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Research Article

Cytogenetic studies in six species of *Scinax* (Anura, Hylidae) clade *Scinax ruber* from northern and northeastern Brazil

Lídia Nogueira[1], Juliani Bruna Zanoni[2], Mirco Solé[3], Paulo Roberto Antunes de Mello Affonso[2], Sérgio Siqueira[2] and Iracilda Sampaio[1]

[1]*Instituto de Estudos Costeiros, Universidade Federal do Pará, Bragança, PA, Brazil.*
[2]*Departamento de Ciências Biológicas, Universidade Estadual do Sudoeste da Bahia, Jequié, BA, Brazil.*
[3]*Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Ilhéus, BA, Brazil.*

Abstract

Scinax species are still underrepresented in cytogenetic studies, mainly with respect to populations from northeastern and northern Brazil. In this study, we provide new chromosomal information on *Scinax boesemani*, *S. camposseabrai*, *S. garbei*, *S. pachycrus*, *S. trilineatus* and *S. x-signatus*, all belonging to clade *S. ruber*. They were collected at two locations in the Caatinga biome (northeastern Brazil) and at one in the Amazon (northern Brazil) biomes. Chromosomes were analyzed by conventional staining, C-banding, Ag-NOR staining, and fluorochrome staining. All species shared a modal diploid value of 2n = 24 and fundamental arm number (FN) of 48. Moreover, both chromosomal size and morphology were similar to other species in this *Scinax* clade. C-banding revealed centromeric heterochromatin in all species, along with terminal species-specific C-bands in some species. Active nucleolar organizer regions (Ag-NORs) were identified at 11q in most species, except for *S. boesemani* and *S. garbei* (Ag-NORs at interstitial region of 8q). Differing from most anurans, GC-rich regions were not restricted to NORs, but also coincident with some centromeric and terminal C-bands. These data contribute to the cytotaxonomy of *Scinax* by providing chromosomal markers and demonstrating the occurrence of microstructural rearrangements and inversions on chromosomal evolution of *Scinax*.

Keywords: amphibians, chromosomes, fluorochromes, heterochromatin, rDNA.

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Introduction

The genus *Scinax* (Anura: Hylidae) comprises 113 species widespread from southern Mexico to Argentina, Uruguay, Trinidad and Tobago and Santa Lucia islands (Frost, 2014). Based on molecular markers, morphology, osteology, myology, reproductive biology and chromosomes, this genus was divided into two clades: *S. catharinae* (which includes two species groups - *S. catharinae* and *S. perpusillus*) and *S. ruber* (species groups *S. rostratus*, *S. uruguayus* and the remaining species) (Faivovich, 2002; Faivovich *et al.*, 2005). The clade *S. ruber* comprises nearly 65 species (Frost, 2014) widespread over open ares of tropical and subtropical regions (Faivovich, 2002).

So far, karyotypes are known for only 39 species of *Scinax* and only few studies included banding methods, such as Ag-NOR staining, C-banding, BrdU, fluorochrome staining, and *in situ* hybridization (Pombal *et al.*, 1995; Kasahara *et al.*, 2003; Nunes and Fagundes, 2008; Cardozo *et al.*, 2011). These cytogenetic studies showed that all species in this genus share a modal diploid value of 2n = 24.

In spite of numerical conservativeness in diploid number, the two clades can be differentiated by cytogenetic analyses. In the clade *S.* catharinae, the first and second chromosomal pairs are submetacentric and NORs are usually located on the sixth pair, while, in the clade *S. ruber*, the first and second pairs are metacentric and NORs have been frequentle been detected on pair 11 (Cardozo *et al.*, 2011).

Since *Scinax* species and populations are still underrepresented in terms of cytogenetic data, and species-specific or population differences might be overlooked, we herein provide the first chromosomal information about samples of the following species within the clade *S. ruber*: *S. boesemani*, *S. camposseabrai*, *S.garbei*, *S. pachycrus*, *S. trilineatus* and *S. x-signatus*, collected in northern and northeastern Brazil.

Sende correspondence to Lídia Nogueira. Instituto de Estudos Costeiros, Universidade Federal do Pará, Alameda Leandro Ribeiro s/n, Aldeia, 68600-000 Bragança, PA, Brazil. E-mail: lidia.nogueira@yahoo.com.br.

Materials and Methods

Twenty one individuals of six species of *Scinax* from the clade *S. ruber* collected in distinct localities in northern (Bragança, PA) and northeastern (Maracás and Jequié, BA) Brazil were cytogenetically analyzed (Table 1). The specimens were deposited in the Herpetological Collection at Universidade Estadual de Santa Cruz (UESC). Metaphasic chromosomes were obtained from cells of intestinal epithelium as described by Schmid (1978). Slides were stained with 10% Giemsa solution in phosphate buffer 0.1 M (pH 6.8) for about 10 min, washed in distilled water and air dried. The best metaphases were selected and photographed for karyotyping and chromosomal measurements. Active nucleolar organizer regions (Ag-NORs) were located by silver nitrate staining (Howell and Black, 1980), and heterochromatin was detected by C-banding according to Sumner (1972), modified by Siqueira *et al.* (2008). Fluorochrome staining using chromomycin A_3 (CMA_3) and 4',6-diamidino-2-phenylindole (DAPI) was performed to detect GC- and AT-rich regions, respectively (Schmid, 1980). All images were captured by epifluorescence microscopy (Olympus BX-51 equipped with digital image software Image Pro-Plus version 6.2). The chromosomes were classified based on centromere position as: m (metacentric), sm (submetacentric) and st (subtelocentric), according to Green and Sessions (1991).

Results

All analyzed species presented a modal diploid number of 2n = 24 and FN = 48. The chromosomal morphology was similar in nearly all species, comprising eight metacentric pairs (1, 2, 7, 8, 9, 10, 11, and 12) and four submetacentric ones (3, 4, 5, and 6). However, pair 7 in *S. boesemani* was submetacentric and pairs 11 and 12 in *S. x-signatus* were submetacentric (Figure 1 and Table 2).

Silver nitrate staining showed that NORs were usually located on the long arms of pair 11 (11q), either at an interstitial position (*S. campossabrai*, *S. trilineatus* and *S. x-signatus*) or in the terminal region (*S. pachycrus*). NORs were also detected at interstitial positions on 8q in *S. boesemani* and *S. garbei*. It is worthy of note that the Ag-NORs were coincident with secondary constrictions observed in Giemsa-stained karyotypes of *S. boesemani*, *S. campossabrai* and *S. x- signatus* (Figure 1).

Heterochromatin was visualized at centromeric regions of most chromosomes in all species (Figure 2). In addition, some species-specific C-bands represented heterochromatin segments at telomeric regions of pairs 3 and 4 in *S. boesemani* (Figure 2A). *S. campossabrai* was characterized by the presence of exclusively centromeric C-bands in all chromosomes (Figure 2B). *S. garbei* and *S. pachycrus* presented terminal C-bands on arms of pairs 1 and 2 (Figure 2C) and pairs 1, 2, 3, 4, 5, 6, 7, and 9 (Figure 2D), respectively. *S. x-signatus* also presented heterochromatin at terminal regions of pair 4 (Figure 2E). Heterochromatic blocks interspersed with NORs were observed in *S. garbei*, *S. pachycrus* and *S. x-signatus* (Figure 2C,D,E).

Fluorochrome staining was successful in *S. boesemani*, *S. pachycrus* and *S. x-signatus*. In these species, GC-rich segments (CMA_3^+ and $DAPI^-$) were coincident with NORs (Figure 3). In addition, all centromeric regions of *S. boesemani* were positively stained, following the C-banding pattern (Figure 3A), while fluorescent signals were observed in several centromeres and terminal regions in 3q and 5 of *S. pachycrus* (Figure 3B) and in most centromeric regions of *S. x-signatus* (Figure 3C).

Interestingly, no chromosomal differences could be found among samples of *S. x-signatus*, in spite of a high degree of isolation by distance of collection sites from distinct biomes (Amazon in Bragança-PA and caatinga in Jequié, BA).

Discussion

We herein provide the first karyotypic data about *S. boesemani*, *S. camposseabrai*, *S. garbei*, *S. pachycrus*, *S. trilineatus* and *S. x-signatus*. The present results confirm the modal diploid values of 24 chromosomes and FN = 48 proposed for *Scinax* and most species in Hylinae (Duellman, 2001; Faivovich, 2002; Kasahara *et al.*, 2003; Cardozo *et al.*, 2011).

In general, the karyotype formulae are similar among species of the *S. ruber* clade. Nonetheless, we noted varia-

Table 1 - Analyzed species, number and sex of specimens (N) and collection site.

Species	Voucher identification	N	Locality
S. boesemani	MZUESC12012(M)	1	Bragança - PA
S. camposseabrai	MZUESC11022(M), MZUESC11023(M), MZUESC11024(M), MZUESC11025 (F)	4	Maracás - BA
S. garbei	MZUESC12006(M), MZUESC12007(F), MZUESC12008(F), MZUESC12009(F)	4	Bragança - PA
S. pachycrus	MZUESC 11008(NI), MZUESC11035 (F), MZUESC11036(M) MZUESC11037(M)	4	Jequié - BA
S. trilineatus	MZUESC12013 (M)	1	Bragança - PA
S. x-signatus	MZUESC11001(M), MZUESC11010(F), MZUESC11018(NI)	3	Jequié - BA
	MZUESC12001 (M), MZUESC12002 (F), MZUESC12003 (F), MZUESC12004 (F)	4	Bragança - PA

M: male. F: female.

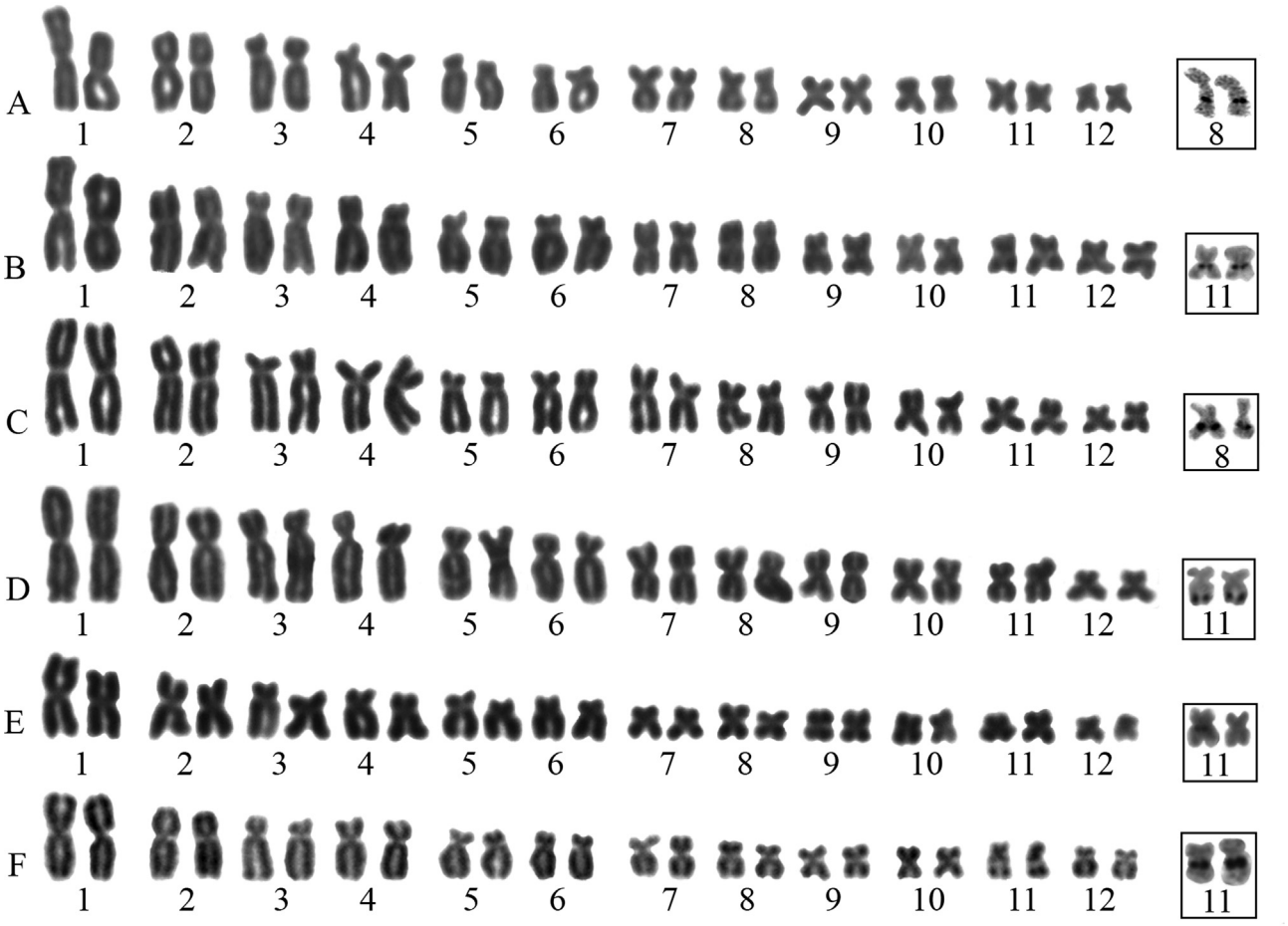

Figure 1 - Giemsa stained karyotypes of *Scinax* species: (A) *S. boesemani*, (B) *S. camposseabrai*, (C) *S. garbei*, (D) *S. pachycrus*, (E) *S. trilineatus* and (F) *S. x-signatus*. The NOR-bearing chromosomes after silver nitrate staining are highlighted in boxes.

tion in morphology has been reported for pairs 7, 11 and 12 from submetacentric to metacentric (Cardozo *et al.*, 2011), as well as in pair 7 of *S. boesemani* (submetacentric) and pairs 11 and 12 (submetacentric) in *S. x-signatus* (Figure 1 and Table 1). These results suggest that microrearrangements such as deletion/duplication of chromosomal segments or inversions may have taken place independently in the abovementioned species.

The results also should prove to be useful for cytotaxonomy and systematics of *Scinax* species. Brusquetti *et al.* (2014) revised *S. fuscomarginatus* and related species, suggesting that *S. trilineatus* is a synonym for *S. fuscomarginatus*, since the genetic differences in 16S and COI sequences could be associated with geographic distance among populations. Moreover, these authors found no signification differences in vocalization and morphometric patterns between the two putative species. On the other hand, we observed chromosomal differences between *S. trilineatus* (8 metacentric and 4 submetacentric pairs) (Table 2), and *S. fuscomarginatus* (6 metacentric and 6 submetacentric pairs) (Cardozo *et al.*, 2011). Thus, the cytogenetic data support the separation of both cryptic species based on structural rearrangements, such as inversions.

The nucleolar organizer regions are also considered efficient markers for the identification of new species (Bruschi *et al.*, 2012) or cryptic forms (Siqueira *et al.*, 2008), as well as to establish infragenus subdivisions (Raber and Carvalho, 2004; Cardozo *et al.*, 2011). In this sense, the Ag-NOR located at 11q is regarded as an ancestral condition in *Scinax* (Cardozo *et al.*, 2011). In the present study, two species of the Amazon region (*S. boesemani* and *S. garbei*) showed GC-rich NORs on 8q, as confirmed by Ag-NOR, C-banding and fluorochrome staining, suggesting a derived feature. An additional nucleolar region in pair 8 was also reported by Cardozo *et al.* (2011) in another species of *S. ruber* clade, *Scinax hayii*. The two species did not come out as closely related in the phylogenetic analysis carried out by Wiens *et al.* (2010) and, hence, the changes in Ag-NOR location should be regarded as independent events leading to convergent distribution pattern of NORs.

A single NOR-bearing chromosome was visualized in *Scinax trilineatus*, and this may be related to the small size of ribosomal cistrons in one homologous or an inactive

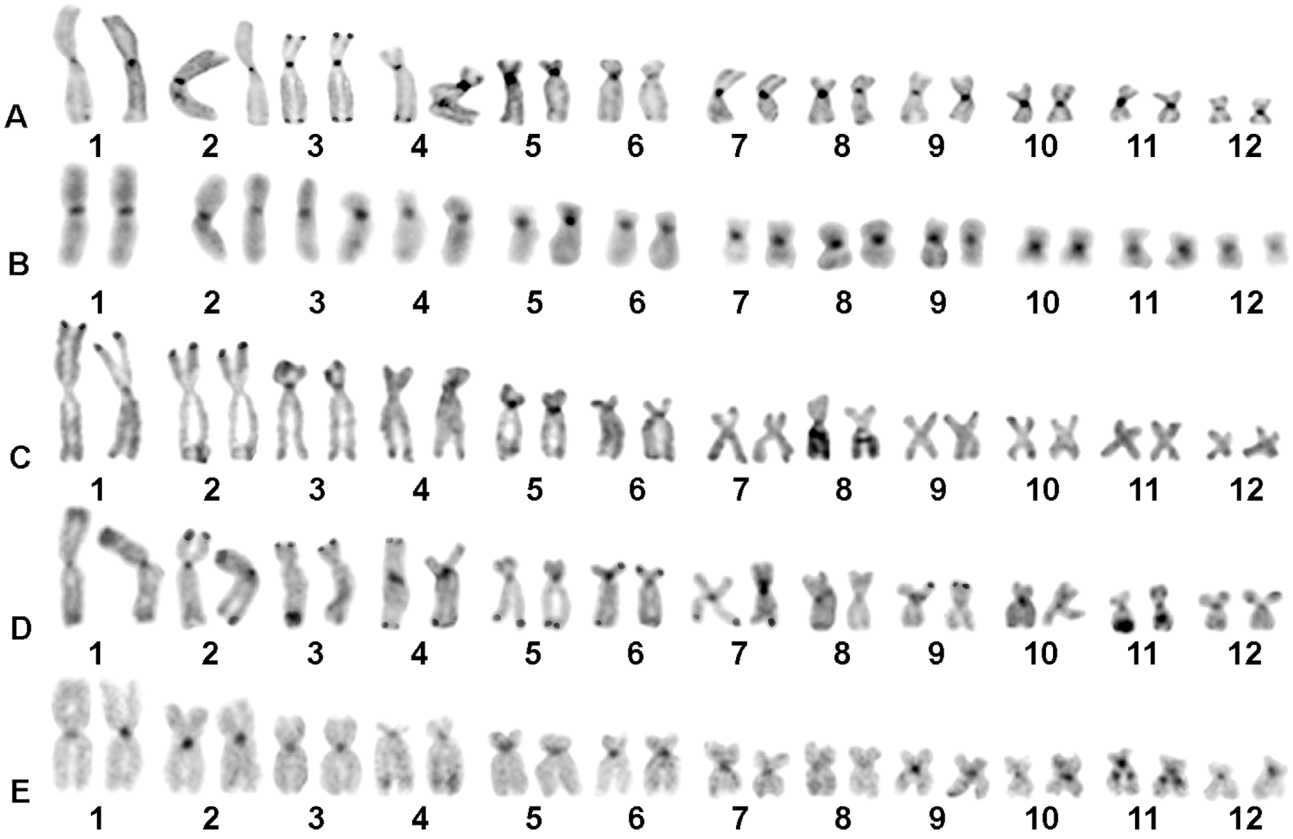

Figure 2 - C-banded karyotypes of *Scinax* species: (A) *S. boesemani*, (B) *S. camposseabrai*, (C) *S. garbei*, (D) *S. pachycrus* and (E) *S. x-signatus*.

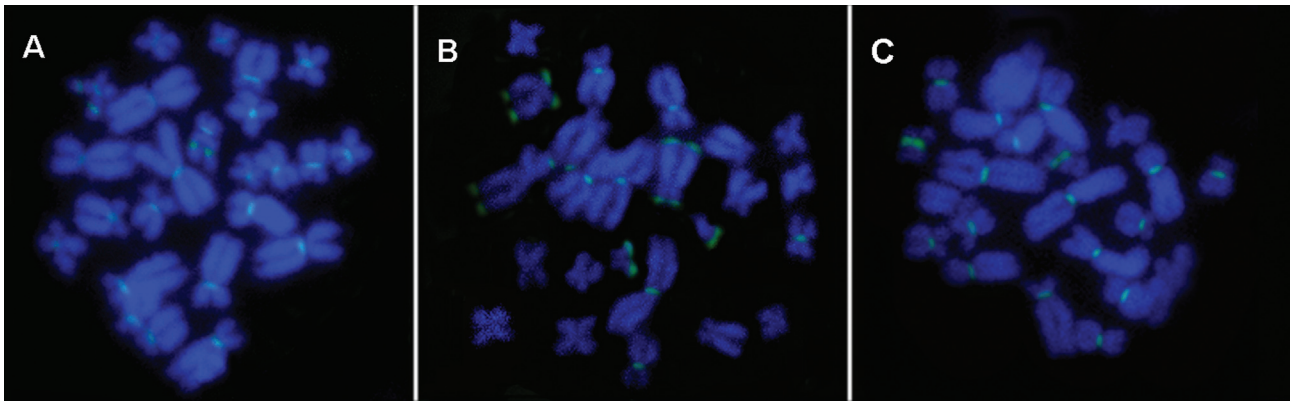

Figure 3 - Mitotic metaphases after fluorochrome staining showing CMA_3^+ signals in green and chromosomes counterstained with DAPI (blue): (A) *S. boesemani*, (B) *S. pachycrus* and (C) *S. x-signatus*.

site, since silver nitrate staining can only reveal previously active NORs (Schmid, 1978; Kasahara, 2009). This Ag-NOR pattern is similar to that reported in *Scinax alter* and *Scinax hiemalis* (Cardozo *et al.*, 2011).

Heterochromatin at centromeric region, as detected in this study, has been commonly reported in species of the *S. ruber* clade, such as *S. acuminatus*, *S. alter*, *S. curicica*, *S. duartei*, *S. eurydice*, *S. fuscovarius*, *S. granulatus*, *S. hayii*, *S. nasicus*, *S. perereca*, *S. similis* and *S. squalirostris* (Kasahara *et al.*, 2003; Cardozo *et al.*, 2011). Another common feature observed in the analyzed species, except for *S. camposseabrai*, was the presence of heterochromatin interspersed with NORs, as described in *S. argyreornatus*, *S. curicica*, *S. eurydice*, *S. hiemalis*, *S. similis* and *S. squalirostris* (Cardozo *et al.*, 2011).

A distinguishable C-banding pattern was observed in *S. boesemani*, *S. garbei*, *S. pachycrus* and *S. x-signatus*, as these species presented additional heterochromatic segments at terminal regions of some chromosomes. Even though terminal C-bands have been reported in other hylids

Table 2 - Chromosomal measurements of analyzed species (RL - relative length; CI = centromeric index and CT = chromosomal type) according to Green and Sessions (1991).

Species		Chromosomal pairs											
		1	2	3	4	5	6	7	8	9	10	11	12
S. boesemani	RL	12.34	10.71	10.05	8.90	7.51	6.21	5.82	5.63	5.62	4.65	4.21	3.84
	CI	0.49	0.41	0.29	0.31	0.25	0.32	0.30	0.42	0.47	0.45	0.47	0.46
	CT	M	M	SM	SM	SM	SM	SM	M	M	M	M	M
S. camposseabrai	RL	14.42	11.58	10.51	9.67	7.45	7.25	6.61	6.30	5.14	4.71	4.52	4.08
	CI	0.46	0.38	0.28	0.31	0.25	0.28	0.41	0.41	0.44	0.46	0.45	0.49
	CT	M	M	SM	SM	SM	SM	M	M	M	M	M	M
S. garbei	RL	16.17	13.57	11.51	10.55	8.96	8.82	8.81	7.02	6.85	5.56	5.47	3.61
	CI	0.46	0.41	0.29	0.33	0.25	0.3	0.38	0.46	0.39	0.48	0.46	0.47
	CT	M	M	SM	SM	SM	SM	M	M	M	M	M	M
S. pachycrus	RL	11.33	9.48	8.85	8.04	6.66	6.61	5.11	4.80	3.88	3.78	3.55	2.60
	CI	0.46	0.39	0.25	0.26	0.26	0.28	0.42	0.45	0.45	0.47	0.4	0.4
	CT	M	M	SM	SM	SM	SM	M	M	M	M	M	M
S. trilineatus	RL	9.76	8.36	7.18	6.76	5.68	5.29	4.24	4.02	3.63	3.43	3.41	3.02
	CI	0.47	0.43	0.31	0.3	0.32	0.28	0.38	0.45	0.44	0.47	0.46	0.49
	CT	M	M	SM	SM	SM	SM	M	M	M	M	M	M
S. x-signatus	RL	11.77	9.26	8.50	8.41	6.76	6.45	5.45	4.80	4.11	4.06	3.87	3.00
	CI	0.48	0.41	0.27	0.35	0.27	0.27	0.41	0.38	0.47	0.42	0.28	0.3
	CT	M	M	SM	SM	SM	SM	M	M	M	M	SM	SM

(Busin *et al.*, 2006; Kasahara *et al.*, 2003; Gruber *et al.*, 2012), this is the first description in *Scinax*. Therefore, C-banding patterns can be potentially used to identify species in this genus, and more studies based on this technique should be performed within *Scinax*.

Fluorochrome staining in amphibians usually identify GC-rich regions associated with NORs and more occasionally C-bands (Ananias *et al.*, 2007; Campos *et al.*, 2008). Indeed, NORs and several centromeric regions in the species anayzed herein were positively stained by CMA_3. In the case of *S. pachycrus*, C-bands at telomeric regions of pairs 3 and 5 were also GC-rich, indicating a homogeneous base composition of heterochromatin. However, there are only few studies on CMA_3/DAPI staining in *Scinax* to provide a reliable scenario of heterochromatin composition.

In conclusion, the present work increased the number of karyotyped species in *Scinax* by providing the first chromosomal data in *S. boesemani*, *S. camposseabrai*, *S. garbei*, *S. pachycrus*, *S. trilineatus* and *S. x-signatus*. Comparisons with previous reports suggest that chromosomal evolution in *Scinax* (*S. ruber* clade) may have been mainly driven by microstructural rearrangements and inversions associated with stable karyotype fomulae, particularly among species within a same clade (*e.g. S. camposseabrai* and *S. pachycrus*). As indicated by Cardozo *et al.* (2011), the chromosomal inversions in the clade *S. ruber* are restricted to pairs 7, 11 and 12. Therefore, banding techniques should prove essential to provide cytotaxonomic markers, as observed in relation to NOR location of the Amazon species and heterochromatin distribution.

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