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Research Report

Chromosomal Analysis of *Lilium longiflorum* x Asiatic Hybrids Using GISH (Genomic *in situ* Hybridization)

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Abstract. In *Lilium*, a popular horticulture crop, the main objective of crossbreeding is introgression of desirable genes and features into new cultivars. Commercial *Lilium* cultivars were produced primarily through 2n-gametes or 2x-gametes as parental plants or backcrossing. The primary genetic difference in 2 methods, is the presence of genomic recombination. Especially, GISH (genomic *in situ* hybridization) among molecular cytogenetic analysis, is the relevant technique to detect genetic information; genome composition, chromosome behavior during meiosis and recombination of hybrids as using their parental DNA as a probe. Based on previous studies, in this research, 12 LA (*L. longiflorum* x *L.* Asiatic hybrids) hybrids were analyzed by GISH (genomic *in situ* hybridization) along with ploidy analysis, and pollen germination tests. The LA hybrids used in this study, showed less pollen germination ability (ranged 0 - 21.74%), but germination of 'Caesars Palace' was significantly higher (59.09%). Interestingly, ploidy analysis showed that 'Caesars Palace' was tetraploid and 'Batistero' had one more additional chromosome from the L genome. Nine of the 12 cultivars exhibited recombination and 3 had only non-recombinant chromosomes; fewer *L. longiflorum* chromosomes were present in the cultivars than Asiatic chromosomes. Consequentially, it is assumed that 2n-gametes were more common methodology than 2x-gametes for producing commercial cultivars. Moreover, backcrossing tended to be performed with the Asiatic hybrid cultivar.

***Additional key words*:** cross-over, cytogenetics, interspecific, microscopy, recombination

Introduction

The genus *Lilium* belongs to the family *Liliaceae* and most of wild *Lilium* species inhabit the Northern Hemisphere and mainly originated in Asia, North America and Europe. *Lilium* is comprised of nearly 100 species which are grouped into 7 sections: *Martagon, Pseudolirium, Archelirion, Sinomartagon, Leucolirion, Oxypetalum* and *Lilium* (Comber, 1949; De Jong, 1974; Lighty, 1968). Lily is one of the main horticulture crop as a potted, garden plant and cut flower. Lily is the fourth popular cut flower (6.14%) at the Dutch auction (Anonymous, 2016) and the Netherlands is a word-renowned the biggest producing country as 76% of total lily cultivation area (Buschman, 2005).

Interspecific Hybrids

In lily breeding, while the breeding history of lily was more than 200 years (Shimizu, 1987), considerable breakthroughs were developed about 50 years ago (MacRae, 1998). The main objective of crossbreeding is introgression of desirable genes and features into new cultivars. To improve physiological and phenotypic characteristics such as fragrance, flower shape, and color, commercial lily breeding focusses on interspecific or intersectional hybrids, due to limitation of gene-pool in same section. However, success rate of cross between species have variation due to distinction among genome. Crossing polygon of genus *Lilium* depending on taxonomic distance, is represented successful possible crossing trend between each section (Fig. 1).

For successful interspecific (intersectional) hybridization

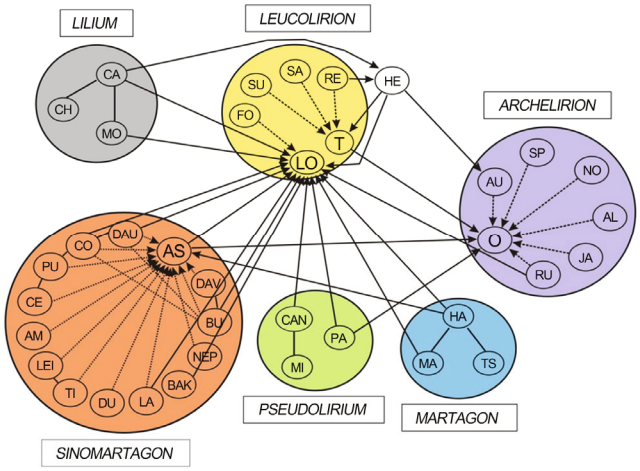

Fig. 1. Schematic successful crosses of species between seven sections of the genus *Lilium* developed by J. M. Van Tuyl at Plant Research International, The Netherlands. In this figure, the connection among the Asiatic (AS), Trumpet(T), and Oriental (O) hybrid groups are represented as dotted lines. In successful crosses between species (small circles) of different section (large circles) the arrows indicated for the female parent. Abbreviations: AL: *L. alexandrae*; AM: *L. amabile*; AS: Asiatic hybrids; AU: *L. auratum; BAK: L. bakerianum; BU: L. bulbiferum;* CA: *L. candidum*; CAN: *L. canadense*; CE: *L. cernuum*; CH: *L. chalcedonicum*; CO: *L. concolor*; DAU: *L. dauricum*; DAV: *L. davidii*; DU: *L. duchartrei*; FO: *L. formosanum*; HA: *L. hansonii*; HE: *L. henryi*; JA: *L. japonicum*; LA: *L. lankongense*; LEI: *L. zleichtlinii*; LO: *L. longiflorum*; MA: *L. martagon*; MI: *L. michiganense*; MO: *L. monadelphum*; NEP: *L. napalense*; NO: *L. nobilissimum*; O: Oriental hybrids; PA: *L. pardalinum*; PU: *L. pumilum*; RE: *L. regale*; RU: *L. rubellum*; SA: *L. sagentiae*; SP: *L. speciosum*; SU: *L. sulphureum*; T: Trumpet hybrid; TI: *L. tigrinum*; TS: *L. tsingtauense*. Modified from van Tuyl et al., 2011 and Lim et al., 2008.

hindrances as pre- and post-fertilization barriers (van Tuyl et al., 1992) caused by genetic or physiological incompatibility and incongruity have to be circumvent. To overcome pre-fertilization barriers, GSM (grafted-style method), CSM (cut-style method) (Asano and Myodo, 1997) and in vitro pollination techniques have been developed. CSM is most reliable method for overcoming pre-fertilization barriers occurred on stigma and in style. Using this technique, the growth inhibitor involved inhibition of pollen tube elongation can be removed by cutting of style and pollinated through placing the pollen. Pollen from the donor plant was germinated on compatible stigma and style with germinating pollen was transfer to ovary cut shortly of mother plants that is GSM methods. However, even if fertilization is succeeded through these methods, post-fertilization barriers can hinder the development of embryos (van Tuyl et al., 1992). In vitro pollination and rescue methods i.e. embryo, ovule, and ovary slice culture (Asano, 1978; Asano, 1980; Asano and Myodo, 1997; Ascher, 1973; North and Wills, 1969; Skirm, 1942) have been developed to circumvent post-fertilization barriers.

Polyploidization in Interspecific Hybrid

Most of F_1 hybrids produced by several techniques e.g. CSM, in vitro pollination and embryo culture, are infertile and sterility of hybrids leads to consequential restriction in breeding. It can be caused by several reasons as chromosome aberrations, genetic difference between sections or other unrecognized factors (Asano, 1982). Polyploidization were used to recover sterility of hybrids, it can be classified as meiotic and mitotic polyploidization (van Tuyl et al., 1989; van Tuyl et al., 1992).

In case of mitotic polyploidization, chromosome doubling is conducted on vegetative tissues by antimitotic reagents; colchicine, oryzalin, amiprophos-methyl (APM) and trifluralin (Blakeslee and Avery, 1937; Emsweller and Brierley, 1940; van Tuyl et al., 1992). Three mechanisms induced 2n-gametes: FDR (first division restitution), SDR (second division restitution) and IMR (indeterminate meiotic restitution). A few of inter-specific, intersectional hybrids rarely produced functional 2n-gametes with somatic chromosome numbers. Meanwhile, due to rarity of 2n-gamaetes in nature, meiotic polyploidization methods e.g. heat shock, injection of caffeine (Lim et al., 2004) and N_2O treatment to flower bud were utilized for artificial induction of 2n-gametes formation.

Somatically doubled chromosomes could not contribute much to introgression breeding, since they produce identical 2x-gametes which induce preferential pairing of homologous chromosomes during meiosis (Ramanna and Jacobsen, 2003; Van Tuyl and Lim, 2003).

Because pairing between homoeologous chromosomes is scarcely occurred, most of hybrids originated from 2x-gametes have non-recombinant chromosomes. On the other hands, backcrossing (BC) progenies derived from 2n-gametes show considerable phenotypic variation because homoeologous pairing and intergenomic recombination between parental genomes can be induced during meiosis I division (Riley, 1965).

Commercial Lily Breeding

There are three frequently used assortments of lilies in commercial breeding industry: *Longiflorum* (*Leucolirion*), Asiatic (*Sinomartagon*) and Oriental (*Archelirion*). Tendency of commercial lily cultivation acreage was shown in Figure 2 and it is possible to infer development of lilies in market. For over 30 years from 1970s, Asiatic hybrids were the leading group characterized by up-facing flower with diverse colors. From 1990s, Oriental hybrids with strong fragrance and impressive flower shapes, were considered the most important group. After advancements in interspecific hybridization techniques: CSM, embryo rescue and ovule culture, diversity of hybrids having combined desirable features from different sections by hybridization has increased.

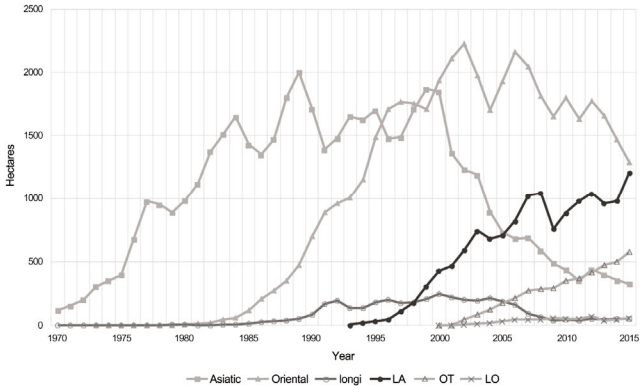

Fig. 2. The chart of annual changes in *Lilium* hybrids acreage in Netherlands was redrawn based on BKD (The Dutch Flower Bulb Inspection Service) publication (BKD, 2015).

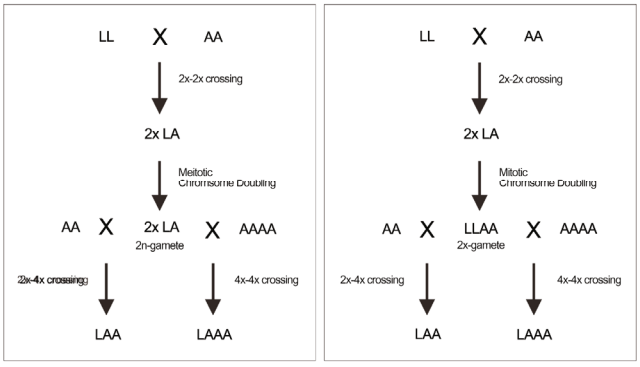

Fig. 3. Schematic illustration of triploid hybrid breeding mechanisms through meiotic polyploidization (2n-gamete) represented with LA hybrid. LL and AA denoted diploid *L. longiflorum* and *L.* Asiatic hybrid genome. A: using 2n-gametes induced by meiotic chromosome doubling, B: using 2x-gamete caused from mitotic chromosome doubling.

The number of somatic chromosomes of wild lilies is 24 (2n = 2x = 24), while, most commercial cultivars produced by interspecific hybridization are polyploid (triploid and tetraploid), as they are developed from back crossing of F_1 interspecific hybrids to a diploid parental plant. Two distinct pathways about triploid LA hybrids production were described in Figure 3.

Genomic *in situ* Hybridization (GISH)

Molecular cytogenetic applications, like *in situ* hybridization (ISH), it is possible to confirm precise physical loci on chromosomes. Therefore, ISH techniques is applied to chromosome mapping, genome analysis, phylogenetic analyses and detection of chromosomal aberrations (Devi et al., 2005).

Genomic *in situ* hybridization (GISH) uses total genomic DNA from one parent species as a probe and genomic DNA from a counterpart species as blocking DNA; in contrast to fluorescence *in situ* hybridization (FISH) which uses ribosomal DNA (e.g., 45s, 18s, 5s), as a probe to detect specific loci on the chromosome. GISH is a powerful method used to analyze hybrids and alloploids, through this technique, detection of translocations between parental genomes and observation of chromosome behavior during meiosis is possible (Lim and Van Tuyl, 2007). Furthermore, GISH provides cytological information, i.e. the genome evolution of partial allopolyploids, polyploid hybrids, and recombinant inbred lines (Kamstra, 1993; Karlov et al., 1999; Lim et al., 2001b). The large genome size of *Lilium* species provides an advantage for cytological analysis on homoeologous recombination and chromosome composition. The improvement of GISH techniques facilitates the depictions of mechanisms underlying 2n gamete production in *Lilium* (Lim et al., 2001a; Barba-Gonzalez et al., 2005).

The mechanisms of introgression breeding to produce interspecific hybrids can be interpreted through genetic analysis, (e.g., GISH, ploidy check and pollen germination tests) in commercial cultivar lilies. Cytogenetic information obtained from commercial cultivars will play a primitive role in modern breeding programs. Therefore, this study used GISH to understand genome evolution through repetitive crossing in lilies.

Materials and Methods

Plant Materials

In this studu, 12 L. *longiflorum*-Asiatic cultivars; ‘Alubuferia’, ‘Batistero’, ‘Breakout’, ‘Caesars palace’, ‘El divo’, ‘Eyeliner’, ‘Fangio’, ‘Miscanti’, ‘Nashville’, ‘Opportunity’, ‘Treasure island’, and ‘ Whist’ developed and released by Dutch breeding companies were analyzed. Two of representative diploid cultivars; *L. longiflorum* “Bright tower” and *L.* Asiaitc hybrid “Dimension” were used as controls to compare the ploidy level and pollen germination ratio.

Ploidy Analysis

Ploidy level was analyzed by flow cytometer (Partec PA, Ploidy Analyzer, Germany) with young and fresh leaves from each cultivar. To release nuclei, leaf tissue was chopped in nuclei extraction buffer (Sysmex, Germany) and incubated. The nuclei suspensions were filtered, before the addition of staining buffer (Sysmex, Germany) through a 30 μm nylon mesh. The solution was then injected into the analyzer.

Pollen Germination

The pollen germination test was conducted following the method adopted by Barba-Gonzalez (2005). Pollen was collected at anthesis and then incubated at room temperature overnight on artificial agar medium, consisting of 20 $mg \cdot L^{-1}$ boric acid (H_3BO_3) (Duchefa, The Netherlands), 100 $g \cdot L^{-1}$ sucrose, 5 $g \cdot L^{-1}$ agar (Duchefa, The Netherlands) at pH 5.8. Germinated pollen was counted under a stereoscopic microscope (Olympus, SZX 16, Japan).

Root Collection and Mitotic Chromosome Preparation

Young and fresh root tips were soaked in α-bromonaphthalene solution for 4 hours at 20°C, after that transfer in acetic acid-ethanol solution (1:3, v/v) for overnight fixation at room temperature. Rinsed with double distilled water and stored in 70% ethanol at -20°C until use. For slide preparations, the materials were washed with distilled water and incubated in an enzyme solvent consisting of 0.3% pectolyase (Duchefa, The Netherlands), 0.3% cellulose (Duchefa, The Netherlands) and 0.3% cytohelicase (Sigma, USA) at 37°C for 1 hour. Digested root tips were squashed on slide with 60% acetic acid and air-dried.

DNA Isolation and Preparation

Genomic DNA was extracted from *L. longiflorum* 'Bright Tower' leaves using a modified C-TAB method (Kanazawa and Tsutsumi, 1992). Extracted genomic DNA was labelled as a probe with biotin-nick translation mixture (Roche, Germany) following manufacturer instruction. Labelling of 45s rDNA was performed by digoxigenin-nick translation mixture (Roche, Germany) as manufacturer protocol. Blocking DNA was herring sperm DNA (Invitrogen, USA), fragmented through autoclaving from 100 bp to 1 kb.

Genomic *in situ* Hybridization and Karyotype Analysis

GISH was conducted according to Lim et al. (2001b). In short, the slides were pretreated with 100 $\mu g \cdot mL^{-1}$ RNase A at 37°C for 1 hour, rinsed in 2X SSC and fixed with 4% paraformaldehyde for 10 minutes. After then, the slides were incubated with 5 $\mu g \cdot mL^{-1}$ pepsin for 10 min at 37°C, dehydrated with a graded ethanol series, and air-dried. The hybridization mixture consisted of formamide, dextran sulfate, SSC, SDS, probes and blocking DNA was denatured at 70°C and cooled on ice. Then, slides were hybridized in a humid chamber overnight for stabilizing. After that, the chromosomes were detected by streptavidin Cy3 (Invitrogen, USA) and anti-digoxigenin fluorescein (Roche, Germany), counterstained with DAPI (4',6-diamidino-2-phenylindole). The slides were photographed with a Nikon BX 61 fluorescent microscope. Chromosomes were classified *L. longiflorum* (L) or Asiatic hybrid (A) and arranged by length of short and long arm (Lim et al., 2001a, Lim et al., 2001b, Stewart, 1947). Recombinant chromosomes were assorted as L/A or A/L chromosome according to the genome of centromere region of chromosome.

Results and Discussions

Commonly, *L. longiflorum*-Asiatic hybrids were simplified as LA hybrids group, however, genome composition of LA hybrids has difference followed their production mechanisms. In this study, by methodology of LA hybrids production, genome of LA hybrids was classified LAA and LAAA. The meaning of LAA is triploid LA hybrid and LAAA is tetraploid produced by backcrossing with *L.* Asiatic hybrids as a recurrent parent.

Ploidy Analysis

Ploidy analysis is imperative to incorporate the parental information and hybridity status of true hybrids. Flow cytometry or chromosome counting are essential analyses to reveal the DNA content in hybrid progenies for the appropriate selection of a male or female 2n-gamete producer (Barba-Gonzalez et al., 2014). Ploidy of each cultivar was investigated using flow-cytometer and chromosomal observation for further confirmation (Table 1).

Representative diploid cultivars (2n = 2x = 24) *L. longiflorum* 'Bright Tower' and Asiatic hybrid 'Dimension' were used as controls in flow cytometry. Microscopic chromosome observation and flow-cytometric analysis confirmed tetraploidy (2n = 4x = 48) in 'Caesars Palace'. Followed flow-cytometry results, 10 cultivars exhibited triploidy with 36 chromosomes: 'Albufeira', 'Breakout', 'El divo', 'Eyeliner', 'Fangio', 'Miscanti', 'Nashville', 'Opportunity', 'Treasure Island'.

Remarkably, although flow-cytometric results indicated triploidy status in 'Batistero', chromosome observation detected 37 chromosomes along with one additional chromosome. It was considered as a B chromosome occurred commonly in plants having large chromosomes (Jones and Houben, 2003). More details about additional chromosome will be discussed in results of GISH and karyotype analysis.

Consequently, with a few exceptions, flow cytometry and chromosomal counting results revealed that most of LA hybrids were triploids (2n = 3x = 36) produced by mechanisms of development of commercial LA hybrids.

Pollen Germination

It is possible to verify fertility of progenies and availability as crossing parents through pollen germination test. Most pollen from interspecific or intersectional hybrids were sterile in this study except few of hybrids producing 2n-gametes. Table 1 shows the pollen germination ratio of polyploid cultivars in contrast with the control diploid cultivars.

Controls: 'Bright Tower' and 'Dimension' had pollen viability as 86.93% and 85.34% of pollen germination percentage. 'Breakout', 'El divo', 'Nashville', 'Opportunity' and 'Whist' exhibited less than 6.00% and four cultivars had 0.00% of pollen germination percentage that is 'Albufeira', 'Batistero', 'Eyeliner' and 'Miscanti'. 'Fangio' and 'Treasure Island' had relatively high pollen germination: 10.71% and 21.74%. It is assumed that triploids produced low- or non-generative pollen. Among 12 LA hybrids, 'Caesars Palace' had the

Table 1. Ploidy analysis based on the results of flow-cytometer and chromosome counting, and pollen germination ratio

Cultivar	Group	Ploidy[z]	The number of chromosomes[y]	Pollen germination (%)
Bright Tower[x]	LL	2x	24	86.93
Dimension[w]	AA	2x	24	85.34
Albufeira	LA	3x	36	0.00
Batistero	LA	3x	36 + 1	0.00
Breakout	LA	3x	36	0.98
El divo	LA	3x	36	2.00
Eyeliner	LA	3x	36	0.00
Fangio	LA	3x	36	10.71
Miscanti	LA	3x	36	0.00
Nashville	LA	3x	36	5.45
Opportunity	LA	3x	36	1.81
Treasure Island	LA	3x	36	21.74
Whist	LA	3x	36	1.00
Caesars Palace	LA	4x	48	59.09

[z]Estimated ploidy by flow-cytometer.
[y]The number of chromosomes by microscopic observation.
[x]*L. longiflorum* 'Bright tower' utilized as a L control.
[w]Asiatic hybrid 'Dimension' was used as a A control.

highest pollen germination ratio (59.09%), and it is expected ability as a pollen parent.

The pollen of interspecific hybrids was predominantly sterile; it caused irregular chromosome disposal or genetic incongruity. Infrequently, BC_1 plants induced by 2n-gamete generating F_1 interspecific hybrids presented different fertility value (Lim and Van Tuyl, 2007).

GISH and Karyotype Analysis

GISH and karyotype analysis was performed to visualize their genome composition between parental genomes and observe crossover in 11 triploids and 1 tetraploid interspecific hybrids. Karyotype results were represented with ideogram for precise perception.

To interpret GISH results in LA hybrids, chromosomes were rearranged by short- and long-arm length. Furthermore, chromosomes were distinguished as non-recombinant chromosomes; L and A genomes (Fig. 4a and b) while 2 types of recombinant chromosomes were classified as A/L and L/A based on centromere region (Fig. 4c and d). L/A signified the chromosomes which has a *L. longiflorum* centromere, it means that Asiatic hybrids segment(s) was introgressed to *L. longiflorum* chromosomes. In contrast, A/L indicated that Asiatic chromosomes translocated with *L. longiflorum* and having Asiatic centromere region. A small and incidental chromosome was observed that was distinct from the standard chromosomes as "A" chromosomes (Fig. 4e).

Based on GISH results, LA hybrids were sorted into non-recombinant and recombinant cultivars. The results of GISH in 'Batistero', 'Opportunity', 'Albufeira' and 'Nashiville' were represented in Fig. 5 and results of karyotype in 'Eyeliner', 'Caesars palace', 'El divo' and 'Fangio' were illustrated with idiogram in Fig. 6. As stated in producing methodologies of commercial cultivars, meiotic polyploidization produced by 2n-gametes contributes in introgression (Ramanna and Jacobsen, 2003; Van Tuyl and Lim, 2003) as chromosome pairing is forced between homoeologous chromosomes during meiosis (Karlov et al., 1999; Lim et al., 2001a; Lim et al., 2001b; Ramanna, 1992; van Tuyl et al., 1989).

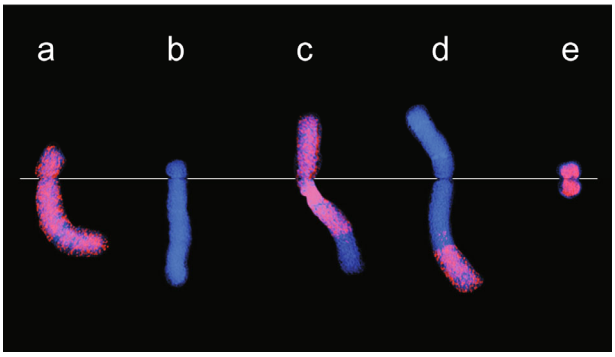

Fig. 4. The classification of chromosomes by genome, homoeologous recombination between *L. longiflorum* and Asiatic hybrid genome and comparative size of B chromosome. *L. longiflorum* was detected with Cy3 (Comber) and Asiatic hybrids were counterstained with DAPI. a: L genome, b: A genome, c: L/A genome d: A/L genome, e: additional chromosome.

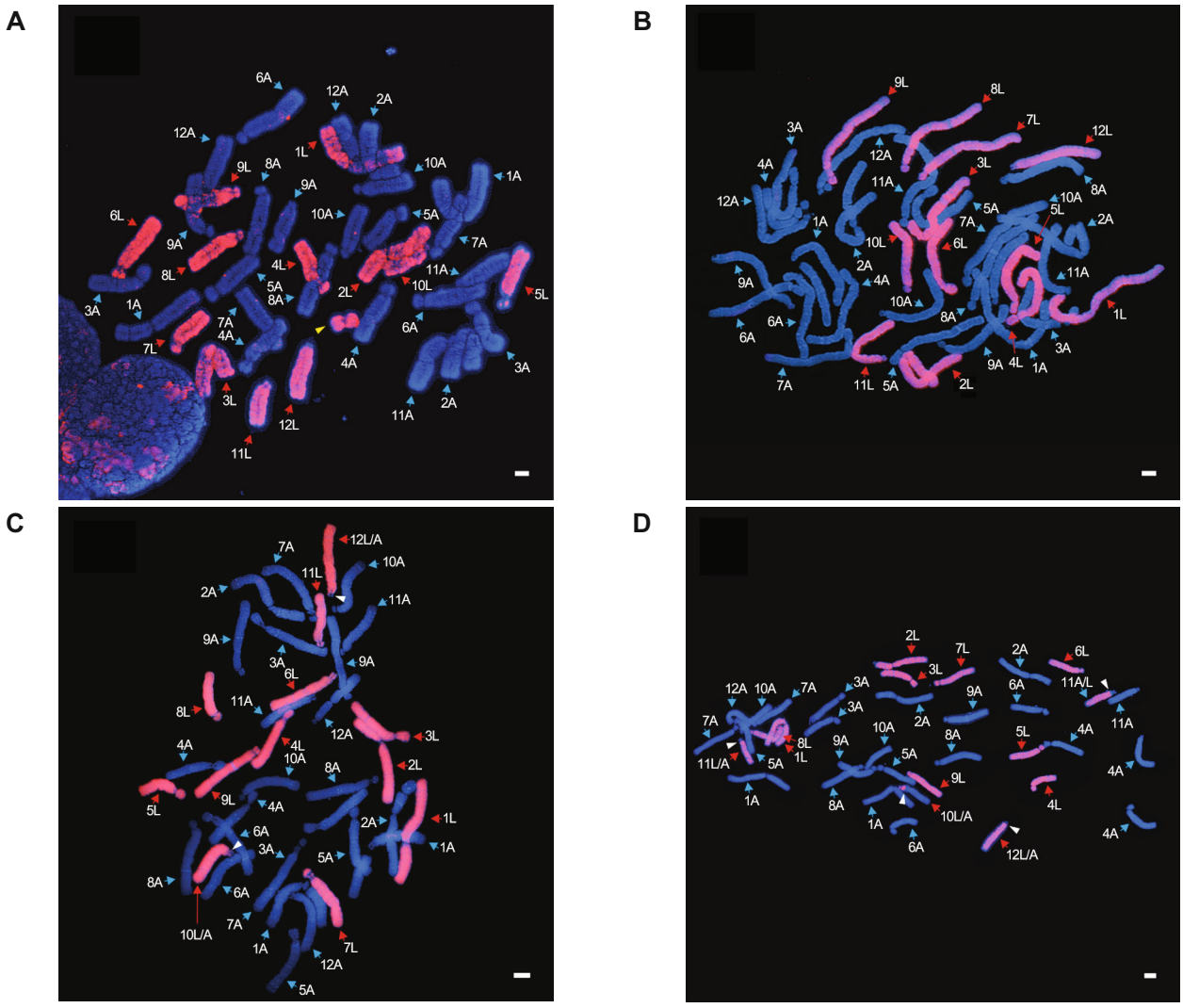

Fig. 5. The Genomic *in situ* hybridization (GISH) results of 4 of *L.longiflorum*-Asiatic cultivars. *L. longiflorum* genomes were represented with red (Cy3) and Asiatic genomes were shown as blue (4',6-diamidino-2-phenylindole). Blue arrows and red arrows indicate Asiatic and *L. longiflorum* chromosomes and yellow arrow head points B chromosome. White arrow head means breakpoint i.e. the locus of recombination point. The homoeologous recombinant chromosomes were subdivided L/A and A/L followed genome of centromere region. Bar (A-D) = 10μm. (A) 'Batistero (2n=3x=36+1)', (B) 'Opportunity (2n=3x=36)', (C) 'Albufeira (2n=3x=36)', (D) 'Nashiville (2n=3x=36).

'Batistero' was implied aneuploidy with 36 chromosomes and 1 small and additional chromosome (Fig. 5A) and chromosomes can be subdivided 24 A chromosomes and 12 + 1 L chromosomes. The extra chromosome (Fig. 5A) was clearly detected as a L genome. Despite total length of a typical chromosome was comparatively smaller for i.e.11.74 ± 20 μm, it significantly showed median centromere, similar to metacentric types. Past researches indicates that B chromosomes are smaller than Achromosomes and contain isochromosomes, telocentric chromosomes, or microchromosomes (Jones and Houben, 2003, Xie et al., 2014). Therefore, the small additional chromosome investigated in 'Batistero' can be regarded as B chromosome.

'Eyeliner' and 'Opportunity' are triploid LA hybrids being 36 chromosomes comprised of 12 red painted L genomes (detected with Cy3) and 24 blue colored A genomes (counterstained with DAPI) without translocation between parental genomes (Figs. 5B and 6A).

As a result, it is possible to confirm that those 3 cultivars have no recombination between parental chromosomes. It can be speculated that F_1 interspecific hybrids generated from chromosome doubling was used for crossing to obtain preferential pairing between homologous chromosomes during meiosis.

The homoeologous recombinant chromosomes were observed in 9 cultivars: 1 tetraploid and 8 triploids. 'Caesars Palace', 'Albuferia' and 'Treasure Island' each had 2 recombinant chromosomes. Interestingly, 'Caesars Palace' i.e. tetraploid was 48 chromosomes distinguished 36 A chromosomes and 12 L chromosomes. Two of homoeologous

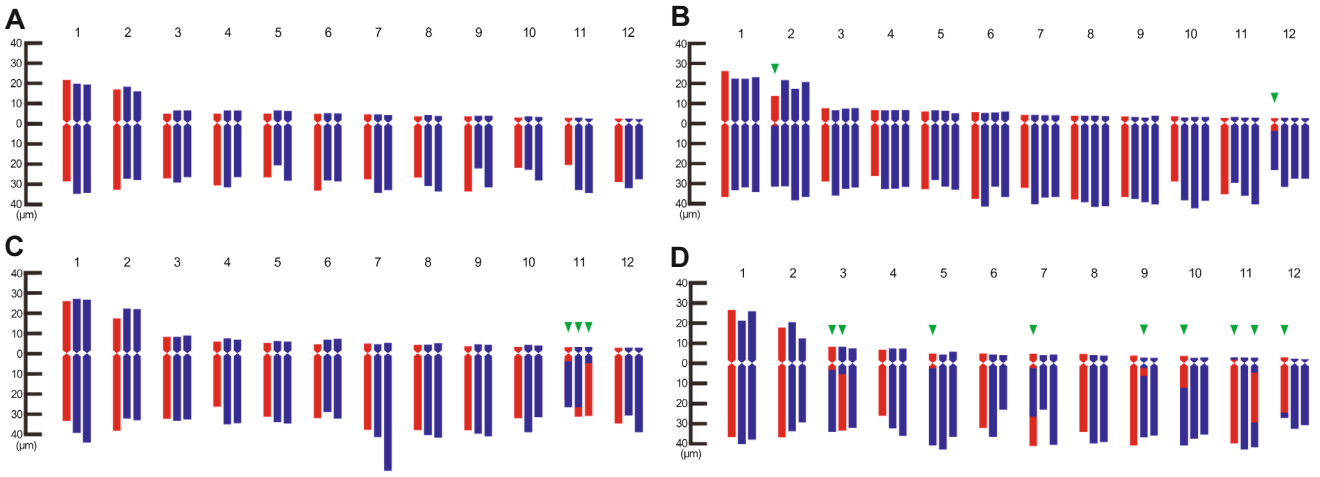

Fig. 6. Idiograms of 4 of *L. longiflorum*-Asiatic cultivars. The red color signifies *L. longiflorum* genome, while blue color means Asiatic hybrid genome and green arrow heads indicate recombinant chromosomes. Chromosomes were arranged by length of long and short arm from 1 to 12. (A) 'Eyeliner (2n=3x=36), (B) 'Caesars palace (2n=4x=48)', (C) 'El divo (2n=3x=36)', (D) 'Fangio (2n=3x=36).

recombinant chromosomes were observed on *L. longiflorum* chromosome #2 and #12. Multiple translocation on was occurred on chromosome #2 having 2 of break-points while chromosome #12 having 1 of break-point (Fig. 6B).

Genome of 'Albuferia' was composed of 12 L and 24 A chromosomes, with 2 of recombination on L chromosome #10 and #12 (Figs. 7B and 8B). Specifically, DNA segments of the Asiatic genome were introgressed on the short-arm of chromosome #10 and #12.

'Treasure Island' was detected 2 of homoeologous translocation on chromosome #6 and #7. Remarkably, one of the translocations was on *L. longiflorum* chromosome (#6) and the other recombinant chromosome was on Asiatic hybrid chromosome (#7).

In the triploid LA hybrid 'El divo', 3 recombinant chromosomes and 3 break-points were represented on chromosome #11 (Fig.6C). One of translocation was occurred on the distal region long-arm on chromosome #11 of Asiatic genome and the others were located on long-arm near the centromere region.

'Miscanti' and 'Nashville' showed 4 translocations respectively, nevertheless, the translocation nature differed between individulas. In case of 'Miscanti', DNA segments of L genomes were changed over to Asiatic genomes on chromosome #4, 7, 11, and 12. Translocated loci were on the distal region of the long arms on chromosome #4 and 11, otherwise, translocation occurred on the end of the short-arms of chromosome #7 and 12. In 'Nashville', recombination between L and A genomes was distributed on chromosomes #10 and 12, two of #11 (Fig. 5D); on respective recombinant chromosome had one breakpoint. One of chromosome #10 (L genome) and the #11 (each of L and A genome) had recombinant site close to the centromere, whereas cross-over was detected on the end of short arm on chromosome # 12 (L genome).

Introgression in 'Whist' was observed on chromosome #6, 10, 12 (L genome) and 8 (A genome); 4 of translocation loci was detected. Recombination was presented close to the centromere region were on chromosome # 6, 8 and 10 whereas, small Asiatic segments were introgressed in the end of L chromosome #11.

The results about 'Breakout' were visualized in Figs. 11B and 12B. *L. longiflorum* DNA segments were substituted for Asiatic genome on chromosome #2, 4 and 11. Translocation was presented on chromosome one set of #2 and one of chromosome #6.

Even if 7 cultivars; 'Albuferia', 'Treasure Island', 'El divo', 'Miscanti', 'Nashville', 'Whist' and 'Breakout' exhibited the same number of recombinant chromosomes and breakpoints, 'Fangio' showed different consequence as 9 of recombinant chromosomes with 12 cross-over loci (Fig. 6D). Totally, ranslocated chromosomes was 4 on Asiatic genome (each of chromosome #3 and 9 and two chromosomes of chromosome #11) and 6 on *Longiflorum* genome (chromosome #3, 5, 7, 10 and 12). Multiple translocation i.e. translocation have breakpoints over than 2 loci were examined on chromosome #7 (L genome), 9 (A genome) and 11 (A genome).

Genome Composition

Table 2 shows the genome constitution of each cultivar concluded from GISH and karyotype analysis. GISH confirmed that polyploid LA hybrids were BC progenies produced by backcrossing F_1 hybrid (Lim et al., 2008). It is demonstrated that 11 of triploid LA hybrids have 2 sets of the Asiatic genome and 1 set of *L. longiflorum* genome and 1 of tetraploid LA hybrid have 3 sets of the Asiatic and 1 set of *Longiflorum*

Table 2. The genome composition and the number of chromosome of 12 cultivars

Cultivar	Genome	No. of chromosome	Chromosome constitution		No of recombinant chromosome			No. of break points
			L	A	L/A[z]	A/L[y]	Total	
Batistero	LAA	36 + 1	12 + 1	24	0	0	0	0
Eyeliner	LAA	36	12	24	0	0	0	0
Opportunity	LAA	36	12	24	0	0	0	0
Albufeira	LAA	36	12	24	2	0	2	2
Treasure Island	LAA	36	12	24	1	1	2	2
El divo	LAA	36	12	24	1	2	3	3
Miscanti	LAA	36	12	24	4	0	4	4
Nashville	LAA	36	12	24	3	1	4	4
Whist	LAA	36	12	24	3	1	4	4
Breakout	LAA	36	12	24	3	3	6	6
Fangio	LAA	36	12	24	6	3	9	12
Caesars Palace	LAAA	48	12	36	2	0	2	3

[z]*L. longiflorum* genome which have Asiatic hybrid segment(s).
[y]Distinguished according to genome of centromere region analyzed by GISH method.

genome. The genome composition of F_1 interspecific hybrids is equal from both parental genome, however, the genome ratio of recurrent parents is increased in subsequent hybrids by repetitive backcrossing (Lim et al., 2008).

Although 'Albuferia' and 'Treasure Island' was observed same number of breakpoints as 2, 'Albuferia' has 2 of L/A recombinant chromosome and 'Treasure Island' was detected aside each of L/A and A/L chromosome. In 'El divo', cross-over occurred on 1 of *Longiflorum* and 2 of Asiatic chromosomes, with 3 of recombinant points. In an instance 'Miscanti' have 4 of L/A recombinant chromosomes and 4 of translocated loci were observed. Identically, 3 of L/A, 1 of A/L genome and 4 of recombinant points were examined in 'Nashville' and 'Whist'. 'Breakout' has 3 of recombination on each translocated genome type having 6 recombinant positions.

'Fangio' has the highest number of translocated chromosomes as 9, it composed 3 of A/L and 6 of L/A genomes and it was inspected 12 recombinant positions. In the tetraploid 'Caesars Palace', only 2 of L/A recombinant chromosomes and 3 of translocation loci were appeared. In these 2 cultivars, more breakpoints were present than translocated chromosomes, it is signified multiple cross-over was happened during meiosis. Reciprocal translocation like pseudolinkage between non-sister chromatid can be caused formation of quadrivalent chromosomes during meiosis which has a complementary translocation lead to multiple translocations (Xie et al., 2014).

Distribution of recombination points had variable trend in each cultivar (Table 3). Among the 9 cultivars with recombinant chromosomes, 8 recombinations were examined on the short arm, in contrast, total 20 translocations were observed on long arm. 'Albuferia' had recombination only on short arm at chromosome #10 and 12, while, 'Whist' (chromosome #6, 8, 10 and 12), 'Albuferia' (chromosome #2 and 12) and 'El divo' (chromosome #11) had genomic translocation only on long arm, other 5 cultivars had recombination on long and short arm both. 'Fangio' showed DNA segments translocation on long arm as well as short arm of chromosome #11, and highest number of recombinations on long arm considering chromosome number.

Chromosome regions are classified as proximal, interstitial and distal region by distance from the centromere and telomere. Proximal region indicates the region near the centromere and distal region signifies the region near the telomere, while interstitial region is located between proximal and distal region. In this study, recombination frequency in each chromosomal region, proximal region had the highest recombination frequency (18 breakpoints), followed by distal region (11), and then interstitial regions (3).

Recombination frequency regarding physical distance demonstrated that the proportion of proximal recombination was less and the proportion of distal recombination was more in p arms than q arms. In q arms, nevertheless repeatedly translocation was focused on in the distal part and absence in proximal district (CIMMYTY Economics Program International Maize & Wheat Improvement Center, 1993). Compared to p arm, the interstitial part of q arms consistently represented some degree of translocation. (Lukaszewski and Curtis, 1993).

In conclusion, 3 cultivars: 'Batistero', 'Eyeliner' and 'Opportunity' without recombinant chromosomes, were produced by crossing with 2x-gametes parents, whereas 9 cultivars having recombination were developed using parents generating 2n-gametes. Compared to the use of F_1 hybrids treated with

Table 3. Distribution of recombination points on chromosome in *L. longiflorum*-Asiatic hybrids having recombinant chromosomes

Cultivar	#1		#2		#3		#4		#5		#6		#7		#8		#9		#10		#11		#12	
	S[x]	L[y]	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L
Albufeira	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D[x]	-	-	-	D	-
Treasure Island	-	-	-	-	-	-	-	-	-	-	-	I[w]	P[v]	-	-	-	-	-	-	-	-	-	-	-
El divo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P, D	-	-
Miscanti	-	-	-	-	-	-	-	D	-	-	-	-	D	-	-	-	-	-	-	-	-	D	D	-
Nashville	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	P	P	-
Whist	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	P	-	-	-	P	-	-	-	D
Breakout	-	-	-	I, D	-	-	-	P	-	-	-	P	-	-	-	-	-	-	-	-	P	-	-	-
Fangio	-	-	-	-	-	P	-	-	-	P	-	-	-	I	-	-	-	P	-	I	P	P, D	-	D
Caesars Palace	-	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P
Total (P)	-	-	-	1	-	1	-	1	-	1	-	2	1	-	-	1	-	1	-	2	2	3	1	1
Total (I)	-	-	-	1	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	1	-	-	-	-
Total (D)	-	-	-	1	-	-	-	1	-	-	-	-	1	-	-	-	-	-	1	-	-	3	2	2
Total	-	-	-	3	-	1	-	2	-	1	-	3	2	1	-	1	-	1	1	3	2	6	3	3

[z]Short arm and [y]Long arm of chromosomes.
[x]Distal region, [w]Proximal region and [v]Interstitial region of chromosomes.

mitotic chromosome doubling, meiotic polyploidization generating 2n-gametes, contributes more to introgression breeding. It is assumed that using 2n-gametes is more common and powerful method than 2x-gamets for the production of commercial cultivars.

Cytogenetic studies assist in understanding breeding mechanisms, that can improve new cultivar development in breeding sciences, facilitating the production of high-quality commercial hybrids. Genetic analysis (cytoflow, chromosome counting and ISH) is a critical way for confirmation of the hybridity status in interspecific and introgressive hybrids. Specially, GISH is an important genetic tool to identify genetic recombination and the parental genomes in interspecific hybrids.

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