

Chromosomal studies on five species of the genus *Leptodactylus* Fitzinger, 1826 (Amphibia, Anura) using differential staining

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Abstract

Cytogenetic studies were carried out on five species of *Leptodactylus*, namely *L. fuscus*, *L. notoaktites*, *L. labyrinthicus*, *L. ocellatus*, and *L. podicipinus*, after standard staining, Ag-NOR and C-banding as well as BrdU incorporation for three of them. The species had $2n = 22$ chromosomes and two basic karyotype patterns. Chromosome 8 was a marker bearing a secondary constriction. In all species, this secondary constriction corresponded to the Ag-NOR site. The species had centromeric C-bands in all chromosomes of the complement, but some interstitial or telomeric bands seemed to differentiate some karyotypes, either at the species or the population level. In *L. ocellatus*, the C-banding pattern confirmed the occurrence of a heteromorphic pericentric inversion in chromosome 8 in specimens from one of the populations. The BrdU incorporation technique showed no detectable difference in the replication patterns of the major bands in the chromosomes of *L. notoaktites*, *L. labyrinthicus*, and *L. ocellatus*.

Introduction

The subfamily Leptodactylinae, comprising eleven genera and 127 species, is widely distributed throughout Brazil, Southeastern Chile, Central America, and Southeastern North America (Frost, 1985; Duellman, 1993). About 53 species, including those of the genus *Leptodactylus*, have been karyotyped, and most of them have $2n = 22$ chromosomes (King, 1990). According to this author, in most species the chromosome complement was formed by metacentric and sub-metacentric chromosomes which gradually decrease in size, except for pair 1, which was much larger than the other elements.

In the family Leptodactylidae the genus *Leptodactylus* has the largest number of representatives studied cytogenetically up to now, totalling 27 species (King, 1990; Kuramoto, 1990; Silva, 1998). However, the most cytogenetic data have been primarily obtained using standard staining techniques. A few studies, like those of Lisanti *et al.* (1990), Baldissera and Batistic (1992), Agostinho (1994), Silva *et al.* (1995), and Amaro and Yonenaga-Yassuda (1998, 1999), used banding techniques and these are, in general, restricted to Ag-NOR localization and detection of C-bands. Bianchi *et al.* (1973)

identified constitutive heterochromatin in the chromosomes of one species of *Leptodactylus* by an autoradiographic method. More recently, Amaro and Yonenaga-Yassuda (1998, 1999) published a brief report about R-banding patterns and fluorescence *in situ* hybridization (FISH) of the telomeric sequence in some species of *Leptodactylus*.

In this paper we describe the results of a cytogenetic analysis of five species of *Leptodactylus* using standard staining, Ag-NOR, and C-band techniques as well as replication banding after BrdU incorporation in three of them. In spite of a very conservative karyotypic pattern, at least among the species *L. fuscus*, *L. notoaktites*, *L. labyrinthicus*, and *L. ocellatus*, C-banding revealed some differences in their karyotypes, at the interspecific and intraspecific level. Additionally, a chromosomal heteromorphism due to pericentric inversion was noticed in one population of *L. ocellatus*.

Materials and methods

Cytogenetic studies of 33 specimens of the genus *Leptodactylus* belonging to the species *L. fuscus*, *L. notoaktites*, *L. labyrinthicus*, *L. ocellatus*, and *L. podicipinus*, were undertaken. Data concerning number of specimens, collection sites, and banding techniques are summarized in Table 1. The voucher specimens were deposited in the amphibian collection of the Departamento de Zoologia, Instituto de Biociências, UNESP, Rio Claro, SP, Brazil.

To obtain mitotic chromosomes, two different procedures were used, namely lymphocyte culture (Kasahara *et al.*, 1998) and direct cytological preparations of bone marrow and liver after *in vivo* colchicine treatment (Baldissera *et al.*, 1993). Chromosome preparations were made of testes.

Some specimens were submitted to *in vivo* or *in vitro* treatment with 5-bromodeoxyuridine plus 5-fluorodeoxyuridine (10 mg BrdU and 0.5 mg FudR in 2 ml 0.9% NaCl solution). For *in vivo* treatment a stock solution of BrdU/FudR, in the proportion of 0.1 ml/10 g body weight, was injected intraperitoneally 18 to 20 h before sacrifice. For *in vitro* treatment, the stock solution of BrdU/FudR was added to each tube at a final concentration of 100 µg/ml, 6 to 15 h before completing the culture time.

Standard staining was undertaken with Giemsa solution. The Ag-NOR staining followed Howell and Black (1980), and the C-banding pattern was according to the technique of Sumner (1972). For the differentiation of replication bands after BrdU incorporation the FPG technique was used according to Dutrillaux and Couturier (1981).

Table 1 Number of specimens, collection sites, and banding techniques used in each species of *Leptodactylus*

Species	Number of specimens	Collection sites	Banding techniques
<i>L. fuscus</i> (Schneider, 1799)	5	Rio Claro, SP	Ag-NOR; C-band
	3	Itirapina, SP	Ag-NOR; C-band
	1	Aracruz, ES	Ag-NOR
	2	Santa Maria, RS	Ag-NOR; C-band
<i>L. notoaktites</i> (Heyer, 1978)	10	Ribeirão Branco, SP	Ag-NOR; C-band; BrdU
	1	Rio Claro, SP	Ag-NOR
<i>L. labyrinthicus</i> (Spix, 1824)	1	Botucatu, SP	Ag-NOR
	4	Rio Claro, SP	Ag-NOR; C-band; BrdU
<i>L. ocellatus</i> (Linnaeus, 1758)	1	Rio Claro, SP	Ag-NOR; C-band; BrdU
	1	Anaurilândia, MS	Ag-NOR; C-band
	2	Guaratuba, PR	Ag-NOR; C-band; BrdU
	1	Santa Maria, RS	Ag-NOR; C-band
<i>L. podicipinus</i> (Cope, 1862)	1	Rio Claro, SP	Ag-NOR; C-band

ES, State of Espírito Santo; SP, State of São Paulo; MS, State of Mato Grosso do Sul; PR, State of Paraná; RS, State of Rio Grande do Sul.

Aracruz, ES 40°16'29'' S, 19°49'13'' W, 60m alt; Rio Claro, SP 22°24'41''S, 47°33'41''W, 625m alt; Itirapina, SP 22°15'10''S, 47°49'22''W, 770m alt; Botucatu, SP 22°53'09''S, 48°26'42''W, 800m alt; Anaurilândia, MS 22°11'15''S, 52°43'04''W, 312m alt; Guaratuba, PR 25°52'58''S, 48°34'29''W, 15m alt; Santa Maria, RS 29°41'S, 53°48'W, 150m alt.

Results

Giemsa staining

The five species of *Leptodactylus* had $2n = 22$ chromosomes. The chromosome pairs of *L. fuscus*, *L. notoaktites*, *L. labyrinthicus*, and *L. ocellatus* were distributed as follows (Figure 1): one large metacentric (chromosome 1), four intermediate submetacentrics (chromosomes 2, 3, 4, and 7), two intermediate metacentrics (chromosomes 5 and 6), and four small metacentrics or submetacentrics (chromosomes 8, 9, 10, and 11). Chromosome 8 showed a secondary constriction at variable sites in the short arms,

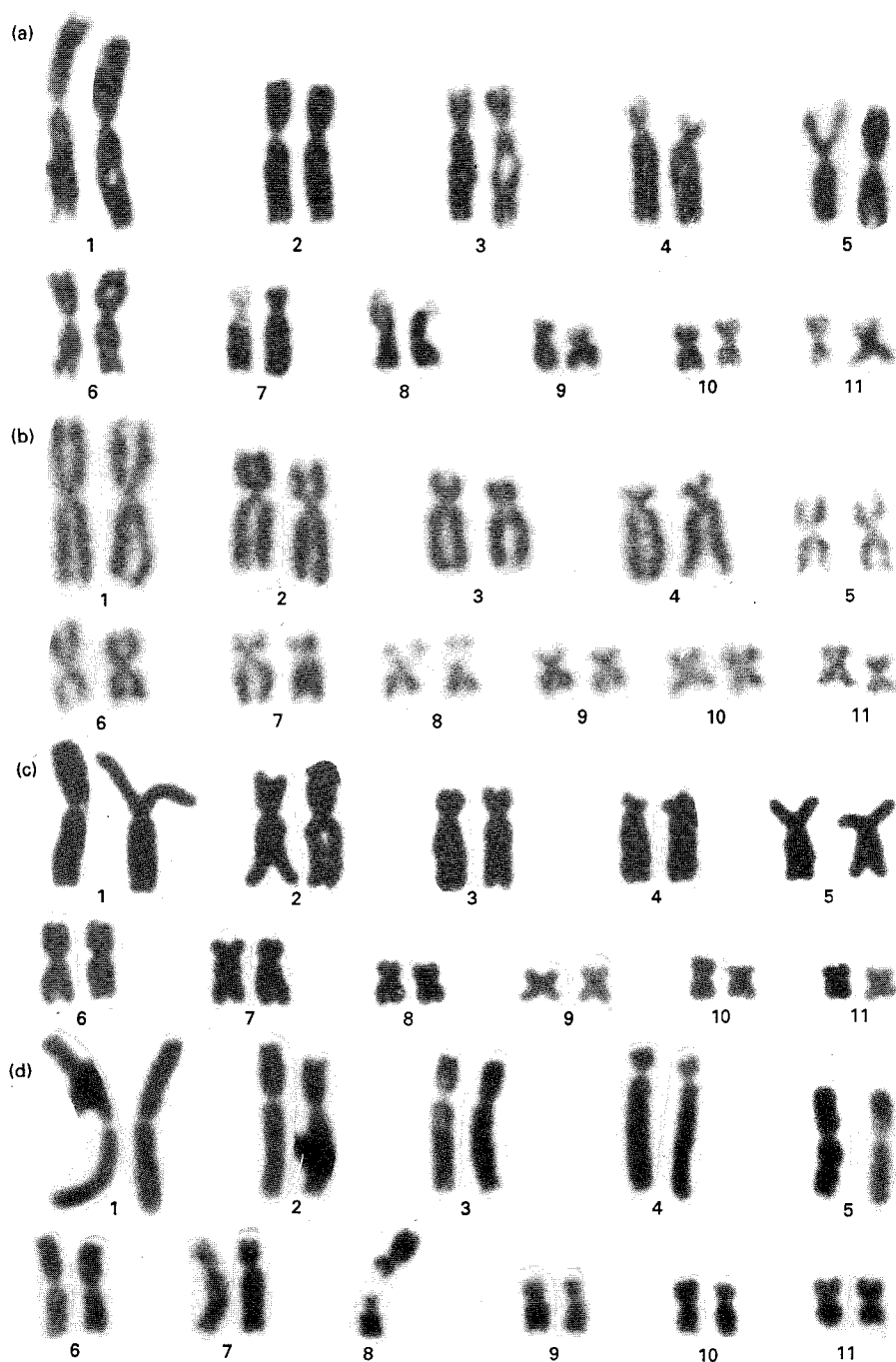


Figure 1 Giemsa-stained karyotypes of male specimens with $2n = 22$ chromosomes. (a) *L. fuscus* from Rio Claro, SP; (b) *L. notoaktites* from Ribeirão Branco, SP; (c) *L. labyrinthicus* from Rio Claro, SP; (d) *L. ocellatus* from Guaratuba, PR. Note the association between heteromorphic chromosome 8 in Figure 1d. x7,500.

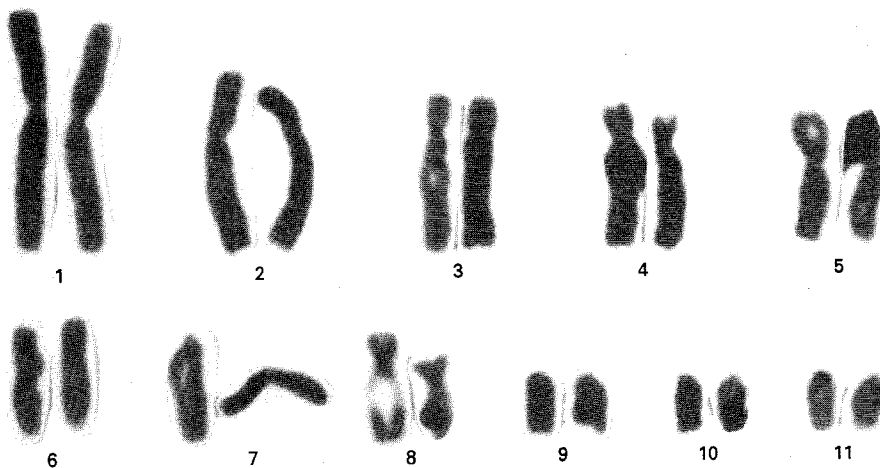


Figure 2 Giemsa-stained karyotype of male *L. podicipinus* with $2n = 22$ chromosomes from Rio Claro SP. $\times 9,300$.

although not always visualized in both homologues. The specimens of *L. ocellatus* from Guaratuba, PR, presented a slight heteromorphism in chromosome 8. The centromere was located more distally in one of the homologues, altering its arm ratio, but both chromosomes frequently appeared in close association (Figure 1d).

The karyotype constitution of *L. podicipinus* was as follows (Figure 2): one large metacentric (chromosome 1), three intermediate submetacentrics (chromosomes 2, 3, and 4), two intermediate metacentrics (chromosomes 5 and 6), one intermediate telocentric (chromosome 7), and four small metacentrics or telocentrics (chromosomes 8, 9, 10, and 11). Chromosome 8 showed a secondary constriction in the long arms.

No heteromorphic sex chromosome pair was recognized in any karyotype. Meiotic analysis of male specimens revealed eleven bivalents in diplotene and metaphase I, and eleven chromosomes in metaphase II cells.

Nucleolus organizer region (NOR) staining

The five species of *Leptodactylus* had a single pair of chromosomes bearing the Ag-NOR, which coincided with the site of the secondary constriction, in the short or long arms of chromosome 8. In *L. fuscus*, *L. notoaktites*, *L. labyrinthicus*, and *L. ocellatus* the Ag-NOR was located in the short arms, whereas in *L. podicipinus* it occurred in the long

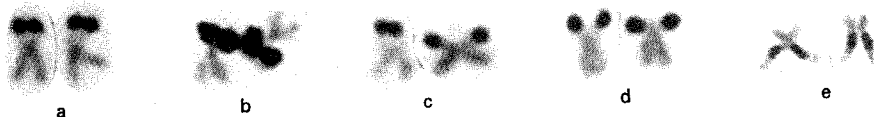


Figure 3 Ag-NOR stained chromosome 8. (a) *Leptodactylus fuscus*; (b) *L. notoaktites*; (c) *L. labyrinthicus*; (d) *L. ocellatus*; (e) *L. podicipinus*. Note chromosome association in Figure 3b. x5,000.

arms (Figure 3). Ag-NOR association (Figure 3b) was frequently seen in the metaphases of all five species, together with variations in Ag-NOR size attributable to differential genetic activity or even to small duplications of rDNA segments.

C-banding

Centromeric C-bands were observed in all chromosomes of the complement, among the five species (Figures 4 and 5). Chromosome 1 manifested positive staining in the terminal region of the long arms, in one or in both homologues, and chromosome 8 showed a large block of C-bands in the short or in the long arms in most species. In *L. labyrinthicus*, chromosome 8 presented a smaller amount of constitutive heterochromatin than in the remaining species.

Other interspecific differences were noticed regarding the distribution of some additional bands. *Leptodactylus labyrinthicus* showed chromosome 2 bearing an interstitial band in the long arms as well as telomeric bands in some chromosomes of the complement (Figure 4c). In *L. fuscus* and *L. ocellatus*, the interstitial and telomeric C-bands seemed to differentiate the karyotypes from distinct localities.

Leptodactylus fuscus from Santa Maria, RS, showed well marked telomeric bands (Figure 6a) not detectable in the specimens from Rio Claro, SP, or Itirapina, SP (Figure 4a). *Leptodactylus ocellatus* from Rio Claro, SP, and Santa Maria, RS, showed a slight C-band in the proximal region of the long arms of chromosome 4 (Figure 5a) while the specimen from Anaurilândia, MS, showed interstitial bands in the short arms of chromosomes 5, 9 and 10, besides telomeric bands in several chromosomes (Figure 6b). The specimens of *L. ocellatus* from Guaratuba, PR, showed an interstitial C-band in the short arms of chromosome 3 and a proximal C-band in the long arms of chromosome 4 (Figure 5b). In these specimens, the homologues of the heteromorphic pair 8 exhibited distinct C-bands, confirming the

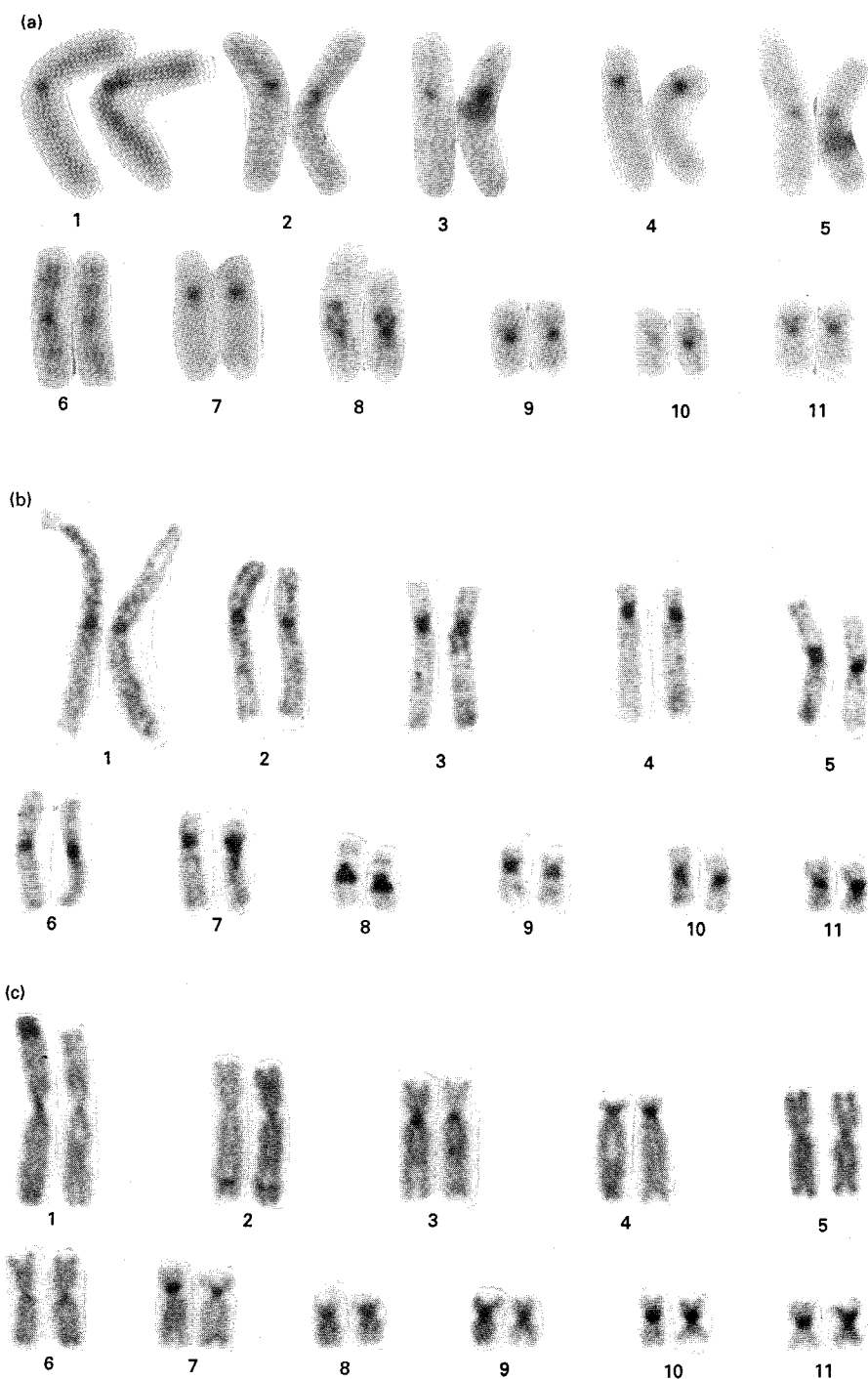


Figure 4 C-banded karyotypes. (a) *L. fuscus* from Rio Claro, SP; (b) *L. notoaktites* from Ribeirão Branco, SP; (c) *L. labyrinthicus* from Rio Claro, SP. x4,750.

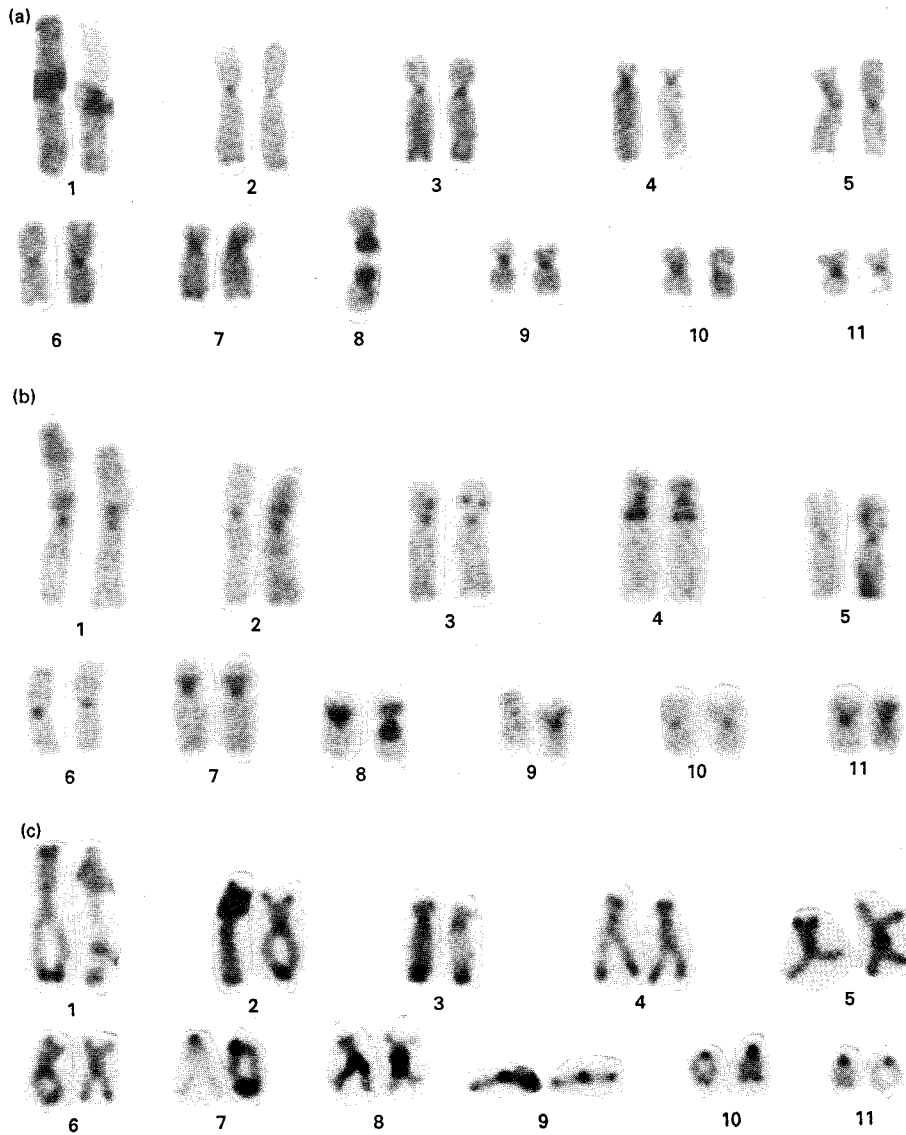


Figure 5 C-banded karyotypes. (a) *L. ocellatus* from Rio Claro, SP; (b) *L. ocellatus* from Guaratuba, PR; (c) *L. podicipinus* from Rio Claro, SP. Note heteromorphic C-banded chromosome 8 due to pericentric inversion in Figure 5b. $\times 13,750$.

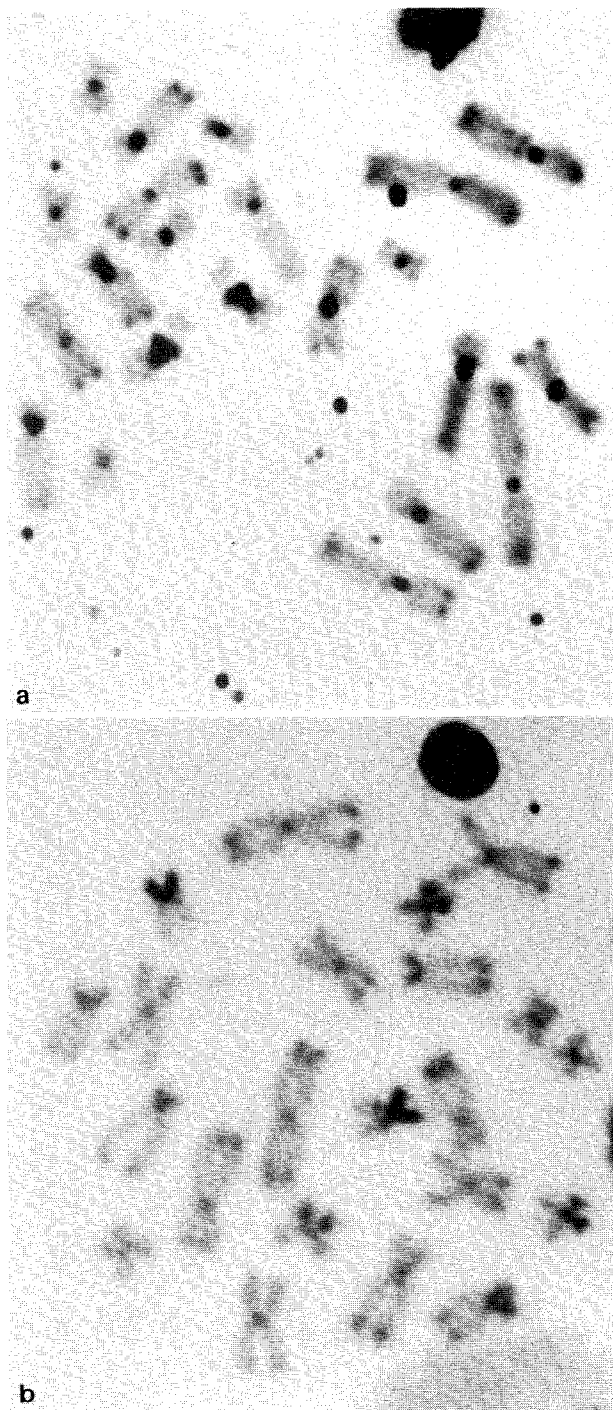


Figure 6 C-banded metaphases. (a) *L. fuscus* from Santa Maria, RS; (b) *L. ocellatus* from Anaurilândia, MS. x6,000.

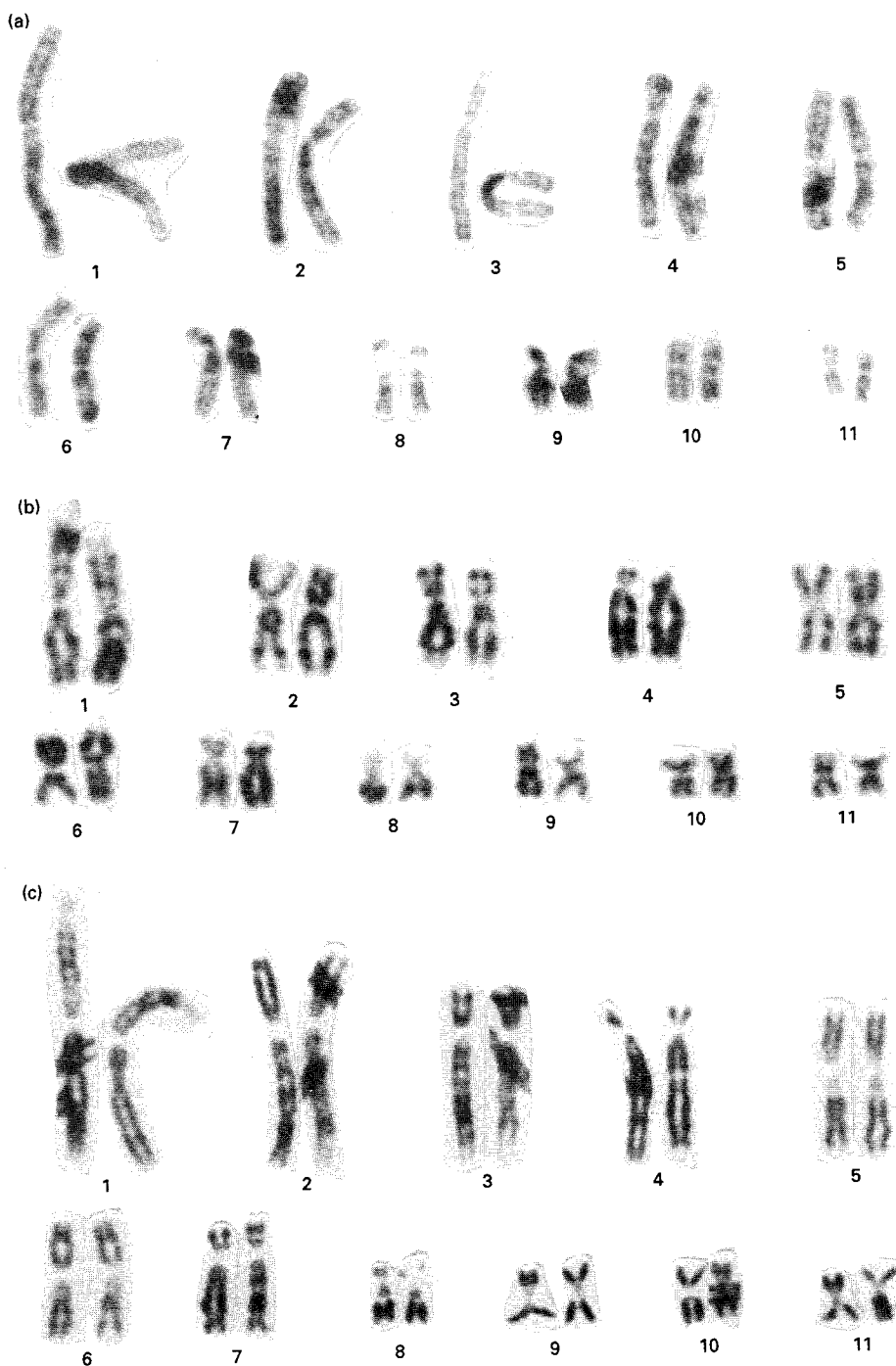


Figure 7 BrdU replication banding karyotypes. (a) *L. notoaktites*, (b) *L. ocellatus*; (c) *L. labyrinthicus*. x12,500.

occurrence of a pericentric inversion: one of them had almost entirely heterochromatic short arms, whereas the other had the C-band shifted to the proximal region of the long arms.

BrdU replication banding

Treatment with 5-bromo-deoxyuridine (BrdU) produced longitudinal differentiation in the chromosomes of *L. notoaktites*, *L. labyrinthicus*, and *L. ocellatus* (Figure 7). The replication bands allowed the homologous pairing of the large and medium-sized chromosomes and even of the small elements in spite of their less evident patterns.

Discussion

The species of *Leptodactylus* examined in the present study belong to each of the morphological groups described by Frost (1985) for the genus: *L. fuscus* and *L. notoaktites* assigned to the *L. fuscus* group, *L. ocellatus* to the *L. ocellatus* group, *L. labyrinthicus* to the *L. pentadactylus* group, and *L. podicipinus* to the *L. melanonotus* group. Using standard staining techniques, most of them, except *L. podicipinus*, showed very similar $2n = 22$ chromosome karyotypes. However, some of these karyotypes were only identified by subtle differences in the position of the secondary constriction in the short arms of chromosome 8. In *L. fuscus* and *L. notoaktites*, this chromosome structure was in an interstitial site, but in the remaining two species it clearly lies at the terminal end. *L. podicipinus* ($2n = 22$) had a very discrepant karyotype due to telocentric elements, rarely found in *Leptodactylus*. In this species, chromosome 8 was a karyotypic marker, bearing a secondary constriction in the proximal region of the long arms.

So far, besides *L. podicipinus*, chromosomes with telocentric morphology have only been observed in the karyotypes of *L. natalensis*, *L. wagneri*, both of the *L. melanonotus* group, and *L. latinasus* of the *L. fuscus* group (Bogart, 1974). Considering that the diploid $2n = 22$ occurs extensively in all species of *Leptodactylus*, it is reasonable to suppose that chromosome rearrangements, mainly of the pericentric inversion type, are responsible for the karyotypic diversification of some *Leptodactylus* species. Unfortunately, BrdU replication banding was not obtained for *L. podicipinus* to test this possibility.

Previous descriptions of the karyotype of *L. fuscus*, *L. ocellatus*, *L. labyrinthicus*, and *L. podicipinus* were made in the 1960's and 1970's based only on standard staining (Brum-Zorilla and Saez, 1968; Beçak *et al.*, 1970; Denaro, 1972; Bogart, 1974). Thus, the present comparative study is the first involving differential staining techniques in species of *Leptodactylus*.

Using silver impregnation, the secondary constriction proved to be the nucleolus organizer region in all five species, so that chromosome 8 should be considered the standard localization of the Ag-NOR among *Leptodactylus* species. Analyses of *L. ocellatus* specimens performed by other workers (Lisanti *et al.*, 1990; Baldissera and Batistic, 1992; Amaro and Yonenaga-Yassuda, 1998) have also shown the occurrence of Ag-NOR in chromosome 8. Nevertheless, in *L. labyrinthicus*, Agostinho (1994) reported chromosome 11 as bearing Ag-NOR. Since chromosomes 8 to 11 were very similar in size and morphology, the discrepant localization of Ag-NOR should be ascribed to distinct ordering of the chromosomes in the karyograms rather than to geographical differentiation.

The position of Ag-NOR in chromosome 8 is variable among *Leptodactylus* species. The shift in localization of Ag-NOR, in the short arms or in the long arms, should be the consequence of minute chromosome rearrangements, like inversions or excision of rDNA segments from one site and reinsertion at another site, without changing the chromosome arm ratio.

Ag-NOR patterns were relatively constant, but the distribution of constitutive heterochromatin was not uniform in the five *Leptodactylus* species. All of them exhibited C-bands at the centromeric region, but the size and the intensity of the positive bands were variable among karyotypes, as also were some of the telomeric or interstitial bands. Although some variability due to technical procedures cannot be completely ruled out, the analysis of several metaphase spreads in each case suggests that a diversification in C-banding patterns occurred at the interspecific or even at the intraspecific level.

Leptodactylus fuscus from Santa Maria, RS, a locality in the South, has a distinct pattern from that found in the specimens collected in Southeastern localities (Rio Claro, SP, and Itirapina, SP). The chief interpopulation difference was observed in *L. ocellatus*, in which three distinct C-banding patterns were noted: one for the specimens from Rio Claro, SP, and Santa Maria, RS; one for the specimen from Anaurilândia, MS; and one for the specimens from Guaratuba, PR. It is premature to ascribe these findings to taxonomically diverse forms, but at least for *L. ocellatus*, there are some differences concerning the external morphology and bioacoustics between animals occurring in the coastal plain and in the plateau areas (unpublished data). A particular C-banding pattern for the specimens from Guaratuba, PR, a locality on the Brazilian coast, might be an additional characteristic indicating that they do not belong to the same species as that of individuals from the plateau areas.

C-banding unequivocally confirmed the occurrence of pericentric inversion in a heteromorphic condition in chromosome 8 in the specimens of *L. ocellatus* from Guaratuba, PR. This rearrangement kept unaltered the site of the Ag-NOR at the distal end of both homologues. Due to the small size of chromosome 8, it was not possible to elucidate the chromosome rearrangement by BrdU replication banding patterns. It is possible that, both homomorphic combinations of chromosome 8 will be found as the sample size of specimens from Guaratuba, PR, is increased.

The preliminary data on BrdU replication banding in *L. notoaktites*, *L. ocellatus* and *L. labyrinthicus* indicate that, although this procedure provided relatively good longitudinal chromosome banding, it was not sufficient to detect differences among the three karyotypes. For this reason, C-banding seems to be the most effective tool for comparative cytogenetic analysis among *Leptodactylus* species with conservative karyotypes.

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