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Molecular cytogenetics of nucleolar organizer regions in *Phyllomedusa* and *Phasmahyla* species (Hylidae, Phyllomedusinae): a cytotaxonomic contribution

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ABSTRACT. Chromosome numbers, morphology, and nucleolus organizer region (NOR) locations are useful cytological characters for taxonomic and evolutionary studies. In this study, we provide the first cytogenetic analysis of *Phyllomedusa bahiana* and *Phasmahyla spectabilis*, and report new cytogenetic data on variation in NOR numbers and positions in *Phyllomedusa rohdei* and *Phyllomedusa nordestina* using conventional staining, AgNOR-banding, and 45S rDNA fluorescence *in situ* hybridization. All 4 species showed 2n = 26 chromosomes. *P. spectabilis* and *P. bahiana* had only 1 pair of NOR-carrying chromosomes. *P. bahiana* showed an NOR length polymorphism, and a rare homomorphic self-compatibility for both NOR lengths in anurans. Variation in the number of NOR-bearing chromosomes was found between the sampled populations of *P. nordestina*, ranging from 3 to 4. This study also clarified previous conflicting results concerning the occurrence of inter- and intra-population NOR variation in *P. rohdei*. The variation, confirmed by 45S

rDNA fluorescence *in situ* hybridization analysis, was congruent with results obtained from AgNOR-banding in all species.

Key words: Chromosome; Evolution; 45S rDNA; AgNOR-banding; Anura

INTRODUCTION

The genus *Phyllomedusa* comprises 30 recognized species, 22 of which are found in Brazil (Frost, 2011). Based on morphological, molecular, and acoustic characters, 26 species have been arranged into 4 species-groups, i.e., the *P. burmeisteri* group (Pombal Jr. and Haddad, 1992), the *P. hypochondrialis* group (Caramaschi, 2006), the *P. perinesos* group (Cannatella, 1982), and the *P. tarsius* group (Barrio-Amorós, 2006). The remaining species are still in need of placement (Faivovich et al., 2009).

Faivovich et al. (2009) pointed out the need for more detailed investigations of some *Phyllomedusa* groups. *P. nordestina* shows a high level of intraspecific sequence divergence (up to 10.4%) in the mitochondrial cytochrome b gene, and *P. rohdei* appears to be paraphyletic by forming a clade with *P. megacephala*. Additionally, *P. rohdei* from different locations showed high p-distance values, suggesting the presence of additional taxa within this group. *P. bahiana* was originally described as a *P. burmeisteri* subspecies by Lutz (1950), and was later elevated to species status by Silva-Filho and Juncá (2006). Faivovich et al. (2009) provided additional support for this placement based on molecular data. However, Pombal Jr. and Haddad (1992) observed specimens with intermediate thigh color patterns across the species range, suggesting the presence of putative fertile hybrids between *P. bahiana* and *P. burmeisteri*.

The genus *Phasmahyla* (Cruz, 1990) contains species that were formerly included in the *Phyllomedusa gutata* group. Morphological and molecular characters have since revealed this genus to be a sister group of *Phyllomedusa* (Faivovich et al., 2009), which makes *Phasmahyla* an important group for understanding the evolution of karyotypes in *Phyllomedusa*.

Cytogenetic analysis is an important complementary approach to assist studies of evolutionary relationships at the species and higher taxonomic levels. However, chromosomal data on *Phyllomedusa* and related genera remain scarce, particularly with respect to the use of molecular cytogenetic techniques. Barth et al. (2009) identified high variation in the number of nucleolus organizer regions (NOR) in populations of *P. rohdei* from Bahia State. Paiva et al. (2010) observed a similar pattern in one population from São Paulo State. Morando and Hernando (1997) also observed this pattern of variation in *P. hypochondrialis*.

The NOR is a chromosomal site containing ribosomal genes, which can be located either directly by fluorescent *in situ* hybridization (FISH) of ribosomal DNA probes or indirectly, by silver nitrate staining (AgNOR-banding). The combined use of these 2 techniques is useful since AgNOR-banding enables detection of only active NORs (Sumner, 1990), while the FISH technique can locate every ribosomal DNA cluster in the genome. Moreover, several studies demonstrated that in some organisms, silver nitrate can bind to some chromosomal regions even in the absence of an NOR (e.g., Utsumi and Takehisa, 1974; Dobigny et al., 2002). Frequently, these rDNA-free silver nitrate-positive regions correspond to heterochromatins (e.g., Fujiwara et al., 1998).

In this study, we analyzed the chromosomal profiles of *P. nordestina* and *P. rohdei*,

which are included in the *P. hypochondrialis* group, *P. bahiana* from the *P. burmeisteri* group, and *Phasmahyla spectabilis*, using conventional and molecular cytogenetic techniques.

MATERIAL AND METHODS

Specimens of *P. bahiana*, *P. rohdei*, *P. nordestina*, and *P. spectabilis* were collected from different localities in the State of Bahia, Brazil. Vouchers were deposited in the Herpetological Collection at Universidade Estadual de Santa Cruz (MZUESC), BA, Brazil (Figure 1 and Table 1).

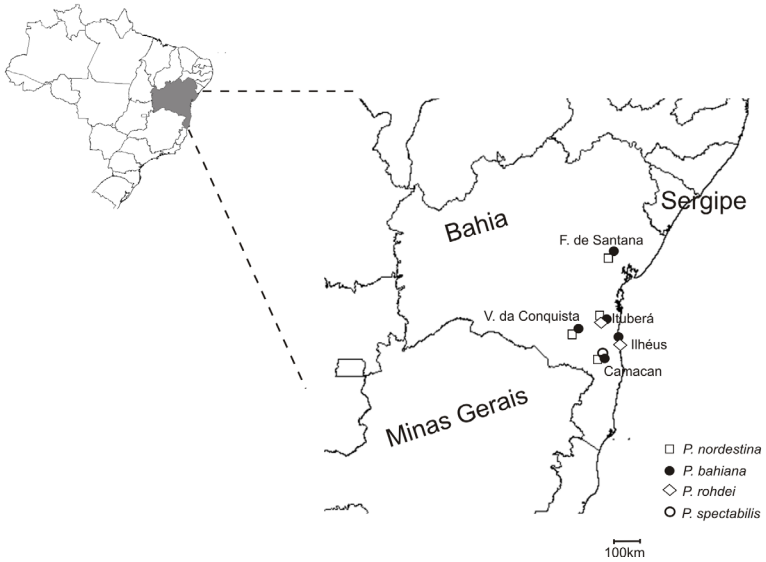

Figure 1. Map of the sampling localities. Feira de Santana (12°06'S 39°02'W), Ilhéus (14°47'S 39°10'W), Camacan (15°24'S 39°30'W), Vitória da Conquista (14°53'S 40°48'W), and Ituberá (13°42'S 39°11'W).

Table 1. Number of specimens of *Phyllomedusa* and *Phasmahyla* analyzed and their respective voucher number and sampling localities in Bahia State.

Genus	No. of specimens	Locality	Voucher number
Phyllomedusa bahiana	12	Feira de Santana	MZUESC7041-MZUESC7052
	1	Vitória da Conquista	MZUESC10862
	2	Ituberá	MZUESC6978; MZUESC6979
	7	Camacan	MZUESC6989; MZUESC6996; MZUESC6998; MZUESC7000; MZUESC7002; MZUESC7014; MZUESC7017
	10	Ilhéus	MZUESC5615; MZUESC6961-MZUESC6965; MZUESC6973; MZUESC6975; MZUESC6993; MZUESC6994
Phyllomedusa rohdei	6	Ituberá	MZUESC7030; MZUESC7032; MZUESC7034; MZUESC7036; MZUESC7056; MZUESC6986
	8	Ilhéus	MZUESC5608-MZUESC5610; MZUESC5612-MZUESC5614; MZUESC6982; MZUESC6985
Phyllomedusa nordestina	5	Vitória da Conquista	MZUESC7003-MZUESC7005; MZUESC7010; MZUESC7013
	1	Camacan	MZUESC6995
	5	Ituberá	MZUESC7027; MZUESC7029; MZUESC7057; MZUESC7059; MZUESC10857
	1	Feira de Santana	MZUESC7040
Phasmahyla spectabilis	3	Camacan	MZUESC10373-MZUESC10375

For cytological preparations, samples were pretreated with 1% colchicine, 10 h prior to dissection. Gut cells were then extracted and prepared following methods described in Schmid (1978). Chromosomes were stained using 5% Giemsa solution. A minimum of 5 slides per specimen and 5 metaphases per slide were analyzed. AgNOR-banding followed methods described in Howell and Black (1980), with a few modifications: we used 25% silver nitrate solution and incubated the slides at 50°C for approximately 30 min. The slides were analyzed and the best quality metaphases were photographed using an Olympus CX-41 microscope equipped with a digital camera. Chromosomes were classified according to Green and Sessions (1991) nomenclature as metacentric, submetacentric, and subtelocentric, and were organized in decreasing order of length.

FISH was performed using the HM123 plasmid probe, containing part of the 45S rDNA sequence from *Xenopus laevis* (Meunier-Rotival et al., 1979), labeled with cyanine 3-dUTP (dUTP-Cy3) by nick translation. FISH procedures followed methods of Moscone et al. (1996), with a few modifications, at 72% stringency. Slides were analyzed in an Olympus BX-51 epifluorescence microscope and the images were captured using the ImageProPlus software.

RESULTS

All *Phyllomedusa* and *P. spectabilis* specimens showed 2n = 26 chromosomes. *P. spectabilis* showed submetacentric chromosomes in pairs 1 to 6, 9, 10, and 12, metacentric chromosomes in pairs 8, 11, and 13, while pair 7 was subtelocentric. *P. bahiana* showed a karyotype with submetacentric chromosomes in pairs 1 to 6, 9, 10, and 12, metacentric chromosomes in pairs 7, 11, and 13, and pair 8 was subtelocentric. *P. nordestina* showed metacentric chromosomes in pairs 1, 4, 6, and 8 to 13, submetacentric chromosomes in pairs 2, 3, and 5, and pair 7 was subtelocentric (Figure 2A-C).

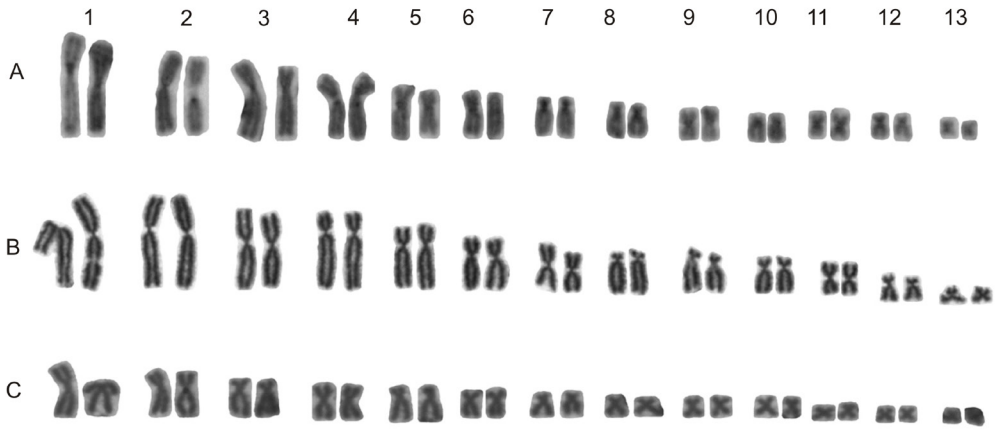

Figure 2. Karyograms of **A.** *Phasmahyla spectabilis* **B.** *Phyllomedusa bahiana* and **C.** *Phyllomedusa nordestina*.

P. spectabilis had the NOR located near the centromere of the 2nd chromosome pair (Figure 3A). *P. bahiana* also showed centromere proximal NORs, which were located in the long arm of the 9th chromosome pair. Most *P. bahiana* individuals showed a heteromorphism in NOR length. Samples with 2 alternative homomorphic states were also observed (Figure 3B and C). *P. nordestina* showed variation in the number of interstitial AgNOR bands across different sampling locations, ranging from 2 to 3 NOR-carrying chromosomes (Figure 3D and E). In specimens of the Ituberá population, 3 AgNOR bands were located in one of the chromosomes of the 9th pair and in both homologues of the 11th pair. However, in Vitória da Conquista and Camacan samples, NORs were only observed in the 11th chromosome pair.

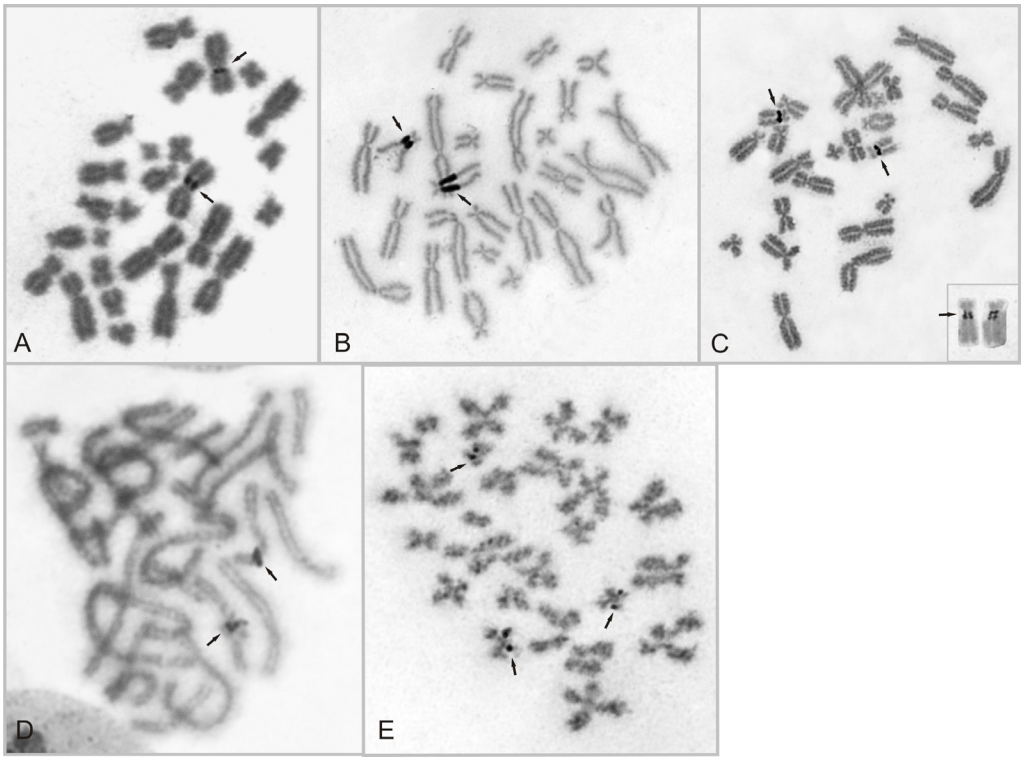

Figure 3. Metaphases labeled by AgNOR-banding. **A.** *Phasmahyla spectabilis* showing 2 chromosomes carrying the NOR; **B. C.** *Phyllomedusa bahiana* showing the heteromorphic NOR and homomorphic short NOR and the duplicated NOR pattern (in the box), respectively; **D. E.** *Phyllomedusa nordestina* showing 2 and 3 chromosomes with NOR, respectively.

Results of 45S rDNA *in situ* hybridization were congruent with the AgNOR-banding pattern in all species analyzed (Figure 4). These results also showed variation in the number of 45S rDNA clusters in *P. rohdei*, which ranged from 1 to 4 chromosomes containing rDNA gene clusters (Figure 4E-G). Their locations were near the telomeric region in the 9th and 10th chromosome pairs.

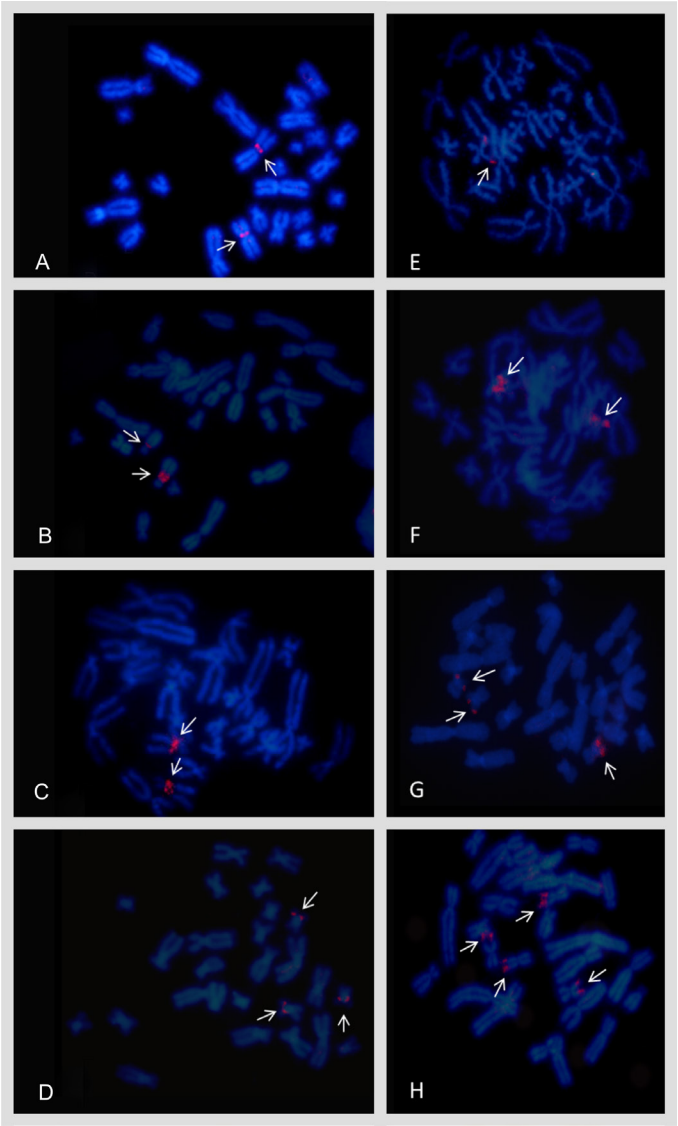

Figure 4. Metaphases labeled by 45S rDNA fluorescence *in situ* hybridization. **A.** *Phasmahyla spectabilis*; **B. C.** *Phyllomedusa bahiana* showing the heteromorphic and homomorphic NOR, respectively; **D.** *Phyllomedusa nordestina*; **E.-H.** *Phyllomedusa rohdei* showing 1 to 4 chromosomes with NORs in different individuals.

DISCUSSION

Previously studied *Phyllomedusa* species exhibited a conservative chromosome number of 2n = 26 (Haddad et al., 1994; Morando and Hernando, 1997; Kasahara et al., 2007; Bruschi et al., 2012), with the exception of *P. tetraploidea*, which had 2n = 52 chromosomes. The increase in chromosome number was most likely due to a polyploidy event in a lineage

with the 2n = 26 karyotype (Kasahara et al., 2007; Brunes et al., 2010). This chromosome number stability among *Phyllomedusa* species was confirmed in the present study and, for the first time, also reported for *P. spectabilis*.

P. spectabilis showed other karyotype similarities with *P. bahiana*. For example, the 7th chromosome pair in *P. spectabilis* showed a very similar morphology to the 8th chromosome pair of *P. bahiana*. One major differentiation was found with respect to the NOR position, which was located in the 2nd chromosome pair in *P. spectabilis* but in the 9th chromosome pair in *P. bahiana*. The karyotype similarities observed between these *Phyllomedusa* and *Phasmahyla* species agree with recent molecular phylogenetic analyses (Faivovich et al., 2005, 2009) that place them as sister groups.

Since most species showed exclusively short NOR bands, the peculiar NOR length heteromorphism exhibited by *P. bahiana* seems to have been more recently derived as a result of a tandem amplification of rDNA clusters in this species. Similar NOR-length heteromorphisms were found in other anurans (Schmid, 1982; Lourenço et al., 2000; Busin et al., 2000; Bruschi et al., 2012), and this variation has been formerly attributed to tandem amplification of rRNA genes or of a whole NOR segment (Schmid, 1982; King et al., 1990).

Schmid (1982) suggested that amplified NORs would be lethal in their homomorphic state, since the chromosome rearrangement could cause additional structural alterations in the NOR-adjacent segments that contain some functionally important genes. This assumption follows from an analysis of 260 individuals from 23 genera that lack the homomorphic amplified state. However, in *P. bahiana*, the amplification seems to be well tolerated, since it showed a feasible rearrangement in homozygosis.

The occurrence of multiple and variable rDNA clusters observed in *P. rohdei* using FISH analysis confirmed the variation previously reported by Barth et al. (2009) and Paiva et al. (2009) based on AgNOR-banding. Morando and Hernando (1997) found similar results in *P. hypochondrialis* using AgNOR-banding. *P. nordestina* is now included in this list as revealed by the present FISH and AgNOR-banding analyses.

Bruschi et al. (2012) found only one pair of chromosomes containing rDNA clusters in populations of *P. rohdei* and *P. nordestina* from Bahia State. The failure to detect variation in this case may have been related to their adoption of a more limited sampling strategy. Moreover, the morphology of the 9th chromosome pair, which was found to contain the NOR in the present analysis, also differs from that observed in Bruschi et al. (2012), who described it as a submetacentric chromosome pair. These differences most likely reflect small morphological variations in the same chromosomes, which can occur because of different chromosomal preparations.

Multiple NOR sites in karyotypes are usually due to chromosomal rearrangements such as inversions and translocations (King et al., 1990), rDNA cistron amplifications (Macgregor and Kezer, 1973), and the presence of genetic transposable elements (Schubert and Wobus, 1985). However, an explanation for differences among species in their susceptibility to molecular reorganization of rDNA clusters is still lacking. Results of the present study contrast with the major pattern of only one chromosome pair with NOR that is found in the other *Phyllomedusa* and related species studied so far; however, multiple NORs have been described in several other anuran species (e.g., Wiley et al., 1989; Lourenço et al., 1998, 2000; Veiga-Menoncello et al., 2003; Amaro-Ghilardi et al., 2006; Silva et al., 1999, 2006), indicating that this variation is common in this group.

This study provides the first cytogenetic data for *P. bahiana* and *P. spectabilis*, and clarified previous conflicting results concerning inter- and intra-population NOR variation in *P. rohdei* and *P. nordestina*. The data shown here improve upon current knowledge about *Phyllomedusa*/*Phasmahyla* karyotype evolution and raise new questions regarding their chromosomal organizations and variation. The new cytogenetic results confirmed higher chromosome variability in the *Phyllomedusa* species, and reiterated the need for taxonomic review of this group combining morphology, bioacoustics, molecular and cytogenetic data.

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