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Short Communication

Interploidy hybridization in *Eriotheca gracilipes* and *E. pubescens* (Malvaceae): experimental evidence, genome and stomatal size

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Abstract

Hybridization and changes in ploidy have been associated with shifts from sexuality to apomixis, and may explain isolation among populations and species. Hybrids resulting from interploidy crosses may contribute to a broader understanding of how these populations and species have originated and evolved. Stomatal morphometrics and flow cytometry analyses were carried out for seedlings from different populations of *Eriotheca estevesiae*, *E. gracilipes* and *E. pubescens*, a group of closely related tree species in the Central Brazilian Cerrados. Controlled cross-pollinations between individuals of different cytotypes of *E. gracilipes* (2n = 2x = 92 and 2n = 6x = 276) and between sexual cytotypes of *E. gracilipes* (2n = 2x = 92) and *E. pubescens* (2n = 4x = 184) were performed. Only one viable seed was obtained from interploidy crosses of *E. gracilipes*. The hybridization between sexual cytotypes did not produce fruits. Genome size analyses indicated that there were apparently no natural hybrids or mixed ploidy populations among the seedlings analyzed. Seedlings stomatal size was consistent with previously reported cytotypes and ploidy levels; and when compared with the stomata of the viable interploidy hybrid of *E. gracilipes*, indicated a tetraploid, intermediate ploidy level. Although the data suggest the possibility of interploidy hybridization, cytotypes appeared relatively stable and natural interploidy hybridization seems to be uncommon among *Eriotheca* trees.

Key words: breeding system, flow cytometry, hybrid, polyploidy, stomata morphometry.

Resumo

Diferentes citótipos na mesma espécie têm sido associados a mudanças na reprodução das plantas, da sexualidade à apomixia, e podem explicar o isolamento entre populações e espécies. Os híbridos resultantes de cruzamentos de interploidia podem contribuir para o entendimento de como essas populações e espécies podem ter se originado e evoluído. A morfometria estomática e as análises de citometria de fluxo foram realizadas para plântulas de diferentes populações de *Eriotheca estevesiae*, *E. gracilipes* e *E. pubescens*, espécies arbóreas dos Cerrados do Brasil Central. Polinizações cruzadas controladas entre indivíduos de citótipos distintos de *E. gracilipes* (2n = 2x = 92 e 2n = 6x = 276) e entre citótipos sexuais de *E. gracilipes* (2n = 2x = 92) e *E. pubescens* (2n = 4x = 184) foram realizadas. Apenas uma semente viável foi obtida a partir de cruzamentos interploidias de *E. gracilipes*. A hibridização entre citótipos sexuais não produziu frutos. As análises do tamanho do genoma indicaram que aparentemente não havia híbridos naturais ou população de ploidia mista entre as plântulas analisadas. O tamanho dos estômatos das plântulas era consistente com os citótipos e o nível de ploidia supostos; e quando comparados com os estômatos do híbrido de interploidia viável de *E. gracilipes*, indicou um nível de ploidia intermediário, tetraplóide. Embora os dados tenham confirmado a possibilidade de hibridização interploidia, os citótipos pareceram relativamente estáveis e a hibridização interploidia natural parece ser menos comum em árvores *Eriotheca*.

Palavras-chave: sistema reprodutivo, citometria de fluxo, híbrido, poliploidia, morfometria estomática.

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Hybridization between species or individuals from different populations is a natural process which occurs in at least 25% of terrestrial species (Chen 2010). One of the consequences is higher vigor among these hybrids in relation to their progenitors (Birchler *et al.* 2003). Hybridization can also be a driver for the occurrence of polyploidy, or moreover, the appearance of new cytotypes within a species. Cytotype is commonly defined as a group of individuals from a species with a particular ploidy level, and studies indicate that at least 16% of plant species exhibit cytotype variation (Rice *et al.* 2015; Soltis *et al.* 2015; Kolář *et al.* 2017). The occurrence of populations or neighbor individuals with different cytotypes is normally considered a barrier for gene exchange among and within species (Hanneman & Peloquin 1968; Levin 2019). This barrier is commonly associated with either geographical or ecological niche separation, or with distinct genetical structure between cytotypes (Balao *et al.* 2009; López-Jurado *et al.* 2019). Populations with multiple cytotypes can help elucidate the polyploidization processes and how they influenced the rise and establishment of such taxa (*e.g.*, Balao *et al.* 2009). Moreover, these mixed-ploidy populations facilitate the study of cytotype-specific ecological relations *in situ* (Kolář *et al.* 2017; Hörandl 2022), enabling performance comparisons of hybrid individuals and explaining their relative importance in these groups (Karunarathne *et al.* 2018).

The ploidy level characterization in species is frequently established by direct chromosome counting. Nonetheless, this method can be impractical for large samples or very laborious. Flow cytometry is currently an alternative for cytogenetic studies due to its precision, efficiency, and possibility of application in a large number of individuals and populations (Jung *et al.* 1993; Balao *et al.* 2009), although it does not allow comparable chromosome number estimates. Flow cytometry can also be expensive and usually depends on fresh plant tissues. For large scale studies, other methods, such as morphometrical analyses using dried material (even from herbaria sheets) could be used. Stomatal morphometry seems to be a feasible alternative for large scale cytotype determination, allowing fairly rapid sample preparation using dry, vegetative material (*e.g.*, Masterson 1994; Vandenhout *et al.* 1995). Pollen or stomatal morphometry have been used as a proxy to ploidy level (*e.g.*, Beck *et al.* 2003) but they show extensive variability and may be affected by other factors (*e.g.*, Jordan *et al.* 2015). So, the use of morphometry as proxies to ploidy requires extensive survey and validation. Stomatal size increased clearly with ploidy in Neotropical *Eriotheca* (Schott & Endl) agamic species complexes (Marinho *et al.* 2014a,b, 2020; Mendes-Rodrigues *et al.* 2019).

Eriotheca has numerous and minute chromosomes (Oliveira *et al.* 1992; Baum & Oginuma 1994; Marinho *et al.* 2014a, b). *Eriotheca gracilipes* (K. Schum.) A. Robyns and *E. pubescens* (Mart. & Zucc.) Schott & Endl present reproductive mosaics with sexual and asexual populations geographically separated (Mendes-Rodrigues *et al.* 2019). *Eriotheca gracilipes* has a diploid sexual cytotype 2n = 2x = 92 (Oliveira *et al.* 1992; Mendes-Rodrigues *et al.* 2005), while *E. pubescens* has a tetraploid sexual cytotype 2n = 4x = 184 (Marinho *et al.* 2014a, b; Mendes-Rodrigues *et al.* 2019). Both species have also hexaploid cytotypes, 2n = 6x = 276, which are apomictic and polyembryonic (Oliveira *et al.* 1992; Marinho *et al.* 2014a, b; Mendes-Rodrigues *et al.* 2005, 2019). A recently described species *Eriotheca estevesiae* Carv.-Sobr. is phylogenetically related to *E. pubescens* (Carvalho-Sobrinho *et al.* 2015). It is diploid, 2n = 2x = 92, and monoembryonic (Marinho *et al.* 2020). Flow cytometry studies and stomatal measurements have been successfully used to corroborate *Eriotheca* ploidy levels (Marinho *et al.* 2014a, b, 2020; Mendes-Rodrigues *et al.* 2019). Despite those cytological and morphological differences, species and cytotypes have quite similar flowers and pollination biology. Cerrado *Eriotheca* are pollinated by large solitary bees which are widely distributed and some of them were observed visiting both *E. gracilipes* and *E. pubescens* (Oliveira *et al.* 1992). Although pollination biology studies for *E. estevesiae* are still lacking, flowers are similar to *E. pubescens* (Carvalho-Sobrinho *et al.* 2015) and there are no clear hybridization barriers among populations and species (Marinho *et al.* 2020). Some cytotypes of *E. gracilipes* and *E. pubescens* occur sympatrically in the Triângulo Mineiro region and both sexual and apomictic individuals require pollination to set fruits and viable seeds (Mendes-Rodrigues *et al.* 2019). As mentioned before, pollen and stomatal sizes are clearly correlated to ploidy levels in the three *Eriotheca* species (Marinho *et al.* 2014a, 2020; Mendes-Rodrigues *et al.* 2019).

We aimed to test hybridization between cytotypes of *Eriotheca* and use cytological methods - flow cytometry and stomatal morphometry - to describe the resulting progeny. In addition, we used flow cytometry to estimate the occurrence of genome size variation in progenies from natural populations of the species, which could provide evidence for the existence of natural hybrids or interploidy cytotypes.

Pollination treatments and hybridization

The hybridization between the two *E. gracilipes* cytotypes was tested by hand pollinations of recently open (mostly first-day) flowers of a diploid individual in Uberlândia, Minas Gerais, Brazil (48°17'W, 18°55'S). Flowers in diploid individuals were previously emasculated and bagged, and pollinated with pollen from three *E. gracilipes* hexaploid individuals from Caldas Novas, Goiás, Brazil (48°40'W, 17°46'S) some 180 km Northwest. We collected branches with pre-anthesis buds which were stored in plastic bags and kept moist with distilled water. We used pollen from buds that opened naturally inside the bags. Due to time-consuming emasculation procedures and limited number of buds, we did only 19 hybridization cross-pollination treatments. To evaluate natural pollination, 35 flowers of the same plant were marked with cotton threads or PVA glue spots at anthesis (as in Oliveira *et al.* 1992).

Hand pollinations between ploidies were made in July 2013 and fruit production was quantified after 30 days. Seeds obtained from the resulting fruits were germinated on distilled water moistened filter paper inside Gerbox plastic boxes. The seedlings were later planted in commercial substrate and maintained in a greenhouse (shading only, no temperature control; temperatures ranged from 25 to 35 ^{o}C). The first pair leaves from the resulting seedlings were collected ca. 45 days after planting and dried in silica gel.

We also evaluated interspecific hybridization between sexual and self-incompatible tetraploid individuals of *E. pubescens* from Cristalina city, Goiás, Brazil (47°44'W, 16°37'S) and sexual and self-incompatible diploid individuals of *E. gracilipes* from the road of access to Sucupira Waterfall, Uberlândia, Minas Gerais, Brazil (48°44'W, 19°30'S). For these hybridization tests, we hand-pollinated 35 previously emasculated flowers in five diploid individuals of *E. gracilipes* using pollen from the tetraploid *E. pubescens*. Pollinations were made on recently open flowers with pollen from three tetraploid individuals of *E. pubescens*. Reciprocal pollinations using tetraploid *E. pubescens* could not be carried out due to logistical problems. Another 51 flowers were marked at the beginning of anthesis for natural pollination and fruit-set estimates. Pollination treatments were carried out in July 2013 and fruit-set evaluated 30 days later.

We did not use hexaploid *E. gracilipes* or *E. pubescens* plants as pollen receptors since they are apomictic and produce clonal embryos independent of the kind of pollination (Mendes-Rodrigues *et al.* 2005, 2019). Taxonomy, ploidy and breeding system had been previously determined for all plants (Marinho *et al.* 2014a, 2014b; Mendes-Rodrigues *et al.* 2005, 2019). Mixed populations of different cytotypes were unknown at the time of the experiments.

The differences in fruit production between hybridization treatments and natural pollinations were tested with Williams' test (G-test). Statistical analyses were carried out using the R program (R Core Team 2020) or in SPSS 20.0 (SPSS, Chicago, IL, USA).

Stomata and genome size measurements

We collected seeds from *E. gracilipes* (diploids from Caldas Novas, 48°40'W, 17°46'S, and hexaploids from Uberlândia 48°44'W, 19°30'S) and *E. pubescens* (tetraploids from Cristalina 47°44'W, 16°37'S and hexaploids from Uberlândia) and also from *E. estevesiae* (putatively diploids from Tocantins state, 49°47'W, 12°25'S). Seeds were extracted manually from natural pollinated mature fruits. The seeds were later sowed in commercial substrate and maintained in natural light and temperature conditions.

The first pair leaves were collected from three seedlings originated from seeds of each cytotype and from the hybrid individuals. The leaves were dried and preserved in silica gel. The morphometric analyses of seedling stomata followed methodology described for stomatal measurements of expanded leaves of adult individuals (Marinho *et al.* 2014a, 2020). We used cyanoacrylate instant glue (SuperBonder-Loctite®) to obtain leaf impressions (Chin *et al.* 1995) of previously re-hydrated leaflets on glass microscope slides. The slides were observed and photographed with a DP70 digital camera on a

BX51 Olympus microscope. Stomatal height (length of guard cells) and width (both guard cells plus stoma) measurements were obtained using ImageJ, version 1.46r (Collins 2007). We measured 30 stomata per seedling, except in the case of the hybrid individual for which we measured 80 stomata.

The ANOVA model residuals from stomatal height and width measurements were approximately normally distributed (tested with Kolmogorov-Smirnov Lilliefors'test), but the cytotypes did not show homoscedastic variances (tested with Levene's test). Therefore, for comparison between cytotypes, regardless of seedling, we used Generalized Linear Models (GLM) adopting Gaussian probability distribution function and identity as link function. For multiple pairwise comparisons we used the least significant difference test (LSD). The variance decomposition of height and width of stomatal measurements was obtained from a Nested ANOVA, as proposed by Neter *et al.* (1985), and carried out using the SAEG program (SAEG 1997).

Other seedlings from each cytotype, cultivated as described above, were used for genome size estimates. For *E. gracilipes*, we used 65 seeds from five polyembryonic/hexaploid mother-plants and 51 seeds from five monoembryonic/diploid mother-plants. From these seeds, we obtained three to 27 and one to 10 seedlings, respectively. Regarding *E. pubescens*, we used 151 seeds from 21 monoembryonic/tetraploid mother-plants and 130 seeds from polyembryonic/hexaploid mother-plants. We obtained three to 11 seedlings from each monoembryonic mother-plant, while 21 to 33 seedlings were obtained from each polyembryonic mother-plant. In all cases, we selected only one seedling from each seed for genome size analysis. Nuclear DNA suspensions from 397 seedlings were measured by flow cytometry using a protocol based on Dolezel *et al.* (1989) and described in detail in Marinho *et al.* (2014b).

For comparison between cytotypes in each species we also used Generalized Linear Models (GLM) adopting Gaussian probability distribution function and identity as link function. Additionally, we present the histograms based on relative frequency for each species construct based on minimum and maximum from each species, but with each ploidy displayed separately. GLM analyses were carried out using the R program (R Core Team 2020).

Despite the limited number of treatments, the hybridization cross-pollinations between the two *E. gracilipes* ploidies (sexual/diploid 2n = 2x = 92 and apomict/hexaploid cytotypes, 2n = 6x = 276) resulted in one fruit out of 19 flowers tested (5.26%). This fruit had five seeds, but only one was well-formed and viable, which germinated and formed an apparently normal seedling. The other seeds presented reduced and malformed embryos. Approximately 30 days after leaf collection, the putative hybrid seedling perished due to fungus infestation, making impossible further monitoring and analyses. For the natural pollinations, although the mother-plant produced fruits and normal viable seeds within the evaluated year, we obtained only one fruit from the 35 marked flowers (2.86%), and there were no significant differences in fruit-set when compared with the interploidy hybridization treatment ($G = 0.14$; $p = 0.6994$). This fruit was not collected due to natural damage, but the tree did produce other fruits from unmarked flowers indicating natural pollination does result in fruits and potentially viable seeds (personal observation). Flowers from diploid individuals of *E. gracilipes* pollinated with pollen from tetraploid individuals of *E. pubescens* did not produce fruits (zero fruits out of 35 treated flowers). However, we did obtain ten fruits from the natural pollination of the same diploid individuals of *E. gracilipes* (19.6%, out of 51 treated flowers). We did not perform reciprocal pollination with the tetraploid *E. pubescens* plants as pollen recipients, but this population had 7.5% natural fruit set in 2010 (Mendes-Rodrigues *et al.* 2019) and pollen was apparently viable.

For morphometric analyses, we considered six cytotypes: *E. estevesiae* (diploid, 2x = 2n = 92), *E. gracilipes* (Eg) diploid (Eg2x) and *E. gracilipes* (Eg) hexaploid (Eg6x), *E. pubescens* (Ep) tetraploid (Ep4x) and *E. pubescens* (Ep) hexaploid (Ep6x). We also analyzed the stomata from the *E. gracilipes* putative interploidy hybrid seedling (Eg 2x6x). The stomatal height differed significantly between cytotypes ($X^2 = 937.06$; d.f. = 5; $p < 0.001$; Fig. 1a) as did the stomatal width ($X^2 = 937.06$; d.f. = 5; $p < 0.001$; Fig. 1b). As for *E. gracilipes*, pairwise comparison for stomatal height measurements of the interploidy hybrid (mean = 33.47 µm; range = 30.31–37.83; 95% confidence interval-CI: 33.03–33.91) did not show significant differences from the *E. gracilipes* hexaploid cytotype (mean = 33.62 µm; range = 27.47–39.83; 95% CI: 33.03–34.21). Nonetheless, it differed from the diploid cytotype of *E. gracilipes*

(mean = 29.89 µm; range = 24.54-34.42; 95% CI: 29.44-30.34). As for stomatal width (Fig. 1b), the analysis resulted in significant differences between the hybrid individual (mean = 15.35 µm; range = 11.21–19.80; 95% CI: 15.05–15.64) and both diploid (mean = 16.45; range = 13.47–19.38; 95% CI: 16.16–16.73), and hexaploid cytotypes (mean = 17.75 µm; range = 12.95–22.60; 95% CI: 17.26–18.23) of *E. gracilipes*.

In *E. pubescens*, the stomatal height differed between tetraploid (mean = 32.36 µm; range = 24.80–42.27; 95% CI: 31.48–33.23) and hexaploid cytotype (mean = 35.76; range = 30.60–42.40; 95% CI: 35.25–36.26), and also from all *E. gracilipes* cytotypes. *Eriotheca pubescens* stomatal width also differed between the tetraploid (mean = 15.37; range = 11.19–19.98; 95% CI: 14.96–15.79) and hexaploid cytotype (mean = 17.17; range = 13.92–20.21; 95% CI: 16.90–17.45). However, the tetraploid cytotype stomatal width did not differ from that of the interploidy hybrid of *E. gracilipes*. The diploid cytotypes of *E. estevesiae* presented the lowest values, both for height (mean = 24.47 µm; range = 19.76–28.69; 95% CI: 24.02–24.91) and width (mean = 14.13 µm; range = 11.43–17.50; 95% CI: 13.87–14.39), and differed from all the other cytotypes measured (Fig. 1).

Comparisons between stomatal sizes from different cytotypes (five cytotypes), showed that cytotype was the most explanatory factor. For stomatal height 61.74% of the variance was explained by the cytotype, 22.94% by the seedlings nested in cytotype and 15.32% by other non-measured factors (residuals), whereas for the width, 31.35% of the variance was explained by the cytotype, 30.26% by the seedlings nested in cytotype, and 38.59% by other non- measured factors (residuals). On a second analysis, where we evaluated the pooled ploidy levels (diploidy versus polyploidy), for stomatal height 56.73% of the variance was explained by ploidy level, 13.69% by cytotype nested in ploidy, 17.74% by seedlings nested in cytotype and 11.84% by non-measured factors (residuals). However, for the width, only 5.62% of the variance was explained by ploidy, 27.08% by cytotype nested in ploidy, 29.58% by seedlings nested in cytotypes and 37.72% by non-measured factors (residuals).

The flow cytometry genome size estimates showed a clear distinction and limited overlap between species cytotypes (Fig. 2). Seedlings from diploid *E. gracilipes* had the smallest DNA content (mean = 3.66 pg; standard deviation = 0.09; range

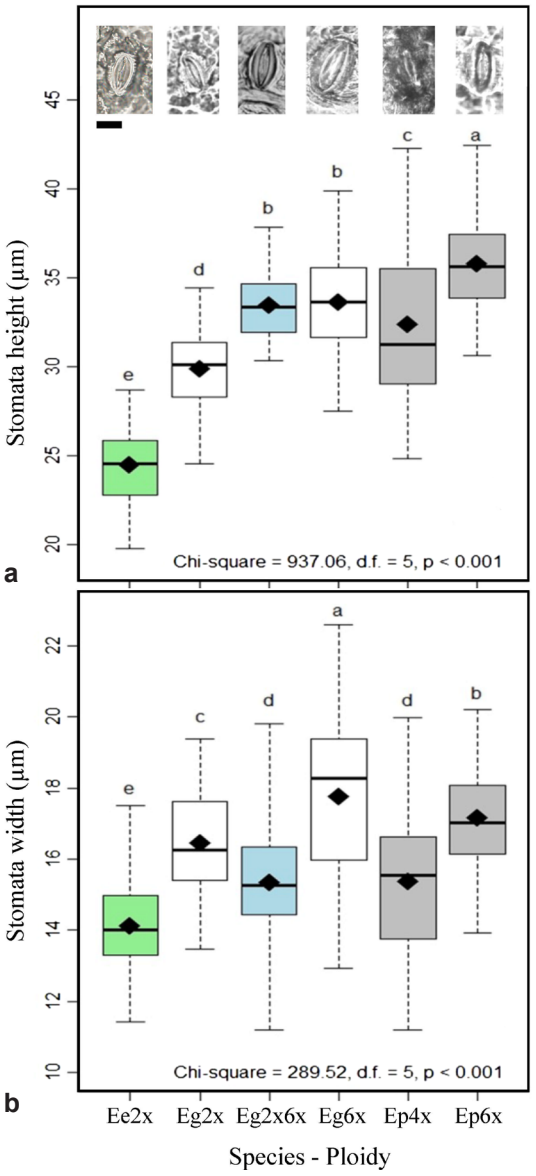

Figure 1 – a-b. Seedling stomatal size of *Eriotheca* spp. (Malvaceae) with different cytotypes – a. stomata reproduced in scale (scale bar = 10 µm) and stomatal height measurements; b. stomatal width measurements. In both panels, seedlings from *Eriotheca estevesiae* from diploid mother-plants (Ee2x, green color); *Eriotheca gracilipes* (Eg) from diploid (Eg2x, white color) and hexaploid (Eg6x, white color) mother- plants and their possible hybrid (Eg2x6x, light blue color); and *Eriotheca pubescens* (Ep) from tetraploid (Ep4x, grey color) and hexaploid (Ep6x, grey color) mother-plants. Means followed by different letters are different based on least significant difference post hoc test ($p < 0.05$). Boxplot represents the minimum, quartile 1, median, quartile 3, maximum and the mean (diamonds).

= 3.45–4.02), while the hexaploid *E. gracilipes* showed the largest (mean = 10.36 pg; standard deviation = 0.58; range = 9.16–11.84); and they were statistically different ($X^2 = 6735.12$; d.f. = 1; $p < 0.001$). Seedlings from tetraploid *E. pubescens* showed intermediate DNA content (mean = 6.92 pg; deviation = 0.11; range = 6.53–7.16), and significantly smaller than hexaploid individuals (mean = 10.20pg; standard deviation = 0.24; range = 9.62–10.82; $X^2 = 23787.53$; d.f. = 1; $p < 0.001$). Hexaploid genome sizes estimates for *E. gracilipes* and *E. pubescens* overlapped broadly but were statistically different ($X^2 = 7.26$; d.f. = 1; $p = 0.007$). As a whole, genome sizes estimates were compatible with ploidy levels, and there were no intermediate DNA content measurements which suggested natural hybrids. The wider genome size variation and a second smaller peak observed for *E. gracilipes* hexaploids (Fig. 2a) may be due to aneuploidy or further anomalies which remain to be studied. But since it represents higher DNA content, it is not possibly related to hybridization with lower ploidy cytotypes.

Hybridization hand pollinations between *E. pubescens* 4x and *E. gracilipes* 2x resulted in no fruit or seed formation, and crossing *E.*

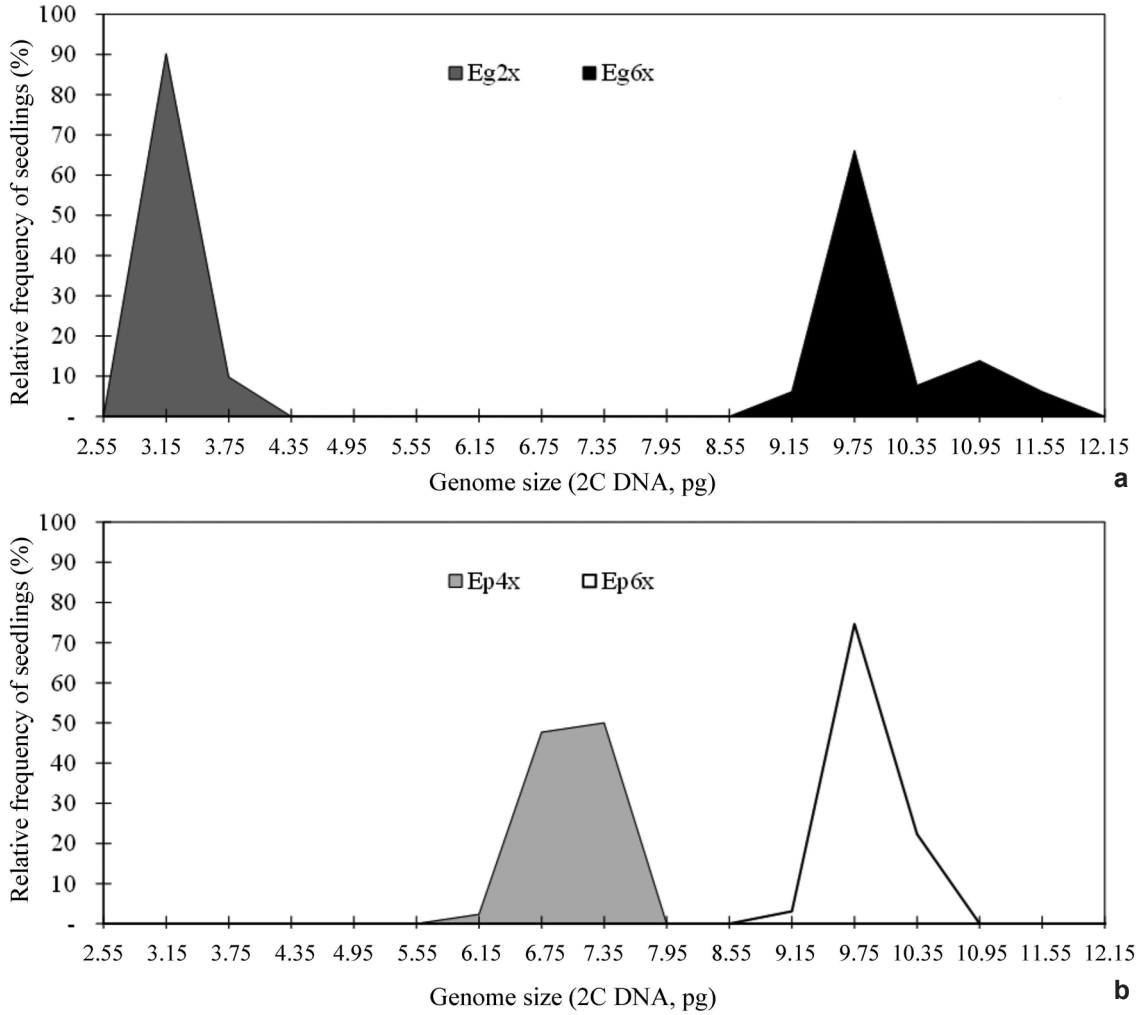

Figure 2 – a-b. Relative frequency of seedlings in different genome sizes (nuclear DNA content estimates) for two *Eriotheca* spp. (Malvaceae) with different cytotypes – a. seedlings from *Eriotheca gracilipes* (Eg) diploid (Eg2x, n = 51) and hexaploid (Eg6x, n = 65) mother-plants; b. seedlings from *Eriotheca pubescens* (Ep) tetraploid (Ep4x, n = 151) and hexaploid (Ep6x, n = 130) mother-plants. Peaks represent prevalent genome size in each species or cytotype. A secondary peak in hexaploidy *E. gracilipes* may be the result of aneuploidy.

gracilipes 2x and *E. gracilipes* 6x resulted in only a single seedling. Despite the limited number of flowers tested, the results are consistent with the idea that differences in ploidy levels represent important reproductive barriers (Coyne & Orr 2004; Schatlowski *et al.* 2014). Furthermore, stomatal morphometrics and genome size analyses showed no evidence of intermediate genome size or ploidy which would indicate natural hybrids. Unsuccessful hybridization is commonly a result of endosperm malformation caused by chromosome imbalance during gamete fusion, which hinders early embryo development (Quarin 1999; Lafon-Placette & Kohler 2016). Such processes would explain the malformed seeds found in the single fruit resulting from *E. gracilipes* cytotypes hybridization tests. Only one seedling actually developed and although the number of hybridization pollinations prevents making generalizations and the early death of the putative hybrid seedling made impossible further ploidy assessment via flow cytometry, stomatal morphometrics suggested an intermediate ploidy level. Stomatal morphometry stomatal was consistent with ploidy level in *Eriotheca* (Marinho *et al.* 2014a, 2020). The Nested ANOVA analyzes showed that the species cytotypes (and pooled ploidy levels) were the most important factors explaining stomatal size variation, possibly because polyploidization affects cell size of *Eriotheca*, as it does in *Citrus* (Costa *et al.* 2004), *Musa* (Vandenhout *et al.* 1995) and other groups (Balao *et al.* 2011).

Seedling screening for stomatal morphometry obtained here were consistent with measurements for fully expanded leaves of adult plants obtained in previous studies (Marinho *et al.* 2014a, 2020; Mendes-Rodrigues *et al.* 2019). They showed clear differences between ploidy levels both in *E. gracilipes* and *E. pubescens*. Moreover, the *E. gracilipes* hybrid individual showed intermediate stomatal size values compatible with intermediate genome size. Other studies involving interploidy and interspecific hybrids, such as in *Hibiscus*, also showed cytotype distinction using stomatal size (Lattier *et al.* 2019). In the case of the *Eriotheca* species studied here, flow cytometry analyses for our large seedling ample (397 seedlings) showed no modifications in genome size which would suggest natural hybridization or genome size variation. Our analyses confirmed relatively stable genome size and ploidy, and possibly non-mixed populations as previously suggested (Marinho *et al.* 2014a, b). In this sense, with both ploidy and stomata smaller than the other species, *E. estevesiae* may share a most recent common ancestor with the diploid ancestor of *E. pubescens* as they share phylogenetic similarities and stellate trichomes (Marinho *et al.* 2020).

Distinct patterns of embryony (monoembryony and polyembryony) and possibly ploidy levels have been recently observed in sympatry for *E. gracilipes* and *E. pubescens* (Marinho *et al.* 2020; C. Mendes-Rodrigues 2019, personal communication). Changes in ploidy have occurred commonly in the group (Costa *et al.* 2017; Mendes-Rodrigues *et al.* 2019). Mixed ploidy populations have been documented for *Handroantus ochraceous* (Bignoniaceae), another agamic polyploid complex in Cerrado (Mendes *et al.* 2018). However, for the *Eriotheca* studied here, we did not find evidence of further admixture or mixed populations. Diploid, tetraploid and haxaploid lineages appear well isolated and stable. Although we confirmed that interploidy hybrids can arise in *E. gracilipes*, it seems natural hybridization is not common.

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References

Balao F, Casimiro-Soriguer R, Talavera M, Herrera J & Talavera S (2009) Distribution and diversity of cytotypes in *Dianthus broteri* as evidenced by genome size variations. Annals of Botany 104: 965-973. <https://doi.org/10.1093/aob/mcp182>

Balao F, Herrera J &Talavera S (2011) Phenotypic consequences of polyploidy and genome size at the microevolutionary scale: a multivariate morphological approach. New Phytologist 192: 256-265. <https://doi.org/10.1111/j.1469-8137.2011.03787.x>

Baum DA & Oginuma K (1994) A review of chromosome numbers in Bombacaceae with new counts for *Adansonia*. Taxon 43: 11-20. <https://doi.org/10.2307/1223456>

Beck SL, Dunlop RW & Fossey A (2003) Stomatal length and frequency as a measure of ploidy level in black wattle, *Acacia mearnsii* (de Wild). Botanical Journal of the Linnean Society 141: 177-181. <https://doi.org/10.1046/j.1095-8339.2003.00132.x>

Birchler JA, Auger DL & Riddle NC (2003) In search of the molecular basis of heterosis. Plant Cell 15: 2236-2239. <https://doi.org/10.1105/tpc.151030>

Carvalho-Sobrinho J, Mota A & Queiroz L (2015) *Eriotheca estevesiae* (Malvaceae: Bombacoideae): a new species from the cerrado vegetation of Brazil. Brittonia 67: 29-36. <https://doi.org/10.1007/s12228-014-9350-4>

Chen ZJ (2010) Molecular mechanisms of polyploidy and hybrid vigor. Trends Plant Science 15: 57-71. <https://doi.org/10.1016/j.tplants.2009.12.003>

Chin J, Wan Y, Smith J & Croxdale J (1995) Linear aggregations of stomata and epidermal cells in *Tradescantia* leaves: evidence for their group patterning as a function of the cell cycle. Developmental Biology 168: 39-46. <https://doi.org/10.1006/dbio.1995.1059>

Collins TJ (2007) ImageJ for microscopy. Biotechniques 43: 25-30. <https://doi.org/10.2144/000112517>

Costa MAPD, Almeida WAB & Mourao FDA (2004) Stomatal analysis of *Citrus* somatic hybrids obtained by protoplast fusion. Pesquisa Agropecuária Brasileira 39: 297-300. <https://doi.org/10.1590/S0100-204X2004000300015>

Costa L, Oliveira Á, Carvalho-Sobrinho J & Souza G (2017) Comparative cytomolecular analyses reveal karyotype variability related to biogeographic and species richness patterns in Bombacoideae (Malvaceae). Plant Systematics and Evolution 303: 1131-1144. <https://doi.org/10.1007/s00606-017-1427-6>

Coyne JA & Orr HA (2004) Speciation. Sinauer Associates, Sunderland. 545p.

Dolezel J, Binarova P & Lucretti S (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. Biologia Plantarum 31: 113-120. <https://doi.org/10.1007/BF02907241>

Hanneman Jr. RE & Peloquin SJ (1968) Ploidy levels of progeny from diploid-tetraploid crosses in potato. American Journal of Potato Research 45: 255-26. <https://doi.org/10.1007/BF02849919>

Hörandl E (2022) Novel approaches for species concepts and delimitation in polyploids and hybrids. Plants 11: 204. <https://doi.org/10.3390/plants11020204>

Jordan GJ, Carpenter RJ, Koutoulis A, Price A & Brodribb T J (2015) Environmental adaptation in stomatal size independent of the effects of genome size. New Phytologist 205: 608-617. <https://doi.org/10.1111/nph.13076>

Jung T, Schauer U, Heusser C, Neuman C & Rieger C (1993) Detection of intracellular cytokines by flow cytometry. Journal of Immunological Methods 159: 197-207. <https://doi.org/10.1016/0022-1759(93)90158-4>

Karunarathne P, Schedler M, Martínez EJ, Honfi AI, Novichkova A & Hojsgaard D (2018) Intraspecific ecological niche divergence and reproductive shifts foster cytotype displacement and provide ecological opportunity to polyploids. Annals of Botany 121: 1183-1196. <https://doi.org/10.1093/aob/mcy004>

Kolář F, Čertner M, Suda J, Schönswetter P & Husband BC (2017) Mixed-ploidy species: progress and opportunities in polyploid research. Trends Plant Science 22: 1041-1055. <https://doi.org/10.1016/j.tplants.2017.09.011>

Lafon-Placette C & Kohler C (2016) Endosperm-based postzygotic hybridization barriers: developmental mechanisms and evolutionary drivers. Molecular Ecology 25: 2620-2629. <https://doi.org/10.1111/mec.13552>

Lattier JD, Chen H & Contreras RN (2019) Variation in genome size, ploidy, stomata, and rDNA signals in *Althea*. Journal of the American Society for Horticultural Science 144: 130-140. <https://doi.org/10.21273/jashs04618-18>

Levin DA (2019) Plant speciation in the age of climate change. Annals of Botany 124: 769-775. <https://doi.org/10.1093/aob/mcz108>

López-Jurado J, Naranjo E & Balao F (2019) Niche divergence and limits to expansion in the high polyploidy *Dianthus broteri* complex. New Phytologist 222: 1076-1087. <https://doi.org/10.1111/nph.15663>

Marinho RC, Mendes-Rodrigues C, Bonetti AM & Oliveira PE (2014a) Pollen and stomata morphometrics and polyploidy in *Eriotheca* (Malvaceae-Bombacoideae). Plant Biology 16: 508-511. <https://doi.org/10.1111/plb.12135>

Marinho RC, Mendes-Rodrigues C, Balao F, Bonetti AM & Oliveira PE (2014b) Do chromosome numbers reflect phylogeny? New counts for Bombacoideae and a review of Malvaceae sl. American Journal of Botany 101: 1456-1465. <https://doi.org/10.3732/ajb.1400248>

Marinho RC, Mendes-Rodrigues C, Bonetti AM & Oliveira PE (2020) Stomatal size, ploidy and polyembryony in *Eriotheca* stellate trichomes species complex (Bombacoideae - Malvaceae) in the Cerrados of Brazil. Plant Biology 23: 91-99. <https://doi.org/10.1111/plb.13177>

Masterson J (1994) Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science 264: 421-424. <https://doi.org/10.1126/science.264.5157.421>

Mendes MG, Oliveira AP, Oliveira PE, Bonetti AM & Sampaio DS (2018) Sexual, apomictic and mixed populations in *Handroanthus ochraceus*

(Bignoniaceae) polyploid complex. Plant Systematics and Evolution 304: 817-829. <https://doi.org/10.1007/s00606-018-1512-5>

Mendes-Rodrigues C, Carmo-Oliveira R, Talavera S, Arista M, Ortiz PL & Oliveira PE (2005) Polyembryony and apomixis in *Eriotheca pubescens* (Malvaceae - Bombacoideae). Plant Biology 7: 533-540. <https://doi.org/10.1055/s-2005-865852>

Mendes-Rodrigues C, Marinho RC, Balao F, Aristad M, Ortiz PL, Carmo-Oliveira R & Oliveira PE (2019) Reproductive diversity, polyploidy, and geographical parthenogenesis in two *Eriotheca* (Malvaceae) species from Brazilian Cerrado. Perspectives in Plant Ecology, Evolution and Systematics 36: 1-12. <https://doi.org/10.1016/j.ppees.2018.11.001>

Neter J, Wasserman W & Kutner MH (1985) Applied linear statistical models: regression, analysis of variance and experimental designs. Richard D. Irwin, Illiniois. 1127p.

Oliveira PE, Gibbs PE, Barbosa AA & Talavera S (1992) Contrasting breeding systems in two *Eriotheca* (Bombacaceae) species of the Brazilian cerrados. Plant Systematics and Evolution 179: 207-219. <https://doi.org/10.1007/BF00937597>

Quarin CL (1999). Effect of pollen source and pollen ploidy on endosperm formation and seed set in pseudogamous apomictic *Paspalum notatum*. Sexual Plant Reproduction 11: 331-335. <https://doi.org/10.1007/s004970050160>

R Core Team (2020) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna: Austria. Available at <https://www.r-project.org/>. Access on 20 January 2020.

Rice A, Glick L, Abadi S, Einhorn M, Kopelman NM, Salman-Minkov A, Mayzel J, Chay O & Mayrose I (2015) The Chromosome Counts Database (CCDB) - a community resource of plant chromosome numbers. New Phytologist 206: 19-26. <https://doi.org/10.1111/nph.13191>

SAEG (1997) Fundação Arthur Bernardes. Versão 7.1. Sistema operacional MS-DOS e manual de codificação. CD. UFV, Viçosa.

Schatlowski N, Wolff P, Santos-Gonzalez J, Schoft V, Siretskiy A, Scott R, Tamaru H & Köhler C (2014) Hypomethylated pollen bypasses the interploidy hybridization barrier in *Arabidopsis*. Plant Cell 26: 3556-3568. <https://doi.org/10.1105/tpc.114.130120>

Soltis PS, Marchant DB, Van de Peer Y & Soltis DE (2015) Polyploidy and genome evolution in plants. Current opinion in genetics & development 35: 119-125. <https://doi.org/10.1016/j.gde.2015.11.003>

Vandenhout H, Ortiz R, Vuylsteke D, Swennen R & Bai KV (1995) Effect of ploidy on stomatal and other quantitative traits in plantain and banana hybrids. Euphytica 83: 117-122. <https://doi.org/10.1007/BF01678038>

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