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Revisiting the polyploidy in the genus *Odontophrynus* (Anura: Odontophrynidae)

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Abstract. The genus *Odontophrynus*, composed of ten species, is found in practically the entire south of South America. *Odontophrynus americanus* was the first vertebrate registered to present natural polyploidy, considering that most individuals have 2N = 4x = 44 chromosomes, although having 2N = 2x = 22 chromosomes is considered the ancestral condition for all genera of the family Odontophrynidae. The present study aimed to analyze the karyotype of *O. americanus*, providing a detailed and comparative description of conventional chromosomal markers, with focus on a possible diploidization process operating in this polyploid genome. The individuals were collected in a fragment of Atlantic Forest in the south-central region of Paraná State, Brazil. The analyzed individuals presented the tetraploid pattern, with biarmed chromosomes. The C-banding showed heterochromatic regions restricted to centromeres and telomeres. Among homologous chromosomes of the same quartet, small differences were observed in morphology, possibly the result of differentiation after the polyploidization event. Finally, the 45S rDNA (Nucleolar Organizer Regions) was mapped in the short arm of quartet 11, showing the nucleolus organizing regions active in the four homologous chromosomes. This genome, although structurally polyploid, may be undergoing a process of diploidization, by becoming functionally equivalent to a diploid genome, via chromosomal rearrangements, epigenetic mechanisms, and/or repetitive DNA dynamics.

Keywords. Amphibian, diploidization, heterochromatin, rDNA.

According to Frost (2023), the family Odontophrynidae currently contains 55 species distributed in three genera *Macrogenioglottus* Carvalho, 1946, *Odontophrynus* Reinhardt and Lütken, 1862, and *Proceratophrys* Miranda-Ribeiro, 1920. Earlier phylogenies validate the monophyly of the family, as well as that *Macrogenioglottus* and *Odontophrynus* are sister taxa (Pyron and Wiens, 2011; Feng et al., 2017). The genus *Odontophrynus* is composed of eleven species widely distributed in southern and eastern South America. *Odontophrynus americanus* (Duméril and Bibron, 1841), a small fossorial anuran with no apparent sexual dimorphism (Quiroga et al., 2015), has the greatest distribution, its range extends to central and southern Argentina, southern Paraguay, southern Brazil, and Uruguay (Frost, 2023).

Odontophrynus americanus was the first case of natural polyploidy found in vertebrates (Beçak et al., 1966). The *Odontophrynus americanus* species group is a complex of morphologically indistinguishable diploid and tetraploid species. It includes currently four diploid species: *O. cordobae* Martino and Sinsch, 2002, *O. juquinha* Rocha, Sena, Pezzuti, Leite, Svartman, Rosset, Baldo, and Garcia, 2017, *O. lavillai* Cei, 1985 and *O. maisuma* Rosset, 2008 with 2N = 2x = 22 chromosomes, and one widely distributed tetraploid species (*O. americanus*) with 2N = 4x = 44 chromosomes (Beçak et al., 1966; Ruiz et

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al., 1981; Martino and Sinsch, 2002; Rosset et al., 2006; Rosset, 2008). Martino et al. (2019) established the existence of cryptic diversity and overestimation of species richness by combining molecular, morphological, and bioacoustic data. Populations known as *O. americanus* comprise at least three species.

Polyploidy plays an important role in speciation and evolution in anurans, with about 50 polyploid species described in several families (Bogart, 1980; Mable et al., 2011; Evans et al., 2012; Schmid et al., 2015). Polyploids originate by autopolyploidization (intraspecies whole-genome duplication) or allopolyploidization (associated with interspecific hybridization). Thus, individuals with an autotetraploid genome can originate by fusion of unreduced (i.e., diploids) gametes, or by suppression of the first mitotic division in fertilized eggs (Schmid et al., 2015). In recently evolved autopolyploids, the homologous chromosomes of a quartet are expected to exhibit identical chromosome banding patterns in somatic metaphases, leading to the multivalent formation during the first meiotic division. On the other hand, in an allopolyploid genome, if there are differences among the karyotypes of the parental species, the banding techniques or the genomic *in situ* hybridization (GISH) allow chromosomes from parental species to be distinguished (Schwarzacher et al., 1989), which will form bivalent configurations in meiosis (Schmid et al., 2015).

In this study, the structure of polyploid karyotype *O. americanus* from a southern Brazilian population is described and subjected to comparative analysis in order to add new data regarding the speculated species complex. Additionally, the data are placed in an evolutionary context, thus contributing to a better understanding of the evolutionary scenario concerning ploidy levels in this group.

Cytogenetic analyses were carried out on six juveniles of *O. americanus* collected in União da Vitória, Paraná State, Brazil (26º13’48”S and 51º05’09”W). Chromosome preparations were performed directly from bone marrow, according to Baldissera et al. (1993). Briefly, the animals received intraperitoneal injection of aqueous solution of colchicine (0.01 ml/g body weight) 1% per 6 h, and then subjected to deep sedation euthanasia by dermal absorption of Lidocaine 5% pomade, following the recommendations of the Ethical Committee in Animal Use from Universidade Estadual do Paraná.

Conventional staining was performed using 5% Giemsa in sodium-phosphate buffer (pH 7.0, for 10 min). Detection of the constitutive heterochromatin was accomplished according to Sumner (1972). Silver staining technique (Ag-NOR detection) was carried out according to Howell and Black (1980). The mitotic metaphases were analyzed under a Carl Zeiss Axiolab A1 microscope equipped with the software Zen Lite and a Zeiss AxioCam ICc1 camera with a resolution of 1.4 megapixels (Carl Zeiss, Oberkochen, Germany). Chromosomes were classified based on the centromeric index according to Green and Sessions (1991) and were arranged in decreasing size.

The specimens of *O. americanus* showed a karyotype of 2N = 4x = 44 chromosomes, distributed in eight metacentric quartets (1, 5–11) and three submetacentric quartets (2–4), thus presenting a fundamental number (FN) = 88 (Fig. 1). There was no variation among the specimens karyotyped. Exclusively between homologous chromosomes of quartets 2, 3, and 4, small differences were observed in terms of chromosomal morphology, which often made it difficult to organize these quartets. The centromeric indexes were established confirming the morphology discrepancies between homologs of the same quartet (Fig. 1). According to the relative size of the chromosomes, the species has a karyotype with four different sizes of chromosomes: one large quartet (1),

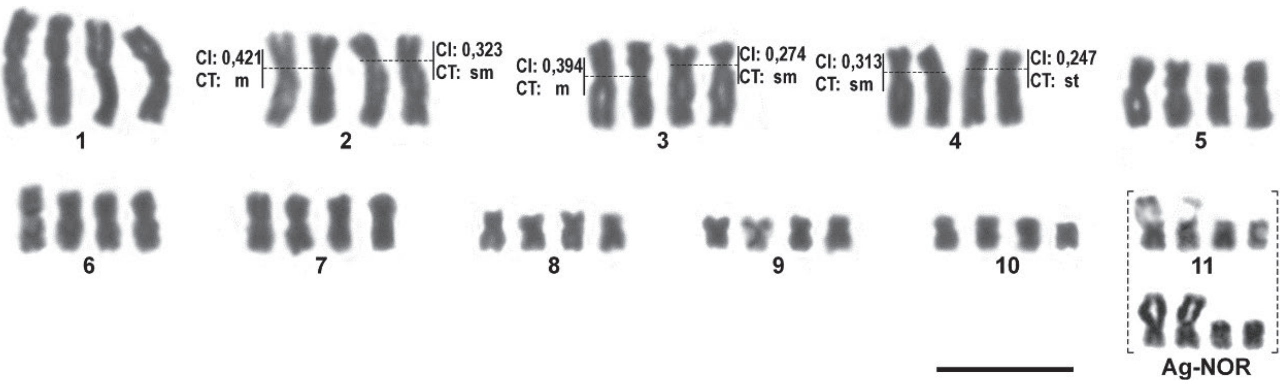

Fig 1. Giemsa-stained karyotype of *O. americanus*. Highlighted the Ag-NORs site localized on the quartet 11. CI: centromeric index; CT: chromosome type; m: metacentric; sm: submetacentric; st: subtelocentric. Bar = 10 μm.

Fig 2. C-banding karyotype of *O. americanus*. Bar = 10 μm.

three medium quartets (2–4), four small (5–8), and three very small (9–11).

Nucleolus Organizer Regions (NORs) were observed on the short arm of quartet 11. Such regions are coincident with secondary constrictions (Fig. 1). A NOR size heteromorphism between homologous chromosomes of the quartet was frequently observed.

The C-banding showed the presence of constitutive heterochromatin in the centromeric and telomeric regions of almost all quartets (absence of centromeric bands in quartets 8 and 9), and coincident with Ag-NOR staining (quartet 11) (Fig. 2).

The family Odontophrynidae was first established as a tribe within the (then) huge family *Leptodactylidae* (Lynch, 1971). The karyotype with 2N = 2x = 22 chromosomes is considered the ancestral condition, given its high frequency in all three genera. This characteristic karyotype is believed to have arisen from the differentiation of the primitive chromosome number of 2N = 26 chromosomes present in the family Leptodactylidae, followed by centric fusions (Bogart, 1973).

Karyotype descriptions of the genus *Odontophrynus* reveal so far a very similar and conserved karyotype, which is composed exclusively of biarmed chromosomes, reflecting in fundamental numbers always twice the 2N, with some constant pairs in morphology between the species (Table 1). These small variations are a consequence of chromosomal rearrangements that only modify the chromosome morphology, such as pericentric inversions, although the centromere repositioning, which alters the chromosome morphology without any accompanying chromosomal rearrangements (Rocchi et al., 2012), could be an alternative pathway leading to chromosomal remodeling.

A special interest has been devoted to the study of the occurrence of diploid (2N = 2x = 22) and tetraploid (2N = 4x = 44) constitutions in the *O. americanus* species group (see Table 1). In this sense, several studies have indicated that it could consist of a complex of species (Rosset et al., 2006; Lanzone et al., 2008; Cianciarullo et al., 2019; Martino et al., 2019) and thus, the *O. americanus* listed with 22 chromosomes are expected to probably be other distinct species.

The difficulty in organizing some quartets (i.e., 2–4) in conventional staining, due to small differences in the position of centromeres, may represent a prognosis for an incipient process of diploidization, as observed in other populations (Ruiz et al., 1981; Schmid et al., 1985). A structural heterogeneity must be created between homologous of quartets in the polyploid karyotype, which can originate even from small rearrangements such as pericentric inversions (Ohno, 1970; Ohno, 1974). Therefore, the differences within the quartets in question can be interpreted as post-polyploid events, indicating a diploidization process operating in this polyploid genome (Ohno, 1970; Schmid et al., 1985; Beçak, 2014).

The variation of NORs location in species of *Odontophrynus* is the result of translocations (Beçak and Beçak, 1974) and/or transposable elements-mediated transpositions events (Gray, 2000; Mandrioli, 2000), which switched these ribosomal genes to other pairs promoting karyotype diversification. The karyotype with NORs on pair 11 is considered as the plesiomorphic condition, found in diploid species from three species groups of *Odontophrynus*, as well as in most individuals studied from tetraploid populations of *O. americanus* (see Table 1).

A size heteromorphism between homologous was frequently observed. The presence of NOR-associated heterochromatin demonstrated that this heteromorphism between homologous of quartet 11 comprises both functional and structural aspects. This condition may have facilitated breaks and transpositions of rRNA genes to other sites in different species and populations of *Odontophrynus* (Wiley et al. 1989; Carvalho et al. 2014).

Table 1. Summary of the chromosome findings of the species of *Odontophrynus*: diploid number (2N), centromeric heterochromatin (Ⓒ), telomeric heterochromatin (Ⓣ), interstitial heterochromatin ⓘ, Nucleolus Organizer Region (NOR), short arm (p), long arm (q), fundamental number (FN), *Artificial hybrid.

Species group	Species	Locality	2N	Ploidy level	C-banding	NOR	FN	Reference
O. americanus	*O. americanus*	Brazil	22	2x	-	4p	44	Ruiz et al. (1981)
		Argentina	22	2x	ⒸⓉⓘ	4p	44	Ruiz et al. (1981)
		Brazil	22	2x	ⒸⓉⓘ	4p, 11p	44	Ruiz et al. (1981)
		Argentina	33	3x	-	-	66	Grenat et al. (2018)
		Brazil	44	4x	-	11p	88	Beçak et al. (1966)
		Argentina	44	4x	-	11q	88	Bogart (1967)
		Uruguay	44	4x	-	4p	88	Ruiz et al. (1981)
		Argentina	44	4x	-	-	88	Grenat et al. (2018)
		Brazil	44	4x	ⒸⓉⓘ	11p	88	Ruiz et al. (1981)
		Uruguay	44	4x	ⒸⓉⓘ	4p, 11p	88	Ruiz et al. (1981)
		Argentina	44	4x	ⒸⓉⓘ	11p	88	Schmid et al. (1985)
		Brazil	44	4x	ⒸⓉ	11p	88	Present study
		Uruguay	66*	6x	-	11p	132	Ruiz et al. (1981)
	O. cordobae	Argentina	22	2x	-	11-	44	Martino and Sinsch (2002)
		Argentina	22	2x	-	4p	44	Salas and Martino (2007)
	O. juquinha	Brazil	22	2x	-	4p	44	Rocha et al. (2017)
	O. lavillai	Argentina	22	2x	-	4p	44	Rosset et al. (2006)
	O. maisuma	Uruguay	22	2x	-	4p	44	Rosset (2008)
		Uruguay	22	2x	Ⓒⓘ	4p	44	Borteiro et al. (2010)
	O. reigi	Argentina, Brazil, Paraguay	22	2x	-	4p	44	Rosset et al. (2021)
O. cultripes	*O. cultripes*	Brazil	22	2x	ⒸⓉⓘ	11p	44	Ruiz and Beçak (1976)
	O. carvalhoi	Brazil	22	2x	ⒸⓉⓘ	8p	44	Ruiz et al. (1981)
O. occidentalis	*O. occidentalis*	Argentina	22	2x	ⒸⓉⓘ	9q, 11p	44	Ruiz et al. (1981)

Heteromorphic NORs could also be related to differences in genetic activity (Amaro-Ghilardi et al., 2008). In fact, in polyploids, while the number of 45S rDNA citrons is proportional to the degree of ploidy, gene expression may be equivalent to a diploid genome (Schmidtke and Engel, 1976). Epigenetic mechanisms are responsible for modulating gene expression through chemical modifications of histones, via methylation, acetylation, and/or phosphorylation (Furey and Sethupathy, 2013). Equalization of gene activity between 2x and 4x species could be at the transcriptional level, probably by rDNA methylation (Hashimshony et al., 2003). Indeed, Ruiz and Brison (1989) found high levels of methylation of ribosomal genes in tetraploid genomes of *O. americanus*. It has been validated by Cianciarullo et al. (2000), who found only 25–30% more ribosomes in *O. americanus* tetraploid than do 2N cells. Therefore, polyploid genomes may become functionally diploid throughout evolution (Schmid et al., 2015).

The presence of constitutive heterochromatin on centromeric and telomeric regions is an expected pattern in *Odontophrynus*. The eventual variation involves the additional presence of interstitial bands that characterize some species/populations (see Table 1). The variation in the distribution pattern of constitutive heterochromatin is generally associated with the dynamics of different classes of repetitive DNA. Heterochromatin is normally rich in repetitive sequences, which can have important functions in speciation and/or adaptation, as they are less subject to selective pressures, favoring the accumulation of differences throughout the evolutionary process (Martins, 2007; Böhne et al., 2008).

In conclusion, the intra- and interpopulation chromosomal variability in *Odontophrynus* is a consequence of its wide geographic distribution throughout South America. Regarding polyploidy within the group, its origin via autopolyploidization seems to be the most accepted, mainly due to the presence of multivalents at meiosis (Beçak et al., 1966; Schmid et al., 1985; Lanzone et al., 2008). However, multivalent formation can also be observed in some allopolyploids, because the structure of chromosomes from different species (i.e., homeologous) can be sufficiently conserved to permit multivalent associations. Autopolyploids, on the other hand, might also have mechanisms that prevent multivalent configuration and thus form bivalents (Gregory and Mable, 2005). Therefore, distinguishing between auto- and allopolyploidization is difficult, since the scenario possibly involves a combination of both mechanisms. The disjunct tetraploid populations are closely associated with several diploid species, which suggests that polyploidy has multiple origins, with putative older lineages accumulating more chromosomal changes within the homologous quartets. The evidence suggests that the benefits of polyploidization are stabilized by epigenetic mechanisms, small structural rearrangements, and repetitive DNA dynamics, which lead the tetraploid genomes to become functionally diploid (diploidization). Given this scenario, the analysis throughout the chromosomal mapping of repetitive elements represents a crucial tool for clarifying the dynamic processes concerned with the karyotype diversification in *Odontoprhynus* species, especially in this group with the uncertain taxonomic assignment.

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