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Divergent subgenome evolution in the allotetraploid frog *Xenopus calcaratus*

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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 have not been investigated in detail. We used 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.

**Keywords:** genome, chromosome, cytogenetics, FISH, chromosome length, 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 “diploidization” of a polyploid genome, wherein chromosomes acquire disomic inheritance, with each having only one partner during cell division. 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](#)).
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, 2015; Session et al, 2016). In X. laevis (subgenus Xenopus) each subgenome contains distinct 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, 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.

Fig. 1 Phylogenetic scenarios of the subgenus *Silurana* adapted from *Evans et al* (2015).
The sister clade, subgenus *Xenopus*, is depicted as an outgroup. (a) A scenario involving one
allopolyploidization 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 allopolyploidization 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*.

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 the ‘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 death. 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 Sci-
eutic, 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 $-80^\circ\text{C}$ 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 $-20^\circ\text{C}$. For laser microdissection, a fresh metaphase suspension was dropped onto a polyethylene-naphthalene membrane. For cytogenetic analysis, a chromosome suspension was dropped onto a microscopic slide according to Courtet et al. (2001). Chromosome preparations were aged at $-20^\circ\text{C}$ for at least one week with the exception of that for the fluorescent in situ hybridization with tyramide signal amplification, FISH-TSA, in which the chromosome suspension was dropped and directly followed by experiment.

For each experiment, mitotic metaphase spreads were counterstained with ProLong$^\text{TM}$ Diamond Antifade Mountant with the fluorescent 4',6-diamidino-2-phenylindole, DAPI stain (Invitrogen by Thermo Fisher Scientific). From ten to twenty 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). Whole chromosome painting probes (WCPs) were prepared according to Krylov et al. (2010). 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 dig probes: 10mM dATP, dGTP, dCTP: 6.5 mM dTTP, bio probes: 10mM 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 CY$^\text{TM}$3-Streptavidin (Invitrogen, Camarillo, CA, USA), respectively, diluted with blocking reagents as used in double-colour rDNA FISH (Knytl et al, 2017). In the 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. calcara- tus* 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. Both 5S and 28S primer sequences (Integrated DNA Technologies, Coralville, IA, USA) are listed in Table S1. Preparation of the 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 70°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), which is 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 in *X. mellotropicalis* as revealed by Zoo-FISH and FISH-TSA (Knytl et al., 2017, 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 NCBI/GenBank (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 (*r*1) (Tymowska, 1973), centromeric index (*i*), and *q/p* arm ratio (*r*2) (Levan et al., 1964).

\[
l = p + q \quad r_1 = \frac{p}{q} \quad r_2 = \frac{q}{p} \quad i = \frac{100}{r_2 + 1}
\]

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) (Team, 2020). R scripts were used and modified from Knytl and Fornaini (2021). One-way analysis of variance (one-way ANOVA) was performed to compare *l* and *i* values between each homoeologous pair of chromosomes. All steps outlining how the measured values were calculated and processed into tables and plots are shown on https://www.github.com/.

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

Based on blastn results, the 5S amplicon showed 96% identity with the
sequence of 5S rDNA of X. tropicalis (accession number X12624.1), and 28S
amplicon showed 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. calcara-
tus 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. tropi-
calis (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. This species diagnosis is also consistent with chromo-
some counts as detailed below. Sequences are deposited to the NCBI/GenBank
database (5S accession number XXX, 28S accession number OM910742, 16S
accession number OM912675).

3.2 Fluorescent in situ hybridization with whole
chromosome painting probes

Ten whole chromosome probes (WCPs) were prepared from each sepa-
rately microdissected chromosome of X. tropicalis. As expected, each probe
hybridized specifically and stained a whole X. tropicalis 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 WCPs derived from this diploid
species.
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 telomeres and centromeres, which presumably is caused by repetitive sequences (red arrowheads). Each scale bar represents 10 µm.
Having confirmed specificity of the *X. tropicalis* WCPs, 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 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 homologous chromosome pair had consistently of higher fluorescent intensity than the other. Based on this, we defined *X. calcaratus* “a” homoeologs (or alpha, α, sensu Evans et al. (2005)) to be the more intensely painted chromosomes and “b” homoeologs (or beta, β, 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) probably caused by accumulation of repetitive sequences on the most portion of the *p* arm.
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 telomeres and centromeres, which presumably is caused by repetitive sequences (red arrowheads). Each scale bar represents 10 µm.
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 intensively 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 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.

![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.](image)

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 7aq (Fig. 5a, red colour). The FISH with 5S probes revealed 8–10 positive loci located on telomeric regions of the XCA 2b, 4a, 5a, 6a, and 8b Fig. 5a, green colour.

Xenopus calcaratus is closely related to X. mellotropicalis 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 1b (Fig. 5b and Fig. 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.

![Fig. 5](image_url) 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 5S rDNA loci. Scale bars represent 10 μm.
3.5 Single-copy gene mapping

To test whether *X. calcaratus* shared a large scale translocation with *X. melettotropicalis*, we mapped the alpha and beta homoeologs of *gyg2*, *cept1*, *sf3b1*, *ndufs1*, and *fn1* in *X. calcaratus*. In *X. melettotropicalis* *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 chromosomes 2b and 9aq (*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 Information (Figs. S1–S5). Thus, similar to the findings from the WCPs discussed above, mapping of single genes in *X. calcaratus* recovered no evidence of a translocation involving chromosomes 9b and 2b as was observed in *X. melettotropicalis* (*Knytl et al*, 2017, 2018b).

![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. White line indicates the centromere position.]

3.6 Mensural analysis of *X. calcaratus* chromosomes

As expected, across all *X. calcaratus* spreads (*n* = 35) we consistently counted 40 chromosomes, indicating that 2*n* = 4*x* = 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 determined as metacentric. If the *i* value was equal to or higher than 25 and lower than 37.5, the chromosome was determined as submetacentric, and if the *i* value was equal to or
greater than 12.5 and lower than 25, the chromosome was determined to be subtelocentric. The karyotype of \textit{X. calcaratus} consists of 12 metacentric, four submetacentric, and four 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 have low centromeric indexes (between 12.5–25) and are therefore subtelocentric. Chromosomes 5a and 5b have centromeric indexes between 25–37.5 and are 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 \(p\) arm (centromeric indexes = 0) are not present in the \textit{X. calcaratus} karyotype.

The \(l\) and \(i\) values were assigned to each individual chromosome 1a–10b from each metaphase and plotted (Fig. 7b and 7c).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{Analysis of \textit{X. calcaratus} chromosomes based on short and long arm length measurements. The a-subgenome is shown in red and b-subgenome 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 corresponding toacrocentric, 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 \textit{X. calcaratus} chromosomes (x axis). (c) intrachromosomal variability of \(i\) value (y axis) on the haploid complement 20 \textit{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.}
\end{figure}
A measure of statistical dispersion, 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 information, Box plot statistics of $l$, matrix $\text{stats}$ 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 information, Box plot statistics of $l$, matrix $\text{stats}$ 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 information, Box plot statistics of $i$, $\text{stats}$ 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 information, Box plot statistics of $i$, $\text{stats}$ 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 easily identifiable by measurements of the arms length.

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 CMA$_3$-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 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.
Table 1  Measured values used for identification of *Xenopus calcaratus* chromosomes (XCA). Median of chromosomal length (l (%)), median of p/q arm ratio (r₁), and median of centromeric index (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 (%) | r₁   | 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        |

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 (Figs. 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 were localized to *X. laevis* chromosome 6L and the oocyte-specific type of 5S genes was localized to telomeres 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 telomeres 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 non-nucleolar secondary constriction, but some of these signals may co-localize with NORs (e.g. 28S-positive signal, Fig. 5a, and CMA3-positive signal on XCA 7a, Fig. 5c). Several *Silurana* tetraploids have one intensively labeled non-nucleolar secondary constriction in the form of heterochromatic block: 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 size and position of the non-nucleolar secondary constrictions within *Silurana* species indicate that these constrictions are very dynamic structures with independent evolution but the most similar position 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 has been 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 (*Knytl et al.*, 2018a) because both include a diversity of diploid and polyploid species. In most diploid and polyploid amphibians, only one chromosome pair bears a NOR (*Schmid et al.*, 1987; *Inafuku et al.*, 2000; *Alves et al.*, 2012; *Schmid*, 1982). 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 (*Diniz et al.*, 2009; *Knytl et al.*, 2013a). One NOR pair may therefore reflect functional diploidization, wherein bivalents

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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). Number of nucleolar loci could be also changed by the 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 corresponds 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 chromosome in *X. calcaratus* subgenome "a" and "b" (1a–10b) 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 built 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 homoeologous pair – chromosome 2 – where the larger chromosome was actually from b-subgenome, warranting revision on 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. mel-

*Xenopus calcaratus* and *X. mellotropicalis* a-subgenomes have no translo-

Table 2 List of *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). The asterisks show chromosome renumbering.

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

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 the heterochromatin transpositions within b-subgenome. The pattern that the nucleolar 28S rDNA 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 a-subgenome being more stable and resembling the *X. tropicalis* diploid genome, and b-subgenome having experienced more chromosomal rearrangements. As discussed above, asymmetric subgenome evolution has also been reported in the allotetraploid *X. laevis* which is in subgenus *Xenopus* (Session et al, 2016), which argues for the plausibility of this phenomenon in subgenus *Silurana*.

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 undetectable by our experimental approaches. It is also possible that intraspecies 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 length, arm ratio, and centromeric indexes along, it was not possible to conclusively distinguish all homoeologous chromosomes from the “a” and “b” 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* WCPs on *X. mellotropicalis* chromosomes and gene mapping using FISH-TSA revealed a large-scale inter-chromosomal translocation of heterochromatic block from chromosome 9b to 2b, abbreviated t(9b;2b) (Knytl et al., 2017, 2018b). This translocation was not identified in *X. calcaratus* based on Zoo-FISH (Fig. 3) and also 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), sf3b1α 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, 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). Also, there is another possible scenario, that the translocation occurred between ancestral chromosomes in a diploid ancestor, abbreviated t(9;2), of *X. epitropicalis* and *X. mellotropicalis*. 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 (CMA$_3$ or C-banding positive, Figs. 5b and 5c) which suggests the repetitive regions associated with the t(9b;2b) of X. mello-tropicalis 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).

Fig. 8 Two potential evolutionary scenarios of the subgenus Silurana modified according to Fig. 1. (a) One allotetraploidization event gave rise to all three species (X. calcaratus, X. epitropicalis, and X. mello-tropicalis). (b) Two independent allotetraploidizations, the first gave rise to X. calcaratus. The second tetraploidization gave rise to a common ancestor of X. epitropicalis and X. mello-tropicalis. Blue spots indicate potential translocation between ancestral chromosomes 9 and 2 (t(9;2)) in a diploid, and between chromosomes 9b and 2b (t(9b;2b)) in a tetraploid common ancestor of X. epitropicalis and X. mello-tropicalis. Red spots indicate translocation occurred separately in X. mello-tropicalis lineage. All scenarios exclude the hypothesis of one shared translocation event in a common ancestor of all Silurana tetraploids.

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 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 advanced cytogenetic investigation of X. epitropicalis, the remaining member of a group of allotetraploid species, similar in scope with this study.

Supplementary information. Supplementary results: Box plot statistics of chromosomal length ($l$); Box plot statistics of centromeric index ($i$); Supplementary table: Table S1: The list of genes used for our study with appropriate gene symbol and name, GenBank accession number, length of the PCR amplicon, and PCR primer sequence.; Supplementary figures: Fig. S1: FISH-TSA with positive red signals on XCA metaphase spreads. The gyg2β and α were
localized on the p arm of XCA 2a and 2b, respectively. Chromosomes were counterstained with DAPI (blue-green). Each scale bar represents 10 µm.; Fig. S2: FISH-TSA with positive red signals on XCA metaphase spreads. The cept1β and α were localized on the p arm of XCA 2a and 2b, respectively. Chromosomes were counterstained with DAPI (blue-green). Each scale bar represents 10 µm.; Fig. S3: FISH-TSA with positive red signals on XCA metaphase spreads. The sf3b1α and β loci were mapped on the q arm of XCA 9a and 9b, respectively. Chromosomes were counterstained with DAPI (blue-green). Each scale bar represents 10 µm.; Fig. S4: FISH-TSA with positive red signals on XCA metaphase spreads. The ndufs1α and β loci were mapped on the q arm of XCA 9a and 9b, respectively. Chromosomes were counterstained with DAPI (blue-green). Each scale bar represents 10 µm.; Fig. S5: FISH-TSA with positive red signals on XCA metaphase spreads. The fn1α and β loci were mapped on the q arm of XCA 9a and 9b, respectively. Chromosomes were counterstained with DAPI (blue-green). Each scale bar represents 10 µm.

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Declarations

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• Conflict of interest The authors declare that they have no conflict of interest.

• 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, permit number XXX issued by the Ministry of Agriculture 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.

• Consent to participate Not applicable

• Consent for publication Not applicable

• Availability of data and materials Sanger sequencing data are available on-line at the NCBI database, accessed on XXX (https://www.ncbi.nlm.nih.gov/). All data generated by RStudio 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 are summarized on-line on GitHub, accessed on 21 May 2022 (https://www.
Raw data of chromosomal arm measurements can be provided in the form of RData file as R workspace, also upon request.

- Code availability
  Not applicable

- Authors’ contributions
  MK conceived and designed research. MK, NRF, BB, VG, and HČ conducted experiments. MK analyzed and validated data and performed statistical analyses. VG performed the fieldwork. MK wrote the initial manuscript and MK, BJE and VK then edited the manuscript. All authors read and approved the manuscript and agreed to the published version of the manuscript.

BJE and VK are joint last authors.

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