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Genetic diversity in *Passiflora* L. species based on pollen and seed morphology and nuclear DNA content

Diversidade genética em espécies de *Passiflora* L. baseado em morfologia do pólen e semente e conteúdo do DNA nuclear

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ABSTRACT

Characterizing and predicting genetic diversity within the genus *Passiflora* is critical for promoting diversified strategies in genetic improvement programs. The present study aimed to characterize the morphology of pollen grains and seeds, and to infer the 2C DNA content using flow cytometry in species of the genus *Passiflora* with ornamental potential. For the morphological characterization of pollen grains and seeds, 11 and 12 descriptors were used, respectively. The data obtained were analyzed using ANOVA and grouped according to the Scott-Knott method. To study genetic diversity, the Mahalanobis distance matrix, and the UPGMA agglomerative method were used. For the morphological characterization of pollen and seeds, the results indicated significant differences across all descriptors. Four descriptors were particularly remarkably divergent: colpus width, equatorial axis, polar axis length, and the number of ornaments on the seeds. The species were grouped into three clusters in the dendrogram using the clustering method. 2C DNA content values demonstrated high interspecific variability, possibly due to chromosomal number and size. These results enabled the detection of genetic diversity, the significance of the biological trait values, and a better knowledge of interspecific gene interactions, all of which aid the selection of ornamental parent plants.

Index terms: Morphological characterization; flow cytometry; germoplasm; ornamental passion flower.

RESUMO

A caracterização e predição da diversidade genética do gênero *Passiflora* são importantes para promover a diversificação de estratégias em programas de melhoramento. O presente estudo objetivou realizar a caracterização morfológica de grãos de pólen e sementes, e inferir o conteúdo de DNA 2C por citometria de fluxo de espécies do gênero *Passiflora* com potencial ornamental. Para a caracterização morfológica de grãos de pólen e de sementes, foram avaliados 11 e 12 descritores, respectivamente. Os dados obtidos foram submetidos à ANOVA e ao agrupamento de Scott-Knott. Para o estudo de diversidade genética utilizou-se a matriz de Mahalanobis e o método aglomerativo UPGMA. Para a caracterização morfológica de pólen e sementes, os resultados indicaram a existência de diferença significativa ($P < 0,05$) para todos os descritores e quatro destes descritores se destacaram para a divergência existente: largura do colpo, eixo equatorial, comprimento total da vista polar e o número de ornamentações nas sementes. As espécies foram distribuídas em três grupos no dendograma pelo método de agrupamento. Os valores de conteúdo de DNA 2C apresentaram alta variabilidade interespecífica. Estes resultados possibilitaram reconhecer a diversidade genética e a importância do valor biológico das características, e melhor compreender as relações gênicas interespecíficas, podendo contribuir para seleção de genitores com potencial ornamental.

Termos para indexação: Caracterização morfológica; citometria de fluxo; germoplasma; passiflora ornamental.

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Introduction

The genus *Passiflora* L. is the largest in the family Passifloraceae A.L. de Jussieu ex Kunth, with over 500 species (Ulmer & MacDougal, 2004). It is divided into four subgenera: *Astrophea (DC.)* Mast., *Decaloba* (DC.) Rchb., *Deidamioides* (Harms) Killip, and *Passiflora* L., the largest of them in number of species (MacDougal & Feuillet, 2004). However, new species are being discovered (Bernacci & Souza, 2012).

The *Passiflora* genus is widely distributed in tropical and subtropical regions that receive high solar radiation (Vanderplank, 2000; Ulmer & MacDougal, 2004) and in vast tracts of humid and shaded tropical forests. Brazil is one of the primary centers of diversity for *Passiflora* L species. In addition to maintaining genetic biodiversity, characterization of *Passiflora* species is also essential at the morphological, agronomic, cytogenetic, and molecular levels. Thus, passion fruit species, a valuable genetic heritage, must be more extensively researched (Junqueira et al., 2008).

The corona, the primary feature of the Passifloraceae family, makes the *Passiflora* flowers unique and complex, with some having strong and bright colors and others having soft and striking colors (Abreu et al., 2009). Interspecific hybridization in *Passiflora* in recent years, both in Brazil and abroad, has primarily focused on ornamental purposes to develop varieties with distinctive floral characteristics (Abreu et al., 2009; Santos et al., 2012; Belo et al., 2018). In Brazil, efforts have been made to identify species with morphological and adaptive characteristics suitable for use as parent plants in interspecific hybrid development and potential ornamental usage (Santos et al., 2012; Belo et al., 2018). The cultivars '*P*. Alva,' '*P*. Priscilla,' '*P*. Aninha,' (Santos et al., 2012) '*P.* Gabriela,' and '*P.* Bella' (Belo et al., 2018) are examples of artificially created hybrids in Brazil. Furthermore, the United States and Europe have registered over 600 *Passiflora* hybrids for ornamental use (Ulmer & MacDougal, 2004; Passiflora Society International, 2021).

The use of morphological descriptors developed exclusively for ornamental *Passiflora* in Brazil (Brasil, 2008) standardizes quantitative and qualitative morphological parameters relating to the shape and size of the plant's leaves, flowers, fruits, and branches. Low genetic variability makes selecting plants for advancement and establishing superior genotypes challenging (Borém & Miranda, 2009). However, the genus *Passiflora* exhibits extensive intra- and interspecific genetic variability (Oliveira, Faleiro, & Junqueira, 2019) as well as significant phenotypic variation and plasticity, as seen in the morphologies of flowers, leaves, and other vegetative parts (Vanderplank, 2000).

Although the palyno-morphological features are of little taxonomic significance in some species (Ullah et al., 2022; Liao et al., 2021), the morphology of pollen grains in *Passiflora*, along with other characteristics, contributes to taxonomic studies at the subgeneric level, indicating a pattern of shapes and ornamentations inherent to each species (Araújo & Santos, 2004; Milward-De-Azevedo & Baumgratz, 2004). The variations in their openings, with diverse pollen wall structures and fusions, are species-specific (Spirlet, 1965). Studies on the role of this reproductive structure in characterizing and differentiating species have validated the significance of morphological analysis of *Passiflora* seeds (Pérez-Cortéz, Escala, & Tillett, 2005). The morphological proximity of ornamentation on pollen grains and seeds among *Passiflora* species suggests a genetic relationship, making selecting potential genes for genetic enhancement easier. However, the morphological and anatomical aspects of pollen grains, particularly in *Passiflora* seeds, have still not been studied.

The nuclear genome size can help with genetic and molecular characterization, taxonomy, systematics, and genetic improvement (Ferreira et al., 2020). Flow cytometry has emerged as the preferred approach for determining 2C DNA content at the pg and ploidy levels. The technique enhances species delineation, which is helpful for phylogenetic studies of a particular genus to infer plant relationships and evolutionary characteristics (Kron, Suda, & Husband, 2007). Flow cytometry is a well-established scientific tool. However, only about 10% of *Passiflora* species have had their 2C DNA content evaluated (Ferreira et al., 2020). Flow cytometry was used to analyze *Passiflora* species, and the size of the nuclear genome varied amongst them. The 2C DNA content values ranged from 0.52 pg in *P. sublanceolata* J. M. MacDougal ex *P. palmeri* Killip to 4.41 pg in *P. alata* Curtis (Yotoko et al., 2011) and up to 5.36 pg in *P. quadrangularis* L. (Souza et al., 2004). These analyses have contributed to genotype characterization (Souza et al., 2004) and evolutionary and taxonomic studies (Yotoko et al., 2011; Amorim et al., 2014; Ferreira et al., 2020).

The current study aims to analyze the morphology of pollen grains and seeds and estimate the 2C DNA content in *Passiflora* species with ornamental potential distributed across the subgenera *Passiflora* and *Decaloba*. The goal was to optimize parental selection in interspecific crosses.

Material and Methods

Plant material

Thirty-one species of the genus *Passiflora* with potential ornamental or agronomic value were evaluated and categorized into two subgenera, *Passiflora* and *Decaloba*. We conducted the 2C DNA content analysis on 28 species, numbered one through 28. Twelve species, numbered 20 to 31, were evaluated for pollen grain and seed morphology (Table 1). The number of species tested varied depending on the sample viability required for each analysis. Thus, the difference in species number reflects the availability and suitability of samples for each type of analysis, such as young leaves, flowers, and seeds. This study used species from the Passiflora Working Collection at Santa Cruz State University (CT-Passiflora/UESC) in Ilhéus, Bahia, Brazil (14º45'15 ''S, 39º13'59''W, 40 m a.s.l.). One species was collected from the state of Bahia (UESC-BA), while the others were obtained from other institutions (Table 1).

Morphological characterization of pollen and seed

Ten flowers of each species were preserved in FAA (Kraus & Arduin, 1997) and refrigerated. The samples were treated according to Bozzola and Russel (1991). They were dehydrated in an ethanol series from 70% to 100%, dried at the critical point, mounted on aluminum stubs with double-sided carbon or silver tape, and kept in a desiccator. Ten stubs were assembled per species, each containing samples from a different flower. Following complete dehydration, the samples were coated with 20 nm of gold and examined under a Zeiss 962 scanning electron microscope (SEM) at the Center for Biosciences

and Biotechnology (CBB), Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Campos dos Goytacazes, Rio de Janeiro, Brazil. Seed samples were processed similarly to pollen grains. Ten seeds from each species were analyzed for their morphological characteristics. The samples were evaluated using eleven descriptors for pollen grains and twelve for seeds (Table 2). The materials were measured using SEM at 5-15 kV and photomicrographed (Figure 1).

Table 1: Plant material, taxonomic position, chromosome number (2*n*) and donor Brazilian institutions of *Passiflora* species.

N°.	Species	Subgenus	2*n*	Donor Institutions
1	*P. misera* HBK	Decaloba	12 (Melo, Cervi, & Guerra, 2001)	UENF, RJ
2	*P. organensis* Gardner	Decaloba	12 (Sader et al., 2021)	IAC, SP
3	*P. coriacea* Juss.	Decaloba	12 (Melo et al., 2014)	IAC, SP
4	*P. ferruginea* Mast.	Decaloba	12 (Melo et al., 2014)	Embrapa Cerrados, DF
5	*P. miersii* Mart.	Decaloba	18 (Melo, Cervi, & Guerra, 2001)	UENF, RJ
6	P. sublanceolata	Passiflora	22 (Belo et al., 2018)	Embrapa Cerrados, DF
8	*P. hatschbachii* Cervi.	Passiflora	18 (Melo, Cervi, & Guerra, 2001)	Embrapa Cerrados, DF
9	P. malacophylla	Passiflora	18 (Souza et al., 2003)	UENF, RJ
10	*P. setacea* D.C.	Passiflora	18 (Melo et l., 2001)	UENF, RJ
11	*P. galbana* Mast.	Passiflora	18 (Storey, 1950)	UENF, RJ
12	*P. nitida* Kunth	Passiflora	18 (Melo et al., 2001)	UENF, RJ
13	*P. filamentosa* Cav.	Passiflora	18 (Beal, 1969)	Embrapa Cerrados, DF
14	*P. serratodigitata* L.	Passiflora	18 (Melo, Cervi, & Guerra, 2001)	UENF, RJ
15	*P. caerulea* Lin.	Passiflora	18 (Nakajima, 1931)	Embrapa Cerrados, DF
16	*P. coccinea* Aubl.	Passiflora	18 (Vieira, Barbosa, & Mayeda, 2004)	UENF, RJ
17	*P. amethystina* J.C. Mikan	Passiflora	12 (Barbosa & Vieira, 1997)	UENF, RJ
18	*P. maliformes* L.	Passiflora	18 (Storey, 1950)	UENF, RJ
19	*P. vitifolia* Kunth	Passiflora	18 (Melo, Cervi, & Guerra, 2001)	Embrapa Cerrados, DF
20	*P. capsularis* L.	Decaloba	12 (Amorim et al., 2014)	UFPR, PR
21	*P. morifolia* Mast	Decaloba	12 (Melo, Cervi, & Guerra, 2001)	Embrapa Cerrados, DF
22	*P. rubra* L	Decaloba	12 (Amorim et al. 2014)	UESC, BA
23	*P. pentagona* Mast.	Passiflora	24 (Melo, Cervi, & Guerra, 2001)	IAC, SP
24	*P. edmundoi* Sacco	Passiflora	18 (Melo, Cervi, & Guerra, 2001)	UENF, RJ
25	*P. alata* Curtis	Passiflora	18 (Souza et al., 2003)	UENF, RJ
26	*P. gibertii* N. E. Brown	Passiflora	18 (Souza & Pereira, 2011)	UNESP, SP
27	*P. cincinnata* Mast.	Passiflora	18 (Souza et al., 2003)	UENF, RJ
28	*P. foetida* L.	Passiflora	22 (Melo, Souza, & Silva, 2017)	UENF, RJ
29	*P. suberosa* L.	Decaloba	12 (Souza et al., 2003)	UENF, RJ
30	*P. edulis* Sims.	Passiflora	18 (Janak Ammal, 1945)	IAC, SP
31	*P. mucronata* Lam.	Passiflora	18 (Guerra, 1986)	UENF, RJ

EMBRAPA Cerrados, DF = Brazilian Agricultural Research Corporation From Cerrado, Federal District; IAC, SP = Agronomical Institute of Campinas, São Paulo; UENF, RJ = State University of Northern Rio de Janeiro, Rio de Janeiro; UESC, BA = State University of Santa Cruz, Bahia; UFPR, PR = Federal University of Paraná, Paraná; UNESP, SP = São Paulo State University, São Paulo.

Table 2: Descriptors used for the morphological characterization of pollen grains and seeds in *Passiflora* species.

Samples	Code	Characteristics[a,b,c]	Unit
Polen	PA	Polar axis	µm
	EA	Equatorial axis	µm
	P/E	Polar/equatorial axis ratio	-
	CW	Colpus width	µm
	Mes	Mesocolpus	µm
	PAL	Polar axis length polar	µm
	Apo	Apocolpus	µm
	Mur	Wall	µm
	Lu	Lumen	µm
	Me	Mesh	µm
	PAI	Polar Area Index	-
Seed	L	Length	mm
	PW	Proximal width	mm
	MW	Median width	mm
	DW	Distal width	mm
	HL	Hilum length	mm
	DO	Distance between ornamentations	mm
	OW	Ornamentation width	mm
	NO	Number of ornamentations	mm
	BW	Brim width	mm
	H/W	Height to median width ratio	mm
	W	Weight*	mg
	T	Thickness	mm

[a] Erdtman (1966), [b]Iversen and Troels-Smith (1950), [c]Barth and Melhem (1988). *Obtained using an analytical balance.

Analysis of nuclear DNA content

Flow cytometry analysis was performed on the first true leaves, with four replicates per taxon. The criteria used by Souza et al. (2004) were followed while selecting standards. Internal controls included *Zea mays* L. cv. 'Kukurice' CE-777 (2C DNA = 5.43 pg; Dolezel, Greilhuber, & Lucretti, 1998); *Solanum lycopersicum* L. (2C DNA = 1.96 pg; Dolezel, Greilhuber, & Lucretti, 1998); *Glycine max* (L.) Merrill (2C DNA = 2.5 pg; Dolezel, Greilhuber, & Lucretti, 1998), and *Passiflora mucronata* Lam. (2C DNA = 3.41 pg; Souza et al., 2004). We utilized the Cystain PI Absolute® kit (PARTEC) to create suspensions of intact nuclei. Approximately 17 mg of leaf tissue from each *Passiflora* species and 20 mg from the selected standard species were chopped at room temperature in a Petri dish containing the kit's extraction buffer. After passing 5.0 mL of the nuclear suspension through a 50 µm mesh nylon filter, we added 2.0 mL of staining solution containing 5.0 mg/mL of RNase and 1.5 mL of propidium iodide. The preparation was incubated at room temperature for at least 30 minutes in a light-shielded container. Following the manufacturer's instructions, the UENF Cytogenetics Laboratory evaluated nuclear suspensions using a Partec CAII flow cytometer (Partec GmbH, Münster, Germany). We adjusted the device's gain to position the peak representing the *Passiflora* nuclei in G_1 at channel 50. About 10,000 nuclei were analyzed for each sample. The fluorescence intensity of nuclei stained with propidium iodide was analyzed at a rate of 20-50 nuclei per second. The peaks, mean areas, and coefficients of variation were determined using DPAC software (Partec®), and the 2C DNA content was calculated according to Dolezel and Gohde (1995) (Equation 1):

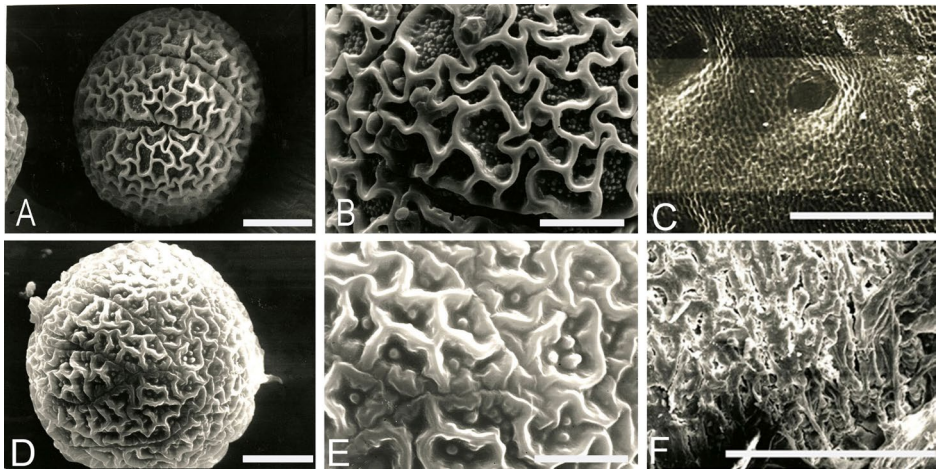

Figure 1: Pollen and seeds characteristics in species of the genus *Passiflora* as observed under a scanning electron microscope. Pollen grain: (A and B) *P. mucronata* and (D and F) *P. capsularis*. Seeds: (C) *P. edulis* and (F) *P. mucronata*. Bar in A and D = 20 µm. Bar in B and E = 5µm. Bar at C and F = 200 µm.

$$2C\ DNA\ content = \frac{\text{Mean } G_0/G_1 \text{ peak DNA content } (2C) \text{ of } \textit{Passiflora} \text{ species x standard plant DNA content } (pg)}{\text{Mean } G_0/G_1 \text{peak of the standard plant}} \quad (1)$$

Statistical analysis

The data acquired from the characterization of pollen grains and seeds were analyzed using a completely randomized design with ten replicates, with ANOVA and the Scott-Knott test for multiple means comparison at a 5% significance level. Species were grouped using the UPGMA hierarchical clustering algorithm, and the average Euclidean dissimilarity matrix was used to predict genetic diversity. We used the GENES software (Cruz, 2013) to analyze pollen and seeds. The 2C DNA content values of the tested species were assessed using analysis of variance in SISVAR software and categorized using the Scott-Knott test for multiple means comparison at a 5% significance level. The histograms were edited with CorelDraw®.

Results and Discussion

Morphological characterization of pollen and seeds

ANOVA revealed significant differences ($P < 0.05$) across species for all evaluated descriptors. The distribution of the mean values of the morphological descriptors from the Scott-Knott test allowed us to compare similar characteristics between species (Tables 3 and 4).

The genetic distance between taxa, as observed from the dissimilarity matrix (Table 5), revealed that the greatest genetic distance occurred between *P. cincinnata* Mast. and *P. capsularis* L., while the smallest distance was found between *P. rubra* L. and *P. capsularis*. In the analysis of the relative contributions of pollen and seed characteristics (Table 6), the characteristics CW (26.23%), NO (24.04%), EA (19.22%), and PAL (14.54%) accounted for 84.04% of the genetic variability observed among the sample group (Table 6). Seven descriptors (Mur, OW, BW, H/W, HL, DO, and PAI) each had a relative contribution of less than 0.00%. The dendrogram, generated using Euclidean distance, identified three groups of species: 'Group I' consisting solely of *P. pentagona* Mast.; 'Group II' comprised of *P. cincinnata*, *P. edulis* Sims*, P. alata, P. gibertii* N. E. Brown*, P. morifolia* Mast.*, P. edmundoi* Sacco, *P. foetida* L., and *P. mucronate*, while 'Group III' included *P. suberosa* L.*, P. capsularis*, and *P. rubra*.

In the mean test of pollen grain morphology, two species from the subgenus *Decaloba* (*P. rubra* and *P. morifolia*) had the highest mean PA values, as did six species from the subgenus *Passiflora* (*P. edmundoi, P. edulis, P. alata, P. pentagona, P. gibertii* and *P. mucronata*) (Table 3). PA ranked fifth descriptor (4.92%), in descending order for the perc entage contribution of genetic divergence (Table 6).

The species *P. mucronata, P. gibertii, P. alata, P. edulis, P. edmundoi* and *P. morifolia* did not differ statistically from each other in the EA characteristic, the third descriptor that most contributed to genetic divergence. In addition, the similarity of mean values for the CW characteristic in *P. alata, P. foetida, P. edulis*, and *P. edmundoi* contributed most to genetic divergence (Table 6), supported the formation of 'Group II' (Figure 2). The

Table 3: Scott-Knott multiple means comparison test on pollen grain morphology characteristics of 12 *Passiflora* species.

Species	PA	EA	P/E	CW	Mes	PAL	Apo	Mur	Lu	Me	PAI
P. mucronata	54.01 c	57.10 b	0.84 e	29.47 c	14.81 a	55.45 b	12.99 c	0.97 b	5.88 c	7.46 c	0.21 c
P. gibertii	57.16 c	57.60 b	0.99 d	28.79 c	9.97 c	55.48 b	16.23 b	0.95 b	7.02 b	8.50 b	0.29 a
P. pentagona	54.29 c	28.76 d	1.88 a	44.67 a	7.25 d	31.60 d	6.20 f	0.53 d	7.71 f	2.26 f	0.19 c
P. alata	55.66 c	58.22 b	0.95 d	35.34 b	9.98 c	56.37 b	12.88 c	0.95 b	6.88 b	8.89 b	0.23 b
P. cincinnata	67.64 a	68.00 a	0.99 d	44.76 a	14.66 a	67.12 a	22.03 a	1.15 a	9.80 a	11.53 a	0.33 a
P. foetida	61.70 b	65.09 a	0.94 d	36.86 b	13.13 b	63.57 a	14.74 b	0.73 c	8.87 a	10.77 a	0.23 b
P. edulis	59.13 c	61.23 b	0.96 d	34.18 b	10.07 c	62.09 a	13.96 c	0.62 e	9.08 a	10.45 a	0.25 b
P. edmundoi	59.84 c	60.19 b	0.99 d	33.23 b	8.46 d	61.11 a	12.71 c	0.66 c	7.39 b	8.51 b	0.20 c
P. suberosa	69.73 a	43.24 c	1.61 b	7.83 d	9.96 c	42.12 c	8.26 e	0.61 c	2.09 f	2.87 f	0.19 c
P. rubra	54.63 c	35.28 d	1.41 c	6.90 d	8.81 d	43.30 c	10.18 d	0.56 d	3.59 e	4.27 e	0.23 b
P. capsularis	46.86 d	44.68 c	1.05 d	9.24 d	7.25 d	45.01 c	8.86 e	0.44 d	4.99 d	5.72 d	0.19 c
P. morifolia	52.37 c	51.56 b	1.01 d	8.79 d	11.62 b	51.45 b	10.58 d	0.64 c	6.02 c	6.97 c	0.20 c

Polar Axis (PA), Equatorial Axis (EA), Polar/Equatorial Axis Ratio (P/E), Colpus Width (CW), Mesocolpus (Mes), polar axis length (PAL), Apocolpus (Apo), Muro (Mur), Lumen (Lu), Mesh (Me), and Polar Area Index (PAI). Means followed by the same letter indicate no statistically different according to the Scott-Knott test at the 5% significance level.

similarity of the means for the PAL characteristic among *P. cincinnata*, *P. foetida*, *P. edulis*, *P. edmundoi*, *P. mucronata*, *P. gibertii*, *P. alata*, and *P. morifolia*, which is the fourth descriptor contributing the most to genetic divergence (Table 6), also contributed to the formation of 'Group II' (Figure 2).

The species *P. capsularis* and *P. suberosa* had the lowest EA values, with no significant differences between them. *P. capsularis*, *P. suberosa*, and *P. rubra*, all from the subgenus *Decaloba*, showed no significant differences among themselves in the characteristics of CW and PAL pollen grains. However, they differed from all species within the *Passiflora* subgenus. It is worth noting that for the species *P. morifolia*, which also belongs to the subgenus *Decaloba*, the descriptors EA and PAL were insufficient to classify this species at the subgeneric level. The remaining descriptors (P/E, Mur, and PAI), ranked in decreasing order of the proportional contribution of characteristics to pollen grains (Table 6), showed minor contributions with values less than 0.02% and did not affect the distribution of groups in the dendrogram (Figure 2).

Table 4: The Scott-Knott multiple means comparison test was conducted on the seed morphology characteristics of 12 *Passiflora* species.

Species	L	PW	MW	DW	HL	DO	WO	NO	BW	H/W	W	T
P. mucronata	4.175 f	1.646 e	2.838 d	1.247 c	0.275 e	0.135 c	0.523 d	19.1 f	0.475 b	1.467 f	9.71 e	1.537 d
P. gibertii	5.648 c	1.244 f	3.402 c	1.251 c	0.42 d	0.153 b	0.368 e	54.0 a	0.00 f	1.655 d	17.74 c	1.890 b
P. pentagona	6.022 b	1,921 d	3.381 c	1.491 b	0.527 b	0.73 d	0.856 a	28.3 d	0.00 f	1.779 c	19.15 b	2.107 a
P. alata	6.113 b	3.199 a	3.772 b	1.686 a	0.680 a	0.145 b	0.714 b	22.7 e	0.317 c	1.622 d	20.86 a	1.939 b
P. cincinnata	5.379 d	2.113 c	3.430 c	1.795 a	0.235 f	0.148 b	0.620 c	31.0 c	0.572 a	1.566 e	20.89 a	2.110 a
P. foetida	4.595 e	1.849 d	2.257 e	1.58 b	0.217 f	0.122 c	0.474 d	23.7 e	0.238 d	2.036 a	10.59 e	1.427 e
P. edulis	6.475 a	2.847 b	4.255 a	1.764 a	0.668 a	0.149 b	0.189 f	40.9 b	0.45 b	1.526 e	19.46 b	1.741 c
P. edmundoi	5.298 d	0.936 g	2.845 d	0.780 f	0.363 d	0.127 e	0.643 c	20.7 f	0.00 f	1.859 b	11.94 d	1.968 b
P. suberosa	3.402 g	1.522 e	2.281 e	0.941 e	0.458 c	0.156 b	0.453 d	14.5 g	0.157 e	1.493 f	6.160 f	1.572 d
P. rubra	3.078 h	1.145 f	1.750 g	1.058 d	0.283 e	0.121 e	0.376 e	6.0 i	0.159 e	1.762 c	2.670 g	1.248 f
P. capsularis	3.257 g	1.192 f	1.891 f	0.956 e	0.278 e	0.264 a	0.215 f	6.70 i	0.145 e	1.714 c	3.650 g	1.382 e
P. morifolia	4.671 e	1.209 f	3.465 c	1.123 d	0.292 e	0.136 c	0.889 a	11.8 h	0.00 f	1.343 g	12.29 d	1.962 b

Length (L), Proximal Width (PW), Median Width (MW), Distal Width (DW), Hilum Length (HL), Distance between Ornamentations (DO), Width of Ornaments (WO), Number of Ornaments (NO), Brim Width (BW), Ratio of Height to Median Width (H/W), Weight (W), and Thickness (T). Means followed by the same letter indicate no statistically different according to the Scott-Knott test at the 5% significance level.

Table 5: Dissimilarity matrix based on morphological descriptors for pollen and seed grains of 12 *Passiflora* species, calculated using the Weighted Euclidean distance.

Species	31	26	23	25	27	28	30	24	29	22	20	21
31	-	0.354193	0.596197	0.381055	0.343682	0.268698	0.413935	0.361084	0.434043	0.442665	0.468985	0.359658
26	-	-	0.493192	0.337825	0.385118	0.346807	0.332664	0.286816	0.47471	0.491511	0.492385	0.360969
23	-	-	-	0.455631	0.677496	0.576125	0.559478	0.458821	0.452504	0.512454	0.576883	0.453388
25	-	-	-	-	0.389125	0.394989	0.242179	0.38661	0.522056	0.573805	0.57112	0.399911
27	-	-	-	-	-	0.387605	0.390795	0.490097	0.658739	0.68254	0.701837	0.532624
28	-	-	-	-	-	-	0.400205	0.304419	0.479722	0.433976	0.450554	0.413075
30	-	-	-	-	-	-	-	0.442222	0.566071	0.605616	0.587558	0.486424
24	-	-	-	-	-	-	-	-	0.402743	0.413481	0.404962	0.279798
29	-	-	-	-	-	-	-	-	-	0.240737	0.333607	0.359438
22	-	-	-	-	-	-	-	-	-	-	0.226645	0.384675
20	-	-	-	-	-	-	-	-	-	-	-	0.389405
21	-	-	-	-	-	-	-	-	-	-	-	-

(31) *P. mucronata,* (26) *P. gibertii,* (23) *P. pentagona,* (25) *P. alata,* (27) *P. cincinnata,* (28) *P. foetida,* (30) *P. edulis,* (24) *P. edmundoi,* (29) *P. suberosa* (22) *P. rubra.* (20) *P. capsularis* and (21) *P. morifolia*.

Table 6: Relative contributions of characteristics related to pollen grain morphology and seed traits in 12 *Passiflora* species (*P. mucronata, P. gibertii, P. pentagona, P. alata, P. cincinnata, P. foetida, P. edulis, P. edmundoi, P. suberosa, P. rubra. P. capsularis* and *P. morifolia*).

Variável	%	(%) acumulada
CW*	26.2359	26.2359
NO**	24.0407	50.2766
EA*	19.2271	69.5037
PAL*	14.5407	84.0444
W**	5.4351	89.4795
PA*	4.9188	94.398
Apo*	2.2146	96.613
Me*	1.1514	97.764
Mes*	1.0097	98.77
Lu*	0.8562	99.630
L**	0.1686	99.799
MW**	0.0759	99.875
PW**	0.0607	99.935
DW**	0.0150	99.950
P/E*	0.0122	99.963
T**	0.0109	99.973
Mur*	0.0067	99.980
OW**	0.0063	99.986
BW**	0.0051	99.992
H/W**	0.0045	99.996
HL**	0.0032	99.999
DO**	0.0002	99.999
PAI*	0.0002	100.000

* Descriptor for pollen grains and ** for seeds. Polar axis (PA), Equatorial axis (EA), Polar/equatorial axis ratio (P/E), Colpus width (CW), Mesocolpus (Mes), Polar Axis Length (PAL), Apocolpus (Apo), Wall (Mur), Lumen (Lu), Mesh (Me), Polar Area Index (PAI), Length (L), Proximal width (PW), Median width (MW), Distal width (DW), Hilum length (HL), Distance between ornamentations (DO), Ornamentation width (OW), Number of ornamentations (NO), Brim width (BW), Height to median width ratio (H/W), Weight (W), and Thickness (T).

The most variable trait in the study of seed morphology was the number of ornamentations (NO), which varied from 54.0 in P. *gibertii* to 6.0 and 6.7 in *P. rubra* and *P. capsularis*. Compared to species from the subgenus *Passiflora*, all species in the subgenus *Decaloba* exhibited the lowest mean NO values (Table 4). With a 5.4% contribution, W was the second most significant characteristic (Table 6). The other descriptors (L, MW, PW, DW, T, OW, BW, H/W, HL, and DO) all contributed less than 0.2% to species divergence and did not affect group formation (Figure 2).

P. pentagona differed from all the other species in terms of the pollen grain descriptors P/E (0.012%), PAL (14.54%), and Apo (2.21%), as well as the seed descriptors NO (24.04%) and HL (0.0032%) (Tables 3 and 4), resulting in 40.80% genetic divergence and the formation of Group I (Figure 2).

P. cincinnata had a different mean value for the descriptor NO than the other species, and it, along with *P. pentagona*, exhibited the highest values for the descriptors CW and T. This most likely contributed to the formation of the isolated subgroup within Group II (Figure 2). The species *P. capsularis*, *P. suberosa*, and *P. rubra* showed no significant differences. The characteristics CW and PAL accounted for 40.77% of the dissimilarity between the species (Table 5), possibly contributing to the formation of Group III. The CW, PAL, and NO characteristics accounted for 64.8% of the observed divergence. *P. capsularis* and *P. rubra* had similar NO variable averages, which differed from other species. However, *P. suberosa* had a different mean than all the other species. Thus, the NO characteristic resulted in the splitting of Group III into two subgroups (Figure 2).

The characterization of pollen grain morphology generates biologically valuable data that can be used in various scientific domains, including taxonomy and phylogeny in *Passiflora* (Mezzonato-Pires et al., 2015). ANOVA analysis of the morphological characteristics of *Passiflora* pollen grains and seeds indicates high interspecific genetic diversity and specificity in pollen grain ornamentation (Spirlet, 1965; Araújo, Silva, & Queiroz, 2008). *Passiflora* seed study often focuses on physiology and ontogeny rather than seed coat morphology. However, some studies employ these traits to assist with botanical identification of species, owing to their high interspecific diversity and low phenotypic plasticity (Perez-Cortéz et al., 2005). Morphological characterization, which primarily employs descriptors that are minimally influenced by the environment and demonstrate interspecific divergence, improves our understanding of gene expressions and genetic relationships between species. This information is crucial for selecting potential species in genetic improvement programs (Abreu et al., 2009).

The genetic dissimilarity identified using various descriptors established three major groups in the dendrogram (groups I, II, and III) to better understand the interspecific diversity of *Passiflora* pollen and seed characteristics in this study. Group I contained only one species, *P. pentagona*, due to the differentiation of the descriptors that contributed the most to species divergence (CW, NO, and PAL). However, the highest genetic distance was observed between *P. cincinnata* and *P. capsularis*, followed by that between *P. pentagona* and *P. cincinnata*. *P. pentagona* has a different chromosome number ($2n = 24$) than other species, possibly related to their genetic distance. Most research on *Passiflora* divergence focuses on intraspecific differences, primarily in commercial passion fruit species (Rodrigues et al., 2017). It is essential to highlight that the high genetic diversity and the large number of species within the genus *Passiflora* make it difficult to find studies correlating the same species.

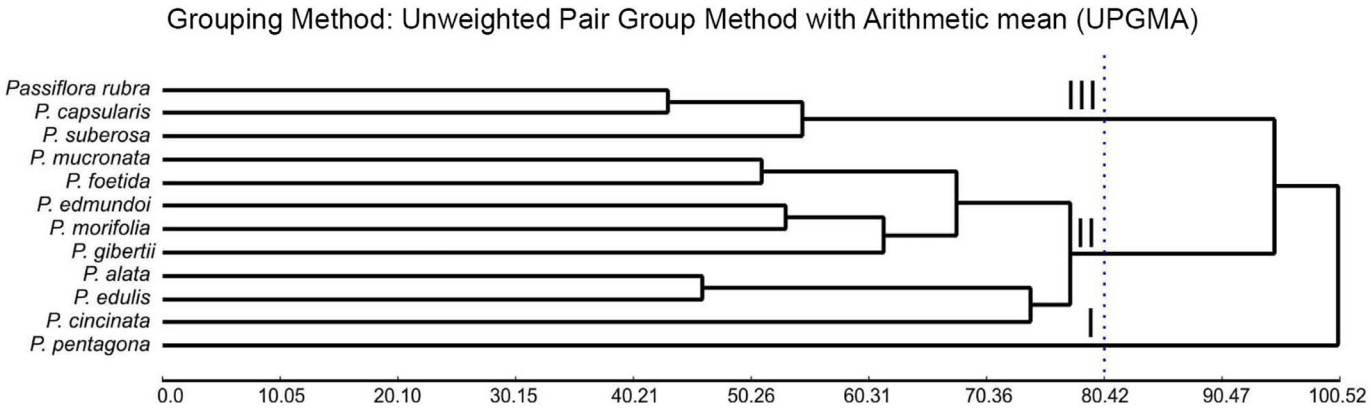

Figure 2: Dendrogram constructed using Euclidean distance and the UPGMA clustering method, based on variables related to the morphology of pollen grains and *Passiflora* seeds.

P. cincinnata was the most divergent species in group II of the dendrogram, forming a subgroup due to variations primarily in two descriptors (CW and NO) that contributed the most to genetic divergence. *P. cincinnata* divergence was also observed in studies based on the genetic dissimilarity of 32 morpho-agronomic descriptors, where it formed a distinct group with the species *P. edulis*, *P. morifolia*, *P. alata*, and *P. foetida* (Marostega, Paiva, & Luz, 2021). *P. cincinnata* demonstrated higher averages for floral descriptors than other groups (Paiva et al., 2014). The current investigation yielded similar results, as the species *P. cincinnata* had higher values for several pollen characteristics (PA, EA, CW, P/E, Apo, Um, Lu, Map, PAI) and seed characteristics (BW, W, T).

Other investigations also revealed genetic affinities between *P. edulis* and *P. alata*, primarily because of their larger average flower structure dimensions and longer androgynophores (Paiva et al., 2014). Our study also found that these species exhibited the highest values for morphological characteristics of pollen grains and seeds, second only to *P. cincinnata*. Crochemore, Molinari and Stenzel (2003) established the classification of *P. alata*, *P. gibertii*, and *P. foetida* into a single group using RAPD molecular markers.

P. morifolia, which belongs to the subgenus *Decaloba*, was the sole species in this study assigned to a different subgenus. Although not included in the same subgenus as *P. suberosa*, the genetic distance between these two was the second smallest. Comparatively speaking, *P. edmundoi* is the species most closely related to *P. morifolia*. Despite being in different groups, these species have not become genetically distant. Previous investigations have also demonstrated that species from different subgenera can be grouped together (Crochemore, Molinari, & Stenzel, 2003). Since the studies do not focus on taxonomy and phylogeny, failing to group the subgenera does not undermine the significance of the results. Understanding the species' morphological characteristics and the interspecific distances determined by these variables is essential.

Additional descriptors might be utilized for phylogenetic or taxonomic studies. Pollen traits such as reticulum pattern (lumen diameter and presence or absence of bacula) and exine thickness are considered fundamental evaluation criteria (Presting, 1964). Pollen types, characterized by variations in operculum (elliptical and pontoperculum opercula) and reticulum lumen sizes, are helpful for taxonomic differentiation (Araújo & Santos, 2004). Genetic diversity studies can also benefit from the pollen grain's apertural pattern and wall properties, including the presence or absence of bacula in the lumen (Soares et al., 2018).

In addition, based on the morphological and molecular characterization of species within the genus *Passiflora*, three species groups were observed for agronomic descriptors. The results of the current study align with the classification of *P. morifolia* among species of the subgenus *Passiflora* (*P. palmeri* Rose*, P. morifolia*, and *P. foetida*). This study also supports the findings of Viana et al. (2010), who identified *P. suberosa* as a distinct *Passiflora* species during cluster analysis using morphological and molecular characteristics. However, the morphological characterization of *P. morifolia* and *P. suberosa* using floral, leaf, and fruit agronomic characteristics revealed that they were genetically close (Viana et al., 2010).

The EA and PAL descriptors for pollen grains were mainly responsible for grouping *P. morifolia* with species from different subgenera. A palynotaxonomic study of species within the subgenus *Decaloba* revealed that only *P. morifolia* had a longer endoaperture in pollen grains of the colporate type (16.0 µm), in contrast to most species that had a value of 10 µm (Milward-De-Azevedo & Baumgratz, 2004). From this perspective, the pollen grains of *P. morifolia* are similar to those of the species in the subgenus *Passiflora*.

The lower genetic distance between the species *P. rubra* and *P. capsularis* in this study is consistent with the findings of Amorim et al. (2011), who showed that these species are genetically quite close. The genetic distances between species can suggest potential parent species for producing interspecific hybrids with high heterosis, resulting in increased hybrid vigor due to genetic divergences (Borém & Miranda, 2009).

In palynological studies, the type of opening is a critical morphological characteristic (Presting, 1964; Spirlet, 1965; Souza, Martins, & Pereira, 2015). Morphological characteristics of seeds, such as size, the number of depressions (foveas) on the surface, and apex and margin features, enable species differentiation. This gives the seed surface of *Passiflora* high diagnostic value (Pérez-Cortéz et al., 2005). Studies of inter- and intraspecific variability in *Passiflora* help to identify and conserve the genus' biodiversity and to identify pairs of divergent individuals, allowing for maximum exploration of existing variability (Oliveira et al., 2018).

The pollen grains and seeds morphometry in *Passiflora* has received little attention, but this study found that these parameters effectively discriminate the existing dissimilarities within the genus. Based on the affinities of the analyzed pollen and seed characteristics, the three groups identified in the dendrogram indicate that the grouped species belong to the same "gene pool." This allows for the possibility of performing convergent crosses to achieve reproductive viability.

All species in the subgenus *Decaloba* analyzed had the same number of chromosomes. However, *P. morifolia* has a wider genetic distance than the others, making it a potential candidate for enhancing heterosis and hybrid vigor in crosses within this subgenus. The most divergent species in the subgenus *Passiflora*, excluding *P. foetida* and *P. pentagona* due to their distinct chromosome numbers, were *P. cincinnata* and *P. edmundoi*, followed by the distance between *P. edulis* and *P. edmundoi*. Therefore, crosses between these species could be beneficial due to their chromosomal compatibility and higher genetic distance. Araújo, Silva and Queiroz (2008) found compatibility between *P. cincinnata* and *P. edulis* in crosses. *P. cincinnata* is gaining popularity in Brazil's ornamental plant industry, particularly since the introduction of the BRS Sertão cultivar (Braga et al., 2016).

2C DNA content

Flow cytometry of isolated nuclei generated histograms depicting the relative 2C DNA content, with two dominant peaks corresponding to the G_0/G_1 nuclei of *Passiflora* and the reference species. Figures 3 and 4 illustrate a selection of the histograms obtained during this study. The ANOVA analysis of the average 2C DNA content of *Passiflora* species yielded highly significant results ($P < 0.01$), with a coefficient of variation of 3.73% and an average 2C DNA value of 2.74 pg.

The Scott-Knott test was used to assess the means of 2C DNA content in pg (Table 7), and differences in genome size allowed 13 statistically distinct mean classes to be identified (Table 3). The 2C DNA content values of all analyzed species varied by up to 87%, with the lowest being 0.62 pg (*P. rubra* and *P. misera* HBK) and the highest being 4.99 pg (*P. alata*). However, the subgenus *Decaloba* had a lower average 2C DNA content (1.01 pg) than the subgenus *Passiflora* (3.23 pg). For species in the subgenus *Decaloba*, there was a discrepancy of up to 69.7% between the lowest 2C DNA content, 0.62 pg (*P. rubra* and *P. misera*), and the highest, 2.05 pg (*P. ferruginea* Mast.). In the case of species within the subgenus *Passiflora*, this variance was up to 81.4%, ranging from 0.93 pg (*P. sublanceolata*) to 4.99 pg (*P. alata*).

The 21 species of the *Passiflora* subgenus were classified into nine groups using the Scott-Knott test, with *P. sublanceolata* being the sole species in its group with the lowest 2C DNA content (0.93 pg). The species of the *Decaloba* subgenus were divided into four groups (Table 7). The 2C DNA content within species and individuals is considered constant (Schifino-Wittmann, 2001). The genus *Passiflora* generally shows stable DNA content, measured in picograms, throughout all taxons, with no intraspecific variations observed thus far (Souza et al., 2004). In this study, the genome size observed in *Passiflora* species had a low coefficient of variation for intraspecific repeats, demonstrating the stability of this biological characteristic within each taxon and the reliability of the flow cytometry results.

Interspecific variation in 2C DNA content was high (87%), and the few studies on passion fruit genome sizes found significant interspecific variation (Souza et al., 2004; Yotoko et al., 2011). According to Greilhuber (1998), these differences in genome size precede significant phenotypic differentiations, like the growth of plant organs.

The subgenus *Decaloba* had a relatively small average genome size (2C DNA value ≤ 2.8 pg), and the seven species investigated were divided into four groups. Species of class "m" can be used as parents in crossbreeding, indicating that their interspecific hybrids may be reproductively viable. This conclusion is based on the equal proportion of DNA content and chromosome number, implying better chromosomal homology than other species with statistically differing DNA content (Baack, Whitney, & Riesebertg, 2005).

P. ferruginea has a small 2C DNA content (2.8 pg ≤ 2C value ≤ 7.0 pg), similar to other species in the subgenus *Passiflora*. The subgenus *Decaloba* species *P. cervii* Milward-de-Azevedo, *P. capsularis*, and *P. rubra* had different 2C DNA contents. Morphological characteristics such as the leaves, flower crown, and pollen shape could help distinguish these species. In *P. cervii*, the pollen is spherical but flattened at the poles, but in the other species, it is spheroidal and elongated (Milward-De-Azevedo, 2008).

Within the *Passiflora* genus, DNA content and genome size correlate with flower size (Yotoko et al., 2011). Species of the subgenus *Decaloba* tended to have smaller flowers than subgenus *Passiflora* (Souza et al., 2004; Yokoto et al., 2011). However, statistical analyses showed no substantial difference among the *Decaloba* subgenus species. Given their shared phylogenetic history, it is vital to examine the relationship between genome size and flower diameter in *Passiflora* (Yokoto et al., 2011).

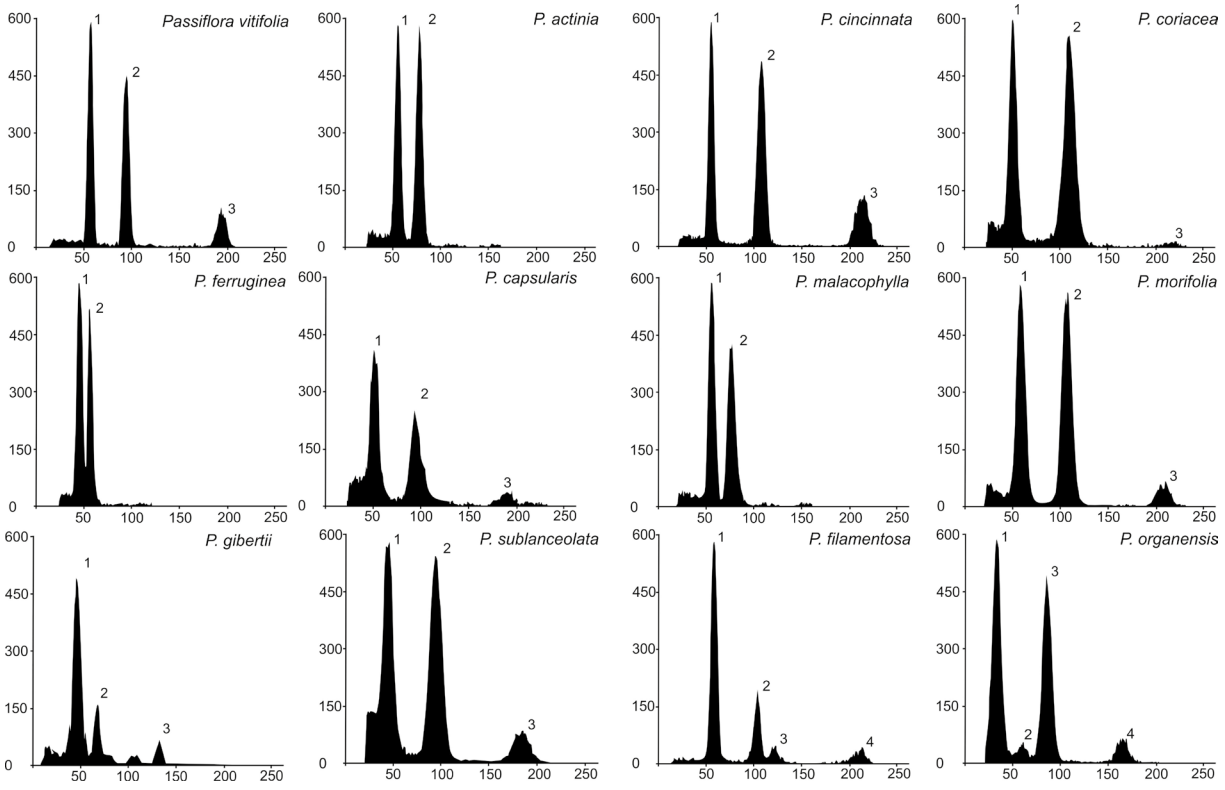

Figure 3: Histograms of 2C DNA content in species of the genus *Passiflora.*

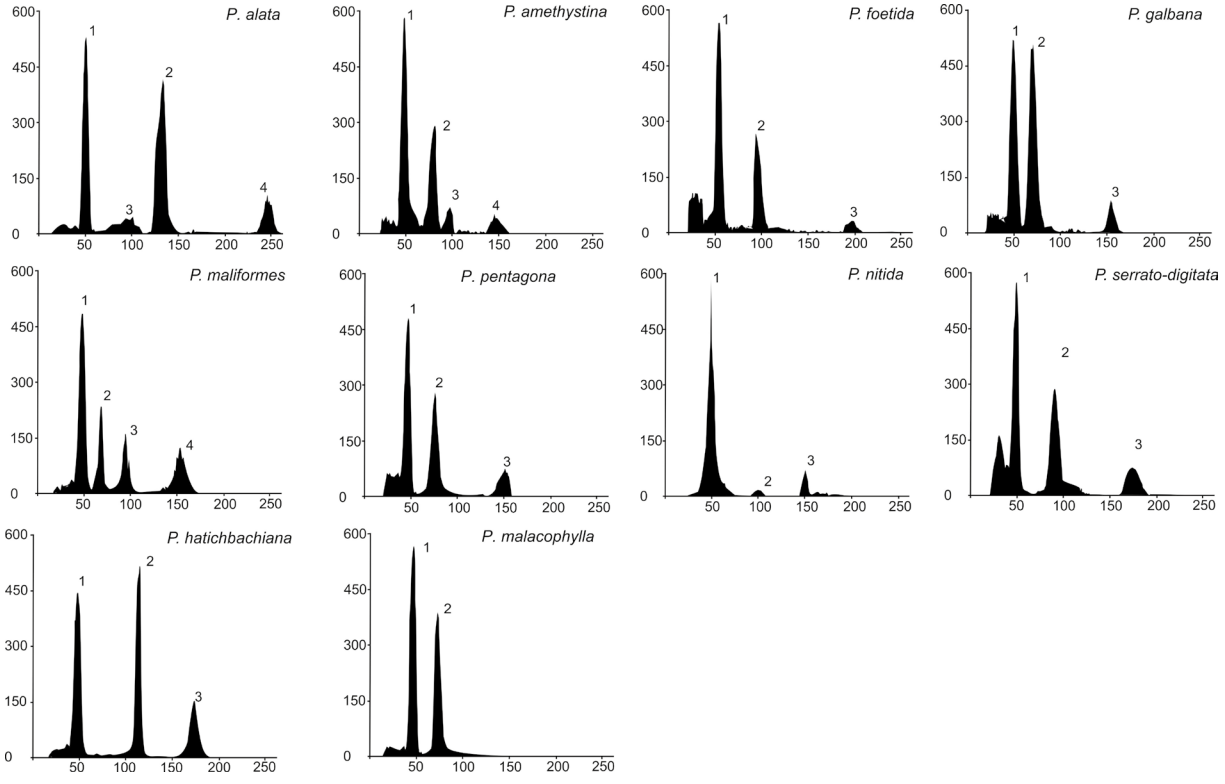

Figure 4: Histograms of 2C DNA content in species of the genus *Passiflora.*

It was observed nine statistical groupings among the 21 species evaluated in the subgenus *Passiflora*. *P. sublanceolata* is the most distinct species in the subgenus *Passiflora*, having the lowest 2C DNA content (0.93 pg) and forming a separate group. There were no other studies on the genome size for this species. However, some species from the subgenus *Passiflora* reportedly have an even lower 2C DNA content, such as *P. palmeri* (0.52 pg) (Yokoto et al., 2011; Ferreira et al., 2020). The species *P. foetida* and *P. sublanceolata* have the same chromosome number, $2n = 20$ or 22 (Belo et al., 2018; Silva & Souza, 2020) and have been utilized as parents in interspecific hybrid progenies with ornamental potential. Their backcrossing produced progeny with chromosome number variations, including triploidy in hybrids (Melo et al., 2017).

Crosses between the species *P. caerulea* L., *P. racemosa* Brot., *P. alata*, *P. amethystina* J. C. Mikan, *P. antioquensis* H. Karst, *P. cincinnata*, *P. gibertii, P. incarnata* L., among others, have been reported to produce potentially ornamental hybrids (Ulmer & MacDougal, 2004; Soares et al., 2020). Despite forming separate groups, interspecific hybridization is possible between various *Passiflora* species, including *P. coccinea* Aubl. and *P. actinia* Hook., *P. galbana* Mast. and *P. actinia*; and *P. coccinea* and *P. setacea* DC. (Junqueira et al., 2008; Santos et al., 2010).

For decades, *Passiflora* researchers have been aware of the distant relationship between the subgenera *Decaloba* ($n = 6$) and *Passiflora* ($n = 9$), as well as the dichotomy between these groupings (Hansen et al., 2006; Silva & Souza, 2020). Cytogenetic data on passion fruit species reveals four distinct chromosome counts across the four subgenera of *Passiflora*: $x = 6$ in *Decaloba*, $x = 9$ and $x = 10$ or 11 in *Passiflora*, $x = 10$ in *Astrophea*, and $x = 12$ in *Deidamioides* (Melo, Cervi, & Guerra, 2001). The basic chromosome number for the entire species cannot be determined precisely, although the most accurate estimates are $x = 6$ or $x = 12$ (Melo, Cervi, & Guerra, 2001; Hansen et al., 2006). Most *Passiflora* species are diploid, having $2n = 12$, 18, or 20 chromosomes (Melo & Guerra, 2003). *P. edulis*, the most commercially important species, is diploid ($2n = 18$), as are most species, favoring genetic improvement to produce interspecific hybrids (Yotoko et al., 2011; Amorim et al., 2014).

Some species groupings show a positive correlation between DNA content, pollen size, and ploidy level. Pollen size is associated with genome size and ploidy levels in sweet potato (*Ipomoea batatas*, Convolvulaceae) and other closely related species, such as *I. trifida* (Srisuwan et al., 2019). However, the chromosome number does not always correlate with the quantity of nuclear DNA and considerable amounts of nuclear DNA can be found in organisms with larger and smaller chromosomal counts (Hanson et al., 2005). Variations in the DNA content within the same plant species have been linked in the literature to environmental change, such as differences in high levels of species dispersion (Basak et al., 2018).

The ploidy level of all related *Passiflora* species is unknown, however, given that most species are diploid ($2n = 12$ or 18), chromosomal numbers $2n = 14$, 20, 22, 24, 36, 72, and 84 (Melo, Cervi, & Guerra, 2001; Melo & Guerra, 2003; Souza et al., 2008) include dysploidies, aneuploidies, and polyploidies. Karyotypic research on the genus *Passiflora* has already revealed four possible basic chromosomal numbers for the genus, $x = 6$, $x = 9$, $x = 10$, and $x = 12$; the three last numbers are considered secondary basic numbers (Melo & Guerra, 2003). Polyploid species in *Passiflora*, such as *P. suberosa* ($2n = 4x = 24$ and 1.84 pg) and *P. quadrangularis* ($2n = 2x = 18$ and 5.36 pg), can have significantly lower DNA content than diploid species (Souza et al., 2004). The discrepancy between DNA content and chromosomal number can be explained by the fact that some species' genomes contain duplicated regions or genes and a large amount of highly repetitive DNA (Lysák & Schubert, 2013). The polar axis of *P. suberosa*, a tetraploid ($2n = 4x = 24$ and 1.84 pg), was 69.73 µm, but that of *P. cincinnata*, a diploid ($2n = 2x = 18$ and 2.78 pg), was 67.64 µm. These findings suggest no positive correlation exists between DNA content, chromosome number, and ploidy level.

The genome size associated with morphological, cytogenetic, and molecular data improves our understanding of the relationships between species. It also assists in selecting potential parents for specific traits in a genetic improvement process (Silva et al., 2014). Similarities in cytogenetic and morphological characteristics can indicate compatibility between species, leading to the grouping of related species. For hybridization to succeed, the species involved must have chromosomal homology (Silva et al., 2014; Silva & Souza, 2020).

Conclusions

The genetic dissimilarities estimated from pollen grain and seed characteristics revealed a high degree of genetic variability in the studied species, which was adequate to predict interspecific genetic divergence. Using flow cytometry to determine DNA content accurately classifies species into distinct taxonomic categories and indicates high interspecific genetic diversity. The analyzed characteristics can aid in selecting potential parents for genetic improvement programs.

Author Contribution

Conceptual idea: Souza, M.M.; Methodology design: Souza, M.M.; Melo, C.A.F; Pereira, T.N.S; Data collection: Souza, M.M.; Melo, C.A.F; Rodrigues. P.S.; Melo, C.A.F.; Data analysis and interpretation: Rolim, R.B.; Melo, C.A.F, Freitas, J.C.O.; Melo, C.A.F.; and Writing and editing: Rolim, R.B.; Melo, C.A.F, Freitas, J.C.O.; Melo, C.A.F.; Souza, M.M.; Pereira, T.N.S.

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