Chromosomal and molecular analyses of *Leptodactylus gracilis gracilis*, *L. gracilis delattini*, and *L. plaumanni* (Anura, Leptodactylidae): taxonomic implications

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Cryptic species, i.e. morphologically undistinguishable ones, are relatively common among anurans of the Brazilian fauna. The characters generally used to separate these species have been, e.g., bioacoustics, colour patterns, ecology, and ethology of live animals (Cardoso, 1985; Scrocchi and Lavilla, 1986; Garcia-Pérez and Heyer, 1993; Heyer et al., 1996; Köhler and Lötters, 1999; Kwet et al., 2001). The group of *Leptodactylus fuscus* has a large distribution, from Panama throughout South America, east of the Andes, south to southern Brazil, Bolivia and Argentina (Frost, 2002). Among the representatives of the group, *Leptodactylus gracilis gracilis* Duméril and Bibron, 1841 and *L. plaumanni* Ahl, 1936 are cryptic species. The former is distributed in subtropical southern Brazil through Uruguay to Paraguay, Bolivia, and northern Argentina, whereas the second species occurs in northeastern Misiones Province (Argentina) and Rio Grande do Sul and Santa Catarina (Brazil). Heyer (1978) considered *Leptodactylus plaumanni* as synonym of *L. gracilis*, but Cardoso (1985) discarded this synonymy after evaluating the vocalization of both species. The latter author suggested the possibility that *L. plaumanni* is a senior synonym of *L. geminus* Barrio, 1973, which was recently confirmed by Kwet et al. (2001). *Leptodactylus gracilis delattini* also included in the *L. fuscus* group was described by Müller (1968), based on specimens collected in Distrito da Ilha do Campeche, Florianópolis municipality, State of Santa Catarina (Brazil). Based on the vocalizations of both *L. gracilis gracilis* and *L. gracilis delattini*, the subspecific status was rejected by Garcia-Pérez and Heyer (1993). Nevertheless, it is important to emphasize that these authors (Garcia-Pérez and Heyer, 1993) did not analyze, in fact, the vocalization of specimens from the Campeche Island, but a sample from the Campeche Beach, located in another site of the Santa Catarina Island.

For this reason, the conclusions about the validity of the subspecies *L. gracilis delattini* remained unsolved. More recently, one of us (P.C.A. Garcia, unpublished data) carried out studies on bioacoustics, morphology and ecology in animals from Campeche Island. The results were distinct from those of Garcia-Pérez and Heyer (1993), suggesting that *L. gracilis delattini* might be considered a valid species.

In 1974, Bogart described the conventionally stained karyotype of specimens referred to *L. gracilis*, without mentioning the collecting site of the animals. In the present work, cytogenetic and molecular data are presented for the first time for *L. gracilis gracilis*, *L. gracilis delattini*, and *L. plaumanni*. We use differential staining techniques, as Ag-NOR, C-banding, CMA3/DA/DAPI, 5-bromodeoxyuridine (BrdU) incorporation, and FISH with rDNA probes. Molecular analyses based on mitochondrial cytochrome b partial sequence were also employed. We discuss the karyological and molecular relationships of the cryptic species *L. gracilis gracilis* and *L. plaumanni* as well as the taxonomic status of *L. gracilis delattini*.

We studied cytogenetically 6 specimens of *Leptodactylus gracilis gracilis* (males CFBH 3795, CFBH 3797, CFBH 3799, CFBH 3801, and females CFBH 3802-03) from Córrego Grande (27°36′S 48°30′W), municipality of Florianópolis; 9 specimens of *L. gracilis delattini* (males CFBH 3805, CFBH 3809-10, CFBH 3812, and females CFBH 3798, CFBH 3804, CFBH 3807-08, CFBH 3811) from Campeche Island (27°42′S 48°28′W), municipality of Florianópolis; and 3 specimens of *L. plaumanni* (males CFBH 3902-03, and 1 juvenile CFBH 3905) from the municipality of Ipuaçú (26°37′S 52°27′W). All the three localities are in the State of Santa Catarina, Brazil. The voucher specimens are in Célio F.B. Haddad collection (CFBH), deposited in the Departamento de Zoologia, Instituto de Biociências, UNESP, Rio Claro, São Paulo, Brazil.

Cell suspensions were obtained directly from bone marrow, liver, spleen, and testis of the animals previously submitted to 0.01% colchicine treatment, in the proportion of 0.1 ml / 10 g animal weight, during 4 to 6 hours (Baldissera et al., 1993). To improve the mitotic index, Phytohemagglutinin-P (Difco) was injected in some specimens, in the same proportion of the colchicine, 48 to 72 hours before the sacrifice (Baker et al., 1971; Wiley, 1982). Lymphocyte cultures were also performed according to the procedures described by Kasahara et al. (1998). Some specimens were submitted to *in vivo* (Silva et al., 2000) or *in vitro* (Kasahara et al., 1998) treatment with 5-bromodeoxyuridine plus 5-fluorodeoroxyuridine.

The chromosome preparations were stained with a 2% Giemsa solution or submitted to the techniques of Ag-NORs (Howell and Black, 1980), C-banding (Sumner, 1972), triple staining CMA3/DA/DAPI (Schweizer, 1980), and *in situ* hybridization with fluorescent rDNA probes (Viegas-Péquignot, 1992) with rDNA probes HM 123 and HM 456 (Meunier-Rotival et al., 1979). For the differentiation of replication bands, after the BrdU treatment, the technique of Fluorochrome plus Giemsa (FPG) was used following Dutrillaux and Couturier (1981), as well as a 3% Giemsa staining prepared with a 2% 4Na-EDTA solution, according to Miura (1995).

Molecular analyses were performed in specimens of *L. gracilis gracilis* (CFBH 3795), *L. gracilis delattini* (CFBH 3798), and *L. plaumanni* (CFBH 3902). DNA sample was obtained from liver and muscle using Genomic Prep Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotec Inc). Mitochondrial cytochrome b gene amplification was obtained through PCR using L-strand MVZ 55 (5′-TGAGGACAAATATCATTCTGAGGGG-CTGCAG-3′) and H-strand MVZ 36 (5′-TCTTCTACTGGTTGTCCTCCGATTCA-3′). Both of them were developed in Museum of Vertebrate Zoology: California University, Bekerley, USA. PCR amplification were performed in 25 μl volumes containing 1.5 U of Taq DNA polymerase, 10 mM Tris-HCL, 50 mM KCl, 2.0 mM MgCl2, 200 μM each dNTP, 10 pM each primer and 200 to 500 of template DNA. PCR reaction was carried out on a PTC-100 MJ-Research according to the program: 95°C for 7 min followed by 35 cycles, each with a denaturation step of 94°C for 1 min; and an annealing step at 45°C for 1 min, and an extension step at 65°C for 8 min. PCR products were purified using GFX™ PCR DNA and Gel band purification Kit (Amersham Pharmacia Biotec Inc). The sequences were obtained in Centro de Estudos de Insetos Sociais (UNESP, Rio Claro) in automatic DNA sequencing ABI 377, dye terminator (Applied Biosystem).

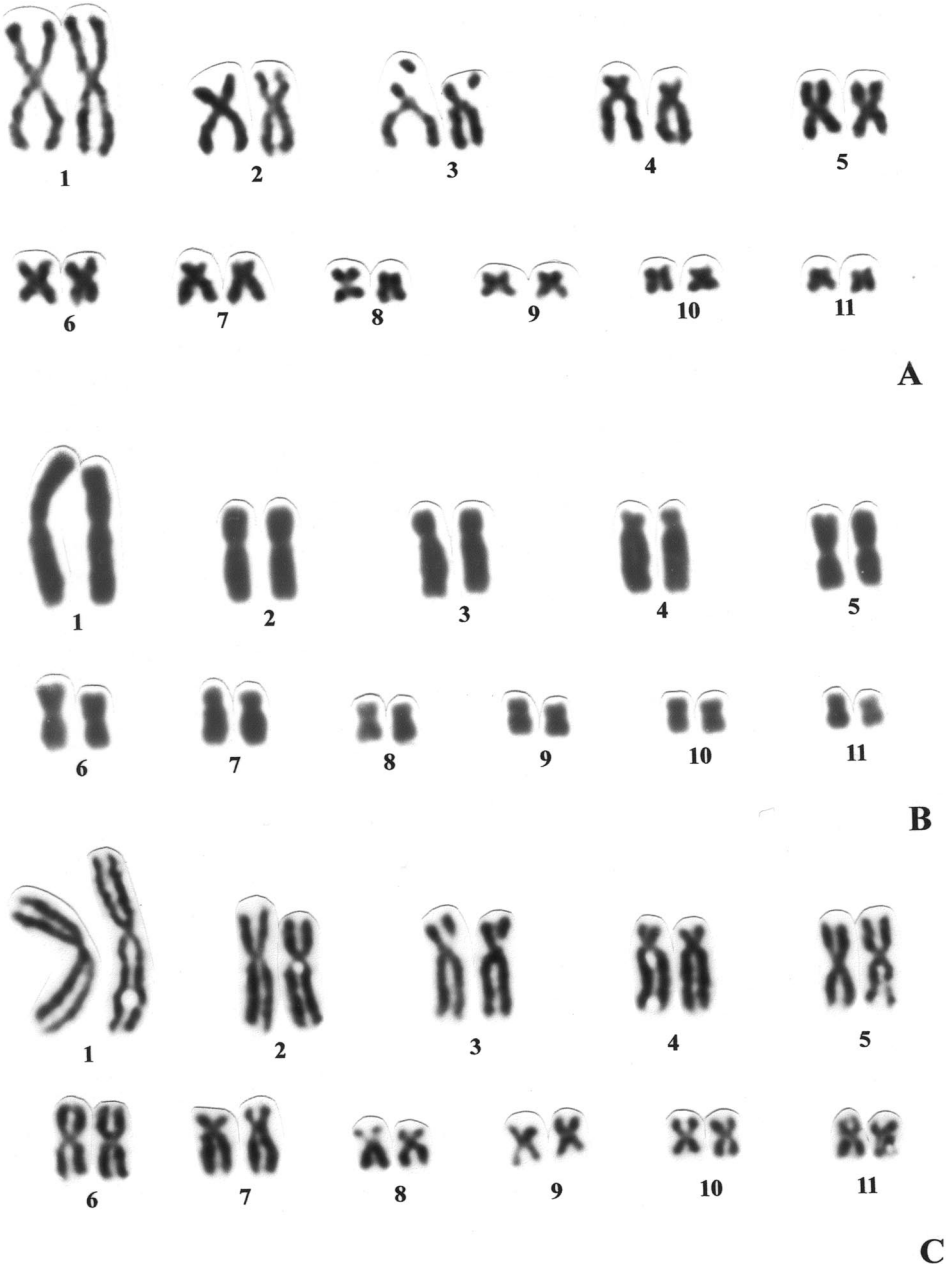

Figure 1. Karyotypes with $2n = 22$ after conventional staining. A: *L. gracilis gracilis* (male), B: *L. gracilis delattini* (female), and C: *L. plaumanni* (male).

Sequences were aligned using Clustal W computer program (Thompson et al., 1994) in BioEdit 5.0.9 (Hall, 1999). Sequences were analysed using maximum-parsimony in PAUP* 4b10 (Swofford, 1998).

Leptodactylus gracilis gracilis, *L. gracilis delattini*, and *L. plaumanni* have $2n = 22$ and similar karyotypes (fig. 1), including: a large metacentric pair (1), four medium submetacentric pairs (2, 3, 4, and 7), two medium metacentric pairs (5 and 6), and four small metacentric or submetacentric pairs (8, 9, 10, and 11). In the three karyotypes, the chromosomes of pair 8 have interstitial secondary constriction in the short arms, although not always visualized in the metaphases. One or both homologues of pair 2 appear in some metaphases with a chromatid break in the proximal region of the short arms, probably due to a fragility site in that region. No heteromorphic sex chromosome pair occurs in any of the karyotypes. The meiotic analysis of the male specimens confirmed the absence of morphologically differentiated sex chromosome pair.

Ag-NOR staining match the secondary constriction of pair 8 (fig. 2A, B, C). The FISH technique confirms the location of the rDNA sequences only in this chromosome pair (fig. 2D, E, F).

C-bands are in the centromeric region of all chromosomes of the three karyotypes. Besides there are a terminal band in the short arms of pair 1, a proximal band in the short arms of pair 8, and a proximal band in the long arms of pair 9 (fig. 3). No particular GC or AT-rich region was identified after CMA3/DA/DAPI staining in *L. gracilis gracilis* and *L. gracilis delattini*, with exception of the nucleolus organizer regions, which were highly CMA3 fluorescent and negatively DAPI stained in both karyotypes (fig. 4).

BrdU treatment differentiated replication bands along the chromosomes of *L. gracilis delattini*, after both FPG technique and 4Na-EDTA Giemsa staining (fig. 5A, B). This last procedure also produced another pattern, in which some heavily stained bands appeared along the chromosomes (fig. 5C).

Our comparative results based on 391 bp of mitochondrial cytochrome b partial sequences showed that *L. gracilis gracilis* (AY332229) and *L. gracilis delattini* (AY332230) form a sister group to *L. plaumanni* (AY332231), which diverged before them (fig. 6). We observed a high identity (99%) between *L. gracilis gracilis* and *L. gracilis delattini*, indicating that these animals can be regarded as a valid subspecies.

Discussion. *Leptodactylus gracilis gracilis*, *L. gracilis delattini*, and *L. plaumanni* have a karyotypic pattern which is commonly observed in the genus (Beçak, 1968; Beçak et al., 1970; Denaro, 1972; Bogart, 1974; Lisanti et al., 1990; Agostinho, 1994; Baldissera and Batistic, 1992; Amaro, 1999; Silva et al., 2000). The *L. fuscus* group comprises a total of 27 species (Heyer, 1969; Heyer, 1978; Frost, 1985; Duellman, 1993; Frost, 2002), of which only nine species have been karyotyped. Among these species, *L. latinasus* has a discrepant karyotype with telocentric elements. Based on this finding, Bogart (1974) suggested the inclusion of *L. latinasus* in the group of *L. melanonotus*, which is formed by species sharing such a feature in the karyotype.

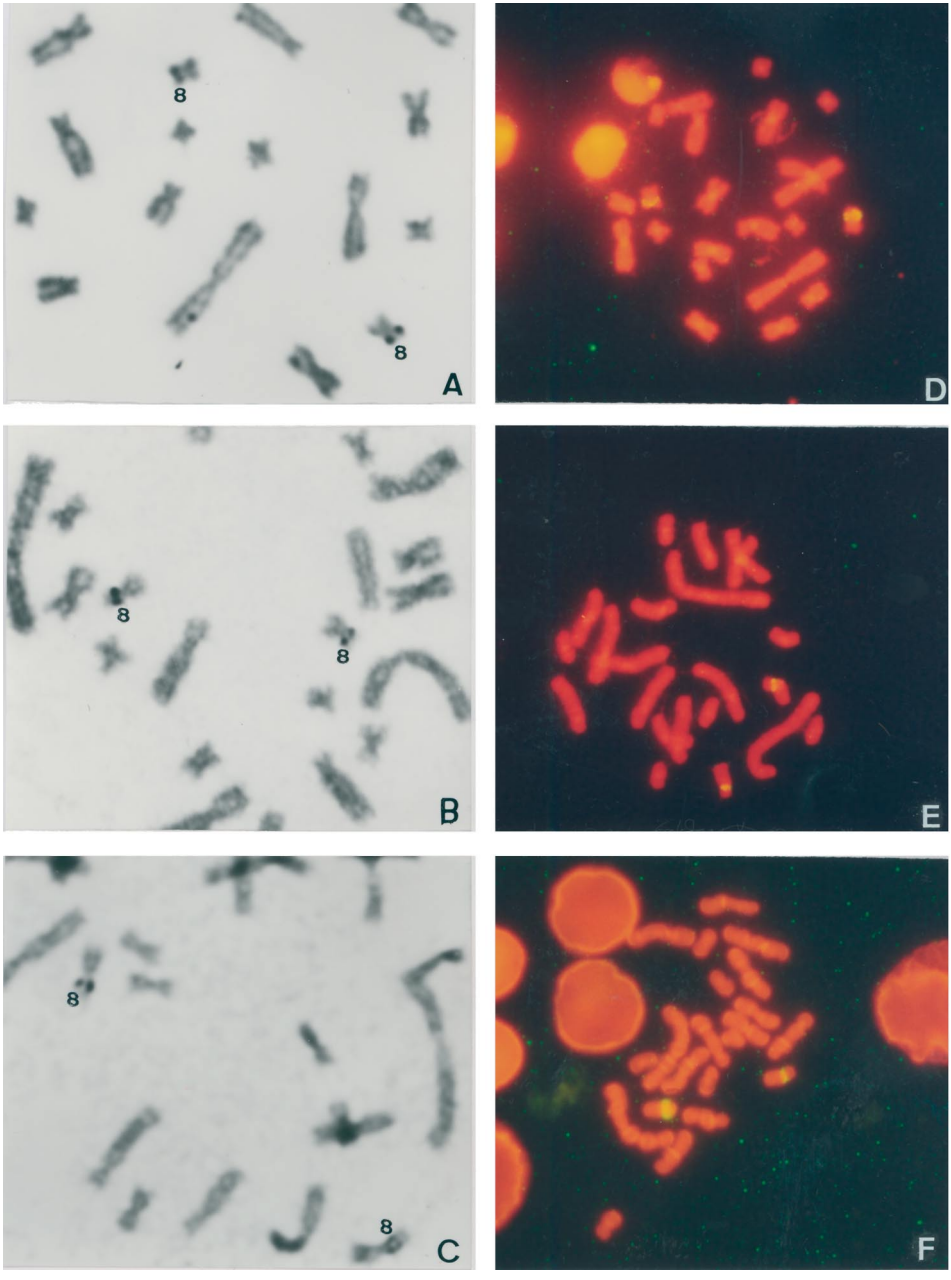

Figure 2. Partial metaphases with Ag-NOR. A: *L. gracilis gracilis*, B: *L. gracilis delattini*, and C: *L. plaumanni*. Metaphases with FISH using rDNA probe. D: *L. gracilis gracilis*, E: *L. gracilis delattini*, and F: *L. plaumanni*.

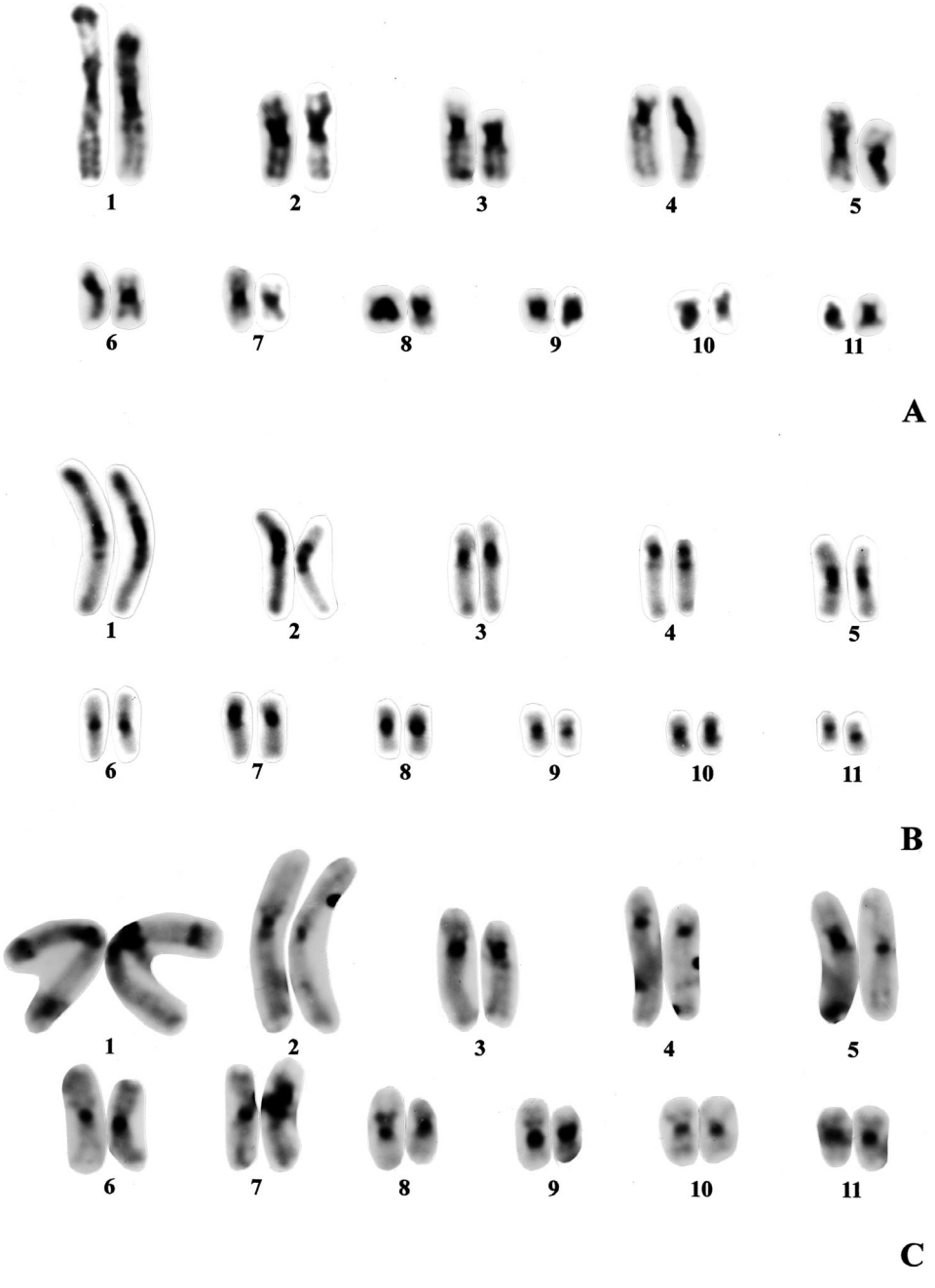

Figure 3. Karyotypes with C-banding. A: *L. gracilis gracilis*, B: *L. gracilis delattini*, and C: *L. plaumanni*. Note the interstitial bands in pairs 1, 8 and 9 in all karyotypes.

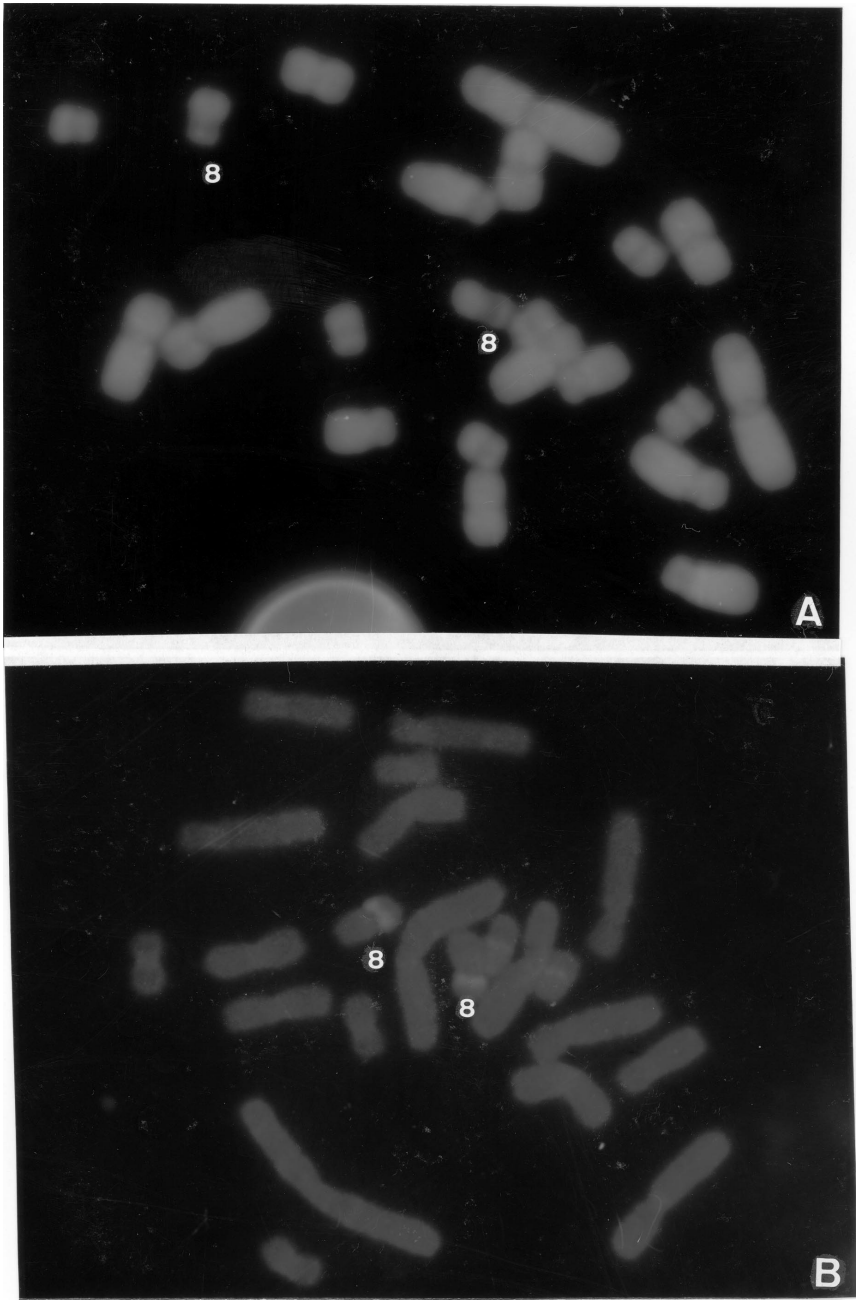

Figure 4. Metaphases of *L. gracilis delattini* with fluorochrome staining. A: DA/DAPI, and B: DA/CMA3. Fluorescent band in pair 8 corresponds in B to the Nucleolar Organizer Region.

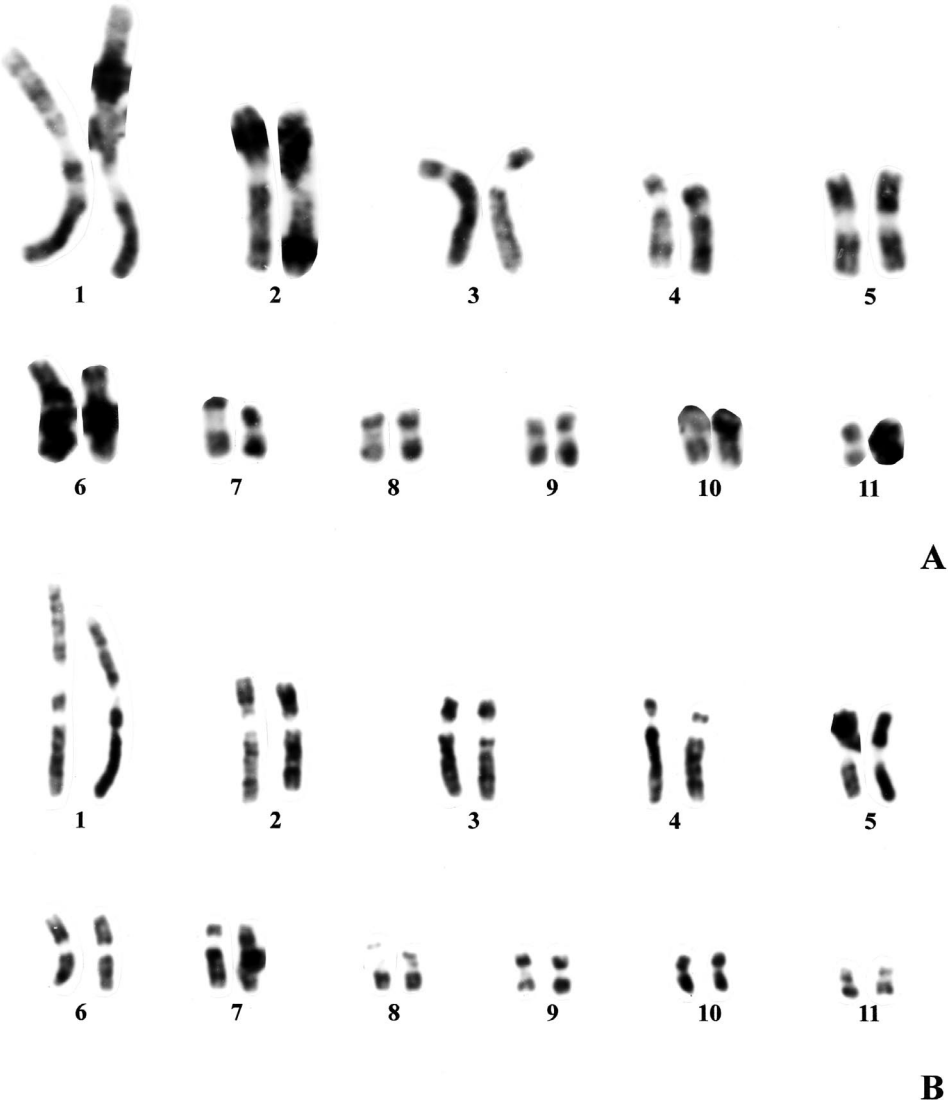

Figure 5. Replication banding patterns. A: *L. gracilis gracilis* after FPG staining, B: *L. gracilis delattini* after Giemsa diluted in 4Na-EDTA staining, C: Metaphase of *L. gracilis delattini* after Giemsa diluted in 4Na-EDTA with distinct pattern observed in B.

Although karyotypically homogeneous, it was possible to recognize a relevant difference regarding to the pairs 8 and 9 in the three taxa. This difference could be due to the occurrence of an interstitial block of constitutive heterochomatin in the chromosomes of pairs 8 and 9; besides the former is Ag-NOR carrier.

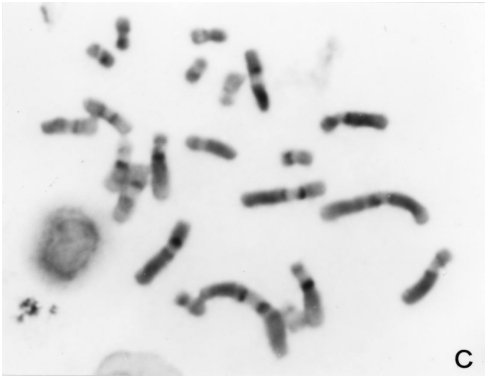

Figure 5. (Continued).

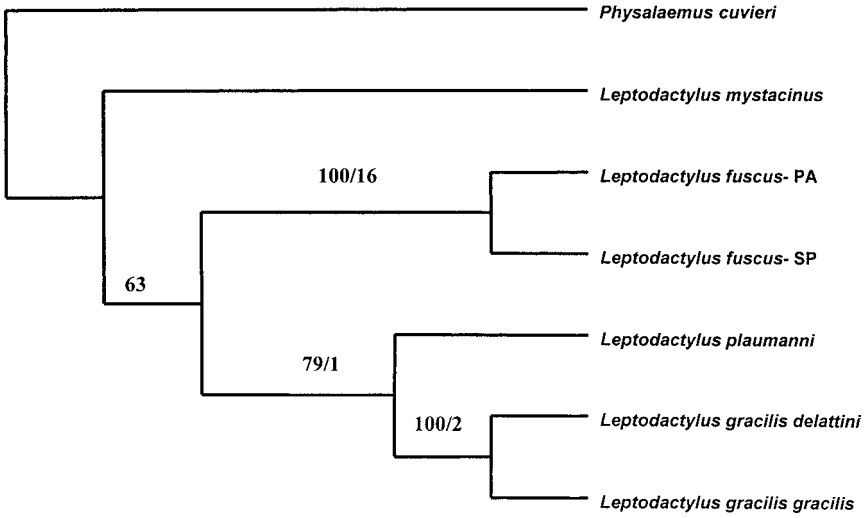

Figure 6. Parsimony phylogenetic tree derived from partial cytochrome b sequence. Numbers indicate bootstrap support followed by Bremer support values.

The Ag-NOR and FISH techniques confirmed the position of the rDNA site in the same chromosome pair, at the interstitial region of the short arms of the chromosomes 8, in the three karyotypes. Although this chromosome pair corresponds frequently to the nucleolus organizer in species of *Leptodactylus*, previous data obtained by Silva et al. (2000) indicated that the position of the Ag-NORs is variable. In some species, they are in the telomeric region of the short arms, delimiting or not satellites, while in others they are in the interstitial region, as observed in the present work. For this reason, the location of NORs has no taxonomic validity for *L. gracilis gracilis*, *L. gracilis delattini*, and *L. plaumanni*, contrary to that observed in other species of amphibians (King, 1990;

Baldissera et al., 1993). C-banding did not allow to find any kind of differentiation among the three karyotypes, as these showed similar patterns with centromeric staining in all chromosomes and secondary bands in the pairs 1, 8, and 9. Some additional C-bands were observed, but their location could not be established with certainty in the karyotypes. Although the presence of extra C-bands had been important to differentiate karyotypically some *Leptodactylus* species or even distinct populations of the same species (Silva et al., 2000), this was not observed in the present work.

Differences in the amount and distribution of heterochromatin have been useful to characterize amphibian species or subspecies, whose karyotypes are highly conserved (Matsui et al., 1985). On the other hand, even in the cases of similar C-banding patterns, taxonomically close anurans species can show heterochromatic regions with distinct molecular composition, as identified by base-specific fluorochrome staining (Schmid et al., 1987; Kasahara et al., 2001). The CMA3/DA/DAPI staining in *L. gracilis gracilis* and *L. gracilis delattini* revealed no particularly fluorescent heterochromatic regions, due to GC or AT richness base pairs. The unique bright CMA3 fluorescence is shown by the NOR, as it is usually observed in amphibian chromosomes (Schmid et al., 1991; Odierna et al., 2001).

The BrdU technique produced the same replication banding patterns after both FPG and 4Na-EDTA Giemsa staining, in the chromosomes of *L. gracilis delattini*. The longitudinal chromosome differentiation has been useful for a more effective comparison of amphibian karyotypes, among species belonging or not to the same genus (Miura, 1995; Wiley and Little, 2000), but in the present work the BrdU technique did not produce satisfactory results in *L. gracilis gracilis* and *L. plaumanni*. Nevertheless, we did not expect any relevant difference between the three taxa of *Leptodactylus*, as their chromosomes are highly homeologous.

The morphologically indistinguishable cryptic species *L. gracilis gracilis* and *L. plaumanni* have distinct geographical distributions, although they are sympatric in some localities in the State of Santa Catarina. The advertisement call (Cardoso, 1985) and the cytochrome b sequence divergence observed in the present study are, therefore, the unique characters that clearly distinguishes the two species. Regarding *L. gracilis gracilis* and *L. gracilis delattini*, the differences in the vocalization are subtle, but they are distinguished by some morphological traits and reproductive patterns. *Leptodactylus gracilis delattini* is geographically isolated: it is confined to a coastal island in the State of Santa Catarina, and has a specific identity, distinct from that of *L. gracilis gracilis* which has a wider distribution. Analysis based on cytochrome b sequence indicates that *L. gracilis gracilis* and *L. gracilis delattini* do not have any divergence, so that they should remain as valid subspecies.

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References

Agostinho, C.A. (1994): Caracterização de populações de rã-pimenta *Leptodactylus labyrinthicus* (Spix, 1824) e avaliação de seu desempenho em cativeiro. Doctoral Thesis, Universidade Federal de São Carlos, São Carlos, São Paulo, Brasil.

Amaro, R.C. (1999): Caracterização citogenética de espécies de *Bufo* e *Leptodactylus* (Amphibia, Anura), utilizando coloração diferencial e hibridação *in situ* fluorescente. Master Dissertation, Universidade de São Paulo, São Paulo, Brasil.

Baker, R.J., Bull, J.J., Mengden G.A. (1971): Chromosomes of *Elaphe subocularis* (Reptilia: Serpentes), with the description of an *in vivo* technique for preparation of snake chromosome. Experientia **27**: 1228-1229.

Baldissera, F.A. Jr., Batistic, R.F. (1992): Estudos citogenéticos em banda C e RON de algumas espécies de anuros brasileiros. Rev. Brasil. Genet. **15**: 78 (Supl.).

Baldissera, F.A. Jr., Oliveira, P.L.S., Kasahara, S. (1993): Cytogenetics of four Brazilian *Hyla* species (Amphibia-Anura) and description of a case with a supernumerary chromosome. Rev. Brasil Genet. **16**: 335-345.

Beçak, M.L. (1968): Chromosomal analysis of eighteen species of anura. Caryologia **21**: 191-208.

Beçak, M.L., Denaro, L., Beçak, W. (1970): Polyploidy and mechanisms of karyotypic diversification in Amphibia. Cytogenetics **9**: 225-238.

Bogart, J.P. (1974): A karyosystematic study of frogs in the genus *Leptodactylus* (Anura, Leptodactylidae). Copeia **3**: 728-737.

Cardoso, A.J. (1985): Revalidation of *Leptodactylus plaumanni* (Amphibia: Leptodactylidae). Pap. Avul. Zool. **36**: 87-90.

Denaro, L. (1972): Karyotypes of Leptodactylidae anurans. J. Herpetol. **6**: 71-74.

Duellman, W.E. (1993): Amphibian species of the world: additions and corrections. Lawrence, University of Kansas Printing Service.

Dutrillaux, B., Couturier, J. (1981): La pratique de l´analyse chromosomique. Paris, Masson.

Frost, D.R. (1985): Amphibian species of the world. A taxonomic and geographical reference. Lawrence, Allen Press and Association of Systematic Collection.

Frost, D.R. (2002): Amphibian Species of the World. a on line reference. V. 2.21.

Garcia-Pérez, L., Heyer, W.R. (1993): Description of the adverstisement call and resolution of the systematic status of *Leptodactylus gracilis delattini* Müller, 1968 (Amphibia: Leptodactylidae). Proc. Biol. Soc. Wash. **106**: 51-56.

Hall, T.A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. **41**: 95-98.

Heyer, W.R. (1969): The adaptative ecology of the species groups of the genus *Leptodactylus* (Amphibia, Leptodactylidae). Evolution **23**: 421-428.

Heyer, W.R. (1978): Systematic of the *fuscus* group of the genus *Leptodactylus* (Amphibia, Leptodactylidae). Nat. Hist. Mus. Los Angeles Sci. Bull. **29**: 1-85.

Heyer, W.R., García-Lopez, J.M., Cardoso, A.J. (1996): Advertisement call variation in the *Leptodactylus mystaceus* species complex (Amphibia: Leptodactylidae) with a description of a new sibling species. Amphibia-Reptilia **17**: 7-31.

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

Kasahara, S., Silva, A.P.Z., Gruber, S.L. (1998): Use of lymphocyte cultures for BrdU replication banding patterns in anuran species (Amphibia). Genet. Mol. Biol. **21**: 471-476.

Kasahara, S., Silva, A.P.Z., Haddad, C.F.B. (2001): Utilização de fluorocromos base-específicos e de técnica de FISH com sondas de DNAr em cromossomos de anfíbios anuros. Genet. Mol. Biol. CdROM (Supl.).

King, M. (1990): Amphibia. v. 4: Chordata 2. In: Animal Cytogenetics. John, B., Ed., Berlin, Gerbrüder Borntraeger.

Köhlers, J., Lötters, S. (1999): Advertisement calls of two Bolivian *Leptodactylus* (Amphibia: Anura: Leptodactylidae). Amphibia-Reptilia **20**: 215-219.

Kwet, A., Di-Bernardo, M., Garcia, P.C.A. (2001): The taxonomic status of *Leptodactylus geminus* Barrio, 1973. J. Herpetol. **35**: 56-62.

Lisanti, J.A., de Barale, G.D., Pinna-Senn, E., Stockert, J.C. (1990): A specimen of *Leptodactylus ocellatus* (Anura, Leptodactylidae) heterozygous for a deletion of the nucleolar organizing region. Comunicaciones Biológicas **9**: 127-131.

Matsui, M., Seto, T., Kohsaka, Y., Borkin, L.J. (1985): Bearing of chromosome C-banding patterns on the classification of Eurasian toads of the *Bufo bufo* complex. Amphibia-Reptilia **6**: 24-34.

Meunier-Rotival, M., Cortadas, J., Macaya, G., Bernardi, G. (1979): Isolation and organization of calf ribosomal DNA. Nucleic Acids Res. **6**: 2109-2123.

Miura, I. (1995): The late replication banding patterns of chromosomes are highly conserved in the genera *Rana*, *Hyla* and *Bufo* (Amphibia-Anura). Chromosoma **103**: 567-574.

Müller, P. (1968): Beitrag zur Herpetofauna der Insel Campeche (27°42′S/48°28′). Salamandra **4**: 47-55.

Odierna, G., Vences, M., Gennaro, A., Lötters, S., Andreone, F. (2001): Chromosome data for Malagasy poison frogs (Amphibia: Ranidae: *Mantella*) and their bearing on taxonomy and phylogeny. Zool. Sci. **18**: 505-514.

Schmid, M., Vitelli, L., Batistoni, R. (1987): Chromosome banding in Amphibia. XI. Constitutive heterochromatin, nucleolus organizer, 18S + 28S and 5S ribosomal RNA genes in Ascaphidae, Pipidae, Discoglossidae and Pelobatidae. Chromosoma **95**: 271-284.

Schmid, M., Nanda, I., Steinlein, C., Kausch, K., Haaf, T. (1991): Sex-determining mechanisms and sex chromosomes in Amphibia. In: Amphibian Cytogenetics and Evolution. Green, D.M., Sessions, S.K., Eds, San Diego, Academic Press.

Schweizer, D. (1980): Simultaneous fluorescent staining of R bands and specific heterochromatin regions (DA-DAPI bands) in human chromosomes. Cytogen. Cell Genet. **27**: 190-193.

Scrocchi, G.J., Lavilla, E.O. (1986): Caracteres para la identificacion de las especies cripticas *Leptodactylus gracilis* y *Leptodactylus geminus* (Anura: Leptodactylidae). Physis **44**: 93-95.

Silva, A.P.Z., Haddad, C.F.B., Kasahara, S. (2000): Chromosomal studies on five species of the genus *Leptodactylus* Fitzinger, 1826 (Amphibia, Anura) using differential staining. Cytobios **103**: 25-38.

Sumner, A.T. (1972): A simple technique for demonstrating centromeric heterochromatin. Exp. Cell Res. **75**: 304-306.

Swofford, D.L. (1999): PAUP*. Phylogenetic Analysis Using Parsimony (and Other Methods), version 4.0. Sunderland, Sinauer Associates.

Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research **22**: 4673-4680.

Viegas-Péquignot, E. (1992): *In situ* hybridization to chromosomes with biotinylated probes. In: *In situ* hybridization: a practical approach**.** Willernson, D., Ed., Oxford, IRL Press.

Wiley, J.E. (1982): Chromosome banding patterns of tree frogs (Hylidae) of the Eastern United States. Herpetologica **38**: 507-520.

Wiley, J.E., Little, M.L. (2000): Replication banding pattern of the diploid-tetraploid treefrogs *Hyla chrysoscelis* and *H. versicolor*. Cytogenet. Cell Genet. **88**: 11-14.

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