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Chromosomal analysis of the leptodactylids *Pleurodema diplolistris* and *Physalaemus nattereri* (Amphibia, Anura)

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Abstract. Detailed characterizations of the karyotypes of the Brazilian leptodactylid frogs *Pleurodema diplolistris*, the only species of *Pleurodema* not studied cytogenetically so far, and *Physalaemus nattereri*, a species in the *Ph. biligonigerus* group, are presented. Both karyotypes had $2n = 22$ and their chromosomes had a very similar morphology, except for pair 11, which was metacentric in *Pl. diplolistris* and telocentric in *Ph. nattereri*. The localization of nucleolar organizer regions (NORs) and heterochromatic bands allowed the differentiation of chromosomes that were morphologically indistinguishable between these species, such as pairs 1, 3 and 10, which showed interstitial C-bands in *Ph. nattereri*, and pair 8, that had an NOR and an adjacent C-band in *Pl. diplolistris*. Pair 8 also has NOR-bearing chromosomes in many other *Pleurodema* species. However, in these species, the NOR is located proximal to the centromere on the short arm, while in *Pl. diplolistris* it occurred distally on the long arm, a condition that may be considered a derived state. In *Ph. nattereri*, the NOR occurred on chromosome 11 and differed from the other species of the *Ph. biligonigerus* group. In contrast, C-banding revealed a heterochromatic block near the centromere on the short arm of pair 3, a characteristic common to all members of this group of *Physalaemus*.

Keywords: Anura, Cytogenetics, Heterochromatin, Leptodactylidae, NOR.

Introduction

The leptodactylid genera *Pleurodema* and *Physalaemus*, currently classified in the subfamily Leptodactylinae (Frost, 2004), are widely distributed in South America and some species of *Plysalaemus* also occur in Mexico. The evolutionary proximity of *Pleurodema* and *Physalaemus* was inferred by Heyer (1975) based on their external and internal morphology, life histories and diploid chromosomal number. These genera were also closely related in the phylogenetic analysis performed by Faivovich et al. (2005), based on mitochondrial and nuclear genes and also on a small data set from foot musculature. Although the aim of this study was the relationships within Hylidae, eight genera of Leptodactylinae were sampled and the representative species of *Pleurodema*, *P. brachyops*, was inferred as the sister group of *Physalaemus cuvieri+Edalorhina perezi*. However, conclusive studies of the phylogenetic relationships of these genera have yet to be done. The non-monophyly of Leptodactylidae has been suggested by several authors (e.g. Ruvinsky and Maxson, 1996; Haas, 2003; Vences et al., 2003; Darst and Cannatella, 2004), but studies with a proper taxon sampling that allows intergeneric analysis were not performed.

Twelve species of *Pleurodema* are recognized (Frost, 2004), and their relationships have been studied by some authors. Based on reproductive characters and on paleogeographic assumptions, Duellman and Veloso (1977) proposed an evolutionary scheme for this genus in which there was a close relationship among *Pl. guayapae*, *Pl. nebulosa*, *Pl. tucumana*, *Pl. diplolistris* and *Pl. marmorata*. These authors also suggested that *Pl. thaul* was closely related to *Pl. bufonina* while *Pl. brachyops* was more related to *Pl. cinerea*, *Pl. borellii*, *Pl. kriegi* and

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Pl. bibroni. Morphological characters or cytogenetic data (chromosome number) supported some of these groups. All of these evolutionary relationships agree with the three groups considered by Cei (1980) in a study of nine species of *Pleurodema* from Argentina, i.e. the *Pl. cinerea* group (*Pl. borellii*, *Pl. bufonina*, *Pl. cinerea*, *Pl. kriegi*, *Pl. thaul*), the *Pl. nebulosa* group (*Pl. guayape*, *Pl. nebulosa*, *Pl. tucumana*) and the *Pl. marmorata* group (*Pl. marmorata marmorata*). However, according to the scheme proposed by Duellman and Veloso, the species considered by Cei to be in the *Pl. marmorata* and *Pl. nebulosa* groups are in the same clade and constitute the sister-group of a clade that includes all species of the *Pl. cinerea* group.

The chromosomal number and morphology of all species of *Pleurodema*, except for *Pl. diplolistris*, have been described. The diploid number for eight of these species is $2n = 22$, while *Pl. bibroni* and *Pl. kriegi* are tetraploid species ($4n = 44$) (Barrio and Rinaldi de Chieri, 1970; Veloso et al., 1973; Duellman and Veloso, 1977). All diploid karyotypes have a very similar chromosomal morphology, but the use of more advanced techniques to study the chromosomes of *Pl. thaul* and *Pl. brachyops* revealed several differences between these karyotypes (Schmid et al., 1993).

The genus *Physalaemus* encompasses 46 species distributed in four groups: *Ph. biligonigerus*, *Ph. cuvieri*, *Ph. signifer* and *Ph. pustulosus* (see review by Frost, 2004; Cruz and Pimenta, 2004; Ron et al., 2005). The *Ph. biligonigerus* group consists of four species: *Ph. nattereri*, *Ph. santafecinus*, *Ph. fuscomaculatus* and *Ph. biligonigerus*. The plylogenetic relationships within this and the other *Physalaemus* groups, except that of *Ph. pustulosus* (Cannatella et al., 1998; Ron et al., 2005), are unknown, as are the relationships among these groups.

Considerable cytogenetic information is available for the *Ph. biligonigerus* group since the karyotypes of *Ph. biligonigerus* and *Ph. fuscomaculatus* have been studied by Giemsa-staining, C-banding and the Ag-NOR method (Amaral et al., 2000). The chromosomal number and morphology of *Ph. nattereri* are also known (see description of *Eupemphix nattereri* [= *Physalaemus nattereri*] in Beçak, 1968), although a detailed description of this karyotype was not provided in the latter report.

In this paper, we describe the karyotype of *Pl. diplolistris*, the only species of *Pleurodema* not studied so far, based on Giemsa-staining, C-banding and the Ag-NOR method. We also provide a detailed characterization of the karyotype of *Ph. nattereri*.

Material and Methods

Specimens

Sixteen specimens of *Pl. diplolistris* (11 males and 5 females) and 12 (10 males and 2 females) of *Ph. nattereri* were analyzed. The *Pl. diplolistris* specimens were collected in March 1999, from Vassouras, in the municipality of Barreirinha, in Maranhão State, Brazil. Eleven of the *Ph. nattereri* specimens were obtained from São José do Rio Preto, São Paulo State, Brazil, from October to December 1997. One *Ph. nattereri* specimen was caught in October 1998, in Rio Claro, also in São Paulo State. All of the specimens studied were deposited in ZUEC (Museu de História Natural, Universidade Estadual de Campinas, Campinas, SP, Brazil).

Chromosomal preparation and techniques

After treatment *in vivo* with colchicine, the specimens were deeply anesthesized and their intestines and testes were processed according to Schmid (1978) and Schmid et al. (1979) for chromosomal preparations. Conventional staining with a 10% Giemsa solution, the C-banding technique (King, 1980) and the Ag-NOR method (Howell and Black, 1980) were done on chromosomal preparations from both species. To investigate further the *Ph. nattereri* karyotype, metaphases from this species were stained with distamycin A/mithramycin A (Schweizer, 1980) and processed for fluorescence *in situ* hybridization (FISH) (Viegas-Péquignot, 1992) using HM 123 as an rDNA probe (Meunier-Rotival et al., 1979). The slides were examined with an Olympus microscope or a BioRad MRC 1024 UV confocal microscope. The morphometric analyses were done manually or using Image Pro-Plus software, version 3 (Media Cybernetics). The chromosomes were classified according to the criteria proposed by Green and Sessions (1991).

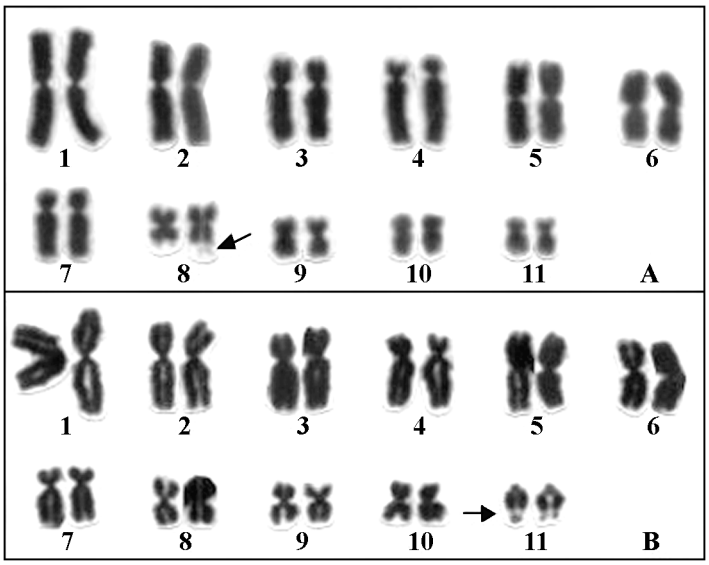

Figure 1. Giemsa-stained karyotypes of *Pleurodema diplolistris* **(A)** and *Physalaemus nattereri* **(B)**. The arrows indicate the secondary constrictions of the NORs.

Results

Pleurodema diplolistris

The full chromosomal complement of this species was $2n = 22$ and consisted of eight pairs of metacentric chromosomes (1, 2, 5, 6, 8, 9, 10 and 11), two submetacentric pairs (3 and 7) and one subtelocentric pair (4). Heteromorphic sex chromosomes were not detected (figs 1 and 5; table 1).

In all specimens analyzed, pair 8 contained the NOR-bearing chromosomes, as detected by the Ag-NOR method (fig. 3). The NOR was located distally on the long arm of this pair and coincided with the secondary constriction in Giemsa-stained metaphases (fig. 3). Size heteromorphism between the NORs was detected (fig. 3) and led to the description of morphs 8a, 8b and 8c.

C-banding detected small amounts of constitutive heterochromatin in the centromeric region of all of the chromosomes of this karyotype. Dark blocks of constitutive heterochromatin were also observed interstitially on the long arm of chromosome 8, adjacent to the NOR (fig. 2). Telomeric heterochromatin was detected in almost all of the chromosomal pairs, although their presence was questionable in the smaller pairs (8-11) (fig. 2).

Physalaemus nattereri

The *Ph. nattereri* karyotype consisted of metacentric (1, 2, 5, 6, 8, 9 and 10), submetacentric (3, 4 and 7) and telocentric (11) chromosomes, and the diploid number was $2n = 22$, both in males and females (figs 1 and 5; table 1). C-banding revealed a small amount of centromeric heterochromatin in all chromosomes. Interstitial C-bands were identified close to the centromere on the short arm of chromosomes 1, 2, 3, 7 and 10 and a dark telomeric heterochromatin was detected on the long arm of chromosome 11 (figs. 2 and 5).

In all specimens analyzed, there was a secondary constriction on the long arm of both homologues of chromosome 11 (fig. 1), coincident with the telomeric C-band (fig. 2), which was detected as an NOR by the Ag-NOR method and in the FISH experiments (fig. 4). The Ag-NOR method also detected the centromeric region of pair 11 and the interstitial C-band on the short arm of pair 1 (fig. 4A), but these were not confirmed as NORs after hybridization

Table 1. Morphometric parameters of the *Pl. diplolistris* and *Ph. nattereri* karyotypes. The measurements were based on 25 metaphases from five specimens of *Pl. diplolistris* and on 30 metaphases from five specimens of *Ph. nattereri*.

	Chromosome number										
	1	2	3	4	5	6	7	8	9	10	11
	Pleurodema diplolistris										
Relative length (%)	15.1	12.7	10.8	10.3	10.6	9.4	8.7	6.6	4.9	4.9	4.3
(range)	(13.5-17.3)	(9.0-14.2)	(8.5-11.9)	(8.3-11.6)	(7.4-11.9)	(7.0-11.6)	(6.1-10.4)	(5.0-8.3)	(4.2-5.9)	(3.3-5.8)	(3.5-5.6)
Arm ratio	1.22	1.54	2.37	3.78	1.34	1.16	2.38	1.66	1.17	1.36	1.61
(range)	(1.08-1.45)	(1.12-1.97)	(1.89-3.0)	(2.98-4.66)	(1.18-1.57)	(1.01-1.44)	(2.05-3.23)	(1.36-2.58)	(1.01-1.54)	(1.12-1.63)	(1.15-1.99)
Classification[a]	m	m	sm	st	m	m	sm	m	m	m	m
	Physalaemus nattereri										
Relative length (%)	13.43	11.37	10.43	9.47	9.27	8.80	7.67	6.43	5.40	5.00	4.30
(range)	(12-16)	(11-14)	(9-12)	(9-11)	(9-10)	(8-10)	(7-9)	(5-8)	(5-7)	(4-6)	(3-6)
Arm ratio	1.12	1.48	1.82	2.64	1.22	1.38	1.91	1.39	1.41	1.40	8.14
(range)	(1.0-1.25)	(2.33-1.2)	(2.33-1.28)	(1.4-3.5)	(1.0-1.8)	(1.0-2.0)	(1.0-2.5)	(1.0-2.5)	(1.0-2.33)	(1.0-1.75)	(8.0-10.0)
Classification[a]	m	m	sm	sm	m	m	sm	m	m	m	t

[a]The chromosomal classification relative to the centromeric position follows Green and Sessions (1991): m: metacentric; sm: submetacentric; st: subtelocentric; t: telocentric.

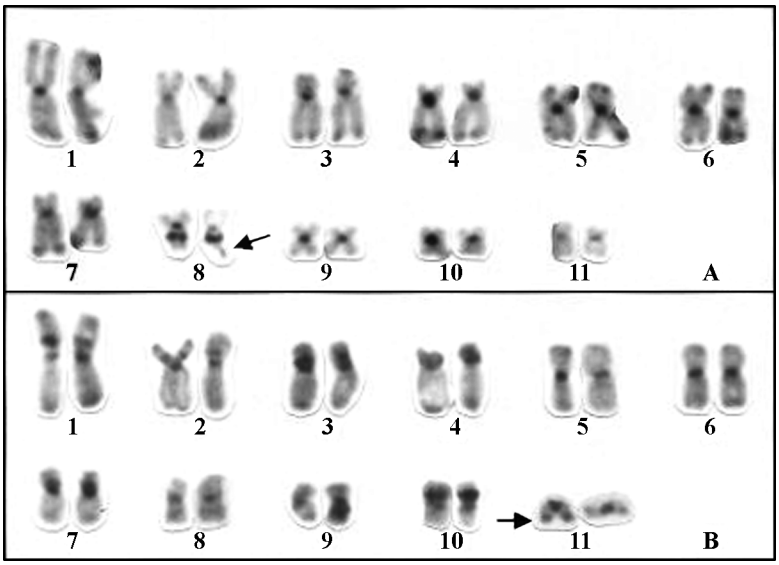

Figure 2. C-banded karyotypes of *Pleurodema diplolistris* **(A)** and *Physalaemus nattereri* **(B)**. Note that the NOR (arrows) appears as a secondary constriction in **A** and as a C-band in **B**.

with the rDNA probe (fig. 4C). All of the heterochromatic blocks detected by silver staining were also seen as bright bands in mithramycin-stained metaphases (fig. 4B). The positive-staining with mithramycin distinguished these regions from the rest of the genome and indicated some similarity in their base composition. This probably accounted for their positive response to silver staining, although only one of them (terminal region of the long arm of chromosome 11) was in fact an NOR.

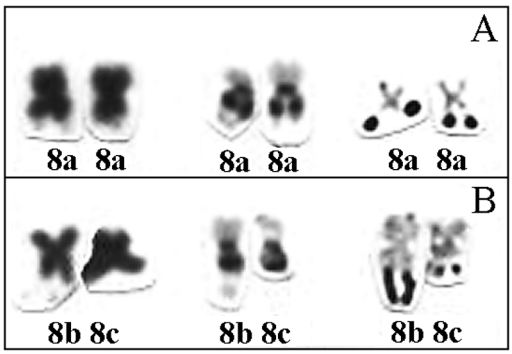

Figure 3. Chromosomal pair 8 of *Pleurodema diplolistris* with homomorphic **(A)** and hetermorphic **(B)** NORs after Giemsa-staining (left column), C-banding (middle column) and Ag-NOR staining (right column).

Discussion

Pleurodema diplolistris

This species had a diploid number of 22 chromosomes, as do all the other diploid *Pleurodema* species (Barrio and Rinaldi de Chieri, 1970; Duellman and Veloso, 1977; Schmid et al., 1993). The morphology of the chromosomes of *Pl. diplolistris* was also very similar to the other species, except for pair 8 of *Pl. brachyops* (Schmid et al., 1993), which was telocentric and distinct from all the other species. The noticeable difference in chromosomal length between pairs 7 and 8 seen in the *Pleurodema* species studied so far was also seen in *Pl. diplolistris* when morph 8a was used for comparison. The presence of considerable telomeric heterochromatin was another typical characteristic found in *Pl. diplolistris* and in the other two *Pleurodema* species, *Pl. thaul* and *Pl. brachyops*, the karyotypes of which were also studied by C-banding (Schmid et al., 1993).

However, *Pl. diplolistris* clearly differed from the other congeneric species in the location of a secondary constriction, which was terminal on the long arm of pair 8 in *Pl. diplolistris* whereas

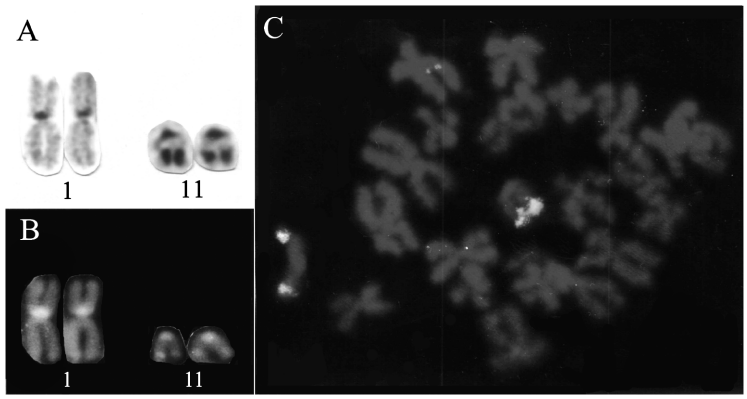

Figure 4. Chromosomal pairs 1 and 11 of *Physalaemus nattereri* after Ag-NOR staining **(A)** and distamycin A/mithramycin A staining **(B)**. Note that the same chromosomal regions were detected by both methods. In **C**, a metaphase of *Ph. nattereri* after hybridization with an rDNA probe. Only the distal site of pair 11 was recognized as an NOR.

in *Pl. borellii* (identified as *Pl. cinerea* from Tucumán by Barrio and Rinaldi de Chieri, 1970, according to Duellman and Veloso, 1977), *Pl. bufonina*, *Pl. cinerea*, *Pl. guayapae*, *Pl. nebulosa*, *Pl. tucumana* (Barrio and Rinaldi de Chieri, 1970), *Pl. marmorata* and *Pl. thaul* (Duellman and Veloso, 1977; Schmid et al., 1993) this constriction occurs on the short arm and more proximal to the centromere, and in *Pl. brachyops* it is placed on pair 7 (Schmid et al., 1993). The Ag-NOR method revealed that in *Pl. diplolistris* (present paper), *Pl. thaul* and *Pl. brachyops* (Schmid et al., 1993) these secondary constrictions are NOR sites. Since *Pl. brachyops* and *Pl. diplolistris* are not basal species relative to the other species of *Pleurodema* (Duellman and Veloso, 1977), it is reasonable to conclude that, in the genus *Pleurodema*, the NOR placed distally on the long arm of pair 8, as occurs in *Pl. diplolistris*, is a derived state for the NOR location, as is that on pair 7 of *Pl. brachyops*, as already suggested by Schmid et al. (1993).

C-banding differentiated pair 8 of *Pl. diplolistris* from that of the other *Pleurodema* studied by this technique (Schmid et al., 1993). Despite the similar morphology, in *Pl. thaul* pair 8 shows no interstitial band, while in *Pl. diplolistris* a conspicuous interstitial C-band occurs adjacent to the NOR. These findings, together with the differential location of the NOR in these chromosomal pairs, suggest that the emergence of pair 8 in *Pl. diplolistris* involved an inversion in a hypothetical ancestral pair 8 with the NOR on its short arm.

Physalaemus nattereri

The karyotype seen here was the same as that described by Beçak (1968) using Giemsa-stained metaphases. The diploid number ($2n = 22$) was the same as in all of the other *Physalaemus* studied to date (see review by Kuramoto, 1990; Lourenço et al., 1999; Silva et al., 1999; Amaral et al., 2000) and the chromosomal morphology also resembled that of the other *Physalaemus* karyotypes (Denaro, 1972; De Lucca et al., 1974; Lourenço et al., 1999; Silva et al., 1999; Amaral et al., 2000). Interspecific differences in chromosomal morphology among *Physalaemus* karyotypes are generally restricted to pairs 8-11. However, simple techniques, such as C-banding and the Ag-NOR method, have revealed conspicuous differences among these karyotypes (see Lourenço et al., 1999; Silva et al., 1999; Amaral et al., 2000).

Comparison of the *Ph. nattereri* karyotype with the karyotypes described for other species of the *Ph. biligonigerus* group (Amaral et al., 2000) showed that the chromosomal morphologies of these karyotypes were very similar, although chromosomes 4 and 5 were arranged

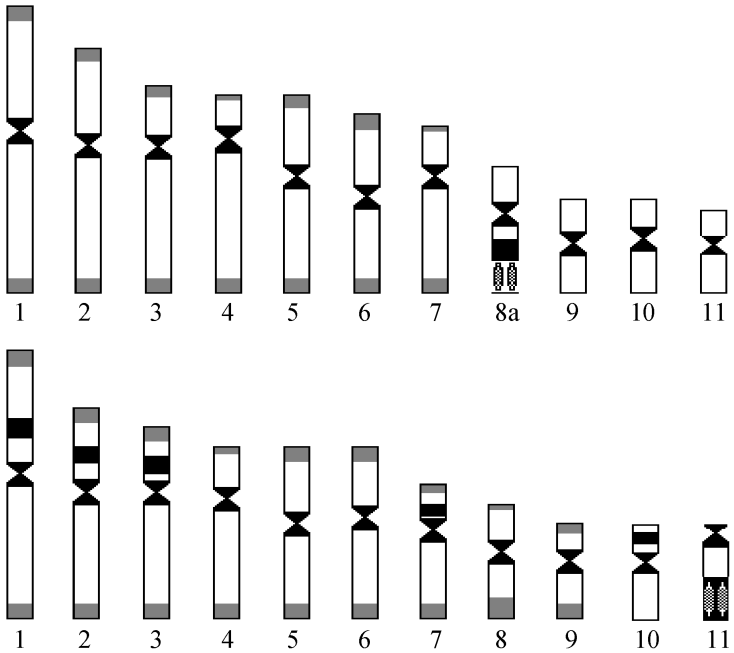

Figure 5. Ideograms of *Pleurodema diplolistris* (top) and *Physalaemus nattereri* (bottom) karyotypes. Solid blocks: dark C-bands. Gray blocks: faint C-bands. Open region: secondary constriction (coincident with the NORs). Checkered circles: NORs. The presence of telomeric C-bands in chromosomes 8-11 of *Pl. diplolistris* is questionable and these bands are not represented in this figure.

in different orders. The distinct arrangement of chromosomes 4 and 5 probably reflected artifactual differences in the measurements done since the sizes of these chromosomes were very similar. Pairs 8-10 of *Ph. nattereri* differed slightly from the other karyotypes of this group. The greatest difference was seen in pair 11, which was telocentric in *Ph. nattereri* and metacentric in *Ph. fuscomaculatus*, *Ph. biligonigerus* and *Physalaemus* sp. (aff. *biligonigerus*). Pair 11 also carried the NOR-bearing chromosomes of the *Ph. nattereri* karyotype, while in the other three *Physalaemus* karyotypes, the NOR was located on pair 9. In all of these cases, the NOR was terminal, and in *Ph. nattereri* and *Ph.* sp. (aff. *biligonigerus*) it coincided with a C-band. These data suggested the occurrence of rearrangement(s) involving pairs 9-11. In *Ph. nattereri*, a faint C-band seen in the telomere of the long arm resembled a band that coincided with the NOR on pair 9 of *Ph.* sp. (aff. *biligonigerus*).

C-banding also indicated homology in pair 3 of all of the karyotypes in the *Ph. biligonigerus* group. In all of these karyotypes, pair 3 showed a conspicuous block of heterochromatin in the short arm, close to the centromere. This band varied in size, being bigger in *Ph. biligonigerus* and *Ph*. sp. (aff. *biligonigerus*). As already postulated by Amaral et al. (2000), this size difference also accounted for variation in the morphology of this pair, which is metacentric in *Ph. biligonigerus* and *Ph.* sp. (aff. *biligonigerus*), and submetacentric in *Ph. fuscomaculatus* and *Ph. nattereri*. The unambiguous identification of this heterochromatic block suggested that this block could be a cytogenetic marker for the *Ph. biligonigerus* group since it has not been found in other *Physalaemus* studied so far (see Lourenço et al., 1999 and Silva et al., 1999).

Despite the similarity between pair 1 of *Ph. nattereri* and that of the other karyotypes of the *Ph. biligonigerus* group suggested by Giemsa staining, C-banding and mithramycin staining revealed a peculiarity in the *Ph. nattereri* kary-

otype. In this complement, pair 1 had a conspicuous interstitial C-band on the short arm that was absent in the other karyotypes. This heterochromatic block and that in the centromere of pair 11 were also detected by silver staining, even though they were not NOR sites, as shown by FISH. The silver impregnation seen in these regions after the Ag-NOR method suggested a similar molecular composition of these heterochromatic blocks and, consequently, a common origin for them.

Intergeneric comparisons

Pleurodema and *Physalaemus* have a diploid number of 22 chromosomes, which is also found in many other leptodactylines, including *Leptodactylus*, *Edalorhina* and *Vanzolinus* species (see list of Kuramoto, 1990). Morphologically, the karyotypes of *Pleurodema* and *Physalaemus* are very similar. An apparent change between pairs 3 and 4 is frequently observed when different karyotypes of these genera are compared. This change could be explained by the similarity in the length of these chromosomes, which makes it difficult to position them properly in the karyograms. Hence, the apparent discrepancy involving pairs 3 and 4 of some species could simply be an artifact of the method of measurement used.

Although only a few *Pleurodema* and *Physalaemus* karyotypes have been studied using C-banding, the Ag-NOR method, FISH with rDNA probes and fluorochrome staining, it is clear that these cytogenetic techniques can reveal many divergences not seen in Giemsa-stained metaphases, even within the same genus (Schmid et al., 1993; Lourenço et al., 1998, 1999; Amaral et al., 2000; Silva et al., 1999; and this paper). This conclusion is confirmed by the interstitial heterochromatic bands in pairs 1 and 10 seen exclusively in the *Ph. nattereri* karyotype, and also by the C-band close to the centromere in pair 3 of all species of the *Ph. biligonigerus* group. The application of these techniques to other species of *Pleurodema* and *Physalaemus* will allow a more accurate comparison of the karyotypes in these genera and will probably improve our understanding of chromosomal behavior during the divergence of these anurans.

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References

Amaral, M.J.L.V., Cardoso, A.J., Recco-Pimentel, S.M. (2000): Cytogenetic analysis of three *Physalaemus* species (Amphibia, Anura). Caryologia **53**: 283-288.

Barrio, A., Rinaldi de Chieri, P. (1970): Estudios citogeneticos sobre el género *Pleurodema* y sus consecuencias evolutivas (Amphibia, Anura, Leptodactylidae). Physis **30**: 309-319.

Beçak, M.L. (1968): Chromosomal analysis of eighteen species of Anura. Caryologia **21**: 191-208.

Cannatella, D.C., Hillis, D.M., Chippindale, P.T., Weigt, L., Rand, A.S., Ryan, M. (1998): Phylogeny of frogs of the *Physalaemus pustulosus* species group, with an examination of data incongruence. Syst. Biol. **47**: 311-335.

Cei, J.M. (1980): Amphibians of Argentina. In: Monitore Zool. Ital (N.S.) Monogr. 2, p. 361-409.

Cruz, C.A.G., Pimenta, B.V.S. (2004): New species of *Physalaemus* Fitzinger, 1826 from Southern Bahia, Brazil (Anura, Leptodactylidae). J. Herpetol. **38**: 480-486.

Darst, C.R., Cannatella, D.C. (2004): Novel relationships among hyloid frogs inferred from12S and 16S mitochondrial DNA sequences. Mol. Phylogenet. Evol. **31**: 462-475.

De Lucca, E.J., Jim, J., Foresti, F. (1974): Chromosomal studies in twelve species of Leptodactylidae and one Brachycephalidae. Caryologia **27**: 183-191.

Denaro, L. (1972): Karyotypes of Leptodactylidae anurans. J. Herpetol. **6**: 71-74.

Duellman, W.E., Veloso, A. (1977): Phylogeny of *Pleurodema* (Anura:Leptodactylidae): a biogeographic model. Occ. Pap. Mus. Nat. Hist. Univ. Kansas **64**: 1-46.

Faivovich, J., Haddad, C.F.B., Garcia, P.C.A., Frost, D.R., Campbell, J.A., Wheeler, W.C. (2005): Systematic review of the frog family Hylidae, with special reference to Hylinae: phylogenetic analysis and taxonomic revision. Bull. Am. Mus. Nat. Hist. **294**: 1-240.

Frost, D.R. (2004): Amphibian species of the world: an online reference. Version 3.0 (22 August, 2004). Electronic database accessible at http://research.amnh.org/herpetology/amphibia/index.html. American Museum of Natural History, New York, USA.

Green, D.M., Sessions, S.K. (1991): Nomenclature for chromosomes. In: Amphibian Cytogenetics and Evolution, p. 431-432. Green, D.M., Sessions, S.K., Eds, San Diego, Academic Press.

Haas, A. (2003): Phylogeny of frogs as inferred from primarily larval characters (Amphibia: Anura). Cladistics **19**: 23–89.

Heyer, W.R. (1975): A preliminary analysis of the intergeneric relationships of the frog family Leptodactylidae. Smith. Contr. Zool. **199**: 1-55.

Howell, W.M., Black, D.A. (1980): Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia **36**: 1014-1015.

King, M. (1980): C-banding studies on Australian hylid frogs: secondary constriction structure and the concept of euchromatin transformation. Chromosoma **80**: 191-217.

Kuramoto, M. (1990): A list of chromosome numbers of anuran amphibians. Bull. Fukuoka Univ. Educ. **39**: 83-127.

Lynch, J.D. (1971): Evolutionary relationships, osteology, and zoogeography of leptodactyloid frogs. Univ. Kans. Mus. Nat. Hist., Misc. Publ. **53**: 1-238.

Lourenço, L.B., Recco-Pimentel, S.M., Cardoso, A.J. (1998): Polymorphism of the nucleolar organizer region (NOR) in *Physalaemus petersi* (Amphibia, Anura, Leptodactylidae) detected by Ag-NOR and FISH. Chromosome Res. **6**: 621-628.

Lourenço, L.B., Recco-Pimentel, S.M., Cardoso, A.J. (1999): Two karyotypes and heteromorphic sex chromosomes in *Physalaemus petersi* (Anura, Leptodactylidae). Can. J. Zool. **77**: 624-631.

Meunier-Rotival, M., Cortadas, J., Macaya, G., Bernardi, G. (1979): Isolation and organization of calf ribosomal DNA. Nucl. Acids Res. **6**: 2109-2123.

Ron, S.R., Coloma, L.A., Cannatella, D.C. (2005): A new, cryptic species of *Physalaemus* (Anura: Leptodactylidae) from Western Ecuador with comments on the call structure of the *P. pustulosus* species group. Herpetologica **61**: 178-198.

Ruvinsky, I., Maxson, L.R. (1996): Phylogenetic relationships among bufonoid frogs (Anura: Neobatrachia) inferred from mitochondrial DNA sequences. Mol. Phylogenet. Evol. **5**: 533–547.

Schmid, M. (1978): Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. Chromosoma **66**: 361-388.

Schmid, M., Olert, J., Klett, C. (1979): Chromosome banding in Amphibia. III. Sex chromosomes in *Triturus*. Chromosoma **71**: 29-55.

Schmid, D., Steinlein, C., Feichtinger, W., Poot, M. (1993): Chromosome banding in Amphibia. XVIII. Karyotype evolution and genomic size variation in *Pleurodema* (Anura, Leptodactylidae). Cytogenet. Cell Genet. **62**: 42-48.

Schweizer, D. (1980): Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma **58**: 307-324.

Silva, A.P.Z., Haddad, C.F.B., Kasahara, S. (1999): Nucleolus organizer regions in *Physalaemus cuvieri* (Anura, Leptodactylidae), with evidence of a unique case of Ag-NOR variability. Hereditas **131**: 135-141.

Vences, M., Kosuch, J., Boistel, R., Haddad, C.F.B., La Marca, E., Lötters, S., Veith, M. (2003) Convergent evolution of aposematic coloration in Neotropical poison frogs: a molecular phylogenetic perspective. Org. Divers. Evol. **3**: 215-226.

Veloso, A., Galleguillos, R., Diaz, N. (1973): Karyotypic analysis of allopatric populations of *Pleurodema thaul* (Lesson). Caryologia **26**: 69-76.

Viegas-Péquignot, E. (1992): *In situ* hybridization to chromosomes with biotinylated probes. In: *In situ* Hybridization: A Practical Approach, p. 137-158. Willernson, D., Ed., Oxford University Press, IRL Press.

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