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Evolution, Epigenetic inheritance, Development – a diplo/tetraploid model for Anura evolution

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ABSTRACT. This review deals with innovative concepts of evolution in vertebrates, such as epigenetic mechanisms and transgenerational inheritance. Evolutionary models based on data of fossil records, cytogenetics and molecular genetics are indicated. The 2R-model of vertebrate evolution is focalized as well as the epigenetic mechanisms of gene regulation and variability of polyploid anurans. It is known that science evolves by routes that are sometimes impelled by puzzling questions. The cytogenetic data here reported for Anurans brought some perplexing considerations involving fundamental concepts of neo-Darwinism regarding slow/fast evolution, ploidy, epigenetics, and transgenerational inheritance. Indeed, a growing body of evidence reveals that besides gene mutations, diversity may also be produced by epigenetic mutations of regulatory segments of DNA. Yet, an intriguing point to be explained is whether these types of mutations can promote evolution via transgenerational inheritance.

Key words: Evolution of polyploid anurans; Epigenetics; Transgenerational inheritance; Development

INTRODUCTION

The animal groups that now inhabit Earth present a great variability of structures and functions. These differences have been investigated in several scientific studies of paleontology, geology, cytogenetics, molecular genetics, and development. These studies

facilitated the knowledge of the evolutive routes of ancestral lineages during the geological scale of time. It has been well established that diverse animal taxa became extinct by geological events and the age of fossils estimated by the study of radioactive minerals in rock sediments.

The first theory of evolution suggested by Lamarck in 1809 indicated that the environment affects the animal structure through the use or disuse of the organs. This theory known as "inheritance of acquired characteristics" did not show convincing results. Based on studies of fossil records, geology, animal morphology and embryology, Charles Darwin published his theory of evolution in the book "The origin of species by natural selection" (Darwin, 1859). This theory came to similar conclusions to the ones of Wallace obtained from studies of animal and plants of the Malay Archipelago. Both scientists and friends published their ideas together in the same year. Later, the Darwin model adjusted with the Mendelian laws of inheritance led geneticists to the well-established neo-Darwinism model.

The advent of molecular techniques for genome analysis enhanced the knowledge of the routes of evolution experienced by organisms of different taxa. The relatively recent field of Biology termed Evo-devo is another attempt to elucidate the mechanisms that control cellular differentiation during ontogeny and the phenotypic innovations created by evolution. In this field new molecular techniques were developed to help the identification of which type of RNA transcription is active in a specific cell differentiation during ontogenesis. Here a brief survey is given on vertebrate evolution, including some innovator suggestions as epigenetic mutations, transgenerational inheritance and development.

The review focuses on the evolution of Anura by genome duplications. The study of polyploid anurans performed in our laboratory supports the 2R-model of vertebrate evolution elaborated by Ohno (1970). Later, this model was confirmed by molecular data, showing the increase in protein complexity of polyploidy in vertebrates. Details of primate evolution obtained by other researchers were included here to better understand the 2R-model traced from fishes to humans.

The early genetic models described here are discussed in the light of actual modern molecular data. These trees were diagrammatically simplified without systematic descriptions and focusing only the main ancestral roots of the actual vertebrates The populational cytogenetic and molecular studies of polyploid anurans were performed with specimens from Brazil and other South American countries. Yet, some biogeological events of Earth were also considered in this evolutive analysis.

EVOLUTION

Bioevolution and the geological scale of time

Our planet started its formation about 4.5 billions years ago according to the geological scale of time. This scale is classified in four Eons: Hadean, Arquean, Proterozoic and Fanerozoic. The Fanerozoic Eon contains three Eras: Paleozoic, Mesozoic and Cenozoic. These Eras are also divided in Periods and Epochs (Figure 1).

According to fossil records early life appeared in the Arquean with the first unicellular organisms. Pluricellular organisms were discovered only in sediments of the Proterozoic Eon. There is a consensus that the prototypes of all extant animals appeared in the Fanerozoic Eon, during the Cambrian period of the Paleozoic Era.

The origin of the vertebrates from Devonian fishes Osteichthyes (about 400 mya) has been indicated in phylogenetic trees according to geological scale of time (Figure 1 and Figure 2).

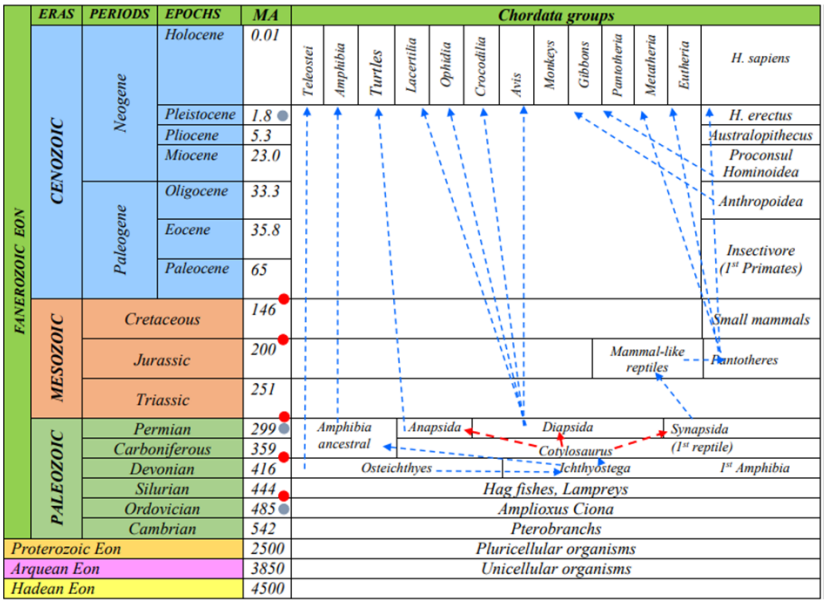

Figure 1. Diagram showing the origin of living Chordata groups (based on Ohno's phylogenetic trees, with modifications). Geological scale of time (based on Teixeira et al., 2009); Mya (million years ago); mass extinction (red spheres); glaciations (blue spheres) (Grotzinger and Jordan, 2013a).

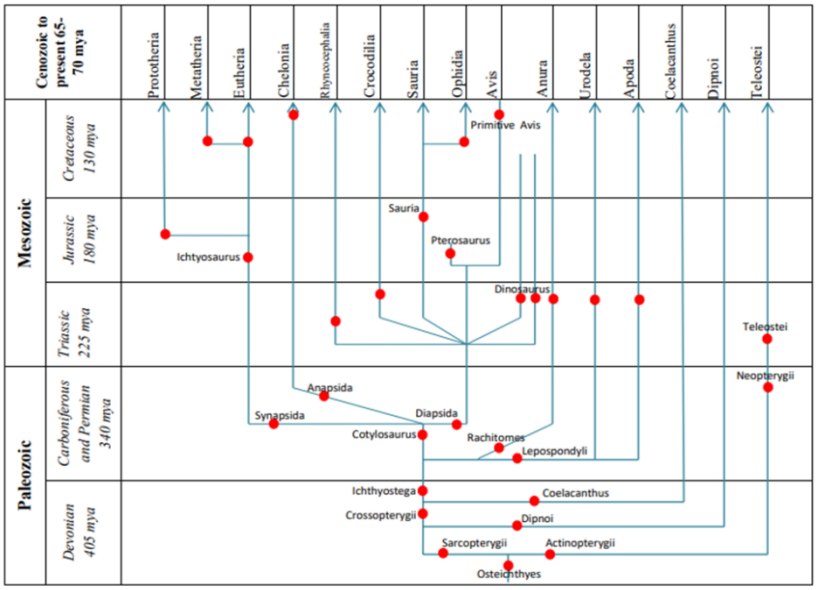

Figure 2. Diagram showing the phylogenetic tree of present vertebrates, based on Ohno (1970).

Mass extinction and evolutive radiation

An analysis of the fossil registers evidenced that during evolution several extinctions of species or a group of species (mass extinctions) occurred followed by the emergence of new species (evolutive radiations). Mass extinction is related to some geological events, such as continental breakages, glaciations, meteors, volcanisms and tectonisms (Table 1 and Figures 3 and 4).

Table 1. Diagram ofsome geological events during Mesozoic periods: continental drift [Gaeta and Martins, 2009]; glaciations and mass extinction [Grotzinger and Jordan, 2013]; geologic times [Teixeira et al, 2009]; meteors [Fairchild, 2009]. M=meteor, E = eruption, G= glaciations, GW= global warming.

Paleozoic	Mesozoic			Cenozoic					
Permian 299-251mya	Triassic 251-300mya	Jurassic 200-146mya	Cretaceous 146-65.5mya	Paleocene 65.5-55.8mya	Eocene 55.8-33.9mya	Oligocene 33.9-23.0 mya	Miocene 23.0-5.3mya	Pliocene 5.3-1.8mya	Pleistocene 1.8-0.01mya
Pangea supercontinent	*Early Amphibia*	*Pangea initial breakages*	*Pangea final final breakages*				*Cooling off periods*	*Glaciations?*	*Glaciations (G)*
Mass extinctions 251 mya (M or E)		*210 mya (E)*	*Mass extinctions – Global warming (GM)*		*65mya (M)*	*55mya*			

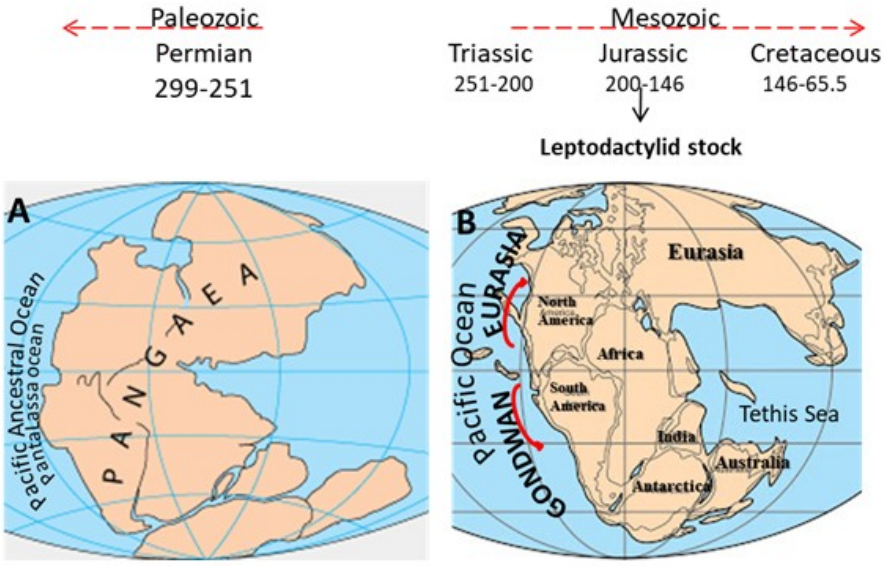

Figure 3. Drawing of the Pangea supercontinent in the Permian period (**A**) and its initial breakage in Laurasia and Gondwana during the Jurassic/Cretaceous (about 150mya). (**B**) followed by new fragmentations at the end of the Cretaceous (65 mya), which led to the current configurations of the continents (based on Grotzinger and Jordan, 2013; Gaeta Tassinari et al., 2009 and geology.com/articles/supercontinent.shtml. The Lepctodactylid stock during the Jurassic period (150 mya) is based on Morescalchi (1973) (from Beçak, 2018).

Figure 4. Meteors positioned in craters in Southeastern Brazil during Cretaceous, Paleocene and Eocene. The larger crater in central Brasil was caused by a meteor during the Triassic (based on Fairchild, 2009), from Beçak (2018).

Five mass extinctions occurred in the periods: Silurian, Carboniferous, Permian/Triassic, Jurassic and Cretaceous (Figure 1). An enormous mass extinction occurred at the end of Permian (about 251 mya) eliminating 95% of species. It is unknown which agent caused this extinction (Grotzinger and Jordan, 2013a).

Another mass extinction in the Cretaceous was caused by environmental changes produced by the impact of a meteor in the Yucatan (Mexico). It is estimated that 75% of the species disappeared, including the dinosaurs (Table 1).

A big geological event occurred in the Jurassic / Cretaceous periods with the fragmentation of a supercontinent and formation of the actual continents. This supercontinent was termed Pangea by Wegener (1915) in his Continental Drift Theory (Figure 3). Later it was described that the continental breakage was caused by the movement of tectonic plates (Wilson, 1968 in Grotzinger and Jordan, 2013b).

A remarkable event called Cambrian Explosion or the "*Big-Bang"* of Biology, occurred in the Paleozoic period, resulting in the emergence of a high diversity of biotypes. These biotypes are considered prototypes of all future fauna (Figure 1). It is not known which agent caused this level of diversity.

However, recent paleoecologic studies showed that complex animals lived millions of years during the Ediacarian pre-Cambrian period. These animals were segmented, with bilateral symmetry and had internal and external skeletons composed of mineralized tissue (Wood, 2019).

Intense climate changes caused by two glaciations occurred before the Cambrian period in the Proterozoic Eon. Other glaciations happened in the Ordovician period and Permian / Carboniferous periods. Glaciations also occurred during the Pleistocene with

cooling in the Miocene (Table 1) (Teixeira et al, 2009; Grotzinger and Jordan, 2013b.In the case of the Paleocene – Eocene (55 mya) a mass extinction was caused by the global warming produced by the elimination of methane gas from the oceans (Table 1).

Since its origin our planet suffered these structural alterations. During a long geological time, the organisms experienced innovations in their structure and functions. These processes allowed adaptations to new ecological niches. Some models were reported to explain the mechanisms creating this evolutive diversity.

Evolution of the Primates

The emergence of *Homo sapiens* in the Pleistocene period includes a captivating history of evolution regarding the appearance of human intelligence. Human evolution stages have been studied through the analysis of the anatomy of the fossils as well as by evolutive cytogenetic experiments of extant mammals. With the advent of molecular methodologies, it was possible to compare some human DNA sequences with remains of ancestral lineages. Despite recent discoveries by molecular researches, more information is needed to decipher the evolutionary history of *H. sapiens*.

According to Ohno's suggestion the first primate lineage, probably represented by insectivores appeared in the Paleocene / Eocene periods (Figure 1 and 5). Members of this order of Mammalia evolved, creating the Hominoidea, a super family that lived in the Miocene. This superfamily split into two branches. One branch, Limnopithecus, evolved creating the actual gibbons. The other branch, Proconsul was the ancestral of the hominids. During the Pliocene, the Proconsul separated into Dryopithecus and the pre-hominid Australopithecus. The Dryopithecus originated the actual groups of chimpanzee, gorilla and orangutang.

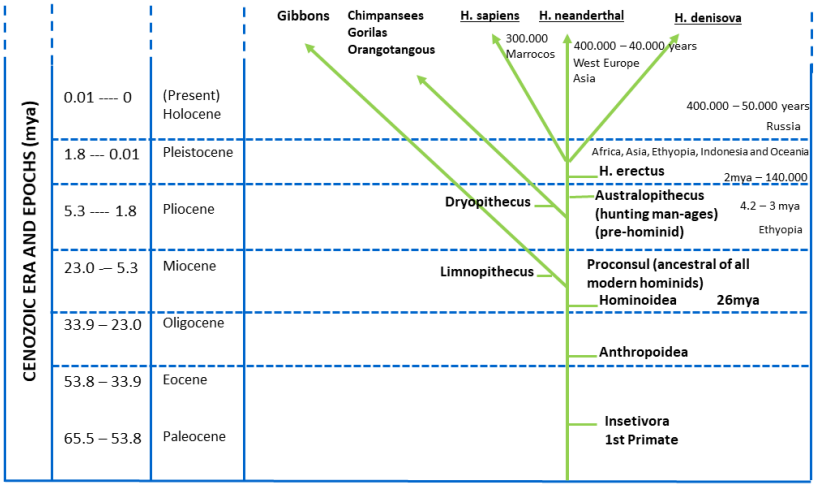

Figure 5. The family tree of *Homo sapiens* (based on Ohno 1970; dos Santos and Lewino, 2019; geologic periods from Teixeira et al., 2009).

The branch Australopithecus is a special lineage that gave origin to the *Homo erectus* in the Pleistocene. In the Pleistocene the *Homo erectus* resulted in three species,

Homo sapiens, *Homo neanderthal* and *Homo denisova* (Ohno, 1970; dos Santos and Lewino, 2019) (Figure 5). DNA sequencing studies showed that modern human share DNA sequences with Neanderthals and Denisovans. This fact indicates that the ancestors of the three groups met and mated with an older hominid, probably *H. erectus* or other contemporary species. Now, there is a consensus that interbreeding between these groups happened before they left Africa (Lowery et al, 2013).

The evolution of the primates was studied using cytogenetic methodologies comparing different species as chimpanzee, orangutang, gorilla and human. The chromosome banding results showed the occurrence of centric fusions, translocations, pericentric inversions correlated with the diversification of the species. Based in these data the authors described phylogenetic trees of the evolution of the primates (de Grouchy, 1974; Dutrillaux, 1981). Since these studies biochemical analysis showed that single-base changes accounted for 1.4% of the differences between chimps / humans. Insertions and deletions accounted for 3.4% (Britten, 2002).

Another model of *Homo sapiens* suggests a cascade of events as described (Freire-Maia, 1988):

Australopithecus afarensis (3.8 mya)
A. africanus (3 mya)
Homo habilis (2 mya)
H. erectus (1.3 – 1.8 mya)
H. sapiens (300 – 500.000 years ago)

Evolution of the Anura (Amphibia) and geological Earth events

Among tetrapods the evolution of Anura has been analyzed by investigators of paleontology, systematic, cytogenetic and molecular experiments. During the course of evolution, these animals experienced some drastic geological Earth events in the Mezosoic periods (Table 1). Morescalchi (1973) reviewed systematic and cytogenetic data and proposed that the anurans emerged from a Leptodactylid lineage in the Jurassic period and diversified since the Cretaceous (Figure 6). This suggestion indicated that some groups spread before the Cenozoic to parts of Gondwanaland and probably other parts. These anurans include: Leptodactylidae, Hylidae, Bufonidae, Ranidae and Ceratophrydidae. The diversification of these forms extended to the Paleocene and Miocene/Pliocene. It is not clear whether Myobatrachidae is related to Bufonidae before Gondwana breakages. The oldest family Pipidae from early Jurassic had specialized forms in early Cretaceous (Savage, 1973) and probably derived from Ascaphid forms (Nobel, 1931) or from a pro-Anura stock (Griffits, 1963; Nevo, 1968). A more recent origin was indicated for Brachicephalidae, Centrolenidae and Pseudidae (Morescalchi, 1973).

The *Odontophrynus* genus classified in the Leptodactylidae family (Savage and Cei, 1965) was reclassified in the family Odontophrynidae (Anura: Neobatrachia) by Pyron and Wiens (2011). Ohno (1970) proposed that the actual amphibians emerged from the Ichthyostega originated from the Crossopterigian fish of the upper Devonian (Figure 7). Through a dichotomy this fish originated the Lepospondyls and Rachitomes in the Carboniferous / Permian periods. The Rachitomes originated the modern anurans and the Lepospondyls gave origin to the Gymnophiona and Urodela.

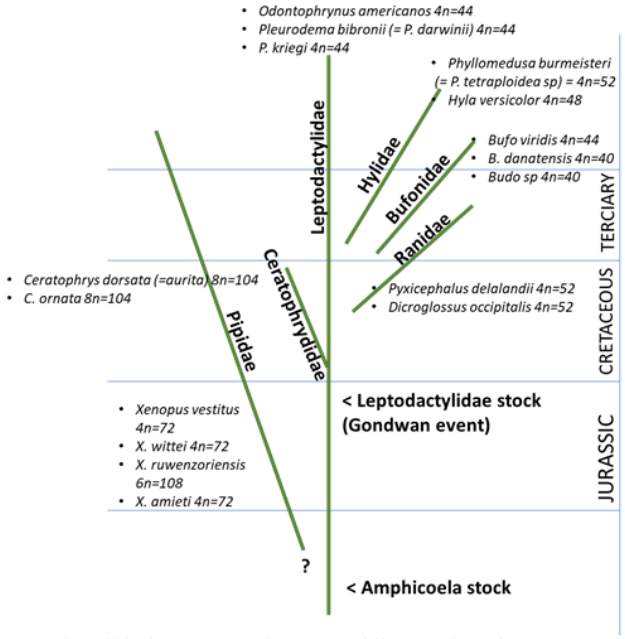

Figure 6. Radiation of Leptodactylids from a Jurassic ancestral lineage (based on Morescalchi, 1973).

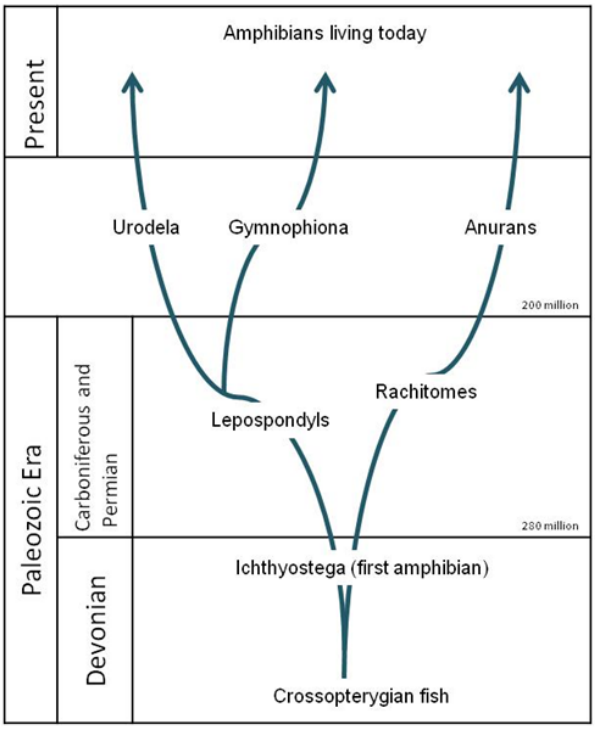

Figure 7. Diagram indicating the origin of anurans, adapted from Ohno (1970) and Beçak (2014).

Recent studies on fish-tetrapods transition using molecular issues and CRISPR techniques give support to Ohno's model of evolution. The results indicated that actual genes controlling the development of limbs, and other organs systems for life on land were created by alterations of genes belonging to fish ancestral lineages (in Pennisi, 2021a).

2R-model of vertebrate evolution

The first model to explain biological evolution was published by Darwin in the famous book *"On the origin of the species by natural selection"* (1859). In it, Darwin assumed that all living individuals of a species descend from a same lineage. Alterations of structure and functions along life would be exposed to natural selection, then incorporated and transmitted to offspring. This assumption was based in studies of comparative embryology.

Today, according to the neo-Darwinism concept of evolution new species emerge by the gradual accumulation of gene mutations producing variability that are exposed to natural selection. In plants new species are quickly created by duplication of hybrid genomes, a process termed allopolyploidy. In animals, it was proposed that gene redundance by autopolyploidy may account for the rapid emergence of new species (Ohno, 1970). This idea was confirmed by later data showing that the number of protein-coding genes in vertebrates is four times that found in invertebrates such as *Caenorhabditis elegans, Ciona intestinalis* and *Drosophila melanogaster* (Spring, 1997).

Coherent with Ohno's idea is the discovery of autotetraploidy in bisexual anurans belonging to Leptodactylidade and Hylidae families. (Beçak et al, 1966; 1967a; Bogart, 1967). Species of these families have different ploidy levels with 2n, 4n and 8n complements.

Pospolyploid species were also reported (Ohno et al, 1968; Wolf et al, 1969) having residual multivalents and high DNA content (Ohno and Atkins, 1966; Atkins and Ohno, 1967; Beçak and Beçak, 1974a). The 2R-model suggested that vertebrates evolved through two rounds of genome duplications. The first duplication in Chordate evolution occurred probably in the Cambrian period of the Paleozoic Era. Later, a second round might have occurred in the Devonian.

Further molecular studies on "Hox" genes confirmed the occurrence of the two polyploidization events and indicated that the second round of genome duplication happened previously and before divergence of Osteichthyes (Postlethwait et al, 1998). A third duplication was also described in fish genomes in the Devonian period after the radiation of ray – finned fish (Actinopterygian and Sarcopterygian lineages) (Meyer and Schartl, 1999).

According to Ohno's model, polyploidization provides the raw material for genome evolution via gene redundance. Diversification of the extra copies may produce new phenotypes and speciation. In conflict with this prediction, it was assumed that gene redundance in vertebrates is caused by tandem duplications (Martin, 1999).

Also, experiments with bacteriophage cannot explain Ohno's suggestion that protein diversification evolved by duplication and mutations. In fact, the results with this virus showed that protein variability may be produced without duplication (Petrie et al., 2018). It may be due to high rate of mutations and small rate of genes repair.

A diplo-tetraploid model for Anura evolution

The first autopolyploid anurans described in cytogenetic studies include *Odontophrynus americanus* (4n=44), *Ceratophrys dorsata* (8n=104), which belong to the Leptodactylidae family and *Phylomedusa burmeisteri* (4n= 52) from the Hylidae family (Beçak et al, 1966;

1967a; 1970b; Bogart, 1967). Also, it was reported that *Eleutherodactylus binotatus* (2n= 22) among the Leptodactylidae is a pospolyploid species in the process of diploidization (Beçak ,1974a).

Today there are about 22 anuran species described as tetraploid, some having related 2n species and other species presenting diploidization (Tables 2 and 3). Multivalent configurations in meiosis of *O. americanus* were previously found, but interpreted as caused by multitranslocations and not polyploidization (Saez and Brum, 1959). Further studies of NORs (nucleolar organizing regions) showed the relationships between *O. americanus 2n* and *4n* species (Ruiz et al, 1981; Almeida et al, 1986; Schmid et al, 1985).

Table 2. Natural polyploidy in bisexual species of Anura.

Families and Species	Level of ploidy	Ancestral number	References
Leptodactylidae			
*Odontophrynus americanus**	4n=44	n=11	Beçak ML et al., 1966; Beçak ML 1967a; 1967b. Bogart, 1967; Barrio and Pistol de Rubel, 1972
Odontophrynuscordobae	4n=44	n=11	Martino and Sinsch, 2002
*Ceratophrysdorsata (= C. aurita)**	8n=104	n=13	Beçak ML, 1967a;Beçak ML et al., 1967.
*Ceratophrysornata**	8n=104	n=13	Bogart, 1967; Barrio andChieri, 1970a, 1970b; Bogart and Wasserman, 1972
*Eleutherodactylusbinotatus (pospolyploid)****	2n=22	n=11	Beçak ML and Beçak W, 1974a
Pleurodemabibroni (= P. darwinii)	4n=44	n=11	Barrio andChieri, 1970a, 1970b.
*Pleurodemakriegii***	4n=44	n=11	Barrio andChieri, 1970a, 1970b.
Myobatrachinae			
*Neobatrachussudelli***	4n=48	n=12	Morescalchi, 1990
*Neobatrachussutor***	4n=48	n=12	Morescalchi, 1990
Hylidae			
Hyla versicolor	4n=48	n=12	Wasserman, 1970; Bogart and Wasserman, 1972.
*Phyllomedusaburmeisteri (P. tetraploideasp)**	4n=52	n=13	Beçak ML et al. 1970a.
Pipidae			
*Xenopusvestitus***	4n=72	n=18	Tymowska and Fischberg, 1973; Tymowskaet al., 1977; Tymowska1991
*Xenopusruwenzoriensis***	6n=108	n=18	FischbergandKobel, 1978
*Xenopussp n (=wittei.sp)***	4n=72	n=18	Fischberg and Kobel, 1978; Tinsley et al., 1979
Xenopusamieti	4n=72	n=18	Kobel et al, 1980
Bufonidae			
Bufodanatensis	4n=44	n=11	Pisanets, 1978
Bufovirides	4n=44	n=11	Mazik et al., 1976
Bufo sp	4n=40	n=10	Bogart and Tandy, 1976
Ranidae			
Dicroglossusoccipitalis	4n=52	n=13	Bogart and Tandy, 1976
Pyxicephalusdelalandii	4n=52	n=13	Bogart and Tandy, 1976

* Meiotic multivalent rings; **Few or absence of multivalent rings; ***Meiotic multivalent rings by multiple translocation pos-polyploidy.

The first diploid species (2n=22) *O. americanus* was cytological described by Beçak et al, 1970. The specimens studied were collected in Botucatu, Brazil. Later, these frogs were designed as *O. moratoi* (Jim and Caramaschi,1980) and reclassified as *Proceratophrys moratoi* (Amaro et al, 2009). Others diploid species were described in *Odontophrynus* genus (Table 3).

Polyploidy was assumed to had happened in the Eocene in *Xenopus* (Knöchel, 1994) and *Odontophrynus* (Pyron and Wiens, 2011). The wide distribution of the *O. americanus* in South American countries was studied by us and other authors (Figure 8).

Table 3. Diploid species with related tetraploid species.

Species	2n	References
Odontophrynus americanus	22	Beçak ML et al., 1970a, 1970b; Beçak ML and Beçak W, 1974b; Barrio and Pistol de Rubel, 1972; Bogart and Wasserman, 1972.
Odontophrynuscultripes	22	Beçak ML 1967a; Beçak ML et al., 1967b; Beçak ML and Beçak W, 1974b.
Odontophrynuscarvalhoi	22	BeçakML et al., 1970a; Beçak ML and Beçak W, 1974b.
Odontophrynusbarrioi	22	Cei et al, 1982.
Odontophrynus occidentalis	22	Saez and Brum 1966; Beçak ML, 1967a; Beçak ML and Beçak W 1974b
Odontophrynuscordobaespnov	22	Martino and Sinsch, 2002; 2008
Odontophrynusmaisumaspnov	22	Rosset, 2008
Odontophrynusjuquinha	22	Caramaschi and Napoli, 2012; Rocha et al, 2017
Odontophrynusreigi	22	Rosset et al, 2021
Ceratophrysornata	26	Barrio and Chieri, 1970a and 1970b.
Phyllomedusaburmeisteri	26	Batistic et al, 1975
Bufoviridis	22	Mazik et al, 1976
Bufosp	20	Bogart and Tandy, 1976
Pyxicephalusdelalandii	26	Bogart and Tandy, 1976
Dicroglossusoccipitalis	26	Bogart and Tandy, 1976
*Hylachrysoscelis**	24	Bogart and Wasserman, 1972; Ralin and Rogers, 1979; Ralin e Selander, 1979
Hylaandersoni	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. chrysoscelis*, (Ralin and Selander, 1979).

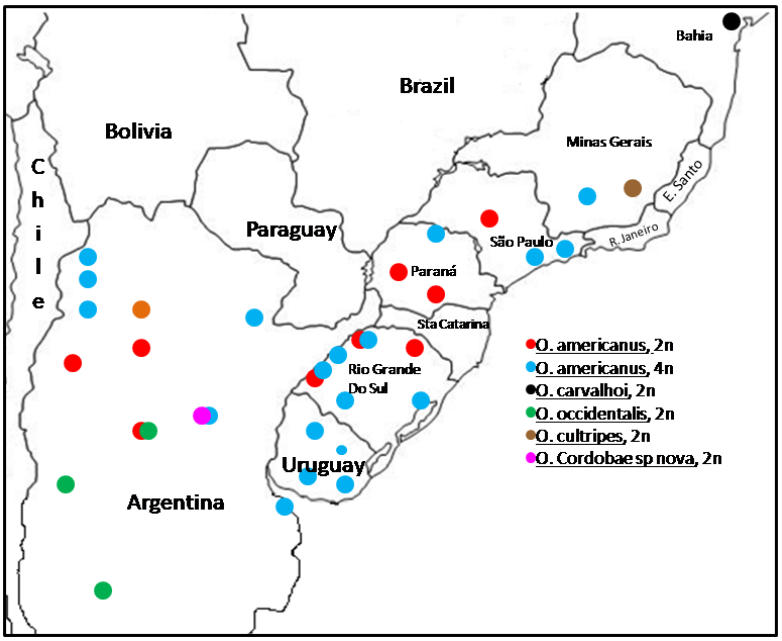

Figure 8. Geographic distribution of diplo and tetraploid species of *Odontophrynus americanus* in South American countries (Table 2 and Table 3) (based on Beçak, 2014).

Several anuran families with polyploid and pospolyploid specimens were described in South America, Africa, Australia and Asia (Figure 9). According to the position of the rDNA genes (Table 4; Ruiz et al, 1981) contained in the chromosomal satellites (Beçak and Beçak, 1974b), we suggested a phylogenetic tree (Figure 10).

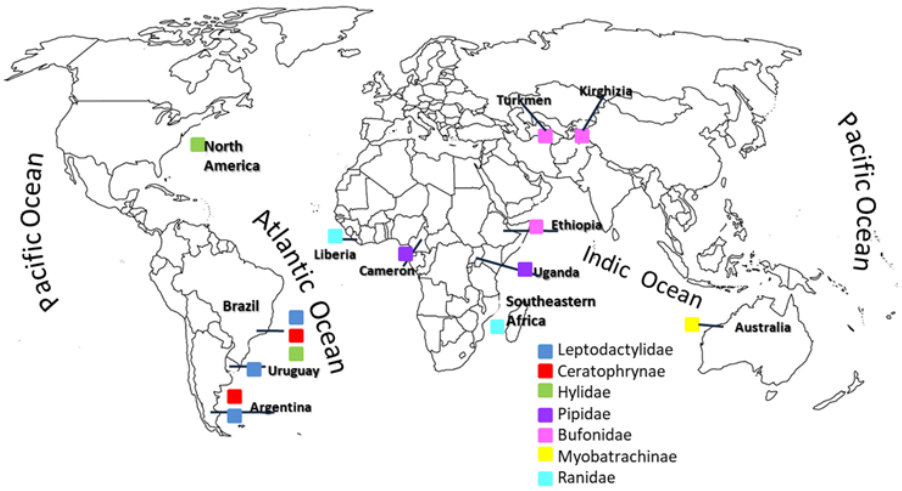

Figure 9: Continental position of the actual polyploidy and pospolyploidLeptodactylis (based in Beçak, 2018).

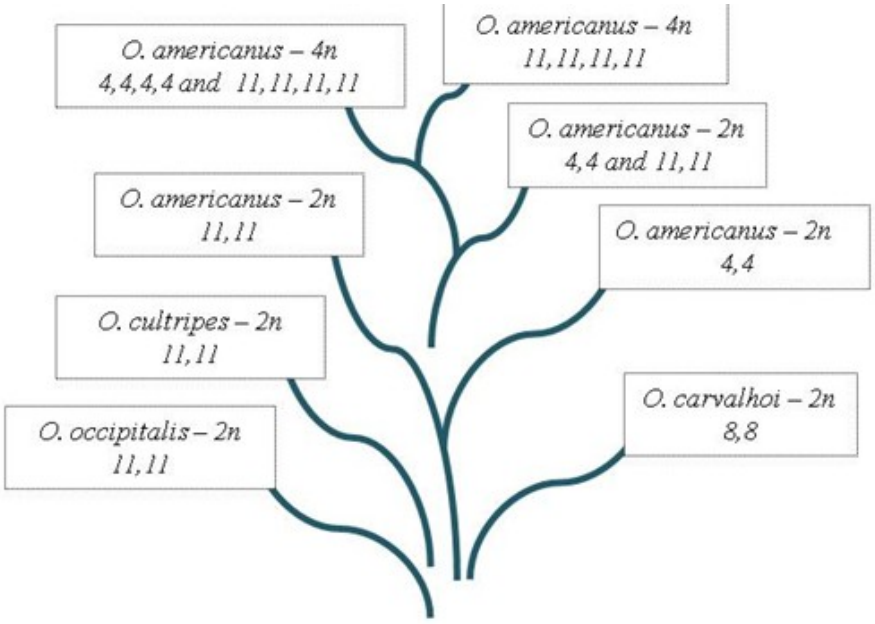

Figure 10. A suggestion to explain the evolution of *Odontophrynus*, based in the polymorphism of 2^{ary} constriction, according to the model by Beçak ML and Beçak W, 1974b.

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	Mendonza (Argentina)	
	-	-	9,9	11,11		
O. carvalhoi, 2n	-	8, 8	-	-	Bahia (Brazil)	
	4,4	-	-	-	São Paulo (Brazil), Cordoba (Argentina)	
O. americanus, 2n	4,4	-	-	11,11	São Paulo (Brazil)	Almeida et al, 1986
	4,4	-	-	-	Cassino, Friburgo (Brazil)	
	4,4	-	-	11,11	Friburgo (Brazil)	
	-	-	-	11,11, 11,11	São Paulo (Brazil)	
O. americanus, 4n	4	-	-	11,11, 11,11	Montevideo and Salto Grande (Uruguay)	Ruiz et al., 1982
	4,4,4,4	-	-	-	Salto Grande (Uruguay)	
	-	-	-	11,11, 11,11	Argentina	Schmidt et al, 1985

Besides autopolyploidy, cytogenetic studies pointed to several chromosome alterations in the evolution of Leptodactilids. In *Pseudopaludicola falcipes*, the diploid number varies in different populations having 2n=16, 2n=18, 2n=20 and 22. These variations were explained by centric fusions (Beçak, 1967; 1968). The karyotypes 2n=16 and 2n=18 were interpreted as caused by fusions or fission of the centromeres. The higher diploid numbers 2n=20 and 2n=22 were explained by pericentric inversion or translocation after centric fusion. Centric fusions were also reported in Hylidae with species having 2n=22, 2n=26, 2n=30, 2n=48 and 2n=52 chromosomes (Beçak, 1968; Rabello, 1970).

Gene regulation in autotetraploid anurans

Gene regulation in autotetrapolyploid anurans was studied in enzymatic and molecular experiments. The results obtained indicated that the amount of RNA produced by both 2n and 4n specimens are similar (Beçak and Goissis, 1971), though the tetraploids have the double amount of chromosomes. Also, it was shown that the amount of rRNA transcribed in the 4n is not the double of that of 2n animals (Beçak and Goissis, 1971) though having double amount of rRNA genes (Schmidtke et al., 1976). Moreover, it was demonstrated that the higher variability of isozymes and other proteins in 4n animals (Beçak, 1969; Schwantes et al., 1969, 1976, 1977) is produced by the expression according to the Hardy-Weinberg equation $(p+q)^4 = (p^4 + 4p^3q+6p^2q^2+4pq^3+q^4)$ instead of $(p+q)^2 = (p^2+2pq+q^2)$ in the 2n status. These data led to the conclusion that though variability of the species is maintained by the two alleles, in the tetraploid the combination of the genes in the four alleles, increases variability enhancing adaptivity and evolution (Beçak and Pueyo, 1970). This conclusion is supported by the idea of 2R-model, by Ohno, 1970. The reduction of RNA expression was confirmed by data obtained using NOR studies (Ruiz et al., 1981).

The reduced expression of rRNA was explained by methylation of rDNAgenes (Ruiz and Brison, 1989). This hypothesis was further supported by data on erythropoiesis and 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

that the 2n cells (Cianciarullo et al., 2000). Further studies on hemoglobin traits between allopatric populations of *O. americanus 4n* showed differences related to the process of diversification (Cianciarullo et al., 2019)

Electron microscope studies showed that there are lower number of nuclear pore complexes (NPC) in the 4n with changes in the transport of products to the cytoplasm (Maul et al, 1980). Also, description by electron microscopy of complex aggregates of neighboring NPC in the 4n species may be related to a lower metabolic activity (Beçak and Fukuda-Pizzocaro, 2007).

Besides rRNA methylation, another epigenetic aspect found in cells of the 4n specimens of *Odontophrynus* was the occurrence of amphyplasty. This picture is characterized by differences on chromosome condensations observed in half of the complement of the 4n anurans during mitosis. The structural differences between the two halves of the genome may be indicative of differences in the replication time of DNA. It is known that differences on chromatin condensation can be explained by histone acetylation (Beçak and Beçak, 1998).

EPIGENETIC

Genome and epigenome concepts

According to the neo-Darwinism theory, each species has a genetic content called a genome that is accounted for by chemical information, which determines the organization of a new organism. This information can mutate, resulting in new phenotypes to be exposed to natural selection.

Moreover, besides the genome each organism contains another DNA content called epigenome which sequences are factors for gene regulation and cell differentiation processes. The mechanisms of DNA methylation, histone modification, chromatin compaction and transposons are some of these factors for gene regulation. During development the epigenetic code determines the pattern of expression or gene inactivation in different tissues.

The term epigenome was created by Waddington (1942) to indicate that even genetically cells similar can develop different structures and functions. Epigenetic research focuses on the differentiation of totipotent cells in embryonic development. It is known that the epigenetic patterns are inherited through mitosis and removed in gametic cells. When the remotion is incomplete the epigenetic marks are transgenerational inherited. This fact that causes a non-mendelian pattern of inheritance can be reversed (Wong and Craig, 2011). Also, some epigenetic alterations are involved in human diseases as cancer, diabetes (Cornacchia et al, 1988). According to Croplay and Suter (2011), epigenetics is the "transmission of non-genetically encoded information".

Epigenetic mechanisms

The lowest level of chromatin organization in eukaryotes is a 100Aº nucleosomal filament. Each nucleosome unit (nu) is formed by 145bp of DNA wrapped into three-quarter turns around one octamere of histones. The octamere contains two molecules each of H_2A, H_2B, H_3 and H_4. Histone H_3 associates with H_4 and H_2A associated with H_2B.

Interconnecting the nucleosomes there is a linker DNA of about 80bp that is associated with one molecule of lysine rich histone (H_1 or H_5). The length of the linker DNA varies (Oudet et al, 1975). This aspect of chromatin fiber is called "beads-on-a-string" model.

During cell divisions the 100Aº fiber condenses into a helix of 300Aº formed by the assembly of six to eight nucleosomes termed solenoid. Others higher levels of chromatin condensation also occur. It is described that the chromatin fibers of human and mouse are arranged in loops during cell divisions (Paulson and Laemmli, 1977; Aiden, 2019) (Figure 11).

Our electron microscopic studies showed that mitotic and meiotic chromatin fibers of anurans and snakes have similar "beads-on-a string" and loops configurations, as in other eukaryotes (Beçak et al., 1977; Beçak and Fukuda, 1979) (Figures 11 and 12).

Some epigenetic mechanisms can alter gene expression without modifying DNA sequences. One mechanism is the methylation of DNA and other is the acetylation of the histones, which modifies the chromatin structure.

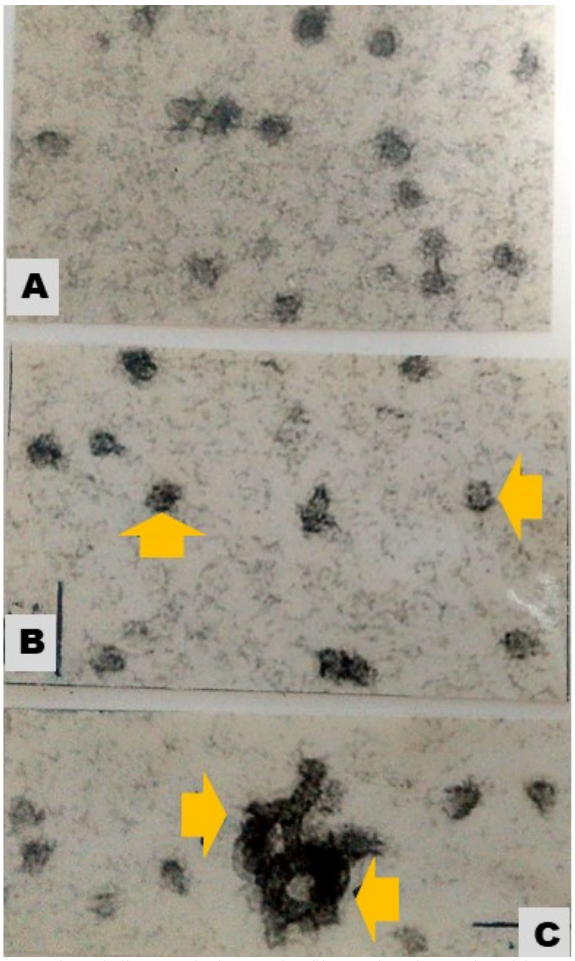

Figure 11. Electron micrographs of disrupted chromatin fibers showing solenoids (A and B) and higher order structures (C); oocytes of *O. americanus* 4n; bars=1.000 Aº (from Beçak and Fukuda, 1979).

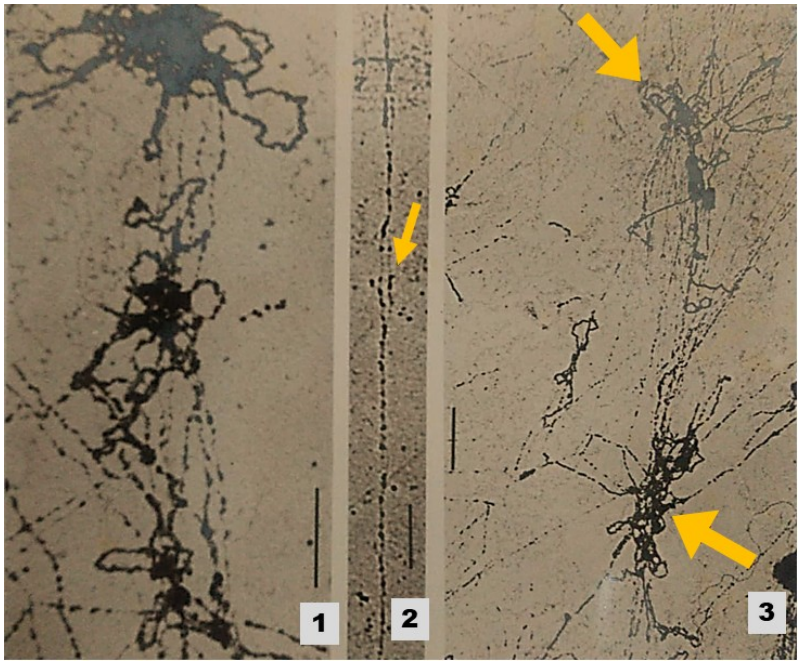

Figure 12. Electron micrographs of solenoidal loops in pachytene nuclei of *Odontophrynus. americanus* 4n, ♂ (1 and 2; bar=0.5µm) and of the snake *Xenodonneuwiedii,* ♂ (3; bar=1µm); from Beçak et al, 1977.

DNA methylation by the addition of methyl residues to cytosine bases in DNA is catalysed by DNA methyltransferase. Several experiments showed that this process is related to chromatin structure and regulation of gene expression. Hypermethylation is associated with inactive chromatin and hypomethylation is correlated with active chromatin (Razin and Cedar, 1977). The methylation process can silence gene expression of regulatory regions as promoters and enhancers being this mechanism cell type specific (Razin and Szyf, 1984; Benvenisty et al., 1985). Differential DNA methylation can also occur between the two alleles of a same gene as in X-inactivation in females (Riggs, 1975), parental imprinting (Swain et al, 1987; Sapienza, 1990) and silencing parasitic elements in the genome (Bestor, 1998). In the case of the histones the enzyme histone-acetyl transferase can add the acetyl group to lisine residues. This process causes decompaction of the chromatin and drives gene expression. By the contrary, the enzymes histone-desacetylase removes the acetyl group causing compaction of the chromatin and repression of transcription.

Molecular studies demonstrated that transposons (TE) are also epigenetic marks altering gene expression without alterations in the DNA sequences. In plants, TE acts in gene regulation promoting protein variability and speciation (Fedoroff, 2012). Recent data on the evolution of the tetrapods indicated that insertions of TE may create new genes. The authors of this experiment showed that DNA transposons promote exon shifting, causing formation of new protein-coding genes (Cosby et al, 2021). Alternatively to these ideas, some researchers suggested that TE could be deleterious for the host (Weiss and Stoye, 2013).

In the case of polyploid anurans the analysis of α-globin genes in 2n and 4n species of *O. americanus* demonstrated that intron 2, which is usually found in vertebrates, is

absent. This fact indicates that these sequences could be pseudogenes related to retrotransposition (Acedo et al., 1997). Later, the study of ribosomal intergenic spacers (IgSs) demonstrated a high level of amplification of these regulatory sequences in the 4n specimens, and that probably a transposon-like sequence was inserted in these IgSs during evolution (Alvares et al, 1998).

Dose compensation in mammals sex-chromosomes

Geneticists have long known that an epigenetic mechanism termed dose compensation inactivates one X-chromosome in female mammals. This process equalizes the expression of X-linked genes between XX females and XY males. The condensed corpuscle (Barr-corpuscle) present in mitotic nuclei of mammalia females correspond to one condensed X-chromosome (Ohno and Hauschka, 1960). This condensed corpuscle was shown to be an inactivated X-chromosome of the females (Lyon, 1961). In *Drosophila* the single X-chromosome of XO males have higher activity lacking dose compensation mechanism.

The inactivation of the X-chromosome in mammals occur by the action of a non-coding RNA that coats the X-chromosome. This RNA is transcribed by the Xist gene from the X-chromosome. Today it is known that X-inactivation involves several steps of epigenetic regulations as DNA methylation, histone modifications, chromatin condensation, non-coding RNA altering replication timing (Blewitt and Gearing, 2011). The DNMTs enzyme and SMCHD1, a coesin / condensing protein maintain the inactivation aspect.

Cytogenetics have indicated that among frogs there are species with homomorphic or heteromorphic sex-chromosomes (Schmid and Steinlein, 2003; Schmid et al., 1983; 1993; 2012). Homomorphic ZW sex-chromosomes were found in *Buergeria buergerii* and XY sex-chromosomes were reported in *Hyla femoralis* (Schmid and Steinlein, 2003). In *Buergeria* NOR is localized in the Z chromosome and in *H. femoralis* NOR is positioned in the X-chromosome. Dose compensation was not observed in these two species. Heteromorphic XY sex-chromosomes were found in *Gastrotheca riobambae* (Schmid et al., 1983). The authors reported the absence of dose compensation in this species.

Sex-chromosomes and dose compensation were not reported in *Odontophrtnus americanus* 2n and 4n (Beçak et al., 1966). Nevertheless, the authors reported silencing of half of the genome in the 4n (Beçak and Kobashi, 2004; Beçak, 2014; 2018).

The mechanism of sex determination in snakes was studied through cytogenetics and genome methodologies. Early cytogenetic data showed different levels of sex determination, since undifferentiated sex-chromosomes in Boidae, homomorphic and heteromorphic (ZZ / ZW) sex chromosomes in Colubridae and heteromorphic pairs in Viperidade (ZZ / ZW) (Beçak et al., 1962; 1969). The differentiation of Z/W chromosomes was attributed to an eventual pericentric inversion followed by gene loss in the W chromosome (Beçak at al., 1969). Yet, it was proposed that sex chromosomes of rattlesnake (Viperidae) are completely heteromorphic at the DNA sequence level being gene recombination absent. These studies also demonstrated that dosage compensation is missing in the snakes (Viscoso et al., 2013)

Due to cytogenetic similarities of snakes and birds regarding the presence of microchromosomes and sex-determination (ZZ / ZW), it was suggested that sex-chromosomes of these two groups had the same origin (Beçak et al., 1964). Regarding avian

sex-chromosomes the experiments indicated that dosage compensation could be quite different from mammals X-inactivation (Graves, 2014).

Epigenetic regulation also occurs in autosomes as in Hox genes. These genes play important role in embryonic development of Bilateria animals. They were first described in *Drosophila*. Mammals genome have four Hox clusters (for review see Soshnikova and Duboule, 2009). Hox genes have a spatio-temporal expression controlled by epigenetic non-coding RNAs.

Transgenerational inheritance

The observations of epigenetic phenotypes in natural populations is explained by the assumption that the pattern of gene transcription can be altered by environment epigenetic agents and transmitted to offspring. This innovator model is termed transgenerational inheritance.

Darwin in his book "The variation of animals and plants under domestication" (1869) postulated that the organization of the body is autoreproduced by means of its parts. Each cell of the organism would produce small granules called *gemulas* that could account for the formation of new cells and tissues. This Pangenese theory assumed that the granules were produced along life and could be affected by environment factors.

Today the fundamental model of inheritance is well established by the neo-Darwinism concept. This theory assumes that evolution is derived through the accumulation of mutations that produce different phenotypes which are exposed to natural selection. Numerous examples of environmental epigenetic events and transgenerational inheritance are being discussed at light of Lamarck concept and neo-Darwinian theory (Jablonka, 1998; 2009; Skinner, 2014; 2015).

Skinner proposed that evolution involves the ability of environment to create epigenetic mutations that can be transmitted to offspring. This theory provides a molecular mechanism for Lamarck's proposal. This neo-Lamarckian concept is not conflicting with neo-Darwinism ideas but adds another route of evolution.

In mammals, experiments using mouse, rat, as well as human, suggested that the epigenetic inheritance may be a common process (Morgan and Whitelaw, 2008). Also, experiments using female rats treated with fungicide, that is antagonic of the reception of androgen, caused alterations of methylation of 15 DNA sequences and abnormalities of testis for four generations (Skinner, 2014).

Evidence was also reported indicating that new species are rapidly created by environment changes. This assumption was based in the findings that the killer whales occur in sympatric populations without geographic barriers but impelled by search of new ecological niches (Riesch, 2016). More recently, it was reported that the adaptative intersexuality in mole was established by the alteration of gene regulation regions (Real et al, 2020).

Besides mammals, it was reported that fast speciation may occur in cavefish by epigenetic mutations. These alterations affect the mechanism of gene regulation of eyes development and can be transgenerational inherited (Rohner et al., 2013).

In agreement with these ideas, molecular data in fishes also indicated that DNA duplication producing genome redundance allows adaptation of these animals to new ecologic niches. In the case of theses fishes the increase of copies of fatty acid desaturase 2

(Fads 2) allowed the adaptation of marine species to survive on a freshwater. The authors showed that transposons were accounted for duplication of Fads 2 gene in freshwater populations (Ishikawa et al., 2019).

In reptiles, experiments on sex-determination mechanisms showed that in the turtle *Trachemys scripta elegans* (*T. scripta*) sex is temperature dependent. In fact, the epigenetic factor kdm6b determines male sex at 26ºC temperature. On the contrary, female sex is produced when this factor is 32ºC (Weber et al., 2020).

Since the discovery of autopolyploidy in anurans (Beçak et al., 1966), we quickly home to *Odontophrynus* model to study evolution. As already mentioned in this review, our studies showed that epigenetic mutations may be related to diversification and radiation of the 4n *Odontophrynus americanus* (Beçak and Kobashi, 2004; Beçak, 2014; 2018). We found that the 4n specimens $(p+q)^4$ have more variability then 2n individuals $(p+q)^2$ (Beçak, 1969). Interesting, also, is that though the number of chromosomes and the amount of DNA content is doubled in the 4n, these animals produce only half amount of protein as found in the diploids. The researchers assumed silencing of half of the genome of the 4n, caused by methylation of rDNA genes (Ruiz et al., 1981; Ruiz and Brisson, 1989) or by amphiplasty of the two halves (Beçak and Beçak, 1998).

Our data on tetraploid anuran are in complete accordance with the 2R-model indicating that gene duplications may create variability (Ohno,1970). Moreover, the expression of half genome by amphiplasty or by methylation of rRNA indicated that speciation was associated with epigenetic evolution (Beçak and Kobashi, 2004; Beçak, 2014; Beçak, 2018).

Previous studies in plants showed that molecular alterations may occur in synthetic polyploids of *Brassica*. The authors assumed that these changes are related to polyploid evolution (Song et al, 1995). Also, later studies in *Brassica* and *Arabidopsis* allopolyploid plants indicated that fast variability in polyploid is drived by epigenetic inheritance (Comai et al., 2000; Pikaard, 2001; Lee and Chen, 2001).

Alterations of flowering time in *Arabidopsis thaliana* are due to methylation changes. New phenotypes were transmitted through eight generations of this plant (Pennisi, 2013). Accordingly, plant investigations indicated that homeology expression in polyploid wheat is associated with epigenetic alterations caused by T.E. within promoters (Ramírez-Gonzalez, et al., 2018).

An interesting case of quick evolution that occur by differences in gene expression was recently reported in jelly fish by evo-devo biologists. With the CRISPR technique the Sox2 gene of this anemone was knock out. As a consequence, the cnidocytes (the cells that deliver the sting) were replaced by the spirocytes. These cells that are known for their stickiness would allow adaptation to other environments (Pennisi, 2021b).

DEVELOPMENT

The development of a pluricellular organism from a zygote is a very complex process of cell differentiation resulting distinct tissues and organs. Though all cells of an organism have the same DNA composition, differential expression of specific genes occurs in each step of cell differentiation. This means that transcription of specific genes is related with each step of development. The aim of researchers on development (Evo-devo) is to elucidate which types of genes are related to body structures and functions.

Early studies on Hox genes contributed to the knowledge of developmental genetics. As it is known these genes promote the orientation and the differentiation of the anterior-posterior axis of animal body. Hox gene were detected in studies of mutations in *Drosophila melanogaster*.

The Hox genes are spatially and temporally arranged in clusters along the chromosome. This means that the ordered position of these genes accounts for the sequential development of specific body segments of the embryo. The patterns of transcription are correlated with gene location in the chromosome (Lewis, 1978). Later studies on Hox genes showed that animals from different levels of complexity share similar genes and mechanisms of gene expression (MacGinnis et al., 1984; Granham et al., 1989: Deboule and Dollé, 1989).

In *Amphioxus* there is only a single dose of Hox genes while in mammals there are four clusters (Garcia and Holland, 1994). Each cluster with several genes is localized in different chromosome. The demonstration that Hox genes are present in the teleost *Danio-rerio* and in human indicated that its origin is anterior to the diversification of Actinopterygii and Sarcopterygii (Postlethwait et al., 1998). The studies on Hox genes gave support to Ohno suggestion that vertebrate evolution is derived through gene redundance via autopolyploidy followed by mutations of the extra copies. This process may produce new functions and speciation.

Drosophila genome has two clusters of Hox genes. Besides *D. melanogaster* there are other models to study development as the *Caenorhabditis elegans*. This small nematode has 60-80% of gene homology with humans (Kalleta and Hengartner, 2006). Also, the zebrafish, *Danio rerio*, is another important model in Evo-devo studies (Kimmel, 1989; Driever et al., 1996; Amores et al., 1998; Postlethwait et al., 1998).

A very important question that remains to be solved in Evo-devo field is to identify the specific relation of genome transcriptome and proteome during ontogenesis. In fact, how could researchers distinguish which type of gene transcription is active in each temporal and spatial stage of development of an embryo? Such challenge is now being overcome through news methodologies as the single-cell assays (Sci-RNA-seq$_3$) and single-cell combinatory indexing chromatin accessibility and mRNA (Sci-CAR) during organogenesis (Cao, 2020). These techniques allow to identify which gene is transcribing and to detect its epigenetic regulatory factor during tissues and organs differentiation. These data were obtained from several animals as worms, mice and human, using a new method called sci-fate (Cao et al., 2020) to distinguish newly synthesized mRNA transcription from “older” in individual cells.

Though enormous progress by molecular research, a complete understanding of the role of epigenetic regulator factors during cell differentiation and evolution is still in its beginning.

The role of epigenetic mechanisms promoting evolution is also reported in invertebrate species. As an example, embryos of parthenogenetic bees (*Apis mellifera*) develop different phenotypes (workers and queens) though having the same genome (Law, 2021).

CONCLUSIONS

We all know that there is still much to be discovered about the mechanisms that drive evolution. Here we reviewed some fundamental ideas on vertebrate evolution based in data described from paleontological, embryological, cytogenetics and molecular methodologies. Also, the findings of an extensive study on anurans obtained by us and other investigators were reported.

Our analysis using the diplo-tetraploidy model of anuran evolution led us to suggest that: the information of both genome and epigenome is written in Earth sediments.

Summarizing our results, we concluded that:

Evolution moves through mutations of extra-copies created by the process of polyploidy. This idea is based in results indicating that although in autotetraploids the original variability is maintained by two alleles the other two extra-copies of homologous genes are free to mutate producing new phenotypes that can be eventually selected (Beçak and Pueyo, 1970). This conclusion in autotetraploid anurans is in perfect accordance with the suggestion in Ohno's 2R-model (1970) to explain the evolution of vertebrates. Indeed, Ohno's model was based in a comparative analysis of DNA content in invertebrates and vertebrates as well as in cytogenetic observations showing the high chromosome number of the complements sometimes associated with residual multivalent configurations at meiosis (Ohno and Atkin, 1966; Atkins and Ohno, 1967). This model considered the description of autotetraploidy in anurans (Beçak et al., 1966) as well as the occurrence of pos-polyploid species of fishes (Ohno et al., 1968; Wolf et al., 1969).

Evolution is created by gene mutation and epigenetic alterations associated with environment. The silence of RNA transcription of half genome of the 4n firstly described by Beçak and Pueyo, 1970 was indicated to be caused by methylation of rRNA genes (Ruiz and Brison, 1989). The repression of half genome activity in 4n was also described as being produced by differential levels of chromatin condensation as observed in amphyplasty configurations (Beçak and Beçak, 1998; Beçak, 2004). The importance of epigenetic mutations during the evolution of the anurans was previously described (Beçak and Kobashi 2004; Beçak 2014 and Beçak 2018).

The evolution of these anurans via transgenerational epigenetic mutations was observed in Brazil and others South Americans countries. Besides autotetraploids, postpolyploid species were also observed in evolutive diploidization processes (Ohno, 1970; Beçak and Beçak, 1974a).

Finally, we concluded that: the history of evolution can be read in the fossil records of Earth sediments and interpreted by the chemical signatures of the genome and epigenome DNA sequences.

This review indicates that both gene regulations are epigenetic mutations are important evolutive factors among diplo/tetraploid anurans. It does not conflict with the well-established neo-Darwinism model, but just adds that besides gene mutations the epigenetic mutations are another factor of variability.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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