Estimation of nuclear DNA content in germoplasm of *Bouteloua curtipendula* (Michx.) Torr. using flow cytometry †

[Estimación del contenido de ADN nuclear en germoplasma de *Bouteloua curtipendula* (Michx.) Torr. mediante citometría de flujo]

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SUMMARY

Background. *Bouteloua curtipendula* (Michx.) Torr. is a grass native to Mexico with a high capacity for adaptation in areas with limited moisture. It has physiological and morphological characteristics that are of interest in genetic improvement programmes for the restoration of degraded areas and the establishment of pastures for livestock grazing. In addition, flow cytometry can provide useful information in the study of the plant genome of this grass, such as nuclear DNA content (2C value) and its correlation with ploidy levels. **Objective.** To determine the nuclear DNA content of 57 ecotypes of *Bouteloua curtipendula* (Michx.) to infer their ploidy level. **Methodology.** Nuclear DNA content was estimated using flow cytometry. The internal standard was *Zea mays* L. The analysis considered average fluorescence intensity indices with a coefficient of variation <5%. Based on the fluorescence index and the 2C DNA value of the internal standard, the absolute DNA content (2C) in pg of each ecotype was obtained. The level of ploidy was inferred from the data obtained. **Results.** The range of nuclear DNA content of the ecotypes analysed ranged from 1.66 to 5.67 pg. Four different ploidy groups were found, with the tetraploid level predominating with 39 ecotypes. **Implications and Conclusion.** There is considerable variation in the nuclear DNA content of *B. curtipendula* (Michx.) Torr., a valuable forage grass for rainfed pastures, and flow cytometry was useful in determining this variation.
Key words: *Bouteloua curtipendula*; DNA; ploidy; chromosomes.

RESUMEN

Antecedentes. *Bouteloua curtipendula* (Michx.) Torr. es una gramínea nativa de México con alta capacidad de adaptación en áreas con restricción de humedad y presenta características fisiológicas y morfológicas de interés en programas de mejora genética para la restauración de áreas degradadas y el establecimiento de pastizales para pastoreo de ganado. Además, la citometría de flujo puede aportar información útil en el estudio de genoma vegetal de esta gramínea, como es el contenido de ADN nuclear (valor 2C) y su correlación con los niveles de ploidía. **Objetivo.** Determinar el contenido de ADN nuclear de 57 ecotipos de *Bouteloua curtipendula* (Michx.) para inferir su nivel de ploidía. **Metodología.** El contenido de ADN nuclear se estimó con la técnica de Citometría de Flujo. El estándar interno

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fue *Zea mays* L. El análisis consideró índices de intensidad de fluorescencia promedio con coeficiente de variación <5 %. A partir del índice de fluorescencia y del valor de ADN 2C del estándar interno, se obtuvo el contenido absoluto de ADN (2C) en pg de cada ecotipo. Con los datos obtenidos se infirió el nivel de ploidía. **Resultados.** El intervalo del contenido del ADN nuclear de los ecotipos analizados osciló de 1.66 a 5.67 pg. Se encontraron cuatro grupos diferentes de ploidía donde predominó el nivel tetraploide con 39 ecotipos. **Implicaciones y Conclusión.** Existe una gran variación en el contenido de ADN nuclear de *B. curtipendula* (Michx.) Torr., gramínea forrajera valiosa para pastizales de secano, y la técnica de citometría de flujo fue útil para determinar esta variación.
Palabras clave: *Bouteloua curtipendula*; ADN; ploídia; cromosomas.

INTRODUCTION

Flow cytometry has emerged as an easily automated, highly sensitive, fast, economical, and safe tool with diverse applications in biological studies across different disciplines (Marsán-Suárez *et al*., 2015; Triana and Marsán, 2020). Today, it is a widely used technique in various laboratories worldwide (Loureiro, 2009). In agriculture, it has been successfully used to study various characteristics of the plant genome, such as nuclear DNA content or 2C value and its correlation with ploidy levels. These data have been very useful for characterising the germplasm of inter- and intraspecific populations of different taxa or to identify genotype-phenotype correlations of interest in programmes for the conservation, improvement and use of genetic resources (Arumuganathan and Earle, 1991; Wilkinson and Stace, 1991; Bonos *et al*., 2002; Oliveira *et al*., 2017). It should be noted that the attributes of flow cytometry offer a significant advantage over other methods of analysis based on classical cytogenetics and fluorescence. Previously, the method for determining the ploidy level of plant cells was limited to karyotype analysis, which involves counting and morphologically characterising chromosomes. However, the procedure is very laborious, time-consuming and requires qualified personnel to obtain quality results. In addition, tissues containing dividing cells (metaphase) are not usually readily available, factors that limit the number of analyses that can be performed in a given time (Ochatt *et al*., 2011).

On the other hand, the genera that make up the Poaceae family exhibit wide inter- and intraspecific diversity, whose genetic, physiological, and morphological characteristics are ecologically important for the balance of various ecosystems, as well as for social actors and the productive or recreational activities they carry out as part of their culture in large regions of the world (Villaseñor, 2016; Soreng *et al*., 2017). In this regard, one of the species most valued for its ecological and economic importance in the Americas, is *Bouteloua curtipendula* (Michx.) Torr. This grass is highly prized for its forage quality. It is distributed in the arid and semi-arid regions of North America, Central America and South America, from Canada to Argentina. In Mexico, this species is popularly known as "banderita" grass. It is currently considered to be undergoing genetic erosion due to various factors, some of which are related to overgrazing, climate change and anthropogenic pressure such as urbanisation (Corrales-Lerma *et al*., 2016; Morales-Nieto *et al*., 2016; Gastelum *et al*., 2020; Martínez *et al*., 2020). However, in northern Mexico, it is one of the most widely used species in grassland restoration, as it adapts to a wide range of climates and has excellent forage value. Due to its potential, recent research has emphasised the selection of outstanding genotypes of *B. curtipendula* for use in grassland restoration in the state of Chihuahua, Mexico (Álvarez-Holguín *et al*., 2022). Identifying ideal genotypes requires analysis of the genetic structure of *B. curtipendula* populations and of their environmental aptitude. Although there are studies that have evaluated populations of this species at the morphological and molecular levels, showing the wide diversity of ecotypes in Mexico (Morales-Nieto *et al*., 2015; Scaglia *et al*., 2021), no reports have been found that indicate the distribution of its cytotypes. In grasses, cytotypes are defined based on the number of chromosomes that indicate the level of ploidy, which correlates with various attributes such as the mode of reproduction. Given that the number of chromosomes (n=x) is directly proportional to the weight of the genome, determining the latter using flow cytometry allows for a more accurate and faster inference of the ploidy level (c=n=x) of the germplasm. The objective of this study was to obtain the absolute nuclear DNA (2C) content in picograms of 57 different ecotypes of *B. curtipendula* to infer the ploidy of each specimen analysed.

MATERIALS AND METHODS

Plant material

Fifty-seven ecotypes of *B. curtipendula* (Michx.) Torr were used from the collection of Native Forage Grasses of PREGEP-Livestock of the Postgraduate College, Montecillo Campus, Mexico. These were collected in arid and semi-arid regions of Mexico in the states of Aguascalientes, Chihuahua, Coahuila, Durango, Guanajuato, Hidalgo, Jalisco, Nuevo León, Oaxaca, Puebla, and Zacatecas (Table 1 and Figure 1). The materials are currently stored ex situ at the same institution. Fresh leaf tissue from *Zea mays* line CML-492 (2C = 5.67 pg) from the International Maize and Wheat Improvement Centre (CIMMYT) was used as an internal reference standard.

Table 1. List of *B. curtipendula* (Michx.) Torr. ecotypes studied.

ID	Origin	ID	Origin	ID	Origin
AQ-061	Aguascalientes	AQ-545	Durango	AQ-752[b]	Coahuila
AQ-073	Aguascalientes	AQ-572	Puebla	AQ-759	Coahuila
AQ-077	Aguascalientes	AQ-574	Zacatecas	AQ-760a	Coahuila
AQ-180	Jalisco	AQ-614	Oaxaca	AQ-760[b]	Coahuila
AQ-202	Guanajuato	AQ-623	Durango	AQ-772	Coahuila
AQ-207	Jalisco	AQ-625	Durango	AQ-773	Coahuila
AQ-251	Aguascalientes	AQ-629	Durango	AQ-774	Coahuila
AQ-252	Aguascalientes	AQ-645	Durango	AQ-792	Nuevo León
AQ-303	Coahuila	AQ-663	Aguascalientes	AQ-870	Hidalgo
AQ-393	Zacatecas	AQ-665	Aguascalientes	AQ-913	Durango
AQ-405	Puebla	AQ-673	Aguascalientes	AQ-927	Durango
AQ-435	Zacatecas	AQ-673[b]	Aguascalientes	AQ-948	Durango
AQ-440	Durango	AQ-680	Zacatecas	AQ-989	Chihuahua
AQ-441	Durango	AQ-685	Hidalgo	AQ-990	Chihuahua
AQ-445	Durango	AQ-722	Zacatecas	AQ-1002	Chihuahua
AQ-453	Durango	AQ-723	Zacatecas	AQ-1010	Chihuahua
AQ-454	Durango	AQ-723[b]	Zacatecas	AQ-1027	Durango
AQ-480	Hidalgo	AQ-741	Zacatecas		
AQ-486	Hidalgo	AQ-750	Coahuila		
AQ-487	Hidalgo	AQ-752	Coahuila		

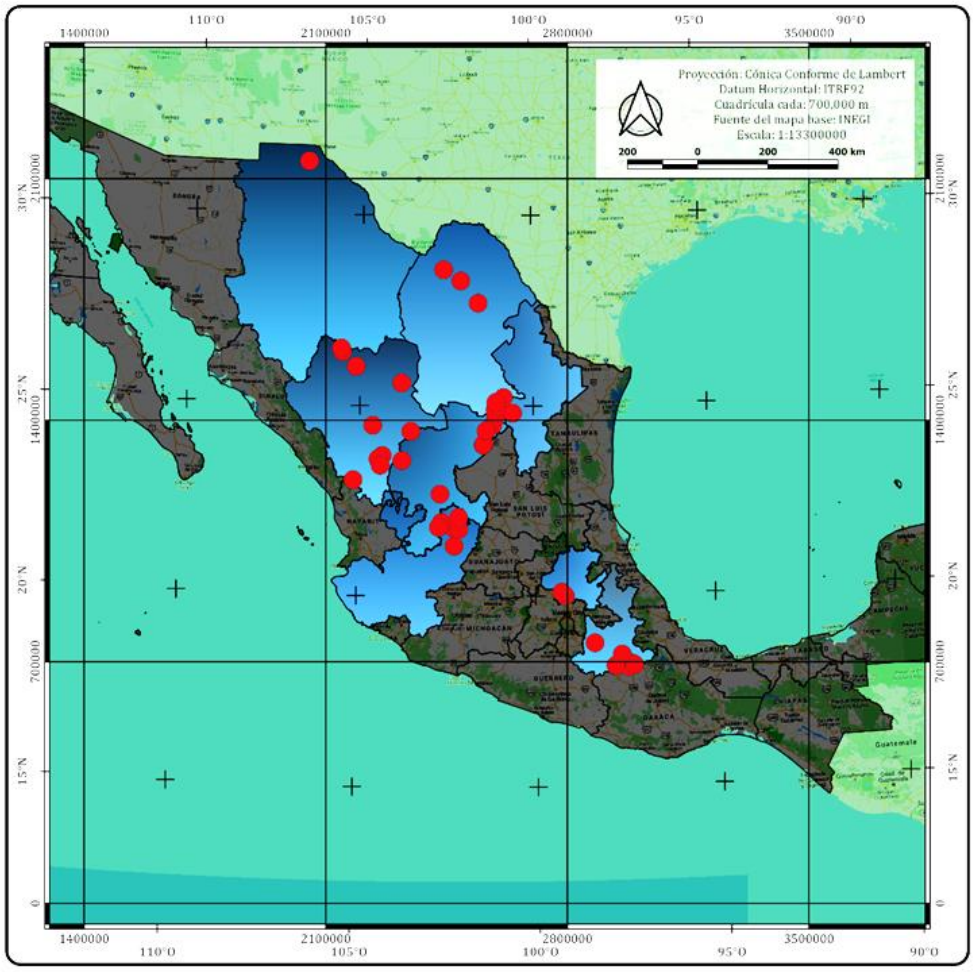

Figure 1. Source of base map: INEGI; Prepared by Dr. Adrián Raymundo Quero Carrillo

Sample preparation

Fragments of leaf tissue (0.5–1.0 cm^2) from the internal reference standard (*Zea mays* line CML-492) were placed in a plastic Petri dish (90×15 mm). in a new Petri dish, overlapping fragments of leaf tissue from B. *curtipendula* and *Zea mays* line CML-492 (internal reference standard) were placed, all immersed in 500 µL of modified Galbraith's buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 0.5% (v/v) TritonX-100, pH 7.0) (Galbraith et al., 1983). Subsequently, the tissue was finely cut with a razor blade in the opposite direction to the orientation of the veins, 500 µL of Galbraith buffer was added, and it was filtered using a 40 µm nylon filter. Finally, 2.5 µl of propidium iodide (PI) (10 mg/ml) was added to the filtered solution and left to incubate for 5 min in the dark for subsequent analysis by flow cytometry (Galbraith *et al*., 1983; Serrato-Cruz *et al*., 2000; Chen *et al*., 2017; Bracho-Gil, 2019).

Estimation of DNA content

To estimate nuclear DNA content, the reference standard (*Zea mays* L. CML-492) was analysed. To do this, the instrumental parameters were configured so that, in the linear Cartesian representation of the histograms obtained, the intensity of the averag forescence of the DNA in G0/G1 stained with IP was positioned at the value 200 on the X-axis. It was verified that the signal emitted by the nuclei in the G2 phase (when the cell replicates the genetic material) was positioned at the value 400 on the same axis. On the other hand, the number of events (cells) with the same fluorescence intensity was recorded on the Y-axis of the ordinates. Subsequently, the germplasm samples of *Bouteloua curtipendula* (Michx.) Torr. were analysed, combined with the internal reference standard to make the necessary comparisons of the mean fluorescence intensity indices, i.e., the sample and the standard were chopped together (at the same time). The first tests carried out only with the standard were essential for determining the location of the peaks in the histograms. With this reference, it was possible to identify the peaks of the G0/G1 phase and the G2 phase of the samples of interest. Once the fluorescence index or relative DNA content of both species was obtained, the absolute nuclear DNA (2C) content was determined in picograms (pg) using the equation described by Dolezel et al. (2007):

$$ADN\ 2C\ ecotype = \left[\frac{(i)\ ecotype}{(i)\ standard}\right] * ADN\ 2C\ standard$$

Where the absolute nuclear DNA content (2C) of the ecotypes of interest was obtained as the quotient of the average fluorescence index of the sample of *B. curtipendula* (Michx.) Torr., divided by the average fluorescence index of the internal standard *Zea mays* L. CML-492, multiplied by the absolute nuclear DNA content of the same standard, whose value is 2C=5.67 pg in this case. More than 8,000 nuclei per sample were analyzed. Three independent replicates were performed for the analysis. An Attune® Acoustic Focus Cytometer (blue/violet configuration, 50 mW laser at 405 nm and a 20 mW laser at 488 nm) was used for the analyses. The signals acquired during the analysis were processed with Attune Cytometric Software v2.1.0.8626.

Ploidy

Ecotypes of *B. curtipendula* with contrasting DNA content were selected and chromosome counts were performed using the methodology proposed by García (1990), which consisted of dissecting growing root tips (approx. 1 cm in length). Briefly, the roots were incubated in 0.05% colchicine for 2 to 3 hours in total darkness at room temperature; they were then fixed in a mixture of acetic acid and alcohol (3:1) for 24 h. The tips were then placed for 10 minutes at 60 °C in 1N HCl and immediately immersed in Schiff's reagent for 5 minutes. The root tip was placed on a microscope slide, a drop of 2% acetic orcein was added, and it was covered with a coverslip before applying the crushing technique. The preparations were observed under a Zeiss optical microscope with a 100x objective to search for cells in metaphase and count the chromosomes. Once the number of chromosomes was obtained, the level of ploidy was inferred based on the basic chromosome number reported for the species *B. curipendula* (n=x=10) according to Kapadia and Gould (1964) and through a simple linear regression plotting the 2C values of six contrasting ecotypes and the number of chromosomes obtained from the count. The estimation of ploidy was based on the assumption that there is a direct proportional correlation between the absolute nuclear DNA content (2C) and the number of complete chromosome sets.

RESULTS AND DISCUSSION

The fluorescence indices of the 57 ecotypes of *B. curtipendula* (Michx.) Torr. ranged from 0.29 to 1.00, indicating that some ecotypes had three times less DNA than the reference standard used (*Zea mays* L. CML-492), while others had the same amount (Figure 2). The absolute content or 2C DNA value obtained from the ecotypes evaluated was 1.66 to 5.67 pg with an average of 3.85 pg (Table 2), which is within the estimated range for some taxa of the Poaceae family (Johnson *et al.*, 1998; Arumuganathan *et al.*, 1999). It is also similar to that reported by Morales *et al.* (2007) in their study of 188 ecotypes of *B. curtipendula,* whose range was 2.04 to 4.31 pg with an average of 3.08 pg. Genotypes with average values of 3.59 pg

were found to predominate, followed by genotypes with 5.67 pg and 2.73 pg. It should be noted that only one genotype with a value of 1.66 pg was found (Table 2). The results showed that the germplasm evaluated presents wide variability (S^2= 0.93) in nuclear DNA content, which is possibly due to phenotypic differences and different collection sites of the ecotypes (Morales, 2007). This variability is valuable for the purposes of using the species; however, because DNA content influences characteristics that may reflect high forage potential, it is important to consider its range when selecting a characteristic before starting a selection programme (Eaton *et al.*, 2004). Similar results have been reported in *Panicum virgatum* L. (Hopkins *et al.*, 1996), *Brachiaria* (Do Valle and Savidan, 1996), *Tripsacum* (Quero *et al.*, 1997), *Paspalum* (Espinoza *et al.*, 2001) and *Poa pratensis* L. (Eaton *et al.*, 2004). On the other hand, changes in DNA content involve the loss or gain of repetitive DNA sequences, modify the nucleotype and are referred to as the effect of the amount of nuclear DNA (functional or not) on the phenotype, such as chromosome size, mitotic cycle, duration of meiosis, life cycle duration, etc. (Bennett, 1987).

The ploidy level of each ecotype inferred by linear regression based on the 2C DNA values of six contrasting samples and the number of chromosomes counted is shown in Table 3. The resulting regression equation was:

$$y = 9.3084x + 6.5418$$

Therefore, it was determined that, in conjunction with the number of chromosomes and the haploid number reported by Kapadia and Gould (1964) for the species studied (1n=1x=10 chromosomes), the ecotype whose 2C DNA value was 1.66 pg is diploid (2n= 2x= 20 chromosomes), those with an interval between 2.54-2.97 pg are triploid (2n= 3X= 30), with an interval of 3.12-3.97 pg are tetraploid (2n= 4X= 40), and those with a 2C DNA content of 5.67 pg are hexaploid (2n= 6X = 60). In total, four ploidy groups were obtained in the germplasm evaluated, with a preponderance of tetraploids (Table 3). This variation was distributed among 1 diploid ecotype, 7 triploid ecotypes, 39 tetraploids, and 10 hexaploids, suggesting that there is broad interspecific genomic diversity, a finding that highlights, based on the geographical regions where this germplasm was collected, that polyploid ecotypes appear to thrive in hostile or disturbed environments, an observation that of course remains to be proven. If this were true, it would be necessary to examine how adaptation to arid and unfavourable environments with limited moisture causes important and decisive responses in the generation of polyploidy, since losses or increases in repetitive DNA, as well as variation in DNA content between diploid and polyploid ecotypes, are considered to be associated with processes of adaptation to changing environmental conditions (Morales *et al*., 2007). Furthermore, in certain families of angiosperms, including grasses, it has been reported that tropical species have smaller genomes than temperate species (Levin and Funderburg, 1979; Bennett, 1987). Levin and Funderburg (1979) indicated that the increase in genome size in temperate species is mainly due to an increase in repetitive DNA, rather than an increase in ploidy level.

Table 2. Fluorescence index, DNA content 2C (pg), mean (pg) and standard deviation of 57 ecotypes of *B. curtipendula*.

No. of ecotypes	Fluorescence index	Range 2C DNA (pg)	Mean DNA 2C (pg)	Standard deviation ADN 2C
1	0.29	1.66	1.66	0
7	0.45-0.52	2.54-2.97	2.73	0.163
39	0.55-0.70	3.12-3.97	3.59	0.308
10	1.00	5.67	5.67	0

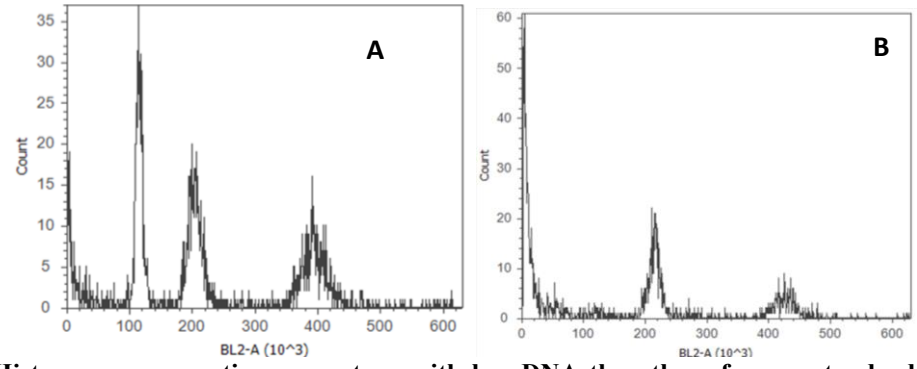

Figure 2. A) Histogram representing an ecotype with less DNA than the reference standard. B) Histogram representing an ecotype with a similar amount of DNA to the reference standard.

The level of ploidy in native ecotypes, representative of the natural richness of *B. cutipendula*, provides basic information for its selection and genetic improvement, such as: establishing the reproductive dynamics between individuals with different levels of ploidy (sexuality and apomixis), identification of ecotypes with forage value and their relationship with the level of ploidy (Quero *et al.,* 2010). The flow cytometry analysis method is a simple way to estimate the ploidy level in a plant species (Riordan *et al.,* 1998), facilitating the expansion of sample size through collection and the inclusion of a greater number of locations and environments in evolutionary and taxonomic studies (Morales, *et al*., 2007). However, classical techniques remain essential for determining ploidy levels when there are no previous studies with specific taxa.

Table 3. Absolute 2C DNA content, approximate number of chromosomes, and ploidy level of 57 ecotypes of *Bouteloua curtipendula* (Michx.) Torr.

ID	2C DNA	No. of chromosomes	Ploidy	ID	DNA 2C	No. of chromosomes	Ploidy
AQ-061	5.67	60	6X	AQ-665	3.71	40	4X
AQ-073	5.67	60	6X	AQ-673[a]	1.66	20	2X
AQ-077	3.12	30	4X	AQ-673[b]	2.84	30	3X
AQ-180	3.97	40	4X	AQ-680	3.40	40	4X
AQ-202	3.21	40	4X	AQ-685	3.40	40	4X
AQ-207	5.67	60	6X	AQ-722	3.40	40	4X
AQ-251	3.97	40	4X	AQ-723[a]	2.84	30	3X
AQ-252	3.32	40	4X	AQ-723[b]	5.67	60	6X
AQ-303	3.65	40	4X	AQ-741	5.67	60	6X
AQ-393	3.83	40	4X	AQ-750	5.67	60	6X
AQ-405	3.54	40	4X	AQ-752[a]	3.94	40	4X
AQ-435	3.97	40	4X	AQ-752b	3.97	40	4X
AQ-440	3.66	40	4X	AQ-759	3.97	40	4X
AQ-441	3.40	40	4X	AQ-760a	2.97	30	3X
AQ-445	3.97	40	4X	AQ-760b	3.97	40	4X
AQ-453	3.12	40	4X	AQ-772	3.12	40	4X
AQ-454	3.12	40	4X	AQ-773	3.37	40	4X
AQ-480	3.62	40	4X	AQ-774	3.97	40	4X
AQ-486	2.54	30	3X	AQ-792	3.40	40	4X
AQ-487	2.72	30	3X	AQ-870	5.67	60	6X
AQ-545	3.34	40	4X	AQ-913	3.69	40	4X
AQ-572	3.97	40	4X	AQ-927	2.55	30	3X
AQ-574	3.92	40	4X	AQ-948	2.63	30	3X
AQ-614	3.31	40	4X	AQ-989	3.97	40	4X
AQ-623	5.67	60	6X	AQ-990	3.97	40	4X
AQ-625	5.67	60	6X	AQ-1002	3.12	40	4X
AQ-629	3.62	40	4X	AQ-1010	3.53	40	4X
AQ-645	5.67	60	6X	AQ-1027	3.40	40	4X
AQ-663	3.40	40	4X				

CONCLUSIONS

The range of nuclear DNA content in the ecotypes analysed from *B. curtipendla* (Michx.) Torr. varied widely, from 1.66 to 5.67 pg. This variation was distributed among 1 diploid ecotype, 7 triploid ecotypes, 39 tetraploids, and 10 hexaploids, suggesting that there is brozad interspecific genomic diversity. The flow cytometry analysis method proved useful for determining the 2C DNA content of *B. curtipendula* and inferring its ploidy in a short time.

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Compliance with ethical standards. Due to the nature of the study this does not apply.

Data availability. Data are available upon request with the corresponding autor at: evillanueva@secihti.mx

Author contribution statement (CRediT). **E. E. Pozos-Méndez –** formal analysis, writing original draft, review., **E. Villanueva-Sánchez –** conceptualization, supervision, formal analysis, writing original draft, review & editing., **M. Hernández-Rodríguez –** conceptualization, formal analysis, writing original draft, review., **A. R. Quero-Carrillo –** formal analysis, writing review & editing., **H. A. Álvarez-Hernández –** formal analysis, writing original draft, review.

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