ARTICLE

Karyotype polymorphism of GC-rich constitutive heterochromatin in *Capsicum* L. pepper accessions

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Breno Machado de Almeida[1*], Lívia do Vale Martins[2], Ângela Celis de Almeida Lopes[2], Regina Lúcia Ferreira Gomes[2], Sérgio Emílio dos Santos Valente[2], Ana Paula Peron[3], Verônica Brito da Silva[2] and Lidiane de Lima Feitoza[2]

Abstract: *Capsicum is represented by peppers and sweet peppers and comprises a group with remarkable genetic variability. Different cultivated* Capsicum *peppers of Brazil were evaluated by using CMA_3 and DAPI specific fluorochromes. There was high polymorphism of highly GC-rich CMA heterochromatic bands among the analyzed species, ranging from six (BAGC 114;* C. annuum*) to 26 blocks (BAGC 81; C.* baccatum*). Heterochromatin percentage ranged from 3.14% (BAGC 114;* C. annuum*) to 8.72% (BAGC 81;* C. baccatum*), corroborating the variation in the number of heterochromatic bands, particularly those distributed in the terminal and subterminal regions of the chromosomes. The information reported in this paper supports the cytogenetic characterization of the domesticated peppers accessions belonging to the* Capsicum *Germplasm Active Bank of the Federal University of Piauí (BAGC-UFPI). Moreover, the present data helped to better understand the karyotype features of peppers and provide additional information that could contribute to the improvement and maintenance of* Capsicum *genetic breeding programs.*

Keywords: *CMA/DAPI staining, genetic diversity, genetic resources*

INTRODUCTION

Capsicum L. genus is native to Tropical and Subtropical America, comprising ~42 species represented by peppers and bell peppers, spices widely used in the worldwide cuisine (Carrizo García et al. 2016, Barboza et al. 2019, Barboza et al. 2020a, Barboza et al. 2020b). The five cultivated and domesticated species of the genus are: *Capsicum annuum* L., *C. chinense* Jacq., *C. frutescens* L., *C. baccatum* L. and *C. pubescens* R. & P. (Ribeiro et al. 2020).

Brazil is considered to be a secondary diversity center of peppers with domesticated, semi- domesticated and wild *Capsicum* species (Barboza et al. 2020a, Ribeiro et al. 2020). Studies of genetic diversity characterization in cultivated plant species are essential for breeding programs. In this context, different methodologies have been applied to explore the genetic divergence among germplasm accessions, e.g., phenotype, biochemical, molecular, and cytogenetic characterization (Costa et al. 2019, Assis et al. 2020, Nankar et al. 2020).

***Corresponding author:**
E-mail: breno.m.almeida@ufv.br
ORCID: 0000-0003-0982-8886

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[1] Universidade Federal de Viçosa, Campus Universitário, Departamento de Agronomia, Viçosa, 36570-900, Minas Gerais, Brasil
[2] Universidade Federal do Piauí, Campus Universitário Ministro Petrônio Portella, 64049-550, Teresina, Piauí, Brasil
3 Universidade Tecnológica Federal do Paraná, Campus de Campo Mourão, 87301-899, Paraná, Brasil

Cytogenetic studies using different approaches have been providing important information about the intra- and interspecific *Capsicum* diversity and have contributed to the systematics, genetics, evolution, and genetic breeding of the genus (Moscone et al. 1993, Moscone et al. 1996, 2007, Scaldaferro et al. 2013, Scaldaferro et al. 2016, Martins et al. 2018, Zhou et al. 2019). Moreover, meiotic analysis has provided essential information regarding reproduction, fertility, recombination, meiotic irregularities and gamete viability. These karyological data, together with molecular and morphoagronomical data, help breeders plan and execute intra- and interspecific crosses of *Capsicum* species (Pozzobon et al. 2006, Pozzobon et al. 2015).

Capsicum species have basic chromosome numbers *n* = 12 and *n* = 13. Species with *2n* = 2x = 24 have symmetric karyotypes, and the chromosome number *n* = 12 is considered to be the ancestral state of *Capsicum* (Carrizo García et al. 2016). This number is present in the domesticated species *C. annuum, C. chinense, C. frutescens, C. baccatum* and *C. pubescens.* On the other hand, the 2n = 2x = 26 group is represented by wild species in South America and exhibits asymmetric karyotype formula, as found in *C. campylopodium* and *C. mirabile* (Pozzobon et al. 2006, Moscone et al. 2007, Barboza et al. 2020b).

The fluorochrome banding with CMA (chromomycin A3) and DAPI (4'-6-diamidino-2- phenylindole) is a classical technique widely used in cytogenetics studies to evaluate the general composition and distribution of the constitutive heterochromatin (CH) (Schweizer 1976). CMA staining reveals the GC (Guanine-Cytosine)-rich regions, while the DAPI staining reveals the AT (Adenine-Thymine)-rich regions in the chromosomes (Guerra 2000).

Previous studies on *Capsicum* identified four types of CH by using the CMA/DAPI banding pattern: highly GC-rich and AT-reduced ($CMA^{++/}DAPI^{-}$); (2) highly AT-rich and GC-reduced ($CMA^{-/}DAPI^{++}$); (3) moderately GC-rich and AT-neutral ($CMA^{+}/DAPI^{0}$); and (4) moderately GC-rich and moderately AT-rich ($CMA^{+}/DAPI^{+}$) (Moscone et al. 2007, Scaldaferro et al. 2013, Romero-da Cruz and Forni-Martins 2015, Romero-da Cruz et al. 2017, Barboza et al. 2019). Most of the CH bands are located in terminal and subterminal regions of the chromosomes, except in *C. flexuosum* and *C. campylopodium,* which show intercalary CH bands. In some cases, the centromeric heterochromatin is visualized as weak $CMA^{+}/DAPI^{0}$ markers in some cultivated taxa, as observed in *C. chinense* and *C. frutescens* (Moscone et al. 2007).

Grabiele et al. (2018) reported a new type of satellite DNA in *Capsicum,* composed of inactive rDNA 18S-25S. The complete unity of rDNA 35S is amplified, disperse and organized in tandem in the genome of the species with *n* = 12, and it is the main component of highly GC-rich heterochromatin, except for *C. recurvatum* and *C. rhomboideum* (both with *n* = 13).

Because of their socioeconomic importance and aiming to preserve the genetic diversity of peppers in the country, the Federal University of Piauí has created the *Capsicum* Germplasm Active Bank (BAGC-UFPI). Currently, the BAGC-UFPI has more than 250 pepper accessions belonging to different Brazilian regions (Northeast, North, Southeast, Midwest and South). Moreover, UFPI performs initiative genetics pre-breeding studies in *Capsicum* (Sousa et al. 2015, Martins et al. 2018, Costa et al. 2019).

In this context, the aim of this study was to increase knowledge of the karyotype constitution of the socioeconomic important *Capsicum* species. This paper described in detail the number, distribution pattern, and percentage of CH bands in the karyotype of 16 pepper accessions of different Brazilian regions (Northeast, Midwest, Southeast and South) belonging to BAGC-UFPI by CMA/DAPI banding technique. The present data contribute to better understanding the dynamics of the CH distribution pattern in *Capsicum* species that can probably be related to the genetic diversity observed in this economically important genus.

MATERIAL AND METHODS

Plant materials

Seeds of 16 pepper accessions were obtained from BAGC-UFPI (Table 1), located in Teresina, Piauí, Brazil. The accessions were selected on the basis of previous intra- and interspecific morphological diversity characterization study (unpublished data).

Chromosome preparation

Root tips obtained from the germinated seeds were pretreated with p-dichlorobenzene (0.015 g mL^{-1}) for 2 hours at room temperature, fixed in solution (ethanol: acetic acid v/v) for at least 24 hours, and stored at -20 °C until use.

CMA/DAPI fluorochrome staining

The protocol described by Schweizer and Ambros (1994) was followed with minor modifications. For each accession, root tips were digested with an enzymatic solution containing 2% cellulase (Onozuka R-10) and 20% pectinase (Sigma-Aldrich). The slides were stained with 10 µL of CMA (0.5 mg mL^{-1}) for 1 hour, counterstained with 10 µL of DAPI (2 mg mL^{-1}) for 30 min, mounted in glycerol/McIlvaine (1:1) and stored for three days before analysis.

Image analyses and morphometry

The five metaphases of each accession were photographed using a DF7000GT digital camera coupled to a Leica DM4B microscope. The images were optimized for brightness and contrast using Adobe Photoshop CS3. Chromosome sizes were measured using the Drawid v0.26 software (Kirov et al. 2017). Idiograms were constructed using Corel DRAW (2017), and chromosome morphologies and parameters (Table 1) were classified according to Guerra (2002). Heterochromatin percentage (%) of GC-rich heterochromatin of each accession and the total size of the heterochromatic blocks were compared to the total size of the chromosome set, as described by Fonsêca et al. (2010).

Table 1. Accession, common name, scientific name, number of diploid chromosomes (2n), range of chromosomal size (RCS), Ratio between long and short arms (R) of each chromosome pair, karyotype formula (KF), total chromosomal length (TCL), chromosomal medium length (CML) and number of CMA/DAPI bands (CMA_3/DAPI). $^{++}$ represents more strongly stained CMA bands, while $^{+}$ represents slightly stained CMA bands. ^{0} represents AT neutral and $^{-}$ AT reduced bands. Heterochromatin percentage (%)

Accession	Common name	Scientific name	*2n*	RCS (µm)	R	KF	TCL (µm)	CML (µm)	CMA_3/DAPI	Heterochromatin (%)
BAGC 81	Pimenta dedo-de-moça	*C. baccatum* var. pendulum	24	2.17-3.14	1.28	11M + 1SM	63.86	2.67	6 CMA^{++}/$DAPI^{-}$ 20 CMA^{+}/$DAPI^{0}$	8.72
BAGC 91	Pimenta-de-cheiro (ardida)	*C. chinense*	24	1.95-2.94	1.26	10M + 2SM	58.33	2.44	6 CMA^{++}/$DAPI^{-}$ 14 CMA^{+}/$DAPI^{0}$	8.53
BAGC 114	Jalapeño Mexicana	*C. annuum* var. *annuum*	24	2.07-3.65	1.13	12M	68.52	2.86	2 CMA^{++}/$DAPI^{-}$ 4 CMA^{+}/$DAPI^{0}$	3.14
BAGC 117	Malagueta preta	*C. frutescens*	24	2.05-3.17	1.33	11M + 1SM	57.61	2.6	8 CMA^{++}/$DAPI^{-}$ 4 CMA^{+}/$DAPI^{0}$	8.57
BAGC 120	Pimenta bunda-de-velho	*C. chinense*	24	2.10-2.93	1.26	11M + 1SM	61.10	2.55	4 CMA^{++}/$DAPI^{-}$ 8 CMA^{++}/$DAPI^{0}$	4.36
BAGC 123	Pimenta bode-amarela	*C. chinense*	24	3.52-4.80	1.24	11M + 1SM	78.46	3.97	4 CMA^{++}/$DAPI^{-}$ 6 CMA^{+}/$DAPI^{0}$	4.71
BAGC 156	Unknown	*C. baccatum* var. *pendulum*	24	2.01-3.01	1.30	10M + 2SM	61.54	2.56	10 CMA^{++}/$DAPI^{-}$ 14 CMA^{++}/$DAPI^{0}$	8.36
BAGC 157	Pimenta	*C. baccatum* var. *pendulum*	24	2.0-3.21	1.34	10M + 2SM	61.20	2.55	8 $CMA^{++/}$/$DAPI^{-}$ 4 CMA^{+}/$DAPI^{0}$	5.65
BAGC 160	Pimenta	*C. chinense*	24	2.03-3.28	1.33	12M	65.07	2.72	6 CMA^{++}/$DAPI^{-}$ 14 CMA^{++}/$DAPI^{0}$	7.79
BAGC 178	Pimenta	*C. baccatum* var. *pendulum*	24	2.11-3.42	1.33	11M + 1SM	66.36	2.76	8 CMA^{++}/$DAPI^{-}$ 10 CMA^{+}/$DAPI^{0}$	6.35
BAGC 208	Pimenta pitanga	*C. baccatum* var. pendulum	24	2.26-3.62	1.29	11M + 1SM	69.20	2.88	6 CMA^{++}/$DAPI^{-}$ 10 CMA^{+}/$DAPI^{0}$	6.44
BAGC 220	Pimenta-Vermelha	*C. annuum* var. *annuum*	24	1.76-3.86	1.24	11M +1SM	71.54	2.98	2 CMA^{++}/$DAPI^{-}$ 8 CMA^{+}/$DAPI^{0}$	4.29
BAGC 242	Pimenta de-cheiro-laranja	*C. chinense*	24	2.59-4.08	1.25	12M	77.03	3.21	6 CMA^{++}/$DAPI^{-}$ 10 CMA^{+}/$DAPI^{0}$	7.70
BAGC 249	Pimenta japonesa	*C. chinense*	24	2.32-3.45	1.25	12M	67.46	2.81	6 CMA^{++}/$DAPI^{-}$ 8 CMA^{++}/$DAPI^{-}$	7.25
BAGC 250	Pimenta-de-cheiro	*C. chinense*	24	2.46-3.61	1.26	12M	70.16	2.92	4 CMA^{++}/$DAPI^{-}$ 6 CMA^{+}/$DAPI^{0}$	5.96
BAGC 252	Pimenta-de-cheiro	*C. chinense*	24	2.10-2.87	1.24	12M	58.20	2.43	6 CMA^{++}/$DAPI^{-}$ 4 CMA^{+}/$DAPI^{0}$	5.77

RESULTS AND DISCUSSION

Capsicum accessions showed *2n* = 24 chromosomes with metacentric (M) and submetacentric (SM) morphologies (Table 1, Figures 1 and 2). There was a *Solanum-like* prophase condensation pattern, with early-condensed proximal regions and late-condensed terminal chromatin (Figure 2i) (Feitoza et al. 2017). Its condensation pattern is related to the chromatin organization at interphase, being also related to the nuclei type. The semi-reticulated nuclei were identified in all analyzed accessions (Figure 2h and 2k), with simple and well-distributed chromocenters, as previously identified by Scaldaferro et al. (2016) and Martins et al. (2018).

Chromosomal Size (RCS) ranged from 1.76 µm in *C. annuum* (BAGC 220) to 4.80 µm in *C. chinense* (BAGC 123), while Total Chromosomal Length (TCL) ranged from 57.61 µm in *C. frutescens* (BAGC 117) to 78.46 µm in *C. chinense* (BAGC 123) (Table 1). Variation regarding RCS and TCL in different *Capsicum* accessions is common and has been previously reported. Sousa et al. (2011) found RCS variation from 2.59 µm to 4.12 µm in *C. chinense* from four accessions collected in different regions of Brazil, while TCL ranged from 82.40 to 84.18 µm. Sousa et al. (2015) identified RCS variation from 3.29 µm in *C. chinense* to 7.48 µm in *C. baccatum.* In *C. frutescens,* the authors found TCL divergence from 105.96 to 144.44 µm. Similarly, Martins et al. (2018) found RCS from 1.96 µm to 5.94 µm, while TCL ranged from 66.55 µm to 117.56 µm in different domesticated *Capsicum* accessions belonging to BACC-UFPI. These differences could be related to unequal degrees of chromosome condensation during cell division (Moscone 1990, Martins et al. 2018), differences in pretreatment (Pozzobon et al. 2006), and/or different classes of repetitive DNA sequences, such as Copia and Gypsy LTR-retrotransposons that can result in genome size variation of the species (Assis et al. 2020).

Three karyotype formulas were identified in the analyzed accessions: seven accessions (BAGC 81, 1117, 120, 123, 178, 208, and 220) exhibited 11M + 1SM, and six (BAGC 114, 160, 242, 249, 250, and 252) exhibited 12M (Table 1). Only three accessions (BAGC 91, 156, and 157) showed 10M + 2SM, which may suggest the presence of three different cytotypes of *Capsicum* accessions at BAGC-UFPI (Sousa et al. 2015, Martins et al. 2018). Further studies using more detailed cytomolecular techniques, e.g., 5S and 35S ribosomal DNA and/or transposable elements such as FISH probes, are needed to investigate the karyotype constitution of these accessions in detail.

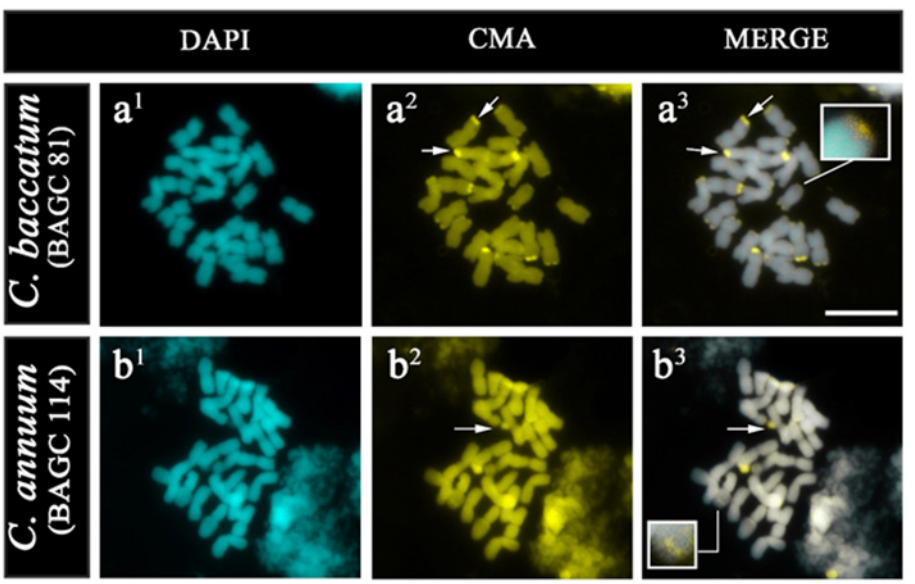

Figure 1. Double staining with CMA/DAPI fluorochromes in *Capsicum* 81 and 114 accessions. a^1 and b^1 represent the chromosomes counterstained with DAPI (in blue). a^2 and b^2 represent the chromosomes stained with CMA. a^3 and b^3 represent the merge of DAPI and CMA images. *C. baccatum* (a^1-a^3) shows the higher number of CMA heterochromatic bands (26), while *C. annuum* (b^1-b^3) had the smallest number of CMA bands (six). Arrows indicate large CMA^{++} blocks. All inserts indicate small CMA^{+} blocks in a region that is difficult to detect. Bar = 10 µm.

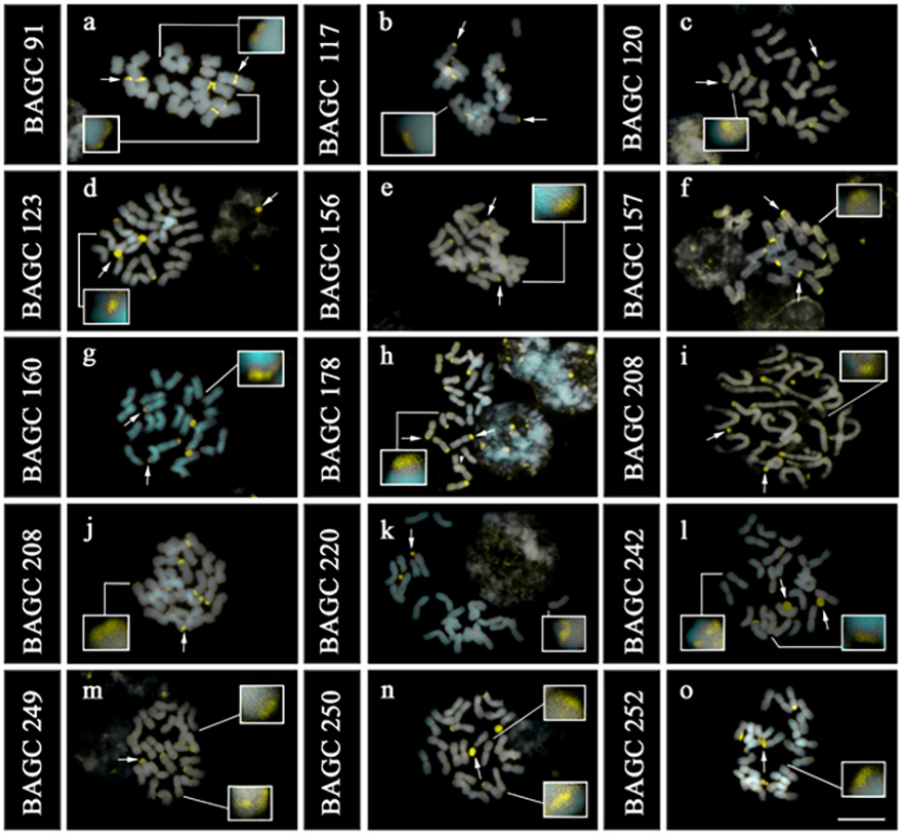

Figure 2. Double staining with CMA/DAPI fluorochromes showing high polymorphism of the CMA heterochromatic bands in different analyzed *Capsicum* accessions. The number of each accession is indicated on the left side of the metaphases. Arrows indicate large CMA^{++} blocks. All inserts indicate small CMA^{+} blocks in a region that is difficult to detect. Bar = 10 µm.

The double-staining technique with CMA and DAPI fluorochromes allowed the identification of two heterochromatin banding patterns: $CMA^{++}/DAPI^{-}$ and $CMA^{+}/DAPI^{0}$ (Figures 1 and 2). No DAPI bands were found in the present study, as opposed to previous works with wild *Capsicum* species (Moscone et al. 1996, Moscone et al. 2007, Scaldaferro et al. 2013). DAPI bands were only found in domesticated (*C. pubescens)* and wild species (*C. campylopodium, C. pereirae* and *C. praetermissum)* (Moscone et al. 2007). For a better understanding of the distribution pattern of the CMA bands in the haploid set, in addition to determining the morphology and size of the chromosomes, all the accessions of the karyotype were schematically represented as idiograms (Figure 3).

All the accessions showed variable number, size, distribution, and type of the heterochromatin blocks. At least two terminal $CMA^{++}/DAPI^{-}$ bands were identified in all accessions, probably corresponding to the nucleolar organizer regions (NORs). The presence of this $CMA^{++}/DAPI^{-}$ pair is commonly described in *Capsicum* species and it seems to be universal within the genus *Capsicum* (Moscone et al. 1996, Martins et al. 2018, Assis et al. 2020).

The accession BAGC 114 (C. *annuum* var. *annuum)* had the smallest number of CH bands, with six CMA blocks. Similar results were found by Moscone et al. (1996) and Martins et al. (2018). The authors highlighted that its species has a smaller number of and simpler heterochromatin banding patterns. On the other hand, the accession BAGC 117, commonly known as "malagueta preta" (C. *frutescens),* showed 12 CMA bands (Figure 2b): four $CMA^{++}/DAPI^{-}$ pairs and two $CMA^{+}/DAPI^{0}$ pairs. Differently, Moscone et al. (1996) identified 18 GC-rich bands and Martins et al. (2018) found four and six GC-rich bands for its species.

According to Carrizo García et al. (2016), *C. annuum, C. chinense,* and *C. frutescens* domesticated species and *C. galapagoense* wild species belong to the *Annuum* clade, previously known as "white-flowered" group. The species

belonging to its clade are closely related regarding karyotype features, including chromosomes with small size, low DNA content, and low GC-rich heterochromatin constitution, mainly distributed at terminal regions of the chromosomes (Moscone et al. 1993, Moscone et al. 1996, Moscone et al. 2007).

C. chinense accessions showed a highly variable number of heterochromatic blocks. For example, the accessions BAGC 123, 250 and 252 (Figure 2c, 2d, 2n and 2o) showed a small number of bands: four $CMA^{++}/DAPI^{-}$ and eight $CMA^{+}/DAPI^{0}$ in BAGC 120, and four $CMA^{++}/DAPI^{-}$ and six $CMA^{+}/DAPI^{0}$ in BAGC 123 and 250, respectively, and six $CMA^{++}/DAPI^{-}$ and four $CMA^{+}/DAPI^{0}$ in BAGC 252. Similar results were found by Romero-da-Cruz and Forni-Martins (2015). The authors found small terminal GC-rich bands in the small and long arms of four chromosome pairs of *C. chinense.*

The accessions belonging to *C. annuum* (BAGC 114 and 220) and *C. chinense* (BAGC 120, 123, 242, 249 and 250) showed an additional small pair of intercalary bands moderately rich in GC ($CMA^{+}/DAPI^{0}$). BAGC 91 and 160 (Figure 2a and 2g) are distinguished from the others because of the presence of 20 GC bands, i.e., six CMA^{++} and 14 CMA^{+} bands. In general, the CH is not homogeneous, varying quantitatively and qualitatively within and between species (Guerra 2000, Roa and Guerra 2015, Mate-Sucre et al. 2020). Moscone et al. (2007) found polymorphism regarding number and size of CMA bands in *C. annuum* cultivars that showed highly GC-rich heterochromatin, with distal and interstitial moderately GC-rich bands distribution among the cultivars.

C. baccatum var. *pendulum* accessions stand out for the number and variation of the heterochromatic bands. The accessions BAGC 157, 178, 208, 156 and 81 have 12, 18, 16, 24 and 26 GC-rich blocks, respectively (Figures 1 and 2). Variations of the number and brightness intensity of the blocks were previously reported for *C. baccatum* cytotypes. Moscone et al. (1996) found variation from 24 to 28 GC blocks, and Aguilera et al. (2017) identified 32 terminal CMA^{+} sites in *C. baccatum* var. *pendulum* chromosomes by the CMA/DA/DAPI banding technique. Similarly, Martins et al. (2018) reported 10 to 18 CMA bands in *C. baccatum* var. *pendulum* accessions belonging to the same BAGC-UFPI germplasm bank. Differently from other domesticated pepper species, its species are differentiated by a larger karyotype length (and larger DNA genome, 3.2 Gb), greater presence of GC-rich heterochromatin and a more complex heterochromatic band pattern (Moscone et al. 2007, Grabiele et al. 2014, Grabiele et al. 2018, Kim et al. 2017, Assis et al. 2020).

Regarding the percentage of GC-rich heterochromatin (Table 1), there was variation from 3.14 - 4.29% in *C. annuum* var. *annuum* (BAGC 114 and 220, respectively) to 5.65 - 8.72% in *C. baccatum* var. *pendulum* (BAGC 157 and 81, respectively). In *C. chinense,* the percentage ranged from 4.36% (BAGC 120) to 8.53% (BAGC 91), while in *C. frutescens* (BAGC 117), it was found that CH composes 8.57% of the total genome.

There is an association between the number and percentage of the CH blocks, despite minor divergences. These differences are probably related to the evolutionary dynamics of the DNA sequences of CH constitution that play an important role in the karyotype evolution of *Capsicum* (Scaldaferro et al. 2013). The accessions with smaller and higher % of CH were the same with smaller and higher CMA banding blocks, namely BAGC 114 (*C. annuum* var. *annuum*) and BAGC 81 *C. baccatum* var. *pendulum,* respectively.

Our results corroborate the variation in CH content (1.72% to 38.91%) previously found within and among species, with an average value of 10.90% (Moscone et al. 1996, Moscone et al. 2003, Moscone et al. 2007, Scaldaferro et al. 2013). In most of the analyzed taxa, there is a positive association between karyotype size and CH amount, indicating that its heterochromatin contributes to the differences found in the chromosome size and probably in the genome size of *Capsicum* (Moscone et al. 1996, Moscone et al. 2007, Scaldaferro et al. 2013, Scaldaferro et al. 2016, Martins et al. 2018). A great variation in genome size is common in plants, probably owing to the large fraction of repetitive DNA found in their genomes. In *Capsicum,* a genus with species of large genome size, this variation could be explained by the large accumulation of transposable elements (Park et al. 2012).

Previous genetic diversity studies using different approaches (morphological, agronomic, cytogenetic, and molecular) have provided additional data about the general genomic/phenotypic features of the species. Cytogenetics is an important tool for understanding pepper karyotypes, as well as serving as a basis for conservation activities and applied research in genetic breeding (Grabiele et al. 2018, Costa et al. 2019, Assis et al. 2020). The CMA/DAPI technique identified the presence and high variation of GC-rich CH in all the analyzed pepper accessions. Polymorphisms of the heterochromatic blocks could be confirmed within and among *Capsicum* domesticated species.

A general pattern was found for CMA marks, particularly located at terminal regions of the chromosomes, with a small number of marks occurring in the intercalary regions of *C. annuum* and *C. chinense* chromosomes. The additional information generated in this study will contribute to a better characterization and understating of karyotype polymorphisms of the Brazilian pepper domesticated accessions belonging to BAGC-UFPI. Moreover, these data will provide additional

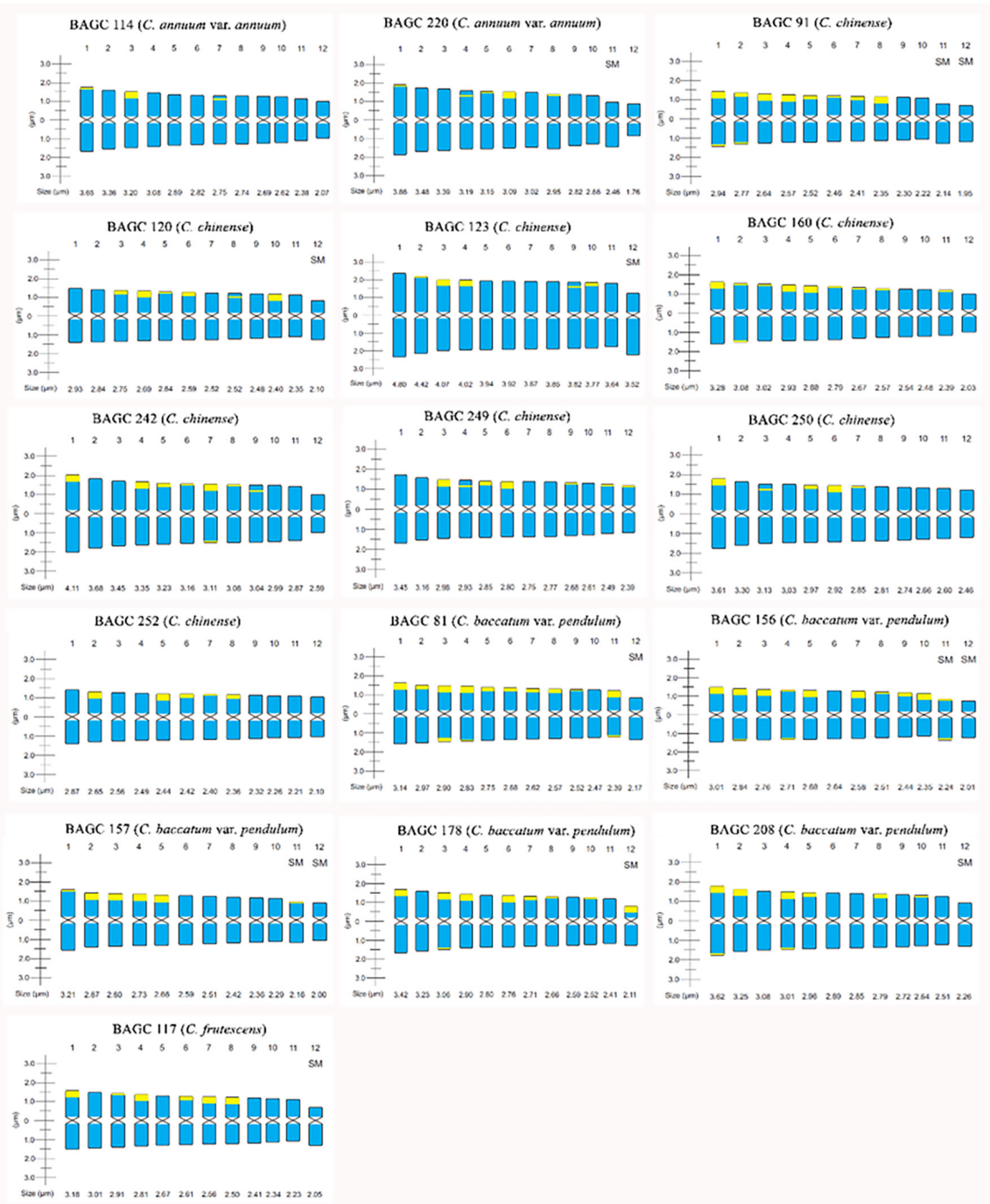

Figure 3. Idiograms representing the size, morphology and distribution of the CMA bands (yellow bands) in the karyotypes of different *Capsicum* pepper accessions belonging to the BAGC-UFPI.

information that can help the genetic breeding programs of *Capsicum* species. *Capsicum* genetic variation is the main support for the genetic breeding program of the genus. Additionally, cytogenetics characterization and molecular and morphological studies are essential for segregated population management and the development of strategies for conservation of pepper germplasms.

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