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# Investigation of *Partamona helleri* (Apidae, Meliponini) B chromosome origin. An approach by microdissection and whole chromosome painting

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**Abstract** – The stingless bee *Partamona helleri* in southeast Brazil shows the regular chromosome number  $2n=34$  and a variable number of up to four minute B1 or B2 chromosomes. Previous cytogenetic analyses have indicated morphological similarities between the B1 chromosome and chromosome segments in the regular karyotype. In this study, microdissection and chromosome painting were employed along with C banding, NOR banding, and base-specific fluorochrome staining to investigate the origin of the B1 chromosome in *P. helleri*. B1-generated probe hybridized exclusively to B1 chromosomes. This result suggests an independent origin from the regular karyotype or, alternatively, that the B chromosome may have suffered substantial genetic alterations along its independent evolution. The absence of higher dosages of these small B chromosomes in this population of *P. helleri* may be related to the existence of either a genetic or cytogenetic constraint in the establishment of such high numbered karyotypes.

Hymenoptera / B chromosome probe / stingless bee

## 1. INTRODUCTION

B Chromosomes are recognized as dispensable, additional chromosomes, usually morphologically different, and possibly arisen from regular (A) chromosomes (Beukeboom 1994). Diverse B chromosome systems occur in a variety of groups of plants and animals. They share other common features such as non-Mendelian inheritance and non-disjunction in

meiosis, which can result in an increase in the chromosome number in the population (Hewitt 1979; Jones and Rees 1982).

The stingless bee *Partamona helleri* (Friese 1900) ranges from southern to northeastern Brazil and exhibits the regular chromosome number  $2n=34$  and a variable number of B chromosomes (Costa et al. 1992; Brito et al. 2005; Tosta et al. 2004; Martins et al. 2009). Although larger B chromosomes were recently registered in northeastern Brazil populations (Martins et al. 2009), Brito et al. (1997) described two diminute B chromosomes, a small submetacentric (B1 chromosome), and a

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small acrocentric (B2 chromosome) occurring in this species restricted to southeastern Brazil.

In several studies carried out on populations from southeastern Brazil (Costa et al. 1992; Brito et al. 1997; Brito et al. 2005; Tosta et al. 2004), individuals with zero and one B chromosome were relatively common. However, higher B chromosome numbers were progressively rarer. So far, a maximum of four B chromosomes per individual was found in only two colonies (Tosta et al. 2004). Such findings raise questions on whether there is a mechanism controlling the dosage of these B chromosomes in *P. helleri* individuals, or possibly a deleterious effect to the organisms, particularly at higher dosages.

Thus far, different cytogenetic analyses have provided the morphological characterization of B chromosomes in *P. helleri*; however, the present knowledge is still scanty to address the mechanisms involved in their origin. Brito et al. (2005) using different cytogenetic techniques showed some morphological similarities between B1 chromosome and small chromosome segments in the regular chromosome pairs 2, 9, 10, and 15. Nevertheless, attempts to establish an association between the presence of these segments and the B1 origin has yielded inconclusive results, and, to date, an evolutionary explanation for the presence of B chromosomes in *P. helleri* is still lacking. Accurate detection of homology between supernumerary chromosomes and segments of regular chromosomes can provide evidence of an intraspecific origin, the types of rearrangements involved, and portability of gene expression in B chromosomes, allowing to correlate it with the mechanisms limiting their accumulation (Camacho et al. 2000; Leach et al. 2005).

Considering that chromosome microdissection and painting have become very effective in elucidating chromosomal rearrangements, the use of such methods is a novel approach to studies on the origin of B chromosomes on bees. This technique has been applied successfully to different studies on the evolution of sexual systems and origin of B chromosomes on different fish species (Diniz et al. 2008; Henning et al. 2008; Silva et

al. 2009; Voltolin et al. 2010). Fernandes et al. (2011) constructed specific probes useful to locate some of the regular centromeric regions of the stingless bee *Tetragonisca fiebrigi*. In this study, we combined microdissection and chromosome painting, C-banding, NOR banding, and base-specific fluorochrome staining to investigate the origin of the chromosome B1 in *P. helleri*.

## 2. MATERIALS AND METHODS

### 2.1. Chromosome preparation and banding

A total of 30 larvae of *P. helleri* in the last instar were collected in the region of Viçosa, Minas Gerais (20°45'12"S and 42°52'53"W). Chromosome preparations by air drying technique (Imai et al. 1988) were obtained using cerebral ganglion cells. C-banding (Sumner 1972) was performed with minor adjustments as proposed by Pompolo and Takahashi (1990). Fluorochrome staining (DAPI/CMA<sub>3</sub>) was performed following Schweizer (1980). The nucleolar organizing regions (NORs) were identified by silver nitrate staining (Howell and Black 1980) with a few modifications (Kavalco and Pazza 2004). We analyzed 20 metaphases per individual, and the best-quality ones were photographed under an Olympus CX-41 microscope. The karyograms were organized in descending order of euchromatic chromosome arm size (Imai 1991) using Adobe Photoshop CS4 version 11.0.2.

### 2.2. Microdissection of B chromosome and probe preparation

The B1 probe was obtained by microdissection of chromosome preparations on glass coverslips stained with 5% Giemsa using a mechanical micromanipulator (Eppendorf, 5171) and an inverted microscope (Axiovert 100—Zeiss). Three B chromosomes were microdissected and transferred to microtubes containing the reaction mix for DOP-PCR (degenerate oligonucleotide-primed polymerase chain reaction). The DOP-PCR was performed according to Diniz et al. (2008) with some modifications as follows. An initial low-stringency PCR was followed by two conventional high-stringency amplifications to obtain

a stock of the first PCR and to mark the PCR products, respectively. The low-stringency steps were performed in microtubes with a 20- $\mu$ L final volume containing 1 $\times$  buffer (260 mM Tris-HCl, pH 9.5, 65 mM MgCl<sub>2</sub>), 0.2 mM dNTP mix, 2.0 mM, DOP primer (5' CCG ACT CGA GNN NNN NAT GTG G 3') (Telenius et al. 1992), microdissected chromosomes, and ultrapure water. The mix was heated at 90°C to denature the chromosomal proteins and then spun briefly before the addition of 4 U *Thermo Sequenase* (*Thermo Sequenase* Cycle Sequencing Kit—USB). PCR reactions were performed in a thermocycler PTC-200™ Peltier (MJResearch, Inc.) under the following conditions: 94°C–3 min, 12 cycles at 94°C–1 min 30 s, 37°C–2 min, with 0.2°C/s increments to 72°C, 72°C–2 min, followed by another 30 cycles of 94°C–1 min 30 s, 56°C–1 min, 72°C–1 min 30 s.

Conventional PCR reactions consisted of 1 $\times$  *Taq* buffer (500 mM KCl, 100 mM Tris-HCl pH 8.4, 1% Triton X-100), 0.04 mM dNTP mix, 2.0 mM MgCl<sub>2</sub>, 0.8 mM DOP primer, 0.05 U *Taq* polymerase (PHT), 2  $\mu$ L of the first PCR product, and ultrapure water 25  $\mu$ L, under the following conditions: 90°C–3 min, 30 cycles at 90°C–1 min 30 s, 56°C–1 min 30 s, 72°C–1 min 30 s. The third PCR solution contained 1 $\times$  *Taq* PCR buffer, 2.0 mM MgCl<sub>2</sub>, 0.04 mM dATP, dCTP, and dGTP, 0.028 mM dTTP, 0.12 mM dUTP labeled (11-dUTP-digoxigenin—Roche Applied Science), 2 mM of DOP primer, 0.05 U of *Taq* polymerase, 3  $\mu$ L of the PCR products from the second reaction, and 50  $\mu$ L of ultrapure water. The amplification conditions were the same as those described for the second reaction. The amplified products were analyzed on 1% agarose gels to verify the size of the fragments, which usually ranged from 300 to 600 bp. The labeled PCR product was precipitated in 100% ethanol and 0.36 mM sodium acetate and diluted in 20  $\mu$ L of water.

### 2.3. Fluorescent in situ hybridization (FISH)

The B1 probe was used for whole chromosome painting (WCP) via fluorescent in situ hybridization. FISH was performed according to the procedures of Pinkel et al. (1986) with a few modifications as follows. The slides were sequentially treated with 100 mg/mL RNase A/2 $\times$  SSC 37°C for 30 min with 0.01% pepsin in 10 mM HCl at 37°C for 10 min and then placed for 10 min in 1 $\times$  PBS, 50 mM MgCl<sub>2</sub>, 1% formaldehyde, and dehydrated in ethanol (70%, 85%, and 100% for 5 min each). Subsequently, the slides

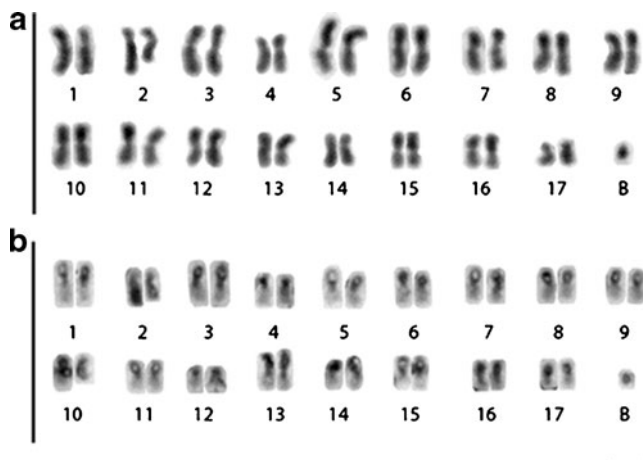
were denatured for 3 min at 70°C in 70% formamide, 2 $\times$  SSC, pH 7.0, and then dehydrated again in cold ethanol. Ten micrograms of B1 probe was added to the hybridization mix (77% stringency and 40  $\mu$ L per slide), which consisted of 50% formamide, 10% dextran sulfate, 2 $\times$  SSC, and water. The hybridization mix was denatured for 10 min at 95°C, applied to the slides, and incubated overnight at 37°C. Slides were then washed three times for 5 min in 50% formamide: 2 $\times$  SSC at 42°C, and for 5 min in 0.1 $\times$  SSC at 60°C. The B1 probe was labeled with digoxigenin-11-dUTP (Roche Applied Science) and signal hybridization detection was performed using anti-digoxigenin-rhodamine (Roche Applied Science). The chromosomes were stained with Vectashield Mounting Medium with DAPI, 1.5 g/mL (VECTOR). The slides were analyzed on an Olympus BX61 epifluorescence microscope and the images were captured separately (using specific UV filters), colored, and superimposed with the Image Pro Plus 6.0 software (Media Cybernetics).

### 3. RESULTS

All *P. helleri* specimens analyzed showed 2n=34+B1 chromosomes (Figure 1a). The heterochromatin was distributed in the centromeric and pericentromeric regions of regular chromosomes and throughout the length of the B1 chromosome (Figure 1b). Six Ag-NOR markings were observed in the telomeric regions of chromosomes of the regular complement. In the second chromosome pair, the NOR also coincided with a large heterochromatic block (Figure 2a). However, no markings were visualized on chromosome B1.

CMA<sub>3</sub> staining marked the telomeric portion of five chromosomes (Figure 2b). It is noteworthy that this species shows a heteromorphism in the second chromosome pair with an extensive CMA<sub>3</sub> marking on the long arm that coincided with the NOR (Figure 2a, b). A DAPI<sup>+</sup>/CMA<sub>3</sub><sup>-</sup> marking was observed on chromosome B1 which reveals the predominance of AT base pairs (Figure 2b).

The probe obtained by microdissection of the B1 chromosome hybridized strongly to the B chromosome only in all metaphases; therefore, no homology was detected with segments of the regular karyotype (Figure 2c, d).

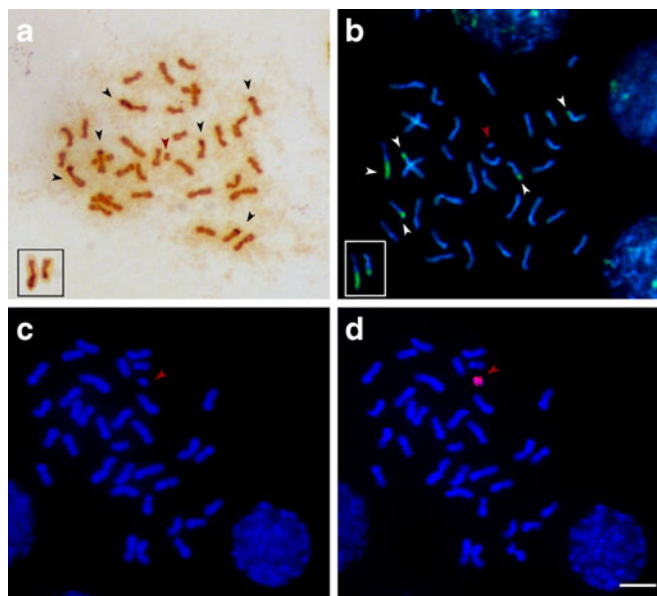


**Figure 1.** Female karyotypes of *P. helleri* with  $2n=34+B1$  stained with **a** Giemsa and **b** C-banding. The *bar* represents 10  $\mu\text{m}$ .

#### 4. DISCUSSION

Previous cytogenetic studies with *P. helleri* were limited to using solely classical techniques

and basically comparing chromosome number and morphology (Costa et al. 1992; Brito et al. 1997; Tosta et al. 2004). In this study, the presence of a heteromorphic segment in the



**Figure 2.** Metaphases of *P. helleri* with  $2n=34+B1$  showing **a** NOR banding (*black arrows*), **b** DAPI/CMA<sub>3</sub> staining (*white arrows*), and **c–d** metaphases before and after the chromosome painting with B1 probe, respectively. The *red arrow* indicates the B1 chromosome. Heteromorphism in the second pair is detached in the metaphase plates. The *bar* represents 10  $\mu\text{m}$ .

second chromosome pair, whose length was equivalent to that of the B1 chromosome (Brito et al. 2005), was confirmed. Moreover, this region is also similar to B1 regarding the presence of heterochromatin. Structural rearrangements involving this segment could be a plausible explanation for the intraspecific origin of the small B1 chromosome. However, in our samples, the heteromorphic chromosome segment that was  $\text{CMA}_3^+$  and  $\text{Ag-NOR}^+$  differed from the B1 chromosome concerning the type of heterochromatin. This length heteromorphism in the chromosomal nucleolus organizer region ( $\text{Ag-NOR}^+$ ) has been observed in different bee species (Duarte et al. 2009; Brito et al. 2005) and seems to be associated to a difference in the number of copies of ribosomal genes in the cluster.

Hybridization of the B1 probe only with the B1 chromosome indicates the lack of homology between this probe and the regular chromosome complement (Figure 2c, d). These results therefore do not lend support to the hypothesis of a regular karyotype origin for this chromosome. Alternatively, this lack of homology may have resulted from an accumulation of genetic alterations in the B chromosome along its independent evolution. Chromosome segments with high heterochromatic content, such as B1, tend to undergo frequent chromosomal mutations (Eichler and Sankoff 2003). Therefore, the changes in DNA sequence might have substantially modified the original chromosome segment. However, so far there is no available time estimate for the origin of the B chromosomes in the population and necessary information to better appraise this matter.

A larger type B chromosome was recently recorded in *Partamona cupira* (Marthe et al. 2010), a species closely related to *P. helleri* (Camargo and Pedro 2003). *P. cupira* occupies a basal position in the so-called Cupira group (*Partamona rustica*, *Partamona criptica*, *Partamona seridoensis*, *Partamona mulata*, *P. cupira*, *Partamona sooretamae*, *Partamona ailyae*, *Partamona littoralis*) (Camargo and Pedro 2003). The presence of B chromosomes may be a plesiomorphic feature in this group of *Partamona* species. Further experiments of heterologous in situ hybridization with B1

probe in the karyotype of *P. cupira* and the karyotyping of these other species may help to clarify this issue.

The rare observation of four Bs per individual (Tosta et al. 2004) and the absence of higher dosages of these small B chromosomes in this population of *P. helleri* is in line with the existence of either a genetic or cytogenetic constraint in the establishment of such higher numbered karyotypes. Thus far, a genetic mechanism underlying this event remains unknown. Multiple developmental gene dosages have been revealed to progressively affect genomic imprinting causing deleterious effects in the animal development (Rocha et al. 2009). The absence of individuals with five or more B chromosomes in this population of *P. helleri* may be caused by restrictions on the development of larvae imposed by higher doses of these genomic elements.

Our cytogenetic analyses were restricted to the fifth larval instar since there is no available technique for karyotyping adult stingless bees. Therefore, it is not possible to assume whether the larvae with a lower dosage of B chromosomes can reach the adult stage. A putative genetic mechanism restricting the fivefold or higher dosage of B1 or B2 chromosomes in this species might probably affect the earlier stages of development prior to the fifth larval stage or even constitute pre-zygotic barriers for germ cells carrying five or more of these chromosomes.

Additional studies need to be conducted in order to better elucidate the effect of B chromosome dosage in *P. helleri*. Considering that the putative genomic imprinting effect of B chromosome may be caused by small genomic segments, as single copy genes, the accuracy in their physical mapping by chromosome painting would be compromised.

The use of another methodology to improve accuracy of in situ hybridization should be tested. Use of Fiber FISH could be an alternative for enabling high resolution in detecting signals smaller than 1 kb (Weier 2001). Future investigations should also consider testing cross-species in situ hybridization of B chromosome probes in order to establish B chromosome homeology



among closely related species and infer the evolutionary origin. Analyses involving larger sampling along the distribution of this species will also help in better understanding the complex system of B chromosomes found in *P. helleri*.

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**Recherche de l'origine du chromosome B de *Partamona helleri* (Apidae, Meliponini) : une approche par microdissection et par peinture du chromosome entier**

**Hymenoptera / abeille sans aiguillon / sonde chromosome B**

**Untersuchungen zum Ursprung der B Chromosomen bei *Partamona helleri* (Apidae, Meliponini) mithilfe von Microdissection und Chromosome Painting.**

**Hymenoptera / B Chromosomen-Sonde / stachellose Bienen**

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