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CHROMOSOME ANALYSIS AND MAPPING OF RIBOSOMAL GENES BY FLUORESCENCE IN SITU HYBRIDIZATION (FISH) IN FOUR ENDEMIC LILY SPECIES (*LILIUM*) IN QINLING MOUNTIANS, CHINA

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Abstract

Detailed karyotypes of 4 lily species (*Lilium regale, L. duchartrei, L. brownii* var *viridium* and *L. leucanthum* var. *centifolium*), native to Qinling Moutains in China, were constructed on the basis of chromosome arm lengths and fluorescence in situ hybridisation (FISH) with the 45S and 5S rDNA sequences as probes. Results showed that the relative length of these 4 genomes were all around 300μm, and the karyotypes were similar, which consisted of 2 pairs of (sub) metacentric and 10 pairs of (sub)telocentric chromosomes. FISH results showed that 4 pairs of homologous chromosomes with 6 45s rDNA and 2 5s rDNA signals in *L. regale*, 7 pairs of homologous chromosomes with 12 45s rDNA and 4 5s rDNA loci in *L. duchartrei*, 5 pairs of homologous chromosomes and one of the chromosome number 2 with 7 45s rDNA loci and 4 5s rDNA loci in *L. brownii* var *viridium*, and 3 pairs of homologous chromosomes with 4 45s rDNA loci and 2 5s rDNA loci in *L. leucanthum* var. *centifolium* could be distinguished. The odd number of 45s rDNA signals in *L. leucanthum* var. *centifolium* reflected its heterozygosity.

Introduction

Lily belongs to genus *Lilium* (2n=2x=24) of family Liliaceae, plants in this genus possess immense ornamental value, and some of them are widely used as a resource of food and medicine in Eastern Asia (Zhang, 2007; Zhao *et al*., 1996). Lily originated in the Himalayan region and they have been extended over the mountain areas in the Northern hemisphere (De Jong, 1974), consists of above 100 species which were classified into seven sections (Comber, 1949; De Jong, 1974).

Although thousands of cultivars have been released for cut-flower production through intra- and inter-specific hybridization (Leslie, 2005), most of the wild lily species are still not utilized in breeding programs. As a result, current commercial breeding aims at combining different desirable traits together via interspecific hybridization and polyploidization, this indicates that it is necessary to discover more useful genetic variation than hitherto has been done.

With a special ecology and complex geographical location, Qinling Mountains (31°42′-34°45′ N,105°46′-111°15′ E), covering around 75 thousand square kilometers, shows transitional weather from subtropical to cold temperate, from humid to semi-humid climate. The region is not only the boundary of south-north climate, but also the mixed centre of temperate and subtropical flora in China. It is also one of the diversity centres of lily species, with 11 species (varieties) widely distributing. However, lily species in this area had already become endangered by continuous threatening of human activity and agriculture production. As a result, preservation, evaluation and utilization are becoming increasingly important. Since lily is an ideal material for cytogenetic analysis, the increased utilization potential of these precious germplasm in lily breeding requires detailed knowledge of their chromosome portraits for cytogenetic studies of their interspecific hybrids and breeding programs. Furthermore, the ability to identify individual chromosomes using fluorescence in situ hybridisation (FISH) will be helpful for detecting the interspecific hybrids and chromosomes in backcross individuals, which has already successfully used in many other plant species (Leitch & Heslop-Harrison, 1992; Jiang & Gill, 1994; Iwano, 1998). In addition, the karyotypes will also be indispensable for mapping genes on the chromosome arms and trace these chromosome segments during the consecutive backcross generations.

The aim of this study was an accurate comparative description of the karyotypes of these 4 lily species, *L. regale, L. duchartrei, L. brownii* var *viridium* and *L. leucanthum* var. *centifolium* using morphometric data and FISH banding with 45S and 5S rDNA sequences as probes.

Materials and Methods

Plant material: During a three-year investigation from 2004 to 2007, a total of 44 counties in Qinling Mountains, accounting for 5000 square kilometers had been systematically investigated for lily distribution. In the 11 lily species reported in the previous research, 8 species were successfully found. 4 species (*L. regale, L. duchartrei L. brownii* var. *viridium and L. leucanthum* var. *centifolium*), which are endemic, were collected in situ (Table 1). Bulbs were then taken back and planted in the nursery of Horticulture College, Northwest A&F University for further identification and materials were also in vitro conserved for experimental use.

Mitotic chromosome preparation: Young roots at a length of 5-10mm were collected and treated with 0.05% colchicine for 4-6 hours at room temperature, then put into freshly prepared Carnoy's solution (ethanol : acetic acid, 3:1 v/v) and stored at 4°C until use. Root tips were washed and incubated by 1% cellulose RS and 1% Pectolyase Y23

(pH 4.5) for 90 minutes at 37°C. chromosome spreading were according to Ross *et al*., (1996).

Table 1. Materials selected and their origin.

Species	Origin of collection
L. regale	Shuanghe Jiuzhaigou County, Sichuan Province
L. duchartrei	Laza valley, Diebu County, Gansu Province
L. brownii var. *viridium*	Sanyuan, Zhen'an County, Shaanxi Province
L. leucanthum var. *centifolium*	Nanfeng, Zhouqu County, Gansu Province

Probes and labeling: Two different probes, 45S and 5S rDNA, were isolated from clone pTa71 and pScT7 respectively (Gerlach & Bedbrook, 1979; Lawrence & Appels, 1986). Then they were labeled with biotin-16-dUTP and digoxigenin-11-dUTP respectively using standard nick translation according to the manufacturer's instruction (Roche Diagnostics GmbH, Mannheim, Germany).

***In situ* hybridization:** FISH was carried out according to Lim *et al*., (2001) with a 40µl hybridization mixture of 50% (v/v) deionized formamide, 10% (w/v) sodium dextran sulphate, 2 × SSC, 0.25% (w/v) sodium dodecyl sulphate, 2-2.5 ng/µL for each probe and 100-200 ng/µL sheared herring sperm DNA, the latter was used as block DNA. The hybridization mixture for FISH was denatured for 10 minutes at 73°C and directly put on ice for at least 10 minutes. As a next step, hybridization mixture was added on the slides and covered with coverslips. After being denatured for 5 minutes at 80°C, slides were transferred to a pre-warmed chamber for overnight hybridization at 37°C. After hybridization, the slides were washed by 0.1 × SSC at 42°C for 30 minutes. The probes labelled with digoxigenin-11-dUTP or biotin-16-dUTP were detected with the anti-digoxigenin-FITC or Cy3 respectively. After detection the slides were counterstained with 1 µg/mL 4',6-diamidino-2-phenyl-indole(DAPI) and mounted with Vectashield . Photographs were taken using a Canon camera attached to a Zeiss Axiophot epifluorescence microscopy.

Karyotyping: The obtained images were analyzed by a free computer program MicroMeasure (Reeves & Tear, 2000). In all four species, the chromosomes were put into sequence according to decreasing short arm lengths (Stewart, 1947; Lim *et al*., 2001; Xie *et al.,* 2010), and the nomenclature used for the discription of the chromosome morphology is that proposed by Levan *et al*., (1965). The abbreviations m, sm, st and t represent metacentric, submetacentric, subtelocentric and telocentric chromosomes respectively. Idiograms were drawn according to the mean centromeric index.

Results

Cytological analysis of somatic metaphase cells indicated that all of these 4 species (*L. regale, L. duchartrei, L. brownii* var. *viridium* and *L. leucanthum* var. *centifolium*) were diploid with a chromosome number 24 respectively. The relative length of the genome, the longest and the shortest chromosome, and the karyotype formula of each species were listed in Table 2. It is evident that all of the 4 species possessed a similar genome size based on the relative length of the genome which were all around 300µm. For *L. regale,* one pair of chromosome was metacentric, one pair was submetacentric, 3 pairs were subtelocentric and 7 pairs of chromosomes were telocentric; For *L. duchartrei*, 2 pairs of chromosome were metacentric, 5 pairs were subtelocentric and 5 pairs were telocentric; *L. brownii* var. *viridium* consisted of one pairs of metacentric chromosomes, one pair of submetacentric, 4 pairs of subtelocentric and 6 pairs of telocentric chromosomes; and for *L. leucanthum* var. *centifolium*, there were one pair of metacentric, 1 pair of submetacentric, 6 pairs of subtelocentric and 4 pairs of telocentric chromosomes. Furthermore, one pair of satellites on chromosome number 2 in *L. duchartrei* was identified in all of the well spread somatic metaphase cells.

In situ hybridization using 45S rDNA and 5s rDNA as probes successfully localized the rDNA positions in all 4 lily species (Fig. 1). Results showed that most of the 45s rDNA loci situated near centromeres or the second constrictions (Fig. 1a, b, c, d). However, the position and number of 45s and 5s rDNA loci differed among the four lily species (Fig. 2). 6 45s rDNA signals on chromosome number 1, 4, 6 and two signals on chromosome 3 were detected and 8 chromosomes were marked in the somatic metaphase cells of *L. regale*; In *L. duchartrei*, 12 45s rDNA loci on 6 pairs of homologous chromosomes and 4 5s rDNA loci on the long arm of chromosome number 7 were mapped and a total of 14 chromosomes were distinguished; 7 45s rDNA signals and two 5s rDNA signals were identified in *L. brownii* var. *viridium*, the signal on one of the chromosome 2 reflected the heterozygous characters of this species; For the left lily species, *L. leucanthum* var. *centifolium*, 6 45s rDNA loci and 2 5s rDNA loci were situated on the somatic metaphase chromosomes. Since there were 2 45s and 2 5s rDNA loci on chromosome number 3, 3 pairs of homologous chromosomes were distinguished (Table 3).

Table 2. Karyotype analysis of four endemic lily species from Qinling Mountains.

species	Chromosome number	Chromosome length (µm)			Karyotype formula
		Total	Longest	Shortest	
L. brownii var. *viridium*	24	301.18	38.97	16.89	4m+8st+12t
L. leucanthum var. *centifolium*	24	300.03	39.48	17.46	2m+2sm+12st+8t
L. duchartrei	24	304.14	38.22	18.72	4m+10st+10t
L. regale	24	299.97	38.91	17.34	2m+2sm+6st+14t

Table 3. number of signals analyzed and the number of chromosomes mapped by FISH with 45s and 5s rDNA as probes in four lily species.

FISH	*L. regale*		*L. duchartrei*		*L. brownii var. viridium*		*L. leucanthum var. centifolium*	
	# of loci	# of chr.	# of loci	# of chr.	# of loci	# of chr.	# of loci	# of chr.
45s	6	6	12	12	7	7	6	6
5s	2	2	4	2	4	4	2	2
Mapped chr.	8	8	16	14	11	11	7	7

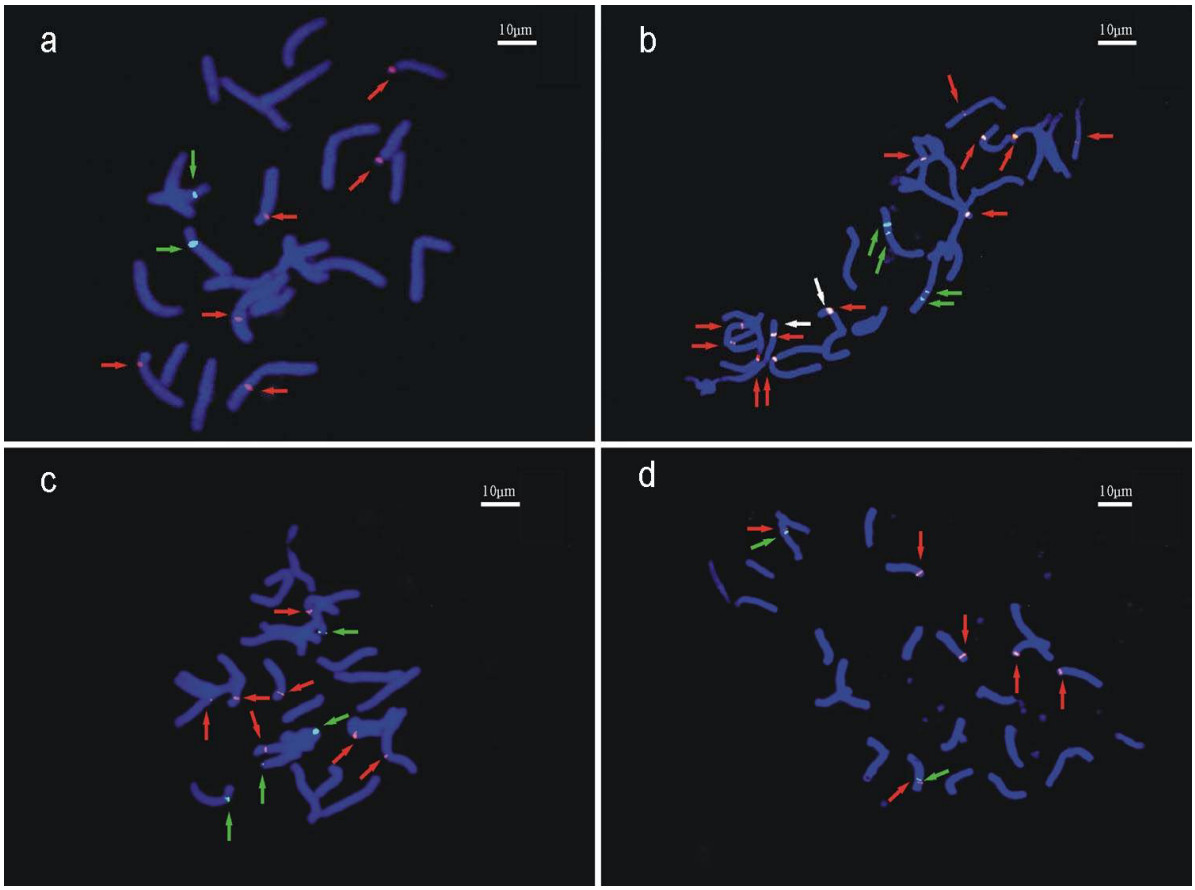

Fig. 1. Somatic metaphase chromosomes stained by FISH using 45s and 5s rDNA as probes. 45s and 5s rDNA signals were marked by red and green arrows respectively. (a) *L. regale*, (b) *L. duchartrei* (white arrows indicate satellites), (c) *L. brownii* var. *viridium*, and (d) *L. leucanthum* var. *centifolium.*

In addition, there was also a variation in terms of the rDNA signal strength which can be well reflected in these lily species. In *L. duchartrei*, the 5s rDNA spot in the relatively distal position on chromosome number 7 were significantly stronger than the other two signals, and the 45s rDNA signals on chromosome number 4 were much weaker compared with other 45s rDNA spots (Fig. 1b); the 5s rDNA signals on chromosome number 12 in *L. brownii* var. *viridium* and the two 45s rDNA signals on chromosome number 3 in *L. leucanthum* var. *centifolium* were also relatively weak (Fig. 1c and d).

Discussion

Lily possesses the largest genome size in angiosperms (Bennett & Smith, 1976; 1991; Van Tuyl & Boon, 1997; Zonneveld *et al*., 2005), plants in genus *Lilium* are diploid with the exception of *L. tigrinum* (*L. lancifolium*) in which both diploids and triploids were found (Noda, 1978; Kim *et al*., 2006). In the four lily species analyzed, the longest chromosomes reached around 39μm and the relative length of genome flunctuated at 300μm (Table 1), which indicated the similar genome size in genus *Lilium* (Van Tuyl & Boon, 1997; Zonneveld *et al*., 2005). The results of this paper revealed that the basic chromosome number of all four species is x=12 with 2 (sub) metacentric and 10 (sub)telocentric chromosomes (Table 1; Fig. 2), which is consistent with many previous report (Noda, 1991; Lim *et al*., 2001; Inceer *et al*., 1999, 2002).

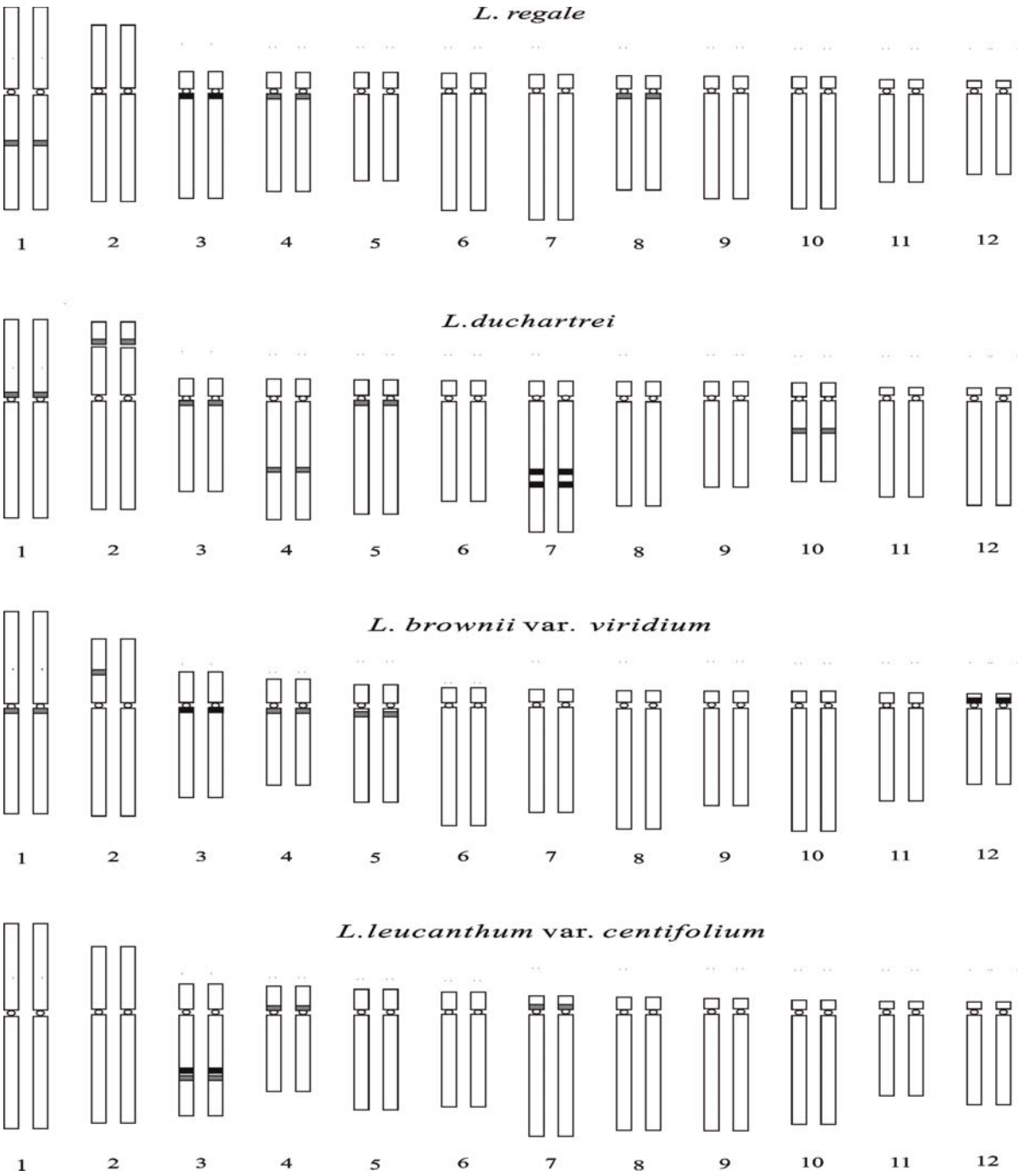

Fig. 2. Karyotype of four lily species after FISH analysis. Note: the strength of signals was not reflected in this idiogram illustration. Balck areas represent 5s rDNA loci and gray areas stand for 45s rDNA loci.

Since Strasburger (1880) who recognised lily species as a particularly suitable model for chromosome research, many studies followed. However, in the standard karyotype of *Lilium* species most chromosomes (4-12) are morphologically too similar to be identified unequivocally without additional diagnostic landmarks. More recently, the introduction of fluorescence in situ hybridisation (FISH) tools using repetitive or single copy sequences complements the traditional banding technologies, along with the use of DNA specific fluorochromes (Peterson *et al*., 1999). The most common application of FISH for karyotype analysis is the localization of ribosomal DNA repeat families. Many reports revealed that the 45S rDNA components are part of the nucleolar organiser region of the satellite chromosomes, whereas the 5S rDNA generally occurrs on the other chromosomes (Gerlach & Dyer, 1980; Mukai *et al*., 1991a, 1991b; Leitch & Heslop-Harrison, 1992; Jiang & Gill, 1994; Kamstra *et al*., 1997;

Fukui *et al*., 1998; Takeda *et al*., 1999). Our FISH detection of 45s and 5s rDNA genes clearly showed that most locations corresponded with the NORs and secondary constrictions (Fig. 1a-d and Fig. 2). It is also noticeable that two 5S rDNA loci can occur on the same chromosome (chromosome number 7 of *L. duchartrei*) (Fig. 1b) and 45S + 5S rDNAs on the chromosome number 3 of *L. leucanthum* var. *centifolium* (Lim *et al*., 2001; Fig. 1d). With the differentiation of chromosomes in different genomes, different individual chromosomes could be identified combining with the morphological data. This results can be used to recognize the identified chromosomes in crossing progenies and sexual polyploidized allopolyploids and hence, is very useful in introgresson breeding, as reported in many other crops (Mukai *et al*., 1991a, 1991b; Leitch & Heslop-Harrison, 1992; Jiang & Gill, 1993, 1994; Iwano, 1998).

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