

## Sex ratio and spatial distribution of pistillate and staminate plants of *Dasyilirion cedrosanum*

### Proporción de sexos y distribución espacial de plantas pistiladas y estaminadas de *Dasyilirion cedrosanum*

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**Abstract.** The genus *Dasyilirion* forms a group of well-represented perennial, dioecious species called “sotoles” in Mexico. This genus is a main component of the arid lands of northern Mexico and southern United States, and plays important ecological and economic roles. Four *D. cedrosanum* populations from northeastern Mexico were surveyed to gather statistics related to sex ratios and spatial distribution. The statistics were performed on an unbiased sample of 448 plants. Tests for sex ratio and spatial distribution were carried out. No evidence of departure from a 1:1 sex ratio proportion was found, both at the global level and within sampling sites. Patterns of spatial sex segregation were absent for all locations. One plant in the global sample could not be classified either as male or female, being anatomically andromonoecious. Both, the staminate and hermaphroditic flowers of this plant produced well-developed pollen. However, the hermaphroditic flowers were unable to produce fruits, even in the presence of pollen sources. The results are consistent with the expected for a well-established dioecious condition at the evolutionary scale, without environmental influences affecting the sex proportions and their spatial distribution.

**Keywords:** Arid land plants; Dioecy; Sex proportion; Sex distribution; Andromonoecy.

**Resumen.** El género *Dasyilirion* forma un grupo bien representado de especies perennes y dióicas, llamadas “sotoles” en México. Este género es un importante componente de las zonas áridas del norte de México y sur de los Estados Unidos, y juega importantes papeles ecológicos y económicos. Cuatro poblaciones de *D. cedrosanum* del noreste de México fueron monitoreadas para obtener estadísticas relacionadas con la distribución espacial y proporción de sexos. Los análisis estadísticos se llevaron a cabo en una muestra no sesgada de 448 plantas. Se hicieron pruebas de proporción de sexos y su distribución espacial. No se encontró evidencia de que exista desviación de una proporción de sexos 1:1 tanto a nivel global como dentro de localidades. Tampoco se encontraron patrones de segregación espacial de sexos. Una planta en la muestra global no pudo ser clasificada como masculina o femenina, ya que tuvo características anatómicas de andromonoica. Las flores estaminadas y hermafroditas de esta planta produjeron polen bien desarrollado. Sin embargo, las flores hermafroditas fueron incapaces de producir frutos, aún en la presencia de fuentes de polen. Los resultados son consistentes con lo esperado para una condición dióica bien establecida en la escala evolutiva, sin efectos ambientales en la proporción de sexos y su distribución espacial.

**Palabras clave:** Plantas de zonas áridas; Dioecia; Proporción de sexos; Distribución de sexos; Andromonoecia.

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## INTRODUCTION

The great majority of flowering plants, around 90%, produce hermaphroditic flowers, which are both staminate and pistillate. Nearly 10% have evolved floral unisexuality, where the male and female organs are carried on separate flowers on the same plant (monoecy) or on separate individuals (dioecy). The later reproductive case, dioecy, involves the presence of individuals with differential sexual roles, either male (staminate) or female (pistillate). However, a number of other sex states or breeding systems exist, that may be intermediate forms during the evolution from hermaphroditism to full unisexuality. Such is the case of ginodioecy, in which populations are composed of female and hermaphroditic plants, or androdioecy, composed by male and hermaphroditic individuals (Ainsworth, 2000).

In dioecious populations, the allocation of sexes in different plants ensures that sexual reproduction occurs between different individuals, thus promoting outcrossing. Furthermore, dioecy allows the allocation of different physiological and morphological traits between sexes, with the consequence of a reproductive specialization and differential investment of resources.

Dioecy is a relatively rare phenomenon in plants. It occurs on 9-10% in terrestrial plants, with approximately 6% incidence in flowering plants (Ainsworth, 2000), and it is patchily distributed among nearly 43% of plant families (Renner, 2014). It tends to be more prevalent in trees and climbing plants than in herbs and shrubs (Ming et al., 2011).

Theory claims that, generally, there are two genomic regions that determine dioecy, a male fertility and a female suppressing region. In species with well-established dioecy these two regions are tightly linked, exhibiting recombination suppression. Absence of recombination suppression promotes the existence of male, female and hermaphroditic plants, as a sign of dioecy in an early stage of evolution (Dellaporta & Calderon-Urrea, 1993).

Sexual dimorphism, arising from dioecy, can be of two types. The primary type refers to the anatomical differentiation in sexual organs between male and female plants, whereas the secondary type refers to differences in structures other than sexual organs (Barrett & Hough, 2012). The secondary sexual dimorphism has a low rate of occurrence. As a result, the sex of the individual plants cannot usually be determined before flowering. Sexual dimorphism in vegetative traits is associated with contrasting strategies of the sexes. Furthermore, a high investment in fruits and seeds can lead to higher rates of mortality in females compared to males, which can bias sex ratios. However, in some wind-pollinated plants, male reproductive costs may match or exceed those of females because of the high investment in nitrogen-rich pollen (Barrett & Hough, 2012).

The theoretical foundation of the sex ratio was first established by Fisher (1930). It states that under equal reproductive costs to produce female versus male offspring, natural

selection will act in the direction of balancing the sex ratio of a population to unity. Despite sex ratios are often close to unity (Dellaporta & Calderon-Urrea, 1993), they might deviate from it, with a bias to males being more common (Barrett & Hough, 2012). The frequent occurrence of male-biased sex ratios is likely to be associated with the greater reproductive investment of females. It has been predicted that long-lived species that experience repeated reproductive episodes should be more likely to develop male-biased sex ratios, which may be especially strong with large investment in fleshy fruits (Barrett & Hough, 2012). Thus, it seems that differences in the degree of sexual dimorphism in reproductive expenditure may influence the sex ratio variation among angiosperm species. Furthermore, some patterns in sex ratio bias seem to exist; for example, an association between male bias and insect-pollinated vines with bird or mammal dispersed fruits was found (Sinclair et al., 2012). Studying sex ratios in dioecious plants is important because this information can provide important clues about the stability and evolutionary timing of dioecy, about biological phenomena related with differential energy contribution between both sexes, and possible environmental influences on sex determination. One of the implications of a departure from the 1:1 sex ratio is its impact on the reduction of the effective population size, which can negatively affect the maintenance of genetic variation (Wright, 1931; Nei, 1975).

A population-level phenomenon that can be encountered in dioecious plants is the so-called spatial segregation of sexes (SSS) (Dawson & Geber, 1999). When it occurs, the spatial structure of the relationship between sexes is not random, but there is a tendency of individuals of the same sex to agglomerate. In a review by Bierzychudek and Eckhart (1988), an SSS pattern was found in 21 of the 32 studied species. Also, in more than 30 dioecious species from 20 families, male-biased sex ratios have been found in more stressful sites. For instance, in the water tupelo tree (*Nyssa aquatica*), it was found that there were more male trees in the shallowest plots; however, there was no evidence of departures from random association of sexes at the smallest spatial scale (i.e., among nearest neighbors: Shea et al., 1993). Spatial segregation of sexes along some environmental gradients is an evidence of ecological differences in the context of males and females in dioecious plants. Other studies have concluded that the non-random distribution of sexes in a given habitat is an evolved response to competition between males and females. The mechanisms that give rise to SSS can be summarized as follows: (i) differential mortality between males and females in different environmental patches, (ii) the ability of individuals to vary their sex according to their physiological condition or environment, the so-called “sex choice”, (iii) skewness of sex ratio in progenies caused by female parents at different sites, the so-called “maternal adjustment”, (iv) active habitat selection by males and females, and (v) differential requirements of male and female seeds (Bierzychudek & Eckhart, 1988).

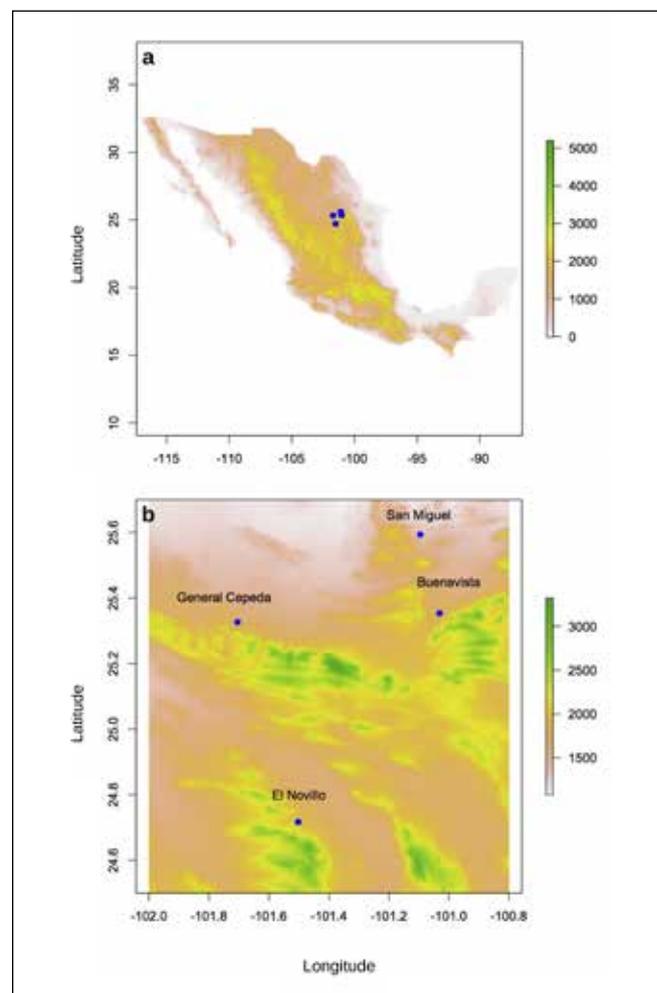
A dioecious genus that has not been studied in relation to sex ratios and spatial sex segregation is *Dasyilirion*. It belongs to the Asparagaceae family and includes 17 species, with *D. micropterum* being the most recently described (Villarreal-Quintanilla et al., 2016). This genus occurs in arid and semiarid lands of north-central Mexico and southwestern United States. Plants are perennial, polycarpic, and bear a rosette of prickly and fibrous leaves and elongate dense inflorescences (Bogler, 2002). Chromosome numbers have been documented for *D. wheeleri*, *D. texanum* (Sato, 1935) and *D. cedrosanum* (Hernández-Quintero et al., 2015), with a homogenous number  $2n = 2x = 38$  and with normal diploid meiotic pairing behavior. However, no evidence has been found of heteromorphic sex chromosomes. Furthermore, male and female plants cannot be distinguished before the flowering stage either morphologically or at the level of chemical composition (Cruz-Requena et al., 2013; Reyes-Valdés et al., 2013).

*Dasyilirion cedrosanum* Trel. is the most abundant species of the genus and occurs in northeastern Mexico, mainly in south-central Coahuila and adjacent areas of the states of Zacatecas and Durango (Villarreal-Quintanilla, 2001). In the state of Coahuila, *D. cedrosanum* grows in mountains and hilly terrains, between 850 and 2,600 m above sea level, in environmental conditions typical of the Chihuahuan Desert (Encina-Domínguez et al., 2013). The only study aimed at understanding dioecy in *Dasyilirion* focuses on the association of phytohormones with sexual development (Rivas-Martínez et al., 2016). There are no systematic studies on frequency and spatial distribution of staminate and pistillate flowers in this genus. The aim of this work was to contribute to the knowledge of dioecy in *Dasyilirion*, through the study of the sex ratio and sex distribution of *D. cedrosanum* in the south-eastern region of the state of Coahuila in Mexico and its adjacent territory in the state of Zacatecas.

## MATERIALS AND METHODS

**Field sampling.** Sotol (*D. cedrosanum*) plants were sampled across four locations in northeastern Mexico; three in the state of Coahuila and one in the state of Zacatecas, with an elevation range from 1,379 m to 1,972 m.a.s.l. (Fig. 1 and Table 1). Data were gathered from 2010 to 2015, and included position (latitude, longitude and elevation) and sex of each individual plant, as well as exposure and average slope for each location. Position data were recorded through a Garmin GPSmap78. All plants were tagged with unique identifiers to keep track of them during the six years of study. The sampling areas consisted of rectangles with areas from 23,625 m<sup>2</sup> to 160,000 m<sup>2</sup>, depending on the size of the population of *D. cedrosanum* at each location. Each location comprised one rectangle, with the exception of Buenavista, where two rectangles were used (Table 1).

The sampled areas at San Miguel, El Novillo and General Cepeda were undisturbed from either urbanization or agricultural activity. The Buenavista sites were areas that belonged to (1) a University campus (Universidad Autónoma Agraria Antonio Narro); here one sampling rectangle was placed in an uncultivated area with a naturally growing population of *D. cedrosanum*, and (2) the botanical garden “Gustavo Aguirre Benavides”; this area contained mainly Chihuahuan Desert species, and where most *D. cedrosanum* plants have grown from natural seed germination. This area was constantly surveyed and weeded. A total of 835 plants were recorded and



**Fig. 1.** Location of the study sites with color-coded elevations scaled in meters. (a) Study sites in the context of the Mexican territory. (b) Close-up map of the region of study with the four locations. Buenavista, General Cepeda and San Miguel are in the state of Coahuila, whereas El Novillo is in the state of Zacatecas.

**Fig. 1.** Sitios de estudio con elevaciones codificadas con color y escala en metros. (a) Sitios de estudio en el contexto del territorio Mexicano. (b) Acercamiento de la región de estudio señalando las cuatro localidades. Buenavista, General Cepeda y San Miguel pertenecen al estado de Coahuila, mientras que El Novillo pertenece al Estado de Zacatecas.

**Table 1.** Geographic locations of the sampled *D. cedrosanum* populations, along with the sizes of the study areas.**Tabla 1.** Ubicaciones geográficas de las poblaciones de *D. cedrosanum* muestreadas, y los tamaños de las áreas de estudio.

Location	State	Latitude (N)	Longitude (W)	Elevation (m)	Sampling area (m <sup>2</sup> )
Buenavista	Coahuila	25° 21.22 '	101° 01.87 '	1794	210,000
El Novillo	Zacatecas	24° 42.99 '	101° 30.15 '	1972	25,000
General Cepeda	Coahuila	25° 19.67 '	101° 42.55 '	1824	23,625
San Miguel	Coahuila	25° 35.62 '	101° 05.70 '	1379	24,000

tagged at the four locations; sampled individual records were rigorously filtered to avoid bias in the sex ratio estimation, as described in the data handling and statistical analysis section.

The climatic and geospatial characteristics of the four locations were as follows. **Buenavista:** This is a University campus (Universidad Autónoma Agraria Antonio Narro) located in the Saltillo Municipality of the state of Coahuila; mean annual temperature = 18.9 °C, with a maximum monthly average of 27.5 °C and minimum of 10.4 °C; mean annual precipitation = 442 mm; NW exposure, slope 3.1%. **El Novillo:** This is a hill by a small dirt road in the Mazapil Municipality of the state of Zacatecas, close to a small town called Los Novillos; mean annual temperature = 18.8 °C, with a maximum monthly average of 28.1 °C and minimum of 9.5 °C; mean annual precipitation = 359 mm; E exposure, slope 9.3%. **General Cepeda:** This is a hill by a secondary road in the General Cepeda Municipality of the state of Coahuila, close to Ejido El Tejocote; mean annual temperature = 20.3 °C, with a maximum monthly average of 27.7 °C and minimum of 13.1 °C; mean annual precipitation = 303 mm; SW exposure, slope 7.3%. **San Miguel:** This is a hill by a secondary road in the Ramos Arizpe Municipality of the state of Coahuila, close to Ejido San Miguel; mean annual temperature = 19.5 °C, with a maximum monthly average of 27.6 °C and minimum of 11.4 °C; mean annual precipitation = 464 mm; NE exposure, slope 17.5%. Climatic data for these sites were obtained from Worldclim v. 1.4. (<http://www.worldclim.org/current>), whose information spans 50 years (1950-2000). For each location in Table 1, the raster of climatic variables were extracted to characterize the sampling sites (García, 2004; Hijmans et al., 2005).

Considering that we found an unusual plant from the reproductive standpoint, the question about its reproductive potential remained to be solved. Particularly, it was interesting to know whether it was a functionally andromonoecious individual or if it was just an anatomical irregularity. To evaluate male fertility, we tested pollen development through staining as follows: flower fascicles were collocated in Petri dishes to extract the pollen grains. The grains were picked with a paintbrush, and deposited in a microscope slide; after adding a drop of acetic carmine stain (1%) it was covered with a coverslip. After 10 to 15 seconds, the preparation was observed in a compound Carl Zeiss microscope, photographed with a Pixera Winder Pro camera, and analyzed with the measuring

software Axion Vision v. 4.8. When pollen grains were rounded and red stained they were considered as well-developed meiotic products (Stone et al., 1995).

**Data handling and statistical analyses.** Data formatting and analysis were carried out with the language and environment for statistical computing R (R Core Team, 2016). Plant sex can be determined only through visual examination of the inflorescences, but single field surveys can lead to biased results, due that female and male inflorescence have different periods of appearance and times of persistence, and most plants are not flowering in a given year. To avoid any source of bias, datasets were filtered, keeping only records fulfilling the following criteria: (i) the year of flowering was recorded, (ii) the year when sex was recorded corresponded to the year of flowering, and (iii) the sex record was the result of at least a whole year's survey. Under those requirements, the data set of 835 plants was reduced to a highly reliable set of 448 plants.

A hypothetical departure from a 1:1 sex ratio was evaluated through a binomial test (Clopper & Pearson, 1934), which was applied to the global counts of pistillate and staminate plants, complemented by an estimate of the proportion confidence interval. To evaluate the heterogeneity of sex ratios among locations, a Pearson Chi-square test was performed, with a *p*-value computed by a Monte Carlo simulation with 2,000 replicates. To complement the heterogeneity test, sex ratios were analyzed for each population through the binomial test.

To evaluate the relative spatial distribution of sexes within each location, a procedure was devised based on a randomization test (Manly, 2006). In order to have accurate three-dimension positions, the individual geographic coordinate data were used to estimate the elevations through the R package for geographic data analysis and modeling *raster* (Hijmans et al, 2016) with bilinear interpolation. Afterwards, coordinate data were converted to UTM scaled in meters through the R package *PBSmapping* (Schnute et al., 2015). With the individual plant locations, a randomization test in the three-dimension space was implemented as follows: for each location, the sex labels for the individual plants were randomized 999 times, and for each randomization the average distance to the nearest neighbor of the same sex was calculated. The experimental mean distance to the nearest

plant with the same sex was added to the 999 values, based on the nonrandomized data, thus obtaining a set of 1000 values to generate an empirical distribution of the mean distance to the nearest neighbor of the same sex, under the null hypothesis of absence of spatial sex segregation. The quantile position of the experimental mean in the empirical distribution was used to estimate the  $p$ -value. The rationale behind this novel test method is that, if individuals of the same sex tend to agglomerate, then the distance between neighbor individuals of the same sex will shorten. Particularly, the average distance from each plant to the nearest individual of the same sex would be reduced. Since there is no theoretical expected distribution of the mean of the nearest individual of the same sex under the null hypothesis of absence of spatial sex segregation (SSS), the randomization test provides an empirical distribution for the null hypothesis, which allows to perform a statistical test. Numerical approaches like Monte Carlo, bootstrap and randomization methods have proved to be highly valuable in the analysis of biological data (Manly, 2006).

Nearest neighbor distance calculations were carried out through the R package *FNN* (Beygelzimer et al., 2013). All the hypotheses tests were complemented with exploratory analyses, based on bar plots, histograms, and two and three-dimensional plots of spatial distribution.

## RESULTS

**Sex proportions.** The close-up map (Fig. 1b) shows that the four study sites were adjacent to mountains with elevations higher than 2000 m.a.s.l. From the 448 individual plant records obtained from those locations, we estimated a global proportion of 47.54% female plants and 52.23% male plants (Table 2). Among the studied areas we discovered in Buenavista an andromonoecious plant (Table 2, see below). Being a single observation and, as we will see below, a plant that seems to be functionally a male, we did not consider it in the test for sex bias. The lowest proportion of females was observed at General Cepeda with 42.71%, and the highest at El Novillo, with

57.14% (Table 2 and Fig. 2). General Cepeda and San Miguel had almost the same estimates for pistillate plants, with 42.7 and 42.3%, respectively, despite the fact that they were the most contrasting sites regarding precipitation. The estimates closest to a 1:1 proportion were observed at Buenavista (Fig. 2), where the largest sample size was obtained; this was also the best surveyed population across the study period, given that it was located at the University campus.

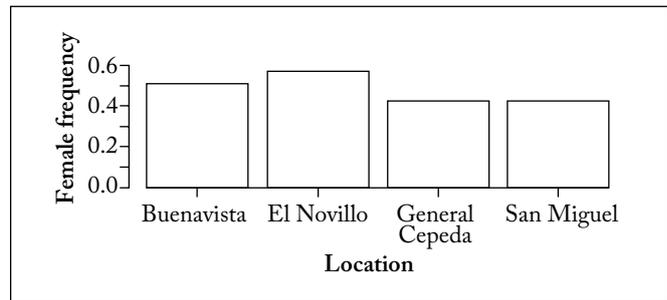


Fig. 2. Female frequencies of *D. cedrosanum* plants across four locations.

Fig. 2. Frecuencias de plantas femeninas de *D. cedrosanum* en cuatro localidades.

The global binomial test for the proportion of pistillate and staminate plants gave a non-significant  $p$ -value of 0.3442, with a 95% confidence interval for the proportion of female plants of 42.94% - 52.40%; thus the null hypothesis of 1:1 sex ratio was not rejected. Regardless of the differences in sample estimates among locations, the Chi-square test with Monte Carlo simulation for a possible heterogeneity of sex ratios gave a  $p$ -value of 0.1869, with no statistical evidence of differences in sex proportions among the four locations. Despite the fact that the non-significant result of the heterogeneity test indicated by itself the absence of departure from the 1:1 sex ratio within locations, individual binomial tests were run for the study sites. The results showed absence of sex bias in all locations, with the minimum Type I error probability ( $p = 0.1285$ ) obtained for San Miguel, and the maximum ( $p = 0.825$ ) for Buenavista.

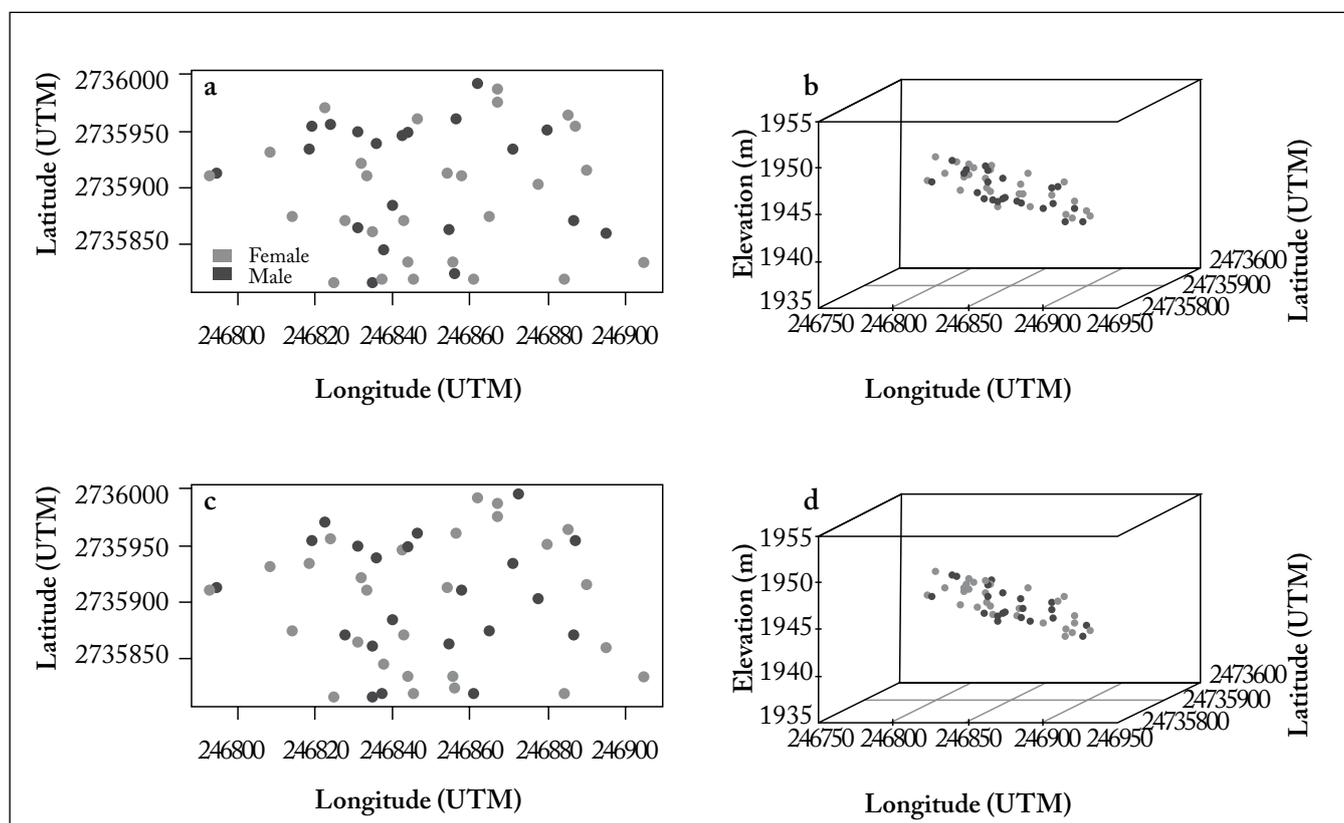
Table 2. Number and percentages of plants of each sex of *D. cedrosanum* in each of the four study sites. Data were gathered from the year 2010 to 2015.

Tabla 2. Números y porcentajes de plantas de cada sexo de *D. cedrosanum* en cada una de las cuatro localidades estudiadas. Los datos fueron obtenidos del año 2010 al 2015.

Location	Female	Female (%)	Male	Male (%)	Andromonoecious	Andromonoecious (%)
Buenavista	94	50.8	90	48.7	1	0.5
El Novillo	28	57.1	21	42.9	0	0.0
General Cepeda	44	42.7	59	57.3	0	0.0
San Miguel	47	42.3	64	57.7	0	0.0
Global	213	47.6	234	52.2	1	0.2

**Spatial sex distribution.** To visualize sex distribution in *D. cedrosanum*, Figure 3a depicts plant positions, color-coded by sex on the horizontal plane, while Fig. 3b represents them in a three-dimension space, at “El Novillo”. There is neither an evident pattern of spatial sex segregation nor a trend relative to elevation, although some rows of plants of the same sex can be misleading. Fig. 3b and 3c depict spatial distribution of the same plants, but with sex labels randomly permuted to generate the empirical distributions under the null hypothesis of absence of spatial sex segregation, which was the basis for the randomization test. Again, some apparent rows of plants of the same sex show up, which are obviously artifacts. The absence of spatial sex segregation was confirmed with the randomization tests, where the experimental mean distances to the nearest individual of the same sex appear in all cases as typical points in the empirical distributions for the null hypothesis of absence of spatial sexual segregation (Fig. 4). If spatial sexual segregation occurred, the triangles in the his-

tograms would be placed significantly to the left, indicating a lower than expected mean distance to the nearest individual of the same sex. The one-tailed P-values ranged from 0.086 in San Miguel to 0.884 in General Cepeda; it means that the hypothesis of random spatial association of sexes cannot be rejected. Furthermore, two locations had the observed distance to the nearest neighbor of the same sex to the left of the distribution, and two to the right, which is also a good indication of absence of a non-random pattern. Two locations, El Novillo and Buenavista, had an estimated distance nearly at the center of the empirical, randomization-based distribution. The global one-tailed P-value, based on a pooled dataset, was 0.455. Thus, there is no evidence of spatial sex segregation. Even when the San Miguel site, with  $P = 0.086$ , is in appearance marginally significant, this is not the case. This is because this is one of a set of four independent tests, and with the Bonferroni approximation a P value of  $0.05/4 = 0.0125$  would be sought to declare a case as significant with  $\alpha = 0.05$ .



**Fig. 3.** Spatial distributions of sexes in plants of *D. cedrosanum* at El Novillo. (a) Horizontal spatial distribution of sexes in UTM coordinates scaled in meters. (b) Three-dimensional representation of the distribution of sexes on the hill at the study site. (c) Horizontal sex distribution after one random permutation of sex labels in the same location, as a part of the randomization approach to test the hypothesis of absence of spatial association of sexes. (d) Three-dimensional distribution of sexes after random permutation.

**Fig. 3.** Distribución espacial de sexos en plantas de *D. cedrosanum* en El Novillo. (a) Distribución espacial horizontal de sexos en coordenadas UTM con escala en metros. (b) Representación tridimensional de la distribución de sexos en el gradiente de altura del sitio de estudio. (c) Distribución horizontal de sexos después de una permutación aleatoria de las etiquetas de sexo en la misma localidad, como parte del enfoque de aleatorización para probar la hipótesis de ausencia de asociación espacial de sexos. (d) Distribución tridimensional de sexos después de una permutación aleatoria.

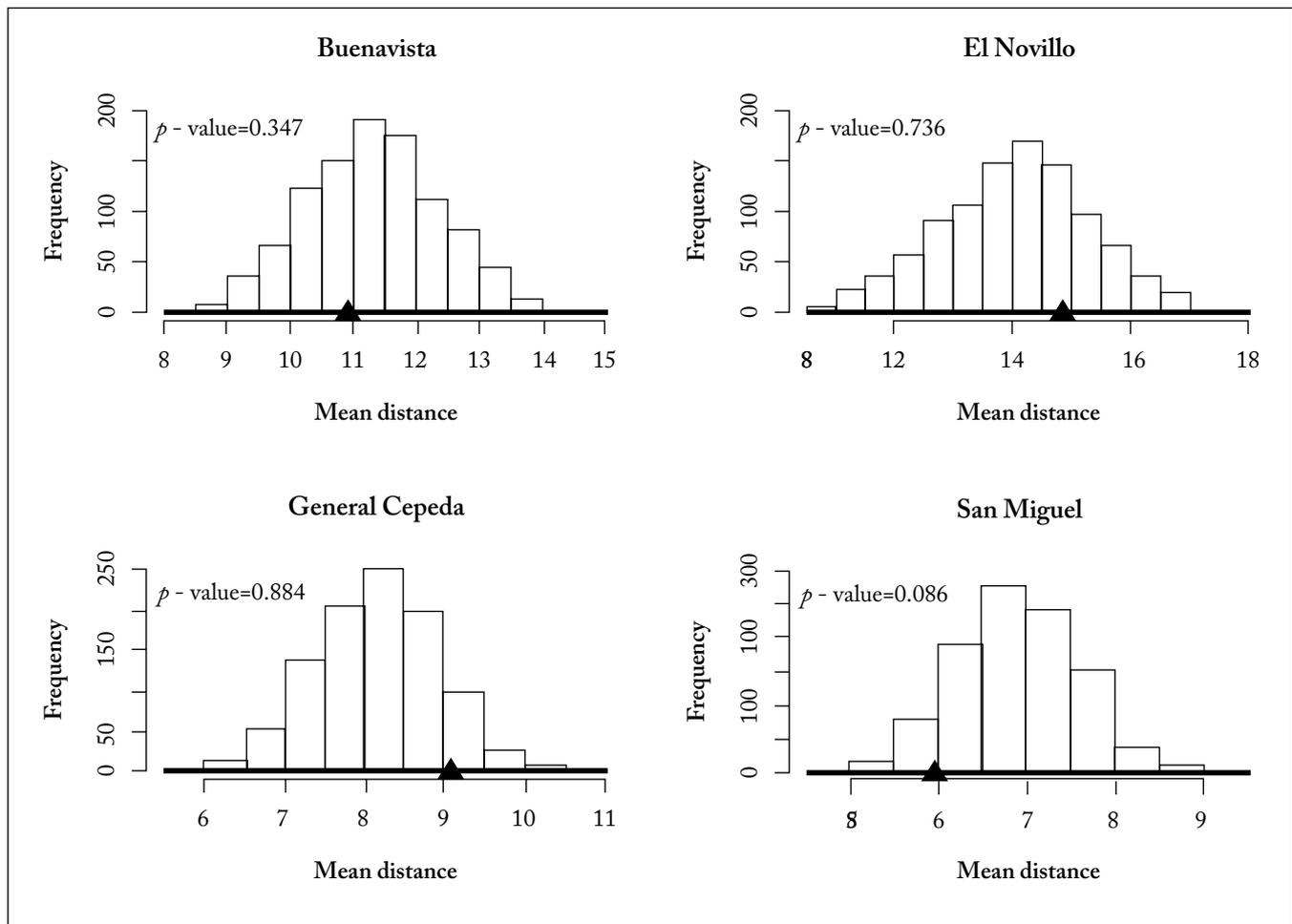


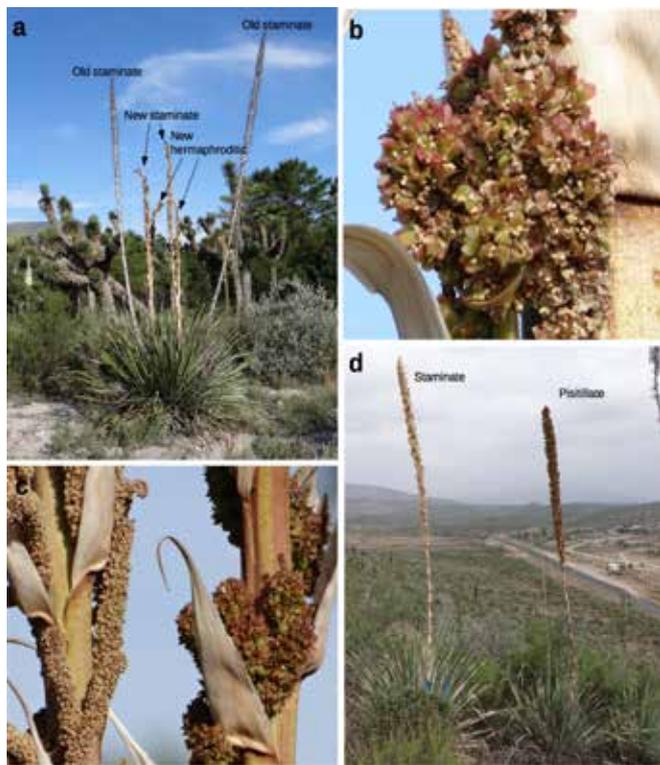
Fig. 4. Empirical distributions of the distance to the nearest-neighbor of the same sex, generated through random permutations of sex labels in each of the four locations. The triangles represent the observed distance in each sample. Note the change of scale in the X and Y axes.  
 Fig. 4. Distribución empírica de la distancia al vecino más cercano del mismo sexo, generada a través de permutaciones aleatorias de etiquetas de sexos en cada una de las cuatro localidades. Los triángulos representan la distancia observada en cada muestra. Note el cambio de escala en los ejes X e Y.

**An andromonoecious plant.** In June 2015, a morphologically andromonoecious sotol plant was found in the botanical garden Gustavo Aguirre Benavides in the Buenavista location. This plant had a stem with two branches, each of them bearing a male inflorescence, probably dating from 2014. Furthermore, each stem exhibited a more recent male inflorescence and a more recent morphologically hermaphroditic inflorescence; thus the plant exhibited four male and two hermaphroditic inflorescences (Fig. 5A to 5C). The height of this plant was 1.48 m and its width 2.51 m; the length of the tallest inflorescence was 2.50 m, which are within the typical dimensions of a *D. cedrosanum* individual. This is the first report of a *Dasyilirion* plant with hermaphroditic flowers (Fig. 5D).

The hermaphroditic flowers (Fig. 6a and 6b) showed the following features: pedicels 1-2 mm long, tepals 6, in 2 series of 3, obovate, 1.5-2.5 mm long, 0.8-2.2 mm wide, apex entire to slightly lacinate, margins hyaline, three tepals cover the

corners of the ovary, and the other three the lateral faces of the ovary, membranaceous, glabrous, greenish, usually with purple; stamens 3, opposite to the tepals, filaments 2.2-3.0 mm long, anthers 2, 1.0-1.2 mm long, nectaries at the ovary base, besides the filaments of the stamens; ovary 3-angled, 2.0-2.5 mm long, 1-loculated, lateral wings with an apical notch shallow, 0.5-0.7 mm long, style 0.4-0.5 mm long, stigma cup shaped with 3 short lobes.

Male inflorescences (Fig. 6c and 6d) had a normal morphology, and the anthers shed pollen as well as those of the hermaphroditic flowers. The latter produced well-developed pollen with a good response to carmine staining (Fig. 6e), similar to the male flowers (Fig. 6f). However, hermaphroditic inflorescences produced only non-developed fruits, with hollow seeds (Fig. 6g). This plant is now being surveyed to carry out more detailed observations regarding inflorescence production.



**Fig. 5.** An andromonoecious plant of *D. cedrosanum*. (a) Andromonoecious plant showing four male and two hermaphroditic inflorescences. The old inflorescences were produced in 2014 and the new ones in 2015. (b) Close-up view of a hermaphroditic inflorescence. (c) A male and hermaphroditic inflorescence in the same plant. (d) Ordinary dioecious plants at San Miguel.

**Fig. 5.** Una planta andromonóica de *D. cedrosanum*. (a) Planta andromonóica que muestra cuatro inflorescencias masculinas y dos hermafroditas. Las inflorescencias antiguas fueron producidas en 2014 y las nuevas en 2015. (b) Acercamiento a una inflorescencia hermafrodita. (c) Una inflorescencia masculina y una hermafrodita de la misma planta. (d) Plantas dióicas normales de San Miguel.

## DISCUSSION

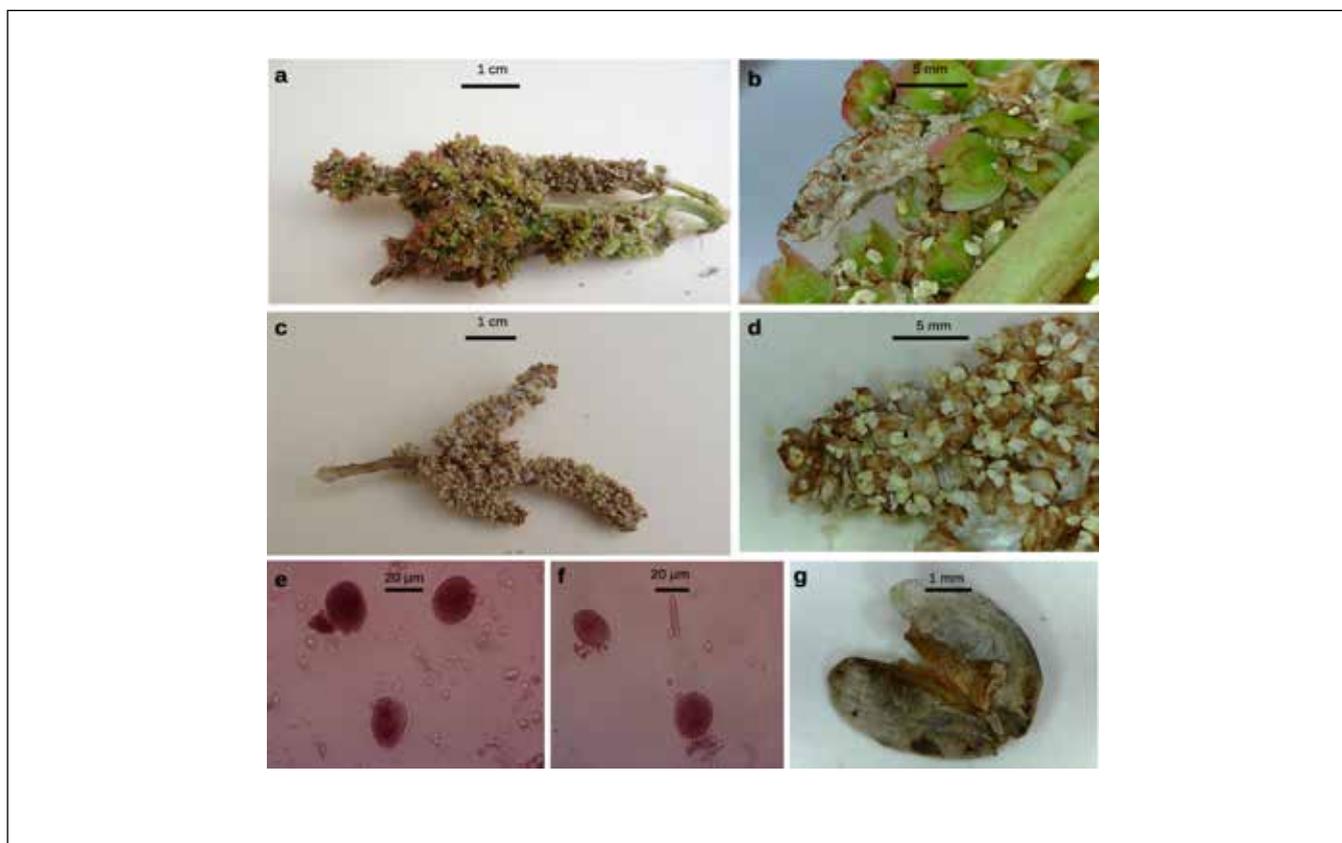
The results indicated that *D. cedrosanum* exhibited a nearly 1:1 sex ratio, with no evidence of bias in sex proportions. Furthermore, there is no evidence of differences in sex ratio among locations. The almost absent presence of bisexual individuals indicates that dioecy in *D. cedrosanum* is well established. On the other hand, the near to 1:1 sex ratio, consistent with the Fisher's theory, suggests an absence of marked differences between male and female investment for pollen and fruit production, respectively. In fact, the fruits of *Dasyliirion* are not fleshy, not requiring a high maternal biomass investment. On the other hand, the need for cross-pollination under harsh conditions with scarcity of insects, may originate a strong sexual selection between males for high pollen production, which may equalize their reproductive investment with that of the females, thus fulfilling Fisher's theory assumption of unbiased sex ratios.

From the stability of dioecy in *D. cedrosanum*, one may hypothesize that the sex-determining region has recombination suppression. Otherwise, dioecy would be unstable, with frequent reversions to hermaphroditism (Dellaporta & Calderon-Urrea, 1993). Our finding of an andromonoecious plant may be considered an extraordinary event, because it occurred only once in all our records. Although the filtered sample contained 448 plants, the original one was composed of 835 plants, being the mentioned individual the only non-unisexual one. Furthermore, if we consider that, in addition to the recorded plants, we have observed nearly 2000 plants at the flowering stage in the field, we can state that bisexuality in *D. cedrosanum* is a highly infrequent event. Furthermore, the aforementioned plant was found to be a non-functionally andromonoecious, because it did not produce developed seeds, even in the close presence of pollen sources.

There seems to be neither environmental effect on sex proportions nor any tendency of plants of certain sex to form clusters or to segregate spatially. The nonsignificant spatial sex segregation result indicates that clustering of plants of the same sex may occur only randomly. There is neither vertical nor horizontal tendency for sex frequencies, and this is true for completely natural populations and for the botanical garden population. Also, when repeated records were available for several flowering years, we did not find a single event of temporal sex change in individual plants, which indicates that each plant sex is hard-defined, hypothetically being the result of the expression of specific genes.

The results of this work suggest that dioecy in *Dasyliirion* is a well-established trait, not being a new evolutionary acquisition. This finding is consistent with the fact that all species of *Dasyliirion* exhibit dioecy (Bogler, 2002), and that the three of the closest relatives of *Dasyliirion* in the subfamily Nolinoideae: *Beucarnea*, *Calibanus* and *Nolina*, also show dioecy or polygamy (Bogler 1998; Ruiz-Sánchez & Specht, 2013). This is a strong indication that dioecy is not a *de novo* evolutionary acquisition of the *Dasyliirion* genus, and may be well established through evolution from a common ancestor of the four genera. This idea is consistent with the stability of the dioecious behavior found in this work for *D. cedrosanum*. One ecological consequence of these results is that fragmentation of the environments of this species will not undermine the adequate sex proportions necessary to maintain the genetic diversity and the reproductive potentials. Furthermore, the finding of homogeneity of sex proportions across locations leads to predict that plantations with reforestation purposes will produce populations with adequate sex ratios for reproductive potential.

With the data examined in this work, there is still no evidence of the existence of an intermediate form that gave rise to dioecy in *Dasyliirion* and its related genera. If the andromonoecious plant reported herein is a reversion to a primitive state, the doubt is still present as to whether the intermediate ancestor was ginodioecious, androdioecious or monoecious.



**Fig. 6.** Details of the andromonoecious plant. (a) Fascicles of a hermaphroditic inflorescence. (b) Close-up view of a hermaphroditic inflorescence showing flowers with anthers and pistil. (c) Fascicles of a male inflorescence from the same plant. (d) Close-up view of a male inflorescence showing flowers with anthers. (e) Carmine-stained pollen from hermaphroditic flowers ( $\times 400$ ). (f) Carmine-stained pollen from male flowers ( $\times 400$ ). (g) Undeveloped fruit showing absence of seed formation.

**Fig. 6.** Detalles de la planta andromonóica. (a) Fascículos de una inflorescencia hermafrodita. (b) Acercamiento a una inflorescencia hermafrodita donde se muestran flores con anteras y pistilo. (c) Fascículo de una inflorescencia masculina de la misma planta. (d) Acercamiento a una inflorescencia masculina donde se muestran flores con anteras. (e) Polen de flores hermafroditas teñido con carmín ( $\times 400$ ). (f) Polen de flores femeninas teñido con carmín. (g) Fruto sin desarrollar donde se muestra ausencia de formación de semilla.

Obviously, more work is needed at the molecular, anatomic and phylogenetic level to understand the nature and origin of dioecy in *Dasyilirion*.

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