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Discovery and Characterization of Novel Fertile Triploids and a New Chromosome Number in Caladium (*Caladium* × *hortulanum*)

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Abstract. **Cultivated caladium (*Caladium* × *hortulanum;* $2n = 2x = 30$) is an important ornamental aroid for the environmental horticulture industry. A better understanding of its genetic diversity is needed for continued improvement of caladium. The caladium germplasm maintained in the University of Florida's caladium breeding program were surveyed for potential variation in chromosome number and nuclear DNA content to gain a better understanding of caladium genetic diversity at the cytogenetic level. For the first time caladium triploidy was discovered in two breeding lines, UF-15-414 and UF-4407 with $2n = 3x = 45$ chromosomes and a nuclear DNA content of 13.86 pg·2C^{-1}. In addition, a new chromosome number ($2n = 2x = 34$) was found in one cultivar, 'White Wing'. Compared with their diploid parents or grandparents, the triploids showed a distinct, attractive leaf morphology with rounder and thicker leaves. The pollen stainability of UF-15-414 and UF-4407 was 63% and 73%, respectively, indicating potential male fertility, which was unexpected. Analysis of simple sequence repeat (SSR) marker banding patterns confirmed that UF-15-414 was a direct progeny of UF-4407 through hybridization with the diploid cultivar Aaron, whereas UF-4407 might result from fusion of an unreduced (female or male) gamete with a reduced gamete. Chromosome counting and SSR marker analysis of 'White Wing' and its progeny cultivars 'White Wonder' and 'White Delight' suggested that 'White Wing' possibly transmitted its 34 chromosomes to progeny during sexual reproduction. The discovery and characterization of these triploids revealed the occurrence of natural sexual polyploidization in caladium and indicated good potential for creating and selecting new triploids for future caladium breeding. The observed new chromosome number in 'White Wing' and its progeny cultivars implies that other chromosome variations may be present among cultivated caladiums. In summary, these results revealed two male-fertile triploid caladiums and a new chromosome number that can enrich the cytogenetic diversity in future caladium cultivar development.**

Caladiums, indigenous to Central and South America, became popular after they were displayed in a plant exposition in Paris in 1867 (Hayward, 1950). By the early 1900s, caladiums were for the first time imported to the United States. In the 1920s, private caladium breeding emerged in Florida, and thousands of new cultivars were created (Wilfret, 1993). Some of these cultivars are still commercially available along with many others produced at the University of Florida (UF). The genus *Caladium* is currently split among a total of 14 recognized species despite much debate in recent decades (Hetterscheid et al., 2009; Mayo et al., 1997). Birdsey (1951) proposed that the cultivated caladium cultivars be listed under the species name *C.* × *hortulanum* due to the interspecific hybridizations made among four caladium species.

Most commercial caladium plants are produced by forcing tubers. Florida growers produce essentially all the caladium tubers used in the United States and in the world for pot plant production and tuber sales (Deng, 2018). To meet the needs of the environmental horticulture industry for new caladium cultivars, UF has maintained a caladium breeding program at the Gulf Coast Research and Education Center (GCREC) since 1976. The program appears to be the only public caladium breeding program in the world. Developing new, novel caladium cultivars has become a challenge to plant breeders because of constraints on the available germplasm. Research has been ongoing to investigate *Caladium* in search of new sources of genetic variability. From this, key species identifiers have been elucidated, specifically chromosome number and nuclear DNA content (Cao et al., 2014).

Chromosome numbers for *Caladium* have been reported as far back as 1940 when the somatic chromosome number for *Caladium bicolor* was reported to be $2n = 30$ (Kurakubo, 1940). Jones (1957) and Pfitzer (1957) reported chromosome numbers of *C.* × *hortulanum* cultivars to be $2n = 30$ as well. Aneuploid chromosome numbers have been reported in some cultivars with $2n = 19$, 26, 28, or 32 (Marchant, 1971; Pfitzer, 1957; Sarkar, 1986; Sharma and Sarkar, 1964). Cao et al. (2014) conducted the most comprehensive study to date analyzing the chromosome number for 39 accessions from 10 caladium species. Differences were observed in certain species and cultivars where chromosome number reports differed by one or two chromosomes. It was suggested that this occurrence could be due to the presence of B chromosomes. Cao et al. (2014) reported a chromosome number of $2n = 30$ for all *C.* × *hortulanum* cultivars investigated and noted that one cell of both 'Candidum' and 'Miss Muffet' had one additional B-like chromosome.

Genome size estimates have become essential for genome sequencing projects (Doležel et al., 2007). Up until 2014, little had been published on nuclear DNA content in caladiums or other aroids (Leitch et al., 2019). Cao et al. (2014) reported the nuclear DNA content for 63 accessions from 10 species of caladium. Genome sizes ranged from 2.98 pg·2C^{-1} in *Caladium lindenii* to 9.89 pg·2C^{-1} in *C.* × *hortulanum* 'Chang Suek'. Interestingly, there was not a strong linear correlation between nuclear DNA content and chromosome number in *Caladium*. The study revealed four different cytotypes in this genus and suggested the possibility of genome duplications followed by loss or fusion of chromosomes in the evolution of *Caladium*.

Induction of polyploids in plants through chromosome doubling have had major effects on plant phenotypes in ornamentals and food crops. Traits such as thicker leaves (Yenchon and Techato, 2014), improved abiotic stress tolerance (Liu et al., 2011), and increased disease resistance (Comai, 2005) have been reported after chromosome doubling. Cai et al. (2015), Cao et al. (2016), and Cao and Deng (2022) successfully regenerated caladium tetraploids in 'Tapestry' and 'Red Flash' cultivars from colchicine treatments and/or leaf segment cultures. These tetraploids had striking differences from their diploid counterparts: Leaves and petioles were thicker, and leaves were distinctly rounded. Although the tetraploids provided unique characteristics, they produce fewer leaves and thus have less ornamental value. Nevertheless, these tetraploids will be valuable breeding parents for producing triploid hybrids.

Many triploids are known to have greater biomass, improved stress resistance, and sterility (Wang et al., 2016). Although absent in wild *Caladium*, triploids have been found among other members of the Araceae family. Triploids occurred in natural *Colocasia* populations in India with larger tubers than that of diploids (Kuruvilla and Singh, 1981). Triploids have also been identified in *Amorphophallus bulbifer* and *Amorphophallus oncophyllus* wild populations (Chauhan and Brandham, 1985). Triploids are becoming more common in certain horticultural

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crops around the world, generated by breeders crossing tetraploids (4*x*) with diploids (2*x*). These interploidy hybrids have made drastic improvements in some crops, such as watermelon and cassava, in which triploids have shown extraordinary increases in plant yield with the added benefit of no seed production (Hoshino et al., 2011). Although many known triploids are produced through interploidy crosses, meiotic abnormalities occur in nature, resulting in the formation of unreduced gametes (2*n*) and subsequently triploids. The most common mechanisms of unreduced gamete formation are first division restitution and second division restitution (De Storme and Geelen, 2013).

Unreduced gamete production and naturally occurring triploids have been reported within the Araceae family. Taro (*Colocasia esculenta*) has been at the center of many of these studies because of its importance as a food crop in some regions of the world. The crop consists of naturally occurring diploids and triploids (Chaïr et al., 2016; Coates et al., 1988). Interestingly, there have been no naturally occurring tetraploids reported in taro, leaving unreduced gametes as the likely source of triploid production. Isshiki et al. (1999) investigated the source of triploids in taro through both hybridizations and selfing of wild taro and showed that 0.56% of the progeny of self-crosses were triploids and 0.58% of hybrid seedlings were also triploids. Isozyme analysis of the hybrid triploids revealed that the female parent contributed unreduced gametes for five of the 1041 progeny and the male parent provided an unreduced gamete for one of the triploids. These studies suggest that unreduced gamete production has occurred in taro within both male and female flowers at low frequencies. Nevertheless, this phenomenon has been rarely observed or reported in other species of Araceae, the aroid family, which comprises ≈3750 species. Viable male gametes in triploids are rare; however, it does occur in some species. Bai et al. (1971) found that in naturally occurring triploid populations of *Colocasia antiquorum*, a close relative of caladiums, viable pollen grains were produced at the same rate and size as diploids. The triploid pollen grains were found to have a range of chromosome numbers from 8 to 25, indicating much variability in pollen grains produced.

To gain a better understanding of the genetic diversity in cultivated *Caladium* at the cytogenetic level, the collection of cultivated *Caladium* germplasm (*C.* × *hortulanum*) maintained by the UF caladium breeding program was surveyed for potential variation in nuclear DNA content and chromosome number. One breeding line (UF-15-414) was found to be a triploid, and a cultivar 'White Wing' was revealed to have a different chromosome number. To understand the potential value of this new ploidy level and this new chromosome number, UF-15-414 and its known parents and grandparents, and 'White Wing' and two of its known progeny cultivars were characterized at the individual, cellular, and molecular levels. Specifically, we examined their leaf morphology, stomata size and density, pollen stainability, chromosome number and nuclear DNA content, and SSR banding pattern. These findings will have major implications on caladium breeding, their possible origins, and caladium genetic research.

Materials and Methods

Plant materials. This study was arranged to make comparisons within two different groups of cultivated caladiums. The first group consisted of triploid lines (UF-15-414 and UF-4407) and five of their parents and grandparents, including 'Aaron', 'Candidum Jr.', 'Florida Moonlight', 'Florida Sweetheart', and 'Red Frill' (Fig. 1). The second group consisted of 'White Wing', its progeny cultivars (White Wonder and White Delight), and a commercial cultivar (White Butterfly). 'White Wonder' is a selfed progeny of 'White Wing'. 'White Delight' is a progeny of 'White Wing' and an older cultivar Jacki Suthers (Fig. 2). 'White Butterfly' shared similar plant and leaf morphologies and coloration pattens with 'White Wing', and it was included as a control for comparison.

Tubers for 'Aaron', 'Florida Moonlight', 'Florida Sweetheart', 'White Butterfly', 'White Wing', UF-15-414, and UF-4407 were collected from the UF/GCREC (Wimauma, FL, USA). Tubers for 'Candidum Jr.' and 'Red Frill' were donated by Florida Boys Caladiums (Lake Placid, FL, USA), and tubers for 'White Delight' and 'White Wonder' by Classic Caladiums (Avon Park, FL, USA). All tubers were asexually propagated through tuber division and produced under field conditions as described by Deng and Harbaugh (2006). Tubers were planted in 3.8-L plastic containers filled with Jolly Gardener Pro-Line C/B Growing Mix (Jolly Gardener, Poland, ME, USA). Plants were hand watered as needed and were fertilized with a controlled-release fertilizer (Osmocote, 15N–3.9P–10K, 5- to 6-month release at 21 °C; The Scotts Company, Marysville, OH, USA) at 6.51 kg·m^{-3}. Pots were arranged in a randomized complete block design (RCBD) with five biological replicates. Plants were grown under natural light in a temperature-controlled greenhouse set between 29.4 °C during the day and 21.1 °C at night.

Morphological and stomata characterization. Fully developed plants (17 weeks after tubers were planted) were used for morphological measurements. Leaf length and width were measured using a stainless-steel ruler from the tip of the leaf apex to the bottom of the basal nodes and across the widest part of the leaf, respectively. Leaf blade thickness, leaf main vein thickness, and petiole diameter were measured using a digital caliper (Fowler & NSK Max-Cal, Tokyo, Japan). The average values from two to three leaves per plant were used for morphological analysis of each of the five biological replicates. Nail polish imprints of the abaxial side of three mature leaves were taken from each accession. Five fields of view were observed for each leaf to estimate the stomata density (no./mm^2). Stomata length and width were measured on three leaves per accession and 10 stomata per leaf using ImageJ (version 1.53e; National Institutes of Health, Bethesda, MD, USA) (Schneider et al., 2012).

Flower induction and pollen grain staining. Tubers of UF-15-414, UF-4407, 'Aaron', 'Florida Moonlight', and 'Florida Sweetheart' were treated for flower induction. Another popular caladium cultivar 'Miss Muffet' was included in this study as a control. Tubers were soaked in a 600 ppm GA_3 solution (ProGibb LV Plus; Valent Biosciences, Libertyville, IL, USA) for 16 h at room temperature. Tubers were then planted in 3.8-L plastic containers filled with Jolly Gardener Pro-Line C/B Growing Mix. Growing conditions for these plants were the same as described previously.

Previous studies have shown that caladium pollen viability declines rapidly after it is shed from staminate flowers on the spathe (Deng and Harbaugh, 2004). To avoid issues with loss of pollen viability due to environmental conditions, pollen was collected and stained on the same day that it was shed from the spathe. The methods described by Xu (2018) were used to prepare the acetocarmine staining solution. Commercial 2% acetocarmine solution (Carolina Biological Supply Company, Burlington, NC, USA) was placed on a hot plate (98 °C) to evaporate acetic acid and generate 4% acetocarmine solution. After collection, pollen was immediately suspended in 4% acetocarmine staining solution and stained overnight. The following day ≈50 µL of the pollen solution was pipetted onto a clean glass slide and covered with a coverslip. Three independent drops were prepared per pollen sample and viewed under an Olympus BH-2 bright field microscope using the ×10 objective lens (Olympus, Center Valley, PA, USA). Photos were taken at random using a Sony DSC-F717 camera (Sony Corporation of America, New York, NY, USA) fixed to the

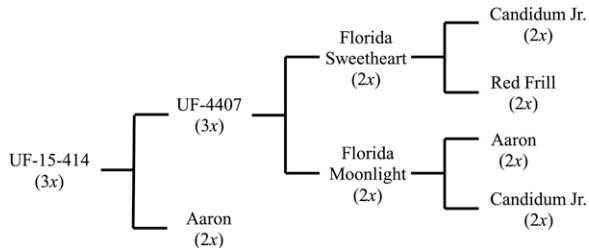

Fig. 1. Pedigree of triploid caladium breeding lines UF-15-414 and UF-4407. All crosses were made at the University of Florida's Gulf Coast Research and Education Center.

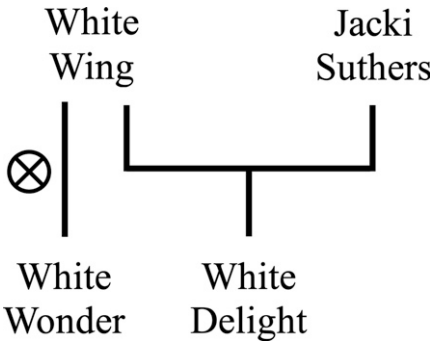

Fig. 2. Pedigree of two 'White Wing' ($2n = 2x = 34$) caladium progeny: 'White Wonder' ($2n = 2x = 34$) and 'White Delight' ($2n = 2x = 34$). These crosses were made by Classic Caladiums LLC in Avon Park, FL, USA.

microscope. Images were used to count all stainable and nonstainable pollen grains per field of view. For each accession, three flowers per plant from three different potted plants were used for pollen stainability analysis. Pollen grain diameter measurements were recorded on 50 pollen grains per accession using ImageJ (version 1.53e; National Institutes of Health) (Schneider et al., 2012).

Determining nuclear DNA content. A Cyflow Ploidy Analyzer (Sysmex Europe GmbH, Norderstedt, Germany) was used to determine nuclear DNA content. The Doležel et al. (2007) protocol was used and rye [*Secale cereal* 'Daňkovské' (16.19 pg·2C^{-1})] was selected as the internal standard. ≈30 mg of fresh caladium leaf tissue and rye were cochopped in 1 mL of cold LBO1 buffer and 50 µL of RNase (Sigma-Aldrich, St. Louis, MO, USA; 1 mg·mL^{-1}). The homogenate was then filtered through a nylon mesh filter (50-µm pore) into a tube with 50 µL of the DNA fluorochrome propidium iodide (Sigma-Aldrich; 1 mg·mL^{-1}). The solution was then fed into the flow cytometer. Three analyses were run on the flow cytometer per plant and three plants were analyzed per cultivar or breeding line. The nuclear DNA contents of caladium samples were calculated as recommended by Doležel et al. (2007): nuclear DNA content of caladium samples = nuclear DNA content of 'Daňkovské' rye (the internal standard; 16.19 pg·2C^{-1}) × (mean fluorescence value of caladium samples ÷ mean fluorescence value of the internal standard).

Chromosome counting. Actively growing caladium root tips (1 cm) were collected from plants grown in the greenhouse, placed in a 2-mM 8-hydroxyquinoline solution, and treated in the dark at 4 °C for 5 hours. Following this pretreatment, root tips were rinsed in deionized water three times and then fixed in a fixative solution (3 methanol: 1 acetic acid, v/v) overnight at 4 °C. Root tips were washed in deionized water three times before being digested (or softened) in 1N HCl at room temperature (≈24 °C) for 23 min. After digestion, the roots were washed three more times in deionized water and then stained in 2% acetocarmine solution (Carolina Biology Supply Company) overnight. The root tips were moved to a glass slide where a scalpel was used to remove the root caps and separate the meristematic tissue. A drop of acetocarmine and coverslip were placed over the meristematic tissue and the cells were squashed by gently tapping the coverslip with a scalpel handle. Slides were observed under an Olympus BH-2 bright field microscope (Olympus, Center Valley, PA, USA). Root tip cells with darkly stained chromosomes were photographed at ×1000 magnification with a Sony DSC-F717 camera (Sony Corporation of America) fixed to the microscope.

SSR marker analysis. All accessions were analyzed using seven caladium-specific SSR markers designed by Gong and Deng (2011). Total genomic DNA was extracted using a Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA was extracted from two different plants for each accession as quality control. A Nanodrop 1000 spectrophotometer (Thermo Scientific, Odessa, TX, USA) was used to estimate sample DNA concentrations. DNA working solutions were created by diluting the preps to 8 ng·µL^{-1}. SSR primers were synthesized by Eurofins Genomics LLC (Louisville, KY, USA) with an M13 tail (5'-CCCAGTCACGACGTTG-3') attached to the 5' end of each forward primer. Polymerase chain reactions (PCRs) each contained 32 ng of DNA template (8 ng·µL^{-1}), 2 mM dNTPs, 0.25 units of *Taq* DNA polymerase, 1 × Standard Taq Reaction Buffer, 2 pmol of reverse primer, 0.2 pmol of forward primer with the M13 tail, and 1.8 pmol of Tide Flour 6-labeled M13 tail primer (New England Biolabs, Ipswich, MA, USA). An Eppendorf Pro S Mastercycler 6325 (Eppendorf, AG, Hamburg, Germany) was used for PCR amplification running a touchdown program: denaturation at 94 °C for 2 min, seven cycles at 94 °C for 45 s, 68 °C (decrease 2 °C progressively each cycle) for 45 s, and 72 °C for 60 s and then 30 cycles of 45 s at 94 °C, 45 s at 54 °C and 60 s at 72 °C, and a final extension at 72 °C for 5 min. PCR products were diluted 1:10 to 1:25 depending on primer pair and then denatured at 94 °C for 3 min before loading into the gel. For each reaction, 0.8 µL of diluted PCR products were separated on an 18-cm long, 6.5% polyacrylamide gel, and visualized on a LI-COR 4300 DNA analyzer (LI-COR, Lincoln, NE, USA). Electrophoresis conditions were 1500 V at 45 °C with a run time of 1 h and 45 min.

Experimental design and statistical analysis. Caladiums planted in 3.8-L pots were arranged in an RCBD with five replicates. Analysis of variance was performed on R 4.1.0 (R Core Team, Vienna, Austria, 2021) to determine if there were significant differences ($P \leq 0.05$) among caladium accessions. When differences were determined significant, means were separated using Tukey's honestly significant difference test. R 4.1.0 was also used to perform linear regression to identify if there is correlation between two traits. Correlation coefficients were calculated using the Pearson correlation test.

Results

Morphological characterization. The caladiums included in this study showed substantial variation in the morphological features accessed. Among UF-15-414, its parents, grandparents, and great-grandparents, three had the fancy leaf type and four had the lance leaf type. Four leaf background and main vein colors were present with caladium leaves colored pink, green, white, and red (Fig. 3). UF-15-414 and UF-4407 both shared a similar leaf appearance, leaf margins, and pink background color with 'Florida Sweetheart'. 'White Wing' and its progeny 'White Delight' and 'White Wonder' shared similar leaf appearances with only subtle differences in leaf margins and the basal lobes. Although similar in white background leaf color, 'White Butterfly' differed from these cultivars with nearly all white leaves and a characteristic pinching of the basal lobes. All four of these cultivars had the lance leaf type.

Within the UF-15-414 lineage there was a wide range in the shape of the leaves, with 'Red Frill' recording the largest leaf length-to-width ratio of 1.77 with more slender leaves and UF-4407 having more rounded leaves with the smallest ratio of 1.16 (Table 1). UF-15-414 and UF-4407 had significantly thicker leaves than their parents and grandparents, with leaves 0.33 and 0.32 mm thick, respectively (Fig. 4). UF-15-414 also had significantly thicker main veins and petioles than the other members of the lineage.

Stomata characterization. Stomata density was found to be genotype-dependent, with a large variation among the triploid lineage (Figs. 5 and 6). Average number of stomata ranged from 96 ('Aaron') to 31 (UF-15-414) per mm^2. UF-15-414 had a significantly lower stomata density and larger stomata length and width when compared with the other diploids in the study. UF-4407, however, had a similar stomata density, length,

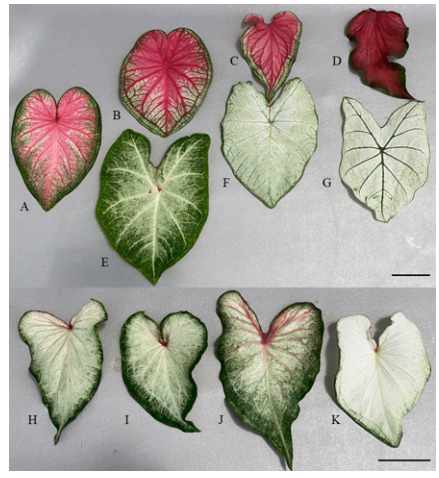

Fig. 3. Typical leaves of caladium triploid lineage (**top**) and white background caladium cultivars (**bottom**). (**A**) UF-15-414. (**B**) UF-4407. (**C**) 'Florida Sweetheart'. (**D**) 'Red Frill'. (**E**) 'Aaron'. (**F**) 'Florida Moonlight'. (**G**) 'Candidum Jr.'. (**H**) 'White Wing'. (**I**) 'White Delight'. (**J**) 'White Wonder'. (**K**) 'White Butterfly'. Scale bar = 5 cm.

Table 1. Leaf morphology measurements of diploid and triploid caladiums. Data were collected on five plants grown at the University of Florida's Gulf Coast Research and Education Center.

Cultivar/breeding line (ploidy level)	Leaf length/width ratio	Leaf main vein thickness (mm) (mean ± SD)	Petiole diam (mm) (mean ± SD)	Stomata length (µm) (mean ± SD)	Stomata width (µm) (mean ± SD)
UF-15-414 (3*x*)	1.28 ± 0.09 bc[z]	2.17 ± 0.46 a	4.90 ± 0.57 a	45.33 ± 4.56 a	19.20 ± 3.72 a
UF-4407 (3*x*)	1.16 ± 0.08 c	1.63 ± 0.32 bc	3.91 ± 0.58 b	28.93 ± 2.86 c	17.27 ± 2.12 b
Aaron (2*x*)	1.35 ± 0.12 bc	1.43 ± 0.30 c	3.22 ± 0.57 c	31.60 ± 3.17 b	15.77 ± 2.25 bcd
Florida Sweetheart (2*x*)	1.32 ± 0.12 bc	1.94 ± 0.63 ab	3.41 ± 0.54 bc	24.97 ± 2.66 d	12.77 ± 1.19 e
Florida Moonlight (2*x*)	1.36 ± 0.15 bc	1.51 ± 0.26 bc	3.60 ± 0.41 bc	33.33 ± 2.77 b	16.23 ± 3.04 bc
Candidum Jr. (2*x*)	1.47 ± 0.12 b	1.72 ± 0.46 bc	3.59 ± 0.54 bc	25.97 ± 2.44 d	14.70 ± 1.53 cd
Red Frill (2*x*)	1.77 ± 0.41 a	1.72 ± 0.18 bc	3.27 ± 0.44 c	27.20 ± 2.58 cd	14.13 ± 2.13 de
White Wing (2*x*)	1.44 ± 0.20 a	1.72 ± 0.42 a	4.31 ± 1.06 a	31.03 ± 3.51 bc	17.37 ± 2.22 a
White Delight (2*x*)	1.47 ± 0.10 a	1.24 ± 0.45 c	2.88 ± 0.66 b	29.37 ± 2.65 c	15.17 ± 2.18 b
White Wonder (2*x*)	1.58 ± 0.28 a	1.35 ± 0.36 bc	2.96 ± 0.62 b	33.03 ± 3.60 ab	17.30 ± 2.73 a
White Butterfly (2*x*)	1.49 ± 0.15 a	1.62 ± 0.39 ab	3.38 ± 0.32 b	34.23 ± 3.49 a	13.63 ± 2.11b

[z]Means followed by the same letter within each column and group are not significantly different by Tukey's honestly significant difference test at the 5% level of significance.

and width to the other diploids. Very small changes were observed between 'White Wing', its progeny, and 'White Butterfly'. There was no significant difference in leaf length-to-width ratios among these white cultivars. Although differing by only 0.04 mm, there was a significant difference in leaf thickness among the four cultivars. White Wing had significantly thicker ($P < 0.05$) petioles than the other cultivars, with nearly 1.0 mm increase from the second thickest White Butterfly.

Pollen size and stainability. More than 32,700 pollen grains were counted from four cultivars and two breeding lines. Stainable pollen grains of all accessions were round to oval and stained a dark red with acetocarmine stain (Fig. 7). Pollen stainability was relatively high among the selected caladium cultivars and breeding lines. UF-4407 parent 'Florida Sweetheart' had the highest pollen stainability with nearly 83% of pollen grains staining bright red (Fig. 7, Table 2). Both UF-15-414 and UF-4407 breeding lines had ≈70% of their pollen grains counted as stainable, which was the average for the group showing little difference between diploids and triploids. Caladium cultivar 'Aaron' recorded the lowest stainability with approximately half of its pollen grains becoming stained. The size of the pollen grains observed was relatively uniform across the samples with small differences between cultivars and breeding lines. UF-15-414 was an exception, with the largest pollen grains nearly 10 µm larger than both of its parents. UF-4407 pollen grain size was nearly identical to the average of both of its parents. Overall, the range of pollen grain lengths was ≈12 µm.

Nuclear DNA content. Flow cytometry resulted in high-quality histograms with sharp peaks among samples. Standard deviation values were very low, not exceeding 0.03 pg among the nine flow-cytometry runs per cultivar or breeding line (Table 3). UF-15-414 and UF-4407 were found to be triploids with nuclear DNA contents of 13.86 and 13.85 pg·2C^{-1}. The diploids within the triploid lineage had similar nuclear DNA contents as previous reports with an average of 9.38 pg·2C^{-1} and a range of 0.37 pg·2C^{-1} (Table 3). When comparing the triploid breeding lines with the diploids, they have a comparable 1Cx DNA content value of 4.62 pg. Although near the bottom of the 1Cx values in the study, these triploids were close to the lowest 1Cx value of 4.59 pg recorded by 'Florida Sweetheart', which is the seed parent of UF-4407. 'Florida Sweetheart' parents 'Red Frill' and 'Candidum Jr.' each recorded a DNA content of 9.36 pg·2C^{-1}. White Wing recorded the highest nuclear DNA content among the four white cultivars with 9.68 pg·2C^{-1}, slightly higher than its two progeny White Wonder and White Delight, which had 9.40 and 9.31 pg·2C^{-1}, respectively. 'White Butterfly' recorded a comparable nuclear DNA content of 9.50 pg·2C^{-1}.

Chromosome counting. The two triploids identified via nuclear DNA content were both confirmed to have $2n = 3x = 45$ chromosomes (Fig. 8, Table 3). 'Florida Sweetheart' has the same chromosome number as previously reported for the species ($2n = 2x = 30$). The white caladium cultivar with the highest nuclear DNA content, White Wing, has four additional chromosomes compared with the known cultivated caladiums. 'White Wing' had $2n = 2x = 34$ chromosomes in all 22 somatic cells that were examined. 'White Wonder' and 'White Delight', the two progenies of 'White Wing', also had the same chromosome number. To determine if this chromosome number was special to white-colored caladiums, 'White Butterfly' was subjected to chromosome squashing and was found to have $2n = 2x = 30$ chromosomes.

SSR marker analysis. Seven caladium-specific SSR markers were analyzed, and variations in banding patterns allowed for the confirmation that UF-15-414 and UF-4407 resulted from sexual hybridizations (Table 4). UF-15-414 exhibited a different banding pattern than UF-4407 in all markers except CaM48 and CaM62, each varying by the gain or loss of one band. UF-15-414 had identical banding to the other parent 'Aaron' in CaM1 and CaM103. Similarly, UF-4407 had the same banding pattern as 'Florida Sweetheart' in CaM18, CaM24, and CaM62, but different in the other four markers and different from 'Florida Moonlight' in all markers. 'White Butterfly', which was generated by the same parents as UF-4407 except in a reciprocal

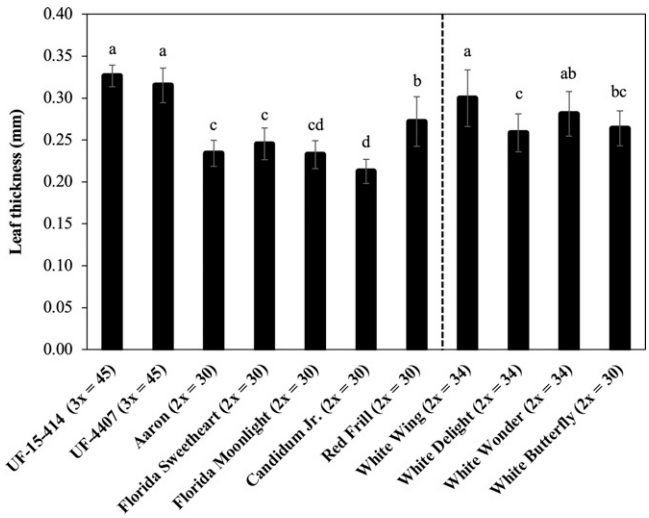

Fig. 4. Average leaf thickness of 11 caladium cultivars and breeding lines. For each, accession measurements were taken on 15 leaves from five plants (three leaves per plant) grown at the University of Florida Gulf Coast Research and Education Center. Means followed by the same letter within each group are not significantly different by Tukey's honestly significant difference test at the 5% level of significance. Dashed line separates statistical analysis of triploids and their parents from the white caladium group.

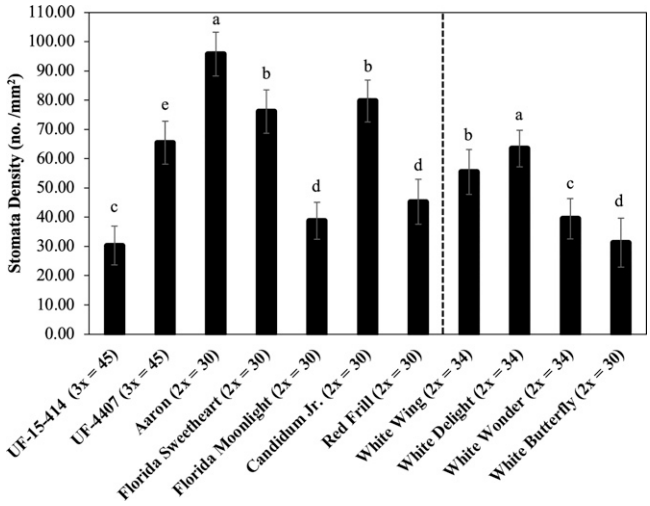

Fig. 5. Average stomata densities for 11 caladium cultivars and breeding lines. Densities were calculated from nail polish imprints taken from the abaxial side of three caladium leaves per accession. Five fields of view were used for counting per leaf. Means followed by the same letter within each group are not significantly different by Tukey's honestly significant difference test at the 5% level of significance. Dashed line separates statistical analysis of triploids and their parents from the white caladium group.

cross, had the same banding as UF-4407 in CaM24, CaM42, and CaM48. Changes in SSR banding patterns also revealed that the two progenies of 'White Wing' did result from hybridizations as well. Few similarities were found between 'White Wing' and its two progeny 'White Delight' and 'White Wonder'. 'White Delight' had the same two bands as 'White Wing' with the CaM24 SSR marker and all three had the same banding with CaM62.

Discussion

This study identified the first two triploids in the genus *Caladium*. Both triploids had ≈13.86 pg·2C^{-1} of DNA and 45 chromosomes, falling in line with expected polyploid values based on 1Cx values of other diploids and the base chromosome number of $x = 15$ in cultivated caladiums.

Triploid UF-4407 originated from a cross between two well-known parents 'Florida Sweetheart' and 'Florida Moonlight'. Both parents in this cross are diploids ($2n = 2x = 30$), as confirmed in this study, thus this triploid likely had resulted from fusion of a reduced gamete with an unreduced gamete ($2n$) from meiotic abnormality. Although naturally occurring polyploidy is not that uncommon in some plant species, triploidy is more sparse (Madlung, 2013). Research in other plant species has shown that $2n$ gamete production is extremely rare, only occurring at rates of 0.1% to 2.0% in natural populations (Kreiner et al., 2017). In plants, unreduced gametes can be produced by female and/or male parents. Our results showed that triploid UF-4407 was highly similar to its maternal parent 'Florida Sweetheart' and rather dissimilar to its paternal parent 'Florida Moonlight' in leaf type and coloration, suggesting that 'Florida Sweetheart' was more likely the contributor of the $2n$ gamete. That is, most likely the triploidy in UF-4407 originated from the fusion of an unreduced female gamete from 'Florida Sweetheart' with a reduced male gamete from 'Florida Moonlight'.

UF-15-414 ($3x$) was a progeny of UF-4407 and 'Aaron'. Strangely, the triploid was able to overcome meiotic abnormalities and produce another triploid progeny. Initially, it was hypothesized that UF-15-414 might have resulted from UF-4407 through apomixis. If so, the two triploids should have the same SSR marker banding pattern. The observed differences in SSR banding patterns between UF-15-414 and UF-4407 rejected our initial hypothesis, instead suggests that UF-15-414 was the result of a sexual hybridization. It remains unknown how a triploid caladium (UF-4407) produced viable female gametes, mated with a diploid parent ('Aaron'), and subsequently produced a progeny (UF-15-414) with three complete sets of chromosomes. Literature searches indicated that defects in genes responsible for the formation of the synaptonemal complex have caused an increase in number of univalents in diploid *Arabidopsis* (Pradillo et al., 2007). It is likely that the mutation leading to the development of UF-4407 is also responsible for the distribution of univalents and trivalents in pollen and ovaries of UF-4407. Triploid flowering pears (*Pyrus* sp.) seem to produce univalents and divalents, with most progeny being triploids and a few diploids (Phillips et al., 2016). In *Hydrangea macrophylla,* it has been observed that triploids produce high rates of univalent pollen and ovaries with all resulting progenies being diploids (Alexander, 2020). The high pollen stainability of UF-4407 and UF-15-414 indicate that there is potentially a high rate of consistency in chromosome segregation during meiosis, but further meiotic studies are needed to confirm the segregation of univalent and trivalents during gamete development.

Induced caladium tetraploids had few leaves in general (Cai et al., 2015; Cao et al., 2016). Compared with induced tetraploids, the triploids identified in this study have significantly improved aesthetic features, including development of multiple leaves and attractive leaf shapes and coloration patterns.

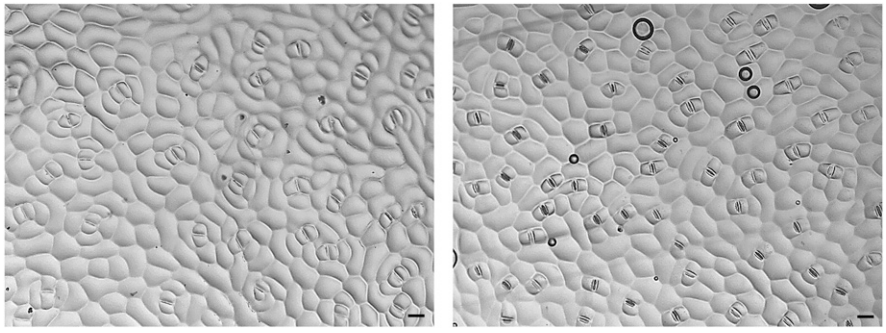

Fig. 6. Caladium stomata micrographs (×100) of UF-15-414 (triploid, left) and 'Florida Moonlight' (diploid, right). Nail polish imprints were taken from the abaxial surface of the leaf and photographed under a bright field microscope. Scale bar = 50 μm.

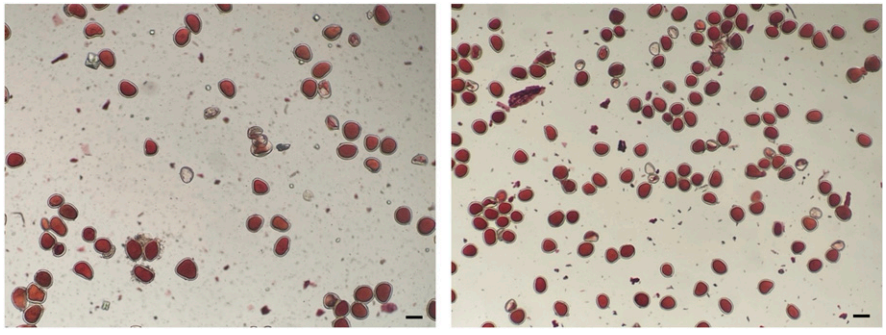

Fig. 7. Micrograph (×100) showing acetocarmine-stained caladium pollen grains from UF-15-414 (triploid, left) and 'Florida Moonlight' (diploid, right). Scale bar = 50 μm.

Table 2. Pollen size and stainability of four caladium cultivars and two triploid breeding lines. Pollen from nine flowers were stained with 4% acetocarmine and analyzed per accession from three separate plants.

Cultivar/breeding line (ploidy level)	Pollen stainability ± SD (%)	Pollen diam (µm)
UF-15-414 (3*x*)	66.97 ± 9.94 d[z]	57.45 ± 5.53 a
UF-4407 (3*x*)	72.92 ± 6.66 c	49.26 ± 4.50 c
Aaron (2*x*)	58.18 ± 10.24 e	48.09 ± 5.56 cd
Florida Sweetheart (2*x*)	82.97 ± 7.68 a	53.48 ± 5.08 b
Florida Moonlight (2*x*)	73.37 ± 5.98 c	46.13 ± 3.78 d
Miss Muffet (2*x*)	78.05 ± 8.94 b	45.50 ± 3.77 d

[z]Means followed by the same letter within each column and group are not significantly different by Tukey's honestly significant difference test at the 5% level of significance.

Table 3. Nuclear DNA content and somatic chromosome numbers of caladium cultivars and breeding lines. Nine flow cytometrical analyses were performed for each selection using three different plants.

Cultivar/breeding line	2C DNA (pg)	1Cx DNA (pg)	Metaphases observed	Chromosome number	Ploidy level
UF-15-414	13.86 ± 0.01 a[z]	4.62	13	45	3*x*
UF-4407	13.85 ± 0.01 a	4.62	11	45	3*x*
Aaron	9.55 ± 0.02 b	4.78	(9)[y]	(30)[y]	2*x*
Florida Sweetheart	9.18 ± 0.02 e	4.59	3 (10)[y]	30 (30)[y]	2*x*
Florida Moonlight	9.43 ± 0.02 c	4.71			2*x*
Candidum Jr.	9.36 ± 0.03 d	4.68			2*x*
Red Frill	9.36 ± 0.02 d	4.68			2*x*
White Wing	9.68 ± 0.02 a	4.84	22	34	2*x*
White Delight	9.31 ± 0.02 d	4.66	9	34	2*x*
White Wonder	9.40 ± 0.01 c	4.70	11	34	2*x*
White Butterfly	9.50 ± 0.01 b	4.75	4	30	2*x*

[z]Means followed by the same letter within each column and group are not significantly different by Tukey's honestly significant difference test at the 5% level of significance.
[y]Number of metaphases and chromosome numbers in parentheses were observed and reported by Cao et al. (2014).

These triploids were also found to have thicker leaves, and thicker petioles and leaf main veins. Together, these characteristics seem to indicate good potential of developing triploids as new cultivars in caladium. UF-15-414 had significantly fewer and larger stomata than the other accessions; however, UF-4407 had stomata comparable to the diploids. Although UF-15-414 followed expectations based on previous studies with caladium tetraploids (Cai et al., 2015), UF-4407 was different with its increased stomata density and reduced size despite its polyploid nature. Thicker leaves and fewer stomata could add potential resistance to two major pathogens in the caladium genus *Xanthomonas axonopodis* and *Colletotrichum caladii,* which affect *Caladium* × *hortulanum*. Further studies will be needed to determine if there is any resistance conferred to these triploids to either pathogen.

Our findings are highly valuable to the future of caladium breeding and may also have considerable impacts on other aroids. Naturally occurring triploids have also been observed in taro (Chaïr et al., 2016; Coates et al., 1988), raising the question of how often unreduced gametes are formed in natural populations and go undetected. The morphological variation observed in *Caladium* due to triploidy has also been observed in *Anthurium andraeanum* that are valued for flower production (Winarto et al., 2018). These anthurium triploids had not only thicker leaves and petioles, but also larger flowers. The current methods for producing triploid anthuriums, however, relies on anther or ovule tissue culture regeneration, which is time-consuming and labor intensive. Other aroids may also benefit from triploidy, such as indoor plants like *Pothos* or *Aglaonema* with potential to produce thicker foliage and petioles. Stricter screening mechanisms for aroid breeding programs in the future could assist in identifying naturally occurring triploids, potentially saving time and effort compared with other techniques.

'White Wing' was found to have a chromosome number of $2n = 2x = 34$ with four additional chromosomes than reports from other commercial *C.* × *hortulanum* caladiums and breeding lines. Unfortunately, the White Wing cultivar has been commercially produced for many years and its lineage is unknown. *Caladium* × *hortulanum* are thought to be a hybrid among *C. bicolor, Caladium marmoratum, Caladium picturatum,* and/or *Caladium schomburgkii* (Birdsey, 1951; Hayward, 1950; Wilfret, 1993). Based on a thorough evaluation of genome sizes and chromosome numbers of caladium species by Cao et al. (2014), it appears that *C. bicolor* and *C. schomburgkii* are strong parent candidates with each having similar genome sizes and the same chromosome number. One species investigated, *C. marmoratum,* had 34 chromosomes; however, the genome size was only 5.57 pg·$2C^{-1}$, nearly half of that of 'White Wing'. Another caladium species, *Caladium clavatum*, was found to have 38 chromosomes, but it too had a small genome size of ≈5.37 pg·$2C^{-1}$. Despite the small genome size, it is possible that 'White Wing' could have originated from a cross between *C. bicolor* and *C. clavatum* adding the four additional chromosomes. *C. clavatum* are known to have distinct morphological differences from cultivated caladiums and *C. bicolor,* such as the production of rhizomatous offsets and a sweet scent given off by the flowers. Although the White Wing cultivar carries neither of these traits, it could be due to the masking of these traits by the other parental haplotype.

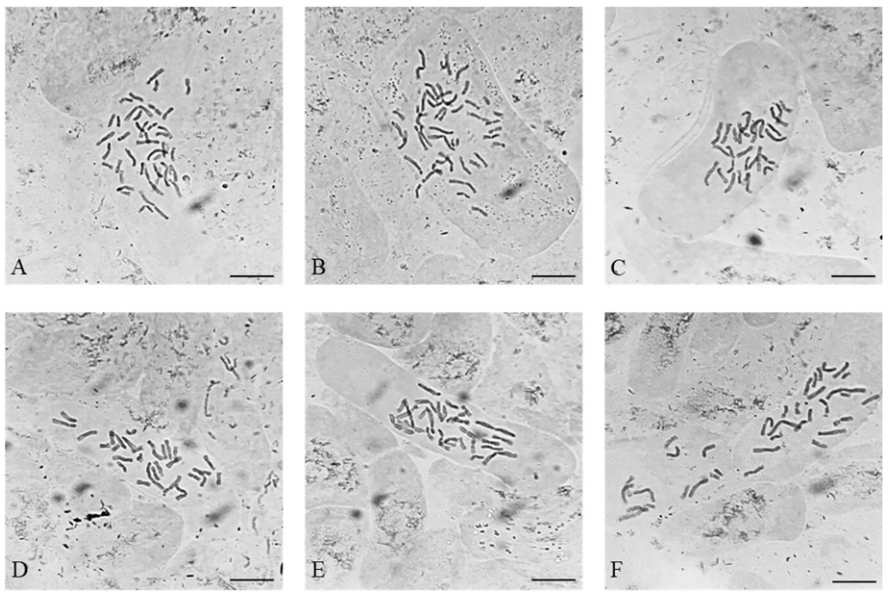

Fig. 8. Micrographs (×1000) of acetocarmine-stained root tip somatic chromosomes of two caladium triploid breeding lines, one cultivar with expected chromosome number, and three cultivars containing four additional chromosomes. (**A**) UF-4407 ($2n = 3x = 45$). (**B**) UF-15-414 ($2n = 3x = 45$). (**C**) 'White Butterfly' ($2n = 2x = 30$). (**D**) 'White Wing' ($2n = 2x = 34$). (**E**) 'White Delight' ($2n = 2x = 34$). (**F**) 'White Wonder' ($2n = 2x = 34$). Scale bar = 10 µm.

Table 4. Simple sequence repeat (SSR) banding patterns from seven caladium-specific SSR markers analyzed on 11 caladium cultivars and breeding lines. Two DNA extractions from separate plants were used for polymerase chain reaction and the experiment was repeated once.

SSR Markers	CaM1	CaM18	CaM24	CaM42	CaM48	CaM62	CaM103
Band Numbers	1 2 3 4	1 2	1 2	1 2 3	1 2 3 4	1 2	1 2 3 4
UF-15-414	2 4[z]	1 2	1 2	1 3	2 4	1	2
UF-4407	2 3 4	1	2	1 3	2 4	1	2 3
Aaron	2 4	2	2	3	2 3	1	2
Florida Sweetheart	2 4	1	2	3	4	1	1 2
Florida Moonlight	1 2	1 2	1 2	3	4	1 2	1 2
Candidum Jr.	1 2	1	1 2	3	4	1	1 2
Red Frill	2 4	1 2	1	2 3	2 4	1	2 4
White Wing	4	1 2	2	2 3	2 3	1	2
White Delight	1 4	2	2	3	2	1	2 4
White Wonder	1 2	2	1 2	3	4	1	2 4
White Butterfly	4	1 2	2	1 3	1 4	1 2	2

[z]Numbers represent the band produced based on relative size with 1 being the heaviest (top) and *n* being the smallest (bottom). For example, a 1 2 indicates that a given accession had two bands located on the gel at position 1 and 2 relative to the other bands present for a given marker.

Primer sequences:

- CaM1 Fwd: CCGAATTATGAACACCCGTAGT Rev: GGACAATTACGGCCTCAAGA
- CaM18 Fwd: TCCTTAGTCCCTGCAGACAGA Rev: GCATTCAGGAACAGGCAAAT
- CaM24 Fwd: CGCAGCAGCTGGAAATG Rev: TCCACCTGCTTTGCTGAAAT
- CaM42 Fwd: CTAGGAGGAGCCCAAGACG Rev: TCCCTTTCTTGTCCCACAAC
- CaM48 Fwd: TCGGTACGATATGTGCAAGG Rev: GCCTTAACATGGAATGAAACG
- CaM62 Fwd: TACCCGCAAGGAAAGGCTAC Rev: GAGCGGTTTGCCTAGTTCGT
- CaM103 Fwd: TTTGTGTAGATTAAAGCTAAATGTG Rev: TTCCTTGAGTCAAAAATTACCT

To investigate how these additional chromosomes segregate, two 'White Wing' progenies were included in the present study. Although nuclear DNA content was reduced in each of the progenies, both cultivars maintained the $2n = 2x = 34$ even though 'White Delight' was developed between a cross of 'White Wing' and 'Jackie Suthers'. At the time of the study, tubers of 'Jackie Suthers' were unavailable for evaluation to determine the presence of 30 or 34 chromosomes. However, Jackie Suthers is another white cultivar with lance leaf type that had been sold commercially for many years. Although its lineage is also unknown, it could be possible that it is of similar origin with 34 chromosomes.

Another plausible explanation for the additional chromosomes could be the presence of B chromosomes. Cao et al. (2014) reported on the potential presence of B chromosomes within the genus, finding the addition of one or two chromosomes in some cells of two cultivated caladiums. There have been many other reports of B chromosomes within the Araceae family (Cusimano et al., 2012). Sharma (1970) reported the presence of four small, rounded B chromosomes in *Arisaema wallichianum;* however, B chromosome presence is usually sporadic among cells and is rarely consistently present among all cells counted. The chromosome squashes of 'White Wing', 'White Delight', and 'White Wonder' in this study consistently had 34 chromosomes. Further analysis of the root tip cells revealed no small, rounded chromosomes in the spreads, indicating that B chromosome presence is unlikely. Crosses between diploid cultivars with 30 chromosomes and 'White Wing' and meiotic cell analyses are needed in the future to determine if the additional four chromosomes are B or A chromosomes.

The use of triploids for cultivar development in caladiums should be considered by plant breeders because of their ability to produce new novel phenotypes. Rounded, thicker leaves are highly associated with the increase in ploidy level in caladium and will add diversity to the current caladiums on the market. Further investigations into the origin of cultivated caladiums are needed with the discovery of 'White Wing' possessing a different chromosome number from other cultivars.

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