

**CHARLES UNIVERSITY
FACULTY OF SCIENCE**

Study programme: Developmental and Cell Biology



Mgr. Nicola Reinaldo Fornaini

*Evolutionary dynamics of chromosomal rearrangements
within the family Pipidae*

Doctoral Thesis

Supervisor: Ing. Martin Knytl, Ph.D.

Prague, 2024

Declaration

I declare that my dissertation thesis represents my original research and that I have cited all sources of information used. This work, or any significant part of it, has not been submitted for any other academic degree or diploma.

Prague, 15.10.2024

Nicola Reinaldo Fornaini

Acknowledgement

I would like to thank all my lab, starting to my supervisor Dr. Martin Knytl for the help during my PhD studies and for the thesis revision, Dr. Vladimir Krylov and Dr. Tereza Tlapakova for the help with bureaucratic and logistic affairs and for the support throughout the years, Dr. Václav Gvoždík for supplying frogs from Africa, Dr. Halina Cernohorska for chromosomal microdissection and Dr Michail Rovatsos for the help with the genomic and bioinformatic part.

First of all I would like to thank Sarah for being next to me even when it was hard and to be my calm when around me was wuthering, your support helped me through the days. I can't no say thanks to my Italian friends from Prague, who made me feel like home when I was kilometres away.

I want to express my gratitude to my father and mother for always letting me do whatever I wanted with my life, to my brother for his weird way of supporting me, my grandmother for always being there and always making me feel loved, and a final thanks also to my augmented family.

I want to say thanks also to all the people I met in the University, and in Prague, the ones who are still here and the ones who took another path. Isn't the PhD the friends we made along the way after all?

I want to say thanks also to my friends from my hometown, to be a fixed point where I can always come back. A thanks also to all the friends I left in Italy, the ones who are still there and the ones who moved abroad as well, it's important to me knowing you're still there after all this time.

A thanks for the ones who passed away too early, you're still in my heart, this one's for you too.

A thought to Africa by Toto, my everlasting soundtrack of all my academic path.

Last but not the least I would like to say thanks to my precious labmate, Baru, the one that helped me to survive the days, thanks for all the time we spent together in the lab and outside the lab, you were the lab partner I always wanted, I wouldn't be here without your support.

And in the end, I bless the *Xenopus* down in Africa.

See you space cowboys.

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Abstract

Xenopus frogs are interesting models for different kinds of studies, due to their particular characteristics. The different levels of polyploidy in different frogs make them also valuable models for cytogenetic and evolutionary analysis. This project investigates the evolutionary dynamics of chromosomal rearrangements within the family Pipidae, focusing on members from the genera *Xenopus*, *Silurana*, and *Hymenochirus*. We examined the chromosome morphology and rearrangements in the allotetraploid frog, *Xenopus calcaratus*, revealing distinct characteristics between its subgenomes. The a-subgenome is more conserved, while the b-subgenome has evolved more rapidly. A specific chromosomal translocation observed in *X. mello tropicalis* but not in *X. calcaratus* highlights differences in chromosomal rearrangements between these species. We suggested that a single allotetraploidization event led to the emergence of *X. mello tropicalis*, *X. epitropicalis*, and *X. calcaratus*, with the translocation occurring post-divergence of *X. calcaratus* but pre-speciation of *X. mello tropicalis* and *X. epitropicalis*. Another scenario involves two independent allotetraploidization events.

We also explored the roles of polyploidization and divergence in the evolution of repetitive elements in six species of African clawed frogs. Combining cytogenetic and genomic data analysis, we mapped U1 and U2 small nuclear RNAs and histone H3 in both diploid and allotetraploid species. The results showed conservation of these elements in diploid and tetraploid species from the subgenus *Silurana*, while variation was observed among allotetraploid species from the subgenus *Xenopus*. These findings suggest that polyploidization initially duplicates tandem repeats, but their copy number can vary over time due to reduction and expansion.

Preliminary analysis of the genome of *X. borealis* revealed around 4-6% differences between its two subgenomes, providing initial insights for future research. Additionally, we discovered that the *Hymenochirus boettgeri* from the Congo is tetraploid, with significant differences from captive diploid populations. This suggests the need for further research to clarify the taxonomy and evolutionary history of *Hymenochirus*. We propose distinguishing the captive population as *Hymenochirus* sp. from the wild population, which retains the name *H. boettgeri*.

Evolutionary dynamics of chromosomal rearrangements within the family Pipidae

1 Introduction

1.1 African clawed frogs (*Xenopus*) as model organism

African clawed frogs, genus *Xenopus*, (family Pipidae), are a population of frogs living in the area of central Africa. They are divided into subgenera *Xenopus* and *Silurana*, *Silurana* includes diploid and tetraploid species, while *Xenopus* includes tetraploid, octoploid, and dodecaploid species in (Tymowska, 1991; Evans et al., 2015; Furman et al., 2018).

Xenopus tropicalis (from subgenus *Silurana*) and *Xenopus laevis* (from subgenus *Xenopus*) are model organisms widely used in medicine and biology, for different purposes. *Xenopus laevis* and *X. tropicalis* are both aquatic animals, and it's easy to store them in an aquarium or in a tank, since they don't need any complicated care or particular environments. The size of the eggs (~1.2mm diameter) makes them easy to manipulate, helped by the large quantities they are laid. Usually *Xenopus* frogs deposit multiple eggs at once, *X. laevis* can produce around 1000 eggs with every single brood, while *X. tropicalis* can lay up to 3000 eggs. Under the right conditions, *Xenopus* females can be mated every two months, while males only once a month. For this reason they have always been considered a valuable tool for studying early embryonic development. Eggs production can be easily stimulated, using hormones like chorionic gonadotropin. Back in the 1930s *Xenopus* frogs were used as a pregnancy test for women, for the presence of a hormone in the urine of pregnant women, that stimulated the laying of the eggs by the frog (Shapiro and Zwarenstein, 1934). Same method is utilized as well in the laboratory, for the production of eggs. After some months of rest, frogs can be induced again. The facilitated control of egg production makes *Xenopus* a valuable model for many studies. *Xenopus* is widely used for studying embryonic development (Figure 1), the changes during the ages and stress response as well as diseases and malformations.

There are many features which make *Xenopus* a unique model organism, such as the possibility to manipulate the embryos easily, they can be injected with different chemicals into the total embryo or into selected cells. Moreover the cell destiny of each early embryonic cell is well known in literature, making it an easy target for gene knock-out, knockdown and overexpression studies. *Xenopus* oocytes are a useful tool for research on ion movement and channel function and in environmental toxicology, extensive genetic screenings that have revealed genes that play roles in various developmental and physiological processes.

Xenopus models are very useful, besides the already mentioned oocyte and embryo characteristics also for the high reproductive capacity, swift external development, and straightforward genomic modification. *Xenopus* are used also as models for human diseases, since they share around 79% genes associated with a disease, in humans (Hellsten et al., 2010; Khokha, 2012; Tandon et al., 2017).

Moreover they are rapid to breed, cheap, easy to manipulate with and have a high successful ratio of gene mutation using CRISPR/Cas, compared to mammalian models. It has been reported in many studies that CRISPR/Cas modifications can be used for phenotype analysis in the F1 generations of both animals, *X. laevis* and *X. tropicalis* (Bhattacharya et al., 2015; Blitz et al., 2013; Wang et al., 2015).

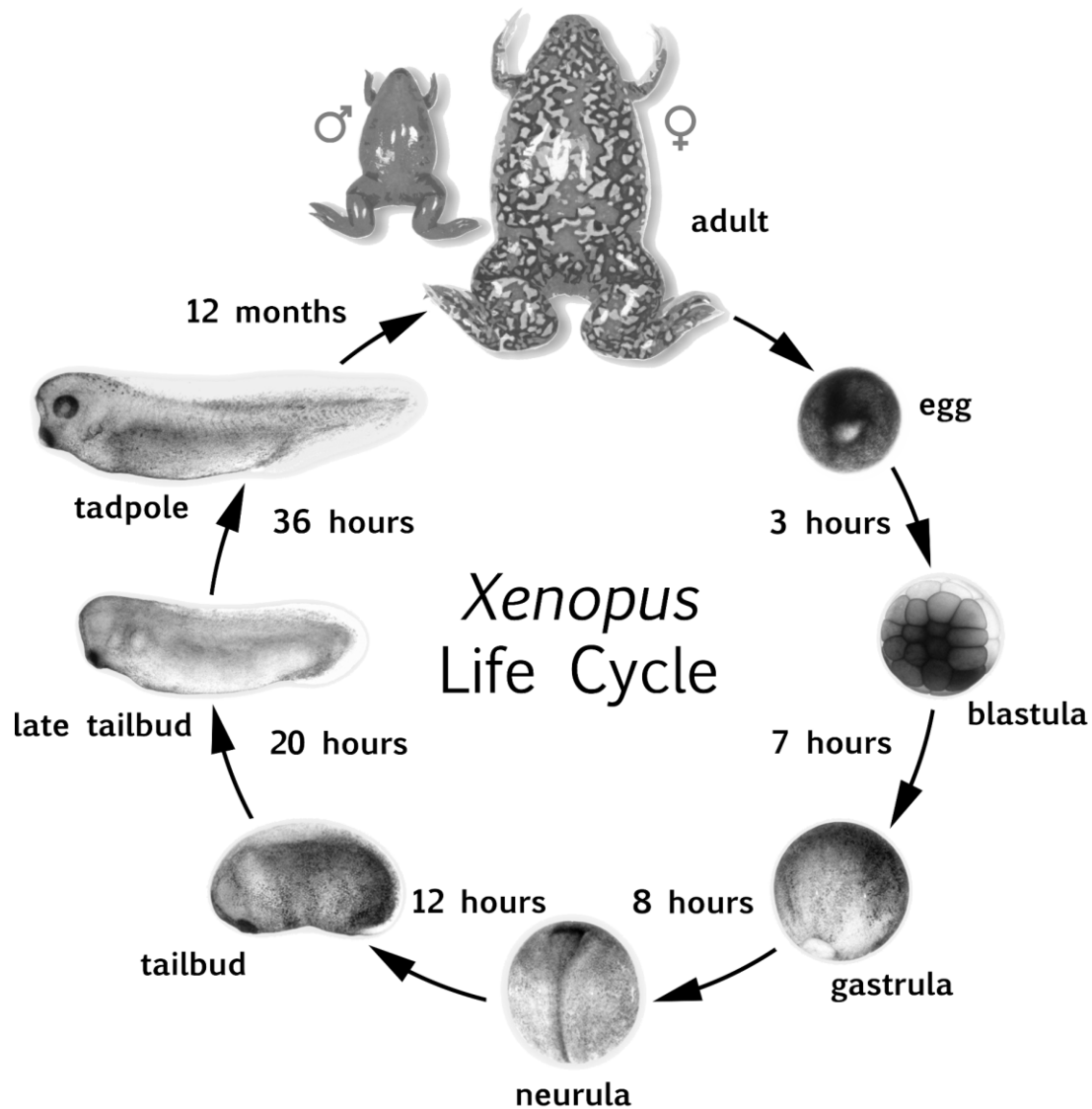


Figure 1. Life cycle of *Xenopus laevis* (Gerhart and Kirschner, 2020).

Lately *Xenopus* frogs were successfully used as a model Investigating spinal cord morphogenesis, functionality, and recovery (Borodinsky, 2017), as demonstration of their great adaptability and wide use for various biological fields. *Xenopus laevis* is also an excellent model for analysis on the development of the heart (Warkman and Krieg, 2007). The large size of the embryo allows the use of microinjections for gene manipulation, an approach used to study the gene function overexpressing or silencing a specific gene of interest.

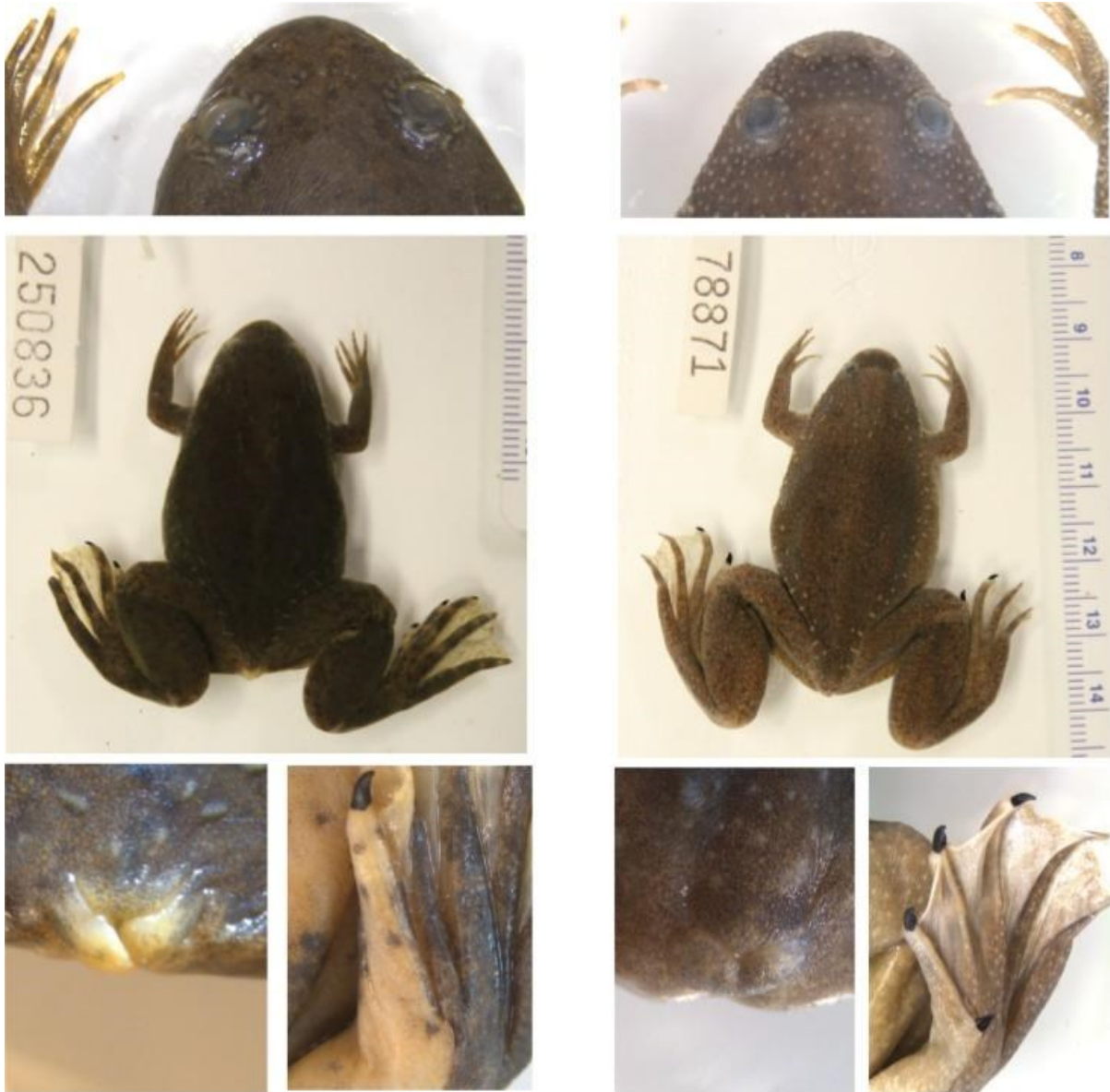


Figure 2. The examination of external features between the subgenera *Xenopus* (right, specimen of *X. victorinus* CAS 250836 [DCB-202]) and *Silurana* (left, *X. calcaratus* CAS 207759) highlights several distinctions. *Silurana* has a smaller portion of the eye covered by the lower eyelid (top), shorter feet (middle), and cloacal lobes that are fused ventrally, compared to *Xenopus*. It also possesses a claw on the prehallux and lacks a skin ridge on the first toe from the prehallux (bottom). In *Silurana*, the skin texture is coarser, the eyes are comparatively smaller, and the subocular tentacle is shorter relative to coexisting *Xenopus* species (Evans et al., 2015).

Both *X. tropicalis* and *X. laevis* genome have been fully sequenced (Hellsten et al., 2010; Roe et al., 1985; Session et al., 2016). Researchers discovered the genome of *X. tropicalis* contains more than 20,000 protein-coding genes, which include orthologs of at least 1,700 genes associated with human diseases. This makes *X. tropicalis* a viable human diseases model. The size of the *X. laevis* genome is 2.7 billion base pairs spread across 18 chromosomes (Session et al., 2016). It also has a longer maturation time, taking 1-2 years to reach the adult form, able to lay eggs. *Xenopus tropicalis* is a simpler alternative. This diploid species has a genome size of about 1.7 billion base pairs on only 10 chromosomes ($n=10$) (Hellsten et al., 2010), and matures much faster than *X. laevis* (Tymowska, 1991).

The availability of the complete genome of *X. tropicalis* and *X. laevis* make them valuable tools for studying human diseases (Nenni et al., 2019) as well as their relatively phylogenetic close distance to humans (Figure 3).

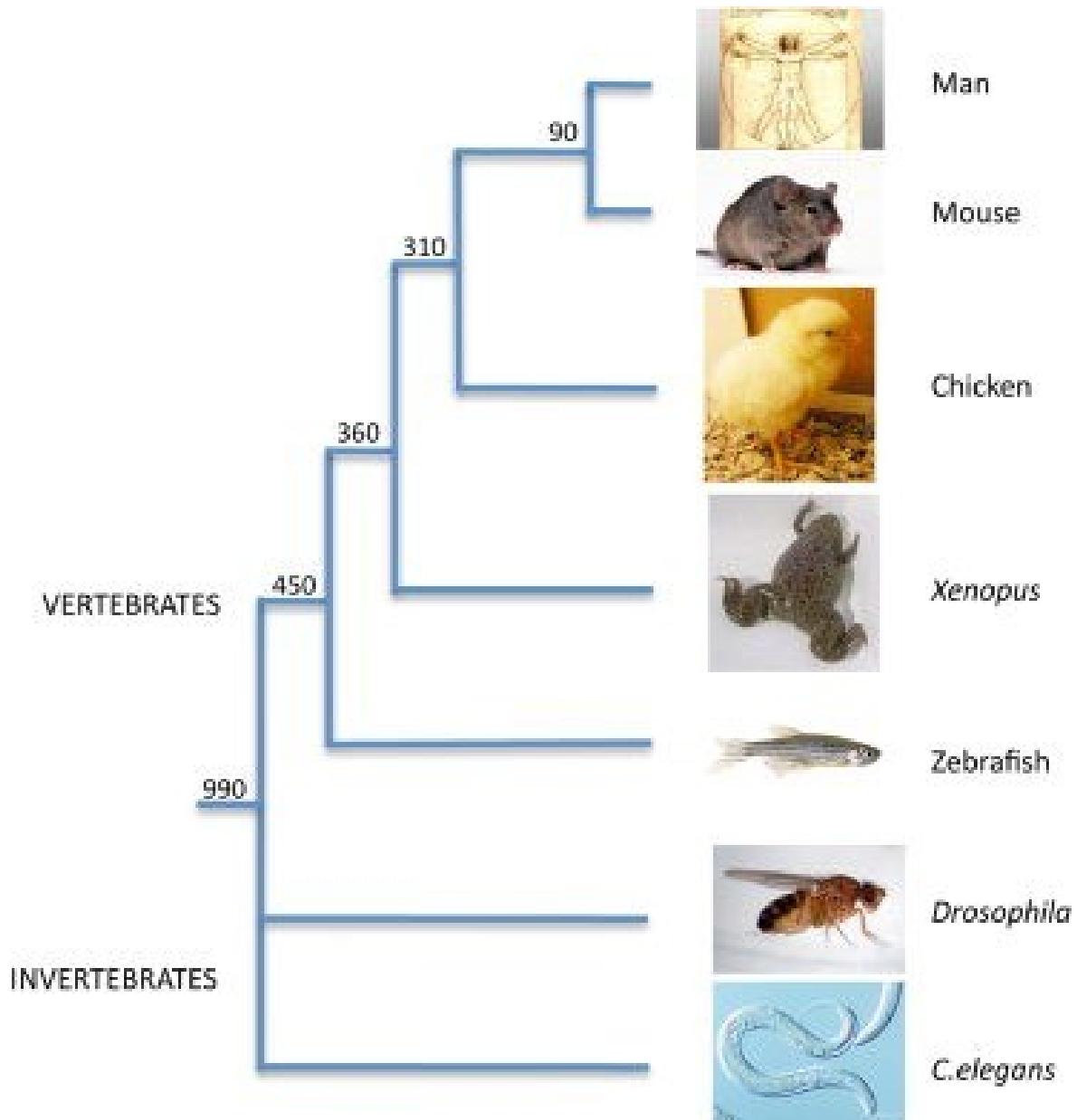


Figure 3. Phylogenetic tree illustrates the evolutionary relationships among the primary animal models frequently utilized in biomedical research. Periods of separation, expressed millions of years ago (Mya), are taken from studies using multi proteins and genes (Hedges, 2002; Hedges and Kumar, 2002; Wheeler and Brändli, 2009). Note that the branch lengths are not proportional to time (Wheeler and Brändli, 2009).

1.2 *Hymenochirus boettgeri* relevance in research

An interesting pipid species for our research is the dwarf clawed frog (*Hymenochirus boettgeri*).

Dwarf clawed frogs, found in captivity, are not commonly used as animals for the research but they are widely used as pets, kept in aquariums. However, the name '*H. boettgeri*' was usually used in laboratory studies and in publications, even when the frogs were taken from captive populations (Cauret et al., 2020; Mezzasalma et al., 2015; Miller et al., 2019; Höbel and Fellows). Nowadays this dwarf clawed frog is considered a valuable model species (Bredeson et al., 2024).

Xenopus and *Hymenochirus* are phylogenetic close, as can be seen in Figure 4.

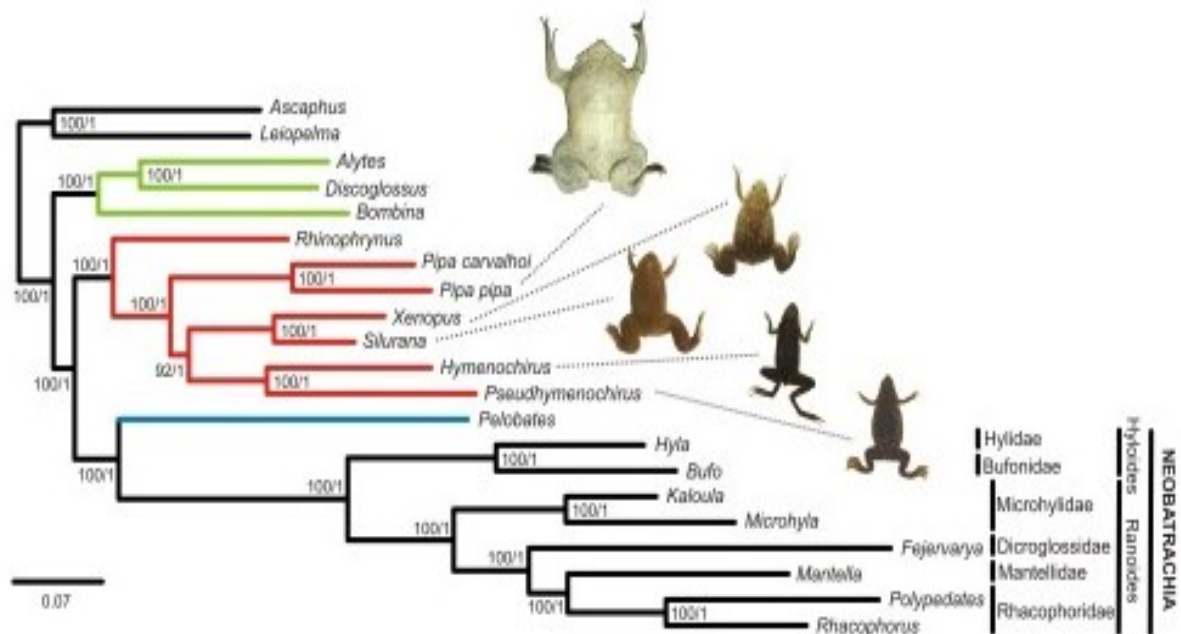


Figure 4. Phylogenetic tree illustrating the various frog species divergence. Adapted from (Irisarri et al., 2011).

Dwarf clawed frogs (*Hymenochirus* sp.) that we have nowadays in aquariums, kept as domestic pets, likely began in the 1950s, when pet fish companies imported these frogs from the wild to Europe and the USA (Olsson and Österdahl, 1960; Sokol, 1962). We don't have precise geographic information about the origin of this species. In addition to documents stating that dwarf clawed frogs were shipped from Leopoldville, which is now known as Kinshasa in the Democratic Republic of the Congo (Rabb and Rabb, 1963) or 'from Stanley Pool' (Sokol, 1962), an area north-east of Kinshasa (Sokol, 1962). However, Leopoldville might have just been the shipping point, and Stanley Pool the arrival point (since collectors could have landed there due to its ports), meaning they may not be the actual areas of origin. There were two species, at least for what it's known, traditionally known with the names *H. boettgeri* and *Hymenochirus curtipes*, that were likely imported in the 1950s and even several years after (Sokol, 1962, 1959), through time *H. boettgeri* became more popular than the frog *H. curtipes* (Rabb and Rabb, 1963; Sokol, 1969). *Hymenochirus curtipes* is known to thrive in open areas and requires higher temperatures in captivity, especially for breeding. Additionally, its tadpoles require big tanks and much more space (Sokol, 1962, 1969b). For that reason, *H. curtipes* might have gradually disappeared from captivity due to its higher breeding specialization and its demanding taking care (Kunz, 2002). Despite both species being mentioned in aquarium and herpetoculture literature, only one species seems to have been observed in aquaria in recent years (Kunz, 2003, 2002). It is usually referred to as *H. boettgeri*, even though it has been registered that the genus needs a taxonomic revision (Kunz, 2004).

The question if the two species analyzed were belonging to the same one of the captive population with *H. boettgeri*. There has been some discussion about certain morphological differences, leading to the hypothesis that the captive population might be a 'domesticated' hybrid between *H. boettgeri* and *H. curtipes* (Cecere, 1998). For example, typical morphological features of *H. boettgeri* include an oval body, broad head, monochromatic dorsum, and sides covered with enlarged tubercles (de Witte; 1930, Arnoult and Lamotte, 1968; Perret, 1966). Aquarium population, showed different morphology, the body sides have homogeneous tubercles without differentiated verrucous, the body is oval to pear-shaped, the head is not distinctly broad, and the dorsum is often mottled (Kunz, 2004) (Figure 5).

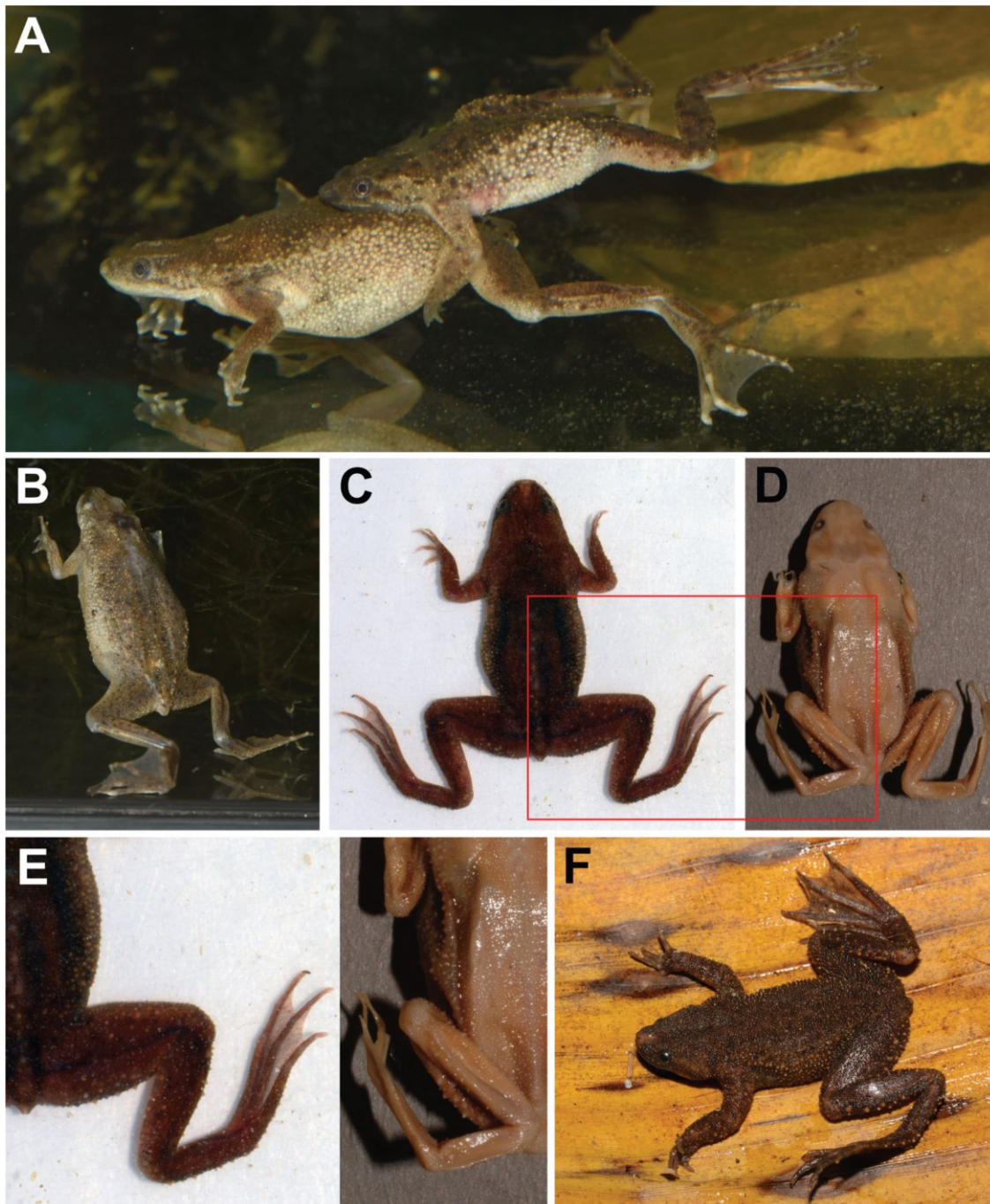


Figure 5. Dwarf clawed frogs, *Hymenochirus* sp. (captive population) and *H. boettgeri*, are shown in various views. In A, a female *Hymenochirus* sp. (IVB-H-Hsp06) and a male (IVB-H-Hsp02) are depicted mating. B shows a female *Hymenochirus* sp. in a dorsolateral view (IVB-H-Hsp04). C features a male *Hymenochirus boettgeri* from the north-western Republic of the Congo (IVB-H-CG17-356). D presents the holotype of *H. boettgeri*, a female (ZMB 11521), with the area marked by the red rectangle detailed in E. Finally, F displays a male *H. boettgeri* from the same locality as the karyotyped individual (IVB-H-CG17-112) (Gvoždík et al., 2024).

1.3 Polyploidization and its role in evolution

Polyploidization is the process of multiplying a complete chromosome set within a species, potentially leading to the creation of a new species. This occurs due to rare mitotic or meiotic events, such as nondisjunction, which result in gametes with a full set of duplicate chromosomes. When a diploid gamete fuses with a haploid gamete, it leads to a formation of a triploid zygote, but these triploids are typically unstable and can cause sterility. Conversely, the fusion of two diploid gametes produces a tetraploid zygote, which is generally stable. In nature, polyploidy can occur at various levels, including tetraploids (four chromosome sets), hexaploids (six chromosome sets), and other multiples of chromosome pairs.

These events allow multiple sets of chromosomes to coexist within a single nucleus, and these sets can be stably inherited by offspring (Comai, 2005). Widespread and multiple polyploidization events produce polyploid species with higher resistance to diseases and environmental stress (Zhang et al., 2019). Polyploidization has a very important role in diversification and evolution, mainly in plants (Falistocco et al., 2024) but it has an important role also in some animal species, like some amphibians, fishes, insects and even in some mammals (Acharya and Ghosh, 2016; Li et al., 2018; Schmid et al., 2015; Zhou and Gui, 2017), anyway, the level of susceptibility for polyploidy it's different according to the species. Polyploids have a relatively high frequency in flowering plants, this can be also found in some species of frogs and fishes. However, in higher vertebrates, as amphibians, reptiles, birds, and mammals, animals that are characterized by having a well-developed brain, a backbone and a more complex structure compared to lower vertebrates like fish, they don't seem to have an high tolerance to polyploidy very well. It is estimated that around 10% of spontaneous abortions in humans are attributed to the presence of polyploid zygotes (Yildirim et al., 2023).

Polyploids can be categorized into two groups: paleo-polyploids and neo-polyploids, based on the status of the parental chromosomes after polyploidization. Paleo-polyploid species evolved from ancestors that experienced polyploidization events in the distant past. Over time, these paleo-polyploid species reverted to having two sets of chromosomes (diploid state) through a process known as diploidization. This involves reshuffling and rearranging the multiple sets of chromosomes inherited from their polyploid ancestors (Figure 6). Contrary, neo-polyploids are species that have multiple sets of chromosomes right from the time of polyploidization. These sets of chromosomes come from merging the chromosomes of their parent species, but they remain independent of each other (Figure 6). The recent improvement of sequencing technologies, made much more complete genomes available, making comparative polyploid analysis easier. Polyploidization events are used also to date the speciation of various species.

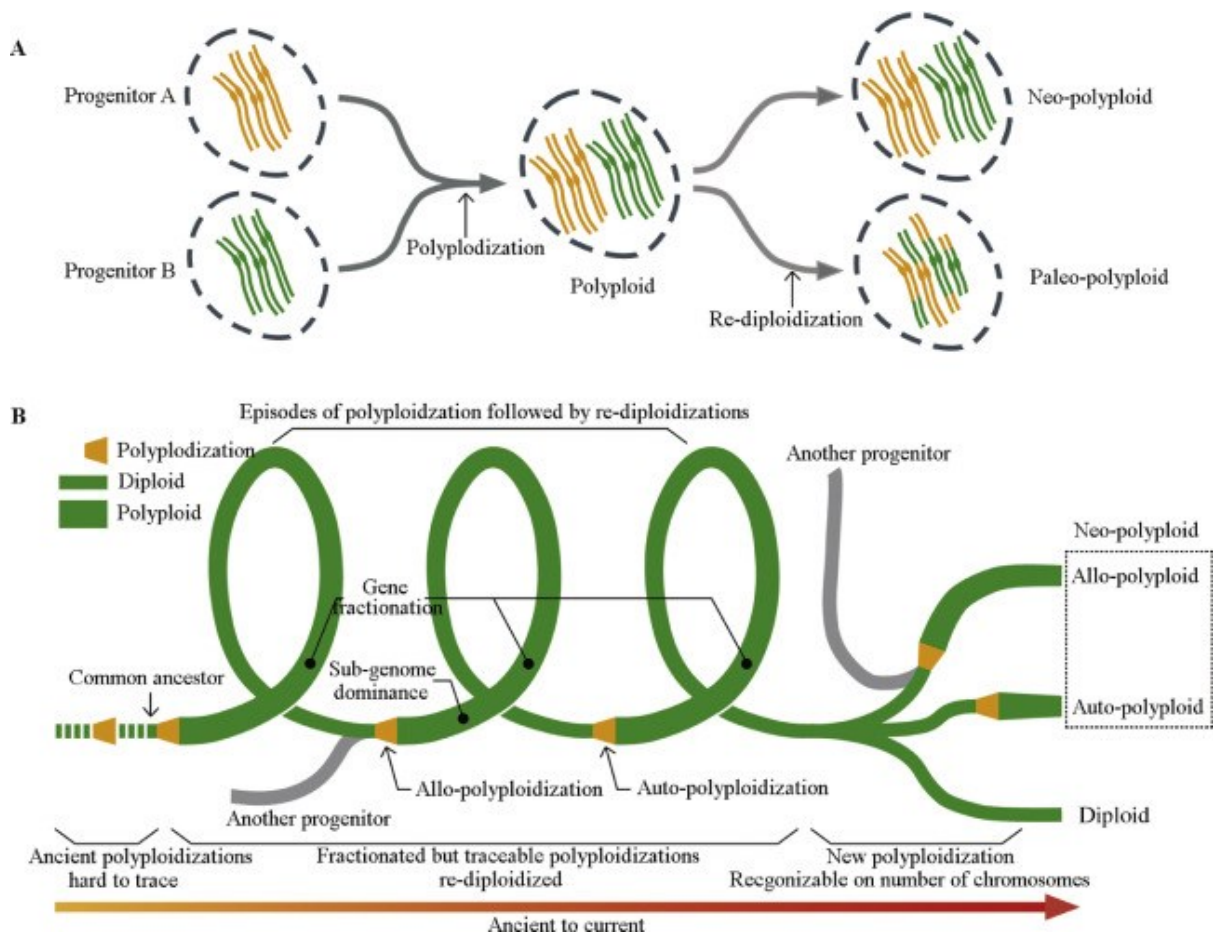


Figure 6. Life cycles of plants experiencing recurrent polyploidization and re-diploidization events (Zhang et al., 2019).

Polyploidy is common in plants, fish, and frogs, indicating clear fitness advantages. In plants, hybrid vigor, or heterosis, is observed, where the polyploid offspring of two diploid progenitors are more vigorous and more resistant to diseases and environmental stress than either diploid parent (Comai, 2005). There are several possible explanations for this phenomenon. One theory suggests that the enforced pairing of homologous chromosomes in an allotetraploid prevents recombination between the genomes of the original progenitors, thereby preserving heterozygosity across generations. This heterozygosity, characterized by multiple gene copies, leads to a reduced accumulation of recessive mutations in the genomes of future generations. Gene redundancy plays a crucial role in the advantages of polyploidization. Offsprings are protected against side effects of recessive mutations, because they have multiple copies, according to the ploidy level, of any gene as in diploids, as mentioned before. This protective aftermath of polyploidy could have a crucial role in small, isolated populations that have as their only mating option inbreeding. Evolution might have selected polyploid individuals, because of their higher resistance to mutations. Gene redundancy offers the potential to diversify gene function over time. Extra copies of non-functional genes can be repurposed in entirely new ways, creating new opportunities for evolutionary selection (Adams and Wendel, 2005). Polyploidy can also influence sexuality by providing selective advantages. For instance, it can break certain self-incompatibility systems, promoting self-fertilization. This could come from interactions between parental genomes in allopolyploids (Comai et al., 2000), asexual reproduction can be promoted as well, which in both plants and animals, is often associated with polyploidy (Heslop-Harrison et al., 2023; Knytl et al., 2022). Allopolyploids are organisms that have more than two sets of chromosomes that originate from at least two different species.

As well with already known advantages, polyploidism comes also with some disadvantages, some of them are confirmed while others are just hypothesized (Comai, 2005). Since cell volume is proportional to the DNA amount in the nucleus, doubling the genome should theoretically double the volume occupied by chromosomes. However, only a 1.6-fold increase in the surface area of the nuclear envelope is observed (Melaragno et al., 1993). This can potentially obstruct the normal balance of elements that are part of the mediation between chromosomes and nuclear components, that includes proteins bound to envelope. The reduced space on the nuclear envelope can also affect the peripheral positioning of centromeric and telomeric heterochromatin (Fransz et al., 2002). Polyploidy can increase the rate of spindle irregularities, leading to disordered segregation of chromatids and the formation of aneuploid cells.

These aneuploid cells, characterized by an irregular number of chromosomes, are more likely to be produced in meiosis involving multiple sets of chromosomes compared to diploid cells. Consequently, polyploidy can also be an obstacle for the normal cycle of mitosis and meiosis.

Autopolyploids are organisms that have more than two sets of chromosomes, all derived from a single ancestral species. They possess the capacity to generate various configurations of homologous chromosomes during meiotic metaphase I, leading to irregular segregation patterns, such as a 3:1 or 2:1 ratio with an additional laggard (laggard chromosomes fail to properly attach to the spindle apparatus, resulting in their random distribution to daughter cells). The resolution of these irregular segregation patterns into balanced products is unattainable, leading to the production of predominantly aneuploid gametes through the random segregation of multiple chromosome types. Allopolyploids exhibit a higher level of constraint in chromosome pairing during meiosis I compared to autopolyploids, yet the stable preservation of both parental chromosomal complements necessitates the production of balanced gametes.

Another drawback of polyploidy involves potential variations in gene expression. The increase in chromosome copy number should affect all genes uniformly, resulting in a consistent increase in gene expression. However, this might change for genes involved in regulatory pathways that don't scale proportionally with ploidy. Experimental evidence for such exceptions exists in several models. For instance, researchers compared mRNA levels per genome for 18 genes across different polyploid levels. As expected, most gene expression patterns increased with ploidy, but some genes exhibited a different expression pattern, not proportional to the level of ploidy. Approximately 10% of these genes were shown to be related to ploidy levels (Guo et al., 1996).

1.4 Polyploidization in *Xenopus* frogs

Amphibians is a vertebrate group composed of over 8,700 species (Frost, 1999), in which around 100 species across 19 families have been found to be polyploid (Mezzasalma et al., 2023). Unlike, for example, teleost fishes where whole genome duplication became an established evolutionary process for the entire group, polyploidy in amphibians has arisen independently on multiple occasions within different families (Schmid et al., 2015). Amphibians stand out among vertebrate lineages because they can form populations and even entire species with both diploid and polyploid individuals capable of sexual reproduction (Bogart, 1980; Mezzasalma et al., 2015).

A notable hallmark setting African clawed frogs different from many other amphibian species is their remarkably elevated frequency of polyploid species (Evans, 2008; Evans et al., 2015; Tymowska, 1991).

So *Xenopus* diploids can be used as system models for evolutionary and cytogenetic research related to polyploidy (Bogart and Bi, 2013; Fornaini et al., 2023; Knytl et al., 2018). Polyploidy can develop through two distinct mechanisms. One is autopolyploidy, which is relatively rare in amphibians and

occurs within a single ancestor due to meiotic incompatibilities triggered by environmental factors. The other mechanism is allopolyploidization, which begins with the hybridization of two or more divergent ancestors, leading to meiotic incompatibilities and subsequently preventing cytokinesis.

Pipid frogs (family Pipidae) represent an ancient evolutionary line of fully aquatic frogs, unlike those that need both aquatic and terrestrial environments to survive. They diverged from their sister lineage, Rhinophrynidae, over 150 Mya. The currently living pipid frogs are divided into two subfamilies, the American Pipinae (*Pipa Laurenti*) and the African Dactylethrinae (split ~110 Mya) containing two deeply divergent tribes (split ~100 Mya), *Dactylethrinini* (*Xenopus* Wagler) and *Hymenochirini* (*Hymenochirus boulengeri* and *Pseudhymenochirus merlini*) (Dubois et al., 2021; Feng et al., 2017; Hime et al., 2021).

For almost thirty years, *Xenopus* frogs have been classified into two genera, *Xenopus* and *Silurana* (Cannatella and De Sa, 1993). These two distinct clades are defined by variations in morphology and the chromosome number of their diploid ancestors (20 for *Silurana* and 18 for *Xenopus*) (Cannatella and Trueb, 1988; Tinsley et al., 1996). However, the previously proposed paraphyletic relationship between *Silurana* and *Xenopus*, in relation to other pipid genera, was defined on morphological evidence (Cannatella and Trueb, 1988), and found no evidence nor confirmation by lately molecular phylogenetic analyses that confronted the predominance of Xenopodinae in relation to other pipid genera (Bewick et al., 2013; Hedtke et al., 2013; Irisarri et al., 2011).

The Pipidae family exhibits a high degree of variability in karyotypes, especially in chromosome numbers and sizes, largely due to the influence of polyploidization throughout their evolutionary history (Schmid et al., 2015; Tymowska, 1991). It has been hypothesized that the ancestral karyotype of Pipidae was $2n = 20$. (Bredeson et al., 2024; Mezzasalma et al., 2015), as is seen in *Xenopus* (subgenus *Silurana*), *Hymenochirus*, *Pseudhymenochirus*, and *Pipa* (*Pipa carvalhoi* (Miranda-Ribeiro, 1937). Mezzasalma et al. (2015) also suggested three mechanisms for the chromosome number increasing in pipid frogs: fission, allopolyploidy and the addition of a B chromosome. In pipids, polyploidy, especially allopolyploidy, was thought to have only been found in *Xenopus* species, including *Silurana* subfamily (Schmid et al., 2015) however our recent studies found that other species outside *Xenopus* are polyploid. I will discuss this topic in the discussion.

In *Silurana* the haploid number is $n = 10$, while in the subgenus *Xenopus*, where the fusion of two chromosomes, 9 and 10, was registered (Session et al., 2016), the haploid number is $n = 9$. Recently, in *Hymenochirus*, was discovered a fusion of chromosomes 8 and 10 (Bredeson et al., 2024). *Pipa* has been hypothesized to occur chromosome fission (Mezzasalma et al., 2015).

Usually, the scientific community that uses African clawed frogs as model in their studies, refers to all the species simply as “*Xenopus*” (Hellsten et al., 2010), but a previous studies on *Xenopus* systematics refer them as two different subgenera, *Silurana* and *Xenopus* (Tinsley et al., 1996), *Silurana* is considered a subgenus within the genus *Xenopus* (Evans et al., 2015). The subgenus *Silurana* encompasses the diploid *X. tropicalis* and the tetraploids *Xenopus epitropicalis*, *Xenopus mellotropicalis* and *Xenopus calcaratus* (Evans, 2008; Evans et al., 2015). Based on a recent taxonomic reassessment of *X. laevis*, the subgenus *Xenopus* comprises 25 described species, including 14 tetraploids (*Xenopus borealis*, *Xenopus clivii*, *Xenopus fraseri*, *Xenopus gilli*, *Xenopus laevis*, *Xenopus largeni*, *Xenopus muelleri*, *Xenopus petersii*, *Xenopus poweri*, *Xenopus pygmaeus*, *Xenopus victorianus*, *Xenopus allofraseri*, *Xenopus parafraseri* and *Xenopus fischbergi*), seven octoploids (*Xenopus amieti*, *Xenopus andrei*, *Xenopus boumbaensis*, *Xenopus itombwensis*, *Xenopus lenduensis*, *Xenopus wittei*, and *Xenopus vestitus*), and four dodecaploids (*Xenopus longipes*, *Xenopus eysoole*, *Xenopus kobeli* and *Xenopus ruwenzoriensis*) (Evans et al., 2015).

The molecular evolutionary history of *Xenopus* frogs is typically analyzed using segments of mitochondrial DNA. This analysis includes most of the mitochondrial 12S and 16S rDNA genes, the intervening tRNA val (tRNA which binds L-valine), cloned homeologs of the autosomal genes RAG1

and DMRT1, and a portion of the mitochondrial cytochrome oxidase I gene (Evans, 2007; Evans et al., 2004).

Thanks to this approach, a research that also used genetic data from four of the new species shown in Evans et al. (2015), it is suggested that tetraploidization occurred at least once in both *Silurana* and *Xenopus*. In contrast, octoploidization and dodecaploidization are believed to have occurred at least three times within the subgenus *Xenopus* (Evans et al; 2015). Phylogenetic analyses prove that these genome duplication events were driven by allopolyploidization rather than autopolyploidization. There is/are an ancestral (2n = 18) diploid(s) that is/are not available for studying, that is/are probably extinct(s) (Figure 7).

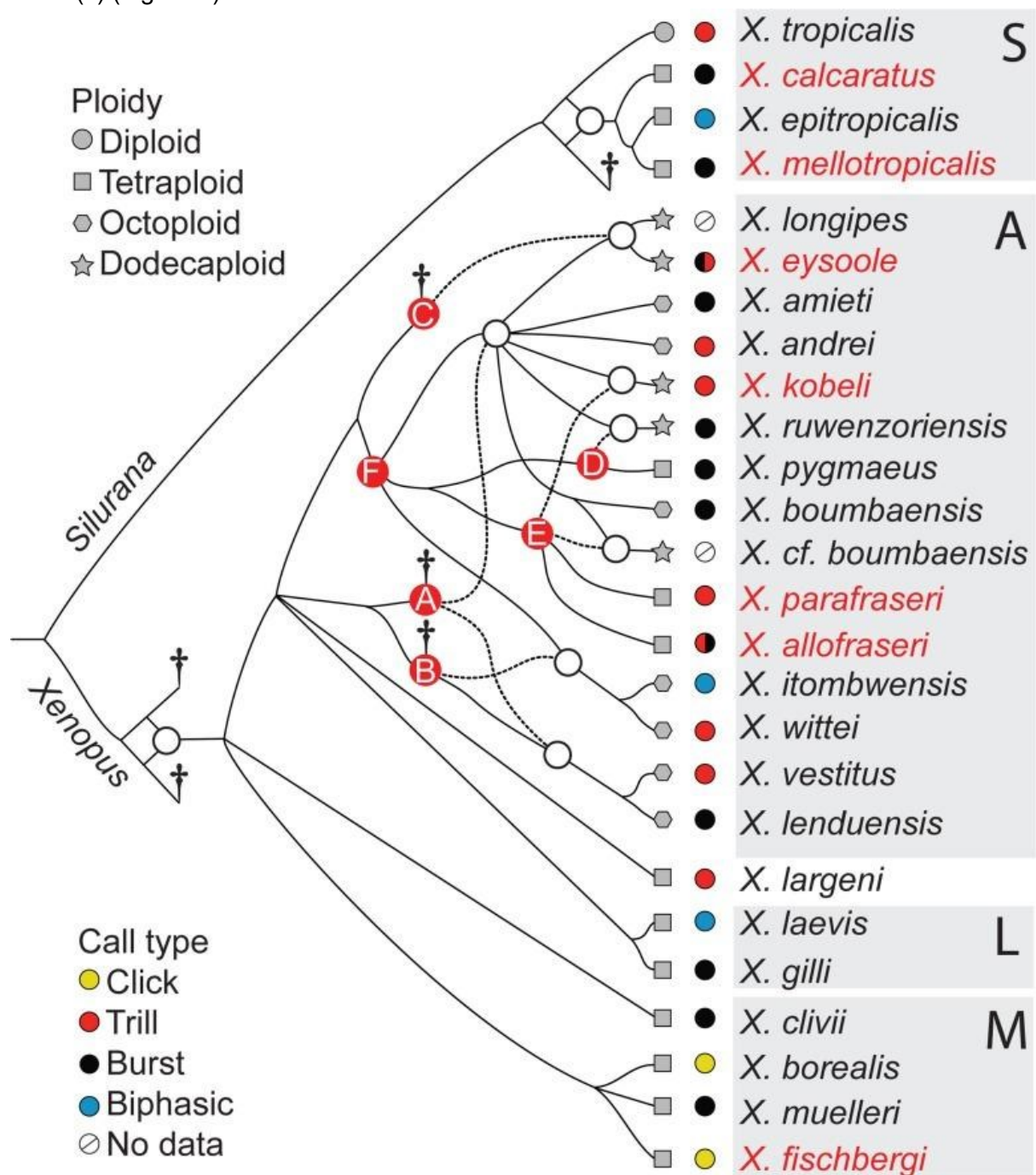


Figure 7. A summary phylogeny was inferred through a comparative analysis of mitochondrial and autosomal gene trees. Newly identified and resurrected species are highlighted in red. The letters S,

A, L, and M represent the subgenus Silurana and the amieti, laevis, and muelleri species groups within the subgenus Xenopus, respectively. Dotted lines indicate paternal ancestral lineages, while circles at internal nodes denote allopolyploidization events. Shapes at branch tips indicate the ploidy of existing species, with colors next to these shapes reflecting vocalization. Daggers mark lost ancestors, including up to three diploid species (assuming allotetraploidization in the subgenus Xenopus) and at least three tetraploid ancestors (A, B, and C). Taken from (Evans et al., 2015).

Hybridization in African clawed frogs does not always lead to genome duplication. Some hybrids retain the same ploidy level as their parental species, such as those between *X. laevis* and *X. muelleri*, *X. laevis* and *X. gilli*, and *X. victorianus* and *X. borealis* (Evans et al., 1998; Yager, 1996).

The high diversity of *Xenopus* species (in total 29 species), makes them a perfect group for studying chromosomal rearrangements and polyploidization. The research on *Xenopus* taxa has also the potential to give some clues if chromosomal rearrangement and polyploidization are linked to speciation (Knytl et al., 2017).

Researchers have investigated the subgenus *Xenopus* and identified significant large-scale rearrangements. Notably, one of the most important findings is the fusion of chromosomes 9 and 10 in a diploid ancestor of allopolyploids, now extinct.

In the subgenus *Xenopus*, a comparison with the well-assembled genome of the diploid species *X. tropicalis* reveals many additional rearrangements in the *X. laevis* S-subgenome. This comparison underscores the relative genomic conservation of the *X. laevis* L-subgenome (Session et al., 2016). Regarding *Silurana* tetraploids, the difference between the a- and b- subgenomes is greater than the divergence between the more conserved a- subgenome and the diploid *X. tropicalis* genome (Evans, 2008; Evans et al., 2015). Also in *Silurana* it has been identified a large-scale rearrangement: in the tetraploid species *X. mellotropicalis*, a nonreciprocal translocation has occurred between the pericentromeric regions of chromosomes 9b and 2b (Knytl et al., 2018, 2017). The rearrangement was identified using fluorescence in situ hybridization (FISH) hybridizing the whole chromosome painting (WCP) probes from *X. tropicalis* to *X. mellotropicalis* chromosomes (Zoo-FISH). It was unknown if the rearrangement occurred also in the progenitor of the three allotetraploid species in *Silurana* or after the divergence, from the other species, of the ancestor of *X. mellotropicalis*, this topic will be explained in section 3.2.

This is due to the unresolved evolutionary relationships among species in the subgenus *Silurana*, mainly because of the limited genomic data available from *X. calcaratus* and *X. epitropicalis* when the study was published. Such data could significantly aid in phylogenetic estimation when combined with genomic data from other *Silurana* species (Cauret et al., 2020; Hellsten et al., 2010).

Regarding polyploidy in *H. boettgeri*, in a striking discovery, B chromosomes have been identified in the captive population of *Hymenochirus* sp. ($2n = 20A + 1B$). Astonishingly, these B chromosomes appeared in nearly half of the karyotypes analyzed from two male specimens (30 out of 66 metaphase spreads from the intestine, spleen, gonads, and lung tips), while the rest exhibited the standard $2n = 20$ configuration (Mezzasalma et al., 2015). However, older studies of '*H. boettgeri*' registered the chromosomal number being $2n = 24$ (Morescalchi, 1981, 1968; Tymowska, 1991), also the karyotype $2n = 22$ was stated (Scheel, 1973). Unfortunately this study only reported the chromosome number and no more information was given. Finally, a recent study recorded $2n = 18$ chromosomes in *H. boettgeri*, with no B chromosomes detected (Bredeson et al., 2024). It remains uncertain whether the variations in chromosome number are due to differences in B chromosome count (except for the $2n = 18$ case, where chromosome fusion occurred), as proposed by Mezzasalma et al. (2015), alternatively, it is possible that earlier researchers examined one or more different species where chromosome fissions had taken place (Morescalchi, 1981, 1968; Scheel, 1973).

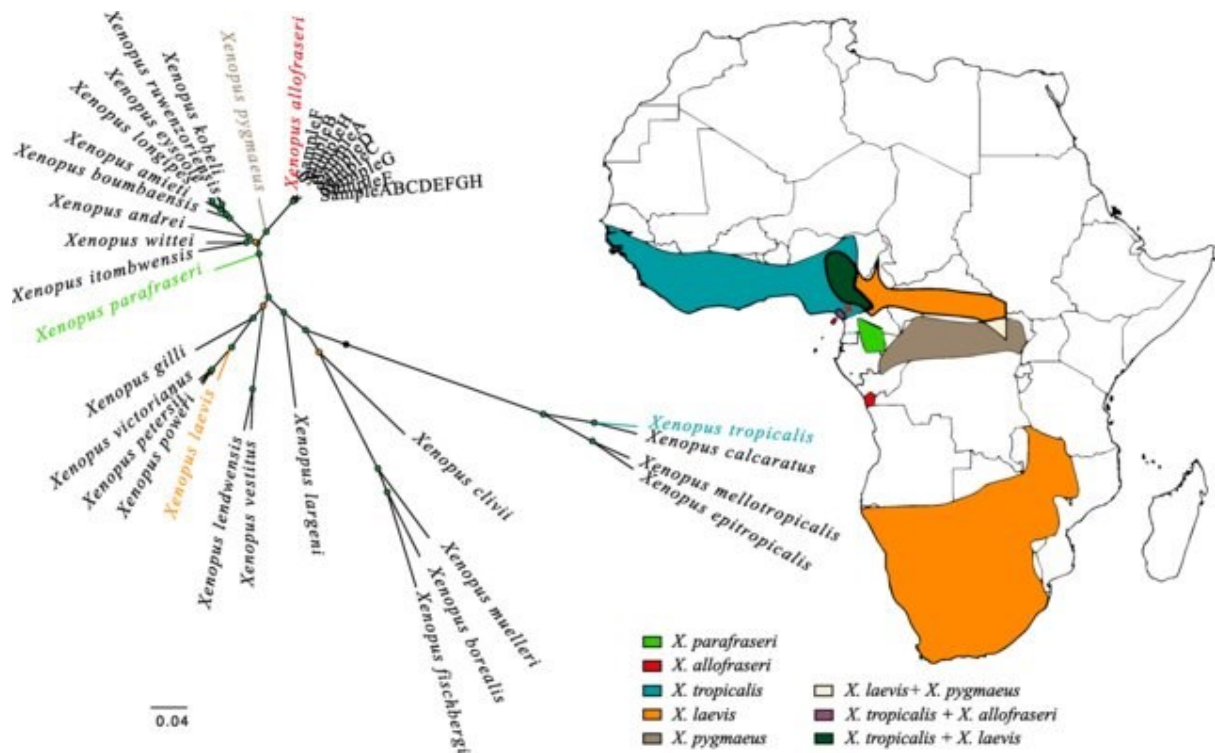


Figure 8. Geographic range of some *Xenopus* species in Africa and maximum-likelihood phylogenetic tree (Ducret et al.; 2021).

2 Materials and Methods

All materials and methods are presented in papers in the supplements part.

2.1 Cytogenetic analysis

All materials and methods used for publications are included in the papers in the section “Supplements”.

2.2 Genomic analysis

We extracted DNA from one *X. epitropicalis* individual using E.Z.N.A HP Total RNA Isolation Kit (Omega-Biotek Inc, Norcross, Georgia 30071, USA). RNA was extracted from three *X. epitropicalis* and six *X. borealis* individuals using E.Z.N.A HP Total RNA Isolation Kit (Omega-Biotek Inc, Norcross, Georgia 30071, USA). RNA was extracted from liver and intestine from all individuals, while heart, muscle (from hind limb) and lungs were extracted from individual 2 and 3. *Xenopus epitropicalis* and *X. borealis* were bred in the Faculty of Science in the Department of Cell Biology, at Charles University in Prague.

RNA-seq and WGS were provided by Novogene Co., Ltd. (Beijing City, China), through the form of raw data.

Adapters, from sequences, were removed by trimming, using the software Trimmomatic (Bolger; 2014).

Two sequences, liver and lungs, of *X. borealis* were taken from Genbank (BioProject PRJNA616217). From *X. borealis* sequences we downloaded the two subgenome S and L and we tried to annotate them with different approaches: we tried to use the software Augustus (Stanke and Waack, 2003) for gene prediction, a tool that predicts genes in eukaryotic genomic sequences but, due to its predictive nature, the results we got were not precise enough so we tried a different approach.

We used BUSCO (Seppey et al., 2019) to annotate only the single copy genes, then we mapped the exons from *X. laevis* genome (GCF_017654675.1) due the high quality of their annotation. From this annotation we selected *X. borealis* exons and we mapped *X. borealis* mRNAseq data from liver and lungs and we found out that both subgenomes were expressed. For mapping of the sequences we used bowtie2 (Langmead and Salzberg, 2012) and Geneious 2024.0 (<https://www.geneious.com/>) mapper. For read counts we used RSEM (Li and Dewey, 2011), a RNA-seq expression quantification tool.

3 Discussion

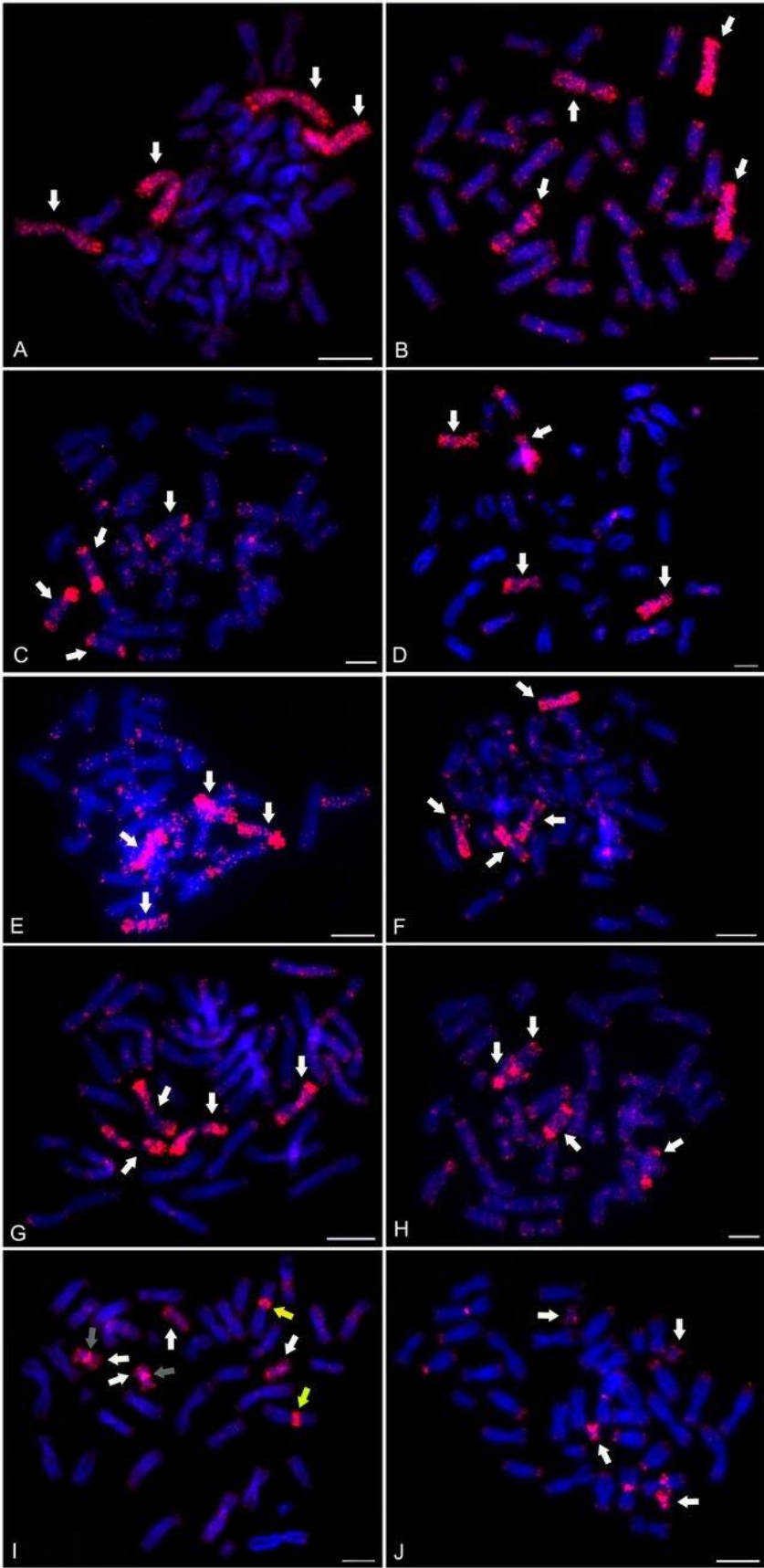
3.1 Divergent subgenome evolution in the allotetraploid frog *Xenopus calcaratus*

In the first Paper, we investigated the subgenome of a species from the *Silurana* subgenus, *X. calcaratus*, to prove the existence of two subgenomes using a different combination of cytogenetic techniques. Similarly, in the *Xenopus* subfamily, like *X. laevis*, two subgenomes have been identified, called S and L (Session et al., 2016).

The first studies analyzed chromosome differences confronting the karyotype of different *Xenopus* species (Tymowska, 1973; Tymowska and Kobel, 1972). They were able to identify that *X. muelleri* and *X. laevis* karyotypes were more similar and more distantly related to *X. tropicalis*. Through modern cytogenetic techniques, like FISH, it was possible to identify the chromosome quartets in a *Silurana* species, *X. mellotropicalis*, (Knytl et al., 2017) however they were not able to distinguish the subgenomes for most of the chromosomes. We wanted to push over this study and we tried to identify the quartets in another *Silurana*, *X. calcaratus*.

We prepared the WCP from *X. tropicalis* chromosomes, in order to hybridize probes with *X. calcaratus* chromosomes and identified two subgenomes, as seen in *X. mellotropicalis* (Knytl et al., 2017). The two subgenomes were easily distinguishable, since we identified two chromosomes with brighter signals as the subgenome “a” and we called “b” the pair that bore the lower intensity signals (Knytl et al., 2017; Tymowska, 1991; Tymowska and Fischberg, 1973). The brighter intensity of the signal in subgenome “a” indicates that this subgenome is more similar to the progenitor *X. tropicalis*, while the lower intensity in the signal for the subgenome “b” indicates that this subgenome is more different than the orthologous pair. This discovery let us understand how polyploidization affected the evolution of the genre *Silurana*. We can easily assume that the two subgenomes evolved independently, while subgenome “a” is more stable, and subgenome “b” underwent through modifications. However with just cytogenetic techniques we are not able to quantify the differences between the two subgenomes, so far we are only able to determine that subgenomes are different in gene expressions, but we can't quantify the differences in expression patterns, since FISH is a qualitative techniques but gives no information about quantity. Thanks to our cytogenetic approach we confirmed that the genome of *X. calcaratus* consists of two different subgenomes, as proved in *X. mellotropicalis* (Knytl et al., 2017). Knytl et al. (2017) were just able to find the quartets in *X. mellotropicalis* using *X. tropicalis* probes. In *X. calcaratus*, we were able to take a step further and find the chromosome quartets, and we also succeeded in identifying the two subgenomes.

Differences between our results and the previous study can be easily seen in Figure 9.



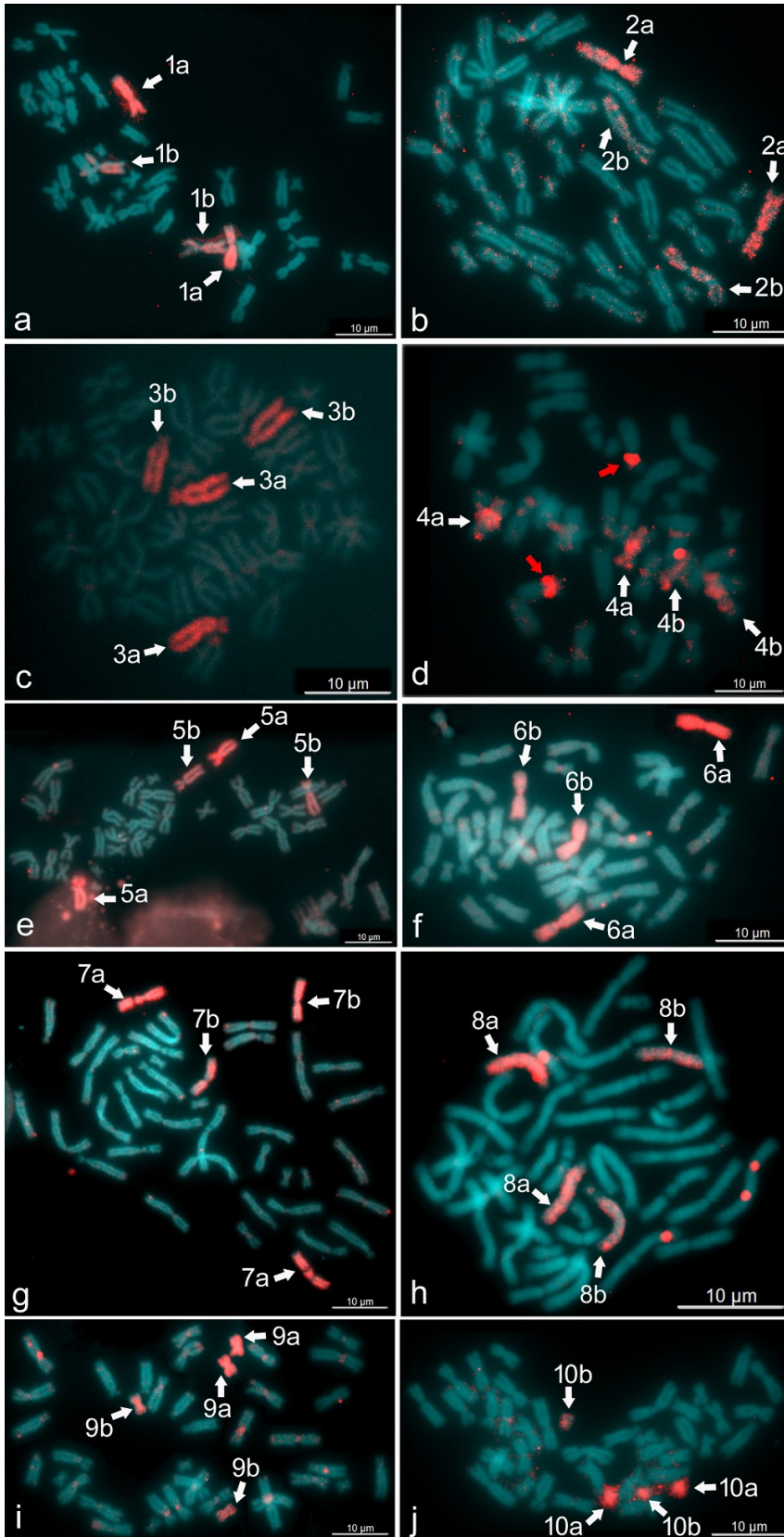


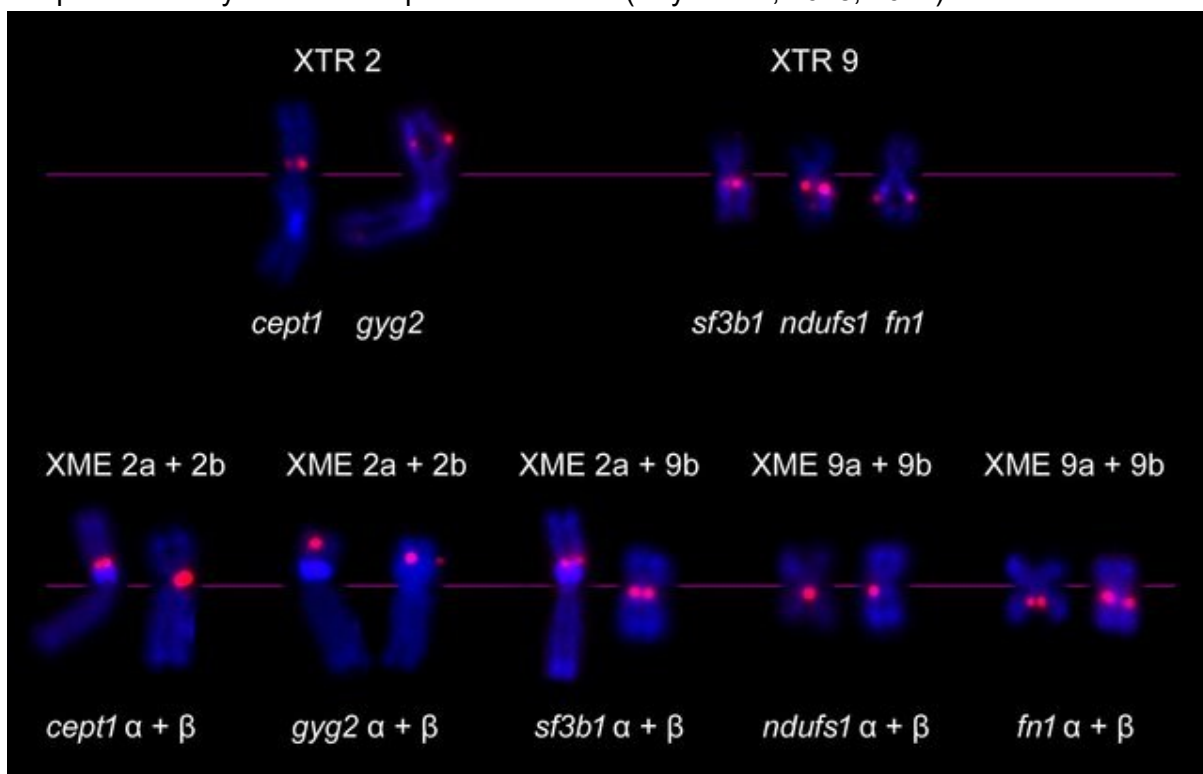
Figure 9. Results from (Knytl et al., 2017) compared with results from (Knytl et al., 2023). In the second picture the two subgenomes are clearly evident, while in the first one it is almost impossible to see any differences between them.

Actually we didn't expect the two subgenomes to be visible after the FISH, this can be given by the fact that one *X. calcaratus* subgenome underwent more mutations while the other was more conserved and remained more similar to *X. tropicalis*. The two subgenomes of *X. mellotropicalis* look very similar, according to FISH results, while our results on *X. calcaratus* showed more difference between the subgenomes. This could have different explanations: first of all that subgenomes of *X. mellotropicalis* are more similar to each other, compared to *X. calcaratus* subgenomes, or that the probes that we used were more efficient, since we dissected more copies of every chromosome, that could have led to clearer results. In the case that the two subgenomes are really that different, this means that in *X. mellotropicalis* occurred different polyploidization events and in a different timing, compared to *X. calcaratus*.

With *X. calcaratus* we were more confident, since the two genomes are clearly visible with cytogenetic approach, that through RNA-seq we could have been able to identify the differences in gene expression between them. We actually started to analyze the last *Silurana*, *X. epitropicalis*, but I will discuss that in section 3.5.

3.2 Finding chromosomal rearrangement within *Xenopus calcaratus*

In *X. mellotropicalis* was found a translocation between chromosome 2 and 9 (Knytl et al., 2018, 2017). In order to find if the same translocation occurred also in *X. calcaratus*, we mapped the alpha and beta homeologs of *gyg2*, *cept1*, *sf3b1*, *ndufs1*, and *fn1* in *X. calcaratus* using FISH with tyramide signal amplification (FISH-TSA). The *ndufs1* α and β , and *fn1* α and β are located on chromosomes 9a and 9b, respectively, while the *sf3b1* α and β genes are found in *X. mellotropicalis* chromosomes 2bp and 9bq, respectively (Knytl et al., 2018). We selected these genes for *X. calcaratus* because they were located near the above mentioned translocation (Knytl et al., 2018). To confirm the translocation we would have expected to find the identical gene positions in *X. calcaratus* and *X. mellotropicalis*, but our results didn't show any proof of translocation that involved chromosomes 9b and 2b, as seen in *X. mellotropicalis* (Knytl et al., 2018, 2017). Gene positions can be seen in figure 10, compared to the positions they found in the previous studies (Knytl et al., 2018, 2017).



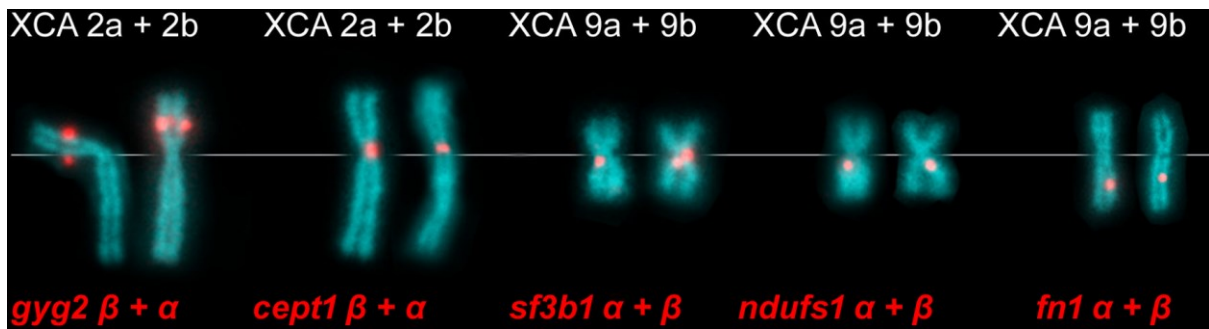


Figure 10. Comparison of XME and XCA FISH-TSA labeling of *gyg2*, *cept1*, *sf3b1*, *ndufs1* and *fn1* genes. The centromere position is indicated by purple lines on XME and is marked by a white line in XCA (Knytl et al., 2023, 2018).

With our results we were able to confirm that the translocation occurred in *X. mellotropicalis* but not in *X. calcaratus*, however we are not able to define if it happened before or after the polyploidization.

Based on Zoo-FISH (Figure 10), this translocation was not found in *X. calcaratus*, nor was it present in *X. tropicalis* (Knytl et al., 2018). Moreover, we discovered that genes involved with rearrangements in *X. mellotropicalis* did not share the chromosomal position with *X. calcaratus*. In Knytl et al. (2018), *sf3b1* was found in *X. mellotropicalis* chromosome 2b but, differently, in *X. calcaratus*, this gene was localized in *X. calcaratus* chromosome 9a, while in *X. tropicalis*, *sf3b1* is mapped on chromosome 9 (Knytl et al., 2018).

The differences observed in the chromosome mapping between these species can be most straightforwardly explained by the translocation (9b;2b) occurring in an ancestor of *X. mellotropicalis* after it diverged from *X. calcaratus*. (Figure 11). Whether the translocation (9b;2b) occurred in the most recent common ancestor of *X. epitropicalis* and *X. mellotropicalis* remains unknown (Figure 11, black and blue spots), or if this translocation involved only *X. mellotropicalis* lineage after diverging from the common ancestor with *X. epitropicalis* (Figure 11, red spot). The translocation might have taken place between ancestral chromosomes in a shared diploid ancestor of *X. epitropicalis* and *X. mellotropicalis*, that would be another possible scenario (Figure 11, black spot).

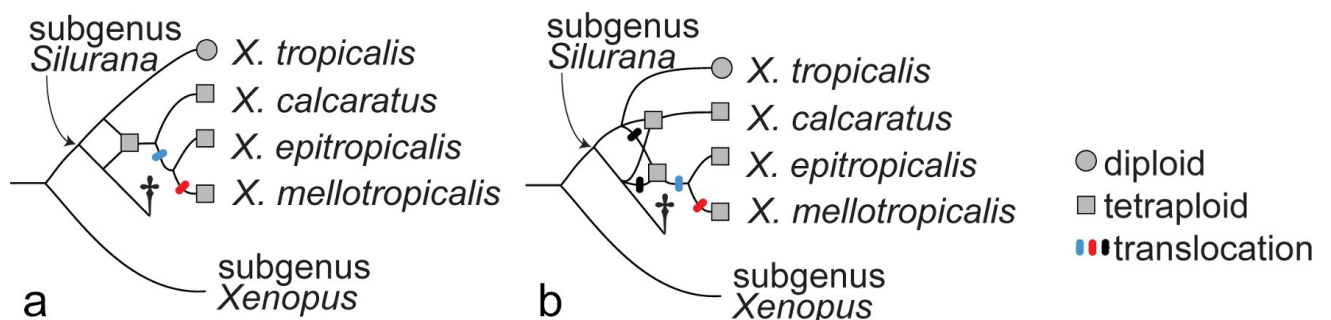


Figure 11. Evolutionary scenarios have been proposed in the subgenus *Silurana* to explain the origin of a translocation found in *X. mellotropicalis* but absent in *X. calcaratus*, with no data available for *X. epitropicalis*. The first scenario suggests that if a single allotetraploidization generated all three tetraploid species, the translocation might have occurred after *X. calcaratus* diverged but before the speciation of *X. mellotropicalis* and *X. epitropicalis* (blue). Alternatively, it could have happened after this speciation in the progenitor of *X. mellotropicalis* (red). Another scenario proposes that if two separate allotetraploidization events took place, this translocation might have happened in both diploid ancestors of the most recent common ancestor of *X. mellotropicalis* and *X. epitropicalis* (both black, but only one is possible) (Knytl et al., 2023).

This interpretation is limited due to the lack of any cytogenetic information about the diploid descendant of the other *Silurana* ancestor.

This lack of data means we cannot exclude the possibility that some rearrangements in the b-subgenome, happened in that ancestor before allotetraploidization, except for the loss of the nucleolus organizer region. Further solutions of this translocation's evolutionary history mystery could be formulated through Zoo-FISH analysis of *X. epitropicalis* chromosomes, which are currently being processed, but no data is available yet.

Using a cytogenetics approach we are able to look for a translocation or a deletion, with WCP probes it's possible to see if chromosomes went through deletion or translocation during evolution. However, small rearrangements such as small translocation, deletion or inversion are hard to identify with FISH, unless we already have a candidate gene, if the mutation is too small, it cannot be detected by this technique. For a wider approach next generation sequence techniques are required, to go through the whole genome and identify some gene alterations or chromosomal abnormalities.

This application is expensive and time consuming, so we kepted as the last step of our research, which will be discussed in section 3.5.

Nucleolus organizer regions (NORs) are specific chromosomal areas essential for nucleolus formation. In the genome of eukaryotes, the nucleolus organizer regions (NORs) include tandem arrays of three ribosomal genes: 18S, 5.8S, and 28S. The ribosome is composed of small and large subunits. The 18S rRNA gene is found in the small subunit, while the 5.8S, 28S, and 5S rRNAs are located in the large subunit. Interestingly, the 5S rRNA gene is not part of the NOR (Vierna et al., 2013). In diploid genomes, NORs typically appear as a single pair (Schmid et al., 1987). The allotetraploid species *X. laevis* shows one NOR mapped on chromosome 3L short arm (p) (Roco et al., 2021; Schmid et al., 1987; Tymowska and Kobel, 1972). During speciation, particularly after allotetraploidization in the subgenus *Xenopus*, it is believed that the homologous NOR on chromosome 3S was lost (Session et al., 2016). In diploid *X. tropicalis*, the NOR is located on chromosome 7 long arm (q) (Roco et al., 2021; Tymowska, 1973; Tymowska and Fischberg, 1973; Uehara et al., 2002). One NOR on chromosome 7aq was also found in the allotetraploid *X. epitropicalis* and *X. mellotropicalis* (Tymowska, 1991; Tymowska and Fischberg, 1973). After allotetraploidization, the homologous NOR on chromosome 7b might have been lost (Knytl et al., 2017).

Using 28S rDNA probes in the FISH experiment, we identified a single pair of NORs on the nucleolar secondary constriction of XCA 7aq (red in Figure 12a). Additionally, FISH analysis with 5S probes revealed approximately 8–10 positive signals located on the telomeric regions of XCA 2b, 4a, 5a, 6a, and 8b (green in Figure 12b).

Given the close relationship between *X. calcaratus* and *X. mellotropicalis*, it was expected that these species would show similar patterns and localization of heterochromatic blocks. To test this, C- and CMA₃-banding techniques were used, which highlight GC-rich chromosomal loci and constitutive heterochromatin. The results showed positive signals on XCA 1bp (Figure 12b and 12c) and faint signals in specific stained regions of XCA 2b, 6b, 7b, and 8b (not indicated by arrows) using C-banding. Furthermore, CMA₃-banding confirmed the presence of a nucleolar secondary constriction on XCA 7aq (Figure 12c). Interestingly, all *X. calcaratus* chromosomes exhibited weak CMA₃-bands on their telomeres, likely due to repetitive sequences.

We proposed at least two plausible explanations for the preserved position and quantity of NORs in *X. mellotropicalis* and *X. calcaratus*. One possibility is that if these frogs originated from separate allotetraploidization events, the loss of one pair of NORs from the same ancestral progenitor could have occurred two times, each time following an event of allotetraploidization. Alternatively, if there was only a single allotetraploidization event in *Silurana*, it is conceivable that, after allopolyploidization within the *Silurana* subgenus, was lost just one pair of NORs.

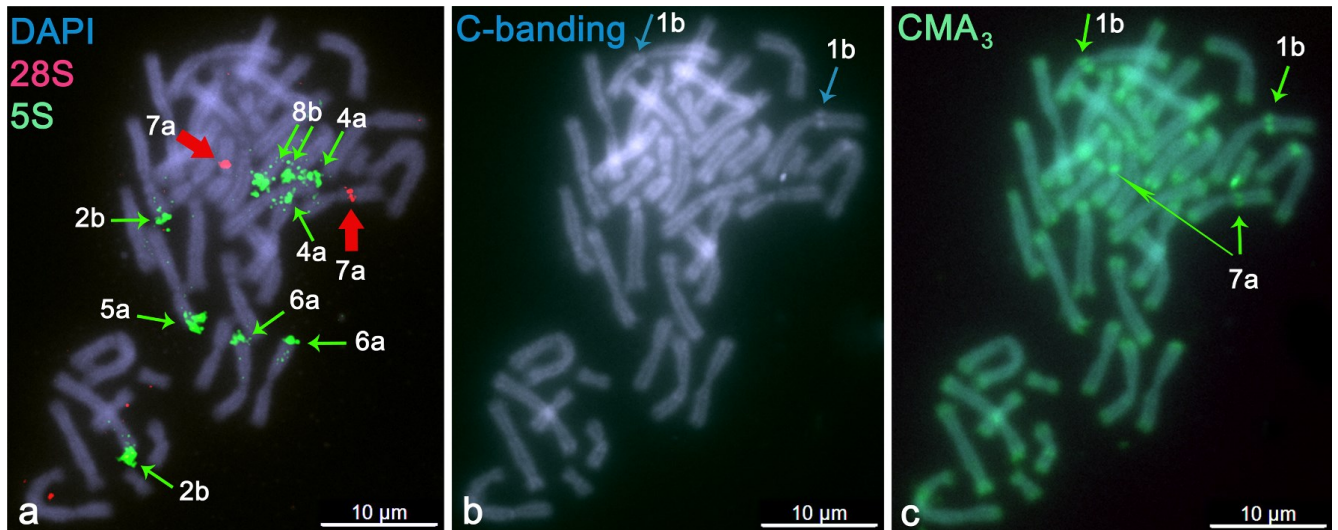


Figure 12. Metaphase spreads of *X. calcaratus*, sequential fluorescent chromosome mapping results (ribosomal DNA ISH/C-banding/Chromomycin A3, CMA3). Scale bars represent 10 μm (Fornaini et al., 2023).

3.3 Cytogenetic Mapping in *Xenopus*

In our second paper we decided to undergo the study of repetitive elements. Short read sequences, due to their repetitive nature, typically present challenges for mapping and repeat quantification, even with whole genome data. However, using a cytogenetic approach like FISH, allowed us to localize repetitive DNA sequences on chromosomes. This approach has been successfully employed to study the distribution of repetitive sequences in the chromosomes of various animal groups, including teleost fish (Bishani et al., 2021; Knytl and Fornaini, 2021), reptiles (Altmanová et al., 2023; Oliveira et al., 2021), birds (De Oliveira Furo et al., 2017; Kretschmer et al., 2021), and mammals (Gerbault-Seureau et al., 2017; Milioto et al., 2019). Cytogenetic mapping of repetitive DNA in amphibians has been crucial for identifying both interspecific and intraspecific rearrangements, as well as other aspects of karyotype evolution (Da Silva et al., 2021; Guzmán et al., 2022; Phimphan et al., 2021). In the *Xenopus* subgenus at least eight independent episodes of allopolyploidization occurred, with each subgenera experiencing at least one allotetraploidization event. The presence of subgenera with different ploidy levels, a result of independent evolutionary events, provides a unique opportunity to investigate how tandem repeats evolve after genome duplications.

Quantifying and localizing repetitive sequences through high-quality genome sequencing is a costly and complex process, especially when compared to employing multiple cytogenetic approaches for gene mapping (Knytl et al., 2018; Symonová et al., 2017). Alternatively, we performed cytogenetic mapping of non-rDNA tandem repeats (U1 and U2 snRNA and H3 histone) in one diploid and five allotetraploid *Xenopus* species to explore the effects of genome duplication and divergence on repetitive elements.

We proceeded with U1, U2 double color FISH, and H3 single color FISH. We picked six species *X. tropicalis*, *X. calcaratus*, *X. pygmaeus*, *X. allofraseri*, *X. laevis* and *X. muelleri*. We chose these species for the availability of the biological samples and to have at least one representative for every species group. As far as U1 and U2, we were expecting to find *X. calcaratus*, the double amount of signals, compared to *X. tropicalis*. Results from FISH confirmed our expectation since we registered one signal for U1 and one for U2 in *X. tropicalis* and, respectively, two signals for each gene, in *X. calcaratus*. All

the signals found in *X. calcaratus*, were homologous to the orthologous U1 and U2 regions of *X. tropicalis*, being consistent with the expected doubling of repeat copy numbers due to allopolyploidization. Contrary to our expectations, the number of repeats was different among the other allotetraploid species. In *X. muelleri*, as in *X. calcaratus*, U1 and U2 snDNA probes mapped to the position expected in homoeologous chromosomes (Figure 13). However, in the allotetraploid species *X. pygmaeus*, *X. allofraseri*, and *X. laevis*, only one signal was detected for U1 and U2 snDNAs. Specifically, in the phylogenetically close species *X. pygmaeus* and *X. allofraseri*, the U1 snDNA probe mapped mostly to the q arms of chromosome 1L, while the U2 snDNA probe targeted the q arms of chromosome 8S (Figure 13c, d). In *X. laevis*, the U1 and U2 snDNA signals were most prominent on the q arms of chromosomes 1S and 8L, each (Figure 13e). Notably, these chromosome regions in *X. laevis* corresponded to the homoeologous chromosomes bearing U1 and U2 snRNA signals in *X. pygmaeus* and *X. allofraseri* (1L and 8S). Instead, a nBLAST (Camacho et al.; 2009) search using the U1 snDNA sequence against *X. laevis* genome revealed 11 copies on chromosome 1L and approximately 35 copies on chromosome 1S. Similarly, the U2 sequence search showed around 40 copies on *X. laevis* chromosome 8L and 12 copies on chromosome 8S. These results prove that a copy number reduction or loss could have occurred.

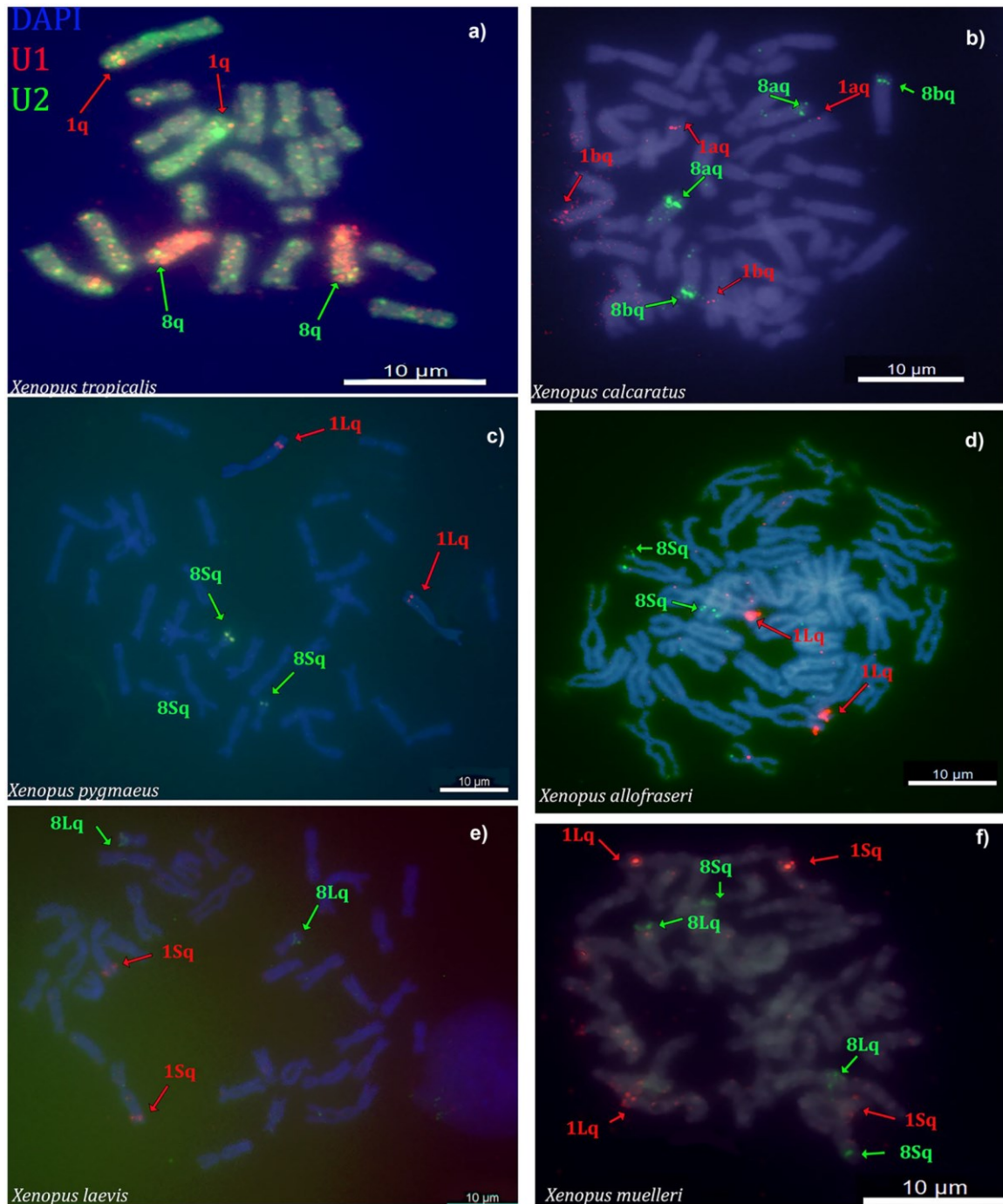


Figure 13. Double-color FISH using U1 and U2 snDNA probes results. Green and red arrows indicate the U2 and U1 repeat loci. Chromosomes were counterstained with DAPI in blue/gray. Scale bars represent 10 μm (Fornaini et al., 2023).

For the mapping of H3, we used the same species and found multiple signals in all of them, located in telomeric and pericentromeric regions (Figure 14). BLAST searches (Camacho et al., 2009) revealed the H3 sequence on *X. tropicalis* chromosomes 3 (11 hits), 6 (11 hits), and 9 (27 hits). On chromosomes 5, 8, and 10, the matches were inferior to 50% of the whole query length. In *X. laevis*, the H3 sequence was found on chromosomes 3S (12 hits), 5L (6 hits), 5S (3 hits), 6L (2 hits), 6S (1 hit), 9_10L (15 hits), and 9_10S (5 hits). Additionally, some hits on *X. laevis* chromosomes 1L and 8L were less than 50% of the query sequence.

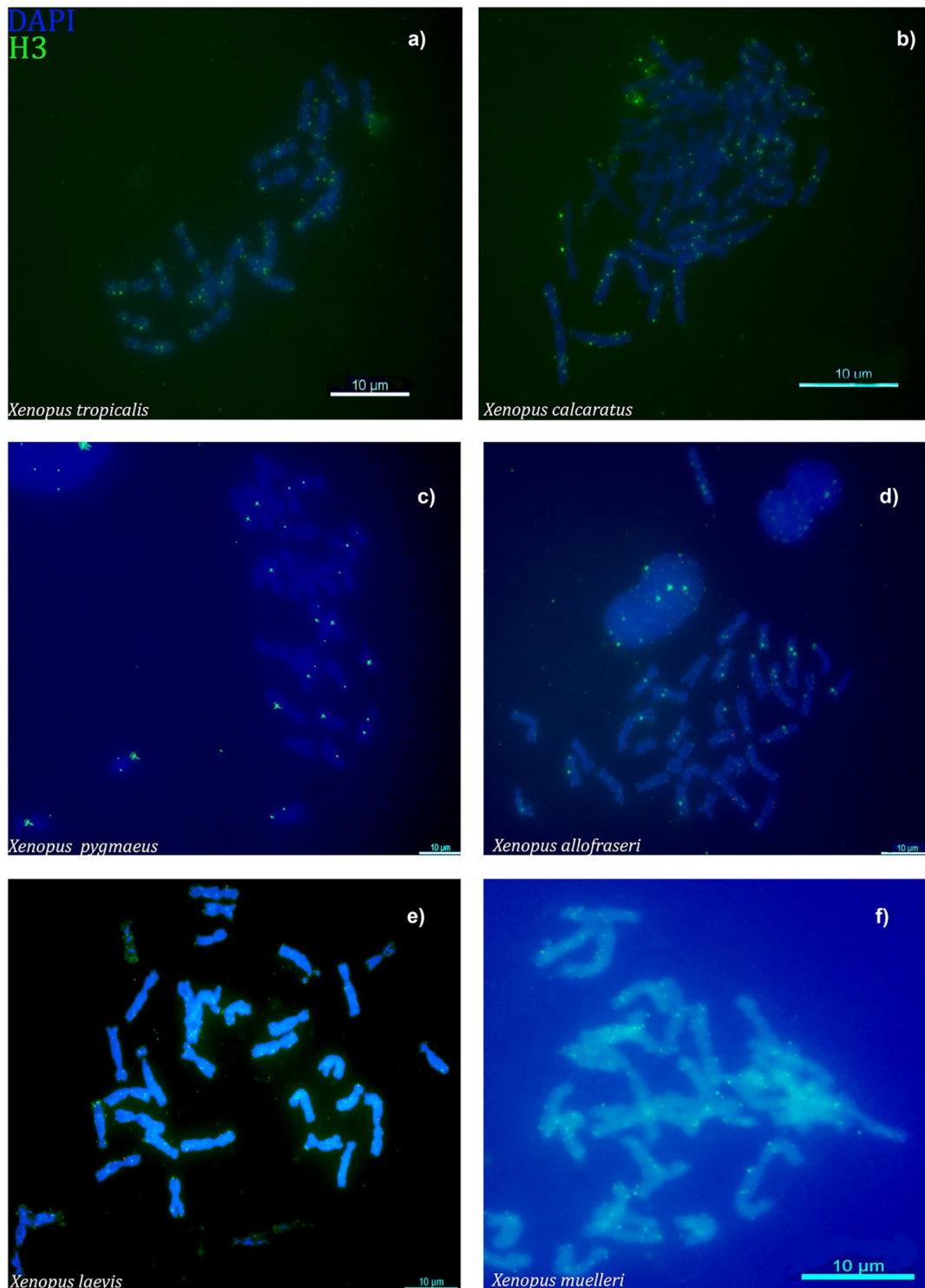


Figure 14. FISH with histone H3 probe. The probe (green) shows multiple signals in all the species. Chromosomes were counterstained with DAPI in blue/gray. Scale bars represent 10 μ M (Fornaini et al., 2023).

We would have expected allotetraploid species to have twice the number of signals compared to the diploid *X. tropicalis*, and also we didn't expect the location on chromosomes to be different. While *X. calcaratus* and *X. muelleri* matched our hypothesis, but the other three allotetraploid species showed the same number of signals as in *X. tropicalis*, that's diploid. Additionally, the snRNA signals in *X. pygmaeus* and *X. allofraseri* were located on a distinct subgenome (U1 on L, U2 on S) compared to

X. laevis (U1 on S, U2 on L). This pattern aligns with an elevated difference in copy number over time after allopolyploidization. Using BLASTn tool (Camacho et al., 2009) on *X. laevis* genome we revealed that these two loci are present in both subgenomes, while, with cytogenetic approach, we revealed the presence in only one subgenome. This could be given by differences in copy number in the two subgenomes and due to FISH sensitivity, that makes this technique incapable to detect small copy number variations. So far we are able to hypothesize this only in one species, *X. laevis*, since genome data are not available for other frogs.

All *Xenopus* species exhibit a NOR on one homologous pair, as described by Tymowska in 1991. Tetraploids from the subgenus *Xenopus* carry NOR on chromosome 3L, while those from the subgenus *Silurana* have it on 7a. Specifically, *X. tropicalis* possesses NOR on chromosome 7 (Knytl et al., 2023; Roco et al., 2021; Session et al., 2016; Tymowska, 1991). We have not detected any small nuclear RNA (snRNA) loci on chromosomes 7, 7a, or 3L, suggesting no genomic features shared between distinct NOR and snRNA repeats. Specifically, U2 snRNA tandem repeats have been mapped in some amphibians, such as in the cycloramphid genus *Thoropa* (Cholak et al., 2020) and the leptodactylid genus *Leptodactylus* (Gazoni et al., 2021), the U2 snRNA repeats were identified. Additionally, in the pipid genus *Pipa* the H3 gene has been mapped as well (Zattera et al., 2020). Notably, there are no other cytogenetic studies that have pinpointed the U1, U2 snRNA, or H3 gene loci within *X. pygmaeus* and *X. allofraseri* group.

In some species of the frog genus *Thoropa*, U2 snRNA was cytogenetically registered on both chromosomes 6 and 7. While, the U2 locus was found exclusively on chromosome 6 or chromosome 7, in other species (Cholak et al., 2020). The observed differences in U2 loci distribution might be attributed to varying copy numbers across these chromosomes. Cytogenetic detection of U1 and U2 snRNA FISH signals was noted on *X. laevis* chromosomes harboring approximately 15 or more copies of U1 and U2 repeats. In the genus *Leptodactylus*, U2 loci were found on chromosome 6 in eight species, with one species showing signals on chromosomes 4, 6, 9, and 10 (Gazoni et al., 2021). They suggested that the additional signals found on chromosomes 4, 9, and 10 could be due to gene transposition by transposable elements (TEs) followed by gene amplification. However, since we did not map TEs on *Xenopus* chromosomes, we are not able to confirm the possibility of TE transposition in this genus.

Regarding the H3 gene, we examined the impact of allotetraploidization and divergence on its evolution. As expected, due to allotetraploidization, both *X. calcaratus* and *X. muelleri*, which are allotetraploids, showed H3 repeats on twice the number of chromosomes compared to the diploid *X. tropicalis*. We observed signals on eight homologous pairs in the allotetraploids *X. pygmaeus*, *X. allofraseri*, and *X. laevis*, which is fewer than expected from allotetraploidization. This may be due to a reduced copy number or deletion of H3 repeats on several chromosomes, possibly in an ancestor of these species, based on their phylogenetic relationships. Despite the variability paired with allopolyploidization followed by evolution of tandem repeats divergence, the relative number of U1, U2, and H3 signals compared to *X. tropicalis* are similar in each allotetraploid species we examined. Prior to our study, only one research had mapped the H3 gene on chromosomes of amphibians (Zattera et al., 2020). In *Pipa carvalhoi*, that's a member of the same family as *Xenopus* but with a phylogenetic distance of about 115–120 million years (Feng et al., 2017; Hime et al., 2021), H3 signals were found on chromosomes 1, 5, 6, 8, and 9, with signals less bright on other chromosomes. Insect species of the family *Acrididae* (Bueno et al., 2013; Oliveira et al., 2011) have shown multiple distributions of the H3 locus across all chromosomes, potentially linked to the transposition of 5S ribosomal RNA (rRNA). 5S rRNA is located on the telomeres of several chromosomes, in *Xenopus* (Knytl et al., 2023, 2017), that might be an insight that the distribution of the H3 locus on different *Xenopus* chromosomes might also be related to the distribution of 5S rRNA. Our research uncovered a fascinating genomic connection between different repetitive elements and rRNA, potentially

providing insights into genome organization and evolution. We observed synteny between As51 satellite DNA, which originates from TEs, and NORs (Vicari et al., 2008), as well as between U1 snRNA and 5S rRNA in the characid fish genus *Astyanax* (Silva et al., 2015). One possible purpose of this co-localization is the TEs silencing of ribosomal genes (Vicari et al., 2008). The 5S ribosomal genes were found at the distal regions of chromosomes 8bq in *X. mellotropicalis* and *X. calcaratus*, which are *Silurana* tetraploids. We did not identify any snRNA loci on chromosomes 7, 7a, or 3L, indicating a lack of genomic relation between distinct NOR and snRNA repeats (Knytl et al., 2023, 2017). In *X. calcaratus*, we mapped U2 snRNA locus, in the same position, giving an evidence of genomic link between 5S rRNA and U2 snRNA. We did not identify any snRNA loci on chromosomes 7, 7a, or 3L, indicating a lack of genomic association between distinct NOR and snRNA repeats. In chromosome 7b in tetraploids frogs belonging to *Silurana* subgenre and on chromosome 3S in tetraploids of the subgenus *Xenopus* it was assumed that the NOR locus was probably deleted (Knytl et al., 2023; Session et al., 2016). In some tetraploids, U1 and U2 loci are mapped to both homoeologs, indicating that the deletion of snRNA in a progenitor of *Xenopus* tetraploids did not occur. This suggests that all the *Xenopus* tetraploids we studied might have the double number of snRNA repeat signals compared to *X. tropicalis*, that's diploid, but with unequal copy numbers in every different subgenome. For example, in one subgenome, the copy number of snRNA repeats was reduced (11 copies of U1 snRNA on *X. laevis* chromosome 1L, compared to 20 copies on *X. tropicalis* chromosome 1) but in the other one we experienced an expansion (35 copies of U1 snRNA on *X. laevis* chromosome 1S versus 20 copies in *X. tropicalis*).

Additionally, a stable number of copies is maintained in some tandem repeats, such as the 40 copies of U2 snRNA on *X. tropicalis* chromosome 8 and *X. laevis* chromosomes 8L. These conserved copy numbers within the U2 gene align with researches that noticed higher conservation of the *X. laevis* subgenome L compared to subgenome S, relative to the *X. tropicalis* genome (Session et al., 2016). Following polyploidization, several events can occur, including the reduction, expansion, and stabilization of non-rDNA tandem repeat copy numbers, as well as the complete loss of the rRNA locus (Knytl et al., 2023; Session et al., 2016). Genes that are preserved as duplicates can follow various evolutionary trajectories. These paths may result in subfunctionalization, where each duplicate retains a portion of the original function, loss of function, or neofunctionalization, where one duplicate acquires a new function. These processes are often subject to strong purifying selection (Force et al., 1999; Lynch et al., 2001). These evolutionary processes are frequently accompanied by chromosomal rearrangements, such as intra- and interchromosomal translocations, inversions, deletions, or degenerations (Evans, 2007; Knytl et al., 2017; Sémon and Wolfe, 2007; Session et al., 2016). Our study highlighted that, in addition to translocation, inversion, deletion, and degeneration, the reduction and expansion of tandem repeat copy numbers play a crucial role in the evolutionary processes following allopolyploidization.

3.4 Genetic Divergence in wild *Hymenochirus boettgeri* and captive *Hymenochirus* sp.

Hymenochirus sp. (previously referred to as *H. boettgeri*) is not part of the *Xenopus* genus, but it shares with *X. tropicalis* a similar karyotype pattern, same chromosome number ($2n = 20$), plus a B chromosome, similar morphology, and similar G-banding patterns (Mezzasalma et al., 2015), this is not surprising, since they are both included in Pipidae family. In a previous study, another chromosome number increasing mechanism was found in this species, besides *Xenopus/Silurana* polyploidization, the addition of an accessory B chromosome (Mezzasalma et al., 2015). Our principal aim was to study cytogenetic differences between the captive population of *Hymenochirus* that can

be easily found in any pet shop, and the wild population from the north-western Congo. We used the classic cytogenetic approach to analyze chromosomes of both populations and we were able to compare the karyotypes of wild and captive *Hymenochirus*. The results confirmed that the common aquarium *Hymenochirus* and the wild one have deep differences in the chromosome asset, since the one who was caught is a tetraploid with 18 chromosomes pairs, while the captive one, is a diploid with ten chromosomes pairs, plus a B chromosome (Figure 15). After this discovery, we decided to identify the captive population as *Hymenochirus* sp. and the wild population as *Hymenochirus boettgeri*. Before our study, there was no evidence of FISH experiments conducted to investigate chromosome evolution in *Hymenochirini*. Our objective was to investigate the presence of polymorphism in chromosome number, morphology, and size. Additionally, we aimed to examine the localization of rDNA and small snDNA multigene families, including tandem repeats, and to identify any potential B chromosomes. To achieve this, we employed various cytogenetic techniques and compared the results with phylogenetic analysis to construct a phylogenetic tree.

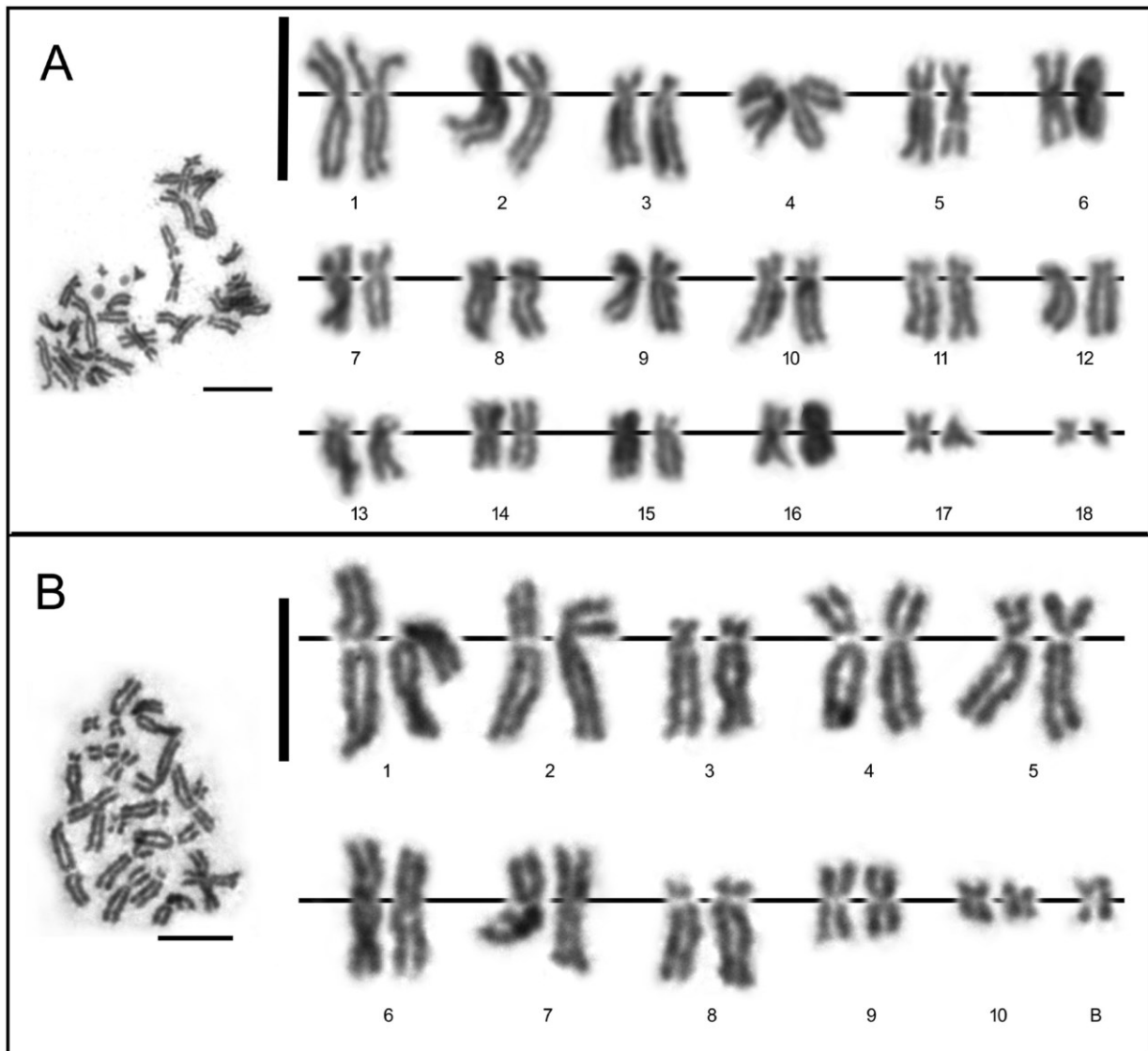


Figure 15. Karyotypes of *Hymenochirus* Species:

(A) *Hymenochirus boettgeri* (IVB-H-CG17-356, male): This karyotype displays 18 homologous chromosome pairs. Short vertical and horizontal lines correspond to the scale, which is 10 μm .

(B) *Hymenochirus* sp. (IVB-H-Hsp06, female): This karyotype shows 10 pairs of A chromosomes and one B chromosome. The long lines indicate the centromere positions, and the short lines represent the scale of 10 μm (Gvoždík et al., 2024)

We did the same cytogenetic analysis on both species. As we mentioned before, we tried to localize U1 and U2 genes with different results: while U2 loci positions are the same *H. boettgeri* and *Hymenochirus* sp. but U1 loci positions are not homologous. Unfortunately we were not able to localize 5S on *H. boettgeri* because the first attempts didn't show us any result and, unfortunately, the continuous cycle of staining and destaining on the slides, led to chromosomal structure damaging, making the detection of any signal impossible, fortunately on *Hymenochirus* sp. we didn't register this problem.

The combination of our new molecular data with the analysis of morphological differences between the two frogs, allowed us to confirm that the species caught in Congo is *H. boettgeri*. The comparison with other molecular data (Deichmann et al.; 2017; Feng et al.; 2017) let us hypothesize that a species

from Central Cameroon was very closely related to our specimen, if not even conspecific. The captive population, which is genetically very different from *H. boettgeri*, couldn't be clearly identified as any known species, based on key characteristics from previous literature (Arnoult and Lamotte, 1968; De Witte, 1930; Perret, 1966) and our data. It was most accurately referred to as *Hymenochirus* sp. However, in both literature and in the aquarium, captive dwarf clawed frogs are often incorrectly labeled as '*H. boettgeri*' (Bredeson et al., 2024; Cauret et al., 2020; Kunz, 2004, 2003; Miller et al., 2019; Sam R. Fellows, 2016) or '*Hymenochirus curtipes*' (Bewick et al., 2013; Kunz, 2002). The molecular phylogenetic analysis we performed suggested that *H. curtipes*, a species once popular in European and American aquariums, probably extinct in captivity (Kunz, 2002; Rabb and Rabb, 1963; Sokol, 1962, 1959). It was a sister lineage to *H. boettgeri* but there is no available data for this species. A thorough revision of the taxonomy of the genus *Hymenochirus* is crucial to understand and describe the relationships within the genus and to correctly identify the *Hymenochirus* frogs from captive populations. As previously noted, our cytogenetic analysis identified 36 chromosomes in *H. boettgeri* from Congo, and 20 A chromosomes along with 1 B chromosome in *Hymenochirus* sp. bred in captivity. These findings are consistent with recent studies (Mezzasalma et al., 2015), but are not consistent with an earlier study (Morescalchi, 1981, 1968; Tymowska, 1991), which reported 24 chromosomes. However the source of Morescalchi's specimen is still unidentified, and another study who registered a '*H. boettgeri*' with 22 chromosomes, provided no details. (Scheel, 1973). To account for this discrepancy, we thought that Morescalchi (1968) and/or Scheel (1973) might have karyotyped one or more different species and misled them. It is possible that they analyzed *H. curtipes*, which was present in captivity during their studies (Kunz, 2002). A possible explanation could be a different chromosome number, that's more evident in counting B chromosomes number, as is found in previously researches (Mezzasalma et al., 2015). Our finding was exceptional, since no study had ever revealed 36 chromosomes before. The chromosome number found in our *H. boettgeri* from the Congo was almost two times that of *Hymenochirus* sp. from the aquarium species, which is believed to have the primordial number of A chromosomes in pipid frogs, $2n = 20$ (Mezzasalma et al., 2015), and exactly double of chromosome number reported by Bredeson et al. (2024). The number, composition and morphology of chromosomes in our *H. boettgeri* indicate that it was either tetraploid or allotetraploid. This discovery represents the first known tetraploidism in frogs that are not part of the genus *Xenopus* (Mezzasalma et al., 2015; Schmid et al., 2015). Size of chromosomes it's another proof of polyploidism, since *H. boettgeri* are smaller than *Hymenochirus* sp. ones. It's known that cells and embryos of polyploid species are smaller than diploid ones (Miller et al., 2023). We used GISH to have another proof of polyploidy, using this technique we hybridized *Hymenochirus* sp. and *H. boettgeri* probe on *H. boettgeri* and *Hymenochirus* sp. chromosomes, and while *Hymenochirus* sp. chromosomes were fully coloured, in *H. boettgeri* some chromosomes were more painted than others (Figure 16F), resembling what we found in *X. calcaratus* (see chapter 3.1), a clear evidence of polyploidy. This might mean that one subgenome of *H. boettgeri* is more similar to *Hymenochirus* sp. while the other went through more modification during evolution, like previously seen in *X. calcaratus* (see chapter 3.1). Our hypothesis was based on a single male individual, so to confirm this, more wild animals need to be tested. Anyway, the genome size of *H. boettgeri* from the northern Democratic Republic of the Congo (DRC) is nearly double (3.77- 4.29 pg) (Liedtke et al., 2018) that reported from previous studies with captive species (2.45 pg) (King, 1990).

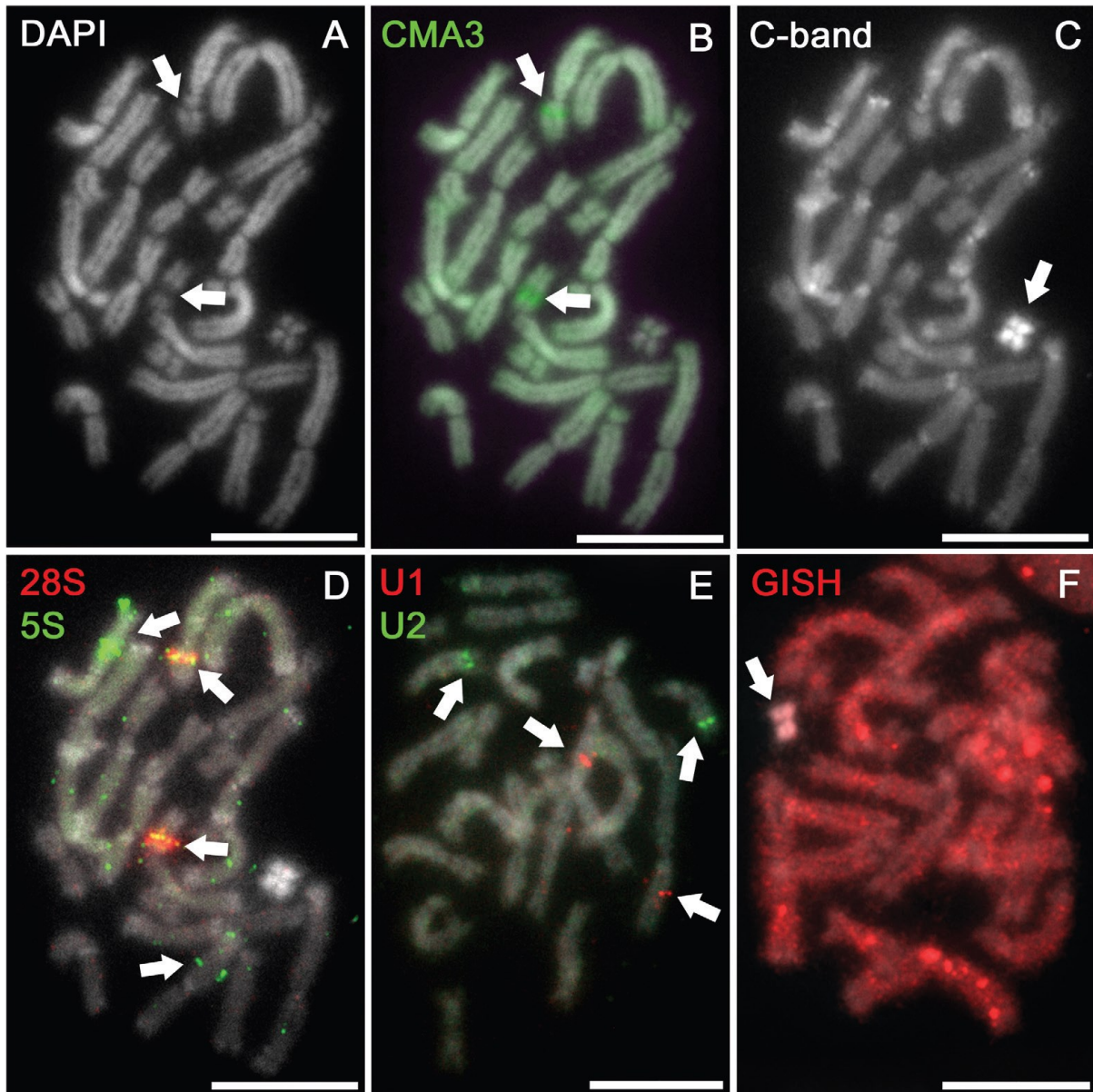


Figure 16. *Hymenochirus* sp. (IVB-H-Hsp06, female sequential fluorescent chromosome mapping using DAPI, CMA3, C-banding, and rDNA FISH, as well as non-sequential snDNA FISH and whole-genome painting on metaphase spreads. Scale bars represent 10 μm (Gvoždík et al., 2024).

We tried to give a possible explanation of the origin of polyploidy in *H. boettgeri*. *Hymenochirus* sp. is believed to have the ancestral pipid karyotype with $2n = 20$ chromosomes, plus one unique B chromosome. In contrast, the tetraploid *H. boettgeri* has 36 chromosomes, likely organized secondarily as diploid ($2n = 4x = 36$). Both species have karyotypes consisting of biarmed chromosomes, with no telocentric chromosomes. However, the evolutionary events behind the chromosome number variations in these two *Hymenochiri* species is unlikely to involve centric fusion of telocentric chromosomes or fission of a metacentric chromosome. A similar difference in chromosome numbers was observed between diploid and allotetraploid pipids in the genus *Xenopus*. We hypothesized that a similar differentiation mechanism, like the fusion between chromosome 9 and 10 that we described in *Xenopus* and *Silurana* (see chapter 1.4), may have occurred in *H. boettgeri* as well.

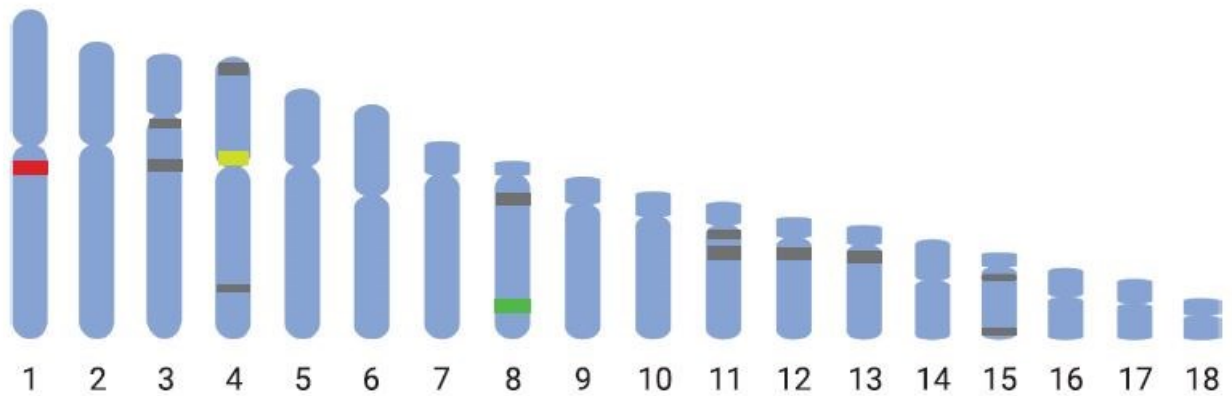
Unfortunately, their molecular phylogenetic data are poorly available and not enough to support the idea that the primordial karyotype of the family *Hymenochirini* is $2n = 20$. This is because *Hymenochirus* sp., with a karyotype with 20 A chromosomes, is for sure the basal lineage of the genus, while the close related genus *Pseudhymenochirus* has $2n = 20$ as well (Mezzasalma et al., 2015). Tetraploidization in *Hymenochirus* is likely a condition that emerged later in the differentiation of this group. However, due to insufficient information and biological samples of karyotypes of other *Hymenochirus* species, we couldn't define a more comprehensive statement about the tetraploidization and its role in the evolutionary scenario of this group. We speculated that *H. curtipes* might be a sister lineage to *H. boettgeri*, but without additional karyotype data, we cannot confirm this hypothesis. We initially thought that the karyotype of '*H. boettgeri*' with $2n = 24$, as presented by Morescalchi, might provide insights, but more information is needed (Morescalchi, 1981, 1968; Tymowska, 1991), could be a misidentified *H. curtipes*, which was available in pet aquaria in 1960s (Rabb and Rabb, 1963; Sokol, 1959; von Filek, 1967). The increased chromosome number could be resulted by fissions, as is seen in *Pipa parva* (Mezzasalma et al., 2015). In any case, this it's just an hypothesis and is unlikely to be confirmed or denied until we won't know the karyotype of *H. curtipes*. In order to understand the functional diploidy of evolutionary tetraploid *H. boettgeri*, we analyzed the number of FISH signals.

The 28S rDNA (NOR), U1, and U2 snDNA loci are located on a single chromosome, that's an evidence of rediploidization of a tetraploid ancestor. Consequently, we would expect a diploid-haploid cycle and crossing-over in tetraploid *H. boettgeri*. This phenomenon has been observed in all *Xenopus* polyploids (Knytl et al., 2023, 2017; Session et al., 2016; Tymowska, 1991). Conversely, in *Xenopus* tetraploids, the number and location of U1 and U2 snDNAs presented more variability with one or two U1 and U2 loci identified by FISH, depending on the copy number of these tandemly repeated elements on the specific chromosome (see chapter 3.2). We didn't find this variability in *Hymenochirus*.

The U1 snDNAs maintained a stable number but exhibited polymorphism in their chromosomal positions in *H. boettgeri* and *Hymenochirus* sp. In *H. boettgeri*, the U1 locus was located on the q arm of chromosome 1, whereas in *Hymenochirus* sp., it was found on the p arm of chromosome 1, differing from our description in chapter 3.2. This rearrangement in *Hymenochirus* could be due to a pericentromeric inversion of a large region or a positional change similar to transposon transposition. Another possibility is that the U1 locus might be present on both chromosome arms but with different copy numbers, which was not revealed by FISH.

The dynamic nature of the U1 locus contrasted with the stable positions of the U2 and NOR loci, which were consistently found on the telomeric part of the q arm of chromosome 8 and the pericentromeric region of the p arm of chromosome 4, respectively. We conducted karyological analysis of both male and female individuals of *Hymenochirus* sp. and male *H. boettgeri*. No differences were found in the karyotypes of the two sexes, indicating that the sex chromosomes are homomorphic. This finding aligns with previous research within the family Pipidae (Tymowska, 1991). We schematize the position of all cytogenetic analyses we did, on the figure 17, to make them more clear.

Hymenochirus boettgeri (Congo)



Hymenochirus sp. (captive population)

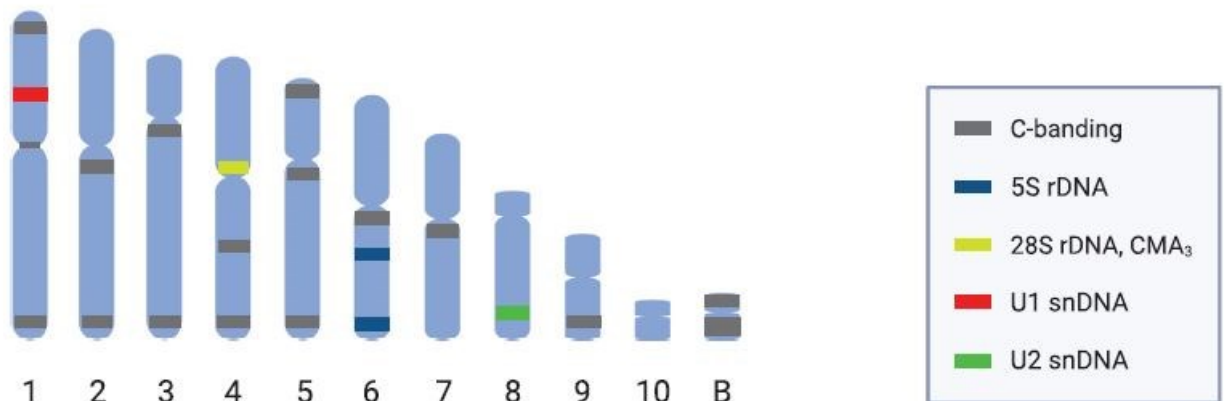


Figure 17. Schematic summarize of the shown results. Created with BioRender (“Scientific Image and Illustration Software | BioRender,” n.d.) (Gvoždík et al., 2024).

3.5 Genomic analysis of *Silurana* and *Xenopus* subgenomes

Working with African Frogs is complicated because of the difficulty of collecting living animals that must be acquired through expeditions in Africa. The animals need to survive the transport and be bred, in order to get living offspring. Moreover, the surviving rate of tadpoles is relatively low. For cytogenetic analysis we needed to cultivate cell cultures, that’s time consuming and with a high chance of failure, due to contaminants or cells death. We first tried to start the cell line using organs but this attempt failed, resulting in very frequent contamination or a low growth rate, so we started to use the hand limbs from tadpoles, with a higher success rate, but the procedure is long and needs high precision and often the final concentration of cell suspension is low, so we had to make sure we had a high initial amount of cells, but it took even more time, leading to potentially new contaminations. Working with *X. epitropicalis* was time consuming since we needed to wait for frogs to being brought from Africa, after that they were injected and, after a couple of days, they laid eggs. In order to have organs ready to be sequenced, when needed adult individuals, unfortunately the death ratio was very high and even the cell cultures were not successful.

At the moment while I'm writing this thesis, we got *X. epitropicalis* sequences, they have been already trimmed but data analysis hasn't been processed yet. *Xenopus borealis* sequences have been sent for sequencing but they are not available yet, at the moment.

After the annotation of the *X. borealis* genome was already available for studying, we noticed that both subgenomes were expressed, this means that no subgenome is silenced. Some genes were expressed mainly from only one subgenome, while some of them were expressed only in the second, however in many cases, we found expression in both genomes for the same genes.

We managed to analyze the difference between the two subgenomes gene expressions and we registered a 6-7% discrepancy.

The next steps of the research will be: check if pattern is the same among tissues or if we will found some issue-specific pattern of subgenome expression, study the the function of the genes using GO term annotation tool (Ashburner et al., 2000; Tinsley et al., 1996) and, finally, checking the alternative splicing between subgenomes.

All these steps will be replicated for *X. borealis* and *X. epitropicalis*.

These first result gave us a preliminary insight and let us confirm our hypothesis that both subgenomes were expressed, as seen with ZOO-FISH (section 3.1), but now it's too early to formulate any hypothesis but the fact we were also able to confirm the differences between the subgenomes and the evidence that we are able to distinguish them, are important steps on what we can work on outside this thesis frame.

4. Conclusions

The primary focus of this project was to investigate the evolutionary dynamics of chromosomal rearrangements within the family Pipidae. To achieve this, we analyzed members from the genera *Xenopus*, *Silurana*, and *Hymenochirus*.

Initially, we examined the dynamics of rearrangements and chromosomal morphology in the allotetraploid frog, *X. calcaratus* and how they affected the evolution of these frogs. Our findings revealed distinct characteristics in the subgenomes of *X. calcaratus*. The a-subgenome is more conserved and resembles the genome of the diploid species *X. tropicalis*, whereas the b-subgenome has differentiated more from the original progenitor and went through more modifications, showing significant differences in the structure of the chromosomes, including different repetitive sequences and heterochromatic blocks. We also discussed a specific chromosomal translocation observed in *X. mellotropicalis* but not found in *X. calcaratus*. This translocation is significant because it highlights the differences in chromosomal rearrangements between these two species. Our study proposed that a single allotetraploidization event led to the emergence of *X. mellotropicalis*, *X. epitropicalis*, and *X. calcaratus*. The translocation happened after the divergence of *X. calcaratus* but before the speciation of *X. mellotropicalis* and *X. epitropicalis*. Alternatively, if two independent allotetraploidization events occurred, probably the translocation occurred either diploid ancestor of the common one of *X. mellotropicalis* and *X. epitropicalis*, the most recent probably. This finding underscores the slow nature of genome evolution in these species and the role of chromosomal rearrangements in their divergence.

We investigated how polyploidization and divergence have influenced the evolution of repetitive elements in six species of African clawed frogs. Combining FISH and genomic data, we were able to map U1 and U2 small nuclear RNAs and histone H3 in both diploid and allotetraploid species. The results showed that the number and position of these repetitive elements were conserved in the diploid and tetraploid species *X. tropicalis* and *X. calcaratus*, both from subgenus *Silurana*, while variation was observed among the allotetraploid species from the subgenus *Xenopus*. We realized that allotetraploid species could have originated from two different independent polyploidization events that exhibited different patterns of repetitive element distribution. Younger allotetraploids, like *X. calcaratus*, have twice as many signals as their diploid relatives, whereas older allotetraploids showed more variation. These findings suggest that polyploidization initially duplicates tandem repeats, but their copy number can vary over time due to reduction and expansion, highlighting the complex evolutionary dynamics of repetitive elements in these frogs.

We began an in-depth analysis of the genome of *X. epitropicalis*. However, due to logistical constraints (waiting for the delivery of the frogs, breeding, sequencing, and analysis time), we were only able to compare our existing *X. borealis* sequence to analyze the factual expression of both subgenomes and to compare the homology between the two subgenomes, which revealed a similarity of around 93-94%. Although this preliminary data does not allow us to make definitive statements, it provides a good initial insight for the continuation of these experiments beyond this thesis frame.

Finally, we turned our attention to another frog, *H. boettgeri*. We discovered that the *Boettgeri* dwarf clawed frog from the Congo is tetraploid, possessing four sets of chromosomes ($2n = 36$). This finding suggests that the wild population is not conspecific with the captive populations, which are diploid ($2n = 20A + 1B$ chromosomes). We indicated that the karyotype of tetraploid frogs could have been evolved through the fusion of two chromosomes followed by allotetraploidization, making it functionally diploid, as seen in the polyploid frogs of *Xenopus* subgenome. These findings highlight significant differences between wild and captive populations, suggesting the need for further research to clarify the taxonomy and evolutionary history of the genus *Hymenochirus*. Furthermore, we proposed

separating the captive population, previously known as *H. boettgeri*, from the wild population, which retained the name *H. boettgeri*. We decided to refer to the captive population as *Hymenochirus* sp.

5. Accompanying sections

5.1 Comments on contributions to co-authored publications

Knytl M.; **Fornaini N.R.** Measurement of Chromosomal Arms and FISH Reveal Complex Genome Architecture

and Standardized Karyotype of Model Fish, Genus *Carassius*. *Cells*, 10, 2343, (2021).

<https://doi.org/10.3390/cells10092343>. IF₂₀₂₁=6.60. Q2 category.

In this publication I measured the length of chromosomes and cytogenetic analysis on chromosomal spreads as well as probes preparations. I also analyzed the pictures.

Knytl M, **Fornaini N.R.** , Bergelová B, Gvoždík V, Černohorská H, Kubičková S, Fokam EB, Evans BJ, Krylov V.

Divergent subgenome evolution in the allotetraploid frog *Xenopus calcaratus*, *Gene*, 2023, Volume 851, 146974.

<https://doi.org/10.1016/j.gene.2022.146974>. IF₂₀₂₃=3.68. Q2 category.

In this publication I prepared the probes for every single chromosome and for the whole genome. I conducted the painting FISH experiments as well as banding experiments. I prepared the ribosomal probes and used them for FISH experiments. I also analyzed FISH experiments and I helped in manuscript preparation.

Fornaini N.R., Bergelová B, Gvoždík V., Černohorská H., Krylov V., Kubičková S., Evans B.J., Knytl M.

Cytogenetic mapping of a repetitive DNA in selected African clawed frogs of the genus *Xenopus* (Pipidae). *Eur J*

Wildl Res 69, 81 (2023). <https://doi.org/10.1007/s10344-023-01709-8>. IF₂₀₂₃=2.24. Q2 category

I conducted the cytogenetic experiments and analysis and I designed and prepared the probes. I also analyzed FISH results and prepared the publication pictures and I participated in manuscript preparation.

Gvoždík V, Knytl M, Zassi-Boulou A.G., **Fornaini N.R.**, Bergelová B. Tetraploidy in the Boettger's dwarf clawed frog (Pipidae: *Hymenochirus boettgeri*) from the Congo indicates non-conspecificity with the captive population, *Zoological Journal of the Linnean Society*, zlad119, (2023)

<https://doi.org/10.1093/zoolin/zlad119> IF₂₀₂₃=3.83. Q1 category.

I prepared ribosomal and small nuclear probes and conducted subsequent FISH experiments. I also analyzed FISH results.

Fornaini N.R., Černohorská H., do Vale Martins L., Knytl M. Cytogenetic analysis of the fish genus *Carassius* Indicates divergence, fission and segmental duplication as drivers of tandem repeat and microchromosome evolution, *Genome Biology and Evolution*, Volume 16, Issue 3 ,evae028, (2024).

<https://doi.org/10.1093/gbe/evae028> . IF₂₀₂₃=3.2. Q2 category.

I prepared the probes for repetitive DNA FISH and I conducted the experiments. I also prepared probes for microchromosome painting FISH and I conducted the experiments. I analyzed the data and edited the pictures, I prepared the phylogenetic tree and I participated in manuscript preparation.

5.2 List of abbreviations

BLAST	Basic local alignment search tool
cept1	Choline/ethanolamine phosphotransferase 1
CMA3	Chromomycin A3
CRISPR	Clustered regularly interspaced short palindromic repeats
DAPI	4',6-diamidino-2-phenylindole
DMRT1	Doublesex and mab-3 related transcription factor 1
DNA	Deoxyribonucleic acid
F1	First filial generation
FISH	Fluorescence in situ hybridization
FISH-TSA	FISH with tyramide signal amplification
fn1	Fibronectin 1
G-banding	Giemsa banding
GISH	Genomic in situ hybridization
GO	Gene Ontology
gyg2	Glycogenin 2
H.	<i>Hymenochirus</i>
H3	Histone H3
mRNA	Messenger ribonucleic acid
Mya	Millions year ago
n	Number of chromosomes in a haploid cell
ndufs1	NADH:ubiquinone oxidoreductase core subunit S1
NOR	Nucleolus organizer region
p arm	Short chromosomal arm
q arm	Long chromosomal arm
RAG1	Recombination activating gene 1
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
rRNA	Ribosomal ribonucleic acid
sf3b1	Splicing factor 3b subunit 1
snRNA	Small nuclear ribonucleic acid
Te	Transposable element
U1	Small nuclear 1
U2	Small nuclear 2
WCP	Whole chromosome painting probe
WGS	Whole genome sequence
X.	<i>Xenopus</i>
XCA	<i>Xenopus calcaratus</i>
XME	<i>Xenopus mellotropicalis</i>
XTR	<i>Xenopus tropicalis</i>
ZOO-FISH	Cross-species chromosome painting

5.3 Reference list

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6. Publications

Paper 1: Measurement of Chromosomal Arms and FISH Reveal Complex Genome Architecture and Standardized Karyotype of Model Fish, Genus *Carassius*



Article

Measurement of Chromosomal Arms and FISH Reveal Complex Genome Architecture and Standardized Karyotype of Model Fish, Genus *Carassius*

Martin Knytl * and Nicola Reinaldo Fornaini

Department of Cell Biology, Faculty of Science, Charles University, 12843 Prague, Czech Republic; fornaini@natur.cuni.cz;

* Correspondence: martin.knytl@natur.cuni.cz

Abstract: The widely distributed ray-finned fish genus *Carassius* is very well known due to its unique biological characteristics such as polyploidy, clonality, and/or interspecies hybridization. These biological characteristics have enabled *Carassius* species to be successfully widespread over relatively short period of evolutionary time. Therefore, this fish model deserves to be the center of attention in the research field. Some studies have already described the *Carassius* karyotype, but results are inconsistent in the number of morphological categories for individual chromosomes. We investigated three focal species: *Carassius auratus*, *C. carassius* and *C. gibelio* with the aim to describe their standardized diploid karyotypes, and to study their evolutionary relationships using cytogenetic tools. We measured length ($q + p$ length) of each chromosome and calculated centromeric index (i value). We found: (i) The relationship between $q + p$ length and i value showed higher similarity of *C. auratus* and *C. carassius*. (ii) The variability of i value within each chromosome expressed by means of the first quartile (Q_1) up to the third quartile (Q_3) showed higher similarity of *C. carassius* and *C. gibelio*. (iii) The fluorescent in situ hybridization (FISH) analysis revealed higher similarity of *C. auratus* and *C. gibelio*. (iv) Standardized karyotype formula described using median value (Q_2) showed differentiation among all investigated species: *C. auratus* had 24 metacentric (m), 40 submetacentric (sm), 2 subtelocentric (st), 2 acrocentric (a) and 32 telocentric (T) chromosomes ($24m + 40sm + 2st + 2a + 32T$); *C. carassius*: $16m + 34sm + 8st + 42T$; and *C. gibelio*: $16m + 22sm + 10st + 2a + 50T$. (v) We developed R scripts applicable for the description of standardized karyotype for any other species. The diverse results indicated unprecedented complex genomic and chromosomal architecture in the genus *Carassius* probably influenced by its unique biological characteristics which make the study of evolutionary relationships more difficult than it has been originally postulated.

Keywords: chromosome; karyogram; in situ hybridization; i value; q/p arm ratio; *Carassius auratus*; *Carassius carassius*; *Carassius gibelio*



Citation: Knytl, M.; Fornaini, N.R. Measurement of Chromosomal Arms and FISH Reveal Complex Genome Architecture and Standardized Karyotype of Model Fish, Genus *Carassius*. *Cells* **2021**, *10*, 2343. <https://doi.org/10.3390/cells10092343>

Academic Editor: Smina Mukhtar

Received: 1 August 2021

Accepted: 29 August 2021

Published: 7 September 2021

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1. Introduction

Karyotype analysis is a fundamental approach by which chromosomes are arranged into homologous pairs with a respect to certain morphological categories. In the broader sense, the term karyotype is also referred to as a complete number of chromosomes describing typical taxon, species, biotype or individual [1]. Homologous chromosome pairs and morphological categories are determined based on the ratio of the long (q) and short (p) arm and the position of the centromere (centromeric index, i value). If the centromere is situated in median, submedian, subterminal or terminal region of the chromosome, the morphological category might be designated as metacentric (M , m), submetacentric (sm), subtelocentric (st) or acrocentric (a)/telocentric (t , T), respectively [2]. Two edge points that are located on median and terminal position sensu stricto have centromeric index 50 (assigned as M) and 0 (assigned as T chromosomes), respectively [2]. In order to maximize the diagnostic information obtainable from a chromosome preparation, the

precise arrangement of individual chromosomes within a single image might be used as a standardized format. The term karyogram can be also used for a graphical depiction of chromosome complements [3,4].

Knowledge of the karyotype is necessary in (cyto)genetics, or related fields, in order to study chromosomal rearrangements and abnormalities [5,6], the identification of sex chromosomes [7,8], and/or chromosome-specific genes [9]. Although chromosomal changes, (i.e., chromosomal losses, duplications, rearrangements), are strictly linked to a certain locus, without the knowledge of detailed karyotype, it is not possible to precisely identify chromosomal aberration, mutation or syndrome [10,11]. Heteromorphic sex chromosomes (morphologically distinct in male or female individuals) can be distinguished by classic cytogenetic procedures based on Giemsa-staining karyogram of both sexes and/or by chromosome painting and fluorescent in situ hybridization (FISH) [12,13]. Cytogenetic mapping and localization of single-copy gene regions in the genome generally reveal both intra- and interchromosomal rearrangements (i.e., inversions, insertions, deletions or duplications) determined using karyograms [9]. Moreover, gene loci, which have not yet been mapped within a genome, can be assigned to a specific chromosome within a standardized karyogram [14].

In some model vertebrate species, a low/moderate number of chromosomes, meaning up to approximately 50 chromosomes, of relatively large-size allows for easy identification of each chromosome according to q/p arm ratio and centromeric index [15]. Each chromosome is usually assigned a numeric code, necessary in evolutionary studies pertaining to patterns of orthologous chromosomes between species. Orthologous chromosomes are inferred to be descended from the same ancestral chromosome separated by a speciation event. This concept is also referred to as shared synteny. In a non-model organism with a high number of relatively small chromosomes, identification of chromosomes into categories are inconsistent (e.g., Knytl et al. [16] vs. Kobayasi et al. [17] vs. Ojima and Takai [18]), as is that case with cyprinid fish from the genus *Carassius*. Although a large number of studies describing *Carassius* karyotype, there is no study proposing a standardized karyogram based on exact measurements of the difference between q and p chromosome arm, arm ratio and centromeric index in any *Carassius* species.

The genus *Carassius* belongs to the monophyletic paleotetraploid tribe, Cyprinini sensu stricto [19], within the family Cyprinidae (ray-finned fishes, Teleostei). Several species have been described within the genus *Carassius* and three of them are widely used in cytogenetic research: (i) *Carassius carassius* (Crucian carp), native and threaten in many European countries [20,21], is diploid with chromosome number $2n = 4x = 100$ [17], where n refers to the number of chromosomes in each gamete of extant species, and x refers to the number of chromosomes in a gamete of the most recent diploid ancestor of the extant species. Most members of the family Cyprinidae contain 25 chromosomes in each gamete, considered as the most recent diploid ancestral state of extant *Carassius* [22]. (ii) *Carassius auratus* (goldfish), well known for its colourful varieties, bizarre shapes of body and formation of domesticated and feral populations [23]. (iii) *Carassius gibelio* (Silver Prussian carp) has recently spread throughout most continental waters, as a result of relocation from native habitats most likely by humans [24–27]. In addition, *Carassius auratus* and *C. gibelio* form diploid ($2n = 4x = 100$), triploid ($3n = 6x \approx 150$), and tetraploid biotypes ($4n = 8x \approx 200$) [28–30].

Here we described standardized karyotype of diploid *Carassius* species with 100 chromosomes (*C. auratus*, *C. carassius* and *C. gibelio*) based on measuring of chromosomal $q + p$ arm length, q/p arm ratio, and i value. We performed FISH experiments with ribosomal probes, and analysed values of $q + p$ length and i in order to characterize the inter-species differences between three *Carassius* species, which commonly co-occur in European waters (*C. carassius* and *C. gibelio*), and bred/distributed worldwide as part of the pet trade (*C. auratus*).

2. Materials and Methods

2.1. Fish Sampling and Origin

Carassius auratus were obtained via the aquarium trade (transported to Czech Republic from Israel). *Carassius carassius* was captured in the Elbe River basin closed to the city Lysá nad Labem, Czech Republic [16,31] and in small pond in Helsinki, Finland [32]. *Carassius gibelio* originated from the Dyje Rive basin, South Moravia, Czech Republic [28].

2.2. Chromosome Analysis

Both males and females of each species (*C. auratus*, *C. carassius*, *C. gibelio*) were used for karyotype analysis. In *C. auratus*, mitotic activity was stimulated by intraperitoneal injection of 0.1% CoCl₂ [32]. Somatic chromosomal complements were synchronized in metaphase of cell mitotic division using 0.1% colchicine (Sigma, St. Louis, MO, USA). Chromosome suspension was generated from the cephalic kidney [33] and stored in fixative solution (methanol: acetic acid, 3:1) at −20 °C, before being observed on a glass slide and stained with 5% Giemsa solution in 1x PBS. The chromosome suspensions used in this study have been used in prior analyses of *C. carassius* and *C. gibelio* [16,28,31,32] meaning that chromosomal suspensions were stored 3–7 years in −20 °C. Chromosomal suspensions were spun in a centrifuge, and fresh methanol and acetic acid were added every three months to maintain the constant ratio of methanol and acetic acid in fixative solution.

2.3. Measurements of *q* + *p* Chromosomal Arm Length

Photos of mitotic metaphase were taken using a Leica DFC 7000T camera and Leica DM6 microscope equipped with a EL6000 (metal halide) fluorescence illumination. A total of ten high-quality photos were taken for each species, five male and five female metaphases, from which measurements of the length of *q* and *p* chromosomal arms were taken. The centromere of each chromosome was identified as the narrowest part of a chromosome (Figure S2). Both arms of each chromatid (i.e., long arm 1 (*q*1), long arm 2 (*q*2), short arm 1 (*p*1), short arm 2 (*p*2)) were measured using ImageJ (V 1.53i) [34]. Chromosomal length (*length*), difference between *q* and *p* arm (*d*), *q*/*p* arm ratio (*r*) and centromeric index (*i*) were calculated according following formulas adopted from Levan et al. [2]:

$$p = \frac{p1 + p2}{2} \quad q = \frac{q1 + q2}{2} \quad (1)$$

$$length = p + q \quad d = q - p \quad r = \frac{q}{p} \quad i = \frac{100}{r + 1} \quad (2)$$

Measurements of *p*, *q*, *length*, *d*, *r*, and *i* were calculated as values in pixels from each image/metaphase and were further analyzed using R software for statistical computing (V 4.1.0) [35] and RStudio environment (V 1.4.1717) [36]. The *i* value was selected as a crucial characteristics for karyotypic analysis because it generally ranges from 0 to 50 [2]. The *r* value generally ranges from 1 to ∞ and thus this value is not suitable for graphical expression of results. The *length* and *i* value were used for calculation of an arithmetic mean of *length* (*mean_length*) and an arithmetic mean of *i* (*mean_i*) separately for each *Carassius* species. The *mean_length* and *mean_i* of each species were plotted (plot function in R).

2.4. Standardized *Carassius* Karyotype

Two highest *i* values (one chromosomal pair) were dissected from each of the metaphases (ten metaphases in total) and put into additional data frame as a numeric vector. This group of *i* values were identified as chromosome 1 (hereafter chr1). The third and fourth highest *i* values were dissected from each metaphase and identified as chr2 and so on up to two chromosomes with the lowest *i* values identified as chr50. This function() is named `Select_chrome` and shown in the supplemental results. All *i* values of each identified chromosome were plotted (boxplot function in R) separately in

each species. The default interquartile range 1.5 was used for elimination of extreme values (errors). Output value ($\$stats$) was called in R. The i values within the the minimum (Q_0), first quartile (Q_1), median (Q_2), third quartile (Q_3) and maximum (Q_4) were investigated in detail, especially if some i values of each individual chromosome were shared among focal *Carassius* species. Chromosomal categories (M , m , sm , st , a and T) were determined according Q_2 of i values in each *Carassius* species. A morphological category of each chromosome was determined according chromosomal nomenclature Levan et al [2]. The following Table 1 shows boundaries between each morphological category:

Table 1. Chromosomal nomenclature used for determination of chromosomal categories according to Levan et al. [2].

Centromeric Position	Arm Ratio	Centromeric Index	Chromosome Category
median sensu stricto	1.00	50	M (metacentric sensu stricto)
median	1.01–1.70	49.9–37.51	m (metacentric)
submedian	1.71–3.00	37.50–25.01	sm (submetacentric)
subterminal	3.01–7.00	25.00–12.51	st (subtelocentric)
terminal	>7.01	12.50–0.01	a/t (acro-/telocentric)
terminal sensu stricto	∞	0	T (telocentric sensu stricto)

2.5. Preparation of 5S and 28S Ribosomal Probes

New 5S PCR primers 5S_F (5'-CAGGGTGGTATGGCCGTAGG-3') and 5S_R (5'-AGCGCCGATCTCGTCTGAT-3') were designed according to the 5S gene of the western clawed frog, *Xenopus tropicalis*. The *X. tropicalis* genomic sequence is available on Xenbase, accessed on 11 June 2020 (<http://www.xenbase.org>). The 28S primers 28S_A (5'-AAACTCTG GTGGAGGTCCGT-3') and 28S_B (5'-CTTACCAAAGTGGCCCACTA-3') used in this study were designed by Naito et al. [37]. Total genomic DNA (gDNA) was extracted from pectoral fin tissue using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. *Carassius gibelio* and *X. tropicalis* gDNA were used as a template for 5S and 28S PCR amplification, respectively. Primers were made by Integrated DNA Technologies (Coralville, IA, USA). The PPP Master Mix (Top-Bio, Prague, Czech Republic) was used for efficient amplification of ribosomal gene fragments. The temperature profile for the non-labelling amplification of the 5S and 28S loci followed Top-Bio instructions: initial denaturation step for 1 min at 94 °C, followed by 35 cycles (94 °C for 15 s, 53 °C for 15 s and 72 °C for 40 s) and a final extension step at 72 °C for 7 min. The obtained PCR products were separated on a 1.25% agarose gel with TA buffer and extracted from the gel using MicroElute Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's instructions. The PCR amplicons were sequenced and mapped using blastn algorithm for finding out of the locus- and species-specificity of amplification. Subsequently, the 5S and 28S ribosomal DNA (rDNA) loci were indirectly labelled by Digoxigenin-11-dUTP (Roche, Mannheim, Germany) and Biotin-16-dUTP (Jena Bioscience, Jena, Germany), respectively, by PCR reaction again. Taq DNA polymerase (Top-Bio) was used for labelling instead of The PPP Master Mix which was used for non-labelling amplification. Conditions for labelling PCR of the 5S and 28S loci were adopted from Sember et al. [38] and slightly modified as follows: initial denaturation step for 3 min at 94 °C, followed by 30 cycles (94 °C for 30 s, 53 °C for 30 s and 72 °C for 40 s) with final extension step at 72 °C for 10 min. The PCR product was separated on a 1% agarose gel with TBE buffer and purified using E.Z.N.A. Cycle Pure Kit (Omega Bio-tek) according to manufacturer's instructions.

2.6. Fluorescent In Situ Hybridization

Cell suspensions prepared from each *Carassius* species were spread onto clean microscopic slides, which were subsequently used for FISH on the same day. The 5S probe from *C. gibelio* was used for chromosomal spreads of *C. auratus*, *C. carassius* and *C. gibelio*.

The 28S probe from *X. tropicalis* was used for *C. auratus*, *C. carassius* and *C. gibelio* chromosomal spreads. In total 44 μ l of the hybridization mixture containing 100 ng of either 5S or 28S rDNA probe, 50% deionized formamide, 2x SSC, 10% dextran sulphate and water was placed on a slide and covered with a 24 \times 50 mm coverslip. Both probe and chromosomal DNA were denatured in a PCR machine with special block for slides at 70 °C for 5 min [9]. Hybridization, post-hybridization washing, blocking reaction and visualization of 5S and 28S rDNA signals were carried out as described in Knytl et al. [39]. The Digoxigenin-11-dUTP/Biotin-16-dUTP labelled probe was detected by Anti-Digoxigenin-Fluorescein (Roche)/CYTM3-Streptavidin (Invitrogen, Camarillo, CA, USA), respectively, diluted according to manufacturer's instructions. Chromosomes were counterstained with ProLongTM Diamond Antifade Mountant with DAPI (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). At least 20 metaphase spreads in total were analysed per individual.

3. Results

3.1. Karyotype Analysis

Number of chromosomes in all studied species (*C. auratus*, *C. carassius* and *C. gibelio*) was uniform ($2n = 4x = 100$). We did not find any differences in chromosomal morphology between male and female individuals. This finding confirmed homomorphic sex chromosomes at least in diploid biotypes of the genus *Carassius*.

3.2. Interspecies Variability Based on Chromosomal Length

Detailed description of R analysis including R scripts are given in Supplementary Results. All steps outlining how the measured values were calculated and processed into tables and plots are shown on the *C. auratus* dataset. The *mean_i* and *mean_length* of each individual chromosome were plotted onto x axis and y axis, respectively, (Figure 1). Morphological chromosomal categories *m*, *sm*, *st*, *a* and *T* are present in each species. Extreme category, such as *T*, is represented by 19, 26, and 25 chromosomes of *C. auratus*, *C. carassius* and *C. gibelio*, respectively (i.e., 19, 26, and 25 points lie on gray dashed vertical lines of interval 0). The *T* chromosomes have small *mean_length*, approaching 0. Other chromosomal categories, such as *M*, are not represented in any species—no *i* value reached 50, with the highest *i* of 48.778 found in chromosome 1 of *C. gibelio*. Chromosomal length of *C. auratus*, *C. carassius*, and *C. gibelio* range from 23.340–40.614, 26.799–44.092, and 15.205–29.496, respectively. It is evident that chromosomes of *C. gibelio* are generally smaller (mean of *q + p* length was 21.36) than those of both *C. auratus* (31.53) and *C. carassius* (35.05). For the minimum of errors we calculated *mean_length* and *mean_i* from a group of ten measured *q + p* values, i.e., each dot on Figure 1 is arithmetic mean resulted from ten measured *q + p* values. Potential influences on chromosomal length are discussed (Section 4).

3.3. Interspecies Variability Based on Centromeric Index Linked to Individual Chromosomes of the Whole Chromosomal Complement, and Standardized *Carassius* Karyotype

The *i* values were assigned to each individual chromosome by dissection of chr1–50 from each metaphase (also Section 2.4, R protocol is described in the section of R analysis of Supplementary Results) and plotted onto x and y axis (Figure 2). Each box depicts a group of chromosomes dependent on *i* values ordered from most metacentric chromosomes on the left side of plots (chr1) to most telocentric chromosomes on the right side of plots (chr50). In general, each species has six to seven chromosomes that were highly variable in *i*, i.e., chr31–37 in *C. auratus*, chr27–33 in *C. carassius* and chr23–29 in *C. gibelio*. The *Q*₁ and *Q*₃ cover *i* values from 0 to 29.342 in *C. auratus*, from 0 to 23.9 in *C. carassius* and from 0 to 25.747 in *C. gibelio*. *Carassius auratus* has the highest variability of *i* value.

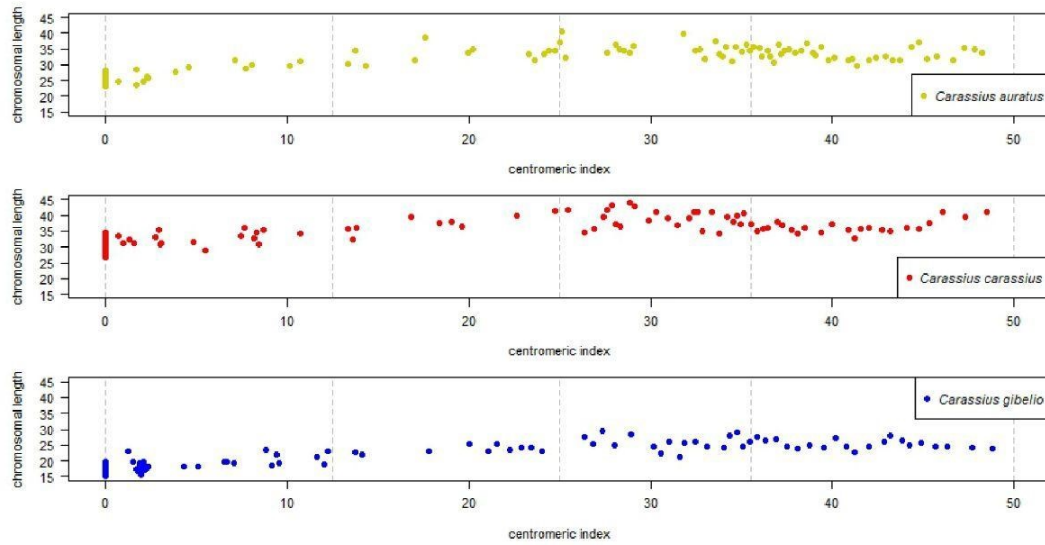


Figure 1. Relationship between centromeric index (i) and chromosomal length ($length$). Top plot shows chromosomes of *Carassius auratus* in yellow, middle plot shows chromosomes of *C. carassius* in red and bottom plot shows chromosomes of *C. gibelio* in blue. Chromosomal categories are bounded by gray dashed vertical lines which define intervals 0–12.5, 12.5–25, 25–37.5 and 37.5–50 corresponding to acrocentric (a), subtelocentric (st), submetacentric (sm) and metacentric (m) chromosomes, respectively. Both plotted i value and $length$ are presented as an arithmetic mean of each chromosome.

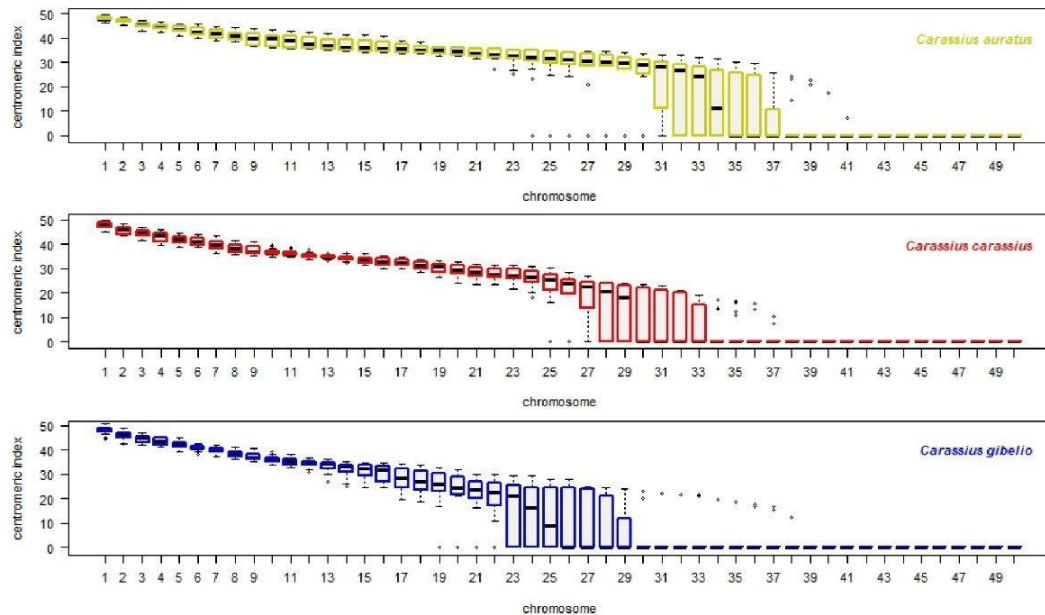


Figure 2. Intrachromosomal variability of i displayed on a whole chromosomal complement. Top plot shows haploid chromosomal complement (50 chromosomes) of *C. auratus* in yellow, middle plot shows 50 chromosomes of *C. carassius* in red and bottom plot shows 50 chromosomes of *C. gibelio* in blue. Each chromosome is linked to i value (y axis). Upper and lower whiskers show extreme values, the minimum (Q_0) and maximum (Q_4), respectively, boxes involve the first quartile (Q_1) and the third quartile (Q_3) group of values. Black line within box indicates *median* value of the dataset (Q_2). Outliers (errors) are drawn as black points.

If some i values are shared among *C. auratus*, *C. carassius* and *C. gibelio*, the range of i values of each individual chromosome (chr1–50) from ten metaphases were compared with the range of i values of other two orthologs of each of other two species (ten metaphases from each species). The Q_1 – Q_3 range of i value within chr1 of *C. auratus* was compared with the Q_1 – Q_3 range of i value within chr1 of *C. carassius* and *C. gibelio*, similarly the Q_1 – Q_3 range of i value within chr2 of *C. auratus* was compared with the Q_1 – Q_3 range of i value of *C. carassius* and *C. gibelio* etc. up to the Q_1 – Q_3 range of i value within chr50 of *C. auratus*, *C. carassius* and *C. gibelio*. As an additional analysis, multi-plot with 50 separate box plots (chr1–50) was generated, each box plot was composed of orthologous chromosomes of all three species (Figure 3).

Based on i value (Figure 1 and 3), we concluded that:

1. Each orthologous chromosome of *C. carassius* and *C. gibelio* shared i values within Q_1 – Q_3 range and therefore we consider that karyotypes of *C. carassius* and *C. gibelio* to be more similar based on i value.
2. Chromosomes 12–30 of *C. auratus* were represented by different i values within Q_1 – Q_3 range those of *C. carassius* and *C. gibelio*. Therefore, we consider the karyotypes of *C. auratus* to be most variable for this parameter.
3. Chromosomes 8, 11 and 31 of *C. auratus* were represented by different i values within Q_1 – Q_3 range those of *C. gibelio* but *C. carassius* shared i values in these chromosomes with both *C. auratus* and *C. gibelio*.

The Q_2 range (median) of i value was used for determination of a standardized karyotype formula. Chromosome1 of *C. auratus*, *C. carassius* and *C. gibelio* with median of i value 47.87 (CAU_median_i column of Table 2), 48.28 (CCA_median_i) and 48.55 (CGI_median_i), respectively, were identified as m chromosomes in all three *Carassius* species (CAU_category, CCA_category, CGI_category columns of Table 2). Karyotypes all three *Carassius* species were different in the number of chromosomal categories sensu Levan et al. [2]. The number of chromosomes in categories found out by arithmetic mean (Figure 1) slightly differed from the number of chromosomes in categories determined by Q_2 range (Figure 2). The Q_2 range eliminated errors and thus we inferred standardized karyotype formula according to the Q_2 range.

$$\text{Carassius auratus} : 24m + 40sm + 2st + 2a + 32T \quad (3)$$

$$\text{Carassius carassius} : 16m + 34sm + 8st + 42T \quad (4)$$

$$\text{Carassius gibelio} : 16m + 22sm + 10st + 2a + 50T \quad (5)$$

3.4. Fluorescent In Situ Hybridization with 5S and 28S Ribosomal Probes

The PCR amplification of 28S and 5S rDNA locus resulted consistently in approximately 300 bp long fragments. Searches, using the blastn algorithm, confirmed the locus and species-specificity of each amplicon: 100% identity with 28S rRNA of *X. tropicalis* (accession numbers XR_004223792–XR_004223798), 95% identity with sequence of 5S rRNA of *C. gibelio* (accession number DQ659261). The amplified 5S rDNA sequence was deposited to the NCBI/GenBank database (accession number BankIt2492026 5s, MZ927820). Mapping of the 5S and 28S loci showed different patterns in the number and position within each investigated *Carassius* species (Figure 4). No differences between males and females were detected. The $q + p$ arms of the FISH images were measured on chromosomes that bear positive rDNA loci because the FISH protocol involves denaturation step after which chromosomal structure is disrupted due to high temperature. The rDNA positive loci are mostly accumulated at the pericentromeric chromosomal region in *Carassius* and another cyprinid fishes [32,40,41] and thus the identification of the centromere and measurement of the $q + p$ arms are precise on rDNA positive chromosomes. The i value of rDNA positive chromosomes (arrows on Figure 4) were assigned to the closest i value of the standardized karyotype from Table 2.

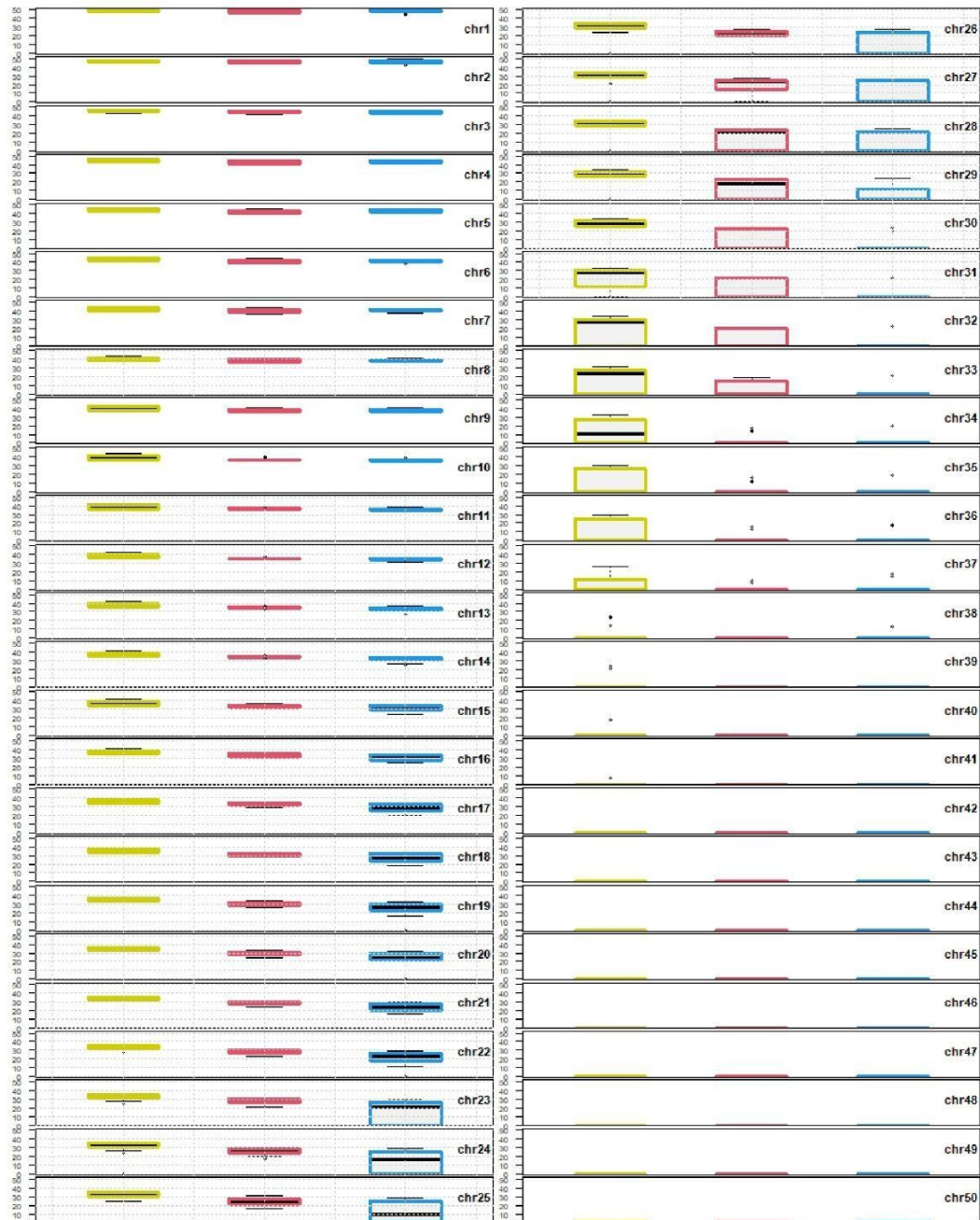


Figure 3. Box plots composed of orthologous chromosomes of each species. Chromosomes are ordered decreasingly according to the *i* value. Chromosomes on the top left of the figure are the most metacentric, chromosome 1 (chr1), chromosomes on the bottom right of the figure are the most telocentric (chr50). Yellow, red and blue boxes represents *C. auratus*, *C. carassius* and *C. gibelio*, respectively. Each chromosome is linked to *i* value (y axis). Upper (Q_0) and lower (Q_4) whiskers show extreme values, boxes involve Q_1 and Q_3 group of values. The black line within boxes indicates median value of the dataset (Q_2). Outliers (errors) are drawn as black points. The grid in some box plots represents significantly different *i* values within the Q_1 – Q_3 range. Box plots without the grid share some *i* values within the Q_1 – Q_3 range.

Table 2. Table of values used for determination of standardized karyotype. Each median (Q_2) of i values corresponds to certain chromosomal category of chr1–50. The medians of i value are sorted decreasingly. CAU = *C. auratus*, CCA = *C. carassius*, CGI = *C. gibelio*, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric, T = telocentric sensu stricto.

	CAU_median_i	CCA_median_i	CGI_median_i	CAU_category	CCA_category	CGI_category
chromosome1	47.87	48.28	48.55	m	m	m
chromosome2	47.07	45.68	46.12	m	m	m
chromosome3	45.74	44.74	44.99	m	m	m
chromosome4	44.81	43.52	43.47	m	m	m
chromosome5	43.46	42.11	42.53	m	m	m
chromosome6	42.43	41.08	40.89	m	m	m
chromosome7	41.63	39.58	39.89	m	m	m
chromosome8	40.84	37.90	38.42	m	m	m
chromosome9	40.13	36.81	36.91	m	sm	sm
chromosome10	39.67	36.17	35.84	m	sm	sm
chromosome11	38.75	35.79	35.41	m	sm	sm
chromosome12	37.65	35.19	34.73	m	sm	sm
chromosome13	36.77	34.86	34.25	sm	sm	sm
chromosome14	36.19	34.36	33.36	sm	sm	sm
chromosome15	35.88	33.66	32.61	sm	sm	sm
chromosome16	35.71	32.70	31.90	sm	sm	sm
chromosome17	35.48	32.22	28.62	sm	sm	sm
chromosome18	35.21	31.23	26.86	sm	sm	sm
chromosome19	34.87	30.54	26.07	sm	sm	sm
chromosome20	34.33	29.11	24.42	sm	sm	st
chromosome21	33.45	28.54	23.45	sm	sm	st
chromosome22	33.03	27.41	22.83	sm	sm	st
chromosome23	32.78	26.86	21.37	sm	sm	st
chromosome24	31.95	26.25	16.52	sm	sm	st
chromosome25	31.41	25.22	9.19	sm	sm	a
chromosome26	31.08	24.00	0.00	sm	st	T
chromosome27	30.70	22.57	0.00	sm	st	T
chromosome28	30.11	20.62	0.00	sm	st	T
chromosome29	29.75	18.13	0.00	sm	st	T
chromosome30	28.96	0.00	0.00	sm	T	T
chromosome31	28.17	0.00	0.00	sm	T	T
chromosome32	26.87	0.00	0.00	sm	T	T
chromosome33	24.41	0.00	0.00	st	T	T
chromosome34	11.19	0.00	0.00	a	T	T
chromosome35	0.00	0.00	0.00	T	T	T
chromosome36	0.00	0.00	0.00	T	T	T
chromosome37	0.00	0.00	0.00	T	T	T
chromosome38	0.00	0.00	0.00	T	T	T
chromosome39	0.00	0.00	0.00	T	T	T
chromosome40	0.00	0.00	0.00	T	T	T
chromosome41	0.00	0.00	0.00	T	T	T
chromosome42	0.00	0.00	0.00	T	T	T
chromosome43	0.00	0.00	0.00	T	T	T
chromosome44	0.00	0.00	0.00	T	T	T
chromosome45	0.00	0.00	0.00	T	T	T
chromosome46	0.00	0.00	0.00	T	T	T
chromosome47	0.00	0.00	0.00	T	T	T
chromosome48	0.00	0.00	0.00	T	T	T
chromosome49	0.00	0.00	0.00	T	T	T
chromosome50	0.00	0.00	0.00	T	T	T

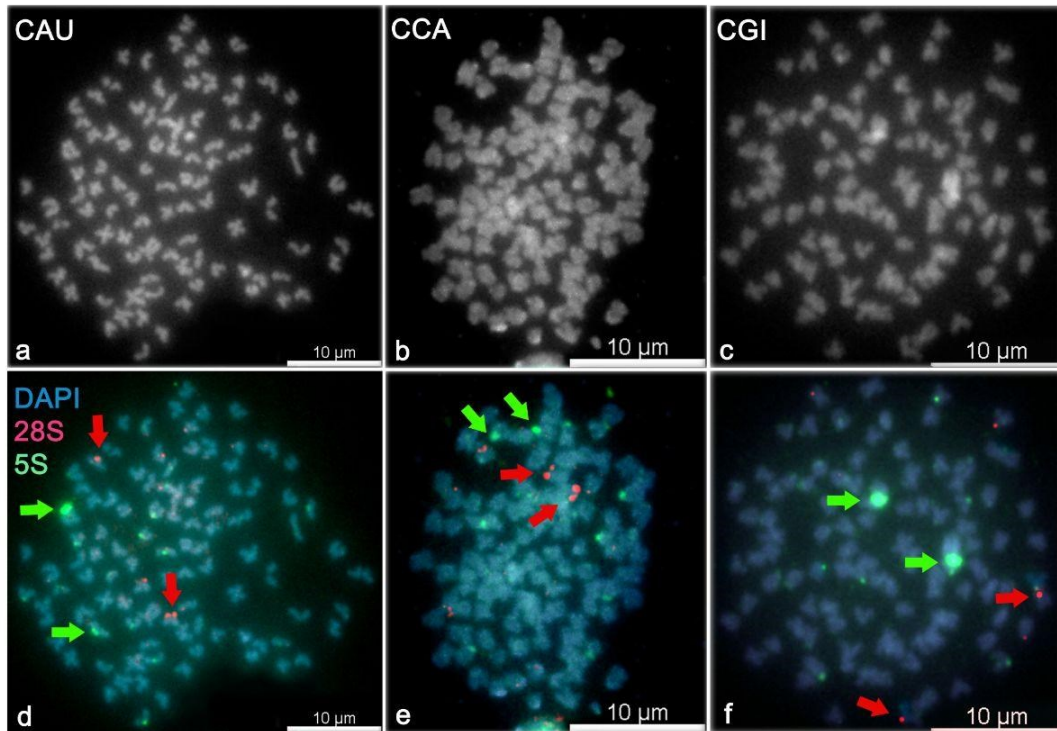


Figure 4. Double-colour fluorescent *in situ* hybridization with 5S and 28S ribosomal probes. DAPI-counterstained metaphase spreads show 100 chromosomes (B&W) in (a) *C. auratus* (CAU), (b) *C. carassius* (CCA) and (c) *C. gibelio* (CGI). The 5S (green) probe shows two more intensive (strong) signals and eight, six and eight less intensive (weak) signals in (d) *C. auratus*, (e) *C. carassius* and (f) *C. gibelio*, respectively. The 28S rDNA probe (red) reveals two strong signals and two, four and two weak signals in (d) *C. auratus*, (e) *C. carassius* and (f) *C. gibelio*, respectively. Green and red arrows correspond to 5S and 28S ribosomal loci, respectively.

Summary of 5S rDNA (corresponding to non-nucleolar region). We found:

- Two more intensive (strong) 5S rDNA signals at the *p* arm of *sm* chromosomes (chr28, *i* value = 30.06) and eight weak signals in *C. auratus*.
- Two strong 5S rDNA signals at the *p* arm of *sm* chromosomes (chr19, *i* value = 30.60) and six weak signals in *C. carassius*.
- Two strong 5S rDNA signals at the *p* arm of *sm* chromosomes (chr17, *i* value = 30.00) and eight weak signals in *C. gibelio*.

Summary of 28S rDNA (corresponding to nucleolar organizer region). We found:

- two strong 28S rDNA signals at the *p* arm of two *sm* chromosomes (chr16, *i* value = 35.69) and two weak signals in *C. auratus*.
- two strong 28S rDNA signals at the *p* arm of two *sm* chromosomes (chr13, *i* value = 34.72) and four weak signals in *C. carassius*.
- two strong 28S rDNA signals at the pericentromeric region of two *T* chromosomes (in the range of chr26–50, *i* value = 0) and two weak 28S rDNA signals in *C. gibelio*.

4. Discussion

Diploid vs. polyploid biotypes—sexual vs. asexual reproduction—genetic vs. environmental sex determination: All these natural phenomena make *Carassius* a valuable experimental model for evolutionary studies. The study of these aforementioned unique characteristics of *Carassius* require basic cytogenetic techniques such as chromosome prepa-

rations and/or karyotype. Diploid biotypes of the genus *Carassius* have a karyotype consisting of 100 chromosomes (e.g., [28], this study), with some exceptions, i.e., 50, 94, 98, 102 or 104 chromosomes [22,42–46]. Karyotype formula of diploid *Carassius* is inconsistently defined by several authors (Table 3) and $q + p$ arms have not been measured. An application of a technique of measurement, and the following determination of standardized karyotype is needed in order to clear up genome architecture and its evolution.

Table 3. Previously published karyotypes of diploid *C. auratus*, *C. carassius* and *C. gibelio* including information about sex and locality of the investigated individuals. NA = information not available, F = female, M = male.

Karyotype	Sex	Locality	References
<i>C. auratus</i>			
$2n = 94$	NA	Japan	[42,47]
$2n = 96-104$	F, M	NA	[48]
$2n = 100(12m + 36sm + 52st-a)$	F, M	Japan	[49,50]
$2n = 104(46m + 16sm + 42a)$	F, M	NA	[22]
$2n = 104(20m + 72sm-st + 12a)$	NA	NA	[44]
$2n = 100(20m + 40sm + 40a)$	F, M	NA	[17]
$2n = 100(16m + 84sm-a)$	NA	NA	[51]
$2n = 100(12m + 36sm + 52st-a)$	F, M	China	[18,52,53]
$2n = 100(22m + 30sm + 48st-a)$	F, M	China	[54–56]
<i>C. carassius</i>			
$2n = 104(20m + 72sm-st + 12a)$	NA	NA	[44]
$2n = 100(20m + 40sm + 40a)$	F, M	Netherlands	[17,57–59]
$2n = 100(20m + 44sm + 36a)$	NA	France	[60]
$2n = 100$	F, M	Bosnia	[61]
$2n = 50(20m + 12sm + 18st-a)$	NA	Romania	[45]
$2n = 100$	F, M	Czech Republic	[62]
$2n = 100(20m + 36sm + 44st-a)$	F, M	Czech Republic	[16,31]
$2n = 100(20m + 36sm + 44st-a)$	F, M	Poland	[40]
$2n = 100(20m + 36sm + 44st-a)$	M	Finland	[32]
<i>C. gibelio</i>			
$2n = 94$	F, M	Belarus	[43]
$2n = 100(20m + 40sm + 40a)$	NA	River Amur	[63]
$2n = 98(48m-st + 50a)$	NA	Romania	[45]
$2n = 102(24m + 36sm-st + 42a)$	F	Yugoslavia	[46]
$2n = 104(24m + 36sm-st + 44a)$	M	Yugoslavia	[46]
$2n = 100(14m + 24sm + 62st-a)$	F, M	Poland	[64]
$2n = 100(26m + 38sm + 36st-a)$	F, M	Poland	[65]

We generated a revised karyotype as a result of novel measurement and using statistical programming we described standardized karyotype of three species (*C. auratus*, *C. carassius* and *C. gibelio*). We measured the length of each individual chromosome of each species and identified each chromosome using i value. Groups of i values were divided into quartiles Q_0 – Q_4 . Our results based on median (Q_2) of i value for each chromosome revealed three different karyotypes; not one of these three karyotypes corresponded to any of the previously published karyotypes from Table 3. The karyotype of *C. auratus* had $24m$, $40sm$, $2st$, $2a$ and $32T$ chromosomes (shortened formula $24m + 40sm + 36st-T$). The karyotype of *C. carassius* had $16m$, $34sm$, $8st$ and $42T$ chromosomes (shortened formula $16m + 34sm + 50st-T$). The karyotype of *C. gibelio* possessed $16m$, $22sm$, $10st$, $2a$ and $50T$ chromosomes (shortened formula $16m + 22sm + 62st-T$). These standardized karyotypes are likely highly reproducible and could be applied to the reconstruction of cytogenetic maps, comparative cytogenetics and genomics, or cytotaxonomy and karyosystematics. The designed R scripts (Supplementary Results) could be applied to the description of

standardized karyotype for another species with relatively high number of chromosomes similar to that of *Carassius*.

In the wider range of i values (Q_1 – Q_3) for each chromosome, we found karyotypes of *C. carassius* and *C. gibelio* more similar, as both *C. carassius* and *C. gibelio* shared some i values within Q_1 – Q_3 range of corresponding orthologous counterparts. The i values in Q_1 – Q_3 of chr8 and chr11–31 in *C. auratus* were significantly different those of i values in Q_1 – Q_3 of *C. carassius* and *C. gibelio* (Figures 2 and 3). This finding indicates higher divergence of *C. auratus* karyotype based on i value. The i value divergence might be supported by distinct origin of the samples used in this investigation—*C. auratus* used in this study was imported from Israel and was domesticated in ancient China [23]. Both *C. carassius* and *C. gibelio* used in this study originated from Europe and mostly from the Czech Republic (only four individuals of *C. carassius* were caught in Finland). A phylogenetic study of Asian–European *Carassius* suggests a closer evolutionary relationship exists between diploid *C. auratus* and diploid *C. gibelio* compared to *C. carassius* and diploid *C. auratus/gibelio* [66]. Thus, phylogenetic distance does not reflect i value divergence in *C. carassius*, as demonstrated in this study. The higher similarity between *C. carassius* and *C. gibelio* i values could be caused by hybridization, as previously confirmed by molecular genetic and cytogenetic tools in several European *Carassius* populations [16,67,68]. Although, we can not exclude a hybrid origin for diploid *Carassius* individuals.

In addition, female meiotic drive might also affect i values [69]. Chromosomes of particular morphology might be preferentially transmitted to the egg during meiosis and the chromosomal complement of new offspring can be rapidly changed. Interestingly, polyploid *Carassius* biotypes have variable numbers of microchromosomes [16] which results in odd or variable chromosome numbers within polyploid *Carassius* [28]. The uniform chromosome numbers, we have described in diploid *Carassius*, are in accordance with expectations that female meiotic drive has never been shown in diploid *Carassius*.

We also showed a relationship between the mean of i value and the mean of $q + p$ chromosomal length. Chromosomes of *C. gibelio* had smaller size (mean of chromosomal length was 21.36) those of *C. auratus* (31.53) and *C. carassius* (35.05, Figure 1). This difference in chromosomal length in *Carassius* might be promoted by altered chromatin spiralisation and condensation during cell cycle, especially during interphase and mitosis [70–72]. In this study, chromosomes were synchronized in metaphase by colchicine but some chromosomes can be fixed in prometaphase, early metaphase or late metaphase and we assume that slight differences in chromosomal length can be present in different *Carassius* species. The length of chromosomes might theoretically be influenced by storage time if the ratio or concentration of acetic acid and methanol is slightly changed [72]. To avoid these inaccuracies and preserving the chromosome suspension, we used a fixative solution with an acetic acid : methanol ratio of 1:3. This ratio was restored through the addition of fresh acetic acid and methanol. In addition, we statistically processed metaphases with different storage times, for which we found no difference in chromosome length. For the case of standardized karyotype, all previous negative effects that influence $q + p$ arm length can be discounted because even if the length of chromosome had changed, the position of the centromere remains identical, thus i values never change [2]. In addition, the i value errors were eliminated through analysis of the median Q_2 range.

The correct order of chromosomes in *Carassius* karyograms remains difficult to determine. For our purposes, we arranged chromosomes according to decreasing i values. As such, chr1 has the highest i value and chr50 has the lowest i value. Using this approach describes a shared synteny of i value among *C. auratus*, *C. carassius* and *C. gibelio* (Figure 3). This method of numbering of chromosomes according i value in *Carassius* can be modified in future studies such as the numbering in human decreasingly according to $q + p$ chromosomal length [73].

FISH analysis revealed two large non-nucleolar 5S loci and two large nucleolar 28S ribosomal loci in each of the *Carassius* species that were investigated; this indicates functional diploidy in evolutionary tetraploid biotype. Two strong rDNA signals were found

in other studies which described *Carassius* karyotype [32,40,74]. The number of 5S rDNA loci (strong and weak signals in total) ranged from eight to ten, and 28S rDNA loci ranged from four to six, with a single chromosome-bearing rDNA locus differing in each *Carassius* species. We found higher similarity in a number of rDNA loci in *C. auratus* and *C. gibelio*, i.e., ten 5S and four 28S rDNA signals in these two species. *Carassius carassius* possessed eight 5s and six 28S rDNA signals. Knytl et al. [32] identified 18 5S and four 28S rDNA loci in diploid *C. carassius* originating from Finland waters. Spoz et al. [40] pointed out ten 5S and four 28S rDNA loci in *C. carassius* from Poland. Chinese *C. auratus* has 2–8 5S rDNA signals [74]. This study brought the first report of the FISH analysis used on diploid European *C. gibelio*, which belongs to phylogenetically very diverse taxonomic group [66]. Our results showed variability in the number and position of ribosomal tandem repeats in *Carassius* which is in accordance with other rDNA studies [32,40,74]. This variability in rDNA loci has been shown between species of the same genus [38] and even between individuals of the same species for 18S and 28S loci [75,76]. The present variability of rDNA loci can be attributed to degree in heterochromatin condensation and nucleolar activity during mitosis [77].

Overall, we have described a standardized karyotype of three species of *Carassius* genus: *C. auratus*, *C. carassius* and *C. gibelio*. We compared these three species using cytogenetic tools: The arithmetic mean of the length of $q + p$ chromosomal arm showed higher similarity of *C. auratus* and *C. carassius*, and a difference of *C. gibelio* (Figure 1). Analysis of median of the i value showed higher similarity of *C. carassius* and *C. gibelio*, and higher difference of *C. auratus* (Figures 2 and 3). FISH confirmed a higher similarity of *C. auratus* to *C. gibelio*, compared to *C. carassius* (Figure 4). Thus, we can conclude that the genus *Carassius* has a very complex cytogenetic background, which can be distinguished using cytogenetic tools, with some inconsistencies in measuring propinquity and congeniality. The findings presented here are consistent with the presence of great genome variability and plasticity. Such genome variability could be attributed to the occurrence of rare natural phenomena such as interspecies hybridization, polyploidization, and/or alternation of reproduction modes within the genus *Carassius* [16,43,78,79].

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/cells10092343/s1>, Figure S1: Giemsa-stained chromosome metaphases (B&W) used for long (q) and short (p) chromosome arms measurement. The Figure shows one representative metaphase for each sex of each species. Each chromosome has a working numeric code 1–100. Metaphases show 100 chromosomes in (a) *Carassius auratus* (CAU) male, (b) *C. auratus* female, (c) *C. carassius* (CCA) male, (d) *C. carassius* female, (e) *C. gibelio* (CGI) male and (f) *C. gibelio* female, white circles in (f) indicate centromeric regions assigned as the narrowest part of chromosome. Figure S2: Giemsa-stained chromosome dissected from metaphase. Graphical example of the measurement of metacentric chromosome with four chromosomal arms (two chromatids). Two thin lines which lead through all four arms cross in the centromeric region—the narrowest part of chromosome. Long arm 1 ($q1$), long arm 2 ($q2$), short arm 1 ($p1$), short arm 2 ($p2$) have 14.8, 13.6, 12.6 and 12.4 pixels, respectively. Calculation of the $q + p$ arm length is shown at the top right of the figure. Table S1: *Carassius auratus* $q + p$ lengths were dissected from each metaphase and added into separate table. The last column (*mean_length*) shows arithmetic mean of length used for dot plot (Figure 1). Table S2: *Carassius auratus* i values were dissected from each metaphase and added into separate table. The last column (*mean_i*) shows arithmetic mean of the i value used for dot plot (Figure 1).

Author Contributions: Conceptualization, M.K.; methodology, M.K. and N.R.F.; software, M.K.; validation, M.K.; formal analysis, M.K.; investigation, M.K. and N.R.F.; data curation, M.K.; writing—original draft preparation, M.K.; writing—review and editing, M.K., N.R.F.; visualization, M.K.; supervision, M.K.; project administration, M.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: All experimental procedures involving fish were approved by the Institutional Animal Care and Use Committee of the Czech Academy of Sciences, Institute of

Animal Physiology and Genetics, according to the directives from the State Veterinary Administration of the Czech Republic, permit number 124/2009, and by the permit number CZ.00221 issued by the Ministry of Agriculture of the Czech Republic. M.K. is a holder of the Certificate of professional competence to design experiments according to §15d(3) of the Czech Republic Act No. 246/1992 coll. on the Protection of Animals against Cruelty (Registration number CZ 03973), provided by the Ministry of Agriculture of the Czech Republic.

Informed Consent Statement: Not applicable.

Data Availability Statement: Sanger sequencing data are available on-line at the NCBI database, accessed on 26 August 2021 (<https://www.ncbi.nlm.nih.gov/>). All data generated by R Studio are not presented in this study and they may be available on request from the corresponding author. All steps generating data frames and plots in R were summarized on-line on GitHub, accessed on 26 August 2021 (<https://www.github.com/>) available on request. Basic $q + p$ arm measurements can be provided in the form of RData file as R workspace, also upon request.

Acknowledgments: We are very grateful to Evans lab (Department of Biology, McMaster University, Canada) which helped us with the basis of computational programming in R (namely Ben J. Evans, Caroline M.S. Cauret and Xue-Ying Song). We thank very much Adrian Forsythe (Department of Zoology, Evolutionary Biology Center, Uppsala University, Sweden) for English correction. We thank Abhishek Koladiya (Department of Immunomonitoring and Flow Cytometry, Institute of Hematology and Blood Transfusion, Czech Republic) for improvement R scripts. Furthermore, we thank MDPI Production Team (especially Liu and Jing) for figuring out L^AT_EX issues, and Editorial Board of Cells for quick and positive feedback.

Conflicts of Interest: The authors declare no conflict of interest.

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Research paper

Divergent subgenome evolution in the allotetraploid frog *Xenopus calcaratus*

Martin Knytl^{a,*}, Nicola R. Fornaini^a, Barbora Bergelová^a, Václav Gvoždík^b,
Halina Černohorská^c, Svatava Kubíčková^c, Eric B. Fokam^d, Ben J. Evans^{e,1}, Vladimír Krylov^{a,1}

^a Department of Cell Biology, Faculty of Science, Charles University, Viničná 7, Prague 12843, Czech Republic

^b Institute of Vertebrate Biology of the Czech Academy of Sciences, Brno, Czech Republic

^c Department of Genetics and Reproduction, CEITEC - Veterinary Research Institute, Hudcova 296/70, Brno 62100, Czech Republic

^d Department of Animal Biology and Conservation, University of Buea, PO Box 63, Buea 00237, Cameroon

^e Department of Biology, McMaster University, 1280 Main Street West, Hamilton L8S4K1, Ontario, Canada

ARTICLE INFO

Edited by Andre van Wijnen

Keywords:

Genome
Chromosome
Cytogenetics
FISH
Chromosome length
Allopolyploidization

ABSTRACT

Allopolyploid genomes are divided into compartments called subgenomes that are derived from lower ploidy ancestors. In African clawed frogs of the subgenus *Xenopus* (genus *Xenopus*), allotetraploid species have two subgenomes (L and S) with morphologically distinct homoeologous chromosomes. In allotetraploid species of the sister subgenus *Silurana*, independently evolved subgenomes also exist, but their cytogenetics has not been investigated in detail. We used a diverse suite of cytogenetic and molecular FISH techniques on an allotetraploid species in *Silurana*—*Xenopus calcaratus*—to explore evolutionary dynamics of chromosome morphology and rearrangements. We find that the subgenomes of *X. calcaratus* have distinctive characteristics, with a more conserved a-subgenome resembling the closely related genome of the diploid species *X. tropicalis*, and a more rapidly evolving b-subgenome having more pronounced changes in chromosome structure, including diverged heterochromatic blocks, repetitive sequences, and deletion of a nucleolar secondary constriction. Based on these cytogenetic differences, we propose a chromosome nomenclature for *X. calcaratus* that may apply to other allotetraploids in subgenus *Silurana*, depending on as yet unresolved details of their evolutionary origins. These findings highlight the potential for large-scale asymmetry in subgenome evolution following allopolyploidization.

1. Introduction

Whole genome duplication (polyploidization) and large-scale chromosomal rearrangements are important evolutionary driving forces that contribute to genome variability, for example by creating duplicate genes and affecting patterns of recombination (Wolfe, 2001). Polyploidization is a potential mode of sympatric speciation wherein reproductive isolation from progenitor species is achieved via inviability or sterility of offspring of a cross between parents of differing ploidy levels (Janko et al., 2018). Polyploidization occurs via genome duplication within a single species (autopolyploidization) or in association with hybridization between two or more distinct species (allopolyploidization). Divergence between homoeologous chromosomes could expedite rediploidization of a polyploid genome, wherein chromosomes acquire disomic inheritance, with each having only one homologous partner during meiosis. This has the effect of creating separate genomic

compartments called "subgenomes"; in allopolyploid genomes, each subgenome is derived mostly or entirely from a different ancestral species (Schiavinato et al., 2021).

Chromosomal changes such as translocations, inversions, insertions, and deletions are divided into small- and large-scale rearrangements. Small-scale rearrangements involve regions smaller than one megabase. Large-scale rearrangements affect regions larger than one megabase, may include fusions and fissions of different chromosomes, and may alter karyotype organization and gene expression. In any genome, the frequency of chromosomal rearrangements is governed by several factors including presence of retrotransposons in genome (Biémont and Vieira, 2006), spatial variation in DNA stability and fragility, and external factors such as radiation or environmental conditions (Feder et al., 2011). Within a polyploid genome, the frequency of rearrangements may differ between subgenomes (Brunet et al., 2006; Session et al., 2016; Li et al., 2021).

* Corresponding author.

E-mail address: martin.knytl@natur.cuni.cz (M. Knytl).

¹ B.J. Evans and V. Krylov are joint last authors.

<https://doi.org/10.1016/j.gene.2022.146974>

Received 23 July 2022; Received in revised form 30 September 2022; Accepted 11 October 2022

Available online 27 October 2022

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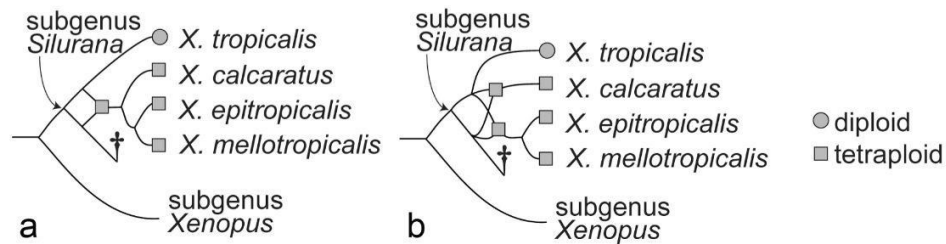


Fig. 1. Phylogenetic scenarios of the subgenus *Silurana*. A sister clade, subgenus *Xenopus*, is depicted as an outgroup. (a) A scenario involving one allotetraploidization via the fusion of two diploid ancestors, one of which was closely related to *X. tropicalis* and the other of which went extinct (dagger), to give rise to the most recent common ancestor of all three allotetraploid species in subgenus *Silurana*. (b) An alternative scenario involving two independent allotetraploidization events; one gave rise to the ancestor of *X. calcaratus* and the other to the most recent common ancestor of *X. epitropicalis* and *X. mellotropicalis*.

1.1. Allopolyploid genomes of African clawed frogs (*Xenopus*)

African clawed frogs, genus *Xenopus* (family Pipidae), are divided into subgenera *Xenopus* and *Silurana*, that include diploid and tetraploid species in *Silurana*, and tetraploid, octoploid, and dodecaploid species in *Xenopus* (Tymowska, 1991; Evans, 2008; Evans et al., 2015). This high species diversity (29 species in total) with a range of ploidy levels makes *Xenopus* a compelling focal group for studying cytogenetics and chromosomal rearrangements in the wake of polyploidization, with the potential to provide insights into whether and how genomic rearrangements are linked to speciation and adaptation.

Available information from extant tetraploids, octoploids, and dodecaploids indicates that they are allopolyploids rather than autopolyploids (Evans et al., 2005; Evans et al., 2015; Session et al., 2016). For example, in *X. laevis* (subgenus *Xenopus*), each subgenome contains different transposable element complements that originate from distinct ancestors (Session et al., 2016).

In subgenus *Xenopus*, several large-scale rearrangements have been identified. The largest involves a fusion between chromosomes 9 and 10 in a diploid ancestor of allopolyploids in subgenus *Xenopus* (Session et al., 2016). Comparison to a high-quality genome assembly of the diploid species *X. tropicalis* evidences several other rearrangements within the *X. laevis* S-subgenome and comparative genomic stability of the *X. laevis* L-subgenome (Session et al., 2016).

In *Silurana* tetraploids, divergence between the homoeologous a- and b-subgenomes is higher than between the a-subgenome and the genome of diploid *X. tropicalis* (Evans, 2008; Evans et al., 2015), as expected by allotetraploidy. In *Silurana*, one large-scale rearrangement has been identified—a nonreciprocal interchromosomal translocation of pericentromeric region between chromosomes 9b and 2a of the tetraploid species *X. mellotropicalis* (sensu Knytl et al., 2017; Knytl et al., 2018b). This rearrangement was identified using fluorescent in situ hybridization (FISH) in which whole chromosome painting (WCP) probes from *X. tropicalis* were hybridized to chromosomes of *X. mellotropicalis* (Zoo-FISH).

It remains unknown whether this rearrangement occurred in the ancestor of all three allotetraploid species in *Silurana* (*X. mellotropicalis*, *X. epitropicalis*, *X. calcaratus*) or more recently after the ancestor of *X. mellotropicalis* diverged from the ancestor(s) of the other species (Fig. 1). This is because evolutionary relationships among species in subgenus *Silurana* remain poorly resolved; very little genomic data has been collected from *X. calcaratus* and *X. epitropicalis* that could be leveraged for phylogenetic estimation along with genomic data from other *Silurana* species (Hellsten et al., 2010; Cauret et al., 2020). Available information from two tightly linked genes (*RAG1* and *RAG2*) suggest that two separate allotetraploidization events occurred in *Silurana*: one generated *X. calcaratus* and the other resulted in the ancestor of *X. mellotropicalis* and *X. epitropicalis* (Evans et al., 2005; Evans, 2007) (Fig. 1b). However, a simpler scenario is also possible

where allotetraploidization occurred only once in *Silurana* (Fig. 1a). Also lacking are cytogenetic characters that can clearly distinguish homoeologous chromosomes of the *Silurana* subgenomes.

Large-scale genomic rearrangements occurred multiple times in genus *Xenopus* and may have been influential in the evolution of *Xenopus*. In this study, we examined cytogenetic evolution of *X. calcaratus* with an aim of better understanding genome evolution in this species and also to further contextualize evolution of the interchromosomal translocation in *X. mellotropicalis* (Knytl et al., 2017). We used a suite of chromosome banding and FISH techniques and statistically processed chromosomal measurement information to provide unprecedented resolution of the karyotype of the allotetraploid Biafran clawed frog, *X. calcaratus*.

2. Methods

2.1. Establishment of primary cell cultures

Primary cell cultures were derived from the hind limb of tadpoles at stage NF55(±1) (Sinzelle et al., 2012) of *X. calcaratus* originated from Bakingili, Cameroon, and *X. tropicalis*, derived from an Ivory Coast laboratory strain. Both species were bred at Charles University, Faculty of Science, Prague, Czech Republic. Briefly, tadpoles were anesthetized by 0.4% MS-222 (Sigma–Aldrich, St. Louis, MO, USA) and then washed with sterile MilliQ water following euthanasia. The hind limbs were removed and homogenized in cultivation medium which was prepared from components as described in Knytl et al. (2017) and modified by addition of Gibco™ Antibiotic–Antimycotic (100X) and 0.1 mM Gibco™ 2-mercaptoethanol (both Thermo Fisher Scientific, Waltham, MA, USA). The explants were then cultivated at 29.5°C with 5.5% CO₂ for five days without disturbance. The medium was then changed every second day. The first and next passages were performed with trypsin-ethylenediaminetetraacetic acid according to Knytl et al. (2017). For cryopreservation, cell aliquots were stored at –80°C in the cultivation medium with the addition of 10% Dimethyl sulfoxid (Sigma–Aldrich).

2.2. Preparation of chromosomal suspension and metaphase spreads

Both *X. tropicalis* and *X. calcaratus* chromosomal suspensions were prepared according to Krylov et al. (2010) and stored in fixative solution (methanol:acetic acid, 3:1, v/v) at –20°C. For laser microdissection, a fresh metaphase suspension was dropped onto a polyethylenephthalene membrane. For cytogenetic analysis, a chromosome suspension was dropped onto a microscope slide according to Courtet et al. (2001). Chromosome preparations were aged at –20°C for at least one week with the exception of that for FISH with tyramide signal amplification, FISH-TSA, in which the chromosome suspension was dropped and directly followed by further experimental procedures.

For each experiment, mitotic metaphase spreads were

counterstained with ProLong™ Diamond Antifade Mountant with the fluorescent 4',6-diamidino-2-phenylindole, DAPI stain (Invitrogen by Thermo Fisher Scientific). Ten to 20 metaphase spreads were analyzed per each banding technique and probe. Microscopy and processing of metaphase images using Leica Microsystem (Wetzlar, Germany) were conducted as detailed in Seroussi et al. (2019).

2.3. Laser microdissection and whole chromosome painting

All ten individual chromosomes from *X. tropicalis* (20 copies of each chromosome) were separately isolated by laser microdissection as previously described in Kubickova et al. (2002) using a PALM Microlaser system (Carl Zeiss MicroImaging GmbH, Munich, Germany). The WCP probes were prepared according to Krylov et al. (2010) with the following modifications in labeling. During whole genome amplification, digoxigenin-11-dUTP and biotin-16-dUTP (both Jena Bioscience, Jena, Germany) were incorporated into the probes (the nucleotide ratio for digoxigenated probes: 10 mM dATP, dGTP, dCTP: 6.5 mM dTTP, biotinylated probes: 10 mM dATP, dGTP, dCTP: 5 mM dTTP). Control double-colour intraspecies painting FISH on *X. tropicalis* and single-colour cross-species Zoo-FISH on *X. calcaratus* chromosomes were carried out as described by Krylov et al. (2010) with minor modifications as detailed in Knytl et al. (2017). Autoclaved *X. tropicalis* genomic DNA was used as a competitor (blocking DNA) according to Bi and Bogart (2006). In the control double-colour painting FISH, the digoxigenin and biotin labeled probe was detected by anti-digoxigenin-fluorescein (Roche, Basel, Switzerland) and CyTM3-streptavidin (Invitrogen, Camarillo, CA, USA), respectively, diluted with blocking reagents as used in double-colour FISH with ribosomal probes in Knytl et al. (2017). In single-colour Zoo-FISH, fluorescent signal of digoxigenin labeled probes was visualized by anti-digoxigenin-rhodamine (Roche).

2.4. Whole genome painting

Xenopus tropicalis genomic DNA (gDNA) was used as a probe for genomic in situ hybridization (GISH) experiments. Whole genome painting (WGP) probes were prepared using the GenomePlex Single Cell Whole Genome Amplification Kit (WGA4), Sigma–Aldrich, according to the manufacturer's whole genome amplification protocol with extracted gDNA. GenomePlex WGA Reamplification Kit (WGA3), Sigma–Aldrich, and labeling with digoxigenin-11-dUTP (Jena Bioscience) was carried out as described in Krylov et al. (2010). A combination of salmon sperm (Knytl et al., 2013b) and autoclaved *X. tropicalis* gDNA (Bi and Bogart, 2006) was used as a competitor DNA. Control GISH was performed on *X. tropicalis* chromosomes as detailed in painting FISH in Krylov et al. (2010), and cross-species GISH was carried out on *X. calcaratus* chromosomes as detailed in Zoo-FISH in Krylov et al. (2010) with minor changes described in Knytl et al. (2017).

2.5. Ribosomal gene mapping and chromosome banding

Double-colour FISH was performed with 5S and 28S ribosomal DNA probes (rDNA FISH), followed by two chromosome banding techniques that were conducted sequentially on the same metaphase spread: C-banding, and then Chromomycin A₃, CMA₃ (Sigma–Aldrich).

Xenopus calcaratus gDNA was used as a template for amplification of both 5S and 28S loci. Total gDNA was extracted from the tail tissue of a tadpole using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. 5S and 28S primer sequences (Integrated DNA Technologies, Coralville, IA, USA) are listed in Table S1. Preparation of 5S and 28S probes including modified PCR conditions (Sember et al., 2015) and labelling with digoxigenin-11-dUTP and biotin-16-dUTP (both Jena Bioscience) is detailed in Knytl and Fornaini (2021). The 5S and 28S probes were hybridized with chromosomal spreads of *X. calcaratus*. In total 22 µL of the hybridization mixture containing 100 ng of either 5S or 28S rDNA probe, and 14 µL

master mix (10% dextran sulfate) was placed on a slide and covered with a 22 × 22 mm coverslip. Both probe and chromosomal DNA were denatured at 72°C for 5 min with subsequent overnight hybridization in a dark wet chamber. Post-hybridization washing and blocking reactions were performed as described for painting FISH in Krylov et al. (2010). Probe signal was visualized as described in Knytl et al. (2017).

The sequential chromosome banding protocol (C-banding, CMA₃) followed Rábová et al. (2015), with the modifications described in Knytl et al. (2017).

2.6. Single-copy gene mapping

We set out to assess whether the *X. mellotropicalis* translocation between chromosomes 9b and 2a (sensu Knytl et al., 2017), with the nomenclature of 2a revised to be chromosome 2b as detailed below in Section 4.2, also was present in *X. calcaratus*. To accomplish this, mapping analysis was performed in *X. calcaratus* for five single-copy genes that flank the translocated region (rearrangement-associated genes) in *X. mellotropicalis* as revealed by Zoo-FISH and FISH-TSA (Knytl et al., 2017; Knytl et al., 2018b). Two of these genes are situated on the short (p) arm of the *X. tropicalis* chromosome 2 (XTR 2) (glycogenin 2, *gyg2* and choline/ethanolamine phosphotransferase1, *cept1*) and three are on the long (q) arm of XTR 9 (splicing factor 3b subunit 1, *sf3b1*, NADH: ubiquinone oxidoreductase core subunit S1, *ndufs1*, and fibronectin 1, *fn1*) (Uno et al., 2012; Seifertova et al., 2013; Knytl et al., 2018b). The same probes from Knytl et al. (2018b) (*gyg2*, *cept1*, *sf3b1*, *ndufs1*, and *fn1*) genes prepared from *X. mellotropicalis* RNA were used and labelled with digoxigenin-11-dUTP (Roche). The FISH-TSA protocol was adapted from Krylov et al. (2007) with minor modifications described in Knytl et al. (2018b). Sequences for alpha and beta single-copy gene loci (*gyg2*, *cept1*, *sf3b1*, *ndufs1*, and *fn1*) that were used as probes for FISH-TSA are deposited in the NCBI GenBank database (Knytl et al., 2018b). Primer sequences are listed in Table S1.

2.7. Measurement and identification of *X. calcaratus* chromosomes

A total of 35 *X. calcaratus* individual metaphase figures were analyzed. Half of the metaphases selected for the identification of individual chromosomes was stained with 5% Giemsa/PBS solution (v/v). The rest were stained with DAPI during FISH experiments. Both arms of each chromatid were measured in pixels using ImageJ, V 1.53i (Schneider et al., 2012). The p and q arm lengths were quantified as described in Knytl and Fornaini (2021). To identify each chromosome, we analyzed chromosomal length (l), p/q arm ratio (r₁) (Tymowska, 1973), centromeric index (i), and q/p arm ratio (r₂) (Levan et al., 1964).

$$l = p + q \quad r_1 = \frac{p}{q} \quad r_2 = \frac{q}{p} \quad i = \frac{100}{r_2 + 1} \quad (1)$$

Chromosomal numbering was adopted from Knytl et al. (2017) with proposed revisions detailed below. Data were analyzed in R software for statistical computing, V 4.1.0 (R Core Team, 2020). One-way analysis of variance (one-way ANOVA) was performed to compare l and i values between each homoeologous pair of chromosomes using R scripts modified from Knytl and Fornaini (2021). All steps outlining how the measured values were calculated and processed into tables and plots are available on <https://github.com/martinknytl/R>.

3. Results

3.1. Species identification

Species of *Xenopus* frogs are notoriously difficult to diagnose based on external anatomy. We therefore used Sanger sequencing of our experimental individual for species diagnosis. PCR amplifications of portions of the 5S and 28S nuclear rRNA genes, and the 16S

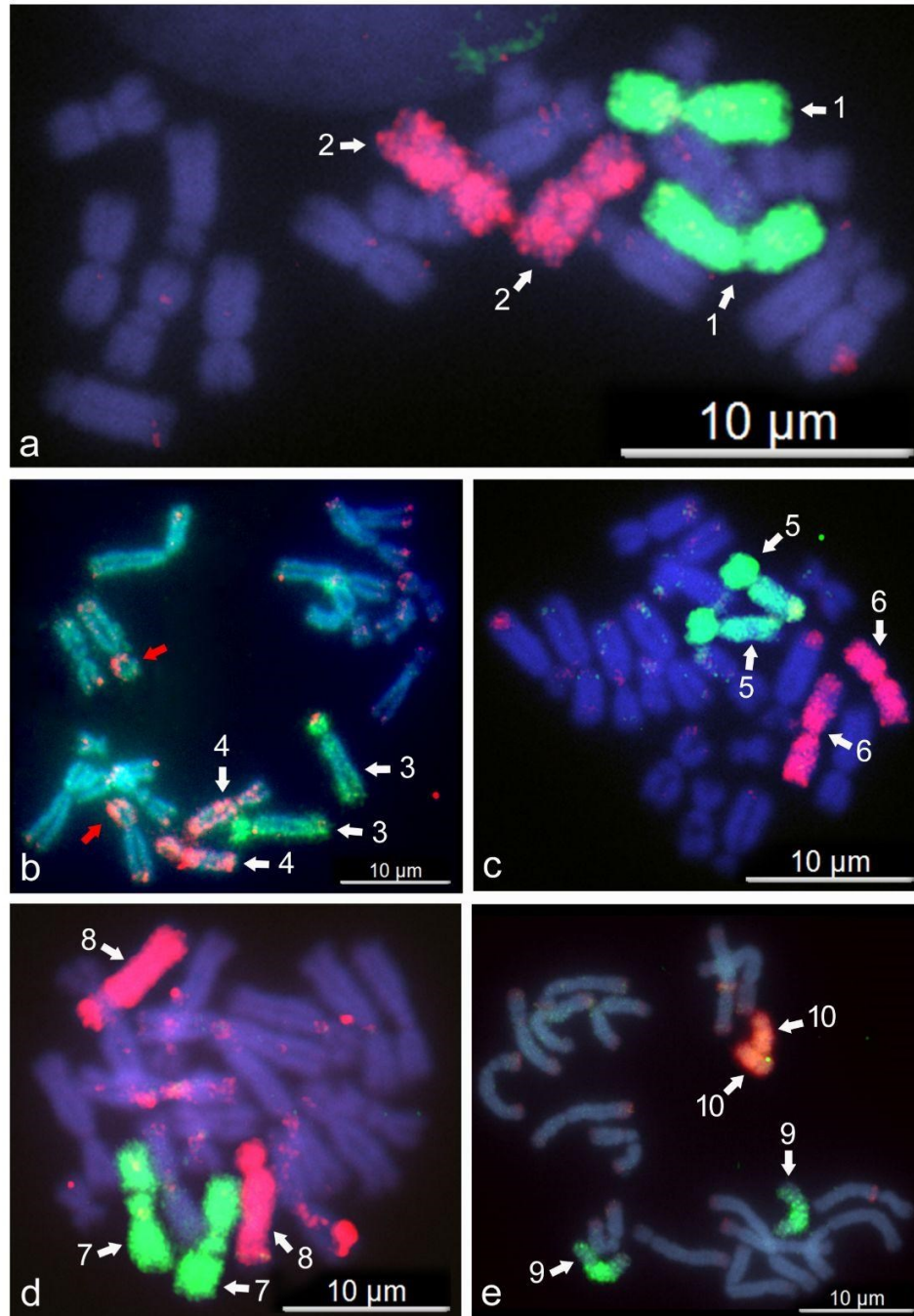


Fig. 2. Double-colour painting fluorescent in situ hybridization on 20 *X. tropicalis* chromosomes (XTR) with *X. tropicalis* whole chromosome painting (WCP) probes (intraspecies painting FISH). White arrowheads indicate painting of whole chromosome pairs using WCP probes derived from (a) XTR 1 (green) and XTR 2 (red), (b) XTR 3 (green) and XTR 4 (red), (c) XTR 5 (green) and XTR 6 (red), (d) XTR 7 (green) and XTR 8 (red), and (e) XTR 9 (green) and XTR 10 (red). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole, DAPI (blue). In addition to stained homologous chromosome pairs, (b) some WCP probes had an additional signal on pericentromeric and telomeric regions, which presumably is caused by repetitive sequences (red arrowheads). Each scale bar represents 10 µm.

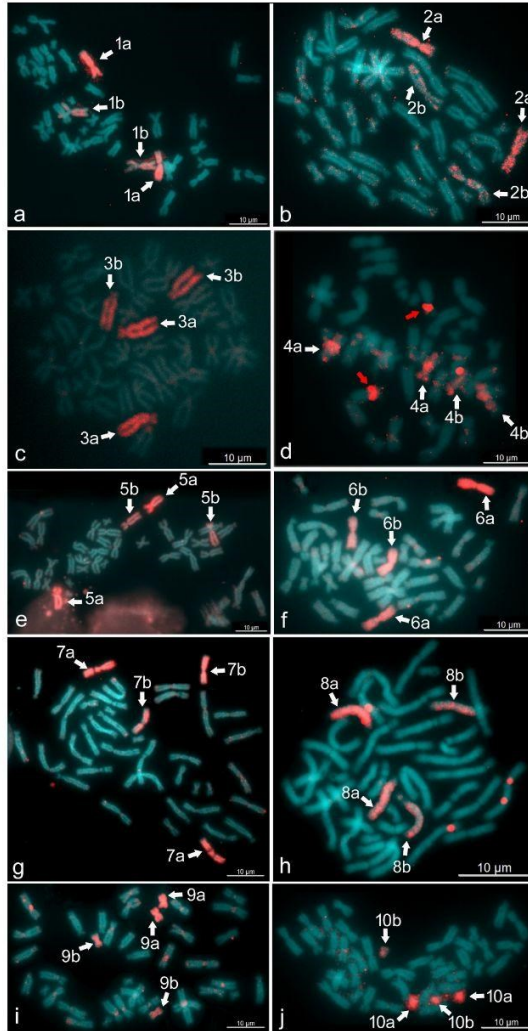


Fig. 3. Cross-species painting FISH (Zoo-FISH) on 40 *X. calcaratus* chromosomes (XCA) using *X. tropicalis* WCP probes. The WCP probes stain the appropriate chromosomal quartets in red. (a) XTR 1–XCA 1a and 1b, (b) XTR 2–XCA 2a and 2b, (c) XTR 3–XCA 3a and 3b, (d) XTR 4–XCA 4a and 4b, (e) XTR 5–XCA 5a and 5b, (f) XTR 6–XCA 6a and 6b, (g) XTR 7–XCA 7a and 7b, (h) XTR 8–XCA 8a and 8b, (i) XTR 9–XCA 9a and 9b and (j) XTR 10–XCA 10a and 10b. White arrowheads show labeled chromosomal quartets. Chromosomes were counterstained with DAPI (blue-green). In addition to red-stained homoeologous chromosomes, some WCP probes also had an additional signal on pericentromeric and telomeric regions, which presumably is caused by repetitive sequences (red arrowheads). Each scale bar represents 10 μm .

mitochondrial rRNA gene resulted in approximately 200 and 300, and 900 bp long amplicons.

Based on *blastn* results, the 5S amplicon had 96% identity with the sequence of 5S rDNA of *X. tropicalis* (accession number X12624.1), and 28S amplicon had 100% identity with 28S rRNA of *X. tropicalis* (accession number XR_004223802.1), which is consistent with membership in subgenus *Silurana* (at the time of this study, sequences of these genes for *X. calcaratus* are not present in the GenBank database). The 16S

amplicon showed 100% identity with 16S rRNA of *X. calcaratus* (accession number KT728037.1) from the same locality in Cameroon but 2.8–3.0% divergence from *X. tropicalis* (accession numbers KT728027.1 and KT728029.1), which is not known to occur in this locality. Together these results confirmed the species identity of the *X. calcaratus* cells. As detailed below, this species diagnosis is also consistent with chromosome counts that also distinguish these two species. Sequences are deposited to the NCBI GenBank database (5S accession number ON908197, 28S accession number OM910742, 16S accession number OM912675).

3.2. Fluorescent in situ hybridization with whole chromosome painting probes

Ten WCP probes were prepared from each separately microdissected chromosome of *X. tropicalis*. As expected, each probe hybridized specifically and stained a whole *X. tropicalis* homologous chromosome pair 1–10 (Fig. 2a–e, with pairs of probes labeled in green and red). This control in *X. tropicalis* demonstrates high specificity of the WCP probes derived from this diploid species.

Having confirmed specificity of the *X. tropicalis* WCP probes, we went on to perform Zoo-FISH experiments on *X. calcaratus* metaphase spreads using each *X. tropicalis* WCP probe. The WCP probes derived from XTR 1–10 hybridized to whole chromosomal quartets (the homoeologous chromosome groups) without evidence of large-scale translocations as follows: XTR 1 to *X. calcaratus* chromosome 1a (XCA 1a) + 1b (Fig. 3a); XTR 2 to XCA 2a + 2b (Fig. 3b); XTR 3 to XCA 3a + 3b (Fig. 3c); XTR 4 to XCA 4a + 4b (Fig. 3d); XTR 5 to XCA 5a + 5b (Fig. 3e); XTR 6 to XCA 6a + 6b (Fig. 3f); XTR 7 to XCA 7a + 7b (Fig. 3g); XTR 8 to XCA 8a + 8b (Fig. 3h); XTR 9 to XCA 9a + 9b (Fig. 3i); and XTR 10 to XCA 10a + 10b (Fig. 3j).

A key finding that emerged from the *X. calcaratus* Zoo-FISH experiments is that one homoeologous chromosome pair had consistently higher fluorescent intensity than the other. Based on this, we defined *X. calcaratus* “a” homoeologs (or *a*, sensu Evans et al. (2005)) to be the more intensely painted chromosomes and “b” homoeologs (or *b*, sensu Evans et al. (2005)) to be the less intensely painted ones. The observation of differential painting intensity is consistent with an allotetraploid origin of *X. calcaratus* from a diploid common ancestor of *X. tropicalis* and another divergent diploid ancestor whose diploid descendants are either extinct or have not yet been discovered (Fig. 1). That these experiments found no evidence of a chromosomal translocation between chromosomes homologous to XTR 9 and 2 in *X. calcaratus* suggests this translocation occurred in an ancestor of *X. mellotropicalis* after divergence from an ancestor of *X. calcaratus*.

Using the WCP probe from *X. tropicalis* chromosome 4, we found an additional signal on XTR 5 (Fig. 2b, red arrowheads) and XCA 5a (Fig. 3d, red arrowheads) which is possibly caused by accumulation of repetitive sequences on a portion of the *p* arm.

3.3. Genomic in situ hybridization with whole genome painting probes

Two GISH experiments were performed with *X. tropicalis* WGP probe. The first one was a control to evaluate the efficacy of the WGP probe against *X. tropicalis* chromosomes (Fig. 4a). All 20 chromosomes were consistently painted, indicating high efficacy. The second GISH experiment hybridized the WGP probe to *X. calcaratus* chromosomes with an aim of providing verification of a distinctive cytogenetic signature of each subgenome. Similar to the WCP experiments shown in Section 3.2, this technique recovered differences in the intensity of fluorescence between several homoeologous pairs (Fig. 4b). In particular, chromosomes XCA 7a, 9a, and 10a were notably more intensely painted than XCA 7b, 9b, and 10b (labeled with arrowheads). These results are consistent with the analysis of *p/q* arm ratio and Zoo-FISH data discussed below, and with a closer evolutionary relationship between the XCA homoeologs “a” and XTR orthologs than between the XCA

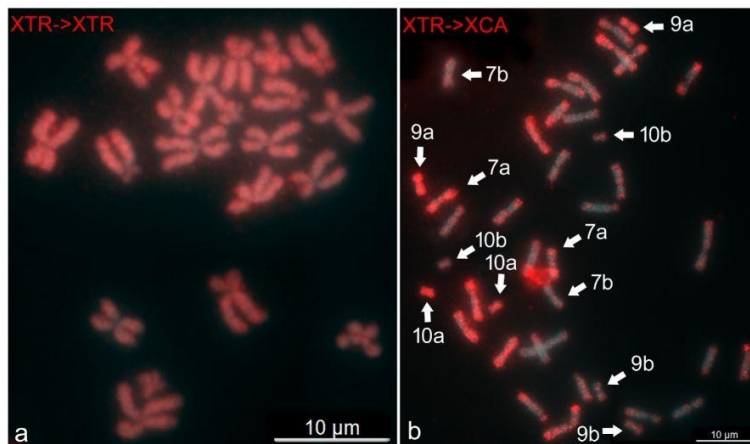


Fig. 4. Genomic in situ hybridization (GISH) using *X. tropicalis* whole genome painting (WGP) probes (in red) against (a) *X. tropicalis* and (b) *X. calcaratus* chromosomes. In (a), the *X. tropicalis* WGP probes hybridized to all 20 *X. tropicalis* chromosomes (XTR→XTR) with a consistent fluorescent signal. In (b), the *X. tropicalis* WGP probes hybridized to all 40 *X. calcaratus* chromosomes but with different intensities between homoeologs—especially between homoeologs XCA 7, 9, and 10 (white arrowheads). Each scale bar represents 10 μm .

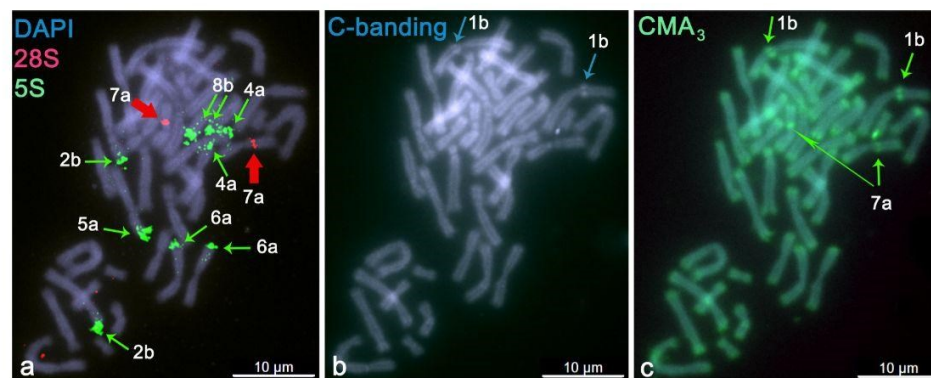


Fig. 5. Sequential fluorescent chromosome mapping (ribosomal DNA FISH/C-banding/Chromomycin A₃, CMA₃) on metaphase spread of *X. calcaratus*. DAPI (blue-green) counter-stained metaphase spreads show all 40 chromosomes. (a) FISH with 5S (green) and 28S (red) ribosomal probes identifies XCA 2b, 4a, 5a, 6a, and 8b (green arrows) and the nucleolar secondary constriction on the *q* arm of XCA 7a (red arrowheads), respectively. (b) C-banding (brighter staining) highlights heterochromatic blocks on the *p* arm of XCA 1b (blue arrows). (c) CMA₃ banding in green (green arrows) shows heterochromatic blocks on the *p* arm of XCA 1b that co-localize with C-bands, and additional CMA₃ positive band on the *q* arm of XCA 7a (nucleolar secondary constriction) that co-localize with 28S rDNA loci. Scale bars represent 10 μm .

homoeologs “b” and XTR orthologs. Other homoeologous chromosomes within the *X. calcaratus* karyotype apart from XCA 7, 9, and 10 also were painted with slightly different but less distinguishable intensities.

3.4. Ribosomal gene mapping and chromosome banding

Nucleolar organizer regions (NORs) are regions in eukaryotic genomes that contain tandem arrays of three ribosomal genes (*18S*, *5.8S*, *28S*). In eukaryotes the ribosome includes small and large subunits; the *18S* rRNA gene is part of the small subunit and the *5.8S*, *28S* and *5S* rRNAs are part of the large subunit, though the *5S* rRNA gene is not contained within the NOR (Vierna et al., 2013). In diploid genomes there is generally only one pair of NORs (Schmid et al., 1987). Building on the findings from Zoo-FISH (Section 3.2), we expected a NOR pair to have been inherited from each of the diploid ancestral species of *X. calcaratus*. However, the FISH experiment with 28S rDNA probes identified only one pair of NORs situated on the nucleolar secondary constriction of XCA 7a (Fig. 5a, in red). The FISH with 5S probes revealed 8–10 positive loci located on telomeric regions of the XCA 2b,

4a, 5a, 6a, and 8b as evidenced in Fig. 5a in green.

Xenopus calcaratus is closely related to *X. melloittropicalis* and for this reason we expected similar patterns and localization of the heterochromatic blocks in these two species. To test this expectation, we used C- and CMA₃-banding, which are staining techniques that highlight GC rich chromosomal loci and blocks of constitutive heterochromatin. These staining methods can be helpful for distinguishing and assigning chromosomes based on banding patterns. C- and CMA₃-banding showed positive signals on XCA 1bp (Figs. 5b and 5c). C-banding also exhibited a faint signal in portions of stained regions on XCA 2b, 6b, 7b, and 8b (not shown by arrows). CMA₃-banding also identified nucleolar secondary constriction of XCA 7aq (Fig. 5c). All *X. calcaratus* chromosomes bore weak CMA₃-bands on telomeres that presumably are caused by repetitive sequences.

3.5. Single-copy gene mapping

To test whether *X. calcaratus* shared a large scale translocation with *X. melloittropicalis*, we mapped the alpha and beta homoeologs of *gyg2*,

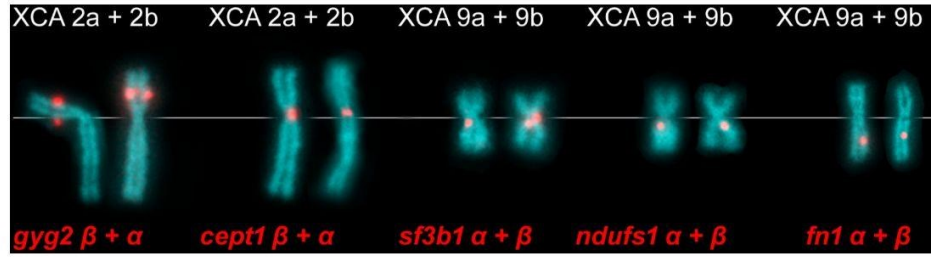


Fig. 6. FISH coupled with tyramide signal amplification (FISH-TSA) with positive red signals on *X. calcaratus* chromosomes. The *gyg2β* and *α*, *cept1β* and *α* loci were localized on the *p* arm of XCA 2a and 2b, respectively. The *sf3b1α* and *β*, *ndufs1α* and *β*, and *fn1α* and *β* loci were mapped on the *q* arm of XCA 9a and 9b, respectively. A white line indicates the centromere position.

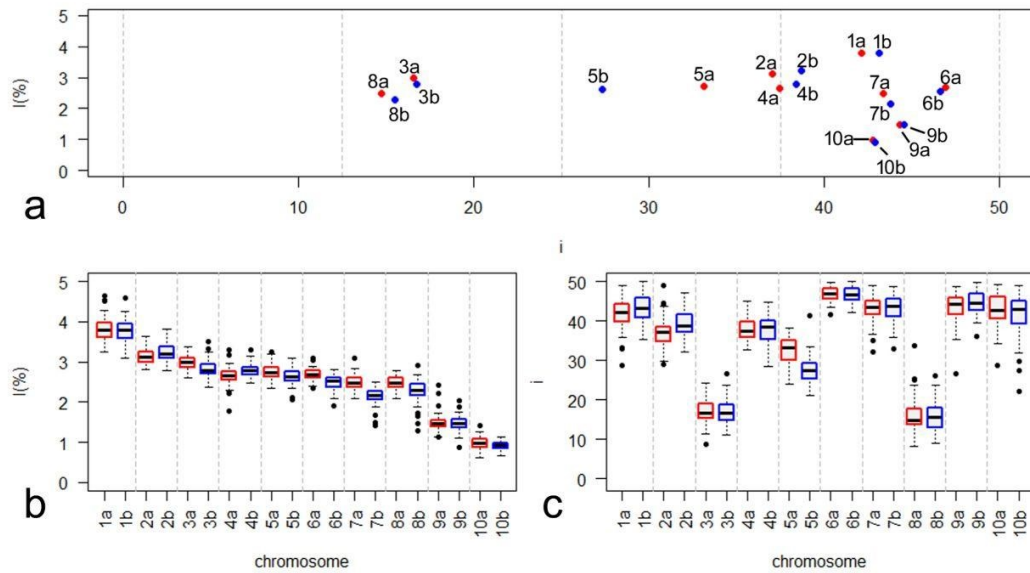


Fig. 7. Analysis of *X. calcaratus* short and long arm chromosome length measurements. The a-subgenome is shown in red and the b-subgenome is in blue. (a) The relationship between centromeric index (*i*), x-axis, and chromosomal length (*l*), y-axis. Gray dashed vertical lines define intervals 0–12.5, 12.5–25, 25–37.5, and 37.5–50 that correspond to acrocentric, subtelocentric, submetacentric, and metacentric chromosomes, respectively. Plotted values of *i* and *l* are medians for each chromosome. (b) shows intrachromosomal variability of *l* value (y axis) for the haploid complement of 20 *X. calcaratus* chromosomes (x axis). (c) intrachromosomal variability of *i* value (y axis) on the haploid complement of 20 *X. calcaratus* chromosomes (x axis). b and c: gray dashed vertical lines delimit homoeologous pairs. The upper and lower whiskers show the minimum and maximum values, respectively, the boxes involve the lower (*Q*₁) and upper (*Q*₃) quartiles, the black lines inside the boxes indicate the median values (*Q*₂); outliers are indicated by black points.

cept1, *sf3b1*, *ndufs1*, and *fn1* in *X. calcaratus*. In *X. mellotropicalis* *gyg2α* and *β*, and *cept1α* and *β* map to chromosomes 2ap and 2bp, respectively. The *ndufs1α* and *β*, and *fn1α* and *β* map to chromosomes 9a and 9b, respectively. The *sf3b1α* and *β* genes map to *X. mellotropicalis* chromosomes 2bp and 9aq, respectively (Knytl et al., 2018b).

In *X. calcaratus* the *gyg2α* and *cept1α* mapped to 2bp, and *gyg2β* and *cept1β* mapped to 2ap. *fn1α*, *ndufs1α*, and *sf3b1α* mapped to XCA 9aq, and *fn1β*, *ndufs1β*, and *sf3b1β* mapped to XCA 9bq, respectively (Fig. 6). The metaphases with 40 chromosomes and FISH-TSA signals are shown in Supplementary material (Figs. S1–S5). Thus, similar to the findings from the WCP probes discussed above, mapping of single-copy genes in *X. calcaratus* recovered no evidence of the translocation involving chromosomes 9b and 2b that was observed in *X. mellotropicalis* (Knytl et al., 2017; Knytl et al., 2018b).

3.6. Mensural analysis of *X. calcaratus* chromosomes

As expected, across all *X. calcaratus* spreads (*n* = 35) we consistently counted 40 chromosomes, indicating that $2n = 4x = 40$, where *n* refers to the haploid number of chromosomes of the extant species, and *x* refers to the haploid number of chromosomes in the most recent diploid ancestor of the extant species.

Each chromosome was measured from 35 metaphases spreads, with each chromosome identified based on comparison to the FISH results, and the median values of *l*, *r*₁, and *i* were calculated. *l* was quantified as a percentage of the sum of *l* across all chromosomes in order to account for variation in resolution and pixel size of our images. Each individual chromosome was also assigned a chromosomal category based on the *i* value. If the *i* value was equal to or greater than 37.5, the chromosome was categorized to be metacentric. If the *i* value was equal to or higher than 25 and lower than 37.5, the chromosome was categorized to be

Table 1

Measurements of *X. calcaratus* chromosomes formulated in medians of l (%), p/q arm ratio (r_1), and i . Chromosomal categories correspond to m = metacentric, sm = submetacentric, and st = subtelocentric chromosomes. Significance codes following l and i values define whether pairs of homoeologous chromosomes are significantly different based on ANOVA test. Significantly different homoeologs are depicted by significance codes "****", "***", and "*" showing the p -value $p < 0.001$, $p < 0.01$, and $p < 0.1$, respectively.

Chromosome	l (%)	r_1	i		Category	
1a	3.78	0.73	42.10		m	
1b	3.79	0.76	43.15	•	m	
2a	3.11	**	0.59	37.04	**	sm
2b	3.20		0.63	38.67		m
3a	2.98	***	0.20	16.58		st
3b	2.80		0.20	16.74		st
4a	2.65	***	0.60	37.48		sm
4b	2.80		0.62	38.42		m
5a	2.73	**	0.49	33.11	***	sm
5b	2.63		0.38	27.33		sm
6a	2.69	***	0.88	46.94		m
6b	2.54		0.87	46.63		m
7a	2.48	***	0.77	43.38		m
7b	2.16		0.78	43.78		m
8a	2.47	***	0.17	14.73		st
8b	2.28		0.18	15.47		st
9a	1.47		0.80	44.31		m
9b	1.48		0.80	44.54		m
10a	0.98	**	0.75	42.78		m
10b	0.93		0.75	42.90		m

submetacentric, and if the i value was equal to or greater than 12.5 and lower than 25, the chromosome was categorized as subtelocentric. The karyotype of *X. calcaratus* consists of 12 pairs of metacentric, four pairs of submetacentric, and four pairs of subtelocentric chromosomes.

We found that pairs of homoeologs tend to have similar chromosomal morphology in terms of l and i values (Fig. 7a). For example, chromosomes 3a, 3b, 8a, and 8b had low centromeric indexes (between 12.5–25) and were therefore both subtelocentric. Chromosomes 5a and 5b had centromeric indexes between 25–37.5 and were therefore both submetacentric. However, chromosomes 2a and 4a were in the submetacentric category whereas their homoeologous chromosomes 2b and 4b were in the metacentric category. The rest of chromosomes fell into the category of metacentric chromosomes (centromeric indexes of 37.5–50). Acrocentric chromosomes (centromeric indexes interval 0–12.5) and telocentric chromosomes without a p arm (centromeric indexes = 0) were not present in the *X. calcaratus* karyotype.

The l and i values were assigned to each individual chromosome 1a–10b from each metaphase and plotted (Figs. 7b and 7c). A measure of statistical dispersion, the interquartile range (Q_1 – Q_3), was used to evaluate the extent of morphological divergence of each homoeologous chromosomal pair. The highest divergence in l between homoeologous chromosomes was found between XCA 7a and 7b. The Q_1 – Q_3 of XCA 7a and 7b l ranged from 2.37% to 2.61% and from 2.06% to 2.27%, respectively (Supplementary material, Box plot statistics of l , matrix \$stats in R output, columns [,13] and [,14], rows [2,]–[4,]). The second highest divergence in l was measured within homoeologous pairs XCA 6a (2.62% to 2.77%) and 6b (2.38% to 2.59%) (Supplementary material, Box plot statistics of l , matrix \$stats in R output, columns [,11] and [,12], rows [2,]–[4,]). The largest difference in i of homoeologous chromosomes was between XCA 5a and 5b, which differed within Q_1 – Q_3 by 29.98–35.08 and 25.39–29.31, respectively (Supplementary material, Box plot statistics of i , \$stats in R output, columns [,9] and [,10], rows [2,]–[4,]). The second largest difference based on the Q_1 – Q_3 interval of i was between XCA 2a (34.69–38.39) and 2b (37.07–41.53, Supplementary material, Box plot statistics of i , \$stats in R output, columns [,3] and [,4], rows [2,]–[4,]). Based on l or i , the four most differentiated homoeologous pairs (2a and 2b, 5a and 5b, 6a and 6b, 7a and 7b) are identifiable based on

arms lengths.

The median values of l (%), r_1 and i are shown in Table 1, where these values are assigned to each chromosome.

Chromosome 7a and 7b of *X. calcaratus* can be distinguished because 7a has a secondary nucleolar constriction in the same position as XTR 7, whereas this feature is absent from XCA 7b. This inference is further supported by a higher intensity of Zoo-FISH and GISH signals with *X. tropicalis* probes on XCA 7a compared to 7b. The l values of some chromosomes were substantially different between "a" and "b" homoeologs (Table 1, significance codes). Based on the cytogenetic information presented here that was obtained from multiple methods, we defined a chromosome nomenclature for *X. calcaratus* that could extend to other tetraploid *Silurana* species depending on their phylogenetic relationships discussed below.

4. Discussion

We used a combination of conventional (C- and CMA₃-banding) and more advanced molecular cytogenetic techniques (rDNA FISH, intra- and cross-species painting FISH, FISH-TSA, and GISH) to study the allotetraploid karyotype architecture of *X. calcaratus*. Our results, together with previous cytogenetic findings (Tymowska and Fischberg, 1982; Schmid and Steinlein, 2015; Knytl et al., 2017), enabled us to distinguish and define the a- and b-subgenomes of *X. calcaratus*. We identified several cytogenetic changes discussed below in the b-subgenome that were not present in the a-subgenome of *X. calcaratus* or in *X. tropicalis*, which indicate that the b-subgenome has undergone more rapid (and asymmetric) evolution compared to the a-subgenome.

4.1. Localization of the heterochromatin, NOR, and other components of the ribosomes

The allotetraploid species *X. laevis* has one NOR on chromosome 3Lp (Tymowska and Kobel, 1972; Schmid et al., 1987; Roco et al., 2021), with a homologous NOR on chromosome 3S thought to have been lost after allotetraploidization in subgenus *Xenopus* (Session et al., 2016). In the diploid *X. tropicalis* the NOR is on chromosome 7q (Tymowska, 1973; Tymowska and Fischberg, 1982; Uehara et al., 2002; Roco et al., 2021), the allotetraploid *X. epitropicalis* and *X. mellotropicalis* also have one NOR on chromosome 7aq (Tymowska and Fischberg, 1982; Tymowska, 1991), with the other on chromosome 7b hypothesized to have been lost after allotetraploidization (Knytl et al., 2017). In *X. calcaratus*, 28S rDNA FISH identified nucleolar (secondary) constriction on XCA 7aq (Fig. 5a). There are at least two possible explanations for the conserved location and number of NORs in *X. mellotropicalis* and *X. calcaratus*. If these two species each evolved via independent allotetraploidization events (Fig. 1b), loss of one pair of NORs from the same ancestral species could have occurred twice independently after each allotetraploidization event. Under a scenario of one allotetraploidization in *Silurana* (Fig. 1a) it is also possible that one pair of NORs was lost only once after allopolyploidization in subgenus *Silurana*.

The 5S ribosomal genes were localized to telomeric regions of most or perhaps all of the chromosomes of *X. laevis* (Pardue et al., 1973). Somatic and oocyte-specific types of 5S ribosomal genes have been identified in *X. laevis* (Peterson et al., 1980). The somatic type of 5S genes was localized to *X. laevis* chromosome 6L and the oocyte-specific type of 5S genes was localized to subtelomeric regions of most chromosomes (Harper et al., 1983). Because somatic and oocyte-specific sequences have 95% sequence similarity (Ford and Brown, 1976), probes to both types of 5S genes co-localize to somatic metaphase spreads (Harper et al., 1983). In *X. tropicalis* the 5S is on subtelomeric regions of most of chromosomes (XTR 2, 3, 4, 5, 6, 7, 8, and 9) with the most intense fluorescent signal on XTR 6 (Knytl et al., 2017). In *X. mellotropicalis* 5S genes were mapped to chromosomes 4a, 5b, and 8b (Knytl et al., 2017) but *X. calcaratus* bears 5S loci on chromosomes 2b, 4a, 5a, 6a, and 8b (Fig. 5a). These findings indicate independent

evolution of the 5S genes in allotetraploid *X. mellotropicalis* and *X. calcaratus* with more frequent deletions in *X. mellotropicalis*.

C- and CMA₃- banding identified a prominent heterochromatic block in the form of a non-nucleolar secondary constriction, but some of these signals may co-localize with NORs (e.g. 28S- and CMA₃-positive signals on XCA 7a, Figs. 5a and 5c). Several *Silurana* tetraploids have one intensely labeled non-nucleolar secondary constriction: for *X. tropicalis* it is on chromosome 9q (Tymowska and Fischberg, 1982; Knytl et al., 2017), for *X. mellotropicalis* it is on 2ap (re-designated below to be 2bp) (Knytl et al., 2017), for *X. epitropicalis* it is on the pericentromeric region of 2a (re-designated below to be 2b) involving both *p* and *q* arms (Tymowska and Fischberg, 1982), and for *X. calcaratus* it is on 1bp. Different sizes and positions of non-nucleolar secondary constrictions within *Silurana* species indicate that these constrictions are dynamic structures with independent evolution; the most similar positions are in *X. epitropicalis* and *X. mellotropicalis* (chromosome 2b), which is consistent with their close evolutionary relationship (Evans et al., 2005).

The number and position of NORs have been extensively explored in both diploid and polyploid animals. Amphibians and fish are good groups with which to explore the effect of whole genome duplication on the evolution of NOR and other ribosomal structures, such as 5S and 45S rRNA genes (Knytl et al., 2017; Knytl et al., 2018a), because both include a diversity of diploid and polyploid species (Evans et al., 2015; Knytl et al., 2022). The number and position of NORs and other ribosomal structures together with cytogenetic mapping of single-copy genes in polyploids may reflect the extent of rediploidization (Symonová et al., 2017). In most diploid and polyploid amphibians, only one homologous chromosome pair bears a NOR (Schmid, 1982; Schmid et al., 1987; Alves et al., 2012). However, in fish there is more extensive variation in the number of NORs, including observations ranging from one (Alves et al., 2012) to seven pairs (Gromicho et al., 2006). For instance, some tetraploid fish possess two pairs of NORs located on one homoeologous set of chromosomes, which is consistent with ancient tetraploidy (Diniz et al., 2009; Knytl et al., 2013a; Symonová et al., 2017). One NOR pair may therefore reflect functional diploidization, wherein bivalents rather than multivalents form during meiosis, which appears to be the case for all *Xenopus* (Tymowska, 1991).

Another possible explanation for among species variation in the number of nucleolar loci is that some have been transcriptionally inactive in the preceding interphase. Therefore, these loci have not been transcribed, and might not be detected by ribosomal probes (Schmid, 1982). The number of nucleolar loci could be also changed via translocation of individual rDNA copies from clusters via viruses or transposons (Gromicho et al., 2006).

4.2. *Silurana* subgenomes with different cytogenetic characteristics

Previous chromosome nomenclature of *X. mellotropicalis* and *X. epitropicalis* designated the larger of the homoeologous pairs as "a" and the smaller pair as "b" (Tymowska and Fischberg, 1982; Tymowska, 1991; Knytl et al., 2017). Although the relative size of homoeologous pairs corresponds to subgenomes in *X. laevis* (Session et al., 2016), this is not necessarily the case in other groups. Because the relative intensities of fluorescence signal in FISH correspond with phylogenetic distance between the probe and the genome being interrogated, evolutionary affinities of chromosomes within each subgenome can be inferred based on the intensity of the probe fluorescence (Markova et al., 2007; Liu et al., 2015). Here, we distinguished homoeologous chromosomes in the *X. calcaratus* a- and b-subgenomes based on measurements of chromosomal *p* and *q* arm length, and comparative cytogenetic findings from Zoo-FISH and GISH using probes from the diploid species *X. tropicalis*. This approach allowed for an evolutionary-based classification system based on divergence from *X. tropicalis* orthologous chromosomes. Chromosomes from the *X. calcaratus* a-subgenome are more similar and closely related to *X. tropicalis* orthologs than chromosomes from the *X. calcaratus* b-subgenome. With this approach, we identified one

Table 2

r_1 values (*p/q* arm ratio) for each chromosome in *X. tropicalis* (Tymowska, 1973), *X. calcaratus* (this study), and *X. mellotropicalis* (Knytl et al., 2017). *Xenopus tropicalis* chromosome nomenclature according Khokha et al. (2009) was used. Each *X. tropicalis* ortholog is divided into the "a" and "b" homoeologous chromosomes in tetraploid *Silurana* karyotypes ("homoeolog" column). Asterisks highlight changes in chromosome nomenclature.

Chromosome	<i>X. tropicalis</i>	Homoeolog	<i>X. calcaratus</i>	<i>X. mellotropicalis</i>
	r_1		r_1	r_1
1	0.72	a	0.73	0.70
		b	0.76	0.67
2	0.61	a	0.59	0.62*
		b	0.63	0.81*
3	0.31	a	0.20	0.20
		b	0.20	0.20
4	0.57	a	0.60	0.60
		b	0.62	0.62
5	0.58	a	0.49	0.42
		b	0.38	0.41
6	0.88	a	0.88	0.88
		b	0.87	0.88
7	0.71	a	0.77	0.66
		b	0.78	0.79
8	0.22	a	0.17	0.21
		b	0.18	0.21
9	0.68	a	0.80	0.88
		b	0.80	0.72
10	0.68	a	0.75	0.70
		b	0.75	0.72

homoeologous pair – chromosome 2 – where the larger chromosome was actually from the b-subgenome, warranting revision of this aspect of the chromosomal nomenclature (Table 2). Consistent with this reappraisal, the intensity of the Zoo-FISH signal on the longer *X. mellotropicalis* chromosome 2 (2a sensu Knytl et al. (2017)) is lower than intensity of the smaller *X. mellotropicalis* chromosome 2 (2b sensu Knytl et al. (2017); see Fig. 4B in Knytl et al. (2017)). Based on results presented here for *X. calcaratus*, *X. mellotropicalis* 2a (XME 2a) and 2b should thus be reclassified as XME 2b and 2a, respectively.

Additionally, our findings indicate that chromosomes XCA 1b and 2b had higher *p/q* arm ratio, centromeric index and chromosomal length than XCA 1a and 2a, respectively. This is also inconsistent with previous designations based on conventional staining such as Giemsa, C-banding, or replication banding (Tymowska, 1991; Schmid and Steinlein, 2015).

Thus far, *X. calcaratus* and *X. mellotropicalis* a-subgenomes have no reported translocations, although the b-subgenome of *X. mellotropicalis* contains large-scale rearrangements between XME 2b and 9b (Knytl et al., 2017). Heterochromatin is evident on XCA 1b (Figs. 5b and 5c) but not on XTR 1 or XME 1b. Knytl et al. (2017) hypothesized that the heterochromatin transpositions occurred within b-subgenome. That the nucleolar 28S rRNA loci (a part of 45S rRNA) were detected only on XCA 7aq but not on 7bq indicates gene loss within *X. calcaratus* b-subgenome. Overall, these results suggest asymmetric subgenome evolution in *Silurana*, with the a-subgenome being more stable and resembling the *X. tropicalis* diploid genome, and the b-subgenome having experienced more chromosomal rearrangements. As discussed above, asymmetric subgenome evolution has also been reported in allotetraploid *X. laevis* which is in subgenus *Xenopus* (Session et al., 2016).

The *p/q* arm ratio of XTR 5 is more similar to the *p/q* arm ratio of XCA 4a, 4b, XME 4a, and 4b than to the *p/q* arm ratio of XCA 5a, 5b, XME 5a, and 5b (Table 2). It is not clear whether *X. tropicalis* chromosomes were identified correctly by Tymowska (1973) and Khokha et al. (2009), or whether both *X. calcaratus* and *X. mellotropicalis* homoeologs 5a and 5b have undergone changes that were undetectable by our experimental approaches. It is also possible that intraspecific variability in chromosome structure exists among geographically separated African populations of *Xenopus*. These possibilities might be distinguished by additional FISH mapping of loci on XTR 5, XCA 5a, 5b, XME 5a, and 5b or by new measurements of the XTR 5 arm length. Using chromosomal

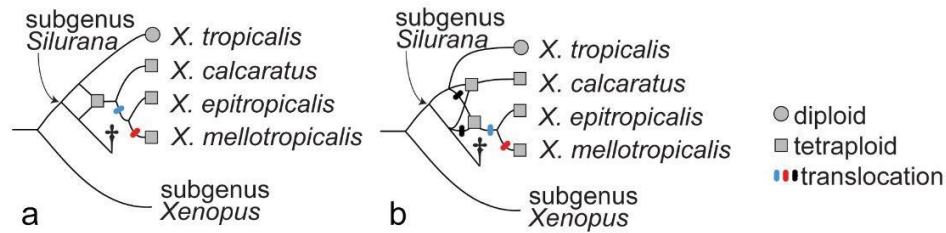


Fig. 8. Evolutionary scenarios in subgenus *Silurana* for the origin of a translocation that is observed in *X. mellotropicalis*, not observed in *X. calcaratus*, and for which no data exists for *X. epitropicalis*. (a) if one allotetraploidization event gave rise to all three tetraploid species then the translocation could have occurred after diverged of *X. calcaratus* but before speciation of *X. mellotropicalis* and *X. epitropicalis* (blue) or after this speciation in the ancestor of *X. mellotropicalis* (red). (b) if instead two independent allotetraploidizations occurred, the translocation could additionally have occurred in either diploid ancestor of the most recent common ancestor of *X. mellotropicalis* and *X. epitropicalis* (both are shown in black, but only one would have occurred).

length, arm ratio, and centromeric indexes alone, it was not possible to conclusively distinguish all homoeologous chromosomes from the a- and b-subgenomes of *X. calcaratus*, although these differences were readily distinguishable by FISH.

The sex chromosomes of *X. tropicalis* and *X. mellotropicalis* are homologous (XTR 7 and probably XME 7a, respectively (Olmstead et al., 2010; Roco et al., 2015; Mitros et al., 2019; Furman et al., 2020; Cauret et al., 2020)). On XTR 7, XME 7a, and XCA 7a the position of the nucleolar secondary constriction on the *q* arm and the *p/q* arm ratio (ranged from 0.66–0.77) are conserved. Because a master sex determining gene has not yet been identified in any *Silurana* species, we were not able to probe this gene and the sex chromosomes of *X. calcaratus* remain unidentified.

4.3. Timing of chromosomal translocation between chromosomes 9 and 2

Zoo-FISH with *X. tropicalis* WCP probes on *X. mellotropicalis* chromosomes and gene mapping using FISH-TSA revealed a large-scale interchromosomal translocation of heterochromatic block from chromosome 9b to 2b, abbreviated t(9b;2b) (Knytl et al., 2017; Knytl et al., 2018b). This translocation was not identified in *X. calcaratus* based on Zoo-FISH (Fig. 3) and also was not present in *X. tropicalis* (Knytl et al., 2018b). More specifically, we found that rearrangement-associated genes in *X. mellotropicalis* did not all map to the corresponding chromosomes of *X. calcaratus*. In Knytl et al. (2018b), *sf3b1a* mapped to XME 2a (which with the proposed revised nomenclature is XME 2b) but in *X. calcaratus* this gene mapped to XCA 9a. In *X. tropicalis*, *sf3b1* is localized on XTR 9 (Knytl et al., 2018b). The most parsimonious explanation for these observations is that the t(9b;2b) occurred in an ancestor of *X. mellotropicalis* after the divergence of *X. calcaratus* (Fig. 8). It is not clear whether t(9b;2b) occurred in the most recent common ancestor of *X. epitropicalis* and *X. mellotropicalis* (Fig. 8, black and blue spots), or if the translocation occurred solely in the *X. mellotropicalis* lineage after the divergence from the common ancestor with *X. epitropicalis* (Fig. 8, red spots). Another possible scenario is that the translocation occurred between ancestral chromosomes in a diploid ancestor of *X. epitropicalis* and *X. mellotropicalis* (Fig. 8b, black spots). A caveat to this interpretation is that we lack cytogenetic information from a diploid descendant of the other ancestor of *Silurana* allotetraploids, and we are therefore unable to rule out the possibility that some of these changes in the b-subgenome (all except the loss of the NOR) occurred in that ancestor before allotetraploidization. Further resolution of the evolutionary history of this translocation could be achieved via Zoo-FISH analysis of *X. epitropicalis* chromosomes. So far, cytogenetic studies of *X. epitropicalis* are limited to conventional banding (Tymowska, 1991; Schmid and Steinlein, 2015). Heterochromatic blocks identified by chromosome banding suggest that the t(9b;2b) of *X. mellotropicalis* is associated with repetitive sequences (Knytl et al., 2017). Extensive rearrangements and GC-rich secondary constrictions were not identified on the XCA 2b (CMA₃ or C-banding

positive, Figs. 5b and 5c) which suggests the repetitive regions associated with the t(9b;2b) of *X. mellotropicalis* also accumulated after divergence from the ancestor of *X. calcaratus*. The effects on recombination of a rearrangement may not be bounded by the breakpoints of the rearrangement (Xia et al., 2020). It is thus conceivable that this translocation influenced speciation through the suppression of chromosomal pairing and recombination (Fishman et al., 2013).

5. Conclusions

Allopolyploid genomes are interesting subjects for cytogenetic investigation because evolutionary phenomena in each subgenome can be distinguished and compared. In this study, we identified two subgenomes in the allotetraploid frog *X. calcaratus*, and found them to be distinguished by cytogenetic characteristics that are consistent with asymmetric evolution. Results are consistent with the proposed allopolyploid origin of *X. calcaratus* (*X. new* tetraploid 2 in (Evans et al., 2005)), with one progenitor species also being an ancestor of *X. tropicalis* and another ancestral species either extinct or undiscovered. To better understand genome evolution in subgenus *Silurana* it is necessary to perform further cytogenetic analyses of *X. epitropicalis*, the remaining member of a group of allotetraploid species.

Ethics approval

Charles University has registered experimental breeding facilities for pipid frogs (16OZ12891/2018–17214, 37428/2019-MZE-18134). All experimental procedures involving frogs were approved by the Institutional Animal Care and Use Committee of Charles University, according to the directives from the State Veterinary Administration of the Czech Republic. MK is a holder of the Certificate of professional competence to design experiments according to §15d(3) of the Czech Republic Act No. 246/1992 coll. on the Protection of Animals against Cruelty (Registration number CZ 03973), provided by the Ministry of Agriculture of the Czech Republic.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The research was funded by the institutional support from the IVB CAS, RVO: 68081766 (VG), institutional support from the Ministry of Agriculture of the Czech Republic, MZE-RO0518 (HC, SK), the Natural Science and Engineering Research Council of Canada, RGPIN-2017-05770 (BJE), and Charles University, Faculty of Science,

Cooperatio (VK). We thank two anonymous reviewers for their helpful comments on this manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.gene.2022.146974>.

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Consequences of polyploidy and divergence as revealed by cytogenetic mapping of tandem repeats in African clawed frogs (*Xenopus*, Pipidae)

Nicola R. Fornaini¹ · Barbora Bergelová¹ · Václav Gvoždík^{2,3} · Halina Černohorská⁴ · Vladimír Krylov¹ · Svatava Kubíčková⁴ · Eric B. Fokam⁵ · Gabriel Badjedjea⁶ · Ben J. Evans⁷ · Martin Knytl^{1,7}

Received: 1 February 2023 / Revised: 13 May 2023 / Accepted: 27 June 2023 / Published online: 21 July 2023
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Abstract

Repetitive elements have been identified in several amphibian genomes using whole genome sequencing, but few studies have used cytogenetic mapping to visualize these elements in this vertebrate group. Here, we used fluorescence in situ hybridization and genomic data to map the U1 and U2 small nuclear RNAs and histone H3 in six species of African clawed frog (genus *Xenopus*), including, from subgenus *Silurana*, the diploid *Xenopus tropicalis* and its close allotetraploid relative *X. calcaratus* and, from subgenus *Xenopus*, the allotetraploid species *X. pygmaeus*, *X. allofraseri*, *X. laevis*, and *X. muelleri*. Results allowed us to qualitatively evaluate the relative roles of polyploidization and divergence in the evolution of repetitive elements because our focal species include allotetraploid species derived from two independent polyploidization events — one that is relatively young that gave rise to *X. calcaratus* and another that is older that gave rise to the other (older) allotetraploids. Our results demonstrated conserved loci number and position of signals in the species from subgenus *Silurana*; allotetraploid *X. calcaratus* has twice as many signals as diploid *X. tropicalis*. However, the content of repeats varied among the other allotetraploid species. We detected almost same number of signals in *X. muelleri* as in *X. calcaratus* and same number of signals in *X. pygmaeus*, *X. allofraseri*, *X. laevis* as in the diploid *X. tropicalis*. Overall, these results are consistent with the proposal that allopolyploidization duplicated these tandem repeats and that variation in their copy number was accumulated over time through reduction and expansion in a subset of the older allopolyploids.

Keywords Amphibians · Anura · snRNA · Histone H3 · Allopolyploidization · In situ hybridization

✉ Martin Knytl
martin.knytl@natur.cuni.cz

¹ Department of Cell Biology, Faculty of Science, Charles University, Viničná 7, Prague 12843, Czech Republic

² Institute of Vertebrate Biology of the Czech Academy of Sciences, Brno, Czech Republic

³ Department of Zoology, National Museum of the Czech Republic, Prague, Czech Republic

⁴ Department of Genetics and Reproduction, CEITEC - Veterinary Research Institute, Hudcova 296/70, Brno 62100, Czech Republic

⁵ Department of Animal Biology and Conservation, University of Buea, PO Box 63, Buea 00237, Cameroon

⁶ Department of Aquatic Ecology, Biodiversity Monitoring Center, University of Kisangani, Kisangani, Democratic Republic of the Congo

⁷ Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON L8S4K1, Canada

Introduction

Repetitive elements are genomic sequences that are present in multiple copies and are found in all eukaryotic genomes, but with varying abundances and genomic distributions. Content of repetitive DNA ranges from less than 10% in some fishes (e.g., *Tetraodon nigroviridis* and *Cynoglossus semilaevis*) and birds (e.g., *Phoenicopterus ruber*, *Struthio camelus*, *Haliaeetus albicilla*) to > 90% in some plants, such as *Allium cepa* (Chalopin et al. 2015; Chen et al. 2014; Zhang et al. 2014; Canapa et al. 2016; Fu et al. 2019).

Repetitive elements in eukaryotes are categorized into transposable elements (TEs) and tandem repeats. TEs, which are scattered throughout the genome, include retroelements/retrotransposons and DNA transposons. Retrotransposons are reversely transcribed to DNA and replicated using a copy and paste mechanism. Therefore, they may increase genome size (Yampolsky 2016; Clark et al. 2019). Among

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retrotransposons, the LINES (Long Interspersed Nuclear Elements) are classified into many subcategories (Valente et al. 2011; Chalopin et al. 2015; Gama et al. 2022). In contrast, tandem repeats are found as tandem arrays or head-to-tail motifs from several to more than a hundred copies (Myers 2007). Tandem repeats are present in clusters within the genome on single chromosomal locus or multiple loci and include microsatellite, minisatellite, satellite DNA, ribosomal genes (minor 5S and major 45S), histone genes, and small nuclear RNA (snRNA) (Symonová et al. 2017b; Knytl et al. 2018a; Sember et al. 2020; Gazoni et al. 2021; Schott et al. 2022). These loci often undergo concerted evolution (Elder and Turner 1995; Liao 1999), causing intraspecific paralogous repetitive elements to be more similar to each other than to their orthologs, even when the origin of repetitive elements precedes speciation (Liao 1999). Repetitive loci on chromosomes show dynamic evolution in terms of where in the genome and how frequently they are found, and they often have a high rate of rearrangement (Bruschi et al. 2014; Liu et al. 2019).

Due to their repetitive nature, short read sequences typically pose challenges to mapping and repeat quantification, including using genomic data. However, localization of repetitive DNA can be achieved using fluorescent in situ hybridization (FISH) cytogenetic techniques. Cytogenetic mapping of repetitive sequences has been studied in teleost fishes (Bishani et al. 2021; Knytl et al. 2022), reptiles (Oliveira et al. 2021; Altmanová et al. 2022), birds (De Oliveira et al. 2017; Kretschmer et al. 2021), and mammals (Milioto et al. 2019; Gerbault-Seureau et al. 2018). In amphibians, cytogenetic mapping of repetitive sequences has identified inter- and intraspecific rearrangements and other features of karyotype evolution (da Silva et al. 2021; Phimphan et al. 2021; Guzmán et al. 2022).

African clawed frogs of the genus *Xenopus* (Pipidae) include almost 30 species and are divided into two subgenera (*Xenopus* and *Silurana*) (Evans et al. 2015) that each is sometimes considered to be a genus (e.g., Dubois et al. 2021). Subgenus *Silurana* includes four described species, the diploid *Xenopus* (*Silurana*) *tropicalis* and three allotetraploids *X. calcaratus*, *X. epitropicalis*, and *X. mellotropicalis*. For practical reasons, we use the name *Silurana* throughout this article, but present it at the subgenus level in accordance with Evans et al. (2015). Subgenus *Xenopus* includes 25 tetraploid, octoploid, or dodecaploid species (Tymowska 1991) that are divided into three species groups: *amieti*, *laevis*, and *muelleri* (Evans et al. 2015). In genus *Xenopus*, at least eight independent allopolyploidization events occurred, including at least one allotetraploidization event in each subgenus (Fig. 1; for an alternative scenario in *Silurana*, see Knytl et al. 2023). This variation in independently evolved ploidy levels offers a compelling opportunity to explore how tandem repeats evolve in the context of genome duplications.

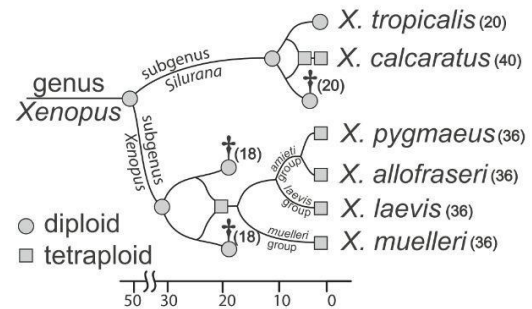


Fig. 1 Inferred phylogenetic relationships and approximate divergence times among our focal taxa based on Evans et al. (2015), Session et al. (2016), and Feng et al. (2017). Allotetraploidization (depicted as reticulating lineages) occurred independently in each subgenus; inferred diploid lineages with no known extant diploid descendants are indicated with daggers. Time scale is in millions of years ago; chromosome numbers are shown in parentheses

About 34.5% and 40% of the *X. tropicalis* and *X. laevis* genomes are repetitive (Hellsten et al. 2010; Session et al. 2016). Ribosomal DNAs (rDNAs) have been cytogenetically mapped on *Xenopus* chromosomes (Schmid and Steinlein 2015; Knytl et al. 2017, 2023; Roco et al. 2021). In allotetraploid species of subgenus *Silurana*, rDNA FISH analysis identified nucleolar organizer region (NOR) on chromosome 7a where the letter “a” refers to the “a subgenome” within the allotetraploid genome (Knytl et al. 2017, 2023). That only one pair of homologous chromosomes contains the NOR locus in *Xenopus* allotetraploids suggests deletion of NOR on chromosome 7b (of the b subgenome) after allotetraploidization (Knytl et al. 2023) because a NOR would have been required in each diploid ancestral species. There is limited cytogenetic information from other repetitive sequences in *Xenopus*, and thus, it is also not known whether non-rDNA repeats vary among *Xenopus* species.

Small nuclear RNAs are components of the spliceosome that perform pre-mRNA splicing. The spliceosome is composed of five RNA tandem-repeat units (U1, U2, U4, U5, and U6 snRNAs) (Valadkhan 2005). Histones are components of the nucleosome that are present in tandem repeat families of five major genes: H1/H5, H2A, H2B, H3, and H4. Here, we used FISH for mapping U1 and U2 snRNA and histone H3 loci on chromosomes from six species of *Xenopus*, including members of both subgenera which diverged from one another approximately 45–50 Mya (Session et al. 2016; Feng et al. 2017). The allotetraploidization event in subgenus *Xenopus* is estimated to have occurred earlier (17–18 Mya) than the one in subgenus *Silurana* and the onset of the polyploid radiation in subgenus *Xenopus* at approximately 17 Mya (Session et al. 2016). Specifically, we examined the diploid *X. tropicalis* and its tetraploid close relative *X. calcaratus*, which are from

subgenus *Silurana*, and the allotetraploids *X. pygmaeus*, *X. allofraseri* (both from the *amieti* species group), *X. laevis* (*laevis* species group), and *X. muelleri* (*muelleri* species group) which belong to subgenus *Xenopus* and arose from the older allotetraploidization. In the subgenus *Xenopus*, the L and S subgenomes diverged from one another about 30–35 Mya, whereas the subgenomes a and b in the tetraploid *Silurana* diverged from one another about 10 Mya (Evans et al. 2015; Session et al. 2016). With an overarching goal of exploring the effect of polyploidization and divergence on the evolution of tandem repeats, we used cytogenetic methods and a genome database to test the hypotheses that (i) the number of the repeat loci in diploid *X. tropicalis* is half that in the allotetraploid species, and (ii) the locations of repeat loci in the studied species are homologous.

Materials and methods

Primary cell cultures and metaphase spread preparations

Primary cell cultures were derived from laboratory strains of *X. tropicalis* (strain ‘Ivory Coast’), *X. laevis*, and *X. muelleri*, and wild-derived strains of *X. calcaratus* and *X. allofraseri* from Cameroon (Bakingili, where they occur in syntopy, 4.0684°N, 9.0682°E) and *X. pygmaeus* from the Democratic Republic of the Congo (Kokolopori, Yalokole, near Luo River, 0.2056°N, 22.8884°E). *Xenopus muelleri* animals were originally obtained from the Institute of Zoology at the University of Geneva (Switzerland). All species were bred at Charles University, Faculty of Science, Prague, Czech Republic. Briefly, tadpoles were anesthetized, hind limbs removed and homogenized (Sinzelle et al. 2012) in a cultivation medium (Knytl et al. 2017) modified according to Knytl et al. (2023). The explants were then cultivated at 29.5 °C with 5.5% CO₂ for five days without disturbance. The media was then changed every day for one week. Passages were performed with trypsin-ethylenediaminetetraacetic acid (Knytl et al. 2017).

Chromosomal suspensions were prepared following Krylov et al. (2010) and stored in fixative solution (methanol/acetic acid, 3:1, v/v) at –20 °C. For cytogenetic analysis, a chromosome suspension was dropped onto a slide (Courtet et al. 2001). Chromosome preparations were aged at –20 °C for at least one week. For each experiment, mitotic metaphase spreads were counterstained with Pro-Long™ Diamond Antifade Mountant with the fluorescent 4',6-diamidino-2-phenylindole, DAPI stain (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). From ten to 20 metaphase spreads were analyzed per probe. Microscopy and processing of metaphase images were conducted using a Leica Microsystem (Wetzlar, Germany) as detailed in Seroussi et al. (2019).

Fluorescent in situ hybridization with repetitive DNA probes

In order to generate probes for FISH, genomic DNA from *X. tropicalis* was used as a template for amplification of the repetitive small nuclear DNA regions (snDNA) U1 and U2, and histone H3. DNA was extracted from tadpole tail tissue using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Primers used for amplification are listed in Table 1. The annealing temperature was 54 °C and the elongation step 30 s for all PCR reactions; other conditions for PCR amplification with PPP Master Mix (Top-Bio, Prague, Czech Republic) followed the manufacturer’s recommendations. Labeling PCR was performed as described in Knytl and Fornaini (2021). Digoxigenin-11-dUTP (Jena Bioscience, Jena, Germany) was used for U2 and H3 labeling, and biotin-16-dUTP (Jena Bioscience) was used for U1 labeling. *Xenopus tropicalis* U1 and U2 snDNA and H3 probes were then hybridized to chromosome spreads of *X. tropicalis*, *X. calcaratus*, *X. pygmaeus*, *X. allofraseri*, *X. laevis*, and *X. muelleri*. The procedures for hybridization mixture preparation, denaturation, and the subsequent overnight hybridization were described previously for rDNA FISH (Knytl et al. 2023). Post-hybridization

Table 1 Genes used for FISH analysis, their GenBank accession numbers, lengths, sequences of primers, and studies in which primers were designed

Gene Symbol	Gene Name	Accession No	Length (bp)	Primer Sequence	Citation
U1	Small nuclear RNA U1	OQ714817	119	U1F: 5'-GCAGTCGAGATCCCACATT-3' U1R: 5'-CTTACCTGGCAGGGGAGATA-3'	Silva et al. (2015)
U2	Small nuclear RNA U2	OQ714818	177	U2F: 5'-ATCGCTTCTCGGCCTTATG-3' U2R: 5'-TCCCGCGGTACTGCAATA-3'	Bueno et al. (2013)
H3	Histone H3	OQ714819	364	H3F: 5'-ATGGCTCGTACCAAGCAGAC(ACG)GC-3' H3R: 5'-ATATCCTT(AG)GGCAT(AG)AT(AG)GTGAC-3'	Colgan et al. (1998)

washing and blocking reactions were performed as described for painting FISH in Krylov et al. (2010). Probe visualization was performed following Knytl et al. (2017). Slides were then de-stained according to the following protocol: nail polish was removed using xylene (2 min) and benzoin (2 min). Slides with cover slip were incubated in 4×SSC/0.1% Tween for 10 min with agitation, and then cover slips were manually removed. Slides without cover slip were then incubated in 4×SSC/0.1% Tween for 30 min with agitation followed by dehydration with methanol series (70, 90, 100% for 3 min each) and then air dried. After slide incubation in fixative solution for 30 min, slides were rinsed with distilled water, air dried, and then aged for 90 min at 60 °C.

Painting FISH

We used whole chromosome painting probes generated by laser microdissection of *X. tropicalis* chromosomes from previous study by Knytl et al. (2023). Whole chromosome painting probes from *X. tropicalis* chromosomes 1 and 8 were newly labeled with digoxigenin-11-dUTP (Krylov et al. 2010) and biotin-16-dUTP (both Jena Bioscience) (Knytl et al. 2023), respectively. De-stained slides (after FISH with the U1 and U2 probes) of *X. tropicalis*, *X. calcaratus*, and *X. laevis* were used for cross-species painting FISH (Zoo-FISH) with a digoxigenin-labeled probe according to the protocols described in Krylov et al. (2010) and modified in Knytl et al. (2017). Subsequently, slides were de-stained again and used for Zoo-FISH with biotin-labeled probe. Detection of signal was carried as detailed for double-color painting in Knytl et al. (2023). The *X. tropicalis* FISH experiments were performed in the reverse order from the other species (i.e., painting FISH first followed by de-staining and snDNA FISH).

Results

Sanger sequencing and BLAST searching

Amplification of the U1 and U2 snDNA and H3 nuclear genes yielded 119 and 177 and 364 bp long amplicons, respectively. Based on BLASTn searches of the *X. tropicalis* “Nigeria” strain genome, the U1 amplicon had 98.3% identity (query cover 100%) with the sequence of U1 spliceosomal RNA, LOC116408489 (accession number XR_004220992.1); U2 amplicon had 98.3% identity (query cover 100%) with U2 spliceosomal RNA, LOC116407440 (accession number XR_004220346.1); and the H3 amplicon had 97.2% identity (query cover 98%) with histone H3, LOC100497127, mRNA (accession number XM_012953339.3). Our sequences were deposited to

Fig. 2 Double-color FISH with U1 and U2 snDNA probes. The U1 probe (red) reveals one clear signal (= a pair of homologous chromosomes) in **a** *X. tropicalis*, **c** *X. pygmaeus*, **d** *X. allofraseri*, and **e** *X. laevis*, while the same FISH shows two signals, each within homoeologous chromosomes in **b** *X. calcaratus* and **f** *X. muelleri*. The U2 (green) probe shows one signal in **a** *X. tropicalis*, **c** *X. pygmaeus*, **d** *X. allofraseri*, and **e** *X. laevis*, while the U2 probe shows two signals, each of them within homoeologous chromosomes in **b** *X. calcaratus* and **f** *X. muelleri*. Green and red arrows correspond to the U2 and U1 repeat loci, respectively. Painting probes were used for identification of chromosomes 1 (green) and 8 (red) in **a** *X. tropicalis*. Chromosomes were counterstained with DAPI in blue/gray. Scale bars represent 10 μM

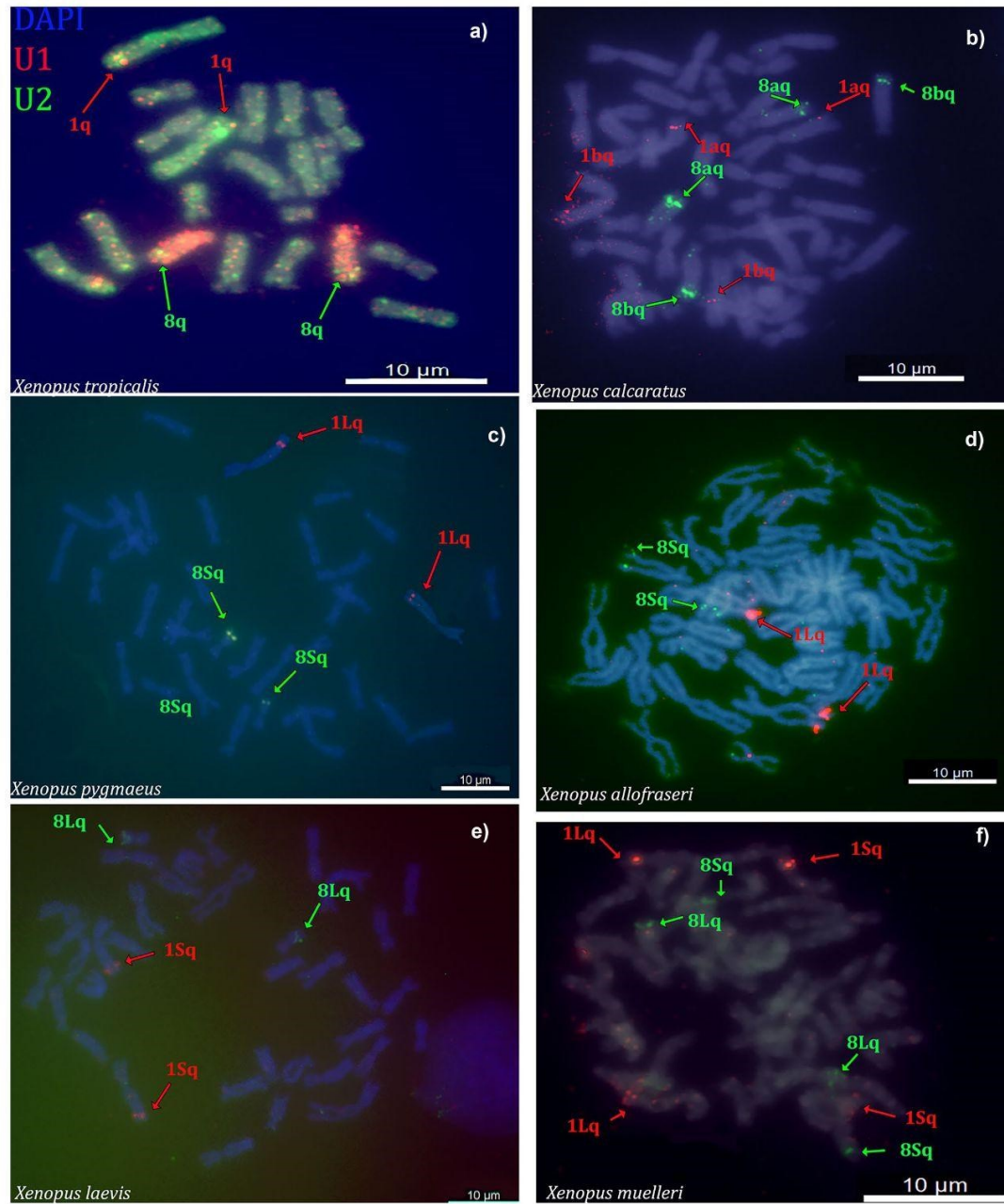
the NCBI GenBank database: U1 snDNA accession number OQ714817, U2 snDNA accession number OQ714818, H3 accession number OQ714819.

FISH with U1 and U2 snDNA probes and their genomic locations in available *Xenopus* genome assemblies

When hybridized on the diploid species *X. tropicalis*, the U1 and U2 snDNA probes had intense signals on the distal region of the long (q) arm of chromosomes 1 and 8 (Fig. 2(a)), respectively. The number of copies was determined based on matches at least 50% of the query sequence length and 85% of the query sequence identity. BLAST results using the U1 sequence as a query to the *X. tropicalis* genome identified ~20 copies of U1 snDNA on chromosome 1. Using the U2 sequence as a query, we identified ~40 copies on *X. tropicalis* chromosome 8. In the allotetraploid species *X. calcaratus*, the U1 and U2 snDNA probes hybridized to the q arms of the chromosomes 1a, 1b and 8a, 8b (Fig. 2(b)), respectively. Chromosomes 1a and 1b are homoeologous to each other, and they are orthologous to *X. tropicalis* chromosome 1, and the mapped U1 region in *X. calcaratus* is thus homologous to the orthologous U1 region of *X. tropicalis* (both species have U1 gene on the distal part of the same chromosome). The U2 snRNA locus also was detected on both homoeologous chromosomes of *X. calcaratus* (chromosomes 8a and 8b) and in a homologous location to the orthologous U2 gene of *X. tropicalis*. For detailed definitions of homologous, homoeologous, and orthologous genes and chromosomes in *Xenopus*, see Song et al. (2021).

These U1 and U2 snDNA probes thus each hybridized to both homoeologous chromosomes in *X. calcaratus* as expected if copy number of each repeat was doubled by allopolyploidization. Similarly, in *X. muelleri*, U1 and U2 snDNA probes mapped to the expected homoeologous chromosomes (U1 on 1Lq and 1Sq; U2 on 8Lq and 8Sq; Fig. 2(f)).

In the allotetraploid species *X. pygmaeus*, *X. allofraseri*, and *X. laevis* only one signal was detected for U1 and U2 snDNAs. For the more closely related pair — *X. pygmaeus* and *X. allofraseri* — the U1 snDNA probe hybridized



most conspicuously to the q arms of chromosome 1L and the U2 snDNA probe hybridized to the q arms of chromosome 8S (Fig. 2(c, d)). In *X. laevis*, the signal of the U1 and U2 snDNA probes was most conspicuous on the q arms of

chromosomes 1S and 8L, respectively (Fig. 2(e)). Both U1 and U2 snRNA signals in *X. laevis* were located on chromosomes (1S and 8L) that are homoeologous to the chromosomes that bear U1 and U2 snRNA signals in *X. pygmaeus*

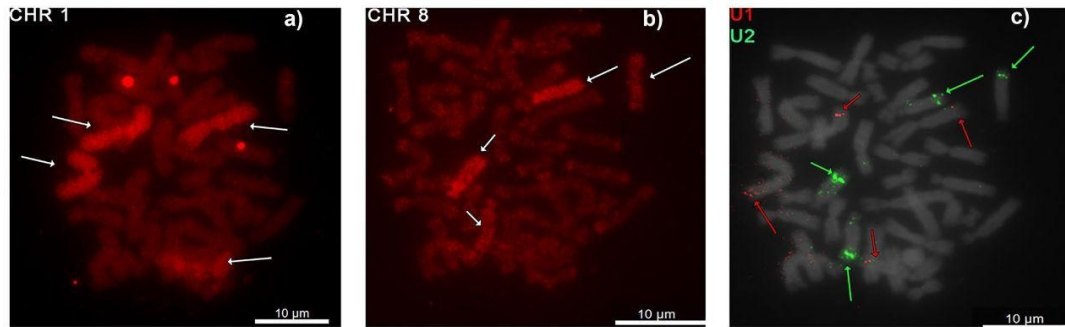


Fig. 3 Sequential FISH in *X. calcaratus* chromosomes. Cross-species painting FISH experiments with the whole chromosome painting probes from *X. tropicalis* **a** chromosome 1 (XTR 1) and **b** XTR 8 highlight chromosome-bearing U1 and U2 snRNA loci, respectively. **c** snDNA FISH illustrates that the U1 locus (in red)

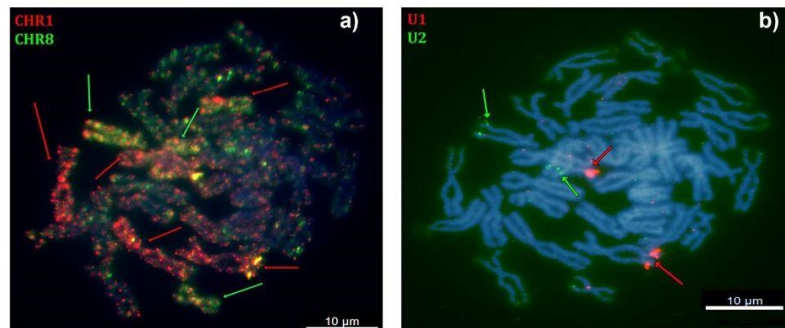
is localized on *X. calcaratus* chromosomes 1 (XCA 1a and 1b) and the U2 locus (in green) on XCA 8a and 8b. **a** and **b** are shown in the red channel, **c** is merged in the red-green-blue (RGB) channels. **c** chromosomes were counterstained with DAPI in gray. Scale bars represent 10 μM

and *X. allofraseri* (1L and 8S) and vice versa. The number of loci did not support the expectation that all tetraploid species should have twice as many snRNA loci as the diploid *X. tropicalis*, a result that could indicate copy number reduction or loss. Consistent with a scenario of reduction in copy number as opposed to complete loss, a BLAST search using U1 snDNA sequence as a query to the *X. laevis* genome recovered 11 copies on chromosome 1L and ~35 copies on chromosome 1S. Using the U2 sequence as a query against the *X. laevis* genome sequence, we identified ~40 copies on *X. laevis* chromosome 8L and 12 copies on chromosome 8S.

Painting FISH

To identify chromosomes bearing U1 and U2 loci, we employed FISH with the whole chromosome painting probes from *X. tropicalis* chromosomes 1 and 8. We successfully identified chromosomes 1 and 8 in *X. tropicalis* (Fig. 2(a)); chromosomes 1a, 1b, 8a, and 8b in *X. calcaratus* (Fig. 3); and chromosomes 1L, 1S, 8L, and 8S in *X. laevis* (Fig. 4).

Fig. 4 Sequential FISH in *X. laevis* chromosomes. Cross-species painting FISH experiments with whole chromosome painting probes from **a** XTR 1 (in red) and XTR 8 (in green) illustrate that **b** the U1 locus (in red) is localized on *X. laevis* chromosomes 1S (XLA 1S), and the U2 locus (in green) on XLA 8L. Both **a** and **b** are merged in the RGB channels. Chromosomes were counterstained with DAPI in blue. Scale bars represent 10 μM



Homoeologous chromosomes of *X. calcaratus* and *X. laevis* were identified based on the intensity of the fluorescence signal (Knytl et al. 2023) and in the other studied species in subgenus *Xenopus* in which the painting FISH approach was not conducted; instead, homoeologous chromosomes were distinguished based on chromosome length (the L homoeologous chromosomes are longer than the S chromosomes; Matsuda et al. 2015; Session et al. 2016).

FISH with histone H3 probe and its genomic locations in available *Xenopus* genome assemblies

We then hybridized H3 probe to chromosome spreads of *X. tropicalis*, *X. calcaratus*, *X. pygmaeus*, *X. allofraseri*, *X. laevis*, and *X. muelleri*. In all species, signals were present in small patches on multiple chromosomes (Fig. 5). We found signals in all chromosomes in *X. tropicalis*, *X. calcaratus*, and *X. muelleri*. However, *X. pygmaeus*, *X. allofraseri*, and *X. laevis* had signals on about half of chromosomes. The H3 probes mapped to telomeric and pericentromeric regions.

Based on BLAST searches, the H3 sequence occurs on *X. tropicalis* chromosomes 3 (11 hits), 6 (11 hits), and 9 (27 hits), and on chromosomes 5, 8, and 10, the matches were less than 50% of the query length. In *Xenopus laevis*, the H3 sequence occurs on chromosomes 3S (12 hits), 5L (6 hits), 5S (3 hits), 6L (2 hits), 6S (1 hit), 9_10L (15 hits), and 9_10S (5 hits). *Xenopus laevis* chromosomes 1L and 8L show some hits being less than 50% of the query sequence. All U1 and U2 snRNA and H3 loci mapped by FISH and BLAST are shown in Table 2.

Discussion

Quantification and localization of repetitive sequences using high-quality genome sequencing and assembly is an expensive and challenging undertaking as compared to using multiple cytogenetic approaches for gene mapping (Knytl et al. 2018b; Symonová et al. 2017a). As an alternative, we cytogenetically mapped non-rDNA tandem repeats (U1 and U2 snRNA and H3 histone) to one diploid and five allotetraploid *Xenopus* species with the aim of testing how repetitive elements were affected by genome duplication and divergence. At least two allotetraploidization events occurred in genus *Xenopus* with respect to our focal species and both — including studied species and their diploid extinct or yet undescribed predecessors — are depicted in Fig. 1.

Barring loss or movement of tandem repeats, we expected allotetraploid species to have twice as many tandem repeats as the diploid *X. tropicalis*, and that the locations of these elements would be unchanged (i.e., on the same region of both homoeologous chromosomes). In general, the cytogenetic results recovered no evidence for long-range movement of the U1, U2 snRNA or H3 loci. In the allotetraploids *X. calcaratus* and *X. muelleri*, the observed number of signals for each of the U1 and U2 snRNA loci matched our expectation based on allopolyploidization. However, for the three other allotetraploids (*X. pygmaeus*, *X. allofraseri*, and *X. laevis*), this expectation was not supported because, although in homologous locations, we found the same number of these tandem repeats as in the diploid *X. tropicalis* (Table 2). Furthermore, the snRNA signals in *X. pygmaeus* and *X. allofraseri* were on a different subgenome (U1 on L, U2 on S) than in *X. laevis* (U1 on S, U2 on L). Overall, this is consistent with an increased variation in copy number over time following allopolyploidization. BLAST searches of the *X. laevis* genome recovered each of these loci in both subgenomes, and it appears that the lower-than-expected number of cytogenetic signals is thus a consequence of differences in copy number, at least in this species. That the signals in *X. pygmaeus* and *X. allofraseri* are on different subgenomes than in *X. laevis* suggests that changes in copy number have been an ongoing phenomenon during *Xenopus*

diversification. During diversification of African clawed frogs, the ancestor of *X. muelleri* diverged from the common ancestor of *X. pygmaeus*, *X. allofraseri*, and *X. laevis* soon after allotetraploidization in subgenus *Xenopus*, around 17 Mya (Fig. 1; Evans et al. 2015; Session et al. 2016). Thus, a parsimonious interpretation of these results posits that changes in copy number arose independently in an ancestor of *X. laevis* and again in the most recent common ancestor of *X. pygmaeus* and *X. allofraseri*.

In amphibians, U2 snRNA tandem repeats have also been localized in the cycloramphid genus *Thoropa* (Cholak et al. 2020) and the leptodactylid genus *Leptodactylus* (Gazoni et al. 2021), and the H3 gene has been localized in the pipid genus *Pipa* (Zattera et al. 2020). To our knowledge, there are no other cytogenetic studies that localize the U1, U2 snRNA, or H3 gene loci in this vertebrate group.

In frog genus *Thoropa*, U2 snRNA was detected cytogenetically on chromosomes 6 and 7 in some species, but others had U2 locus only on chromosome 6 or only on chromosome 7 (Cholak et al. 2020). The explanation for this variation might be that U2 loci are universally situated on both chromosomes 6 and 7 in all investigated *Thoropa* species but with different copy numbers on each of these chromosomes. We found similar variability in the location and number of FISH signals. Using BLAST, we identified locations where the sequence of our probe matched the *X. laevis* genome sequence and found that the number of U1 and U2 snRNAs detected by BLAST did not match the number of snRNAs detected by FISH. This inconsistency may be due to variation in the copy number of tandem repeats in each *X. laevis* subgenome and the lack of sensitivity of FISH to detect the locus with a low copy number of repeats. The U1 and U2 snRNA FISH signals were cytogenetically detected on those *X. laevis* chromosomes that contain ~ 15 and more copies of U1 and U2 repeats. In *Leptodactylus*, the U2 loci were identified on chromosome 6 in eight species, but one of them had signals on chromosomes 4, 6, 9, and 10 (Gazoni et al. 2021). These authors proposed that the additional signals on chromosomes 4, 9, and 10 can be the result of transposition by TEs followed amplification of the gene. We did not map TEs on *Xenopus* chromosomes and are thus unable to evaluate the possibility of TE transposition in this genus.

We also evaluated the effects of allotetraploidization and divergence on the evolution of H3 repeats. As expected, due to allotetraploidization, the allotetraploids *X. calcaratus* and *X. muelleri* possessed H3 repeats on twice as many chromosomes as the diploid *X. tropicalis*. However, in the allotetraploid *X. pygmaeus*, *X. allofraseri*, and *X. laevis*, we found signals on eight homologous pairs, which are fewer than expected based on allotetraploidization. This presumably is due to a decreased copy number or deletion of H3 repeats on several chromosomes, possibly in an ancestor of *X. pygmaeus*, *X. allofraseri*, and *X. laevis* based on phylogenetic

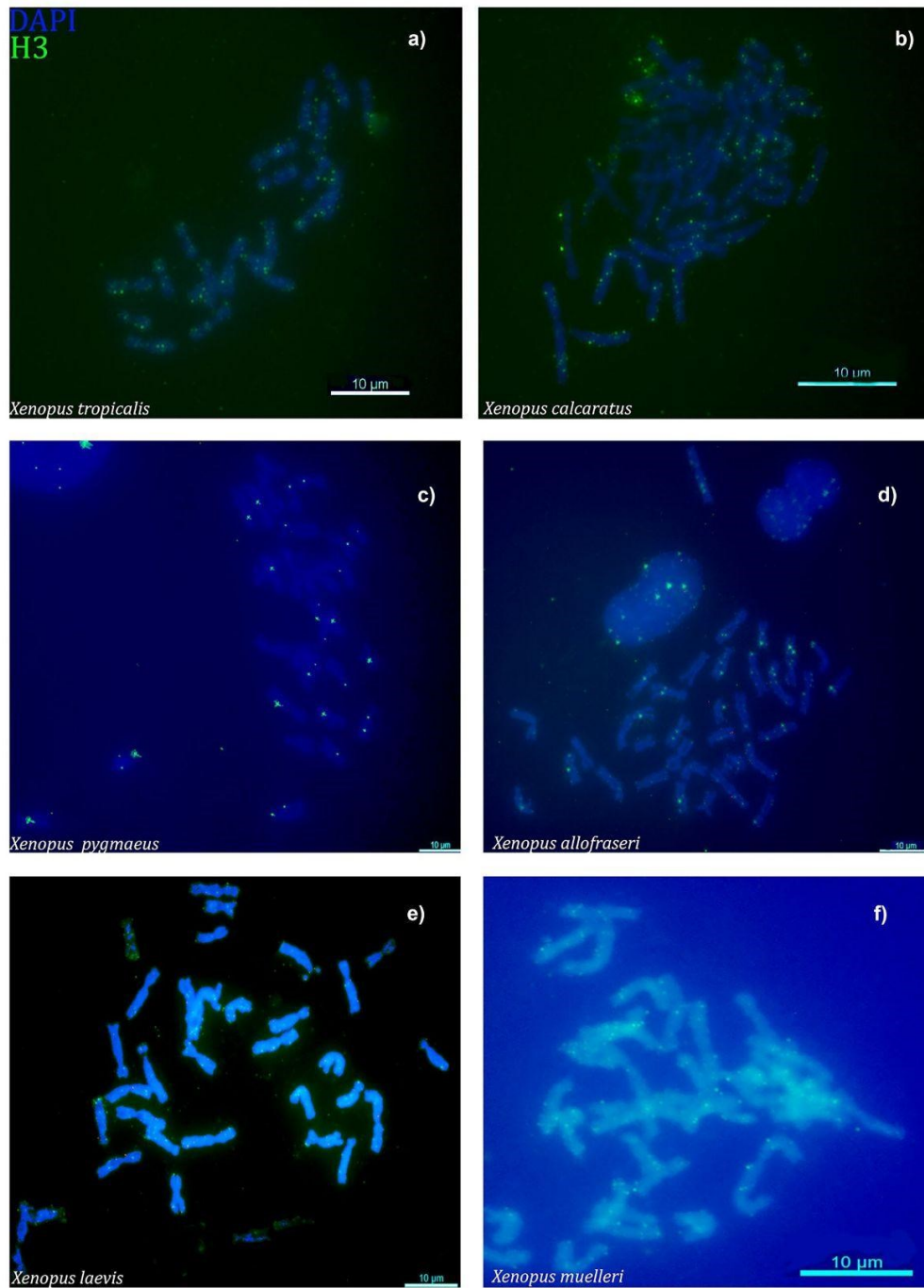


Fig. 5 FISH with histone H3 probe. The probe (green) shows multiple signals in **a** *X. tropicalis*, **b** *X. calcaratus*, **c** *X. pygmaeus*, **d** *X. allofraseri*, **e** *X. laevis*, and **f** *X. muelleri*. Chromosomes were counterstained with DAPI in blue/gray. Scale bars represent 10 µM

Table 2 Numbers and chromosomal positions of U1 and U2 snRNA and H3 loci per haploid genome in diploid *X. tropicalis* (subgenus *Silurana*) and reduced genome (half of the somatic set) in allotetraploid *X. calcaratus* (subgenus *Silurana*), *X. pygmaeus*, *X. allofraseri* (both in the *amieti* species group), *X. laevis* (*laevis* species group), and *X. muelleri* (*muelleri* species group). The numbers of loci are

given according to the FISH results and the genomic database. The XTR, XCA, XPY, XAL, XLA, and XMU abbreviations correspond to chromosome of *X. tropicalis*, *X. calcaratus*, *X. pygmaeus*, *X. allofraseri*, *X. laevis*, and *X. muelleri*, respectively. NA means that information is not available in the genome database

Species	Number of chromosomes	U1 snRNA by FISH	U1 snRNA genomic database	U2 snRNA revealed by FISH	U2 snRNA genomic database	H3 by FISH	H3 genomic database
<i>X. tropicalis</i>	n=10	1 (XTR 1)	1 (XTR 1)	1 (XTR 8)	1 (XTR 8)	10	6
<i>X. calcaratus</i>	n=20	2 (XCA 1a and 1b)	NA	2 (XCA 8a and 8b)	NA	20	NA
<i>X. pygmaeus</i>	n=18	1 (XPY 1L)	NA	1 (XPY 8S)	NA	8	NA
<i>X. allofraseri</i>	n=18	1 (XAL 1L)	NA	1 (XAL 8S)	NA	8	NA
<i>X. laevis</i>	n=18	1 (XLA 1S)	2 (XLA 1L, XLA 1S)	1 (XLA 8L)	2 (XLA 8L, XLA 8S)	8	9
<i>X. muelleri</i>	n=18	2 (XMU 1L and 1S)	NA	2 (XMU 8L and 8S)	NA	18	NA

relationships among these species. Despite the variability associated with allopolyploidization followed by divergence in evolution of tandem repeats, the relative dosages of signals of U1, U2, and H3 with respect to *X. tropicalis* are similar in each allotetraploid species we examined.

Until recently, there was only one study in which the H3 gene was mapped on amphibian chromosomes (Zattera et al. 2020). In *Pipa carvalhoi*, a representative of the same family as *Xenopus* but deeply phylogenetically divergent by about 115–120 My (Feng et al. 2017; Hime et al. 2021), H3 signals were detected on chromosomes 1, 5, 6, 8, and 9, and less intense signals were visible on other chromosomes. Multiple distributions of H3 locus on all chromosomes have been observed, for example, in insect species of the family Acrididae (Oliveira et al. 2011; Bueno et al. 2013) where the distribution of the H3 locus may be related to the transposition of 5S ribosomal RNA (rRNA). In *Xenopus*, 5S rRNA is situated on telomeres of multiple chromosomes (Knytl et al. 2017, 2023), and therefore, the observed distribution of the H3 locus on multiple *Xenopus* chromosomes may also be associated to the distribution of 5S rRNA.

Co-localization of repetitive elements

An interesting finding that emerged from this study is a genomic association of various repetitive elements with rRNA; this can potentially provide evolutionary insights into genome organization. Synteny exists between As51 satellite DNA (originating from TEs) and NORs (Vicari et al. 2008) or between U1 snRNA and 5S rRNA in the characid fish genus *Astyanax* (Silva et al. 2015). One of possible roles of a co-localization can be a silencing of ribosomal genes by TEs (Vicari et al. 2008). All *Xenopus* species have NOR on a single homologous pair (Tymowska 1991). *Xenopus tropicalis* has NOR on chromosome 7, tetraploids from subgenus *Silurana* on 7a, and tetraploids from subgenus *Xenopus* on

3L (Tymowska 1991; Session et al. 2016; Roco et al. 2021; Knytl et al. 2023). We have not identified any snRNA loci on chromosomes 7, 7a, or 3L, and this finding argues against a genomic association between distinct NOR and snRNA repeats. The 5S ribosomal genes have been detected at the distal regions of the *X. melnotropicalis* and *X. calcaratus* (*Silurana* tetraploids) chromosomes 8bq (Knytl et al. 2017, 2023) which is the same position as the U2 snRNA locus in *X. calcaratus* (this study), supporting a genomic association between 5S rRNA and U2 snRNA. The NOR locus presumably was deleted on chromosome 7b in tetraploids of subgenus *Silurana* and on chromosome 3S in tetraploids of subgenus *Xenopus* (Session et al. 2016; Knytl et al. 2023). However, U1 and U2 loci map to both homoeologs in some tetraploids, and thus, deletion of snRNA in an ancestor of *Xenopus* tetraploids did not take place. There is the possibility that all our investigated *Xenopus* tetraploids have twice as many snRNA repeat loci as the diploid *X. tropicalis* but that these loci have unequal copy number within individual subgenomes, meaning that copy number of snRNA repeats was reduced in one subgenome (11 copies of U1 snRNA on *X. laevis* chromosome 1L is less than 20 copies on *X. tropicalis* chromosome 1; thus, the copy number of U1 snRNA on *X. laevis* chromosome 1L was reduced) but expanded in the other subgenome (35 copies of U1 snRNA on *X. laevis* chromosome 1S versus 20 copies in *X. tropicalis*). Moreover, some tandem repeat loci maintain a stable number of copies, for example, 40 copies of U2 snRNA on *X. tropicalis* chromosome 8 and *X. laevis* chromosomes 8L). These stable copy numbers within the U2 gene are consistent with studies that observed a higher evolutionary conservation of the *X. laevis* subgenome L than subgenome S, compared to *X. tropicalis* genome (Session et al. 2016). Reduction, expansion, and stability in the copy number of non-rDNA tandem repeats and complete loss of rRNA locus are the possible events that occur after polyploidization (Session et al. 2016;

Knytl et al. 2023). Genes retained as duplicates have distinct evolution leading to loss of function, subfunctionalization, or neofunctionalization and are under strong purifying selection (Force et al. 1999; Lynch et al. 2001), often followed by intra- and/or interchromosomal translocations, inversions, deletions, or degenerations (Evans 2007; Sémon and Wolfe 2007; Session et al. 2016; Knytl et al. 2017). Our study highlighted that copy number reduction and expansion of tandem repeats can be an important driver of evolution following allopolyploidization such as translocation, inversion, deletion, and degeneration.

Conclusion

Repetitive elements are integral components of a genome, and their evolution can be affected by mechanism of polyploidization or divergence among species. We used cytogenetic approaches to study repetitive elements in six species of *Xenopus* frogs (one diploid, five allotetraploids) with an aim of localizing and quantifying tandem repeats in their genomes. For U1 and U2 snRNA, and H3 tandem repeats, the locations were generally homologous, but we detected variation in copy numbers that resulted from reduction and expansion after allotetraploidization. The dynamic evolution of tandem repeats was most apparent in allotetraploid species that arose from the older allotetraploidization event.

Acknowledgements We thank two anonymous reviewers for their constructive comments on early versions of the manuscript. Also, we thank Xenbase (Karimi and Vize 2014) for making available the high-quality genome assemblies of *X. tropicalis* (v10.1) and *X. laevis* (v9.2).

Author contributions Laboratory experiments [Nicola R. Fornaini], [Barbora Bergelová], [Václav Gvoždík] and [Halina Černohorská]; Graphic design [Nicola R. Fornaini] and [Ben J. Evans]; Fieldwork [Václav Gvoždík], [Eric B. Fokam], [Gabriel Badjedjea] and [Ben J. Evans]; Funding acquisition [Václav Gvoždík], [Halina Černohorská], [Svatava Kubíčková], [Ben J. Evans] and [Martin Knytl]; Conceptualization of the study [Václav Gvoždík], [Ben J. Evans], [Martin Knytl]; Visualization [Martin Knytl]; Data curation and validation [Martin Knytl]; Project administration [Martin Knytl]; Writing – original draft preparation [Martin Knytl]; Supervision [Martin Knytl]; All authors reviewed, edited, and approved the final version of the manuscript.

Funding Open access publishing supported by the National Technical Library in Prague. The research was supported by the institutional support from the IVB CAS (RVO: 68081766); the Ministry of Culture of the Czech Republic (DKRVO 2019–2023/6.VII.e, National Museum, 00023272) (both VG); institutional support from the Ministry of Agriculture of the Czech Republic (MZE-RO0518) (HČ, SK); the Natural Science and Engineering Research Council of Canada (RGPIN-2017–05770) (BJE); the P JAC project CZ.02.01.01/00/22_010/0002902 MSCA Fellowships CZ – UK (MK).

Data availability The Sanger sequences that support the findings of this study are openly available in GenBank (accession numbers OQ714817–9).

Declarations

Statement of ethics Charles University has registered experimental breeding facilities for pipid frogs (16OZ12891/2018–17214, 37428/2019-MZE-18134). All experimental procedures involving frogs were approved by the Institutional Animal Care and Use Committee of Charles University, according to the directives from the State Veterinary Administration of the Czech Republic, reference number MSMT-20585/2022–4 issued by the Ministry of Education, Youth and Sport of the Czech Republic (MK is a manager of the experimental project on living *Xenopus* animals). MK is a holder of the Certificate of professional competence to design experiments according to §15d(3) of the Czech Republic Act No. 246/1992 coll. on the Protection of Animals against Cruelty (Registration number CZ 03973), provided by the Ministry of Agriculture of the Czech Republic.

Conflict of interest The authors declare no competing interests.

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Original Article

Tetraploidy in the Boettger's dwarf clawed frog (Pipidae: *Hymenochirus boettgeri*) from the Congo indicates non-conspecificity with the captive population

Václav Gvoždík^{1,2,*}, Martin Knytl^{3,4,*}, Ange-Ghislain Zassi-Boulou⁵, Nicola R. Fornaini³, Barbora Bergelová³

¹Institute of Vertebrate Biology of the Czech Academy of Sciences, Brno, Czech Republic

²National Museum of the Czech Republic, Department of Zoology, Prague, Czech Republic

³Department of Cell Biology, Faculty of Science, Charles University, Viničná 7, Prague 128 43, Czech Republic

⁴Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, L8S 4K1, Canada

⁵Department of Biology, National Institute for Research in Exact and Natural Sciences, Brazzaville, Republic of the Congo

*V. Gvoždík and M. Knytl are co-first and co-senior authors in alphabetical order.

Corresponding authors. Institute of Vertebrate Biology of the Czech Academy of Sciences, Studenec 122, 675 02 Studenec, Czech Republic. E-mail: vaclav.gvozdik@gmail.com; Department of Cell Biology, Faculty of Science, Charles University, Viničná 7, 128 43 Prague, Czech Republic. E-mail: martin.knytl@natur.cuni.cz

ABSTRACT

Cytogenetics can be used as a tool to study the evolution of polyploidy and taxonomy. Here we focus on aquatic African pipids, dwarf clawed frogs (*Hymenochirus*). Our study reveals that dwarf clawed frogs, present for decades in captivity, are best referred to as *Hymenochirus* sp. instead of the commonly used name '*H. boettgeri*' or sometimes '*H. curtipes*'. We present the first karyotype from a morphologically identified specimen of *H. boettgeri* with a known locality in the north-western Congo, which is tetraploid with $2n = 36$. The captive *Hymenochirus* species has been found diploid in previous studies with different reported chromosome numbers; here we reveal $2n = 20A + 1B$ chromosomes. Our findings suggest that the tetraploid *H. boettgeri* karyotype evolved through fusion of two binned chromosomes and subsequent allotetraploidization, and is functionally diploid, similar to the origin of tetraploid clawed frogs in the subgenus *Xenopus*. We observed the stable presence of a single B chromosome in both sexes of our individuals from the captive population of *Hymenochirus* sp. However, additional investigation is necessary to clarify whether there is variation in the number of A and B chromosomes among populations, individuals, and/or tissues. Further research is also needed to understand the evolution and taxonomy of the genus *Hymenochirus*.

Keywords: amphibia; Anura; chromosome; cytogenetics; FISH; karyotype; polyploidy; taxonomy

INTRODUCTION

Amphibians represent a vertebrate clade that contains >8600 species (AmphibiaWeb 2023; Frost 2023) and approximately 100 species from 19 families have been identified as polyploid (Mezzasalma *et al.* 2023). Polyploidy in amphibians has evolved independently several times in multiple families, unlike, for example, teleost fishes, where whole genome duplication arose in the whole group as an ancestral evolutionary process (Schmid *et al.* 2015). Amphibians form both diploid and polyploid populations/species with sexual reproduction, making them a unique group compared to other vertebrate lineages (Bogart 1980, Mezzasalma *et al.* 2023). There is, therefore, a great advantage

that diploids can serve as reference material for evolutionary and cytogenetic studies of related polyploids (Bogart and Bi 2013, Knytl *et al.* 2018, Fornaini *et al.* 2023). Polyploidy can arise in two different ways. Autopolyploidy, less common in amphibians, arises within a single ancestor in which meiotic incompatibilities are caused by environmental factors. A second possible origin is allopolyploidization, initiated by hybridization of two or more ancestors divergent enough to promote meiotic incompatibilities that prevent following cytokinesis.

Pipid frogs (family Pipidae) represent an ancient evolutionary lineage of fully aquatic frogs that split from its sister-lineage Rhinophrynidae more than 150 Mya. The extant pipid frogs

Received 28 April 2023; revised 27 July 2023; accepted 2 August 2023
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are divided into two subfamilies, the American Pipinae (*Pipa* Laurenti, 1768) and the African Dactylethrinae (split ~110 Mya) containing two deeply divergent tribes (split ~100 Mya), Dactylethriini (*Xenopus* Wagler, 1827) and Hymenochirini (*Hymenochirus* Boulenger, 1896 and *Pseudhymenochirus* Chabanaud, 1920); for evolutionary relationships, see *Feng et al.* (2017), *Dubois et al.* (2021), and *Hime et al.* (2021). The genus *Xenopus* is divided into two subgenera, *Xenopus* and *Silurana* Gray, 1864, which are sometimes considered valid genera (e.g. *Dubois et al.* 2021). Pipids are known as a group with highly variable karyotypes, particularly chromosome numbers, primarily due to the role of polyploidization in their evolutionary history (*Tymowska* 1991, *Schmid et al.* 2015). *Mezzasalma et al.* (2015) suggested that the ancestral karyotype of pipids was $2n = 20$, which can be observed in *Xenopus* (subgenus *Silurana*), *Hymenochirus*, *Pseudhymenochirus*, and *Pipa* (*P. carvalhoi* (Miranda-Ribeiro, 1937)). These authors also proposed three mechanisms for the increase in chromosome number in pipid frogs: allopolyploidy, fission, and addition of B chromosomes. In pipids, polyploidy, especially allopolyploidy, has so far only been found in *Xenopus*, including *Silurana* (*Schmid et al.* 2015). The haploid number $n = 10$ is present in *Silurana* and $n = 9$ in the subgenus *Xenopus* in which fusion of chromosome 9 and 10 occurred (*Session et al.* 2016). Recently, a fusion of chromosome 8 and 10 was documented in *Hymenochirus* (*Bredeson et al.* 2021). It has been hypothesized that chromosome fissions occurred in *Pipa* (*Mezzasalma et al.* 2015), and potentially also occurred in Central African *Hymenochirus* (see results in: *Morescalchi* 1968, 1981, *Scheel* 1973).

The addition of B chromosomes was found in the captive (pet trade) population of '*Hymenochirus boettgeri*' ($2n = 20A + 1B$), with B chromosomes detected in approximately half of the karyotypes scored in the two males studied (30/66 metaphase spreads obtained from the intestine, spleen, gonads, and lung tips; the remainder had $2n = 20$; *Mezzasalma et al.* 2015). On the other hand, previous, relatively old studies of '*H. boettgeri*' reported $2n = 24$ chromosomes (*Morescalchi* 1968; republished by: *Morescalchi* 1981, *Tymowska* 1991), or *Scheel* (1973) stated karyotype $2n = 22$. The latter only stated the chromosome number and provided no further details. Finally, a recent study found $2n = 18$ chromosomes and no B chromosomes in '*H. boettgeri*' (*Bredeson et al.* 2021). It is unclear whether the differences in chromosome number represent variation in B chromosome number (with the exception of $2n = 18$, where chromosome fusion occurred), as hypothesized by *Mezzasalma et al.* (2015), or whether earlier researchers (*Morescalchi* 1968, 1981, *Scheel* 1973) studied one or more different species in which chromosome fissions occurred.

The captive stock of dwarf clawed frogs (*Hymenochirus* spp.) probably originated in the 1950s, when dwarf clawed frogs were imported from the wild by pet fish companies to Europe and the USA (*Sokol* 1959, *Olsson* and *Österdahl* 1960). The only available geographic information states that dwarf clawed frogs 'were shipped from Leopoldville'—present-day Kinshasa in the Democratic Republic of the Congo (*Rabb and Rabb* 1963)—or 'from Stanley Pool' (*Sokol* 1962), an area north-east of Kinshasa. However, Leopoldville could only have been the place of shipment or Stanley Pool the place of arrival (collectors could have landed here, as it is an area with ports), not the actual

area of origin. At least two species, traditionally referred to as *H. boettgeri* (Tornier, 1896) and *H. curtipes* Noble, 1924, were probably imported in the 1950s and possibly later (e.g. *Sokol* 1959, 1962), the latter species being less common (*Rabb and Rabb* 1963, *Sokol* 1969). *Hymenochirus curtipes* has been reported to be an open area species (*Noble* 1924), requiring higher temperature in captivity and especially for breeding, and its tadpoles require more space (*Sokol* 1962). Therefore, it has been hypothesized that *H. curtipes* gradually disappeared from captivity due to its more specialised breeding requirements (*Kunz* 2002). However, names of both species can still be found in the aquarium/herpetoculture literature, although only one species seems to have been found in aquaria in recent decades (*Kunz* 2002, 2003). It is usually called *H. boettgeri*, although it has been noted that a taxonomic revision of the genus is needed (*Kunz* 2004).

The conspecificity of the aquarium population with *H. boettgeri* has sometimes been questioned based on certain morphological differences, and it has even been hypothesized that the aquarium population may represent a 'domesticated' hybrid between *H. boettgeri* and *H. curtipes* (*Cecere* 1998). Typical morphological features of *H. boettgeri* are, for example, the sides of the body covered with enlarged tubercles, the body rather oval, the head broad, and the dorsum monochromatic (*de Witte* 1930, *Perret* 1966, *Arnoult and Lamotte* 1968). This does not correspond much to the aquarium population, which is characterized by the body sides with homogeneous tubercles, without differentiated verrucosity, the body oval to pear-shaped, the head not distinctly broad, and the dorsum often mottled (*Kunz* 2004). Dwarf clawed frogs from the aquarium population are usually kept as a hobby animal and usually not as a laboratory animal. Nevertheless, it has been used in several laboratory studies where frogs were typically obtained from the pet trade and the name '*H. boettgeri*' is usually used in publications (e.g. *Mezzasalma et al.* 2015, *Höbel and Fellows* 2016, *Miller et al.* 2019, *Cauret et al.* 2020). *Bredeson et al.* (2021) referred to this dwarf clawed frog as an emerging model species. However, given the confusion with the species identification of the captive population since its establishment in aquaria (*Rabb and Rabb* 1963, *von Filek* 1967, *Cecere* 1998, *Kunz* 2002), we recommend naming the captive population of dwarf clawed frogs as *Hymenochirus* sp. We will use this name hereafter.

To date, all cytogenetic findings in the tribe Hymenochirini have been based on conventional and Giemsa staining, and banding techniques (*Morescalchi* 1968, *Mezzasalma et al.* 2015). No fluorescent *in situ* hybridization (FISH) experiments have been performed to study chromosome evolution in Hymenochirini. Furthermore, sex chromosomes were unknown in the genus *Hymenochirus* until *Cauret et al.* (2020) identified the male heterogametic system on chromosome 4 of '*H. boettgeri*' (= *Hymenochirus* sp.) using whole-genome sequencing. However, no study has focused on the cytogenetic delineation of sex chromosomes in the tribe Hymenochirini. Pipid frogs have homomorphic sex chromosomes, meaning that the sex chromosomes look identical and are morphologically indistinguishable from each other, e.g. the Z chromosome from the W chromosome in *Xenopus laevis* (Daudin, 1802) (see: *Tymowska* 1991). Despite the homomorphic nature of the sex chromosomes, there is great diversity in sex determination systems/mechanisms and

genetic differentiation of sex chromosomes in pipids (Roco *et al.* 2015, Cauret *et al.* 2020, Furman *et al.* 2020, Song *et al.* 2021).

Here, for the first time, we cytogenetically investigated the Boettger's dwarf clawed frog (*H. boettgeri*) from the wild with a known locality in comparison to *Hymenochirus* sp. from the captive population, including the first information on karyotype in females. We aimed to test whether polymorphism exists in the chromosome number, morphology and size, localization of ribosomal DNA (rDNA) and small nuclear DNA (snDNA) multigene families, i.e. tandem repeats, and occurrence of B chromosome(s) within our material. Our findings reveal the presence of allopolyploidization in the genus *Hymenochirus*, and also point to previously discussed uncertainties in the species' identification of the captive population. The unclear taxonomy is discussed in the context of new karyotype data, available molecular data, and comparison with type material.

MATERIAL AND METHODS

Karyotype sampling

One male of the Boettger's dwarf clawed frog (*H. boettgeri*) from the north-western part of the Republic of the Congo (Mindjong, Mbemba Forest; 1.4701°N 14.3556°E, 520 m) was compared with three males and three females from the aquarium population of *Hymenochirus* sp. obtained from a private breeder in the Czech Republic (Fig. 1; Supporting Information, Table S1). Voucher specimens are deposited in the herpetological collection of the Institute of Vertebrate Biology of the Czech Academy of Sciences (IVB-H), Research Facility Studenec, Brno, Czech Republic (IVB-H-CG17-356, IVB-H-Hsp01—Hsp06). Dwarf clawed frogs were identified following available keys (de Witte 1930, Perret 1966, Arnoult and Lamotte 1968) and by comparison with type material of all *Hymenochirus* species. Chromosomes were prepared according to Völker *et al.* (2006). A piece of foot interdigital webbing between the second and third toes was cut from each hind limb of an adult using fine laboratory scissors. After 2 weeks, a section of regenerated webbing tissue, about 2 mm wide, was cut off and incubated in Ringer's solution with colchicine, as described in detail, including subsequent hypotonization, in Völker *et al.* (2006). Tissue fixation was performed and metaphases were spread on glass slides in the same manner as described in the embryo preparation protocol (Völker and Kullmann 2006). Microscopy and processing of metaphase images were conducted using Leica Microsystem (Wetzlar, Germany) as detailed in Seroussi *et al.* (2019). Short (p) and long (q) arms were measured in pixels using ImageJ, v.1.53i (Schneider *et al.* 2012) and chromosome length was quantified as proposed by Knytl and Fornaini (2021). Chromosome size in μm was estimated using a scale bar generated by chromosome photography software. Chromosomes were identified according to a nomenclature based on the ratio of the p and q arms (Levan *et al.* 1964).

DNA barcoding

The seven karyotyped individuals were DNA barcoded targeting one mitochondrial DNA (mtDNA) and one nuclear DNA (nDNA) marker. A fragment of the mitochondrial 16S rRNA gene (16S) was amplified using a primer pair (16SL1 and 16SH1;

adapted from: Palumbi *et al.* 1991; for details see: Gvoždik *et al.* 2010) commonly used in amphibian DNA barcoding (Vences *et al.* 2005, 2012). Consensus sequences were obtained from sequencing in both directions, resulting in a 579-bp long fragment. Our new sequences were combined with all available homologous 16S fragments of *Hymenochirus* from GenBank, adding 11 specimens, and two outgroups—representatives of two other African pipid genera [*Pseudhymenochirus merlini* Chabanaud, 1920 and *Xenopus tropicalis* (Gray, 1864)] (see Supporting Information, Table S1). The sequences were aligned using the MAFFT algorithm (Katoh and Standley 2013), resulting in an alignment length of 583 bp. A best-fit model of nucleotide evolution (TIM2+F+G) was inferred in parallel with the maximum-likelihood phylogenetic tree, and branch support was obtained using 1000 standard bootstrap pseudoreplicates, all using IQ-TREE 2 (Kalyaanamoorthy *et al.* 2017, Minh *et al.* 2020).

A similar methodology was also applied to collect nuclear DNA data, a fragment of the recombination activating protein 1 (*rag1*) gene, using a pair of primers RAG-1_MartF11 and RAG-1_AmpR1 (Pramuk 2006). All seven karyotyped individuals were sequenced from both sides, resulting in consensus sequences 933-bp long, but low-quality ends were trimmed in the Congo specimen of *H. boettgeri*, resulting in a fragment 879-bp long. No stop codons were present. Six additional *Hymenochirus* sequences and the same outgroups as defined above were added from GenBank (for details see Supporting Information, Table S1). The IQ-TREE 2 maximum likelihood tree was inferred using partitioned models (Chernomor *et al.* 2016) based on codon positions (best-fit models: F81 for codon positions 1 and 2, TPM2 for codon position 3). Branch support was obtained using 1000 bootstrap pseudoreplicates.

Since all six individuals from the aquarium population (*Hymenochirus* sp.) had the same haplotype, in both 16S and *rag1*, only one representative sequence per each marker was deposited in the GenBank database along with data on *H. boettgeri* from the Congo (for Acc. Nos. see Supporting Information, Table S1).

Chromosome banding, and repetitive FISH with snDNA and rDNA probes

Five cytogenetic techniques were conducted sequentially on the same metaphase spread in the following order: (i) 5% Giemsa/PBS solution (v/v), (ii) chromomycin A₃ CMA₃ (Sigma-Aldrich, St. Louis, MO, USA), (iii) C-banding, (iv) double-colour FISH with the 5S and 28S rDNA probes, and (v) double-colour FISH with the U1 and U2 snDNA probes. The CMA₃/C-banding protocol followed Rábová *et al.* (2015) with the modifications described in Knytl *et al.* (2017). In order to generate probes for repetitive FISH, *X. tropicalis* and *H. boettgeri* were used as templates for amplification of U1 and U2 snDNAs, respectively, while *X. tropicalis* and *Hymenochirus* sp. were used for the 5S and 28S rDNA regions, respectively. The same *X. tropicalis* U1 amplicon from Fornaini *et al.* (2023) was used and labelled. Total genomic DNA (gDNA) was extracted from the webbing tissue of adult frogs using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Conditions for polymerase chain reaction (PCR) amplification

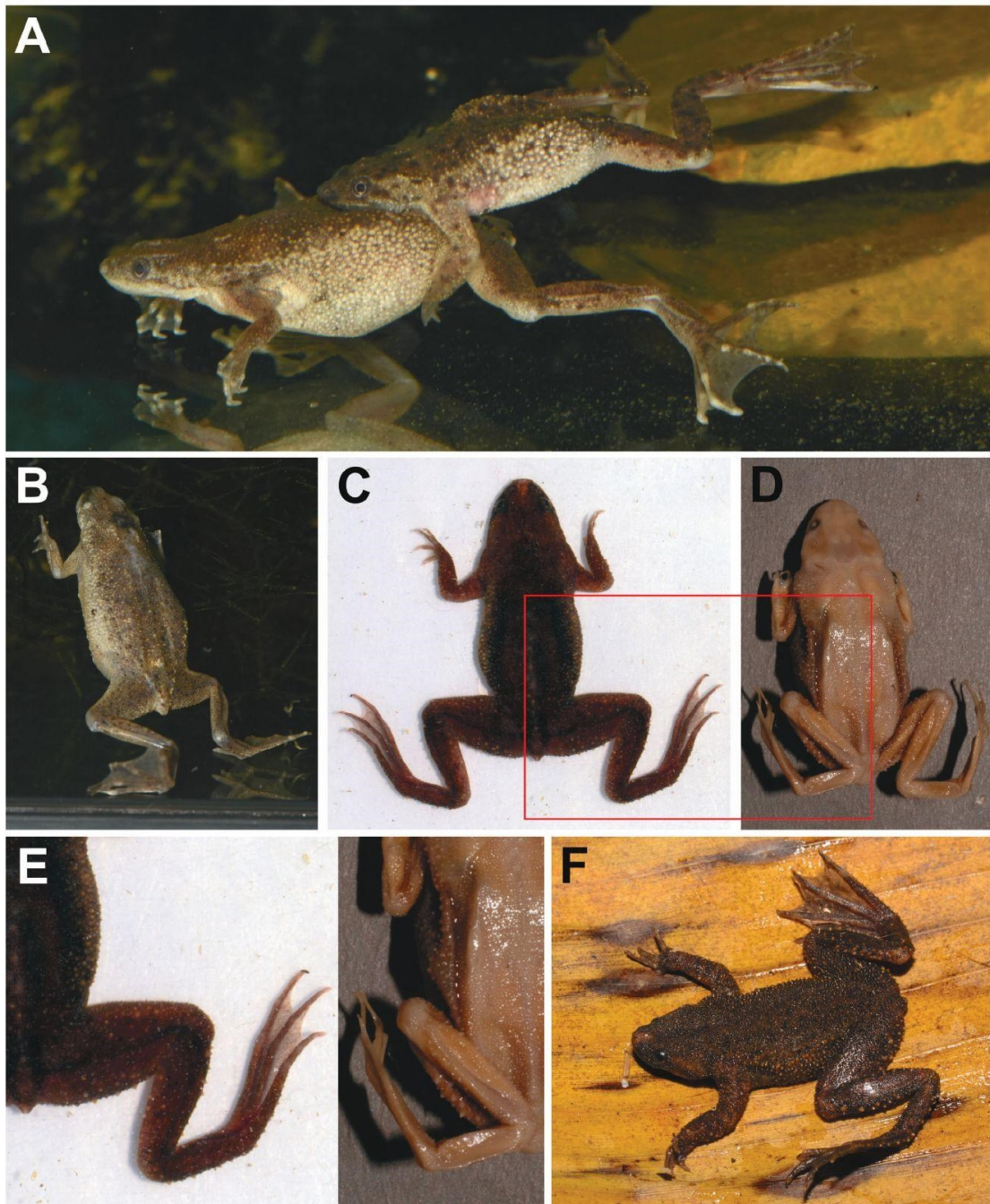


Figure 1. Dwarf clawed frogs, *Hymenochirus* sp. (captive population) and *H. boettgeri*. A, *Hymenochirus* sp., female (IVB-H-Hsp06) and male (IVB-H-Hsp02) in amplexus. B, *Hymenochirus* sp., female in dorsolateral view (IVB-H-Hsp04). In (A) and (B), note the relatively smooth flanks and hindlegs with homogeneous, unenlarged tubercles. C, *Hymenochirus boettgeri* from the north-western part of the Republic of the Congo (male, IVB-H-CG17-356). D, holotype of *H. boettgeri*, female (ZMB 11521). The area marked by the red rectangle is detailed in (E). E, *Hymenochirus boettgeri* from the same locality as the karyotyped individual (IVB-H-CG17-112, male). F, *Hymenochirus boettgeri* from the same locality as the karyotyped individual (IVB-H-CG17-112, male). In (C–F), note the enlarged and spiny tubercles on the flanks and hindlegs typical for *H. boettgeri*.

were used according to PPP Master Mix (Top-Bio, Prague, Czech Republic) supplier recommendations. Primers used for amplification are listed in the **Supporting Information, Table S2**. The PCR conditions for the U1 and U2 snDNA amplifications were taken from [Fornaini et al. \(2023\)](#). Amplifications of the 5S and 28S rDNA loci and PCR labelling (5S, 28S, U1, and U2 probes) with digoxigenin-11-dUTP (Roche, Mannheim, Germany) and biotin-16-dUTP (Jena Bioscience, Jena, Germany), including purification steps, are detailed in [Knytl and Fornaini \(2021\)](#). Digoxigenin-11-dUTP was used for 5S and U2 labelling. Biotin-16-dUTP was used for 28S and U1 labelling. The 5S, 28S, U1, and U2 probes were hybridized with chromosome spreads of *H. boettgeri* and *Hymenochirus* sp. The contents of the hybridization mixture, denaturation, and the subsequent overnight hybridization were conducted as it was described in the rDNA FISH protocol ([Knytl et al. 2023](#)). Post-hybridization washing and blocking reactions were performed as described for painting FISH in [Krylov et al. \(2010\)](#). Biotin and digoxigenin probe signals were detected using CY3™-streptavidin (Invitrogen, Camarillo, CA, USA) and anti-digoxigenin-fluorescein (Roche), respectively ([Knytl et al. 2017](#)). After Giemsa staining, slides were de-stained in methanol and acetic acid solution ([Rábová et al. 2015](#)). Between techniques (ii)–(v), slides were de-stained as given in the protocol in [Fornaini et al. \(2023\)](#) and counterstained with ProLong™ Diamond Antifade Mountant with the fluorescent 4',6-diamidino-2-phenylindole, DAPI stain (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA).

Genomic *in situ* hybridization

Hymenochirus boettgeri and *Hymenochirus* sp. gDNAs were used as probes for genomic *in situ* hybridization (GISH) experiments. Whole-genome painting (WGP) probes were prepared using the GenomePlex Single Cell Whole Genome Amplification Kit (WGA4), Sigma–Aldrich, according to the manufacturer's whole genome amplification protocol with extracted gDNA. GenomePlex WGA Reamplification Kit (WGA3), Sigma–Aldrich, was used for labelling with digoxigenin-11-dUTP (Jena Bioscience), as described in [Krylov et al. \(2010\)](#). A combination of salmon sperm ([Knytl et al. 2013](#)) and autoclaved *H. boettgeri* and *Hymenochirus* sp. gDNAs ([Bi and Bogart 2006](#)) were used as a competitor DNA conspecifically to a probe. The highly concentrated competitor gDNAs were extracted using the DNeasy Blood and Tissue Kit (Qiagen) from liver and used 5 µg per ~500 ng of a probe. A total of ~500 ng of probe was used per hybridization mixture. A control GISH was performed with the *Hymenochirus* sp. probe hybridized on *Hymenochirus* sp. chromosomes, as detailed in the painting FISH method ([Krylov et al. 2010](#)), and cross-species GISH experiments were carried out with *Hymenochirus* sp. and *H. boettgeri* on *H. boettgeri* and *Hymenochirus* sp. chromosomes, respectively, as technically detailed in the Zoo-FISH methodology ([Krylov et al. 2010](#)), with minor changes ([Knytl et al. 2017](#)).

RESULTS

Species identification

In mtDNA (16S), the specimen obtained from the north-western Congo (IVB-H-CG17-356) closely matches with 99.5% identity the specimen from the south-western Congo retrieved from

GenBank and originally identified as '*Hymenochirus* sp.' and deposited under the museum catalogue number USNM 584175 ([Deichmann et al. 2017](#)). Here, based on comparisons with type material ([Fig. 1](#)), we identify this lineage as *H. boettgeri*, as opposed to *Hymenochirus* sp., the naming we apply to the captive population (our new specimens IVB-H-Hsp01 to IVB-H-Hsp06; [Fig. 2](#)). *Hymenochirus boettgeri* forms a well-supported (bootstrap 82%) common clade with *H. curtipes* (from the wild, north-eastern Rep. Congo; [Jackson and Beier 2006](#), [Jackson et al. 2007](#), [Deichmann et al. 2017](#)) and this clade is sister to the captive population of *Hymenochirus* sp. Uncorrected *p*-distance in 16S between *H. boettgeri* and *Hymenochirus* sp. is 8.6%.

For nDNA (*rag1*), less comparative data were available, and only two lineages (species) are identified ([Fig. 2](#)). Our specimens from the captive population (*Hymenochirus* sp.) share a clade with other specimens originating, or probably originating, from the pet trade, while our Congo *H. boettgeri* specimen shares a clade with a specimen originating from Cameroon (museum number CAS 249965), originally identified as '*Hymenochirus* sp.' (GenBank, online museum catalogue) or '*H. boettgeri*' ([Feng et al. 2017](#)). Interestingly, the *rag1* sequence of our *H. boettgeri* from the Congo has an unusually high proportion of heterozygous positions (3.4%), indicating—in agreement with the karyotype results—the allotetraploid nature of this specimen.

Morphologically, our specimen from the Congo corresponds closely with the type of *H. boettgeri* (e.g. enlarged and spiny tubercles on the flanks and hindlegs), while the specimens from the captive population do not correspond to any of the described species ([Fig. 1](#)).

Cytogenetic characterization of *Hymenochirus boettgeri* from the Congo

For both species of the genus *Hymenochirus*, we carried out the same methods. Giemsa staining shows better results than DAPI, thus we present Giemsa-stained karyotypes with consistently stained chromosomes. *Hymenochirus boettgeri* possesses 36 chromosomes with eight pairs of metacentric, two pairs of submetacentric, and seven pairs of subtelocentric chromosomes ([Fig. 3A](#)). All chromosomes are biarmed, the fundamental arm number (FN) is 72. The size of chromosome 1 is shown as the vertical scale bar and is estimated to be less than 10 µm.

DAPI (negative black band) and CMA₃ (positive green band) highlight the NOR locus on a single homologous pair, which is morphologically similar to *Hymenochirus* sp. chromosome 4 ([Fig. 4A, B](#)). C-banding identifies 14 heterochromatic blocks (on seven homologous pairs) ([Fig. 4C](#)). The rDNA FISH analysis shows the 28S NOR locus on the same chromosome on which the CMA₃ is displayed. The 5S probe does not highlight any locus ([Fig. 4D](#)), probably because of low efficacy of the used 5S probe. Unfortunately, we could not have repeated FISH with the 5S probe because chromosome structure on slide is broken after several attempts of staining and de-staining. The U1 locus is identified in the pericentromeric region of the q arm of the largest chromosome 1 ([Fig. 4E](#)), which is not homologous to the U1 locus of *Hymenochirus* sp. (the pericentromeric region of the p arm of chromosome 1). The U2 locus is found in the telomeric region of the q arm of chromosome 8 ([Fig. 4E](#)), which is homologous to the U2 locus of *Hymenochirus* sp. GISH analysis with the *Hymenochirus* sp. WGP probe and competitor

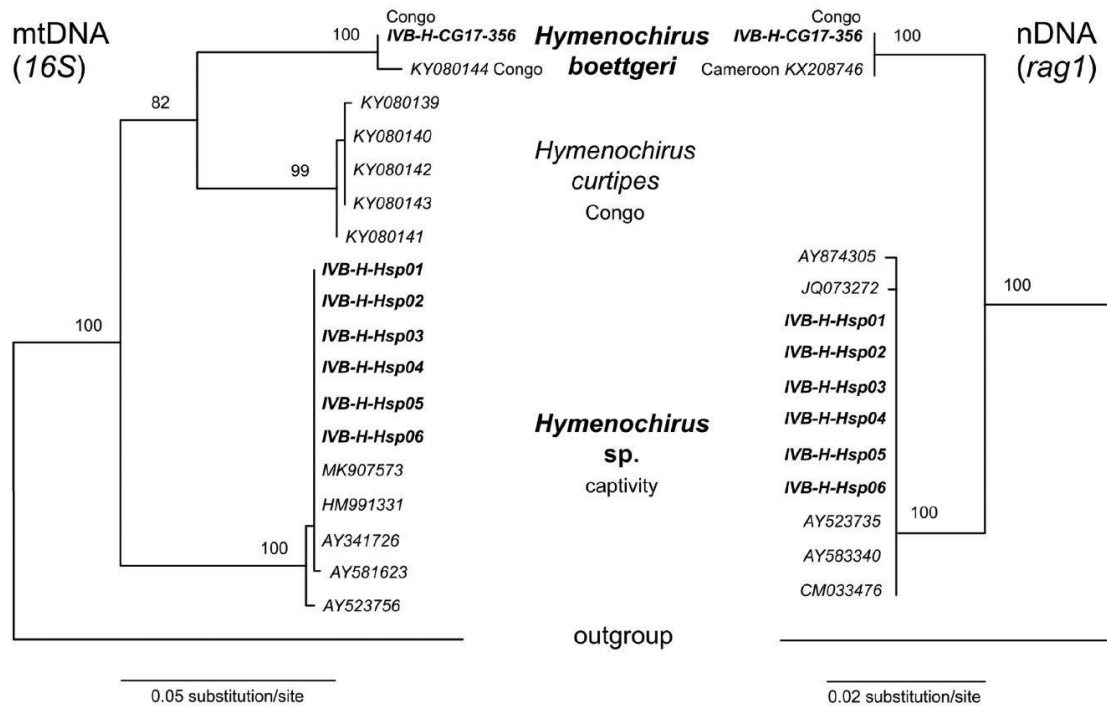


Figure 2. Phylogenetic trees of dwarf clawed frogs. Maximum likelihood mtDNA (16S, left) and nDNA (*rag1*, right) trees showing the positions of karyotyped individuals (in bold) in the context of available molecular sampling retrieved from GenBank (acc. nos. listed). For sampling details, see [Supporting Information, Table S1](#).

Hymenochirus sp. DNA paint all 36 chromosomes of *H. boettgeri*, with some chromosomes having less intense signals (arrows in [Fig. 4F](#)). The difference in signal intensity is one of the other clues to determine the level of ploidy and homoeologous chromosomes. We find no heterochromatic B chromosomes in the karyotype of *H. boettgeri*.

Cytogenetic characterization of the captive *Hymenochirus* sp.

The number of chromosomes in *Hymenochirus* sp. is 21 (20A + 1B) with seven pairs of metacentric, one pair of submetacentric, and two pairs of subtelocentric chromosomes, and one extra B chromosome ([Fig. 3B](#)). All chromosomes are biarmed, FN = 42. The size of chromosome 1 is estimated to be 12–13 μm . Both males and females have the same number and general morphology of chromosomes. Sequential DAPI and CMA₃ analyses ([Fig. 5A, B](#)) show a single NOR on the p arm of submetacentric chromosome 4. DAPI consistently stains all 21 chromosomes but without staining of the NOR locus, which is CMA₃-positive. C-banding highlights telomeric and pericentromeric bands on most chromosomes, which helps to identify homologous chromosome pairs ([Fig. 5C](#)). Interestingly, the NOR locus is not stained using C-banding. The B chromosome is entirely painted and shows a high degree of heterochromatization ([Fig. 5C, D, F; Supporting Information, Fig. S1A, B, D, E](#)). FISH with rDNA probes identifies the 5S locus on the telomere and interstitial region of the q arm of

chromosome 6. The 28S (NOR) locus is mapped to chromosome 4 ([Fig. 5D](#)) and is identical with CMA₃ bands. FISH with snDNA probes identifies the U1 locus on chromosome 1 and the U2 locus on chromosome 8 ([Fig. 5E](#)). GISH with the *H. boettgeri* WGP probe paints 20 chromosomes. Accessory B chromosome is stained with DAPI (DAPI-positive) but is not painted with the *H. boettgeri* WGP probe ([Fig. 5F; Supporting Information, Fig. S1A](#)). We performed two control GISH experiments with the *Hymenochirus* sp. WGP probe: the first with ([Supporting Information, Fig. S1B](#)) and the second without ([Supporting Information, Fig. S1C](#)) *Hymenochirus* sp. competitor DNA. Surprisingly, the WGP probe does not paint the B chromosome of *Hymenochirus* sp. in either control experiment (no red signal on the B chromosome). DAPI staining shows no signal on the B chromosome in the GISH experiment without the competitor ([Supporting Information, Fig. S1C, F](#)) compared to GISH with the competitor DNA where DAPI stains the entire B chromosome ([Supporting Information, Fig. S1A, B, D, E](#)).

DISCUSSION

Taxonomy

Our new molecular data, combined with the morphological characteristics of the specimen, such as enlarged tubercles on its body sides and hindlegs, confirms that our specimen from the northern Republic of the Congo is *H. boettgeri* ([Fig. 1](#)).

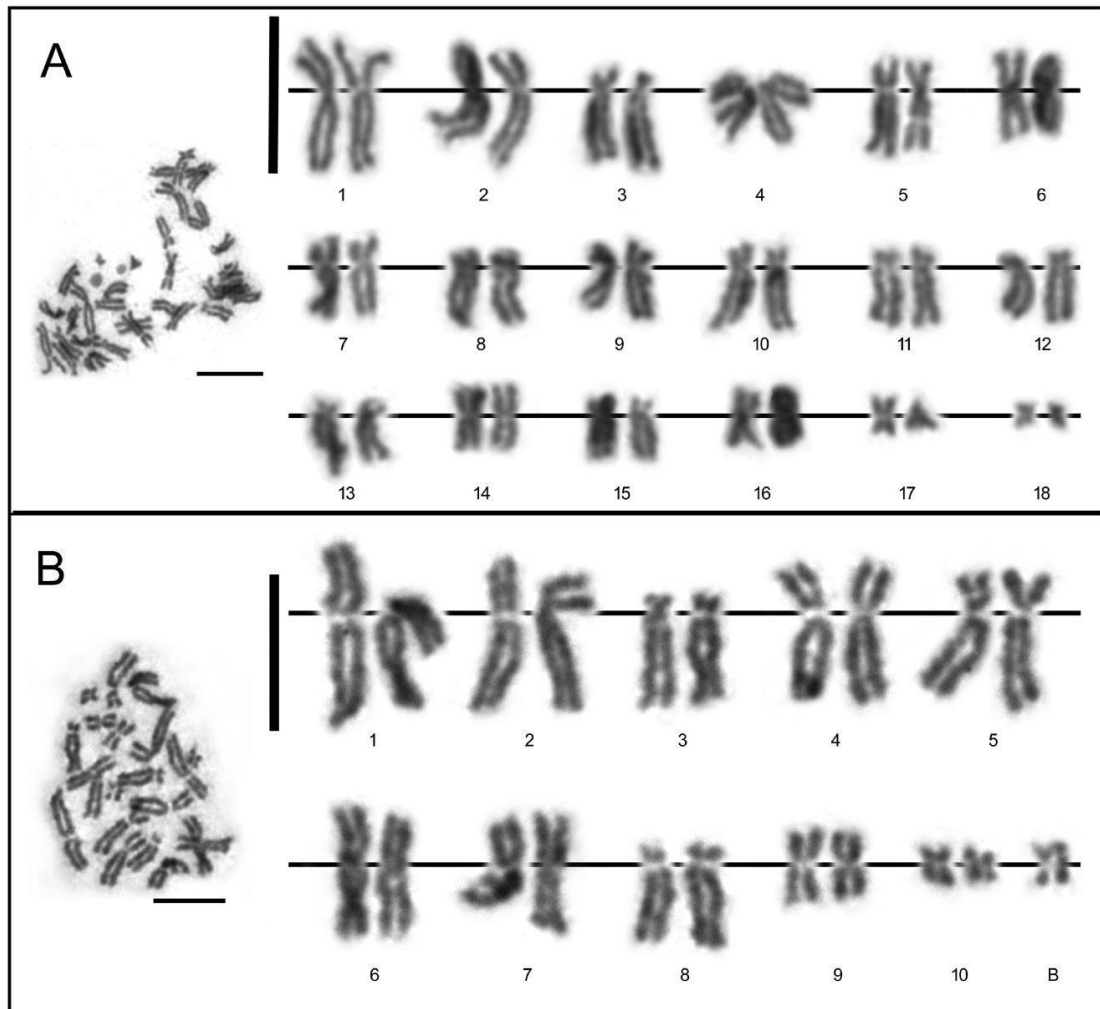


Figure 3. Karyotypes of (A) *Hymenochirus boettgeri* (IVB-H-CG17-356, male) with 18 homologous chromosome pairs, and (B) *Hymenochirus* sp. (IVB-H-Hsp06, female) with 10 pairs of A chromosomes and one B chromosome, arranged from Giemsa-stained chromosomes. Chromosomes were cut from metaphase spreads on the left. Long lines in karyotype arrangements indicate the position of chromosome centromere. Short vertical and horizontal lines correspond to the scale = 10 μ m.

Two other specimens from the southern part of the Republic of the Congo and central Cameroon are very closely related with our specimen, and thus most probably conspecific, according to available molecular data (Deichmann *et al.* 2017, Feng *et al.* 2017; Fig. 2; Supporting Information, Table S1). The captive population, genetically deeply divergent from *H. boettgeri*, is not clearly identifiable as any described species (following identification keys in: de Witte 1930, Perret 1966, Arnoult and Lamotte 1968; and our unpublished data) and is most appropriately named as *Hymenochirus* sp. However, in literature and practice, captive dwarf clawed frogs are usually mistakenly referred to as '*H. boettgeri*' (e.g. Kunz 2003, 2004, Mezzasalma *et al.* 2015, Höbel and Fellows 2016, Miller *et al.* 2019, Cauret *et al.* 2020, Bredeson *et al.* 2021) or '*H. curtipes*' (e.g. Bewick *et al.*

2012; but see: Kunz 2002). Our molecular phylogenetic analysis suggests that *H. curtipes*, which used to occur in European and American aquaria in the past but probably became extinct in captivity (Sokol 1959, 1962, Rabb and Rabb 1963, Kunz 2002), is a sister-lineage of *H. boettgeri* (Fig. 2). However, data for other species are currently missing. A taxonomic revision is needed to properly understand the diversity and relationships within the genus *Hymenochirus* and to name the dwarf clawed frogs from the captive population.

Tetraploidy in *Hymenochirus boettgeri*

Our cytogenetic analysis detected 36 chromosomes in *H. boettgeri* from the Congo and confirmed 20 A chromosomes and one B chromosome in *Hymenochirus* sp. from the captive

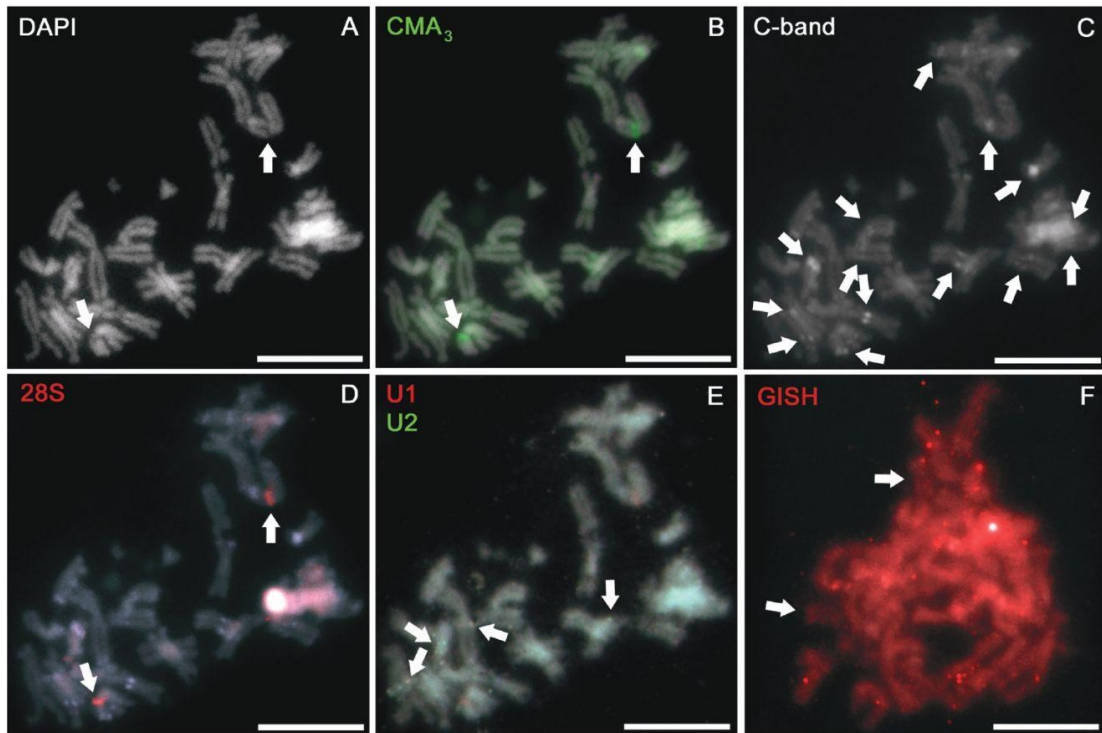


Figure 4. *Hymenochirus boettgeri* (IVB-H-CG17-356, male), sequential fluorescent chromosome mapping—DAPI, CMA₃, C-banding, ribosomal DNA (rDNA) FISH, small nuclear DNA (snDNA) FISH; and non-sequential whole-genome painting on metaphase spread. A, DAPI (black and white, B&W) consistently stains all 36 chromosomes. B, CMA₃ banding in green shows nucleolar secondary constriction (NOR locus) on the p arm of chromosome 4 that co-localizes with 28S. C, C-banding (B&W, brighter staining) highlights heterochromatic blocks on telomeric and pericentromeric regions of seven homologous chromosomes (14 arrows). D, FISH with 28S (red) ribosomal probes shows the p arm of chromosome 4. E, FISH with U1 (red) and U2 (green) snDNA probes shows very weak signals. The U1 probe maps to the q arm of chromosome 1, the U2 probe maps to the q arm of chromosome 8. F, genomic *in situ* hybridization (GISH) with *Hymenochirus* sp. whole-genome painting DNA probe that hybridizes to all 36 chromosomes with different intensity. Arrows show the less intensely painted chromosome pair. Scale bars represent 10 μ m.

population (Fig. 3). The latter finding is consistent with that of Mezzasalma *et al.* (2015), but in contrast to an earlier study by Morescalchi (1968; republished by: Morescalchi 1981, Tymowska 1991), which found 24 chromosomes. However, the origin of Morescalchi's specimen is unknown and no details at all were given by Scheel (1973), who reported 22 chromosomes in '*H. boettgeri*'. As a possible explanation of this discrepancy, we hypothesize that Morescalchi (1968) and/or Scheel (1973) may have karyotyped one or eventually more different species, possibly *H. curtipes* that was present in captivity at the time of Morescalchi's and Scheel's analyses (Kunz 2002). Another explanation could be variation in chromosome number, especially in the number of B chromosomes, as suggested by Mezzasalma *et al.* (2015). See further discussion below, in the section about the B chromosomes.

Compared to all previous studies, our finding of 36 chromosomes is exceptional. The chromosome number of our *H. boettgeri* from the Congo is almost twice that of *Hymenochirus* sp. from the captive population, which has the putative

ancestral number of A chromosomes in pipid frogs of $2n = 20$ (Mezzasalma *et al.* 2015), and exactly twice the number of chromosomes found by Bredeson *et al.* (2021). The number, morphology, and composition of chromosomes of our *H. boettgeri* provide evidence that our specimen is tetraploid or allotetraploid, respectively, which is the first case of tetraploidy in pipids outside the genus *Xenopus* (Mezzasalma *et al.* 2015, Schmid *et al.* 2015). The size of the chromosomes also indirectly demonstrates the tetraploid nature of *H. boettgeri*, as its chromosomes are smaller than those of *Hymenochirus* sp. (Fig. 3). Polymorphism in chromosome size is also visible in the karyotypes of diploid *X. tropicalis* compared to allotetraploid *X. laevis*, *X. mellotropicalis* Evans, Carter, Greenbaum, *et al.*, 2015, or *X. calcaratus* Peters, 1875 (see: Session *et al.* 2016, Knytl *et al.* 2017, 2023). In *X. laevis*, the chromosomes of the S-subgenome (shorter homoeologues) have undergone losses and deletions, and, therefore, the size of these chromosomes has decreased (Session *et al.* 2016). In cyprinid fishes, for example, polyploids have chromosomes that are smaller than those of their

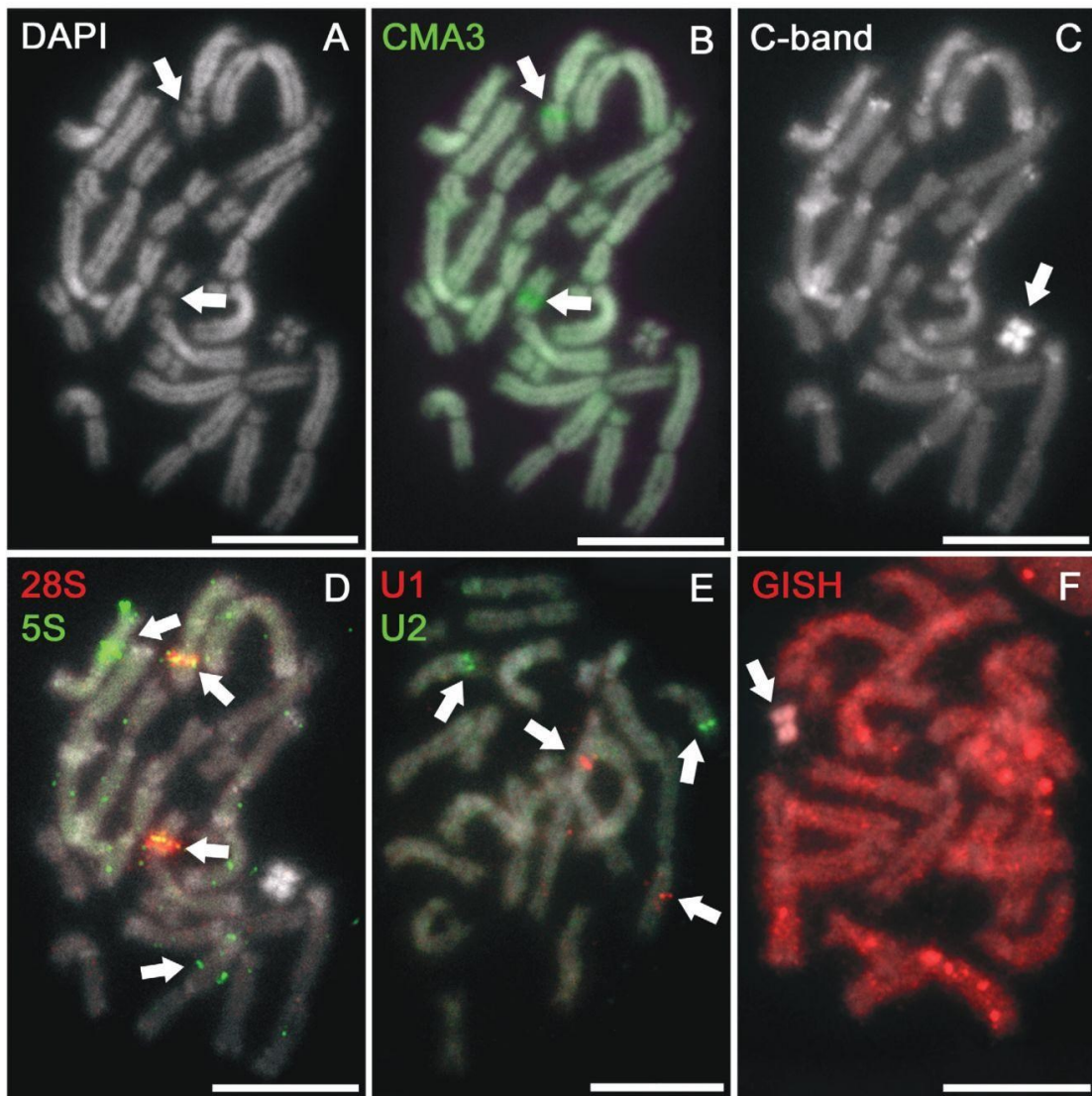


Figure 5. *Hymenochirus* sp. (IVB-H-Hsp06, female), sequential fluorescent chromosome mapping (DAPI, CMA₃, C-banding, rDNA FISH), non-sequential snDNA FISH, and whole-genome painting on metaphase spread. A, DAPI (B&W) counter-stained metaphase spread shows all 21 chromosomes. B, CMA₃ banding in green shows NOR locus on the p arm of chromosome 4. CMA₃ signal co-localizes with 28S locus. C, C-banding (B&W, brighter staining) highlights heterochromatic blocks on telomeric and pericentromeric regions of almost all chromosomes. In addition, the whole B chromosome is intensely banded (arrow). D, 5S (green) and 28S (red) rDNA loci are located on the q arm of chromosome 6 and p arm of chromosome 4, respectively. The 5S rDNA is situated on two different chromosomal loci within the single q arm. E, the snDNA loci U1 (red) and U2 (green) are located on the p arm of chromosome 1 and the q arm of chromosome 8, respectively. F, the GISH experiment of the *H. boettgeri* whole-genome painting probe, which hybridizes on *Hymenochirus* sp. chromosomes. All chromosomes are painted (red) except one, B chromosome, which shows no GISH signal and is DAPI-positive (arrow). Scale bars represent 10 μ m.

diploid relatives (e.g. visible on karyotypes of diploid and triploid/tetraploid *Carassius* Nilsson, 1832; see: Kalous and Knytl 2011, Knytl *et al.* 2013, 2022). Further support for tetraploidy comes from GISH, where the tetraploid *H. boettgeri* probe consistently painted all 20 A chromosomes of *Hymenochirus*

sp., while the diploid *Hymenochirus* sp. probe painted some *H. boettgeri* chromosomes more intensely than others, similar to what was observed in diploid and allotetraploid *Xenopus* (Knytl *et al.* 2023). This suggests that one subgenome of *H. boettgeri* may be evolutionarily closer to *Hymenochirus* sp. than the other

subgenome, indicating an allotetraploid origin. Further indirect evidence of allotetraploidy is the high frequency of heterozygous positions in the *rag1* nucleotide sequence, where a potential divergence of 3.4% between two paralogues is indicative of an allotetraploid origin.

The discovery of tetraploidy is based on a single male individual, which may open the question of whether tetraploidy is an autapomorphy of *H. boettgeri*, or whether there is geographic variation at the ploidy level within this supposedly widespread Central African species (Channing and Rödel 2019). To answer this question, multiple populations of *H. boettgeri* need to be cytogenetically examined. However, the genome size of *H. boettgeri* from the northern Democratic Republic of the Congo (DRC) is almost twice as large (3.77–4.29 pg; Liedtke *et al.* 2018) as that reported by King (1990; 2.45 pg). The latter most probably originated from the captive population, as virtually all dwarf clawed frogs used in research in recent decades have come from the captive population. Given that the locality of the DRC samples is approximately 800 km east of our karyotyped individual [one of us (V.G.) provided material for the Liedtke *et al.* (2018) study from a locality at 2.1228°N 21.3891°E], this is indicative of *H. boettgeri* being tetraploid throughout its range, or at least in a substantial portion of its populations.

Potential evolutionary origin of tetraploidy in *Hymenochirus boettgeri*

Hymenochirus sp. has the presumed pipid ancestral karyotype $2n = 20$ (plus one B chromosome unique in this species), while tetraploid *H. boettgeri* has 36 chromosomes, which are probably secondarily organized as diploid ($2n = 4x = 36$; see below). Both karyotypes consist of biarmed chromosomes (no telocentrics), and, therefore, neither centric fusion of two telocentric chromosomes nor fission of a metacentric chromosome may be the evolutionary mechanism underlying the different chromosome numbers of the two *Hymenochirus* species. The same difference in chromosome number between diploid and allotetraploid pipids is known between two subgenera of the genus *Xenopus*: diploid *X. (Silurana) tropicalis* ($2n = 20$) and tetraploid species of the subgenus *Xenopus* ($2n = 36$), e.g. *X. laevis* (Tymowska 1991; Evans *et al.* 2004). The 36-chromosome karyotype of the subgenus *Xenopus* arose after the fusion of two metacentric chromosomes 9 and 10 in a common ancestor of both subgenomes ($2n = 18$) and subsequent allotetraploidization (Evans *et al.* 2005; Session *et al.* 2016). We assume that a similar mechanism may have occurred in *H. boettgeri*. Interesting, and still poorly understood, is a recent finding pointing to a polymorphism in the number of chromosomes in the captive population, where karyotype $2n = 18$ was found in *Hymenochirus* sp. (see Fig. 2 and Supporting Information, Table S1 for molecular identification) after the fusion of chromosomes 8 and 10 (Bredeson *et al.* 2021).

The limited molecular phylogenetic data available support the hypothesis that the ancestral karyotype of the tribe Hymenochirini is $2n = 20$, because *Hymenochirus* sp. with 20 A chromosomes represents the first diverging branch of the genus and the sister-genus *Pseudhymenochirus* also has $2n = 20$ (Mezzasalma *et al.* 2015). Thus, tetraploidization in *Hymenochirus* is probably a derived state that occurred later in the evolution of the genus. However, because we lack information on karyotypes of other *Hymenochirus* species, we cannot formulate

a more precise hypothesis about the evolutionary scenario of tetraploidization in this genus. For example, *H. curtipes* appears to be a sister-lineage to *H. boettgeri* (Fig. 2), but we lack information on its karyotype. We can only speculate that the karyotype of '*H. boettgeri*' with $2n = 24$ presented by Morescalchi (1968; republished by: Morescalchi 1981, Tymowska 1991) could be misidentified *H. curtipes*, which was available in hobby aquaria in the 1960s (Sokol 1959, Rabb and Rabb 1963, von Filek 1967), and the increased chromosome number could have arisen by fissions (e.g. as in *Pipa parva* Ruthven and Gaige, 1923; see: Mezzasalma *et al.* 2015). However, this is only a speculation that is unlikely to be confirmed or refuted unless we know the karyotype of *H. curtipes*.

FISH in tetraploid *Hymenochirus boettgeri* and diploid *Hymenochirus* sp.

The numbers of FISH signals of multigene families are helpful to understand the functional diploidy of evolutionarily tetraploid *H. boettgeri*. The 28S rDNA (NOR), U1 and U2 snDNA loci are situated on a single chromosome, indicating rediploidization of a tetraploid ancestor, probably associated with a sexual mode of reproduction, and, therefore, the presence of a diploid–haploid cycle and crossing-over are expected in tetraploid *H. boettgeri*. This phenomenon has been described in all *Xenopus* polyploids (Tymowska 1991, Session *et al.* 2016, Knytl *et al.* 2017, 2023). On the other hand, the number and localization of U1 and U2 snDNAs are more diverse in *Xenopus* tetraploids, with one or two U1 and U2 loci detected by FISH, depending on the copy number of these tandemly repeated elements on the exact chromosome (Fornaini *et al.* 2023), which is not the case for the tetraploid *Hymenochirus* in this study.

The U1 snDNAs show the stable number but polymorphism in their position on *H. boettgeri* and *Hymenochirus* sp. chromosomes. *Hymenochirus boettgeri* has the U1 locus on the q arm of chromosome 1 and *Hymenochirus* sp. has this locus on the p arm of chromosome 1. *Xenopus* has the U1 locus close to the telomere on the q arm of chromosome 1 (Fornaini *et al.* 2023). A responsible mechanism for the rearrangement of the U1 locus in *Hymenochirus* can be pericentromeric inversion of a massive region, or a change of the position by a mechanism similar to transposition of transposons. An alternative explanation of this polymorphism is that the U1 locus is present on both chromosome arms but with a different number of copies—reduced copy number on the p arm and expanded copy number on the q arm of chromosome 1 in *H. boettgeri*. The diversity of the U1 locus localization points to its dynamic character, which is not the case of the U2 and NOR loci found constantly on the telomere of the q arm of chromosome 8 and pericentromeric region of the p arm of chromosome 4, respectively. All mapped loci are shown in Figure 6.

We analysed karyologically male and female individuals of *Hymenochirus* sp. (females for the first time) and male *H. boettgeri*. No sex-specific differences in karyotype and/or chromosome morphology have been found, suggesting that the sex chromosomes [most likely chromosome 4 as identified in *Hymenochirus* sp. by Cauret *et al.* (2020)] are homomorphic. This is consistent with previous findings within the family Pipidae (Tymowska 1991).

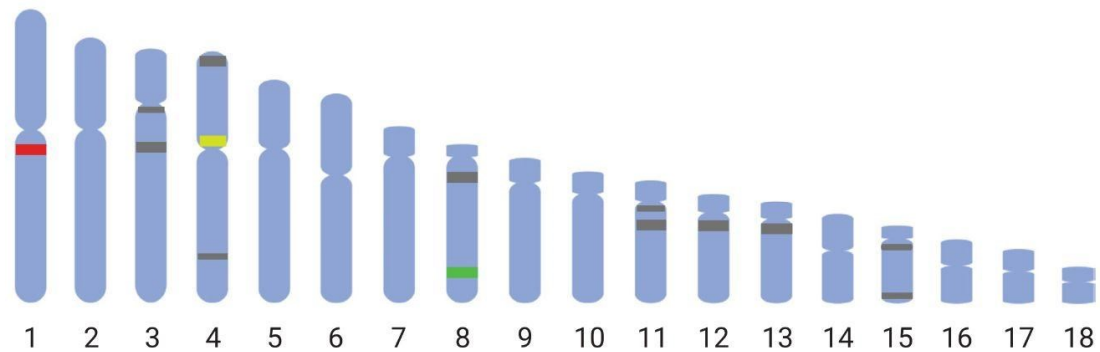
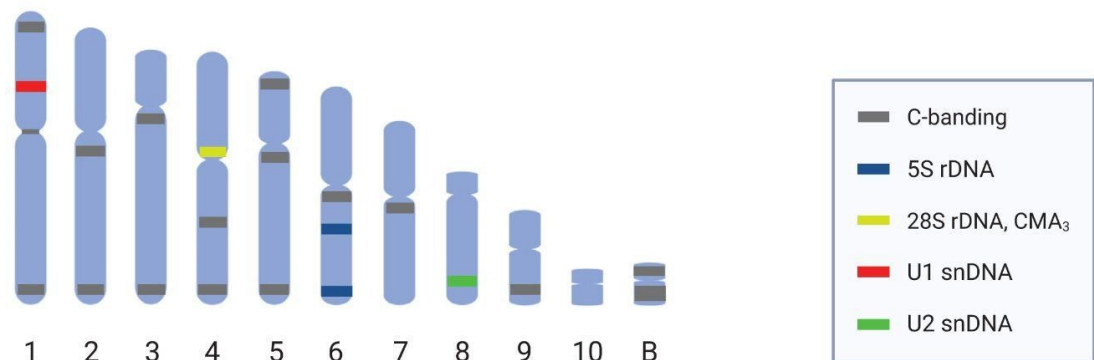
Hymenochirus boettgeri (Congo)*Hymenochirus* sp. (captive population)

Figure 6. Schematic representation of the chromosomal location of the U1 (red) and U2 (green) snDNAs, 5S (dark blue) and 28S (yellow) rDNAs, and C-bands (dark grey) in *H. boettgeri* (Congo) and *Hymenochirus* sp. (captive population). The haploid A chromosome set of each species, 18 chromosomes in *H. boettgeri* and 10 chromosomes in *Hymenochirus* sp., is arranged in descending order of size. The B chromosome of *Hymenochirus* sp. is depicted separately from the A chromosomes and is entirely covered in grey, as revealed by C-banding. The 5S rDNA locus was not detected in *H. boettgeri* and is only depicted in the *Hymenochirus* sp. karyotype. Mapping of the U1 snDNA locus identified a pericentric inversion or copy number reduction/expansion visible on non-homologous regions of chromosome 1. Created with BioRender.com.

B chromosomes

B chromosomes, also known as accessory, supernumerary, or they may sometimes be referred to as microchromosomes, are often considered selfish or parasitic in nature. They are maintained in a cell through meiotic or mitotic drive, have a low gene content, and tend to have a high degree of heterochromatization and repetitive elements. In contrast to redundant traits, B chromosomes may be important for sex determination or protecting against parasites (reviewed in: Camacho *et al.* 2000, Johnson Pokorná and Reifová 2021). We were able to identify B chromosomes using C-banding, as the B chromosomes in *Hymenochirus* sp. are highly heterochromatized, as noted by Mezzasalma *et al.* (2015). An intensely highlighted B chromosome is visible in each nucleus, including nuclei where chromosomes are not fully condensed (data not shown). In amphibians, the number of B chromosomes can be stable (e.g. *Uraeotyphlus* Peters, 1880; see:

Venu *et al.* 2021), or variable between different populations (e.g. *Leiopelma* Fitzinger, 1861; see: Green 1991), or even variable between tissues within the same individual (e.g. *Dicamptodon* Strauch, 1870; see: Green 1991). In their study, Mezzasalma *et al.* (2015) found either the presence of a B chromosome or no B chromosome in various tissues of *Hymenochirus* sp. (previously referred to as '*H. boettgeri*'). We consistently found one B chromosome in all six individuals of both sexes (three males, three females) of *Hymenochirus* sp. However, we used only one source of somatic tissue—foot webbing. The variability in the presence/absence of B chromosomes in different tissues found by Mezzasalma *et al.* (2015) may be caused by uneven cell division of B chromosomes in some cells/tissues (Johnson Pokorná and Reifová 2021). During meiotic division, B chromosomes can be preferentially transferred to the germline (Camacho *et al.* 2000), but irregular chromosome pairing and nondisjunctions

can lead to polymorphisms, as found by Mezzasalma *et al.* (2015). The latter authors also discussed whether four of the 24 chromosomes found by Morescalchi (1968) in '*H. boettgeri*' could possibly represent B chromosomes. If this were true, then *Hymenochirus* sp. may contain a variable number of B chromosomes from zero to at least four, as can also be inferred from the uncommented brief report by Scheel (1973) on $2n = 22$, where two chromosomes could potentially be B chromosomes. This hypothesis assumes that the material processed in the previous studies was conspecific with our *Hymenochirus* sp. However, another explanation may be that earlier studies (Morescalchi 1968, Scheel 1973) karyotyped (an) other species, as discussed above, and supernumerary chromosomes above the presumed ancestral number of $2n = 20$ may or may not represent B chromosomes.

The preferred hypothesis for the evolutionary origin of B chromosomes is that they were derived from A chromosomes by rearrangements (Camacho *et al.* 2000). It is unclear whether B chromosomes are also present in other *Hymenochirus* species, but we have not detected them in tetraploid *H. boettgeri*. B chromosomes have not yet been identified in other genera of the family Pipidae. We attempted to reveal the origin of the B chromosome in *Hymenochirus* sp. using GISH analysis with a whole-genome painting probe from *H. boettgeri* hybridized on a chromosome spread of *Hymenochirus* sp. and vice versa. Neither B chromosome was painted by probe in any of the GISH experiments, including the control experiment with a conspecific whole-genome painting probe, which indicates that the *Hymenochirus* B chromosome may be highly stable during the denaturation process due to the high heterochromatin (shown by C-banding, Fig. 5C) and AT-specific (shown by DAPI in GISH, Fig. 5F) content and the low permeability of the B chromosome to the painting probe during hybridization. These characteristics make the *Hymenochirus* B chromosome unsuitable for painting *in situ* hybridization techniques. Other methodological approaches, such as high-throughput sequencing, may help to better study their evolution.

CONCLUSION

In this study, we demonstrate that the captive population of dwarf clawed frogs is best taxonomically named *Hymenochirus* sp. and not *H. boettgeri*, as is usually the case. We present for the first time the karyotype from a specimen with a known locality (NW Congo) that we identified as *H. boettgeri*. This species is tetraploid with $2n = 36$, in contrast to diploid *Hymenochirus* sp. with $2n = 20$ A chromosomes and one B chromosome according to our results. Based on several cytogenetic and molecular tools used, we hypothesize that the *H. boettgeri* karyotype evolved from a diploid ancestor with the original chromosome number $2n = 20$ through fusion of two biarmed chromosomes and subsequent allotetraploidization resulting from hybridization of two diploid ancestors with $2n = 18$. The scenario is similar to that proposed for the origin of tetraploid clawed frogs from the subgenus *Xenopus* (Evans *et al.* 2005, Session *et al.* 2016), and its probable relevance is supported by the recent finding of a karyotype with $2n = 18$ with an identified fusion of two chromosomes in *Hymenochirus* sp. (Bredeson *et al.* 2021). Based on a comparison of the two *Hymenochirus* species and mapping of the U1 snDNA locus, we revealed that either pericentromeric inversion

or reduction/expansion of U1 copies occurred on chromosome 1. In the captive population of *Hymenochirus* sp. we found the stable presence of a single B chromosome in both sexes, in contrast to the presence (one B chromosome) or absence found by Mezzasalma *et al.* (2015). Results of previous studies also indicate a possible higher number of B chromosomes in *Hymenochirus* (four or two; Morescalchi 1968, Scheel 1973). Further research is needed to assess patterns of variation in the number of A and B chromosomes among populations, individuals and/or tissues, as well as to better understand the (karyotype) evolution and taxonomy of the genus *Hymenochirus*.

SUPPLEMENTARY DATA

Supplementary data are available at *Zoological Journal of the Linnean Society* online.

ACKNOWLEDGEMENTS

We would like to thank the IRSEN (Brazzaville) authorities for issuing permits and logistical support, and Zdeňka Dittrichová (Brandýs nad Labem) for providing the material of *Hymenochirus* sp. We further thank M.-O. Rödel and F. Tillack (ZMB, Berlin) for providing access to the type specimen of *Hymenochirus boettgeri*, Matej Dolinay, Jérémy Thomas, and Alain S. Nzila for help in the field, and F. Snitýl for help in the laboratory.

ETHICS

Charles University has registered experimental breeding facilities for pipid frogs (16OZ12891/2018–17214, 37428/2019-MZE-18134). All experimental procedures involving frogs were approved by the Institutional Animal Care and Use Committee of Charles University, according to the directives from the State Veterinary Administration of the Czech Republic, reference number MSMT-20585/2022–4 issued by the Ministry of Education, Youth and Sport of the Czech Republic (M.K. is a manager of the experimental project on living *Xenopus* and *Hymenochirus* animals). M.K. and V.G. are holders of the certificate of professional competence to design experiments according to §15d(3) of the Czech Republic Act No. 246/1992 Coll., on the Protection of Animals against Cruelty (Registration numbers CZ.03973, CZ.02519), provided by the Ministry of Agriculture of the Czech Republic.

FUNDING

This study was supported by the P JAC project CZ.02.01.01/00/22_010/0002902 MSCA Fellowships CZ—UK, Ministry of Culture of the Czech Republic (DKRVO 2019–2023/6.VII.e, National Museum, 00023272), and the Czech Science Foundation (23-07331S). Open access publishing was supported by the National Technical Library in Prague.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

All the data supporting the findings of this study are available within the article and its supplementary materials. DNA sequences are

deposited in GenBank (see Supporting Information, Table S1 for accession numbers).

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Supporting Information

Tetraploidy in the Boettger's dwarf clawed frog (Pipidae: *Hymenochirus boettgeri*) from the Congo indicates non-conspicuity with the captive population

Václav GVOŽDÍK^{‡,*}, Martin KNYTL^{‡,*}, Ange-Ghislain ZASSI-BOULOU, Nicola R. FORNAINI, Barbora BERGELOVÁ

[‡]V. Gvoždík and M. Knytl are co-first and co-senior authors in alphabetical order.

*Corresponding authors. E-mail: vaclav.gvozdik@gmail.com (V. Gvoždík), martin.knytl@natur.cuni.cz (M. Knytl)

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Table S1. Material used in cytogenetic and molecular analyses.

Species (updated identification)	16S GenBank	rag1 GenBank	Specimen	Origin	Reference	Original identification	Remark
<i>Hymenochirus boettgeri</i>	OR360735	OR352008	IVB-H-CG17-356	Congo, Mindjong (Mbemba)	This study	-	Male, karyotyped
<i>H. boettgeri</i>	KY080144		USNM 584175	Congo, Simombondo	Deichmann <i>et al.</i> (2017)	<i>Hymenochirus</i> sp.	
<i>H. boettgeri</i>		KX208746	CAS 249965	Cameroon, Ndombam	Feng <i>et al.</i> (2017)	<i>Hymenochirus</i> sp. / <i>H. boettgeri</i>	
<i>H. curtipes</i>	KY080139		USNM 576617	Congo, Impongui	Deichmann <i>et al.</i> (2017)	<i>H. curtipes</i>	
<i>H. curtipes</i>	KY080140		USNM 576620	Congo, Impongui	Deichmann <i>et al.</i> (2017)	<i>H. curtipes</i>	
<i>H. curtipes</i>	KY080141		USNM 576618	Congo, Impongui	Deichmann <i>et al.</i> (2017)	<i>H. curtipes</i>	
<i>H. curtipes</i>	KY080142		USNM 563881	Congo, Ganganya Brousse	Deichmann <i>et al.</i> (2017)	<i>H. curtipes</i>	Jackson & Beier (2006)
<i>H. curtipes</i>	KY080143		USNM 576619	Congo, Impongui	Deichmann <i>et al.</i> (2017)	<i>H. curtipes</i>	
<i>Hymenochirus</i> sp.	OR360736*	OR352009*	IVB-H-Hsp01	Laboratory/Pet trade	This study	-	Male, karyotyped
<i>Hymenochirus</i> sp.	OR360736*	OR352009*	IVB-H-Hsp02	Laboratory/Pet trade	This study	-	Male, karyotyped
<i>Hymenochirus</i> sp.	OR360736*	OR352009*	IVB-H-Hsp03	Laboratory/Pet trade	This study	-	Male, karyotyped
<i>Hymenochirus</i> sp.	OR360736*	OR352009*	IVB-H-Hsp04	Laboratory/Pet trade	This study	-	Female, karyotyped
<i>Hymenochirus</i> sp.	OR360736*	OR352009*	IVB-H-Hsp05	Laboratory/Pet trade	This study	-	Female, karyotyped
<i>Hymenochirus</i> sp.	OR360736*	OR352009*	IVB-H-Hsp06/DNA	Laboratory/Pet trade	This study	-	Female, karyotyped
<i>Hymenochirus</i> sp.	AY341726		?	Laboratory/Pet trade	Vences <i>et al.</i> (2003)	<i>H. boettgeri</i>	
<i>Hymenochirus</i> sp.	AY523756	AY523735	VUB 0092	Laboratory/Pet trade	Roelants & Bossuyt (2005)	<i>H. boettgeri</i>	
<i>Hymenochirus</i> sp.	AY583340		?	Laboratory/Pet trade	San Mauro <i>et al.</i> (2005)	<i>H. boettgeri</i>	
<i>Hymenochirus</i> sp.	AY581623		Isolate BJE-2004	Laboratory/Pet trade	Evans <i>et al.</i> (2004)	<i>Hymenochirus</i> sp.	
<i>Hymenochirus</i> sp.		AY874305	Isolate BJE-2004 [‡]	Laboratory/Pet trade	Evans <i>et al.</i> (2005)	<i>Hymenochirus</i> sp. / <i>H. curtipes</i>	
<i>Hymenochirus</i> sp.	HM991331		MNCN/ADN 28465	Laboratory/Pet trade	Irisarri <i>et al.</i> (2011)	<i>H. boettgeri</i>	
<i>Hymenochirus</i> sp.	MK907573		Isolate BJE3814	Laboratory/Pet trade	Cauret <i>et al.</i> (2020)	<i>H. boettgeri</i>	
<i>Hymenochirus</i> sp.		JQ073272	ZCMV 12658	Laboratory/Pet trade	Crotini <i>et al.</i> (2012)	<i>H. boettgeri</i>	
<i>Hymenochirus</i> sp.		CM033476 [†]	Isolate Female2	Laboratory/Pet trade	Bredeson <i>et al.</i> (2021)	<i>H. boettgeri</i>	
<i>Pseudhymenochirus merlini</i>	HM991333	HM998975	MNCN/ADN 28467	Guinea Bissau	Irisarri <i>et al.</i> (2011)	-	Outgroup
<i>Xenopus (Silurana) tropicalis</i>	MN259067		Isolate R7931_AMNH17274	Sierra Leone	Evans <i>et al.</i> (2019)	-	Outgroup
<i>X. (S.) tropicalis</i>		AY874306	MHNG 2644.55	Sierra Leone	Evans <i>et al.</i> (2005)	-	Outgroup

* The same haplotype in all individuals within a genetic marker (16S, rag1).

[‡] The voucher specimen is erroneously identified as KU 205801 in GenBank, but this represents a specimen of *Pipa pipa* (B.J. Evans, pers. comm., 7 August 2023).

[†] rag1 extracted from the assembly of chromosome 8_10 (Bredeson *et al.*, 2021), genome UCB_Hboe_1.0 (University of California, Berkeley, 2021, GenBank: GCA_019447015.1).

1

Table S2. Markers used in FISH analysis, amplified gDNA templates, GenBank accession numbers, lengths, PCR primer sequences, and references to studies in which the primers were designed. The *U1* snDNA amplicon was used from Fornaini *et al.* (2023).

Gene Symbol	Gene Name	gDNA Template	Accession No.	Length (bp)	Primer Sequence	Reference
<i>5S rRNA</i>	5S ribosomal RNA	<i>Xenopus (Silurana) tropicalis</i>	OR360596	251	XTR 5S F: 5'-CAGGGTGGTATGGCCGTAGG-3' XTR 5S R: 5'-AGCGCCGATCTCGTCTGAT-3'	Knytl & Fornaini (2021)
<i>28S rRNA</i>	28S ribosomal RNA	<i>Hymenochirus</i> sp.	OR389490	290	28SA: 5'-AAACTGTGGTGGAGGTCCGT-3' 28SB: 5'-CTTACAAAAGTGGCCCACTA-3'	Naito <i>et al.</i> (1992)
<i>U1 snRNA</i>	U1 small nuclear RNA	<i>X. (S.) tropicalis</i>	OQ714817	119	U1F: 5'-GCAGTCGAGATTCCCACATT-3' U1R: 5'-CTTACCTGGCAGGGGAGATA-3'	Silva <i>et al.</i> (2015)
<i>U2 snRNA</i>	U2 small nuclear RNA	<i>Hymenochirus boettgeri</i>	OR359290	177	U2F: 5'-ATCGCTTCTCGGCCTTATG-3' U2R: 5'-TCCCGCGGTACTGCAATA-3'	Bueno <i>et al.</i> (2013)

2

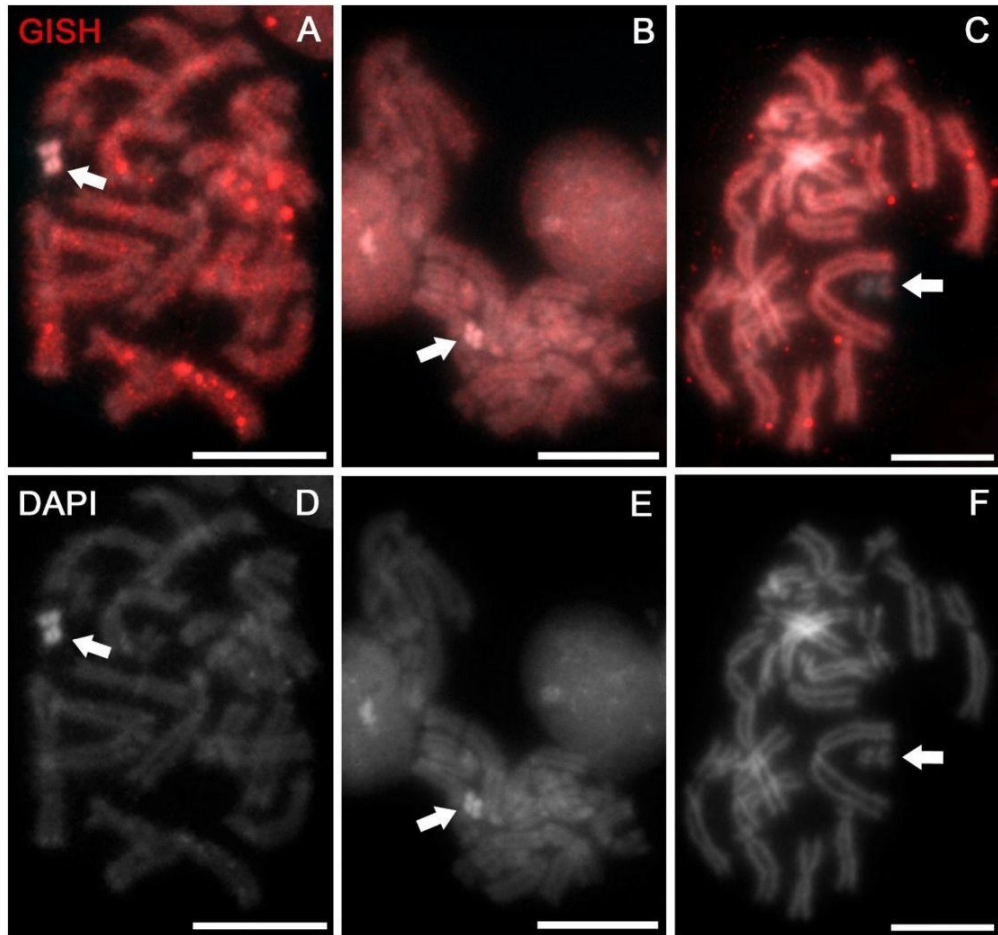


Figure S1. Genomic in situ hybridization (GISH) on *Hymenochirus* sp. (IVB-H-Hsp06) metaphase spreads. (A) The *H. boettgeri* whole genome painting (WGP) probe and *H. boettgeri* competitor DNA hybridize on all *Hymenochirus* sp. chromosomes (red) except one – the B chromosome shows no GISH signal (arrow). This is an identical figure as Fig. 5F. (B) Control GISH with the *Hymenochirus* sp. WGP probe and *Hymenochirus* sp. competitor DNA hybridized on all *Hymenochirus* sp. chromosomes (red) except one – the B chromosome shows no GISH signal (arrow). (C) Control GISH with the *Hymenochirus* sp. WGP probe without competitor DNA hybridized on all *Hymenochirus* sp. chromosomes (red) except one – the B chromosome shows no GISH signal (arrow). (D–F) DAPI channel (B&W) shows intensely painted B chromosomes (D, E, arrows) but no DAPI signal was found in the GISH experiment in which no competitor DNA was used (F, arrow). Scale bars represent 10 μm .

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Cytogenetic Analysis of the Fish Genus *Carassius* Indicates Divergence, Fission, and Segmental Duplication as Drivers of Tandem Repeat and Microchromosome Evolution

Nicola R. Fornaini¹, Halina Černohorská², Livia do Vale Martins³, and Martin Knytl ^{1,4,*}

¹Department of Cell Biology, Faculty of Science, Charles University, Prague 12843, Czech Republic

²Genetics and Reproductive Biotechnologies, CEITEC—Veterinary Research Institute, Brno 62100, Czech Republic

³Department of Biology, Federal University of Piauí, Floriano, Piauí, Brazil

⁴Department of Biology, McMaster University, Hamilton, Ontario L8S4K1, Canada

*Corresponding author: E-mail: martin.knytl@natur.cuni.cz.

Accepted: February 03, 2024

Abstract

Fishes of the genus *Carassius* are useful experimental vertebrate models for the study of evolutionary biology and cytogenetics. *Carassius* demonstrates diverse biological characteristics, such as variation in ploidy levels and chromosome numbers, and presence of microchromosomes. Those *Carassius* polyploids with ≥ 150 chromosomes have microchromosomes, but the origin of microchromosomes, especially in European populations, is unknown. We used cytogenetics to study evolution of tandem repeats (U1 and U2 small nuclear DNAs and H3 histone) and microchromosomes in *Carassius* from the Czech Republic. We tested the hypotheses whether the number of tandem repeats was affected by polyploidization or divergence between species and what mechanism drives evolution of microchromosomes. Tandem repeats were found in tetraploid and hexaploid *Carassius gibelio*, and tetraploid *Carassius auratus* and *Carassius carassius* in conserved numbers, with the exception of U1 small nuclear DNA in *C. auratus*. This conservation indicates reduction and/or loss in the number of copies per locus in hexaploids and may have occurred by divergence rather than polyploidization. To study the evolution of microchromosomes, we used the whole microchromosome painting probe from hexaploid *C. gibelio* and hybridized it to tetraploid and hexaploid *C. gibelio*, and tetraploid *C. auratus* and *C. carassius*. Our results revealed variation in the number of microchromosomes in hexaploids and indicated that the evolution of the *Carassius* karyotype is governed by macrochromosome fissions followed by segmental duplication in pericentromeric areas. These are potential mechanisms responsible for the presence of microchromosomes in *Carassius* hexaploids. Differential efficacy of one or both of these mechanisms in different tetraploids could ensure variability in chromosome number in polyploids in general.

Key words: teleost fish, polyploidy, U1 and U2 snDNAs, histone H3, chromosome painting, FISH.

Significance

Fish of the genus *Carassius* (Teleostei: Cyprinidae) are very popular models for studying genome and karyotype evolution, but there is a large gap in knowledge of microchromosome evolution and non-nucleolar tandemly repeated sequences at the level of experimental multispecies comparative studies. Based on cytogenetic investigation of closely related species with different ploidies, we propose three major processes that drive the evolution of microchromosomes and tandem arrays—divergence, fission, and post-polyploidization segmental duplication. All three drivers have a crucial impact on the evolution of *Carassius* and may trigger diversification into a plethora of individual extant species in the world.

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Introduction

In the fish family Cyprinidae (Cypriniformes, Teleostei), several independent polyploidization events (multiplications of haploid set of chromosomes) have occurred, giving rise to species with a large diversity of ploidy levels (Cherfas 1966). Ancestral number of chromosomes for the entire group of cyprinids (cyprinids, cyprinid group = the whole family Cyprinidae) is estimated to be 50 (Winfield and Nelson 2012).

Within Cyprinidae, the paleotetraploid clade Cyprinini (sensu Yang et al. 2010) includes the *Carassius* and *Cyprinus* genera, both of which have 100 chromosomes and solely *Carassius* has ~150 and ~200 chromosomes. It is clear that at least three polyploidization events occurred in this genus (since the independent evolution of the clade Cyprinini). The first polyploidization occurred in the common ancestor of Cyprinini and resulted in a chromosome number equal to 100 (Yang et al. 2010). The second polyploidization gave rise to the chromosome number ~150 and the third to ~200. Species from the clade Cyprinini that have 100 chromosomes are considered evolutionary tetraploids because of the most recent diploid ancestor did have 50 chromosomes (Ohno et al. 1967). In terms of biological development, these evolutionary tetraploids produce reduced gametes with 50 chromosomes that fuse during fertilization and the offspring continues to develop with recombinant and restored unreduced genetic information. The karyotype formula of a diploid member of Cyprinini shows biological and evolutionary ploidy by means of $2n = 4x$, where n defines the number of chromosomes in a gamete of the extant species (biological ploidy), and x refers to the number of chromosomes in a gamete of the most recent diploid ancestor of the extant species (evolutionary ploidy) (Knytl et al. 2017). In the following text, ploidy levels will be referred to the evolutionary term of ploidy, i.e. tetraploid, hexaploid, and octoploid *Carassius* are those with 100, ~150, and ~200 chromosomes, respectively.

Carassius is the most commonly used Cyprinini model for biological research (e.g. Pang et al. 2017; Ağdamar et al. 2020; Khosravi et al. 2022; Pavlov 2022a, 2022b; Tapkir et al. 2022; Wang et al. 2022a; Fedorčák et al. 2023; Jan et al. 2023; Tapkir et al. 2023). Its exceptional diversity of skills represents possible biological phenomena such as the presence of three ploidy levels—tetraploid, hexaploid, and octoploid (Kalous and Knytl 2011; Xiao et al. 2011; Knytl et al. 2022). The alternation of sexual and asexual (gynogenetic) mode of reproduction (Cherfas 1966; Przybył et al. 2020; Fuad et al. 2021) gives *Carassius* a competitive advantage in the rate of spatial expansion of asexuals. In addition, sexual reproduction should ensure higher resistance to parasites than gynogenesis due to recombination processes (Hakoyama et al. 2001). Another biological phenomenon is sex determination, which in *Carassius* is

governed by sex determining genes (Wen et al. 2020) and environmental temperature (Li et al. 2018). Variation in the number of chromosomes in *Carassius* polyploids (biologically speaking, individuals who possess ≥ 150 chromosomes) was revealed as another exceptional trait and may be caused by male genetic contribution into the egg/embryo (i.e. paternal leakage) during gynogenesis, leading to the presence of different numbers of microchromosomes in karyotypes (Yi et al. 2003; Ding et al. 2021). Macrochromosomes are larger than microchromosomes and possess clearly visible centromere, chromatids, and telomeres at both ends (Nanda and Schmid 1994).

Microchromosomes were originally thought to be redundant components of genomes, but have been found to be gene-rich and low in the content of the repetitive fraction (International Chicken Genome Sequencing Consortium 2004). In birds they occur in relatively high numbers (30–40 pairs) and their numbers are extremely conserved across various species (Waters et al. 2021; de Souza et al. 2023). However, this is not the case for *Carassius*. In *Carassius*, the number of microchromosomes ranges between 6 (Zhou and Gui 2002; Knytl et al. 2013b, 2018) and 18 (Zhao et al. 2021). There are only a few studies focusing on microchromosome painting, which usually crossed two strains of *Carassius* and analyzed their artificial offspring (Li et al. 2016, 2018; Zhao et al. 2021). The effect of paternal leakage in artificial *Carassius* progeny was evidenced by inseminating the *Carassius* egg with heterologous (i.e. from a species other than maternal one) sperm. The newly arisen offspring contained microchromosomes in karyotypes unlike the maternal karyotype, which did not contain them (Yi et al. 2003).

In the Czech Republic, *Carassius* is represented by four species. Commonly occurring invasive *Carassius gibelio* consists of tetraploid, hexaploid, and octoploid ploidy levels (Lusk et al. 2010; Knytl et al. 2013b). *Carassius auratus*, very well-known due to its colorful varieties as goldfish. This species also forms tetraploids, hexaploids, and octoploids (Xiao et al. 2011; Rylková et al. 2013). The third species, *Carassius carassius*, native to the Czech Republic, has been considered critically endangered since 2017 (Chobot and Němec 2017) and is strictly tetraploid (Knytl et al. 2013a). The fourth, *Carassius langsdorfii*, was discovered in the Czech Republic in 2007 by Kalous et al. (2007). The discovered female was hexaploid, but no cytogenetic examination other than conventional Giemsa staining has been conducted. Additionally, several *Carassius* hybrids were identified in natural Czech waters (Papoušek et al. 2008; Knytl et al. 2013b, 2018).

Due to the very complex characteristics and possible cryptic hybridization between *Carassius* species, it is difficult to reveal the origin of polyploids in the sense of allopolyploidy (more ancestors) or autopolyploidy (single ancestral species). Nevertheless, mixed allo- and autopolyploid origin was revealed and subgenomes were identified in hexaploid

C. gibelio by whole genome sequencing (Kuhl et al. 2022). Subgenomes are genomic units that originate from lower ploidy ancestors. Genome of hexaploid *C. gibelio* consists of three subgenomes (Kuhl et al. 2022; Wang et al. 2022b). It is clear that hybridization and an allopolyploid event caused changes in the number of chromosomes and microchromosomes, but the origin and function of the microchromosomes in *Carassius* remain obscure, as well as the number and localization of the U1 and U2 loci of small nuclear DNA (snDNA) and/or histone H3, which represent the repetitive fraction of a genome by tandemly repeated arrays and are not associated with nucleolus (Huang and Spector 1992), hereafter referred to as (non-nucleolar) tandem repeats.

We used *Carassius* from natural waters of the Czech Republic to study inter-ploidy relationships within a species and between multiple species, specifically tetraploid and hexaploid *C. gibelio*, and tetraploid *C. auratus* and *C. carassius*. We used fluorescent in situ hybridization (FISH) to map U1 and U2 snDNAs and H3 histone in tetraploids and hexaploids. Moreover, we used painting FISH to map microchromosomes in hexaploids and genomic regions associated with microchromosomes in tetraploids. We addressed to answer the following questions: (i) Do interspecific *Carassius* tetraploids share the same number of non-nucleolar tandem repeats examined? (ii) Do hexaploid females have one and a half times higher proportion of these tandem repeat loci than tetraploids? (iii) Are there any differences in the number and position of FISH signals for microchromosomes in hexaploids? (iv) What are the possible origins of microchromosomes and the evolutionary forces behind their evolution? Here, we consider the distribution of tandem repeats and microchromosomes in *Carassius* tetraploid and hexaploid karyotypes in an evolutionary context.

Results

FISH with Repetitive DNA Probes

We hybridized *C. gibelio* U1, U2, and H3 probes in four different groups: tetraploid ($2n = 4x = 100$) and hexaploid ($3n = 6n = 157$) *C. gibelio*, tetraploid *C. auratus* ($2n = 4x = 100$), and tetraploid *C. carassius* ($2n = 4x = 100$) (Fig. 1). Evolutionary relationships among the *Carassius* species examined are shown in the phylogenetic tree with *Cyprinus carpio* as an outgroup. *Carassius gibelio* and *C. auratus* form a single mitochondrial clade, while *C. carassius* is more distant to the others. FISH using U1 and U2 probes showed two signals (on one homologous pair) for each gene, except for the U1 gene in *C. auratus*, which showed four clear signals, while two signals for the U2 gene are consistent with the other species. FISH using H3 probe showed four signals in each species. The same number of loci did not support the expectation that hexaploid *C. gibelio* should have one and a half as many snDNA loci as tetraploid *C. gibelio*. These results indicate a

copy number reduction or loss of the entire U1, U2, and H3 loci in a subgenome (after polyploidization). The variation in the number of FISH signals in individuals with the same ploidy level and the chromosome number may be explained by a variation in the copy numbers of tandem repeats per locus, which is explained in detail in section “snDNA Tandem Repeats”.

Intra-ploidy Painting FISH with Whole Microchromosome Painting Probe

Microchromosomes selected for microdissection and subsequent FISH procedure were identified based on the following criteria: they are tiny, have an unclear centromere position and indistinguishable chromatids, and may be unpaired in a karyotype.

The first painting FISH experiment was conspecific (*C. gibelio* probe against *C. gibelio* metaphases) and within individuals with the same ploidy level (hexaploid probe against hexaploid metaphases), referred to as intra-ploidy conspecific painting FISH. We conducted these experiments as a control to see if the whole microchromosome painting probe worked well, and also to see if the number of microchromosomes differed among *Carassius* populations. We used *Carassius* chromosomes from three different river basins in the Czech Republic. Conspecific painting FISH revealed variation in the number of microchromosomes, which ranged from six to nine (Fig. 2).

Five investigated females possessed 150 (Odra River basin, Fig. 2A), 149, 150 (both Elbe River basin, Fig. 2B, C), 150 (Mrlina River basin, Fig. 2D), and 153 (Elbe River basin, Fig. 2E) chromosomes with 6, 6, 7, 8, and 9 microchromosomes, respectively. *Carassius* used to generate the whole chromosome painting probe originated from the Odra River basin. The variation in the number of microchromosomes was consistent with previous studies (Zhou and Gui 2002; Knytl et al. 2013b; Li et al. 2016; Knytl et al. 2018; Li et al. 2018). The FISH signal was consistently spread over the entire surface of the microchromosomes, indicating the high efficacy of the FISH technique conducted. The presence of clearly highlighted chromosomes indicates that the probe and competitor DNA collaborated properly and that nonspecific hybridization of the probe to repetitive regions was inhibited. The *Carassius* microchromosomes did not show a high degree of heterochromatization, as is typical for B chromosomes, which are sometimes considered to be microchromosomes (Bishani et al. 2021; Gvoždík et al. 2023). Our finding is consistent with a high gene content and a low proportion of a repetitive fraction within the microchromosomes.

Inter-ploidy Painting FISH with Whole Microchromosome Painting Probe

The other aim of the study was to trace the origin of microchromosomes using tetraploid *Carassius* relatives and to

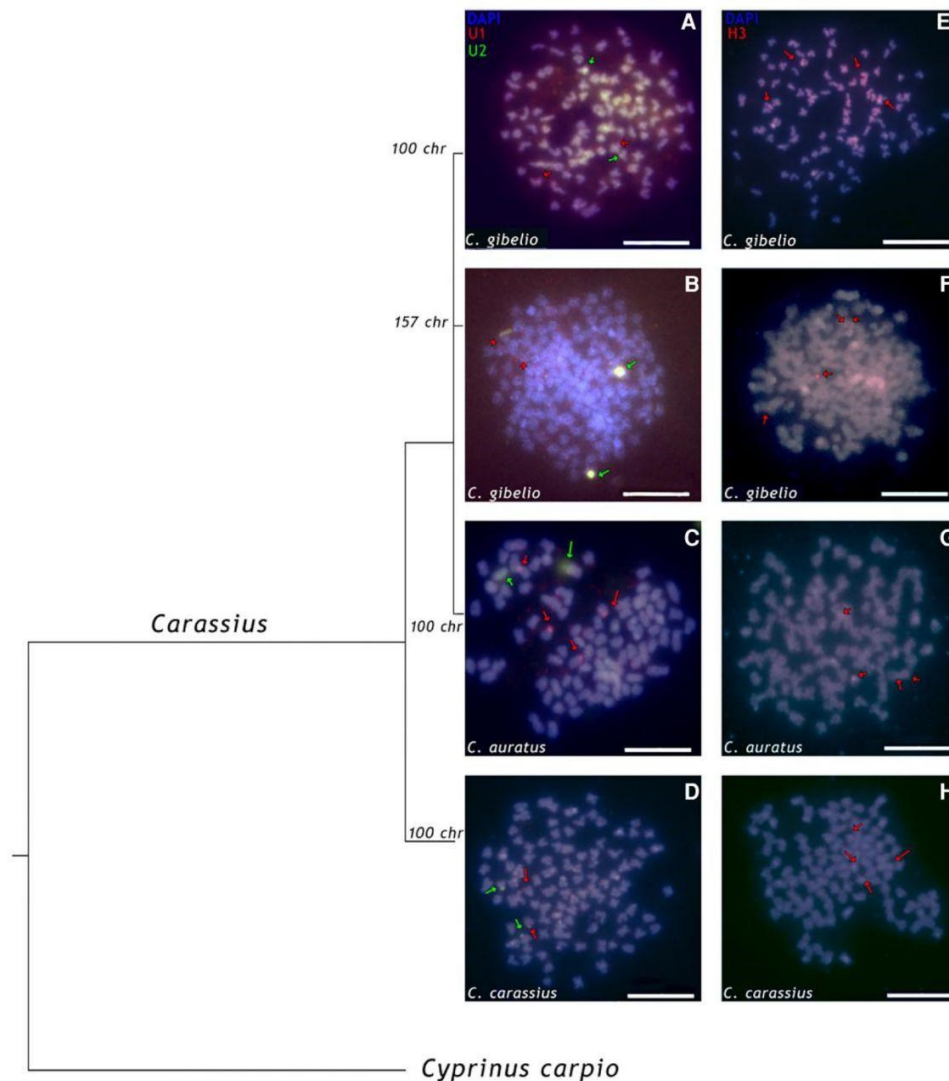


FIG. 1.—Double-color FISH with U1 and U2 snDNA probes (A, B, C, D). The U1 probe reveals one clear signal (= a pair of homologous chromosomes) in tetraploid (A) and hexaploid (B) *C. gibelio*, tetraploid *C. carassius* (D), while the same FISH shows two signals in tetraploid *C. auratus* (C). The U2 probe shows one signal in all species. The green and red arrows correspond to the U2 and U1 repeat loci, respectively. The U1 and U2 signals are located in the telomeric part of chromosomes as well as the H3 signals. Single-color FISH with the H3 snDNA probe (E, F, G, H). The probe shows two clear signals for all species: *C. gibelio* (E, F), *C. auratus* (G), and *C. carassius* (H). Chromosomes were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) in blue/gray. Some DAPI-intensive spots are also visible at centromere positions or cover entire chromosomes (B, D, E, no arrows). Scale bars represent 10 μm . Each *Carassius* metaphase is anchored in the phylogenetic tree to visualize the phylogenetic distance between specimens. Each branch depicts the number of chromosomes that corresponds to the appropriate individual. *Cyprinus carpio* is used as outgroup at the bottom of the figure.

determine whether some of the genome sequences in tetraploids are similar to those sequences in microchromosomes in hexaploids. We hybridized the whole microchromosome

painting probe (the same one as we used for intra-ploidy painting FISH) to metaphase spreads of tetraploid male and female *C. gibelio* (inter-ploidy conspecific painting

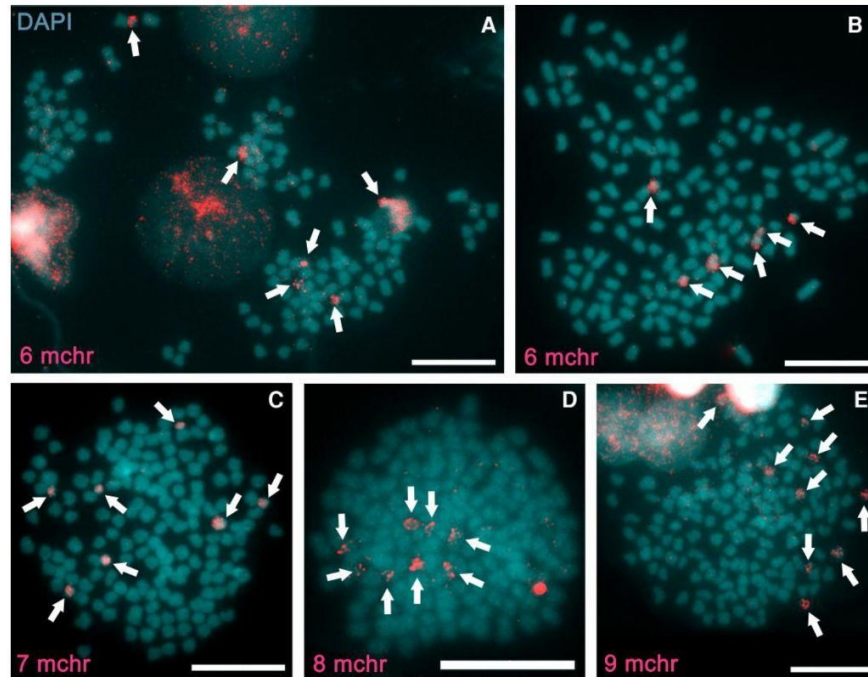


Fig. 2.—Intra-ploidy conspecific painting FISH with the *C. gibelio* whole microchromosome painting probe hybridized to the hexaploid *C. gibelio* females. Each metaphase represents a different individual. The microchromosome probe (red signal, arrows) shows different numbers of microchromosomes in each individual, respectively, six (A, B), seven (C), eight (D), and nine (E). Chromosomes were counterstained with DAPI in blue/gray. Nuclei with unfragmented chromatin are visible (top left, A, E). Scale bars represent 10 μ m.

FISH), *C. auratus*, *C. carassius*, and *C. carpio* (inter-ploidy interspecific painting FISH). Inter-ploidy painting FISH showed the similarity of microchromosomes to some genomic regions in tetraploids (microchromosome-associated regions). Fluorescent signals do not cover the entire chromosome lengths and are usually located in pericentromeric regions of mapped chromosomes. The highlighted pericentromeric region indicates that this portion gave rise to the microchromosomes by fission of macrochromosomes, followed by segmental duplication of specific pericentromeric regions (see in “Mechanism of Origin of *Carassius* Microchromosomes”). The number and intensity of the FISH signals decrease with increasing phylogenetic distance (Fig. 3). As expected, *C. gibelio* (Fig. 3A) showed the highest intensity and the highest number of the FISH signals. On the other hand, *C. carpio* (Fig. 3D), the most distant relative, showed very low intensity of the FISH signals. The number of signals ranges from 8 to 10 in *C. gibelio*, from 6 to 8 in *C. auratus*, and from 4 to 6 in *C. carassius*. The number of signals in *C. carpio* was stable and showed four loci related to the microchromosome-associated regions. No differences between males and females were found.

Considering the mapping of tandem repeats and microchromosomes together, we can exclude co-localization of the investigated tandem repeats with microchromosome-associated regions, and thus we can exclude mutual interactions of U1 or U2 snDNAs or H3 histones with microchromosome-associated regions within a single chromosome. The FISH signals of tandem repeats are situated in pericentromeric regions of telocentric and/or subtelocentric chromosomes with measured centromeric index 0–25. Microchromosome-associated fragments are also located in pericentromeric regions but on submetacentric and metacentric chromosomes (centromeric index 25–50). In each *Carassius* species, a pair of chromosomes carrying the microchromosome-associated locus is one of the largest chromosomes in the karyotype.

Discussion

snDNA Tandem Repeats

Carassius is a widely used experimental model for its extraordinary characteristics, but is rarely used for tandem repeat mapping or microchromosome painting. The consequences of genome duplication and divergence can be studied if we

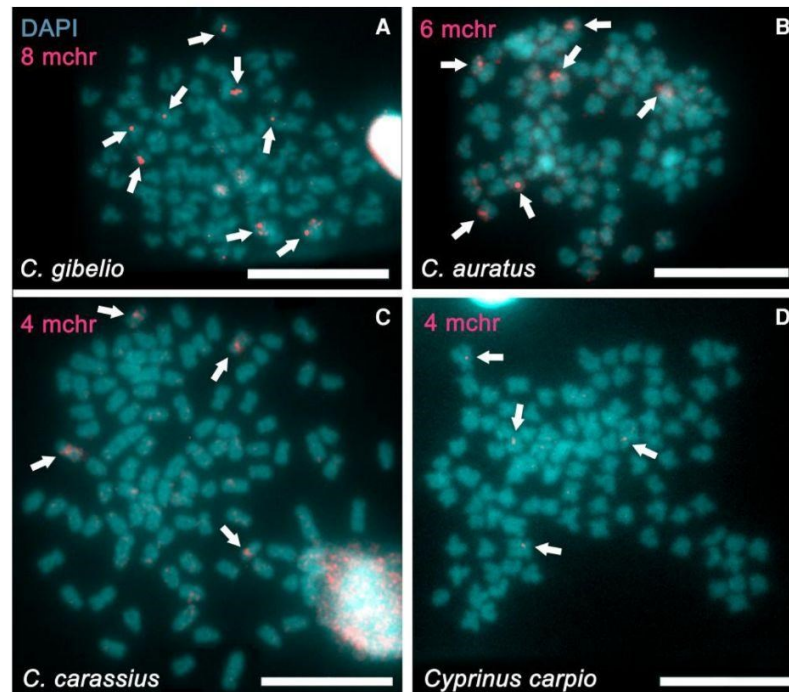


Fig. 3.—Inter-ploidy painting FISH with the *C. gibelio* whole microchromosome painting probe mapped to chromosomes of four tetraploid species, *C. gibelio*, *C. auratus*, *C. carassius*, and *C. carpio*. The microchromosome probe (red) shows signals on portions of the pericentromeric chromosomal regions, indicated by arrows. The number of FISH signals varies by species: eight in *C. gibelio* (A), six in *C. auratus* (B), four in *C. carassius* (C), and four in *C. carpio* (D). Chromosomes were counterstained with DAPI in blue/gray. Scale bars represent 10 μm .

can compare the number of tandem repeat loci between different ploidy levels of closely related species (Fornaini et al. 2023), and this is precisely what can be examined in *Carassius*.

One way to find out these evolutionary consequences is to map tandem repeats on chromosomes using FISH. We compared the number of FISH signals of non-nucleolar tandem repeats (U1 and U2 snDNA and H3 histone) in tetraploid *C. gibelio* with hexaploid *C. gibelio*. Our hypothesis was that hexaploid *C. gibelio* would have one and a half as many signals as tetraploid *C. gibelio*. However, we found equal numbers of all tandem repeat loci in both tetraploids and hexaploids (Fig. 1). The expectations were not met, and we found out that divergence affected the number of tandem repeats more effectively than polyploidization. Since we found the same number of tandem repeats in *C. gibelio* tetraploids and hexaploids, hexaploids may experience a reduction in the number of tandem repeat copies per locus or a complete loss of a tandem repeat locus, likely due to an ongoing evolutionary process of re-diploidization with no need to duplicate and produce more snRNA or H3

histones than in tetraploids (Fornaini et al. 2023). In the other investigated tetraploids, *C. auratus* and *C. carassius*, we found the same number of tandem repeats as in hexaploid *C. gibelio*, except for the U1 locus in *C. auratus* localized on two chromosome pairs. The number of mapped loci is summarized in Table 1.

Mapping of snDNAs is rare throughout Cyprinidae. To our knowledge, this is the first cytogenetic localization of U1 snDNA within the clade Cyprinini. For instance, in the cyprinid genus *Hypophthalmichthys*, which is not paleotetraploid as Cyprinini, a single chromosome pair carries the U1 snDNA locus (Sember et al. 2020). Why the U1 locus was retained duplicated in *C. auratus* and why this locus was reduced in other *Carassius* species is not yet clear, but few other known U1 mapping studies carried out on species other than cyprinids show that the U1 locus is present in a karyotype on single chromosome pair (Cabral-De-Mello et al. 2012; Carvalho et al. 2017; Malimpensa et al. 2020) as well as on six chromosome pairs (Silva et al. 2015). The U2 snDNA locus was previously mapped to *C. carassius* and *C. gibelio* (Bishani et al.

2021). The number of the U2 snDNA signals in *C. carassius* agrees with our results, however, Bishani et al. (2021) identified six U2 signals (two triplets) in the hexaploid *C. gibelio* versus two signals we found in this study. This inconsistency may be due to genetic variation in the copy number of U2 tandem repeats per locus between different *C. gibelio* populations because *C. gibelio* is a complex of individuals with diverse biological skills and different origins (Knytl et al. 2022; Lu et al. 2023).

Another type of tandem repeats is ribosomal RNA which underpins and organizes the nucleolar organizer structure (NOR) and contains 18S, 5.8S, and 28S clusters of ribosomal DNA, rDNA (nucleolar tandem repeats) (Symonová and Howell 2018). The number of nucleolar tandem repeats in tetraploid *Carassius* appears to be more complex. Tetraploid *Carassius* has NORs on two chromosome pairs (Spoz et al. 2014; Knytl et al. 2018; Knytl and Fornaini 2021) which indicates the multiplication of the NOR number due to whole genome duplication and is consistent with paleotetraploid origin (Yang et al. 2010). Cytogenetic mapping of nucleolar tandem repeats in *Carassius* hexaploids indicates a complete/incomplete loss of this locus in one subgenome because their number in hexaploid is equal to the number of NORs in tetraploids (Knytl et al. 2018).

Both nucleolar and non-nucleolar tandem repeats have similar evolutionary destinies leading to loss and/or deletion of redundant copies or reduced copy number per locus—one pair of U1, one pair of U2 snDNAs, and two pairs of H3 histones in tetraploid and hexaploid *C. gibelio* (this study); two pairs of NORs in tetraploid and hexaploid *Carassius* (Knytl et al. 2018).

Table 1

Numbers of U1 and U2 SnDNA, histone H3, and microchromosome-associated (Mchr.) loci in *Carassius* found in this study

Specimen	Ploidy	U1	U2	H3	Mchr.
<i>C. gibelio</i>	2n = 4x	2	2	4	8–10
<i>C. gibelio</i>	3n = 6x	2	2	4	6–9
<i>C. auratus</i>	2n = 4x	4	2	4	6–8
<i>C. carassius</i>	2n = 4x	2	2	4	4–6
<i>C. carpio</i>	2n = 4x	NA	NA	NA	4

Note. Signals are counted per whole genome (one locus = signal on one chromosome regardless of homologous or non-homologous). NA = information not available from this study.

Table 2

Tandem repeats used for FISH analysis, their GenBank accession numbers, lengths, sequences of primers, and studies in which primers were designed

Gene name	Accession no.	Length (bp)	Primer sequence	Reference
Small nuclear RNA U1	OR366029	112	U1F 5'--GCAGTCGAGATTCCCACATT--3'	Silva et al. (2015)
			U1R 5'--CTTACCTGGCAGGGGAGATA--3'	
Small nuclear RNA U2	OR366030	140	U2F 5'--ATCGCTTCTCGGCCTTAT--3'	Bueno et al. (2013)
			U2R 5'--TCCCGGCGGTAAGCAATA--3'	
			H3F 5'--ATGGCTCGTACCAAGCAGAC (ACG) GC--3'	
Histone H3	OR596332	375	H3R 5'--ATATCCCT (AG) GGCAAT (AG) AT (AG) GTGAC--3'	Colgan et al. (1998)

Evolution of *Carassius* Microchromosomes

The complex of the genus *Carassius* is a group of representatives with different characteristics and in some cases they do not meet the definition of a species (Kalous et al. 2012; Knytl et al. 2022). Therefore, it is important to comprehend the phylogeny of this group. For this reason, we generated a phylogenetic tree from the known mitochondrial sequences of the cytochrome b gene. This analysis confirmed the closest relationship between *C. gibelio* and *C. auratus* and a greater evolutionary distance between the latter two species and *C. carassius* (phylogenetic tree in Fig. 1). Chromosome painting with the whole microchromosome painting probe coincides with the phylogenetic distance and shows the highest intensity and number of signals in the most closely related tetraploid *C. gibelio* and *C. auratus* (Fig. 3A, B and Table 1). Tetraploid and hexaploid *C. gibelio* can form polyphyletic lineage (Kalous et al. 2012), and since there is a diversity of abilities between these ploidy levels, such as origin and modes of reproduction, definition of species is somewhat of controversial (Kalous et al. 2012). When we used the whole microchromosome painting probe on *C. carassius* chromosomes, the intensity and number of fluorescent signals are obviously lower than on *C. gibelio* and *C. auratus* chromosomes, but spatially the signal in *C. carassius* covers at least the same-size or even larger chromosomal region than in *C. gibelio/C. auratus* (Fig. 3A–C). In the most distant *C. carpio*, intensity of the signal is the lowest from all specimens tested, and the number of signals is equal to the number of signals in *C. carassius*.

High intensity and strength of the FISH signal may be indicative of repetitive elements, as is the case of tandem repeats in this study or a different type of repetitions in other studies (e.g. Knytl et al. 2018). We tried to avoid a repetitive signal by using autoclaved competitor DNA. This DNA blocking approach inhibits binding of a probe to repetitions. We successfully used the same DNA blocking technique for painting FISH to map unique genomic sequences (Knytl et al. 2013b, 2017, 2023). We also increased stringency washing because signals were not detected using the previously verified FISH protocol. Using the described DNA blocking and protocol modifications,

we exclude random nonspecific binding of the probe to chromosomes.

It has been proposed that the number of microchromosomes varies within *Carassius* species (Yi et al. 2003; Li et al. 2016, 2018) and microchromosomes are one of the sources associated with variation in chromosome number in *Carassius*. Yi et al. (2003) carried out a crossbreeding experiment in which *C. gibelio* eggs were fertilized with heterologous sperm of *Megalobrama amblycephala*. The newly produced artificial progeny possessed from 5 to 15 microchromosomes. The microchromosomes were microdissected and used for whole microchromosome painting probe. The probe was hybridized with chromosomes of both parental species. The maternal karyotype did not show any FISH signals, indicating absence of microchromosomes. The paternal karyotype of *M. amblycephala* contained microchromosome-associated regions on four chromosome pairs and the signal was spread over parts of the chromosomes. Two signals were situated on telomeres and the remaining two signals in pericentromeric regions. The study brought evidence of paternal leakage into the allogynogenetic offspring. Similarly, we observed signals in the pericentromeric regions of two to four pairs of chromosomes in *Carassius* tetraploids and *C. carpio*.

Mechanism of Origin of *Carassius* Microchromosomes

If the microchromosomes in the hexaploid females we examined were derived from the paternal leakage, all six to nine microchromosomes would have originated from the father, as suggested by the artificial crosses conducted by Yi et al. (2003). There is no evidence as to what species was the donor of the microchromosomal genome complement in our study. Both maternal genome complement and paternal leakage could have led to the synergistic collaboration and microchromosome formation in hexaploids. The number of microchromosomes can shape/change independently multiple times due to paternal leakage through gynogenesis (Yi et al. 2003; Lamatsch and Stöck 2009; Knytl et al. 2013b). It was found that microchromosomes are building blocks in birds, reptiles, and mammals that undergo the mechanism of macrochromosome fission and/or fusion of two micro- or micro- and macrochromosomes (Kretschmer et al. 2020; Waters et al. 2021). For instance, mammals do not have microchromosomes (Srikulnath et al. 2021) and therefore fusions involving microchromosomes occurred in their common progenitor(s), but after divergence with birds and reptiles that do have microchromosomes (Waters et al. 2021). In *Carassius*, the most likely mechanism for the origin of microchromosome is fission of macrochromosomes. Our results indicate that microchromosomes arose by fission from the pericentromeric regions of the submeta- and metacentric chromosomes of tetraploid *Carassius* species.

The number of microchromosomes in hexaploid females (6–9) corresponds to the number of microchromosome-associated regions in tetraploids (4–10), but the painting signal in tetraploids covers only a portion of the chromosomal area compared to the signal on entire chromosomes in hexaploids, so there should be an additional mechanism besides fission that amplifies microchromosome-like regions. Duplication of an entire block of gene(s) translocated from the macrochromosome to the microchromosome after genome duplication, i.e. segmental duplication (Leister 2004), might be an accompanying mechanism that played an important role in the evolution of *Carassius* microchromosomes. Presumably, tandem duplication, a process in which gene(s) is/are replicated at the original chromosomal locus without subsequent relocation (Blanc et al. 2000), might collaborate with fission after whole genome duplication in *Carassius*. Segmental or tandem duplications can be followed by gene loss at the original locus (Leister 2004) due to divergence (see less widespread FISH signals in tetraploids than in hexaploids; Figs. 2 and 3). Segmental/tandem duplications were evidenced, for example, in plants of the genus *Arabidopsis* (Blanc et al. 2000; Cannon et al. 2004; Leister 2004).

Direct proof of our proposed mechanisms can be provided by sequencing of *Carassius* microchromosomes. This analysis requires microdissection, library preparation, and sequencing of every single microchromosome. While this analysis is time consuming and costly, future evidence is desirable for a better mechanistic understanding of the evolution of *Carassius* microchromosomes. Since *Carassius* genomes have already been sequenced (Li et al. 2021; Kuhl et al. 2022; Wang et al. 2022b), it is possible to map sequences from microdissected microchromosomes and compare them with microchromosome sequences from Chinese *Carassius* populations (Ding et al. 2021).

Back in Time: Microchromosomes in the Most Recent Common Ancestor of Bony Vertebrates

Bony vertebrates (Euteleostomi, sensu lato Osteichthyes) are a clade of vertebrates that formed after divergence from agnathans and chondrichthyans. It is estimated that the ancestral karyotype of bony vertebrates is similar to that of spotted gar (*Lepisosteus oculatus*). The present-day karyotype of this species contains 58 chromosomes, involving macrochromosomes and from 18 to 20 microchromosomes (Braasch et al. 2016; Symonová et al. 2017). *Lepisosteus oculatus* represents a strong conservation of microchromosome structure and synteny over 450 million years, for example, compared to the chicken genome (Braasch et al. 2016). It is assumed that the ancestral karyotype of bony vertebrates contained 12 microchromosomes (Sacerdot et al. 2018), a number similar to that of *Carassius*. It is questionable whether *Carassius* microchromosomes arise independently from macrochromosomes of *Carassius*

tetraploids, or whether these microchromosomes are highly conserved and homologous to the microchromosomes of ancestor of bony vertebrates, eventually to those of chicken and gar. Our results suggest an independent origin of the *Carassius* microchromosomes, as neither *Carassius* tetraploids nor *C. carpio* have any microchromosomes, but this does not exclude the hypothesis that *Carassius* microchromosomes are homologous to microchromosomes of chicken and/or gar. Microchromosomes can arise and disappear spontaneously during evolution due to the fusion–fission model of macro- and microchromosomes (reviewed in Srikulnath et al. 2021). Mapping *Carassius* microchromosomes to genomes that are not only closely related to *Carassius* genomes, but also to genomes related to the most recent common ancestor of bony vertebrates, i.e. the basal lineages of gars, is a challenge for further *Carassius* research.

Conclusion

Both repetitive elements and microchromosomes are integral components of the *Carassius* genome and their evolution can be affected by polyploidization or divergence between species. Analysis of tandem repeat mapping showed evolutionary conservation in the number of these mapped repeats between *Carassius* species with different ploidy levels, except for the U1 snDNA locus in *C. auratus*. This conservation suggests a reduction and loss of copy number per locus (particularly in hexaploids) that may have occurred by divergence after polyploidization. For further examples of reduction, loss, and/or expansion of copy number of tandem repeats per locus, see, e.g. Fornaini et al. (2023). Analysis of the whole microchromosome painting revealed evolution by fission followed by post-polyploidization segmental duplication of pericentromeric macrochromosomal regions as a potential mechanism responsible for the presence of microchromosomes in *Carassius* hexaploids. Other examples of these latter evolutionary mechanisms are Cannon et al. (2004) and Kretschmer et al. (2020). We did not find co-localization of tandem repeats with microchromosome-associated regions, so we ruled out their cooperation within the same chromosome locus. Both structures evolved independently in *Carassius*. All of our findings are consistent with structural genomic changes that follow genome duplication.

Materials and Methods

Fish Sampling and Origin

Carassius auratus was obtained via the aquarium trade (transported to the Czech Republic from Israel). *Carassius carassius* was collected in alluvial ponds and old oxbows of the Elbe River basin close to the city Lysá nad Labem, the Bohemia region. *Carassius gibelio* was captured in the Elbe

River basin close to the city Lysá nad Labem, the Bohemia region (hexaploids); in the Mrlina River basin, Global Positioning System: 50°15'35.8"N, 15°08'43.4"E, the village Zábřovice, the Bohemia region (hexaploids); in the Odra River basin, the Moravian-Silesian region (tetraploids, hexaploids). All three river basin districts are located in the Czech Republic. Field surveys of ichthyofauna were performed in 2010–2012 and then in the River Mrlina in 2021. More detailed information on morphological analysis, voucher specimens, and location coordinates are given in Knytl et al. (2013b). Sex of individuals was identified based on dissection of gonads. Males and females of *Carassius* tetraploids and females of *Carassius* hexaploids were used in this study. *Cyprinus carpio* gonads were not inspected by dissection and sex of this individual was not identified. Because *C. carpio* was used as an outgroup for our analyses, knowledge of sex and origin is not crucial to our investigation.

Chromosome Preparations

Chromosome spreads were prepared from tetraploid males and females (*C. gibelio*, *C. auratus*, and *C. carassius*) and hexaploid *C. gibelio* females. For all *Carassius* species, we used a method in which chromosome suspension was prepared directly from the cephalic kidney (Bertollo and Cioffi 2015). Mitotic activity was stimulated by intraperitoneal injection of 0.1% CoCl₂ per 100 g of weight 24 h before colchicine (Sigma, St. Louis, MO, USA) application (Knytl et al. 2018). Ready-to-use chromosome suspension was stored in fixative solution (methanol:acetic acid, 3:1, v/v) at –20 °C as described in Knytl and Fornaini (2021). *Cyprinus carpio* chromosomes were obtained from regenerating fin tissues according to Kalous et al. (2010). The methods of colchicine treatment and hypotonization were originally adopted from *Chromaphysemon* killifishes (Cyprinodontiformes, Nothobranchiidae) (Völker et al. 2006). Tissue fixation was performed and metaphases were spread on slides in the same manner as described in the embryo preparation protocol in Völker and Kullmann (2006). Microscopy and processing of metaphase images were conducted using Leica Microsystem (Wetzlar, Germany) as detailed in Seroussi et al. (2019). At least 20 metaphases were analyzed per each individual and five individuals were investigated per each analysis.

FISH with Repetitive DNA Probes

In order to generate probes for FISH, genomic DNA (gDNA) from hexaploid *C. gibelio* was used as a template for amplification of the U1 and U2 snDNA regions, and H3 histone. DNA was extracted from adult fish tissues using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primers used for amplification are listed in Table 2. The annealing temperature was 54 °C and the elongation step 30 s for all polymerase

chain reactions (PCR); other conditions for PCR amplification with PPP Master Mix (Top-Bio, Prague, Czech Republic) followed the manufacturer's recommendations. PCR amplification of the U1, U2, and H3 genes consistently resulted in 112, 140, and 375 bp long fragments, respectively. The search using the `blastn` algorithm confirmed the locus- and species-specificity of each amplicon: 98.98% identity with the U1 DNA sequence of *C. gibelio* (accession number XR_008182931.1), 97.10% identity with the U2 DNA sequence of *C. gibelio* (accession number XR_008154662.1), and 97.53% identity with H3 DNA of *C. gibelio* (accession number XM_052561945.1). Labeling PCR was performed as described in Knytl and Fornaini (2021). Digoxigenin-11-deoxyuridine triphosphate (dUTP) (Jena Bioscience, Jena, Germany) was used for U2 and H3 labeling, and biotin-16-dUTP (Jena Bioscience) was used for U1 labeling. *Carassius gibelio* U1 and U2 snDNA and H3 probes were then hybridized to chromosome spreads of *C. carassius* and *C. auratus* and tetraploid and hexaploid *C. gibelio*. The procedures for hybridization mixture preparation, denaturation, and subsequent overnight hybridization were previously described for rDNA FISH (Knytl et al. 2023). Post-hybridization stringency washing and blocking reactions were performed as described for painting FISH in Krylov et al. (2010). Probe signal was visualized following Knytl et al. (2017).

Phylogenetic Tree

We used mitochondrial cytochrome b of *C. carassius* (accession number KR131839), *C. auratus* (accession number KX688781), diploid (tetraploid) *C. gibelio* (accession number KX688784), and triploid (hexaploid) *C. gibelio* (accession number KX601125.1) to construct the phylogenetic tree. The *C. carpio* mitochondrial genome (accession number NC_001606) was used as an outgroup. Sequences were aligned using the multiple sequence comparison by log-expectation (MUSCLE) algorithm (Edgar 2004) through MEGA11 software (Tamura et al. 2021). After alignment, Iq-tree2 software (Minh et al. 2020) was used to predict the nucleotide substitution model using ModelFinder (Kalyaanamoorthy et al. 2017) and to create the tree. The best fitting model was found to be Hasegawa-Kishino-Yano (HKY+F) (Hasegawa et al. 1985). The tree was displayed using FigTree <http://tree.bio.ed.ac.uk/software/figtree/>.

Painting FISH with Whole Microchromosome Painting Probe

Microchromosomes from a single *C. gibelio* female were isolated individually by laser microdissection as previously described in Kubickova et al. (2002) using a PALM Microlaser system (Carl Zeiss MicroImaging GmbH, Munich, Germany). A total of 10 single microchromosomes

were dissected from multiple metaphases (approximately two microchromosomes from each metaphase). These microchromosomes were then pooled and used to paint a whole microchromosome FISH probe, subsequently completed using the GenomePlex Single Cell whole genome amplification Kit (WGA4), Sigma-Aldrich, according to the manufacturer's protocol for whole genome amplification with extracted gDNA. GenomePlex WGA Reamplification (WGA3), Sigma-Aldrich, and labeling with digoxigenin-11-dUTP (Jena Bioscience) were carried out as described in Krylov et al. (2010). Autoclaved *C. gibelio* gDNA (Bi and Bogart 2006) was used as a competitor (blocking DNA). The digoxigenin-labeled probe was detected by anti-digoxigenin-fluorescein (Roche, Basel, Switzerland). Conspecific painting FISH *C. gibelio*-*C. gibelio* was conducted as detailed in painting FISH in Krylov et al. (2010). Inter-ploidy painting FISH was carried out as described in Zoo-FISH in Krylov et al. (2010), with minor changes (Knytl et al. 2017). In addition, the current protocol was modified by increasing the total salt concentration from 2xSCC to 4xSCC (8xSCC with 50% formamide, 1:1, v/v) for less effective stringency washing. Chromosomes were counterstained with ProLong™ Diamond Antifade Mountant with the fluorescent *4ifinmath*,6-diamidino-2-phenylindole (DAPI) stain (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA).

Acknowledgments

The authors thank Lukáš Kalous, Štěpán Romočuský, and Jiří Knytl for help with sample collection and storage. Authors also thank two anonymous reviewers for their valuable suggestions in early version of the manuscript. This work is supported in part by funds from the P JAC project # CZ.02.01.01/00/22_010/0002902 MSCA Fellowships CZ-UK (M.K.), Grant Agency of Charles University # 54123 (N.R.F., M.K.), and Ministry of Agriculture of the Czech Republic, institutional support # MZE-RO0523 (H.C.). Open access publishing was supported by the National Technical Library in Prague (CzechELib).

Author Contributions

N.R.F., H.C., and M.K. conducted the experiments, received funding; N.R.F. and M.K. analyzed the results, designed graphics, prepared original draft; L.V.M. and M.K. conceptualized the study; M.K. is responsible for resources, visualization, data curation and validation, project administration, and supervision. All authors reviewed, edited, and approved the final version of the manuscript.

Conflict of Interest

No competing interest is declared.

Data Availability

All the data supporting the findings of this study are available within the article. Sanger sequencing data are deposited in the GenBank NCBI database, <https://www.ncbi.nlm.nih.gov/genbank/> (see Table 2 for accession numbers).

Statement of Ethics

M.K. is a holder of the certificate of professional competence to design experiments according to § 15d(3) of the Czech Republic Act No. 246/1992 coll. on the Protection of Animals against Cruelty (Registration number CZ 03973), provided by the Ministry of Agriculture of the Czech Republic.

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Associate editor: Federico Hoffmann