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Dynamics of chromosomal evolution in the genus *Hypsiboas* (Anura: Hylidae)

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ABSTRACT. Hylidae is one of the most species-rich families of anurans, and 40% of representatives in this group occur in Brazil. In spite of such remarkable diversity, little is known about this family and its taxonomical and systematic features. Most hylids have 2n = 24, even though most of the cytogenetic data are mainly obtained based on the conventional chromosomal staining and are available for only 16% of *Hypsiboas* species, a genus accounting for about 10% of the hylid diversity. In this study, cytogenetic data of distinct species and populations of *Hypsiboas* were analyzed, and the evolutionary dynamics of chromosomal macro- and microstructure of these amphibians were discussed. Contrary to the conservativeness of 2n = 24, this genus is characterized by a high variation of chromosomal morphology with as much as 8 karyotype patterns. Differences in the number and location of nucleolus organizer regions and C-bands allowed the identification of geographical variants within nominal

species and cytotaxonomical chromosomal markers. Comparative analyses revealed a strong phylogeographic relationship between chromosomal patterns in this group.

Key words: Ag-NORs; C-banding; Chromosomes; Karyotype evolution; Pericentric inversion

INTRODUCTION

Hylidae is recognized as one of the most species-rich families in the order Anura. These amphibians are mainly distinguished by the presence of adhesive disks in their fingers and by their arboreal behavior; they are found in all continents except Antarctica, with predominance over the Neotropical region (Frost, 2012).

Hylids comprise 47 genera and 907 species (Amphibiaweb, 2012), commonly subdivided into 3 subfamilies: Phyllomedusinae, Pelodryadinae, and Hylinae (Faivovich et al., 2005; Wiens et al., 2010). Among these, the subfamily Hylinae is the most diverse one, encompassing about 650 species (Frost, 2012).

The species richness and wide geographical range account for the controversial taxonomy and systematics of Hylinae. Therefore, phylogenetic studies have focused on this subfamily to verify their monophyletism and the taxonomic validation of their members (Faivovich et al., 2005; Salducci et al., 2005; Wiens et al., 2010). One of the groundbreaking studies using this approach was carried out by Faivovich et al. (2005); they revealed a remarkable change in taxonomic relationships of Hylinae. On the basis of sequencing data of mitochondrial and nuclear genes as well as morphological data of 226 Hylidae species, these authors reallocated two-third of the species formerly recognized in the genus *Hyla* into other 15 genera, including *Hypsiboas*.

Members of the genus *Hypsiboas* are popularly known as tree frogs and widespread throughout humid areas of Atlantic Forest, Amazon, Brazilian savannah, and Caatinga; this genus includes 86 species, accounting for about 10% of the total hylid diversity (Amphibiaweb, 2012). According to genetic, morphological, behavioral, and ecological data, *Hypsiboas* frogs are divided into 7 species groups: *Hypsiboas albopunctatus*, *Hypsiboas benitezi*, *Hypsiboas faber*, *Hypsiboas pellucens*, *Hypsiboas pulchellus*, *Hypsiboas punctatus*, and *Hypsiboas semilineatus* (Faivovich et al., 2005).

Such groupings are supported by morphological synapomorphies that are useful to define monophyletic units. However, some species have characters that hinder their allocation in any of the currently recognized groups (Faivovich et al., 2005).

Recently, Wiens et al. (2010) identified 7 monophyletic groups in this family by gene sequencing; however, their evolutionary relationships were slightly divergent from those proposed by Faivovich et al. (2005).

Despite the continuous efforts to clarify the systematic relationships in Hylidae, few karyotypic reports are available. Less than 17% of anurans have been cytogenetically analyzed, and most of these studies are restricted to karyotype macrostructure determined using conventional staining techniques (King, 1990).

For several years, amphibians have been regarded as one of the few vertebrate groups characterized by highly conserved karyotypes. Nonetheless, with improvements in

the methods of chromosomal banding and molecular cytogenetics, a remarkable variability in chromosomal microstructure has been reported, which allowed a refined identification of species and their geographical variants, thereby serving as a reliable tool for taxonomic studies (Kasahara et al., 2003; Siqueira et al., 2004).

Usually, hylids have 2n = 24, with a karyotype that mainly consists of biarmed chromosomes (King, 1990), and variations in chromosomal number have been regarded as apomorphies (Catroli, 2008). One exception is the genus *Dendropsophus*, separated from the genus *Hyla* to comprise species with 30 chromosomes despite their incipient morphological diagnosis, thus showing the importance of cytogenetics to analyze systematics in this group (Chek et al., 2001).

Within *Hypsiboas*, cytogenetic data have been shown for only 16% of the species, most of which have been derived based on conventional karyotype descriptions, and thus, some inferences about their chromosomal evolution that need to be determined using banding techniques are missing (Raber et al., 2004; Ananias et al., 2004; Gruber et al., 2006; Nunes and Fagundes, 2008; Carvalho et al., 2009).

The present study aimed to perform a thorough cytogenetic analysis in distinct species of *Hypsiboas* and compare these data to those obtained in previous studies in order to identify chromosomal markers that could be used to outline the evolutionary dynamics of chromosomal macro- and microstructure of these amphibians.

MATERIAL AND METHODS

Cytogenetic analyses were performed in specimens of *Hypsiboas crepitans*, *Hypsiboas pombali,* and *H. semilineatus* from Estação Ecológica Estadual Wenceslau Guimarães (EEEWG; municipality of Wenceslau Guimarães) and *Hypsiboas atlanticus* from an Atlantic forest fragment in the municipality of Itacaré; both sites are located in Bahia state, northeastern Brazil (Figure 1). Voucher specimens are deposited in the Cytogenetics Laboratory at Universidade Estadual do Sudoeste da Bahia and Museu de Zoologia, Universidade de São Paulo. Besides the data obtained in the present study, those obtained in previous studies in other *Hypsiboas* species and populations were also analyzed.

Mitotic chromosomes were obtained from the intestinal epithelium as described by King and Rofe (1976) with modifications (hypotonic treatment in distilled water for 20 min, followed by fixation in 3:1 Carnoy's solution for 24 h) or else by dissociation of liver tissue according to Garcia C. (unpublished data).

Chromosomal number and morphology were determined using conventional Giemsa staining, and the number of chromosomal arms (FN) was established taking into account that metacentric, submetacentric, and subtelocentric chromosomes bear 2 arms and acrocentric chromosomes are one-armed.

Idiograms were constructed on the basis of the results obtained in this study as well as those reported previously. Each karyotype figure was digitalized, and chromosomes were digitally measured according to Levan et al. (1964). The chromosomal measurements were used to build the idiograms. This standardization of chromosomal morphology pattern was required to allow a reliable comparison of distinct karyotype formulae and sizes, since different authors follow different criteria of chromosomal classification.

C-banding was performed according to Sumner (1972) with modifications. The

slides were initially immersed in 3:1 Carmoy's fixative for 10 min and then treated with 0.2 N HCl for 13 min at room temperature. Subsequently, the slides were immersed in $Ba(OH)_2$ solution at 60ºC for 60 s, followed by incubation in a 2X standard saline citrate bath at 60ºC for 30 min. Finally, the slides were stained with 5% Giemsa solution in phosphate buffer (pH 6.8).

Active nucleolus organizer regions (Ag-NORs) were detected using silver nitrate staining as proposed by Howell and Black (1980).

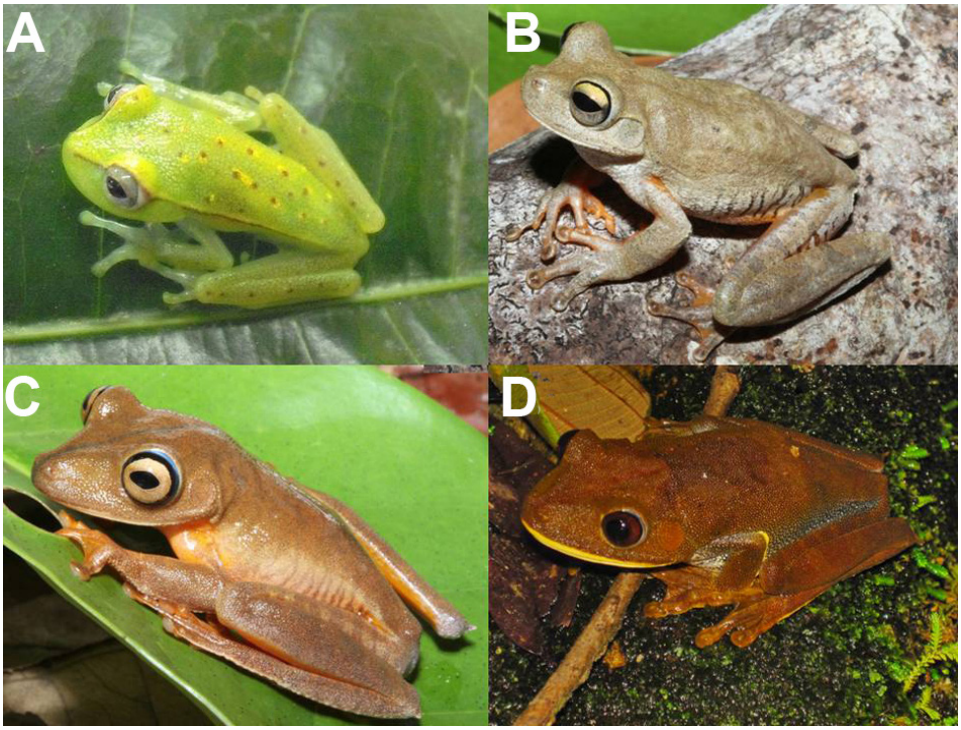

Figure 1. Photographs of individuals of *Hypsiboas* species analyzed in this study: **A.** *H. atlanticus*. **B.** *H. crepitans*. **C.** *H. pombali*. **D.** *H. semilineatus*. Photos by: Sérgio Siqueira Jr (A) and Mauro Teixeira Jr (B, C, D).

RESULTS

All species of *Hypsiboas* analyzed in this study had 2n = 24 and FN = 48, with distinct karyotype formulae (Figure 2, Tables 1 and 2).

Single Ag-NORs were identified at interstitial position on long arms of the seventh chromosomal pair in *H. crepitans, H. pombali*, and *H. semilineatus*. All silver-stained metaphases in *H. pombali* and *H. semilineatus* showed Ag-NOR marks on only 1 homologous chromosome from such a pair. *H. atlanticus* presented multiple and terminal NORs on long arms of 2 chromosomal pairs (10 and 12; Figure 2, boxes).

C-banding revealed heterochromatin blocks near centromeres in all chromosomes of *H. crepitans*, *H. semilineatus*, and *H. pombali*, as well as in pairs 1-5 of *H. atlanticus*. Additional C-bands were observed at subterminal and interstitial positions on long arms of pairs 1, 2, 3, 6, 7, and 8 of *H. atlanticus* and on the short arms of pair 3 in *H. crepitans*. Terminal heterochromatin segments coincident to NORs were detected only in *H. atlanticus*. In the other species, the Ag-NORs proved to be euchromatic (Figures 2 and 3).

The association of present data with the available cytogenetic reports in *Hypsiboas* assured a detailed comparison of karyotypic formulae in these amphibians (Table 1). After the chromosomal measurements were standardized, 8 karyotype groups could be defined, many of which were shared by distinct species (Table 2 and Figure 3).

Figure 2. Giemsa stained (I) and C-banded (II) karyotypes of: **a.** *H. atlanticus*; **b.** *H. crepitans*; **c.** *H. pombali*, and **d.** *H. semilineatus*. In boxes, the NOR-bearing chromosomes after silver nitrate staining and C-banding.

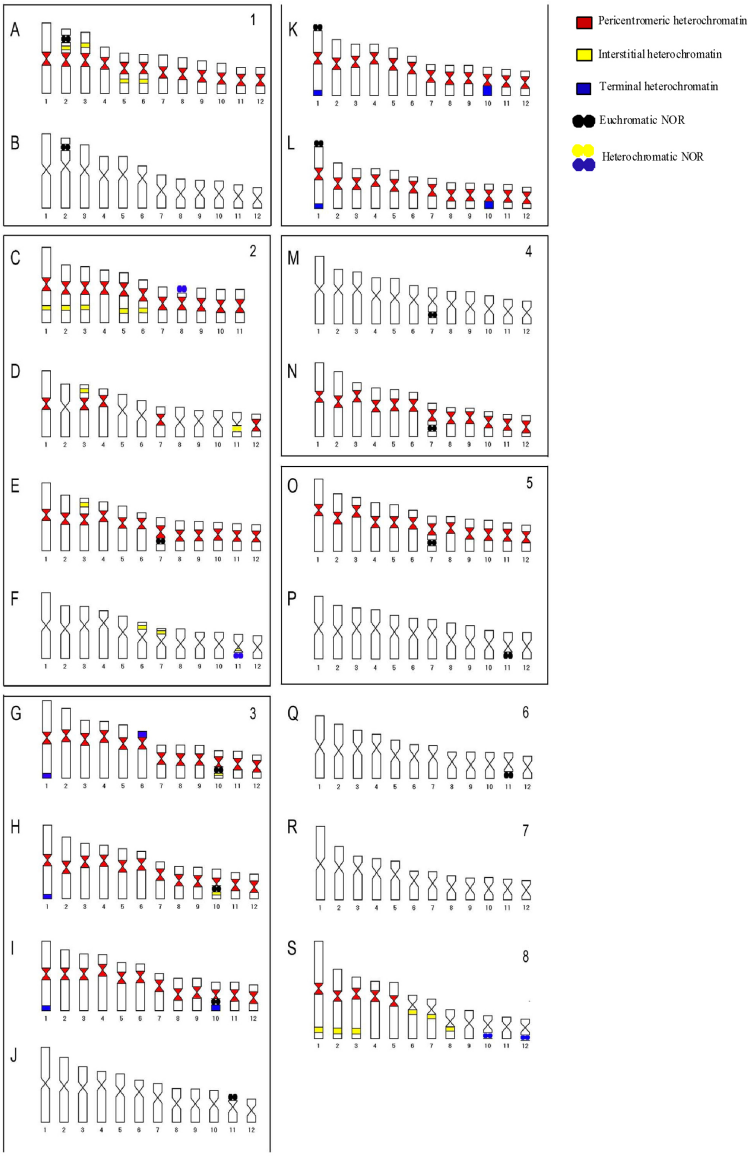

Figure 3. Idiograms of cytogenetically analyzed *Hypsiboas* species. **A.** *H. albomarginatus* (Carvalho et al., 2009); **B.** *H. albomarginatus* (Nunes and Fagundes, 2008); **C.** *H. albopunctatus* (Gruber et al., 2006); **D.** *H. crepitans* (Gruber et al., 2006); **E.** *H. crepitans* (present study); **F.** *H. raniceps* (Gruber et al., 2006); **G.** *H. bischoffi* (Raber et al., 2004); **H.** *H. guentheri* (Raber et al., 2004); **I.** *H. marginatus* (Ananias et al., 2004); **J.** *H. pardalis* (Nunes and Fagundes, 2008); **K.** *H. semiguttatus* (Ananias et al., 2004); **L.** *H.* sp aff *semiguttatus* (Ananias et al., 2004); **M.** *H. semilineatus* (Nunes and Fagundes, 2008); **N.** *H. semilineatus* (present study); **O.** *H. pombali* (present study); **P.** *H. faber* (Carvalho et al., 2009); **Q.** *H. faber* (Nunes and Fagundes, 2008); **R.** *H. polytaenius* (Nunes and Fagundes, 2008); **S.** *H. atlanticus* (present study).

Table 1. Summary of available cytogenetic reports in *Hypsiboas*.

Species	Species group	Locality	2n	Karyotype formula	C-banding	Supernumeraries	NORs	Reference
H. albomarginatus	*H. faber*	Anchieta and Cariacica/ES	24	m = 1, 2, 3, 10, 11, 12 sm = 4, 5, 7, 8, 9 st = 6	Unavailable	*	IB 2p	Nunes and Fagundes (2008)
H. albomarginatus	*H. faber*	Bertioga, Mogi das Cruzes and Picinguaba/SP	24	m = 1, 2, 3, 7, 8, 9, 10, 11, 12 sm = 4, 5, 6	PB = all chromosomes IB = 2p and 3p; 5q and 6q NORs = euchromatic	*	IB 2p	Carvalho et al. (2009)
H. albopunctatus	*H. albopunctatus*	Rio Claro/SP	22	m = 1, 8, 10, 11 sm = 2, 3, 4, 5, 6, 7, 9	PB = all chromosomes IB = 1q, 2q, 3q, 7q NORs = heterochromatic S = partially heterochromatic	(1) small m	TB 8p	Gruber et al. (2006)
H. atlanticus	*H. punctatus*		24	m = 1, 9 sm = 2, 4, 8, 10, 11, 12 st = 3, 5, 6, 7	-	-	TB 10q e 12q	Present study
H. bischoffi	*H. pulchellus*	S. Francisco de Paula/RS Rancho Queimado/RS	24	m = 1, 2, 8, 11, 12 sm = 3, 5, 7, 9, 10 st = 4, 6	PB = all chromosomes TB = 1q IB = 10q 6 = short arms entirely heterochromatic NORs euchromatic	*	IB 10q	Raber et al. (2004)
H. crepitans	*H. faber*	Piranhas/AL	24	m = 1, 8, 10 sm = 2, 3, 4, 5, 6, 7, 9, 11, 12	PB = most chromosomes IB = 3p NORs = heterochromatic	*	IB 11q	Gruber et al. (2006)
H. crepitans	*H. faber*	Estação Ecológica de Wenceslau Guimarães/BA	24	m = 1, 2, 8, 9, 11, 12 sm = 3, 5, 6, 7, 10 st = 4	-	-	IB 7q	Present study
H. faber	*H. faber*	Pedra Azul/ ES	24	m = 1, 2, 9, 12 sm = 3, 5, 8, 10 st = 4, 6, 7, 11	-	*	IB 11q	Nunes and Fagundes (2008)
H. faber	*H. faber*	Mogi das Cruzes and Picinguaba/SP	24	m = 1, 2, 8, 9, 10 11, 12 sm = 3, 4, 5, 6, 7	-	*	TB 11q	Carvalho et al. (2009)
H. guentheri	*H. pulchellus*	Terra de Areia/SC	24	m = 1, 2, 8, 11, 12 sm = 3, 5, 7, 9, 10 st = 4, 6	PB = all chromosomes TB = 1q IB = 10q NORs euchromatic	*	IB 10q	Raber et al. (2004)
H. marginatus	*H. pulchellus*	S. Francisco de Paula/RS	24	m = 1, 2, 8, 11, 12 sm = 3, 5, 7, 9, 10 st = 4, 6	PB = all chromosomes 10q = entirely heterochromatic TB = 1q NORs = heterochromatic NORs = euchromatic	*	IB 10q	Ananias et al. (2004)

Continued on next page

Table 1. Continued.

Species	Species group	Locality	2n	Karyotype formula	C-banding	Supernumeraries	NORs	Reference
H. joaquini	*H. pulchellus*	-	24	m = 1, 2, 8, 11, 12 sm = 3, 5, 7, 9, 10 st = 4, 6	PB = all chromosomes 10q = entirely heterochromatic TB = 1q NORs = euchromatic	*	TB 1p	Ananias et al. (2004)
H. paradalis	*H. faber*	Cariacica/ES	24	m = 1, 2, 9, 11, 12 sm = 3, 5, 7, 8, 10 st = 4, 6	Unavailable	*	TB 11p	Nunes and Fagundes (2008)
H. pombalis	*H. semilineatus*	Estação Ecológica de Wenceslau Guimarães/BA	24	m = 1, 4, 8, 9, 10, 11, 12 sm = 2, 3, 5, 6, 7	-	-	IB 7q	Present study
H. raniceps	*H. albopunctatus*	Brasilândia/MS	24	m = 1, 8, 10, 11 sm = 2, 3, 4, 5, 6, 7, 9, 12	PB = 11q IB = 6p and 7p NORs = heterochromatic	*	TB 11q	Gruber et al. (2006)
H. semiguttatus	*H. pulchellus*	Carambá do Sul/SC S. Francisco de Paula/RS Piraquara/PR	24	m = 1, 2, 8, 11, 12 sm = 3, 5, 7, 9, 10 st = 4, 6	PB = all chromosomes 10q = entirely heterochromatic TB = 1q NORs = euchromatic	*	TB 1 p	Ananias et al. (2004)
H. semilineatus	*H. semilineatus*	Santa Teresa/ES	24	m = 1, 2, 10, 11, 12 sm = 4, 5, 8 st = 3, 6, 7, 9	Unavailable	*	IB 7q	Nunes and Fagundes (2008)
H. semilineatus	*H. semilineatus*	Estação Ecológica de Wenceslau Guimarães/BA	24		-	-	IB 7q	Present study
H. sp aff *semiguttatus*	*H. pulchellus*	Misiones/Argentina	24	m = 1, 2, 8, 11, 12 sm = 3, 5, 7, 9, 10 st = 4, 6	PB = all chromosomes 10q = entirely heterochromatic TB = 1q NORs = euchromatic	*	TB 1p	Ananias et al. (2004)

m = metacentric; sm = submetacentric; st = subtelocentric; PB = pericentromeric band; TB = terminal band; IB = interstitial band; NORs = nucleolus organizer regions; q = long arm; p = short arm; * no data

Table 2. Karyotype formulae in species of *Hypsiboas* after standardization of chromosomal measurements.

Species	Chromosomal Pairs												Reference
	1	2	3	4	5	6	7	8	9	10	11	12	
Karyotype group 1													
H. albomarginatus	m	m	m	sm	sm	sm	m	m	m	m	m	m	Carvalho et al. (2009)
H. albomarginatus	m	m	m	sm	sm	st	m	m	m	m	m	m	Nunes and Fagundes (2008)
Karyotype group 2													
H. albopunctatus	m	m	sm	st	sm	sm	sm	m	sm	m	m	-	Gruber et al. (2006)
H. crepitans	m	m	sm	st	sm	sm	sm	m	m	sm	m	m	Gruber et al. (2006)
H. crepitans	m	m	sm	st	sm	sm	sm	m	sm	m	m	m	Present study
H. raniceps	m	m	sm	st	sm	sm	sm	m	sm	m	m	m	Gruber et al. (2006)
Karyotype group 3													
H. bischoffi	m	m	sm	st	sm	st	sm	m	m	m	m	m	Raber et al. (2004)
H. guentheri	m	m	sm	st	sm	st	sm	m	m	m	m	m	Raber et al. (2004)
H. marginatus	m	m	sm	st	sm	st	sm	m	m	m	m	m	Ananias et al. (2004)
H. pardalis	m	m	sm	st	sm	st	sm	m	m	m	m	m	Nunes and Fagundes (2008)
H. semiguttatus	m	m	sm	st	sm	st	sm	m	m	m	m	m	Ananias et al. (2004)
H. sp aff *semiguttatus*	m	m	sm	st	sm	st	sm	m	m	m	m	m	Ananias et al. (2004)
Karyotype group 4													
H. semilineatus	m	m	st	sm	sm	st	sm	sm	sm	m	m	m	Nunes and Fagundes (2008)
H. semilineatus	m	m	st	sm	sm	st	sm	sm	sm	m	m	m	Present study
Karyotype group 5													
H. pombali	m	m	sm	m	sm	sm	sm	m	m	m	m	m	Present study
H. faber	m	m	sm	sm	sm	sm	sm	m	m	m	m	m	Carvalho et al. (2009)
Karyotype group 6													
H. faber	m	m	sm	st	sm	st	st	sm	m	m	sm	m	Nunes and Fagundes (2008)
Karyotype group 7													
H. polytaenius	m	sm	sm	sm	sm	st	sm	m	m	m	m	m	Nunes and Fagundes (2008)
Karyotype group 8													
H. atlanticus	m	m	st	st	sm	st	m	sm	m	sm	sm	sm	Present study

m = metacentric; sm = submetacentric; st = subtelocentric.

With regard to the karyotype macrostructure, only chromosomal pairs 1 and 5 (metacentric and submetacentric, respectively) remained unchanged in all species analyzed thus far. The remaining chromosomal pairs were characterized by variations in centromere position, although pairs 2, 10, 11, and 12 were less variable in morphology (Table 2).

Comparison of the distribution pattern of constitutive heterochromatin and Ag-NORs revealed that some chromosomal markers could be identified in *Hypsiboas albomarginatus* (interstitial NORs on short arms of pair 2), *H. crepitans* (interstitial and subterminal heterochromatin on short arms of pair 3), and in species of the *H. pulchellus* group (terminal heterochromatin on long arms of pair 1; Figure 3).

DISCUSSION

Of the 86 species recognized of the genus *Hypsiboas*, only 15 have been cytogenetically analyzed, including those analyzed in the present data. All species share 2n = 24 chromosomes and FN = 48, except *H. albopunctatus* (2n = 22; Table 1). The predominance of a diploid number of 24 chromosomes is one of the main cytogenetic features within Hylidae, even though higher and lower diploid values have already been reported (2n = 18 in *Aplastodiscus leucopygius* up to 2n = 52 in *Phyllomedusa tretraploidea*; Catroli, 2008). The karyotype consisting of 24 chromosomes or lower diploid values present in derived anuran families such as Dendrobatidae, Hylidae, Leptodactylidae, and Ranidae have been thought to have evolved through numerical reduction from a putative ancestor with 2n = 26 (Bogart, 1973), as

corroborated by the phylogeny proposed by Faivovich et al. (2005).

According to Gruber et al. (2006), 2 mechanisms could account for the reduction in the diploid number of *H. albopunctatus*: tandem fusions or translocations between the smallest chromosomes that also likely resulted in the differential NOR position. These authors have also argued that the occurrence of a supernumerary chromosome reported in this species could be indicative of the former presence of 24 chromosomes in *H. albopunctatus*.

As for chromosomal morphology, the karyotypes of *Hypsiboas* mainly consist of several pairs of biarmed chromosomes that are thought to have evolved dynamically. While both morphology and size of pairs 1 and 5 remain constant in all species/populations studied, pairs 3, 4, 6, and 7 are morphologically variable between samples ranging from metacentric and submetacentric to subtelocentric (Table 2). This behavior, coupled with the size maintenance of chromosomal pairs, is strong evidence that pericentric inversions are the main mechanism responsible for the differentiation of the karyotype macrostructure in this group.

On the basis of such chromosomal macrostructure, we clustered the species and/or populations analyzed into 8 groups, distinguished by their karyotype formulae (herein called karyotype groups; Figure 3 and Table 2). These karyotype groups were not composed of populations from a single species or species from the same group established by Faivovich et al. (2005) and Wiens et al. (2010). For instance, in the *H. faber* species group, 5 karyotype formulae were identified (karyotype groups 1, 2, 3, 5, and 6). Similarly, *H. semilineatus* and *H. pulchellus* showed 2 karyotype groups each (karyotype groups 4-5 and 3-7, respectively). On the other hand, the groups *H. albopunctatus* and *H. punctatus* comprised a single chromosomal pattern (karyotype groups 2 and 8, respectively). Thus, in spite of sharing a similar karyotype pattern, the chromosomal microstructure within *Hypsiboas* was highly variable and could be used to differentiate species and their geographical variants.

In general, NORs are a useful cytogenetic marker in amphibians, and the number and location of these sites remain constant within most species of anurans (Lourenço et al., 1998).

On the other hand, NORs in species and populations of *Hypsiboas* are variable, although most species presented single NORs located interstitially on long arms of medium-sized to small chromosomes (pairs 7, 10, and 11; present study; Ananias et al., 2004; Gruber et al., 2006; Nunes and Fagundes, 2008). Besides interspecific variation, 2 populations of *H. crepitans* (present study; Gruber et al., 2006) also differed in relation to the NOR-bearing pair. These data indicate that NORs are weak chromosomal markers for species identification of the genus *Hypsiboas* when used separately from other techniques or, alternatively, they might indicate the presence of cryptic species. Nonetheless, NORs located on the largest chromosomes (pairs 1 and 2) or terminal NORs were restricted to certain groups, serving as species-specific markers, such as in *H. albomarginatus* and *Hypsiboas semiguttatus* (Ananias et al., 2004; Nunes and Fagundes, 2008).

To our knowledge, this is the first report of multiple NORs in *H. atlanticus*, unlike the predominance of terminal single NORs in anurans (King et al., 1990). The available phylogenetic studies in *Hypsiboas* suggest that interstitial single NORs are likely a plesiomorphic trait (Nunes and Fagundes, 2008), while variant conditions would be apomorphic.

The variability in NOR location could be explained by translocation or transposition events; both the number and size of chromosomes remain unchanged among the distinct karyotypes analyzed. Similar mechanisms can be inferred to determine the multiple NORs in *H. atlanticus*. Putatively, the ancestor species would bear terminal NORs on pair 10. A portion

of rDNA sites would have been transferred to the terminal region of pair 12 via translocation or transposition, resulting in the small silver-stained signals observed in this species. Likewise, transposition and/or translocation events have been regarded as the main mechanisms of dispersal of 45S ribosomal sites in anurans with multiple NORs (Schmid et al., 1995).

The application of fluorescent in situ hybridization (FISH) using 45S rDNA performed in 4 *Hypsiboas* species (*H. albomarginatus*, *H. faber*, *Hypsiboas pardalis*, and *H. semilineatus*) invariably confirmed the occurrence of single NORs (Nunes and Fagundes, 2008). This technique would be very useful in the present study to corroborate this pattern once a single homologous chromosome was stained with silver nitrate in both *H. pombali* and *H. semilineatus*, indicating preferential activation of ribosomal sites in the preceding interphase. Moreover, FISH could be useful to verify the actual occurrence of multiple NORs in *H. atlanticus*, since there are reports of intraspecific silver-stained regions equivalent to heterochromatin segments with acidic nature in other animal groups (Sumner, 1990; Sánchez et al., 1995).

Heterochromatin distribution is one of the main features used in chromosomal differentiation between species or populations of anurans. Thus, these regions represent one of the most widely used chromosomal markers in cytotaxonomic studies of species within the genera *Hyla* (Anderson, 1991), *Telmatobufo* (Formas and Cuevas, 2000), and *Allobates* (Veiga-Menoncello et al., 2003).

The few available reports in *Hypsiboas* show that most species present large blocks of pericentromeric heterochromatin through the karyotype. Interstitial C-bands are observed in some chromosomes of a few species, and terminal blocks are even less frequent (Table 1 and Figure 2). However, a thorough comparison of the pattern of heterochromatin distribution among populations is hindered because most reports lack C-banding data.

The evolutionary relationships of the species studied in the molecular phyaslogeny established by Faivovich et al. (2005) and the data compiled in Figure 3, as well as those from closely related species, suggest that the karyotypes exclusively comprising pericentromeric heterochromatin should reflect a plesiomorphic feature in this group. Therefore, interstitial or terminal heterochromatic blocks would be considered variation of the ancestral condition.

The species of *Hypsiboas* with interstitial/terminal heterochromatin are known to present reduced amounts of pericentromeric blocks, as observed in *H. albomarginatus*, *H. albopunctatus*, *H. crepitans*, *Hypsiboas raniceps*, and *H. atlanticus* (Gruber et al., 2006, Carvalho et al., 2009; present study). This association can be the result of chromosomal inversions that determined the transference of heterochromatic regions to chromosomal arms, thereby changing the pair morphology and reinforcing that inversions are the main mechanism of karyotype evolution in *Hypsiboas*. Such inversions, involving heterochromatic segments, could also account for the existence of some species with heterochromatic NORs, while most of the species present euchromatic NORs. These regions possibly became interspersed or associated with heterochromatic after the occurrence of such structural reorganization of chromosomes.

The interstitial heterochromatin in pair 3 of *H. crepitans* can be a potential species-specific marker, since it is observed only in populations of this species and is conserved throughout geographical variants. A similar situation is found in relation to the C-terminal band on the long arms of pair 1 in species from the group *H. pulchellus* and for the terminal block on the short arms of pair 6 in *Hypsiboas bischoffi* (Figure 3).

The pattern of heterochromatin and NOR distribution among populations of a single species or closely related species is thought to be similar. However, according to the data

shown in figure 3 and the collection sites of populations analyzed, we can infer that the evolution of karyotype microstructure is related to geography than to phylogenetic relationships. For instance, the species studied from EEEWG shared the same C-banding and Ag-NOR pattern despite the differences in the karyotype formulae. This pattern was also reported for *Hypsiboas marginatus*, *H. semiguttatus*, and *H.* aff. *semiguttatus* (Ananias et al., 2004) and for *H. bischoffi* and *Hypsiboas guentheri* (Raber et al., 2004). This relationship between evolutionary dynamics of chromosomal microstructure and geographic location could be determined by unique environmental features that favored an optimal karyotype organization. Similar inferences have been proposed to explain geographical variation in lizards of the genus *Tropidurus* (Kasahara et al., 1996) and some fish groups, such as *Leporinus* (Galetti Jr et al., 1981, 1984). Nonetheless, this hypothesis can only be confirmed with increased cytogenetic studies in the genus *Hypsiboas*.

Our data suggest that *Hypsiboas* represents an interesting model to study chromosomal evolution in anurans, because this genus presents a remarkable karyotype variation, although most of its diversity remains unexplored karyologically. The identification of cytotaxonomic markers and the putative phylogeographic relationships in the karyotype microstructure indicate that further chromosomal studies are required in this genus. Due to taxonomical controversy of this frog group, population studies using cytogenetic and molecular techniques should also be performed to identify the cryptic diversity in some species, such as those detected in *H. crepitans* and *H. faber*.

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