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Sex chromosome differentiation in *Belostoma* (Insecta: Heteroptera: Belostomatidae)

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ABSTRACT. *Belostoma*, a genus of the family Belostomatidae, includes species of great ecological importance as biocontrol agents. Few species of these species have been the subject of cytogenetic analyses. Karyotypic evolution in this genus involves agmatoploidy and simploidy; there are also different sex chromosome systems. We examined two *Belostoma* species (*B. dilatatum* and *B. candidulum*) collected from the Paranapanema River Basin (Brazil). Mitotic and meiotic analysis revealed $2n(♂) = 26 + X_1X_2X_3Y$ for *B. dilatatum* and $2n(♂) = 14 + XY$ for *B. candidulum*; both karyotypes have holokinetic chromosomes. Differences in heterochromatin distribution were also observed between the species, besides variation in the localization of CMA_3^+/$DAPI^-$ blocks. The existence of different types of sex

chromosome systems in these species was confirmed based on arrangements of the chromosomes in different meiotic stages. We identified a new sex system in *B. dilatatum*, and make the first cytogenetic report on *B. candidulum*.

Key words: Holokinetic chromosomes; Heterochromatin; Simploidy; Sex chromosomes; Agmatoploidy

INTRODUCTION

The family Belostomatidae Leach, 1815, comprises the largest heteropterans, where it is a group with a worldwide distribution and a high diversity in the tropical region (Merritt and Cummins, 1996; Ribeiro, 2007). These are animals with predatory habits, and their ecological importance lies in the role they play as biocontrol agents, where aquatic arthropods, fish, and amphibians are its main prey (Cullen et al., 1969; Saha et al., 2010).

According to Ribeiro (2007), the family Belostomatidae is composed of eight genera with approximately 150 species, distributed in three subfamilies: Belostomatinae, Horvathiniinae and Lethocerinae (Lauck and Menke, 1961). The first subfamily has the highest number of genera, among which *Belostoma* is the most diverse (Lauck and Menke, 1961; Ribeiro, 2005). The systematics of this genus is confusing since the different species of *Belostoma* have a similar morphology, and also because few studies have been conducted in Brazil (Ribeiro, 2007).

Most of the chromosomal analyses in species of the family Belostomatidae have been restricted to conventional analyses (Table 1). The cytogenetic data show that most species have males with chromosome numbers ranging from $2n = 4$ in *Lethocerus* sp. (Chickering, 1927, 1932) to $2n = 29$ in some species of *Belostoma* (Papeschi and Bidau, 1985; Papeschi, 1988, 1991). Nevertheless, analyzed species showed holokinetic chromosomes, a characteristic common to all species of heteropterans (Papeschi and Bressa, 2006). Besides the wide variation in diploid number, most species have different sex chromosome systems (Table 1). Simple and multiple systems have been reported in the genus *Belostoma*, and in all cases the male was the heterogametic sex.

Papeschi and Bressa (2006) proposed an ancestral karyotype with $2n = 26 + XY$ for the genus, substantiating that in the genus *Belostoma*, chromosomal evolution supposedly occurred by simploidy (fusion) and agmatoploidy (fission), as happens in many organisms with holokinetic chromosomes. Simploidy events would be related to a decrease in the diploid number without changes in the type of sex chromosome system. The formation of a multiple sex chromosome would be attributed to agmatoploidy, by which the X chromosome would undergo fragmentations resulting in two new X chromosomes (X_1X_2).

Although there is evidence of the mode of karyotypic evolution in the genus *Belostoma*, much about these events still remains unknown. In order to provide a more detailed characterization of chromosomal diversity and a better understanding of the karyotypic evolution in this group, we investigated two species of *Belostoma* through chromosomal markers. The cytogenetic data for one of the species are previously unpublished, and so are data on the occurrence of a new sex chromosome system in the other. The mechanisms involved in the evolution of these systems are also discussed.

Table 1. Cytogenetic data for the family Belostomatidae.

Subfamily	Species	Diploid number (2n)	References
Belostomatinae	*Belostoma elegans*	29 (26 + X_1X_2Y♂)	Papeschi (1988), Papeschi (1991)
		29/30 (26 + X_1X_2Y♂/ $X_1X_1X_2X_2$ ♀)	Papeschi and Bidau (1985)
	B. bergi	29 (26 + X_1X_2Y♂)	Papeschi and Bressa (2004) *apud* Papeschi and Bressa (2006)
	B. bifoveolatum	29 (26 + X_1X_2Y♂)	Papeschi (1991)
	B. cummingsi	29/30 (26 + X_1X_2Y♂/ $X_1X_1X_2X_2$ ♀)	Papeschi and Bidau (1985)
	B. dentatum	29 (26 + X_1X_2Y♂)	Papeschi and Bidau (1985), Papeschi (1991)
	B. dilatatum	29 (26 + X_1X_2Y♂)	Papeschi (1992)
		30 (26 + $X_1X_2X_3Y$♂)	Present study
	B. discretum	29 (26 + X_1X_2Y♂)	Papeschi and Bressa (2004) *apud* Papeschi and Bressa (2006)
	B. elongatum	29 (26 + X_1X_2Y♂)	Papeschi (1992)
	B. gestroi	29 (26 + X_1X_2Y♂)	Papeschi (1992)
	B. martini	29 (26 + X_1X_2Y♂)	Papeschi (1991)
	B. indentatum	29 (26 + X_1X_2Y♂)	Ueshima (1979)
	B. plebejum	16 (14 + XY♂)	Papeschi (1994)
		15 (13 + XY♂)	
		17 (14 + X_1X_2Y♂)	
	Belostoma sp	16 (14 + XY♂)	Papeschi (1996)
		17 (14 + X_1X_2Y♂)	
	B. flumineum	24 (22 + XY♂)	Chickering (1916, 1927b) *apud* Papeschi (1988)
	Belostoma sp	24 (22 + XY♂)	Montgomery (1901, 1906) *apud* Papeschi (1988)
	B. candidulum	16 (14 + XY♂)	Present study
	B. micantulum	16 (14 + XY♂)	Papeschi (1988)
	B. orbiculatum	16 (14 + XY♂)	Papeschi (1996)
	B. oxyurum	8 (6 + XY♂)	Papeschi (1988), Papeschi and Bidau (1985)
	Diplonuchus annulatus	28 (26 + XY♂)	Jande (1959) *apud* Papeschi (1988)
	D. rusticus	28 (26 + XY♂)	Bawa (1953), Jande (1959) *apud* Papeschi (1988)
	D. subrhombeus	28 (26 + XY♂)	Jande (1959) *apud* Papeschi (1988)
Lethocerinae	*L. uhleri*	30	Chickering and Bacorn (1933) *apud* Papeschi (1988)
	L. annulipes	28 (26 + XY♂)	Papeschi (1992)
	Lethocerus sp1	28	Chichering (1932) *apud* Papeschi (1988)
	L. griseus	28 (26 + XY♂)	Chichering (1927a) *apud* Papeschi (1988)
	L. melloleitaoi	28 (26 + XY♂)	Papeschi and Bressa (2004) *apud* Papeschi and Bressa (2006)
	L. indicum	26 (24 + neo-X-neo-Y♂)	Banerjee (1958), Bagga (1959), Jande (1959) *apud* Papeschi (1988)
	Lethocerus americanus	8 (6 + XY♂)	Chickering (1927a,b) Chickering and Bacorn (1933) *apud* Papeschi (1988)
	Lethocerus sp2	4 (2+neo-X-neoY♂)	Chichering (1927a) *apud* Papeschi (1988)

Adapted from Papeschi and Bressa (2006).

MATERIAL AND METHODS

Samples and collection sites

Two species of the genus *Belostoma* [*B. dilatatum* (Dufour, 1863) (10 males) and *B. candidulum* Montandon, 1903 (15 males)] were collected from Água do Macuco Stream, Lower Paranapanema River basin (22°54'15.3"S and 50°23'47.98"W), between the states of Paraná and São Paulo, Brazil. Individuals from each species were deposited at Universidade Federal do Pampa (UNIPAMPA).

Chromosome preparations and conventional staining

The gonads of the adult specimens were dissected in physiological solution for insects (7.5 g NaCl, 2.38 g Na_2HPO_4 and 2.72 g KH_2PO_4 in 1 L distilled water). The testicles were treated with a hypotonic solution (tap water) for 3 min and fixed in Carnoy I (3:1, methanol:acetic acid) for 30 min. Chromosome preparations were made by cellular suspension by maceration in one drop of 45% acetic acid, with each gonad previously treated with 45% acetic acid. These preparations were submitted to conventional staining with 3% Giemsa and also to chromosome banding techniques.

Chromosome banding

Silver nitrate staining of active nucleolar organizer regions (Ag-NOR) was performed according to Howell and Black (1980). The distribution of heterochromatin was analyzed by Giemsa C-banding after treatments with 0.2 M HCl, $Ba(OH)_2$ and 2X SSC (Sumner, 1972). The GC- and AT-rich bands were detected with chromomycin A_3 (CMA_3) and 4'-6-diamino-2-phenylindole (DAPI), respectively, according to Schweizer (1976). The slides were stained with 0.5 mg/mL CMA_3 for 1.5 h, washed in distilled water and sequentially stained with 2 µg/mL DAPI for 30 min. Slides were mounted with a medium composed of glycerol/McIlvaine buffer, pH 7.0, 1:1, plus 2.5 mM $MgCl_2$.

RESULTS

B. dilatatum

The chromosomal number observed in all samples of *B. dilatatum* was $2n(♂) = 30 = 26 + X_1X_2X_3Y$ (Figure 1). By conventional analysis, large interphasic nuclei with several well-defined chromocenters were observed (Figure 1a). In pachytene, the homologues were associated, and curved filament-like structures were observed, where it was difficult to distinguish the bivalents (Figure 1b and c). Heterochromatic blocks were located terminally on all bivalents, and interstitial dots were also observed. Sex chromosomes were positively heteropycnotic and usually associated with each other (Figure 1b and c). During diplotene/diakinesis, 1 or 2 chiasmata per bivalent were observed, while the separate sex chromosomes remained univalent (Figure 1d).

The results of the analyses of meiocytes in metaphases I and II confirmed that bivalents divide reductionally, and sex chromosomes, equationally (Figure 1e and f). In metaphase II, the bivalents were arranged in a ring and the univalent sex chromosomes were positioned inside the ring as a pseudo-tetravalent. This was confirmed by the fact that during that period, 17 structures corresponding to 13 autosomal and four sex chromosomes were observed. The Y chromosome probably corresponded to the large structure evidenced in the pseudo-tetravalent, while the other three smaller chromosomes corresponded to the X_1, X_2 and X_3 (Figure 1f).

Staining with fluorochromes showed chromocenters $CMA_3^+/DAPI^+$ (Figure 2a and f); however, in interphasic nucleus, two $CMA_3^+/DAPI^-$ regions were observed (Figure 2). In pachytene, heterochromatic blocks were observed, confirming the $CMA_3^+/DAPI^+$ characteristic of the heterochromatin (Figure 2b and g). A $CMA_3^+/DAPI^-$ block was observed in one of the

bivalents, probably corresponding to the staining evidenced in the interphasic nucleus (Figure 2l). This block impregnated by silver nitrate, confirmed the existence of Ag-NORs (Figure 1g).

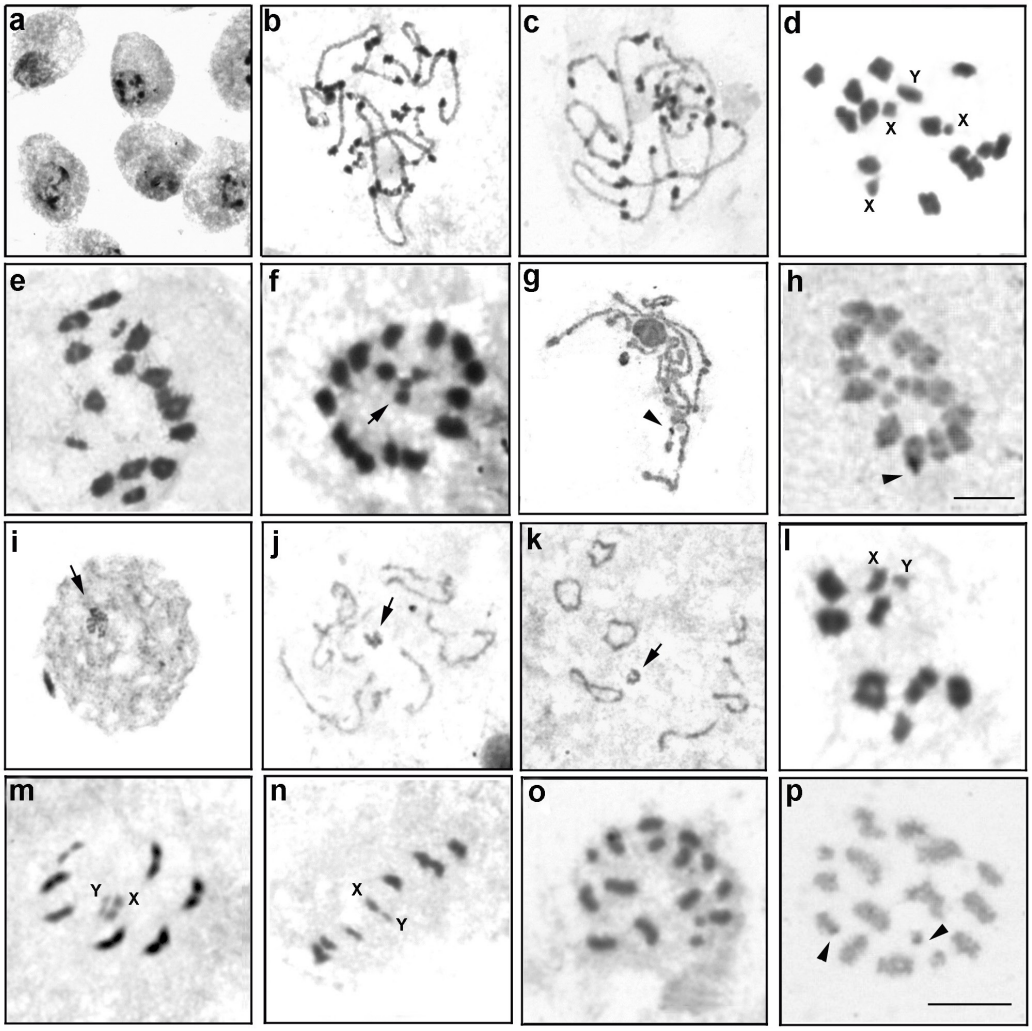

Figure 1. Meiotic stages in *Belostoma dilatatum* (**a-h**) and *B. candidulum* (**i-p**): **a.** Interphasic nucleus; note the quantity and distribution of chromocenters. **b** and **c.** Pachytene; note the distribution of heterochromatic blocks. **d.** Diplotene/diakinesis; sex chromosomes are viewed as univalents. **e.** Transition between diakinesis and metaphase I. **f.** Metaphase II; arrows show the sex chromosomes inside the ring formed by autosomes. **g** and **h.** Pachytene and metaphase II staining by silver nitrate, respectively; arrowheads show Ag-NOR. **i.** Interphasic nucleus; arrows show the chromocenter corresponding to NOR. **j.** Pachytene; arrow indicates the association between sex chromosomes. **k.** Diplotene; note the chiasmata; arrow indicates associated sex chromosome. **l.** Diakinesis; note sex chromosome-like univalents. **m.** Metaphase I; sex chromosome inside ring autosomes. **n.** Metaphase II; note the touch-and-go pairing between X and Y. **o** and **p.** Mitotic metaphase in conventional staining and Ag-NOR, respectively; arrowheads show the NOR-bearing chromosomes. Bars = 10 μm.

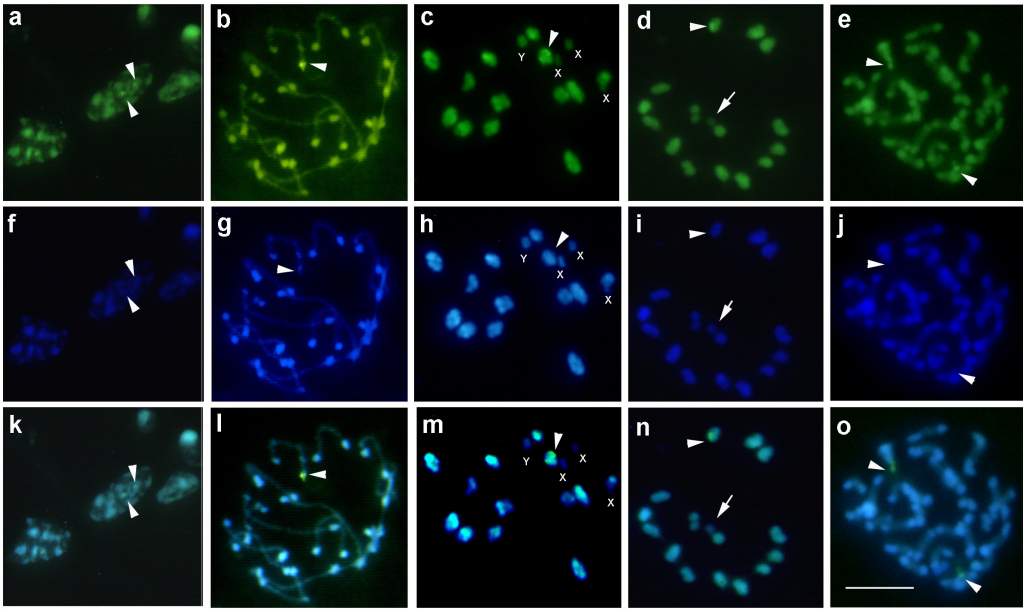

Figure 2. Fluorochrome staining in *Belostoma dilatatum*: (**a-e**) CMA_3; (**f-j**) DAPI; (**k-o**) overlapping CMA_3/DAPI. **a. f. k.** Interphasic nucleus. **b. g. l.** Pachytene. **c. h. m.** Diakinesis; note the distribution of heterochromatic blocks. **d. i. n.** Metaphase II. **e. j. o.** Mitotic metaphase. Arrows show the sex chromosomes. Arrowheads indicate CMA_3^+/$DAPI^-$ sites; note the presence of these sites in autosomes. Bars = 10 μm.

In diakinesis, the presence of heterochromatic blocks in autosomes was confirmed (Figure 2c, h and m). In metaphase II, sex chromosomes showed no highlighted blocks, while one of the autosomes displayed a CMA_3^+/$DAPI^-$ block (Figure 2d, i and n) and also Ag-NORs (Figure 1h).

The analysis of spermatogonial cells in mitotic metaphase showed the terminal and interstitial distribution of heterochromatic blocks, and the occurrence of a CMA_3^+/$DAPI^-$ block in the terminal region of a medium-sized chromosomal pair (Figure 2e, j and o).

B. candidulum

All *B. candidulum* samples showed $2n(♂) = 16 = 14 + XY$, with the karyotype composed of 1 small, 9 medium, and 4 large chromosomes, all holokinetic (Figure 1p). The X chromosome was characterized as medium-sized chromosome, and the Y, as small-sized chromosome (Figure 1m and n). In the analysis of the nuclei, only one chromocenter and one region with positive heteropycnosis were observed (Figure 1i).

Pachytene showed the association of bivalents, which, in addition, showed little heterochromatin located in the region of association among sex chromosomes (Figure 1j and k). In diplotene, 1 or 2 chiasmata per bivalent were observed, and the sex chromosomes were associated (Figure 1k). In diakinesis, the sex chromosomes were dissociated and became univalent (Figure 1l). In metaphase I, bivalents were arranged in a ring in the periphery, while the sex chromosomes were positioned inside in lateral conformation (Figure 1m). In metaphase II, X and Y

chromosomes displayed negative heteropycnosis and in touch-and-go conformation (Figure 1n).

After C-banding and CMA_3/DAPI staining, the CMA_3^+/$DAPI^-$ blocks were localized. CMA_3^+/$DAPI^-$ blocks were observed in the chromocenter (Figure 3a, e and i), and these blocks were also observed in pachytene (Figure 3b, f and j). In diplotene, these blocks were distributed in the sex chromosomes (Figure 3c, g and k), where X showed one block in the terminal region and Y showed staining in both terminations (Figure 3 box). In the mitotic metaphases, CMA_3^+/$DAPI^-$ blocks (Figure 3d, h and l), which were coincident with Ag-NORs, could also be seen (Figure 1p). Thus, we demonstrated the presence of these sequences on the sex chromosomes and their total absence on the autosomes.

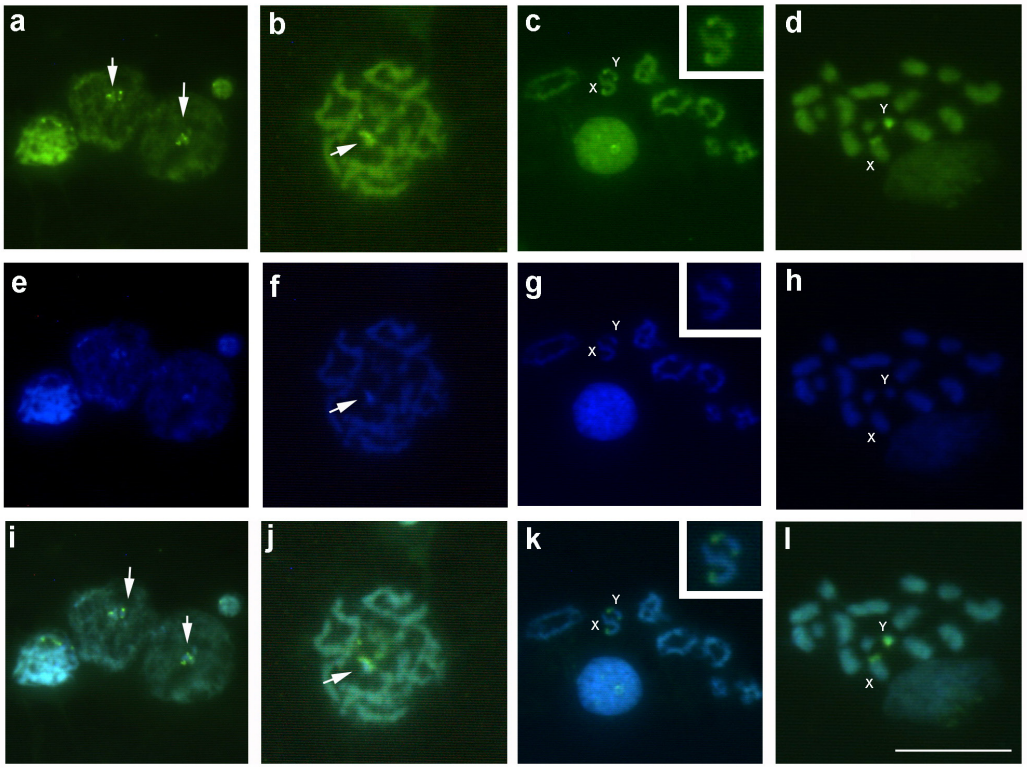

Figure 3. Fluorochrome staining in *Belostoma candidulum*: (**a-d**) CMA_3; (**e-h**) DAPI; (**i-l**) overlapping CMA_3/DAPI. **a. e. i.** Interphasic nucleus. **b. f. j.** Pachytene. **c. g. k.** Diplotene; box: association between X and Y chromosomes. **d. h. l.** Mitotic metaphase. Arrows indicate CMA_3^+/$DAPI^-$ sites; note the presence of these sites in sex chromosomes. Bars = 10 µm.

DISCUSSION

Conventional analysis of the two species of *Belostoma* revealed $2n(♂) = 30 = 26 + X_1X_2X_3Y$ for *B. dilatatum* and $2n(♂) = 16 = 14 + XY$ for *B. candidulum*. Similar karyotypes were reported by different authors for other species of the genus (Table 1). In addition to defin-

ing the diploid number, conventional mitotic and meiotic analysis confirmed the occurrence of holokinetic chromosomes in the species studied, a common feature of all heteropterans (Jacobs, 2004; Papeschi and Bressa, 2006; Bardella et al., 2010).

The arrangement of univalent sex chromosomes in a pseudo-tetravalent, with autosomes forming a ring in the periphery during metaphase II, confirmed the existence of three X and one Y chromosomes, where the Y corresponds to the largest, and the X to smaller chromosomes arranged in a semicircle around the Y (Figure 1f). Fragmentation of the X chromosome is recurrent in this genus, and this arrangement has already been described for other species of *Belostoma* with a multiple sex chromosome system, such as *B. elegans* Mayr, 1871, *B. cummingsi* De Carlo, 1930, and *B. dentatum* Nieser, 1975 (Papeschi and Bidau, 1985).

A great variation in the diploid number is observed in the genus, with $2n = 8$ (6 + XY) to $2n = 29$ (26 + X_1X_2Y), the latter being the most common (table 1). All males of *B. dilatatum* analyzed in this work showed 30 chromosomes, suggesting $2n = 32$(26 + $X_1X_1X_2X_2X_3X_3$) for the females, considering that this diploid number is probably fixed in the population. Thus far, *B. dilatatum* is the species with the highest number of chromosomes in the genus. This variation in chromosome number occurs because holokinetic chromosomes that undergo a breakdown (agmatoploidy) or fusion (simploidy) (Luceño and Guerra, 1997) remain viable in subsequent cell divisions. This was demonstrated in experiments with X- and γ-rays in *Rhynchospora pubera* (Vahl) Boeck (Vanzela and Colaço, 2002).

The cytogenetic data available for the family Belostomatidae so far have led to the proposal of an ancestral karyotype $2n = 26 + XY$ (Papeschi and Bressa, 2006). Data on this ancestral karyotype suggest that agmatoploidy events have originated the common karyotype found in the genus *Belostoma* ($2n = 26 + X_1X_2Y$). A second agmatoploidy event has probably occurred in the sex chromosomes of *B. dilatatum* samples analyzed in this work, resulting in the karyotype $2n = 26 + X_1X_2X_3Y$ (Figure 4). Sex chromosome systems with multiple X chromosomes are common in Heteroptera, but the occurrence of $X_1X_2X_3Y$ was observed in the Belostomatidae for the first time. Other reports of this system were described in a few species of Pentatomidae, Aradidae, and Reduviidae (Heizer, 1950; Jacobs, 1986; Severi-Aguiar et al. 2006).

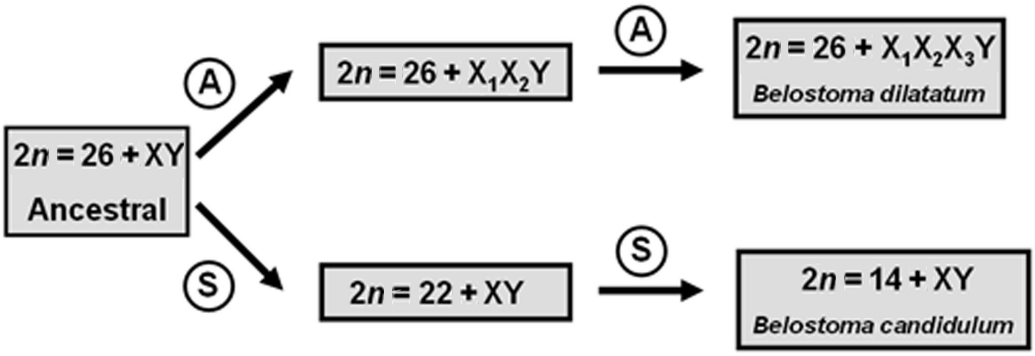

Figure 4. Scheme suggesting the karyotype differentiation and evolution in *Belostoma dilatatum* and *B. candidulum*. $2n = 26 + XY$: ancestral karyotype suggested by Papeschi and Bressa (2006). A = Agmatoploidy. The first event involved the X chromosome resulting in the multiple system of sex chromosomes; the second event of agmatoploidy probably occurred giving rise to the multiple system $X_1X_2X_3Y$ of *B. dilatatum*. S = Simploidy. Simploidy seems to have occurred between the autosomal chromosomes in the ancestral karyotype; other simploidy events possibly occurred between autosomes, and also autosomes and sex chromosomes, resulting in the karyotype found in *B. candidulum*.

Papeschi (1996), in analyzing different populations of *Belostoma* sp, found an interspecific polymorphism of sex chromosomes, where two karyotypes could be observed: 1) $2n = 16 = 14 + XY$, in most males; and 2) $2n = 17 = 14 + X_1X_2Y$. The author attributed the origin of the multiple sex system to the fragmentation of the X chromosome of the first karyotype. Furthermore, the samples with $2n = 17$ showed meiotic instability with three different arrangements in metaphase II. Therefore, the *Belostoma* sp samples represented direct evidence of the mechanism of the origin of sex chromosomes by agmatoploidy, which probably also occurred in *B. dilatatum* samples, the subject of this study.

While agmatoploidy is probably the most important event in karyotypic evolution in *B. dilatatum*, simploidy seems to be a major chromosomal rearrangement in *B. candidulum*, as well as in other species of *Belostoma* with reduced diploid numbers (Figure 4 and Table 1).

B. candidulum showed $2n(♂) = 16 = 14 + XY$ and a karyotype composed of holokinetic chromosomes. The X chromosome corresponds to a medium-sized chromosome, and the Y, to small-sized chromosome. This karyotypic organization has been observed in other species of the genus, such as *B. micantulum* (Stål, 1860) (Papeschi, 1988), *B. plebejum* (Stål, 1860) (Papeschi, 1994), and *B. orbiculatum* Estévez and Polhemus, 2001 (Papeschi, 1996).

During the meiotic divisions, it was possible to observe the association between the sex chromosomes of *B. candidulum*, mainly in prophase I (pachytene and diplotene). This association is probably related to the fact that these chromosomes are bearers of nucleolus organizer regions. This was evident by impregnation with silver nitrate and staining with fluorochromes. The interphasic nucleus showed a nucleolus with $CMA3^+/DAPI^-$ blocks, and during the other phases, sex chromosomes with Ag-NORs and $CMA3^+/DAPI^-$ were observed. In diplotene, the association between these chromosomes and the location of $CMA3^+/DAPI^-$ blocks was observed (Figure 3 box).

Ueshima (1979) proposed that the sex chromosome system XX/XY is ancestral in the suborder Heteroptera. If we consider this proposal, together with the ancestral karyotype of *Belostoma* ($2n = 26 + XY$) (Papeschi and Bressa, 2006), we can propose that there was no agmatoploidy in the origin of sex chromosomes in *B. candidulum.* However, a reduction in the diploid number leads us to infer the occurrence of various simploidy events in the species (Figure 4), as proposed by Papeschi and Bressa (2006) for other species of the genus with a reduced diploid number.

Evidence is supported by the occurrence of simploidy in karyotypic evolution of *Belostoma*. Analyses by fluorescent *in situ* hybridization with 18S rDNA probes in three species of the genus showed the presence of rDNA sites, which correspond to $CMA3^+/DAPI^-$ blocks (Papeschi and Bressa, 2006) on autosomes of *B. elegans* ($2n = 26 + X_1X_2Y$) and on sex chromosomes of *B. oxyurum* Lauck, 1962 ($2n = 6 + XY$) and *B. micantulum* ($2n = 14 + XY$). The authors proposed that the reduction in the diploid number probably involved the fusion of an ancestral pair of sex chromosomes with a NOR-bearing autosomal pair, and that there was a loss of heterochromatin during the fusions.

For *B. candidulum*, evidence is supported by the presence of Ag-NOR and $CMA3^+/DAPI^-$ blocks in sex chromosomes, unlike what occurred in *B. dilatatum*, where these stainings were observed in autosomes.

The simploidy between sex chromosomes and autosomes is also evidenced in other heteropteran species. In *Dysdercus albofasciatus* Berg 1878, cytogenetic analyses by different techniques revealed the occurrence of a neo-X and neo-Y sex chromosome system, which

would have evolved by the insertion of an ancestral X chromosome in an autosome, and as a result, the autosome homologue became the neo-Y chromosome (Bressa et al., 2009).

These events probably also occurred in the samples of the genus *Belostoma* studied here. A wide difference in the distribution of heterochromatic blocks could be observed between the species analyzed. While *B. dilatatum* showed a large number of heterochromatic blocks distributed in the terminal regions of the chromosomes, *B. candidulum* showed little heterochromatin, which was restricted to the sex chromosomes. This supported the hypothesis of Papeschi and Bressa (2006) that species with a low diploid number have reduced heterochromatin. Furthermore, the distribution of Ag-NOR and $CMA3^{+}$/$DAPI^{-}$ blocks allows us to infer that *B. dilatatum* had a karyotype evolution driven mainly by the agmatoploidy of sex chromosomes, while the karyotype of *B. candidulum* evolved by simploidy between autosomes and sex chromosomes.

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