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.


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
member of the family Ceratophrynidae, which is currently recognized as subfamily Ceratophryinae and included in the family Leptodactylidae. That author considered Macrogenioglossus and Odontophrynus Reinhartd and Lütken, 1862 as closely related. Lynch (1971) considered Macrogenioglossus as a junior synonym of Odontophrynus in the leptodactylid subfamily Telmatobiiinae. Later, Heyer (1975) considered Macrogenioglossus and Odontophrynus as distinct genera. According to Frost (2002), Macrogenioglossus is allocated in the leptodactylid subfamily Ceratophryinae, with other five genera, Ceratophrys Reinhartd and Lütken, 1862, Chacophryx 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 Macrogenioglossus 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 Ceratophryinae, as formerly proposed by Carvalho (1946). Nevertheless, due to the fact that Macrogenioglossus 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 Macrogenioglossidae. In 1978, Abrawaya & Jackson observed high similarity of larval morphology and mating call among Macrogenioglossus alipioi, Odontophrynus americanus, and O. occidentalis Berg, 1896, and considered these data indicative of a new evidence of the close relationship between Macrogenioglossus and Odontophrynus.

Macrogenioglossus 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 (Bécaiak, 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 Macrogenioglossus 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 Macrogenioglossus 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 phytohemagglutinin P (Difco) for mitosis stimulation, according to the procedures described in BALDISSEIRA 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 (BALDISSEIRA 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](image-url)  
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).

![Image of chromosome bands](image1)

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

![Image of metaphase with DA/CMA3 staining](image2)

**Fig.3- Metaphase of Macrogenioglossus 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 (RUÍZ, CEI & BEÇAK, 1982; ALMEIDA, RUÍZ & 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 localities 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 & KASHAR, 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 0 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 (KASHAR, 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 & KASHAR, 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 *Macrogenioglossus 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.

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LITERATURE CITED


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.


