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Short Communication

Comparative cytogenetics of nine populations of the *Rhinella* genus (Anura, Bufonidae) with a highlight on their conservative karyotype

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Abstract

The genus *Rhinella* is one of the most diverse groups of bufonid toads, currently composed by 93 valid species and naturally distributed throughout different Neotropical ecoregions. Here, we analyze nine Brazilian populations of toads representing species of the *Rhinella margaritifera* and *Rhinella marina* groups. These new data include the first description of the *R. hoogmoedi* and *R. proboscidae* karyotypes, as well as other taxonomically unresolved forms. Chromosomal analysis of the populations revealed pronounced chromosomal uniformity (2n=22), including the diploid number and chromosomal morphology. Three different NOR-bearing chromosomes were identified: in the subterminal region of pair 10q in *R. hoogmoedi*, *Rhinella* sp. 1 and *Rhinella* sp. 2, in subterminal region of 7p in *R. proboscidae* and *Rhinella* cf. *margaritifera* while in *R. henseli* and *R. icterica* was detected in interstitial region of 7p. Karyotypic uniformity of the genus permits the inference of interspecific chromosome homologies and evolutionary changes in the NOR-bearing chromosome may represent an informative character in species group level. The review of the cytogenetic data of the *Rhinella* species together with the new karyotypes reported here contributes to the understanding of the chromosomal evolution of these toads, which karyotypes are highly conserved despite the ample distribution of many forms.

Keywords: Bufonid toads, nucleolar organizing region, chromosomal evolution.

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Cytogenetic data provide a powerful tool for the evaluation of the taxonomic (Cuevas, 2008; Fávero *et al.*, 2011; Funk *et al.*, 2012) and evolutionary relationships (Veiga-Menoncello *et al.*, 2014) among anuran species. The understanding of chromosomal characters helps to identify synapomorphies (Cunningham and Cherry, 2004; Targueta *et al.*, 2012; Suárez *et al.*, 2013; Ferro *et al.*, 2018) and homoplasies (Cardozo *et al.*, 2011), and when combined with molecular phylogenetic inferences, these can contribute to the understanding of the role of chromosomal rearrangements in the diversification of a lineage (Veiga-Menoncello *et al.*, 2014).

Evolutionary analysis of anuran cytogenetics has provided important additional insights for phylogenetic inferences (Lourenço *et al.*, 2015; Targueta *et al.*, 2018). Recent shifts in analytical approaches have allowed more systematic evaluations,that have traced evolutionary changes in the chromosomal complement (*e.g.*, Lourenço *et al.*, 2008), both within and among anuran groups. However, the lack of cytogenetic data for many groups remains a limiting factor, especially if taking into account the considerable taxonomic richness of the order Anura, which has at least 7,058

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species (*sensu* Frost, 2019). An interesting example is the genus *Rhinella*, a cosmopolitan group currently composed of 92 valid species (*sensu* Frost, 2019), although there is considerable evidence of a number of cryptic species and undescribed taxa (Fouquet *et al.*, 2007a). Most of the species of this genus are arranged in species groups (*R. crucifer, R. festae, R. granulosa, R. margaritifera, R. marina, R. spinulosa*, and *R. veraguensis*), but some taxa have not been assigned to any existing group (Chaparro *et al.*, 2007; Moravec *et al.*, 2014).

Cytogenetic analyses of the genus *Rhinella* revealed a pronounced chromosomal uniformity, including the diploid number and chromosomal morphology, and some species, such as *Rhinella icterica*, *Rhinella jimi*, and *Rhinella schneideri*, cannot even be distinguished by their C-banding or the distribution of their NOR (Kasahara *et al.*, 1996; Amaro-Ghilardi *et al.*, 2007). However, alternative NOR-bearing chromosomes have been identified in other species of the genus (Silva, 2010; Baraquet *et al.*, 2011). The present study is based on a compilation of the available chromosomal data for *Rhinella*, combined with karyotypes obtained from nine Brazilian populations of toads representing species of the *Rhinella margaritifera* and *Rhinella marina* groups. These new data include the first description of the karyotypes of two species of the *R. margaritifera* group (*Rhinella hoogmoedi* and *Rhinella proboscidae*), as well as other taxonomically unresolved forms.

The specimens and their respective collecting localities are listed in Table 1. The collection of specimens was authorized by SISBIO/Chico Mendes Institute for the Conservation of Biodiversity, through license number 20266-1. Voucher specimens were deposited in the Zoology Museum (ZUEC) "Prof. Dr. Adão José Cardoso" at Campinas State University (UNICAMP) in Campinas, SP, Brazil. The chromosomal samples were prepared from suspensions of intestinal epithelial cells, following King and Rofe (1976) and Schmid (1978). The chromosomes were stained with 10% Giemsa or submitted to the Ag-NOR technique (Howell and Black, 1980). The chromosomes were ranked and classified according to the criteria of Green and Session (1991). In addition to these primary data, the Web of Science (Institute of Scientific Information, Thomson Scientific) bibliographic database was searched for all the published cytogenetic data available on the genus *Rhinella*.

Chromosomal analysis of the nine *Rhinella* populations revealed conservative karyotype features, beginning with the diploid number (2n = 22), which was consistent across all species (see Table 2; Figure 1). All karyotypes consist of six metacentric (pairs 1–3, 5, 8 and 9; Fig. 1) and five submetacentric pairs (pairs 4, 6, 7, 10 and 11; Fig.1). While relatively few *Rhinella* species have been analyzed cytogenetically (Table 2), the available data are remarkable for their uniformity, with only metacentric and submetacentric pairs being found in any species. Small differences in the number of metacentric and submetacentric pairs are found in some studies (Amaro-Ghilardi *et al.*, 2008; Baraquet *et al.*, 2011), however, it seems likely that this has been due to the application of different criteria for the classification of the chromosomes, rather than any real variation among species in their karyotypes.

Three different NOR-bearing chromosomes were identified in the present study. In *R. hoogmoedi* (Bertioga, SP), *Rhinella* sp. 1 (Bacabeira, MA), and *Rhinella* sp. 2 (Parque Viruá, RR), the silver impregnation method detected a NOR site in the subterminal region of the long arm of pair 10, while in *R. proboscidae* (Reserva Ducke, AM) and *Rhinella* cf. *margaritifera* (Laranjal do Jari, AP) the NOR was located in the subterminal region of the short arm of the homologs of pair 7 (Figure 2). In two species of the *R. marina* group, *Rhinella henseli*, and *Rhinella icterica*, from both Passo Fundo and Sertão (RS), NOR-bearing chromosomes were detected in the interstitial region of the short arm of the homologs of pair 7, in both sampled populations (Figure 2).

A similar degree of uniformity has been observed in inter-population cytogenetic studies of other *Rhinella* species. For example, considerable karyological uniformity has been found in the *R. icterica* populations from three

Table 1 - Number of *Rhinella* specimens analyzed and their localities in Brazil.

Species	Number of specimens analyzed	Collection locality	Geographical coordinates
R. hoogmoedi	2 M + 3 F	Bertioga, SP	23º48'23.15"S; 46º03'32.23"O
R. proboscidae	2 M + 1 F	Reserva Florestal Adolpho Ducke, Manaus, AM	2º57'48.04"S; 59º55'22.20"O
Rhinella sp. 1	2 M + 1 F	Bacabeira, MA	2º56'28.32"S; 44º21'41.35"O
Rhinella sp. 2	5 M	Parque Viruá, RR	1º17'26.82"N; 61º09'09.20"O
Rhinella cf. *margaritiffera*	5 M + 1 F	Laranjal do Jari, AP	1º05'39.49"N; 53º13'04.98"O
R. henseli	2 M + 5 F	FLONA,sPasso Fundo, RS	28º18'59.10"S; 52º11'26.42'O
R. henseli	3 M + 3 F	Sertão, RS	28º02'33.46"S; 52º12'58.56"O
R. icterica	3 M + 2 F	FLONA, Passo Fundo, RS	28º18'59.10"S; 52º11'26.42'O
R. icterica	8 M	Sertão, RS	28º02'33.46"S;52º12'58.56"O

M: male; F: female

Table 2 - Detailed cytogenetic data available for species of the *Rhinella* genus.

Species	2n	FN	NOR-bearing chromosome	Reference
R. margaritifera group				
R. hoogmoedi	22	44	St 10(p)	Present study
R. margaritifera	22	44	St 10(q)	Baldissera *et al.* (1999)
Rhinella sp. 1	22	44	St 10(q)	Present study
Rhinella sp. 2	22	44	St 10(q)	Present study
Rhinella cf.margaritifera	22	44	St 7(p)	Present study
R. proboscidea	22	44	St 7(p)	Present study
R. crucifer group				
R. crucifer	22	44	St 7(p)	Kasahara *et al*., (1996) Silva (2010)
R. ornata	22	44	St 7(p)	Silva (2010)
R. pombali	22	44	St 7(p)	Silva (2010)
R. marina group				
R. arenarum	22	44	Int 7 (p)	Baldissera *et al.* (1999) Baraqueti *et al.* (2011)
R. icterica	22	44	Int 7 (p)	Kasahara *et al.* (1996) Baldissera *et al.* (1999) Azevedo *et al.* (2003) Present study
R. marina	22	44	Int 7 (p)	Baldissera *et al,* (1999)
R. schneideri	22	44	Int 7 (p)	Kasahara *et al,* (1996) Azevedo *et al.* (2003) Amaro-Ghilardi *et al.* (2008) Baraquet *et al.,* (2011)
R. rubescens	22	44	Int 7 (p)	Amaro-Ghilardi *et al.,* (2008)
R. jimi	22	44	Int 7 (p)	Amaro-Ghilardi *et al.* (2008)
R. achavali	22	44	Int 7 (p)	Kolenc *et al.* (2013)
R. henseli	22	44	Int 7 (p)	Present study
R. granulosa group				
R. granulosa	22	44	Ter 5(q)	Baldissera *et al.* (1999)
R. pygmaea	22	44	Ter 5(q)	Baldissera *et al.* (1999)
R. fernandezae	22	44	n.e.	Baraquet *et al.* (2011)
R. spinulosa group	n.e.	n.e.	n.e.	n.e.
R. veraguensis group	n.e.	n.e.	n.e.	n.e.
R. festae group	n.e.	n.e.	n.e.	n.e.
R. achalensis (not assigned to any group)	22	44	Int10(p)*	Baraquet *et al.* (2011)

q = long arm; p = short arm; St= subtelocentric; int =interstitial position; per = pericentromeric position; ter = terminal position in the chromosome; n.e. = not examined; *only secondary constriction information.

sites in the Brazilian state of São Paulo (Kasahara *et al.*, 1996; Baldissera *et al.*, 1999; Azevedo *et al.*, 2003) and in the two populations from Rio Grande do Sul (present study). The *cururu* toad, *R. icterica*, occurs in southern Brazil, ranging from Rio Grande do Sul in the South to Bahia in the Northeast, as well as Minas Gerais and Goiás, eastern Paraguay, and extending westward to Missiones in Argentina. It is considered a cosmopolitan species, occurring in different habitats and altitudes, including within the Atlantic Forest biome. The cytogenetic data available on the populations of *R. schneideri*, another species distributed widely in South America, indicate a similar pattern of uniformity (Kasahara *et al.*, 1996; Azevedo *et al.*, 2003; Amaro-Ghilardi *et al.*, 2007; Baraquet *et al.*, 2011).

The overview of the data available for the different *Rhinella* species (Table 2) indicates that the position of the NOR site is relatively stable within phenotypic groups in the genus. The *Rhinella* species groups are determined based on phenotypic criteria (Frost, 2018), and while the monophyly of the *Rhinella granulosa* group has received support from molecular phylogenetic analyses (Pereyra *et al.*, 2016), this has not been confirmed in the others. Karyo-

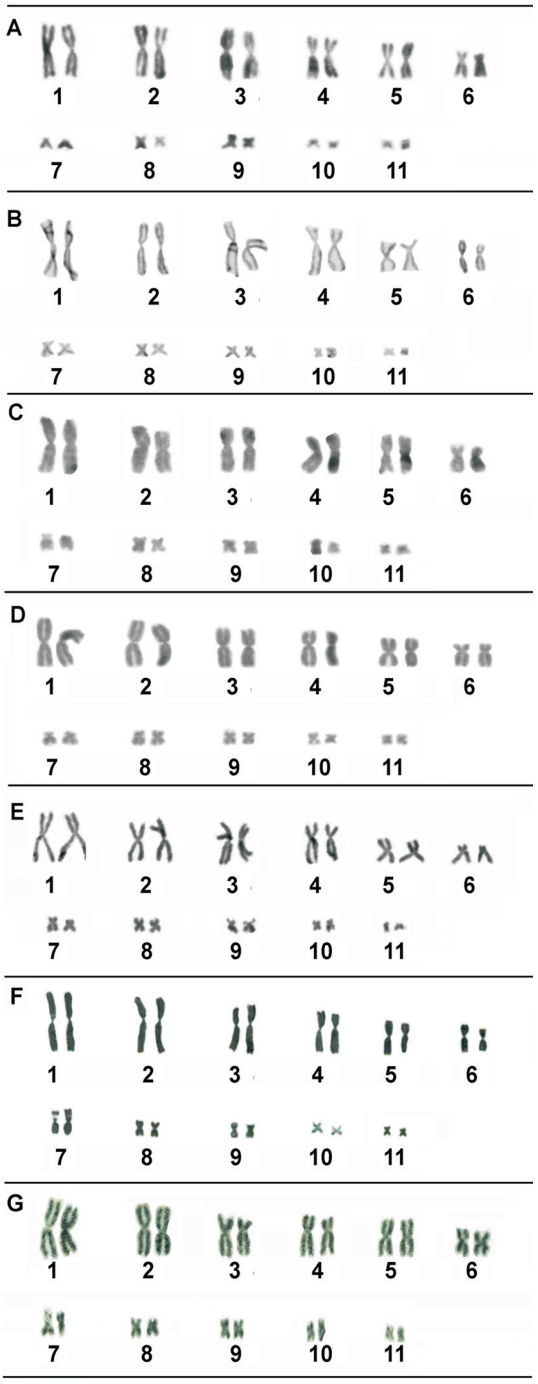

Figure 1 - Karyotypes of five species of the *Rhinella margaritifera* (A-E) and *Rhinella marina* (F-G) groups from Brazil, based on Giemsa staining: (A) *R. hoogmoedi*; (B) *R. proboscidae*; (C) *Rhinella* sp. 1; (D) *Rhinella* sp. 2 and (E) *Rhinella* cf. *margaritifera*; (F) *R. ictarica* from Sertão, RS; (G) *R. henseli,* from Sertão, RS.

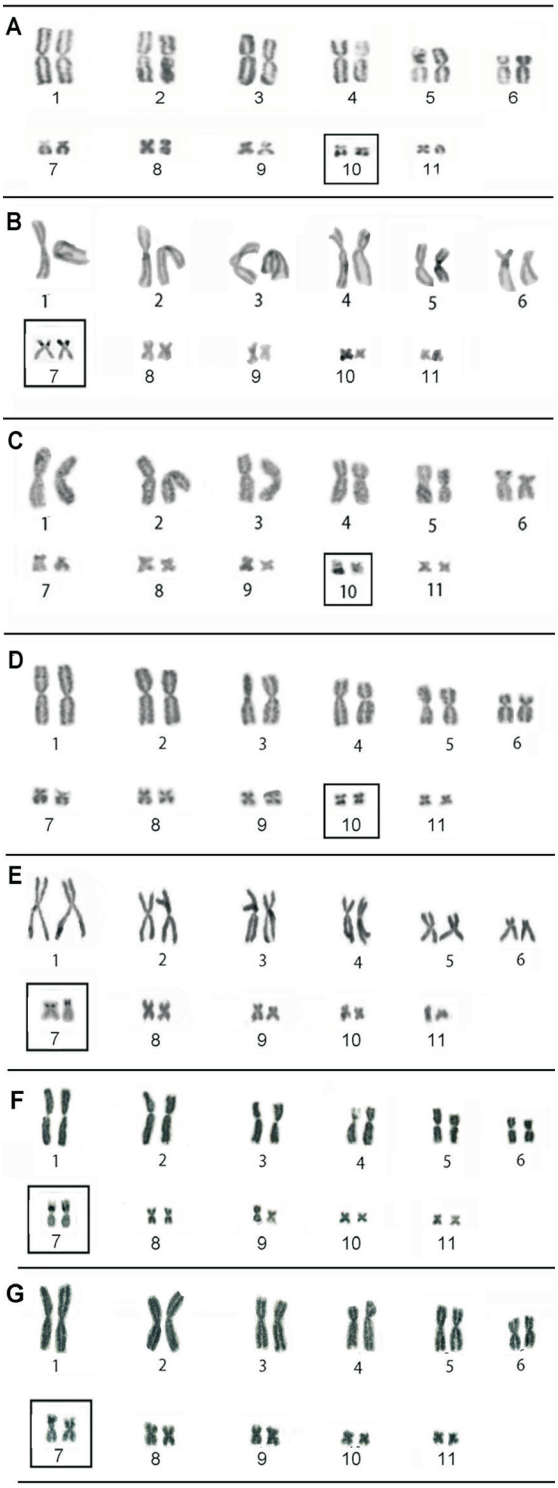

Figure 2 - Karyotypes of five species of the *Rhinella margaritifera* (A-E) and *Rhinella marina* (F-G) groups from Brazil stained by the Ag-NOR method: (A) *R. hoogmoedi*; (B) *R. proboscidae*; (C) *Rhinella* sp. 1; (D) *Rhinella* sp. 2, and (E) *Rhinella* cf. *margaritifera*; (F) *R. ictarica,* from Sertão, RS; (G) *R. henseli,* from Sertão, RS. The boxes indicate the NOR-bearing chromosomes.

type data are available for four of these species groups (Table 2). In all the species of the *Rhinella crucifer* group, the NOR is found in the subtelocentric region of pair 7p, whereas in the *R. marina* group, it is found in an interstitial region of the 7p, and in the *R. granulosa* group, it is located in the terminal region of the homologs of pair 5q. In the *R. margaritifera* group, by contrast, the NOR site is found in the subterminal region of the homologs of pair 10q or the interstitial region of pair 7p (Table 2).

As the karyotypic uniformity of the genus permits the inference of interspecific chromosome homologies, it seems reasonable to conclude that the evolutionary changes in the NOR-bearing chromosome may represent a putative synapomorphy in each species group. However, the confirmation of this hypothesis would require the analysis of a much larger dataset, for a more reliable evaluation of potential synapomorphies. If confirmed, the variation in the characteristics of the NOR-bearing chromosomes would provide valuable insights for the understanding of the phylogenetic relationships among the *Rhinella* species groups. In the *R. granulosa* group, for example, Pereyra *et al.* (2016) identified NORs in the homologs of pair 5 as an additional synapomorphy in this group. A similar scenario can be inferred for both the *R. crucifer* and *R. marina* groups, but not the *R. margaritifera* group, given the variation already observed in the NOR-bearing chromosomes of the different species of this group.

The *R. margaritifera* group is currently composed of 19 species (Vaz-Silva *et al.*, 2015), with the NOR being found in the homologs of pair 10 (long arm) in *R. hoogmoedi* (present study), and in *Rhinella* sp. 1 from Maranhão and *Rhinella* sp. 2 from Roraima. Baldissera *et al.* (1999) described the karyotype in specimens of the *R. margaritifera* group from Tucuruí, in the Brazilian state of Pará. In the present study, three populations (*Rhinella* sp. 1, *Rhinella* sp. 2, and *Rhinella* cf. *margaritifera*) were assigned to the *R. margaritifera* group based on morphological and biogeographical criteria, although it was not possible to determine the taxonomic status of these populations based on their karyotypes. Despite their morphological similarities, the *Rhinella* cf. *margaritifera* specimens from Laranjal do Jari can be distinguished from all the other populations assigned to the *R. margaritifera* group based on the NOR-bearing chromosome and by phenotypic features (Lima - personal observation), which may indicate the presence of a novel taxonomic entity, which requires further investigation.

The taxonomic status of the species of the *R. margaritifera* group remains uncertain, and phylogenetic inferences indicate the existence of a number of cryptic lineages, and a possible species complex within this group (Fouquet *et al.*, 2007a). This reinforces the need for a thorough taxonomic review of the arrangement of the *R. margaritifera* group in the Amazon region. Molecular approaches have been effectively applied to the recognition and description of many new *Rhinella* species (Fouquet *et al.*, 2007b; Moravec *et al.*, 2014), and it would almost certainly provide important insights into the delimitation of the species within the *R. margaritifera* group.

The species of the *Rhinella margaritifera* group are distributed in northern South America and the Central America forest domain, except for *R. hoogmoedi* (Caramaschi and Niemeyer, 2003), which inhabits the Atlantic Forest biome, and *R. scitulla*, *R. ocellata, R. sebbeni*, and *R. paraguayensis*, which occur in the Brazilian Cerrado savanna (Ávila *et al.*, 2010; Vaz-Silva *et al.*, 2015). However, the forest-dwelling species tend to present high levels of individual variation in morphological features, which limits the usefulness of these attributes for the discrimination of species. Santos *et al.* (2015) identified populations from western Ecuador and Panama, frequently assigned to *R. margaritifera* species, as *R. alata*, which has helped to resolve the confusing zoogeography of the *R. margaritifera* complex. However, the status of the populations of the *R. margaritifera* group from the east of the Andes remains unresolved, and the phylogenetic inferences of Fouquet *et al.* (2007a) indicated the potential existence of at least five distinct taxa identified as *R. margaritifera* in Brazil and French Guiana.

The *R. proboscidae* karyotype is described here for the first time, and it presents a NOR on the homologs of pair 7, a condition different from that of the other species of the *R. margaritifera* group. While the taxonomy and arrangement of the species in this group are complex (Fouquet *et al.*, 2007a), it has been diagnosed by the presence of an expansion of the posterior ramus of the pterygoid (Pramuk, 2006), a putative morphological synapomorphy that supports the *R. margaritifera* group. However, this putative phenotypic synapomorphy has not been formally tested. *Rhinella proboscidae* occurs along the Amazon River between Peru and Manaus, in Brazil, the locality sampled in the present study. The morphological characters of these populations support their inclusion in the *R. margaritifera* group. In contrast with the other species groups, however, in which the NOR-bearing chromosome represents a putative synapomorphy, two distinct scenarios are equally possible for the *R. margaritifera* group: (1) the NOR on pair 10 is a chromosomal synapomorphy in this group and the NOR on pair 7 of *R. proboscidae* represent a character reversion; or (2) the retention of an ancestral polymorphism. Unfortunately, the lack of a complete phylogenetic reconstruction that includes representatives of all the *Rhinella* groups hampers more conclusive inferences on the chromosomal evolution of this genus. In the specific case of the *R. margaritifera* group, a more systematic analysis of monophyly based on the investigation of specific molecular markers would likely provide decisive insights into the evolution of this group.

The conservative arrangement of the NOR-bearing chromosomes in the different *Rhinella* groups highlights the potential contribution of cytogenetic data for the identification of diagnostic synapomorphies in species groups or clades. A similar approach has been applied successfully in other amphibian groups (see Grant *et al.*, 2017; Targueta *et al.*, 2018). For example, comparative cytogenetics and the allocation of chromosomal characters (morphology and NOR sites) in a phylogenetic tree inferred from molecular markers allowed Cardozo *et al.* (2011) to identify three potential synapomorphies in the genus *Ololygon* (*Scinax*

catharinae clade – Duellman *et al.*, 2016). Lourenço *et al.* (2015) also identified the interstitial C-band in chromosome pair 5 as a synapomorphy of the *Physalaemus cuvieri* species group.

Another relevant feature of *Rhinella* genus is the high frequency of hybridization and introgression events (Azevedo *et al.*, 2003; Narvaes and Rodrigues 2009; Sequeira *et al.*, 2011; Guerra *et al.*, 2011; Pereyra *et al.*, 2015) in areas of sympatry, mainly in the *Rhinella marina* group. As chromosomal features may represent important pre- or post-zygotic barriers to reproduction, groups with a uniform karyotype, such as those found in *Rhinella*, may reflect the relaxation of any isolation mechanism, which would further contribute to the high frequency of hybridization events observed in this genus. For example, Azevedo *et al.* (2003) identified an intermediate form between *Rhinella icterica* and *Rhinella schneideri* in a sympatric zone, based on the banding patterns of seroproteins analyzed by electrophoresis, even though the intermediate form presented no modification of the karyotype in comparison with the parental species.

Overall, it is hoped that this review of the cytogenetic data available for the *Rhinella* species, together with the new karyotypes reported here, will contribute to the understanding of the mechanisms of evolutionary changes that led to the diversification of these toads. Despite the ample distribution of many forms, karyotypes are highly conserved in most cases.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

DPB conduced the experiments, conceived and designed the study and wrote; DYS, AS and NCF analyzed the data; APL and GVA collected specimens and helped to identify the specimens; KAC processed material of specimens; CSB and SMR designed the study and wrote the manuscript. All authors read and approved the final version.

References

Amaro-Ghilardi RC, Silva MJ, Rodrigues MT and Yonenaga-Yassuda Y (2007) Chromosomal studies in four species of genus *Chaunus* (Bufonidae, Anura): localization of telomeric and ribosomal sequences after fluorescence *in situ* hybridization (FISH). Genetica 134:159-168.

Ávila RW, Pansonato A and Strüssmann C (2010) A new species of the *Rhinella margaritifera* group (Anura: Bufonidae) from Brazilian Pantanal. Zootaxa 2339:57–68.

Azevedo MFC, Foresti, F, Ramos PRR and Jim J (2003) Comparative cytogenetic studies of *Bufo ictericus*, *B. paracnemis* (Amphibia, Anura) and an intermediate form in sympatry. Genet Mol Biol 26:289-294.

Baldissera FA, Batistic RF and Haddad CFB (1999) Cytotaxonomic considerations with the description of two new NOR locations for South American toads, genus *Bufo* (Anura: Bufonidae). Amphibia-Reptilia 20:413-420.

Baraquet M, Valetti JA, Salas N and Martino A (2011) Redescription of the karyotypes of five species of the family Bufonidae (Amphibia: Anura) from central area of Argentina. Biologia 66:543-547.

Caramaschi U and Niemeyer H (2003) Nova espécie do complexo de *Bufo margaritifer* (Laurenti, 1768) do Estado do Mato Grosso do Sul, Brasil (Amphibia, Anura, Bufonidae). Bol Mus Nac Sér Zool 501:1-16.

Cardozo DE, Leme DM, Bortoleto JF, Catroli GF, Baldo D, Faivovich J, Kolenc F, Silva APZ, Borteiro C, Haddad CFB *et al.* (2011) Karyotypic data on 28 species of *Scinax* (Amphibia: Anura: Hylidae): diversity and informative variation. Copeia 2:251-263.

Chaparro JC, Pramuk JB and Gluesenkamp AG (2007) A new species of arboreal *Rhinella* (Anura: Bufonidae) from cloud forest of southeastern Peru. Herpetologica 63:203-212.

Cuevas CC (2008) A new species of the genus *Alsodes* (Anura: Neobatrachia) from the Nothofagus forest, Coastal Range, Southern Chile, identified by its karyotype. Zootaxa 1771:43-53.

Cunningham MJ and Cherry MI (2004) Molecular systematics of African 20-chromosome toads (Anura: Bufonidae). Mol Phylogenet Evol 32:671-685.

Duellman WE, Marion AB and Hedges SB (2016) Phylogenetics, classification, and biogeography of the treefrogs (Amphibia: Anura: Arboranae). Zootaxa 4104:1-109.

Fávero ER, Veiga-Menoncello ACP, Rossa-Feres DC, Strüssmann C, Giaretta AA, Andrade GV, Colombo P and Recco-Pimentel SM (2011) Intrageneric karyotypic variation in *Pseudopaludicola* (Anura: Leiuperidae) and its taxonomic relatedness. Zool Stud 50:826-836.

Ferro JM, Cardozo DE, Suárez P, Boeris JM, Blasco-Zúniga A, Barbero G, Gomes A, Gazoni T, Costa W, Nagamachi CY *et al.* (2018) Chromosome evolution in Cophomantini (Amphibia, Anura, Hylinae). PLoS One 13:e0192861.

Fouquet A, Gilles A, Vences M, Marty C, Blanc M and Gemmell NJ (2007a) Underestimation of species richness in Neotropical frogs revealed by rDNA analyses. PLoS One 2:e1109.

Fouquet A, Vences M, Salducci MD, Meyer A, Marty C, Blanc M and Gilles A (2007b) Revealing cryptic diversity using molecular phylogenetics and phylogeography in frogs of the *Scinax ruber* and *Rhinella margaritifera* species groups. Mol Phylogenet Evol 43:567-682.

Funk WC, Caminer M and Ron SR (2012) High levels of cryptic species diversity uncovered in Amazonian frogs. Proc R Soc B Biol Sci 279:1806-1814.

Grant T, Rada, M, Anganoy-Criollo M, Batista A, Dias PH, Jeckel AM, Machado DJ and Rueda-Almonacid JV (2017) Phylogenetic systematics of dart poison frogs and their relatives revisited (Anura: Dendrobatoidea). South Am J Herpetol 12:1-90.

Green DM and Sessions SK (1991) Amphibian Cytogenetics and Evolution. Academic Press, San Diego, 456 p.

Guerra C, Baldo D, Rosset S, Borteiro C and Kolenc F (2011) Advertisement and release calls in Neotropical toads of the *Rhinella granulosa* group and evidence of natural hybridization between *R. bergi* and *R. major* (Anura: Bufonidae). Zootaxa 3092:26–42.

Howell WM and Black DA (1980) Controlled silver staining of nucleolar organizer regions with a protective colloidal developer: A 1 step method. Experientia 36:1014-1015.

Kasahara S, Silva APZ and Haddad CFB (1996) Chromossome banding in three species of Brazilian toads (Amphibia-Bufonidae). Braz J Genet 19:237-242.

King M and Rofe R (1976) Karyotypic variation in the Australian gecko *Phyllodactylus marmoratus* (Gray) (Gekkonidae, Reptilia). Chromosoma 54:75-87.

Kolenc F, Borteiro C, Cotichelli L, Baldo D, Debat CM and Candioti FV (2013) The tadpole and karyotype of *Rhinella achavali* (Anura: Bufonidae). J Herpetol 47:599-606.

Lourenço LB, Bacci Jr. M, Martins VG, Recco-Pimentel SM and Haddad CFB (2008) Molecular phylogeny and karyotype differentiation in *Paratelmatobius* and *Scythrophrys* (Anura, Leptodactylidae). Genetica 132:255-266.

Lourenço LB, Targueta CP, Baldo D, Nascimento J, Garcia PCA, Andrade GV, Haddad CFB and Recco-Pimentel SM (2015) Phylogeny of frogs from the genus *Physalaemus* (Anura, Leptodactylidae) inferred from mitochondrial and nuclear gene sequences. Mol Phylogenet Evol 92:204-216.

Moravec J, Lehr E, Cusi JC, Córdova JH and Gvodík V (2014) A new species of the *Rhinella margaritifera* species group (Anura, Bufonidae) from the montane forest of the Selva Central, Peru. ZooKeys 371:35–56.

Narvaes P and Rodrigues MT (2009) Taxonomic revision of *Rhinella granulosa* species group (Amphibia, Anura, Bufonidae), with a description of a new species. Arq Zool 40:1-73.

Pereyra MO, Baldo D, Blotto BL, Iglesias PP, Thomé MTC, Haddad CFB, Barrio—Amorós C, Ibáñez R and Faivovich J (2015) Phylogenetic relationships of toads of the *Rhinellagranulosa* group (Anura: Bufonidae): A molecular perspective with comments on hybridization and introgression. Cladistics 32:36-53.

Pramuk JB (2006) Phylogeny of South American Bufo (Anura: Bufonidae) inferred from combined evidence. Zool J Linn Soc 146:407–452.

Santos SP, Ibáñez R and Ron SR (2015) Systematics of the *Rhinella margaritifera* complex (Anura, Bufonidae) from western Ecuador and Panama with insights in the biogeography of *Rhinella alata*. Zookeys 501:109-145.

Schmid M (1978) Chromosome banding in Amphibia. II. Constitutive heterochromatin and nucleolus organizer regions in Ranidae, Microhylidae and Rhacophoridae. Chromosoma 68:131-148.

Sequeira F, Sodré D, Ferrand N, Bernardi JAR, Sampaio I, Schneider H and Vallinoto M (2011) Hybridization and massive mtDNA unidirectional introgression between the closely related Neotropical toads *Rhinella marina* and *R. schneideri* inferred from mtDNA and nuclear markers. BMC Evol Biol 11:264.

Silva DJS (2010) Análise citogenética e morfométrica em populações de *Rhinella pombali* (Baldissera Jr., Caramaschi e Haddad, 2004) e *Rhinella crucifer* (Wied- Neuwied, 1821) (Anura, Bufonidae). M. Sc. Thesis, Universidade Federal de Viçosa, Viçosa.

Suárez P, Cardozo D, Baldo D, Pereyra MO, Faivovich J, Orrico VGD, Catroli GF, Grabiele M, Bernarde PS, Nagamachi C *et al.* (2013) Chromosome evolution in *Dendropsophini* (Amphibia, Anura, Hylinae). Cytogenetics Genome Res 141:295-308.

Targueta CP, Rivera M and Lourenço LB (2012) Karyotypic differentiation via 2n reduction and a finding of a case of triploidy in anurans of the genus *Engystomops* (Anura, Leiuperidae). Genetica 139:1339-1347.

Targueta CP, Vittorazzi SE, Gatto KP, Bruschi DP, Veiga-Menoncello ACP, Recco-Pimentel SMR and Lourenço LB (2018) Anuran cytogenetics: An overview. In: Norris N and Miller C (eds) An Essential Guide to Cytogenetics. Nova Science Publishers, New York, pp 1-64.

Veiga-Menoncello ACP, Lourenço LB, Strussmann C, Rossa-Feres DC, Andrade GV, Giaretta A and Recco-Pimentel SM (2014) A phylogenetic analysis of *Pseudopaludicola* (Anura) providing evidence of progressive chromosome reduction. Zool Scripta, 43:261-272.

Vaz-Silva W, Maciel NM, Bastos RP and Pombal Jr JP (2015) Revealing two new species of the *Rhinella margaritifera* species group (Anura, Bufonidae): An enigmatic taxonomic group of Neotropical toads. Herpetologica, 71:212-222.

Internet resources

Frost DR (2019) Amphibian Species of the World: an Online Reference. American Museum of Natural History, New York, USA. http://research.amnh.org/herpetology/amphibia/index.html (April 27, 2019).

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