

# 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*, *Paratelmatoebius*, *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*, *Paratelmatoebius*, 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 C-banding patterns in *Leptodactylus gracilis gracilis*, *L. gracilis delattini*, *L. knudseni*, *L. labyrinthicus*, *L. notoakites*, *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 polymorphism 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.*

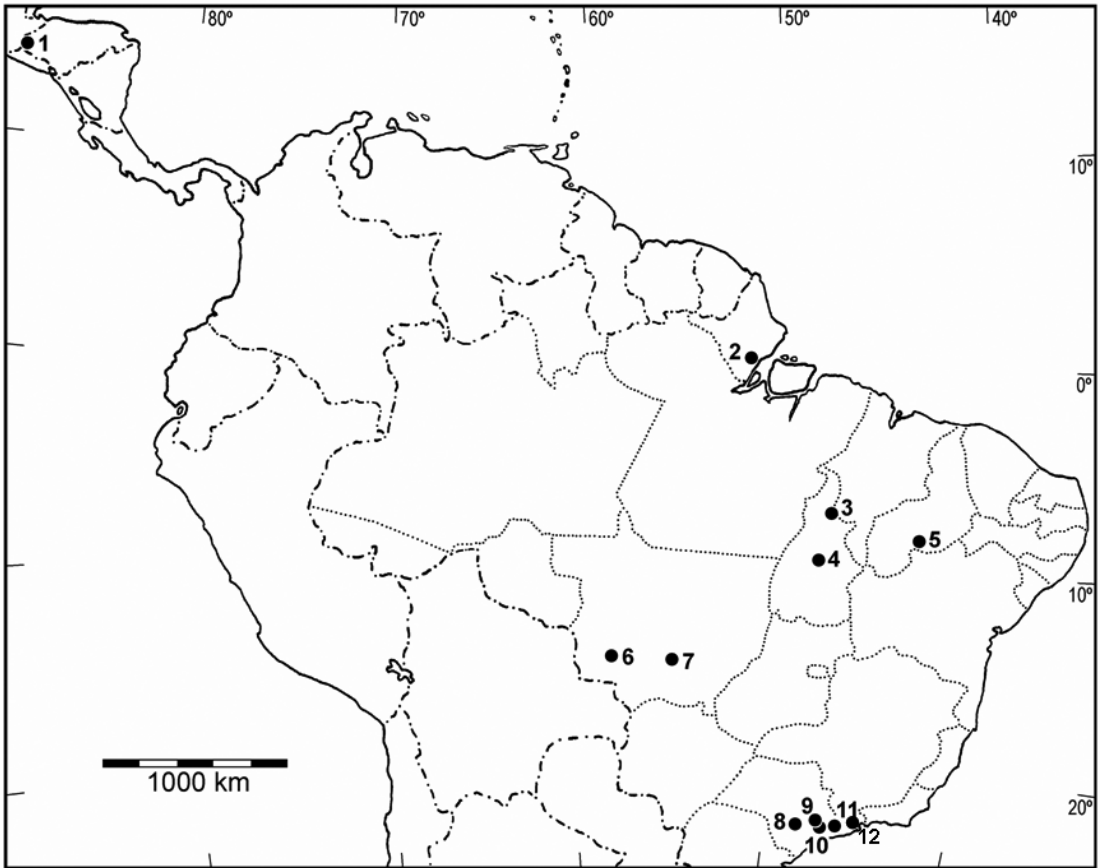


**Figure 1** - (A) *Leptodactylus silvanimbus* (from Heyer *et al.* 2002), (B) *L. gracilis*, (C) *L. labyrinthicus*, (D) *L. macrosternum*, (E) *L. mystacinus*, (F) *L. ocellatus*, (G) *L. petersii*, (H) *L. pustulatus*. Photos: Felipe F. Curcio (B, C, D, E, G), Gabriel Skuk (F) and Vinicius Xavier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

*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) Ocatepeque, 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) Juititaba, SP - *L. ocellatus*; (11) São Bernardo do Campo, SP - *L. gracilis*; (12) Jambreiro, 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).

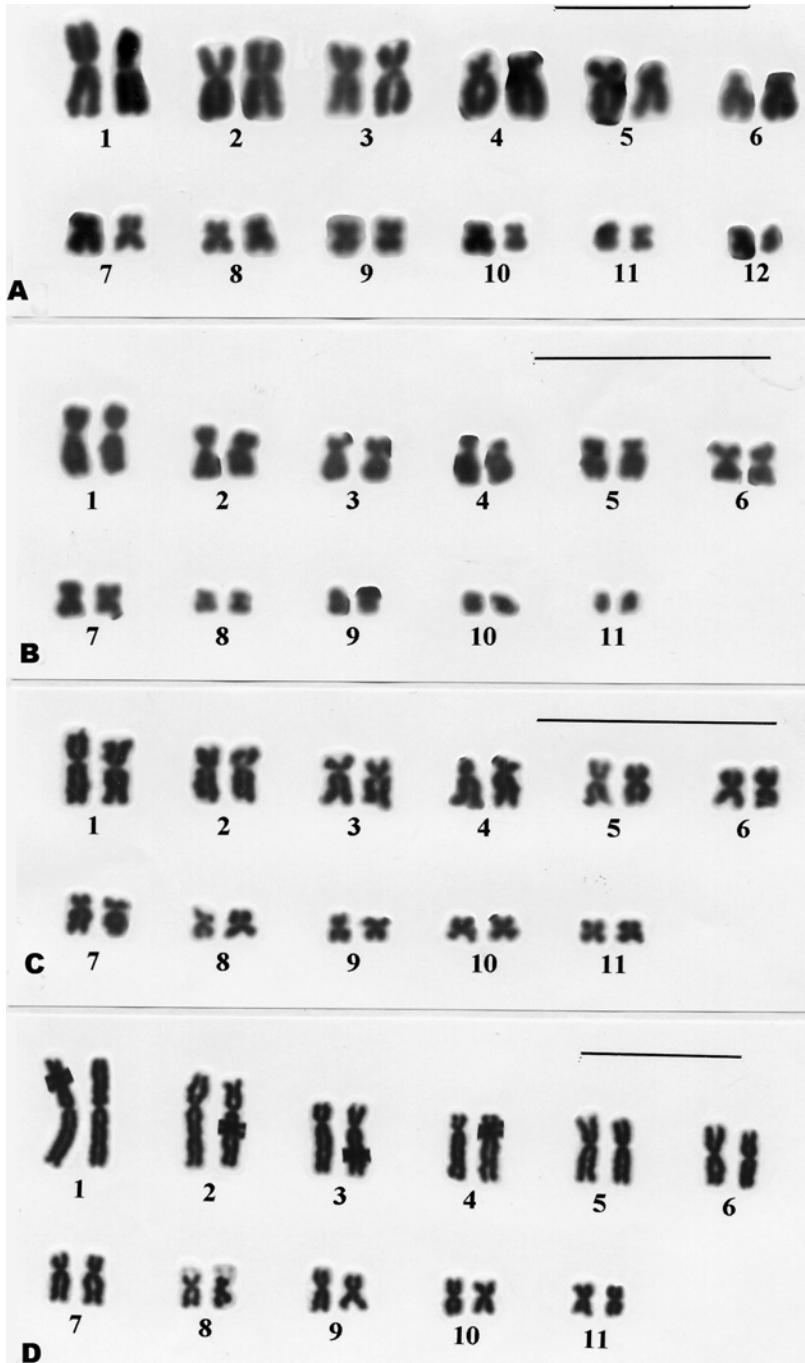
Presence of a secondary constriction was detected in different positions of pair 8: at the terminal region of the short arm of *L. labyrinthicus* and *L. ocellatus* (Figure 3D and Figure 4A); at the interstitial region of the short arm of *L. gracilis* (Figure 4D) and at interstitial portion of the long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

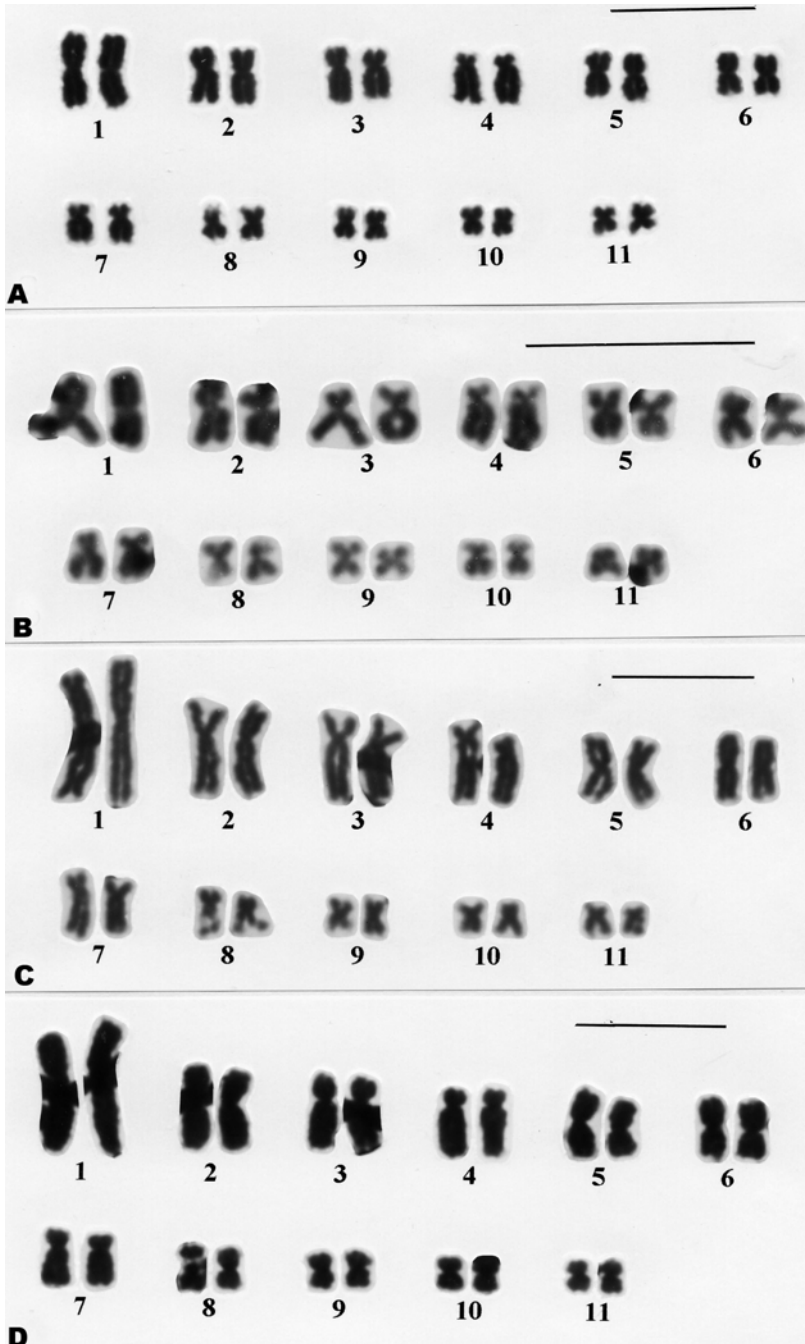
## Discussion

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

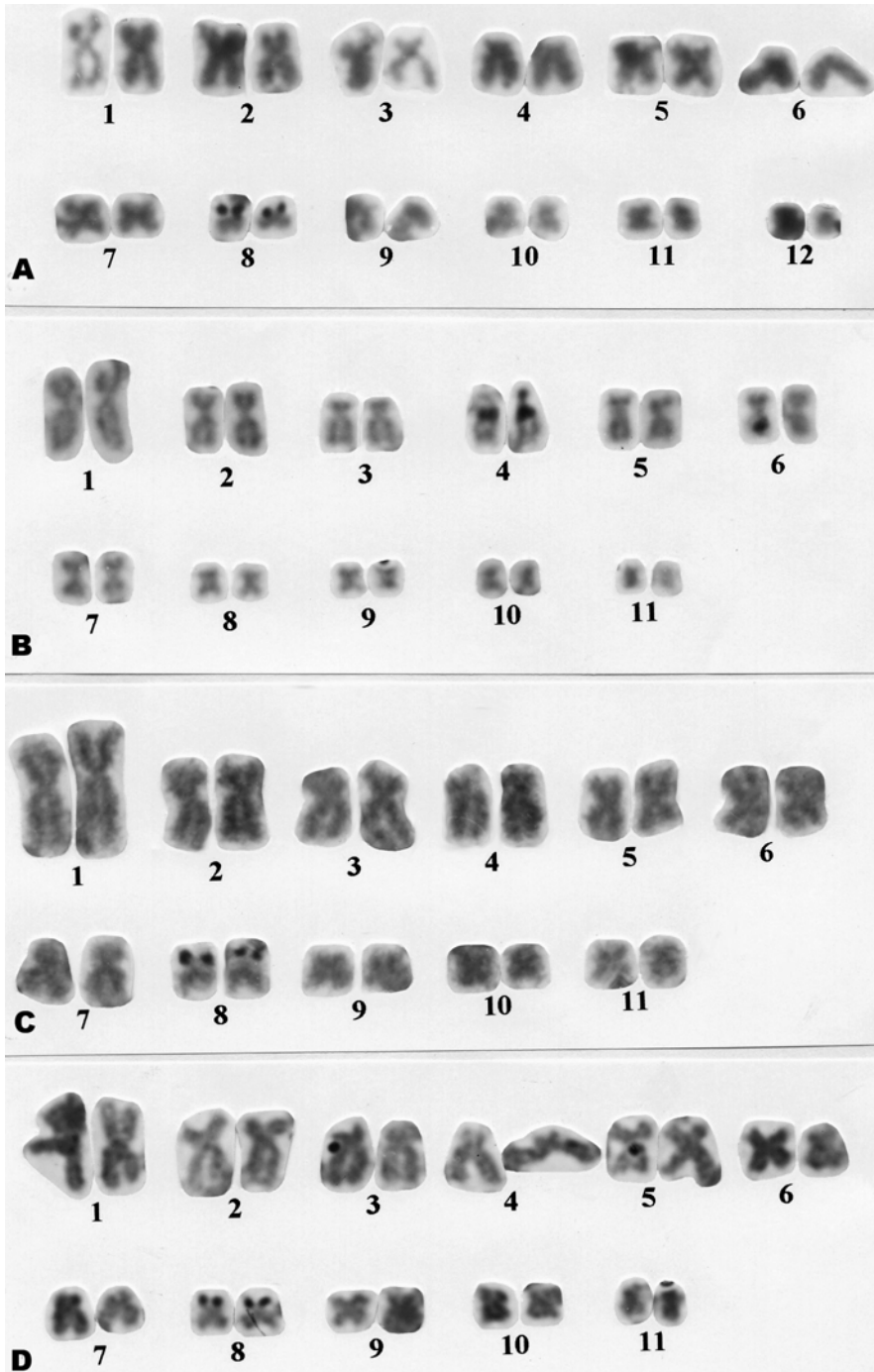


**Figure 3** - Conventional stained karyotypes of *Leptodactylus*. (A) *L. silvanimbus* (2n=24), (B) *L. petersii* (2n=22), (C) *L. pustulatus* (2n=22), (D) *L. labyrinthicus* (2n=22). Note the presence of terminal secondary constriction in the short arm of pair 8 of *L. labyrinthicus* (D). Bar=10 $\mu$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



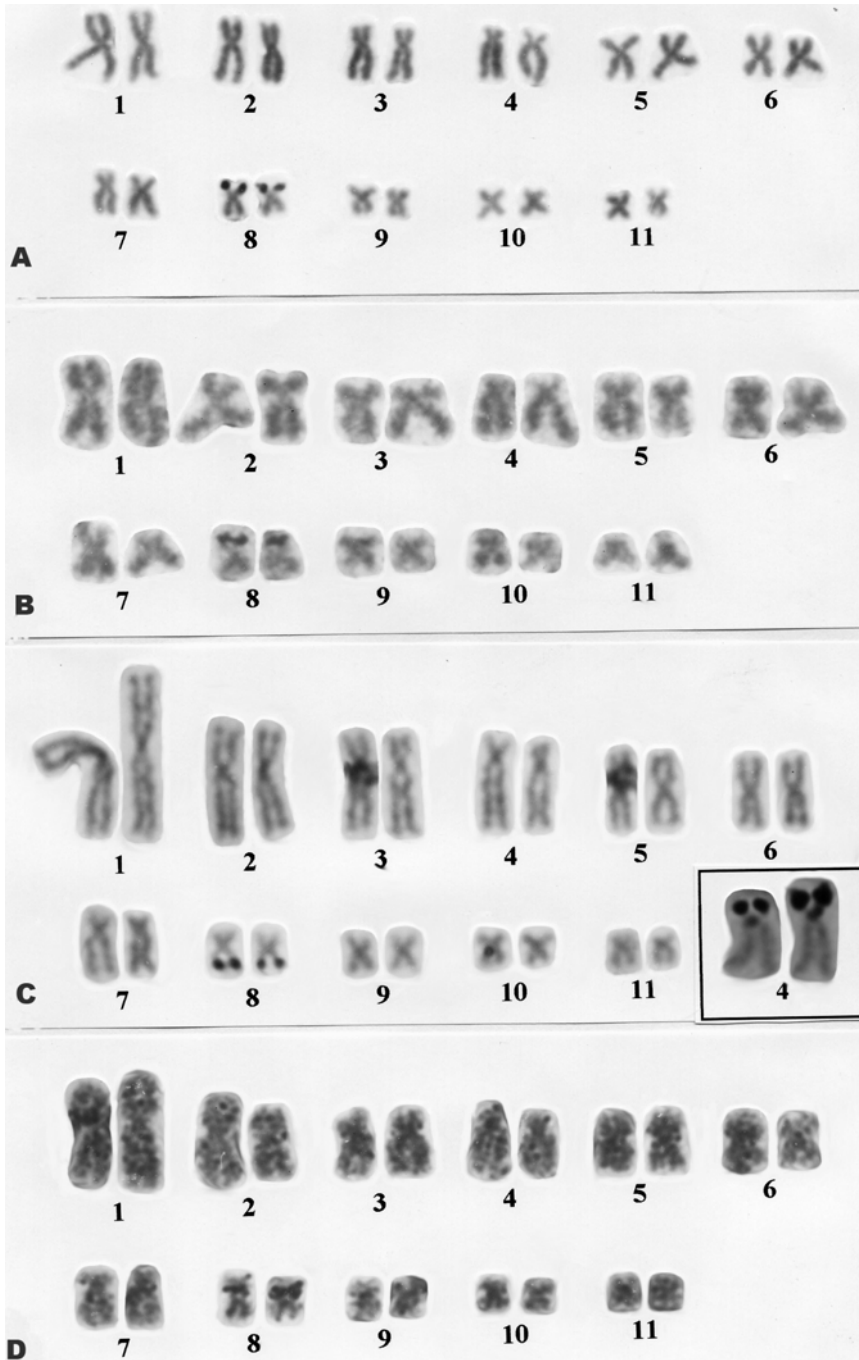


**Figure 4** - Conventional stained karyotypes with  $2n=22$  of *Leptodactylus*. (A) *L. ocellatus*, (B) *L. macrosternum*, (C) *L. mystacinus*, (D) *L. gracilis*. Note the presence of terminal secondary constriction in one homologue of short arm of pair 8 of *L. ocellatus* (A), at interstitial portion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



**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 *Vanazolinius*) 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*, currently only reported for *L. silvanimbus*, 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 intrapopulation 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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### References

- Amaro-Ghilardi, R. C., M. T. Rodrigues and Y. Yonenaga-Yassuda. 2004. Chromosomal studies after differential staining and fluorescence *in situ* hybridization using telomeric probe in three *Leptodactylus* species (Leptodactylidae, Anura). *Caryologia* 57: 53–65.
- Baldo, J. D. 2002. Estudios cromossômicos de las especies argentinas del género *Leptodactylus* Fitzinger 1826 (Amphibia, Anura). Unpublished Ph.D. Thesis. Universidad Nacional de Misiones, Argentina.
- Barbieri, F. D. 1950. Observaciones sobre los cromosomas y espermatozoides en algunos batracios del genero *Leptodactylus*. *Acta Zoologica Lilloana* IX: 455–463.
- Barrio, A. 1973. *Leptodactylus geminus*, una nueva especie del grupo *fuscus* (Anura, Leptodactylidae). *Physis* 32: 199–206.
- Beçak, M. L. 1968. Chromosomal analysis of eighteen species of Anura. *Caryologia* 21: 191–208.
- Beçak, M. L., L. Denaro and W. Beçak. 1970. Polyploidy and mechanisms of karyotypic diversification in Amphibia. *Cytogenetics* 9: 225–238.
- Bianchi, N. O. and J. O. Molina. 1967. DNA replication patterns in somatic chromosomes of *Leptodactylus ocellatus* (Amphibia, Anura). *Chromosoma* 22: 391–400.
- Bianchi, N. O., M. S. Bianchi and L. Vidal-Rioja. 1973. Heterochromatin late replication and secondary constrictions in the chromosome complement of *Leptodactylus ocellatus*. *Caryologia* 26: 397–403.
- Bogart, J. P. 1967. Chromosomes of the South American amphibians family Ceratophryidae with a reconsideration of the taxonomic status of *Odontophrynus americanus*. *Canadian Journal of Genetics and Cytology* 9: 531–542.
- Bogart, J. P. 1970. Los cromosomas de anfibios anuros del genero *Eleutherodactylus*. *Acta IV Congresso Latinoamericano de Zoologia* 1: 65–78.
- Bogart, J. P. 1973a. Evolution of anuran karyotypes. Pp. 337–349 in J. L. (ed.), *Evolutionary Biology of the Anurans*. Columbia, University of Missouri Press.
- Bogart, J. P. 1973b. Method for obtaining chromosomes. *Caldasia* XI: 29–40.
- Bogart, J. P. 1974. A karyosystematic study of frogs in the genus *Leptodactylus* (Anura: Leptodactylidae). *Copeia* 1974: 728–737.

- Brum-Zorilla, N. and F. A. Saez. 1968. Chromosomes of Leptodactylidae (Amphibia Anura). *Experientia* 24: 969.
- De Lucca, E. J., J. Jim and F. Foresti. 1974. Chromosomal studies in twelve species of Leptodactylidae and one Brachycephalidae. *Caryologia* 27: 183–192.
- de Sá, R. O., W. R. Heyer and A. Camargo. 2005. A phylogenetic analysis of *Vanzolinius* Heyer, 1974 (Amphibia, Anura, Leptodactylidae): Taxonomic and life history implications. *Arquivos do Museu Nacional do Rio de Janeiro* 63: 707–726.
- Denaro, L. 1972. Karyotypes of Leptodactylidae frogs. *Journal of Herpetology* 6: 71–74.
- Footo, D. L., J. E. Wiley, M. L. Little, and J. Meyne. 1991. Ribosomal RNA gene site polymorphism in *Bufo terrestris*. *Cytogenetics and Cell Genetics* 57: 196–199.
- Frost, D. R. 2004. *Amphibian Species of the World: an Online Reference*. Version 3.0. URL: <http://research.amnh.org/herpetology/amphibia/index.html>. American Museum of Natural History, New York, USA. Captured on 22 August 2004.
- Frost, D. R., T. Grant, J. Faivovich, R. H. Bain, A. Haas, C. F. B. Haddad, R. O. de Sá, A. Channing, M. Wilkinson, S. C. Donnellan, C. J. Raxworthy, J. A. Campbell, B. L. Blotto, P. Moler, R. C. Drewes, R. A. Nussbaum, J. D. Lynch, D. M. Green, and W. C. Wheeler. 2006. The amphibian tree of life. *Bulletin of the American Museum of Natural History* 297: 1–370.
- 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.
- Heyer, R. 1969. The adaptive ecology of the species groups of the genus *Leptodactylus* (Amphibia, Leptodactylidae). *Evolution* 23: 421–428.
- Heyer, W. R. 1972. The status of *Leptodactylus pumilio* Boulenger (Amphibia, Leptodactylidae) and the description of a new species of *Leptodactylus* from Ecuador. *Contributions in Science of the Natural History Museum of Los Angeles County* 231: 1–8.
- Heyer, W. R. 1978. Systematics of *fuscus* group of the frog genus *Leptodactylus* (Amphibia, Leptodactylidae). *Science Bulletin of the Natural History Museum of Los Angeles County* 29: 1–85.
- Heyer, W. R. and M. J. Diment. 1974. The karyotype of *Vanzolinius discodactylus* and comments on usefulness of karyotypes in determining relationships in the *Leptodactylus*-complex (Amphibia, Leptodactylidae). *Proceedings of the Biological Society of Washington* 87: 327–336.
- Heyer, W. R., R. de Sá, J. R. Mc Craine, and L. D. Wilson. 1996. *Leptodactylus silvanimbus* (Amphibia: Anura: Leptodactylidae): natural history notes, advertisement call, and relationships. *Herpetological Natural History* 4: 169–174.
- Heyer, W. R., R. de Sá and S. Muller. 2002. *Leptodactylus silvanimbus*. *Catalogue of American Amphibians and Reptiles* 743: 1–3.
- Heyer, W. R., R. O. de Sá and S. Muller. 2005. The enigmatic distribution of the Honduran endemic *Leptodactylus silvanimbus* (Amphibia: Anura: Leptodactylidae). Pp 81–101 in M. A. Donnelly, C. Guyer and M. H. Wake (eds.), *Ecology and Evolution in the Tropics: a herpetological perspective*. Chicago, University of Chicago Press.
- 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.
- Kaiser, H., C. Mais, F. Bolaños, and C. Steinlein. 1996. Chromosomal investigation of three Costa Rican frogs from the 30-chromosome radiation of *Hyla* with description of a unique geographic variation in the nucleolus organizer regions. *Genetica* 98: 95–102.
- Kasahara, S., A. P. Z. Silva and S. L. Gruber. 1998. Use of lymphocyte cultures for BrdU replication banding patterns in anuran species (Amphibia). *Genetics and Molecular Biology* 21: 471–476.
- King, M. 1990. Amphibia in B. John and C. Gwent (eds.), *Animal Cytogenetics*. Volume 4. Chordata 2. Berlin, Gebrüder Borntraeger.
- Kuramoto, M. 1990. A list of chromosome numbers of anuran amphibians. *Bulletin of Fukuoka University of Education* 39: 83–127.
- Larson, P. M. and R. de Sá. 1998. Chondrocranial morphology of *Leptodactylus* larvae (Leptodactylidae: Leptodactylinae): its utility in phylogenetic reconstruction. *Journal of Morphology* 238: 287–305.
- León, P. E. 1970. Report of the chromosome numbers of some Costa Rican anurans. *Revista de Biología Tropical* 17: 119–124.
- Lourenço, L. B., P. C. A. Garcia and S. M. Recco-Pimentel. 2000. Cytogenetics of two species of *Paratelmatobius* (Anura: Leptodactylidae), with phylogenetic comments. *Hereditas* 133: 201–209.
- Lourenço, L. B., P. C. A. Garcia and S. M. Recco-Pimentel. 2001. A cytogenetic and molecular analysis of the genera *Paratelmatobius* and *Scythrophrys* (Anura, Leptodactylidae), with emphasis on the nucleolar organizer region (NOR). *Chromosome Research* 9: 71.
- Lourenço, L. B., P. C. A. Garcia and S. M. Recco-Pimentel. 2003. Cytogenetics of a new species of *Paratelmatobius cardosoi* group (Anura: Leptodactylidae), with

- the description of an apparent case of pericentromeric inversion. *Amphibia-Reptilia* 24: 47–55.
- Lourenço, L. B., S. M. Recco-Pimentel and A. J. Cardoso. 1998. Polymorphism of the nucleolus organizer regions (NORs) in *Physalaemus petersi* (Amphibia, Anura, Leptodactylidae) detected by silver staining and fluorescence *in situ* hybridization. *Chromosome Research* 6: 621–628.
- Mc Craine, J. R., L. D. Wilson and K. L. Williams. 1986. The tadpole of *Leptodactylus silvanimbus*, with comments on the relationships of the species. *Journal of Herpetology* 20: 560–562.
- Miura, I. 1994. Sex chromosome differentiation in the Japanese brown frog, *Rana japonica*. II. Sex-linkage analyses of the nucleolar organizer regions in chromosome no. 4 of the Hiroshima and Saeki populations. *Zoological Science* 11: 807–815.
- Morescalchi, A. 1970. Some trends of karyological evolution in the anura. *Acta do IV Congresso Latino de Zoologia* 1: 159–166.
- Morescalchi, A. and G. Gargiulo. 1968. Su alcune relazioni cariologiche del genere *Bufo* (Amphibia, Salientia). *Rendiconto dell'Accademia delle Scienze Fisiche e Matematiche* 35: 117–120.
- Morescalchi, A., G. Gargiulo and E. Olmo. 1968. Note citotassonomiche sui Leptodactylidae (Amphibia, Salientia). *Bollettino di Zoologia* 35: 333–334.
- Saez, F. A. and N. Brum. 1960. Chromosomes of South American amphibians. *Nature* 185: 945.
- Savage, J. M. and J. E. De Weese. 1979. A new species of leptodactylid frog, genus *Eleutherodactylus*, from Cordillera de Talamanca, Costa Rica. *Bulletin of the Southern California Academy of Sciences* 78: 107–115.
- Schmid, M. 1978. Chromosome banding in Amphibia I: Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. *Chromosoma* 66: 361–388.
- Schmid, M., W. Feichtinger, R. Weimwer, C. Mais, F. Bolaños, and P. León. 1995. Chromosome banding in Amphibia. XXI. Inversion polymorphism and multiple nucleolus organizer regions in *Agalychnis callidryas* (Anura, Hylidae). *Cytogenetics and Cell Genetics* 69: 18–26.
- Silva, A. P. Z., C. F. B. Haddad and S. Kasahara. 1999. Nucleolus organizer regions in *Physalaemus cuvieri* (Anura, Leptodactylidae), with evidence of a unique case of Ag-NOR variability. *Hereditas* 131: 135–141.
- Silva, A. P. Z., C. F. B. Haddad and S. Kasahara. 2000. Chromosomal studies on five species of the genus *Leptodactylus* Fitzinger, 1826 (Amphibia, Anura) using differential staining. *Cytobios* 103: 25–38.
- Silva, A. P. Z., P. C. A. Garcia, V. G. Martins, M. Bacci, and S. Kasahara. 2004. Chromosomal and molecular analyses of *Leptodactylus gracilis gracilis*, *L. gracilis delattini* and *L. plaumanni* (Anura, Leptodactylidae): taxonomic implications. *Amphibia-Reptilia* 25: 185–196.
- Silva, A. P. Z., C. F. H. Haddad, G. G. Galassi, and S. Kasahara. 2006. Multiple nucleolus organizer in *Leptodactylus mystacinus* (Amphibia, Anura) and comments on its systematic position in the *L. fuscus* group based on cytogenetic and molecular analyses. *Genetica* 127: 35–44.
- Wiley, J. E., M. L. Little, M. A. Romano, D. A. Blount, and G. R. Cline. 1989. Polymorphism in the location of the 18S and 28S rRNA genes on the chromosomes of the diploid-tetraploid tree frogs *Hyla chrysoscelis* and *H. versicolor*. *Chromosoma* 97: 481–487.

Appendix I - Available information on the karyotypes of *Leptodactylus*

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



## 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); Juititaba, 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).