Cytogenet Genome Res 103:155–162 (2003)
DOI: 10.1159/000076304

Cytogenetic and Genome Research

Comparative cytogenetic analysis on four tree frog species (Anura, Hylidae, Hylinae) from Brazil

S. Kasahara,[a] A.P. Zampieri Silva,[a] S.L. Gruber[a] and C.F.B. Haddad[b]

[a] Departamento de Biologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro, SP; [b] Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro, SP (Brasil)

Abstract. A comparative cytogenetic analysis was carried out on four Hylinae tree frogs from Brazil (*Aparasphenodon brunoi, Corythomantis greeningi, Osteocephalus langsdorffii*, and *Scinax fuscovarius*) using Giemsa staining, BrdU replication banding, Ag-NOR staining, C-banding, DAPI and CMA_3 fluorochrome staining, and fluorescence in situ hybridization (FISH) with an rDNA probe. All the species share closely similar 2n = 24 karyotypes, almost indistinguishable by standard staining. The technique of BrdU incorporation allowed the identification of each pair of homologs and the establishment of extensive homeology for the great majority of the chromosomes, mainly of *A. brunoi, C. greeningi,* and *O. langsdorffii*. Despite highly conserved replication banding patterns, the use of the other banding techniques disclosed some minor differences, which reinforces the importance of extensive cytogenetic analyses for the karyotypic characterization of Anuran species. The present cytogenetic data confirm the closer proximity of *A. brunoi, C. greeningi,* and *O. langsdorffii*, whereas *S. fuscovarius* is phylogenetically more distant.

Amphibians belonging to the order Anura have been frequently described as a karyotypically conservative group. In fact, there are a number of cases, either at the genus or at the family level, in which several species share almost indistinguishable karyotypes by standard staining. The differential staining methodologies developed since the 1970's have provided a more detailed characterization of the karyotypes of anuran species (Schmid et al., 1990). As a consequence, the idea of karyotypic uniformity can no longer be extensively supported and for this reason analyses performed with banding techniques are an important tool for establishing patterns of chromosome evolution and phylogenetic relationships among anuran species.

The family Hylidae comprises five subfamilies, of which Hylinae is the most diversified. The systematics of this subfamily has been subject to several revisions in the last years and, according to Frost (2002), it includes 26 genera and 551 species. Except for *Hyla* with 335 representatives and *Scinax* with 84, the remaining genera, *i.e. Aparasphenodon* (three species), *Corythomantis* (one species), and *Osteocephalus* (17 species), include a small number of species. Reviews published in 1990 (King, 1990; Kuramoto, 1990) have shown that about 140 species of Hylinae have been chromosomally examined so far. For the majority 2n = 24 karyotypes are predominant and these were studied in general only after standard staining.

Taking into account that few Hylinae species from Brazil have been karyotyped, we performed comparative cytogenetic analysis on *Aparasphenodon brunoi, Corythomantis greeningi, Osteocephalus langsdorffii*, and *Scinax fuscovarius*, based on conventional Giemsa technique and various differential staining procedures, as BrdU replication banding, Ag-NOR staining, C-banding, AT-specific DAPI and GC-specific CMA_3 fluorochromes counterstained with DA, and fluorescence in situ hybridization (FISH) with an rDNA probe.

The standard karyotypes of both *A. brunoi* and *O. langsdorffii* were described previously by Foresti (1972) and also by

Supported by FAPESP and CNPq, and PROPP-FUNDUNESP.

Received 13 June 2003; manuscript accepted 21 August 2003.

Request reprints from Sanae Kasahara, Departamento de Biologia
Instituto de Biociências. Universidade Estadual Paulista (UNESP), CP 199
13506-900 Rio Claro, SP (Brasil)
telephone: 55-19-3526-4150; fax: 55-19-3526-4136
e-mail: kasahara@rc.unesp.br

0301–0171/03/1032–0155$19.50/0

Accessible online at:
www.karger.com/cgr

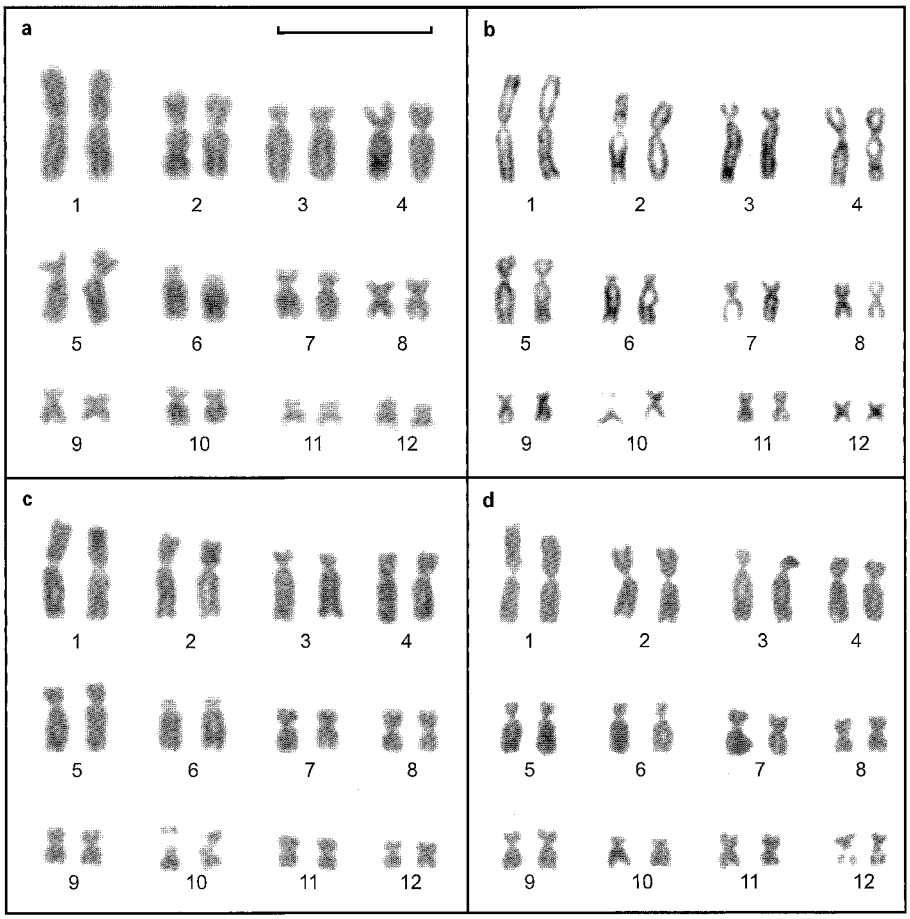

Fig. 1. Conventionally Giemsa-stained karyotypes from (**a**) a male *Aparasphenodon brunoi*, (**b**) female *Corythomantis greeningi*, (**c**) male *Osteocephalus langsdorffii*, and (**d**) female *Scinax fuscovarius*. Bar represents 10 μm.

Bogart (1973) who referred to *A. brunoi* as *Corythomanthis brunoi* in his study. According to the compilation made by King (1990), *S. fuscovarius (Ololygon fuscovaria)* from Central America was analyzed by C-banding and by Ag-NOR staining and the corresponding ideogram was reported by Anderson (1991). More recently, Baldissera et al. (1993) described the conventionally stained karyotype and the Ag-NOR localization in specimens of *S. fuscovarius (Hyla fuscovaria)* from Brazil, but no other banding data have been obtained so far. Representatives of *C. greeningi* have never been karyotyped before.

Aparasphenodon brunoi, C. greeningi, and *O. langsdorffii* were selected in the present study because they are considered related species, rarely found in Brazilian fauna, belonging to less diversified Hylinae genera, among which there are doubts about their systematic relationships. *S. fuscovarius*, a very common Hylinae in the southeastern region, was taken as an outgroup, in order to provide more reliability in our cytogenetic comparison within a group which is supposed to share a common ancestor.

Materials and methods

The sample of Hylinae tree frogs comprised two males of *Aparasphenodon brunoi* from Aracruz, State of Espírito Santo; two females of *Corythomantis greeningi* from Piranhas, State of Alagoas; two males of *Osteocephalus langsdorffii*, from Rio Claro and Ubatuba, State of São Paulo; five males, four females, and two juveniles of *Scinax fuscovarius* from Rio Claro (four males, four females, and one juvenile), Santa Maria da Serra (one male), and Itatiba (one juvenile), State of São Paulo. Alagoas is in northeastern Brazil, whereas Espírito Santo and São Paulo are in the southeastern region. The voucher specimens are deposited in the amphibian collection of the Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro, State of São Paulo, with the registration numbers CFBH 5918-5919 *(A. brunoi)*, A354, A360 *(C. greeningi)*, CFBH 2982, CFBH 5920 *(O. langsdorffii)*, and CFBH 3984, CFBH 5921-5924, CFBH 5925-5928, CFBH 5930-5931 *(S. fuscovarius)*.

Chromosome spreads were obtained from blood cells cultured according to Kasahara et al. (1998). 5-bromodeoxyuridine (BrdU) and 5-fluorodeoxyuridine (FudR) were added to lymphocyte cultures prior to colchicine treatment in order to obtain replication banding patterns. For some specimens of *S. fuscovarius*, direct chromosome preparations from bone marrow and liver were obtained after in vivo colchicine treatment (Baldissera et al., 1993). In these cases, in vivo BrdU/FudR treatment was performed (Silva et al., 2000). Meiotic chromosome preparations were also obtained from the testes of *A. brunoi, O. langsdorffii,* and *S. fuscovarius* males.

Conventional staining was performed with Giemsa diluted in phosphate-buffered saline, pH 6.8. Replication banding was achieved by FPG staining as described by Dutrillaux and Couturier (1981). C-banding and Ag-NOR staining were carried out according to the methods of Sumner (1972) and Howell and Black (1980), respectively. Fluorescent staining was obtained with AT-specific 4′,6-diamidino-2-phenylindole (DAPI) and GC-specific chromomycin A_3 (CMA_3), both combined with the counterstain distamycin A (DA) by the method of Schweizer (1980). Fluorescence in situ hybridization (FISH) with an rDNA probe (Meunier-Rotival et al., 1979) was performed following the method of Viegas-Péquignot (1992).

Metaphases of each species were selected for chromosome measurements in order to determine the relative length and centromeric ratio. The chromosome morphology followed the nomenclature of Green and Sessions (1991).

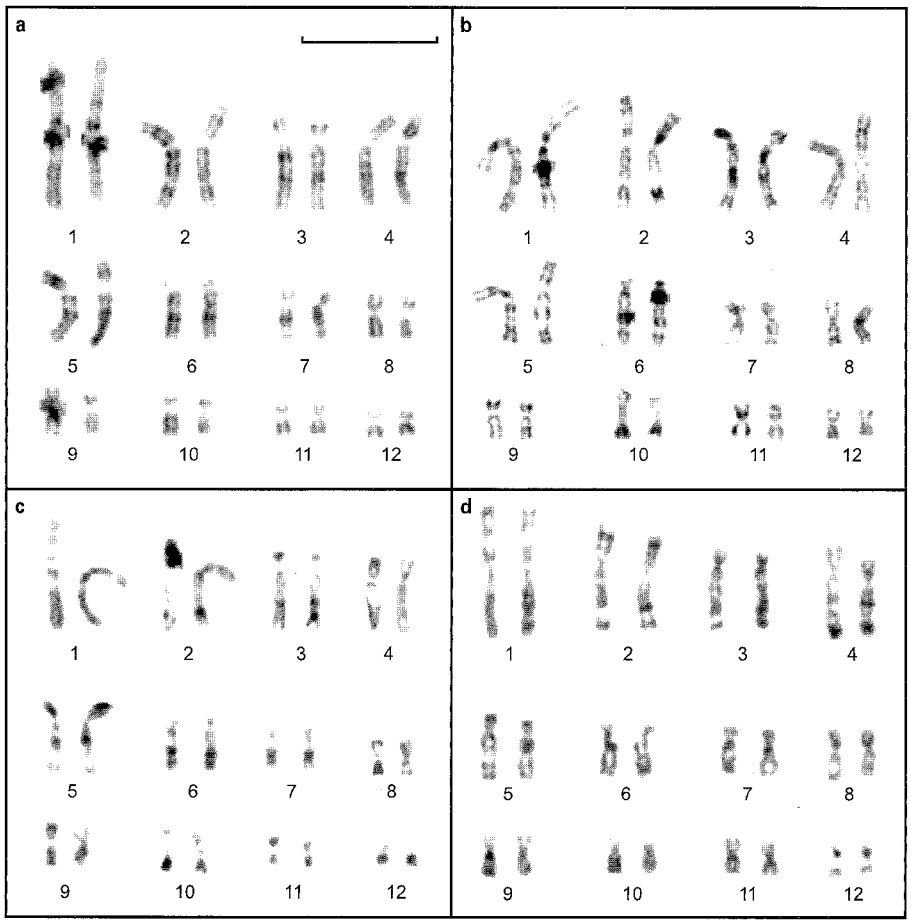

Fig. 2. BrdU replication banding patterns in karytoypes from (**a**) a male *Aparasphenodon brunoi*, (**b**) female *Corythomantis greeningi*, (**c**) male *Osteocephalus langsdorffii*, and (**d**) juvenile *Scinax fuscovarius*. Bar represents 10 μm.

Table 1. Relative length (RL), centromeric ratio (CR) and nomenclature for centromeric position (CP) on mitotic chromosomes according to Green and Sessions (1991)

Species		Chromosome No.											
		1	2	3	4	5	6	7	8	9	10	11	12
Aparasphenodon brunoi	RL	17.73	13.20	11.84	11.77	10.74	7.50	6.27	5.24	4.72	4.46	3.62	2.84
	CR	1.09	1.61	3.81	1.98	1.96	4.80	2.73	1.45	2.17	2.0	1.66	1.75
	CP[a]	m	m	st	sm	sm	st	sm	m	sm	sm	m	sm
Corythomantis greeningi	RL	17.50	15.98	11.97	11.11	9.53	7.63	6.18	4.80	4.34	4.34	3.48	3.09
	CR	1.19	1.05	4.05	1.91	2.22	4.80	2.61	1.21	2.14	1.53	1.65	1.23
	CP[a]	m	m	st	sm	sm	st	sm	m	sm	m	m	m
Osteocephalus langsdorffii	RL	16.53	13.10	11.33	11.33	10.91	7.58	6.47	5.96	5.02	4.77	3.92	2.98
	CR	1.25	1.75	3.12	2.32	2.52	5.84	1.53	1.33	1.26	1.43	1.00	1.33
	CP[a]	m	sm	st	sm	sm	st	m	m	m	m	m	m
Scinax fuscovarius	RL	18.42	13.01	10.39	9.51	8.12	7.51	7.24	5.93	5.58	4.89	4.80	4.54
	CR	1.00	1.66	3.57	2.02	3.04	2.73	1.76	1.06	1.46	2.50	1.20	2.25
	CP[a]	m	m	st	sm	st	sm	sm	m	m	sm	m	sm

[a] m = metacentric; sm = submetacentric; st = subtelocentric.

Results

All four Hylinae species, *A. brunoi, C. greeningi, O. langsdorffii*, and *S. fuscovarius*, have diploid chromosome numbers of 2n = 24 and similar conventionally stained karyotypes, with some differences in the relative length and chromosome morphology (Fig. 1a–d; Table 1). Pair 10 is a marker in *A. brunoi, C. greeningi*, and *O. langsdorffii*, bearing a secondary constriction not always visualized in both homologs. In *A. brunoi*, the secondary constriction is hardly seen at the distal end of the

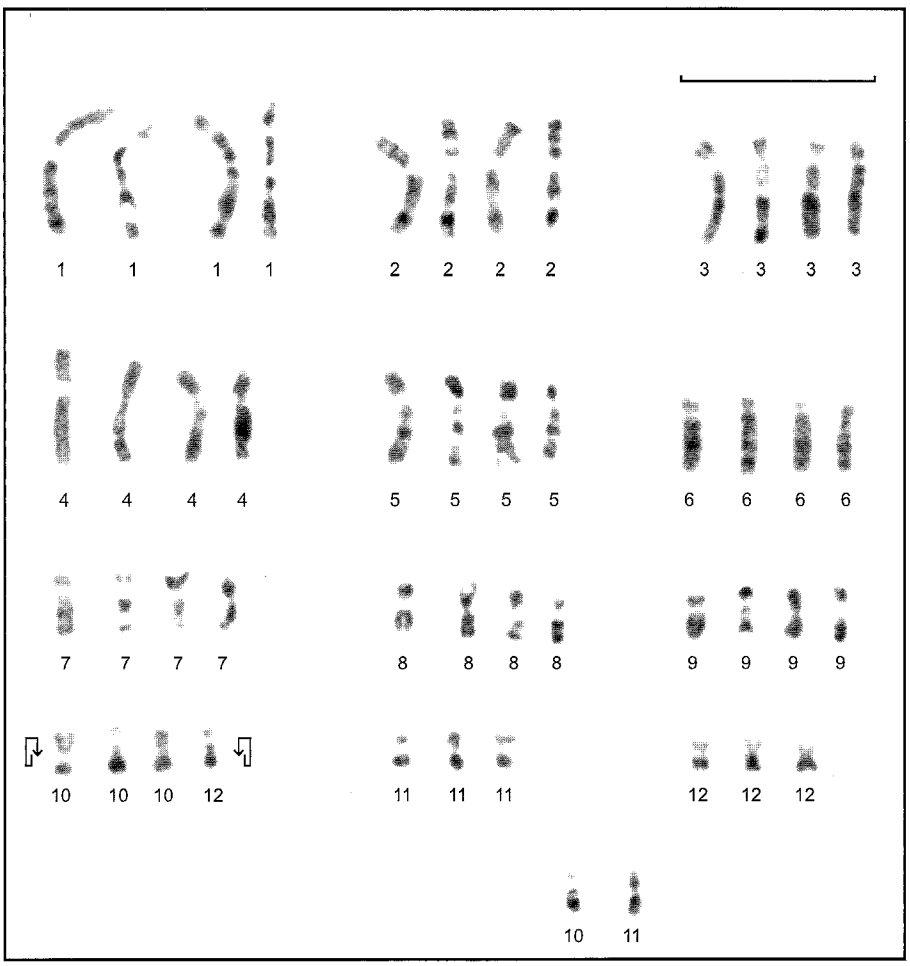

Fig. 3. Composite partial karyotypes of the four tree frog species after BrdU replication banding. From left to right, chromosomes of *Aparasphenodon brunoi*, *Corythomantis greeningi*, *Osteocephalus langsdorffii*, and *Scinax fuscovarius*. Chromosomes 10 and 11 of *Scinax fuscovarius* have no counterpart. Bar represents 10 μm.

long arms and could be documented only in overexposed photograph copies (Fig. 4a). In the other two species, the secondary constriction appears interstitially in the short arms of one or both chromosomes 10 (Figs. 1b, c, 4e, i). In *S. fuscovarius*, pair 12 is the marker, with the secondary constriction at the interstitial region of the long arms (Figs. 1d, 4k). The size of this constriction is variable, being occasionally larger in one of the homologs.

BrdU replication banding allowed the precise pairing of the chromosomes in *A. brunoi, C. greeningi, O. langsdorffii*, and *S. fuscovarius* (Fig. 2a–d). In Fig. 3, the haploid sets of each species were arranged side by side to allow a more detailed comparison. There is a close correspondence in the banding patterns of the first six chromosomes of each species, but chromosome 5 in *S. fuscovarius* has smaller short arms. With the exception of chromosomes 10 and 11 of *S. fuscovarius,* which were found to have no counterpart, the remaining small-sized chromosomes are also apparently homeologous in the four species. The NOR-bearing chromosomes 10 in *C. greeningi* and *O. langsdorffii* have a similar banding pattern to chromosomes 10 in *A. brunoi* and 12 in *S. fuscovarius*, considering these chromosomes in an inverted position.

Ag-NORs are found at the sites of the secondary constrictions (Fig. 4b–d, f–h, j, l, m). Ag-NORs are frequently heteromorphic and in some metaphases of *C. greeningi*, they appeared as tandem duplicated blocks (Fig. 4g, h). In this species and in *A. brunoi*, metaphases with a unique Ag-NOR were also observed (Fig. 4d, h).

Figure 5a–d shows the C-banded karyotypes of *A. brunoi, C. greeningi, O. langsdorffii*, and *S. fuscovarius*, respectively. They have positively stained heterochromatin distributed at the centromeric region, but *O. langsdorffii* also exhibited positive staining in the telomeric region of some chromosomes. An interstitial C-band was also observed on the long arms of chromosome pair 10 in *C. greeningi*, near the centromere (Figs. 4n, o, 5b), and distally on the long arms of chromosome pair 5 in *O. langsdorffii* (Figs. 4p, 5c). Faint interstitial bands were sporadically noticed, as those on the short arms of chromosomes 4 in *A. brunoi* and *C. greeningi*. Depending on the metaphases, there was C-positive staining at the NOR site itself or in its adjacent areas (Fig. 5c) in all four species.

The AT-specific fluorochrome DAPI counterstained with DA produced a uniform staining with no particular bright region in the chromosomes of the four species. However, in *A. brunoi* DAPI-negative fluorescence was observed in the centromeric region and interstitially on the long arms of the homologs of pair 5 (Fig. 6a). These DAPI-negative regions, on the other hand, exhibit a very brilliant fluorescence after staining

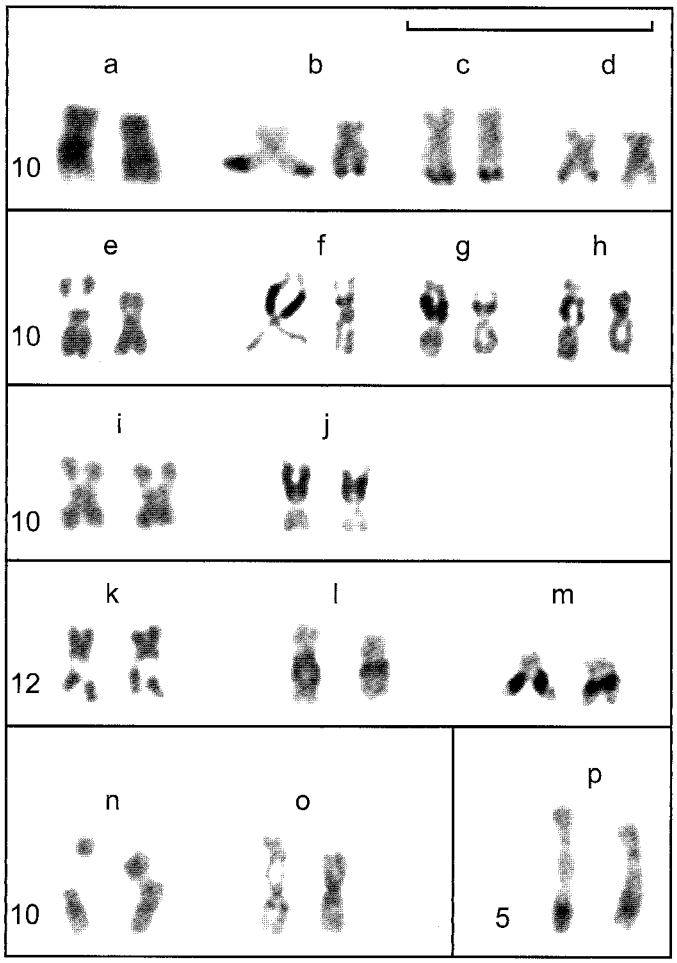

Fig. 4. Conventional Giemsa staining (**a**, **e**, **k**), Ag-NOR staining (**b–d**, **f–h**, **j**, **l**, **m**), and C-banding (**n–p**) of chromosomes 10 of *Aparasphenodon brunoi* (**a–d**), chromosomes 10 of *Corythomantis greeningi* (**e–h**, **n**, **o**), chromosomes 10 (**i**, **j**) and 5 (**p**) of *Osteocephalus langsdorffii*, and chromosomes 12 (**k–m**) of *Scinax fuscovarius*. Note unique Ag-NORs in **d** and **h**. Bar represents 10 μm.

with the GC-specific fluorochrome CMA_3 and DA (Fig. 6b). In *C. greeningi*, *O. langsdorffii*, and *S. fuscovarius*, this staining technique produced bright fluorescence in the NOR sites (Fig. 6c–f), although in most of the metaphases of the former species one of the NOR-bearing chromosomes showed fluorescence in the areas adjacent to the secondary constriction (Fig. 6c). *C. greeningi* also showed a slightly bright fluorescence in the centromeric region of the chromosomes with DA/CMA_3.

In situ hybridization (FISH) with an rDNA probe confirmed the occurrence of the nucleolus organizer sequences at the distal end of the long arms of chromosomes 10 in *A. brunoi* and interstitially in the short arms of chromosomes 10 in *C. greeningi* and *O. langsdorffii* or in the long arms of chromosomes 12 in *S. fuscovarius* (Fig. 7a–d).

No distinct heteromorphic sex chromosomes or apparently sex-specific banded chromosomes were noticed in any of the karyotypes. Meiotic analyses of male specimens of *A. brunoi, O. langsdorffii*, and *S. fuscovarius* showed 12 bivalents in diplotene or metaphase I cells and 12 chromosomes in metaphase II cells, also with no cytological evidence of sex chromosomes.

Discussion

The karyotypes of the tree frogs of the present study, belonging to four distinct genera, largely match the general ideogram presented by King (1990) for the 2n = 24 Hylinae group, comprising metacentrics or submetacentrics/subtelocentrics and a small-sized marker chromosome pair, bearing a secondary constriction. With conventional staining, *A. brunoi*, *C. greeningi*, and *O. langsdorffii* have nearly identical karyotypes, with a clear discontinuity in the chromosome lengths of the first five pairs and the remaining seven pairs. The least similarity was seen in the karyotype of *S. fuscovarius,* which presents a gradual decrease in chromosome lengths due to the slight difference in the size of pairs 5 and 6.

Amphibian chromosomes have been described with complete absence or poor longitudinal differentiation after conventional G- or R-banding techniques, but multiple reproducible euchromatic bands have been successfully obtained when BrdU is incorporated into chromosomes during DNA replication (Schmid et al., 1990; Kasahara et al., 1998). Replication banding obtained for the first time in *A. brunoi, C. greeningi, O. langsdorffii*, and *S. fuscovarius* allowed precise pairing and the establishment of extensive homeology among the chromosomes of the four species, even among those with non-coincident morphology. No major structural rearrangement was recognized causing the repatterning of the four karyotypes. Although chromosome 5 of *S. fuscovarius* was confirmed to have lost a small region of its short arms, at present we have no indication if it was rearranged to another chromosome in the complement. Conservative replication banding in different species is not surprising since it was also found by Miura (1995) in *Rana*, *Hyla*, and *Bufo*, three genera belonging to distinct Anura families. Undoubtedly, more refined procedures, like M-FISH, are necessary to confirm with certainty the homeology of the whole chromosome complements of the four Hylinae species.

Although the species *A. brunoi, C. greeningi, O. langsdorffii*, and *S. fuscovarius* have chromosomes with highly conserved replication banding patterns, the use of additional banding techniques disclosed some differences in their karyotypes, supporting the idea that each of them are species-specific. In the four species, NORs are not at the same position in homeologous NOR-bearing chromosomes, but the occurrence of gross structural rearrangements is apparently ruled out. The shift in the NOR position in homeologous chromosomes at interspecific level is not infrequent and this may be important to characterize individually species with similar karyotypes (Silva et al., 2000). The same is true in regard to C-banding, which has been useful to distinguish some Anuran species with nearly identical conventionally stained karyotypes (Matsui et al., 1985; Schmid et al., 1990; Silva et al., 2000; Schmid et al., 2002). The four Hylinae species share similar centromeric C-banding patterns, but *O. langsdorffii* displayed some additional telomeric positive staining in some chromosomes, besides an unequivocal interstitial band in chromosomes 5.

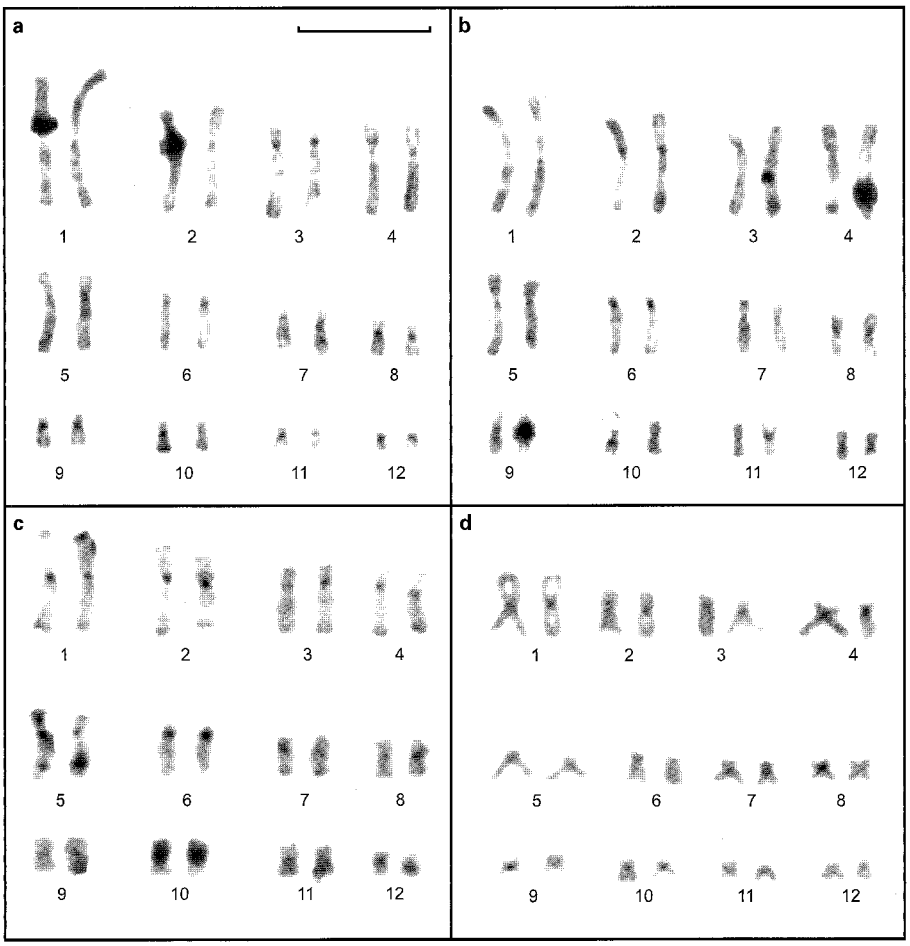

Fig. 5. C-banded karyotypes from (**a**) a male *Aparasphenodon brunoi,* (**b**) female *Corythomantis greeningi,* (**c**) male *Osteocephalus langsdorffii,* and (**d**) female *Scinax fuscovarius.* Bar represents 10 μm.

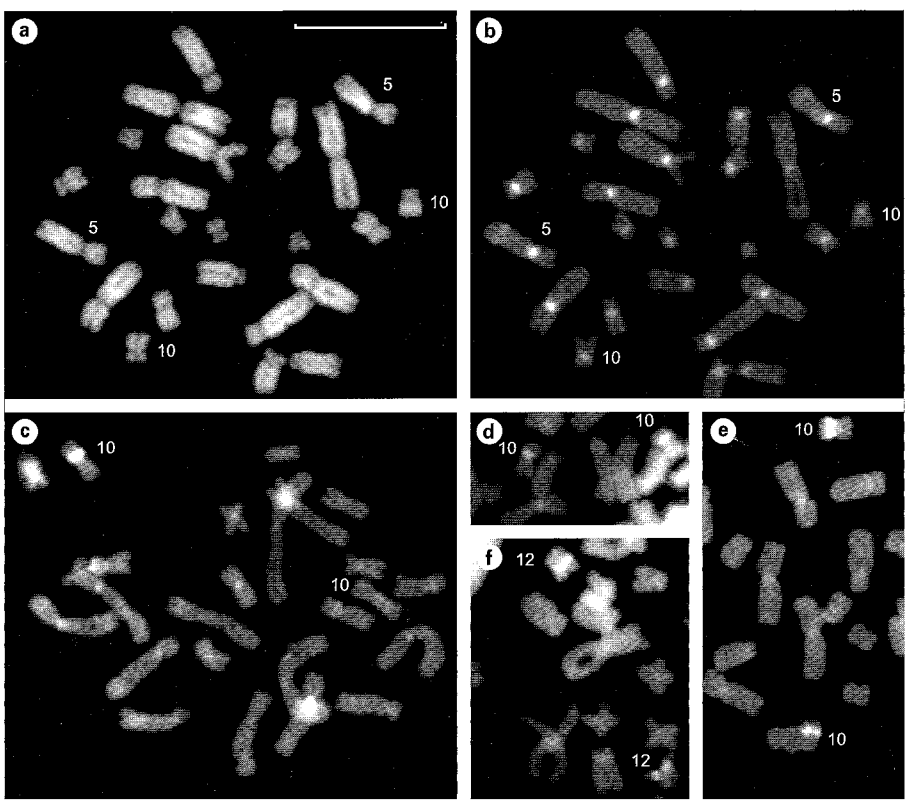

Fig. 6. DA/DAPI (**a**) and DA/CMA_3 (**b–f**) stained metaphases from (**a**, **b**) a male *Aparasphenodon brunoi,* (**c**, **d**) female *Corythomantis greeningi,* (**e**) male *Osteocephalus langsdorffii,* and (**f**) juvenile *Scinax fuscovarius.* Partial metaphases in **d–f**. Bar represents 10 μm.

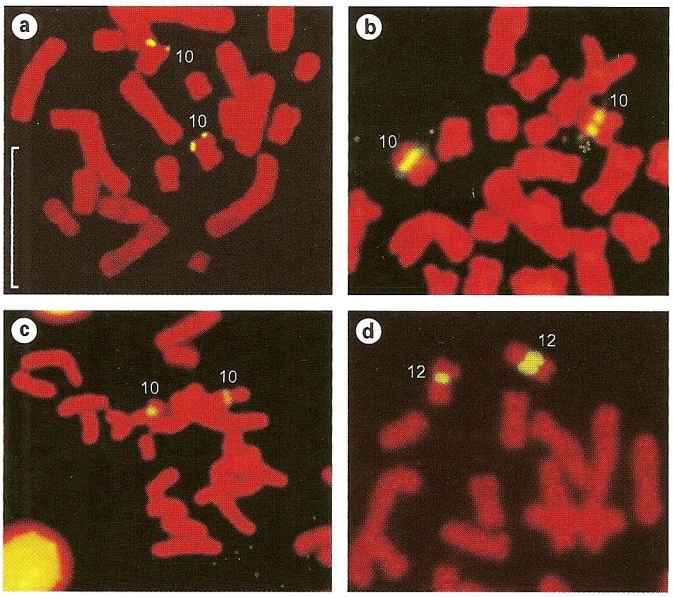

Fig. 7. Partial metaphases showing NOR-bearing chromosomes after fluorescence in situ hybridization with an rDNA probe. (**a**) *Aparasphenodon brunoi*, (**b**) *Corythomantis greeningi*, (**c**) *Osteocephalus langsdorffii*, and (**d**) *Scinax fuscovarius*. Bar represents 10 μm.

The most remarkable karyotypic difference was revealed by fluorochrome CMA_3 staining. There is evidence of a high GC content in the heterochromatin at the centromeric regions and interstitially in chromosome pair 5 of *A. brunoi*, although this latter region is not identified by C-banding. It is interesting to remark that the corresponding chromosomes 5 in *O. langsdorffii* displayed a C-band at approximately the same site, but this heterochromatin is not stained with CMA_3.

DA/DAPI staining revealed no specific bright AT-rich class of repetitive DNA sequences in any of the four species of Hylinae, even though heterochromatin with a high content of AT base pairs has been frequently described in amphibian genomes (Schmid et al., 1990; Schmid et al., 2002). In two Australian *Mixophyes*, Schmid et al. (2002) observed that an extremely brightly fluorescing heterochromatin with DAPI, Hoechst 33258, and actinomycin D/DAPI presented a quenched fluorescence after DA/DAPI staining. We have no indication that the same occurs in the four species of Hylinae, but it would be interesting to use some other procedures, in order to eventually detect this class of heterochromatin.

In contrast to the observations in *C. greeningi, O. langsdorffii*, and *S. fuscovarius* and frequently in lower vertebrates, especially fishes and amphibians (Amemiya and Gold, 1986; Schmid et al., 1990), the NOR site of *A. brunoi* is not stained with DA/CMA_3. This finds a parallel in human NOR-bearing chromosomes, in which this cytological marker is not identified by GC base pair-specific fluorochromes, probably as consequence of the small number of repeating units (Schmid et al., 1990). The tiny hybridization signal with an rDNA probe in chromosomes 10 of *A. brunoi*, strongly suggests that the number of rDNA repeats is actually low, impairing the stainability and consequently the visualization of this site with CMA_3. The shorter rDNA sequence would also explain the fact that the secondary constriction of these chromosomes is hardly visible.

The result observed in one of the NORs of *C. greeningi*, in which the adjacent areas and not the NOR itself exhibited bright fluorescence, provides another important clue for understanding the mechanism of the CMA_3 staining. Possibly, the stainability of the NOR site also depends on the high GC-content of the heterochromatin, which appears frequently associated with this chromosome region.

Undoubtedly, the most intriguing result with DA/CMA_3 is the difference in the molecular nature of the heterochromatin in *A. brunoi*, in spite of the similarity of the centromeric C-banding patterns in the four Hylinae species. In *C. greeningi*, however, there is a slight increase in GC-content as shown by the vestige of bright fluorescence at the centromeric region, not observed in the other two species. This finding is not so unexpected if we take into account the close proximity of *Aparasphenodon* and *Corythomantis*, two genera probably sharing a common ancestor (Trueb, 1970). Formerly, *A. brunoi* was included in the genus *Corythomantis* (see Frost, 2002), since species in both genera have similar external morphology. Nevertheless, it is known that they have distinct geographical distributions and habitats. The genus *Corythomantis* occurs in Brazil, from the State of Maranhão to the State of Bahia, and probably north of the State of Minas Gerais, inhabiting open and xeric areas in "caatinga" or "cerrado" formations. The genus *Aparasphenodon* has a wider distribution, from the coastal region of southeastern Brazil to south of State of Bahia; upper Orinoco Basin, Venezuela, and adjacent Colombia (Sazima and Cardoso, 1980; Frost, 2002; Pimenta and Haddad, unpublished data).

In conclusion, the cytogenetic study on four tree frogs species indicates that there are more similarities among *A. brunoi*, *C. greeningi*, and *O. langsdorffii*, which might form a monophyletic group, whereas *S. fuscovarius* has a more discrepant karyotype. Besides that extensive chromosome homeology based in replication banding does not preclude minor karyotypic differences. This reinforces the importance of using various banding techniques for the improvement of karyotype characterization of Brazilian anuran species in further analyses on phylogenetic relationships.

Acknowledgements

The authors are grateful to Dr. O. Moreira Filho and Dr. S. Recco-Pimentel for providing facilities in their laboratories in order to carry out fluorochrome staining and FISH technique. We also thank Drs. E. Zefa, A. Abe, A. Mesa, and C. Fontanetti for collecting some of the hylin specimens, and Cristiane Mileo for helping us with the figures.

References

Amemiya CT, Gold JR: Chromomycin A_3 stains nucleolus organizer regions of fish chromosomes. Copeia 1:226–231 (1986).

Anderson K: Chromosome evolution in holarctic *Hyla* treefrogs, in Green DM, Sessions SK (eds): Amphibian Cytogenetics and Evolution, pp 299–331 (Academic Press, San Diego 1991).

Baldissera Jr FA, Oliveira PSL, Kasahara S: Cytogenetics of four Brazilian *Hyla* species (Amphibia-Anura) and description of a case of supernumerary chromosome. Rev Brasil Genet 16:335–345 (1993).

Bogart JP: Evolution of anuran karyotypes, in Vial JL (ed): Evolutionary Biology of Anurans, pp 337–349 (University of Missouri Press, Columbia 1973).

Dutrillaux B, Couturier J: La Pratique de l'Analyse Chromosomique (Masson, Paris 1981).

Foresti F: Aspectos Cromossômicos da Família Hylidae (Amphibia-Anura). Master Thesis, University of São Paulo (Piracicaba 1972).

Frost DR: Amphibian species of the world: an online reference. V2.21 (15 July 2002). http:/research.amnh.org/herpetology/amphibian/index.html.

Green DM, Sessions SK: Nomenclature for chromosomes, in Green DM, Sessions SK (eds): Amphibian Cytogenetics and Evolution, pp 431–432 (Academic Press, San Diego 1991).

Howell WM, Black DA: Controlling silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36:1014–1015 (1980).

Kasahara S, Silva APZ, Gruber SL: Use of lymphocyte cultures for BrdU replication banding patterns in anuran species (Amphibia). Genet molec Biol 21:471–476 (1998).

King M: Amphibia, in John B (ed): Animal Cytogenetics, Vol 4/2 (Gerbrüder Borntraeger, Berlin 1990).

Kuramoto M: A list of chromosome number of anuran amphibians. Bull Fukuoka Univ Educ 39:83–127 (1990).

Matsui M, Seto T, Kohsaka Y, Borkin L: Bearing of chromosome C-banding patterns on the classification of Eurasian toads of the *Bufo bufo* complex. Amphibia-Reptilia 6:23–33 (1985).

Meunier-Rotival M, Cortadas J, Macaya G, Bernardi G: Isolation and organization of calf ribosomal DNA. Nucl Acids Res 6:2109–2123 (1979).

Miura I: The late replication banding patterns of chromosomes are highly conserved in the genera *Rana*, *Hyla* and *Bufo* (Amphibia-Anura). Chromosoma 103:567–574 (1995).

Sazima I, Cardoso AJ: Notas sobre a distribuição de *Corythomantis greeningi* Boulenger, 1896 e *Aparasphenodon brunoi* Miranda-Ribeiro, 1920 (Amphibia, Hylidae). Iheringia, Ser Zool Porto Alegre 55:3–7 (1980).

Schmid M, Steinlein C, Nanda I, Epplen JT: Chromosome banding in Amphibia, in Olmo E (ed): Cytogenetics of Amphibians and Reptiles, pp 21–45 (Birkhäuser, Basel 1990).

Schmid M, Haaf T, Steinlein C, Nanda I, Mahony M: Chromosome banding in Amphibia. XXV. Karyotype evolution and heterochromatin characterization in Australian *Mixophyes* (Anura, Myobatrachidae). Cytogenet Genome Res 97:239–253 (2002).

Schweizer D: Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58:307–324 (1980).

Silva APZ, Haddad CFB, Kasahara S: Chromosomal studies on five species of the genus *Leptodactylus* Fitzinger, 1826 (Amphibia, Anura) using differential staining. Cytobios 103:25–38 (2000).

Sumner AT: A simple technique for demonstrating centromeric heterochromatin. Expl Cell Res 75:304–306 (1972).

Trueb L: The evolutionary relationships of casque-head tree frogs with co-ossified skulls (Family Hylidae). Univ Kansas Publ Mus Nat Hist 18:547–716 (1970).

Viegas-Péquignot E: *In situ* hybridization to chromosomes with biotinylated probes, in Wilkinson DG (ed): *In Situ* Hybridization: A Practical Approach, pp 137–158 (IRL Press, Oxford 1992).