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Full Length Article

Hybridization and Identification of Asiatic Lily Hybrids using Fluorescence *In Situ* Hybridization

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Abstract

In this study, hybridization between lily (*Lilium* sp.) Asiatica Hybrida 'Carolina' and 'Melanie' was conducted with cut style pollination and embryo rescue. Progenies were obtained, and the parents and 2 progenies were analyzed using fluorescence *in situ* hybridization (FISH) with 45S rDNA as probe. The results showed that all of them are tetraploid (4n=4x=48); 'Carolina' has 17 signals of 45 S rDNA distributed in chromosome 1, 2, 4, 5, 7 and 11; 'Melanie' has 21 signals of 45S rDNA distributed in chromosome 1, 2, 4, 5, 6, 7 and 11; two progenies have 18 and 19 loci of 45S rDNA, respectively coming from 'Carolina' and 'Melanie'. This result revealed that the generation is true progenies and FISH could accurately identify the authenticity of lily hybrids. Meanwhile, the chromosome slides used for FISH and the distribution of 45S rDNA loci among different Asiatic hybrids have been discussed in this paper. © 2017 Friends Science Publishers

Keywords: Lily; Hybridization; Hybrids identification; 45S rDNA; Fish

Introduction

Asiatic lily is the biggest cultivar groups of lily varieties belongs to the family Liliaceae (Ren *et al.*, 2012; Hwang *et al.*, 2015). Royal Horticulture Society based on lily varietal character put the lily varieties into nine hybrid systems, mainly contained Asiatic hybrids, *Martagon* hybrids, *Candidum* hybrids, Oriental hybrids, *Longiflorum* hybrids, American hybrids, Trumpet hybrids, Miscellaneous hybrids, and lily species (Comber, 1949). Asiatic hybrids have some excellent traits as large, bright-colored flowers, highly ornamental aesthetic values and pleasant fragrance. It also possesses high application values and economical values. China is the origin center of lily with half species of the world and almost 50 endemic lily species (Zhao *et al.*, 2000). Lily breeding is very late and with rarely independent of intellectual property rights compared with western country. Therefore, integrating the good traits of the parents and developing some new commercial varieties is an urgent task of lily breeding.

In the crossbreeding process, not all hybrids obtained are true hybrid off springs. The identification methods of *Lilium* hybrids include morphology, cytology and molecular markers (Hu *et al.*, 2009). Comparison of parent traits and hybrids traits is the quite direct method but it takes too much time. Thus, a quick way to identify hybrids is useful and helpful before the hybrid progenies reach the flowering period. The hybrid progenies obtained by embryo rescue or seed collection were given in early hybrid identification. This includes accurate and rapid identification of the authenticity of F1 generation (Zhou *et al.*, 2011).

Identification of *Lilium* hybrids is a basic work in lily breeding program, and it can improve breeding efficiency. There are several methods of hybrid identification such as Fluorescence *In situ* Hybridization (FISH), Genome *In situ* Hybridization (GISH), random amplified polymorphic DNA (RAPD) and Giemsa C-band (Akio *et al.*, 2005). Rayburn and Gill (1985) first used chromosome *in situ* hybridization labeled with biotin (biotin-dUTP) as a probe, and made a great breakthrough in probe labeling method. Using fluorescent substances like digoxigenin or biotin as a probe, *in situ* hybridization was carried out in root tip sections or some other plant tissue slices. Then determining the position of probe in chromosomes, which was observed by fluorescence microscopy before the antibodies combine with fluorescein reaction with probe marker. This technology has many good characteristics of safety, high sensitivity, and hybridization within a short time. FISH technology is an effective method to determine the distribution of rDNA in the genome. And It also has a wide range of applications. FISH and RAPD were used to analyze the evolution of *V. ramuliflora* at the diploid and tetraploid levels (Han *et al.*, 2017). At present, rDNA has been successfully mapped to many animals and plant

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species. It is also applied in various fields of genome researches such as chromosome structure analysis and quantitative trait loci (QTL) studies (He *et al.*, 2014).

In recent years, FISH is widely used in the classification and identification of lily. Zhou *et al.* (2008) used FISH and found that 45S rDNA was localized near the centromere region on the chromosomes of *Lilium longiflorum*, *L. leichtlinii*, *L. auratum* and *L. pardalinum* as probe. Liu *et al.* (2008) analyzed the root tip chromosomes of Minjianglily using Giemsa C-banding and FISH. In the meantime, GISH technology also played an important role in the field of cytogenetics. The composition of polyploidy genes formed natural or artificial cultivation could be detected by GISH. Lim *et al.* (2000) confirmed the generation is true progenies of 'L. *longiflorum* Thumb' and '*L. rubellum* Baker' by using GISH. Marasek *et al.* (2004) analyzed '*L. henryi*', 'Marco Polo' and 'Expression' combination of two technologies FISH and GISH with 5S, 25S rDNA as probe. GISH can hybridize with the whole chromosome and it could be observed at any time during the division of the cell.

The Chinese market, as well as the scientific application of lily species are mainly imported due to the complex genetic background of lily species (Wang *et al.*, 2012). When used as male or female parent in cross-breeding, FISH is more direct and convenient to identify the authenticity of lily hybrids and it will not affect the seedling growth. In this study FISH was adopted to identify Asiatic hybrids female parent 'Carolina', male parent 'Melanie' and their hybrid offspring. The distribution of 45S rDNA in parents and hybrid progeny chromosomes was analyzed.

Materials and Methods

Plant Materials

Asiatic hybrids 'Carolina', 'Melanie' and their hybrid offspring growing in the nursery of science Park, Beijing University of Agriculture. The 45 S rDNA is provided by ZHOU, Zhejiang University.

Embryo Rescue

Firstly, female parent 'Carolina' stamen was removed before the maturation of anther. Ensure that there is no self-pollination or other pollen polluted. Pollinating 'Carolina' with 'Melanie' pollen and wrapping female parent's stigma with foil. Then hung up signs recording the pollination combination number and date. Finally, Collecting the inflationary fruits after about 60 days.

The fruit of hybrid progenies about 60 days of age were put in clean bench after pollination, washed by clean water and soaked in 75% ethanol for 1 min and sterilization with ultraviolet lamp for 30 min. Then it was cut by blade after burning in the sprit lamp. Putting the white translucent embryo, which could be clearly observed directly into the embryo culture medium for 6~8 weeks. After emergence of seedlings, rooting culture in tissue was made. Rooting at 25℃ and 2000 lux W/m^2 for few weeks until the root is enough sturdy to be carried through FISH. Embryo culture medium: half-strength MS culture medium, 60 g/L sucrose, 4.8 g/L agar. Rooting culture medium: half-strength MS culture medium, 0.2 mg/L NAA, 60 g/L sucrose, 5 g/L agar.

Chromosome Preparation

Root tips (cut about 1 cm) were collected in the morning about ten o'clock, prepared in a saturated cycloheximide solution, and kept in this solution for 6h at 4℃. The material was then rinsed in tap water before being fixed in acetic acid-ethanol (1:3) for 12 h at 4℃. Root tips were stored at -20℃ in 70% ethanol until used. The materials were rinsed thoroughly before incubation in a pectolytic enzyme mixture (1% pectolyase Y23, 1% celluase RS) for 1 h at 37℃. Squash preparations were made in a drop of 45% acetic acid. Then root tips were broken with tweezers. The microscopic slides were frozen in liquid nitrogen and the cover slips removed with a razor blade quickly. Slides were then finally dehydrated in absolute ethanol for 2 min, air-dried, and stored at -20℃ in a freezer until used.

Probe DNA

Clone pTa71 contains a 9 kb Eco RI fragment of the 45S rDNA from wheat as probe (Gerlach and Bedbrook, 1979). Isolated DNA of 45S rDNA sequences from pTa71 and pScT7 were labelled with digoxigenin-11-Dutp by nick translation for *in situ* hybridization using DIG-Nick Translation Mix (Roche 11745816910). 16 µL DDW and 1 µg 45Sr DNA were prepared into a centrifuge tube. Then added 4 µL DIG-Nick translation Mix and shake it with no light before incubation in this mixture for 90 min at 15℃. Lastly, added 1 µL 0.5 M EDTA (pH 8.0) for 10 min at 65℃ and stored at -20℃ in a freezer until used.

Fish

The microscope slides were left overnight at 37℃ before treatment with 100 µg/mL RNase (each slide drops about 100 µL) parafilm sealing at 37℃ for 1 h. The slides were washed three times in 2×SSC at 20℃ for 5 min, treated with 100 µL pepsin (5µg/mL) at 37℃ for 10 min. Each slide washed 2 times in 2×SSC for 5 min, treated with 4% (W/V) paraformaldehyde parafilm sealing at 37℃ for 10 min, washed another three times in 2×SSC for 5 min, dehydrated through a graded ethanol series (70%, 90% and 100% for 3 min). Finally, the microscope slides were air-dried for 30 min until used.

The composition of hybridization mixture (40 µL) includes 50% deionized formamide, 10% (W/V) sodium dextran sulphate (sigma), 20×SSC, 0.25% (W/V) SDS, and 1.25~2.5 ng/µg probe DNA with foil packs. The samples

were denatured for10 min at 70℃ in water bath and put rapidly on ice at least for 5 min. Each slide with 40 μL of the hybridization mixture was covered with a slip of plastic sheet, denatured 5 min at 83℃, and left overnight(12~14 h) at 37℃ in a closed humidified container without light.

When the hybridization was accomplished, slides were washed in 20×SSC 3 times for 15 min, transferred to 0.1×SSC at 42℃ 3 times for 30 min, 2×SSC at indoor temperature 2 times for 5 min, each slide drops 200 μL blocking reagent with parafilm sealing at 37℃ for 5 min and incubated for 1 h at 37℃ in blocking buffer 100 μL (0.1M maleic acid, 0.15M NaCl, 1% (w/v) blocking reagent) including 200 ng anti-DIG-FITC. Slides with PBS buffer solution were washed 3 times at 37℃ for 5 min. Then each slide was counterstained with 5 mg/mL DAPI for 5 min. At last, root tips slides were observed by an Olympus-B41 fluorescence microscope equipped with epifluorescence illumination (digoxigenin labeled probe DNA was detected by FITC). Images were captured and photographed by Spot Cooled CCD. Use Photoshop software for image processing.

Results

Hybridization between Asiatic Hybrids

In this experiment, we conducted a total of 11 combinations of hybrid experiments between Asiatic hybrids. Hybrid embryos were obtained from the fruit in 10 combinations. Only one combination did not get the hybrid embryo. Eight combinations of tetraploid and tetraploid (4x×4x) hybridization, and the seed setting percentage was high and a large amount of embryo seeds were obtained. Three combinations of tetraploid and diploid (4x×2x) or diploid and tetraploid (2x×4x) hybridization, but the seed setting rate was low with less embryo seeds. The results are shown in (Table 1).

Identification of Hybrid Progeny

Asiatic hybrids female parent 'Carolina', male parent 'Melanie' and their hybrid progenies were analyzed by using FISH with 45 S rDNA as probe. 'Carolina' 'Melanie' and their hybrid progenies are all tetraploid. (4n=4x=48) as shown in Fig. 1.

Female parent 'Carolina' has 17 signals of 45 S rDNA distributed in chromosome 1, 2, 4, 5, 7 and 11, respectively located near the centromere in chromosome 1, 5, and 7, at the end of short arm of chromosome 2, at the middle of long arm of chromosome 11, and at the end of long arm of chromosome 4 (Fig. 2a).

Male parent of 'Melanie' has 21 signals of 45S rDNA distributed in chromosome 1, 2, 4, 5, 6, 7 and 11, respectively located near the centromere in chromosome 1, 5, and 7, at the end of short arm of chromosome 2, at the middle of long arm of chromosome 11, and at the end of long arm of chromosome 4 and 6 (Fig. 2b).

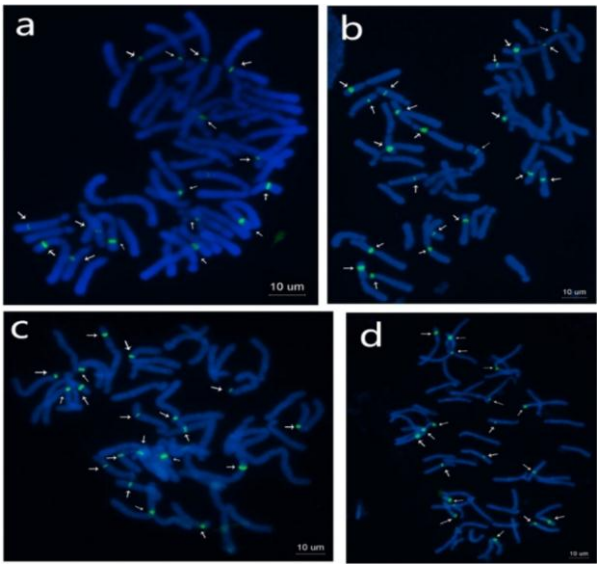

Fig. 1: The results of FISH on mitotic metaphase chromosomes using 45S rDNA as probe. (a) *'Carolina'*; (b) *'Melanie'*; (c) *Progenies of 'Carolina'× 'Melanie' 15-189-43*; (d) *Progenies of 'Carolina'× 'Melanie' 15-189-45*. Bar=10 μm

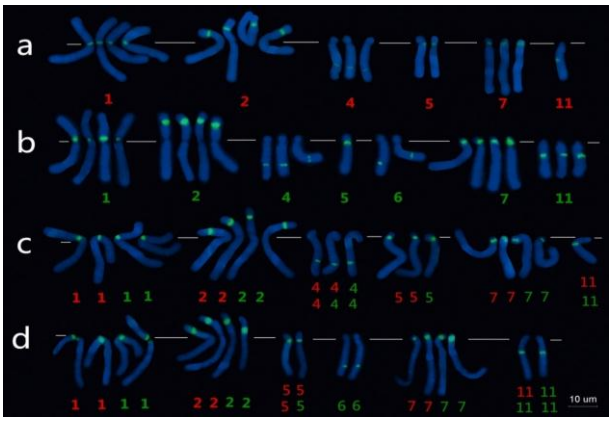

Fig. 2: The metaphase chromosomes aligning the chromosomes with 45S rDNA loci. (a) *'Carolina'*; (b) *'Melanie'*; (c)*Progenies of 'Carolina'× 'Melanie' 15-189-43*; (d) *Progenies of 'Carolina'× 'Melanie' 15-189-45*. Bar=10 μm

Hybrid progeny (15-189-43) has 19 signals of 45S rDNA distributed in chromosome 1, 2, 4, 5, 7 and 11. Hybrid progeny (15-189-45) has 18 signals of 45S rDNA distributed in chromosome 1, 2, 5, 6, 7 and 11 (Fig. 2). Based on the hybridization site of parents, it can be judged that hybrid progeny 15-189-43 has 11 chromosomes (signals distributed in chromosome 1, 2, 4, 5, 7 and 11) originated from 'Carolina' labeled with red digital, another 8 chromosomes (signals distributed in chromosome 1, 2, 5 and 7) originated from 'Melanie' labeled with green digital (Fig. 2c).

Table 1: Hybridization result in Asiatic hybrids successful varieties

Chromosome ploidy	Male parent	Female parent	Number of pollinated flowers	Number of fruit	Setting percentage	Number of embryo
4x×4x	Carolina	Easy Dance	63	59	94	2425
4x×4x	Red Life	Easy Dance	53	53	100	2126
4x×4x	Red Life	Melanie	40	30	75	752
4x×4x	Red Life	Carolina	5	5	100	32
4x×4x	Melanie	Carolina	50	31	62	965
4x×4x	Pearl Justien	Carolina	33	30	91	1105
4x×4x	Pearl Jennifei	Carolina	33	23	70	923
4x×4x	Red Life	Pearl Justien	2	2	100	54
4x×2x	Purple Eye	Pearl Jennifei	23	2	9	9
4x×2x	Purple Eye	Pearl Justien	21	7	33	0
2x×4x	Pearl Justien	Purple Eye	10	5	50	2

Hybrid progeny 15-189-45 has 8 chromosomes (signals distributed in chromosome 1, 2, 5, 7 and 11) originated from 'Carolina' labeled with red digital, another 10 chromosomes (signals distributed in chromosome 1, 2, 5, 6, 7 and 11) originated from 'Melanie' labeled with green digital (Fig. 2d). All these findings clearly confirmed that F1 generation (15-189-45, 15-189-43) is true progeny of 'Carolina' and 'Melanie'.

Discussion

The 45S rDNA is highly tandem repeat sequences located in nucleolus organizer region (NOR) small constriction (Long and Daw, 1980). Fig. 2 shows that 45S rDNA fluorescence loci were mostly near the centromere in chromosome and few at the end of short or long arm of chromosome, it is consistent with previous findings (Gao *et al.*, 2012). Most obvious is the constant number of single NORs on chromosomes 1, 2, 4 and 5 found in many species of *Lilium* studied so far (Stewart, 1947). The previous and present study confirmed that the tribe Lilieae has the basic chromosome number as x=12 (diploid) lily (Gao *et al.*, 2009; Muratovic´ *et al.*, 2010). Asiatic hybrids have different ploidy like chromosome number as triploid (3n=3x=36) and tetraploid (4n=4x=48). Accompanied with the increase in chromosome number, 45S rDNA hybridization signal loci also increase. The distribution of 45S rDNA loci in different Asiatic hybrids is basically the same. Tetraploid varieties may have originated from the parents with different 45S rDNA sites in the process of hybridization, and some sites only by the male or female parent genetic. If we can find a specific repeat sequence, the typical chromosome can be distinguished quickly.

Only male parent 'Melanie' and progeny 15-189-45 showed same signals of 45S rDNA in chromosome 6 (Fig. 2). This shows that progeny's chromosome originated from 'Melanie' specifically from 'Melanie'. Progeny chromosome 5, 11 could have originated from 'Carolina', 'Melanie' or both Progenies. 15-189-43 and 15-189-45 have two chromosomes (distributed in chromosome 1, 2 and 7) respectively originated from female parent of 'Carolina' and male parent of 'Melanie'. Progeny (15-189-43) chromosome 4 may have 2 situations: two chromosomes are derived from the same parent or each chromosome originated from male or female parents. We can quickly identify the authenticity of offspring and judge whether the progeny has the typical chromosome same as their parents by comparing the results of FISH. Meanwhile, the source of the typical chromosomes with fluorescence signals can be traced.

Key to successful *in situ* hybridization is to obtain good chromosome. Excellent chromosome preparation requires clean background, few impurities, and more mitotic metaphase figure of chromosomes. Lily chromosome is larger than usual plant materials and more suitable for chromosome production (Kibyung, 2000). Root-tip squashing method is convenient to produce good chromosome morphology. When the cell is in mitotic metaphase, the chromosomes are most easily observed, so it is necessary for to prepare the appropriate treatment solution and the reasonable control of processing time. Pretreatment (with cycloheximide) can inhibit the formation of the spindle fiber. Chromosomes can be shortened, which is convenient for dispersing and counting. Generally, any meristems undergoing cell division can be used as a material for chromosome production. Kirov *et al.* (2014) studied the dynamics of plant chromosome spreading after dropping cell suspension on slides. Keeping root tips in cycloheximide for 6 h at 4℃ is the best solution. Root tips were rinsed in a pectolytic enzyme mixture for about 1 h can make enzymatic hydrolysis thoroughly. We can adjust time according to the size of root tips. The chromosome slides need to be put in the refrigerator to prevent bacterial contamination.

Femandez *et al.* (2010) showed that hybridization between relative species has good cross compatibility, while interspecific hybridization between lily groups generally requires cutting stigma and embryo rescue techniques to overcome incompatibility. The cross compatibility between the same ploidy is greater than that of different ploidy (Wang *et al.*, 2015). In this study, two materials are Asiatic hybrids with the same ploidy (4n=4x=48). Asiatic hybrids 'Carolina', 'Melanie' and 2 progenies respectively have 17, 21, 19, 18 signals of 45 S rDNA. Many studies have demonstrated that diploid angiosperms have probably 10 45S rDNA loci

(Mousavizadeh *et al.*, 2016; Totta *et al.*, 2016). The tetraploid signal loci are double to that of diploid. This indicated that the number of homologous chromosome was increased during the formation of polyploidy in lily hybrid breeding. Above all, 45S rDNA FISH can be used for identifying the authenticity of *Lilium* hybrids and polyploidy ploidy detection.

In this experiment, hybrid combinations had close hybridization among Asiatic hybrids, so it is convenient to identify the authenticity of hybrid progeny by FISH. Regardless of parental genetic relationship, as long as their rDNA loci exist differences, any types of hybrid progenies can be identified (Xiang *et al.*, 2007; Ding *et al.*, 2016). For the past of few years, GISH technology is also widely used, but it requires that the genome of the parents had been differentiating to a certain extent before distinguishing different parental genome from hybrid progeny (Ren *et al*., 2012). Combination of two technologies FISH and GISH (Karlov *et al*., 1999; Qi *et al.*, 2016; Xu *et al.*, 2016) to analyze the genetic relationships between genomes is very meaningful. The genomic constitution of polyploid varieties and rapid determinationof genetic makeup of hybridsis worthy of in-depth study.

Conclusion

The hybrid seed setting rate between the same ploidy is higher than that of different ploidy in Asiatic hybrids. This can prove that hybridization between the same ploidy has a better cross compatibility. Although lily chromosome ploidy is complex, FISH as a molecular marker technique can also quickly and accurately detect the authenticity of hybrid offspring. This technology can help molecular-assisted breeding and identify the authenticity of many plant progeny not only in lily.

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