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Original article

Inter- and intra-population B chromosome variability in *Partamona helleri* (Apidae: Meliponini)

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Abstract – The stingless bee *Partamona helleri* (Meliponini) is distributed across the Atlantic rainforest biome in Brazil. Cytogenetic studies on *P. helleri* have shown a conserved diploid number and the presence of several B chromosome types. Our goal was to investigate the intraspecific karyotypic variation among 21 colonies of *P. helleri* from 14 different localities across four Brazilian states, using classical and molecular cytogenetics, as well as image cytometry (ICM). All populations had $2n = 34$ and up to 5B chromosomes, which showed inter- and intra-population variation in number and size. C-banding evidenced the centromeric/pericentromeric heterochromatin and the partially heterochromatic B chromosomes. $GA_{(15)}$ microsatellite hybridized in the euchromatic arm of the A chromosomes and did not mark the B chromosomes, whereas $TTAGG_{(6)}$ corresponded to the telomeric sequence of the A and B chromosomes. The 18S rDNA and CMA_3^+ sites varied among the colonies, indicating the karyotype variability of the species. The ICM demonstrated an increase in nuclear genome size regarding the presence of B chromosomes. We highlighted the importance of population studies in species with a wide range of distributions, and how the B chromosomes contribute to the intra- and inter-population variability of *P. helleri*.

B chromosome / flow cytometry / image cytometry / repetitive DNA / stingless bee

1. INTRODUCTION

Bees of the genus *Partamona* Schwarz, 1939 are social insects belonging to the Meliponini tribe (Apidae), distributed throughout the Neotropical region from southern Mexico to southern Brazil (Pedro and Camargo 2003). This genus had a recent divergence 4 mya (Rasmussen and Cameron 2010) and currently comprises 33 described species (Camargo and Pedro 2013). They are widely represented in behavioral, ecological, and ecotoxicological studies (Bernardes et al. 2018; Araujo et al. 2019; Pereira et al. 2020), as well as in studies involving genetics and cytogenetics (Brito and Arias 2010; Rasmussen and Cameron 2010; Martins et al. 2013, 2014; Tosta et al. 2014; Gonçalves et al. 2020; Lopes et al. 2020).

To date, 11 *Partamona* species have been cytogenetically analyzed, showing a conserved chromosome number of $2n = 34$ in females

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and $n = 17$ in males (Brito-Ribon et al. 1999; Brito et al. 2003, 2005; Marthe et al. 2010; Tosta et al. 2014; Gonçalves et al. 2020; Lopes et al. 2020), including the description of several types of B chromosomes (Martins et al. 2009, 2013, 2014). *Partamona helleri* (Friese 1900) holds the record of the highest number of supernumerary chromosomes in bees, with up to 7B being found in the same individual (Martins et al. 2014; Cunha et al. 2021a). This species is distributed across the Atlantic rainforest biome in Brazil, encompassing the states of Bahia, Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, and Santa Catarina (Camargo and Pedro 2013).

Martins et al. (2013) highlighted the lack of homology of the *P. helleri* B chromosomes with any of the regular set of chromosomes of the A complement through chromosome painting, whereas the discussion about its origin in the genus still requires further investigation (Tosta et al. 2014; Machado et al. 2016). Population studies have demonstrated variations in the number (0–7) and morphology (submetacentric-acrocentric) of these B chromosomes in *P. helleri* from different localities (Brito et al. 1997; Tosta et al. 2007; Martins et al. 2009, 2013, 2014), highlighting the intraspecific genetic diversity of this taxon.

We aimed to investigate the intraspecific karyotypic variation in *P. helleri* using classical and molecular cytogenetics, as well as by image cytometry (ICM). The current sampling area was expanded to four Brazilian states. The use of repetitive DNA probes can provide valuable information on the genome organization and B characterization (Munoz-Pajares et al. 2011; Ruiz-Estévez et al. 2012; Milani and Cabral-de-Mello 2014; Ruiz-Ruano et al. 2015; Jetybayev et al. 2018). Additionally, the image cytometry associates cytogenetic and cytometric tools to quantify the DNA content of individual chromosomes and can be used to compare A and B complements, which are ideal for differentiating small chromosomes (Rosado et al. 2009; Ruiz-Ruano et al. 2011; Mendonça et al. 2016; Silva et al. 2018a).

2. MATERIAL AND METHODS

2.1. Sampling and cytogenetic techniques

Samples of 21 natural colonies of *P. helleri* were collected from 14 localities encompassing the states of Minas Gerais — MG, Espírito Santo — ES, Rio de Janeiro — RJ, and Bahia — BA (Table I; Figure 1). Collecting permit number SISBIO-ICMBio 52,690–1 was issued to Camila Moura Novaes. Voucher specimens were deposited in the scientific collection of the Apiário Central at Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

The cerebral ganglia were dissected from 10 larvae of each colony to obtain metaphasic chromosomes (Imai et al. 1988). Giemsa staining was used to determine the chromosome number and the presence of B chromosomes. To determine the general heterochromatic patterns, the C-banding technique was performed on some of the colonies, representing the four Brazilian states (Sumner 1972). On average, 10 metaphases of each individual were photographed using a photomicroscope BX 60 coupled with a Q-Color3 Olympus® image capture system.

Fluorescence in situ hybridization (FISH) was performed following Pinkel et al. (1986), using $GA_{(15)}$ and $TTAGG_{(6)}$ microsatellite probes, as well as the 18S rDNA probe. $GA_{(15)}$ and $TTAGG_{(6)}$ probes were synthesized and labeled with Cy3 fluorochrome at the 5′ end (Sigma-Aldrich). The 18S ribosomal probe was obtained through polymerase chain reaction (PCR) using the primers: 5′-TAATTCCAGCTCCAATAG-3′ and 5′-CCACCCATAGAATCAAGA-3′ (Pereira 2006). This probe was labeled with digoxigenin-11-dUTP (Roche Applied Science), and the signal was detected using anti-digoxigenin-rhodamine (Roche Applied Science). Sequential staining with the fluorochromes DAPI/CMA_3 (4′-6-diamidino-2-phenylindole/chromomycin A_3) was performed following Schweizer (1980). Digital images of the fluorescence images were captured using a BX53F Olympus microscope equipped with an MX10 Olympus camera using CellSens imaging software.

Table I
GPS localities of the *Partamona helleri* samples, maximum of B chromosomes found per locality, and number of marked chromosomes by the CMA_3 and 18S rDNA techniques.

Locality	GPS	Number of B chromosomes	CMA_3^+	18S
Cataguases, MG	21°14′36.12″ 42°43′57.40″	Up to 3 B on females and 1 B on males	4	5
Viçosa, MG	21°14′36.12″ 42°43′57.40″	Up to 3 B	6[#]	9
Linhares, ES	19°24′58.0″ 040°04′31.8″	Up to 5 B	7	5
Governador Lindemberg, ES	19°17′18.5″ 040°30′08.4″	Up to 3 B on females and 2 B on males	6	7
Itarana, ES	19°50′54.4″ 040°53′13.5″	Up to 2 B	4	6
Santa Teresa, ES	19°57′13.0″ 040°36′18.2″	Up to 4 B on females and 2 B on males	4	5
Natividade, RJ	21°2′32.65″ 41°58′16.94″	1 B	7	4
Carangola, MG	20°39′59.71″ 42°1′12.33″	Up to 4 B	7[#]	6
Porciúncula, RJ	20°59′16.45″ 42°6′8.23″	1 B	8	7
Antônio Prado de Minas, MG	20°58′55.12″ 42°09′47.49″	Up to 2 B	5	7
Ponte Nova, MG	20°25′08.56″ 42°55′41.87″	1 B	6	6
Jaguaraçú, MG	19°39′7.07″ 42°42′57.59″	0 B	9	9
Marliéria, MG	19°43′13.03″ 42°44′9.24″	0 B	3	–
Macarani, BA	15°49′22.0″ 40°31′52.3″	Up to 3 B on females and 1 B on males	4	6

[#]B chromosomes with positive signals

2.2. Image cytometry

Five colonies were chosen for chromosomal DNA content measurements using ICM. The Feulgen procedure followed the method described by Carvalho et al. (2011). After fixation, the slides were washed in distilled water, air-dried, and hydrolyzed in HCl 5 M (Merck) for 18 min at 25 ºC. The slides were washed again in distilled water and stained with Schiff reagent for 12 h at 4 ºC. Subsequently, the slides were washed three times for 3 min in 0.5% SO_2-water (sulfur dioxide solution) (Merck) and then with distilled water.

Metaphase images were captured with a Photometrics CoolSNAP Pro (Roper Scientific, Tucson, AZ, EUA) monochromatic CCD video camera of 12 bits gray on an Olympus BX-60 microscope. The microscope was equipped with a source of stabilized light, PlanApo objective magnification of 100× with a 1.4 numeric aperture, an aplanat achromat condenser with an aperture of 1.4, a neutral density filter (ND6), and a green interference filter (IF550). The Köhler method was applied prior to each slide capture session to adjust the optimal light path and reduce stray light.

The images were digitalized with CoolSNAP Pro kit (MD, EUA), and ICM analyses were performed using the Image Pro-Plus1 6.1 analysis system (Media Cybernetics, MD, EUA). Calibration and evaluation of the image analysis system were performed and consisted of three tests: stability, linearity, and uniformity (reviewed in Carvalho et al. 2011). Spatial calibration was performed using a micrometer slide (Olympus). The ICM tools stabilized after 12 min, and the software of the image analysis system automatically calculated $R^2 = 0.9978$ in the linearity test and CV = 0.68% in the uniformity test.

The 12 bits gray image of each metaphase was taken, and all chromosomes were simultaneously

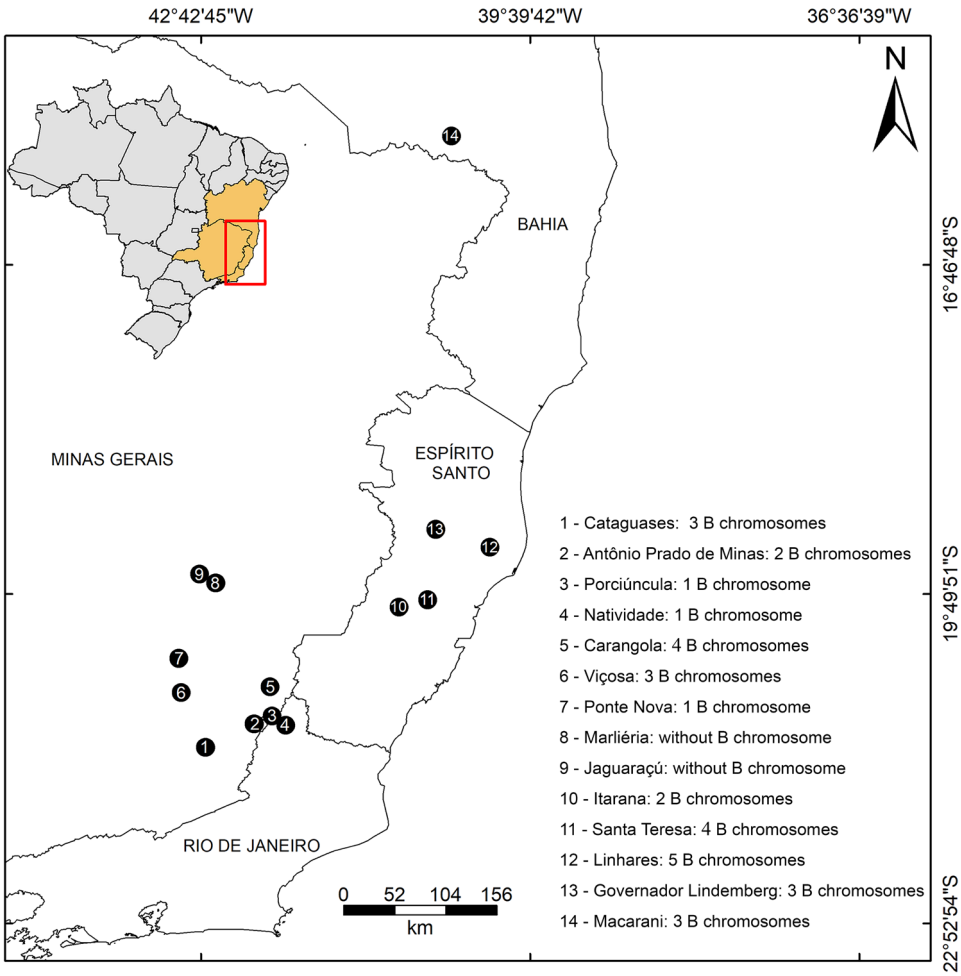

Figure 1. Geographical locations of the *Partamona helleri* samples.

and completely individualized using Image Pro Plus 6.1 software. Because the pixels do not have an intrinsic value, spatial and optical density (OD) values were attributed to them. The software algorithm automatically multiplied the chromosome area (μm^2) by the average OD, resulting in the integrated optical density (IOD). The images were then selected, and the homologous chromosomes of each colony were paired.

One of the colonies without B chromosomes was chosen for the flow cytometry (FCM) experiment to convert the IOD values of each individual chromosome to picograms (pg). FCM was performed according to Cunha et al. (2021b), using adult individuals fixed in absolute ethanol and stored at −20 °C. *Scaptotrigona xanthotricha* was used as the internal standard. The 1C value was measured according to the formula: (G_0/G_1 peak channel of the species × 1C value in pg of the standard)/G_0/G_1 peak channel of the standard.

3. RESULTS

3.1. Cytogenetic analyses

The chromosome number was the same in every colony of *P. helleri*: $2n = 34$ in females and

$n = 17$ in males. Male individuals were found in Cataguases, MG; Governador Lindemberg, ES; Santa Teresa, ES; and Macarani, BA. B chromosomes were found in 17 out of the 21 colonies and varied in number from 0 to 5 (Table I; Figure S1). They represented the smallest chromosomes in the karyotype, except in Macarani, BA, where they were among the largest in the karyotype (Figure S1). The number of B chromosomes also varied within each colony, showing a prevalence of less than 3B among the sampled area, and included some B-free colonies from Minas Gerais and Espírito Santo (Table II). The Bs were classified as submetacentric or subtelocentric, and the colonies had just one of the morphologies or both (Table II). Heterochromatin was distributed mainly in the centromeric and pericentromeric regions of the A complement, whereas the B chromosomes were partially heterochromatic with a euchromatic portion (Figure 2A).

The microsatellite $GA_{(15)}$ probe showed markings in the euchromatic portion of one chromosome arm of all A complement chromosomes and did not mark the B chromosomes (Figures 2B and S2), whereas the $TTAGG_{(6)}$ probe marked only the telomeric region of all chromosomes, including the B chromosomes (Figures 2C and S3). The 18S rDNA probe only marked the A chromosomes, varying from 4 to 9 positive signals (Table I; Figure 3), and diverged from the number of CMA_3^+ in some colonies, which varied from 3 to 9 markings, including some B chromosomes (Table I; Figure S4). No variation within the colony was found regarding the molecular techniques.

Table II
Sample localities of the 21 colonies of *Partamona helleri* with the morphology and frequencies of B chromosomes.

Locality	Morphology of the B	0B	1B	2B	3B	4B	5B
Cataguases, MG	Subtelocentric	19.23%	36.54%	40.38%	3.85%	–	–
Viçosa, MG	Submetacentric, subtelocentric	33.33%	57.41%	5.55%	3.71%	–	–
Linhares, ES	Submetacentric, subtelocentric,	12.50%	33.94%	12.50%	37.50%	1.78%	1.78%
Linhares, ES	Submetacentric, subtelocentric,	47.89%	35.21%	12.68%	4.22%	–	–
Governador Lindemberg, ES	Submetacentric, subtelocentric	35.71%	34.28%	25.71%	4.30%	–	–
Governador Lindemberg, ES	Submetacentric	25.76%	56.06%	18.18%	–	–	–
Governador Lindemberg, ES	Submetacentric	49.25%	44.78%	5.97%	–	–	–
Governador Lindemberg, ES	–	100%	–	–	–	–	–
Governador Lindemberg, ES	–	100%	–	–	–	–	–
Itarana, ES	Submetacentric	79.31%	13.79%	6.90%	–	–	–
Itarana, ES	Submetacentric	48.98%	48.98%	2.04%	–	–	–
Santa Teresa, ES	Submetacentric, subtelocentric	10.15%	14.49%	39.13%	30.43%	5.80%	–
Natividade, RJ	Subtelocentric	70.00%	30.00%	–	–	–	–
Carangola, MG	Submetacentric, subtelocentric	30.65%	30.65%	17.74%	17.74%	3.22%	–
Porciúncula, RJ	Submetacentric	75.00%	25.00%	–	–	–	–
Antônio Prado de Minas, MG	Submetacentric	47.69%	36.92%	15.39%	–	–	–
Ponte Nova, MG	Submetacentric	32.73%	67.27%	–	–	–	–
Jaguaraçú, MG	–	100%	–	–	–	–	–
Marliéria, MG	–	100%	–	–	–	–	–
Macarani, BA	Subtelocentric	27.78%	55.56%	16.66%	–	–	–
Macarani, BA	Subtelocentric	61.54%	25.00%	11.54%	1.92%	–	–

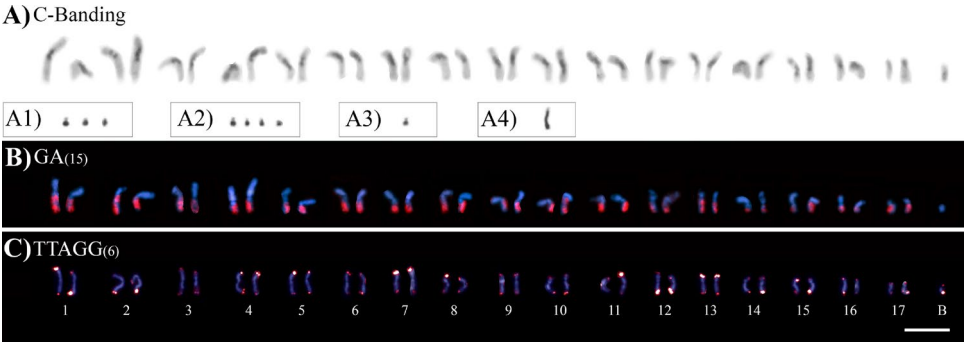

Figure 2. The colony from Viçosa, MG was used as a representative of the cytogenetic techniques performed in *Partamona helleri*. **A** C-Banding. The boxes display the B chromosomes from A1 Carangola, MG; A2 Santa Teresa, ES; A3 Porciúncula, RJ; A4 Macarani, BA; **B** microsatellite GA(15) probe; **C** microsatellite TTAGG(6) probe. Scale bar 5 μm.

3.2. Chromosomal DNA content

The slides prepared for the ICM, hydrolyzed in 5 M HCl solution for 18 min at 25 ºC and maintained in the Schiff reagent for 12 h at 4 ºC, showed stoichiometrically stained chromosomes. The telomeric and centromeric regions were well defined, which are prerequisites for morphometric characterization and determination of the OD and IOD. The Governador Lindemberg-ES colony did not show B chromosomes (Figure 4); therefore, it was chosen to measure the

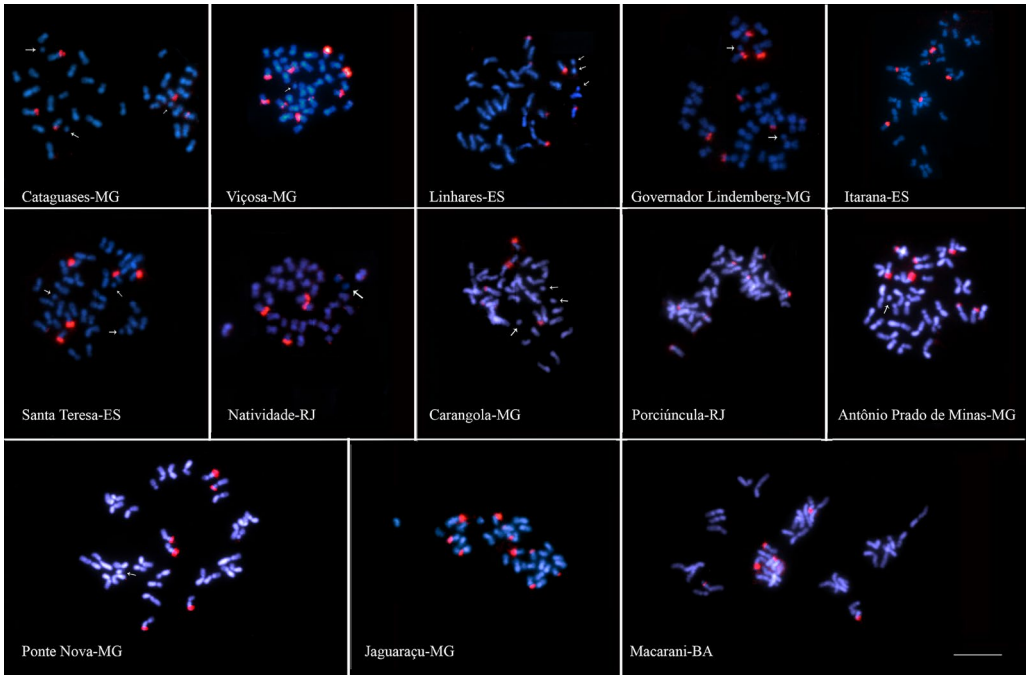

Figure 3. Fluorescence in situ hybridization (FISH) pattern with 18S rDNA probe in different *Partamona helleri* colonies. B chromosomes are indicated by arrows. Scale bar 5 μm.

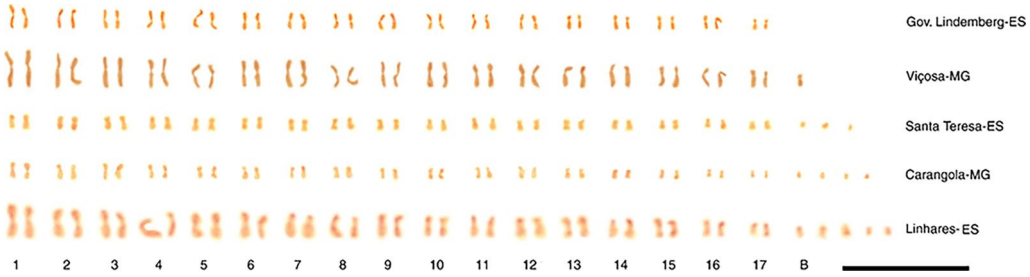

Figure 4. Chromosomes stoichiometrically stained using samples from different *Partamona helleri* colonies showing well-defined telomeric and centromeric portions, which are prerequisites for morphometric characterization and determination of area, optical density (OD), and integrated optical density (IOD). Scale bar 20 µm.

nuclear DNA content by FCM. The FCM histograms showed a G_0/G_1 peak with a coefficient of variation below 5% and at least 5000 nuclei. The mean nuclear genome size of *P. helleri* was 1C = 0.609 pg.

All karyotypes had $2n = 34$ and up to 5B chromosomes. The chromosomes from the A complement were ordered in decreasing size according to the total chromosome length. Using all IOD values of each A chromosome of the five colonies (22 metaphases), we calculated the mean IOD value of the 17 chromosomes and converted it to picograms (pg) using the 1C nuclear DNA content (1C = 0.609 pg). Both results are shown in Table III. The mean values oscillated from 0.0514 pg (chromosome 1) to 0.0297 pg (chromosome 17) (Table III). The mean values of chromosome 5, 8, and 14 were slightly smaller than those of chromosome 6, 9, and 15, respectively (Table III).

The IOD values of the B chromosomes were measured individually from one individual of each colony, and the values were converted to picograms (Table III). The specimen from Viçosa, MG, showed only one B chromosome (0.0146 pg), which represents an increase of 2.40% in the total genome size. The three B chromosomes found in the specimen from Santa Teresa, ES, represent a total of 0.0275 pg and an addition of 4.51% in the total genome size. The four B chromosomes found in the specimen from Carangola, MG, had a total of 0.0591 pg, representing an increase of 9.70% in the total genome size. The specimen from Linhares, ES, had five B chromosomes with a total of 0.0879 pg, which represents an addition of 14.43% in the total genome size content.

4. DISCUSSION

The $2n = 34$ observed in all *P. helleri* colonies is the most common diploid number found among the Meliponini genera (reviewed in Cunha et al. 2021a). The numerical variation regarding the presence of B chromosomes is rare among bees and is found in only a handful of species: *Melipona quinquefasciata* (Silva and Rocha 2018b), *Melipona rufiventris* (Lopes et al. 2008), *Tetragonisca fiebrigi* (Barth et al. 2011), *Partamona cupira*, *Partamona rustica*, and *P. helleri* (Marthe et al. 2010; Tosta et al. 2014). However, only *P. helleri* displays diversity in the number and morphology of these supernumerary chromosomes (Costa et al. 1992; Brito et al. 1997, 2005; Martins et al. 2009, 2013, 2014; Tosta et al. 2004, 2014; Present study).

The observed intra-individual variation in the number of B chromosomes may indicate that they are mitotically unstable. The mitotic nondisjunction creates mosaic individuals (Camacho et al. 2004), and the fact that we observed a lower frequency of 4B and 5B within the colonies may indicate an absence of an accumulation mechanism (Table II). Chromosome meiotic behavior is an interesting feature for investigating the presence of B-drive in *P. helleri*. Additionally, we report the presence of B-free colonies in Marliéria/Jaguaraçú, MG, and in Governador Lindemberg, ES, a fact that has only been reported

Table III
Integrated optical density (IOD) of the A metaphasic chromosomes of *Partamona helleri* and 1C values of the A and B complement.

Chromosome	A chromosome[a]	A chromosome[b]	B chromosome[c]
1	0.0672 ± 0.0015	0.0514	Viçosa-MG
2	0.635 ± 0.0009	0.0486	0.0146
3	0.5506 ± 0.0015	0.0425	Santa Teresa-ES
4	0.548 ± 0.0067	0.0419	0.0115
5	0.453 ± 0.0003	0.0347	0.0099
6	0.484 ± 0.0044	0.0370	0.0061
7	0.437 ± 0.0021	0.0334	Carangola-MG
8	0.432 ± 0.0005	0.0330	0.0214
9	0.452 ± 0.0004	0.0346	0.0171
10	0.448 ± 0.0011	0.0343	0.0122
11	0.422 ± 0.0013	0.0323	0.0084
12	0.418 ± 0.00007	0.0320	Linhares-ES
13	0.416 ± 0.0088	0.0318	0.0252
14	0.392 ± 0.0125	0.0300	0.0214
15	0.419 ± 0.00007	0.0320	0.0171
16	0.390 ± 0.00237	0.0298	0.0137
17	0.388 ± 0.00006	0.0297	0.0105
Total	7.969	0.609	

[a]Mean IOD values of the 17 A chromosomes obtained from 22 metaphases ± the standard deviation

[b]Mean 1C values exhibited in picograms — pg

[c]Chromosomal DNA content of the individual B chromosomes. Values exhibited in pg (1C)

in the Viçosa, MG, region (Costa et al. 1992). The variability in the size and morphology of these extra elements indicates the presence of different types of B chromosomes in this species, suggesting distinct evolutionary pathways due to geographic vicariance since their origin or even distinct origins (Martins et al. 2009, 2014). The microdissection approach (Martins et al. 2013) of the different B types would be an interesting avenue for further investigation.

Heterochromatin, mainly in the centromeric/pericentromeric regions with only a few terminal blocks, seems to be a conserved trait among *P. helleri* populations, whereas the B chromosomes vary from completely to partially heterochromatic with euchromatic extremities (Brito et al. 2005; Martins et al. 2013, 2014). These euchromatic portions of some B chromosomes are often related to gene activity (Ruiz-Estevez et al. 2012; Banaei-Moghaddam et al. 2015; Valente et al. 2017; Jetybayev et al. 2018). In *P. helleri*, the presence of ribosomal sites in some B chromosomes may be an evidence of gene activity in these extra elements (Martins et al. 2014).

In this study, although some colonies showed CMA_3^+ in the B chromosomes, they were not corroborated by the 18S rDNA FISH probe, which only marked A chromosomes (Figure 3). This result indicates that not all CMA_3^+ markings are related to the nucleolar organizing region in this species. The lack of complete homology between CMA_3 and 18S rDNA sites (Table I) calls for attention to the specificity of the CMA_3 fluorochrome as an indicator of the nucleolar organizing regions in this species, as well as in other *Partamona* species (Gonçalves et al. 2020).

The expansion of the 18S rDNA to numerous chromosomes, from four to nine terminal sites, could occur due to an unequal exchange between non-homologous chromosomes or transposition mediated by transposable elements (Sochorová et al. 2018; Hirai 2020), constituting another indicator of the genetic diversity among *P. helleri* populations. The presence of odd numbers of chromosomes marked with this probe (i.e., 5, 7, 9) suggests the occurrence of different rearrangements between homologous chromosomes.

Microsatellite $GA_{(15)}$ marked the euchromatic region of the A chromosomes, a trait that has been observed in other stingless bees (Piccoli et al. 2018; Travenzoli et al. 2019; Lopes et al. 2020); however, the function of these sequences in euchromatin is still poorly understood (Li et al. 2004; Oliveira et al. 2006). On the other hand, this microsatellite did not mark the euchromatic portions of the B chromosomes, whereas euchromatic or partially euchromatic B chromosomes in grasshoppers show several microsatellite markings (Milani and Cabral-de-Mello 2014; Ruiz-Ruano et al. 2015). The microsatellite $TTAGG_{(n)}$ is considered the ancestral telomeric sequence of insects (Kuznetsova et al. 2020). This sequence comprises the telomeric region of *P. helleri* A and B chromosomes and has been found in several *Melipona* species (Travenzoli et al. 2019), as well as in various ant genera (Pereira et al. 2018; Micolino et al. 2019, 2020; Castro et al. 2020).

Differences in heterochromatin content between chromosomes could explain some smaller chromosomes with slightly higher IOD values compared to larger ones (Silva et al. 2018a). In addition, B chromosomes were usually the smallest chromosomes of the karyotype and exhibited lower DNA content than the A chromosomes. Nevertheless, they could influence the nuclear genome size in *P. helleri* depending on their DNA contents and numbers, representing an increase of 2 to 15% of the total genome size depending on the analyzed population.

Brito and Arias (2010) showed the presence of genetic structure among *P. helleri* populations along its distribution area, which could have favored the fixation of different chromosomal rearrangements, leading to the cytogenetic diversity observed in this species. Additionally, our extensive sampling showed the prevalence of just a few B chromosomes per colony, including some B-free colonies. At lower frequencies, a higher number of B chromosomes are found in different geographical locations; this should be taken into account while designing flow cytometry studies. The intra-population variability highlights the importance of this type of study in species with a wide range of distribution. Future studies should focus on the sampling of southern *P. helleri* nests, that is, São Paulo, Paraná, and Santa Catarina, which have never been studied from a cytogenetic point of view.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

Study conception and design: CMN and DML. Data collection and analyses: CMN, MSC, and WRC. Supervision: TMSF and DML. Literature review and the first draft of the manuscript: CMN and MSC. All the authors commented on previous versions and approved the final manuscript.

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CONSENT, DATA AND/OR CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable.

Conflict of interest The authors declare no competing interests.

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