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Author(s): Glaucilene Ferreira Catroli, Julián Faivovich, Célio F. B. Haddad, and Sanae Kasahara
Source: Journal of Herpetology, 45(1):120-128. 2011.
Published By: The Society for the Study of Amphibians and Reptiles
DOI: 10.1670/09-249.1
URL: http://www.bioone.org/doi/full/10.1670/09-249.1

Journal of Herpetology, Vol. 45, No. 1, pp. 120–128, 2011

Conserved Karyotypes in Cophomantini: Cytogenetic Analysis of 12 Species from 3 Species Groups of *Bokermannohyla* (Amphibia: Anura: Hylidae)

GLAUCILENE FERREIRA CATROLI,[1,2] JULIÁN FAIVOVICH,[1,3] CÉLIO F. B. HADDAD,[1,4] AND SANAE KASAHARA[1,5]

[1]*Departamentos de Biologia e Zoologia, Instituto de Biociências, Universidade Estadual Paulista, Avenida 24A, 1515, Rio Claro, CEP 13506-900, São Paulo, Brazil*
[3]*División Herpetología, Museo Argentino de Ciencias Naturales-CONICET, Ángel Gallardo 470, C1405DJR, Buenos Aires, Argentina; E-mail: julian@macn.gov.ar*

ABSTRACT.—***Bokermannohyla* is one of the five genera included in the recently recognized tribe Cophomantini, of the hylid frog subfamily Hylinae. Although karyotypic diversity is relatively well known in two genera of Cophomantini, *Aplastodiscus* and *Hypsiboas*, in *Bokermannohyla* chromosome data are restricted to only two of its 28 species. In this paper, we describe the karyotypes of 12 species of *Bokermannohyla* using standard staining, Ag-NOR, C-banding, DAPI, CMA_3, and BrdU incorporation. The 12 species share a similar diploid karyotype with 2n = 24 biarmed chromosomes; most observed differences involved the NOR-bearing chromosomes (and the NOR position within these chromosomes) and C-banding patterns. The overall similarity of these karyotypes with those of *Aplastodiscus* and *Hypsiboas* widens the notion of remarkable morphological homogeneity among Cophomantini karyotypes. The results obtained thus far are promising for comparative studies on the genus *Bokermannohyla* and, in a wider sense, will allow a better understanding of karyotype differentiation and chromosomal evolution in Cophomantini.**

Hylidae is the most species-rich family of anurans, having now 891 described species (updated from Frost, 2010). Faivovich et al. (2005) presented a phylogenetic analysis of Hylidae, with particular emphasis in the subfamily Hylinae. They included 228 hylids, exemplars of 40 of the 41 genera then included in the family. This analysis allowed testing the monophyly of 24 of these genera and of 25 of the 41 species groups included in the then enormous genus *Hyla*. Its results revealed the nonmonophyly of several genera, among which *Hyla* stands out particularly for having its fragments spread throughout Hylinae. To remedy this situation, several taxonomic changes were introduced, including the recognition of four tribes in the subfamily Hylinae (Cophomantini, Dendropsophini, Hylini, and Lophiohylini), the erection of several new genera, and the reallocation of most species formerly included in *Hyla* in 15 genera, some already recognized, some resurrected, and others new. These results were corroborated by Wiens et al. (2006).

A phylogenetic hypothesis for Hylidae and the associated monophyletic taxonomy provides a historical framework for the understanding of karyotypic diversity and chromosome evolution. There is a long history of karyotypic studies in hylids (Green and Sessions, 2007; Catroli and Kasahara, 2010).

However, the lack of a historical context never allowed a clear understanding of the real gaps in the knowledge of chromosomes and their consequences for understanding the big picture of hylid karyotypic evolution; having a phylogenetic hypothesis now allows us to pinpoint areas of the tree where knowledge about chromosomes is particularly important and needed.

Cophomantini is the sister taxon of the other three tribes of subfamily Hylinae and is composed of the genera *Aplastodiscus*, *Bokermannohyla*, *Hyloscirtus*, *Hypsiboas*, and *Myersiohyla*. The latter is the sister group of the other four genera, followed by *Hyloscirtus* and then by *Bokermannohyla*, which is the sister taxon of *Aplastodiscus* + *Hypsiboas* (Faivovich et al., 2005). Knowledge about karyotypes in this tribe is restricted to some species groups of *Hypsiboas* (e.g., Rabello, 1970; Bogart, 1973; for a review, see Catroli and Kasahara, 2010), *Aplastodiscus* (Bogart, 1973; Carvalho et al., 2009a,b), and two species of *Bokermannohyla* (Foresti, 1972; Baldissera et al., 1993). No karyotype has been described from any species of *Hyloscirtus* and *Myersiohyla*. In this context, chromosomal information about these latter three genera, together with the available data on *Aplastodiscus* and *Hypsiboas*, would allow a better understanding of chromosome diversification and possible underlying evolutionary mechanisms in Cophomantini.

Bokermannohyla has recently been erected to accommodate the former *Hyla circumdata*, *Hyla claresignata*, *Hyla martinsi*, and *Hyla pseudopseudis* groups. On the basis of the individual monophyly of the available exemplars of two of these species groups or on hypotheses by previous authors, Faivovich et al. (2005) kept recognizing these groups in *Bokermannohyla*. The genus currently comprises 29 species (Frost, 2010): 18 in the *Bokermannohyla circumdata* group, two in the *Bokermannohyla claresignata* group, three in the *Bokermannohyla martinsi* group, and six in the *Bokermannohyla pseudopseudis* group. All the species are distributed only in Brazil, where they occur in the Atlantic Forest and Cerrado formations.

The monophyly of *Bokermannohyla* is supported thus far by molecular data, and a thorough morphological study of the genus remains to be done. Evidence for the monophyly of the *B. circumdata* and *B. pseudopseudis* group is mostly restricted to molecular data resulting from the analysis of Faivovich et al. (2005). Species of the former *H. claresignata* group were unavailable to Faivovich et al. (2005), but they were tentatively associated with *Bokermannohyla* on the basis of previous association of this group with the former *H. circumdata* group (Bokermann 1972; Jim and Caramaschi 1979). Faivovich et al. (2005) included only one of the two species of the *B. martinsi* group, but its monophyly was recognized on the basis of the presence of a well-developed humeral spine and a bifid distal element of the prepollex in adult males of the group (Bokermann, 1965); more recently a third species was described (Faivovich et al., 2009). A more rigorous test of the monophyly of *Bokermannohyla*, as well as of the individual monophyly of each of it species groups, remains to be done.

Karyotypic information has been published for only one species of the *B. circumdata* group, *Bokermannohyla luctuosa*, by Baldiserra et al. (1993), and there are references to a second species, *Bokermannohyla izecksohni*, in an unpublished thesis (Foresti, 1972). Therefore, the cytogenetic knowledge in relation to the taxonomic diversity of *Bokermannohyla* is nearly null. In this paper, we describe chromosome morphology and NOR

[2]Corresponding Author. Present address: Laboratório de Biologia Celular, Instituto Butantan, Avenida Vital Brazil 1500, São Paulo, CEP 05503-900, São Paulo, Brazil; E-mail: gcatroli@gmail.com
[4]E-mail: haddad1000@gmail.com
[5]E-mail: kasahara@rc.unesp.br

(Nucleolar Organizer Region) in 12 species of *Bokermannohyla* and centromeric bands (C-bands) pattern for nine of them; and also we use other fine-grained techniques like fluorochrome AT or GC-specifics and incorporation of BrdU in some exemplars from three of the currently recognized species groups, the *B. circumdata*, *B. martinsi*, and *B. pseudopseudis* group. The results are discussed in the context of current knowledge of karyotypic diversity and evolution of the tribe Cophomantini.

Materials and Methods

We analyzed 33 specimens from 12 species: eight from the *B. circumdata* group (*Bokermannohyla ahenea, B. circumdata, B. luctuosa, Bokermannohyla hylax, Bokermannohyla* sp. 1, *Bokermannohyla* sp. 2, *Bokermannohyla* sp. 3, and *Bokermannohyla* sp. 4); these latter four are unidentified species, tentatively included in the *B. circumdata* group on the basis of overall similarity with most species of the group; one from the *B. martinsi* group (*B. martinsi*); and three from the *B. pseudopseudis* group (*Bokermannohyla alvarengai, Bokermannohyla ibitiguara, Bokermannohyla saxicola*). Vouchers were fixed in 10% formalin and stored in 70% ethanol and are housed in the Célio F. B. Haddad collection (CFBH), Departamento de Zoologia, Instituto de Biociências, UNESP, Rio Claro, São Paulo, Brazil. For voucher information, collecting locality data, sex, and techniques applied to each specimen, see Appendix 1.

Chromosome spreads were prepared directly from bone marrow, liver, and spleen or intestine epithelium after in vivo treatment with colchicine following the protocol proposed by Schmid (1978). In vivo 5-bromodeoxyuridine (BrdU) and 5-fluorodeoxyuridine (FudR) treatment was performed in some exemplars (Silva et al., 2000). Mitotic chromosomes of one exemplar of *B. hylax* were obtained from lymphocytes cultured according to Kasahara et al. (1998). Meiotic chromosome preparations were also obtained from testes. 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 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), following the method of Christian et al. (1998). Chromosome morphological nomenclature (Table 1) is that of Green and Sessions (1991, 2007).

Results

Standard Karyotypes.—All specimens available from the three species groups of *Bokermannohyla* have diploid karyotype with 2n = 24 chromosomes. They are all biarmed such that the FN is unequivocally 48 in all species (Figs. 1A–H, 2A–D; for Centromeric Indexes, see Table 1). Pairs 1 to 6 are distinctly longer than pairs 7 to 12; within this latter group, chromosomes decrease gradually in size. Morphologically, pairs 1, 8, 10, 11, and 12 were metacentric, pairs 2, 3, 5, 7, and 9 submetacentric, and pairs 4 and 6 subtelocentric. Differences concerning this classification were observed in pair 5 of *B. luctuosa* and *B. ahenea*, which are metacentric and in pair 2 of *B. alvarengai* and *B. saxicola*, which also are metacentric instead of submetacentric as are the remaining 10 species. A subtle secondary constriction was observed only in *B. alvarengai,* at the terminal region of the short arms of pair 4 (Fig. 2A). Testicular cells from males of *B. ahenea*, *B. circumdata, B. ibitiguara*, *B. luctuosa*, *B. martinsi*, *B. saxicola*, *Bokermannohyla* sp. 3, and *Bokermannohyla* sp. 4, stained with Giemsa, showed 12 bivalents in metaphase I and 12 chromosomes in metaphase II.

Ag-NOR Staining.—The silver staining technique revealed only one Ag-NOR bearing pair in the 12 species (Figs. 1A–H, 2A–D, insets). In all species of the *B. circumdata* group, in *B. martinsi,* from *B. martinsi* group, and in *B. saxicola*, from the *B. pseudopseudis* group, the Ag-NOR was detected in the terminal region of the long arms of pair 11. In *B. alvarengai*, the sequential use of conventional and Ag-NOR staining revealed that the labelling is in the terminal region of the short arms of pair 4, coincidently with the secondary constriction. *Bokermannohyla ibitiguara* has the Ag-NOR located at the terminal region of the pair 1.

Although *B. ahenea*, *B. martinsi* and *Bokermannohyla* sp. 1 have more prominent labelling, the Ag+ region is subtle in the remaining species. In *B. saxicola*, the Ag-NOR is heteromorphic in size, and in some metaphases, the NOR site could not be seen in one of the homologues. In some metaphases of *B. alvarengai*, the centromeric region of the Ag-NOR bearing pair and of some other chromosomes is Ag-positive. This was also observed in metaphases of *B. saxicola*, but in this species all pairs exhibited labelling in the centromeric regions of all chromosomes (Fig. 3).

C-Banding.—Satisfactory results with this technique were achieved in all but three species (Fig. 4A–I). In *B. hylax* and *Bokermannohyla* sp. 4, attempts to obtain C-bands in the available specimens were unsuccessful. In *Bokermannohyla* sp. 1, despite attempts to obtain C-bands from several plates of the same individual, the results were inconclusive. Only in a few metaphases, blocks of heterochromatin were evident in the centromeric region of the chromosomes, and in some pairs, they extend to the pericentromeric region as well. In one of the homologues of pair 11, there was a C-band in the terminal region of the long arms, coincident with the Ag-NOR site.

In all the other available species, heterochromatic blocks were concentrated in the centromeric region of most pairs; interstitial, pericentromeric, or terminal bands were detected in some chromosomes. Pair 4 of *B. circumdata* and *B. luctuosa* and pairs 11–12 of all studied species from the *B. circumdata* group rarely showed C-bands in the analyzed metaphases. Centromeric bands are more conspicuous in all chromosomes of *B. martinsi* than in all other studied species.

Bokermannohyla circumdata and *B. luctuosa* have a slightly C-positive staining in the terminal region of the long arms of pair 10. *Bokermannohyla ibitiguara* shows a slight C-band in the terminal region of the long arms of pair 1, also coincidently with the Ag-NOR and *B. luctuosa* has a terminal band in the short arms of the pair 7.

Interstitial bands were observed in the long arms of pair 7 of *B. alvarengai*, *B. martinsi*, and *B. saxicola*, as a more prominent block in the short arms of pair 8 of *B. alvarengai*, in the short arms of pair 1 in *B. martinsi*, and in only one homologue of this same pair in *B. saxicola*.

In the chromosome pairs 1 and 2 of *B. circumdata* and 1 of *B. luctuosa*, heterochromatin is also present in the pericentromeric region. A single, large band was also seen in pair 3 in *B. martinsi* as well as in *B. alvarengai* and *B. saxicola*, but these latter two share a more prominent pericentromeric band in its short arms. *Bokermannohyla saxicola* also has heterochromatin in the short arms of pair 8 and in the long and short arms of pair 1. Pericentromeric heterochromatin is observed in pairs 6 of *B. ahenea*, and a more prominent one is in long arms of this same pair of *B. martinsi*.

Base-Specific Fluorochromes.—The fluorochrome staining provided satisfactory results in 10 species of *Bokermannohyla*; all attempts to use this technique in *B. hylax* and *B. alvarengai* were unsuccessful. The fluorochrome AT-specific DAPI did not reveal any fluorescent signal in the chromosomes of most species. The single exception is *B. circumdata*, which presented a bright DAPI signal in the terminal region of the long arms of a single, small metacentric pair, likely pair 10 (Fig. 5A). With

TABLE 1. Centromeric indexes of the species studied in this paper.

Species		Chromosome number											
		1	2	3	4	5	6	7	8	9	10	11	12
Bokermannohyla ahenea	CR[a]	1.20	2.00	2.00	5.00	1.60	4.00	2.27	1.30	2.00	1.00	1.50	1.00
	CP[b]	m	sm	sm	st	m	st	sm	m	sm	m	m	m
Bokermannohyla circumdata	CR	1.16	1.69	1.70	4.00	1.78	5.00	1.75	1.25	2.00	1.00	1.00	1.50
	CP	m	sm	sm	st	sm	st	sm	m	sm	m	m	m
Bokermannohyla hylax	CR	1.16	2.00	1.78	5.00	2.00	6.25	1.92	1.25	1.80	1.00	1.50	1.00
	CP	m	sm	sm	st	sm	st	sm	m	sm	m	m	m
Bokermannohyla luctuosa	CR	1.10	1.78	2.13	3.10	1.60	4.00	1.84	1.25	1.70	1.00	1.66	1.00
	CP	m	sm	sm	st	m	st	sm	m	sm	m	m	m
Bokermannohyla sp.1 (gr. *circumdata*)	CR	1.16	2.00	2.32	6.36	2.17	5.00	1.76	1.05	2.2	1.05	1.00	1.00
	CP	m	sm	sm	st	sm	st	sm	m	sm	m	m	m
Bokermannohyla sp.2 (gr. *circumdata*)	CR	1.40	1.71	2.27	5.00	2.00	3.50	2.00	1.00	2.00	1.00	1.00	1.28
	CP	m	sm	sm	st	sm	st	sm	m	sm	m	m	m
Bokermannohyla sp.3 (gr. *circumdata*)	CR	1.05	3.00	1.76	4.00	2.00	4.44	2.25	1.00	1.71	1.00	1.00	1.00
	CP	m	sm	sm	st	sm	st	sm	m	sm	m	m	m
Bokermannohyla sp.4 (gr. *circumdata*)	CR	1.05	1.72	2.00	5.00	2.80	3.12	2.11	1.00	2.50	1.2	1.42	1.28
	CP	m	sm	sm	st	sm	st	sm	m	sm	m	m	m
Bokermannohyla alvarengai	CR	1.15	1.65	1.75	4.50	1.85	4.00	2.00	1.30	2.53	1.00	1.05	1.00
	CP	m	m	sm	st	sm	st	sm	m	sm	m	m	m
Bokermannohyla ibitiguara	CR	1.14	1.90	2.11	4.00	1.86	3.50	2.00	1.00	2.50	1.20	1.10	1.25
	CP	m	sm	sm	st	sm	st	sm	m	sm	m	m	m
Bokermannohyla saxicola	CR	1.30	1.57	1.78	4.00	1.72	4.50	2.66	1.47	2.00	1.00	1.10	1.25
	CP	m	m	sm	st	sm	st	sm	m	sm	m	m	m
Bokermannohyla martinsi	CR	1.25	2.03	1.78	4.00	1.73	5.00	2.00	1.40	2.50	1.00	1.00	1.00
	CP	m	sm	sm	st	sm	st	sm	m	sm	m	m	m

[a] Centromeric ratio (length of long arm : length of short arm).
[b] Chromosome type according to centromeric position; m = metacentric, sm = submetacentric, st = subtelocentric.

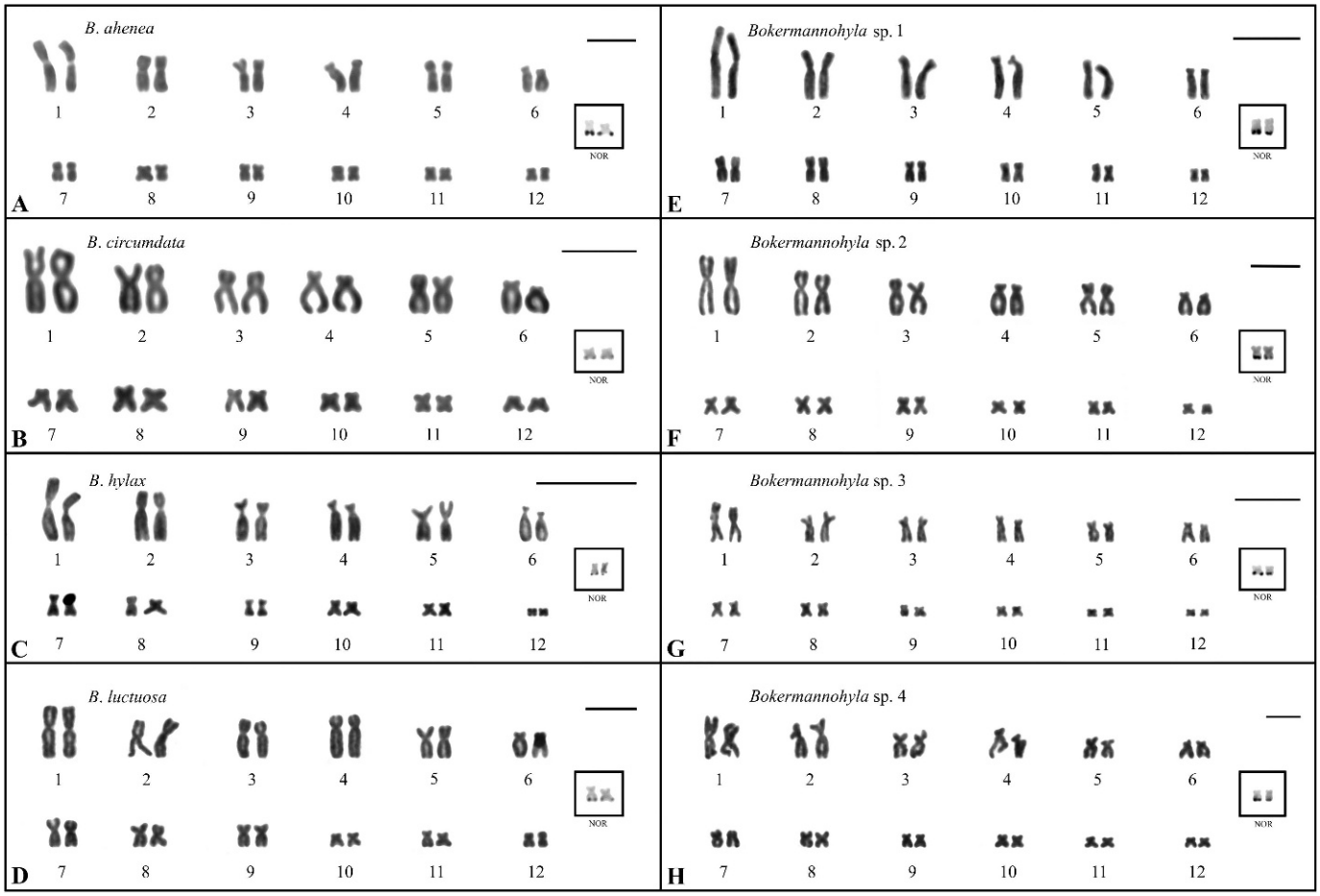

FIG. 1. Standard stained karyotypes from eight species of the *Bokermannohyla circumdata* group. Insets: Ag-NOR in chromosome pairs 11. Bar = 10 μm.

CMA_3, brilliant fluorescence was detected at the centromere of all chromosomes in the analyzed species and, coincidently with the Ag-NOR sites, at the terminal region of the long arms of pair 11, in species of the *B. circumdata* group, *B. martinsi*, and *B. saxicola*, and in the long arms of pair 1 in *B. ibitiguara*. Considering this conserved pattern, figures illustrating the fluorochrome staining of only *B. circumdata* and *B. luctuosa* from the *B. circumdata* group, of *B. martinsi* from the *B. martinsi* group, and of *B. saxicola* from the *B. pseudopseudis* group are presented here (Fig. 5A–H).

Replication Bands in Bokermannohyla.—The FPG technique after BrdU incorporation was applied in metaphases of *B. circumdata* and *B. ahenea* (*B. circumdata* group), in *B. martinsi* (*B. martinsi* group), and in *B. alvarengai* (*B. pseudopseudis* group). Although poor replication banding patterns were obtained, mainly among the small-sized pairs, the chromosomes of each haploid set were tentatively arranged side by side (Fig. 6). The replication banding patterns are very similar among the chromosomes that had a better incorporation of BrdU, mainly those of pairs 1, 2, 4, 6, 9, and 10, reinforcing the idea of chromosome homeologies of the species belonging to the three groups.

DISCUSSION

Information on the karyotypes of *Bokermannohyla* thus far has been limited to *B. izecksohni* (Foresti, 1972, using the name *B. circumdata* in an unpublished thesis) and *B. luctuosa* (Baldissera et al., 1993). In the latter species, a supernumerary chromosome was identified in two specimens that were 2n = 24 + 1B.

On the basis of the cytogenetic information available for the subfamily Hylinae summarized by King (1990), Kuramoto (1990), Green and Sessions (2007), and Catroli and Kasahara (2010), it is evident that the chromosome number 2n = 24 and the chromosome morphology reported in the species of *Bokermannohyla* are highly conserved in Cophomantini and, in general, in Hylinae. A comparison of known karyotypes of three of the five genera included in Cophomantini, *Aplastodiscus*, *Bokermannohyla*, and *Hypsiboas* corroborates the remarkable similarity among them.

In *Hypsiboas* that together with *Aplastodiscus* is the sister-taxon of *Bokermannohyla*, the chromosome number is most frequently 2n = 24, and the morphology of the pairs is similar among species, with few or almost no differences among most of them. The only known species with a different chromosome number in *Hypsiboas* is *Hypsiboas albopunctatus* with 2n = 22, a fact that has been explained by rearrangements involving the smaller pairs of the karyotype, with a reduction of one pair (Bogart, 1973; Gruber et al., 2007).

However, in *Aplastodiscus*, chromosome number varies between 2n =18 and 2n = 24, with species having karyotypes with 2n = 20 and 2n = 22 (Bogart, 1973; Carvalho et al., 2009a,b). In the species of *Aplastodiscus* whose karyotype is 2n = 24 (Carvalho et al., 2009a), there are no evident morphological differences between them and the karyotypes of *Bokermannohyla*. Among the *Aplastodiscus* species with 2n lower than 24, besides differences in their chromosomal number, the morphology of the pairs is very similar to those with 2n = 24. This suggests that karyotypic differentiation had occurred by reduction of the chromosome number, apparently resultant

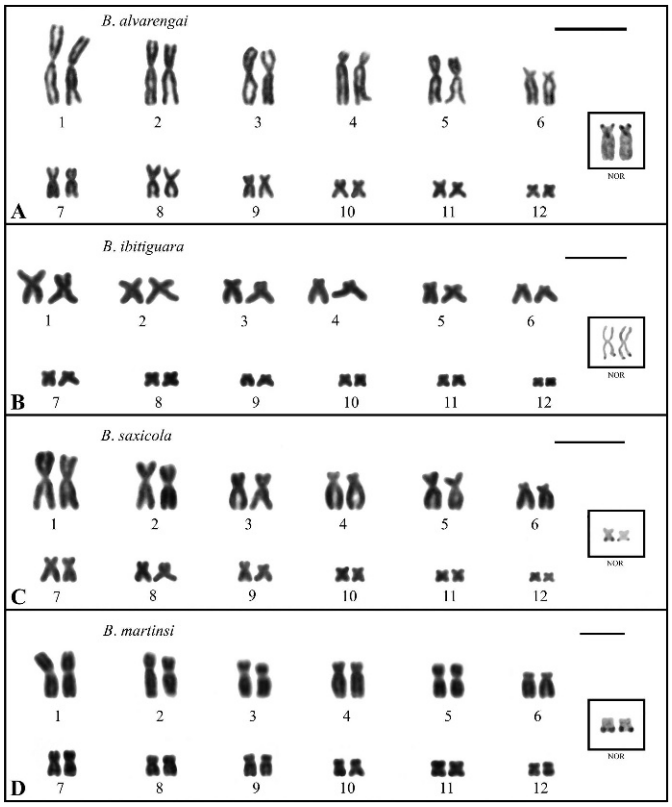

FIG. 2. Standard stained karyotypes from three species of the *Bokermannohyla pseudopseudis* group (A–C) and from *Bokermannohyla martinsi* (D). Insets: Ag-NOR in chromosome pairs 4, 1, 11, and 11, respectively. Bar = 10 μm.

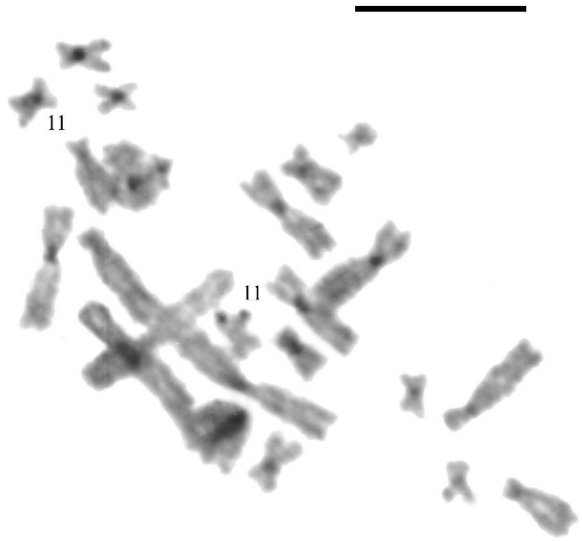

FIG. 3. *Bokermannohyla saxicola* metaphase submitted to the silver staining technique presenting marks in the centromeric regions of the chromosomes. Note NORs on chromosome pair 11. Bar = 10 μm.

from the loss of one or more pairs (Carvalho et al., 2009a,b). The homogeneity in chromosome morphology observed in Cophomantini extends also to another tribe of Hylinae, Lophiohylini, as has been analyzed by Kasahara et al. (2003).

The species of *Bokermannohyla* do not show heteromorphic sex chromosomes, either after standard staining or with some banding techniques; however, only specimens from a single sex (or only juveniles) of some species were available for the present study. Meiotic cells showed no differentiated sex chromosomes in males of *B. ahenea*, *B. circumdata*, *B. ibitiguara*, *B. luctuosa*, *B. saxicola*, *B. martinsi*, *Bokermannohyla* sp. 3, and *Bokermannohyla* sp. 4, which exhibited all 12 bivalents with ring configuration, indicative of terminal chiasmata, during diakinesis. The absence of sexually heteromorphic pairs in *Bokermannohyla* is common in most anurans, in which sex chromosomes are not differentiated morphologically (Schmid et al., 1991). Among hylids the occurrence of heteromorphic sexual pairs is rare and there are records for only eight species (see Catroli and Kasahara, 2010).

As in most anurans (Schmid et al., 1990), the species of *Bokermannohyla* present a single pair of Ag-NOR. In the exemplars of the *B. circumdata* group, *B. ahenea*, *B. circumdata*, *B. hylax*, *B. luctuosa*, *Bokermannohyla* sp. 1, *Bokermannohyla* sp. 2, *Bokermannohyla* sp. 3, and *Bokermannohyla* sp. 4, and in the available species of the *B. martinsi* group, the position of the NOR is conserved in the terminal region of the long arms of chromosome 11. In the three studied species from the *B. pseudopseudis* group, the position of the NOR is variable and unique for each species, occurring on pairs 1 (*B. ibitiguara*), 4 (*B. alvarengai*), or 11 (*B. saxicola*). A possible explanation for these differences is the occurrence of chromosomal rearrangements in the NOR-bearing pairs (Schmid et al., 1990), but possible mechanisms that would be involved are not clear, because no evident alterations in the chromosomes morphology or size have been noticed. Although currently considered a species of the *B. pseudopseudis* group, the relationships of *B. saxicola* are still unclear, and still unpublished molecular analyses suggest that this species might actually be closely related to the *B. circumdata* group, which have the Ag-NOR in pair 11 (J. Faivovich, pers. obs.).

The Ag-NORs located in small-sized pairs in the karyotypes of *Aplastodiscus*, *Hypsiboas*, and the *B. circumdata* and *B. martinsi* groups, and the difficulties in establishing the exact identities of these pairs suggest that the Ag-NOR in all these the Ag-NOR-bearing chromosomes are homeologous. At the same time in species from other hyline tribes with known Ag-NOR (Wiley, 1982; King et al., 1990; Anderson, 1991; Kasahara et al., 2003), its occurrence on pair 11 or on other smaller pairs is very frequent, indicating the possibility that the homeology of the NOR-bearing chromosome could be extended more inclusively in the hylid tree.

With the exception of *B. ahenea, B. martinsi*, and *Bokermannohyla* sp. 1, the Ag-NOR labelling are tiny, with subtle staining, probably as a consequence of a small number of rDNA cistrons within the NORs or as a result of differential activity. In *Bokermannohyla* sp. 1 and in *B. saxicola*, the sizes of the Ag-NOR are very distinctive, being larger in one of the homologues. In the latter species, the heteromorphism is prominent, and in some metaphases the Ag-NOR is very small or practically absent in one of the homologues. Ag-NOR heteromorphism is a frequent finding, and it has been reported before in *B. luctuosa* (Baldissera et al., 1993). A lack of Ag-staining may indicate no-transcribing NOR in the preceding interphase, not possessing the associated Ag-stainable protein, or even that the rDNA sequences were completely lost (Schmid, 1982). However, in some cases, it is evident that a given NOR shows more intense transcriptional activity than the one from its homolog chromosome, as observed in genus *Rhinella* (Amaro-Ghilardi et al., 2008). Heteromorphism in the size of NOR signals was observed in some species, but by using FISH with ribosomal probes, it was clearly demonstrated that the difference observed after Ag-staining should be attributed to differences in chromosomal condensation or gene activity.

The Ag-positive staining of heterochromatic regions with silver nitrate observed in *B. alvarengai* and *B. saxicola* also has been reported in other anurans, such as *Rhinella schneideri* (as

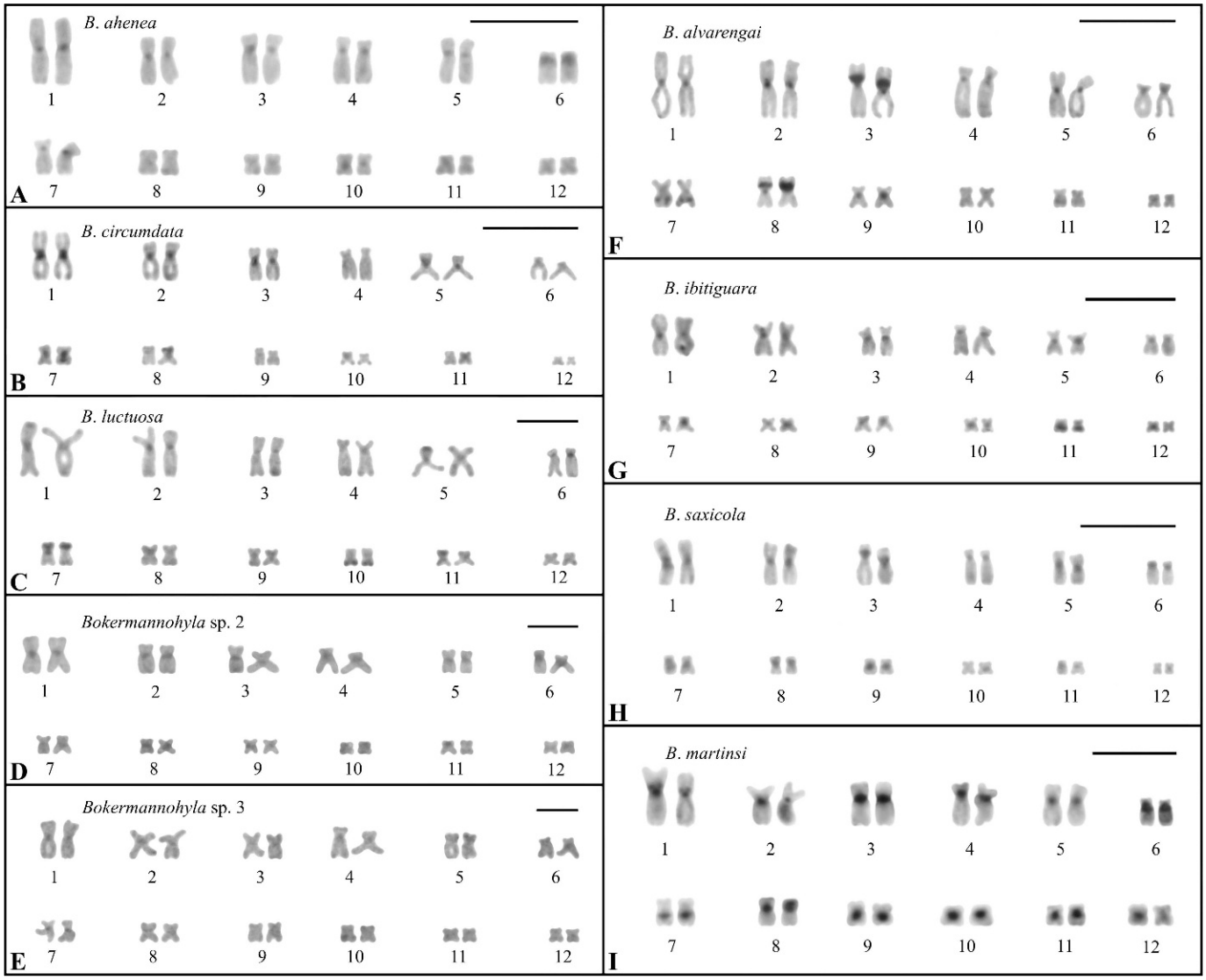

FIG. 4. C-banded karyotypes from five species of the *Bokermannohyla circumdata* group (A–E), three of the *Bokermannohyla pseudopseudis* group (F–H) and one from the *Bokermannohyla martinsi* group (I). Bar = 10 μm.

Bufo paracnemis) by Kasahara et al. (1996) and Azevedo et al. (2003), where they also found a pattern coincident with C-bands. Labelling with silver nitrate could indicate the occurrence of multiple NORs as has been shown in some anurans (Silva et al., 2006; Ananias et al., 2007). However, the co-occurrence with heterochromatic regions suggests that the labelling in these areas could be unspecific, as was shown in *R. schneideri* and *Eupemphix nattereri* with the use of in situ hybridization techniques (Kasahara et al., 1996; Azevedo et al., 2003; Ananias et al., 2007). In this way, it can be predicted that this is also the case of *Bokermannohyla*.

C-bands were obtained for the first time in *B. ahenea*, *B. alvarengai*, *B. circumdata*, *B. ibitiguara*, *B. luctuosa*, *B. martinsi*, *B. saxicola*, *Bokermannohyla* sp. 1, *Bokermannohyla* sp. 2, and *Bokermannohyla* sp. 3. In all of these species, the bands are mostly centromeric, but in a few chromosomes they are pericentromeric, interstitial as well. Although the metaphases of the only exemplar of *Bokermannohyla* sp. 1 are of low quality, the C-banding pattern can be established with some certainty, indicating C-bands blocks that are larger than those seen in other species of the *B. circumdata* group, which showed a smaller amount of heterochromatin. In *B. luctuosa*, another species of the *B. circumdata* group, Baldissera et al. (1993) previously described a weak C-band in the karyotype, with positive staining restricted to the B-chromosome found in some specimens.

In the metaphases of *B. circumdata* and *B. luctuosa*, besides the centromeric region, an evident C-band was observed in the terminal region of the long arms of pair 10. Although in principle it was interpreted as the same pair that contains the Ag-NOR, the sequential staining with silver nitrate revealed that the two pairs are different, with the C-bands on pair 10 and the Ag-NOR in pair 11. This heterochromatin was subsequently characterized by fluorochrome staining as AT rich in *B. circumdata* and the same is presumed to *B. luctuosa*.

In the three species of the *B. pseudopseudis* group, there is variation regarding quantity and distribution of heterochromatin, including presence of pericentromeric heterochromatin in some chromosomes, with the exception of those of *B. ibitiguara*. The karyotypes of *B. alvarengai* and *B. saxicola* have C-bands that are clearly larger than *B. ibitiguara*, which has subtle pericentromeric bands. In *B. alvarengai*, pair 3 shows a large pericentromeric block of heterochromatin in the small arms, and a similar band can be distinguished in this pair in *B. saxicola* and *B. martinsi*; smaller bands occur in the interstitial region of the long arms of pair 7 in these three species. *Bokermannohyla saxicola* and *B. martinsi* also have pair 1 as a common marker, with an interstitial band in its short arms.

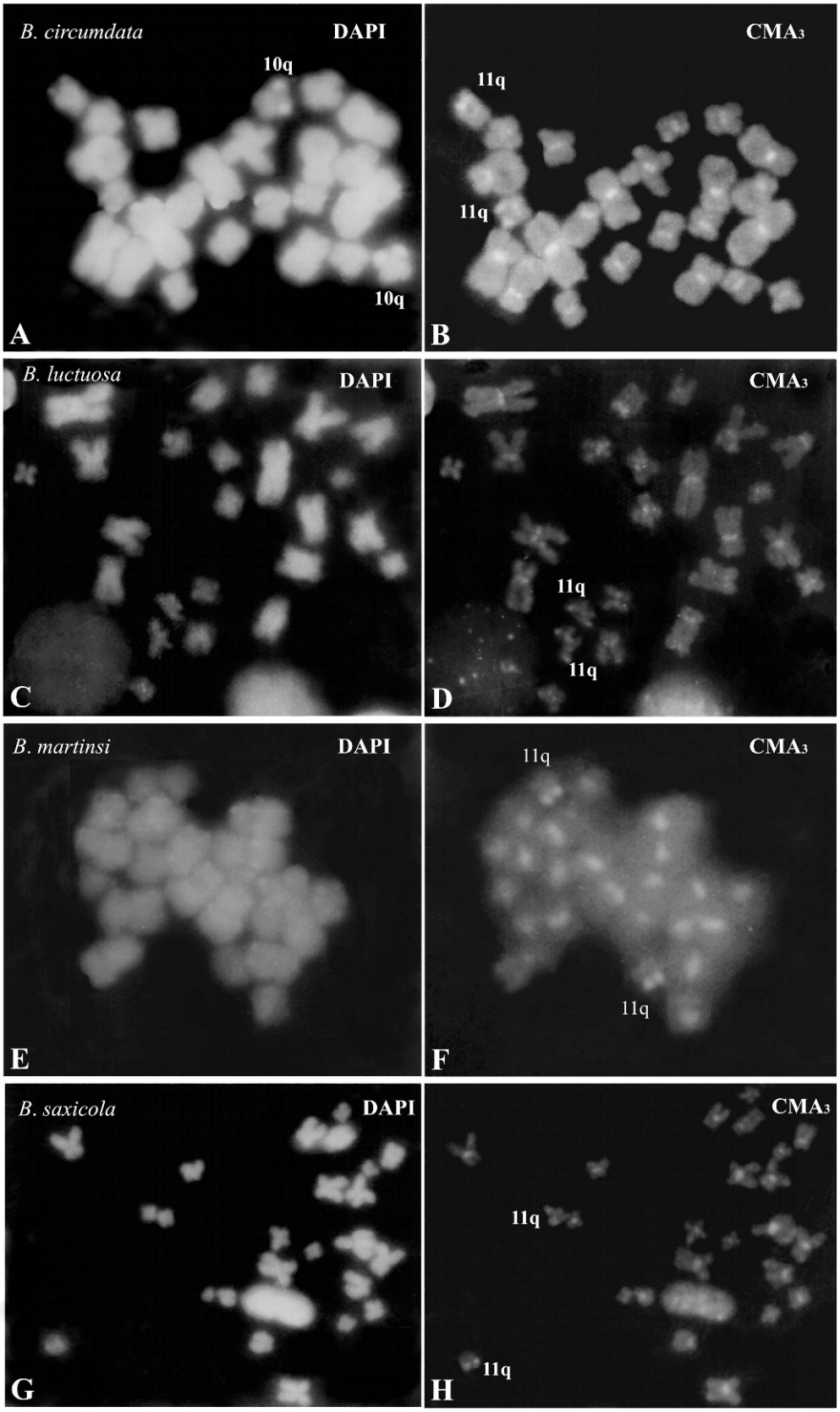

FIG. 5. DAPI and CMA_3 stained metaphases from *Bokermannohyla circumdata* (A–B), *Bokermannohyla luctuosa* (C–D), *Bokermannohyla saxicola* (E–F) and *Bokermannohyla martinsi* (G–H). Bar = 10 μm.

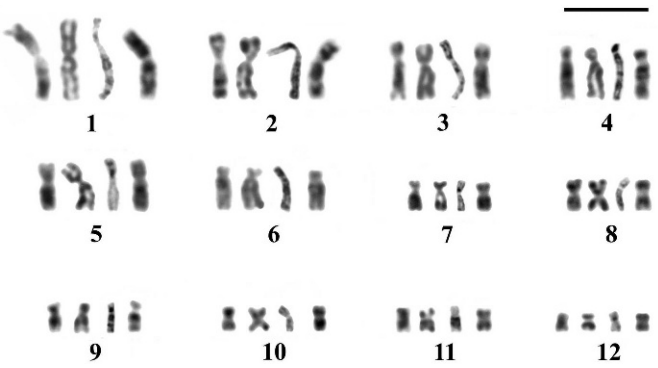

FIG. 6. Partial karyotypes (haploid sets) from four species of *Bokermannohyla* after BrdU replication banding. From left to right, *Bokermannohyla ahenea*, *Bokermannohyla circumdata*, *Bokermannohyla alvarengai*, and *Bokermannohyla martinsi*. Bar = 10 μm.

Subtle C-bands coincident with the Ag-NOR were observed in *B. ibitiguara* and more strongly stained in *Bokermannohyla* sp. 1, although these were not evident in all metaphases. According to Schmid (1982), the chromosomal segment adjacent to the NOR is of heterochromatic nature, being strongly stained by the C-band. However, in these two species the C-band does not seem to be restricted to the adjacent region, but also the NOR appears stained, as was previously observed in *R. schneideri* (Kasahara et al., 1996). For species in other groups of Hylidae, no common pattern of C-bands has been established, but the centromeric blocks are common to most of the studied species, as it often occurs in most amphibians (Gruber et al., 2007; Green and Sessions, 2007).

By using the GC-specific fluorochrome, CMA_3, besides the bright signal in the NORs, fluorescent signals were evident in the centromeres of all *Bokermannohyla* species studied, indicating the occurrence of GC-rich heterochromatin in that region. Curiously, it was found that the centromeric signal obtained with CMA_3 is not coincident with the centromeric C-bands. Fluorescent signals were detected only in the centromere but do not occur in the pericentromeric or interstitial regions, where there are, in some species, prominent heterochromatic blocks. Thus, it can be inferred that different classes of heterochromatin are present in chromosomes of the species of this genus.

This was also observed in *Agalychnis callidryas*, from the hylid subfamily Phyllomedusinae, in which fluorochromes revealed differences in base content between the centromeric and pericentromeric heterochromatin identified with C-banding. In this species the centromeric region has GC-rich areas (as observed in *Bokermannohyla*), and the pericentromeric region shows subtle or strong brilliant AT-rich signals depending on whether fluorochromes were combined with counterstaining (Schmid et al., 1995).

There are few records in the literature of the use of base-specific fluorochromes in Hylidae, but similar results to those obtained with CMA_3 in *Bokermannohyla* have already been recorded. A CMA_3-positive centromeric region has been observed in other hylids, such as *Aparasphenodon brunoi*, whose centromeres are brightly stained, and in other two species *Corythomantis greeningi* and *Itapotihyla langsdorffii* in which there are more subtle fluorescent signals in the centromeric region of some chromosome pairs (Kasahara et al., 2003).

The use of DAPI revealed only a single AT site in the terminal region of the long arms of pair 10 in *B. circumdata*, the same that was C-positive. The observation of metaphases sequentially stained with both fluorochromes, AT and GC specific, in this species confirmed that pairs stained with DAPI and CMA_3 are different, despite the similar pattern. It is evident then that, in the repetitive site of the long arm of pair 10 there is a predominance of AT-rich sequences, whereas that of pair 11, in which the NOR is located, is GC rich, as already expected.

Replication banding was obtained in *B. ahenea*, *B. alvarengai*, *B. circumdata*, and *B. martinsi*. Comparison between the four species haploid sets indicates that the chromosomes must be homeologous. This is more evident in the first six chromosomes in which bands can be more easily distinguished. In the other chromosomes, mainly among the smallest pairs, the degree of differentiation obtained did not allow a more detailed analysis, even though there are clear signals of the incorporation of BrdU. In species of Hylidae, the FPG technique has not been commonly employed (Wiley, 2003; Miura, 1995; Wiley and Little, 2000; Kasahara et al., 2003), but comparison of the replication patterns from some species showed that the chromosome sets are highly conserved (Kasahara et al., 2003; Gruber et al., 2007).

The information on chromosome morphology reported here for several species of *Bokermannohyla* strengthens the notion of a conserved pattern in karyotypes of Cophomantini. Thus far this idea has been based only on *Aplastadiscus* and *Hypsiboas*: The morphological homogeneity is extended one node down the tree in Cophomantini.

Acknowledgments.—D. Andrade (Instituto de Biociências, UNESP, Rio Claro, SP) collected the specimens of *B. alvarengai*, and F. Toledo (UNICAMP, Campinas, SP) the specimens of *Bokermannohyla* sp 2. For their help in the field in various trips looking for species of *Bokermannohyla*, we thank J. Genini, L. O. M. Giasson, F. S. F. Leite, V. G. D. Orrico, B. G. Pacheco, I. C. Tiburcio, F. Toledo, K. Vieira, and J. Zina. D. Baldo and M. Pereyra read the manuscript and provided useful comments. GFC thanks to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support. For financial support during different stages of this project, JF thanks FAPESP procs. 05/56756-0 and 06/52088-5, ANPCyT PICT-2006-223, and 2007-2202 and Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET). CFBH acknowledges support from FAPESP (procs. 01/13341-3 and 08/50928-1) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). SK acknowledges support from FAPESP (proc. 07/54374-8) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Collection permits were issued by IBAMA-RAN.

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Accepted: 30 May 2010.

Appendix 1

List of specimens examined and techniques applied to each of them (a = conventional staining; b = Ag–NOR staining; c = C-banding; d = fluorochrome staining (DAPI + CMA3); e = FPG staining f = conventional staining of meiotic cells).

Bokermannohyla ahenea.—BRAZIL: São Paulo: São José do Barreiro, CFBH 21240 (a, b, c, f; male); CFBH 21241 (a, b, c, d; male); CFBH 21243 (a, e; male).

Bokermannohyla alvarengai.—BRAZIL: Minas Gerais: Congonhas do Norte, CFBH 16716 (e; juvenile); CFBH 16717 (a, b, c; juvenile).

Bokermannohyla circumdata.—BRAZIL: São Paulo: Salesópolis, CFBH 16700 (a; female); 16701 (a; juvenile). Campos do Jordão, CFBH 16702 (a, b; female). BRAZIL: Minas Gerais: Camanducaia, CFBH 16705 (b; male); CFBH 16706 (a; female); CFBH 16707 (f; male); CFBH 16708 (a, b, d, e, f; male); CFBH 16709 (c, e; female); CFBH 16710 (b, f; male); CFBH 16711 (e; male); CFBH 16712 (f; male); and CFBH 16713 (a, c, e; male).

Bokermannohyla hylax.—BRAZIL: São Paulo: Salesópolis, CFBH 16698 (a, b; juvenile); CFBH 16699 (a, b; female).

Bokermannohyla ibitiguara.—BRAZIL: Minas Gerais: Furnas, CFBH 16718 (a, b, c, f; male); CFBH 16719 (d, f; male).

Bokermannohyla luctuosa.—BRAZIL: São Paulo: Jundiaí, CFBH 21238 (a, b, c, d; male); CFBH 21239 (b, c, d, f; male).

Bokermannohyla martinsi.—BRAZIL: Minas Gerais: Nova Lima, CFBH 21235 (b, f; male); CFBH 21236 (b, c, d, f; male); CFBH 21237 (a, e; male).

Bokermannohyla saxicola.—BRAZIL: Minas Gerais: Serra do Cipó, CFBH 16714 (a, b, c, d, f; male); CFBH 16715 (a, b, c, f; male).

Bokermannohyla sp. 1.—BRAZIL: Minas Gerais: Furnas, CFBH 16721 (a, b, d; female).

Bokermannohyla sp. 2.—Brazil: Paraná: Morretes, CFBH 21242 (a, b, c , d; female).

Bokermannohyla sp. 3.—BRAZIL: Minas Gerais: Nova Lima, CFBH 21244 (a, b, c, d, f; male).

Bokermannohyla sp. 4.—BRAZIL: Minas Gerais: Santa Bárbara, CFBH 21245 (a, b; female); CFBH 21246 (b, d, f; male).