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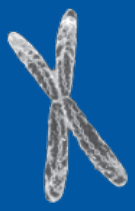

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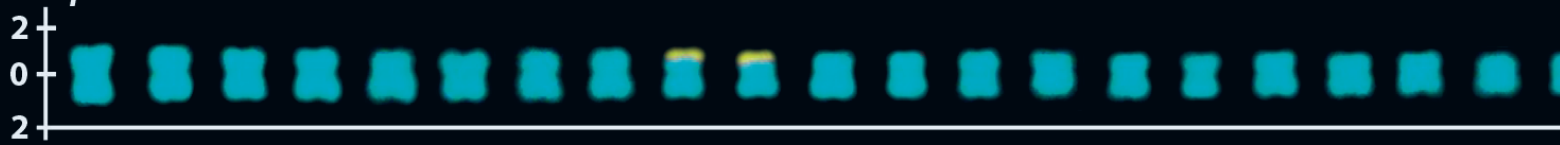
Opuntia cochenillifera

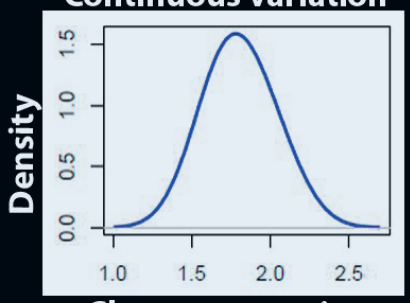


Symmetric karyotype

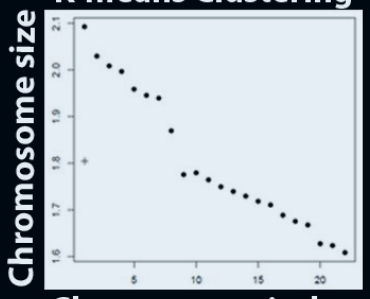
Unimodal

Classification of Stebbins: 1A
A1 = 0,08
A2 = 0,09

Continuous variation



K-means Clustering



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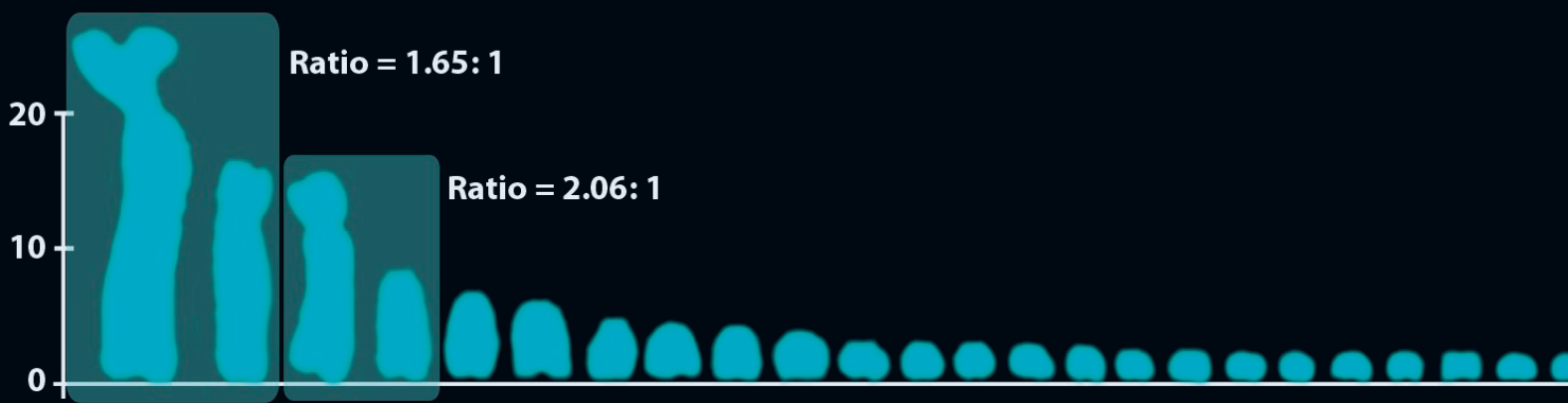

Asymmetric karyotype

Trimodal

Classification of Stebbins: 4C
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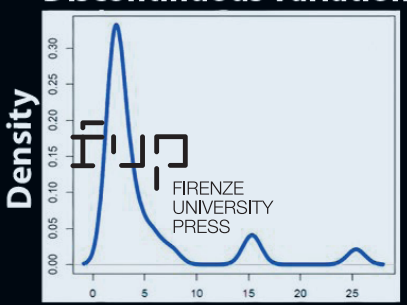
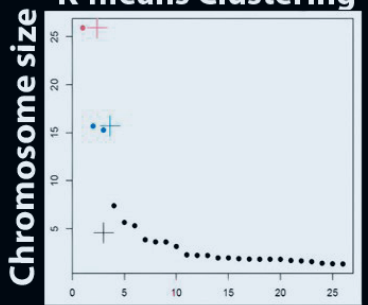
A2 = 1

Discontinuous variation



K-means Clustering



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Cover: figure from the article inside by Praween Supanuam et al. The genome of the southern short-horned tree dragon *Acanthosaura meridiona* Trivalairat, Sumontha, Kunya & Chaingkul, 2022 (Squamata, Draconinae) was analyzed using classical and molecular techniques to identify and study its chromosomal and repetitive elements.

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Gamma Irradiation Effects on *Salvia hispanica* L. seeds in M2 Generation: A comprehensive study of genetic variation and phytochemical responses

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Abstract. Gamma irradiation is a powerful tool in mutation breeding, promising to boost plant productivity and yield while influencing phytochemical composition and morphological traits. This study focuses on understanding the effects of gamma irradiation on Chia (*Salvia hispanica* L.) seed development, encompassing germination, growth, and photochemical properties. Ionizing radiation has proven to be a potent physical agent in mutation breeding initiatives, potentially enhancing plant productivity and yield. A comprehensive analysis was conducted, encompassing the application of distinct gamma irradiation doses ranging from (0, 50,100, 150, 200, and 250 Gy) in M_1(2021-22) and M_2 (2022-23) and oils were extracted in M_2 generation using the Soxhlet technique. Various parameters, including sterol composition, fatty acid composition, tocopherol content, and fatty acid value (FAV), were meticulously analyzed using the Gas Chromatography-Mass Spectrometry (GC-MS) technique. Increased phytochemical viz., Alpha-linolenic acid (60.23%), Linoleic acid methyl ester (19.78%), and Palmitic acid (11.96%) were obtained at 100 Gy irradiation that had not been reported in earlier research. Therefore, the potential of gamma irradiation to enhance chia seeds' nutritional and phytochemical properties exists. This insight holds promise for advancing seed development and overall plant performance, offering valuable prospects for crop improvement and the creation of nutrient-rich agricultural products.

Keywords: *Salvia hispanica* L., Gamma irradiation, GC-MS analysis, Ionizing radiation, Alpha-linolenic acid.

1. INTRODUCTION

In recent years, there has been a notable surge in the utilization of chia seeds within the food, dietary supplement, and cosmetic industries. This surge in popularity can be attributed not only to the seed's valuable chemical composition and biological activity but also to its widespread availability. Chia (*Salvia hispanica* L.), commonly known as Mexican chia or Spanish sage and a member of the Lamiaceae family, is grown in tropical and subtropical countries (Ixtaina et al., 2008). Chia seeds are frequently used in the functional food industry due to their rich composition. They typically con-

tain approximately 30–33% lipids, 15–25% proteins, and 26–41% carbohydrates, along with various vitamins, essential minerals, and a substantial dietary fiber content ranging from 18–30% (Ullah et al., 2016). Furthermore, chia seeds contain a wide range of polyphenols, known for their antioxidant properties and potential health benefits (Ixtaina et al., 2011). This is due to their rich omega-3 fatty acid content and a favorable omega-3 to omega-6 fatty acid ratio popular among individuals adhering to plant-based diets, including vegetarians and vegans (Sebastiani et al., 2019).

Mutation breeding techniques are being employed to introduce genetic diversity into chia due to its limited genetic base. In recent years, they have gained widespread use, particularly for enhancing the genetic diversity of vegetatively propagated crop plants. Gamma irradiation is a form of ionizing radiation widely used in various scientific disciplines, including agriculture and plant breeding. Its ability to induce genetic variation and stimulate physiological responses in living organisms makes it a valuable tool (Ali *et al*., 2015). In our experimental design, we employed triplicate treatments, subjecting chia seeds to gamma irradiation, while a control group was closely monitored for changes in morphology, cytology, and phytochemical responses.

2. MATERIAL AND METHOD

2.1 Seed procurement

Inbred seeds of the chia plant were obtained from NutriPlanet Private Limited, Bengaluru-520068, Karnataka, India. Two varieties of chia plants, namely Black and White, were provided. Given the heightened economic significance of black seeds and their associated properties, these were selected as the focal variety for this study.

2.2 Seed irradiation treatment

Inbred chia seeds were enclosed within individual pockets and subjected to distinct irradiation doses (50, 100, 150, 200, and 250 Gy) using a Cobalt-60 source at NBRI, Lucknow. The irradiation process was carried out using gamma rays at a dose rate of 7.247 kGy.

2.3 Seed sowing

After treatment, all the irradiated seeds were sown in the triplicate set of pots following a randomized complete block design (RCBD) in the field. The temperature and humidity were recorded at 25±2 °C and 76% respectively.

2.4 Morphological traits

Seven days after seed sowing the germination percentage of the plants was calculated and plant survival rates were recorded after 30 days. Plant height was gauged at 45-day intervals, accompanied by the observation of various leaf mutants arising from diverse exposures. The onset of plant flowering, occurring around day 120, was scrutinized, encompassing an examination of variations in their inflorescence patterns.

2.5Meiotic study- To facilitate the cytological observations, the young floral buds of plants were fixed in carnoy's fixative (Alcohol 3: Glacial Acetic Acid 1) for 24 hours. These buds were subsequently preserved in 90% alcohol. Taken small size anther and gently teased using needle and forceps. Staining was accomplished using a 2% acetocarmine solution. Microscopic observations were carried out using a Nikon phase-contrast microscope (Nikon Eclipse E200, Japan). The identification of cytological abnormalities was undertaken and the total abnormality percentage (%) within the treated sets was calculated.

2.6 Extract extraction

For GC-MS studies, harvested mature seeds from the M_2 generation subjected to gamma treatment were utilized, with corresponding control sets. Methanolic extraction was prepared by placing 10 grams of seeds in 250 ml of methanol solvent within a Soxhlet apparatus for oil extraction. Filtered samples were stored in an Eppendorf tube and labeled with different irradiation doses.

2.7 GC-MS Analysis

Methanolic extracts were prepared for both gamma-treated and control seeds separately by adding 10gram seeds to 250 ml of methanol transferring them to the Soxhlet apparatus and extracting the essential oils out of it. Extracts were further filtered by using Whatman filter paper. Model GCMS-QP2010 serial no. 0205251 SHIMADZU was used for GC-MS analysis. After filtration 6 µl of Methanolic extract was injected into the column and analyzed. The conditions were set as under Injection temp: 260 °C, column oven temperature 100°, injection mode split, total flow -16.3 mL/min, Pressure -90.5

kPa, Ion Source Temp. 220 °C, Interface Temp. -270 °C, Solvent ut time: 3.50 min, Detector gain mode: relative, Relative Detector Gain: +0.00 kV, Threshold: 1000. The chemical composition was elucidated, encompassing saturated and unsaturated fatty acids, sterols, steroids, vitamins, and other metabolites. Identification of different metabolites was based on their fatty acid content, characterized by area percentage and retention time.

2.8 Statistical analysis

Observed data underwent analysis utilizing SPSS 16.0 software. A one-way analysis of variance (ANOVA) was conducted, followed by Duncan's Multiple Range Test (DMRT, with significance at $P < 0.05$) for mean separation. For Graphical representations using Sigma Plot 10.0 software. Actual means and standard errors were computed and the dataset was subjected to further analysis of variance.

3. RESULTS AND DISCUSSION

3.1 Germination and survival rates

After 30 days differences in parameters were observed in M_1 generation. Various morphological parameters such as germination rate, survival rate, plant height, and inflorescence axis were meticulously recorded. Comparative data of germination and survival of both the M_1 (90.38±1.49% at 50 Gy to 73.45±2.85% at 100 Gy) and M_2 (92.16±1.74% at 50 Gy to 75.58±2.83 100 Gy) generations are shown in (Fig. 1). Control germination was recorded 95.36±0.84%. In all treatment sets the germination percentage of M_2 were higher compared to M_1. This demonstrates a dose-dependent response in chia plant germination to gamma irradiation. This result is consistent with (Hanafy and Akladious 2018) regarding the negative impact of high doses of gamma rays on plant morphology and growth. A similar finding was reported by (Aparna *et al*., 2013 in *Arachis hypogaea* L.).

Survival rates exhibited a negative correlation with increasing irradiation doses, with the control group showing the highest survival rate (93.56± 0.1.02). In treatment sets at 50 recorded 85.23±1.41 to the lowest survival rate observed at 250 Gy irradiation (65.24 ± 2.77) shown M_1 generation and increased from 88.65±1.15% at 50 Gy to 69.35±2.54% at 100 Gy in M_2 generation. These findings align with the notion that ionizing irradiation can have adverse effects on various plant traits, including germination and survival (Mittler, R. 2002). Similar trends were observed in *Cuminum cyminum* seedlings by (Verma *et al*. 2017).

3.2 Morphological traits

Plant height increased significantly at 100 Gy (75.48 ± 1.68 cm) compared to the control (72.46±0.98 cm) in the M_1 generation. (Fig. 2A) In the M_2 generation plant height increased prominently as compared to M_1 recorded with bushy mutants recorded at lower doses of exposure as shown in (Fig. 3 L). Inflorescence axis length also increased at 100 Gy (12.42±0.21 cm) M_1 and (13.25±0.24 cm) enhanced in M_2 but declined with higher radiation doses (Fig. 2B). Different types of leaf mutants were characterized, including color, shape, and size variations.

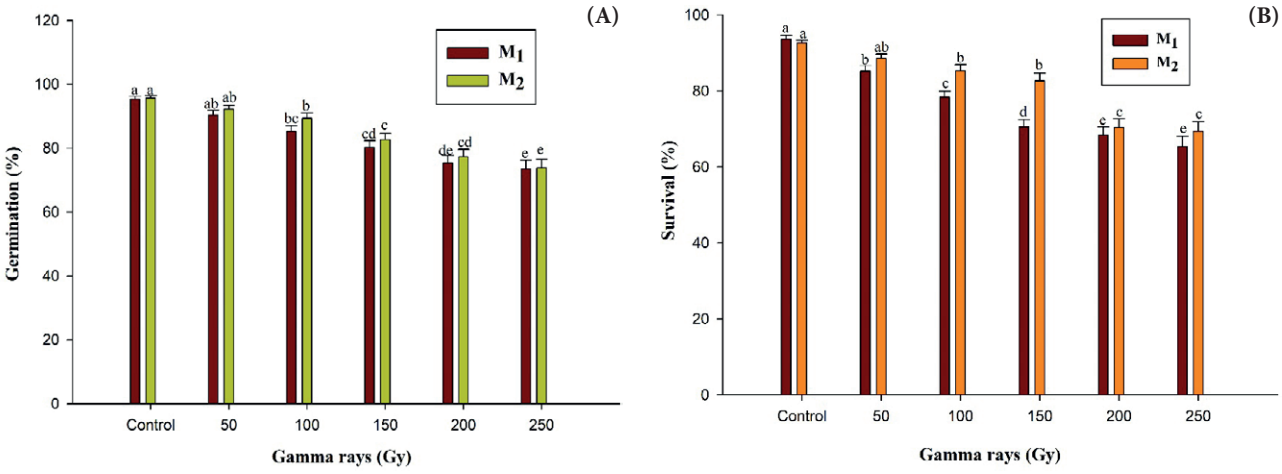

Figure 1. The morphological parameters Germination percentage (A) and Survival percentage (B) of the M1 and M2 generation about gamma radiosensitivity were investigated through seed treatment at $P < 0.05$ significance enhancement as in ANOVA .

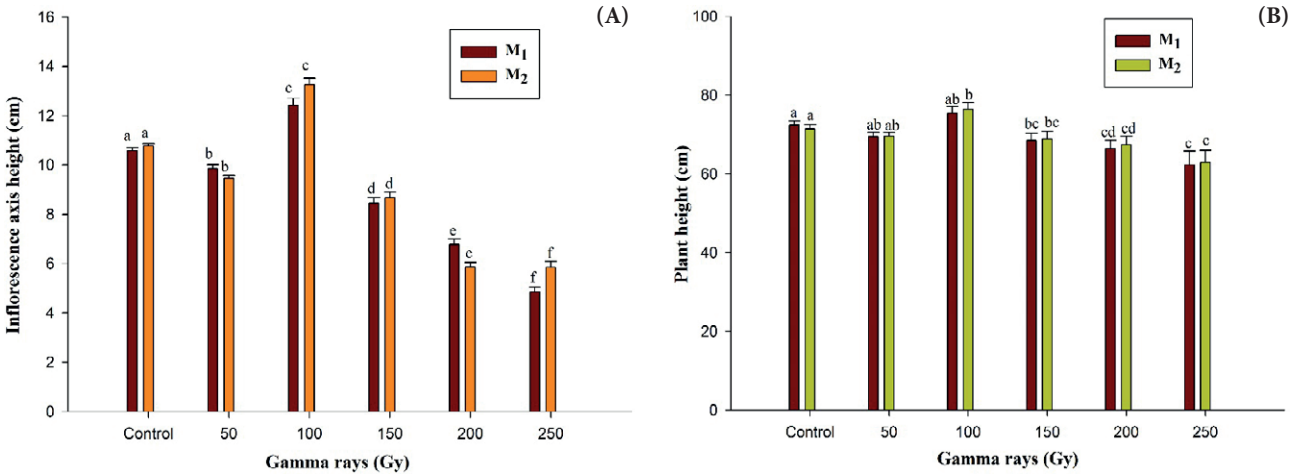

Figure 2. Graph representing morphological observations of plant height in M1 and M2 generation (A) and inflorescence axis in M1 and M2 generation (B) difference at $P < 0.05$ significance enhancement as in ANOVA .

Various leaf mutants were observed in the M_2 generation, including Semi-xantha mutants, Albo-viridis, Semi Albina, Yellow-viridis, Maculata mutants, Tricotyledonous leaves, Bifurcated leaves and single axes with three inflorescences in M_2 generation depicted in (Fig. 3). The hypothesis proposed by Wi *et al.* (2007) suggests that lower doses of gamma irradiation may induce growth by influencing hormonal activities and bolstering antioxidant defenses in plant cells. This could enable plants to better withstand daily stress factors. The underlying reasons for these chlorophyll mutants and the genes and proteins involved remain subjects of ongoing research, as noted by Ahumada-Flores *et al.* (2020). Several authors have previously reported different types of chlorophyll mutations, such as Xantha, Albina, Viridis, and Chlorine, among others (Kolar *et al.*, 2011; Arisha *et al.*, 2015; Verma *et al.*, 2018).

A novel observation was made regarding tricotyledonary true leaves at a specific node in *Salvia hispanica L.* plants (Fig 3D). Similarly, tricotyledonary seedlings have been reported in sunflowers by (Hu *et al.* 2006), who suggested that this phenotype is controlled by a few recessive genes, which are typically masked by dominant traits but occasionally manifest due to the lethality of masking genotypes. These tricotyledonary seedlings bear three true leaves at each internode. In the case of Kalmegh, Dwivedi *et al.* (2021) also reported the presence of trimeric true leaves in the M_2 generation. It has been observed that lower-dose gamma irradiation has a stimulatory effect and enhances various traits, consistent with findings by Kim *et al.* (2001). However, it should be noted that higher doses of gamma radiation beyond 100 Gy had detrimental effects, consistent with the findings of Hanafy and Akladious (2015), who explained that the highest gamma-ray dosage negatively impacted fenugreek morphology and growth when compared to control plants. Seed weight increased in the treatment group compared to the control, with the most significant change observed at 100 Gy (1.76±0.02 g of 250 seeds) compared to the control (1.32±0.031 g of 250 seeds) in Fig. 4B. Seed weight (in 1 cm square) increased, as depicted in (Fig. 4B).

3.3 Cytological abnormalities

Meiotic studies of pollen mother cells (PMCs) revealed various cytological abnormalities, including scattering, stickiness, laggard movement, and bridge formation depicted in (Fig. 5). The percentage of abnormal PMCs (Tab %) increased with higher doses of gamma irradiation, ranging from (4.62 ± 0.14 to 12.59 ± 0.31) in Table 1. Pollen sterility also increased with irradiation dose, with the control group showing the highest fertility rate (97.47 ± 0.99 %) compared to the lowest at 64.96 ± 2.48 % mentioned in Table 1. The inhibitory effect on the cell cycle of gamma irradiation at higher doses has also been reported earlier in *Allium cepa* by (Ahirwar, 2015). Furthermore, (Kumar and Dwivedi *et al.* 2021) have reported that bridge formation can result from spindle dysfunction induced by higher-dose mutations. (Kumar and Gupta 2009) suggested that gene mutations or the direct action of mutagens on target proteins responsible for chiasma terminalization during diakinesis at meiosis-I can lead to structural defects in these proteins. These defects ultimately impair their proper functioning, resulting in the formation of chromosomal

Figure 3. Leaf mutants after gamma irradiation observed in M_2 generation **A.** control; **B.** Semi-xanthan**; C.** Alboviridis; **D**. Tricotyledonous leaf; **E.** Bifurcated; **F** single axis with three inflorescence bud; **G.** Semi-albina; **H.** Yellow-Viridis; **I.** Xantha mutant; **J.** Maculata; **K.** plant height variation in M2 generation: control group with treatment sets in M_2 generation; **L.** control with Bushy mutant; **M.** Control Inflorescence; **N.** inflorescence with fused axis; **O.** Seed size in treatment and control.

bridges. Stickiness, for example, may result from imbalances in spindle fibers caused by mutagenic treatment. (Jabee *et al.,* 2008).

Furthermore, the study noted a decline in pollen fertility due to the formation of sterile pollen as a side effect of mutagenic treatment. This increase in pollen sterility with higher irradiation doses poses a risk to the survival of plant genotypes. A decline in pollen fertility was attributed to the formation of sterile pollen, primarily resulting from the adverse effects of mutagens on the male reproductive organs. It was observed that the rate of pollen sterility increased with escalating doses of irradiation, ultimately leading to the production of non-via-

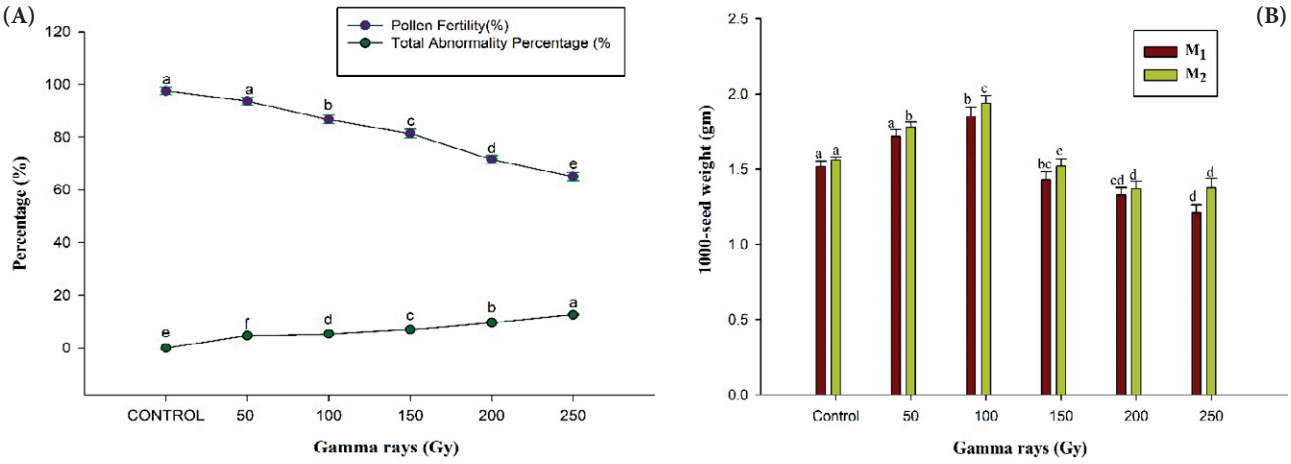

Figure 4. Graph (A) shows a negative correlation between Pollen fertility and Total Abnormality Percentage (%) with increased doses of gamma irradiation and (B) represents seed weight difference in M1 and M2 generation .

Table 1. Gamma irradiation-induced cytological abnormalities and their percentage in *Salvia hispanica* L.(2n=12) during Meiosis.

Treatment	PMC	METAPHASIC ABNORMALITY			ANAPHASIC ABNORMALITY				OTH	TAB	POLLEN FERTILITY
		SC	ST	PM	AST	ASC	AUN	BG			
CONTROL	458	-	-	-	-	-	-	-	-		97.47±0.99
50 Gy	441	0.98±0.09	0.53±0.07	0.61±0.08	0.61±0.15	0.61±0.07	0.46±0.01	0.22±0.01	0.00±0.00	4.62±0.14	93.65±0.59
100 Gy	443	0.97±0.06	0.83±0.07	0.67±0.22	0.60±0.15	0.53±0.07	0.67±0.12	0.22±0.13	0.22±0.13	5.26±0.39	86.67±2.97
150 Gy	393	1.44±0.09	0.93±0.09	0.93±0.30	0.85±0.08	1.19±0.09	1.23±0.16	0.26±0.15	0.25±0.15	6.95±0.21	81.36±2.32
200 Gy	379	1.85±0.14	1.14±0.18	1.41±0.10	1.32±0.16	1.32±0.16	1.23±0.16	0.35±±0.09	0.36±0.24	9.57±0.71	71.49±2.13
250 Gy	354	1.98±0.17	1.50±0.07	1.79±0.27	1.60±0.25	1.78±0.07	1.60±0.20	1.02±0.08	0.61±0.23	12.59±0.31	64.96±2.48

Where, PMC's- Pollen mother cells, Sc- Scattering of chromosomes, Pm- Precocious movement of chromosomes, St- Stickiness of chromosomes, Ast- Anaphasic stickiness, Aun- Anaphase Unorientation, Oth- Others, Tab- Total abnormality percentage (p= <0.5).

ble pollen. This phenomenon poses a potential threat to the survival of plant genotypes within the system. Singh and Kumar (2020) reported a similar pattern in *Artemisia annua*, observing an increase in pollen sterility proportional to the irradiation dose. (Jagtap and More 2014) conducted an analysis for *Lablab purposes* and arrived at a similar conclusion: plant sterility intensifies as the dose of physical or chemical mutagens increases.

3.4 Biochemical composition

Gamma irradiation led to alterations in the fatty acid composition of chia seed oil in the treatment and control set analyzed in M_2 generation Table 2. Saturated fatty acids, such as Palmitic acid (16:0), showed an increase at 100 Gy (8.42%) compared to the control (7.52%). Unsaturated fatty acids, including Alpha-linolenic acid, Linolenic acid methyl ester, and Alpha-Monosterin, exhibited enhancements at various irradiation doses depicted in Table 2Vitamin E was the predominant vitamin observed, with a significant increase of 0.36% following gamma irradiation at 100 Gy. Additionally, gamma-sitosterol, a sterol compound, showed a notable increase from 0.06% in the control group to 9.89% in the treated samples. Other sterols exhibited variable responses to the irradiation treatment. The presence of fumaric acid, triterpenoids, and corticosteroids was also detected and showed alterations with gamma irradiation. The metabolites were categorized into five main groups: saturated fatty acids, unsaturated fatty acids, esters, vitamins, and stigmasterol with their area percentages. The GC-MS analysis of gamma-treated seeds of M_2 has revealed noteworthy changes, including a significant enhancement in the content of unsaturated fatty acids. Significant increases were observed in various phytochemicals of chia seeds following gamma irradiation at 100 Gy, including Alpha-linolenic acid

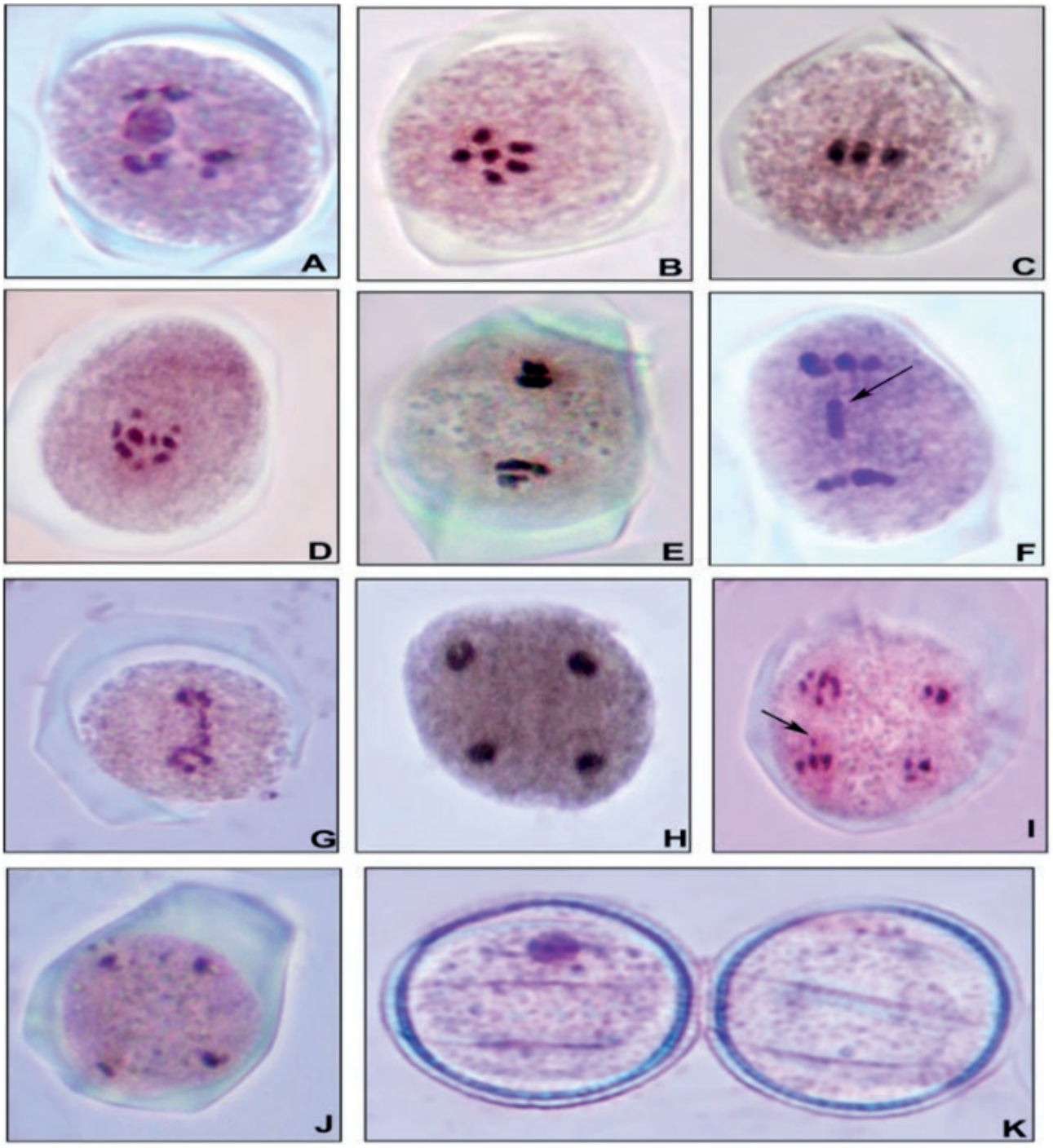

Figure 5. Cytological Meiotic anomalies induced by gamma irradiation: *Salvia hispanica* L. (2n=12) A. Diplotene Stage; B. Normal Metaphase (2n=12); C. Stckiness at metaphase I; D. Scattering at metaphase I; E. Normal Anaphase I; F. laggard at Anaphase I; G. Bridge formation at Anaphase I; H. Normal Anaphase II; I. Laggard at Anaphase II; H. Normal Telophase II; J. Normal Pollen and sterile pollen grains (Scale=10μ).

(60.23%), Linoleic acid methyl ester (19.78%), Palmitic acid (11.96%), vitamin E (5%), and gamma-sitostenone (9.89%) (Table 2). These enhancements in the phytochemical profile were notably higher compared to the control group, representing a novel finding not previously documented in existing literature. In contrast,

Table 2. GC-MS analysis was conducted to compare the concentration percentages and retention times (in minutes) of the treatment and control groups in M2 generation seed of gamma irradiation treatment.

Compound	Metabolites detected	Molecular formula	RT	Control Area %	T1 Area %	T2 Area %	T3 Area %	T4 Area %	T5 Area %
Saturated fatty acids	Myristic acid	C14H28O2	12.53	0.15	0.1	0.19	0.19	0.14	0.13
	Palmitic acid	C16H32O2	14.72	7.52	0.26	8.42	-	5	4.73
	Stearic acid	C18H36O2	16.62	3.67	3.06	3.21	0.33	0.37	1.98
	Beta. Monoglyceride	C19H38O4	19.54	7.27	7.73	6.16	0.14	7.02	6.76
	Stearic acid methyl ester	C19H38O2	16.62	3.67	3.06	3.21	0.18	2.07	0.4
	Lauric acid	C12H24O2	19.91	0.09	0.14	0.28	0.13	0.16	0.1
Unsaturated fatty acid	Linoleic acid, methyl ester	C19H34O2	15.9	1.87	1.18	2.24	0.95	2.28	2.37
	Linolenic acid, methyl ester	C19H32O2	15.97	6	4.52	7.44	7.37	6.9	4.22
	Alpha-Linolenic acid	C18H30O2	16.45	5.46	7.05	17.73	11.6	5.07	0.38
	Linolenic acid, ethyl ester	C20H34O2	21.04	20.2	10.84	13.32	9.45	7.44	4.22
	Alpha-Monostearin	C21H42O4	21.12	2.02	1.7	6.24	1.29	0.17	0.17
	Linolein, 2-mono-	C21H38O4	21.54	0.51	0.08	0.4	0.1	0.37	0.27
	Linolenic acid, methyl ester	C19H32O2	21.61	0.52	0.5	12.59	7.37	4.22	0.3
	Alpha-Linolenic acid	C18H30O2	16.45	5.46	7.05	17.73	11.6	0.38	0.04
Vitamins	Gamma. -Tocopherol	C18H28O3	23.62	1.91	1.81	2.04	1.3	1.81	0.98
	Delta. -Tocopherol	C28H48O2	22.72	0.19	-	6.13	0.17	0.16	0.17
	Vitamin E	C29H50O2	24.34	0.06	0.12	0.36	-	0.16	0.15
Acid ester	Fumaric acid	C25H46O4	17.602	0.82	0.1	0.11	0.37	0.37	0.11
	3-Cyclopentylpropionic acid, 2-dimethylamino ethyl ester	C12H23NO2	19.02	1.56	0.79	0.82	0.71	0.14	1.29
Phytosterol	Stigmasterol	C29H48O	25.91	1.58	0.79	1.04	0.86	1.44	1.59
	Compesterol	C29H48O	25.61	2.09	-	-	-	0.5	0.53
	Gamma-Sitosterol	C29H50O	26.8	8.65	5.26	6.13	9.89	4.53	0.38
	Fucosterol	C30H50O	26.93	0.54	0.34	0.41	-	0.5	0.53
Corticosteroids	11-Dehydrocorticosterone	C21H28O4	21.95	0.3	0.43	0.33	0.44	0.26	0.1

decreases were recorded in the levels of Beta-monoglyceride (15%), 11-dehydrocorticosterone (66.67%), and stearic acid (46%) within the irradiated samples.

It's worth noting that GC-MS analysis was previously conducted by (B. de Falco et al., 2018) under different irradiations of Chia plants; however, their analysis was focused solely on polar and non-polar compounds. The specific response of gamma-treated seeds profiling enhancement in unsaturated fatty acid had not been previously studied. This phenomenon plays a pivotal role in the production of diverse plant varieties.

CONCLUSION

In conclusion, the M_2 generation exhibited more pronounced results across all aspects of the study morphological, cytological, and biochemical when compared to the M_1 generation. The findings suggest that lower doses of gamma irradiation have a stimulating effect on the chia plant's morphological traits and phytochemical properties. The GC-MS analysis of chia seeds showed a notable enhancement in unsaturated fatty acid content at 100 Gy irradiation, which can have positive implications for the plant's medicinal and nutritional properties. However, higher doses were found to be detrimental to these phytochemical properties. This finding is significant as it has the potential to play a pivotal role in enhancing plant productivity and promoting the enlargement of seed size in the treated plants. "The discovery of a notable increase in unsaturated fatty acids, particularly Alpha-linolenic acid, following exposure to 100 Gy of gamma irradiation represents a novel and previously unreported finding. These observations have implications for the potential use of gamma irradiation in crop improvement and seed quality enhancement.

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Cytogenetics of *Cheniella* (Leguminosae: Cercidoideae) from China and Vietnam

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Abstract. The cytogenetics of *Cheniella*, a recently segregated genus of the early-diverging subfamily Cercidoideae of Leguminosae, remain understudied, hindering our understanding of the cytological evolution and the utilization of this important plant group. Here we conducted comparative cytogenetic studies on 11 species and one subspecies of *Cheniella* and one species of its sister genus *Phanera*. Unlike earlier reports which recovered $2n$=28 for two *Cheniella* species, we consistently observed chromosome counts of $2n$=26 for all 11 species of *Cheniella*, supporting the segregation of *Cheniella* from *Phanera*, which consistently exhibited $2n$=28 in this and previous studies. Our analyses, along with previous cytogenetic data, indicates that $2n$=14, $2n$=26 and $2n$=28 are the predominant chromosome numbers in the basal-most genus *Cercis*, *Cheniella* and the remainder genera, respectively. The ancestor of the subfamily is most probably a diploid with $2n$=14, with subsequent polyploidization followed by chromosome reduction events leading to $2n$=28 and $2n$=26 in the other lineages. Our results provide new insight into the cytotaxonomy and chromosome evolution of Cercidoideae, also lay the foundation for future genomics research.

Keywords: *Bauhinia* s.l., chromosome counts, cytology, Fabaceae, *Phanera*, Southeast Asia.

INTRODUCTION

The plant family Leguminosae Juss. (or Fabaceae Lindl.) is currently recognised by the Legume Phylogeny Working Group to consist of six subfamilies (LPWG, 2017), of which the Cercidoideae LPWG contains about 14 genera and 340 species distributed pantropically and in some subtropical regions. Various species of Cercidoideae are used for food, timber, dyes, ropes and medicine, and widely cultivated as ornamental trees in many areas of the world (Clark et al., 2017; Gu et al., 2024). The flowers of many Cerci-

doideae species are highly attractive and fragrant, with great value or potential as garden ornamental plants. The subfamily Cercidoideae currently contains 14 genera including *Adenolobus* (Harv. ex Benth. & Hook.f.) Torre. & Hillc., *Barklya* F.Muell., *Bauhinia* L., *Brenierea* Humbert, *Cercis* L., *Cheniella* R.Clark & Mackinder, *Gigasiphon* Drake, *Griffonia* Baill, *Lysiphyllum* (Benth.) de Wit, *Phanera* Lour., *Piliostigma* Hochst., *Schnella* Raddi; *Tournaya* A.Schmitz, and *Tylosema* (Schweinf.) Torre & Hillc. (Wunderlin, 1976; Lewis & Forest, 2005; LPWG, 2017; Clark et al., 2017; Sinou et al., 2020).

The initially diverged lineage of Cercidoideae, *Cercis*, exhibits a somatic chromosome number of 2*n*=14, whereas most other lineages in this subfamily were consistently reported to have a somatic chromosomal count of 2*n*=28, with a few exceptions of 2*n*=24, 2*n*=26, or even 2*n*=42, 2*n*=56 found in several species of *Barklya*, *Bauhinia*, *Gigasiphon*, *Lysiphyllum*, and *Piliostigma* (Table 1) (Sharma & Raju, 1968; Goldblatt, 1981; Yeh et al., 1986; Kumari & Bir, 1989). Intraspecific chromosomal variations are also observed. For example, *Bauhinia monandra* Kurz exhibits counts of 2*n*=24, 2*n*=28, and 2*n*=42 (Sharma & Raju, 1968; Gill & Husaini, 1982; Darlington & Wylie, 1955), *Bauhinia acuminata* L. has 2*n*=26 and 2*n*=28, and *Lysiphyllum hookeri* (F.Muell.) Pedley shows both 2*n*=26 and 2*n*=28 (Sharma & Raju, 1968; Singhal et al., 1980b; Goldblatt, 1981; Sarkar et al., 1982; Basumatari & Das, 2017).

Cheniella R.Clark & Mackinder, a recently segregated genus from *Bauhinia* s.l., contains 16 species and three subspecies, and is closely related to *Phanera* (Clark et al., 2017; Gu et al., 2024; Peng et al., 2024). The centre of diversity of *Cheniella* is in southern China, and its full distribution range extends westward to India and southeast through Indochina into Malesia (Clark et al., 2017). The genus is characterised as being tendrilled lianas with a deeply to slightly bilobed or emarginate leaf blade, elongate hypanthia, a fleshy disc on which the staminodes are mounted, glabrous or densly hirsute, oblong and compressed, indehiscent or tardily dehiscent pods with numerous seeds (Fig. 1). The chromosome numbers of two species in *Cheniella* have been previously reported, *C. corymbosa* (Roxb.) R.Clark & Mackinder and *C. quinnanensis* (Benth.) R.Clark & Mackinder, both with 2*n*=28 chromosomes (Sharma & Raju, 1968; Singhal et al., 1980a). It must be noted that the initial identifications of *C. corymbosa* and *C. quinnanensis* by Sharma & Raju (1968) and Singhal et al. (1980a) were *Bauhinia corymbosa* and (probably) *Bauhinia glauca* respectively, of which the former name was synonymised to *C. corymbosa* and the latter was probably erroneously identified, the correct name being *C. quinnanensis*. Beside the misidentification, the accuracy and reliability of the chromosome numbers in previous studies needed to be tested especially for those groups that were poorly studied or for those that have various chromosome counts reported.

To test the cytogenetics of *Cheniella*, we counted the chromosome numbers of 11 species and one subspecies of *Cheniella*, as well as one species of *Phanera*. By combining evidence from cytology and morphology, this study aims to provide the chromosomal data and cytotaxonomy of *Cheniella* and to compare these with other members of subfamily Cercidoideae.

MATERIALS AND METHODS

All seeds or transplanted living plants studied were collected in the field of southern and southwestern China and adjacent regions except for one sample was collected from Vietnam. Detailed collection information is shown in Table 1. The vouchers of all collections and permanent slides are deposited in the herbarium of South China Botanical Garden, Chinese Academy of Sciences (IBSC).

All cytological observations were made from root tip cells obtained either from seeds or from transplanted living individuals. All root tips were obtained from germinating seeds, mature and dry seeds were cut the seed coat and placed in petri dishes lined with moist filter paper and cultured at room temperature until 1–2 cm root sprouted. Root tips were pretreated in a saturated 1,4-dichlorobenzene solution for 150 min, then fixed with Carnoy's fluid (absolute alcohol: glacial acetic acid, 3:1, v/v) at 4 °C for at least 30 min. The fixed roots were hydrolysed in 1 N HCl solution at 60 °C for 4 min, stained with modified phenol magenta stain for 2 h and squashed for cytological observation. The best metaphase plates were photographed using a Nikon DS-Fi2 digital camera attached to the BX41 Olympus microscope. Permanent slides were made using the standard liquid nitrogen method.

RESULTS AND DISCUSSION

The interphase nuclei of 11 species from *Cheniella* and one species from *Phanera* studied in this paper show the similar shape and distribution pattern of chromatin, which are dispersed evenly throughout the nuclei (Fig. 1, A). According to Tanaka (1971, 1977), they can be categorised as the complex chromocentre type, which is characterised by darkly stained chromocentres of irregular shape and lightly stained chromatin threads. The

Table 1. Statistics on chromosome numbers in Cercidoideae.

Genus	Species	Chromosome number (2*n*)	Locality	Voucher	References	Notes
Adenolobus	*A. pechuelii* (Kuntze) Korcz. & Hillc.	28	Walvis Bay, Namibia	Seely *s.n.*	Goldblatt (1981)	
Barklya	*B. syringifolia* F.Muell.	26	Cult. in Australia	Pedley A1772 (MO)	Goldblatt (1981)	
Bauhinia	*B. acuminata* L.	28	-	-	Sharma & Raju (1968)	
		26	Uttar Pradesh, India	Singhal 23001	Singhal et al. (1980b)	*
		28	Howrah, India	CBLH 4512	Sarkar et al. (1982)	
		28	-	-	Kumari & Bir (1989)	
		28	-	-	Sinha & Singh (2013b)	
		28	Guwahati, Assam	-	Basumatari & Das (2017)	
	B. corniculata Benth.	28	-	-	Sharma & Raju (1968)	
	B. divaricata L.	28	-	-	Sandhu & Mann (1988)	
	B. forficata Link	28	-	-	Gill & Husaini (1986)	
		28	Paraná, Brazil	E. Biondo 303 (ICN)	Biondo et al. (2005)	
	B. lunarioides A.Gray ex S.Watson	28	-	Tharp 44159 (TEX)	Turner (1956)	
	B. galpinii N.E.Br.	28	-	-	Sharma & Raju (1968)	
		28	-	-	Paiva & Leitao (1989)	
		28	Uttar Pradesh, India	Singhal 23502	Singhal et al. (1990)	
	B. monandra Kurz	28	-	-	Sharma & Raju (1968)	
		24	-	-	Gill & Husaini (1982)	*
		42	-	-	Darlington & Wylie (1955)	*
	B. petersiana Bolle	28	-	-	Sharma & Raju (1968)	
	B. purpurea L.	28	-	-	Sharma & Raju (1968)	
		28	-	-	Yeh et al. (1986)	
		28	-	-	Kumari & Bir (1989)	
		28	-	-	Sinha & Singh (2013a)	
		28	-	-	Sinha & Singh (2013b)	
	B. racemosa Lam.	28	-	-	Sharma & Raju (1968)	
		28	-	-	Kumari & Bir (1989)	
		28	-	-	Sinha & Singh (2013b)	
	B. rufescens Lam.	28	-	-	Sharma & Raju (1968)	
		56	-	-	Sharma & Raju (1968)	*
	B. tomentosa L.	28	-	-	Sharma & Raju (1968)	
		28	-	-	Gill & Husaini (1982)	
		28	-	-	Kumari & Bir (1989)	
	B. ungulata L.	28	Belém, Brazil	Souza 14	Souza & Benko-Iseppon (2004)	

(Continued)

Table 1. (Continued).

Genus	Species	Chromosome number (2*n*)	Locality	Voucher	References	Notes
	B. variegata L.	28	-	-	Atchison (1951)	
		28	-	-	Sharma & Raju (1968)	
		28	-	-	Bir & Kumari (1979)	
		28	-	-	Sinha & Singh (2013a)	
		28	-	-	Sinha & Singh (2013b)	
		28	Sirmaur, India	56688 (PUN)	Rani et al. (2013)	
		28	Kangra, India	56276 (PUN)	Rani et al. (2013)	
		28	Nagaon, Assam	-	Basumatari and Das (2017)	
		28	Guangzhou, China	-	Zhong et al. (2022)	
	B.× blakeana Dunn	28	-	-	Sharma & Raju (1968)	
		28	-	-	Sinha & Singh (2013a)	
Cercis	*C. canadensis* L.	14	Cult. in USA	Curtis 101 (MO)	Curtis (1976)	
		14	-	-	Hill (1989)	
		14	-	-	Blackwell (1990)	
	C. chinensis Bunge	14	-	-	Yeh et al. (1986)	
		14	-	-	Chen et al. (2003)	
		14	Guangxi, China	-	Li et al. (2023)	
	C. chingii Chun	14	Jiangsu, China	-	Chen et al. (1991)	
	C. siliquastrum L.	14	-	-	Fernandes et al. (1975)	
		14	Prebalkan, Bulgaria	BK 73192	Kuzmanov (1975)	
Cheniella	*C. clemensiorum* (Merr.) R.Clark & Mackinder	26	Da Hang Pro, Vietnam	LBo779 (IBSC)	This study	
	C. corymbosa (Roxb.) R.Clark & Mackinder	26	Hainan, China	ZQB59 (IBSC)	This study	
		28	-	-	Sharma & Raju (1968)	*
	C. didyma (H.Y.Chen) R.Clark & Mackinder	26	Guangdong, China	TuTY4691 (IBSC)	This study	
	C. hupehana comb. nov. ined.	26	Hubei, China	ZQB68 (IBSC)	This study	
	C. longipes (Hosok.) S.R.Gu, T.Y.Tu & D.X.Zhang	26	Hainan, China	ZQB56 (IBSC)	This study	
	C. longistaminea S.R.Gu, T.Y.Tu & D.X.Zhang	26	Guangxi, China	ZengQB198 (IBSC)	This study	
	C. ovatifolia (T.C.Chen) R.Clark & Mackinder	26	Guangxi, China	TuTY4799 (IBSC)	This study	
	C. paraglauca sp. nov. nom. ined.	26	Guangdong, China	GuSR125 (IBSC)	This study	
	C. quinnanensis (T.C.Chen) R.Clark & Mackinder subsp. *quinnanensis*	26	Guangxi, China	TuTY4816 (IBSC)	This study	
		28	Uttar Pradesh, India	Singhal 22812	Singhal et al. (1980a)	*
	C. quinnanensis subsp. *villosa* R.Clark & Mackinder	26	Guangdong, China	TuTY4848 (IBSC)	This study	
	C. tenuiflora (Watt ex C.B.Clarke) R.Clark & Mackinder	26	Yunnan, China	GuSR021 (IBSC)	This study	
	C. tianlinensis × *ovatifolia*	26	Guangxi, China	ZQB32 (IBSC)	This study	
	C. touranensis (Gagnep.) R.Clark & Mackinder	26	Guangxi, China	TuTY4795 (IBSC)	This study	

(Continued)

Table 1. (Continued).

Genus	Species	Chromosome number (2*n*)	Locality	Voucher	References	Notes
Gigasiphon	*G. macrosiphon* (Harms) Brenan	26	Mua hills, Kenya	Gachathi *s.n.*	Goldblatt (1981)	
Lysiphyllum	*L. diphyllum* (Banks) de Wit	28	-	-	Sharma & Raju (1968)	
		28	-	-	Bir & Kumari (1979)	
		28	-	-	Kumari & Bir (1989)	
	L. hookeri (F.Muell.) Pedley	28	-	-	Sharma & Raju (1968)	
		26	-	-	Sharma & Raju (1968)	
		26	Cult. in Australia	Pedley A7771 (MO)	Goldblatt (1981)	
Phanera	*P. championii* Benth.	28	Taiwan, China	4728	Peng et al. (1986)	
		28	Guangxi, China	-	Lu et al. (2024)	
	P. integrifolia (Roxb.) Benth.	28	-	-	Sharma & Raju (1968)	
	P. roxburghiana (Voigt) Bandyop., Anand Kumar & Chakrab.	28	-	-	Sharma & Raju (1968)	
		28	Uttarakhand, India	Singhal 23281	Singhal et al. (1990)	
	P. semibifida (Roxb.) Benth.	28	-	-	Sharma & Raju (1968)	
	P. vahlii (Wight & Arn.) Benth.	28	-	-	Sandhu & Mann (1988)	
		28	Sirmaur, India	56004 (PUN)	Rani et al. (2013)	
	P. yunnanensis (Franch.) Wunderlin	28	Yunnan, China	GuSR097 (IBSC)	This study	
Piliostigma	*P. malabaricum* (Roxb.) Benth.	28	-	-	Sharma & Raju (1968)	
		28	-	-	Kumari & Bir (1989)	
	P. thonningii (Schumach.) Milne-Redh.	26	-	-	Yeh et al. (1986)	

* indicates the chromosome numbers should be interpreted with caution because they are different from other studies or from the usual perception.

similar pattern is consistent with the other reported Cercidoideae species.

Heterochromatin and euchromatin segments are clearly seen at mitotic prophase in all samples. The heterochromatin segments are located in the proximal regions that are deeply stained, indicating early condensation, while the euchromatin segments in the distal regions of chromosomes are lightly stained and extended, indicating late condensation (Fig. 1, B–C). According to Tanaka (1971, 1977), the prophase chromosomes of all species in this study are of the proximal type.

The prochromosomes in the pro-metaphase are curly and gradually arranged on the equator of the spindle with indistinct edges (Fig. 1, D). Paired sister chromatids are clearly visible during late metaphase stage (Fig. 1, E). Successful separation of daughter chromosomes is visible in late anaphase stage, moving from the equatorial plate to the poles of the spindle, but new nuclear membranes have not yet formed (Fig. 1, F).

There was little difference in size between the chromosomes in each species of *Cheniella* and *Phanera* (Fig. 1, G–T). Chromosomes in all species of *Cheniella* were rod-shaped or oblong in mitotic metaphase nuclei, whereas they were round or punctate in *Phanera yunnanensis* (Franch.) Wunderlin. The cell size and mitotic metaphase nuclei chromosome size of *P. yunnanensis* were smaller in comparison with *Cheniella*. Chromosomes in species of both *Cheniella* and *Phanera* are so small at mitotic metaphase nuclei that karyotypes cannot be clearly distinguished, but the number can be clearly counted. All studied *Cheniella* species have the same chromosome number $2n$=26 (Fig. 2, G–R), while the chromosome number of *P. yunnanensis* is $2n$=28 (Fig. 2, S–T). These results demonstrate the differences between *Cheniella* and *P. yunnanensis* in cytological characters. The chromosome count of *Cheniella* species is here determined to be $2n$=26, suggesting that the previous reported chromosome number of $2n$=28 (Singhal et al., 1980a; Sharma & Raju, 1968) might be erroneous. Consistency in chromosome numbers between different species within the genus indicates that speciation within *Cheniella* is not driven by polyploidy or chromosomal number variation.

The seeds of the artificial hybrid *Cheniella tianlinensis* × *ovatifolia* were harvested from the field, hand-pollinated and bagged, the mature legumes were collected for cytological analysis, revealing a chromosome number of $2n$=26 (Fig. 2, R). The maternal parent of the hybrid was *Cheniella tianlinensis* (T.C.Chen & D.X.Zhang) S.R.Gu, T.Y.Tu & D.X.Zhang and the paternal parent was *Cheniella ovatifolia* (T.C.Chen) R.Clark & Mackinder. Although chromosome counts for *C. tianlinensis* were not obtained, the chromosome number of *C. ovatifolia* was $2n$=26. Given the successful production of hybrid seeds with $2n$=26, it is reasonable to infer that *C. tianlinensis* also has a chromosome number of $2n$=26. These findings support the inclusion of *C. tianlinensis* within *Cheniella*, and are consistent with Gu et al. (2024).

Taxonomy of Cheniella *and* Phanera

Based on derived floral characters, palynology and previous molecular evidence, Clark et al. (2017) established the genus *Cheniella* to include 10 species and three subspecies. This was supported by the prior study of Hao et al. (2003) which presented a phylogenetic analysis of the nuclear ITS region, recovering a clade of five species later reassigned to *Cheniella*. However, in a phylogenetic study by Sinou et al. (2020) which sequenced *Legcyc1*, *Legcyc2*, *matK* and *trnL-F* for 17 liana species from Asia, a polytomy resulted, including *Cheniella* and *Phanera*. *Cheniella* appeared non-monophyletic, with sampled species dispersed in two clades, raising questions about the validity of the genus.

In contrast, Gu et al. (2024) analysed the concatenated sequences of 77 CDS, 103 IGS, 19 introns, and 4 rRNA genes, recovering two distinct clades for *Cheniella* and *Phanera*, and presenting a sister relationship between them. Unlike Sinou et al. (2020), *P. yunnanensis* grouped with other *Phanera* species rather than *Cheniella corymbosa*. Moreover, *P. yunnanensis* differs morphologically from *Cheniella* in characters that are informative at the generic level, having a raceme or simple cyme of two flowers, staminodes not joined at the base on a fleshy disc (Fig. 1, O–P), and a coriaceous legume that dehisces along both sutures.

In the treatment of Clark et al. (2017), *P. tianlinensis* was not included in *Cheniella* due to its pubescent legumes and rarity in herbaria. Gu et al. (2024) found that the fruit traits and flower structures of *P. tianlinensis* align with *Cheniella*. Additionally, *P. tianlinensis* also cluster with the *Cheniella* clade phylogenetically. Intergrating evidence of the morphological and molecular studies, Gu et al. (2024) concluded that *Cheniella* is a natural group that includes *P. tianlinensis*.

In the present study, all *Cheniella* species exhibited rod-shaped or oblong chromosomes in mitotic metaphase nuclei, unlike *Phanera yunnanensis*, which displayed round or punctate chromosomes. Additionally, cell size and mitotic metaphase chromosome size in *P. yunnanensis* were smaller in comparison with *Cheniella*. All examined *Cheniella* species possessed a chromosome number of $2n$=26, whereas *P. yunnanensis* had a chromosome number of $2n$=28, which consistent with numbers reported from other studies of *Phanera* (Sharma & Raju, 1968; Peng et

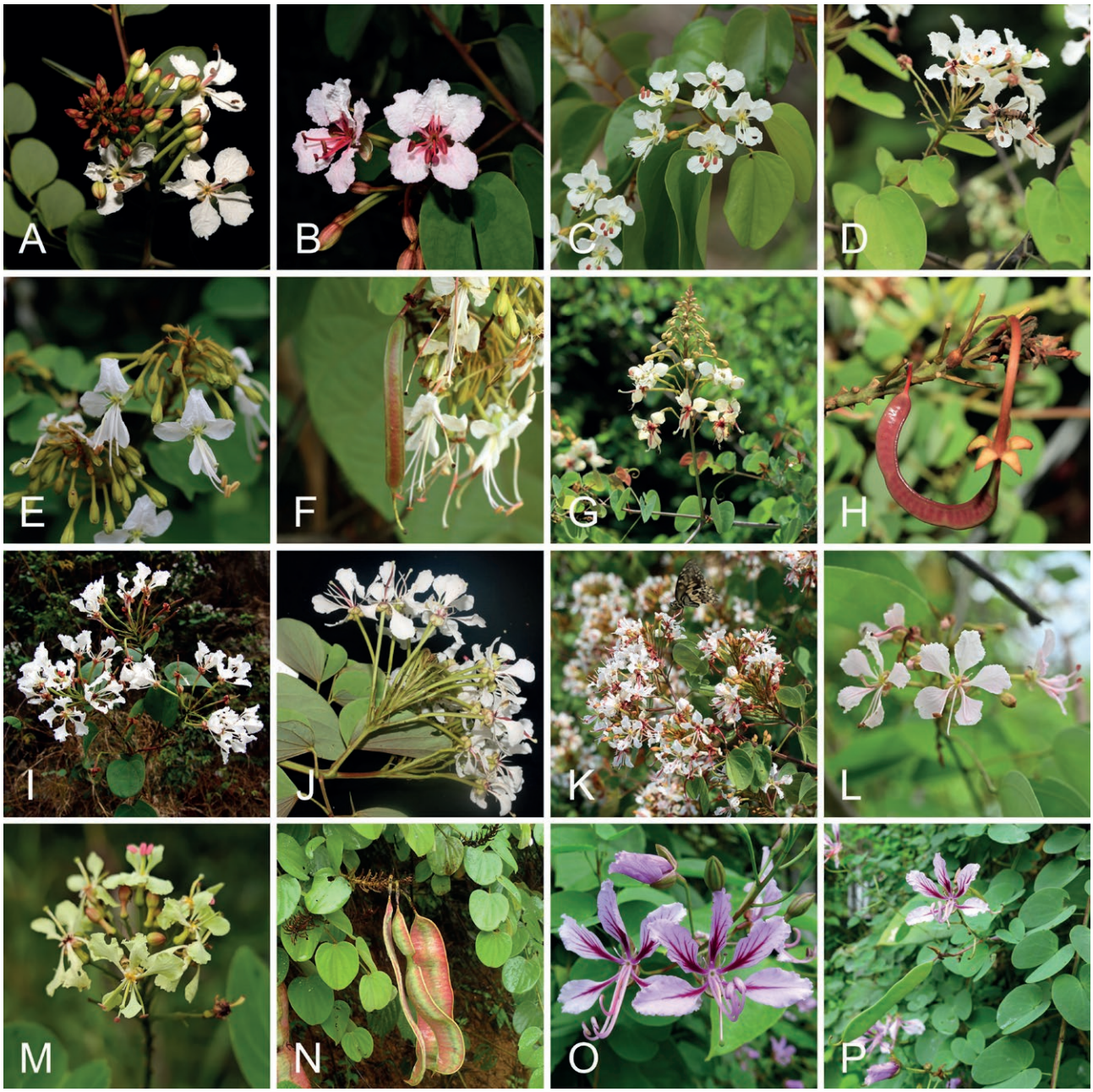

Figure 1. Morphological diversity in *Cheniella* and comparison with *Phanera*. A: *Cheniella didyma*; B: *C. corymbosa*; C: *C. quinnanensis* subsp. *villosa*; D: *C. quinnanensis* subsp. *quinnanensis*; E–F: *C. longistaminea*; G–H: *C. longipes*; I: *C. ovatifolia*; J: *C. tenuiflora*; K: *C. paraglauca* sp. nov. nom. ined.; L: *C. hupehana* comb. nov. ined.; M: *C. touranensis*; N: *C. clemensiorum*; O–P: *Phanera yunnanensis*. Photos: A, G–H & O–P, Qiu-Biao Zeng; B–F & I, Tie-Yao Tu; J & K, Shi-Ran Gu; L, Yi-Chen Zhang; M, Kai-Wen Jiang; N, Bo Li.

al., 1986; Singhal et al., 1990; Lu et al., 2024). *Cheniella tianlinensis* has a chromosome number of 2*n*=26, as it can hybridize with *C. ovatifolia* (2*n*=26), producing offspring with chromosome number of 2*n*=26. These findings highlight the differences between *Cheniella* and *Phanera*, and confirm that *C. tianlinensis* belongs to *Cheniella*.

Chromosome number evolution within Cercidoideae

The subfamily Cercidoideae of Leguminosae contains 14 genera and a diverse array of species, many of which exhibit significant intraspecific or interspecific variability in chromosome numbers, 2*n*=14, 24, 26, 28

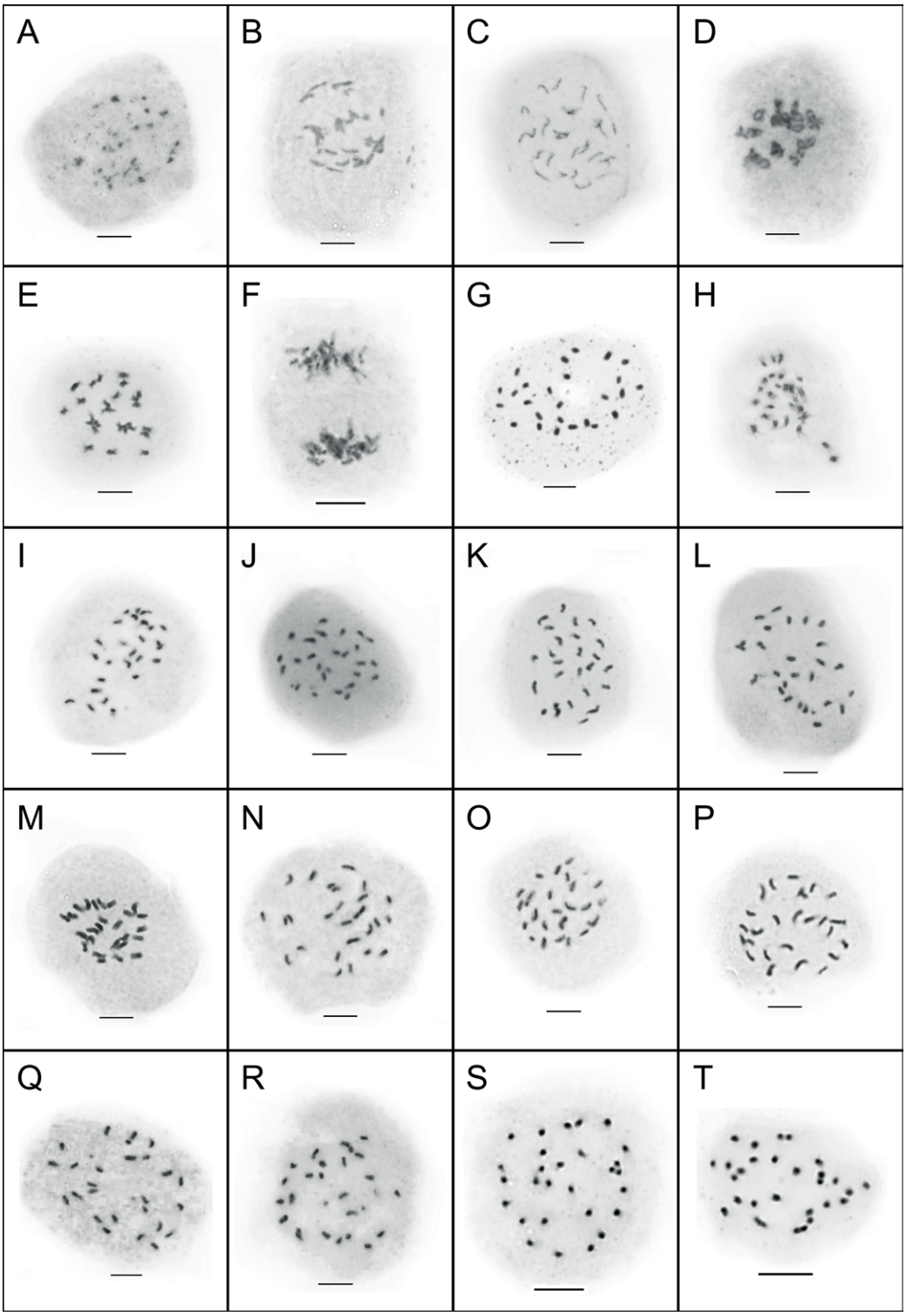

Figure 2. Comparative cytological features between *Cheniella* and *Phanera*. Scale bars=2 μm. A: Mitotic interphase of *Cheniella didyma*. B: Early prophase of *C. ovatifolia*. C: Late prophase of *C. corymbosa*. D: Pro-metaphase of *C. quinnanensis* subsp. *villosa*. E: Late metaphase of *C. longistaminea*. F: Mitotic anaphase of *Phanera yunnanensis*. G–T: Mitotic metaphases, G: *C. longipes*, 2*n*=26; H: *C. touranensis*, 2*n*=26; I: *C. hupehana* comb. nov. ined., 2*n*=26; J: *C. clemensiorum*, 2*n*=26; K: *C. longistaminea*, 2*n*=26; L: *C. corymbosa*, 2*n*=26; M: *C. paraglauca* nom. ined., 2*n*=26; N: *C. quinnanensis* subsp. *villosa*, 2*n*=26; O: *C. ovatifolia*, 2*n*=26; P: *C. quinnanensis*, 2*n*=26; Q: *C. tenuiflora*, 2*n*=26; R: *Cheniella tianlinensis* × *ovatifolia*, 2*n*=26; S–T: *P. yunnanensis*, 2*n*=28.

(42, 56) (Doyle, 2012; Steven et al., 2015; Roberts & Werner, 2016; LPWG, 2017). The earliest diverging lineage within Cercidoideae, *Cercis*, has a somatic chromosome number of 2*n*=14, whilst most other lineages in this subfamily share the chromosome number 2*n*=28, including *Adenolobus*, *Griffonia*, *Phanera*, *Piliostigma*, and most species of *Bauhinia* (Table1). Our study has confirmed that *Cheniella* possesses a somatic chromosome number of 2*n*=26, which is the same as several species of *Barklya*, *Bauhinia*, *Gigasiphon*, *Lysiphyllum*, and *Piliostigma* (Table1). Exceptions to the predominant chromosome numbers have been observed occasionally in *B. monandra* (2*n*=24 and 2*n*=42) and *B. rufescens* (2*n*=56) (Darlington & Wylie, 1955; Sharma & Raju, 1968; Gill & Husaini, 1982).

Given the basal-most phylogenetic position of *Cercis* within Cercidoideae (Hao et al., 2003; LPWG, 2017; Gu et al., 2019; Sinou et al., 2020; Gu et al., 2024), it is reasonable to infer that the ancestral state of chromosome number for this subfamily was likely a diploid with 2*n*=14. *Cercis* retains the characteristics of the diploid ancestors, whereas the ancestor of the sister clade of Cercis, which comprises all the remaining genera experienced a whole genome duplication event, resulting in the chromosome number of 2*n*=28, with probably a few undergoing further duplications to achieve higher chromosome numbers. This was followed by at least three independent aneuploidy chromosomal variation events, reducing the chromosome numbers to 2*n*=26. Reported chromosome counts of 2*n*=24, 2*n*=42 and 2*n*=56 in certain genera or species should be interpreted with caution. Understanding chromosomal evolution within this group is crucial for elucidating the broader evolutionary patterns that shape its biodiversity.

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Karyotype variability of the genus *Colocasia* (Araceae) of Assam, North East India

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Abstract. Cytological study in four species of *Colocasia* (Araceae) of Assam showed a variation of chromosome numbers. Basic chromosome number of the species was reported as n= 14. Dominance of metacentric chromosomes in all the four species and symmetric karyotypes indicate the primitive evolutionary status of the species. Analysis of chromosome asymmetry indices indicate the karyotype homogeneity. Deviation of basic chromosome numbers in *Colocasia manii* Hook. f. and *Colocasia fallax* Schott. reflects the possible existence of aneuploidy. Presence of secondary constriction indicates the chromosomal plasticity.

Keywords: asymmetry indices, chromosome number, *Colocasia*, karyotype.

INTRODUCTION

Araceae is a diverse plant group that comprises of 114 genera and 3750 species (Petruzzello 2018). Most of the genera under the family Araceae are predominantly found in tropical Asia (Grayum, 1990). The genus *Colocasia* under the family Araceae is predominantly found in Asia and South-East Asia is the primary centre of origin of this genus (Plucknett 1979). Large number of species and ecotypes of *Colocasia* have been reported from North Eastern part of India (Anbazhagan et al. 2015; Angami et al. 2015). The genus comprises of tropical, evergreen, perennial herb and are important sources of food and medicine. *Colocasia* has been used as an integral part of cuisine by various communities of Assam, India since time immemorial (Baro et al. 2023). The genus is highly polymorphic. Li and Boyce (2010) reported twenty (20) species of *Colocasia* over the world out of which six (6) species are found in Assam (Menla et al. 2019). Several species and ecotypes under this genus have been reported by Ahmed et al. (2020) on the basis of morphological characters of corm and aerial parts of the plant. Devaraju et al. (2023) reported considerable degree of variability in *Colocasia esculenta*. (L.) Schott. Cytological investigations have also been reported by some workers on few species of the genus *Colocasia* (Cao and Long 2004; Senavongse et al. 2018) from different parts of the world.

Chromosome number and genome size are important cytological characters that significantly influence various organismal traits. Karyo-morphological information can open a new direction for evaluating evolutionary status of different species of the genus. The chromosome number in *Colocasia* is reported as 2n=28 and 42 by Wulansari et al. (2021). Variations of chromosome numbers have also been reported in the genus *Colocasia* (Saensouk et al. 2019). Presence of intra-specific variation of chromosome numbers in *Colocasia* species had been reported by Coates (1988). Chromosome diversity at the intra and inter-specific level is necessary for adaptation and survival of species in changing environmental conditions. Chromosome study and karyotype analysis have been described as important parametres to estimate the inter and intra-specific diversity of various species within a genus (Stebbins 1971; Young et al. 2012) and can be a major aid for distinguishing taxonomic groups, deducing taxonomic relatedness and evolutionary status (Lavania 1985; Lorenzo and Eroglu 2013). Chromosomal polymorphism, potent promoters of reproductive isolation and speciation can be further correlated with differences in morphological parameters. The aim of this study is to establish the cytotaxonomic relationship among four (4) different species of *Colocasia* found in Assam.

MATERIALS AND METHODS

Four species of *Colocasia* Schott viz. *Colocasia manii* Hook. f., *Colocasia esculenta* (L.) Schott., *Colocasia fallax* Schott. and *Colocasia gigantea* (Blume) Hook. f. was collected from various locations of Assam, India and were maintained under optimal conditions for root initiation. Newly emerging healthy roots of 5-7 mm size were treated with 0.008 M (aq.) 8-Hydroxyquinoline (oxine) for one (1) hour (Tlaskal 1979; Nair 2016). The root tips were then washed thoroughly with distilled water, treated with 0.075 M KCl and were macerated in a mixture 1.5 % acetocarmine and 1 N HCl in 9:1 (v/v) ratio. The slides were observed under microscope and well separated metaphase stages were photographed at magnification of 100X × 45X and proceeded with karyotyping using the software assisted imaging application. Length of long arm (L), length of short arm (S), length of chromosome (CL), total chromosome length of diploid complement (TCL), arm ratio (AR), relative length percentage (RL%), centromeric index (CI) were considered for the characterization of the karyotypes. The nomenclature of the chromosome type and morphology was done following the standard system proposed.

Chromosome asymmetry indices was measured by considering TF% (Huziwara 1962), Stebbins' classes A-C (Stebbins 1971), Ask% (Arano 1963), karyotype asymmetry A (Watanabe et al. 1999), intrachromosomal and asymmetry index (A_1) and interchromosomal asymmetry index (A_2) (Zarco 1986), co-efficient of variation of chromosome length (CV_{CL}), co-efficient of variation of centromeric index (CV_{CI}) and asymmetry index (AI) (Paszko 2006).

RESULTS AND DISCUSSION

The genus showed variations in chromosome number and morphology. Detailed chromosome and karyotype parameters of four species of *Colocasia* are shown in Table 1, Figure 1.

Colocasia manii Hook.f.

In *Colocasia manii* Hook. f. somatic number was 2n=30 with karyotype formula 2n=30=24m+4sm+2st. Range of single chromosome length was 5.71-21.42 µm. Total chromosome length was 343.57 µm, relative chromosome length ranges from 2.07 to 6.23 µm. The arm ratio and centromeric index were recorded as 1.00–2.72 and 0.36–0.50 respectively.

Colocasia fallax Schott

Colocasia fallax Schott showed chromosome number 2n= 24 and 2n=28 with somatic karyotype formula 2n=24=18m+4sm+2st and 2n=28=2M+18m+8sm respectively. The genotype with chromosome number 2n= 24 has total chromosome length (TCL) 683.48 µm with relative chromosome length ranges from 2.18-7.15 µm. Range of arm ratio was 1.00– 2.16 and centromeric index was recorded as 0.31-0.50. While in the genotype with 2n=28 chromosome number, the total chromosome length (TCL) was recorded as 635.12 µm. The relative chromosome length, range of arm ratio and centromeric index were 2.20-4.95 µm, 1.02-3.25 and 0.31-0.49 respectively.

C. esculenta (L.) Schott

In *C. esculenta (L.)* Schott somatic chromosome number was recorded as 2n=28 with somatic karyotype formula 2n=28= 22m+6sm. Range of single chromosome length was recorded as 11.00–34.66 µm. Total chromosome length was 618.99 µm. The relative length of chro-

Table 1. Chromosome parameters of four genotypes of *the genus Colocasia*.

Species	Chromosome number (2n)	Karyotype formula	Length of short arm (µm)	Length of long arm (µm)	Single chromosome length (µm)	Relative length of chromosome (%)	Arm Ratio (AR)	Centromeric index (CI)	Karyotype Symmetry/ asymmetry (S/AI) Index
Colocasia manii Hook.f.	2n=30	24m+4sm+2st	2.14–9.64	3.57–11.78	5.71–21.42	2.07–6.23	1.00–2.72	0.36–0.50	1.3
	2n= 24	18m+4sm+2st	4.98–21.95	8.98–25.94	13.96–47.89	2.18–7.15	1.00–2.16	0.31–0.50	1.4
C.fallax Schott	2n= 28	2M+18m+8sm	1.92–7.91	3.33–8.52	6.9–15.9	2.20–4.95	1.02–3.25	0.31–0.49	1.3
C. esculenta (L.) Schott.	2n=28	22m+6sm	5.00–12.66	6.00 –22.00	11.00–34.66	1.88–5.37	1.00–2.07	0.31–0.52	1.2
	2n=28	2M+22m+4sm	5.60–12.80	6.56 –13.43	12.16–26.22	2.57–5.40	1.00–1.57	0.38–0.50	1.1
C. gigantea (Blume) Hook.f.	2n=30	4M +20m+ 4sm+2st	3.32–8.46	3.80–13.39	7.21–21.83	2.01–5.53	1.00–3.75	0.211–0.483	1.3
	2n=32	2M+22m+4sm+4st	2.84–5.72	3.21–10.20	6.06–15.91	2.05–4.704	1.00–3.50	0.222–0.51	1.4

mosome, arm ratio was recorded as 1.88-5.37, 1.00–2.07 respectively and centromeric index was 0.31–0.52.

C. gigantea (Blume) Hook.f.

Somatic number was recorded as 2n=28, 2n=30 and 2n=32 with karyotype formula 2n=28=2M+22m+4sm, 2n=30=4M+20m+4sm+2st and 2n=32=2M+22m+4sm+4st respectively. In genotype with 2n=28, single chromosome length ranges from 12.16 to 26.22 µm, total chromosome length was 567.49 µm; relative chromosome length ranges from 2.57 to 5.40; arm ratio and centromere index were recorded as 1.00–1.57 and 0.38–0.50 respectively.

For genotype with 2n=30, total chromosome length was recorded as 525.01 µm, relative chromosome length, arm ratio and centromeric index were found as 2.01–5.53, 1.00–3.75 and 0.211–0.483 respectively. In genotype 2n=32 the total chromosome length was 632.51 µm. Relative length of chromosome was 2.05–4.704 µm, arm ratio and centromeric index were recorded as 1.00-3.50 and 0.222-0.51 respectively.

Chromosome symmetry/asymmetry index (S/AI) for all seven (7) chromosomes complement showed symmetric karyotypes with the range from 1.1 to 1.4 (Table 1). All seven chromosome complements showed chromosome symmetry.

Karyotype asymmetry indices

Karyotype asymmetry indices of all the seven karyotypes and scattered diagrams are presented in Table 2, Figs 2, 3 & 4.

Among the karyotypes the highest TF% was recorded for *C. gigantea* (Blume) Hook.f. 2n=28 and lowest was also in the same genotype with chromosome complement 2n= 32. TF% and AsK% showed perfect negative corelation for all seven karyotypes. Except for the chromosome complements 2n=30 and 2n=32 of *C. gigantea* (Blume) Hook.f. TF% values fall in 41.08–45.39 which indicates symmetric karyotype. Lower value of TF% in chromosome complements 2n=30 and 2n=32 of *C. gigantea* (Blume) Hook.f. were recorded as 39.69 and 39.20 respectively and reflected karyotype asymmetry for these two complements. A1 and A2 values ranges from 0.15–0.30 and 0.19–0.44 respectively. A1 and A2 showed negative correlation for the complements 2n=32 of *C. gigantea*, (Blume) Hook.f. 2n=24 and 2n=28 of *C. fallax* Schott. and 2n=28 of *C. esculenta* (L.) Schott. While for 2n=28 of *C. gigantea* (Blume) Hook.f. A1 showed positive correlation with A2. CVcl and CVci values fall in the range of 19.82–44.02 and 9.45–20.73 respectively. Highest value of

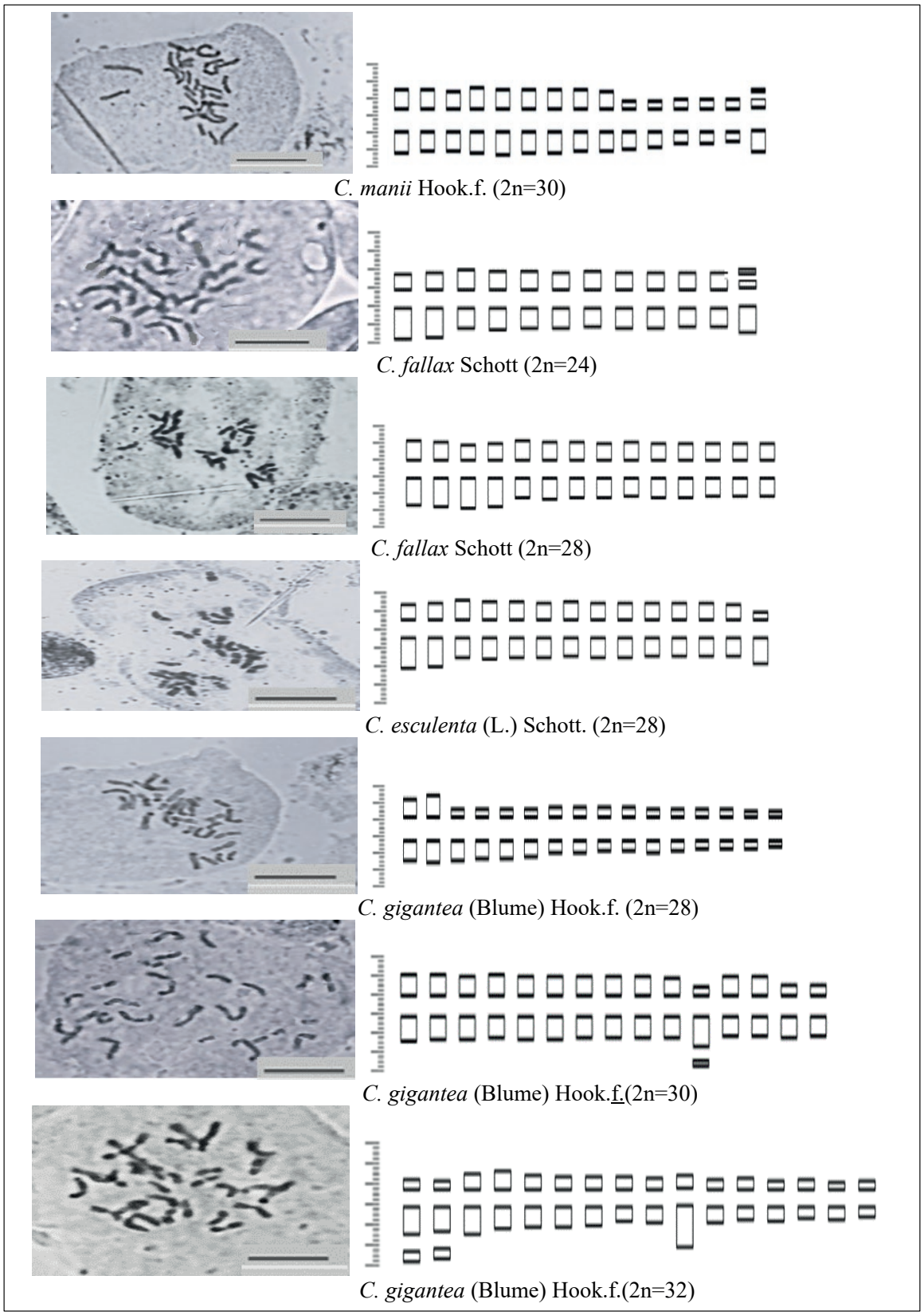

Figure 1. Karyotypes and ideogram of *Colocasia* genotypes of Assam.

Table 2. Karyotype asymmetry indices of four Species of *Colocasia* species.

Karyotype indices	*C. mannii* Hook. f.	*C.fallax* Schott		*C. esculenta* (L.) Schott	*C. gigantea* (Blume) Hook. f.		
	2n =30	2n=24	2n=28	2n=28	2n=28	2n=30	2n=32
TF%	43.99	43.64	41.08	41.73	45.39	39.69	39.20
ASK%	55.99	56.35	58.01	58.26	46.0	60.30	60.79
A	0.118	0.129	0.17	0.149	0.45	0.190	0.199
A_1	0.20	0.216	0.28	0.245	0.15	0.28	0.303
A_2	0.44	0.325	0.23	0.276	0.19	0.253	0.230
CV_{CL}	44.02	32.55	23.58	25.62	19.82	25.33	23.04
CV_{CI}	9.45	11.99	12.91	12.67	8.36	19.17	20.73
AI	26.56	27.13	28.21	21.93	23.69	23.21	21.14
Stebbins' Type	1C	2B	1B	2B	1B	1B	1B

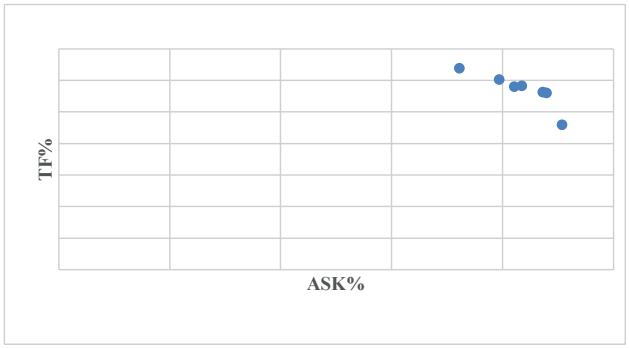

Figure 2. Scattered diagram for AsK % against TF %.

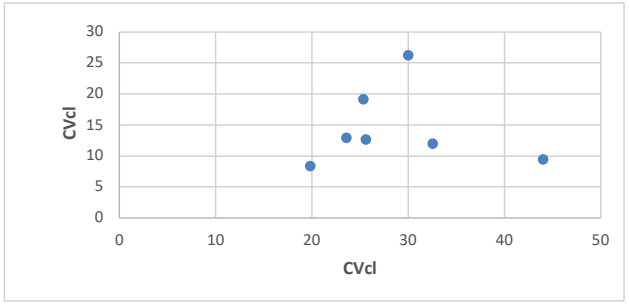

Figure 3. Scattered diagram for CVCL against CVCI.

CVcl & CVci were recorded in 2n=30 of *C. manii* Hook. f. and 2n=32 of *C. gigantea* (Blume) Hook.f. The CVcl and CVci was evident for positive correlation and their higher range of values indicated the heterogeneous karyotypes. AI values range from 21.14 –28.21.

Under the present investigation, *C. manii* Hook. f. (2n=30); *C. fallax* Schott. (2n=24 and 2n=28), *C. esculenta* (L.) Schott. (2n=28) and *C. gigantea* (Blume) Hook.f.

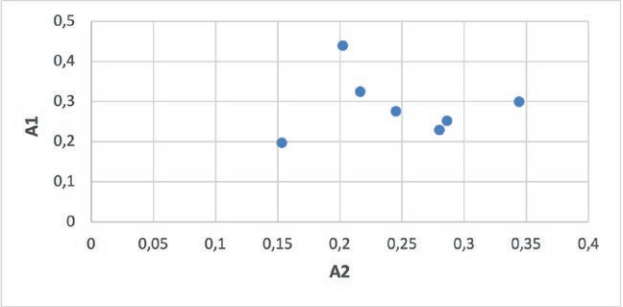

Figure 4. Scattered diagram for A2 against A1.

(2n=28) showed asymmetric karyotype with respect to TF%, A1, A2 and AI; but showed symmetric karyotype with accordance to AsK%, A, CVcl for the complement 2n=28 of *C. fallax* Schott.and 2n=28 of *C. esculenta* (L.) Schott. The complements 2n=30 and 2n=32 of *C. gigantea* (Blume) Hook.f. showed symmetric karyotype for the indices TF%, CVcl, AI and asymmetric karyotype for the indices AsK%, A, A1, A2 and CVci. All the genotypes with different chromosome complements showed Stebbins' asymmetry class 2B and 1B except for *Colocasia manii* Hook. f. which exhibited 1C.

Detail chromosome morphology and karyotype analysis is the potential source to establish the relationship among the genotypes and also to find out the divergence among the genotypes. Diversity of chromosome numbers (2*n*=26, 28, 36, 38, 42 and 56) in the genus *Colocasia* and also the presence of polyploidy cytotype has been reported (Yang 2003; Wang et al. 2017). Das et al. (2015) also reported ploidy level in *Colocasia* species with chromosome number 2n=42 (triploid). Under the present investigation the basic chromosome number n = 14 was found in all the four (4) species. Chair

et al. (2016) also reported genomic number n = 14 in the genus *Colocasia*. However intraspecific variation of chromosome numbers was found in *C.fallax* Schott (2n=24 & 2n=28) and in *C. gigantea* (Blume) Hook.f. (2n=28, 2n=30 & 2n=32) . Wang et al. (2017) reported inter and intraspecific chromosomal variation in five species of *Colocasia* with chromosome count 2n= 26, 28, 38, 42, and 56. Variation in somatic chromosome numbers in mitotic cells of many angiosperm species under the genus *Phalaris* (Poaceae) was reported by Winterfeld et al. (2018). Karyotype analysis may be good tools for identification of intra and inter-species variation but under the present investigation, the diversity in chromosome number within a species became a hindrance to establish the karyotype evolution with related taxa.

CONCLUSION

Karyotype analysis of *Colocasia* genotypes under present investigation showed basic chromosome number n=14. The prevalence of more metacentric chromosomes indicates primitiveness and chromosome symmetry. TF % and uniform Stebbins' chromosome type of the species also indicate karyotype symmetry and karyotype homogeneity implying primitiveness of the genus. Lower and almost uniform values of AI, CV_{CL}, $CV_{CI,}$ A1 and A2 also reflect karyotype homogeneity. Deviation of basic chromosome number from n=14 in some genotypes under two species viz. *C. fallax* Schott.and *C. gigantea* (Blume) Hook.f. *gives* an indication towards the occurrence of aneuploidy and chromosomal plasticity. Aneuploid in the species may widen the genetic variations which may lead to the formation of different diagnostic morphological and floral characters in the species. Presence of secondary constriction in the certain genotypes indicates high chromosomal plasticity and their potential relevance to chromosome evolution. Variation of chromosome number which may be due to aneuploidy or euploidy may cause variety of phenotypic changes in the species including plant architecture. These variations may cause dosage imbalance of gene on the affected chromosomes that may alter the phenotypic alteration of the species. In *Colocasia* under present investigation, variation of chromosome number within the species may contribute towards the establishment of new species.

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Robertsonian rearrangements in the genome of the azure damselfish, *Chrysiptera hemicyanea* (Perciformes, Pomacentridae)

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Abstract. Cytogenetic studies on the azure damselfish, *Chrysiptera hemicyanea* (Weber, 1913), revealed a karyotype with 2n = 31 chromosomes (17 metacentric, 2 submetacentric, and 14 telocentric; FN = 52). The study found Robertsonian polymorphisms, which involve small heterochromatic regions at the centromeres. Nucleolar organizer regions (NORs) were observed near the ends of the long arms on the large metacentric chromosome pair (pair 2). FISH analysis, which detects specific DNA sequences, revealed notable variability in the distribution of ribosomal DNA (5S and 18S rDNAs) along the chromosomes. Specifically, the 18S rDNA was located at the ends of the long arms on the large metacentric chromosomes (pair 2), while the 5S rRNA genes were found near the centromere of another large metacentric chromosome pair (pair 3). The analysis also showed that repetitive DNA sequences $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$ were spread across the subtelomeric and telomeric regions of various chromosomes. The study suggests that the structure of the karyotype and chromosome number are linked to the Robertsonian rearrangements observed, highlighting their significant role in the evolutionary changes in the karyotype of the genus *Chrysiptera*.

Keywords: azure damselfish, Chromosome, ribosomal DNA, Robertsonian rearrangements.

INTRODUCTION

When comparing the karyotypic (chromosomal) diversity of freshwater and marine fish, freshwater species exhibit more chromosomal changes. This increased diversity in freshwater environments is due to the numerous physical barriers present in continental waters, which limit gene flow and lead to greater karyotypic variation. In contrast, marine environments present complex and harder-to-define barriers, shaped by dynamic factors like ocean currents and winds, which can facilitate wider dispersal of species and lead to less distinct karyotypic differentiation. Genetic connectivity among marine fish popula-

tions, linked to changes in their karyotypes, is influenced not only by physical and ecological conditions but also by the pelagic larval stage, which is crucial for their dispersal (Brum & Galetti, 1997; Molina & Galetti, 2004; Martinez et al., 2015; Santos et al., 2024). Among Perciformes, the Pomacentridae family, which includes damselfish and clownfish, is particularly notable due to its close association with coral reefs (Allen and Werner 2002; Bellwood and Wainwright 2002; Tang et al., 2021). This family displays significant diversity in forms and biological traits, making it a useful model for studying how the pelagic (open water) period of larvae affects karyotypic evolution (Molina and Galetti, 2004; McCord et al., 2021).

As we advance into the era of genomic natural history, where genomic technologies offer significantly greater detail and statistical power, a reference genome will be crucial for enhancing our understanding of animal biology (Hotaling et al., 2021). Currently, there are 14 reference genomes available for Pomacentrids (Roberts et al., 2023), genetic variation in reef fish populations has been well-documented through studies of allozymes (variant forms of enzymes) and mitochondrial DNA (Knowlton et al., 1993; Shulman and Bermingham, 1995; Molina and Galetti, 2002; Limon et al., 2023). Despite this variation at the genetic level, many reef fish species maintain relatively stable chromosomal structures, even over extensive geographical ranges. A key factor in karyotypic differentiation among fish is Robertsonian chromosomal rearrangements, such as centric fusion (where two acrocentric chromosomes fuse at their centromeres) and fission (where a single chromosome splits into two). These rearrangements contribute significantly to chromosomal diversity and differentiation in various fish groups (Molina and Galetti, 2002; Getlekha et al., 2017).

In Pomacentridae reef fish, Robertsonian chromosomal rearrangements, particularly centric fusion, have been observed in various contexts. For example: *Dascyllus*: Centric fusion appears as a polymorphic trait, meaning different individuals or populations within the genus may exhibit this rearrangement (Ojima and Kashiwagi, 1981; Takai A., 2012; Getlekha et al., 2016a; Getlekha et al., 2017). *Chromis*: This genus also shows centric fusion, indicating its role in karyotypic diversity (Molina and Galetti, 2002). *Chrysiptera*: Centric fusion has been established in the derived karyotypes of specific species, highlighting its role in evolutionary changes (Takai and Ojima, 1995; Galetti et al. 2000; Hardie and Hebert, 2004; Molina and Galetti 2004). Moreover, new fish sex chromosomal systems have been associated with Robertsonian rearrangements (Brum et al., 1992). These rearrangements might contribute to the formation of genetically distinct populations by impeding gene flow.

In this study, we investigated the karyotype, heterochromatin pattern, and nucleolar organizer regions of the fish species *C. hemicyanea*. We employed several methods to analyze and identify chromosomal features: Microsatellite Sequences: Specific microsatellite markers, including $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$, were used to examine chromosomal patterns and identify specific chromosomes involved in the rearrangements. Repetitive DNA Probes: Fluorescence *in situ* hybridization (FISH) with probes for 18S rDNA and 5S rDNA was used to localize ribosomal RNA genes and further characterize chromosomal structures. These techniques helped identify chromosomes involved in Robertsonian rearrangements, contributing to a deeper understanding of the chromosomal evolution and structure in *C. hemicyanea*.

MATERIAL AND METHODS

For the cytogenetic study, ten male and ten female azure damselfish (*Chrysiptera hemicyanea*) samples were collected from the Gulf of Thailand (Pacific Ocean). Chromosomes were prepared using the techniques outlined by Getlekha et al. (2016a). The method for detecting nucleolar organizer regions (Ag-NORs) was based on Howell and Black (1980), while the visualization of heterochromatin bands (C-bands) followed the method described by Sumner (1972).

Fluorescence *in situ* hybridization (FISH) was carried out on the chromosomes of *C. hemicyanea* following the procedure described by Martins and Galetti (1998). For this, two DNA sequences, 5S rDNA and 18S rDNA, were used as probes. The sequences were amplified from nuclear DNA using PCR and derived from the genome of the fish species *Hoplias malabaricus*, which belongs to the Erythrinidae family. The probes were cloned into plas-

Figure 1. General characteristic of the azure damselfish (*Chrysiptera hemicyanea* (Weber, 1913)).

mid vectors using competent *Escherichia coli* DH5α cells (Invitrogen, San Diego, CA, USA). After cloning, the 5S and 18S rDNA probes were labeled with Spectrum Green-dUTP and Spectrum Orange-dUTP, respectively, following Roche's protocols (Mannheim, Germany) for nick translation. Additionally, Sigma (St. Louis, MO, USA) synthesized the microsatellites $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$, which were directly tagged with Cy3 at the 5' end.

High stringency conditions were used for fluorescence *in situ* hybridization (FISH) to ensure precise results. Initially, metaphase chromosomal slides were treated with 40 µg/ml RNase for 1.5 hours at 37°C to eliminate background interference and remove RNA. The hybridization solution was prepared with 10% dextran sulfate to enhance probe penetration, 2.5 ng/µl of labeled probes (microsatellites and rDNA), 2 µg/µl salmon sperm DNA to block non-specific binding, and 50% deionized formamide to minimize non-specific interactions. This mixture was then applied to the slides. To prepare the chromosomes for hybridization, the chromosomomal DNA was denatured in a solution of 70% formamide/2x SSC at 70°C for 4 minutes to prepare them for hybridization. The slides were then incubated overnight at 37°C in a moist chamber with 2x SSC buffer to allow the probes to bind to their target sequences. To reduce non-specific binding, excess unbound probes were removed by washing the slides first at 65°C for 5 minutes with 2x SSC and then at room temperature for 5 minutes with 1x SSC. The slides were mounted in Vectashield antifade solution to prevent photobleaching and enhance fluorescence, and counterstained with DAPI to visualize the DNA. This careful preparation ensured that the FISH analysis could detect microsatellite and rDNA sequences with high specificity and clarity.

After that, metaphase spreads were captured with a CoolSNAP camera and analyzed using Image Pro Plus 4.1 software. Imaging was performed with an Olympus BX50 microscope.

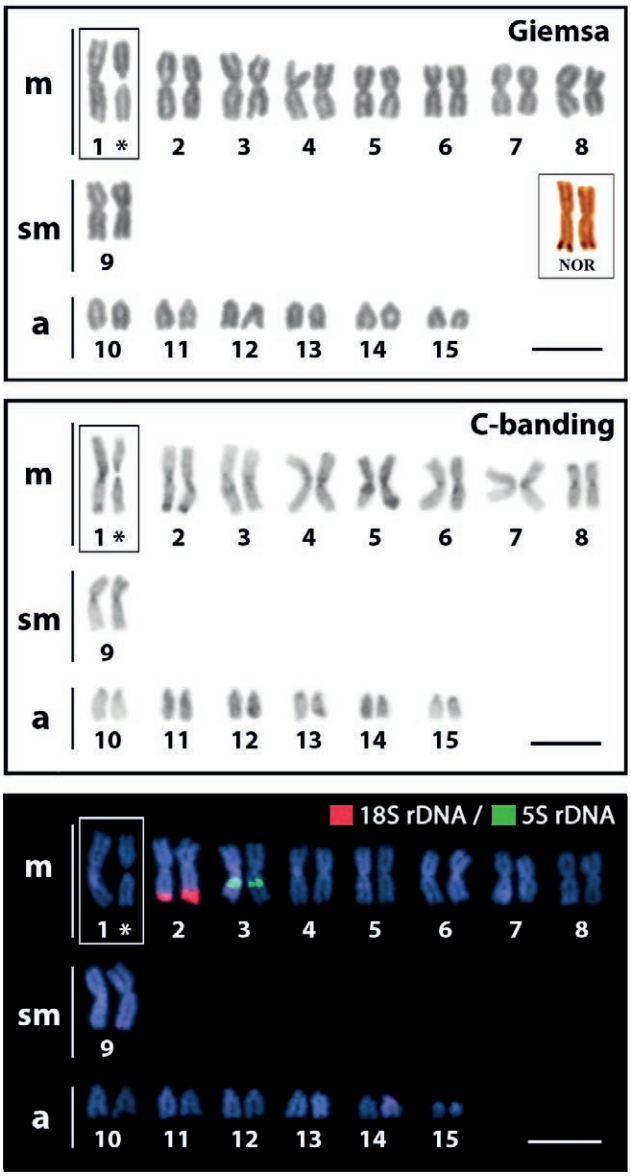

Figure 2. Karyotypes of *Chrysiptera hemicyanea* were analyzed using several methods: Giemsa staining, Ag-NOR banding (highlighted in the boxes), C-banding, and fluorescence *in situ* hybridization with 5S and 18S rDNA probes. Chromosomes involved in centric fusions are indicated in the larger boxes. Scale bar = 5 µm.

RESULTS

The azure damselfish (*Chrysiptera hemicyanea*) has a karyotype with 31 chromosomes and a fundamental number of 52 in both male and female, including 17 metacentric, 2 submetacentric, and 14 acrocentric chromosomes. Notably, the karyotype features large metacentric chromosomes that are nearly twice as large as the others. Nucleolar organizer regions (Ag-NORs) were located at the ends of the long arms on one pair of these large metacentric chromosomes. The C-banding analysis showed small blocks of heterochromatin primarily at the centromeres of most chromosomes.

FISH analysis showed that 18S rDNA clusters were located at the telomeric ends of two large metacentric chromosome pairs (pair No. 2). On the other hand, 5S rDNA genes were exclusively present in the pericentromeric regions of two different large metacentric chromosomes (pair No. 3). These results offer valuable information about the genomic arrangement of the azure dam-

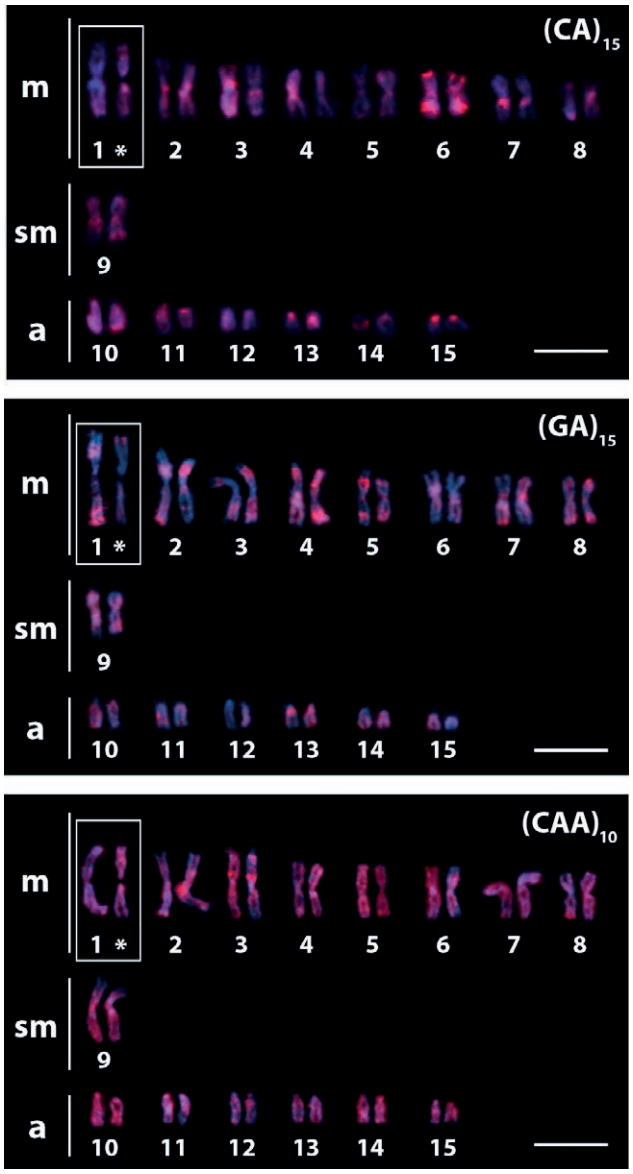

Figure 3. Fluorescence *in situ* hybridization was used to map the chromosomes of *Chrysiptera hemicyanea* with di- and tri-nucleotide microsatellites. The distribution patterns for (CA)15, (GA)15, and (CAA)10 microsatellites as probes are illustrated. Scale bar = 5 μm.

selfish, particularly in relation to the placement of ribosomal DNA sequences on its chromosomes.

Microsatellite sequences in *C. hemicyanea* are distributed unevenly across its chromosomes, as revealed by chromosomal mapping. Strong hybridization signals were observed in both the telomeric and interstitial regions of many chromosomes, indicating a broad distribution. Specifically, the $(CA)_{15}$ microsatellite sequence was primarily found on metacentric chromosome No. 6. Conversely, the $(GA)_{15}$ sequence, though weaker than $(CA)_{15}$, was distributed throughout the interstitial regions of all chromosomes. The $(CAA)_{10}$ sequence showed strong signals on several chromosomes. No significant differences were observed between males and females, suggesting that microsatellite distribution is similar in both sexes. These findings, which highlight the varied and uneven distribution of microsatellite sequences, provide valuable insights into the genomic organization and diversity of *C. hemicyanea*.

DISCUSSION

Recent genetic research on marine fish has shed light on how speciation occurs, emphasizing the importance of chromosome rearrangements in this process (King, 1987; Crandall et al., 2019). These chromosomal changes are thought to play a key role in the formation of new species. In freshwater fish, species with limited mobility and smaller reproductive populations often show greater karyotypic diversity, with a wider range of chromosome structures (Bertollo et al., 1979; Moreira-Filho and Bertollo, 1991; Sribenja and Getlekha, 2024a,b). In contrast, marine fish generally display more stable karyotypes due to fewer physical barriers, higher mobility, larger populations, and more consistent environmental conditions (Brum, 1995; Liggins et al., 2016).

In some families of Perciformes, such as the Gobiidae, distinct cytogenetic traits can help differentiate populations, with notable chromosome polymorphism linked to centric fusions and fissions (Giles et al., 1985; Vitturi and Catalano, 1989; Amores et al., 1990). However, in other fish groups, cytogenetic data may not provide clear markers for species identification, even in widely distributed species (Rossi et al., 1996). This lack of clear differentiation due to significant chromosomal rearrangements might be countered by internal changes within linkage groups, which can contribute to post-zygotic barriers essential for speciation (Molina et al., 2002).

The patterns of chromosomal rearrangements between Pomacentridae genome assemblies remain unclear. Numerous cytogenetic studies have explored how variations in chromosome number influence fish mobility, revealing an inverse relationship between chromosome diversity and mobility. Furthermore, chromosome rearrangements have been found to either facilitate or inhibit recombination events (Galetti et al., 2000; Molina and Galetti, 2004; Kirkpatrick and Barton, 2006; Martinez et al., 2015). Robertsonian polymorphisms play a significant role in karyotype variation across different Perciformes families, including Pomacentridae, Gobiidae, and Cichlidae. In addition to verifying variations in chromosome number, the dot-plot

comparison between this genome and that of the closest relative with a chromosome-scale assembly uncovered multiple rearrangements across all corresponding chromosomes (Roberts et al., 2023). For instance, in the Pomacentridae family, most species have a relatively stable karyotype with 48 chromosomes. However, Chromosome formulae vary widely (FN = 48–90), often due to pericentric inversions that contribute to karyotype diversification (Takai and Ojima, 1987). In contrast, the Chrominae subfamily, which includes genera such as *Acanthochromis*, *Azurina*, *Chromis*, and *Dascyllus*, exhibits notable Robertsonian polymorphisms. Diploid numbers within this subfamily vary considerably: *D. trimaculatus* (47–48), *D. reticulatus* (34–37), *D. aruanus* (27–33), *C. insolata* (46–47), and *C. flavicauda* (39) (Ojima and Kashiwagi, 1981; Molina and Galetti, 2002; Getlekha et al., 2016a,2017; Yuan et al., 2018). Although asynchronic hermaphroditism is prevalent among these species, the existence of multiple sexual chromosomes does not seem to correlate with the karyotypic variations observed (Ojima and Kashiwagi, 1981; Getlekha and Tanomtong, 2020). This assembly will provide a crucial basis for examining how genome structure varies at a metapopulation level and how these variations influence recombination and adaptation.

Gene flow among Pomacentridae populations mainly occurs through the drifting or active migration of pelagic larvae, as the adult fish are generally nonmigratory (Allen, 1991; Robitzch et al., 2016). Current evidence does not indicate a direct relationship between the length of the larval stage and the geographic range of these fish. Some researchers suggest that the complex behaviors of fish larvae (Leis and Carson-Ewart, 1998) might result in hydrodynamic movements that could actually limit their dispersal rather than facilitate it (Doherty et al., 1994; Salas et al., 2020).

Previous research on the genus *Chrysiptera* have revealed differences in the diploid chromosome number and fundamental number among species, which are caused by chromosomal rearrangements (Table 1). Centric fusions likely account for the species with fewer chromosomes and distinctive metacentric chromosomes. This chromosomal variation could be a transient phenomenon, reflecting a specific stage in the evolutionary process within the *Chrysiptera* species, potentially indicative of karyotypic orthoselection (White, 1973; Artoni et al., 2015; Santos et al., 2024).

In *C. hemicyanea*, the nucleolar organizer regions (NORs) reflect their evolutionary connections. Although the NORs are positioned at the ends of metacentric chromosomes in *C. hemicyanea*, their distribution pattern is quite similar to that observed in other Pomacentrid species, despite being located at a subterminal position on the metacentric chromosome (2nd pair) (Takai and Ojima, 1987; Takai and Ojima, 1995; Artoni et al., 2015).

Chromosomal regions that are differentially stained using C-banding techniques, known as C-bands, highlight localized areas of constitutive heterochromatin. In fish, C-bands are primarily found in centromeric regions, and occasionally in telomeric and interstitial regions (Gold et al., 1986; Takai and Ojima, 1988; Kashiwagi et al., 2005). Although there is considerable variation in the distribution of C-banded heterochromatin among chromosomes and species, many fish species exhibit C-bands as small dot-like formations predominantly in centromeric regions, with often weak staining. This relatively simple distribution of C-bands appears to be a fundamental characteristic in fish. In *C. hemicyanea*, the distribution patterns of centromeric C-bands, appearing as small dot-like spots in centromeric and telomeric regions, are consistent with previous cytogenetic studies. However, *C. hemicyanea* also displays distinctive C-bands in the terminal regions of the long arms of NOR-bearing chromosomes (pair no. 2). These observations suggest that *C. hemicyanea* has a notably differentiated karyotype with respect to the distribution of constitutive heterochromatin.

Table 1. Karyotype data of some genus *Chrysiptera*.

Species	2n	NF	Formula	NORs	Reference
Chrysiptera cyanea	42	66	6m+16sm+2st+18a	2	Takai and Ojima, 1995
C. leucopoma	48	80	4m+22sm+6st+16a	2	Takai and Ojima, 1995
C. rex	36	58	12m+10sm+14st-a	2	Takai and Ojima, 1995
C. rollandi	46	50	2sm+2st+42a	2	Kasiroek et al., 2014
C. starckii	48	60	2m+10sm+36a	2	Takai and Ojima, 1987
C. hemicyanea	48	78	30sm+10st+8a	2	Takai and Ojima, 1999
	31	56	8m+10sm+32a	2	present study

2n = diploid number, NF = fundamental number, NORs = nucleolar organizer regions, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric chromosome.

The distribution of C-bands in pomacentrids varies widely, ranging from simple to complex patterns (Takai and Ojima, 1999). These variations highlight the significance of constitutive heterochromatin in chromosome evolution. The C-band distribution in *C. hemicyanea*, which exhibits G-band-like patterns (Takai and Ojima, 1999), appears to be a rare phenomenon among fish chromosomes. This pattern represents one of the most differentiated conditions not only within Pomacentridae but also among teleostean fish.

The alignment of 18S rDNA probes with the Ag-NOR clusters in *C. hemicyanea* suggests that having ribosomal DNA clusters on a single chromosomal pair is a fundamental trait for the Pomacentridae family (Molina, 2000; Getlekha et al., 2016a, b). In contrast, 5S rDNA clusters were located on a different pair of metacentric chromosomes, specifically subterminally on the long arm, and did not overlap with the chromosome pair containing the NORs. Previous research on 5S rRNA gene mapping has been conducted in genera like *Chromis*, *Dascyllus*, *Abudefduf*, and *Pomacentrus* (Molina and Galetti, 2002; Getlekha et al., 2016a, b; 2018; Zhang et al., 2021). The 5S rDNA loci appear to be more conserved in Pomacentridae, usually positioned in the pericentromeric region of a chromosome. In summary, various FISH applications using 18S and 5S ribosomal genes have proven effective in establishing phylogenetic relationships, distinguishing species, and understanding historical population dynamics in both freshwater and marine environments (Artoni et al., 2015).

Microsatellite sequences like $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$ show significant variation in their distribution across the chromosomes of *C. hemicyanea*. Most of these sequences are concentrated in telomeric regions, which are rich in DNA repeats. Their clustering on specific chromosomes suggests that repetitive DNA dynamics could influence divergence among pomacentrid fish species. Research has shown that repetitive DNAs are crucial in the evolution of genomes across different fish species (Cioffi and Bertollo, 2012; Moraes et al., 2017; Saenjundaeng et al., 2018, 2020; Sassi et al., 2019; Yano et al., 2014; Yüksel and Gaffaroglu, 2008). In this study, microsatellites $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$ are present on all chromosomes of *C. hemicyanea*, with notable clusters showing strong signals in specific regions (Figure 3). Recent and previous studies indicate that microsatellites are commonly located in heterochromatic regions of fish genomes (Getlekha et al., 2016a, b; 2018).

Investigating evolutionary relationships within the genus *Chrysiptera* necessitates analyzing these data. Despite differences in chromosomal architecture, similar microsatellite distribution patterns might indicate shared evolutionary histories. Conversely, variations in these patterns could highlight rapid changes in microsatellite sequences (Cioffi et al., 2011; Molina et al., 2014a, b). Analyzing microsatellite sequence structures provides valuable insights into the functional diversification and organization of repeated DNAs across species. However, studies examining how chromosome structure influences ecology, population dynamics, and adaptive evolution are facilitated by chromosome-scale genomes, which provide more detailed gene sequences and their locations (Roberts et al., 2023). Understanding the distribution and evolution of microsatellite sequences in *C. hemicyanea* enhances our knowledge of genomic dynamics and evolutionary processes in pomacentrid fishes. This insight also aids in more thorough investigations of genetic diversity and speciation.

Our focus is on Robertsonian rearrangements in the karyotypes of *C. hemicyanea*. According to Ojima (1983), in higher teleostean groups, including Pomacentridae, the average number of subtelo- and acrocentric (A-type) chromosomes is 38.3, while meta- and submetacentric (M-type) chromosomes average 7.5. In contrast, the pomacentrids studied show an average of 30.2 A-type chromosomes and 17.1 M-type chromosomes. This indicates that Pomacentridae has undergone significant structural changes, with a notable transition from A-type to M-type chromosomes.

CONCLUSION

Teleostean fishes frequently display a notable karyotypic characteristic: many species possess 48 diploid chromosomes, with a majority being acrocentric. This observation suggests a conservative pattern in the evolution of fish karyotypes (Ohno, 1974; Ojima, 1983). Gosline (1971) and Ojima (1983) noted that this karyotypic feature is especially prominent in intermediate and higher teleostean groups. Ohno (1974) proposed that a karyotype consisting of 48 acrocentric chromosomes (48A karyotype) might represent the ancestral form in fish evolution, as it appears across various fish families and orders.

In the Pomacentridae family, diploid chromosome numbers vary from 26 to 48, and fundamental numbers range from 48 to 84. Some species within this family also exhibit the 48A karyotype. It is hypothesized that the 48A karyotype could have been ancestral for Pomacentridae, with subsequent diversification occurring primarily through pericentric inversions and Robertsonian rearrangements (Takai and Ojima, 1987, 1991, 1995). Furthermore, this study proposes that the karyotypic diversification in pomacentrids has been influenced not

only by structural chromosomal changes but also by variations in the amount and placement of constitutive heterochromatin. In the future, telomeric probes will be tested to further enhance the understanding characterization of the karyotype of *Chrysiptera hemicyanea*.

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What defines a bimodal karyotype? Bimodality revisited

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Abstract. Bimodal karyotypes, initially defined by Avdulov, are characterized by one large and one small set of chromosomes, reflecting a particular type of karyotype asymmetry. Despite later discussions by Stebbins, the absence of a quantitative criterion has led to subjective classifications. This study revisits the concept of bimodality through a literature review and proposes an objective criterion based on the ratio between the smallest chromosome of the larger set and the largest of the smaller set. Chromosome morphology and asymmetry were analyzed in 32 species previously classified as bimodal. Statistical tests were applied to detect size discontinuities and assess bimodality. We propose two forms of bimodality, interchromosomal and intrachromosomal, considering differences in size and morphology. Our results show that *Drosophila melanogaster* and *Scaphura nigra* exhibit trimodal karyotypes. A ratio of ≥1.5:1 between chromosomal subsets provides a clear and objective criterion for defining bimodality, aligning with the original concepts of Avdulov and Stebbins.

Keywords: Avdulov, bimodal karyotype, chromosome asymmetry, chromosome variation, cytogenetics, Stebbins.

INTRODUCTION

Delaunay (1923) is credited with possibly coining the term "karyotype," which refers to the complete set of chromosomes found in the nucleus of a somatic cell. Each functional metaphase chromosome is equipped with telomeres and replication origins, as well as a primary constriction known as the centromere, which plays a crucial role in cell division by anchoring to a molecular structure called the kinetochore (Bodor et al. 2014). The centromere divides the chromosome into two parts, typically a short arm and a long arm. Its position determines the classification of each chromosome based on the ratio of their arms, which can be categorized as metacentric, submetacentric, acrocentric, or telocentric (Guerra 1986). However, variations of this classification can be found in the literature (Levan et al. 1964).

The exception is holokinetic chromosomes, which lack a primary constriction because they have kinetochores distributed along the entire length of the chromosomes (Wrensch et al. 1994), as in some genera of the families Cyperaceae and Juncaceae (Greilhuber 1995; Balslev 1996; Guerra et al. 2019).

The karyotype represents the first phenotypic expression of the genotype (Guerra 2008), exhibiting remarkable diversity that reflects evolutionary processes (Carta et al. 2018). Karyotype evolution involves multiple levels of variation, resulting from changes in both chromosome number and structure (Mayrose and Lysak 2021). These changes often exhibit phylogenetic correlations, as evidenced by the multitude of traits commonly observed in comparative analyses (Oliveira et al. 2015; Moraes et al. 2017; Chase et al. 2023). Karyotypes exhibit variations in terms of chromosome number, size, and centromere positioning, as well as the presence and positioning of secondary constrictions. These differences encompass aspects of chromosome morphology and molecular composition (Weiss-Schneeweiss and Schneeweiss 2013).

In eukaryotes, the smallest chromosome number is $2n = 2$. This has been documented in the helminth *Parascaris univalens* (Nielsen et al. 2014) and the ant *Myrmecia pilosula* (Crosland and Crozier 1986). In plants, the smallest chromosome number is $2n = 4$, as seen in *Haplopappus gracilis* A.Gray and *Brachyscome dichromosomatica* C.R. Carter (Asteraceae) (Tanaka 1967; Leach et al. 2004), along with certain Poaceae, Cyperaceae, and Asparagaceae species (Bennett et al. 1986; Vanzella et al. 2003; Violetta et al. 2005). On the opposite end of the spectrum, *Sedum suaveolens* Kimnach. (Crassulaceae), with $2n$ = ca. 640 between the angiosperms, and the monilophyte *Ophioglossum reticulatum* L. have the highest chromosome count recorded with $2n = 1{,}260$ (Guerra 1988a).

A symmetrical karyotype is characterized by the predominance of metacentric and submetacentric chromosomes of relatively uniform sizes, a trait observed in different groups (Bertollo et al. 1983; Castro et al. 2016). Asymmetrical karyotypes exhibit an increasing number of acrocentric chromosomes, along with greater variation in chromosome size, making the karyotype more heterogeneous (Levitsky 1931; Stebbins 1971; Paszko 2006), exemplified by *Welwitschia mirabilis* Hook. with $2n = 42$ acrocentric chromosomes (Khoshoo and Ahuja 1962) and several species of *Oxalis* L. (De Azkue and Martinez 1983), insects as *Frankliniella* and *Selenothrips* (Brito et al. 2010) and mammals (Yang et al. 1997).

Typically, variations in chromosome size and morphology are evaluated using inter- and intrachromosomal asymmetry indices, respectively (Paszko 2006; Chiarini and Barboza 2008; Souza et al. 2010; Pierozzi 2011; Alves et al. 2011; Assis et al. 2013; Medeiros-Neto et al. 2017). Chromosome size and morphology varies considerably and, according to Stebbins (1971), asymmetric karyotypes originated from symmetrical ones. There must be definite limits to the number, size, and morphology of chromosomes within a karyotype; exceeding these limits could impair processes like mitosis and meiosis. However, these limits exhibit remarkable flexibility.

Occasionally, this asymmetry becomes extreme, showcasing pronounced differences in chromosome size and shape, thus allowing for the formation of two distinct subsets of chromosomes within the karyotype. Concerning interchromosomal asymmetry specifically in terms of chromosome size, these subsets emerge: one comprising larger chromosomes and the other smaller ones. Avdulov (1931) coined the term "bimodal" to describe karyotypes that consist of two sharply discontinuous chromosomal subsets: one with large chromosomes and the other with small chromosomes. Although asymmetry and bimodality are related concepts, they are distinct. A bimodal karyotype always exhibits some level of asymmetry; however, an asymmetrical karyotype is not necessarily bimodal.

Bimodality is evident in certain cases, such as *Eleutherine bulbosa* Urb. and species within the family Asparagaceae, where classifying the karyotype as bimodal is straightforward (Goldblatt and Snow 1991). However, in other plant groups like certain orchids, karyotypes are classified as bimodal, such as *Vanilla planifolia* Andrews (Piet et al. 2022), where a gradual variation in chromosome size is observed. In this case, the variation in chromosome size differs significantly from traditionally recognized bimodal karyotypes (Avdulov 1931; Watkins 1936; Stebbins 1971). It is evident that the concept of bimodality is primarily related to interchromosomal variation. On the other hand, could karyotypes characterized by a predominance of metacentric and acrocentric chromosomes, without submetacentric ones, be considered as a form of intrachromosomal bimodality?

All These questions arise due to the absence of a clear criterion defining a bimodal karyotype. For instance, in some representatives of *Drosophila melanogaster* Meigen, one chromosome pair is notably smaller than the others, leading to a distinct discontinuous variation in size among the chromosomes. Although this karyotype exhibits clear discontinuity, it is not classified as bimodal in the literature, illustrating instances where bimodal karyotypes are overlooked. Conversely, there are cases where karyotypes exhibit continuous variations in chromosome size but are classified as bimodal. Some karyotypes feature three sets of chromosomes in terms of size, a trait observed in many grasshopper spe-

cies, which are referred to as bimodal (Mesa et al. 2010). Additionally, there are karyotypes composed of metacentric and acrocentric only, as in *Chaetanthera renifolia* (J.Rémy) Cabrera (Asteraceae) with 2*n* = 44, being two metacentric and 42 acrocentric chromosomes only (Baeza et al. 2010), opening the possibility of being considered bimodal with respect to chromosome morphology.

The objective of this work is to reassess the concept of bimodal karyotypes. We conducted a thorough review of the literature to examine the usage of the term and to identify any deviations from Avdulov's original concept. Additionally, we delved into the primary theories concerning the evolutionary origins of bimodal karyotypes, supported by clear evidence in the literature. Furthermore, we undertook a comparative statistical analysis of bimodal karyotypes. This was done with the aim of establishing a clear criterion for defining bimodality, consistent with the framework established by Avdulov (1931) and later expanded upon by Stebbins (1971).

MATERIALS AND METHODS

Data collection

A literature review was conducted by searching for articles containing the keywords "Bimodal Karyotype or Bimodality". In each article, the concept of bimodal karyotype was highlighted when available, along with the species whose karyotypes were classified as bimodal. All concepts, including the criteria used for the application of the term, were compared and discussed with the definition of bimodal karyotype as originally established by Avdulov (1931) and Stebbins (1971).

Images of the karyotypes of some species recorded in the papers as presenting bimodal karyotypes were selected for analysis, provided they included a micrometer scale for comparison and clear chromosome morphology. For each karyotype, the size of all chromosomes was measured using the software Imagetool® version 3.0 (available at http://compdent.uthscsa.edu/dig/itdesc.html), calibrated with the scale available in the selected images. Additionally, the morphology of all chromosomes per karyotype was established based on Guerra (1986).

Among the asymmetry indices, the A_1 and A_2 by Romero-Zarco (1986) were utilized in our analyses as they are considered the most accurate in assessing dissimilarity among chromosomes in a karyotype (Paszko 2006). The classification of karyotypic asymmetry by Stebbins (1971) was also employed for karyotype comparisons (Paszko 2006). Ideal karyotypes according to Stebbins (1971), representing the theoretically possible extremes of symmetry and asymmetry, were constructed using Photoshop CS3 (Figure 1). Real karyotypes close to the ideal schematic karyotypes were also presented to demonstrate the analyses (Figure 2).

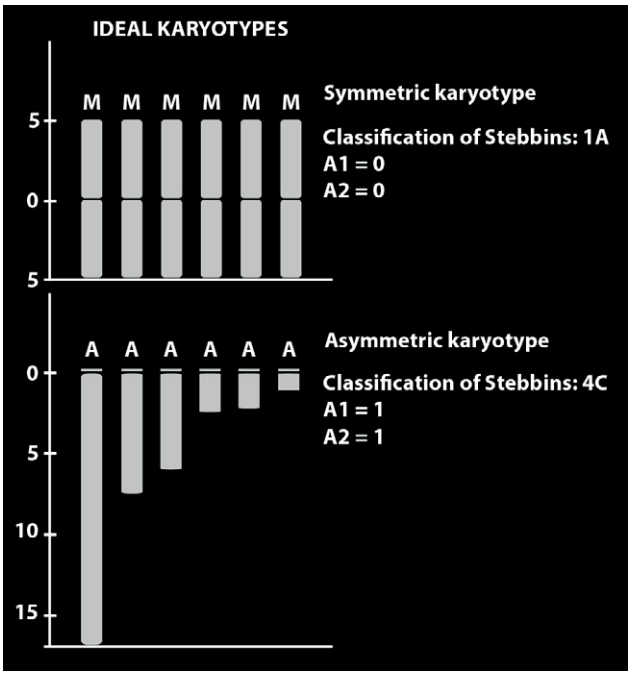

Figure 1. Idiograms of the theoretically possible ideal karyotypes with *n* = 6. The first represents the extreme of symmetry, composed of exactly identical metacentric chromosomes (M), classified by Stebbins as 1A, and Romero-Zarco (1986) indices A1 = 0 and A2 = 0. The second represents the extreme of asymmetry, composed of acrocentric chromosomes (A), classified by Stebbins as 4C, and Romero-Zarco (1986) indices A1 = 1 and A2 = 1. The chromosomes are aligned at the centromere position. A scale in μm is displayed on the left.

Inter- and intrachromosomal asymmetry, as well as the discontinuity in size between chromosome groups of the analyzed species, were compared with three species classified by Stebbins (1971) as presenting bimodal karyotypes: *Aloe zebrina* Baker and *Consolida regalis* Gray (now *Delphinium consolida* L.) and *Muscari comosum* (L.) Mill. (Figure 3). Based on this information, clear quantitive and qualitative criteria were established to better define the bimodality of a karyotype.

Statistical analyses

The chromosome size data were collected and organized into a vector containing measurements in micrometers. These measurements were subsequently converted into a data frame to facilitate subsequent analyses in the R 4.4.1 statistical environment. To compare the efficien-

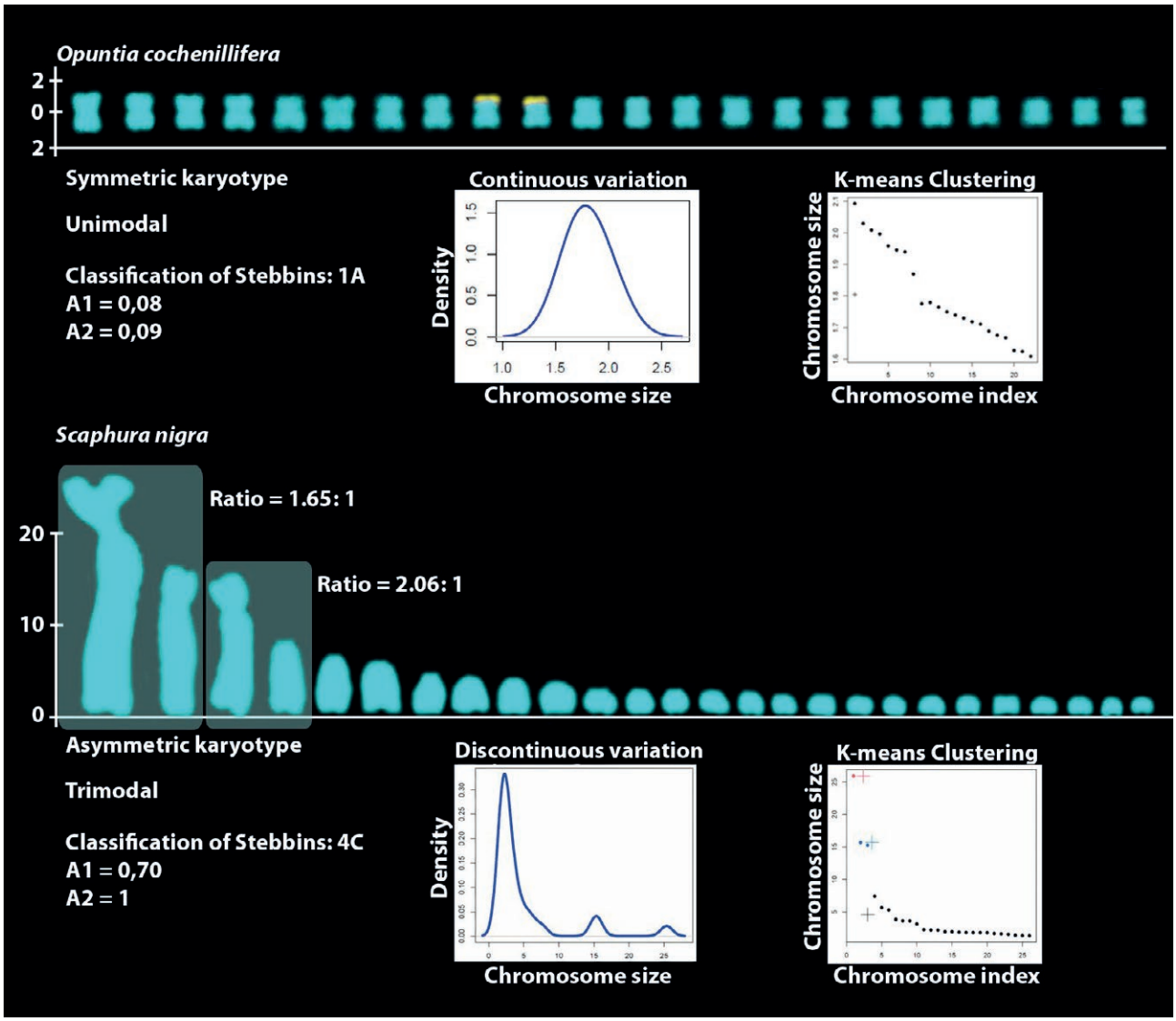

Figure 2. Karyograms of a real symmetric karyotype (*Opuntia cochenillifera* (L.) Mill with 2*n* = 22) and an asymmetric Trimodal karyotype (*Scaphura nigra* Stål with 2*n* = 26). The first karyogram represents symmetry, composed of very similar metacentric chromosomes (M), classified by Stebbins as 1A, with Romero-Zarco (1986) indices of A1 = 0.08 and A2 = 0.09. The second karyogram (schematic drawing based in Mesa et al. 2010) represents almost extreme asymmetry, composed of submeta and acrocentric chromosomes, classified by Stebbins as 4C, with Romero-Zarco (1986) indices of A1 = 0.70 and A2 = 1. The number of peaks in the density plot indicates continuous or discontinuous variation, respectively. The k-means clustering displays the number of chromosome subsets in different colors based on discontinuity. The proportion between subsets is shown for *Scaphura nigra*. A scale in μm is displayed on the left.

cy of different statistical methods in detecting discontinuities and bimodality in chromosome size within each karyotype, Hartigan's Dip Test, Silverman's Test and proportionality analysis were also utilized. All analyses were conducted using the statistical software R 4.4.1. Criteria such as sensitivity in detecting bimodality, robustness to different distribution patterns of chromosome sizes, and interpretability of the results were considered to compare the efficiency of each method. The results were analyzed based on the consistency and interpretation of evidence provided by Stebbins (1971) and Avdulov (1931).

Histograms and density plots

To verify the continuous or discontinuous variation in chromosome sizes, histograms and density plots were used. The histogram allowed the observation of the fre-

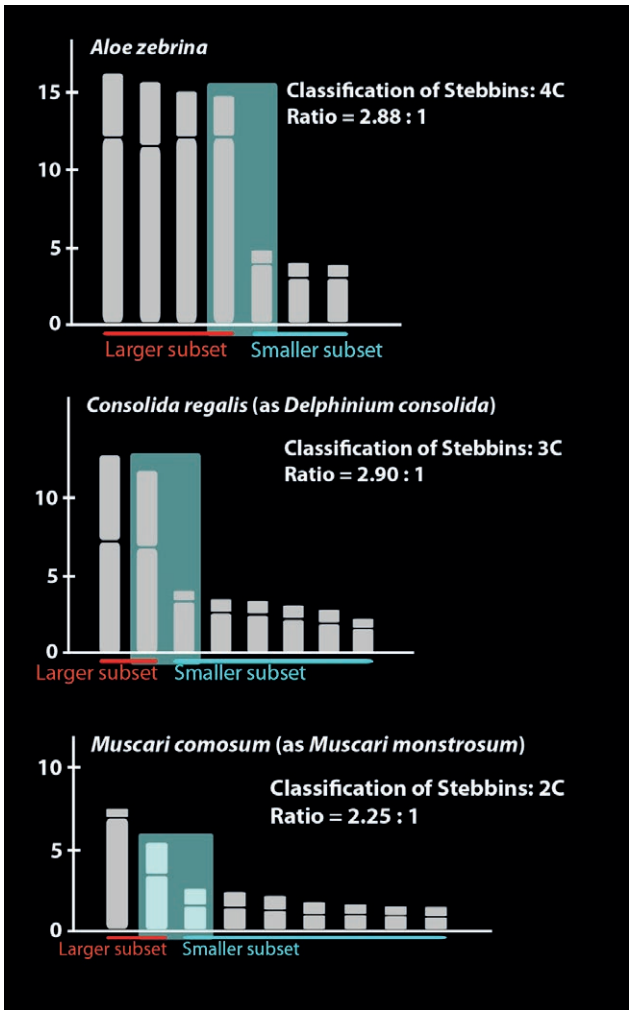

Figure 3. Idiograms of the species *Aloe zebrina* with $n = 7$ (classified by Stebbins as 4C), *Consolida regalis* with $n = 8$ (classified by Stebbins as 3C) and *Muscari comosum* with $n = 9$ (classified by Stebbins as 2C). Chromosomes are aligned by the base and in descending order. The gray box highlights the smallest chromosome of the larger subset next to the largest chromosome of the smaller subset. The ratio between these highlighted chromosomes is given alongside. A scale in μm is displayed on the left.

quency of different size measurements, while the density plot provided a continuous visualization of the data distribution. The density plot was used to provide a continuous estimate of the distribution of chromosome sizes, helping to identify the presence of chromosomes subsets (Thrun et al. 2020).

K-means clustering analysis

The K-means clustering analysis is a statistical technique used to partition a dataset into k clusters, where each observation belongs to the cluster with the nearest mean (Jain 2010). This technique can reveal distinct patterns in the variation of chromosome sizes, indicating whether the distribution is continuous and unimodal or discontinuous and bimodal (Wu 2012). When the variation in chromosome size is continuous and unimodal, the data tend to distribute smoothly and gradually, forming a straight line. Statistically, this means that the data density shows a single main peak. A greater number of clusters with distant centroids indicate the presence of multiple modes (or chromosome subsets). Thus, the variation within clusters is smaller, but the variation between clusters is larger.

The Hartigan's Dip Test

Hartigan's Dip Test was applied to assess the unimodality of chromosome sizes. This statistical test evaluates whether the data distribution can be considered unimodal or if there is evidence of bimodality (Hartigan and Hartigan 1985). Hartigans' Dip Test is effective at detecting multimodality in a data distribution, and it does not assume a specific distribution of the data (such as normality), making it flexible for several distribution shapes. However, it requires a sufficient number of observations to accurately detect multimodality. With small samples, it may not be able to distinguish between closely spaced modes. The choice of significance level can affect the interpretation of results, leading to some subjectivity in determining multimodality.

The Silverman Test

Silverman's Test complemented Hartigan's Dip Test by offering an alternative approach to detecting bimodality in chromosome sizes using kernel density estimates to assess data distribution shape (Silverman 2017). The Silverman Test is specifically designed to test the hypothesis of bimodality *versus* unimodality, being highly sensitive to detect two distinct peaks in a distribution. This test may be more effective in detecting bimodality in smaller samples compared to Hartigan's Dip Test. However, although it is more flexible than many parametric tests, it still assumes that the underlying shape of the distribution is smooth, which may not be suitable for all distributions.

Regression analysis

Regression was conducted to examine the relationship of the ratio between the smallest chromosome of the

larger subset and the largest chromosome of the smaller subset in putative bimodal karyotypes and the p-values from the Silverman Test. No specific transformations were necessary as the variables were ready for analysis. For the Welch's t-test, the data were divided into two groups based on the chromosome ratio: one group with ratios < 1.50:1 and another with ratios ≥ 1.50:1. A simple linear regression model was chosen to assess the relationship between the chromosome ratio (independent variable) and the p-values from the Silverman Test (dependent variable). The analysis was performed using R software. The regression results were visualized in a scatter plot with the following characteristics: The x-axis represents the chromosome ratio, and the y-axis represents the p-values from the Silverman Test. Blue points represent species with p-values ≤ 0.05, while black points represent species with p-values > 0.05. The vertical blue line represents the 1.50:1 ratio, and the horizontal red line indicates the significance level (p = 0.05).

RESULTS

Kariomorphometry data and karyotype asymmetry

In this study, we analyzed 32 species identified as having bimodal karyotypes in scientific articles. The species, along with their respective diploid chromosome numbers, the size of the largest and smallest chromosome in the complement, intra- (A_1) and interchromosomal asymmetry (A_2) according to Romero-Zarco (1986), asymmetry classification of Stebbins (1971), the size of the smallest chromosome of subset 1 and the largest chromosome of subset 2 (SCh1-LCh2), and when it occurred, the smallest chromosome of subset 2 and the largest chromosome of subset 3 (SCh2-LCh3), as well as the ratio between subsets are summarized in Table 1.

The chromosome numbers of the analyzed species ranged from 2*n* = 8 in *Drosophila melanogaster* and *H. chillensis* (Kunth) Britton to 2*n* = 90 in *Agave fourcroydes* Lem. (Table 1). The smallest chromosome among the analyzed species was recorded for *Puya mirabilis* (Mez) L.B.Sm. with 0.53 µm, while the largest was recorded for *Scaphura nigra* Stål (Orthoptera) with 25.90 µm (Table 1), which also exhibited the greatest discrepancy between the largest and smallest chromosome in the complement (27.30 times). The smallest difference was observed in *Oxalis linarantha* Lourteig, which varied only 2.14 times (Table 1). Most species (13 taxa) showed a variation between 3 to 3.99 times.

According to Romero-Zarco's asymmetry index (1986), *Aloe zebrina* exhibited the most intrachromosomal asymmetric karyotype with A_1 = 0.77, while *Bixa orellana* L. showed the most symmetric karyotype with A_1 = 0.09 (Table 1). *Scaphura nigra* displayed the most interchromosomal asymmetric karyotype with A_2 = 1.0, whereas *Calydorea crocoides* Ravenna was the most symmetric with A_2 = 0.24 (Table 1). Fifteen species demonstrated moderately asymmetric karyotypes ranging from A_1 = 0.40 to 0.60, while eight species displayed slightly asymmetric karyotypes with $A_1 \leq 0.39$. Only six species exhibited highly asymmetric karyotypes with $A_1 \geq 0.61$ (Table 1). Regarding A_2, thirteen species had moderately asymmetric karyotypes ranging from A_2 = 0.40 to 0.60, while eleven species showed slightly asymmetric karyotypes with $A_2 \leq 0.39$. Eight species displayed highly asymmetric karyotypes with $A_2 \geq 0.61$ (Table 1). According to Stebbins' (1971) classification of asymmetry categories, *Bixa orellana* exhibited the most symmetric karyotype classified as 1B, while *Aloe zebrina* and *Scaphura nigra*♂ were classified as 4C, highly asymmetric (Table 1).

Histograms, density plots and K-means clustering analysis

The analyses of the histograms reveal a variety of patterns in chromosome size distributions among the studied species, with clear examples of unimodality, bimodality, and more complex distributions. K-means cluster graphs complement these observations by identifying distinct subgroups within the chromosome distributions. Out of the 32 species analyzed, 24 exhibited two distinct peaks in the density histograms, suggesting a bimodal distribution. The K-means cluster graphs of these species show two distinct clusters (Figures 4-5).

On the other hand, species such as *Calydorea crocoides*, *Cephalanthera rubra* (L.) Rich. (Figure 4), *Gastrodia gracilis* Blume, *Herbertia darwinii* Roitman & J.A.Castillo, *Hyacinthella dalmatica (*Avé-Lall.) Trinajstic, and *Puya mirabilis* (Figure 5) display a single peak in their density histograms, indicating a unimodal distribution of chromosome sizes. The K-means cluster graphs of these species present a single cluster of points. The species *Drosophila melanogaster* (Figure 4) and *Scaphura nigra* (Figure 5), showed three peaks in their density histograms, indicating a trimodal distribution. The K-means cluster graphs of these species reflect this complexity with three clusters.

Hartigans' Dip Test

The Hartigans' Dip Test revealed that seven out of the 30 species analyzed (23.33%) have a bimodal distribution of chromosome sizes (Table 2). The species considered bimodal by the Hartigans' Dip Test, with p-values ≤ 0.05, were: *Agave angustifolia* Haw., *A. parviflora* Torr.,

Table 1. Species mentioned in scientific articles as having bimodal karyotypes, chromosome number (2*n*), size of the largest and smallest chromosome in the complement (in micrometers - μm), the intra- (A1) and interchromosomal (A2) asymmetry index (Romero-Zarco, 1986) and Stebbins' Classification (1971), size of the smallest chromosome in Subset 1 and largest chromosome in Subset 2 (SCh1-LCh2), and when present, the smallest chromosome in Subset 2 and largest chromosome in Subset 3 (SCh2-LCh3), the ratio between the largest and smallest chromosomes of the subsets.

Species*	2*n*	Size (μm)	Asymmetry Index		Classification of Stebbins	SCh1-LCh2	SCh2-LCh3	Ratio
		Largest/ smallest	A_1	A_2				
Agave angustifólia	60	6.48-2.16	0.39	0.62	2C	6.18-4.10		1.50:1
A. cupreata	60	5.87-1.26	0.28	0.65	2C	4.85-2.75		1.76:1
A. fourcroydes	90	16.74-2.39	0.31	0.59	2C	12.02-6.83		1.75:1
A. parviflora	60	11.51-1.21	0.22	0.55	2C	9.09-5.10		1.78:1
A. tequilana	60	6.35-0.92	0.36	0.69	2C	5.32-3.30		1.61:1
Aloe tenuior	14	9.17-2.99	0.57	0.42	3B	7.87-4.33		1.81:1
A. vera	14	16.95-3.25	0.58	0.43	3B	13.23-4.85		2.72:1
A. zebrina	14	15.58-4.04	0.77	0.49	4C	14.16-4.90		2.88:1
Bixa orellana	14	3.64-1.47	0.09	0.36	1B	3.53-2.34		1.50:1
Calydorea crocoides	14	8.55-3.34	0.39	0.24	2B	7.12-5.58		1.27:1
C. undulata	14	8.55-3.34	0.35	0.36	2B	7.26-4.50		1.61:1
Cephalanthera longifolia	32	9.54-1.88	0.46	0.53	2B	8.55-4.53		1.88:1
C. rubra	44	12.14-2.40	0.38	0.48	2C	10.71-8.72		1.22:1
Consolida regalis	16	13.76-2.14	0.49	0.51	3C	11.91-4.10		2.90:1
Cuscuta nitida	28	6.25-0.97	0.14	0.80	2C	5.18-1.65		3.13:1
Drosophila melanogaster ♂	8	6.79-0.69	0.42	0.55	3C	6.57-4.12	4.10-0.70	1.59:1/5.85:1
Eleutherine bulbosa	12	6.19-1.49	0.18	0.67	2C	6.08-3.17		1.91:1
Epidendrum fulgens	24	3.15-1.20	0.35	0.25	2B	3.10-1.90		1.63:1
Gastrodia gracilis	22	3.10-1.00	0.28	0.26	2B	3.00-2.36		1.27:1
Herbertia darwinii	14	4.17-1.86	0.41	0.30	2B	3.55-2.44		1.45:1
Hyacinthella dalmatica	20	4.69-1.45	0.42	0.33	2B	4.54-3.16		1.43:1
H. chillensis	8	7.23-1.98	0.56	0.50	3B	5.28-2.62		2.00:1
Leopoldia comosa	18	7.48-1.21	0.30	0.72	2C	5.49-2.68		2.04:1
Luzuriaga radicans	20	11.43-3.35	0.56	0.46	3B	10.82-6.54		1.65:1
Milium montianum	22	6.00-1.81	0.34	0.55	2C	5.40-2.40		2.25:1
Oxalis linarantha	14	1.87-0.87	0.33	0.29	2B	1.84-1.19		1.54:1
Puya mirabilis	50	1.52-0.53	-	0.25	C			
Scaphura nigra♂	26	25.90-1.34	0.70	1.00	4C	25.90-15.68	15.28-7.40	1.65:1/2.06:1
Sellocharis paradoxa	20	5.70-2.20	0.70	0.27	3B	5.05-2.96		1.70:1
Sprekelia formosissima	60	11.76-2.89	0.44	0.30	3C	11.66-7.72		1.51:1
Tigridia pavonia	28	7.85-1.90	0.31	0.72	2B	7.22-4.06		1.77:1

* Species classified by Stebbins (1971) as representing four different levels of karyotypic bimodality are highlighted in bold.

Aloe tenuior Haw., *A. vera*, *A. zebrina* Baker and *Milium montianum* (now *Milium vernale* M.Bieb.). The other 23 species (76.67%) were considered unimodal, with p-values greater than 0.05, indicating the absence of bimodality.

Silverman Test

The Silverman Test indicated that 20 out of the 30 species (66.67%) have a bimodal distribution of chromosome sizes (Table 2). The species considered bimodal by the Silverman Test, with p-values ≤ 0.05, were: *Agave angustifolia* Hw., *A. cupreata* Trel. & A.Berger, *A. fourcroydes*, *A. parviflora*, *A. tequilana* F.A.C.Weber, *Aloe tenuior*, *A. vera*, *A. zebrina*, *Cephalanthera longifolia*, *Consolida regalis*, *Cuscuta nitida* E.Meyer., *Epidendrum fulgens* Brongner, *H. chillensis*, *Muscari comosum*, *Luzuriaga radicans* Ruiz & Pav., *Milium montianum*, *Sellocharis paradoxa* Taub., *Sprekelia formosissima* (L.) Herb., and

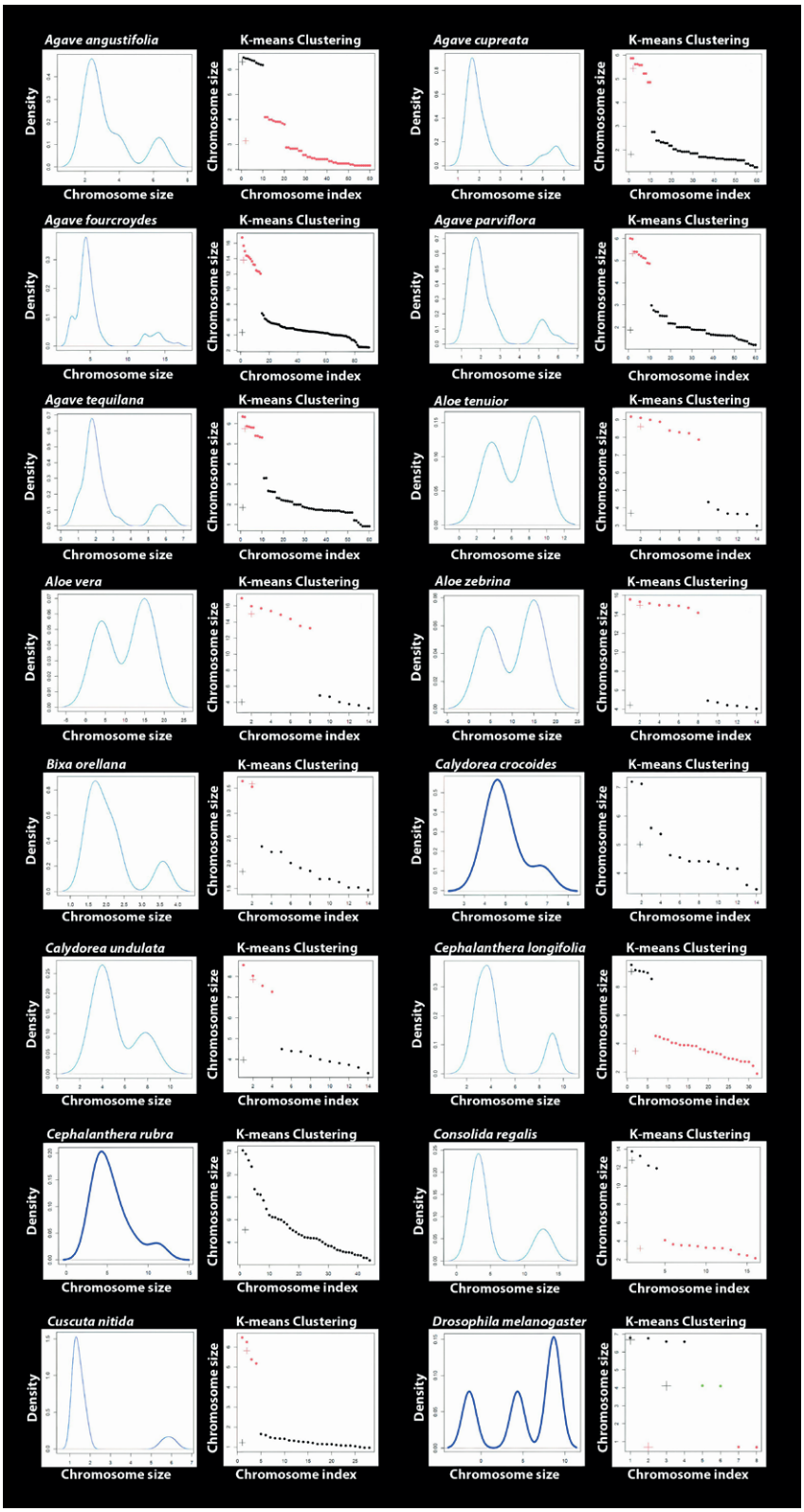

Figure 4. Density histograms and K-means clustering analysis of chromosome size variation. Karyotypes with continuous chromosome size variation exhibit a single peak. Bimodal karyotypes display two peaks, while trimodal karyotypes show three peaks. K-means clusters indicate the chromosomal subsets. Unimodal and trimodal karyotypes are highlighted with thicker blue lines.

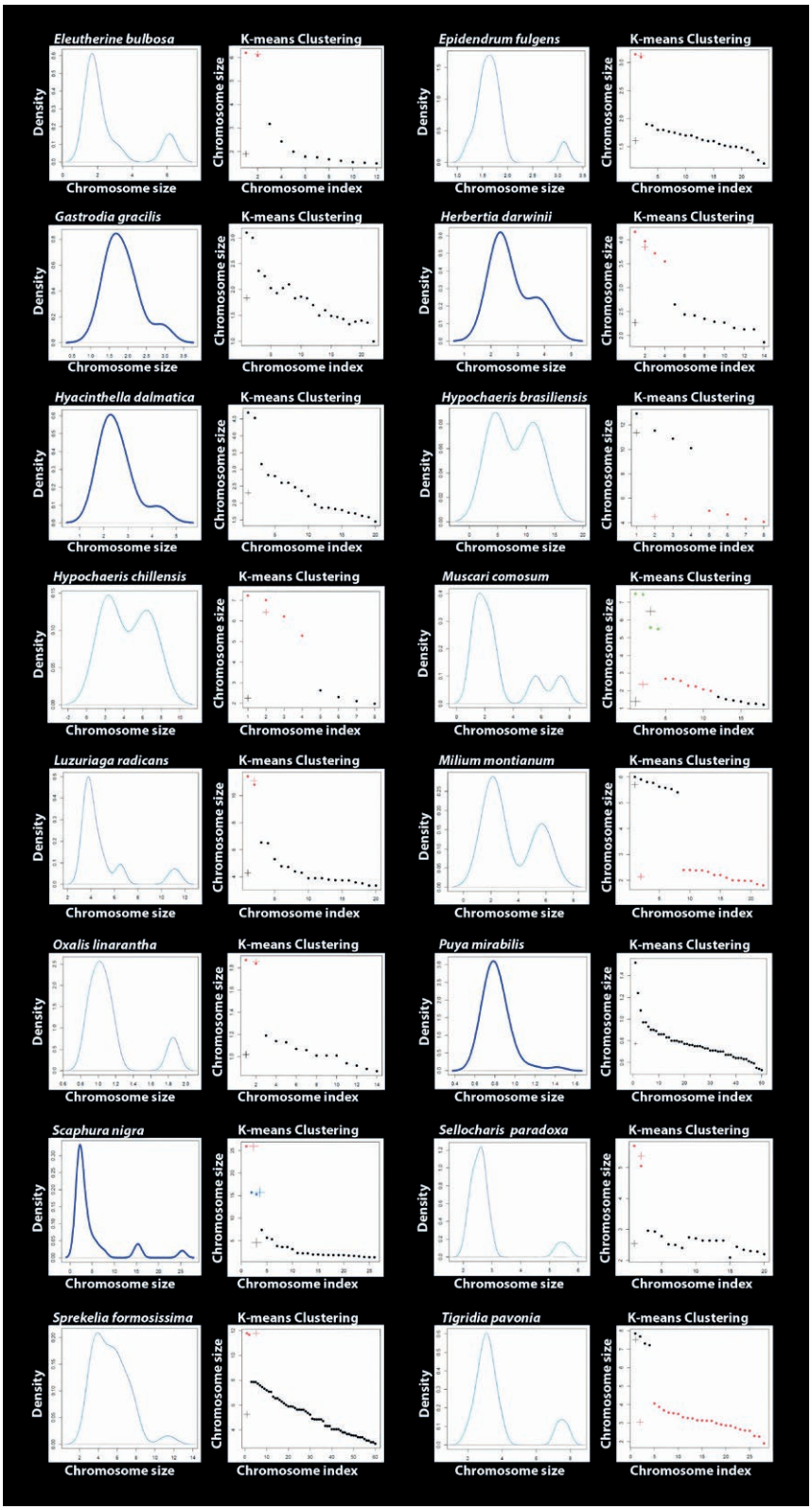

Figure 5. Density histograms and K-means clustering analysis of chromosome size variation. Karyotypes with continuous chromosome size variation exhibit a single peak. Bimodal karyotypes display two peaks, while trimodal karyotypes show three peaks. K-means clusters indicate the chromosomal subsets. Unimodal and trimodal karyotypes are highlighted with thicker blue lines.

Table 2. Results of Hartigans' Dip Test and Silverman Test for bimodality assessment in different species, with their respective diploid chromosome numbers (2*n*), Hartigan's Dip Test statistic (D), and associated p-values, indicating the probability of unimodality or bimodality. P-values less than 0.05 suggest bimodality.

Species	2*n*	Hartigans' dip test	Silverman test
Agave angustifolia	60	D = 0.072829 p-value = 0.01416 Bimodal	p-value = 0.002002002 Bimodal
A. cupreata	60	D = 0.056188 p-value = 0.1607 Unimodal	p-value = 0.00 Bimodal
A. fourcroydes	90	D = 0.042581 p-value = 0.2724 Unimodal	p-value = 0.00 Bimodal
A. parviflora	60	D = 0.065686 p-value = 0.04409 Bimodal	p-value = 0.00 Bimodal
A. tequilana	60	D = 0.055344 p-value = 0.1758 Unimodal	p-value = 0.00 Bimodal
Aloe tenuior	14	D = 0.15544 p-value = 0.001726 Bimodal	p-value = 0.02002002 Bimodal
A. vera	14	D = 0.17993 p-value = 0.00004786 Bimodal	p-value = 0.01101101 Bimodal
A. zebrina	14	D = 0.19608 p-value = 0.0000007587 Bimodal	p-value = 0.01601602 Bimodal
Bixa orellana	14	D = 0.071429 p-value = 0.8058 Unimodal	p-value = 0.1011011 Unimodal
Calydorea crocoides	14	D = 0.067901 p-value = 0.8718 Unimodal	p-value = 0.1711712 Unimodal
C. undulata	14	D = 0.097354 p-value = 0.2761 Unimodal	p-value = 0.08008008 Unimodal
Cephalanthera longifolia	32	D = 0.075225 p-value = 0.153 Unimodal	p-value = 0.008008008 Bimodal
C. rubra	44	D = 0.043544 p-value = 0.7966 Unimodal	p-value = 0.1941942 Unimodal
Consolida regalis	16	D = 0.10106 p-value = 0.1592 Unimodal	p-value = 0.05505506 Bimodal
Cuscuta nitida	28	D = 0.052203 p-value = 0.8241 Unimodal	p-value = 0.01201201 Bimodal
Drosophila melanogaster♂	8	D = 0.12463 p-value = 0.2185 Unimodal	p-value = 0.3153153 Unimodal
Eleutherine bulbosa	12	D = 0.083333 p-value = 0.6877 Unimodal	p-value = 0.08708709 Unimodal
Epidendrum fulgens	24	D = 0.049242 p-value = 0.9431 Unimodal	p-value = 0.009009009 Bimodal
Gastrodia gracilis	22	D = 0.067753 p-value = 0.5714 Unimodal	p-value = 0.1551552 Unimodal
Herbertia darwinii	14	D = 0.084586 p-value = 0.528 Unimodal	p-value = 0.1131131 Unimodal
Hyacinthella dalmatica	20	D = 0.056534 p-value = 0.903 Unimodal	p-value = 0.08408408 Unimodal
H. Chillensis	8	D = 0.14425 p-value = 0.09007 Unimodal	p-value = 0.05405405 Bimodal
Luzuriaga radicans	20	D = 0.064706 p-value = 0.7327 Unimodal	p-value = 0.05105105 Bimodal
Milium montianum	22	D = 0.15152 p-value = 0.00004228 Bimodal	p-value = 0.02002002 Bimodal
Muscari comosum	18	D = 0.065046 p-value = 0.7877 Unimodal	p-value = 0.02502503 Bimodal
Oxalis linarantha	14	D = 0.071429 p-value = 0.8058 Unimodal	p-value = 0.06906907 Unimodal
Puya mirabilis	50	D = 0.038571 p-value = 0.8748 Unimodal	p-value = 0.1921922 Unimodal
Scaphura nigra♂	26	D = 0.046423 p-value = 0.9567 Unimodal	p-value = 0.3153153 Unimodal
Sellocharis paradoxa	20	D = 0.058333 p-value = 0.8703 Unimodal	p-value = 0.02502503 Bimodal
Sprekelia formosissima	60	D = 0.042304 p-value = 0.6078 Unimodal	p-value = 0.03803804 Bimodal
Tigridia pavonia	28	D = 0.059555 p-value = 0.6144 Unimodal	p-value = 0.007007007 Bimodal

Tigridia pavonia (L.f.) DC. The remaining 10 species (33.33%) were considered unimodal by the Silverman Test, with p-values greater than 0.05.

Comparison between Hartigans' Dip Test and Silverman Test

Comparing the two tests, we observed that the Silverman Test was more sensitive in detecting bimodality. This difference in sensitivity suggests that the Silverman Test is less stringent in identifying bimodal distributions. On the other hand, the species that were considered bimodal by both tests are: *Agave angustifolia*, *A. parviflora*, *Aloe tenuior*, *A. vera*, *A. zebrina*, *Hypochaeris brasiliensis*, and *Milium montianum*. The species considered unimodal by both tests were: *Bixa orellana*, *Calydorea crocoides*, *C. undulata*, *Cephalanthera rubra*, *Eleutherine bulbosa*, *Gastrodia gracilis*, *Herbertia darwinii*, *Hyacinthella dalmatica*, *Oxalis linarantha*, and *Puya mirabilis*.

The results indicate that the Silverman Test is more effective in detecting bimodality compared to the Hartigans' Dip Test, identifying a higher proportion of species with a bimodal distribution of chromosome sizes. This sensitivity can be particularly useful in studies aiming to identify bimodality in chromosomal data sets, although the Hartigans' Dip Test may be preferred in contexts where specificity and the reduction of false positives are crucial.

Regression Analysis

A regression analysis was conducted to examine the relationship between the ratio of chromosome subsets (according to the diagrams illustrated in the Figure 3, see Table 1) and the p-values of the Silverman test (Table 2). The results of the linear regression as follows: Intercept: 0.02004 (Standard Error: 0.03729, t = 0.538, p = 0.595) and Proportion Coefficient: 0.02619 (Standard Error: 0.01752, t = 1.495, p = 0.145). The residuals showed the following distribution: Minimum: -0.09001, 1st Quartile: -0.05987, Median: -0.03335, 3rd Quartile: 0.03038, and Maximum: 0.24132. The residual standard error was 0.0842 with 30 degrees of freedom. The multiple R-squared was 0.06936, indicating that approximately 6.94% of the variability in the Silverman test p-values can be explained by the ratio of chromosome subsets. The adjusted R-squared was 0.03834. The F-statistic value was 2.236 with a p-value of 0.1453, suggesting that the relationship between the 1.50:1 ratio and the p-values is not statistically significant.

To compare the means of the Silverman test p-values between the ratios less than and greater than 1.50:1, a Welch's t-test was performed. The results were as follows: Mean of p-values for ratios less than 1.50:1 = 0.1516517. Mean of p-values for ratios greater than 1.50:1 = 0.05259105. Welch's t-test indicated the following results: t-statistic: 4.0689, Degrees of Freedom: 14.321, p-value: 0.001101, with a 95% Confidence Interval for the Difference in Means (0.04695375, 0.15116747). These results indicate a significant difference in the means of the p-values between the ratio groups, with a p-value less than 0.05.

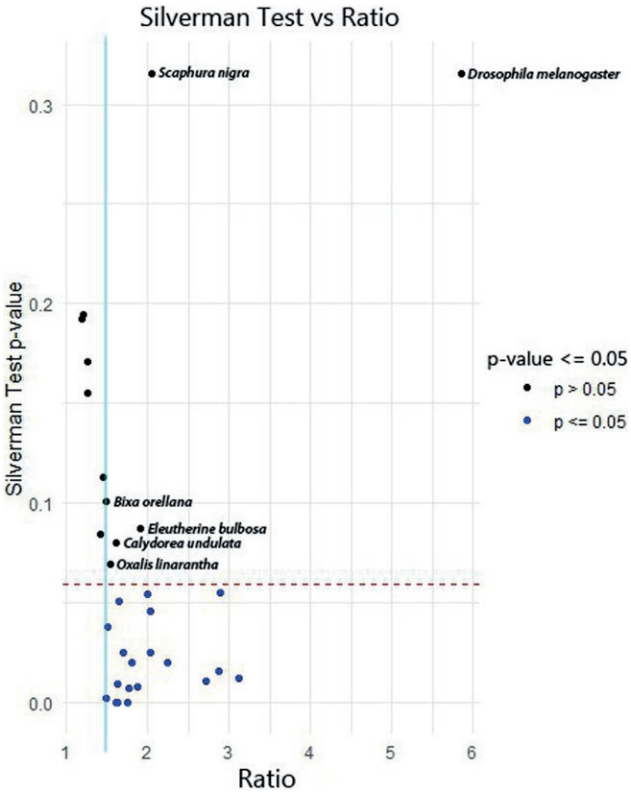

Figure 6. Scatter plot with regression lines shows the relationship between the chromosome ratio and the Silverman test p-values. The vertical blue line represents the 1.50:1 ratio, and the horizontal red line indicates the significance level (p = 0.05). Blue points represent species with p-values ≤ 0.05, while black points represent species with p-values > 0.05.

According to the scatter plot (Figure 6???), most ratios less than 1.50:1 have higher p-values, indicating a greater tendency to be considered unimodal, while ratios greater than 1.50:1 tend to have lower p-values, indicating a greater tendency to be considered bimodal. Species highlighted such as *Scaphura nigra* and *Drosophila melanogaster* have significantly higher p-values because they have trimodal karyotypes (not bimodal), while the species *Bixa orellana*, *Eleutherine bulbosa*, *Calydorea undulata*, and *Oxalis linarantha* are closer to the significance line, making them more difficult to classify statistically.

The regression analysis results indicate that the chromosome ratio does not have a statistically significant relationship with the Silverman test p-values. However, Welch's t-test suggests that there is a significant

difference in the mean p-values between ratios less than and greater than 1.50:1. These results suggest that ratios greater than 1.50:1 are associated with lower p-values, indicating a higher tendency to consider bimodality in karyotypes from this ratio. The graph corroborates these results (Figure 6), showing a clear distinction between the p-values for ratios less than and greater than 1.50:1.

DISCUSSION

Applying the original concept and its variations in the literature

The concept of bimodal karyotype was coined by Avdulov (1931) and extensively discussed by Stebbins (1971). It describes karyotypes with two distinct classes of chromosomes: one composed of large chromosomes and the other of small chromosomes, with a distinctly significant difference between the classes, representing a special type of karyotype asymmetry. Therefore, the concept of karyotypic bimodality involves several explicit criteria: 1. The formation of two subsets (or classes) of chromosomes; 2. It is a concept exclusively related to chromosome size, disregarding chromosome number and morphology (centromere position); 3. The difference between the two subsets is distinctly significant, not merely discontinuous; 4. The concept is not related to the largest and smallest chromosome in the complement, which can have a significant difference but still show continuous variation between extremes. The discrepancy specifically refers to the difference between the classes of large and small chromosomes, i.e., the smallest chromosome in the larger subset and the largest chromosome in the smaller subset. However, the challenge lies in establishing how significant this difference between subsets must be, making the concept's application somewhat impractical and often subjective.

The literature presents various applications and/or variations of the original concept (see, for example, Báez et al. 2019; Ibiapino et al. 2022), which perfectly meet the criteria originally established and discussed (Avdulov 1931; Stebbins 1971). However, some publications present fundamentally different concepts, which can explain the divergence in interpreting the criteria related to bimodality when applying the term to a given karyotype under analysis.

In most cases, the misapplication of the concept is related to the occurrence of large and small chromosomes in the same karyotype, classifying them as bimodal. The ambiguity here is that while every bimodal karyotype indeed has large and small chromosomes, not every karyotype with large and small chromosomes can be considered bimodal. For instance, in *Calydorea crocoides* (largest chromosome = 8.55 μm, smallest = 3.34 μm), *Cephalanthera rubra* (largest chromosome = 12.14 μm, smallest = 2.40 μm), *Gastrodia gracilis* (largest chromosome = 3.10 μm, smallest = 1.00 μm), *Herbertia darwinii* (largest chromosome = 4.17 μm, smallest = 1.86 μm), *Hyacinthella dalmatica* (largest chromosome = 4.69 μm, smallest = 1.45 μm) and *Puya mirabilis* (largest chromosome = 1.52 μm, smallest = 0.53 μm), the size variation between the two extremes is continuous (Figures 4-5). Thus, it is not possible to determine the larger and smaller chromosome subsets due to the absence of a marked discontinuity between them.

Another common inconsistency is considering a karyotype bimodal when discontinuities occur multiple times throughout the complement. If more than one discontinuous and significant interval exists between chromosome sizes, there will be more than two subsets in the complement, deviating from the concept of bimodal karyotype. This is the case with the cytotype analyzed of *Drosophila melanogaster* (Figure 4) and *Scaphura nigra* (Figure 5), which have three distinct subsets of chromosomes and are therefore trimodal (see Table 1).

Another problem in applying the concept is related to the inclusion of criteria that were not established by Avdulov (1931) or Stebbins (1971), nor tested statistically, such as the inclusion of relative chromosome size. Relative chromosome size is a measure that expresses the size of a chromosome in relation to the total size of the chromosome set of a karyotype. Including relative size as a criterion for establishing bimodality is problematic because karyotypes with high chromosome numbers will reduce the levels of discontinuity, depending on the total chromosome size, the extremes might be overvalued, disregarding whether the variation between them is continuous or discontinuous (Table 3).

Intrachromosomal Bimodality: a special case

The original idea of characterizing a bimodal karyotype is clearly interchromosomal, meaning it is related to the strong discontinuity in chromosome size within a complement. For example, some *Oxalis* species, such as *O. linarantha*, exhibit clear bimodality in chromosome size (Vaio et al. 2016). On the other hand, *O. eriocarpa* DC. has chromosomes with continuously varying sizes and karyotypes formed exclusively by metacentric and acrocentric chromosomes (Vaio et al. 2013). Regarding morphology, metacentric and acrocentric chromosomes are considered evolutionary extremes, based on the hypothesis that asymmetric karyotypes originate from symmetric ones (Stebbins 1971; Medeiros-Neto et al. 2017).

Table 3. Relative size of each metaphase chromosome of the species analyzed.

Species	Relative sizes
Agave angustifolia	0.05 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.03 0.03 0.03 0.03 0.02 0.02 0.02 0.02 0.02 0.02 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01
A. cupreata	0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01
A. fourcroydes	0.03 0.03 0.03 0.03 0.03 0.03 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.00 0.00 0.00 0.00 0.00 0.00
A. parviflora	0.07 0.07 0.08 0.08 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03
A. tequilana	0.05 0.05 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.00
Aloe tenuior	0.10 0.10 0.10 0.10 0.10 0.09 0.09 0.09 0.05 0.04 0.04 0.04 0.03 0.03
A. vera	0.12 0.10 0.10 0.10 0.09 0.09 0.09 0.09 0.04 0.04 0.04 0.04 0.04 0.03
A. zebrina	0.24 0.22 0.22 0.22 0.22 0.21 0.20 0.20 0.07 0.07 0.07 0.07 0.07 0.06
Bixa orellana	0.12 0.12 0.08 0.08 0.07 0.07 0.07 0.07 0.06 0.06 0.06 0.06 0.05 0.05
Calydorea crocoides	0.12 0.10 0.08 0.08 0.07 0.07 0.06 0.06 0.06 0.06 0.06 0.06 0.06 0.06
C. undulata	0.12 0.11 0.11 0.10 0.06 0.06 0.06 0.06 0.06 0.05 0.05 0.05 0.05 0.05
Cephalanthera longifolia	0.07 0.07 0.07 0.06 0.06 0.06 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02
C. rubra	0.05 0.05 0.05 0.04 0.04 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01
Consolida regalis	0.12 0.12 0.11 0.11 0.05 0.05 0.04 0.05 0.05 0.05 0.05 0.05 0.05 0.05 0.02 0.02
Cuscuta nitida	0.12 0.11 0.11 0.07 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02
Drosophila melanogaster♂	0.19 0.18 0.18 0.18 0.11 0.11 0.02 0.02
Eleutherine bulbosa	0.21 0.20 0.08 0.08 0.06 0.06 0.06 0.05 0.05 0.05 0.05 0.05
Epidendrum fulgens	0.07 0.07 0.05 0.05 0.05 0.05 0.05 0.05 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.03 0.03 0.03 0.03 0.03 0.03 0.03
Gastrodia gracilis	0.08 0.07 0.06 0.05 0.05 0.05 0.05 0.05 0.04 0.05 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.03 0.03 0.03 0.03
Herbertia darwinii	0.11 0.11 0.10 0.09 0.07 0.07 0.06 0.06 0.06 0.06 0.06 0.05 0.05 0.05
Hyacinthella dalmatica	0.10 0.09 0.06 0.06 0.05 0.05 0.05 0.05 0.05 0.05 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04
H. Chillensis	0.21 0.21 0.16 0.15 0.08 0.08 0.07 0.06
Luzuriaga radicans	0.11 0.11 0.07 0.07 0.05 0.05 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.04 0.03
Milium montianum	0.09 0.09 0.08 0.08 0.07 0.07 0.07 0.07 0.04 0.04 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.02 0.02 0.02 0.02
Muscari comosum	0.16 0.15 0.09 0.07 0.05 0.05 0.05 0.05 0.04 0.04 0.04 0.03 0.03 0.03 0.03 0.03 0.03 0.03
Oxalis linarantha	0.12 0.12 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.06 0.06 0.06 0.06 0.04
Puya mirabilis	0.04 0.04 0.03 0.03 0.03 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.01
Scaphura nigra♂	0.22 0.13 0.12 0.06 0.04 0.04 0.03 0.03 0.03 0.03 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.01 0.01 0.01
Sellocharis paradoxa	0.09 0.08 0.05 0.05 0.05 0.05 0.05 0.05 0.05 0.05 0.05 0.05 0.05 0.04 0.04 0.04 0.04 0.04 0.04 0.04
Sprekelia formosissima	0.04 0.03 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.02 0.01 0.01 0.02 0.02 0.02 0.01 0.01 0.02 0.02 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01 0.01
Tigridia pavonia	0.07 0.07 0.08 0.08 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03

Chromosome changes, especially centric fusions/fissions, are the main causes of the direct transition between meta- and acrocentric chromosomes. Chromosome fusions occur when two chromosomes unite, forming a single metacentric chromosome. In contrast, chromosome fissions involve the breakage of a chromosome, resulting in two smaller acrocentric chromosomes (Guerra 2008). This transition related to centric fission/fusion events frequently occurs without changes in the fundamental number (without changes in the number of chromosome arms between related species with different chromosome numbers), as seen in the genera *Nothoscordum* (Souza et al. 2012) and *Ipheion* (Souza et al. 2010). These structural changes are important in speciation, as they can affect chromosome segregation during meiosis and generate reproductive barriers between populations.

Submetacentric chromosomes are considered intermediate in the evolution of chromosome morphology (Stebbins 1971). In this context, karyotypes composed solely of metacentric and acrocentric chromosomes, with a complete absence of submetacentric chromosomes, exhibit intrachromosomal asymmetry. We propose here to classify these karyotypes as a form of intrachromosomal bimodality. This is exemplified in *Oxalis eriocarpa*, which displays a bimodal karyotype in terms of chromosome morphology. Additionally, some species of *Oxalis* exhibit two levels of bimodality: one interchromosomal and the other intrachromosomal (Vaio et al. 2013).

Evolutionary hypotheses for the origin of bimodal karyotypes

The debate on the origin of bimodal karyotypes began in the 1930s with Avdulov and was later expanded upon by Stebbins (1971). Since then, several causes have been identified for the origin of bimodal karyotypes. Structural chromosomal alterations, especially unequal translocations, fusions, and fissions, can result in the formation of chromosomal subsets of contrasting sizes within a complement. Generally, asymmetric karyotypes are the result of chromosomal rearrangements, which can occur separately involving a single chromosome, as seen in *Nothoscordum* Kunth (Souza et al. 2012), or simultaneously involving different chromosomes, as observed in *Arabidopsis thaliana* (L.) Heynh. (Lysak et al. 2007). Numerous examples in the literature demonstrate how rearrangements lead to distinct discontinuities in chromosome size, such as in the genus *Ornithogalum* L. (Liliaceae), where some species exhibit bimodal karyotypes due to fusions and fissions (Stedje 1989; Vosa 1997). Chromosome fusions are also involved in the origin of bimodal karyotypes in some reptile groups, like the genus *Sceloporus* Wiegmann (Lisachov et al. 2020). In the allotetraploid *Tragopogon* × *miscellus* Ownbey (Asteraceae), intergenomic translocations result in chromosomes of variable sizes, with individuals displaying different karyotypes exhibiting various levels of interchromosomal asymmetry, some of which are clearly bimodal (Chester et al. 2012).

Another factor clearly demonstrated in the differentiation between chromosomal subsets is the amplification of certain repetitive DNA sequences. Two well-studied examples in the literature include *Cuscuta* subgenus *Pachystigma* (Convolvulaceae), which has $2n$ = 28-30 chromosomes with one set of large chromosomes and another set of small chromosomes. The large chromosomes contain a wide variety of abundant repetitive sequences, such as 5S and 35S ribosomal DNAs, a satellite DNA superfamily SF1, and LTR retrotransposons, which are absent in the smaller chromosome subset (Ibiapino et al. 2022). The second example is *Eleutherine bulbosa* Urb., with $2n$ = 12 and a pair of large chromosomes four times larger than the other chromosomes in the complement. The larger pair is heteromorphic, with one chromosome having a pericentric inversion and a proximal duplication within the inversion (Guerra 1988b). Differential accumulation of the most abundant genome retroelements, occurs only in the larger pair, explaining the cause of bimodality in *E. bulbosa* (Báez et al. 2019).

Another possibility for the origin of bimodality is hybridization, as suggested for certain classic bimodal karyotypes like *Agave* L. (McKain et al. 2012), and the tetraploid *Emilia fosbergii* Nicolson (Guerra and Nogueira 1990; Moraes and Guerra 2010), allopolyploids with parents having significantly different chromosome sizes (McKain et al. 2012). In such cases involving hybridization, minimal or no chromosomal rearrangements between subgenomes are necessary to maintain the difference between the inherited chromosomal subsets. Although this is not a common scenario, as allopolyploids generally exhibit rapid rearrangements between subgenomes, it has been demonstrated in *Milium montianum* (Poaceae - Bennett et al. 1992) and *E. fosbergii* (Moraes and Guerra 2010). It is possible that many other bimodal karyotypes have a hybrid origin, related or not to polyploidy, whose analyses may be hampered by ancient events obscured over time. While we are now well-informed about the possible causes of bimodality, understanding why evolution often maintains bimodality in entire clades remains challenging.

Method for identifying bimodal karyotypes

The interchromosomal asymmetry index (Romero-Zarco 1986) and Stebbins' categories (1971) showed diver-

gent results for the same species, a direct consequence of the different factors each test considers regarding variation. While the A_2 index is based on the standard deviation of the entire chromosomal complement, Stebbins' categories consider only the ratio between the smallest and largest chromosome in the complement (Medeiros-Neto et al. 2017). Thus, although both indicate interchromosomal asymmetry, the indices provide information about different levels within chromosomal variation, often resulting in divergent responses for the same species.

However, none of the tested interchromosomal asymmetry indices showed a consistent pattern to indicate a karyotype as bimodal. This is clearly observed in *Puya mirabilis*, whose karyotype is bimodal, but it is classified as symmetric by the Romero-Zarco index (A_2 = 0.25) and asymmetric by Stebbins' categorization (see Table 1). Stebbins' categorization also classified species with bimodal karyotypes as moderately asymmetric, such as *Tigridia pavonia* in 2B, with A_2 = 0.72 (Table 1), thus being inadequate for assessing bimodality.

Statistical tests also yielded divergent results in identifying bimodal karyotypes. While Hartigans' Dip Test identified 23.33% of species as bimodal, the Silverman Test identified 66.67% (Table 2). Due to this high divergence, the proposal to define bimodal karyotypes based on the ratio between the smallest chromosome of the larger subset and the largest chromosome of the smaller subset may be more objective and practical than relying solely on statistical tests. This method can provide an intuitive and direct indicator of bimodality, helping to avoid ambiguities.

Stebbins' (1971) observations about bimodal karyotypes are useful because they convey a consistent idea about the operational concept of bimodality. Although he did not formally propose a limit between large and small chromosomal subsets, Stebbins compared bimodal karyotypes of various species with other related karyotypes, defined only as asymmetric, in his discussion on "the origin of bimodal karyotypes." According to Stebbins (1971), the karyotypes of species belonging to the genera *Aloe*, *Yucca*, and *Gasteria*, as well as *Consolida regalis* and *Muscari comosum* are bimodal (see Figure 3). In this study, we represented the bimodal karyotypes of these species in idiograms and analyzed them comparatively. We observed that all karyotypes considered bimodal by Stebbins (1971) exhibit a ratio ≥ 1.5:1 between the smallest chromosome of the larger subset and the largest chromosome of the smaller subset.

We evaluated two approaches: the first considers karyotypes as bimodal based on a ratio ≥ 2:1. We found that this criterion can be more stringent, identifying karyotypes with a clearer distinction between the two subsets, which reduces the risk of false positives but may fail to identify some bimodal karyotypes with less pronounced differences. On the other hand, the ratio ≥ 1.5:1 is more inclusive, identifying a larger proportion of karyotypes as bimodal, aligning with the greater sensitivity observed in the Silverman Test. This criterion can include karyotypes with less extreme differences that are still distinctly bimodal.

Based on the results of statistical analysis, the ratio of ≥ 1.5:1 seems to be the best approach for defining bimodal karyotypes. Regression analysis and Welch's t-test suggest that the 1.5:1 ratio is associated with lower p-values, indicating a greater tendency to detect bimodality (Figure 6, Table 1). While the 2:1 ratio is more stringent, the 1.5:1 ratio offers a balance between rigor and sensitivity, avoiding false negatives and still representing a distinctly discrepant difference between chromosomal subsets, capturing the essence of the original definition of bimodality proposed by Avdulov (1931) and later discussed by Stebbins (1971).

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