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Polymorphism of the nucleolus organizer regions (NORs) in *Physalaemus petersi* (Amphibia, Anura, Leptodactylidae) detected by silver staining and fluorescence *in situ* hybridization

Luciana B. Lourenço, Shirlei M. Recco-Pimentel & Adão J. Cardoso*

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The nucleolus organizer regions (NORs) of both karyotypes I and II of *Physalaemus petersi* (Jiménez de la Espada, 1872) from the Brazilian Amazon were studied by Giemsa staining, and by the Ag-NOR method. Karyological group I specimens were also studied by the fluorescence *in situ* hybridization (FISH) technique. Multiple NOR-bearing chromosomes were detected in both karyotypes. The coincident results of the Ag-NOR and FISH methods rule out the occurrence of silent NORs in this anuran. There was no intraindividual NOR variability in either group, but interindividual variability of NORs was high in group I. Seven different patterns of active NOR distribution were definitely recognized among fifteen specimens. This was considered to be a NOR site polymorphism. These results, combined with the C-band polymorphism previously reported for *P. petersi*, demonstrate a high rate of chromosome evolution in this group.

Key words: Amphibia, cytogenetics, *in situ* hybridization, nucleolus organizer region polymorphism

Introduction

Nucleolus organizer regions (NORs) are important markers for the study of chromosome evolution. The number and position of NORs are usually characteristic of species or populations, although interindividual variability of these regions has been observed within populations of various organisms. In Anura, NOR analysis by silver staining has shown that most species, in both primitive and derived families, possess only one pair of NORs in their diploid karyotypes (see Schmid 1982, Mahony & Robinson 1986). This observation led King *et al.* (1990) to suggest the presence of only a single pair of NORs in diploid karyotypes as an ancestral condition in Anura, a hypothesis previously proposed by Schmid (1978a) for bufonids and hylids.

The leptodactylid *Physalaemus petersi* (Jiménez de la Espada, 1872) is an interesting species for cytogenetic studies. Previous analysis revealed the occurrence of two distinct karyotypes, called karyotypes I and II, among sympatric specimens from the Brazilian Amazon, morphologically identified as *P. petersi*, suggesting the occurrence of cryptic species. We also detected heteromorphic sex chromosomes (XX/XY) and C-band polymorphism among specimens with karyotype I (L. Lourenço *et al.*, in preparation). In the present paper, we describe the presence of multiple NORs and NOR polymorphism in *P. petersi* from the same locality as those studied previously.

Materials and methods

Specimens

Sixteen specimens of *P. petersi* (Table 1) from the Brazilian Amazon forest were studied. One male and one female were collected from the Humaitá Reserve in April 1995, two females and eight males were obtained from the Tejo estuary, and four males from the Restauração municipality (Marechal Thaumaturgo), both in the alto Juruá Reserve, in January 1996. These forest reserves are in Acre State, Brazil. All animals were deposited in the Natural History Museum 'Professor Adão José Cardoso' of the State University of Campinas (ZUEC), in Brazil.

The specimens were classified as members of the karyological group I or II after the comparative analysis of Giemsa-stained metaphases with those karyotypes previously described (L. Lourenço *et al.*, in preparation).

Chromosome preparation and NOR analysis

Mitotic metaphases were obtained from intestine and testis cell suspensions according to the methods of Schmid (1978a) and Schmid *et al.* (1979). The Ag-NOR method was carried out as described by Howell & Black (1980) using slides stored at −20°C or previously treated by the FISH technique and washed in distilled water, as proposed by Hirai *et al.* (1994). Secondary constrictions were recorded from metaphases stained with Giemsa. Two recombinant plasmids (HM123, HM456), containing fragments of *Xenopus laevis* rDNA (Meunier-Rotival *et al.* 1979), were used as individual probes or together in a probe mix to identify the ribosomal genes in FISH experiments performed according to the protocol of Viegas-Péquignot (1992). These plasmids were biotin labelled by the nick translation reaction according to the GIBCO protocol. After FISH,

L. B. Lourenço and S. M. Recco-Pimentel (corresponding author) are at the Department of Cell Biology, and Adão J. Cardoso is at the Department of Zoology, Biology Institute, Unicamp, 13083-970, Campinas, SP, Brazil. Tel: (+55) 19 788 7786; Fax: (+55) 19 788 7821; Email: Shirlei@obelix.unicamp.br.
*In memoriam

Table 1. Museum catologue number, sex, origin and number of silver-stained metaphases that support the identification of the NOR pattern of *Physalaemus petersi*

Animals	sex	location	NOR pattern	Number of Ag-stained metaphases	
				From intestine	From testis
Karyological group I					
ZUEC 9610	F	Humaitá Reserve	VII	11	–
ZUEC 9628	M	Humaitá Reserve	VII	16	–
ZUEC 9650	M	Restauração	III	13	–
ZUEC 9652	M	Restauração	III	21	16
ZUEC 9653	M	Restauração	III	4	–
ZUEC 9620	F	Tejo estuary	IV	8	–
ZUEC 9624	F	Tejo estuary	IV	9	–
ZUEC 9625	M	Tejo estuary	I	23	20
ZUEC 9639	M	Tejo estuary	V	52	–
ZUEC 9641	M	Tejo estuary	VI	12	–
ZUEC 9642	M	Tejo estuary	III	4	–
ZUEC 9643	M	Tejo estuary	IV	16	–
ZUEC 9645	M	Tejo estuary	V	20	–
ZUEC 9647	M	Tejo estuary	V	10	–
ZUEC 9649	M	Tejo estuary	V	115	–
Karyological group II					
ZUEC 9654	M	Restauração	VIII	25	–

ZUEC, Natural History Museum Professor Adão José Cardoso, State University of Campinas, Brazil.

slides were screened on a Zeiss Axiophot Microscope equipped with a FITC filter or on a Zeiss Confocal Microscope coupled to BioRad software.

Results

Fifteen of the animals used in the present study belong to karyological group I, and one male (ZUEC 9654) from Restauração municipality (Alto Juruá Reserve from Brazil) belongs to karyological group II.

The analysis of karyological group I specimens of *P. petersi* showed multiple NOR-bearing chromosome pairs and high interindividual variability in NOR number and localization. NORs were observed in autosome pairs 6, 8 and 10, both in the homozygous and in the heterozygous condition, and in sex chromosome Y in 11 of 13 males of this karyological group. Among the 15 specimens of this group analysed, seven different patterns of active NOR distribution were undoubtedly recognized by the Ag-NOR method (Figure 1, Table 1), and no intraindividual variability was observed, even between intestine and testis cells (Table 1). All the NORs detected by this technique correspond to secondary constrictions visualized in Giemsa-stained metaphases (Figure 2). As expected, the number of silver-stained nucleoli in diploid nuclei varied between one and the number of NORs of that genome (Figure 3). FISH experiments using HM 123 and/or HM 456 as probes performed in three specimens showing different active NOR patterns revealed the same regions detected by silver method (Figure 4).

In addition to NORs, the Ag-NOR technique also detected a peculiar heterochromatic region in one chromosome 8 of some metaphases from three specimens. Such regions are easily distinguished from the NORs by the intensity of silver impregnation, which results in black dots in NORs and brown sectors in heterochromatic regions (Figure 5). In Giemsa-stained metaphases these regions constitute secondary constrictions (Figure 2d).

In the single specimen of karyological group II analysed, active NORs were observed in both chromosomes of pair 8 and in one chromosome 9 (Figure 1h). These regions also correspond to secondary constrictions (Figure 2h) and the number of nucleoli per diploid nucleus varies between one and three (Figure 3e).

Discussion

The Ag-NOR method revealed high interindividual variability of active NOR patterns in *P. petersi*. Analysis of numerous mitotic metaphases (Table 1) from each individual, obtained from two different tissues in some males, showed no intraindividual variability in NOR activity. Thus, the different number and size of nucleoli in interphase diploid nuclei could result from NOR association or dissociation, events already observed in various organisms (see review by Schwarzacher &

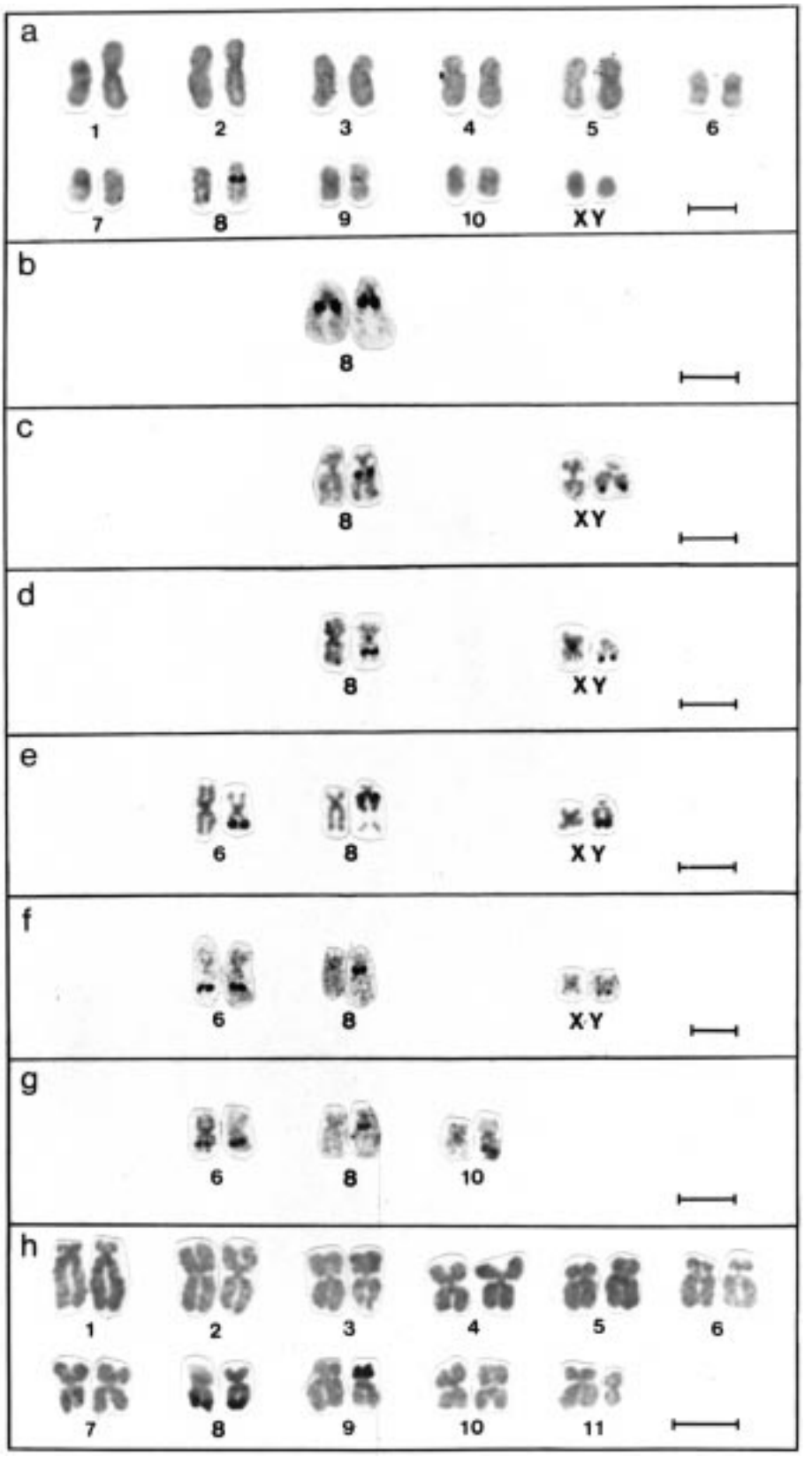


Figure 1. NOR patterns observed in the karyological group I (**a–g**) and group II (**h**) specimens of *P. petersi* by the Ag-NOR method. **a–g** Patterns I–VII respectively. **h** Pattern VIII. Bar = 5 μm.

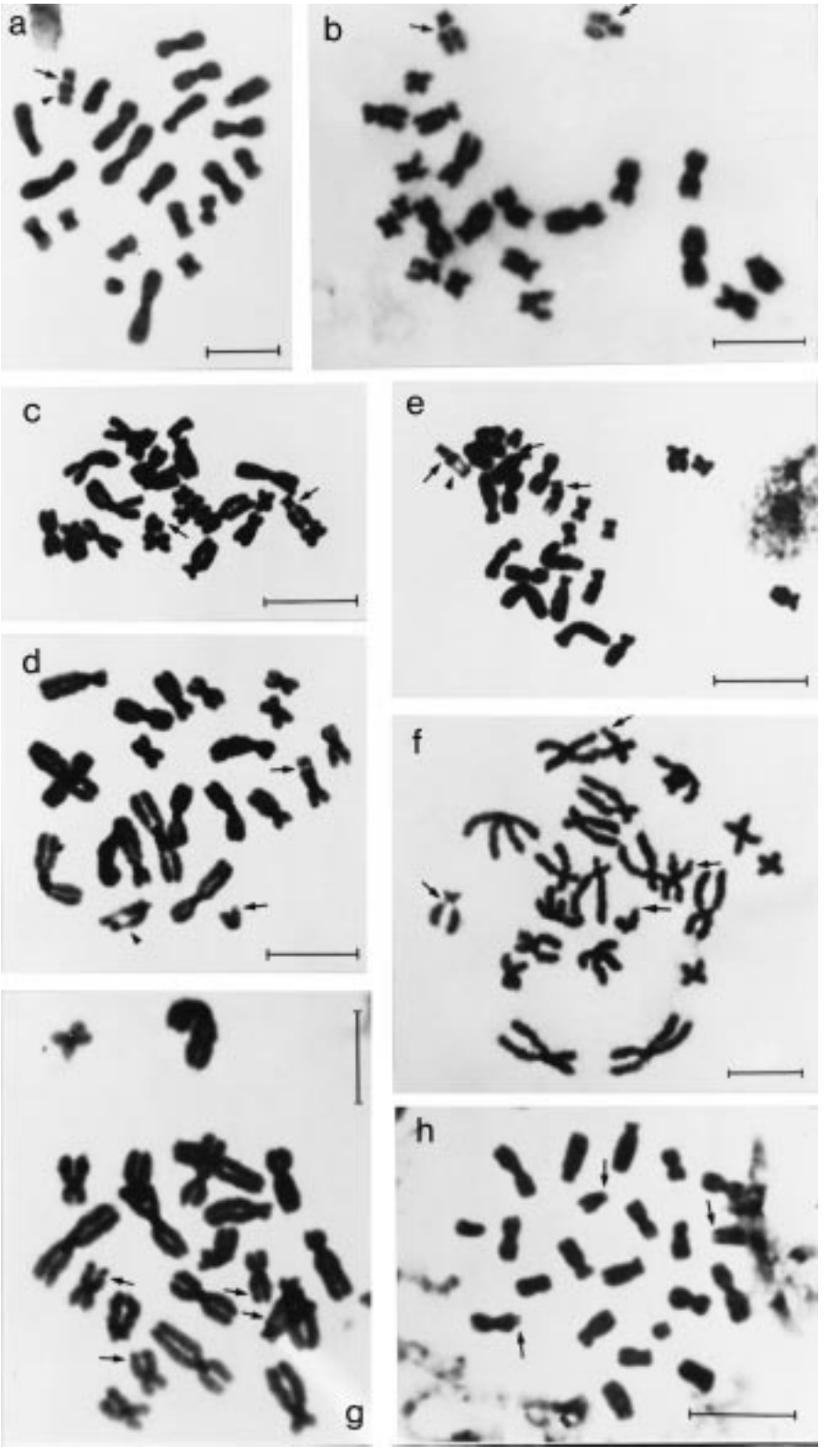


Figure 2. a–g Giemsa-stained metaphases corresponding to NOR patterns I–VII respectively. **h** Metaphase stained by Giemsa from karyological group II specimen with NOR pattern VIII. The arrows point to secondary constrictions coincident to NORs. The arrowhead on **d** indicates the secondary constriction non coincident to NORs that showed interesting behaviour after C-banding and Ag-NOR methods. Bar = 10 μm.

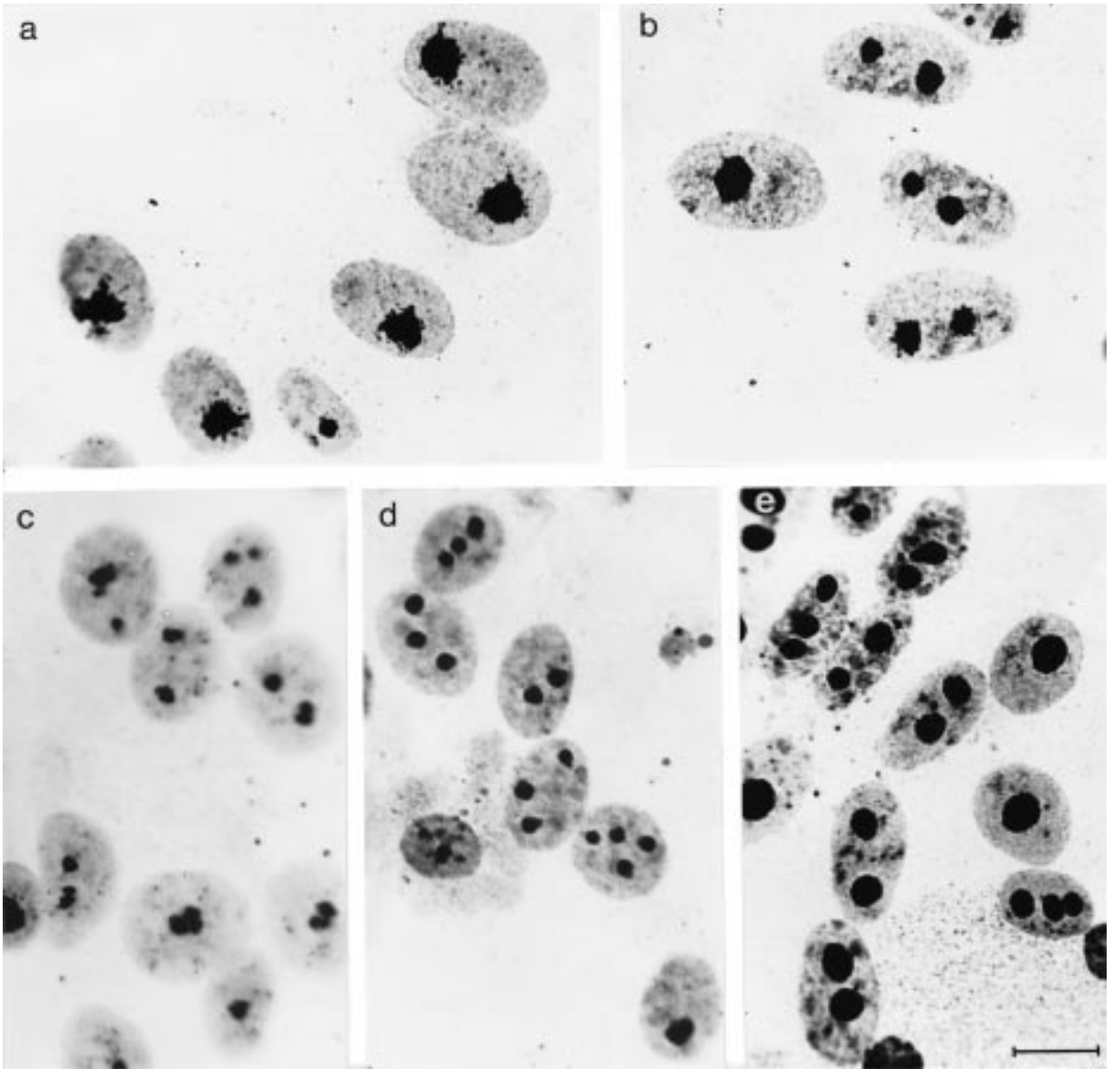
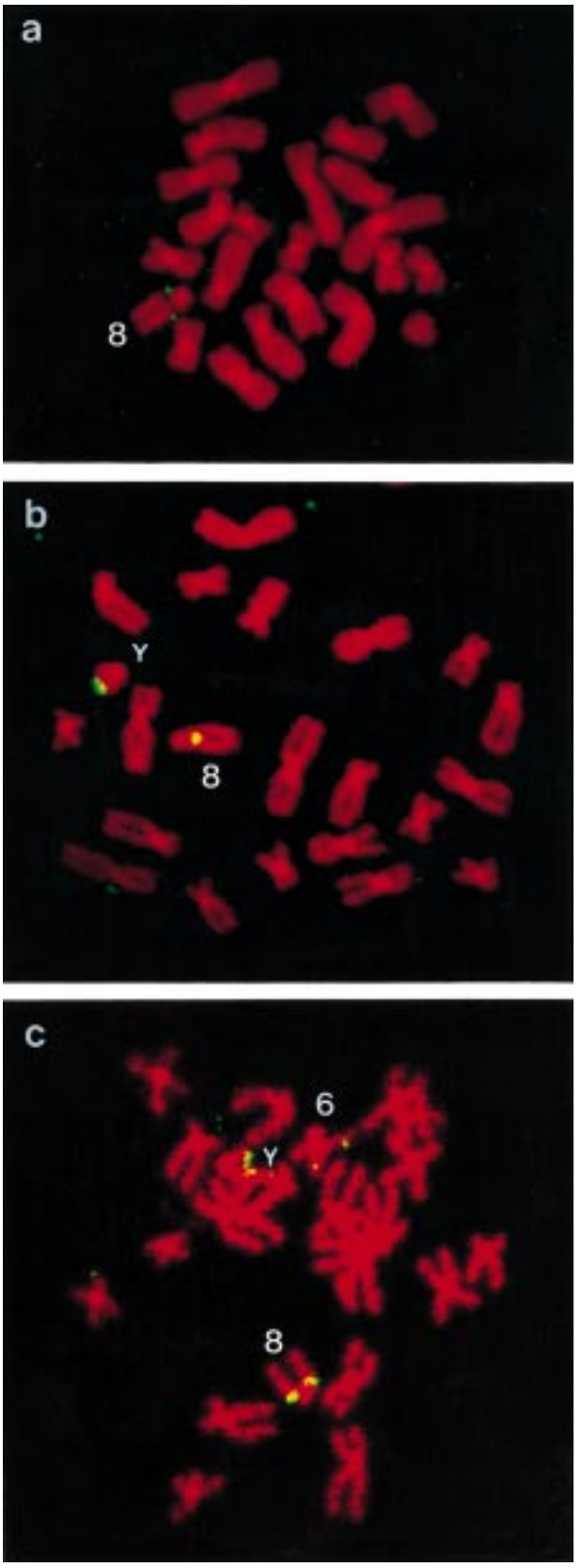


Figure 3. Interphase nuclei submitted to the Ag-NOR method. **a–d** Karyological group I specimens with 1–4 NOR per diploid genome respectively. **e** Karyological group II specimen with 3 NORs per diploid genome. Bar = 10 μm.

Wachtler 1993). The patterns observed by the Ag-NOR method correspond to those detected by FISH experiments, suggesting NOR site variability in *P. petersi* instead of an interindividual phenomenon of differential NOR expression. On this basis, we propose that the Ag-NOR method detects all the NORs present in the genome of this anuran. This observation is in agreement with the results obtained by *in situ* hybridization experiments performed with most of the anurans studied by these methods to date (Schmid *et al* 1986, 1993, 1995, King *et al.* 1990, Foote *et al.* 1991) and differs from those presented to *Hyla chrysoscelis* and *H. versicolor* (Wiley *et al.* 1989). In these hylids, *in situ* hybridization allowed detection of additional sites that did not stain with silver in any of the specimens analysed.

The two animals from the Humaitá Reserve presented the same NOR distribution pattern, as was also the case for the karyological group I specimens from the Restauração municipality. In contrast, among the specimens from the Tejo estuary, six patterns could be observed, including that shown by the Restauração animals. These results suggest a marked NOR poly-



morphism in the Tejo population of karyological group I *P. petersi*. The small samples from the other two locations analysed prevent any conclusion about the presence/absence of NOR polymorphism in those populations.

In anurans, interindividual size heteromorphisms of homologous NORs are widely observed (Schmid 1978a,b 1982). On the other hand, heteromorphisms related to the presence/absence of NORs are rarer. Intrapopulational variability of NOR number and localization was observed in diploid and tetraploid *Odontophrynus* species (Ruiz *et al.* 1981), in *H. chrysoscelis* and *H. versicolor* (Wiley *et al.* 1989), in *Bufo terrestris* (Foote *et al.* 1991) and in *Agalychnis callidryas* (Schmid *et al.* 1995). The polymorphisms detected in diploid *Odontophrynus* species, in *B. terrestris* and in *A. callidryas* consist of the variability of additional NOR sites occurring besides a fixed NOR pair. On the other hand, the tetraploid species *O. americanus* and *H. versicolor* do not show fixed NOR in any homologous chromosomes. With respect to the polyploid *Odontophrynus* species, Ruiz *et al.* (1981) argued that the absence of NOR detection by silver staining could also be due to NOR inactivation instead of the absence of a NOR site, because *in situ* hybridization experiments were not performed. The polymorphism observed in *H. chrysoscelis* most closely resembles that of *P. petersi* from the Tejo estuary region, in which no fixed pair of NOR was found by hybridization techniques. Thus, the polymorphism of NORs in *P. petersi* indicates a high genome variability in this genome, which could also be detected by the C-banding technique (L. Lourenço *et al.*, in preparation).

The presence of multiple NOR-bearing chromosomes is a derived character in Anura according to King *et al.* (1990), and it was observed in both karyological groups of *P. petersi*. Besides this derived character, at least group I of *P. petersi* presents sex chromosomes and large amounts of heterochromatin (L. Lourenço *et al.*, in preparation) that are also derived traits in Anura (King 1991).

Possible mechanisms involved in the dispersion of NOR sites in anuran genomes have been discussed by some authors (Wiley *et al.* 1989, King *et al.* 1990, Foote *et al.* 1991, Schmid *et al.* 1995, Kaiser *et al.* 1996). They comprise the transposition of NORs by mobile genetic elements closely linked to NOR cistrons, amplifications of 'orphan' rDNA cistrons, reinsertion errors during extrachromosomal amplification of ribosomal cistrons, translocations and inversions involving chromosome segments containing NORs.

The variable relative size and arm ratio of the different morphs of chromosomes 6 and 8 (L. Lourenço *et al.*, in preparation; Figures 1 & 6) is in agreement with the hypothesis of translocations, because such chromosome rearrangements are expected to change the morphology

Figure 4. Metaphases after *in situ* hybridization with the HM 456 probe (**a** & **b**) and the HM 123 and 456 probe mix (**c**). **a** ZUEC 9652. **b** ZUEC 9625. **c** ZUEC 9639.

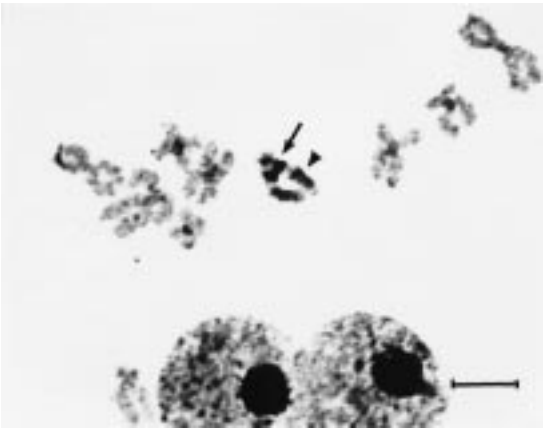


Figure 5. Silver-impregnated metaphases from ZUEC 9649. The arrows point to NORs and the arrowhead indicates an interesting region on chromosome 8. Note that some centromeric regions were also detected. Bar = 5 μm.

of the chromosomes. A model for heterochromatin dispersion based on translocation events encouraged by chromosomal proximity in mitotic anaphase, proposed by Schweizer & Loidl (1987), was already considered in the study of multiple non-centromeric C-bands in *P. petersi* (L. Lourenço *et al.*, in preparation). These events can also be involved in NOR dispersion in this species. Although chromosomes 6, 8 and 10 may present polymorphic NOR and non-centromeric C-bands, probably no linkage occurs between NOR and any non-centromeric C-band, at least in chromosomes 6 and 8. This suggests that some events, resulting exclusively in the dispersion of NOR or C-band blocks, have occurred, although some rearrangements could involve both chromosome regions. Nevertheless, molecular techniques are requried for the understanding of NOR dispersion in the *P. petersi* genome.

Another interesting finding in karyological group I of *P. petersi* was the presence of NOR in the Y chromosome of most males. The presence of NOR in sex chromosomes has already been described in some anurans (Schmid 1980, Schmid *et al.* 1986, 1993, Green 1988, Green & Sharbel 1988, Mahony 1991), but dimorphism related to the presence/absence of NOR in the NOR-bearing sex chromosome was described only in *Pyxicephalus adspersus* (Schmid 1980). The author proposed that the NOR observed in the W chromosome from a single female in the sample was derived from the other fixed NOR of the genome of this species. In *P. petersi* the presence of multiple NORs in the genome does not allow this kind of interpretation. Thus, the polarization of NOR dispersion in this *P. petersi* group genome could be explained only after karyological studies of ancestral groups. In any case, the NOR heteromorphism may have played some role in sex chromosome differentiation in this group.

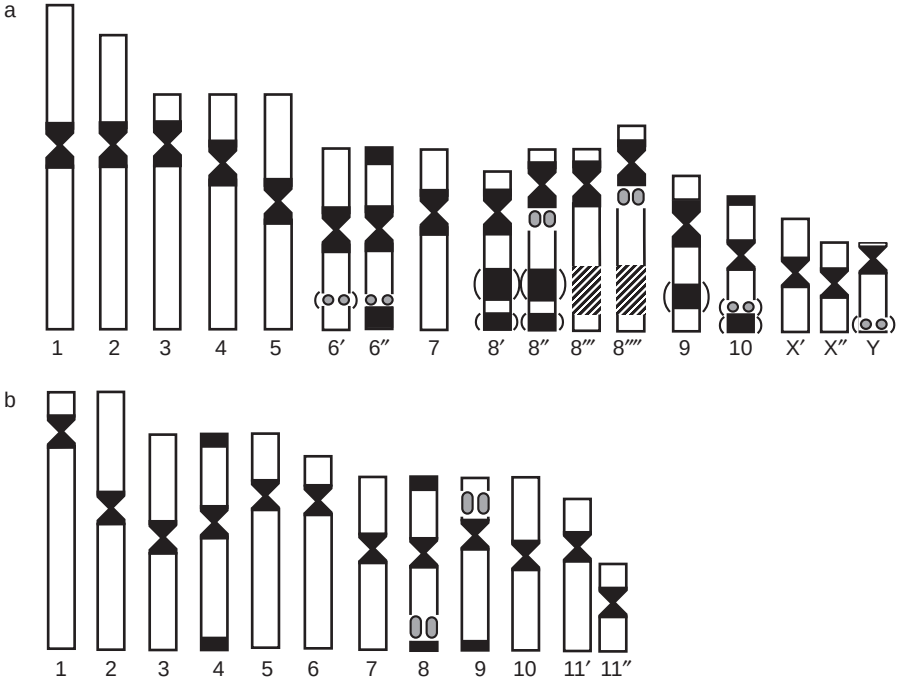


Figure 6. Idiograms of karyotypes I (**a**) and II (**b**) of *P. petersi*. Solid blocks, dark C-bands. Striped blocks, heterochromatin with a curious behaviour. Open regions, secondary constrictions. Checkered circles, NORs. Brackets, highly polymorphic regions.

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