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DETECTION THE PLOIDY LEVELS IN ASIATIC LILY CROSS-BREEDING THROUGH KARYOTYPE ANALYSIS AND FISH

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Abstract

Conventional hybrid breeding methods were used to hybridize and evaluate the affinity of different ploids lily varieties as parents, and abundant hybrid offspring were obtained by embryo rescue. The karyotype analysis and 45S rDNA-based fluorescence in situ hybridization (FISH) were applied to identify the lily chromosomes. The genetic variation of chromosomes between progenies was analyzed and the 45S rDNA distribution was analyzed. It was found that the parental ploidy had some influence on the hybridization affinity. Diploid and tetraploid interploidy hybridization (Intraploidy hybridization) was stronger than between different ploidy hybridization (Interploidy hybridization). Triploid lily could be used as a successful female with diploid or tetraploid and 3x×4x hybridization was easier to hybridize than 3x×2x. The chromosome ploidy of Asian lily was rich in diploid, triploid, and tetraploid. The 45S rDNA signal loci of lily were usually not in pairs but increased with the ploidy of chromosome. Asian lily varieties had a pair of chromosomes on chromosome 1, which could be used as the characteristics of Asian lilies. The hybrid chromosomes were identified by the hybridization progenies by hybridization with FISH. The chromosomes were identified as true hybrids from all parents, and there were differences between different genotypes of hybrid progeny. Karyotype combined with fluorescence in situ hybridization could trace the origin of characteristic chromosomes in hybrid progeny, and identify hybrid authentically quickly and effectively.

Key words: Asiatic lily; Chromosome ploidy; Karyotype analysis; FISH.

Abbreviations: DAPI – 4',6-diamidino-2-phenylindole, EBN – endosperm balance number, FISH – fluorescence in situ hybridization FISH, LD – 'Loreto'× 'Tresor', GISH – genomic in situ hybridization, GR – 'Gironde' × 'Renoir', GT – 'Gironde'× 'Tresor', rDNA – ribosomal DNA, RG – 'Renoir' × 'Gironde', ND –'Navona' × 'Detroit', NL – 'Gironde' × 'Detroit', NT – 'Navona'× 'Tresor', SSC – salinesodium citrate

Introduction

There are two common features of some economically important ornamental crops, polyploidy and interspecific hybrid origin, which are also found in lily (Hwang *et al.*, 2011). Lilies are one of the major bulbous plants that are grown to produce cut flowers and potted plants. Producing new cultivars that combine desirable horticultural characters of species or cultivars of lilies is an important breeding objective (Akutsu *et al.*, 2007). Lily (Lilium) is rich in color, especially Asiatic lilies, which are important parents for flower color in flower breeding (Hwang *et al.*, 2011). Asiatic lilies are rich in ploidies, including hybrids of diploid, triploid and tetraploid. Hence, using Asiatic lilies to produce new cultivars that combine desirable horticultural characters of species or cultivars is a more important breeding objective (Zhou *et al.*, 2012). Hybridization affinity was directly related to the availability of hybrid progeny, but there were many factors affecting the affinity (Wang *et al.*, 2015). Therefore, understanding the correlation between the ploidy and cross-compatibility to analyse the diversity of offspring chromosome numbers obtained from hybridization of different ploidy lilies and crossbreed with triploid lily as female parent could provide the basis for lily ploidy crossbreeding and lay a theoretical foundation (Younis *et al.*, 2014).

FISH and karyotype analysis are important means to study chromosomes (Figueroa *et al.*, 2010). Karyotype analysis was based on the morphological characteristics of chromosomes, mainly in the number of chromosomes, the relative length and arm ratio (Rong *et al.*, 2011). However, studies have shown that karyotype differences of lily chromosomes were not very obvious, it was difficult to distinguish chromosomes by single karyotype (Younis *et al.*, 2015). It is an amenable molecular cytogenetic approach of FISH to detect the position of specific genes with chromosomal markers (Contreras *et al.*, 2012). Fluorescence in situ hybridization could localize the target gene to somatic metaphase chromosomes, so the application of the combination of fluorescence in situ hybridization and karyotype analysis could be more accurate in identification of chromosome morphology to carryout hybrid identification and study the chromosomal genetic variation, guiding the breeding from the cytogenetic chromosomes level (Gao *et al.*, 2014; Hwang *et al.*, 2014).

As a probe 45S rDNA was usually used in the fluorescence in situ hybridization of lily (Hwang *et al.*, 2011). With hundreds to thousands of tandem repeats, tremendous intra- or interspecies in location, , hybridization signal intensity, and number, 45S ribosomal DNA (rDNA)) gene is a vital chromosomal marker for characterization and chromosome identification (Lim *et al.*, 2001). Marasek *et al.*, (2004) identified hybrids of 'Royal Lace' × 'High Class' by 45S rDNA FISH and Giemsa C-banding. Hwang *et al.* (2015) identified the hybrid offspring of Asian lily 'Petit Brigitte' × Qingdao lily (L. tsingtauense) by 45S rDNA probe. In this study, to compare the chromosomes in lily hybrids, and trace their origin, the structure of these chromosomes is analyzed using FISH and karyotype analysis techniques and the relevance of these structures to the probable origin is discussed.

Materials and Methods

Plant materials: In the current study, Asiatic hybrid cultivars 'Renoir' (2n=2x=24), 'Gironde' (2n=2x=24), 'Navona' (2n=3x=36), 'Detroit' (2n=4x=48), 'Loreto' (2n=4x=48) and 'Tresor' (2n=4x=48) were imported from the Netherlands at the end of the growing season. Bulbs were selected with an even size of ca. 20 g, sanitized and stored in humid standard pot medium at a cooling room with temperature controlled at 4°C for 8 weeks. Then they were planted in a greenhouse, maintained at 22–25/17–20°C day/night temperature, under natural light conditions at the Genetic Improvement Centre of Agricultural and Forest Crops in Ludong Unversity, Yantai, China (116.3° E, 40.0° N) from March 1 to July 15, 2017.

Hybridization pollination, embryo rescue, chromosome preparation and FISH: The female parent was defoliated during bud stage to prevent self-pollination, Pollination was carried out at 8:00 am to 10:00 am after flowering and was bagged at once in order to avert pollen contamination. Then we took embryo rescue after the fruits weaken and become yellow. We washed off the fruits then disinfected in the clean bench with 75% alcohol and 1% NaClO with the seed coat removed and was inoculated into a culture medium in a conventional tissue culture room.

We cut the fresh root tips when 1 ~3 cm long, pretreated them with saturated a-bromonaphtalene solution for 4~6 hrs at room temperature, after which we fixed they in an acetic acid: ethanol (1:3 v/v) solution and kept with 70% ethanol solution at -20°C. Then we washed the fixed root tips thoroughly with distilled water, excised the growing points from the root tips and treated them with enzymes (0.3% pectolyase, 0.3% cytohelicase, 0.3% cellulase) in 150 mM citrate buffer solution at 37°C for 60 min. After that, we transferred them onto a glass slide and squashed in a drop of acetic acid solution (60%) (Peterson *et al.*, 1999), then air-dried them overnight at 37°C and kept at -20°C prior to FISH.

The chromosomal DNA was treated on the slide with an enzyme mixture (2% pectolyase - Sigma, 2% cellulase -Yakult, USA) at 60℃ for 30 min according to the method described by Lim *et al*., (2001) with some modifications. The slides were washed in 2×salinesodium citrate (SSC) three times and then postfixed in paraformaldehyde solution (4%) for 10 min after we pre-treated the slides with RNase A in 2×SSC (100 uL·mL−1, DNase free; Cayman Chemical, Ann Arbor, MI, USA) for 60 min at 37°C, then washed in 2×SSC for 5 min completely, after which we incubated the slide in 0.01M HCl for 2 min. Next, we treated the samples with 100 μg/ml pepsin (Sigma, USA) in 0.01M HCl at 37℃ for 10 min, after which they were washed with distilled water for 2 min and 2× SSC two times each for 5 min. The hybridization mixture contained deionized formamide (50%), dextransulfate (10%), 2× SSC, and 20 μg·mL−1 of probe DNA. The mixture was denatured for 10 min at 70℃. The hybridization mixture was transferred to slides and covered with cover slips. The slides were then denatured for 5 min at 80℃, after which they were incubated in a humid chamber for 1 h at 37℃. After the step of hybridization, we washed each slide once with with 0.1× SSC at 42℃ for 30 min followed by the detection of biotinylated probe by using fluorescein isothiocyanate FITC-conjugated anti-digoxygenin antibody (Roche, Mannheim, Germany) and Cy™3-streptavidin conjugate (Invitrogen, USA) for 60 min at 37℃ each. Then we counterstained the chromosomes with 2μl·mL -1 of 4',6-diamidino-2-phenylindole (DAPI) in Vecta shield (Vecta Laboratories, Miamisburg, OH, USA) and observed under the fluorescence Microscope (Nikon BX 61, Huntington, New York, USA). Next, we took image through a charge coupled device (CCD) and the images processing was done through Genus Image Analysis imaging system (Applied Imaging Corporation, genus version 3.8 program, Santa Clara, CA, USA). Finally, we completed the confirmation of putative homologous chromosomes on the basis of FISH results and morphological characteristics.

Results and Discussion

Karyotype diversity and FISH of lily varieties: As we know, a majority of wild lilies are diploid but the varieties of lily are abundant in chromosome ploidy. Looking at the output from Figs. 1 and 2, we can see that the chromosomes of lily hybrids 'Renoir', and 'Gironde' were 2n = 2x = 24, 'Navona' was 2n = 3x = 36, 'Detroit', 'Loreto' and 'Tresor' were 2n = 4x = 48. The karyotypes of 'Renoir' and 'Gironde' were 3A, and the karyotypes of 'Navona', 'Detroit', 'Tresor' and 'Loreto' were 3B.

The chromosomal karyotype analysis of lily cultivars was similar, but there existed a large difference among the 45S rDNA distribution of different cultivars (Figs. 1 and 2). It was shown that the karyotype formulas of Asian lily cultivars were similar of 2 pairs of centromere chromosomes and 10 pairs of proximal centromeric chromosomes, except that 'Navona' and 'Loreto' which had one pair of terminal centromeric chromosomes, 'Tresor' had 1 pair of centromere chromosomes. The ratio of the longest chromosome to the shortest one ranged in 4.08 ('Tresor') to 4.96 ('Navona'). The asymmetry coefficient of six lily varieties was high, about 80%, and the variation range was from76.75% to 81.61%. Among all lily varieties, 'Navona' was the highest, followed by 'Gironde'、'Loreto'、'Detroit'、'Tresor'、'Renoir'.

From Table 1, we can see there were much different among karyotype characteristics of Asiatic lily cultivars that there were six 45S rDNA signal loci existed in both 'Gironde' and 'Renoir', the triploid lily cultivar 'Navona' had 13 45S rDNA signal sites. In addition, there were 16 45S rDNA signal loci in tetraploid lily cultivars Detroit, Tresor and Loreto, and 45S rDNA signal loci number increased with the ploidy of chromosome. Furthermore, the 45S rDNA signal loci of Asian lily varieties were common on chromosomes 1, 2, 3, 5, 6, 8, 10 and 11, respectively. Further analysis of the results showed that there were a pair of 45S rDNA signals existing on chromosome 1 in all 6 species, one or a pair of 45S rDNA signals on chromosome 2, one or two signals on chromosome 6. It was found that there were 45s rDNA signals on chromosome 3, except for 'Tresor'. Triploid and tetraploid varieties had two or three 45S rDNA signals on chromosome 11.

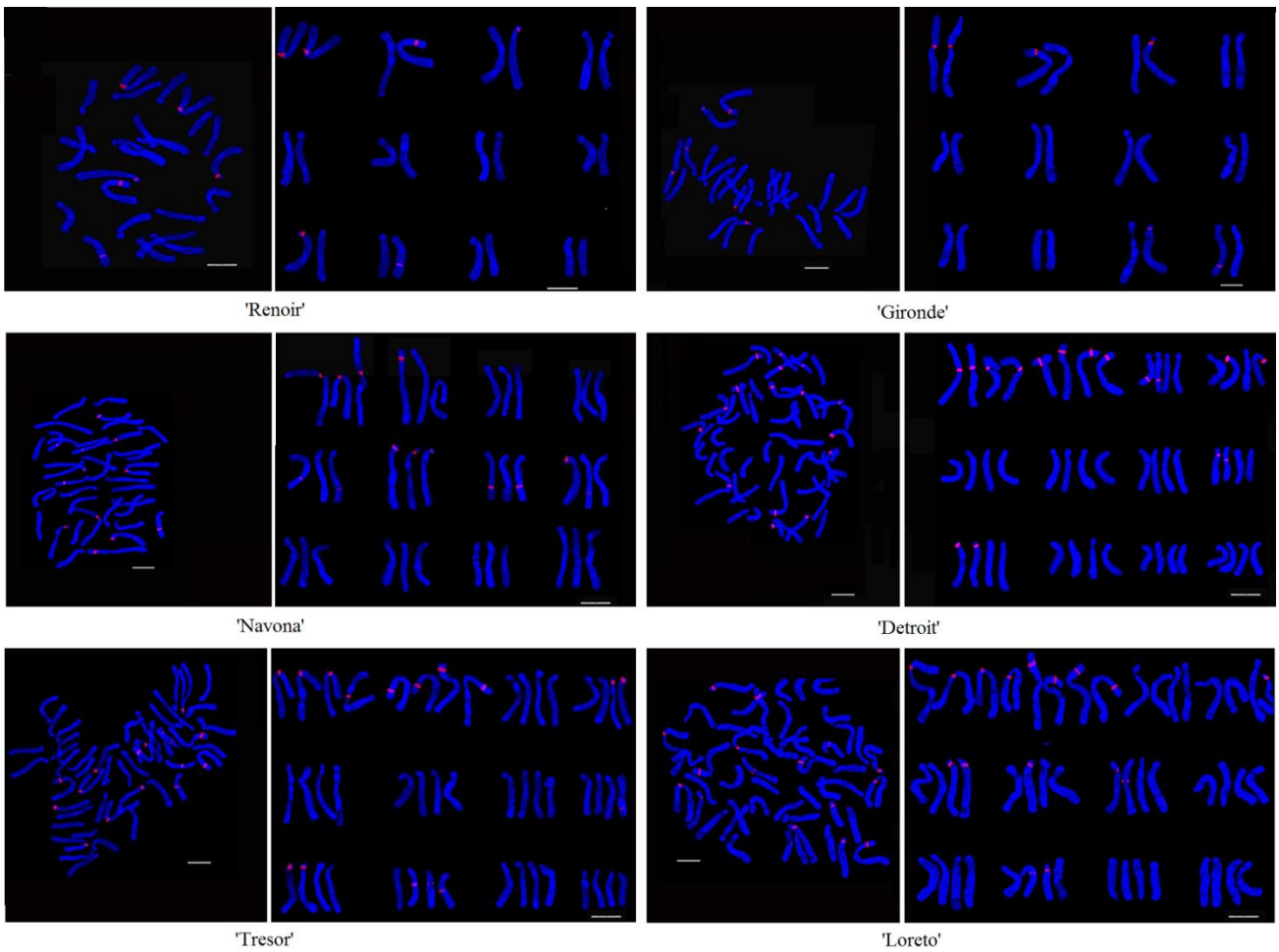

Fig. 1. The FISH result on karyograms and metaphase chromosomes of lily cultivars with 45S rDNA as probe.

Table 1. The karyotype analysis result and morphological data of six Asian lily cultivars.

Cultivars	Karyotype formula	Karyotype type	Arm length of aberrant chr. (um)	As. K/%	45S rDNA	
					Number	No.
'Gironde'	2n=2x=24=4m+20st	3A	4.57	78.35	6	1,2,3,6,8
'Renoir'	2n=2x=24=4m+20st	3A	4.23	76.75	6	1,2,3,6,10
'Navona'	2n=3x=36=3m+3sm+27st+3t	3B	4.96	79.01	13	1,2,3,6,8,10
'Loreto'	2n=4x=48=8m+36st+4t	3B	4.79	78.23	16	1,2,3,5,6,8,11
'Tresor'	2n=4x=48=4m+4sm+40st	3B	4.08	77.43	16	1,2,5,6,10,11
'Detroit'	2n=4x=48=8m+40st	3B	4.34	77.79	16	1,2,3,6,8,11

Hybridization diversity of different lily ploidy parents: 'Gironde' ×'Renoir' and 'Renoir'× 'Gironde' hybrids were both diploid with 2 metacentric chromosomes and 10 subtelocentric chromosomes, both of which were 3A karyotype with the karyotype asymmetry coefficient and the average arm ratio close to parents (Table 2). It was shown that 2x×2x hybrid combinations had higher affinity as well as 4x × 4x hybrid combinations, only one from the four 2x×4 x crosses was obtained and the percentage of seed setting and embryo of 2x×2x cross combinations were higher than that of 2x×4 x cross combinations. It was followed that the 2x×2x hybridization affinity was higher than the 2x×4x hybridization. On the other side, the 2x×3x hybridization had no result proving that the triploid hybrids was cross incompatible as a sire, which was caused by pollen abortion in meiosis of triploid lily. Finally the embryo seeds of 4x×4x cross combinations were not got although the fruit expanded, indicating that as a female parent, the tetraploid had high hybridization affinity only with the same ploidy male parent, and the hybridization was not compatible with diploid parent. The 4x×3x crosses had only one seed but had no embryo seeds inside, which proved that the triploid lily was not suitable as a male parent in hybridization.

The 3x×2x/4x hybrid combination showed high affinity, indicating that the triploid hybrids could be crossed as female parent. 3x×4x hybrid combinations had higher seed setting rate and higher embryo ratio than those of 3x×2x crosses. It was presumed that triploid hybrids as female parents had higher cross-compatibility with tetraploid lilies as male parents than diploid lilies.

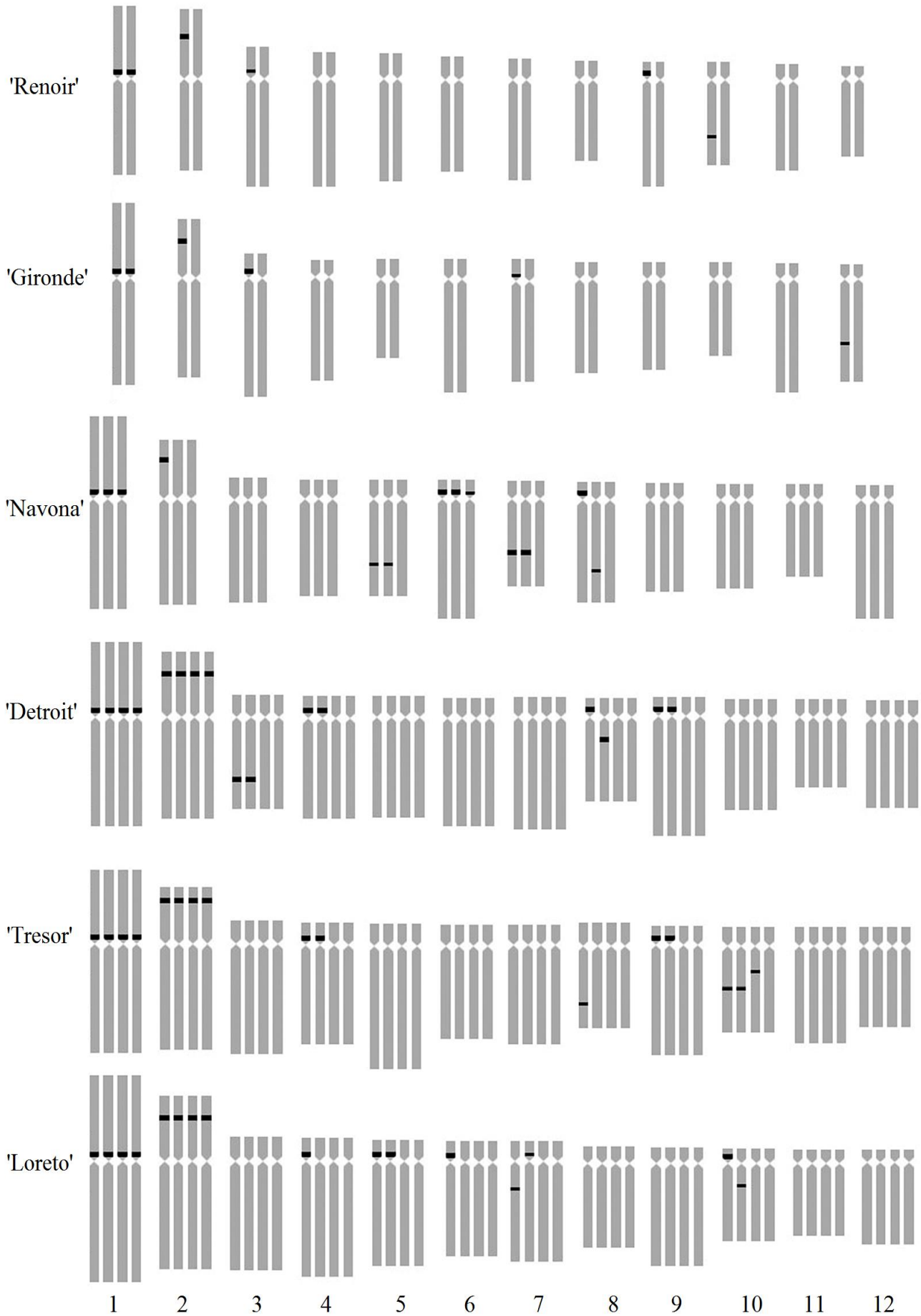

Fig. 2. The result of 45S rDNA distribution on chromosomes of lily cultivars. Bar = 10 um.

Table 2. The cross combination of all parents.

Cross	Mean fruit rate (%)	Mean embryo rate (%)	Cross	Mean fruit rate (%)	Mean embryo rate (%)
2x×2x	80	10	3x×4x	93.3	8.3
2x×3x	0	0	4x×2x	58.3	0
2x×4x	40	1.7	4x×3x	5	0
3x×2x	77.5	1.1	4x×4x	88.3	2.1

According to classical genetic laws, diploids usually produce x gametes, tetraploids usually produce 2x gametes, so the hybrid chromosome ploidy of diploid or tetraploid female parents are stable, the offspring of 2x × 2x hybrid was diploid (2n = 4x = 48) and 4x × 4x hybrids were triploid (2n = 3x = 36) (Fig. 3).

The results of chromosome analysis showed that the hybrids of triploid as female parent were aneuploid and the number of 'Navona' × 'Detroit' was between 38 and 47 with an average of 42.5. the number of 'Navona' × ''Tresor' was between 36 and 45 with an average of 40.2, the number of 'Navona' × 'Loreto' was between 38 and 47 with an average of 42.1 (Table 1 and Fig. 3). As the tetraploid lily provided 2x gametes, we could infer that the gametes of the triploid hybrids 'Navona' were heterozygous when they were crossed as female parent. The average number of chromosomes was 17.6, similar to (x+6). The results showed that the chromosome number varied with different progenies obtained from the triploid female parent. By observing the ploidy, we could only see the change of the chromosomes number between parents and offspring. In order to study the chromosomal behavior between parents and progeny, chromosome structure should be studied more deeply through karyotype analysis and fluorescence in situ hybridization.

The ploidies of lily varieties were more abundant. The hybrids (Asian lily, Oriental lily and Longiflorum hyrids) were mostly diploid, and the inter-group hybrids (LA, LO and OT) were mostly triploid. Dubouzet *et al.* (1999) showed that Asian lily cultivars were mainly diploid, triploid, tetraploid and aneuploid. In our study, two of the six Asian lily cultivars were diploid, one triploid and three tetraploid, which were consistent with the reports. The crosses of different ploidy hybrids were not only ploidy, and aneuploidy. Polyploid plants with many excellent horticultural traits, are an important trend of breeding. The chromosomes of the Lilium species were large, and the karyotypes were relatively stable. They were usually composed of two pairs of large central (m) or near central (sm) centromeric chromosomes and 10 pairs of end (t) or proximal (st). The chromosome karyotypes of six lily hybrids and their hybrids were consistent with this rule. In general, the karyotype of Lilium was a stable type 3B (Nishikawa *et al.*, 1999), such as *Lilium osthornii*, *L. tsingtauense* and *L. regale* (Lee *et al.*, 2011) 3A type was also found in *L. davidii*, L. *leucanthum* and *L. lophophorum* (Hu *et al.*, 2017). In our study, the karyotypes of two lily cultivars were 3A and other four lilies were 3B, and the karyotypes of the crosses were 3A or 3B. The asymmetric coefficient of Lilium L. was about 80%, which was very asymmetric. Wang *et al.* (2015) reported that the asymmetric coefficient of Asian lily varieties was 78.54% ~ 84.05% 77.04% ~ 86.08% of oriental lily varieties. In our study, the asymmetric coefficient of lily variety and hybrid progeny varied were from 76.29% to 81.68%, which was in accordance with the above conclusions. At present, only karyotypic data could not reflect the karyotype characteristics of each cultivar population, and it was difficult to trace the chromosomes between parents and offspring in cross breeding genetic behavior. So the combination of the karyotype analysis and chromosome banding analysis or fluorescence in situ hybridization and other technologies were important to obtain more information on chromosomes, providing more reliable cytogenetic basis for the genetic relationship analysis and identification of hybrid.

Breeding potential of triploid lily and diversity of lily hybrid progeny: At present, autotriploid and heterotriploid triploid had been extensively applied to genetics and cross-breeding of many plants (Ramanna & Jacobsen, 2003). Different from other triploid plants, for instance bananas and seedless watermelons, although the majority of male sterile, triploid lilies could be crossed as a female parent with a suitable male parent, since the embryo sac of lily was a Fritillaria type (Zhou *et al*., 2012). In our study, three crosses were successfully obtained from triploid hybrids. According to Zhou *et al*., (2012), the level of endosperm was the reason for the development of seed or failure, the endosperm ploidy of 3x×2x hybrid group was 7x, 3x×4x was 8x, so, the endoplasmic amphiploid of their endosperm was the key to the survival of the aneuploidy embryos (Zhou *et al.*, 2011). Zhou *et al*., (2012) hypothesized that in the hybridization of the triploid lily as female parent to other ploidy lilies, it was a necessary condition for obtaining hybrid progeny with at least five identical genomes in the endosperm of hybrids, indicating that the success of 2x/4x hybridization had greater significance for lily breeding. As female parent, triploid lily was an ideal material for breeding. As male parent, triploid lily could also obtain hybrid progenies, which could be used for lily breeding in hybrid breeding (Du *et al.*, 2014). 3x×2x hybrids generally produced diploid offspring in other plants, whereas 3x×4x hybrids usually produced tetraploid offspring (Carputo *et al.*, 2005), because the progeny could survive only when the gametes provided by the triploid female were aneuploid (Xie *et al.*, 2010). However, in lilies 3x×2x/4x hybrid progeny were usually aneuploid (Barba-Gonzalez *et al.*, 2005). In our study, triploid maternal hybrids were also aneuploid, which could be explained by the formation of the megasporocyte embryo sac of Fritillaria type. In addition, it was shown that when the triploid hybrids were as female parent, the gametes were usually aneuploidy, approximately x+6 (Zhou *et al.*, 2012). It could obtain a variety of chromosome number of euploid or aneuploid by different ploidy lilies hybridization, producing a wealth of species and could be cut through the scale or tissue culture stabilized, so as to cultivate lily breeding new varieties of resources for breeding new lily varieties.

Fig. 3. The chromosome at metaphase of representative progenies (Bar=10 μm).

It could be seen from Table 3 that for 'Renoir' × 'Gironde' hybridization, the parents and the three cross progenies RG1, RG2, RG3 were all diploid and the karyotype were all 3A. From the karyotype formula, the hybrid progenies and parents both consisted of 10 pairs of proximal centromeric chromosome and 2 pairs of centromeric chromosomes, the karyotype asymmetry coefficient and the average arm ratio were close to parents, and most of which were intermediate.

Relevance between hybridization compatibility and the ploidy of parents: For 'Gironde' × 'Renoir' hybridization, the parents and the two offspring, GR1 and GR2, were all diploid with a karyotype of 3A. From the karyotype formula, the parents and hybrid progenies consisted of 10 pairs of proximal centromere staining and 2 pairs of centromeric chromosomes, the karyotype asymmetry coefficient and the average arm ratio were close to their parents (Fig. 4). For 'Gironde' × 'Tresor' hybridization, the female parent 'Gironde' was diploid, the male parent 'Tresor' was tetraploid, the two crossed offspring GT1 and GT2 were all triploid, and the karyotype of the female parent was 3A, but the karyotypes of the male parent and offspring were all 3B. From the karyotype formula, the hybrid progeny was consistent with the male parents. The asymmetry coefficient and average arm ratio of GT1 were similar to those of female parent, but GT2 was similar to male parent. For 'Navona' × 'Tresor' hybridization, the female parent 'Navona' was triploid and the male parent 'Tresor' was tetraploid. The hybrids NT1, NT2, NT3 were aneuploid or triploid, the number of chromosomes was 36-42. The karyotype of parental and genotypes NT2 and NT3 was 3B and the karyotype of NT1 was 3A. From the karyotype formula, NT1 and NT2 were consistent with the male parent, consisted of a pair of central centromeric dyes, a pair of proximal centromeric chromosomes and 10 pairs of proximal centromeric chromosomes. The asymmetry coefficient was close to that of parents, but the average arm ratio of hybrid progenies was only similar to that of female parent (Table 3 and Fig. 4).

For 'Loreto' × 'Detroit' hybridization, the parents and two offspring LD1, LD2 were all tetraploid, the karyotype were all 3B. From the karyotype formula, LD1 and the female parent had the same karyotype formula with chromosome 9 for the end chromosomes, LD2 and the male parent had two pairs of centromeric chromosomes and 10 pairs of proximal chromosome composition. The asymmetry coefficients were both higher than their parents, but the average arm ratios were different, LD1 between parents, but LD2 was less than parents. For 'Navona' × 'Loreto' hybridization, the female parent 'Navona' was triploid and the male parent 'Loreto' was tetraploid. Four hybrid offspring NL1, NL2, NL3 and NL4 were all aneuploid, and the number of chromosomes was 42-47. The karyotype of parents and offspring were all 3B. The karyotype type, asymmetry coefficient and average arm ratio of hybrid progenies were similar to parents with no significant difference. For 'Navona' × 'Detroit' hybridization, the female parent 'Navona' was triploid and the male parent 'Detroit' was tetraploid, four hybrid offspring ND1, ND2, ND3 and ND4 were all aneuploid, the number of chromosomes was 37-47. The ND1karyotype was 3A but the karyotype of parents and genotypes of the other three filial generations were 3B, and. From the karyotype formula, the chromosome 4 of genotype ND3 and female 'Navona' were end centromere, genotype ND1 and ND4 were consistent to the male 'Detroit', composed of two pairs of central filaments chromosome and 10 pairs of proximal centromeric chromosomes. Chromosome 2 of genotype ND2 was near the centromere chromosome, consistent to the female parent 'Navona'. The asymmetry coefficient and average arm ratio of hybrid progeny were similar to that of parent, but no significant difference was found (Table 3 and Fig. 5).

The results of FISH **(**Figs. 4, 5 and 6) showed that 45S rDNA loci numbers of RG1, RG2 and RG3 genotypes were 7, 6 and 5, respectively, GT1 and GT2 genotypes were 12 and 13, respectively, LD1 and LD2 were 14 and 15, separately. Although the number of chromosomes of genotype NL2 and genotype NL4 were the same, the numbers of 45S rDNA were 14 and 16, respectively, NT1, NT2 and NT3 were 11, 12 and 13, separately. Each hybrid progeny as true hybrids could be detected chromosomes from both parents.

Table 3. The FISH comparison and chromosome karyotypes of filial generations.

Genotype	Karyotype formulate	Average arm ratio	Karyotype type	As.K/%	45S rDNA		
					Number	From male	From female
RG1	2n=2x=24=4m+20st	4.96	3A	79.01	7	1,2,3,8	1,2,6
RG2	2n=2x=24=4m+20st	4.34	3A	77.79	6	1,2,3,8	1,3
RG3	2n=2x=24=4m+20st	4.08	3A	77.43	5	1,2	1,6,10
GR1	2n=2x=24=4m+20st	4.56	3A	77.89	6	1,3,6,10	1,2
GR2	2n=2x=24=4m+20st	4.24	3A	76.80	6	1,3,10	1,2,3
GT1	2n=3x=36=3m+3sm+30st	4.51	3B	78.19	12	1,3,5,11	1,2,6,8
GT2	2n=3x=36=3m+3sm+30st	4.07	3B	76.29	13	1,3,5,11	1,2,3,8
NT1	2n=3x=36=3m+3sm+30st	4.92	3A	79.54	11	1,2,5,10,11	1,3,10
NT2	2n=3x+4=40=3m+4sm+33st	4.92	3B	76.69	12	1,2,5,10,11	1,3,6,8
NT3	2n=3x+6=42=7m+35st	5.26	3B	79.37	13	1,2,5,10,11	1,3,6,8
LD1	2n=4x=48=8m+36st+4t	4.51	3B	79.44	14	1,2,3	1,2,5,8,11
LD2	2n=4x=48=8m+40st	4.07	3B	78.90	15	1,2,3	1,2,3,5,11
NL1	2n=4x-1=47=4m+4sm+31st+4t	4.83	3B	79.11	16	1,2,3,6,11	1,3,6,8,10
NL2	2n=4x-6=42=8m+34st	4.43	3B	77.46	14	1,2,6,11	1,3,6,8,10
NL3	2n=4x-4=44=3m+4sm+37st	4.26	3B	77.65	13	1,2,3,5,11	1,2,3,6,10
NL4	2n=4x-6=42=8m+34st	4.78	3B	78.66	16	1,2,5,6,8,11	1,2,3,6,10
ND1	2n=3x+2=38=6m+32st	4.80	3A	78.56	12	1,2,3,6,8	1,3,6,10
ND2	2n=3x+4=40=4m+3sm+33st	4.80	3B	79.24	15	1,2,3,6	1,2,3,8,10
ND3	2n=3x+1=37=6m+28st+3t	4.87	3B	78.75	13	1,2,3,6,11	1,2,3,6,10
ND4	2n=4x-1=47=7m+40st	4.55	3B	78.34	15	1,2,6	1,2,3,6,8,10

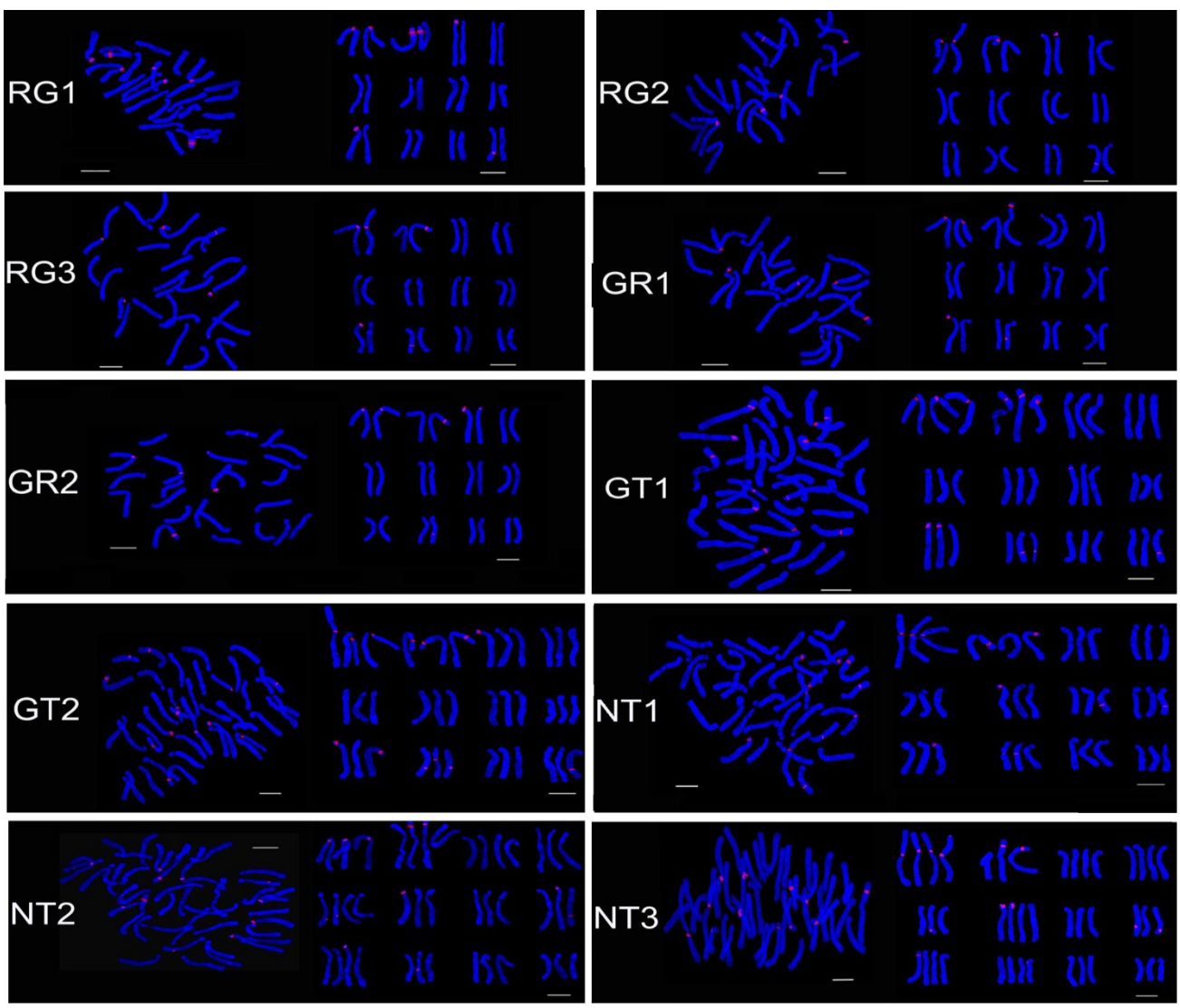

Fig. 4. FISH with 45S rDNA as probe on metaphase chromosomes and karyograms of hybrid progenies. RG1, RG2, RG3: Hybrid progenies of 'Renoir' ×'Gironde'; GR1, GR2: Hybrid progenies of 'Gironde'× 'Renoir'; GT1, GT2: Hybrid progenies of 'Gironde' × 'Tresor'; NT1, NT2, NT3: Hybrid progenies of 'Navona' × 'Tresor'. (Bar=10μm).

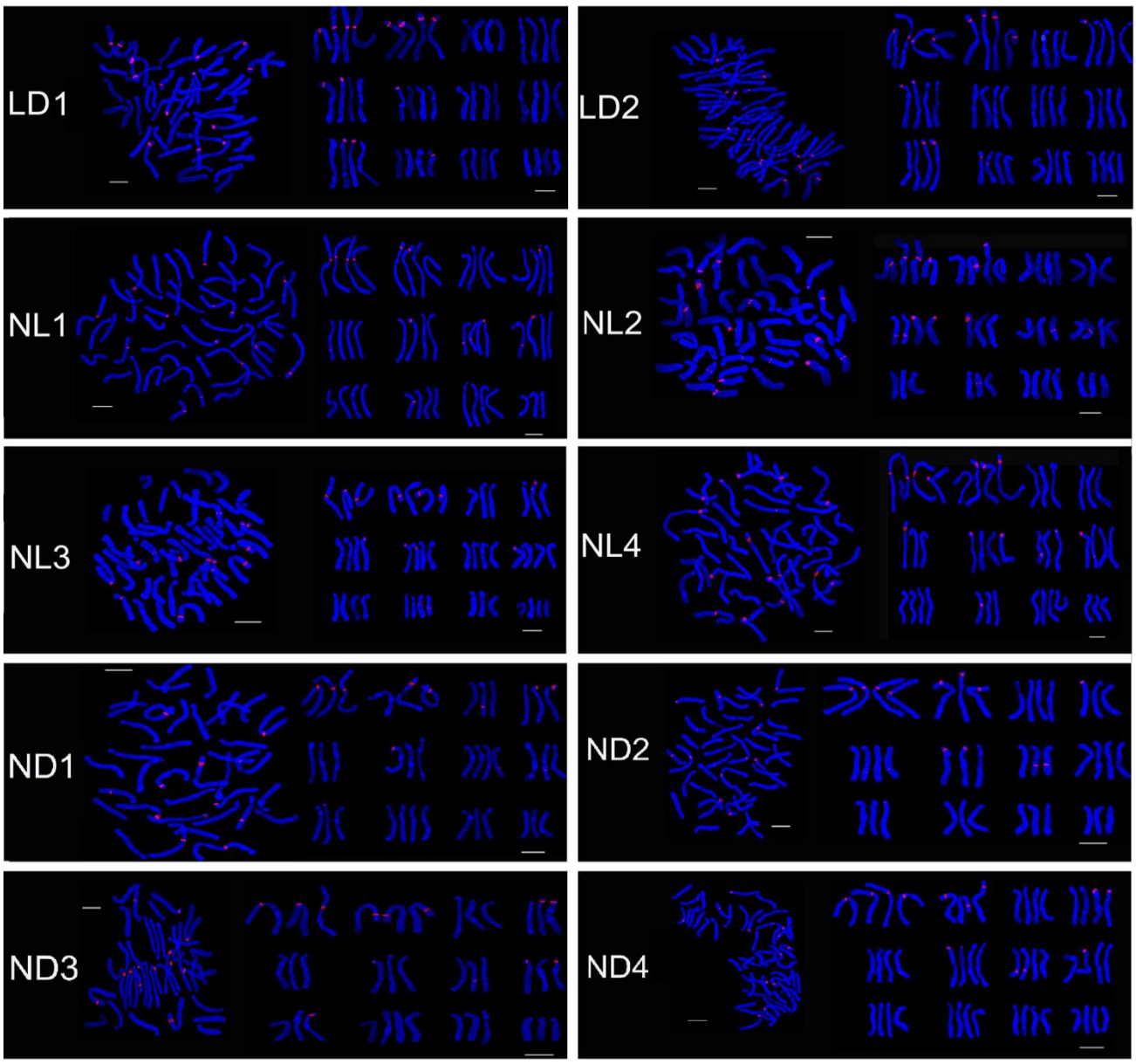

Fig. 5. FISH with 45S rDNA as probe on metaphase chromosomes and karyograms of hybrid progenies (LD1, LD2; NL1, NL2, NL3, NL4; ND1, ND2, ND3, ND4) LD1, LD2: Hybrid progenies of 'Loreto' × 'Detroit'; NL1, NL2, NL3, NL4: Hybrid progenies of 'Navona' × 'Loreto'; ND1, ND2, ND3, ND4 are the progenies of 'Navona' × 'Detroit'. (Bar=10μm).

Hybridization incompatibility was due to the large difference of amphoteric components between parents. The chromosome number of parents had a great influence on the hybridization affinity. It is shown that the hybridization was easier when the parental ploidy was same (Azadi *et al.*, 2010). Wiejacha *et al*., (2001) proposed a hypothesis EBN (endosperm balance number) that the endosperm could develop normally only when the ratio of parental gene of hybrid endosperm was 1: 2, which could explain the success of interspecific hybridization and ploidy interspecific hybrid (Carputo & Barone, 2005). In the 2x×4x and 4x×2x hybrid progeny, the rate of the proportion of parents in the endosperm was 1: 1 and 1: 4, respectively, the development of endosperm was not complete, so it was difficult to produce seeds leading to a low seed setting rate. Yamagishi *et al*., (2012) found that the hybrids of oriental hybrid lily had lower cross-compatibility, and tetraploid hybrids had higher cross-compatibility than diploid hybrids in the hybridization combinations. In our study, the cross-compatibility between diploid and tetraploid was poor (Table 3), only two group was obtained triploid hybrid progeny (GT1 and GT2), consistent with previous studies. The cross-compatibility of grape varieties with different ploidy hybridization was low and diploid female parent had higher affinity than tetraploid female (Mizuochi *et al.*, 2007). Studies had shown that triploid hybrids could be used as mothers to produce aneuploid progenies during hybridization, providing abundant breeding opportunities, and it was easier for 3x×4x hybridization than 3x×2x hybridization (Zhou *et al.*, 2011, 2012), which was consistent with our results (Table 3). This might be due to the extra chromosome number provided by the male parent (Köhler *et al.*, 2010). There was a relationship between ploidy and cross-compatibility of lily varieties, and it was clear that parental ploidy had positive directive significance for lily breeding.

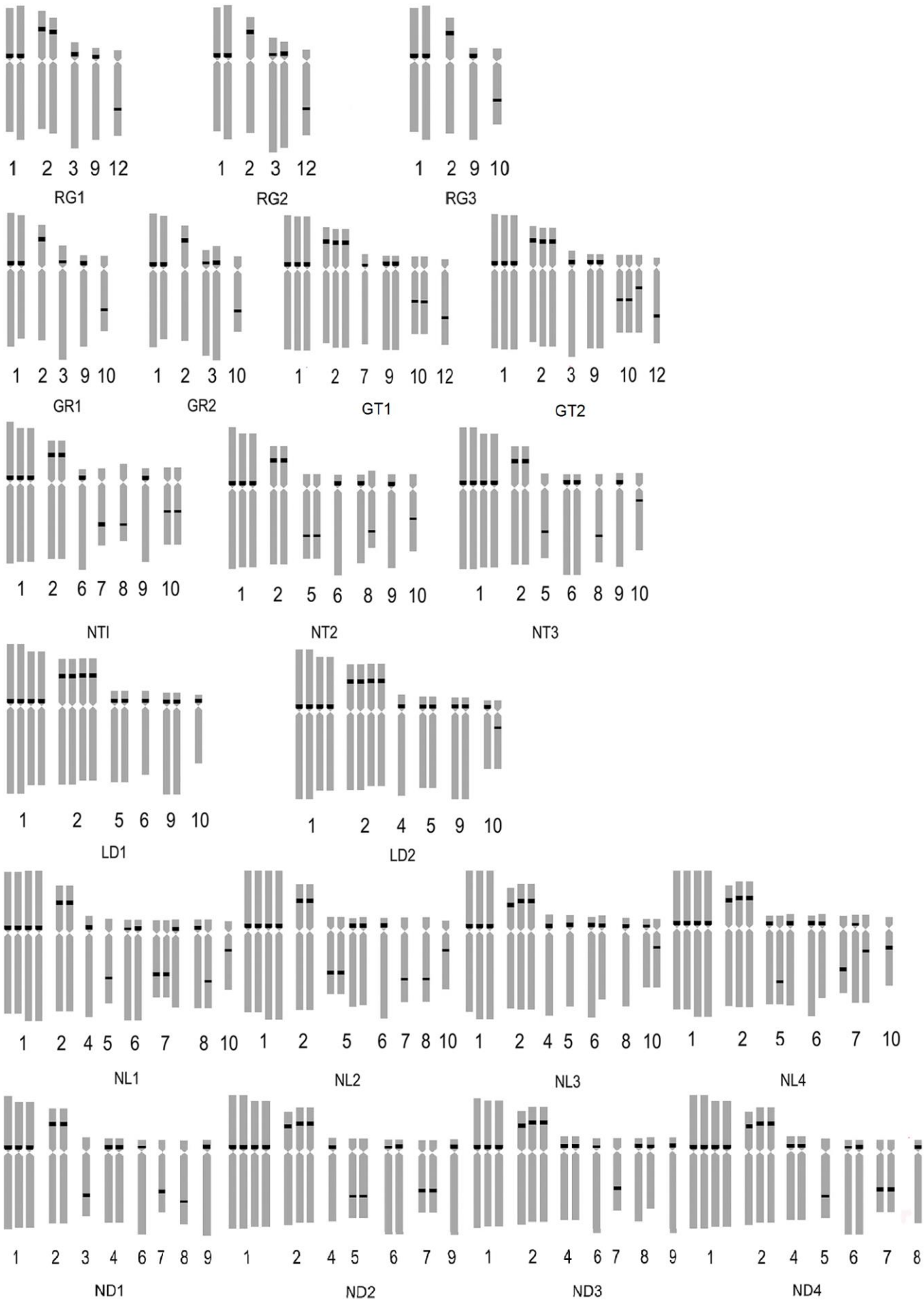

Fig. 6. Idiogram analysis of all lily progenies (The black parts indicate the sites of 45S rDNA).

45S rDNA fluorescence in FISH and the method of lily hybrid identification: The Asian lily varieties loci on chromosome 1 and their hybrid offspring were the same, which indicated that it was very stabilized which could serve as a characteristic for Asian lily. There was a pair of signals on chromosome 2 for 'Tresor', 'Detroit' and 'Loreto', suggesting that their parents all had a pair of loci on chromosome 2. There was only one signal on chromosome 2 for 'Navona', 'Gironde' and 'Renoir', suggesting that the parents were a chromosome 2 with a species on the site and a species without a site. The distributions of 45S rDNA loci on L chromosomes 3 to 12 were not very obvious, and different genotypes had signal distribution on different chromosomes. Fluorescence localization of 45S rDNA in other lily species had also been reported. For example, *L. brownii* had three pairs of signals, located on chromosomes 3, 6 and 9 (Yin *et al.*, 2013); the *L. tsingtauense* of the Martagon vole group had four pairs of signals located on chromosomes 3, 4, 5, and 10 (Lee *et al.*, 2014); The L. longiflorum had three pairs of signals located on chromosomes 3, 5, and 7 (Hu *et al.*, 2017);. *L. pardalinum* had three pairs of signals, located on chromosomes 4, 5 and 10 (Hu *et al.*, 2017); *L. rubellum* had two signals on chromosome 2 (Lim *et al.*, 2001). The distribution and number of signal loci in various Lilium species showed their karyotype differences, which provided some biological basis for the genetic relationship. The methods of lily hybrid identification were mainly included morphological identification, cytological methods and molecular markers. Morphological identification was an intuitive method, but Lilium cultivation had a lasting breeding cycle with at least 2 to 3 years for hybrid plants from seeding to blooming. Cytology and molecular markers were used for early hybrid identification, shorten the breeding cycle. GISH genomic in situ hybridization could be used to identify the hybrids between groups, and also to analyze the recombination of chromosomes between genomes, but FISH only needed the distribution of rDNA on the parents. Therefore, the combination of conventional karyotype analysis and FISH fluorescence in situ hybridization could identify the hybrids authentically by judging whether they had typical parental chromosomes, and could also track the characteristic chromosomes with the signal loci in the hybrid.

Conclusions

We used conventional hybrid breeding methods to hybridize and evaluate the affinity of different ploidy lily varieties as parents, and abundant hybrid offspring were obtained by embryo rescue. The karyotype analysis and 45S rDNA-based FISH were applied to identify the lily chromosomes. The genetic variation of chromosomes between progeny progenies was analyzed and the 45S rDNA distribution was analyzed. It was found that the parental ploidy had some influence on the hybridization affinity. Diploid and tetraploid interploidy hybridization (Intraploidy hybridization) was stronger than between different ploidy hybridization (Interploidy hybridization) affinity, triploid lily could be used as a female successfully with diploid or tetraploid and 3x×4x hybridization was easier to hybridize than 3x×2x. The chromosome ploidy of Asian lily was rich in diploid, triploid, and tetraploid. The 45S rDNA signal loci of lily were usually not in pairs but increased with the ploidy of chromosome. The hybrid chromosomes were identified by the hybridization progenies by hybridization with FISH. The chromosomes were identified as true hybrids from all parents, and there were differences between different genotypes of hybrid progeny. Karyotype combined with FISH could trace the origin of characteristic chromosomes in hybrid progeny, and identify hybrid authentically quickly and effectively

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