Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
https://riviste.fupress.net/index.php/caryologia

OPEN ACCESS

Citation: Araújo, L. F., Dornelas, C. S. M., Felix, L. P. & Nollet, F. (2024). What defines a bimodal karyotype? Bimodality revisited. *Caryologia* 77(4): 43-60. doi: 10.36253/caryologia-3034

Received: October 15, 2024

Accepted: April 18, 2025

Published: July 15, 2025

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

What defines a bimodal karyotype? Bimodality revisited

Leylson Ferreira Araújo[1], Charlys Seixas Maia Dornelas[2], Leonardo P. Felix[2], Felipe Nollet[1,*]

[1] *Laboratório de Citogenética e Evolução Vegetal, Departamento de Botânica, Universidade Federal de Pernambuco, Recife, PE, Brasil*
[2] *Departamento de Biociências, Laboratório de Citogenética Vegetal, Centro de Ciências Agrárias, Universidade Federal da Paraíba, Campus II, Areia, PB, Brasil*
*Corresponding author. Email: nolletmedeiros@yahoo.com.br

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).

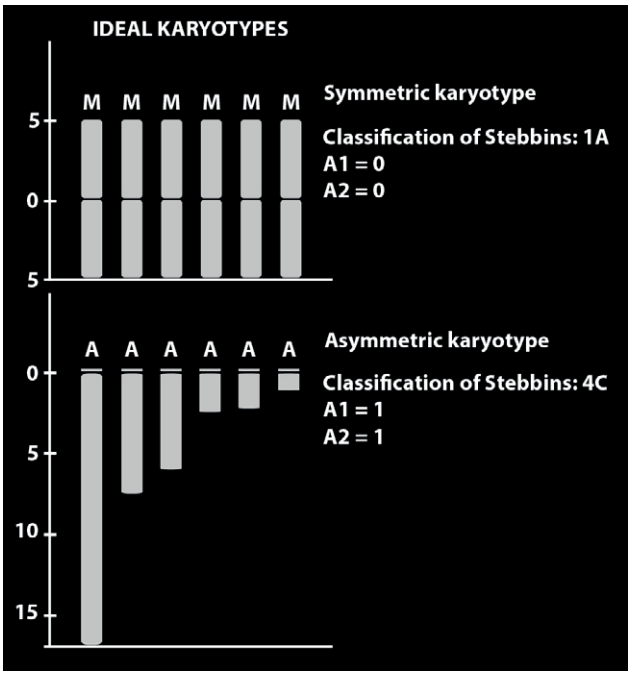

Figure 1. Idiograms of the theoretically possible ideal karyotypes with *n* = 6. The first represents the extreme of symmetry, composed of exactly identical metacentric chromosomes (M), classified by Stebbins as 1A, and Romero-Zarco (1986) indices A1 = 0 and A2 = 0. The second represents the extreme 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-

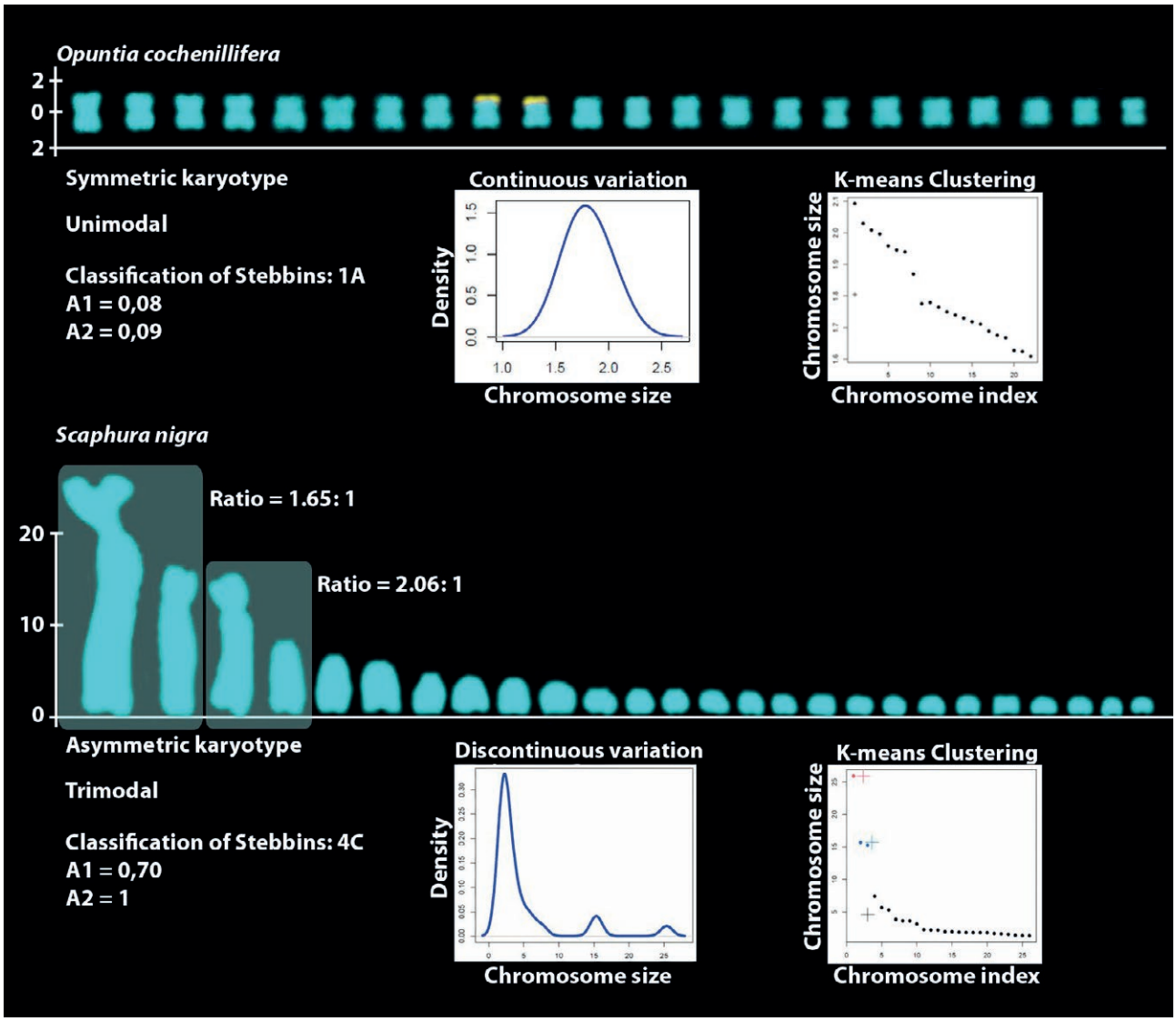

Figure 2. Karyograms of a real symmetric karyotype (*Opuntia cochenillifera* (L.) Mill with 2*n* = 22) and an asymmetric Trimodal karyotype (*Scaphura nigra* Stål with 2*n* = 26). The first karyogram represents symmetry, composed of very similar metacentric chromosomes (M), classified by Stebbins as 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-

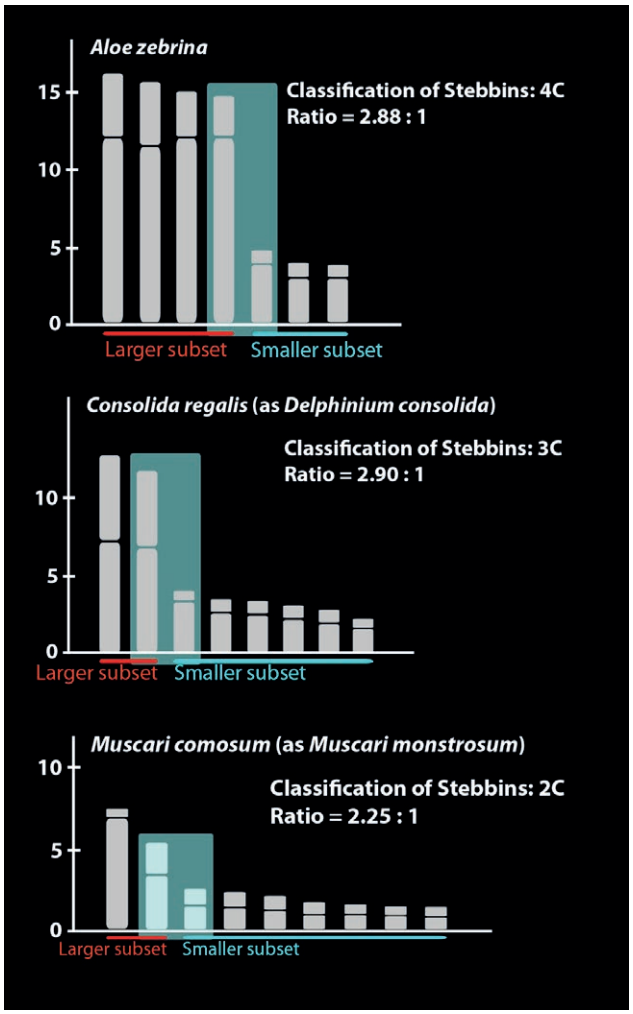

Figure 3. Idiograms of the species *Aloe zebrina* with $n = 7$ (classified by Stebbins as 4C), *Consolida regalis* with $n = 8$ (classified by Stebbins as 3C) and *Muscari comosum* with $n = 9$ (classified by Stebbins as 2C). Chromosomes are aligned by the base and in descending order. The gray box highlights 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

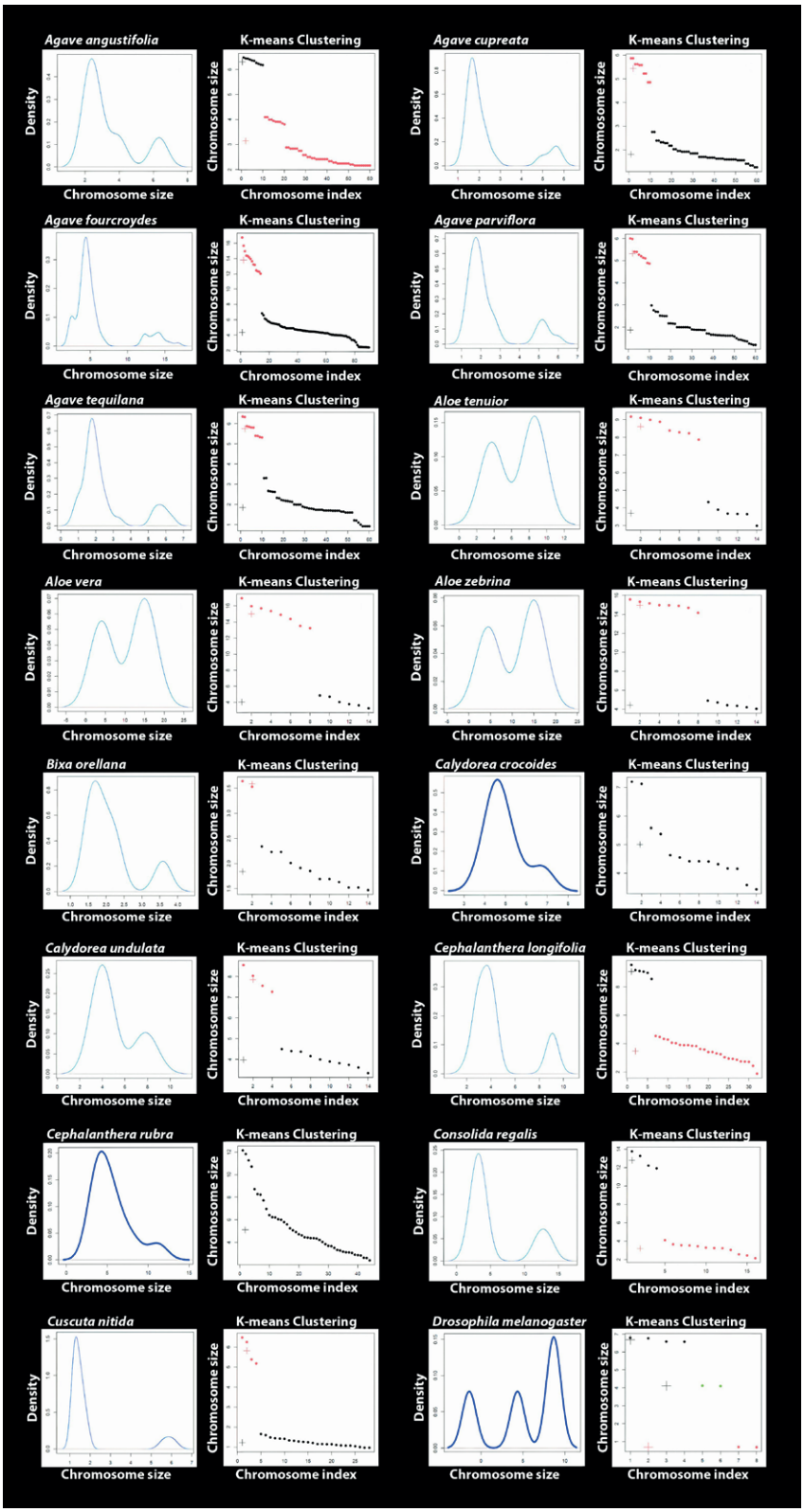

Figure 4. Density histograms and K-means clustering analysis of chromosome size variation. Karyotypes with continuous chromosome size variation exhibit a single peak. Bimodal karyotypes display two peaks, while trimodal karyotypes show three peaks. K-means clusters indicate the chromosomal subsets. Unimodal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

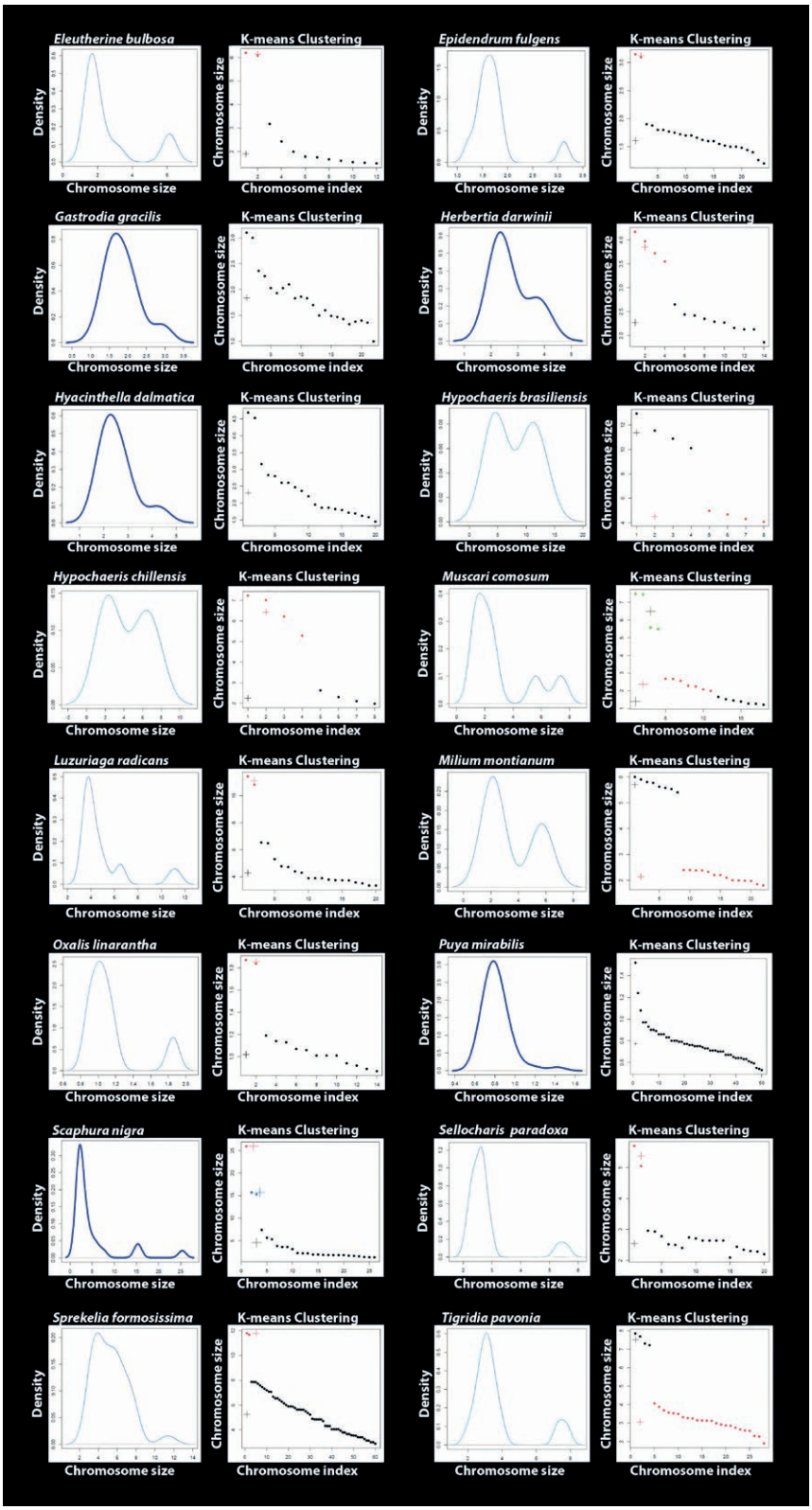

Figure 5. Density histograms and K-means clustering analysis of chromosome size variation. Karyotypes with continuous chromosome size variation exhibit a single peak. Bimodal karyotypes display two peaks, while trimodal karyotypes show three peaks. K-means clusters indicate the chromosomal subsets. Unimodal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

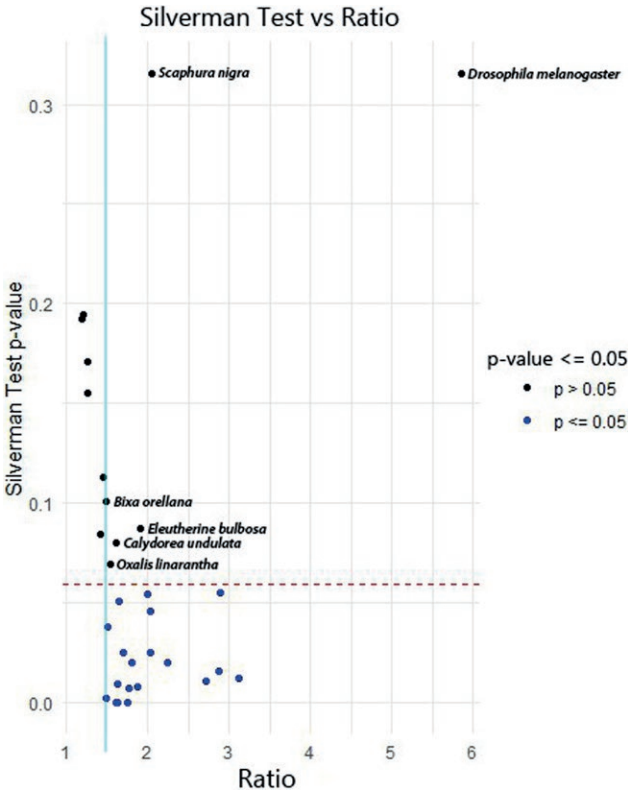

Figure 6. Scatter plot with regression lines shows the relationship between the chromosome ratio and the Silverman test p-values. The vertical blue line represents the 1.50:1 ratio, and the horizontal red line indicates the significance level (p = 0.05). Blue points represent species with p-values ≤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

ACKNOWLEDGEMENTS

We thank Fundação de Amparo à Ciência e Tecnologia de Pernambuco – FACEPE and Instituto Nacional do Semiárido – INSA for technical support.

REFERENCES

Alves LIF, Lima SAA, Felix LP. 2011. Chromosome characterization and variability in some Iridaceae from Northeastern Brazil. Genet Mol Biol 34(2):259-267. https://doi.org/10.1590/S1415-47572011000200016

Assis FNM, Souza BCQ, Medeiros-Neto E, Pinheiro F, Silva AEB, Felix LP. 2013. Karyology of the genus *Epidendrum* (Orchidaceae: Laeliinae) with emphasis on subgenus *Amphiglottium* and chromosome number variability in *Epidendrum secundum*. Bot J Linn Soc 172(3):329-344. https://doi.org/10.1111/boj.12045

Avdulov NP. 1931. Karyosystematische Untersuchung der Familie Gramineen. Bull Appl Bot Genet Plant Breed Suppl 44: 1-428.

Baeza M, Ruiz E, Negritto M. 2010. Comparative analysis in the *Alstroemeria hookeri* Lodd. (Alstroemeriaceae) complex Sensu Bayer (1987). Genet Mol Biol 33(1): 119-124. https://doi.org/10.1590/S1415-47572010005000012

Báez M, Vaio M, Dreissig S, Schubert V, Houben A, Pedrosa-Harand A. 2019. Together but different: The

subgenomes of the bimodal *Eleutherine* karyotypes are differentially organized. Front Plant Sci 10:1170. https://doi.org/10.3389/fpls.2019.01170

Balslev H. 1996. Juncaceae. Fl Neotrop 68: 1-167. https://www.jstor.org/stable/4393863

Bennett MD, Smith JB, Seal AG. 1986. The karyotype of the grass *Zingeria biebersteiniana* (2*n* = 4) by light and electron microscopy. Can J Genet Cytol 28(4): 554-562. https://doi.org/10.1139/g86-081

Bennett ST, Kenton AY, Bennett MD. 1992. Genomic *in situ* hybridization reveals the allopolyploid nature of *Milium montianum* (Gramineae). Chromosoma 101: 420-424. https://doi.org/10.1007/BF00582836

Bertollo LAC, Takahashi CS, Moreira-Filho O. 1983. Multiple sex chromosomes in the genus Hoplias (pisces: Erythrinidae). Cytologia 48:1-12. DOI: https://doi.org/10.1508/cytologia.48.1

Bodor DL, Mata JF, Sergeev M, David AF, Salimian KJ, Panchenko T, Cleveland DW, Black BE, Shah JV, Jansen LET. 2014. The quantitative architecture of centromeric chromatin. eLife 3:e02137. https://doi.org/10.7554/eLife.02137

Brito RO, Affonso PRAM, Silva Jr JC. 2010. Chromosomal diversity and phylogenetic inferences concerning thrips (Insecta, Thysanoptera) in a semi-arid region of Brazil. Genet Mol Res 9(4): 2230-2238.

Carta A, Bedini G, Peruzzi L. 2018. Unscrambling phylogenetic effects and ecological determinants of chromosome number in major angiosperm clades. Sci Rep 8(1): 1-14. https://doi.org/10.1038/s41598-018-32515-x

Castro JP, Medeiros-Neto E, Souza G, Alves LI, Batista FR, Felix LP. 2016. CMA band variability and physical mapping of 5S and 45S rDNA sites in Brazilian Cactaceae: Pereskioideae and Opuntioideae. Braz J Bot 39: 613-620. https://doi.org/10.1007/s40415-015-0248-5

Chase MW, Samuel R, Leitch AR, Guignard MS, Conran JG, Nollet F, Fletcher P, Jakob A, Cauz-Santos LA, Vignolle G, et al. 2023. Down, then up: non-parallel genome size changes and a descending chromosome series in a recent radiation of the Australian allotetraploid plant species, *Nicotiana* section *Suaveolentes* (Solanaceae). Ann Bot 131(1): 123–142. https://doi.org/10.1093/aob/mcac006

Chester M, Gallagher JP, Symonds VV, Silva AVC, Mavrodiev EV, Leitch AR, Soltis PS, Soltis DE. 2012. Extensive chromosomal variation in a recently formed natural allopolyploid species, *Tragopogon miscellus* (Asteraceae). PNAS 109(4): 1176–1181. https://doi.org/10.1073/pnas.1112041109

Chiarini FE, Barboza GE. 2008. Karyological studies in *Jaborosa* (Solanaceae). Bot J Lin Soc 156(3):467-478. https://doi.org/10.1111/j.1095-8339.2007.00734.x

Crosland MWJ, Crozier RH. 1986. *Myrmecia pilosula*, an Ant with Only One Pair of Chromosomes. Science 231(4743): 1278. https://doi.org/10.1126/science.231.4743.1278.

de Azkue D, Martinez A. 1983. The chromosome complements of shrubby *Oxalis* species from South America. Plant Syst Evol 141: 187–197. https://doi.org/10.1007/BF00989001

Delaunay LN. 1923. Vergleichende karyologische Untersuchungen einiger *Muscari* Mill. und *Bellevalia Lapeyr* Arten. Monit Jard Bot Tbilisi 2(1): 24-55.

Goldblatt P, Snow N. 1991. Systematics and chromosome cytology of *Eleutherine* Herbert (Iridaceae). Ann Mo Bot Gard 78(4): 942–949. https://doi.org/10.2307/2399735

Greilhuber J. 1995. Chromosomes of the monocotyledons (general aspects). In: Randall PJ, Cribb PJ, Cutler DF, Humphries CJ. editors. Monocotyledons: systematics and evolution. Kew: Royal Botanic Gardens; p. 379–414.

Guerra M. 1986. Reviewing the chromosome nomenclature of Levan et al. Rev Bras Genet 9(4): 741–743.

Guerra M. 1988a. Introdução à citogenética geral. Rio de Janeiro: Guanabara koogan.

Guerra M. 1988b. Mitotic and meiotic analysis of a pericentric inversion associated with a tandem duplication in *Eleutherine bulbosa*. Chromosoma 97: 80-87. https://doi.org/10.1007/BF00331797

Guerra M. 2008. Chromosome numbers in plant cytotaxonomy: concepts and implications. Cytogenet Genome Res 120(3-4): 339–350. https://doi.org/10.1159/000121083

Guerra MS, Nogueira MTM. 1990. The cytotaxonomy of *Emilia* spp. (Asteraceae: Senecioneae) occurring in Brazil. Plant Syst Evol 170(3-4): 229–236. http://www.jstor.org/stable/23674239

Guerra M, Ribeiro T, Felix L. 2019. Monocentric chromosomes in *Juncus* (Juncaceae) and implications for the chromosome evolution of the family. Bot J Linn Soc 191(4): 475-483. https://doi.org/10.1093/botlinnean/boz065

Hartigan JA, Hartigan PM. 1985. The Dip Test of unimodality. Ann Statist 13(1): 70-84. https://doi.org/10.1214/aos/1176346577

Ibiapino A, Báez M, García MA, Costea M, Stefanović S, Pedrosa-Harand A. 2022. Karyotype asymmetry in *Cuscuta* L. subgenus *Pachystigma* reflects its repeat DNA composition. Chromosome Res 30(1):91-107. doi: https://doi.org/10.1007/s10577-021-09683-0.

Jain AK. 2010. Data clustering: 50 years beyond K-means. Pattern Recognit Lett 31(8): 651-666. https://doi.org/10.1016/j.patrec.2009.09.011

Khoshoo TN, Ahuja MR. 1962. The Karyotype in *Welwitschia mirabilis*. Nature 193: 356–357. https://doi.org/10.1038/193356a0

Leach CR, Houben A, Timmisa JN. 2004. The B chromosomes in *Brachycome*. Cytogenet Genome Res 106(2-4):199–209. https://doi.org/10.1159/000079288.

Levan A, Fredga K, Sandberg AA. 1964. Nomenclature for centromeric position on chromosomes. Hereditas 52(2): 201-220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Lisachov AP, Tishakova KV, Romanenko SA, Molodtseva AS, Prokopov DY, Pereira JC, Ferguson-Smith MA, Borodin PM, Trifonov VA. 2021. Whole-chromosome fusions in the karyotype evolution of *Sceloporus* (Iguania, Reptilia) are more frequent in sex chromosomes than autosomes. Philos Trans R Soc Lond B Biol Sci 376(1833):20200099. https://doi.org/10.1098/rstb.2020.0099

Lysak MA, Cheung K, Kitschke M, Bureš P. 2007. Ancestral chromosomal blocks are triplicated in Brassiceae species with varying chromosome number and genome size. Plant Physiol 145(2): 402–410. https://doi.org/10.1104/pp.107.104380

Mayrose I, Lysak MA. 2021. The evolution of chromosome numbers: mechanistic models and experimental approaches. Genome Biol Evol 13(2):evaa220. https://doi.org/10.1093/gbe/evaa220

McKain MR, Wickett N, Zhang Y, Ayyampalayam S, McCombie WR, Chase MW, Pires JC, dePamphilis CW, Leebens-Mack J. 2012. Phylogenomic analysis of transcriptome data elucidates co-occurrence of a paleopolyploid event and the origin of bimodal karyotypes in Agavoideae (Asparagaceae). Amer J Bot 99(2):397–406. https://doi.org/10.3732/ajb.1100537

Medeiros-Neto E, Nollet F, Moraes AP, Felix LP. 2017. Intrachromosomal karyotype asymmetry in Orchidaceae. Genet Mol Biol 40(3): 610–619. https://doi.org/10.1590/1678-4685-GMB-2016-0264

Mesa A, Fontanetti CS, Ferreira A. 2010. The chromosomes and the sex determining mechanism of *Scaphura nigra* (Orthoptera, Ensifera, Tettigoniidae, Phaneropterinae). J Orthoptera Res19(2):239-242. http://hdl.handle.net/11449/19826

Moraes AP, Guerra M. 2010. Cytological differentiation between the two subgenomes of the tetraploid *Emilia fosbergii* Nicolson and its relationship with *E. sonchifolia* (L.) DC. (Asteraceae). Plant Syst Evol 287:113–118. https://doi.org/10.1007/s00606-010-0302-5

Moraes AP, Koehler S, Cabral JS, Gomes SS, Viccini LF, Barros F, Felix LP, Guerra M, Forni-Martins ER. 2017. Karyotype diversity and genome size variation in Neotropical Maxillariinae orchids. Plant Biol 19(2):298-308. https://doi.org/10.1111/plb.12527.

Nielsen MK, Wang J, Davis R, Bellaw JL, Lyons ET, Lear TL, Goday C. 2014. *Parascaris univalens*—a victim of large-scale misidentification? Parasitol Res 113(12): 4485–4490. https://doi.org/10.1007/s00436-014-4135-y.

Oliveira IG, Moraes AP, Almeida EM, Assis FNM, Cabral JS, Barros F, Felix LP. 2015. Chromosomal evolution in Pleurothallidinae (Orchidaceae: Epidendroideae) with an emphasis on the genus *Acianthera*: chromosome numbers and heterochromatin. Bot J Linn Soc 178(1): 102–120. https://doi.org/10.1111/boj.12273

Paszko B. 2006. A critical review and a new proposal of karyotype asymmetry indices. Plant Syst Evol 258(1-2): 39-48. https://doi.org/10.1007/s00606-005-0389-2

Pierozzi NI. 2011. Karyotype and NOR-banding of mitotic chromosomes of some *Vitis* L. species. Rev Bras Frutic 33:564-570. https://doi.org/10.1590/S0100-29452011000500077

Piet Q, Droc G, Marande W, Sarah G, Bocs S, Klopp C, Bourge M, Siljak-Yakovlev S, Bouchez O, Lopez-Roques C, et al. 2022. A chromosome-level, haplotype-phased *Vanilla planifolia* genome highlights the challenge of partial endoreplication for accurate wholegenome assembly. Plant Commun 3(5): 100330. https://doi.org/10.1016/j.xplc.2022.100330

Romero-Zarco C. 1986. A new method for estimating karyotype asymmetry. Taxon 35(3):526-530. https://doi.org/10.2307/1221906

Silverman BW. 2017. Density estimation for statistics and data analysis. New York (NY): Chapman & Hall.

Souza LGR, Crosa O, Guerra M. 2010. Karyological circumscription of *Ipheion* Rafinesque (Gilliesioideae, Alliaceae). Plant Syst Evol 287:119-127. https://doi.org/10.1007/s00606-010-0304-3

Souza LGR, Crosa O, Speranza P, Guerra M. 2012. Cytogenetic and molecular evidence suggest multiple origins and geographical parthenogenesis in *Nothoscordum gracile* (Alliaceae). Ann Bot 109(5): 987-999. https://doi.org/10.1093/aob/mcs020

Stebbins GL. 1971. Chromosomal evolution in higherplants. London: Edward Arnold.

Stedje B. 1989. Chromosome evolution within the *Ornithoghlum tenuifolium* complex (Hyacinthaceae), with special emphasis on the evolution of bimodal karyotypes. Plant Syst Evol 166: 79-89. https://doi.org/10.1007/BF00937877

Tanaka R. 1967. A comparative karyotype aAnalysis in *Haplopappus gracilis* (2*n*=4) and *H. ravenii* (2*n*=8). Cytologia 32(3-4): 542-552. https://doi.org/10.1508/cytologia.32.542

Thrun MC, Gehlert T, Ultsch A. 2020. Analyzing the fine structure of distributions. PLoS ONE 15(10): e0238835. https://doi.org/10.1371/journal.pone.0238835

Vaio M, Gardner A, Emshwiller E, Guerra M. 2013. Molecular phylogeny and chromosome evolution among the creeping herbaceous *Oxalis* species of sections *Corniculatae* and *Ripariae* (Oxalidaceae). Mol Phylogenet Evol 68(2):199–211. https://doi.org/10.1016/j.ympev.2013.03.019

Vaio M, Gardner A, Speranza P, Emshwiller E, Guerra M. 2016. Phylogenetic and cytogenetic relationships among species of *Oxalis* section *Articulatae* (Oxalidaceae). Plant Syst Evol 302:1253–1265. https://doi.org/10.1007/s00606-016-1330-6

Vanzella ALL, Cuadrado A, Guerra M. 2003. Localization of 45S rDNA and telomeric sites ob holocentric chromosomes of *Rhynchospora tenuis* Link (Cyperaceae). Genet Mol Biol 26(2): 199-201. https://doi.org/10.1590/S1415-47572003000200014

Violetta K, Pistrick K, Gernand D, Meister A, Ghukasyan A, Gabrielyan I, Houben A. 2005. Characterisation of the low-chromosome number grass *Colpodium versicolor* (Stev.) Schmalh. (2*n* = 4) by molecular cytogenetics. Caryologia 58(3): 241-245. https://doi.org/10.1080/00087114.2005.10589457

Vosa C. 1997. Heterochromatin and ecological adaptation in southern African *Ornithogalum* (Liliaceae). Caryologia 50(2): 97–103. https://doi.org/10.1080/00087114.1997.10797389

Watkins GM. 1936. Chromosome numbers and species characters in *Yucca*. Am J Bot 23(5): 328–333. https://doi.org/10.2307/2436092

Weiss-Schneeweiss H, Schneeweiss GM. 2013. Karyotype diversity and evolutionary trends in angiosperms. In: Greilhuber J, Dolezel J, Wendel J, editors. Plant genome diversity. Volume 2. Vienna: Springer. p. 209–230.

Wrensch DL, Kethley JB, Norton RA. 1994. Cytogenetics of holokinetic chromosomes and inverted meiosis: keys to the evolutionary success of mites, with generalizations on eukaryotes. In: Houck MA, editor. Mites. Boston: Springer. p. 282–343.

Wu J. 2012. Advances in K-means clustering: a data mining thinking. Springer Theses. Heidelberg: Springer Berlin.

Yang F, O'Brien PCM, Wienberg J, Neitzel H, Lin CC, Ferguson-Smith MA. Chromosomal evolution of the Chinese muntjac (*Muntiacus reevesi*) Chromosoma 106(1): 37-43. https://doi.org/10.1007/s004120050222