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CHROMOSOMA

Chromosome Banding in Amphibia

I. Constitutive Heterochromatin and Nucleolus Organizer Regions in *Bufo* and *Hyla*

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Abstract. The distribution and quantity of constitutive heterochromatin and of the nucleolus organizer regions (NORs) on the chromosomes of 22 species of bufonids and hylids (Amphibia, Anura) was investigated. Three different kinds of constitutive heterochromatin were found and the frequency of brightly fluorescing heterochromatic regions was remarkably high. On almost all chromosomes there is centric and telomeric heterochromatin. Quantitative estimates of heterochromatin demonstrate that large DNA differences among closely related species can not be attributed to differing quantities of constitutive heterochromatin. In all species investigated, only one homologous pair of NORs was found, which lies preferentially in the proximal and interstitial segments of the long chromosome arms. The NORs are always associated with constitutive heterochromatin on both sides. The size variability between homologous NORs is very high. In the euchromatic regions of the metaphase chromosomes, neither Q- nor G-bands can be demonstrated; this can be attributed to an extremely strong contraction of the anuran chromosomes. On the basis of these results various mechanism of the chromosomal evolution in Anura are discussed.

Introduction

Although banding techniques have become an essential part of cytogenetic analysis, little has been reported in the literature concerning banded chromosomes in the anuran amphibians. Until now, the majority of the results have been obtained by comparative chromosome investigations in the Anura with conventionally stained chromosomes. Isolated instances of banding analysis can be found for *Leptodactylus ocellatus* (Bianchi et al., 1973), *Odontophrynus americanus* and *O. cultripes* (Garcia Ruiz and Beçak, 1976), and *Xenopus* (Matsui, 1974; Stock and Mengden, 1975). Meticulous comparative cytogenetic observations have been made on conventionally stained chromosomes between individual anuran families (Morescalchi, 1971; 1973) and, in particular, among many

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species of the genus *Bufo* (Bogart, 1972) and *Xenopus* (Tymowska, 1973, 1977; Tymowska and Kobel, 1972; Tymowska and Fischberg, 1973). In these reports, the criteria for comparison taken into consideration were primarily the number and morphology of the chromosomes (relative length, centromere index and arm ratio), and the number and position of secondary constrictions. The data obtained from these investigations gave the first indications of the processes that had taken place during the chromosomal evolution in the anuran amphibians. Furthermore, the degree of relatedness between individual species could be determined. In order to obtain a more detailed knowledge of the characteristics of anuran chromosomes, the use of differential staining techniques is obligatory. These methods allow the demonstration and analysis of multiple bands and can provide valuable indications of the molecular structure of the chromosomes (e.g. various DNA-fractions, repetitive sequences).

Biochemical investigations of the genomes of the Anura have been performed in great number and with numerous techniques. There have been abundant quantitative analyses of the DNA-content in many anuran species (Sexsmith, 1968; Goin et al., 1968; Beçak et al., 1970; Olmo, 1973) as well as different kinetic studies on DNA reassociation (Straus, 1971; Davidson et al., 1972, 1973; Baldari and Amaldi, 1976). Beyond these, the genes for nucleolar ribosomal (5S, 18S and 28S) RNA have been localized on the chromosomes of *Xenopus laevis* by in situ hybridization studies (Pardue and Birnstiel, 1973; Pardue et al., 1973) and quantitatively characterized with DNA-RNA filter hybridization (e.g. Brown and Weber, 1968; Brown et al., 1971; Birnstiel et al., 1972) Complementary cytogenetic studies using the differential banding methods are, therefore, especially necessary in the anuran groups in which results have accumulated and questions have arisen from the biochemical investigations.

The work presented here is a primary report about the results of the differential banding techniques applied to the chromosomes of 70 amphibian species. It was attempted to determine whether constitutive heterochromatin is always present in the genomes of the Anura, in which chromosomal regions it is accumulated, what portion it represents of the total genome, and into how many different categories it can be divided using the banding techniques. Furthermore, an investigation was made to determine to what extent the very similar karyotypes of closely related anuran species can be differentiated from one another by Q-banding, C-banding and AgAS-staining. For this purpose, several species were chosen from the families Bufonidae (toads) and Hylidae (tree-frogs), which belong to the most highly developed anuran groups (Procoela).

The Bufonidae and Hylidae occur in the largest variety and are among the most wide-spread families of the Anura. With the exception of Australia (for the *Bufonidae*) and Africa (for the Hylidae) they are cosmopolitan. Knowledge about the evolution of these Anura, especially of the genus *Bufo*, has been furthered by a large number of different investigations. In this way, aside from the purely karyological work (Bogart, 1972; Morescalchi, 1973), an overwhelming amount of genetic-compatibility data has been collected: intra- and intercontinental hybridizations between species (Blair, 1959, 1963, 1972); biochemical investigations of the DNA-content (Ullerich, 1966, 1967; Olmo, 1973), studies on hemoglobins and transferrins (Guttman, 1967), and about the secretions of the parotoid-glands (Low, 1967; anatomical and physiological studies of the skeleton (Martin, 1972), type of testes (Blair, 1972), and vocal mechanisms (Martin, 1972). All these results indicate that the genus *Bufo* arose in the South American zone from a primitive ancestor of

the family Leptodactylidae having 22 chromosomes. Through the central-american corridor, the ancestral *Bufo* species reached the southern part of North America and experienced explosive speciation in the northern continent during the pliocene and pleistocene ages. In at least 3 different migrations through western North America and over the land-bridge in the Bering strait during the tertiary period, the toads reached Europe and Asia. Further movements brought the toads to North Africa where they attained new speciations radiating forth from various geographic centres. The Hylidae reached their greatest differentiation in the neotropics. Although their evolution from the family Leptodactylidae is considered definite, the possibility of a polyphyletic origin from various stems of the Leptodactylidae can not be excluded with certainty (Griffiths, 1963).

Material and Methods

Animals. 22 species of the families Bufonidae and Hylidae were available for this investigation (Table 1). The 47 animals were obtained from specialized animal dealers and were kept in aquaterraria at 18–20° C. The Bufonids were fed once a week with minced liver; the Hylids were fed several times a week with flies.

Tissues Studied. All animals received an intraperitoneal injection of 0.3% colchicine solution (Merck) 14–16 h before they were sacrificed with diethyl-ether; the amount of injected solution ranged from 0.25–1.0 ml, depending on the size of the animals. The chromosomes were prepared both from the bone marrow and testes (direct methods), and from the blood (PHA-stimulated cultures). As to the method of chromosome preparation, this will be covered in somewhat more detail in the following sections, since what has been described to date in the literature has not always proven to be reliable.

Bone Marrow. Femur, tibia and humerus were freed from the musculature with a scalpel, and the cartilageneous epiphyses cut off. The bone marrow was flushed out into a centrifuge tube with 8–10 ml of hypotonic KCl solution (0.075 M) using a fine hypodermic needle. In the bone cavities of many species, fat deposits have accumulated; these clumps must be removed after the flushing-out procedure from the uppermost layers of the hypotonic solution. The bone marrow was then vigorously resuspended with a Pasteur pipette, and afterwards left in the hypotonic KCl-solution at room temperature for 1 h.

Testes. These were cut as finely as possible in a Petri dish and suspended vigorously with a Pasteur pipette in 8–10 ml hypotonic KCl-solution. The hypotonic treatment lasted for 45 min at room temperature.

Blood Cultures. During ether narcosis, the animal was cut open ventrally under sterile conditions and the pericardium removed. From the ventricle of the beating heart, 0.1–0.5 ml of blood was removed using a heparinized syringe. In order to obtain the maximum volume of blood, the needle must be inserted exactly at the point of the ventricle. The blood was cultured in 4 ml of amphibian medium (Wolf and Quimby, 1964; Gibco), 0.1 ml phytohemagglutinin M (Difco), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Incubation was for 72 h at 26° C; 2–4 h before termination of the cultures, 0.15 ml vinblastine sulfate (1 μg/ml) was added. Then, the cell suspension was centrifuged and, after resuspension in 8 ml of 0.046 M KCl solution, the cells were incubated for 15 min at 26° C.

Fixation and Preparation of Slides. After hypotonic treatment, the cell suspension was centrifuged at 1000 rpm and fixed with 8 ml of an ice-cold acetic acid – methanol (1:3) solution. The first ml of fixative was added drop-wise under constant shaking. The fixed material was left overnight at 4° C, then washed with freshly prepared fixative; bone marrow and testes were washed once, blood cultures, twice. Finally the cells were resuspended in 1–2 ml fixative, and 3–6 drops of this suspension dropped on slides previously cleaned with concentrated chromic-sulfuric acid and rinsed well with distilled water. The slides were either allowed to dry at room temperature (for later C-banding) or briefly passed 3 times over a Bunsen burner (for orcein staining, Q-banding

Table 1. Summary of material studied and methods used

Species	2n	Origin[a]	Animals		Number of metaphases analysed[b]			
			♂	♀	O	Q	C	AgAS
Bufonidae								
Bufo bufo	22	Eur.	1	1	30	16; 4	46; 6	5
B. calamita	22	Eur.	2	2	35	16; 5	56; 7	5
B. parvus	22	Eur.	2	0	29	11; 8	22; 5	9
B. viridis	22	Eur.	1	1	115	12; 5	40; 7	5
B. americanus	22	N. Am.	0	2	27	10; 9	28; 8	9
B. boreas	22	N. Am.	0	1	17	5; 4	32; 4	6
B. compactilis	22	N. Am.	1	0	15	5; 5	12; 3	5
B. fowleri	22	N. Am.	1	1	29	11; 9	60; 9	7
B. punctatus	22	N. Am.	0	1	25	7; 6	10; 2	5
B. terrestris	22	N. Am.	1	1	61	37; 14	41; 8	12
B. valliceps	22	N. Am.	1	2	114	47; 9	67; 8	8
B. arenarum	22	S. Am.	2	1	46	39; 16	–	3
B. marinus	22	S. Am.	1	0	31	29; 10	–	4
B. garmani	20	Afr.	1	1	132	15; 10	77; 9	7
B. mauritanicus	22	Afr.	1	1	172	59; 12	28; 4	11
B. poweri	20	Afr.	4	0	79	45; 18	47; 10	4
B. poweri(?)	30	Afr.	0	1	20	10; 5	10; 5	4
B. regularis	20	Afr.	2	0	33	15; 8	25; 5	5
Pedostibes hosii	22	Bor.	0	1	81	8; 5	33; 4	5
Hylidae								
Hyla arborea	24	Eur.	1	2	20	12; 5	–	2
H. cinerea	24	N. Am.	2	1	34	19; 5	10; 3	2
H. septentrionalis	24	B., C.	1	1	45	12; 5	42; 4	2
Pseudacris ornata	24	N. Am.	0	1	57	16; 5	43; 4	5

[a] Eur. = Eurasia; N. Am. = North America; S. Am. = South America; Afr. = Africa; Bor. = Borneo; B., C. = Bahamas, Cuba

[b] O: Orcein stained metaphases. In these preparations, the chromosome number, position of secondary constrictions, association frequency of nucleolus organizer regions, and absolute lengths of chromosomes were investigated. The analysis was performed in a bright-field microscope, without photography. Q: Quinacrine mustard staining. C: C-bands. The first number indicates the mitoses photographed, the second indicates the karyotypes laid out. AgAS: Ammoniacal $AgNO_3$ staining. All metaphases were photographed and karyotyped

or ammoniacal $AgNO_3$ staining). The slides were treated by the various staining methods within 4 weeks.

Aceto-Orcein Staining. For the analysis of the chromosome number, as well as for the demonstration of secondary constrictions the slides were stained for 30 min in 3% orcein (in 50% acetic acid). Then, they were washed in absolute ethanol and permanently mounted with Eukitt.

C-Banding. The staining of constitutive heterochromatin was performed according to the method of Sumner (1972). The slides were incubated for 5 min at 30°C in a saturated $Ba(OH)_2$ solution. The staining was done either in 8% Giemsa (pH 6.8, 5 min) or in a solution of quinacrine mustard for observation under the fluorescence-microscope.

Fluorescence. The aqueous solution of the fluorochrome quinacrine mustard (Serva) was always freshly-prepared (0.05 mg/ml). The slides were stained either directly or after a pretreatment in

the saturated $Ba(OH)_2$-solution. The stained slides were washed in phosphate buffer (pH 5.5) and mounted in a saturated saccharose solution. For the microscopic analysis, a Zeiss fluorescence microscope was used, equipped with an HBO 50W/AC lamp, BG 12 exciting filter and a 53/44 barrier filter. Each metaphase, from which a karyogram was made, was photographed at 3 different exposure times; by this means, it is also possible to detect the very small, brightly fluorescing heterochromatic regions as well as the extremely weakly fluorescing satellites of many chromosomes (Overton et al., 1976).

Staining of the Nucleolus Organizer Regions (NORs). The quinacrine mustard-stained slides were thoroughly rinsed with distilled water and stained according to the method of Goodpasture and Bloom (1975). Those metaphases were photographed in which the specific silver staining at the nucleolus organizer regions of the chromosomes could be identified. The comparison between $AgNO_3$ and fluorescence staining for a given metaphase allows the exact localization of the nucleolus organizer regions on the chromosomes.

Analysis of the Banding Patterns. The slides were systematically searched for high-quality mitoses; metaphases were selected for photography with respect to number of chromosome-overlappings, clarity of bands and contraction of chromatids. From each of the 47 individuals studied, several karyotypes (Table 1) with different banding patterns (Q-, C- and $AgNO_3$-banding) were made. The chromosome pairs cut out from photographic prints were arranged, according to size, in parallel rows, so that the conformity of the banding pattern could be observed. In this way, the normal banding pattern of a species, as well as intra- and interindividual variations of specific bands, can be very easily analysed. In addition several chromosomal aberrations in the Anura could be identified with this banding analysis procedure (Schmid, in preparation).

Results

Euchromatin

In contrast to the situation in the other vertebrate classes, the staining with quinacrine did not reveal multiple banding patterns in any of the investigated anuran species. The euchromatic parts of the chromosomes all fluoresce with equal intensity. All of those chromosome regions which markedly fluoresce more strongly or weakly than the euchromatin, can be identified upon closer analysis as constitutive heterochromatin (Figs. 1–8). The demonstration of characteristic Q-bands in the euchromatin seems indeed to be dependent upon the state of contraction of the chromosomes. On the extremely long prophase chromosomes, the fluorochrome reveals throughout a great many single bands (Fig. 1 d); even on those prophase chromosomes stained conventionally with orcein, there exist density differences along the chromatids. The more the chromosomes are contracted, the more uniform is the fluorescence in the euchromatic regions. It also was impossible to induce G-bands in euchromatin by prolonged enzymatic treatment with trypsin (Seabright, 1971) and pronase (Dutrillaux, 1971).

Heterochromatin

The constitutive heterochromatic chromosomal regions were detected predominantly by three staining techniques: direct staining with quinacrine (Q-bands,

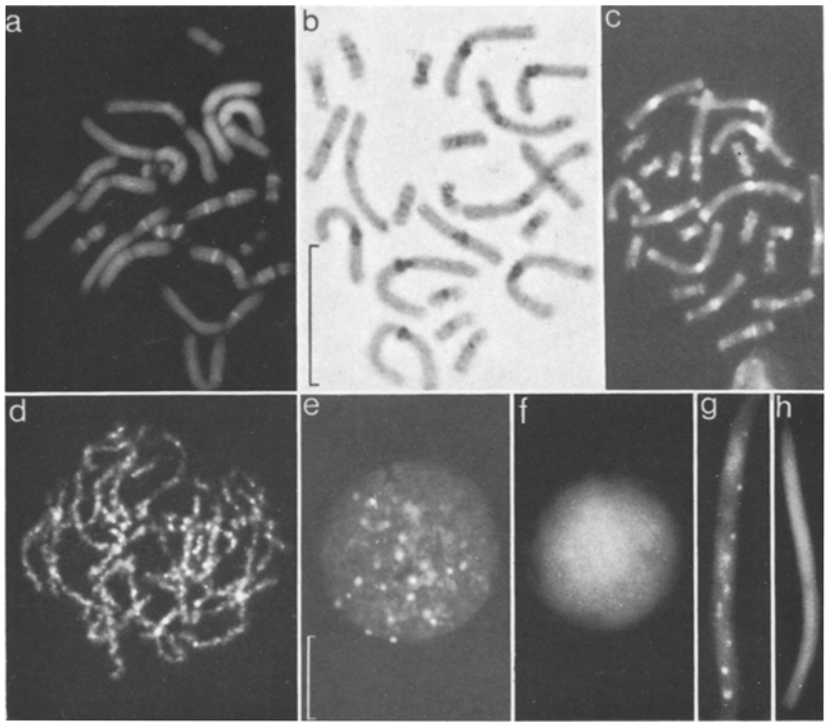

Fig. 1a–h[1]. Metaphases and interphase nuclei of male *Bufo mauritanicus* (**a–e, g**) and *Bufo arenarum,* demonstrating the staining techniques for constitutive heterochromatin. **a** Direct staining with quinacrine showing brightly fluorescing interstitial heterochromatin and weakly fluorescing centromeres and telomeres. **b** Giemsa stained C-bands. **c** Quinacrine stained C-bands showing centric, interstitial, and telomeric heterochromatin. **d** Prophase chromosomes of bone marrow; note brightly fluorescing heterochromatin and Q-bands. **e–h** Cell nuclei of bone marrow and sperm heads with and without strongly fluorescing heterochromatic blocks

Fig. 1a), Giemsa-staining after $Ba(OH)_2$ denaturation (C-bands, Fig. 1b), and staining with quinacrine after $Ba(OH)_2$ denaturation (fluorescing C-bands, Fig. 1c). The fluorescing C-bands, as opposed to the Giemsa-stained C-bands, have the advantage of a greater distinctness of the terminal and interstitial heterochromatin. The presence of a definite heterochromatic chromosome region was only considered certain when it was clearly visible in at least 10 of the analysed karyotypes (Table 1). In addition, the presence or absence of brightly fluorescing heterochromatic areas was sought in quinacrine stained somatic cell nuclei and mature spermatozoa (Fig. 1e–h). This turned out to be especially necessary when the heterochromatic regions were so small as to be overlooked on metaphase chromosomes (*Hyla cinerea, H. septentrionalis*). The number of chromocenters in the cell nucleus is usually less than the number of heterochromatic chromosomal regions, probably owing to fusion of several heterochromatic areas in the interphase nucleus which leads to the formation of larger chromocenters (Hsu et al., 1971; Schmid and Krone, 1976).

[1] Bars in Figures 1–8 represent 10 μm

In the karyotypes of 12 of the 18 bufonids investigated, brightly fluorescing heterochromatin was present (Figs. 2–10, Table 2). On the chromosomes of the genus *Bufo*, the brightly fluorescing regions lie predominantly on the short and long arms of the chromosomes. In *Pedostibes hosii* the centromere fluoresces on chromosome pairs 4 and 5. In the *Bufo* species of the North American continent, the especially large, brightly fluorescing heterochromatic areas lie almost without exception in the telomeric regions of chromosome 7 to 11. Of the 3 Euro-asiatic toad species investigated, only *Bufo viridis* possesses brightly fluorescing segments which are also confined to the small chromosomes. In the African toads, the narrow bands of strongly fluorescing heterochromatin are localised in the interstitial areas of the medium-sized and small chromosomes. Only chromosome pair 10 of *Bufo mauritanicus* possesses in addition a terminally-situated heterochromatic band which fluoresces strongly. In the African area, the toads differ from one another with respect to their chromosome number. While *B. mauritanicus* has, as is usual for bufonids, 22 chromosomes, the *B. regularis* group of species has 20 (Bogart, 1968, 1972). The single investigated female classified as *Bufo poweri* had 30 chromosomes in all the tissues studied (Fig. 7a). It is very likely that this represents a spontaneously occurring triploidy (see Discussion).

On the quinacrine-stained metaphase chromosomes of the hylids examined, no brightly fluorescing heterochromatin could be found (Figs. 8b–d, 10). In the diploid interphase nuclei of the American species *Hyla cinerea* and *H. septentrionalis*, however, there were always 4 to 6 very small regions which displayed an obviously stronger fluorescence than the rest of the chromatin. In addition, in the pachytene stage of meiosis and in mature spermatozoa of these 2 species usually 3 blocks of strongly fluorescing heterochromatin are observed. These findings warrant the assumption that there are 6 strongly fluorescing chromosomal regions in the diploid cells of *Hyla cinerea* and *H. septentrionalis*, which can not, however, be localised on the metaphase chromosomes because of their extremely small size.

The comparison between Q- and C-banding karyotypes (Figs. 2–8) shows that the brightly fluorescing heterochromatic chromosomal regions, with 3 exceptions, are all C-band-positive (Figs. 9, 10). The exceptions involve the interstitial bands on chromosome 7 of *Bufo terrestris* (on the long arm) and on chromosome 9 of *B. viridis* (on the short and long arms). However, since these cases involve relatively small bands (Figs. 2, 5), there remains the possibility that they can not be differentiated clearly enough by the C-banding technique.

In the karyotypes of all species investigated, there are many chromosomal regions which fluoresce more weakly than the euchromatin. Such areas lie preferentially at the centromeres and telomeres, but can also be found not infrequently in the interstitial region of the chromosome arms (Figs. 2–8). Apart from the strongly reduced fluorescence of the nucleolus organizer regions, the difference in intensity of the fluorescence between these regions and the euchromatin is usually only minimal. For this reason they are more obviously recognizable by direct analysis in the fluorescence microscope than by analysis in photographs. The brightly and weakly fluorescing heterochromatic regions never lie directly side by side on a chromosome, but rather are always clearly separated from

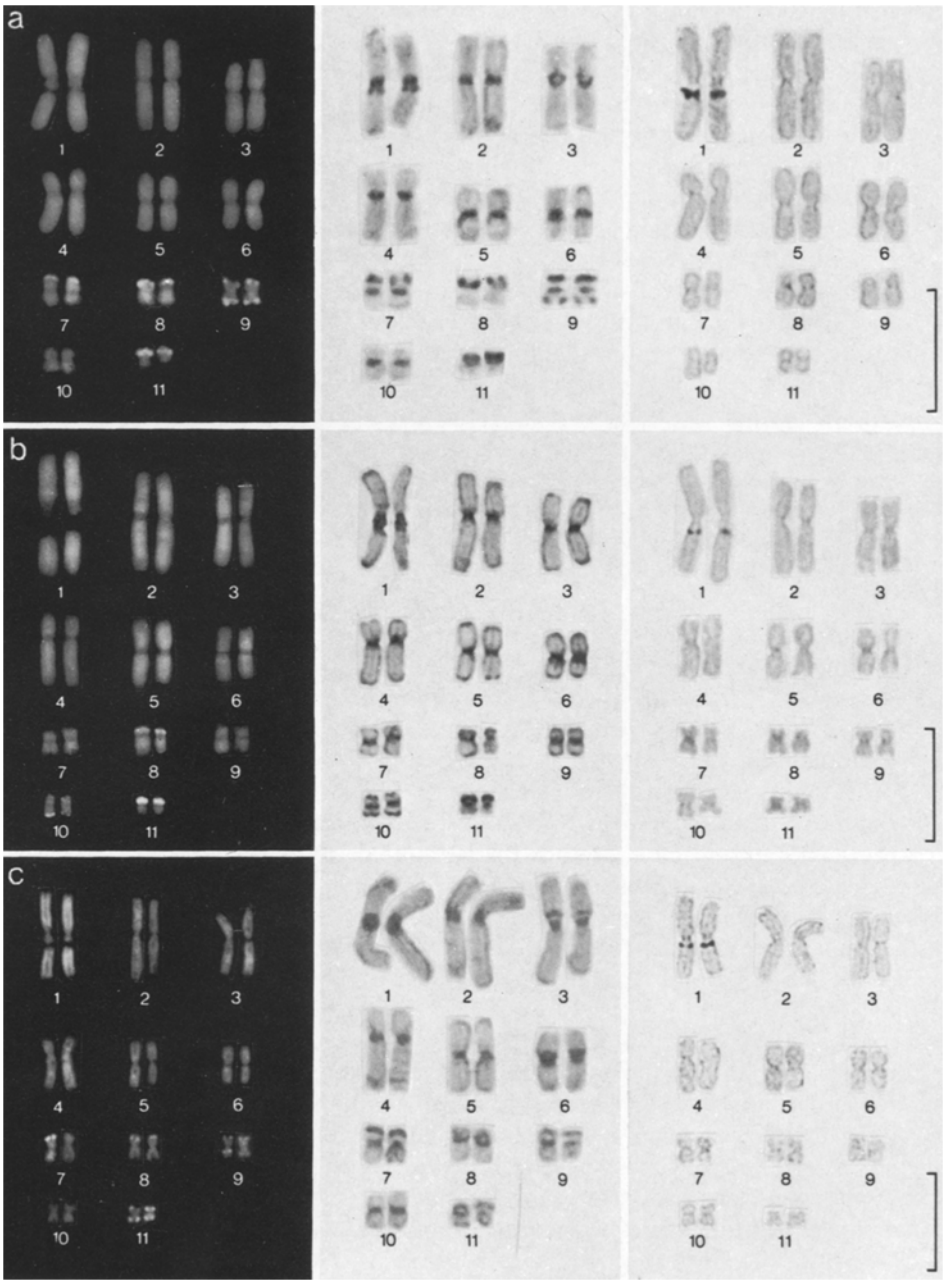

Fig. 2a–c. Karyotypes of **a** *Bufo americanus*, **b** *B. fowleri* and **c** *B. terrestris*, showing quinacrine staining (left), C-bands (middle) and AgAS stained NORs (right)

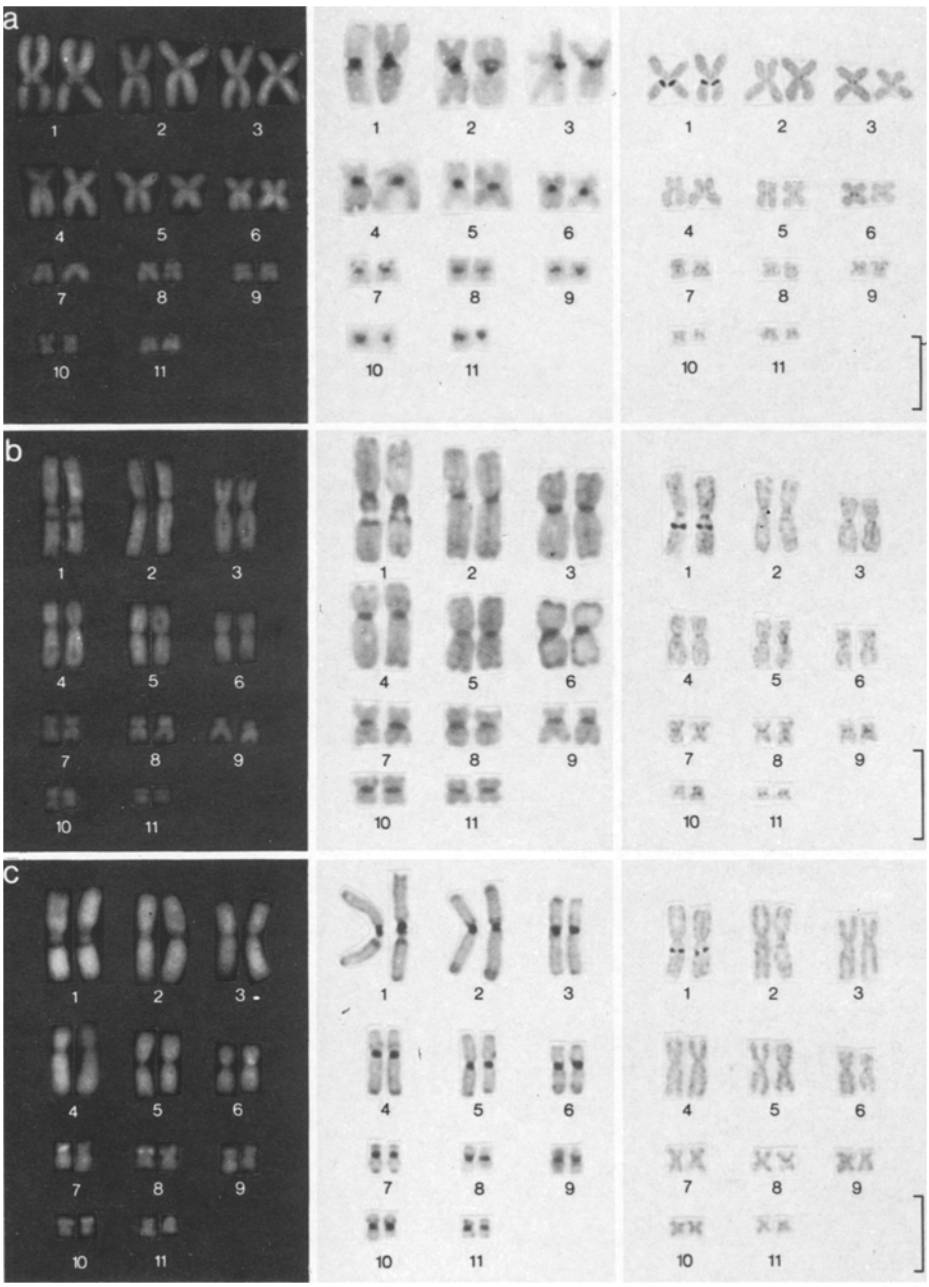

Fig. 3a–c. Karyotypes of **a** *Bufo punctatus*, **b** *B. valliceps* and **c** *B. compactilis* showing quinacrine staining (left), C-bands (middle) and AgAS stained NORs (right)

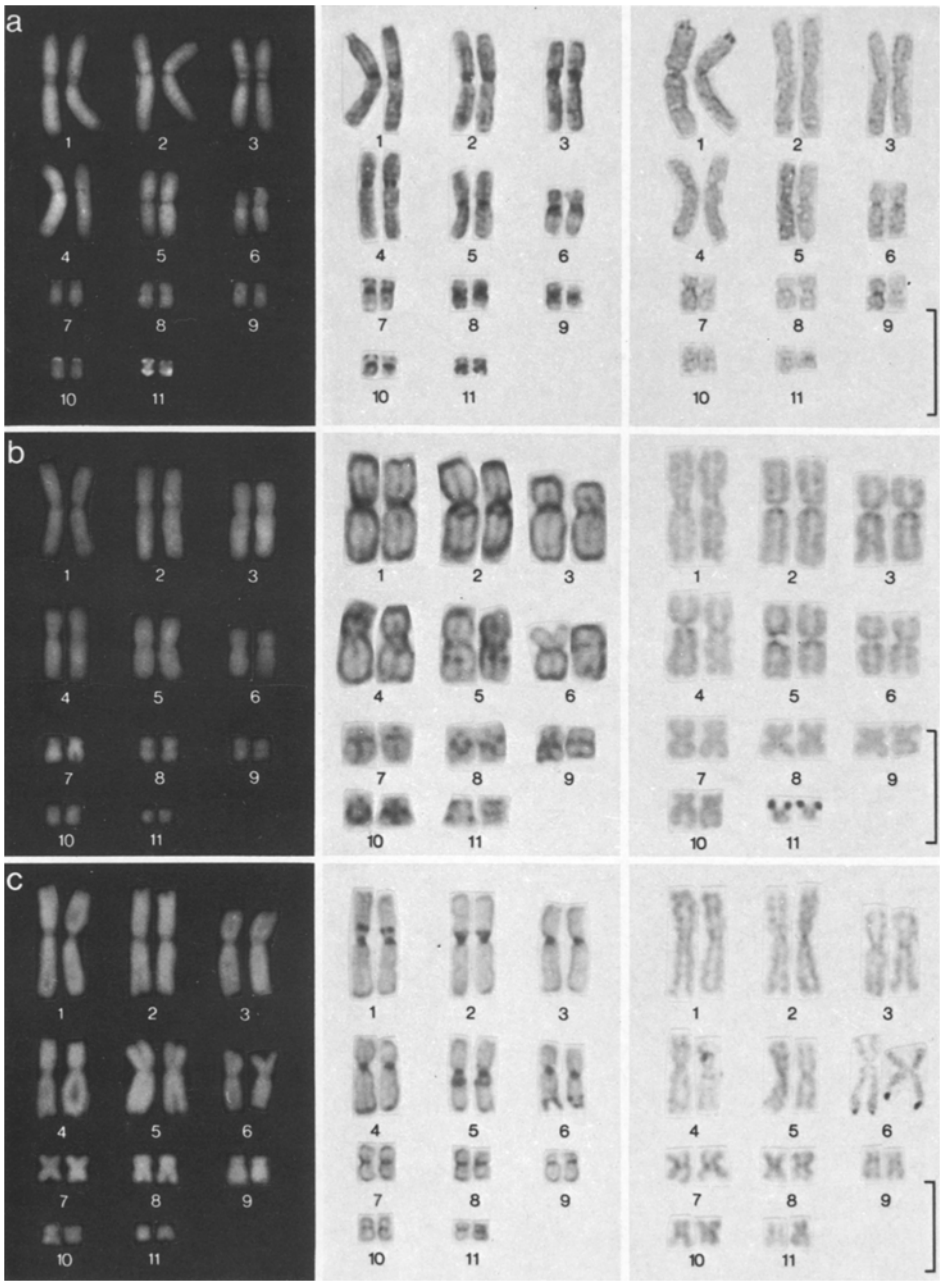

Fig. 4a–c. Karyotypes of **a** *Bufo boreas*, **b** *B. parvus* and **c** *B. bufo* showing quinacrine staining (left), C-bands (middle) and AgAS stained NORs (right)

one another by euchromatin (Figs. 9, 10). The weakly fluorescing, interstitially located heterochromatic regions are sometimes discernable as secondary constrictions using conventionally (aceto-orcein) stained chromosomes. In the C-banded preparations, the weakly fluorescing heterochromatin reacts differently as compared with the strongly fluorescing material: the centromeres and the nucleolus organizer regions, as a rule, become more strongly stained C-bands than the other interstitial bands and the telomeres (Figs. 2–10). Only in isolated instances (*Bufo calamita* and *Pedostibes hosii*) are the weakly fluorescing telomeres stained by C-banding as intensely as the pericentromeric regions (Figs. 5b, 8a).

In most of the analysed karyotypes there is no visible difference between the heterochromatic regions of homologous chromosome pairs. Only in 8 animals (7 species), there was a size difference between definable homologous heterochromatic bands which could be found in all mitoses (Table 2). These size variations coincided in 7 cases with weakly fluorescing heterochromatin (centromeres, interstitial C-bands) and in 2 cases with strongly fluorescing heterochromatin (telomeres). Aside from this size variability of particular heterochromatic bands, which also occurs in other vertebrates including man, no heteromorphism on homologous chromosomes could be demonstrated. The differential banding techniques did not reveal any indication of the existence of sex-chromosomes in any of the anuran species studied. Similarly, no irregularities in the pairing configuration of homologous chromosomes were detectable with banded chromosomes in the meiotic stages of male individuals.

With mammalian chromosomes, it could often be shown that the heterochromatic regions have a clear apposition of the chromatids in mid-metaphase (Schmid, 1967). This is also true for the constitutive heterochromatic chromosome regions of the amphibians (Figs. 1–8); the close apposition of the interstitial and terminal heterochromatin appears to be one of the causes of the typical parallel position of the chromatids of amphibian chromosomes.

The chromosomes of *Bufo arenarum, B. marinus* and *Hyla arborea* gave no C-banding, even though the method was varied many times. The only C-bands which were observed in just a few mitoses of these 3 species, lay in the nucleolus organizer regions. On the quinacrine-stained chromosomes, however, several heterochromatic bands, more weakly fluorescing than the euchromatin, were detected (Fig. 7b, c). It is assumed that these weakly fluorescing bands represent C-band-positive heterochromatin, which only becomes stainable after a suitable pre-treatment that has not yet been achieved.

Nucleolus Organizer Regions (NORs)

All species of bufonids and hylids investigated had in their karyotypes 2 NORs which could be specifically demonstrated by using the AgAS technique (Figs. 2–8). In the interphase nuclei of various diploid tissue cells, never more than 2 $AgNO_3$-stained nucleoli could be detected. The chromosomal position of the NORs did not vary between individuals of the same species that were examined, but did often differ considerably between species. In 9 species, the

Table 2. DNA-values, chromosome lengths and heteromorphisms of the heterochromatin and of the Ag-stained nucleolus organizer regions in the anuran species studied

Species	pg DNA (2C)[a]	µm length of chromosomes (2n)[b]	pg DNA per µm chromosome	Q-bright heterochromatin	C-band heteromorphisms[c]	Positions of Ag-NOR	Animals with Ag-NOR heteromorphisms
Homo sapiens	7.3	176.0	0.04				
Bufo bufo	15.5	139.0	0.11	–	6q NOR	6q ter	1
B. calamita	6.7	110.0	0.06	–		11q ter	1
B. parvus				–	11p NOR	11p ter	1
B. viridis	8.6	118.8	0.07	+		6q tel	1
B. americanus	12.7	111.0	0.11	+		1q per	1
B. boreas				+		1p ter	1
B. compactilis				+		1q per	
B. fowleri	14.2	122.8	0.12	+	1 cen, 11p ter	1q per	1
B. punctatus	10.6	95.8	0.11	–		1q per	
B. terrestris	11.1	96.0	0.12	+	7p ter	1q per	2
B. valliceps	9.2	109.0	0.08	–		1q per	1
B. arenarum				–		7p inter	1
B. marinus	11.3	100.8	0.11	–		7p inter	
B. garmani				+	3 cen	6q inter	1
B. mauritanicus				+		10q inter	1
B. poweri				+		6q inter	3
B. poweri(?), 3n				+		7q inter	1
B. regularis	8.9	74.4	0.12	+		6q inter	1
Pedostibes hosii				+	6q per	10q ter	
Hyla arborea				–		10q inter	2
H. cinerea				+		10q inter	
H. septentrionalis				+		10q inter	1
Pseudacris ornata				–	1 cen, 11p inter	11q ter	1

[a] The human DNA-value was determined by Bachmann (1972), and the values for the *Bufo* species by Ullerich (1966), Bachmann (1970), and Olmo (1973)

[b] The total length of the 46 human chromosomes was measured by Lubs et al. (1971); for the measurements of the *Bufo* chromosomes the sexes were not evaluated separately

[c] p: short arm; q: long arm; cen: centric; inter: interstitial; tel: telomeric; ter: terminal; per: pericentric or proximal

NORs were found in a pericentric position, while in 6 other species they lay in an interstitial or terminal position. In one species (*Bufo viridis*) the NORs occupy a telomeric position (Figs. 9, 10). The NORs were localized on the long arms of the chromosomes in 18 species, and on the short arms in only 4 species; it must be taken into consideration here that the location of the NORs on almost metacentric chromosomes is difficult to assign to either the short or long arm.

In metaphases of the triploid female classified as *Bufo poweri*, 3 chromosomes with specifically stained NORs are always found (Fig. 7a). The maximum number of nucleoli in interphase nuclei of this animal was 3. Analysis of the

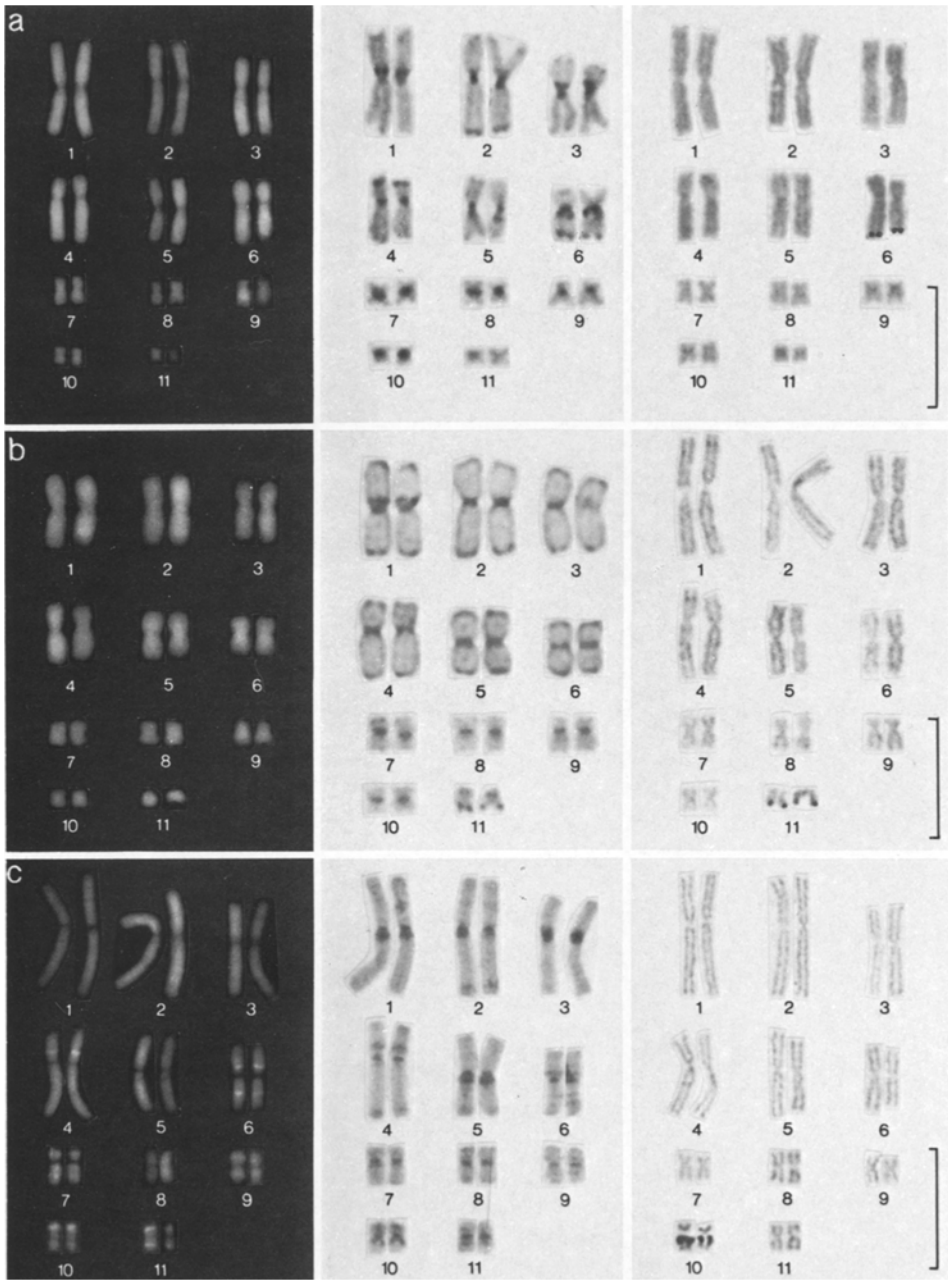

Fig. 5a–c. Karyotypes of **a** *Bufo viridis*, **b** *B. calamita* and **c** *B. mauritanicus*, showing quinacrine staining (left), C-bands (middle) and AgAS stained NORs (right)

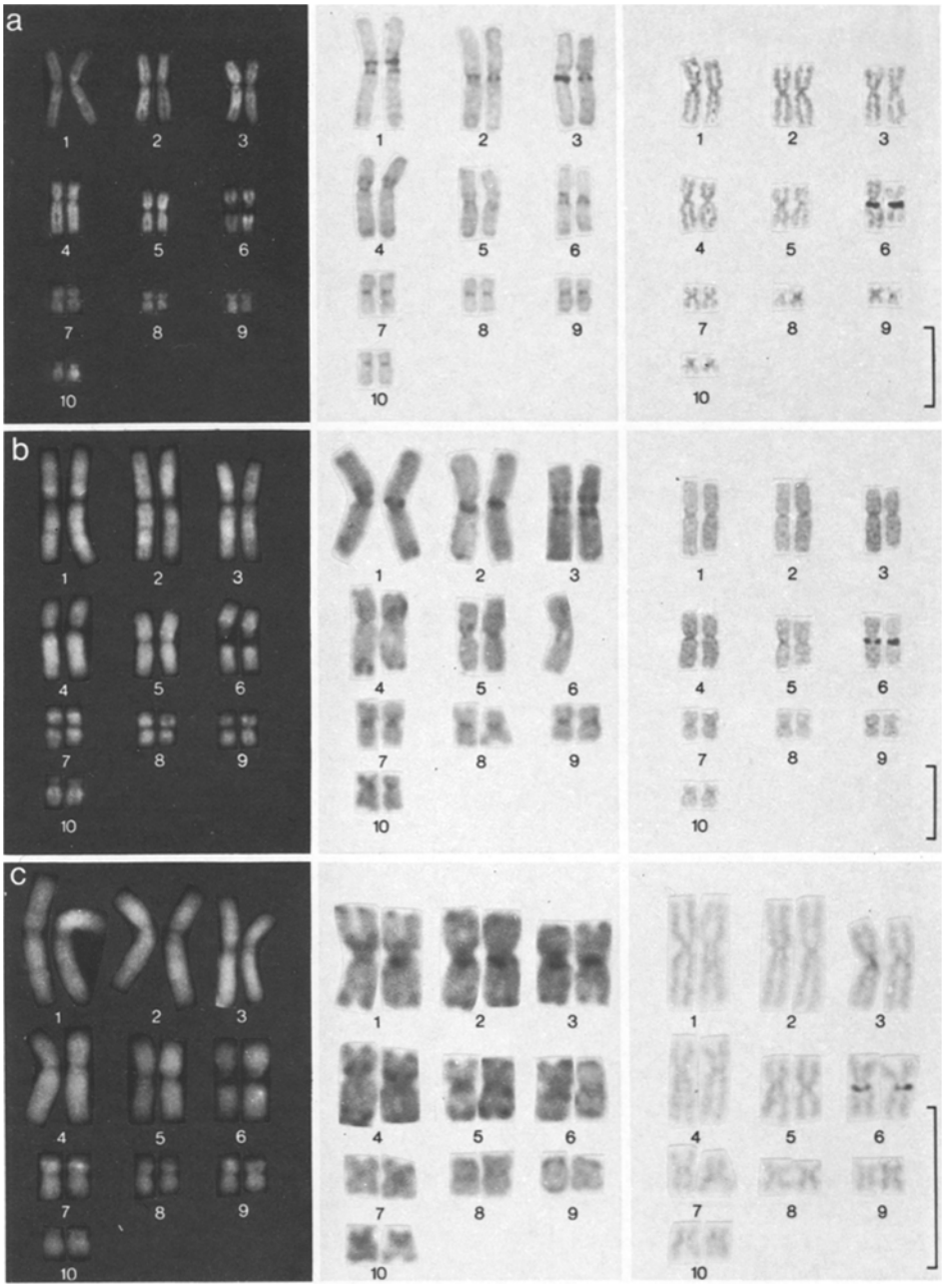

Fig. 6a–c. Karyotypes of **a** *Bufo garmani*, **b** *B. poweri* and **c** *B. regularis*, showing quinacrine staining (left), C-bands (middle) and AgAS stained NORs (right)

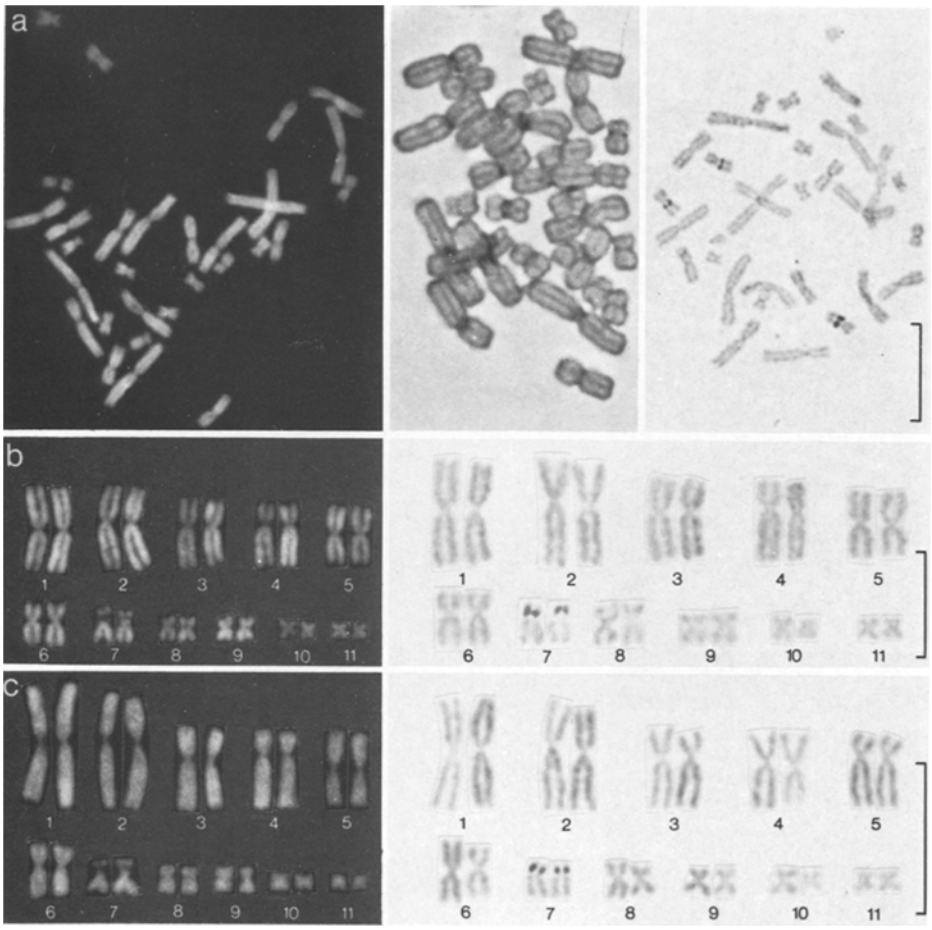

Fig. 7a–c. Metaphases of **a** a 3n female classified as *Bufo poweri*, showing quinacrine staining (left), C-bands (middle) and AgAS stained NORs (right). **b, c** Karyotypes of *B. arenarum* and *B. marinus* showing quinacrine-staining (left) and AgAS stained NORs (right)

karyotypes showed that the NORs of this female lie on chromosome 7, and not on chromosome 6 as in the four males of this species examined (Fig. 6b). Either this female belonged to another strain of *B. poweri* or to another species of the *B. regularis* group. The triploid female displayed no conspicuous morphological features. Unfortunately, no further females were available for this investigation, so it can not yet be determined whether this triploidy arose spontaneously, or whether it is the normal chromosome constitution for females of this species.

The NORs are always accompanied by constitutive heterochromatin which is strongly C-band-positive and which fluoresces more weakly than euchromatin. In many C-banded metaphases, it appears that the nucleolar constriction itself is C-band-positive. Nevertheless, on optimally stained chromosomes the consti-

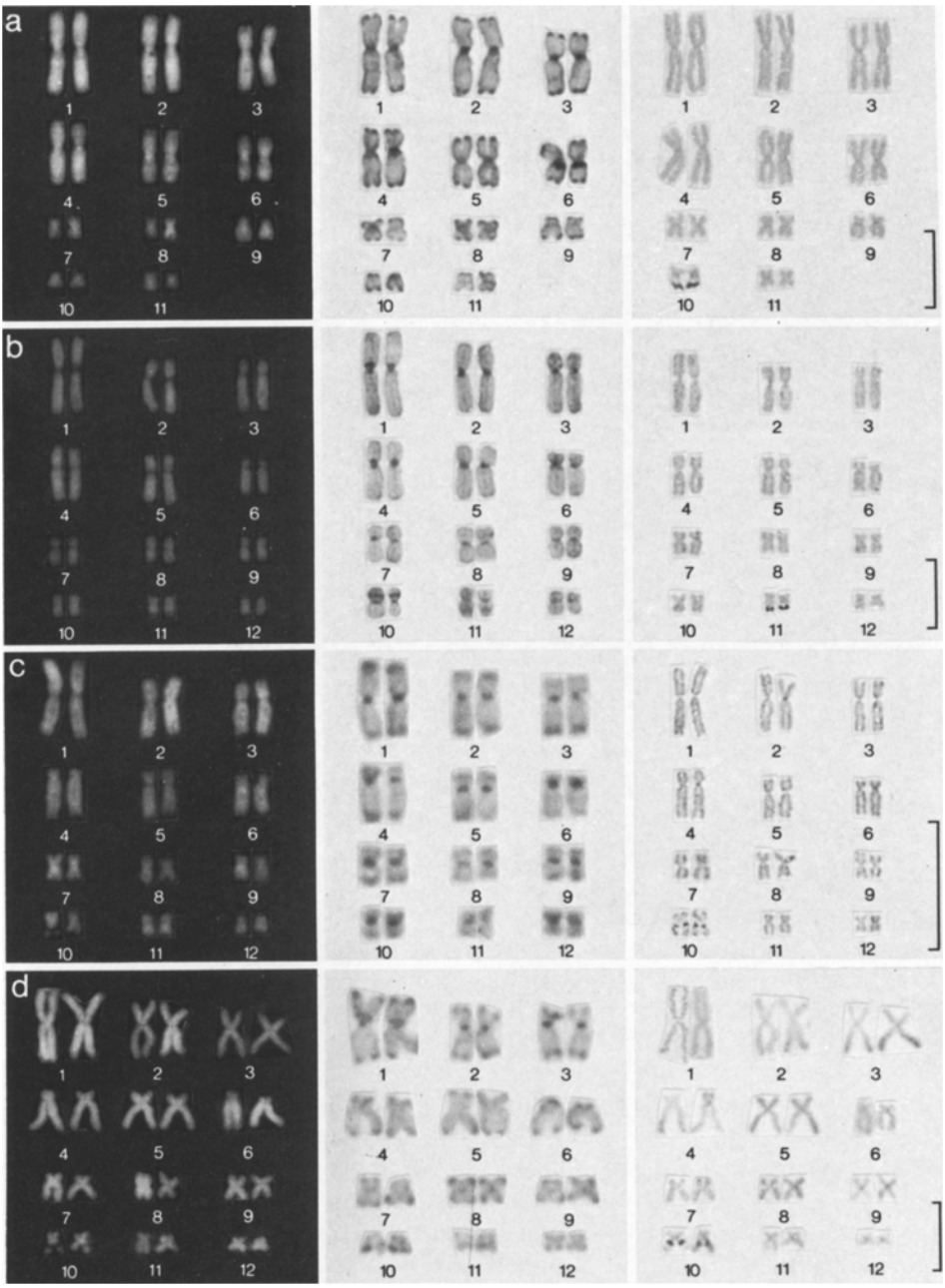

Fig. 8a–d. Karyotypes of **a** *Pedostibes hosii*, **b** *Pseudacris ornata*, **c** *Hyla septentrionalis* and **d** *H. cinerea*, showing quinacrine staining (left), C-bands (middle) and AgAS stained NORs (right)

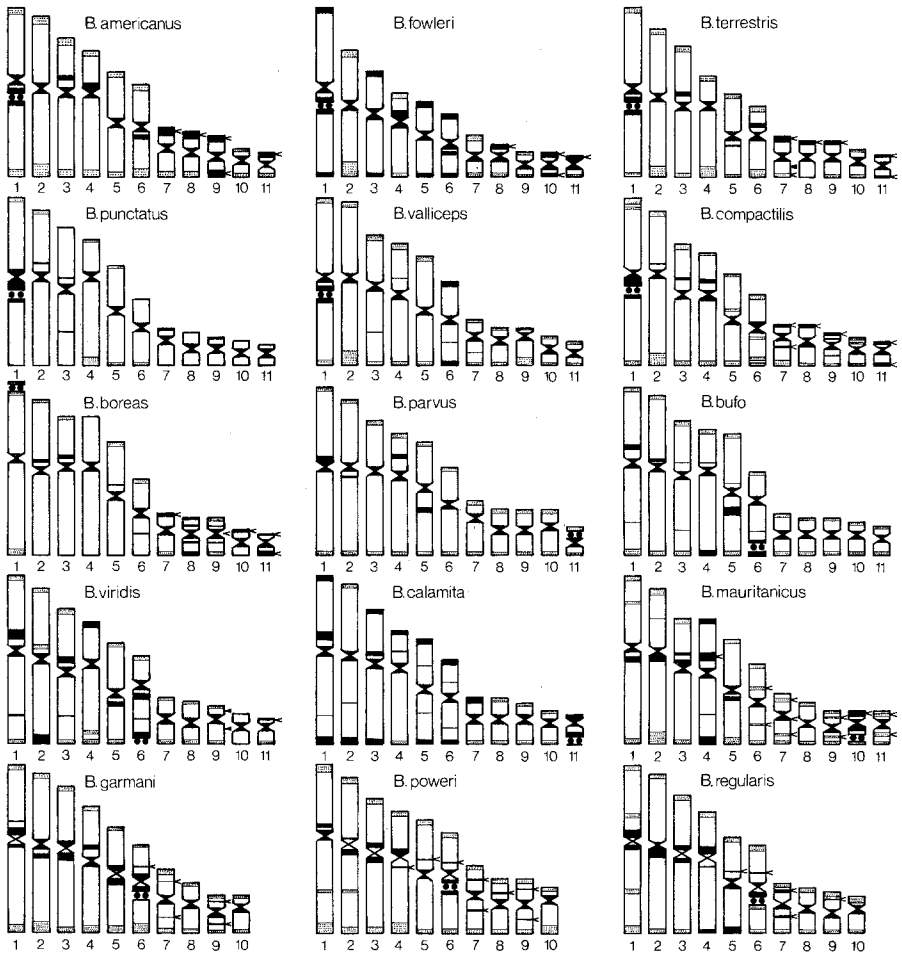

Fig. 9. Diagrammatic representation of *Bufo* karyotypes showing the position of the constitutive heterochromatin and NORs. Dark circles: NORs. Dark sectors: C-band positive heterochromatin. Spotted sectors: heterochromatin, only weakly stainable by C-banding. Open arrows: brightly fluorescing, C-band-positive, regions. Filled arrows: brightly fluorescing, C-band-negative, regions

tutive heterochromatin can be localized with certainty in the segments adjacent to the NORs (e.g. *Bufo valliceps, B. compactilis,* Fig. 3b, c). In 2 animals (*B. parvus* and *B. bufo*) a different large amount of heterochromatin could be detected on the respective homologous NORs in all metaphases analysed (Table 2, Fig. 4b, c). The occasionally heteromorphism of C-bands in the NORs of other species is attributed to different degrees of contraction of the homologous chromosomes.

The karyotypes shown in Figures 2–8 were derived from animals in which in most of the metaphases investigated, both NORs appear as nucleolar constrictions of equal length. With the AgAS technique about equal amounts of silver are deposited in the homologous NORs of these animals. Accordingly, both

Ag-stained nucleoli have the same area in interphase. However, most of the 47 animals studied had heteromorphic NORs (Table 2). The size of the nucleolar constrictions and of the Ag-blocks in the NORs appeared to be extremely variable not only in the bufonids and hylids, but in all 70 anuran species so far investigated; a more detailed description of this variability of the NORs in the Anura is in preparation.

The nucleolar constriction is in all species the most conspicuous secondary constriction of the karyotype and the only one that reacts specifically with $AgNO_3$. In no other secondary constriction was an Ag-block ever detected. A further indication that the Ag-negative constrictions do not represent NORs is the lack of associations among these constrictions in the metaphase plate. The Ag-positive nucleolar constrictions were associated in at least 10–20% of all mitoses from bone marrow and from stimulated lymphocytes. This is in agreement with the results obtained with conventionally stained chromosomes from *Xenopus muelleri* and *X. tropicalis,* where only one pair of homologous constrictions are associated consistently with one another, even though 2 pairs of secondary constrictions are present in the karyotype (Tymowska and Kobel, 1972; Tymowska, 1973).

Discussion

Euchromatin

The lack of Q- and G-bands in the euchromatic segments of anuran chromosomes can be attributed to the extremely strong contraction of the chromosomes in metaphase. A similar situation is found in the chromosomes of the higher plants. Greilhuber (1977) was able to determine that the hypothetical distance between the bands on metaphase chromosomes in plants is too small to be resolved with the light microscope. The comparison of human chromosomes with those of anurans (Table 2) shows that the mean DNA content per μm of chromosome length in metaphase is 1.5 to 3 times greater in the Anura analysed. This is true not only for the bufonids and hylids, but also for 48 other species investigated (Schmid, in preparation). Anura may, however, exist on whose metaphase chromosomes Q- or G-bands could be demonstrated. In the plant genus *Lilium,* 2 species have been found in the meantime, which show easily identifiable Q-band patterns (Kongsuwan and Smyth, 1977). It would be of interest to determine their DNA content and the amount of DNA per μm chromosome length.

Heterochromatin

In the bufonids and hylids analysed, 3 classes of constitutive heterochromatin can be differentiated: C-band-positive, fluorescing very strongly; C-band-positive, fluorescing more weakly than euchromatin; and telomeric heterochromatin that shows only weak C-banding. With respect to the 3 C-band-negative, but

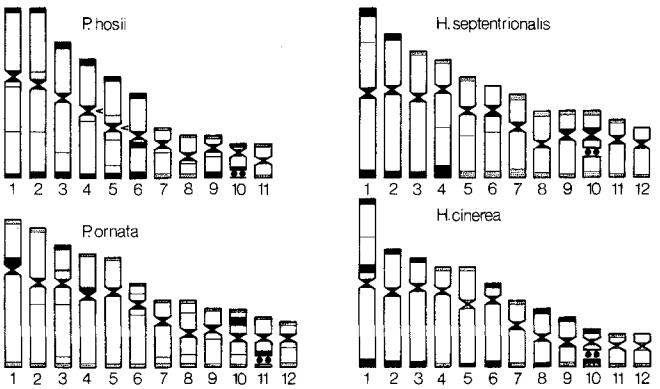

Fig. 10. Diagrammatic representation of the karyotypes of *Pedostibes hosii* (Bufonidae) and the Hylidae showing the position of constitutive heterochromatin and NORs. Dark circles: NORs. Dark sectors: C-band positive heterochromatin. Spotted sectors: heterochromatin, only weakly stainable by C-banding. Open arrows: brightly fluorescing, C-band positive, regions

strongly fluorescing heterochromatic regions of *Bufo terrestris* and *B. viridis*, a special heterochromatin class does not have to be assumed; it is also possible that these small heterochromatic bands stand out clearly with quinacrine staining, without being detectable by the C-band-technique. However, that there is constitutive heterochromatin which is C-band-negative but fluoresces very strongly when stained with fluorochromes could be shown in the case of the unusually large pair of autosomes in *Drosophila nasutoides* (Lee and Collins, 1977). It is conceivable that the three classes of heterochromatin identified in the Anura can be further characterized by employing other differential staining techniques. This has been done for instance with mammalian chromosomes where various C-band-positive regions can be differentiated from one another by their fluorescence intensity obtained with the fluorochromes Hoechst 33258, or chromomycin A_3, or mithramycin (Jalal et al., 1974; Schweizer, 1976).

There are several characteristic features of the constitutive heterochromatin of eukaryotic chromosomes known: late DNA-replication in the S-phase, condensed state in interphase, large amount of repetitive DNA-sequences, differential staining with banding techniques, association with secondary constrictions, enhanced frequency of spontaneous or induced breakages, and genetic inertness. It turns out that no class of constitutive heterochromatin possesses all of these features. Therefore, it can not be excluded that some heterochromatic chromosomal regions in the Anura studied were not revealed by the banding techniques employed. One such case was found previously in *Leptodactylus ocellatus* (Anura, Leptodactylidae); of the 21 heterochromatic bands of this species, 4 have a late DNA-replication, but no differential staining with the C-banding technique (Bianchi et al., 1973).

Experimental data have indicated that AT base-pairs enhance fluorescence with quinacrine, while GC base-pairs quench this fluorescence (Weisblum and

de Haseth, 1972; Weisblum, 1973). It therefore would appear that the DNA in the strongly fluorescing heterochromatic chromosome regions is richer in AT pairs than the DNA in the heterochromatin which fluoresces more weakly than euchromatin. The demonstration of this correlation was indeed possible in mammals by analytical density gradient centrifugation of the DNA (Jalal et al., 1974): in species with strongly fluorescing heterochromatin, the satellite DNA is lighter (AT-rich) than the main-band; in species in which weakly fluorescing heterochromatin predominates, the satellite DNA is heavier (GC-rich) than the main-band DNA. Also in the Anura, a biochemical investigation of the satellite-DNA would yield valuable data concerning the nature of the three kinds of heterochromatin found. This would be of special interest in the case of the terminal heterochromatin that is weakly stained by C-banding. Such heterochromatic regions have also been found on the chromosomes of plants (Vosa, 1976), *Drosophila melanogaster* (Hsu, 1971), and mammals (Arrighi et al., 1974; Jalal et al., 1974). As already suggested by Hsu and Arrighi (1971), the difference in staining intensity between C-band positive regions could be due to variable proportions of repetitive sequences. Accordingly, very highly repetitive sequences would reassociate better after alkaline denaturation and would be stained with Giemsa more strongly than DNA with a minor proportion of repetitive sequences.

The chromosomes of the bufonids and hylids are strikingly different from most of the chromosomes in other vertebrate classes with regard to their high frequency of constitutive heterochromatic regions. It is remarkable that the number of heterochromatic bands on most of the chromosomes analysed is independent of the length or DNA-content of the chromosomes (Figs. 11, 12). Thus, just as many or even more heterochromatic regions can lie on the smaller as on the largest chromosomes of the karyotypes. Only a few of the chromosomes have less than 3 heterochromatic regions, the majority have between 3 and 5 (Figs. 11, 12). The 3 chromosomal regions in which constitutive heterochromatin is almost always demonstrable are the pericentromeric regions and both telomeres. The telomeric heterochromatin could promote, the long-lasting terminal pairing of the bivalents in meiosis, which was observed in all higher Anura (Morescalchi, 1973). This would indicate that the terminal pairing of the ring-like bivalents is rarely due to chiasmata which are dissolved until late in meiosis, but rather to the close apposition of the telomeric heterochromatin of the bivalents. In the early stages of male meiosis the chromosomes of the Anura have a diffuse appearance; thus, it could not be determined whether the interstitial and proximal heterochromatin exerts an influence on the frequency of chiasmata. However, investigations of the lampbrush chromosomes in female meiosis (Morescalchi, 1973), displayed no preferential position of chiasmata.

Of the 22 species investigated, 14 have strongly fluorescing heterochromatin in their karyotypes. This is a remarkably high number compared with the results in other vertebrates in which strongly fluorescing heterochromatin occurs much more rarely (Jalal et al., 1974). Since brightly fluorescing heterochromatic bands are also quite frequent in the Urodela (Ragghianti et al., 1973; Bailly et al., 1976; Schmid and Krone, 1976), it is possible that this is a general characteristic of amphibian genomes.

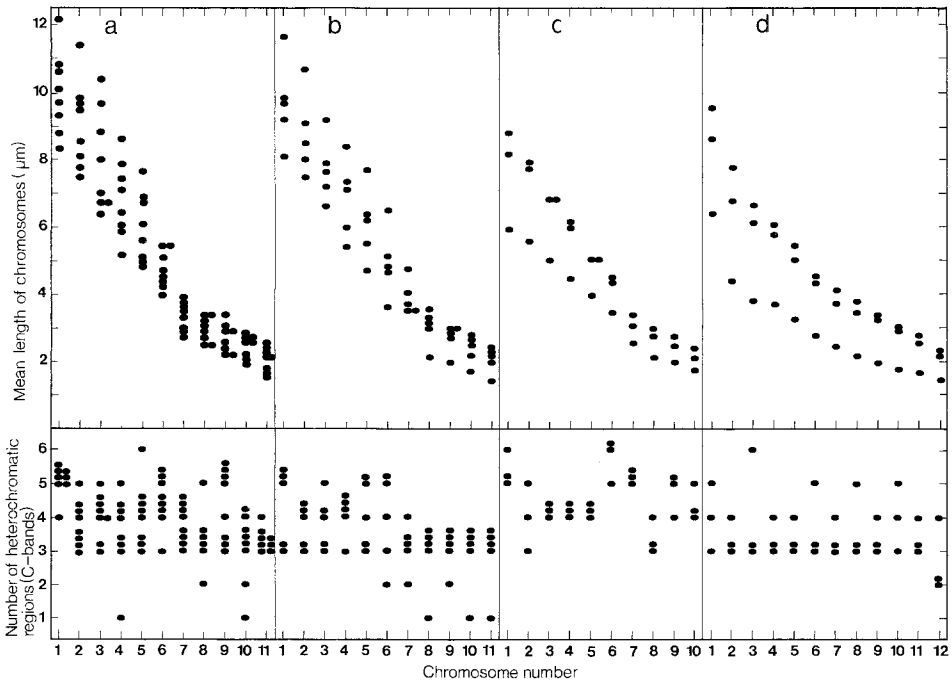

Fig. 11 a–d. Chromosome lengths and number of C-bands in **a** Bufonidae with 2n = 22 and brightly fluorescing heterochromatin (eight species), **b** Bufonidae with 2n = 22 and without brightly fluorescing heterochromatin (five species), **c** Bufonidae with 2n = 20 (three species) and **d** Hylidae with 2n = 24 (three species). Each symbol represents the value for one single chromosome in a given species. The individual species are not represented by distinct symbols

The chromosomes of both South American toads, *Bufo arenarum* and *Bufo marinus,* have no brightly fluorescing heterochromatin. Closely related to the South American bufonids are the leptodactylids *Odontophrynus americanus* and *O. cultripes* which also have only heterochromatin which fluoresces more weakly than the euchromatin (Garcia Ruiz and Beçak, 1976). While in North America and in Eurasia, species occur with, as well as without brightly fluorescing heterochromatin, all African species studied have brightly fluorescing heterochromatin. These data apparently indicate that the brightly fluorescing heterochromatin arose first after the colonization of the North American habitat, during the strong speciation in the pliocene and pleistocene ages. By migrations across the Bering Strait (Blair, 1972), several species of bufonids reached Eurasia, some of which had strongly fluorescing heterochromatin and some not. Only such Euroasiatic species possessing strongly fluorescing heterochromatin in their genomes finally reached Africa; this is supported by the fact that the area of dissemination of the only European toad with brightly fluorescing heterochromatin (*Bufo viridis*) reaches as far as North Africa (Inger, 1972). As far as the hylids are concerned more information on a wider variety of species must be gathered before concrete statements can be made with regard to the individual quantity and the localization of constitutive heterochromatin.

The investigations of Ullerich (1965, 1966) have shown that, among the chromosomes of the three european toad species (*Bufo bufo, B. viridis* and *B. calamita*), measurable differences in chromosome length exist. Each of the chromosomes of *B. bufo* contains more DNA than its respective homologues of *B. viridis* and *B. calamita* (Fig. 12b). In total, *B. bufo* has 49% more DNA

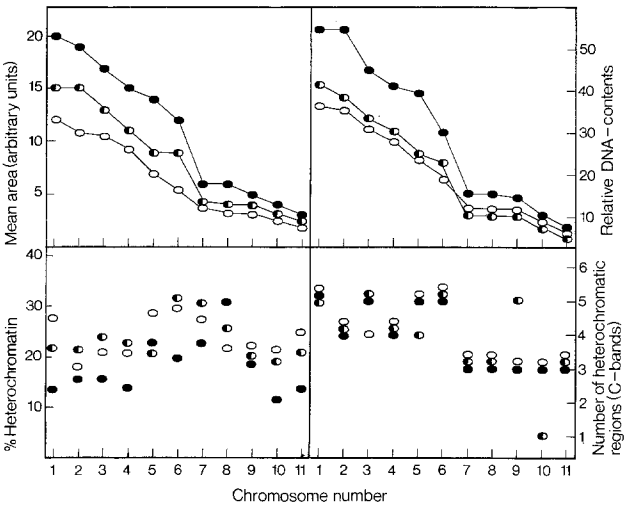

Fig. 12. Total area, DNA-values, percentages of constitutive heterochromatin and number of C-bands of the 11 chromosomes of *Bufo bufo* (●), *B. viridis* (◐), and *B. calamita* (○). The relative DNA-values were determined by Ullerich (1966)

than *B. viridis* and 39% more than the *B. calamita,* respectively (Ullerich, 1966). The relative DNA-contents of homologous chromosomes of the three species correspond approximately to their relative length, with the exception of the five small chromosomes (Fig. 12a). It is difficult to believe that the large differences in DNA-content among these 3 *Bufo* species are due to significant differences in important genetic information. A more plausible explanation would be that, in the evolution of these species, different amounts of redundant DNA-sequences were formed. These should, however, be detectable by staining the constitutive heterochromatin. The comparison of the amounts of measurable constitutive heterochromatin in the karyotypes of the three species (Fig. 12c) shows that no such differences exist. *B. bufo* does not have any more constitutive heterochromatin than *B. viridis* or *B. calamita.* Also, the number of heterochromatic regions per chromosome is essentially the same in all three species (Fig. 12d). It must certainly be kept in mind that the weakly stained telomeric and interstitial heterochromatic regions can not be measured with the same exactness as the strongly stained heterochromatin at the centromeres and NORs. The range of this inaccuracy of measurement is, however, well below the range of the differences in DNA contents among the three species. An explanation for the finding, that the differences in DNA-content are not attributable to differing amounts of visible constitutive heterochromatin is presented by the results of Baldari and Amaldi (1976) on DNA reassociation kinetics for amphibian genomes. These authors have demonstrated that the large difference in DNA-content among amphibian genomes was not attributable alone to differing quantities of highly repetitive and foldback sequences, but rather to a far greater extent to differing amounts of the intermediate repetitive sequences. Aside from this, it was discovered that the variations in the amount of intermedi-

ate repetitive sequences between species is due to variations in the number of copies in the various sequence-classes (Straus, 1971; Baldari and Amaldi, 1976). Since the C-banding pattern only reveals those chromosome regions with highly repetitive or rapidly reassociating sequences, the differences in the intermediate repetitive sequences remain unrecognized by the measurements performed. From DNA-reassociation kinetics in amphibian genomes it could also be concluded that, as in all other vertebrate genomes, the various sequence classes possess an interspersed organisation (Davidson et al., 1973; Baldari and Amaldi, 1976). The increase in the number of copies of intermediate repetitive sequences (i.e. the repetition frequency) would thus have changed only the absolute size of the chromosomes, but not the length-ratio of their arms. This would explain why the morphology of the ten or eleven chromosomes of the bufonids has remained constant during evolution. On the other hand, considerable increases of DNA in definite chromosome segments (constitutive heterochromatin) must have distinctly changed the form of individual chromosomes in various species.

All primitive groups of the present-day Anura have, in addition to the meta- and submetacentric chromosomes, smaller acrocentric chromosomes and microchromosomes. In the evolutionary process, the number of acrocentric chromosomes and microchromosomes was successively reduced by centric fusion and translocations involving entire chromosomes (Morescalchi, 1971, 1973). In the karyotypes of the more highly evolved families, meta- and submetacentric chromosomes occur almost exclusively. The proximal, interstitial and telomeric C-bands could be, in part, relics of constitutive heterochromatin at the ancestral centromeres of acrocentric chromosomes and microchromosomes. The formation of interstitial C-bands from the pericentric heterochromatin of inactivated centromeres after translocations involving whole chromosomes has also been taken into consideration for mammalian chromosomes (Hsu and Arrighi, 1971). Also in support of this are the examples of chromosomal aberrations cited by Hsu et al. (1975), by which translocated centromeres were apparently inactivated (latent centromeres).

Nucleolus Organizer Regions (NORs)

Only such NORs on metaphase chromosomes are stained specifically with the AgAS-technique which were active in the preceding interphase (Miller et al., 1976a, b; Engel et al., 1977). Thus, inactive or "dormant" NORs would remain undetected by this method. Results that are independent of the state of activity of the NORs can only be expected from in situ hybridization studies with highly labelled rRNA. The specificity, speed, and simplicity of this method nevertheless justify its application for the localization of genes for 18S+28S rRNA in comparative studies of the karyotypes of many species even though the possibility remains that the complete set of NORs might not be covered.

In none of the 47 bufonids and hylids occur more than two Ag-stained

NORs. Interindividual differences were not present, neglecting the frequent size variability of Ag-stained NORs. This is a clear indication that, in the karyotypes of these Anurans studied, actually only two NORs occur. One can speculate that karyotypes with a single large NOR pair are more ancestral than those in which the NORs are distributed over several chromosomes (Hsu et al., 1975). Accordingly, the number of NORs would have remained constant during the evolution of the bufonids and hylids, despite abundant speciation. By means of translocations and inversions, the NORs would have been transferred to other chromosomes as complete packages, rather than as multiple smaller units distributed throughout the genome. These assumptions are supported by the finding that, in the genus *Bufo* chromosome breaks occur preferentially in constitutive heterochromatin (Bogart, 1972). In the present work, 22 spontaneous aberrations were identified in 2300 analysed mitoses of the bufonids (these observations will be reported in a following contribution to this series). These aberrations were found without exception in the constitutive heterochromatic regions. Since the NORs of bufonids are always associated with constitutive heterochromatin, the probability of breaks within the nucleolar constriction is slight. This conservative distribution pattern of the 2 large NORs was not always maintained during the evolution of chromosomes in all groups of Amphibia. Investigations about the NORs of frogs of the genus *Rana* (Suborder Diplasiocoela) have revealed that, in the karyotypes of many species, there are up to 16 (!) smaller NORs (Schmid, in preparation).

In the karyotypes of species that belong to the same or to closely related species-groups, the Ag-stained NORs always lie in comparable chromosomal regions. In the North American toads, the pericentric region on the largest chromosome pair appears to be preferred as the position of the NOR. A single paracentric inversion on the same chromosome arm seems to have brought the NOR into a terminal position (*Bufo boreas*). The NOR-bearing submetacentric chromosome 6 of the african *B. regularis* group could have arisen by the fusion of two small chromosomes, one of which contained the original NOR. This is supported by the results with experimentally obtained interspecies hybrids: most of the *Bufo* species with 22 chromosomes, which form viable hybrids with species of the *B. regularis* group, have an obvious secondary constriction in the short arm of chromosome 7 or in the long arm of chromosome 11 (Bogart, 1972; Blair, 1972).

On the basis of the results described the question arises whether there are any regularities in the positions of the NORs and of the heterochromatic regions, respectively. The NOR of the 22 anuran species studied lie preferentially in the long arms of the chromosomes, where they tend to occupy a proximal position. This is also the case for most species of 14 further anuran genera (unpublished results). On the other hand, the results of Lima-de-Faria (1973, 1976) indicate that the ribosomal genes in most of the eukaryotes studied by him lie preferentially in the terminal segments of the short chromosome arms. This conclusion is based on the analysis of the nucleolar constrictions in conventionally stained karyotypes, predominantly of plants. The analysis of a representative sample of anuran species shows, that there are factors, which favor

a non-terminal position of the NORs in their chromosomes. Thus, owing to the telomeric heterochromatin of the anuran chromosomes, the terminal or telomeric position of the NORs could be unstable, since breaks also occur preferentially in the genetically inert heterochromatic regions. By means of inversions, the NORs would end up in a more stable interstitial chromosomal region.

The non-coincidental distribution of constitutive heterochromatin in the karyotypes of these Anura, especially of the brightly fluorescing heterochromatic bands, suggests an interaction between the individual chromosomal regions. Although the functional significance of the linear arrangement of the various kinds of chromatin is still unknown, the observed patterns are reminiscent of the proposition of a "chromosome-field" as formulated by Lima-de-Faria (1954, 1976). The use of banding techniques on the chromosomes of further anuran species should indicate according to which specific and general rules the evolution of the karyotypes in the Anura has taken place.

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References

Arrighi, F.E., Hsu, T.C., Pathak, S., Sawada, H.: The sex chromosomes of the chinese hamster: constitutive heterochromatin deficient in repetitive DNA sequences. Cytogenet. Cell Genet. **13**, 268–274 (1974)

Bachmann, K.: Specific nuclear DNA amounts in toads of the genus Bufo. Chromosoma (Berl.) **29**, 365–374 (1970)

Bachmann, K.: Genome size in mammals. Chromosoma (Berl.) **37**, 85–93 (1972)

Bailly, S.: Localisation et signification des zones Q observeés sur les chromosomes mitotiques de l'amphibien Pleurodeles waltlii Michah. après coloration par la moutarde de quinacrine. Chromosoma (Berl.) **54**, 61–68 (1976)

Baldari, C.T., Amaldi, F.: DNA reassociation kinetics in relation to genome size in four amphibian species. Chromosoma (Berl.) **59**, 13–22 (1976)

Beçak, W., Beçak, M.L., Schreiber, G., Lavalle, D., Amorim, F.O.: Interspecific variability of DNA content in Amphibia. Experientia (Basel) **26**, 204–206 (1970)

Bianchi, N.O., Bianchi, M.S., Vidal-Rioja, L.: Heterochromatin late replication and secondary constrictions in the chromosome complement of Leptodactylus ocellatus. Caryologia (Firenze) **26**, 397–403 (1973)

Birnstiel, M.L., Sells, B.H., Purdom, I.F.: Kinetic complexity of RNA molecules. J. molec. Biol. **63**, 21–39 (1972)

Blair, W.F.: Genetic compatibility and species groups in U.S. toads (Bufo). Texas J. Sci. **11**, 427–453 (1959)

Blair, W.F.: Evolutionary relationships of North American toads of the genus Bufo: a progress report. Evolution (Lawrence, Kans.) **17**, 1–16 (1963)

Blair, W.F.: Characteristics of the testes. In: Evolution in the genus Bufo (W.F. Blair, ed.), pp. 324–328. Austin-London: University of Texas Press 1972

Blair, W.F.: Evidence from hybridization. In: Evolution in the genus Bufo (W.F. Blair, ed.), pp. 196–232. Austin-London: University of Texas Press 1972

Bogart, J.P.: Chromosome number difference in the amphibian genus Bufo: the Bufo regularis species group. Evolution (Lawrence, Kans.) **22**, 42–45 (1968)
Bogart, J.P.: Karyotypes. In: Evolution in the genus Bufo (W.F.Blair, ed.), pp. 171–195. Austin-London: University of Texas Press 1972
Brown, D.D., Weber, C.S.: Gene linkage by RNA-DNA hybridization. I. Unique DNA sequences homologous to 4S RNA, 5S RNA and ribosomal RNA. J. molec. Biol. **34**, 661–680 (1968)
Brown, D.D., Wensink, P.C., Jordan, E.: Purification and some characteristics of 5S DNA from Xenopus laevis. Proc. nat. Acad. Sci. (Wash.) 3175–3179 (1971)
Cei, J.M., Erspamer, V., Roseghini, M.: Biogenic amines. In: Evolution in the genus Bufo (W.F. Blair, ed.), pp. 233–243. Austin-London: University of Texas Press 1972
Davidson, E.H., Amenson, C., Hough, B., Britten, R.J.: Interspersion of repetitive sequences in Xenopus DNA. Carnegie Inst. Wash. Year Book **71**, 273–276 (1972)
Davidson, E.H., Hough, B.R., Amenson, C.S., Britten, R.J.: General interspersion of repetitive with non-repetitive sequence elements in the DNA of Xenopus. J. molec. Biol. **77**, 1–25 (1973)
Dutrillaux, B., Grouchy, J. de, Finaz, C., Lejeune, J.: Mise en évidence de la structure fine des chromosomes humains par digestion enzymatique (pronase en particulier). C.R. Acad. Sci. (Paris) **273**, 587–588 (1971)
Engel, W., Zenzes, M.T., Schmid, M.: Activation of mouse ribosomal RNA genes at the 2-cell stage. Hum. Genet. **38**, 57–63 (1977)
Garcia Ruiz, I.R., Beçak, W.: Further studies on polyploid amphibians. V. C-banding in diploid and tetraploid species of Odontophrynus. Chromosoma (Berl.) **54**, 69–74 (1976)
Goin, O.B., Goin, C.J., Bachmann, K.: DNA and amphibian life history. Copeia (Wash.) 532–540 (1968)
Goodpasture, C., Bloom, S.E.: Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma (Berl.) **53**, 37–50 (1975)
Greilhuber, J.: Why plant chromosomes do not show G-bands. Theor. Appl. Genet. **50**, 121–124
Griffiths, I.: The phylogeny of the Salientia. Biol. Rev. **38**, 241–292 (1963)
Guttman, S.I.: Evolution of blood proteins in the cosmopolitan toad genus Bufo. Ph. D. Thesis, Univ. Texas (1967)
Hsu, T.C.: Heterochromatin pattern in metaphase chromosomes of Drosophila melanogaster. J. Hered. **62**, 285–287 (1971)
Hsu, T.C., Arrighi, F.E.: Distribution of constitutive heterochromatin in mammalian chromosomes. Chromosoma (Berl.) **34**, 243–253 (1971)
Hsu, T.C., Cooper, J.E.K., Mace, M.L., Brinkley, B.R.: Arrangement of centromeres in mouse cells. Chromosoma (Berl.) **34**, 73–87 (1971)
Hsu, T.C., Pathak, S., Chen, T.R.: The possibility of latent centromeres and a proposed nomenclature system for total chromosome and whole arm translocations. Cytogenet. Cell Genet. **15**, 41–49 (1975)
Hsu, T.C., Spirito, S.E., Pardue, M.L.: Distribution of 18+28S ribosomal genes in mammalian genomes. Chromosoma (Berl.) **53**, 25–36 (1975)
Inger, R.F.: Bufo of Eurasia. In: Evolution in the genus Bufo (W.F. Blair, ed.), pp. 102–118. Austin-London: University of Texas Press 1972
Jalal, S.M., Clark, R.W., Hsu, T.C., Pathak, S.: Cytological differentiation of constitutive heterochromatin. Chromosoma (Berl.) **48**, 391–403 (1974)
Kongsuwan, K., Smyth, D.R.: Q-bands in Lilium and their relationship to C-banded heterochromatin. Chromosoma (Berl.) **60**, 169–178 (1977)
Lee, C.S., Collins, L.: Q- and C-bands in the metaphase chromosomes of Drosophila nasutoides. Chromosoma (Berl.) **61**, 57–60 (1977)
Lima-de-Faria, A.: Chromosome gradient and chromosome field in Agapanthus. Chromosoma (Berl.) **6**, 330–370 (1954)
Lima-de-Faria, A.: The specific location of ribosomal genes in the eukaryotic chromosome. In: Modern aspects of cytogenetics: constitutive heterochromatin in man (R.A. Pfeiffer, ed.). Symp. Med. Hoechst **6**, pp. 39–44. Stuttgart-New York: Schattauer 1973
Lima-de-Faria, A.: The chromosome field. I. Prediction of the location of ribosomal cistrons. Hereditas (Lund) **83**, 1–22 (1976)
Low, B.S.: Evolution in the genus Bufo: evidence from parathoid glands. Ph. D. Thesis, Univ. Texas (1967)

Lubs, H., Hostetter, T., Ewing, L.: Cited in Paris Conference (1971): Standardization in human cytogenetics. Birth defects: original article series, vol. **8**, No. 7. New York: The National Foundation 1972

Martin, R.F.: Evidence from osteology. In: Evolution in the genus Bufo (W.F. Blair, ed.), pp. 37–70. Austin-London: University of Texas Press 1972

Martin, W.F.: Evolution of vocalization in the genus Bufo. In: Evolution in the genus Bufo (W.F. Blair, ed.), pp. 279–309. Austin-London: University of Texas Press 1972

Matsui, S.: Structural proteins associated with ribosomal cistrons in Xenopus laevis chromosomes. Exp. Cell Res. **88**, 88–94 (1974)

Miller, D.A., Dev, V.G., Tantravahi, R., Miller, O.J.: Suppression of human nucleolus organizer activity in mouse-human somatic hybrid cells. Exp. Cell Res. **101**, 235–243 (1976)

Miller, O.J., Miller, D.A., Dev., V.G., Tantravahi, R., Croce, C.M.: Expression of human and suppression of mouse nucleolus organizer activity in mouse-human somatic cell hybrids. Proc. nat. Acad. Sci. (Wash.) **73**, 4531–4535 (1976)

Morescalchi, A.: Comparative karyology of the Amphibia. Boll. Zool. **38**, 317–320 (1971)

Morescalchi, A.: Amphibia. In: Cytotaxonomy and vertebrate evolution (A.B. Chiarelli and E. Capanna, eds.), pp. 233–348. London-New York: Academic Press 1973

Olmo, E.: Quantitative variations in the nuclear DNA and phylogenesis of the Amphibia. Caryologia (Firenze) **26**, 43–68 (1973)

Overton, K.M., Magenis, R.E., Brady, T., Chamberlin, J., Parks, M.: Cytogenetic darkroom magic: now you see them, now you don't. Amer. J. Hum. Genet. **28**, 417–419 (1976)

Pardue, M.L., Birnstiel, M.L.: Cytological localization of repeated gene sequences. In: Modern aspects of cytogenetics: constitutive heterochromatin in man (R.A. Pfeiffer, ed.), pp. 75–85. Stuttgart-New York: Schattauer 1973

Pardue, M.L., Brown, D.D., Birnstiel, M.L.: Location of the genes for 5S ribosomal RNA in Xenopus laevis. Chromosoma (Berl.) **42**, 191–203 (1973)

Ragghianti, M., Bucci Innocenti, S., Mancino, G.: Bandeggiatura indotta da "C-, G- e Q-staining methods" e pattern di replicazione dei cromosomi di Triturus. Rend. Accad. naz. Lincei **55**, 764–770 (1973)

Schmid, W.: Heterochromatin in mammals. Arch. Klaus-Stift. Vererb.-Forsch. **42**, 1–60 (1967)

Schmid, M., Krone, W.: The relationship of a specific chromosomal region to the development of the acrosome. Chromosoma (Berl.) **56**, 327–347 (1976)

Schweizer, D.: Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma (Berl.) **58**, 307–324 (1976)

Seabright, M.: The use of proteolytic enzymes for the mapping of structural rearrangements in the chromosomes of man. Chromosoma (Berl.) **36**, 204–210 (1972)

Sexsmith, E.: DNA values and karyotypes of Amphibia. Ph. D. Thesis, Dept. of Botany, University of Toronto 1968

Stock, A.D., Mengden, G.A.: Chromosome banding pattern conservatism in birds and nonhomology of chromosome banding patterns between birds, turtles, snakes and amphibians. Chromosoma (Berl.) **50**, 69–77 (1975)

Straus, N.A.: Comparative DNA renaturation kinetics in amphibians. Proc. nat. Acad. Sci. (Wash.) **68**, 799–802 (1971)

Sumner, A.T.: A simple technique for demonstrating centromeric heterochromatin. Exp. Cell Res. **75**, 304–306 (1972)

Tymowska, J.: Karyotype analysis of Xenopus tropicalis Gray, Pipidae. Cytogenet. Cell Genet. **12**, 297–304 (1973)

Tymowska, J.: A comparative study of the karyotypes of eight Xenopus species and subspecies possessing a 36-chromosome complement. Cytogenet. Cell Genet. **18**, 165–181 (1977)

Tymowska, J., Fischberg, M.: Chromosome complements of the genus Xenopus. Chromosoma (Berl.) **44**, 335–342 (1973)

Tymowska, J., Kobel, H.R.: Karyotype analysis of Xenopus muelleri (Peters) and Xenopus laevis (Daudin), Pipidae. Cytogenet. Cell Genet. **11**, 270–278 (1972)

Ullerich, F.-H.: Unterschiede im DNS-Gehalt der Genome von Bufo bufo und Bufo viridis. Z. Naturforsch. **20b**, 720–722 (1965)

Ullerich, F.-H.: Karyotyp und DNS-Gehalt von Bufo bufo, B. viridis, B. bufo × B. viridis und B. calamita (Amphibia, Anura). Chromosoma (Berl.) **18**, 316–342 (1966)

Ullerich, F.-H.: Weitere Untersuchungen über Chromosomenverhältnisse und DNS-Gehalt bei Anuren (Amphibia). Chromosoma (Berl.) **21**, 345–368 (1967)
Vosa, C.G.: Heterochromatic banding patterns in Allium. II. Heterochromatin variation in species of the paniculatum group. Chromosoma (Berl.) **57**, 119–133 (1976)
Weisblum, B.: Fluorescent probes of chromosomal DNA structure: three classes of acridines. Cold. Spr. Harb. Symp. quant. Biol. **38**, 441–449 (1973)
Weisblum, B., Haseth, de, P.L.: Quinacrine, a chromosome stain specific for deoxyadenylate-deoxythymidylate-rich regions in DNA. Proc. nat. Acad. Sci. (Wash.) **69**, 629–632 (1972)
Wolf, K., Quimby, M.C.: Amphibian cell culture: permanent cell line from the bullfrog (Rana catesbeiana). Science **114**, 1578–1580 (1964)

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