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SHORT COMMUNICATION

Karyotypes and Ag-NORs in *Phyllomedusa camba* De La Riva, 1999 and *P. rhodei* Mertens, 1926 (Anura, Hylidae, Phyllomedusinae): cytotaxonomic considerations

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Abstract
The karyotypes of *Phyllomedusa camba* De La Riva, 1999 and *P. rhodei* Mertens, 1926 are presented and the chromosome pairs with Ag-NORs are identified. Both karyotypes have 2n=26 chromosomes with similar morphology, an exception being the presence of three acrocentric pairs in *P. camba*. In this species the Ag-NORs are found in the proximal region of pairs 1 and 5 whilst in *P. rhodei* an extensive inter-individual variation was observed in the number and position of the Ag-NORs (1p, 3q, 5p, 8p, 11q, and 12q). Based on comparative cytogenetic data of *P. camba* and *P. rhodei*, we discuss the phenetic groups proposed for *Phyllomedusa* genus.

Keywords: *Cytogenetic, chromosome, Amphibia,* Phyllomedusa*, phenetic group*

Introduction

The family Hylidae has about 870 species, currently distributed in the subfamilies Hylinae, Pelodryadinae and Phyllomedusinae (Faivovich et al. 2005; Frost 2007). Molecular and morphological studies obtained by Faivovich et al. (2005) show a sister relationship between Pelodryadinae and Phyllomedusinae, which together correspond to the sister taxon of Hylinae. The subfamily Phyllomedusinae is comprised of seven nominal genera: *Agalychnis*, *Hylomantis*, *Cruziohyla*, *Pachymedusa*, *Phasmahyla*, *Phrynomedusa*, and *Phyllomedusa* (Frost 2007). Faivovich et al. (2005) discussed several other characters which are likely to be synapomorphies of Phyllomedusinae, and demonstrated on the basis of molecular data that *Cruziohyla* is the sister taxon of the remaining genera. Two clades were observed: one containing *Phasmahyla* and *Phyllomedusa*, and the other containing *Agalychnis*, *Hylomantis*, *Cruziohyla*, *Pachymedusa* and *Phrynomedusa*. The 30 species of *Phyllomedusa* form a monophyletic group and most of the species can be distributed amongst five species groups: *burmeisteri*, *hypochondrialis*, *buckeli*, *perinesos* and *tarsius* (Faivovich et al. 2005; Caramaschi 2006; Frost 2007). A few of the Brazilian species were allocated to other genera such as *Hylomanthis*, *Phasmahyla* and *Phrynomedusa* by Cruz (1990). The phylogenetic relationships between the species in these groupings are hitherto not well established.

Phyllomedusa camba De la Riva, 1999 distributed throughout the southeastern Amazon Basin from southweastern Peru, western Brazil (states of Amazon, Acre, and Rondônia) to eastern Bolivia, is included in the *P. tarsius* species group together with *P. boliviana*, *P. coelestis*, *P. tarsius*, *P. trinitatis* and *P. venusta*. Barrio-Amorós (2006) defined the species belonging to the *P. tarsius* group using morphological characters (*P. coelestis*, *P. tarsius*, *P. neildi* sp. nov., *P. trinitatis*, and *P. venusta*) and proposed that *P. boliviana*, *P. camba* and *P. sauvagii* (considered in the group by De la Riva 1999, and Faivovich et al. 2005) should be excluded from the

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group because they do not share the most striking feature (herein considered as a synapomorphy) of the group, that is, the golden iris with black reticulations. Although Barrio-Amorós (2006) proposed the exclusion of *P. camba* from the *P. tarsius* species group, its position in the group is maintained (Frost 2007).

Phyllomedusa rohdei Mertens, 1926 is distributed throughout the lowlands of southern Brazil and is included in the *P. hypochondrialis* species group with *P. ayeaye*, *P. azurea*, *P. centralis*, *P. hypochondrialis*, *P. megacephala*, *P. nordestina*, *P. oreades*, and *P. palliata* (Caramaschi 2006; Frost 2007). The species *P. rhodei* has the muscle epicoracoideus, considered a synapomorphy for the group by Faivovich et al. (2005). Although *P. rhodei* was included in the study by Faivovich et al. (2005), the authors did not present a phylogenetic relationship between this species and others in the *P. hypochondrialis* species group, nor with species belonging to other species groups.

With regard to cytogenetic data, most of the information available about the species of *Phyllomedusa* is limited to the diploid number, which is 2n=26 for most of the species (Beçak et al. 1970; Batistic et al. 1975; Batistic 1989; Kuramoto 1990). Concerning differential staining technique, Batistic (1989) presented C-banding and Ag-NOR data and suggested a hypothesis for the origin of polyploidy in *Phyllomedusa* based on the position of the Ag-NORs. Morando and Hernando (1997) applied the Ag-NOR technique to chromosomes of *P. sauvagii* and *P. hypochondrialis* and detected differences in the number of Ag-NORs between the species as well as a heteromorphism in *P. hypochondrialis*. Kasahara et al. (2007) demonstrated the occurrence of Ag-NORs in *P. distincta* and *P. tetraploidea* and registered the occurrence of a triploid hybrid. In addition, these authors described the BrdU replication banding pattern in *P. distincta*.

Considering the difficulty in defining the groups of *Phyllomedusa* species as well as the uncertain relationships among species of the *P. tarsius* group, we present for the first time the karyotypes of *P. camba* and *P. rohdei*. Additionally, we also provide Ag-NOR staining for the two species and observe multiple Ag-NORs patterns in the chromosomes of both species.

Materials and methods

Cytogenetic analysis was carried out on two specimens of *Phyllomedusa camba* collected in Ministro Andreazza, state of Rôndonia (RO) (11°04′27″S; 61°31′01″W) north of Brazil and on seven specimens of *Phyllomedusa rohdei* collected in Biritiba-Mirim, state of São Paulo (SP) (23°34′21″S; 46°02′19″W), southeastern Brazil (Figures 1A,B). The voucher specimens were deposited in the Amphibian collection (CFBH) of the Departamento de Zoologia, Instituto de Biociências, UNESP, Rio Claro, SP, Brazil.

The mitotic chromosomes were obtained from direct preparations of bone marrow, liver and testis treated with 0.01% colchicine at a proportion of 0.1 ml/10 g body weight, as described in Baldissera et al. (1993) and Silva et al. (2000), or from the intestine using the technique in Schmid (1978). To improve the mitotic index, we injected phytohemagglutinin in some specimens before the colchicine treatment, at the proportion of 0.1 ml/10 g body weight, 48–72 h before sacrifice. Conventional staining was made with Giemsa 10% diluted in phosphate buffer pH 6.8, and silver nitrate labeling

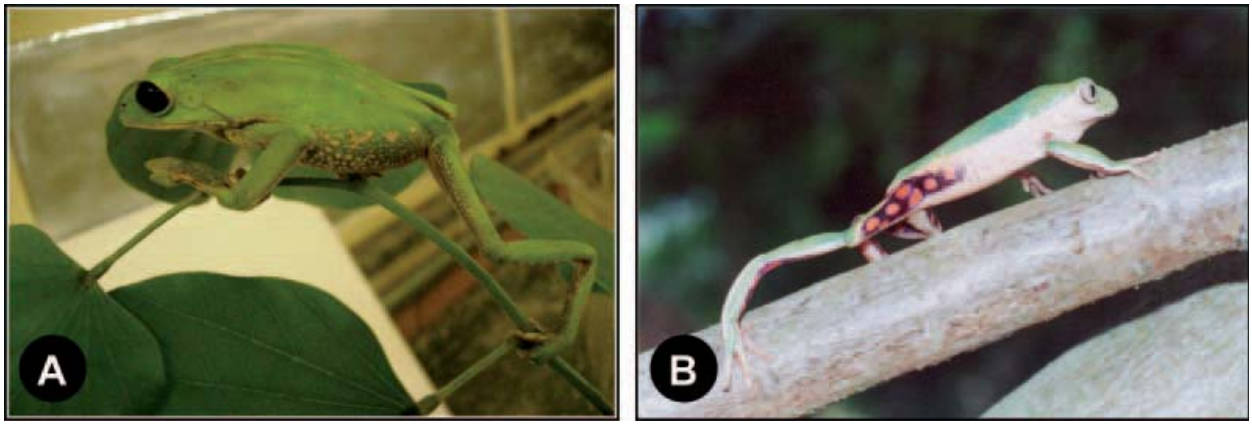

Figure 1. **A**, adult male of *Phyllomedusa camba* from Ministro Andreazza, Rôndonia (RO); **B**, *Phyllomedusa rohdei* from Biritiba-Mirim, São Paulo (SP).

of the nucleolar organizer regions (Ag-NOR) was obtained by the technique of Howell and Black (1980).

Results and discussion

The species *P. camba* and *P. rhodei* have a diploid number corresponding to 2n=26, and a karyotype similar to that found for the majority of the Phyllomedusinae species: *Phyllomedusa* (Beçak et al. 1970; Batistic et al. 1975; Batistic 1989; Morando & Hernando 1997; Kasahara et al. 2007); *Agalychnis* (Schmid et al. 1995); and *Pachymedusa* (Schmid 1980). An exception was observed in several tetraploid populations of *P. burmeisteri* from Brazil, with 4n=52 (Beçak et al. 1970; Batistic et al. 1975; Batistic 1989). The karyotype of *P. rhodei* is made up of metacentric pairs (1, 4, 10, and 13), and submetacentric pairs (2, 3, 5–9, 11 and 12), while in *P. camba*, pairs 1, 4, 7, 10, and 13 are metacentric, 2, 3, 5, 6, and 9 are submetacentric, and 8, 11 and 12 are acrocentric (Figures 2A,B). The occurrence of acrocentric pairs in *P. tarsius* observed by Batistic (1989) suggests the proximity of this specie and *P. camba*. Nevertheless, these data refute the proposal of Barrio-Amorós (2006), which excluded *P. camba* from the *P. tarsius* group. If the presence of acrocentric pairs is considered a synapomorphy, *P. sauvagii*, which possesses no acrocentric chromosomes (Batistic 1989), might be excluded from the *P. tarsius* group. The karyotype with metacentric and submetacentric chromosomes in *P. rohdei* is compatible to that found by Batistic (1989) for this species and for *P. ayeaye* and *P. hypochondrialis* (all allocated to the *P. hypochondrialis* species group).

As stated by Bogart (1991), centric fusions and fissions are the most likely mechanism for changes in chromosomal number. The subfamily Phyllomedusinae is considered basal in the Hylidae family due to morphological characters and the diploid number of 26 chromosomes (Bogart 1973; Morescalchi 1990). Nevertheless, in the species of *Phyllomedusa* considered to be derived, no alterations have occurred in the number of chromosomes, although the presence of acrocentric chromosomes and polyploidization in some species suggest the presence of speciation events in this anuran group. The species *P. tarsius* and *P. camba*, both of them with acrocentric chromosomes, can be considered very closely related and derived in the *Phyllomedusa* genera.

In *P. camba*, 20 metaphases showed Ag-NORs in the proximal regions of the short arms of pair 1, and in the proximal regions of the long arms of pair 5 coincident with secondary constriction (Figure 3B). In *P. rohdei*, the silver staining showed an extensive inter-individual variation in the number and position of Ag-positive regions, in 1p, 3q, 5p, 8p, 11q and 12q (Figure 3A). Inter-individual variation in single or multiple Ag-NOR patterns was described by Wiley et al. (1989) in *Hyla chrysoscelis* and *H. versicolor*, Foote et al. (1991) in *Bufo terrestris*, Miura (1994) in *Rana japonica*, Schmid et al. (1995) in *Agalychnis callidryas*, Kaiser et al. (1996) in *Dendropsophus ebraccatus*, Silva et al. (1999) in *Physalaemus cuvieri*, and Silva et al. (2006) in *Leptodactylus mystacinus*.

In Anura, NOR analysis by silver staining has shown that species, in both primitive and derived families, possess only one pair of Ag-NORs in their diploid karyotype (Schmid 1982; Mahony & Robinson 1986). This observation led King et al. (1990) to suggest the presence of only a single pair of Ag-NORs in diploid karyotypes as an ancestral

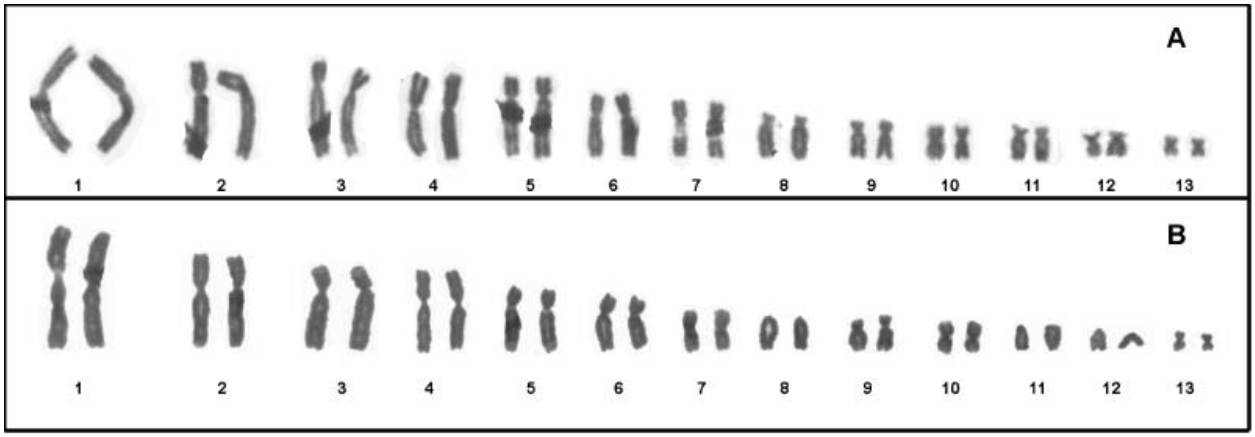

Figure 2. **A**, Giemsa-stained karyotypes of *Phyllomedusa rohdei*; **B**, *Phyllomedusa camba*. Note three acrocentric pairs (8, 11 and 12) in *P. camba*.

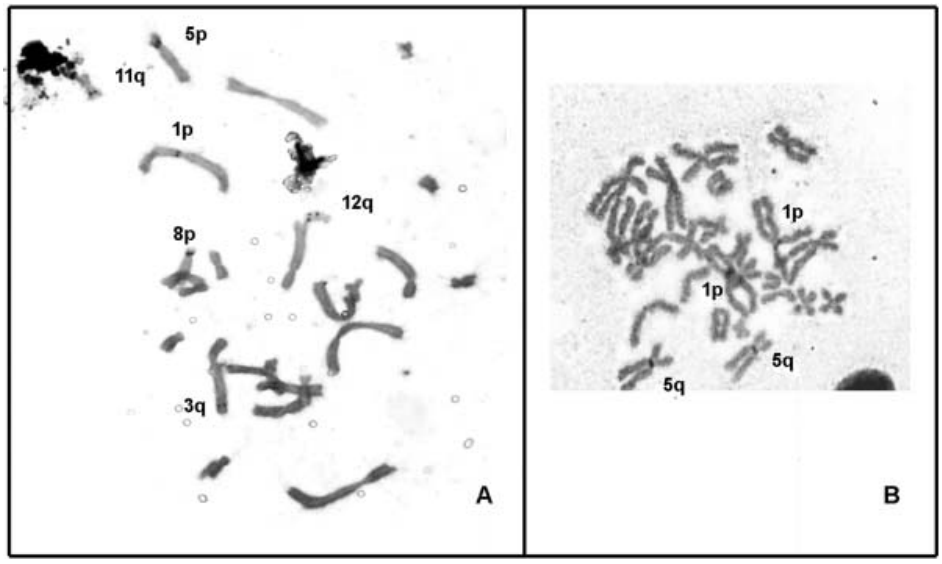

Figure 3. **A**, Metaphases with Ag-NOR bearing chromosomes of *Phyllomedusa rohdei*; **B**, *Phyllomedusa camba*.

condition in Anura, a hypothesis previously proposed by Schmid (1978) for bufonids and hylids. In the genus *Phyllomedusa*, the occurrence of more than one chromosome with Ag-NORs was observed in *P. burmeisteri*, *Phyllomedusa* sp., *P. distincta*, *P. iheringi*, *P. hypochondrialis*, *P. ayeaye*, *P. tarsius*, *P. rohdei* and *P. tetraploidea* (Table I).

Cytochemical tests have revealed that silver does not attach to the ribosomal DNA itself, but rather to proteins specifically associated with this region and to the heterochromatin (Nardi et al. 1978; Sánchez et al. 1995; Dobigny et al. 2002). Nevertheless, in some anuran species, multiple variable Ag-NOR positive region was confirmed using more suitable techniques, such as *in situ* hybridization with a fluorescent rDNA probe (Foote et al. 1991; Kaiser et al. 1996; Lourenço et al. 1998, 2000). These authors suggest that some mechanisms involved in NOR dispersion in anuran genomes may include inversions and translocations involving chromosomal segments containing NORs, transpositions by mobile genetic elements, amplifications of 'orphan' rDNA cistron, and reinsertion errors during extrachromosomal amplifications of ribosomal cistrons. Incongruous FISH with rDNA probe data with Ag-staining findings were reported in *Leptodactylus mystacinus* by Silva et al. (2006), and these authors suggested that some Ag-positive sites in the genome of *L. mystacinus* are not true Ag-NORs, but might be related to some peculiarities in the heterochromatin located at these sites, although they are not always identified with C-banding.

The present cytogenetic data are not conclusive for deciding the taxonomic status of *Phyllomedusa* at phenetic species group. Nevertheless, we think that our cytogenetic data are toward a closer proximity

Table I. Multiples Ag-NORs sites in *Phyllomedusa* species.

Species	Location of the Ag-NORs	Reference
Phyllomedusa burmeisteri	1p 1p 9q 9q	Batistic 1989
Phyllomedusa sp. 4n	1p 1p 8p 8p	Batistic 1989; Kasahara et al. 2007
Phyllomedusa distincta	1p 1p 9q 9q	Batistic 1989; Kasahara et al. 2007
Phyllomedusa iheringii	1p 1p 9q 9q	Batistic 1989
Phyllomedusa sauvagii	9q 9q	Batistic 1989; Morand & Hernando 1997
Phyllomedusa hypochondrialis	9–10q 11q	Batistic 1989
	7p 8q 11q 13q	Morand & Hernando 1997
Phyllomedusa ayeaye	1p 1p 9q 9q	Batistic 1989
Phyllomedusa tarsius	1p 1p 9q 9q	Batistic 1989
Phyllomedusa camba	1p 1p 5q 5q	This report
Phyllomedusa rohdei	1p 3q 5p 8p 11q 12q	This report
	2p 8p 8p	Batistic 1989

between *P. camba* and *P. tarsius*. Cytogenetic studies of other species of the *P. tarsius* group (sensu De la Riva 1999) are necessary to confirm our hypothesis.

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