A Combination of the “Squash” and “Splash” Techniques to Obtain the Karyotype and Assess Meiotic Behavior of *Prosopis laevigata* L. (Fabaceae: Mimosoideae)

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Summary  A combination of modified squash and splash techniques was used for a cytogenetic study of mitotic and meiotic cells of *Prosopis laevigata*. The mitotic karyotype was obtained for the species, and meiotic behavior was monitored from prophase I through the synaptonemal complex (SC). Analysis using light microscopy showed $2n=28$, with chromosomes that, depending on the technique employed, varied in length from 0.84 to 1.84 µm in squashed specimens and from 1.0 to 1.82 µm in splashed specimens. These values are larger than those previously recorded for other South American species of the genus. The karyotype was studied by the splash method. The frequency of tetraploid cells was about 11% of 2052 metaphase cells examined. Meiosis was normal, because 14 bivalents were seen at metaphase I. The SC in pachytene showed regular features and an asynchronous pairing pattern with some short segments of lateral elements apically or interstitially unpaired, and occasional partner exchanges between three lateral elements. A constant association of the telomeric end of a bivalent with the nucleolus in mid- and late-prophase suggests the location of an active nucleolus organizer region (NOR) and its relation with a unique pair of mitotic chromosomes with satellites. The techniques employed here are recommended for species not amenable to cytogenetic studies with conventional techniques.

Key words  Leguminosae/Fabaceae, *Prosopis*, Karyotype analysis, Squash, Splash, Meiosis, Synaptonemal complex, Nucleolus organizer region.

*Prosopis* L. (Fabaceae) is a primitive mimosoid genus that is notable because of its disjunct distribution pattern and the difficulties in elucidating its phylogeny. Forty-five species have been described, of which 41 are American, three Asiatic, and one African (Burkart 1976, Schinini 1981). The genus probably originated in tropical Africa, where at present only the unspecialized, mesic species *P. Africana occurs* (Burkart 1976). The extant North American species of the genus belong to 4 evolutionary lines, all of which are also represented in South America; 3 of these lines show a pronounced geographic disjunction, lacking representatives between the equator and $22^\circ$N, latitude (Rzedowski 1988).

In Mexico, the nine species of *Prosopis* (mesquites) make up communities of broad geographic and ecological distribution, ranging from sea level to 2500 m of altitude. These communities form the dominant tree canopy in many of the arid and semi-arid regions in Mexico. *Prosopis laevigata* (Willd.) M. C. Johnst, belongs to sect. *Algarobia* DC, and is the most common mesquite of central and southern Mexico (Rzedowski 1988).

The genus is taxonomically difficult because most species lack well-marked morphological discontinuities and transitional forms are frequently found. Hybridization and perhaps introgression seem to be frequent within sect. *Algarobia*, between some of the sympatric species with broad dis-
tributions (Hunziker et al. 1975). The recorded chromosome counts reveal that Prosopis is diploid, with one possible exception (P. juliflora, which is probably a tetraploid race (Hunziker et al. 1975)). In all cases, the basic chromosome number is \( x = 14 \), with the diploid condition \( 2n = 28 \). Most of the cytogenetic studies in this group have failed to establish the karyotypic formula of the species, mainly due to the small chromosome size, which ranges between 0.5 and 1.3 \( \mu \text{m} \) (Hunziker et al. 1986). The present study is the first cytogenetic study of \( P. laevigata \) in which both squash and splash techniques are used to obtain the karyotypic formula and its relationship to the dynamics of meiotic chromosomes. Since cytogenetic studies play an important role in the interpretation and elucidation of the relationships of genera and species of plants, as well as of their centers of origin and evolution (Grant 1987, Kenton et al. 1986, Poggio et al. 1989), the present study is undertaken to learn the cytological features of \( P. laevigata \). These features will contribute to an understanding of the genetic system and taxonomic relationships of \( P. laevigata \).

Materials and methods

Seeds were collected from 5 trees of \( P. laevigata \), in Municipio Santiago de Anaya, Actopan Valley, State of Hidalgo, Mexico, during July and August, 1997. These seeds were germinated at 30°C in a constant-temperature incubator, and the primary roots (1–2 cm in length) were used for mitotic study. Immature inflorescences for meiotic study were collected from the same locality in February, 1998.

**Mitotic slides prepared by the squash method**

After a pre-treatment for 5 h in 0.002 M 8-hydroxyquinoline at room temperature, the roots were fixed in Farmer’s solution (3 : 1 ethanol : acetic acid) for at least 2 h, hydrolyzed in 1 N HCl for 12 min at 60°C, and stained in Feulgen for 1 h. To obtain more contrast of the chromosomes, the squashing was done in 2% aceto-orcein. Coverslips were removed by the dry ice method (Conger and Fairchild 1953).

**Mitotic slides prepared by the splash method**

Sixty roots were pre-treated and fixed using the above method. The meristems were then excised and macerated in a mixture of 20% pectinase + 2% cellulase for 2 h in the oven at 37°C. The cell pellet was then separated by centrifugation at 1500 rpm for 10 min, then placed in fresh solution of 0.075 M KCl for 20 min (which causes hypotoric shock), and rinsed twice in 0.075 M KCl. The pellet was then fixed again in Farmer’s solution and washed twice in Farmer’s solution. Two or three drops of the pellet were placed on a slide and stained with 10% Giemsa.

The chromosomes were measured in the 5 best metaphase cells obtained by the squash method and in an equal number of metaphase cells obtained by splash. A digital vernier ruler (Mitutoyo Digimatic Caliber CD-G"BS) was employed for measuring chromosomes from photographs taken at the same magnification. For obvious reasons, only pictures with chromosomes that could be measured accurately were taken into account.

**Meiotic slides prepared by the squash method**

Pollen mother cells were obtained from immature anthers and stained with 1% aceto-carmin. The slides were made temporarily with Hoyer mounting medium. Cells in the substages of prophase, MI, AI and All were analyzed.

**Meiotic slides prepared by the splash method**

Inflorescences in the appropriate stage of development were fixed in freshly made Farmer’s solution for 48 h, then transferred to 70% ethyl alcohol and stored at 4°C until analyzed. Anthers were
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Dissected using fine tweezers and processed in a similar way to the meristems in the mitotic slides prepared using the splash method. To monitor the pairing pattern of the homologous chromosomes through the visualization of the synaptonemal complex (SC), and to locate active NORs, a method of silver staining (AgNO₃ + ammoniacal silver) was used following modified techniques (Bloom and Goodpasture 1976, Quack and Noel 1977, Tapia et al. 1992).

All the slides were made permanent using synthetic resin. Photographs were taken in a Carl Zeiss Axioskop, using Kodak Technical Pan film. For the morphological identification of the chromosomes and to obtain the karyotypic formula of Prosopis laevigata, the system proposed by Levan et al. (1964) was used.

Results

The distinctness of the mitotic metaphases, the separation of the chromosomes inside the cells, and the number of cells analyzed confirm the diploid number of the species (2n = 28), previously reported by Hunziker et al. (1975). Other fine features of the karyotypic structure were revealed, such as the constancy of a secondary constriction and a noticeable satellite portion in a pair of chromosomes in the nuclei (Fig. 1a, b). The analysis of mean differences t = 0.95 (n = 5) shows significant differences between the total chromosome lengths obtained by the two methods, and the comparison of variances F = 0.95 (n = 5) shows a larger variability in the splash method of the average chromosome size. However, it must be pointed out that the adequate definition of chromosome morphology, essential in classifying the individual chromosomes, was consistently possible only using the splash technique. Those obtained by the squash technique, even though of good quality, did not lend themselves to a fine analysis.

In the metaphase cells prepared by the splash technique, the primary constriction could be discriminated to construct the idiogram (Fig. 2b). The 28 chromosomes of P. laevigata are shown in Fig. 2a. Values shown in Table 1 are mean values of 5 metaphase plates. The karyotype formula includes 2 pairs of metacentrics (m), 10 pairs of submetacentrics (sm), and 2 pairs of subtelocentrics (st). The larger of the subtelocentrics bears a secondary constriction with its corresponding satellite, which is evident because of its large size. Thus, 2m+10sm+2st is the first karyotype formula proposed for a species of Prosopis. Additionally, the ploidy level and its frequency in cells of the primary root meristem could be established in cells with well condensed chromosomes: 223 tetraploid cells out of a total of 2052 metaphase cells were recorded, showing a rate of approximately 11%.

Fig. 1. Mitotic chromosome complements 2n=28 in Prosopis laevigata. a) squash technique. b) splash technique. Arrows show chromosomes with satellite portion. Bar=10 µm.
The difference in length between the longest and the shortest chromosomes, 0.81 μm, points toward an asymmetric karyotype (Levitsky 1931). In the present study, using the squash technique, the chromosome length ranged from 0.84 to 1.84 μm, whereas with the splash technique, the length ranged from 1.01 to 1.82 μm. The average total chromosome length was larger in the splashed specimens (38.16 ± 2.52 μm) than in the squashed ones (31.92 ± 1.92 μm) (Table 2).

Table 1. Mean chromosome measures in Prosopis laevigata

<table>
<thead>
<tr>
<th>N</th>
<th>Total length (μm)</th>
<th>Length L arm (μm)</th>
<th>Length S arm (μm)</th>
<th>r</th>
<th>Nomenclature</th>
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<tr>
<td>1</td>
<td>1.42</td>
<td>0.81</td>
<td>0.60</td>
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<td>2</td>
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<td>0.51</td>
<td>2.07</td>
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</tr>
<tr>
<td>5</td>
<td>1.54</td>
<td>1.01</td>
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<td>1.94</td>
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</tr>
<tr>
<td>6</td>
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<td>0.99</td>
<td>0.44</td>
<td>2.25</td>
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</tr>
<tr>
<td>7</td>
<td>1.36</td>
<td>0.90</td>
<td>0.46</td>
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</tr>
<tr>
<td>8</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>13</td>
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<td>1.22</td>
<td>0.29</td>
<td>4.20</td>
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</tr>
<tr>
<td>14</td>
<td>1.01</td>
<td>0.79</td>
<td>0.19</td>
<td>4.15</td>
<td>st</td>
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</table>

Obtained from 5 metaphase cells using the splash technique. N=Number of chromosome, r=Arms ratio, after Levan et al. 1964. L=Long, S=Short. * satellite-bearing chromosome.

Table 2. Comparison of total chromosome lengths (μm) obtained by two techniques

<table>
<thead>
<tr>
<th>Cell</th>
<th>Squash</th>
<th>Splash</th>
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</thead>
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<tr>
<td>1</td>
<td>34.94</td>
<td>41.99</td>
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<td>2</td>
<td>30.47</td>
<td>39.03</td>
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<tr>
<td>3</td>
<td>33.50</td>
<td>37.13</td>
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<td>4</td>
<td>30.55</td>
<td>35.22</td>
</tr>
<tr>
<td>5</td>
<td>30.42</td>
<td>37.46</td>
</tr>
<tr>
<td>Σ</td>
<td>159.63</td>
<td>190.83</td>
</tr>
<tr>
<td>X</td>
<td>31.92</td>
<td>38.16</td>
</tr>
<tr>
<td>S</td>
<td>1.92</td>
<td>2.52</td>
</tr>
</tbody>
</table>
The meiotic slides obtained using the squash technique confirm the haploid chromosome number of 14. Furthermore the chromosome behavior in meiotic prophase conformed to that of a diploid species and was normal regarding pairing in pachytene, chiasmata formation, 14 bivalents in equatorial plate, and segregation in anaphase I and II. In some images it was possible to observe very clearly the insertion of the satellite portions in a nucleolus (Fig. 3a). It must be pointed out that no evidence of polysomaty was found. The slides prepared using the splash technique showed perfectly spread out chromosomes, with good silver impregnation and the SC behavior could be followed without difficulty. However, owing to the absence of swellings or other nuclear-membrane-adhering structures the beginning and end of individual bivalents was very difficult to observe. The meiotic pairing pattern was asynchronous: during mid-pachytene some bivalents frequently showed 3 to 4 short segments of SC with terminal or interstitial asynapsis, while other bivalents had complete synopsis (Fig. 3b).

During mid- and late-prophase, there were constant images of a bivalent clearly connected by its telomeric region to the single nucleolus, showing an intense staining at the point of contact (Fig. 3c, d). Taken as a whole, the images of meiotic prophase confirm the existence of an active NOR and its relationship with the pair of chromosomes with the satellite portion seen in the mitotic complements. Occasionally, during zygotene, partner exchanges between meiotic bivalents involving three lateral elements were observed (Fig. 3e), however these do not result in the formation of multivalents, since only bivalents were found in metaphase I. This could be explained by the existence of correcting mechanisms that rearrange the SC at the beginning of pachytene to form bivalents

Fig. 3. Meiotic prophase in *P. laevigata*. a) Detail of an early pachytene nucleus stained with aceto-carmine. The arrows show the telomeric region of the bivalent with satellites immersed in a nucleolar vacuole. b) Asynchronous pairing in pachytene. The long arrows show completely paired bivalents. The short arrows show unpaired regions. c) and d) Nucleolus-associated bivalents in mid- and late prophase, respectively. The arrows show the contact points. e) Partner exchange involving 3 lateral elements (b–e) (splash technique, with silver staining). Bar=20 μm.
Discussion

In general, our results agree with those previously reported of chromosome morphology and meiotic behavior for other species of the genus (Hunziker et al. 1975). Regarding chromosome size, we report greater ranges in specimens obtained both by squash and splash techniques, than in those obtained by Hunziker et al. (1986) for South American species of Prosopis (0.8–1.3 μm). While the resulting differences might be due to the intrinsic factors involved in the different techniques employed, they could also point out a certain genomic specialization of P. laevigata, which may be associated with its distribution range. Furthermore, the percentage of tetraploid cells found in the root meristems of P. laevigata, suggesting the diploid nature of this species, can be a character for comparison with in future intra- and interspecific studies and for ecogeographic studies.

Even though the statistic analysis shows significant differences between the total chromosome length obtained by the conventional and the splash techniques, we prefer the values obtained by the latter. The main advantage of the splash technique is that it allows the chromosomes to spread out separately in the cytoplasmatic space, facilitating their counting, observation, and analysis. Furthermore, since it has already been demonstrated that the enzymatic treatment does not denature the chromosomal DNA in root meristems (Jamieson et al. 1986), there is accuracy in evaluating chromosome size. This is because, even in the most minute chromosomes the size does not depend on the pressure applied upon the tissue, as is the case in squashing, which sometimes distorts chromosome morphology.

We believe that the frequency of regions of unpaired SC observed in the pachytene nuclei of P. laevigata is a trait common in the meiotic process of the species, and not due to alterations produced by failures in the formation of the SC. This is supported by the observation that these regions appeared to occur more or less randomly and by the presence of partner exchanges that, by resolving themselves into rupture and reunion, hindered the synapsis in pachytene (Rasmussen and Holm 1980). However, these traits will have to be evaluated in other species of the genus, especially in those of purported hybrid origin, to assess if they are common in other species.

Concerning the techniques followed herein, we point out that the enzymatic treatments in the preparation of plant chromosomes have already been previously employed (Hizume et al. 1980, Jamieson et al. 1986, Geber and Schweizer 1987). However, because of the success we have achieved in the splash method (operative simplicity, high production both in quality and quantity of mitotic metaphases, and perfectly spread-out meiotic nuclei) we recommend its use in other groups not amenable to cytogenetic study.

The chromosome number of most species and hybrids included in the Prosopis complex has been determined. However, for lack of detailed karyotypic analysis, the chromosomal evolution in the genus has been poorly understood. On the other hand, the meiotic studies have been limited to the results obtained by the squash technique. This is the first report on the karyotype of a species of Prosopis, and we hope that, the use of the new technique will open the possibility of studying chromosome architecture, banding, and in situ hybridization. Furthermore, the possibility of analyzing the meiotic pairing patterns through visualizing the SC will offer another point of comparison between isolated populations and sympatric ones where hybridization processes are thought to occur.

The elucidation of the phylogeny of the Prosopis complex has been for a long time the objective of taxonomists, cytogeneticists, and evolutionists. However the taxonomic positions of some members of the genus are not clear. The cytogenetic study of the group is incomplete, and the ecogeographic distribution patterns are still to be solved. Part of this problem will be solved when we can establish reliable comparisons between chromosome changes, if any, and the evolution of the included in the group.
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References


