BOLETIM DO MUSEU NACIONAL

NOVA SÉRIE
RIO DE JANEIRO - BRASIL

ISSN 0080-312X

ZOOLOGIA | Nº 499 | 08 DE ABRIL DE 2003

CHROMOSOME BANDING IN *MACROGENIOGLOTTUS ALIPIOI* CARVALHO, 1946 (AMPHIBIA, ANURA, LEPTODACTYLIDAE), WITH COMMENTS ON ITS TAXONOMIC POSITION [1]

(With 6 figures)

ANA PAULA ZAMPIERI SILVA[2, 3]
CÉLIO F. B. HADDAD[2, 4]
SANAE KASAHARA[2, 5]

ABSTRACT: We performed cytogenetic studies on *Macrogenioglottus alipioi* using chromosome banding techniques. A discussion on the taxonomic status of the genus *Macrogenioglottus*, which is still controversial because it shares morphological characters typical of both Leptodactylidae and Bufonidae, is presented. Based on comparative cytogenetic data of *Macrogenioglottus alipioi, Proceratophrys boiei,* and *Bufo paracnemis,* we suggest that *M. alipioi* is karyologically more related to *P. boiei,* allocated in the family Leptodactylidae.

Key words: Amphibia, Anura, *Macrogenioglottus alipioi,* taxonomy, chromosomes.

RESUMO: Bandamento cromossômico em *Macrogenioglottus alipioi* Carvalho, 1946 (Amphibia, Anura, Leptodactylidae), com comentários sobre sua posição taxonômica.

São apresentados dados citogenéticos de *Macrogenioglottus alipioi* com técnicas de bandamento cromossômico. É apresentada uma discussão acerca da posição taxonômica da espécie que gera, ainda, muitas controvérsias, pois trata-se de um animal com características morfológicas típicas de Leptodactylidae e Bufonidae. Com base na análise citogenética comparativa entre *Macrogenioglottus alipioi, Proceratophrys boiei* e *Bufo paracnemis,* verificou-se que *M. alipioi* compartilha características cromossômicas que a aproxima de *P. boiei,* alocada na família Leptodactylidae.

Palavras-chave: Amphibia, *Macrogenioglottus alipioi,* Anura, taxonomia, cromossomos.

INTRODUCTION

Macrogenioglottus Carvalho, 1946 is a monotypic genus that includes the species named *M. alipioi* Carvalho, 1946, which has a geographical distribution limited to the Atlantic Rain Forest, from the South of the State of Bahia to the State of São Paulo (FROST, 2002). The species was described by CARVALHO (1946) as a

[1] Submitted on May 22, 2002 Accepted on November 12, 2002.

[2] Universidade Estadual Paulista, IB, Pós-Graduação em Biologia Celular e Molecular. Caixa Postal 199, Rio Claro, 13506-900, SP, Brasil.
[3] E-mail: zampierisilva@aol.com.
[4] E-mail: haddad@rc.unesp.br.
[5] E-mail: kasahara@rc.unesp.br. Corresponding author.

member of the family Ceratophrynidae, which is currently recognized as subfamily Ceratophryinae and included in the family Leptodactylidae. That author considered *Macrogenioglottus* and *Odontophrynus* Reinhardt and Lütken, 1862 as closely related. LYNCH (1971) considered *Macrogenioglottus* as a junior synonym of *Odontophrynus* in the leptodactylid subfamily Telmatobiinae. Later, HEYER (1975) considered *Macrogenioglottus* and *Odontophrynus* as distinct genera. According to FROST (2002), *Macrogenioglottus* is allocated in the leptodactylid subfamily Ceratophryinae, with other five genera, *Ceratophrys* Reinhardt and Lütken, 1862, *Chacophrys* Reig & Limeses, 1963, *Lepidobatrachus* Budgett, 1899, *Odontophrynus*, and *Proceratophrys* Miranda-Ribeiro, 1920, but the correct taxonomic classification of *M. alipioi* is still controversial, because the animal shares morphological characters typical of both Leptodactylidae and Bufonidae.

One of the most complete comparative analysis of *M. alipioi* was carried out by REIG (1972), who studied external as well as internal characters, including the skeleton and musculature. Based on these data, the author concluded that *Macrogenioglottus* is phenetically more closely related to the bufonids, in particular to the genus *Bufo* Laurenti, 1768, and rejected completely the inclusion of the species in the family Leptodactylidae or Ceratophrynidae, as formerly proposed by CARVALHO (1946). Nevertheless, due to the fact that *Macrogenioglottus* presents some peculiar features, not shown in Bufonidae, REIG (1972) suggested that this genus must be allocated in a new monotypic family, which was designated by him as Macrogenioglottidae. In 1978, ABRAWAYA & JACKSON observed high similarity of larval morphology and mating call among *Macrogenioglottus alipioi*, *Odontophrynus americanus*, and *O. occidentalis* Berg, 1896, and considered these data indicative of a new evidence of the close relationship between *Macrogenioglottus* and *Odontophrynus*.

Macrogenioglottus alipioi is a relatively rare species, poorly represented in collections. The former cytogenetic data are restricted to the description of a 2n=22 karyotype in a specimen collected in the State of São Paulo (BEÇAK, DENARO & BEÇAK, 1970). Later, a brief report on the diploid number and Ag-NOR site in specimens from the States of São Paulo and Bahia was presented by BALDISSERA & BASTOS (1993) and AMARO-GHILARDI & YONENAGA-YASSUDA (2001), respectively. In the present paper, we present a careful chromosome analysis of *Macrogenioglottus alipioi* through differential staining techniques, in order to provide new cytogenetic data as a way to understand the taxonomic status of the species. Additionally, karyological data on the leptodactylid *Proceratophrys boiei* Wied-Neuwied, 1824 and the bufonid *Bufo paracnemis* Lutz, 1925 are introduced to support our discussion.

MATERIAL AND METHODS

Two specimens (male CFBH 3998 and juvenile CFBH 3999) of *Macrogenioglottus alipioi* were collected in Picinguaba (23°25'S, 44°50'W), Municipality of Ubatuba, State of São Paulo, Brazil. 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.

Cytological preparations were obtained from bone marrow and testis after *in vivo* colchicine treatment and previous injection with phytohemagglutin P (Difco) for mitosis stimulation, according to the procedures described in BALDISSERA JÚNIOR, OLIVEIRA & KASAHARA (1993). One of the animals was also injected with a solution of 5-bromodeoxyuridine plus 5-fluorodeoxyuridine (SILVA, HADDAD & KASAHARA, 2000). Analyses were performed after routine Giemsa staining, C-banding (SUMNER, 1972), Ag-NOR staining (HOWELL & BLACK, 1980), triple staining CMA3/DA/DAPI (SCHWEIZER, 1980), and FPG (Fluorochrome Plus Giemsa) technique (DUTRILLAUX & COUTURIER, 1981). The chromosome nomenclature followed GREEN & SESSIONS (1991).

The sample of *Proceratophrys boiei* (CFBH 4532, CFBH 4533, CFBH 4534) is constituted by three males collected in Ribeirão Branco (24°21'S, 48°44'W), State of São Paulo, Brazil, which were treated according to routine procedures for obtaining direct chromosome preparations (BALDISSERA JÚNIOR, OLIVEIRA & KASAHARA, 1993). A female of *Bufo paracnemis* (CFBH 4536) from Rio Claro (22°24'S, 47°33'W), State of São Paulo, Brazil, provided metaphase cells after lymphocyte cultures, with *in vitro* treatment with BrdU (KASAHARA, SILVA & GRUBER, 1998).

RESULTS

The karyotype of *Macrogenioglottus alipioi* includes 22 chromosomes grouped into five large pairs, two medium, and four small pairs. Pairs 1, 5, 6, and 7 are metacentric, pairs 2, 3, and 4, submetacentric, and the remaining four small chromosome pairs of metacentric or submetacentric type (Fig.1). Pair 8 posses an interstitial secondary constriction in the short arms, visualized in one or both of the homologues. No heteromorphic chromosome pair was recognized in the karyotype, also confirmed by meiotic analysis in male specimen. Diplotene and metaphase I cells revealed 11 bivalents and metaphase II cells, 11 chromosomes.

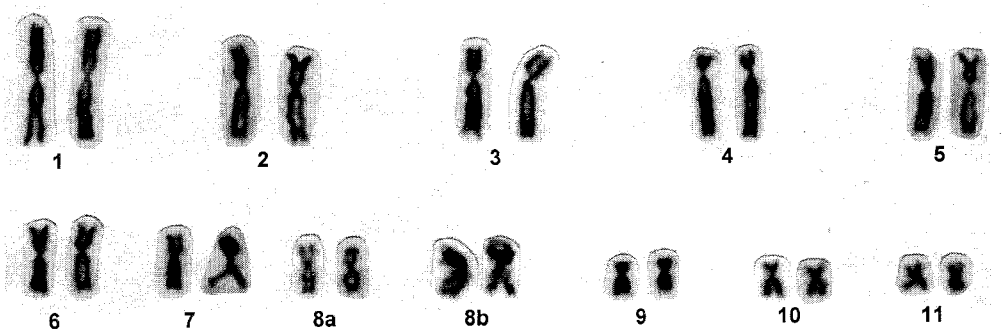

Fig.1- Giemsa-stained karyotype of *Macrogenioglottus alipioi* Carvalho, 1946, ♂, 2n=22. Observe chromosome pair 8 after conventional (8a) or Ag-NOR staining (8b).

The single pair of Ag-NORs of *M. alipioi* is located in the same site of the secondary constriction, in the short arms of pair 8 (Fig.1). All the chromosomes showed well marked

centromeric heterochromatin plus interstitial or telomeric C-bands in some elements (Fig.2). The most evident of these interstitial bands are located in pairs 8 and 11. The CMA3/DA/DAPI technique showed one pair of fluorescent NOR and centromeric heterochromatin after staining with the fluorochrome CMA3 (Fig.3). The BrdU treatment produced longitudinal replication bands in the chromosomes of *M. alipioi* (Fig.4).

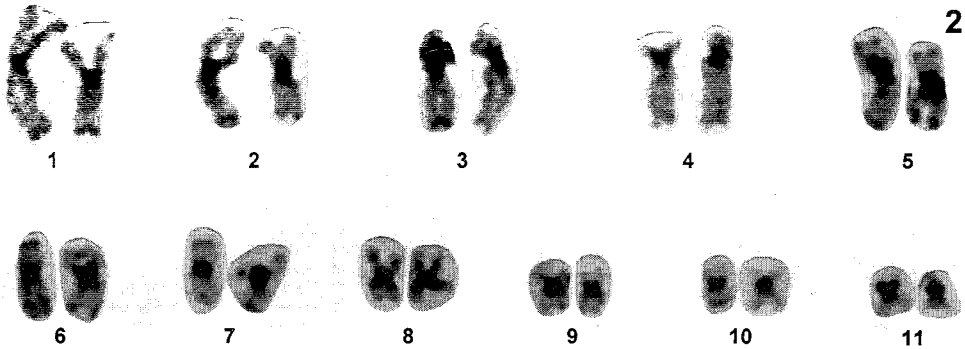

Fig.2- C-banded karyotype of *Macrogenioglottus alipioi* Carvalho, 1946, 2n=22.

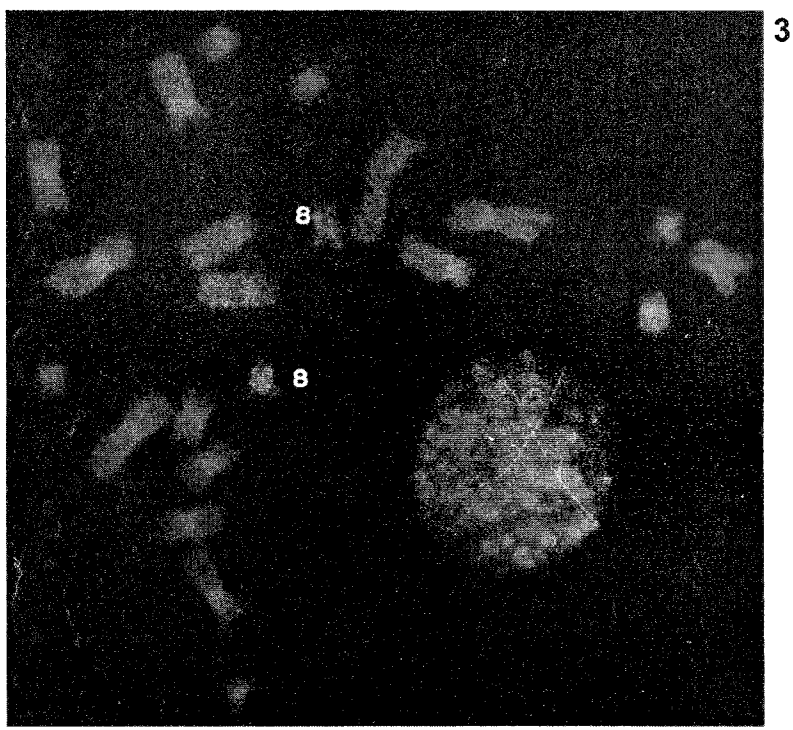

Fig.3- Metaphase of *Macrogenioglottus alipioi* Carvalho, 1946, 2n=22, with DA/CMA3 staining, showing fluorescent NOR (pair 8) and centromeric heterochromatin.

Cytogenetic data of *Proceratophrys boiei* (conventional stained karyotype and Ag-NOR) and of *Bufo paracnemis* (conventional stained karyotype and Ag-NOR, BrdU-banded karyotypes) are presented in figure 5 and figure 6, respectively.

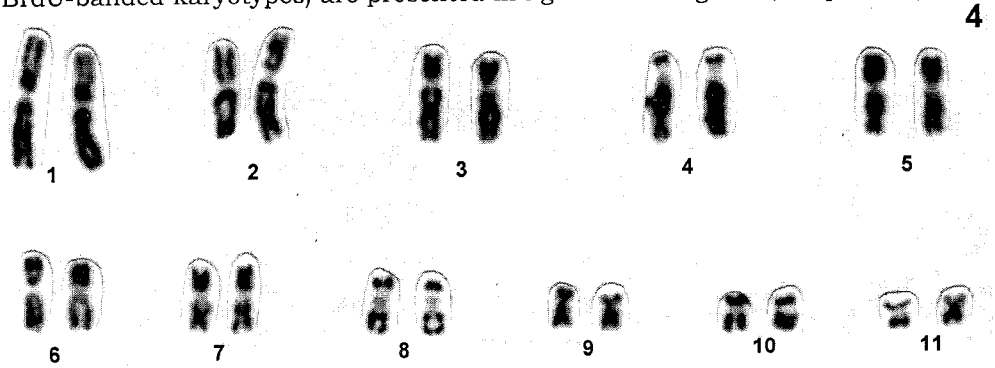

Fig.4- Replication bands in the karyotype of *Macrogenioglottus alipioi* Carvalho, 1946, 2n=22, after BrdU incorporation.

DISCUSSION

The cytogenetic analysis of *Macrogenioglottus alipioi* confirms the previous data obtained by BEÇAK, DENARO & BEÇAK (1970), on the basis of a unique specimen collected in a locality of the State of São Paulo not mentioned by the authors. Some divergences between the karyotypes may be largely attributed to distinct criteria of chromosome nomenclature adopted by the authors, although BEÇAK, DENARO & BEÇAK (*op.cit.*) have reported a secondary constriction also in pair 11. This marker was not observed in the present study, but in the corresponding site an interstitial C-band was noticed. Our data are compatible with the occurrence of a single pair of nucleolar organizer region in *M. alipioi,* but variation involving this cytological marker can not be completely ruled out. Among amphibian species, interpopulational differences in number of NORs have been described in which, besides a fixed pair of NOR, other secondary sites occur (RUIZ, CEI & BEÇAK, 1982; ALMEIDA, RUIZ & BEÇAK, 1986; KAISER *et al.*, 1996; SILVA, HADDAD & KASAHARA, 1999).

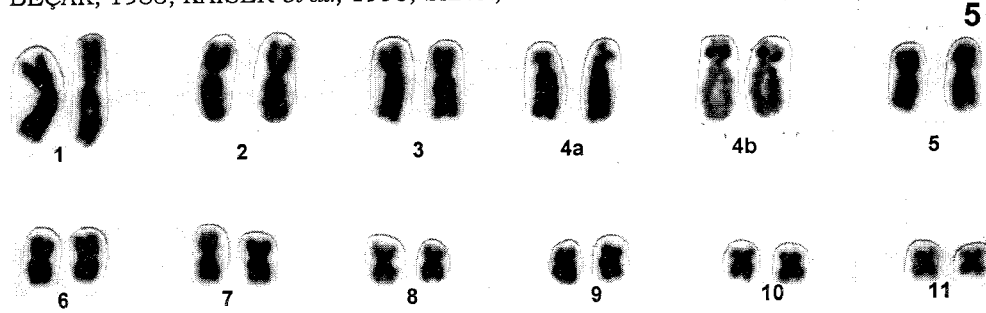

Fig.5- Giemsa-stained karyotype of *Proceratophrys boiei* Wied-Neuwied, 1824, ♂, 2n=22. Observe chromosome pair 4 after conventional (4a) or Ag-NOR staining (4b).

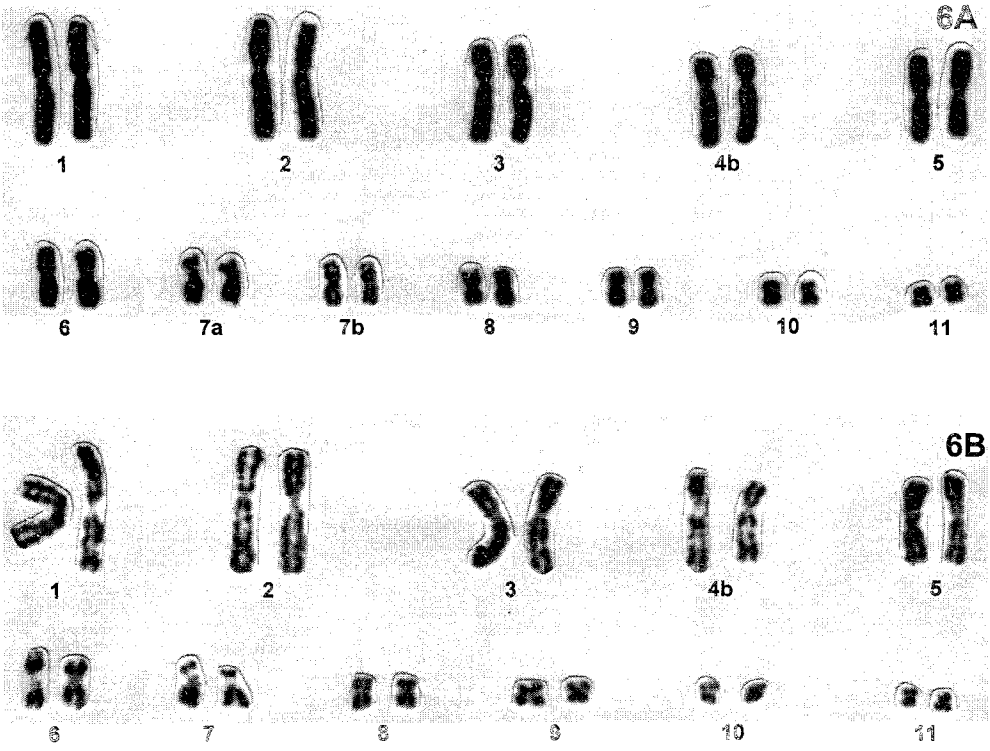

Fig.6- Karyotype of *Bufo paracnemis* Lutz, 1925, ♀, 2n=22: (A) Giemsa staining. Observe chromosome pair 7 after conventional (7a) or Ag-NOR staining (7b); (B) Replication bands, after BrdU incorporation.

In this paper, a detailed karyotypic characterization of *M. alipioi* using differential staining techniques is presented for the first time. With both Ag-NOR and CMA3/DA techniques, a single pair of NORs, located in chromosomes 8, was confirmed for the species. C-banding showed heterochromatin distributed mainly in the centromeric regions, which have a slight predominance of GC content. Observing several FPG stained metaphases, it was evident that the chromosomes of *M. alipioi*, except for the small elements, exhibited relatively well defined replication bands, which allowed the correct pairing of the homologues.

As formerly stressed by BEÇAK, DENARO & BEÇAK (1970), the karyotype of *M. alipioi* shows great similarity with those presented by species of Leptodactylidae belonging to the genera *Odontophrynus* (*O. carvalhoi* and *O. occidentalis*) and *Proceratophrys* (*P. boiei* and *P. appendiculata*). In fact, it is possible to recognize in the karyotypes of both *M. alipioi* and *P. boiei* of the present study a clear discontinuity between the seven pairs of chromosomes of large or medium size and the four pairs of small-sized chromosomes. A close correspondence in the gross

morphology of each chromosome pair, at least regarding to the seven first chromosome pairs of the two species, was also observed. Although no *Odontophrynus* species was available to us for cytogenetic analysis, we also found a close correspondence between the karyotype of *Macrogenioglottus alipioi* and those described by RUIZ, CEI & BEÇAK (1982) and ALMEIDA, RUIZ & BEÇAK (1986) for *Odontophrynus* species from Argentina and Brazil.

Macrogenioglottus alipioi and *Bufo paracnemis* also share a similar karyotypic pattern, but some divergences may be pointed out. In the latter species, there are six pairs of large or medium-sized chromosomes and its karyotype clearly includes five pairs of small size and not four like in *M. alipioi.* Additionally, submetacentric pairs 3 and 4 present distinct chromosome arm ratios in both species.

Taking into account the site of the nucleolar organizer regions, the three karyotypes are quite divergent, because Ag-NORs occupy the chromosomes of pair 4 in *P. boiei,* pair 7 in *B. paracnemis,* and pair 8 in *M. alipioi.* Nevertheless, according to the report of AMARO-GHILARDI & YONENAGA-YASSUDA (2001), specimens of *P. boiei* from two other locaties of the State of São Paulo have Ag-NORs in the short arms of the chromosome 8 like in *M. alipioi.* Chromosome pair 8 bearing Ag-NOR has also been described in other 2n=22 representatives in Leptodactylidae, as *O. carvalhoi* (RUIZ, SOMA & BEÇAK, 1981) and the totality of the species belonging to the genus *Leptodactylus* analysed up to now for this cytological marker (SILVA, HADDAD & KASAHARA, 2000; SILVA *et al.* in prep.). Regarding to the other known karyotypes of *Bufo,* BALDISSERA JÚNIOR, BATISTIC & HADDAD (1999) summarized the occurrence of Ag-NORs on pairs 7, 5 or 10 for the species from South America, on pair 1 for North American species, on pairs 6 and 11 for Eurasian species, and on pairs 1 and 6 for African species, this last group with 2n=20 chromosomes.

C-banding pattern of *B. paracnemis* as well as of two other species of *Bufo* was previously obtained (KASAHARA, SILVA & HADDAD, 1996). A relatively small amount of centromeric heterochromatin was observed in the three karyotypes as well as some secondary bands. The chromosome 7 possesses a C positive band matching to the secondary constriction. No suitable C-banded metaphases were available for *P. boiei,* preventing a comparative analysis with *M. alipioi.*

The next step was the comparison of the karyotypes with BrdU replication banding. As *Proceratophrys boiei* did not provide FPG banded chromosomes, a comparative analysis was carried out between *M. alipioi* and some *Leptodactylus* species (SILVA, HADDAD & KASAHARA, 2000; SILVA *et al.*, in prep.) because they present the same karyotypic pattern observed in *P. boiei.* The karyotypes of the representatives of the three genera, *Macrogenioglottus, Leptodactylus* and *Bufo,* showed distinct levels of band differentiation not allowing a detailed analysis. A rough correspondence of replication banding, at least for some chromosomes or chromosome segments, was noticed in the three karyotypes, but it was not possible to establish any evolutive relationship among them. This is not surprising, as a high degree of conservativeness in the replication banding patterns was detected in the larger chromosomes of *Rana, Hyla, Bufo,* and *Xenopus* (MIURA, 1995), although the results indicate that *Hyla* is more closely related to *Bufo* than to *Rana* Linnaeus, 1758.

The present cytogenetic data are not conclusive for deciding about the taxonomic status of *Macrogenioglottus alipioi* at family level. Nevertheless, we think that our cytogenetic data are toward a closer proximity between this and those included in the family Leptodactylidae, which strengthens the former position of *M. alipioi* into this family. The study of sequences of mitochondrial DNA, which is under way (S.F. dos Reis, A.C.R. Alves, and C.F.B. Haddad) might be a more useful tool to clarify this question.

ACKNOWLEDGEMENTS

We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support; Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) for permission to collect the specimens.

LITERATURE CITED

ABRAWAYA, J.P. & JACKSON, J.F., 1978 – Reproduction in *Macrogenioglottus alipioi* Carvalho (Anura, Leptodactylidae). **Natural History Museum Contribution of Science**, Los Angeles, **298**:1-9.

ALMEIDA, T.M.; RUIZ, I.R.G. & BEÇAK, W., 1986 – Ribosomal gene activity detected by silver staining in two diploid populations of *Odontophrynus americanus* (Amphibia, Anura) from Southern Brazil. **Revista Brasileira de Genética**, Ribeirão Preto, **9**:433-437.

AMARO-GHILARDI, R.C. & YONENAGA-YASSUDA, Y., 2001 – Análise citogenética com identificação de Ag-RONs em três espécies da subfamília Telmatobiinae (Leptodactylidae, Anura). In: CONGRESSO NACIONAL DE GENÉTICA. 47., Águas de Lindóia. **Resumos...**, CDROM.

BALDISSERA, F. & BASTOS, R.P., 1993 – Análise cariotípica de *Macrogenioglottus alipioi* (Anura). In: CONGRESSO LATINO AMERICANO DE HERPETOLOGIA 3., Campinas. **Resumos...**, p.55.

BALDISSERA JÚNIOR, F.A.; OLIVEIRA, P.L.S. & KASAHARA, S., 1993 – Cytogenetics of four Brazilian *Hyla* species (Amphibia-Anura) and description of a case with a supernumerary chromosome. **Revista Brasileira de Genética**, Ribeirão Preto, **16**:335-345.

BALDISSERA JÚNIOR, F.A.; BATISTIC, R.F. & HADDAD, C.F.B., 1999 – Cytotaxonomy considerations with the description of two new NOR locations for South American toads, genus *Bufo* (Anura: Bufonidae). **Amphibia-Reptilia**, Leiden, **20**:413-420.

BEÇAK, M.L.; DENARO, L. & BEÇAK, W., 1970 – Polyploidy and mechanisms of karyotypic diversification in Amphibia. **Cytogenetics**, Basel, **9**:225-238.

CARVALHO, A.L., 1946 – Um novo gênero de ceratofrídeo do Sudeste Baiano. **Boletim do Museu Nacional, Nova Série, Zoologia**, Rio de Janeiro (73):1-5.

DUTRILLAUX, B. & COUTURIER, J., 1981 – **La Pratique de l´Analyse Chromosomique**. Paris: Masson. 87p.

FROST, D.R., 2002 – **Amphibian Species of the World. An Online Reference**. v.2.21. Availlable at: http://research.amnh.org/herpetology/amphibia/index.html. Captured on 02 October 2002].

GREEN, D.M. & SESSIONS, S.K, 1991 – Nomenclature for chromosomes. In: GREEN, D.M. & SESSIONS, S.K. (Eds.) **Amphibian Cytogenetics and Evolution**. San Diego: Academic Press. p.431-432.

HEYER, W.R., 1975 – A preliminary analysis of the intergeneric relationships of the frog family Leptodactylidae. **Smithsonian Contribution in Zoology**, Washington, **199**:1-55.

HOWELL, W.M. & BLACK, D.A., 1980 – Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. **Experientia**, Basel, **36**:1014-1015.

KAISER, H.; MAIS, C.; BOLAÑOS, F.; STEINLEIN, C.; FEICHTINGER, W. & SCHMID, M., 1996 – Chromosomal investigation of three Costa Rican frogs from the 30-chromosome radiation of *Hyla* with the description of a unique geographic variation in nucleolus organizer regions. **Genetica**, Dordrecht, **98**:95-102.

KASAHARA, S.; SILVA, A.P.Z. & HADDAD, C.F.B., 1996 – Chromosome banding in three species of Brazilian toads (Amphibia-Bufonidae). **Brazilian Journal of Genetics.**, Ribeirão Preto, **19**:237-242.

KASAHARA, S.; SILVA, A.P.Z. & GRUBER, S.L., 1998 – Use of lymphocyte cultures for BrdU replication banding patterns in anuran species (Amphibia). **Genetics and Molecular Biology**, Ribeirão Preto, **21**:471-476.

LYNCH, J.D., 1971 – Evolutionary relationships, osteology, and zoogeography of leptodactilid frogs. **Miscellaneous Publications of Museum of Natural History University of Kansas**, Lawrence, **53**:1-238.

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

REIG, O.A., 1972 – *Macrogenioglottus* and the South American bufonid toads. In: BLAIR, W.F. (Ed.) **Evolution in the Genus *Bufo***. Austin and London: University of Texas Press. p.14-36.

RUIZ, I.R.G.; SOMA, M. & BEÇAK, W., 1981 – Nucleolar organizer regions and constitutive heterochromatin in polyploid species of the genus *Odontophrynus* (Amphibia, Anura). **Cytogenetics and Cell Genetics**, Basel, **29**:84-98.

RUIZ, I.R.G.; CEI, J.M. & BEÇAK, W.,1982 – Chromosomal evolution in allopatric popultions of the *Odontophrynus occidentalis* group (Amphibia, Anura) from western Argentina. **Cytogenetics and Cell Genetics**, Basel, **33**:303-311.

SCHWEIZER, D., 1980 – Simultaneous fluorescent staining of R bands and specific heterochromatin regions (DA-DAPI bands) in human chromosomes. **Cytogenetics and Cell Genetics**, Basel, **27**:190-193.

SILVA, A.P.Z.; GARCIA, P.C.A; MARTINS, V.G. & KASAHARA, S., (in prep.?) – Chromosomal and molecular analysis os *Leptodactylus gracilis gracilis, Leptodactylus gracilis delattini* and *Leptodactylus plaumanni* (Anura, Leptodactylidae): taxonomy implications.

SILVA, A.P.Z.; HADDAD, C.F.B. & KASAHARA, S., 1999 – Nucleolus organizer regions in *Physalaemus cuvieri* (Anura, Leptodactylidae), with evidence of a unique case of Ag-NOR variability. **Hereditas**, Lund, **131**:135-141.

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**, Cambridge, **103**:25-38.

SUMNER, A.T., 1972 – A simple technique for demonstrating centromeric heterochromatin. **Experimental Cell Research**, New York, **75**:304-306.