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Chromosome banding in three species of Brazilian toads (Amphibia-Bufonidae)

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ABSTRACT

The chromosomes of *Bufo crucifer*, *B. ictericus*, and *B. paracnemis* were studied by conventional staining as well as with C banding and NOR techniques. These species have a diploid number of 2n = 22 and identical karyotypes, composed of metacentric and submetacentric chromosomes. The C banding patterns and NOR data indicate that these species of *Bufo* are not differentiated by the distribution and amount of constitutive heterochromatin or the position of the nucleolar organizer regions.

INTRODUCTION

The genus *Bufo* is the most extensive group of the family Bufonidae, comprising more than 200 species distributed all over the world, excepting Artic regions, New Guinea, Australia, and adjacent islands (Frost, 1985). According to the reviews made by King (1990) and Kuramoto (1990), chromosomal analyses have been performed on about 95 species, from Europe, Asia, Africa and America, and the majority of these studies were based on conventionally stained cytological preparations.

The *Bufo* species have great karyotypic uniformity and, with few exceptions, they have shown 2n = 22 chromosomes. Another basic diploid number is 2n = 20 which has been found exclusively among African species. Some cases of tetraploidy with 4n = 44, 4n = 40 and a case of natural triploidy with 30 chromosomes have also been described (revisions in King, 1990 and Kuramoto, 1990).

Former comparative analyses were based primarily on the number and position of secondary constrictions (Cole *et al.*, 1968; Doyle and Beckert, 1970; Bogart, 1972). Differential staining techniques of chromosomes have also been employed (Schmid, 1978, 1980, 1982; Beck and Mahan, 1979; Matsui *et al.*, 1985, Schmid and Almeida, 1988; Schmid and Guttenbach, 1988; Foote *et al.*, 1991; Herrero *et al.*, 1993) and some of these studies have shown that taxonomically different forms sharing the same basic karyotype can have distinct banding patterns.

The purpose of this study was to characterize the NORs and the C banding patterns of *Bufo crucifer*, *B. ictericus* and *B. paracnemis*, three common toads of Southeastern Brazil. Although the conventionally stained karyotypes of these species have been previously described by Beçak (1968), chromosome banding is restricted to a brief report of NORs localization in *B. crucifer* (Baldissera Jr. and Batistic, 1992).

MATERIAL AND METHODS

The sample comprises four males of *B. crucifer* and one male of *B. ictericus* collected in Serra do Japi,

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Jundiaí, State of São Paulo; two males and three females of *B. paracnemis* collected in Rio Claro, State of São Paulo, and one male of this latter species from Corumbataí, State of São Paulo. All the voucher specimens were deposited in the amphibian collection of the Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Campus de Rio Claro.

The procedures for obtaining direct mitotic chromosomes from bone marrow, liver and spleen as well as meiotic preparations have been described in Baldissera Jr. *et al.* (1993). For one male of *B. paracnemis*, chromosomes were obtained from lymphocyte culture. In this case, a blood sample was obtained from the ventricule with a heparinized syringe and transferred to a flask containing complete 199 culture medium plus phytohemagglutinin (0.5 ml of blood in 5 ml of medium). Cultures were incubated for 4-5 days, at 30°C. Two drops of 0.01% colchicine were added to each 5 ml of culture, 30 to 90 min before harvesting. After that, a hypotonic treatment with 0.075 M KCl and fixation with methanol and acetic acid (3:1) were applied. Conventional staining was performed with Giemsa in phosphate-buffered saline. C banding patterns were obtained by the technique of Sumner (1972) and Ag-NOR staining, according to Howell and Black (1980).

In this study, attempts were made to obtain replication banding patterns. So, before colchicine treatment, specimens of *B. crucifer* and *B. paracnemis* were also injected intraperitoneally with a solution of 5-bromodeoxyuridine and 5-fluordeoxyuridine (10 mg BrdU and 0.5 mg FudR in 2 ml 0.9% NaCl solution), about 0.1 ml/10 g body weight, 18 to 24 h before they were killed (Schempp and Schmid, 1981). The chromosome differentiation followed the technique of Dutrillaux and Couturier (1981) for FPG staining with 33258 Hoechst-Giemsa (fluorochrome plus Giemsa).

RESULTS

Conventional staining

All specimens of *B. crucifer*, *B. ictericus*, and *B. paracnemis* had a diploid number of 2n = 22. The karyotypes of these three species (Figures 1a, 1b and 1c) are formed by metacentric or submetacentric chromosomes, which may be grouped into five large pairs, one intermediate, and five small pairs. Pair 7 has a secondary constriction which is located interstitially on the short arms of the chromosomes. In some metaphases, it appeared as a very enlarged unstained region or it was not visualized at all (Figure 5a).

Males and females of *B. paracnemis* had identical karyotypes, with no morphologically recognized sex-chromosomes. Also, no heteromorphic pair was found in the male karyotypes of the other two species.

Two metaphases with a discrepant diploid number of 2n = 23, both with an extra, small acrocentric chromosome were observed in a sample of 50 cells of the male specimen of *B. ictericus*. Meiotic analyses of male specimens of the three species revealed, in diplotene and metaphase I cells, 11 bivalents, the majority of them ring shaped, exhibiting two terminal chiasmata (Figures 2a, 2b and 2c). The metaphase II cells had 11 chromosomes (Figure 2d).

NOR staining

In the three species of *Bufo*, NORs occupied the same position, at the secondary constriction on the short arms of chromosome pair 7 (Figures 3a, 3b, 3c and 3d). Nevertheless, inter and intraindividual variations in the

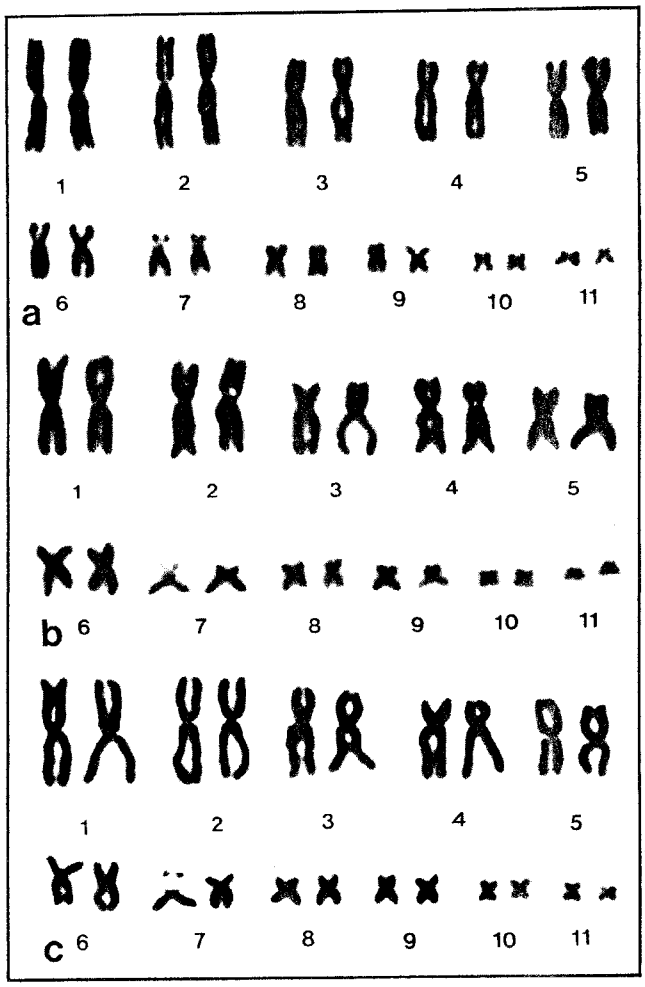

Figure 1 - Karyotypes after conventional staining of three species of *Bufo*, with 2n = 22. a, *B. crucifer*, male; b, *B. ictericus*, male; c, *B. paracnemis*, female.

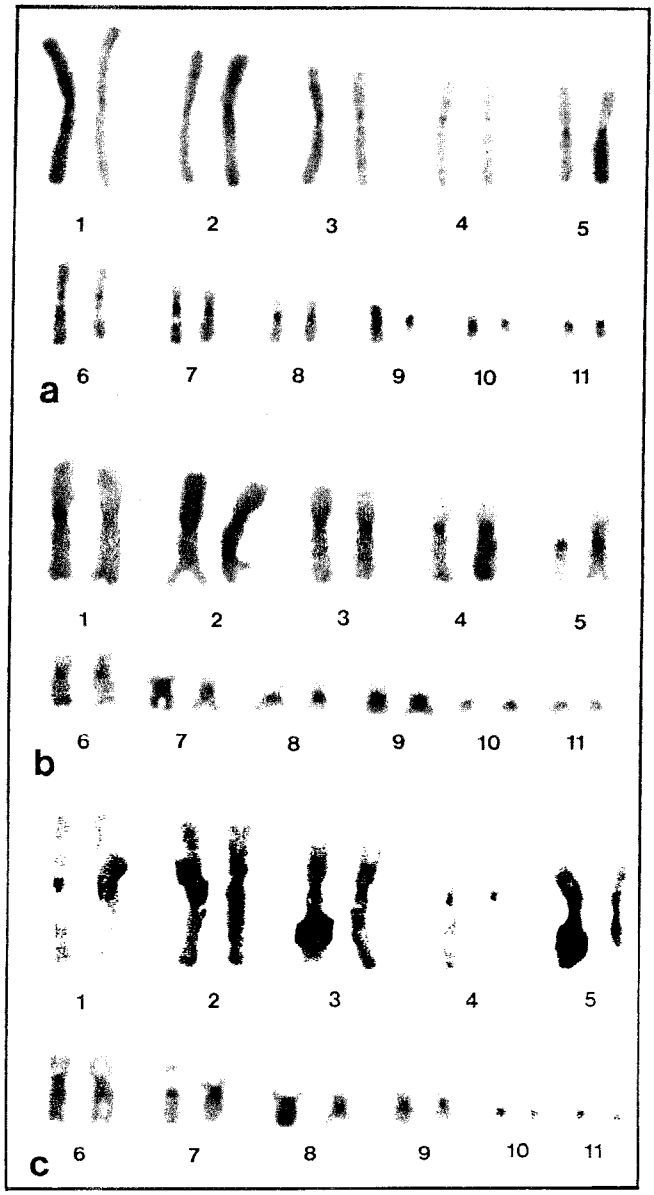

Figure 4 - C-banded karyotypes. a, *Bufo crucifer*, male; b, *B. ictericus*, male; c, *B. paracnemis*, female.

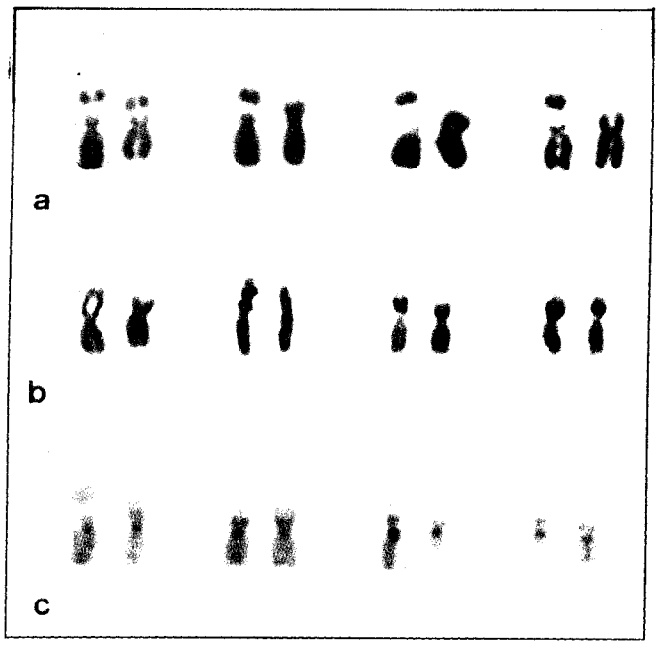

Figure 5 - Pair 7 from different metaphases of *Bufo crucifer* and *B. paracnemis*. a, Conventional staining; b, Ag-NOR staining; c, C banding.

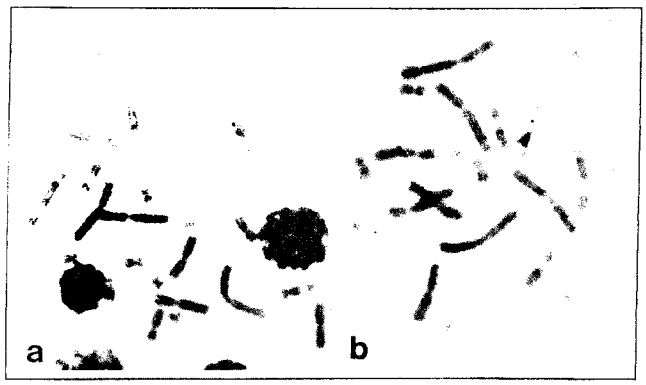

Figure 6 - a and b, Metaphases of *Bufo crucifer* after BrdU treatment, showing vestiges of longitudinal banding.

size between the larger elements (pairs 1 to 6) and the remaining five pairs (King, 1990), the present karyotypes have a medium sized chromosome pair separating the groups of large and small chromosomes.

The two cells with 2n = 23 of *B. ictericus* might be a sporadic finding or represent a second cell line, exhibiting a supernumerary chromosome. Although supernumeraries are not frequent among anurans, some species have already been described with additional chromosomes in the karyotype (Baldissera Jr. *et al.*, 1993).

In the three species, the Ag-NOR staining confirmed the role of the secondary constriction of chromosome pair 7 in nucleolus organization. This region was also C positive stained, indicating that the constitutive heterochromatin is associated with rDNA. Nevertheless, there was no evidence of C-banded heterochromatic segments, adjacent to the NORs, as usually described for anuran species (Schmid, 1978, 1982).

The variability of number and size of the NORs indicates that, besides a different level of activity between homologous NORs, differences in rDNA content also occur, as shown by the tandem duplicated NOR in *B. crucifer*. On the other hand, the lack of a second NOR in all 80 metaphases of a female *B. paracnemis* is suggestive of complete deletion of the ribosomal genes in one of the chromosomes.

Figure 2 - Meiotic cells from male specimens of *Bufo crucifer* (a and d), *B. ictericus* (b), and *B. paracnemis* (c), with 2n = 22. a and b, Diplotene; c, metaphase I; d, metaphase II.

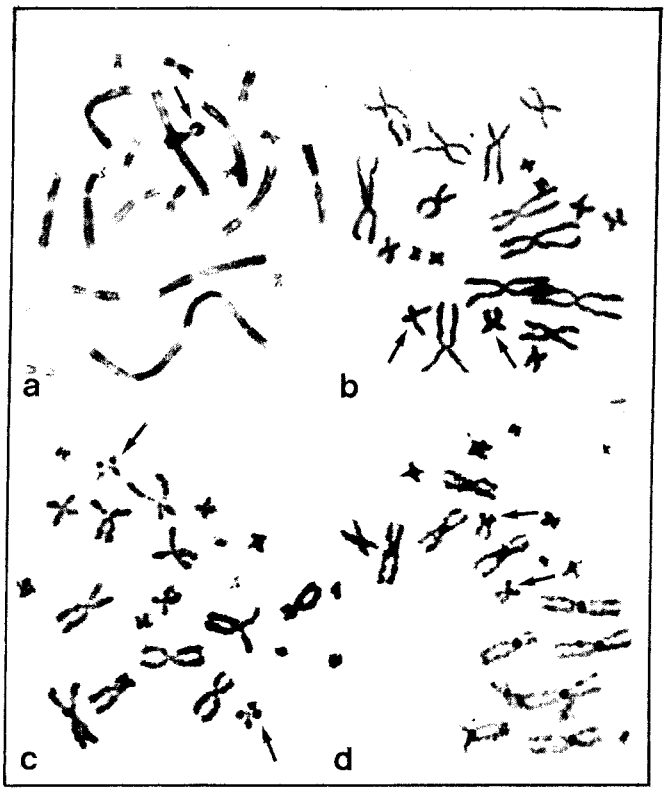

Figure 3 - Ag-NOR stained metaphases. a, *Bufo crucifer*, with one NOR (arrow); b, *B. ictericus*, with 2 NORs (arrows); c and d, *B. paracnemis*, with 2 NORs (arrows). Note the differentiation of the heterochromatic regions in d.

number as well as in the size of NORs were observed among the specimens of *B. crucifer* and *B. paracnemis* (Figure 5b).

In the former species, while two males had two NORs in all metaphases, the other two specimens had only one NOR in all or almost all the metaphases. In these two males, a duplicated NOR was frequently recognized in some cells (Figure 5b).

Four specimens of *B. paracnemis* had two NORs per metaphase in all or almost all of the samples. On the other hand, two other individuals exhibited only one NOR in all metaphases. One of the specimens of B. *paracnemis* showed differentiation of the centromeric heterochromatin in some metaphases, similar to that observed with the NORs (Figure 3d), suggesting that proteins with affinity to Ag-NOR staining also occur in the heterochromatin.

C banding

The three species of *Bufo* presented positively stained constitutive heterochromatin in the centromeric region of all chromosomes (Figures 4a, 4b and 4c). In some metaphases, the chromosomes belonging to pair 7 had an interstitial C band matching the region of the secondary constriction (Figure 5c). Furthermore, in these species weak telomeric spots in some chromosomes and a slight proximal band in the long arms of chromosome pair 6 could occasionally be observed.

BrdU incorporation

Among six specimens submitted to *in vivo* BrdU treatment, three showed chromosome modifications. These were characterized by vestiges of longitudinal banding (Figures 6a and 6b), slight sister chromatid differentiation, highly lengthened chromosomes with breaks, gaps and marked secondary constrictions in several regions, as well as late replicating centromeric segments.

DISCUSSION

The karyotypes of *B. crucifer*, *B. ictericus*, and *B. paracnemis* were indistinguishable with conventional staining. Unlike the basic karyotype of the *Bufo* species with 2n = 22, in which there is a distinct demarcation in

The species of *Bufo* with 2n = 22 have shown NORs in variable locations (Schmid, 1978; Beck and Mahan, 1979; Matsui *et al.*, 1985; Herrero *et al.*, 1993). However, the use of this chromosome marker is largely limited to distinguishing each of the different forms, because the species belonging to the same or closely related species groups frequently show NORs in the same chromosome region. *B. ictericus* and *B. paracnemis* are two closely related species of the *marinus* group, while *B. crucifer*, the most ancestral form of the genus, belongs to the *crucifer* group. The two other species of the *marinus* group, *B. marinus* and *B. arenarum*, that have been karyotyped up to now have also shown NORs on the short arms of pair 7 (Schmid, 1978, 1982; Beck and Mahan, 1979).

Contrary to that found with NORs, the distribution and amount of constitutive heterochromatin, visualized by C banding and by its reactivity after fluorochrome staining or restriction enzyme digestion, have a specific pattern in some of the species or subspecies of *Bufo* (Schmid, 1978, 1980, 1982; Matsui *et al.*, 1985; Schmid and Almeida, 1988; Schmid and Guttenbach, 1988; Herrero *et al.*, 1993). Nevertheless, the present study showed no noticeable difference in the C banding patterns of *B. crucifer*, *B. ictericus*, and *B. paracnemis*, indicating that the karyotypes of these three species are also indistinguishable with regard to this parameter. *B. marinus* and *B. arenarum* exhibited a relatively small amount of centromeric heterochromatin (Schmid, 1980; Schmid and Almeida, 1988). Although Schmid (1980) has referred to the presence of telomeric and interstitial spots, the C-banded karyotypes of these species are not consistently different from that observed in our species of *Bufo*.

In conclusion, our findings showed that although NORs and C banding do not differentiate any of the present species of *Bufo*, these patterns appear to characterize the *marinus* species group. Undoubtedly, improved replication banding patterns obtained with BrdU incorporation into chromosomal DNA are needed to better characterize the chromosomes of *B. crucifer*, *B. ictericus*, and *B. paracnemis*.

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RESUMO

Foram estudados os cromossomos de *Bufo crucifer*, *B. ictericus* e *B. paracnemis*, com coloração convencional, bandas C e RONs. Observou-se nas três espécies número diplóide de 22 cromossomos e cariótipos idênticos, constituídos de metacêntricos e submetacêntricos. Os padrões de bandas C e RONs indicam que estas espécies de *Bufo* não são diferenciadas tampouco pela distribuição e quantidade de heterocromatina constitutiva nem pela localização das regiões organizadoras de nucléolos.

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