Jong, J.H., Ramanna, M.S. (1997) Molecular cytogenetics of *Alstroemeria*: identification of parental genomes in interspecific hybrids and characterization of repetitive DNA families in constitutive heterochromatin. Chromosome Res 5: 31 – 39.
Lim, K.B., Chung, J.D., van Kronenburg, B.C.E., Ramanna, M.S., de Jong, J.H., and. van Tuyl, J.M. 2000. Introgression of *Lilium rubellum* Baker chromosomes into *L. longiflorum* Thunb.: a genome painting study of the F_1 hybrid, BC_1 and BC_2 progenies. Chromosome Res 8:119–125.
Mukai, Y., Endo, T.R. and Gill, B.S. 1991. Physical mapping of the 5 S rRNA multigene family in common wheat. J. Hered. 81:290–295.
Noda, S. 1991. Chromosomal variation and evolution in the Genus *Lilium*. In: Tsuchiya T, Gupta PK (eds) Chromosome engineering in plants: genetics, breeding, evolution. Part B, Elsevier, Amsterdam, pp. 507–524.
Ogihara, R. 1968. Karyotypes of *Lilium rubellum* Baker. La Kromosomo. 74: 2415–2418.
Stewart, R.N. 1947. The morphology of somatic chromosomes in *Lilium*. Amer. J. Bot. 34: 9–26.
Strasburger, E. 1880. Zellbildung und Zelltheilung. Fischer Verlag, Jena, 3rd Edition
Van Tuyl, J.M., van Diën, M.P., van Creij, M.G.M., van Kleinwee, T.C.M., Franken, J., and Bino, R.J. 1991. Application of in vitro pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Sci. 74: 115–126.

Tables

Table 1. Possible origin of NOR bearing chromosomes of **OLO**-hybrid '972729-4' revealed by FISH.

Chromosome number	Number of Oriental chromosomes possessing NORs	Possible origin species
Ch #1	2	*L. alexandrae* *L. auratum* *L. nobillisimum* *L. rubellum* *L. speciosum*
Ch #2	1	*L. auratum* *L. japonicum* *L. rubellum*
Ch #3	0	*L. alexandrae* *L. auratum* *L. japonicum* *L. nobillisimum* *L. speciosum*
Ch #4	1	*L. alexandrae* *L. auratum* *L. japonicum* *L. rubellum* *L. speciosum*
Ch #5	2	*L. speciosum*
Ch #12	2	*L. alexandrae* *L. auratum* *L. japonicum* *L. rubellum* *L. speciosum*

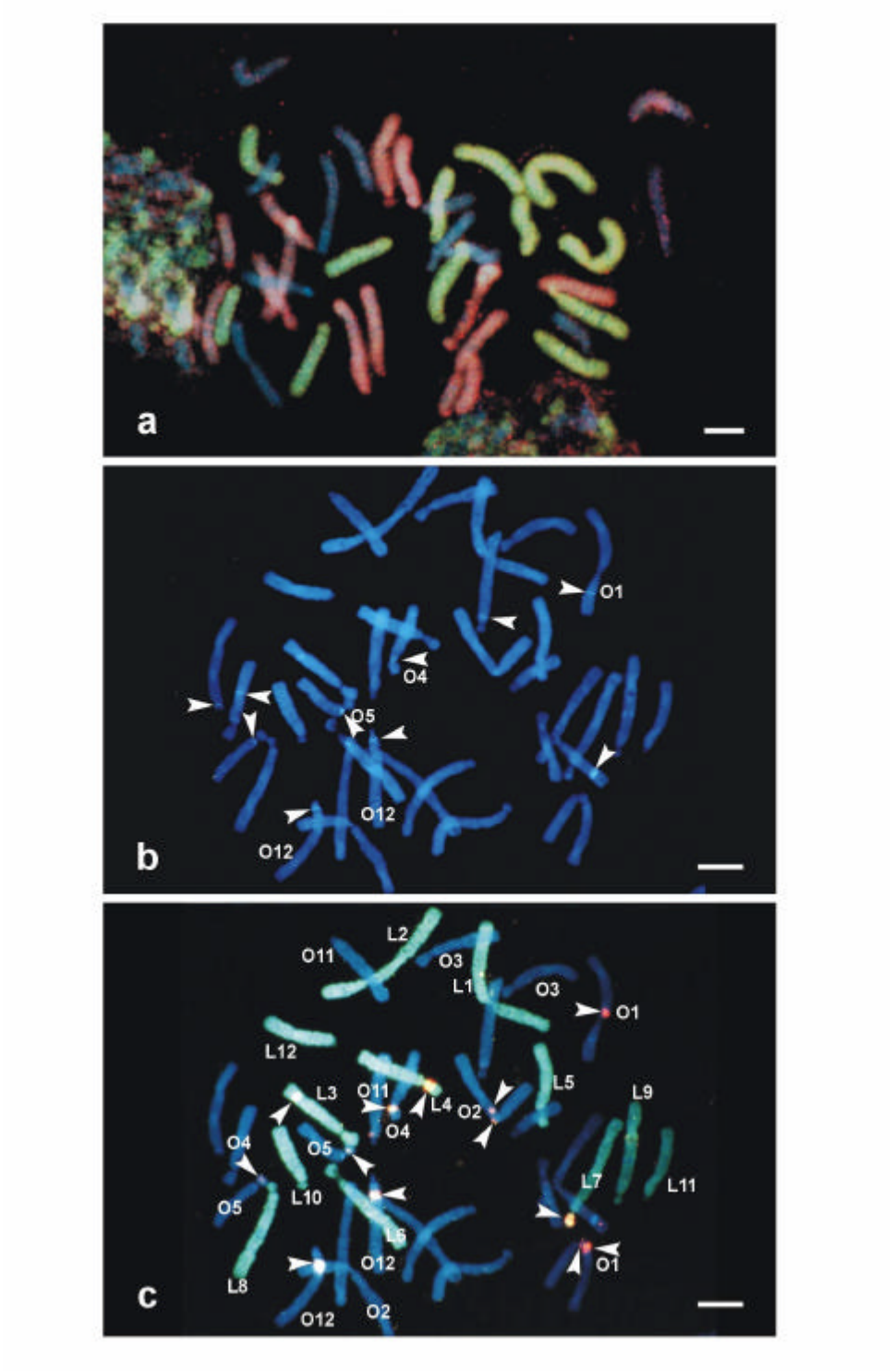

Fig.1. (a) Multicolor GISH of **OLA**-hybrid '942653-1' on mitotic metaphase chromosome complement. Green-, red- and blue-fluorescence were detected by anti-Dig FITC, Cy3 Avidin-streptavidin and DAPI counterstaining. (b) Mitotic metaphase complement of **OLO**-hybrid '972729-4'. DAPI staining showed few DAPI-bands is enable to distinguish some of chromosome numbers. (c) GISH with detection of 45S rDNA sequence (red signals) allowed to identify parental chromosomes and 45S rDNA bearing chromosomes. Bars indicate 10 μm.