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Chromosomal differentiation of populations of *Lysapsus limellus limellus*, *L. l. bolivianus*, and of *Lysapsus caraya* (Hylinae, Hylidae)

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Abstract

Cytogenetic analysis were done on specimens from two populations of *Lysapsus limellus limellus*, three of *L. l. bolivianus* and of one of *Lysapsus* caraya. All animals showed a diploid chromosomal number of $2n=24$. The karyotypes of the two *L. limellus* subspecies were very similar, differing only by the larger amount of telomeric heterochromatin and a small pericentromeric C-band on the short arms of pair 2 in *L. l. limellus* specimens. The karyotype of *L. caraya* differed from those of the two *L. limellus* subspecies in terms of chromosomal morphology, C-banding pattern and location of the main NOR on chromosomes 7 and 6, respectively. The karyotype of the *L. l. bolivianus* population from Guajará-Mirim/RO differed from those of the other populations of the same subspecies in morphology and heterochromatin pattern of chromosomes 7 and 8. Additional NORs were detected by silver staining and confirmed by FISH in one of the homologues of pairs 1 and 8 in *L. l. bolivianus* and in pair 7 in *L.* caraya. These results suggest that a reassessment of the taxonomic status of *L. limellus* subspecies, especially of the *L. l. bolivianus* populations, may be necessary.

Keywords: Lysapsus; Karyotype; NOR; Heterochromatin; In situ hybridization

1. Introduction

The genus *Lysapsus* includes small, slender, semiaquatic anurans ranging in length from 16 to 21 mm, with a distribution restricted to South America (Duellman and Trueb, 1986; Garda et al., 2004). The taxonomic position and phylogenetic relationships of this genus have always been a matter of debate, with successive discussions about their relationship to the Hylidae (Savage and Carvalho, 1953; Terrini et al., 2002) and/or Leptodactylidae (Morescalchi, 1973; Garda et al., 2004). Together with the genus *Pseudis*, *Lysapsus* was, for a long time, included in the family Pseudidae. Subsequently, *Pseudis* and *Lysapsus* were included in the subfamily Pseudinae, a sister group of Hylinae (Frost, 2002). Frost (2004) included the two genera in the subfamily Hylinae based on molecular studies by Darst and Canatella (2004). Recently, Faivovich et al. (2005), in an extensive re-analysis of the family Hylidae, showed the sister-group relationship between *Scarthyla goinorum* and *Lysapsus*+*Pseudis*.

The genus *Lysapsus* includes the species *L. limellus* Cope 1862, *L. laevis* Parker, 1935, and *L. caraya* Gallardo 1961 (Frost, 2004). Currently, *Lysapsus limellus* has been divided into the subspecies *L. l. limellus*, *L. l. bolivianus* and *L. l. bolivianus*. Klappenbach (1985) discussed that *L. l. bolivianus* should be considered a subspecies of *L. laevis*, but according to Carlos Alberto Cruz (personal information) *L. l. bolivianus* and *L. laevis* show important morphological external differences and they should be included in distinct taxa.

In addition to the discussions regarding the taxonomic situation of *Lysapsus*, synonymous species and the maintenance of subspecific classification, the intergeneric relationships with *Pseudis* are also confusing. Parker (1935) considered *L. laevis* and *L. limellus* as belonging to the genus *Pseudis*,

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whereas until the studies of Gallardo (1961), the species *P. minuta* was included in the genus *Lysapsus* as *L. mantidactylus*.

To obtain cytogenetic data that could contribute to our understanding of the taxonomic position, phylogenetic and intraspecific relationships, and subspecies classification of the genus *Lysapsus*, in this study we analyzed the karyotype, C-banding pattern and location and number of nucleolar-organizer regions (NORs) in populations of *L. l. limellus*, *L. l. bolivianus* and *L. caraya*.

2. Material and methods

2.1. Specimens

The following populations were analyzed: *L. l. limellus* from Corumbá/MS (nine males and six females) and Nossa Senhora do Livramento/MT (seven males and five females); *L. l. bolivianus* from Santarém/PA (15 males and 12 females), Manaus/AM (12 males and 10 females) and Guajará-Mirim/RO (12 males and 14 females), and one *L. caraya* population from Santa Terezinha/MT (12 males and eight females). Voucher specimens were deposited at the Museu Nacional do Rio de Janeiro (MNRJ) and the Museu de História Natural 'Prof. Adão José Cardoso', Universidade Estadual de Campinas (ZUEC), and are registered under the accession numbers: MNRJ 34056–34070 (*L. l. limellus*—Corumbá), MNRJ 34071–34082 (*L. l. limellus*—Nossa Senhora do Livramanto), MNRJ 33860–33886 (*L. l. bolivianus*—Santarém), MNRJ 33907–33927 (*L. l. bolivianus*—Manaus), MNRJ 33942–33968 (*L. l. bolivianus*—Guajará-Mirim) and ZUEC 13200–13219 (*L. caraya*). All specimens were collected under a permit issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA—process 02001008875/01–11, collection license number 083/04 and genetical material access, authorization number 022/2005).

2.2. Chromosome preparations

The chromosomes were obtained from intestinal epithelium and testicular cell suspensions, as described by King and Rofe (1976) and Schmid (1978a). The material was processed for staining with 10% Giemsa (pH 7.0, for 10 min), C-banding according to Sumner (1972) with modifications in the length of treatment with barium hydroxide, silver staining (Ag-NOR) according to Howell and Black (1980), and fluorescence in situ hybridization (FISH) according to Viégas-Péquignot (1992), using a recombinant plasmid (HM123) containing a fragment of *Xenopus laevis* rDNA (Meunier-Rotival et al., 1979) to localize ribosomal genes. The probe was biotin-labeled by a nick-translation reaction according to the manufacturer's protocol (Gibco). The chromosomes were classified according to Green and Sessions (1991).

3. Results

3.1. Karyotype description

All karyotypes had $2n = 24$ chromosomes (Fig. 1A–F), as confirmed by the presence of 12 bivalents during meiotic diakinesis. None of the species analyzed showed sex determination-related heteromorphic chromosomes.

In the karyotypes of the *L. l. limellus* populations from Corumbá and Nossa Senhora do Livramento and of the *L. l. bolivianus* populations from Santarém and Manaus, pairs 1, 3, 8, 9, 10, 11 and 12 were metacentric, pairs 2, 4, 5 and 7 were submetacentric and pair 6 was subtelocentric (Fig. 1A–D; Table 1). A similar karyotype was observed for *L. l. bolivianus* specimens from the Guajará-Mirim population, but chromosome 7 was metacentric and chromosome 8 was submetacentric. In addition, seven of the 26 specimens analyzed showed heteromorphism in the relative chromosome size and morphology of the homologues of pair 1 (morphs 1a and 1b) (Fig. 1E; Table 1). All chromosomes of the *L. caraya* karyotype were metacentric (Fig. 1F; Table 1).

Most metaphases of the *L. limellus* subspecies showed secondary constrictions in the telomeric regions of the short arms of homologues 2, 7, and of the short and long arms of pair 8, in addition to a subtelomeric constriction in the long arms of pair 7 (Fig. 1A–E). In the karyotype of *L. caraya*, secondary constrictions were detected in the telomeric regions of the short and long arms of most chromosomes, except for pairs 1, 3, 4 and 5. In pair 6, a secondary constriction was observed only in the short chromosome arms (Fig. 1F). The measurements of the chromosomes include the secondary constrictions (Table 1).

3.2. Heterochromatin pattern

Centromeric heterochromatin bands were detected in all chromosomes of the species analyzed. Telomeric heterochromatin blocks were seen in the chromosomes 2, 8, 9, 10 and 11 of *L. l. limellus*, in contrast to the karyotype of *L. l. bolivianus* from Santarém and Manaus in which the telomeric bands strongly stained only in pairs 2, 4, 8 and 9 while in the Guajará-Mirim population they were detected in chromosomes 1, 2 and 7. The karyotype of *L. caraya* were also characterized by heterochromatin blocks in the telomeric regions of the chromosomes, except for pairs 3, 4 and 5. A small interstitial band on the short arms of the homologues of pair 5 were observed in *L. l. limellus* e in *L. caraya*. *Lysapsus l. bolivianus* from Manaus had an homologue with an heterochromatic block in the chromosomes of pair 4. In *L. l. limellus* and *L. l. bolivianus*, a heterochromatin segment was detected interstitially on the short arms of the homologues of pair 1, but was absent in the karyotype of L. caraya. An additional heterochromatin band was observed in the pericentromeric region of the short arms of pair 2 in *L. l. limellus* specimens, but was absent in *L. l. bolivianus* and *L. caraya*. The chromosomes of pair 6 showed a small heterochromatin block in the

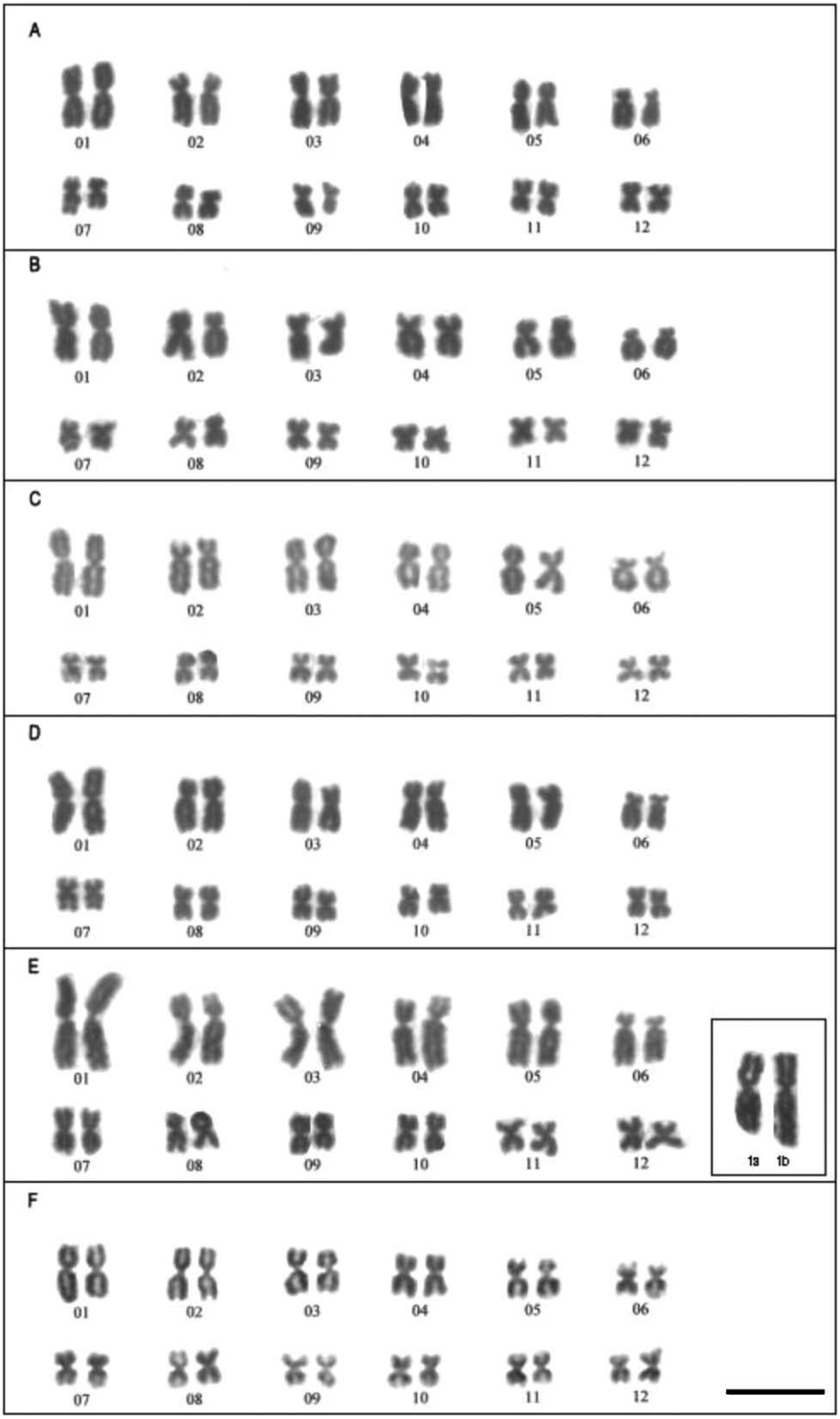

Fig. 1. Karyotypes stained with Giemsa. (A) *Lysapsus limellus limellus*, Corumbá/MS; (B) *L. l. limellus*, Nossa Senhora do Livramento/MT; (C) *L. l. bolivianus*, Santarém/PA; (D) *L. l. bolivianus*, Manaus/AM; (E) *L. l. bolivianus*, Guajará-Mirim/RO; (F) *L. caraya*, Santa Terezinha/MT. Bar = 5 μm.

pericentromeric region of the short arms in all species, with this block being more strongly stained in *L. caraya*. The karyotype of the *L. l. bolivianus* population from Guajará-Mirim differed from the karyotypes of the Santarém and Manaus populations by having an additional positive C-band interstitial on the short and long arms of pair 7 and on the long arms of pair 8. In seven of 26 specimens of the Guajará-Mirim population, in which heteromorphic morphs of pair 1 were observed in the Giemsa-

Table 1
Morphometric parameters of the karyotypes of *L. l. limellus* (Corumbá/MS and Nossa Senhora do Livramento/MT), *L. l. bolivianus* (Santarém/PA, Manaus/AM and Guajará-Mirim/RO) and *L. caraya* (Santa Terezinha/RO)

	Chromosomes												
	1a	1b	2	3	4	5	6	7	8	9	10	11	12
L. l. limellus—Corumbá/MS ($n=29$)													
r.s.	13.79	–	11.56	10.28	9.69	9.33	8.27	7.25	6.76	6.42	5.94	5.75	4.96
c.i.	0.41	–	0.30	0.38	0.34	0.37	0.23	0.31	0.38	0.38	0.41	0.44	0.44
c.p.	M	–	SM	M	SM	SM	ST	SM	M	M	M	M	M
L. l. limellus—Nossa Sra. do Livramento/MT ($n=24$)													
r.s.	14.53	–	11.01	10.27	9.63	9.15	7.86	7.34	6.71	6.29	5.99	5.84	5.38
c.i.	0.40	–	0.36	0.37	0.35	0.36	0.23	0.33	0.38	0.38	0.39	0.43	0.42
c.p.	M	–	SM	M	SM	SM	ST	SM	M	M	M	M	M
L. l. bolivianus—Santarém/PA ($n=28$)													
r.s.	13.69	–	11.48	10.51	9.93	9.37	7.59	7.04	6.72	6.31	6.06	5.87	5.43
c.i.	0.42	–	0.30	0.38	0.35	0.36	0.23	0.30	0.42	0.38	0.45	0.44	0.42
c.p.	M	–	SM	M	SM	SM	ST	SM	M	M	M	M	M
L. l. bolivianus—Manaus/AM ($n=26$)													
r.s.	14.10	–	11.55	11.08	10.02	9.41	7.74	7.07	6.38	6.07	5.89	5.54	5.15
c.i.	0.40	–	0.37	0.39	0.34	0.34	0.22	0.36	0.38	0.39	0.42	0.42	0.44
c.p.	M	–	SM	M	SM	SM	ST	SM	M	M	M	M	M
L. l. bolivianus—Guajará-Mirim/RO ($n=26$)													
r.s.	14.80	15.22	11.85	10.67	9.91	8.70	8.17	7.53	6.47	6.04	5.77	5.42	4.67
c.i.	0.39	0.36	0.36	0.38	0.35	0.36	0.23	0.38	0.36	0.41	0.43	0.38	0.40
c.p.	M	SM	SM	M	SM	SM	ST	M	SM	M	M	M	M
L. caraya—Sta.Terezinha/MT ($n=22$)													
r.s.	12.63	–	10.96	9.91	9.39	9.04	7.98	7.54	7.16	6.84	6.49	6.11	5.95
c.i.	0.41	–	0.40	0.40	0.41	0.44	0.38	0.42	0.46	0.47	0.50	0.44	0.45
c.p.	M	–	M	M	M	M	M	M	M	M	M	M	M

Classification according to Green and Sessions (1991). n, number of metaphases analyzed; r.s., relative size (%); c.i., centromeric index; c.p., centromere position; M, metacentric; SM, submetacentric; ST, subtelocentric; MS, Mato Grosso do Sul; MT, Mato Grosso; PA, Pará; AM, Amazonas; RO, Rondônia.

stained karyotype, an additional heterochromatin block was detected on the larger arm of the 1b morph (Fig. 2A–F).

3.3. Nucleolar organizer regions (NORs)

NORs were detected in the subtelomeric region of the long arms of pair 7 in all karyotypes of the *L. l. limellus* and *L. l. bolivianus* populations, whereas in *L. caraya* the NOR was observed in the telomeric region of the shorts arms of chromosome 6. In all of the species analyzed, heteromorphism in the size of one of the NORs was observed between homologues in some specimens (Fig. 3A–F).

An additional NOR was detected in the subtelomeric region of the long arms of one of the homologues of pair 8 in five of the 22 *L. l. bolivianus* specimens from the Manaus population and in the long arms of one of the homologues of pair 1 in seven of the 26 *L. l. bolivianus* specimens from Guajará-Mirim, which coincided with the heterochromatin block (morph 1b) (Fig. 3D and E). Furthermore, an additional NOR was detected in the telomeric region of the long arms in one of the homologues of pair 7 in 60% of *L. caraya* specimens (Fig. 3F). All of the results obtained by silver staining were confirmed by in situ hybridization with the HM123 probe (Fig. 3A–F).

4. Discussion

The diploid chromosome number ($2n=24$) seen in the *Lysapsus* species analyzed here agreed with that reported by Barrio and Rubel (1970) for *L. l. limellus* from Santa Fé, Argentina. Discrete differences were observed in *L. l. limellus* only in the morphology of chromosomes 3, 6, 8 and 9, and may have resulted from the use of different chromosome classification tables. Another possibility is that these differences were characteristic of the karyotypes of the populations and could be indicative of the preliminary stages of chromosomal differentiation among these populations. However, since no differential chromosome labeling technique was used by Barrio and Rubel (1970), neither of these hypotheses can be confirmed.

Characteristically, when submitted to conventional Giemsa staining, many *Lysapsus* chromosomes showed a secondary constriction in the telomeric regions, in addition to nucleolar constrictions. These secondary constrictions are larger in *L. caraya* than in the other species. Since the secondary constrictions were considered in the morphometry, the chromosomes were classified as metacentric in *L. caraya*. According to Schmid (1978a,b), heterochromatin segments occasionally acquire the appearance of secondary constrictions and are not necessarily nucleolar constrictions. King (1990) identified five classes of secondary constrictions in 12 *Litoria* species (Hylidae) based on C-banding and silver staining. According to this author, positive C-band constrictions that are not stained by silver, such as those observed in *Lysapsus* are common in anurans, although their expression may vary among chromosomes, cells and individuals.

As discussed by Schmid (1978a,b) and Schmid et al. (1990), a general characteristic of anuran karyotypes is the presence of

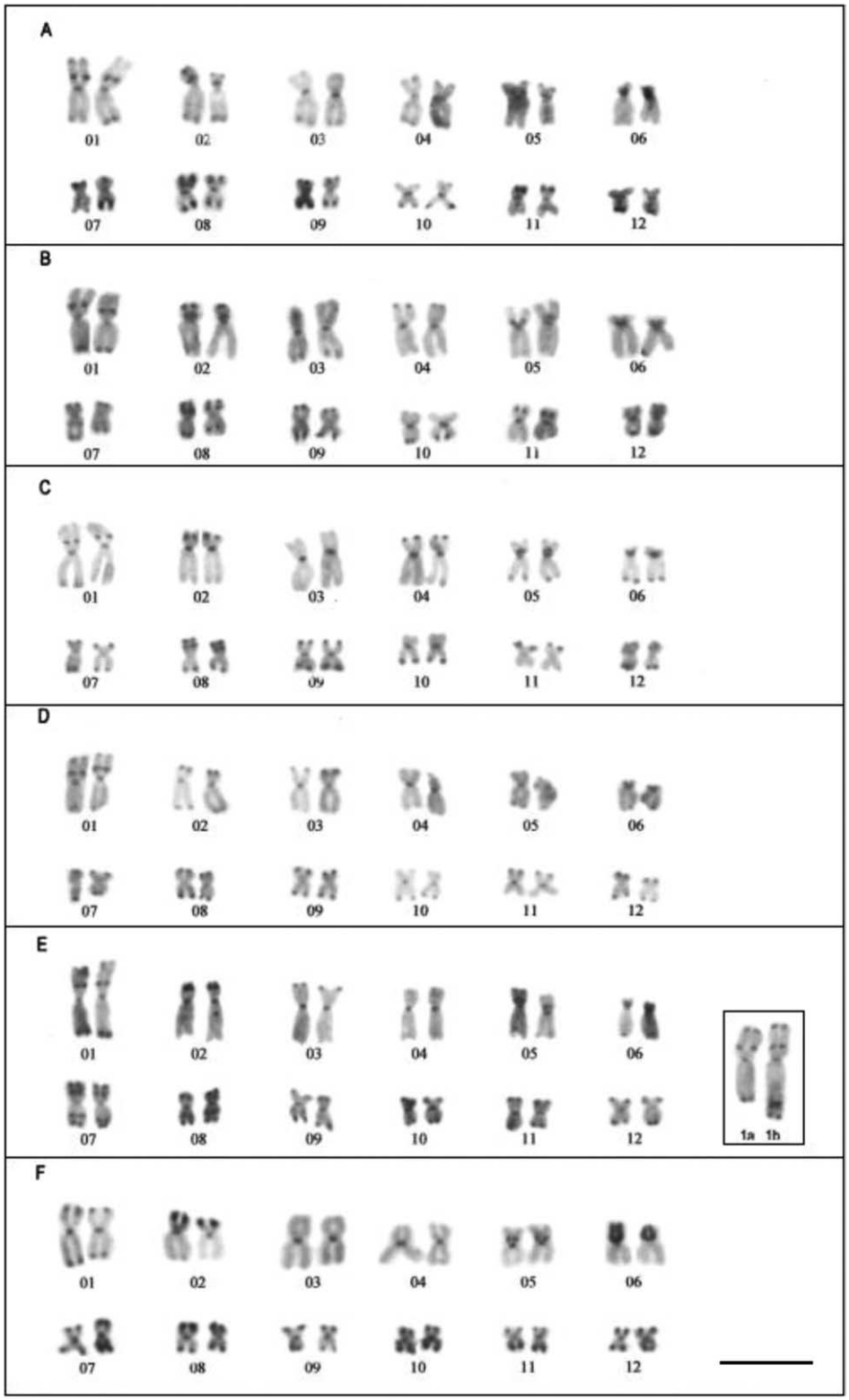

Fig. 2. Karyotypes stained for C-banding. (A) *Lysapsus l. limellus*, Corumbá/MS; (B) *L. l. limellus*, Nossa Senhora do Livramento/MT; (C) *L. l. bolivianus*, Santarém/PA; (D) *L. l. bolivianus*, Manaus/AM; (E) *L. l. bolivianus*, Guajará-Mirim/RO; (F) *L. caraya*, Santa Terezinha/MT. Bar = 5 μm.

only one pair of NOR per diploid genome, which is normally located in the same chromosome pair in species belonging to the same group or to closely related groups. In the *L. limellus* subspecies and in *L. caraya*, the NOR were located on different chromosome pairs (7 and 6, respectively). Similar interspecies variation has been described for some hylid (Schmid, 1978a,b; Wiley et al., 1989; Anderson, 1991; Medeiros et al., 2003), bufonid (Anderson, 1991; Baldissera et al., 1999), and

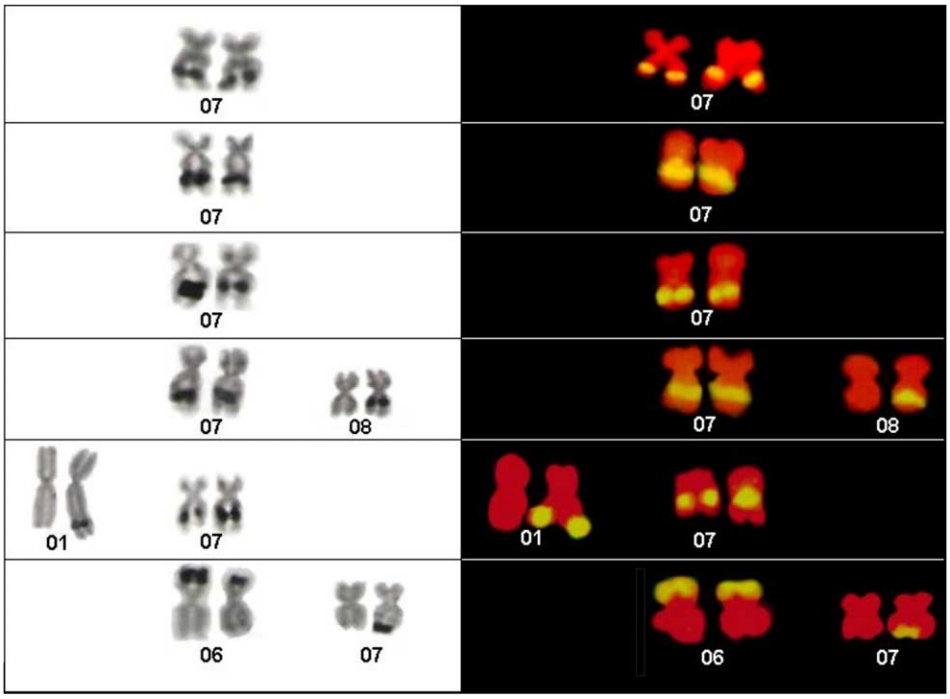

Fig. 3. Chomosomes bearing the main and the additional NORs detected by AgNOR (first column) and by in situ hybridization with HM123 rDNA probe (second column). (A) *Lysapsus l. limellus*, Corumbá/MS; (B) *L. l. limellus*, Nossa Senhora do Livramento/MT; (C) *L. l. bolivianus*, Santarém/PA; (D) *L. l. bolivianus*, Manaus/AM; (E) *L. l. bolivianus*, Guajará-Mirim/RO; (F) *L. caraya*, Santa Terezinha/MT. Bar = 5 μm.

dendrobatid (Aguiar-Jr et al., 2002; Veiga-Menoncello et al., 2003) species. According to Schmid et al. (1990), interspecies variations in NOR location indicate that chromosomal rearrangements in the segments containing sequences of rDNA have occurred during chromosomal evolution and that translocations or inversions are the most probable mechanisms in the dispersal of these genes in the genome, especially in the case of alterations in relative chromosome size or morphology.

In the karyotypes of the *L. limellus* subspecies, pair 7 carrying the NOR on the long arms is submetacentric in *L. l. limellus*, and metacentric in *L. l. bolivianus* from Guajará and pair 6 is subtelocentric in all *L. limellus*. In contrast, in *L. caraya* the homologues of pair 6 carrying the NOR in the telomeric region of the short arms, as well as the homologues of pair 7, are metacentric. These data suggest the probable occurrence of a translocation mechanism of ribosomal cistrons during the evolution of these chromosomes, thereby altering their morphology. Since, in the *L. limellus* subspecies the NOR is located on submetacentric pair 7, as also observed for *Pseudis* with $2n = 24$ (Busin et al., 2001, in preparation), a genus that, together with *Lysapsus*, used to be placed in the subfamily Pseudinae, it seems likely that the NOR located on pair 7 represents a plesiomorphic condition and that during the evolution of the *L. caraya* karyotype ribosomal genes were translocated from chromosome 7 to chromosome 6. Future study of the NOR location in *L. laevis* would be helpful in the analysis of this genus and could confirm this hypothesis. The observation of an additional active NOR on one of the homologues of pair 7 in 60% of the *L. caraya* karyotypes analyzed may indicate the persistence of some rDNA copies at this plesiomorphic location.

According to Schmid (1978b), Odierna et al. (2000) and Andreone et al., (2003), the location of the NOR is a useful marker in the taxonomy and phylogeny of anurans. The karyotypic differences in NOR location and chromosomal morphology among the two *L. limellus* subspecies and *L. caraya*, and the distribution of some interstitial heterochromatin bands, such as those detected on the short arms of the homologues of pair 1 as a marker of the *L. limellus* subspecies but absent in *L. caraya*, cytogenetically differentiated the *L. limellus* subspecies from *L. caraya*.

Although most anurans possess only one pair of NOR per diploid genome, additional active NORs were detected by the Ag-NOR method and FISH on one of the homologues of one chromosome pair in 22.3% of the *L. l. bolivianus* specimens from Manaus and in 26.9% of the Guajará-Mirim specimens. In *L. l. bolivianus* from Guajará-Mirim, an additional active NOR was observed on the long arms of morph 1b, coincident with the heterochromatin block, a finding clearly indicating the addition of a segment on this arm. Since, this extra segment led to alterations in the relative chromosome size, arm ratio and, consequently, chromosomal morphology (M to SM), mechanisms of translocation involving the NOR-containing chromosomal segment with subsequent addition of heterochromatin could be acting on the evolution of this chromosome. However, in the *L. l. bolivianus* specimens from Manaus, translocation is less supported because there was no alteration in the relative size or arm ratio in chromosomes with or without NORs.

The presence of different telomeric secondary constrictions distributed among various chromosomes of the *Lysapsus* karyotypes, together with the heterochromatin pattern of large amounts of C-band positive segments in the telomeric

regions, characterize the genus and clearly differentiate it from representatives of the genus *Pseudis* analyzed by Busin et al. (2001, in preparation).

At the subspecific level, the *L. l. limellus* karyotypes differed from those of *L. l. bolivianus* from Santarém and Manaus by their higher amount of telomeric heterochromatin and a small heterochromatin block on the short arms of chromosome 2 in *L. l. limellus*, although the chromosomal morphology and NOR location were similar in both subspecies.

The karyotype of the *L. l. bolivianus* population from Guajará-Mirim differed from that of other populations of the same subspecies by the presence of additional heterochromatin blocks and alterations in the morphology of chromosomes 7 and 8. The percentage of the chromosomes of pair 7 in the genome was higher in the Guajará-Mirim population compared to the populations from Santarém and Manaus, with these chromosomes also showing an altered morphology (SM/M). In contrast, the chromosomes of pair 8 showed no increase in relative size, although the arm ratio was altered (M/SM), probably because of the heterochromatin block. These additional blocks may have originated from other regions of the genome or are related to amplification of repetitive DNA segments, which can be the cause of increase in the size of the chromosomes. However, the hypothesis of a transformation of euchromatin into heterochromatin suggested by King (1991) cannot be ruled out.

According to John (1988), the amount and distribution of heterochromatin are variable factors, which have contributed significantly to the mechanism of chromosomal isolation since they change at an extremely rapid rate during evolution. In addition, the pattern of heterochromatin distribution has been determined for a large number of a large number of anuran species and populations in studies that have investigated the possible polymorphisms and/or variations, in an attempt to understand phyletic interrelationships. These distribution patterns have also been used to characterize different taxa (Kasahara et al., 1996; Lourenço et al., 1998; Silva et al., 1999; Busin et al., 2001).

In conclusion, the species *L. caraya* and the subspecies of *L. limellus* can easily be distinguished by chromosome morphology, C-banding pattern and NOR locations. *Lysapsus limellus bolivianus* from Santarém and Manaus differed slightly from *L. l. limellus* in the heterochromatin pattern, but they showed identical chromosome morphology and NOR location, indicating that they are very closely related. Also, the differences in the C-banding pattern and in the morphology of chromosomes 7 and 8 for the populations of *L. l. bolivianus* from Santarém + Manaus and for that of Guajará-Mirim, suggest a taxonomic reassessment of these populations.

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