

# CHROMOSOME NUMBERS, NUCLEAR DNA CONTENT, AND POLYPLOIDY IN *CONSOLEA* (CACTACEAE), AN ENDEMIC CACTUS OF THE CARIBBEAN ISLANDS<sup>1</sup>

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Polyploidy, an important mechanism of plant evolution, was investigated in *Consolea*, an endemic Caribbean opuntoid genus represented by nine subdioecious species with very narrow distributions, including species classified as rare or threatened. Standard chromosome counting and flow cytometric analyses were used to determine chromosome numbers and ploidy of each taxon. Compared to the base number ( $x = 11$ ), the mitotic and meiotic counts indicated that there are seven hexaploid ( $2n = 66$ ) and two octoploid species ( $2n = 88$ ); no diploids were found. Histograms of intact nuclei confirmed that all species are polyploid, with C-DNA values ranging from 4.88–9.50 pg. The variation of DNA content was significantly higher for the octoploids than for the hexaploids. Male and female sexual morphs had similar DNA content, suggesting that there are no sex chromosomes. Cytomixis between cells and microsporocytes with no chromatin were observed. This provides a mechanism whereby gametes with variable chromosome numbers are produced, influencing reproduction and promoting speciation. In conclusion, C-DNA content and chromosome number separated *Consolea* species into two groups, which may correspond to two phylogenetic lineages or indicate that polyploidization occurred independently, with comparable effects on C-DNA content.

**Key words:** Cactaceae; Caribbean; chromosomes; *Consolea*; cytomixis; DNA content; flow cytometry; polyploidy.

Polyploidy, the possession of three or more sets of chromosomes representing the haploid genome, is an important genetic mechanism that has contributed to the adaptation and speciation of plants, animals, and other eukaryotes (Masterson, 1994; Ramsey and Schemske, 1998; Coghlan et al., 2005). Evidence suggests that polyploidy occurs in all major plant groups, accounting for 50–70% of flowering plants (Coghlan et al., 2005) and for ~2–4% of their speciation events (Otto and Whitton, 2000). Polyploidy modifies both the genotype and the phenotype of an organism, generating morphological and physiological changes that consequently alter habitat and geographical distributions (Thompson et al., 2004), reproductive systems (e.g., polyploidy can cause shifts to asexual reproduction; Roche et al., 2001), and breeding systems (e.g., polyploidy can breakdown self-incompatibility and change plant–animal pollination; Thompson et al., 2004). This mechanism is characterized by a variation in the amount of nuclear DNA (C-value), with simultaneous changes in chromosome number and structure (Murray et al., 2005). It can arise via genome doubling, gametic nonreduction, and polyspermy (Otto and Whitton, 2000). Polyploidy can be classified by its mode of origin into (1) autopolyploidy, derived from one individual, or from crosses within or between populations of a single species, and 2) allopolyploidy, arising from

crosses between species forming hybrids (Ramsey and Schemske, 1998). Consequently, an autopolyploid is monophyletic in origin, whereas an allopolyploid is polyphyletic (Bretagnolle and Thompson, 1995).

Along with hybridization and cryptic chromosomal rearrangements, polyploidy is one of the major causes of speciation in the Cactaceae (Grant and Grant, 1980; Parfitt, 1980; Pinkava et al., 1985; Cota and Philbrick, 1994). Cytological studies have shown that polyploid cytotypes are common in the Opuntioideae (64%) and Cactoideae (13%) (Pinkava et al., 1985, 1998), with the highest levels of ploidy in the South American opuntoids *Austrocylindropuntia* Backeberg, *Mique-liopuntia* Fric ex F. Ritter, and *Tephrocactus* Lem. (Rebman and Pinkava, 2001). In North America, polyploid cacti are reported for species of opuntoids such as *Cylindropuntia* (Engelmann) F. M. Knuth, *Opuntia* Miller, and *Pereskopsis* Britton & Rose, and for the cactoid *Echinocereus* Engelmann (Pinkava, 2002; Parfitt and Gibson, 2004). Studies have also revealed interspecific variation in chromosome size, genome length, and chromosomes with satellites in several cactus species, e.g., *Astrophytum* Lemaire (Das et al., 2000a), *Mammillaria* Haworth (Briones et al., 2004), and *Opuntia* (Bandyopadhyay and Sharma, 2000; Palomino and Heras, 2001). Cota and Philbrick (1994) suggested a correlation among polyploidy, higher latitudes, and geographical elevation for *Echinocereus* (Cactoideae). Parfitt (1980) indicated the presence of euploids and provided information for documenting both autotetraploids and autoallotetraploids in *Opuntia* (Parfitt, 1980).

Standard meiotic and mitotic chromosome counting have been commonly used to determine ploidy levels (e.g., Stockwell, 1935; Parfitt, 1980; Ross, 1981; Pinkava et al., 1985; Cota and Philbrick, 1994; Cid and Palomino, 1996; Palomino et al., 1998; Palomino and Heras, 2001), hybridization (Parfitt, 1980; Pinkava et al., 1985; Tel-Zur et al., 2004), relationships between species (Cota and Wallace, 1995; Bandyopadhyay and Sharma, 2000), karyotype analyses (Palomino et al., 1998; Das et al., 2000b; Briones et al., 2004), and translocations (Pinkava

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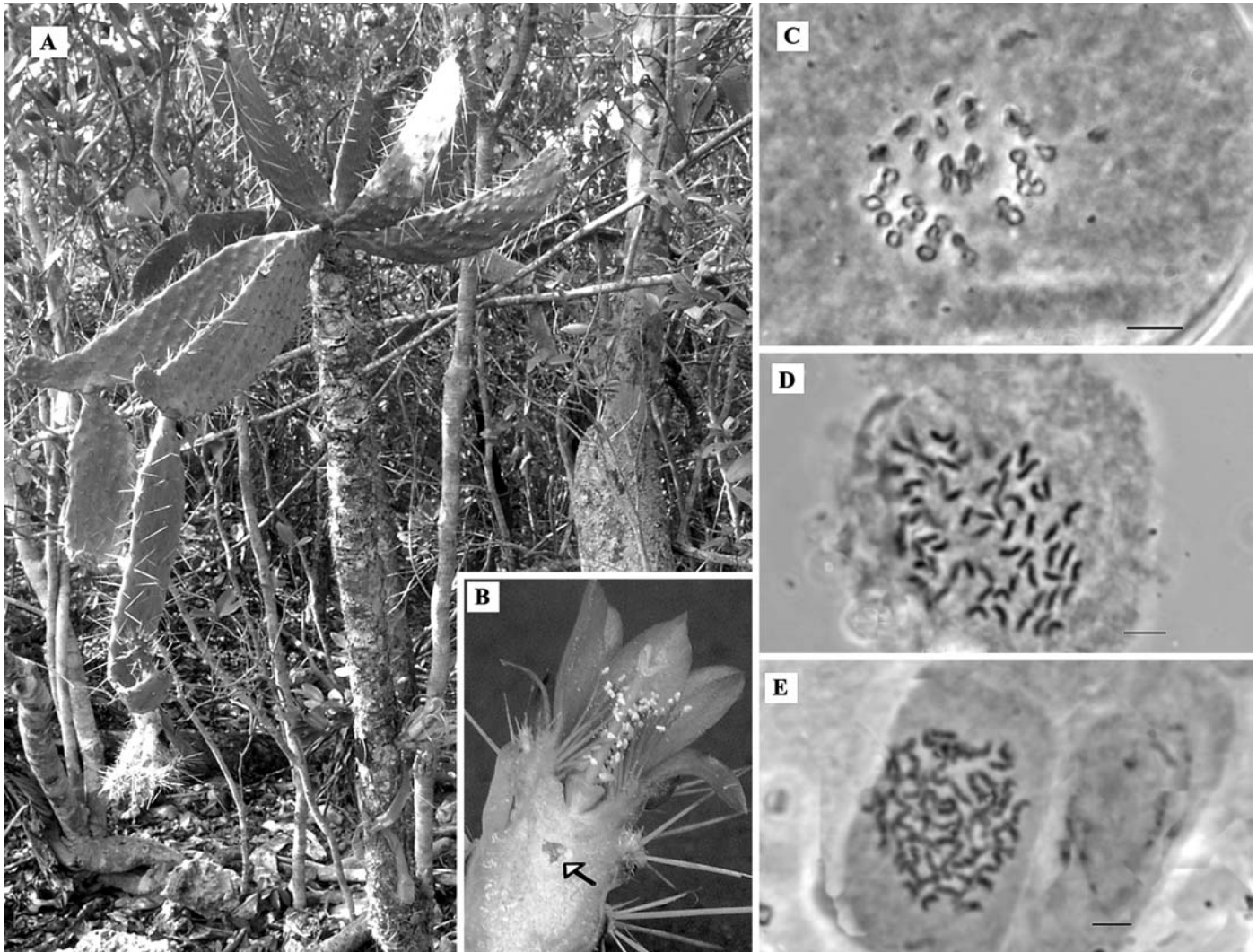


Fig. 1. Growth habit and light micrographs of representative hexaploid species of *Consolea*. (A) Growth habit of *C. corallicola*; (B) longitudinal section of *C. corallicola* flower showing aborted ovules (arrow); (C–E) Chromosome stains of (C) *C. corallicola* ( $2n = 33\text{II}$ , Florida), (D) *C. millspaughii* ( $2n = 66$ , Long Island, Bahamas), and (E) *C. moniliformis* ( $2n = 66$ , Dominican Republic). Bars = 5  $\mu\text{m}$ .

et al., 1985) within the Cactaceae. Standard chromosome counting is the most direct way to establish ploidy levels, but it can be labor intensive and in many cases not completely reliable if the chromosomes are small and numerous (Tuna et al., 2001). Because chromosomes are located in the cell nucleus, nuclear DNA content can be also used to estimate ploidy level. Several approaches have been used to measure DNA content, but two have been regularly used in the Cactaceae: cytophotometry (Mohanty et al., 1997; Das et al., 1998; Das et al., 2000a, b) and Feulgen microdensitometry (www.rbgkew.org.uk/cvalues; Bennet et al., 2000).

While the techniques described have been used for measuring DNA content, the most widely accepted approach is flow cytometry (FC), a cytological method for plants with small chromosomes that allows determination of DNA content of isolated nuclei stained with DNA-specific fluorochromes. In plant breeding, FC is useful for analyzing ploidy level, detecting intraspecific hybrids and apomixis, determining genome size, determining the effect of endoreduplication, and

more recently, for identifying sex (Costich et al., 1991; Dolezel, 1991). The major advantages of FC are its precision and rapidity, which allow large-scale and rapid analyses of cells (Dolezel, 1991). At present this method has been used on only *Rebutia albiflora* F. Ritter & Buining, *Opuntia microdasys* (Lehm.) Pfeiffer (Zonneveld et al., 2005), and several species of *Mammillaria* (Palomino et al., 1999; Del Angel et al., 2006).

The present study uses standard meiotic and mitotic chromosome techniques as well as FC analyses to assess whether speciation in *Consolea* Lem. (Opuntioideae, Cactaceae) has occurred through polyploidization. *Consolea* comprises nine tree-like species (Figs. 1A, 2A) (Areces-Mallea, 1996, 2000, 2001; Anderson, 2001) distributed in the Florida Keys, the Bahamas, and the Greater and Lesser Antilles. Hunt et al. (2006) grouped these into three species. However, because these nine species can be easily distinguished in the field by distinctive features of their cladodes, flowers, and seeds (Areces-Mallea, 1996, 2000, 2001; Anderson, 2001; V.

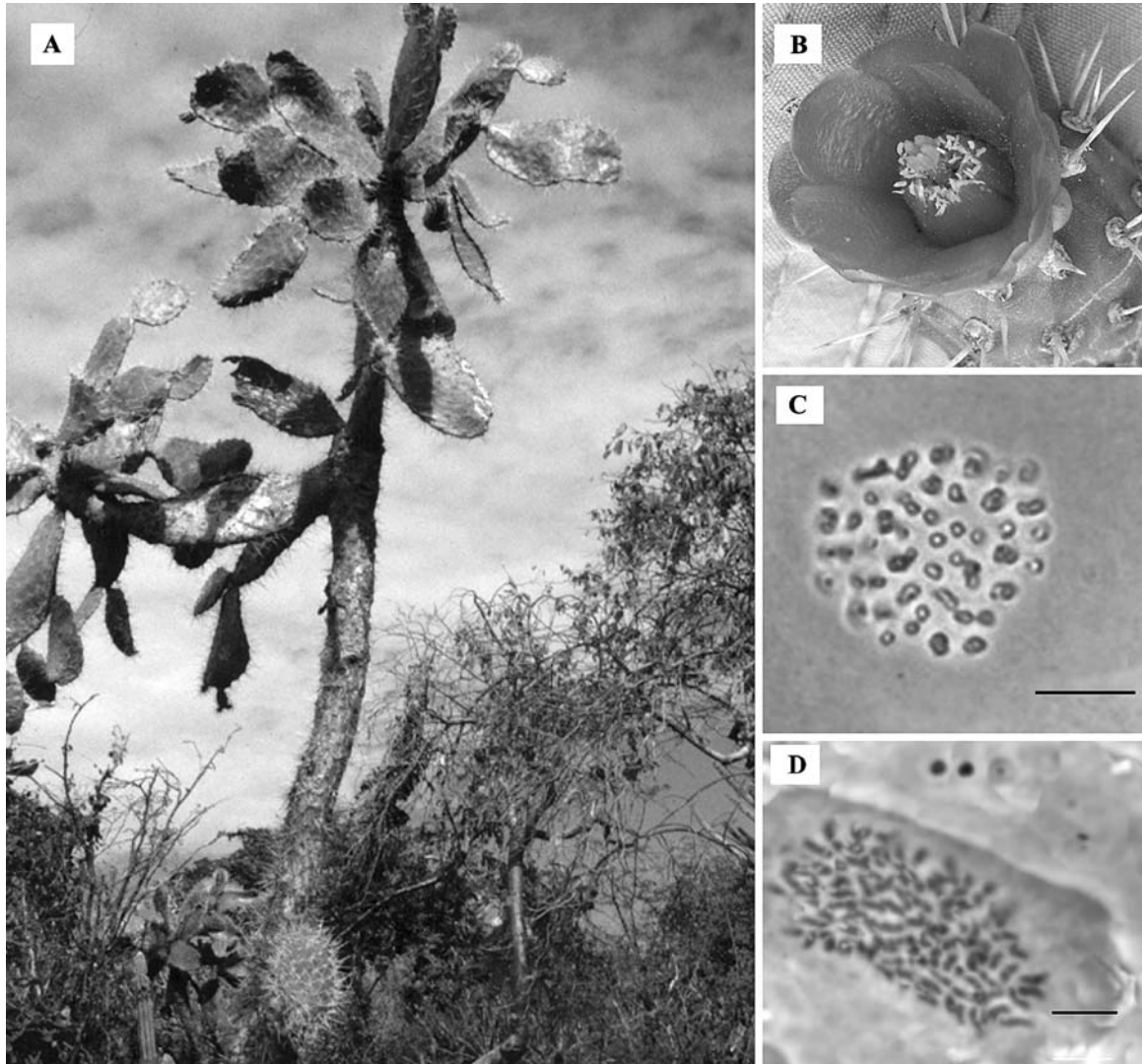


Fig. 2. Growth habit and light micrographs of representative octoploid species of *Consolea*. (A) Growth habit of male *C. rubescens* (Guánica Dry Forest Reserve, Puerto Rico); (B) female flower of *C. rubescens* (Cabo Rojo, Puerto Rico); (C, D) Chromosome stains of (C) *C. rubescens* ( $2n = 44II$ , Cabo Rojo, Puerto Rico), bar = 10  $\mu m$ , and (D) *C. falcata* ( $2n = 88$ , Haiti), bar = 5  $\mu m$ .

Negrón-Ortiz, personal observation), this study recognizes nine species.

In addition to being endemic to the Caribbean Basin, *Consolea* contains species classified as rare or threatened. For example, Florida semaphore cactus, *C. corallicola*, has a State Protected Status (Coile and Garland, 2003) and is represented by two extant small populations of only functionally male individuals (Fig. 1A, B) (Negrón-Ortiz and Strittmatter, 2004; V. Negrón-Ortiz, unpublished data). *Consolea* is cryptically dioecious/subdioecious (Strittmatter et al., 2006), with male, female, and, in a few species, fruiting male (sensu Barrett and Case, 2006) plants, and with significantly male-biased populations for most species (V. Negrón-Ortiz, personal observation). To date, chromosome number in *Consolea* have been reported to be either hexaploid [*C. millspaughii* (Britton) A Berger ( $2n = 66$ , Yuasa et al., 1973), *C. corallicola* Small ( $2n = 66$ , Pinkava, 2002)], dodecaploid [*C. rubescens* (Salm-Dyck ex A.P. de Candolle) Lem. ( $2n = 132$ , Katagiri, 1952; Yuasa et al., 1973)], or diploid [*C. moniliformis* (Linn.) A

Berger and *C. rubescens* ( $2n = 22$ , Spencer, 1955)]. The specific objectives of this study were to (1) determine/confirm the chromosome number and ploidy level for the species of *Consolea*, (2) describe FC protocols to characterize the nuclear DNA content as an estimation of ploidy level, and (3) determine whether FC can detect gender, i.e., male and female sexual morphs.

## MATERIALS AND METHODS

**Meiotic and mitotic chromosome counts**—Root tips representing all taxa were excised from cladodes kept indoors in sunlight. The root tips were collected between 0820–0900 hours, pretreated with paradichlorobenzene for 2 h (Singh, 1993), rinsed with distilled water, and transferred to Farmers solution (3 ethanol : 1 glacial acetic acid, v/v) for a minimum of 48 h, then rinsed and stored in 70% ethanol under refrigeration. The root tips were hydrolyzed in 1.2 N HCl at room temperature for 40 min, rinsed with distilled water, and stained with 1% of either iron-aceto-carmin or aceto-orcin for 24 to 48 h. The

TABLE 1. The 2C nuclear DNA content, megabase pairs (1 pg = 980 Mbp), chromosome number (2n), ploidy level, and internal standard used for the studied species of *Consolea*.

Species and gender	Locality	Voucher information	2C DNA ± SE (pg)	No. of Mbp	2n	Ploidy level (x)	Standard
<i>Consolea</i> sp.	Cayman Island	Strittmatter, s.n., 2003, MU	7.76 ± 0.0	3802	–	8	P
<i>Consolea</i> sp.	St. Croix, VI	Strittmatter, s.n., 2003, MU	9.50 ± 0.103	4655	–	9 or 10	L, P
<i>C. corallicola</i>	Florida						
Male	BNP	Negrón-Ortiz, s.n., 2005, MU	5.14 ± 0.095	2519	66	6	P
Male	LTK	DBG, 1997039701, 2000	5.16 ± 0.813	2528	66	6	P, Pg
<i>C. falcata</i>	Dept. Nord Ouest, Haiti	Strittmatter, 29, 2000, MU	7.68 ± 0.0	3763	88	8	P
<i>C. macracantha</i>	Yabacoa, Cuba	DBG, 03830101, 1995	4.88 ± 0.07	2391	66	6	P
<i>C. millspaughii</i> , male	Long Island, Bahamas	Strittmatter, 42, 2000, MU	4.92 ± 0.0	2411	66	6	P
	Cayman Island	Cayman Brac Gemini, Bot. Garden, M9300088	4.88 ± 0.0	2390	–	6	P
subsp. <i>caymanensis</i>	Cayman Island	Strittmatter, 108, 2003, MU	7.67 ± 0.134	3758	–	8	P
<i>C. moniliformis</i> , male	Lago Enriquillo, Dominican Republic	Strittmatter, 39, 2000, MU	5.07 ± 0.0	2484	66	6	P
<i>C. nashii</i>	Great Inagua, Bahamas	Strittmatter, 43, 2000, MU	5.09 ± 0.0	2494	66	6	P
<i>C. picardea</i> , male	Dominican Republic	Strittmatter, 36, 2000, MU	4.92 ± 0.06	2411	66	6	P
<i>C. rubescens</i>	Puerto Rico						
Male	CR	Negrón-Ortiz, 815, MU	7.70 ± 0.169	3773	88	8	L
Female	CR	Negrón-Ortiz, s.n., 2005, MU	7.93 ± 0.070	3886	88	8	L
Male	Guánica	Negrón-Ortiz, s.n., 1995, MU	7.85 ± 0.0	3847	88	8	L
<i>C. spinosissima</i>	Hellshire Hills, Jamaica	Strittmatter, 1, 1999, MU	5.04 ± 0.0	2479	66	6	P
<i>Leptocereus quadricostatus</i> , hermaphrodite	CR	Negrón-Ortiz, s.n., 2006, MU	1.59 ± 0.05	779	22	2	P
<i>Nopalea cochinellifera</i> , protandrous	CR	Negrón-Ortiz, s.n., 2005, MU	1.96 ± 0.018	959	22	2	P
<i>Opuntia acaulis</i> , hermaphrodite	Dominican Republic	DBG, 1997040201	7.60 ± 0.0	3724	–	8	P, L
<i>O. dillenii</i> , hermaphrodite	Ecuador	DBG, 1995031401	4.55 ± 0.0	2229	–	6	P
<i>Pilosocereus royenii</i> , hermaphrodite	CR	Negrón-Ortiz, s.n., 2006, MU	6.51 ± 0.0	3185	–	6	P
<i>Pereskia grandifolia</i>	–	–	1.96 ± 0.0	960	22	2	P

Note: L = *Lycopersicon esculentum* ‘Stupické’; P = *Pisum sativum* ‘Citrad’; Pg = *Pereskia grandifolia*; Bot. = Botanical; BNP = Biscayne National Park; LKT = Little Torch Key; CR = Cabo Rojo; DBG = Desert Botanical Garden; MU = W. S. Turrell Herbarium, Miami University; Dept. = Department; VI = U.S. Virgin Islands; – = no data.

material was washed in 45% acetic acid, then mounted and squashed in Hoyer’s medium (Willey, 1971).

Meiotic chromosomes were prepared following Malallah and Attia (2003), with some modification specified by Singh (1993). A total of 86 flower buds of *C. corallicola* (N = 33), *C. millspaughii* (N = 17), and *C. rubescens* (N = 36) at different developmental stages were collected and fixed in a solution of either 1 propionic acid : 3 ethanol and 1 g of ferric chloride/100 mL (Singh, 1993) or ethanol-chloroform-acetic acid (6 : 3 : 1) for at least 24 h. Ten anthers per bud were stained in either a drop of aceto-carmin (then immediately squashed by the conventional method) or in Snow’s carmin (Singh, 1993) for 2–3 d at room temperature. Pollen mother cells (PMCs) stained with Snow’s carmin were released in a drop of 45% acetic acid, mounted, and squashed in Hoyer’s medium.

Slides were examined using phase contrast optics and Zeiss microscope (Oberkochen, Germany) or a Nikon Labophot-2 microscope at ×100 (oil immersion). Photographs were taken with a Retiga 1300i digital camera (QImaging, Canada) using Image Pro-Plus version 5.1.1 computer software (Media Cybernetics, Bethesda, MD). A composite image, including 3–15 photographs in sequence, was generated for most mitotic cells observed using the extended depth of field of Image Pro-Plus, with maximum local contrast as the focus analysis option. The resulting image was enhanced using the Gauss filter. Counts were made by (1) printing digital images and assigning a number to each chromosome observed in the image, and (2) creating a point feature (under the measurement tool of Image Pro-Plus) to each chromosome observed in the image or series of images. Chromosome numbers were determined by counting 3–5 squashes per slide (total of three slides) per species. The format used for reporting chromosome numbers follows Strother (1972) and Strother and Nesom (1997). The bivalent symbol (II) represents meiotic counts, as in 2n = 33 II. Mitotic counts are indicated as in 2n = 66. Ploidy level was assigned on the basis of a basal chromosome number of 11 (Ross, 1981; Lichtenzeig et al., 2000). Voucher information is given in Table 1.

**Flow cytometry**—Preliminary attempts using the two-step Otto buffer, as reported for the cactus *Mammillaria san-angelensis* (Palomino et al., 1999), did

not produce results presumably because of abundant mucilage and acidity of the tissue. Subsequently, a suspension of intact nuclei of the (1) epidermal layer of the stem and of (2) root tissue were analyzed by flow cytometry using the protocol of Dolezel et al. (1989) with some modifications. About 60–90 mg of tissue was chopped in 1 mL ice-cold lysis buffer LBO1 with pH unadjusted for the epidermal layer (as suggested by J. Suda, Charles University, Prague, personal communication) and with pH adjusted to 7.5 for the root tissue. In a few occasions as little as 12 mg of root tissue was used with adequate results. About 19–27 mg of leaf tissue of *Pereskia grandifolia* Haworth, *Lycopersicon esculentum* Mill. cv. Stupické, or *Pisum sativum* L. cv. Citrad, with 2C DNA content of 2.05, 1.96, and 9.09 pg, respectively, was added as internal reference (2C values represent the DNA content of a diploid somatic nucleus) (Dolezel et al., 1989; De Rocher et al., 1990). Because of the possibility of endopolyploidy (*L. esculentum* exhibited endopolyploidy when young leaves were analyzed), the G<sub>0-1</sub> peaks of the sample and the standard may overlap. Therefore, appropriate standards were selected by first analyzing the samples without the internal reference to determine the position of the lowest ploidy peak corresponding to the G<sub>0-1</sub> phase. The suspension was filtered with 37-µm nylon mesh and stained with 50 µL (1 mg/mL) of propidium iodide (PI). Given that PI stains RNA in addition to DNA, the stained solution was treated with 50 µL (1 mg/mL) of RNase. Samples were incubated in darkness for 5–60 min before analysis.

Flow cytometric analysis was also used to determine the DNA content of male and female sexual morphs of *C. rubescens*. Ideally, all the species should have been tested, but *C. rubescens* was selected because I have samples representing both sexual morphs, and the other species were not available. Three male and three female plant cladodes were collected from a Cabo Rojo, Puerto Rico population and grown in a greenhouse. For each sexual morph approximately 70–85 mg of root tissue was analyzed simultaneously with about 19 mg of *L. esculentum* cv. Stupické, the internal reference, following the protocol described earlier. In addition, FC was used to test whether more than one peak representing each gender could be detected after simultaneous analysis of male and female sexual morphs. For this objective, three different analyses, each containing 140, 150, and 200 mg of root tissue of each sexual

morph, were chopped together in the same petri dish and analyzed as described earlier.

**Data acquisition and analysis**—Fluorescence intensity of stained nuclei was first analyzed in the Lombardi Center of Georgetown Medical School, then at Miami University using a FACSort flow cytometer (Becton-Dickinson, NJ, USA). Pulse area was detected using FL2-A. ModFit (Verity Software version 3.1) or CellQuest (Becton Dickinson Immunocytometry system, San Jose, California, USA) software was used to visualize and measure histograms peaks. Three to four replicas were prepared per sample and run on low pressure to acquire clear peaks. The mean DNA content per plant was usually based on 5000–8000 nuclei but sometimes based on 10 000 nuclei. Mean positions of the  $G_{0-1}$  (nuclei) peak of the sample and the internal standard were determined by analyzing the data with the software specified.

The formula used for converting fluorescence values to DNA content follows Dolezel and Bartos (2005): sample DNA content = (sample  $G_{0-1}$  peak mean)/(standard  $G_{0-1}$  peak mean)  $\times$  DNA content of known standard. The amount of DNA in plant cells is expressed in picograms (pg) as a C value (Bennett and Smith, 1976). Values of nuclear DNA (in pg) were converted to megabase pairs (Mbp), given that 1 pg = 980 Mbp (Bennett et al., 2000).

**Statistical analysis**—One-way ANOVA was used to determine differences in 2C DNA content (Del Angel et al., 2006) (1) for *Consolea* species grouped by ploidy level, (2) between male and female *C. rubescens* individuals from the Cabo Rojo population, and (3) between populations of *C. corallicola* from Little Torch Key and Biscayne National Park (Florida). Analyses were performed using StatView version 4.5 software.

## RESULTS

**Meiotic and mitotic chromosome counts**—The mitotic technique successfully stained *Consolea* chromosomes in actively dividing root cells (Figs. 1, 2). However, it was not always possible to accurately count the mitotic spreads in the cells because the chromosomes were numerous and tended to stick together. This may explain the great variation in the counts. Morphologically, most chromosomes had metacentric centromeres, but chromosomes with distinctly unequal arm lengths were found within the same species (Figs. 1, 2).

Compared to the base number, the chromosome counts (Table 1) indicated that *Consolea* is polyploid. The species were grouped into two main ploidy levels based on the chromosome counts. The counts indicated that seven of the nine species are hexaploids (Fig. 1) and two are octoploid (Fig. 2).

For the endangered *C. corallicola*, the chromosome number determined with meiotic and somatic counts for the population in Biscayne National Park (BNP) was equal to the somatic counts for the Little Torch Key (LTK) population (Table 1). The PMCs of *C. corallicola* from BNP had shortened and thickened bivalents, suggesting that the cells were in prophase I of diakinesis, a favorable stage to count the chromosomes (Fig. 1C). The meiotic counts indicated that this endangered species is hexaploid ( $2n = 33$  II; Table 1), consistent with the somatic number of  $2n = 66$  determined for both populations and confirming the published counts (Pinkava, 2002). One of 36 flower buds of *C. rubescens* revealed PMCs in prophase I of diakinesis and prophase II, indicating that this species is an octoploid ( $2n = 44$  II,  $2n = 88$ , Fig. 2C). Meiotic counts for *C. millspaughii* were attempted, but the cells were in prophase I of pachynema, a stage in which the nucleoli were visible with some chromosomes attached to them, making counts impossible. However, the mitotic counts indicated that *C. millspaughii* from the Bahamas is a hexaploid species (Table 1), confirming the published counts of Yuasa et al. (1973).

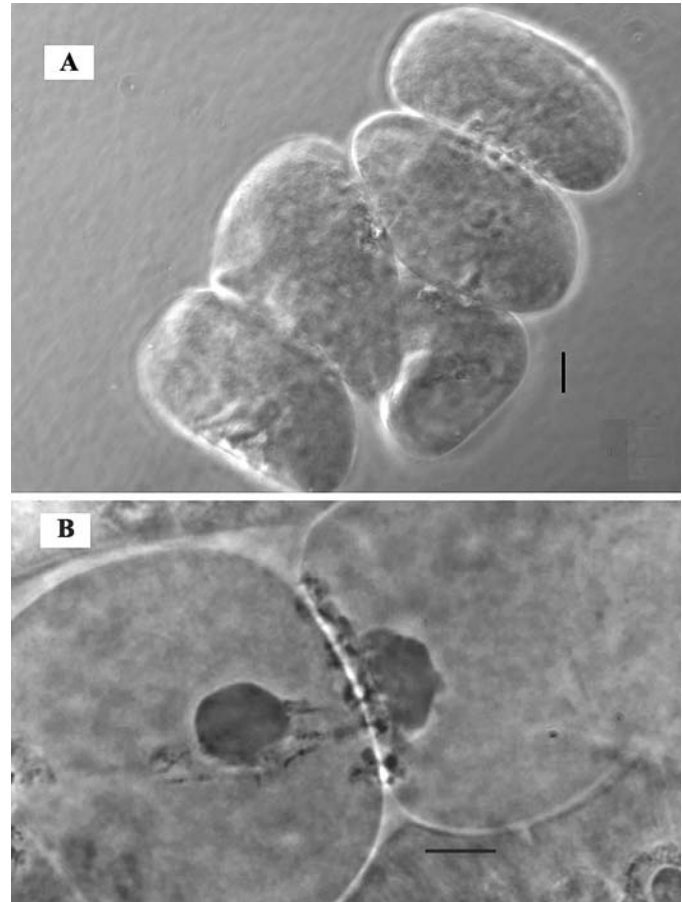


Fig. 3. Pollen mother cells and cytoplasmic connections (A) among cells of *Consolea corallicola* and (B) in *C. millspaughii* (Bahamas). Bar = 10  $\mu$ m.

**Cytomixis**—During inspection of immature anthers of *C. corallicola*, *C. millspaughii*, and *C. rubescens*, multiple chromatin bridges between microsporocytes were observed in prophase I; such bridges allow chromatin transfer between cells (Fig. 3). The number of cells involved in the phenomenon varied from two to five in *C. corallicola*, four to seven in *C. millspaughii*, and four to nine in *C. rubescens*. Occasionally, microsporocytes were completely empty subsequent to total chromatin migration into another microsporocyte. In some PMCs of *C. corallicola*, the location of some chromosomes indicated migration from a donor to its attached recipient cell. In many slides of these three species, chromosomes were clearly oriented toward the entrance of the channel or positioned within the channel, indicating continued transfer toward the recipient cell.

**Cytometric analyses**— $G_{0-1}$  and  $G_2$  phases—DNA content of nuclei isolated from the roots of *Consolea* species and analyzed simultaneously with the standard shows that most of the nuclei were in phase  $G_{0-1}$ , which represents 2C DNA content (Fig. 4A, B). For each species of *Consolea*, the histograms displayed one peak of 2C. In several samples of *C. corallicola* BNP, *C. falcata*, *C. picardea*, and *C. spinosissima*, a second peak corresponded to  $G_2$  (4C DNA content).

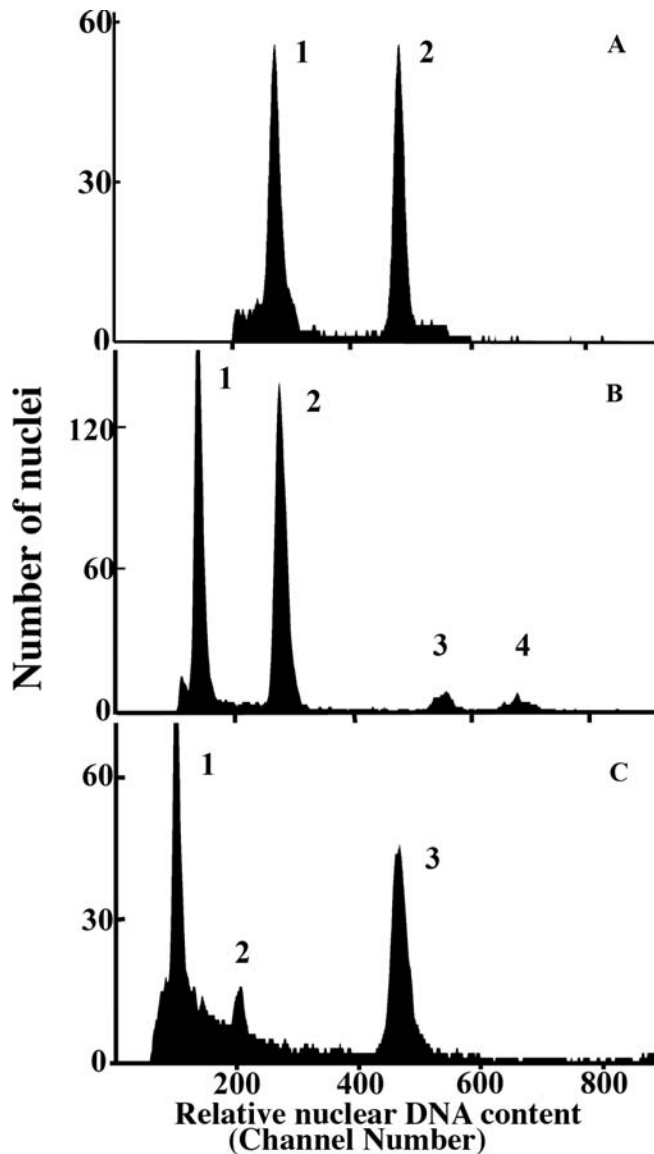


Fig. 4. Histograms of DNA content obtained after analyses of nuclei isolated from root tissue of (A) hexaploid *Consoulea nashii*, (B) octoploid female *C. rubescens*, and (C) diploid *Nopalea cochinellifera*. In histogram A, peak 1 represents nuclei with 2C ( $G_1$ ) DNA content of *C. nashii*, and peak 2 represents 2C nuclei for *Pisum sativum* as internal standard. In histogram B, peak 3 represents 2C nuclei of *C. rubescens*; peaks 1, 2, and 4 represent 2C, 4C, and 16C nuclei, respectively, of *Lycopersicon esculentum* as internal standard. Peaks 1 and 2 of histogram C represent 2C and 4C nuclei of *N. cochinellifera*, respectively; peak 3 represents 2C nuclei for *Pisum sativum* as internal standard.

In *C. corallicola*, *C. picardea*, and *C. moniliformis*, cytometric analyses of cladode epidermal cells revealed mixtures of multiploid nuclei with three peaks of 2C, 4C, and 8C, suggesting endopolyploidy, a frequent process of cell DNA synthesis without cell division (Palomino et al., 1999). The results for the other six species were incomprehensible because of the abundant mucilage of the tissue. In general, analyses of histograms of intact nuclei extracted from the roots indicated that root is a better tissue for cytometric analysis; endoreduplication was not observed.

**DNA content**—The 2C DNA content obtained by FC varied two fold (from 4.88 to 9.50 pg) among the nine *Consoulea* species (Table 1). The average DNA content (2C) was significantly lower for the hexaploids (5.03 pg) than for the octoploids (7.75 pg) (ANOVA  $F = 1208.11$ ,  $df = 1$ ,  $P < 0.0001$ ). DNA content for *Consoulea* sp. and *C. millspaughii* subsp. *caymanensis* of Cayman Islands (Table 1) were within the range obtained for the octoploid *Consoulea* species, suggesting that they are octoploid. The highest C value (9.5 pg) was for *Consoulea* sp. of St. Croix, Virgin Islands, suggesting nanoploid or decaploid levels. Unfortunately, the lack of chromosome counts for this taxon precludes an accurate ploidy determination.

The 2C DNA contents for *C. corallicola* collected from two widely separated populations in the Florida Keys were similar (ANOVA  $F = 0.604$ ,  $df = 1$ ,  $P = 0.49$ ); in addition, the ploidy level was identical. In contrast, the DNA content for two subspecies of *C. millspaughii* located on widely distant islands of different longitude coordinates differed greatly (Fig. 3, Table 1), but sample sizes were inadequate to test for significance.

Females of *C. rubescens* tended to have greater mean DNA content than the males, but this difference (0.23 pg) was not statistically significant (ANOVA  $F = 0.34$ ,  $df = 1$ ,  $P = 0.59$ ). Therefore, when male and female individuals of *C. rubescens* were analyzed simultaneously by FC, it was not possible to discriminate peaks of each sexual morph nuclei.

**Other species**—Cytometric analyses of *Pereskia grandifolia* leaves with and without pH adjusted to 7.5 consistently showed mixtures of multiploid nuclei, with three peaks of 2C, 4C, and 8C. Occasionally, a fourth peak of 9C was present when pH was not adjusted. The 4C peak overlapped with the 2C peak for *C. corallicola*, *C. falcata*, *C. moniliformis*, *C. nashii*, *C. picardea*, and *C. spinosissima*, indicating that *P. grandifolia* was not an ideal standard for those species and necessitating alternative standards. Histograms generated from root tissue of *Nopalea cochinellifera* (L.) Salm-Dyck (Fig. 4C) and *Pilosocereus royerii* (L.) Byles & G.D. Royley had two peaks corresponding to phases  $G_{0-1}$  and  $G_2$ . Histograms of *Leptocereus quadricostatus* (Bello) Britton & Rose also had two peaks of 2C and 4C, but in some cases four peaks of 2C, 4C, 8C, and 16C were obtained, suggesting the presence of endopolyploid nuclei. Histograms of *Opuntia acaulis* Ekman & Werdermann and *O. dillenii* (Ker Gawler) Haworth consistently had one peak, indicating that the isolated nuclei were in phase  $G_{0-1}$ .

The range in 2C DNA content for the diploid species *Leptocereus quadricostatus* and *N. cochinellifera* was similar to the DNA content of the diploid cactus species reported for *Mammillaria* (Del Angel et al., 2006) and *Pereskia grandifolia* (this study; Del Angel et al., 2006). The DNA content of the other species was within the range of 4.55 pg in *O. dillenii* to 7.6 pg in *Opuntia acaulis*, similar to values obtained for *Consoulea* (Table 1).

## DISCUSSION

**Polyploidy and C-DNA levels**—Combined data from chromosome counts and FC confirmed that all *Consoulea* species are polyploid. These data showed distinctive ploidy/C-DNA levels among species and between populations of *C. millspaughii* from widely separated geographical regions

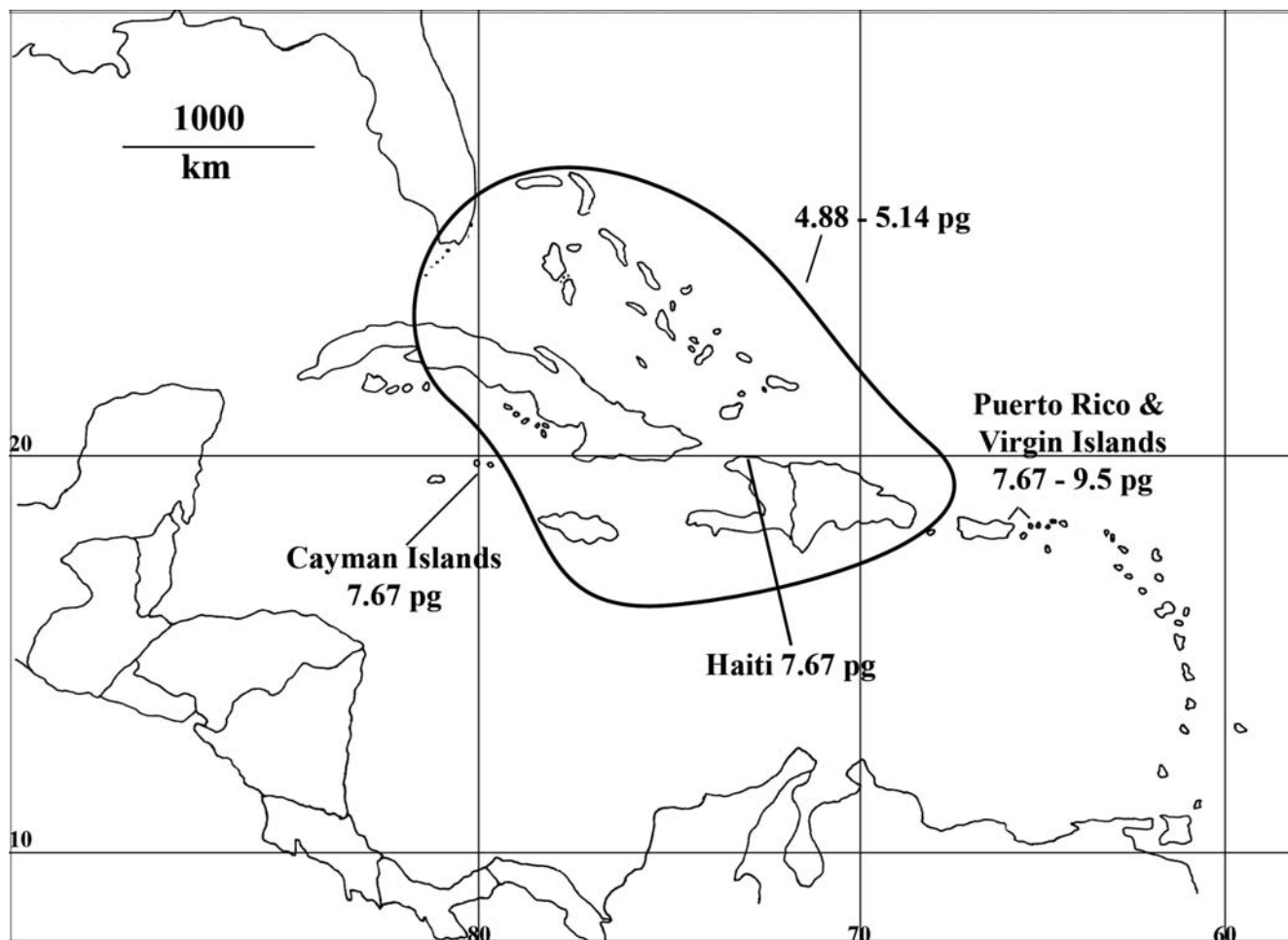


Fig. 5. Map of the Caribbean region showing the distribution of hexaploid (encircled islands, except Haiti) and octoploid species and their DNA content.

(Long Island, Bahamas, and Cayman Islands). Most of the hexaploid species are distributed in the Bahamas, Florida Keys, and Greater Antilles, specifically on Cuba, Hispaniola, and Jamaica (Fig. 5). *Consolea falcata*, an octoploid species endemic to Haiti (Hispaniola), is in a group with the highest DNA content, along with *C. rubescens* from the island of Puerto Rico, *Consolea* sp. from St. Croix, Virgin Islands, as well as *Consolea* sp. and *C. millspaughii* subsp. *caymanensis* from the Cayman Islands (Fig. 5). *Consolea rubescens* and *Consolea* sp. (from St. Croix) are found outside the geographical range of most hexaploids, between longitudes 60° to 67° (Fig. 5), and they share a similar dark greenish-red color for the stem. With the information to date, there seems to be no correlation between polyploidy and latitude and/or longitude.

Diploidy was not observed as reported by Spencer (1955) for two species in Puerto Rico: *C. moniliformis* (as *O. moniliformis*) for the town of Cabo Rojo, and *C. rubescens* (as *O. rubescens*) for Mona Island. Subsequent work has not confirmed the species in these locations, and vouchers were not listed in his publication. My field work in Cabo Rojo and conversations with G. Breckon (U. Puerto Rico), indicated that the species identifications were switched. Chromosome counts for *C. rubescens* of Cabo Rojo showed that it is a polyploid

species, and C-DNA values fall within the ploidy range when compared to diploid cactus species (Palomino et al., 1999; Del Angel et al., 2006). Unfortunately, I didn't have access to the population of *C. moniliformis* from Mona Island. But based on counts conducted throughout the whole range of the genus and from populations of *C. moniliformis* occurring allopatrically in Hispaniola, it is most logical to assume that diploidy is absent.

These results were not surprising because of the high ploidy level reported for the Opuntioideae (Baker, 2002; Pinkava, 2002). Differences in DNA content have been interpreted as an adaptation to different environmental conditions (Walker et al., 2005). Dart et al. (2004) indicated that differences in DNA content between and within species could be caused by the loss or gain of entire chromosomes or by change in chromosome size. Chromosomes are quite homogenous among the *Consolea* species. Therefore, it is obvious that the differences in DNA content between *Consolea* species is caused by gaining chromosomes. Thus, polyploidy is clearly a possible contributor to C-value variation, but the relationship between these is not straightforward (Murray et al., 2005). Several authors have shown that an increase of the mean C-value is not proportional to ploidy and mean genome size (Tuna et al., 2001; Leitch and Bennett, 2004). Tuna et al. (2001) found a slight reduction of DNA content as ploidy level increases. This reduction could be

a necessary adaptation for the establishment and stabilization of polyploid genomes (Ozkan et al., 2003).

**Speciation by polyploidy**—The data strongly support that polyploidy is one of the major causes of diversity in the current *Consolea* species, which have at least two main ploidy levels. Because most of the species are hexaploids, it is reasonable to infer that the ancestral condition was a plant with a haploid ( $n$ ) number of 33 and a diploid ( $2n$ ) number of 66, from which evolved the different polyploid descendants. But how can a new cytotype with higher ploidy be formed? Studies on *Beta vulgaris* and alfalfa have provided evidence for one-step ploidy formation of autohexaploids via the union of reduced ( $2\times$ ) and unreduced gametes ( $4\times$ ) (Ramsey and Schemske, 1998). Alternatively, hybridization between different cytotypes could generate allohexaploids via a two-step pathway. First, the cross would form intermediates possessing odd ploidy numbers; subsequently, production of new cytotypes with even ploidy level would be generated (Ramsey and Schemske, 1998). Examples for the last pathway are reported for crosses between *Nicotiana paniculata*  $\times$  *N. rustica*, and *Triticum cassum*  $\times$  *T. turgidum* (Ramsey and Schemske, 1998).

Natural hybridization among the opuntioids is common (Parfitt and Gibson, 2004), resulting in plants with unique, combined, or intermediate character states. On the island of Bermuda, Howard (1982) observed plants that appeared to be hybrids between *C. rubescens* and *O. dillenii*. The intermediate plants possessed the growth habit of *C. rubescens* and the spines of *O. dillenii*. Similarly, *Opuntia*  $\times$  *Lucayana* Britton, a native of Turks and Caicos Islands, is reported to be a hybrid between *C. nashii* and *O. dillenii* (Procter and Fleming, 1999). The hybrid exhibits a tree-like growth form of *C. nashii* and floral features of *O. dillenii*. The hybridization that is occurring between this genus and some *Opuntia* spp., combined with phylogenetic studies that have documented reticulate evolution in the Opuntioideae (Mayer et al., 2000; Griffith, 2003), suggest the hypothesis that the genus *Consolea* could be of hybrid origin. Griffith (2005) suggested the possibility of reticulate evolution for this genus. In his analyses, the ITS (nuclear marker) sequence DNA data suggest the inclusion of *Consolea* in *Opuntia* s.s. Whereas the chloroplast (*trnL-F*) sequence data place *Consolea* outside *Opuntia* s.s. Pattern of incongruent placement, based on two different markers, is considered evidence of a reticulation event (Linder and Rieseberg, 2004) and, in this case, perhaps of ancient reticulation. This could have been achieved through a variety of pathways (Ramsey and Schemske, 1998).

The current data also suggest that cytomixis may have caused polyploidy in *Consolea*. Cytomixis has been observed in diploid species (Sapre, 1978) and in plants with genetic instability, such as haploids, aneuploids, hybrids, mutants, triploids, and apomicts (Falistocco et al., 1995). Thus, its occurrence in *Consolea* is not surprising. Cytomixis could explain the formation of microsporocytes with no chromatin, resulting in pollen abortion, and the presence of various pollen grain sizes in *Consolea*. Cytomixis could be considered a possible source of polyploid or aneuploid pollen, a process of evolutionary significance if these pollen grains sire progeny.

Cytomixis may result in  $2n$  gametes (Falistocco et al., 1995). This might occur in *Consolea*, because I observed large microsporocytes, both nucleated and enucleated. According to Ramsey and Schemske (1998), typically  $2n$  gametes can be identified by the large diameter of the pollen grains. Pinkava et

al. (1998) suggested why most polyploid species within the Cactaceae originate from fertilization involving unreduced gametes. In summary, they (1) observed viable macropollen in diploid species, (2) indicated that  $2x$  and  $3x$  opuntioids are more common than  $4x$  taxa, and (3) pointed out the significance of the genome dosage effects on the morphology of interspecific and intergeneric hybrids.

Polyploidy can be also explained by meiotic irregularities (e.g., Lichtenzweig et al., 2000; Diggle et al., 2002), although their association is not well established, and many polyploids have normal chromosome pairing at meiosis (e.g., Law et al., 1983; Diggle et al., 2002). Although meiotic irregularities were not observed in the few samples of *C. corallicola*, *C. millspaughii*, and *C. rubescens* examined, this mechanism cannot be ruled out. Irregular chromosome disjunction during anaphase I in pollen mother cells of *Selenicereus megalanthus* (K. Schumann ex Vaupel) Moran, a tetraploid cactus species, has been reported to be one of the major causes of reduced pollen viability (Lichtenzweig et al., 2000). Because of the high rates of pollen germination in *Consolea*, meiotic aberrations may not be that frequent. It is also possible that meiotic aberrations are not found in plants of higher ploidy; further examination of meiosis in *Consolea* is warranted.

Phylogenetic reconstructions indicated that small genome size (referred to 1C-value) represents the ancestral condition for most major angiosperm clades (Soltis et al., 2003). If this statement is accurate, then the mean DNA content for the hexaploids, 5.01 pg, represents the ancestral DNA content because the mean DNA content of the octoploids was 7.78 pg. A bidirectional mode of genome size evolution, however, occurs in the Gossypieae (Wendel et al., 2002) and to some extent in *Sorghum* (Price et al., 2005). Unfortunately, the lack of a comprehensive phylogenetic study prevents further interpretation of the direction(s) of evolution of genome size in *Consolea*.

**Significance of high levels of polyploidy**—*Endopolyploidy*—Endopolyploidy, a process where the cells undergo repeated cycles of DNA synthesis without cell division, resulting in cells with multiple ploidy levels, can vary among individuals of one species in response to different environmental conditions (Barow, 2006). Common in both animals and plants, this process is extremely regulated, is known to occur in active development and differentiation of somatic tissue and is assumed to increase the nuclear DNA content (D'Amato, 1989). The degree of endopolyploidization varies among plant organs and is primarily related to specific families (Barow and Meister, 2003), including the Cactaceae (Palomino et al., 1999; Del Angel et al., 2006). In *Consolea*, endopolyploidization of water storage parenchyma cells of stem and cladodes could be an adaptation to promote cell expansion but inhibit mitosis, allowing faster growth and development during favorable conditions. Several authors have stated that endopolyploidy is beneficial for succulents and cacti possessing small genome size (Palomino et al., 1999; Del Angel et al., 2006); therefore, this mechanism is ecologically important when water is scarce and temperatures are high.

**Reproduction**—Several authors have proposed polyploidy as a mechanism that can trigger different reproductive strategies. Polyploidy has been linked with mating systems such as asexual reproduction, i.e., apomixis and vegetative, and self-



fertility (Ramsey and Schemske, 1998; Rosquist, 2001), all of which help in the establishment, survival, and dispersal of recently arisen polyploid species. Vegetative propagation by stem, cladodes, and abscised ovaries is common in *Consolea* and in many other cactus species (Rebman and Pinkava, 2001). To date, all known pentaploid cactus species produce seeds by apomixis (Pinkava et al., 1998). Two types of apomixes, sporophytic agamospermy (adventive embryony) and diplosporic parthenogenesis (development of embryos from an unfertilized egg), occur in *Opuntia* (Naumova, 1993; Rebman and Pinkava, 2001; Reyes-Agüero et al., 2006). Apomixis is the mode of seed production in the endangered species *C. corallicola* from LTK (as *O. spinosissima*, Negrón-Ortiz, 1998).

Rebman and Pinkava (2001) determined that gynodioecious and trioecious cactus species in the Cactaceae are polyploid. Therefore, in addition to the mating systems described, sexual dimorphism may have arisen via polyploidy (Miller and Venable, 2000; Gorelick, 2005). But how can sexual dimorphism be triggered? In a model proposed by Miller and Venable (2000), polyploidy disrupts self-incompatibility (Richards, 1997; Miller and Venable, 2000), leading to inbreeding depression. Male sterile mutants (female sexual morphs) are favored because they avoid inbreeding; later, female sterile mutants invade the population, giving rise to dioecy. *Consolea* has all the conditions to fit the model: the genus belongs to the Cactaceae, a family predominantly hermaphroditic with a widespread self-incompatibility genetic condition (Boyle, 1997); all the species are polyploids; and all the species possess a subdioecious/cryptically dioecious mating system. Despite this compelling scenario, Brunet and Liston (2001) stated that the supporting evidence for this model is weak.

Flow cytometry has been used for sex identification of dioecious plants with heteromorphic sex chromosomes (Costich et al., 1991; Dolezel and Göhde, 1995). However, heteromorphic sex chromosomes are extremely rare in flowering plants (Gorelick, 2005), and because *Consolea* and most Cactaceae chromosomes are mostly monomorphic, these types of chromosomes are not likely to be found in this genus. Theoretically, dioecy and sex chromosomes evolved from hermaphrodites that only possessed autosomes, and signals such as cytosine methylation enforced Muller's ratchet, resulting in shorter Y chromosomes (Gorelick, 2005). But in plants with high rates of polyploidy and polysomy, such as bryophytes and homosporous pteridophytes, chromosomal rearrangements provide an alternative explanation for the origin of heteromorphic sex chromosomes (Gorelick, 2005). For dioecious species lacking heteromorphic sex chromosomes, Gorelick (2005) concurs that the model described in the previous paragraph provides a reasonable mechanism for the origin of this mating system.

In summary, the present study illustrates the usefulness of classic cytological techniques and FC for detection of ploidy level in *Consolea*. DNA content for the diploid cactus species included in the study is similar to that of diploid *Mammillaria* species (Palomino et al., 1999; Del Angel et al., 2006). The application of FC to diagnose gender, which could be important in conservation, suggested that females tend to have more DNA content than males, although this difference was not significant. More comparisons of males vs. females of dioecious species is needed to determine whether sex can be determined from DNA content at early stages of plant

development. In the present study, mean C-DNA content and chromosome number separated *Consolea* species into two main groups, which could be interpreted as two phylogenetic lineages. Alternatively, polyploidization may have occurred independently more than twice, with comparable effects on C-DNA content.

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