



Short Communication

## Cytogenetics of the Brazilian *Bolitoglossa paraensis* (Unterstein, 1930) salamanders (Caudata, Plethodontidae)

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### Abstract

Plethodontid salamanders of genus *Bolitoglossa* constitute the largest and most diverse group of salamanders, including around 20% of living caudate species. Recent studies have indicated the occurrence of five recognized species in the Brazilian Amazon Rainforest. We present here the first cytogenetic data of a Brazilian salamander, which may prove to be a useful by contribution to the cytotaxonomy of the genus. Specimens were collected near the “type” locality (Utinga, Belém, PA, Brazil). Chromosomal preparations from duodenal epithelial cells and testes were subjected to Giemsa staining, C-banding and DAPI/CMA<sub>3</sub> fluorochrome staining. All specimens showed a karyotype with 13 bi-armed chromosome pairs (2n = 26). Nucleolar Organizer Regions, evidenced by CMA<sub>3</sub>, were located distally on the long arm of pair 7 (7q). DAPI+ heterochromatin was predominantly centromeric, with some small pericentromeric bands. Although the C-banding patterns of other *Bolitoglossa* species are so far unknown, cytogenetic studies conducted in other Plethodontid salamanders have demonstrated that pericentromeric heterochromatin is a useful cytological marker for identifying interspecific homeologies. Species diversification is usually accompanied by chromosomal changes. Therefore, the cytogenetic characterization of *Bolitoglossa* populations from the middle and western Brazilian Amazon Basin could identify differences which may lead to the identification of new species.

**Keywords:** Salamanders, *Bolitoglossa*, karyotypes, Brazilian Amazon, cytogenetics.

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The Plethodontid salamander genus *Bolitoglossa* is, by far, the most species-rich and diverse genus of salamanders. Its 124 assigned species represent around 20% of the living caudate species (Sessions, 2008; Rovito *et al.*, 2012; Frost, 2013; Brcko *et al.*, 2013). Widespread over the American tropics, these salamanders are found from northeastern Mexico to the South American Rainforest, and Middle America is the most species-rich region (Parra-Olea *et al.*, 2004). Cytogenetic information on *Bolitoglossa* species is scarce, being limited to 14 species (Green and Sessions, 2007). Like several other genera of the Plethodontidae family, the *Bolitoglossa* species studied show karyotypes with 2n = 26 bi-armed chromosomes and slight cytogenetic differences, basically in the location of Nucleolar Organizer Regions (NORs), with no available C-banding data (Sessions and Kezer, 1991). Based on a phylogenetic analysis of molecular data, Parra-Olea *et al.* (2004)

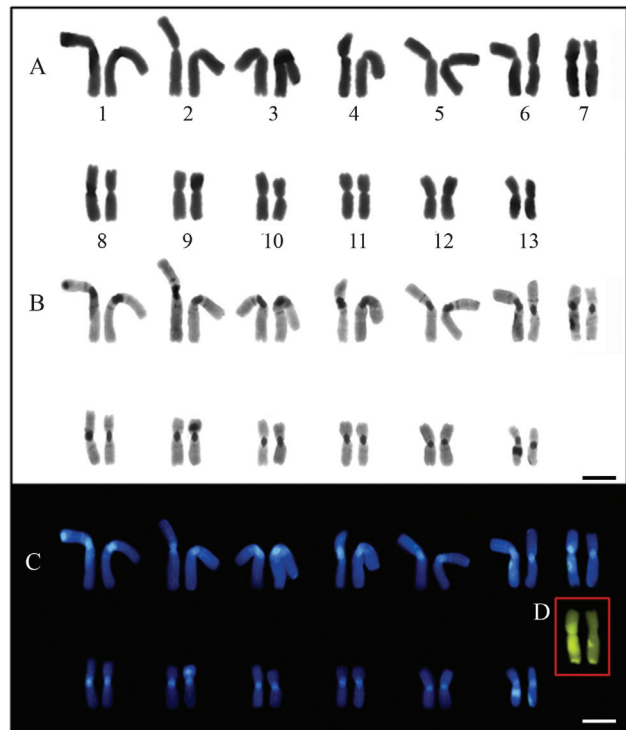
removed the Brazilian salamander populations from the synonymy of *B. altamazonica*, resurrecting *B. paraensis*, which remain as the only formally recognized species in the Brazilian Amazon Rainforest. Furthermore, based on the large genetic divergences found among the Brazilian salamander samples studied by them, these authors also suggest the existence of a species complex under this taxon name, stating the need of more detailed studies. Recently, Brcko *et al.* (2013) analyzed the external morphological characteristics of 278 *Bolitoglossa* specimens and confirmed the presence of a species complex in the Brazilian Amazon, suggesting an increase of species from one to five (*B. caldwella* sp. nov., *B. madeira* sp. nov., *B. tapajonica* sp. nov., *B. paraensis* and *B. altamazonica*). In an attempt to find cytological markers capable of identifying interspecific differences, we present here a cytogenetic analysis of *B. paraensis* specimens from a type locality, aiming to find characteristics which might complement the cytogenetic data available for the genus and contribute to its cytotaxonomy.

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Twelve individuals belonging to the species *Bolitoglossa paraensis* were cytogenetically analyzed, ten of which were males (MPEG 21316-7, 21370, 22177-8, 31654-7, 31665; PS-518) and one female (MPEG 31656). Specimens collected in the Gunma Ecologic Park (municipality of Santa Barbara, State of Pará, Brazil; 1°12'46" S/48°17'18" W) were sacrificed with 5% lidocaine, fixed in 10% formalin, stored in 70% ethanol, and deposited in the herpetological collection of the "Museu Paraense Emilio Goeldi" (MPEG) and in the herpetological collection of the Cytogenetics Laboratory at the Federal University of Pará in Belém/PA. Specimens were collected under permanent permit number IBAMA 13248-1.

Preparations of mitotic and meiotic chromosomes were obtained, respectively, from intestine fragments and testes, according to Kezer *et al.* (1980). Chromosome preparations were placed onto slides and stained with a 5% Giemsa solution with phosphate buffer. Metaphase chromosomes were treated for C-banding (Sumner, 1972), submitted to AgNO<sub>3</sub> impregnation (Howell and Black, 1980), staining with CMA<sub>3</sub>/DAPI fluorochromes (Schweizer and Ambros, 1994), and fluorescent *in situ* hybridization (FISH) (Viegas-Péquignot, 1992). Staining with CMA<sub>3</sub>/DAPI fluorochromes was done on cells previously submitted to C-banding or FISH procedures. Telomeric FISH experiments were performed with a DAKO FITC telomere PNA FISH kit (K532511-8; DAKO, Glostrup, Denmark), following the manufacturer's protocol. The karyotypes were organized using Adobe Photoshop CS5, and the chromosomes classified according to Green and Sessions (2007). Chromosome measurements and centromeric index (CI) calculation of each chromosome pair were performed in 10 metaphases, using the Micromesure v. 3.3 software (Reeves and Tear, 2000).

All *B. paraensis* specimens analyzed showed a karyotype with 13 chromosome pairs ( $2n = 26$ , NF = 52), most of them metacentric, with the exception of pairs 9 and 12, which were submetacentric (Figure 1A, Table 1). Their C-banding pattern is characterized by a large amount of heterochromatin in all centromeric regions and small pericentromeric bands on both arms of pairs 1-9 (Figure 1B). There were no differences in C-banding patterns between males and females, indicating that there are no differentiated sex chromosomes in this species. Fluorochrome heterochromatin characterization with CMA<sub>3</sub>/DAPI techniques showed only DAPI+ bands (Figure 1C). Nucleolar Organizer Regions can also be evidenced by CMA<sub>3</sub> (Green and Sessions, 2007), and in *B. paraensis* they were located distally on 7q, where the CMA<sub>3</sub>+ regions (Figure 1D) are coincident with the Ag-NORs labeling (Figure 2A-C). The meiotic cells analyzed showed 13 bivalents in diakinesis, with the same DAPI/CMA<sub>3</sub> banding pattern as the mitotic chromosomes (Figure 2B,C). FISH staining with telomere PNA probes showed a signal in the terminal regions of all



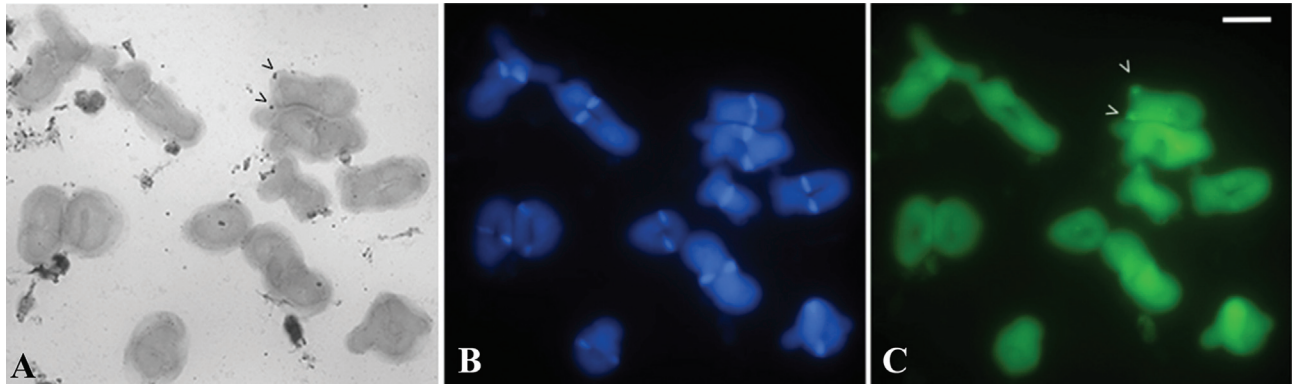
**Figure 1** - Sequential karyotypes of a male specimen of *Bolitoglossa paraensis*: A) conventional Giemsa staining, B) C-banding pattern, C) DAPI staining; D) NOR CMA<sub>3</sub>+ staining. Bar = 10  $\mu$ m.

**Table 1** - Morphometric data of *Bolitoglossa paraensis* chromosomes. (CI = centromeric index; % set = relative size; chromosome type: m = metacentric, sm = submetacentric; \* = NOR-bearing chromosome pair).

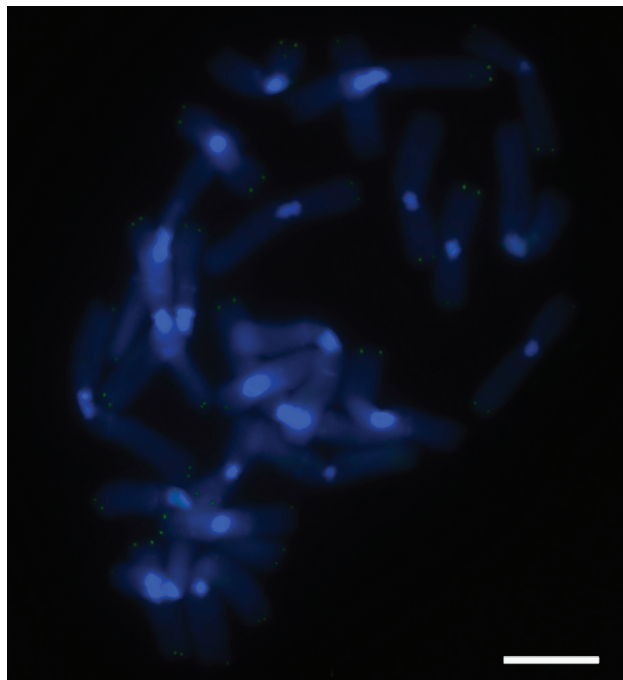
Chromosome pair	% set	CI	Type
1	11.13	0.434 $\pm$ 0.025	m
2	10.63	0.442 $\pm$ 0.021	m
3	10.23	0.451 $\pm$ 0.029	M
4	9.27	0.46 $\pm$ 0.038	m
5	8.71	0.462 $\pm$ 0.025	m
6	8.27	0.445 $\pm$ 0.037	m
7	7.1	0.375 $\pm$ 0.01	m*
8	6.56	0.455 $\pm$ 0.015	m
9	5.94	0.373 $\pm$ 0.01	sm
10	5.77	0.467 $\pm$ 0.015	m
11	5.77	0.396 $\pm$ 0.025	m
12	5.48	0.362 $\pm$ 0.015	sm
13	5.13	0.445 $\pm$ 0.02	m

chromosomes (Figure 3). Again, no heteromorphic sex chromosomes were identified.

The cytogenetic findings described here for *B. paraensis* are consistent with the  $2n$  and FN previously reported for other *Bolitoglossa* species (Green and Sessions, 2007), indicating great karyotype uniformity among species of this genus.



**Figure 2** - Diakinesis: A) Ag-NOR, B) DAPI and C) CMA<sub>3</sub> staining patterns of *Bolitoglossa paraensis*. Arrowheads indicate the locations of NORs. Bar = 10  $\mu$ m.



**Figure 3** - FISH signals obtained with telomere PNA probes in the terminal regions of all chromosomes. Bar = 10  $\mu$ m.

Sessions and Kezer (1991) analyzed the location of NORs in 26 species of Plethodontid salamanders, eight of them belonging to genus *Bolitoglossa*. These authors found 13 different NOR locations, which they classified from A to M, also discriminating primary (present on both homologous chromosomes) and secondary (present on only one homolog) NORs. In *Bolitoglossa* species, primary NORs were found at locations A (*B. adspersa*), B (*B. engelhardti* and *B. helmrichi*), and H (*B. flaviventris*, *B. subpalmata*, *B. dofleini*, *B. franklini*, and *B. rostrata*). The labeling location, relative size, and chromosome morphology of the NOR-bearing pair of *B. paraensis* resemble type H in the classification of Sessions and Kezer (1991). However, it is difficult to make comparisons, because the classification

used by those authors did not associate NOR sites to any chromosome pair.

Although the C-banding patterns of other *Bolitoglossa* species are unknown, cytogenetic studies performed in other Plethodontid salamanders have demonstrated that pericentromeric heterochromatin is a useful cytological marker for identifying interspecific homeologies (Sessions, 2008). Several hypotheses have been proposed to explain the origin of pericentromeric heterochromatin in Caudata, and their relationship with satellite DNA (stDNA) appears to be well established. According to Macgregor and Sessions (1986), the growth and dispersion of stDNA located at centromeres are involved in the formation of pericentromeric bands. Under this model, stDNA would be accumulated in the centromeres by tandem duplication mechanisms as a first step, and chromosomal rearrangements, such as inversions and/or unequal exchanges at these sites, would cause random breaks and dispersion of stDNA over the chromosome arms, thus producing the pericentromeric bands. Hence, the age of stDNA is related to its chromosomal location, because stDNA present only in closely related species, considered “new”, is found in centromeric regions, whereas “older” stDNA, shared by distant lineages, is located in interstitial or pericentromeric regions (for review, see Sessions 2008 and references therein). The available stDNA sequencing data show them to be AT-rich regions (Barsacchi-Pilone *et al.*, 1986), so it is not surprising that the heterochromatin present in *B. paraensis* was DAPI+. Detailed studies of stDNA, such as its isolation and sequencing from pericentromeric and centromeric heterochromatic regions in *B. paraensis* by cloning and fluorescent *in situ* hybridization, can provide useful information about the karyotype evolution in this genus.

Telomeres are considered a conserved sequence (TTAGGG) among invertebrate and vertebrate species (Meyne *et al.*, 1989). However, the absence of FISH hybridization signals using telomere probes in the newt *Cynops pyrrhogaster* led Murakami *et al.* (2007) to suggest that there could be differences in the telomeric repetitive



consensus sequences of Caudata species, as observed in *Drosophila* species and plants of family Alliaceae (Fuchs *et al.*, 1995; Biessmann *et al.*, 2000). The results shown here for *B. paraensis* (Figure 3), however, do not support this assumption, or at least indicate that it is not extensive to all Caudata species.

The diversity of primary and secondary NOR sites, together with the qualitative and quantitative variability of stDNA, lead us to believe that a detailed cytogenetic and molecular characterization of *Bolitoglossa* species from the middle and western Brazilian Amazon Basin should disclose useful differences, not only improving our knowledge on chromosome evolution in *Bolitoglossa* species, but also helping to identify new species of this genus.

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