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Cytogenetic study of *Leptodactylus fuscus* and *L. latrans* (Anura: Leptodactylidae) from the semiarid Brazilian Caatinga scrublands

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Abstract

Cytogenetic study of *Leptodactylus fuscus* and *L. latrans* (Anura: Leptodactylidae) from the semiarid Brazilian Caatinga scrublands. Frogs in the family Leptodactylidae have diploid chromosome numbers ranging from 2n = 18 to 2n = 26, although there is a predominance of 2n = 22 karyotypes. In the present study, 52 specimens of *Leptodactylus fuscus* and *Leptodactylus latrans* were obtained from three municipalities (Carira, Poço Redondo, and Tobias Barreto) of the Brazilian state of Sergipe for karyotype analysis using conventional Giemsa staining techniques, and C- and Ag-NOR banding of the chromosomes obtained from the intestinal epithelium. The results of this study show that the individuals analyzed have highly similar karyotypes, with no evidence of any pronounced species-specific markers. However, some differences were observed in the chromosome morphology and C-bands in comparison with the karyotypes described previously for these species, which may represent intraspecific geographic variation in both taxa.

Keywords: amphibians**,** chromosomes, C-bands, Ag-NOR.

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Resumo

Análise citogenética de *Leptodactylus fuscus* e *L. latrans* (Anura: Leptodactylidae) de áreas de Caatinga do Brasil. A família Leptodactylidae apresenta números diplóides que variam de 2n = 18 a 2n = 26, porém, na maioria dos gêneros descritos na literatura, há uma predominância de cariótipos com 2n = 22. Neste trabalho foram analisados citogeneticamente 52 exemplares de *Leptodactylus fuscus* e *Leptodactylus latrans* coletados pela primeira vez no estado de Sergipe, norte do Brasil, nos municípios de Carira, Poço Redondo e Tobias Barreto, por meio de técnicas de coloração convencional, Banda C e Ag-RON, empregadas em preparações do epitélio intestinal. Os dados mostraram que as espécies aqui analisadas apresentaram padrões cariotípicos muito similares, sem evidência de marcadores específicos das espécies. Contudo, algumas variações de morfologia cromossômica e bandas C observadas divergiram de cariótipos já descritos na literatura e podem indicar diferenças cromossômicas geográficas intraespecíficas para ambas as espécies.

Palavras-chave: anfíbios**,** cromossomos, Banda C, Ag-RON.

Introduction

The genus *Leptodactylus* Fitzinger, 1826 currently includes 89 frog species, which are found between southern North America and southern South America (Frost 2013). In Brazil, a total of 67 species are presently included in this genus (Pyron and Wiens 2011). Over the past few years, the systematics of the family Leptodactylidae has been reviewed by a number of authors (Hedges *et al*. 2008). For example, *Adenomera*, whose member species were originally included in *Leptodactylus*, was classified by Pyron and Wiens (2011) as a valid genus, a position supported by Frost (2013).

There is a predominance of 2n = 22 karyotypes in leptodactylid species. Approximately 40 species of *Leptodactylus* have been karyotyped (Zaracho and Hernando 2011), and most share very similar features, with the emphasis on metacentric and submetacentric chromosomes (Silva *et al*. 2004, Amaro-Ghilardi *et al*. 2006, Gazoni *et al*. 2012). A number of cytogenetic studies have reinforced the conservative nature of the karyotypes of the representatives of this genus (Denaro 1972, Bianchi *et al*. 1973, Bogart 1974, Silva *et al*. 2000, Amaro-Ghilardi *et al*. 2006, Arruda and Morielle-Versute 2008, Gazoni *et al*. 2012). The taxonomic position of a number of species groups is, however, still controversial (Angulo *et al*. 2003, Heyer 2005, Lavilla *et al*. 2010).

Based on a molecular study of the mitochondrial DNA of *Leptodactylus fuscus*, Camargo *et al*. (2006) confirmed that this taxon probably includes a number of cryptic species. These analyses also revealed the presence of a number of unrelated clades representing different, genetically isolated populations. Isozyme data from this same study confirmed the molecular analysis, indicating considerable differentiation in the two main clades, and the absence of gene flow between them.

The species now classified as *Leptodactylus latrans* (Steffen, 1815) has a long and controversial taxonomic history, beginning with the original description of *Rana ocellata* Linnaeus, 1758. Lavilla *et al*. (2010) concluded that this nomen is in fact a senior synonym of the Jamaican species *Osteopilus brunneus* (Gosse, 1851), whereas the taxon *Leptodactylus ocellatus* corresponds to *Rana latrans* Steffen, 1815. Given this, *Leptodactylus latrans* (Steffen, 1815) was revalidated as a new combination, and designated the new combination *Osteopilus ocellata* (Linnaeus, 1758), a senior synonym of *Osteopilus brunneus* (Gosse, 1851).

Detailed data on the evolutionary relationships of amphibian populations from different regions may be especially important for the understanding of the history of Caatinga ecosystems, as well as the development of effective conservation strategies (Rodrigues 2005).

The species *L. fuscus* and *L. latrans* are found in the Caatinga scrublands of the Brazilian state of Sergipe, and little is known about the ecology or diversity of the populations that inhabit this semiarid biome. The present study provides a cytogenetic analysis of specimens of the two species from Sergipe, based on conventional staining and C- and Ag-NOR banding patterns.

Materials and Methods

The present study was based on the cytogenetic analysis of 14 specimens of *L. fuscus* and 38 specimens of *L. latrans* collected in the Brazilian state of Sergipe (Table 1, Figure 1), in the municipalities of Poço Redondo (9°48'29.73" S, 37°41'09.59" W), Carira (10°22'54.18" S, 37°41'06.33" W), and Tobias Barreto (11°10'24.55" S, 38° 00'50.14" W). All the specimens were deposited in the herpetological collection of the Laboratório de Herpetologia e Ictiologia, Departamento de Biologia, Universidade Federal de Sergipe, Brazil.

Metaphase chromosomes were obtained from the intestinal epithelium, following the protocol of Schmid (1978), with slight modifications. An average of five slides was prepared for each specimen, which were treated with traditional Giemsa staining, and C- and Ag-NOR banding.

The conventional staining was conducted using Giemsa stain diluted in 6.8 pH phosphate buffer for 10 minutes. The C- and Ag-NOR banding used methods provided by Sumner (1972) and Howell and Black (1980), respectively. The metaphase spreads were analyzed by light microscopy, and photographed using a Moticam 2500 digital camera. Karyotypes were constructed and chromosome classification followed the nomenclature suggested by Green and Sessions (1991, 2007).

Results

All the specimens of both species have a karyotype of 2n = 22 chromosomes, and FN = 44 chromosome arms (Figures 2 and 3). The karyotype consists of seven pairs of large and medium chromosomes, and four pairs of small chromosomes. Chromosomes 1, 5, and 6 are metacentric, 2, 3, and 7 are submetacentric, and chromosome 4 is subtelocentric, while chromosomes 8, 9, 10, and 11 are either metacentric or submetacentric.

Table 1. Number of specimens of *L. fuscus* and *L. latrans* of different age-sex classes collected at the three study sites in Sergipe, northern Brazil. Legend: J = juvenile, F = adult female, M = adult male.

Species	Locality	Number of specimens by age/sex class
Leptodactylus fuscus	Poço Redondo	4J
	Tobias Barreto	4J, 5F, 1M
Leptodactylus latrans	Carira	2J, 8F, 10M
	Poço Redondo	7J, 1F, 2M
	Tobias Barreto	1J, 4F, 3M

Figure 1. Location of the three municipalities in the Brazilian state of Sergipe (Carira, Poço Redondo, and Tobias Barreto), northern Brazil, where the *Leptodactylus* specimens were collected for the present study.

A secondary constriction in the terminal region of the short arm of both homologues of chromosome 8 was observed in *L. fuscus*. C-banding in this species revealed that the constitutive heterochromatin is found principally in the centromeric and/or pericentromeric regions of all the chromosomes (Figure 4). In some metaphases, however, blocs of heterochromatin of varying length were observed in the interstitial regions of the arms of some of the chromosomes of certain individuals. Chromosome 8 demonstrated C-positive heterochromatin in the proximal region of the short arms.

Centromeric/pericentromeric C-banding was observed in all the chromosomes of *L. latrans,* although C-positive telomeres were found only in the specimens from Tobias Barreto (Figure 5). Chromosomes 4, 7, 8, and 9 of the specimens from Poço Redondo and Tobias Barreto show pericentromeric C-positive blocs on the short arms. Chromosomes 10 and 11 were all similar in the specimens from Tobias Barreto, with subtle terminal markings on the short arms, while these marks were not apparent in the specimens from Poço Redondo. In general, the *L. latrans* specimens from Tobias Barreto presented a more accentuated banding pattern, with technically better results than the specimens from other sites. It was not possible to obtain C-bands for the specimens from Carira.

In *L. fuscus*, Ag-NORs were observed in the interstitial region of the short arm of chromosome 8, coinciding with the site of the secondary constriction (Figure 6), while in *L. latrans*, these bands were found in the terminal region of the short arms of this chromosome (Figures 7 and 8). In some cases, Ag-NORs were observed in association with one another (Figure 7, B.1).

Discussion

The present cytogenetic study was based on conventional staining and C- and Ag-NOR banding of specimens of *L. fuscus* and *L. latrans* from the semiarid Caatinga scrublands of the Brazilian state of Sergipe.

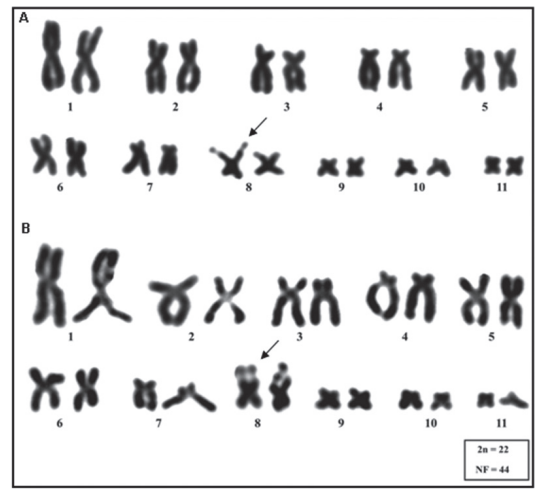

Figure 2. Karyograms for *Leptodactylus fuscus* based on conventional Giemsa staining. Specimens obtained in (**A**) Poço Redondo; (**B**) Tobias Barreto. The arrows indicate the location of the secondary constriction in chromosome 8. Sex and voucher of specimens: (**A**) juvenile, FA5 and (**B**) adult female, FT2.

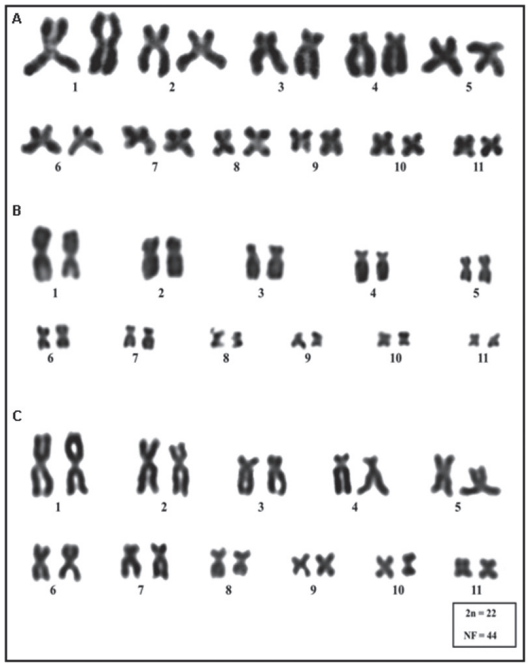

Figure 3. Karyograms of *Leptodactylus latrans* based on conventional Giemsa staining. Specimens obtained in (**A**) Carira; (**B**) Poço Redondo, and (**C**) Tobias Barreto. Sex and voucher of specimens: (**A**) adult male, OC15; (**B**) juvenile, OA1, and (**C**) adult female, OT3.

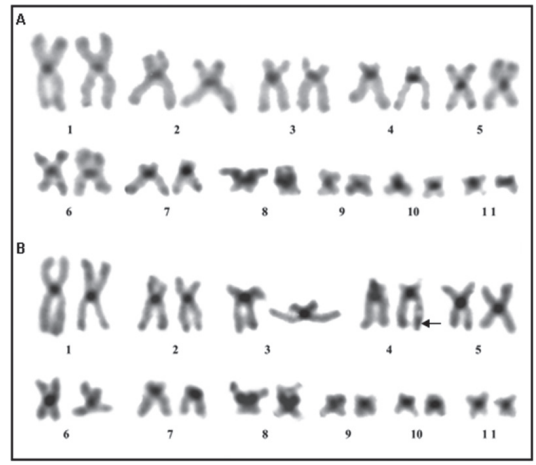

Figure 4. Distribution of C-bands in *Leptodactylus fuscus* specimens from (**A**) Poço Redondo and (**B**) Tobias Barreto. The arrow in (**B**) indicates variation in the distribution of interstitial heterochromatin between the homologues of chromosome 4. Sex and voucher of specimens: (**A**) juvenile, FA3 and (**B**) juvenile, FT2.

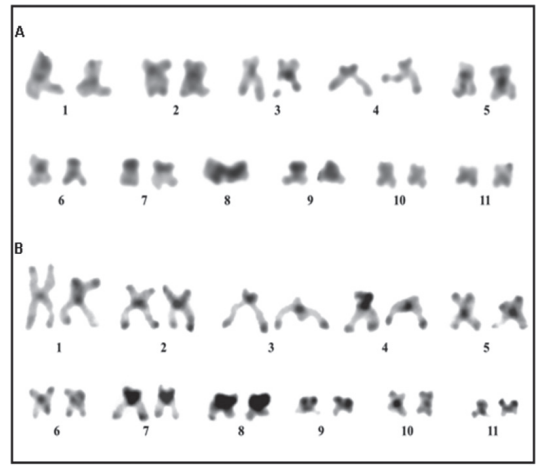

Figure 5. Distribution of C-bands in *Leptodactylus latrans* specimens from (**A**) Poço Redondo and (**B**) Tobias Barreto. Note the association between the chromosome 8 homologues in (**A**). Sex and voucher of specimens: (**A**) juvenile, OA5 and (**B**) adult female, OT2.

While both the species analyzed in the present study had been previously karyotyped, beginning in the 1970s (Denaro 1972, Bianchi *et al*. 1973, Bogart 1974), and revised more recently (Amaro-Ghilardi *et al*. 2006, Silva *et al*. 2006), this is the first cytogenetic study of populations from the Caatinga, the least well protected Brazilian biome (Leal *et al*. 2005).

The diploid chromosome number of various leptodactylid species range from 18 to 26, although there is a predominance of 2n = 22, which is almost exclusive to the genus *Leptodactylus*. Most *Leptodactylus* species have highly similar karyotypes, with a predominance of metacentric and submetacentric chromsomes

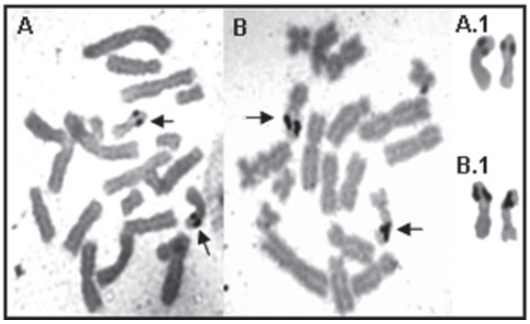

Figure 6. Metaphases following Ag-NOR banding in *Leptodactylus fuscus* specimens from (**A**) Poço Redondo and (**B**) Tobias Barreto. The arrows indicate the Ag-NORs. Highlighted: (A.1 and B.1) Pairs of chromosome 8 showing NORs. Sex and voucher of specimens: (**A**) juvenile, FA1 and (**B**) adult female, FT1.

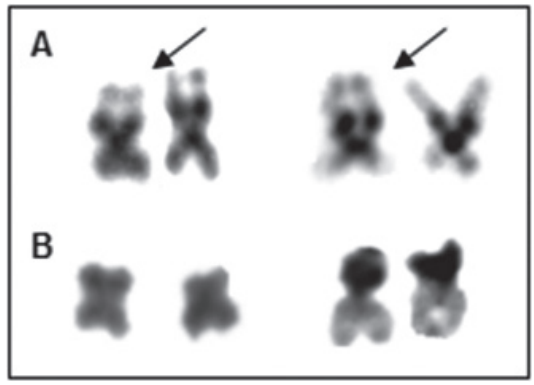

Figure 8. Secondary constriction located in the (**A**) intermediate region of the short arm of chromosome 8 in *L. fuscus*, and (**B**) terminal region of the short arm of chromosome 8 in *L. latrans*.

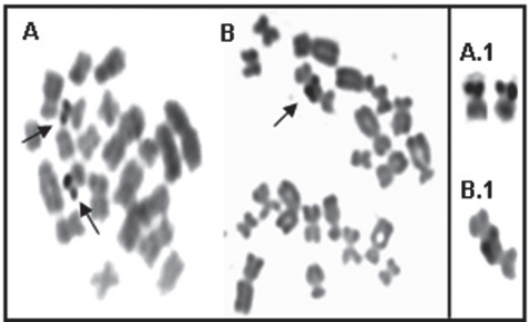

Figure 7. Metaphases of *Leptodactylus latrans* after Ag-NOR banding in specimens from (**A**) Poço Redondo and (**B**) Tobias Barreto. Highlighted: (A.1) NORs in the short arm of chromosome 8 and (B.1) presence of associated NORs. Sex and voucher of specimens: (**A**) juvenile, OA4 and (**B**) adult male, OT1.

(Beçak *et al*. 1970, Silva *et al*. 2000, 2004, 2006; Amaro-Ghilardi *et al*. 2004), as observed in the present study. There are some divergent species, however, such as *Leptodactylus silvanimbus* and *Leptodactylus marmoratus* (= *Adenomera marmorata*), which are 2n = 24 (Amaro-Ghilardi *et al*. 2006, Campos *et al*. 2009, Gazoni *et al*. 2012), and *Leptodactylus hylaedactylus* (= *Adenomera hylaedactyla*), which is 2n = 26 (Campos *et al*. 2009).

A marked secondary constriction was observed in the short arm of chromosome 8 in both populations of *L. fuscus*, coinciding with the Ag-NOR site. This structure was, however, not observed in *L. latrans*,

In closely-related species, the C-banding pattern may vary in terms of the quantity of heterochromatin, number of bands, and the location and density of the heterochromatin, which may represent an important cytotaxonomic parameter by reflecting possible chromosomal rearrangements arising during the evolution of the group (Sumner 1990)**.** The C-banding is centromeric in both *L. fuscus* and *L. latrans*, although the constitutive heterochromatin was centro/pericentromeric in all the specimens of both species, as well as additional C-positive regions in some chromosomes, such as the telomeric or interstitial regions, or even the Ag-NOR sites.

No between-population differences were found in the C-banding of *L. fuscus*. C-positive telomeric regions were not observed in specimens of this species from Rio Claro in the southeastern Brazilian state of São Paulo (Silva *et al*. 2000). The homologues of chromosome 4 in the specimens from Tobias Barreto showed varying quantities of heterochromatin, which is typical of C-banding patterns. Such differences in the quantity and distribution of the heterochromatin have been important for the definition of species- and subspecies-level differentiation in amphibians (Matsui *et al*. 1985). The heterochromatin distribution pattern observed in the specimens of *L. latrans* from Tobias Barreto was similar to that described by Amaro-Ghilardi *et al*. (2006) in specimens from Igarassu (Pernambuco, Brazil), which presented C-positive pericentromeric blocs on the short arms of chromosomes 4, 7 and 8.

Studies of other *L. latrans* populations have revealed three distinct patterns in the distribution of the heterochromatin, which have not been specifically attributed to distinct taxa, but rather geographic variation in the karyotype or possibly indicative of a species complex, given observed individual variation in external morphology, bioacoustics, and habitat use (Silva *et al*. 2000, 2006).

Most *Leptodactylus* species have a single pair of NORs, generally located on chromosome 8, albeit in distinct parts of this chromosome (Lisanti *et al*. 1990, Silva *et al*. 2004, Amaro-Ghilardi *et al*. 2006). However, in some species, the NORs are located on chromosomes 3 or 4, while *L. marmoratus* (= *Adenomera marmorata*) presents multiple NORs on the telocentric chromosomes 6 and 8 (Gazoni *et al*. 2012).

In the *L. fuscus* specimens analyzed in the present study, the NORs were observed in the interstitial region of the short arm of chromosome 8, coinciding with the site of the secondary constriction. In *L. latrans*, NORs were also observed in the terminal region of chromosome 8, with a small euchromatic region at the end of the constriction.

All the specimens of *L. fuscus* and *L. latrans* analyzed here presented karyotypes highly similar to those described in the literature, except for some variation in the C-banding. It seems likely that new patterns will be discovered as further cytogenetic studies are performed on species of *Leptodactylus*, principally those using more refined techniques capable of identifying species-specific chromosome markers, as well as a wider range of specimens representing new sites in Sergipe and other regions of the Caatinga. Such data should provide additional insights in the evolutionary relationships within the group.

The leptodactylid frogs *Leptodactylus fuscus* and *L. latrans* analyzed in the present study both have a 2n = 22 diploid chromosome number, with metacentric, submetacentric, and subtelocentric chromosomes. No marked differentiation was found in the karyotypes of either species. In addition, no between-population variation in the C-banding pattern was observed in *L. fuscus*, whereas some differences were found in the banding pattern of the *L. latrans* specimens from Tobias Barreto and Poço Redondo, as well as between these specimens and those described from other sites, which indicates that this widely-distributed species may either represent part of a species complex or an ongoing radiation.

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