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RESEARCH ARTICLE

Genome sizes and ploidy levels in Mexican cactus pear species *Opuntia* (Tourn.) Mill. series *Streptacanthae* Britton et Rose, *Leucotrichae* DC., *Heliabravoanae* Scheinvar and *Robustae* Britton et Rose

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Abstract The ploidy levels and amounts of DNA of 23 *Opuntia* species from Mexico were determined by flow cytometry. Four different ploidy levels (2n = 2x, 2n = 4x, 2n = 6x, 2n = 8x) with 2C-DNA amount ranging from 4.17 pg (*Opuntia incarnadilla* Griffiths) to 6.53 pg (*Opuntia heliabravoana* Scheinvar) were determined among the samples analyzed. Polyploidy is widespread (93%) among these *Opuntia* species. *Opuntia heliabravoana* Scheinvar was the sole diploid species. *Opuntia leucotricha* DC. (2C = 5.71 pg), *Opuntia spinulifera* Salm-Dyck (2C = 5.51 pg), *Opuntia robusta* Wendl. ex Pfeiff. var. *larreyi* (F. A. C. Weber) Bravo (2C = 4.98 pg), and *Opuntia elizondoana* E. Sánchez et Villaseñor (2C = 5.29 pg) were tetraploids. *Opuntia oligacantha* C.F. Först. (2C = 5.33 pg), *Opuntia incarnadilla* Griffiths and *Opuntia matudae* Scheinvar (2C = 5.25 pg) were hexaploids. *Opuntia zamundioi* Scheinvar (2C = 4.35 pg), *Opuntia lasiacantha* Pfeiff. (2C = 4.88 pg), *Opuntia hyptiacantha* F.A.C. Weber (2C = 4.84 pg), *Opuntia streptacantha* Lem. ssp. *streptacantha* (2C = 4.64 pg) and *Opuntia streptacantha* Lem. subsp. *aguirrana* Scheinvar et A. Rodríguez (2C = 4.43 pg), *Opuntia megacantha* Salm-Dyck (2C = 5.01 pg), *Opuntia joconostle* F.A.C. Weber. ex Diguet (2C = 4.70 pg), *Opuntia ficus-indica* (L.) Miller (2C = 4.90 pg), *Opuntia albicarpa* Scheinvar (2C = 4.80 pg), *Opuntia amarilla* Griffiths (2C = 4.84 pg), *Opuntia chavena* Griffiths (2C = 4.70 pg), *Opuntia cochinera* Griffiths (2C = 5.10 pg), *Opuntia fuliginosa* Griffiths (2C = 4.64 pg), *Opuntia pachona* Griffiths (2C = 4.70 pg), *Opuntia cretochaeta* Griffiths (2C = 4.35 pg), *Opuntia rzedowskii* Scheinvar (2C = 4.77 pg), *Opuntia robusta* Wendl. ex Pfeiff. ssp. *robusta* (2C = 4.98 pg) and *Opuntia robusta* Wendl. ex Pfeiff. var. *guerrana* (Griffiths) Sánchez-Mejorada (2C = 5.05 pg) were all octoploids. The

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series *Streptacanthae* Britton *et* Rose showed a high level of ploidy with octoploid species except for *Opuntia heliabravoanae* Scheinvar (2n = 2x), *Opuntia elizondoana* E. Sánchez and Villaseñor (2n = 4x) and *O. matudae* Scheinvar (2n = 6x). *Opuntia spinulifera* Salm-Dyck was determined to be as a tetraploid species. Series *Leucotrichae* DC. grouped tetraploid and hexaploid species. The monospecific series *Heliabravoanae* Scheinvar has one species: *Opuntia heliabravoana* Scheinvar diploid (2n = 2x). The monospecific series *Robustae* Britton et Rose seems to be a contradictory group; containing three varieties: *Opuntia robusta* Wendl. ex Pfeiff. ssp. *robusta* and *Opuntia robusta* Wendl. ex Pfeiff. var. *guerrana* (Griffiths) Sánchez-Mejorada as octoploid taxa and: *Opuntia robusta* Wendl. ex Pfeiff. var. *larreyi* (F.A.C. Weber) Bravo which is tetraploid. In earlier report Rafael del Castillo and Mario González-Espinosa (1988) indicate that the arborescent varieties of this species are diploid and the postrate variety tetraploid. Implications for botanical systematics, genetic resources and breeding are discussed.

Keywords Nopal · Xoconostle · DNA · Content flow cytometry · Genetic resources · Opuntia

Introduction

Opuntia is the largest genus of Cactaceae with more than 360 species, ranging in geographic distribution from Peace River, in the north of Canada, at 59° North latitude, south to Patagonia in Argentina, at 52° of South latitude and from sea level coastal dunes, up to 5,100 masl in Peru (Bravo-Hollis and Scheinvar 1995). This genus includes an important group of edible fruit species. These plants are commonly known as Bunny Ears, Prickly Pear, Cactus Pear, Figue de Babarie in Europe, Africa, Tuna (Caribean, Chile) and Palma (Brazil). The fruits: tuna and higo de la India in México. Some species and Indian Fig in Mexico, some are grown for their edible pads, called nopales or nopalitos, or for their fruits, called tunas and xoconostles. Nopales are the fifth most consumed vegetable in the central states of Mexico and its fruits, the third most consumed fruit in Mexico (Mondragón 2001).

The species of *Opuntia* in Mexico are divided into 17 series (Bravo-Hollis 1978). The series *Streptacanthae* Britton et Rose includes the main species producing nopalitos, tunas and xoconostles. The species of series *Leucotrichae* DC. and *Heliabravoanae* Scheinvar also produce xoconostles; they are sympatrically or allopatrically distributed with eighteen species of the series *Streptacanthae. Robustae* Britto et Rose is a monospecific series with three varieties: *O. robusta* Wendl. ex Pfeiff. ssp. *robusta, O. robusta* Wendl. ex Pfeiff. var. *guerrana* (Griffiths) Sánchez-Mejorada, and, *O. robusta* Wendl. ex Pfeiff var. *larreyi* (F.A.C. Weber) Bravo. They produce sweet tunas for fresh consumption and can be used to add flavor to pulque, a fermented drink produced from the juice of *Agave*. The majority of species of *Opuntia* of these four series are thought to be polyploid but reports for only eight species have been published (Palomino and Heras 2001; Pinkava 2002).

Currently it is accepted that the base number for *Opuntia* is $x = 11$ (Pinkava et al. 1985), but reports of chromosomes counts are scarce. Determining ploidy levels in cactus pear by counting chromosomes is tedious, difficult and time consuming, as they are small, generally dot-like and most often clumped together (Palomino and Heras 2001; Soza and Acosta 1966). To overcome these difficulties, flow cytometry has been used to determine the ploidy levels of cactus pear in Mexico (Palomino et al. 2003).

In the present study, we looked for a quantitative base component to assess differentiation among the nuclear genomes of the main *Opuntia* species. Despite the number of sympatric zones, many factors limit interspecific recombination. Information about within species variation in ploidy could help to improve breeding and conservation strategies.

Flow cytometry is a technique of genome quantification developed first for biomedical researches and adapted for genetic plant analysis. This technique provides an estimation of volume and intensity of fluorescence of the nuclei of

isolated cells. A great number of nuclei can be analyzed with ease (>100/min). The use of a appropriate parameter of absolute genome size can be used to evaluate ploidy levels or to report genome sizes. Application examples at the interspecific level are numerous in the literature as for the genera *Citrus, Dioscorea, Zea* and *Sorghum* (Dansi et al. 2001; Laurie and Bennett 1985; Ollitrault et al. 1995). General reports of nuclear amounts in gymnosperms has been presented by Murray (1998) and Bennett et al. (2000), and implications of this method for systematics presented by Ohri (1998).

The objective of this study was to use flow cytometry to determine the DNA amounts and ploidy levels of cactus pear (*Opuntia)* species focusing on fruit-edible species (Series *Leucotrichae, Streptacanthae, Heliabravoanae* and *Robustae)* from México.

Materials and methods

Plant material

Plants studied consisted of 23 principal edible fruit species from Mexico (Table 1). This species are members of four botanical series *(Leucotrichae, Streptacanthae, Heliabravoanae* and *Robustae)* collected in 2003 in different localities of Mexico and maintained as a field collection at the Botanical Garden of the Biological Institut of the Universidad Nacional Autónoma de México (UNAM), México city, with a replicate at Universidad Autónoma de Chapingo, Estado de México, México (Agronomy University, UACH). Herbarium vouchers were deposited in the Herbarium of UNAM (MEXU) and in the herbarium of UACH (CHAP) (Segura et al. 2003). Assignment of these species to series (Table 1)

Table 1 *Opuntia* species included in genome size determination by flow cytometry

Series	Species	Accession number (MEXU)	Cytology report[1]
Leucotrichae	*O. leucotricha* DC.	6515-B	4X
	O. spinulifera Salm-Dyck	6508	6X
	O. oligacantha Förster	6501-F	N/R
	O. zamudioi Scheinvar	Extint	N/R
Streptacanthae	*O. scheeri* F.A.C.Weber	6892	N/R
	O. lasiacantha Pfeiff.	6813, 6861	N/R
	O. hyptiacantha F.A.C.Weber	6856, 6878	6X?,8X
	O. streptacantha Lem. ssp. *streptacantha*	6527, 6810, 6811, 6865, 6877	8X
	O. streptacantha ssp. *aguirrana* Scheinvar et A. Rodriguez	6880	8X
	O. megacantha Salm-Dyck	6857, 6901	N/R
	O. joconostle F.A.C. Weber ex Diguet	6512, 6516	N/R
	O. ficus-indica (L.) Mill.	6870, 6889	2X,6X,7X,8X
	O. albicarpa Scheinvar	6859	N/R
	O. amarilla Griff.	6815, 6820, 6821	N/R
	O. chavena Griff.	6888	N/R
	O. cochinera Griff.	6902	8X
	O. incarnadilla Griff.	6862	N/R
	O. fuliginosa Griff.	6881	N/R
	O. pachona Griff.	6888, 6900	N/R
	O. matudae Scheinvar	6883, 6884, 6886	N/R
	O. cretochaeta Griff.	6887	N/R
	O. rzedowskii Scheinvar	6874, 6875	N/R
	O. elizondoana Sánchez et Villaseñor	6541	N/R
Heliabravoanae	*O. heliabravoana* Scheinvar	6525	N/R
	O. robusta Wendland ex Pfeiff. ssp. *robusta*	12	N/R
Robustae	*O. robusta* Wendland ex Pfeiff. var. *larreyi* (F.A.C. Weber) Bravo	6871	2X?,4X
	O. robusta Wendland ex Pfeiff var. *guerrana* (Griff.) Sánchez-Mejorada	41	N/R

[1]Palomino and Heras (2001); Pinkava (2002) N/R: Not reported

follows a current systematic arrangement proposed by Scheinvar and Rodríguez-Fuentes (2003); Scheinvar (2004a,b, 2005) on the basis of variation in epidermal, glochids, pollen grains; protein characteristics among taxa were studied by Estrada et al. (2001). This arrangement served as a source of testable hypothesis.

DNA isolation

For this purpose, the epidermis of pads was sampled, following the procedure described by Galbrait et al. (1983) and utilized by Leblanc et al. (2002) for maize to prepare and stain the nuclei. To release nuclei, epidermal tissue was cut in a Petri glass dish. Following addition of 1.5 ml of ice-cold extraction buffer (0.1 M citric acid containing 0.5% Tween 20), the epidermis was finely chopped with a sharp razor blade. The homogenate was filtered through a 40 μm nylon gauze filter to remove cellular debris, and immediately analyzed by flow cytometry.

Flow cytometry

Samples were analyzed at the Apomixis Laboratory of Centro Internacional de Mejoramiento del Maiz y Trigo (CIMMyT). Flow cytometry measurements were performed with a Partec CA II flow cytometer (Partec Gmbh, Münster, Germany). The cytometer is equipped with a combination of filters to determine staining by using phytochrome bis-Benzimide (2′[4 -etoxiphenyl1]-5-[4-methyl-1 -piperazinyl]-2,5′-bi-1H-benzimidazol) with the assistance of DPAC software (Partec Gmbh). The cytometer was adjusted to the G1 peak of nuclei isolated from three control species: *O. heliabravoana* (2n = 2x = 22 chromosomes), *O. matudae* (2n = 6x = 66 chromosomes) and, *O. streptacantha* ssp. *streptacantha* and ssp. *aguirrana* (2n = 8x = 88 chromosomes). For each species, evaluations (values) relative to *O. heliabravoana* (diploid) were done in triplicate. *O. heliabravoana* sample was set at channel 50. This calibration was checked periodically to minimize variation due to runs and keep it constant during analysis of samples prepared from plants of unknown ploidy. Two measurements were made for each isolation and at least 2000 nuclei were examined each time. To estimate ploidy level of remaining species, the position of the G1 peak on the histogram obtained was compared to that of the three control species. A ratio produced by the software package was used to determine ploidy levels. The size of genome in *Opuntia* species was determined relative a maize accession (*Zea mays* ssp. *mays*, race Zapalote Chico, Oaxaca 50 Tep.60 A 5269) and to a barley cultivar (*Hordeum vulgare* cv. Sultan) from Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMyT), for which genome sizes were reported by Laurie and Bennett (1985) and by Johnson et al. (1999), respectively. At least three replicates per species were measured. Fluorescence intensity obtained for each species served to estimate 2C-DNA amount and were analyzed by means of Newman-Keuls test with the goal of grouping ploidy levels. Ollitraut et al. (1995) used similar homogeneous groups test to compare DNA amount among *Citrus* cultivars.

Results and discussion

After release and staining, isolated nuclei of epidermal tissue irradiated with UV radiation emitted fluorescence which was easily measured by the flow cytometer. For each sample, analysis of the relative fluorescence intensity of the isolated nuclei yielded a histogram showing a dominant peak corresponding to the nuclei in the G1 phase of the cell cycle and a minor peak corresponding to G2 nuclei.

The amount of debris in the samples was negligible. The dominant G1 peak simplified ploidy estimation. Fig. 1 shows a DNA histogram derivated from *O. ficus-indica* (2n = 8x) and maize as control, by using the protocol previously described.

The 2C-DNA amount of the *Opuntia* species analyzed ranged from 4.17 pg for *O. incarnadilla* to 6.53 pg for the diploid *O. heliabravoana*. The former species belongs to the series *Streptacanthae* and the last one to series *Heliabravoanae*.

Values for genome sizes, of species are reported in Table 2. Coefficients of variation (cv) for G1 peaks for all species were $<7\%$. The cvs reflected the narrowness of the peaks indicative of reliability. Clear separation of peaks was even

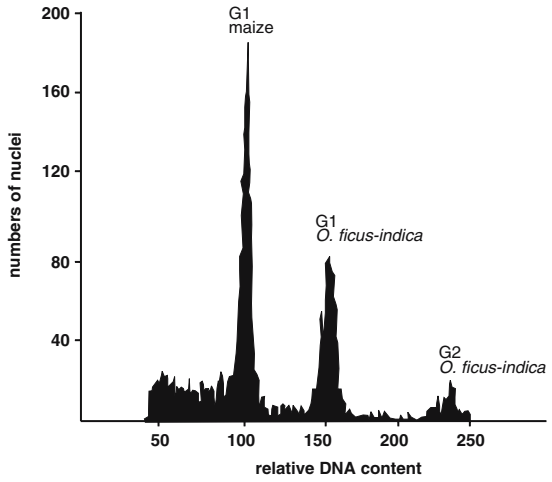

Fig. 1 Histogram obtained from a sample containing nuclei of *Zea mays* race Zapalote chico and *Opuntia ficus-indica*. The G2 of *Z. mays* is masked by the G1 peak of *O. ficus-indica*

obtained when a sample was prepared from a mixture of epidermis from diploid (2x), tetraploid (4x), hexaploid (6x) and octoploid (8x) individuals (Fig. 2).

Four ploidy levels, diploid, tetraploid, hexaploid and octoploid were identified among the 23 species of *Opuntia* (Table 2). Of the 18 species from series *Streptacanthae* as determined by Scheinvar et al. (Estrada et al. 2001), 15 were octoploid, two were hexaploids (*O. incarnadilla* and *O. matudae)* and one (*O. elizondoana)* produced tetraploid values (Table 2). The relative nuclear DNA content in arbitrary units (AU), expressed as channel numbers, ranged from 50.700 to 53.01 for the diploids, and 80.92–91.22 AU for the tetrapolids, from 120.71 to 127.60 AU for hexaploids, and from 133.18 to 156.07 AU for hexaploids, and from 133.18 to 156.07 AU for the octoploids. The wide range of variation among octoploids shows in Fig. 2 was notable.

Evaluation of nuclear genome size and ploidy levels among Opuntia species

The mean 2C genome of the group of principal edible species of *Opuntia* analyzed here is 5.05 pg. In comparison to 2C-values for the cacti by Bennet and Leitch (2003), the mean of *Opuntia* species analyzed here exceeded those of *Escobaria bella* Britton et Rose (2C = 3.05 pg), *Pseudolobivia* sp. (2C = 3.25 pg), *Borzicactus aurivillus* K. Schum. (2C = 3.35 pg), *Cleistocactus smaragidifolius* (F.A.C. Weber) Speg. (2C = 3.35 pg), *Aporocactus flagelliformis* Lem. (2C = 3.80 pg), and *Trichocereus werdermannianus* Backeb. (2C = 3.90 pg), but less than most of *Mammillaria* species reported or *Weberbauerocereus winterianus* Anthony (2C = 14.20 pg).

Variations of ploidy levels estimated for these *Opuntia* species are consistent with their assignement to series, except to *O. heliabravoana, O. elizondoana, O. matudae* and *O. zamudioi.*

The relative fluorescence intensity values for these four species indicate that their estimated ploidy level has probably reached a different way or stage towards speciation. *Opuntia* species are predisposed for rapid successful speciation due to small isolated populations via peripatric speciation, sexual reproduction, perennial habit, and, apomixis (Pinkava 2002). Different combinations of these four characteristics are determinate by environmental variables (if no human selection occurs) and produce different pathways of speciation. Indeed genome size variation of these four species certainly contributes to limit recombination in interspecific hybrids where they are simpatrically distributed with other opuntias. Species of *Opuntia* have a chromosome base number $x = 11$ (Bravo-Hollis and Scheinvar 1995) and Pinkava (2002) reported that unreduced gametes and chromosomal duplication account for the origins of most polyploid North American opuntias. We suggest that c-DNA markers could help to verify our results (Fig. 3).

From these results, we can verify that these species form a special group where polyploidy has played an important role in evolution as Pinkava (op. cit.) suggests. If nearly 6% of *Opuntia* species are currently reported as octoploid (Palomino and Heras 2001; Pinkava op. cit.), in our results all but three species of series *Streptacanthae* are octoploids suggesting that they share a similar evolutionary pathway in Mexico. Our results for *O. streptacantha* differ with Yuassa et al. (1973) (in Pinkava 2002), who reported this species as a diploid, but agree with Pinkava and Parfitt (1982), who reported *O. streptacantha* from Mexico as an octoploid. *O. zamudioi* may possibly be related to species of series *Streptacanthae* by its degree of

Table 2 Estimated DNA amounts, species, subspecies and varieties groups and ploidy levels as determined for *Opuntia* species by flow cytometry

Species	Relative flourescense intensity mean (AU)	Coefficient of Variation	Newman-Keulus Homogeneous group	2C-DNA amount estimated (pg)	Interpretation (ploidy level)
O. leucotricha	87.32	2.48	b	5.71	4X
O. spinulifera	84.25	4.65	b	5.51	4X
O. oligacantha	122.34	6.18	c	5.33	6X
O. zamudioi	133.18	4.83	de	4.35	8X
O. lasiacantha	149.45	3.81	e	4.88	8X
O. hyptiacantha	148.22	3.67	e	4.84	8X
O. streptacantha ssp. *streptacantha*	142.09	2.71	de	4.64	8X
O. streptacantha ssp. *aguirrana*	135.77	4.72	de	4.43	8X
O. megacantha	153.36	3.95	e	5.01	8X
O. joconostle	143.96	3.97	de	4.70	8X
O. ficus-indica	150.16	4.11	e	4.90	8X
O. albicarpa	146.95	3.73	e	4.80	8X
O. amarilla	148.05	3.84	e	4.84	8X
O. chavena	143.95	4.84	e	4.70	8X
O. cochinera	156.07	5.52	e	5.10	8X
O. incarnadilla	127.60	4.63	cd	4.17	6X
O. fuliginosa	141.93	3.68	de	4.64	8X
O.pachona	143.87	2.63	e	4.70	8X
O. matudae	120.71	6.02	c	5.25	6X
O. cretochaeta	133.18	5.03	de	4.35	8X
O. rzedowskii	146.01	3.66	e	4.77	8X
O. elizondoana	80.92	4.27	de	5.29	4X
O. heliabravoana	50.00	3.69	a	6.53	2X
O. robusta var. *robusta*	152.42	6.30	e	4.98	8X
O. robusta var. *larreyi*	91.22	2.67	b	5.96	4X
O. robusta var. *guerrana*	154.61	6.08	e	5.05	8X
Cylindropuntia imbricata	53.01	4.04		6.92	2X
Zea mays ssp. *mays* race Zapalote chico Oaxaca 50 5269[1]	123.26	3.12		13.49 [1]	
Hordeum vulgare cv. Sultan[2]	87.05	2.01		11.12[2]	

[1] Laurie and Bennett 1985; [2] Johnston et al. 1999

ploidy. Genetic divergence among species has been estimated using DNA markers by our team (Segura et al. 2003). Such markers could test the relatedness of *O. zamudioi* to members of series *Streptacanthae*.

Intraspecific variation of the relative fluorescence intensity of accessions of *O. ficus-indica, O. pachona, O. amarilla* and *O. streptacantha*, was high (10.13 AU, 12.86 AU, and, 10.32 AU, 6.36 AU, respectively). This finding generally concurs with those of Palomino and Heras (2001), who studied *O. cochinera, O. hyptiacantha* and *O. streptacantha* (all these from series *Streptacanthae)* and observed important internal karyotypic variation for genome length and structure.

The Neuman–Keuls interspecific means test conformed the tetramodal distribution of ploidy determinations with overlap between tetraploid and hexaploid levels and hexaploid and octoploid levels (Table 2). We lack cytological evidence determined if aneuploidy is implicated in these results, but the group identified as ''d'' in the means test displayed continuous variation between the hexaploid and octoploid levels, indicating possible duplication, deletion or aneuploidy. Heptaploids are scarce in *Opuntia*,

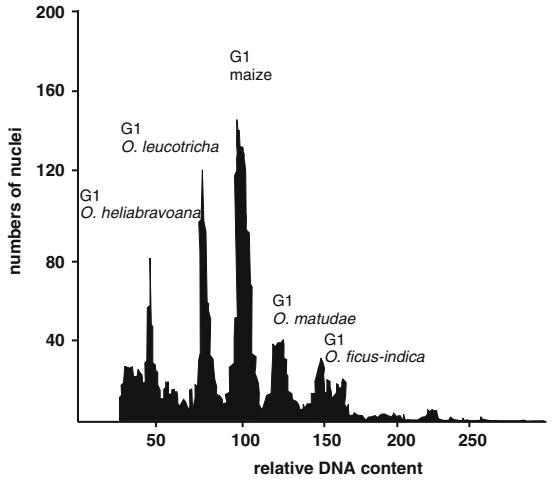

Fig. 2 Histogram obtained from a sample containing nuclei of *O. heliabravoana* (2n = 2X), *O. leucoctricha* (2n = 4X) *O. matudae* (2n = 6X), *Z. mays* race Zapalote chico and *Opuntia ficus-indica* (2n = 8X)

only *O. ficus-indica* has been reported also as heptaploid (Pinkava 2002).

For purposes of interpretation, *O. incarnadilla* was grouped into the ''c'' or hexaploid species group. *O. zamudioi, O. streptacantha* ssp. *streptacantha, O. streptacantha* ssp. *aguirrana, O. joconostle, O. fuliginosa*, and *O. cretochaeta* were grouped in accordance. Octoploids were grouped into the ''e'' or octoploid group in accordance with Palomino and Heras's (2001) chromosome counts. We used the same *O. streptacantha* ssp. *streptacantha* accession, LS6810, determined to be octoploid by these authors. It would be worthwhile to count the chromosomes of *O. zamudioi* and *O. cretochaeta* to confirm our results from cytometry.

Implications for genetic-diversity assessment and breeding

In this study, no correlation was found between ploidy level and geographic origin of these *Opuntia* species in contrast to Pinkava (2002) who demonstrated a cytological relationship to geographical distribution for the *Opuntia basilaris* Engelm. et Bigel. complex, with four varieties and two ploidy levels related to saline soils, and for the route of evolution for the *Opuntia polyacantha*

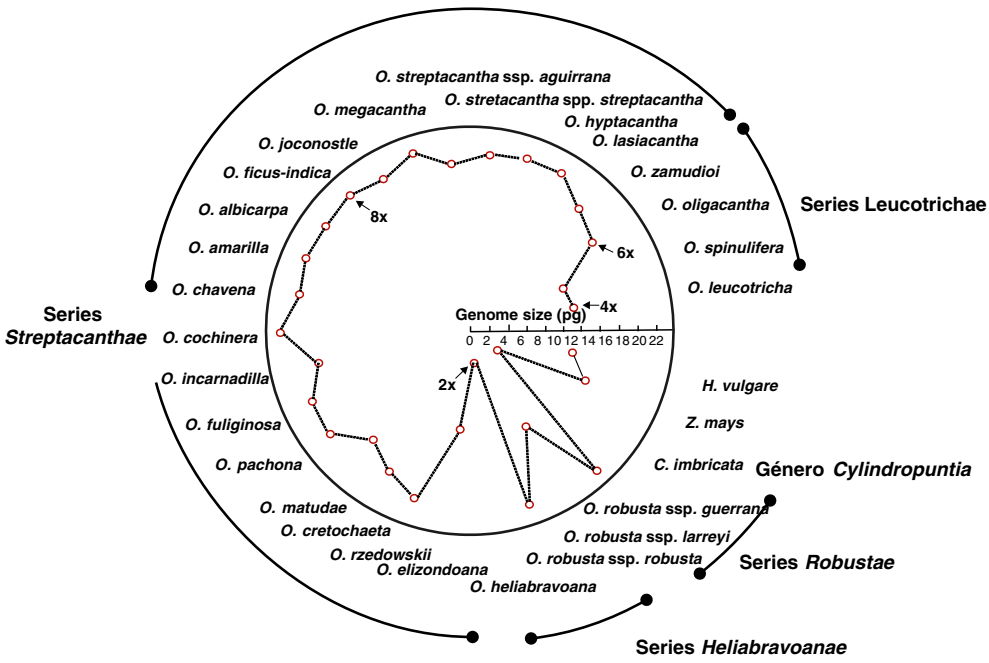

Fig. 3 Variation in the size of nuclear genomes of *Opuntia* species of series *Streptacanthae, Leucotrichae* and *Robustae*, and of *Cylindropuntia imbricata*, *Zea mays* race Zapalote chico and *Hordeum vulgare* cv. Sultan. Arrows indicate for the four differenct ploidy levels for *Opuntia*

Haw. complex, where the diploid source is in northern Mexico and tetraploid and hexaploid derivatives in the United States and Canada respectively.

This topic might be better understood either through more comprehensive analyses or a more intensive examination of certain taxa. For examples, this would be an interesting goal for the *Opuntia robusta* complex, which contain three sympatric varieties with two different ploidy levels (tetraploid and octoploid).

Opportunities are already available to test the value of *Opuntia* genome size as a predictor of responses to environment and evolution. It is likely that the full value of nuclear DNA amounts will be realized only when these determinations can be applied in association with other measurable plant traits (Grime 1998; Otto and Whitton, 2000; Leich et al., 1998).

Variations in chromosome size and subsequent meiotic abnormalities have disrupt breeding programs. *Opuntia ficus-indica* × *Opuntia robusta* ssp. *robusta* has been used as the basis of recombinant populations, and such variation could explain some of the segregationnal disturbances observed by Rodriguez (2002).

Increasing genome size may affect PCR amplification success negatively because of a decrease in target: non target DNA or by dilution of the available primer pool by nonspecific binding (Garner 2002).

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