Rev. Brasil. Genet. 16, 2, 335-345 (1993)
(Brazil. J. Genetics)

CYTOGENETICS OF FOUR BRAZILIAN *Hyla* SPECIES (AMPHIBIA - ANURA) AND DESCRIPTION OF A CASE WITH A SUPERNUMERARY CHROMOSOME

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ABSTRACT

The chromosomes of *Hyla fuscovaria, H. hayii* and *H. prasina*, with 2n=24, and of *Hyla sp.* (*aff. circumdata*), a new species, with 2n=24 and 2n=25, were studied.

The karyotypes with 2n=24 in the four species were very similar, with almost no differences in the size and morphology of the chromosomes. The numerical variability found in *Hyla sp.* (*aff. circumdata*) is due to the occurrence of a supernumerary chromosome in some specimens. NOR data obtained for the first time in the four species and C banding analysis of *H. prasina* indicate that such types of banding may be useful to differentiate species with very similar karyotypes, contributing to the understanding of chromosome evolution and the establishment of phylogenetic relationships among Brazilian *Hyla* species.

INTRODUCTION

To date, a few more than 90 species of frogs, belonging to the genus *Hyla*, have been cytogenetically analysed and the majority of the studies were based on conventionally stained chromosomes. The most widespread diploid numbers among these species (Beçak, 1968; Rabello, 1970; Foresti, 1972; Morescalchi, 1973, 1979; King, 1990; Anderson, 1991) are 2n=24 and 2n=30, which indicates a dichotomy within the genus *Hyla*; discrepant diploid numbers, as 2n=18, 2n=20, 2n=22, 2n=26, 2n=32 and 2n=34, have also been found, although sporadically. Additionally, a case with 2n=48, due to polyploidy, has been found (Wasserman, 1970).

The nondifferentially stained karyotypes with 2n=24 are very similar, with small differences in the morphology and size of some chromosomes. The most important

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distinction among these karyotypes is the position of a secondary constriction, not always recognizable, which may be located in different chromosome pairs or different regions of the same chromosome pair.

Although chromosome banding is a very important tool in comparative cytogenetics, relatively few *Hyla* species have been studied. Schmid (1978) presented the Q and C banding patterns and the nucleolar organizer regions (NORs) in *H. arborea, H. cinerea* and *H. septentrionalis*. Later, Wiley (1982) described the G banding patterns of nine *Hyla* species from the United States (*H. squirella, H. crucifer, H. gratiosa, H. cinerea, H. andersonii, H. femoralis, H. avivoca, H. chrysoscelis* and *H. versicolor*) and Wiley *et al.* (1989) identified a polymorphism in the location of the NORs in *H. chrysoscelis* and *H. versicolor*. Replication banding patterns with BrdU incorporation were also reported by these authors in the chromosomes of *H. chrysoscelis*. Recently, C banding and NOR staining in a sample of 23 species of *Hyla* (*H. andersonii, H. arborea, H. arenicolor, H. avivoca, H. cadaverina, H. chinensis, H. chrysoscelis, H. cinerea, H. crucifer, H. euphorbiacea, H. eximia, H. femoralis, H. gratiosa, H. japonica, H. lancasteri, H. meridionalis, H. microcephala, H. punctata, H. regilla, H. savignyi, H. smithii, H. squirella* and *H. versicolor*), as well as *in situ* restriction enzyme analysis on the chromosomes of eight of these species, were reported by Anderson (1991), who summarized data obtained previously in 1986. Based on these banding data, the author described presumptive sex chromosomes in *H. femoralis* and *H. squirella*, and discussed the chromosome evolution in Holarctic *Hyla* treefrogs.

Our study provides information about mitotic and meiotic chromosomes of *Hyla prasina, H. hayii, H. fuscovaria* and a new species referred to as *Hyla sp. (aff. circumdata)*. Although the conventionally stained karyotypes of the two former species were previously described (Beçak, 1968), those of *H. fuscovaria* and *Hyla sp. (aff. circumdata)* as well as the data on NORs in the four species and on C banding in *H. prasina* are reported for the first time.

MATERIAL AND METHODS

Our sample was comprised of specimens of *H. prasina* (13 males and one female), *H. hayii* (two males), and *Hyla sp. (aff. circumdata)* (three males and one female) collected in Serra do Japi, Jundiaí, State of São Paulo, and of *H. fuscovaria* from Rio Casca, State of Minas Gerais (three males) and Rio Claro, State of São Paulo (one female).

Taxonomic identification of the specimens was made by Dr. Célio F.B. Haddad, from Departamento de Zoologia, Instituto de Biociências, UNESP (Rio Claro Campus).

In order to improve the mitotic index, the specimens were injected intraperitoneally with an aqueous solution of Fleischmann's yeast plus dextrose about

48h before sacrifice (Cole and Leavens, 1971). Then, 0.1% colchicine solution (0.1 ml/10 g of body weight) was injected and the animal was killed 2 h later.

Preparations of the bone marrow, liver and testes were obtained. The cells from all tissues were treated with a hypotonic solution of 0.075 M KCl for 30 min., at 37°C. A 3:1 mixture of methanol and acetic acid was used as fixative. First, six drops and then 2 ml of fixative were gently added to the cell suspension within the hypotonic solution. Two or three changes of fresh fixative were made, and the cell suspension was dropped on a slide over a water bath at 60°C. The slide was air-dried at room temperature. Conventional staining was performed with 1 ml of Giemsa in 29 ml of phosphate-buffered saline, pH 6.8, for 7 min. C banding patterns were obtained according to the method of Sumner (1972) and NOR staining by the technique of Howell and Black (1980).

RESULTS

Conventional staining

A diploid number of 2n=24 chromosomes was found in all specimens of *H. prasina, H. hayii, H. fuscovaria*, and in two males of *Hyla sp.* (*aff. circumdata*). One male and one female of this last species showed an odd diploid number of 25 chromosomes.

The karyotypes with 2n=24 chromosomes in the four species (Figures 1a, 1b, 1c and 2a) are formed mainly by metacentrics and submetacentrics. The first five chromosome pairs are of great size, the sixth is of medium size and the remainder are small. A conspicous secondary constriction was visualized in the proximal region of the long arms of chromosome pair 12 in *H. fuscovaria*. A less marked secondary constriction at the end of the long arms of both or one of the chromosomes belonging to pair 12 was observed in *H. prasina* and *H. hayii*, and to pair 11 in *Hyla sp.* (*aff. circumdata*).

The karyotype with 2n=25, found in two specimens of *Hyla sp.* (*aff. circumdata*), is similar to that with 2n=24 chromosomes except for the presence of an additional small metacentric (Figure 2b). This extra chromosome was found in a sample of 62 mitotic cells of the male specimen and 40 cells of the female. There was no evidence of mosaicism, with distinct cell lines, in either specimen.

Meiotic analysis, in males with 2n=24 in all four species of *Hyla*, revealed 12 bivalents in metaphase I cells (Figure 3a) and 12 chromosomes in metaphase II cells (Figure 3b). The majority of the bivalents were ring shaped, exhibiting two terminal chiasmata but frequently one and even two of the greatest bivalents presented only one terminal chiasmata. In these cases the bivalent showed a particular open configuration. The male with 2n=25 presented 12 bivalents in metaphase I cells plus one univalent corresponding to the extra chromosome (Figure 3c). Only four metaphase II cells were

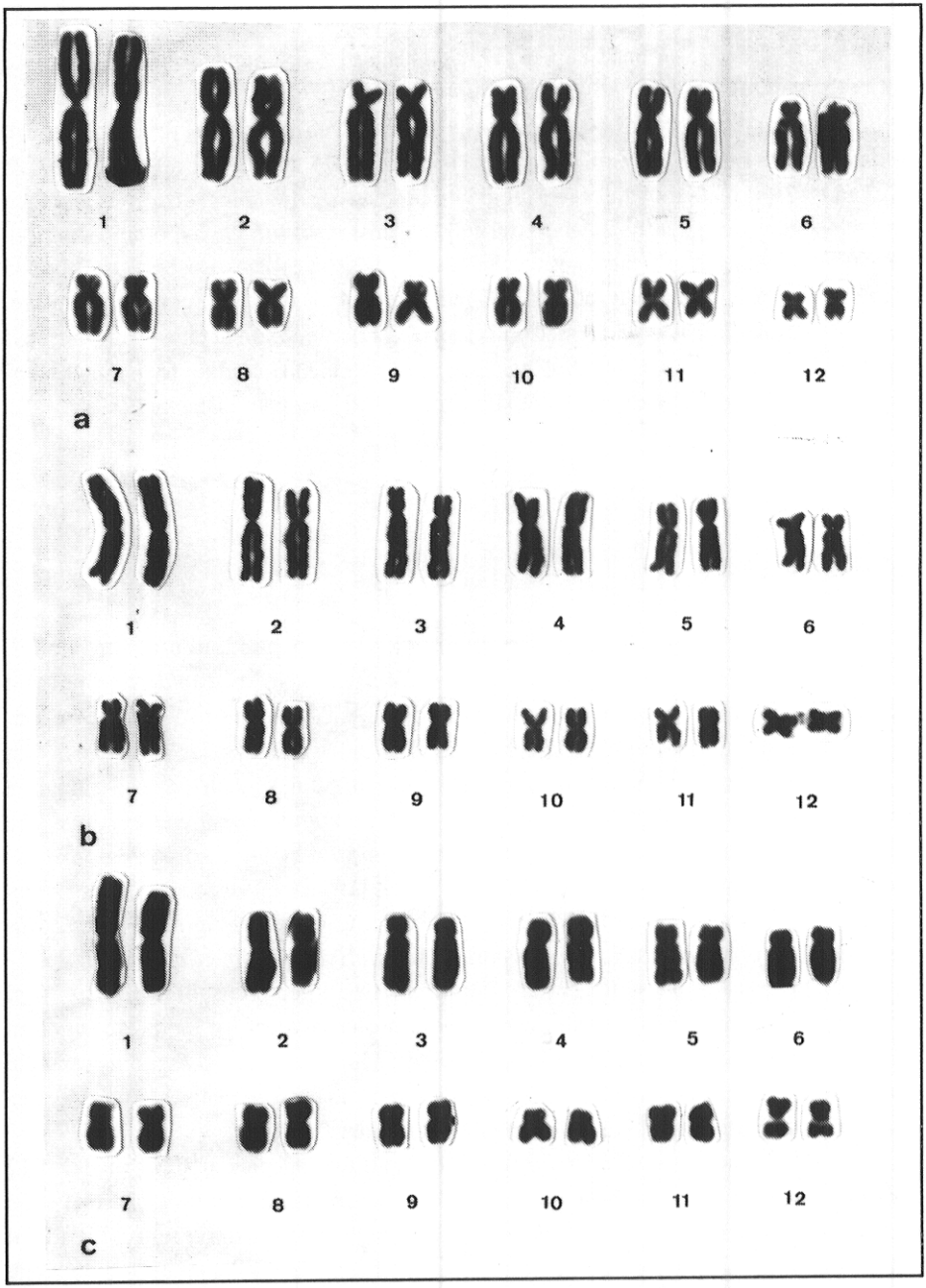

Figure 1 - Karyotypes after conventional staining of males with 2n=24. (a) *Hyla prasina*; (b) *H. hayii*; (c) *H. fuscovaria*. Note association of satellited chromosomes 12 in b and interstitial secondary constriction in chromosome pair 12 in c.

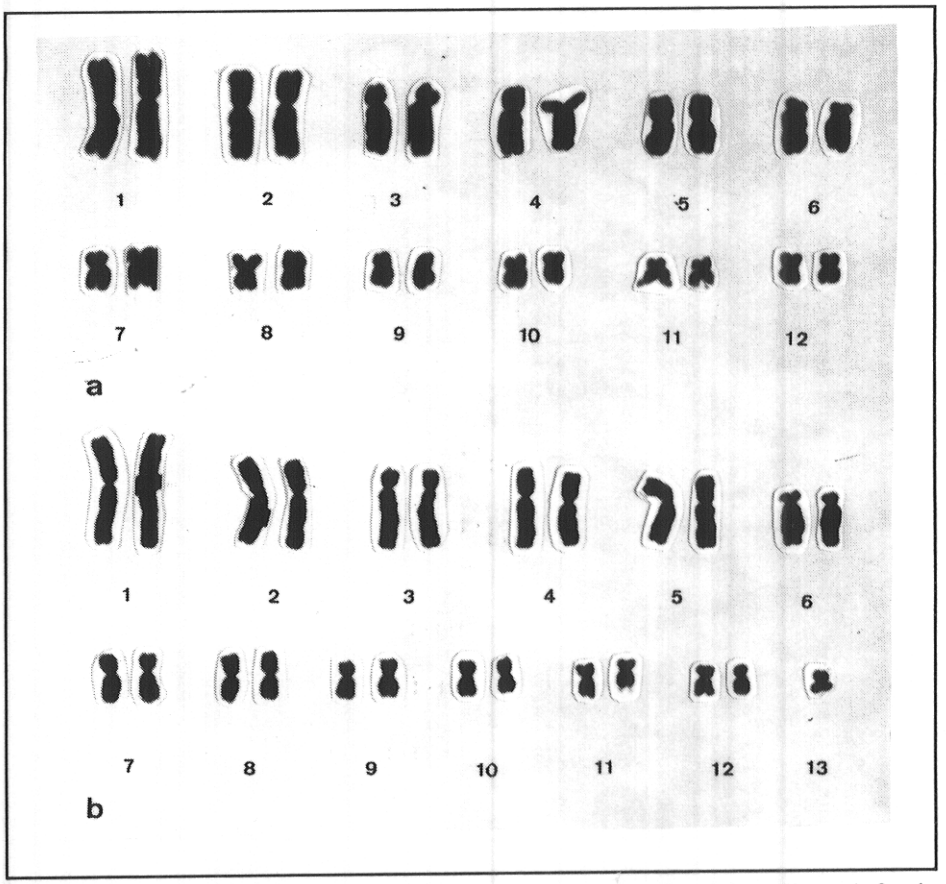

Figure 2 - Karyotypes after conventional staining of *Hyla sp.* (*aff. circumdata*). (a) male with 2n=24; (b) female with 2n=25, with supernumerary chromosome (no. 13).

available for analysis. Two of them revealed 12 chromosomes and the other two, 13 chromosomes, including the small extra metacentric (Figure 3d).

NOR staining

Hyla sp. (*aff. circumdata*), *H. hayii* and *H. fuscovaria* have in their karyotypes a pair of chromosomes bearing NORs which appear stained after Ag-NOR technique. In *Hyla sp.* (*aff. circumdata*) and *H. hayii*, NORs are at the distal end of the long arms of the chromosome pairs 11 and 12, respectively (Figures 4a and 4b), while in *H. fuscovaria* NORs appear at the interstitial region of the long arms of the chromosome pair 12 (Figure

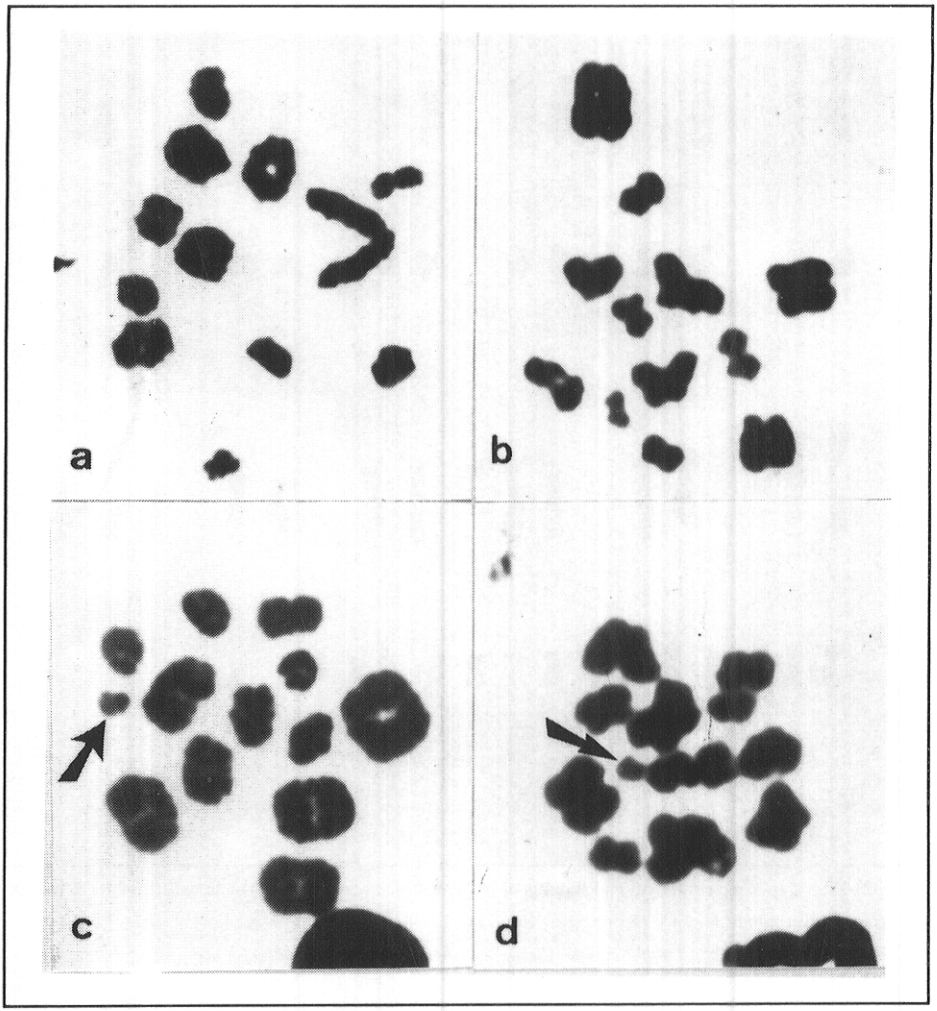

Figure 3 - Meiotic cells of males of *Hyla prasina* with 2n=24 (a and b) and *Hyla sp.* (*aff. circumdata*) with 2n=25 (c and d). (a) metaphase I with 12 bivalents; (b) metaphase II with 12 chromosomes; (c) metaphase I with 12 bivalents plus one univalent (arrow) corresponding to the supernumerary chromosome; (d) metaphase II with 13 chromosomes, including the supernumerary (arrow).

4c). In *Hyla sp.* (*aff. circumdata*) NORs differ considerably in size, one of them being twice as large as the other.

H. prasina showed NORs at the end of the long arms of chromosome pair 12 but an additional pair bearing NORs occurs in this species. In one individual only the

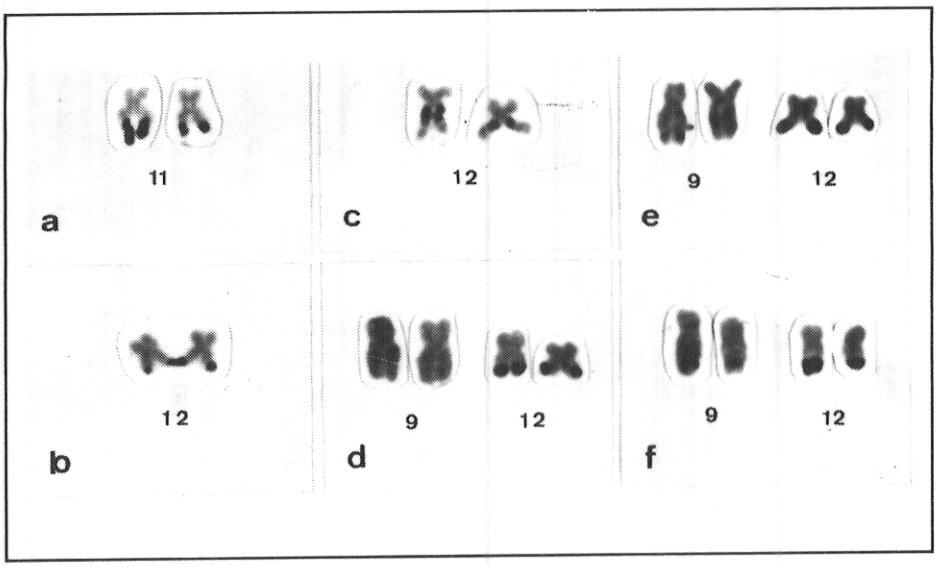

Figure 4 - Chromosome pairs from metaphase cells after Ag-NOR staining. (a) *Hyla sp.* (*aff. circumdata*): 2 NORs; (b) *H. hayii*: 2 NORs; (c) *H. fuscovaria*: 2 NORs; (d) (e) and (f) *H. prasina*: 2, 3 and 4 NORs.

NORs of chromosome pair 12 were stained (Figure 4d). In three other specimens, besides these two NORs, a third and even a fourth NOR located in chromosome pair 9 were observed (Figures 4e and 4f).

C banding

C banding technique was applied to the chromosome preparations of *H. prasina*.

In the karyotype of *H. prasina* (Figure 5) constitutive heterochromatin is in the centromeric region of all chromosomes except chromosome pair 12 which shows a slight telomeric band in the long arms. Chromosome pairs 7 and 8 also had conspicous heterochromatic blocks along their arms while less markedly dot-like C-positive bands appear interstitially in the chromosome arms of pairs 1, 3, 4 and 5.

DISCUSSION

The karyotypes of *H. fuscovaria* and *Hyla sp.* (*aff. circumdata*) have 2n=24 chromosomes. This diploid number is the same as that observed in *H. prasina* and *H.*

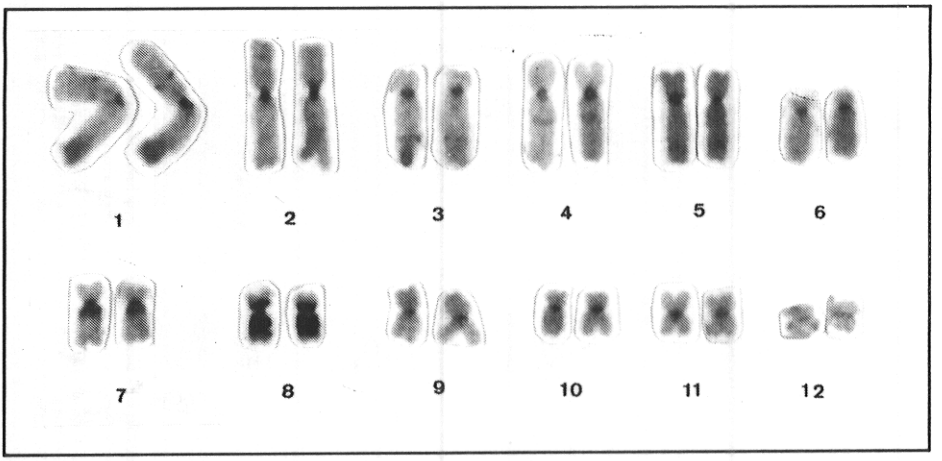

Figure 5 - C-banded karyotype of male of *Hyla prasina* with 2n=24.

hayii as well as in the great majority of the *Hyla* species cytogenetically studied up to now.

Heteromorphic sex-chromosomes were not identified in any of the karyotypes, so we conclude that in *H. prasina, H. fuscovaria* and *Hyla sp.* (*aff. circumdata*), male and female karyotypes are morphologically identical.

Hyla sp. (*aff. circumdata*) is a newly described species which occurs in the State of São Paulo. Its taxonomic position was determined on the basis of the morphological traits which are characteristic of the *Hyla* species belonging to the *circumdata* group (Haddad, personnal communication). The description of this species will be published elsewhere (Pombal-Jr. and Haddad, submitted to Herpetologica).

The odd element found in some specimens of *Hyla sp.* (*aff. circumdata*) with 2n=25 may be interpreted as a supernumerary on the basis of some characteristics, which are generally attributed to this class of chromosome (Jones and Rees, 1982). It is the smallest of the complement, with a morphology different from that of the remaining chromosomes, and this extra chromosome always formed a univalent in metaphase I cells. Although no conclusive C banding preparation was obtained for the specimens of *Hyla sp.* (*aff. circumdata*), some C banding metaphases could be examined. The supernumerary appeared more intensely stained than the other chromosomes, which would be indicative of its heterochromatic nature.

Few anuran species with additional chromosomes in the karyotype have been reported. According to the revision made by Green (1991), they occur in *Leiopelma hochstetteri, Discoglossus pictus, Scaphiopus hammondi, Acris crepitans, Amolops*

liangshanensis, Rana everetti and *R. temporaria*, and 17 other amphibian species, belonging to the Order Caudata, have also exhibited supernumerary chromosomes.

Secondary constriction in one or two chromosomes was identified in the conventionally stained karyotypes. Though *H. fuscovaria* presented a conspicous secondary constriction located interstially in the chromosome arm, in the three other species this structure was not always discernible, depending greatly on the size of the constriction and degree of chromosome condensation. Among the 14 specimens of *H. prasina*, for example, the secondary constriction was hardly noticed in one of the number 12 chromosomes, and even so only in rare metaphases.

In the Ag-stained karyotypes the chromosomes bearing NORs, which are at the same position as the secondary constrictions, were easily identified. Contrary to what is commonly observed in anuran species, which have only one chromosome pair with NORs (Schmid *et al.*, 1990), *H. prasina* showed two chromosome pairs: one of them is the 12th and the other is presumably the 9th. *H. hayii* and *H. fuscovaria* also presented NORs in chromosome pair 12, but in *H. fuscovaria* they are located in a different site than in the other two species. In *Hyla sp.* (*aff. circumdata*) NORs are in a distinct chromosome pair, the 11th, at the distal end of the long arm.

The difference in NORs sizes, observed mainly in *Hyla sp.* (*aff. circumdata*), may be considered as a result of differential genetic activity or small duplications/ deletions of rDNA sequences. The figures obtained for this species, however, are more compatible with the idea that duplication of rDNA segments took place in one of the chromosomes of *Hyla sp.* (*aff. circumdata*).

Our data on the distribution and amount of constitutive heterochromatin in *H. prasina* showed that this species has a C banding pattern different from those described for *Hyla* species in the literature (Schmid, 1978; Anderson, 1991).

The C banding pattern of *H. prasina* is typical of that found in anuran species which have, according to Schmid *et al.* (1990), the constitutive heterochromatin located preferentially at the pericentromeric/telomeric regions and also interstitially. This species showed positive C bands at the telomeres of the long arms of chromosome pair 12, confirming that NORs are always found associated with constitutive heterochromatin (Schmid *et al.*, 1990).

ACKNOWLEDGMENTS

The authors are grateful to CNPq for financial support, to Dr. Célio F.B. Haddad for providing the specimens and to Mrs. Lucila de L. Segalla Franco for typing the manuscript.

Publication supported by FAPESP.

RESUMO

Foram estudados os cromossomos de *Hyla fuscovaria*, *H. hayii* e *H. prasina*, com 2n=24, e de *Hyla sp.* (*aff. circumdata*), uma espécie nova com 2n=24 e 2n=25. Com coloração convencional, os cariótipos com 2n=24 nas quatro espécies são muito semelhantes entre si, não existindo praticamente diferenças no tamanho e morfologia dos cromossomos. A variação numérica observada em *Hyla sp.* (*aff. circumdata*) é devida à ocorrência de um cromossomo supernumerário em alguns examplares da espécie. Os dados de RONs obtidos pela primeira vez para as quatro espécies e os de bandas C em *H. prasina* indicam que esses tipos de marcação cromossômica podem ser úteis para diferenciar espécies com cariótipos similares, contribuindo para o entendimento da evolução cromossômica e para o estabelecimento das relações filogenéticas de espécies brasileiras de *Hyla*.

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(Received May 25, 1992)