Genetica (2007) 130:281–291
DOI 10.1007/s10709-006-9105-6

ORIGINAL PAPER

Chromosome banding in three species of *Hypsiboas* (Hylidae, Hylinae), with special reference to a new case of B-chromosome in anuran frogs and to the reduction of the diploid number of 2n = 24 to 2n = 22 in the genus

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Received: 31 May 2006 / Accepted: 21 August 2006 / Published online: 20 September 2006
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Abstract The chromosomes of hylids *Hypsiboas albopunctatus*, *H. raniceps*, and *H. crepitans* from Brazil were analyzed with standard and differential staining techniques. The former species presented 2n = 22 and 2n = 23 karyotypes, the odd diploid number is due to the presence of an extra element interpreted as B chromosome. Although morphologically very similar to the small-sized chromosomes of the A complement, the B was promptly recognized, even under standard staining, on the basis of some characteristics that are usually attributed to this particular class of chromosomes. The two other species have 2n = 24, which is the chromosome number usually found in the species of *Hypsiboas* karyotyped so far. This means that 2n = 22 is a deviant diploid number, resulted from a structural rearrangement, altering the chromosome number of 2n = 24 to 2n = 22. Based on new chromosome data, some possibilities were evaluated for the origin of B chromosome in *Hypsiboas albopunctatus*, as well as the karyotypic evolution in the genus, leading to the reduction in the diploid number of 2n = 24 to 2n = 22.

Supported by FAPESP and CNPq

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Keywords Ag-NOR · BrdU incorporation · C banding · Fluorochrome staining · Replication banding · Supernumerary chromosome

Introduction

Hylidae is one of the most diversified family among anurans, with approximately 870 species, formerly grouped into the subfamilies Hemiphractinae, Hylinae, Pelodryadinae, and Phyllomedusinae, and with a wide geographical distribution in American, Australopapuan, and Eurasian continents (Frost 2004). Due to many taxonomic and systematic questions, extensive revisions have been carried out in this taxon, the most recent one by Faivovich et al. (2005). In this study, along with a systematic review of Hylidae, a comprehensive phylogenetic analysis was presented, on the basis of existing taxonomic evidences and molecular sequencing of mitochondrial as well as nuclear genes. Among the important conclusions, Faivovich et al. (2005) removed Hemiphractinae from the family, reducing the number of subfamilies to three, and presented a new monophyletic taxonomy for Hylinae, so that the Brazilian species previously included in the genus *Hyla* were assigned to another distinct genera. According to this, *Hyla albopunctata*, *Hyla raniceps*, and *Hyla crepitans* belong now to the genus *Hypsiboas*, and must be referred as *Hypsiboas albopunctatus* (Spix, 1824), *Hypsiboas raniceps* Cope, 1862, and *Hypsiboas crepitans* (Wied-Neuwied, 1824). The two former species are currently included in the *Hypsiboas albopunctatus* group, whereas the later in the *Hypsiboas faber* group (Faivovich et al. 2005).

Specimens of *Hypsiboas albopunctatus* from Brazil were karyotyped previously by Beçak (1968), Bogart (1973), Soma et al. (1974), Cardoso (1986), Baldissera and Batistic (1992), Feitosa et al. (1995), and from Argentina, by Insaurralde et al. (1991), all of them reporting diploid number of 2n = 22. Cardoso (1986) also found 2n = 23, due to the presence of B chromosome. *Hypsiboas raniceps* from Brazil and Argentina, and *H. crepitans* from Brazil and Colombia have been analyzed only with standard staining, both species showing 2n = 24 (Rabello 1970; Rabello et al. 1971; Bogart 1973; Insaurralde et al. 1991). The 2n = 22 had already been considered a deviant diploid number, taking into account that the referred species was a part of a dichotomy of the genus *Hyla* characterized by 2n = 24 chromosomes (King 1990).

Specimens of *Hypsiboas albopunctatus*, *H. raniceps*, and *H. crepitans* were karyotyped using both conventional and differential staining techniques, for obtaining new data to explain the reduction in the diploid number of 2n = 24 to 2n = 22. In this study, the karyotype of some individuals in the sample of *H. albopunctatus* showed an extra element interpreted as B chromosome, leading to the variation in diploid numbers of 2n = 22 and 2n = 23 in the species.

Material and methods

Our sample of *Hypsiboas albopunctatus* comprises 17 male specimens (CFBH07436-37-38, CFBH07441-42-43-44-45-46-47, CFBH07449-50-51, CFBH07453-54-55, and A337) collected in Rio Claro, state of São Paulo, in Southeastern Brazil. The unique female (CFBH07425) of *H. raniceps* was from Brasilândia, state of Mato Grosso do Sul, in Central Brazil, whereas the two males (CFBH02966-67) of *H. crepitans*, from Piranhas, state of Alagoas, in Northeastern Brazil. The voucher specimens are deposited in the Collection Célio F. B. Haddad, Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro, SP, Brazil.

Direct chromosome preparations of bone marrow, liver and testis were obtained from the specimens of *H. albopunctatus* previously injected with phytohemagglutinin to improve the mitotic index and 0.1% to 0.01% colchicine solution, according to the technique described in Baldissera et al. (1993). In some cases, *in vivo* treatment with 5-bromodeoxyuridine and 5-fluorodeoxyuridine was also performed (Silva et al. 2000), in order to obtain replication-banding patterns. From some specimens of *H. albopunctatus*, as well as from the female of *H. raniceps* and the males of *H. crepitans*, chromosome spreads were obtained from blood cells cultured according to Kasahara et al. (1998). In these cases, *in vitro* BrdU/FudR treatments were performed.

Conventional staining was carried out with Giemsa diluted in phosphate-buffered saline, pH 6.8. C banding and Ag-NOR labeling were performed according to Sumner (1972) and Howell and Black (1980), respectively. Fluorescence 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 (DA), according to Schweizer (1980), whereas replication banding was differentiated by Fluorochrome plus Giemsa (FPG) staining as described by Dutrillaux and Couturier (1981).

Results

Conventional staining

Two distinct karyotypes were found in the sample of *Hypsiboas albopunctatus*. Eight specimens have 2n = 22 karyotype (Fig. 1(a)), in which the first five pairs are of large size, pair 6 is medium-sized, and the remaining five pairs are of small size. Pairs 1, 8, 10, and 11 are metacentric, whereas pairs 2, 3, 4, 5, 6, 7, and 9 are submetacentric. Six specimens exhibited a similar karyotype, but with an odd diploid number of 2n = 23, due to the presence of a small-sized metacentric B chromosome (Fig. 1(b)). Although three specimens provided no mitotic metaphases their diploid numbers were determined by the analysis of meiotic phases as 2n = 22 in two and 2n = 23 in one male. *Hypsiboas raniceps* and *H. crepitans* have 2n = 24, with pairs 1 to 5 of large size, pair 6 of medium size, and pairs 7 to 12 of small size. In the former species, the pairs 1, 8, 10, and 11 are metacentric, and the remainder are submetacentric (Fig. 1(c)). *Hypsiboas crepitans* has an equivalent karyotype, but slight discrepancy was noticed as regard to the morphology of pair 11, which is submetacentric (Fig. 1(d)), contrary to that observed in *H. raniceps*. This difference may be explained by the occurrence of an interstitial secondary constriction in the long arms (Fig. 3 (c)), although not always clearly visible in all metaphases of *H. crepitans*.

Meiotic analyses of 2n = 22 male specimens of *Hypsiboas albopunctatus* revealed 11 bivalents in diplotene or metaphase I cells (Fig. 2(a) and (b)), and 11 chromosomes in metaphase II. In the case of 2n = 23 males, 11 bivalents were also observed plus one univalent corresponding to the B chromosome (Fig. 2(c)

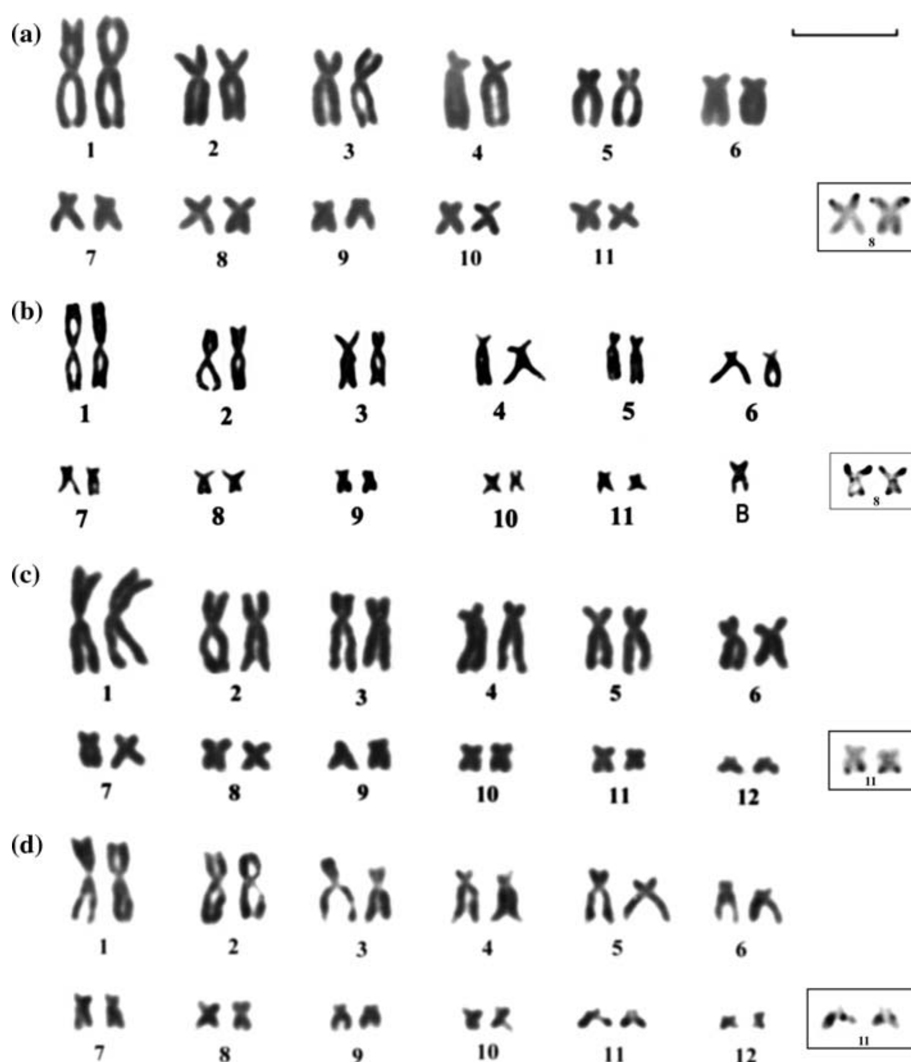

Fig. 1 Conventional Giemsa-stained karyotypes. Insets: chromosome pairs from the same metaphases after sequential silver staining. (**a**, **b**) *H. albopunctatus* with 2n = 22 (**a**) and 2n = 23 (**b**), and Ag-NOR in pair 8. (**c**) *H. raniceps* with 2n = 24 and Ag-NOR in pair 11. (**d**) *H. crepitans* with 2n = 24 and Ag-NOR in pair 11. Bar = 10 μm

and (d)). Metaphase II cells of these individuals had either 11 or 12 chromosomes (Fig. 2(e) and (f)).

There is no cytological evidence of heteromorphic sex chromosome pair in the males of *Hypsiboas albopunctatus*. The same is true for the female of *H. raniceps* and for the males of *H. crepitans*.

Differential staining

Both 2n = 22 and 2n = 23 karyotypes of *Hypsiboas albopunctatus* labeled Ag-NOR in one of the small-sized chromosome pairs (Fig. 1 (a) and (b), Fig. 3(b)). Sequential silver impregnation technique used in the previously standard stained metaphases confirmed the Ag-NOR site in the end of the short arms of the homologs of pair 8. Occasionally, these chromosomes appeared associated by silver stained nucleolar material (Fig. 3(a) and (b)). In *H. raniceps* and *H. crepitans*, Ag-NORs are located in the long arms of the chromosomes of pair 11, at the terminal and interstitial regions, respectively (Fig. 1(c) and (d), Fig. 3(d)). It is important to remark that in *H. raniceps*, similarly to *H. albopunctatus*, the site of the telomeric Ag-NOR was never observed as a secondary constriction.

C banding in *Hypsiboas albopunctatus* (Fig. 4(a)) revealed positively stained centromeric heterochromatin, but the chromosomes 1, 2, 3, and 7 also showed interstitial bands. In some metaphases, the chromosomes of pair 8 exhibited telomeric band, coincident to the site of Ag-NOR, and large block of C banded heterochromatin, extending through the pericentromeric region of both short and long arms. The B-chromosome presented a peculiar C banding pattern, with dark positive staining seen in almost the whole chromosome, with exception of the terminal regions of its short and long arms. In *H. raniceps*, C banded heterochromatin is almost absent, but the chromosomes 11 had a proximal positive band in the long arms, very prominent in one of the homologs; interstitial bands in the short arms of the chromosome

Fig. 2 Meiotic cells of *H. albopunctatus*. (**a**, **b**) diplotene (**a**) and metaphase I (**b**) with 11 bivalents of a male with 2n = 22. (**c**, **d**) metaphases I with 11 bivalents and one univalent corresponding to the B chromosome of a male with 2n = 23. (**e**, **f**) metaphases II with n = 11 (**e**) and n = 12 (**f**) chromosomes of a male with 2n = 23. Bar = 10 μm

pair 6 and occasionally of the chromosome pair 7 are also seen (Fig. 4(b)). In *H. crepitans*, C banding pattern is predominantly centromeric, with some interstitial bands, like in the short arms of chromosome pair 3 (Fig. 4(c)). The C positive labelings observed in the end of some chromosomes of this species, and occasionally of *H. raniceps*, are most probably due to the telomere staining, not corresponding actually to heterochromatic regions.

The CMA_3/DA/DAPI staining was performed in cytological preparations of the three species, but with reliable results only in *Hypsiboas albopunctatus*. In this species, the GC-specific CMA_3 counterstained with DA revealed brilliant fluorescence at the proximal region of short arms of chromosomes 2 and in the end

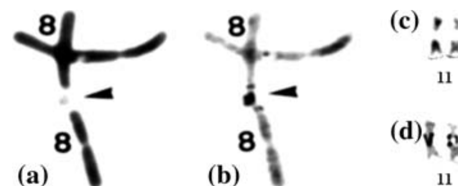

Fig. 3 (**a**, **b**) Chromosome pair 8 after Giemsa (**a**) and Ag-NOR staining (**b**) of *H. albopunctatus*. (**c**, **d**) chromosome pair 11 after Giemsa (**a**) and Ag-NOR staining (**b**) of *H. crepitans*

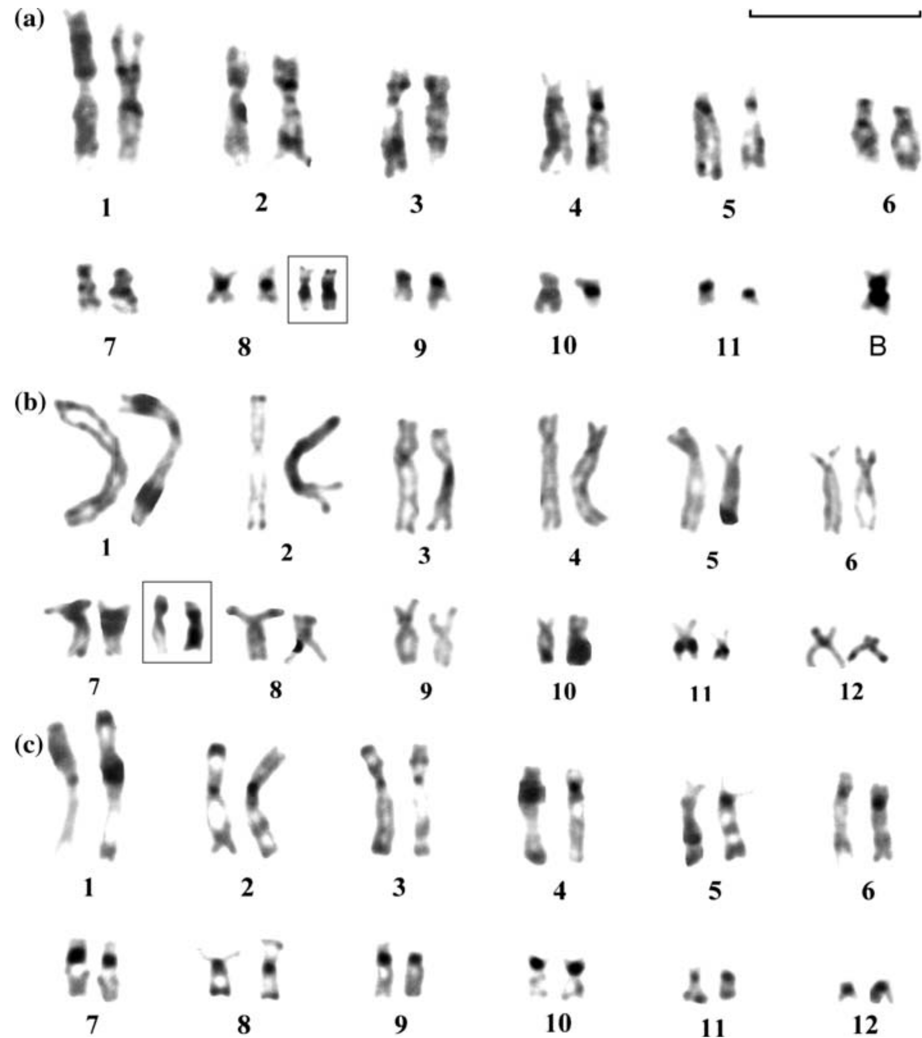

Fig. 4 C-banded karyotypes. (**a**) *H. albopunctatus* with 2n = 23. Inset: chromosome pair 8 from another metaphase. (**b**) *H. raniceps*. Inset: chromosome pair 7 from another metaphase. (**c**) *H. crepitans*. Bar = 10 μm

of the short arms of the chromosomes 8, coincident to the NOR (Fig. 5(a)). With this fluorochrome, the B chromosome showed a narrow negative band in the proximal short arms (Fig. 5(c)), but not clearly visualized in all metaphases, because it is very subtle. On the other hand, only this region exhibited bright fluorescence with AT-specific DAPI counterstained with DA (Fig. 5(b) and (d). In *H. raniceps* there is no indication of brilliant fluorescence with any fluorochrome, whereas in *H. crepitans* some bright fluorescent regions could be recognized after CMA_3/DA staining, like in the NOR-bearing chromosomes and in one of the large-sized pair.

BrdU replication banding patterns were obtained in the chromosomes of the three species of *Hypsiboas*, allowing precise identification of the large and medium-sized pairs (Fig. 6(a), (b), and (d)). The small-sized chromosomes showed in general poor longitudinal banding. The B-chromosome of *H. albopunctatus* exhibited positive bands in the terminal regions of both short and long arms, and negative staining in the proximal regions (Fig. 6(a)). In some metaphases, in which the band differentiation was not properly achieved, such chromosome had proximal darkly stained regions in both short and long arms, and this pattern was similar to the C banding of the chromosomes 8 as seen in Fig. 4(a).

The comparison of the banded karyotypes revealed almost full homeology of the large and medium-sized chromosomes (Fig. 7). Some of the discrepancies, like the larger short arms of the chromosomes 6 of *H. raniceps*, may be explained by the presence of the C banded interstitial heterochromatic region. Among the small-sized chromosomes of the three species, the correspondence in the replication banding is not so clear. Nevertheless, observing several distinct metaphases, not always suitable for the whole chromosome pairs, and even on the basis of indirect comparisons with our previous data obtained in four Hylinae species of the *Aparasphenodon*, *Corythomantis*, *Itapotihyla* (as *Osteocephalus*), and *Scinax* genera (Kasahara et al.

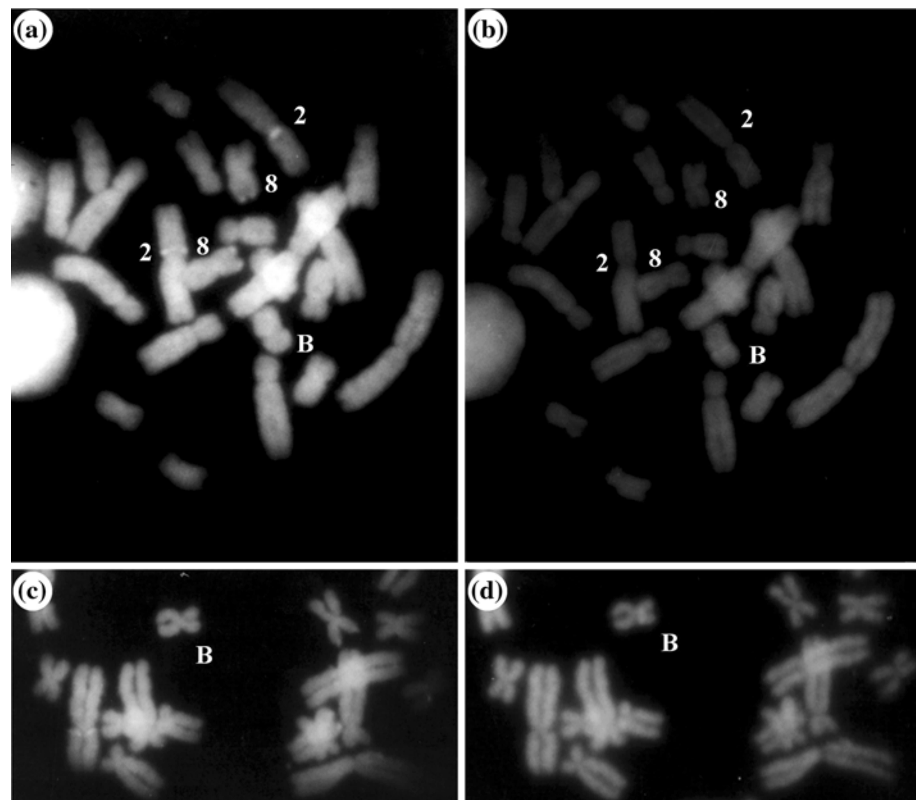

Fig. 5 CMA_3/DA (**a**, **c**) and DAPI/DA (**b**, **d**) stainings in metaphases (**a**, **b**) or partial metaphases (**c**, **d**) of *H. albopunctatus*

2003), all of them with 2n = 24 and very conservative replication banding patterns, homeology of the chromosome pairs of small size seems to exist as shown in Fig. 7.

Discussion

B chromosome in *Hypsiboas albopunctatus*

The 2n = 22 diploid number in *Hypsiboas albopunctatus* of the present study, confirmed previous cytogenetic findings in this species (Beçak 1968; Bogart 1973; Soma et al. 1974; Cardoso 1986; Baldissera and Batistic 1992; Insaurralde et al. 1991; Feitosa et al. 1995). Some individuals exhibited karyotype with 2n = 23, as observed formerly by Cardoso (1986) in specimens from two distinct localities also in Southeastern Brazil. This variant was not described in any other of the remaining samples, most probably because of a reduced number of analyzed individuals, or due to populational differences in the karyotypic constitution.

The extra element in the 2n = 23 karyotype of *Hypsiboas albopunctatus* was interpreted doubtless as B chromosome, considering some features frequently associated to supernumeraries, as listed and commented by several authors, among which Jones and Rees (1982), Green (1991, 2004), Beukeboon (1994), and Camacho et al. (2000) may be cited. Particularly, Camacho (2004) edited the most recent publications on Bs, occurring in a variety of organisms, and analyzed under distinct approaches. According to Jones and Rees (1982), who had performed an extensive revision and dozens of reports on supernumeraries, B chromosomes share several characteristics, but it is important to emphasize that excluding the numerical variability and the dispensability, the others have not been found universally. In our case, the most remarkable features are: presence restricted to some individuals, distinct morphology with regard to the regular A chromosomes, heterochromatic nature, and lack of chromosome pairing with any other chromosome during meiosis.

The B chromosome was found in seven specimens of our sample, corresponding to a relatively high percentage of more than 40% of B carrier. Nevertheless, out of 17 specimens, 10 were not found bearing B chromosome, which is in accordance to the idea of dispensability attributed to it. In fact, no apparent difference exists between the adaptability of the individuals with or without B. In nature, size difference among individuals belonging to the same sex has been ever noticed, and in a very preliminary morphometric

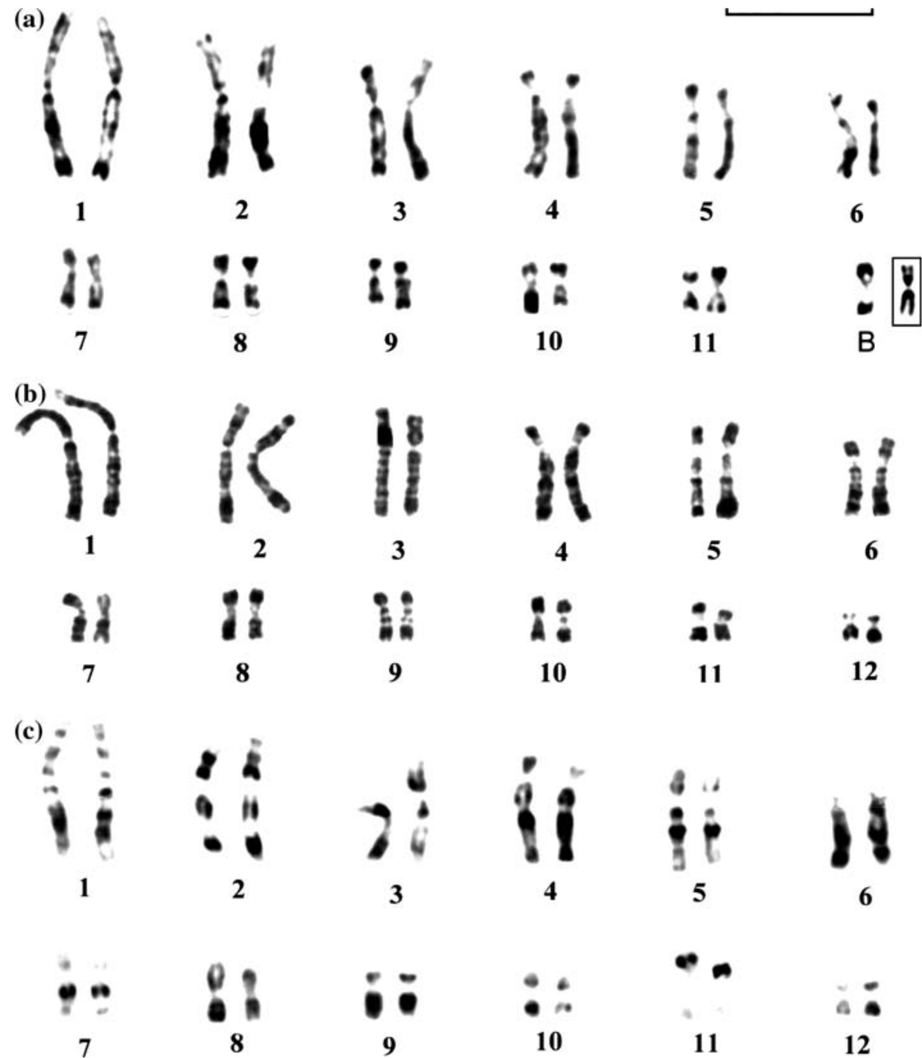

Fig. 6 BrdU replication banding patterns. (**a**) *H. albopunctatus* with 2n = 23. Inset: B chromosome from another metaphase. (**b**) *H. raniceps*. (**c**) *H. crepitans*. Bar = 10 µm

evaluation, there was a suggestion that individuals bearing B chromosome were slightly larger than those without supernumerary, with regard to at least the snout-vent length, arm width, and tympanum diameter. Evidently, our samples are still too small to consider these data as significative, but this matter might be carefully investigated, since the assumption is controversial in the literature.

The presence of B chromosome exclusively in male specimens of *Hypsiboas albopunctatus* is rather a biased finding, due to our collecting procedures based on the advertisement call during the breeding season, than a new case of supernumerary related to sex, similarly to what was noticed in the frog *Leiopelma hochstetteri* (Green 1991, 2004). Both males and females of this species exhibited supernumeraries, but one particular B chromosome was found only in females, which was interpreted to be the W chromosome, in a sex determination mechanism of 0W:00 type. Further karyotype screenings of females, as well as of specimens from other populations of *H. albopunctatus*, would be interesting to be carried out for evaluating the extension of B chromosome occurrence in the species.

As usual, the B chromosome of *Hypsiboas albopunctatus* is small-sized, being very close morphologically to the 8th pair, but it is promptly recognized even under standard staining. C banding suggests heterochromatic nature, but not in its entirety, because the distal ends of the chromosome are not C-positive stained. This pattern is in accordance to what was observed with BrdU incorporation, suggestive of late replication only for the proximal regions in both arms of B chromosome. Among amphibians, studies at the molecular level of supernumerary chromosomes are relatively rare, with exceptions of those performed in the frog *Leiopelma hochstetteri* and in the salamander *Dicamptodon tenebrosus* (Green 2004). The analyses

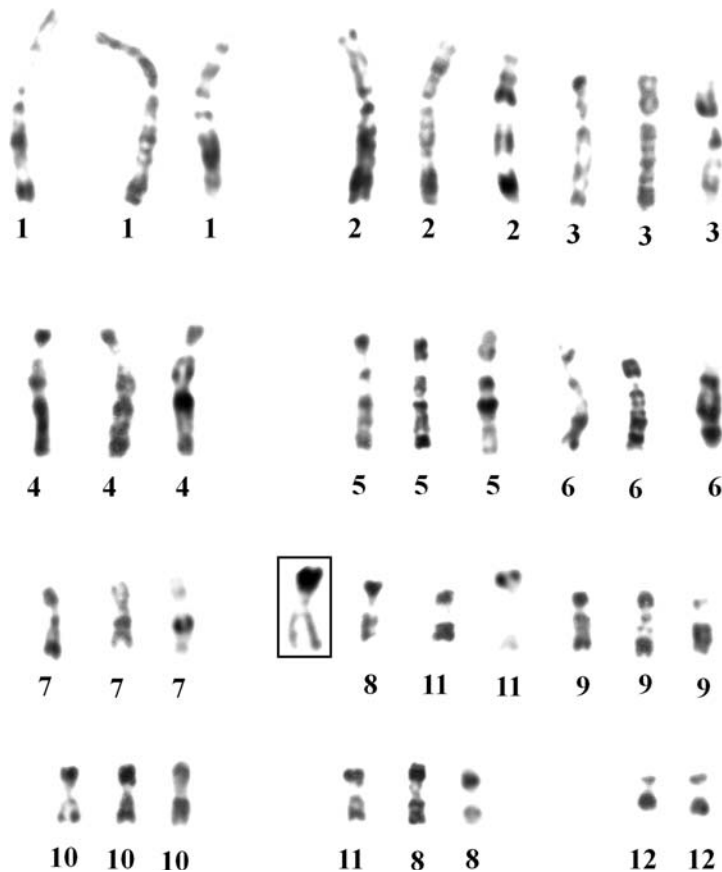

Fig. 7 Composite partial karyotypes after BrdU replication banding patterns. From left to right, chromosomes of *H. albopunctatus, H. raniceps*, and *H. crepitans*. Inset: chromosome 8 of *H. albopunctatus* from another metaphase

showed that their Bs are partly composed of repetitive DNA that is found throughout the A chromosomes, strengthening the hypothesis of intraspecific origin of supernumeraries. In the case of *H. albopunctatus*, although not using improved molecular techniques, DAPI/DA staining provided an unequivocal evidence of AT richness in the short arm proximal heterochromatin of B, not observed in any other chromosome site.

The origin of the B chromosomes is difficult to be determined, but the hypothesis of trisomy of A chromosome is very attractive. If true, the morphology of B chromosome of *Hypsiboas albopunctatu*s and the staining pattern in some metaphases after FPG technique similar to that seen after C banding in chromosome 8, lead us to think this B arising by means of total or partial duplication of this chromosome. In any case, it is supposed that the supernumerary has undergone subsequent differentiation, preventing meiotic pairing with chromosomes 8, and additionally loss of the NOR site, characteristic of these chromosome pair. The univalent condition was always observed in the meiosis of specimens of *H. albopunctatu*s, all of them presenting a unique B; this was also true in the Brazilian anuran *Bokermannohyla luctuosa* (as *Hyla* sp. in Baldissera et al. 1993), bearing one B per metaphase. When numerous, Bs have been observed either in meiotic association or not; even though they were homologous in the species, like the two extra chromosome in the rodent *Akodon montensis* (*Akodon* sp.), that were observed either paired, forming a bivalent or unpaired, as two univalents (Kasahara and Yonenaga-Yassuda 1982). This unusual behavior during meiosis supports the idea of Bs as a particular class of chromosomes, with a non-mendelian mode of segregation, and this may lead eventually to the numerical variability of supernumeraries among individuals.

The B chromosome of the present sample of *Hypsiboas albopunctatu*s is most likely the same to that observed by Cardoso (1986), although this author referred to it as non-heterochromatic, and probably as isochromosome, due to its ring conformation during meiosis. These characteristics are not in conformity with our observations, and for this reason, the possibility of two distinct types of Bs in the species cannot be completely excluded.

According to the recent survey of Green (2004), Bs were described in 26 amphibian species, corresponding to about 2% of the total number of the species karyotyped so far. There are four Brazilian anurans

with B chromosomes, including *Hypsiboas albopunctatus* (present study): *Bokermannohyla luctuosa* (as *Hyla* sp. in Baldissera et al. 1993), *Megaelosia massarti* (Rosa et al. 2003), and *Dendropsophus nanus* (Medeiros et al. 2006). Undoubtedly, this number will increase as more species and even more individuals from known or new localities are screened. Also, additional information on several aspects of Bs is expected, contributing to elucidate one of the most intriguing classes of chromosomes.

The karyotypes of *Hypsiboas albopunctatus*, *H. raniceps*, and *H. crepitans*: reduction in the chromosome number of 2n = 24 to 2n = 22

The karyotypes of *Hypsiboas albopunctatus*, *H. raniceps*, and *H. crepitans* are largely in accordance with those previously described in the literature for each species (Beçak 1968; Rabello 1970; Bogart 1973; Cardoso 1986), even though the chromosome orderings in each karyograms or the nomenclatures of their morphology adopted by the distinct authors were not always coincident. Nevertheless, slight discrepancies in the morphology of some chromosomes exist, as in the case of *H. albopunctatus* analyzed by Bogart (1973) and *H. crepitans* by Rabello (1970), so that karyotypic geographical differences cannot be completely ruled out.

Aside the smaller diploid number of *Hypsiboas albopunctatus*, the three species share the same karyotypic constitution, with chromosome pairs 1 to 11 morphologically almost identical. This similarity was also evident by the comparative analyses of the replication banding patterns of their chromosomes, which revealed a high degree of conservativeness. For this reason, it is important to emphasize that the use of the Ag-NOR and C banding techniques, and in a certain extension the fluorochrome staining, were fundamental for characterizing individually each species on the cytogenetic point of view.

The three species presented a single pair of Ag-NORs, in the chromosomes 11 of *Hypsiboas raniceps* and *H. crepitans*, but at distinct sites, and in the chromosomes 8 of *H. albopunctatus*. In the case of the last species, the Ag-NOR bearing chromosomes are not coincident with those previously reported by Baldissera and Batistic (1992), who found Ag-NOR in the chromosomes 7. This might be due to populational differences or, most probably, to unmatched ordering of the chromosomes in the karyograms. C banding patterns of the *Hypsiboas* species are quite distinct and may be also referred as species-specific. It is remarkable the least amount of C banded heterochromatin in *H. raniceps*, with practically only a conspicuous proximal labeling in the long arms of the chromosomes 11, adjacent to the NOR. Fluorochrome staining provided additional data on the molecular content of some repetitive regions of the chromosomes. Particularly, the use of CMA_3 was relevant not only to identify the site of the NOR in *H. albopunctatus*, but also for detect other GC-rich repetitive regions in the species. These regions were also important for strengthening the karyotypic differences of the three species of *Hypsiboas* on the level of some specific cytological markers.

Relatively good replication banding patterns were obtained in *Hypsiboas raniceps* and *H. crepitans*, but in *H. albopunctatus* the chromosomes were less differentiated, probably due to incomplete BrdU incorporation. Nevertheless, although preliminary, our comparison of the three karyotypes revealing almost full homeology of the large and medium-sized chromosomes and close correspondence of the small-sized elements can be taken as reliable. This fact is not surprising, since Miura (1995) demonstrated a high level of interfamily chromosome homeology by comparing the replication banding in the karyotypes of some species of bufonid, hylid, pipid, and ranid.

It is remarkable that 2n = 22 has been observed only in *Hypsiboas albopunctatus*. Two other karyotyped species of the *H. albopunctatus* morphological group, that are *H. raniceps* and *H. fasciatus*, this last one analyzed by Bogart and Bogart (1971), and all the other species of the genus *Hypsiboas* studied so far, presented 24 chromosomes (King 1990; Kuramoto 1990; Gruber 2002). This fact strongly suggests that the karyotypic evolution was toward a reduction in the chromosome number of 2n = 24 to 2n = 22, and this possibility, as well as the probable mechanisms responsible for it, had been considered before by Bogart (1973). In the present paper, these proposals were evaluated, on the basis of the new chromosome data.

One of the mechanisms is an end-to end fusion occurred in a presumed ancestral 2n = 24 karyotype, similar to those described for *H. raniceps* and *H. crepitans*. Since the large and medium-sized pairs exhibited a close correspondence in the replication banding patterns in the three species of *Hypsiboas*, the rearrangement must have involved chromosomes of small size, most probably one of the NOR-bearing chromosomes 11, altering the position of this marker in the 2n = 22 karyotype. The hypothesis of a tanden fusion between the chromosomes 11 and 12 seems to be attractive, because a chromosome pair equivalent to the smallest 12th pair of the *H. raniceps* and *H. crepitans*, also observed by us in other 2n = 24 Hylinae species (Kasahara et al. 2003), is absent in the 2n = 22

karyotype of *H. albopunctatus*. The possibility that the rearrangement has occurred with any of the other small-sized chromosomes is not ruled out, due to a certain uniformity in their banding patterns. Nevertheless, it is interesting to remark that the NOR-bearing chromosomes 8 of *H. albopunctatus* present conservatively the same replication patterns of the marker chromosomes of *H. raniceps* and *H. crepitans*, as well as of those of the four Hylinae previously karyotyped, being most probably homeologous to them.

An alternative mechanism proposed by Bogart (1973) for reducing the diploid number in *H. albopunctatus* was the translocation of the genetic material from the smallest chromosome in the ancestral 2n = 24 karyotype to one or more chromosomes. In this case, its homologue would have undergone heterochromatinization, giving rise to a B chromosome, which was eventually lost. This hypothesis sounds now a concrete possibility, as a true almost heterochromatic extra chromosome was observed in some individuals of *Hypsiboas albopunctatus* of our sample. The idea of disappearance or loss of one small-sized chromosome pair is, indeed, rather more suitable with the almost complete correspondence of the 2n = 24 and 2n = 22 karyotypes, excepting by the lack of the 12th pair in *H. albopunctatus*, visualized even with standard staining. The question that should be answered is, therefore, whether the 2n = 23 diploid number bearing a single B is not fortuitous, but might represent a vestige of a 2n = 24 karyotype that existed before in such species.

The assumption related to the mechanism of reduction in the chromosome number in *Hypsiboas albopunctatus*, along with the origin of B chromosome in the species, remains unsolved, and, undoubtedly, it deserves further cytogenetic investigations using more resolutive approaches.

Acknowledgements The authors are greatly indebted to FAPESP and CNPq for financial support; to Dr. A.S. Abe and Dr. P.C. Garcia for helping in the collection of some specimens used in this study; and to Rogilene Aparecida Prado for technical assistance.

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