Polyploidy and epigenetic events in the evolution of Anura

M.L. Beçak

Laboratório de Genética, Instituto Butantan, São Paulo, SP, Brasil

Corresponding author: M.L. Beçak
E-mail: mlbecak@yahoo.com.br

ABSTRACT. This article reviews the polyploidy events that have long been demonstrated to play a role in the evolution of Anura, while also discussing the importance of epigenetic control of gene expression and diversity. Findings on Brazilian autopolyploid anurans, mainly of the genus *Odontophrynus*, obtained in previous studies on their cytogenetics, chromatin ultrastructure, and molecular gene regulation are discussed here. Our data on genome duplication and on epigenetic events were analyzed here regarding phylogenetic trees, including the classic 2R model for vertebrate evolution and the growing evidence of similar epigenetic mechanisms in animal and allopolyploid plants. We propose that polyploidy and epigenetic events led to rapid Anura diversity and speciation. Also, recent advances in molecular studies in other organisms led us to revisit some controversial models of evolution.

Key words: Anuran evolution; Genetic and epigenetic mutations; Autopolyploidy

Autopolyploidy as a driving force in anuran evolution

An intriguing challenge for evolutionists is to explain how new and complex structures evolved in the course of evolution. Among the vertebrates, the emergence of amphibians from fishes during the late Devonian required anatomical and functional alterations necessary for terrestrial life. The Neo-Darwinism theory explains that these changes result from the accumulation of random mutations exposed to natural selection. Accordingly, intraspecific diversity would be a by-product of a gradual selective process of microevolution. Nevertheless, major interspecies alterations, which occurred in amphibian evolution, for example, may indicate the involvement of other mechanisms of macroevolution, leading to the creation of higher levels of organization as new genera, families, and phyla.

It is known that haploid DNA content ("genome size" or "C-value") is not associated with organism complexity. The findings of non-coding DNA, including "junk" DNA (pseudogenes) and selfish DNA (transposable elements), explained the differences in DNA content between species or other groups (Ohno, 1972; Doolittle and Sapienza, 1980). The data on genome sizes indicate great variability among animal groups (overall 130-fold, from 1C = 0.95 to 120 pg) (Gregory, 2003). Morescalchi and Olmo (1982) estimated genome sizes and repetitive and non-repetitive nucleotide fractions in some reptiles, amphibians, and selachians, and hypothesized that DNA variation reflects an adaptive role in the course of evolution of the amniotes.

The increase in DNA content in some species of anurans was demonstrated to result from autopolyploidy (Beçak et al., 1966, 1967b). This event led to the idea that the vertebrate genome evolved through two DNA duplications (2R), one in the transition of the invertebrates to the vertebrates, and the other in the divergence of the Osteichthyes (Ohno, 1970, 1999). Further molecular studies on Hox genes confirmed the occurrence of two polyploidizations and indicated that the second R occurred prior to the divergence of Osteichthyes (Postlethwait et al., 1998).

According to Ohno (1970, 1999), polyploidization provides the raw material for genome evolution through the production of gene redundancy. Later alterations of these copies would result in the acquisition of new functions and in speciation.

The idea that vertebrate evolution proceeded through polyploidizations was further confirmed by other molecular findings of paralogous genes in several gene families. Paralogous genes are related genes in the same species descendant from a genome duplication. Conversely, orthologous genes are related genes belonging to different species that descended from a speciation event.

Undoubtedly, random mutation and selection had a fundamental role in the creation of new species, as predicted in the Neo-Darwinism theory. This model of evolution assumes that mutations are random events with the same probability of occurring in natural populations. According to this idea, random mutations produce genetic differences whose frequencies would be the result of the selection of advantageous phenotypes. Despite its universal acceptance, this model of evolution is questioned regarding the way natural selection operates random mutations. The controversial idea dictated through molecular experiments assumes that nonrandom mutations could create new species. This model proposes that the genomes have already silenced polymorphisms that can reveal new phenotypes by chemicals or environmental changes (Waddington, 1942; Rutherford and Lindquist, 1998). Evidence favoring this hypothesis was presented by Rohner et al. (2013), showing that inhibition of the heat shock protein 90 (HSP 90) reveals silent polymorphisms of eye size in cavefish. This idea still remains an open question among Neo-Darwinists.

Here, we suggest that in Anura, polyploidization followed by genetic and epigenetic events led to fast diversification. This idea does not depreciate the important role of natural selection operating in single mutations, and also does not conflict with the relevant involvement of tandem duplications creating DNA increase and diversity. It adds another perspective to explain how evolution operates.

Phylogeny of the anurans

A model for the origin and evolution of the amphibians was schematically traced by Ohno (1970). On the surface, the first amphibian Ichthyostega emerged from the Crossopterygian fish in the upper Devonian. Through a dichotomy, the Ichthyostega originated the Lepospondyls and Rachitomes in the Carboniferous and Permian periods. Modern anurans descended from the Rachitomes, while living Urodela and probably Gymnophiona descended from Lepospondyls (Figure 1).
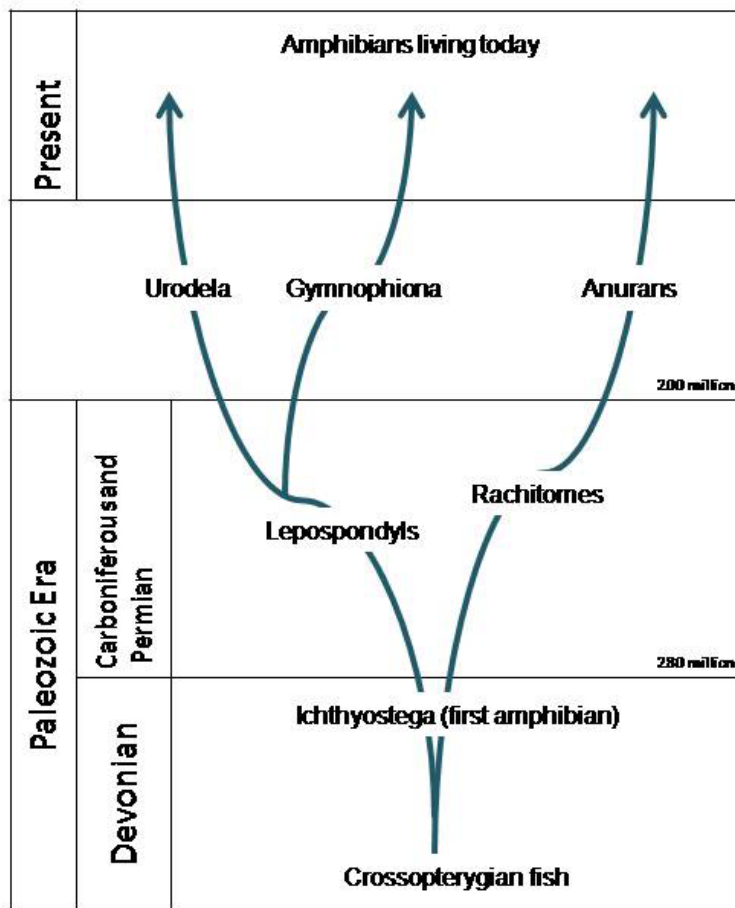

Figure 1. Diagram indicating the origin of anurans, adapted from Ohno (1970).

Another fundamental role of the Ichthyostega is that they originated the first reptile lineage, the Cotylosaurus, which evolved to Anapsida, Diapsida, and Synapsida. The Diapsida gave origin to Avis, Crocodilia, Lacertilia, and Ophidia. The Anapsida evolved into Chelonia and the Synapsida originated the mammals.

The model proposed by Morescalchi (1973) for the evolution of Amphibia was based on systematic and cytogenetic data and indicated that the recent amphibians, including Pelobatidae and higher Anura, may have originated from a Leptodactylid stock. Members of this stock had dispersed to different continents from the Gondwanaland at the end of Jurassic or afterwards (Figure 2).

Cytogenetic findings reported the occurrence of autopolyploid species in several Anura families (Figure 2 and Table 1) as well as several diploid related species (Table 2).
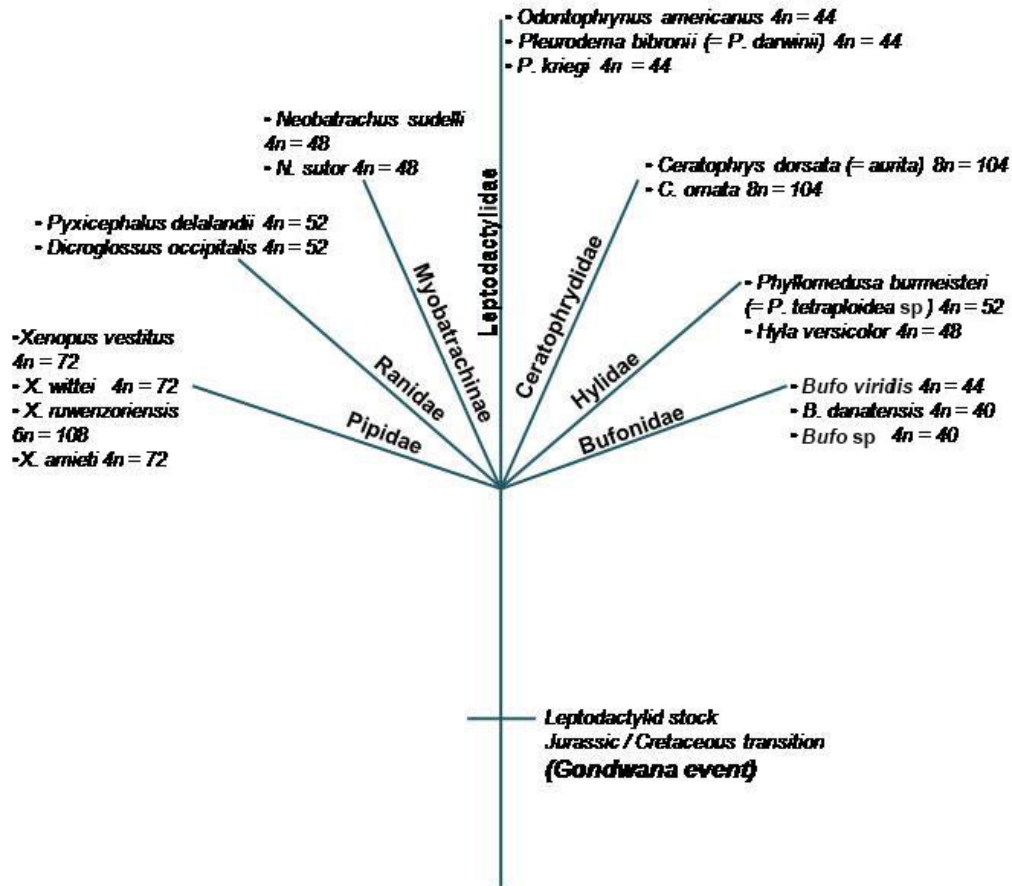

Figure 2. A schematic drawing showing the dispersion of Leptodactylid species in the Tertiary period. This event occurred before the Cretaceous, prior to the final breaking-up of the Gondwanian super-continent, based and modified from Morescalchi (1973) and complemented with further polyploidy descriptions. Time when polyploidy phenomena occurred is not determined.

Table 1. Natural polyploidy in bisexual species of Anura.

Families and species	Level of ploidy	Ancestral number	References
Leptodactylidae			
Odontophrynus americanus*	4n = 44	N = 11	Beçak, 1967a; Beçak et al., 1966, 1967b; Bogart, 1967; Barrio and Pistol de Rubel, 1972
Odontophrynus cordobae	4n = 44	N = 11	Martino and Sinsch, 2002
Ceratophrys dorsata (= *C. aurita*)*	8n = 104	N = 13	Beçak, 1967a; Beçak et al., 1967b
Ceratophrys ornata*	8n = 104	N = 13	Bogart, 1967; Barrio and de Chieri, 1970a,b; Bogart and Wasserman, 1972
Eleutherodactylus binotatus (post-polyploidy)***	2n = 22	N = 11	Beçak and Beçak, 1974a
Pleurodema bibroni (= *P. darwinii*)**	4n = 44	N = 11	Barrio and de Chieri, 1970a,b
Pleurodema kriegi**	4n = 44	N = 11	Barrio and de Chieri, 1970a,b
Myobatrachinae			
Neobatrachus sudelli**	4n = 48	N = 12	Morescalchi, 1970
Neobatrachus sutor**	4n = 48	N = 12	Morescalchi, 1970
Hylidae			
Hyla versicolor	4n = 48	N = 12	Wasserman, 1970; Bogart and Wasserman, 1972
Phyllomedusa burmeisteri (*P. tetraploidea*)*	4n = 52	N = 13	Beçak et al., 1970a
Pipidae			
Xenopus vestitus**	4n = 72	N = 18	Tymowska and Fischberg, 1973; Tymowska et al., 1977; Tymowska, 1991
Xenopus ruwenzoriensis**	6n = 108	N = 18	Fischberg and Kobel, 1978
Xenopus sp (= *wittei*)**	4n = 72	N = 18	Fischberg and Kobel, 1978; Tinsley et al., 1979
Xenopus amieti	4n = 72	N = 18	Kobel et al., 1980
Bufonidae			
Bufo danatensis	4n = 44	N = 11	Pisanetz, 1978; In Kawamura, 1984
Bufo viridis	4n = 44	N = 11	Mazik et al., 1976
Bufo sp	4n = 40	N = 10	Bogart and Tandy, 1976
Ranidae			
Dicroglossus occipitalis	4n = 52	N = 13	Bogart and Tandy, 1976
Pyxicephalus delalandii	4n = 52	N = 13	Bogart and Tandy, 1976

*Meiotic multivalent rings; **few or absence of multivalent rings; ***meiotic multivalent rings by multiple translocation post-polyploidy.

Table 2. Diploid species with related tetraploid species.

Species	2n	References
Odontophrynus americanus	22	Beçak et al., 1970a,b; Barrio and Pistol de Rubel, 1972; Bogart and Wasserman, 1972; Beçak and Beçak, 1974b
Odontophrynus cultripes	22	Beçak, 1967; Beçak et al., 1966, 1967b; Beçak and Beçak, 1974b; Beçak and Kobashi, 2004
Odontophrynus carvalhoi	22	Beçak et al., 1970a; Beçak and Beçak, 1974b
Odontophrynus barrioi	22	Cei et al., 1982
Odontophrynus occidentalis	22	Saez and Brum-Zorilla, 1966; Beçak, 1967; Beçak and Beçak, 1974b
Odontophrynus cordobae (sp)	22	Martino and Sinsch, 2002
Ceratophrys ornata	26	Barrio and de Chieri, 1970a,b
Phyllomedusa burmeisteri	26	Batistic et al., 1975
Bufo viridis	22	Mazik et al., 1976
Bufo sp	20	Bogart and Tandy, 1976
Pyxicephalus delalandii	26	Bogart and Tandy, 1976
Dicroglossus occipitalis	26	Bogart and Tandy, 1976
*Hyla chrysoscelis**	24	Bogart and Wasserman, 1972; Ralin and Selander, 1979
Hyla andersoni	24	Wasserman, 1970

**H. versicolor* was also considered to be an allopolyploid or autopolyploid species that arose from hybridization between eastern and western populations of *H. chrysosceles* (Ralin and Selander, 1979).

In the case of the family Pipidae, different levels of ploidy were found in the genus *Xenopus* from Africa. It is not known whether *Xenopus fraseri* (2n = 36), *Xenopus amieti* (2n = 72) and *Xenopus ruwenzoriensis* (2n = 108) are auto- or allopolyploid species (Kobel et al., 1980; supplemented with previous references).

As pointed out by Martino and Sinsch (2002), the data on mitochondrial DNA in *Hyla chrysoscelis*/*Hyla versicolor* indicated that the tetraploid forms had multiple origins and that there are tetraploid lineages without related diploids (Ptacek et al., 1994; Mable and Roberts, 1997).

Figure 3 shows the geographical distribution of the species of the genus *Odontophrynus* (Beçak and Beçak, 1974b; supplemented with data from Ruiz et al., 1981, 1982, 1984; Barrio and Pistol de Rubel, 1972; Saez and Brum-Zorrilla, 1966). In these studies, the sympatry of both diploid and tetraploid specimens of *Odontophrynus americanus* was found in Santa Barbara do Sul, Brazil (Ruiz et al., 1984). The latter authors reported another map with the geographical distribution of *O. americanus* in Southern Brazil.
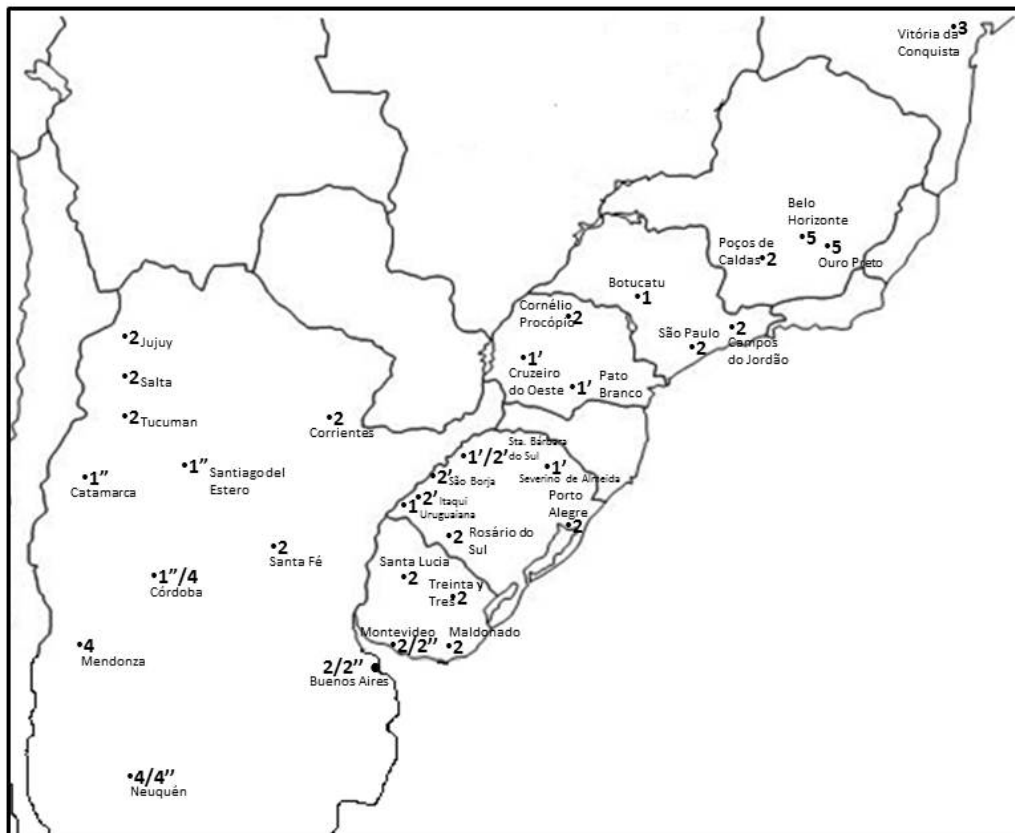

Figure 3. Geographical distribution of *Odontophrynus* species from several regions of Brazil, Uruguay and Argentina, as follows: 1) *O. americanus*, 2n; 2) *O. americanus*, 4n; 3) *O. carvalhoi*, 2n; 4) *O. occidentalis*, 2n; 5) *O. cultripes*, 2n. Beçak and Beçak, 1974b (1 to 5), Ruiz et al., 1984 (1' and 2'); Barrio and Pistol de Rubel, 1972 (1"); Saez and Brum-Zorrilla, 1966 (2"); Ruiz et al., 1982 (4").

Polyploidy in amphibians was also reviewed by Kawamura (1984). Although allopolyploidy occurred in plants and in parthenogenetic vertebrates, it was once believed that autopolyploidy would be incompatible with the sex-determination mechanisms in bisexual vertebrates (Muller, 1925). This assumption changed with the demonstration of autopolyploidy in bisexual anurans (Beçak, 1967a; Beçak et al., 1966, 1967b, 1970a; Beçak and Beçak, 1974a,b).

Although heteromorphic sex chromosomes are not commonly present in anurans, some cytogenetic studies have reported male and female heterogamety. *Pyxicephalus adspersus* has female heterogamety (ZZ/ZW) (Schmid, 1980; Schmid and Bachman, 1981); *Eusophus migueli* and *Rana esculenta* have male heterogamety (XX, XY) (Iturra and Veloso, 1981; Schempp and Schmid 1981, respectively).

Intra- and interspecific chromosomal polymorphisms in *Odontophrynus*

Several investigations have examined the polymorphisms of secondary constrictions (Table 3) and of C and NOR bands (Table 4) in species of South American *Odontophrynus*. Hypothetical phylogenies based on these polymorphisms were proposed to explain the evolution of this genus (Beçak and Beçak, 1974b; Ruiz and Beçak, 1976; Ruiz et al., 1980, 1981; Cortadas and Ruiz, 1988).

In accordance with the geographical distribution and the polymorphism of secondary constrictions, the karyotype having satellites in pair 11 (*O. americanus*, 2n; *Odontophrynus cultripes*, 2n; and *Odontophrynus occidentalis*, 2n) was assumed as basic (Figure 4).

An eventual translocation in 2n *O. americanus* probably moved these satellites to pair 4. Further hybridization of this population could originate the karyotype with satellites in both pairs 4 and 11, as described by Beçak et al. (1974b). Duplication of this genome would originate the tetraploid population with satellites on the same pairs. A loss of the satellites on pair 4 could originate the tetraploid karyotype with satellites only on pair 11.

Another suggestion also indicated that the 4n species with satellites on pairs 4 and 11 resulted from the hybridization of two 2n populations with different positions of the satellites. The presence of satellite in pair 8 of 2n *Odontophrynus carvalhoi* was explained by an eventual translocation from pair 11 (Beçak and Beçak, 1974b).

On the basis of NOR banding, another phylogenetic line was assumed to explain the evolution and diversification of the genus *Odontophrynus* (Ruiz et al., 1981). This idea proposes that an original ancestor with satellites on pair 11 gave rise to the contemporary species. The authors also assumed the suggestion by Beçak and Beçak (1974b) that translocation may explain the appearance of satellites in chromosomes 4 and 8.

Chromosomal rearrangements as multiple translocations in post-polyploids occurred in Southern Brazil and Uruguay. These alterations, reducing the number of chromosomes, led to the diploidization process. Also, some cytogenetic polymorphisms at secondary constrictions (Beçak, 1969; Beçak and Beçak, 1974b) or in NOR bandings or C-bandings in *O. americanus* (Ruiz et al., 1980; 1981; Schmid et al., 1985; Almeida et al., 1986) and in *Ceratophrys ornata* (Schmid et al., 1985), as well as in *Ceratophrys dorsata* (Soares-Scott et al., 1988), have been interpreted as post-polyploid events.

Interspecific variability of DNA content has also been reported in Amphibia, including *Odontophrynus* species (Beçak et al., 1970a).

Table 3. Chromosome position of secondary constrictions in 2n and 4n species of *Odontophrynus*.

Species	Chromosome types				Localities	References
	4	8	10	11		
O. cultripes, 2n	-	-	-	11,11	Minas Gerais, Goiás (Brazil)	Beçak et al., 1966, 1967a,b, 1970a
O. occidentalis, 2n	-	-	-	11,11	Argentina	Beçak et al., 1970a, 1974b
O. carvalhoi, 2n	-	8,8	-	-	Bahia (Brazil)	
O. americanus, 2n	4,4	-	-	11,11	São Paulo (Brazil)	Beçak et al., 1970b
	4,4	-	-	-	Santiago del Estero, Catamarca, Cordoba (Argentina)	Barrio and Pistol, 1972; Ruiz et al., 1982
	-	-	-	11,11	Cordoba (Argentina)	Bogart and Wasserman, 1972
O. americanus, 4n	-	-	-	11,11,11,11	São Paulo, Minas Gerais, Paraná (Brazil), Argentina	Beçak et al., 1966; Beçak and Beçak, 1974b; Saez and Brum-Zorrilla, 1966; Schmidt et al., 1985
	-	8	10	11,11,11,11	São Paulo (Brazil)	Beçak and Beçak, 1974b
	4,4,4,4	-	-	11,11,11,11	Rio Grande do Sul (Brazil), Santa Lúcia (Uruguay)	Beçak and Beçak, 1974b
	-	-	-	11,11,11,11	TreintayTres and Maldonado (Uruguay) and Tucuman and Azul (Argentina)	Barrio and Pistol de Rubel, 1972
	4,4,4,4	-	-	11,11,11,11	Uruguay and Argentina	Barrio and Pistol de Rubel, 1972

Table 4. NOR position in 2n and 4n species of *Odontophrynus*.

Species	Chromosome types				Localities	References
	4	8	9	11		
O. cultripes, 2n	-	-	-	11,11	Minas Gerais (Brazil)	Ruiz et al., 1982
O. occidentalis, 2n	-	-	-	11,11	Mendoza (Argentina)	
	-	-	9,9	11,11		
O. carvalhoi, 2n	-	8,8	-	-	Bahia (Brazil)	
O. americanus, 2n	4,4	-	-	-	São Paulo (Brazil), Cordoba (Argentina)	
	4,4	-	-	11,11	São Paulo (Brazil)	
	4,4	-	-	-	Cassino, Friburgo (Brazil)	Almeida et al., 1986
	4,4	-	-	11,11	Friburgo (Brazil)	
O. americanus, 4n	-	-	-	11,11, 11,11	São Paulo (Brazil)	Ruiz et al., 1982
	4	-	-	11,11, 11,11	Montevideo and Salto Grande (Uruguay)	
	4,4,4,4	-	-	-	Salto Grande (Uruguay)	
	-	-	-	11,11, 11,11	Argentina	Schmidt et al., 1985
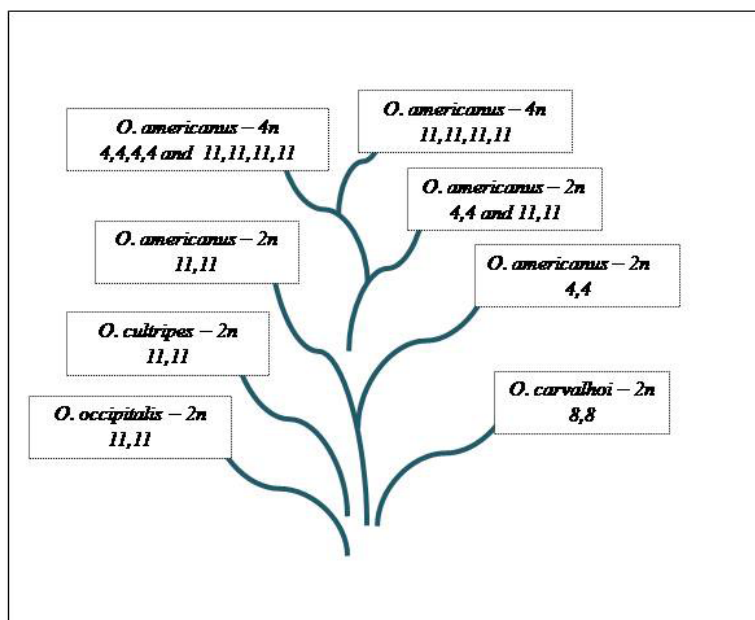
Figure 4. A suggestion to explain the evolution of *Odontophrynus*, based in the polymorphism of secondary constriction, according to the model by Beçak and Beçak, 1974b.

Besides polyploidy and translocations, other chromosome alterations have been observed in the anurans. In the family Leptodactylidae, the great interpopulation variation of the diploid number in *Pseudopaludicola falcipes*, with 2n = 16, 2n = 18, 2n = 20, and 2n = 22, was attributed to centric fusions (Beçak, 1967, 1968; Batistic, 1970). Fusion or fission of the centromeres accounted for the alterations between the karyotypes with 2n = 16 and 2n = 18. The higher diploid numbers (2n = 20 and 2n = 22) were explained by the probable occurrence of chromosome changes such as pericentric inversion or translocation after centric fusion (Batistic, 1970). Centric fusions were also described in Hylidae, with species having 2n = 22, 2n = 26, 2n = 30, 2n = 48, and 2n = 52 chromosomes (Beçak, 1968; Rabello, 1970).

Structural alterations of the genomes have also been observed in the post-polyploid anuran *Eleutherodactylus binotatus*, a diploid species (2n = 22) with 4-fold DNA content and possessing a dodecavalent ring in the diplotene. These post-polyploid multiple translocations were interpreted as signals of diploidization (Ohno, 1970; Beçak and Beçak, 1974a). Translocation events detected through the presence of multivalent meiotic configurations also occurred in other anuran genus, *Physalaemus petersi*, 2n (Lourenço et al., 2000).

According to Morescalchi (1973), the more primitive karyotypes belonging to Ascaphids and Discoglossids contain acrocentric chromosomes and microchromosomes. Probably centric fusions occurred during evolution, reducing the chromosome number. The origin of the Pipidae is still controversial: this family is probably derived from primitive ascaphid forms (Morescalchi, 1973).

Time of divergence of Amphibia

Different methods have been used to estimate the time of divergence of vertebrates during the course of evolution. The paleontological data based on fossil records (Ohno, 1970) were compared with molecular clock results based on models of nucleotide or amino acid substitution (Hedges and Kumar, 2002). An interesting conclusion obtained from these molecular studies was that the molecular clock data to explain animal diversity agree with those of paleontological analysis of fossil records (Hedges and Kumar, 2002).

Analysis of the divergence time estimated using the molecular and fossil records of the Paleozoic and Cenozoic showed some differences regarding the origin of placental mammals. The results indicated that these animal groups originated before the Cretaceous and extinction of dinosaurs (Kumar and Hedges, 1998).

A new method was recently developed using DNA markers to estimate divergence time during evolution (Pennisi, 2013b). The data obtained showed that turtles are closer to crocodiles and birds than to lizards and snakes. This conclusion disagrees with Ohno's suggestion (1970) that turtles (descendants of Anapsida) diverged in time from crocodiles, alligators, lizards, snakes, and birds (descendants of Diapsida) in the early Triassic of the Mesozoic Era.

According to Gould (1995) and Ohno (1996, 1997), with the exception of Porifera and Coelenterata, almost all phyla appeared simultaneously within a 6 to 10 million-year window in the 550-million year Cambrian period. This great detonation of diversity is known as the Cambrian Explosion. According to Gould (1995), the Cambrian Explosion indicates the abrupt formation of the animal kingdom, in which all the structural archetypes arose during the same period.

Regarding the origin of present day amphibians, there are relatively scarce phylogenetic studies, because there is an evolutionary gap of fossils in the Mesozoic Era. Studies based on comparative DNA content showed that the Urodela as well as some Anura and Apoda have high DNA content. Great variations in nuclear DNA quantity were described between species of a single family as well as between families. Some interspecific differences in nuclear DNA content were attributed to polyploidy (Beçak et al., 1967b; Morescalchi, 1973).

The time when polyploidy occurred among Anura species is not known. Considering the high frequency of quadrivalent and octovalent chromosomes in meiosis of the 4n *O. americanus* and *Phyllomedura burmeisteri* (= *P. tetraploidea* sp) and in the 8n *C. dorsata*, it seems that polyploidization in these species was a more recent event compared to other polyploid species.

By to the time when tetraploidy occurred, studies of α- and β-globin sequences indicated that the diversification of *Xeropus laevis* and *Xeropus tropicalis* happened at 110-120 million years ago (MYA). Moreover, a probable duplication of the *X. laevis* genome occurred at 40-60 MYA (Knöchel, 1994). These conclusions are based on previous data showing that *X. leavis* is a probable tetraploid (2n = 36), having two clusters of α- and β-globin genes, while *X. tropicalis* (2n = 20) has only one cluster (Jeffreys et al., 1980; Hosbach et al., 1983).

To study the level of diversity among diploid and tetraploid species, we performed interspecific mating of *O. americanus* (4n) with several 2n species of the same genus. Artificial triploid (3n = 33) hybrids were obtained from *Odontophrynus* by the mating of 4n *O. americanus* females with 2n males of *O. americanus*, *O. cultripes* and *O. carvalhoi* (Beçak et al., 1968a; Beçak and Beçak, 1970b). The observations in meiosis that 3n produces n, 2n and 3n gametes and aneuploids led the authors to assume that 3n may be a stepping stone for the production of different ploidy levels (Beçak and Beçak, 1970) (Tables 5 and 6). This assumption later found support on the description of 3n allopolyploids in populations of *P. burmeisteri* (= *P. tetraploidea* sp) (Haddad et al., 1994).

Table 5. Induced polyploid hybrids in intra- and interspecific mating.

Species				Authors
O. americanus ♀ (4n) São Paulo - Brazil	*O. cultripes* x♂ (2n) Minas Gerais - Brazil F₁: viable (up to 5 years old) 3n = 33 (spermatocytes with III, II, I configurations)	*O. americanus* x♂ (2n) São Paulo - Brazil F₁: viable 3n = 33 x(2n) Montevideo (Uruguay) F₁: viable 6n = 66	*O. carvalhoi* x♂ (2n) Bahia - Brazil F₁: viable 3n = 33	Beçak et al., 1968a Beçak et al., 1970b Beçak and Beçak, 1970 Ruiz et al., 1981
O. americanus ♂ (4n) São Paulo - Brazil	*O. cultripes* x♀ (2n) Minas Gerais - Brazil F₁: unviable 3n (postgastrula stage)	*O. americanus* x♀ (2n) São Paulo - Brazil F₁: unviable 3n		Beçak et al., 1968a Beçak et al., 1970b Beçak and Beçak, 1970

Table 6. Natural polyploid hybrids from interspecific mating.

Species		Authors
O. americanus (4n) Minas Gerais - Brazil	*O. cultripes* x (unknown sex) (2n) F₁: 1 viable young 3n = 33	Ruiz et al., 1980
P. burmeisteri (= *tetraploidea*) (4n) São Paulo - Brazil	*P. distincta* x (unknown sex) (2n) F₁: 7 viable 3n (with III, II, I configurations)	Haddad et al., 1994

Epigenome: epigenetic control of genome expression

Epigenetic events are mechanisms that allow different expression of the genome in different cells and stages of development without change in the original DNA sequences. The variation of gene expression is caused by molecular mechanisms such as methylation of cytosine residues, histone modification, and non-protein coding RNA (ncRNA). The mechanisms of epigenetic control constitute the epigenome.

Methylation signals

Ongoing biochemical studies indicate that the steps in animal development are controlled by genetic and epigenetic signals. Epigenetic marks are those that are inheritable without DNA sequence alteration. These signals identify active and silenced genes. DNA methylation and histone alterations are epigenetic marks.

It is known that DNA methylation signals are erased in the initial steps of development and a *de novo* methylation pattern is restored during implantation (Hashimshony et al., 2003). In animal cells, DNA methylation can account for epigenetic silencing of gene activity through chromatin condensation. Higher order chromatin fibers contain inactive genes. Conversely, open chromatin fibers unfolded into nucleosomes display active DNA sequences. Chromosome regions with condensed heterochromatin contain a low density of active genes and high levels of transposons and repeats. It was also pointed that DNA methylation is required to prevent DNA damage caused by transposons (Yoder et al., 1997).

The presence of methyl groups is now accepted to be necessary for chromatin condensation and gene silencing. In the chromosomes, the methyl group binds preferentially to H_1 histone (Jost and Hofsteenge, 1992), which is associated with the DNA linker between nucleosomes. The condensed chromatin fibers characterize the heterochromatic regions of the chromosomes. Histones H_3 and H_4 are mostly hypoacetylated in these heterochromatic regions, which carry silenced genes (Hashimshony et al., 2003).

Furthermore, regulatory proteins were found to bind preferentially to DNA palindromes (Mezquita et al., 1985). Long palindromes formed by smaller ones were found in the amino and carboxyl terminal regions of H_1 belonging to five species of vertebrates (trout, chicken, human, rat, and mouse) (Ohno and Beçak, 1993).

Relatively few investigations of chromatin structure have been performed in lower vertebrates. Comparative studies evidenced that in snakes and in the tetraploid anuran *O. americanus*, the chromatin fibers from spermatocytes I have a similar appearance as the ones of mammalian spermatocytes in regard to nucleosomes and higher order structures (Beçak et al., 1977).

An epigenetic aspect found in *O. americanus* 4n was the occurrence of amphiplasty, characterized by diphasic stages of the cell cycle revealed in the two halves of the genomes. This phenomenon may indicate differences in the DNA replication time, probably related to methylation (Beçak and Beçak, 1998). Epigenetic mechanisms silencing extra copies of genes were also proposed (Schmidtke et al., 1976; Ruiz et al., 1989; Beçak and Kobashi, 2004 for review).

Comparative studies using autoradiography demonstrated that, although there were differences in satellite replication time between the parental chromosomes of triploid interspecific hybrids, asynchronous DNA replication did not occur in tetraploids (Batistic et al., 1973).

Structural studies using restriction-enzyme banding in *Odontophrynus* (Beçak et al., 1988; Beçak and Kobashi, 2004 for review) suggested that the difficulty in obtaining G-bands in anurans would be caused by the lower number of CG sequences (probably arranged in small agglomerations) or due to higher chromatin packing, as previously proposed (Schmid, 1978; Birstein, 1982; Bernardi et al., 1985; Schmid and de Almeida, 1988).

In the anuran *O. americanus*, pioneer papers reported that genetic variability of autopolyploidy specimens results from the tetrasomic expression of the duplicated alleles. Indeed, in comparing the expression of homologous genes in natural autopolyploid specimens of *O. americanus* with the diploid ones, research indicated that the electrophoretic patterns

of several enzymes exhibit three phenotypes in diploids, while there are five in tetraploids, as expected by Hardy-Weinberg $(p+q)^4$ (Beçak et al., 1968b; Schwantes et al., 1969, 1976, 1977).

The tetrasomic expression of the duplicated alleles and the silencing of extra copies in the tetraploids support the further "subfunctional" model that partitioning of ancestral functions among duplicated copies may occur after genome doubling (Lynch and Conery, 2000, Adams et al., 2003).

Previous experiments were performed to clarify whether or not the greater genetic variability observed in the 4n species resulted from the redundant copies or from gene regulation. Accordingly, it was shown that in 2n and 4n *O. americanus*, autotetraploidy does not result in doubling total RNA content (Beçak and Goissis, 1971), even though 4n have twice the number of 18S and 28S ribosomal genes (Schmidtke et al., 1976). Furthermore, the quantitative synthesis of lactate dehydrogenase and hemoglobin has been demonstrated to be reduced in 4n *O. americanus*, being similar to what had been found in 2n species (Beçak and Pueyo, 1970).This was confirmed by data obtained using NOR studies (Ruiz et al., 1981).

It was stated that the reduced genetic activity in the tetraploid could be at the transcriptional level, probably by methylation of rDNA (Ruiz and Brison, 1989). This hypothesis was further supported by data on erythropoiesis and on the transcription of DNA coding for hemoglobin in 2n and 4n *O. americanus*, which revealed that the 4n cells have only 30% more hemoglobin and 25-30% more ribosomes than do 2n cells (Cianciarullo et al., 2000).

Besides the analysis of chromatin structure, it was also observed in electron microscopic studies that the reduced transcription activity of the 4n specimens, when compared to the 2n, may be the result of either the lower number of nuclear pore complexes (NPC) in the tetraploids or changes in the transport of products to the cytoplasm (Maul et al., 1980). Along the same line of investigation, further electron microscopic description of complex aggregates of neighboring NPCs in the 4n species indicated a phenotype of low metabolic activity (Beçak and Fukuda-Pizzocaro, 2007).

Comparative studies in diploid *Hyla chrysoscelis* and tetraploid *H. versicolor* (Bachmann and Bogart, 1975) revealed that the mechanism of gene regulation is similar to that found for 2n and 4n *Odontophrynus*.

Recently, several reports claimed that epigenetic mechanisms account for alterations of gene expression in human cells through chemical modifications of the histones by either methylation, acetylation or phosphorylation (Furey and Sethupathy, 2013; Kasowski et al., 2013; Kilpinen et al., 2013).

Rapid genome changes after polyploidy were demonstrated to occur in molecular experiments using synthetic polyploids of *Brassica* (Song et al., 1995). It was suggested that these genome alterations could accelerate the process of evolution. The data obtained also showed that alterations in DNA methylation may account for genome changes. Also, in plants, fast alteration of flowering time due to methylation changes was reported in *Arabidopsis thaliana* (Pennisi, 2013a). Curiously, they observed that the new phenotypes were transmitted through eight generations of this plant.

Transposable element (TE) signals

Molecular experiments have demonstrated that eukaryote genomes display only a fraction of protein-coding DNA. The human genome with 3 billion DNA bases has only

21,000 structural genes. Two-thirds of the human genome consists of TEs, which can move throughout the genome (Lorenc and Makalowski, 2003). TEs are considered to originate from virus insertion (Ovchinnikov et al., 2002). They were first named "parasites" or "selfish" DNA because of their ability to out-replicate independently and for being considered unnecessary DNA to hosts (Doolittle and Sapienza, 1980).

The first reports showing that DNA amount does not correlate with organism complexity (the C-value paradox) led to the previous assumption of "junk" DNA, without function and considered to be "remains of extinct genes" (Ohno, 1972).

Accumulating evidence obtained from molecular studies shows that TEs are epigenetic marks involved in alterations of gene expression without changes of DNA sequences. Now they account for important roles regarding gene regulation and protein diversification, driving rapid species divergence (Makalowsky et al., 1994; Nekrutenko and Li, 2001; Ovchinnikov et al., 2002; Fedoroff, 2012). In plants with high DNA content, species divergence seems to be caused by TEs. Fedoroff (2012) pointed out that speciation in plants is a consequence of TE activation caused by DNA damaging agents, interspecific hybridization, and allopolyploidization. Conversely, it was proposed that the insertion of retroviral DNA in host genome may affect the control of RNA transcription and splicing, and could be either deleterious or useful to the host (Weiss and Stoye, 2013).

Recently, comparative studies of the mechanism in dosage compensation in *Drosophila melanogaster, D. pseudoobscura* and *D. miranda* demonstrated that two waves of TE amplification allowed the evolution of dosage compensation in neo-X-chromosomes (Ellison and Bachtrog, 2013; Chuong and Feschotte, 2013).

In the case of anurans, experiments on the molecular organization of α-globin genes in 2n and 4n species of *O. americanus* revealed that intron 2, which is usually found in vertebrates, is absent in both cases, indicating that these sequences may be pseudogenes related to retrotransposition (Acedo et al., 1997). Yet, analysis of ribosomal intergenic spacers (IgSs) demonstrated a high level of amplification of these regulatory sequences in the 4n anurans, and that probably a transposon-like sequence was inserted in these IgSs during evolution (Alvares et al., 1998).

CONCLUSION

Here, we discuss our data on cytogenetic and population studies dealing with the evolution of the anurans while also focusing on molecular biology data obtained from other organisms.

Among the anurans, the genus *Odontophrynus* is a pivotal reference for understanding the diversification of the species. Population studies using cytogenetic and phylogenetic data showed that polyploidy had a fundamental role in the process of speciation. Moreover, the detection of post-polyploid alterations, such as multitranslocations, indicated how genome evolution has proceeded in different families after genome duplication. Also, molecular studies on gene regulation of hemoglobin, several enzymes, and ribosomal RNA have demonstrated that methylation is an effective epigenetic factor, silencing half of the duplicated genome and equalizing gene activity of 2n and 4n species.

The central dogma of biological evolution according to the Neo-Darwinism theory establishes that the accumulation of random mutations causes differences among organisms which will be exposed to natural selection. Nevertheless, variability and speciation in an-

urans by genome duplication modulated by genetic and epigenetic events poses the validity of a complementary mechanism of evolution. Indeed, polyploidization and epigenetic events diverge from the classic conviction that variability is due only to the concatenated process: coding DNA sequences → mRNAs → proteins.

Our assumption is based on several approaches dealing with the level of gene expression of either rDNA, several enzymes, or hemoglobin comparatively analyzed in diploid and tetraploid species of anurans. It was assumed that the reduced expression of these genes detected in the tetraploid species was caused by methylation silencing of half of their genomes. Also, the tetrasomic gene expression determined in the tetraploids indicated post-polyploid diversification. This idea is supported by the subfunctionalization model proposed for allopolyploid plants. Moreover, the identification of pseudogenes and remains of TEs in ribosomal genes indicated that these signals had a role in promoting gene variability after polyploidy.

This scenario poses the conclusion that polyploidy modulated by genetic and epigenetic agents has been a dynamic force driving rapid variability among anurans.

This assumption is supported by emerging data in *Brassica* and *Arabidopsis* allopolyploid plants, indicating that genetic and epigenetic events stabilize duplicated genomes (Comai et al., 2000; Pikaard, 2001; Lee and Chen, 2001).

Current researchers propose that alterations of gene activity without changes in DNA sequences can be inheritable in animal evolution. Rohner et al. (2013) showed that alterations in HSP 90 can reveal silenced polymorphisms in cavefish. This means that masked polymorphisms present in genomes could reveal new phenotypes by stress created by environmental changes. The advantageous traits would be positively selected. Along the same line, Skinner (2014) proposes that "transgeneration epigenetics" occurs in rats.

In the meantime, whether epigenetic mutations are really heritable or not remains to be determined and demand further studies.

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