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CELLULAR AND MOLECULAR BIOLOGY

The chromosome number and heterochromatin distribution pattern suggest differentiation among species of the *Stylosanthes scabra-seabrana* complex

IRLANE CRISTINE S.A. LIRA, NATONIEL F. DE MELO, RAFAELA PRISCILA ANTONIO, RONALDO S. DE OLIVEIRA & MANOEL A. DE QUEIROZ

Abstract: The genus *Stylosanthes* has economic importance in semi-arid regions and requires studies that reveal complex relationships between species involving different ploidies. The aim of this study was to cytogenetically investigate accessions of *Stylosanthes* identified as *S. scabra*, in order to properly identify the number, morphology, and pattern of distribution of heterochromatin, analyzing the karyological variability of these species. Accessions with 2*n*=40 and 2*n*=20 were identified, exhibiting semi-reticulate interphase nuclei, symmetric karyotype, varied morphology, with differences in average chromosomal size, and genome length. The analysis with the fluorochromes chromomycin (CMA) and 4',6-diamidino-2-phenylindole (DAPI) allowed the visualization of two CMA^+ blocks in the subterminal region of the short arm in all accessions, and $DAPI^+/CMA^-$ bands in *S. scabra* accessions. This suggests that not only chromosomal rearrangements, but also changes in the composition of heterochromatin, may have occurred during the speciation of this genus, and that *S. scabra* may be undergoing chromosomal evolution based on the observed karyological differences. In addition to the ploidy level, the distribution pattern of CMA^+ heterochromatin reinforces the separation between *S. scabra* and *S. seabrana*. Thus, this genus represents an interesting group of plants for further studies on the content and quantity of repetitive and non-repetitive DNA sequences.

Key words: Staining CMA/DAPI, chromosome banding, fluorochromes, genetic diversity.

INTRODUCTION

Some legumes, especially of the genus *Stylosanthes*, are commercially successfully used, either for direct grazing, alone or intercropped with grasses, in hay production, and they are still considered promising for the establishment and recovery of degraded areas. Besides their forage use, they play other roles, whether as suppliers of wood and firewood, as apicultural pasture, for medicinal, landscaping, or artisanal purposes. (Vieira & Barros 2008, Costa & Coradin 2016, Tropical Forages 2024). The genus *Stylosanthes* is composed of about 50 species, of which approximately 25 occur in Brazil (Costa et al. 2011). In various reports, species distinction is mainly based on morphological characteristics, although a series of other techniques have been used to assist in the taxonomy of the genus, such as electrophoresis, cytogenetic characterization, and variability analysis, among others (Gillies & Abbott 1996, Liu et al. 1999, Vander Stappen et al. 2002, Chandra & Kaushal 2009, Date 2016). Studies involving cytogenetic characterization of the genus *Stylosanthes* are still incipient, which has been a gap for breeding programs. In this case,

there are reports of species with chromosome numbers ranging from 2*n*=20 (diploids), 2*n*=40 (tetraploids) and 2*n*=60 (hexaploids) (Cameron 1967, Polido et al. 2015, Franco et al. 2020).

On the other hand, genetic improvement activities with this forage legume in northeastern Brazil were initiated by Embrapa Semiárido (CPATSA) using accessions originating from the Embrapa Cerrados Active Germplasm Bank (CPAC). These accessions were collected between the 1970s and 1990s, initially identified as *S. scabra*. However, among these accessions, there arose a doubt about the possible presence of the species *S. seabrana*, considering that this second species was taxonomically described only in Maass & 't Mannetje (2002). The two species have different chromosomal numbers: *S. scabra* is tetraploid (2n=40) and *S. seabrana* is diploid (2n=20).

Despite *S. seabrana* being described in 2002 as a different species from *S. scabra*, Vanni & Fernandez (2011) proposed the synonymization of *S. seabrana* as a cytotype of *S. scabra*, as they found both diploids and tetraploids in the roots of seedlings grown from a commercial sample of *S. seabrana* cv. Única from Australia. Conversely, Cook & Schultze-Kraft (2020), based on a review of several works utilizing various approaches such as morphology, agronomy, ploidy, and phylogeny, definitively refuted the synonymization of *S. seabrana* with *S. scabra*. According to Tropical Forages (2024) it is observed that the morphological, agronomic, rhizobial, cytological, and phylogenetic differences between *S. seabrana* and *S. scabra* are clear, indicating that taxonomic logic requires treating them as distinct species.

From a morphological point of view, *S. seabrana* presents leaflet shape narrowly elliptical, leaflet indumentum glabrous except for long bristles on the margins and midrib, leaflet venation prominently raised veins on the lower surface, length of axis rudiment 7-8 mm, ploidy diploid (2*n* = 20), genome A, soil pH acidic to neutral. Already, *S. scabra* presents leaflet shape elliptical to obovate, leaflet indumentum pubescent with bristles at least underneath or on the margins, leaflet venation without prominently raised veins on the lower surface, length of axis rudiment 4-5 mm, ploidy tetraploid (2*n*=40), genome AB, soil pH acid, soil texture light, soil fertility low, rhizobial specificity promiscuous.

A clear and precise characterization of the karyotype is fundamental when comparing cytogenetically different species or examining variation among individuals of the same species, as one of the most important evolutionary mechanisms promoting speciation is chromosomal rearrangements and heterochromatin polymorphisms (Huang & Rieseberg 2020, Vimala et al. 2021). Thus, the present study aims to correctly characterize cytogenetically 13 *Stylosanthes* accessions occurring in Brazil, identifying the number, chromosomal morphology, and distribution pattern of heterochromatin.

MATERIALS AND METHODS

The present study was conducted using 13 accessions (Table I) primarily identified as *S. scabra*, which were collected in several Brazilian regions and maintained in the Active Germplasm Bank of Embrapa Cerrados (Planaltina-DF). The analyses were conducted at the Plant Biotechnology Laboratory of Embrapa Semi-arid (Petrolina-PE) using root meristems obtained from seeds germinated in gerbox boxes and kept in BOD at 25 °C for 24 h.

The mitotic analyses were performed according to the protocol described by Guerra & Souza (2002) where root tips were pretreated with 0.002 M 8-hydroxyquinoline (8HQ) for 24

Table I. List of accessions collection data of *Stylosanthes* studied, Chromosomal number (NC), mean chromosomal length (mCL), karyotypic formula (CF), total haploid chromosomal length (TCL) of *Stylosanthes* accessions analyzed in mitotic metaphases.

Accessions	Species	Collection location	Year of collection	NC (*2n*)	mCL (µm)	FC	TCL (µm)
CPAC 397	*S. scabra*	Campo Grande - MS, Rodovia BR-163, km 184 (8 km N)	1979	40	1.6	18 m + 2 sm	32.02
CPAC 564	*S. scabra*	São Gabriel do Oeste – MT, Rodovia BR-163, km 603	1979	40	2.48	18 m + 2 sm	49.67
CPAC 1244	*S. scabra*	PA	1981	40	2.1	19 m + 1 sm	41.94
CPAC 1261	*S. scabra*	MA	1981	40	1.68	19 m + 1 sm	33.66
CPAC 4947	*S. scabra*	Pajeú - MG	1997	40	1.31	17 m + 3 sm	26.27
CPAC 4950	*S. seabrana*	Andaraí - BA	1997	20	1.58	10 m	15.88
CPAC 4963	*S. seabrana*	Morro do Chapéu - BA	1997	20	1.6	10 m	16.05
CPAC 4966	*S. seabrana*	Oliveira dos Brejinhos - BA	1997	20	1.57	10 m	15.68
CPAC 5114	*S. scabra*	Estrada Diamantina/Mendanha – MG, after the police station, approximately 12 km on the right	1999	40	1.51	19 m + 1 sm	30.26
CPAC 5196	*S. scabra*	Datas - MG	2000	40	2.26	20 m	45.29
CPAC 5205	*S. scabra*	Santa Vitória - MG	2000	40	1.83	19 m + 1 sm	36.56
CPAC 5228	*S. scabra*	Estrada Piracicaba/Charqueada - SP, Rodovia SP-308, km 187	2000	40	1.38	18 m + 2 sm	27.52
CPAC 5234	*S. scabra*	Espírito Santo do Pinhal - SP, Rodovia SP-147, km 63 (-200m)	2000	40	1.66	18 m + 2 sm	33.13

m= metacentric; sm= submetacentric.

hours at 8 °C and fixed in Carnoy 3:1 solution (ethanol: acetic acid), and then stored at -20 °C until its use.

In the preparation of the slides, the fixed roots were washed in distilled water, and then their digestion was performed with a mixture of cellulase (2% w / v) and pectinase (20% v / v) at 37 °C for 90 min, being crushed in acetic acid at 45%. The coverslip was removed after freezing in liquid nitrogen and the slides were dried at room temperature.

Chromosomal banding was performed three days after slide preparation, using chromomycin - CMA (0.5 mg/ml) for 1 hour and 4',6-diamidino-2-fenilindol - DAPI (2 µg/ml) for 30 minutes, followed by assembly in McIlvaine medium (glycerol/$MgCl_2$). The best cells were captured through the Leica QFish program, coupled to a Leica DM 2000 fluorescence microscope.

To identify the chromosomal number and morphology, at least three metaphases were examined per individual, in up to five individuals per accession from which the best metaphases were used for chromosome measurements and preparation of idiograms. The measurements were performed using the Leica QFish program,

and chromosomes from well-dispersed metaphases were measured, calculating the length of short arms (SA), length of long arms (LA), and total or absolute chromosomal length (L).

The idiograms were arranged in order by decreasing size of the short arm. The chromosomal nomenclature suggested by Guerra (1988) was adopted, where the position of the centromeres was defined numerically by calculating the ratio between the long arm (l) and the short arm (c), through the formula: r = l/c, using the descriptors to define the karyotypic formula: Metacentric (M); Submetacentric (SM); Acrocentric (A); Telocentric (T). In addition, the following karyologic parameters were estimated: total haploid chromosomal length (TCL); mean chromosomal length (mCL); ratio between the largest and smallest arm (BL/BC or RA); and relative chromosomal length (CA/TCL or RL).

RESULTS AND DISCUSSION

Table I presents the collection information and data regarding the number and chromosomal morphology of the 13 *Stylosanthes* germplasm accessions evaluated, of which three exhibited karyotypes with 2*n*=20 and ten exhibited karyotypes with 2*n*=40 (Figures 1 and 2). In general, a symmetrical karyologic pattern was observed, very conserved with metacentric to

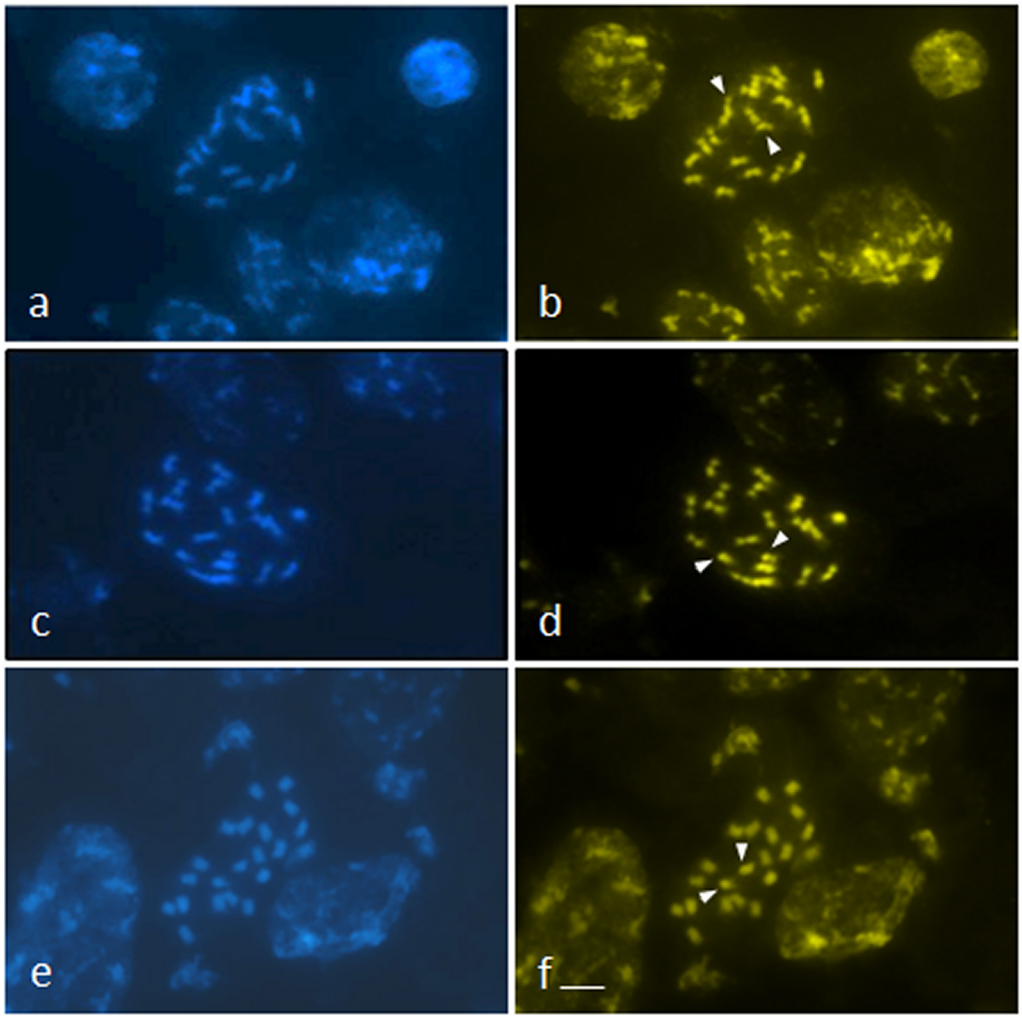


Figure 1. DAPI/CMA_3 staining in *S. seabrana* with 2*n*=20. Accession CPAC 4966 ("a" and "b"); accession 4963 ("c" and "d"); accession 4950 ("e" and "f"). Arrowheads indicate the location of CMA^+/$DAPI^-$ blocks in the terminal regions of two small chromosomes. Bar in "f" represents 5 µm.

submetacentric chromosomes and interphase nuclei of the semireticulate type.

The analysis of the relationship values between the arms, the length of the haploid complement, the average length of the chromosomes and karyotypic asymmetry index, as well as the presence of satellites, allowed the identification and comparison of common characteristics among the species. In several species, interspecific variations in these characters are important, and provide substantial information for establishing relationships between taxa, regarding chromosomes organization (Soares- Scott et al. 2005, Bernardes et al. 2013).

The mean chromosomal length showed variations among the diploid accessions (2*n*=20), ranging from 1.57 µm in the CPAC 4966 accession, to 1.60 µm in the CPAC 4963 accession, while in the tetraploid accession (2*n*=40) it was from 1.31

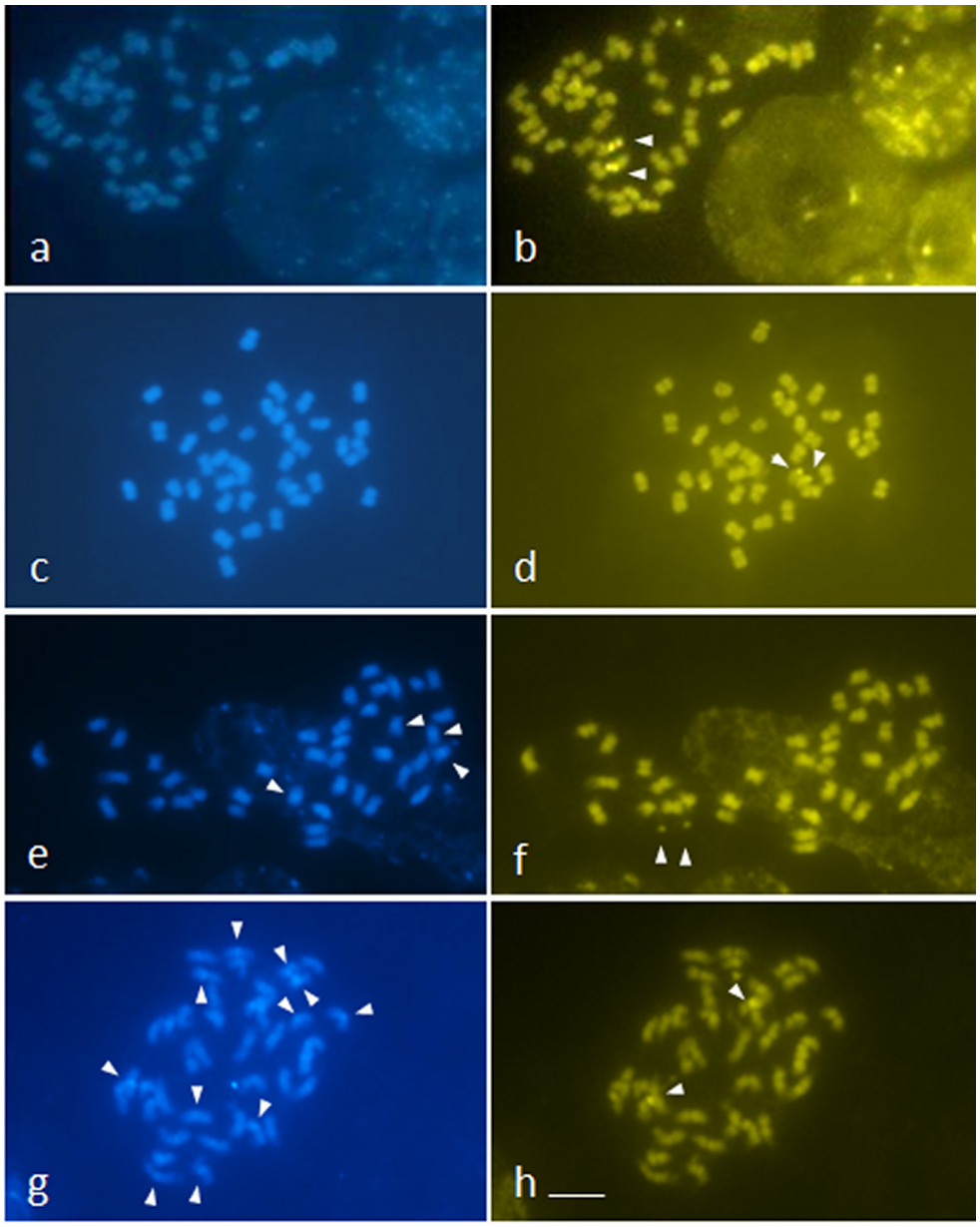


Figure 2. DAPI/CMA_3 staining in *S. scabra* with 2*n*=40. Accession CPAC 4394 ("a" and "b"); accession CPAC 5234 ("c" and "d"); accession CPAC 1261 ("e" and "f"); accession CPAC 5205 ("g" and "h"). Arrowheads indicate the location of $CMA^+/DAPI^-$ blocks in "b, d, f, h" and $DAPI^+/CMA^-$ in "e, g". Bar in "h" represents 5 µm.

µm in the CPAC 4947 accession, to 2.48 µm in the CPAC 564 access (Table I). In *Stylosanthes*, Franco et al. (2020) evaluating eight different species of the genus (*S. acuminata, S. gracilis, S. grandifolia, S. guianensis, S. hippocampoides, S. macrocephala*, S. *pilosa* and S. *ruellioides*) and five accessions *S. guianensis* (1480, 4171, 1463, LC2538 and cv. Mineirão) observed that all *Stylosanthes* species studied were diploid (2*n*=20) with predominantly metacentric chromosomes measuring on average 2.7 µm.

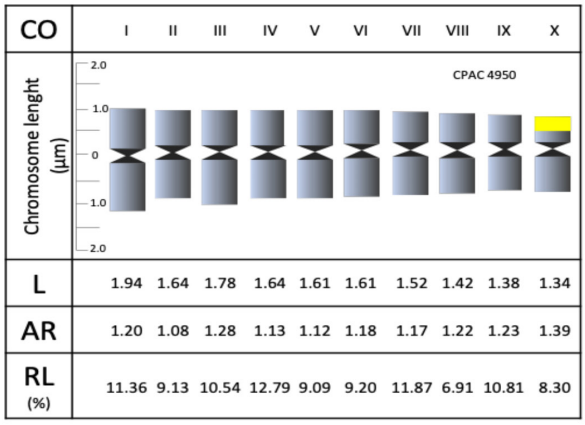
Similarly, when comparing the average chromosomes size of the two *Stylosanthes* species studied in this work with the values reported for other genera of the same tribe Papilionoideae (*Phaseolus*, *Vigna* and *Macroptilium*), it was observed that the average chromosome size of these species varies from 1 µm to 3 µm (Forni-Martins 1989). There is a trend towards increased chromosomal size in polyploids reported in several other species, such as in the family Velloziaceae (Melo et al. 1997) and Passifloraceae (Melo et al. 2001).

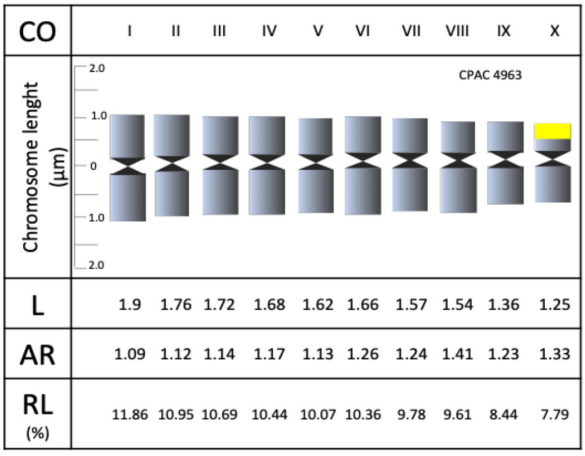
Regarding the karyotypic formula, all diploid accessions presented chromosomes with metacentric morphology. Among the tetraploid accessions, nine out of the 10 accessions of *S. scabra* analyzed showed a karyotype with metacentric and submetacentric chromosomes, generally with one to three submetacentric ones. Only the *S. scabra* accession CPAC 5196 presented all metacentric chromosomes (Table l), confirming, albeit to a small degree, a karyomorphological variability in the studied species. The predominance of metacentric chromosomes in the accessions of the two species indicated a tendency to maintain a symmetrical karyotype. This tendency towards karyotypic symmetry in members of the Fabaceae family was also observed by Kumari & Bir (1989) in some representatives of the subfamily *Caesalpinoideae,* and by Franco et al. (2020) in at least seven species *of Stylosanthes*.

The detailed study of karyotypic asymmetry in certain plant groups provides a clear understanding of the significance of karyologic evolution. In this context, karyotypic asymmetry indices have been extensively utilized to infer mechanisms of chromosomal evolution in plants (Paszko 2006). Increases in karyotypic asymmetry are primarily attributed to Robertsonian translocations (common form of chromosomal rearrangement), inversions and uneven translocations, which are detectable through the analysis of meiotic behavior (Romero 1986).

The karyological differences observed among the of *Stylosanthes* accessions suggest that two mechanisms may have contributed to chromosomal alterations: I) chromosomal rearrangements, such as, pericentric inversions or unequal translocations, in the case of corresponding chromosomes from different accessions, showing similar lengths, but different proportions of arm length; II) changes in DNA content, as could be the case of an increase observed in the accessions *S. scabra* CPAC 564 (mean chromosomal length, mCL, 2.48 µm), CPAC 1244 (mCL 2.10 µm) and CPAC 5196 (mCL 2.26 µm), or decrease as observed in the accession CPAC 4947 (mCL 1.31 µm).

The relative length (CR or RL) of the chromosomes, representing the proportion of each chromosome within the total length of the haploid set (CA/TCL or RL), is an important measure for comparing different accessions and even different species. Figure 3 shows that in the three diploid accessions of *S. seabrana* there was no significant variation in the chromosomal sizes of pairs I to X, the chromosomes with the greatest variation were chromosome VIII and chromosome IX, with values ranging for chromosome VIII from 6.91%, in the CPAC 4950





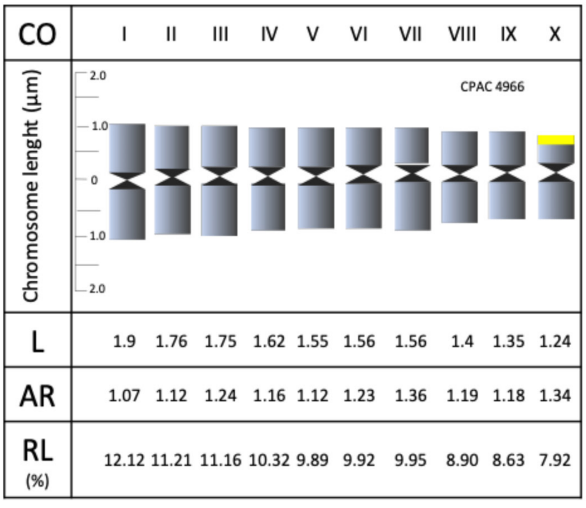


Figure 3. Idiograms of the accessions of *S. seabrana* (2*n*=20) showing the chromosomal length (L), the ratio between arms (AR) and the relative chromosomal length (RL) and CMA+ bands in yellow. CO = ordering of chromosomal pairs.

accession, to 9.61% in the CPAC 4963 accession, and for chromosome IX from 8.44%, in the CPAC 4963 accession, to 10.81% in the CPAC 4950 accession.

Vieira et al. (1993) observed in 12 diploid species of the genus *Stylosanthes* a variation between 11.15% to 12.16% for the size of chromosome I and 7.08% to 9.77% for the size of the chromosome X, also demonstrating significant variation among these diploid accessions.

On the other hand, among the 10 tetraploid accessions analyzed from *S. scabra* there was a greater variation in relative chromosomal length, especially in pair I, with variation ranging from 4.51% in the CPAC 5196 accession, to 7.02% in the CPAC 564 accession. This represents a difference of more than 50% between the accessions, and this chromosomal pair is highly important for differentiation in the comparative analysis of these genotypes (Figures 4 to 7). For the chromosomal pair XX, the variation ranged from 2.55% in the CPAC 5196 accession, to 4.50% in the CPAC 4387 accession.

Other chromosomes also exhibited significant variation in relative lengths, with variations ranging from3.70% to 6.09% in pair V, and from 3.49% to 6.25% in pair VII, for the accessions CPAC 1244 and CPAC 564, respectively. In this scenario, the observation of a predominance of metacentric chromosomes is an indication that the karyotype is symmetrical, a common observation in several members of the Leguminosae family (Pinto et al. 2016, Van-Lume et al. 2017).

The analysis using double CMA/DAPI staining enabled the visualization of two CMA^+ blocks in the terminal region of the smallest chromosomal pair in the accessions of both *S. seabrana* and *S. scabra* (Figures 2 and 3). $DAPI^+$ blocks were observed in two accessions of *S. scabra* (CPAC 1261 and CPAC 5205) but were not

observed in the other eight accessions of the species.

Vieira et al. (1993) also reported the presence of secondary constrictions in the smallest chromosomal pair of some diploid species, which corroborates the existence of the CMA^+ blocks observed in the terminal region of the last metacentric chromosomal pair of the diploid cytotypes of our study.

CMA^+ bands were reported by Franco et al. (2020) for the species *S. hippocampoides*, *S. gracilis, S. macrocephala, S. pilosa, S. ruellioides* and *S. guianensis* (accessions 1480, 1463, 4171 and LC2538) observing two signs CMA^+ bands in the short arms of the smallest chromosomal pair. Conversely, *S. acuminata, S. grandifolia* and *S. guianensis* cv. Mineirão presented four CMA^+ bands, with two in the short arms of the smallest chromosomal pair and the other two in the proximal region of a large chromosomal pair. In *S. ruellioides*, these authors observed proximal $CMA^-/DAPI^+$ bands in all chromosomes of this species. Marques et al. (2018) reported $DAPI^+$ pericentromeric heterochromatin on all chromosomes of *S. hamata, S. viscosa* (2*n*=20) and *S. scabra* (2*n*=40), although sometimes the signal was very weak, especially in *S. scabra*.

The results obtained suggest that not only chromosomal rearrangements, but also changes in the composition of heterochromatin may have occurred during the speciation of these species of *Stylosanthes* with *S. scabra* being a recent allotetraploid. According to Marques et al. (2018), the species may be undergoing chromosomal evolution as can be observed in the accessions in relation to the reported karyological differences. Regarding the CMA^+ bands described in the present work, it was possible to observe them in only one pair of chromosomes in both species. In this case, considering that *S. scabra* is a tetraploid, we would expect to observe two pairs of chromosomes with CMA^+ bands, but only one pair was observed in the 10 accessions analyzed. This observation, added to the ploidy level, reinforces the separation between *S. scabra* and *S. seabrana*. Therefore, this genus represents an intriguing group of plants for further studies on the content and quantity of repetitive and non-repetitive DNA sequences.

CONCLUSIONS

The numerical chromosomal cytogenetic analysis conducted in this study is one of the tools that allows differentiation between *S. seabrana* and *S. scabra* accessions. Through comparative chromosomal morphological evaluation, it may be possible to provide insights for better differentiation among *Stylosanthes* accessions. Using chromosomal staining with fluorochromes, it was possible to observe the presence of different heterochromatic regions among the studied accessions.

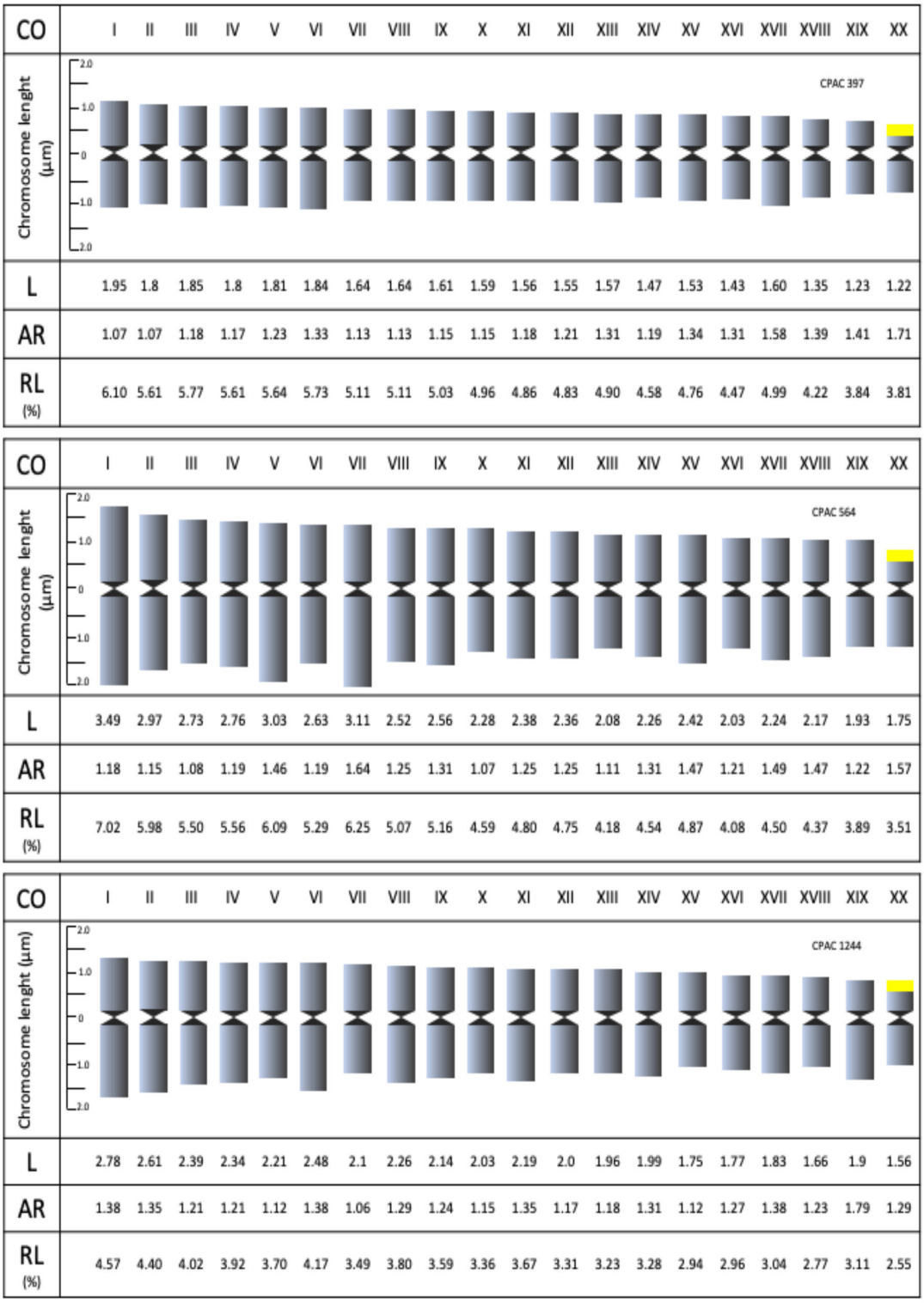


Figure 4. Idiograms of the accessions CPAC 397, CPAC 564 and CPAC 1244 of *S. scabra* (2*n* = 40) showing the chromosomal length (L), the ratio between arms (AR) and the relative chromosomal length (RL) and CMA+ bands in yellow. CO = ordering of chromosomal pairs.

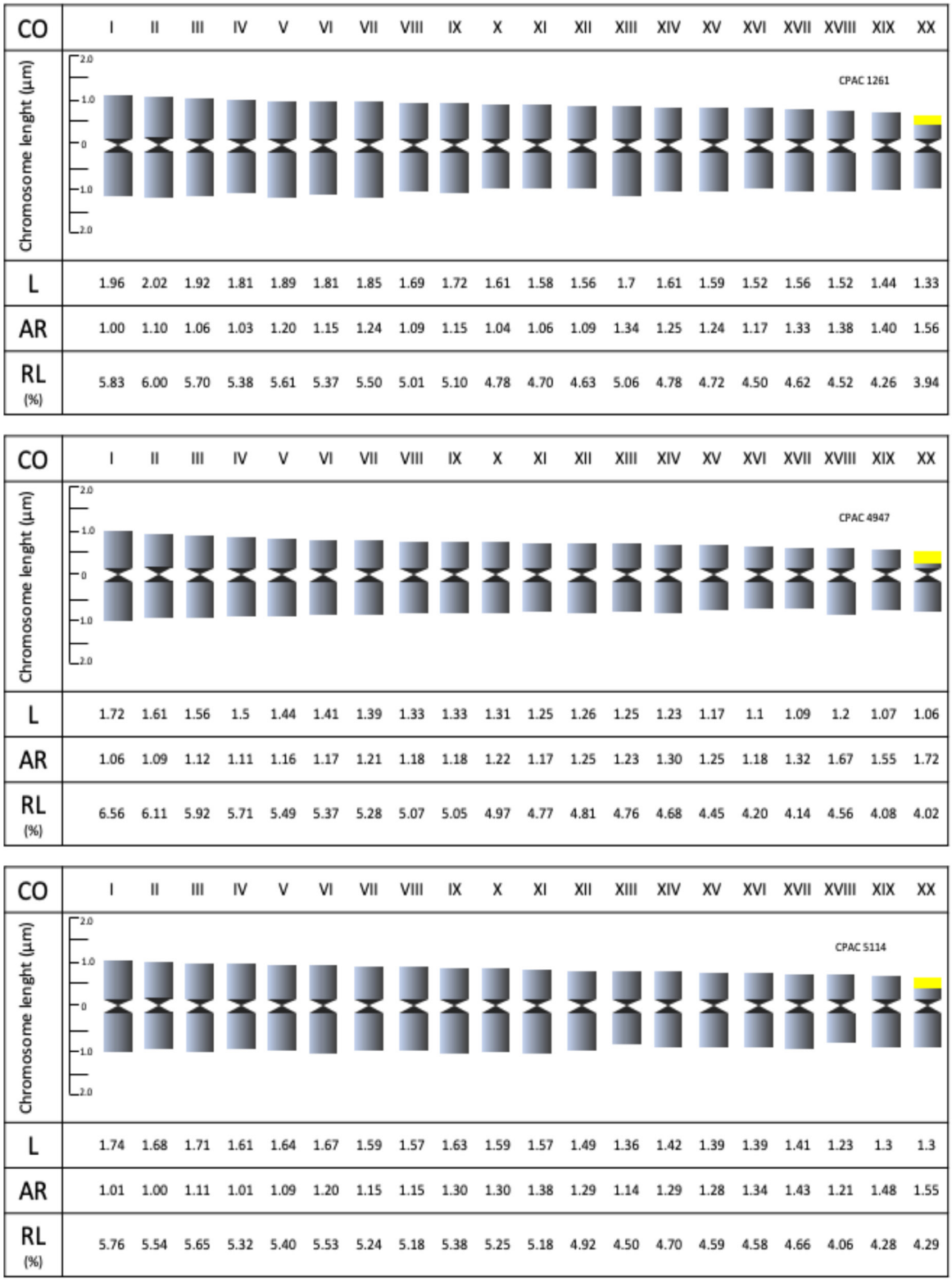


Figure 5. Idiograms of the accessions CPAC 1261, CPAC 4947 and CPAC 5114 of *S. scabra* (2*n*=40) showing the chromosomal length (L), the ratio between arms (AR) and the relative chromosomal length (RL) and DAPI+ bands in blue and CMA+ in yellow. CO = ordering of chromosomal pairs.

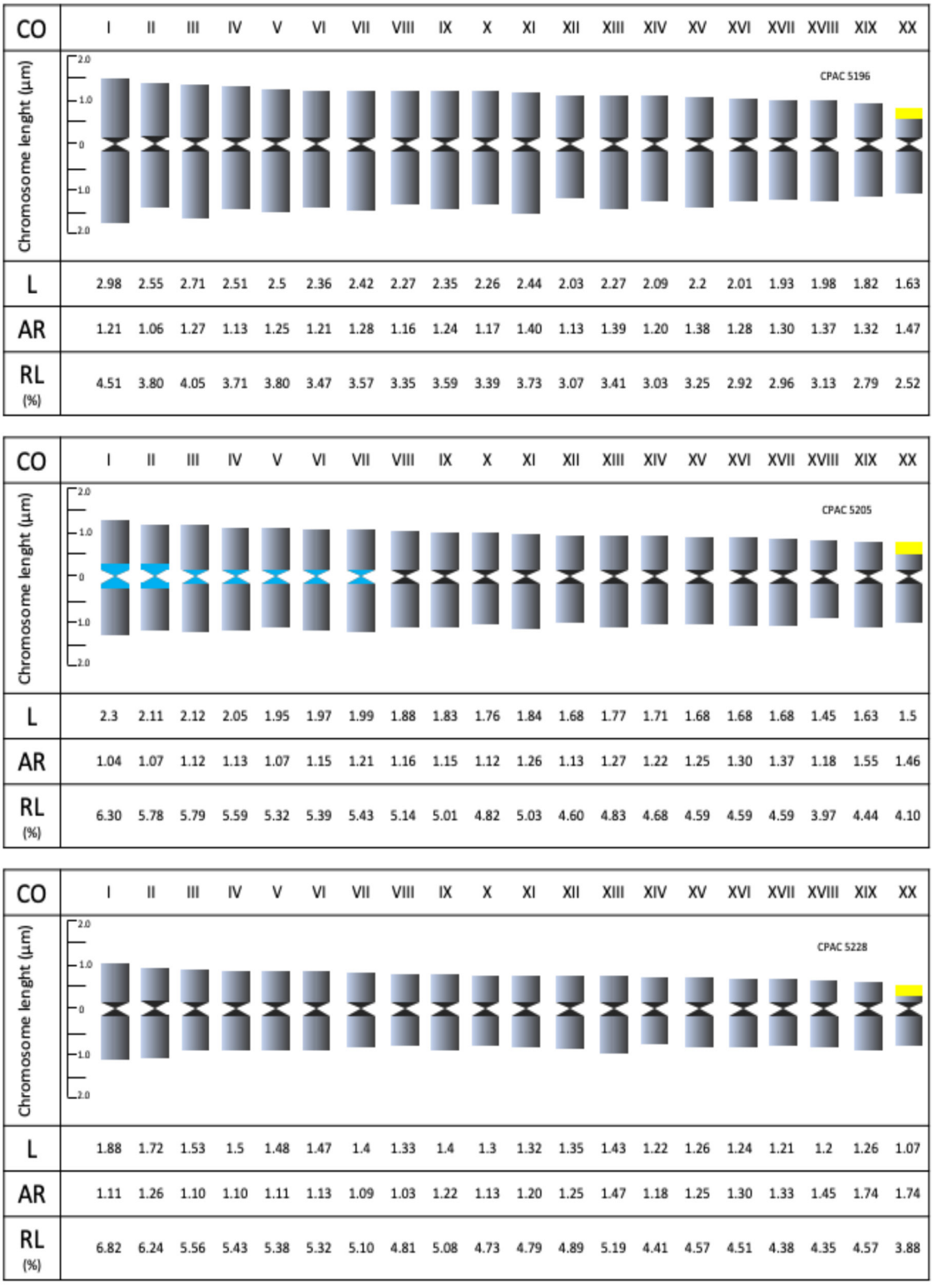


Figure 6. Idiograms of accessions CPAC 5196, CPAC 5205 and CPAC 5228 of *S. scabra* (2n = 40) showing chromosome length (L), arm ratio (AR) and relative chromosome length (RL) and DAPI$^+$ bands in blue and CMA$^+$ in yellow. CO = ordering of chromosome pairs.

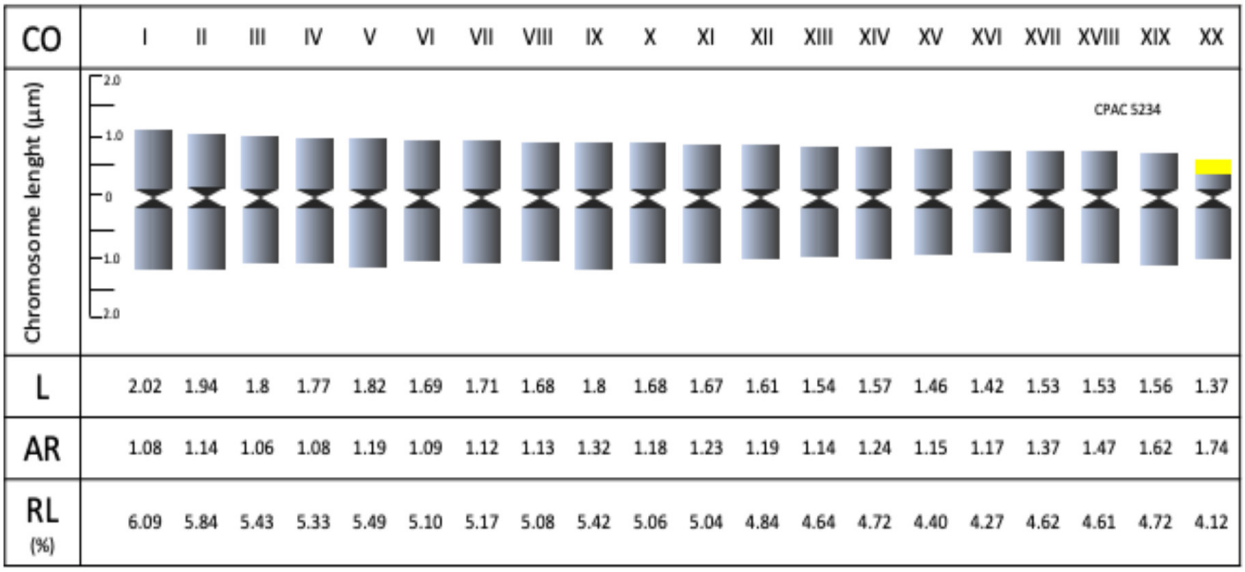


Figure 7. Idiogram of the CPAC 5234 accession of *S. scabra* (2*n*=40) showing chromosomal length (L), ratio between arms (AR) and relative chromosomal length (RL) and CMA+ bands in yellow. CO = ordering of chromosomal pairs.

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IRLANE CRISTINE S.A. LIRA[1]
https://orcid.org/0000-0001-6938-7037

NATONIEL F. DE MELO[2]
https://orcid.org/0000-0001-6888-4090

RAFAELA PRISCILA ANTONIO[2]
https://orcid.org/0000-0003-0913-0541

RONALDO S. DE OLIVEIRA[3]
https://orcid.org/0000-0003-0996-9144

MANOEL A. DE QUEIROZ[4]
https://orcid.org/0000-0001-9501-2343

[1]Universidade Estadual de Feira de Santana (UEFS), Programa de Pós-Graduação em Recursos Genéticos Vegetais/PPG-RGV, Avenida Transnordestina, s/n, Novo Horizonte, 44036-900 Feira de Santana, BA, Brazil

[2]Empresa Brasileira de Pesquisa Agropecuária, Embrapa Semiárido, BR-428, Km 152, s/n, Zona Rural, 56302-970 Petrolina, PE, Brazil

[3]Instituto Federal de Educação, Ciência e Tecnologia da Bahia, IF Baiano, Campus Xique-xique, BA 052, Km 468, Zona Rural, 47400-000 Xique-Xique, BA, Brazil

[4]Universidade do Estado da Bahia, Departamento de Tecnologia e Ciências Sociais/DTCS, Rua Edgard Chastinet Guimarães, s/n, São Geraldo, 48904-711 Juazeiro, BA, Brazil

Correspondence to: **Irlane Cristine de Souza Andrade Lira**
E-mail: irlane.cristine@gmail.com

Author contributions

IRLANE CRISTINE DE S.A. LIRA: conceptualization, investigation, methodology, resources, validation, visualization, writing – original draft, writing – review & editing. NATONIEL F. DE MELO: conceptualization, investigation, methodology, resources, supervision, validation, visualization, writing – original draft, writing – review & editing. RAFAELA PRISCILA ANTONIO: resources, validation, visualization, writing – original draft, writing – review & editing. RONALDO S. DE OLIVEIRA and MANOEL A. DE QUEIROZ: resources, validation.