

Complex Ploidy Level Variation in Guayule Breeding Programs

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ABSTRACT

Guayule (*Parthenium argentatum* A. Gray) is a potential source of natural rubber, but attempts to domesticate and cultivate this perennial crop for large-scale production in the southwestern United States have been intermittent over the past century. Genetic improvement through modern plant breeding is needed to increase its yield potential and suitability for commercialization. Natural variation for ploidy level is extensive among individuals in wild guayule populations, but less is known about the extent of ploidy level variation in guayule breeding germplasm. Because ploidy variation is among the factors that slow the rate of genetic gain in guayule breeding programs, determining the ploidy level of publicly available guayule accessions would help to accelerate the development of stable, high yielding cultivars. To that end, we adapted flow cytometry to examine the ploidy of 34 guayule accessions available from the National Plant Germplasm System. The data revealed a natural polyploid series ranging from diploid ($2n = 2x = 36$) to pentaploid ($2n = 5x = 90$), with $4x$ being the predominant ploidy. Interestingly, not all plants sampled from an accession had the same ploidy level (mixed ploidy). Notably, the integration of ploidy and pedigree data uncovered complex ploidy variation in guayule breeding programs. The frequency and range of ploidy variation observed in this germplasm will help to direct future breeding efforts as well as linkage analysis and genome-wide association studies.

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Abbreviations: NALPGRU, National Arid Land Plant Genetics Resources Unit; NPGS, National Plant Germplasm System; WPB, woody plant buffer.

POLYPLOIDY is a profound but pervasive evolutionary force in angiosperms (reviewed in Soltis and Soltis, 2009). Multiplication of an entire chromosome set due to unreduced gametes or interspecific hybridization increases the copies of the basic chromosome set or ploidy level, but it also confers to polyploid individuals the potential to gain novel phenotypes and exhibit transgressive variation for adaptive traits relative to their diploid progenitors (reviewed in Leitch and Leitch, 2008; Levin, 1983; Rieseberg and Willis, 2007). In natural populations, ploidy differences among individuals can act as a reproductive barrier and serve as a driving force for speciation. Thus, polyploidy directly impacts mating patterns and gene flow and, as such, the level of genetic diversity within and among individuals. In the context of a plant breeding program, knowledge of the type and extent of ploidy level variation in a crop species is important for studying patterns of inheritance, estimating the copy number of alleles, and selecting parents that when crossed will produce fertile offspring.

Guayule (*Parthenium argentatum* A. Gray), a member of the family Compositae (Asteraceae), is a xerophytic, woody perennial shrub native to desert regions of the southwestern United States and northern Mexico. Throughout the 20th century there have been periodic

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efforts in the United States to domesticate and commercialize guayule as a renewable source of natural rubber (reviewed in Ray et al., 2005). The prevalence of allergies attributed to latex from the rubber tree [*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.]—the predominant commercial source of natural rubber—has spurred renewed interest in guayule because its latex is hypoallergenic (Siler and Cornish, 1994). In addition, concerns about the vulnerability of the rubber tree to leaf blight, coupled with projected shortages of natural rubber by 2020 (Mann, 2009), have contributed to current efforts to develop guayule as a domestic source of natural rubber.

Guayule reproduces by facultative apomixis (diplospory type) and has a highly variable number of chromosomes (Bergner, 1944; Powers, 1945; Stebbins and Kodani, 1944), but to our knowledge the nuclear genome size (C-value) of diploid or polyploid guayule has never been estimated. With a base chromosome number of 18, wild populations of guayule consist of a natural polyploid series that ranges from diploid ($2n = 2x = 36$) to pentaploid ($2n = 5x = 90$) (Kuruvadi et al., 1997). In general, diploids reproduce sexually and are self-incompatible, whereas polyploids are facultative apomicts (reviewed in Thompson and Ray, 1988). Higher yielding, adapted cultivars are needed if guayule is to be an economically viable crop, but in combination with wild characteristics, its mode of reproduction and ploidy level variation have hindered genetic improvement.

The genetic diversity of guayule can be more effectively exploited in a breeding program when the ploidy level of the germplasm is known. Because guayule diploids reproduce sexually, they have been more amenable than apomictic polyploids to plant breeding methodologies that require interbreeding of reselected plants, such as recurrent selection (Ray et al., 1995). However, polyploids have been reported to be superior to diploids for important traits such as vigor and architecture of plants as well as yield of rubber and biomass (Kuruvadi et al., 1997). Specialized breeding strategies have been designed to unite genetic variation from both diploids and polyploids into a single guayule cultivar (Ray et al., 2005; Thompson and Ray, 1988). Previous studies on ploidy in guayule relied on cytological counts of chromosomes in root meristematic or pollen mother cells using a light microscope (Hashemai et al., 1989; Herickhoff et al., 1994). This is a protracted procedure for even a modest number of samples and certainly not suitable for large-scale screening, as would be required in guayule breeding programs. Thus, a faster method for evaluating ploidy levels of guayule plants would be valuable for efficient improvement of germplasm.

Flow cytometry has been used for cytological applications such as determination of ploidy levels, detection of aneuploidy, screening of reproductive mode, analysis of cell cycle, and estimation of absolute nuclear DNA amount (nuclear genome size). Compared to chromosome counting, flow cytometry is a rapid and simple method for the

determination of ploidy levels in crop species. With a high level of accuracy, flow cytometry indirectly estimates the number of chromosomes in somatic cells by measuring the DNA content of fluorescence stained nuclei (Arumuganathan and Earle, 1991a, b). As evidence of its robustness, flow cytometry has been used to measure the nuclear DNA content of more than 3500 diverse plant species (Loureiro et al., 2007b). Recently, Kelley et al. (2009) used flow cytometry to characterize 33 different *Poa* species for ploidy level and reproductive behavior. Notably, the mode of reproduction and range of ploidy level variation in *Poa* are similar in complexity to those reported in guayule (Bergner, 1946; Kelley et al., 2009; Powers, 1945; Stebbins and Kodani, 1944). Thus, flow cytometry also has potential to be suitable for ploidy level analysis in guayule.

Guayule breeding efforts will benefit from a rapid, accurate method for ploidy level analysis and knowledge of ploidy in available guayule germplasm. We conducted the present study to (i) adapt flow cytometry for ploidy level analysis in guayule and (ii) evaluate the extent of ploidy level variation within and among guayule cultivars and germplasm lines that are available from the U.S. National Plant Germplasm System (NPGS) (available at <http://www.ars-grin.gov>, verified 28 Sept. 2010).

MATERIALS AND METHODS

Plant Material

We determined the ploidy level of 34 guayule accessions (Table 1) that are in the NPGS and maintained by the National Arid Land Plant Genetics Resources Unit (NALPGRU) at Parlier, CA. Achenes (hereafter seeds) for 33 out of the 34 accessions were obtained from NALPGRU; AZ-2 (PI 599675) was from an in-house seed source (Ray et al., 1999). Seeds were germinated on moist vermiculite in a controlled growth chamber environment with 14 h of light ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 28°C) and 10 h of darkness (at 21°C).

We separately planted each 7-d-old seedling into a 10.16 cm diameter pot containing moistened Sunshine Mix #1 (Sun Gro Horticulture Inc., Bellevue, WA) and perlite (4:1 ratio). Seedlings were grown in a controlled growth chamber environment under conditions identical to those for germination. All plants in the growth chamber were fertilized every 2 wk with 20–20–20 (50 ppm N) Peters Professional plant nutrient solution (The Scotts Company, Marysville, OH). After 2 mo in the growth chamber, we separately transplanted each guayule plant into a 3.8 L (one gallon) pot containing the previously described soil mixture. Plants were grown under natural light in a glasshouse with daytime and nighttime temperatures at 28 and 21°C , respectively. In the glasshouse, all plants were fertilized every two-weeks with 20–20–20 (200 ppm N) Peters Professional plant nutrient solution.

Sample Collection and Preparation for Flow Cytometry

The 36-Chromosome (PI 478663) plant that was repeatedly used as the internal diploid standard for ploidy level analysis (Supplementary Fig. 1) and four additional 36-chromosome plants evaluated in this

Table 1. Ploidy levels of 34 guayule accessions.

Accession	Cultivar or germplasm line	n [†]	Average peak ratios	Estimated ploidy [‡]	Published literature ploidy [§]
PI 478663	36-Chromosome	7	1.00	2x	2x; Ray et al. (1995), this study
PI 478664	Cal-3	3	1.00	2x	2x; Tysdal et al. (1983)
W6 550		4	1.52	3x	
W6 2244		5	1.51	3x	3x; this study
W6 2245		6	1.52	3x	
W6 2247		4	1.55	3x	
PI 478640	11591	5	1.52	3x	3x; this study
PI 478644	11609	3	1.52	3x	
PI 478646	11633	2	1.52	3x	
PI 478655	N565	1	1.50	3x	
PI 478656	N565-II	1	1.52	3x	
PI 478658	N575	1	1.52	3x	
		3	1.99	4x	
PI 478665	Cal-4	1	1.54	3x	
		1	2.59	5x	
PI 599674	AZ-1	1	1.55	3x	
		3	2.04	4x	
PI 478653	12231	2	1.52	3x	
		1	2.48	5x	
W6 549		1	2.01	4x	4x; Estilai (1986)
W6 2189		6	2.01	4x	
W6 2192		3	1.99	4x	
W6 2253		2	2.00	4x	4x; this study
W6 2260		2	1.97	4x	
PI 478639	593	3	2.02	4x	
PI 478641	11600	1	1.98	4x	
PI 478642	11604	5	1.99	4x	
PI 478643	11605	4	1.96	4x	
PI 478645	11619	1	1.91	4x	
PI 478647	11634	2	1.93	4x	4x; Thompson and Ray (1988)
PI 478648	11635	7	2.00	4x	4x; Thompson and Ray (1988)
PI 478650	11693	2	1.97	4x	4x; Thompson and Ray (1988)
PI 478660	4265-X	7	1.99	4x	4x; Thompson and Ray (1988)
PI 599675	AZ-2	4	2.02	4x	
PI 599676	AZ-3	3	2.04	4x	
PI 599678	AZ-5	6	2.01	4x	4x; this study
PI 599679	AZ-6	5	2.01	4x	
PI 478657	N566	6	2.48	5x	5x; this study

[†]Number of single plants with a specified ploidy level.

[‡]Ploidy levels estimated by flow cytometry with an internal diploid standard.

[§]Ploidy levels determined by cytological chromosome counting.

study were confirmed as diploid ($2n = 2x = 36$) based on mitotic chromosome counts in root tip cells. Root tips were pretreated with ice water for 24 h and fixed in ethanol:glacial acetic acid (3:1), followed by staining in 1% acetocarmine and squash preparation as previously described by Costich et al. (2010). Counts of metaphase chromosomes from images were independently obtained and confirmed by two people (authors Michael A. Gore and Bernd Friebe).

We separately collected fresh, intact leaf tissue (40–60 mg) in triplicate (technical replication) from individual plants (biological replication) of each unknown accession and the 36-Chromosome plant used as the diploid standard. Tissue samples consisted of young, fully expanded leaves that subtended immature flower stalks because young, rapidly growing leaves at this developmental stage gave the best results (data not shown). Collected tissue samples were preserved at 4°C until same-day preparation.

Samples were prepared for ploidy analysis as previously described by Loureiro et al. (2007a), with various modifications. For the preparation of each individual sample, equal amounts of tissue from an unknown accession and the diploid standard (internal control) were combined and added to a Petri dish containing 1 mL of woody plant buffer (WPB) (Loureiro et al., 2007a). The combined leaf tissue was coarsely chopped in the WPB using a sharp razor blade for approximately 30 s. The resulting homogenate was passed through a Partec 30 µm CellTrics (Partec GmbH, Münster, Germany) disposable nylon filter to remove tissue debris. Nuclei from somatic cells were stained with 4 µg mL⁻¹ of the DNA-specific fluorochrome DAPI (4µ, 6-diamidino-2-phenylindole) (Sigma-Aldrich, St. Louis, MO). Samples were incubated on wet ice for 10 min, followed immediately by flow cytometry.

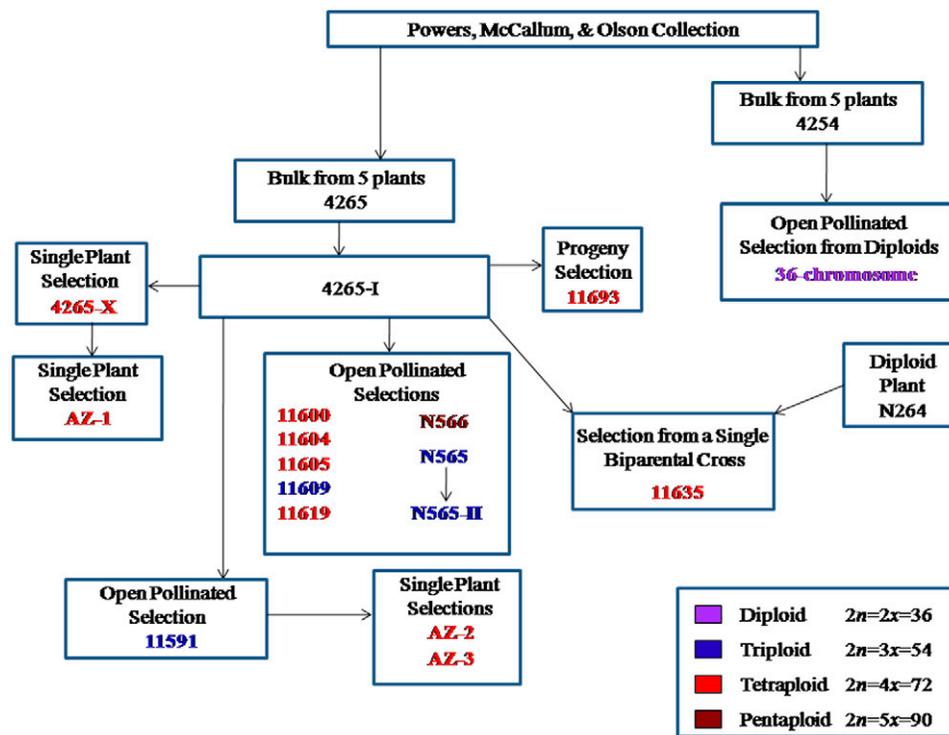


Figure 1. Pedigrees, plant breeding selection methods, and ploidy levels of guayule cultivars and germplasm lines. Accessions with a colored font were examined with flow cytometry in this study and had pedigree information available from Thompson and Ray (1988) and Ray et al. (1999). Although not all sampled plants from AZ-1 had the same ploidy level, it is considered a tetraploid based on the ploidy of its parent (4265-X) and majority of sampled AZ-1 plants.

Analysis of Ploidy Level with Flow Cytometry

Fluorescence of DAPI-stained nuclei was analyzed using a Partec Ploidy Analyser flow cytometer with an HBO-100W mercury lamp (Partec GmbH). The gain of the instrument was adjusted to ensure that the mean position of the G1 peak for the diploid standard (36-Chromosome) was set at channel 100. Ploidy level of each unknown guayule accession was calculated per Doležel et al. (2007) as follows: mean position of the G1 peak for an unknown guayule accession (“sample”) divided by the mean position of the G1 peak for the diploid standard 36-Chromosome (“reference”) multiplied by 2 (the ploidy of 36-Chromosome). The quality of the data was assessed by calculating the coefficient of variation for each measurement with the Partec CA3 analysis software.

RESULTS AND DISCUSSION

We adapted the DNA flow cytometry method of Loureiro et al. (2007a) to examine ploidy level variation within and among the 34 guayule accessions available from the NPGS. There are additional guayule accessions in the NPGS, but a substantial number of them were not available at the time of this experiment (Germplasm Resources Information Network, http://www.ars-grin.gov/cgi-bin/npgs/html/tax_site_acc.pl?PARL%20Parthenium%20argentatum, verified 28 Sept. 2010) or may have inviable seed (Ray et al., 2005). Extensive variability in seed germination and seedling emergence and mortality was observed among accessions (data not shown), which was presumably due to the reduced viability and lower quality of older seed lots rather than inadequacies of seed conditioning treatments (Jorge et al., 2006). As a result, the total

number of emergent seedlings that survived for each accession ranged from one to seven, but we were still able to examine ploidy levels in 124 individual plants. Further research is needed to improve germination and reduce seedling mortality in guayule, but this is beyond the scope of this work.

Of the 34 guayule accessions, 32 were polyploid and two were diploid. Ploidy ranged from diploid ($2n = 2x = 36$) to pentaploid ($2n = 5x = 90$), with a median and mode ploidy of $4x$ (Table 1). This observed ploidy series ($2x$ to $5x$) and the preponderance of polyploids, especially tetraploids, are consistent with reports for wild populations (Kuruvadi et al., 1997). Although individual plants with ploidy levels up to $8x$ have been identified within cultivated guayule populations (Powers, 1945; Thompson and Ray, 1988), ploidy levels greater than $5x$ were not observed in this study. Polyploid plants with an irregular number of chromosomes (i.e., aneuploidy) are also found in guayule populations (Bergner, 1946; Kuruvadi et al., 1997; Powers, 1945), but we did not evaluate the sensitivity of flow cytometry for detecting aneuploidy. In addition, many guayule plants contain one to several very small B or supernumerary chromosomes (Bergner, 1946; Catcheside, 1950) but if present are not expected to contribute significantly to differences in nuclear DNA content among guayule accessions.

Within four accessions, we observed more than one ploidy level among individuals (mixed ploidy). For example, we detected triploid and pentaploid plants within Cal-4 (PI 478665), which is a composite of open-pollinated seeds

that were bulk harvested from several diploid plants with resistance to *Verticillium* wilt (Tysdal et al., 1983). These findings are not unexpected given that Tysdal et al. (1983) had suspected that most of the resistant diploid plants were contaminated with pollen from neighboring polyploid plants. Similarly, AZ-1 (PI 599674) contained both triploid and tetraploid plants (Table 1), but in contrast to Cal-4 it was developed from a single plant selected from 4265-X (PI 478660; Fig. 1). We believe that the identified triploid plant from AZ-1 is a contaminant from unintended outcrossing to a diploid, because 4265-X is a tetraploid and also the product of a single-plant selection. The other two mixed ploidy accessions, 12231 (PI 478653) and N575 (PI 478658), were collected from wild populations in Durango and Zacatecas, Mexico, respectively (Thompson and Ray, 1988). In these cases, natural variability for ploidy level has been most likely retained within these two accessions.

The guayule accessions evaluated in this study represent cultivars and germplasm lines as well as parental stocks from which some of the cultivars and lines were derived. This allowed us to compare the ploidy level of cultivars and lines to that of their source. Exemplifying complex familial relationships and a narrow germplasm base, 15 out of the 34 accessions have a lineage that traces back to 4265-I (Fig. 1). Johnson (1950) selected 4265-I from 4265, a bulked collection of five wild plants from Durango, Mexico. Open-pollinated selections from 4265-I and its descendent N565 (PI 478655) resulted in the development of nine accessions that included triploid, tetraploid, and pentaploid plants. On the other hand, 4265-X and 11693 (PI 478650) were developed through controlled pollination of 4265-I plants, but both 4265-X and 11693 are tetraploid. Taken together, these findings suggest that 4265-I was a mixture of ploidy levels. The lack of viable 4265-I seed prevented our exploration of this hypothesis, but nonetheless it agrees with previous chromosome counts made on individual plants from 4265-I (Johnson 1950).

Interestingly, our data indicated 11591 (PI 478640) was triploid, but single-plant selections, AZ-2 (PI 599675) and AZ-3 (PI 599676), both derived from 11591, were tetraploid (Fig. 1 and 2). AZ-2 and AZ-3 were selected due to their higher rubber and resin content relative to 11591 (Ray et al., 1999), which could be partly attributed to higher ploidy level. Although 11591 is a phenotypically uniform cultivar, off types have been occasionally observed within large field plantings (Terry Coffelt and Dennis Ray, unpublished data, 2005). Therefore, it is possible that tetraploid plants from 11591 exist at very low frequency and, thus, would have a higher probability of being observed when a large number of individuals are evaluated. This explanation is plausible given that 11591 is an open-pollinated selection and nearly one thousand single-plant families were field-evaluated for phenotypic variation during the development of AZ-2 and AZ-3 (Ray et al., 1999). In contrast, only five plants from 11591 were analyzed for ploidy levels in this study.

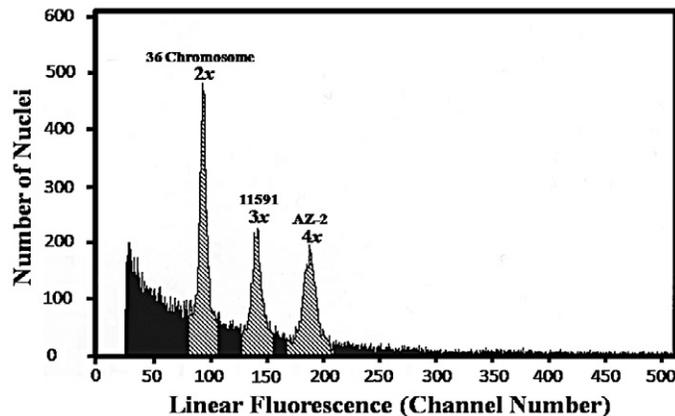


Figure 2. Flow cytometry histogram of 36-Chromosome (diploid), 11591 (triploid), and AZ-2 (tetraploid) plants. The 36-Chromosome plant was repeatedly used as the internal diploid standard for flow cytometry. AZ-2 is a single plant selection from 11591 but differs in ploidy to that of 11591.

The accuracy of ploidy level data for six (36-Chromosome, W6 2244, 11591, W6 2253, AZ-5, and N566) of the 34 polyploid guayule accessions was directly assessed by cytological chromosome counting—the most definitive method for ploidy level analysis (Fig. 3 and Supplementary Fig. 2). The flow cytometry estimated ploidy level of each accession was identical to that determined by chromosome

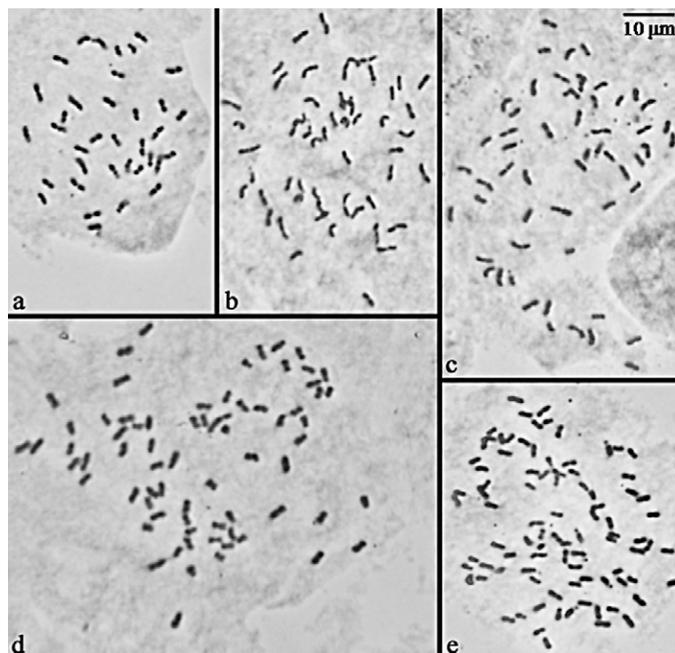


Figure 3. Mitotic metaphase chromosomes in meristematic root tip cells of five guayule accessions. (a) 36-Chromosome ($2n = 2x = 36$); (b) W6 2244 ($2n = 3x = 54$); (c) 11591 ($2n = 3x = 56$); (d) W6 2253 ($2n = 4x = 72$); and (e) N566 ($2n = 5x = 90$). A single plant from each accession was evaluated. All plants had an exact multiple of the base chromosome number in guayule ($x = 18$), except for the plant from 11591. We were unable to determine if this plant had an extra pair of homologous chromosomes (tetrasomic, $2n + 2$) or two B chromosomes. Even though two additional chromosomes were present, the 11591 plant was correctly scored as triploid ($3x$) with flow cytometry (average peak ratio = 1.517).

counting (Table 1). In addition, published ploidy level data based on cytological chromosome counting were available for another six different guayule accessions (Cal-3, W6 549, 11634, 11635, 11693, and 4265-X). These data were valuable for indirectly evaluating the accuracy of flow cytometry measurements. Similarly, the flow cytometry-estimated ploidy levels of these six accessions were identical to those obtained by chromosome counting in prior published studies (Table 1).

Although perfect concordance exists for all of the attempted direct and indirect comparisons, meiotic or mitotic chromosome counts in pollen mother or root meristematic cells, respectively, should be conducted to confirm ploidy levels. Nonetheless, taken together, these data clearly indicate that flow cytometry is a highly accurate alternative to chromosome counting when it is necessary to screen large numbers of single plants, as would be necessary for the establishment of guayule breeding populations with a specific ploidy level. In addition, flow cytometry can be used to rapidly identify the predominant mode of reproduction (apomictic vs. sexual) in selected polyploid breeding lines that are facultative apomicts by analyzing the difference in DNA content among embryo and endosperm cells in mature seeds (Matzk et al., 2000).

CONCLUSIONS

This work constitutes the first flow cytometry study of variability in ploidy levels within and among guayule accessions maintained by the NPGS. We detected a natural ploidy series among guayule accessions comparable to that of wild populations, but mixed ploidy occurred within four polyploid accessions. Given that a substantial number of the available guayule accessions are open-pollinated selections, it is within reason that a higher frequency of mixed ploidy would have been detected if larger sample sizes had been evaluated. Thus, we highly recommend that ploidy analysis be conducted on individual guayule plants if ploidy level is a factor that needs to be considered in genetic and transgenic studies as well as in breeding programs. In addition to ploidy level analysis, the adapted flow cytometry method in combination with appropriate reference standards can be used to evaluate the extent of nuclear genome size (C-value) variation among guayule and other *Parthenium* species, which has important implications for taxonomy, intra- and interspecific molecular breeding, and whole-genome sequencing. Importantly, the ploidy level data reported in this study will support future efforts to construct linkage (diploid \times diploid) or association mapping populations in guayule. It is important to assemble association mapping populations with guayule accessions that have the same ploidy, because it circumvents the challenge of differentiating the effects of functional variants from that of allele dosage (Zhu et al., 2008). Not only is ploidy level analysis fundamental to

the initial characterization of guayule germplasm, but it also lays the foundation for accelerating the breeding of this perennial species through genomewide (or genomic) selection (Bernardo and Yu, 2007; Meuwissen et al., 2001).

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