Cytogenetic Characterization of Two Partamona Species (Hymenoptera, Apinae, Meliponini) by Fluorochrome Staining and Localization of 18S rDNA Clusters by FISH

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Summary The tribe Meliponini comprises several hundred species of stingless bees which are major pollinators of many tree species. In the present work we studied two Partamona species: Partamona helleri and Partamona seridoensis. Both species presented similar karyotypes with 2n=34 chromosomes for females, pericentricomic heterochromatic blocks and terminal GC rich heterochromatic segments in chromosome pairs 2, 9, 10 and 15, as shown by sequential C banding-DA/CMA3 staining. The heterochromatin is heterogeneous: pericentromeric and the terminal blocks behave quite differently. FISH with 18S rDNA probe showed that NORs were in terminal blocks. In addition, B chromosomes were observed in P. helleri. The results of the different treatments employed such as C banding and fluorochrome staining led us to believe that their origin was not due to non-disjunction of A genome chromosomes. Supernumerary pericentricomic heterochromatin and the non-homologous portion of a heteromorphic pair behaved in a similar fashion, indicating that may be originated by fission. The FISH assay showed, however that there was no sequence correspondence between these segments.

Key words Partamona, Meliponini, 18S rDNA, B chromosomes.

The Meliponini are known as stingless bees and are spread over the Pantropical region. This tribe presents a great diversity comprising several hundred species. However, the precise number is not known because of the abundance of cryptic species (Michener 2000). Meliponini are believed to pollinate from 40 to 90% of tree species depending on the ecosystem (Kerr et al. 1996). According to Kerr et al. (1996) almost 100 species of this tribe are already endangered, and the destruction of native vegetation in Brazil endangers bee species that nest in tree holes. As a consequence, many Meliponini are frequently observed nesting in wall crevices and even in plant vases in urban areas.

Kerr and Silveira (1972) hypothesized that the different bee chromosome numbers resulted from polyploidization of a basic number, n=8 of bee species. Alternatively, the Minimum Interaction Theory (Imai et al. 1986, 1988, 1994), based on ants, has also been proposed to explain the karyotype evolution of Meliponini. According to this theory, primitive karyotypes had a small number of large chromosomes and, with time the chromosomes got smaller and increased in number by centric fission. This process would prevent deleterious interactions such as translocations within the interphase nucleus.

For Partamona, seven species have been studied. All presented 2n=34 chromosomes for females: P. pearsoni and P. helleri (cited as cupira) (Tarelho 1973), P. mulata, P. aiylae, P. vicina and P. sp. (Brito-Ribon et al. 1999), P. peckolti (Brito et al. 2003). In P. helleri a system of B (or super-
numerary) chromosomes was observed, in addition to the normal complement, varying from 0 to 4 B chromosome inside and among colonies (Costa et al. 1992, Brito et al. 1997, Tosta et al. 2004).

B chromosomes are quite rare in Hymenoptera. They have been observed in eight ant species (Crozier 1975, Imai 1974, Imai et al. 1977, 1984, Mariano et al. 2001, Lorite et al. 2000, Palomeque et al. 1990); in wasps Nasonia vitripennis wasps (Nur et al. 1988); Trichogramma kaykai (Stouthamer et al. 2001) Trypoxylon albitarse (Araújo et al. 2000); and in another Meliponini bee Melipona quinquefasciata, with a system varying from 0 to 4 (Pompolo et al. 2004).

According to Zurita et al. (1998), B chromosomes may behave as selfish genetic elements, which would increase their transmission beyond the Mendelian rate of 0.5. Over time, these chromosomes would lose the sequences responsible for their selfish behavior, being neutralized by the A genome. The cycle would then be closed with the loss of neutral or near-neutral Bs over the generations, and the appearance of another selfish B by mutation. Previously we have detected a heteromorphism in the second pair of the karyotype of some colonies of P. helleri that was hypothesized to be related to the origin of B chromosomes (Brito et al. 1997). Nevertheless, a thorough chromosomal characterization of the A genome of this species is needed to reinforce or refute this possibility.

In this work, we described the karyotype of Partamona seridoensis and P. helleri, from the Atlantic Forest bioma. We have also investigated the structure of the B chromosomes and their relation with the A genome in P. helleri, using several techniques such as C banding, fluorochrome staining and fluorescent in situ hybridization.

Materials and methods

We have analyzed 11 colonies of Partamona helleri: six from the town of Viçosa (20°45’LS, 42°52’LW), one from São Miguel do Anta (20°42’LS, 42°43’LW), one from Argirita (21°36’LS, 46°08’LW), and three from Porto Firme (20°40’LS, 43°05’LW), all in Minas Gerais state, Brazil. Three Partamona seridoensis nests were collected at Sant’Ana do Seridó city (6°35’LS, 36°46’LW) in the Rio Grande do Norte state, Brazil; two of them were orphans. The species were identified by Dr. Silvia Regina de Menezes Pedro, University of São Paulo, Ribeirão Preto, SP (Brazil).

Cerebral ganglion metaphase slides were obtained of post-defecating larvae as described by Imai et al. (1988). The following chromosome banding techniques were applied: C banding according to Sumner (1972), GTG banding (Seabright 1971), fluorochrome staining with DA/Chromomycin A3 (Schweizer, 1980) and Q banding with mustard quinacrine (Schmid 1980). Sequential treatments, BS-DA/CMA3, with C banding followed by DA/CMA3 and Giemsa staining were performed using the same procedures used in the individual treatments.

Fluorescent in situ hybridization (FISH) was performed by the method of Pinkel et al. (1986) using an 18S rDNA probe from maize, Zea mays, kindly provided by Dr. Newton Carneiro, EM- BRAPA, Sete Lagoas, MG (Brazil). This probe was labelled with fluorescein (fd-12-dUTP) by random priming with a STRATAGENE labeling kit as recommended by manufacturer. The probe was denatured at 70°C for 5 min and the chromosomal DNA was denatured in a solution of 70% formamide 2×SSC, at 80°C for 5 min. Hybridization was performed overnight in a moist chamber at 37°C in the presence of 60% formamide. After hybridization, three 0.1×SSC washes were performed at 37°C.

Chromosomes were classified according to Imai (1991) and mounted by pairing the chromosomes in decreasing size order.

Results and discussion

General features of P. helleri and P. seridoensis karyotypes

From eleven analyzed colonies of P. helleri, five had B chromosomes (2K=
20M\(^{cc}\)+4M\(^{r}\)+8M\(^{ct}\)+2M\(^{cc}\)) of B1 type. This B type was described by Brito et al. (1997). \textit{P. seridoensis} had 2n=34 chromosomes (2K=22M\(^{cc}\)+4M\(^{r}\)+4M\(^{ct}\)+4M\(^{cc}\)) in females and n=17 for males. The orphan colonies of \textit{P. seridoensis} had only haploid larvae, probably sons of workers. Workers may develop ovaries when the queen is absent; a fact observed by Sakagami et al. (1963) for the \textit{Partamona}.

The C banding procedure revealed that the heterochromatin localization is similar in both species, with most of chromosomes with metacentric morphology (M\(^{cc}\) and M\(^{r}\)) i.e. heterochromatic blocks at the pericentromeric region (Figs. 1A, 2A). Chromosome pairs 2, 4, 9, 10 and 15, in both species presented terminal heterochromatic blocks, (Figs. 1A, 2A). The presence of this chromatin, around the centromere had been previously observed in \textit{P. sp.} (Brito-Ribon et al. 1999) and in \textit{P. peckolti} (Brito et al. 2003).

The heteromorphism observed by Brito et al. (1997) in \textit{P. helleri} has been detected in some of the colonies analyzed. This fact could not be associated with the presence of B chromosomes in the karyotype as it was also been observed in regular 2n=34 cells. As the heteromorphism was easily visible in colony G15, we subjected this colony to other cytogenetic techniques to verify the possibility of this structural feature was directly related with the B chromosome origin.

According to the Minimum Interaction Theory of Imai et al. (1986, 1988, 1994) metacentric chromosomes (M) such as those of \textit{P. helleri} and \textit{P. seridoensis} would have evolved by centric fissions from large euchromatic metacentric chromosomes of an ancestral karyotype, followed by \textit{in tandem} heterochromatin increase, and posterior chromosomal rearrangements such as pericentric inversions and fusions with inactivation of one of the centromeres. This heterochromatin growth would be the result of telomerase action, unequal crossing-over, genic amplification or telomere-telomere recombination (revised by Imai 1991).

Hoshiba and Imai (1993) have proposed the Minimum Interaction Theory to explain the karyotype evolution of hymenopterans as a whole. It has been supported by data obtained from wasps of the genus \textit{Microstigmus} (Costa et al. 1993) and \textit{Trypoxylon} (Gomes et al. 1995, Scher and Pompolo
We wish to gain a better understanding of the nature of heterochromatin in the tribe Meliponini to verify if the Minimum Interaction Theory can be applied to stingless bees. Pertinent studies on Meliponini bees include those of Pompolo and Campos (1995) in *Leurotrigona*, Rocha et al. (2003) in *Friesomiellita* and *Tetragonisca angustula*, Caixeiro (1999) in *Plebeia*, and Costa et al. (2004) in *Trigona*. However, rather few species (about 80 out of 400 species) of stingless bees have been studied.

**Localization of GC rich regions**

To characterize the karyotypes of *P. helleri* and *P. seridoensis* we have applied GTG banding according to Seabright (1971). Trypsin digested proteins that allowed us to recognize easily the chromosome pairs: 2, 9, 10 and 15 in both species. Even though as good as G band pattern cannot be achieved in insects as in mammals, the digestion of the terminal portions of those chromosome pairs matched to the positive GC bands highlighted by the DA/CMA$_3$ staining and also to the faint bands of mustard quinacrine coloration (Figs. 1B, C, D and 2B, C, D). We conclude that the proteins digested by the trypsin have affinity for AT rich regions of *Partamona*. The sequential treatment BS-DA/CMA$_3$ revealed that not all positive DA/CMA$_3$ bands were heterochromatic. Previous C banding treatment prevented the appearance of the bright terminal bands of chromosome pair number 4, which were observed when the fluorochrome was applied separately in *P. helleri*.

These results showed that heterochromatin is not homogeneous in the karyotype of the two species. This has also been previously demonstrated in *Eufriesea violacea* (Gomes et al. 1998), an orchid bee species which possesses large and predominantly heterochromatic chromosomes and has three to four GTG bands per chromosome when treated with trypsin. The composition difference between the pericentromeric heterochromatin and the ones located on distal portions of chromosome pairs 2, 9, 10 and 15, in *P. helleri* and *P. seridoensis* (Table 1), led us to conclude that it is improbable that these chromatin regions have the same origin.

**Number and localization of NOR sites by FISH**

With the objective to further study the DNA composition of the CMA$_3$ positive heterochromatic regions we used an in situ hybridization assay, since there are many examples on the literature of coincidental C$^+/$/CMA$_3^+/$/NOR$^+$ marks. The FISH results with rDNA 18S probe confirmed that pairs number 2, 9, 10 and 15 carry cistrons for the ribosomal RNA. The same correspondence of CMA$_3^+/$/NOR has been observed in the coleopteran *Olla v-nigrum* (Maffei et al. 2001a); and in the wasp *Trypoxyylon albitarse* (Araújo et al. 2000); the stingless bees *Plebeia* and *Melipona* (Maffei et al. 2001b); *Friesella schrottkyi* (Mampumbu 2002) and *Partamona peckolti* (Brito et al. 2003).

However, we have noticed a difference in the number of rDNA carrier chromosomes among colonies of the same species and between species. In *P. helleri*, individuals of colony PK had six

<p>| Table 1. | Comparison of the techniques applied to chromosomes of <em>Partamona helleri</em> and <em>P. seridoensis</em>. (+ and – mean positive and negative responses to the treatments employed, respectively) |</p>
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<thead>
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<th>Technique</th>
<th>Chromosome pairs (Terminal portion of the large arm)</th>
<th>Pericentromeric heterochromatin</th>
<th>B chromosomes of <em>P. helleri</em></th>
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<td>C banding</td>
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<td>FISH (18S rDNA)</td>
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carrier chromosomes and the workers of colony G15 had eight carriers chromosomes, while in the P. seridoensis non-orphan colonies individuals, seven chromosomes showed hybridization with the 18S rDNA probe (Fig. 3). The odd numbers of NORs can be explained by heterozygosity due to NOR presence in two chromosome pairs of P. helleri in colony PK and in one pair of colony 153 of P. seridoensis.

Hirai et al. (1994, 1996) have reported interspecific numerical variation of NORs among Myrmecia ants. Their results showed that species presenting higher chromosome numbers also presented higher numbers of 28S rDNA positive hybridization regions. These results allowed the authors to infer that the karyotype evolution theory proposed by Imai et al. (1986, 1988, 1994) could explain the pattern observed and, also to propose that the fission-fusion cycle would also be responsible for the rDNA dispersion observed in Myrmecia. The difference in rDNA chromosome carrier number observed between P. helleri and P. seridoensis may be related to the chromosome evolutionary process that the species have undergone. However, we believe that more species must be assayed before an analogy to the Myrmecia case may be proposed.

The largest chromosome of the heteromorphic pair of P. helleri of colony G15 individuals showed an intense hybridization with the probe on the heterochromatic segment of the long arm. That may represent a larger rDNA gene copy number, if one considers the intensity of the signals in the other two colonies (Fig. 3A).

NOR heteromorphisms have been reported in the literature for many species such as Glossina flies (Willhoft 1997); in two Myzus aphids (Bizzaro et al. 1999) and in the fish Pinirampus (Swarça et al. 2001). Unequal crossing-over between the homologous chromosomes and in tandem gene amplification of rDNA sequences may be a possible explanation for such heteromorphisms. This idea has gained support by the results of Mandrioli et al. (1999) with the analysis of the rDNA intergenic spacers of the aphid Acyrthosiphonpisum.

Another fact that could increase the rearrangement frequency in P. helleri cells would be the presence of B chromosomes as seen in the grasshopper Eyprepocnemisplorans (Henriques-Gil et al. 1984) and in rye, Secale cereale (Jones 1991). There were modifications not only in frequency but also in chiasma distribution at the normal complement homologous chromosomes. In the case of Partamona helleri, this event must have happened in the queen gametic cells, since we have noticed such heteromorphism in all cells of the individual of colony G15. A population study combined with molecular cytogenetics should be applied to show if the structural abnormality on the P.
The 18S rDNA nuclear genes were the first to be mapped in the *Partamona* genus. The analysis of this chromosome marker can aid the construction of a particular model for the karyotype evolution of the Meliponini bees. However, analyses of the 18S rDNA region in Meliponini are still scarce. Only *Melipona* (Rocha *et al.* 2002) and one *Friesella* species (Mampumbu 2002) have been analyzed so far.

**B chromosomes and the structure of the A genome of *P. helleri***

Even though B chromosomes have been studied since 1907, when they were first described (for revision see Camacho *et al.* 2000). Knowledge about their origin is still quite controversial.

The various treatments applied in this work allowed us to conclude that the pericentromeric heterochromatin of the normal complement and that of B chromosomes of *P. helleri* are not similar. It suggests that the B chromosome have not originated from the A genome by non-disjunction followed by accumulation. However, the B chromosomes heterochromatin showed the same responses to the treatments of C, G, CMA₃ and mustard quinacrine banding when compared to the terminal portions of the chromosome pairs 2, 9, 10 and 15 (Fig. 1 and Table 1).

Such numerical alterations could have appeared from the A genome of *P. helleri* through fission, in particular at the heteromorphic pair. It was supported by the similar responses of the heterochromatin of the non-homologous portion of heteromorphic pair and of the supernumerary chromosome to the treatments used. However, the *FISH* data showed that the terminal portions of chromosome pair number two and the B chromosome have different DNA compositions (Fig. 3A).

A RAPD marker band associated with the B chromosome has already been found in individuals carrying only one B chromosome (Tosta *et al.* 2004). The hybridization of this sequence as a probe on *P. helleri* chromosomes may show homologies between A and B chromosomes, if the supernumeraries were originated by fissions of the normal complement. However, if homologies would be observed between chromosomes of the different *Partamona* species, it will suggest an origin by interspecific hybridization for the Bs, as noticed for the extra PSR chromosome of *Nasonia vitripennis* (McAllister and Werren 1997, Perfecciti and Werren 2001).

The genus *Partamona* has been morphologically characterized as a monophyletic group but there are many unsolved questions concerning to the phylogeny of this genus (Camargo and Pedro 2003, Pedro and Camargo 2003). Cytogenetics and molecular approaches of nuclear and mitochondrial genomes can clarify these relations within the genus. We believe that cytogenetic data are strong phylogenetical tools, and are indispensable in the study of evolutionary pathways. Such data include: chromosome number and length; arm number and length; heterochromatin position; NORs number, and GC and AT rich “islands” position, among others. A good beginning has been done with *Frieseomelitta* (Rocha *et al.* 2003); *Partamona* (Brito-Ribon *et al.* 1999, Brito *et al.* 2003); *Plebeia* (Caixeiro, 1999); and *Melipona* (Rocha 2002, Rocha and Pompolo 1998, Rocha *et al.* 2002). Our expectations are that our results will contribute for the evolutionary study of the *Partamona* genus.

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