# Study on the homology of the genomes of tetraploid Asiatic lilies (Lilium) using FISH (Fluorescence in situ hybridization)

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Study on the homology of the genomes of tetraploid Asiatic lilies (*Lilium*) using FISH (Fluorescence *in situ* hybridization)

Shujun Zhou*1,2), Lei Zhong1), Lu Zhang1), Zhenghua Xu1), Xuxin Liu1), Kehu Li2), Guixue Zhou2)  

1) College of Forestry, Jiangxi Agricultural University, 1101 of Zhimindayao, Nanchang 330045, Jiangxi Province, China  
2) Department of Horticulture, College of Agriculture and Biotechnology, Zhejiang University, 866 Yuhangtang Road, Hangzhou, Zhejiang Province, 310058, China

Running title: Homology of the genomes of tetraploid Asiatic lilies (*Lilium*)

*For correspondence: Shujun Zhou  
College of Landscape and Art, Jiangxi Agricultural University, 1101 of Zhimindayao, Nanchang 330045, Jiangxi Province, China

Email: zhou2014@jxau.edu.cn; shujunzhou@msn.com

Tel and fax: 0086-791-83813243

Mobile: 0086-15179172568
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Abstract

Asiatic lily cultivars, bred by hybridization and/or chromosome doubling of species of section Sinomartagon of Lilium, are diploid, triploid or tetraploid, but the homology of the genomes among Sinomartagon species and Asiatic lilies remains unclear. In the present research, two tetraploid Asiatic cultivars were analyzed, using 45S rDNA as probe, for their FISH karyotypes and their chromosomal association, anaphase I, telophase II and pollen viability were surveyed to assess the multivalent segregation. Chromosomal assortment of six progenies of the two tetraploid cultivars were also investigated. The results showed that the tetraploid cultivars had similar FISH karyotypes, they predominantly formed multivalents, and these were equally separated because their anaphase I, telophase II and their pollen viability are normal, as those of diploid species; apart from minor variations, FISH karyotypes of progenies were similar to each other and to their parents. Based on these results and considering the high crossability among Sinomartagon species and/or Asiatic lilies, we concluded that Sinomartagon species and their resulting cultivars share a common genome, so polyploidy Asiatic lilies are autopolyploid.
Introduction

Asiatic lily, the largest group of modern lily cultivars, is bred by hybridization and/or chromosome doubling from species of section Sinomartagon in *Lilium* L. of Liliaceae (McRae 1998, Van Tuyl et al. 2000). The wild species are diploid (2n = 2x = 24) while their resulting Asiatic lily cultivars are diploid, triploid (2n = 3x =36) and tetraploid (2n = 4x = 48) (Li et al. 2011). In *Lilium*, interspecific hybridizations within Sinomartagon are easy to carry out and the hybrids are usually fertile, in contrast to interspecific hybridizations between different sections, which are difficult and the F1 hybrids are usually sterile (Okazaki et al. 1994, Obata et al. 2000, Van Tuyl et al. 1991, 2011). The former is similar to intraspecific hybridizations and the latter similar to most other interspecific or intergeneric hybridizations, as in *Alstroemeria* (Kamastra et al. 1999) and *Hordeum-Triticum* hybrids (Jiang & Liu 1987). Sinomartagon species, while they have their own morphological or habitual characters and geographical isolation (De Jong 1974), do not appear to have evolved sufficiently to cause their sexual isolation under cultivation. FISH, florescence in situ hybridization, with 45S rDNA probes also indicates that the genomes of the species of Sinomartagon are mainly similar to each other with small variations (Lim et al. 2001, Sultana et al. 2010). These Sinomartagon species appear to share a common genome, but this seems to be debatable. It is known that botanists prefer the ‘morphological’ species concept, in which similar characters and geographical isolation are the main criterion, while zoologists accept more the ‘biological’ species concept which emphasizes interbreeding capability among individuals or populations (Soltis & Soltis 2009). Similarly, some *Lilium* researchers prefer to consider morphological species, coding each species with specific letter(s) for its genome (Lim et al. 2001, Chung et al. 2013, Xi et al. 2014). Others emphasize biological species; when different species
within one section are easily crossable and the F1 hybrids are fertile, the species and their resulting cultivars should share a common genome (Zhou et al. 2011, 2012, 2013, 2014). To confirm the homology of the Asiatic lily genomes, two tetraploid Asiatic cultivars, ‘Tresor’ and ‘Val di Sole’, and their progenies were analyzed using FISH with 45S rDNA as probe on meiotic and mitotic chromosomes, and the genomic differentiation among Asiatic lilies was discussed.

Materials and methods

Plant Materials

Two tetraploid Asiatic lily cultivars, ‘Tresor’ and ‘Val di Sole’, and their six progenies were investigated (Table 1). The two cultivars were bought from Hongyue Company, Zhejiang Province, China. The progenies were from a cross (090023) between ‘Tresor’ as female and ‘Val di Sole’ as male.

Mitotic Chromosome Preparation

Mitotic chromosomes from root tips were prepared on slides, according to Zhou et al. (2008a). Four to six root tips were cut off and incubated in 0.7 mM cycloheximide (Amresco) at room temperature for 4 h, then stored in a fixative, composed of ethanol and acetic acid (3:1 vol:vol). Prior to chromosome preparation, the root tips were incubated in an enzyme mix of 1% (w/v) cellulase RS (Duchefa Biochemie) and 1% (w/v) pectinase Y23 (Duchefa Biochemie), at 37 °C for about 1 h. The meristem of the root tips was mixed with 16 μL 45% acetic acid on a glass slide, covered with a glass cover slip and then squashed. Chromosome preparations were checked with a phase contrast microscope (Olympus BH-2) for FISH. Chromosomes were numbered according to
Lim et al. (2001). As the centromeres are not clear, only chromosomes with rDNA loci were coded (Ren et al. 2012).

Meiotic Chromosome Preparation

Meiotic chromosome slides were prepared according to Zhou et al. (2008b). A one mm anther section at metaphase I was put on a slide and the pollen mother cells (PMCs) were gently mixed, after removing unnecessary debris with fine forceps, with a drop of 45% acetic acid and covered with a cover glass. The slides were examined under a phase microscope (Olympus BH-2).

Fluorescence in situ hybridization (FISH)

The protocol was according to Kuipers et al. (2002) with minor modifications. Clone pTa71 (Gerlach and Bedbrook, 1979), containing 45S rDNA, was labeled with biotin-16-dUTP by nick translation (Roche) as the probe for in situ hybridization. The hybridization mix (40 µL) contained 50% deionized formamide, 10% dextran sulphate (Amresco), 2x SSC (0.3 M NaCl plus 30 mM sodium citrate, pH 7.0), 0.25% SDS, and 25-50 ng probe DNA. Signal was detected with streptavidin-CY3 (Invitrogen) and biotinylated anti-streptavidin (Vector Laboratories). Having been counterstained with DAPI (Roche), the slides were observed under a fluorescence microscope (Olympus BH-41). Images were taken with an attached CCD (Micropublisher 3.3 RTV) driven by Image-Pro® (MediaCybernetics).

Anaphase I and Telophase II

Anthers at anaphase I were sectioned in a drop of 2% acetocarmine (Fluka 22000) on a slide, and the pollen was covered with a cover glass and checked under a microscope. Similarly, PMCs at
telophase II were stained with 1% Carbol-Fuchsin (Fluka 21820) and examined under a microscope.

Pollen viability

Pollen viability was measured using the percentage of stained and germinated pollen. Ripe, unopened anthers were fixed with ethanol and acetic acid (3:1 vol:vol) and the pollen stained with 2% acetocarmine and then checked with a microscope (Olympus BH-41) (Zhou et al. 2011). Only fresh pollen was used for germinating on a medium containing 100 g·L⁻¹ sucrose (Sinopharm Chemical Reagent Co.), 5 g·L⁻¹ bacteriological agar (Sinopharm Chemical Reagent Co.), 20 mg·L⁻¹ boric acid (Shanghai Yunling Refinery) and 200 mg·L⁻¹ Ca(NO₃)₂ (Shanghai Naihui Pengzheng-Yingfang Refinery). Pollen grains were scattered on the medium and cultured at 25 °C overnight, then observed under a trinocular stereo microscope (Oplenic SZM745T).

Results

FISH Karyotypes of ‘Tresor’ and ‘Val di Sole’

As claimed by lily breeders, FISH confirmed that the two parent Asiatic cultivars are tetraploid (2n = 4x = 48) (Fig. 1: a1 and b1). The chromosomes bearing 45S rDNA loci were extracted to form the FISH karyotypes (Fig. 1: a2 and b2). All chromosomes 1, 2, 4 and 7 had similar 45 rDNA loci, suggesting that, among different Asiatic genomes, these chromosomes are very homologous. Meanwhile, FISH signals varied on the homologues of chromosomes 5, 6 and 11. On the sets of chromosome 5 and 6 of ‘Tresor’ or ‘Val di Sole’, only one homologue had rDNA loci, indicating that the two cultivars did not originate directly from chromosome doubling, because it could make
any chromosome occur as a pair. With chromosomes 11, three homologues had the 45S rDNA loci in ‘Tresor’ but two in ‘Val di Sole’. It was concluded that the FISH karyotypes of the two Asiatic cultivars were very similar to each other, with only minor variations.

**Chromosome Association**

The chromosome association was seemingly quite complex, however the associated chromosomes with rDNA loci fit their FISH karyotypes very well. All four homologues of chromosomes 1, 2, 4, 7, and 11 were associated and thus formed multivalents respectively, tight or loose (Fig. 2: a3, a4, b3, and b4). Multivalents were predominant, suggesting that the Asiatic cultivars are autotetraploid.

**Separation of the Multivalents**

It is difficult to directly observe how the multivalents separate and how many chromosomes are distributed at each pole. However, the problem was solved by analyzing the key phases of microsporogenesis, the percentage of pollen germination and the chromosome numbers of the progenies. Figure 2a shows that chromosomes moving to the two poles were balanced due to the similar size of nuclei at anaphase I. At anaphase II, 98% PMCs formed tetrads, which were very similar to those of diploid species (Fig. 2b). Most pollen grains also appeared normal (Fig. 2c) and their germination was up to 80%. These results indicated that the associated chromosomes at metaphase I, whether or not multivalents, usually had a balanced separation and finally produced pollen with balanced chromosomes.
**FISH Karyotypes of Progenies**

The karyotypes of eight progenies analyzed with FISH were similar to their parents with minor variations. Three were tetraploid (2n = 4x = 48) and the other three were aneuploid (2n = 4x - 1 = 47). Clearly, 090023-29 (Fig. 3c) lost one homologue of chromosomes 4, 090023-30 (Fig. 3d) lost one of chromosomes 1, and 090023-31 (Fig. 3e) added one homologue of chromosomes 1 (Figs. 3 and 4). However, it is uncertain which chromosome was lost in 090023-22 and 090023-31. Both chromosome addition and subtraction are caused by unbalanced segregation (Fig. 5). In addition, one homologue of chromosomes 4 in 090023-11 (Fig. 3a) and 090023-33 (Fig. 3f) had two rDNA loci, which was different from their parent. This resulted from translocation between nonhomologous chromosomes (Fig. 4). Both chromosome 5 and 6 with rDNA loci simultaneously occurred in 090023-11 (Fig. 3a), -22 (Fig. 3b), -30 (Fig. 3d), and -31 (Fig. 3e), but only on chromosome 5 in 090023-29 (Fig. 3c) and neither 5 nor 6 in 090023-33 (Fig. 3f). The distribution of rDNA loci on chromosomes 5 or 6 could be explained by tetrasomic inheritance; however, the possibility (4/6) of rDNA loci on chromosome 5 and 6 occurring simultaneously in the progenies is much higher than that theoretically expected (1/4) (Fig. 7). This could indicate that the chromosomes 5 and 6 having rDNA loci is important for survival of the progenies.

**Discussion**

*Genomic Differentiation of Sinomartagon Species and Asiatic Cultivars*

Asiatic lilies originated from hybridization and/or chromosome doubling within Sinomartagon species, e.g., *L. dauricum, L. concolor, L. pumilum, L. cernuum, L. amabile, L. leichtlinii, L. tigrinum, L. lankongense, L. duchartrei, L. bulbiferum, L. davidii* or others (Van Tuyl et al. 2011).
Their relationship has been confirmed by FISH (Sultana *et al.* 2010, Ren *et al.* 2012). As illustrated in Figure 4, most chromosomes bearing 45S rDNA loci of cultivars, 1, 2, 7, and 11, could be traced directly to wild species. The chromosomes 4 of the cultivars could not be found in the species; they should in fact correspond to chromosomes 6 of the species (Sultana *et al.* 2010).

This is possibly caused by the methodology of chromosome nomenclature due to the similarity in chromosomes from 3 to 7 in *Lilium*. Variations were observed mainly on chromosomes 5 and 6. The FISH karyotypes not only show the relationship of Sinomartagon species and Asiatic cultivars, but also indicate that genomic differentiation among the diploid Sinomartagon species or cultivar is very limited. This is confirmed by the chromosome association of tetraploid Asiatic lilies, which is similar to that observed in autotetraploid *Arabidopsis thaliana* (Santos 2003), *Crepis capillaris* (Jones 1994, Jones and Vincent 1994) and cucumber (Chen *et al.* 2010), but different from allotetraploid plants, such as *Alstroemeria*, in which bivalents are predominant (Ramanna *et al.* 2003, Ramanna & Jacobson 2003). Clearly, the reports support Asiatic tetraploid lilies being autotetraploid. This is also supported by hybridizations within Sinomartagon or Asiatic cultivars, which is easy and the hybrids are fertile (Van Tuyl *et al.* 2011), implying that they may be considered a ‘biological’ species complex. So far, GISH (genomic in situ hybridization) has been extensively used to distinguish the parental genomes or intergenomic recombination of hybrids between different sections of *Lilium*, but this does not work with hybrids between different species within Sinomartagon. In addition, rDNA loci of other species or cultivars of Leucolirion or Achelirion are significantly different from those of Sinomartagon (Karlov *et al.* 1999, Marasek *et al.* 2004, Xie S *et al.* 2010). Therefore, it is concluded that the genomic differentiation of Sinomartagon species is not sufficient to cause essential sexual isolation and the polyploid Asiatic
cultivars are autopolyploid.

*Genomic Variations of the Progenies*

Based on the results, as discussed above, we consider both ‘Tresor’ and ‘Val di Sole’ to be autotetraploid. Autotetraploids usually have tetrasomic inheritance when their genic loci segregate during gamete formation (Parisod *et al.* 2010), while multivalents may separate unevenly and form aneuploid gametes (Dagne 2001). The loss of one chromosome of the gametes formed in diploids or allopolyploids usually results in lack of viability or weak progeny, however, the loss is more tolerated or compensated for in autotetraploids (Wei and Zhang 2009). 45S rDNA has been extensively used as a marker for certain chromosomes at metaphase I of meiosis and mitosis in plants, e.g., *Alstroemeria* (Kamstra *et al.* 2004) and *Arabidopsis* (Santos *et al.* 2003). It has been found that rDNA loci are transferred from chromosome 3 to 4 after 20-30 generations of autotetraploid *A. thaliana* (Weiss & Maluszynska 2000). These findings are strongly supported by the present results.

*The Significance of ‘Biological’ Species Concept for Hybridization*

That Sinomartagon species can easily hybridize with each other under cultivation implies that there is little differentiation between their genomes, so they could not be regarded as different biological species, even though they have evolved their own characters due to geographical isolation. The most likely reason for this is that vegetative propagation is more important than sexual propagation for *Lilium* survival. This causes most *Lilium* to be sparsely distributed in their favorable native areas. Some lily researchers have seemingly preferred to regard these as
morphological species possessing different genomes (Lim et al. 2001, Chung et al. 2013, Xi et al. 2014). This can lead to confusion, especially, when discussing the compatibility of lily hybridizations. It is more reasonable for the Sinomartagon species to be regarded as having a common genome despite their different ‘morphological’ traits. For lily hybridization breeding, it is very useful to consider that all Asiatic cultivars and their original Sinomartagon species share a common genome (A), the same as for Longiflorum cultivars and their original species (L), Oriental cultivars and their species (O), Trumpet cultivars and their species (T) (Van Tuyl et al. 2000). Based on this assumption, the success or failure of lily interploid hybridizations can be well explained by the new hypothesis, “Five same genomes of endosperm are essential for its development in lily interploid hybridizations” (Zhou et al. 2012), and the new theory could guide selection of parents to combine different lily genomes (Zhou et al. 2014, Zhou 2014).

**Conclusion**

While Sinomartagon species of *Lilium* have distinct morphological characters and natural geographical isolation, they have similar FISH karyotypes, with minor variations, they can be easily hybridized with each other to produce fertile hybrids under cultivation, and their synthetic tetraploids show chromosomal association similar to other autotetraploid plants. All the results show that genomic differentiation among Sinomartagon species and their resulting cultivars is very limited, so they may share a common genome. This is of great use to facilitate analyzing compatibility of parents in lily breeding.

**Acknowledgements**
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References


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Tables

Table 1. Plant materials

Figure Legends

_Figure 1_ FISH on metaphase chromosomes of root tips and at metaphase I of anthers of ‘Tresor’ and ‘Valid di Sole’. As the rDNA was labeled with biotin and detected with Cy3, the 45S rDNA loci are pink, while the slides were counterstained with DAPI so chromosomes are in blue-purple. Only the chromosomes with rDNA loci were coded according to nomenclature (Lim 2001). ‘Tresor’, a1-4: a1, mitotic chromosomes; a2, FISH karyotype; a3-4, chromosome association at metaphase I. ‘Val di Sole’, b1-4 are as in a1-4. Bar = 100 µm

_Figure 2_ The main phases of microsporogenesis: a) anaphase I, at which the separated chromosomes of multivalents have completely moved to the opposite poles; b) telophase II, at which the second division of meiosis has been completed and tetriads formed; c) Most pollen grains of the tetraploid Asiatic lily ‘Tresor’ appear normal. Bar = 20µm

_Figure 3_ Mitotic chromosomal morphology of six progenies and their FISH karyotypes based on 45S rDNA loci. White arrows indicate that the signals are of noise or there is a chromosome overlapping. a1-2, 090023-11; b1-2, 090023-22; c1-2, 090023-29; d1-2, 090023-30; e1-2, 090023-31; f1-2, 090023-33. Bar = 10 µm.

_Figure 4_ Comparison of FISH karyotypes of Sinomartagon species and Asiatic cultivars. * The
missing chromosome is uncertain.

Figure 5 Ideogram of segregation and consequence of mutivalents. Most mutivalents formed by four homologous chromosomes evenly segregate and move to the opposite poles, and thus the gametes are normal (N). However, a very few tetravalents may unevenly divide and result in one chromosome missing (M) in half of the gametes and one chromosome added (A) in the other half.

Figure 6 A possible form of translocation between nonhomologous chromosomes. As illustrated, a set of four homologous chromosomes (black) and another set of two homologous chromosomes may be associated together. If a crossover occurs between the nonhomologous chromosomes, consequentially, normal (N) gametes and translocation (T) gametes are produced. T* indicates the gametes which fit chromosome 4 of 090023-11 and -33 (Fig. 4a & f, red arrow)

Figure 7 Separation and assortment of one set of chromosomes with only one rDNA homologue.
Figure 1 FISH on metaphase chromosomes of root tips and at metaphase I of anthers of 'Tresor' and 'Valid di Sole'. As the rDNA was labeled with biotin and detected with Cy3, the 45S rDNA loci are pink, while the slides were counterstained with DAPI so chromosomes are in blue-purple. Only the chromosomes with rDNA loci were coded according to nomenclature (Lim 2001). 'Tresor', a1-4: a1, mitotic chromosomes; a2, FISH karyotype; a3-4, chromosome association at metaphase I. 'Val di Sole', b1-4 are as in a1-4. Bar = 100 µm 185x159mm (100 x 100 DPI)
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Figure 3 Mitotic chromosomal morphology of six progenies and their FISH karyotypes based on 45S rDNA loci. White arrows indicate that the signals are of noise or there is a chromosome overlapping. a1-2, 090023-11; b1-2, 090023-22; c1-2, 090023-29; d1-2, 090023-30; e1-2, 090023 – 31; f1-2, 090023-33. Bar = 10 µm.

173x277mm (100 x 100 DPI)
Figure 4 Comparison of FISH karyotypes of Sinomartagon species and Asiatic cultivars. * The missing chromosome is uncertain.
170x284mm (100 x 100 DPI)
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