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A Cytotaxonomic Survey of the Genus *Melanophryniscus* Gallardo, 1961 (Anura: Bufonidae)

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Abstract.—We present a cytogenetic survey of the basal bufonid genus *Melanophryniscus* that covered 14 of the 25 species currently recognized, representing the three phenetic species groups: *M. moreirae*, *M. stelzneri*, and *M. tumifrons*. All species presented a diploid chromosome complement constituted by 11 bi-armed chromosome pairs ($2n = 2x = 22$; $FN = 44$). Some remarkable differences were observed between species groups: chromosome pair 4 was metacentric in species of the *M. tumifrons* group (also with a distinctive C-positive block) but submetacentric in the *M. stelzneri* group and *M. sanmartini* (*M. moreirae* group); pair 5 was submetacentric in *M. sanmartini* and metacentric in the rest. Chromosome secondary constrictions and silver-stained nucleolar organizer regions were located either in pair 5, 7, or 8 in the *M. tumifrons* group, *M. sanmartini* and *M. krauczuki* (*M. stelzneri* group), and *M. stelzneri* group, respectively; and pair 7 was relatively larger in *M. sanmartini* and *M. krauczuki*. Studied cytogenetic characters support the *M. tumifrons* group and suggest a close relationship between *M. krauczuki* and *M. sanmartini*. These results call for a reassessment of species relations within *Melanophryniscus* under an inclusive phylogenetic study.

The genus *Melanophryniscus* is composed of small species of toads distributed in central eastern South America, with 25 species being currently recognized (Frost, 2010). These toads are the more basal genus of Bufonidae (Frost et al., 2006) and are considered a monophyletic taxon due to several synapomorphies (Graybeal and Cannatella, 1995; Larson et al., 2003; Daly et al., 2008). However, to date there are no phylogenetic hypotheses regarding species relationships. Three phenetic groups are currently recognized according to Cruz and Caramaschi (2003). The *M. stelzneri* and *M. tumifrons* groups are characterized by a dorsal coloration pattern with contrasting spots or blotches, and a noticeable frontal glandular swelling, respectively, whereas the *M. moreirae* group includes some species that lack the frontal swelling and the conspicuous coloration pattern.

Cytogenetic data about basal bufonids is scarce, and very fragmentary for *Melanophryniscus*, for which there were no previous inclusive studies addressing banding patterns and location of nucleolar organizer regions (NORs). The cytogenetic data available for *Melanophryniscus* include the karyotype description of only a small number of species. The first published karyological studies are those of Sáez (1937, 1939), who based his observations on histological sections of testes obtained from a species of the *M. stelzneri* group (mentioned as *Atelopus stelzneri*). Currently, it is not possible to assign this material to any species because the locality of origin of studied specimens was not reported. Sáez described a karyotype comprising 22 chromosomes, with six large and five small chromosome pairs. Morescalchi and Gargiulo (1968) described the karyotype of a species of the *M. stelzneri* group (also as *A. stelzneri*), with 22 bi-armed chromosomes and reported distal secondary constrictions in the long arm of pair 11. Beçak et al. (1970) studied a male specimen of *M. moreirae* from São Paulo state, Brazil, and its karyotype consisted of 11 somatic chromosome pairs. The fourth and fifth pairs were submetacentric, whereas the rest were metacentric. Based on chromosome number and morphology, these early observations and those of de Lucca et al. (1974) on *M. moreirae*, suggested a close relationship between the bufonids and the genus *Melanophryniscus*, which had been placed formerly outside the Bufonidae.

More recently, Morand and Hernando (2002) described the karyotype of *M. cupreuscapularis* and *M. klappenbachi* and reported that NORs were present in the long arms of pair 8, terminal in *M. klappenbachi* but subterminal in *M. cupreuscapularis*. Soon afterward, Baldo and Basso (2004) described the karyotype of a male specimen of *M. krauczuki*, in which a secondary constriction was present in only one chromosome of pair 11 and also observed 11 bivalents in the meiotic prophase.

The purpose of this work is to characterize the karyotypes of 14 species of *Melanophryniscus* from Argentina, Brazil, Paraguay, and Uruguay, by using conventional and differential staining methods (C-banding and silver staining of NORs [Ag-NOR]) to gain insight on the cytotaxonomy of this genus.

Materials and Methods

Karyotypes were obtained from cell preparations of intestines and testes after in vivo colchicine treatment, following Schmid (1978, 1980). Chromosome number and morphology were recorded on conventional preparations stained with Giemsa solution (10%) and C-banding and Ag-NOR impregnation were performed after Sumner (1972) and Howell and Black (1980), respectively. We used x (basic chromosome number), n (gametic chromosome number), $2n$ (somatic chromosome number), and FN (fundamental number, or number of chromosomal arms) as suggested by White (1954). Morphometric measurements of chromosomes were made using Micromeasure version 3.3 software (Reeves and Tear, 2000). The relative length (*RL*) and centromeric ratio (*CR*) were calculated. We used the term ''homoeology'' to designate the homology present between chromosomes of different species that have descended from a common ancestral chromosome (Huskins, 1932). Terminology of chromosome classification according to their *CR* is that of Levan et al. (1964), as modified by Green and Sessions (1991, 2007): *m*, metacentric; *sm*,

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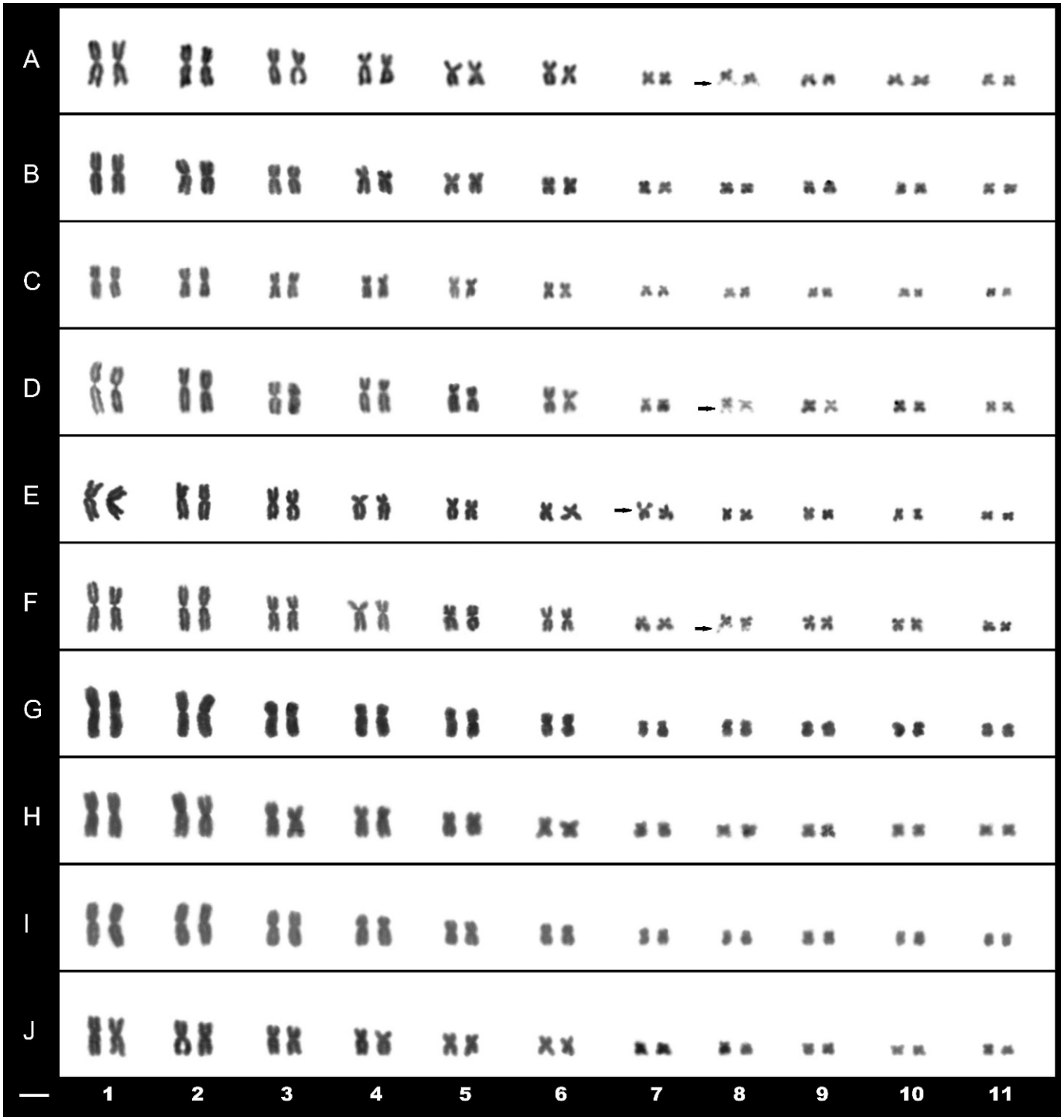

FIG. 1. Giemsa-stained karyotypes of 10 species of the *Melanophryniscus stelzneri* group: (A) *M. atroluteus*, (B) *M. cupreuscapularis*, (C) *M. fulvoguttatus*, (D) *M. klappenbachi*, (E) *M. krauczuki*, (F) *M. montevidensis*, (G) *M. paraguayensis*, (H) *M. rubriventris*, (I) *M. stelzneri stelzneri*, and (J) *M.* sp. aff. *montevidensis*. The arrows indicate the secondary constrictions. Scale = 10 μm.

submetacentric; *st*, subtelocentric; and *t*, telocentric. We studied 65 specimens of 14 *Melanophryniscus* species from Argentina, Brazil, Paraguay, and Uruguay (Appendix 1). Vouchers are stored in the personal collection of DB, at Museo de La Plata, Argentina (MLP DB); Célio F. B. Haddad amphibian collection, Departamento de Zoologia, Universidade Estadual Paulista ''Julio de Mesquita Filho,'' Rio Claro, São Paulo (CFBH), Brazil; herpetological collection of Laboratorio de Genética Evolutiva, Universidad Nacional de Misiones (LGE); herpetological collection of Instituto de Investigación Biológica del Paraguay (IIBP-H); and the herpetological collection of Museo Nacional de Historia Natural (MNHN), Montevideo, Uruguay.

RESULTS AND DISCUSSION

All studied specimens of *Melanophryniscus* presented a chromosome complement including 11 bi-armed chromosome pairs ($2n = 2x = 22$; $FN = 44$). This basic chromosome number, already reported for *Melanophryniscus*, is conserved among all studied members of 25 genera of Bufonidae (Kuramoto, 1990),

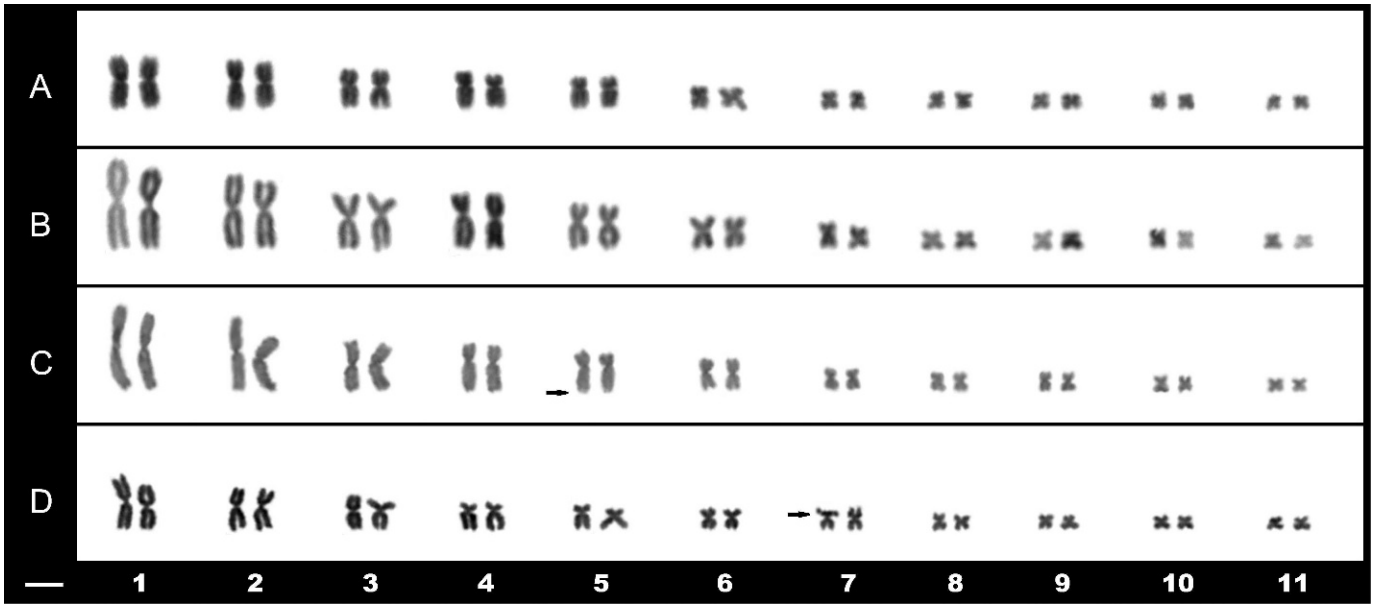

FIG. 2. Giemsa-stained karyotypes of three species of *Melanophryniscus tumifrons* group: *M. devincenzii* (A), *M. orejasmirandai* (B), and *M.* cf. *tumifrons* (C), and one species of the *Melanophryniscus moreirae* group, *M. sanmartini* (D). The arrows indicate the secondary constrictions. Scale = 10 μm.

except for some species of *Amietophrynus* that present $x = 10$ (Bogart, 1968; Bogart, 1972; Morescalchi and Gargiulo, 1968; Schmid, 1978). Many other Leptodactyliformes such as Leiuperidae (Tomatis et al., 2009), and some members of Leptodactylidae (subgenus *Leptodactylus*; Amaro et al., 2006), Cycloramphidae (*Alsodes nodosus*, *Odontophrynus*, *Proceratophrys*, and *Macrogenioglotus*; Kuramoto, 1990), and Aromobatidae (some species of *Allobates*; Veiga-Menoncello et al., 2003) share this same basic chromosome number. The reduction of a diploid chromosome number from 26 to 22 chromosomes has been proposed as a synapomorphy of Bufonidae (Grant et al., 2006). The fundamental chromosome number of 44 is also quite conserved in the 22-chromosome bufonids, the basal genus *Osornophryne* being the only exception, with an $FN = 42$ due to the presence of a pair of telocentric chromosomes (Ardila-Robayo et al., 1988).

In the karyotypes of *Melanophryniscus*, the chromosome pairs 1–6 were large, whereas pairs 7–11 were small, and no heteromorphic sex chromosomes were observed (Figs. 1–2; Table 1). The karyotypes of all nine species of the *M. stelzneri* group were quite similar, with only minor interspecific differences (Fig. 1). In most of these species pairs 4 and 8 were *sm*, with the rest being *m*. Exceptions are *M. atroluteus* and *M. paraguayensis* which pair 9 is *sm* and *M. s. stelzneri* with pairs 10 and 11 being *sm*; in addition, in *M. krauczuki* pair 8 is *m* and pair 7 is relatively larger than in the other studied species. In the *M. tumifrons* group, all chromosomes are *m*, except for the *sm* pair 8 of *M. orejasmirandai* (Fig. 2A–C). In the single studied

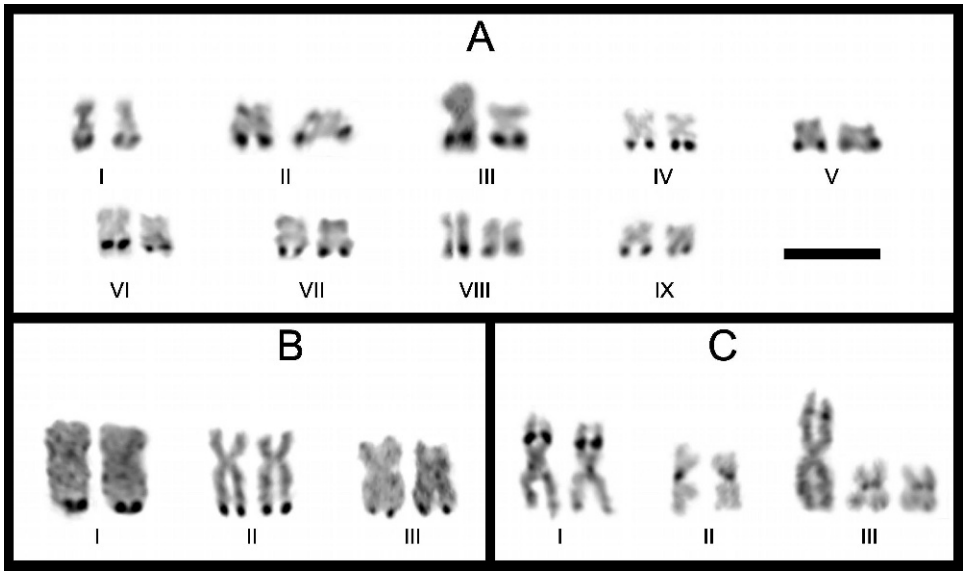

FIG. 3. NOR-bearing chromosome pairs stained by Ag-NOR. (A) Pair 8 of nine species of the *Melanophryniscus stelzneri* group: (I) *M. atroluteus*, (II) *M. cupreuscapularis*, (III) *M. fulvoguttatus*, (IV) *M. klappenbachi*, (V) *M. montevidensis*, (VI) *M. paraguayensis*, (VII) *M. rubriventris*, (VIII) *M. stelzneri stelzneri*, and (IX) *M.* sp. aff. *montevidensis*. (B) Pair 5 of three species of *M. tumifrons* group: (I) *M. devincenzii*, (II) *M. orejasmirandai*, and (III) *M.* cf. *tumifrons*. (C) Pair 7 of (I) *M. sanmartini*, (II) *M. krauczuki*, and (III) *M. krauczuki* (MLP DB 8760) with a single additional Ag-NOR in pair 3. Scale = 10 μm.

TABLE 1. Morphometric analysis of the chromosomes of species of *Melanophryniscus* analyzed in this study, including phenetic groups. RL = relative length; CR = centromeric ratio; CT = chromosome type; *m* = metacentric; *sm* = submetacentric.

Phenetic group	Species		Chromosome pair										
			1	2	3	4	5	6	7	8	9	10	11
M. stelzneri	*M. atroluteus*	RL	17.46	14.95	13.01	11.11	10.06	8.1	5.64	4.97	5.29	4.98	4.44
		CR	1.31 ± .13	1.33 ± .16	1.36 ± .16	1.89 ± .3	1.44 ± .15	1.3 ± .15	1.46 ± .19	2.23 ± .46	1.71 ± .41	1.51 ± .23	1.46 ± .29
		CT	*m*	*m*	*m*	*sm*	*m*	*m*	*m*	*sm*	*sm*	*m*	*m*
	M. cupreuscapularis	RL	16.57	15.23	12.48	11.28	9.57	8.2	5.93	5.51	5.23	5.08	4.92
		CR	1.25 ± .08	1.33 ± .07	1.37 ± .1	1.76 ± .19	1.46 ± .16	1.19 ± .13	1.33 ± .14	2.01 ± .28	1.63 ± .19	1.43 ± .16	1.44 ± .18
		CT	*m*	*m*	*m*	*sm*	*m*	*m*	*m*	*sm*	*m*	*m*	*m*
	M. fulvoguttatus	RL	17.61	15.72	12.38	10.94	10.03	7.9	5.61	5.39	4.71	5.2	4.52
		CR	1.3 ± .11	1.24 ± .11	1.37 ± .14	1.84 ± .23	1.62 ± .17	1.13 ± .09	1.46 ± .24	2.31 ± .52	1.39 ± .18	1.36 ± .28	1.25 ± .12
		CT	*m*	*m*	*m*	*sm*	*m*	*m*	*m*	*sm*	*m*	*m*	*m*
	M. klappenbachi	RL	17.59	15.63	12.83	10.79	9.77	8.32	5.88	5.07	5.14	4.72	4.26
		CR	1.33 ± .11	1.31 ± .19	1.46 ± .21	1.92 ± .19	1.33 ± .13	1.21 ± .14	1.39 ± .21	2.15 ± .28	1.63 ± .15	1.5 ± .18	1.36 ± .16
		CT	*m*	*m*	*m*	*sm*	*m*	*m*	*m*	*sm*	*m*	*m*	*m*
	M. krauczuki	RL	17.25	15.63	14.2	11.19	9.55	8.33	6.25	5.05	4.66	4.15	3.75
		CR	1.36 ± .12	1.25 ± .13	1.18 ± .09	2.33 ± .21	1.68 ± .19	1.48 ± .19	1.5 ± .15	1.54 ± .27	1.46 ± .31	1.32 ± .17	1.36 ± .27
		CT	*m*	*m*	*m*	*sm*	*m*	*m*	*m*	*m*	*m*	*m*	*m*
	M. montevidensis	RL	17.5	15.99	12.88	11.14	9.62	8.46	5.34	5.07	4.9	4.86	4.23
		CR	1.36 ± .18	1.32 ± .15	1.41 ± .18	1.98 ± .32	1.47 ± .21	1.36 ± .21	1.61 ± .36	2.3 ± .47	1.64 ± .33	1.62 ± .43	1.48 ± .21
		CT	*m*	*m*	*m*	*sm*	*m*	*m*	*m*	*sm*	*sm*	*m*	*m*
	M. paraguayensis	RL	16.93	15.22	11.88	11.33	9.46	8.04	5.93	5.64	5.5	5.11	4.96
		CR	1.35 ± .08	1.21 ± .12	1.48 ± .12	1.82 ± .22	1.59 ± .23	1.22 ± .09	1.54 ± .27	2.06 ± .47	1.72 ± .35	1.47 ± .31	1.51 ± .08
		CT	*m*	*m*	*m*	*sm*	*m*	*m*	*m*	*sm*	*m*	*m*	*m*
	M. rubriventris	RL	16.6	15.48	12.47	11.55	9.8	8.02	5.76	5.48	5.17	5.04	4.63
		CR	1.32 ± .14	1.39 ± .15	1.45 ± .11	1.77 ± .26	1.57 ± .17	1.27 ± .15	1.4 ± .21	2.17 ± .34	1.59 ± .23	1.49 ± .29	1.52 ± .38
		CT	*m*	*m*	*m*	*sm*	*m*	*m*	*m*	*sm*	*m*	*m*	*m*
	M. sp. aff. *montevidensis*	RL	16.16	15.75	13.55	11.51	8.87	7.94	6.10	5.30	4.94	5.25	4.72
		CR	1.28 ± .04	1.25 ± .15	1.43 ± .21	1.90 ± .16	1.36 ± .01	1.46 ± .24	1.34 ± .45	2.08 ± .03	1.39 ± .23	1.52 ± .38	1.4 ± .21
		CT	*m*	*m*	*m*	*sm*	*m*	*m*	*m*	*sm*	*m*	*m*	*m*
	M. s. stelzneri	RL	17.51	15.77	13.11	11.39	9.67	8.02	5.28	5.04	5.09	4.78	4.35
		CR	1.35 ± .10	1.26 ± .15	1.32 ± .10	1.78 ± .14	1.44 ± .12	1.29 ± .19	1.65 ± .28	2.13 ± .44	1.54 ± .34	2.09 ± .53	1.8 ± .52
		CT	*m*	*m*	*m*	*sm*	*m*	*m*	*m*	*sm*	*m*	*sm*	*sm*
M. moreirae	*M. moreirae*[a]	RL	20.16	17.57	12.84	11.72	10.41	7.34	6.16	5.36	4.92	4.34	4.16
		CR	1.33 ± .12	1.45 ± .16	1.37 ± .17	1.96 ± .40	1.89 ± .36	1.36 ± .19	1.46 ± .2	1.39 ± .13	1.31 ± .23	1.47 ± .17	1.22 ± .14
		CT	*m*	*m*	*m*	*sm*	*sm*	*m*	*m*	*m*	*m*	*m*	*m*
	M. sanmartini	RL	15.94	14.48	12.49	10.86	9.11	8.19	7.29	5.78	5.55	5.25	5.05
		CR	1.21 ± .19	1.35 ± .18	1.24 ± .15	2.52 ± .43	2.05 ± .51	1.50 ± .21	1.46 ± .24	1.53 ± .2	1.53 ± .25	1.48 ± .17	1.46 ± .19
		CT	*m*	*m*	*m*	*sm*	*sm*	*m*	*m*	*m*	*m*	*m*	*m*
M. tumiforns	*M. devincenzii*	RL	18.07	16.01	12.67	11.3	9.77	7.66	5.57	5.26	5.03	4.54	4.11
		CR	1.18 ± .12	1.24 ± .11	1.46 ± .15	1.65 ± .23	1.69 ± .27	1.31 ± .23	1.39 ± .14	1.44 ± .24	1.46 ± .22	1.35 ± .23	1.37 ± .22
		CT	*m*	*m*	*m*	*m*	*m*	*m*	*m*	*m*	*m*	*m*	*m*
	M. orejasmirandai	RL	18.38	15.71	12.59	12.11	9.61	7.74	5.48	5.04	5.02	4.27	4.05
		CR	1.26 ± .12	1.22 ± .08	1.45 ± .15	1.34 ± .18	1.49 ± .19	1.29 ± .18	1.3 ± .17	1.99 ± .34	1.3 ± .19	1.43 ± .22	1.37 ± .21
		CT	*m*	*m*	*m*	*m*	*m*	*m*	*m*	*sm*	*m*	*m*	*m*
	M. cf. *tumifrons*	RL	17.92	16.01	12.71	11.05	9.51	7.42	6.04	5.57	5.12	4.6	4.04
		CR	1.23 ± .11	1.27 ± .16	1.3 ± .2	1.65 ± .23	1.55 ± .25	1.33 ± .23	1.41 ± .3	1.45 ± .35	1.45 ± .25	1.35 ± .24	1.21 ± .12
		CT	*m*	*m*	*m*	*m*	*m*	*m*	*m*	*m*	*m*	*m*	*m*

[a] Data from published karyotypes by Beçak et al. (1970) and Lucca et al. (1974).

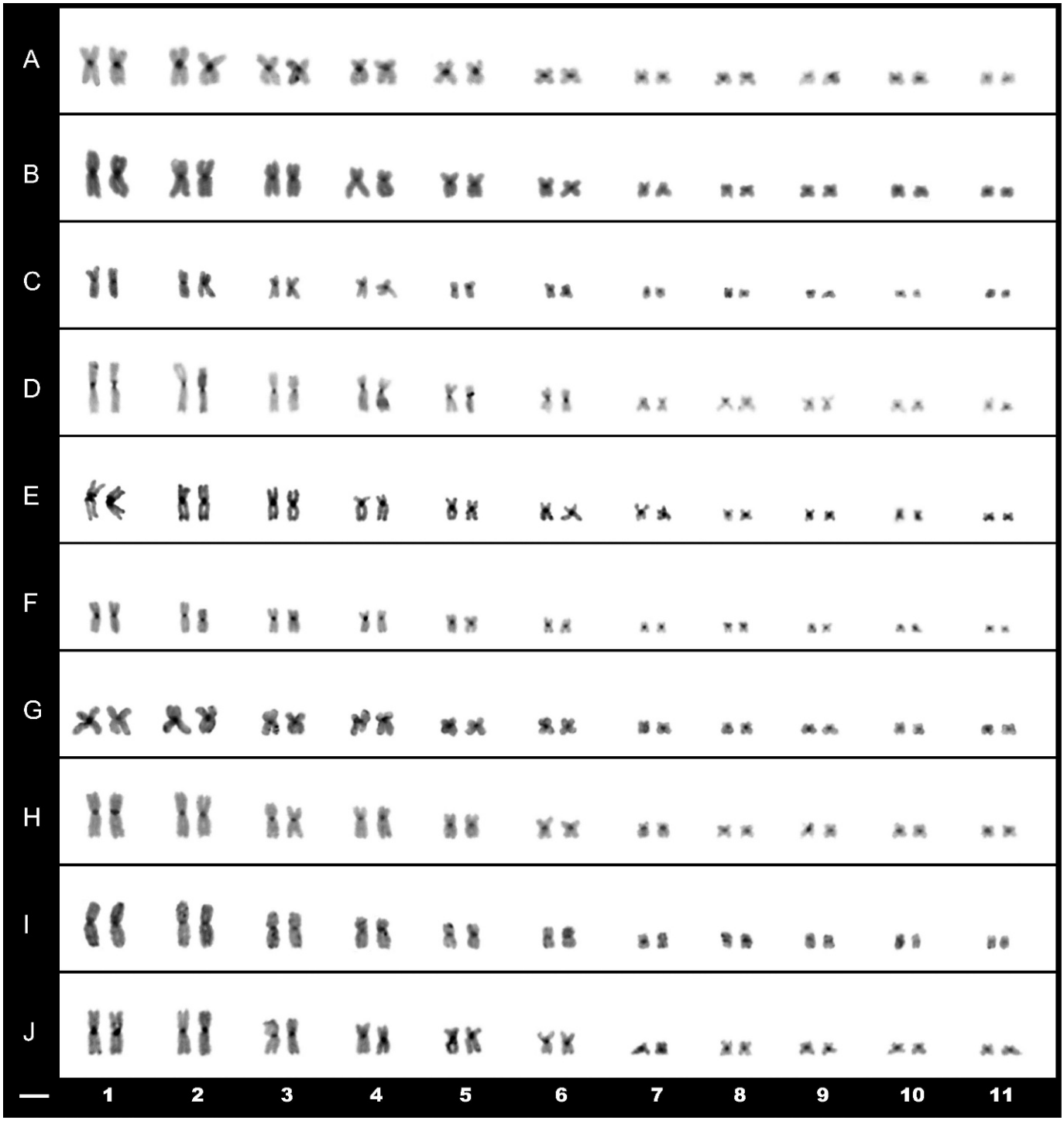

FIG. 4. C-banded karyotypes of 10 species of the *Melanophryniscus stelzneri* group: (A) *M. atroluteus*, (B) *M. cupreuscapularis*, (C) *M. fulvoguttatus*, (D) *M. klappenbachi*, (E) *M. krauczuki*, (F) *M. montevidensis*, (G) *M. paraguayensis*, (H) *M. rubriventris*, (I) *M. stelzneri stelzneri*, and (J) *M.* sp. aff. *montevidensis*. Scale = 10 μm.

species of the *M. moreirae* group, *M. sanmartini*, chromosomes are *m* except for the *sm* pairs 3 and 4 (Fig. 2D).

There are only slight variations in chromosome morphology between *Melanophryniscus* species groups. Chromosome pair 4 is *sm* in all species of the *M. moreirae* and *M. stelzneri* groups, whereas the observed centromeric ratios indicate that it is *m* in the *M. tumifrons* group (this study). Pair 5 is *sm* in both species of the *M. moreirae* group (*M. moreirae* and *M. sanmartini*; Beçak et al., 1970; de Lucca et al., 1974; this study), but *m* in the *M. stelzneri* and *M. tumifrons* groups (this study). It is noteworthy that chromosome size is markedly different between the first six (large) chromosome pairs and the remaining five pairs in almost all species of the *M. stelzneri* and *M. tumifrons* groups, but it gradually decreases in *M. krauczuki* (*M. stelzneri* group) and *M. sanmartini* (*M. moreirae* group) as chromosome pair 7 is relatively larger in these two species (Table 1).

The location of NORs were coincident with the secondary constrictions in all species: subterminal on the long arm of pair

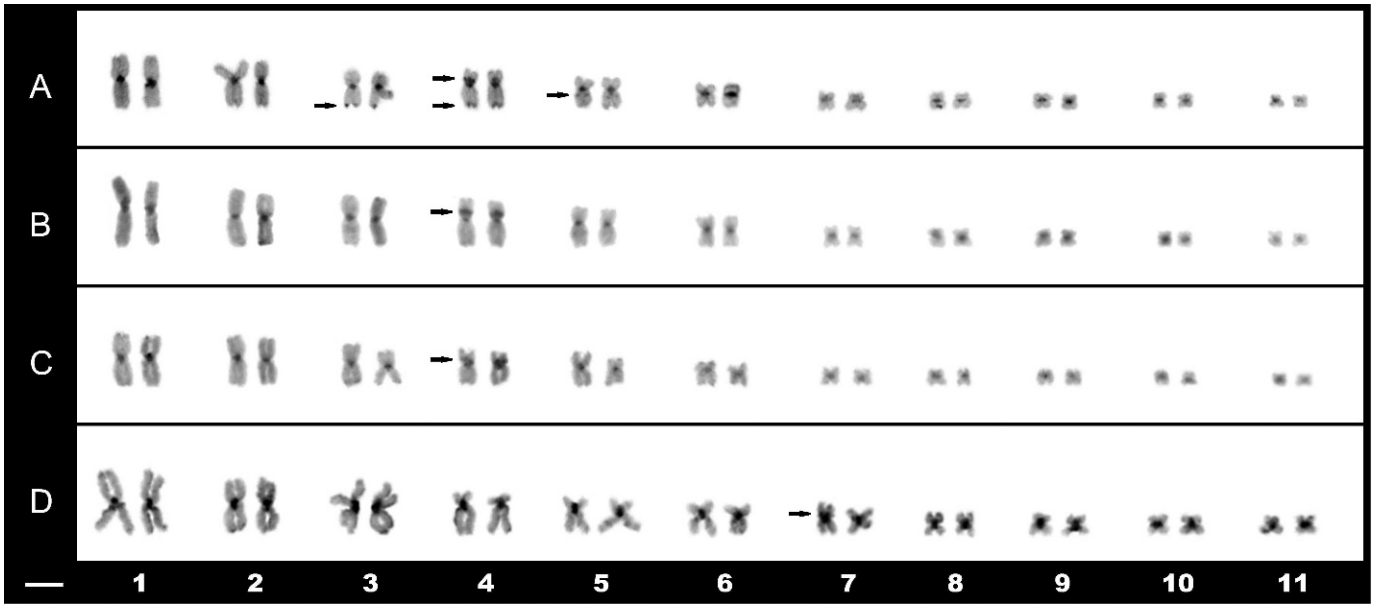

FIG. 5. C-banded karyotypes of three species of the *Melanophryniscus tumifrons* group: *M. devincenzii* (A), *M. orejasmirandai* (B), and *M.* cf. *tumifrons* (C), and one species of the *Melanophryniscus moreirae* group, *M. sanmartini* (D). The arrows indicate the telomeric and interstitial bands. Scale = 10 μm.

8 in the *M. stelzneri* group (Fig. 3A) but proximal in the short arm of pair 7 in *M. krauczuki* (Fig. 3C, II), distal in the long arms of pair 5 in the *M. tumifrons* group (Fig. 3B), and interstitial in the short arm of pair 7 in *M. sanmartini* (Fig. 3 C, I). Another variation was observed in one female of *M. krauczuki* (MLP DB 8760) that in addition to the NORs-bearing pair 7 presented interstitial labeling in the short arm of one of the homologues of pair 3 (Fig. 3C, III). All studied species exhibited a pericentromeric pattern of C-positive bands in all chromosomes, with the NOR regions being C-negative (Figs. 4–5). The three species of the *M. tumifrons* group also have a heterochromatic block on the short arm of pair 4 (Fig. 5); and in addition, *M. devincenzii* has some telomeric C-positive signals on the long arms of pairs 3 and 4 and interstitially on the long arms of pair 5. *Melanophryniscus sanmartini* also have a heterochromatic block contiguous to NOR regions on the short arm of the pair 7 (Fig. 5D).

The *M. moreirae* and *M. stelzneri* groups are weakly defined and to date there are no putative synapomorphies proposed for them. The presence of NORs in pair 8 exhibited by the *M. stelzneri* group (Morand and Hernando, 2002; this study) is unique among Bufonidae but shared by some other Leptodactyliformes with $2n = 22$ chromosomes like *Physalaemus* and *Leptodactylus* (Amaro et al., 2006; Tomatis et al., 2009). Although some studies have indicated the occurrence of secondary constrictions both in pairs 10 and 11 in the *M. stelzneri* group (Morescalchi and Gargiulo, 1968; Bogart, 1972; Baldo and Basso, 2004), this can be explained by differences in homoeology assessment as the size and morphology of the last five pairs are very alike. The presence of secondary constrictions and NORs on the short arm of chromosome pair 7 in *M. sanmartini* and *M. krauczuki* also were reported for *Atelopus zeteki* (only as secondary constrictions evident, Ramos et al., 2002), *Nannophryne* (only as secondary constrictions evident, Bogart, 1972; Formas, 1978; Córdova, 1999), and some phenetic groups of *Rhinella* (Baldissera et al., 1999). *Melanophryniscus krauczuki* was originally included in the *M. stelzneri* group by Baldo and Basso (2004), and *M. sanmartini* was grouped with *M. moreirae*, in the *M. moreirae* group (Cruz and Caramaschi, 2003). Similar chromosome morphology in *M. krauczuki* and in the species of the *M. moreirae* group (larger *RL* and NOR bearing pair 7) and also the recent study of exosomatic characters (Borteiro et al., unpubl. data), indicate that *M. krauczuki* would be phylogenetically more closely related to *M. sanmartini* than to the species currently included in the *M. stelzneri* group.

The *M. tumifrons* group is composed of species with the presence of an evident frontal swelling that consists of a skin macrogland, a putative synapomorphy of this group (Baldo and Basso, 2004). Cytogenetic data presented herein also support this grouping. The presence of distal NORs in the long arm of pair 5 is shared by the three species of the *M. tumifrons* group studied here. A similar condition is observed only in species of the *Rhinella granulosa* group among Bufonidae (Baldissera et al., 1999; Cotichelli et al., unpubl. data). Thus, the location of NORs in pair 5 is probably synapomorphic for the *M. tumifrons* group or a less inclusive group, and homoplastic with the condition in the species of the *R. granulosa* group. These species also have a heterochromatic C-positive block in the short arm of pair 4 that is not present in other *Melanophryniscus* already studied.

This study provides previously unknown evidence for variation that is cytotaxonomically relevant for *Melanophryniscus*. The similarities between species in the *M. moreirae* group and *M. krauczuki*, and within the *M. stelzneri* and the *M. tumifrons* groups, must be tested in an inclusive phylogenetic analysis to assess species relationships. Finally, cytogenetic information of two recently described species, *M. admirabilis* and *M. vilavelhensis*, which are currently unassigned to any group (Di-Bernardo et al., 2006; Steinbach-Padilha, 2008) may suggest their intrageneric relationships.

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Literature Cited

Amaro, C. R., G. Skuk, R. O. de Sá, M. Trefaut Rodrigues, and Y. Yonenaga-Yassuda. 2006. Karyotypes of eight species of *Leptodactylus* (Anura, Leptodactylidae) with a description of a new karyotype for the genus. Phyllomedusa 5:119–133.

Ardila-Robayo, M. C., P. M. Ruiz-Carranza, and J. I. Hernandez-Camacho. 1988. Cariotipos de dos especies de *Osornophryne* (Amphibia: Anura: Bufonidae). Trianea (Act Cient Tecn INDERENA) 2:551–562.

Baldissera, F. A., Jr., R. F. Batistic, and C. F. B. Haddad. 1999. Cytotaxonomic considerations with the description of two new NOR locations for South American toads, genus *Bufo* (Anura: Bufonidae). Amphibia-Reptilia 20:413–420.

Baldo, D., and N. G. Basso. 2004. A new species of *Melanophryniscus* Gallardo, 1961 (Anura: Bufonidae), with comments on the species of the genus reported for Misiones, northeastern Argentina. Journal of Herpetology 38:393–403.

Beçak, M. L., L. Denaro, and W. Beçak. 1970. Polyploidy and mechanism of karyotypic diversification in Amphibia. Cytogenetics 9:225–238.

Bogart, J. P. 1968. Chromosome number difference in the amphibian genus *Bufo*: the *Bufo regularis* species group. Evolution 22:42–45.

———. 1972. Karyotypes. *In* W. F. Blair (ed.), Evolution of the Genus *Bufo*, pp. 171–195. University Texas Press, Austin.

Córdova, J. H. 1999. On karyomorphs, cladistics and taxonomic status of the *Bufo spinulosus* species group (Amphibia, Anura) in Peru. Stuttgarter Beiträge zur Naturkunde, Serie A (Biologie) 600: 1–28.

Cruz, C. A. G., and U. Caramaschi. 2003. Taxonomic status of *Melanophryniscus stelzneri dorsalis* (Mertens, 1933) and *Melanophryniscus stelzneri fulvoguttatus* (Mertens, 1937) (Amphibia, Anura, Bufonidae). Boletim do Museu Nacional 500:1–11.

Daly, J. W., H. M. Garraffo, T. F. Spande, H. J. C. Yeh, P. M. Peltzer, P. M. Cacivio, J. D. Baldo, and J. Faivovich. 2008. Indolizidine 239Q and quinolizidine 275I. Major alkaloids in two Argentinian bufonid toads (*Melanophryniscus*). Toxicon 52:858–870.

De Lucca, E. J., J. Jim, and F. Foresti. 1974. Chromosomal studies in twelve species of Leptodactylidae and one Brachycephalidae. Caryologia 27:183–192.

Di-Bernardo, M., R. Maneyro, and H. Grillo. 2006. New species of *Melanophryniscus* (Anura: Bufonidae) from Rio Grande do Sul, Southern Brazil. Journal of Herpetology 40:261–266.

Formas, J. R. 1978. The chromosomes of *Bufo rubropunctatus* and *Bufo chilensis* (Anura, Bufonidae) and other species of the *spinolosus* group. Experientia 34:452–454.

Frost, D. R. 2010. Amphibian species of the world: an online reference. Version 5.4. New York: American Museum of Natural History, [cited 2010 Nov 8]. Available from: http://research.amnh.org/vz/herpetology/amphibia/.

Grant, T., D. R. Frost, J. P. Caldwell, R. Gagliardo, C. F. B. Haddad, P. J. R. Kok, D. B. Means, B. P. Noonan, W. E. Schargel, and W. C. Wheeler. 2006. Phylogenetic systematics of dart poison frogs and their relatives (Amphibia: Athesphatanura: Dendrobatidae). Bulletin of the American Museum of Natural History 299:1–262.

Graybeal, A., and D. C. Cannatella. 1995. A new taxon of Bufonidae from Peru, with descriptions of two new species and a review of phylogenetic status of supraspecific bufonid taxa. Herpetologica 51:105–131.

Green, D. M., and S. K. Sessions. 1991. Nomenclature for chromosomes. *In* D. M. Green and S. K. Sessions (eds.), Amphibian cytogenetics and Evolution, pp. 431–432. Academic Press, San Diego, CA.

———. 2007. Karyology and cytogenetics. *In* H. Heatwole and M. Tyler (eds.), Amphibian Biology. Vol. 7, pp. 2756–2841. Surrey Beatty and Sons, Chipping Norton.

Howell, W. M., and D. A. Black. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36:1014–1015.

Huskins, C. L. 1932. A cytological study of Vilmorin's unfixable dwarf wheat. Journal of Genetics 25:113–124.

Kuramoto, M. 1990. A list of chromosome numbers of anuran amphibians. Bulletin of Fukuoka University of Education 39:83–127.

Larson, P. M., R. O. de Sa, and D. Arrieta. 2003. Chondrocranial, hyobranchial and internal oral morphology in larvae of the basal bufonid genus *Melanophryniscus* (Amphibia: Anura). Acta Zoologica 84:145–154.

Levan, A., K. Fredga, and A. Sandberg. 1964. Nomenclature for centromeric position on chromosomes. Hereditas 52:201–220.

Morand, M., and A. Hernando. 2002. Análisis citogenético de dos especies de *Melanophryniscus* (Amphibia, Anura, Bufonidae). Revista Española de Herpetología 16:93–99.

Morescalchi, A., and G. Gargiulo. 1968. Su alcune relazioni cariologiche del genero *Bufo* (Amphibia, Salientia). Rendiconto dell'Accademia delle Scienze Fisiche e Matematiche Società Nazionale di Scienze, Lettere e Arti in Napoli 35:117–120.

Ramos, C. W., N. Pimentel, and V. Martínez-Córtes. 2002. Karyotype of the endemic golden frog *Atelopus zeteki* (Dunn) from Panama. Caribbean Journal of Science 38:268–270.

Reeves, A., and J. Tear. 2000. Micromeasure version 3.3 [Internet]. [cited 2010 March 3]. Available from: http://www.colostate.edu/Depts/Biology/MicroMeasure.

Sáez, F. A. 1937. Sobre la citología de *Atelopus stelzneri* (Weyenb.) (Anfibios: Anuros). Archivo Fitotécnico del Uruguay 2:452–454.

———. 1939. Nota sobre la citología de *Atelopus stelzneri* (Weyenbergh). *Physis* 18:161–164.

Schmid, M. 1978. Chromosome banding in Amphibia I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. Chromosoma 66:361–388.

———. 1980. Chromosome banding in Amphibia. IV. Differentiation of GC- and AT-rich chromosome regions in Anura. Chromosoma 77:83–103.

Steinbach-Padilha, G. C. 2008. A new species of *Melanophryniscus* (Anura, Bufonidae) from the Campos Gerais region of southern Brazil. Phyllomedusa 7:99–108.

Sumner, A. T. 1972. A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75:304–306.

Tomatis, C. G., D. Baldo, F. Kolenc, and C. Borteiro. 2009. Chromosomal variation in the species of the *Physalaemus henselii* group (Anura, Leiuperidae). Journal of Herpetology 43:555–560.

Veiga-Menoncello, A. C., A. P. Lima, and S. M. Recco-Pimentel. 2003. Cytogenetic analysis of four central Amazonian species of *Colostethus* (Anura - Dendrobatidae) with a diploid complement of 22 chromosomes. Hereditas 139:189–198.

White, M. J. D. 1954. Animal Cytology and Evolution. Cambridge University Press, Cambridge, U.K.

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Appendix 1

Specimens Examined

Melanophryniscus atroluteus.—ARGENTINA: Provincia de Corrientes: Departamento Ituzaingó: Ruta provincial N°39, 10 km from Ruta Nacional N°14 (27°50′15″S; 55°59′12″W), MLP DB 8938 (female); Provincia de Misiones: Departamento Candelaria: Ruta Nacional N°12 and Ruta Provincial N°3 (27°27′46″S; 55°40′55″W), MLP DB 3554 (female); Ñu Pyahú, Ruta Provincial N°3, 3.5 km from Ruta Nacional N°12 (27°29′25″S; 55°40′06″W), MLP DB 8762–3 (females). URUGUAY: Departamento Treinta y Tres: Bañado de los Oliveras (33°11′57″S; 54°27′18″W), MNHN 9361 (male), Novena sección, Vergara (32°56′48″S; 53°56′27″W), MNHN 9362 (male).

Melanophryniscus cupreuscapularis.—ARGENTINA: Provincia de Corrientes: Departamento Capital: Paraje Perichón (27°26′31″S; 58°45′08″W), MLP DB 7123 (female).

Melanophryniscus devincenzii.—ARGENTINA: Provincia de Corrientes: Departamento Ituzaingó: Ruta provincial N°39 10 km from Ruta Nacional N°14 (27°50′15″S; 55°59′12″W), MLP DB 8935 (male), 8758–9 (females); Provincia de Misiones: Departamento Candelaria: Cerro Corá (27°29′21.3″S; 55°36′31.2″W), MLP DB 5740 (male); Ñu Pyahú, Ruta Provincial N°3, 3.5 km from Ruta Nacional N°12 (27°29′25″S; 55°40′06″W), MLP DB 5694 (male), LGE 046 (female); Departamento San Pedro: Forestal Montreal, Arroyo Competidor, near to Arroyo Yabotí (26°52′00″S; 54°02′00″W), MLP DB 2300 (male); Parque Provincial Esmeralda, Estación Biológica (26°53′36″S; 53°52′42″W), MLP DB 3477, 3536 (males).

Melanophryniscus fulvoguttatus.—PARAGUAY: Departamento Concepción: surroundings of Concepción city, Estancia Ybú (23°27′17.9″S; 57°26′01.6″W), IIBP–H 737 (male), 738 (hembra); Colonia San Alfredo, Estancia Garay Kué, Reserva Natural Privada Cerrados del Tagatiyá (22°38′29.2″S; 57°22′46″W), MLP DB 6762 (male).

Melanophryniscus klappenbachi.—ARGENTINA: Provincia de Chaco: Departamento San Fernando: Resistencia, Club Sixty (27°25′17″S; 58°56′20″W), MLP DB 3491–2 (males), 3494, 3553 (females).

Melanophryniscus krauczuki.—ARGENTINA: Provincia de Misiones: Departamento Candelaria Ñu Pyahú, Ruta Provincial N°3, 3.5 km from Ruta Nacional N°12 (27°29′25″S; 55°40′06″W), MLP DB 5689, 5690 (males); 5736, 8760–1 (females).

Melanophryniscus montevidensis.—URUGUAY: Departamento Rocha: Cabo Polonio (34°24′00″S;53°48′00″W), MNHN 9363 (female); La Pedrera (34°35′24″S; 54°07′30″W), MNHN 9364 (female); Laguna de Rocha (34°39′45″S; 54°13′19″W), MNHN 9365, 9367–8 (males), 9366 (female); Punta Rubia (34°35′05″S; 54°07′14″W), MNHN 9369 (male).

Melanophryniscus orejasmirandai.—URUGUAY: Departamento Maldonado: Sierra de las Ánimas (34°42′00″S; 55°19′00″W), MNHN 9370–2 (males), 9373 (female).

M. paraguayensis.—PARAGUAY: Departamento Central: Municipalidad de Mariano Roque Alonso, Urbanización Surubìí (25°11′12″S; 57°30′50″W), IIBP–H 1443, MLP DB 6766–8 (males).

Melanophryniscus rubriventris.—ARGENTINA: Jujuy: Departamento Doctor Manuel Belgrano: Tiraxi (24°00′34″S; 65°23′00″W) MLP DB 8816, 8820 (males), 8817, 8819 (females).

Melanophryniscus sanmartini.—URUGUAY: Departamento Lavalleja: Salto del Penitente (34°22′34″S; 55°03′23″W), MNHN 9376–7 (males); Departamento Rivera: Cerro Trindade (30°57′21″S; 55°27′08″W), MNHN 9379 (male); Curticeras (31°00′24″S; 55°35′18″W), MNHN 9380 (male); Establecimiento Trinidad (30°58′49″S; 55°25′45″W), MNHN 9381 (female); Departamento Rocha: Santa Teresa (33°59′55″S; 53°34′38″W), MNHN 9378 (male).

Melanophryniscus stelzneri stelzneri.—ARGENTINA: Provincia de Córdoba: Departamento Colón: Pozo Azul (30°57′00″S; 64°19′48″W), MLP DB 1396 (male); Provincia de San Luis: Departamento La Capital: Potrero de Los Funes MLP DB 3474 (male); Departamento Libertador General San Martín: Las Chacras (32°33′00″S; 65°47′00″W), MLP DB 4688 (female); 5021–2 (males).

Melanophryniscus cf. *tumifrons*.—BRAZIL: Rio Grande do Sul: Municipio de Gravataí, close to Campus Palavra da Vida Sul (29°48′03″S; 50°55′43″W), CFBH 27238–40 (males); CFBH 27241–2 (females).

Melanophryniscus sp. aff. *montevidensis*—ARGENTINA: Provincia de Santa Fe: Departamento Colonias: Esperanza (31°23′13″S; 60°55′03″W) MLP DB 3442 (male).