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Genome size variation in guayule and mariola: Fundamental descriptors for polyploid plant taxa

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ABSTRACT

Guayule (*Parthenium argentatum* A. Gray) has tremendous potential as a domestic source of natural rubber production in the southwestern United States. However, genetic improvement of guayule has been slowed by its complex mode of reproduction, natural ploidy series, and lack of genetic and genomic resources. The interspecific hybridization of guayule with its closest sister taxon mariola (*P. incanum* Kunth) offers an opportunity to access novel genetic variation for guayule breeding programs, but mariola accessions available from the U.S. National Plant Germplasm System (NPGS) have never been evaluated for natural variation in ploidy level. In addition, the nuclear genome sizes for guayule and mariola at any ploidy level are unknown. To that end, we examined the ploidy of 10 mariola accessions, which revealed a natural polyploid series ranging from triploid ($2n = 3x = 54$) to pentaploid ($2n = 5x = 90$). In contrast, a ploidy analysis of five guayule accessions uncovered a natural polyploid series that ranged from diploid ($2n = 2x = 36$) to hexaploid ($2n = 6x = 108$). More than one ploidy level among individual plants (mixed ploidy) and instances of aneuploid plants were observed for accessions of both guayule and mariola. The nuclear genome sizes of guayule and mariola were similar at identical ploidy levels, and the genome size of diploid guayule (1624 Mb) was almost twofold smaller than the genomes of sunflower (*H. annuus* L. $2n = 2x = 34$) and lettuce (*L. sativa* L.; $2n = 2x = 18$), two other Compositae (Asteraceae) species that are being genome-sequenced. The results from this study will serve as a foundation for interspecific breeding and genome sequencing of guayule and mariola.

1. Introduction

Guayule (*Parthenium argentatum* A. Gray) is a woody perennial shrub indigenous to the deserts of the southwestern United States and Northern Mexico. This member of the Compositae (Asteraceae) family has been commercialized as a renewable source of natural rubber and hypoallergenic latex (see review in Ray et al., 2005). Even though the rubber tree [*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.] is the predominant commercial source of natural rubber in the world, interest in guayule as a sustainable domestic source of natural rubber has intensified partly due to the projected global shortages of rubber by the next decade and the vulnerability of rubber tree plantations to South American leaf blight caused by the fungus *Microcyclus ulei* (Mann, 2009). Not only is guayule more amenable to traditional agronomic practices than the rubber tree, but it also produces a resin that resists termites when impregnated into wood (Holt et al., 2012). Guayule bagasse (plant residue after rubber and latex extraction) has the added potential of serving as a source of biofuel in the southwestern United States (Boateng et al., 2009).

Tremendous ploidy level variation exists within and among wild populations, cultivars, and germplasm lines of guayule (Bergner, 1946; Kuruvadi et al., 1997; Gore et al., 2011). This natural polyploid series typical ranges from diploid ($2n = 2x = 36$) to pentaploid ($2n = 5x = 90$), but even higher ploidy levels such as octoploid are possible (Powers, 1945; Thompson and Ray, 1988). However, tetraploid plants predominate in wild stands (Kuruvadi et al., 1997) and the U.S. germplasm collection (Gore et al., 2011). In addition, there is a low incidence of aneuploids within natural populations (Powers, 1945; Bergner, 1946; Kuruvadi et al., 1997).

Abbreviations: Mb, megabase; NPGS, National Plant Germplasm System.

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Guayule diploid plants principally have a sexual mode of reproduction and are obligate outcrossers by virtue of a sporophytic self-incompatibility system (see review in Thompson and Ray, 1988; Ray et al., 2005). In contrast, the facultative expression of apomixis in polyploid plants results in the production of both apomictic and sexual offspring. This complex mode of reproduction and a highly variable number of chromosomes have unquestionably slowed the genetic progress for a crop that is still in the process of domestication.

Genetic improvement for rubber yield has primarily resulted from single-plant selections in genetically narrow populations of apomictic polyploid plants, thus new avenues for exploitation of genetic diversity should be considered (Ray, 1993). The extent to which interspecific hybridization has been used in guayule breeding programs is limited, but the 13 other species of the genus *Parthenium* (www.theplantlist.org) with only trace amounts of rubber are potential sources of genetic variation for increased vigor, resin content, biomass, disease resistance, and cold tolerance (Ray et al., 2010). Of these 13 species, the facultative apomictic species *P. incanum* Kunth (mariola) with a natural ploidy series is postulated to be the closest sister taxon of guayule (Powers and Rollins, 1945). Indeed, interspecific hybrids between guayule and mariola are found at low frequency where both species naturally coexist in the wild (Rollins, 1944) and have been produced by controlled crosses for broadening the genetic base of guayule (Rollins, 1945).

The genetic diversity of guayule and mariola can be more effectively exploited in an interspecific breeding program when the ploidy level and chromosome number variation of the germplasm are known. We have previously collected ploidy level data from guayule germplasm available in the U.S. National Plant Germplasm System (NPGS) (Gore et al., 2011), but identical information for mariola germplasm is still needed. In addition, having nuclear genome size estimates of guayule and mariola is critical knowledge for helping to understand genome evolution and develop genome sequencing projects. We conducted the present study to (i) examine ploidy level variation within and among mariola germplasm available from NPGS, and (ii) measure nuclear genome size of guayule and mariola accessions at several ploidy levels.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of six guayule and 10 mariola accessions, as well as an interspecific hybrid of these two species, were obtained from the National Arid Land Plant Genetics Resources Unit (NALPGRU) at Parlier, CA (Table 1). Of the six guayule accessions, CFS-21 and CFS-24 are wild material collected from Texas, while the other four accessions are improved lines developed by guayule breeding programs in California (W6 429, 11591, N566) and Arizona (AZ-5). The pedigrees of the four improved cultivars and breeding lines are available from Thompson and Ray (1988), Ray et al. (1999), and Gore et al. (2011). All of the mariola accessions are wild material collected from native stands in Texas. The natural interspecific hybrid was collected from the wild in the Mexican state of Coahuila. Seeds of pea (*Pisum sativum* L.; Ctirad) were obtained from J. Doležel at the Institute of Experimental Botany in the Czech Republic. Sunflower (*Helianthus annuus* L.; PI 642777; HA 412 HO) and lettuce (*Lactuca sativa* L.; PI 536851; Salinas) seeds were obtained from the North Central Plant Introduction Station in Ames, IA, and R. Michelmore at University of California, Davis, respectively.

Seeds were sown on moist vermiculite inside a growth chamber (14 h light, 250 μmol m^{-2} s^{-1} at 28 °C and 10 h dark at 21 °C). Seven-day-old seedlings were planted individually into 10 cm diameter pots containing moistened Sunshine Mix #1 (Sun Gro Horticulture Inc., Bellevue, WA) and perlite (4:1 ratio). Plants were fertilized every 2 weeks with 20–20–20 (50 ppm N) Peters Professional plant nutrient solution (The Scotts Company, Marysville, OH, USA). Two months later, seedlings were transplanted separately into 1 gal pots with Sunshine Mix #1. Plants were grown under natural light in a greenhouse with daytime and nighttime mean temperatures at 28 and 21 °C, respectively. Plants were fertilized every two-weeks with 20–20–20 (200 ppm N) Peters Professional plant nutrient solution.

2.2. Sample collection and preparation for ploidy analysis

Leaf tissue samples were collected and prepared for ploidy analysis as previously described (Gore et al., 2011). Briefly, young, fully expanded leaves were collected from immature flower stalks of individual plants with unknown ploidy and a *P. argentatum* diploid plant (PI 478663; W6 429) that was repeatedly used as an internal diploid standard and confirmed to be diploid ($2n = 2x = 36$) via mitotic chromosome counts in root tip cells in this (data not shown) and our previous study (Gore et al., 2011). Leaf tissue samples were collected the morning of the experiment and maintained at 4 °C until preparation later in the same day. To prepare each sample for ploidy analysis, we added 1 mL of woody plant buffer (Loureiro et al., 2007) to a Petri dish that contained equivalent amounts of leaf tissue from an individual plant with unknown ploidy and the *P. argentatum* diploid plant, followed by coarse chopping of the combined leaf tissue with a razor blade for 30 s. The resultant homogenate was filtered through a Partec 30 μm CellTrics (Partec GmbH, Münster, Germany) disposable nylon filter. Nuclei were stained with 4 μg mL^{-1} of 4′,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich, St. Louis, MO, USA) and incubated on wet ice for 10 min before immediate analysis by the flow cytometer.

2.3. Analysis of ploidy level with flow cytometry

A Partec Ploidy Analyser flow cytometer (Partec GmbH) was used to analyze the fluorescence of nuclei stained with DAPI as previously described (Gore et al., 2011). Briefly, the mean position of the G1 peak for the *P. argentatum* diploid standard was set at channel 100. The order in which samples were run on the instrument was randomized. In the first experiment, we evaluated 15 plants from each of 16 accessions ('unknowns') listed in Table 1. One plant from each accession was randomly assigned to one of 15 blocks or runs on the flow cytometer. The entire experiment was replicated twice, for a total of two measurements per plant. To assess the quality of the data, the Partec CA3 analysis software was used to calculate the coefficient of variation for each measurement.

The peak ratio for each plant of unknown ploidy was calculated per Doležel et al. (2007) as the mean position of the G1 peak for the unknown ploidy plant ('sample') divided by the mean position of the G1 peak for the *P. argentatum* diploid standard plant ('reference'). For each experiment, we screened for statistical outliers in SAS version 9.2 (SAS Institute, 2011) by examining the Studentized deleted residuals obtained from a mixed linear model fitted with peak ratio as the dependent variable, plant as a fixed effect, and replicate nested within plant as a random effect. Degrees of freedom were calculated via the Satterthwaite approximation. With outliers removed, the same mixed linear models were used to obtain least square means for peak ratios with the LSMEANS statement in PROC MIXED. For each accession, the overall average and standard deviation of least square means for peak ratios of plants within a ploidy level were calculated. The ploidy level within an accession was estimated by multiplying the average peak ratio by two (the ploidy of the *P. argentatum* diploid plant, $2x$).

Table 1
Ploidy levels and chromosome numbers of 17 guayule and mariola accessions.

Species	Accession number	Germplasm name	Ploidy analysis			Cytogenetic analysis			
			No. plants[a]	Average peak ratio	SD[b]	Estimated ploidy level	No. plants	Chromosome counts	
P. argentatum	PI 478663	W6 429	1	1.00		2*x*	1	2*n* = 36	
P. argentatum	PI 478640	11591	14	1.51	0.01	3*x*	1	2*n* = 54	
P. argentatum	PI 478640	11591	1	2.46		5*x*			
P. argentatum	PARL 820	CFS-24	5	1.51	0.01	3*x*			
P. argentatum	PARL 820	CFS-24	7	1.98	0.03	4*x*			
P. argentatum	PARL 820	CFS-24	3	2.50	0.12	5*x*			
P. argentatum	PI 599678	AZ-5	15	2.01	0.04	4*x*	1	2*n* = 72	
P. argentatum	PI 478657	N566	3	1.96	0.02	4*x*	1	2*n* = 72	
P. argentatum	PI 478657	N566	12	2.48	0.02	5*x*	1	2*n* = 90	
P. argentatum	PARL 816	CFS-21	14	1.97	0.02	4*x*			
P. argentatum	PARL 816	CFS-21	1	2.94		6*x*	1	2*n* = 108	
P. incanum	PARL 790	CFS03-2005	15	1.56	0.01	3*x*			
P. incanum	PARL 794	CFS07-2005	15	1.57	0.01	3*x*			
P. incanum	PARL 795	CFS08-2005	15	1.56	0.01	3*x*			
P. incanum	PARL 799	CFS12-2005	15	1.56	0.01	3*x*			
P. incanum	PARL 800	CFS13-2005	15	1.55	0.01	3*x*			
P. incanum	PARL 791	CFS04-2005	14	1.56	0.01	3*x*	1	2*n* = 54	
P. incanum	PARL 791	CFS04-2005	1	2.20		4*x*	1	2*n* = 76[c]	
P. incanum	PARL 798	CFS11-2005	14	1.56	0.01	3*x*	1	2*n* = 55[c]	
P. incanum	PARL 798	CFS11-2005	1	2.35		5*x*	1	2*n* = 84[c]	
P. incanum	PARL 793	CFS06-2005	3	1.57	0.01	3*x*	1	2*n* = 55[c]	
P. incanum	PARL 793	CFS06-2005	12	2.06	0.01	4*x*	1	2*n* = 72	
P. incanum	PARL 792	CFS05-2005	15	2.05	0.02	4*x*			
P. incanum	PARL 788	CFS01-2005	14	2.04	0.02	4*x*			
P. incanum	PARL 788	CFS01-2005	1	2.46		5*x*	1	2*n* = 90	
P. argentatum/*P. incanum*	W6 2271	R1109	15	2.00	0.03	4*x*	2	2*n* = 70[c]	2*n* = 73[c]

[a] Number of plants.
[b] SD, standard deviation.
[c] Aneuploid plant.

2.4. Cytological preparation and image analysis

We conducted mitotic chromosome counts in root tip cells from representative plants within a ploidy level of several accessions per Gore et al. (2011). Briefly, actively growing root tips were pretreated with ice water for 24 h, followed by fixation in ethanol:glacial acetic acid (3:1), staining in 1% acetocarmine and squash preparation as previously described by Costich et al. (2010). Images were captured using an Olympus DP71 camera attached to a Zeiss Photomicroscope III and processed with Adobe Photoshop CS5 Version 12.0 × 64 (Adobe Systems Incorporated, San Jose, CA, USA). Counts of metaphase chromosomes from images were independently confirmed by two people (authors Paul L. Sanchez and Bernd Friebe).

2.5. Sample collection and preparation for genome size analysis

The protocol for the preparation of leaf samples for flow cytometry used in this analysis of genome size is based on that of Arumuganathan and Earle (1991), with various modifications. Leaves from the unknown plants, the *P. argentatum* diploid standard, and a pea standard were harvested the day before the experiment and sent via overnight courier to the collaborating lab at Cornell University (Ithaca, NY USA). Upon arrival the following morning, a 2 cm^2 piece of leaf of the unknown plus the same amount of *P. argentatum* diploid standard leaf were finely chopped together in 900 μL of extraction buffer (Solution "A") in a Petri dish kept on ice. Solution A was prepared fresh on the day of the experiment as follows (per sample): 877.5 μL Woody Plant Buffer (Loureiro et al., 2007), 0.9 mg DTT, and 22.5 μL Triton X-100 solution (Sigma–Aldrich, St. Louis, MO, USA). The resulting slurry was poured through a 30 μm CellTrics (Partec GmbH) nylon filter. The following staining solution was then added to the filtrate: 11.25 μL propidium iodide-RNase solution [7.5 μL propidium iodide (5 mg mL^{-1}), 0.375 μL RNase stock (100 mg mL^{-1}), and 3.375 μL Solution A]. Samples were kept in a light-protected cooler and transported to the flow cytometer.

2.6. Analysis of genome size with flow cytometry

The samples were run on an LSRII (BD Biosciences, San Jose, CA, USA) within 2 h after completion of preparation. The stained nuclei samples were excited by a 488 nm laser. Propidium iodide fluorescence was measured with a 610/20 nm band pass filter and doublets were excluded using a linear PI fluorescence area and width plot. We evaluated 1, 3, 4, or 6 plants each of 11 *Parthenium* accessions (i.e., unknown samples), for a total of 38 plants, that captured ploidy levels representative of those shown in Table 1. To expand the comparison of genome size within the Compositae (Asteraceae) family, we also evaluated three plants each of lettuce and sunflower. Each plant was randomly assigned to one of three blocks, but if a *Parthenium* accession had only one plant for a ploidy level, that one plant was assigned to all three blocks. In addition, the *P. argentatum* plant used as the diploid control for ploidy analysis was included twice in each block. Thus, each block consisted of 20 samples. We also randomized the order in which samples were run on the instrument. The entire experiment was replicated two times, for a total of two, six, or 12 measurements per plant. We calculated the genome size of the *P. argentatum* diploid standard by running it twice in each block along with pea (2C content = 9.09 pg DNA), a reference genome size standard provided by Doležel et al. (2007), taking the mean of the two values, and then using that number to calculate the genome sizes of the 18 unknown samples in the block.

With statistical outliers removed per the statistical approach described in Section 2.3, a mixed linear model was used to obtain the least square means for genome size with the LSMEANS statement in PROC MIXED. For each accession, the overall average and standard deviation of the least square means for nuclear genome size of plants within a ploidy level were calculated.

3. Results and discussion

3.1. Ploidy level variation

The extent of ploidy level variation among and within five guayule accessions was evaluated. Of the five guayule accessions evaluated, three of them were selected based on the results of Gore et al. (2011) to capture a natural ploidy series for eventual nuclear genome size estimation. As predicted, the ploidy ranged from triploid ($2n = 3x = 54$) to pentaploid ($2n = 5x = 90$), but a larger sampling of plants revealed mixed ploidy within N566 and 11591 (i.e., not all plants sampled from an accession had the same ploidy level) (Table 1). Two wild accessions, CFS-21 and CFS-24, were also surveyed in an attempt to extend the upper range of the ploidy series. Through the mixed ploidy of these two wild accessions, a series of four ploidy levels was detected that included the successful identification of a hexaploid plant. Cytological chromosome counting confirmed the accuracy of estimated ploidy levels for five representative plants ($3x$–$6x$). Even though aneuploidy occurs at an appreciable level in guayule (Kuruvadi et al., 1997; Gore et al., 2011), there was no evidence of aneuploidy for these five plants. However, aneuploidy may have been uncovered if additional guayule plants had been examined cytologically, especially plants of wild accessions (CFS-21 and CFS-24).

We also explored ploidy level variation within and among 10 accessions of mariola—the closest sister taxon of guayule (Rollins, 1944; Powers and Rollins, 1945; Rollins, 1945). As with guayule, a natural ploidy series, although not as extensive, was detected for mariola that ranged from triploid ($2n = 3x = 54$) to pentaploid ($2n = 5x = 90$) (Table 1). Such a ploidy series ($3x$ to $5x$) was also detected for wild mariola accessions by Stebbins and Kodani (1944), but diploid mariola plants were not identified in our study. In addition, mixed ploidy was detected for four of the mariola accessions, which likely resulted from a mixture of asexual and sexual seed produced by facultative apomictic plants (Powers, 1945; Powers and Rollins, 1945). Notably, four of six plants across the three ploidy levels showed evidence for aneuploidy, with $2n = 55$, 76, and 82 chromosomes. In addition, we evaluated ploidy variation within an interspecific hybrid accession produced from a natural cross between guayule and mariola that was collected from the wild in Coahuila, Mexico (www.ars-grin.gov). The hybrid was found to be tetraploid and have at least two aneuploid plants. Although the ploidy of parental plants for the interspecific hybrid could not be accurately predicted based on these data alone, interspecific hybridization between tetraploid plants of guayule and mariola are more likely to produce tetraploid offspring (Rollins, 1945).

3.2. Nuclear genome size variation

The nuclear genome sizes of 12 guayule and mariola accessions that captured the ploidy level variation of these two *Parthenium* species were estimated (Table 2). Estimates of nuclear genome sizes (haploid) for the $2x$ to $6x$ guayule ploidy series ranged from 1624 to 4812 Mb, with an incremental increase of 779 Mb per ploidy level based on a linear regression model ($R^2 = 0.99$). With a range from 2616 to 4181 Mb, the $3x$–$5x$ ploidy series of mariola extended slightly beyond the $3x$–$5x$ range of guayule. With the exclusion of the two aneuploid plants that had 76 and 84 chromosomes, a linear regression model ($R^2 = 0.99$) showed that genome size increased 763 Mb per ploidy level for mariola. Thus, the genome sizes of guayule and mariola at the same ploidy level were highly similar. Additionally, the estimated nuclear genome size of the interspecific hybrid was similar to the genome sizes of the guayule and mariola tetraploids (Table 2).

We found that the nuclear genome size of the diploid guayule accession was 1.9–2.2-fold smaller than that of lettuce ($2n = 2x = 18$) and sunflower ($2n = 2x = 34$), respectively, which are two diploid species also in the Compositae family (Table 2). This suggests that the genome of the diploid guayule accession had a lower repetitive DNA content than the genomes of the lettuce (Salinas) and sunflower (HA 412 HO) accessions that are being genome-sequenced. Our estimates of 3130 ± 147 and 3589 ± 215 Mb for the nuclear

Table 2
Nuclear genome sizes (megabase; Mb) of 12 guayule and mariola accessions and two comparative species, lettuce, and sunflower.

Species	Germplasm name	Ploidy and chromosome number	Nuclear genome size analysis			
			No. plants	Average picogram DNA/nucleus	SD[g]	Estimated haploid genome size (Mb)[h]
P. argentatum	W6 429	$2n = 2x = 36$[a]	1	3.32		1624
P. argentatum	11591	$2n = 3x = 54$[a]	3	4.98	0.04	2435
P. argentatum	11591	$2n = 5x = 90$[b]	1	7.89		3858
P. argentatum	N566	$2n = 4x = 72$[b]	3	6.28	0.08	3071
P. argentatum	CFS-21	$2n = 4x = 72$[b]	3	6.56	0.07	3208
P. argentatum	AZ-5	$2n = 4x = 72$[a]	3	6.56	0.06	3208
P. argentatum	N566	$2n = 5x = 90$[a]	3	8.06	0.05	3941
P. argentatum	CFS-21	$2n = 6x = 108$[a]	1	9.84		4812
P. incanum	CFS04-2005	$2n = 3x = 54$[a]	3	5.53	0.03	2704
P. incanum	CFS04-2005	$2n = 4x = 76$[c]	1	7.67		3751
P. incanum	CFS11-2005	$2n = 3x = 55$[c]	3	5.35	0.16	2616
P. incanum	CFS11-2005	$2n = 5x = 84$[c]	1	8.16		3990
P. incanum	CFS06-2005	$2n = 3x = 55$[c]	3	5.48	0.14	2680
P. incanum	CFS06-2005	$2n = 4x = 72$[a]	3	7.02	0.05	3433
P. incanum	CFS01-2005	$2n = 4x = 72$[b]	3	7.10	0.13	3472
P. incanum	CFS01-2005	$2n = 5x = 90$[a]	1	8.55		4181
P. argentatum/*P. incanum*	R1109	$2n = 4x = 70$–73[d]	3	6.50	0.05	3179
L. sativa	Salinas	$2n = 2x = 18$[e]	3	6.40	0.15	3130
H. annuus	HA 412 HO	$2n = 2x = 34$[f]	3	7.34	0.22	3589

[a] Chromosome number based on one plant.
[b] Chromosome number inferred from chromosome counts on euploid plants of the same species with an identical ploidy level.
[c] Chromosome number based on one aneuploid plant.
[d] Chromosome numbers based on two aneuploid plants.
[e] Chromosome number from Truco et al. (2013).
[f] Chromosome number from Kane et al. (2011).
[g] SD, standard deviation.
[h] Haploid nuclear genome size was estimated as follows: [(average picogram DNA/nucleus × 978 Mb)/2] (Doležel et al., 2003).

genome size of lettuce and sunflower were close to the previously reported estimates of 2700 and 3500 Mb (Kane et al., 2011; Truco et al., 2013).

4. Conclusions

This is the first flow cytometry study of variability in ploidy levels within and among mariola accessions from the NPGS, as well as the first estimation of nuclear genome size for guayule, mariola, and an interspecific hybrid of these two species. Similar to the findings of Gore et al. (2011) for guayule, we detected a natural ploidy series for mariola (3*x*–5*x*) and aneuploid plants, with evidence for mixed ploidy within accessions. When combined with ploidy level data from more than 30 guayule accessions (Gore et al., 2011), these ploidy results from the 10 mariola accessions could be immediately used by breeders to support the development of interspecific hybrids with specific ploidy levels. Given that ploidy level also is of critical importance to genetic and transgenic studies of mariola, the ploidy of experimental plants must be determined before primary experiments are initiated.

The diploid guayule accession had an estimated nuclear genome size that was about twofold smaller than lettuce and sunflower, thus indicating that it may be less complex to sequence than the lettuce and sunflower genomes, with their likely higher repetitive DNA contents. Similarly, mariola would be less complicated to sequence if a diploid accession was available, given the likely similar genome sizes of mariola and guayule diploids. Importantly, the availability of guayule and mariola genome sequences would help to: (i) enhance the study of genome structure and function within the Compositae, (ii) serve as a framework for the development of molecular breeding tools for the genetic improvement of guayule, and (iii) facilitate the transfer of novel traits from mariola to guayule.

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