Herpetology Notes, volume 13: 679-683 (2020) (published online on 16 August 2020)

First description of the karyotype of *Ololygon machadoi* (Anura: Hylidae)

Luísa P. Reis[1], Naiara P. Araújo[2,3], Matias M. Malleret[2], Mirela P. Valeri[2], Paulo C. A. Garcia[1,4], and Marta Svartman[2,*]

Abstract. We describe for the first time the karyotype of *Ololygon machadoi* (Amphibia, Anura, Hylidae) collected in southeastern Espinhaço Mountain Range, Brazil. The chromosome preparations were obtained from tadpoles cultured fibroblasts and this population presented a diploid number 2n=24, and the absence of heteromorphic sex chromosomes. The karyotype included 12 subtelocentric chromosomes, four submetacentrics and eight metacentrics, the CBG-banding revealed heterochromatin in the pericentromeric regions of all chromosomes, and silver nitrate staining showed that the nucleolar organiser regions (Ag-NORs) are located on the short arms of pair 6. The cytogenetic information on *O. machadoi* shall contribute to future phylogenetic studies of this genus.

Keywords. cytogenetics, chromosome variation, heterochromatin, Ag-NOR

Introduction

The treefrogs of the family Hylidae Rafinesque, 1815, are remarkable by their great diversity, with over 700 valid species, representing just over 8% of all known frogs in the world (Frost, 2020). Hylid frogs are divided into seven subfamilies, including Scinaxinae, which reunites the genus *Julianus* Duellman et al., 2016, *Sphaenorhynchus* Tschudi, 1838, *Ololygon* Fitzinger, 1843, and *Scinax* Wagler, 1830. The genus *Ololygon* is composed of 50 species found from the Atlantic Forest in eastern Brazil and extending southward to northeastern Argentina and westward into gallery forests in the Brazilian Cerrado (Frost, 2020).

Cytogenetic data are available for only 24 *Ololygon* species (Bogart, 1973; Cardozo et al., 2011; Peixoto et al., 2015, 2016; Gruber et al., 2017; Targueta et al., 2018). All of them presented a diploid number 2n=24, and the absence of heteromorphic sex chromosomes. The nucleolar organiser regions (NOR) were observed on pair 6, with the exception of *O. canastrensis*, which had an additional NOR in pair 11, *O. tripui*, with an additional NOR in pair 3 (Cardozo et al., 2011; Peixoto et al., 2016) and *O. centralis*, in which the NOR is located at the proximal region of the long arm of pair 1 (Targueta et al., 2018). The heterochromatin evidenced after CBG-banding was predominantly pericentromeric, with variable amounts among species (Cardozo et al., 2011; Peixoto et al., 2015, 2016).

Therefore, we describe for the first time the karyotype of the species *Ololygon machadoi* Bokermann and Sazima, 1973, which is distributed in the large rocky meadow formation of Serra do Espinhaço (Espinhaço Mountain Range; Gontijo, 2009; Frost, 2020). The species reproduces throughout the year in permanent streams surrounded by riparian vegetation and males call during day and night (Eterovick and Sazima, 2004). Tadpoles of this species are very conspicuous with respect to the colour pattern, having recently been used for camouflage studies against predators (Eterovick et al., 2018; Gontijo et al., 2018).

[1] Programa de Pós-Graduação em Zoologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos 6627, Belo Horizonte, Minas Gerais 31270-901, Brazil.

[2] Departamento de Genética, Ecologia e Evolução, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos 6627, Belo Horizonte, Minas Gerais 31270-901, Brazil.

[3] Instituto Federal de Educação, Ciência e Tecnologia de Rondônia, Avenida Vereador Otaviano Pereira Neto 874, Jaru, Rondônia 76890-000, Brazil.

[4] Departamento de Zoologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos 6627, Belo Horizonte, Minas Gerais 31270-901, Brazil.

[*] Corresponding author. E-mail: svartmanm@icb.ufmg.br

In addition, we standardised a protocol used to obtain cell culture from tadpole tissue. Although most of the cytogenetic work conducted for this group is based on direct chromosome preparations obtained from adult´s tissues, we worked on obtaining cell cultures, which offer some advantages, as a larger number of high-quality metaphases and the possibility of repeating cell harvest in the desired conditions (Stanyon and Galleni, 1991). The optimal conditions for Amphibia cell culture were well established for the model-species *Rana catesbeiana* (Wolf and Quimby, 1964) and their protocols have been used integrally or with slight modifications in several studies (e.g. Schmid and Steinlein, 2015). Our protocol is more similar to that described by Schmid and Steinlein (2015) but with the following modifications: we used tadpole tissue, and we diluted the culture medium in water, as described by Auclair (1961) and applied in aquatic vertebrate cultures (e.g. Wolf and Quimby, 1964).

Materials and Methods

We analysed ten individuals of *Ololygon machadoi* captured in the municipality of Mariana, state of Minas Gerais, Brazil (20º09'12"S, 43º31'14"W). For the taxonomic identification of the tadpoles, we compared the specimens with others deposited in the tadpole collection (Coleção de Girinos do Centro de Coleções Taxonômicas da Universidade Federal de Minas Gerais - https://www2.icb.ufmg.br/cct/col-zoologica.html), with the original description of Bokermann and Sazima (1973), and the interactive identification key for the tadpoles of the iron quadrangle, Minas Gerais, Southeast Brazil, available at: http://biodiversus.com.br/saglab/aqf/chave/girinos/ (Pezzuti et al., 2019). Other specimens of the lot were deposited in the tadpoles collection, voucher UFMG 1857.

We maintained tadpoles in aquaria until they reached stage 42 (Gosner, 1960), at which point they were euthanised being immersed in a solution with 5% lidocaine dissolved in water. The specimens were then washed once with 10% sodium hypochlorite for two seconds and then 70% absolute alcohol for one minute, at room temperature (approximately 23-25 °C). This procedure proved to be efficient to avoid contamination during cell culture. A third of the terminal portion of the right hind limb was removed, transferred to a Petri dish, minced with sterile scalpels and scissors, and incubated in 2 mL of a collagenase solution (1 mg/mL) dissolved in Dulbeccos's Modified Eagle Medium (DMEM), supplemented with foetal bovine serum (48% DMEM, 12% FBS and 40% distilled water).

After some tests, collagenase treatment for four hours at room temperature was found to be ideal. The cells were cultured in DMEM diluted in sterile distilled water at room temperature. The pH of the medium was maintained at 7.5 using Hepes (1M) as necessary. The culture medium was changed at two or three-day intervals and the cells propagated at a 1:2 volume ratio when cell confluency reached about 70-80%. After cell multiplication, they were harvested and frozen in liquid nitrogen for future use. To obtain chromosome preparations, cells were harvested according to the following procedure: colcemid (0.01 µg/mL) was added to the culture flask and after one hour the cells were washed with phosphate saline buffer (PBS) and removed by standard trypsinisation (Stanyon and Galleni, 1991). The cells were transferred to a 15 mL test tube and centrifuged at 1000 rpm for 7 min, the supernatant was removed and the pellet was resuspended in a hypotonic solution (0.075M KCl) at room temperature for 8 minutes, after which 0.5 mL of fixative (3:1 methanol: acetic acid) was added (pre-fixation) and the cells were centrifuged again at 800 rpm for 8 min. The supernatant was discarded and the pellet was slowly resuspended in 5 mL of fixative (fixation). After fixation for 20 minutes at room temperature, the samples were centrifuged for 5 min at 800 rpm, the supernatant was removed and the cells resuspended in 5 mL of fixative. This last step was repeated twice. The chromosome preparations were dropped onto cleaned glass slides following standard procedures and stained with Giemsa's solution (Schmid and Steinlein, 2015). Untreated chromosomes were also submitted to the techniques of CBG-banding (Sumner, 1972), Ag-NOR (Howell and Black, 1980) and staining with a 4′,6-diamidino-2-phenylindole (DAPI)

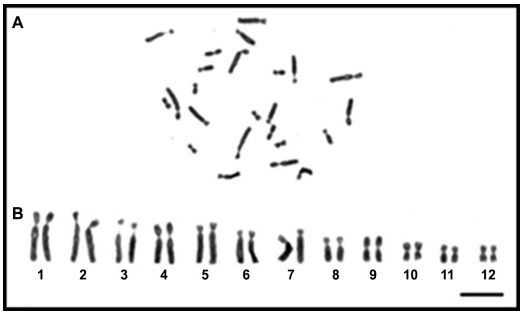

Figure 1. (A) Metaphase and **(B)** karyotype of *Ololygon machadoi* (2n=24). Bar=10µM.

Table 1. Chromosome measurements of *Ololygon machadoi*. Relative length (RL), arm ratio (AR) and classification (CP) according to Green and Sessions (1991); st: subtelocentric; sm: submetacentric; m: metacentric.

Chromosome pair	1	2	3	4	5	6	7	8	9	10	11	12
RL (%)	13,12	12,68	10,7	10,49	10,4	9,49	8,31	6,7	5,9	4,7	3,86	3,64
AR	3,06	3,92	5,1	2,15	4,05	3,77	3,03	2,68	1,18	1,22	1,65	1,15
CP	st	st	st	sm	st	st	st	sm	m	m	m	m

solution (0.8ng/μL) in Slowfade (Invitrogen). At least 15 metaphases from each individual were analysed. Analyses were performed under a Zeiss AxioImager 2 epifluorescence microscope and images captured with the AxioVision software (Zeiss). The chromosomes were aligned in decreasing order of size using Adobe Photoshop and ImageJ v.2. The chromosomes were classified according to Green and Session (1991) as metacentric, submetacentric, subtelocentric, and telocentric (Table 1), allowing the comparison with previously published work.

Results

The resulting chromosome preparations had many well-defined metaphase cells with elongated chromosomes (Fig. 1). The use of tadpoles seemed useful probably because young specimens have more dividing cells than adult tissues.

Ololygon machadoi presented a 2n=24 and the absence of heteromorphic sex chromosomes. The karyotype was composed of six pairs of subtelocentric chromosomes (pairs 1, 2, 3, 5, 6, and 7), two submetacentric pairs (pairs 4 and 8), and four pairs of metacentrics (pairs 9, 10, 11, and 12)

CBG-banding revealed pericentromeric blocks of heterochromatin in all chromosomes (Fig. 2A) and DAPI staining (Fig. 2B) showed brighter labelling in the same regions. Silver nitrate staining showed the presence of a single pair of Ag-NORs located interstitially in the short arms of chromosome pair 6 (Fig. 3).

Discussion

The 2n=24 in *Ololygon machadoi* is the same described for all species of the genus and is considered a highly conserved character. Nevertheless, the chromosomes morphology (12 subtelocentrics, four submetacentrics, and eight metacentric chromosomes) differs from that reported for most species of the genus, which presented two subtelocentrics, 12 submetacentrics, and ten metacentrics (Cardozo et al., 2011; Gruber et

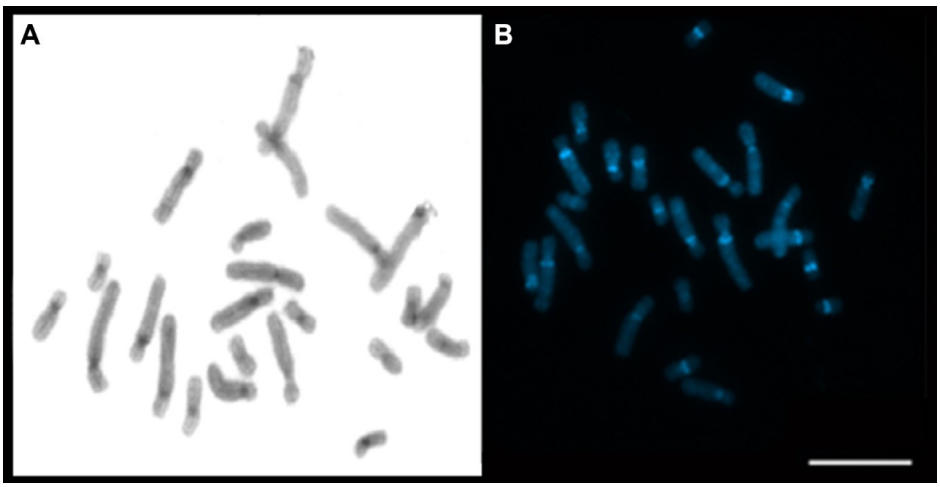

Figure 2. Metaphase of *Ololygon machadoi* (2n=24) after: **(A)** CBG-banding and **(B)** DAPI staining. Bar=10μM.

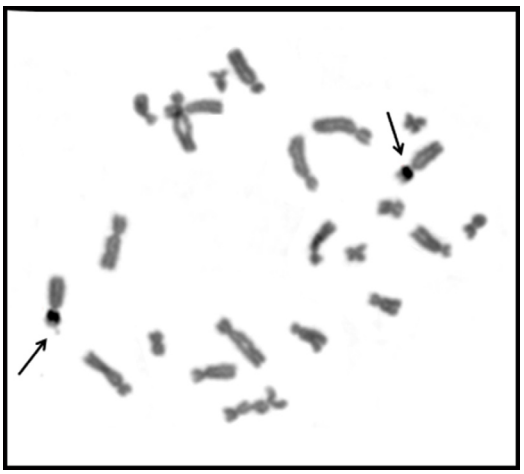

Figure 3. Metaphase of *Ololygon machadoi* (2n=24) after silver nitrate staining. Arrows indicate the Ag-NORs on pair 6.

al., 2017). The diversity of chromosome morphology is evident in other species of the genus. Nogueira et al. (2015) pointed out that rearrangements such as chromosome inversions or deletions/duplications of chromosome segments could explain this variation, while Peixoto et al. (2015) suggested that species with similar karyotypes are phylogenetically closer. The two largest submetacentric chromosomes (pairs 1 and 2) were considered a synapomorphic trait of the genus (Faivovich, 2002; Cardozo et al., 2011; Peixoto et al., 2015, 2016; Gruber et al., 2017; Targueta et al., 2018). However, Peixoto et al. (2015) reported a metacentric pair 1 in *O. belloni*. In this study, pairs 1 and 2 were subtelocentric, which may be an autapomorphy.

CBG-banding (Fig. 2A) revealed a pattern similar to that described for other *Ololygon* species (Cardozo et al., 2011; Peixoto et al., 2015, 2016) and the pattern revealed by DAPI staining (Fig. 2B) suggests that the heterochromatin of this species is AT-rich (Schweizer et al., 1979). A divergent pattern was observed only in *O. consezai*, in which some chromosome pairs had additional subtelomeric heterochromatic blocks in their long arms.

Silver nitrate staining also showed a pattern typical of the genus, in which the Ag-NORs are located in the sixth larger chromosome pair, suggesting a possible synapomorphy (Cardozo et al., 2011; Peixoto et al., 2015, 2016; Gruber et al., 2017). Divergent results were observed in *O. canastrensis* and *O. tripui*, which have an additional Ag-NOR in pair 11 and pair 3, respectively (Cardozo et al., 2011; Peixoto et al., 2016), and in *O. centralis*, in which the Ag-NOR is located at the proximal region of the long arm of pair 1 (Targueta et al., 2018).

In summary, we provide the first description of the karyotype of a population of *Ololygon machadoi* obtained from cultured fibroblasts of tadpoles. Our results indicate that the quality of chromosome preparations obtained from cell cultures improves the analysis. The protocol described may be useful for other amphibian species.

Acknowledgments. LPR, MMM and MPV were recipients of master's fellowships and NPA of a doctoral fellowship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). PCAG and MS thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for research productivity fellowships. The specimens analysed were collected under the permit 028.11/2015/MG from Instituto Estadual de Florestas de Minas Gerais. It is important to notice that a new genus *Gabohyla* has been recently created and is not cited in the Introduction: Araújo-Vieira et al. (2020). A new genus of lime treefrogs (Anura: Hylidae: Sphaenorhynchini). Zoologischer Anzeiger **286**: 81–89 (https://doi.org/10.1016/j.jcz.2020.04.002).

References

Auclair, W. (1961): Cultivation of monolayer cultures of frog renal cells. Nature **192**: 467–468.

Bogart, J.P. (1973): Evolution of anuran karyotypes. In: Evolutionary Biology of the Anurans, p. 337–349. Vial, J.L. Ed., Columbia, USA, University of Missouri Press.

Bokermann, W.C., Sazima, I. (1973): Anfíbios da Serra do Cipó, Minas Gerais, Brasil. 1- Espécies novas de "Hyla" (Anura, Hylidae). Revista Brasileira de Biologia **33**: 329–336.

Cardozo, D.E., Leme, D.M., Bortoleto, J.F., Catroli, G.F., Baldo, D., Faivovich, J., et al. (2011): Karyotypic data on 28 species of *Scinax* (Amphibia: Anura: Hylidae) diversity and informative variation. Copeia **2**: 251–263.

Eterovick, P.C., Sazima, I. (2004): Anfíbios da Serra do Cipó: Minas Gerais - Brasil. First Edition. Belo Horizonte, Minas Gerais, Brazil, Editora PUC Minas.

Eterovick, P.C., Mendes, I.S., Kloh, J.S., Pinheiro, L.T., Václav, A.B.H.P., Santos, T., Gontijo, A.S.B. (2018): Tadpoles respond to background colour under threat. Scientific Reports **8**: 1–8.

Faivovich, J. (2002): A cladistic analysis of *Scinax* (Anura: Hylidae). Cladistics **18**: 367–393.

Frost, D.R. (2020): Amphibian Species of the World: an Online Reference, version 6.1. Available at: http://research.amnh.org/herpetology/amphibia/index.html. Accessed on 10 Jun 2020.

Gontijo, B.M. (2009): Uma geografia para a Cadeia do Espinhaço. Megadiversidade **4**: 7–14.

Gontijo, A.S.B., Espanha, J., Eterovick, P.C. (2018): Is tadpole coloration adaptive against bird predation? Acta Ethologica **21**:

69–79.

Gosner, K.L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica **16**: 183–190.

Green, D.M., Sessions, S.K. (1991): Nomenclature for chromosomes. In: Amphibian Cytogenetics and Evolution, p. 431–432. Green, D.M., Sessions, S.K., Ed., San Diego, USA, San Diego Academic Press.

Gruber, S.L., de Oliveira, G.I.G., Silva, A.P.Z., Narimatsu, H., Haddad, C.F.B., Kasahara, S. (2017): Comparative analysis based on replication banding reveals the mechanism responsible for the difference in the karyotype constitution of treefrogs *Ololygon* and *Scinax* (Arboranae, Hylidae, Scinaxinae). Comparative Cytogenetics **11**: 267–283.

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

Nogueira, L., Paim, F., Diniz D., Sole M., Affonso, P.R., Siqueira, S., Sampaio, I. (2015): Cytogenetic analysis of *Scinax auratus* and *Scinax eurydice* (Anura, Hylidae) with emphasis on cytotaxonomy. Comparative Cytogenetics **9**: 227–236.

Peixoto, M.A.A., Lacerda, J.V.A., Coelho-Augusto, C., Feio, R.N., Dergam, J.A. (2015): The karyotypes of five species of the *Scinax perpusillus* group (Amphibia, Anura, Hylidae) of southeastern Brazil show high levels of chromosomal stabilization in this taxon. Genetics **143**: 729–739.

Peixoto, M.A.A., Oliveira, M.P., Feio, R.N., Dergam, J.A. (2016): Karyological study of *Ololygon tripui* (Lourenço, Nascimento and Pires, 2009), (Anura, Hylidae) with comments on chromosomal traits among populations. Comparative Cytogenetics **10**: 505–516.

Pezzuti, T.L., Leite, F.S.F., Garcia, P.C.A. (2019): Chave de identificação interativa para os girinos do Quadrilátero Ferrífero, Minas Gerais, Sudeste do Brasil. Available at: http://biodiversus.com.br/saglab/aqf/chave/girinos/**.** Accessed on 10 June 2020.

Schmid, M., Steinlein, C. (2015): Chromosome Banding in Amphibia XXXII. The genus *Xenopus* (Anura, Pipidae). Cytogenetic and Genome Research **145**: 201–217.

Schweizer, D., Ambros, P., Andrle, M., Rett, A., Fiedler, W. (1979): Demonstration of specific heterochromatin segments in the orangutan (*Pongo pygmaeus*) by a distamycin/DAPI double staining technique. Cytogenetic and Genome Research **24**: 7–14.

Stanyon, R., Galleni, L. (1991): A rapid fibroblast culture technique for high resolution karyotypes. Italian Journal of Zoology **58**: 81–83.

Sumner, A.T. (1972): A simple technique for demonstrating centromeric heterochromatin.Experimental Cell Research **75**: 304–306.

Targueta, C.P., Guerra, V., Gambale, P.G., Bastos, R.P., Melo e Silva, D., Telles, M.P.C. (2018): Cytogenetics of two hylid frogs from Brazilian Cerrado. Genetics and Molecular Biology **41**: 814–819.

Wolf, K., Quimby, M.C. (1964): Amphibian cell culture: permanent cell line from the bullfrog (*Rana catesbeiana*). Science **144:** 1578–1580.

Accepted by Clarissa Canedo