Cytogenetics of two species of *Paratelmatobius* (Anura: Leptodactylidae), with phylogenetic comments

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In this paper we provide a cytogenetic analysis of *Paratelmatobius cardosoi* and *Paratelmatobius poecilogaster*. The karyotypes of both species showed a diploid number of 24 chromosomes and shared some similarity in the morphology of some pairs. On the other hand, pairs 4 and 6 widely differed between these complements. These karyotypes also differed in their NOR number and location. Size heteromorphism was seen in all NOR-bearing chromosomes of the two karyotypes. In addition, both karyotypes showed small centromeric C-bands and a conspicuous heterochromatic band in the short arm of chromosome 1, although with a different size in each species. The *P. cardosoi* complement also showed other strongly stained non-centromeric C-bands, with no counterparts in the *P. cardosoi* karyotype. Chromosome staining with fluorochromes revealed heterogeneity in the base composition of two of the non-centromeric C-bands of *P. cardosoi*. Comparison of the chromosomal morphology of these *Paratelmatobius* karyotypes with that of *P. lutzii* showed that the *P. poecilogaster* karyotype is more similar to that of *P. lutzii* than *P. cardosoi*. These cytogenetic results agree with the proposed species arrangements in the *P. cardosoi* and *P. lutzii* groups based on morphological and ecological data.

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The genus *Paratelmatobius* Lutz and Carvalho is endemic to Brazil and comprises the species *P. lutzii*, *P. gaigeae*, *P. poecilogaster*, *P. cardosoi* and *P. mantiqueira* (see review by POMBAL JR. and HADDAD 1999). *Paratelmatobius* species are usually rare, and *P. gaigeae*, *P. lutzii* and *P. mantiqueira* may be considered as missing species (POMBAL JR. and HADDAD 1999). Based on morphological and ecological data, POMBAL JR. and HADDAD (1999) recognized two species groups, the *P. cardosoi* group and the *P. lutzii* group, in this genus. The former group contains *P. cardosoi* and *P. mantiqueira* and the *P. lutzii* group encompasses *P. lutzii*, *P. poecilogaster*, and probably *P. gaigeae*.

The intergeneric phylogenetic relationships of this group are not yet clear. LYNCH (1971) considered *Paratelmatobius* a member of the subfamily Leptodactylinae and pointed out some morphological similarities among *Paratelmatobius*, *Scythrophrys*, *Physalaemus* and, to a lesser extent, *Zachaenus*. This classification was followed by FROST (1999). HEYER (1975) *Paratelmatobius* grouped together with the genera *Scythrophrys*, *Zachaenus*, *Thoropa*, *Megaelosia*, *Hylodes*, *Cycloramphus*, *Crossodactylus*, *Crossodactylodes* and *Craspedoglossa*. The proximity between the genera *Paratelmatobius* and *Scythrophrys*, suggested by LYNCH (1971) and HEYER (1975), was also indicated by GARCIA (1996). Based on ecological and morphological characters, GARCIA (1996) suggested a closer relationship between *Scythrophrys* and *P. gaigeae* (= *P. cardosoi* sensu POMBAL JR. and HADDAD 1999) than between this species of *Paratelmatobius* and *P. lutzii*, and suggested a revision of both of these genera.

The cytogenetic information on *Paratelmatobius* is restricted to the chromosome number and morphology of the karyotype of *P. lutzii* (DE LUCCA et al. 1974). To provide more data for a phylogenetic analysis of this group, we have done a cytogenetic study of the living species of *Paratelmatobius*, *P. cardosoi* Pombal Jr. and Haddad and *P. poecilogaster* Giaretta and Castanho (see review by POMBAL JR. and HADDAD 1999).

MATERIALS AND METHODS

Animals

Fifteen males of *P. cardosoi* and 21 specimens (3 males, 3 females, 11 tadpoles and 4 juveniles) of *P. poecilogaster* from Paranapiacaba, São Paulo State (Brazil) were studied. The *P. cardosoi* specimens were

collected in November and December 1997 and the *P. poecilogaster* individuals were collected in December 1994, March 1995, November and December 1997. All the animals, except the tadpoles, one female and two juveniles of *P. poecilogaster*, were deposited in ZUEC (Museu de História Natural "Prof. Dr. Adão José Cardoso", Universidade Estadual de Campinas, Campinas, SP, Brasil) or in CFBH (Célio F.B. Haddad's collection, deposited in Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro, SP, Brasil).

Chromosome preparation and techniques

Mitotic chromosomes were obtained from intestinal and testes cell suspensions as described by SCHMID (1978a) and SCHMID et al. (1979). Conventional staining with a 10 % Giemsa solution, the C-banding technique (KING 1980), the Ag-NOR method (HOWELL and BLACK 1980), staining with distamycin A/DAPI (SCHWEIZER 1980), and fluorescence in situ hybridization (FISH) with an rDNA probe (VIEGAS-PÉQUIGNOT 1992) were performed in chromosomal preparations from both species. The rDNA probe used in FISH experiments consisted in a mixture of two recombinant plasmids, HM123 and HM456 (MEUNIER-ROTIVAL et al. 1979), which were biotin-labeled by a nick translation reaction according to the GIBCO protocol. To investigate further the *P. cardosoi* heterochromatin, metaphases from this species were stained with distamycin A/mithramycin A (SCHWEIZER 1980). The slides were examined on an Olympus microscope or on a BioRad MRC 1024 UV confocal microscope. Some of the photographs were obtained using the software Image Pro-Plus (Media Cybernetics), Version 3.

RESULTS

Paratelmatobius cardosoi

The full chromosomal complement of this species was 2n = 24 and consisted of eight pairs of metacentric chromosomes (1–3, 5, 6, 9, 11 and 12), three submetacentric pairs (7, 8, and 10) and one subtelocentric pair (4) (Fig. 1A, 1C, 2 and Table 1). In all specimens analyzed, there was a secondary constriction in the long arm of both homologues of chromosome 7 (Fig. 1A), which were detected as NORs by the Ag-NOR method (Fig. 2A and 2B) and in situ hybridization (Fig. 2C). These techniques also identified a size heteromorphism between the homologous NORs in five of the specimens studied (Fig. 2A and C; Table 2). After distamycin A/mithramycin A staining, the NORs showed a bright fluorescence (Fig. 3A)

Table 1. *Morphometric parameters of the Paratelmatobius cardosoi and Paratelmatobius poecilogaster karyotypes. The measurements were based on 26 metaphases from five specimens of P. cardosoi and 25 metaphases from five specimens of P. poecilogaster. The chromosome nomenclature relative to the centromeric position follows* GREEN *and* SESSIONS *(1991)*

		Chromosomes												
		1	2	3	4	5	6	7	8a	8b	9	10a	10b	10c	11	12
P. cardosoi	r. l.	12.42	12.12	11.38	9.83	9.50	9.12	8.27	6.83	–	5.88	5.34	–	–	4.38	4.08
	a. r.	1.62	1.56	1.57	6.76	1.30	1.25	2.17	2.01	–	1.18	1.86	–	–	1.62	1.20
	c.c.	m	m	m	st	m	m	sm	sm	–	m	sm	–	–	m	m
P. poecilogaster	r. l.	13.79	11.80	10.96	10.50	9.75	8.80	7.70	6.29	5.68	5.46	4.96	4.16	5.58	4.70	4.40
	a. r.	1.51	2.06	2.54	1.45	1.10	3.03	2.35	2.67	2.33	1.48	1.58	2.02	1.18	1.64	1.40
	c.c.	m	sm	sm	m	m	st	sm	sm	sm	m	m	sm	m	m	m

r.l.: relative length (%); a.r.: arm ratio; c.c.: centromeric classification; m: metacentric; sm: submetacentric; st: subtelocentric

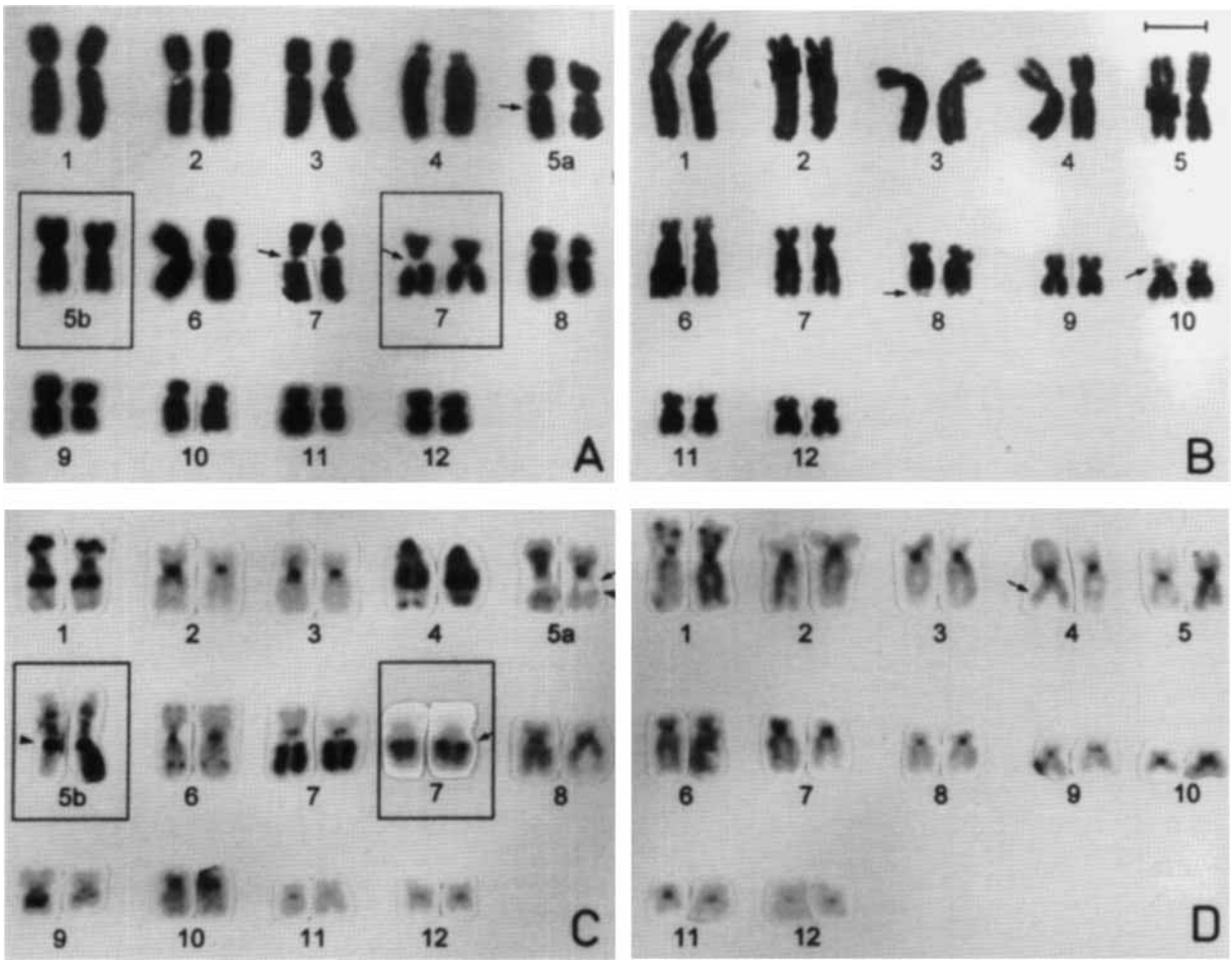

Fig. 1A–D. Karyotypes of *Paratelmatobius cardosoi* (**A**,**C**) and *Paratelmatobius poecilogaster* (**B**,**D**) after Giemsa staining (**A**,**B**) and C-banding (**C**,**D**). In **A**, the secondary constrictions corresponding to homomorphic and heteromorphic NORs (pair 7 in the inset) are indicated by small arrows. An arrow indicates the secondary constriction in chromosome 5a (not equivalent to a NOR in **A** and **C**). In **B**, the arrows indicate secondary constrictions of NORs. In **C**, the arrowhead indicates a small C-band in chromosome 5a and a large one in 5b. The inset shows pair 7 from another metaphase. Note that a heterochromatic block adjacent to the NOR (small arrow) can be distinguished from the remaining arm. In **D**, the arrow indicates a faint band in the long arm of chromosome 4. Bar = 5 μm (A, C) and 5.4 μm (B, D).

while in distamycin A/DAPI stained metaphases, they were negatively stained (Fig. 3B).

C-banding detected small amounts of constitutive heterochromatin in the centromeric region of all the chromosomes of this karyotype. Dark blocks of constitutive heterochromatin were also observed interstitially in both arms of chromosomes 1 and in the long arm of chromosomes 4, 5 and 9, and in the telomeric region of the long arm of chromosome 4. The whole short arm of chromosome 4 and the long arm of chromosome 7, except for the NOR, were revealed by C-banding. A heterogeneous intensity of staining was observed throughout this heterochromatic segment in the long arm of chromosome 7. A faintly stained C-band was seen in the short arm of chromosome 5 (Fig. 1C).

Variation in size was seen in the dark C-band of the long arm of chromosome 5. In two individuals, this band was smaller in both homologues (morph 5a), while three other specimens showed a bigger band (morph 5b) which was also homozygous. A heterozygous state for pair 5 was seen in one specimen. In Giemsa-stained metaphases, a secondary constriction was observed interstitially in the long arm of chromosome 5a (Fig. 1A). This constriction was also detected after C-banding (Fig. 1C). All of the seven specimens analyzed by C-banding showed size heteromorphism for the interstitial band of pair 9 (Fig. 1C).

Fluorescent staining with distamycin A/mithramycin A and distamycin A/DAPI revealed some heterochromatic blocks as bright segments in

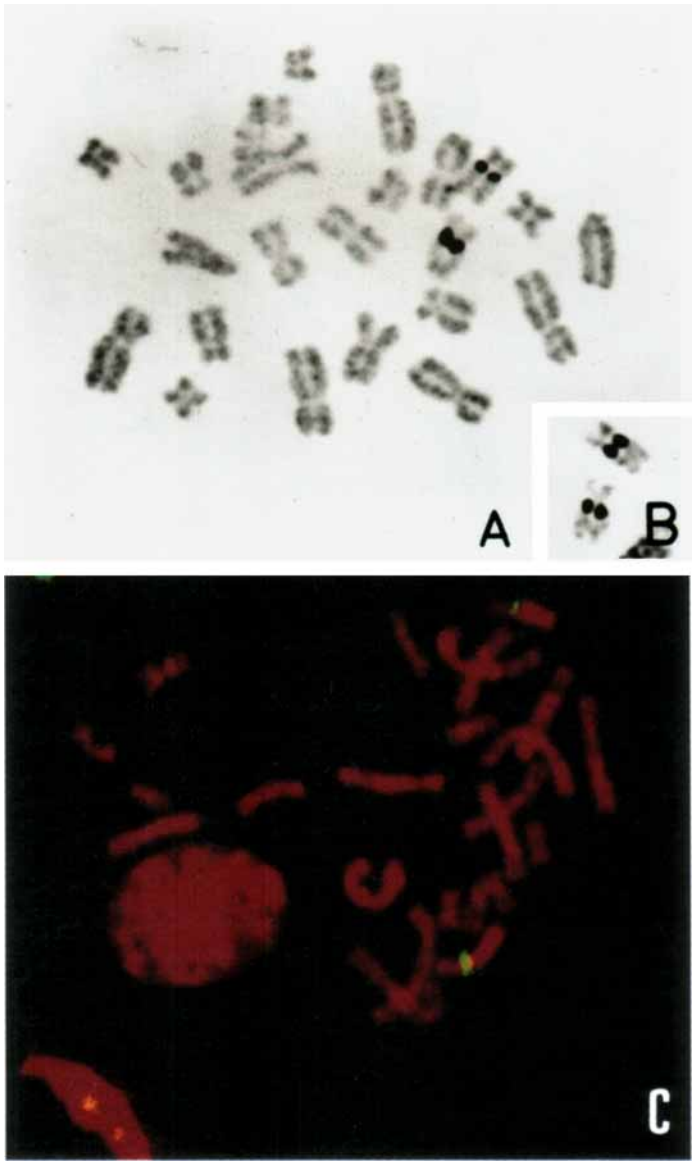

Fig. 2A–C. *P. cardosoi* chromosomes treated with the Ag-NOR method (**A**, **B**) and FISH with rDNA probes (**C**). Note the size heteromorphism of the NORs detected in A and C. In **B**, a pair of homomorphic NORs.

the *P. cardosoi* karyotype. The C-band in the long arm of chromosome 1 was brightly fluorescent in mithramycin-stained metaphases. Additionally, the C-band in the short arm of chromosome 1 showed a mithramycin-positive and DAPI-negative block adjacent to a mithramycin-negative and DAPI-positive block. In chromosome 7, the interstitial dark C-band adjacent to the NOR was more fluorescent after distamicyn A/DAPI staining than the remaining heterochromatic arm. In contrast, the NOR was only slightly fluorescent after mithramycin staining. Also, in one individual, a slightly brighter region coincident with a large C-band block was detected by distamycin/mithramycin A staining in the long arm of chromosome 5. No bright fluorescence was detected in any centromeric region or heterochromatic segments of chromosome 4 (Fig. 3).

Paratelmatobius poecilogaster

The *P. poecilogaster* karyotype consisted of metacentric and submetacentric chromosomes, and the diploid number was $2n = 24$ (Fig. 1B and 1D; Table 1). Two NOR-bearing chromosomal pairs (8 and 10) were identified in all specimens examined by the

Ag-NOR method (Fig. 4A–D) and by fluorescence in situ hybridization (Fig. 4E and F). Variation in NOR size was detected for both pairs of NOR-bearing chromosomes (Fig. 4). Four patterns of NOR distribution were seen in the 12 specimens examined (Fig. 4A–D). The analysis of a considerable number of metaphases (Table 2) showed no intraindividual variation in the NOR pattern, although the smaller NOR of the pair 8 of BC 33.09 (Fig. 4C) was hardly visualized in some metaphases. The NORs were detected as secondary constrictions in Giemsa-stained and distamycin A/DAPI-stained metaphases (Figs 1B and 5).

Table 2. *Number of silver-stained and C-banded metaphases analyzed from each specimen. CFBH: Prof. Célio F. B. Haddad's collection. ZUEC: Museu de História Natural "Prof. Dr. Adão José Cardoso", Universidade Estadual de Campinas, Campinas, SP, Brasil. BC: symbol attributed in the Cytogenetic Laboratory of the Department of Cell Biology for the identification of the specimens that were not deposited in museum. The asterisks indicate specimens with evident heteromorphic NORs in their genomes*

Specimen	Metaphases analyzed by	
	Ag-NOR	C-banding
P. cardosoi		
CFBH 3255	40	31
CFBH 3256	4	–
CFBH 3257	6	4
CFBH 3258	9	–
CFBH 3259	41*	37
CFBH 3260	17	–
CFBH 3261	50	–
CFBH 3262	15*	8
CFBH 3263	10	8
CFBH 3264	24*	61
CFBH 3265	11	–
CFBH 3266	83*	115
CFBH 3267	70*	44
CFBH 3268	13	5
CFBH 3269	24	29
P. poecilogaster		
ZUEC 9599	12	–
BC 33.09	111*	37
BC 33.10	51*	20
BC 33.11	7*	3
BC 33.13	6*	3
ZUEC 9747	11*	41
BC 33.16	7*	–
BC 33.17	10*	–
BC 33.18	15	–
CFBH 3251	26	7
CFBH 3253	31	–
CFBH 3254	61	–

C-banding revealed a small amount of centromeric heterochromatin in all chromosomes. A dark interstitial C-band was identified in the short arm of chromosome 1 and a faint C-band was observed interstitially in the long arm of chromosome 4. No interindividual variation was detected among the five specimens analyzed by this technique (Fig. 1D). Fluorescent staining with DAPI did not produce bright regions in *P. poecilogaster* chromosomes.

DISCUSSION

The *Paratelmatobius* karyotypes described here and that of *P. lutzii* (De Lucca et al. 1974) have the same chromosome number (2n = 24). Comparison of the relative length and the centromeric position of the chromosomes of *P. lutzii* (De Lucca et al. 1974) and *P. poecilogaster* shows that these karyotypes are very similar. The divergence between the arm ratio of chromosome pairs 3 and 4 of *P. lutzii* and the corresponding pairs in *P. poecilogaster* may be an artifact. This apparent divergence can be explained by an exchange between the arm ratios for pairs 3 and 4 of *P. lutzii* in the table reported by De Lucca et al. (1974), what can be supported by the figure of the chromosomes of this species shown in the same paper. Thus, chromosome pairs 3 and 4 of *P. lutzii* can be considered to be homologous to the corresponding pairs in *P. poecilogaster*.

Similarly, pairs 6 and 7 in *P. lutzii* are probably homologous to pairs 7 and 6 in *P. poecilogaster*, respectively, despite the different relative lengths attributed to these pairs in these two species. Such differential classification can be considered a technical artifact caused by divergent methods of measurement adopted in these karyotypes descriptions, but may also reflect small interspecific variations in the relative length of these chromosomes. The same argument applies to pairs 11 and 12 of these *Paratelmatobius* species.

The *P. lutzii* and *P. poecilogaster* karyotypes actually differed in the morphology of chromosome 8, which was metacentric in the *P. lutzii* and submetacentric in *P. poecilogaster*. In the later species, pair 8 was one of the NOR-bearing chromosome pairs. The location of the NOR in the *P. lutzii* karyotype is unknown.

Based on chromosomal morphology, the *P. cardosoi* karyotype resembled that of *P. lutzii* and *P. poecilogaster*, but pairs 4 and 6 are widely divergent in the *P. cardosoi* complement (Fig. 6). NOR analyses also showed large differences between the *P. poecilogaster* and *P. cardosoi* karyotypes. While in *P. cardosoi* the NOR-bearing chromosome was a medium sized submetacentric pair (pair 7), in *P. poe-*

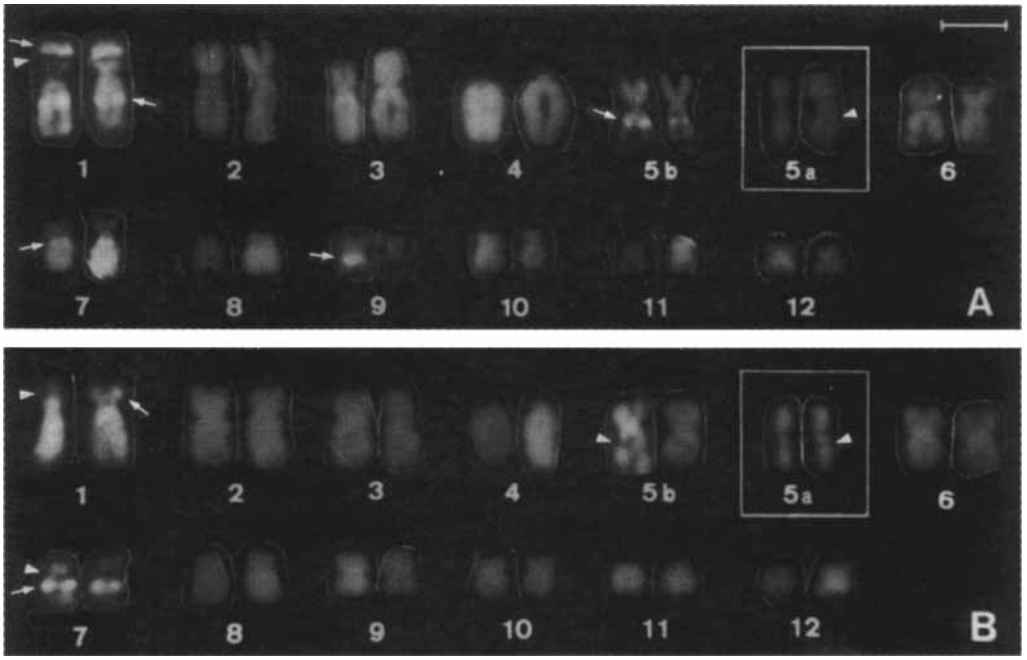

Fig. 3A and B. Karyotype of *P. cardosoi* stained with DA/mithramycin A (**A**) and DA/DAPI (**B**). The arrows indicate positively stained regions and the arrowheads indicate negatively stained segments. Note that the NOR is faintly stained in A and negatively stained in B. Bar = 5 μm.

cilogaster, NORs are observed in two smaller chromosome pairs (pairs 8 and 10).

Multiple NOR-bearing chromosome pairs have been considered a derived state in Anura (KING et al. 1990), and have been found in species from different families (SCHMID 1978b; MAHONY and ROBINSON 1986; WILEY et al. 1989; SCHMID et al. 1995; LOURENÇO et al. 1998a). The evolutionary origin of multiple NORs in anurans probably includes events such as inversions, translocations, transposition by mobile genetic elements, amplification of "orphan" rDNA cistrons, and reinsertion errors during extrachromosomal amplification of ribosomal cistrons, as have been discussed by several authors (WILEY et al. 1989; KING et al. 1990; FOOTE et al. 1991; SCHMID et al. 1995; LOURENÇO et al. 1998a).

Although no intraspecific variation was seen in the localization of NOR in the two species examined, size heteromorphism was detected in all NOR-bearing chromosome pairs in *P. cardosoi* and *P. poecilogaster*. In situ hybridization experiments with rDNA probes and silver staining revealed the same regions in all metaphases, and both techniques were able to detect homologous NOR size heteromorphism in some specimens. The coincident labeling suggested the absence of silent NOR in these karyotypes, and that the size heteromorphisms reflect different numbers of rDNA genes in homologous NOR rather than a differential expression of these regions.

In anurans, size heteromorphism of homologous NORs has been described extensively (SCHMID 1978a and b, SCHMID 1980a and b, SCHMID 1982), with most cases being attributed to NOR amplification, although deletion events have been reported (SCHMID 1982). The correlation between in situ labeling and the amount of rDNA genes has been noted by others (KING 1988; KING et al. 1990). Additionally, the existence of individuals with different amounts of rDNA has been shown by molecular analyses, such as those performed by KNOWLAND and MILLER (1970a,b) which involved normal individuals and mutants of *Xenopus laevis* with a partial nucleolus.

P. cardosoi and *P. poecilogaster* had a small amount of centromeric heterochromatin, but *P. cardosoi* showed a larger quantity of non-centromeric heterochromatin. The similar morphology of chromosome pair 1 from both species allows the recognition of a probable homology between the interstitial C-band in the short arm of *P. poecilogaster* chromosome 1 with part of the interstitial C-band in the short arm of *P. cardosoi* chromosome 1, since this band is bigger than in *P. poecilogaster*. The other non-centromeric C-bands of *P. cardosoi* have no counterparts in the *P. poecilogaster* karyotype.

Fluorochrome staining of *P. cardosoi* metaphases revealed heterogeneity in two heterochromatic blocks in this karyotype. One of the blocks corresponded to the C-band in the short arm of chromosome 1, which

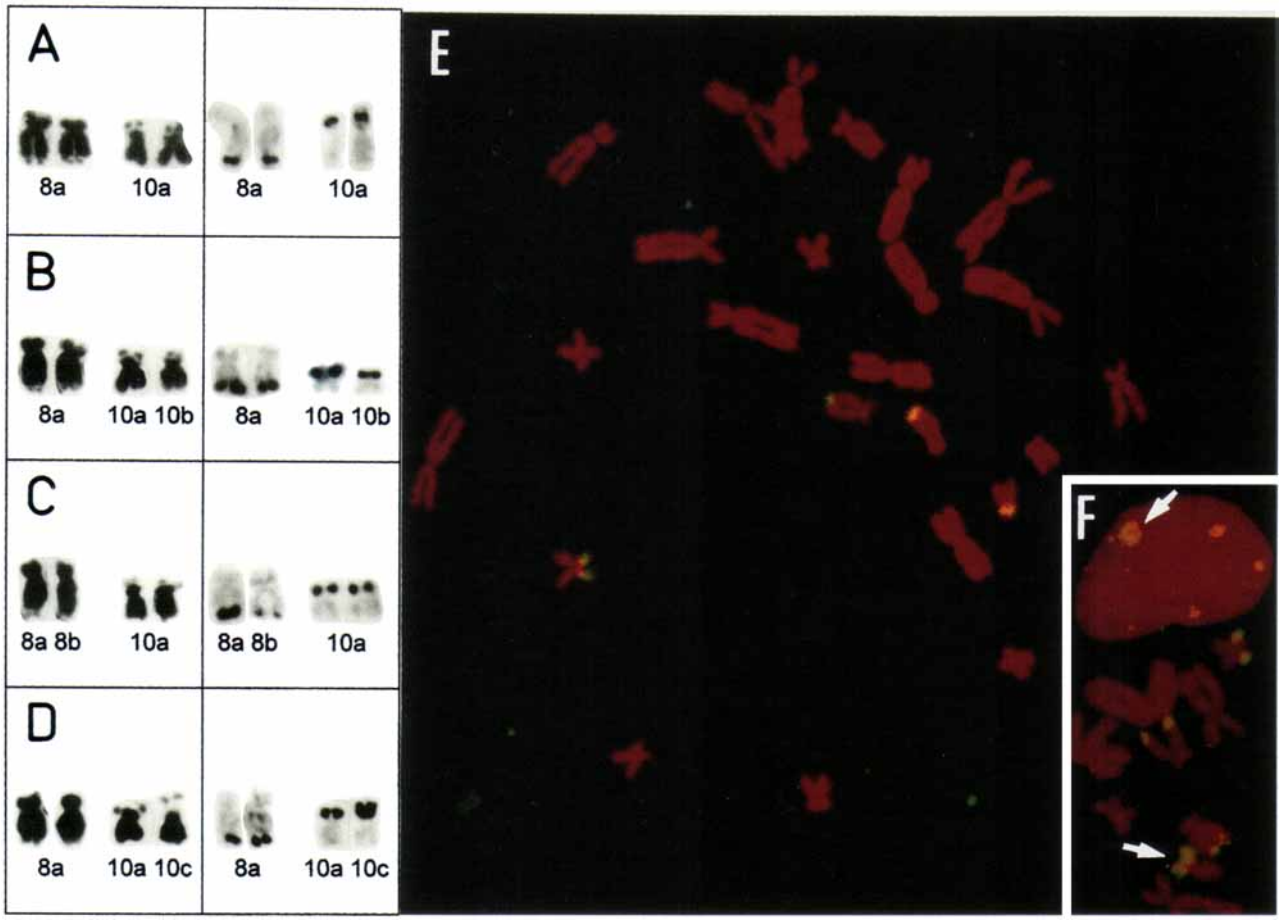

Fig. 4A–F. Fig. A–D. NOR-bearing chromosomes of *P. poecilogaster* stained with Giemsa (left squares) and the Ag-NOR method (right squares). The four patterns of NOR distribution are shown. In **E**, a metaphase with two pairs of homomorphic NORs after FISH with rDNA probes. In **F**, the same pattern shown in D, after hybridization with rDNA probes. Note the larger label seen in the chromosome and nucleus (arrow).

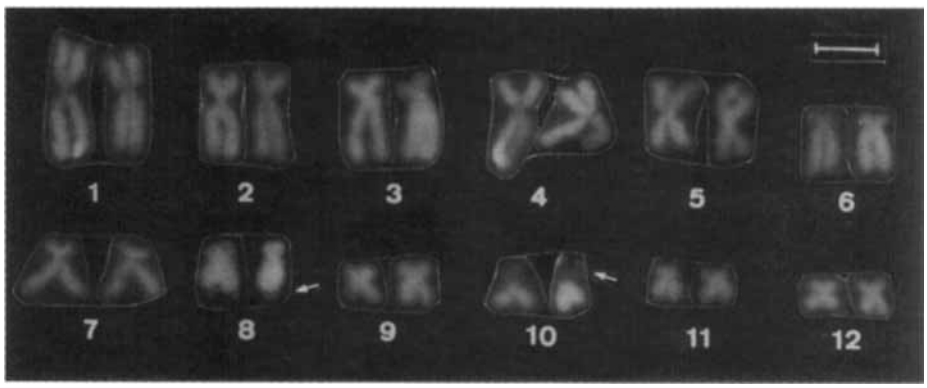

Fig. 5. Karyotype of *P. poecilogaster* stained with DA/DAPI. The NORs are negatively stained (arrows). Bar = 5 μm.

showed an AT-rich region adjacent to a GC-rich one. The second region corresponds to the heterochromatic block located between the NOR and the telomere in chromosome 7. Only a small region adjacent to this NOR was AT-rich, as shown by distamycin/DAPI staining. The remaining heterochromatic arm of this chromosome showed no specific base-pair richness. This heterogeneity in the composition of

chromosome 7 heterochromatin may be responsible for the differential staining of this segment in some C-banded metaphases, in which a darker region was seen between the NOR constriction and the remaining long arm of the chromosome.

The analysis of *P. cardosoi* heterochromatin also revealed size variation in the interstitial bands of the long arms of chromosomes 5 and 9. Two morphs of each pair were identified. Interindividual variation in the C-banding pattern was observed, with three combinations of morphs 5a and 5b found in the specimens studies. In all C-banded specimens, chromosome pair 9 was heteromorphic.

This comparative cytogenetic analysis of *Paratelmatobius* species indicates that the *P. poecilogaster* karyotype is more similar to that of *P. lutzii* than to *P. cardosoi*. This conclusion agrees with the proposal of POMBAL JR. and HADDAD (1999) concerning the *P. lutzii* and *P. cardosoi* groups.

The diploid number of 2n = 24 seen in *Paratelmatobius* is rarely found in the family Leptodactylidae (see KING 1990 and KURAMOTO 1990). Among the genera considered by LYNCH (1971) and HEYER (1975) to be closely related to *Paratelmatobius*, only one species of the genus *Hylodes*, *H. nasus* (BOGART 1991), and the monotypic genus *Scythrophrys* (LOURENÇO et al. 1998b, and manuscript in preparation) have 24-chromosome karyotypes. However, based on the chromosomal morphology, the *H. nasus* karyotype (BOGART 1991) does not share many similarities with those of *Paratelmatobius* described here. A complete chromosomal evaluation of *Paratelmatobius* requires a better characterization of the *H. nasus* karyotype and, specially, a cytogenetic analysis of *Scythrophrys*. Other leptodactylid species with 24-chromosome karyotype is *Adenomera marmorata* (BOGART 1974), but this karyotype, with seven telocentric, two subtelocentric, one submetacentric and 2 metacentric chromosome pairs, widely differs from those described in the present paper.

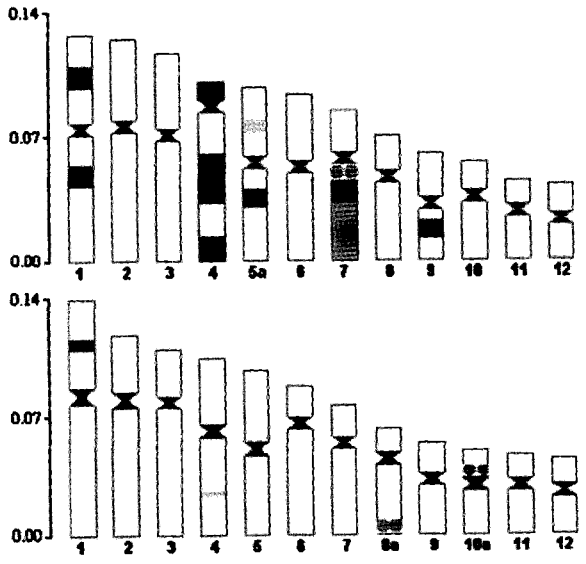

Fig. 6. Idiograms of the *Paratelmatobius cardosoi* (top) and *Paratelmatobius poecilogaster* (bottom) karyotypes. Solid blocks: dark C-bands. Dark gray block: C-band seen as a clearer region in some C-banded metaphases. Light gray block: faint C-band. Open regions: secondary constrictions. Checkered circles: NORs.

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