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Characterization of the chromosomes of sotol (*Dasylirion cedrosanum* Trel.) using cytogenetic banding techniques

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Abstract. Sotol (*Dasylirion cedrosanum* Trel.) is a perennial species with numerous grayish-to-green leaves that grow symmetrically from the base of the stem outward. Its inflorescences can measure up to 3 m in height and contain membranous bracts that enclose seeds. It is a species that has been scarcely studied at the cytogenetic level, with only one report available in the literature. In Mexico, it has economic importance because it is used to prepare the alcoholic beverage sotol. In the present work, the chromosomes of *Dasylirion cedrosanum* Trel. were obtained and analyzed using different cytogenetic banding techniques and morphometric analysis to construct the first karyotype for this species. Chromosomes were obtained by germinating plant seeds collected in the locality of Las Adjuntas, Santiago, Nuevo León, Mexico. Treatment with colchicine as an antimitotic was performed, followed by enzymatic treatment with pectinase and cellulase, to eliminate the cell walls. Chromosome slides were stained with Giemsa, GTG banding technique, CBW banding, and the 4',6-diamidino-2-phenylindole fluorescence dye, and observed under a microscope. A chromosomal number 2n of 38 chromosomes, as previously reported, was confirmed. Using the different banding techniques, we observed that all chromosomes exhibited a submetacentric morphology with a fundamental number of 76, and it was possible to visualize the pattern of GTG and CBW bands; these findings are reported for the first time for this species. Morphometric analysis established that the average length of the chromosomes was between 5.09 and 9.84 mm.

Keywords: *Dasylirion cedrosanum* Trel., sotol, karyotype, morphology, cytogenetics.

INTRODUCTION

Sotol (*Dasylirion cedrosanum* Trel.) belongs to the *Asparagaceae* family and was first described in 1838. Sotol is perennial and polycarpic in nature, with a semicylindrical and apical morphology (Zuccarini, 1838). The name of the genus means "thick lily" and it has numerous pointed and thorny

grayish-to-green leaves measuring from 30 to 170 cm with a spoon shape in their lower part that grows from the base of the stem outward in a symmetrical way. Its stem is thick and fibrous, and up to 1 m in height (Sierra Tristán, 2008). It presents dioecious inflorescences with a single type of gametes in stamens (male scape) or pistils (female scape) that reach 3 m in height (Flores-Gallegos, 2019) (Figure 1).

The geographic distribution of sotol ranges from the southwestern United States to Oaxaca, Mexico. Sixteen species were originally described, most of which are endemic to Mexico (Bogler, 1995), and additional species were later identified in northeastern Mexico (Bogler, 1998). *Dasylirion cedrosanum* Trel. is the species with the greatest economic importance in Mexico, from which a drink called sotol is prepared via the fermentation of the stem of the plant (Hernández-Quintero, 2015). This drink is used for recreational purposes and as a medicinal remedy for diabetes and stomach ailments (Goverment of Mexico, 2022). The genus *Dasylirion* has rarely been studied at the cytogenetic level. Previous studies reported a diploid chromosome number of 38 in the species *Dasylirion texanum* and *Dasylirion wheeler* in specimens grown under greenhouse conditions, and also described multiple submetacentric chromosomes and two acrocentric chromosomes exclusively in *Dasylirion texanum* (Sato, 1935). For *Dasylirion cedrosanum* Trel., there is only one report of the gametic (n = 19) and somatic (2n = 38) chromosome number in plants collected in Saltillo, Coahuila, Mexico (Hernández-Quintero, 2015). Moreover, it has not been reported in the plant chromosomal number index (IPCN: Index to Plant Chromosome Numbers) of the Missouri Botanical Garden, USA, where most of the records of the chromosome numbers of various plant species worldwide can be found (Goldblatt, 2021).

Figure 1. *Dasylirion cedrosanum* Trel. (Photograph by Arturo Cruz Anaya, https://www.naturalista.mx/observations/21031288)

Using different cytogenetic staining techniques, the chromosomes of a species can be observed and characterized. The usual Giemsa and 4',6-diamidino-2-phenylindole (DAPI) staining allows the observation of the number and structure of each chromosome. The GTG banding technique (G bands with trypsin and Giemsa) helps to visualize the shape and pattern of the light bands (euchromatin) and dark bands (heterochromatin) present in each chromosome; and the CBW banding technique (C bands with barium and Wright's stain) specifically stains the centromeres and heterochromatic regions of the chromosomes (Barch, 1997).

The morphometric measurements of chromosomes are another important tool in this context. Images of the chromosomes are acquired under a bright-field or fluorescence microscope, and are analyzed using software that reports the results of the measurements in micrometers. The morphometric measurements that can be used are as follows: the total length of the chromosome (TCL), the length of the short (SAL) and long (LAL) arms, and the average length of the short (ASAL) and long (ALAL) arms. The centromere index (CI), which is the relationship between the length of the short arm of the chromosome and the TCL (Peruzzi, 2013), is expressed as a percentage (0%–50%). The relative longitude (RL), which is defined as the TCL divided by the sum of the total length of the karyotype, is also expressed as a percentage (Jabeen, 2012), whereas the fundamental number (FN) is the number of short and long arms present in a karyotype (Matthey, 1945; Matthey, 1965) and helps to determine the type of chromosomes present (Nirchio, 2014). The objective of this study was to obtain and characterize the chromosomes of *Dasylirion cedrosanum* Trel. to develop a karyotype of the species based on the morphological characteristics of its chromosomes.

MATERIALS AND METHODS

Collection site

We used *Dasylirion cedrosanum* Trel. seeds collected in the locality of Las Adjuntas, Santiago, Nuevo León, Mexico (25°18'03.6"N, 100°08'27.3"W) (Figure 2).

Preparation seeds

The seeds were washed and disinfected with 1% sodium hypochlorite (SIGMA-Aldrich, St. Louis, USA), and 10 seeds were placed in each petri dish (Pyrex, Tehama, CA, USA) in triplicate, and transferred to a bioclimatic chamber (Stemcells technologies, Vancouver, Canada) at 25-30°C for approximately 10 days for germination.

Cytogenetics

To obtain chromosomes, the technique of Hernández-Quintero et al. (2015), with some modifications, was used. The apical meristems were cut with a scalpel, preferably between 08:00 and 10:00 am, and incubated in 2% colchicine (Sigma-Aldrich) at 37°C for 48 h, followed by washing for 15 min with distilled water. Farmer's fixative solution (methanol:glacial acetic acid, 3:1) (CTR Scientific, Mexico) was then added, and the solution was incubated for 24 h at 4°C, washed in distilled water for 15 min, vortexed for 5 min, and centrifuged at 7440' *g* for 1 min. Subsequently, the supernatant was decanted and a mixture of pectinase (PlantMedia, Ohio, USA) and cellulase (PlantMedia) at a ratio of 1:1 at 0.2% was added, followed by resuspension of the pellet in distilled water and incubation at 37°C for 2 h. The cell button was dripped onto slides with a layer of glacial acetic acid, and heat was then applied.

Different staining and cytogenetic banding techniques were performed on the chromosomes obtained. 1) Giemsa staining: the chromosomes were incubated in Giemsa solution (Sigma-Aldrich) (1:20) for 5 min, rinsed in distilled water, and allowed to dry. 2) GTG banding: the chromosomes were incubated in 0.025 M trypsin at 37°C for 1 min, stained with Giemsa stain 1:20 for 5 min, rinsed, and allowed to dry. 3) CBW banding: the

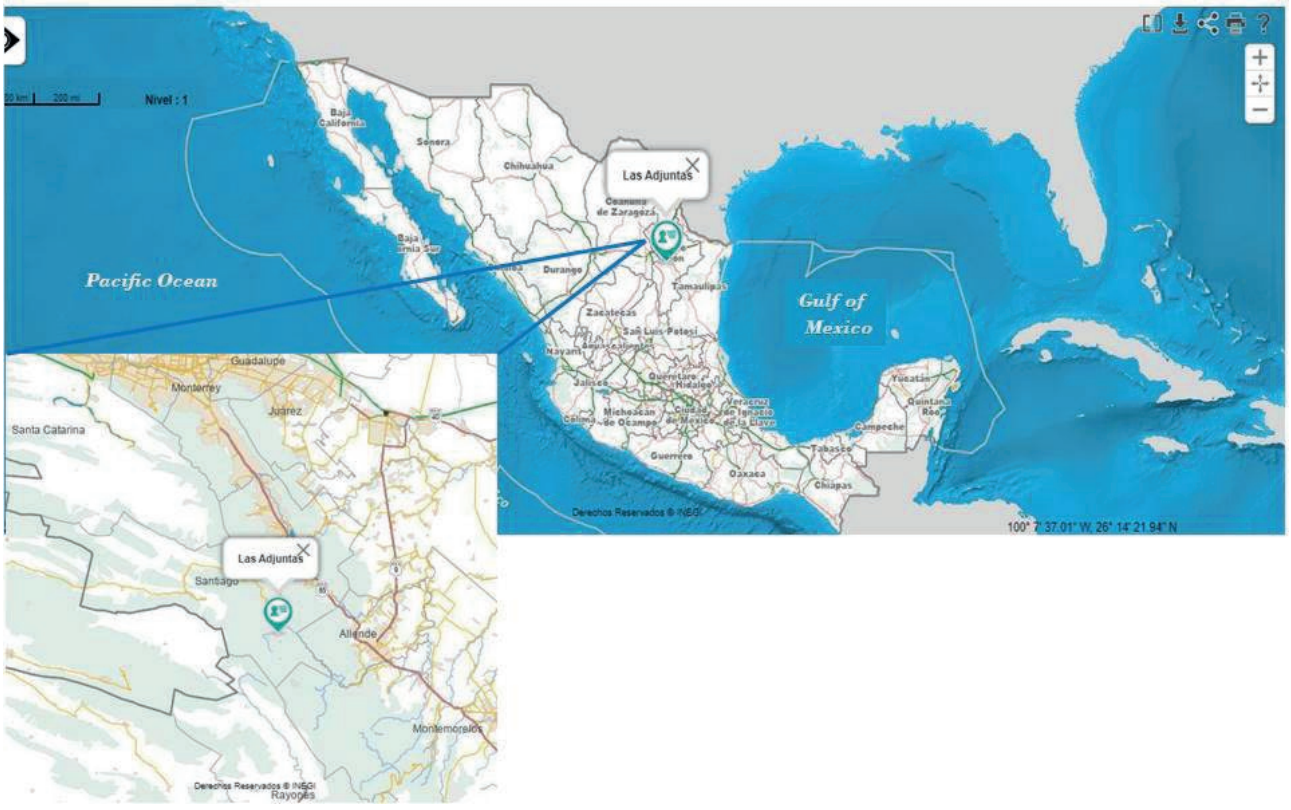

Figure 2. *Dasylirion cedrosanum* Trel. seed collection site, Las Adjuntas, Santiago, N.L., Mexico (25°18'03.6"N, 100°08'27.3"W), as indicated by the red globe (INEGI Map, 2021 https://www.inegi.org.mx/app/mapas/).

chromosomes were incubated in 0.2 N HCl (SIGMA-Aldrich) for 60 min at room temperature, rinsed with DNase-free water, and allowed to dry; then they were immersed in 5% BaOH (SIGMA-Aldrich) for 40 min and rinsed, passed through 70% and 100% ethanol, immersed in 2× SSC (3 M Sodium chloride and 300 mM Sodium citrate dihydrate, pH 7.0, SIGMA-Aldrich) for 60 min at 60°C, rinsed, dried, and stained with 1:3 Wright's stain for 2 min. 4) Fluorescent staining with DAPI (SIGMA-Aldrich): 7 µl of DAPI at 2.5 mg/ml were added to chromosomes, which were then incubated for 15 min at 4°C. The slides were observed using an AxioScope A1 microscope (Zeiss, Göttingen, Germany) with a 100× objective and coupled to an Axiocam 502 mono camera (Zeiss) coupled to Zen blue (version 3.3.89.0000) software (Zeiss). Images were acquired with an Axiophot HXP 120 V fluorescence lamp with a DAPI filter (Zeiss). The karyotype was organized based on the size of the chromosomes, from longest to shortest, in descending order, and pairing the homologues based on the observed GTG bands (Levan, 1964).

Morphometry

DRAWID (version 0.26) software (Kirov et al., 2017) was used to perform the morphological measurements of TCL, SAL, LAL, ASAL, ALAL and CI, and for the elaboration of the ideogram of the chromosomes. The images of the metaphases were opened in the software and each chromosome was measured, starting from the long arm toward the middle region, where the position of the centromere was marked (Centromere button), and continuing with the short arm at the lower end, thus ending the measurement. The process was repeated individually for each chromosome. RL was obtained using the formula: RL = (TCL / STCL) (100); TCL was obtained by adding the measurements of SAL and LAL; ASAL and ALAL were obtained based on the lengths determined for the 38 chromosomes; whereas CI was obtained from the formula: CI = SAL / (SAL + LAL). All measurements were performed individually for each of the 38 chromosomes in each of the cells analyzed. The FN of the karyotype was calculated using as a criterion the presence of two arms in the chromosomes. Descriptive statistics were applied, such as mean and standard deviation, using SPSS Statistics, version 22 (IBM, Armonk, NY, USA).

RESULTS

Cytogenetics

Thirty metaphases were analyzed, which led to the identification of 38 chromosomes (2n) in 100% of the analyzed cells. All chromosomes were of the submetacentric type, that is, with the centromere displaced toward one of the ends, in which the arms differed in length. Using the GTG banding technique, it was possible to establish a pattern of bands in each chromosome, with the presence of dark bands in the telomeric regions of most of them. Based on the GTG banding patterns, the karyotype depicted in Figure 3 was established.

Using CBW banding, the centromere was observed in a displaced position toward one of the ends of the arms of the chromosomes. Concomitantly, heterochromatin regions were observed at the ends of some chromosomes (Figure 4).

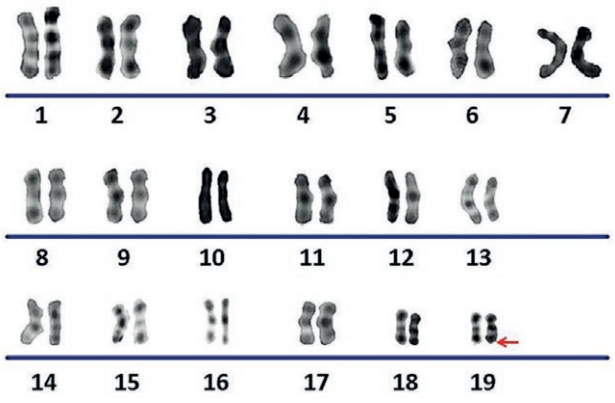

Figure 3. Karyotype of *Dasylirion cedrosanum* Trel. stained with GTG banding. The pattern of bands in each chromosome and the dark bands in the telomeric region (arrow) are shown.

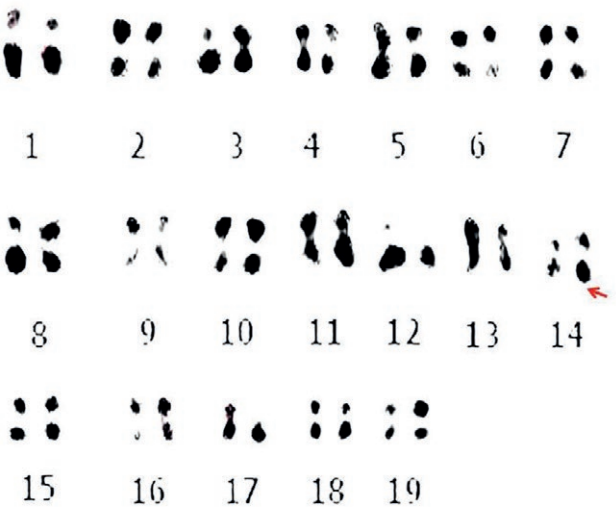

Figure 4. Chromosomes of *Dasylirion cedrosanum* Trel. stained with CBW banding, in which the presence of telomeric heterochromatin is confirmed (arrow).

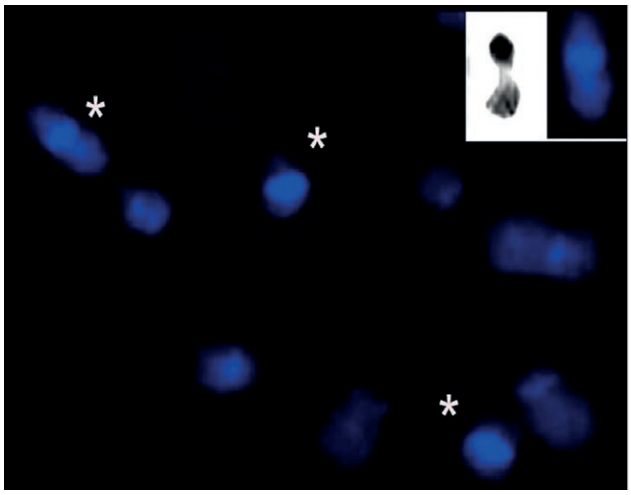

Figure 5. Chromosomes of *Dasylirion cedrosanum* Trel. stained with DAPI. More intensely marked areas of constitutive heterochromatin can be observed (chromosomes marked with an asterisk). In the upper-right corner, the CBW bands can be compared with the zones detected using DAPI, thus confirming the distribution in the chromosomes of the constitutive heterochromatin.

Visualization using DAPI allowed us to observe the morphology of the chromosomes in greater detail, while also confirming their number and submetacentric shape (Figure 5).

Morphometry

TCL average of the chromosomes was in the range of 5.09 mm for the shortest chromosome and 9.84 mm for the longest. The remaining chromosomes ranged from 5.47 to 8.62 mm in length. The average of the SAL indicated a length of 2.96 ± 0.58 mm, whereas the average of the LAL was 3.93 ±0.73 mm. The RL average of the 38 chromosomes ranged from 1.94% to 3.76%, whereas the CI average was between 39.98% and 44.66 %. FN of the karyotype of *Dasylirion cedrosanum* Trel. was 76, considering as a criterion that each chromosome had two arms (Table 1).

Based on the results of staining, banding, and previous measurements, and using the image analysis software, an ideogram of the *Dasylirion cedrosanum* Trel. chromosomes was constructed with GTG bands (Figure 6).

Table 1. Mean length of short arm chromosome (SAL), long arm chromosome (LAL), total arm chromosome (TCL), relative length (RL), and centromeric index (CI) from 30 metaphases of *Dasylirion cedrosanum* Trel (2n=38).

Chromosome	SAL x̄±SD (µm)	LAL x̄±SD (µm)	TCL x̄ (µm)	RL %	CI %	Type of chromosome
1	4,44±1,42	5,79±1,63	9,84	3,76	44,66	Submetacentric
2	3,66±0,44	5,06±1,44	8,62	3,29	43,76	Submetacentric
3	3,81±1,25	4,54±0,46	8,30	3,17	43,24	Submetacentric
4	3,48±0,83	4,64±0,84	8,01	3,06	42,21	Submetacentric
5	3,48±0,77	4,31±0,68	7,72	2,95	43,31	Submetacentric
6	3,26±0,72	4,32±0,64	7,52	2,87	43,64	Submetacentric
7	3,04±0,84	4,23±0,45	7,19	2,74	43,11	Submetacentric
8	2,77±0,66	4,22±0,51	6,96	2,66	39,98	Submetacentric
9	2,73±0,30	4,07±0,90	6,77	2,59	41,96	Submetacentric
10	2,89±0,49	3,78±0,66	6,64	2,54	44,47	Submetacentric
11	2,81±0,47	3,74±0,66	6,53	2,49	43,34	Submetacentric
12	2,80±0,63	3,63±0,52	6,41	2,45	42,57	Submetacentric
13	2,58±0,27	3,72±0,76	6,26	2,39	42,73	Submetacentric
14	2,70±0,49	3,48±0,57	6,16	2,35	43,12	Submetacentric
15	2,66±0,33	3,38±0,70	5,99	2,29	43,27	Submetacentric
16	2,39±0,10	3,48±1,03	5,84	2,23	41,65	Submetacentric
17	2,31±0,70	3,44±0,34	5,69	2,17	41,64	Submetacentric
18	2,49±0,56	3,02±0,44	5,47	2,09	43,79	Submetacentric
19	2,23±0,60	2,94±0,52	5,09	1,94	43,85	Submetacentric
ASAL= 2.96±0.58		ALAL=3.93±0.73			ΣTCL=261.88	

SD = Standard deviation, ASAL= average short arm, ALAL= average long arms.

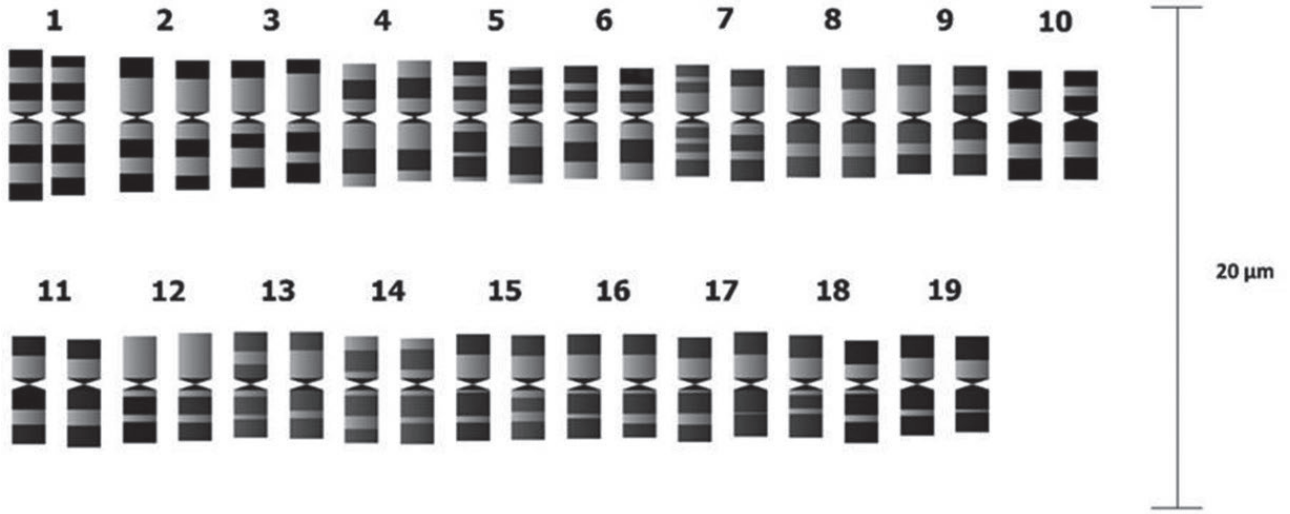

Figure 6. Ideogram of the chromosomes of *Dasylirion cedrosanum* Trel., with GTG bands.

DISCUSSION

In the present work, we found a somatic number of 38 chromosomes in the studied specimens of *Dasylirion cedrosanum* Trel. These results coincide with those reported by Hernández-Quintero et al. (2015), thus reaffirming that the chromosome number is highly conserved among the species of the genus *Dasylirion* (Sato, 1935). In some plant species, there is a natural selection process, karyotypic orthoselection, which consists of the presence of the same type of chromosome rearrangement in specimens of the same species, thus maintaining the chromosome number, which is involved in the process of evolution (Palomino, 2010). This process occurs via the amplification of noncoding regions of DNA at chromosome crossover sites, resulting in a uniform basic number and karyotypes that maintain chromosome number and structure (Flores-Maya et al., 2015). Additional cytogenetic and molecular biology studies are required to determine if the same process is present in *Dasylirion cedrosanum* Trel.

In plants, it is difficult to obtain chromosomal bands because of the presence of a cell wall and the characteristics of the tissue (Chattopadhyay, 1988). In plant chromosomes, the pattern of GTG bands observed in mammals has not been reported. However, in this work, modifications of the original technique allowed the resolution of light and dark chromosomal bands, leading to the identification of seven bands on large chromosomes and five bands on smaller chromosomes. In addition, dark telomeric bands were detected in most of the chromosomes, which represents the first report of a banding pattern for this species. Previous work on *C. pubescens* indicated the presence of dark bands at the telomere level (Guevara, 2000) and suggested that these bands represent the constitutive heterochromatin—as corroborated here in *Dasylirion cedrosanum* Trel.—with the implementation of the CBW bands. Plant chromosomes contain much more DNA than do vertebrate chromosomes, with a comparable length and with a higher degree of compaction, which explains the presence of these dark bands at the telomere level (Argüelles Saenz, 2018).

Using different cytogenetic banding techniques and morphometric analysis, we established that all chromosomes were of the submetacentric type, which differs from the report of subtelocentric chromosomes previously (Hernández-Quintero et al., 2015). Therefore, it was possible to establish an FN of 76 for *Dasylirion cedrosanum* Trel., and represents the first report defining the FN in this species. The TCL of the chromosomes was significantly higher than those obtained in previous work (Hernández-Quintero et al., 2015). Although hydroxyquinoline is widely used in plants and is especially suitable for species with large chromosomes (Sharma, 2014), in the present work, colchicine was used as an antimitotic agent, with modification of the exposure time and concentration, and afforded more elongated chromosomes, which assisted observation of the bands in each of them. The CI average was between 39.98 % and 44.66 %, and the RL was between 1.94 % and 3.76 % for each chromosome, which can be considered as an approximation of the contribution of each of them to the total content of the *Dasylirion cedrosanum* Trel. genome.

Although all chromosomes present in the karyotype of *Dasylirion cedrosanum* Trel. were of the submetacentric type, in some plant species, transcriptional activity has been observed in nonacrocentric chromosomes. As a future perspective of this study, AgNORs banding or fluorescent *in situ* hybridization using specific probes for the 5S and 45S regions should be carried out.

It is important to determine the number of chromosomes and the ploidy level of a species, especially in those of economic importance. In the case of *Dasylirion cedrosanum* Trel., the lack of cytogenetic studies is well known. To our knowledge, this is the first time that chromosomal characteristics have been reported and a karyotype constructed for this species in specimens from the state of Nuevo León. In addition, these data are very useful in the phylogenetic and taxonomic study of a species to analyze the evolutionary mechanisms involved in speciation and diversity.

AUTHOR'S CONTRIBUTIONS

C.G.V, E.I.C.G and S.M.L designed the study, K.R.M. and C.G.V. performed analyses, K.R.M., C.G.V, E.I.C.G and S.M.L collected data, K.R.M., C.G.V and E.I.C.G led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

GEOLOCATION INFORMATION

https://www.journalmap.org/search#list?bounds=57.98481,164.35547|-24.36711,44.47266&precision=1&query=sotol

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