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Unprecedented record of ten novel B chromosomes in the stingless bee *Partamona helleri* (Apidae, Meliponini)

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Abstract – *Partamona helleri* from southeastern Brazil has shown the presence of up to four minute supernumerary chromosomes of the B1 or B2 type and a numeric variation from $2n=34$ to 38 chromosomes. Since the maximum dosage of four of these Bs per individual has been observed, the existence of genetic mechanisms controlling their accumulation has been suggested. In this study, several new types of B chromosomes were found in other localities of the geographic distribution of this species, especially nearby the northern end of the species range, showing a numeric variation of $n=18$ to 19 in males and $2n=34$ to $2n=41$ in females. Ten novel types of acrocentric and metacentric B chromosomes were classified as B3 to B12. Eight types were found in the state of Bahia and two types in the state of Espírito Santo. The results suggest that these Bs have had different evolutionary mechanisms involved in their origin and diversification. A variation within and between geographic samples in the number of chromomycin A3 and Ag-NOR markings was observed as a result of the presence or absence of different Bs. Six different B chromosomes showed Ag-NOR markings, which denote presence of gene activity. The karyotype variation and the patterns of B chromosome distribution observed indicate that this species is likely undergoing a process of genetic differentiation.

stingless bee / geographical differentiation / B chromosome / Ag-NOR banding

1. INTRODUCTION

In stingless bees, B chromosomes have been reported in a few species such as *Melipona rufiventris* (Lopes et al. 2008), *Tetragonisca fiebrigi* (Barth et al. 2011), *Partamona helleri*—formerly named as *Partamona cupira* (Costa et al. 1992; Brito et al. 1997; Tosta et al. 2004; Martins et al. 2009), and *P. cupira* (Marthe et al. 2010). In the genus *Partamona*,

these chromosomes occur in addition to the standard karyotype, with $2n=34$ chromosomes.

Previous cytogenetic analysis of *P. helleri* (Friese 1900) was mostly restricted to the state of Minas Gerais in southeastern Brazil, where two types of minute B chromosomes were found and classified as B1 and B2 by Brito et al. (1997). Tosta et al. (2004) reported the highest variation from one to four of these minute Bs per individual. Martins et al. (2009) found a new larger B chromosome (herein named B3 chromosome) in two locations of the state of Bahia, near the northernmost end of the species range.

Since in the previous studies there was no record of dosages higher than four B1 or B2 chromosomes

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per individual in this species, the existence of genetic mechanisms controlling their accumulation has been suggested by Costa et al. (1992) and Martins et al. (2012). Martins et al. (2012) also argued that potential constraints to higher accumulation would likely take place in the earlier instars of the bee larvae having more than four B chromosomes. Such effect, blocking their development in an earlier stage, would prevent B chromosome detection in the specimens, since the sampling stage for cytogenetic analysis in social stingless bees is technically limited to the fifth larval instar.

A number of different explanations have been proposed for the formation of B chromosomes in different organisms, e.g., as a byproduct chromosomal alterations in the regular karyotype (Camacho et al. 2000), formation from centric fragments by unequal translocation, or even as a byproduct of a hybridization process (Jones 1995; Jones et al. 2008). Martins et al. (2012) investigated the origin of B1 chromosome in populations of *P. helleri* from southeastern Brazil using microdissection and chromosome painting and found no homology with any of the regular chromosomes. This result can be interpreted as either due to an independent origin from the regular genome or that B1 chromosome has suffered substantial changes during the karyotype evolution process.

Camacho et al. (2000) pointed out that nucleolar organizer regions (NORs) could provide mechanisms for generating new B chromosomes. rRNA gene activity in B chromosomes has been found in both plants (Leach et al. 2005) and animals (Ruiz-Estévez et al. 2012). Brito et al. (2005) using 18S rDNA in situ hybridization in *P. helleri* did not find presence of rDNA clusters in the B chromosomes. Martins et al. (2012) found similar result with Ag-NOR banding. In this study, we report several new types of B chromosomes and higher chromosome numbers in other localities of the geographic distribution of the species, especially nearby the northern end of the species range, which showed the highest karyotypic diversity. The new B chromosomes were analyzed and described using different cytogenetic techniques.

2. MATERIALS AND METHODS

We analyzed 20 individuals per colony. One colony was collected in Cruz das Almas (S12°40'/W30°06'); two colonies in Itacaré, both localities are in the state of Bahia (S14°16'/W38°59'); and one colony in Santa Teresa, in the state of Espírito Santo (S19°56'/W40°36'). The chromosome preparations were obtained from brain ganglion cells in the fifth larval instar, following Imai et al. (1988). C-banding was performed according to Sumner (1972) with modifications by Pompolo and Takahashi (1990). The location of nucleolar organizer regions using silver nitrate (Ag-NOR banding) was performed according to Howell and Black (1980) with modifications suggested by Kavalco and Pazza (2004). Fluorochrome staining (4',6-diamidino-2-phenylindole (DAPI)/chromomycin A3 (CMA₃)) was performed following Schweizer (1980). Fluorescence in situ hybridization (FISH) was performed using a 45S rDNA probe of *Arabidopsis thaliana*, according to Schwarzacher and Heslop-Harrison (1999). The probe was labeled with cyanine 3-dUTP (Cy3) via nick translation (Invitrogen). The chromosomes were stained with DAPI. We analyzed 20 metaphase spreads per individual, and the best spreads were photographed in an Olympus CX-41 photomicroscope. Fluorescence images were captured in a Leica DRMA2 photomicroscope using IM50 software. The karyograms were organized following the methodology of Imai (1991) in decreasing order of size of euchromatic arms, using Adobe Photoshop CS4 version 11.0.2.

3. RESULTS

Present results revealed a higher variation in the chromosome number of *P. helleri*, which included males with $n=18$ to 19 and females with $2n=35$ to 41 chromosomes. This was caused by a variation in the presence/absence of ten different types of B chromosomes, varying in number from one to seven per individual (Table I). In the colony from Cruz das Almas (Figures 1a, 2a, 3a, and 4a, d), we found females with $2n=35$ to $2n=37$ and males with $n=18$ to $n=19$ chromosomes (Table I). In one colony from Itacaré (colony 1) (Figures 1b,

2b, 3b, and 4b), we found a variation from $2n=36$ to $2n=39$ (Table I) and, in the other (colony 2) (Figures 1c, e, 2c, 3d, and 4c, e, f), a variation from $2n=37$ to $2n=41$. The colony from Santa Teresa (Figures 1d, 2d, and 3c) showed a numerical variation from $2n=35$ to $2n=36$ chromosomes.

The C-banding revealed metacentric chromosomes (sensu Imai 1991) in regular karyotype with heterochromatin distributed in the centromeric and pericentromeric regions of regular chromosomes (Figure 2). We also found morphological variation in regular chromosomes, which resulted in the three karyotypic formulae: $2k=4M^{ct}+10M^c+20M^{cc}$ in Itacaré and $k=3M^{ct}+5M^c+9M^{cc}$ in Cruz das Almas in the state of Bahia and $2k=10M^c+24M^{cc}$ in the samples from Espírito Santo (Table I, Figure 2).

The ten novel B chromosomes were classified in morphological types that could be arranged into acrocentrics (hereafter named B3, B4, B5, and B7) and metacentrics (B6, B8, B9, B10, B11, and B12) (Table II and Figures 1 and 2). The B3 chromosome classified here has been previously observed by Martins et al. (2009) in two other localities in the state of Bahia. The heterochromatin is distributed in almost the entire chromosome length, with euchromatin restricted to the chromosomal extremities on B4 and B5 (Figure 2a–c) and on B11 and B12 chromosomes (Figures 1d and 2d). The B chromosomes exhibited a high size variation from large, with length similar to regular chromosomes (B3, B4, B5, B6, B7, B11), to small B chromosomes (B8, B9, B10, B12) (Figures 1 and 2).

Fluorochrome staining showed a variation from five to eight CMA_3 positive markings (CMA_3^+) that correspond to chromosome segments rich in GC base pairs (Figure 3). This variation was due to the presence of CMA_3 markings mostly in the short arms on both B (Table II) and regular chromosomes. DAPI staining (Figure 3e) marked the centromeric and pericentromeric regions of all chromosomes showing that these heterochromatins are rich in AT base pairs.

The silver nitrate staining (Ag-NOR banding) in turn varied within and between geographic samples from 3 markings, as observed in a haploid male, to 11 positive markings as observed in a diploid female in the terminal regions of the chromosomes (Figure 4). In total, six different B chromosomes had terminal Ag-NOR markings (Table II and Figure 4). Fluorescence in situ hybridization using rDNA 45S probe in colony 2 confirmed the presence of rDNA clusters (NORs) in B chromosomes (Figure 3).

A size heteromorphism was observed on the sixth chromosome pair in samples from Itacaré and Santa Teresa (Figure 1a, b, e). This heteromorphic segment had a long Ag-NOR positive block, which coincided with the CMA_3^+ marking on the long arm (Figures 3c, d and 4b).

4. DISCUSSION

The record of seven B chromosomes per individual and the $2n=41$ karyotype are the highest numbers so far found in a stingless bee. A total of ten morphological types of B chromosomes were reported in this study which differ substantially from what have been previously registered in southeastern Brazil, where only the minute B1 and B2 chromosomes were found (Brito et al. 1997).

The novel B chromosomes were characterized mainly by the large variation in number, size, and structure, including C-band patterns and molecular composition of the heterochromatin. The types and number of B chromosomes per individual varied geographically, having been found a greater variation and dosage in Itacaré in southern Bahia, with the occurrence of up to seven B chromosomes per individual and the B3, B4, B5, B6, B7, B8, B9, and B10 chromosome types. On the other hand, individuals from Santa Teresa in the state of Espírito Santo showed only the types B1, B7, B11, and B12.

The high number of B chromosomes per specimen found in this study indicates a different pattern of tolerance to the accumula-

Table I. Collecting locations, karyotypic formulae, number of Bs, and CMA₃ or Ag-NOR markings on the regular and B chromosomes of *P. helleri*.

Sampling locations	Karyotypic formulae (A chromosomes)	Number of B chromosome per individual	B chromosome type
Itacaré-BA (1)	2k=4M ^{ct} +10M ^c +20M ^{cc}	2, 3, 4, or 5	B4 B5 B6 B9 B10
Itacaré-BA (2)	2k=4M ^{ct} +10M ^c +20M ^{cc}	3, 5, 6, or 7	B3 B4 B5 B6 B7 B8 B9 B10
Cruz das Almas-BA	k=3M ^{ct} +5M ^c +9M ^{cc}	1, 2, or 3	B4 B5 B6 B9 B10
Santa Teresa-ES	2k=10M ^c +24M ^{cc}	1 or 2	B1 B7 B11 B12

tion of these newly described B chromosomes. This observation reinforces the genetic differences of these chromosomes when compared to the minute B chromosomes previously recorded in southeast Brazil, for which Martins et al. (2012) suggested the existence of cytogenetic or genetic constraints in establishing karyotypes with high B numbers.

The samples from Northeast region showed some B chromosomes with size comparable to the largest chromosomes in the regular karyotype, as well as smaller Bs (Figures 1 and 2). The record of one large B chromosome in *P. helleri* was first made by Martins et al. (2009) in two localities near the northern end of the species distribution, in the state of Bahia.

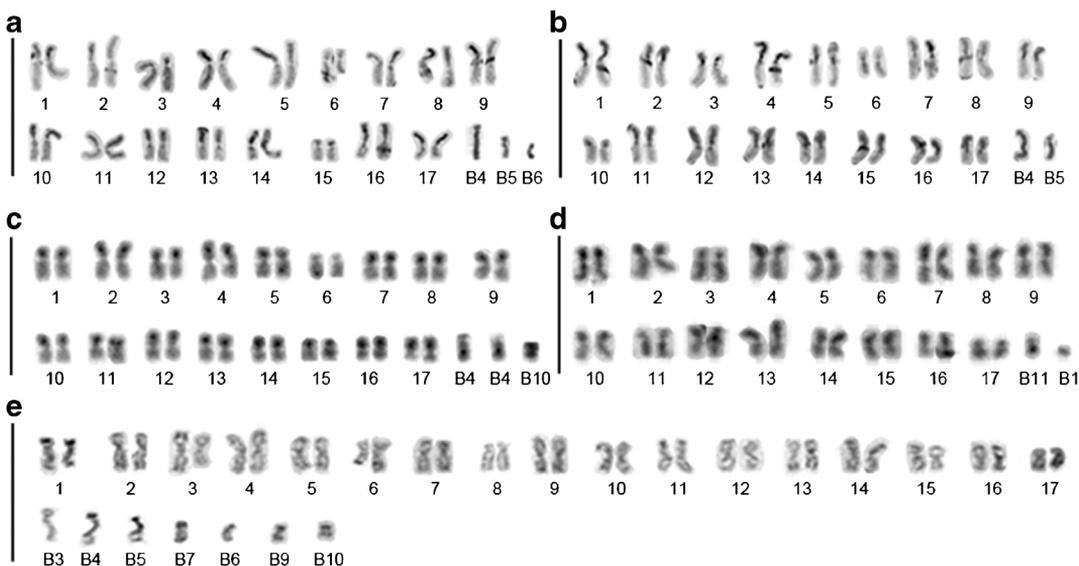


Figure 1. Karyotype of *P. helleri* submitted to Giemsa staining: **a** female from Cruz das Almas, BA with 2n=34+3B; **b** female from Itacaré, BA (colony 1) with 2n=34+2B; **c** female from Itacaré, BA (colony 2) with 2n=34+3B; **d** female from Santa Teresa, ES with 2n=34+2B; and **e** female from Itacaré, BA, colony 2 with 2n=34+7B (metaphase submitted to Ag-NOR staining). Heteromorphism in the sixth chromosome pair of regular karyotype is shown in **a**, **b**, and **e**. The bar represents 10 µm.

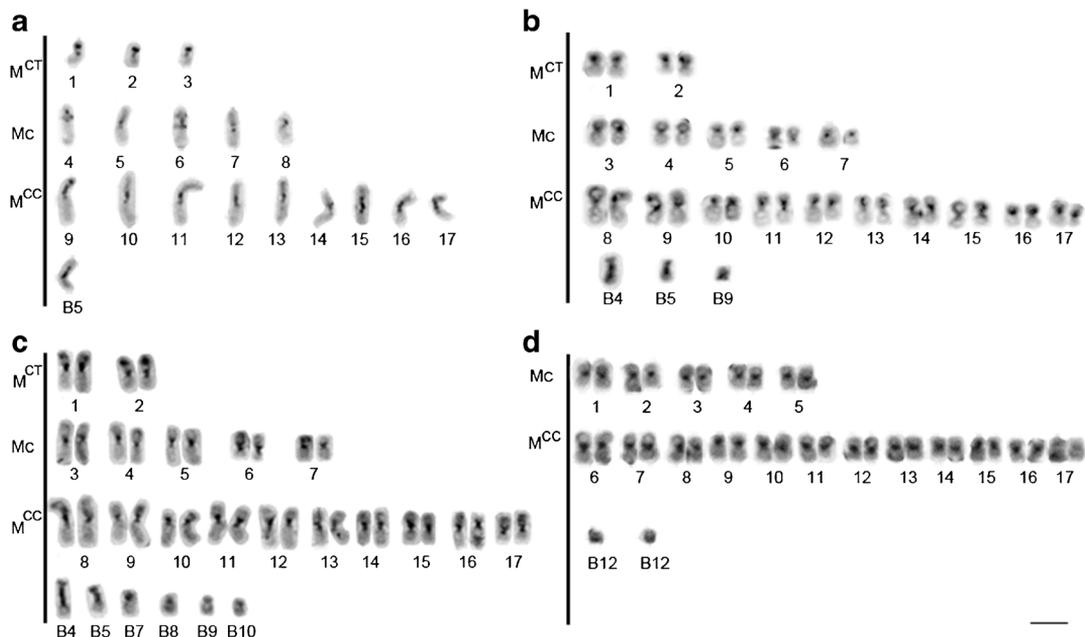


Figure 2. Karyotype of *P. helleri* submitted to C-banding: **a** male from Cruz das Almas, BA with $n=17+1B$; **b** female from Itacaré, BA (colony 1) with $2n=34+3B$, **c** female from Itacaré, BA (colony 2) with $2n=34+6B$; and **d** female Santa Teresa, ES with $2n=34+2B$. The bar represents 10 μm .

Likewise, a large B chromosome with a short arm rich in GC base pairs was described by Marthe et al. (2010) in a closely related species, *P. cupira* which showed morphological similarity with B4 and B5 types found in *P. helleri*. Camargo and Pedro (2003) suggest that *P. helleri* is a sister group of the “termitophilous” *Partamona* species, being closely related to the cupira group (*Partamona aylae*, *Partamona littoralis*, *Partamona sooretamae*, *Partamona criptica*, *Partamona rustica*, *Partamona mulata*, *Partamona seridoenses*, *P. cupira*). Considering the proximity between these species, a possible homeology between these similar B chromosomes must be further investigated.

The expressive variation within and between geographic samples in the number of Ag-NOR and CMA₃⁺ markings was mainly due to the presence or absence of B chromosomes carrying these markings in the individuals. Six distinct types of Bs carrying ribosomal sites were found.

Since silver staining can be indicative of ribosomal gene activity (Hubbell 1985), presence of additional active NORs can provide selective advantage for B-carriers (Beukeboom 1994) and favor the maintenance of these B chromosomes in the population. B chromosomes with rDNA clusters have been observed in diverse animal species such as the grasshopper *Eyprepocnemis plorans* (Cabrero et al. 1987; López-León et al. 1991; Ruiz-Estévez et al. 2012), the anuran *Leiopelma hochstetteri* (Green 1988), and the rodent *Apodemus peninsulae* (Boeskorov et al. 1995) and in plants (*Crepis capillaris*) (Leach et al. 2005), among others.

Although microdissection analysis of the minute B1 chromosome in *P. helleri* populations from southeastern Brazil was inconclusive about its origin, Martins et al. (2012) suggested either an independent origin from the rest of the genome or the occurrence of substantial changes in the chromosomal segment during its process of evolution in the karyotype. The

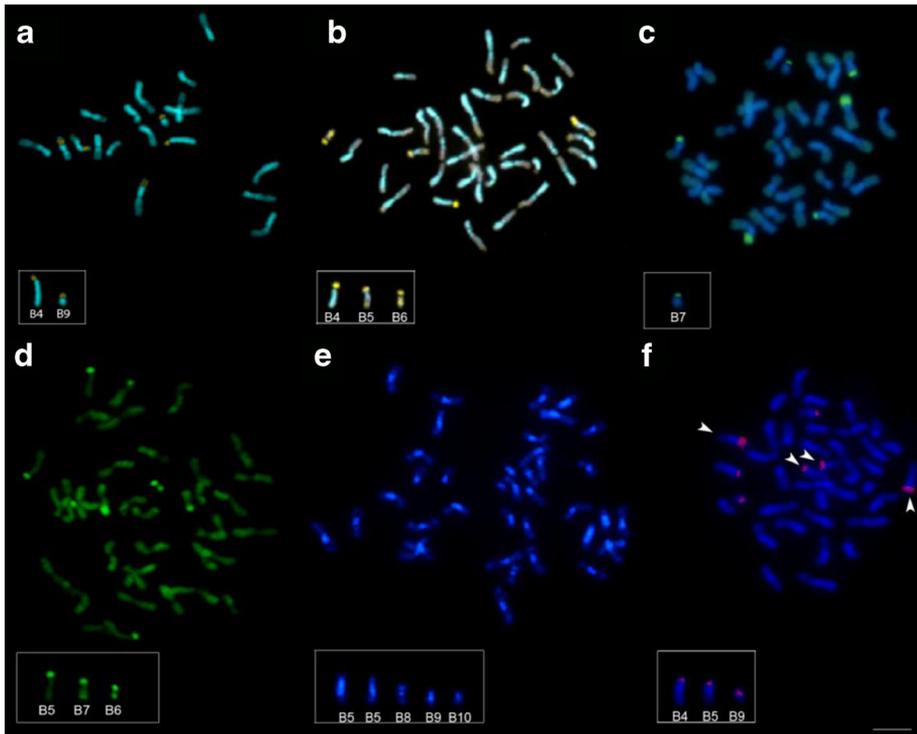


Figure 3. Metaphases of *P. helleri* stained with DAPI/CMA₃: **a** male from Cruz das Almas, BA with $n=17+2B$; **b** female from Itacaré, BA (colony 1) with $2n=34+3B$; **c** female Santa Teresa, ES with $2n=34+1B$; **d** CMA₃ staining in female from Itacaré, Bahia (colony 2) with $2n=34+5B$; and **e** DAPI staining in female from Itacaré, BA (colony 2) with $2n=34+5B$. Metaphase submitted to rDNA 45S FISH: **f** female from Itacaré, BA (colony 2) with $2n=39$. The B chromosomes marked with DAPI, CMA₃, or rDNA 45S FISH are detached in each metaphase. The *arrowheads* indicate regular heteromorphic chromosome with rDNA blocks. The *bar* represents 10 μm .

novel set of B chromosomes of *P. helleri*, especially those in the samples from Bahia, does not seem to share a common origin and their structural differences suggest the existence of different evolutionary mechanisms involved in their origin and diversification. The mention that the rDNA clusters are prone to breakage and provide mechanism for B chromosome formation (Camacho et al. 2000), and the discovery of such clusters in six of the B chromosomes reaffirms this as a likely mechanism involved in the formation of some Bs carrying NORs. Subsequent rearrangements involving the existing B chromosomes may have generated variants in the population.

The similarity between the B4 and B5 chromosomes, for example, suggests that these chromosomes are variant forms resulting from small structural changes in the interstitial heterochromatin content. Bakkali and Camacho (2004) in studies of grasshopper, *Eyprepocnemis plorans*, revealed 16 variants for B chromosomes in nine populations from Morocco, many being derived from the other Bs. Among the mutations involved in the process are rDNA deletion, translocations, duplications, and chromatid nondisjunction with consequent formation of isochromosomes.

Another relevant aspect observed in the karyotypes of *P. helleri* was the geographical variation

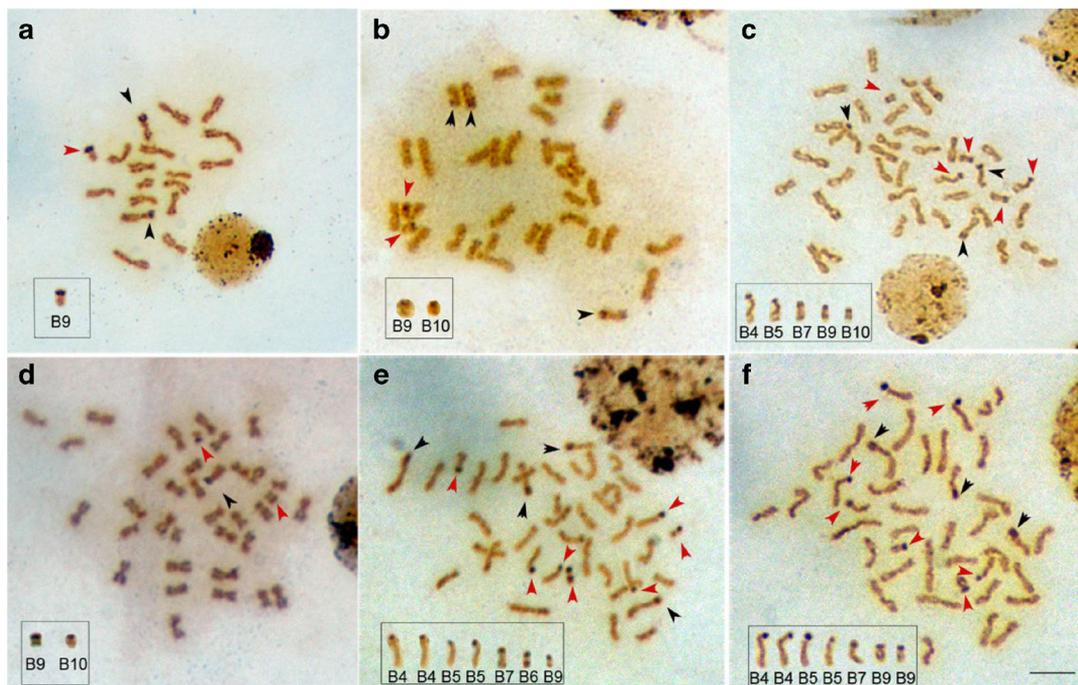


Figure 4. Ag-NOR staining in metaphase of *P. helleri*: **a, d** male and female from Cruz das Almas, BA with $n=17+1B$ and $2n=34+2B$, respectively; **b** female from Itacaré, BA (colony 1) with $2n=34+2B$; **c, e, f** female from Itacaré, BA (colony 2) with $2n=34+5B$ or $2n=34+7B$. B chromosomes stained with Ag-NOR are detached in each metaphase. *Black and red arrowheads* indicate Ag-NOR markings on regular and B chromosomes, respectively. The *bar* represents 10 μ m.

Table II. *P. helleri* B chromosome characterization by different cytogenetic techniques.

B chromosome type	Karyotypic description	Heterochromatin	Euchromatin	CMA ₃ staining	Ag-NOR banding
B1	–	+	–	–	–
B2	A	+	– ^a	–	– ^a
B3	A	+	–	+	– ^a
B4	A	+	+	+	+
B5	A	+	+	+	+
B6	M	+	+	+	+
B7	A	+	+	+	+
B8	M	+	+	– ^a	– ^a
B9	M	+	+	+	+
B10	M	+	+	+	+
B11	M	+	+	– ^a	– ^a
B12	M	+	+	– ^a	– ^a

+ and – indicate positive and negative response to treatment, respectively. B1 and B2 morphology followed Brito et al. (1997). B1 and B2 fluorochrome data followed Brito et al. (2005) and Martins et al. (2012)

^aData not analyzed

in the regular karyotype characterized by the presence or absence of a M^{ct} chromosome pair (Figure 2). Furthermore, the length heteromorphism observed in the sixth chromosome pair differed from that of Brito et al. (1997, 2005) which showed a heteromorphism in the second pair. Length heteromorphisms can be formed by translocations, unequal crossing over, and heterochromatin tandem growth or deletion (Hatanaka et al. 1998). These and other variations found in the regular chromosomes of *P. helleri* may be involved in the origin of some of the Bs found in different geographic regions, and the several pieces of evidence of chromosome rearrangements found suggest a high level of restructuring in the regular karyotype in this species.

From these and previous results, it is apparent that *P. helleri* shows B chromosomes differentiated by geographical regions in accordance with the distribution pattern of the species, with more B chromosomes varying in size, morphology, and dosage in *P. helleri* population from the state of Bahia, the northernmost part of the species range, when compared to those obtained in *P. helleri* populations from Espírito Santo and Minas Gerais.

The diversity of B chromosomes can be used as a model to understand the dynamics of these populations and the mechanisms involved in the karyotype evolution. The karyotype variation and the patterns of B chromosome distribution indicate that this species is likely undergoing a process of genetic differentiation. More detailed studies must be developed to determine the probable center of origin of B chromosomes, the different mechanisms involved in their origin, and the presence of transcriptional gene activity. Within this perspective, the fact that regular and B chromosomes contain rDNA is a good start for further investigations of their origin.

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Observation inédite de 10 nouveaux chromosomes B chez l'abeille sans aiguillon *Partamona helleri* (Apidae, Meliponini)

Abeille sans aiguillon / différenciation géographique / chromosome B / bandes Ag-NOR

Ein unerwarteter Befund mit zehn neuen B-Chromosomen bei der stachellosen Biene *Partamona helleri* (Apidae, Meliponini)

Stachellose Biene / geographische Differenzierung / B-Chromosom / Ag-NOR

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