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B CHROMOSOMES IN THE TREE FROG *HYPSIBOAS ALBOPUNCTATUS* (ANURA: HYLIDAE)

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ABSTRACT: Supernumerary or B chromosomes are one of the main causes for numerical chromosomal variation in higher eukaryotes. These extragenetic elements have been studied for more than a century, with the goal of trying to understand their origin, and how they survive as a polymorphism in natural populations. *Hypsiboas albopunctatus* is a nocturnal hylid frog distributed in the central–eastern part of South America. Previously, variation in chromosome numbers was described for a population from Rio Claro, São Paulo, Brazil, in which a single small-sized, metacentric B chromosome was present in ca. 40% of the analyzed individuals ($n = 17$). We herein describe the presence of B chromosomes in populations of *H. albopunctatus* from northeastern Argentina (Corrientes and Misiones provinces), with unusual morphological and structural characteristics. The frequency of B chromosomes varied significantly among analyzed populations. We found four diploid numbers ($2N = 22$, $22 + 1B$, $22 + 2B$, and $22 + 3B$), and in a few individuals mitotic instability occurred. C banding revealed variations in the heterochromatin (DAPI+) pattern between Bs with similar morphology, indicating the existence of two new structural variants of these supernumerary elements in *H. albopunctatus* (B1 and B2). Nucleolar organizer regions marked positively on the eighth pair, coincident with the location of ribosomal DNA as demonstrated with fluorescent in situ hybridization, but Bs did not mark positive with these two techniques. Also, fluorescent in situ hybridization with telomeric probes showed no differences in location and intensity between Bs and autosomal chromosomes. The present communication is the first case of B chromosome polymorphisms in hylid frogs and the sixth reported in Anura.

Key words: FISH; *Hypsiboas albopunctatus*; Polymorphism; Supernumerary chromosome

B CHROMOSOMES (Bs) are, by definition, dispensable supernumerary chromosomes that do not recombine with autosomes and follow their own evolutionary pathway (definition from J. P. M. Camacho and J. S. Parker; cited in Beukeboom, 1994). The presence of these accessory elements is one of the causes of intraspecific numerical chromosomal variation in higher organisms, and their occurrence has been reported for a vast amount of species (Jones and Rees, 1982; Beukeboom, 1994; Camacho et al., 2000). In anurans, B chromosome descriptions are available for 12 families: Alytidae, Cycloramphidae, Craugastoridae, Eleutherodactylidae, Hemiphractidae, Hylidae, Hylodidae, Leiopelmatidae, Leiuperidae, Ranidae, Scaphiopodidae, and Strabomantidae (Green, 2004; Medeiros et al., 2006; Gruber et al., 2007; Lanzone et al., 2008; Milani et al., 2010; Schmid et al., 2010). The family Hylidae is the largest in Anura, currently comprising 901 species, with an extraordinary diversity (Frost, 2011). More than 280 species have been karyotyped in this group (Catroli and Kasahara, 2009), and only four of them are known to carry Bs: *Acris crepitans*, *Bokermannohyla luctuosa*, *Dendropsophus nanus*, and *Hypsiboas albopunctatus* (Green, 2004; Medeiros et al., 2006; Gruber et al., 2007; Catroli and Kasahara, 2009). *Hypsiboas albopunctatus* was cytogenetically studied by Gruber et al. (2007), who analyzed 17 males from a population in Rio Claro, São Paulo, Brazil, reporting the existence of a metacentric, small-sized, and mitotically stable B. This supernumerary element was mainly heterochromatic (C+), with the exception of the distal region of both arms; its size and shape were similar to the eighth autosomal pair. It reached a maximum number of one per cell and remained as an univalent during meiosis.

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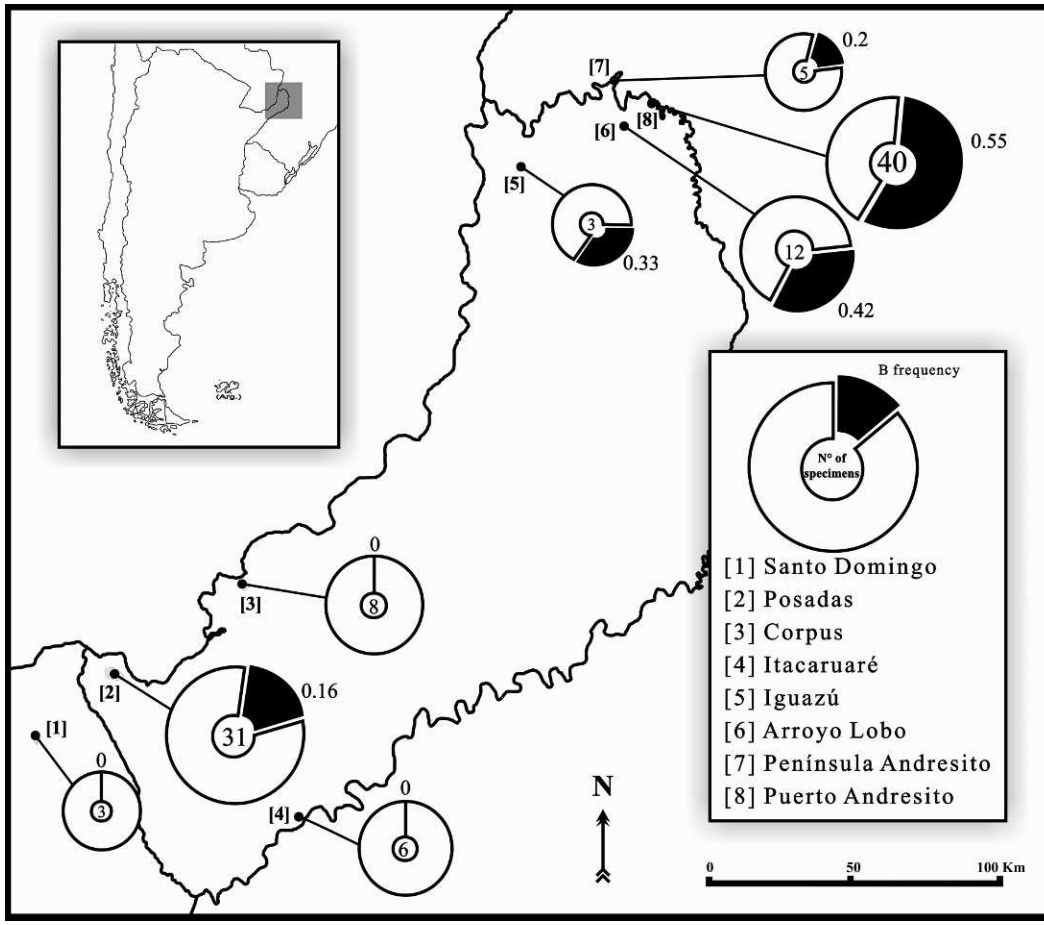

FIG. 1.—Map of distribution of B chromosomes of *Hypsiboas albopunctatus* and its prevalence per locality.

Despite being distributed in many frog taxa, the geographic coverage and frequency, the genomic nature, and the genetic behavior of B chromosomes are still poorly understood. In this work, we studied variations in the chromosome number of Argentinean populations of *H. albopunctatus* due to the presence of B chromosomes. In order to identify a possible autosomal candidate for its origin, we describe their morphology and structure performing different banding techniques and fluorescent in situ hybridization (FISH). We discuss the markedly different frequencies of Bs observed among populations of *H. albopunctatus,* noting the existence of Bs in Anura as a rare event, with special reference to Hylidae.

MATERIALS AND METHODS

We analyzed cytogenetically 106 males and 2 females of *H. albopunctatus* collected from natural populations in the provinces of Corrientes and Misiones, northeastern Argentina, (Fig. 1, Table 1). Cytological preparations from bone marrow and gut were obtained following cellular suspension techniques (Schmid et al., 2010). Voucher specimens were deposited in the herpetological collection of the Museo de La Plata (MLP DB), Buenos Aires, Argentina; and at the Laboratorio de Genética Evolutiva (LGE), Posadas, Misiones, Argentina. Slides were conventionally stained with 10% phosphate-buffered Giemsa (pH = 6.8). Chromosomes were ordered and classified by their centromeric

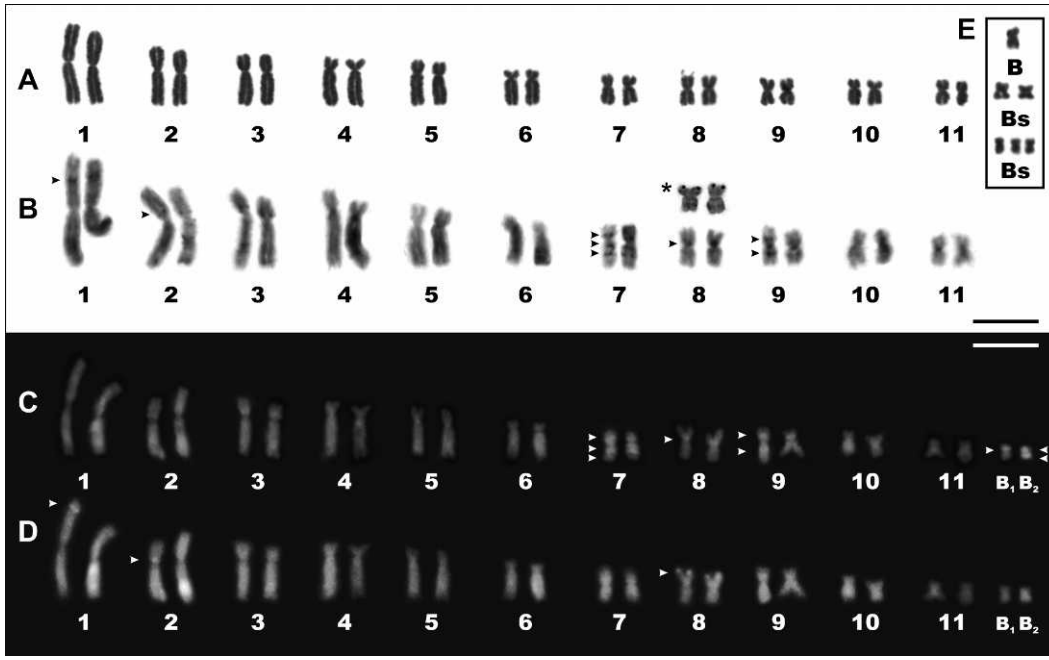

FIG. 2.—Karyotypes of *Hypsiboas albopunctatus* conventionally stained with Giemsa (A), C banding (B), DAPI (C) and CMA3 (D) staining pretreated with a C-banding–like protocol. Pair eight carrying nucleolar organizer regions is inset (*) as well as B chromosomes (E). Arrowheads indicate heterochromatin C+ (B), DAPI+ (C), and CMA3+ (D). Bar = 10 μm.

index (CI), with the use of the nomenclature proposed by Levan et al. (1964), taking into account the considerations made by Green and Sessions (1991), and chromosome morphology was analyzed with the use of the Micromeasure v3.3 software (Reeves and Tear, 2000). Bs were easily identified by their size, which is smaller than the smallest pair of

TABLE 1.—Specimens of *Hypsiboas albopunctatus* analyzed per locality, and the chromosome numbers found therein. F_B = frequency of Bs per locality (prevalence). Abbreviations: CO = Corrientes Province, Argentina; MI = Misiones Province, Argentina. Geographic coordinates are based on the datum WGS84.

			Maximum number of Bs and chromosome number (2N)				
Locality	Coordinates	N	0B (22)	1B (23)	2B (24)	3B (25)	F_B
Santo Domingo (CO)	27°39′S 56°08′W	3	3	–	–	–	0
Posadas (MI)	27°26′S 55°53′W	31	26	5	–	–	0.16
Itacaruaré (MI)	27°54′S 55°16′W	6	6	–	–	–	0
Corpus (MI)	27°08′S 55°27′W	8	8	–	–	–	0
Iguazú (MI)	25°47′S 54°32′W	3	2	1	–	–	0.33
Península Andresito (MI)	25°32′S 54°07′W	5	4	–	1	–	0.2
Puerto Andresito (MI)	25°35′S 53°59′W	40	18	18	3	1	0.55
Arroyo Lobo (MI)	25°42′S 54°05′W	12	7	5	–	–	0.42
Total		108	74	29	4	1	0.31

TABLE 2.—Chromosome morphology in *Hypsiboas albopunctatus*. Abbreviations: CN = chromosome number; CI = centromeric index; SD = standard deviation; m = metacentric; sm = submetacentric; st = subtelocentric.

Hypsiboas albopunctatus												
$2N = 22 + B_1B_2$												
CN	1	2	3	4	5	6	7	8	9	10	11	B1	B2
% set	16.13%	12.44%	11.7%	11.15%	9.74%	8.1%	6.8%	6.66%	6.56%	5.75%	4.96%	3.95%	3.64%
CI ± SD	0.49 ± 0.01	0.40 ± 0.04	0.32 ± 0.01	0.24 ± 0.04	0.29 ± 0.01	0.26 ± 0.02	0.36 ± 0.03	0.47 ± 0.03	0.37 ± 0.05	0.34 ± 0.04	0.47 ± 0.03	0.46 ± 0.05	0.45 ± 0.03
Type	m	m	sm	st	sm	sm	sm	m	m	sm	m	m	m

the chromosome complement. Nucleolar organizer region (NOR) detection was performed following silver impregnation (Howell and Black, 1980), and location of heterochromatin was made by standard C-banding (Sumner, 1972). Heterochromatin AT-rich and GC-rich sequences were evidenced with DAPI and Chromomycin A3 staining (CMA3; Schweizer, 1976), respectively; and as well with the modifications introduced by Barros e Silva and Guerra (2010). Fluorescent in situ hybridization (FISH) was carried out with a biotin-labeled 18S rDNA probe (Viegas-Péquignot, 1992), and an all-human telomere probe marked with digoxigenin (Oncor P4097-DG5) following the manufacturer's protocol, to detect, respectively, with avidin-Cy3 and anti-digoxigenin-fluorescein.

RESULTS

Hypsiboas albopunctatus presented a standard diploid set of 2N = 22 chromosomes, with no distinguishable sex chromosomes. Pairs 1, 2, 8, 9, and 11 are metacentric; 3, 5, 6, 7, and 10 submetacentric, whereas the fourth pair is subtelocentric (Fig. 2, Table 2). We observed a C+ interstitial band on chromosome 1 and on the proximal region of 2p, as well as 8p, whereas Pair 7 was C positive in the proximal regions of both arms, and interstitially in the q arm. Also, Pair 9 showed an interstitial C+ band on 9p9q (Fig. 2B).

After performing a C-banding–like pretreatment, bright DAPI+ signals appeared proximally on 7p7q plus interstitially on 7q, proximally on 8p, in addition to 9p9q (Fig. 2C). Also, CMA3+-rich CG heterochromatin was evident on the interstitial and proximal regions of chromosomes 1 and 2, respectively, and at the end of the p arm of Pair 8 (Fig. 2D) coinciding with the location of Ag-NORs (DAPI-; Fig. 2B). Nevertheless, DAPI/CMA3 staining without pretreatment did not reveal evident signals in any autosome (Fig. 3A,B). FISH with the ribosomal DNA probe showed specificity only for the eighth pair, matching with NORs, observed with silver impregnation (Fig. 3C), and the telomeric probe produced signals on all telomeres of all chromosomes, with no

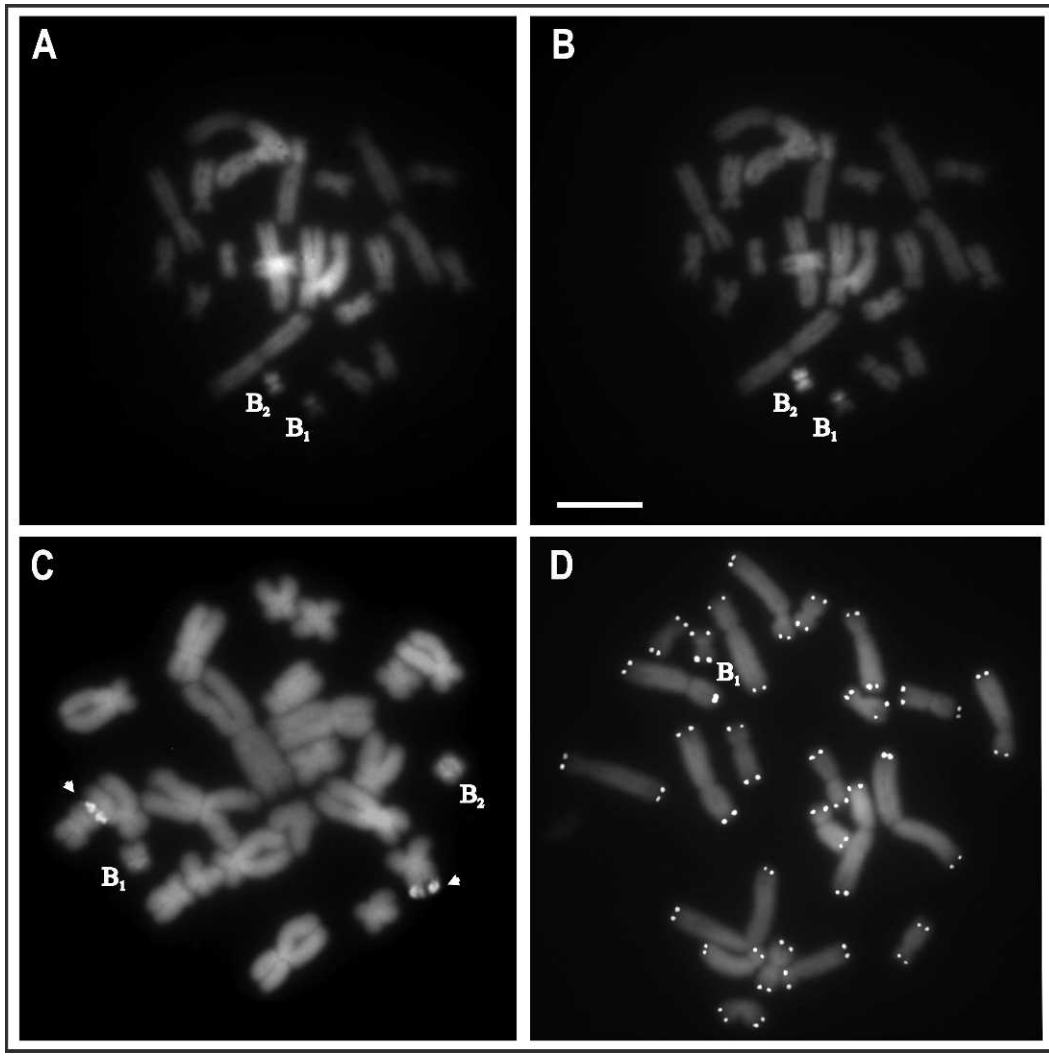

FIG. 3.—CMA3 and DAPI banding (A and B, respectively) in *Hypsiboas albopunctatus* chromosomes; FISH with 18S DNA probe (C), and telomeric DNA probe (D). Arrowheads indicate eighth pair marking rDNA. B1 and B2 are indicated. Bar = 10 μm.

ectopic bright fluorescence signals in any chromosome (Fig. 3D).

Four different chromosome numbers with 0, 1, 2, and 3 B chromosomes were found. Supernumeraries revealed mostly a euchromatic nature, with little pericentromeric AT-rich heterochromatin (DAPI+) and a lack of bright CMA3. C banding plus DAPI was performed on 28 individuals from a total of 34 B+ frogs; all of them showed this pattern. Nonetheless, in an individual with two Bs (MLP DB 8214), each one showed different amounts and location of heterochromatin, allowing us to identify two kinds of supernumeraries. One of them presented scarce heterochromatin in the proximal region of the p arm (B1), and the other C+ bands in the proximal regions of both arms (B2), and also DAPI+ (Fig. 2C, 3B). Both of them were metacentric (B1: CI = 0.46 ± 0.05; B2: CI = 0.45 ± 0.03), and smaller in size than the smallest autosome pair (11), representing approximately 3.95% (B1) and 3.64% (B2) of the diploid complement (Table 2).

Unlike the eighth pair, accessories had no affinity for silver staining, showing an absence of active Ag-NOR sites. The same was observed when we performed FISH with 18s rDNA, where B1 and B2 did not show Cy3 fluorescence (Fig. 3C). Telomeric DNA hybridization was made on an individual with only one B (B1); it showed bright signals on its telomeres (Fig. 3D).

As shown in Table 1, 74 specimens (68.52%) presented the standard karyotypic formula 2N = 22, 29 (26.85%) had one B, 4 of them two Bs (3.7%), and just 1 individual showed three Bs (0.9%). Bs were detected only in five localities (Posadas, Iguazú, Arroyo Lobo, Peninsula Andresito, and Puerto Andresito); they were absent in Santo Domingo, Itacaruaré, and Corpus. It is worth noting the significant contrast in the frequency of Bs among the larger samples from Posadas and Puerto Andresito (Fig. 1). In addition, we found two B+ specimens from Puerto Andresito and one from Arroyo Lobo with intraindividual variation in their chromosome numbers; all of them presented cellular mosaics 2N = 22 (0 B) and 2N = 23 (1 B) and less than 20% of the cells were B+. Of these, only a single male from Puerto Andresito carried the B1 type, whereas in the other specimens the B type were not determined because of their rarity, which made their characterization difficult.

Discussion

The *H. albopunctatus* group is composed of nine species (Faivovich et al., 2005), of which only three have been cytogenetically analyzed: *Hypsiboas raniceps* and *Hypsiboas fasciatus* with 2N = 24 chromosomes, and *H. albopunctatus* with 2N = 22 (Bogart and Bogart, 1971; Bogart, 1973; Gruber et al., 2007; this article). Herein, we present differences in the chromosome morphology of the standard karyotype from previous reports for *H. albopunctatus* (for review see Gruber et al., 2007). We also report additional heterochromatin compared to the data of Gruber et al. (2007) on chromosomes 7, 8, and 9. These discrepancies in chromosome shape and heterochromatin content may possibly be a consequence of the measuring procedure and technique resolution respectively, and not necessarily interpopulation differences. Gruber et al. (2007) studied the chromosomes of 17 males of *H. albopunctatus*, observing interindividual variations in the chromosome number due to the presence of a single small-sized metacentric and mitotically stable supernumerary chromosome, which is almost completely heterochromatic except for its distal regions. The authors explained that this B could have originated from a partial or complete duplication of the eighth chromosome pair of the complement of *H. albopunctatus*, subsequently degenerating, losing homology with its precursor, and undergoing the elimination of the NOR. This hypothesis is plausible, but the actual evidence available is insufficient to speculate about the origin of this chromosome, and many other different scenarios are possible.

In the specimens from Argentina studied by us, Bs are morphologically similar to each other and have little heterochromatin. Even so, in relation to the quantity of C+ heterochromatin (DAPI+), we identified more frequently a B type with a relatively lesser amount of heterochromatin (B1). B1 was present in all B+ individuals we observed, whereas richer heterochromatic B (B2) was only seen in a frog B1-B2. It was not possible to determine whether the existence of B2 is indeed a low-frequency polymorphism in the studied population or a new chromosomal mutation. Whatever the situation, its presence raises a question about the origin of this variant, which probably involved rearrangement of B1 (perhaps by centric misdivision). This is supported by the nearly perfect metacentric shape and DAPI+ heterochromatin disposition on both arms.

Hylidae is a very large family representing over 15% of anuran species (Frost, 2011). Chromosomes have been studied in approximately 35% of its taxa, with the vast majority of studies limited to conventional techniques (Catroli and Kasahara, 2009). So far only four species within this group are reported to have supernumeraries (Schmid et al., 2010). Hernandez-Guzmán et al. (2011) recently reported a B in the Mexican tree frog *Smilisca baudinii*, but only two specimens from different sexes were analyzed, one of them carrying an accessory chromosome. In the

genus *Hypsiboas*, the 2N = 24 chromosome complement is highly conserved, and karyotypes with fewer chromosomes such as the 2N = 22 observed in *H. albopunctatus* could be the result of different chromosomal rearrangements that would have led to a reduction in the basic number (Bogart, 1973; Kasahara et al., 2003; Gruber et al., 2007). Bogart (1973) stated that this reduction may have occurred by an end-to-end fusion that involved two chromosomes of the complement, or by the translocation of genetic material to different chromosomes from one of the smallest chromosomes of the 2N = 24 type (e.g., 11 or 12). These rearrangements would have changed the NOR marker position from the 11th chromosome in the standard karyotype, to Pair 8 in *H. albopunctatus*, leaving the homologue of the small chromosome involved in this rearrangement in a heteromorphic state, leading to degeneration and heterochromatinization, thus becoming a small metacentric B (Bogart, 1973; Gruber et al., 2007). On the other hand, FISH with rDNA showed specificity for Pair 8 but not for Bs, which is the opposite of what would be expected if these Bs originated from an ancestral degenerated NOR carrier. Although structural degeneration and gene silencing are very common and essential features of Bs (Green, 1990), more research is necessary to strengthen this hypothesis. High-resolution techniques are required to uncover the source of B chromosomes (Sharbel et al., 1998; Teruel et al., 2009) and to test for genetic homology between these elements and the genome of *H. albopunctatus* and related species. In addition, the telomeric probe demonstrated signals in the distal parts of all chromosomes including the supernumerary B1. Remarkably, we did not observe any ectopic telomeric signal either on the autosomes or on the Bs.

The available data demonstrate that it is not possible to make generalizations about B characteristics among anurans, because of the enormous diversity in their Bs. They can be large or small, with heterochromatin or entirely euchromatic; some may be completely inert, others transcriptionally active (Green, 1988, 1991, 2004; Schmid et al., 2010). Nonetheless, a common characteristic of most Bs in this order has been their somatic mitotic stability, maintaining a constant number per nucleus within individuals. Despite this, in *Odontophrynus americanus* (Rosset et al., 2006; Lanzone et al., 2008) and *Eleutherodactylus gundlachi* (Schmid et al., 2010) unstable supernumeraries were observed. Of 34 B+ *H. albopunctatus* analyzed in this article, 3 had intraindividual numeric differences on their chromosomal constitutions, with the presence of 1 B in less than 20% of analyzed cells. It is important to emphasize that in one of these three frogs, we could determine the kind of B as B1, but we do not know whether the other accessories were of the same type. Nur (1963, 1969), proposed that the maintenance of a B chromosome in a population may be highly conditioned by its instability, being essential to this an accumulation mechanism in germinal cells (in gonads), for instance. However, if B1 is stable in most studied individuals, why is there instability in one? In the three frogs that had unstable Bs, why did they have a very low occurrence (less than 0.2)? New studies including germinal cells are necessary to understand if this instability phenomenon could be counteracted by accumulation of B chromosome in gonads.

In the population of *H. albopunctatus* from Rio Claro, the frequency of supernumeraries was over 0.4 (Gruber et al., 2007). In our case, the frequency of the Bs differs significantly between populations. Northern populations exhibited a high frequency of Bs, contrary to what we observed in more southern ones as in Posadas (0.16). The lack of supernumerary chromosomes in some populations could be simply because of the small size of the samples and not because of their real absence.

B chromosomes are present in nearly 15% of the eukaryotic organisms, and have been reported to date in many species of plants, animals, and some fungi (Jones and Rees, 1982; Beukeboom, 1994; Camacho et al., 2000; Camacho, 2005). Amphibians are not an exception (see the review by Schmid et al., 2010). Even so, the chances of finding Bs in a group is perhaps limited by important factors such as the availability to researchers of techniques able to detect them in certain taxa, or the number of individuals per population analyzed (Beukeboom, 1994; Pal-

estis et al., 2004; Trivers et al., 2004; Camacho, 2005). In anurans, including *Hypsiboas albopunctatus*, other cases of B's polymorphism have been described: *Megaelosia massarti*, *Odontophrynus americanus*, *Gastrotheca espelettia*, *Eleutherodactylus gundlachi*, *Craugastor* sp. (as *Craugastor* sp. B in Schmid et al., 2010), and *Leiopelma hochstetteri* (Green, 1991, 2004; Lanzone et al., 2008; Schmid et al., 2010), with the latter frog containing the greatest diversity of B chromosomes described for vertebrates (more than 30 variants; Green, 2004). The divergence of Bs from a single ancestral B by structural changes represents a characteristic phenomenon of this type of chromosome (Green, 1990; Camacho et al., 2000). Even so, we do not know whether the diversity of Bs observed in *H. albopunctatus* is consequence of divergence from a common origin or if they have arisen independently.

Hypsiboas albopunctatus has a wide geographical distribution; from eastern Bolivia, to central–southern Brazil, eastern Paraguay, the northern part of Uruguay, and northeastern Argentina (Frost, 2011). The still-tiny portion of its geographic distribution that has been cytogenetically studied exhibited a rich diversity of B chromosomes, with large differences in their frequencies of occurrence. This situation strongly encourages further studies, both those with broader geographic coverage and those aimed at elucidating the evolutionary role Bs play in natural populations.

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