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Chromosome Banding in Amphibia. XXXII. The Genus *Xenopus* (Anura, Pipidae)

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Key Words

BrdU/dT replication banding · Chromosome staining · FISH · Polyploidy · *Xenopus* · *X. laevis*-type karyotype · *X. tropicalis*-type karyotype

Abstract

Mitotic chromosomes of 16 species of the frog genus *Xenopus* were prepared from kidney and lung cell cultures. In the chromosomes of 7 species, high-resolution replication banding patterns could be induced by treating the cultures with 5-bromodeoxyuridine (BrdU) and deoxythymidine (dT) in succession, and in 6 of these species the BrdU/dT-banded chromosomes could be arranged into karyotypes. In the 3 species of the clade with 2n = 20 and 4n = 40 chromosomes (*X. tropicalis*, *X. epitropicalis*, *X.* new tetraploid 1), as well as in the 3 species with 4n = 36 chromosomes *(X. laevis*, *X. borealis*, *X. muelleri)*, the BrdU/dT-banded karyotypes show a high degree of homoeology, though differences were detected between these groups. Translocations, inversions, insertions or sex-specific replication bands were not observed. Minor replication asynchronies found between chromosomes probably involve heterochromatic regions. BrdU/dT replication banding of *Xenopus* chromosomes provides the landmarks necessary for the exact physical mapping of genes and repetitive sequences. FISH with an *X. laevis* 5S rDNA probe detected multiple hybridization sites at or near the long-arm telomeric regions in most chromosomes of *X. laevis* and *X. borealis*, whereas in *X. muelleri*, the 5S rDNA sequences are located exclusively at the long-arm telomeres of a single chromosome pair. Staining with the AT base pair-specific fluorochrome quinacrine mustard revealed brightly fluorescing heterochromatic regions in the majority of *X. borealis* chromosomes which are absent in other *Xenopus* species.

Clawed frogs of the genus *Xenopus* belong to the tongueless frogs of the anuran family Pipidae. All species occur exclusively in Africa where they are generally parapatric, although in many regions different species occur sympatrically [Tinsley et al., 1996]. The species *Xenopus laevis* is the amphibian most widely used in biological research because of the ease with which it reproduces and can be reared in the laboratory [Gurdon, 1996]. The most remarkable cytogenetic fact in the genus *Xenopus* is that polyploidization has played a primary role in evolution and speciation. Two major clades, one consisting of 4 species and chromosome numbers of 2n = 20 and 4n = 40, the other one with 22 species and chromosome numbers ranging from 4n = 36 to 12n = 108, have been discovered (table 1). In some previous publications, the spe-

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Table 1. Species of the genus *Xenopus*, their ploidy level and relevant initial cytogenetic studies

Species	Ploidy level	References
X. tropicalis-type karyotypes		
X. tropicalis	2n = 20	Tymowska, 1973
X. epitropicalis	4n = 40	Tymowska and Fischberg, 1982; Tymowska, 1991
X. new tetraploid 1	4n = 40	Tymowska, 1991; Evans et al., 2004
X. new tetraploid 2	4n = 40	Evans et al., 2004
X. laevis type-karyotypes		
X. borealis	4n = 36	Tymowska and Fischberg, 1973; Tymowska, 1976, 1977, 1991
X. clivii	4n = 36	Tymowska and Fischberg, 1973; Tymowska, 1977
X. fraseri	4n = 36	Tymowska and Fischberg, 1973; Tymowska, 1977
X. gilli	4n = 36	Tymowska and Fischberg, 1973; Tymowska, 1977, 1991
X. laevis laevis	4n = 36	Wickbom, 1945; Weiler and Ohno, 1962; Morescalchi, 1963; Tymowska and Kobel, 1972; Tymowska, 1977, 1991
X. laevis bunyoniensis	4n = 36	Tymowska, 1991
X. laevis petersi	4n = 36	Tymowska and Fischberg, 1973; Tymowska, 1977, 1991
X. laevis poweri	4n = 36	Tymowska, 1991
X. laevis sudanensis	4n = 36	Tymowska, 1991
X. laevis victorianus	4n = 36	Tymowska and Fischberg, 1973; Tymowska, 1977, 1991
X. largeni	4n = 36	Tymowska, 1991
X. muelleri	4n = 36	Tymowska and Kobel, 1972; Tymowska and Fischberg, 1973; Tymowska, 1991
X. pygmaeus	4n = 36	Loumont, 1986
X. sp. nov. VI	4n = 36	Tymowska, 1991
X. sp. nov. IX	4n = 36	Tymowska, 1991
X. amieti	8n = 72	Kobel et al., 1980
X. andrei	8n = 72	Loumont, 1983
X. boumbaensis	8n = 72	Loumont, 1983
X. itombwensis	8n = 72	Evans et al., 2008
X. lenduensis	8n = 72	Evans et al., 2011
X. vestitus	8n = 72	Tymowska, 1976; Tymowska et al., 1977
X. wittei	8n = 72	Tymowska, 1976; Tymowska and Fischberg, 1980
X. sp. nov. X	8n = 72	Tymowska, 1991
X. longipes	12n = 108	Loumont and Kobel, 1991
X. ruwenzoriensis	12n = 108	Tymowska and Fischberg, 1973; Tymowska, 1991
X. cf. *boumbaensis*	12n = 108	Evans, 2007
X. sp. nov. VIII[a]	12n = 108	Tymowska, 1991

[a] According to Evans et al. [2012], the species status is uncertain. This species may be the same as *X.* cf. *boumbaensis*.

cies belonging to the former clade have been separated from the others and placed in the genus *Silurana* [e.g. Evans, 2008]. Polyploidization events have occurred independently in the 2 clades after they diverged 50–65 Mya [Evans et al., 2004; Chain and Evans, 2006; Hellsten et al., 2007].

In contrast to other amphibian groups, relatively few chromosome banding analyses have been performed on species of *Xenopus* [Matsui, 1974; Pardue, 1974; Fukui et al., 1975; Stock and Mengden, 1975; Tymowska and Fischberg, 1982; Sekiya and Nakagawa, 1983; Stock, 1984; Schmid et al., 1987; Schmid and Steinlein, 1991; Tymowska, 1991; Uehara et al., 2002]. Some of these focused on the experimental possibility to induce multiple G-bands in mitotic chromosomes of these frogs. The work presented here describes the results of various banding techniques, mainly 5-bromodeoxyuridine (BrdU) replication banding and quinacrine staining, as well as fluorescence in situ hybridizations (FISH) applied to the chromosomes of several species of *Xenopus*.

Table 2. The *Xenopus* species examined in the present study, collection sites, number of specimens analyzed, degree of ploidy, and cytogenetic techniques applied

Species	Country	Locality of sampling	Specimens	Ploidy	Techniques[a]
X. tropicalis	Ivory Coast	Adiopodoume	2♂, 1♀	2n = 20	1–3
	Ivory Coast	Adiopodoume	1♀	3n = 30[b]	1–3
X. epitropicalis	Congo	Kinshasa	7♂, 6♀	4n = 40	1, 2
X. new tetraploid 1[c]	Cameroon	Longyi	2♂, 2♀	4n = 40	1, 2
X. laevis	Republic of South Africa	unknown	2♂, 2♀	4n = 36	1–5
	unknown	breed (University of Würzburg)	4♂, 20♀	4n = 36	1–5
X. borealis	Kenya	Marsabit	4♂, 3♀	4n = 36	1–4
X. muelleri	Tanzania	Ifakara	5♂, 4♀	4n = 36	1–4
	unknown	animal dealer	10 juvenile	4n = 36	1–4
X. clivii	unknown	breed (University of Geneva)	3♂, 2♀	4n = 36	1, 3
X. ruwenzoriensis	unknown	animal dealer	2♂	12n = 108	1, 2

[a] 1 = Conventional staining; 2 = BrdU/dT replication banding patterns; 3 = quinacrine fluorescence; 4 = FISH with 5S rDNA probe; 5 = FISH with repetitive DNA probe (SAT-79).

[b] This single *X. tropicalis* was an occasional triploid individual.

[c] *X.* new tetraploid 1 was first known as *X.* species nova VII [Graf and Fischberg, 1986] and later as *X. paratropicalis* [Flajnik et al., 1993; Sato et al., 1993; Shum et al., 1993]. However, the latter does not represent a valid name [Blackburn and Beier, 2011].

Materials and Methods

Animals

Adult individuals of *X. tropicalis* (2♂, 2♀), *X. epitropicalis* (7♂, 6♀), *X.* new tetraploid 1 (2♂, 2♀), *X. laevis* (6♂, 22♀), *X. borealis* (4♂, 3♀), *X. muelleri* (5♂, 4♀, 10 juveniles), *X. clivii* (3♂, 2♀), and *X. ruwenzoriensis* (2♂) were obtained from Dr. Charles H. Thiébaud (Station de Zoologie Expérimentale, University of Geneva, Switzerland), Dr. Ulrich Scheer (Biocenter, University of Würzburg, Germany), and an animal dealer (Germany). The original collection sites of the animals, as far as known, are shown in table 2. All procedures with the living animals strictly conformed to the guidelines established by the Animal Care Committees of the respective countries. Eight additional species were obtained by Dr. Charles H. Thiébaud, but their fibroblast cultures yielded no satisfactory cell growth so that only conventional chromosome staining could be performed. These species were: *X. fraseri* (4n = 36), *X. largeni* (4n = 36), *X. pygmaeus* (4n = 36), *X. amieti* (8n = 72), *X. andrei* (8n = 72), *X.* species nova X (according to Tymowska [1991]; 8n = 72), *X. wittei* (8n = 72), and *X. boumbaensis* (8n = 72).

Xenopus new tetraploid 1 was first mentioned by Graf and Fischberg [1986] as *Xenopus* species nova VII, a tetraploid species with 4n = 40 chromosomes, which was initially collected in Longyi and Nkoemvone (Cameroon). Subsequently, Tymowska [1991] described its karyotype and recognized that this species is karyologically closely related to the tetraploid *X. epitropicalis* and diploid *X. tropicalis*. Only 2 years later, Flajnik et al. [1993], Sato et al. [1993] and Shum et al. [1993] used the name *X. paratropicalis* for this species. However, as outlined by Blackburn and Beier [2011], the name *X. paratropicalis* does not represent a valid name according to the criteria established by the International Commission on Zoological Nomenclature [1985]. Two decades later, this species was referred to as *S.* new tetraploid 1 by Evans et al. [2004], and this designation has been used in a number of subsequent publications [Evans et al., 2005, 2008, 2011; Evans, 2007; Chain et al., 2008; Bewick et al., 2010]. In the meantime, there are nearly 30 nucleotide sequences deposited in GenBank as *Xenopus* new tetraploid 1 (GenBank taxon ID: 451443). Therefore, this name is also used in the present study.

Cell Cultures and Chromosome Preparation

The animals were anesthetized with 0.05% MS222 (3-aminobenzoic acid ethyl ester) in water, sacrificed by cervical cleavage, and the kidneys and lungs aseptically removed and minced. Small pieces from both tissues (about 1 × 1 mm in size) were cultured in MEM (Gibco) supplemented with 13% fetal calf serum (Boehringer) and 0.9% penicillin-streptomycin (stock solution: 10,000 U/ml, Gibco). The cell cultures were kept for 4 weeks as monolayer cultures in 25-cm^2 flasks (Nunc) and incubated at 26°C under ordinary atmospheric conditions. Cells were harvested for chromosome analysis during the second subculture using a 0.05% trypsin/0.02% EDTA solution. Colcemid (Gibco) at a final concentration of 0.15 μg/ml of culture medium was added 2 h prior to harvesting the cells. Hypotonic treatment lasted 35 min at 37°C in 0.027 M sodium citrate. After hypotonic treatment, the cells were centrifuged for 10 min at 800 rpm, the hypotonic solution was discarded with a Pasteur pipette, and the cell pellet fixed drop-wise with 5–8 ml of ice-cold acetic acid:methanol (1:3). The fixed material was left overnight at 4°C and was then again centrifuged for 10 min at 800 rpm. Finally, the cells were resuspended in 1–2 ml fixative, and 3–6 drops of this suspension were dropped onto slides previously cleaned either in concentrated chromic-sulfuric acid or ethanol and rinsed well with deionized H_2O. The chromosome slides were air-dried at room temperature for 3 days and were not heated because this significantly reduced the quality of BrdU replication banding patterns.

BrdU/dT Replication Banding

All experiments were performed on non-synchronized kidney and lung cells from the second passage of the cell cultures. The highest rate of metaphases showing a clear BrdU replication banding was obtained when the cells were treated with BrdU and deoxythymidine (dT) within the last 24 h of culture. The following experimental procedure was used for all species of *Xenopus*.

Twenty-four hours before cell harvest, 100 µg/ml BrdU (Sigma) was added to the subcultures. After 16 h, the cells were washed twice with conventional MEM culture medium and fed with medium containing 48 µg/ml dT (Sigma). The subcultures were kept in this medium for the last 8 h.

Differential staining of the BrdU/dT replication bands was obtained with a modified fluorescence-plus-Giemsa (FPG) technique [Perry and Wolff, 1974]. Chromosome slides were aged for 3 days at room temperature and then immersed in buffered 0.03 µg/ml eosin Y solution for 30 min [Hazen et al., 1985]. Eosin Y (standard yellow, Fluka) was utilized in preference to Hoechst 33258, which is commonly used for the FPG technique, because it significantly enhances the microscopic resolution of the replication bands. After rinsing the slides in distilled water, they were UV-irradiated for 30 min in a freshly prepared buffer solution [Latt, 1973] at a distance of 10 cm from the UV lamp (254 nm) and submerged 1 cm below the buffer level. Then, the slides were rinsed in buffer [Latt, 1973] and incubated in 2× SSC for 90 min. Finally, the slides were stained for 6 min in 5% Giemsa solution (pH 6.8), washed twice in distilled water, air-dried, and permanently mounted in Eukitt.

Fluorochrome Staining of Chromosomes

Staining of metaphase chromosomes with the fluorochromes quinacrine mustard and DAPI was performed according to the methods in Schmid et al. [2010].

FISH Experiments

A 79-bp-long satellite DNA probe of *X. laevis* was provided by Dr. Joseph G. Gall (Carnegie Institution of Washington, Baltimore, Md., USA). The vector used was TOPO-PCRII (3.9 kb), which can be linearized with the restriction endonucleases *Spe*I, *Kpn*I or *Hind*III. Sequence details, organization, and transcription of this repetitive DNA were analyzed by Spohr et al. [1981].

The hybrid plasmid Xlo, containing *X. laevis* 5S rDNA, was used for the chromosomal localization of the 5S rRNA genes. The plasmid Xlo is a derivative of pMB9 into which one single repeating unit of 5S rDNA (0.7 kb) has been inserted. The inserted DNA sequence was obtained by cleaving *X. laevis* 5S rDNA with restriction endonuclease *Hin*dIII. The 5S rRNA gene itself comprises 120 bp of the total 6.1 kb in the hybrid plasmid [Brown and Gurdon, 1978].

Labeling of both probes with biotin, denaturation of the probes, FISH, and detection of the hybridized probes were as described in Schmid et al. [2010].

Microscopy

Microscopic analyses were conducted using Zeiss photomicroscopes III, Zeiss fluorescence microscopes and Zeiss Axiophot microscopes equipped with incident HBO 50W mercury lamp illumination. The filter combinations necessary for the analyses of metaphases stained with the various fluorochromes or for FISH were described by Schmid et al. [2010].

Results and Discussion

Karyotypes and Nomenclature

Xenopus chromosomes can be divided into 3 classes on the basis of centromere location: metacentrics, submetacentrics and subtelocentrics (acrocentrics). However, within these classes, chromosomes show a continuous gradient from large to small, and homologs can be paired up only with difficulty. Therefore, in conventionally stained preparations, it is not possible to identify with certainty all chromosome pairs. In their pioneering cytogenetic work on the genus *Xenopus*, Janina Tymowska and co-workers (for references, see table 1) did not arrange the ordinarily stained chromosomes according to decreasing lengths, but established sophisticated standard karyotypes which were based on the relative sizes of short and long chromosome arms. Subsequently, these unconventional chromosome groupings were adopted by others in studies on chromosome banding [Sekiya and Nakagawa, 1983; Schmid et al., 1987; Schmid and Steinlein, 1991], chromosome painting [Krylov et al., 2010] and mapping of genes and repetitive DNA sequences [Courtet et al., 2001; Krylov et al., 2003, 2007; Tlapakova et al., 2005; Nanda et al., 2008; Uno et al., 2008, 2013]. In the present study, this system is simplified and the chromosomes are arranged according to the new nomenclature of *Xenopus* chromosomes established by the *Xenopus* Gene Nomenclature Committee [Matsuda et al., this issue].

General Characteristics of BrdU/dT Replication Banding Patterns in Xenopus Chromosomes

BrdU substitution during the first half of the S-phase, followed by dT incorporation during the remainder of the S-phase, produces chromosomal replication banding patterns which resemble G-bands (figs. 1–5). The BrdU-containing (early-replicating) chromosome regions are stained pale blue, whereas the dT-containing (late-replicating) regions are stained dark red by the Giemsa staining solution (as observed in bright-field microscopy without color filters). The contrast between early- and late-replicating bands achieved by Giemsa staining is by far higher than that observed after staining with fluorochromes like DAPI (fig. 6). The late-replicating bands in *Xenopus* chromosomes are classified as G-bands in analogy to the BrdU/dT labeling studies performed on chromosomes of mammals and birds [for review, see Sumner, 1990].

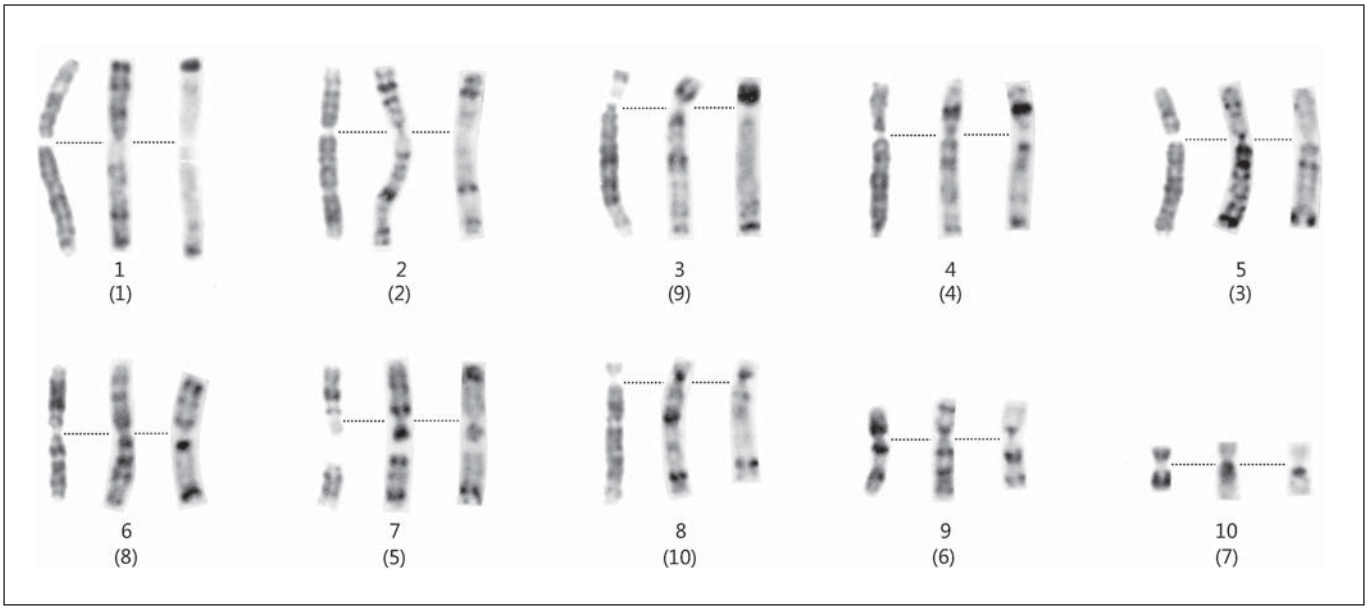


Fig. 1. Chronology of BrdU/dT replication banding patterns in the chromosomes of *X. tropicalis*. The arrangement of the chromosomes from left to right corresponds to the time sequence of replication. Left: early-replicating R-bands. Middle: G-bands which replicate in the second half of the S-phase. Right: very late-replicating G-bands and C-bands which replicate at the end of the S-phase. Chromosomes are arranged according to the new nomenclature of *Xenopus* chromosomes [Matsuda et al., this issue]. Numbers in parentheses correspond to the old chromosome numbering [Tymowska, 1991]. Centromeric regions are connected by dotted lines.

Time Sequence of BrdU/dT Replication Banding Patterns in Xenopus Chromosomes

Due to the asynchronous DNA replication, early- and late-replicating regions alternate along *Xenopus* chromosomes. Such replication patterns exist in the chromosomes of all vertebrates [e.g. Dutrillaux, 1975; Almeida Toledo et al., 1988; Yonenaga-Yassuda et al., 1988; Schmid et al., 1989]. Figure 1 illustrates the chronological replication patterns in the 10 chromosomes of *X. tropicalis* from the midstage to near the end of S-phase. The chromosomes are arranged from left to right according to increasing BrdU substitution (pale regions) and decreasing dT incorporation (dark bands). Each chromosome displays a particular chronological replication pattern. In the triplets shown in figure 1, the chromosomes on the left show the R-bands which replicate in the first half of the S-phase, the chromosomes in the middle exhibit the G-bands which replicate in the second half of the S-phase, and in the chromosomes on the right, the very late-replicating G-bands as well as the C-bands which replicate at the end of the S-phase can be recognized. The same chronology of replication was determined in the chromosomes of *X. laevis* [Schmid and Steinlein, 1991].

BrdU/dT Replication Bands in Xenopus Species with X. tropicalis-Type Karyotypes

The BrdU replication banding patterns in the metaphase chromosomes of *X. tropicalis* (2n = 20 and 3n = 30), *X. epitropicalis* (4n = 40) and *X.* new tetraploid 1 (4n = 40) are shown in figure 2. The triploid *X. tropicalis* was an occasional female triploid individual collected in Adiopodoume, Ivory Coast (table 2). Comparisons of the karyotypes from these 3 species of *Xenopus* show the existence of perfect homoeologies. Minor differences of the replication banding patterns between the karyotypes can be traced back to differing BrdU/dT treatment times and/or to differences in the sizes and locations of late-replicating heterochromatic chromosome regions. This, of course, does not exclude the possibility that very small chromosome rearrangements, like terminal reciprocal translocations, insertions and inversions, exist. Minor replication asynchronies found between chromosomes of individual triplets or quartets most probably involve heterochromatic regions. Similar asynchronous replication of homologous heterochromatic regions, also described as 'kinetic polymorphisms', has also been observed in hemiphractid frogs [Schmid et al., 2012], birds [Schmid et al.,

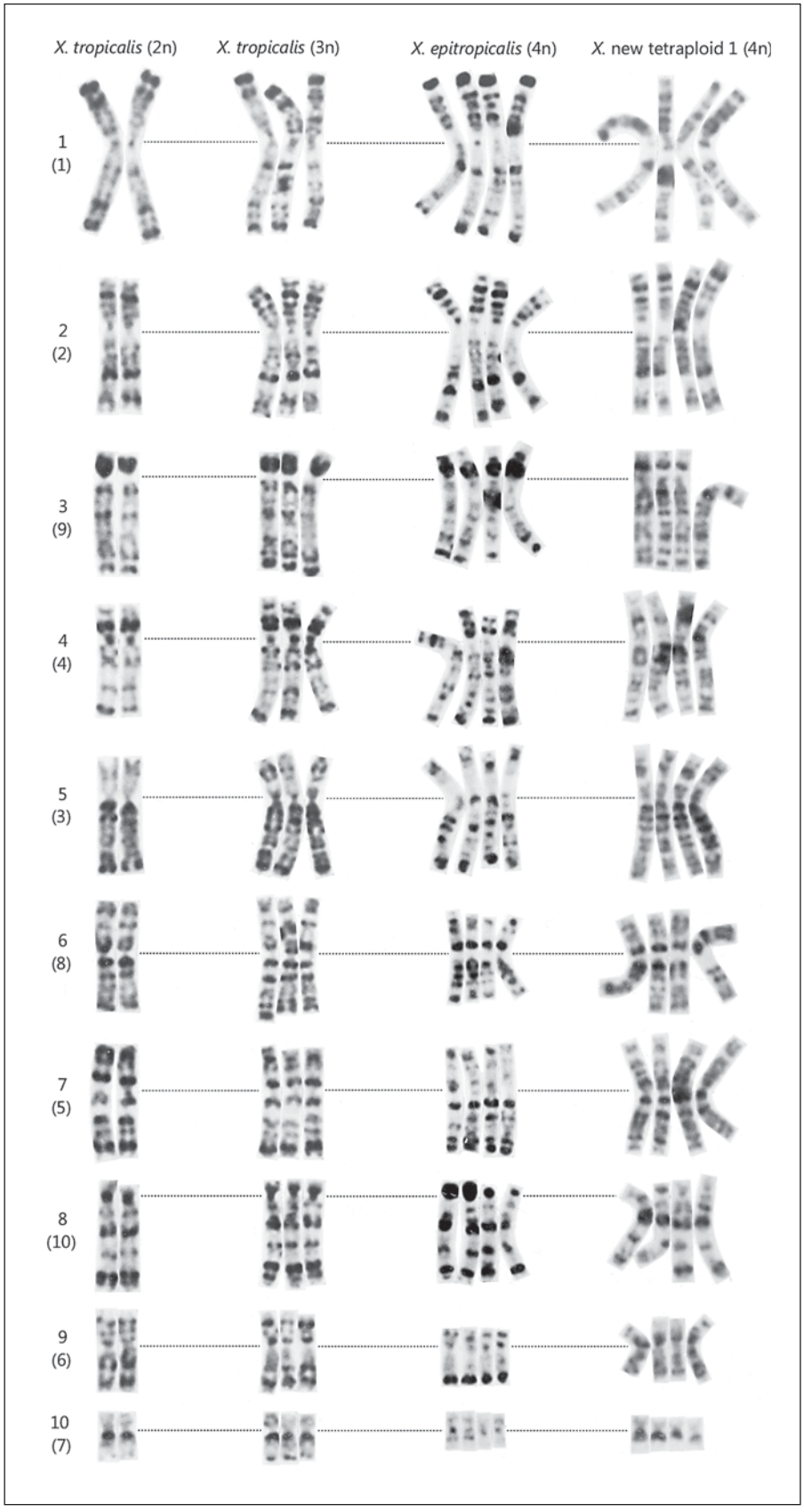


Fig. 2. BrdU/dT replication banding patterns in karyotypes of diploid and triploid *X. tropicalis*, tetraploid *X. epitropicalis* and tetraploid *X.* new tetraploid 1. BrdU was incorporated during the first half of the S-phase, followed by dT treatment during the remainder of the cell cycle. The pale-stained (BrdU-substituted) regions correspond to early-replicating R-bands, the dark-stained (dT-containing) regions to G-bands. Chromosomes are arranged according to the new nomenclature of *Xenopus* chromosomes [Matsuda et al., this issue]. Numbers in parentheses correspond to the old chromosome numbering system [Tymowska, 1991]. Centromeric regions are connected by dotted lines.

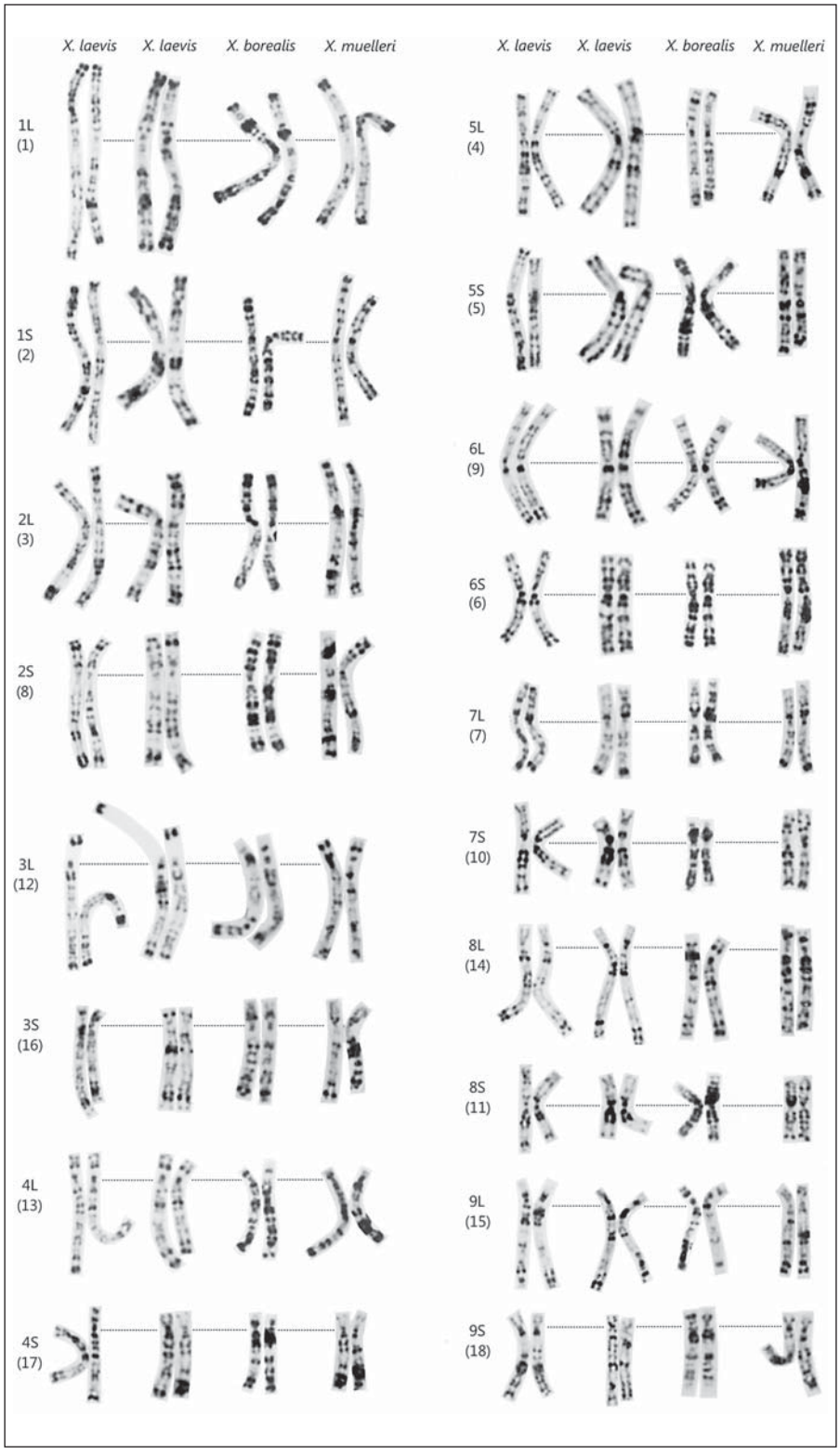


Fig. 3. BrdU/dT replication banding patterns in karyotypes of tetraploid *X. laevis*, *X. borealis* and *X. muelleri*. BrdU was incorporated during the first half of the S-phase, followed by dT treatment during the remainder of the cell cycle. The pale-stained (BrdU-substituted) regions correspond to early-replicating R-bands, the dark-stained (dT-containing) regions to G-bands. Chromosomes are arranged according to the new nomenclature of *Xenopus* chromosomes [Matsuda et al., this issue]. Numbers in parentheses correspond to the old chromosome numbering system [Tymowska, 1991]. Centromeric regions are connected by dotted lines.

Fig. 4. Metaphase chromosomes of the dodecaploid *X. ruwenzoriensis* (12n = 108) showing BrdU/dT replication banding patterns. BrdU was incorporated during the first half of the S-phase, followed by dT treatment during the remainder of the cell cycle.

1989], chimpanzees [Seuánez, 1979], and humans [Grzeschik et al., 1975].

Neither heteromorphic sex chromosomes nor sex chromosome-specific replication bands were detected in *X. tropicalis, X. epitropicalis* and *X.* new tetraploid 1 in the present study. Differences in number, size and staining intensity detected between replication bands in homologous chromosomes were inconsistent and not restricted to female or male specimens. The BrdU/dT replication bands give evidence that the sex chromosomes of these 3 *Xenopus* species are still in an undifferentiated, homomorphic stage.

The nucleolar constrictions of the 3 species are located in the long-arm paracentromeric region of chromosomes 7. These appear as non-stained gaps regardless when BrdU and dT are incorporated during the cell cycle (figs. 1, 2).

All cytogenetic data available, i.e. chromosome lengths, centromere positions, location of nucleolus organizer regions (NORs), and replication bands, indicate that, with the exception of the ploidy level (2n = 20 and 4n = 40), the karyotypes of this *Xenopus* clade were highly conserved during evolution.

BrdU/dT Replication Bands in Xenopus Species with X. laevis-Type Karyotypes

The BrdU/dT replication banding patterns in the metaphase chromosomes of the tetraploids (4n = 36) *X. laevis*, *X. borealis* and *X. muelleri* are depicted in figure 3. The banding patterns reveal a clear homoeology of all 18 chromosome pairs in the 3 species. For some chromosome pairs, however, the banding pattern seems not to be identical. This is probably due to the fact that the chromosomes compared with each other are from cells that were obtained from non-synchronized cell cultures. Small time differences in the incorporation of BrdU and/or dT in different cells can lead to visible displacements or disappearances of certain bands and thus fake structural non-homoeologies. Such minor differences can even be seen between the 2 *X. laevis* karyotypes shown in figure 3. Furthermore, some of the differences between the replication bands in the karyotypes of the 3 species could be caused by differing amounts and locations of constitutive heterochromatin. No sex-specific replication bands were observed in the BrdU/dT-banded karyotypes of the 3 species analyzed.

Similar to the *Xenopus* species with an *X. tropicalis*-type karyotype (see above), the 3 species with an *X. laevis*-type karyotype analyzed in the present study show evolutionarily conserved chromosome complements. Furthermore, as already shown by classical cytogenetic investigations [reviewed by Tymowska, 1991], this holds true for the other species of this clade with an increasing degree of ploidy (table 1). The NORs can be located in different positions of the karyotypes, but this is also the case in many other amphibian taxa with highly conserved karyotypes [reviewed by Schmid et al., 2010, 2012].

As demonstrated previously, BrdU/dT labeling induces reproducible and reliable replication bands along the metaphase chromosomes irrespective of the genome size of the species [Schmid et al., 2003]. Thus, BrdU replication banding is also easily induced in *Xenopus* species with high ploidy level and large genomes. Figure 4 shows a metaphase of the dodecaploid *X. ruwenzoriensis* (12n = 108) with distinct replication bands in all chromosomes. Unfortunately, in all available preparations, the high number of chromosome overlaps inhibited the construction of karyotypes showing the 12 homologs of all 9 chromosomes.

Reconstruction of the Tetraploid Karyotype of X. laevis

In the first study on BrdU/dT replication banding in *X. laevis*, Schmid and Steinlein [1991] made an attempt to arrange the 4n = 36 chromosomes in a tetraploid karyo-

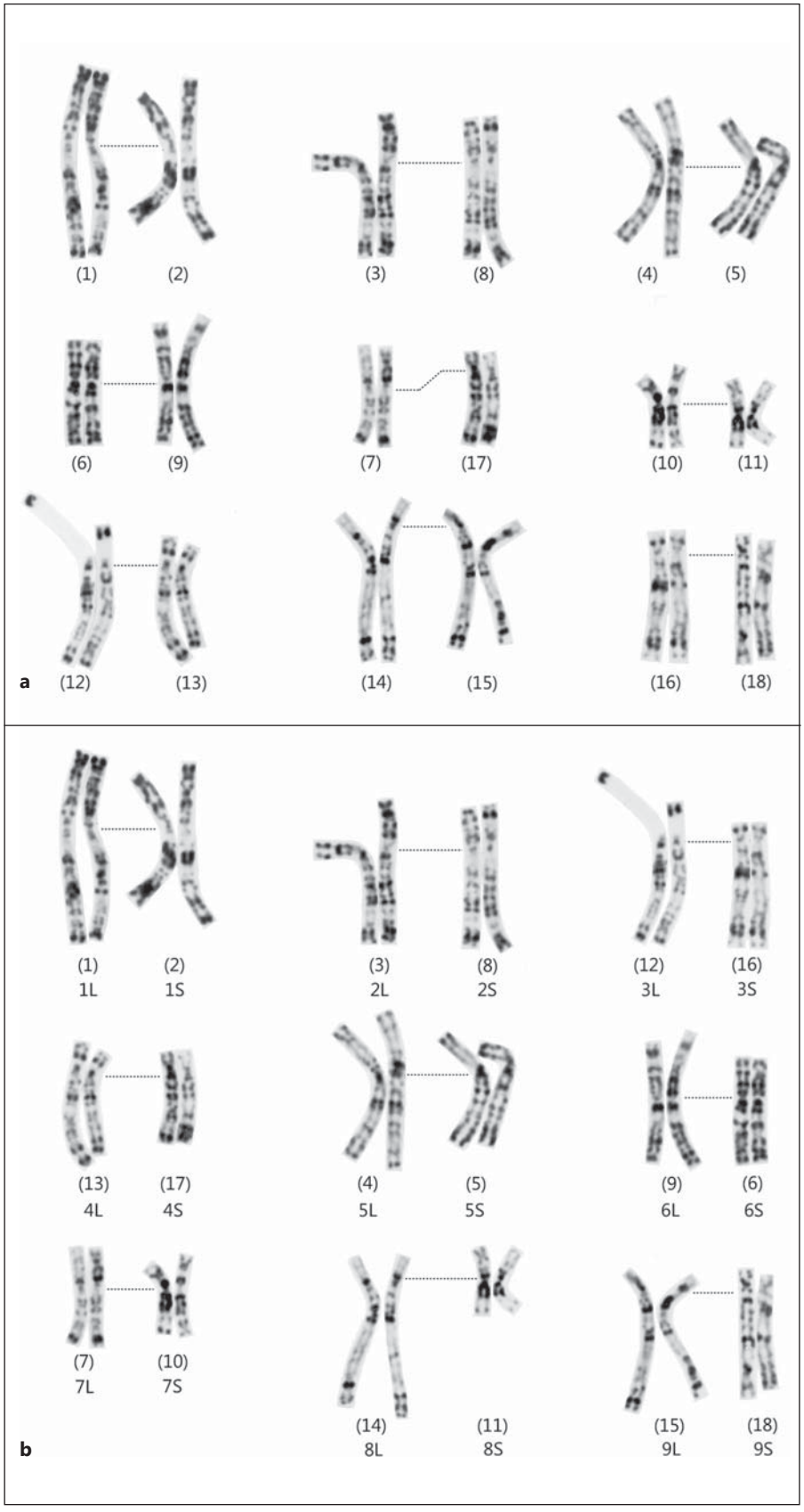


Fig. 5. BrdU/dT-banded chromosomes of *X. laevis* showing replication banding patterns at the mid S-phase. Chromosomes are arranged in quartets to reconstruct the most probable ancestral tetraploid karyotype. Centromeric regions are connected by dotted lines. Numbers in parentheses correspond to the old chromosome numbering [Tymowska, 1991], the other numbers to the new nomenclature of *Xenopus* chromosomes [Matsuda et al., this issue]. **a** Quartets formed after Schmid and Steinlein [1991]. **b** Quartets formed after Uno et al. [2013].

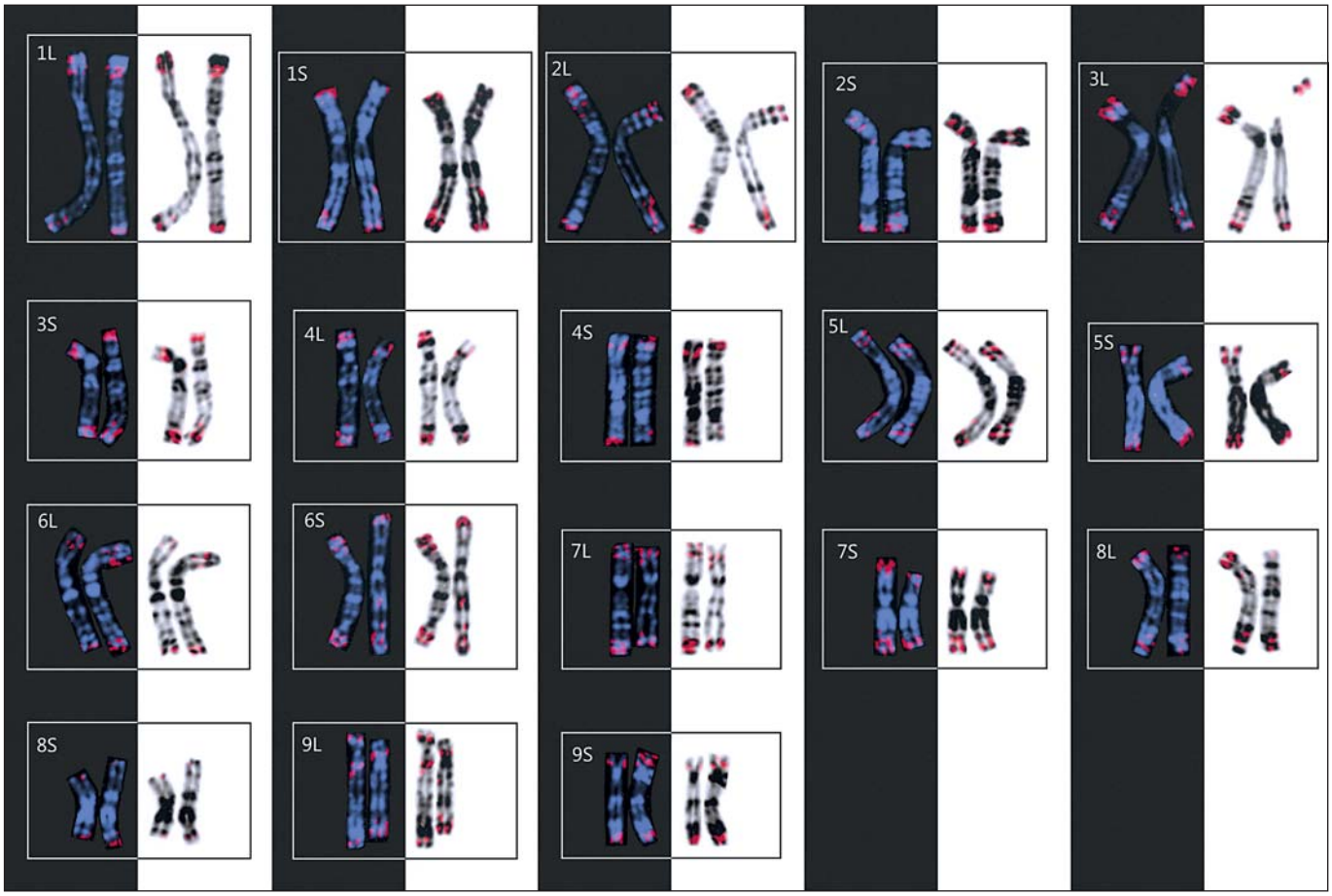


Fig. 6. Left columns: karyotype of *X. laevis* showing simultaneous high-resolution BrdU/dT replication banding patterns and FISH mapping of the cloned 79-bp-long repetitive probe. BrdU was incorporated for the first half of the S-phase, followed by dT treatment during the remainder of the cell cycle. Chromosomes are stained with DAPI. Right columns: the same karyotype with replication banding patterns electronically converted to black and white bands. Chromosomes are arranged according to the new nomenclature of *Xenopus* chromosomes [Matsuda et al., this issue].

type showing groups of 4 chromosomes (quartets). In doing so, the length of the chromosomes, the position of the centromeres and the size and relative location of high-resolution replication bands were considered. The result of that analysis is presented in figure 5a.

Following this very first trial, studies using Zoo-FISH with 8 probes obtained from *X. tropicalis* [Krylov et al., 2010], as well as FISH with 60 cDNA clones of *X. laevis* [Uno et al., 2013] were used to identify the exact tetraploid *X. laevis* karyotype. The 9 quartets identified by Uno et al. [2013] are shown in figure 5b. Not all of these are consistent with those identified in the studies of Schmid and Steinlein [1991] and Krylov et al. [2010]. The 4 quartets recognized as homoeologous by BrdU/dT replication banding and FISH with cDNA probes are 1 + 2, 3 + 8, 4 + 5, and 6 + 9. The remaining 5 quartets are formed in a different way in both studies (fig. 5). Uno et al. [2013] stated that this discrepancy may be explained by a difference in the *X. laevis* chromosome numbering system used in these studies. While this argument may apply to 2 of these 5 quartets, it can be called into question for quartet 11 + 14 and, to a lesser extent, for the quartets 13 + 17 and 15 + 18 established by Uno et al. [2013] (fig. 5b). It seems difficult to explain the large size differences between chromosome pairs 11 and 14. Since *Xenopus* chromosomes are evolutionarily largely conserved (see above), it must be assumed that in the ancestral hybrid allopolyploid genome all homoeologous chromosomes had very similar morphologies. Therefore, the large size difference between chromosomes 11 and 14 is

enigmatic, as it is not caused by significantly differing amounts of constitutive heterochromatin [Schmid and Steinlein, 1991]. Thus, if the results of Uno et al. [2013] are not substantially confirmed by further studies, the possibility cannot be excluded that in that study the identification of some chromosomes is inconsistent and resulted from the replication banding technique employed by these authors. It should be noted that Uno et al. [2013] did not apply BrdU in combination with dT, did not incubate the chromosome preparations in a buffered eosin Y solution, and stained the chromosomes with the fluorochrome Hoechst 33258 instead of Giemsa solution. These are all factors that reduce the quality of the replication bands and, in turn, the correct identification of chromosomes. Furthermore, most of the BrdU-banded metaphases with hybridized cDNA probes depicted in the publication of Uno et al. [2013] show over-condensed chromosomes in mid-metaphase. Since the submetacentric chromosomes 12–18 of *X. laevis* (Tymowska nomenclature) are quite similar, their correct identification is difficult in such preparations.

Polyploidy in Xenopus and Origin of the Two Chromosome Lineages

The genus *Xenopus* is composed of 2 different lineages, one consisting of species with *X. tropicalis*-type karyotypes (2n = 20 and 4n = 40), and the second of species with *X. laevis*-type karyotypes (4n = 36, 8n = 72 and 12n = 108) (table 1). The only extant *Xenopus* species still possessing the original diploid karyotype is *X. tropicalis* (2n = 20). The 3 tetraploid species with 4n = 40 chromosomes most likely are of allotetraploid origin [Tymowska and Fischberg, 1982; Tymowska, 1991]. Their karyotypes derived from 2 extinct, closely related and diploid (2n = 20) species.

Concerning the origin of the species with *X. laevis*-type karyotypes, the following explanation was advanced by Schmid and Steinlein [1991]. The karyotypes of all species with *X. tropicalis*-type karyotypes contain a typical chromosome which is absent in the species with *X. laevis*-type karyotypes. This submetacentric chromosome is the smallest in the karyotypes and was numbered as No. 7 (figs. 1, 2) by Tymowska and co-workers (table 1). Paleontological data indicate that the *X. tropicalis* clade is very ancient [Estes, 1975]. It is conceivable that several diploid species with 2n = 20 chromosomes coexisted in the African Miocene. In the ancestor of the lineage with *X. laevis*-type karyotypes, the chromosome No. 7 was translocated by a non-reciprocal, tandem-like rearrangement onto another chromosome. This reduced the diploid chromosome number to 2n = 18. Subsequently, 2 different species derived from this ancestor with 2n = 18 hybridized and created the first allotetraploid (2n = 36) individuals. This occurred either by direct fusion of 2 diploid gametes to give an allotetraploid zygote or, alternatively, by fusion of 2 haploid gametes to form a diploid zygote followed by autopolyploidization resulting in a tetraploid zygote. The assumption that the parental karyotypes were sufficiently different plausibly explains why the BrdU/dT replication bands are not the same within the quartets of *X. laevis*. A clear support for the allopolyploid origin of the various *Xenopus* species is that analysis of meiosis almost exclusively demonstrates bivalents [Tymowska, 1991].

The non-reciprocal translocation involving the chromosome No. 7 present in the *X. tropicalis*-type karyotypes (2n = 20 and 4n = 40) explains why this chromosome is missing in all other species of *Xenopus*. This translocation has been confirmed by studies using comparative gene mapping [Uno et al., 2013] and Zoo-FISH [Krylov et al., 2010].

Localization of a 79-bp-Long Satellite DNA to BrdU/dT-Banded Chromosomes

BrdU replication banding of *Xenopus* chromosomes provides the landmarks (bands) necessary for the exact physical mapping of genes. This has been demonstrated by the analyses of Uno et al. [2008, 2013] who located many cDNA clones to individual replication bands in *X. laevis* and *X. tropicalis* chromosomes. In our experience, however, a considerably higher contrast and resolution of the bands is obtained when BrdU substitution is followed by dT incorporation, and when the chromosome preparations are treated with a buffered eosin Y solution prior to staining. This procedure was employed in the present study (see Materials and Methods), and the result is exemplified in figure 6. It shows the signals of a 79-bp-long satellite DNA [Spohr et al., 1981] obtained by FISH to the BrdU/dT-banded chromosomes of *X. laevis*. Although in the chromosomes this repetitive DNA is organized as interspersed short repeats flanked by unique sequences, the bands to which the probe hybridizes are well distinguishable.

Localization of 5S rDNA Sequences by FISH

As shown previously by radioactive in situ hybridization on mitotic and lampbrush chromosomes of *X. laevis* [Pardue et al., 1973; Pardue, 1974; Callan et al., 1987, 1988], FISH demonstrates 5S rDNA sequences at or near the telomeric regions of the long arms of most chromosomes (fig. 7a). No 5S rDNA hybridization signals were

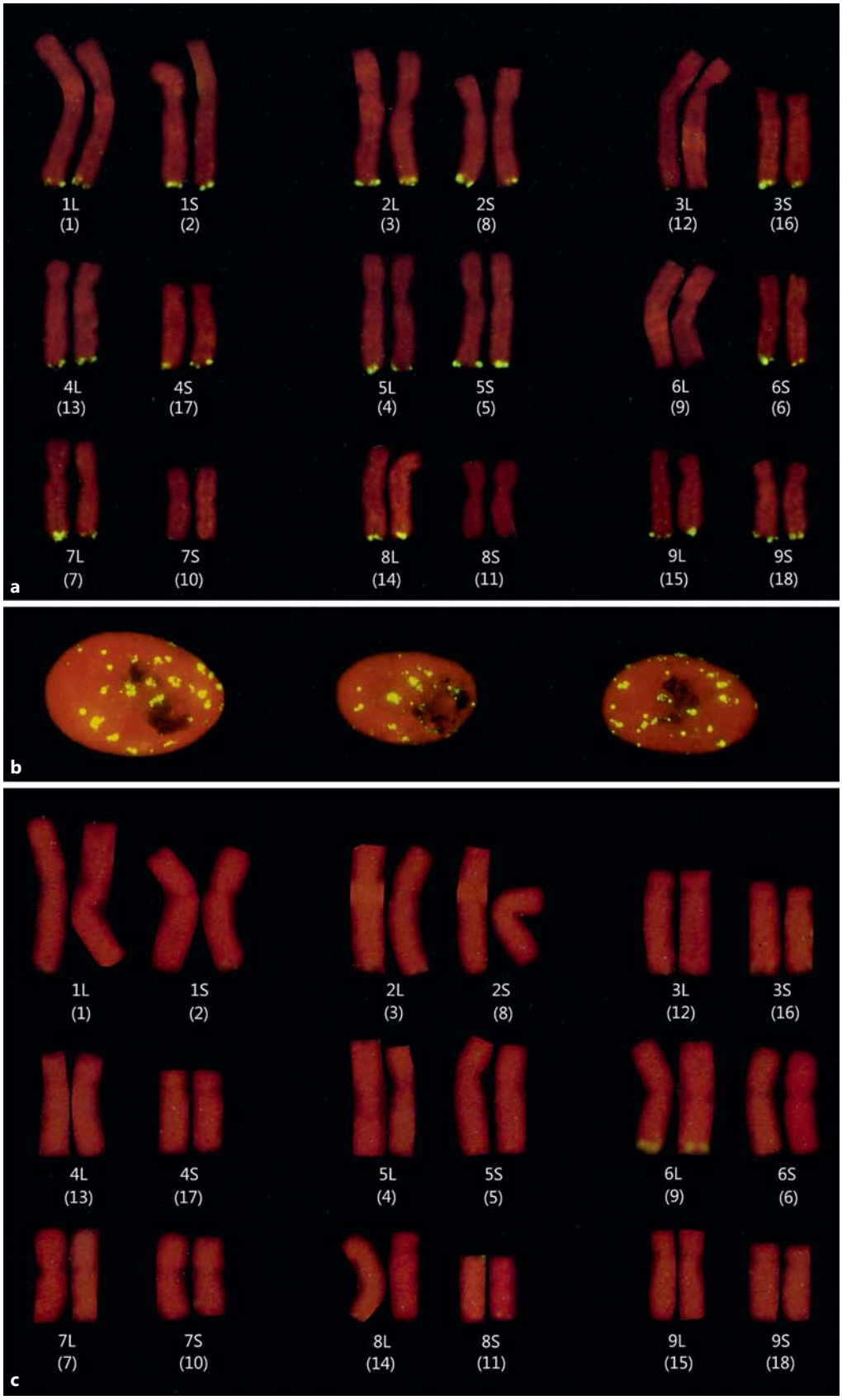


Fig. 7. FISH of the 5S rDNA probe to chromosomes (**a**) and fibroblast nuclei (**b**) of *X. laevis* and chromosomes of *X. muelleri* (**c**). Chromosomes are arranged according to the new nomenclature of *Xenopus* chromosomes [Matsuda et al., this issue]. Numbers in parentheses correspond to the old chromosome numbering [Tymowska, 1991]. In *X. laevis*, 5S rDNA clusters are located in the long-arm telomeric regions of almost all chromosome pairs, whereas in *X. muelleri* these are confined to the long-arm telomeric region of chromosomes 6L. Note multiple 5S rDNA hybridization signals in the fibroblast nuclei of *X. laevis*.

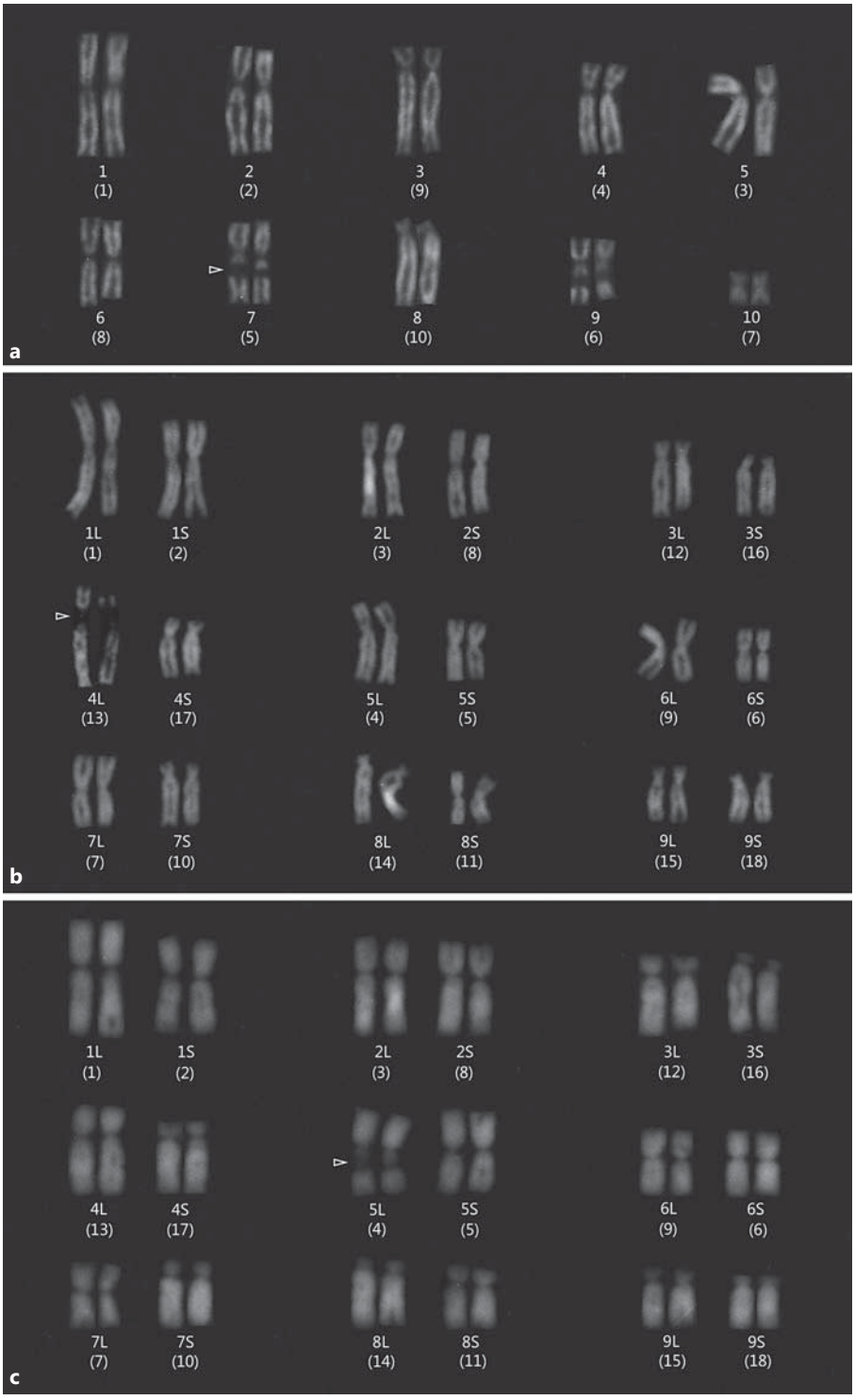
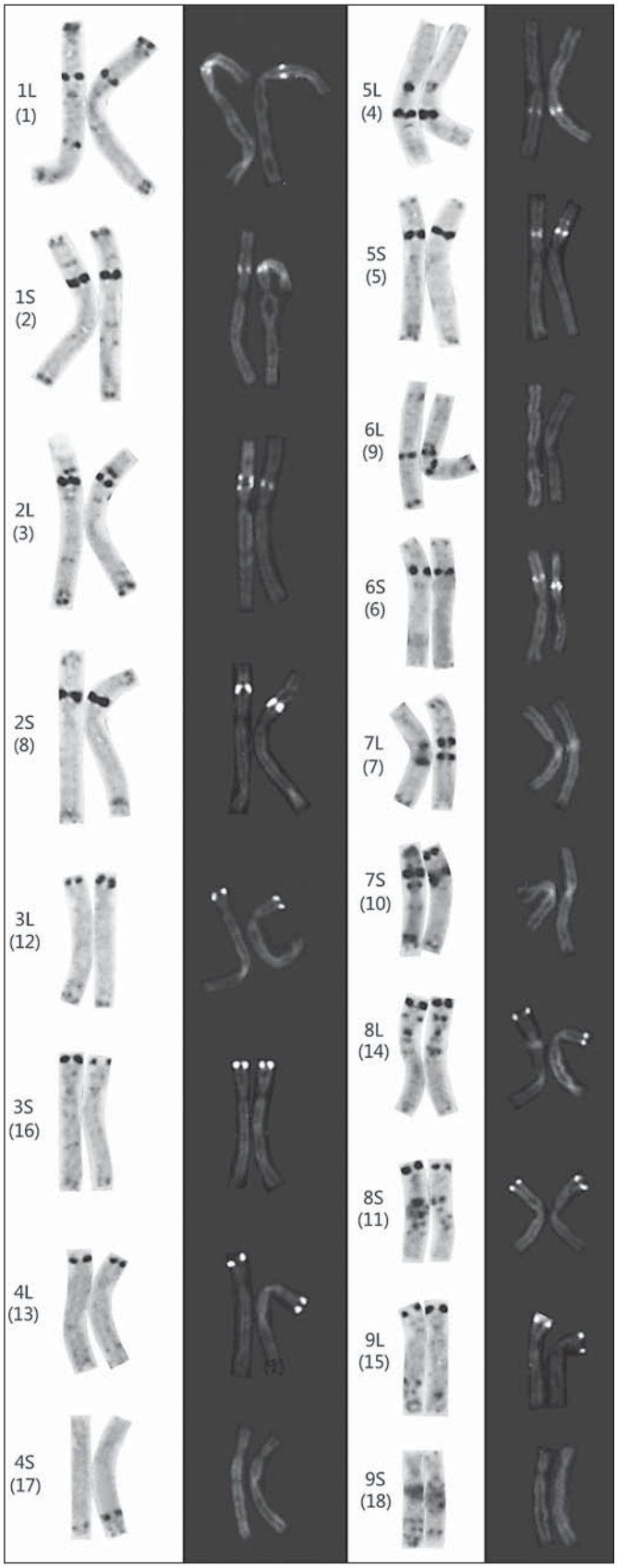


Fig. 8. Chromosomes of *X. tropicalis* (**a**), *X. clivii* (**b**) and *X. muelleri* (**c**) showing quinacrine mustard fluorescence. Arrowheads mark nucleolar constrictions. Chromosomes are arranged according to the new nomenclature of *Xenopus* chromosomes [Matsuda et al., this issue]. Numbers in parentheses correspond to the old chromosome numbering [Tymowska, 1991].

detected in the chromosomes 3L, 6L, 7S, and 8S. In fibroblast cells, the 5S rDNA shows a rather uniform distribution of small clusters over the nuclei (fig. 7b). The chromosomes of *X. borealis* exhibit a very similar or identical distribution of multiple 5S rDNA loci at the long-arm telomeres of many chromosomes (not shown). Such high numbers of 5S rDNA locations as found in *X. laevis* and *X. borealis* are an exception among the cytogenetically



studied amphibians and may be unique to the family Pipidae [for review, see Schmid et al., 2012].

In contrast to the *X. laevis* genome, the 5S rDNA sequences are located exclusively at the long-arm telomeres of the chromosomes 6L in *X. muelleri* (fig. 7c). This is in clear contradiction to the results obtained by Pardue [1974] who claimed that *X. muelleri* and *X. laevis* show the same multiple chromosomal distribution of 5S rRNA loci. As explained below, the specimen(s) used in that study were probably *X. borealis* and not *X. muelleri*.

Quinacrine Staining

As expected from the results obtained in the chromosomes of other anuran families [for reviews, see Schmid et al., 2010, 2012], staining with the fluorochrome quinacrine mustard shows that the large euchromatic segments in the chromosomes of *X. tropicalis, X. clivii* and *X. muelleri* fluoresce with a uniform and moderate intensity (fig. 8). All chromosome regions fluorescing distinctly weaker than the euchromatin consist of constitutive heterochromatin or NORs (as confirmed by C-banding and Ag-staining). The same result was shown for *X. laevis* [Pardue, 1974; Schmid et al., 1987].

The quinacrine-stained karyotype of *X. borealis* is remarkable because of the very brightly fluorescing quinacrine-positive (Q^+) heterochromatic regions located in interstitial or telomeric positions of chromosomes 1L–4L, 5L and 5S, 6S, and 8L–9L (fig. 9, right columns). With the exception of chromosome 5L, all these Q^+ bands are located in the short arms. The comparison with BrdU/dT replication bands reveals that the Q^+ bands consist of late-replicating heterochromatin (fig. 9, left columns). It should be mentioned that in the study of Pardue [1974], a quinacrine-stained karyotype of *X. muelleri* is presented which shows the same Q^+ band patterns as the karyotype of the *X. borealis* in the present study. The 9 specimens examined here were collected in Ifakara, Tanzania (table 2) and identified by Charles H. Thiébaud (Station de Zoologie Expérimentale, University of Geneva, Switzerland), whereas there is no information on the origin and identification of the specimen(s) studied by Pardue

Fig. 9. Chromosomes of *X. borealis* showing very late BrdU/dT replication banding patterns (left columns) and quinacrine mustard staining (right columns). Note that many of the very late-replicating heterochromatic C-bands show extremely bright quinacrine fluorescence. Chromosomes are arranged according to the new nomenclature of *Xenopus* chromosomes [Matsuda et al., this issue]. Numbers in parentheses correspond to the old chromosome numbering [Tymowska, 1991].

[1974]. Moreover, Brown et al. [1977] provided evidence that *X. borealis* is easily misidentified as *X. muelleri*. Therefore, it seems likely that the specimen(s) used by Pardue [1974] were actually *X. borealis*.

Similar Q^+ heterochromatic bands as in *X. borealis* were detected in 11 chromosomes of *X. fraseri* [Schmid et al., 1987], although the correct identification of the 2 specimens in that study was again uncertain. Similar to *X. borealis*, most of the Q^+ bands in *X. fraseri* are located in interstitial and terminal sites of the chromosomes' short arms.

Like in all other anuran species [for review, see Schmid et al., 2012], quinacrine mustard does not induce multiple Q-bands in the chromosomes of *Xenopus*. An explanation for the almost complete absence of multiple Q-, G- and R-band patterns in chromosomes of cold-blooded vertebrates is that their genomes are not strongly compartmentalized by DNA base composition and, therefore, do not contain the so-called isochores which correspond to DNA sequences in Q-, G- and R-bands of warm-blooded vertebrates [for reviews, see Bickmore and Craig, 1997 and Schmid et al., 2012]. It came as a surprise that treatment of *X. laevis* and *X. muelleri* chromosomes with trypsin or a trypsin-urea combination yielded G-band-like patterns [Stock and Mengden, 1975; Sekiya and Nakagawa, 1983; Stock, 1984; Uehara et al., 2002]. As outlined by Stock [1984], very slight changes in the fixation protocol of amphibian chromosomes can drastically influence a banding success. Different proteins associated along the chromosomes may vary between vertebrate groups, and this could account for different responses to fixation and banding treatments. An additional factor, which may affect a successful banding, is thought to be the source of the analyzed metaphases, and it appeared that metaphases obtained from tissue culture are more suitable for banding than metaphases obtained by direct bone marrow preparation [Stock, 1984]. In the present study, however, all chromosomes of *Xenopus* that were analyzed were obtained from tissue cultures and no multiple banding patterns could be induced with quinacrine mustard. Finally, most authors agree that none of the banding patterns that have been induced in the chromosomes of a few amphibians are of the same quality as those obtained under identical conditions in warm-blooded vertebrates [for review, see Schmid et al., 2012].

The comparison of the karyotypes of *X. clivii* and *X. muelleri* with those of the other *Xenopus* species with *X. laevis*-type karyotypes examined in the present study shows some distinct differences. The chromosomes 7S of *X. clivii* and *X. muelleri* and the chromosomes 8S of *X. muelleri* are not metacentric as in the other species, but have a submetacentric morphology (fig. 8b, c). This can be easily explained by the occurrence of pericentric inversions in the chromosomes 7 and 8 in the ancestors of *X. clivii* and *X. muelleri*.

It is mandatory that additional chromosome banding experiments on the other species of *Xenopus* (table 1) must be performed before any general conclusions can be drawn. Such studies should include BrdU/dT replication banding, direct fluorescence stainings with AT base pair-specific fluorochromes (quinacrine mustard, DAPI, Hoechst 33258) and GC base pair-specific fluorochromes (mithramycin, chromomycin A_3), fluorescence counterstainings (distamycin A/mithramycin, distamycin A/DAPI), and FISH using 5S and 18S + 28S rDNA and other repetitive DNA probes. These studies should also be extended to representative species in the other genera of the family Pipidae.

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