Karyotypes of eight species of *Leptodactylus* (Anura, Leptodactylidae) with a description of a new karyotype for the genus

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Abstract

Karyotypes of eight species of Leptodactylus (Anura, Leptodactylidae) with a description of a new karyotype for the genus. Eight species of the Neotropical genus Leptodactylus were karyologically studied: seven of them (L. gracilis, L. mystacinus, L. petersii, L. pustulatus, L. macrosternum, L. ocellatus, L. labyrinthicus) presented 2n=22 and L. silvanimbus showed a distinctive karyotype with 2n=24. Nucleolar organizer regions (Ag-NORs) were detected in two different pairs of chromosomes: pair 4 at the proximal region of the long arm of one individual of L. mystacinus from São Paulo state and of L. petersii; and pair 8 of all other species (located terminally at the short arm of L. silvanimbus, L. ocellatus, L. macrosternum, L. pustulatus, and L. labyrinthicus; interstitially at the short arm in L. gracilis; and at the long arm in L. mystacinus from Mato Grosso state). The diploid number reported here for L. silvanimbus shared with Scythrophrys and Paratelmatobius could represent the ancestral chromosome number for Leptodactylus (sensu Frost et al. 2006); in this case the 2n=22 karyotypes would then represent the derivative condition for the genus. Nevertheless, the distinctive karyotype of L. silvanimbus lead us to preclude a final decision on its relationships. Additional studies including morphological and molecular approaches are needed in order to clarify the phylogenetic position of *L. silvanimbus*.

Keywords: Anura, Leptodactylidae, *Leptodactylus, Leptodactylus silvanimbus,* karyotypes, Ag-NORs.

Introduction

The genus *Leptodactylus* is distributed from southern Texas throughout lowland (< 1800 m)

Mexico, Central America, portions of the Greater and Lesser Antilles, and in South America on both sides of the Andes to Peru on the west and Argentina on the east. As conceived until recently (Frost 2004), about 64 species arranged in four traditionally recognized species groups were recognized for the genus (Heyer

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1969): fuscus, melanonotus, ocellatus, and pentadactylus. Recently, de Sá et al. (2005) proposed that the monotypic genus Vanzolinius should be a synonym of Leptodactylus, based on molecular and morphological data. This concept of Leptodactylus changed again more recently following a comprehensive molecular and morphological study on the relationships among major groups of amphibians (Frost et al. 2006). In this paper, Vanzolinius is deeply imbedded within Leptodactylus supporting its synonymy with Leptodactylus, Adenomera is placed in the synonymy of Lithodytes, and Lithodytes is placed as a subgenus Leptodactylus. The same paper restricted the content of Leptodactylidae to Leptodactylus, Edalorhina, Engystomops, Eupemphix, Hidrolaetare, Paratelmatobius, Physalaemus, Pleurodema, Pseudopaludicola, Scythrophrys, and Somuncuria. A more recent publication (Grant et al. 2006), based on the same molecular data used by Frost et al. (2006) but on a different taxonomic sampling, restricted the leptodactylids to Leptodactylus, Hidrolaetare, Paratelmatobius, and Scythrophrys.

As frog systematics is undergoing a period of active revision and change affecting the contents of families and genera, we prefer to take a conservative approach and follow the "traditional" concept of *Leptodactylus* to present our data.

The karyotypes of 25 species of Leptodactylus (sensu Frost 2004) have been described (Appendix I); most of them were exclusively based on conventional staining and description of number and chromosome morphology. Leptodactylus species have 22 chromosomes with slight differences in morphology among the species (King 1990, Kuramoto 1990, Amaro-Ghilardi et al. 2004). Studies including differential staining showed species-specific Cbanding patterns in Leptodactylus gracilis gracilis, L. gracilis delattini, L. knudseni, L. labyrinthicus, L. notoaktites, L. pentadactylus, L. plaumanni, and L. podicipinus; furthermore differences in the distribution of constitutive heterochromatin among populations of L.

fuscus, L. mystacinus, and L. ocellatus were also observed (Silva et al. 2000, 2004, 2006, Baldo 2002, Amaro-Ghilardi et al. 2004). Nucleolar organizer regions (NORs) were detected in the short arm of pair 8 of all species studied, except for L. latinasus and L. podicipinus, which showed NORs in the long arm of pair 8 (Silva et al. 2000, Baldo 2002); and for L. mystacinus, which presented a polimorphism of NORs: individuals from Misiones, Argentina, showed NORs in the interstitial region of the short arm of pair 4 and specimens from São Paulo state, Brazil, presented interindividual variation of NORs in both pairs 4 and 8 (Baldo 2002, Silva et al. 2006).

Leptodactylus silvanimbus is a species with an interesting distribution, occurring in cloud forest and moderate elevation pine forest habitats in extreme southwestern Honduras. The species is known from three localities at moderate and intermediate elevations (1470-2000 m) along the Continental Divide of the Cordilleras de Celaque and del Merendón in Departamento Ototepeque, Honduras (Heyer et al. 2002). Leptodactylus silvanimbus was not clearly allocated to any previously defined species group of Leptodactylus in its original description. Subsequently, L. silvanimbus was considered a member of the *melanonotus* group (Mc Craine et al. 1986, Heyer et al. 1996), or as a part of a melanonotus-ocellatus clade (Larson and de Sá 1998). Molecular data analyses of 12S and 16S mitochondrial genes indicated that L. silvanimbus does not show a clear relationship with the "traditional" species groups of Leptodactylus, however these data suggested that L. silvanimbus may have a basal position within Leptodactylus (Heyer et al. 2005, de Sá et al. 2005).

Herein we describe the karyotype of *L.* silvanimbus, which presents a diploid number not reported to any species allocated in the former genus *Leptodactylus*. The karyotype of *L. silvanimbus* is compared to the karyotypes of *L. gracilis, L. mystacinus (fuscus group), L.* petersii, *L. pustulatus (melanonotus group), L.*



Figure 1 - (A) Leptodactylus silvanimbus (from Heyer et al. 2002), (B) L. gracilis, (C) L. labyrinthicus, (D) L. macrosternum, (E) L. mystacinus, (F) L. ocellatus, (G) L. petersii, (H) L. pustulatus. Photos: Felipe F. Curcio (B, C, D, E, G), Gabriel Skuk (F) and Vinicius Xavier (H).

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macrosternum, L. ocellatus (ocellatus group), and L. labyrinthicus (pentadactylus group). The karyotypes of L. petersii, L. pustulatus, L. macrosternum, and L. silvanimbus are here reported for the first time and those of L. gracilis, L. mystacinus, L. ocellatus, and L. labyrinthicus are reported from new localities. Finally we compare the new karyological data reported with those know for Adenomera, Lithodytes, and Vanzolinius, as well as to other related Leptodactylidae.

Material and Methods

Cytogenetic analyses were carried out on 17 specimens of Leptodactylus: L. gracilis, L. mystacinus (fuscus group), L. petersii, L. pustulatus (melanonotus group), L. macrosternum, L. ocellatus (ocellatus group), L. labyrinthicus (pentadactylus group), and L. silvanimbus (Figure 1). Localities, number and sex of the individuals are provided in Appendix II and Figure 2.



Figure 2 - Localities of Leptodactylus analyzed in this paper. (1) Ocotepeque, Belén Gualcho, Honduras - L. silvanimbus;
(2) Igarapé Camaipi, AP - L. petersii; (3) Palmeirante, TO - L. macrosternum and L. pustulatus; (4) Palmas, TO - L. macrosternum; (5). Caracol, PI - L. labyrinthicus; (6) Jauru, MT - L. mystacinus; (7) Chapada dos Guimarães, MT - L. mystacinus; (8) Buri, SP - L. ocellatus; (9) Piedade, SP - L. ocellatus; (10) Juquitiba, SP - L. ocellatus; (11) São Bernardo do Campo, SP - L. gracilis; (12) Jambeiro, SP - L. mystacinus.

Mitotic metaphases were obtained either from bone marrow, liver, spleen or intestine after *in vivo* colchicine treatment according to conventional protocols (Bogart 1973b, Schmid 1978) or from fibroblast culture (Amaro-Ghilardi *et al.* 2004). Chromosome studies were performed after Giemsa and Ag-NOR staining (Howell and Black 1980).

Results

The karyotype of L. silvanimbus consists of 24 chromosomes, with pairs 1 to 6 submetacentrics and pairs 7 to 12 metacentrics (Figure 3A). All other Leptodactylus have karyotypes with 2n=22. In L. petersii pairs 1 to 5 are submetacentrics and pairs 6 to 11 are metacentrics (Figure 3B). In L. pustulatus pairs 1 to 4 and pair 7 are submetacentrics; pairs 5, 6, and 8 to 11 are metacentrics (Figure 3C). In L. labyrinthicus and L. ocellatus pairs 1, 5, 6, 9, 10 and 11 are metacentrics and pairs 2, 3, 4, 7 and 8 are submetacentrics (Figure 3D and 4A). The karyotype of L. macrosternum is similar to that of L. labyrinthicus and L. ocellatus, except for pair 8 that is metacentric instead of submetacentric (Figure 4B). In L. mystacinus pairs 1 to 4 and 7 and 8 are submetacentrics and pairs 5, 6, 9, 10 and 11 are metacentrics (Figure 4C). Leptodactylus gracilis has a karyotype similar to L. mystacinus, except for pair 8 that is metacentric (Figure 4D).

Presence of a secondary constriction was detected in different positions of pair 8: at the terminal region of the short arm of *L. labyrinthicus* and *L. ocellatus* (Figure 3D and Figure 4A); at the interstitial region of the short arm of *L. gracilis* (Figure 4D) and at interstitial portion of the long arm of *L. mystacinus* from Mato Grosso State (Figure 4C).

Nucleolar organizer regions (Ag-NORs) were detected in two chromosome pairs: pair 4 at the proximal region of the long arm of in *L. petersii* (Figure 5B) and *L. mystacinus* from Jambeiro, São Paulo state (Figure 6C) and pair 8 of the other species, including individuals of *L.*

mystacinus from Chapada dos Guimarães e Jauru, Mato Grosso state (Figures 5A-C, 6). In *L. silvanimbus, L. ocellatus, L. macrosternum, L. pustulatus,* and *L. labyrinthicus,* Ag-NORs were detected at the terminal region of the short arm of pair 8 (Figures 5A-C, 6A,B) whereas in *L. gracilis* they were found interstitially at the short arm of this pair (Figure 6D). *Leptodactylus mystacinus* showed Ag-NORs interstitially at the long arm of pair 8 in the individuals from Mato Grosso state (Figure 6C).

Discussion

Heretofore all species of Leptodactylus (sensu Frost 2004) cytogenetically studied had 2n=22. Karyotypes presented here for L. gracilis, L. labyrinthicus, L. mystacinus, and L. ocellatus are similar to those previously reported (Barbieri 1950, Saez and Brum 1960, Bianchi and Molina 1967, Beçak 1968, Brum-Zorrilla and Saez 1968, Denaro 1972, Barrio 1973, Bianchi et al. 1973, Bogart 1974, Kasahara et al. 1998, Silva et al. 2000, 2004, 2006, Baldo 2002, Amaro-Ghilardi et al. 2004). Leptodactylus silvanimbus is unique among the species previously included in Leptodactylus showing a karyotype consisting of 24 chromosomes. Nevertheless this diploid number has been reported for Adenomera marmorata, a taxon now included in Leptodactylus. As Adenomera marmorata has six pairs of telocentric chromosomes (Bogart 1974), its karyotype shows striking differences when compared with that of L. silvanimbus which encompasses only biarmed chromosomes. In Adenomera andreae and A. hylaedactyla, another diploid number (2n=26) was reported (Bogart 1970, 1973a; 1974). The karyotype of Lithodytes lineatus, another taxon included in Leptodactylus by Frost et al. (2006), are composed of 18 biarmed chromosomes (Bogart 1970) and Vanzolinius discodactylus has a 2n=22 karyotype very similar to the other Leptodactylus above referred (Heyer and Diment 1974). Results of Frost et al. (2006) and Amaro-Ghilardi et al.



Figure 3 - Conventional stained karyotypes of *Leptodactylus*. (A) *L. silvanimbus* (2n=24), (B) *L. petersii* (2n=22), (C) *L. pustulatus* (2n=22), (D) *L. labyrinthicus* (2n=22). Note the presence of terminal secondary constriction in the short arm of pair 8 of *L. labyrinthicus* (D). Bar=10μm.

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Figure 4 - Conventional stained karyotypes with 2n=22 of Leptodactylus. (A) L. ocellatus, (B) L. macrosternum, (C) L. mystacinus, (D) L. gracilis. Note the presence of terminal secondary constriction in one homologue of short arm of pair 8 of L. ocellatus (A), at interstitial portion of long arm of pair 8 of L. mystacinus (C) and in the interstitial region of short arm of pair 8 of L. gracilis (D). Bar=10µm.

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Figure 5 - Localization of NORs in Leptodactylus. (A) L. silvanimbus (pair 8), (B) L. petersii (pair 4), (C) L. pustulatus (pair 8), (D) L. labyrinthicus (pair 8).



Figure 6 - Localization of NORs in Leptodactylus. (A) L. ocellatus (pair 8), (B) L. macrosternum (pair 8), (C) L. mystacinus [pair 8 to animals from Mato Grosso state and pair 4 (in the rectangle) to the individual from São Paulo state], (D) L. gracilis (pair 8).

Grant *et al.* (2006) suggest that the clade including *Paratelmatobius* and *Scythrophrys* is the sister group of *Leptodactylus*, and in both genus karyotypes with 24 chromosomes were observed (De Lucca *et al.* 1974, Lourenço *et al.* 2000, 2001, 2003).

Taking a conservative approach to the contents of Leptodactylus (i.e., excluding Adenomera, Lithodytes, and Vanzolinius) the distinct diploid number of L. silvanimbus could be interpreted according to two different hypotheses. Firstly, the 2n=24 karyotype in Leptodactylus could be derived from an ancestral karyotype with 2n=22, similar to that of other species of Leptodactylus (sensu Frost 2004), due to one event of chromosomal fission, followed by pericentric inversions in the pairs resulted from the fission, since only biarmed chromosomes are found in the 2n=24 karyotype. Alternatively, the 2n=24 karyotype of L. silvanimbus could represent the ancestral diploid number for Leptodactylus and the 2n=22 karyotypes would represent the derived condition in the genus. This second hypothesis would require a pericentric inversion followed by centric fusion. Phylogenetic reconstructions based on sequences of 12S and 16S mitochondrial genes was not able to solve the relationship of L. silvanimbus to other species of Leptodactylus; however it suggested that L. silvanimbus could have a basal position within the genus (de Sá et al. 2005, Heyer et al. 2005).

Considering the recent systematic changes proposed for *Leptodactylus* (Frost *et al.* 2006), we then have four diploid numbers in the genus: 2n=18, 2n=22, 2n=24, and 2n=26. Under this scenario, the sister group of *Leptodactylus* is the clade formed by *Paratelmatobius-Scythrophrys* both with 2n=24. Under this hypothesis, a diploid number of 2n=24 would be considered the ancestral diploid number for *Leptodactylus*, and the karyotypes of most *Leptodactylus* (2n=22) and the subgenus *Lithodytes* (2n=18 and 2n=26) would represent derived conditions within *Leptodactylus*.

NORs were detected in two distinct sites: pair 4 in L. petersii and L. mystacinus from São Paulo state and pair 8 in L. gracilis, L. labyrinthicus, L. macrosternum, L. mystacinus from Mato Grosso state, L. pustulatus, L. ocellatus, and L. silvanimbus. Previously, Ag-NORs were described in pair 8 for many species of Leptodactylus and in pair 4 of L. mystacinus from Misiones, Argentina and from Descalvado e Mogi das Cruzes, São Paulo state (Silva et al. 2000, 2004, 2006, Baldo 2002, Amaro-Ghilardi et al. 2004). In the individuals of L. mystacinus from Descalvado analyzed by Silva et al. (2006) were also detected an inter- and intra-individual variation of number and location of NORs, in pairs 4 and 8, and pair 4 were considered the main NOR-bearing pair. This intraspecific polymorphism in the position of Ag-NORs observed in L. mystacinus is uncommon among amphibians and was observed in few cases, both in inter- and intrapopulational levels, e.g. Hyla chrysoscelis and H. versicolor (Willey et al. 1989), Bufo terrestris (Foote et al. 1991), Rana japonica (Miura 1994), Agalychnis callidryas (Schmid et al. 1995), Hyla ebraccata (Kaiser et al. 1996), Physalaemus cuvieri (Silva et al. 1999), and Physalaemus petersi (Lourenço et al. 1998). Our sample of L. mystacinus is not sufficient to determine if all individuals from the populations studied show only one NOR-bearing pair; more individuals must be studied to confirm the fixation of only one pair of NORs in these populations.

Species with NORs in pair 8 showed differences in the exact location along the chromosome. In L. chaquensis, L. fuscus, L. knudseni, L. labyrinthicus, L. macrosternum, L. notoaktites, L. ocellatus, L. pentadactylus, L. pustulatus, and L. silvanimbus Ag-NORs were detected terminally at the short arm. In L. bufonius, L. elenae, L. fuscus from Argentina, L. gracilis, L. mystacinus from Cordoba, Salta and Tucuman, Argentina and L. plaumanni NORs were found interstitially at the short arm and in L. mystacinus from Mato Grosso state and L. podicipinus they were observed interstitially at the long arm of pair 8 (Silva *et al.* 2000, 2004, Baldo 2002, Amaro-Ghilardi *et al.* 2004, present study). These differences in the localization of NORs could be explained by inversion, in the cases of *L. gracilis*, *L. mystacinus* from Mato Grosso state, and *L. podicipinus*, or translocation or transposition, in the case of *L. petersii* and *L. mystacinus* from Misiones and São Paulo, as previously proposed by Silva *et al.* (2000), for example.

The cytogenetic data obtained for L. silvanimbus found a new chromosomal number, 2n=24, for the genus Leptodactylus (sensu stricto, i.e., besides the subgenus Lithodytes). Furthermore, under the most recent molecular hypothesis, and heretofore the largest available analysis of amphibian relationships, this karyotype would represent the ancestral diploid number for the genus.

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Species	2n	Reference
L. albilabris	22	Bogart 1974
L. bolivianus	22	León 1970; Heyer and Diment 1974
L. bufonius	22	Barbieri 1950, Bogart 1967, 1974, Brum-Zorrilla and Saez
0		1968, Heyer and Diment 1974, Baldo 2002
L. chaquensis	22	Barbieri 1950, Brum-Zorrilla and Saez 1968, Heyer and Diment
*		1974, Baldo 2002
L. elenae	22	Heyer 1978, Baldo 2002
L. fuscus	22	Beçak et al. 1970, Bogart 1974, Heyer and Diment 1974, Silva
v		et al. 2000, Baldo 2002
L. geminus	22	Barrio 1973
L. gracilis	22	Denaro 1972, Barrio 1973, Bogart 1974, Baldo 2002, Silva et
0		al. 2004, present study
L. insularum	22	Bogart 1974
L. knudseni	22	Heyer 1972, Amaro-Ghilardi et al. 2004
L. labialis	22	León 1970, Bogart 1974
L. labyrinthicus		
(L. pentadactylus	22	Denaro 1972, Kasahara et al. 1998, Silva et al. 2000, Baldo
labyrinthicus)		2002, present study
L. laticeps	22	Brum-Zorrilla and Saez 1968
L. latinasus	22	Barbieri 1950, Brum-Zorrilla and Saez 1968, Bogart 1974,
(L. prognathus)		Baldo 2002
L. macrosternum	22	Present study
L. melanonotus	22	Bogart 1967, 1974, Heyer and Diment 1974
L. mystaceus	22	Bogart 1974, Heyer and Diment 1974
L. mystacinus	22	Bogart 1974, Baldo 2002, Silva et al. 2006, present study
L. natalensis	22	Bogart 1974
L. notoaktites	22	Silva et al. 2000
L. ocellatus	22	Barbieri 1950, Saez and Brum 1960, Bianchi and Molina 1967,
		Beçak 1968, Brum-Zorrilla and Saez 1968, Bianchi et al. 1973,
		Bogart 1974, Kasahara et al. 1998, Silva et al. 2000, Baldo
		2002, Amaro-Ghilardi et al. 2004, present study
L. pentadactylus	22	Brum-Zorrilla and Saez 1968, Morescalchi and Gargiulo 1968,
		Morescalchi et al. 1968, Morescalchi 1970, Bogart 1974, Heyer
		and Diment 1974, Amaro-Ghilardi et al. 2004
L. petersii	22	Present study
L. plaumanni	22	Baldo 2002, Silva et al. 2004
L. podicipinus	22	Denaro 1972, Bogart 1974, Savage and De Weese 1979, Silva
		et al. 2000, Baldo 2002
L. pustulatus	22	Present study
L. rhodonotus	22	Bogart 1974
L. silvanimbus	24	Present study
L. wagneri	22	Bogart 1974, Heyer and Diment 1974, Savage and De Weese 1979

Appendix I - Available information on the karyotypes of Leptodactylus

Karyotypes of eight species of Leptodactylus (Anura, Leptodactylidae) with a description of a new karyotype

Appendix II - Specimens Studied

Leptodactylus silvanimbus – HONDURAS: Ocotepeque, Belén Gualcho (14°19'N, 88°47'W), AF 95 (female).

Leptodactylus gracilis – BRAZIL: São Bernardo do Campo, SP (23°41'S, 46°33'W), AF 1002, 1003 (males).

Leptodactylus petersii – BRAZIL: Igarapé Camaipi, AP (0°010'S, 51°53'W), AF 938 (female).

Leptodactylus mystacinus – BRAZIL: Aproveitamento Múltiplo de Manso, Chapada dos Guimarães, MT (15°27'S, 55°44'W), AF 742 (male); Usina Hidrelétrica Guaporé, Jauru, MT (15°20'S, 58° 51'W), AF 1416 (female); Jambeiro, SP (23°18' S, 45°41'W), AF 1641 (male).

Leptodactylus ocellatus – BRAZIL: Piedade,

SP (23°42'S, 47°25'W), AF 1227 (female); Juquitiba, SP (23°55'S, 47°04'W), AF 1308 (female); Buri, SP (23°47'S, 48°35'W), AF 1374 (female).

Leptodactylus labyrinthicus – BRAZIL: Caracol, PI (09°16'S, 43°19'W), AF 876 (male); Usina Hidrelétrica Guaporé, Jauru, MT (15°20'S, 58, 51'W), AF 1417 (male).

Leptodactylus macrosternum – BRAZIL: Usina Hidrelétrica Lajeado, Palmas, TO (10°12'S, 48°21'W), AF 1266 (male); Palmeirante, TO (07°51'S, 47°55'W), AF 1565 (female), AF 1568 (female).

Leptodactylus pustulatus – BRAZIL: Palmeirante, TO (07°51'S, 47°55'W), AF 1566 (male), AF 1567 (female).