Short Communication

Cytogenetics and sperm ultrastructure of *Atelopus spumarius* (Anura, Bufonidae) from the Brazilian Amazon

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Abstract

The current taxonomy of most *Atelopus* species is based on morphological and color data only. Recent studies suggest that *A. spumarius* may represent a species complex assigned under the same name. Karyotypic data and description of sperm ultrastructure for 13 specimens of *A. spumarius* are presented here for the first time. A chromosomal analysis revealed 2n = 22 chromosomes, with centromeric heterochromatin in all pairs and a nucleolar organizer region (NOR) on the telomere of pair 7. The sperm was of the bufonoid type, presenting a filiform nucleus covered by an acrosomal complex and a mitochondrial collar in the neck region. The tail was composed of an axoneme, an undulating membrane and an axial rod. A karyotype analysis of *A. spumarius* showed the same chromosome number and similar chromosomal morphology as described for congeneric species, with slight differences probably resulting from pericentric inversions. The NOR location (on pair 7) was the same as that observed for species belonging to the genus *Rhinella*. The spermatological findings indicate a close relationship between *Atelopus* and the bufonoid lineage. The present data are useful for reference in future studies to determine whether more than one species are assigned to *A. spumarius.*

Keywords: Ag-NOR, *Atelopus spumarius*, C-band, chromosome, sperm ultrastructure.

The harlequin frog, *Atelopus spumarius* Cope, 1871, is found in the Amazon region throughout Ecuador, Peru, the Guianas and Brazil (Amazonas, Para and Amapá states) (Frost, 2013). Cocroft *et al.* (1990) and Lötters *et al.* (2002) suggested that this name may refer to a species complex, with the true *A. spumarius* being found only in the upper Amazon Basin (Peru, Colombia and Brazil). This suggestion implies that the populations in southern Peru, Ecuador and central Brazil may represent undescribed taxa. Furthermore, Lötters *et al.* (2002) suggested that the populations from Guiana and the eastern Amazon may belong to yet another species complex: *Atelopus hoogmoedi*.

The current taxonomy of this genus is solely based on morphological and color data (Ramos *et al.*, 2002), with the presence of toxins also being considered a taxonomic tool (Kim *et al.*, 1975). Other potentially useful criteria, such as osteology, larval morphology, molecular data, cytogenetics and sperm ultrastructure, have not been applied to this genus. In anurans in general, cytogenetics has been used increasingly as a tool to investigate chromosomal evolution and to make taxonomic inferences, indicating the existence of new species and differentiating between cryptic ones (Giaretta and Aguiar-Jr 1998, Medeiros *et al.* 2003; Lourenço *et al.*, 2006; Siqueira *et al.*, 2008), and data from sperm ultrastructure have been used as additional characters in systematic studies (see Jamieson and Leung, 1991; Garda *et al.*, 2002, Aguiar-Jr *et al.*, 2006; Veiga-Menoncello *et al.* 2006, 2007).

The only cytogenetic studies in *Atelopus* done so far are those on *A. varius* (Duellman, 1967; Schmid, 1980), *A. ignescens* and *A. guanujo* (Barrera *et al.* 1984), and *A. zeteki* (Ramos *et al.*, 2002), which described 2n = 22 chromosomes with highly similar morphology. No differential staining (C-banding or Ag-NOR) studies have been done in this genus. Sperm ultrastructure data are available for other genera in the family Bufonidae, but not for *Atelopus*. Species within the genera *Ansonia*, *Bufo*, *Nimbaphrynoides* and *Melanophriniscus* have been studied using sperm ultra-

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structure data (Jamieson, 2003) and show a morphological pattern that characterizes the superfamily Bufonoidea.

We present unique chromosomal data, including banding patterns, for *A. spumarius* and the first ultrastructural sperm characteristics for the genus *Atelopus*. Our aim was to expand the number of characteristics available for making taxonomic inferences in future studies.

Nine males and four females of *A. spumarius* from Reserva Florestal Adolpho Ducke, Manaus, Amazonas, were analyzed. The frogs were collected with the authorization of the Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (IBAMA - Proc. 02010.000025/2005-51). Voucher specimens were deposited in the Museu de Zoologia "Prof. Adão José Cardoso" of Instituto de Biologia at Universidade Estadual de Campinas, Brazil, under the following accession numbers: 13274-13298.

Mitotic metaphases were obtained from testicular and intestinal epithelial cell suspensions, as described by King and Rofe (1976) and Schmid (1978). The chromosomes were stained with 10% Giemsa solution and subjected to C-banding (Sumner, 1972), with slight modifications as suggested by Siqueira *et al.* (2008), and to Ag-NOR (Howell and Black, 1980) techniques. All slides were analyzed with an Olympus BX60 microscope, and images were processed using Image Pro-Plus 5.1 software. The chromosomes of at least five metaphases were measured and classified as described by Green and Sessions (1991).

Testes were removed, cut into small pieces and fixed overnight at 4 °C in 0.1 M sodium cacodylate buffer, pH 7.2, containing 2% paraformaldehyde, 2% glutaraldehyde, 3% sucrose and 5 mM $CaCl_2$. Postfixation was performed for 1 h in the same buffer containing 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM $CaCl_2$. The tissues were subsequently rinsed in sodium cacodylate buffer and incubated *en bloc* with 0.5% uranyl acetate. After rinsing in buffer, the tissue fragments were dehydrated in an increasing ethanol series and embedded in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate (Watson, 1958; Venable and Coggeshall, 1965) and examined with a LEO 906 transmission electron microscope. Scanning electron microscopy was performed following the protocol of Veiga-Menoncello *et al.* (2007).

Atelopus spumarius (Figure 1) presented a diploid number of 22 chromosomes; pairs 1, 2, 5-7 and 9-11 were metacentrics, and pairs 3, 4 and 8 were submetacentrics (Figure 2A-C). This species has the same chromosome number and chromosomal morphology similar to *A. varius*, *A.* zeteki, *A. ignescens* and *A. guanujo* (Duellman, 1967; Schmid, 1980; Barrera *et al*. 1984; Ramos *et al*., 2002). Pairs 8 and 9 have a very similar size, accounting for their inverted position when different karyotypes are compared. Such inverted position and some variation in chromosomal morphology between the karyotyped species may be due to
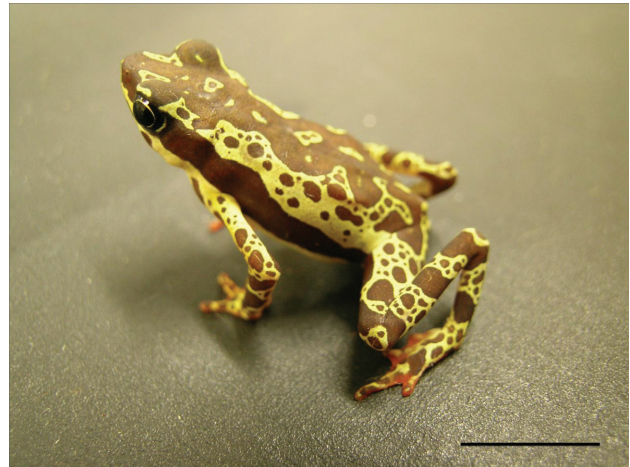
Figure 1 - Photograph of a specimen of *Atelopus spumarius* from Reserva Florestal Adolpho Ducke, Manaus, Amazonas. Photograph by S. Siqueira. Bar = 1 cm.
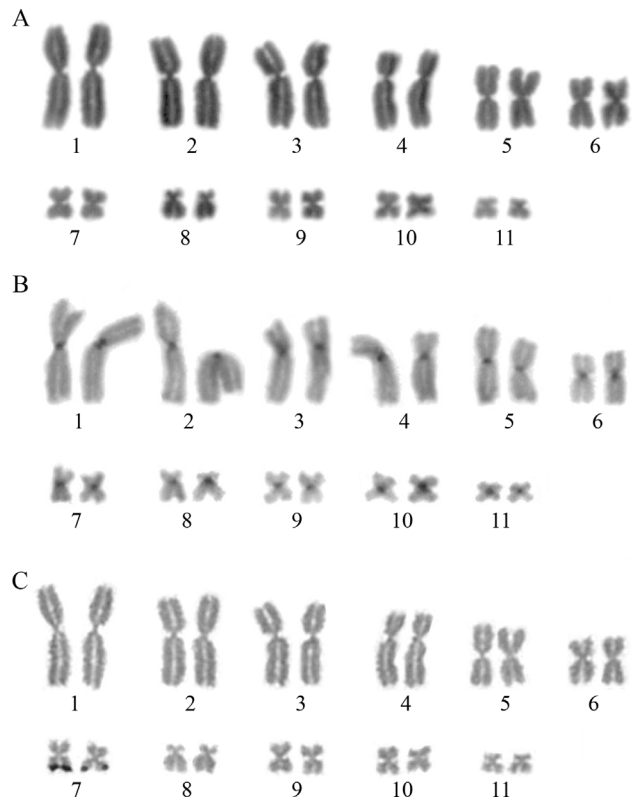
Figure 2 - Karyotypes of *Atelopus spumarius* subjected to: (A) Giemsa staining; (B) C-banding; (C) Ag-NOR staining. Note the telomeric NOR in the long arms of pair 7 (arrow).

pericentric inversions, a mechanism suggested for chromosomal evolution in *Atelopus* (Ramos *et al.,* 2002).

According to Kuramoto (1990), the majority of Bufonidae species have 2n = 22 chromosomes, suggesting that chromosome number is highly conserved in this family. This assumption was corroborated by Bush *et al*. (1977), who postulate that the bufonids have a slow rate of

chromosomal evolution in comparison with other verte-brate groups. This hypothesis is supported by karyotype analyses of other bufonid taxa, including *Rhinella crucifer*, *R. icterica* and *R. schneideri* (Kasahara *et al.*, 1996), *Melanophryniscus* species (King, 1990; Kuramoto, 1990), and the species of the genus *Bufo* studied by Baldissera *et al.* (1999), whose karyotypes are very similar to each other and to those of *Atelopus*.

All the karyotypes described so far in this genus had only been analyzed by conventional Giemsa staining. The C-banding technique here employed detected heterochro-matin only in the centromeres of all pairs (Figure 2B). The lack of C-banding patterns for other *Atelopus* species pre-cludes intrageneric comparisons. Nonetheless, the patterns described here are very similar to those found for *Chaunus* species by Amaro-Ghilardi *et al.* (2008). The shared cen-tromeric-pericentromeric banding pattern in these species reinforces the idea of a conserved karyotypic nature of this pattern, but the significance of such similarity cannot be as-sessed until the evolutionary state of such character (heterochromatin distribution) is defined.

NOR regions were detected on the telomeres of the long arm of pair 7 (Figure 2C). In *A. zeteki* (Ramos *et al.*, 2002), the pericentromeric constriction observed in pair 7 is probably the NOR site, which coincides with the location found in *A. spumarius*.

On the basis of studies of NOR location, Baldissera *et al.* (1999) found three groups within six *Bufo* species ana-lyzed. The first two groups were composed of species with Ag-NOR labeling on pair 5 and/or 10 (*B. arenarium*, *B. rufus*, *B. ictericus* and *B. paracnemis*). The third group in-cluded *B. marinus* and *B. crucifer*, where NORs were lo-cated on pair 7, which is characteristic of the *crucifer* and *marinus* groups. The last two species (*B. ictericus* and *B. paracnemis)* were later separated from the other *Bufo* spe-cies and included in the new genus *Rhinella* (Chaparro *et al.*, 2007; Frost, 2013) The shared presence of a NOR in pair 7 could indicate a degree of proximity between these taxa and the *Atelopus* species.

Under scanning electron microscopy, the spermato-zoon of *Atelopus spumarius* presented a 19 μm long fili-form head and a 40 μm long tail. The tail had a very large undulating membrane (Figure 3A). In longitudinal sections the nucleus appeared cylindrical, with the anterior portion covered by the acrosomal complex (Figure 3B). The acro-some consists of a conical electron-dense vesicle covering 6 μm of the anterior portion of the nucleus.

A subacrosomal cone filled with a diffuse, thick ma-terial is observed under the acrosome (Figure 3B). In trans-verse sections the acrosomal complex appeared circular and became progressively narrower posteriorly, together with the subacrosomal cone (Figure 3D-H). Also in these sections the nucleus appeared circular with a 0.9 μm width at its base (Fig. 3I). The chromatin was highly electron

dense and had a fibrillar-like structure, with differentially condensed areas (Figure 3H-J).

The features of the acrosomal complex here observed (*i.e.*, the nucleus covered by an acrosomal vesicle above the subacrosomal cone) are characteristic of the neobatrachian bufonoid lineage and have been described for most species of the previous genus *Bufo* (Jamieson, 2003).
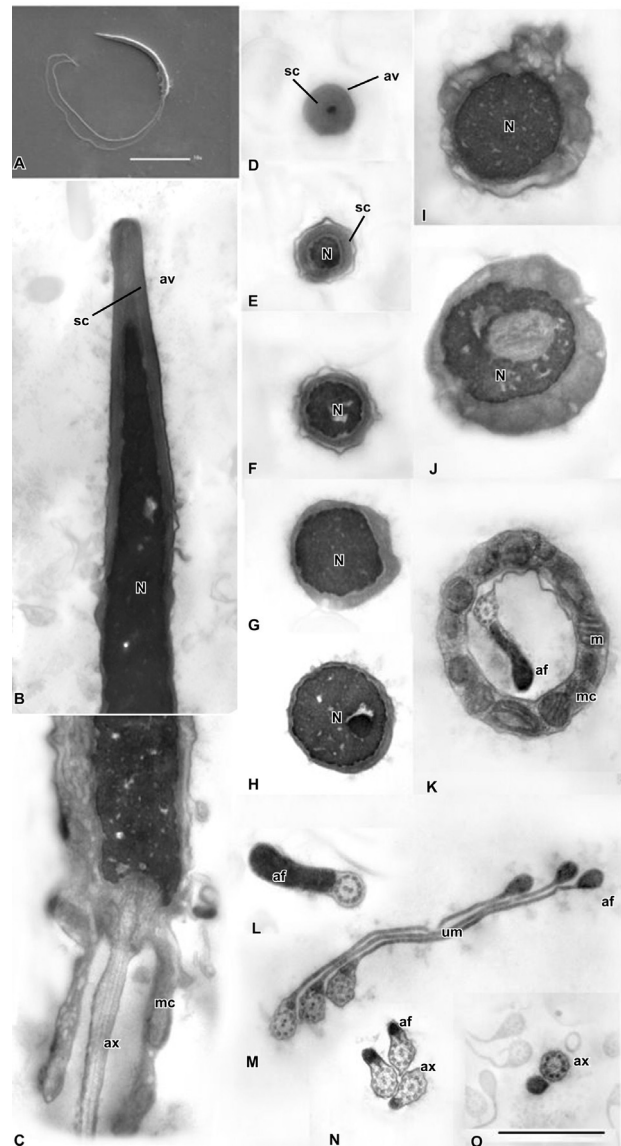
Figure 3 - Spermatozoa of *Atelopus spumarius*. Scanning (A) and trans-mission (B-O) electron micrographs. B and C: longitudinal sections of the head and flagellum, respectively. In B, note the acrosomal complex, with the acrosomal vesicle (av) and the subacrosomal cone (sc), covering the nucleus. D-H: transverse sections of the head showing the circular shape of the acrosomal complex. I and J: basal region of the nucleus showing the nucleus (N) with fibrillar-like chromatin. K: mitochondrial collar (mc) surrounding the initial portion of the flagellum. L-O: transverse sections of the flagellum, showing the axial fiber (af) and undulating membrane (um) (L and M), which become closer in the distal portion (N) until only the axoneme (ax) is observed in the final portion (O). Bar = 1 μm. Bar = 10 μm (A), 1 μm (B-O).

In the midpiece, a nuclear fossa contained the proximal and distal centrioles, which lie at an angle of 90° to each other (Figure 3C). The axoneme originates from the distal centriole. A cytoplasmic expansion containing mitochondria extended from the nuclear base to the initial portion of the flagellum, forming a collar-like structure also observed in transverse sections (Figure 3C and K).

In transverse sections, the flagellum consisted of an axoneme, an undulating membrane and a highly electron-dense axial fiber with a width similar to that of the axoneme (Figure 3K and L). The axoneme and axial fiber merge in the distal portion of the tail (Figure 3M). The fiber appeared as a protein bridge that narrows at the final portion of the tail (Figure 3N). In the distal portion of the flagellum, the axial fiber became closer to the axoneme and then disappeared together with the undulating membrane (Figure 3N). At the end of the flagellum, only the axoneme was observed (Figure 3O).

The spermatological patterns of the flagellum (*i.e.*, composed of an axoneme and axial rod, presence of a mitochondrial collar and undulating membrane) are also characteristic of the neobatrachian bufonoid lineage, as found by Jamieson (2003) in most species of the previous genus *Bufo*.

Using molecular data, Grant *et al.* (2006) presented a cladogram indicating as the *Atelopus* sister groups the genus *Melanoprhyniscus* and the three genera now included in the family Hylodidae (*Hylodes*, *Crossodactylus* and *Megaelosia*). These proposed relationships are supported by the present data, as *Melanophryniscus cambaraensis* (Báo *et al.*, 2001) and the hylodids *Hylodes phyllodes*, *Crossodactylus* sp. and *Megaelosia massarti* display the same sperm ultrastructural pattern observed in *A. spumarius.*

Taken together, the sperm ultrastructural characteristics of *A. spumarius* and the karyotype data support its close proximity to the bufonoid lineage.

The present findings better characterized the species *Atelopus spumarius* and allow future comparative studies to determine whether more than one species is assigned under this name. The unique C-banding, Ag-NOR and ultrastructural data of *A. spumarius* corroborate its position within the family Bufonidae. In addition, the numerically and morphologically conserved karyotypes within *Atelopus* species studied so far attest to the well-documented monophyly postulated by Lötters *et al.* (2010).

Acknowledgments

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