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Physical mapping of 45S and 5S rDNA in two *Sprekelia formosissima* cytotypes (Amaryllidaceae) through Fluorescent *In Situ* Hybridization (FISH)

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Abstract. Chromosome number and position of rDNA were studied in plants of *Sprekelia formosissima* (Amaryllidaceae) collected in two populations with different ploidy level (2*n*=2*x*=60 and 2*n*=5*x*=150). The 5S and 45S rRNA *loci* were localized and physically mapped using two-color fluorescence in situ hybridization probes. The diploid (2*n*=2*x*=60) cytotype showed four *loci* for the 45S rDNA in two chromosome pairs (11 and 25) in telomeric position. The 5S rDNA was present in six *loci* of three homologous chromosome pairs (3, 13 and 19) in subtelomeric and telomeric positions. The chromosomes of the pentaploid cytotype (2*n*=5*x*=150) showed five *loci* for the 45S rDNA in telomeric position and five *loci* for the 5S rDNA in subtelomeric position. The karyotypic formula is 13m + 16sm + 1 st and the karyotype symmetry/asymmetry index is TF % = 34.67, AsK % = 65.32 and Syi % = 54.81, concluding that it is an asymmetric karyotype, bimodal with one distinctively large pair of chromosomes (10.42 μm) and a gradual decrease in the size of the other chromosome pairs, from the longest of 6.84 μm, to the shortest of 2.61 μm.

Keywords: bulbous genus, karyogram, karyotypic formula, ornamental, plant chromosomes, ploidy level.

INTRODUCTION

Sprekelia Heist, is a bulbous genus of the monocotyledonous family Amaryllidaceae, it is a perennial, herbaceous monotypic genus commonly known as Aztec lily or Jacobean lily represented by the sole species *Sprekelia formosissima* (Flory 1977; Sánchez 1979); it is distributed through Mexico and Guatemala (López-Ferrari and Espejo-Serna 2002). Its scarlet flowers with curved petals have made of it an exceptional ornamental pot plant.

The technique of Fluorescent *In Situ* hybridization (FISH) has become a very useful tool for the detection of specific DNA in the genome of organisms. In this technique, the genes encoding the 5S ribosomal RNA (rDNA

5S) and the 18S-5.8S-26S ribosomal RNA (45S rDNA) are commonly used as markers for the physical mapping of plant chromosomes due to the high number of their repeating units, specific position on chromosomes and highly conserved sequences (Liu and Davis 2011). The 45S ribosomal RNA genes are clustered in tandem arrays of repeating units of the genes 18S, 5.8S and 26S, internal transcribed spacer sequences (ITS) and external non-transcribed spacer sequences (NTS), with an approximate size of 7.5-18.5 Kb in plants (Mizuochi *et al.* 2007). The 5S ribosomal RNA genes are also found in tandem repeats with an approximate size of 0.2-0.9 Kb, with a highly conserved region (120 bp long) separated by a NTS sequence (Specht *et al.* 1997). Physical mapping of rDNA has been performed in many plants such as: maize (Li and Arumuganathan 2001), orchids (Cabral *et al.* 2006), *Crotalaria juncea* (Mondin *et al.* 2007), *Agave* (Robert *et al.* 2008; Gomez-Rodriguez *et al.* 2013), asparagus (Deng *et al.* 2012), *Lilium* (Lim *et al.* 2001; Hwang *et al.* 2015), *Tigridia pavonia* (Arroyo-Martínez *et al.* 2018), among many others, however, there is little work in plants of the Amaryllidaceae family using these modern techniques of molecular cytogenetics. The objective of this study was the physical mapping of rDNA 45S and 5S in two cytotypes (diploid and pentaploid) of *Sprekelia formosissima*.

MATERIAL AND METHODS

Plant material

Sprekelia formosissima bulbs were collected from two different populations and maintained in an *in vivo* collection at the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C. (CIATEJ). Two cytotypes were found, a diploid population ($2n=2x=60$) from the road Villa-Corona-Cocula, and a pentaploid population ($2n=5x=150$) from a private collection in Zapopan, both in the state of Jalisco, México. The bulbs were placed in a container with moist substrate composed by a mixture of Peat Moss and Vermiculite (7:3) for the bulbs to produce roots. Sampling was performed in ten bulbs of each of the two populations.

Chromosome spreads

Obtaining metaphasic chromosomes was performed as described by Rodríguez-Domínguez *et al.* (2017), in brief, the root apex were pretreated in a saturated solution of α-bromonaphthalene (0.1%) for 48 h at 4 °C and fixed in a methanol:acetic acid (3:1) solution for 24 h at 4 °C. Afterwards, the root apex were washed with milli-Q water, macerated in an enzyme mixture containing each 0.2% (w/v) of pectolyase Y23, RS cellulase and cytohelicase in 10 mM citrate buffer (pH 4.5) and incubated at 37 °C for 3 h. Later, a cell suspension was obtained by vortexing, followed by washing with distilled water, centrifuging at 10,000 rpm for 45 s, washed with methanol, then, centrifuged at 11,000 rpm during 30 s. The cell pellet was resuspended in 100 µl methanol. Finally, in a fume hood, the slides were covered with a layer of pure acetic acid and 10 µl of cell suspension were added to each slide. The slides were immediately inclined at an angle of 45 degrees, and two drops of pure acetic acid were added; the slides were exposed (facing down) to vapor from a water bath at 55 °C for 5 s, finally, a drop of pure acetic acid was added and allowed to air dry.

DNA probes

Two different clones were utilized as probe, clones pTa71 and pTa794 which contains the *EcoRI* fragment of 45S and 5S ribosomal DNA from wheat respectively (Gerlach and Bedbrook 1979; Gerlach and Dyer 1980). The bacteria were cultured for 24 hours under constant agitation (225 rpm) at 37 °C in LB medium supplemented with 0.1 mg/ml ampicillin. Isolation of wheat ribosomal DNA was performed using the kit High Pure Plasmid Isolation Kit (Roche®) according to the supplier's instructions. In brief, 4 ml of the inoculated culture medium were transferred to 15 ml tubes and centrifuged at 9,000 rpm for 1 minute, the supernatant was discarded; the bacterial pellet was resuspended in 250 µl of suspension buffer and transferred to a 1.5 ml tube. 250 µl of lysis buffer were added and mixed by inversion, then incubated for 5 min and 350 µl of binding buffer were added and incubated for 5 min on ice; followed by centrifugation at 13,000 rpm for 10 min. The supernatant was transferred to a filtration membrane and centrifuged at 13,000 rpm for 1 minute; the membrane was transferred to a new tube and 500 µl of washing buffer I was added and centrifuged at 13,000 rpm for 1 minute. The column was transferred to a new tube and 700 µl of washing buffer II was added; centrifuged at 13,000 rpm for 1 minute. The flowthrough liquid was discarded and the column was centrifuged at 13,000 for 1 minute to dry the membrane, which was transferred to a new 1.5 ml tube and 100 µl of elution buffer was added and centrifuged at 13,000 rpm for 1 minute.

Labeling the probes

Fluorescent in situ hybridization (FISH) was performed using two different probes (45S and 5S wheat rDNA) which were direct labelled as follows: 1 µg of rDNA in 12 µl mQ water, 4 µl Nick translation mix (Roche®) and 4 µl of 5x fluorophore mix (5 µl of each 2.5 mM dATP, dCTP, dGTP, 3.4 µl 2.5 mM dTTP, 4 µl 1 mM labeled dUTP and 27.6 µl mQ water) were incubated at 15 °C for 90 min. Reaction was stopped by adding 1 µl of 0.5 M EDTA (pH 8.0) and heating at 65 °C for 10 min. 45S rDNA was labeled with Tetramethylrhodamine-5-dUTP and 5S rDNA with Fluorescein-12-dUTP.

Fluorescent in situ hybridization

Slides with metaphase chromosomes were incubated at 37 °C overnight, the next day, each slide was incubated in 200 µl RNase A (100 µg/ml) in 2xSSC at 37 °C for 1 h and washed three times for 5 min each in 2x SSC, incubated in 0.01 M HCl for 2 min. 200 µl Pepsin (5 µg/ml) were added and incubated at 37 °C for 10 min, washed in mQ water for 2 min and in 2x SSC for 5 min each. The slides were incubated in 4% paraformaldehyde for 10 min and washed three times in 2x SSC for 5 min each. The slides were dehydrated in 70%, 90% and absolute ethanol series for 3 min each and air-dried. Hybridization followed using a mixture consisting of 20x SSC, 50% formamide, 10% sodium dextran sulphate, 10% SDS and 25-50 ng of each probe. The DNA was denatured by heating the hybridization mixture at 70 °C for 10 min and then placed on ice for at least 10 min. For each slide, 40 µl of hybridization mixture was used. The preparations were denatured at 80 °C for 10 min and incubated overnight in a humid chamber at 37 °C. After overnight hybridization, the slides were washed three times in 2x SSC at 37 °C for 5 min each, in the last wash temperature was increased to 42 °C and the slides were then washed three times in 0.1x SSC at 42 °C each followed by three washes in 2x SSC at RT for 5 min each. Chromosomes were counterstained with 1µg/ml DAPI (4,6-diamidino-2-phenylindole) and a drop of Vectashield anti-fade (Vector laboratories) was added for its examination under a Leica DMRA2 microscope equipped with epi-fluorescent illumination, filter sets of DAPI, FITC and Cy3. Images were captured by a Evolution QEi (Media Cybernetics) monochrome digital camera and processed with Image-Pro Plus software. The DAPI images were sharpened with a 7x7 High Gauss spatial filter. DAPI, FITC and Tetramethylrhodamine fluorescence were pseudo-colored with their respective dye tint for the FISH analyses.

Karyotype analysis

Karyotype analysis was based on at least 10 high-quality metaphase plates with almost similar degree of chromatin condensation, chromosome measurements were made using the freeware software Micromeasure (Reeves 2001) vers 3.3. The following measurements of each pair of chromosomes were made: Chromosome length, long arm (L), short arm (S), arm ratio (L/S). The chromosomes were assorted into different categories based on arm's ratio arranged according to Levan *et al.* (1964) and the karyogram was performed; the chromosome homology was assigned according to similarities in length, morphology and centromere position. The number of homologous chromosomes was assigned sequentially according to the reduction of the chromosomal length and in base to centromere position. Three different methods of evaluating karyotype asymmetry were used: the TF% (Huziwara, 1962), the AsK% (Arano, 1963) and the Syi (Greilhuber and Speta, 1976; Venora *et al.* 2002).

RESULTS

The physical distribution of the 45S and 5S rDNA were investigated by fluorescent *in situ* hybridization (FISH) (Figures 1 to 3) in two cytotypes of *Sprekelia formosissima*, a diploid ($2n=2x=60$) and a pentaploid ($2n=5x=150$). The diploid ($2n=2x=60$) cytotype showed

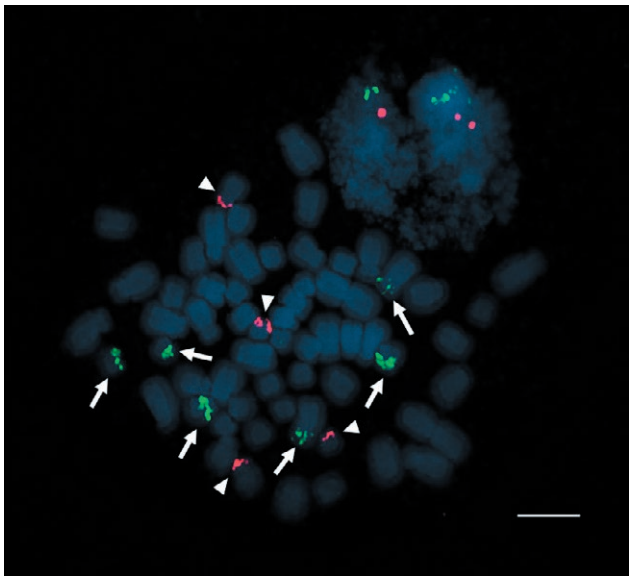

Figure 1. Fluorescent *in situ* hybridization of wheat 45S (red signals, arrowheads) and 5S rDNA (green signals, arrows) in a diploid *Sprekelia formosissima* ($2n=2x=60$) cytotype. The chromosomes were counterstained with DAPI. Bar=10µm.

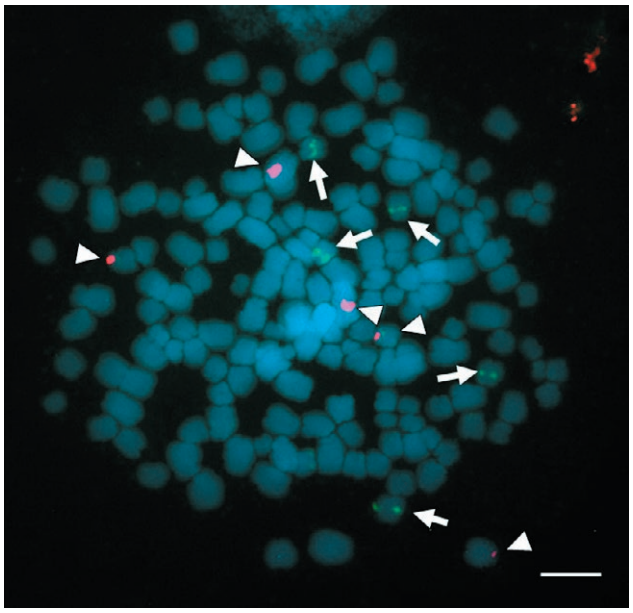

Figure 2. Fluorescent *in situ* hybridization of wheat 45S (red signals, arrowheads) and 5S rDNA (green signals, arrows) in a pentaploid *Sprekelia formosissima* (2*n*=5*x*=150) cytotype. The chromosomes were counterstained with DAPI. Bar=10μm.

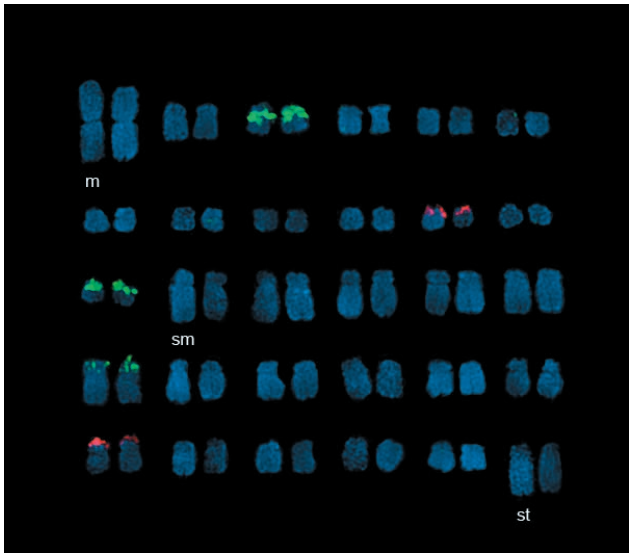

Figure 3. Karyogram of diploid *Sprekelia formosissima*. The wheat 45S rDNA hybridization *loci* (red signal) are present in chromosome pairs 11 and 25, while the wheat 5S rDNA *loci* (green signal) are present in chromosome pairs 3, 13 and 19.

four *loci* for the 45S rDNA in two chromosome pairs (11 and 25) in telomeric position. The 5S rDNA was present in six *loci* of three homologous chromosome pairs (3, 13 and 19) in subtelomeric and telomeric positions (Figures 1 and 3). Chromosome pair 1 was identified due to its unmistakable large size. The chromosomes of the pentaploid cytotypes (2*n*=5*x*=150) showed five *loci* for the 45S rDNA in telomeric position and five *loci* for the 5S rDNA in subtelomeric position (Figure 2). The hybridization *loci* for each probe was found always in different chromosomes. Thirty chromosome pairs (60 chromosomes in total) were identified in the diploid cytotype of *S. formosissima* and were arranged based on arm's ratio according to Levan *et al.* (1964) in the karyogram shown in Figure 3. The karyotypic formula was 13m + 16sm + 1 st with predominance of m and sm chromosomes. The longest per shortest chromosome ratio (L/S) ranges from 1.00 to 5.12. The karyotype symmetry/asymmetry index is TF % = 34.67, AsK % = 65.32 and Syi % = 54.81, concluding that it is an asymmetric karyotype, bimodal

Table 1. Chromosome parameters of *Sprekelia formosissima*

Chromosome pair no.	Total length (μm)	Long arm (L) (μm)	Short arm (S) (μm)	Arm ratio L/S	Centromeric position
1	10.42	5.40	5.02	1.08	M
2	5.02	3.10	1.92	1.61	M
3	3.98	2.29	1.69	1.36	M
4	3.86	2.22	1.64	1.35	M
5	3.60	1.96	1.64	1.20	M
6	3.41	1.95	1.46	1.34	M
7	3.26	1.71	1.55	1.10	M
8	3.24	1.89	1.35	1.40	M
9	3.19	1.81	1.38	1.31	M
10	3.14	1.89	1.25	1.51	M
11	3.06	1.55	1.51	1.03	M
12	2.87	1.57	1.30	1.21	M
13	2.61	1.36	1.25	1.09	M
14	6.84	4.77	2.07	2.30	Sm
15	6.46	4.70	1.76	2.67	Sm
16	6.15	4.25	1.90	2.24	Sm
17	6.08	4.46	1.62	2.75	Sm
18	6.04	4.3	1.74	2.47	Sm
19	5.50	4.12	1.38	2.99	Sm
20	5.43	4.07	1.36	2.99	Sm
21	5.22	3.77	1.45	2.60	Sm
22	5.16	3.63	1.53	2.37	Sm
23	5.14	3.63	1.51	2.40	Sm
24	5.04	3.32	1.72	1.93	Sm
25	4.91	3.19	1.72	1.85	Sm
26	4.61	3.15	1.46	2.16	Sm
27	4.31	2.92	1.39	2.10	Sm
28	4.16	2.68	1.48	1.81	Sm
29	3.68	2.50	1.18	2.12	Sm
30	6.84	5.41	1.43	3.78	St

with one distinctively large pair of chromosomes (10.42 µm) and a gradual decrease in the size of the other chromosome pairs, from the longest of 6.84 µm, to the shortest of 2.61 µm. (Table 1).

DISCUSSION

According to Roa and Guerra (2012), the most frequent *loci* number of 45S rDNA in angiosperms is two or four per diploid karyotype and they are generally located in terminal regions of the chromosomes, found more frequently in the short arms. In the cytotypes of *S. formosissima* analyzed in this work, both signals (45S and 5S rDNA) were detected in the short arms of the chromosomes. Even though the localization sites and the number of copies of the 5S and 45S *loci* present variation, it is probably not related to the ploidy level as has been suggested in some studies (Weiss-Schneeweiss and Schneeweiss 2013). This coincides with our results where four 45S rDNA sites and six 5S rDNA sites were detected in diploid plants, while five 45S rDNA sites and five 5S rDNA sites were detected in the pentaploid cytotype. There may be a different number of signals in species that have the same ploidy level, for example, García (2015) reported four 45S sites and nine 5S sites for diploid *Sprekelia howardii* Lehmiller, while in our work only four 45S sites were detected and six 5S sites for diploids analyzed. Differences in signal sizes were also detected even when they were in homologous chromosomes, which may be due to the existence of different copy numbers of the rDNA genes in each chromosome. There was a difference regarding the relation of the number of signals of rDNA in the cytotypes analyzed in this study; in the diploid cytotype there was less 45S signals with respect to 5S signals, while a similar number of rDNA signals were detected in the pentaploid cytotype. Based on the chromosome number of the diploid cytotype ($2n$=60) it can be considered as a polyploid due to its high chromosome number (Stebbins 1971), with this in mind, the lower 45S rDNA *loci* could be explained since these are generally more prone to experience rapid homogenization, silencing, and loss of *loci*, especially in polyploids (Clarkson *et al.* 2005; Kovařík *et al.* 2005; Weiss-Schneeweiss *et al.* 2008; Kotseruba *et al.* 2010). According the symmetry/asymmetry indexes used, the analyzes established that it is an asymmetric karyotype, however due to the predominance of metacentric and submetacentric chromosomes, it was impossible to differentiate them only by their size, for this reason the number of individual chromosomes identified was relatively low. In *S. formosissima* it was observed that the number of signals in the pentaploid cytotype does not correspond to a multiple of the number of signals present in the diploid cytotype, so it is concluded that at least for the wheat rDNA probes used in this work, the Ploidy level of *S. formosissima* is not related to the number of observed signals. The cytotype where the greatest number of chromosomes could be identified was the diploid *S. formosissima* ($2n=2x=60$), where two chromosomal pairs were identified with the 45S rDNA probe, three pairs with 5S rDNA probe and pair 1 due to its unmistakable large size (Figure 3), so a total of six chromosomal pairs could be identified.

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