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Redescription of the karyotype of five species of the family Bufonidae (Amphibia: Anura) from central area of Argentina

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Abstract: In this study karyotypic features of the five species of the family Bufonidae from the central area of Argentina are described. The species are *Rhinella achalensis*, *Rhinella arenarum*, *Rhinella fernandezae*, *Rhinella schneideri* and *Melanophryniscus stelzneri*. The metaphases were obtained from intestinal and testis cells, using conventional techniques. Twenty metaphasic figures per individual were analyzed and the total length of each chromosome and the length of the four arms were measured. The obtained measurements were processed using Excel 2000 to obtain the average length of the arms p and q, the arm ratio, the centromeric index, the relative chromosome length and the relative arm length. All species showed karyotype $2n = 22$, and karyotype formula of 6 : 5. Pairs one to six were large, with a relative chromosome length between 18.64–7.59%; pairs seven to eleven were small, with a relative chromosome length between 7.18–2.42%. In all species the chromosome morphology was metacentric or submetacentric. Karyotype and ideograms were made for all species, based on morphometric parameters of the chromosome complement. Finally, discriminant analysis was used to separate the five species analyzed, with a highly significant classification rate of 80% and $P < 0.0001$. These results agree, in general, with those presented by other authors, however, in *M. stelzneri* detailed karyological studied have not been made so far, thus this work represents a significant contribution to the karyotypic decryption features of this species and the *Rhinellla* species from central area of Argentina.

Key words: *Rhinella*; cytogenetics; Argentina

Introduction

In general, amphibian anurans despite the large number of species and habitat diversity, show conserved morphological characteristics, which make the use of such features difficult in phylogenetic investigations (Hillis 1991). In Anura, there is a general trend towards supplementing morphological data with alternative data sets, including karyological data, allozyme patterns and mitochondrial gene sequences, all of which have provided new insights to the taxonomy and phylogeny of this group (Aguiar et al. 2004).

The morphological characteristics of chromosomes, such as number, shape, size, presence of secondary constrictions, which together comprise the karyotype, give new insight for chromosome analysis of individuals or populations of a particular species (Rodriguez Piazze 1995), allowing to re-evaluate the systematic of anurans, improving current ideas about their life histories (Morescalchi 1990). Numerous studies about chromosome analyses have provided information for studies in taxonomy and phylogeny, and are also used as a fundamental characteristic in species identification (Busin et al. 2001; Aguiar et al.

2002, 2004; Busin et al. 2006, 2008; Lourenço et al. 2008).

The family Bufonidae (Gray, 1825) is one of the anurans taxa with worldwide distribution, except for Australia, Madagascar and Oceania (Morescalchi 1973; Baldissera et al. 1999; Frost 2010). According to Frost (2010) the genus *Rhinella* comprises 86 species, while in *Melanophryniscus* 25 species have been recognized. In Argentina, Bufonidae are represented by the genera *Rhinella* (Fitzinger, 1826) and *Melanophryniscus* (Gallardo, 1961) (Manzano et al. 2004).

Rhinella is one of the genera of Anura that has been most studied karyologically (Morescalchi 1973). In about 50% species conventional cytogenetic studies have been carried out, and only 20% studies have been performed using chromosome banding techniques (Rodriguez Piazze 1995; Córdova 1999). The karyotypes are also known for other species of the family Bufonidae, such as *Melanophryniscus* (Morescalchi 1973).

This study was carried out in a Bufonidae population that has not previously been described from the central area of Argentina and we redescribe and compare karyotypic characteristics of the species *Rhinella achalensis* (Cei, 1972), *Rhinella arenarum*

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Fig. 1. Karyotype of five Bufonidae species: A – *Rhinella achalensis;* B – *Rhinella arenarum*; C – *Rhinella fernandezae*; D – *Rhinella schneider*i; E – *Melanophryniscus stelzneri.*

(Hensel, 1867), *Rhinella fernandezae* (Gallardo, 1957), *Rhinella schneideri* (Werner, 1894) and *Melanophryniscus stelzneri* (Weyenbergh, 1875).

Material and methods

Cytogenetic analyses were carried out on ten individuals of five species of the family Bufonidae collected from different regions of Córdoba province: 2 ♂♂ *R. achalensis* from La Posta, Pampa de Achala, San Alberto Department (31°36′ S, 64°52′ W); 2 ♂♂ *R. arenarum* and 1 ♂ *R. fernandezae* from Alejandro Roca, Juárez Celman Department (33°21′ S, 63°42′ W); 2 ♂♂ *Rhinella schneideri* from Lucio V. Mansilla, Tulumba Department (29°48′ S, 64°43′ W) and 3 ♂♂ *Melanophryniscus stelzneri* from Achiras, Río Cuarto Department (33°10′ S, 64°59′ W).

Each specimen was injected intraperitoneally with 0.1 ml/10 g of body weight of a 0.3% colchicine solution. Eight hours later, animals were sacrificed using 0.1% tricaine metasulfonate (MS-222). Chromosomes were obtained from intestinal and testicular cells after treatment with 1% sodium citrate, fixed in a 3 : 1 solution of methanol : acetic acid. The material was stained with 10% Giemsa in phosphate buffer 6.8 pH for 10 minutes. All these techniques were performed according to Schmid (1978a, b); Schmid et al. (1979); Salas (2006) and Salas & Martino (2007).

Chromosomes were visualized using a Zeiss Axiophot-Axiolab and photographed using Axiocam HRc Zeiss. We analyzed 20 metaphasic figures per individual. On the metaphases the total length of each chromosome and the length of the four arms were measured, using image analysis by Adobe® Photoshop® 9.0.

The data obtained were processed using Microsoft Excel® 2000, and the length of the arms p and q, centromeric index, the arm ratio, the relative chromosome length, and the relative arm length were calculated using the following formulas:

Average length of the arms p and q: $q = (q^1 + q^2)/2$
$p = (p^1 + p^2)/2$

Centromeric index (i): $i = $ (length of the short arms of chromosome (p) / total length total of chromosome ($p + q$)) $\times 100$

Arm ratio (r): $r = q/p$

Relative chromosome length (rl): $rl = $ (total length of chromosome / $\sum$ of the length of the chromosome of the haploid joint) $\times 100$

Relative arm length (rlq or rlp): rlq or $rlp = $ (length of the arms (q or p) / $\sum$ of the length of the chromosome of the haploid joint) $\times 100$

The data were processed to get the average for species. The ideograms were elaborated using these morphometrical data, which was necessary because in the karyotypes the differences between chromosomes are not observable to the naked eye.

The chromosome morphology was established according to Levan et al. (1964) and Aiassa et al. (2001): metacentric (M) (r between 1–1.5; i between 50–40), submetacentric (SM) (r between 1.51–7; i between 39.9–12.5) and acrocentric or telocentric (A–T) ($r > 7.1$; i between 12.4–0). The location of the secondary constriction was determined when possible.

Also discriminant analysis with all karyometric variables was performed to detect interspecific differences using the program Statgraphics Plus 5.0.

Results

The five species studied have the same chromosome number, $2n = 22$ (Fig. 1), with a fundamental number NF = 44. The eleven chromosomal pairs can be classified into two groups. The first six chromosomes comprise a group of large chromosomes, with a relative chromosome length between 18.64% for *M. stelzneri* and

Table 1. Morphometric parameters of the karyotypes for elaboration of the ideogram of five Bufonidae species studied.

P	*R. achalensis*				*R. arenarum*				*R. fernandezae*				*R. schneideri*				*M. stelzneri*			
	rl	*r*	*i*	T	*rl*	*r*	*i*	T	*rl*	*r*	*i*	T	*rl*	*r*	*i*	T	*rl*	*r*	*i*	T
1	16.95	1.03	50.01	M	16.46	1.09	47.78	M	18.00	1.28	43.97	M	15.80	1.20	45.50	M	18.64	1.30	43.47	M
2	16.70	1.39	41.83	M	15.91	1.42	41.23	M	16.47	1.33	43.21	M	15.65	1.43	41.15	M	16.11	1.32	43.12	M
3	12.96	1.43	41.29	M	12.83	1.36	41.43	M	13.80	1.28	44.05	M	12.81	1.53	39.52	SM	13.34	1.38	41.97	M
4	12.35	1.88	34.81	SM	11.36	1.74	37.69	SM	11.33	1.42	41.66	M	12.03	2.02	33.16	SM	11.62	1.75	36.52	SM
5	10.89	1.10	47.64	M	10.86	1.21	45.09	M	10.20	1.37	42.70	M	11.45	1.21	45.25	M	10.04	1.48	40.53	M
6	7.59	1.57	38.90	SM	7.97	1.17	47.74	M	8.93	1.35	42.67	M	8.70	1.47	40.76	M	7.75	1.29	43.77	M
7	5.20	1.35	42.71	M	7.18	1.24	45.89	M	5.07	2.21	31.23	SM	6.32	1.59	38.79	SM	4.83	1.68	38.96	SM
8	4.97	1.23	45.04	M	5.24	1.31	42.50	M	4.67	1.50	40.00	M	5.98	1.38	42.60	M	4.73	1.41	41.63	M
9	4.69	1.99	33.90	SM	4.93	1.31	43.78	M	4.67	1.58	38.87	SM	5.25	1.24	44.71	M	4.47	1.69	38.37	SM
10	3.84	1.05	48.91	M	3.68	1.22	44.75	M	3.47	1.29	43.92	M	3.38	1.18	45.86	M	4.42	1.49	40.39	M
11	3.86	1.00	49.53	M	3.27	1.63	39.95	SM	3.40	1.24	44.58	M	2.42	1.20	45.63	M	4.05	1.51	40.21	M

Explanations: P – pair*; rl* – chromosomal relative length; *r* – the arms ratio; *i* – centromeric index; T – Type, M – metacentric, SM – submetacentric.
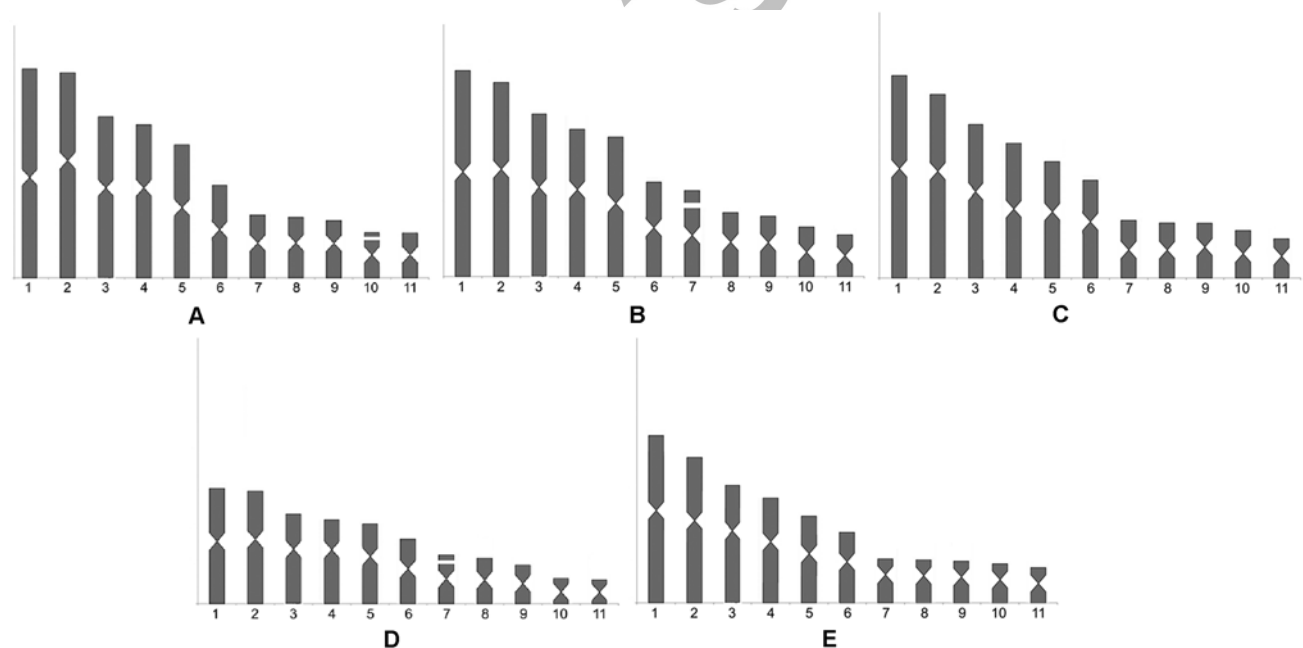

Fig. 2. Ideograms based on the morphometrical data of Table 1 for: A – *Rhinella achalensis;* B – *Rhinella arenarum*; C – *Rhinella fernandezae*; D – *Rhinella schneideri*; E – *Melanophryniscus stelzneri.*

7.59% for *R. achalensis*; and the remaining five chromosome pairs comprise a group of small chromosomes with a relative chromosome length between 7.18% for *R. arenarum* and 2.42% for *R. schneideri* (karyotype formula 6:5).

The chromosome morphology was always metacentric or submetacentric in the five species studied. The ideograms were elaborated using the length of the chromosomes, the arm ratio, relative chromosome length, relative arm length and centromeric index (Table 1).

Rhinella achalensis
In the six large pairs, 1–3 and 5 were metacentric, whereas the pairs 4 and 6 were submetacentric; in the five small pairs, 7–8 and 10–11 were metacentric, while pair 9 was submetacentric (Table 1). In the karyotype of *R. achalensis* we noted the presence of a secondary constriction on the short arm of chromosome 10 (Fig. 2A).

Rhinella arenarum
Chromosomal large pairs 1–6 were metacentric, except for pair 4 with submetacentric morphology. The small pairs 7–10 were metacentric, except for pair 11 with submetacentric morphology (Table 1, Fig. 2B).

For this species, a secondary constriction on the short arm of chromosome 7 was detected.

Rhinella fernandezae
The karyotype of *Rhinella fernandezae* consisted of six large pairs of metacentric chromosomes, whereas in the small pairs, there were only two submetacentric chromosomes (7 and 9) and the remaining were all metacentric (Table 1, Fig. 2C).

Rhinella schneideri
In the group of large chromosomes, pairs 3 and 4 were of submetacentric morphology, and pairs 1–2 and 5–6 were of metacentric morphology, whereas in the five

small chromosomes only pair 7 was submetacentric, and pairs 8–11 were metacentric (Table 1, Fig. 2D). Also in *R. schneideri* a secondary constriction in the short arm of chromosome 7 was detected.

Melanophryniscus stelzneri

The species had six pairs of large chromosomes, only pair 4 was metacentric and pairs 1–3, 5 and 6 were metacentric, whereas in five small pairs, the pairs 7 and 9 were of submetacentric morphology, and the pairs 8, 10 and 11 were of metacentric morphology (Table 1, Fig. 2E).

Discriminant analysis showed four functions, the first with an Eigenvalue of 4.30 explained 81.16% of the observed total variation. The discriminant function was highly significant ($P < 0.0001$). The canonical correlation of 0.90 (close to 1) indicated that the function had a high weight, while the Wilks Lambda of 0.085 (close to 0) indicated that the two selected variables (length of the chromosome and rl) were good variables to discriminate species.

The percentage of cases correctly classified was of 80%: *R. achalensis* 81.82%, *R. arenarum* 63.64%, *R. fernandezae* 90.91%, *R. schneideri* 72.73% and *M. stelzneri* 90.91% (Fig. 3).

Discussion and conclusion

The diploid chromosome number ($2n = 22$) of the five *Rhinella* species analyzed here agree with those presented by other authors (Beçak 1968; Morescalchi 1973; Duellman & Trueb 1994; King 1990; Azevedo et al. 2003; Amaro-Ghilardi et al. 2008).

For the five species we obtained the same karyotype formula (6 : 5), all metacentric or submetacentric coinciding with King's (1990) findings for species of *Atelopus*, *Melanophryniscus*, *Nectophryne*, *Nectophrynoides*, *Pedostibes* and species of European, Asian and American *Rhinella*. Morescalchi (1973) in his description of the chromosomes of the family Bufonidae also indicated that there are morphological differences in the 22 chromosomes of *Bufo* (*Rhinella*), but they usually have six large pairs and five small pairs, all metacentric and submetacentric, except one or two pairs of subtelocentric among the small chromosomes (NF = 44), and *Melanophryniscus* chromosomes have been of the same karyotype formula.

For *R. achalensis*, our results agree with those proposed by Rahn (1982), with respect to diploid number $2n = 22$. However, the cited author, based on the relative chromosome length, ordered the chromosomes in three groups: pairs 1–5 of large size, pair 6 of medium size and pairs 7–11 of small size, in contrast to the two groups determined in this work. According to Rahn (1982) only chromosome pairs 4 and 7 are submetacentric, and the others chromosomes are metacentric, whereas in this study the morphology was submetacentric for pair four, six and nine.

Rhinella arenarum and *R. schneideri*, according to Rahn (1982) and Baldissera et al. (1999), represent
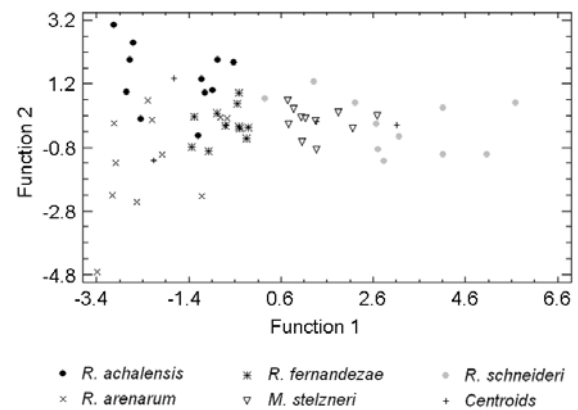

Fig. 3. Canonical functions 1 and 2 from discriminant analysis performed on karyometric variables for five Bufonidae species.

diploid number $2n = 22$, arranged in the same three groups as in this study. In addition, Baldissera et al. (1999) identified chromosomes 3, 4 and 6 as submetacentric, and the rest of the chromosomes as metacentric. In this study, the results differed for *R. arenarum* as submetacentric chromosomes were the pairs 4 and 11.

Baldissera et al. (1999) observed also secondary constrictions in the interstitial region of the short arm of chromosome 7 in *R. schneideri*. We observed the same secondary constriction in the same pair for *R. schneideri*, and also for *R. arenarum*. In fact, according to Baldissera et al. (1999), the secondary constrictions in the interstitial region of the short arm in chromosome 7 are characteristic of the South American *Bufo* (*Rhinella*) species. However, in *R. achalensis* secondary constriction was observed in the short arm of chromosome 10, as shown by Rhan (1982).

In this study, the large chromosomes of *R. schneideri*, pairs 3–4 are submetacentric and in the small chromosomes only pair 7 shows this morphology. The remaining pairs have metacentric morphology. These results differ from those presented by Amaro-Ghilardi et al. (2008) who observed that pairs 2–4 and 6 have submetacentric morphology and the rest of pairs are metacentric. Baldissera et al. (1999) and Beçak (1968) only described the submetacentric morphology for pairs 3–4 and 6. The results of relative chromosome length, centromeric index and arm ratio presented by Beçak (1968) are partially congruent with the observations presented in this paper.

Finally, our results on the relation to diploid number ($2n = 22$) and the 6 : 5 chromosomal formula and chromosome morphology (particularly metacentric pairs 1–3, 5–6, 8, 10–11) of *Melanophryniscus stelzneri* is in full agreement with Morescalchi (1973) and King (1990). However, detailed karyological studied of *M. stelzneri* have not been made so far, thus our work is a significant contribution to the description of karyotypic features of this species and the *Rhinellla* species from the central area of Argentina.

Acknowledgements

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